AUTOIMMUNE SERA AS PROBES FOR NUCLEAR SUBSTRUCTURE

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DECLARATION

I declare,

(a) that this thesis has been composed by myself, and

(b) that the work is my own, except where stated.

ABSTRACT

Systemic rheumatic diseases have been shown to produce antinuclear antibodies in many previous studies. This property has proved useful both for diagnostic purposes and for the identification and characterisation of novel nuclear antigens.

In a blind study, a panel of 24 sera from patients with a wide range of systemic rheumatic disease was assayed for autoantibodies specific for the nucleus by whole cell immunofluorescence and Western blotting. Some well characterised clinical conditions were identified by the specific immunofluorescence staining patterns observed, and by the molecular weights of the polypeptides reacting on blots. Apparently novel staining patterns in cells and on blots were also observed and two sera in particular were chosen for further study.

The autoantibodies present in these sera and in sera from additional patients with the same conditions allowed the identification of cDNA clones from a human expression library. A group of sera from scleroderma patients which recognised a polypeptide of 95kDa led to the isolation of 3 clones which cross-hybridised with each other, and when sequenced proved to code for DNA topoisomerase I. This protein has been shown to be identical to the marker antigen Scl-70 originally estimated to have a molecular weight of 70kDa (Shero *et al*, 1986). The second group of sera from MCTD patients recognising a polypeptide of 38kDa on Western blots led to the isolation of a single human cDNA showing ~75% nucleic acid sequence identity and ~85% amino acid sequence identity with

the m9 transcript of the *Drosophila* developmental gene *Enhancer of Split E(spl)*. *E(spl)* m9 forms part of a complex locus in *Drosophila* which interacts with other neurogenic genes during the initial stages of neurogenesis. Loss or a mutation in any one of those genes results in neural hyperplasia due to incorrect determination of cell fate.

However this protein is not the 38kDa antigen detected on Western blots, as the cDNA is incorporated in the reverse orientation relative to the direction of transcription within the λ vector. The isolation of the human homologue of *E(spl)* m9 clone was probably due to the selection of a spurious negative clone, which had not been eliminated in the rounds of screening and which remained on the final plate.

Further studies to investigate the significance of isolating an *E(spl)* analogue from a human cDNA library were carried out. The cDNA cross-hybridised with the *Drosophila* cDNA and with a genetic construct which rescues flies with otherwise lethal mutations within the locus. It also hybridises to human genomic DNA. Using this cDNA as a probe, three mouse cDNAs have been isolated which appear to encode the full length mouse equivalent. This opens many avenues of study for this interesting and previously unidentified gene.

ABBREVIATIONS

A	amps
ACA	anticentromere antibody
ADP	adenosine diphosphate
ANA	antinuclear antibody
Amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
β-gal	β-galactosidase
βme	b-mercaptoethanol
CNS	central nervous system
cpm	counts per minute
CREST	calcinosis, Raynauds phenomenon, esophageal dismotility, sclerodactyly, telangiectasia
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanidine triphosphate
dTTP	deoxythymidine triphosphate
ddNTP	dideoxynucleotide triphosphate
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
ds	double stranded

DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
E(spl)	Enhancer of split
ER	endoplasmic reticulum
FCS	foetal calf serum
HAT	hypoxanthine aminopterin thymidine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMG	high mobility group
hr(s)	hour(s)
HS	horse serum
HVR	hypervariable regions
ICR	internal control region
IDDM	insulin-dependent diabetes mellitus
IF	intermediate filament
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-β-D-thio-galactopyranoside
K	1000 revolutions per minute
kb	kilobase pairs
KCM	potassium chromosome medium
kDa	kiloDaltons
LGT	low gelling temperature
Mb	megabase
MCTD	mixed connective tissue disease
MG	myasthenia gravis

MHC	major histocompatibility complex
min(s)	minute(s)
NOR	nucleolar organising region
NZB/W	New Zealand black/white
OD	optical density
o/n	overnight
p	short chromosome arm
PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
pfu	plaque forming unit
Pm-Scl	polymyositis-scleroderma
PMSF	phenylmethyl-sulphonyl fluoride
PV	pemphigus vulgaris
PVP	polyvinyl propylene
q	long chromosome arm
RA	rheumatoid arthritis
RANA	rheumatoid arthritis-associated nuclear antigen
Rh	rhesus
RN	recombination nodule
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	revolutions per minute

RT	room temperature
SAR	scaffold associated region
SC	synaptonemal complex
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
secs	seconds
SLE	systemic lupus erythematosus
snRNP	small nuclear ribonucleoprotein
SRP	signal recognition particle
ss	single stranded
SSC	sodium saline citrate
TBS	tris buffered saline
TCA	trichloroacetic acid
TE	10mM Tris pH 7.5, 1mM EDTA pH 8.0
TEMED	N, N, N',N'-tetramethylethylenediamine
TESPA	3-aminopropyl triethoxy silane
TF	transcription factor
Tris	Tris (hydroxymethyl) aminomethan
TSH	thyroid stimulating hormone
Tw	Tween-20
UV	ultra violet
vol	volume
VP	viral protein
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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CHAPTER ONE

INTRODUCTION

1 INTRODUCTION

1.1 Autoimmunity

Autoimmunity is defined as a condition in which an immune response is directed against a constituent of an organism's own body (Blakiston's Pocket Medical Dictionary). Autoimmune diseases affect between 5 and 7% of the population and consist of a wide spectrum of disorders diverse in terms of symptoms, systems involved and their degree of manifestation (Sinha *et al.*, 1990). In spite of this complexity, they can be subdivided into three basic categories. Firstly, there are those characterised by specific organ damage and the production of autoantibodies which react with that particular organ alone. An example of this type of disease is Graves disease in which the thyroid gland is hyperfunctional and autoantibodies are produced to the thyroid stimulating hormone (TSH) receptors (Strakosh *et al.*, 1982). The second category consists of diseases characterised by specific organ damage but the autoantibodies produced are not specific for that organ. Primary biliary cirrhosis (PBC) is a disease in which the small bile ducts within the liver are destroyed leading ultimately to liver failure (Taal *et al.*, 1983) but the characteristic autoantibody reacts with the mitochondria in all cells (Frazer *et al.*, 1985). The final group of diseases are those involving multisystem damage and autoantibodies which react with a systemic antigen. The classic disease in this group is systemic lupus erythematosus (SLE) in which the joints,

kidneys, skin, central nervous system (CNS), lungs, heart and skeletal muscle in any combination are the target organs. Autoantibodies produced are characteristically reactive towards the nucleus; the defined antigens being deoxyribonucleic acid (DNA), histones, nuclear enzymes and protein and ribonucleic acid (RNA) complexes (Tan, 1982). SLE is probably the most severe condition of this type. Milder conditions in this category affect fewer systems, for example, dermato/polymyositis involves skin and muscle only, but all produce the characteristic antinuclear antibodies (ANA). These few examples clearly indicate the wide range of autoimmune disease; the aetiology is similarly diverse.

1.1.1 The Aetiology of Autoimmune Diseases.

Autoimmunity has been described as a "mosaic" (Shoenfeld and Isenberg, 1989), implying that the aetiology involves many different factors. These factors can be classifed as genetic, immune defects, hormonal, environmental, plus other factors which do not seem to qualify for the groups listed above.

Familial studies first indicated the importance of genetic factors in autoimmunity. The effects were determined as genetic rather than environmental by examining the incidence of disease in monozygotic and dizygotic twins. The concordance of SLE in monozygotic twins is approximately 70% whereas that of dizygotic twins is no more than that of first degree relatives at approximately 5% (Block *et al.*, 1976). Symptom free family members have often been shown to have an increased incidence of autoantibody production (Isenberg *et al.*, 1985; Shoenfeld *et al.*, 1987).

