ANALYSIS OF THE MOLECULAR STRUCTURE OF CENTROMERES OF CHINESE HAMSTER AND HUMAN CHROMOSOMES

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ABSTRACT

Monoclonal anti-CHO chromosome scaffold, and polyclonal anti-centromere autoimmune antibodies were employed to investigate the molecular organisation of the centromeres of Chinese hamster and human chromosomes. The presence of a novel ring-like structure was revealed, which was conserved amongst the metaphase centromeres of two rodents, namely Chinese hamster and mouse, and in humans. In metaphase the antigen had a molecular weight of 170kD, this polypeptide may be processed from, or the stable degradation product of, a 200kD interphase precursor full-length polypeptide. The antigen could not be detected in interphase centromeres, but the ring-like structure was detected in chromosome scaffold preparations, indicating that the ring antigen resisted the 2M NaCl extraction, and hence, tightly bound the DNA of metaphase chromosomes. The possible relationship of the centromere antigen with another high molecular weight and major chromosome scaffold protein, DNA topoisomerase II, was also explored.

The centromere antigen was also localised apparently to the interdigitating microtubules of the midzone, in anaphase; seemingly to the contractile ring at cytokinesis, and also to the interphase centrioles. The antigen was shown to be distinct from the microtubule-associated proteins and had non-identity with another high molecular weight protein component of the contractile ring, namely myosin. The dramatic relocation of the antigen, however, at the metaphase/anaphase transition suggests that it falls into a class of recently identified dual chromosomal and cytoplasmic components, termed the "passenger proteins".

The ring antigen is postulated to be involved in holding the sister chromatids together by a strong interaction with the centromeric DNA through prometaphase to the metaphase-anaphase transition. It is suggested that the ring antigen is disengaged from the centromere prior to anaphase, and may be involved in the regulation of the metaphase-anaphase transition.

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ABBREVIATIONS

BSA	bovine serum albumin
CDC	cell division cycle
CDE	centromere DNA element
CEN	centromere
CENP	centromere protein
CHO	Chinese hamster ovary
CLiP	chromatid linking protein
Colo	colon, carcinoma, human cell line
CREST	Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly and Telangiectasia
DMSO	dimethyl sulphoxide
EDTA	disodium ethylene-diaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
F12	basic culture medium for human, CHO cells
FITC	fluorescein isothiocyanate
GH	hypotonic glycine-hexylene glycol buffer
GHT	GH buffer containing Triton-X-100
HeLa	epithelioid carcinoma, cervix, human cell line
HRPO	horse radish peroxidase
INCENP	inner centromere protein
IPTG	isopropyl ß-D-thiogalactopyranoside
K20	ovary, Chinese hamster cell line
LMTK$^-$	connective tissue, mouse, thymidine kinase mutant, bromodeoxyuridine resistant cell line
Mabs	monoclonal antibodies
MAP	microtubule associated protein
MAR	matrix associated region
MTOC	microtubule organising centre
NOR	nucleolar organiser region
PBS	phosphate buffered saline
PMSF	phenylmethyl sulfonylfluoride
RPMI	basic growth medium for hybridoma cells
SAR	scaffold associated region
SDS	sodium dodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SnRNP	small nuclear ribonucleoprotein particles
TBS	Tris buffered saline
TCA	trichloroacetic acid
TRIC	rhodamine isothiocyanate

Notes:

The **centromere** is the term generally used to denote the primary constriction of chromosomes and the proteins associating with the DNA located there. The **kinetochore** on the other hand is the site of microtubule attachment. Some authors use these two terms synonymously, nevertheless, in this thesis the afore-mentioned definitions will be adhered to.

The **scaffolds** referred to in this thesis, unless otherwise stated, are from **metaphase chromosomes**, and not **nuclear matrices** (sometimes also termed nuclear scaffolds, or nuclear cage), which are prepared from **interphase nuclei**, and the protein composition of these two types of samples are distinct.

CONTENTS

CHAPTER I 1 THE CELL CYCLE AND NUCLEAR ORGANISATION

Overview

The aim of this project was to analyse the molecular nature of Chinese hamster and if possible human centromeres. Thus, the literature pertaining directly to this area and relevant background is reviewed in this and the subsequent Introduction chapters.

In order to appreciate the processes occurring at the mammalian centromere the current view of the molecular regulation the cell cycle, and a relevant background of the organisation of the interphase nucleus of animal cells will be outlined in this Chapter I 1. This is only a global overview, with particular references to features potentially pertinent to the centromere and mammalian chromatin. For a more in depth analysis the reader should refer to the references therein.

Since the chromosomes are directly influenced by the spindle machinery, then the relevant components of the cytoskeleton are treated in Chapter I 2. Finally, the chromosome structure and in particular the centromere architecture is discussed in Chapter I 3

As the cell progresses from interphase into mitosis, the nuclear DNA dissociates from the nuclear envelope, and the chromatin condenses into the compact chromosomes. Meanwhile, the nuclear envelope vesicularises, and the nuclear lamins depolymerise. The cell then undergoes mitosis, whereby the chromosomes segregate to the poles. Towards the end of mitosis the membrane vesicles and lamins bind the surface of the condensed DNA, then re-establish the nuclear membrane, and finally the cells then go on to divide. For a review see Newport and Forbes (1987), Gerace and Burke (1988) and more recently Murray and Kirschner (1991).

I.1.1 THE MOLECULAR REGULATION OF THE CELL CYCLE

Nearly twenty years ago Hartwell and coworkers identified several cell division cycle (cdc) mutants in *Saccharomyces cerevisiae* on the basis of arrest at certain morphologically distinct points of the cell cycle (Hartwell et al, 1974). Thus, temperature-sensitive mutants allowed the first identification of the genes required for progress through the cell cycle, furthermore, they facilitated the identification of a crucial point in the cell cycle, namely "START" (Hartwell et al, 1974), at which the cell is sensitive to the extracellular nutrient levels and intracellular signals.

I 1.1.1 p34(cdc2)

The two regulators, and later shown to be homologues, of the yeast cell cycle, which were shown to be important at the START stage of G1 (the point after which the cell is committed to division), are CDC28 (Hartwell et al, 1974) and cdc2 (Nurse et al, 1976) identified in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively. In addition to its regulation of START, cdc2+ is, also, required at the G2-mitosis transition (Nurse & Bissett, 1981) i.e. G1/S and G2/M transitions, respectively. It was later discovered that the CDC28 and cdc2 were functionally interchangeable (Beach et al, 1982), an observation which was later explained on the basis of considerable sequence homology and that both genes encode a protein kinase of approximately 34kD, termed p34 (Hindley & Phear, 1984; Lorincz & Reed, 1984).

The effects of p34(cdc2) are cell cycle specific but its levels do not dramatically fluctuate during the cycle, and modulation of activity occurs via interactions with cyclins (see next section, I 1.1.2). That a p34(cdc2) homologue plays a role in the G1/S transition in higher eukaryotes comes from the following lines of evidence reviewed by Huberman (1991). A *Xenopus* homologue of p34(cdc2) that is essential for DNA replication in *Xenopus* egg extracts has been identified (Blow and Nurse, 1990). Also, it has been found that a cDNA human homologue of the yeast p34(cdc2) could replace the *CDC28* gene in *S. cerivisiae* without detriment to the G1/S transition (reviewed by Reed, 1991). Additionally, an antisense oligonucleotide for the human homologue was found to block human T lymphocytes at G1/S (Furukawa et al, 1990). A family of p34(cdc2) related genes has also been identified in many animal cells (Pines and Hunter, 1991) and is a key regulatory molecule of the eukaryotic cell cycle, and coordinates cell growth, DNA replication and entry into mitosis.

I 1.1.2 Cyclin

The modulation of p34(cdc2) activity occurs via the association of regulatory subunits, namely the cyclins (for a review see Hunter and Pines, 1991). The cyclins appear and are degraded during the cell cycle in an orderly fashion. The cell cycle specific activity of p34 is achieved by the phosphorylation and dephosphorylation of specific sites (reviewed by Draetta, 1990).

Transition from phase G1 to S in *S. cerivisiae* is regulated by three cyclins (refer to Reed, 1991; Norbury and Nurse, 1991). However, cell cycle control is primarily achieved by the p34 modification state, rather than the types of cyclin present (Broek et al, 1991). The G1 to S transition is achieved in *S. cerivisiae* by these G1 cyclins in a G1 cyclin-p34(cdc2) complex. Subsequently the gene products of CDC4, CDC7, (reviewed by Newlon, 1988) and CDC46 (Hennessy et al, 1990) have to act for DNA replication to occur. Of these CDC7 has also been shown to be a protein kinase (Hollingsworth and Sclafani, 1990).

It is thought that p34(cdc2) activation during cell division occurs as follows. During G1, the activation, by dephosphorylation, of p34(cdc2), in response to changes in the external environment and to cell growth, results in DNA replication being initiated (Murray and Kirschner, 1989). The activation process involves the interaction of cyclins with p34(cdc2) to form a complex with kinase activity.

In animal cells there is a unique cyclin, cyclin A, which has been identified so far, and this has been implicated as having a role in mitosis, by the genetic analysis of *Drosophila melanogaster* (Lehner and O'Farrell, 1989). In human cells the kinase activity associated with cyclin A appears to be at a maximum in S and G2 phases (Pines and Hunter, 1990). During S phase and G2 a complex of cyclin A and p34(cdc2) is activated (which might monitor DNA replication and down-regulate the cyclin B - p34(cdc2) complex until the end of G2).

In budding yeast the predominant cyclin at the G2/M transition is termed cyclin B, and is rapidly proteolysed at the onset of anaphase (Evans et al, 1983; Ghiara et al, 1991, Surana et al, 1991). At the end of G2 the cyclin B - p34(cdc2) complex is activated, by dephosphorylation, and initiates events such as chromosome condensation, nuclear membrane breakdown and the reorganisation of the microtubules.

I 1.1.3 Maturation Promoting Factor

In 1971 Masui and Smith both found meiotically dividing frog oocytes to contain a substance capable of inducing meiosis in immature oocytes. In *Xenopus* oocytes this maturation promoting factor (MPF) has been found to be a protein kinase with a principal subunit of p34(cdc2), and which has extensive homology to cyclin. MPF activity in the nucleus fluctuated from a maximum in mitosis and reached a minimum in interphase (Gerhart et al, 1984).

The mitotic transition from metaphase to anaphase is induced by the degradation of cyclin, which in turn leads to the inactivation of MPF. Thus, the cell exits mitosis and interphase ensues, when cyclin degradation ceases leading to increasing levels, post-translational modifications of MPF, and the eventual initiation of another round of mitosis. For a review refer to Murray (1988), and more recently Meikrantz and Schlegel (1992).

I 1.1.4 Clocks Versus Dominoes

The two approaches to the study of the cell cycle led to two theories. The experiments on marine and amphibian eggs resulted in the cell cycle being viewed as a biochemical oscillator, in which the events were independent of each other, and, thus, was termed the "clock" theory.

The yeast experiments resulted in the cell cycle being treated as a set of inter-dependent reactions, and, therefore was called the "domino" theory. The advances in the research in this field has lead to the unification of these two theories (Murray and Kirschner, 1989).

Therefore, the molecular events occurring during entry and exit of mitosis is now beginning to be understood. For a recent review of cyclin, p34(cdc2) and the holoenzyme complex cyclin-dependent protein kinase refer to Meikrantz and Schlegel (1992).

I 1.2 THE ORGANISATION OF THE NUCLEUS

The nucleus is a highly complex and regulated cellular structure. Research into its organisation has been extensive (for a review refer to Newport and Forbes, 1987; Nigg, 1988), however, only those aspects relevant to this project will be described here.

With the development of the electron microscope in the early 1950s the exploration of cellular substructures became more accessable. In particular the complexity of the eukaryotic nucleus became apparent. A structure within the nucleus, which is pertinent to this project (some of the antibodies used reacted with this) is the nucleolus.

I 1.2.1 The Nucleolus

The activity of the genes encoding ribosomal RNA (rRNA) results in the formation of a distinct nuclear substructure, namely the nucleolus. The nucleoli are the sites of ribosome biogenesis, where rRNA precursor molecules are synthesised, processed and associate with ribosomal and non-ribosomal proteins, coordinated in a time- and space-dependent manner (Fischer et al, 1991). For a review of ribosome formation and morphologically distinct nucleolar components refer to Fakan and Puvion (1980). The electron microscope has allowed the differentiation of three nucleolar domains defined by differences in morphology: the fibrillar centre, the dense fibrillar component, and the granular component (for a definition of the nomenclature see Jordan, 1984). The latter constitutes the bulk of the nucleolus in metabolically active cells (Fischer et al, 1991).

Cytologically the nucleolus disappears in late prophase and reforms during telophase (Benavente, 1991). After mitosis the nucleolus reforms by the coalescence of the prenucleolar bodies around the chromosomal nucleolar organising centre (NOR), which contains tandemly repeated rRNA genes (see I 1.2.2). During interphase the nucleolus visibly interacts with one or more chromosomes, of the NOR, where many copies of DNA direct the synthesis of ribosomal RNA (reviewed by Fischer et al, 1991).

A 116kD nucleolar antigen of the dense fibrillar centres of nucleoli has been identified in HeLa cells, using a human anti-nucleolar autoimmune serum (Masson et al, 1990). During mitosis this antigen disperses to the cytoplasm until late telophase, when it localises to the prenucleolar body. Another nucleolar antigen of the dense fibrillar component, of molecular weight 34kD and termed "fibrillarin", has been detected (Ochs et al, 1985). This antigen reacted with autoantibodies against the U3 small nuclear ribonucleoprotein particle (SnRNP) (Reddy et al, 1983; Lischwe et al, 1985) indicating that the dense fibrillar centre is the site of pre-rRNA packaging (see I 1.2.2).

I 1.2.2 Nucleolar Organiser Regions and Small Nuclear Ribonucleoprotein Particles

Amongst the many proteins associated with the nucleolus, there is a subset, which is located specifically to the nucleolar organiser regions (NOR) during mitosis (Fischer et al, 1991). These proteins were identified because of their ability to reduce silver under acid conditions, whilst most other cellular proteins remained unstained (Goodpasture & Bloom, 1975).

The staining of mammalian cells with antinuclear autoimmune sera frequently results in a speckled or granular appearance of the nucleus, due to two major antigenss termed Sm and RNP. Sm antibodies precipitate RNA- protein complexes containing small nuclear ribonucleoprotein particles (snRNPs): U1, U2, U4, U5 and U6, whilst RNP antibodies only precipitate U1 complexes (Lerner & Steitz, 1979). The snRNPs are known to function in intron splicing of mRNA processing (Fischer et al, 1991). Since interactions only occur with certain chromosomes and non-centromeric regions, then the NOR and SnRNPs are of little direct interest for this project, and so will not be dealt with further.

I 1.2.3 Lamins

The nuclear lamins line the nucleoplasmic face of the inner nuclear membrane. They are biochemically and structurally related to the intermediate filament protein family (Franke, 1987) and have been implicated as interphase sites of anchorage for higher order chromatin domains (Gerace, 1986). Whereas, during mitosis the nuclear envelope disassembles and the nuclear lamins become diffusely distributed throughout the cytoplasm.

There are three major lamins, lamin A, B and C of molecular weights in the range of 60-75kD (Gerace et al, 1978). Type A lamins are recovered in the soluble phase, while type B lamins remain associated with the membrane vesicles, little is known about type C (for a review refer to Stick, 1988; Gerace and Burke, 1988; Nigg, 1988).

Condensed chromosomes become enclosed by a tightly associated nuclear envelope which non-selectively excludes all macromolecules not attached to the chromosomes (Benavente, 1991; Bouvier et al, 1985). Microinjection experiments of specific antibodies to nuclear lamins (Benavente and Krohne, 1986) into metaphase cells resulted in the chromosomes being blocked in a condensed state. The authors suggested that the lamins play a role in establishing and maintaining a specific topological organisation of the chromatin in the interphase nucleus. Further such microinjection studies have led to the notion that nuclear membrane reformation, and nuclear import of proteins is required for chromatin decondensation and also nucleolus reformation (Benavente et al, 1989).

I 1.3 THE NUCLEAR MATRIX

There has been controversy over the reality of nuclear matrices as physiological structures (Cooke, 1988). Whether or not they reflect a real *in vivo* state is arguable, however, their preparation does result in a considerable enrichment for a specific subset of proteins remaining in the residual structures. For a review turn to Verheijen et al, (1988) or a more recent study von Kries et al, (1991).

I 1.3.1 Matrix Associated Regions

Note! The **scaffolds** referred to in this thesis, unless otherwise stated, are from **metaphase chromosomes**, and not **nuclear matrices** (sometimes also termed nuclear scaffolds, or nuclear cage), which are prepared from **interphase nuclei**, and the protein composition of these two types of samples are distinct.

The loop model of chromosome structure, in which the chromatin is organised into loops stabilised at their bases by non-histone proteins (Laemmli et al, 1978), outlined in I 3.4 might predict that there are specific regions along the interphase chromosome at which the nuclear matrix (cage or scaffold) attach (Cooke and Brazell, 1980; Mirkovitch et al, 1984). The nuclear matrix (an expression first introduced by Berezney and Coffey, 1974) is itself a residual structure remaining after particular biochemical treatments, such as nuclease and sequential salt extractions or detergent protocols, have been used to remove the majority of the DNA and a substantial proportion of the histones (Capco et al, 1982). In the residual matter there are specific nuclear matrix-DNA interactions, sometimes confusingly termed "scaffold associated regions" (SARs), or more appropriately "matrix associated regions" (MARs) (Gasser and Laemmli, 1986a). These MARs were isolated by a very mild extraction procedure applied to isolated nuclei (low concentration of lithium 3', 5-diidosalicylate in physiological salt buffers) that apparently preserve the specific matrix-DNA interactions (MacGillivray & Birnie, 1986). The extracted nuclei were then digested with nucleases, and the MARs isolated, by co-sedimentation with the nuclear matrix proteins. For a review of MARs see Gasser & Laemmli, 1987.

The MARs have been mapped to DNA fragments of lengths 0.3 to several kb (Gasser & Laemmli, 1986b), and each fragment contains multiple sites of matrix-DNA interactions. It has been demonstrated that many MARs contain AT rich motifs, similar to the DNA topoisomerase II cleavage consensus sequences (Mirkovitch et al, 1984; Gasser & Laemmli, 1986b; Cockerill & Garrad, 1986), and in fact topoisomerase II preferentially binds MAR fragments (Adachi et al, 1989). There are approximately three copies of the consensus sequence per average loop length of the DNA found in both nuclear matrix and chromosome scaffolds (see I 3.4.3). This is consistent with a possible role of topoisomerase II as a "loop fastener" as in the loop model mentioned above. Consistent with this is the finding that MARs are non-coding sequences, and generally non-transcribed regions (Gasser & Laemmli, 1987).

The identification of the MARs is indicative of higher-order organisation in the interphase nucleus and is consistent with specific interactions occurring between the chromatin and the nuclear matrix (or cage; Cooke & Brazell, 1980; Verheijen et al, 1988).

There are some proteins which have been identified in both chromosome scaffolds and nuclear matrices, one of which, topoisomerase II is discussed elsewhere (see I 3.4.3). Nuclear matrix preparations obtained by a modified procedure in order to stabilise the proteins by SH cross-linking (Kaufman & Sharp, 1984), led to the identification of a 62kD non-lamin, non-histone protein. By immunolocalisation the antigen was shown to reside at the periphery of the interphase nuclei of rat liver hepatocytes, and be present along the length of the chromatin. During mitosis the antigen remains associated with the chromatin, and fractionates with a crude chromosome scaffold preparation. This behaviour of the antigen led to the suggestion that it was involved in the maintenance of some aspect of chromosomal structure (Fields & Shaper, 1988). However, further evidence and the elucidation of its exact role has yet to be determined.

Several proteins have been identified as fractionating together with the nuclear matrix. These include c- and v-myc (Blackwood and Eisenman, 1991; Evan & Hancock, 1985), nuc2 in yeast (Hirano et al, 1985), and repressor-activator binding protein in yeast (Shore & Nasmyth, 1987), however, research in this area is still in its early stages as there is much more yet to be deduced.

CONTENTS

CHAPTER I 2 THE CYTOSKELETON

The emphasis of this thesis concerns the molecular components of the mammalian centromere, therefore, only those elements of the cytoskeleton relevant to this will be treated here. Hence, the spindle apparatus, division machinery and their various components will be reviewed in this chapter.

I 2.1 THE SPINDLE POLE

Visualised by light microscopy the interphase cell has no obvious polarity, whereas, in mitosis, there is an evident polarity, with the microtubules focusing at the polar regions. The centrosome consists of the centrioles and surrounding pericentriolar material.

I 2.1.1 The Centrosome

The major microtubule-organising centre (MTOC) in almost all animal cells is the centrosome. In 1901, Boveri defined the centrosome as "an autonomous permanent organ of the cell... the true division centre of the cell (whose) division creates the centres of the daughter cells. It coordinates nuclear and cytoplasmic division." It is now known that the centrosome contains a pair of centrioles orientated at right angles to each other, one of which migrates to the opposite side of the nucleus before mitosis. The molecular composition of this structure is, however, poorly characterised, although, it is thought possible that gamma-tubulin is associated with, or a component of the centrosome (Zheng et al, 1991). In animal cells it consists of the centrioles and an associated region of pericentriolar material. In interphase the animal centrosome acts as the primary microtubule-organising centre (MTOC), with the ends of the microtubules embedded there (refer to I 2.2).

The dependence of a cell on the presence of a centrosome for division is a subject of controversy, since some mammalian cells lack detectable centrosomes at critical stages of the cycle (Hiraoka et al, 1989; Maro et al, 1988). Moreover, in the mitotic cells of higher plants the microtubules terminate in a poorly defined region completely lacking centrioles (Karsenti and Maro, 1986). Also centrioles are absent from the meiotic spindles of mouse oocytes, but later appear in the developing embryo (Karsenti and Maro, 1986). Thus, it appears that the microtubules are nucleated not by the centrioles but by the amorphous, poorly defined, pericentriolar material. Despite the inconsistencies in morphological data, there is a MTOC, although not necessarily centrioles, in all cells that forms the spindle poles in mitosis, and is involved in microtubule organisation in interphase.

The study of MTOCs has advanced considerably with the advent of isolation procedures, which maintain microtubule nucleating activity (Verde et al, 1990). However, the most successful approach has been in using human autoantisera, and the identification of homologues in mammals, plants and yeast (e.g. Hiraoka et al, 1989). Two genes encoding

such antigens have been isolated from budding yeast and are termed spindle pole antigens 1 and 2 (SPA1 and SPA2, respectively), see Winey & Byers, (1993). However, despite these advances, the molecular nature of the centrosomes is still relatively unclear.

The regulation of microtubule activity at the centrosome has been correlated with centrosomal phosphorylation, which reaches a maximum just prior to anaphase, then decreases rapidly at the onset of anaphase, and cdc2 kinase activity is associated with centrosomes in mitosis (Bailly et al, 1989; Riabowol et al, 1989). However, the exact control of function at the centrosome is still to be elucidated.

I 2.1.2 The Centriole

The centriole is a small cylindrical organelle (approximately 0.2 um x 0.4 um) consisting of nine groups of three microtubules forming the wall of the centriole (refer to Lefebvre and Rosenbaum, 1986). Most animal cells have a pair of centrioles that act as the focal point for the centrosome. The centrosome organises the cytoplasmic microtubules in interphase and duplicates in mitosis to nucleate the two poles of the mitotic spindle. The centriole duplicates at around the time of DNA synthesis, with the paired centrioles separating and then each forms a daughter centriole, which is perpendicular to the parental centriole. The immature centriole consists of a ninefold array of single microtubules, which then may act as the template for the assembly of the triplet microtubules of the mature structure (Karsenti and Maro, 1986; Ramanis and Luck, 1986; Vorobjev and Chenstov. 1982).

There has been speculation that the centriole may be a fully autonomous self-replicating organelle. It is possible that some information necessary for centriole duplication is carried in the centriole as for mitochondria and chloroplasts. In support of this is the study carried out by Hall and coworkers in 1989, who reported that they had isolated a 6-9 megabase linear DNA molecule, which was localised to the basal bodies of *Chlamydomonas reinhardtii* . This DNA was shown to encode proteins involved in basal body structure and flagellar assembly (both containing centrioles) which segregate independently of the chromosomal DNA. However, such centriolar DNA has not been isolated from any other organism, thus, it cannot be excluded that this DNA element is a species specific anomaly and not a general property.

I 2.2 SPINDLE MICROTUBULES AND CHROMOSOME MOVEMENT

Microtubules are very labile structures, and in fact depend on this lability for their function. In particular this is true for the microtubules of the mitotic spindle, which is formed at the onset of mitosis after the disassembly of the cytoplasmic microtubules. The spindle microtubules themselves are in a state of rapid assembly and disassembly, thus, making them particularly sensitive to tubulin binding drugs, for example colchicine.

Each molecule of this alkaloid binds one molecule of tubulin and, thus, prevents its polymerisation. Therefore, a dividing cell exposed to colchicine causes the disappearance of the mitotic spindle and the cell being blocked in mitosis (Inoue, 1981; Salmon et al, 1984).

Microtubules have an inherent polarity due to the tubulin subunit arrangement. *In vitro* reconstitution experiments have shown that one end elongates three times as fast as the other, the plus and the minus ends, respectively (McIntosh and Euteneuer, 1984; Farrel et al, 1987). The orientation of a microtubule can be determined by the so-called "tubulin hook decoration" procedure. Depending on the polarity, the tubulin hooks on the microtubule outer surface will either orientate in a clockwise or anticlockwise direction, when the microtubule is observed in cross-section (Euteneuer, 1984). Such a hook decoration procedure revealed that the microtubules of the microtubule-organising centre (MTOC) have their plus ends facing outwards (Karsenti and Maro, 1986). Thus, the kinetochore and interdigitating microtubules (see later, this section) have their minus ends in the centrosomal region, and their plus ends attached to the kinetochores or lying on the midzone, respectively.

For a general review of microtubules see McIntosh & McDonald (1989). Depending on the cytological localisation, the spindle microtubules can be divided into aster, kinetochore, and interdigitating microtubules, and these will be treated below.

The alignment of the chromosomes on the metaphase plate is dependent upon two major events. Firstly, the kinetochores have to make bipolar attachments, whereby sister kinetochores are attached by microtubules, termed kinetochore microtubules, to the opposite poles (Ault et al, 1991). This process begins in late prophase when a mature kinetochore assembles on each centromere. After the nuclear membrane has broken down the microtubules can access the chromosomes, and by metaphase, microtubules attach to the kinetochores, the number depending on the species, e.g. in humans there are 20-40 (Rieder 1982), whereas, in yeast there is only one per kinetochore (Peterson and Ris, 1976). Only the plus ends of the microtubules extend from the poles, and it is these ends of the kinetochore microtubules which attach to the kinetochores. The kinetochore is thus said to "cap" the microtubule, and thereby protects the plus end from depolymerisation, as does the centrosome the minus end (Mitchison and Kirschner, 1985). Therefore, the kinetochore-capped kinetochore microtubules are unusually stable.

Once bipolar attachment has occurred, an oscillation like movement commences between the two poles, with the chromosome moving first towards and then away from a given pole (Ault et al, 1991). Eventually an equilibrium is reached, when all the chromosomes are relatively motionless and aligned on the metaphase plate (Nicklas 1988).

In addition to the microtubules which attach to the kinetochores, there are two other types of microtubules. The astral microtubules originate from the poles, or asters, as do the other two types, and apparently are not capped. These do not interact with kinetochores nor closely with other microtubules. Some of the microtubules do not interact with kinetochores, and overlap the microtubules originating from the opposite pole at the central region of the spindle. These are termed the interdigitating microtubules.

Subsequent to metaphase there are two types of movement directly involving the chromosomes, namely that observed in anaphase A and anaphase B. Movement towards the poles is mediated by the kinetochores and the kinetochore microtubules, whilst movement of the spindle and chromosomes towards opposite poles is probably facilitated by the astral and interdigitating microtubules (Rieder et al, 1986; Salmon, 1989), termed anaphase A and B, respectively.

The regulation of the transition from metaphase to anaphase is relatively poorly understood. Anaphase begins with an abrupt, but synchronous, splitting of each chromosome into its sister chromatids. The signal to initiate anaphase is not a force exerted by the spindle, since chromosomes detached from the spindle will separate into chromatids at the same time (Murray and Szostak, 1985). There is some evidence that cytosolic calcium ions are involved in the "anaphase" trigger (Hepler and Callahan, 1987). The use of a fluorescent Ca^{2+} indicator dye has shown that there is a tenfold increase in the intracellular Ca^{2+} level at anaphase in some cells. Also the microinjection of low levels of Ca^{2+} into cultured cells at metaphase has been shown to be able to cause premature anaphase. Furthermore, membrane vesicles, which are rich in Ca^{2+}, have been demonstrated to accumulate at the spindle poles (Hepler and Callahan, 1987). Thus, Ca^{2+} appears to be a potentially candidate molecule in regulating the onset of anaphase, though further work is required to clarify this important regulatory phase of mitosis.

I 2.3 MOTOR PROTEINS

From 1883 (Van Beneden) until relatively recently, it was thought that chromosomes were pulled towards the spindle poles by the shortening of the microtubules, by disassembly, at the pole end. However, ingenious experiments using photobleaching of microtubules has demonstrated that microtubule disassembly occurs at the kinetochore, at the plus end, and not at the polar end (Gorbsky et al, 1988; Nichlas, 1989).

In addition to the poleward directed movement there is also a force which tends to push a given chromosome away from the nearest pole, this is sometimes referred to as "polar wind" (Rieder et al, 1986).

As is the case for kinetochore and interdigitating microtubules, the astral microtubules interact with chromosomes at their plus ends, and could possibly drive the polar ejection movement of the prometaphase chromosomes (i.e. chromosome alignment on the metaphase plate).

Elegant experiments have been carried out determining the deflection of fine glass needles in order to estimate the force exerted in the movement from the metaphase plate to the pole, and this was found to be 10^{-5} dynes per chromosome (Nichlas, 1983). In spite of this relatively large force, chromosome movement is not as fast as might be expected, indicating that some factor might in fact limit the progress of the chromosomes.

The magnitude of the force exerted on a chromosome, suggests that a biochemical motor is involved in this process. There are two models for motor function. In one model, there is a postulated ATP driven microtubule "walking" protein which disassembles the microtubule. In the other model movement is driven by microtubule disassembly (see Alberts et al, 1989).

I 2.4 MICROTUBULE-DEPENDENT ATPASES

Three microtubule dependent ATPases closely associated with microtubules have been identified in a variety of cells, and species. These are dynein, kinesin and dynamin (reviewed by Vallee and Shpetner, 1990).

I 2.4.1 Dynein

The first microtubule-associated, force producing molecule to be identified, was dynein (Gibbons, 1963), a complex of several polypeptides, of which the molecular weight of the heavy chain has been estimated to be 410kD (Paschal et al, 1987). It is thought that there are four polypeptides in the range of 50-60kD, and three molecules of molecular weight 74kD; and assuming two heavy chains, gives a total molecular weight in the region of 1300kD (Vallee & Shpetner, 1990). Amongst other species, dynein has also been purified from human (HeLa) cells (Pfarr & McIntosh, 1988). It is thought that cytoplasmic dynein plays a role in retrograde organelle transport (Vallee et al, 1989). However, its potential role in chromosome movement is even less clear, although, dynein might be involved in the movement of the chromosomes toward the poles (Vallee & Shpetner, 1990). Relatively recently, cytoplasmic dynein has been localised at the primary constriction (Pfarr et al,1990; Steuer et al, 1990) and in the kinetochore plate Wordeman et al, 1991, and refer to I 3.2.2).

Dynein has also been implicated as a pole directed motor (Vallee, 1990) at the kinetochore. Dynein has been localised to the kinetochore of CHO cells, chicken embryonic fibroblasts, human HeLa cells and kangaroo rat PtK1 cells. Thus, in these experiments anti-dynein staining was shown to decorate both tubulin and the centromeres on isolated metaphase chromosomes (Pfarr et al, 1990; Steuer et al, 1990).

I 2.4.2 Kinesin

Kinesin has been shown to have microtubule-dependent motor activity (Vale et al, 1985). Kinesin consists of two heavy (124kD) and two light (64kD) chains. Studies concerned with the cellular localisation of this protein have yielded conflicting results. In sea urchin eggs, anti-kinesin antibodies have given a staining pattern seemingly the same as that obtained with anti-tubulin antibodies (Scholey et al, 1985). In contrast, after tubulin staining was abolished, the kinesin staining persisted (Leslie et al, 1987), suggesting a non-microtubular binding region of the spindle. Unfortunately, the results were not unequivocal, and the localisation in mammalian cells is even less clear.

I 2.4.3 Dynamin

Dynamin has been identified in calf brain cytosol (Schpetner & Vallee, 1989), and has a molecular weight of 100kD. Its role is relatively poorly characterised, although it has been postulated that dynamin has a role in anaphase B of mitosis, where it may be involved in facilitating sliding of the microtubules of the opposite poles, i.e. interdigitating microtubules (Masuda et al, 1988).

I 2.5 MICROTUBULE ASSOCIATED PROTEINS

Over the years an ever increasing number of proteins have been identified, which interact with the microtubules to realise their function. The best characterised of these microtubule associated proteins (MAPs) are: the tau proteins, 190kD MAP, MAPs 1 and 2, kinesin, cytoplasmic dynein, buttonin and syncolin. For a recent review turn to Wiche et al, 1991. Those MAPs, which are not microtubule-dependent ATP-ases (treated in the previous section) will now be detailed.

I 2.5.1 Tau Proteins

The first such MAP was isolated by copurification with tubulin (Wiche et al, 1991), and displays considerable size heterogeneity (Cleveland et al, 1977) due to different extents of protein phosphorylation (Flament and Delacourte, 1989; Himmler, 1989), with molecular weights of 55-65kD. The tau proteins have a rod-like structure with arm-like projections (Hirokawa et al, 1984), and it is thought that they cross-link microtubules. A repeat region of 31 or 32 amino acids at the C-terminus was shown to comprise the microtubule binding domain, in both the tau protein and MAP 2 (I 2.5.3) (Wiche et al, 1991).

Using synthetic peptides it was demonstrated that the acidic C-terminals of alpha and beta tubulins specifically bound tau and MAP2 (Littauer et al, 1986; Maccioni et al, 1988). Tau proteins have been shown to bind actin in a calmodulin inhibited manner (Kotani et al, 1985). However, in spite of the relatively good characterisation, the tau proteins are found almost exclusively in axons (Binder et al, 1985) of brains, and so are of limited interest for general mitotic processes.

I 2.5.2 190kD MAP

Unlike the tau proteins, the heterogeneous MAPs in the range of 200kD are found predominantly in non-neuronal cells (Wiche et al, 1991). The best characterised of these is the bovine adrenal 190kD protein, and this protein has been shown to promote microtubule assembly (Kotani et al, 1986). It has been suggested that this class of MAPs is ubiquitous in mammalian tissues, and the differences due to species-specific homologues (Kotani et al, 1988).

I 2.5.3 MAP 2

Together tau and MAP 2 are the most extensively characterised MAPs. Based on electrophoretic mobility, MAP 2 appears to have a molecular weight of 300kD, however, on the basis of its nucleotide sequence this is only 199kD (Lewis et al, 1988). Electrophoresis at high resolution has shown MAP 2 to consist of two bands, termed MAP 2A and 2B (Kim et al, 1979). These components and a third, smaller isoform, MAP 2C, of molecular weight 70kD are derived by differential splicing of the transcripts from the same gene (Garner and Matus, 1988). It is thought that MAP 2 constitutes a component of the cross-bridges of microtubules (Hirokawa et al, 1988). In addition to tubulin binding, MAP 2 has been shown to bind calmodulin (Lee and Wolf, 1984) and actin filaments (Sattilaro, 1986).

I 2.5.4 MAP 1

This MAP has a molecular weight of 350kD (Sloboda et al, 1975), making it the largest of the MAPs. After its initial discovery, this protein was resolved by SDS-PAGE, and shown to resolve into two (termed MAP 1A and B) or even four bands, depending on the gel system and species of origin of the protein to be examined. MAP 1A has a slightly higher molecular weight than MAP 1B, which is one of the most abundant MAPs of brain tissue, but is also found in non-neuronal cells (Wiche et al, 1991). MAP 1B has been sequenced in its entirety in mouse and the microtubule-binding domain has been shown to be a highly basic region consisting of multiple copies of the motif Lys-Lys-Glu-X, where X is almost always any acid or hydrophobic residue (Noble et al, 1989). This binding domain is completely distinct from the highly similar tau and MAP 2 microtubule-binding domains. All three binding domains do, however, have a net positive charge and consist of repeated elements (Wiche et al, 1991).

The MAPs referred to in this section are all fibrous proteins. The only clearly established function of such MAPs is that they stabilise microtubules. Another function is thought to be the promotion of microtubule assembly. There are two non-fibrous, MAPs, both detected in non-neuronal cell types and are introduced below.

I 2.5.5 Buttonin

Buttonin was first observed on sea urchin egg microtubules (Hirokawa et al, 1985), received its name because of its shape and was shown to have a molecular weight of 75kD (Hirokawa & Hisanaga, 1987). Buttonin is apparently arranged in close relationship to the tubulin lattice of mitotic spindles, such that one "button" partially covers three tubulin dimers (Hirokawa et al, 1985). The physiological function of buttonin is still unknown, though it may function in spindle formation and stabilisation (Wiche et al, 1991).

I 2.5.6 Syncolin

This MAP was first detected in chicken erythrocytes and was predominantly located to the poles of mitotic spindles (Feick personal communication). Syncolin has the same mobility in SDS-PAGE as MAP2, but has different properties, e.g. it does not promote microtubule assembly (Wiche et al, 1991). It is thought that this MAP may cross-link microtubules (Wiche et al, 1991).

I 2.5.7 Dynasomes

Kinesin, cytoplasmic dynein, and "dynasomes" (Weisenberg et al, 1987) are microtubule associated proteins which are capable of generating motile force. Dynein and kinesin are dealt with elsewhere (I 2.4.1 & 2, respectively). It has been suggested that dynasomes, which appear to contain dynamin, are involved in microtubule gelation-contraction, and transport in neural cells, and have been discussed earlier (I 2.4.3).

I 2.6 MIDBODY

The midbody is formed from bundles of spindle microtubules, containing alpha and beta tubulins, which span across the interzone, the central region of the spindle where interdigitating microtubules (see earlier I 2.2) overlap, at the end of anaphase (Mullins and McIntosh, 1982). Thus, these microtubules originate from opposing spindle poles and overlap in this interzone region (McIntosh et al, 1979; McIntosh et al, 1975), but do not capture kinetochores (see I 2.2). A dense matrix substance coats the microtubules in this region of overlap (Mullins and McIntosh, 1982). The formation of the cleavage furrow concentrates the microtubules into a compact region, which forms the midbody (Krishan & Buck, 1965). This midbody region may act to keep the cytoplasm, of the regions partitioned by the furrow, separate (Mullins & Biesele, 1977). Alternatively the midbody may in fact maintain the cytoplasm continuous between the two cells until actual physical division (Sanger et al, 1985; Schulze and Blose, 1984).

The midbody matrix itself is a spindle associated substance that appears to bind the microtubules together at the region of overlap. A 115kD doublet has been shown to be retained on isolated midbodies from CHO (Mullins & McIntosh, 1982). Proteins of molecular weights 95kD and 105kD were also detected in CHO cell, by a monoclonal antibody raised against isolated spindles (Sellito & Kuriyama, 1988). These antibodies are located preferentially to the equatorial region of the spindle at metaphase, and as the cell cycle progresses, become organised into short lines along the spindle axis corresponding to the midbody matrix, and finally are condensed into a dot at the midzone. The antigens also cross-reacted with the interphase centrosomes, and with the nuclei in a speckled pattern in a cell cycle-dependent manner (Sellito & Kuriyama, 1988).

An antigen has been identified by a monoclonal antibody, which was produced by the immunisation of bovine thymocytes treated with DNase I and RNase A, and 0.8M NaCl (Pankov et al, 1990). The antigen was located to the outer corona region of the kinetochore at metaphase, but at anaphase was found on the plus ends of the polar microtubules in the midbody region (see I 3 for a more in depth treatment of a recently identified class of kinetochore and cytoplasmic antigens).

I 2.7 THE TELOPHASE DISC

The use of an autoimmune serum has recently led to the identification of a structure in human cells, termed by the authors as the "telophase disc" (Andreassen et al, 1991). The antigen was detected at the centromeres in prophase, then at the spindle equator in mid-anaphase and telophase. As the cell progresses from mid-anaphase the structure formed a disc bisecting the two cell hemispheres prior to telophase. During telophase the disc became constricted at the position of the cleavage furrow.

This disc appeared to be distinct from the spindle microtubules, and it was shown to contain myosin but not actin. The autoimmune serum detected an antigen of molecular weight 60kD. The disc structure has been proposed to function in cytokinesis, possibly containing mechanical elements for cell cleavage. However, the exact function and nature of this structure has still to be elucidated, and also its conservation to other species.

I 2.8 CLEAVAGE FURROW

The first visible sign of cleavage in animal cells is the "furrowing" of the plasma membrane during anaphase, which occurs in the plane of the metaphase plate, at right angles to the long axis of the mitotic spindle. Microinjection experiments in which the spindle is relocated demonstrated that the spindle *per se* determines the position of the furrow (Rappaport, 1986). Cleavage itself is accomplished by the contraction of a ring composed mainly of actin filaments (Schroeder, 1972), a structure termed the contractile ring. This ring is attached to the plasma membrane by as yet uncharacterised proteins.

During normal division the ring does not thicken, implying that its volume is reduced as the furrow invaginates. When cleavage ends the ring disappears and the cleavage furrow narrows to form a structure also termed as the "midbody", which contains the remnants of both sets of polar microtubules together with the dense matrix material (see earlier, I 2.6).

Actin and myosin II have been implicated in the cleavage furrow formation process (Cao & Wang, 1990; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Mabuchi and Okuno, 1977; Schroeder, 1973). Actin filaments have been shown to be present in parallel bundles in the contractile ring (Cao & Wang, 1990; Perry et al, 1971). Whilst myosin II molecules are in the form of minifilaments in this region (Fujiwara & Pollard, 1976). Immunocytochemical approaches have demonstrated the presence of microtubule associated proteins (Bulinski and Borisy, 1988; Connoly et al 1977; Connoly et al 1978; Izant et al, 1982; Sherline and Shiavone, 1978), myosin (Aubin et al, 1979; Fujiwara and Pollard, 1976), actin (Aubin et al, 1979; Fujiwara and Pollard, 1976), actin-binding proteins (Aubin et al, 1979; Fujiwara et al, 1978; Nunnally et al, 1980) and calmodulin (Welsh et al, 1979). However, further experimentation is required in this area to determine the exact molecular processes occurring.

CONTENTS

CHAPTER 3 CHROMOSOME STRUCTURE

In 1903 both Sutton and Boveri independently proposed the "Chomosome Theory of Inheritance". According to this theory, the chromosomes ("coloured bodies", originally observed in the 1870s) carry the heredity factors, and the behaviour of these at cell division, provides the molecular explanation for Mendelian inheritance. Since then the study of chromosome structure and behaviour has been of fundamental interest to scientists.

I 3.1 CHROMATIN STRUCTURE

Several biochemical techniques have been exploited to investigate the properties of chromatin. An examination of mitotic chromosomes by microscopy after banding (Harrison et al, 1981; Holmquist, 1988), for instance, reveals that each chromosome has an individual pattern, reproducible between different samples of the same species. This suggests that the chromatin is packaged, not randomly, but in a precise manner into mitotic chromosomes. The organisation of chromatin has been approached at the DNA, or fine-level, and also at a higher-level structure, which culminates in the mitotic chromosome (Bostock and Sumner, 1978).

I 3.1.1 Fine Structure

The amount of DNA which is packaged into a nucleus of diameter of about 5um, in a human haploid cell, is approximately 6pg. It has been estimated that this is equivalent to a length of 1.74m if it were unravelled into a continuous linear molecule. However, in its most condensed form, the metaphase chromosome is packaged into a length of only about 150um, a compaction of about 8,000 to 10,000- fold (Paulson, 1988). The packaging of such an extraordinary length of DNA into cells has been of considerable interest for many years.

The currently accepted theory of the organisation of the eukaryotic chromosome involves several orders of coiling (Laemmli et al, 1978; Rattner and Lin, 1985). The DNA helix is initially coiled around the histone octamer, forming the nucleosome core particle (Kornberg and Klug, 1981; Richmond et al, 1984). The presence of histone H1 and linker DNA then results in the formation of the 10nm nucleosome fibre ("beads-on-a-string"). Further coiling results in the formation of the 25-30nm chromatin fibre, giving a compaction of about 40-fold (Belmont et al, 1987; Pederson et al, 1986). At this higher-level of packaging the chromatin organisation is rather unclear. A model for this level of structure, is the "solenoid" (Finch & Klug, 1976). This model proposes that the 10nm fibre is folded so that there are 6 nucleosomes per turn of the coil, with H1 molecules associating down the centre of the solenoid (Thoma & Koller, 1977). However, this and the subsequent levels of packaging are relatively poorly understood.

I 3.1.2 Higher-Order Structure

Of the many models for the higher-order organisation (further condensation of the 25-30nm fibre) of chromatin, the loop model (Laemmli et al, 1978) has received the most experimental support. In this model the chromatin is organised into loops of 5-100kb. These loops are stabilised at their bases by non-histone proteins. In the condensed chromosome the loops are thought to be held together by protein-protein or protein-DNA interactions. This level of organisation may form an internal "scaffold" (see I 3.4) along the chromosome axis, with the scaffold proteins organising the bases of the loops.

I 3.1.3 Mitotic Versus Meiotic Chromosome Structure

Since the general function of the chromosome is to transmit hereditary information from the parent to daughter cells, then the general structure required of the chromosome might reflect this, irrespective of the type of cell division. The differences between the two types of division is due to the generation of genetic variation occurring in meiosis, and the consequent structural changes required to facilitate this. Specifically in meiosis I the homologous chromosomes associate, the synaptonemal complex forms and recombination events can occur. These are all processes, which under normal circumstances, are peculiar to meiosis (reviewed by Chandley, 1988) and presumably, therefore, mediated by factors peculiar to meiosis. It is reasonable to expect that the molecular structure of the meiotic chromosome has features quite distinct from the mitotic chromosome. However, the fundamental chromosome higher-order structure is most probably very similar if not essentially the same for both cases.

Unfortunately, it has been difficult to isolate and purify meiotic chromosomes for molecular analyses (Walmsey and Moses, 1984; Ierardi et al, 1983). These difficulties were due, for instance, to the property of meiotic chromosomes to attach via the ends of the sister chromatids to the nuclear envelope. Therefore, initial progress in studying chromosome structure was primarily facilitated by studies of somatic metaphase chromosomes (Moens, 1990).

For a review of the molecular architecture of meiotic chromosomes, e.g. the presence and role of topoisomerase II and other proteins, refer to Moens, 1990. The literature presented in this chapter are for mitotic chromosomes, and also, unless otherwise stated, pertain to the animal structure.

I 3.2 MITOTIC METAPHASE CHROMOSOME STRUCTURE

The bulk of the experimental data presented in this thesis is based on Chinese hamster chromosomes, since these are relatively robust and withstand isolation procedures, and are a good model system for mammalian chromosome structure. It is their molecular nature which

is of interest for this thesis, however, if the reader is interested in their cytological properties, there is an excellent review by Stubblefield and Wray, 1971.

Chromosomes reach their most compacted state by metaphase and in order to examine chromosome packaging it has been this stage which has been most intensively studied. Additionally, in this compacted state the chromosomes are more resistant to damage occurring during experimentation. Moreover, to examine the molecular components of mammalian chromosomes, mass isolation techniques have been developed (e.g. Gooderham and Jeppesen, 1983; Hadlaczky et al, 1982; Stubblefield and Wray, 1970). In order to obtain chromosomes most closely resembling their *in vivo* state, the relatively robust metaphase chromosomes are generally the material of choice.

The metaphase chromosome viewed with the light microscope can be seen to consist of the major regions: the sister chromatid arms, the kinetochore, and the telomeres. Our present knowledge of the molecular organisation of these is presented below.

I 3.2.1 Sister Chromatid Arms

The metaphase chromosome consists of two strands of DNA, along its length, with associated proteins, highly coiled, and each forming a sister chromatid. The sister chromatids are connected at the primary constriction (see Rieder, 1982; Rattner, 1991), the region onto which kinetochore molecular components build and can interact with microtubule proteins. The molecular nature of the organisation within the chromatid arms at the higher-order level is only gradually being determined. Various treatments, resulting in chromosome banding (reviewed by Holmquist, 1988), indirectly imply the presence of order at the higher organisational level, since it is thought that the banding appearance is brought about by the removal of certain proteins (Holmquist, 1988). However, the nature and identity of the proteins, which bring about and maintain condensation of the sister chromatids, i.e. higher-level organisation, are relatively unknown.

A major component of the chromosome scaffold (see I 3.4) is DNA topoisomerase II. By using antisera against this protein, on gently expanded chromosomes, it has been shown that topoisomerase II is located along the entire central axis of each chromatid, extending through the kinetochore (Earnshaw et al, 1985; Gasser et al, 1986). The enzymatic activity of DNA topoisomerase II has been extensively investigated (Wang, 1987; Drake et al, 1989). However, its exact role in the metaphase chromosome is unknown (refer to I 1.4.4.3 for further details of topoisomerase II as a scaffold protein), although its abundance may suggest that it has a structural role in addition to its enzymatic functions.

I 3.2.2 The Kinetochore

It is important to clarify at this point the difference in two terms occasionally inappropriately used. The centromere is the term generally used to denote the primary constriction of chromosomes and the proteins associating with the DNA located there. The kinetochore on the other hand is, a more peripheral region, and is the site of microtubule attachment. Some authors use these two terms synonymously, nevertheless, in this thesis the aforementioned definitions shall be adhered to. The molecular nature of the centromere will be treated in a later section (I 3.3), here the kinetochore will be reviewed.

The segregation of chromosomes during cell division is visibly a highly coordinated activity. For a study of the prometaphase chromosome configuration and order in early mitosis see Chaly and Brown (1988). The kinetochore is crucial to this process, since it attaches to the spindle microtubules, which align the chromosomes on the metaphase plate and then pull these towards their respective poles. Extensive cytological studies of the kinetochore have been carried out (reviewed by Rieder, 1982). This region is also termed the trilaminar kinetochore, and consists of inner, middle and outer layers. Using electron microscopy the kinetochore appears physically as two parallel plates on either side of the primary constriction region of the chromosome (Rieder, 1982).

In addition to its role in microtubule attachment, the kinetochore may also contain the mechanochemical motor, which powers chromosome movement to the poles in anaphase (Pluta et al, 1990). Disassembly of the microtubules occurs at the kinetochore during anaphase, and it is this which requires force generation (Mitchison & Sawin, 1990). It has been suggested that this "motor" may be located in the fibrous corona next to the outer kinetochore plate (Rieder et al, 1989). In support of this is the recent finding of cytoplasmic dynein and tubulin being localised to the kinetochore and interacting with other proteins therein (Wordeman et al, 1991). For a review of the mammalian kinetochore structure refer to Rieder, 1982; Ris and Witt, 1981; Valvidia and Brinkley, 1985.

I 3.2.3 The Telomere

Telomeres are the regions at the ends of eukaryotic chromosomes, which are essential for both stability and complete replication of the chromosomal termini at the end of each cell cycle. Chromosomes with internal breakages are "sticky" i.e. the internal exposed surface attaches with great affinity to other chromosomal regions. Thus, this indicates that some components or conformation peculiar to the normal chromosome ends usually "seal" and stabilise these regions.

