MONOCLONAL ANTIBODIES AS PROBES OF CHROMOSOME STRUCTURE

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I hereby declare that this thesis has been composed by myself and that the work described in it is my own.

CONTENTS

Acknowledgments

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ABSTRACT

This thesis describes investigations into the structure of eukaryotic chromosomes using monoclonal antibodies. Of particular interest was to determine the relationship of the chromosome core, a residual structure after the dehistonisation and nuclease treatment of mitotic chromosomes, to any substructure within the intact chromosome.

In the first chapter, current knowledge of chromosome structure is reviewed, with particular emphasis on the higher order folding of chromatin and the structures proposed to mediate this.

In the second chapter, an electrophoretic study of the non-histone proteins of the chromosome core is described. A possible relationship with proteins in the interphase nucleus is shown.

The third chapter concerns the raising of monoclonal antibodies to chromosome core proteins. The preliminary characterisation of two of these antibodies, 4G8 and 2C6 is described.

In the fourth chapter, the detailed characterisation of the 4G8 and 2C6 antigens is described, using the techniques of "western-blotting", immunocytology and immuno-electron-microscopy. Antibody 4G8 reacted with a protein exclusively found in the chromosome core. This protein was also shown to part of a substructure within intact metaphase chromosomes and probably also involved in interphase nuclear organisation. Antibody 2C6 reacted with a chromosome core protein showing an altogether different distribution, being abundantly distributed throughout the chromosome and also part of a cytoplasmic filament system. Taken together, the results provide strong support for a non-histone-protein core being involved in the higher organisation of eukaryotic chromosomes.

In the final chapter, the value of the monoclonal antibody approach in the study of chromosome structure is considered, and some speculations concerning the two antigens characterised are made.

Abbreviations

BSA- bovine serum albumin

DTT- dithiothreitol

EDTA- ethylenediamine tetracetic acid disodium salt

EtOH- ethanol

FCS- fetal calf serum

MeOH- methanol

PAGE- polyacrylamide gel electrophoresis

PBS- phosphate buffered saline (Dulbecco's)

PEG- polyethylene glycol

SDS- sodium dodecyl sulphate

Chapter 1 INTRODUCTION

It is now nearly 100 years since Waldeyer proposed the name chromosome for the thread like objects seen in the cell at mitosis and the chromatic elements of the nucleus discovered by Boveri (history reviewed by Darlington, 1977). Since the 1960's, with the advent of high resolution electron microscopy and a knowledge of the primary structure of DNA, the organisation and ultrastructure of chromosomes has been the subject of much investigation. The chromosome is a tremendous example of biological packaging, for there is nearly 2m of double helical DNA within the human haploid genome (Bostock and Sumner, 1978), and yet this is contained in a nucleus of about 5μm diameter or in mitotic chromosomes which are less than 10μm long (Mullinger and Johnson, 1980).

However, chromosomes are more than mere packages, and they were better described by Mullinger and Johnson (1980) as "vehicles of gene segregation and frameworks for gene operation". Associated with or integral to the chromosome must be a host of molecular systems involved in gene operation and the control of gene expression. The mode in which the DNA is packaged must account for the spatial constancy of different parts of the genome, not only in terms of classical linkage, but also within particular regions of chromosomes seen at metaphase using in

situ hybridisation. This organisation is not immutable, for chromosome aberrations in which fragments "break off" or translocate occur (see Bostock and Sumner, 1978), and knowledge of the way in which the chromosome is organised will no doubt lead to a better understanding of these phenomena.

In this introduction I shall review our current understanding of the organisation of the chromosome. The word chromosome, when unqualified, refers equally to the mitotic and interphase states of the cell.

Before looking at whole chromosomes, it is necessary to discuss the first two levels of organisation of double helical DNA, the 10 and 25nm chromatin fibres.

1.1 THE 10 AND 25nm CHROMATIN FIBRES.

These two fibres represent the first two steps of the packaging of DNA. They refer to the thickness of fibres first found in isolated chromatin using electron microscopy and X-ray diffraction, the presence of which has now been confirmed in intact metaphase chromosomes and nuclei using low angle X-ray diffraction (Langmore and Paulson, 1983). In these fibres the DNA is organised by nucleosomes, disc shaped octameric complexes of the histones H2a, H2b, H3 and H4. The structure of the nucleosome and its interaction with DNA have been thoroughly reviewed by Lilley and Pardon (1979) and McGhee and Felsenfeld (1980).

The basic histone octamer has been called the "core

particle" and around this about 146bp of the DNA fibre is wrapped. This particle complexes with a molecule of histone H1, so that in the nucleosome the DNA makes two turns round the core particle with H1 stabilising the entry and exit point. The linker DNA joining the nucleosomes is between 20 and 100bp. The entire "string of beads" assembly has a fibre diameter of about 10nm.

It was found that in the presence of divalent cations, these fibres would contract and form a fibre of 25nm. Thoma et al (1979) investigated this phenomenon using electron microscopy, and formulated a model for the 25nm fibre in which the 10nm fibre coiled into a solenoid through the interaction of neighbouring nucleosomes. This model has been convincingly validated by the X-ray diffraction studies of Langmore and Paulson (1983).

The above two levels of packaging lead to a 40-fold contraction in the length of the DNA duplex (Chambon, 1978), but another 1000-fold contraction is required to pack this DNA into mitotic chromosomes (Mullinger and Johnson, 1980). This "higher order folding" brings the chromosome as a whole into view.

1.2 HIGHER ORDER FOLDING OF CHROMATIN

Until recently the sole means of investigating the folding of chromatin within chromosomes was by electron microscopy, and many models have been proposed, which in retrospect, appear to be based on very uncertain observations. There were two main

schools of thought. One, typified by the folded fibre model of DuPraw (see DuPraw, 1970) envisaged the chromatin fibre to fold back and forth upon itself in a manner akin to the folding of globular proteins. The other view of chromosome organisation involved a very ordered hierarchy of helical foldings. This was probably provoked by the fact that under certain conditions isolated chromosomes adopted a coiled chromatid appearance (Ohnuki, 1968), although there is no evidence to suggest that this is more than an artefact. This model is typified by that of Bak (Bak et al and Zeuthen, 1978), who observed 0.4μm fibres in fragmented metaphase chromosome preparations, which were called "unit fibres". These were proposed to be formed from the cylindrical coiling of 25nm chromatin fibres, and these unit fibres would then spiralise to give the final chromatid.

The use of biochemical approaches together with more refined electron microscopic techniques has led us to a new understanding of chromosome organisation which is reviewed below. The chromatin fibre is envisaged as being organised into many independent looped domains, anchored to the chromatid axis at mitosis and the nuclear matrix at interphase.

1.3 LOOPED CHROMATIN DOMAINS IN INTERPHASE NUCLEI

The first suggestion of looped chromatin organisation in interphase came from sedimentation studies with "nucleoids"(Cook and Brazell, 1975). These were cells which were directly lysed by detergent into a high salt concentration. In such structures

the DNA is completely dehistonised, and therefore in an extended duplex configuration. In experiments with nucleoids from mouse cells (Ide et al, 1975), HeLa cells (Cook and Brazell, 1975), Drosophila cells (Benyajati and Worcel, 1976) or Saccharomyces (Pinon and Salts, 1977) common sedimentation properties in sucrose gradients were found. First the sedimentation rate exhibited a biphasic response to ethidium bromide intercalation, indicating it was behaving as circular supercoiled DNA. X-irradiation abolished this response, and target size analysis was used by Cook and Brazell (1975) to show that the DNA was organised as many independent supercoiled domains of 80-100,000b.p..

This phenomenon was also observed by fluorescence microscopy by Vogelstein et al (1980) who followed the change of the diameter of 3T3 cell nucleoids with varying ethidium concentration. At the ethidium concentration where the DNA was completely relaxed, the nucleoid diameter was used to estimate the loop length, and a value of 84-96000b.p. was obtained.

Another approach which yielded a similar result came from Zachau and co-workers (Igo-Kemenes and Zachau, 1978), who isolated nuclei from rat liver and subjected them to varying degrees of nuclease treatment. By relating the time course of digestion to the maximum sized fragments obtained, they proposed that the chromatin fibre was organised in the form of 30-70,000b.p. domains.

The looped organisation of the chromatin fibre seems

therefore to be well established in interphase nuclei. The anchorages which must constrain these loops are discussed later.

1.4 LOOPED DOMAINS OF CHROMATIN IN METAPHASE CHROMOSOMES

In 1977 Laemlli and co-workers (Adolph et al, 1977; Paulson and Laemmli, 1977) showed that metaphase chromosomes, isolated from HeLa cells and dehistonised with either 2M NaCl, or dextran sulphate with heparin, resembled interphase nucleoids (eg. Cook and Brazell, 1975), in that they exhibited low sedimentation rates in sucrose gradients and a halo of DNA when stained with DNA fluorochromes. When such dehistonised chromosomes were surface spread for electron microscopy, the halo could be resolved into individual fibres which appeared to take the form of loops anchored to a structure axial to the chromatid axes which was named the "scaffold". Contour measurement on such loops indicated a size of 45-95,000 b.p..

Mullinger and Johnson (1980) prepared metaphase nucleoids, in which metaphase cells were lysed directly into high salt solution and spread for electron microscopy. In this case DNA integrity was maintained, and the loops which were observed radiating from the chromatid axes were supercoiled. Again, loops were estimated to be of the order of 50-100,000 b.p.(20-40μm) and in chromosomes which were very contracted it was possible to estimate 600-1000 loops per chromatid, which fitted well with the known DNA content of human chromatids.

Other evidence for the looped organisation of mitotic chromosomes came from electron microscopy on thin sections of isolated HeLa cell metaphase chromosomes. Marsden and Laemmli (1979) showed that when such chromosomes were sectioned transversely, radial loops of the 25nm chromatin fibre could be seen, forming a "star" like arrangement. If the chromosomes were pretreated with EDTA (the chelation of Mg^{++} causes the 25nm fibre to uncoil giving a 10nm beads on string fibre, Thoma et al 1979) the pattern was the same but the loops were longer and were formed from the 10nm chromatin fibre. Calculation of the fully extended length of such loops was in close agreement with those obtained with spread dehistonised chromosomes (80,000 b.p.).

More recently, scanning electron microscopy studies of the surface structure of isolated metaphase chromosomes showed protruberances which were equated with the ends of chromatin loops (Adolph and Kreisman, 1983).

Thus, the higher order folding of chromatin throughout the cell cycle appears to involve the folding of the 25nm fibre into loops. The next question is to determine the nature of the structure which anchors these loops, and I will first deal with what is known about this for metaphase chromosomes.

1.5 SCAFFOLDS AND CORES IN MITOTIC CHROMOSOMES.

As the chromatin loops of metaphase nucleoids were shown to retain supercoiling by Mullinger and Johnson (1980), there must be some form of rigid anchorage at the chromatid axes. Experiments carried out by Laemmli and co-workers on dehistonised metaphase HeLa cell chromosomes showed that the integrity of such structures was destroyed by proteases, but not RNase (Adolph et al, 1977). When the dehistonised chromosomes were subjected to nuclease digestion, and the resultant "scaffold" viewed by electron microscopy, it was found to retain basic chromosome morphology (Laemmli et al, 1978). Thus it appeared that the scaffold represented a proteinaceous "backbone" within the metaphase chromosome. Electron microscopy of dehistonised chromosomes which had been surface spread (Paulson and Laemmli, 1977) did indeed show this rather coarse, fibrous scaffold running along the chromatid axes, surrounded by a halo of DNA loops apparently anchored to it. The concept of a proteinaceous core within each chromatid was lent further support by the discovery that cores could be resolved by light microscopy of chromosomes after silver staining (Howell and Hsu, 1979; Satya-Prakash et al,1980). Jeppesen et al (1978) demonstrated non-histone protein cores could be generated from Chinese hamster metaphase chromosomes which were dehistonised with 0.2N HCl and digested with DNase. These structures contained less than 0.5% chromosomal DNA, but retained characteristic chromosome morphology, and hence are analogous to the scaffold of Laemmli et al(1978).

The concept of a non-histone protein core actually had been first suggested in the early 1970's by Stubblefield (1973), who on the basis of electron microscope observations formulated a model in which DNA and protein formed a backbone fibre out of which loops of DNA (epichromatin) emerged. Dounce et al (1973) and Sobell (1973) also suggested the existence of a non-histone protein core. In both these models the core was invoked on mainly theoretical grounds in order to provide a mechanism for mitotic chromosome condensation (1.7) , however they both predicted loops of DNA emerging from the core as observed by Paulson and Laemmli (1977).

This model of chromosome organisation received much criticism, it being argued that the scaffold or core is an artefact caused by the collapse and aggregation of regions of high chromatin concentration during dehistonisation and electron-microscope spreading (Okada and Comings, 1980; Hadlaczky et al, 1981 and Burkholder, 1983). While such studies have shown that a certain amount of chromatin aggregation may occur in the preparation of cores or scaffolds, particularly in surface spread preparations for electron microscopy, against this must be set the following points. First, cores consistently retain chromosome morphology. Second, this morphology can be obtained by three different dehistonisation procedures (dextran-sulphate/heparin and high salt, Laemmli et al, 1978; 0.2N HCl, Jeppesen et al,1978). Finally, the use of improved chromosome preparations and the analysis of the proteins found in cores shows a remarkably simple composition of non-histone proteins.

Both Gooderham and Jeppesen (1983) and Lewis and Laemmli (1982) have recently reported new mitotic chromosome isolation procedures which result in much "cleaner" preparations of chromosomes from Chinese hamster or HeLa cells. In conjunction with much gentler preparations of cores (for example the avoidance of pelleting chromosomes by Gooderham and Jeppesen, 1983), these workers have shown that cores have a very simple polypeptide composition, not to be expected if cores are taken to be artefacts of chromatin aggregation. Gooderham and Jeppesen (1983) found about ten main polypeptides, and interestingly, one of them appeared to be the intermediate filament protein vimentin. This suggested the possibility that the proteins involved in the cytoskeleton might also be involved in chromosome organisation. Lewis and Laemmli (1982) also produced cores with a simple protein composition. They argued that core structure was maintained by only two different polypeptides, and from the investigation of the effects of various chelating agents on core preparation, suggested that metalloprotein interactions, either through Cu^{++} or Ca^{++}, were involved. When these structures were viewed by electron microscopy, they were found to retain basic chromosome outline and were of the form of a "fibrous scaffolding" (Earnshaw and Laemmli, 1983). Interestingly, the remnants of the kinetochores were found in these scaffolds (see also Earnshaw et al, 1984).

Assuming that the looped DNA of mitotic chromosomes is organised by a non-histone protein core, Jeppesen and Bankier (1979) suggested that sequence specific DNA:core-protein

interactions may occur at the base of the loops. They isolated the small amount of protected core DNA and found a slight enrichment in repetitive sequences from observations on reassociation kinetics. However, similar results were obtained by Tien-Kuo (1982), who questioned whether the enrichment was significant. Razin et al (1978) found an enrichment of satellite sequences in chromosome core protected DNA, however this group prepared cores by restriction enzyme nucleolysis of dehistonised chromosomes, enabling large repeated sequences without cleavage sites to remain attached to the cores. Consequently, it is not certain whether specific DNA:protein interactions do occur at the chromosome core, although Jeppesen and Bankier (1979) suggest that the sequences could be as short as 10bp and hence very difficult to detect by reassociation kinetics. Sequence analysis of chromosome core DNA currently in progress (P.Jeppesen, pers. comm.) should clarify this matter.

The following section investigates the relationship between the mitotic chromosome core and analogous structures found at interphase.

1.6 INTERPHASE CHROMOSOME ORGANISATION- NUCLEAR SUBSTRUCTURES

The cage and matrix

As with mitotic chromosomes, some form of anchorage must be present in the nucleus in order to maintain the looped chromatin

organisation as described above. Moreover, there is evidence for a discrete territorial organisation of each chromosome within the nucleus (Hens et al, 1983), again indicating a high degree of organisation within the nucleus.

When McCready et al (1980) produced nucleoids from HeLa cells (see 1.3), the DNA loops were found to be anchored to a structure resembling the nucleus, composed mainly of protein, which was termed the nuclear cage. Vogelstein et al (1980) demonstrated a similar structure in 3T3 cell nucleoids. When nuclei are prepared and then extracted with high salt and nuclease digested, a residual nuclear structure called the matrix is obtained (Agutter and Richardson, 1980; Kaufmann et al, 1981) which seems to be equivalent to the cage. This will be discussed further below, however the nucleoid and derived cage have been of great value in the search for the components involved in and the mechanism of anchorage of looped chromatin within the nucleus.

Three types of DNA association with the nuclear cage have been found.

1 Specific DNA sequences are associated with the cage: By subjecting nucleoids with intact DNA to varying degrees of nuclease digestion, a quantitative enrichment of DNA associated with the cage (i.e. close to the base of a loop) can be achieved, and these DNAs can be filter hybridised with specific probes. Using this technique, evidence has been obtained for the specific association of the alpha-globin gene in HeLa cells (Cook and

Brazell 1980), the ovalbumin gene in chicken oviduct cells (Robinson et al, 1982), and integrated SV40 in 3T3 cells (Nelkin et al, 1980) with the nuclear cage. Robinson et al (1982) suggested that cage attachment is a general feature of active genes, but as the loops of DNA attached to the cage are all of about 80,000 b.p., it seems hard to imagine active genes so evenly distributed. Two other discoveries about the DNA association with the cage described below may clarify this matter.

2 DNA replication is associated with the cage: Vogelstein et al,(1980) showed by pulse labelling and autoradiography of nucleoid preparations, that in 3T3 cells DNA synthesis occured at the nuclear cage. This was confirmed in HeLa cells by McCready et al (1980) who showed that newly synthesised DNA resists detachment from the nuclear cage.

3 Transcription is associated with the cage : Jackson et al (1981) showed that newly synthesised RNA is bound to the nuclear cage. This finding could now explain the apparent associations of active genes with the cage, and a model for these different interactions could be formulated (McCready et al, 1982). The model recognised three kinds of DNA:cage interaction. First, the structural loops, of the type originally noted in nucleoids. Within these loops, temporary loops formed by the attachment of DNA to either transcription or replication complexes on the nuclear cage.

These results showed the nuclear cage to be a complex structure upon which the looped chromatin domains are anchored. Another approach to understanding this organisation was to

examine the protein composition of the nuclear matrix. The composition of the nuclear matrix has been found to be very sensitive to the isolation procedure used (Kaufmann et al, 1981), and it is evidently a complex structure associated with other activities apart from the organisation of chromatin. These include providing hormone receptor sites and hnRNA processing. However, there are protein components of the nuclear matrix which are very similar to those of the chromosome core, and the role of these in the organisation of nuclear chromatin is of great interest.

Adolph (1980) showed similarities in the electrophoretic profiles of the chromosome core and nuclear matrix proteins. This indicated the possibility that at least part of a structural component of the nucleus remained as part of a chromosome substructure during mitosis.

More recently, Lebkowski and Laemmli (1982a, 1982b) prepared nuclear matrices from HeLa cells and showed that they had common proteins and behaved in a similar manner to metaphase chromosome scaffolds (Lewis and Laemmli, 1982). In particular, one level of organisation in these structures was based on a metalloprotein. They suggested that nuclear chromatin is organised at two levels. During interphase the nuclear lamina was proposed to provide one level of organisation which dissolves during mitosis leaving the scaffold type proteins to organise chromosome condensation, possibly via metalloprotein interactions.

1.7 CHROMOSOMES AND THE CELL CYCLE

The chromosome undergoes a major gross structure change for the duration of mitosis, and a major problem is to relate the metaphase and interphase models of chromosome organisation described above. Mullinger and Johnson (1980) have shown that the fundamental organisation through the cell cycle is probably constant, as G_1 cells can give prematurely condensed chromosomes with axial cores when dehistonised.

It has long been thought that during mitosis there is a low level of chromosomal protein sulphydryl groups (Bostock and Sumner, 1978), and this led to the suggestion of Dounce *et al* (1973) and Sobell (1973) that chromosome condensation is driven by the formation of disulphide bonds between chromosome core proteins which are dispersed at interphase. While this model is attractive, it has recently been pointed out by Sumner (1983) that the apparent increase in disulphides during mitosis may be an artefact of preparation. There are many other changes in chromosomal protein modification upon entry into mitosis. The best known is the phosphorylation of histone H1 (Bostock and Sumner, 1978), but probably more significant is the observation of Detke and Keller (1982) who showed an as yet unidentified covalent modification to two proteins common to the nuclear matrix and chromosome scaffold upon entry into mitosis.

Recently, there have been suggestions that the familiar cytoskeletal proteins may also be involved in organising nuclear structure and effecting chromosome condensation. Armbruster *et*

al (1982) showed the presence of both actin and tubulin within metaphase chromosomes and interphase nuclei by immuno-electronmicroscopy. More significantly, Gooderham and Jeppesen (1983) demonstrated the intermediate filament protein vimentin amongst the proteins of the chromosome core. Finally, the observations of Capco and Penman (1983), using long-focal-length electron microscopy on detergent-extracted fibroblasts, have shown that at at mitosis, the chromosomes are associated with a complex filamentous system, connected to both chromosomes and what is conventionally regarded as the cytoskeleton. This result suggests that part of chromosome organisation may be determined by systems that extend throughout the cell, however we are still very ignorant about these processes.

1.8 THE OBJECTIVES OF THIS THESIS

The aim of this work was to examine the proteins of the chromosome core, to characterise them and look for homologies with nuclear substructure. In the search for the components of chromosome substructure, a major problem is the relationship between the isolated "components" (protein, DNA), the isolated substructure (e.g. the scaffold), and chromosome structure in vivo. For example, concentrating on metaphase chromosomes, the currently favoured core model is derived from work on isolated chromosome cores, and the only conclusive way of showing that these structures are not artefacts will be to show a scaffold type distribution of core proteins within native chromosomes. This was the limitation of the electrophoretic characterisation

of core proteins which is described in Chapter 2 and from which the approach to the study of chromosome structure using monoclonal antibodies was developed.

Before this study, the use of antibodies to study chromosome structure was largely limited to the work of Elgin's group on polytene chromosomes(e.g. Silver and Elgin 1977,1978) who raised antisera to proteins associated with specific bands. This approach was elegantly followed up by Saumweber et al (1980) who systematically extracted Drosophila chromosomal proteins and produced a library of hybridomas to them. Again, the antibodies were used to stain polytene chromosomes and could then be associated with specific proteins. Apart from this type of work, Bustin et al (1976) has raised antisera to the histones and successfully used these to stain human metaphase chromosomes, and Turner (1982) has produced a monoclonal antibody to histone 2b which has been used to observe its distribution and accessibility in chromosomes. However, there have been no reports of studies particularly aimed at the structural non-histone chromosomal proteins using monoclonal antibodies.

Although much work remains before the organisation of chromosomes will be understood properly, I hope this thesis will demonstrate the enormous potential of the antibody approach in dealing with such problems.

Chapter 2 CHROMOSOME PREPARATIONS, GEL ANALYSIS AND QUESTIONS RAISED.

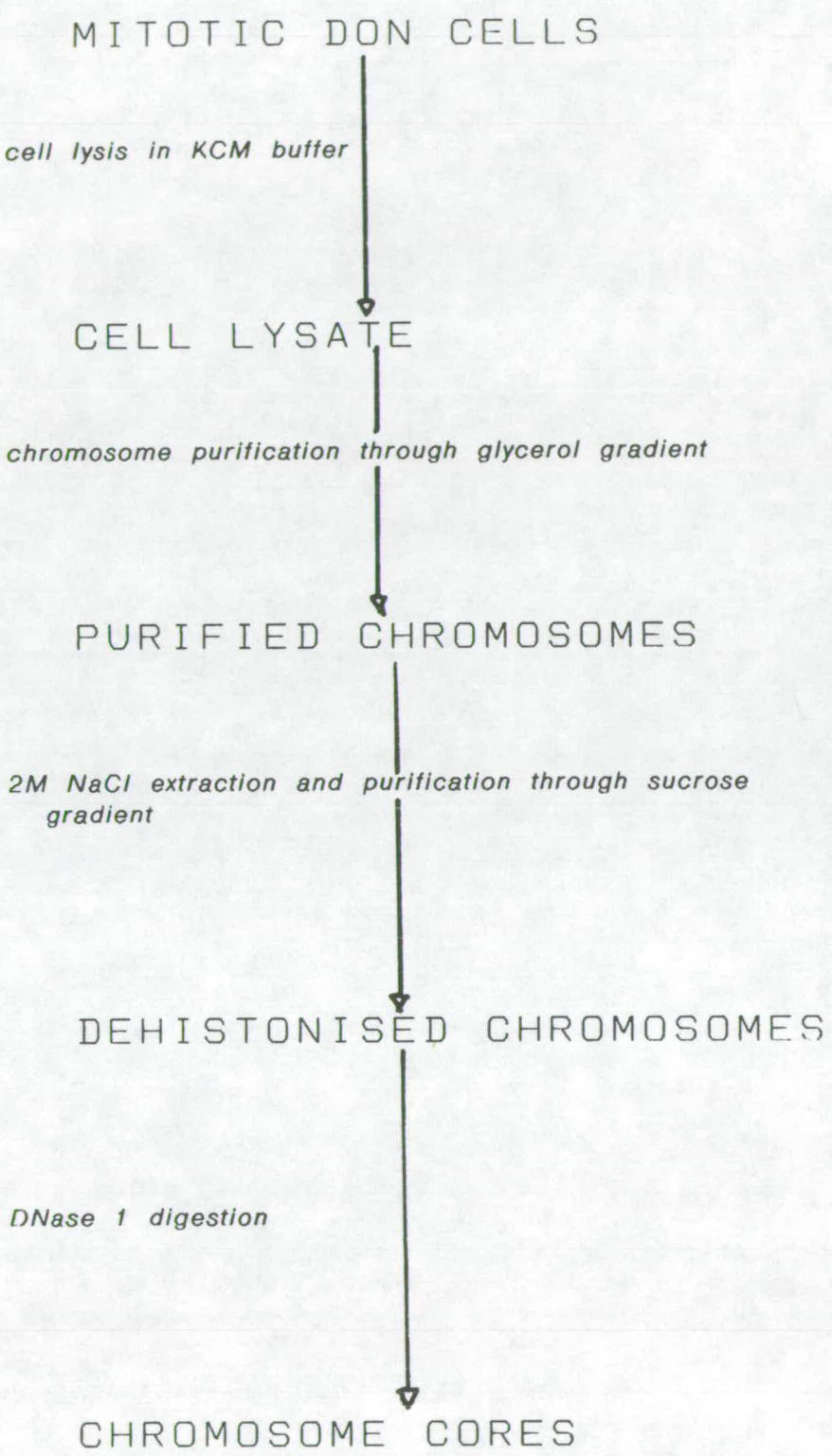
In the Introduction the evidence for a non-histone protein core in the mitotic chromosome along with a related structure in the interphase nucleus was described. In this section I will briefly discuss the methods used for the preparation of chromosomes, nuclei and cores; the raw materials of this work; and consider some experiments carried out to determine their protein composition.

2.1 THE ISOLATION OF METAPHASE CHROMOSOMES AND CHROMOSOME CORES

The basic rationale for the preparation of chromosome cores has been discussed in the Introduction (Ch.1.5). Two methods have been used in this work, that described by Gooderham and Jeppesen (1983) for the majority of experiments, and in some early experiments that of Jeppesen et al (1978). The preparation procedure of Gooderham and Jeppesen (1983), known as the "KCM" method, involves the isolation of metaphase chromosomes from mitotic cells under gentle conditions in a quasi-physiological isolation medium based on potassium chloride. This is followed by purification of the chromosomes by glycerol -gradient centrifugation, dehistonisation of the chromosomes in a sucrose/salt gradient and finally DNase 1 digestion to give chromosome cores. The procedure is shown schematically in diag.2.1.

The earlier isolation procedure for chromosome cores was described by Jeppesen et al (1978). In this case chromosomes are

Diag. 2.1 Outline of chromosome core preparation.



liberated from mitotic cells into a medium based on the buffer CAPS (cyclohexylaminopropane sulfonic acid) at pH 10 and the stabilising agent hexylene glycol. These chromosomes are dehistonised by incubation with 0.2N HCl and digested with DNase 1 to give cores, and will be referred to as CAPS pH 10 chromosomes and cores. This method gives less "clean" core preparations than the KCM technique, probably because hexylene glycol can promote the aggregation of chromosome components during dehistonisation (Mullinger and Johnson, 1980).

2.2 THE ISOLATION OF NUCLEI AND NUCLEAR CORES

Nuclei were prepared in KCM or CAPS pH10 buffer systems, and nuclear cores were prepared by the dehistonisation and nuclease treatment of nuclei in similar procedures to the preparation of chromosome cores. This preparation is similar to that of the nuclear matrix (Kaufmann et al, 1981); however as minor differences in procedure can lead to completely different sub-nuclear structures, a separate nomenclature seems warranted.

2.3 GEL ANALYSIS OF CHROMOSOME AND NUCLEAR CORE PROTEINS.

Gooderham and Jeppesen (1983) presented a thorough gel electrophoretic analysis of the chromosome core, showing a reproducible simple subset of chromosomal proteins. The experiments described here were designed to extend these observations to the polypeptide composition of the nuclear core and compare this to the chromosome core.

Fig. 2.1 shows the gel profile of chromosome, chromosome core, nuclear and nuclear core proteins prepared by the CAPS pH 10 method. Two points are clear- first that the core proteins represent a small subset of total chromosomal and nuclear proteins, mainly in the 55-70,000 M_r range, and secondly that most polypeptides in this range are common to both the chromosome core and nuclear core. To examine this question further, chromosome and nuclear core proteins were analysed by 2-dimensional gel electrophoresis and the results are shown in fig.2.2.

The 2-dimensional gels of chromosome and nuclear core proteins show that the major polypeptides of both structures (about 10) are shared. This result was a strong indication that the chromosome core proteins were more than just aggregation artefacts as suggested by Okada and Comings (1980) and moreover, that they played a major structural role in the chromosome throughout the cell cycle.

On the other hand, the 2-dimensional gel results could be explained in terms of a common set of cytoplasmic contaminants in chromosome and nuclear cores, and two observations suggested that this must be taken seriously. First, with the adoption of "KCM" chromosome and nuclei preparations, Gooderham and Jeppesen (1983) demonstrated that it was possible to obtain much simpler gel patterns from both chromosomes and cores. Second, examination of the CAPS pH 10 preparations of chromosomes and cores showed they were contaminated with nuclear lamina proteins. This conclusion was first suggested by an electron microscopic

Figure 2.1 SDS-PAGE separation of chromosome and nuclear core polypeptides (CAPS preps)

lane 1: Total Don chromosome proteins

lanes 2+3: Don nuclear core proteins

lanes 4-6: Don chromosome core proteins

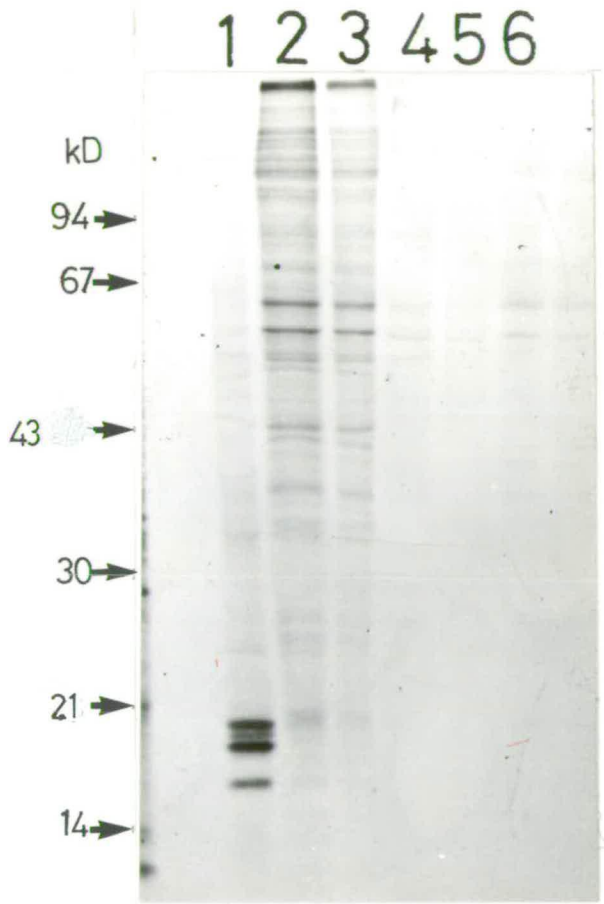

1 2 3 4 5 6
kD
94
67
43
30
21
14

Figure 2.2 2-D electrophoresis of chromosome core and nuclear core proteins

2-dimensional gels were run according to the method of O'Farrell (1977).