Genetic predisposition was also studied by the analysis of major histocompatibility complex (MHC) associations. More than 40 diseases are known or thought to be autoimmune and almost all of these are influenced by the genes encoded in the MHC, the human leukocyte antigen (HLA) region on the short arm of chromosome 6. The first associations reported were with human class I alleles, but with methods for typing class II alleles, most diseases have been shown to be more strongly associated with class II. The class II region, HLA-D, is complex, spans approximately 1.1 Mb and is located centromeric to classes I and III. Polymerase chain reaction technology (Saiki *et al.*, 1988) has allowed the analysis of the nucleotide sequences of class II genes of autoimmune patients and of normal individuals. For some diseases short stretches of sequence or critical residues were identified as playing a role in predisposition. For example, the HLA DRβ chain has been associated with the development of pemphigus vulgaris (PV) in DR4 positive individuals if it contains negatively charged residues in the third of its four hypervariable regions (HVR). Some diseases appear to be associated strongly with a particular chromosomal combination of alleles. For example, individuals that inherit A1, B8 and DR3 HLA alleles together on one chromosome by linkage disequilibrium, have an increased risk of developing insulin-dependent diabetes mellitus (IDDM), myasthenia gravis (MG), SLE and coeliac disease (Dawkins *et al.*, 1982). However most individuals who carry the class II epitopes which confer predisposition to certain diseases do not develop the condition. This emphasises that although MHC class II

antigens are important in the development of some autoimmune diseases, other factors are required.

Immune defects are the second class of factors reported to be involved in the induction of autoimmune disease. When stimulated, the immune system is capable of enhanced replication of the components involved in an immune response. Therefore, it is clear that strict and specific control mechanisms must be available to minimise the production of autoantibodies. A breakdown in these can result in a loss of tolerance to self antigens. The mechanisms by which self-tolerance is lost are not known, but possible critical steps have been identified (Sinha *et al.*, 1990). Susceptibility is conferred by the MHC if its gene products bind self antigen and present it to the immune system in such a way as to induce an immune response. The activation of T cell subsets is also controlled by MHC molecules, as they select epitopes for the induction of antigen-specific suppressor T cells.

T cells with anti-self reactivities are also required for autoimmunity to develop. In normal individuals this type of T cell is present but it is thought that antigen-specific suppressor T cells decrease their numbers and "clonal paralysis" of anti-self T cells (Burkly *et al.*, 1989) assists in maintaining tolerance. Cytokines are involved in controlling the duration and magnitude of an immune response. (They include lymphokines, interleukins and interferons.) They are produced locally by a wide range of cell types but almost all act on B and T cells in picomolar concentrations (Balkwill and Burke, 1989). T cells infiltrating

lesions in autoimmune disease secrete IL-2 and have the IL-2 cell surface receptor. IL-2 is produced after specific or nonspecific activation of T cells and enhances the level of stimulation of many of the cells involved in the immune response (Smith, 1980). These examples demonstrate how a loss of regulation of production of these potent proteins could result in the induction of an autoimmune disease.

Hormones, particularly sex hormones, have an established effect on the incidence of autoimmune diseases (El-Roeiy and Shoenfeld, 1985). MHC class II-associated diseases, which form the largest group, are generally suffered by female patients. MHC class I-associated types show an association with male patients. Thymic hormones (Horowitz *et al.*, 1977), corticosteroids and vitamin D (Rigby, 1988) have all been linked with autoimmune disease. It is thought that testosterone and thymic hormones enhance, whilst oestrogens may inhibit the function of $CD8^+$ T cell clones (Lahita and Kunkel, 1983), which act as immune suppressors. (Autoantibodies and suppressor T cell defects are common in asymptomatic, first degree female relatives of patients with certain types of autoimmune disease (Shoenfeld *et al.*, 1987.) Patients suffering from Klinefelter's syndrome (males with an extra X chromosome) have an increased incidence of autoimmune disease when compared to the normal male population (Lavalle *et al.*, 1987). Studies in New Zealand Black/White (NZB/W) mice have assisted in the determination of the effects of sex hormones on autoimmune disease. These mice have been bred specifically so that the female F_1 hybrids spontaneously develop an autoimmune disease similar to human SLE. Castrated male mice

develop a disease similar to that suffered by the female mice. Administration of androgens to an affected female mouse significantly increased its lifespan and little or no evidence of the autoimmune disease was found (Theofilopoulous and Dixon, 1985).

The genetic predisposition, immunological defects and correct hormonal background are often present in an individual but the disease does not manifest itself until a 'trigger' event occurs. (Inbred mice are the exception to this; they have been bred to exaggerate a specific weakness in order to provide an animal model to assist in the study of autoimmune disease.) In many diseases this trigger seems to take the form of some environmental insult. The major factors involved in this process are the sun, which may alter autoantigens by ultra-violet (UV) irradiation, and certain drugs which can induce almost any type of autoimmune disease. Penicillamine is known to induce scleroderma, myasthenia gravis, SLE, rheumatoid arthritis (RA), pemphigus and myositis and drugs such as procainamide and hydralazine used in the therapy of certain autoimmune diseases can also induce an autoimmune response. Procainamide has been studied more than other types of drugs. After one year of treatment, approximately 50% of the patients have developed antibodies to denatured DNA and histones. 15 to 20% of these patients may develop some lupus like symptoms (Rubin, 1988).

Infectious agents, (Shoenfeld and Cohen, 1987) such as hepatitis B and human immunodeficiency virus (HIV), are the third group of factors involved in the initiation of certain autoimmune diseases. Mycobacteria are thought to induce autoimmunity by 'molecular mimicry' between the bacterial antigens and those of the

host; antigenic similarities have been shown between a measles virus protein, a herpes simplex virus protein and vimentin (despite their different molecular weights) (Fujinami *et al.*, 1983) and also between *Trypanosoma cruzi* and mammalian neurone antigens (Wood *et al.*, 1982). However, infection by mycobacteria is not sufficient to initiate an autoimmune disease unless the appropriate immunogenetic background is also present; infection by atypical mycobacteria is extremely common in certain environmental areas, but the percentage of the population with mycobacterial infection is less than that which develops autoimmune disease.

The last category of factors involved in the induction of autoimmunity comprises those which fail to fit into any of the four above.

Cancer and ageing have an effect on the immune system which may be responsible for the induction of autoimmune disease. Studies in mice have shown that autoantibodies to red blood cells are produced after certain types of viral infection. Old T cells can lose cell surface receptors and changes in their receptors are also observed which may explain the presentation of self antigens to the immune system and the mounting of an immune response. (Walford, 1980) Autoantibodies are more prevelant in the elderly when compared to a young control population; 19 to 42% in the elderly compared to 2% in the young control group (Tomer and Shoenfeld, 1988). Malignant transformation of cells resulting in membrane changes may provoke an immune response. Expression of proto-oncogenes associated with abnormal growth and differentiation of sub-populations of

lymphocytes has been observed in autoimmune-prone mice (Mountz *et al.*, 1985).

The aetiology of autoimmune diseases is clearly complex. The range of this type of disease is similarly diverse. There is, however, a common link between these diseases; the production of autoantibodies.

1.1.2 The Origin of Autoantibodies

Is the generation of autoantibodies the cause or the consequence of autoimmune disease? Autoantibodies are produced by asymptomatic individuals with no apparent harmful effects. However the titres of autoantibodies produced by such individuals are much lower than those in symptomatic patients. It is suggested that these "natural" autoantibodies might even prevent autoimmune disease (Cohen and Cooke, 1986). An example of the use of the "blinding" effects that antibodies may have, is the administration of preformed IgG anti-Rhesus (anti-Rh) antibodies to Rh^- mothers who have given birth to Rh^+ babies. These antibodies prevent the mother's immune system from developing competent T cells specific for the foreign Rh antigens by binding to them. During a second Rh^+ pregnancy, the mother will not produce anti-Rhesus factor antibodies, which would cause foetal red cell damage. "Natural" autoantibodies tend to be of the IgM class whilst those associated with diseases are of the IgG class (Clough and Valenzuela, 1980).