An interesting question is how the telomeric DNA is replicated without loss of the terminal bases at the 5′ end of each strand. This problem is predicted by the fact that conventional semiconservative replication occurs in the 5′to 3′direction, and that cellular DNA polymerase requires an RNA primer.

The molecular structure of this region, at the ends of the higher eukaryotic chromosomes, is gradually being better understood. For a recent study of telomere cloning and analyses of mammalian chromosomes, refer to Brown et al (1990). At the telomere there are short tandem repeats, one strand being G-rich, the other C-rich. There has been a suggestion that non-Watson Crick base pairing occurs at the telomeres (Henderson et al, 1987), and that these G.G base pairings are involved in telomerase priming, or are recognised by telomere-binding proteins. An alternative possibility is that the G-DNA may stabilise the ends against degradation or recombination (for a review of telomeres see Blackburn & Szostak, 1984; Zakian, 1989).

DNA sequences constituting the telomere are similar amongst relatively diverse eukaryotes, however, few telomere specific proteins in these species, and none in the human telomeres, have been identified to date. Two non-histone proteins that bind the telomeres of *Oxytricha* have been identified (Gottschling & Zakian, 1986; Price & Cech, 1987). These proteins have molecular weights of 55 and 43kD and bind as a heterodimer (Price & Cech, 1989). Terminal binding proteins have been isolated from yeast (Berman et al, 1986).

The telomeric DNA consists of tandem repeats of relatively simple DNA sequences. For instance, in humans, other vertebrates, slime molds and trypanosomes the telomeric repeat unit is AGGGTT; for ciliate protozoa *Tetrahymena* and *Euplotes* it is GGGGTT and GGGGTTTT, respectively; for the malarial parasite *Plasmodium* AGGGTT(C/T); for Baker's yeast and the slime mould *Dictyostelium* $G_{1\text{-}3}T$ and $G_{1\text{-}8}A$, respectively (reviewed by Blackburn, 1990). A terminal tract of these sequences, a few hundred base pairs in yeasts or ciliates, or thousands of base pairs in vertebrates is apparently sufficient to stabilise the telomere. Recently, the human telomere has successfully been reintroduced into mammalian cells and has been shown to be functional (Farr et al, 1991).

A ribonucleoprotein reverse transcriptase, telomerase, (reviewed by Blackburn, 1984, see also Greider, 1990) has been isolated, which is responsible for the synthesis of telomere DNA, and apparently contains the required RNA template intrinsically within the enzyme itself (Greider and Blackburn, 1987; Lundblad and Blackburn, 1990).

I 3.3 CENTROMERE STRUCTURE

The analysis of the structure of eukaryotic chromosomes has made considerable progress in recent years in two major systems: yeast and humans. For completeness and for the information it yields about centromere structure, the non-mammalian, lower eukaryotic system will therefore also be reviewed here.

I 3.3.1 Yeast Centromeric DNA

Chromosome "walking" experiments involving centromere-linked marker genes of *Saccharomyces cerevisiae* genomic libraries, and the screening for recombinant plasmids led to the isolation of the first yeast centromere DNA segment (Clarke & Carbon, 1980). This approach, and a procedure of direct selection for DNA segments capable of imparting stable inheritance on plasmids, normally lost at high frequencies, resulted in the isolation of the centromeres of 12 of the 16 yeast chromosomes (Bloom & Carbon, 1982).

There was no cross-hybridisation found between the centromeres at the DNA sequence level, however, a comparison of the sequences resulted in the identification of three consensus elements (Fitzgerald-Hayes et al, 1982) containing two conserved sequences of 8bp and 25bp lements I and III, respectively. The three components are centromere DNA elements (CDE) I, II and III.

CDE I	CDE II	CDE III
PuTCACPuTG	78-86 bp (>90% A+T)	TGTTT(T/A)TGNTTTCCGAAANNNNAAA

Pu, purine; N, no consensus

These sequence elements reside within a 125bp stretch (Cottarel et al, 1989), which contain all of the molecular components required for centromere function. Of the three sequence elements, CDE III has proven the most interesting. It is a highly conserved 25bp sequence containing a region of partial dyad symmetry. Deletion and point mutation analyses have shown that CDE III is crucial to centromere function (Fitzgerald-Hayes, 1987; Hegemann et al, 1988; Saunders et al, 1988), whereas, within certain limits, mutations in CDE I and II can be tolerated. CDE III is positioned at the centre of a centromere core particle within the 250bp region.

This core particle is larger than the normal nucleosome core particle (160bp) of the bulk of the chromatin in yeast and higher eukaryotes (Bloom et al, 1988). The AT-rich CDE II may correspond to the spacer DNA, fascilitating winding around the core particle. For a review of the yeast centromere structure refer to Bloom et al, 1989; Clarke & Carbon, 1985.

I 3.3.2 Yeast Centromeric Proteins

An exonuclease (exoIII) protection assay has demonstrated the presence of specific centromere binding proteins (CBPs) on CDE III of CEN 3 (centromere of *Saccharomyces cerevisiae* chromosome number 3) (Gaudet & Fitzgerald-Hayes, 1987). One such CBP has been purified and its recognition site identified (Cai and Davies, 1989). The action of a centromere protein, CPF1, a helix-loop-helix protein, has been shown to be necessary for optimal centromere function (Mellor, et al, 1991) and its DNA binding site, CDE I, has been characterised (Niedenthal et al, 1991) see reviews of yeast centromere structure by Bloom et al (1989), Clarke and Carbon (1985).

Recently, a 240kD multisubunit protein complex was isolated, which specifically binds to the CDE III sequence of budding yeast. This centromere binding complex (CBF3) consists of three subunits of molecular weights 58kD, 64kD and 110kD, and it is thought to be a major component of the budding yeast centromere (Lechner, Carbon, 1991).

In spite of the advances in this area of research, the application of this knowledge to the understanding of the mammalian centromere may be of limited value. There are significant differences between the centromeres of yeast and those of higher eukaryotes, and in particular those of mammals. In *Saccharomyces cerevisiae* one microtubule attaches per kinetochore, whereas, in mammalian systems 20 to 50 attach per kinetochore. Furthermore, in yeast there is no repetitive DNA in the centromere, whereas in higher eukaryotes this region is in fact satellite rich.

I 3.3.3 Alphoid DNA

The primary constriction of mammalian chromosomes consists of heterochromatin composed of highly repeated DNA sequences (satellite DNA, see Willard (1989). However, such satellite DNA families in different mammals are largely unrelated in sequence and length. In human chromosomes the primary constriction contains alpha-satellite (alphoid) DNA, composed of tandem repeats of 170 bp in groups within a larger unit repeated in a 300-5000kD area (Mannuelidis, 1978; Waye and Willard, 1987; Willard and Waye, 1987, Wu and Mannuelidis, 1980).

Thus, human alphoid DNA differs in amount and sequence from chromosome to chromosome (Willard, 1989; Willard and Waye, 1987) and, hence, a kinetochore related DNA in such sequences has not been identified. However, there has been a report of a minor satellite DNA sequence consisting of 120 bp monomers on the outer surface of the centromere, corresponding to the kinetochore region (Wong and Rattner, 1988) of some of the chromosomes. However, as yet no satellite consensus DNA sequence has been identified which is present at the centromeres of all the chromosomes in humans.

I 3.3.4 Human Centromeric Proteins

The study of human centromere proteins has been considerably facilitated by utilising anticentromere autoantibodies found in the sera of patients with rheumatic diseases (Tan, 1982). Specifically, the sera of patients with the CREST (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly and Telangiectasia) variant of Scleroderma contain autoantibodies, which have led to the identification of several centromeric antigens (reviewed recently by Earnshaw & Bernat, 1991; Rattner 1992). These fall into the following categories, and molecular weights, as determined by Western blotting, are shown.

Centromere Proteins (CENPs)

CENP-A: 17 kD; CENP-B: 80kD; CENP-C: 140kD; CENP-D: 50kD (in rats only); 59kD mitosis specific

Chromatid Linking Proteins (CLiPs)

These have molecular weights of 50kD and 63kD

It is important to note that other centromeric proteins have been identified by the use of monoclonal antibodies, raised against various immunogens, and these are dealt with elsewhere (I 3.4.2).

The centromere proteins (CENPs) were localised on chromosome spreads of human cells to two dots at the centromeres (Fritzler et al, 1980; Moroi et al, 1980). Microinjection experiments using autoimmune sera against CENPs A, B and C, indicated that they are required for the stuctural stability of the kinetochore (Bernat et al, 1991). Of the CENPs, CENP-B has been the most extensively studied (Brinkley, 1990; Earnshaw et al, 1987; Rattner, 1991).

The functions of these three centromere proteins is gradually being elucidated. CENP-A is thought to be a centromere specific histone, or a modified histone (Palmer et al, 1987; Pluta et al, 1990). Recently, CENP-A was purified to homogeneity from bovine sperm (in which CENP-A is the only histone-like molecule of the chromatin) (Palmer et al, 1987). Specifically, it is thought that CENP-A is a variant of H3 and, thus, may be interacting with the centromeric DNA, possibly in an altered, centromere specific nucleosome core particle, to fulfill some structural function.

The DNA sequence encoding the most abundant of the centromere proteins, CENP-B, has been cloned (Earnshaw et al, 1987), on the basis of which the molecular weight of this protein has been determined as 65kD and not 80kD as it appears to be in SDS-PAGE, where it presumably runs anomolously. CENP-B has been shown to be a multidomain protein and can specifically bind DNA. Its DNA sequence indicates that the gene product itself is highly acidic and associates with repetitive alphoid DNA sequences, the CENP-B box, found at the centromeres of most human chromosomes (Masumoto et al, 1989; Wevrick et al, 1990). The alpha-satellite DNA, to which CENP-B specifically binds, is the 17bp sequence:

CTTCGTTGGAAPuCGGGA

The CENP-B box contained within some alphoid monomers, but not others (Masumoto et al, 1989), are found at the centromeres of all the human centromeres, with the apparent exception of the Y chromosome (Masumoto et al, 1989), and in mouse minor satellite DNA (Masumoto et al, 1989; Wong and Rattner, 1988). The amount of CENP-B protein per chromosome apparently varies, since the amount of alpha-satellite DNA varies between chromosomes (Willard and Waye, 1989). That CENP-B actually specifically binds the CENP-B box DNA sequence was recently confirmed by Muro et al (1992).

The CENP-B protein itself contains a protease sensitive region near the N-terminus; an N-terminus DNA binding domain (but lacking the typical residues for helix-turn-helix binding) recognising the CENP-B box (Pluta et al, 1992), and the C-terminus contains 87% acidic residues (Sullivan and Glass, 1991). Immunoelectron microscopy has shown that the CENP-B protein is not located in the kinetochore, but to the heterochromatin beneath the plate and it has been estimated that there are 200-5000 copies of the protein per chromosome (Cooke et al, 1990). Recently, it was reported that CENP-B is a highly conserved protein amongst mammalian cells (Sullivan and Glass, 1991).

Human anti-centromere autoantibody injection experiments led to mitotic arrest, with the chromosomes unable to align properly. Such cells embedded and prepared for transmission electron microscopy demonstrated that the chromosomes attached to the microtubules via the chromatin (Bernat et al, 1991), but not at the kinetochores.

From immunoelectron microscopy it has been suggested that the largest molecular weight centromere protein, CENP-C, may localise to the kinetochore domain or to the kinetochore itself (Cooke et al, 1990), and may thus interact with the microtubules. It has been suggested that CENP-C may interact with the motor proteins at the kinetochore. There is some evidence that CENP-C is required for centromere activity, inasmuch as CENP-C has been shown to be absent from the inactive centromere of a dicentric (number 13) in every cell of a human foetus, whereas CENPs -A and -B were present (Earnshaw et al, 1989).

The use of CREST sera has led to the identification of another set of centromeric proteins, the CLiPs. In immunofluorescence microscopy studies these antigens have, however, been possibly localised to a region of the kinetochore, not to the centromere, and to patches along the inner surfaces of the sister chromatids in Indian muntjac chromosomes (Rattner et al, 1988). The antigen in the kinetochore was distributed as though along a kinetochore plate. However, it is important to note that in this species there is an X-autosome fusion chromosome, in which the centromere is a compound structure, and therefore, unusually extended (less than or equal to 1.45um) (Comings and Okada, 1971). Therefore, the "plate" may in fact be many points fused together. It is more likely that the CLiPs are involved in the association of the sister chromatids, since the antigens are also distributed along the inner surfaces of the sister chromatids.

I 3.3.5 Human Centromeric DNA

Recently, in this laboratory a 14kb length of DNA was isolated from human metaphase chromosomes by co-immunoprecipitation using a human anti-centromere autoimmune serum (Hadlaczky et al, 1991). Co-transfection of mouse ($LMTK^-$) cells with this DNA cloned into a lambda vector together with a selectable marker gene (conferring neomycin resistance, termed aminoglycoside 3/-phosphotransferase type II, APH-II), resulted in a cell line containing a dicentric chromosome (Hadlaczky et al, 1991). The extra centromere of the dicentric was demonstrated to contain human DNA, marker and vector sequences as shown by *in situ* hybridisation. That the additional, artificial centromere was functional, was indicated by its ability to bind mouse centromere antigens, as shown by immunofluorescence microscopy with an anticentromere autoimmune serum detecting these antigens. More importantly, frequent breakage of the dicentric chromosome at anaphase, indicating that both centromeres were bound by microtubules, was observed, resulting in a mini, monocentric chromosome break-off product. The centromere of the resulting minichromosome was also shown to contain centromere proteins, human DNA, marker and vector sequences as did the dicentric from which it originated.

The minichromosome was maintained in the cell line over many generations, and, thus, proved to be stable, i.e. its centromere was functioning normally and the breakage region from the dicentric had presumably acquired a terminal region. The breakage region was, therefore, not "sticky" (a property of breakage fragments), demonstrated by its not being unusually recombinogenic, and attaching to other chromosomes. Thus, this study is a major development in the *in vivo* construction of a minichromosome, from human DNA (possibly centromeric), marker and vector sequences, and possibly mouse sequence, which were functional in mouse cells. For a discussion of mouse DNA sequences at the centromeres refer to Joseph et al, 1989. The DNA transfected into the mouse cells alone, or together with mouse sequences inherent therein, were capable of generating a functional centromere. The minichromosome, therefore, could be analysed further in order to try to assertain what sequences are the minimum required for centromere function.

The sequence of the 14kb DNA has been determined and is deposited in the GenBank data base (see Hadlaczky et al, 1991). It does not contain any alphoid DNA sequences, and therefore, does not contain the CENP-B box. However, no alphoid DNA sequence consensus has been identified which is present at the centromeres of all human chromosomes, whereas, an exciting result of this study is that hybridisation was observed with the DNA sequences on the majority of human chromosomes.

I 3.4 CHROMOSOME SCAFFOLDS

Another approach which has yielded information about chromosome architecture, to a certain extent the centromere, is a biochemical treatment of chromosomes giving so-called "chromosome scaffolds". As indicated previously, it is important to discriminate between the two types of "scaffolds" referred to in cell biology. The **scaffolds** referred to in this thesis, unless otherwise stated, are from **metaphase chromosomes**, and **not nuclear matrices** (sometimes also termed nuclear scaffolds, or nuclear cage), which are prepared from **interphase nuclei**, and since the protein composition of these two types of samples are distinct.

I 3.4.1 General Properties of Chromosome Scaffolds

Isolated metaphase chromosomes, when digested with nucleases and treated with high salt (2M NaCl), result in residual structures, termed chromosome scaffolds (Adolph et al, 1977a). The treatment of metaphase chromosomes with 2M NaCl alone, or other methods which deplete the chromosomes of histones (Adolph et al, 1977b) results in a histone-depleted residual structure surrounded by a halo of DNA. The protein profile of 2M NaCl, or 2M NaCl treated and nuclease digested, chromosomes is apparently identical, the only difference being the absence of non-tightly bound DNA in the latter.

When viewed with the electron microscope the chromosome scaffold is seen to consist of a fibrous network that is about 4-5 times the length of intact untreated chromosomes (Hadlaczky et al, 1982).

Chromosome scaffolds can be prepared by the stabilisation of isolated chromosomes in buffers containing Cu^{2+} or Ag^{+} at 4°C, followed by the extraction of histones by one of several possible procedures: 2M NaCl, dextran sulphate-heparin, or low concentrations of lithium 3′, 5′-diiodosalicylate (LIS) in low or medium-salt buffers. The metal stabilisation step can be substituted by the isolation of the chromosomes in buffers containing polyamines and EDTA. Irrespective of the method employed to obtain histone depletion, the residual structure appears the same both biochemically and microscopically. In all procedures a 170kD protein, shown to be topoisomerase II (see later I 4.4.3) is enriched for by comparison with native chromosomes. (Lewis & Laemmli, 1982; Gasser et al, 1986).

I 3.4.2 The Residual Centromere

The most electron dense region of the chromosome scaffold is the residual centromere, and to a lesser extent the telomeres (Hadlaczky, 1985). The greater resistance of the centromeres (and telomeres) to this harsh treatment suggests that they contain very stable DNA-protein complexes.

Use of chromosome scaffolds for the immunisation of mice has resulted in the detection of centromere associated antigens. Monoclonals raised against chicken chromosome scaffolds have been produced, which react with antigens of molecular weights 135 and 155kD (Cooke et al, 1990). These so-called inner centromere proteins (INCENPs) are localised to two dots in the pericentromeric region of the chromosomes, and not to the centromere. Unlike the CENPs, a line drawn joining the INCENP antigenic dots, is parallel (and not perpendicular) to the long axis of the chromosome, and lies between the sister chromatids. These antigens behave differently to the CENPs at anaphase.

The CENPs remain at the centromere throughout the cell cycle, whereas, the INCENPs actually dissociate from the pericentromeric region and remain in the midzone during anaphase.

The other, recently discovered, centromere associated protein is termed CENP-E, and was isolated using a Mab raised against human chromosome scaffolds (Compton et al, 1991). This antigen is localised to two dots on human chromosomes in a manner similar to the CENPs -A, -B, and -C, but, which is more peripheral, i.e. more distal from the point of connection of the sister chromatids.

I 3.4.3 DNA Topoisomerase II

This ubiquitous enzyme is a homodimer with a molecular weight between 166-180kD, species depending (Goto and Wang, 1982; Hsieh, 1983; Miller et al, 1981; Shelton et al, 1983;). In addition to its enzymatic function, DNA topoisomerase II has also been shown to be a major component of both the chromosome scaffold and the nuclear matrix (Berrios et al, 1985; Mirkovitch et al, 1988; Berrios & Fisher, 1988). Scaffolds prepared from human Hela metaphase chromosomes contain two major proteins, termed Sc1 and Sc2, of molecular weights 170kD and 135kD, respectively (Lewis & Laemmli, 1982). The 170kD, Sc1 protein, was shown to be DNA topoisomerase II (Earnshaw and Heck, 1985; Gasser et al, 1986). A role has been suggested for topoisomerase II in mitosis in allowing normal sister chromatid segregation by bringing about DNA topological changes (Holm et al, 1985). Furthermore, immunolocalisation shows that topoisomerase II occurs at discrete foci, and in some regions appears in the form of a zig-zag along the length of the sister chromatids (Gasser et al, 1986). Thus, it is considered possible that this protein serves some structural role in chromosome architecture (Uemura and Yanagida, 1984; Adachi et al, 1991; Wood and Earnshaw, 1990). Recently, topoisomerase II has been proven to be necessary for chromosome condensation (Adachi et al, 1991). Topoisomerase II is estimated to be present in approximately 3 copies per 70kb loops of DNA, this together with its axial core localisation, makes it a candidate molecule for stabilising loops in metaphase chromosomes (Gasser et al, 1986; Earnshaw & Heck, 1985).

This potential structural role for topoisomerase II extends to interphase nuclei, also. Evidence for such a role comes from the isolation of DNA from nuclear matrices samples, and the sequence analysis of the DNA of such matrix associated regions (MARs see earlier I 1.3.1). MARs in fact are enriched for the topoisomerase II cleavage consensus sequence (Sander & Hsieh, 1985), and topoisomerase II indeed cleaves two such sequences *in vitro* (Udvardy et al, 1985). However, although cleavage requires sequence recognition, cleavage and binding are not synonymous. Hence, this is only indirect evidence of a possible topoisomerase II-nuclear matrix interaction.

I 3.5 RESTRICTION ENDONUCLEASE DIGESTION OF CHROMOSOMES

Various biochemical treatments have been tested and shown to result in chromosome banding patterns. The use of such techniques has shown that mitotic chromosomes of both animals and plants contain regions of structural organisation. Such banding techniques apparently reflect the normal structural organisation of the chromosomes, presumably brought about by DNA elements and associated proteins (Burkholder, 1989). One such banding pattern, namely C-banding, allows the localisation of constitutive heterochromatin, particularly evident in the centromeric region (Arrighi and Hsu, 1971).

There are restriction endonucleases which cleave the DNA at sequence specific sites and bring about G- (Giemsa) and C-like banding in mouse and human chromosomes (Mezzanotte et al, 1983; Miller et al, 1983; Kaelbling et al, 1984; Burkholder and Schmidt, 1986) and it was hoped that the use of such techniques would shed light on the relationship of the underlying DNA to higher-order structure.

Until relatively recently restriction endonuclease digestion of chromosomes and their study was limited to fixed chromosomes, which hindered a biochemical analysis. However, it has been shown that banding can also be produced *in vitro* on isolated unfixed chromosomes (Burkholder and Schmidt, 1986). and this technique provided further evidence that regional chromosomal DNA specificity underlies banding (Burkholder, 1989).

Mouse chromosomes have been studied as a useful model for chromosome structure. The arms of mouse chromosomes contain main band DNA, whilst the centromeres are comprised of main band and satellite sequences (Lica and Hamkalo, 1983). The satellite DNA constitutes 6% of the total genome (Southern, 1975) and is localised to all of the centromeres with the notable exception of the Y chromosome (Pardue and Gall, 1970). For a recent study of the changes in chromosome structure in response to digestion by various endonucleases determined by electron microscopy refer to Gosalvez et al, (1990).

Attempts have been made to characterise the mammalian centromeric region by utilising restriction endonuclease digestion of chromosomes (Lica and Hamkalo, 1983; Rattner, 1986). However, in all such studies only the gross morphology of the chromosomes could be approached and in order to try to study the centromere DNA and proteins in particular, which are responsible for centromere function, other approaches have been exploited (see I 3.3.4 and I 3.3.3).

CONTENTS

MATERIALS AND METHODS

M1 MATERIALS

M1.1 STANDARD SOLUTIONS

PBS

137mM NaCl
2.7mM KCl
4.3mM Na_2HPO_4
1.4mM KH_2PO_4, pH 7.4

TBS

10mM TRIS
0.9% NaCl, pH 7.4 (HCl)

RPMI 1640

L-Amino Acids	mg/ml
Arginine	200.0
Asparagine	50.0
Aspartic Acid	20.0
Cyctine	50.0
Glutamic Acid	20.0
Glutamine	300.0
Glycine	10.0
Histidine	15.0
Hydroxyproline	20.0
Isoleucine (allo-free)	50.0
Leucine (methionine-free)	50.0
Lysine HCl	40.0
Methionine	15.0
Phenylalanine	15.0
Proline (hydroxyl-L-proline-free)	20.0
Serine	30.0
Threonine (allo-free)	20.0
Valine	20.0

Vitamins	
Biotin	0.2
Vitamin B_{12}	0.005
Calcium pantothenate	0.25
Coline chloride	3.0
Folic acid	1.0
i-Inositol	35.0
Nicotinamide	1.0
p-Aminobenzoic acid	1.0
Pyridoxine HCl	1.0
Riboflavine	0.2
Thiamine HCl	1.0

Inorganic Salts	
NaCl	6000.0
KCl	400.0
$Na_2HPO_4.7H_2O$	1512.0
$MgSO_4.7H_2O$	100.0
$NaHCO_3$	2000.0
$Ca(NO_3)_2.4H_2O$	100.0

Other Components	
Glucose	2000.0
Glutathione (reduced)	1.0
Phenol red	5.0

Ham's F12

L-Amino Acids	mg/ml
Alanine	8.9
Arginine HCl	211.0
Asparagine.H_2O	15.0
Aspartic Acid	13.3
Cycteine HCl	31.5
Glutamic Acid	14.7
Glutamine	146.0

Glycine	7.5
HistidineHCl	21.0
Isoleucine	3.9
Leucine	13.1
Lysine HCl	36.5
Methionine	4.5
Phenylalanine	5.0
Proline	34.5
Serine	10.5
Threonine	11.9
Tryptophan	2.0
Tyrosine	5.4
Valine	11.7
Vitamins	
Biotin	0.007
Calcium D-pantothenate	0.48
Coline chloride	14.0
Folic acid	1.3
myo-Inositol	18.0
Nicotinamide (Niacinamide)	0.04
Pyridoxine HCl	0.06
Riboflavine	0.04
Thiamine HCl	0.34
Vitamin B_{12}	1.36
Inorganic Salts	
NaCl	7600.0
KCl	223.6
$Na_2HPO_4.7H_2O$	268.0
$MgCl_2.6H_2O$	100.0
$CaCl_2.2H_2O$	44.1
$NaHCO_3$	1176.0
$FeSO_4.7H_2O$	0.83
$CuSO_4.5H_2O$	0.002
$ZnSO_4.7H_2O$	0.86
Other Components	
Glucose	1802.0
Hypoxanthine, sodium salt	4.1
Linoleic acid	0.08
Phenol red	1.2
Putrescine 2HCl	0.16
Sodium pyruvate	110.0
Thioctic acid (Lipoic acid)	0.21
Thymidine	0.73

M1.2 CELLS

Mammalian Cells

K_{20} Chinese Hamster Ovary cell line.

LMTK$^-$ (connective tissue, mouse, thymidine kinase mutant, bromodeoxyuridine resistant, 2n=40) this mouse cell line was derived from a subline of a 5-bromo-2-deoxyuridine resistant strain of the L-M mouse fibroblast cell line (Hsu and Somers, 1962).

HeLa (epithelioid carcinoma, cervix, human, 2n=46) this human cell line was the first aneuploid, epithelial-like cell line derived from human tissue and maintained in cell culture.

COLO 320HSR (colon, adenocarcinoma, human 2n=46) this human cell line contains marker chromosomes with homogeneously staining regions.

EJ30 human cell line.

All cells were incubated at 37°C with 5% CO_2 Chinese hamster ovary (CHO) cells (K_{20}), and human (HeLa, EJ30 and Colo HSR) were cultured every two days in F-12 medium (10.63g/1000ml F-12 nutrient mix, 1.176g/1000ml $NaHCO_3$, 10ml/1000ml antibiotic; Gibco, Scotland) supplemented with 10% foetal calf serum (Human, Hungary). Cells were loosened from petri dishes by incubation in trypsin for 3-4 min, the trypsin was removed and the cells tranferred to a new dish by pipetting with 10ml fresh medium. Hybridoma clones, and mouse (LMTK$^-$) cells were cultured daily or every two days depending on their state in RPMI medium (containing 2g/1000ml $NaHCO_3$) supplemented with 10% foetal calf serum and 630mg/1000ml glutamine. Mouse cells, cultured in RPMI, were loosened from petri-dishes and passaged as CHO and human cells. Hybridoma cells were not loosened by trypsin treatment since they do not adhere to culture dish surface. For storage, hybridoma cells were grown in 10cm petri dishes for 3-4 days to a high titre, concentrated for 5 min at 1000rpm in an MSE bench centrifuge, and transferred to 1.5ml complete RPMI medium containing 10% DMSO and stored in liquid nitrogen. To thaw, ampullae were warmed under running hot tap water, pipetted gently, diluted in fresh 5ml RPMI medium and spun for 5 min at 1000rpm in an MSE centrifuge and cultured in either 2cm or 10cm (depending on the titre) petri dishes. For large scale Colo nuclei isolation cells were grown in suspension.

M 1.3 ANTIBODIES

Mouse anti-CHO chromosome scaffold monoclonal antibodies.
Mouse anti-Drosophila topoisomerase II monoclonal antibodies
Mouse anti-Drosophila topoisomerase II polyclonal antibodies (a kind gift from Dr. A. Udvardy, BRC)
Human anti-centromere autoimmune serum LU851 was obtained from a patient at the Department of Dermatology, Karolinska Hospital, Sweden.

M 2. METHODS

M 2.1 IMMUNOLOGICAL TECHNIQUES

M 2.1.1 Harvesting of Hybridoma Supernatants

To obtain monoclonal antibodies, appropriate hybridoma cells (for the initial production of anti-CHO chromosome scaffold hybridomas see Appendix) were cultured in complete RPMI medium, and when of high titre in a 10cm, or 18cm culture dish, were starved of media for 3-4 days. Cells were monitored by light microscopy to determine their state. To obtain the maximal immunoglobulin concentration cells have to be left for as long as possible to secrete immunoglobulin into the medium, however, leaving them too long will have a detrimental effect, due to cell lysis and the action of acidic conditions and proteases on the antibodies. The state of the cells can be determined microscopically, so that the stage when excessive cell death occurs can be avoided. Thus, when deemed ready, the supernatants were harvested, 0.1% sodium azide added, and stored at 4°C.

M 2.1.2 Spot Test for Immunoglobulin Production

To test for the presence of immunoglobulin secreted into the supernatant by the hybridoma cells, 10µl aliquots of supernatant were transferred to nitrocellulose filters and allowed to dry, before soaking in blocking solution (1% bovine serum albumin, 20% foetal calf serum, in 0.05% Tween-20-PBS) for 10 min. Filters were washed and incubated in blocking solution, containing anti-mouse HPRO conjugated secondary antibody, for 10 min with agitation at room temp and protected from light. Filters were washed in PBS, then TBS and colour developed using colour developing solution (2ml cold methanol, 6mg 4-chloro-1-napthol, 10ml TBS, 10µl H_2O_2). The colour reaction was stopped by transfer to distilled water, the filters dried and stored, protected from light.

M 2.1.3 Immunoglobulin Subtyping

For the determination of mouse immunoglobulin types an Amersham sub-typing kit was employed. Strips with anti-mouse immunoglobulin sub-types immobilised onto them were incubated with the supernatant to be tested, with agitation for 20min at room temperature.

Strips were then washed with 3 changes of TBS and incubated with HRPO-conjugated anti-mouse secondary antibodies, and incubated with agitation for 20min at room temperature protected from light. Strips were then washed with 3 changes of TBS and colour developed as in M 2.1.2.

M 2.1.4 Production of Mouse Ascites

Hybridoma cells were grown to high titre in two 15cm culture dishes. Cells were asceptically harvested and concentrated by spinning for 5 min at 1500rpm in a bench MSE centrifuge. Cells were resuspended in 300µl sterile PBS. 100µl were injected per BalbC mouse into the intraperitoneal cavity using a 21 gauge needle. The mice were observed daily for presence of tumours, and more frequently towards 10 days to two weeks as tumours grew. When in a terminal condition the mice were sacrificed and the ascitic fluid removed from the intraperitoneal cavity. To remove hybridoma and red blood cells, the blood was spun for 5 min at 1500rpm in an MSE centrifuge and the pellet discarded. Ascites were diluted in 1:1 glycerol and stored at -20°C.

M 2.1.5 Affinity Column Purification of Immunoglobulin

The pH of each hybridoma supernatant was adjusted by TRIS if necessary to pH 7.0. The column, Protein A CL4B or anti-mouse 4B (Pharmacia), was first washed with 25 x volume elution buffer, 0.2M glycine, pH adjusted to 2.5 with 0.2M HCl, then washed with 25 x volume PBS, pH 8.0. Hybridoma supernatant containing immunoglobulin to be purified was passed through the column 4-5 times, under a closed circuit system using a peristaltic pump, at a flow rate of 1g. Unbound immunoglobulin was removed by rinsing the column with PBS, pH 7.0, and elution buffer (0.2M glycine, 0.2M HCl, pH 2.5) applied to the column. Neutralising buffer (1.5M TRIS) was added to 1ml eppendorf tubes, so that 1ml of eluate would return to neutral pH. 1ml fractions were collected, and the column topped up with PBS, pH7.0, until elution buffer could no longer be detected (using pH paper). The column was washed with PBS, pH7.0, 0.05% NaN_3 added, and the samples stored at 4°C. The fractions were tested for presence of immunoglobulin by immobilisation onto nitrocellulose as for supernatants. Similar fractions were collated and tested for specificity on CHO metaphase cells by immunofluorescence microscopy.

M 2.1.6 Immonofluorescence Microscopy

To block cells at metaphase, cells were cultured and incubated in a 10cm culture dish as for normal culturing, overnight. Then 5µg/ml colchicine was added to 10ml of medium and incubated for 3-4h.

Microscope slides were cleaned with 100% methanol, washed in distilled water and dried.

The titre of the cells to be used for immunofluorescence was estimated microscopically in a 10µl sample. Cells were added to 150µl hypotonic Stenman (10mM HEPES or PIPES, 30mM glycerol, 1mM $CaCl_2$, 0.8mM $MgCl_2$ in distilled water, pH 7.0 (NaOH)) or GH solution per cytocentrifugation chamber, for 10 min at room temperature. Then an equal volume of 0.2% Triton-X-100 (in distilled water) was added and incubated for 5 min to make the cell membranes permeable. 300µl of cells in solution were added per cytocentrifugation chamber, and spun for 8 min. in a cytocentrifuge. Slides were immersed in fixative (type stated in text) for 10 min at -20°C, and washed twice in PBS. Since different antigens react differently to different fixatives, three different fixative were utilised. Two of these were the organic solvents, 100% methanol and 100% acetone, which achieve fixation by removing lipids, dehydrating cells and precipitating the proteins. The third fixative used was 4% paraformaldehyde, which is a protein cross-linking reagent and forms a network of inter-molecular bridges via free amino groups.

Slides were transferred to a wet chamber, primary antibody added, and coverslip applied. Slides were incubated for 1 h at room temp, coverslip removed, and washed in PBS. FITC conjugated secondary antibody was added (concentration as in text), coverslip applied and incubated in the dark for 1 h at room temp. Slides were then washed three times in PBS and counterstained with HOECHST 33258 DNA binding dye or propidium iodide depending on requirements.

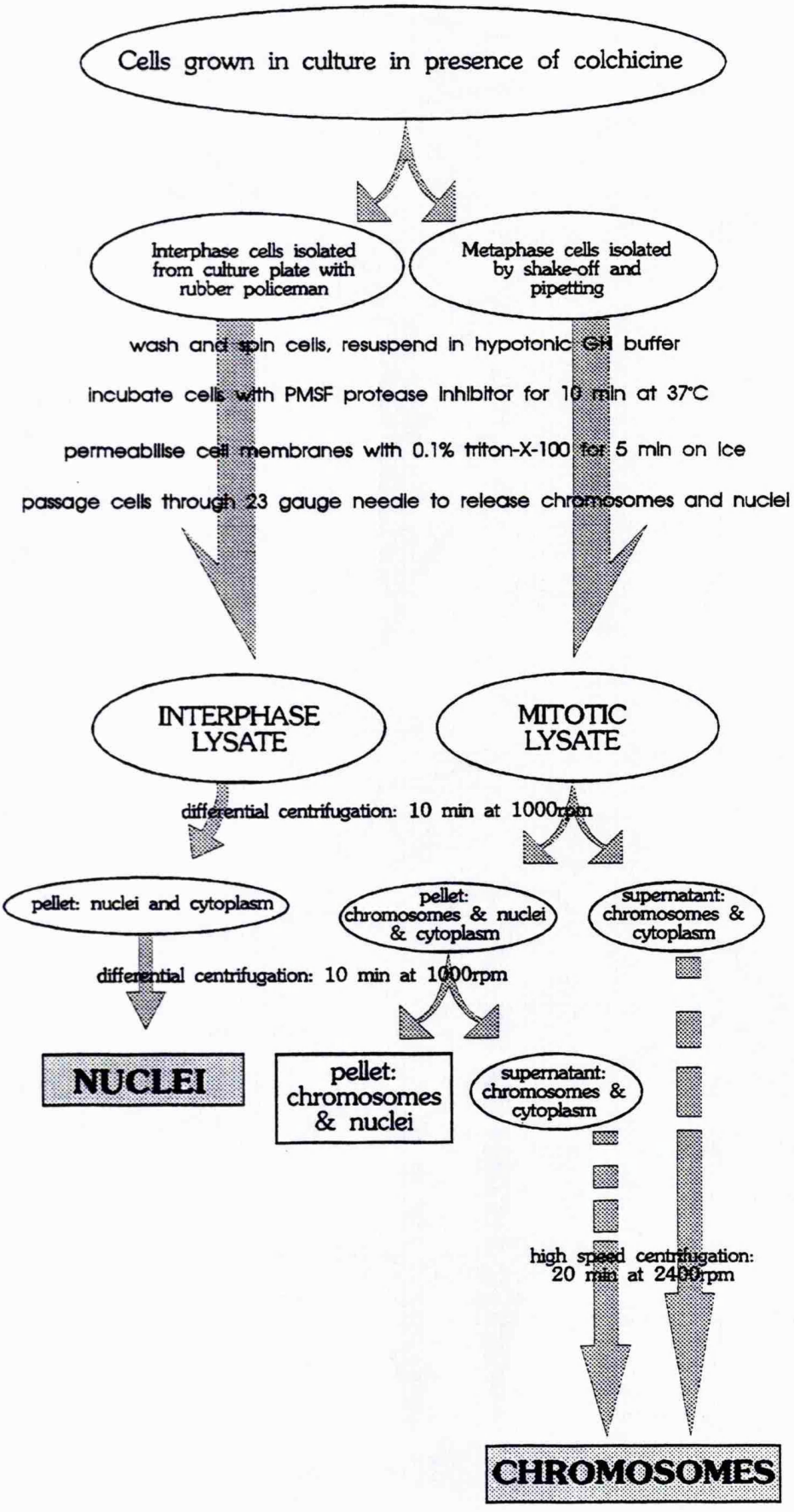
M 2.1.7 Immonological Treatment of Proteins Electrophoretically Transferred to Nitrocellulose Filters

Filters were incubated overnight at 4°C in blocking solution (1% bovine serum albumin, 20% foetal calf serum, in PBS containing 0.2% Tween-20) and then processed. Depending on the type of primary antibody used the concentration varied (see text). Unless otherwise stated supernatants from hybridoma clones were used undiluted. Filters were incubated with primary antibody for 1h with agitation at room temp in petri dishes. Filters were washed in PBS and incubated in HRPO conjugated secondary antibody for 1h with aggitation at room temperature in the dark. Filters were washed in TBS and colour developed (2ml cold methanol, 6mg 4-chloro-1-napthol, 10ml TBS, 10µl H_2O_2). The colour reaction was stopped by transfer to distilled water, dried and stored protected from light.

M 2.1.8 Immunoprecipitation of CHO Proteins

Chromosome, nuclei, metaphase cytoplasm, and interphase cytoplasm fractions were obtained as described above and radiolabelled as described in M 2.2.5. 300µl of Sepharose was added 1:1 to protein fractions and incubated with agitation at room temperature for 2h.

MASS ISOLATION OF CHROMOSOMES AND NUCLEI



100µl of electrophoresis sample buffer was then added to each fraction, boiled and 10µl loaded onto 5-20% SDS PAGE, and electrophoretically transferred to a nitrocellulose filter following the standard procedures. The subsequent Western blot was incubated with a photographic film for 16h and 48h at room temperature, without a screen and for 24h at -80°C with an intensifying screen and developed.

M 2.2 PROTEIN SAMPLE PREPARATION TECHNIQUES

M 2.2.1 Mass Isolation of Chromosomes and Nuclei

Chromosome and nuclei isolations were carried out using a glycine-hexylene glycol isolation buffer (Hadlaczky et al. 1982). Cells were grown in 50ml media in 10-12, 18 cm dishes and blocked at metaphase by addition of 250µl of 1000µg/ml colchicine and incubated overnight (12-14 h). Mitotic cells were gently separated from interphase cells still adhering to the petri dish surface using a wide mouth 25ml pipette. Interphase cells were gently removed using a rubber policeman. To determine the success of metaphase blocking, mitotic cells were fixed onto slides using cold 1:3 acetic acid: methanol, stained with carbol fuchsin and the mitotic index determined.

Both mitotic and interphase fractions were centrifuged in 80ml tubes for 10 min at 2000rpm. The supernatant was removed and cells were washed in hypotonic glycine buffer (100mM glycine, 1% hexylene glycol (also known as 2-methyl-2, 4-pentanediol) pH 8.4-8.6 $Ca(OH)_2$). Cells were respun in 10ml tubes for 10 min at 2000rpm, the supernatant discarded, and 9ml hypotonic glycine buffer (GH buffer) added. The pellet was resuspended and incubated for 10 min at 37°C with 100µl of protease inhibitor, PMSF (100mM phenylmethyl sulfonylfluoride in iso-propanol alcohol). To make the cell membranes fragile, cells were transferred to ice and incubated for 5 min with 100µl of Triton water to final concentration 0.1% Triton-X-100. In order to liberate the chromosomes and nuclei, cells were passaged through a 23-gauge needle. The success of this treatment was monitored by light microscopy. When the chromosomes and nuclei were deemed to be liberated from the cell membranes, tubes were topped up with GH buffer containing 0.1% Triton-X-100 (GHT buffer) and centrifuged as follows. Nuclei were washed twice (or more depending on how successful the liberation from the cytoplasm was, as determined by phase contrast microscopy) in GHT buffer with centrifugation for 10 min at 1000rpm. Nuclei are not disrupted at this speed, and can be separated from cell debris and cytoplasm. Mitotic cells were differentially centrifuged in GHT buffer for 10 min at 1000rpm to separate the chromosomes (in supernatant) from the contaminating nuclei (in pellet). Centrifugation was repeated until chromosomes were sufficiently free of nuclei, as determined by light microscopy, and chromosome fractions collated. The final pellet contained chromosomes and nuclei and was kept as a mixed fraction.

Pure chromosomes and mixed fraction were washed twice in GHT to remove cytoplasmic contaminants with centrifugation for 20 min at 2400rpm, and monitored by phase contrast microscopy. Depending on the required fate of the isolated chromosomes and nuclei, they were either resuspended in electrophoresis sample buffer or nuclease buffer (see appropriate sections).

M 2.2.2 Preparation of Chromosome Scaffold and Nuclear Matrix Samples for Western Blotting

Isolated chromosomes and nuclei were resuspended in nuclease buffer (2.5 mM $MgCl_2$, 100 mM NaCl, 10 mM TRIS, pH 7.5), 100µg DNase I and 100µg RNase A added, and incubated for 1 h at 37°C. To this solution an equal volume of 4 M Oudet buffer (4 M NaCl, 40 mM TRIS, 20 mM $Na_2S_2O_5$, 4 mM $MgCl_2$, pH 8.0) was added and incubated for 1 hr on ice. The solution was then layered on 10ml of a 2 M Oudet buffer containing 7.5% sucrose in a centrifuge tube and spun for 30 min at 15000rpm at 0°C. The resulting pellet was resuspended in electrophoresis sample buffer.

M 2.2.3 Preparation of Chromosome Scaffold and Nuclear Matrix Samples for Immunofluorescence Microscopy

Previously isolated CHO chromosomes and nuclei were stored at 4°C in 1:1 glycerol:GHT buffer. Prior to use they were washed in GHT buffer and spun for 10 min at 2400rpm and 1000rpm for chromosomes and nuclei, respectively. Round, 1cm diameter slides were cleaned and placed on teflon semi-sphere supports in 20ml centrifuge tubes. 2M Oudet buffer (containing 2M NaCl) was transferred to the tubes. 1-2µl of isolated chromosomes or nuclei were resuspended in 1ml of GHT buffer, mixed and layered onto the Oudet buffer. Tubes were spun for 30 min at 4000rpm at 0°C in a K23 centrifuge. Slides were then removed and fixed for 10 min in methanol at -20°C. For colour photography cells were counter-stained for DNA with propidium iodide (0.1% para-phenilene diamine in PBS containing 3ng propidium iodide). 100µl of this solution was added to each slide and incubated for 3 min at room temp. Slides were then washed in PBS and mounted in 1:9 propidium iodide solution: glycerol.

M 2.2.4 TCA Precipitation of Proteins

Protein were precipitated using equal volumes of ice-cold 20% trichloroacetic acid, vortexed and incubated on ice for 30 min. The suspension was filtered through Whatman paper, washed with excess 10% TCA with 100% ethanol and allowed to air dry.

M 2.2.5 Radioactive Labelling of CHO Proteins

CHO (K_{20}) cells in 4 x 18cm dishes, in 40ml culture medium were incubated for 24h with 250Ci ^{35}S methionine per dish. Then 250µl 1000µg/ml colchicine and 10ml of fresh culture medium was added and the cells incubated for a further 12h.

Phase contrast microscopy revealed a mitotic index of 50%, chromosomes and nuclei were isolated by the standard procedure, and metaphase and interphase cytoplasms were obtained by retaining supernatants after high speed centrifugation.

M 2.2.6 Isolation of Chinese Hamster Tubulin

Chinese hamster was sacrificed, brain removed and added to an equal volume of ice cold buffer containing 100mM MES, 1mM EGTA, 0.5mM $MgCl_2$, 1mM mercaptoethanol, 1mM EDTA, 1mM GTP, pH 6.4. The solution was made homogeneous in a Waring blendor. The homogenate was centrifuged at 20,000rpm (45,000g) at 4°C for 1h and the supernatant (crude tubulin extract) retained.

M 2.3 PROTEIN ANALYSIS TECHNIQUES

M 2.3.1 Gel Electrophoresis

Proteins were disolved in electrophoresis sample buffer (300mM TRIS, 5% SDS, 10% beta-mercaptoethanol, 50mM EDTA, 10% glycerol, pH 6.8) and loaded onto gels at a concentration of about 75-100µg protein per lane. Unless otherwise stated protein samples were separated on 5-20% gradient SDS-polyacrylamide slab gels (see Laemmli et al, 1970). Gradient gels have the following advantages over linear gels: the leading edge is more retarded than the trailing edge giving sharper protein bands; the gradient in pore size increases the range of the molecular weights which can be fractionated; the proteins with close molecular weights are more likely to be separated. Particularly the second point was important in the choice of the gradient gel system over the linear one, since the range of the mammalian proteins used in the experiments was large. Bis-acrylamide was used at 50% acrylamide, 1.4% bis (ratio of 36:1). Mini gels (4ml) were electrophoresed in electrophoresis buffer (0.025M TRIS, 0.192M glycine, 0.1% SDS, pH 8.3 without adjustment) at 80V for 90 min, and 0.01% pyroninė G included to stain and locate the lanes on the subsequent nitrocellulose filter. Gels were stained for proteins in Coomassie Brilliant Blue (0.1% Coomassie, 50% methanol, 10% acetic acid in distilled water) for 30 min with agitation at room temp. Gels were destained using several changes of 7% acetic acid in distilled water, absorption of excessive stain was aided by the addition of a small piece of sponge.

M 2.3.2 Western Blotting

Proteins separated by SDS-PAGE, as detailed elsewhere, are in a dense gel matrix and thus not available for further study. There are techniques available for *in situ* analysis of such proteins, though these are limited and rather cumbersome (see Bowen et al, 1980), and the method of choice is usually transfer to nitrocellulose filters via protein blotting.

Passive diffusion of proteins to filters (Bowen et al, 1980) has certain drawbacks, such as the considerable time required and the incomplete transfer of proteins, due to the inevitable relatively fast diffusion of SDS out of the gel, however, an advantage to this technique is that the proteins have more time to renature. However, on the whole another transfer procedure, namely electroblotting, was chosen due to the less lengthy process, and the better rate of transfer, passive diffusion gives only 1% to 10% success of protein transfer by comparison to electroblotting (Bowen et al, 1980).

Electrophoresis gels were transferred to 4 (8cm x 10cm) Whatman filters soaked in SIGM transfer buffer (30mM glycil glycine, 10mM imidazole, 0.1% SDS, 20% methanol). The nitrocellulose filter was soaked in SIGM buffer and placed on the gel, another 4 pre-soaked Whatman filters was then placed on top, and the whole sandwiched between plastic plates. Transfer was made for 2h at 100mA at room temperature. Transferred molecular weight markers were stained with amido black (0.2% amido black, 10% acetic acid, 45% methanol in distilled water), and destained (20% acetic acid, 70% methanol, 10% distilled water).

The nitrocellulose filter with transferred proteins for immunological treatment was transferred to blocking solution (1% bovine serum albumin, 20% foetal calf serum in Tween-PBS containing 0.05% Tween 20) and incubated overnight at 4°C.

M 2.4 LAMBDA GT11 EXPRESSION LIBRARY SCREENING TECHNIQUES

M 2.4.1 Immunological Screening of Lambda gt11 Expression Libraries

E.coli Y1090r- was streaked for single colonies on LB plates, pH 7.5, containing 100µg/ml ampicillin and incubated overnight at 37°C. Starting with a single colony the bacteria were grown to saturation in LB, pH 7.5 at 37°C with agitation.

For 90mm plates 0.2ml Y1090r- exponentially growing cells were mixed with 0.1ml lambda diluent (10mM Tris-HCl, pH 7.5, 10mM $MgCl_2$) containing $3x10^4$ pfu of the lambda gt11 library for each plate. For 150mm plates 0.6ml of the host culture was used with 10^5 pfu in lambda diluent for each plate. The phage was adsorbed to the cells at room temperature for 20 min.

2.5ml or 7.5ml of LB, pH 7.5 soft agar was added to 90mm and 150mm plates, respectively, to the culture and poured onto LB plates, pH 7.5. After 3.5 hr growth at 42°C the plates were carefully (to avoid air bubbles) overlaid with dry nitrocellulose filters, previously saturated with 10 mM isopropyl ß-D-thiogalactopyranoside in H_2O (IPTG, which is a *lac* Z gene expression inducer).

These were then incubated for a further 3.5 hr at 37°C (to induce protein expression). During this time plaques and the ß-galactosidase fusion proteins, released from the lytically growing cells, were transferred to the filters.

The plates were then removed to room temperature, and the position of the filters marked with a needle. The filters were then removed to blocking solution to saturate any non-specific binding sites on the nitrocellulose. The primary antibody (monoclonal supernatant used undiluted or polyclonal diluted as for Western blotting) against the centromere antigen of interest was incubated with the filters as for Western blotting, as was the secondary antibody and colour development described elsewhere.

M 2.4.2 Procedure for Making Positive Control Filters

Host cells were grown as described above. Control phages used were lambda gt11 recombinants containing an ovalbumin/ß-galactosidase fusion in a 1:in 500 dilution of a background of lambda gt10, to give negative plaques. Infection, lysis and transfer to filters was carried out as described above. The primary antibody used was 1:5000 dilution of mouse anti-ß-galactosidase monoclonal antibody.

CONTENTS

RESULTS: R 1. SCREENING OF ANTI-CHROMOSOME SCAFFOLD MABS

General Overview

The mammalian centromeric region consists of constitutive heterochromatin (Arrighi & Hsu, 1971). Consequently, this region of the chromosome does not contain any proximal flanking selectable genetic markers. Therefore, the analysis of the centromere DNA sequence cannot be approached by standard genetical methods. However, immunological techniques have facilitated certain studies of mammalian centromeres. For instance, the sera of patients with the CREST variant of Scleroderma contain autoantibodies (Moroi et al, 1980; Fritzler at al, 1980), which react with centromere proteins. The DNA binding sequence of one of these centromere proteins, CENP-B, has been determined (Masumoto et al, 1989).