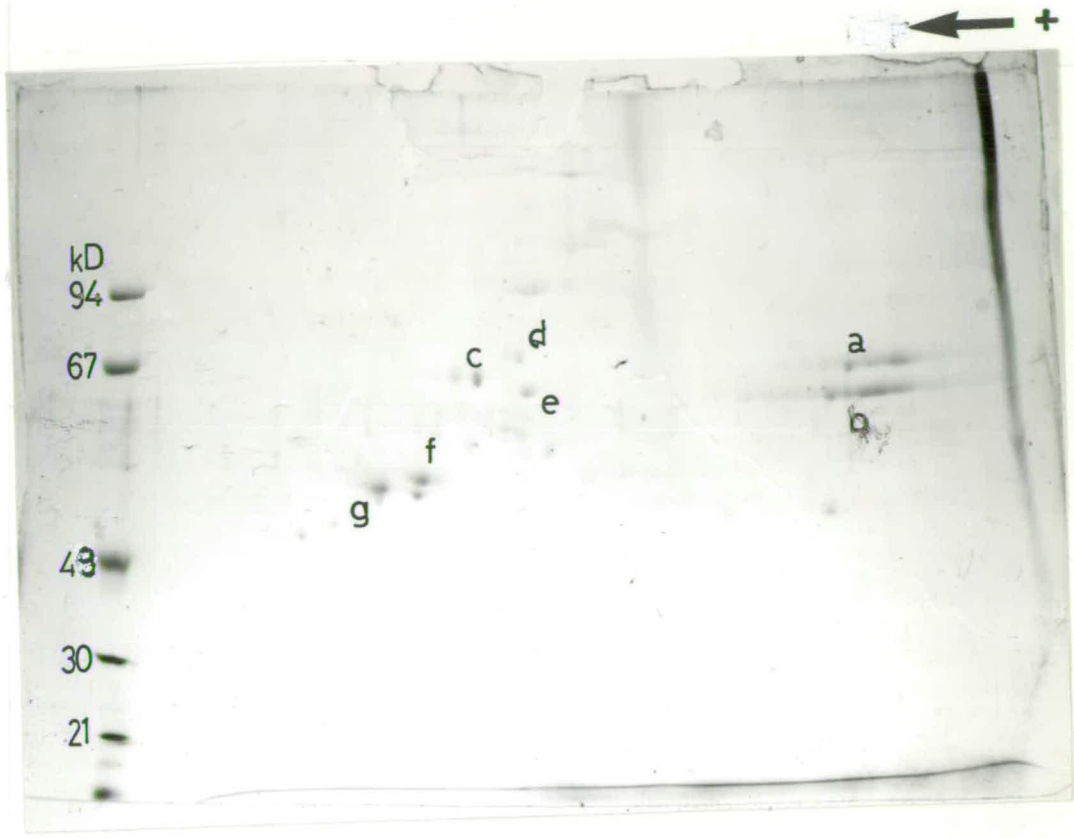
Gel a) Don cell nuclear cores isolated by the CAPS pH10 procedure

Gel b) Don chromosome cores isolated by the CAPS pH10 procedure

Gel (a) also contains a lane of molecular weight markers.

The most evident common polypeptides are marked.

a



b

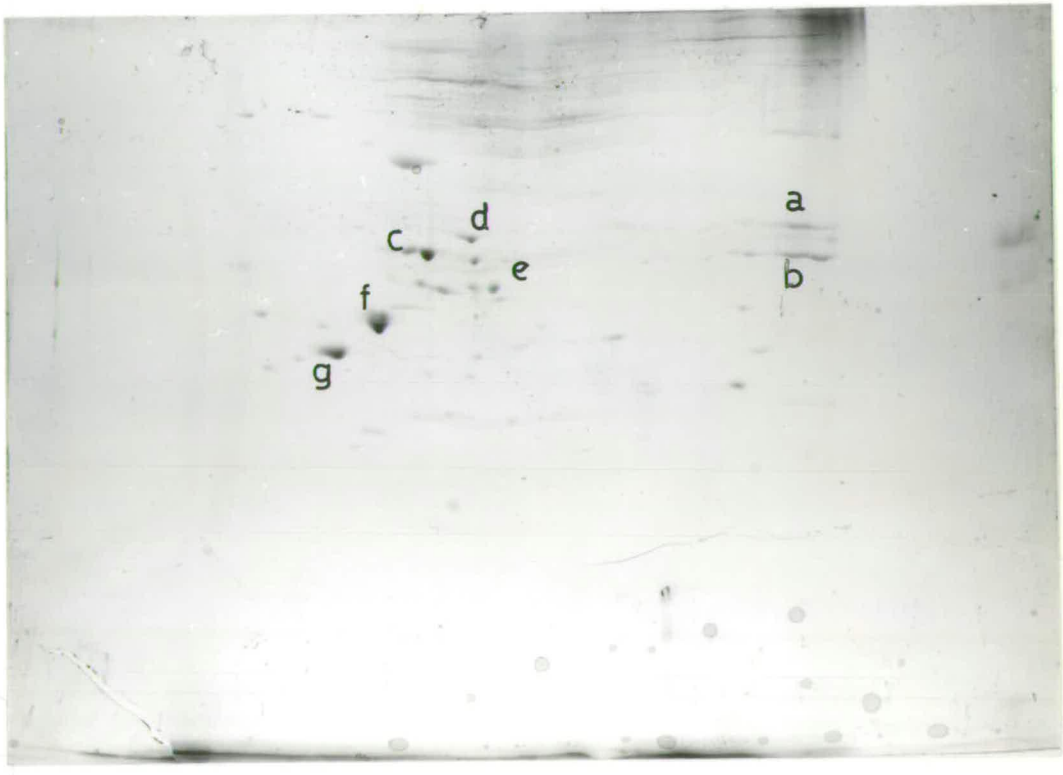


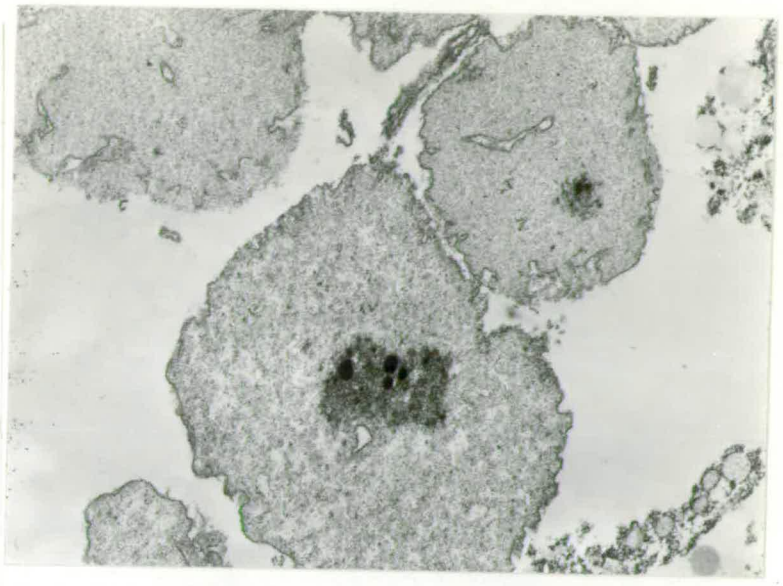
Figure 2.3 Electron microscopy of nuclear cores

a) Isolated Don cell nuclei: Nuclei were fixed in 2.5% glutaraldehyde in 0.1M cacodylate pH 7.5 and embedded in araldite. Thin sections were stained with uranyl acetate (saturated solution in 50% EtOH) followed by Reynolds lead citrate (Reynolds, 1963). X 6,300

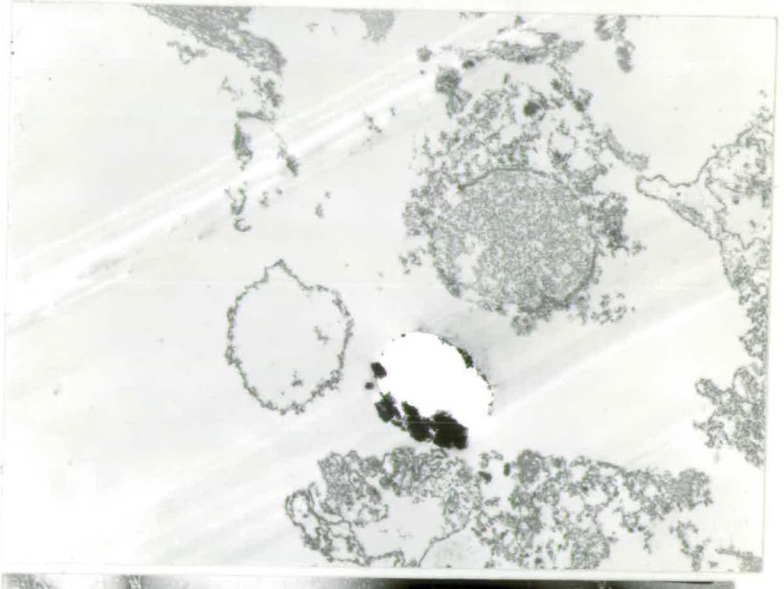
b) Isolated Don cell nuclear cores prepared as above. Although the morphology was very variable, the residual structures were distinguished from nuclei by their small size and loss of internal material. One residual structure in this micrograph is a completely empty shell, presumably consisting of nuclear lamina. X 6,300

c) Isolated Don nuclear cores settled onto carbon coated electron-microscope grids and negatively stained using 3.0% Na-phosphotungstate pH6.6. Showing detail of nuclear lamina-pore complex. X 11,000.

a



b



c

Figure 2.4 SDS-PAGE of KCM chromosome and nuclear core preparations

Electrophoresis was carried out as described in methods using a 15% acrylamide separating gel.

lane 1: Marker proteins (Pharmacia): Phosphorylase b-94kD, bovine serum albumin-67kD, ovalbumin-43kD, carbonic anhydrase-30kD, soybean trypsin inhibitor-20kD& alpha lactalbumin-14.4kD.

lane 2: Myosin

lane 3: Don cell nuclei

lane 4: """""""""""""""""

lane 5: Don cell nuclear cores

lane 6: """""""""""""""""""""""""

lane 7: Don cell nuclei

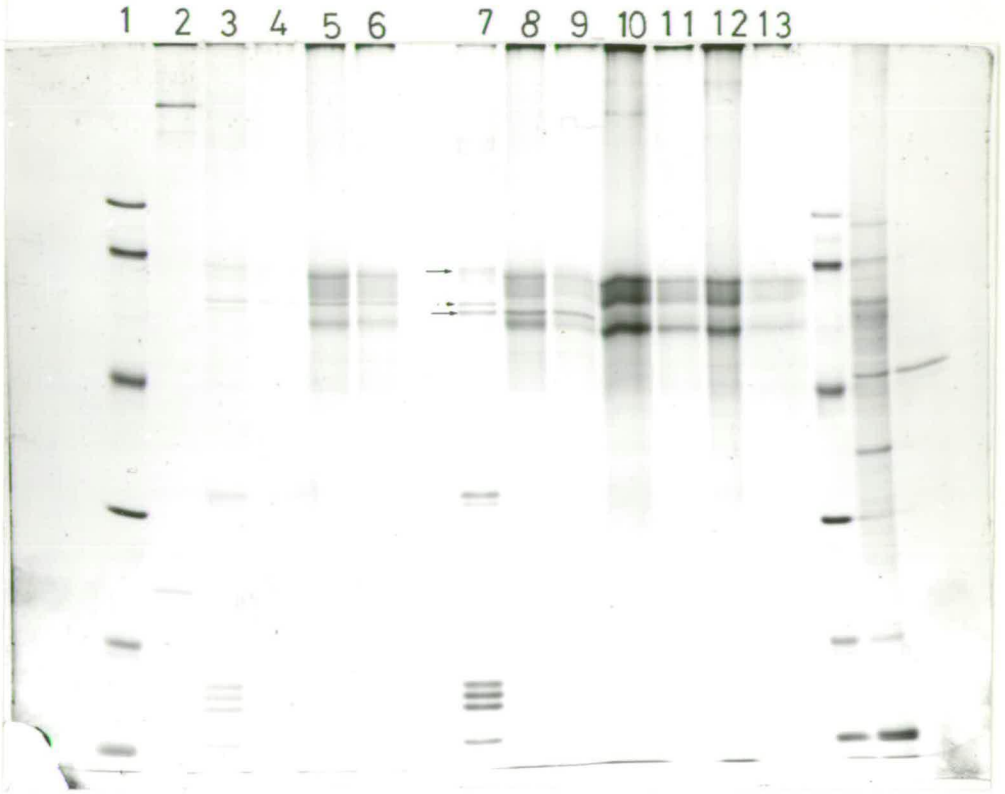
lane 8: Don cell nuclear cores

lane 9: """""""""""""""""""""""""

lane 10:Don cell metaphase chromosome cores

lane 11: CHO cell nuclear cores- prepared in exactly the same manner as Don cells.

lane 12+13:"""


1 2 3 4 5 6 7 8 9 10 11 12 13

analysis of CAPS pH10 nuclear cores, as in figure 2.3. Figures 2.3a+b are thin sections of nuclei and nuclear cores respectively, and show that the nuclear core morphology is very similar to the nuclear matrix as reported elsewhere (Kaufmann et al, 1981). Fig. 2.3c shows the nuclear cores prepared as negatively stained whole mounts, and from this it is clear that the nuclear core is still bounded by the nuclear lamina-pore complex (compare with electron micrographs in Krohne et al, 1978). The polypeptides of the nuclear lamina have been well characterised and 2-dimensional gel maps have been published by Kaufmann et al (1983). Comparing the gels in the Kaufmann paper with those in fig.2.2 it was clear that spots a,b and c represent the lamina polypeptides A, B and C in both chromosome and nuclear core preparations. While the lamina proteins have been implicated in the organisation of nuclear chromatin (Lebkowski and Laemmli, 1982b), it has been shown that they are disassembled and dispersed in the cytoplasm at mitosis (Gerace and Blobel, 1980). Their presence in chromosome cores prepared by the CAPS pH 10 method is best explained on the basis of the observations of Jost and Johnson (1981) who showed that when mitotic cells were colcemid or cold treated, the lamina polypeptides condensed over the surface of the chromosome. Both these treatments are used in the preparation of mitotic chromosomes, colcemid being essential for a good yield of mitotic cells, and cold treatment necessary to disrupt the mitotic spindle. This result demonstrated that cytoplasmic contamination was the cause of three of the common polypeptides in CAPS pH10 chromosome and nuclear cores. Because

of the uncertainties of determining whether a particular protein in the chromosome core was a contaminant or not, the comparison of nuclear core and chromosome core polypeptides was limited. This led to the strategy of raising antibodies to the proteins of the chromosome core, with the aim of demonstrating the distribution of such proteins within the intact chromosome, and this is the subject matter of the chapters to follow. However, from a recent study, Gooderham and Jeppesen (1983) have shown that chromosome core proteins prepared by the KCM procedure are free of nuclear lamins and probably represent chromosome cores with minimal cytoplasmic contamination, although the presence of vimentin should be noted. While much work is required in terms of protein characterisation with these preparations, in figure 2.4 some gel data is shown. There is still an apparently high degree of homology between the nuclear and chromosome core polypeptides. This can be best observed in lanes 9, 10 and 11. Lane 10, containing Don cell chromosome cores is very similar to the adjacent lanes containing Don and CHO cell nuclear core proteins. It should also be pointed out that the core proteins are very minor species in terms of concentration within total nuclear proteins. They are not observed in the lane containing total nuclear protein (lane 7), the three prominent bands in this case corresponding to the nuclear lamina proteins (arrowed).

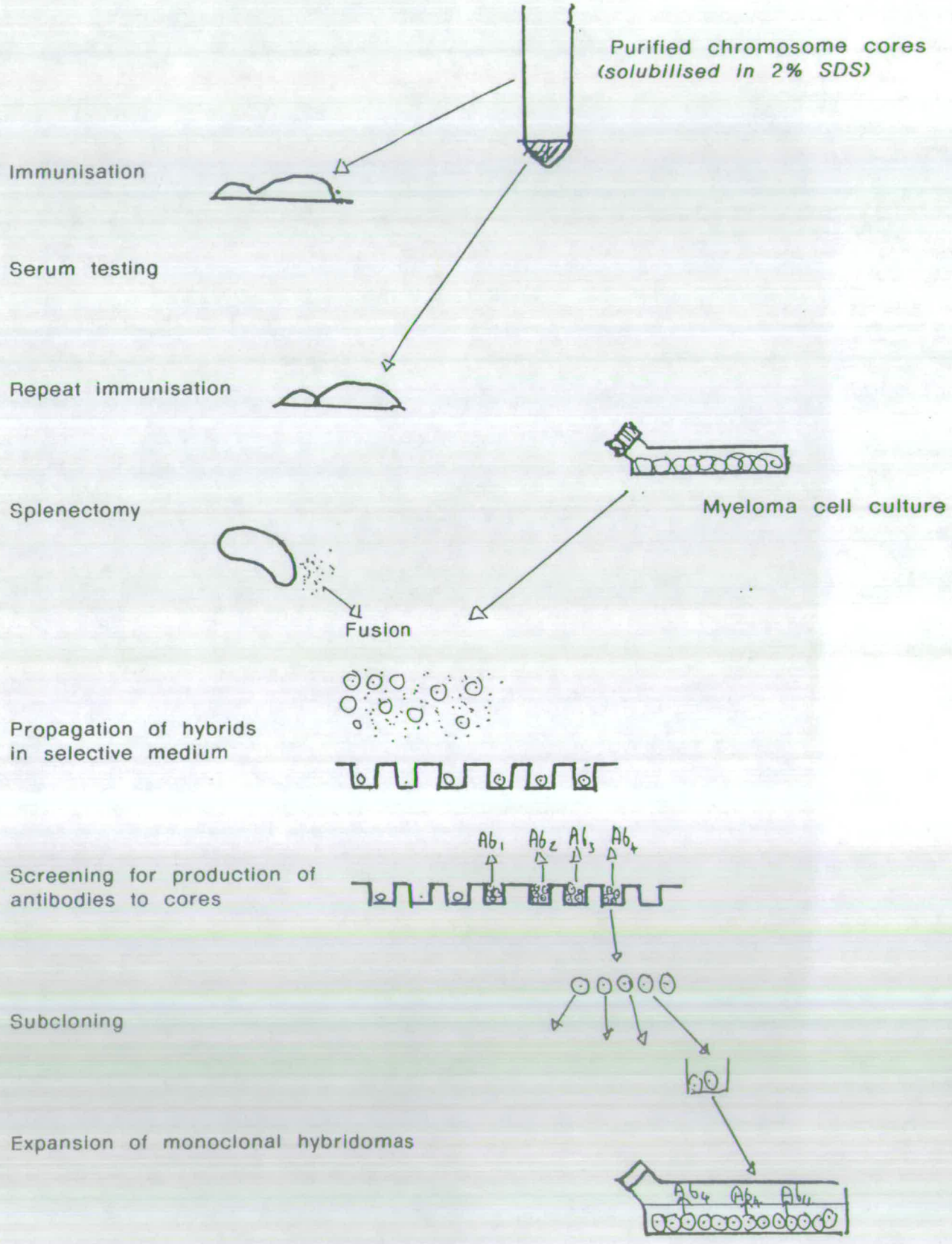
Chapter 3 HYBRIDOMA GENERATION

3.1 Introduction

From the results shown in the previous chapter, it can be seen that chromosome cores are composed of a rather simple subset of chromosomal proteins. However, many questions arise from this observation. For example, are these proteins restricted to the core or are they distributed throughout the chromosome? This is related to the question of whether chromosome cores, like the scaffold described by Laemmli and co-workers (Lewis and Laemmli, 1982; Paulson and Laemmli, 1977), are substructures within the metaphase chromosome *in vivo* or are generated by aggregation phenomena during the isolation procedure as suggested by Okada and Comings(1980) and Hadzlacky *et al* (1981). Although the biochemical characterisations by Gooderham and Jeppesen (1983) and Lewis and Laemmli (1982) argue against this, there is still much debate about the reality of the core type model for chromosome organisation. Another question concerns the fate of the chromosome core in the interphase nucleus, and this leads on to the relationship of nuclear cores to chromosome cores, as they appear to have a similar polypeptide composition(Ch.2.3).

With the advent of hybridoma technology, developed by Kohler and Milstein(1975), it became possible to generate monospecific antibodies to components of heterogeneous antigens,and thus opened up an avenue for viewing cellular ultrastructure at

Diag. 3.1 The preparation of monoclonal antibodies against chromosome cores–schematic summary



a molecular level. Such monoclonal antibodies seemed to be an ideal approach for gaining further insight into the nature and distribution of chromosome core proteins, and the remaining part of this thesis describes the generation and application of two such antibodies. In this chapter, the preparation of the antibodies is described. In essence, this involves the immunisation of mice with chromosome cores; the immortalisation of antibody producing spleen cells from the mice by fusion with a myeloma cell line; and a selection system to isolate hybrid cell or hybridoma producing a desired antibody: this is summarised in diag.3.1. The procedures will be dealt with in sequence in the following sections.

3.2 Immunisation

There are many approaches to immunisation (see Goding, 1980; Zola and Brooks, 1982), the main aim being to present the antigen in its most antigenic form, to expand the clones of progenitor spleen cells for the relevant antibody, and to ensure they are at the right stage of differentiation for successful fusion. The last two requirements have largely been met by empirical approaches, for little is known about the mechanisms of development of antibody producing B-cells. The development of *in vitro* immunisation procedures may allow a greater understanding of these processes and a more efficient method of producing desired monoclonal antibodies (Reading, 1982).

In the work described here, the immunogen was composed of isolated chromosome cores solubilised in a denaturing buffer containing SDS. There were two reasons for presenting chromosome cores in this manner. First, cores are highly insoluble, presumably being composed of hydrophobic proteins, and it was thought that some proteins would be inaccessible to the mouse immune system when complexed in such structures. Secondly, Dr.P.Jeppesen(pers. comm.) found no detectable antibody reacting with chromosome cores after immunising rabbits with intact cores. This suggested that cores are poor antigens and it was considered that denaturation might increase the antigenicity.

The immunisation program consisted of 3 injections at 12wk intervals, each of 10-50µg chromosome core proteins(fig.3.1)

3.3 Analysis of mouse serum antibodies after immunisation

After the second immunisation, the mouse serum was tested for antibodies to chromosome proteins by "dot-blotting"(see below, 3.4) and "western-blotting"(see 4.1). This demonstrated the effectiveness of the immunisations and allowed the most strongly responding mice to be selected for the third immunisation and subsequent fusion. Fig.3.2a shows a dot blot of sera from five immunised mice and one non-immunised mouse versus chromosome core proteins. The control non-immune serum showed little reactivity but the sera from all the immunised mice clearly reacted.

Figure 3.1: SDS-PAGE analysis of the chromosome core immunogen

Chromosome cores were taken up in 2%(w/v) SDS, 40mM DTT, 10mM Tris-HCl pH7.5 and heated at 100° for 5min. An aliquot was taken for electrophoresis as shown:

Lane A= marker proteins (Pharmacia-see fig 2.4 for details).

Lane B= tubulin and actin markers

Lane C= core protein immunogen

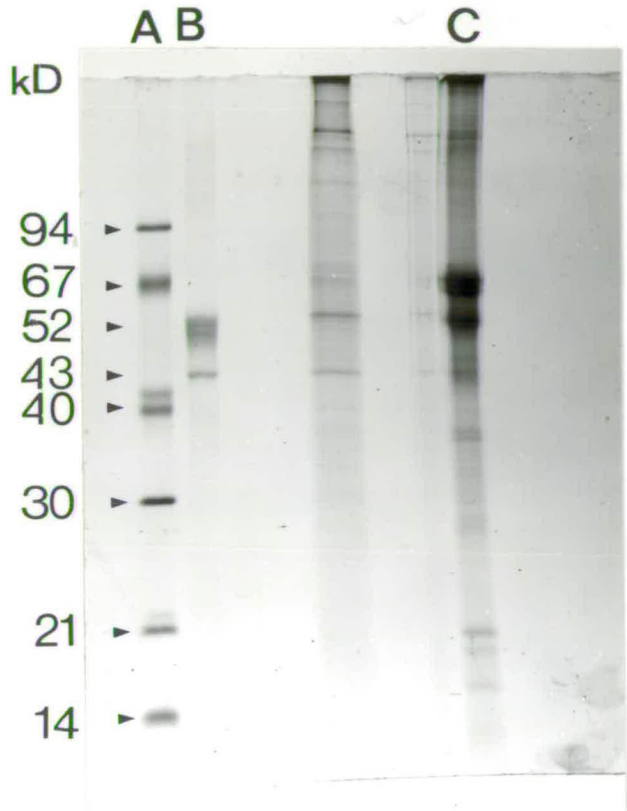

A B
C
kD
94
67
52
43
40
30
21
14

Figure 3.2: Detection of serum antibodies to chromosome core proteins in immunised mice

a) Dot Blotting

Serum from the immunised mice and a non-immunised control were applied to preabsorbed dots of antigen bound to the nitrocellulose filter. The antigens were loaded as follows: Rows A+B= control (Pharmacia low mol. wt. marker proteins); rows C+D= chromosome cores; rows E+F= control (no protein loaded). The sera were loaded as marked (1-6). Serum (1) was control non-immune serum.

b) Western Blotting

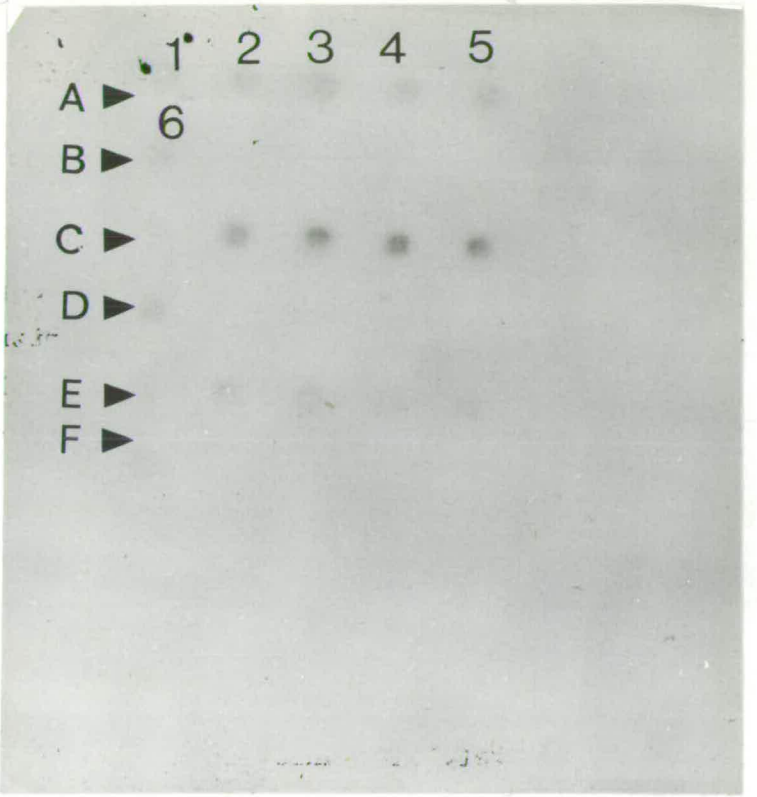
Total chromosomal and chromosome core proteins were electrophoresed in a 15% acrylamide SDS-PAGE gel. The bands were transferred to nitrocellulose electrophoretically, probed with mouse serum, and then with I^{125}-labelled second antibody.

A-D: Coomasie blue stained gel replica; A= markers (Pharmacia)
B= Chromosome core protein
C= Total chromosomal protein
D= Chromosome core protein

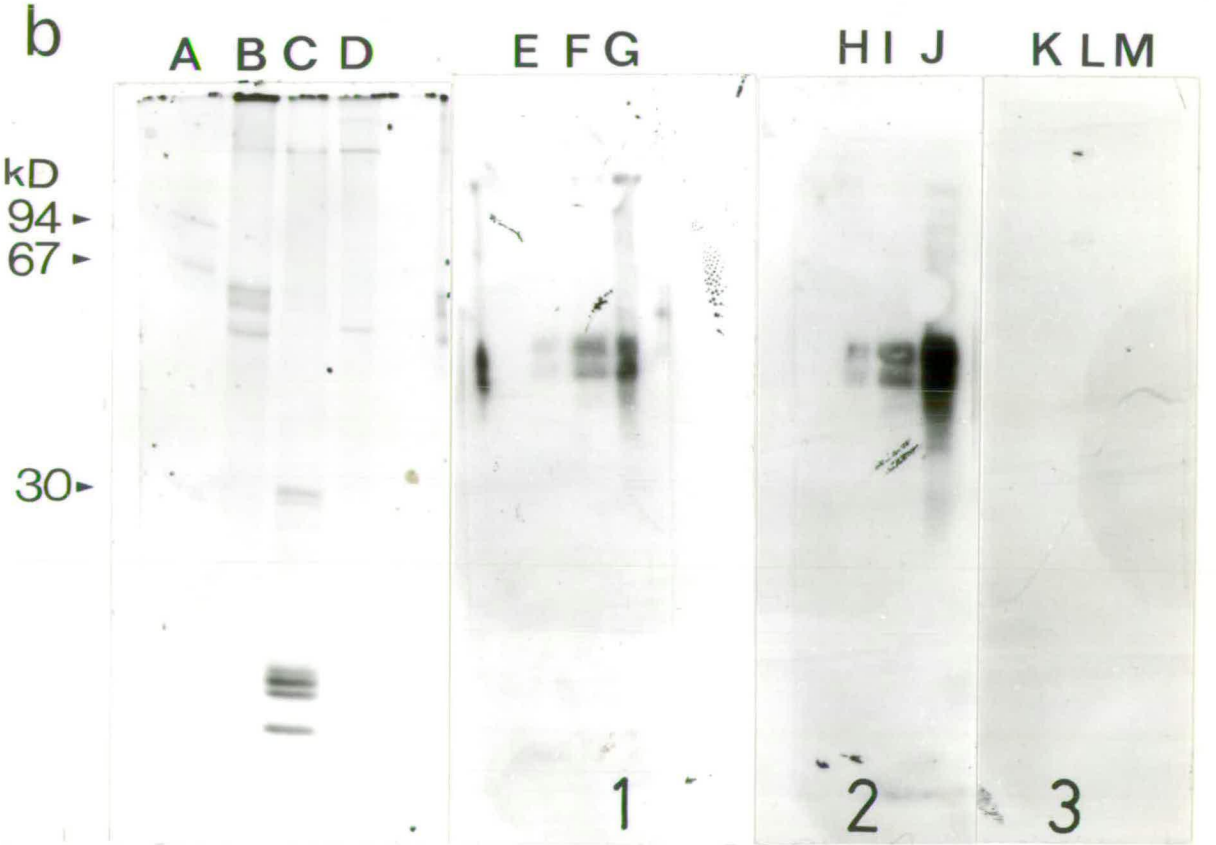
E-M: Autoradiograms of probed filters; E, H, K as in B
F, I, L as in C
G, J, M as in D

Filter 3 was probed with control non-immune serum, filters 2 and 4 were probed with sera from two of the immunised mice.

a



b



This result was confirmed by western-blotting as shown in fig.3.2b. This shows the presence of antibodies against the main set of chromosome core polypeptides in sera from two immunised mice but not in the control non-immune serum. Sera from the other immunised mice gave a similar reaction, but the reaction illustrated was the strongest and these two mice were selected for further immunisation.

3.4 Fusion and the isolation of hybridomas.

In order to produce a hybridoma, an antibody producing spleen cell must be fused with a myeloma cell line. Fusion of the two types of cell in close contact can be brought about by Sendai virus, lysolecithin, DMSO or polyethyleneglycol(PEG). At the subsequent division of the resulting heterokaryon, the two nuclei fuse to produce a hybrid cell, or hybridoma. This has the characteristics of an immortalised cell line, derived from the myeloma parent,and also the capacity to produce the spleen cell coded immunoglobulin. The basis of this procedure has been extensively reviewed by Goding(1980) and Zola and Brooks(1983).