There are two models proposed to explain the production of autoantibodies in disease. The first is that of polyclonal B cell activation. Hyperactivity in B cells is observed in mouse models of SLE (Theofilopoulos *et al.*, 1983) and in human SLE also. B lymphocytes taken from patients or animals with active SLE do not respond to further stimulation with polyclonal activators *in vitro*, presumably due to prior stimulation *in vivo* (Cohen and Ziff, 1977). Polyclonal activation has been shown to increase the number of autoantibody secreting cells, as a proportion of all antibody secreting cells, which may account for the increase in some types of autoantibody (Portanova *et al.*, 1985). However polyclonal activation is not thought to initiate the disease alone (Hang *et al.*, 1985). The second model is that of selected autoantibody induction. This mechanism is driven by the antigens themselves. The disproportionate increase in autoantibodies in the sera of autoimmune patients and mice suggests the preferential activation of autoreactive B cells (Portanova *et al.*, 1985). Ineffective removal of cellular breakdown products which are present due to ageing effects or as the result of cancer, for example, may stimulate this response. Genes coding for anti-DNA antibodies in SLE show many somatic mutations which suggest that selection is driven by the antigens themselves (Shlomchik *et al.*, 1987). B cell hyper-responsitivity and the polyclonal activation observed in SLE can be explained as a secondary effect brought on by circulating lymphokines. A recent hypothesis proposed has combined the two models (Dziarski, 1988). It proposes that initial polyclonal activators and additional signals stimulate the B cell population to

undergo polyclonal expansion and differentiation into antibody (IgM) secreting cells. Autoantigens exposed and lymphokines produced by this initial response then stimulate a further expansion of clones causing an immunoglobulin class switch and antigen driven selection of auto-reactive clones (Dziarski, 1988). A similar model was proposed by Klinman *et al.* for the hyperproliferation of B cells in autoimmune-prone mice (Klinman *et al.*, 1988).

1.1.3 Anti-Nuclear Antibodies (ANA) and Associated Diseases

Systemic rheumatic disease is a term used to describe a group of autoimmune disorders which affect the connective tissues. A common feature of this group of related diseases is the presence of anti-nuclear antibodies (ANA) in the sera of a high proportion of patients.

The presence of ANA's in the serum of systemic rheumatic patients has been shown by many workers to be a useful diagnostic aid, as the antigenic specificities of these antibodies are often associated with particular diseases. ANAs can be detected by indirect immunofluoresence. Early studies were performed on sections of preserved tissues, but now cell lines tend to be used as they are more easily prepared and are readily available. Using this method ANAs were observed to produce different staining patterns within the nucleus and it was concluded that this was due to their differing reactivities towards the nuclear components. However some nonhistone nuclear antigens are readily solubilised by some buffers used in the preparatory stages of staining (for example, nuclear ribonucleoprotein (nRNP); Northway and Tan, 1972) and this

initially caused conflicting reports of the activities present in some autoimmune sera.

There are three broad classes of ANA; those which recognise DNA, those which recognise histones and those which recognise nonhistone proteins. Antibodies to DNA can themselves be grouped according to the form of DNA with which they react. The first group react only with double-stranded (ds) DNA and do not cross react with single-stranded (ss) DNA (Gilliam *et al.*, 1980). It is not known what forms the antigenic site of these antibodies. The second group of antibodies react with only ss DNA and do not cross react with ds DNA. The antigenic sites of this type of antibody are polymers of purine and pyrimidine bases which are accessible in the ss form but not in the ds form of DNA (Tan, 1989). The final group of DNA antibodies is that which reacts with both ds and ss DNA. These antibodies mainly recognise the deoxyribose phosphate backbone but are occasionally against the helical structure of ds DNA and react with ss DNA where it has folded into a secondary helical structure (Tan, 1989). Antibodies to DNA are thought to play a role in the pathogenesis of some diseases. In some SLE patients it has been demonstrated that the appearance of circulating DNA antigen is followed by the occurence of autoantibodies to DNA. The antigen then precipitates with the antibody to form circulating immune complexes *in vivo* (Tan *et al.*, 1966). DNA is an intranuclear component and in order to form these immune complexes it has to become 'exteriorised'. This is probably the result of cellular breakdown and is only known to occur in the case of DNA. These complexes then become deposited in the vascular organs and this

correlates with the decreasing titres of the antibody in the serum and with worsening symptoms (Koffler *et al.*, 1967) of the disease. Antibodies to native or ds DNA only occur in SLE in readily detectable levels. They therefore provide a useful diagnostic test for cases of SLE. Antibodies to ss DNA are prevelant in drug induced lupus erythematosus. Low titres are observed in SLE, RA and some non-rheumatic diseases such as chronic active hepatitis and infectious mononucleosis (Koffler *et al.*, 1971). This limits the use of anti-ss DNA for diagnostic purposes.

The second class of ANAs recognise histones. There are antibodies to all the classes of histone; H1, H2A, H2B, H3 and H4, and they are found in SLE, drug induced autoimmunity, RA and undifferentiated forms of connective tissue disease (Aitcheson *et al.*, 1980; Rekvig and Hannestad, 1980; Molden *et al.*, 1967). There are antibodies in this group which also react specifically with dimers of H2A and H2B and with tetramers of H3 and H4 (Rubin and Waga, 1987; Thomas *et al.*, 1984; Gohill *et al.*, 1985; Portanova *et al.*, 1987). The dimer form H2A-H2B is the antigen with which the majority of antibodies in patients with SLE and procainamide-induced LE react. In LE induced by hydralazine, H2A-H2B dimers are recognised as a target antigen but the tetramer form of H3-H4 is more reactive.

The final class of antibodies are those which react with the nonhistone proteins present within the nucleus. Most of the antibodies in this group are specific to a particular disease. Those which are specific are useful diagnostically.

Following DNA and histones, Sm was the next antigen to be identified and characterised using autoimmune sera (Tan and Kunkel, 1966) and has been shown to be associated with SLE. It has also been shown to be present complexed with another autoantigen, nuclear RNP (nRNP). Antibodies to nRNP and ribosomal RNP (rRNP) are also found in the sera of SLE patients. However nRNP is more frequently associated with mixed connective tissue disease (MCTD) patients (Mattioli and Reichlin, 1973). This particular condition was not defined as a disease in its own right until the discovery of the autoantibodies that reacted with nRNP. Antibodies to this particular antigen are found in lower frequencies in the sera of patients with discoid lupus, scleroderma, RA, and Sjogrens syndrome. These autoantigens were shown to be composed of a species of small nuclear (sn) RNAs, also known as U RNAs complexed with proteins (Lerner and Steitz, 1979). The autoantibodies produced by the patients with these diseases proved invaluable in showing that the snRNPs were involved in the splicing of precursor messenger RNA (mRNA) (Yang *et al.*, 1981; Padgett *et al.*, 1983; Bozzoni *et al.*, 1984). Although these antigens are complexes of RNA and protein, the antigenic sites are exclusively found on the proteins. This was demonstrated by the fact that the autoantibodies do not precipitate snRNAs when dissociated from protein and by the identification of the antigenic polypeptides by Western blotting. Sm has four antigenic polypeptides of 29kDa (B'), 28kDa (B), 16kDa (D), and 13kDa (E). Antibodies to B and B' cross react with D and *vice versa*. The presence of serum antibodies has allowed the isolation of a cDNA clone coding for E (Weiben *et al.*, 1985). nRNP has

antigenic proteins of 70kDa, 33kDa (A), and 22kDa (C). There are also rarely occurring antibodies which react with polypeptides of 31-32kDa (A') and 28.5kDa (B'') (Mimori *et al.*, 1984; Habets *et al.*, 1985). These autoantibodies have been used to show that the core proteins of U1, U2, U5 and the U4/U6 complex are made up of combinations of B, B', D, and E polypeptides, in addition to a trimer of E, F and G that all run at approximately 12-13kDa on electrophoresis. U1 also contains A and C, whilst U2 snRNP contains A' and B''. The 70kDa polypeptide has been shown to be a nuclear matrix protein and not an intrinsic component of U1 snRNPs (Verheijen *et al.*, 1986). However it appears that for the U1 RNP antigen to be recognised by the autoantibodies, it has to be associated with the 70kDa protein of the nuclear matrix.