Another approach has involved the biochemical treatment (high salt and restriction enzyme digestion) of isolated chromosomes, which results in an enrichment for so-called "chromosome scaffold" proteins (of which the centromere is a prominent component). Such protein samples have been used for immunisation and resulted in the production of anti-chromosome scaffold monoclonal antibodies (Cooke et al, 1987; Yen et al, 1991). Since antibodies have been the only tools successful in approaching the molecular components of the mammalian centromere the afore-mentioned two types of antibodies were employed in this study, namely: 1. monoclonal antibodies (Mabs) raised against CHO chromosome scaffolds and 2. human autoimmune sera recognising centromere proteins.

1. CHO Chromosome Scaffold Mabs

The region of the mammalian chromosome which is most resistant to the treatment which results in the production of "chromosome scaffolds" has been shown to be the centromere (Hadlaczky et al, 1981). Thus, Mabs were raised against CHO chromosome scaffolds in an attempt to isolate antigens of the mammalian centromere. Anti-chromosome scaffold Mabs have been extensively used in these studies and the results obtained with these are presented in Chapters R1 to R9.

Using such anti-chromosome scaffold antibodies, a Mab, secreted by hybridoma clone AC1, was identified, which recognises a ring-like structure at the centromeres of CHO, mouse and human chromosomes in immunofluorescence microscopy (Chapters R2 and R4). The antigen has an apparent molecular weight of 170kD, as determined by Western blotting (Chapter R3), and this centromere antigen is distinct from the other centromere antigens identified to date (Chapter R4). The ring structure is conserved to mouse ($LMTK^-$) centromeres, to the centromeres of the hybridoma clones themselves, AC1 and BA2, and human (EJ30) centromeres, also. (Chapter R5).

The centromere ring is present on chromosome scaffolds indicating that it binds DNA (Chapter R6). The relationship of this antigen to the chromosome scaffold antigen topoisomerase II (Chapter R7) and molecules involved in motility, namely microtubules (Chapter R8) and myosin (Chapter R9), were also investigated.

2. Human Autoimmune Sera

The majority of centromere antigens isolated to date have been identified by using anti-centromere autoantibodies present in the sera of patients with the autoimmune disease scleroderma (Moroi et al, 1980). A similar approach was also utilised in this study to investigate human centromeres. There are three centromere proteins identified by the use of autoimmune sera: CENP-A, 17kD; CENP-B, 80kD; and CENP-C, 140kD. The gene encoding the most abundant centromere protein, CENP-B, has been cloned (Earnshaw et al, 1987), but the gene for the two remaining prominent centromere proteins, CENPs -A and -C, have not yet been isolated. Therefore, human autoimmune sera were isolated which react with either CENP-A or -C, and a human fibroblast lambda gt11 cDNA library screened in order to try to isolate the genes encoding either of these (Chapter R10).

R 1.1 ISOLATION OF ANTI-CHROMOSOME SCAFFOLD MABS

Introduction

Various biochemical treatments of isolated chromosomes have been used to obtain a residual chromosome structure termed the "chromosome scaffold" (Laemmli et al, 1978). The chromosome scaffold is depleted of histones (Paulson & Laemmli, 1977) and, thus, is enriched for other more tightly bound proteins. In electron microscopy studies it has been shown that the structure which is most resistant to high salt and restriction enzyme digestion treatment is the centromere (Hadlaczky et al, 1981; Earnshaw et al, 1984). Therefore, in order to try to isolate antigens of the centromeric region, monoclonal antibodies were raised against Chinese hamster chromosome scaffolds (by J. Keresô, of this laboratory). Thus, a previously generated panel of 37 immunoglobulin secreting, hybridoma clones was screened in this study, to identify those which detect components involved in CHO chromosome higher-order structure.

In order to prepare mammalian chromosome scaffolds, CHO chromosomes were isolated in bulk and treated with 2M NaCl and DNase I. The resulting chromosome scaffolds were then used to immunise mice, and subsequently hybridoma clones were generated. 116 clones were found to be positive on the basis of ELISA on CHO chromosome scaffolds. Since these procedures were carried out prior to the start of this project these are dealt with in the appendix. The subsequent retesting of the immunoglobulins secreted by the hybridoma clones, for immunopositivity on chromosomal antigens is given below.

Spot Test for Immunoglobulin Production, Immunofluorescence Microscopy and Western Blotting Analysis of Anti-Chromosome Scaffold Mabs

Of the 116 originally frozen hybridoma clones (in November 1984), 46 were no longer viable, i.e. they could not be thawed and maintained in culture. The remaining 70 were successfully recultured and the supernatants generated by these were tested for immunoglobulin production, as described in Materials and Methods. Of the 70 clones, 33 no longer secreted immunoglobulin, whereas 37 were shown to still secrete immunoglobulin on the basis of spot tests of supernatants immobilised onto nitrocellulose filters and treated with anti-mouse secondary antibodies (see Materials and Methods). In order to determine what, if any, antigens were detected by the Mabs on native, i.e. not treated with 2M NaCl and DNase I, CHO chromosomes, cells were blocked at metaphase with colchicine and the cells harvested by shake-off. These mitotic cells were then made permeable to immunoglobulin by treatment with the detergent Triton-X-100, swollen by incubation in hypotonic solution and cytocentrifuged onto microscope slides. The resulting slides were fixed and incubated with undiluted supernatants from each clone. After washing, secondary antibody coupled to FITC was incubated with each sample, washed and then counter-stained with the DNA binding dye, HOECHST 33258, and mounted. Only 14 of the 37 hybridoma clones gave a detectable signal, and so these were analysed further (for an overall summary of the screening, and a summary of the types of antigens detected by the 14 immunopositive Mabs refer to Table 1.1). Any Mab detecting only a cytoplasmic antigen was discarded from the study (see R 1.1.1). All of the clones were tested on CHO preparations fixed with 100% methanol. If using this fixation procedure the results were unclear then replicate samples were fixed with 4% paraformaldehyde and were treated for indirect immunofluorescence. If under these fixation conditions the staining pattern was still inconclusive then an additional, third, fixation of 100% acetone was made. For a summary of the nature and localisation of the signal detected by the 14 immunopositive Mabs in immunofluorescence microscopy using the three different fixative procedures see Table 1.2.

In parallel to the immunofluorescence studies Western blots were carried out with the 14 clones to determine the antigenic bands, if any, detected by the Mabs in CHO chromosome and nuclear preparations. Where relevant the evolutionary conservation was determined on Western blots with human cell line (Colo) chromosomes and nuclei, *Drosophila melanogaster* S3 cells and *Saccharomyces cerevisiae* .

Table 1.1 Summary of Screening of Anti-Chromosome Scaffold Mabs

A: Hybridoma clones were generated by the immunisation of mice with CHO chromosome scaffold preparations. 116 positive Mabs were selected on the basis of ELISA on CHO chromosome scaffold samples. Of these 70 could be maintained in a viable state in culture, and of these only 37 were still capable of secreting immunoglobulin. Using immunofluorescence microscopy 14 clones were identified which gave an acceptably strong signal on CHO cells. Of these 8 gave signals on metaphase chromosomes or on the interphase nuclei. The remaining 6 only reacted with the cytoplasm and so were discarded from the study.
B: The breakdown of the types of antigens detected by the 14 immunopositive Mabs by immunofluorescence microscopy is shown.

A	SUMMARY: Screening of Hybridoma Mabs		
	Total no. clones	116	
	Successfully cultured	70	(116-46)
	Secreting immunoglobulin	37	(70-33)
	IMMUNOPOSITIVE CLONES	14	(37-23)
	Cytoplasm positive	11	
	Nucleus positive	2	
	Nucleolus positive	2	
	CENTROMERE POSITIVE	6	

B	Type of antigen detected				
clone	cytoplasm	CENTROMERE	nucleolus	nuclear	centriole
AB1	Yes	No	No	No	No
AB4	Yes	No	Yes	No	No
AC1	No	YES	No	No	Yes
BA1	No	No	Yes	No	No
BA2	No	YES	No	No	Yes
BB1	Yes	YES	No	No	No
BC6	Yes	No	No	No	No
BD6	Yes	?	No	No	No
CB3	Yes	No	No	Yes	No
CC5	Yes	No	No	Yes	No
DD3	Yes	No	No	No	No
EA5	Yes	?	No	No	No
EB5	Yes	No	No	No	No
ED3	Yes	?	No	No	No

? Faint centromere positive, possible artefact

R 1.1.1 Cytoplasmic Positive Mabs

Of the 14 immunopositive Mabs, 6 were identified as only reacting with cytoplasmic spots or contours in the cytoplasm of the CHO cells (AB1, BC6, BD6, DD3, EA5 and EB5), tested by immunofluorescence microscopy (see Materials and Methods). Since the aim of this project was to analyse the molecular structure of CHO and, if possible, human centromeres, these cytoplasmic positive Mabs were not of relevance, and hence not pursued.

R 1.2 CHARACTERISATION OF ANTI-CHROMOSOME SCAFFOLD MABS

A detailed account of the strongest reacting and potentially interesting clones on the basis of immunofluorescence microscopy and Western blotting analysis follows in this and the subsequent chapter R2.

R 1.2.1 Clones AB2 and CC1

Note: These two clones are not amongst the 14 immunopositive Mabs, since they were later shown to only give a background reaction in immunofluorescence microscopy studies. However, the study of these two Mabs did demonstrate the sensitivity of the immunofluorescence microscopy detection system, and the importance of distinguishing between immunoglobulin production and secretion, and, thus are included here.

Introduction

Of the 70 clones successfully recultured, two gave borderline results in spot tests for the presence of immunoglobulin in the supernatant (see Materials and Methods). Specifically, supernatants from hybridoma clones AB2 and CC1 gave very faint positive reactions when immobilised onto nitrocellulose filters, incubated with anti-mouse secondary antibodies coupled to HRPO and colour developed. In order not to exclude any possibly interesting clones from the study these were then tested by immunofluorescence and Western blotting.

Immunofluorescence Microscopy

In order to determine if Mabs AB2 and CC1 reacted with CHO antigens, cells were blocked at metaphase by incubation with colchicine, collected by shake-off, washed and concentrated by centrifugation, and prepared for immunofluorescence microscopy. Cells were made permeable to immunoglobulin by incubation in Triton-X-100, and swollen in hypotonic solution. The cells were then cytocentrifuged onto microscope slides and fixed in 100% methanol. Fixed samples were incubated in undiluted supernatant, washed and incubated in FITC-conjugated secondary antibody, washed, and counter-stained with HOECHST 33258 and mounted. In the subsequent immunofluorescence microscopy it appeared that both supernatants contained immunoglobulins reacting weakly with some, but not all of the centromeres of the CHO chromosomes.

Figure 1.1 Indirect Immunofluorescence Microscopy of CHO Colchicine Treated Cells Stained with Supernatants from Mab CC1, Showing Immunonegative Background Staining

CHO cells were colchicine treated to obtain metaphases, cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation, incubated with supernatant from hybridoma clone CC1, and then anti-mouse FITC conjugated secondary antibody, and counter-stained for DNA with HOECHST 33258.

Supernatant CC1 contains no secreted immunoglobulin, hence **A** shows the background staining obtained by immunofluorescence. The faint centromere (large arrows) and even fainter telomere (smaller arrows) staining are brought about by the difference in decondensation of the various regions of the chromosome. The hypotonic treatment of the chromosomes results in an overall chromatin decondensation; the telomeres and the centromeres are more densely packaged than the chromosome arms. The faint centromere and telomere staining is due to a background staining of these more densely packaged regions by the FITC secondary antibody. **A'** is the staining for DNA of **A**.

Magnification: x5,000.

A
v
V
v
V
A'

(Fig 1.1. Apparent faint centromere, and very faint telomere staining of CHO metaphase chromosomes by CC1, AB2, not shown). However, these result could not be observed on every slide prepared and so placed the validity of this observation in doubt.

Western Blotting

In order to determine if Mabs AB2 and CC1 reacted with protein bands of CHO, chromosomes and nuclei were isolated in bulk, the proteins separated by 5-20% gradient SDS-PAGE, and electrophoretically transferred to nitrocellulose filters. In the resulting Western blots using supernatants from clones AB2 and CC1, no antigen could be detected in the CHO chromosome and nuclei protein samples (results not shown).

Immunoglobulin Production versus Secretion

Due to the faint reaction in the spot test for immunoglobulin presence and the difficulty in reproducing the staining pattern detected by immunofluorescence microscopy, it was considered possible that clones AB2 and CC1 were still producing immunoglobulin but no longer able to secrete this product into the surrounding medium. To determine if this was the case for these two clones, AB2, CC1 and ED3 (positive control clone known to secrete immunoglobulin, see R1.2.2), hybridoma clone cells were collected by centrifugation, incubated with Triton-X-100 and passed through a gauge 23 needle to break the cell walls and liberate any intracellular immunoglobulin. Cell debris were then removed by centrifugation and the intracellular fraction tested in parallel with normal supernatants from the two clones by spot tests for immunoglobulin production. It was found that there was no longer a detectable level of immunoglobulin secreted by clones AB2 and CC1 (results not shown). However, the lysates of AB2, CC1 and ED3 all contain immunoglobulin, (not shown). Therefore, it can be concluded that AB2 and CC1 are still producing immunoglobulin but are no longer able to secrete this product to the extracellular medium.

ELISA of AB2 and CC1 Supernatants

Consistent with the above finding was the result of an ELISA carried out in another laboratory (Dr. Éva Monostori, Immunology, BRC, personal communication). Supernatants from clones AB2, CC1, and AC1 as a positive control (see R2.4) were tested by ELISA, and AC1 was shown to secrete low, but significant levels of immunoglobulin, whereas AB2 and CC1 gave a result of the same order as that obtained for the negative control, RPMI (hybridoma culture medium).

Discussion

From these initial screening experiments of a panel of monoclonal antibodies raised against CHO chromosome scaffolds, the following was found. Two of the hybridoma clones, AB2 and CC1, amongst the 70 tested, and the 33 not secreting, were still in fact producing immunoglobulin but no longer able to export it to the extracellular medium. The initial very weak reaction obtained in the spot test may have been due to a small percentage of the population at that time still secreting immunoglobulin, or alternatively cell lysis in culture liberating low levels of immunoglobulin. This may also explain the inconsistent reaction obtained in immunofluorescence microscopy.

It is, however more probable that the faint centromere and even fainter telomere staining was brought about by a varying level of decondensation of the different regions of the chromosomes. The hypotonic treatment of the chromosomes, required for immunofluorescence microscopy, gives an overall decondensation of the chromatin, and the telomeres and the centromeres are more densely packaged than the chromosome arms. Thus, the faint centromere and telomere staining may be due to a background staining of these more densely packaged regions, by the anti-mouse FITC conjugated secondary antibody.

However, since it is not practical to exploit non-immunoglobulin secreting clones, these two clones were not pursued further.

R 1.2.2 Mab ED3 Detects a 116kD Cytoplasmic Protein

Immunofluorescence Microscopy

In order to determine the cellular localisation of the antigen detected by hybridoma clone ED3, which was one of the 14 immunopositive clones selected for further study (see Table 1.2), CHO cells were colchicine treated, cytocentrifuged onto slides and incubated with supernatant. Upon examining these slides the results were difficult to interpret. Using both 100% methanol (Fig 1.2.A), which precipitates proteins, and 4% paraformaldehyde (not shown), which is a protein cross-linking reagent (Harlow and Lane, 1988), the cytoplasm but not contours associating with the chromosomes, could be observed. In order to try to reduce the cytoplasmic staining a 100% acetone (which decreases the cytoplasmic background more than the other organic solvent used) fixation was made. This did reduce the intensity of this cytoplasmic reaction, and made visible pairs of dots, which could be observed at the centromeres on some preparations (Fig 1.2.B). These were not present on every chromosome, but numbered between 0 and 8 per cell. This result was difficult to consistently replicate, thus raising the possibility of this not being a specific reaction but an artefact. Nevertheless, an indication of the possible nature of the antigen detected by Mab ED3 was indicated by the concurrent Western blot analysis detailed below.

Figure 1.2 Indirect Immunofluorescence Microscopy of CHO Colchicine Treated Cells Stained with Supernatant from Mab ED3

CHO cells were colchicine treated to obtain metaphases, cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation, incubated with supernatant from hybridoma clone ED3, and then anti-mouse FITC conjugated secondary antibody, and counter-stained for DNA with HOECHST 33258.

In **A** the faint cytoplasmic contours (**cc**) can be seen on the metaphase spread, using 100% methanol as the fixative. Using 100% acetone as the fixative, resulted in several pairs of centromere dots (**cd**) being detected on some metaphase spreads, as in **B**. **A'** and **B'** are the HOECHST 33258 counter-staining for DNA images of **A** and **B**, respectively.

Magnification: x2,500.

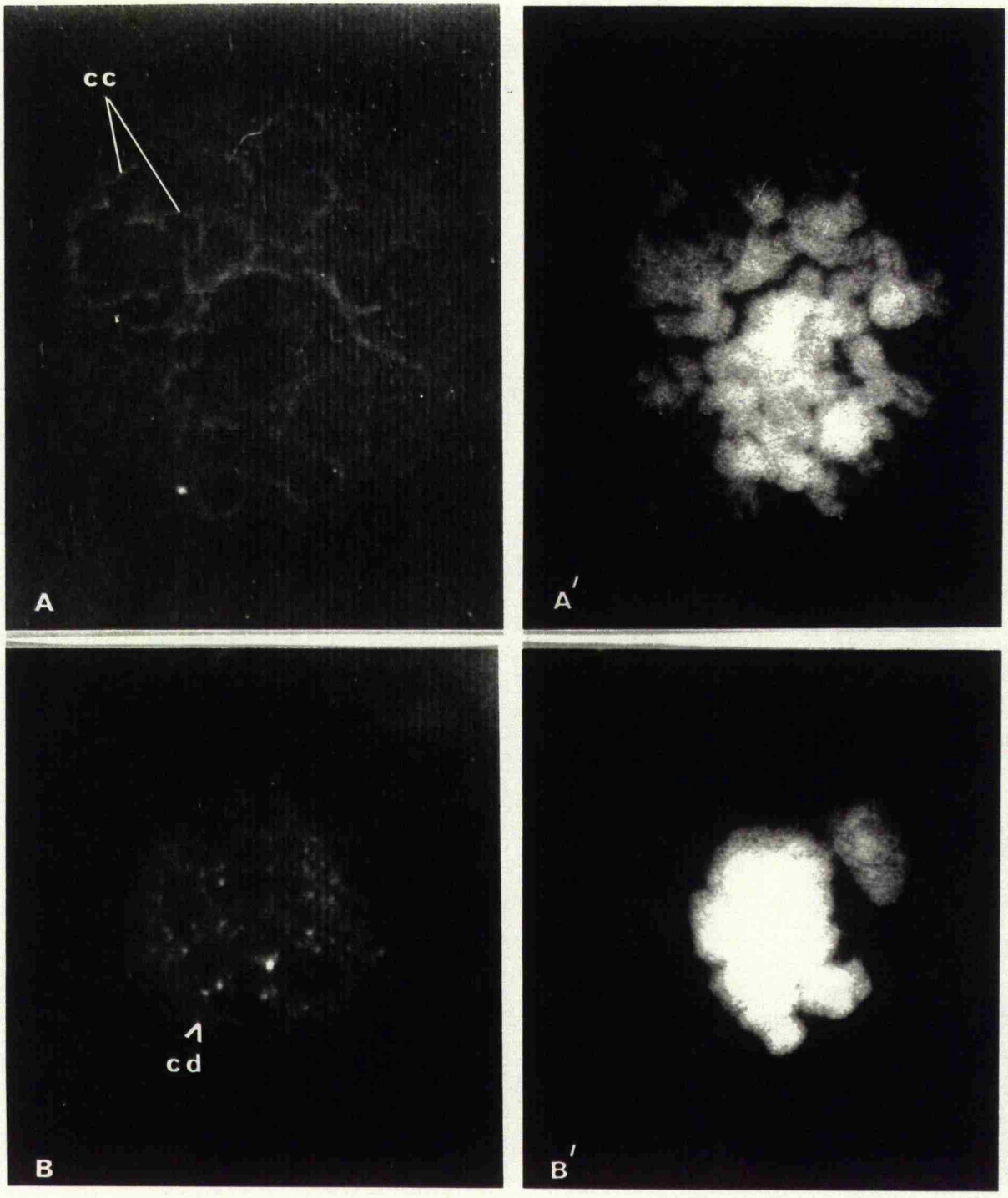
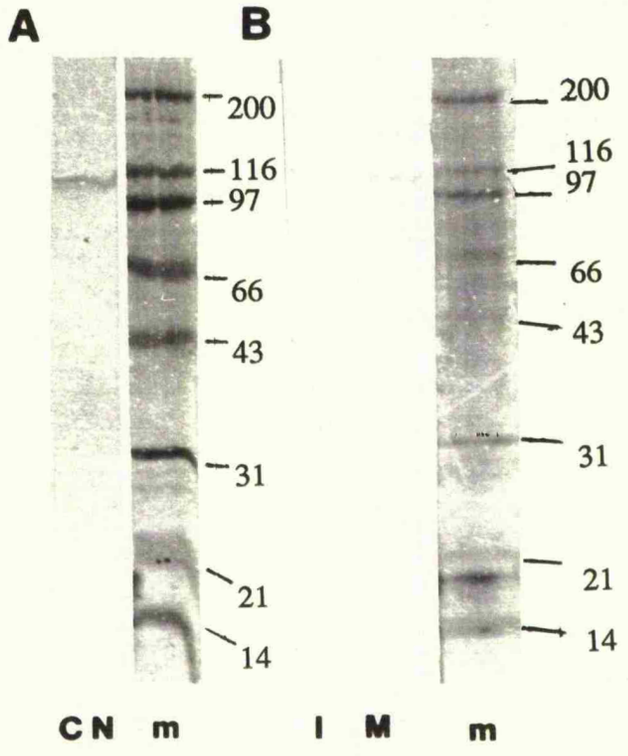
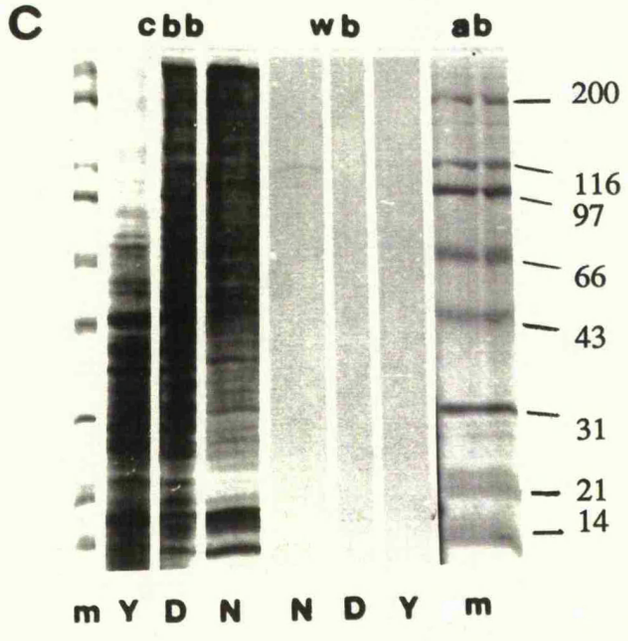

cc
A
A'
cd
B
B'

Figure 1.3 Western Blots of CHO, Human Colo, Drosophila and Yeast Protein Samples with Mab ED3

To determine the molecular weight of the antigen detected by Mab ED3, CHO chromosome scaffold and nuclear matrix proteins were separated by 5-20% SDS-PAGE and electrophoretically transferred to nitrocellulose filters. The subsequent Western blots showed that the antigen detected by the ED3 antibodies in the supernatant has a molecular weight of about 116kD on CHO and human protein samples.

A: Immunological treatment of CHO chromosome scaffold (**C**) and nuclear matrix (**N**) proteins using supernatant secreted by hybridoma clone ED3. **m** are Coomassie Blue stained high and low molecular weight markers of the following values: myosin 200kD, *E.coli* beta-galactosidase 116.25kD, rabbit muscle phosphorylase b 97.4kD, bovine serum albumin 66.2kD, hen egg white ovalbumin 42.699kD, bovine carbonic anhydrase 31.0kD, soybean trypsin inhibitor 21.5kD, hen egg white lysozyme 14.4kD. Notice the doublet detected on both chromosome scaffold and nuclear matrix proteins.
B: Immunological treatment CHO interphase cytoplasm proteins (**I**), obtained by TCA precipitation of interphase lysate, nuclear matrix (**M**) proteins using supernatant secreted by hybridoma clone ED3. **m** are Coomassie Blue stained high and low molecular weight markers as in **A**. An antigen of weight 116kD can be detected in the interphase cytoplasm in addition to its distribution in the nuclear matrix.
C: Immunological treatment of human (Colo) nuclear extract (**N**), *Drosophila melanogaster* S_3 total cell (**D**), and *Saccharomyces cerevisiae* (**Y**) proteins. **m** are Coomassie Blue stained high and low molecular weight markers as in **A**. **ccb** is Coomassie Brilliant Blue staining of proteins in the gel, **wb** is the immunological treatment of the Western blot of the proteins, and tested with ED3 supernatant, **ab** is the Amido Black staining of the proteins transferred to the nitrocellulose filter. The 116kD antigen (doublet) is conserved between human and CHO cells, but not to Drosophila or yeast.


A
B
200
116
97
66
43
31
21
14
200
116
97
66
43
31
21
14
C N m
I M m


C
cbb
wb
ab
200
116
97
66
43
31
21
14
m Y D N
N D Y m

Western Blotting

In order to determine the antigen(s) detected by the Mab secreted by hybridoma clone ED3, CHO chromosome and nuclear extracts (2M NaCl treated), and interphase cytoplasmic proteins (since the antigen appeared to be cytoplasmic in location) were separated on 5-20% gradient SDS-polyacrylamide gels (Fig 1.3). The separated proteins were then electrophoretically transferred to a nitrocellulose filter. The filter was incubated with supernatant from hybridoma clone ED3, washed, incubated with anti-mouse HRPO-conjugated secondary antibody, washed, and colour developed. In CHO chromosome and nuclear extracts, and interphase cytoplasm TCA precipitated proteins, an antigen of molecular weight 116kD was detected. In order to examine the evolutionary conservation of this antigen, a Western blot of human (Colo) nuclear extract, *Drosophila melanogaster* and *Saccharomyces cerevisiae* total cell proteins was made (Fig 1.3C). In a Colo nuclear extract a doublet of molecular weight 116kD, as with the CHO samples, could be observed. However, no positive signal was obtained for blots of Drosophila or yeast. Thus, the 116kD antigen is only conserved between the two mammalian species analysed.

Discussion

It has been previously suggested that a 115kD doublet, identified by SDS-PAGE, is a component of the CHO midbody matrix (Mullins & McIntosh, 1982). The midbody matrix itself is the central zone in anaphase which corresponds to the region connecting the daughter cells at the cleavage furrow formed as the cell completes division. Since colchicine treated cells are blocked at metaphase no anaphase or telophase cells were available for observation. Therefore, an analysis of anaphase and telophase cells would be required to determine if this Mab actually does recognise a component of the midbody matrix. In addition to its cytoplasmic location, the antigen detected by ED3, however, was shown to be present in CHO 2M NaCl treated chromsomes and nuclei, and human (Colo) nuclear extract proteins. These results are most consistent with the antigen being a cytoplasmic component, which is resistant to high salt treatment or a contaminant of the salt treated sample. However, since the centromere staining was doubtful, and since the antigen appeared to be primarily cytoplasmic in location, clone ED3 was not pursued further.

R 1.2.3 Unidentified Fluorescing Objects (UFOs): Mabs CB3 and CC5

Immunofluorescence Microscopy

The immunofluorescence studies of CHO cells blocked at metaphase and treated as previously for microscopy, revealed that the antibodies secreted by hybridoma clones CB3 and CC5 recognised some, as yet undefined, components of the nucleus.

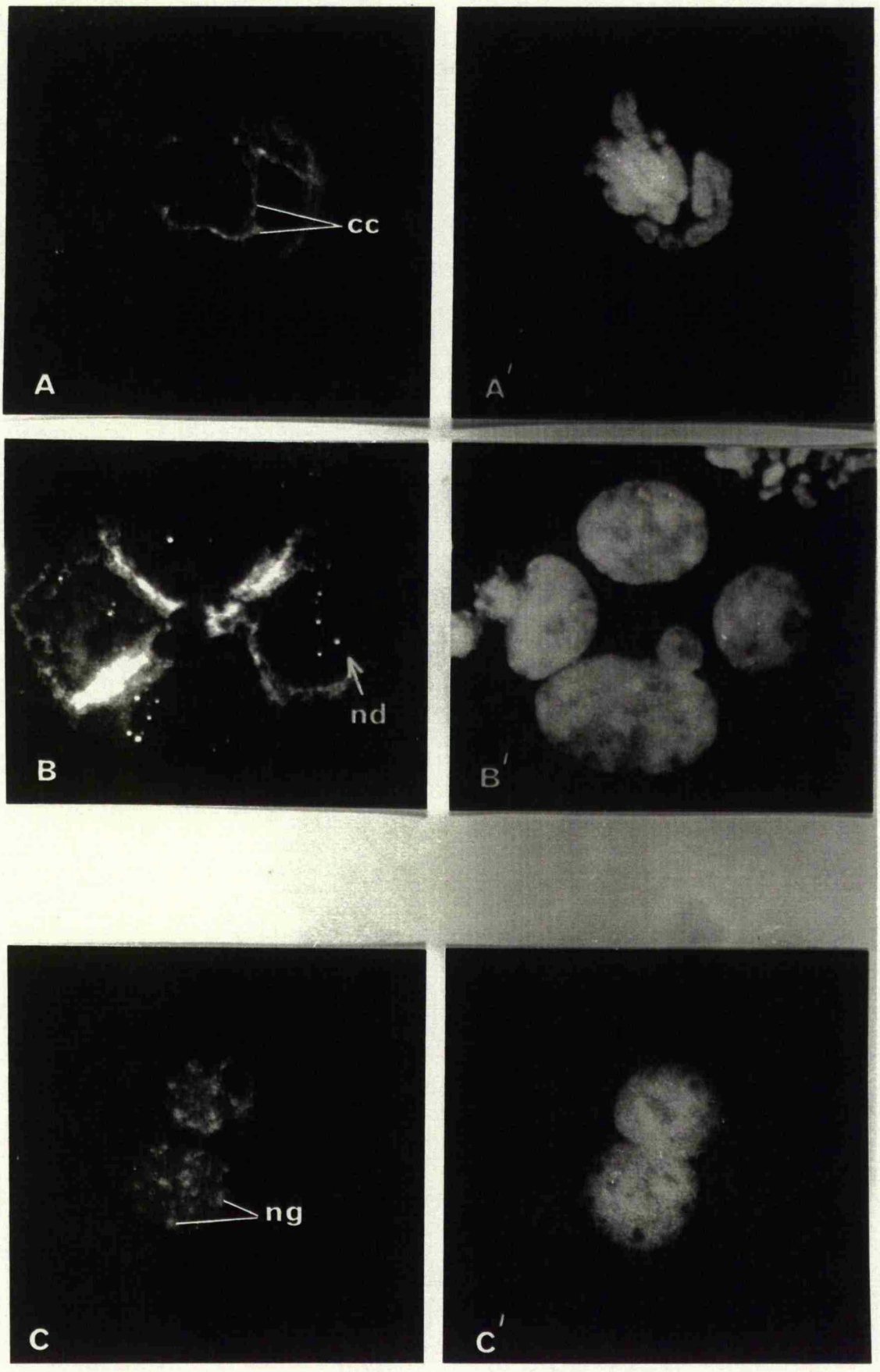
Figure 1.4 Indirect Immunofluorescence Microscopy of CHO Colchicine Treated Cells Stained with Supernatants from Mabs CC5 and CB3

CHO cells were colchicine treated to obtain metaphases, cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation, incubated with supernatants from hybridoma clones CC5 and CB3, then anti-mouse FITC conjugated secondary antibody, and counter-stained for DNA with HOECHST 33258.

The typical cytoplasmic contours (**cc**) stained by Mab CC5 on metaphase preparations can be seen in **A**. Notice the fine speckles in interphase in the cytoplasm, the nucleus, which is immunonegative, and the small brightly decorated dots (**nd**) in **B**. **A'** and **B'** are the DNA counter-stained images of **A** and **B**, respectively.

The staining pattern obtained using Mab CB3 is shown in **C**. The cells are decorated with various sized nuclear granules (**ng**). **C'** is the counter-staining for DNA with HOECHST 33258 of **C**.

Magnifications: A: x3,000; B: x3,000; C: x3,000.


cc
A
A'
nd
B
B'
ng
C
C'

Mab CC5

Using 100% methanol fixation Mab CC5 gave a cytoplasmic staining, such that contours and fine speckles were observed on both interphase and metaphase preparations. In metaphase the contours followed the outline, not of individual, but groups of chromosomes (Fig 1.4.A). In interphase the nucleus as a whole was immunonegative, however, within each nucleus between 3 to about 10 very small brightly stained dots could be observed (Fig 1.4B). The same staining pattern was obtained in parallel immunofluorescence studies on HeLa cells using 100% methanol fixation (not shown), demonstrating that the antigen is conserved between CHO and humans. CHO cells when fixed with 4% paraformaldeyde gave a much stronger contour and general cytoplasmic staining, so much so that it was not possible to determine if the nuclear dots still occurred (results not shown).

Mab CB3

Mab CB3, stained various sized fine granules in the nuclei of interphase CHO cells fixed onto slides with 100% methanol (Fig 1.4.C). The pattern resembled that obtained by anti-snRNP antibodies (Tan, 1966; Nyman et al, 1986). In the surrounding cytoplasm very fine spots were stained, and this Mab also gave an apparently positive signal on metaphase spreads. Spots appeared to be present on the chromosome arms, but these could not be detected on every chromosome of a given spread (data not presented). Furthermore, this result was difficult to reproduce, thus placing the validity of this observation in doubt.

Western Blotting

On Western blots of CHO chromosomes, nuclei and nuclear matrix protein samples no antigen could be detected by clone CB3, whereas clone CC5 did react with a protein band of molecular weight 20kD on CHO nuclear matrix (Fig 2.3A, Chapter R2).

Discussion

Since the exact nature of the antigens detected by hybridoma clones CB3 and CC5 cannot be stated they have been termed "unidentified fluorescing objects". The granules decorated in the nuclei by Mab CB3 appear similar to the pattern observed for anti-snRNP autoimmune sera staining of human cells (Tan, 1966; Nyman et al, 1986).

However, the inability to detect a band, by Western blotting using Mab CB3, makes this mere speculation. Since the two Mabs react with antigens that are difficult to define, and did not appear to be involved in higher-order chromosome structure they were noted but not pursued further.

R 1.2.4 Nucleolus Positive Mabs AB4 and BA1

Immunofluorescence Microscopy

To determine the *in vivo* localisation, if any, of the antigens detected by the antibodies secreted by hybridoma clones AB4 and BA1 on metaphase preparations, CHO cells were colchicine treated and cytocentrifuged onto slides. Using 100% methanol fixation and incubation of slides with supernatants from both clones AB4 and BA1, antigens were observed in the following locations. For both Mabs very fine, and abundant speckles occurred in the cytoplasm, and slightly larger and less frequent speckles occurred in the nucleolus and in the nucleoplasm of interphase cells (Fig 1.5). The staining pattern was apparently identical for both clones. The fine speckles found in the nucleus appeared to occur at a particular, but undefined stage of interphase, since this speckled staining was only detected on some of the interphase cells. However, it cannot be ruled out that this speckled pattern could possibly be an artefact of preparation. In contrast it was possible to consistently reproduce the nucleolus and cytoplasmic staining (Fig 1.5) in replicate experiments, indicating that these were not artefacts. In addition to the above staining pattern, Mab AB4, but not BA1, also detected a contour following the outline of the chromosomes. This pattern was also obtained with paraformaldehyde fixation with Mab AB4, but Mab BA1 did not give an immunopositive reaction using 4% paraformaldehyde fixation, suggesting that this type of treatment denatures the antigen or epitope.

The antigen detected by Mab AB4 was apparently localised to the periphery of the chromosomes (as usual for anti-nucleolar antibodies, see discussion later in this chapter), however, this staining decreased in intensity as the amount of cytoplasm remaining associated with the chromosomes decreased. Thus, this indicates that the antigen is in the cytoplasm surrounding the chromosomes and not on the chromosomes themselves. This conclusion is consistent with the acetone fixation data. For 100% acetone fixation (which is more effective in removing the cytoplasm from preparations than either methanol or paraformaldehyde) the chromosome contours could no longer be detected (not shown), and only the nuclei were stained by Mab AB4.

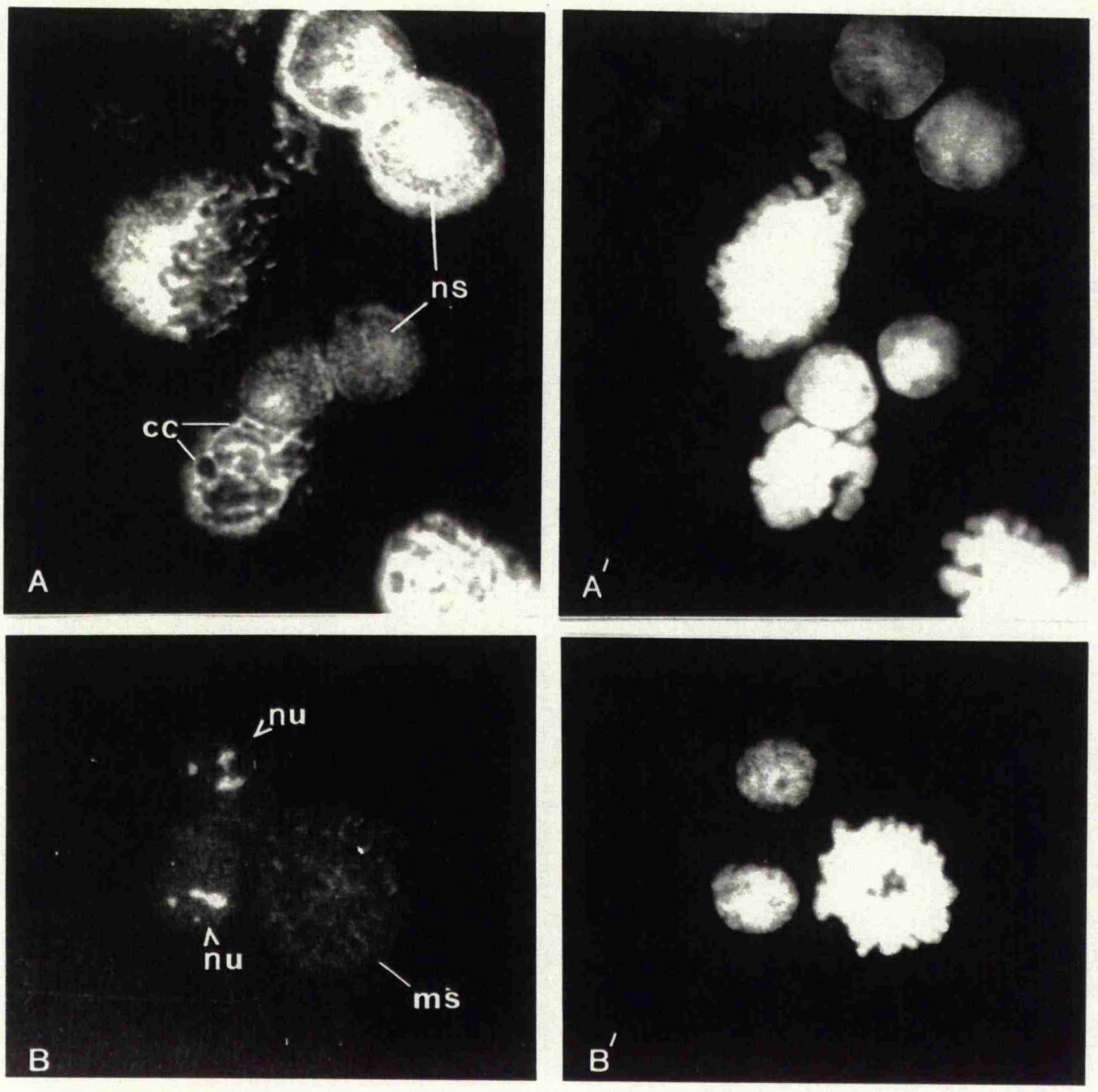
In order to determine if this AB4 antigen is conserved between CHO and humans, HeLa cells were prepared for immunofluorescence.
An identical staining pattern of the nucleolus, cytoplasm and chromosome contours was obtained by 100% methanol fixation of HeLa cells (results not shown), thus showing that the antigen is conserved between CHO and HeLa cells.

Figure 1.5 Indirect Immunofluorescence Microscopy of CHO Colchicine Treated Cells Stained with Supernatant from Mab AB4

CHO cells were colchicine treated to obtain metaphases, cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation, incubated with supernatant from hybridoma clone AB4, then anti-mouse FITC conjugated secondary antibody, and counter-stained for DNA with HOECHST 33258.

Notice the strong chromosome contours (**cc**) stained by Mab AB4 on the metaphase spreads (**ms**) in **A** and **B**. At different stages of interphase either nuclear speckles (**ns**) or the nucleoli (**nu**) are decorated by antibody AB4. **A'** and **B'** show the DNA staining patterns of **A** and **B**, respectively.

Magnification: x3,000.


ns
cc
A
A′
nu
nu
ms
B
B′

Western Blotting

Since both AB4 and BA1 Mabs react with the nuceolus and components of the cytoplasm then it was anticipated that a positive signal be observed on nuclei, but not on chromosomes, since in a good chromosome isolation the cytoplasm is removed. To investigate this, CHO protein samples were separated by 5-20% gradient SDS-PAGE, and electrophoretically transferred to nitrocellulose. The subsequent Western blots of the proteins of the CHO chromosomes and nuclei treated with supernatant from clone BA1 did not give a positive signal. However, blots incubated with AB4 did reveal an antigen of molecular weight 40kD on CHO nuclear proteins (data not shown), but no antigen could be detected on the chromosome sample (not shown). To determine if the antigen(s) detected by clone BA1 in immunofluorescence on CHO is (are) present in another species a further Western blot of a Colo nuclear extract was prepared. In spite of the negative result obtained for clone BA1 on CHO samples, an antigen of molecular weight 30kD was weakly detected on Colo nuclear extracts (results not shown), suggesting that the antigen in CHO may have been denatured.

Discussion

Hybridoma clones BA1 and AB4 stain antigens found in the cytoplasm, the nucleolus and the nucleoplasm. In addition AB4 recognises an antigen which is cytoplasmic but associates with the periphery of the chromosomes. Mab AB4 detects an antigen of molecular weight 40kD in nuclei from CHO, and BA1 30kD in human (Colo) nuclear extract proteins. Considering the molecular weight of the antigen detected by AB4 on CHO protein samples, and BA1 on a human sample, and the chromosome contour staining exhibited by AB4, then a relationship with "perichromin" (McKeon et al, 1984) may be indicated. The perichromin antigen is located to the periphery of chromosomes. However, perichromin is also found on the nuclear envelope, a staining pattern which was not observed for either clone BA1 or AB4. Perichromin has a molecular weight of 33kD in CHO cells. Thus, perichromin, although not the same as the antigens detected by BA1 and AB4, may be related to the antigens detected by these two Mabs.

Alternatively, a nucleolar antigen detected by an autoimmune serum has been localised to fine grains in the cytoplasm, the nucleolus and the periphery of chromosomes and has a molecular weight of 38kD in CHO cells (Hadlaczky & Praznovszky, 1988). Therefore, the antigens detected by the two hybridoma clones AB4 and BA1 may be such nucleolar antigens. However, since neither Mab detects a chromosome specific structure, then they were deemed of limited interest for this research.

R1 SCREENING OF ANTI-CHROMOSOME SCAFFOLD MABS

Summary

Of the 14 immunopositive clones, 6 Mabs detected solely cytoplasmic components; ED3 detected an apparently primarily cytolasmic antigen, but possibly a chromosome specific centromere antigen; CB3 and CC5 detected obscure nuclear and cytoplasmic antigens; AB4 and BA1 decorated nucleolar antigens. The remaining three Mabs detected antigens of a very similar nature and so are presented together in a separate, subsequent chapter, R2.

Table 1.2 Summary of Nature and Localisation of Signal in Immunofluorescence Microscopy of Immunopositive Mabs

To determine the cellular localisation of the antigens detected by the Mabs, CHO cells were blocked at metaphase by colchicine treatment and prepared for immunofluorescence microscopy, as described in Materials and Methods. All Mabs were tested using 100% methanol as the fixative. If the result was ambiguous, or further details required two other fixation procedures were utilised, 4% paraformaldehyde (which cross-links cytoplasmic proteins) and 100% acetone (which removes a greater degree of cytoplasmic proteins than does 100% methanol). Of the 14 Mabs screened in this manner, 6 were shown to only detect cytoplasmic antigens (AB1, BC6, BD6, DD3, EA5 and EB5), and thus were discarded from the study. The remaining 8 decorated components of the chromosomes or nuclei and are described in R 1.2.2-4 and R 2.

CLONE	100% methanol	4% paraformaldeyde	100% acetone
AB1	Cytoplasm spots Nucleolus	_	ND
AB4	Cytoplasm spots Nucleolus Chromosome contours	Chromosome contours	Nucleolus
AC1	Centromere rings Centriole	Centromere rings	Centromere rings
BA1	_	Cytoplasm Nucleolus	ND
BA2	Centromere rings Centriole	ND	ND
BB1	Cytoplasm spots	Cytoplasm contours	Centromere spots
BC6	Cytoplasm	Cytoplasm contours	Cytoplasm
BD6	_	Cytoplasm contours Centromere?	Cytoplasm contours
CB3	Large nuclear spheres	ND	ND
CC5	Cytoplasm contours Nuclear spots	Cytoplasm contours	ND
DD3	Cytoplasm	Cytoplasm	_
EA5	_	Cytoplasm Centromere? Telomere	Chromosome contours
EB5	Cytoplasm contours	Cytoplasm contours	ND
ED3	Cytoplasm contours	Cytoplasm contours	Cytoplasm contours Centromere?

? Staining was difficult to replicate _ No positive staining observed ND Not done

CONTENTS

R 2 CHARACTERISATION OF ANTI-CENTROMERE MABS

On the basis of the screening of the anti-chromosome scaffold Mabs by immunofluorescence microscopy, of the 14 clones analysed, three centromere positive clones were identified, hybridoma clones: AC1, BA2 and BB1, and the experiments using these are described here. Centromere positivity *per se* , however, was not a sufficient requirement for a Mab to be used in further analyses of the centromere. Cross-reactions with cytoplasmic proteins, for instance, would make studies more difficult to interpret. Thus, the general characterisation of the centromere antigens detected by these Mabs, and their suitability for further studies of the centromere, will now be treated in turn.

R 2.1 ANTI-CENTROMERE MAB BB1

Immunofluorescence Microscopy

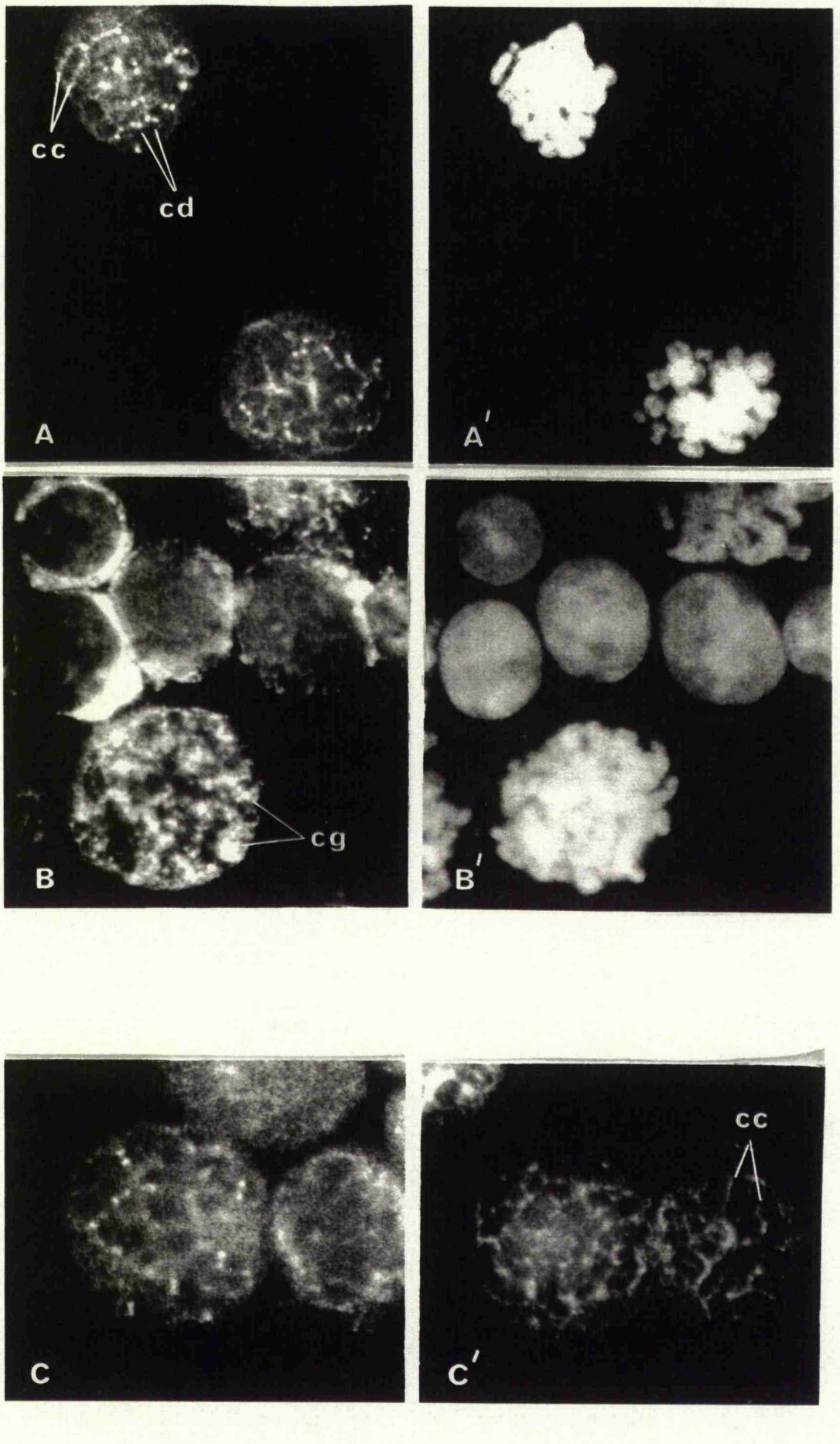
In order to determine the *in vivo* location of the antigens detected by the antibody secreted by hybridoma clone BB1, CHO colchicine treated cells were cytocentrifuged onto slides to be processed for immunofluorescence microscopy (Fig. 2.1), as previously described. When 100% methanol fixation was used the following pattern was observed for interphase cells. A faint uniform nuclear staining was obtained, and this was surrounded by a strong staining of numerous speckles in the cytoplasm (not shown). In metaphase spreads the overall intensity of the immunofluorescence was reduced. There was a faint staining of the cytoplasm surrounding the chromosomes, giving a contour appearance similar, or the same, as that obtained with Mab AB4 (see Fig. 2.1C). A possible relationship between the antigen(s) detected by the two different clones is dealt with in the subsequent section (Chapter R2.1.1). In addition to the cytoplasmic pattern a faint positive signal was observed at the centromeres (Fig. 2.1). Two small dots could be detected at each centromere in a manner similar to that given by anti-centromere autoimmune sera (Fig. 2.2B and D). However, this positive reaction could not always be faithfully reproduced. In order to determine if this pattern were perhaps artifactual, different fixation procedures were attempted. As might be expected, the immunofluorescence observed using 4% paraformaldehyde (which cross-links proteins) as the fixative, revealed the cytoplasmic contours. The presence of a strong cytoplasmic staining, however, meant that any potential centromere reaction would have been obscured. If the centromere antigen were possibly masked, or the epitope altered using the methanol fixation then a different fixation method, which reduces the amount of cytoplasm, might give a stronger reaction for the centromere. Therefore, to reduce the cytoplasmic staining a 100% acetone fixation was carried out. This revealed that the centromere reaction did indeed become stronger (Fig. 2.1A), and this was shown to be consistently reproducible under these conditions. As would be expected the cytoplasmic staining was reduced, although still detectable.