The fusion technique used in this work was based on the method of Claflin and Williams (1978). PEG was used as the fusion agent and the myeloma cell line was P3 NS1/1-Ag4-1(NS1). The fusion mixture was plated over 576 culture wells(0.2ml) in order to introduce a degree of cloning; for if two or more hybridomas were plated in the same well, not only would one

overgrow the other, but also screening would be made rather difficult. Fungal contamination led to the loss of 50% of these and the remainder were scored for growth after 10 days. Culture supernatants from growing colonies of cells were screened by dot blotting (Sternberg and Jeppesen, 1983). This is a screening assay, developed over the course of this work, which offers considerable advantages in terms of ease, versatility and clarity of results, over the traditional radioassay carried out on microtitre plates (e.g. Turner, 1981). In dot-blotting, the solid phase to which the antigen is bound is a nitrocellulose filter. The high adsorbancy of nitrocellulose means that a wide variety of antigens can be bound, including intact chromosomes or chromosomal proteins solubilised in SDS-polyacrylamide-gel loading buffer (see 6.2.1). The antigen is bound to the filter as an array of small spots, and after blocking the filter to further protein binding, each antigen spot is overlayed with an appropriate culture supernatant. If an antibody specific to the antigen is present, it binds and is not removed during the subsequent washings. Finally, antigen spots with bound antibody are detected with a radioactive anti mouse-immunoglobulin "second-antibody". 9 cell lines out of a total of 60 were found to be producing antibodies against chromosomal proteins. Fig.3.3 shows the identification of two of these cell lines, 4G8 and 2C6. They were of interest because they reacted with chromosome core proteins as well as total chromosomal protein. The other 7 cell lines producing antibodies to chromosome protein were cryopreserved for future investigation and the above 2

antibodies were used in all the work to follow.

4G8 and 2C6 were subcloned at limiting dilution twice to ensure stability and monoclonality of the hybridomas. In the first subcloning they gave 100% and 90% positive subclones respectively, and in the second subcloning each hybrid gave 100% positive subclones(screening against core and total chromosomal proteins)

3.5 Antibody subclass determination

Antibody subclass was determined by double diffusion analysis(Ouchterlony and Nielsen,1975). The results, shown in fig 3.4 demonstrate that 2C6 is an IgM and 4G8 an IgG1. Light chains were not typed.

3.6 Summary of 2C6 and 4G8 specificities as determined by dot-blotting

Experiments of the type illustrated in fig.3.5 allowed some preliminary characterisation of monoclonal antibodies 2C6 and 4G8. In these, a modification of the "dot-blotting" assay was used in which a variety of different antigens were applied to a nitrocellulose filter, and the binding of each antibody to them was examined. Column A, containing gel electrophoresis marker proteins, acted as a control to determine a background level of antibody binding. The results are summarised in table 3.1.

Figure 3.3: Detection of antibodies against chromosome and chromosome core protein in the supernatants of hybridomas 4G8 and 2C6.

Culture supernatants were tested by dot-blotting. The filter had been loaded with chromosome core proteins solubilised in SDS gel loading buffer (upper half) and total chromosome proteins in the same buffer (lower half).

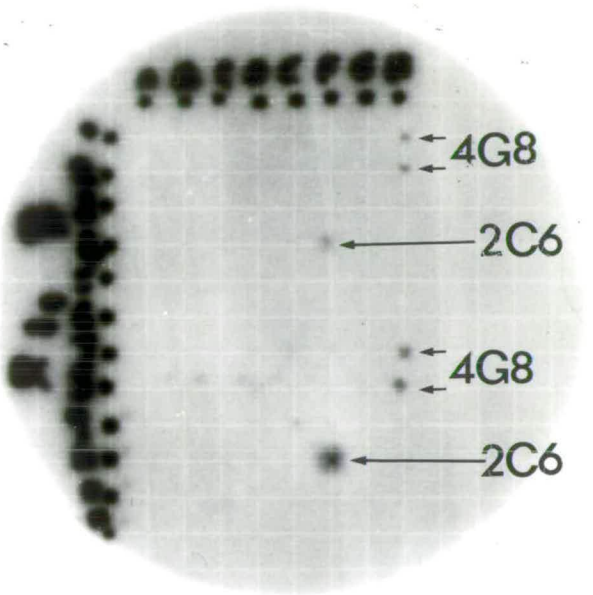

4G8
2C6
4G8
2C6

Figure 3.4: Antibody subclass determination

Ouchterlony double diffusion gel stained with amido-black to reveal precipitin bands. The central wells were loaded with hybridoma culture supernatant concentrated 10X using a Minicon spinal-fluid concentrator (Amicon). The surrounding wells contained subclass-specific antisera as marked.

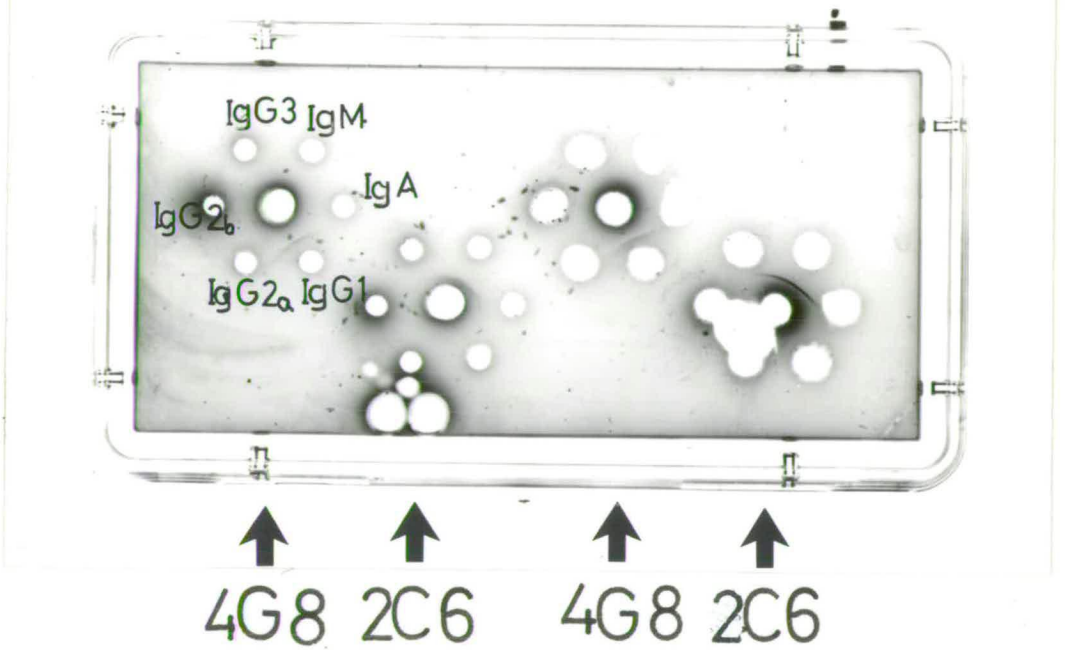

IgG3
IgM
IgA
IgG2b
IgG2a
IgG1
4G8
2C6
4G8
2C6

Figure 3.5: Dot-blotting analysis of the reactivities of 2C6 and 4G8

The antigens were loaded along columns as follows:

A= control (Pharmacia low molecular weight marker proteins)

B= Chromosomes

C= chromosome cores

D= nuclei

E= nuclear cores

F=tubulin and actin

G= chromosomes

H= nuclei

A-F were loaded from SDS gel loading buffer and G-H were loaded from KCM buffer.

The antibodies were loaded across the rows of the filter as marked.

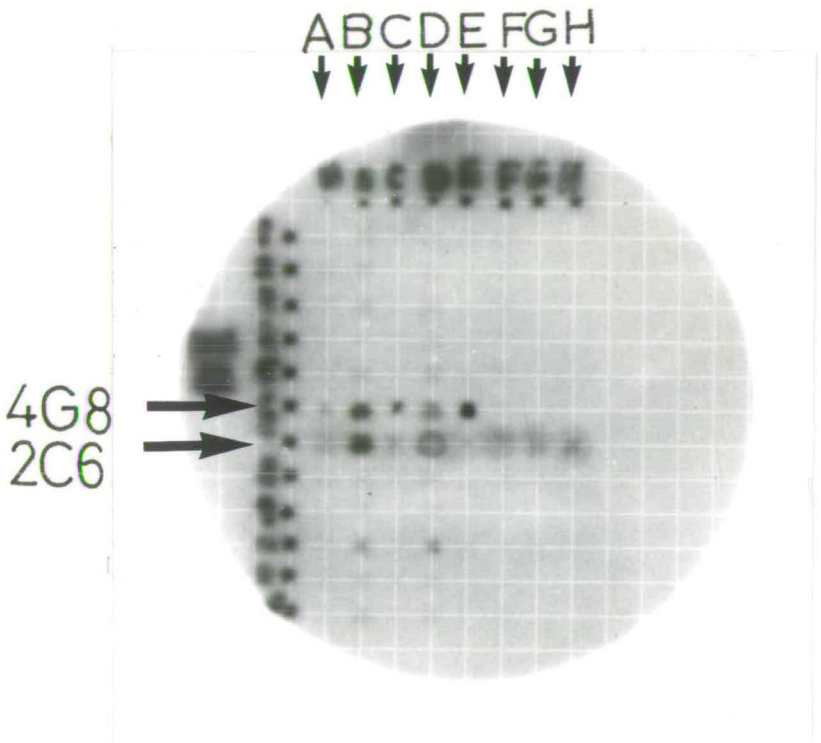

A B C D E F G H
4G8
2C6

Table 3.1 Summary of the specificities of monoclonal antibodies 2C6 and 4G8.

antibody	subclass	reactivity against:(+<++<+++)					
		chromo-somes	nuclei	chromo-somes*	chromo-cores*	nuclei*	nuc-cores*
4G8	IgG1	-	-	+	+++	+	+++
2C6	IgM	++	++	++	+	++	+

*- *these preparations were SDS solubilised.*

4G8 appeared to react with a chromosomal antigen, enriched in the core. The antigen was also found in the interphase nucleus and was enriched in the nuclear core. However, these results were obtained in SDS solubilised preparations only. This could be accounted for in two ways; first that the antibody reacts with a denatured epitope, and second, that the antigen is inaccessible to antibody in intact chromosomes and nuclei. These possibilities are distinguished in Ch.4.

2C6 reacted with an antigen in both 'native' and denatured chromosomes and nuclei. The antigen was also detected in cores, but at a diminished level relative to its chromosomal abundance.

These results suggest that 2C6 reacts with a component which is evenly distributed throughout the chromosome, and that 4G8 reacts with an antigen which has a differential distribution, being enriched in the core. Whether this enrichment reflects the in vivo situation or is a product of the isolation procedure is a question dealt with in Ch4, in which these two antibodies and their target antigens are characterised in greater detail.

Chapter 4 STUDIES ON CHROMOSOME STRUCTURE USING MONOCLONAL ANTIBODIES

The remainder of this thesis is concerned with the use of the monoclonal antibodies to chromosome cores in the investigation of the nature of the antigens they recognise and their ultrastructural organisation, in order to gain an insight into the structure of the chromosome and the significance of cores. The following three approaches were used

(1) "Western-blotting" was carried out to determine whether the antigens are proteins, and if so, to characterise them. The technique has been reviewed by Burnette(1981), and Towbin and Gordon (1984) amongst others.

(2) Immunocytological probing of light microscopic preparations of chromosomes and whole cells was carried out to determine the distribution of the core protein antigens. Both immunofluorescence (Osborn and Weber,1977) and immunoperoxidase(Nakane and Pierce, 1966) techniques were used.

(3) Immunoelectronmicroscopy was carried out to investigate the role of the chromosome core antigens with respect to cell ultrastructure. A colloidal-gold-labelled "second-antibody" probe was used as described by DeMey(1983).

4.1 WESTERN-BLOTTING EXPERIMENTS

Antibodies 4G8 and 2C6 were earlier shown (3.6) to bind to SDS solubilised components of nuclei and chromosomes, the 4G8 antigen apparently being enriched in chromosome cores. The fact that the antigens were SDS soluble suggested that they were

proteins, and this was tested more rigorously in the following section by "western-blotting". This technique involves the transfer of polypeptides resolved on a SDS-PAGE gel onto a sheet of nitrocellulose. The nitrocellulose can then be exposed to antibody followed by a labelled "second-antibody" enabling a particular polypeptide to be identified as the antigen.

4.1.1 Western-blotting identification of the 4G8 antigen

Some results of western-blotting experiments using 4G8 are compiled in fig.4.1. 4G8 was found to bind a polypeptide of apparent molecular weight 67,000 in Don cell chromosome cores (fig. 4.1b, lane C), Don cell nuclear cores (fig.4.1b, lane D), Don cell nuclei (fig.4.1a, lane E; fig. 4.1a, lane C). Also shown in fig.4.1a (lanes A+B) is the very weak reaction of 4G8 with total cell proteins from Don and HeLa cells. This indicates that the antigen is a very minor cellular protein. The results agree with the distribution of the 4G8 antigen found by dot-blotting (see Ch.3.6), although an enrichment of the antigen in chromosome cores cannot be shown because the loadings on the gels were not quantitative. The reaction with the marker protein, bovine serum albumin (BSA, fig.4.1b lane A) was observed in some blots, but is considered to be non-specific for the same reasons as are outlined below for 2C6.

4.1.2 Western-blotting identification of the 2C6 antigen

2C6 was also found to bind a polypeptide of 67,000 M_r in SDS-polyacrylamide gels of chromosomal and nuclear proteins. The antibody gave consistently weak reactions in western blotting, suggesting a very low affinity for the blotted antigen. Fig 4.2 shows a typical result. The only obvious bands on the autoradiogram are in the total Chinese hamster Don cell proteins (lane A'), the chromosome core proteins (lane C'), and on the 67,000M_r marker protein (BSA, lane H'). The reaction with BSA was observed in experiments with other antibodies and appears to be a non-specific reaction. Closer examination revealed a weak reaction with nuclear proteins from Chinese hamster Don and human FL cells. This does not reproduce well in the photograph, but is faintly visible in lane F'. Obviously, as all the blotting experiments using 2C6 gave very weak reactions, any conclusions drawn from this work must be very tentative. However the reaction observed does appear to be a genuine one as the molecular weight was consistently 67,000. The reaction with BSA at a similar molecular weight raises the possibility that the 67,000 M_r reaction might be with a contaminating serum albumin from the cell culture stage of the chromosome preparation. This possibility cannot be ruled out at this stage, however if any serum proteins did contaminate the chromosome preparations, they would be present in minute quantities compared to those found in the gel marker proteins. Thus, if the "western-blot"-band in chromosome preparations was BSA, the binding to BSA in the marker protein lanes would have been very much stronger than observed.

Figure 4.1 Western blots with 4G8

a) 4G8 binds a 67,000 M_r polypeptide in Don and HeLa cells

(i)Nitrocellulose blot from gel probed with 4G8

(ii) Coomassie stained gel after blotting

(iii)Control blot probed with second antibody only

Lanes: A, A': total Don cell proteins, B, B': total HeLa cell proteins, C, C': Don cell nuclear proteins.

b) The 67,000 M_r antigen is found in chromosome cores.

Autoradiogram of nitrocellulose filter probed with 4G8-

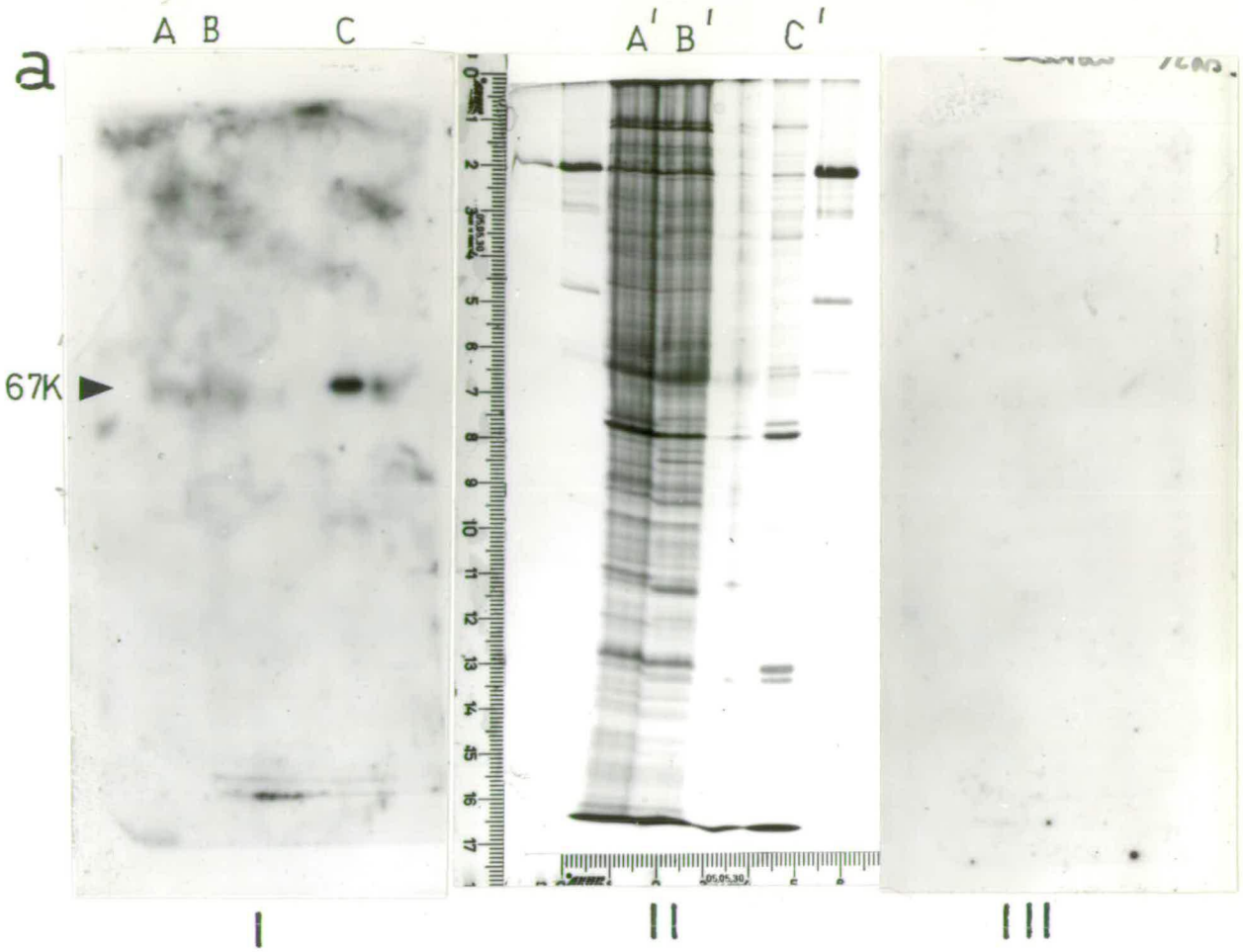
Lane A: 67,000 M_r standard (BSA gives non-specific reaction)

Lane B: FL cell nuclear proteins

Lane C: Don cell chromosome core proteins

Lane D: Don cell nuclear core proteins

Lane E: Don cell nuclear proteins.


a
A B C
A' B' C'
67K
I
II
III

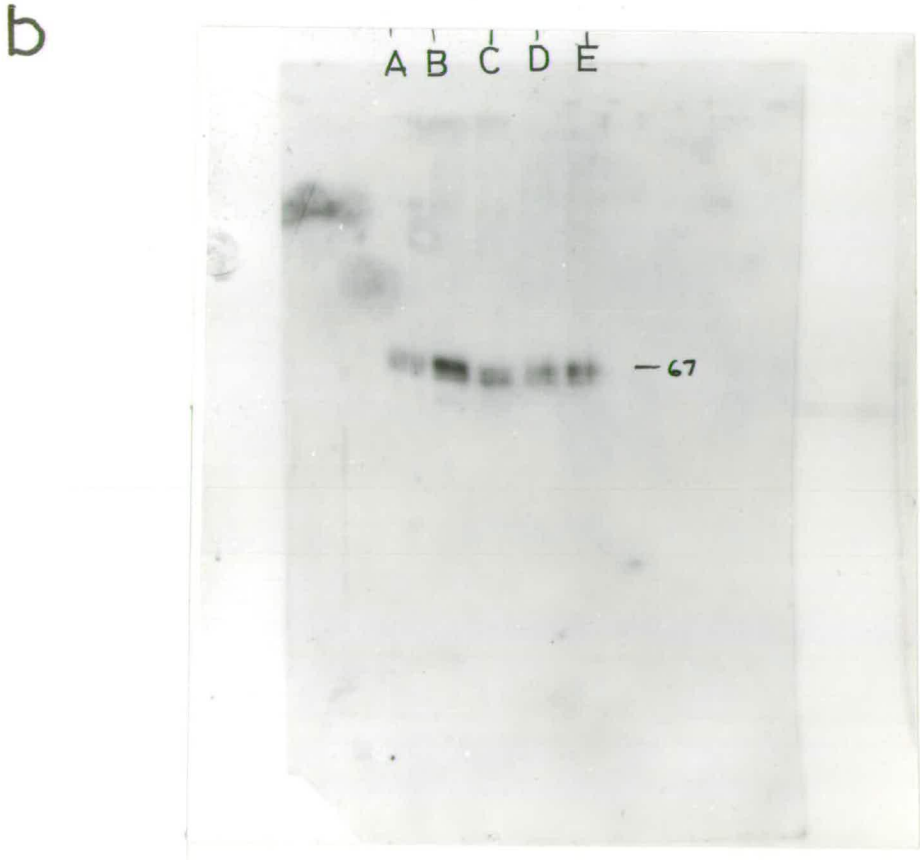

b
A B C D E
67

Figure 4.2 Western blots with 2C6

A-H: amido-black stained replica filter

A'-H': autoradiogram after probing with 2C6

Lane A+A': total Don cell proteins

B : FL cell nuclear proteins

C : Don cell chromosome core proteins

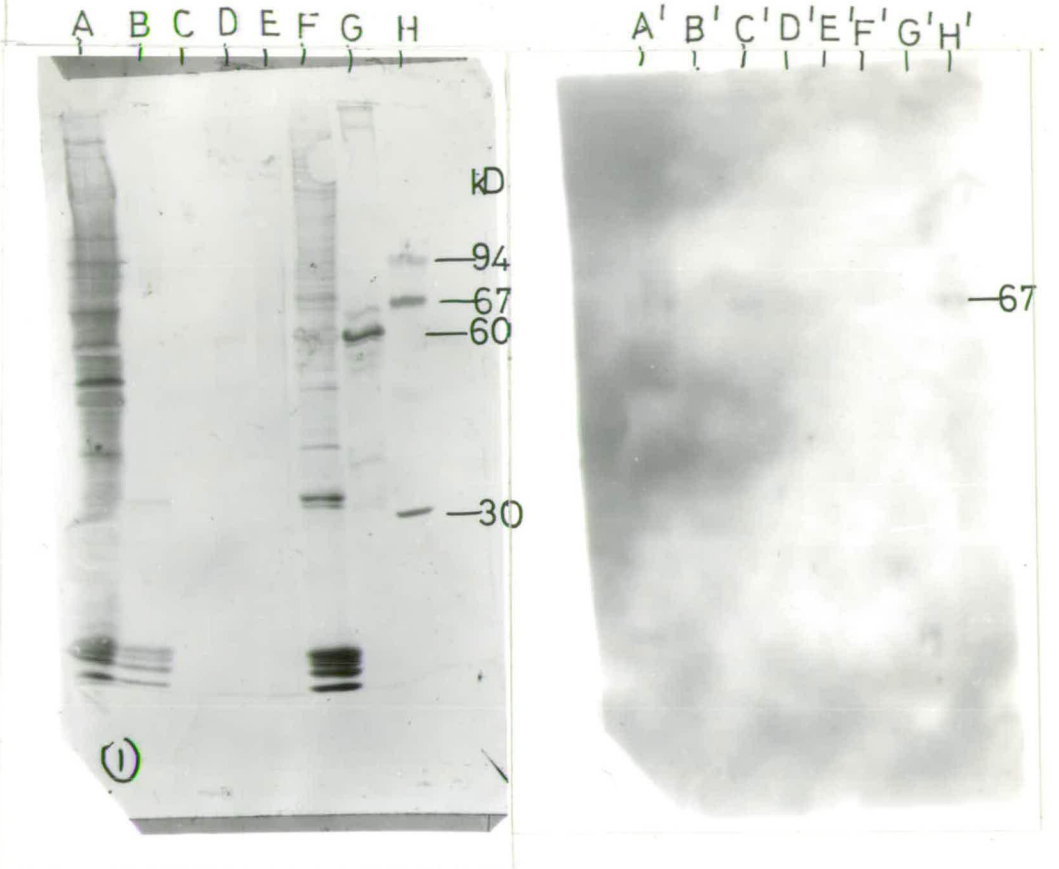
D : Don cell nuclear core proteins

E : Don cell nuclear core proteins

F : Don cell nuclear proteins

G : Markers (Pharmacia high molecular wt. marker proteins)

H : Markers (Pharmacia low molecular wt. marker proteins)


A B C D E F G H
kD
94
67
60
30
(I)
A' B' C' D' E' F' G' H'
67

4.1.3 Summary of western-blotting results

Both antibodies were found to bind polypeptides with an apparent molecular weight of 67,000, although the data on 2C6 must be treated with some caution. On SDS gels of chromosomal proteins this region contains the major chromosome core proteins; however owing to the poor resolution of such gels, neither antibody can be ascribed to a specific polypeptide. It should be noted that although the 2C6 and 4G8 antigens band at similar positions on 1D gels, the results in the section to follow clearly show them to be distinct species. As chromosomal and chromosome core proteins had been mapped at high resolution by Gooderham and Jeppesen(1983) using 2-dimensional gels, immunoblotting of such gels seemed to be the best approach for the further characterisation of the two antigens. However, this approach has been unsuccessful, apparently owing to the very small amounts of polypeptides transferred to nitrocellulose after 2-dimensional gel electrophoresis.

4.2 IMMUNOCYTOLOGICAL EXPERIMENTS

Antibodies 2C6 and 4G8 were used to probe preparations of chromosomes and culture cells on microscope cover-slips. Two methods were used to visualise antibody binding. Immunofluorescence, in which a fluorescein-conjugated second antibody against murine immunoglobulin is used, has been fully reviewed by Osborn and Weber (1977). In the immunoperoxidase technique, described by Nakane and Pierce (1966), the second antibody is conjugated with horse-radish peroxidase. In the presence of hydrogen peroxide, the enzyme catalyses the oxidation of soluble diaminobenzidine tetrahydrochloride to an insoluble polymer, which is deposited as a brown precipitate at the sites of enzyme activity. The chemistry of this reaction is discussed by Pearse (1968). While Osborn and Weber (1977) reported the immunofluorescent technique to be more sensitive than immunoperoxidase, in the experiments described here the reverse was often the case, particularly where very small structures were visualised. The disadvantage of immunofluorescence lies in the rapid decrease of fluorescein fluorescence under prolonged illumination. On the other hand, immunofluorescence was found to give a much cleaner background than immunoperoxidase, and was always used for unambiguous demonstration of antibody binding to fine subcellular structures.

Before either method of immunoprobing could be applied, specimen preparation and fixation methods had to be established. Fixation can have major effects on the immunoreactivity of

Table 4.1 FIXATION PROCEDURES FOR IMMUNOCYTOLOGY

MATERIAL	FIXATIVE	ATTACHMENT TO COVERSLIP	REFERENCE
culture cells	formaldehyde then acetone permeabilisation	cells grown on coverslips	Osborn&Weber 1977
culture cells	methanol then acetone	cells grown on coverslips	Osborn&Weber 1977
chromos--omes and cores	formaldehyde	chromosomes centrifuged onto coverslip and fixed	Gooderham& Jeppesen 1983
chromos--omes etc.	ethanol	centrifuged onto cover--slip and fixed	

proteins, and the chemistry of the different types of fixative is discussed by Pearse (1968). The methods used in the experiments described below are summarised in table 4.1.

In the initial experiments, 2C6 and 4G8 were used to probe fixed culture cells, and the 2C6 antigen was found to display an unexpected nucleocytoplasmic distribution. Following this, the antibodies were applied to chromosomes, dehistonised chromosomes and chromosome cores.

Unless otherwise noted in the text or figure legends, all chromosome preparations were derived from Don chinese hamster fibroblasts by the KCM method described in Ch.2. Controls, in which antibody-free culture medium was used instead of hybridoma supernatant, were run to determine the level of any non-specific reaction.

RESULTS

4.2.1 2C6 antigen- distribution in cultured cells

2C6 reacted with Don chinese hamster, HeLa, mouse myeloma(NS1), and human FL cells, revealing a punctate cytoplasmic system, radiating from, and concentrated at the nucleus. The results for Don and HeLa cells are shown in fig.4.3. Surprisingly, as the antigen had been shown to be present in nuclei and chromosomes by dot blotting(Ch.3.6), there was no reaction with the nucleus. Also, in mitotic cells, the antibody appeared to have a much greater cytoplasmic abundance than in

interphase(fig.4.3d) and although it is not clear in fig.4.3d, immunofluorescence experiments showed that the metaphase plate was not stained. Consequently, a further immunoblotting experiment was carried out, in which the reaction of 2C6 with a post-nuclear fraction obtained during the preparation of Don cell nuclei (Ch.2) was examined. The results, as shown in fig.4.3e indicated that the 2C6 antigen is indeed present in the cytoplasm of Don cells. This material was pelletable, probably representing the filamentous system observed in fig.4.3a-d, and there is much scope for detailed investigation of this material by further subcellular fractionation procedures.

To gain further insight into the nature of the antigen recognised by 2C6, the staining patterns of antibodies to cytoskeletal proteins and also an antibody to histone 2b were examined(fig4.4). While anti-actin, anti-intermediate filament and anti-tubulin (not ill.) all bound to diffuse filamentous systems in the cytoplasm of Don cells, none exhibited the intense perinuclear reaction given by 2C6. It therefore appeared likely that 2C6 was not reacting with a cytoskeletal protein. The antibody to histone 2b gave a faint cytoplasmic reaction, but in most cells the nucleus was unstained. This antibody was a monoclonal generously provided by Dr. B.Turner, and had been shown to bind chromosomes, but only after salt extractions(Turner,1982). It was suggested that the antibody bound a masked epitope which was exposed after salt extraction, and this seemed to be the reason for the lack of reactivity against the nucleus shown here.

This observation suggested two explanations for the lack of reaction of 2C6 with chromosomes and nuclei. First, that the antigen had a nucleocytoplasmic distribution, but was inaccessible to the antibody in the nucleus, and second, that the antigen was a cytoplasmic protein which artefactually contaminated the nuclei and chromosomes during their isolation. The first explanation was shown to be most likely, owing to the fortuitous observation that treatment of fixed cells with urea resulted in a strong nuclear reaction with 2C6. This result was examined in greater detail in the following experiments.

Fig.4.5 shows the result of probing urea treated Don cells with 2C6, anti-histone 2b, anti-actin and anti-intermediate filament antibodies. In urea treated cells 2C6 intensely stained nuclei and chromosomes, although a low level of cytoplasmic staining remained. It is possible that this result was due to the collapse or aggregation of the 2C6 antigen onto the chromosomes and nuclei, but the following observations suggested this was not so:

(i) The intensity of reaction was much greater than could be accounted for by the reorganisation of the cytoplasmic antigen;

(ii) The staining patterns of antibodies to intermediate filaments (fig.4.5b), actin (fig.4.5d) and tubulin(not ill.) were not altered by urea treatment;

(iii) The urea treatment resulted in the unmasking of the histone 2b antigen.

Thus, it appeared that urea treatment resulted in the exposure of the 2C6 antigen in nuclei and chromosomes. This antigen

retained a nucleocytoplasmic distribution, although this is unclear in Fig4.5a owing to the short photographic exposure which the nuclear fluorescence required.

Following this observation, various other treatments were carried out on Don cells, and the results are shown in fig.4.6. Treatment of cells with trypsin after fixation(fig 4.6a) also enabled 2C6 to stain the nucleus, although at a lower level than that after urea treatment. Post-fixation treatment with the disulphide reducing agent, dithiothreitol (DTT), resulted in the selective unmasking of the 2C6 antigen on chromosomes but not on the nucleus(fig.4.6b). This result is compatible with the finding of Dounce et al (1973) that 2-mercaptoethanol leads to the swelling of fixed chromosomes, possibly opening up antigenic sites. Fig.4.6c shows the reaction of 2C6 on cells extracted with Triton X-100 and 2M KCl. This was a modification of the method described by Cook and Brazell(1975) for the production of nucleoids(see Ch.1.3). The residual nuclei and radiating fibres were intensely stained. The results obtained by Cook and Brazell(1975,1976) suggest that the fibres are composed of nuclear DNA, and experiments on dehistonised chromosomes described in the next section demonstrated that the 2C6 antigen is associated with DNA.