SS-A/Ro and SS-B/La were the next pair of antigens to be isolated. Ro was isolated from extracts of spleen from humans and identified using antibodies from SLE patients (Clark *et al.*, 1969). La was found by similar studies (Mattioli and Reichlin, 1974). Both of these antigens were reported to be cytoplasmic. Subsequently two antigens were isolated from soluble extracts of a human lymphocyte line using serum antibodies from patients with Sjogren's syndrome and called SS-A and SS-B (Asplaugh and Tan, 1975). These antigens were localised to the nucleus. In 1979, they were shown to be identical; Ro was in fact SS-A and La was SS-B, leaving the differing reports of their cellular location to be reconciled. La was found to be extremely soluble in physiological saline and could therefore be leached out of the nucleus and into the cytoplasm during preparation for cell fractionation. However further studies

have indicated that these antigens may be present in the nucleus or in the cytoplasm depending on the functional state of the cell (Tan, 1989). Autoantibodies to these antigens are frequently present in patients with Sjogren's syndrome, and sometimes in SLE and in RA.

Two proteins of 60kDa and 52kDa associated with four or five types of small cytoplasmic RNAs (Y RNAs) are precipitated by anti-SS-A/Ro antibody. The Y RNAs range in size from 80 to 112 bases in size (Wolin and Steitz, 1984). The precipitation of these two types of protein is due to two antibody populations present in the sera. This was proved when affinity purified antibodies from the 60kDa protein did not cross react and *vice versa*. There are also sera which contain only one species of antibody and therefore precipitate only one of the protein antigens. SS-B/La antibodies precipitate a 48kDa protein complexed with nascent RNA polymerase III transcripts but the antigenic site is present on the protein as with Sm and nRNP antigens (Rinke and Steitz, 1982; Chan *et al.*, 1986). This protein has two distinct structural domains; a 28kDa methionine rich region and a 23kDa domain containing phosphorylated amino acids. These are thought to provide two distinct antigenic binding sites on this protein. The 48kDa protein forms a 43kDa degradation product which is also recognised by the antibodies. Anti-SS-A/Ro is often present without anti-SS-B/La but not *vice versa*. Anti-SS-B/La is useful as a serological marker for Sjogrens syndrome when associated with sicca syndrome; the antibody is present in approximately 60% of such patients. Anti-SS-A/Ro has been shown to be a good marker for neonatal lupus (Franco *et al.*, 1981).

Ku is an antigen associated with 55% of scleroderma/ polymyositis overlap, 33% of SLE/polymyositis/scleroderma overlap and more rarely in SLE and scleroderma alone (Tan, 1982). It consists of a pair of proteins 60-66kDa and 80-86kDa also known as p70/p80. These proteins have been shown to be DNA binding (Mimori *et al.*, 1986) *in vivo* and *in vitro*. They have been localised to condensing chromosomes in prophase and telophase (Reeves, 1985). However their function is unknown. There is one common antigenic site on each protein and one that distinguishes them from each other (Francoeur *et al.*, 1986).

3% of SLE patients have autoantibodies to an antigen which shows distinct expression patterns within a single population of cells, dependent on whether they are proliferating or not. This antigen is hence known as proliferating cell nuclear antigen (PCNA) and is at the peak of expression in late G and early S phases of the cell cycle. It has been localised to the nucleolus and also to the nucleus in general (Takasaki *et al.*, 1981). A protein called cyclin isolated independently from this study, was found to have a molecular weight of 35-36kDa and its expression was also related to the cell cycle (Bravo *et al.*, 1981). These proteins were subsequently shown to be identical (Mathews *et al.*, 1984). It is thought that this protein is required for chain elongation during DNA synthesis and that it is the auxiliary protein to DNA polymerase δ (Bravo *et al.*, 1987).

There are several autoantigens which are specifically associated with scleroderma and its many related forms. ANAs have been reported to be present in frequencies from 40 to 90% in this

group. With the advent of tissue culture and the use of cell line material as substrate for immunofluoresence, the higher figure seems to be more accurate. This group of diseases produces quite a wide range of cell staining patterns and autoantibodies to particular antigens have been related to these observations.

A diffuse granular pattern in the nucleus has been correlated with sera containing autoantibodies to Scl-70, the marker antigen for scleroderma (Douvas *et al.*, 1979). This antigen was isolated from chromatin and found to be a basic protein of 70kDa. Further studies found that sera from scleroderma patients with antibodies to Scl-70 also recognised DNA topoisomerase I which has a molecular weight of approximately 100kDa (Shero *et al.*, 1986; Guldner *et al.*, 1986). The discrepency in molecular weights was resolved by the discovery that the 100kDa protein could be spontaneously degraded if protease inhibitors were not used in the extraction procedure. The autoantibodies produced by scleroderma patients were shown to inhibit the relaxation of supercoiled DNA by DNA topoisomerase I. It has been found in the cells of man, rabbit and rat indicating a degree of cross species conservation (Tan *et al.*, 1980).

The presence of large granules within an interphase cell and the localisation of these granules to the centromeres of condensed mitotic chromosomes, is the characteristic staining pattern observed with anti-centromere autoantibodies (ACA) (Moroi *et al.*, 1980). The actual antigenic region was initially determined to be the inner and outer layers of the trilaminar kinetochore (Brenner *et al.*, 1981). There was no reactivity with the centromeric DNA itself. The antigens of the kinetochore have been shown to be proteins of

molecular weights 17-19kDa (CENP-A), 80kDa (CENP-B), and 140kDa (CENP-C) (Cox *et al.*, 1983; Guldner *et al.*, 1985; Earnshaw *et al.*, 1986). Recently immunoelectron microscopy has clarified the position of some of these antigens (Cooke *et al.*, 1990). 95% of CENP-B is distributed along the centromeric heterochromatin beneath the kinetochore, indicating that it may be associated with the α-satellite heterochromatin. CENP-A and/or CENP-C are thought to associate with a "collar" of chromatin encircling the kinetochore plates. 95% of CENP-B has been cloned (Earnshaw *et al.*, 1987). Autoantibodies to the centromeres of chromosomes are characteristic of a particular subset of scleroderma patients with a syndrome known as CREST (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly and telangiectasia). The antibody is highly specific to this disease and is found in the sera of 70 to 80% of CREST patients. Anticentromere antibodies have been found in 25% of patients with Raynaud's phenomenon but no other symptoms of CREST, but these individuals may have the condition in an early form and do not yet show the other characterisic symptoms.

Antinucleolar antibodies of varying types are present in the serum of 45% of scleroderma patients and these were used to try to characterise the structural organisation of the nucleolus itself (Hernandez-Verdun *et al.*, 1979). In 4% of patients there are antibodies to RNA polymerase I and this type of antibody produces punctate staining of interphase nucleoli. In dividing cells the nucleolar organising regions (NOR) are stained (Reimer *et al.*, 1986; Reimer *et al.*, 1987). Thirteen polypeptides ranging in size from 210 to 12.5kDa are precipitated by this type of antibody. There

were four phosphoproteins of 180kDa, 80kDa, 64kDa and 18kDa present in the complex which is thought to contain subunits of RNA polymerase I and other factors necessary for transcription. 8% of scleroderma sera contain antibodies to fibrillarin and produce a clumpy nucleolar staining pattern on immunofluoresence of whole cells. This 34kDa antigen is a component of U3 RNP (Parker and Steitz, 1987) and the epitope is highly conserved between species (Vaughan, 1987). Homogeneous nucleolar staining associated with diffuse granular staining of the nucleus itself is the observed pattern given by autoantibodies to the Pm-Scl antigen. The antigen is recognised by patients with polymyositis/scleroderma overlap disease and consists of a complex of eleven proteins with molecular weights ranging from 110 to 20kDa. The localisation of the antibodies suggests that the antigen is associated with preribosomes. The antigenic determinants were found on polypeptides of 100 and/or 80kDa (Reimer *et al.*, 1986). Rarely, scleroderma sera contains autoantibodies to a 40kDa protein which is complexed to a 7.2S RNA in the nucleolus (Reddy *et al.*, 1983). This 7.2S RNA has been shown to be an RNA polymerase III transcript and is also associated with SS-B/La (Hashimoto and Steitz, 1983). A 90kDa protein has been localised to the NOR by a previously undetected autoantibody found in scleroderma sera (Rodriguez-Sanchez *et al.*, 1987). The anti-nucleolar antibodies described here are not encountered to any significant extent other than in scleroderma and its overlap conditions and is therefore specific for this group of diseases.