Figure 2.1 Indirect Immunofluorescence Microscopy of CHO Colchicine Treated Cells Stained with Supernatants from Mabs BB1 and AB4

CHO cells were colchicine treated, cytocentrifuged, prepared for immunofluorescence, and incubated with supernatants from hybridoma clone BB1 (**A, B** and **C**) or AB4 (**C'**); then anti-mouse FITC conjugated secondary antibody, and counter-stained for DNA with HOECHST 33258 (**A'** and **B'** of **A** and **B**, respectively).

A: Using 100% acetone as the fixative the cytoplasmic contour staining (**cc**) was reduced (compare with **B**) and the centromere dots (**cd**) were more prominant.
B: Using 100% methanol the cytoplasmic staining was much stronger than in **A**, and cytoplasmic granules (**cg**) were observed.
C: Notice the similarity between the cytoplasmic contours (**cc**) obtained with antibodies from hybridomas BB1 (**C**) and AB4 (**C'**). For staining with BB1 100% acetone, and with AB4 100% methanol was used.

Magnifications: A: x2,500; B: x3,000; C: x3,000.


cc
cd
A
A′
cg
B
B′
C
cc
C′

Refer to Fig. 2.1B to compare this reduced cytoplasm, and stronger centromere pattern with the strong cytoplasmic staining obtained, using 100% methanol as the fixative.

To demonstrate that the BB1 antigen was novel, i.e. not the same as the centromere proteins detected by human anti-centromere autoimmune sera, the staining pattern obtained for this Mab was compared with that obtained by one such serum available in this laboratory. Therefore, CHO cells were prepared for immunofluorescence using the serum LU851 (Hadlaczky et al, 1989) on CHO colchicine treated cells. After incubation with the human anti-centromere serum, slides were treated with anti-human FITC conjugated secondary antibody and prepared for microscopy by the standard procedure. The staining pattern exhibited by Mab BB1 was different to that of the anti-centromere serum as can be seen in Fig. 2.2. Mab BB1 decorates a cytoplasmic antigen, and although both antibodies react with the metaphase centromeres, BB1 does not stain the interphase centromeres, whereas serum LU851 does. Therefore, Mab BB1 does not react with the major centromere proteins isolated to date.

Western Blotting

To determine if Mab BB1 was able to detect antigenic bands in CHO chromosomal and nuclear proteins, Western blots were prepared of these samples by the standard procedure and tested with supernatant from clone BB1. However, no positive signal could be detected (results not shown). Since the original immunising material resulting in the generation of the panel of Mabs was chromosome scaffolds, it was possible that the antigen might be retained, and enriched for in 2M NaCl and DNase I treated CHO protein samples. To determine the molecular weights of any such antigen(s) detected by immunoglobulin secreted by hybridoma clone BB1, CHO chromosome scaffold and nuclear matrix proteins were separated by 5-20% gradient SDS-PAGE and electrophoretically transferred to nitrocellulose (Fig. 2.3). When this blot was incubated with supernatant from clone BB1 bands of molecular weights 40 and 66kD could be detected in the nuclear matrix proteins (Fig. 2.3B). However, no positive signal was produced in the chromosome scaffold proteins (data not shown). This indicated that the antigen or epitope is not present in detectable quantities, or is degraded in the chromosome scaffold fraction.

Discussion

On the basis of the immunofluorescence data, hybridoma clone BB1 has been shown to react with the CHO centromere, as well as a component of the cytoplasm. Since the Western blot of CHO chromosome scaffolds did not give a positive result, the molecular weight of the centromere antigen detected by clone BB1 could not be determined.

Figure 2.2 Comparative Indirect Immunofluorescence Microscopy of Mouse Anti-Chromosome Scaffold Centromere Mab BB1, and Human Anti-Centromere Autoimmune Serum LU851 on CHO Colchicine Treated Cells

To differentiate the staining obtained with Mab BB1 and the human anti-centromere autoimmune serum, LU851 (which detects the major human centromere proteins), CHO cells were stained with both these antibodies. CHO cells were colchicine treated, cytocentrifuged and prepared for immunofluorescence.

Both antibodies decorated dots (**d**) at the centromeres, in an apparently identical manner, compare **A** (BB1 staining pattern) and **B** (LU851 staining pattern) metaphases. Mab BB1, however, additionally stained a cytoplasmic antigen in metaphase (**A**) and in interphase (**C**), which LU851 did not (**B** and **D** respectively). Moreover, BB1 did not detect the interphase centromeres (**ic**) (**C**), in contrast to LU851, which does (**D**). **A'**, **B'**, **C'** and **D'** are the HOECHST 33258 DNA counter-stained images of **A**, **B**, **C** and **D**, respectively.

Magnifications: A&B: x3,000; C&D: x2,000.

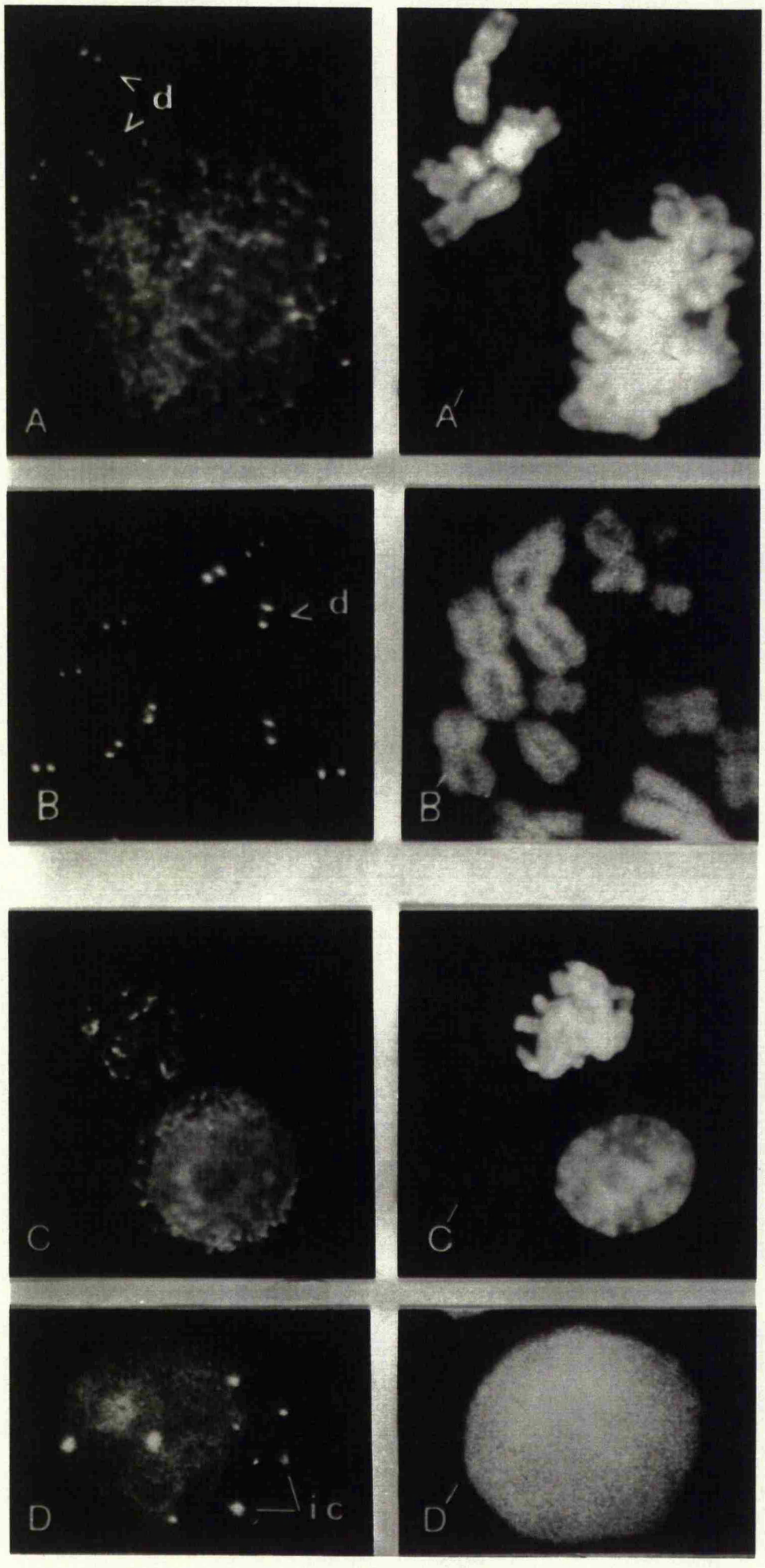
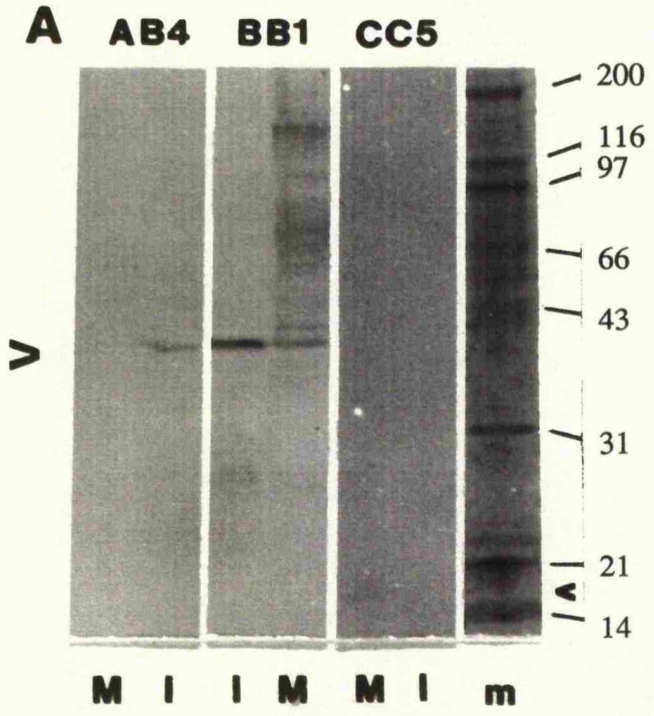

d
A
A'
d
B
B'
C
C'
ic
D
D'

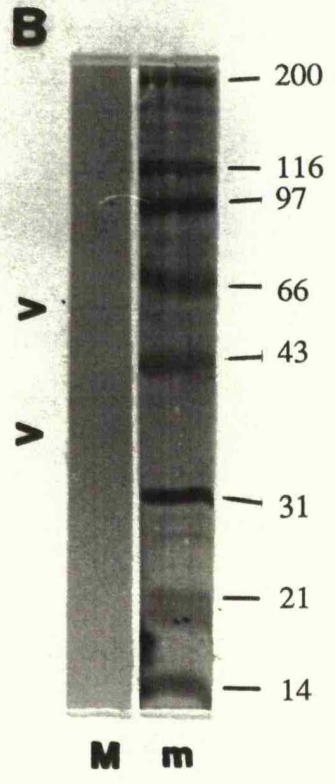
Figure 2.3 Western Blot of CHO Nucear Matrix and Interphase Cytoplasm Proteins Treated with AB4 (anti-cytoplasm Mab), BB1 (anti-centromere and -cytoplasm Mab) and CC5 (unidentified anti-nuclear Mab)

To determine the molecular weight of the antigen detected by Mabs AB4, BB1, and CC5, CHO nuclear matrix (**M**) proteins were separated by 5-20% SDS-PAGE and electrophoretically transferred to nitrocellulose filters. Since the cytoplasmic contour staining patterns exhibited by Mabs AB4 and BB1 appeared identical, interphase cells were lysed and proteins isolated by TCA precipitation, and this fraction (**I**) included in the Western blot. **m** are Coomassie Blue stained high and low molecular weight markers of the following values: myosin 200kD, *E.coli* beta-galactosidase 116.25kD, rabbit muscle phosphorylase b 97.4kD, bovine serum albumin 66.2kD, hen egg white ovalbumin 42.699kD, bovine carbonic anhydrase 31.0kD, soybean trypsin inhibitor 21.5kD, hen egg white lysozyme 14.4kD.

A: Mab CC5 weakly detected an antigen of molecular weight of about 20kD, see small arrowhead. The anti-centromere and -cytoplasm Mab, BB1 (undiluted supernatant), cross-reacted with several high molecular weight antigens in the nuclear matrix. In the interphase cytoplasm this Mab gave a single strong signal of a protein band of approximate molecular weight 40kD, a band which the anti-cytoplasm Mab, AB4, also appeared to detect (large arrowhead).

B: Using a 1/10 dilution of supernatant from hybridoma clone BB1 two antigens, of molecular weights 40kD and 66kD were detected, see arrowheads, in CHO nuclear matrix (**M**) proteins.


A
AB4
BB1
CC5
200
116
97
66
43
31
21
14
M I I M M I m


B
200
116
97
66
43
31
21
14
M m

Nor is it possible to state that the bands obtained in nuclear matrix proteins (which should be free of cytoplasm) correspond to the cytoplasmic contour antigen(s). This point will be dealt with in the next section.

R 2.1.1 Possible Relationship between Cytoplasmic Contours of Mabs AB4 and BB1

When the immunofluorescence patterns obtained for Mabs AB4 and BB1 are compared, then the cytoplasmic contours around the periphery of the chromosomes appear to be very similar if not the same (Fig. 2.1C). Therefore, it was considered possible that the antigen responsible for the cytoplasmic staining was common to both clones. To test this hypothesis total CHO interphase cells were harvested and the cytoplasm was precipitated using TCA. A Western blot of this interphase cytoplasm and a nuclear matrix protein sample was tested with supernatants from hybridoma clones AB4 and BB1 (Fig. 2.3A). The results of this showed that both clones did indeed detect an antigen of molecular weight 40kD in the interphase cytoplasm. Thus, the 40kD antigen may give the chromosome contour staining detected by both clones. Since a 40kD antigen could be detected on the nuclear matrix then this may indicate that the 40kD antigen may have been a cytoplasmic contaminant of the nuclear matrix, or, alternatively, that there are two antigens of molecular weight 40kD, one is a component of the nuclear matrix, the other a component of the cytoplasm.

Discussion

The results indicate that a 40kD cytoplasmic antigen may give the chromosome contour staining common to both Mabs AB4 and BB1. Since the 66kD antigen recognised by Mab BB1 could not be detected in the interphase cytoplasm, this raises the question of its localisation. Of several possibilities to explain this, the TCA precipitation procedure may have denatured the epitope in the cytoplasmic fraction. Thus, it cannot be excluded that this antigen is cytoplasmic also, but could not be detected. Another possibility is that this antigen is responsible for the centromere staining given by BB1, and was not present in sufficient quantities to be detectable in the chromosome fraction tested. In order to show conclusively the cellular localisation of the 66kD an antibody elution should be carried out and followed by immunofluorescence microscopy. However, as follows, two other Mabs were identified, which were shown to be more suitable for the analysis of the centromere, and therefore, Mab BB1 was not exploited further.

R 2.2 ANTI-CENTROMERE MAB BA2

Immunofluorescence Microscopy

On the basis of immunofluorescence microscopy studies of 100% methanol fixed, colchicine treated, CHO cells, Mab BA2 gave a centromere positive staining pattern. On interphase cells one or two dots (not shown) could always be observed which corresponded to the centrioles (depending on the stage of interphase, the centriole had either divided or not). The interphase centromeres were not labelled, however, on metaphase spreads the centromeres did react with the monoclonal antibody secreted by hybridoma clone BA2. The reaction was such that two dots (Fig. 2.4), or a line perpendicular to the long axis of the chromosomes, at the centromeres was visible on each chromosome. Occasionally a ring could be seen encircling the centromere (Fig. 2.4A). The relevance of these centromere staining patterns, particularly the intriguing ring structure, will be dealt with later (Chapter R4).

Western Blotting

To attempt to establish the molecular weight of the antigen(s) detected by clone BA2, CHO nuclear matrix and chromosome scaffold proteins were prepared for Western blotting. The resulting nitrocellulose filters, when incubated with supernatant from BA2 gave a weak signal on the chromosome scaffold but not on the nuclear matrix. The band on the chromosome scaffold, had an apparent molecular weight of 43kD (results not shown). However, attempts to repeat this result failed, suggesting that this was an aspecific binding or some other anomaly. The difficulties encountered in detecting the antigens in Western blots by this Mab, and the other anti-centromere Mab AC1, will be treated later.

Discussion

The above data demonstrated that hybridoma clone BA2 did detect both a centromere antigen, and a centriole antigen. However, the actual molecular weight of the antigen(s) proved difficult to identify. A value for the weight cannot be stated for CHO. This appears to be due to the epitope, or more likely the antigen itself, being very labile. This possibility is developed further in a later chapter on clone AC1 (Chapter R3). In conclusion, BA2 is a potentially interesting anti-centromere Mab, it does not cross-react with cytoplasmic proteins, and so was studied further, these experiments can be found in the subsequent chapters.

Figure 2.4 Indirect Immunofluorescence Microscopy of CHO Colchicine Treated Cells Stained with Column Purified BA2 Immunoglobulin

CHO cells were colchicine treated to obtain metaphases, cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation, incubated with column purified immunoglobulin from hybridoma clone BA2 and then anti-mouse FITC conjugated secondary antibody.

Immunoglobulin from clone BA2 gave a strong signal on the centromeres of CHO metaphase chromosomes. This Mab reacted with the two dots at the centromeres of the chromosomes in a manner similar to that observed for Mab BB1 and human anti-centromere autoimmune serum LU851. Unlike these antibodies, Mab BA2 detected a ring-like antigen at the centromeres on some of the centromeres (*arrow in **A**). On more flattened metaphase spreads, where the chromosomes were more spread out, only the two dots could be seen at the centromeres (**B**). **B'** is the HOECHST 33258 counter-stained image for DNA.

Magnification: x4,000.


A


B


B'

R 2.3 ANTI-CENTROMERE MAB AC1

Immunofluorescence Microscopy

Upon using any of the three fixation procedures, detailed previously, essentially the same centromere staining pattern was obtained on CHO metaphase spreads by Mab AC1, as for BA2 (R2.4). Thus, the dots (Fig. 2.5A), lines and rings (see R.4.2) were all detected with Mab AC1, in a manner apparently identical to that obtained for Mab BA2. When fixed with 4% paraformaldehyde the cytoplasmic background staining obscured any centriole staining that may have been present. However, both 100% methanol and 100% acetone fixation gave centriole positive reactions (see Fig. 2.5B/). Since Mabs AC1 and BA2 gave a strong centromere signal and were not obscured by any cytoplasmic cross-reaction, they were chosen for further analyses of the centromere. Hence, the immunofluorescence staining pattern will be analysed more fully in a later chapter (R.4.2), where a detailed account of, in particular the AC1 centromere staining, and comparisons with other centromere staining patterns will be given.

Western Blotting

Western blots of CHO chromosome scaffold and nuclear matrix proteins initially failed to give a positive band, when incubated with supernatants prepared from AC1 by the standard procedure. The possibility arose, that the inability to detect a positive antigenic band was due to a low titre of the antibody in the supernatant. Therefore, in order to increase the titre of the AC1 immunoglobulins, mice were injected with AC1 (and BA2, independently and for the same reasons) hybridoma cells. The resulting ascitic fluid was then harvested and tested for centromere positivity on CHO colchicine treated cells. One such centromere positive AC1 ascites (all of the BA2 ascites were found to contain unacceptable background reactions by immunofluorescence microscopy, and so were not persued further), was then tested on a preliminary Western blot with CHO chromosomal proteins, and this appeared to give a positive signal with a band of molecular weight 66kD. However, this result could not be subsequently reproduced. Several technical difficulties were encountered when using this Mab and, therefore, the antigen detected by this clone proved extremely difficult to characterise. This problem and its solution will be the subject of the next chapter.

Discussion

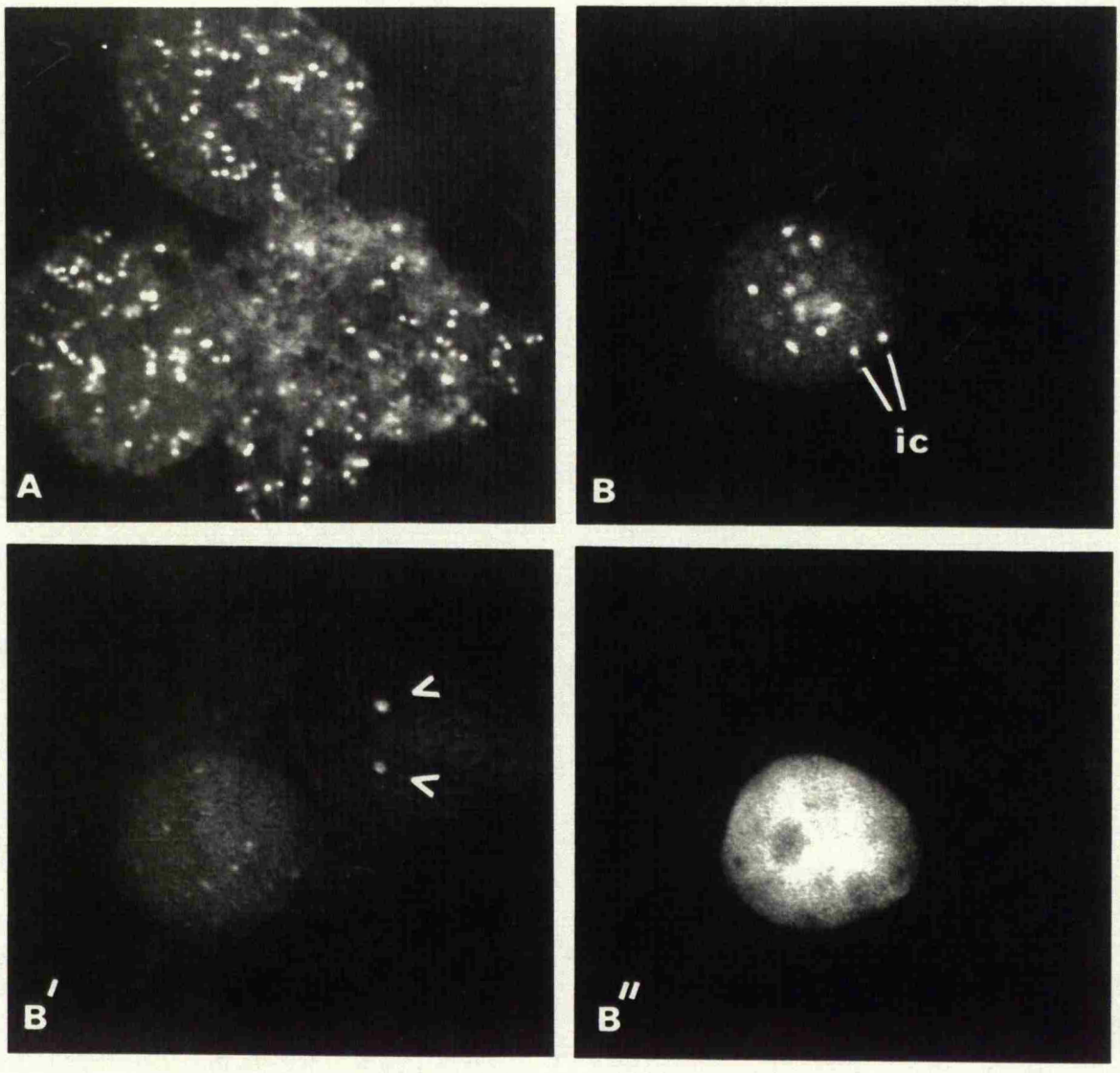
The above results indicated that clone AC1 appeared to detect a similar, if not the same, antigen as clone BA2. These clones both react with dots, lines and rings at the centromeres of CHO chromosomes, as well as a component of the centriole, as judged by immunofluorescence microscopy. As was the case for clone BA2, the antigen detected by AC1 proved to be very difficult to determine in Western blots.

Figure 2.5 Double Indirect Immunofluorescence Microscopy with Mouse Mab AC1, and Human Anti-Centromere Autoimmune Serum LU851 on CHO Colchicine Treated Cells

CHO cells were colchicine treated to obtain metaphases, cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation, incubated with either supernatant from hybridoma clone AC1 (**A** and **B'**) and then anti-mouse FITC conjugated secondary antibody; or human anti-centromere autoimmune serum LU851 (**B**), anti-human TRITC conjugated secondary antibody, and counter-stained for DNA with HOECHST 33258 (**B''**).

A shows that Mab AC1 stains the centromere dots in a manner identical to Mab BA2 on metaphase spreads.
B, **B'**, and **B''** are the LU851, AC1 and HOECHST staining patterns, respectively, obtained on the same interphase cell. Unlike the human anticentromere autoimmune serum LU851 (**B**), Mab AC1 did not give a positive signal on the interphase centromeres (see **ic** on **B**), whereas AC1 did decorate the interphase centrioles (see the two arrowheads), whilst LU851 did not.

Magnifications: A: x2,000; B: x3,000.


A
B
ic
B'
B''

However, both Mabs AC1 and BA2 decorated a centromere substructure, which was a very exciting result, in particular the ring, and thus, on the basis of the immunofluorescence data the antigen detected by these Mabs were explored further. The technical difficulties experienced in determining the molecular weight of the centromere ring antigen, and the novelty of the ring, form the subject of the next two chapters.

Summary of Anti-Chromosome Scaffold Mab Screening

In summary of the overall screening of the anti-chromosome scaffold Mabs, of the 70 viable hybridoma clones, 37 were shown to still be secreting immunoglobulin, and of these 14 gave a significant immunopositive reaction detected by immunofluorescence microscopy. Of the 14 immunopositives, 3 were shown to be centromere positive. Of these 3, 2 (BA2 and AC1) reacted with the centromere and centriole, without detecting the cytoplasm. The remaining clone (BB1) reacted with an apparently different component of the centromere, but also gave a positive signal on the cytoplasm. Since this Mab, BB1, gave such a strong reaction with the cytoplasm it was decided that this clone should not be exploited further. Furthermore, it was generally observed that Mab BA2 proved to be even more difficult to characterise by Western blotting than AC1, and so it was decided to place the emphasis on clone AC1 in the subsequent studies of the centromere.

A summary of the Western blots of the anti-chromosome scaffold hybridoma clone screening can be found in Table 2.1.

Overall Observations

The diversity of the antigens detected by the panel of anti-CHO chromosome scaffold Mabs demonstrated the heterogeneous nature of the initial immunising material. Native chromosomal material contains a great diversity of proteins, and although high salt treatment results in an enrichment for certain proteins, the resulting material is by no means homogeneous. Thus, it was not surprising that many of the Mabs detected antigens not later found on CHO chromosomes, for instance 6 of the 14 Mabs detected exclusively (using three possible fixation procedures) cytoplasmic antigens. Although cytoplasmic contamination of the original immunising material could have resulted in these Mabs, it is more probable that there are epitopes common to both cytoplasmic and chromosomal scaffold proteins.

The initial assumption, based on the fact that chromosomal scaffold material is enriched for centromeric components (Hadlaczky et al, 1981; Earnshaw et al, 1984), could lead to the isolation of Mabs reacting with the centromeres proved to be valid. Thus, the identification of 3 of 14 Mabs which were centromere positive (21.4%) validates this premise.

Now attention will be focused on the analysis of the centromere utilising Mabs AC1, and where relevant BA2.

Table 2.1 Summary of Western Blot Analysis of Anti-Chromosome Scaffold Hybridoma Clones on CHO, Human, Drosophila and Yeast Proteins

Proteins separated by 5-20% SDS PAGE and electrophoretically transferred to nitrocellulose filters. Western blots were tested with various antibodies, the positive signals are listed below.
Proteins preparations used were: CHO chromosomes, nuclei, interphase cytoplasm, chromosome scaffold, nuclear matrix, human Colo nuclear extract, *Drosophila melanogaster* S_3 total cells, and *Saccharomyces cerevisiae* .

Figures shown are molecular weights in kilodaltons.

CHO Protein Sample						Other Species Protein Sample		
CLONE	Chrom.	Nucl.	Int. Cyto.	Chrom. Scaff.	Nucl. Matrix	Colo NE	Dros. S3 cells	Sac. cerev.
AB4	-	40	40		40			
AC1	(66?)			-	-			
BA1	-	-				30		
BA2				(43?)	-			
BB1	-	-		-	66			
			40	-	40			
CC5			-		20			
ED3			116	116	116	116	-	-

No positive signal obtained -

Blank: not done

(?): results not possible to replicate

CONTENTS

SECTION B: ANALYSIS OF CENTROMERE USING MABS AC1 AND BA2

R 3 ATTEMPTS TO DETERMINE THE MOLECULAR WEIGHT OF THE AC1 ANTIGEN

General Introduction

As was described in the previous chapter, Mab AC1 was deemed worthy of use in further experiments, in terms of its detecting an antigen of the CHO centromere. In the subsequent chapters the characterisation of this centromere antigen is presented. It will be demonstrated that the monoclonal antibody secreted by this hybridoma clone detects a novel ring structure at the centromeres of CHO chromosomes (Chapter R4), and that this structure is conserved to the centromeres of the mouse (LMTK$^-$), the hybridoma cells themselves, and human (EJ30) (Chapter R5). It will be demonstrated that the ring structure binds the CHO chromosome scaffolds, indicating that the antigen is closely associated with chromosome scaffold DNA (Chapter R6). The relationship of this chromosome scaffold and centromere antigen to another chromosome scaffold antigen, topoisomerase II, is also investigated (Chapter R7). In addition, a possible relationship of this antigen to two classes of proteins involved in cellular movement, namely tubulin, microtubule associated proteins (Chapter R8) and myosin (Chapter R9), is considered.

After many experiments to optimise the Western blotting procedures, it was possible to conclude that the antigen had an apparent molecular weight of 170kD (this Chapter), on the basis of its electrophoretic mobility. Although this chapter primarily concerns experiments using Mab AC1, where relevant, details of studies using the other anti-centromere ring Mab, BA2, are included.

R 3.1 INITIAL ATTEMPTS TO DETERMINE THE MOLECULAR WEIGHT OF THE AC1 ANTIGEN

Introduction

After hybridoma clone AC1 had been identified as staining an antigen of CHO centromeres, it was of interest to determine the molecular weight of the antigen detected by this Mab. As had already been indicated in the previous chapter this proved difficult to achieve, in as much as the antigen band proved difficult to detect. The exact problems regarding this and their solution is the subject of this chapter.

In initial Western blots of CHO protein samples no positive reactive band could be consistently detected in replicate experiments. In one experiment a 66kD band was detected on a CHO chromosome scaffold protein sample using AC1 antibodies (results not shown). However, in replicate experiments no signal was detectable.

To understand this problem and in order to attempt a solution, a consideration of the original material used to immunise the mice, which gave rise to the hybridoma clones, should be made. For this purpose chromosomes were isolated in bulk from colchicine treated CHO cells. The chromosomes were treated with 2M NaCl and digested with DNase I (100ug/ml), and the resulting chromosome scaffold fraction used to immunise mice. The chromosome scaffold itself is depleted of histones (Adolph et al, 1977) and contains tightly bound protein-DNA complexes, which form a relatively minor proportion of the bulk chromatin. Therefore, the minor quantities of such an antigen may limit detection. Moreover, the epitope may be altered by the denaturing effect of the SDS-PAGE used to separate the proteins prior to Western blotting. It is also possible that the stability of the relevant protein may influence the result, hence a labile protein, under the particular conditions used for Western blot analysis, may prove difficult to detect. Thus, for these reasons an antigen visible in immunofluorescence microscopy may not be possible to detect in a Western blot. Bearing such considerations in mind the following strategies were employed in trying to detect the antigen in Western blots. The two limiting factors which were considered and tackled were the quantity and quality of the antigen. These two are dealt with in turn below.

R 3.2 QUANTITY OF PROTEIN LIMITING DETECTION OF AC1 ANTIGEN

Various strategies were employed in trying to detect the antigen in Western blots assuming that the low level of this antigen in the protein samples was limiting its detection. Two aspects were focused on, to improve detection. One involved the preparation of the protein sample and the gel electrophoresis conditions, and the other, the immunological procedures used for Western blotting.

R 3.2.1 Protein Condition, Gel and Transfer Procedures

All of the appropriate positive controls carried out for the Western blots were satisfactory (not shown). Specifically, Coomassie brilliant blue staining of the gels showed the protein sample and protein markers on the gradient gels to be in good condition and separated correctly. The amido black staining of molecular weight protein markers showed that the electrophoretic transfer of these proteins was also satisfactory. Furthermore, various positive control antibodies used: anti-topoisomerase II mouse polyclonal and monoclonals; rabbit anti-MAP (microtubule associated proteins); and rabbit anti-muscle myosin antibodies on: *Drosophila* proteins containing high levels of topoisomerase II (Hadlaczky et al, 1988; see Chapter R7); Chinese hamster brain tubulin containing MAPs (Chapter R8); and bovine myosin (Chapter R9), respectively, all gave strong positive signals. The standard techniques of SDS-PAGE and Western blotting were all working satisfactorily, and, therefore, any difficulties in detecting the AC1 antigen had to be due to some problem with the antigen *per se* .

The limits of detection of the Western blotting procedure is between 10 and 100 fmoles, which corresponds to about 1-10ng of protein (Harlow and Lane, 1988) In order to increase the sensitivity of detection, gels were deliberately overloaded. Approximately double (of the order of 150-200ug) the normal amount (about 100ug) of protein was loaded per lane, as judged by the appearance of the Coomassie staining of the gels. In addition, thicker gels (1.5mm as opposed to 0.8mm) were poured to increase the concentration of each protein band. It has been shown that certain proteins resist or are poorly transferred electrophoretically (Bowen et al, 1980). Coomassie brilliant blue staining of gels after electrophoretic transfer to nitrocellulose filters showed that minor quantities of certain proteins did indeed remain behind in the gels (data not shown). Furthermore, larger proteins take longer to transfer than smaller proteins (Harlow and Lane, 1988), and anticipating that the AC1 antigen was one such protein (for instance the chromosome scaffold protein topoisomerase II has a molecular weight of 170kD), longer transfers were made. In place of the normal 100 mA for 2 hours for small gels, overnight transfers of 8 to 12 h at the same current settings were made. Despite exhaustive attempts none of these strategies led to the detection of the AC1 antigen (results not shown).

R 3.2.1.1 Enrichment for the AC1 Antigen

The above experiments all involved non-scaffold chromosomal protein samples. Whereas, the immunising material resulting in the production of hybridoma clone AC1 was in fact CHO chromosome scaffold samples, a material which is enriched for certain proteins. Therefore, CHO isolated chromosomes were treated with 2M NaCl in order to enrich the protein sample for chromosome scaffold antigens and increases the possibility of detection if the quantity of the antigen was a limiting factor. When Western blots were repeated using such protein samples it was still not possible to detect a protein band (results not presented). Since none of the above approaches led to a positive signal being detected on such Western blots, attention was focused onto the immunological treatment of the blots.

R 3.2.2 Optimising Immunological Procedures: Attempt to Detect the Antigen by Increasing the Titre of the Primary Antibody

A possibility was considered that the titre of the primary antibody may be too low to allow detection of the AC1 antigen. Thus, certain strategies were employed to increase this titre.

R 3.2.2.1 Increasing the Immunoglobulin Titre in the Supernatant

Spot tests of AC1 supernatants for immunglobulin presence (see Materials and Methods) always showed that this hybridoma clone was a poor immunoglobulin producer. An undiluted hybridoma supernatant has an antibody concentration in the range of 1-55ug/ml of immunoglobulin, which is ideal for Western blotting (Harlow and Lane, 1988).

If the AC1 antibody concentration in the supernatant was less than this, then this could have limited detection of the antigen. It is known that it is possible to increase the titre of the immunoglobulin in the supernatant by various cell culture techniques (Harlow & Lane, 1988). Supernatants are normally harvested 3 to 4 days after the last fresh culture medium is provided. The cells carry on secreting immunoglobulin for a few days in the absence of fresh medium, and so the immunoglobulin titre increases further, without being diluted by the medium. After such a "starvation" period the medium becomes depleted of nutrients, and it has been shown that an addition of glucose (Harlow & Lane, 1988) can prolong the life of the hybridoma cells. Hence, glucose to a final concentration of 1% was added to the culture medium, thus increasing the hybridoma cell lifespan without significantly diluting the supernatant, and the immunoglobulin therein. At the same time HEPES, pH 7.2, to a final concentration of 25mM was also added in order to return the pH to a more physiological one, which should also allow the cells to carry on secreting immunoglobulin for longer periods. Finally, when the cells were no longer in an ideal condition (before reaching death stage when cells lyse and liberate proteases into the medium which would degrade the immunoglobulin), the cells were separated from the supernatant by centrifugation, the cells discarded and freshly cultured new cells added to the supernatant, and incubated further, for 2-3 days. Such an enriched supernatant was tested for the presence of immunoglobulins by the normal spot test procedure. The intensity of the colour reaction showed that the titre of the immunoglobulin had indeed increased. This AC1 supernatant, produced under these modified conditions, was harvested and incubated with Western blots of CHO chromosome scaffold and nuclear matrix protein samples. However, still no positive band could be observed (not shown).

R 3.2.2.2 Recloning of the AC1 Hybridoma Clone

It is common for hybridoma clones, after a period of time of being maintained in culture, to become a mixed population of cells. Some of these cells are no longer secreting immunoglobulin or the immunoglobulin genes have undergone rearrangement, resulting in a reduced yield of immunoglobulin of the original, required specificity (Harlow and Lane, 1988). Thus, it is common practice to reclone important hybridoma clones, i.e. grow up colonies from single cells and retest the supernatants for the required immunopositivity. Recloning of AC1 was attempted and achieved by J. Keresô in this laboratory, for further details see appendix. However, no significant difference could be observed in a Western blot (results not shown) using supernatant from such a recloned AC1 supernatant as opposed to the non-recloned original with CHO chromosome scaffold and nuclear matrix proteins. Therefore, an alternative attempt was made to enrich for AC1 immunoglobulin.

R 3.2.2.3 Production of Ascitic Fluid

The titre of a desired immunoglobulin can be increased by growing the hybridoma cells *in vivo*. A typical tissue culture supernatant contains 1-50μg/ml of antibody, whereas for ascites the concentration is increased to 0.9-9mg/ml (Harlow and Lane, 1988) with a much lower contaminating protein concentration (foetal bovine serum for the former and mouse protein for the latter). Hence, a 100 times dilution of an ascites should give an antibody concentration of 90ug/ml, thus, the antibody concentration should not be a limiting factor. In order to enrich for the AC1 and BA2 (which apparently reacts with the same centromere ring and centriole antigen as does AC1) immunoglobulins, mice were injected into the abdominal cavity with freshly cultured, aseptically harvested, hybridoma cells in order to obtain ascites. When tumours became evident and the mice were in a terminal condition the ascitic fluid was collected. The ascites were then tested on CHO, colchicine treated cells by immunofluorescence for centromere positivity. The AC1 ascites deemed to react with the centromere, and which gave a minimal amount of background (from endogenous, non-hybridoma mouse immunoglobulins) were then used on Western blots of CHO scaffold protein samples. Unfortunately, the BA2 ascites gave unacceptably high background reactions and, hence, were not utilised. The AC1 ascites did not, however, give a positive, specific, reproducible signal. In parallel to generating the ascites another method of concentrating the immunoglobulins was employed. This is described below.

R 3.2.2.4 Affinity Column Purification of Immunoglobulin

Harvested AC1 and BA2 supernatants were applied to columns in order to purify the immunoglobulin from the culture media and to concentrate the immunoglobulins. BA2 immunoglobulin was purified on a CNBr anti-mouse sepharose column, and AC1 on an anti-mouse Protein-A column. However, in an earlier ELISA by others in this group, it was mistakenly determined that clone AC1 secretes IgG, whereas it in fact secretes IgM (see R3.2.3). This inaccurate result led to AC1 antibody being column purified using Protein A, which shows poor or no affinity for IgM. The resulting column purification fractions were assayed by spot tests for the presence of mouse immunoglobulin. These spot tests for both AC1 and BA2 showed that the affinity column purification of BA2, but (as expected) not of AC1, had increased the concentration of the immunoglobulin in the eluate by comparison with the supernatant from which the purification was made (not shown). The BA2 fractions giving the strongest reaction in spot tests, were examined by immunofluorescence on colchicine blocked CHO cells for centromere activity (see Chapter R2). All fractions were found to be centromere positive (therefore, the acid elution had not affected the specificity of the immunoglobulin), and so the fractions giving the strongest reactions in spot tests were pooled. BA2 purified immunoglobulins were incubated with Western blots of CHO scaffold proteins. However, again no positive band could be detected (results not given).

R 3.2.3 Optimising Immunological Procedures: Optimising Sensitivity of Detection of Secondary Antibody

Since no positive signal could be obtained by the above approaches, when (according to standard procedures, Harlow & Lane, 1988) the primary antibody should have been provided in excess, this indicated that it was not the low titre of the primary antibody, but perhaps more probably the minor quantities of the antigen which was less than the level required for detection. Therefore, two alternative strategies were attempted. The sensitivity of detection of an antigen in Western blots can be increased by radioactively labelling proteins and immunoprecipitating the antigen of interest (Harlow & Lane, 1988). The limits of detection of Western blotting can also be influenced by the type of secondary antibody used, and so the following experiments were carried out.

R 3.2.3.1 Radioactive 35S Labelling of CHO Proteins

By radioactively labelling CHO total proteins of cells grown in culture containing (^{35}S) it was hoped to also label the ring antigen. BA2 anti-centromere immunoglobulin immobilised on Sepharose beads was used in an attempt to immunoprecipitate the centromere antigen. In this way it was hoped possible to obtain radioactively labelled BA2 antigen, and to determine the molecular weight of this by autoradiography. Thus, by these means it was hoped to increase the sensitivity of the detection system.

In a preliminary experiment the incubation of rapidly growing CHO cells with (^{35}S) labelled methionine was carried out. In order to block the cells at metaphase, CHO cells were treated with colchicine and the chromosomes and nuclei isolated. An immunoprecipitation with CNBr anti-mouse Sepharose bound immunoglobulins, from the other anti-centromere ring hybridoma clone, BA2, was then attempted. In retrospect, as might have been expected, the immunoglobulin cross-reacted with many of the CHO mitotic cell proteins. In order to proceed with this approach it would have been necessary, therefore, to preadsorb the BA2 immunoglobulin with cold CHO proteins. However, at this stage, it became evident from parallel experiments, that the centromere ring antigen could be detected in Western blots by other, more straight forward means. Therefore, this approach was abandoned.

R 3.2.3.2 Specific Anti-Immunoglobulin Subtype Secondary Antibody

In order to try to increase the sensitivity of detection of the AC1 antigen by Western blotting it was decided to use a specific anti-immunoglobulin sub-type secondary antibody. The secondary antibody used thus far was polyvalent HRPO conjugated i.e. containing immunoglobulins raised against all immunoglobulin sub-types.

Although unlikely to be the limiting factor, it was considered possible to increase the sensitivity of detection by the use of a secondary antibody specifically reacting with the sub-type of the Mab secreted by hybridoma clone AC1 (and BA2). In order to determine the immunoglobulin sub-types, supernatants from hybridoma clones AC1 and BA2 were tested by a mouse immunoglobulin subtyping "dipstick" kit (Amersham, as described in Materials and Methods). The result of this demonstrated that clones AC1 and BA2 both secreted IgM side chain kappa (see Fig. 3.1A). Therefore, anti-mouse IgM-HRPO conjugated secondary antibody, as opposed to the polyvalent anti-mouse immunoglobulins used so far, was tested on a Western blot of CHO scaffold proteins, treated with AC1 supernatant (data not shown). Use of such a secondary antibody did not, however, give a detectable signal.

R 3.2.3.3 Anti-Mouse IgM Streptavidin-Biotin HRPO

Since it was considered probable that the AC1 antigen was only present in minor quantities in chromosome samples, then the more sensitive system of streptavidin-biotin complex conjugated to HRPO was employed in an attempt to detect the AC1 antigen. However, as for the previous experiment no positive signal could be obtained (not shown).

R 3.2.3.4 Successful Detection of 170kD AC1 Antigen Using Anti-Mouse IgM Alkaline Phosphatase

An alternative strategy which was used for increasing the sensitivity of detection of the AC1 antigen in Western blots, was alkaline phosphatase coupled secondary antibody as opposed to HRPO (horse radish peroxidase). Western blots of CHO chromosomes and nuclei, were first incubated with AC1 supernatant, and then treated with anti-mouse IgM immunoglobulin conjugated to alkaline phosphatase. A positive signal was finally obtained in CHO chromosomes, but not nuclei, by this procedure. This Western blot demonstrated the presence of a band of molecular weight 170kD (Fig. 3.1B) in chromosomes. This signal, however, was only slightly stronger than the background.

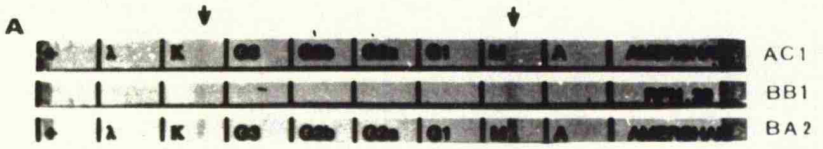
Figure 3.1 Determination of Molecular Weight of AC1 Antigen

The subtypes of the three anti-centromere monoclonal antibodies was determined using an immunoglobulin subtyping "dipstick" kit in **A**. Supernatants from clones **AC1**, **BA2** and **BB1** were tested with this kit. All three clones secrete Mabs of IgM kappa light chain, see arrowheads.

B: A 170kD antigen (see arrowhead) was detected in CHO chromosomal proteins (**c**), but not in CHO nuclei (**n**) on Western blots of these protein samples separated by 5-20% SDS PAGE, tested with supernatant from **AC1**, and anti-mouse IgM alkaline phosphatase conjugated secondary antibody. Notice the signal detected by AC1 is very faint by comparison with the aspecific reaction of the alkaline phosphatase conjugated secondary antibody, demonstrated when AC1 supernatant was replaced by culture medium **RPMI** containing no immunoglobulin. **m** are amido black stained high and low molecular weight markers of the following values: myosin 200kD, *E.coli* beta-galactosidase 116.25kD, rabbit muscle phosphorylase b 97.4kD, bovine serum albumin 66.2kD, hen egg white ovalbumin 42.699kD, bovine carbonic anhydrase 31.0kD, soybean trypsin inhibitor 21.5kD, hen egg white lysozyme 14.4kD.

C: Western blot of proteins from CHO nuclei separated as in **A** and tested with either SIGMA or Dakopatts, HRPO or alkaline phosphatase conjugated secondary antibody. Lanes **1** to **5** inclusive were treated with HRPO conjugated secondary antibody, and **6** to **12** inclusive with alkaline phosphatase conjugated secondary antibody. The specificity and concentration of the secondary antibodies follows. Lane **1**, SIGMA polyvalent 1/200; **2**, SIGMA anti-IgM 1/200; **3**, Dakopatts polyvalent 1/500; **4**, Dakopatts polyvalent 1/1000; **5**, Dakopatts polyvalent 1/1500; **6**, SIGMA polyvalent 1/5000; **7**, SIGMA polyvalent 1/10000; **8**, SIGMA anti-IgM 1/10000; **9**, SIGMA anti-IgM 1/15000; **10**, Dakopatts polyvalent 1/500; **11**, Dakopatts polyvalent 1/1000; **12**, Dakopatts polyvalent 1/1500. Dakopatts anti-IgM 1/500 was found to give the most acceptable signal to noise ratio. Depending on the type of antibody, concentration and manufacturer the signal to noise ratio varied. The arrowheads show the position of the 170kD antigen detected by AC1 supernatant, on CHO nuclei (**n**), **m** are Coomassie Blue stained molecular weight markers as used in **B**.

D: Colo nuclear extract proteins separated by 5-20% SDS PAGE, blotted and incubated with anti-IgM alkaline phosphatase conjugated secondary antibody. The secondary antibody was preadsorbed with different quantities of Colo nuclear extract proteins, and washed with either the normal Tween-20 PBS washing solution, or with 0.5% DOC, 0.1% SDS, 1% NP-40 and 150mM NaCl washing solution. Lanes **1** to **8** inclusive were incubated with AC1 supernatant. Lanes **9** and **10** were incubated with blocking solution without primary antibody. Lanes **1**, **3**, **4**, **5**, and **10** were washed with the normal Tween-20 PBS washing solution, lanes **2**, **6**, **7**, **8**, and **9** were washed with the alternative solution. For lanes **3** and **8** secondary antibodies were preadsorbed with 1µl protein extract; **4** and **7** 10µl of protein; **5** and **6** 25µl of protein. Arrowheads show the position of the 170kD antigen. The alternative washing solution appears to remove the AC1 170kD band, and there is no significant difference brought about by preadsorption with these concentrations of competing Colo protein. **m** is the same as above.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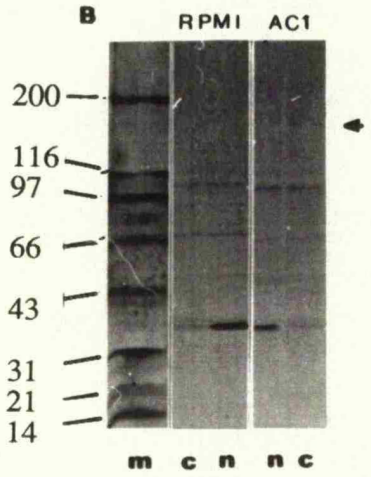
BA2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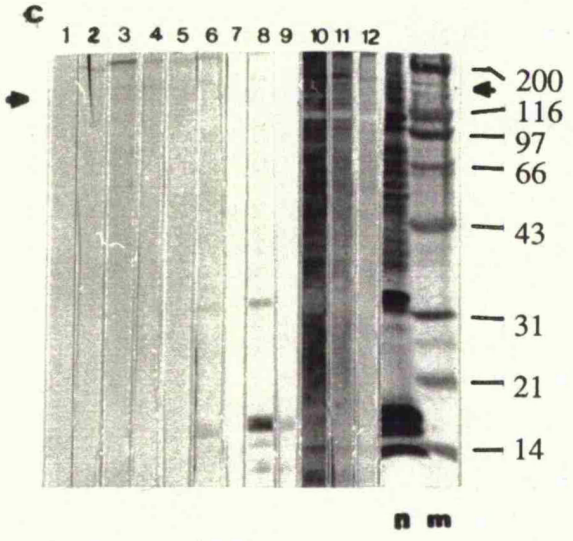
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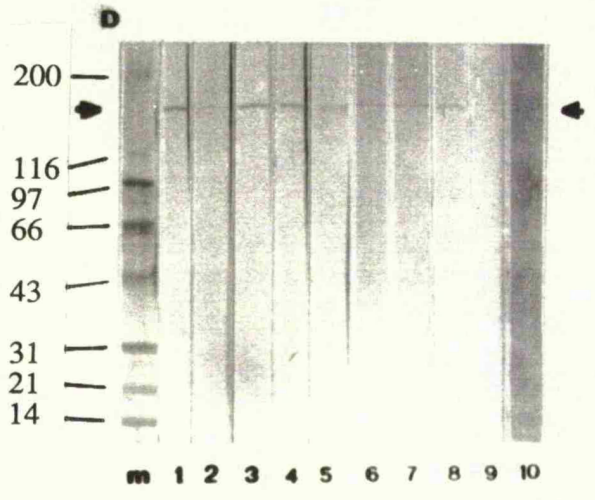
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Conclusion

From these experiments it is possible to conclude that the original assumption that the antigen detected by AC1 is only present in minute quantities is indeed apparently correct and the more sensitive detection method of alkaline phosphatase labelling was required before the postulated positive band could be visualised. However, the alkaline phosphatase conjugated secondary antibody resulted in a considerably increased background noise. Since the apparently positive signal was only slightly stronger than the relatively high background, the signal to noise ratio was then optimised to demonstrate conclusively that the 170kD band was actually a positive signal, and these experiments are described below.