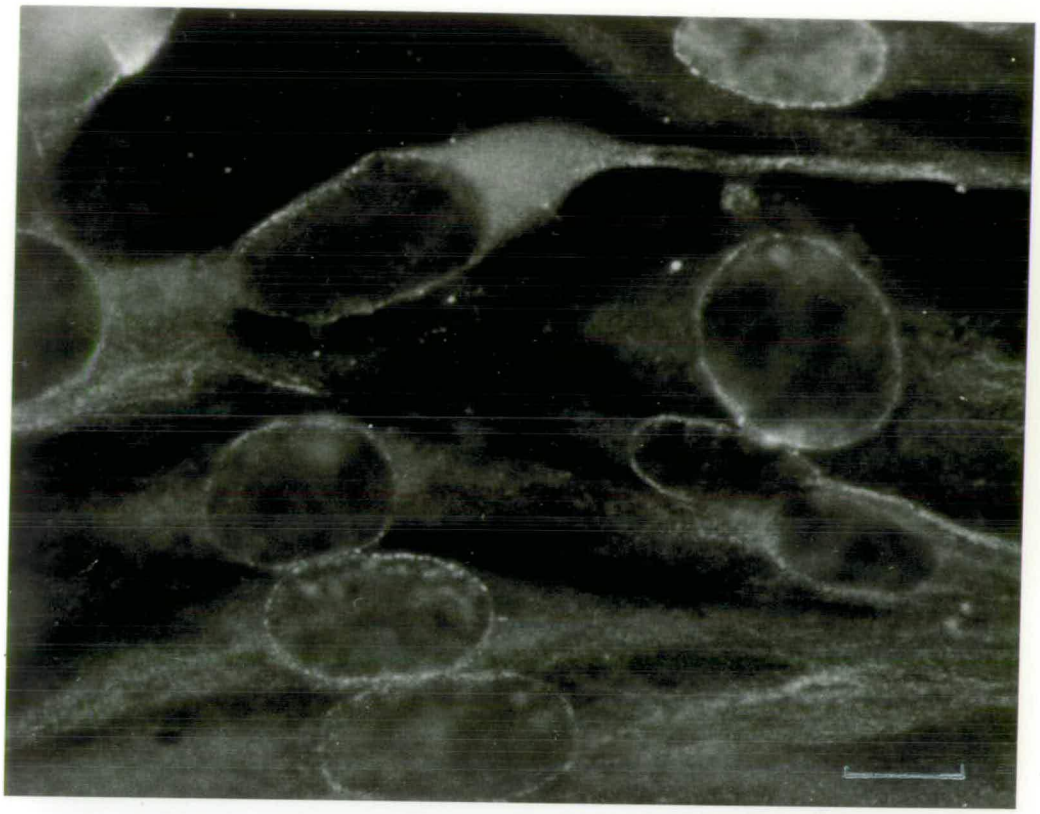
FIG.4.3 Antibody 2C6 binding to Don and HeLa cells

a-d) immunofluorescence and immunoperoxidase visualisation of 2C6 binding in fixed cells. Bar= 10μm.

a) Don cells, fixed for 5min in MeOH and 1min in acetone
b,c,d) HeLa cells, fixed for 5min in 3.7% formaldehyde in PBS and 10s in acetone.
In (d) the mitotic cells are arrowed.

e) Western blot showing nucleocytoplasmic distribution of the 2C6 antigen: lanes A+C= total Don nuclear protein; lane B=post-nuclear material (after the cell lysis and nuclear pelleting step{2.2}, the supernatant was centrifuged at 2,500 r.p.m. for 10min to remove any further nuclear contamination and then was centrifuged at 90,000g for 30min. The pellet was taken up in gel loading buffer for this experiment).

a



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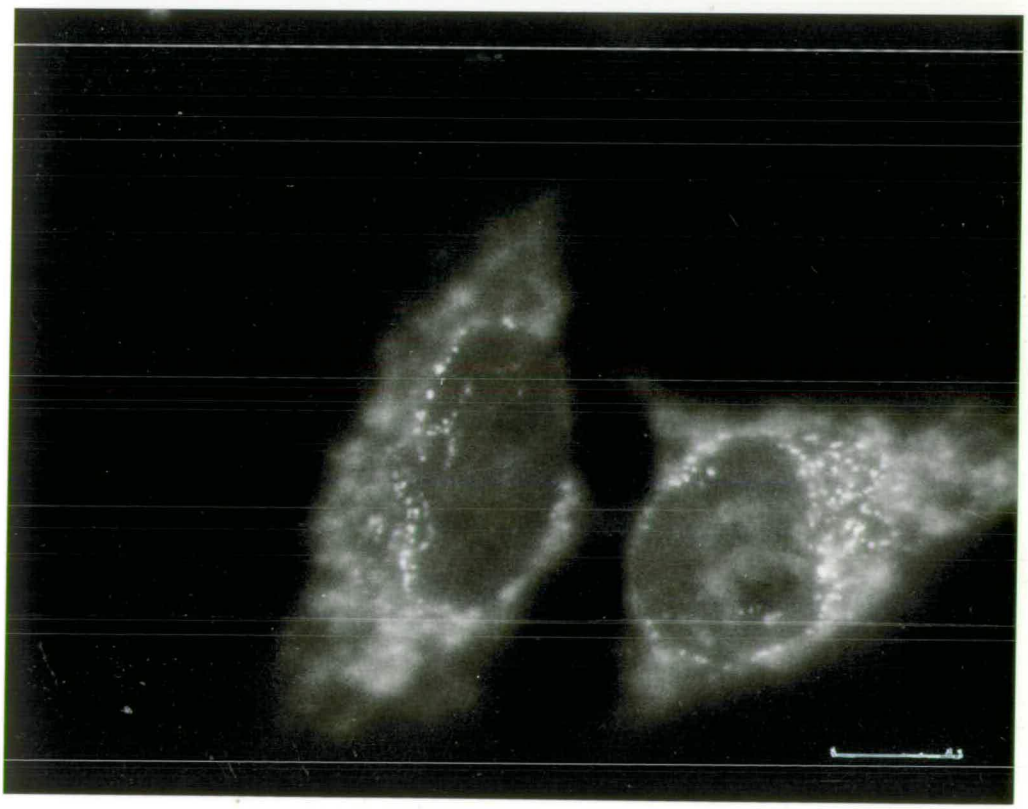

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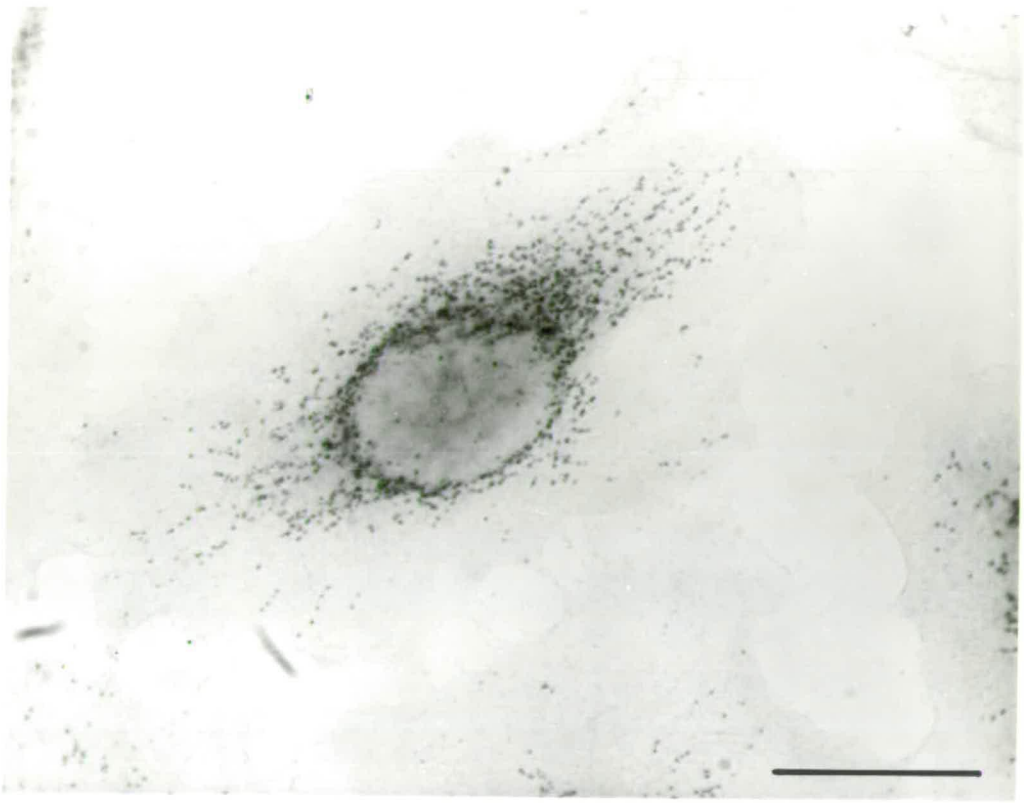

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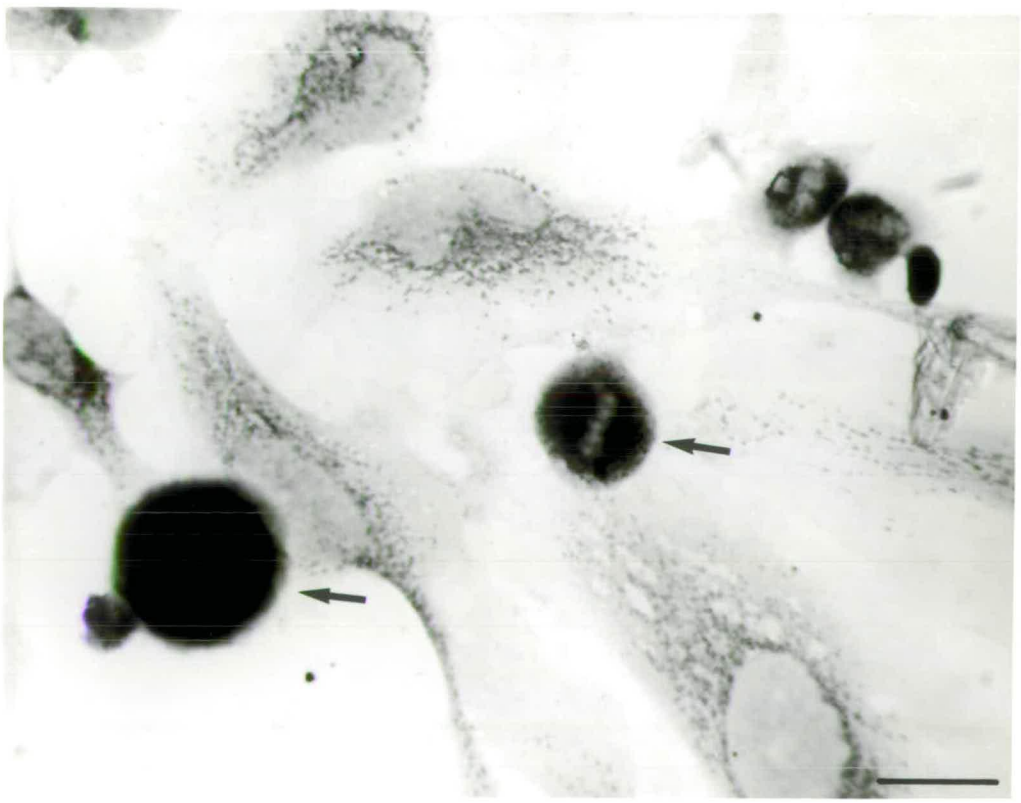

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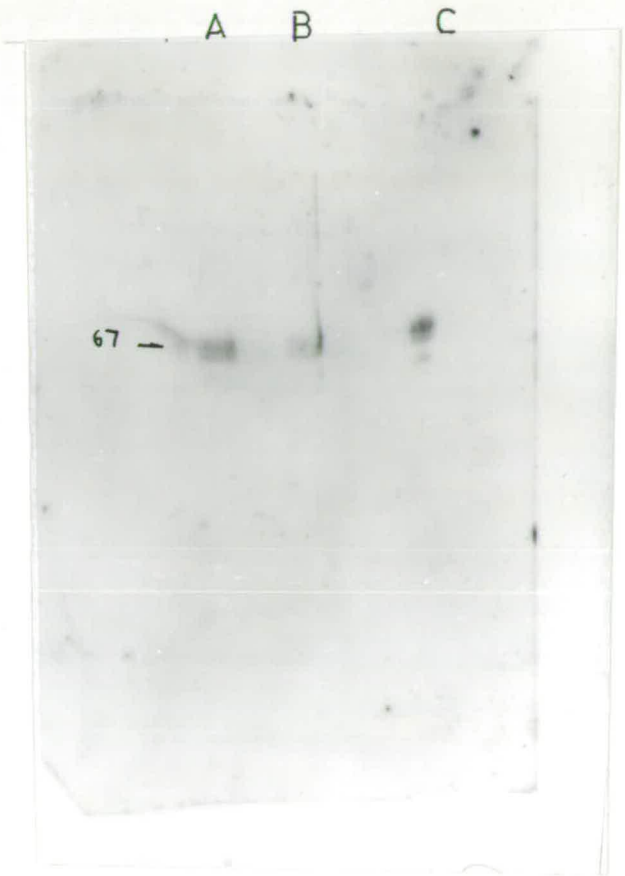


FIG. 4.4 Comparison of 2C6 binding to Don cells with cytoskeleton and histone antibodies

Don cells were grown on cover slips and fixed for 5min in MeOH and 1min in acetone. Immunofluorescence with:

a) 2C6

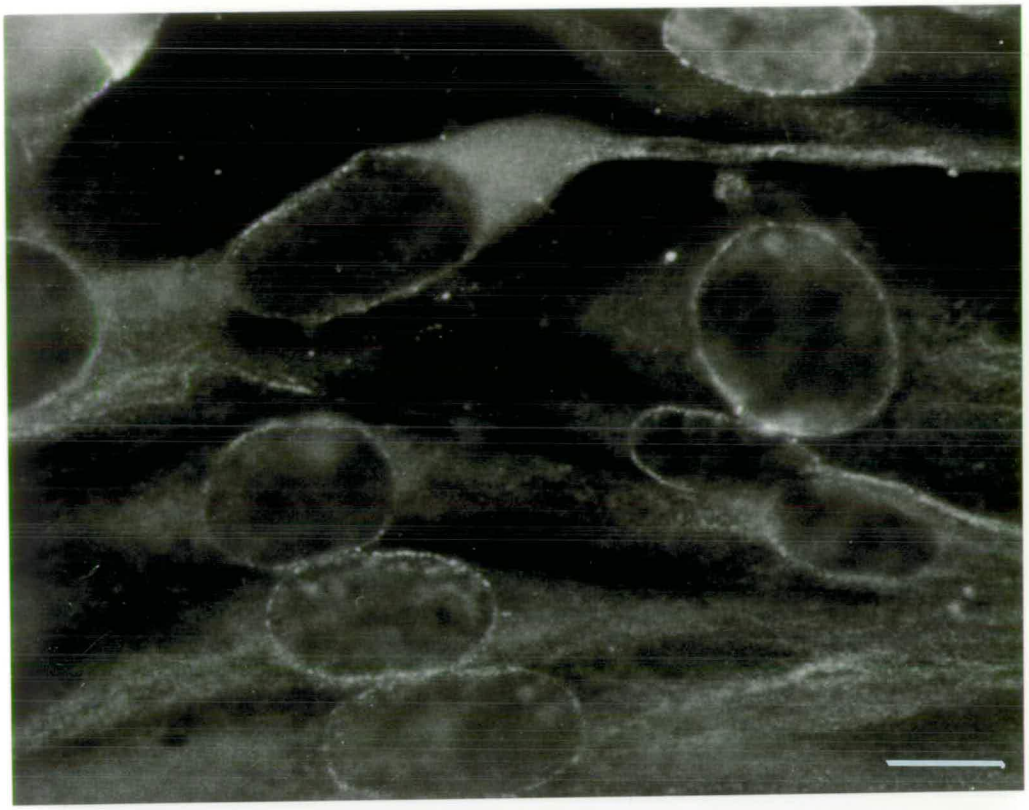
b) anti-intermediate filament

c) anti histone 2b

d) anti-actin

Bar= 10µm.

a



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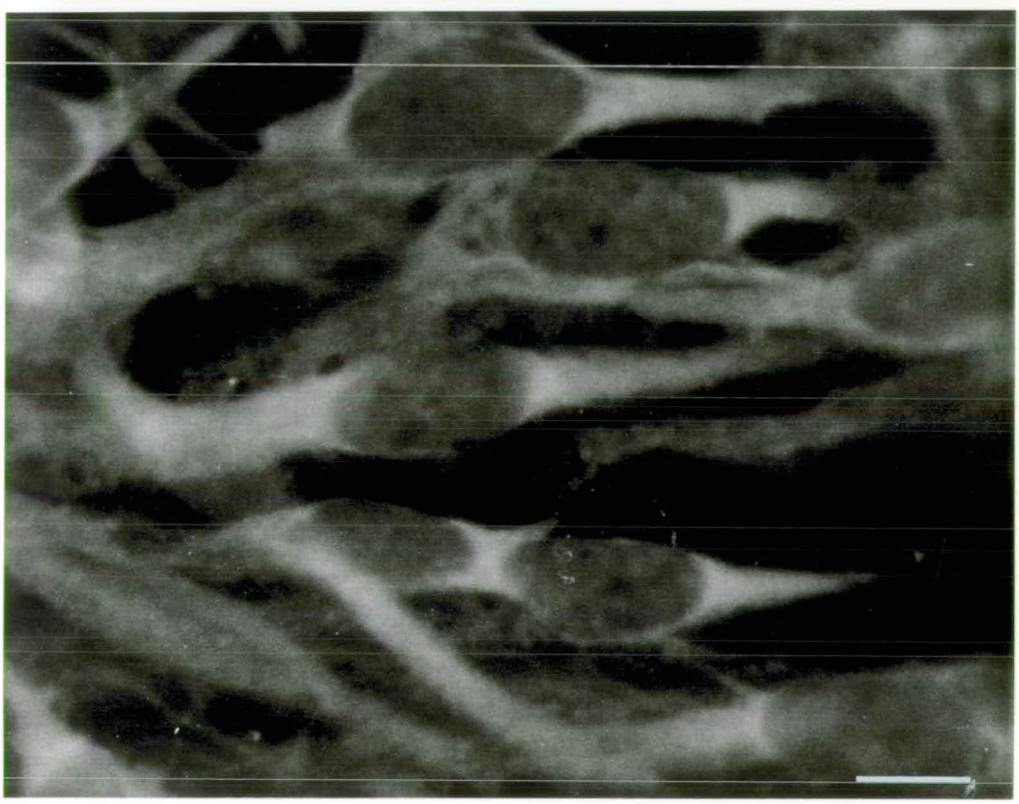

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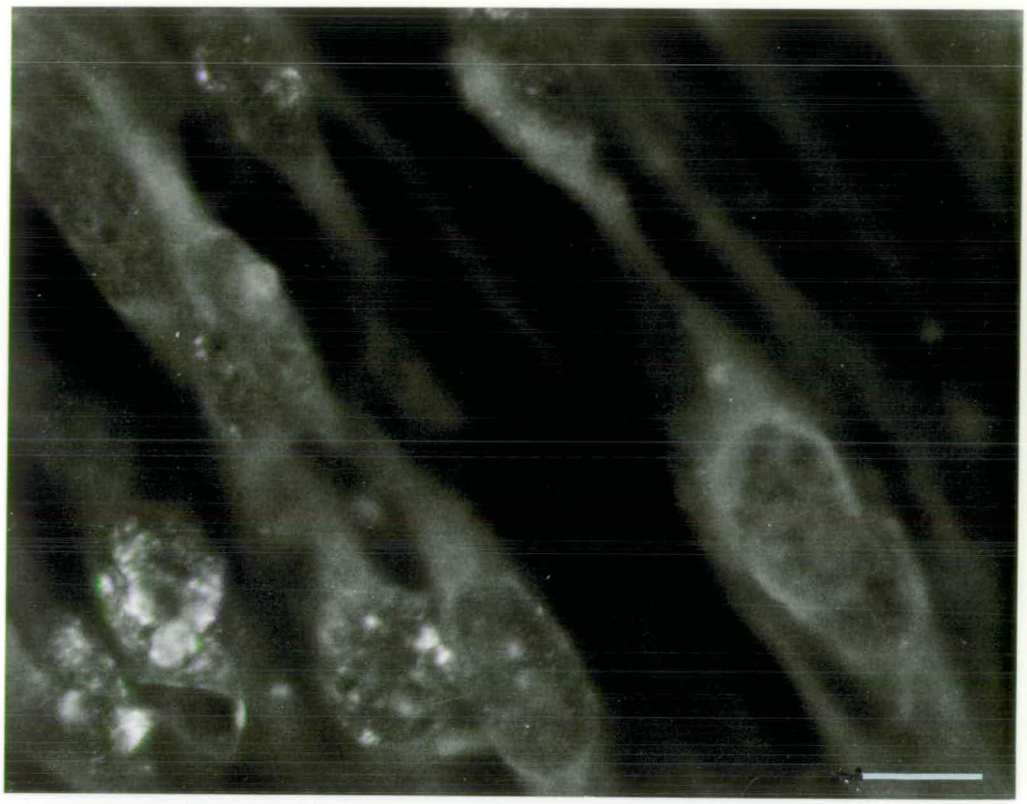


d

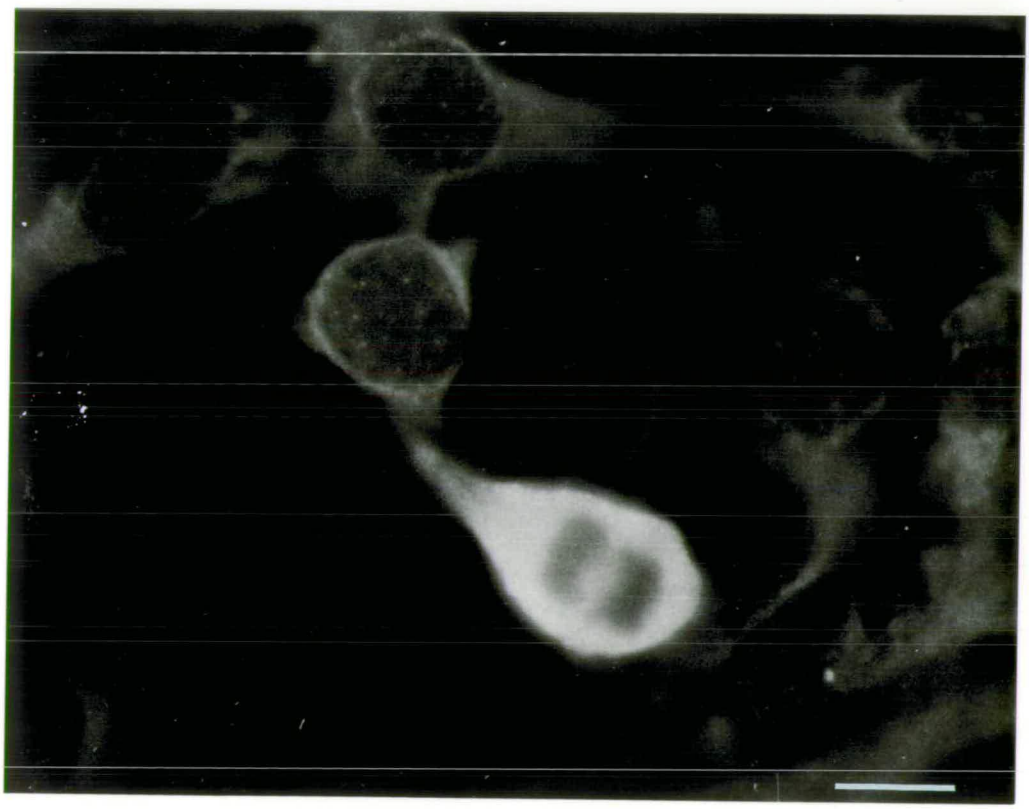


FIG 4.5 Effect of post-fixation urea treatment on Don cell immunofluorescence

Don cells were grown on cover slips, fixed for 5min in MeOH and 1min in acetone, followed by 10s in PBS containing 2M urea and a PBS wash. They were then processed for immunofluorescence with the following antibodies:

a) 2C6*

b) anti-intermediate filament

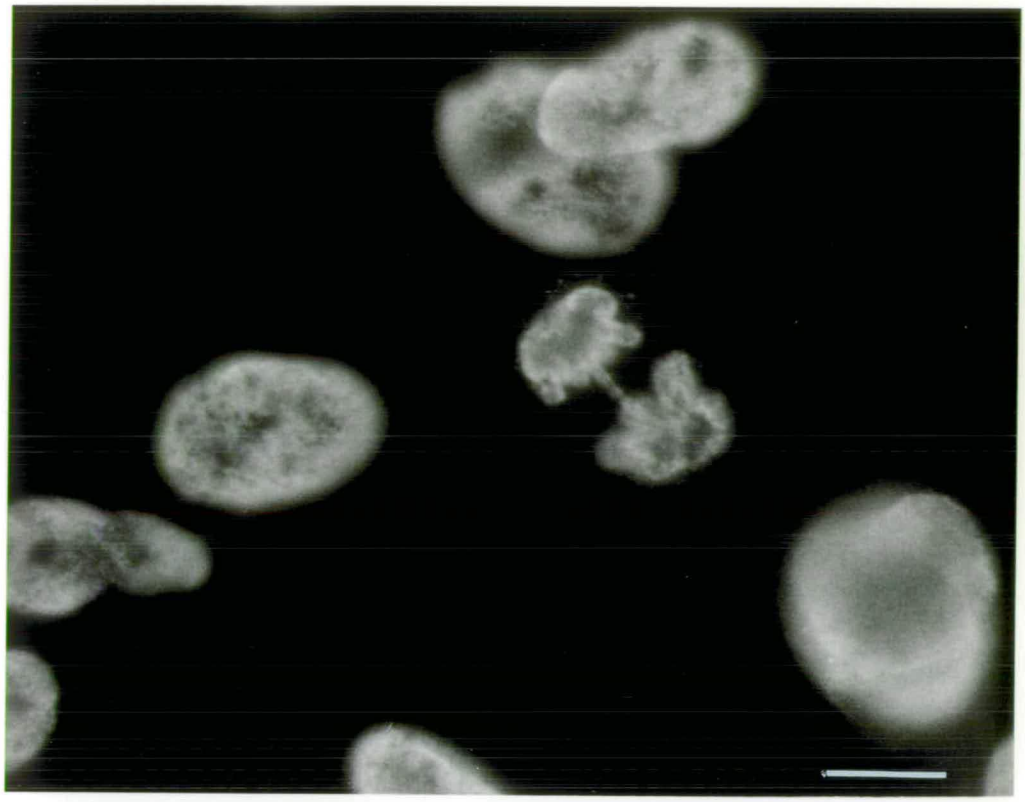
c) anti-histone 2b

d) anti-actin

Bar= 10μm.

*The intensity of nuclear fluorescence with 2C6 (a) required a very short photographic exposure, and so a significant level of cytoplasmic fluorescence is not apparent.

a



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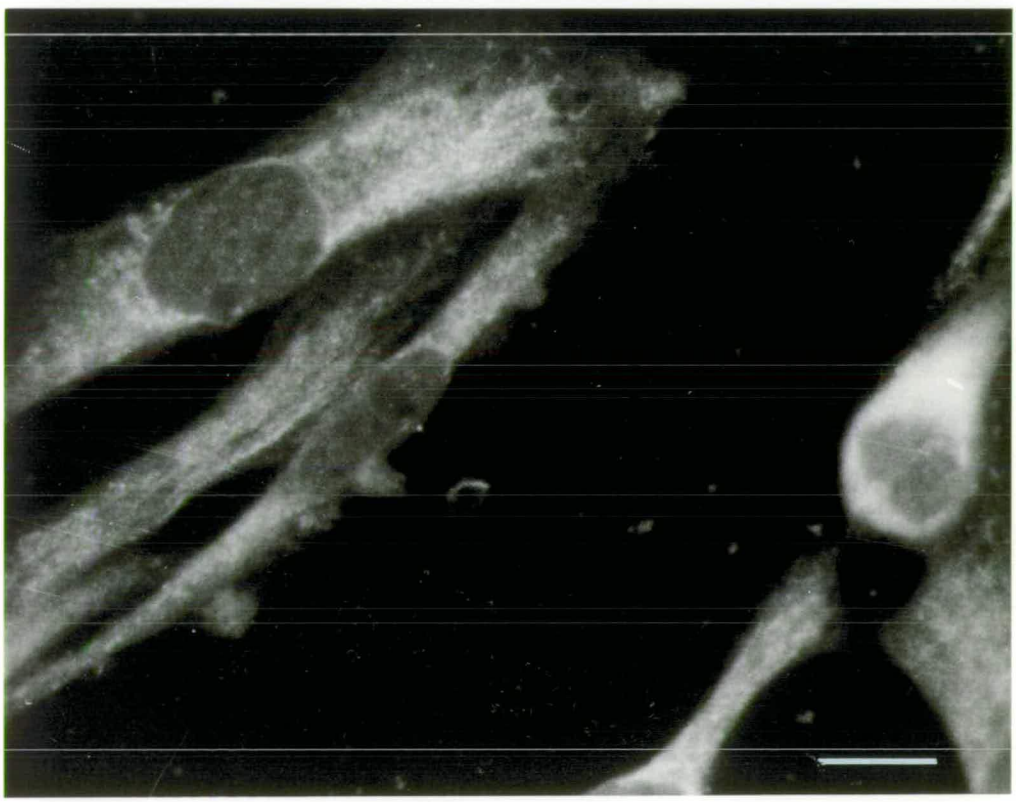


c

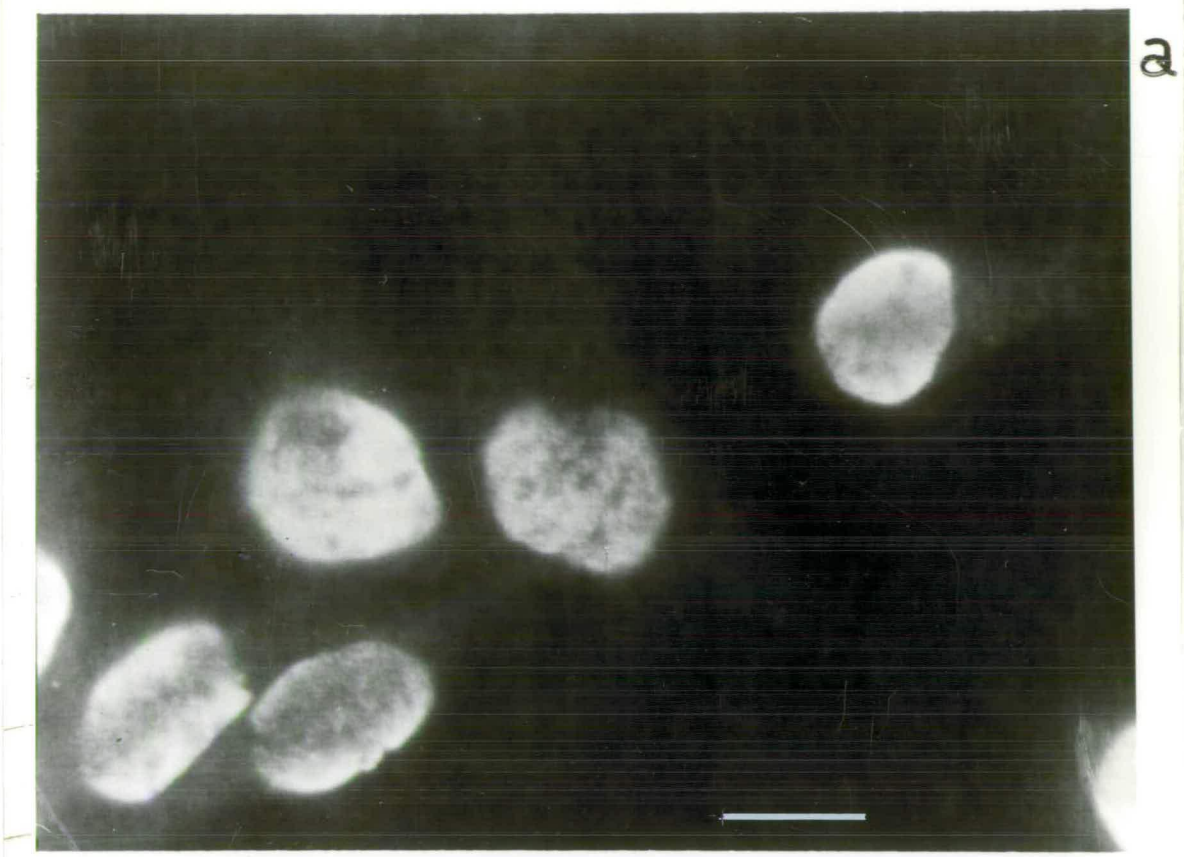
d

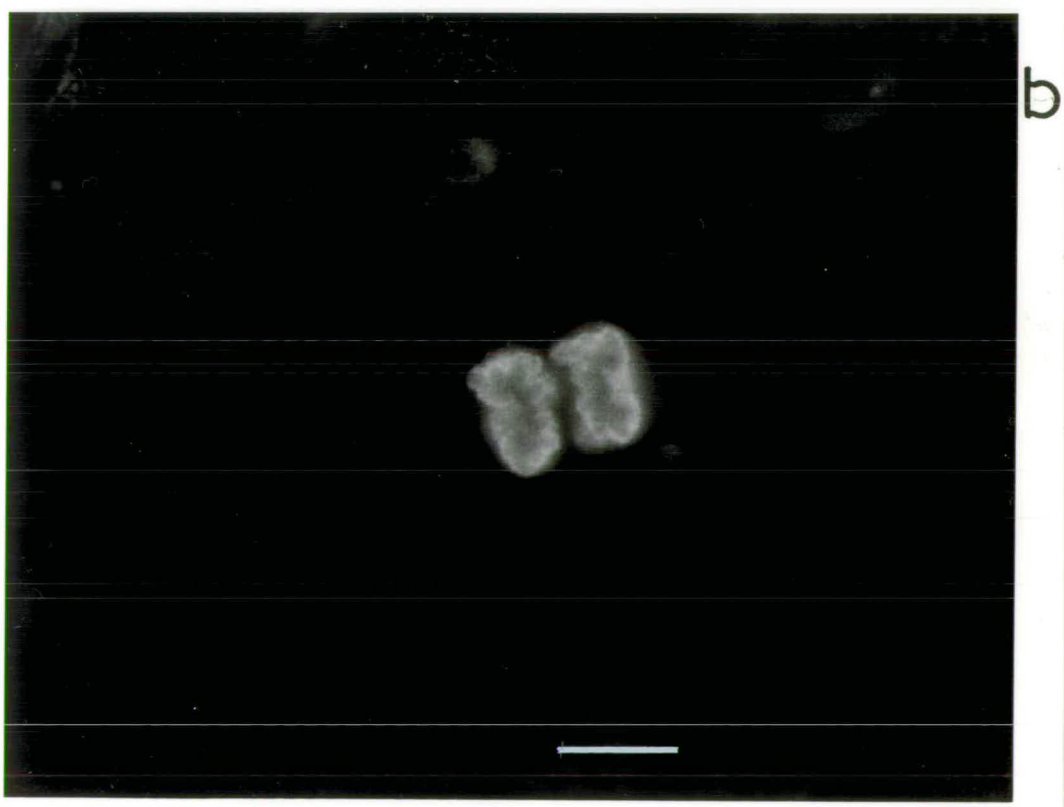
FIG 4.6 Treatments revealing the 2C6 nuclear antigen

a) Don cells were grown on cover slips, fixed for 5min in MeOH and 1min in acetone, and were then treated for 5s with PBS containing 20µg/ml trypsin. They were then washed with PBS and processed for 2C6 immunofluorescence. Bar= 10µm.