Rheumatoid arthritis-associated nuclear antigen (RANA) has been detected with autoantibodies in the sera of 90% of patients with RA. Normal controls have the antibodies with a frequency of 6 to 25%, equivalent to that found in the sera of patients with other types of autoimmune disease, such as SLE, scleroderma and MCTD. However the antigen and its function have not yet been characterised.

Certain species of transfer RNA (tRNA) synthetases are recognised by autoantibodies specific to dermatomyositis and polymyositis. The antigen of the first of these autoantibodies was found to be the protein part of histidyl-tRNA synthetase with a molecular weight of 50kDa (Mathews and Bernstein, 1983). Threonyl-tRNA synthetase (PL-7) was detected by a second autoantibody in the sera of myositis patients. The antigen was a protein of 80kDa (Mathews *et al.*, 1984). Two further autoantibodies recognised naked alanyl-tRNA and alanyl-tRNA synthetase (Bunn *et al.*, 1986). The epitopes recognised by the autoantibodies appear to be the catalytic sites of these enzymes as the autoantibodies can be shown to inhibit their function (Dang *et al.*, 1988)

The Mi antigen was isolated from calf thymus extract and consists of two proteins of 61 and 53kDa, however its structure and function are as yet uncharacterised (Targoff and Reichlin, 1985).

This group of diseases can give rise to autoantibodies in the sera of its patients which recognise a 54kDa protein which is part of the signal recognition particle (SRP). This protein contains the antigenic site and has been shown to be involved in polypeptide

synthesis and the translocation across the endoplasmic reticulum in preparation for secretion (Reeves *et al.*, 1986).

31% of patients with polymyositis contain antibodies in their serum which recognise an antigen of 150kDa, Jo-1. It appears to be a protein and is localised to the nucleus (Nishikai and Reichlin, 1980).

There are some autoantibodies which occur very rarely and are difficult to associate with specific diseases. Autoantibodies to nuclear lamins recognise three proteins of 74kDa (A), 68kDa (B), and 60kDa (C). The antibodies produce a linear staining pattern of the nuclear envelope. The lamins are thought to provide a point of anchorage for interphase chromosomes to the nuclear envelope (Gerace, 1986). Antibodies to poly(ADP-ribose)polymerase (ADPRP) are found in the sera of patients with Sjogren's syndome and sometimes in ill-defined autoimmune disease (Yamanaka *et al.*, 1987). The antigen is a DNA binding protein and is involved in DNA repair in eukaryotes.

The anti-nuclear antibody diversity is clearly demonstrated by the range of antigens and antibodies discussed here. ANAs have aided in the sometimes difficult task of diagnosis, but the question of why they arise in these diseases remains unresolved. The reasons are possibly as diverse as the diseases themselves but answers to this question would probably aid in the discovery of more effective treatments and, possibly prevention.

A practical role for ANAs that has emerged more recently is in the isolation and characterisation of nuclear antigens at functional and structural levels. The use of isolated nuclear antigens as

stimulants to raise antibodies in normal animals has had very limited success as they appear to be only poorly immunogenic. Therefore serum autoantibodies are of particular importance for studying this group of diseases. ANAs have proved invaluable as probes for components of the nucleus, as shown by the discovery of Sm, its association with nRNPs and their functions. The use of ANAs combined with other experimental methods have greatly added to our knowledge of the structure and function of the nucleus over the past decade or so. This knowledge is valuable for basic scientific research, but will hopefully be of clinical value by providing insights defects involved in the development of autoimmune diseases in patients.

1.2 The Nucleus

The nucleus is the feature distinguishing eukaryotic cells from prokaryotes and akaryotes, with the notable exception of mammalian red blood cells. (These specialised cells lose their nuclei during maturation in order to increase their haemoglobin content and optimise their oxygen carrying capacity.) Fossil evidence suggests that eukaryotes evolved between 1500 and 900 million years ago and so, therefore, did nuclei (Cavalier-Smith, 1988).

The nucleus is defined by a nuclear envelope which consists of a double membrane, complete with pore complexes allowing active transport and passive diffusion to and from the cytoplasm. The inner and outer membranes perform different functions. The nucleus

contains two basic classes of components, nucleic acids and proteins. The proteins can be further sub-divided into histone and non-histone proteins whilst the nucleic acids are present in ribosylated and deoxyribosylated forms. The major part of the volume of the nucleus is occupied by DNA. Unlike prokaryotes, it is not in the form of a single circular chromosome but is contained in a much more complex form of packaging called chromatin. The structure of chromatin is observed in differing states which vary with the cell cycle. A relatively decondensed form of chromatin is found in interphase cells, whilst those undergoing mitosis and meiosis condense this structure further to form chromosomes.

1.2.1 Chromatin, Chromosomes and Associated Proteins

The somatic mammalian cell passes through two clearly identifiable states during its cycle. These are interphase and mitosis. Mitosis is the shorter of the two stages, lasting approximately 2 hours of a typical 24 hour cycle. During interphase the DNA has to be accessible to enzymes and other effector molecules so that proteins necessary for the day to day running of the cell can be produced. Therefore the active DNA is packaged in the form of decondensed chromatin during interphase.

DNA in the linear double helical form is too bulky to be organised inside the nucleus, so packaging it into chromatin solves this. Chromatin structure is largely determined by the histones present within the nucleus. The primary unit of chromatin is the nucleosome. This consists of a well protected core structure which takes the form of 146 base pairs (bp) of DNA (Prunell *et al.*, 1979) coiled 1.75 times around a histone octamer, comprising two each of

H2A, H2B, H3 and H4 proteins(Weintraub *et al.*, 1976). Under an electron microscope, when prepared under low salt conditions, these core units can be seen as beads joined together by a variable length of linker DNA.

The next level of chromatin structure is the mononucleosome, which is basically the nucleosome core unit with a linking stretch of DNA and histone H1 associated with it. The linker piece of DNA can be absent or up to approximately 80bp in sea urchin sperm (Morris, 1976; Spadafora et al., 1976). A run of 22bp of DNA completes two turns around the nuleosome core (Simpson, 1978). The remainder joins the mononucleosomes together. Histone H1 is bound to one side of this core particle at the point where the DNA strand enters and exits its helical turns around the octamer of histones (Noll and Kornberg, 1977). It is thought that H1 is crosslinked to the core histones. Experiments performed *in vitro* have indicated that H1 can cross link to H2A and H3 (Boulikas *et al.*, 1980; Ring and Cole, 1979). A single molecule of H1 is associated with each nucleosome core. This 10nm structure is often referred to as beads on a string and has a packing ratio of 6-7:1 relative to naked DNA.

The structure of the next level of organisation of chromatin is still somewhat disputed and there have been three models proposed. All models accept that the 10nm nucleosome string is arranged into a 30nm fibre. The first of these, the solenoid model, was proposed in 1976 and postulates that the nucleosomes are arranged face to face (with respect to the disc-like histone octamers) and are coiled with 4 - 10 nucleosomes per each turn. The H1 is thought to fill the hollow space inside the coil (Finch and Klug, 1976). In 1977, Renz, Nehls and Hozier proposed a superbead

model in which nucleosomes associate in globular clusters of heterogeneous sizes and that H1 crosslinking assisted in the maintainence of this type of structure (Renz *et al.*, 1977). The third model, the helical ribbon model, proposes that the nucleosomes arrange themselves into a zig-zag formation with each nucleosome edge to edge with the next. This relaxed form compacts by twisting the nucleosomes by 90° so that they lie face to face forming a ribbon-like structure and the ribbon then coils into a helix which corresponds in size to the accepted 30nm structure (Woodcock *et al.*, 1984). Further data has suggested that the superbead model has only been observed due to artifacts produced by techniques preparing chromatin for study in low ionic strength buffers. It has also been suggested that the work done on the helical coil models was carried out on native chromatin and is therefore more representative of chromatin structure *in vivo*. The number of nucleosomes per turn in the solenoid model was later postulated to be 12 (Walker and Sikorska, 1979; Walker and Sikorska, 1979) but it is now accepted to be in the region of 5.9 per solenoid turn (Butler, 1988). This level of packing using either model increases the packing ratio to approximately 40:1.