R 3.2.4 Optimising Immunological Procedures: Reducing Background Noise

Various approaches were attempted in order to reduce the background noise of the Western blots treated with alkaline phosphatase-conjugated antibodies.

R 3.2.4.1 Varying the Blocking Protein

Depending on the types of proteins used to block the aspecific binding sites of the nitrocellulose filters, antibodies may still be able to bind to the filters. A different type of blocking solution was, therefore, attempted. Horse serum, which is a preferred blocking agent (Harlow & Lane; 1988) was utilised instead of foetal calf serum (normally employed in this laboratory). However, when Western blots (carried out as previously) were repeated with this modified blocking solution and alkaline phosphatase coupled secondary antibody used, the difference in the amount of background was only marginal (data not presented).

R 3.2.4.2 Optimum Anti-Mouse Alkaline Phosphatase Conjugated Secondary Antibody Concentration

By systematically decreasing the secondary antibody concentration, an optimum was found: 1 in 10,000 for SIGMA polyvalent, and 1 in 15,000 for SIGMA anti-IgM-alkaline phosphatase conjugate which gave the minimal amount of background noise (not shown) over the signal.

R 3.2.4.3 Source of Secondary Antibody

It has been found in this laboratory that depending on the manufacturer, different secondary antibodies can show different signal to noise ratios. Specifically, it was found that Dakopatts secondary antibodies gave a significantly lower background than Sigma (Fig. 3.1C), which had given the high background noises in the previous experiments. The optimum concentration was then determined for this antibody, and found to be 1 in 500, and this was used in all subsequent experiments.

R 3.2.4.4 Incubation of Secondary Antibody with Competing Protein

To try to reduce the aspecific binding exhibited by the secondary antibody for the sample proteins, human (Colo cell line) nuclear protein extracts at concentrations 1, 10 and 25 µl per 2ml of solution were incubated with such antibodies (Fig. 3.1D). However, these did not noticeably reduce the background noise.

R 3.2.4.5 Alternative Washing Agent

A stronger washing solution consisting of 0.5% DOC, 0.1% SDS, 1.0% NP-40, and 150mM NaCl instead of the usual Tween-PBS was used (Fig. 3.1D). Unfortunately, the washing solution was so successful that the blocking proteins were also removed by this procedure, resulting in a greater background noise.

R 3.2.4.6 Varying the Incubation Time

Instead of the 1h at room temperature with agitation used normally in this laboratory, the secondary antibody was provided for different incubation times of either 45 or 30 min, and even 30 min proved to be sufficient to allow binding of the primary antibody while reducing slightly the background (data not presented).

Discussion

From all these experiments it transpired that a concentration of 1 in 500 for Dakopatts alkaline phosphatase-conjugated secondary antibody incubated for 30min, washing with Tween-PBS proved to give the most satisfactory results, and allowed the conclusive demonstration that AC1 reproducibly detects an antigen of molecular weight 170kD in CHO.

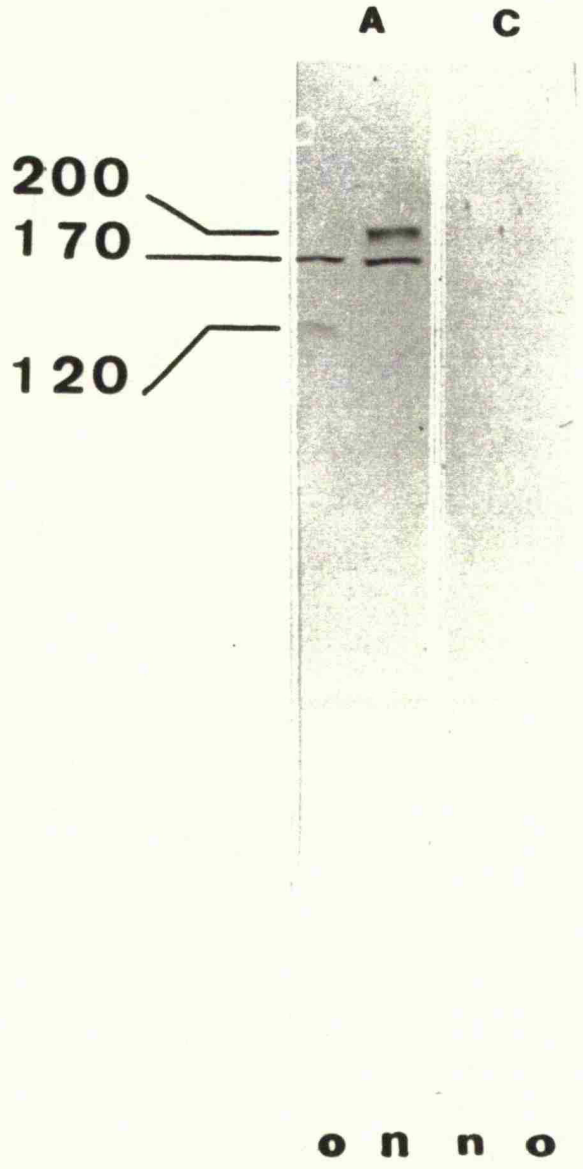
When originally considering the potential problems limiting detection of the AC1 antigen two major factors were taken into account: quantity and quality. The former has been dealt with above. Now attention will be turned to the latter.

R 3.3 STATE OF PROTEIN LIMITING DETECTION OF BA2 AND AC1 ANTIGEN

In order to test the possibility of an *in vitro* degradation of the antigen, two different human (Colo) nuclear protein extracts were used for a Western blot (see Fig. 3.2) tested with purified immunoglobulin from the other Mab, BA2, which also detects the centromere ring. The difference between the two human nuclear extract samples was the length of time they had been stored prior to use. In the fresher sample an additional higher molecular weight band, 200kD could be detected, whereas in the older extract this was no longer present. In both extracts, a band of 170kD could be detected. However, in the older extract the 170kD band was much fainter, indicating that degradation had occurred, and a possible degradation product of 120kD was also observed.

Figure 3.2 Western Blot of "Old"and "New" Colo Nuclear Extracts Tested Mab BA2

Depending on the length of time the proteins had been stored at -20°C the antigen bands detected by antibody (**a**) BA2 with Colo nuclear extracts varied. Proteins were separated by the standard 5-20% gradient SDS PAGE procedure. The control RPMI culture medium containing no hybridoma immunoglobulins (**c**) shows the minimal amount of background noise. In the "old" (**o**) sample the higher molecular weight band of 200kD could no longer be detected, and a presumed 120kD breakdown product could be faintly observed. In the "new" (**n**) extract both 200kD and 170kD antigens could be detected.


A
C
200
170
120
o n n o

A similar experiment, using CHO mitotic total cells tested with AC1 supernatant, 3 days after dissolving in sample buffer, showed the presence of the 170kD band, whereas only 5 and then 7 days after dissolving in sample buffer this antigen could no longer be detected (not shown).

Discussion

Extensive experiments were required before it was possible to demonstrate that Mab AC1 detects an antigen of molecular weight 170kD on CHO proteins. The same difficulties were encountered for the apparently identical centromere ring antigen stained by the other hybridoma clone, BA2 (see chapter R2 on anti-centromere clone BA2). Mab BA2 detected 170kD, and 200kD antigenic bands on human (Colo) protein samples.

Further, alternative strategies in addition to those described above, were considered to try to detect the AC1 antigen in Western blots, however, since the difficulties were resolved these were not pursued. For completeness these alternatives are briefly discussed here. Nuclear matrix proteins have been shown to be poorly electrophoretically transferred to nitrocellulose filters for immunological detection (Bowen et al, 1980). Such proteins have been more successfully transferred by simple passive diffusion (Bowen et al, 1980). If none of the methods outlined in the results chapter above had given a positive result, then such a diffusion transfer would have been the next step. An alternative factor which was considered was the denaturing effect of SDS-PAGE. This could have resulted in the antigen or epitope being denatured, or altered so that the antibody could no longer react with it. Again, as for the previous point the appropriate steps would have been taken if necessary, specifically non-denaturing gels for protein separation would have been utilised prior to blotting.

The difficulties of detection of the antigen in Western blots can be attributed to two characteristics of the centromere ring antigen. The antigen is only present in very minor quantities in CHO chromosome protein samples, and in chromosome scaffold protein samples. The other factor is that the antigen is apparently very labile in sample buffer.

AC1 Antigen Detected in Western Blots is a Minor Chromosomal Protein

The experiments aimed at increasing the sensitivity of antigen detection in Western blots of CHO protein samples revealed that the antigen detected by the antibody secreted by hybridoma clone AC1 (and BA2) is only present in minor quantities. Even in chromosome scaffold preparations, which should be enriched for this and other proteins, the 170kD signal detected by AC1 was relatively weak.

AC1 Antigen is Very Labile

The experiments aimed at investigating the stability of the AC1 antigen *in vitro* demonstrated that the antigen or epitope is very labile. This lability explains the difficulty of detecting the antigen in Western blots at all, and made it very difficult to obtain a positive signal in the replicate experiments, even with otherwise optimal conditions. Since the length of time of storage of the proteins in sample buffer was an important factor influencing detection, then it is possible to conclude that the epitope or the entire antigen was degraded *in vitro* .

Conservation of the 170kD Antigen Between CHO and Human Cells

Mab AC1 detected an antigen of 170kD in both CHO proteins and human (Colo) nuclear extracts. Also, from the experiment testing human (Colo) nuclear extracts with Mab BA2 it is possible to conclude that the Mab detects an antigen of apparent molecular weight 170kD, and a possible high molecular weight form (since it is unusual for monoclonals to detect more than one polypeptide) of molecular weight 200kD. Thus, the 170kD antigenic band is conserved between CHO and humans. This is rather unusual for centrome antigens, for instance the CENPs -A, -B, and-C do have CHO homologues (Chapter R4), but these are of different molecular weights, indicating that, although the epitopes are conserved, the antigens are of different electrophoretic mobilities. Thus, the 170kD antigen is more conserved than these CENPs, possibly indicating that the antigen in this form has been under a stronger selection pressure.

Correlation of Centromere Ring Antigen and 170kD Antigen

Having established the molecular weight of the antigen detected by AC1 in Western blots, it was important to try to determine if this antigen is actually responsible for the centromere ring staining observed by immunofluorescence microscopy. Since the AC1 antigen is present only in very minor quantities the standard procedure in such cases, antibody elution, could not be carried out. This procedure entails the elution of a given antibody from a band on a Western blot. The given antibody is then used in immunofluorescence microscopy to show the cellular localisation of the antigen. However, the AC1 antigen was present only in very low levels, and thus it was deemed impractical to carry out such an experiment. Therefore, another approach was required to establish the relationship between the ring antigen and the 170kD antigen. Indirect evidence indicating that the 170kD antigen was indeed responsible for the centromere staining came from the experiments using the other anti-centromere clone, BA2. The immunofluorescence microscopy data showed that the two Mabs stained an apparently identical antigen on metaphase spreads. Both AC1 and BA2 proved to be very difficult to characterise by Western blotting. Both detected antigens which proved to be very labile and present in low levels.

Thus, it appears likely that both Mabs are reacting with the same antigen, and unlikely that both are cross-reacting with a protein not responsible for the centromere staining. Moreover, considering the fact that 170kD (or 200kD) represents a comparatively large protein, presumably containing several possible epitopes, although possible, it seems unlikely that two different Mabs should react with the same epitope. If both Mabs detect different epitopes, then it is improbable that two different epitopes of the AC1 170kD antigen are both present on the same cross-reacting protein. Therefore, this is a good indication, though not definitive, that the 170kD antigen corresponds to the centromere ring antigen, and appears not to be a fortuitous cross-reaction.

The fact that Mab BA2 detects an additional higher molecular weight band implies that this 200kD antigen may be a high molecular weight form (since Mabs rarely react with more than one polypeptide). AC1 had also given a positive signal on a 200kD antigenic band in a Western blot of CHO proteins (not shown), suggesting that this also detects a high molecular weight form in CHO cells. The fact that AC1 did not detect the 200kD antigen on human protein preparations is most probably due to these samples having undergone *in vitro* degradation, and is not necessarily a difference in behaviour of the two Mabs, AC1 and BA2.

CONTENTS

R.4 AC1 ANTIGEN IS A NOVEL CENTROMERE ANTIGEN

Introduction

There are four categories of proteins identified to date which have been localised to the centromeric region of the mammalian chromosome. These are the "centromere proteins" (CENP-A, -B, -C, Earnshaw and Rothfield. 1985), the "inner centromere proteins" (INCENPs, Cooke et al, 1987), the "chromatid linking proteins" (CLiPs, Rattner et al, 1988), a mitosis specific centromere antigen (Hadlaczky et al, 1989) and a "centromere associated protein" (CENP-E, Yen et al, 1991). Since Mab AC1 (described in the previous chapters R2 and R3) recognises an antigen of the centromere, it was important to differentiate this centromere antigen from the centromeric proteins identified to date. Two lines of evidence will be presented, which demonstrate that the antigen detected by AC1 is in fact a novel antigen, ie distinct from the CLiPs (Rattner et al, 1988), INCENPs (Cooke et al. 1987) and most importantly the CENPs, which most resemble the AC1 antigen in metaphase chromosomal localisation.

The first line of evidence is based on the staining patterns, produced by the antibodies detecting these proteins, observed using immunofluorescence microscopy. The INCENPs and CLiPs (Rattner et al, 1988) are all localised to regions distinctly different to that occupied by the CENPs and the AC1 antigen. The INCENPs are localised to the pericentromeric region between the sister chromatids at metaphase (Cooke et al. 1987). The CLiP antigens are localised to two dots in the outer region where the microtubules bind the kinetochore (Rattner et al, 1988).

The second line of evidence is that, in addition to the differences in chromosomal localisation, the apparent molecular weight of the AC1 antigen, 170 000 (with a possible precursor of 200 000) is different. This indicates that the antigen detected by Mab AC1 is distinct from the INCENPs, CLiPs and CENPs and the mitosis specific antigen. The INCENPs have molecular weights of 155 000 and 135 000 (Cooke et al. 1987), the CLiPs 50 000 and 63 000 (Rattner et al, 1988) and the mitosis specific antigen 59 000 (Hadlaczky et al, 1989). The CENPs have molecular weights of 17 000, 80 000 and 140 000 (Earnshaw and Rothfield. 1985), and CENP-E a molecular weight of 250-300 000 (Yen et al, 1991).

Since the CENPs -A, -B, and -C are physically located to a region of the centromere which is apparently very close to or the same as that occupied by the antigen detected by Mab AC1, a comparison of the localisation of these two types of antigens was made in order to differentiate between these two centromeric regions, and also to determine the positional relationship between them at the centromere.

R 4.1 THE MOLECULAR WEIGHT OF THE AC1 ANTIGEN IS DIFFERENT TO THAT OF THE CENPs

The molecular weights of the CENPs (A, B, and C) have been determined on human protein fractions. In order to distinguish the AC1 antigen from these CENPs, the band detected by AC1 was compared on the same Western blot using CHO proteins, with the bands detected by an anti-centromere autoimmune serum (which, amongst other antigens, reacts with the CENPs-A, -B and -C). These Western blots were of proteins from isolated CHO chromosomes and nuclei, and were tested with AC1 supernatant and LU851 (an autoimmune serum detecting the CENPs -A, -B, -C and a 59kD mitosis specific centromere antigen, Hadlaczky et al, 1989). In these experiments a 170kD molecular weight band was detected amongst the CHO proteins, by Mab AC1, in greater quantities with chromosomal proteins than with nuclear proteins (data not presented in this chapter, refer to chapter R7, Fig 7.1). In contrast, LU851 detected two antigenic bands of molecular weights 46kD and 83kD in both chromosomal and nuclear proteins (Fig 4.1A), demonstrating that the molecular weights of the CENP antigens are not conserved between humans and CHO.

An alternative approach was also employed to further substantiate that the AC1 antigen was distinct from the CENPs. Human (Colo) chromosomal proteins were isolated and separated by 5-20% SDS-PAGE, and electrophoretically transferred to nitrocellulose filters. The resulting Western blots were incubated with AC1 supernatant and LU851. In these human chromosomal proteins, the supernatant from hybridoma clone AC1 again reacted with a 170kD band, whilst, in contrast, LU851 reacted with a 140kD, a 80kD and a 17kD band (Fig 4.1B), as expected (Hadlaczky et al, 1989). Therefore, the Western blotting data provided further evidence that the AC1 antigen was indeed a novel centromere antigen, distinct from the CENPs, CLiPs and INCENPs.

R 4.2 AC1 ANTIGEN IS LOCALISED TO A NOVEL CENTROMERE RING STRUCTURE IN IMMUNOFLUORESCENCE MICROSCOPY

In the preliminary studies (described in chapter R2) using Mabs AC1 and BA2 on cytocentrifuged, colchicine treated, CHO cells, both antibodies stained two dots at the centromeres. An axis drawn connecting the two dots was perpendicular to the long axis of the chromosome, as is the case for the antigens detected by anticentromere autoantibodies (Earnshaw and Rothfield. 1985). In contrast, however, to the AC1 and BA2 antibodies (chapter R2), the autoimmune sera have been shown to stain the interphase centromeres (Earnshaw and Rothfield. 1985), but not the interphase centrioles.

Upon closer observation, using immunofluorescence microscopy, a further difference between the CENP antigens detected by the autoimmune serum, and the AC1 antigen became evident.

Figure 4.1 Western Blots of CHO and Human Colo Chromosomal and Nuclear Proteins Tested with AC1 and LU851, to Differentiate the AC1 Antigen from CENPs -A, -B, -C

Chromosomes and nuclei were isolated from CHO and human colchicine treated cells, their proteins extracted and separated by 5-20% SDS-PAGE. Western blots of the CHO proteins were tested with human anti-centromere autoimmune serum LU851 to determine the molecular weights of the antigens in CHO.

In **A** it can be seen that **LU851** reacts with antigenic bands of molecular weights 46kD and 83kD (see arrowheads) in chromosomal proteins (**c**) and nuclei (**n**). Shown are Coomassie Brilliant Blue (**cbb**) stained, chromosomal and nuclear proteins and molecular weight markers (**m**) of the following values: myosin 200kD, *E.coli* beta-galactosidase 116.25kD, rabbit muscle phosphorylase b 97.4kD, bovine serum albumin 66.2kD, hen egg white ovalbumin 42.699kD, bovine carbonic anhydrase 31.0kD, soybean trypsin inhibitor 21.5kD, hen egg white lysozyme 14.4kD. **a** is the Amido Black staining of the markers after electrophoretic transfer to nitrocellulose filters.

In **B** the Western blot (prepared as above) of proteins of human chromosomal (**c**), mixed fraction containing both chromosomal and nuclear proteins (**M**) and nuclear (**n**) origin were tested with **LU851** serum and **AC1** supernatant. LU851 detects bands of molecular weights 17kD (arrowhead **A**), 80kD (arrowhead **B**), and (arrowhead **C**) in greater quantities in nuclei and progressively less as the proportion of chromosomes increases; whereas, AC1 stains a band of molecular weight 170kD (starred arrow) in greater quantities in chromosomes, less so in the mixed fraction, and least in the nuclear fraction. In the nuclear fraction tested with AC1, an additional band of molecular weight 200kD (small arrowhead) could be detected, as seen in chapter R3, when precautions were taken to avoid the degradation of the AC1 antigen. **a** is the Amido Black staining of the markers after electrophoretic transfer to nitrocellulose filters.

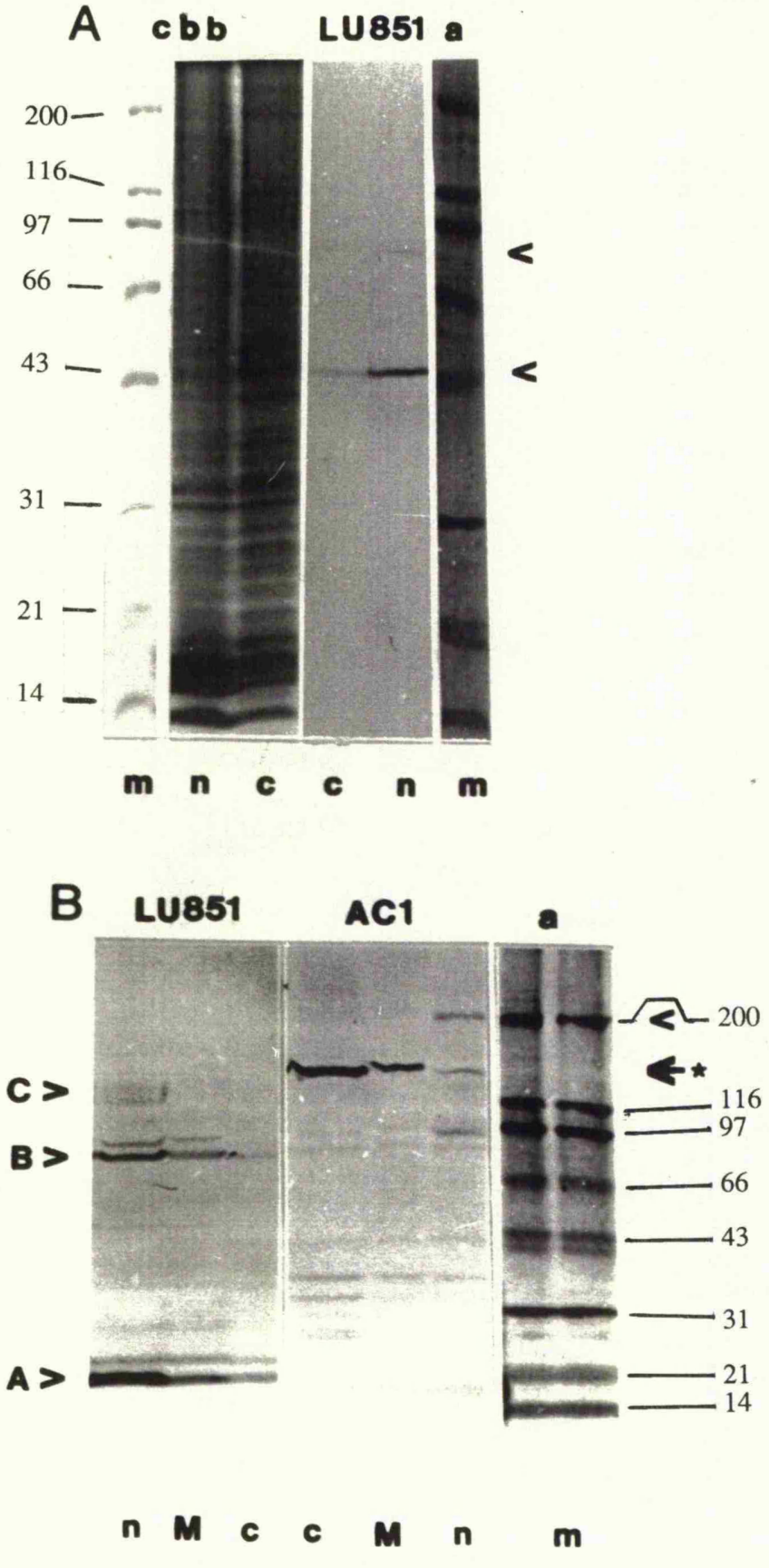
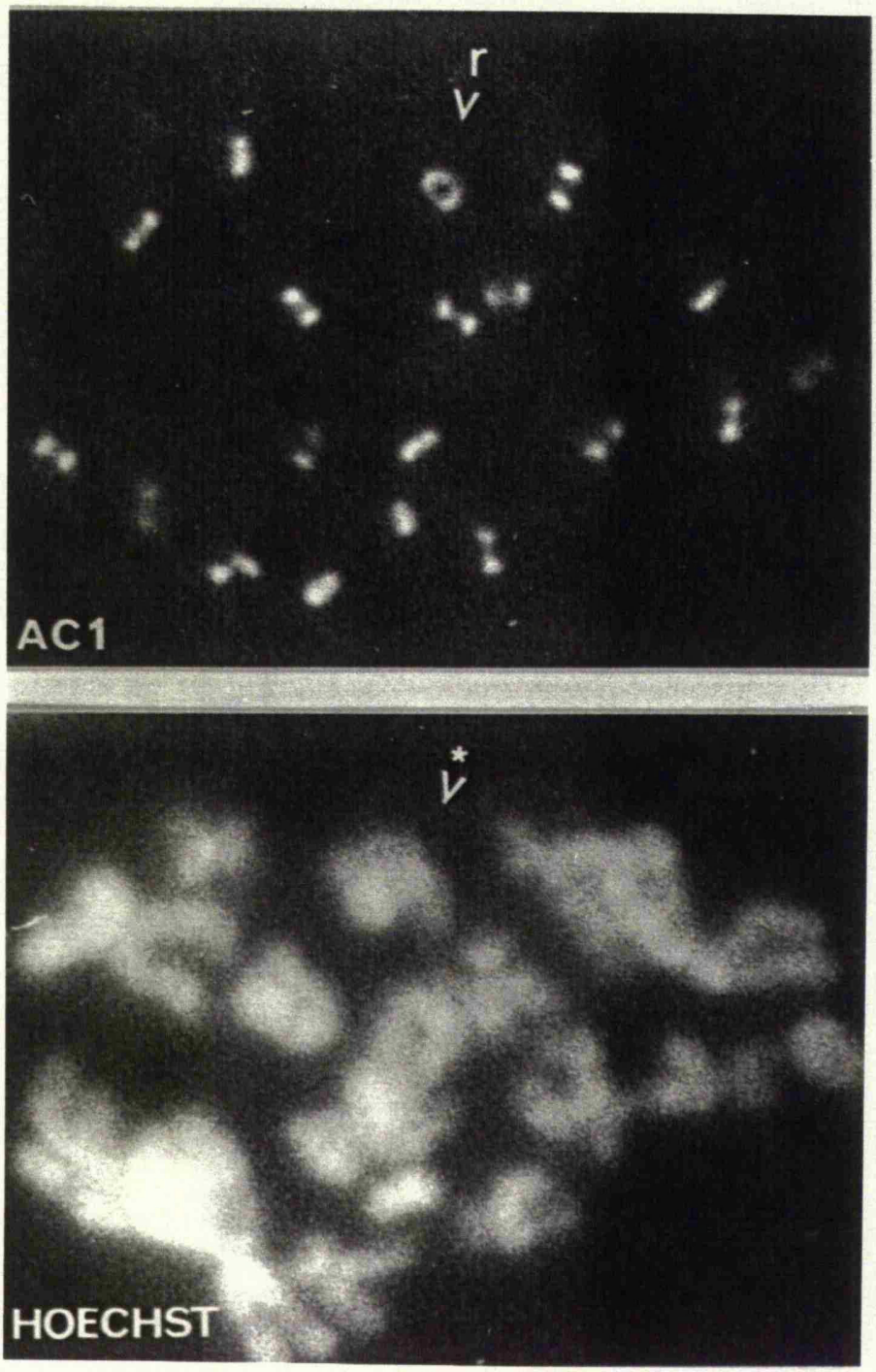

A
cbb
LU851
a
200
116
97
66
43
31
21
14
m n c c n m
B
LU851
AC1
a
C >
B >
A >
200
116
97
66
43
31
21
14
n M c c M n m

Figure 4.2 Indirect Immunofluorescence Microscopy of Low Speed Cytocentrifuged, CHO Colchicine Treated Cells, Tested with AC1 Supernatant

CHO cells were colchicine treated to obtain metaphases, cytocentrifuged at 800rpm (as opposed to the standard 1200rpm) and prepared for immunofluorescence by 100% methanol fixation, incubated with supernatant from hybridoma clone **AC1** and then anti-mouse FITC conjugated secondary antibody. A comparison of the AC1 staining with the DNA counter-stained **HOECHST** 33258 image, shows that the AC1 antigens are located to the centromeres. The lower speed centrifugation resulted in the AC1 antigen being less distorted than for the standard procedure. The line and "dumbbell" forms are more evident than for the higher speed cytocentrifugation. In addition a complete ring can be observed (**r**), the chromosome of which is labelled with the starred arrowhead.

Magnification: 4,000.


r
AC1
*
HOECHST

In a minority of the preparations stained with Mab AC1, instead of the two dots, distorted dots connected by a line or "dumbbell", a "horse-shoe" shape encircling the centromere, and occasionally a complete ring around the centromere could in fact be observed (Chapter R2.3 & R2.4.). Since these ring-structures could be consistently reproduced on all slides, but with varying frequency, then it appeared possible that this structure was the normal form of the antigen *in vivo* . The rings were observed preferentially on chromosomes that did not lie flat on the slide, but were orientated so that the sister chromatids pointed upwards, out of the plane of the slide, thus allowing them to be observed in transverse section. The dots, lines, "dumbbell" and "horse-shoe" forms could, thus, perhaps be the result of damage occurring during the preparation of the slides for immunofluorescence, and/or the orientation of the chromosomes after being cytocentrifuged onto the slides. In order to test this possibility CHO cells were cytocentrifuged at a lower speed (800rpm instead of 1200rpm), and the incidence of the rings stained by Mab AC1 did increase (Fig 4.2). Furthermore, cells spotted onto slides without cytocentrifugation (a procedure not normally employed, since it is very difficult to view chromosomes in multiple focal planes) and processed for immunofluorescence showed an even greater number of rings stained by AC1 (not shown). Such structures have, however, never been reported for studies of centromere staining of CENPs (for instance described by Earnshaw and Rothfield. 1985).

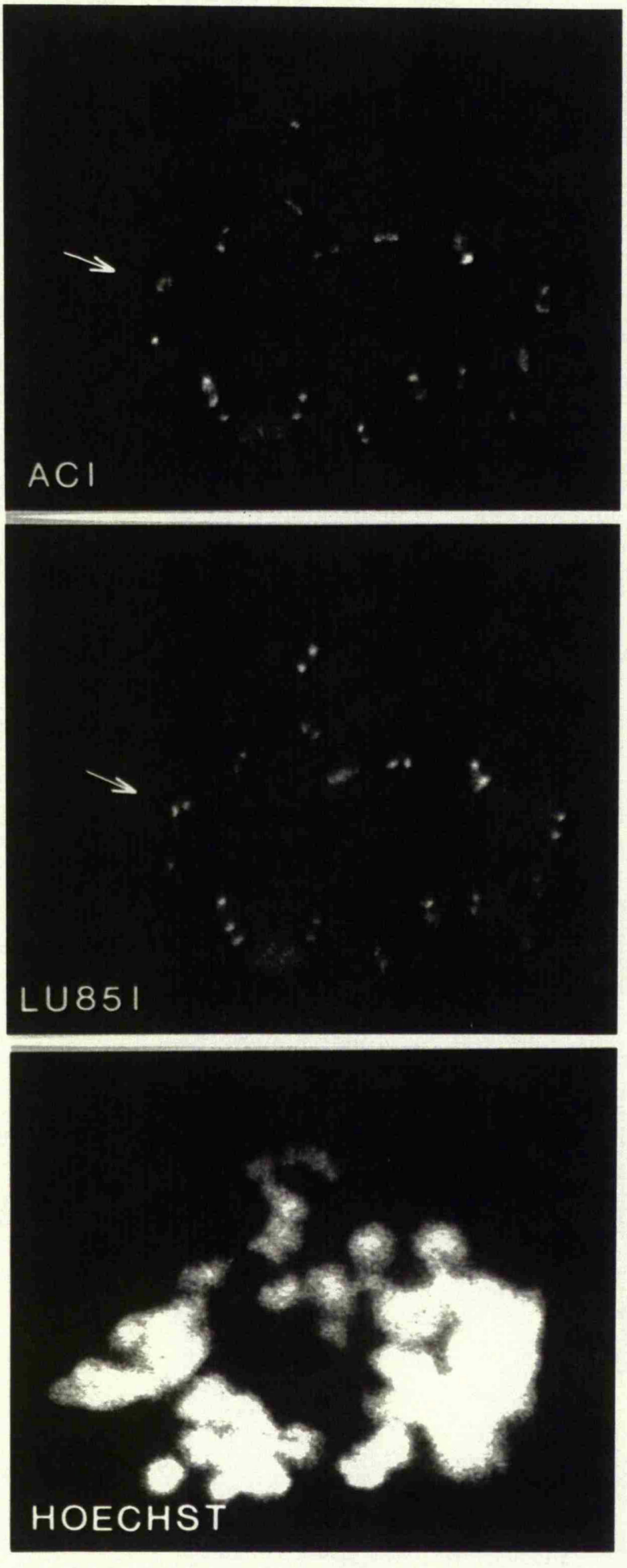
R 4.3 DIFFERENTIAL STAINING WITH AC1 AND LU851

Thus, on the basis of their different centromere structural organisation inferred from the staining patterns, the antigen detected by AC1 can be said to be distinct from the corresponding features of the CENPs, the CLiPs and the INCENPs. However, it is conceivable that the autoimmune sera do also react with the centromeric ring structure, but that the preparation conditions appertaining in other laboratories were such that the rings were destroyed, thus only leaving the two dots. Thus, it was important to determine the exact positional relationship of the CENPs to the AC1 antigen, under the same preparation conditions. To investigate this relationship a differential staining experiment was carried out using AC1 and an autoimmune serum available in this laboratory, LU851. These antibodies were used on CHO colchicine treated cells in immunofluorescence studies as follows. AC1 supernatant contains mouse immunoglobulin, and LU851 serum contains human immunoglobulin. Therefore, it was possible to carry out a differential staining using anti-mouse FITC conjugated secondary antibody and anti-human TRIC conjugated secondary antibody. Using different filters for viewing the slides it was possible to distinguish the staining patterns given by the two different primary antibodies on the same preparation. This experiment clearly demonstrated that the centromere ring detected by Mab AC1 encircles a region upon which the LU851 two centromere dots colocalise (see Fig 4.3). By altering the focal plane it was possible to observe that the LU851 dots are localised on the ring, but lie 180^{o} from each other, see Fig 4.4.

Figure 4.3 Double Staining of CHO Colchicine Treated Cells Tested with FITC-Anti-Mouse and TRITC-Anti-Human Antibodies, to Differentiate the Centromeric Localisation of the AC1 Antigen from the CENP's -A, -B, and -C

CHO cells were prepared for immunofluorescence microscopy by the standard procedure, with the exception that the primary antibody was **AC1** (mouse) supernatant and **LU851** (human) serum; and the secondary antibody was anti-mouse FITC conjugate and anti-human TRITC conjugate. Counter-staining for DNA was with **HOECHST** 33258.
The different chromosomal localisations of the autoimmune serum antigens and the AC1 antigen can be observed on the same preparation. LU851 only detected the paired centromere dots. The arrow indicates an AC1 ring antigen, onto which the LU851 antigens colocalised.

Magnification: x3,000.


ACI
LU851
HOECHST

Thus, this result confirms that the centromere ring antigen detected by AC1 is different from the antigens stained by anticentromere autoimmune sera, and shows the positional relationship between them.

Discussion

Two lines of evidence presented in this Chapter confirmed that the antigen, detected by antibodies produced by hybridoma clone AC1, are distinct from the other antigens identified to date in the centromeric region of mammalian chromosomes. Both the molecular weight and the centromeric localisation of the AC1 antigen demonstrate that the AC1 ring antigen is in fact a novel centromere structure.

The Cellular Localisation of the AC1 Antigen is Different to that of Other Proteins in the Centromeric Region

AC1 Antigen Versus INCENPs

Two other antigens isolated by exploiting anti-chromosome scaffold monoclonal antibodies, INCENPs (Cooke et al. 1987), gave a completely different cellular localisation in immunofluorescence studies. The INCENPs are localised to the pericentromeric region between the sister chromatids at metaphase (Fig 4.4). These antigens are not located in the ring structure, nor in the centriole. Therefore, the AC1 antigen is distinct from these.

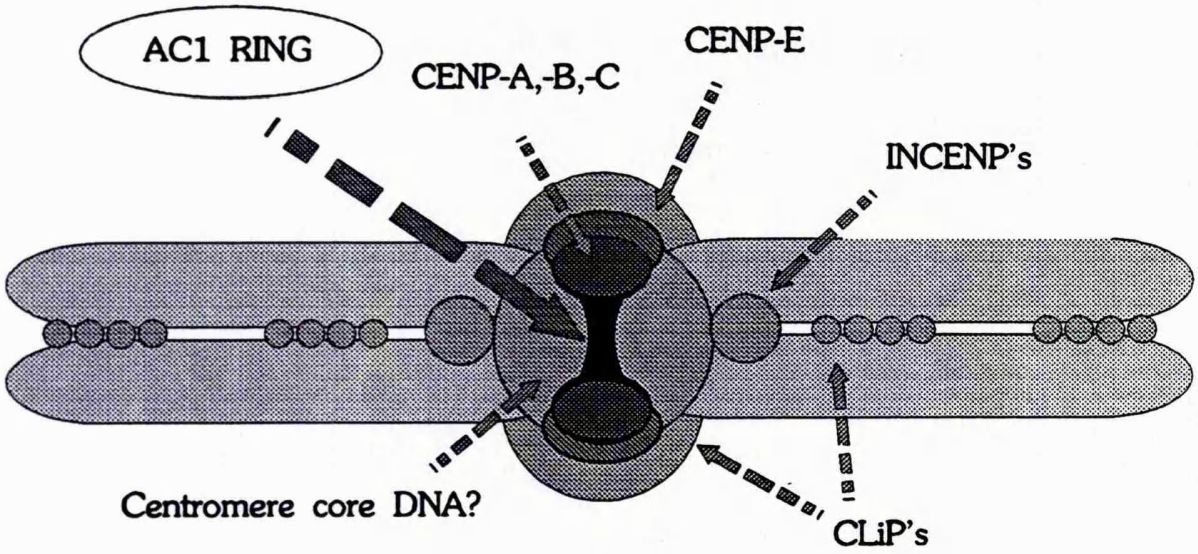
AC1 Antigen Versus CLiPs

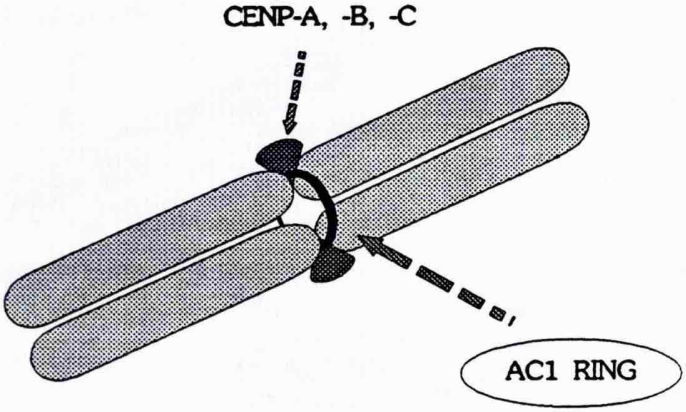
Another monoclonal antibody, raised by immunisation with extracts from sodium chloride treated bovine thymocyte samples (Rattner et al, 1988), did not detect an antigen localised to either the ring, or to the centriole, using immunofluorescence microscopy. These CLiP antigens are localised to two dots in the outer region where the microtubules bind the kinetochore (see Fig 4.4). Furthermore, these antigens could be removed by 0.4 M NaCl treatment, thus providing further evidence that they are different from the AC1 antigen (this remains associated with CHO chromosomes treated with 2M NaCl, see later Chapter, R6).

AC1 Antigen Versus CENPs -A, -B, -C

The double staining experiment, using AC1 antibodies and autoimmune serum LU851 on the same preparation demonstrated that the CENPs localise onto, but are distinct from, the centromere ring structure detected by antibodies from hybridoma clone AC1. This is illustrated in the 3 dimensional representation of the positional relationship of these two types of antigens given in Fig. 4.4.

Figure 4.4 Diagrammatic representation of the major centromeric, and associated proteins of the mammalian chromosome in 2D, and 3D to show AC1 centromere ring position in relation to the chromatid arms and CENP-A, -B, and -C





Molecular weights of centromere and associated proteins

CENP-A:	17 000
CENP-B:	80 000
CENP-C:	140 000
CENP-E:	250-300 000
INCENP:	135 000 & 155 000
CLiP's:	63 000 & 50 000
AC1:	170 000

AC1 AntigenVersus CENP-E

CENP-E is a recently discovered (Yen et al, 1991) centromere associated protein (using a Mab raised against human chromosome scaffolds), which is localised to two dots in a manner similar to the CENPs -A, -B, and C, but, which is more peripheral (refer to Fig. 4.4). This antigen has not been reported to form part of a centromeric ring structure, and therefore, appears to be unrelated to the AC1 antigen.

The Molecular Weight Obtained for the AC1 Antigen is Different to that of Other Proteins Associated with the Centromeric Region

The AC1 antigen detected a band of apparent molecular weight of 170kD in both CHO and human protein samples. Therefore, the AC1 antigen is conserved between these two species. In contrast, the molecular weight of the antigens detected by the human anticentromere autoimmune serum were not conserved. In CHO, LU851 detected bands of molecular weights 46kD and 83kD, whilst in human proteins the CENPs -A, -B, -C had molecular weights of 17kD, 80kD, 140kD, respectively (Earnshaw and Rothfield. 1985). The molecular weights of the other centromeric antigens are: the INCENPs 155kD and 135kD (Cooke et al. 1987); the CLiPs 50 and 63 kD (Rattner et al, 1988); a mitosis specific centromere antigen 59kD (Hadlaczky et al,1989) and CENP-E 250-300kD (Yen et al, 1991). Therefore, these data support the proposal that the AC1 antigen is a novel centromeric antigen.

Possible Function of the Centromere Ring Antigen

Having established that the antigen detected by Mab AC1 is a novel centromeric structure, the question arises as to its function. Since the form of the structure, in which the AC1 antigen is located, is circular, then this makes a role in microtubule binding highly unlikely; if microtubule binding were its function then a plate structure would be more likely. Rather this implies a possible function in holding the sister chromatids together. For instance, in prometaphase the alignment of the chromosomes on the metaphase plate involves a to- and fro- motion between opposing spindle poles, until an equilibrium is reached, when the kinetochores face the pole to which they are attached. Thus, at this stage of mitosis the chromosomes are under considerable torsional stress, and some means of maintaining the sister chromatid association is required. Therefore, a possible function of the centromere ring antigen may be to hold the sister chromatids together in prometaphase, whilst the kinetochore microtubules of the opposing poles partake in a type of "tug of war".

Having established that the AC1 antigen is a novel centromere antigen, it was of interest to investigate further its evolutionary conservation, and these experiments are presented in the next Chapter.

CONTENTS

R.5 EVOLUTIONARY CONSERVATION OF THE AC1 ANTIGEN BETWEEN RODENT AND HUMAN CELLS

Introduction

The overall aim of this project was to analyse the molecular structure of mammalian centromeres using CHO as a model system, but if homologues of centromere components could be found it was of particular interest to examine these on human centromeres. In the previous Chapter it was demonstrated that the AC1 antibody detected a novel centromere antigen on CHO centromeres (by immunofluorescence microscopy), and that the 170kD antigen was conserved between CHO and human (Colo) chromosomal proteins (by Western blotting). A study of the ring structure containing the AC1 antigen in CHO, *per se*, or as a potential model for human centromere organisation is worthwhile. However, it would be of even more interest if it could be shown by immunofluorescence microscopy that the AC1 antigen were conserved between other mammalian species, primarily if the centromere antigen could be detected on human chromosomes.

R 5.1 MAB BA2 DECORATES MOUSE (LMTK-) CENTROMERES

Since CHO and mouse cells are reasonably closely related species, it was thought possible that the centromere antigen detected by the Mabs AC1 and BA2 would be conserved between these two species. To examine this possibility, mouse ($LMTK^-$) cells were prepared for immunofluorescence microscopy by the standard procedure, and tested with supernatant from hybridoma clone BA2 (which apparently reacts with the same centromere and centriole components as does AC1). As was anticipated a centromere positive staining pattern could be observed on the $LMTK^-$ cells (Fig. 5.1C) demonstrating that the BA2 antigen was conserved in this mouse cell line. The same experiment carried out using Mab AC1 on mouse ($LMTK^-$) cells gave an identical staining pattern (data not presented). Mouse chromosomes have a particular extensive region of pericentric heterochromatin, which results in the strong HOECHST signal observed. The centromere staining pattern revealed that the positive signal occured within this region of heterochromatin.

Conclusions

The $LMTK^-$ chromosomes have long tracts of satellite DNA in the pericentromeric region, and this heterochromatic region remains condensed under the normal immunofluorescence preparation procedures, making it more heavily stained by the DNA binding dye, HOECHST 33258 (Fig. 5.1B/ and C/). The BA2 staining appeared to be within this region, indicating that the DNA interacted with by this antigen may be within this region, but not a sequence predominantly repeated in the mouse satellite DNA.

Figure 5.1 Immunofluorescence Microscopy of BA2 Staining of the Hybridoma Cells Own Centromeres, and BA2 Staining of Mouse (LMTK-) Cells

A and **B**: BA2 hybridoma cells were colchicine treated to obtain metaphases, cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation, and then incubated with anti-mouse FITC conjugated secondary antibody.

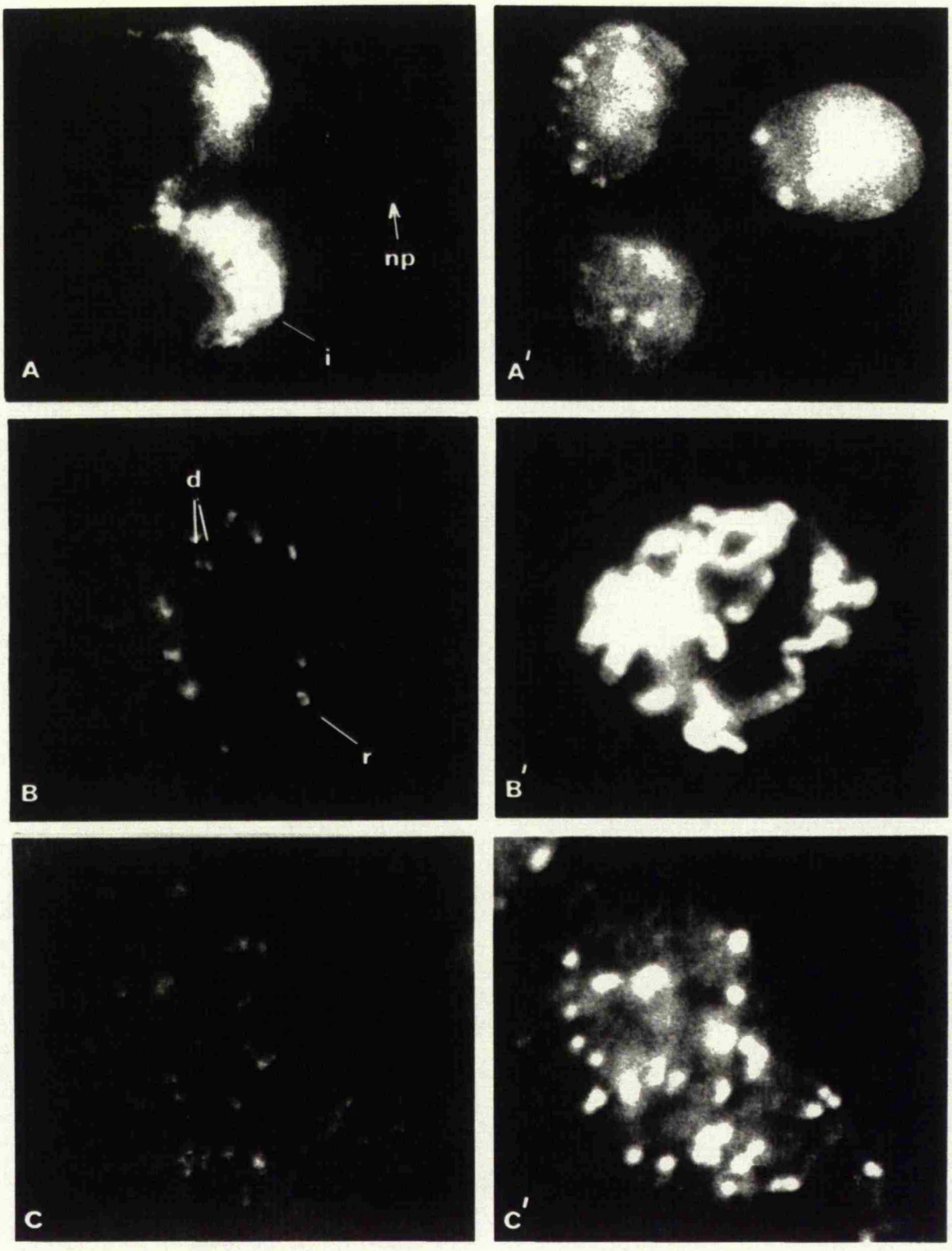
In interphase cells the cellular localisation of the immunoglobulin (**i**) can be observed (**A**). Not all of the cells are producing immunoglobulin, one such non-producer (**np**) is presented (**A**).
B shows a late prophase/ early prometaphase with two rings (**r**) next to each other, or a doublet (**d**).

C: Mouse ($LMTK^-$) cells were colchicine treated to obtain metaphases, cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation, and then incubated with column purified immunoglobulin from hybridoma clone BA2 and then anti-mouse FITC conjugated secondary antibody.

C shows a prophase mouse preparation. The double dots of the centromere are weakly stained. Notice the strongly stained heterochromatic centromeric regions of the mouse chromosomes. This is feature is normally characteristic of mouse chromosomes, due to the lengthy tracts of satellite DNA in the centromeric region.

A′, **B′**, and **C′** are the HOECHST 33258 staining patterns for DNA for **A**, **B**, and **C**, respectively.

Magnification: x3,000.


np
i
A
A′
d
r
B
B′
C
C′

R 5.2 AC1 ANTIBODY BINDS HYBRIDOMA CENTROMERE

The monoclonal antibodies produced by hybridoma clone cells are frequently raised against proteins of organisms other than mice, or its related species. Therefore, there is a limited danger of the immunoglobulins being generated actually binding to the hybridoma clone's own cellular proteins. However, if a Mab is required, which reacts with a mouse protein, or a protein from a related species, then a number of factors normally prevent the immunoglobulin being toxic to its own cells. After production, the immunoglobulin is normally compartmentalised, thereby preventing contact of the immunoglobulin with its own cellular proteins (Fig. 5.1A). Furthermore, the nuclear membrane acts as an additional barrier preventing access of immunoglobulins to nuclear proteins. However, during mitosis the nuclear matrix breaks down during late prophase. Therefore, during this phase of the cell cycle mouse nuclear proteins are potentially (though no data supports such a hypothesis) exposed to any "stray" monoclonal antibodies.