b) Don cells were fixed as above and treated with 5mM DTT in PBS for 60s prior to immunofluorescent probing. Bar= 10µm.

c) Don cells were grown on cover slips, incubated in TBS(150mM NaCl, 10mM Tris-HCl pH7.5) containing 1.0%(v/v) Triton X-100 for 2min. They were then washed in TBS, treated for 10min with TBS containing 1.5M KCl, and further washed in PBS before MeOH-Acetone fixation as above. Bar= 50µm.


a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C

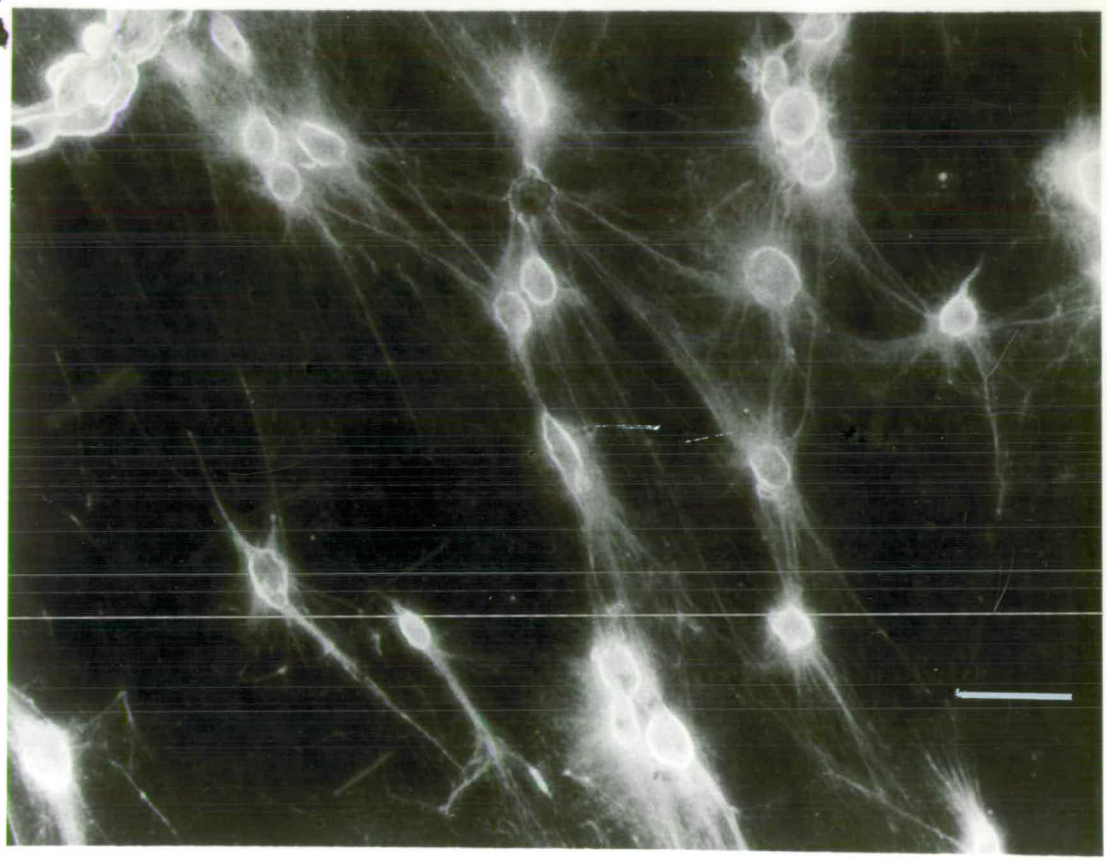


FIG 4.7 2C6: staining pattern on isolated Don chromosomes.

Don cell chromosomes, isolated by the KCM procedure, were either formaldehyde(a,b) or ethanol(c,d) fixed.

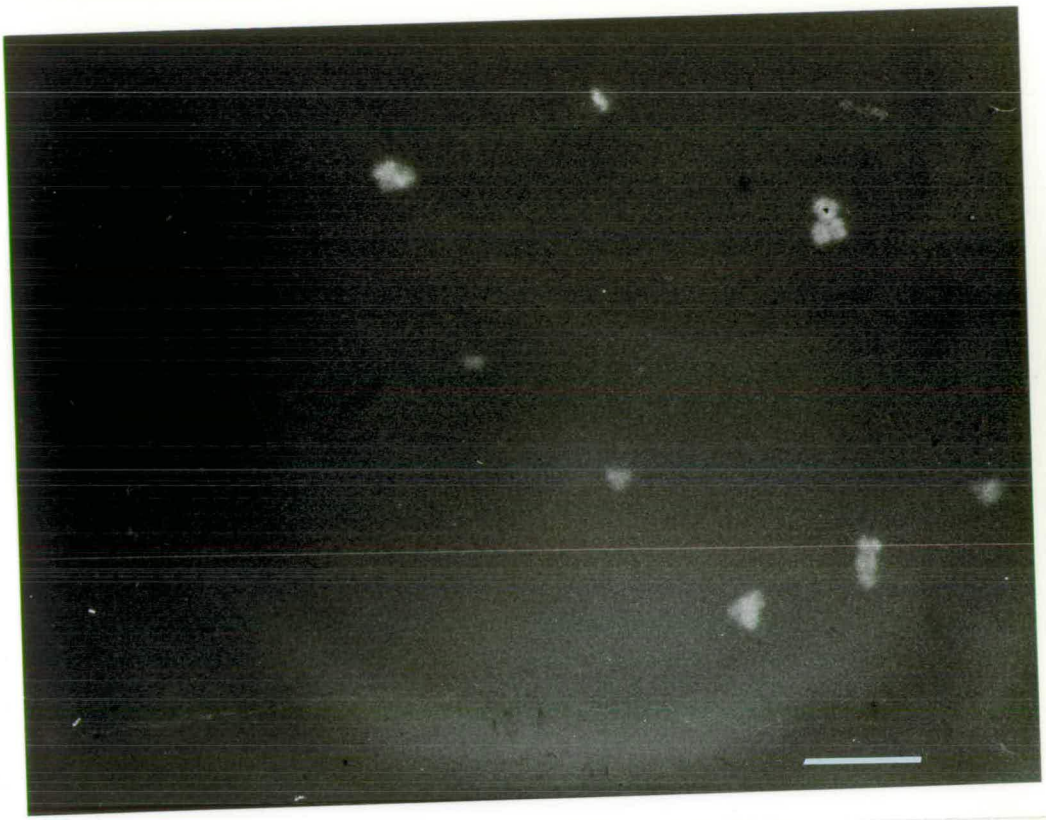
a) formaldehyde fixation, Hoechst 33258 stain.

b) formaldehyde fixation, 2C6 immunoperoxidase.

c) ethanol fixation, Hoechst 33258 stain.

d) ethanol fixation, 2C6 immunofluorescence.

Bar= 10μm.



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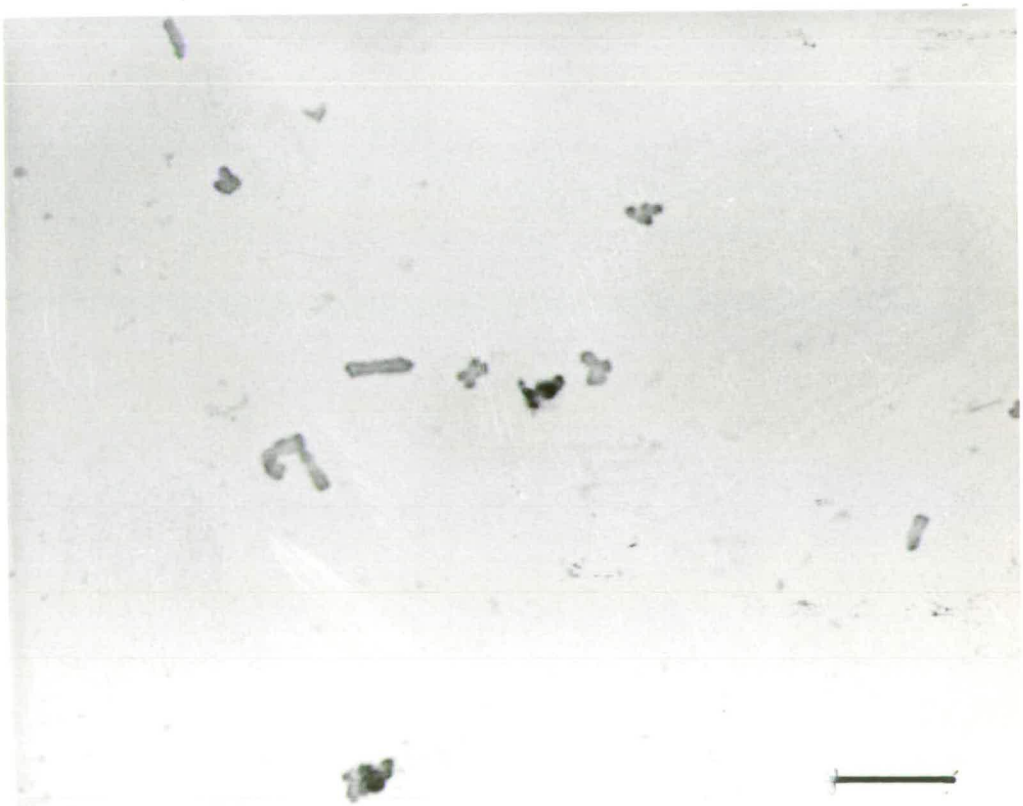

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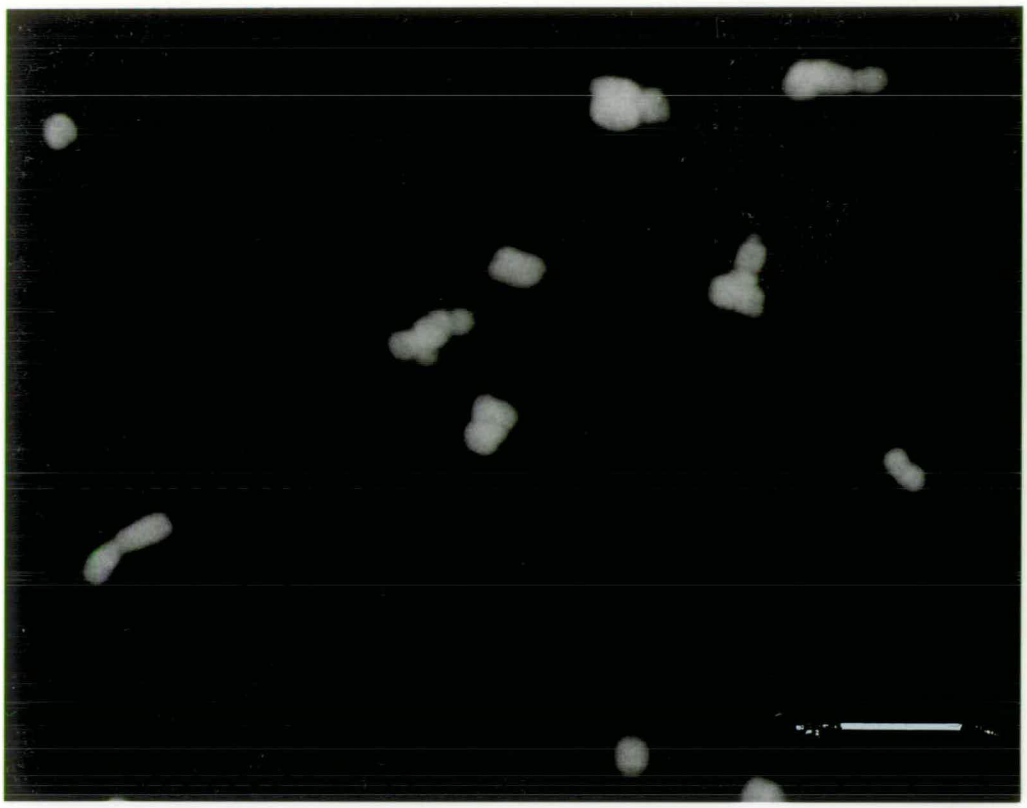

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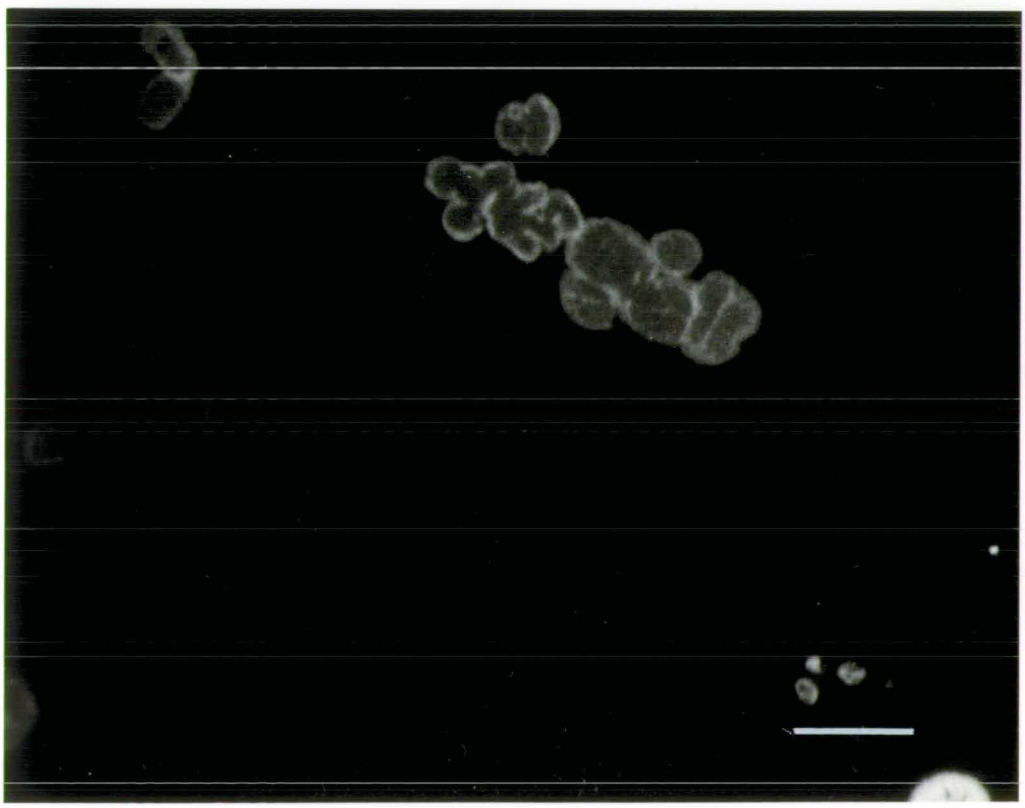


d

FIG 4.8 2C6: staining of dehistonised chromosomes

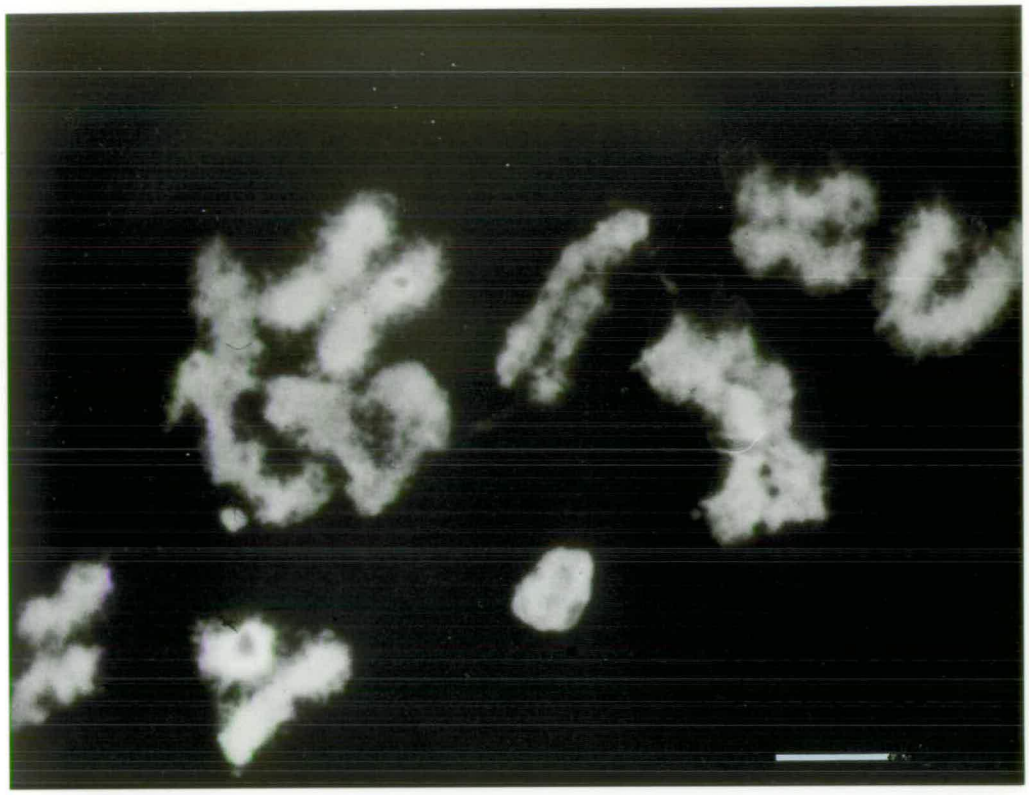
Dehistonised chromosomes (after the NaCl extraction step described in 6.1.3) were fixed either in formaldehyde or ethanol and processed for 2C6 immunofluorescence.

a) formaldehyde fixed

b) ethanol fixed— arrows mark decorated DNA fibres.

c) formaldehyde fixed dehistonised chromosomes stained with Hoechst 33258.

Bar= 10μm.



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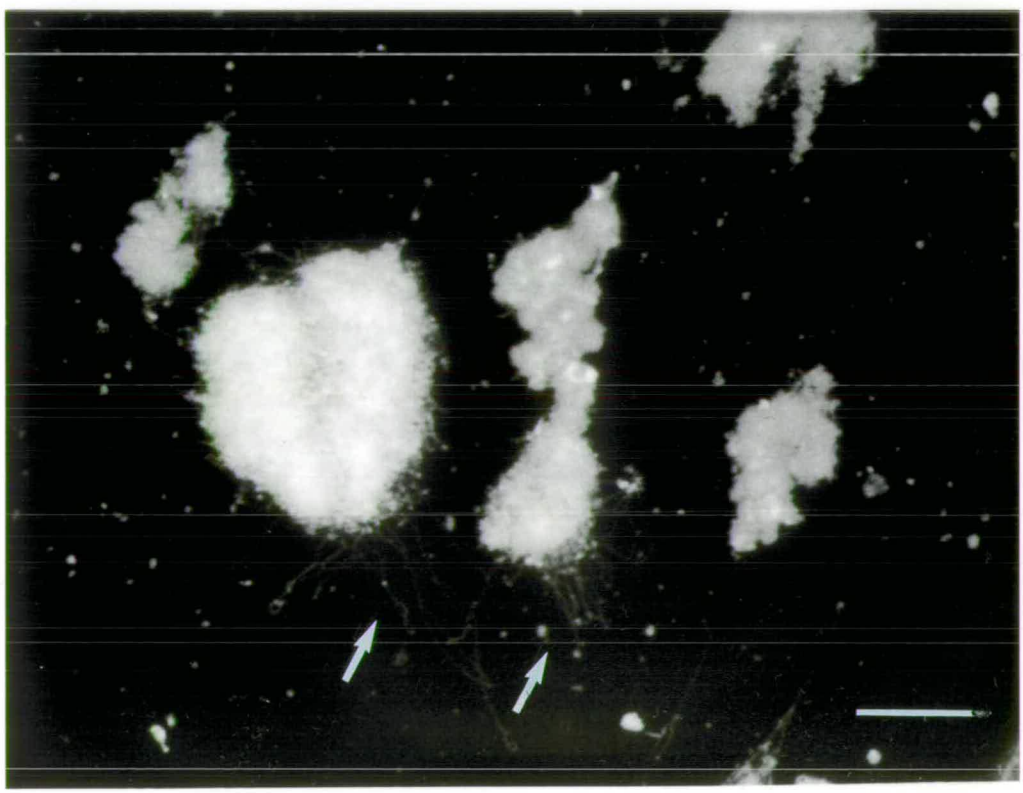


b

C

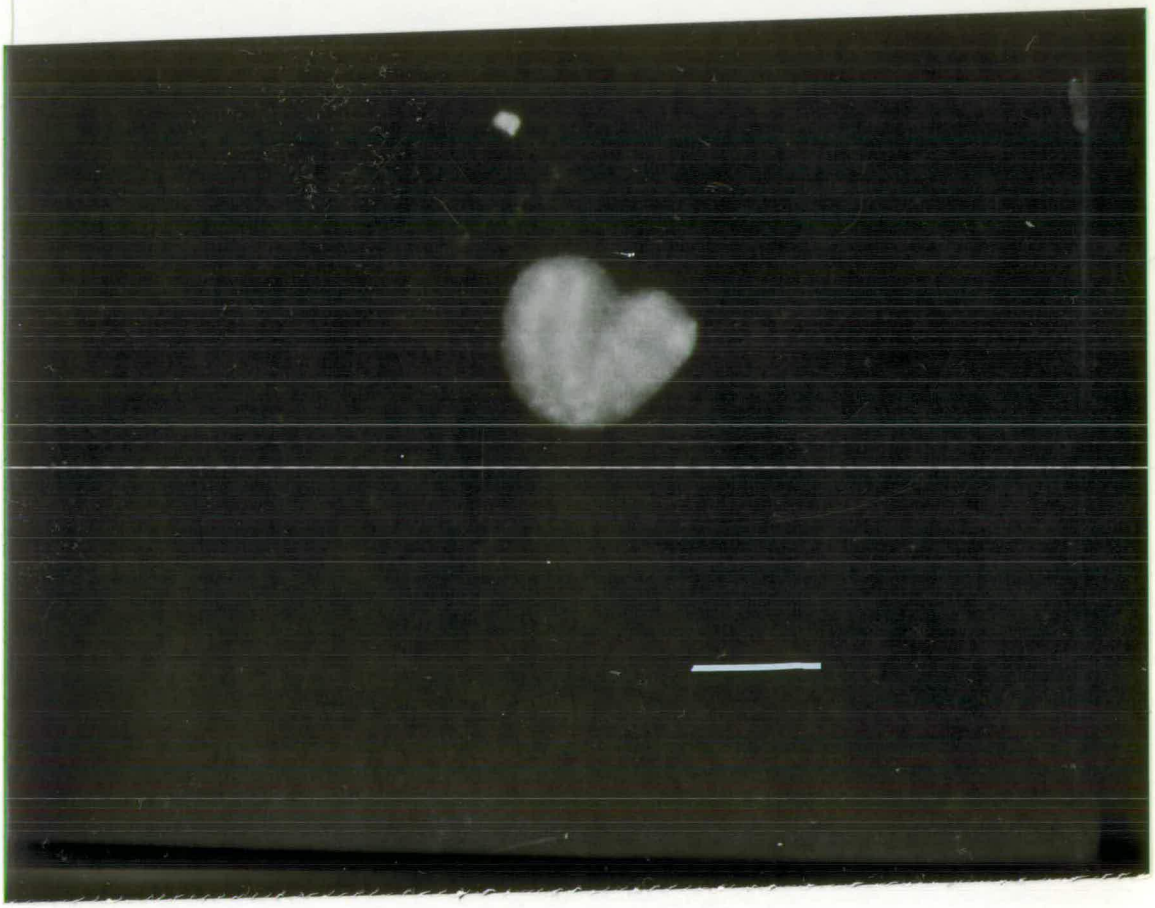
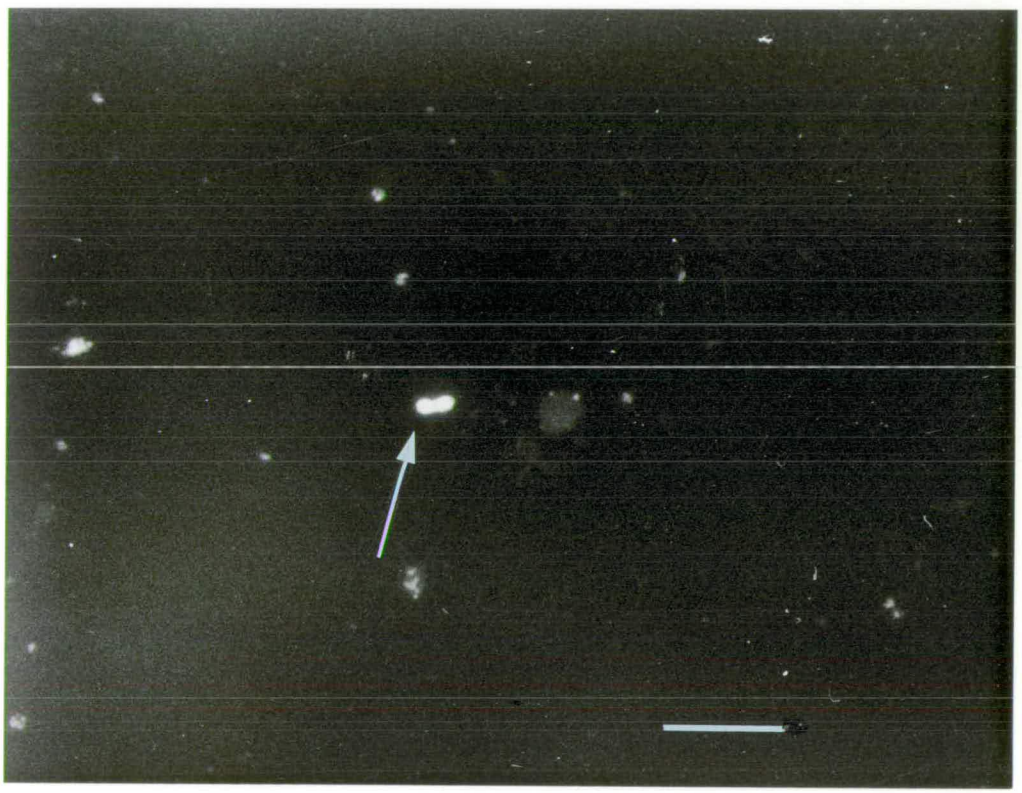


FIG 4.9 2C6: staining of chromosome cores

Don chromosome cores were formaldehyde fixed and processed for immunofluorescence. The arrow marks a typical core. The smaller particles are thought to be disintegrated cores (see Gooderham and Jeppesen, 1983).

Bar= 10μm.



4.2.2 Antibody 2C6-binding to chromosomes, dehistonised chromosomes and cores.

While the experiments described in the above section using whole cells indicated a complex distribution of the 2C6 antigen and a possible association with nuclear DNA, immuno-blotting experiments(see Ch.4.1 above) and dot-blotting (Ch.3.6) had shown the antibody to recognise a protein found in isolated nuclei and chromosomes. The experiments described in this section were directed towards characterising the distribution of the antigen in chromosomes.

Fig.4.7 shows the reaction of 2C6 with Don cell chromosomes, isolated by the KCM procedure and centrifuged onto coverslips. The reaction can be compared with specimens prepared in parallel and stained with the DNA binding fluorochrome Hoechst 33258. In fig.4.7c+d, the chromosomes were fixed in ethanol, and it is evident that this causes a considerable swelling , probably due to the rapid dehydration this fixative causes. This phenomenon will be further considered in 4.2.4 below.

Fig.4.8 shows the reaction of 2C6 with dehistonised chromosomes which were centrifuged onto coverslips and fixed in formaldehyde(a) or ethanol(b). The staining of the formaldehyde-fixed dehistonised chromosomes correlated with the distribution of DNA revealed by Hoechst 33258 staining (fig.4.8c) and suggested that the 2C6 antigen was tightly bound to chromosomal DNA. This was more apparent when ethanol fixed

dehistonised chromosomes were stained. As in the case of chromosomes, the fixative created a very swollen structure, but in many cases chromatid morphology was still visible. The antibody intensely stained the residual chromatid axes, and also a halo of fine filaments around these. The filaments are are only poorly resolved in fig.4.8b owing to the photographic conditions. The distribution of the filaments suggested they were dehistonised DNA fibres, arranged around the chromatid axes in the looped manner described by Paulson and Laemmli(1977).

Finally, as shown in fig.4.9, 2C6 also reacted with chromosome cores, confirming the result obtained by dot blotting(Ch.3.6) and the significance of this result will be further discussed in 4.2.4 below.