The 30nm chromatin conformation folded into loops (Benyajati and Worcel, 1976; Paulson and Laemmli, 1977) or domains (Igo-Kemenes and Zachau, 1977) is associated with the nuclear lamina which lines the nuclear envelope of interphase nuclei (Aaronson and Blobel, 1975; Stick and Hausen, 1980). Other terms for the supporting structure in interphase nuclei have been suggested, such as matrix (Comings and Wallach, 1978; Long *et al.*, 1979; Capco *et al.*, 1982), envelope (Murray and Davies, 1979), ghost (Riley et al., 1975) and

cage (Cook and Brazell, 1980). Most of the DNA is thought to be in the looped form of chromatin (Lepault *et al.*, 1980) associated with this support structure. It is proposed that the presence of the nucleosome packaging and the higher ordering into loops has a role in the control of transcription of genes. Active genes are thought to be folded into loops more loosely than inactive ones giving rise to hetero- and euchromatin forms. It is thought that active genes are preferentially positioned within these looped domains such as the puffs seen in the Balbiani rings of *Chironomus* (Daneholt *et al.*, 1982). The active genes are thought to be able to unravel themselves from the polynucleosome formation reversibly without the dissocation of histones (McKnight *et al.*, 1978). This reversible change may be triggered by varying rates of polymerase initiation and transcription or by non histone proteins, such as SV40 virus VP1 (Moyne *et al.*,1982). The transcribing genes for rRNA in amphibian oocytes are not compacted into nucleosomes at all (Scheer, 1980).

Internal enhancer sequences play a role in gene regulation. Mouse immunoglobulin genes are known to contain these enhancer sequences (Gillies *et al.*, 1982; Mercola *et al.*, 1983). Alone, however, they are not sufficient to promote transcription and are thought to act in conjunction with the positional effects already discussed above.

Modifications of the DNA itself have been suggested as mechanisms for altering the transcriptional activity of genes. These modifications may affect only single bases such as postsynthetic methylation. Active genes are relatively unmethylated whilst inactive genes are usually methylated, but this is not always the case and the true role of methylation in gene regulation is not

clear. Methylation may induce structural changes which are specifically recognised by factors involved in transcription. However, methylation alone is not capable of transcriptional control (Waalwijk and Flavell, 1978).

Histones, and modified forms of histone proteins, and non-histone proteins, are also known to be associated with DNA in chromatin, and are thought to be involved in gene regulation. Proteins associated with DNA may be recognising specific sequences or more general classes of sequence present within the genome. A non-histone protein D1 of Drosophila is a component of the transcriptionally inactive satellite 1.688 and has a molecular weight of 50kDa (Varshavsky *et al.*, 1983). A 37kDa protein, TFIIIA, has been isolated from *Xenopus* oocytes and shown to bind to the intragenic regulatory sequence of the 5S DNA (Engelke *et al.*, 1980). Examples of proteins which bind to general sequences are RNA polymerase II, which binds to nucleosome cores, and a group of proteins called the high mobility group (HMG) proteins which associate with transcriptionally active chromatin (Johns, 1982). All nucleosomes, whether their constituent DNA is active or not, appear to have two binding sites for HMGs 14 and 17 at each of the DNA entrance and exit points (Mardian *et al.*, 1980; Sandeen *et al.*, 1980). HMGs 1 and 2 are thought to bind to the linker DNA (Jackson *et al.*, 1979). However the exact role of HMG proteins is not clear; they cannot be directly associated with transcriptionally active genes, yet they are sometimes found to be present in increased amounts. Modifications of histones are thought to affect the nucleosome and chromatin structure. Variant forms have been found at different stages of development and the cell cycle. These forms

have been found to make the chromatin more suceptible to thermal denaturation and nuclease digestion in sea urchins (Simpson, 1981). This may affect rates of transcription simply by producing heterogeneous forms of chromatin. Post synthetic modifications of histones affect gene activation, such as acetylation of their amino-terminal portions. This is thought to destabilise the 30nm structure of chromatin and have conformational effects on the nucleosome itself (Bode *et al.*, 1980; Bode *et al.*, 1983; Muller *et al.*, 1982). Ubiquitination of histones has been suggested to prevent the formation of higher order structures, either by modifying nucleosome-nucleosome interactions (Levinger and Varshavsky, 1982) or by allowing recognition of active genes by proteolytic enzymes which subsequently remove the nucleosome cores (Varshavsky *et al.*, 1983).

The alternative state in which the somatic cell can be observed is that of mitosis. This is a short stage of the cell cycle, as mentioned previously, when the DNA duplicated during S phase, is segregated between the two resultant daughter cells. Obviously the segregation must be very accurate otherwise one or both of the daughter cells would not contain the complete complement of genetic information coded for in the genome. In order to do this the DNA must be further condensed from the looped 30nm structure and detached from the nuclear lamina, as the nuclear envelope breaks down during mitosis. This further condensation of the interphase chromatin is observed as the production of distinct chromosomes.

Electron microscopy suggested that the compact chromosomes were produced by the looping of the 30nm fibre (Dupraw, 1970). In 1976, it was shown that the long continuous strings of interphase

DNA were organised into looped domains containing, on average, 80kb lengths held in place by RNA and proteins (Cook and Brazell, 1975; Benyajati and Worcel, 1976). These domains become radially positioned within the chromosome to form a structure known as a miniband with a packing ratio of 1.2×10^4:1 relative to naked DNA. The minibands now stack on top of one another to form the sister chromatid of the mitotic chromosome. The loops were found to form supercoils thus compacting the DNA even further. The final packing ratio is the same as that of the minibands, *i.e.* 1.2×10^4:1. The structure of the metaphase chromosome has been shown not to be dependent on histones for its morphology. When the histones are selectively removed, the DNA remains bound to a central scaffold composed of non-histone proteins (Adolph *et al.*, 1977). The binding sites of these proteins are located at specific DNA sequences known as scaffold-associated regions (SARs) which were first observed in interphase cells. These regions appear to have been conserved through evolution. Some SARs have been shown to be closely linked to enhancer-like regulatory elements and most of those analysed have been found to contain sequences homologous to topoisomerase II binding sites (Gasser and Laemmli, 1986; Cockerill and Garrard, 1986). A major protein of scaffolds has subsequently been shown to be topoisomerase II (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986). Another major protein was found to be β-tubulin with a molecular weight of 53kDa (Adolph, 1977). Later studies by Gooderham and Jeppesen (1983) did not detect any β-tubulin within scaffolds. The DNA projects from the scaffold as a series of loops (Stubblefield and Wray, 1971; Adolph *et al.*, 1977; Paulson and Laemmli, 1977; Marsden and Laemmli, 1979). The loops which are observed in

interphase nuclei are essentially similar to those projecting from the chromosomal scaffold following dehistonisation. Topoisomerase II is found in both these scaffolds and the nuclear matrix of interphase cells (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986; Mirkovitch *et al.*, 1984). Therefore the structure of chromatin observed in these stages of the cell cycle is postulated to be virtually the same; the difference being the location of the stabilising structures to which the chromatin attaches. The scaffold is thought to behave dynamically under the regulation of the cell cycle. This would allow the condensation of chromatin into chromosomes to be controlled by the self-assembling scaffold alone (Earnshaw and Laemmli, 1983).