The material with which the mice were originally immunised for the production of anti-chromosome Mabs, was Chinese hamster chromosomal proteins. CHO and mice are reasonably close species evolutionarily. Therefore, it was considered possible that the anti-CHO mouse monoclonal antibodies may bind hybridoma cell proteins, and in particular the possibility arose that these antibodies may bind the mouse hybridoma cell's own centromeres.

Antibodies Produced by AC1 and BA2 Bind Hybridoma Centromeres

In order to test the possibility that the antibodies bind the hybridoma clones' own centromeres, AC1 and BA2 hybridoma cells were blocked at metaphase by colchicine treatment, after 3-4 hours the mitotic cells were harvested by shake-off and concentrated by centrifugation. These mitotic cells were prepared for immunofluorescence microscopy as for other cells, with one important exception. Unlike slides prepared for immunofluorescence microscopy in previous experiments, no primary antibody was used. Thus, only anti-mouse secondary antibody conjugated to FITC was applied. Therefore, any of the hybridoma cells' own immunoglobulins which had bound to their own cellular proteins prior to, or more likely during treatment for immunofluorescence microscopy, would be labelled by the FITC-conjugated secondary antibody. Hence, this may determine whether or not the immunoglobulin present normally in the cell is capable of binding its own centromeres. The result of this experiment showed that for both hybridoma clones AC1 (data not presented) and BA2 (Fig. 5.1B) centromere positive, in fact ring positive, reactions were detected for both clones.

Conclusions

From these results it can be concluded that the antigens detected by the Mabs produced by the hybridoma clones AC1 and BA2 are conserved between CHO and these mouse hybridoma cells, in addition to the other mouse cell line ($LMTK^-$).

Interestingly, the immunoglobulins produced by the hybridoma clones AC1 and BA2 were capable of binding their own centromeres, and these results can be interpreted as being due to one of two possible explanations. It is possible that during cytocentrifugation and before fixation, the intracellular immunoglobulins bind the centromeres, i.e. *in vitro* centromere binding. It is theoretically possible that the immunoglobulins indigenously present in the hybridoma cells, bound the antigens during the short period of cytocentrifugation (5 min, see Materials and Methods) and before fixation. Alternatively, although unlikely, it is possible that the immunoglobulins bound the centromeres prior to preparation for immunofluoresence microscopy, i.e. *in vivo* .

It seems more probable that centromere binding occurred *in vitro* . Normally protein synthesis, intracellular translocation and compartmentalisation of polypeptides is a highly ordered activity. However, if centromere binding had occurred *in vivo* , and since these cells were still capable of undergoing mitosis, then it can be interpreted that the antigen-antibody complex did not interfere drastically with centromere function.

Indirectly it can be assumed that only those clones remained viable, at the initial selection and the subsequent culturing stages, which produced immunoglobulins either not binding to regions essential for normal cell function, or binding without loss of function. If the Mabs bound an essential region, for instance in the endoplasmic reticulum, cytoplasm, or even the nucleus, then this would lead to the suicide of the cells producing them. Thus, the production of an anti-centromere hybridoma clone inherently involves the selection for antibodies which if able to bind hybridoma antigens, do so without functional inactivation.

R 5.3 THE AC1 CENTROMERE ANTIGEN IS CONSERVED TO HUMAN CENTROMERES

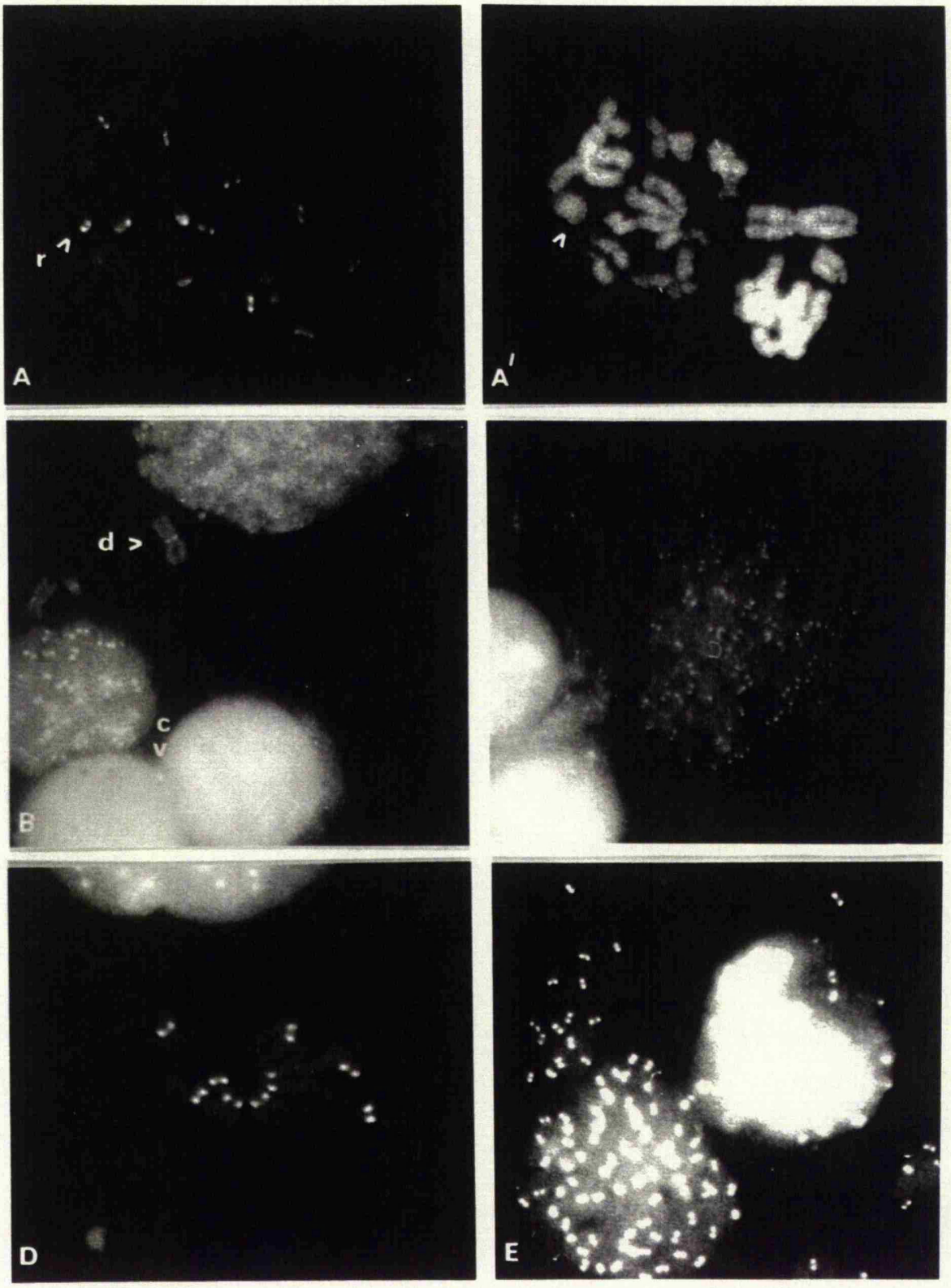
The molecular weights of the centromere proteins CENP-A, -B, and -C, are not conserved between CHO and humans (Chapter R4), although there are CHO homologues of different sizes. However, the AC1 antigen appeared to be conserved between these two species on the basis of Western blotting data, and it was reasonable to expect that the centromere antigen may be detectable on human chromosomes in immunofluorescence studies. In order to test this hypothesis human (EJ30) cells were prepared for immunofluorescence microscopy and tested with AC1 Mab by the standard protocol (Fig. 5.2). As was anticipated, the antigen was indeed conserved to these human cells.

Figure 5.2 Immunofluorescence Microscopy of CHO and Human (EJ30) Cells Stained with Mab AC1, and LU851 of Human (EJ30) Cells

CHO and human (EJ30) cells were colchicine treated to obtain metaphases, cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation. CHO preparations were incubated with supernatant from hybridoma clone AC1, and human preparations were tested with such an AC1 supernatant or human anticentromere autoimmune serum LU851; and then anti-mouse or anti-human FITC conjugated secondary antibodies, respectively.

A shows the usual anticentromere staining pattern obtained by Mab AC1 on a CHO metaphase spread. **A'** is the HOECHST 33258 staining for DNA of **A**. Notice the ring (**r**) of the chromosome lying in transverse orientation. The arrowhead in **A'** shows the transverse orientation of the chromosome, on which the ring can be detected.
B and **C** give the AC1 staining pattern on human (EJ30) metaphase and interphase cells. Notice the interphase centrioles (**c**), and that AC1 decorates the pair of dots (**d**) of the metaphase centromeres, but does not react with the interphase centromeres.
D and **E** demonstrate the centromere staining pattern obtained with the anticentromere autoimmune serum LU851 on human (EJ30) preparations. Both the interphase and metaphase centromeres are stained.

Magnifications: A, B,C, E: x2,000; D: x3,000.


r
A
A'
d >
c
v
B
D
E

In fact both the double dots of the metaphase centromeres (Fig. 5.2B and C), single or pair of dots (depending on centriole replication) interphase centrioles (Fig. 5.2B), but not the interphase centromeres (Fig. 5.2B), were decorated. Therefore, essentially the same pattern was obtained as for the mouse and hamster cells. Moreover, the centromere ring could also be detected (see Chapter R 7.2) on the human cells, demonstrating the conservation of the ring structure, amongst the three mammalian species examined.

Conclusions

These experiments demonstrated that the centromere antigen detected by Mabs AC1 and BA2, is conserved between the mammalian species tested, i.e. hamster (K20), mouse (hybridoma and LMTK$^-$) and most importantly human (EJ30). This suggests that the antigen is serving a function common to and of fundamental importance in these species.

Since the centromeres of all animal cells carry out essentially the same function, such as maintaining the prometaphase sister chromatid association as suggested in Chapter R4, then it may be anticipated that some of the molecular components be conserved, or have close homologues. Thus, the ring structure detected by Mab AC1 in CHO, mouse and human cells may be one such component.

One of the long term practical/applied aims of chromosome structure research (in addition to its basic research appeal) is, by seeking an understanding of its molecular nature, the construction of artificial chromosomes. The construction of artificial chromosomes has at least two purposes. One is to test the theories of structure and function upon which their production was based, and the other is to produce a potential tool for the treatment of diseases (for an explantion of the latter see main Discussion). Once the important structural elements have been identified and characterised, the creation of a human artificial chromosome becomes more realistic. Thus, the presence of the AC1 antigen on human chromosomes is all the more exciting (see main Discussion).

CONTENTS

R 6 THE CENTROMERE RING IS A COMPONENT OF THE CHO CHROMOSOME SCAFFOLD

Introduction

As described previously, the AC1 antigen was shown to be localised to a ring structure encircling the centromere (Chapter R4). It was considered, therefore, possible that this substructure might serve some structural role, for instance the antigen may physically stabilise the centromere, by holding the sister chromatids together at metaphase. If the AC1 antigen were to serve a structural function, then it might be anticipated that the antigen interacts with other molecular components of the centromere. Since the position of the centromere (shown by C-dot staining or banding, Bostock & Sumner, 1978, primary constriction localisation and anti-centromere autoimmune sera staining, Fritzler et al, 1980; Moroi et al, 1980) on a given chromosome is constant, this suggests that there is an underlying DNA sequence determining the location of the mammalian centromere proteins. The AC1 antigen could interact with centromere proteins or possibly could interact with the centromeric DNA itself.

Chromosome scaffold fractions are depleted of H1 and the core histones, and thus, are enriched for certain other proteins (Adolph et al, 1977). The chromosome scaffold is a residual structure consisting of a core of non-histone proteins thought to tightly interact with the DNA at the bases of DNA loops (Paulson & Laemmli, 1977). The preparation of scaffolds from isolated chromosomes by high salt treatment alone, results in this residual core being surrounded by a halo of these loops of DNA. The additional treatment of these structures with restriction endonucleases results in the removal of the DNA halo, a structure in which the DNA content has been estimated at 0.1 to 0.5% of the chromosomal DNA (Adolph et al, 1977; Bowen et al, 1981). Since the non-histone proteins strongly interact with the scaffold DNA it is thought that they serve a structural function (Hadlaczky et al, 1982).

Since the original immunising material resulting in the production of the hybridoma clone AC1 was such a chromosome scaffold fraction, it is conceivable that the AC1 antigen be a component of the chromosome scaffold. To investigate this, it was decided to examine, by immunofluorescence microscopy, if the ring structure itself remained detectable on chromosome scaffolds preparations. The high salt treatment (high ionic strength, used to obtain scaffolds) disrupts the protein-protein interactions, and the weaker (histone-DNA) nucleoprotein complexes, by the dissociation of electrostatic interactions (Gasser at al, 1989). If detectable on the chromosome scaffolds, this would indicate the AC1 antigen tightly associates with a protein bound to DNA or to the DNA itself.

R 6.1 THE CENTROMERE RING IS DETECTED IN THE CHO CHROMOSOME SCAFFOLD BY IMMUNOFLUORESCENCE MICROSCOPY

To determine if the ring antigen was present in chromosome scaffold preparations, CHO cells were blocked at metaphase by colchicine treatment, and were harvested by shake-off and concentrated by centrifugation by the standard method. Chromosomes were then liberated from cells by passage through a 23G needle, and separated from contaminating nuclei by differential centrifugation. The chromosomes were then separated from contaminating cytoplasm by repeated washing and high speed centrifugation (as described in Materials and Methods). The chromosomes (in 1ml isolation buffer) were then layered onto 2M NaCl and 5% sucrose in chromosome isolation buffer, and centrifuged at high speed onto slides. The resulting slides were then processed for immunofluorescence with AC1 antibodies. These procedures were the same as for previous immunofluorescence with the exception that counter-staining for DNA was achieved with propidium iodide).

An examination of these slides revealed that the two dots, lines, and most importantly rings, could indeed be detected on the CHO chromosome scaffolds (see Fig. 6.1). The 2M NaCl treatment resulted in the chromosomes being expanded, i.e. more loosely packaged and consequently larger than under more physiological conditions, and surrounded by a halo of DNA. Therefore, the centromere was no longer an obvious constriction on the chromosomes. The antigen stained by Mab AC1 did, however, localise to the residual chromosomes and not to the DNA halo, strongly suggesting that the antigen tightly binds to the DNA of these residual structures.

Discussion

Since the original immunising material used to generate Mab AC1 was CHO chromosomes resistant to DNase I and 2M NaCl treatment, then such residual matter would have been the most appropriate material for the immunofluorescnce experiments. However, it has been demonstrated that the protein profiles of such high salt and restriction enzyme treated, and only high salt treated chromosomes, without enzyme digestion, are identical within the limits of detection (Hadlaczky et al, 1981). Furthermore, a DNase I digestion of the chromosomes in addition to the 2M NaCl treatment was not considered practical, since such material would be so far removed from normal chromosome morphology, that it would not be possible to determine where the antigen localised.

This experiment shows that the ring structure is retained in CHO chromosome scaffolds, showing that it is remarkably stable, which is consistent with the antigen being a protein tightly interacting with another protein or directly bound to DNA. The ring may, therefore, be a key point of structural stability of mammalian centromeres.

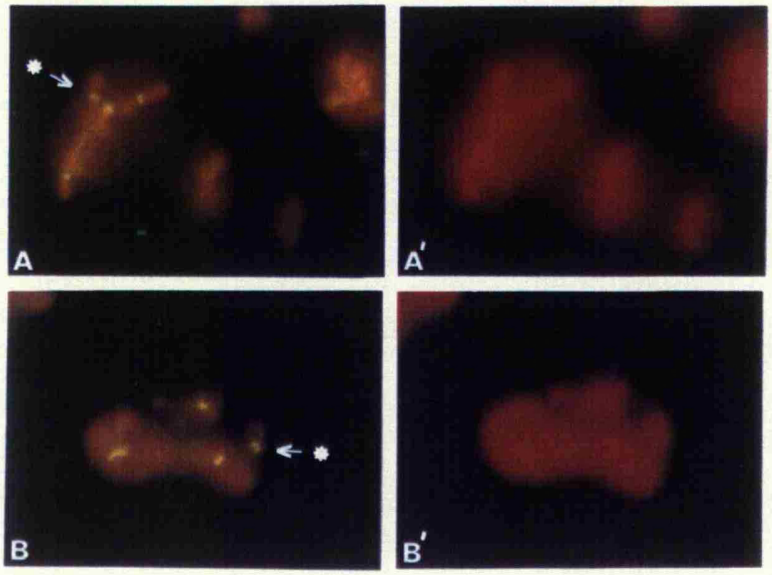
Figure 6.1 Indirect Immunofluorescence Microscopy of CHO Isolated Chromosomes Centrifuged Through 2M NaCl onto Slides and Tested with AC1 Supernatant: Demonstration that the Centromere Ring Antigen is a Component of the Isolated Chromosome Scaffold Preparations

Isolated CHO chromosomes were centrifuged at high speed through isolation buffer containing 2M NaCl onto slides in order to obtain chromosome scaffolds for immunofluorescence microscopy. Immunological procedures were carried out according to the standard protocol. Counter-staining for DNA was with propidium iodide (**A'** and **B'**) of AC1 treated preparations (**A** and **B**, respectively).

In **A**, a distorted ring (starred arrow) can be seen on the metaphase chromosomes. The chromatid arms consist of high salt resistant, ie scaffold proteins, and the surrounding halo is the expanded DNA.

In **B**, another distorted ring (starred arrow) can be observed on a late prophase/ early metaphase chromosome.

Magnification: x1,000.


A
A'
B
B'

Thus, the centromere ring antigen, alone or in association with other proteins, may bind centromeric DNA sequences and might play a role in physically stabilising the sister chromatids at the centromere, as discussed earlier (Chapter R4).

A possible interaction of the AC1 antigen directly with DNA may explain the difficulties encountered in its detection by the method of Western blotting. For instance, the tight association of the antigens with DNA may limit its solubility and ability to enter the polyacrylamide gel, or result in its poor electrophoretic transfer to nitrocellulose filters (Bowen et al, 1980). Thus, a DNA interaction may severely limit the quantity of the antigen available for detection by this system. This could possibly be tested by digesting CHO chromosomal proteins with DNase prior to Western blotting with Mab AC1.

Assuming that the AC1 antigen interacts with the metaphase centromeric DNA, the staining of the interphase, and cytoplasmic structures, namely the centrioles, is a puzzling matter. Indirectly there is reasonable evidence that the antigen detected by AC1 at the centromere is the same as that stained at the centriole. Two different monoclonal antibodies, AC1 and BA2, (possibly recognising different epitopes) both detected the centromere and the centriole, and produced an identical staining pattern in each case. Furthermore, the same staining pattern of both the centromere and centrioles was obtained with human chromosomes using the AC1 antibody. Although possible, it is questionable that the two different antibodies, AC1 and BA2, were both cross-reacting with the same protein of the centriole; and more unlikely still that Mab AC1 was cross-reacting with this same protein in two different species. If the centromere ring and the centriole both contain the same antigen, then the questions arise as to the role of the antigen in the centriole, and the relationship of this antigen in the two decidedly different cellular localisations. It has been shown that centrioles (although not in mammalian cells) contain DNA (Hall et al, 1989). Therefore, it is conceivable that the AC1 antigen binds a protein-DNA complex, or the DNA directly, in the centriole and not just at the centromere.

In interphase the AC1 antigen was not detected on the centromere by immunofluorescence microscopy. Moreover, by Western blotting, the AC1 antigen was shown to be more abundant in metaphase than in interphase. Since the AC1 antigen appears to strongly interact with a protein-DNA complex, or directly to the DNA in metaphase, then it is interesting to speculate on the fate of the antigen after metaphase and, in particular, in interphase. It is plausible that the centromeric antigen is either degraded *in vivo* after it has fulfilled its function in mitosis, or that the epitope is altered and so the antigen is no longer detectable in interphase at the centromeres.

The behavior of the AC1 antigen during the cell cycle, and its possible relationship to the spindle apparatus will be addressed later (Chapter R8). However, in the next Chapter attention will be turned to the relationship of the AC1 antigen to another high molecular weight chromosome scaffold (DNA binding) protein, namely DNA topoisomerase II.

CONTENTS

R 7 RELATIONSHIP BETWEEN CENTROMERE AND TOPOISOMERASE II

Introduction

The enzymatic properties of topoisomerase II have been well characterised (for a review see Wang, 1985) and this is the most abundant protein of the chromosome scaffold (Earnshaw & Heck, 1985; Gasser et al, 1986). This protein has been localised to the chromatid arms on gently expanded chromosomes (Gasser et al, 1986). Light and electron microscopy studies have shown that topoisomerase II is localised to the radial loop domains of chromosomes (Earnshaw & Heck, 1985) and nuclei (Berrios et al, 1985). The importance of topoisomerase II has been shown for the correct disjunction of chromosomes at anaphase (DiNardo et al, 1984; Holm et al, 1985, Uemura & Yanagida, 1986) and a role in chromosome condensation has been suggested (Newport & Spann, 1987). Also, electron microscopy studies have shown that the most resistant region of the chromosome to 2M NaCl treatment is the centromere (Hadlaczky et al, 1982).

Both the centromere antigen, detected by antibody AC1, and topoisomerase II have molecular weights of about 170kD. In view of these facts, but, despite the observation that topoisomerase II is relatively abundant in chromosome scaffolds, whereas, the AC1 antigen is a minor chromosomal protein, a possible relationship between the centromere and topoisomerase II may be proposed. Thus, it may be postulated that the topological changes which occur at the centromere during mitosis might be due to a centromere specific variant of topoisomerase II. In order to investigate these possibilities, first it was deemed a priority to show by Western blotting that the anti-topoisomerase II antibodies really did detect an antigen in the same molecular weight range as the AC1 antigen, since it has been suggested that topoisomerase II undergoes considerable *in vivo* and *in vitro* degradations (Hadlaczky et al, 1988). Two of the anti-topoisomerase II antibodies currently available in this laboratory, were tested, therefore, on Western blots of chromosomal and nuclear proteins, from CHO, and human cells. CHO and human (Colo) chromosomes and nuclei were isolated, separated by SDS-PAGE and prepared for Western blotting by the normal procedures and the results are detailed below.

R 7.1 DETECTION OF THE AC1 ANTIGEN AND TOPOISOMERASE II BY WESTERN BLOTTING

Western blots of CHO chromosomal and nuclear protein samples were made in order to demonstrate that the available anti-*Drosophila* topoisomerase II antibodies did indeed react with the CHO topoisomerase, that bands of the expected molecular weight could be detected by the anti-topoisomerase antibodies, and to compare the pattern of bands given by these antibodies with those obtained with AC1.

CHO Chromosomal and Nuclear Proteins Probed with AC1 and Anti-Topoisomerase II (1C2) Mabs

The anti-*Drosophila melanogaster* topoisomerase II mouse monoclonal antibody, termed 1C2, was shown previously, to detect the 166kD DNA topoisomerase II canonical form in *Drosophila* embryo total proteins, a 166kD doublet in freshly frozen embryos, and multiple bands in the range 170kD to 132kD in purified enzyme preparations from embryos and pupae (Hadlaczky et al, 1988). In the Western blot in this experiment, both AC1 and 1C2, monoclonal antibodies, detected antigenic bands of about 170kD (Fig. R7.1A) with preparations of chromosomal proteins. The signal obtained with AC1 was, as anticipated, much stronger with the chromosomal rather than with nuclear proteins, consistent with its being enriched in chromosomes. The 1C2 Mab only faintly detected a 170kD antigenic band in the chromosome fraction, but no detectable signal could be observed in this region in the nuclear fraction. Unlike the AC1 Mab, 1C2 reacted with additional bands of molecular weights 200kD and 120kD with the chromosomal proteins; and gave the strongest signal on a 66kD antigen with both CHO protein fractions Thus, this anti-topoisomerase II antibody does detect a band in apparently the same molecular weight range as that detected by AC1 on these and previous CHO protein samples (in Chapter R3).

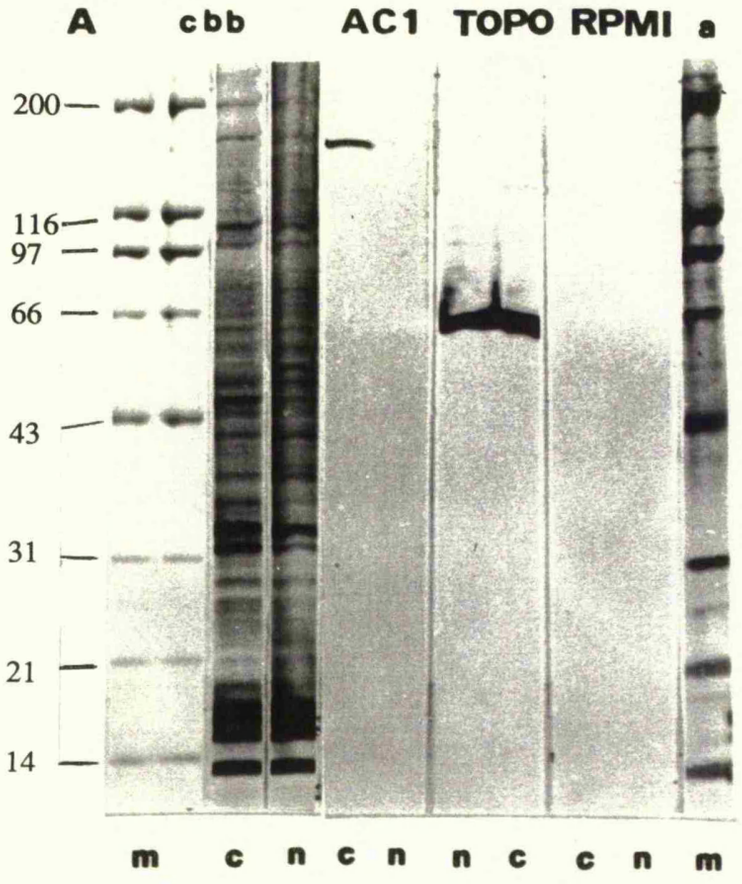
Discussion

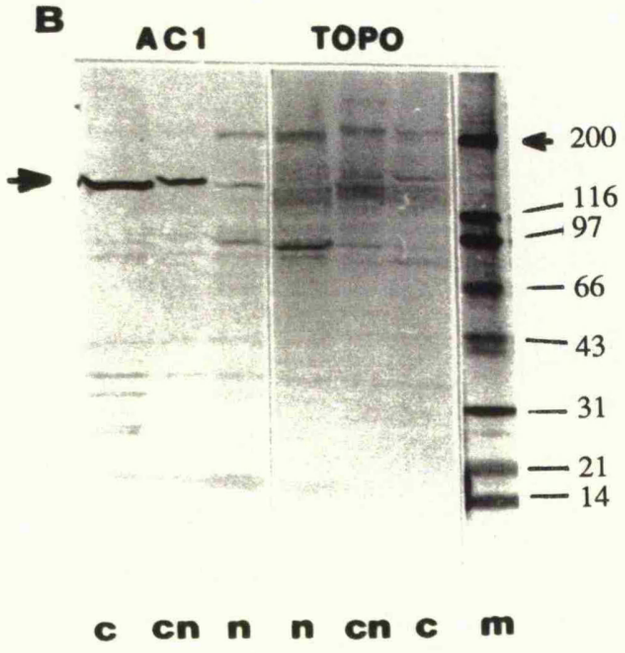
The stronger signal of the 170kD AC1 antigen in CHO chromosomal compared with nuclear proteins is consistent with the immunofluorescence data (Chapter R4) and the postulated role of the AC1 antigen in chromosome structure. The weak signal obtained with the anti-topoisomerase II Mab 1C2 in CHO chromosomes may indicate that this epitope is rare (only one or few epitopes per antigen) on CHO topoisomerase II, or that the polypeptide or epitope had suffered *in vitro* and/or *in vivo* degradation, which has been observed in *Drosophila* (Hsieh, 1983; Shelton et al, 1983). The strongly staining 66kD antigenic band may correspond to a stable degradation product as also observed in *Drosophila* (38kD antigen) by Hadlaczky et al, 1988 (see later, R 7.4), raising the possibility that the 120kD band (Fig. 7.1 A) may also be an *in vitro* degradation product. The fact that 1C2 does not react with the higher molecular weight antigens (120kD and above) in the CHO nuclear proteins may suggest that the antigen or epitope is only present in sub-detectable levels, or has been degraded.

Since in contrast, the AC1 Mab only detected the higher molecular 170kD antigen the results indicated in fact that topoisomerase II and the AC1 antigen were distinct molecules. Nevertheless, depending on the difference in distribution, quantity or sensitivity to degradation of the epitopes, different monoclonal antibodies can give different signal intensities on the same antigen.

Figure 7.1 Western Blots of CHO and Human, Chromosomal and Nuclear Proteins with Mab AC1 and Anti-Topoisomerase II Antibodies

A: CHO chromosomes (**c**) and nuclei (**n**) were Western blotted by the standard procedure and tested with supernatant from hybridoma clone **AC1**, and supernatant from an anti-topoisomerase II (**TOPO**) monoclonal antibody, 1C2. **RPMI** represents the negative control, culture medium not containing hybridoma immunoglobulin. Coomassie Brilliant Blue (**cbb**) stained chromosome, nuclear proteins are shown, and molecular weight markers (**m**) are of the following values: myosin 200kD, *E.coli* beta-galactosidase 116.25kD, rabbit muscle phosphorylase b 97.4kD, bovine serum albumin 66.2kD, hen egg white ovalbumin 42.699kD, bovine carbonic anhydrase 31.0kD, soybean trypsin inhibitor 21.5kD, hen egg white lysozyme 14.4kD. **a** is the Amido Black staining of the markers after electrophoretic transfer to nitrocellulose filters.
Notice the 170kD band detected by AC1 in the chromosomal proteins, and in lesser quantities in the nuclear proteins. 1C2 also stains a band of the same molecular weight in chromosomal proteins, in addition to 200kD, 120kD bands. This Mab also gives a strong signal with a 66kD antigenic band in both chromosomes and nuclei.
B: Human (Colo) chromosomes (**c**), nuclei (**n**) and mixed fraction of chromosomes and nuclei (**cn**) were Western blotted by the standard procedure and tested with supernatant from hybridoma clone **AC1**, and in this case with serum from an anti-topoisomerase II (**TOPO**) mouse polyclonal antibody. **m** is the amido black staining of the molecular weight markers electrophoretically transferred to nitrocellulose filters as in **A**. The larger arrowhead indicates the 170kD antigen which is detected by both AC1 and the polyclonal antibody. The distribution of the AC1 170kD antigen, between the different protein fractions, is different to that exhibited by topoisomerase II. The AC1 antigen is more abundant in the chromosome fraction and the signal decreases in intensity as the proportion of nuclei increases. In contrast, the topoisomerase II 170kD is present in equal quantities in all three fractions. In addition to the 170kD antigen, the anti-topoisomerase II polyclonal antibody detects a band of molecular weight 200kD in all three fractions (smaller arrowhead), as does the AC1 antibody, but in nuclei only.


A
cbb
AC1
TOPO
RPMI
a
200
116
97
66
43
31
21
14
m
c
n
c
n
n
c
c
n
m


B
AC1
TOPO
200
116
97
66
43
31
21
14
c
cn
n
n
cn
c
m

Since both the antibodies used were monoclonal, then even the differences in signal strength, between the protein fractions, does not exclude the possibility of the AC1 antigen being related to or even identical to topoisomerase II.

R 7.2 COMPARISON OF THE DISTRIBUTION OF THE AC1 ANTIGEN AND TOPOISOMERASE II BETWEEN HUMAN CHROMOSOMAL AND NUCLEAR PROTEINS BY WESTERN BLOTTING

In parallel to the Western blot with CHO proteins, AC1 and another anti-topoisomerase II, mouse polyclonal serum, available in the laboratory, were tested on human protein samples.

Human Chromosomal and Nuclear Protein Fractions Probed with Mab AC1 and an Anti-Topoisomerase II Polyclonal Antibody

To compare the antigenic bands detected on human (Colo) proteins, isolated chromosomes and nuclei were prepared for Western blotting by the standard protocol. Since the AC1 antigen, was previously found to be (Chapters R3 & R4) apparently more abundant in chromosomal fractions (consistent with this was the result in Fig. 7.1A), then a mixed fraction of approximately 1:1 chromosomes: nuclei was included in the Western blot. The other anti-topoisomerase II antibody available in this laboratory was a mouse polyclonal (from which the 1C2 monoclonal was derived) serum reacts with an approximately 170kD (the 166kD canonical form of) topoisomerase II, and a 38kD antigen, possible *in vivo* degradation product, on *Drosophila* total embryo proteins (Hadlaczky et al, 1988).

The result of the Western blot, probed with supernatant from hybridoma AC1, and the anti-topoisomerase II mouse polyclonal serum is shown in Fig. 7.1B. Both types of antibodies again reacted with an apparently similar molecular weight antigen of 170kD. However, the intensity of the staining of the bands between the protein samples was markedly different for the two antibodies. As was anticipated, the anti-centromere Mab AC1 detected a band, which was more abundant in the chromosome fraction than in the mixed fraction, which in turn was more abundant than in the nuclear fraction. In contrast, the 170kD antigen detected by the anti-topoisomerase II polyclonal was present in approximately equal quantities in all three fractions as would, in fact, be expected for topoisomerase II. Therefore, although both antibodies recognise an apparently similar high molecular weight polypeptide, the quantitative distributions of the antigens and/or the epitopes are different.

Discussion

Mab AC1 stained a 170kD antigen in the human proteins in a similar manner as to that observed with CHO (R7.1), i.e. the signal is stronger in the chromosomal proteins by comparison with the nuclear proteins. However, an additional antigenic band of apparent molecular weight 200kD detected by Mab AC1 was observed in the nuclear fraction. A band of apparently the same higher molecular weight was also detected by the Mab BA2 (Chapter R3), in addition to the 170kD when, as in this experiment also, freshly prepared Colo fractions were tested. Therefore, this result may indicate that in interphase there is a 200kD precursor to the 170kD antigen. The polyclonal anti-topoisomerase II antibody staining of both the 170kD and the 200kD, suggests that in human cells there is a larger polypeptide that may also be processed to give the 170kD form of topoisomerase II. Again, however, the differences in the signal strengths with AC1 observed between the different protein preparations may be due to conformational changes or distribution of epitopes, hence these results cannot exclude the possibility of identity between the AC1 antigen and topoisomerase II.

R 7.3 HUMAN INTERPHASE AND METAPHASE EXTRACTS PROBED WITH AC1 AND 1C2 MABS

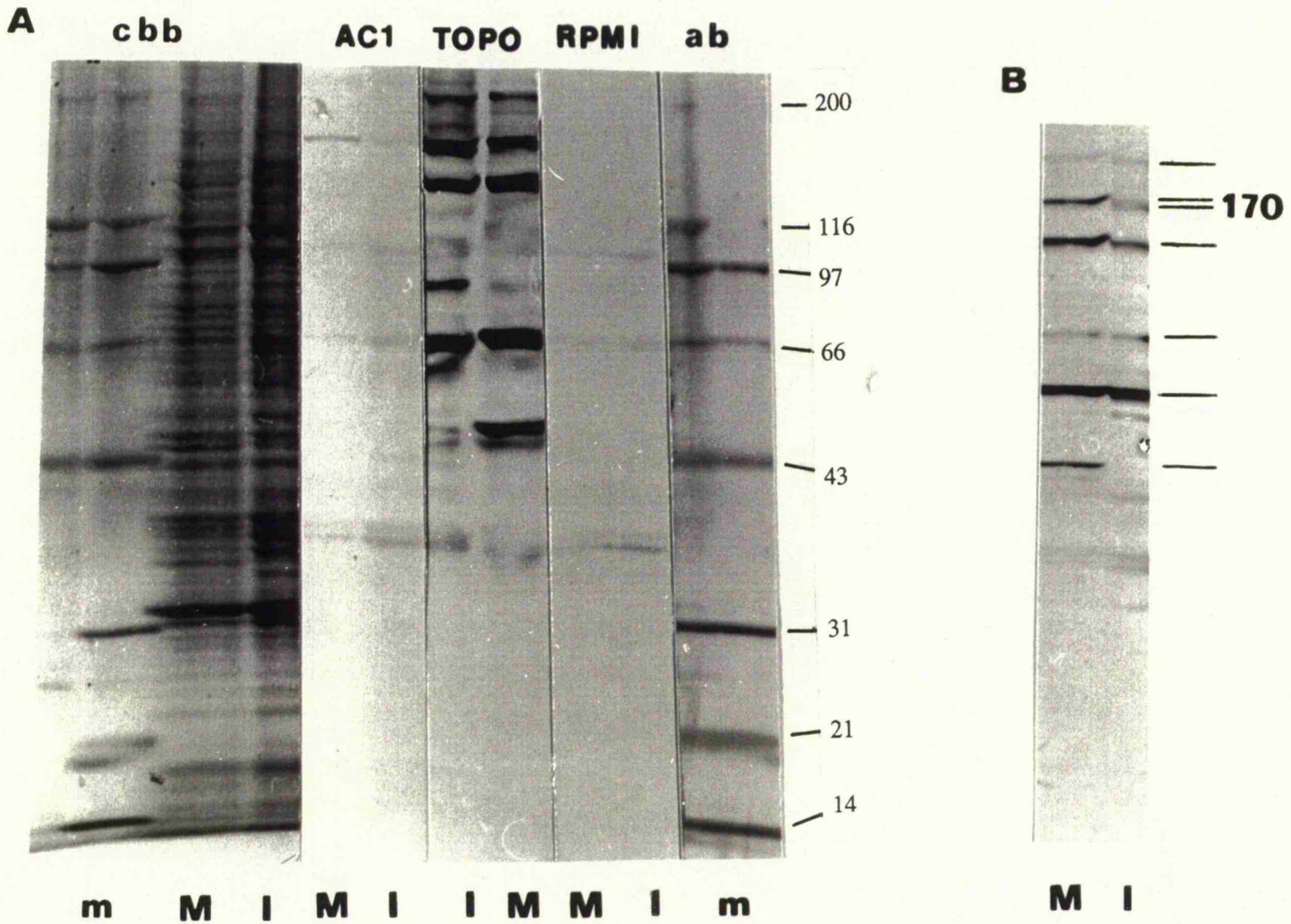
Due to the known interaction of eukaryotic topoisomerase II with DNA, and the previously demonstrated affinity of the AC1 antigen for DNA (Chapter R4), the behaviour of the AC1 and topoisomerase II antigens was investigated on salt treated human (Colo) metaphase and interphase extracts. Western blots were prepared as previously, and tested with supernatants from AC1 and 1C2. As was expected, the AC1 antibody stained an antigen of apparent molecular weight 170kD in the metaphase extract proteins, and gave a weaker signal with the interphase extract proteins (see Fig. 7.2). In contrast, the 1C2 Mab detected several bands on both metaphase and interphase proteins: 200kD, 170kD, 135kD, 85kD and 66kD. Interestingly, this Mab apparently reacts with a doublet in the 170kD weight region with the interphase preparation, but not with the metaphase preparations (in a similar manner to that observed on *Drosophila* fresh embryos, Hadlaczky et al, 1988). Furthermore, an additional, lower molecular weight antigenic band appeared in the metaphase fraction, of weight 45kD. It is tempting to consider this to be a breakdown product of one of the 170kD doublet bands.

Discussion

The same trend for AC1, enrichment in metaphase over interphase fractions, was observed with the salt treated proteins, as was also noticed for the native chromosomal by comparison with the nuclear proteins. The 1C2 anti-topoisomerase II Mab detected several bands in both the chromosomal and nuclear fractions with equal intensities, with two interesting features.

Figure 7.2 Western blot of Human (Colo) Interphase and Metaphase Extracts Tested with AC1 Supernatant and Anti-topoisomerase II Mab

Human (Colo) chromosomes and nuclei were isolated by the standard procedure and treated to obtain NaCl extracts. Proteins were separated by 5-20% SDS PAGE and electrophoretically transferred to nitrocellulose filters. Western blots were tested with undiluted supernatant from hybridoma clone **AC1** and the anti-topoisomerase II hybridoma clone 1C2 (**TOPO**), and a negative control of **RPMI** medium containing no hybridoma immunoglobulins. Coomassie Brilliant Blue (**cbb**) stained metaphase (**M**), and interphase (**I**) extract proteins are shown, and molecular weight markers (**m**) are of the following values: myosin 200kD, *E.coli* beta-galactosidase 116.25kD, rabbit muscle phosphorylase b 97.4kD, bovine serum albumin 66.2kD, hen egg white ovalbumin 42.699kD, bovine carbonic anhydrase 31.0kD, soybean trypsin inhibitor 21.5kD, hen egg white lysozyme 14.4kD. **ab** is the Amido Black staining of the markers after electrophoretic transfer to nitrocellulose filters.
In **A** it is possible to observe that the AC1 antigen is more abundant in the metaphase extract compared with the interphase one, in contrast to the level of topoisomerase II. Amongst the antigenic bands detected by the anti-topoisomerase Mab the 200kD band was detected in both interphase and metaphase extracts; and a 170kD doublet was present in the interphase, but not in the metaphase extract. In **B** a 1/10 dilution of the 1C2 Mab is shown, which more clearly presents the 170kD doublet.


A
cbb
AC1
TOPO
RPMI
ab
200
116
97
66
43
31
21
14
m
M
I
M
I
I
M
M
I
m
B
170
M
I

In the interphase protein preparation, a doublet was detected in the 170kD molecular weight region, which was not detected in the metaphase fraction, and an additional band of 45kD appeared when the metaphase fraction was analysed. This may be a degradation product of the topoisomerase II 170kD form. The existence of a doublet in this molecular weight region in interphase is in itself interesting. The fact that one of these two antigenic bands may be degraded, and the other not, is consistent with the presence of multiple forms of topoisomerase II in the cell (Hadlaczky et al, 1988), which possibly have different functions (for instance as a centromere specific variant).

These results, as did the previous ones, demonstrate that both Mabs detected antigens with similar properties, but supports the conclusion that they are indeed distinct.

R 7.4 TOTAL PROTEINS OF CHO INTERPHASE CELL AND DROSOPHILA FRESH EMBRYO PROTEINS PROBED WITH AC1 AND ANTI-TOPOISOMERASE II ANTIBODIES

In an attempt to discriminate more specifically between the AC1 antigen and topoisomerase II, a protein sample enriched in topoisomerase II, *Drosophila* fresh embryos, was tested by Western blotting with AC1 and with both of the anti-topoisomerase II antibodies. If the AC1 Mab reacted with a 170kD antigen in such *Drosophila* proteins this would be an indication of the identity of the AC1 antigen with topoisomerase II. Conversely, it is reasonable to expect the anti-topoisomerase II antibodies should react with the same molecular weight antigenic band, as does the AC1 Mab, in CHO proteins, if there is identity between the two.

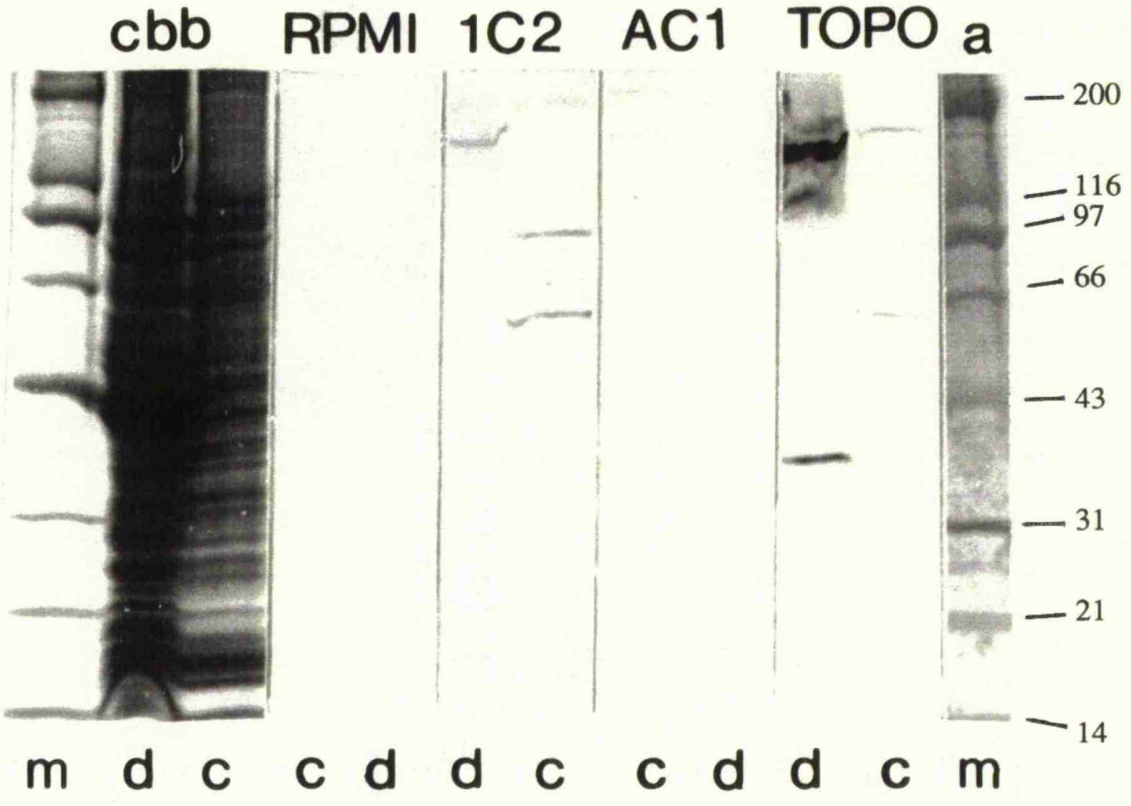
Since topoisomerase II is normally constitutively expressed, CHO interphase cells were isolated from non-colchicine treated cells, and these total cells were dissolved in electrophoresis sample buffer. *Drosophila* fresh embryos, which contain increased levels of topoisomerase II, were kindly provided by Dr. A. Udvardy and dissolved in sample buffer. These two protein samples were separated by 5-20% SDS-PAGE and electrophoretically transferred to a nitrocellulose filter by the standard procedure. The resulting Western blot was tested with the two types of anti-topoisomerase II antibodies, Mab 1C2 and the mouse polyclonal serum, together with Mab AC1 and RPMI (culture medium containing no hybridoma immunoglobulin) as a negative control (see Fig. 7.3).

The polyclonal anti-topoisomerase II serum detected antigenic bands of 170kD and 60kD, and AC1 reacted with bands in the range 220 to 200kD and 170kD in the CHO interphase total proteins. The monoclonal anti-topoisomerase II, 1C2, detected bands of weights 200kD, 95kD and 60kD, but in this experiment not 170kD in CHO interphase proteins.

Figure 7.3 Western Blot of CHO Interphase Total Cell and Drosophila Fresh Embryo Proteins Tested with AC1 and Anti-Topoisomerase II Antibodies

CHO cells were isolated as normal, with the exception that colchicine was not added to the culture medium. These CHO interphase cells (**c**) were dissolved in electrophoresis sample buffer. Drosophila fresh embryos (**d**) were dissolved in sample buffer. These two protein samples were Western blotted by the standard protocol. **RPMI** represents the culture medium, which does not contain hybridoma immunoglobulins, **1C2** the anti-topoisomerase II Mab, **AC1** anti-centromere Mab and **TOPO** the anti-topoisomerase II mouse polyclonal. Shown are Coomassie Brilliant Blue (**cbb**) stained chromosome, nuclear proteins and molecular weight markers (**m**) of the following values: myosin 200kD, *E.coli* beta-galactosidase 116.25kD, rabbit muscle phosphorylase b 97.4kD, bovine serum albumin 66.2kD, hen egg white ovalbumin 42.699kD, bovine carbonic anhydrase 31.0kD, soybean trypsin inhibitor 21.5kD, hen egg white lysozyme 14.4kD. **a** is the Amido Black staining of the markers after electrophoretic transfer to nitrocellulose filters.

Notice that Mab 1C2 does not react with a 170kD antigen in CHO interphase proteins. The anti-topoisomerase II polyclonal does react with a 170kD antigenic band in the CHO interphase total cell proteins. AC1 reacts with the 170kD band in the CHO proteins but does not react with the 170kD topoisomerase II band in the Drosophila proteins.


cbb
RPMI
1C2
AC1
TOPO
a
200
116
97
66
43
31
21
14
m d c c d d c c d d c m

Since the polyclonal did detect a 170kD antigen, then the most probable explanation of this anomaly is that the epitope usually bound by 1C2 was altered or degraded under the conditions used. With the *Drosophila* protein extract, enriched for topoisomerase II, Mab 1C2 reacted with 200kD, and 170kD bands, and the polyclonal with 200kD, 170kD and 38kD bands. Whereas, the AC1 Mab did not give a positive signal with the *Drosophila* sample at all.

Discussion

The absence of a positive signal with AC1 with the *Drosophila* proteins, although consistent with the nonidentity with topoisomerase II, does not exclude the possibility that the epitope detected by the AC1 Mab is not conserved between CHO and *Drosophila.* Alternatively, the epitope, or antigen, may have been degraded, or only present in sub-detectable levels. Therefore, these results again do not allow topoisomerase II to be unequivically distinguished from the AC1 antigen.

The explanation for the anomaly that Mab 1C2 detects a 200kD antigen, but the polyclonal did not, was probably because the antigen was present in low levels and or the epitope detected by 1C2 is more prevelant than those detected by the majority of the immunoglobulins in the polyclonal serum. Conversely, that the 1C2 Mab did not react with a 170kD antigenic band is probably due to the epitope (not antigen) being degraded under the particular conditions used. As was expected both anti-topoisomerase antibodies detected antigenic bands of molecular weights 170kD and 200kD in *Drosophila* proteins.

Hadlaczky et al (1988) described an apparently stable 38kD (possible degradation product) antigenic band *Drosophila* which could also be detected by the polyclonal antibody to topoisomerase II. Then it is possible that the low molecular weight bands, 60kD and 66kD, detected by 1C2, on CHO and human, respectively, may also be stable breakdown products of the topoisomerase II molecule.

R 7.5 COMPARISON OF THE CHROMOSOMAL LOCALISATION OF THE AC1 ANTIGEN AND TOPOISOMERASE II ON HUMAN CHROMOSOMES

Since the molecular weight of the AC1 centromere antigen appeared to be the same, within the limits of sensitivity of the Western blotting system, as that of topoisomerase II, and that any differences between the two data obtained might be due to different distributions and sensitivities of the epitopes, further experiments were carried out. Using immunofluorescence microscopy, the chromosomal localisation of the antigen detected by this anti-topoisomerase II antibody, under the same experimental conditions, as used to reveal the AC1 centromere ring antigen, was determined. This experiment was performed on human (EJ30) colchicine treated chromosomes cytocentrifuged onto slides (as described in Materials and Methods).

In the resulting immunofluorescence studies using the anti-topoisomerase II polyclonal serum, it transpired, as shown in Fig. 7.4, that the these antibodies reacted with the centromeres in a manner sharing many common features as those obtained with Mabs AC1 and BA2. The anti-topoisomerase II serum stained two dots at the metaphase centromeres in a manner similar to that observed for Mabs AC1 and BA2. In addition to the centromere dots, apparent lines spanned the centromere, or occasionally a third dot connecting the two centromere dots were observed (see Fig. 7.4). In addition, like the AC1 and BA2 Mabs, the anti-topoisomerase II serum did not stain the interphase centromeres, but unlike these Mabs, the polyclonal anti-topoisomerase II antibody did not decorate the centrioles.

Parallel to this immunofluorescence study, EJ30 cells were tested with another two anti-topoisomerase II Mabs, 2C5 (Hadlaczky et al, 1988), and 2C4 (unpublished anti-topoisomerase II antibody originating from the same source as 2C5 and 1C2) under the same experimental conditions. Importantly, neither of these Mabs stained the centromeres (results not shown) making it highly unlikely that the 170kD canonical form of topoisomerase II was responsible for the centromere staining pattern observed for the polyclonal antibody.