4.2.3 Antibody 4G8- binding to fixed cells

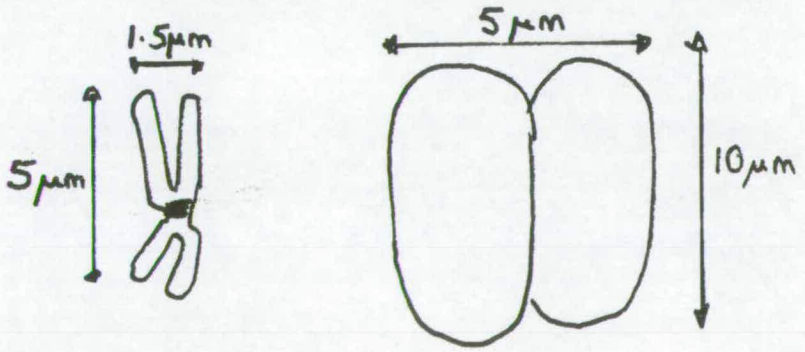
No binding of 4G8 was detected in fixed cells, despite attempting all the treatments described in 4.2.1 above.

4.2.4 Antibody 4G8-Binding to chromosomes and chromosome cores

4G8 was shown by dot blotting to bind a chromosomal antigen, enriched in the chromosome core (Ch.3.6). The experiments described in the following section were aimed at defining the organisation of this antigen within the chromosome.

When formaldehyde-fixed Don chromosomes were probed with 4G8, there was no detectable reaction. However, when the

chromosomes were fixed in ethanol, binding was detected within the more swollen chromosomes. The antibody appeared to recognise an axial element within the chromosome, and this is shown in fig.4.10 . This can be compared with the 2C6-stained ethanol-fixed chromosomes in fig.4.7d. Whilst this substructure was poorly resolved by immunofluorescence, immunoperoxidase gave a much clearer reaction(fig.4.11) and a background level of reaction throughout the chromosome allowed the location of the 4G8 binding element to be visualised. The structure observed was rather variable, but there were two consistent features. First, it was only observed in the most swollen chromosomes, suggesting an internal location normally inaccessible to antibody probing. Note that 2C6 (fig.4.11c) revealed no such substructure. The diagram below compares the typical size of metacentric chromosomes in which 4G8 staining was observed with that of formaldehyde-fixed or unfixed chromosomes.



formaldehyde ethanol

Secondly, the staining appeared to follow an axial element

running along the chromatids. The structure in most cases appeared to be a single element, although in some chromosomes it branched(fig4.11b arrow), possibly at the centromere. Thus, it is possible that this structure represented an axial element within each chromatid, and that these aggregated during fixation. This interpretation, influenced by the models of chromosome structure involving a central protein core within each chromatid(see Ch.1.4) is supported by the results of staining isolated chromosome cores with 4G8 described below. It is unlikely that the axial element was an artefact generated by the ethanol fixation, as the distribution of 2C6 and Hoechst 33258 staining in these chromosomes shows no such substructure.

Fig.4.12 shows the result of staining isolated chromosome cores with 4G8, along with the 2C6 results presented earlier for comparison. The results show that both 4G8 and 2C6 react with isolated chromosome cores. The cores appeared as previously described by Gooderham and Jeppesen(1983), with clear residual chromosome morphology. The antibodies reacted with both ethanol- and formaldehyde-fixed cores, supporting the conclusion reached above that the non-reaction of 4G8 with formaldehyde fixed chromosomes was due to inaccessibility of the antigen, rather than denaturation of the 4G8 binding epitope. As the ubiquitous protein to which 2C6 bound was found in the chromosome core, this structure could not be regarded as containing an exclusive set of proteins forming a chromosome backbone. However, the results clearly suggest that at least one protein of the isolated chromosome core, the 4G8 antigen, has a distinct "core" type of

distribution in intact chromosomes.

Figure 4.10 4G8 staining of ethanol fixed chromosomes

Ethanol fixed Don chromosomes were processed for immunofluorescence as described in 6.4.2. The faint axial staining is arrowed. Bar= 10μm.

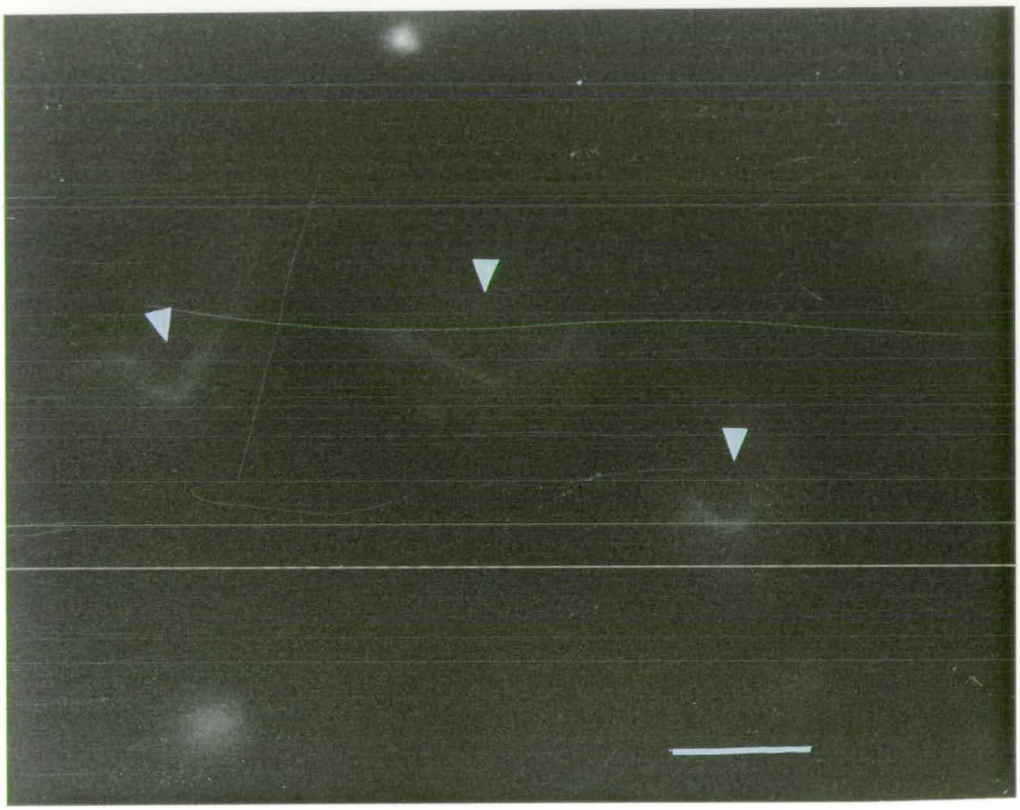


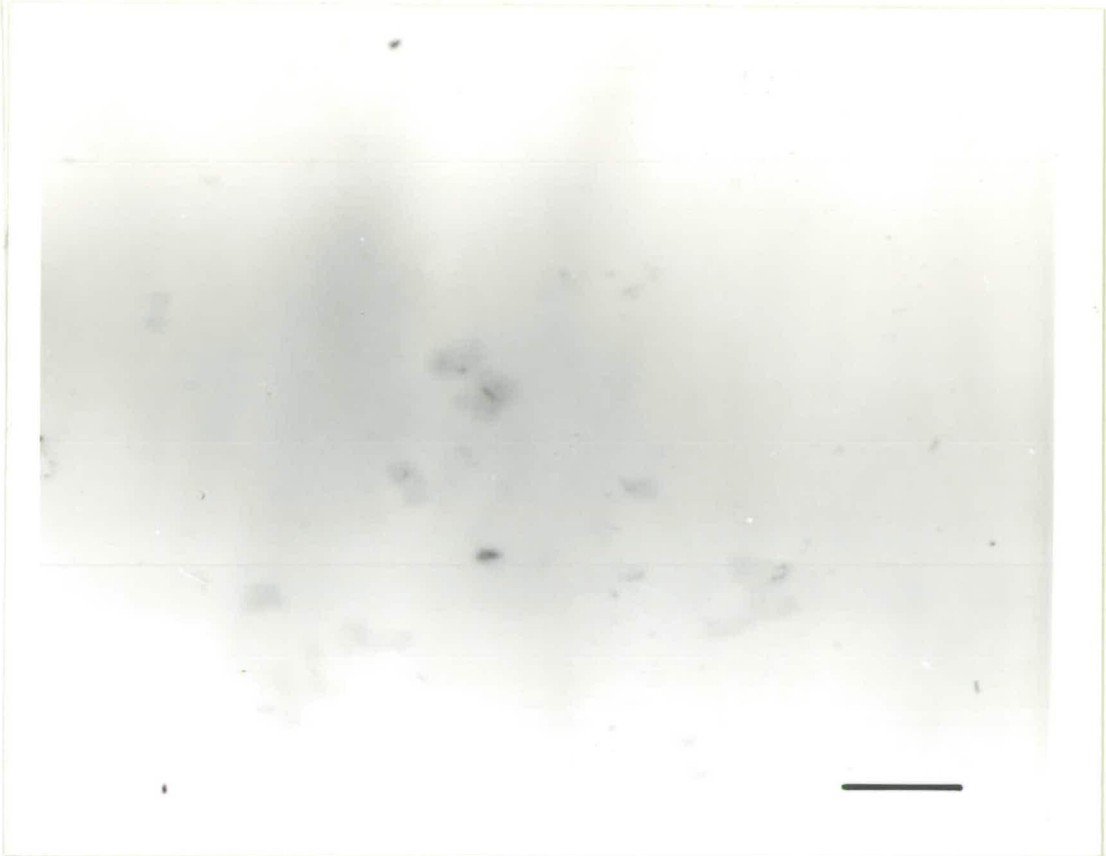
Figure 4.11 4G8 staining of ethanol fixed chromosomes

Ethanol fixed Don chromosomes were processed for immunoperoxidase as described in 6.4.2

a+b) 4G8 stain

c) 2C6 stain

Bar= 10μm.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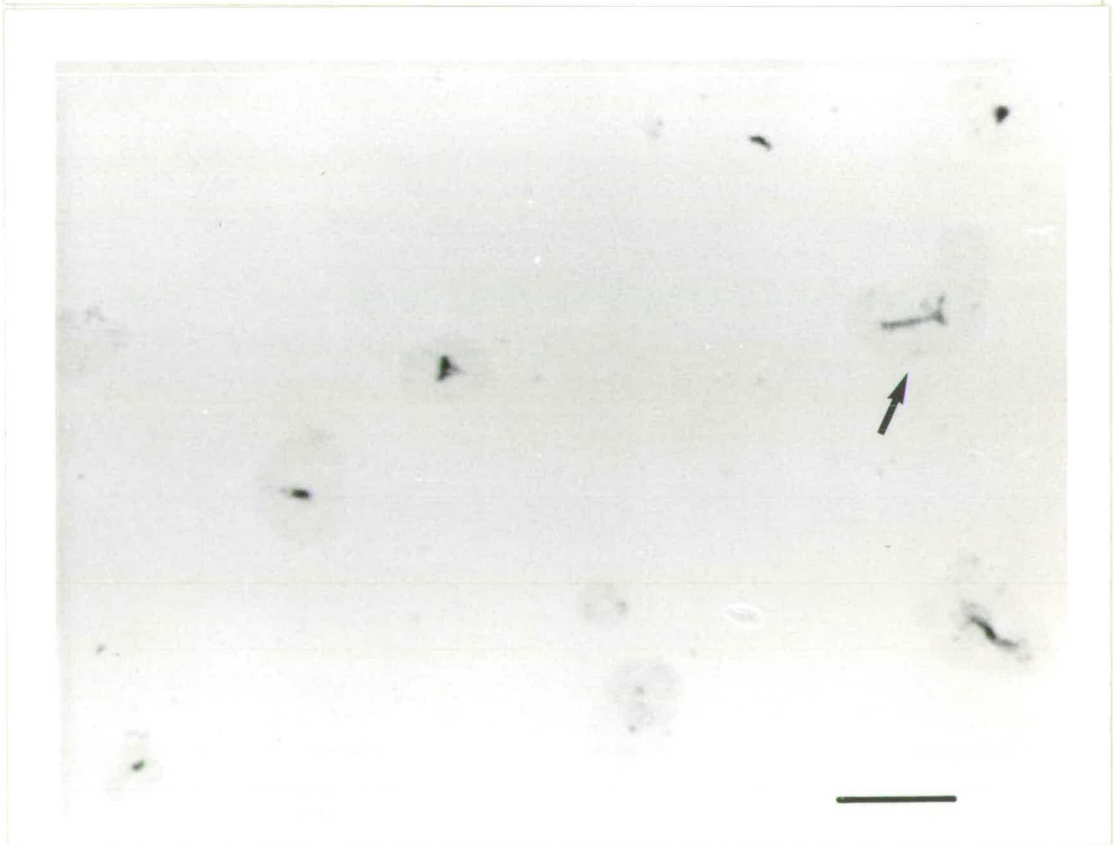

b

C

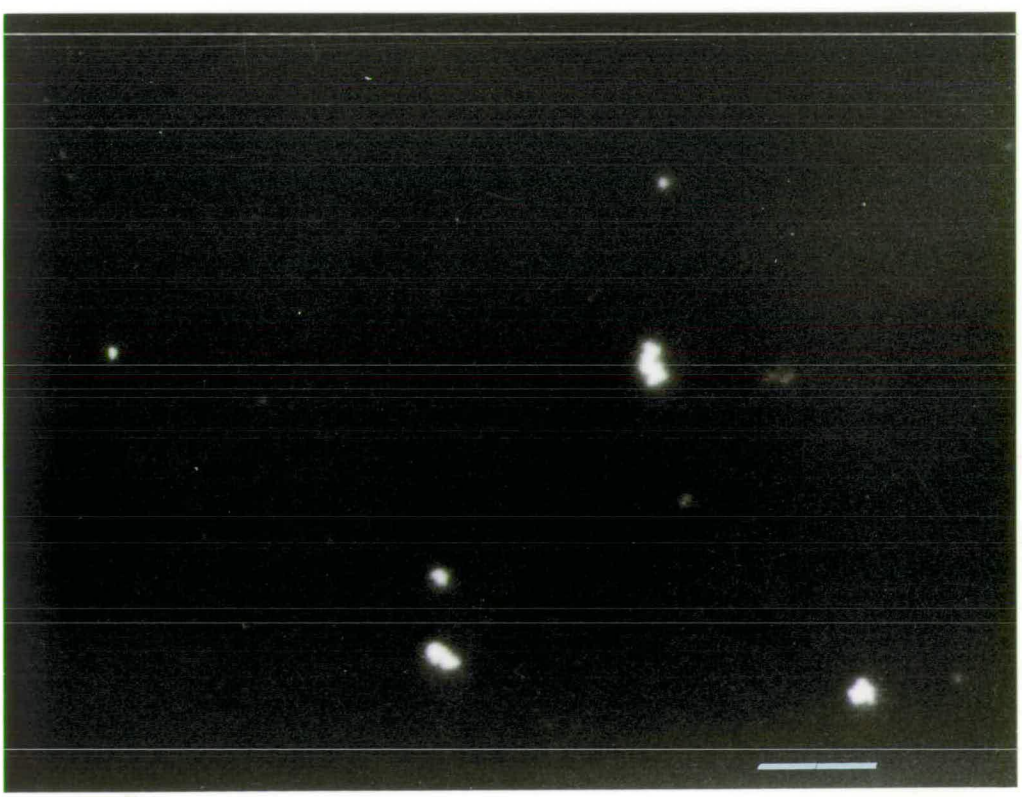
Figure 4.12 4G8 staining of Don chromosome cores

Chromosome cores were fixed and stained as described in 6.4.2.

a) 4G8 staining after ethanol fixation (immunoperoxidase).
b) 4G8 staining after formaldehyde fixation (immunofluorescence).
c) 2C6 staining after ethanol fixation (immunofluorescence).

Bar= 10μm.

a



b

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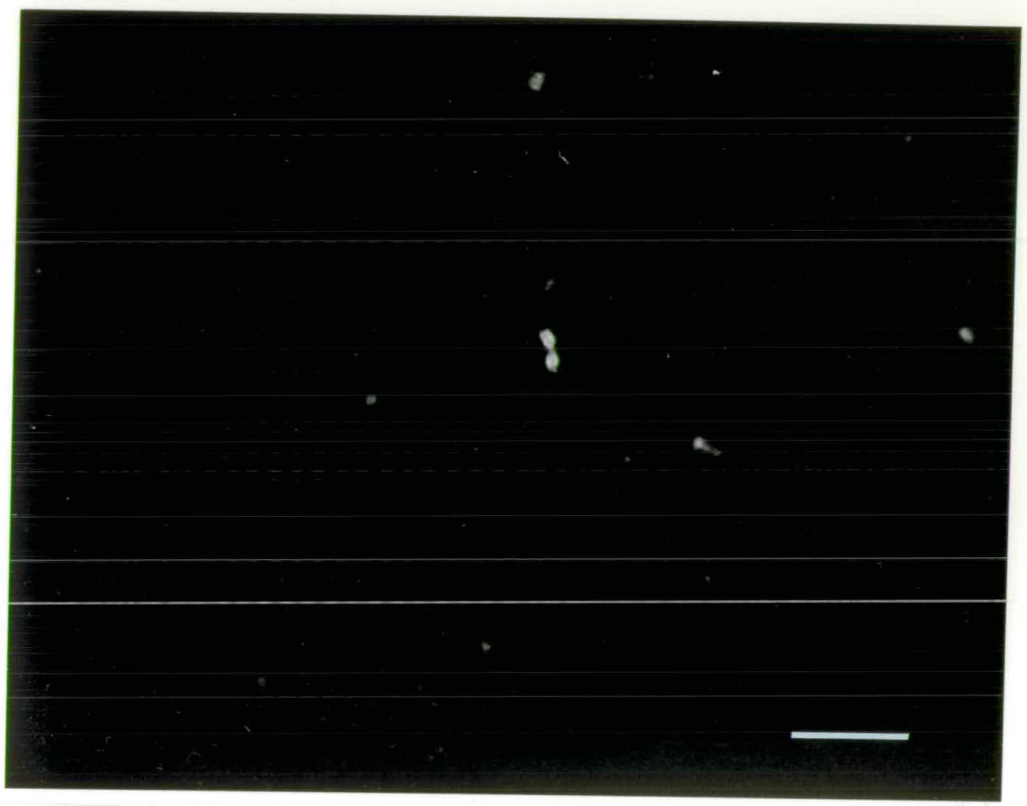


4.3 IMMUNO-ELECTRON MICROSCOPY

In the previous section, two types of chromosomal protein distribution were defined; the ubiquitous 2C6 antigen, and the core localised 4G8 antigen. In order to extend these observations, electron microscope specimens of isolated chromosomes and sectioned mitotic cells were probed with the monoclonal antibodies. Monoclonal-antibody binding was visualised with an antibody to murine immunoglobulins, itself labelled with colloidal-gold. The chemistry and applications of colloidal gold probes have been reviewed by DeMey (1983).

Thin sections of mitotic Chinese hamster CHO cells were first probed. Okada and Comings (1980) argued against the existence *in vivo* of the chromosome core because no such structure had been observed in preparations of this kind. Their findings relied on conventional heavy metal staining of thin E-M sections and in view of the high protein concentration in metaphase chromosomes, the result is not surprising. The immunoprobing of such preparations with a monoclonal antibody to a putative core protein seemed to offer the ideal method to demonstrate the presence or absence of a chromosome core.

A second series of experiments involved the probing of whole mount isolated Don chromosomes, and attempted to reproduce the immunofluorescence and immunoperoxidase results presented in the previous section.

4.3.1 4G8 and 2C6 binding to CHO cell thin sections

CHO cells, enriched for mitoses with colcemid, were fixed, impregnated and embedded in Lowicryl K4-M (Roth et al, 1981). Thin sections were probed with monoclonal antibodies and 10nm-colloidal-gold-particle —labelled second antibodies. In all experiments antibody-free culture medium was used instead of hybridoma supernatant on control specimens.

When thin sections were probed with 2C6, a heavy labelling of chromosomes was found, with very little cytoplasmic reaction. Fig.4.13 shows a representative cell, and although a region of denser labelling can be seen near the telomere of a presumptive large metacentric chromosome, in the majority of chromosomes the labelling was uniform. Some clusters of gold particles were observed over the cytoplasm eg. fig.4.13b-arrow, but it was not possible to determine whether these were significant, control specimens showing similar levels of cytoplasmic labelling (not ill.). The gold particles also appeared to bind in clusters to chromosomes, but this was most probably due to inhomogeneities in the surface of the resin and to the fact that the antibodies could only penetrate to antigens on or very close to the surface of the section.

Fig 4.14 shows the binding of 2C6 to an interphase CHO cell. The nucleus was less heavily labelled than mitotic chromosomes, but there was still a clear specificity for the nucleus. Fig 4.14b shows a close up view of an invagination of the nuclear membrane, and demonstrates the 2C6 antigen to be

intranuclear and not on the membrane, lamina or pore complex. This result supports the results of immunofluorescence on fixed culture cells in 4.2.1., in which an intranuclear location was suggested by the antigen's inaccessibility to probing.

Sectioned CHO cells were probed with 4G8 with the aim of providing evidence for the location of the 4G8 antigen at the core of the chromosome, and hence for the presence of the chromosome core *in vivo*. The findings, as shown in figs.4.15 and 4.16, were inconclusive. Fig 4.15a+b show the most frequently obtained result (from approx 500 cells examined), with virtually no labelling at all apart from the control level. However, a few cells were found with clusters of gold labelling upon apparently transversely cut chromosomes. The problem in interpreting these results is that upon close examination, similar clusters were found in the cytoplasm of the same and other cells, suggesting that the few cells showing chromosomal labelling represented non-specific antibody binding. The results are illustrated in figs.4.15c+d and 4.16. In fig 4.15c, the chromosome arrowed appears to be cut transversely through the two chromatids, and at high magnification a striking cluster of gold particles can be seen upon the "core" of one chromatid. Another example of this type of result is shown in fig.4.16, and in this case extrachromosomal labelling is evident, demonstrating the difficulty in assessing the significance of the "core" labelling.

If the few examples of labelling upon the axis of chromosomes represent the core visualised in 4.2.4 by

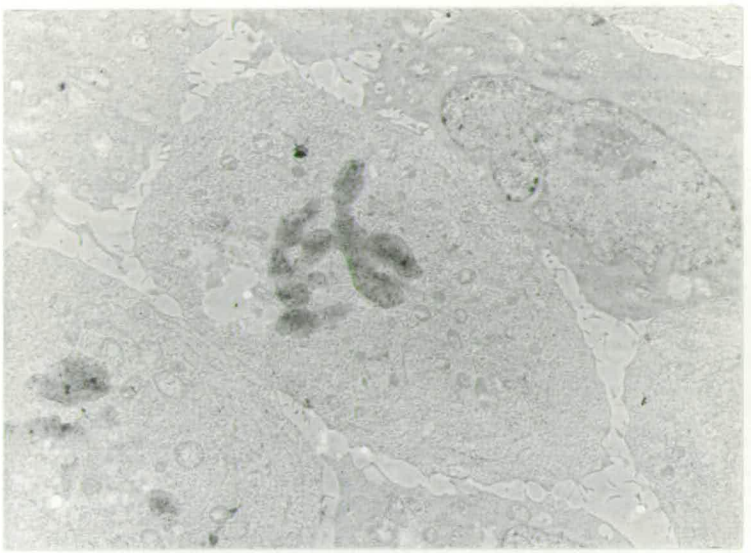
Figure 4.13 Immunogold staining of CHO cell Lowicryl sections-antibody 2C6

Series of magnifications on one cell. Stained for 2min in uranyl acetate (saturated in 50% ethanol) before viewing.

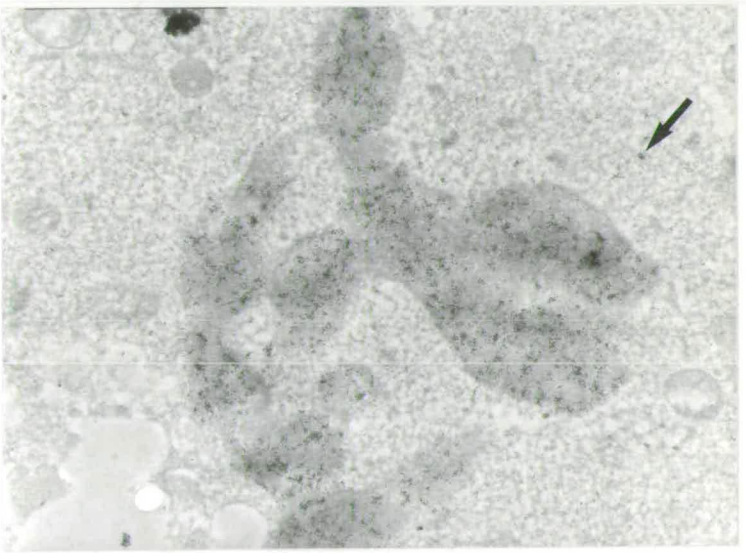
a) x3650

b) x11770

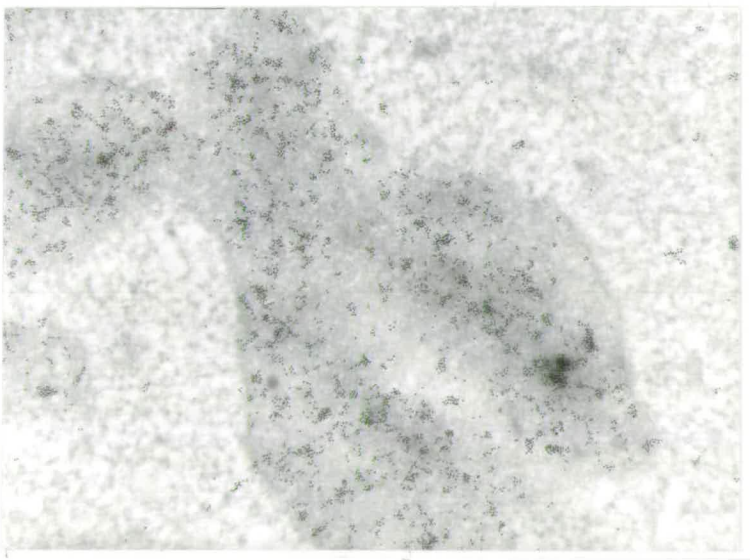
c) x20880



a



b



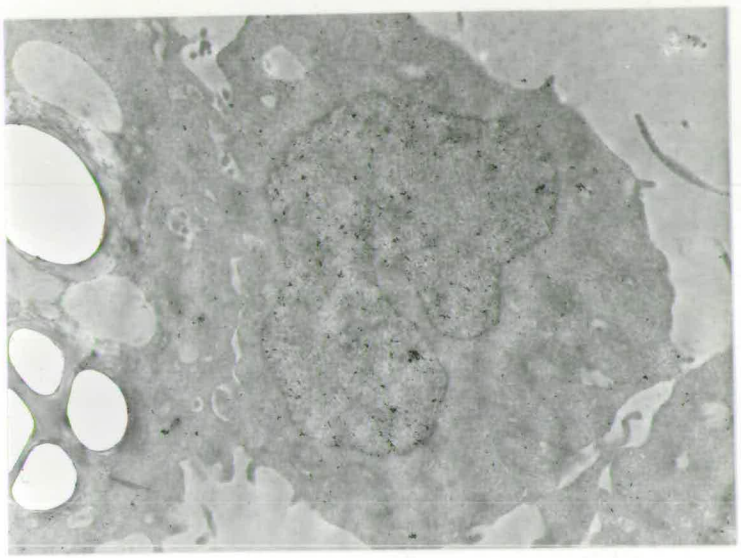
c

Figure 4.14 Immunogold staining of CHO cell Lowicryl sections-antibody 2C6

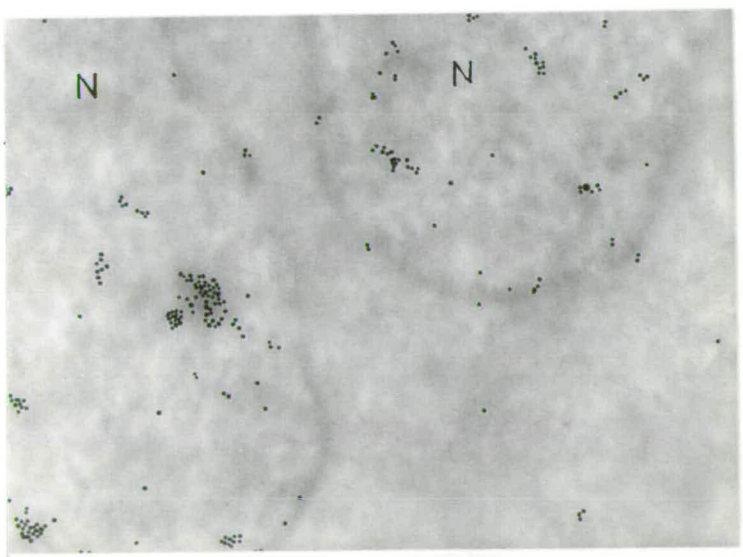
2C6 binding to the nucleus:

a) view of whole cell x9300

b) detail on nuclear membrane. N= nucleus x50000



a



b

Figure 4.15 Immunogold staining of CHO cell Lowicryl sections

Reaction with 4G8.

a+b) Two views of a metaphase cell showing the most frequently obtained result, with no significant reaction. a=x6400, b=x18600.

c+d) Two views of a cell exhibiting chromosome "core" reaction. c=x4700, d=x20500.