Another group of non-histone proteins have been shown to be associated with the chromosome; those which form the kinetochore at the primary constriction or centromere. The kinetochore is the site of initiation and formation of the spindle microtubules which pull the sister chromatids apart during mitosis (Pepper and Brinkley, 1977; Mitchison and Kirschner, 1985). It has been shown to be a tri-lamellar structure of two electron dense plates separated by a lucent zone, positioned along the surface of the chromosome (Commings and Okada, 1971; Roos, 1973; Reider, 1982). The study of kinetochores free from the metaphase chromosome has determined that the outer plate is composed of 30nm fibres. It is thought that these fibres are a subclass of chromatin fibrils extending from the chromosome itself (Rattner, 1986). Proteins associated with these plate structures are often recognised by the serum antibodies of scleroderma/CREST patients (Moroi *et al.*, 1980; Cooke *et al.*, 1990). Later it was shown that there is in fact a family of proteins CENP-A

(14- 19.5kDa), CENP-B (70-80kDa), CENP-C (110-140kDa), CENP-D (20-25kDa) and CENP-E (33-34kDa) (Cox et al., 1983; Valdivia and Brinkley, 1985; Earnshaw et al., 1986; Kingwell and Rattner, 1987) localised to the kinetochore. Autoimmune sera have assisted in the discovery of the functions of some of these proteins. The 17kDa CENP-A is present in pre-mitotic and mitotic centromeres and has been shown to be associated with mononucleosomes (Palmer et al., 1987). It is thought that CENP-A may be a modified histone species. The 80kDa CENP-B and 140kDa CENP-C are associated with the scaffolds of mitotic chromosomes (Earnshaw et al., 1984), whilst a 50kDa protein (later included into the class of CENP-E) has been localised to the kinetochore of two mammalian species, cells from the Indian Muntjac deer and human HeLa cells, indicating the conserved nature of this group of proteins (Kingwell and Rattner, 1987). However the function of CENP-E is not yet known. The 80kDa protein of class CENP-B has been cloned (Earnshaw et al., 1987) and the analysis of this protein is the most complete in the group. As the distribution of this protein varies from chromosome to chromosome, it is thought that its distribution may reflect the presence of alphoid satellite sequences (Cooke *et al.*, 1990). A 2.9kb mRNA codes for the protein which is characterised by two acidic domains comprised almost entirely of glutamic and aspartic acids. It may also contain a tubulin binding domain (Balczon and Brinkley, 1987), but its major function is thought to be in mediating chromosome and mitotic spindle interactions. Inner centromere proteins of 155kDa (INCENP-A) and 135kDa (INCENP-B) have also been identified (Cooke et al., 1987) and they are thought to be responsible for maintaining sister chromatid pairing until division at anaphase. During interphase the

kinetochores are associated with the inner surface of the nuclear membrane (Moroi *et al.*, 1981).

The ends of the chromosomes, telomeres, have also been shown to be associated with the nuclear membrane during interphase (Moens, 1987). The telomeres are specialised structures found at the end of a linear chromosome which are responsible for the stability and complete replication of that chromosome. They consist of an array of short tandem repeats at the very ends, with longer more complex repeats inside these. The sub-telomeric repeats are thought to account for the associations with the nuclear membrane and each other. These tandem repeats show similarities between the species (Allshire et al., 1988) and human telomeres have now been cloned (Cross et al., 1989). Telomeres have also been shown to have non-histone proteins associated with them. In *Oxytricha* a 55kDa and a 43kDa species have been identified (Price and Cech, 1989). Another associated protein, telomerase, has been identified in *Tetrahymena* and is responsible for the addition of the repeated sequences of nucleotides base by base (Greider and Blackburn, 1987; Greider and Blackburn, 1989). This ensures the complete replication of the chromosome.

There is another type of division which cells can undergo, but this does not occur in the somatic cell population. Meiosis is a type of segregation which is exclusive to the germ line cells within an organism. The organisation of the DNA during meiosis is essentially similar to that during mitosis. The DNA condenses to form chromosomes by the looping of the chromatid and its attachment to the meiotic scaffold. This scaffold is thought to be unique to meiosis in its protein composition, although it contains some of the

structural proteins found within the scaffold formed by mitotic cells. The folding of the loops onto the core is very precise and they are positioned within the chromosome so that recombination can occur during meiosis I when the homologous chromosomes align in parallel by the formation of synaptonemal complexes (SC) (Moens and Pearlman, 1988). The structure and sequences of the DNA involved in forming these complexes is as yet undetermined. Recombination is correlated with the occurrence of a recombination nodule (RN) on the SC (Carpenter, 1979) and therefore positions at which chiasma are formed are dependent on the position of the SC. As stated this type of cellular division occurs in the germ line cells and relative to the rest of the body is a rare event. For the purposes of this introduction, structure of chromosomes and their associated proteins within somatic cells is more important.

The other form of nucleic acids present within the nucleus are those in the ribosylated form, the RNAs. They also have proteins specifically associated with them or involved in their functions. RNA is associated with chromatin at specific times during the cell cycle as a result of transcription of the genetic code of the DNA. Unlike prokaryotes, this process is isolated inside the nucleus by the nuclear envelope, so transcription is not accompanied by simultaneous translation. Therefore the RNA initially transcribed can be modified prior to translation and so the structure of the translated product is very different from the initial transcript. The processing it undergoes will be described below.

1.2.2 RNA and Associated Proteins

RNA is the intermediate which carries the information coded for by the DNA into the cytoplasm so that it can be translated giving rise to a functional protein. There are three RNA polymerases (I, II and III) involved in the process of transcribing the code of the DNA into RNA: RNA polymerase I is responsible for synthesising the large ribosomal RNAs. The primary transcript is known as the 45S RNA and this gives rise to the 28S RNA, 18S RNA and the 5.8S RNA. RNA polymerase III is responsible for the transcription of the tRNAs, the small ribosomal 5S subunit and the small nuclear RNAs, whilst polymerase II is involved in the transcription of the genes which give rise to the proteins required by the cell. Polymerases all begin and end transcription at specific places which are specified by a group of proteins known as transcription factors (Bogenhagen, 1985; Bieker *et al.*, 1985). RNA polymerase II produces precursor mRNA, also known as heterogeneous nuclear (hn) RNA, which is variable in size according to the genes from which they were transcribed. Most of these transcripts will be translated into protein in the cytoplasm, but before this they are packaged by specific nuclear proteins into a form known as hnRNP (Martin *et al.*, 1980; Dreyfuss *et al.*, 1984). It is in this form that the transcripts undergo a series of modifications within the nucleus. The first modification is the capping of the 5' end of the molecule with an additional guanine nucleotide (Adams and Cory, 1975; Perry and Kelly, 1976). The second modification involves the addition of 100-200 adenylic acid residues to the 3' end of the transcript by poly-A polymerase. However the 3' end is not that specified by the actual transcription termination site but one is

produced by the cleaving of the mRNA 11-30 nucleotide bases after a signal sequence AAUAAA, by a specific endonuclease (McLaughlin *et al.*, 1973; Zaret and Sherman, 1982; Wickens, 1990). The mRNA is now further processed and long stretches of RNA are removed from the middle of the molecules (Crick, 1979). This process is known as splicing and in most eukaryotic cells, it is mediated by a group of particles known as snRNPs (Lerner and Steitz, 1981; Lerner et al., 1980). The 5' end of the RNA sequence to be removed is bound by U1 snRNP and the 3' end is possibly bound by U5 snRNP at this point. The presence of ATP allows U2 to complex to the intron at its 3' end which precipitates the formation of a spliceosome by the addition of U4/U6 and U5 snRNPs. Another energy dependent step releases the intron RNA as a lariat structure and the two exons become bound together (from a review by Sharp, 1987). The snRNPs form a major group of autoimmune antigens as previously discussed and this property was of great use in establishing which snRNPs were required for splicing. The mRNA is transported out of the nucleus in this processed form to be translated into protein. The RNA polymerase I product is a 45S RNA and this too is modified post-transcriptionally. It is broken down into its component 28S, 18S and 5.8S structures by nucleolar ribonuclease activities (Denoya *et al.*, 1981) and U3, which is only found in the nucleolus, is thought to be involved (Crouch *et al.*, 1983; Bachellerie *et al.*, 1983). New ribosomes are assembled in the nucleolus from precursor molecules which have been packaged as RNP particles. 5S RNA, which is transcribed by RNA polymerase III does not undergo post-transcriptional modification (Birkenmeier *et al.*, 1978). The tRNAs which are also a product of polymerase III are modified after

transcription. The primary transcripts of the tRNAs are produced with extra sequences at their 5' and 3' ends and these sequences are "trimmed" by RNase type activities. The 5' end is trimmed first. The 3' end then has a CCA terminus added by an energy dependent reaction. The enzyme catalysing this reaction is tRNA nucleotidyltransferase (Deutscher, 1982). The tRNAs also contain introns which are spliced out by cleaving the precursor tRNA at the boundaries of the intron and then ligating the two halves of the tRNA molecule together again (Peebles *et al.*, 1979). The final post-transcriptional proccessing of tRNA is that of base modification or transposition which is thought to be necessary for stabilising the tRNA in preparation for translation.