Discussion

Some of the data obtained in these experiments, described in this section, revealed certain similarities between the centromere ring antigen detected by the monoclonal antibody AC1 and eukaryotic topoisomerase II. The immunofluorescence microscopy data showed that under the standard preparation conditions used in this laboratory, the staining pattern given by the anti-topoisomerase II polyclonal antibody was similar to that obtained with the anti-centromere Mabs AC1 and BA2. Topoisomerase II normally interacts with DNA through an initial covalent binding. If, for example, the AC1 antigen is a specific "variant" of topoisomerase II that binds the centromere then it might mediate changes required to bring about sister chromatid separation at anaphase. This hypothesis is consistent with the observed characteristics, demonstrated by the AC1 antigen in its interaction with DNA. Nonidentity of the AC1 antigen and the DNA topoisomerase II canonical form, would also, be consistent with this hypothesis of there being a centromere variant of a topoisomerase isoform.

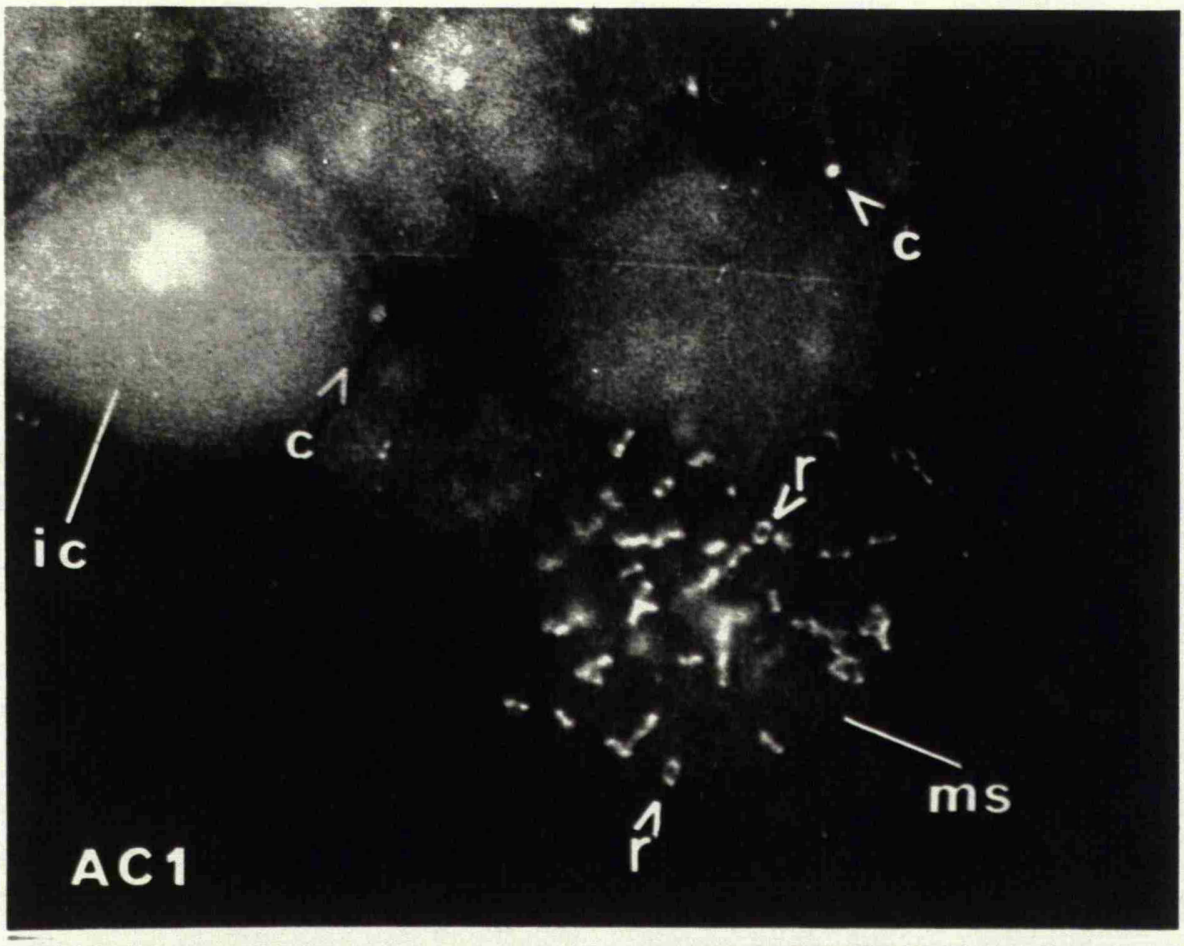
Possible Relationship of the AC1 Antigen to DNA Topoisomerase II

In summary, experiments 7.1 to 7.4 do not absolutely exclude a possible relationship between the centromere antigen and topoisomerase II, since the molecular weight of the AC1 antigen is apparently the same as, or very similar to, that of the topoisomerase II 170kD canonical form.

Figure 7.4 Immunofluorescence Microscopy of Human (EJ30) Colchicine Treated Cells Tested with AC1 Mab and Anti-Topoisomerase II Polyclonal Serum

Slides were prepared by the standard protocol for immunofluorescence. The **AC1** Mab decorates the centromere dots, lines and rings (**r**) on the human chromosomes of the metaphase spread (**ms**), and the interphase centrioles (**c**) of interphase cells (**ic**).
The anti-topoisomerase II mouse polyclonal antibody stains two dots and occasionally a third dot between these two (starred arrowhead).

Magnification: x4,000.


c
c
ic
r
ms
r
AC1


TOPO

Figure 7.5

ANTIBODY	PROTEIN SAMPLE			
	CHINESE HAMSTER (K20)			FRUIT FLY
	INTERPHASE TOTAL CELL	NUCLEAR	CHROMO – SOMAL	FRESH EMBRYO
AC1	200 170	– 170	– 170	– –
1C2	200 – – 95 60 –	– – – – 66 –	200 170 120 – 66 –	200 – – – – –
II/2	– 170 60 –	ND	ND	200 170 – 38
RESULTS NUMBER	R 7,4	R 7,1	R 7,1	R 7,4

Figure 7.5 Legend

Summary of the molecular weights of the antigens determined by Western blotting, in kDa.

Antibodies used are:

AC1 monoclonal anticentromere antibody
1C2 monoclonal antitopoisomerase II antibody
II/2 polyclonal antitopoisomerase II antibody

Results number refers to the section of Chapter R7.
- no positive signal at this molecular weight.
ND not done
* double antigenic band

Figure 7.5

ANTIBODY	PROTEIN SAMPLE			
	HUMAN (COLO)			
	NUCLEAR	CHROMO – SOMAL	METAPHASE EXTRACT	INTERPHASE EXTRACT
AC1	200 170	— 170	— 170	— 170
1C2	ND	ND	200 170 135 85 66 45	200 *170 135 85 66 —
II/2	200 170 — —	200 170 — —	ND	ND
RESULTS NUMBER	R 7,2	R 7,2	R 7,3	R 7,3

However, it appears unlikely that the two antigens are one and the same since topoisomerase II has been shown to be a prominent protein in chromosome scaffold fractions, whilst the AC1 antigen is only a minor protein. Furthermore, the immunofluorescence data with the two anti-topoisomerase II Mabs, 2C5 and 2C4, which failed to detect the exact same structures reacting with the anti-centromere Mab AC1, are consistent with the AC1 antigen being distinct from the normal cellular topoisomerase II.

If the AC1 antigen has nonidentity with topoisomerase II, then the results in section R7.5 still remain to be explained. The centromere positive staining reaction obtained with the anti-topoisomerase II polyclonal could be explained on the basis of multiple forms of topoisomerase II. It is possible that there exists a population of immunoglobulins in the polyclonal serum, which recognised an epitope present on one of the isoforms, which is more prevalent on a centromere specific variant.

A centromere variant would, if it exists, bind DNA, and it could be this centromere DNA binding domain itself, which is close to or at the epitope detected by a subpopulation of immunoglobulins in the polyclonal serum, and by the monoclonal antibodies AC1 and BA2.

In addition to examining the potential relationship with topoisomerase II, these experiments have exposed certain other interesting characteristics of these two proteins. For a summary of the Western blotting data refer to Fig. 7.5. The 200kD antigen will be discussed below.

The 200kD Antigen Detected by AC1 in CHO is Apparently Cytoplasmic

If the results of experiments 7.1 and 7.4 are compared, then it appears probable that the 200kD antigen detected by AC1 is cytoplasmic in location. In total CHO cells (interphase nuclei and cytoplasm) the 200kD and 170kD antigens could be detected. Whereas, in isolated nuclei only the 170kD antigen could be detected, and that weakly. The 170kD antigen amongst the nuclear proteins could also, arguably be due to either cytoplasmic or chromosomal contamination. In the intact cell the 200kD protein could be an independent gene product or alternatively a precursor which may be proteolytically cleaved, resulting in the 170kD antigen. Such a cleavage could occur at the interphase-metaphase transition, and may, for example, regulate its DNA binding affinity at this stage. Alternatively, the 170kD antigen may already have a DNA binding capacity, but is localised to the cytoplasm, until nuclear membrane breakdown occurs, when it could gain access to the condensing chromosomes and bind the centromeric DNA.

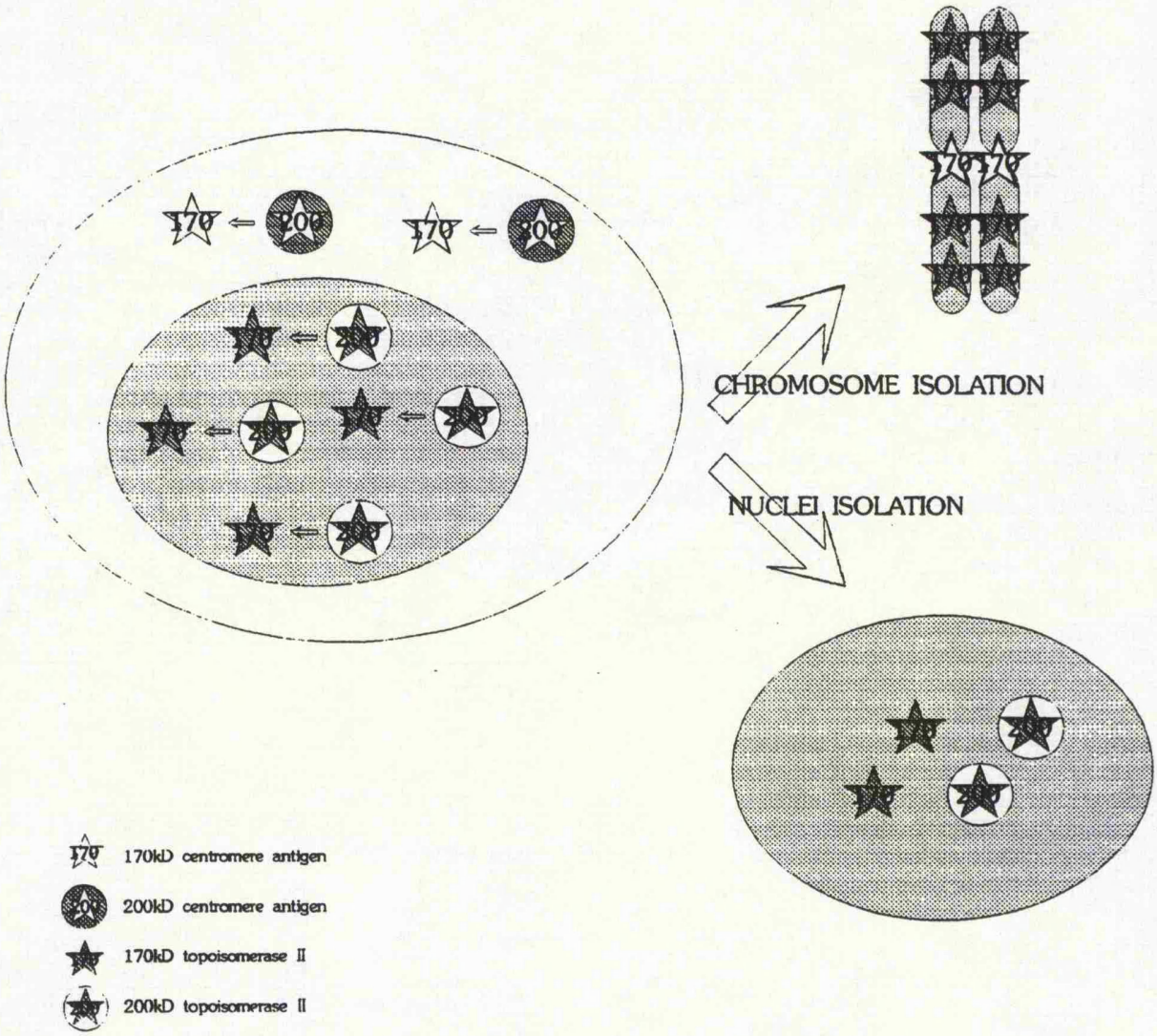
The 200kD Antigen Detected by AC1 May Loosely Interact with the Chromosomes and Nuclei in Human Cells

If the experiments 7.2 and 7.3 are considered together, it appears possible that the 200kD antigen may not be a protein tightly bound to DNA, as the ring and 170kD antigen have been shown to be. This interpretation arises from the observation that the 200kD antigen could be detected amongst the nuclear proteins, but not on the interphase extracts. Consistent with its not being a tight DNA binding protein, is the behaviour of the 200kD antigen detailed above in CHO, which is apparently cytoplasmic. However, this is in contradiction with the BA2 Western blot of such Colo interphase extracts (Chapter R3), in which both the 170kD and 200kD antigens could be detected. Therefore, the absence of the 200kD antigen in the interphase extract may be due to *in vitro* degradation or its presence, when detected by BA2, due to cytoplasmic, and/or chromosomal contamination of the nuclear fraction. This question remains unresolved. Amongst the possible interpretations, a model for the 170kD and 200kD distribution in the interphase cell and subsequently, after mass chromosome and nuclei isolation procedures, is presented in Fig. 7.6.

Figure 7.6 Legend

In this model, the topoisomerase II 200kD and 170kD forms are located primarily to the nucleus, where processing of the 200kD into the 170kD form may occur. In interphase the 170kD topoisomerase II form interacts with the decondensed chromatin. However, in interphase the AC1 centromere 200kD is primarily compartmentalised to the cytoplasm, where it to may also undergo processing, giving the 170kD form just prior to mitosis. Upon breakdown of the nuclear membrane the 170kD centromere form could then gain access to the centromeric DNA to which it binds.

Figure 7.6 Hypothetical Distribution of AC1 Centromere Antigen and Topoisomerase II During Interphase and After Isolation of Chromosomes and Nuclei



CONTENTS

R 8 RELATIONSHIP BETWEEN THE CENTROMERE AND MICROTUBULES

Introduction

In colchicine treated metaphase cells the distribution of the antigen detected by the AC1 Mab was characterised in detail in previous Chapters. One aspect, however, that was consistently observed and puzzling was the reaction of the antibody with the interphase centrioles. The centromeres and the centrioles are both components of the spindle apparatus, but are located far from each other at metaphase. The centromeric region mediates the alignment of the chromosomes on the metaphase plate, whereas the centrioles are found at the spindle poles. Furthermore, the centriole is a cytoplasmic component, whereas the AC1 Mab was generated as a result of immunisation with chromosome scaffold material. Hence, the reaction of the AC1 antibody with both the metaphase centromeres and interphase centrioles was intriguing. However, there is an important common feature to both cellular substructures, namely that they both interact with the spindle microtubules.

Recently several antigens have been described, which are present at the centromere, but later in the cell cycle redistribute to the interzone of what is later to become the cleavage furrow (Cooke et al, 1987: Kingwell, 1987; Pankov et al, 1990; Yen et al, 1991). Even though the main aim of this project was to investigate centromere structure it was considered appropriate to investigate the distribution of the AC1 antigen at other stages of the cell cycle, in order to give a clearer picture of its role within mitosis.

The staining of both the metaphase centromeres and interphase centrioles by the AC1 antibody could be due to one or other of the following. The AC1 antibody may have cross-reacted with a related epitope on a different antigen, or alternatively the same antigen could be present at the two sites, i.e. at the centromere and at the centriole. It is less likely, although cannot be excluded, that a cross-reaction with a related but distinct epitope was the cause of this staining pattern, since a different Mab, BA2, also detected the centromere and the centrioles in the same manner as AC1. Furthermore, AC1 detected both the centromere and centrioles in human cells, also, making a non-specific cross-reaction unlikely. Thus, it was of interest to investigate the possible relationship of the AC1 antigen with the microtubules and associated proteins of the spindle apparatus.

R 8.1 MAB AC1 DECORATES THE SPINDLE MICROTUBULES, MIDZONE AND CONTRACTILE RING

The method by which the CHO cells were blocked at metaphase, namely colchicine treatment, influences the state of the spindle microtubules.

Colchicine inhibits the further polymerisation of the microtubules, and since the microtubules are in a constant flux of polymerisation and depolymerisation, the net result of this treatment is the depolymerisation of the microtubules. Consequently, in the colchicine treated preparations the spindle apparatus is absent, whilst the centrioles, which are resistant to this treatment, remain.

Therefore, in order to investigate the possible relationship of the AC1 antigen to the spindle apparatus, spontaneous mitotic cells were prepared for immunofluorescence microscopy (Fig. 8.1). In such spontaneous mitotic cells the majority of the cells are in anaphase, and metaphase is less frequent. On such CHO spontaneous mitotic cells, in anaphase a positive signal was obtained generally over the spindle microtubules, when stained with AC1 antibody. This may have been a cross-reaction, however, a particular area, the interdigitating microtubules in the midzone region, appeared to strongly be decorated with antibody. Due to the overall microtubule reaction obtained it was not possible to determine if the centromeres were also stained.

Amongst the mitotic cells telophase cells were also present, and the antibody in such cases localised to a double collar form across the region of cytoplasm still connecting the two daughter cells, which may correspond to the contractile ring of the cleavage furrow. Thus, the AC1 antibody apparently has a high affinity (possibly aspecific) for the spindle microtubules in general, but preferentially stains the midzone in anaphase, and apparently the contractile ring of the cleavage furrow at cytokinesis.

R 8.2 AC1 ANTIGEN HAS NONIDENTITY WITH TUBULIN

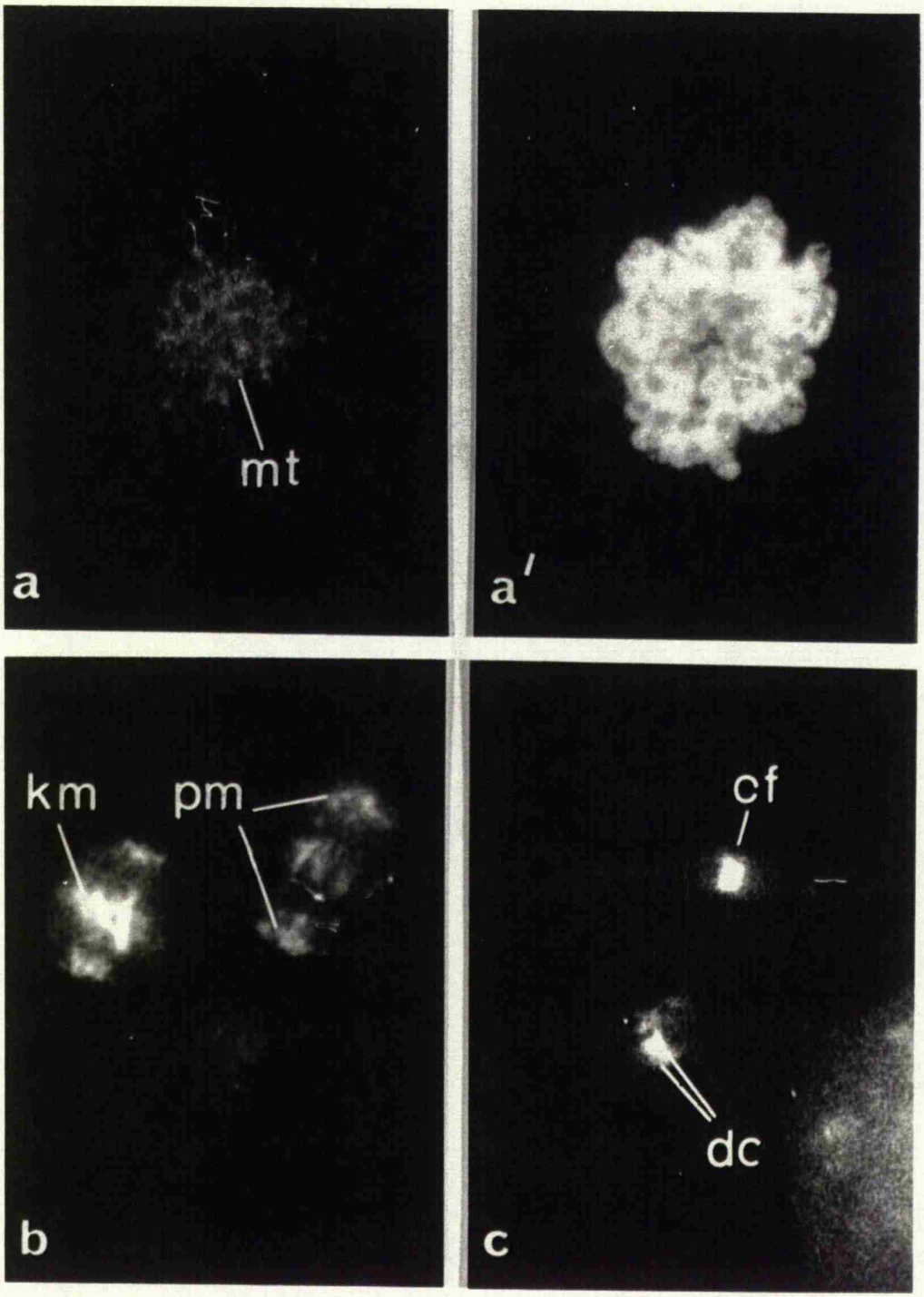
These somewhat surprising results, led to a further consideration of the spindle apparatus. The major component of the spindle is tubulin, and this has been shown to be present in the outer corona of the kinetochore. Unlike the AC1 antigen, however, tubulin can be removed by 150mM NaCl. Furthermore, tubulin has a molecular weight of 55kD, therefore, on the basis of this difference in the molecular weights and cellular distribution patterns (tubulin has been shown to be absent from the centromere), the AC1 antigen can be assumed to have nonidentity with tubulin. Moreover, tubulin is a component of the cytoplasm, and total CHO cells were tested by Western blotting with AC1 previously (Chapter 7, Fig. 7.4) and no positive signal was obtained in the region of 55kD.

In spite of this, the antigen detected by AC1 may still be associated with a component of the spindle microtubules. Tubulin isolation procedures involve repeated cycles of polymerisation- depolymerisations, of material rich in microtubules, such as brain. After such treatment tubulin is not homogeneous, but contains a minor class of associating proteins. Previous studies have characterised this class of proteins and termed them microtubule associated proteins (MAPs).

Figure 8.1 Immunofluorescence of CHO Spontaneous Mitotic Cells Stained with AC1 Mab

CHO cells were frequently cultured to ensure a higher rate of spontaneous mitotic cells. Mitotic cells were cytocentrifuged and prepared for immunofluorescence by 100% methanol fixation, incubated with supernatant from hybridoma clone AC1 and then anti-mouse FITC conjugated secondary antibody.
In anaphase AC1 decorates the microtubules (**mt**), in **a** the microtubules in transverse section can be visualised, **a'** is the HOECHST 33258 DNA staining of **a**. In **b** the microtubules are presented at anaphase in longitudinal section. Both the kinetochore (**km**), polar microtubules and centrioles (**pm**) are stained. In **c** the cleavage furrow (**cf**) at late telophase is presented, the cleavage furrow is stained with a double collar (**dc**).

Magnifications: A: x4,000; B&C: x3,000.


mt
a
a'
km
pm
b
cf
dc
c

If the spindle component recognised by AC1 is a MAP then the antigen should remain detectable after treatment resulting in the loss of proteins other than tubulin and the MAPs. The two major MAPs that have been characterised in detail have molecular weights of 350kD and 280kD, and are designated MAPs 1 and 2, respectively.

R 8.3 AC1 ANTIGEN HAS NONIDENTITY WITH THE MICROTUBULE ASSOCIATED PROTEINS

In order to investigate the possible relationship of the AC1 antigen to the microtubule associated proteins, a crude extract of tubulin was made from Chinese hamster brain. This together with a pig brain crude extract, and one and two cycles of polymerisation from pig brain proteins (currently available in this laboratory) were separated by SDS-PAGE and were tested in a Western blot. The Western blot was incubated with AC1 supernatant and anti-MAP 2 as a control (see Fig. 8.2). A band of molecular weight in the range of 160 to 170kD was detected with hamster and pig brain crude extracts by the AC1 antibody and the anti-MAP 2 control. However, the band detected by AC1 disappeared by the first cycle of polymerisation, whilst MAP 2 could still be detected by the control antibody. Therefore, the AC1 antigen is not a MAP by standard definition.

Discussion

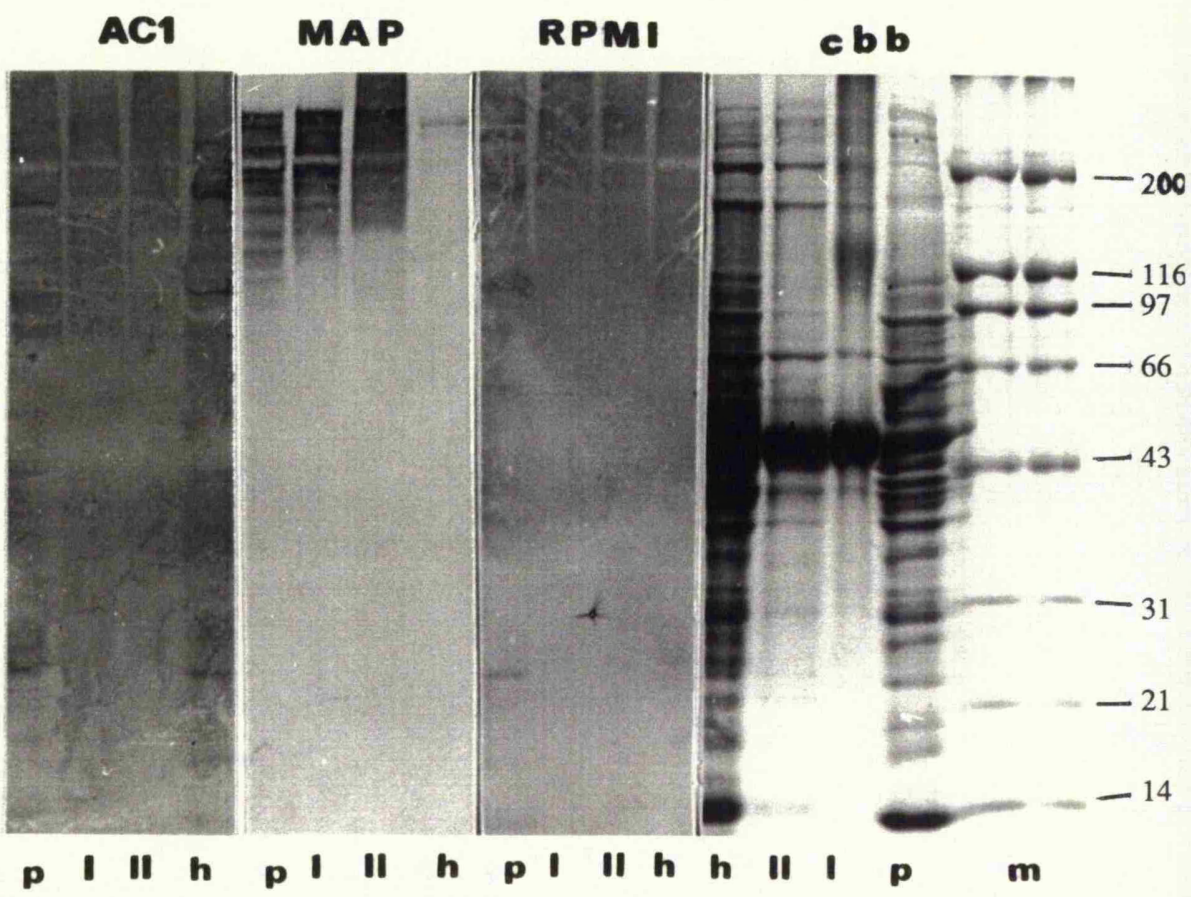
That the AC1 antigen can be detected at all on the crude brain extracts (which is not a proliferating tissue and so should not be expected to contain high levels of a mitotic antigen) suggests that the antigen may be present in all tissues at a low, but detectable level. Since the antigen cannot be detected after two cycles of tubulin polymerisation, it can be concluded that the AC1 antigen is not a MAP as currently defined. Nevertheless, the immunofluorescence data do suggest that the antigen is also a component of the spindle, thus, it may be a protein which is more loosely associated with the microtubules, than the MAPs.

AC1 Ring Antigen May Bind the Centromere DNA Reversibly

There are two, unexpected characteristics of the AC1 antigen, which have interesting theoretical implications. One characteristic of the antigen is its strong interaction with DNA, indicated by its presence on chromosome scaffolds. If the antigen strongly and irreversibly binds DNA it might be possible to detect it on the interphase centromeres. It could be argued that the antigen remains associated with the DNA, but that the epitope has been altered or masked and, thus, can no longer be detected. However, this is unlikely in light of the fact that another Mab, BA2, behaves in an identical manner in mitosis on CHO, and AC1 on human cells. Therefore, an alternative possibility arises, that the AC1 antigen reversibly binds the centromeric DNA.

Figure 8.2 Western Blot of Chinese Hamster Brain Crude Tubulin Extract, and Pig Brain Crude Tubulin Extract, One and Two Cycles of Tubulin Polymerisation Tested with Anti-MAP 2 and AC1 Antibodies

Chinese hamster (**h**) crude brain tubulin extract, pig (**p**) crude brain tubulin extract, one cycle of tubulin polymerisation (**I**), and two cycles of polymerisation (**II**) were separated by 5-20% SDS PAGE and electrophoretically transferred to a nitrocellulose filter. The Western blot was tested with undiluted supernatant from hybridoma clone **AC1**, anti-**MAP** 2 serum, and a negative control of **RPMI** medium containing no hybridoma immunoglobulins. Coomassie Brilliant Blue (**cbb**) stained proteins are shown, and molecular weight markers (**m**) are of the following values: myosin 200kD, *E.coli* beta-galactosidase 116.25kD, rabbit muscle phosphorylase b 97.4kD, bovine serum albumin 66.2kD, hen egg white ovalbumin 42.699kD, bovine carbonic anhydrase 31.0kD, soybean trypsin inhibitor 21.5kD, hen egg white lysozyme 14.4kD.
AC1 reacts with the 170kD antigen in pig and hamster crude tubulin extracts, but the antigen is no longer detectable after two or even one cycle of polymerisation. An additional band of about 100kD is detected on the hamster crude tubulin extract. The anti-MAP 2 antibody gives a positive signal on the 280kD band, on all four protein fractions.


AC1
MAP
RPMI
cbb
200
116
97
66
43
31
21
14
p I II h p I II h p I II h h II I p m

AC1 Antigen Interacts with Components of the Cytoskeleton

Another unexpected characteristic of the AC1 antibody is that it stains the spindle microtubules. Thus, in metaphase the antigen is tightly associated with DNA, but at anaphase may dissociate (only inferred, but not observed directly), and localise to the midzone, and at telophase collect at the contractile ring. Finally, in interphase the AC1 antigen may be concentrated at the centrioles, in reduced quantities (indicated by the Western blotting data, or in a conformation which does not readily allow access to immunoglobulins), by comparison with the quantities detectable in metaphase.

Other Antibodies Stain the Centromeric Region at Metaphase, but Dissociate at Anaphase

There are several antigens isolated recently which have a centromeric localisation, but which later localise to the midzone. The INCENPs (Cooke et al, 1987) which are located pericentromerically, between the sister chromatids at metaphase (see R4 table 4), but dissociate and accrue on the midzone at anaphase. A mitosis specific mammalian antigen of molecular weight 38kD with a similar appearance to the INCENPs has been identified using autoimmune sera (Kingwell, 1987). The use of monoclonal antibodies raised against bovine thymocytes has demonstrated the existence of antigens of molecular weights 140 and 155kD, which are found on the region where the microtubules attach the kinetochore at metaphase, and at the midbody in anaphase (Pankov et al, 1990). Another antigen, recently identified, is CENP-E (Yen et al, 1991), which was generated against human chromosome scaffolds. This antigen is located peripherally to the CENPs -A, -B, and -C at metaphase, suggesting a looser association with the centromere. CENP-E then dissociates at anaphase to the midzone, and concentrates at the midbody at telophase. Therefore, the AC1 Mab is not the only one which stains the centromere at metaphase, and also cytoplasmic components later in the cell cycle.

There are two possible conclusions from these experiments, that the midzone and contractile ring staining patterns are either cross-reactions with another protein(s) (not detectable by Mab AC1 in Western blots), or that the patterns are specific reactions with the same antigen at different cell-stage dependent cellular locations. If the latter is considered, then the AC1 antigen may have pleiotropic functions. One such role is at the prometaphase and metaphase mitotic centromere, perhaps stabilising the interaction between sister chromatids, or for instance, possibly mediating DNA topological changes. Another function of this antigen could be in spindle microtubule movement, for instance it may mediate protein-protein interactions involved in chromosome movement at the spindle. If correct this suggests that there may be two domains of the AC1 protein antigen, detected by Mab AC1, one interacts with the centromeric DNA, the other with the spindle microtubules. Alternatively, the association of the antigen with the microtubules, may merely be a method of dissociating it from the centromere DNA and redistributing it to the daughter cells.

Localisation of the AC1 Antigen to the Cleavage Furrow

Recently, a "telophase disc" structure was identified using an autoimmune serum, which was also anticentromeric (Andreassen et al, 1991), reacting with a 60kD antigen of human (HeLa) cells. The antigen colocalised with CENP-B at prophase, metaphase and early anaphase. At mid-anaphase the antigen relocalised to the interdigitating microtubules of the midzone, and was continuous, but distinct from the tubulin there. At telophase the antigen localised to the telophase disc structure, which spanned the midline of the cell, beyond the region of the microtubules to the cell membrane, which the authors suggest is important in cell cleavage. This antigen apparently is in the form of a solid disc, making it distinct from the region stained by AC1 at this stage in the cell cycle.

Therefore, this finding, observed by using Mab AC1, demonstrates that, together with the Andreassen data, there are at least two antigens of the centromeric region which are also, apparently, involved in cleavage furrow formation. Considering the cellular localisation of the AC1 antigen at telophase, then it is possible that this antigen interacts with the outer region of the telophase disc where the cell membrane invaginates. These exciting results may also suggest that proteins of the centromeric region may also play a role in cytokinesis.

A further consideration, although very tentative, of the possible relationship of the AC1 antigen with a protein involved in cell motiliy, and identified in the contractile ring, namely myosin, is treated in the next Chapter.

CONTENTS

R 9 POSSIBLE RELATIONSHIP OF CENTROMERE ANTIGEN TO MYOSIN

Introduction

Although tentative, certain observations led to experiments to determine if the antigen detected by Mab AC1 were possibly related to myosin. Myosin has been shown to be a component of the contractile ring (Fujiwara & Pollard, 1976; Yumura & Fukui, 1985; Schroeder, 1987), and the AC1 antigen is apparently found in the contractile ring (previous Chapter, R8). The molecular weight of the AC1 antigen is 170kD, but on some blots an antigen of 200kD could also be detected (refer to R3, Fig 3.2). The 170kD antigen could conceivably be a stable breakdown product of the 200kD antigen. Thus, the AC1 antigen is in the same molecular weight range as the heavy chain myosin polypeptide. Myosin is also an exceedingly labile protein, as the AC1 antigen has proven to be (unstable in gel electrophoresis sample buffer).

Therefore, there are certain characteristics shared by the AC1 antigen and myosin, and in order to address the possibility that these two proteins are related, two approaches were used. One was to present Mab AC1 (anti-CHO) with rabbit and bovine myosins in Western blots, and if conserved between these species, the AC1 Mab should give a positive signal when tested on these myosins. Additionally, myosin is a relatively abundant protein throughout the cell cycle, so the other approach was to use the anti-myosin antibodies currently available in this laboratory, and to test these on CHO interphase proteins, by Western blotting.

R 9.1 MAB AC1 DOES NOT REACT WITH BOVINE OR RABBIT MYOSIN

Western blots were prepared by the standard procedure (see Materials and Methods) using bovine and rabbit myosins and incubated with AC1 monoclonal antibody. However, AC1 did not give a positive signal on either the rabbit or the bovine myosins, thereby demonstrating that the CHO and human antigen detected by AC1 does not cross-react with the rabbit or bovine myosins.

R 9.2 ANTI-*E.COLI* 170KD AND 180KD AND YEAST MYOSIN ANTIBODIES DO NOT REACT WITH THE 170KD CHO ANTIGEN

Since myosin is a relatively abundant cytoplasmic protein, interphase cells were harvested, without colchicine treatment, and dissolved in electrophoresis sample buffer. Then a Western blot was prepared of these CHO interphase proteins and tested with rabbit anti-*E.coli* 170kD and 180kD heavy chain myosin, and anti-yeast fusion heavy chain myosin polyclonals (the kind gifts of Dr. E. Orr, Leicester University). However, neither the anti-*E.coli* nor the anti-yeast myosin rabbit sera reacted with the 170kD antigen in the CHO interphase proteins.

Fig 9.1 Western Blots of *E.coli* , CHO and Myosin Proteins Tested with AC1 and Anti-*E.coli* Myosin, and Anti-Yeast Myosin

E.coli total (**E**), Bovine myosin (**M**), and CHO total interphase cell (**C**) proteins were prepared for Western blotting by the standard procedure. Coomassie Brilliant Blue (**cbb**) stained proteins are shown, and molecular weight markers (**m**) are of the following values: myosin 200kD, *E.coli* beta-galactosidase 116.25kD, rabbit muscle phosphorylase b 97.4kD, bovine serum albumin 66.2kD, hen egg white ovalbumin 42.699kD, bovine carbonic anhydrase 31.0kD, soybean trypsin inhibitor 21.5kD, hen egg white lysozyme 14.4kD. **ab** is the Amido Black staining of the markers in **B** after electrophoretic transfer to the nitrocellulose filter.

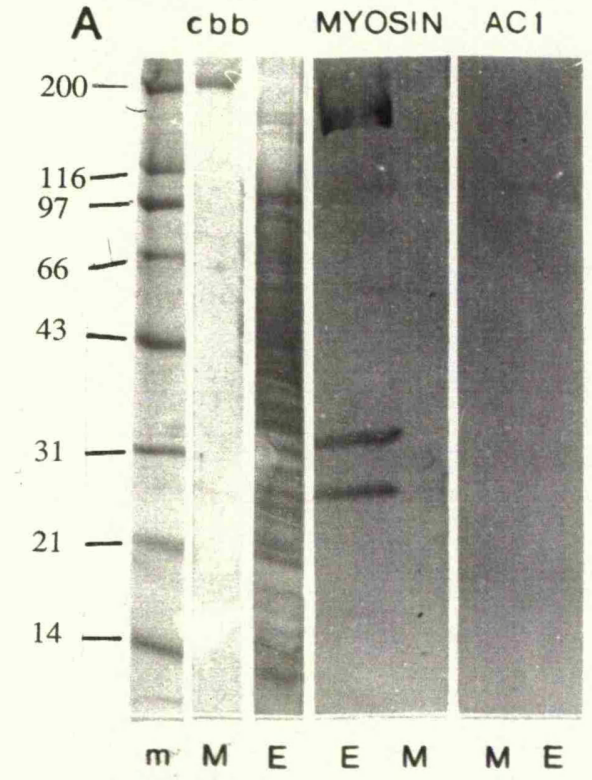
A: *E.coli* total (**E**) and Bovine myosin (**M**) proteins were tested with anti-*E.coli* 180kD myosin (**MYOSIN**) and supernatant from hybridoma clone AC1. The anti-myosin detects 180kD, 34kD and 29kD bands with the *E.coli* proteins, but does not cross-react with the bovine myosin. The AC1 Mab does not give a positive signal with either the *E.coli* proteins, or with the bovine myosin.

B: *E.coli* total (**E**) and CHO total interphase cell (**C**) proteins were tested with various antibodies. Lanes **1** to **4** on the left hand-side are blots of *E.coli* proteins, and lanes **1** to **4** on the right-hand side of the Western are blots of CHO total interphase cell proteins.

Antibodies tested on *E.coli* : lane **1**, anti-*E.coli* 170kD myosin; **2**, anti-*E.coli* 180kD myosin; **3**, anti-yeast fusion myosin; **4**, AC1 Mab.

Antibodies tested on CHO total interphase cells: lanes **1** to **4** same as for *E.coli* .

Notice AC1 does not cross-react with the *E.coli* proteins, but does detect the 170kD antigen in the CHO proteins. None of the anti-myosin antibodies give positive signals in the CHO proteins.


A
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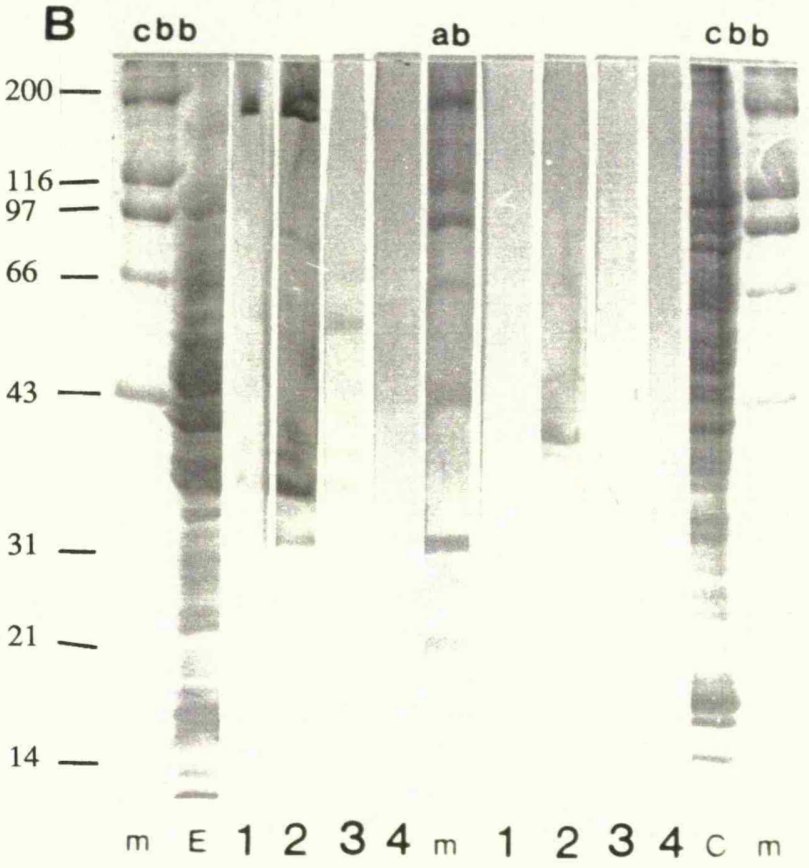
B
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Discussion

Myosin is a very heterogeneous molecule, with many species, and cell type specific isoforms. Unfortunately, in these studies antibodies to authentic hamster, mouse or human myosin was not available for testing. Therefore, the negative results obtained, although consistent with the non-identity of the AC1 antigen and heavy chain myosin, are not by any means definitive.

Cytoplasmic myosin has been shown to be localised in a recently identified, telophase disc, structure of the cleavage furrow in human cells (Andreassen et al, 1991). The authors postulated a model for cell cleavage, in which the disc is generated to concentrate and align myosin at the position where the cleavage furrow will form. The disc-associated myosin could then interact with actin at its periphery (since actin is not present in the disc), and this could generate a radial (and not tangential as would be expected for a contractile ring) force for cleavage.

There is no data to suggest that myosin is a component of the centromere. However, the AC1 antigen does appear to localise to a region, which could correspond to the peripheral region of telophase disc structure. Therefore, it is feasable that it interacts with either, or both, actin and myosin at this site. In order to demonstrate this potential interaction, double anti-myosin (species specific) and AC1 immunofluorescence staining experiments would have to be carried out.

CONTENTS

RESULTS: R 10 SCREENING OF LAMBDA GT11 LIBRARIES

Introduction

There were two main aims of the screening of lambda gt11 expression libraries for centromere antigens. Since the antigen detected by monoclonal antibodies AC1 was deemed to be potentially very interesting, but shown to be a minor component of chromosomes (chapter R3) it was decided to try to obtain larger quantities of the antigen, by first attempting to isolate the gene encoding it. However, the possibility of success of such an approach was considered relatively limited, since the bacterial system (*E. coli*) does not carry out the post-translational modifications frequently present on eukaryotic proteins, and these antibodies were monoclonal. Therefore, if the Mab detected a post-translational modification, or protein-DNA complex, then the prokaryotic gene expression system would not produce a protein, which Mab AC1 could react with.

The second aim for the expression library screening was to try to isolate the genes encoding the human centromere proteins CENPs A, B and C. For the isolation of genes by the immunological screening of expression libraries the antibodies of preference are polyclonal, for the reasons indicated above. Thus, it was decided that in parallel to the screening with the monoclonal antibodies, polyclonal antibodies to human centromere antigens currently available in this laboratory would be utilised. The majority of the information about the human centromere was first obtained by using anti-centromere autoantibodies, which are present in the sera of patients with the autoimmune disease scleroderma (Moroi et al, 1980). There are three main centromere proteins identified by the use of autoimmune sera: CENP-A, 17kD; CENP-B, 80kD; and CENP-C, 140kD (refer to chapter I 4.3.4). The gene encoding the most abundant centromere protein, CENP-B, has been cloned (Earnshaw et al, 1987), but the gene for the two remaining prominent centromere proteins, CENPs -A and -C, have not yet been isolated (see Introduction Chapter I 4). Therefore, human autoimmune sera were sought, which reacted with either CENP-A or -C, and then a human fibroblast λgt11 cDNA library was screened in order to try to isolate either of the corresponding genes.

The human fibroblast cDNA library chosen consisted of 2.2×10^6 independent clones (Clontech) and the ratio of recombinant to parental phages was 89%. The mRNA source of the library was from human lung fibroblast tissue culture cells (ATCC# CCL186), inserted into the λgt11 unique Eco R1 cloning site, with an average insert size of 1.3kb in the range of 0.5 to 3.0kb. The insert is cloned into the *lac Z* gene allowing the overexpression of the foreign DNA as part of the ß-galactosidase fusion protein, in response to induction by addition of isopropyl ß-D-thiogalactopyranoside.

The phage encodes a temperature-sensitive repressor (cI857), which is inactive at 42°C, and carries an amber mutation (S100), which renders it lysis defective, allowing infected cells to accumulate high levels of phage DNA following a temperature shift.

In order to allow phage adsorption to bacteria, cells are grown in media containing maltose, so that the receptor required for infection is expressed on the surface of the cells. Also, bacteria rapidly degrade most unusual polypeptides, therefore, the host cell used for library screening, *E.coli* Y1090, contains a *lon* mutation, i.e. it is defective in the protein degradation pathways. This, together with the expression of the foreign DNA as a fusion to the prokaryotic ß-galactosidase, increases the lifetime of the novel protein produced by the induced lysogens. Proper expression of the foreign DNA in λgt11 recombinant lysogens depends on the orientation and reading frame of the inserted DNA with respect to *lac Z* . Therefore, a maximum of one-sixth of the recombinants carrying a specific DNA will produce a fusion polypeptide which can be detected by the corresponding antibody.

R10.1 SCREENING FOR ANTI-CENP-A AND CENP-C SPECIFIC ANTISERA

As a prerequisite to the antibody screening of the lambda expression library, it was necessary to identify which of the anti-centromere autoimmune sera already currently available in this laboratory, reacted with either CENP-A or -B. Five autoimmune sera, which detect human centromere proteins, determined by immunofluorescence microscopy previously by T. Praznovszky of this laboratory, were tested in Western blots of human (Colo) nuclear proteins, to determine which centromere antigenic polypeptide bands they reacted with. The five sera, denoted and referred to as CT1 (termed as LU851 in Chapter R4 and elsewhere, e.g. Hadlaczky, et al, 1989), CT6, CT13, CT14 and CT20 in this chapter, were shown to react with the following molecular weight polypeptides:

Antigen	Mol. Wt	CT1	CT6	CT13	CT14	CT20
CENP-C	140 000	+	-	+	-	+
CENP-B	80 000	+	-	+	-	+
CENP-A	17 000	+	-	+	+	-

From these results it transpired that two of the sera detected all three major centromere proteins, namely CT1 and CT13. Apparently the immunoglobulin in serum CT6 had undergone degradation, since none of the three antigens were detected, an interpretation which is consistent with later findings in immunofluorescence studies, in which this serum no longer decorated the centromeres of human (HeLa) chromosomes (data not shown). CT14 only reacted with one of the CENPs, namely CENP-A, molecular weight: 17 000, and CT20 with the other two polypeptides, CENP-B and CENP-C, of molecular weights 80 000 and 140 000, respectively.

Since the gene encoding human CENP-B has already been cloned, serum CT14 was deemed the most interesting of the sera since this only reacted with CENP-A. Serum CT13 was used in subsequent studies since the two polypeptides, CENPs -A and -C were detected, in addition to -B; but the possibility that an already cloned gene (CENP-B) could be isolated, was kept in mind. CT1 was not persued, since this serum recognised the same three CENPs that CT13 did with the human proteins due to the limited quantities of this serum remaining after extensive previous (Hadlaczky et al, 1989) and the then anticipated experiments (Hadlaczky et al, 1991) and CT20 was not persued, since only CENP-C was detected in addition to -B.

Six additional antinuclear autoimmune sera, recently obtained from patients in Debrecen, were also screened for potentially useful sera for the expression library screening. Of these, the following sera, termed: MI, KJ, SJ, BB, LZ and VB were found to be centromere positive by immunofluorescence microscopy carried out by the standard procedure, previously on human chromosomes (HeLa) by T. Praznovszky of this laboratory. These six sera when tested by Western blotting carried out by the standard procedure (see Materials and Methods) on human nuclear proteins (Colo) detected all three CENPs A, B and C, with the exception that SJ only reacted with a 17kD antigenic band, namely CENP-A. Therefore, this serum was also used in the lambda gt11 expression library screening.

Summary: Antibodies Used in Lambda gt11 Expression Library Screening

Antibody	Antigen Detected in Humans
mouse monoclonal AC1	170kD
human polyclonal CT13	17kD, 80kD, 140kD
human polyclonal CT14	17kD
human polyclonal SJ	17kD

R10.2 Optimisation of Lambda gt11 Screening

Before beginning screening it was important to optimise the experimental parameters. Thus, determination of the optimal primary antibody concentration, exclusion of antibodies cross-reacting with bacterial proteins, and positive controls to demonstrate that protein expression was being induced, were carried out.

R10.2.1 Optimum Serum Dilution

To try to avoid obtaining misleading false positives, the anticentromere antibodies to be used, were tested on trial filters of human brain lambda gt11 plaques, to determine the optimum primary antibody concentration.

For human sera dilutions of 1 in 100, 1 in 200, 1 in 500, 1 in 1000 and 1 in 2000 in PBS were made; for the mouse monoclonal antibodies undiluted supernatant and dilutions of 1 in 2, 1 in 5 and 1 in 10 in blocking solution were made. As a result of these experiments the antibodies were used in subsequent studies at the following concentrations: CT13, CT14 and SJ - 1 in 1000, and AC1 - undiluted.