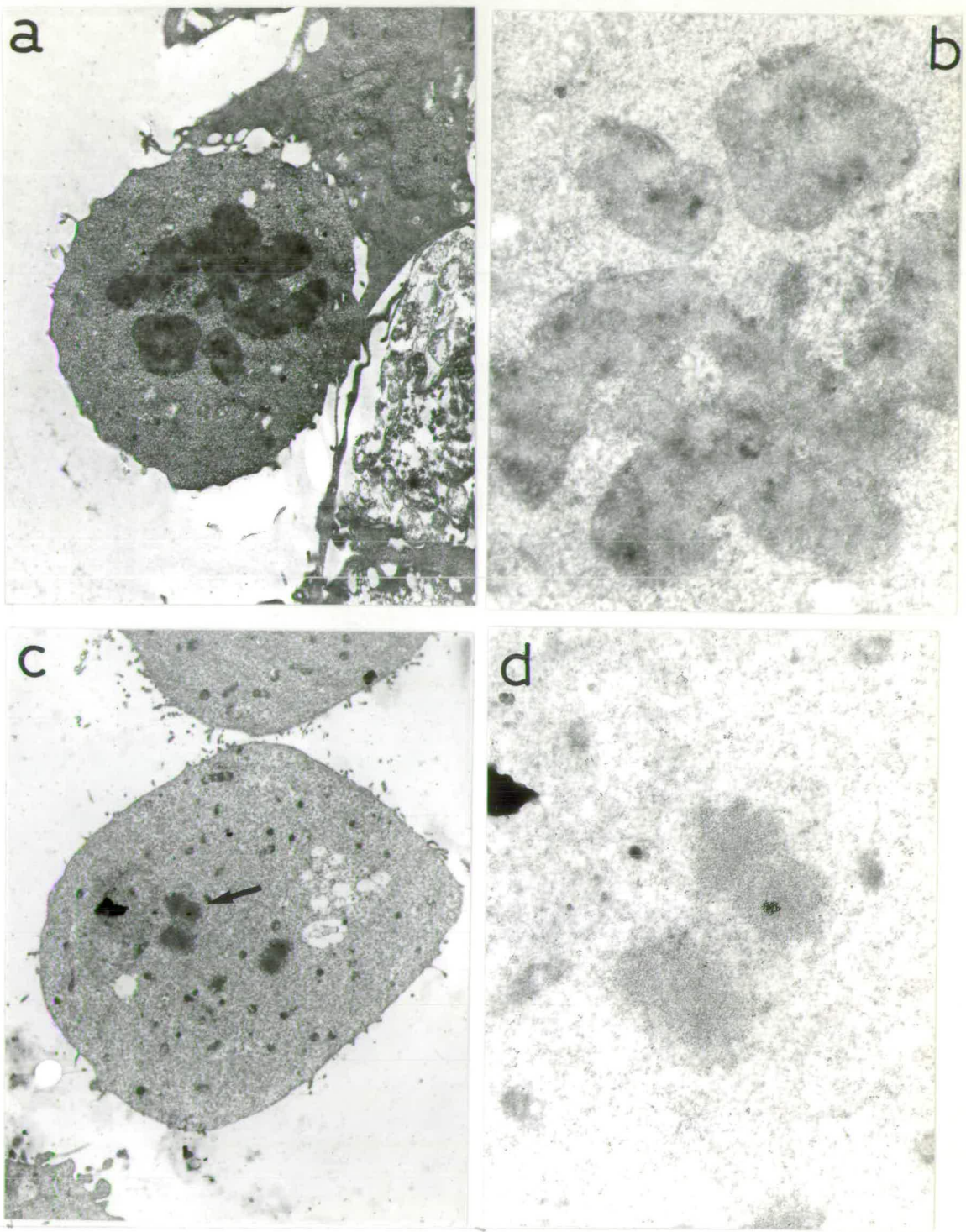
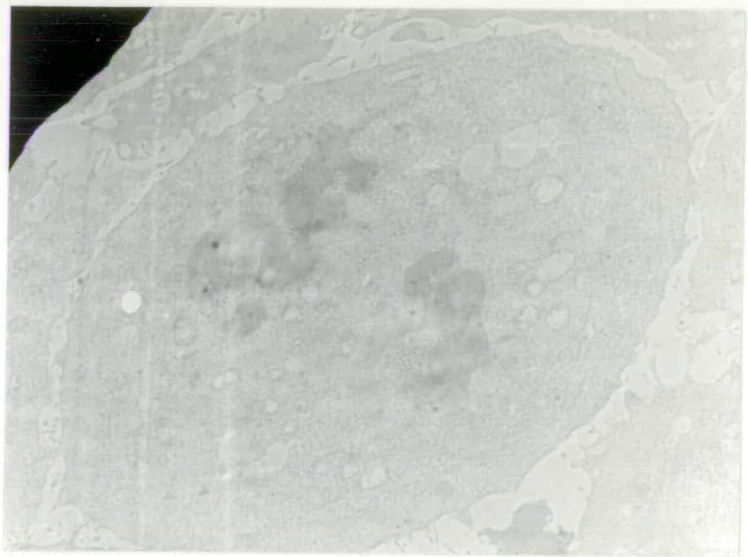
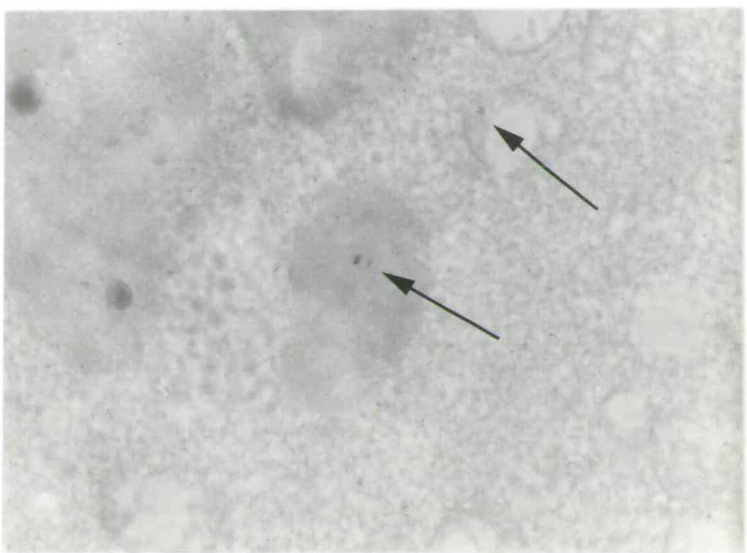

a
b
c
d

Figure 4.16 Immunogold staining of CHO cell Lowicryl sections

a+b) Another example of 4G8 staining a possible chromosome core structure. a=x3650, b=x18600.
In (b) arrows mark clusters of gold particles both on the core of a chromosome and on the cytoplasm.



a

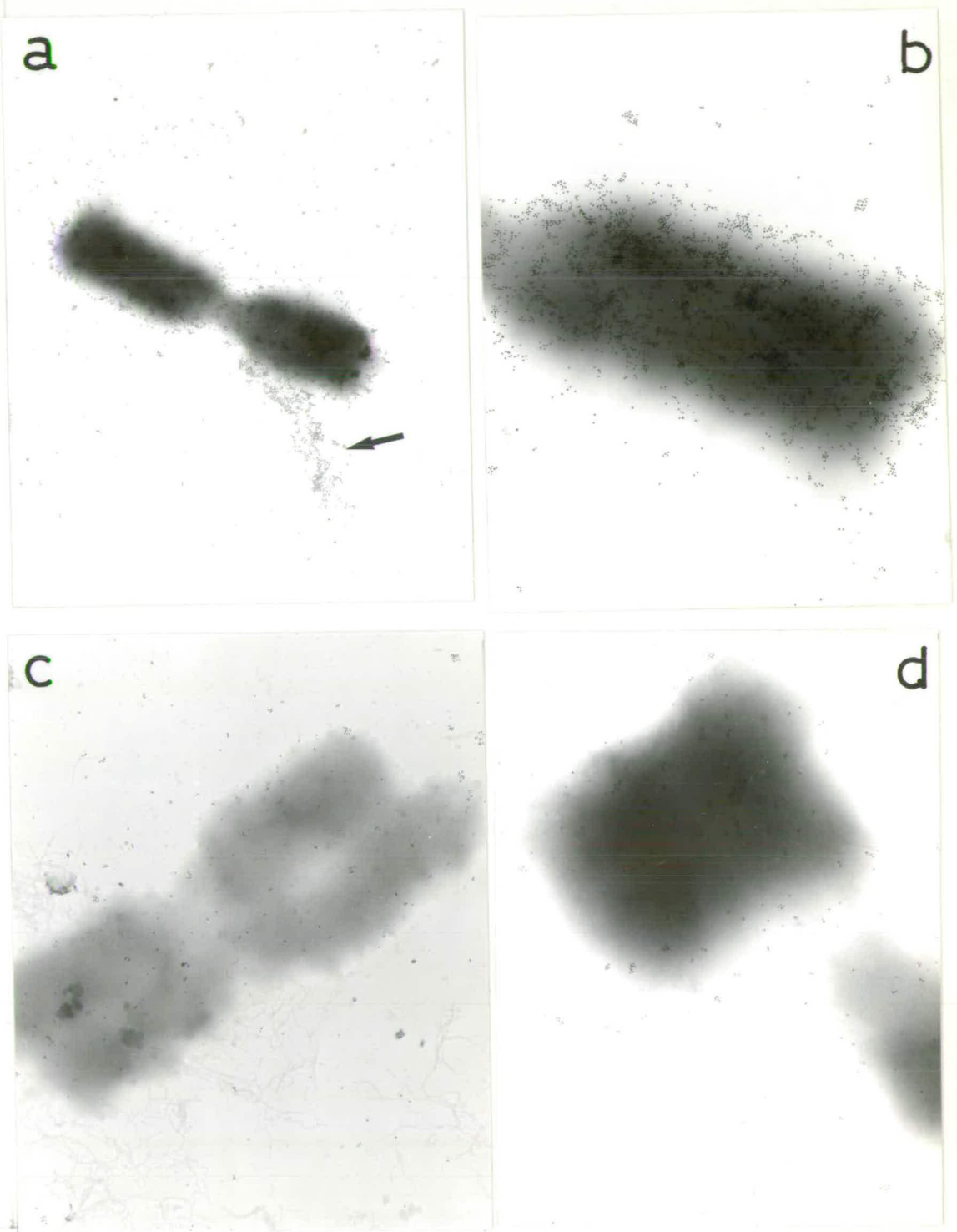


b

Figure 4.17 Immunogold staining of whole mount Don cell chromosomes

Reaction with 2C6: a) x15800, b) x26200.

Reaction with 4G8: c) x15800, d) x26300.


a
b
c
d

immunofluorescence, the rarity of such results could be explained on the following grounds. First, the immunofluorescence results suggested that cores are very fine structures, and may therefore be below the limit of sensitivity of the immunogold technique. Also, at this level of ultrastructure, roughness in the surface of the section may make some observed structures inaccessible to antibody probing, as seems to be the case for the experiments using 2C6 described above. Finally, the 4G8 antigen may not be stable to the treatments used in the preparation of the electron-microscope sections.

On the other hand, the core labelling may have been due to chance non-specific binding, and this question can only be resolved by further analysis of many more cells.

4.3.2 4G8 and 2C6 binding to isolated Don chromosomes

Probing isolated chromosomes deposited on electron-microscope-grids offered an alternative approach to immunoelectronmicroscopy, and it was attempted to reproduce the conditions used in Ch4.2.4 for the visualisation of the 4G8 antigen using ethanol fixed chromosomes mounted on electron-microscope grids instead of glass cover slips.

Chromosomes, prepared by the KCM method (Ch.2.2), were picked up on carbon coated electron-microscope grids and fixed with ethanol. This fixation procedure had been shown to open up the chromosome sufficiently for immunofluorescent visualisation of a "core" structure (Ch.4.2.4). Formaldehyde fixed chromosomes

were too electron-dense to allow good contrast between them and the gold particles, and were therefore unsuitable for this work.

Experiments using 2C6 confirmed the sensitivity of the technique. Fig 4.17a+b show two views of a metacentric chromosome which was heavily labelled with 2C6. Electron transparent, but heavily labelled material, was often seen "spilling" out of chromosomes (fig 4.17a-arrow), and as the 2C6 antigen had been shown to be tightly bound to chromatin fibres (Ch.4.2.3), it appeared that these regions represented a degree of loss of chromosome integrity during preparation.

When 4G8 was used to probe chromosomes in the same manner, only a background level of labelling was found (fig 4.17c+d), equivalent to that found in controls using the gold labelled second-antibody alone. The most likely explanation of this result is that the chromosomes were insufficiently expanded to allow antibody access to intrachromosomal material. In Ch.4.2.4, ethanol-fixed chromosomes on glass cover slips were vastly expanded compared to formaldehyde-fixation, and "core" structures were visualised in metacentric chromosomes with lengths greater than 10μm and widths greater than 5μm. The largest metacentric chromosomes found in the E-M specimens were much less expanded, with lengths up to 5μm and widths less than 1μm. This difference in the response of chromosomes to ethanol fixation can only be explained in terms of the difference in interaction between glass and carbon substrates with chromosomes. Thus, further experiments using alternative substrates

such as parlodion are required.

4.4 SUMMARY

The 4G8 antigen is a protein located within the core of the chromosome.

The results described in this chapter are probably the first direct demonstration of a core type structure within an intact mammalian metaphase chromosome, without recourse to harsh treatments such as silver staining (Satya-Prakash et al, 1980). The argument that 4G8 binds a protein in the core of the chromosome rests on three pieces of evidence.

i) 4G8 antigen was found to be enriched in isolated cores compared to intact chromosomes (Ch.3.6).

ii) 4G8 antigen was found to be inaccessible to antibody except under conditions where the chromosome was vastly swollen.

iii) In the most swollen chromosomes, the antibody appeared to bind to an axial substructure.

These results also validate the use of isolated chromosome cores or scaffolds as representations of a genuine chromosome substructure, and hence as a source of proteins potentially involved in chromosome organisation. However, one reservation must be noted. The 2C6 antigen, which was shown to be a generally distributed chromosome protein, was also found within the core. In other words, the core probably consists of two types of protein, those exclusive to it and also some that are found

throughout the chromosome. This type of organisation together with the possible functions of the proteins will be discussed in chapter 5.

The 2C6 antigen is an abundant nucleocytoplasmic protein of unknown function.

The immunofluorescence results both on the cytoplasmic distribution and chromosomal distribution of 2C6 indicated that it was very abundant. However, this protein appears to be completely unknown. It forms a cytoplasmic filamentous system and also seems to be tightly bound to chromatin. The main value of the antibody in this work was to provide a control marker against which the binding of 4G8 to chromosomes could be compared. The 2C6 antigen requires further research in its own right, and some ideas with respect to this and its function are given in the general discussion.

Chapter 5 GENERAL DISCUSSION

At the outset of the work described in this thesis, the emphasis was on the characterisation of the proteins found in the isolated chromosome core or scaffold, argued by Jeppesen and co-workers (Gooderham and Jeppesen, 1983) and Laemmli (Lewis and Laemmli, 1982) to represent a non-histone protein substructure involved in maintaining the gross structure of the chromosome and the organisation of the packaged DNA molecule.

This approach was complicated by the fact that cores could only be generated in small quantities, that they were highly refractile multiprotein complexes and that there was a fundamental uncertainty as to whether a certain fraction of the core proteins were merely the products of the aggregation of the dense chromatin during chromosome dehistonisation as suggested by Okada and Comings (1980), Hadlaczky *et al* (1981) and Burkholder (1983). The above difficulties lead to the approach of developing monoclonal antibodies as probes for the components of chromosome cores, in an attempt to characterise them and define their relationship to *in vivo* chromosome structure.

5.1 The use of monoclonal antibodies as probes of chromosome structure.

The hybridoma technique seemed ideally suited to the analysis of the chromosome core, in that it enabled monospecific antibodies to be raised against individual antigens in a complex mixture of proteins. Once obtained, antibodies could be used in two ways: first to locate their antigens within chromosomes using immunocytological procedures, and secondly to identify by "western-blotting" which polypeptides of the chromosome core these were. As described here, only two antibodies have been characterised in detail and these provide strong evidence for the conclusion that at least one protein of the chromosome core occupies an exclusively axial (core type) location within the intact chromosome and that conversely, at least one more protein of the core is distributed more generally throughout the chromosome and in this case the cell also. The main problem encountered using this approach for 4G8 and 2C6 was the poor quality of the "western-blotting" data, the possible reasons for which are discussed in Ch.4.1.. While this meant that the biochemical characterisation of the chromosome core proteins has been limited, the ultrastructural data obtained has been very valuable-particularly that for 4G8 which has provided very strong evidence for the core as a morphological entity in the mitotic chromosome in vivo.

In recent years, various groups have published results of research into chromatin organisation and nuclear structure using

monoclonal antibodies (see Bhorjee et al, 1983 & Kane et al, 1982), however in these reports the approach taken was very much of a "shotgun" type, in which nuclei or chromatin extracts were used to raise random libraries of hybridomas.

The work described here shows that monoclonal antibodies can be used with great success in dissecting the structure of the eukaryotic chromosome. In the following discussion, the nature of the antigen to which 4G8 and 2C6 bind will be further examined, and some speculation will be made as to their function in relation to the wealth of information on chromosome structure available from other approaches.

5.2 The 4G8 antigen: a chromosome core protein.

The evidence for the location of the 4G8 antigen at the core of the chromosome has already been discussed in Ch.4. It is unfortunate that the "western blotting" data has yet to reach the quality required to make an identification of the polypeptide involved, however at a molecular weight of 67000 it fits within a group of bands seen on SDS gels of isolated chromosome cores (see Ch.2 and Gooderham and Jeppesen, 1983). These proteins are very minor constituents of the intact chromosome, for example note the extremely weak immunofluorescence obtained in fig.4.10.

The function of the 4G8 antigen is harder to deduce. Its presence at the core of the chromosome does not require it to

have a structural role, such as the anchoring of looped DNA. It may for example be involved in a regulatory process, perhaps being stored at the core of the chromosome as a pool available for use when the nucleus reforms. However, a structural function was suggested by the finding that the 4G8 antigen was located in interphase nuclei and enriched in the nuclear matrix. The matrix has long been associated with at least one level of higher order chromatin organisation (see Ch.1 & Lebkowski and Laemmli, 1982), and thus this protein satisfies a basic requirement for a component involved in chromatin organisation throughout the cell cycle. On the other hand, its presence in the nucleus may be simply as an inactive pool in storage for a functional role in mitosis, and this would explain why in the immuno E-M probing of sectioned cells, no significant binding of 4G8 to the nucleus was observed. Another possible function of the 4G8 antigen may be as envisaged by Dounce et al (1973) for a chromosome core protein. In this model the core protein is bound to DNA at regular intervals and promotes chromosome condensation by disulphide cross-linking into a proteinaceous core. Indeed, Jeppesen and Morten (1984) have provided evidence that this type of cross-linked protein core may be involved in maintaining the metaphase state. The model of Dounce et al (1973) envisaged the core proteins to be covalently linked in the DNA primary sequence, and although this idea runs against current thinking, it is noteworthy that Werner and co-workers have found a set of proteins which are tightly bound to DNA, behaving exactly as would be expected of internucleotide covalent linkers

(Werner et al, 1980). Moreover the estimated spacing between these linkers is 30,000bp, similar to the size of looped domains of DNA in interphase and metaphase (see Ch1.3). Interestingly, one of these proteins has a molecular weight almost identical to the 4G8 antigen and is located in the nuclear matrix (Werner et al, 1984). These findings have been recently supported by those of Bodnar et al (1983), who found an apparently identical set of tightly bound proteins at 40,000bp spacing along HeLa DNA, and with a nuclear matrix enrichment, although they argued against the proteins being covalently bound to DNA. It will be of interest to examine the relationship of these tightly bound proteins to the chromosome core proteins, and 4G8 seems to be the ideal tool for this kind of comparison.

If a relationship between the chromosome core proteins and the above mentioned tightly bound proteins exists, there is one very speculative experiment which would be of interest. Both Bodnar et al,(1983) and Werner and Petzelt (1981) found evidence of the tightly bound proteins also being found associated with bacterial DNA. The bacterial chromosome is known to be organised as independent loops radiating from a membrane bound ribonucleoprotein focus or core (Kavenoff and Ryder, 1976). Could this be the fundamental unit of, and evolutionary precursor of the eukaryotic chromosome? One way in which this could be investigated would be to look for proteins common to the eukaryotic and prokaryotic chromosome core, using antibodies such as 4G8.

The true function of the 4G8 antigen will require much

further work. The experiments described in this thesis pave the ground for a detailed study of a chromosome core protein, and the following approaches now seem likely to be of value:

i) Comprehensive analysis of the 4G8 antigen by "western blotting". Once an unequivocal identification of the polypeptide involved has been made it will be possible to investigate its DNA binding properties and any cell-cycle-dependent covalent modifications. The possible sequence- specific DNA binding of the chromosome core proteins has previously been discussed by Jeppesen and Bankier (1979) and would seem to be a strong candidate for the mechanism of anchorage of DNA loops at constant points both on the nuclear matrix and chromosome core. As the core proteins might well also be involved in the condensation of chromosomes, they might undergo covalent modifications to trigger this process, leading to conformational changes which allow cross-linking to form a core.

ii) Further immuno-electronmicroscopy is required to confirm the core location of the 4G8 antigen in sectioned chromosomes and to locate its presence in the nucleus.

iii) If the 4G8 antigen plays a key role in chromosome structure one would expect it to conserved over a wide range if not all eukaryotes. The data given here shows it to be found in man and chinese hamster. This can now be extended to see if this protein is highly conserved.

5.3 The 2C6 antigen is an unknown nucleocytoplasmic protein.

The distribution of the 2C6 antigen is quite remarkable. In intact cells it constitutes a cytoplasmic filamentous system and also is a major component of the nucleus and chromosomes, albeit masked to immuno-probing without the use of urea and trypsin pretreatments. Interestingly, Turner (1982) found that trypsin treatment improved the immunofluorescent staining of nuclei by a monoclonal anti-histone 2B. What this means in molecular terms is unclear. The fixation treatments used would be expected to disrupt, if not remove, the nuclear membrane altogether. However, there may be other structures at the nuclear cortex, such as the lamina, which obstruct antibody access. The abundance of the 2C6 antigen is made clear from the intensity of immunofluorescence obtained from the nucleus (fig.4.5a), or cytoplasm (fig 4.3a). The cytoplasmic fluorescence alone was qualitatively estimated to be of the same magnitude as obtained with antibodies to the major cytoskeletal proteins, tubulin and actin. The evidence from figs.4.6 and 4.8 on dehistonised nuclei and chromosomes show that the antigen was tightly bound to DNA fibres. This could be a result of its dissociation from the chromosome under high salt treatment and non-specific reassociation with the rather "sticky" free DNA, however the intensity of staining of the DNA fibres argues against this. Finally, the 2C6 antigen is found in the chromosome core, and if it is uniformly distributed along DNA, this result is not surprising, as 0.1-0.5% chromosomal DNA remains within the core (Jeppesen and Bankier, 1979).

Before adding any further interpretation to these results, a note of caution must be repeated. The "western-blotting" data on the 2C6 antigen was very poor. This was interpreted in Ch.4 as being due to the antibody exhibiting a low affinity for blotted proteins, possibly due to epitope denaturation. This is also suggested to some extent by the "dot-blotting" results in Ch.3-fig.3.4 , where 4G8 gave a much stronger signal on SDS solubilised chromosomal proteins than 2C6, even though the immunocytology results of Ch.4 showed the 2C6 antigen to be much more abundant. However, another possibility exists- namely that the weak binding observed to a 67,000 M_r polypeptide was artefactual, and that the 2C6 antigen is not a protein at all. In view of its abundance, it could be a trivial antigen, such as a carbohydrate moiety on a glycoprotein. Alternatively, 2C6 may react with an epitope common to several proteins.

I will continue this discussion by assuming that the 2C6 antigen is a single protein, for it is not uncommon to find monoclonal antibodies which are totally unreactive in "western-blotting" type experiments. If more reliable "western-blotting" data cannot be obtained, the only approach to demonstrating that the 2C6 antigen is a single protein will be to isolate it. For such an abundant protein, this should be possible either by immunoprecipitation (Davis et al, 1983), or by the screening of cDNA expression libraries (Helfman et al, 1983). This approach was given a preliminary attempt during the course of this work, using a human liver poly-A RNA library in E.coli (isolated by Woods et al, 1982), and although no clones were found producing

the 2C6 antigen, only a small fraction of the library was examined. It should be noted that although all the work reported in this thesis has been on cultured cells, the 2C6 antigen is present in human liver cells (unpublished immunohistochemical observations by Miss C. McCauley).

There seem to be two possibilities for the function of the 2C6 antigen. First, it may be an as yet unidentified cytoskeletal and karyoskeletal protein. Capco and Penman (1983) have observed continuous filaments between the chromosomes, the mitotic apparatus and the plasma membrane in mitotic cells using long-depth-of-field whole-mount electron-microscopy. Also Maul (1977) has produced electron micrographs showing filaments continuous from within to without the nucleus. This would explain the distribution of the protein, but not its chromosomal abundance. A more likely explanation is that the 2C6 antigen is a fundamental protein required for chromatin organisation or the control of transcription/replication processes. This would explain its uniform distribution along the DNA fibre. The cytoplasmic filamentous system stained by 2C6 might then represent this protein in transit from ribosomal synthesis to the nucleus, for such an abundant protein would require a high rate of synthesis.

5.4 CONCLUSIONS

Taken together, the results reported here lead to the following conclusions:

1 The metaphase chromosome contains at least one protein, the 4G8 antigen, distributed along a substructure occupying an axial location.

2 This protein is a major component of isolated chromosome cores or scaffolds, and therefore provides strong evidence that these isolated substructures represent their proposed counterparts *in vivo*.

3 The chromosome core and interphase nuclear matrix contain common components, and this probably reflects an underlying common form of higher order chromatin organisation throughout the cell cycle.

4 A hitherto undiscovered, highly abundant nucleocytoplasmic protein (the 2C6 antigen) is a major component of metaphase chromosomes.

5.4.1 Eukaryotic chromosome structure in light of the above conclusions.

While the basic principles of higher order DNA folding within the eukaryotic chromosome are now understood (see Ch. 1.3), we are still only able to build relatively simple models for what must be a complex system; encompassing DNA loop

anchorage, the organisation of specific DNA sequences and a mechanism to effect chromosome condensation. Bearing these factors in mind, the following model can be constructed for chromosome organisation. While it is very speculative, it synthesises the results of this thesis and of other workers into a catalyst for further research.

The proposed organisation of the metaphase chromosome is of the core type as proposed by Lewis and Laemmli (1982) and Jeppesen and Gooderham (1983) ; involving a chain of non-histone protein running along the chromatid axes and anchoring the looped DNA. Possibly these proteins are cross-linked by disulphides (see Jeppesen and Morten, 1984) or metalloprotein interactions (Lewis and Laemmli, 1982), which would allow the disassembly of the core as the chromosomes decondense. In this case, the core would be associated with a variety of other components, such as enzymes involved in its formation. The isolated core used in this work, prepared by the nuclease digestion of dehistonised chromosomes, would be expected to contain all these components plus a certain amount of residual DNA. Also co-purifying with this DNA will be any tightly bound proteins- such as the 2C6 antigen. Also some association between the core and the kinetochore, upon which the mitotic spindle acts, would be expected. Earnshaw et al(1984) have used autoimmune sera to two kinetochore proteins to show that they are retained in isolated chromosome cores. These proteins are probably not part of the core structure running along the chromatid axis in intact chromosomes, but probably tightly interact with the axial core,

which then provides a framework over which the "pull" of the spindle microtubules can act at anaphase.

The fate of the core at interphase is unclear. The core proteins evidently dissociate (this provides a mechanism for chromosome decondensation) and become associated with the nuclear matrix (Lebkowski and Laemmli, 1982). While the looped organisation of chromatin is retained in the nucleus, it is not possible to predict whether these loops remain anchored and organised by the chromosome core proteins or by a new set of matrix proteins.

This model provides a direction for future research, and the following types of approach are likely to be of value.

(i) Using monoclonal antibodies as described in this thesis. The potential of monoclonal antibodies has already been discussed, and one can easily envisage that a well characterised antibody to a core protein could be mapped right through the cell cycle.

(ii) The investigation of the residual DNA sequences associated with the core, and any resultant DNA:protein interactions (Jeppesen and Bankier, 1979; Lebkowski and Laemmli, 1982b).

(iii) The investigations of chromosomes in lower eukaryotes, such as yeasts, where much DNA sequence information on sites of structural importance is becoming available (telomeric sequences- Szostak and Blackburn, 1982; centromeric sequences, Carbon, 1984). The understanding of these very small chromosomes should give us some indication of the basic elements of higher eukaryotic chromosome structure.

Chapter 6 MATERIALS AND METHODS

MATERIALS

Chemicals: unless specifically marked, all chemicals were of analytical grade purchased from Sigma or B.D.H.

Radiochemicals were purchased from Amersham International.

Antibodies: Anti-mouse immunoglobulin conjugated to fluorescein, horse-radish-peroxidase, also rabbit antisera to tubulin and actin, from Miles-Yebda Ltd.

The following monoclonals apart from 2C6 and 4G8 were used: HBC-7, against histone 2B and also HLC-1 against intermediate filaments, were generously provided by Dr. Bryan Turner, Dept. of Anatomy, Univ. of Birmingham. DA6-112, against actin was provided by Dr. Veronica van Heyningen, MRC Clinical and Population Cytogenetics Unit.

Cell culture media and sera were purchased from Flow Labs.

Cell Lines used:

Chinese hamster- Don K2 (ATCC CCL 16) and CHO K1 (ATCC CCL 61)

Human- FL (ATCC CCL 62) and HeLa S3 (ATCC CCL 2.2)

Mouse (myeloma)- P3 NS1/1-Ag4-1 (Kohler et al, 1976)

All cell lines were obtained from local stocks.

Most frequently used buffers

KCM pH8- chromosome medium: 120mM KCl; 20mM NaCl; 10mM Tris-HCl pH8

KCM pH7.5- nuclear isolation medium- as above but titrated to pH7.5

Tris-saline- 150mM NaCl, 10mM Tris-HCl pH7.4

PBS- Dulbeccos phosphate buffered saline: composition per litre; 8g NaCl, 0.2g KCl, 0.2g KH_2HPO_4, 1.15g Na_2HPO_4, adjusted to pH 7.3 with NaOH.

A note on centrifuges

All low speed centrifugation work during cell work and chromosome isolations etc. was carried out in an MSE Chilspin centrifuge equiped with a rotor no.34121-613., unless otherwise noted.

6.1 CHROMOSOME AND NUCLEI PREPARATIONS

6.1.1 Cell culture and harvesting

Don chinese hamster cells (ATCC. no. CCL16) were cultured in Dulbecco's modified Eagles medium(DMEM) supplemented with 10% fetal calf serum (FCS), upon 98mm Petri dishes in a 37°C humidified incubator with a 10% CO_2 atmosphere.

For the preparation of metaphase cells, the procedure of Jeppesen et al (1978) was used. Semi-synchronous culture was initiated by subdividing confluent contact inhibited cells at a ratio of 1:3. Routinely this was done by trypsinising 13 plates with trypsin-EDTA solution (0.05%(w/v) trypsin, 0.02%(w/v) EDTA in modified Puck's Saline A (Flow Labs.). The cells were washed off the plates and resuspended in 400ml fresh culture medium, and were then divided over 40 plates. After 23h growth, Colcemid (Fluka) was added to the cells to give 0.1µg/ml of medium. After 6h incubation, mitotic cells were dislodged with a gentle stream of medium and centrifuged at 1000rpm for 15min in an MSE Chilspin centrifuge. The cell pellet was resuspended in 10ml fresh culture medium and cooled on ice for between 30min and 16h. The cells were then pelleted at 1000rpm for 10min at 4°C and resuspended in 10ml fresh culture medium (4°C) prior to chromosome isolation.

Interphase cells were prepared by harvesting 5-10 confluent plates of Don cells ($\sim 10^7$ cells per plate) using trypsinisation. The cells were dislodged after 5min incubation at 37°C with 4ml

trypsin-EDTA per plate, and were collected by centrifugation at 1000rpm for 10min at 4°C. The cell pellet was then resuspended in 10ml fresh culture medium at 4°C prior to the preparation of nuclei.

6.1.2 "KCM" Chromosome Isolation

Metaphase chromosomes were isolated according to the method of Gooderham and Jeppesen (1983). All steps were carried out at 4°C unless otherwise indicated.

Mitotic cells suspended in culture medium (see above) were pelleted at 1000rpm for 10min and the pellet was gently resuspended in 10ml of 50mM KCl using a plastic pasteur pipette. The suspension was incubated at 37°C for 10min, cooled on ice for 5min and then centrifuged at 1000rpm for 10min. The pellet was then resuspended in 10ml KCM pH 8 buffer (120mM KCl, 20mM NaCl, 10mM Tris-HCl pH 8) containing 2mM $CaCl_2$, and to this was added 100µl 10%(v/v) Triton X-100. After 5min incubation on ice, cell disruption was effected by passing the suspension gently through an 11cm, 20ga hypodermic needle, the lysis being monitored by taking small samples for phase contrast microscopy. Usually, ten passages back and forth were sufficient, and when completed 200µl of 0.5M EDTA pH 8.5 was added. The suspension was then carefully layered above a precooled 30ml 10-50% linear glycerol-gradient in KCM pH 8 containing 0.5mM EDTA, 0.1%(v/v) Triton X-100, within a 50ml polypropylene centrifuge tube. This was centrifuged in a Sorvall HB4 swinging-bucket rotor for 50min

at 1000rpm. After centrifugation, 5ml fractions were withdrawn from the top of the gradient using a pipette and aliquots from each fraction were fixed in formalin and centrifuged onto coverslips for phase contrast microscopy (See 6.4.2 for details). Purified chromosomes usually banded in fractions 4-7 and these were pooled and dialysed overnight against 2l of KCM pH 8 containing 1mM $MgCl_2$ and 0.1%(v/v) Triton X-100. After dialyses the suspension was taken on for the preparation of chromosome cores (see 6.1.3 below), or pelleted at 3000rpm for 30min and taken up in loading buffer for polyacrylamide gel electrophoresis (see 6.2.1).

6.1.3 "KCM" Preparation of chromosome cores.

The method of Gooderham and Jeppesen (1983) was followed. All steps were at $4^{o}C$.

After dialyses, the chromosome suspension was gently mixed with 2/3 vol 5M NaCl and then incubated on ice for 30min with occasional swirling. Then the suspension (vol. 60-70ml) was divided into three equal lots and each was layered upon a step gradient consisting of:

10ml 2.5%(w/v) sucrose supported on a cushion of 0.3ml 0.6M metrizamide (Nyegaard Ltd.) ; both in 2M NaCl, 10mM Tris-HCl pH 8, 0.1%(v/v) Triton X-100; contained in a 38ml polyallomer tube (2.5x8.75cm). The gradients were centrifuged for 4h at 3000rpm in a Sorvall AH627 swinging bucket rotor. After the

centrifugation, dehistonised chromosomes were collected in a 2ml fraction taken from the sucrose-metrizamide boundary using a Pasteur pipette inserted from above. The fractions were pooled and dialysed overnight against 1l 10mM Tris-HCl pH 8, 1mM $MgCl_2$, 0.1%(v/v) Triton X-100. At this stage dehistonised chromosomes could be prepared for microscopy by fixing aliquots of the dialysate onto coverslips (see 6.4.2).

The dehistonised chromosome suspension was then incubated with 10µg/ml deoxyribonuclease 1 (DNase1, Sigma) for 30min, followed by the addition of EDTA to 10mM. Samples from the resulting suspension of chromosome cores were sometimes then taken for microscopy (6.4.2). The chromosome cores were collected by centrifugation for 30min at 20,000rpm in a Sorvall AH627 swinging bucket rotor and used for gel electrophoresis (see 6.4.2) or immunisation of mice (see 6.3.2).

6.1.4 "KCM" Preparation of nuclei

The method of Gooderham and Jeppesen(1983) was followed. The harvested Don cells (see 6.1.1) were pelleted at 1000rpm for 10min and resuspended in 10ml 50mM KCl. This suspension was incubated at 37°C for 10min, then cooled on ice for 5min and centrifuged at 1000rpm for 10min. This and all further procedures were carried out at 4°C. The pellet was resuspended in 10ml KCM pH 7.5 containing 2mM $CaCl_2$ and to this was added 100µl 10%(v/v) Triton X-100. Cell disruption was effected by

passage through a hypodermic needle as described for chromosome preparation (see 6.1.2). The lysate was centrifuged at 1000rpm for 10min and the pellet of nuclei resuspended in 10ml KCM pH 7.5 2mM $CaCl_2$ 0.1%(v/v) Triton X-100. The suspension was passed through the hypodermic needle again, and the nuclei were pelleted at 1000rpm once more. The nuclei were either taken for polyacrylamide gel electrophoresis (see 6.2.1) or used for the preparation of nuclear cores.

6.1.5 "KCM" Preparation of nuclear cores.

Nuclei derived from approximately $5x10^7$ cells were resuspended in 40ml KCM pH7.5 with 0.1%(v/v) Triton X-100 and 26ml 5M NaCl was added to the suspension. Dehistonisation and nuclease digestion were performed in exactly the same manner as for chromosomes (see 6.1.3).

CAPS PREPARATIONS

6.1.6 CAPS Preparation of Chromosomes and cores

The isolation of chromosomes in the CAPS (cyclohexylaminopropane sulfonic acid) pH 10 buffer system, their dehistonisation in 0.2N HCl and DNase 1 digestion was as described by Jeppesen et al (1978). Metaphase Don cells were harvested,

trypsinised and resuspended in fresh culture medium as described in 6.1.1. All subsequent steps were carried out at 4°C. After centrifugation at 1000rpm for 5min the cells were resuspended in 10ml CAPS pH10 buffer (2mM $CaCl_2$;1mM CAPS pH10.1; 1%(v/v) hexylene glycol). The suspension was incubated for 10min at 37°C, 5min on ice and 100µl 10%(v/v) Triton X-100 was added. After 5min on ice, cell lysis was effected by 10-20 passages through an 11cm 20ga hypodermic needle. The lysate was centrifuged at 1000rpm for 10min, and the supernatant was collected and centrifuged at 2400rpm for 30min to pellet the chromosomes. The chromosomes were washed twice in CAPS pH10 0.1% Triton X-100 and finally resuspended and washed three times in 2ml TCM (2mM $CaCl_2$; 1%(v/v) hexylene glycol; 0.1%(v/v) Triton X-100; 10mM Tris-HCl pH 8.

Dehistonisation was carried out as follows. To the chromosome suspension in 2ml TCM was added 0.5ml 1N HCl. This extraction was allowed to proceed for 4h at 4°C with agitation, and then the dehistonised chromosomes were pelleted at 2400rpm for 10min. This was washed three times in 0.2N HCl-0.1% Triton X-100 and finally resuspended in 0.2ml 0.2N HCl-0.1% Triton X-100. To this, 10ml TCM containing 1mM $MgCl_2$ was added (TCM.Mg). The dehistonised chromosomes were pelleted at 2400rpm for 30min and resuspended in 2ml TCM.Mg, followed by the addition of 50µl 1mg/ml DNase 1. The digestion was allowed to proceed for 60min on ice, followed by centrifugation at 3400rpm for 10min. The pellet was washed three times in: 10mM Tris-HCl pH8; 1mM EDTA; 1% hexylene glycol; 1% Triton X-100; to yield purified

chromosome cores.

6.1.7 CAPS Preparation of nuclei and nuclear cores

This followed the method for chromosomes with the following modifications. Interphase cells were harvested, swollen and lysed as described for metaphase cells. After cell lysis, the differential centrifugation step used in the chromosome preparation was omitted and the isolated nuclei pelleted directly and resuspended three times in CAPS pH10 using several passages through a 20ga hypodermic needle.

6.2 POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins of the chromosome core and the various chromosome and nuclear subfractions were analysed either by SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970), or by the two dimensional gel system of O'Farrell and O'Farrell (1977), combining isoelectric focusing and SDS-PAGE.

6.2.1 Sample preparation

Samples were taken up in loading buffer (62.5mM Tris-HCl pH 6.8, 2%(w/v) SDS, 10%(v/v) glycerol, 50mM DTT, 0.05%(w/v) bromophenol blue and incubated for 5min on a boiling water bath. After this step samples were often stored for up to 6months at -20°C, and immediately before use were vortexed and then centrifuged for 2min at full speed in an Eppendorf 3200 centrifuge.

6.2.2 SDS Polyacrylamide gel electrophoresis

Gels were cast in a 20x20x0.1cm cassette. The separating gel was cast first, leaving the upper 2.5cm of the cassette for the stacking gel. Routinely 15% acrylamide separating gels were used (15%(w/v) acrylamide, 0.05%(w/v) N,N'-methylene-bisacrylamide(bis)) although on some occasions 8% and 20% acrylamide gels were used. Recipes for all of these are given in table 6.2.1. Samples were loaded into 0.5x1.0cm wells cast in the stacking gel (2.5%(w/v) acrylamide, 0.12%(w/v) bis).

Table 6.2.1 Polyacrylamide gel recipes

Stock solution	Gel Recipe 8%	15%	20%	Stacking
Acrylamide 40%(w/v)	6ml	11.25ml	15ml	1.25ml
Bis-acrylamide 2%(w/v)	2.5ml	1.31ml	1ml	1.2ml
1.875M Tris-HClpH 8.8	6ml	6ml	6ml	-----
1.25M Tris-HClpH6.8	----	----	----	2ml
H_2O	15.2ml	11.1ml	7.7ml	15.35ml
Mixture degassed before adding				
SDS (10%w/v)	0.3ml	0.3ml	0.3ml	0.2ml
TEMED(tetramethylenediamine)	8µl	8µl	8µl	20µl
Ammonium persulphate(10%w/v)	80µl	80µl	80µl	50µl

(the recipe for the 10-16% acrylamide exponential gradient gel used as the second dimension in 2D gels is exactly as set out in O'Farrell and O'Farrell (1977))

followed by 1h at 800V.

The gels were then expelled from their tubes with compressed air and equilibrated for 2h with 3.0%(w/v) SDS, 10%(v/v) glycerol, 50mM DTT, 62.5mM Tris-HCl pH 6.8; using 2x5ml per gel. The gels were often stored for up to 6 months at -70°C at this stage.

The second dimension of electrophoresis was carried out using a 10-16% concave exponential gradient acrylamide slab gel using the exact recipes of O'Farrell (1977). The isoelectric-focusing gel was annealed to the top of the stacking gel using a molten solution of 1%(w/v) agarose in the above equilibration buffer. Electrophoresis was at 220V and a few drops of 0.05%(w/v) bromophenol blue added to the upper electrode buffer served as a marker. After electrophoresis gels were stained as above (6.2.3).

6.3 MONOCLONAL ANTIBODY PRODUCTION

6.3.1 Antigen preparation

Chromosome cores were prepared as described in 6.1.3. The final pellet of cores was solubilised in 120µl of immunisation buffer (20mM DTT, 2%(w/v) SDS, 10mM Tris-HCl pH7.4) by incubation on a boiling water bath for 5min. An aliquot of this solution was taken for SDS-polyacrylamide gel electrophoresis to

assess the quality of the preparation and to allow an approximate protein quantitation. The remainder was stored at -70°C until required for immunisation.

6.3.2 Immunisation of mice

Chromosome cores, solubilised as above, were made up to 0.6ml with immunisation buffer. 2 vol (1.2ml) complete Freunds Adjuvant (GIBCO Ltd.) were added and an emulsion prepared by several passages through a 19ga hypodermic needle. The emulsion was used to immunise six 8wk old female balb/c mice subcutaneously(~10µg protein/mouse)

After 4 weeks, a second immunisation was given as described above except that incomplete Freunds Adjuvant was used. Five days later 0.2ml of blood was taken by tail bleeding, and the serum was analysed by "western-blotting" (see 6.4.2) for reaction with chromosome core proteins. The two mice with strongest serum reaction to core proteins were given a third immunisation, identical to, and 4 weeks after the second one. After a further three months, and 3 days prior to fusion (see below) a booster immunisation was administered to the two selected mice. This consisted of ~50µg chromosome core protein in immunisation buffer without adjuvant and was given intraperitoneally.

6.3.3 Myeloma cell culture

Mouse myeloma cells- P3 NS1/1-Ag4-1 (NS-1, Kohler et al,1976) were grown in RPMI 1640 medium containing 10% FCS. The culture was maintained in a humidified incubator at 37°C with a 5% CO_2 in air atmosphere. Cells were subcultured when their density approached $3x10^5$ cells/ml. From time to time medium including 10µg/ml 6-thioguanine was used in order to select against HPRT revertants, but this was excluded for at least two subcultures prior to fusion.

6.3.4 Fusion

a) spleen cells Spleen cells were isolated as follows. The mice were killed and the spleens removed. Each spleen was then placed in 5ml RPMI 10%FCS; and after several holes were poked in it with a 26ga hypodermic needle, medium was forced through the spleen displacing the cells. The resulting suspension was collected and a further 5ml medium was forced through each spleen in the same manner. The extracts were pooled and centrifuged at 1000rpm for 10min. The pellet was then suspended in 5ml ice cold 0.17M NH_4Cl and after 10min incubation on ice 15ml serum free RPMI was added and the suspension centrifuged at 1000rpm for 10min. The cell pellet was then resuspended in 10ml serum free RPMI at room temperature, giving a total of $5x10^7$ cells.

b) myeloma cells 10^7 NS1 cells in exponential growth were harvested by flushing the growth flasks with a jet of medium from a Pasteur pipette. The cells were collected by

centrifugation at 1000rpm for 10min, and were washed twice in serum free RPMI before being suspended in 10ml serum free RPMI at room temperature.

c) fusion $5x10^7$ spleen cells and 10^7 NS1, both in serum free RPMI at room temp. were mixed in a "universal" vial (Sterilin Ltd) and centrifuged at 1200rpm for 5min. The supernatant was removed and the pellet of cells tapped loose. 0.5ml of 35%(w/v) PEG1540 was then added to the pellet and the cells were resuspended by gentle swirling.

35% PEG1540 was prepared by autoclaving 3.5g polyethylene glycol mol.wt. 1540 (Koch-light) with 1.3ml RPMI. The solution was made slightly alkaline by the addition of 5µl Tris base.

The suspension was centrifuged at 700rpm for 5min, and precisely 8min after the addition of the PEG, 5ml of RPMI was added slowly over 2min. The pellet was then gently resuspended by swirling for 4min, and then centrifuged at 1200rpm for 5min. The supernatant was then removed and 5ml of HAT medium was added without disturbing the pellet. The composition of HAT medium is given in table 6.3.4. After 10min the pellet was gently resuspended and the suspension diluted to 60ml with HAT medium. To this was added a suspension of $6x10^6$ feeder cells (see below-6.3.6) in 60ml of HAT medium and the combined suspension was distributed over six 96 well microculture plates (Falcon 3040F), delivering 0.2ml per well. The plates were then placed in a humidified 37°C incubator with a 5% CO_2 atmosphere.

TABLE 6.3.4 "HAT" MEDIUM

RPMI 1640 medium (Flow) *plus*

CONSTITUENT	CONCENTRATION	100X STOCK	
fetal calf serum	15%		
hypoxanthine	50uM	34mg	
aminopterin	0.45uM	0.98mg	in 100ml
thymidine	10uM	12.1mg	
oxaloacetic acid	1mM	0.67g	in 100ml
sodium pyruvate	0.45mM	0.25g	
L-glutamine	0.03%	3% (GIBCO)	
Porcine insulin (NOVO- Actrapid MC)	0.2U/ml	80U/ml	
MOPS (3 (N-morpholino) propane sulphonic acid)	12.5mM		

6.3.5 Initial growth and subcloning of hybrids

Seven days after fusion, each culture plate well was half emptied with a plastic Pasteur pipette and refilled with fresh HAT medium. This feeding was thenceforth carried out every 2-3 days. By 14 days after fusion, colonies of cells in wells showing growth had occupied 50% of the growth area and culture supernatants were removed for screening by "dot-blotting" (see below-6.3.8).

Posotive cell lines were subcultured in 2ml wells (24 well culture plates-Linbro) and when near confluent were suspended in 2ml culture medium. 1.8ml of this suspension was taken for cryopreservation (see below-6.3.7). The remainder was subcloned at limit dilution as follows. Serial dilutions of the suspension were made until 100 cells in 10ml HAT medium were obtained. To this a suspension of 10^6 feeder cells in 10ml HAT medium was added, and the mixture distributed over a 96 well microculture plate, 0.2ml per well.

The culture plates were incubated and fed with fresh medium as described above, and the clones screened by "dot-blotting". Positive clones were subcultured into $25cm^2$ culture flasks (Falcon) and the supernatants collected. Culture supernatant was centrifuged at 2000rpm to remove cells, and was stored with the addition of sodium azide to 0.1%(w/v) at 4^oC or at -20^oC for long term storage.

6.3.6 Preparation of feeder cells

6 mice were killed, and the skin was folded back on the ventral side to expose the peritoneal cavity. 5ml of ice cold PBS was injected into the peritoneal cavity , which was then lightly pummeled. The lavage was withdrawn but discarded if any blood or gut contamination was observed. The lavage was then centrifuged at 2000rpm for 10min and the pellet washed twice in PBS. The cells were resuspended in RPMI HAT medium at 10^5 cells per ml and this was added to the suspension of cells obtained after fusion or subcloning, prior to distribution over culture plates. Alternatively, the cells were cryopreserved as described below.

6.3.7

Cryopreservation

Suspensions of cells were centrifuged at 1500rpm for 10min. The pellet was resuspended in ice cold FCS containing 5%(v/v) Dimethylsulphoxide (DMSO) at $5x10^6$ cells/ml. 1ml aliquots were transferred to plastic ampoules which were cooled overnight in a polystyrene block within a -70°C freezer. The ampoules were then transferred to liquid nitrogen.

For the recovery of cells, the relevant ampoule was removed from liquid nitrogen and placed in warm water to thaw the contents, which were then gently diluted into 10ml culture medium. The cells were pelleted at 1000rpm for 10min and resuspended again at an appropriate dilution in culture medium, ready for

plating.

If hybridoma cells were thawed, they were always subcloned as soon as cell growth was reestablished.

6.3.8 Screening-"dot-blotting"

The "dot blot" screening assay has been fully described elsewhere (Sternberg and Jeppesen, 1983). The antigen against which the culture supernatant was to be screened was loaded as a grid of spots on a nitrocellulose filter (Schleicher and Schull BA85, 92mm circles with 5mm grid). Routinely, chromosomes or nuclei were either suspended in KCM buffer or solubilised in SDS polyacrylamide gel loading buffer (see 6.2.1), and 1µl spots were applied to the nitrocellulose filter with a glass capillary tube. The preprinted grid was used to locate the spots. When the spots were dry, the nitrocellulose filter was incubated in 50ml Tris-Saline (150mM NaCl, 10mM Tris-HCl pH 7.4) containing 5% bovine serum albumin (BSA- Sigma frac.V) for 30min at room temperature, in order to block the remaining protein binding capacity. The nitrocellulose filter was then laid upon a 90mm circle of filter paper (Whatman grade 1), which had been saturated in Tris-saline 5% BSA and placed in the lid of a 90mm plastic petri dish. 1µl aliquots of hybridoma culture supernatant applied to the spots of antigen loaded on the filter, and the petri dish was covered to prevent evaporation. After 30min incubation at room temperature, the nitrocellulose filter was washed briefly in 100ml Tris-saline, then for 10min in 100ml Tris-

saline, a further 10min in 100ml Tris-saline containing 0.05%(v/v) NP40 (NonIdet P40), and finally for 10min in Tris-saline alone. The nitrocellulose filter was then incubated in 30ml Tris-saline 5% BSA containing $2x10^5$ cpm/ml $[I^{125}]$ $F(ab)'_2$ rabbit anti-mouse immunoglobulin (40μCi/μg) for 60min. The washings described above were then repeated, the nitrocellulose filter dried and autoradiographed. Clear positive supernatants were usually detectable after 12h autoradiography.

6.3.9 Antibody subclass determination

Ouchterlony double diffusion analysis (Ouchterlony and Nielsen,1975) was carried out in Miles immunodiffusion plates with Miles subclass specific antisera. The gel consisted of 1% agar(DIFCO-Noble) in PBS. Precipitin bands usually were visible after 48h at 4°C.

6.4 IMMUNOLOGICAL METHODS

6.4.1 Western-blotting

Electrophoretic transfer of proteins from a polyacrylamide gel to a nitrocellulose filter and subsequent antibody probing was carried out essentially as described by Burnette(1981).

The proteins were first separated on a 15% polyacrylamide SDS gel(see 6.2.2) which was then equilibrated for 30min at room

temperature with transfer buffer (20mM tris base, 150mM glycine in 20% methanol). The polypeptides were then electrophoretically transferred onto a nitrocellulose-filter (Schleicher and Schull, BA85) using an Electroblot apparatus (E-C Apparatus Corp) with a current of 0.5A for 16h at $4^{o}C$. After transfer, the nitrocellulose-filter was incubated in tris-saline (10mM Tris-HCl pH7.4, 150mM NaCl) with 5%(w/v) BSA(Sigma frac.V) for 30min to saturate protein binding capacity. The nitrocellulose-filter was then incubated in undiluted hybridoma culture supernatant for 1-4 hours at room temperature, and washed for 10min in tris-saline, 20min with two changes of tris-saline 0.05% NP40(NonIdet P40,BDH) and finally a further 10min in tris-saline. The nitrocellulose filter was then incubated for 1 hour at room temperature in tris-saline 5%BSA containing $2\text{-}5x10^{5}$ cpm/ml ^{125}I conjugated second-antibody(sheep anti-mouse Ig, 8µCi/µg, Amersham Int.; or rabbit anti mouse Ig, 40µCi/µg, generously provided by Dr.K.Guy) followed by washing as described above. The nitrocellulose-filter was dried and autoradiographed in a Kodak X-ray cassette with intensifying screens against Kodak X-omat AR film at $-70^{o}C$.

In some experiments, replica gels were transferred to nitrocellulose and the duplicate filter was stained in 0.1% amido-black, 20% ethanol, 7% acetic acid for 10min and destained through several changes of 20% ethanol, 7% acetic acid.

6.4.2 Immunocytology

Fixation

Cultured cells (Don, CHO, NS1 and HeLa) were subcultured into petri dishes containing 12mm diameter glass cover-slips. After 24 hours the cover-slips were removed, rinsed in PBS and fixed with either (1) 3.7%(v/v) formaldehyde in PBS for 5 min followed by 30 sec in acetone and air drying or (2) 10 sec in 50%(v/v) ethanol, 20 sec in 90%(v/v) ethanol and 20 sec in acetone followed by air drying. Immuno-staining is described below.

Chromosomes, dehistonised chromosomes and cores were centrifuged onto glass cover slips and fixed by one of the following methods:(a) Formaldehyde fixation- Aliquots(10-50µl) were taken at the appropriate stage of chromosome core preparation and diluted in 1ml of the appropriate buffer containing 3.7% formaldehyde. The suspension was centrifuged at 2500rpm for 10 min in a flat bottom glass tube containing a 12mm diameter cover slip. The coverslip was then rinsed in water and air dried. (b) Ethanol fixation-chromosomes were centrifuged onto coverslips as described above but without the formaldehyde. After the centrifugation the coverslip was immediately immersed in 50%(v/v) ethanol for 10 sec and then 90%(v/v) ethanol for 20sec .

Immuno-staining

All the incubations and washings described below were conveniently carried out with each coverslip at the bottom of a well in a 24x2ml well cell culture plate(Linbro). Monoclonal antibodies 4G8 and 2C6 were generated as described in ch.3. Monoclonal antibodies to histone 2b and intermediate filaments were generously provided by Dr. Bryan Turner, and their preparation is described by Turner (1981). Rabbit antisera to actin and tubulin were obtained from Miles-Yebda.

The coverslip was incubated under a drop of hybridoma culture supernatant(~0.1ml) or antiserum diluted in PBS for 1-4 hours at 37°C in a humidified incubator after which it was rinsed three times with PBS. Second antibody incubation was carried out in the same manner,using 1/10 diluted fluorescein conjugated rabbit-anti-mouse-Ig or 1/40 diluted horse -radish-peroxidase conjugated rabbit-anti-mouse-Ig (both from Miles-Yebda). The coverslip was washed three times in PBS, and in the case of the fluorescein label was rinsed in water and mounted on a microscope slide in 0.25M Na_2CO_3, 50% glycerol pH9. Fluorescence was observed using a Wild M20 microscope with a quartz-halogen illuminator in transmitted dark field mode. A Wild FITC filter set with an additional BG23 barrier filter was used. Photomicrographs were taken on Kodak Tri-X film.

When peroxidase conjugated second-antibody was used, the coverslip was washed three times in PBS as above, and then incubated in the following developer solution: 0.05%(w/v)

diaminobenzidine tetrahydrochloride in PBS with 1/500 vol 30% H_2O_2. The reaction was terminated after 10 min by several PBS washes, and the coverslip was rinsed in water, dried, and mounted on a microscope slide in DPX(Gurr). Observation and photography was carried out using a Wild M20 microscope and Kodak technical-pan film.

Hoechst staining

Staining with Hoechst 33258 (Bisbenzimide) was carried out as described by Jeppesen et.al.(1978). Coverslips were incubated for 10min in SSC (0.15M NaCl, 0.015M NaCitrate), 10min in SSC containing 0.5µg/ml Hoechst 33258, 10min in SSC and finally 10min in water. Stained coverslips were mounted on a drop of 5mM NaCitrate pH 4 in 50% glycerol and viewed using a Wild M20 microscope with a quartz-halogen illuminator in transmitted bright field mode. A BG12 exciting filter and OG1c barrier filter were used, and photography was on Kodak Tri-X film.

6.4.3 Immunoelectron microscopy

Whole mount chromosomes

Isolated Don cell chromosomes, prepared by the KCM method(see 6.1.2) were deposited on carbon coated 200 mesh E-M grids by placing the grids under a drop of appropriately diluted chromosome suspension for 15min. The grids were then immersed in 50% ethanol for 10s and 90% ethanol for 10s, followed by air

drying.

CHO cell thin sections

CHO cells, enriched for mitoses by colcemid arrest were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH7.5 with 0.1M sucrose. The pellets were then dehydrated through an ethanol series, and infiltrated at 4°C with Lowicryl K4M resin(PolySciences Ltd). The resin was polymerised under UV light for 24h at 4°C followed by 48h at room temperature. Thin sections were cut with a glass knife and mounted on nickel 200 E-M grids.

Immunoprobing

Grids were immersed in TBS (20mM tris-HCl pH8.2, 150mM NaCl) containing 0.1%(w/v) BSA(Sigma frac.V) for 10min to saturate their protein binding capacity, and then incubated under a drop of hybridoma culture supernatant(for incubation conditions see below). The grids were washed with 3 changes of TBS pH8.2 and incubated under a drop of gold -labelled (10nm particle) goat-anti-mouse-Ig(Janseen, Belgium) in TBS 0.1% BSA . For this and the monoclonal antibody incubations the conditions were,(1) for whole mounts,2h at 37°C;(2) for thin sections 24h at room temperature. The grids were then washed with three changes of TBS, postfixed in TBS containing 1% glutaraldehyde, washed in water and air dried. Heavy metal staining for 4min in uranyl acetate(saturated solution in 50% ethanol) and 2min in

Reynolds lead citrate (Reynolds, 1963) was carried out on certain thin sections. Specimens were viewed on a Philips EM-300 electron microscope operated at 80kV.

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Dot-Blotting — a Novel Screening Assay for Antibodies in Hybridoma Cultures

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A novel solid phase radioimmunoassay using nitrocellulose filters, is described. The method is of particular value in screening hybridoma cultures for monoclonal antibodies against chromosomal protein. Also it permits detection of antibodies directed against epitopes stable to SDS denaturation.

Key words: *radioimmunoassay – monoclonal antibody – chromosome protein*

Introduction

One of the most critical factors in monoclonal antibody production is the ability to screen many culture supernatants rapidly for the production of relevant antibody. Most commonly some type of solid-phase assay with the antigen bound to microtitre plates is used (Goding, 1980). Success with such methods depends upon the ability of antigen to become adsorbed to the plastic surface.

We are producing monoclonal antibodies against a subset of Chinese hamster chromosomal proteins which remain after metaphase chromosomes have been extracted with 2 M NaCl and digested with nuclease, the 'core' proteins (Gooderham and Jeppesen, 1983). This work has led to the development of a novel screening technique, in which the antigens are bound to nitrocellulose filters. By this method, particulate antigens such as chromosomes are easily bound to the solid phase without resorting to fixatives such as glutaraldehyde (Stocker and Heusser, 1979) or polylysine coated surfaces (Turner, 1981). Moreover, antigens solubilised in SDS solution may be bound directly, and this is of particular value where highly insoluble antigens such as subchromosomal structures are used. The method also has the advantage of distinguishing antibodies which bind denatured polypeptides and are thus effective for probing Western blots from SDS gels (Burnette, 1981).

The assay procedure in essence involves immobilising dissolved or suspended

Abbreviations: SDS, sodium dodecyl sulphate; BSA, bovine serum albumin.

antigen as a spot on a nitrocellulose filter, blocking the filter to further protein binding and overlaying the spot with a drop of culture supernatant. After washing, the filter is probed with radioiodinated second antibody.

Materials and Methods

Chromosome, chromosome core and nuclear isolation

Chromosomes, chromosome cores and nuclei were prepared from Don K2 Chinese hamster cells as described by Gooderham and Jeppesen (1983).

Antibodies

Monoclonal antibodies 4G8 and 2C6 were derived from the fusion of mouse myeloma cell line P3-NS1-Ag4-1 with spleen cells from BALB/c mice immunised with SDS solubilised chromosome core protein. Their preparation will be described elsewhere. Iodine-125 conjugated $F(ab)_2$ rabbit anti-mouse immunoglobulin (20 μCi/μg) was generously provided by Mr. Keith Guy.

Assay procedure

The antigen against which culture supernatants are to be screened is dissolved or suspended in a suitable buffer. Routinely, chromosomes and nuclei were either suspended in KCM buffer (120 mM, KCl, 20 mM NaCl, 0.1% Triton X-100, 10 mM Tris-HCl, pH8) as described by Gooderham and Jeppesen (1983) or solubilised in SDS polyacrylamide gel electrophoresis loading buffer (1.0% SDS, 50 mM DTT, 62.5 mM Tris-HCl, pH 6.8, modified from Laemmli (1970)). The antigen was then loaded as a series of spots on a nitrocellulose filter with a drawn out glass capillary tube. 92 mm circles of 0.45 μM nitrocellulose with a preprinted 5 mm grid (Schleicher and Schull BA85) were used and the grid line intersections were used to locate the spots. It was found helpful to mark out the lines at the perimeter of the grid with a black 'magic marker' felt tip pen. (This was also found to bind radiolabelled second antibody nonspecifically and thus allow rapid identification of spots on the autoradiogram.) The volume used per spot was ~ 0.5 μl, representing ~ 0.1–1.0 μg protein.

After loading, the filter was washed in 50 ml Tris-saline (0.9% NaCl, 10 mM Tris-HCl pH 7.4 (Burnette, 1981)) containing 5% bovine serum albumin (BSA, Sigma fraction V) for 30 min at room temperature, in order to block the remaining protein binding capacity.

The nitrocellulose filter was then laid upon a 90 mm circle of filter paper (Whatman grade 1), which had been saturated with Tris-saline 5% BSA and placed in the lid of a 90 mm plastic petri dish (Sterilin). With reference to the grid markings, 1 μl aliquots of culture supernatants were placed on grid intersections, thus overlaying sites of bound antigen. The dish was covered to prevent evaporation and after 30 min at room temperature the nitrocellulose filter was washed briefly in 100 ml Tris-saline, then for 10 min in 100 ml Tris-saline, a further 10 min in 100 ml Tris-saline with 0.05% NP40 (B.D.H.) and finally for 10 min in Tris-saline alone.

The nitrocellulose filter was then transferred to 20 ml Tris-saline 5% BSA containing $\sim 5 \times 10^5$ cpm/ml ^{125}I-labelled $F(ab)_2$ rabbit anti-mouse IgG and incubated at room temperature for 45 min with shaking, followed by the washing procedure described above.

The filter was then vacuum dried and autoradiographed at $-70°C$ with pre-flashed Kodak X-omat AR film in a Kodak X-ray cassette.

Results and Discussion

Fig. 1. shows an autoradiograph of 2 filters, one loaded with total chromosome protein and the other with chromosome core protein, each filter being used to test 80 culture supernatants from a fusion mixture plating. The spot on each filter cor-

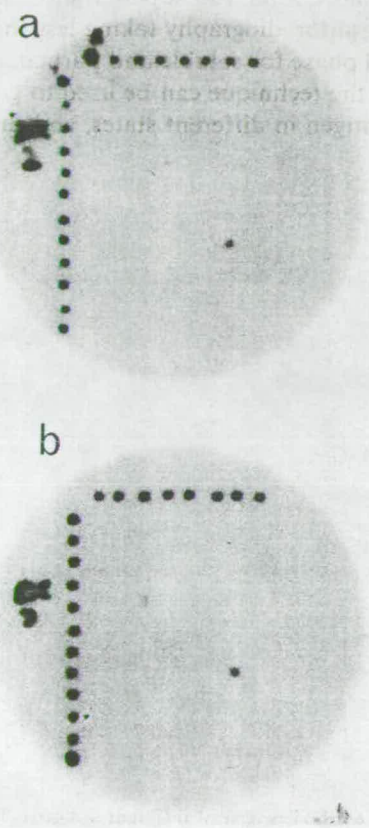

Fig. 1. Dot blotting detection of a monoclonal antibody against chromosome cores. Eighty different culture supernatants were screened against (a) chromosome cores, (b) total chromosome protein, both applied to the nitrocellulose from SDS loading buffer.

responded with a supernatant which has since been shown by Western blotting (Burnette, 1981) to react with a chromosome core antigen (unpublished data). The grid on the filter was readily visualised, by means of the 'magic marker' delineation and also a lower background radioactivity on the printed grid lines.

The dot blotting technique has also proved useful for preliminary analysis of antibody specificity. In the experiment illustrated in Fig. 2, a series of different antigens was loaded as dots along grid columns and various antibodies applied across grid rows. Antibody 4G8 showed binding to SDS denatured chromosomes and nuclei but not to either in their non-denatured state. This result accurately predicted that this antibody would bind Western blots but not bind chromosome preparations for immunofluorescence. On the other hand antibody 2C6 bound chromosomes and nuclei in both states and was subsequently found to bind both formalin fixed chromosomes and chromosome protein Western Blots (unpublished data).

The dot blotting technique has been successfully used to screen culture supernatants for monoclonal antibodies. On 1 nitrocellulose filter 100 supernatants may be rapidly screened, the whole procedure, including autoradiography taking less than 24 h. The method gives reliable binding to the solid phase for soluble and particulate antigens, even in the presence of SDS. In addition the technique can be used to give preliminary information on antibody binding to antigen in different states, and thus on epitope stability.

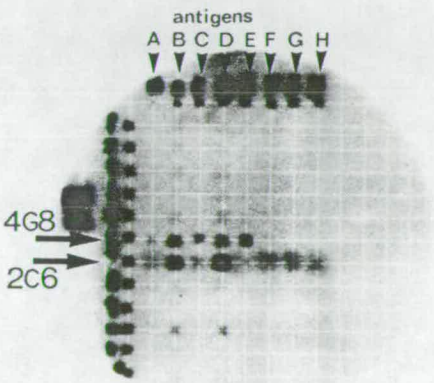

Fig. 2. Dot blotting analysis of the reactivity of 2 monoclonal antibodies against different antigens. The antigens were loaded along columns as follows: (A) control (Pharmacia low molecular weight standard proteins); (B) chromosomes; (C) chromosome cores; (D) nuclei, (E) nuclei treated with 2 M NaCl and DNaseI; (F) tubulin and actin; (G) chromosomes; (H) nuclei. A–F were loaded from SDS loading buffer, G–H were loaded from KCM buffer. Antibodies were loaded across rows of the filter as marked.

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