R10.2.2 Bacterial Protein Preabsorption

To test for the possibility of cross-reaction of the anticentromere antibodies with bacterial proteins, Y1090 cells were lysed by boiling in electrophoresis sample buffer, separated by SDS-PAGE, Western blotted and probed with the antibodies. It was found that all antibodies, except AC1, showed a faint cross-reaction with *E.coli* proteins in the molecular weight range of 18kD to 80kD. Therefore, CT13, CT14 and SJ were preincubated with *E.coli* proteins (Promega) at a concentration of 5.0 mg/ml prior to a trial screening on a human fibroblast library. The background was only slightly reduced, indicating that the cross-reaction obtained with Western blotting may have only been marginal anyway.

R10.2.3 Efficiency of IPTG Induction

In order to demonstrate that the expression of fusion proteins was occurring as expected two positive control primary antibodies, anti-ß-galactosidase mouse Mab and anti-chicken ovalbumin rabbit serum were used (Promega). Plaque lifts were prepared as normal, and half of the filter was saturated in 10mM IPTG, whereas the other half was soaked in distilled water and then air dried. A lambda plaque forming unit dilution was chosen such that 40 plaques were obtained per filter. On the non-induced half of the filters only a very faint signal was obtained, whereas for the induced halves significantly strong signals were observed. For the anti-ß-galactosidase antibody 100% recovery of positive plaques was obtained, for the anti-chicken ovalbumin antibody approximately 50% was recovered. Thus, the IPTG induction and fusion protein expression was working as expected.

R10.4 Screening of Human Fibroblast Lambda gt11 Expression Library with Anti-Centromere Antibodies

The human fibroblast cDNA library chosen consisted of 2.2×10^6 independent clones (Clontech) and the ratio of recombinant to parental phages was 89%. The mRNA source of the library was from human lung fibroblast tissue culture cells (ATCC# CCL186), which is a cell line from transformed tissue. Therefore, it was anticipated that the library would contain genes encoding proteins expressed in mitotic actively dividing cells, i.e. potentially containing genes encoding centromere proteins.

R 10 SCREENING OF LAMBDA GT11 LIBRARY

The only gene encoding a centromere protein (CENP-B) to date was isolated by screening a human endothelial cell derived λgt11 expression library with an anticentromere autoimmune serum (Earnshaw et al, 1987). These authors obtained immunopositive plaques at a frequency of about 1/50,000, when a total of 9 x 10^5 phages were screened. Therefore, a screening of a similar magnitude was anticipated in order to isolate a centromere protein encoding gene using one of the designated antibodies.

Thus, having optimised the experimental conditions, the four antibodies, CT13, CT14, SJ and AC1, were used to screen the human fibroblast λgt11 library. The number of phages screened are shown in the table below:

Antibody	No. phages screened	No positive clones
AC1	1.0 x 10^5	0
CT13	1.6 x 10^5	0
CT14	1.2 x 10^5	0
SJ	2.0 x 10^5	2?

Discussion

Based on an anticipated possible frequency of 1 positive plaque in 5 x 10^4, there was a reasonable probability that in the numbers of phages screened, one or more of the genes encoding centromere proteins, if present in the library, should have been picked up. Since only two "false" positives and no "real" positives were found, then, this approach was abandoned. The observation that no positive plaques were detected by antibody AC1 can be explained by the fact that this reacts with one epitope only, since it is monoclonal. It is reasonable to anticipate a low probability of the prokaryotic system expressing a fusion polypeptide which is detectable by such an antibody (see introduction).

Neither human autoimmune serum CT13, nor CT14 gave a positive signal when tested on the number of plaques indicated above. This suggests that the gene may not be present in the lambda library. The antigen detected by human autoimmune serum SJ, however, apparently gave two positive signals, which could not be confirmed on rescreening attempts. Due to lack of time before conclusion of the experimental period this could not be pursued further. However, it may be fruitful if other workers carried on the screening of the fibroblast expression library with serum SJ, in order to possibly isolate the gene encoding CENP-A.

CONTENTS

CHAPTER D 1 THE MAMMALIAN CENTROMERE

Overview of Discussion Chapters

In the subsequent discussion Chapters the research on the mammalian centromere will be subdivided into four areas. Firstly, a consideration of the fundamental role of the mammalian centromere from basic principles is developed (Chapter D1). Secondly, the role of the AC1 antigen, which has been characterised during these studies (R2 to R9), in the functioning of the mammalian centromere is discussed (Chapter D2). Thirdly, the potential future research directions regarding the AC1 antigen and the centromere in general are considered (Chapter D3).

D 1.1 THE FUNCTIONAL REQUIREMENTS OF A CHROMOSOME

In order to appreciate the role of the centromere in the cell cycle, the requirements of the entire chromosome will first be considered in global terms. In order to maintain information about a cell in existence, the cell copies, divides, and passes on that information to the daughter cells. Here, however, there is a paradox, that in the meantime the environment has changed and the original information content may not be so appropriate in the new environment. Therefore, an inbuilt mechanism leading to variation, via mutations, also allows the cell a possibility of change.

In precise terms it is the chromosome which is the vector capable of transmitting the information content to the daughter cells. Since most environments are relatively stable, this information transfer process has to have a high degree of fidelity to maintain the information content, but some low level of mutation in order to bring about the possibility of change (Coyne, 1992; Wilson, 1985). This change can occur in a variety of ways, namely mutations at the gene level and/or the chromosomal level. Chromosomal mutations can involve rearrangements within a given chromosome, between different chromosomes, and are also possible for sets of chromosomes (consider non-disjunction theories of speciation) (Wilson, 1985) when, for whatever reason, the mitotic machinery "malfunctions". For instance, chromosome rearrangements are thought to play a major role in primate evolution (de Grouchy et al, 1978), and chromosome doubling in hybrids (allopolyploidy) is a main cause of speciation in plants (Coyne, 1992). Since faithful distribution of chromosomes is the rule rather than the exception, then research in this area has logically placed the emphasis on the process of accurate cell division, and it is this which is considered in this thesis. The chromosomal sub-region which is responsible, by interacting with the spindle microtubules, for chromosome distribution, is the centromere. The requirements of which are considered below.

D 1.2 THE REQUIREMENTS OF A FUNCTIONAL CENTROMERE

Introduction

From basic principles the following essential properties of a centromere can be anticipated:

1.- Contains centromere DNA sequence onto which centromeric proteins package to build the basic centromere of interphase.

2.- Replication of centromeric DNA.

3.- Regulation of centromere state (interphase vs mitotic) depending on stage of cell cycle, and addition of specific proteins to give rise to the active centromere of mitosis.

4.- Interaction with surrounding chromatid arms to maintain structural integrity whilst exposed to torsional stresses.

5.- Interaction with spindle microtubules to bring about chromosome locomotion involving:

a\ bipolar attachment

b\ synchronised arrangement on metaphase plate

c\ regulation to give synchronised separation of centromeres and sister chromatids i.e. anaphase

d\ deposition of chromosomes at the polar destination in daughter cells i.e. telophase

e\ possible non-random spatial distribution during telophase-interphase.

If the behaviour of the mammalian centromere is considered in global terms there are two states, an apparently inactive state, whilst the cell carries out its normal house-keeping functions, when not involved in cell division and the centromere is apparently "dormant" (points 1 & 2). The other state is an active one in which the centromere is involved in cell division (points 3-5). These two states are considered below.

D 1.3. THE INTERPHASE CENTROMERE

D 1.3.1 Centromere DNA Sequence

What evidence is there that there is a specific centromere DNA at all? It is feasible that various species of DNA are sufficient to give binding sites for centromere proteins, without there being any specific DNA sequence at a centromere "core". However, the specific nature of the molecular processes occurring at the centromere make this seem unlikely.

Proteins detected at the interphase centromere include the centromere proteins (CENPs), which are localised on chromosome spreads of human cells to two dots at the centromeres (Fritzler et al, 1980; Moroi et al, 1980) at both interphase and metaphase. One of these CENPs has been shown to associate with repetitive alphoid DNA sequences. A consensus sequence has been determined, namely the CENP-B box, which is found at the centromeres of most human chromosomes (Masumoto et al, 1989; Wevrick et al, 1990). The DNA sequence of alpha-satellite DNA, to which CENP-B specifically binds, is the 17bp: CTTCGTTGGAAPuCGGGA. The CENP-B box is contained within some alphoid monomers, but not others (Masumoto et al, 1989), and is found at the centromeres of all the human centromeres, with the apparent exception of the Y chromosome (Masumoto et al, 1989). Therefore, apparently the satellite DNA sequences do not fulfill the expectations of centromeric DNA.

After replication the interphase centromeric DNA interacts with certain proteins to build up the interphase centromere. Of particular potential importance in the interphase centromere is CENP-A, which is thought to be a centromere specific histone, or a modified histone (Palmer et al, 1987, 1991; Pluta et al, 1990). Specifically, it is thought that CENP-A is a variant of H3 and, thus, may be interacting with the centromeric DNA, possibly in an altered, centromere specific nucleosome core particle, to fulfill some structural function.

Although some of the component DNA sequences are being identified, the minimal, but entire sequence of DNA required to build up the interphase and subsequent metaphase centromere has not been elucidated. A potential solution to this problem may have been approached by Hadlaczky and co-workers. They recently isolated and sequenced a 14kb length of human DNA, by immunoprecipitation with an anticentromere autoimmune serum. This human DNA fragment was cotransfected into mouse cells, together with a dominant marker gene and lambda vector DNA, and resulted in the formation of a functional centromere (Hadlaczky et al, 1991). It is not yet clear what role the human DNA plays in this artificially created centromere, but its study may reveal enlightening information about the mammalian centromere. The sequence data shows that there are several inverted repeat sequences, potentially capable of forming stem and loop structures, as possible protein binding sites. However, the identity of a protein(s) potentially bound by such palindromes has not been elucidated.

D 1.3.2 Replication of Centromeric DNA

It has been proposed, though not widely accepted, that the replication of the centromeric DNA itself is delayed until after the pericentric heterochromatin has been replicated (Vig & Broccoli, 1988).

During such a delayed replication mechanism it is possible that the physical separation of the sister chromatids allows access of the replication machinery to the centromeric DNA. Then the synchronous separation of the sister chromatids could be regulated by the synchronous action of a decatenating molecule liberating the centromeres of sister chromatids, thus, allowing anaphase to proceed. An obvious candidate for such a function is DNA topoisomerase II, or a centromere specific variant thereof.

D 1.4 THE TRANSITION FROM THE INTERPHASE CENTROMERE TO THE MITOTICALLY ACTIVE CENTROMERE

As detailed above, there are two main states of the centromere. The transition between these two states, namely interphase to mitosis, would appear to be highly regulated. It would seem logical that some feedback mechanism is required to monitor the cell state, i.e. should mitotic division be triggered or not. Depending on this feedback mechanism, centromere activation is initiated or supressed. Possible modes of regulation may involve, for instance, cyclin dependent kinase p34(cdc2) or cytosolic Ca^{2+}, for details refer to Introduction Chapters I 1.1 and I 2.2. However, this regulatory process is still controversial, and awaits further analysis.

D 1.4.1 The Molecular Components of the Mitotically Active Centromere

The molecular components of the kinetochore are built onto the underlying centromere. The subsequent kinetochore-microtubule interaction separates and distributes the sister chromatids to the daughter cells. This process is a general one and appears by no means to be chromosome specific. Therefore, the molecular components involved in this kinetochore-mediated process would seem to be common to all of the chromosomes of a given species.

That there are centromere specific proteins at all is indicated by the following. Biochemical treatments resulting in chromosome banding suggest the presence of structural regions of the chromosomes underlined by specific protein-protein, and protein-DNA interactions; one such region is the centromeric heterochromatin observed by C-banding. Furthermore, since cell division is a highly orchestrated activity then it is logical to expect that certain proteins are responsible for mediating this process. There is experimental evidence that certain, presumably evolutionarily conserved, proteins are present at the centromeres of all higher eukaryotic chromosomes.

Considering the fact that the chromosomes of mice cells, for instance, can be transferred to human cells (and *vice versa*) and these are all still capable of undergoing segregation, suggests that the molecular components of the centromere are either conserved or can be substituted by the components from the other species.

This is pure speculation yet it is likely that as the molecular structure of mammalian centromeres is better characterised, then the underlying mechanisms and structure will be shown to be remarkably similar.

In the light of this it is not surprising that certain proteins have been found to package onto the centromeres to eventually form the kinetochore plates. Although present on the interphase centromeres, an additional role for the centromere proteins (CENPs) in metaphase is not excluded. The use of anticentromere autoimmune sera have demontrated the presence of the CENPs on the standardly termed active centromeres (Earnshaw & Rothfield, 1985) (on the basis of the ability to interact with microtubules and bring about chromosome segregation). Of the centromere proteins (see I 3.3.5), it has been suggested that CENP-C may interact with the motor proteins at the kinetochore. There is some evidence that CENP-C is required for centromere activity, inasmuch as CENP-C has been shown to be absent from the inactive centromere of a dicentric (number 13) in every cell of a human foetus, whereas CENPs -A and -B were present (Earnshaw et al, 1989).

Another antigen, which was identified by the use of a human anticentromere autoimmune serum, is exclusively located on centromeres in mitosis. This antigen has a molecular weight of 59kD (Hadlaczky et al, 1989), and appears to interact with the centromeric DNA, although no specific DNA sequence has yet been identified. Therefore, this antigen appears to be a marker for, and a component of, the active centromere.

D 1.5 THE CENTROMERE AND SISTER CHROMATID ARMS

During mitosis there are considerable torsional stresses which are incurred by the chromosomes. In particular the integrity of the entire length of the chromosome depends on the physical stability of the interaction between the centromere and the chromosome arms. This is particularly relevant at the metaphase/anaphase transition, when the forces exerted by the microtubules on the chromosomes are very strong (see I 3). Therefore, it appears probable that the area connecting the sister chromatid arms to the centromeres, i.e. the pericentromeric heterochromatic region, be responsible for this physical integrity of the chromosome.

D 1.6 THE KINETOCHORE-MICROTUBULE INTERACTIONS

For correct chromosome disjunction, bipolar attachment of the centromeres (Ault et al, 1991) and alignment of the chromosomes on the metaphase plate (Nicklas, 1988), precede sister chromatid (and therefore centromere) separation. This stage, therefore, must be monitored, possibly by some mechanism detecting the relative tensile state of the microtubules in the two hemispheres of the cell, or the positional relationship of the chromosomes to the equator, although the molecular processes regulating this are still unknown.

The kinetochore and microtubules together motor the movement of the chromosomes through prometaphase to their destination in telophase. This has been detailed in depth in the Introduction Chapter I 2.2, and although far from completely characterised is now reasonably well understood, and so will not be dealt with further here.

D 1.6.1 The Centromere and Sister Chromatid Association

There is a very important regulatory step in mitosis, whereby the association of the sister chromatids is apparently highly controlled. At the metaphase-anaphase transition the segregation of the centromeres of the sister chromatids appears to be highly coordinated. Thus, all of the chromosomes apparently enter anaphase at the same time and the sister chromatids separate synchronously (Murray and Szostak, 1985). It is likely that the separation at the centromere is a precise mechanism and not a passive "ripping" apart. However, the molecular mechanisms required in this process are not understood, although there is some evidence that cytosolic Ca^{2+} is involved (Hepler and Callahan, 1987; see Introduction Chapter I 2.2).

Since not only proteins, but also DNA is contained in the centromere, the conformation of which has to be altered, then it appears possible that topoisomerase II, or a centromere specific variant thereof, may be involved in mediating the topological transformations required. Furthermore, some means of signalling that the chromosomes have reached the appropriate stage for the onset of anaphase is required to initiate separation of the sister chromatids, which similarly should involve the centromere.

The inner centromere proteins (INCENPs) Mr=155,000 and Mr=135,000, detected by Mabs raised against chicken chromosome scaffolds, are localised to two dots at the pericentromeric region between the sister chromatids during metaphase, whereas at anaphase they remain associated with the metaphase plate, and at telophase are focused in the midbody (Cooke et al. 1987). The INCENPs are the most tightly bound chromosomal antigens reported to date, since they resist 2M NaCl extraction. Thus, these proteins may interact with the DNA of the active mitotic metaphase pericentromeric region, but also may be involved in the regulation of the sister chromatid association-disassociation process of the metaphase/anaphase transition.

There have been several antigens which have been observed in the kinetochore at metaphase, which then are strikingly relocated to the midzone of the spindle at anaphase, in a manner similar to the INCENPs.

These antigens are CENP-E, of molecular weight 250-300kD, identified by monoclonal antibodies raised against human chromosome scaffolds (Yen et al, 1991), another or possibly the same antigen of molecular weight 250kD (Compton et al, 1991), and antigens of molecular weights 140kD and 155kD, identified by monoclonal antibodies raised against bovine nuclear thymocyte extracts (Pankov et al, 1990). The role of these centromere (and cytoplasmic antigens) is still a unclear, although their rapid disassociation at the metaphase/anaphase transition suggests a role in the coordinated separation of the sister chromatids at anaphase (for a further discussion of this class of proteins, see Earnshaw and Bernat, 1991)

D 1.7 POSSIBLE SPATIAL ORGANISATION OF THE CHROMATIN IN INTERPHASE

During the majority of the cell cycle the centromere is apparently inactive, inasmuch as there is no obvious centromere-mediated chromosome movement. Some authors consider it possible that there is organisation of the chromatin within the interphase nucleus (Cooke and Brazell, 1980), chromosome order in prometaphase (Chaly and Brown, 1988), non-random association of homologous and non-homologous chromosomes in interphase (Hadlaczky et al, 1986), maintenance of the anaphase and telophase arrangement of chromosomes in interphase and prophase (Rabl, 1885). Therefore, if an interphase order of chromatin exists, then the centromerere, which as a means of mediating chromosome movement at this stage is inactive, may provide nuclear attachment sites, as is thought for MARs (Gasser et al, 1986). Therefore, it is possible that the interphase centromere may serve as one such, or a major MAR-like attachment site. If fulfilling such a function (which is far from proven) then this attachment would probably occur in late telophase or early interphase.

Due to the structural stability of centromeres (e.g. resistance to high salt treatment (I 3.4) it has been suggested that the centromere may have an additional structural importance in chromosome architecture (Hadlaczky et al, 1982). It has been postulated that the centromere may act as an initiation site or "foot-stone" providing a structural basis for the maintenance of the integrity of the chromosome (Hadlaczky, 1985), or for compaction.

CONTENTS

D 2 POSSIBLE FUNCTIONS OF THE AC1 ANTIGEN

Introduction

The Mammalian Centromere

During the course of these studies many interesting aspects of the mammalian centromere have come to light, refer to Results Chapters. The exact identity and nature of the molecular components required for a functional mammalian centromere still elude us, although, the picture is becoming clearer. During interphase the centromere is compacted but not visible normally by light microscopy, and apparently inactive. During DNA replication the centromeric DNA is also replicated, though there is some evidence that this region is late replicating (Vig & Broccoli, 1988).

There are two overall functions of the centromere in mitosis: the coordinated synchronous movement of the chromosomes, and regulating the structural integrity of the chromosomes. In the former the kinetochore interacts with the spindle microtubules to bring about chromosome movement from prometaphase through metaphase and anaphase to their destination in telophase.

During mitosis the kinetochore interacts with the microtubules in order to bring about bipolar attachment of the kinetochores and the alignment of the chromosomes on the metaphase plate. The microtubule interaction directs the movement of the two chromosome sets to the spindle poles. Movement of the chromosomes by the microtubules involves considerable mechanical forces, and it is the centromeric region, which is also responsible for maintaining the integrity of the chromosomes, during the entire movement process, through prometaphase until the metaphase-anaphase transition. At this stage the sister chromatids separate, a process involving structural changes at the centromere, which are not yet understood. As the cycle proceeds through anaphase to telophase the kinetochore and captured microtubules translocate the chromosomes to the poles.

In addition to its role in chromosome movement, and maintenance of sister chromatid association, the centromere may play a role in higher-order chromosome organisation (Hadlaczky et al, 1982; Hadlaczky, 1985). Thus, there is an opinion that the centromere may serve as a focal point, onto which the rest of the chromatin begins to condense, at the late interphase early prophase stage.

The AC1 Antigen

Considering the above overall functions of the mammalian centromere and the properties of the AC1 antigen then the following can be deduced.

Firstly, since the AC1 antigen only associates with the centromere during prophase and metaphase, but disassociates at anaphase, then a function of the antigen in chromosome locomotion may be excluded. Secondly, many protein and DNA sequences interact to physically stabilise the chromatin against the physical stresses incurred during chromosome movement. It is in such a capacity that the AC1 antigen is likely to play a crucial role. Thirdly, if the interphase centromere serves as a focal point for higher-order chromosome organisation then it is unlikely that the AC1 antigen is involved, since it cannot be detected on the interphase centromeres.

D 2.1 POSSIBLE FUNCTIONS OF THE CENTROMERE RING IN STABILISING THE SISTER CHROMATID ASSOCIATION IN PROMETAPHASE

In higher eukaryotes, during prometaphase, two important events occur. The sister kinetochore plates, on the opposite sides of a given chromosome, are attached to the microtubules of opposite poles, i.e. bipolar attachment (as opposed to both plates to the same pole). This is followed by polar ejection, involving the movement of the chromosomes away from the nearest pole, until a dynamic equilibrium is reached when the chromosomes are equidistant from both poles. It is possible that the AC1 ring antigen plays an important part in either one or both of these two events. Before, this is discussed, the processes of bipolar attachment and alignment on the metaphase plate are briefly outlined.

D 2.1.1 Bipolar Attachment

Initially, microtubule attachment to the kinetochore is weak, however, once bipolar attachment is realised, spindle attachment becomes strong. Therefore, it appears that microtubule binding at the kinetochore becomes stable when the sister kinetochores are pulled in opposite directions. Conversely, if the sister kinetochores are attached to microtubules of the same pole, the chromosome spontaneously detaches from the spindle. The process by which this occurs is, however, unknown. Hence, bipolar attachment of the microtubules to the kinetochores inherently involves forces tending to pull the sister chromatids in opposite directions, putting the connection between them under considerable stress.

D 2.1.2 Alignment on the Metaphase Plate

The other important event in prometaphase is the alignment of the chromosomes, with bipolar attachments, on the metaphase plate, a process which involves polar ejection forces. During this process a given chromosome moves towards the closest pole, but is then expelled from the polar region by the non-kinetochore, astral microtubules (Ault et al, 1991) and the chromosomes oscillate until an equilibrium is reached. It has been postulated that the magnitude of the force exerted on the chromosome is proportional to the length and the number of the microtubules bound to the kinetochores.

Figure D 1. Legend

This figure shows the changes in the location of the AC1 antigen as the cell progresses from prometaphase through metaphase and to anaphase.

Prometaphase-metaphase

The AC1 antigen is shown as a ring (in black) encircling the sister chromatids at the centromere. Microtubules are shown attached to the chromosomes or overlapping at the midbody. The pair of centrioles are shown at right angles to each other (also in black).

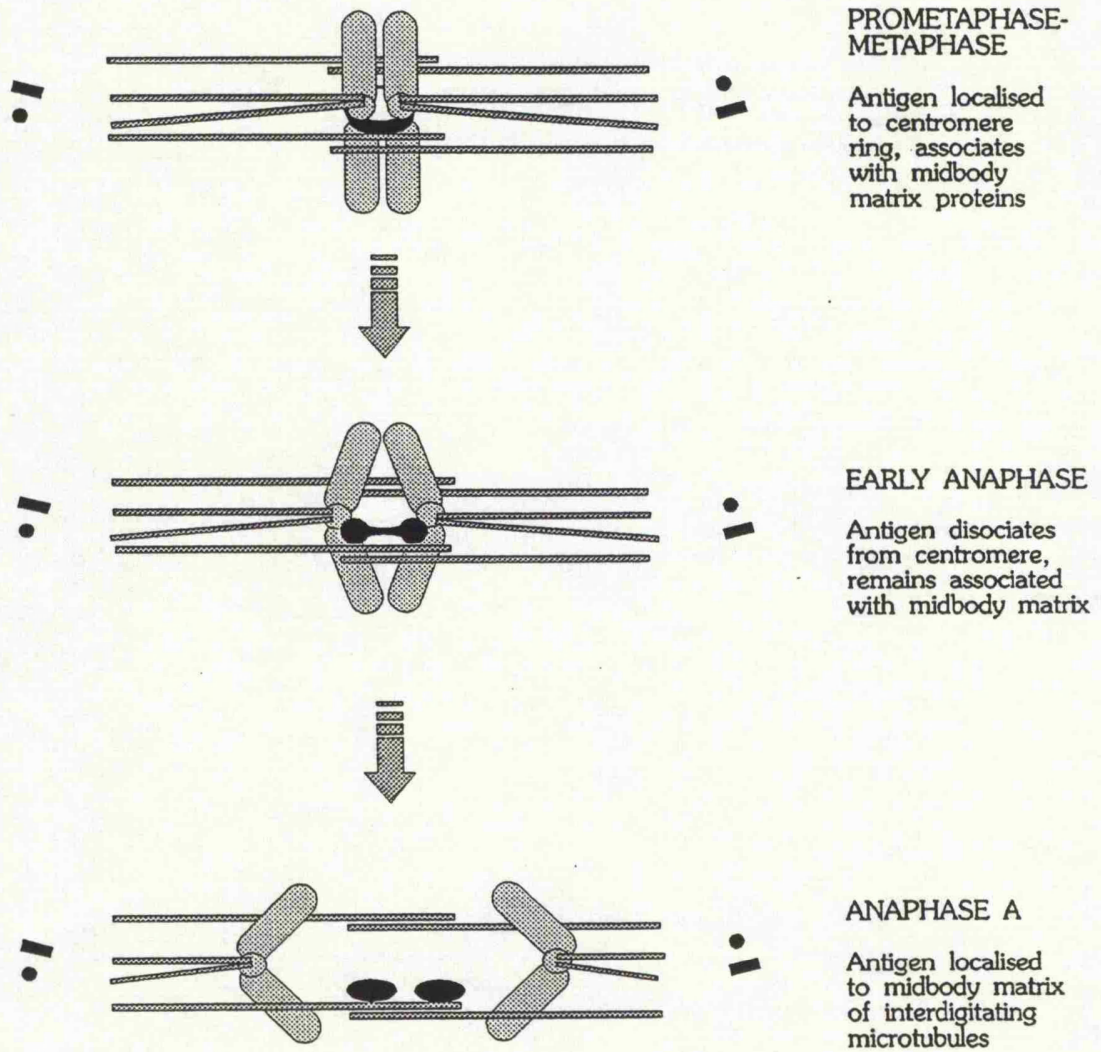
Early Anaphase

As the sister chromatids move apart the AC1 antigen is distorted (shown in black as a dumbbell shape) as it disociates from the centromeres.

Anaphase A

As the chromosomes are moved further apart the AC1 antigen remains on the midbody matrix (shown as black oval shapes).

Figure D.1. Diagrammatic representation of the apparent fate of the AC1 antigen from prometaphase to anaphase



Thus, the chromosomes move to and fro, between the poles until they arrange on the metaphase plate.

D 2.1.3 The AC1 Antigen May Maintain Structural Stability at the Centromere

As indicated above, the bipolar attachment and alignment on the metaphase plate place the connections between the sister chromatids under considerable tensional forces, tending to pull or possibly push them apart. Thus, physically the centromeric region is important in keeping the sister chromatids together as a prerequisite for bipolar attachment and alignment of the chromosomes on the metaphase plate. The centromere ring identified in this project, detected by the monoclonal antibody AC1, appears to be a reasonable candidate for bringing about such physical stability, against these physical strains. Thus, the ring could stabilise and/or maintain the connection between the sister chromatids, by encircling this region and interacting with the centromeric DNA. Such a strong DNA interaction (refer to Chapter R6), therefore, may provide the means to withstand the tensional forces tending to pull the sister centromeres apart. See Fig. D1.

D 2.2 CONTROL OF SISTER CHROMATID ASSOCIATION AT THE METAPHASE/ANAPHASE TRANSITION

In metaphase, the kinetochore plates are connected by the microtubules to the two poles. At this stage all the chromosomes are apparently subject to approximately equal forces from the two opposing poles. Therefore, some molecular mechanism, as yet undefined, may detect this dynamic tensional equilibrium and trigger the microtubules at the kinetochore to begin sister chromatid movement to their facing poles. At this stage the connection between the chromatids may be disengaged and/or the torsion provided by the microtubules so great that the sister chromatids are pulled apart. It is tempting to think that some precise molecular mechansim is involved in disengaging the connection between the sister chromatids, rather than their being merely ripped apart.

A possible candidate for the metaphase/anaphase trigger is a process mediated by cytosolic calcium ions. The evidence supporting this idea, that cytosolic calcium ions are involved in the "anaphase" trigger (Hepler and Callahan, 1987), is as follows. The use of a fluorescent Ca^{2+} indicator dye has shown that there is a tenfold increase in the intracellular Ca^{2+} level at anaphase in some cells. Also the microinjection of low levels of Ca^{2+} into cultured cells at metaphase has been shown to be able to cause premature anaphase. Furthermore, membrane vesicles, which are rich in Ca^{2+}, have been demonstrated to accumulate at the spindle poles (Hepler and Callahan, 1987). Thus, Ca^{2+} appears to be a potentially candidate for a role in bringing about anaphase, though further work is required to clarify this important regulatory phase of mitosis.

It is possible that the process preventing premature sister chromatid separation is linked to some signal, for instance depending upon the completion of centromere DNA replication.

In support of this, it has been suggested that the replication of the centromeric DNA itself is delayed until after the pericentric heterochromatin has been replicated (Vig & Broccoli, 1988). However, there is very little information about the timing of centromere replication in mammalian cells. Alternatively, it is possible that the DNA topoisomerase II mediated decatenation of the replicated DNA of the sister chromatids is the trigger which allows the chromosomes to proceed into anaphase. In this context it may be more plausable to presume that a molecule, as yet unidentified, interacts with the AC1 antigen, reducing its affinity for the centromeric DNA, or even cleaving its DNA binding domain, thus releasing the sister chromatids from each other, when replication, decatenation and correct positioning on the spindle all have occurred.

The possibility that a DNA-binding protein may be a regulator in triggering a transitional phase of mitosis, would be strengthened if other DNA-binding proteins were to be shown to have a similar function. Indeed there is an example of one such DNA-binding protein which has been shown to regulate a transition of mitosis. This protein, termed RCC1, regulates not the metaphase/anaphase transition, but the interphase/mitosis one. However, this example does strengthen the possibility that a DNA-binding protein can carry out such a regulatory function. In order to understand the analogy the RCC1 mediated regulation of the interphase-mitosis transition (Nishitani et al, 1991), will be outlined briefly. At the interphase-mitosis transition, mitosis is normally delayed until the DNA has been replicated. This mitosis delay mechanism involves a 45kD DNA binding protein, identified in hamster cells, which is encoded by the RCC1 gene. If the RCC1 gene is mutated then the cells in S or G2 (but not G1) proceed into mitosis without replicating their DNA. It has been proposed that when RCC1 is mutated, tyrosine dephosphorylation and, hence, activation of the cdc2 kinase occurs and so the cell enters premature mitosis. It has been suggested that the RCC1 gene product plays a structural role in decondensed chromosomes. When the gene is mutated premature chromatin condensation may occur. If M-phase regulatory proteins are sensitive to the state of chromatin condensation, then the structural change may trigger mitosis.

The RCC1 gene product is one example of a DNA-binding protein, which is involved in the transition from interphase into M-phase. It is, therefore, possible that a process similar to that involving the RCC1 gene product may stall the transition from metaphase to anaphase, until all of the chromosomes are appropriately orientated and aligned, and once the cell has reached this stage, anaphase can proceed.

However, the question then arises as to what molecular changes bring this trigger about, and whether the AC1 antigen, for example, is actively or passively dissociated from the centromere at this stage. This remains to be determined.

D 2.3 DIFFERENT CELLULAR LOCATIONS OF THE AC1 ANTIGEN

At late anaphase the AC1 antigen appears to be localised to the midzone, and later to a double collar at cleavage furrow in late telophase. In interphase, Mab AC1 did not react with the centromeres but did decorate the centrioles. Thus, the AC1 antigen is apparently appearing at different cellular locations at different stages in the cell cycle, see Fig. D2. These observations can be explained by at least three alternatives.

There could be a fortuitous cross-reaction of the anticentromere antibody, AC1, with a component of the spindle machinery, which is present in the midzone, cleavage furrow and centriole. A monoclonal antibody is not necessarily monospecific. The same 3D conformation could potentially be found on different, even unrelated proteins. Therefore, it is possible, although unlikely, that the same 3D conformation is present on two or more quite distinct proteins associated with both the centromere and the mitotic spindle. Since the midzone, cleavage furrow and centriole are cytoplasmic components, and the original immunising material was chromosomal scaffold proteins, then its detection is presumably due to the presence of the same 3D conformation on the cytoplasmic elements as on the centromere antigen and not *vice versa.*

Alternatively, Mab AC1 may react with a conserved epitope on two different, but evolutionarily, and, therefore, perhaps functionally, related antigens, namely one being centromeric and the other cytoplasmic. It was observed that a larger molecular weight antigen, 200kD, could be detected in CHO and human interphase, but not metaphase, protein fractions (Chapters R3 and R7). Thus, it cannot be excluded, if the cytoplasmic component staining is of a different protein, and presumably, of a different molecular weight, and that this may correspond to the 200kD antigenic band.

The third possibility is that the antigen detected at the centromere and the midzone, contractile ring and centriole are one and the same. Consistent with this possibility is the behaviour of the AC1 antigen in metaphase and interphase, observed by immunofluorescence microscopy, whereby the AC1 Mab stains the centromeres in metaphase, but in interphase only decorates the centrioles. This observation is consistent with the Western blotting data that the level of 170kD antigen is greater in metaphase than in interphase protein samples.

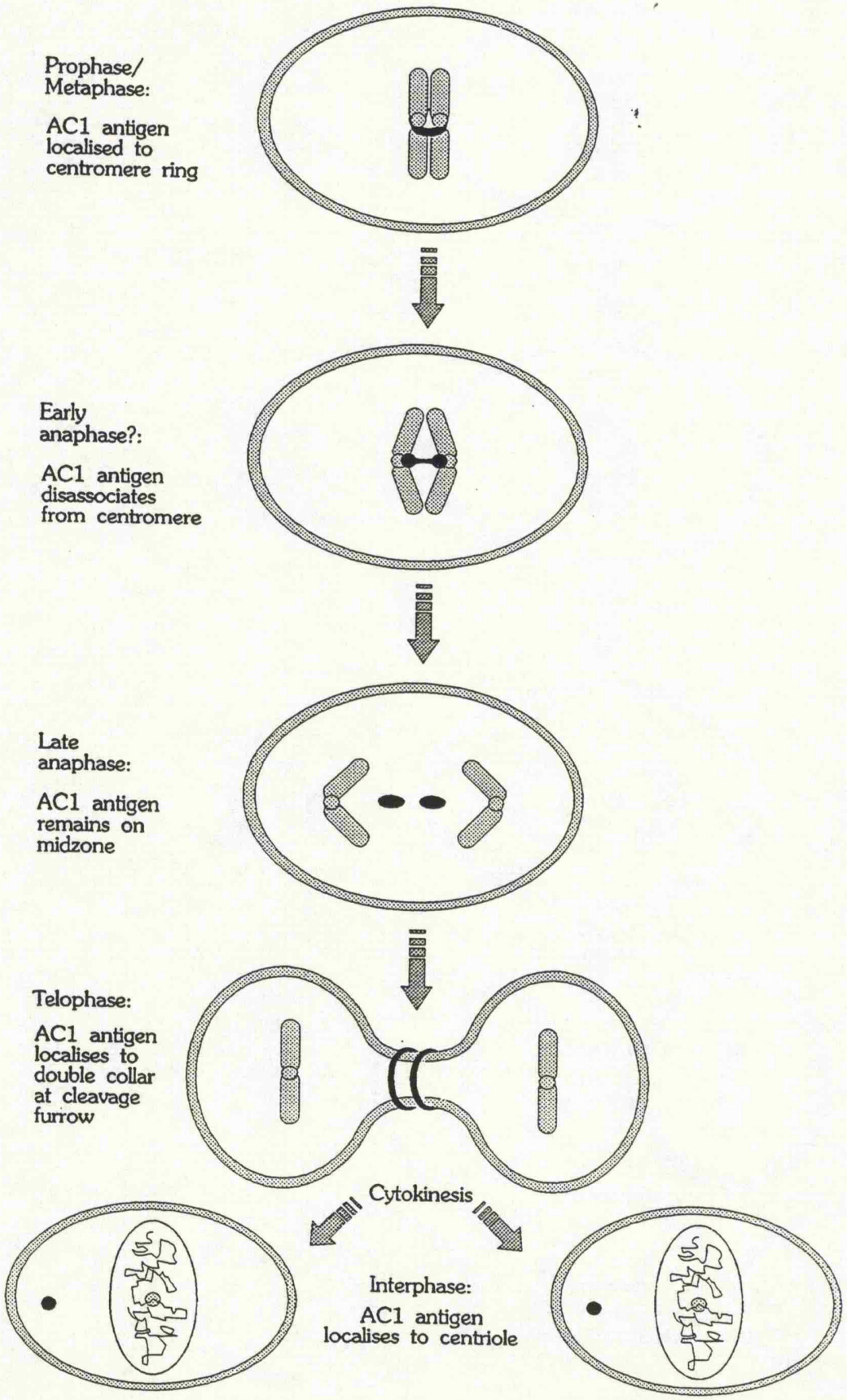
Figure D 2. Legend

This figure shows the changes in the location of the AC1 antigen at various stages throughout the cell cycle

The AC1 antigen is shown in black.

In prophase/metaphase the antigen is located to a ring encircling the sister chromatids. In early anaphase the ring becomes distorted and dumbbell shaped as the sister chromatids are pulled apart and the antigen remains associated with the midbody matrix. By late anaphase the antigen is completely disociated from the centromeres and remains on the midbody matrix. At telophase the antigen forms a double collar at the cleavage furrow and by interphase is concentrated at the centriole.

Figure D2. Postulated Distribution of the AC1 Antigen Throughout the Cell Cycle



That the different cellular locations of the AC1 antigen is not due to a cross-reaction is supported by the data obtained using another Mab, BA2. The two different Mabs, AC1 and BA2, both react with the centromere ring and the centriole in an apparently identical manner in CHO and mouse samples. AC1 also detects these structures in human cells.

Furthermore, AC1 detects an antigen of molecular weight 170kD in metaphase chromosomal proteins and 170kD and 200kD in interphase proteins from CHO, whilst BA2 reacted with 170kD and 200kD antigenic bands in human nuclear protein extracts. Considering the fact that the 170kD and 200kD antigens are relatively large, then there is a greater probability of their carrying many potential immunogenic epitopes, than for a smaller molecule. Therefore, it is possible that Mabs AC1 and BA2 are reacting with different epitopes. In this case it is very unlikely that two different Mabs are cross-reacting with different proteins, and more likely that the different staining patterns are due to the same antigen redistributing to different sites at different stages of the cell cycle. However, these observations are by no means conclusive proof, since two separate monoclonal antibodies may react with the same epitope.

D 2.4 POSSIBLE FUNCTION OF THE AC1 ANTIGEN ON THE MIDZONE IN ANAPHASE AND THE CONTRACTILE RING IN TELOPHASE

At late anaphase the AC1 antigen appears to be localised to the midzone, and later to a double collar at cleavage furrow in late telophase (refer to Chapter R8). Cleavage furrow formation in animal cells is relatively poorly understood, although cytokinesis appears to require the formation of an actin containing contractile ring across the bridge of cytoplasm between the two daughter cells. Interestingly, in spite of the centromeric localisation and DNA binding of the AC1 antigen in metaphase, in telophase the antigen appears to be relocated to just such a double collar, which could correspond to the contractile ring. Thus, the antigen appears to have a dual identity, on the one hand the antigen strongly interacts with the centromeric DNA (shown by the presence of the antigen on chromosome scaffolds), on the other it appears to be a component of the cytoplasm.

This, however, leads to a somewhat paradoxical siuation. What is a DNA-binding protein doing in the (cytoplasmic) contractile ring? Molecular structural/ conformational changes of the spindle machinery and intracellular matrix occur in both prometaphase and cytokinesis. The antigen may serve some function in these changes. If the AC1 antigen has an additional affinity for the midbody matrix, then it is possible that at anaphase the DNA-binding domain of the antigen is conformationally changed, or the DNA with which it interacts is altered by, for example, topoisomerase II, thus, releasing the protein from the centromeric DNA. Alternatively, the DNA-binding domain may be cleaved, releasing a portion of the protein (retaining the AC1 epitope).

Either way this could leave the antigen on the midzone, ensuring that the antigen does not remain on the centromere, and no longer holds the sister chromatids together. The antigen in this model, therefore, may only be present in the contractile ring in a passive capacity, as a residue of the spindle, chromosome segregation process.

D 2.5 RELATIONSHIP BETWEEN THE CENTROMERE RING AND THE INTERPHASE CENTRIOLE

It has been shown by Hall et al, (1989) that a 6-9 megabase, linear DNA molecule is localised to the basal bodies of *Chlamydomonas reinhardtii* . Thus, it is possible that the same, AC1, antigen is present at both extremes of the spindle apparatus, namely at the centromere and at the centriole, and mediates a DNA-protein interaction at both sites. For instance the antigen may link another protein directly, or other proteins indirectly to the centromeric region. This postulated DNA interaction at both locations is consistent with the observation that the antigen can be detected on the residual structures, the chromosome scaffolds, by both the immunofluorescence microscopy and Western blotting techniques (implicating a DNA interaction). That the AC1 antigen has an affinity for other proteins is possibly implied by the antibody staining of the midzone microtubules. It is conceptually feasible that one of the domains of the antigen binds DNA, and another domain (since the antigen is a relatively large molecule at 170kD) may have a loose interaction with the microtubules, or, more likely, is indirectly interacting with the microtubules via, for instance, a microtubule associating protein, or midbody matrix protein (coating the interdigitating microtubules at the midzone region). This would also be consistent, in particular, with the AC1 antibody staining of microtubules in the midzone.

Centrosome (centrioles and surrounding spindle matter) isolation procedures have been developed, therefore, it may be worthwhile in the future to test such a protein fraction with Mab AC1, to determine if the AC1 antigen can be detected on such spindle proteins.

D 2.6 CHROMOSOMAL PASSENGER PROTEINS

Well after the completion of the experimental part of this thesis work, other researchers have reconsidered certain aspects of the centromeric and chromosomal proteins. The property of apparently tightly bound (shown by their resistance to high salt extraction) chromosomal proteins in metaphase, which relocate to cytoplasmic locations in anaphase, has been demonstrated by an increasing number of researchers (for recent and excellent reviews, see Earnshaw and Bernat, 1991; Rattner, 1992).

Metaphase chromosome associated antigens have been identified, which relocate to the spindle interzone at anaphase. One of these was identified by a monoclonal antibody raised against chicken chromosome scaffolds.

The antibodies decorate the so-called inner centromere proteins (INCENPs), since they are located to the inner sister chromatid area outside of the centromere, and these were shown to have molecular weights of 135kD and 150kD (Cooke et al, 1987). These antigens are tightly bound, since they resist 2M NaCl extraction. The other such antigen was identified by a human autoimmune serum which reacted with an antigen of molecular weight 38kD, with a similar staining pattern to the INCENPs on Indian muntjac chromosomes (Kingswell et al, 1987).

The metaphase kinetochore antigens of molecular weights 140kD and 155kD, detected by monoclonal antibodies raised against bovine nuclear thymocyte extracts, have also been shown to relocate to the spindle interzone of Chinese hamster cells at anaphase. These antigens are extracted by 0.4 M NaCl (Pankov et al, 1990). More recently a so-called CENP-E, of molecular weight 250-300kD has been identified by the use of a monoclonal antibody raised against human chromosome scaffolds (Yen et al, 1991) and possibly the same antigen of molecular weight 250kD has been recognised by other researchers (Compton et al, 1991). Both these antigens also relocate from the centromere to the spindle interzone at anaphase.

Earnshaw and Bernat explain this class of proteins as follows:
"... the chromosomes may make important structural contributions to the anaphase spindle and cleavage furrow, which are normally thought of as "cytoskeletal" functions. These structural contributions may be made by members of a new class of "chromosomal passenger" proteins that use the chromosome as a means of conveyance so that they are correctly positioned at the metaphase plate to carry out their nonchromosomal functions during anaphase and the subsequent mitotic events."

Therefore, it appears that the AC1 antigen is a member of this class of proteins, and may carry out its function at the centromere, and "hitch a ride" to the interzone where it dissociates from the chromosome. On the spindle it may carry out some as yet unidentified cytoplasmic function.

D 2.7 FUTURE RESEARCH DIRECTIONS

Two of the possible future directions of research on mammalian centromere structure are considered here. The first is in relation to the ring antigen presented in this thesis, and the second is more general regarding centromere structure in global terms.

There are two outstanding question areas regarding the centromere ring antigen detected by Mab AC1. The first is what other proteins there are, which the AC1 antigen interacts with, in the centromeric region, in order to bring about its function. The second is what DNA sequence(s) does the antigen interact with.

D 2.7.1 Relationship of the AC1 Antigen to Other Proteins of the Centromeric Region

An experiment worth attempting would be to immunoprecipitate the AC1 antigen under conditions that maintain protein-protein interaction.

In order to identifiy other proteins complexed with the AC1 antigen. Since it has already been shown (Chapter R3) that the AC1 antigen is a relatively labile, minor chromosomal component, and its detection therefore difficult the antibody should be preadsorbed with cold chromosomal proteins, before the immunoprecipitation of radiolabelled proteins. The use of cross-linking agents might also be used to achieve ultimately a more global picture of protein: protein interactions in the centromere, rather than the relatively vague positional, spatial relationship of individual proteins which is currently accepted.

Useful information about the AC1 antigen itself could be gained by determining the amino acid sequence and/or the DNA sequence of the gene encoding this polypeptide. The low levels of the antigen and its apparent labile nature, make protein sequencing difficult, although not impossible. Moreover, the standard approaches for isolating the gene encoding this polypeptide are difficult. The fact that the antibody reacting with the centromere ring is monoclonal makes the isolation of the gene encoding the polypeptide detected by AC1, rather unlikely, by standard methods of immunological screening of expression libraries, since the bacterial hosts do not make the post-translational modifications frequently present on eukaryotic epitopes. Therefore, the most appropriate strategy may still be to try to immunoprecipitate the antigen in large quantities, keeping its labile nature in mind, and attempting protein sequencing. If successful a synthetic oligonucleotide could be made and the gene encoding the antigen isolated from a genomic library.

D 2.7.2 Sequence of the DNA that Interacts with the AC1 Antigen

To attempt to isolate the DNA interacting with the AC1 antigen affinity column purification could be attempted. Chromosomes could be isolated, DNase and 2M NaCl extracted, and the residual chromosome scaffold incubated with Mab AC1 which has been immobilised onto an affinity column. Since it has already been shown (Chapter R3) that the AC1 Mab has a high aspecific affinity for chromosomal and nuclear proteins, and that the AC1 antigen is a minor chromosomal component, the antibody should be preadsorbed with cold chromosomal proteins, before the radioactively, or biotin-labelled ones. The resulting fraction, containing AC1 polypeptide potentially bound to DNA, should be end-labelled for instance with [alpha-^{32}P] dATP by Klenow polymerase.

D 2.7.3 Possible Reconstitution Experiments Using Isolated Components of the Mammalian Chromosome

Assuming an assortment of DNA and protein components could be identified and purified to homogeneity, then chromosome reconstitution experiments could be attempted in order to attempt to construct a mammalian artificial chromosome.

Summary of Thesis

The studies carried out in this PhD thesis resulted in the identification of a novel ring-like structure, which was conserved amongst the metaphase centromeres of two rodents, namely Chinese hamster and mouse, and in humans. The ring-like antigen was detected in chromosome scaffold preparations and, thus, tightly bound the DNA of metaphase chromosomes. The antigen was also detected on the interdigitating microtubules of the midzone, in anaphase, to the contractile ring at cytokinesis, and also to the interphase centrioles. This dramatic relocation of the antigen at the metaphase/anaphase transition suggested that it falls into a class of recently identified dual chromosomal and cytoplasmic components, termed the "passenger proteins". In this thesis the ring antigen was postulated to be involved in holding the sister chromatids together by a strong interaction with the centromeric DNA through prometaphase to the metaphase-anaphase transition. It was also suggested that the ring antigen is disengaged from the centromere prior to anaphase, and may be involved in the regulation of the metaphase-anaphase transition.

Therefore, the experimental work carried out during this thesis has brought to light an intriguing antigen interacting with the chromosomes and and the cytoplasm of mitotic cells. The future experimentation suggested above, using the monoclonal antibody AC1, could lead to further interesting insights into the architecture of the mammalian metaphase centromere.

APPENDIX

A1 Pig Brain Tubulin Extraction

(Taken from Weingarten et al, 1974) 240ml of freshly slaughtered pig brains were transferred to 180ml PB (100mM MES, 1mM EGTA, 0.5mM $MgCl_2$, 1mM mercaptoethanol, 1mM EDTA, 1mM GTP, pH 6.4) on ice. A homogeneous suspension was obtained using a Waring Blendor. The homogenate was centrifuged at 45,000g for 1-2.5 h at 4°C, the supernatant diluted 1:1 in PBG (PB containing 8M glycerol) and warmed to 37°C for 20min. The sample was then centrifuged at 45,000g for 1-2.5h at 25°C and the supernatant discarded. The pellet was dissolved in 48ml ice cold PB and chilled for 30min using a magnetic stirrer and kept on ice. The sample was centrifuged at 75,000g for 1h at 4C and the subsequent pellet discarded.The supernatant was diluted 1:1 in PBG and warmed to 37°C for 20min. The sample was then centrifuged at 75,000g at 25°C for 1h and the supernatant discarded. The pellet was dissolved in PB to a final protein concentration of 3.5-6.0mg/ml, and stored in small aliquots in liquid nitrogen. In order to have active tubulin the aliquot was repolymerised.

A 2 ELISA

This technique was used after generation of the anti-CHO chromosome scaffold Mabs, before the start of this project, however for completeness the ELISA procedure is included here.

A sample of 30µg CHO chromosome scaffold proteins was diluted in 10ml blocking solution (1% bovine serum albumin, 20% foetal calf serum in Tween-PBS containing 0.0% Tween 20) and 100µl distributed per well of a microtiter plate, incubated overnight at 4°C. Non-bound solution was shaken off and the wells washed with three changes of Tween-PBS (containing 0.05% Tween 20). The antibody solution (hybridoma supernatant undiluted) was added and incubated for 2h at 37°C, then washed with three changes of Tween-PBS as above. 100µl of HRPO conjugated anti-mouse secondary antibody in 0.5% bovine serum albumin in Tween-PBS was added per well. This was incubated protected from light for 1h at 37°C, then washed as above. Subsequently 200µl of NPP substrate was added per well and incubated for 30 min at room temperature protected from light. 25µl of 0.5M NaOH was used to stop the hydrolysis and the intensity of the reaction determined with a spectrophotometer.

A 3 AC1 Hybridoma Cell Recloning

Hybridoma clone AC1 was recloned as in Harlow and Lane (1988), cells were diluted in a 96 well microtitre plate at a cell density of 2, 1 and 0.5 cells per well. Cells were incubated as described for hybridoma cells in Materials and Methods and tested by ELISA for immunoglobulin production.

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