This concludes the discussion of the nucleic acids, their structure and their associations with proteins, the other major class of molecule present within the nucleus. These proteins perform both structural and catalytic functions in association with the DNA and RNA of the cell. The final characteristic of the nucleus is the envelope which isolates the contents and their functions from the cytoplasm. I have mentioned it briefly but will now discuss its properties and structure in more detail.

1.2.3 The Nuclear Envelope

The nuclear envelope is a double membrane formed by a specialised region of the endoplasmic reticulum (ER). However these membranes do not entirely isolate the nucleus from the surrounding cytoplasm, due to the presence of pore complexes. These provide a diffusion channel for ions and small molecules and are able to actively select and transport larger proteins and RNP particles into

the cytoplasm (Schroder *et al.*, 1988). The nuclear pores are large complex structures composed of proteins of sizes ranging from 45-210kDa. However the arrangement and function of the individual proteins is not known. The inner and outer membranes differ functionally; the outer membrane is involved in membrane bound protein synthesis and has been shown to be continuous with the cellular ER. The inner membrane is lined by a fibrillar mesh known as the nuclear lamina. This is believed to function in the attachment and spatial organisation of the interphase chromatin and rearrangements of the nuclear membrane structure during the cell cycle. The nuclear lamina consists of intermediate filament (IF) - like proteins comprised of a central helical rod flanked by non-helical head and tail domains. they also contain a signal which localises them to the nucleus (Loewinger and McKeon, 1988). There are three types of lamins in vertebrates: A, which is completely solubilised during mitosis and B, which remain attached to the nuclear membrane throughout the cell cycle and lamin C(Burke and Gerace, 1986). The lamina is associated with the inner membrane due to its interaction with the integral membrane proteins (Senior and Gerace, 1988). Three proteins of 75kDa, 68kDa and 55kDa have been identified and shown to be exclusively located at the inner membrane and tightly associated with the insoluble lamina. A 58kDa membrane protein from avian nuclear envelope has been postulated to be a lamin B receptor (Worman *et al.*, 1988). The association of the lamina to the inner membrane has also been suggested to be due to post-translational modifications of the lamins (Wolda and Glomset, 1988; Beck et al., 1988). The nuclear membrane breaks down during mitosis and reassembles when division is complete. The mechanisms

are poorly understood but the process of reassembly is thought to be related to the phosphorylation of the lamin proteins. The reformation of the nuclear membrane has been suggested to control DNA replication itself (Blow and Laskey, 1988).

This section has discussed the major components of the nucleus and their structures. The nucleus is a very complex organelle and the processes that occur within it are essential for the existence of the eukaryotic cell. It is not possible to discuss all the minor nuclear components due to lack of space in this introduction and, indeed, due to the lack of knowledge. The areas that I have discussed show how the complexity of the structure correlates with the vast range of anti-nuclear autoantibodies which have been isolated to date (from the sera of patients). Hopefully these, and further autoantibodies yet to be identified, will continue to aid our research into the structure of the nucleus, and at the same time, our expanding knowledge of the antigenic targets may help our understanding of autoimmune disease.

1.3 The Aims of the Project

Many previous studies have established the use of autoimmune anti-nuclear antibodies for the identification and characterisation of nuclear antigens. A previous observation by Jeppesen and Nicol showed that serum from a scleroderma/CREST patient contained autoantibodies to an antigen which was localised to the non-histone scaffold region of the metaphase chromosome (Jeppesen and Nicol,

1986). This clearly demonstrates the use of autoimmune sera for research purposes. Therefore, in the light of this discovery, the aim of my project was to use the sera from patients with a range of autoimmune diseases, to identify further antigens, hopefully playing structural roles within the nucleus.

CHAPTER TWO

MATERIALS AND METHODS

Table 2.1 giving details of the sera used in the course of this project.

Batch No	Patient No	Initials	Disease
1	#1	MJ	SLE
1	#2	MN	SLE
1	#3	JM	MCTD
1	#4	JM	dermatomyositis
1	#5	JP	SLE
1	#6	AG	SLE
1	#7	ML	scleroderma
1	#8	LH	Sicca syndrome
1	#9	MM	(ideopathic thrombocytopaenic
1	#10	KM	(purpura (ITP))
1	#11	JaL	polymyositis
1	#12	JG	Raynaud's phenomenon only
1	#13	H	scleroderma
1	#14	R	MCTD
1	#15	FF	MCTD
1	#16	LS	SLE
1	#17	SS	MCTD
1	#18	CL	ITP
1	#19	M	osteoarthritis
1	#20	B	Osteoarthritis
1	#21	EP	CREST
1	#22	SC	CREST
1	#23	BB	SLE
1	#24	NF	SLE
2	1-12		scleroderma
2	13-24		MCTD

2 MATERIALS AND METHODS

2.1 Patients and Sera

Autoimmune sera used in the course of this project were obtained from patients attending the Northern General Hospital, Edinburgh and gifted to me by Professor G. Nuki. The control serum JL was obtained from an apparently healthy female volunteer who worked in this unit.

Antibody IC2

This antibody was given to me by D. Hartley. It is a mouse polyclonal serum antibody which had been precipitated with ammonium sulphate. The antibody was raised against a fusion protein of glutathione S-transferase and a 1.2kb Eco RI fragment from the C-terminal end of the *Drosophila melanogaster E(spl)* clone, NB5. The fusion protein was produced by the vector pGEX-1N in the host cell XL-1 blue.

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the cells were frozen in 10% glycerol, the culture medium was changed 24 hours (hrs) after thawing.

2.2.2 Maintenance of Cell Lines in Culture

Three types of cell line were used in this project and each required different conditions.

Don K_2

This is a Chinese hamster lung fibroblast cell line. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Biocult) supplemented with 7.5% foetal calf serum (FCS)

(Gibco Biocult) which had been heat inactivated for 30 minutes (mins), and grown as an attached culture at 37°C on 9cm Petri dishes (Nunc) in a 10% CO_2 atmosphere.

L cells

This is a mouse fibroblast cell line (Flow Laboratories; NCTC L929, passage number 564). They were cultured in DMEM supplemented with 10% FCS and 1x non-essential amino acids from a 100x stock (Gibco Biocult). As before these cells were grown as an attached culture on 9cm Petri dishes at 37°C in a 10% CO_2 atmosphere.

Both of these cell lines were passaged routinely once a week and were detached from the dishes by 0.25% trypsin solution from a 10x stock (Gibco Biocult) in 0.02% ethylenediaminetetraacetic acid (EDTA). The cells were split at 1:10 and 1:100 before replating, and were also fed once a week between passages.

PARIT

This is a human lymphoblastoid cell line and is cultured in RPMI 1640 (Gibco Bio-cult) supplemented with 10% FCS as a suspension culture in 9cm Petri dishes (Sterilin) again at 37°C in a 10% CO_2 atmosphere.

These cells were passaged twice a week, split at a ratio of 1:3.

2.2.3 Preparation of Whole Cells for Indirect Immunofluorescence

Cell lines which were growing as attached cultures (ie. Don K_2 and Mouse L fibroblasts) were plated out onto Petri dishes

containing glass coverslips and grown up for 24 hrs. The coverslips were then rinsed in cold Dulbecco's A phosphate buffered saline (PBS) (Oxoid) and fixed for 6 mins in 100% methanol at -20°C (Aubin *et al.*, 1980). They were then rinsed in either PBS or "KCM" (chromosome isolation medium as described in Gooderham and Jeppesen, 1983; 120mM KCl, 20mM NaCl, 10mM Tris pH 8.0, 0.1% Triton X-100, 0.5mM EDTA pH 8.0) and were then taken through the indirect immunofluorescence procedure which will be described later in this chapter.

The PARIT cell line which grows in suspension was resuspended at 1 x 10^5 cells/ml in RPMI and 500µl was spun onto slides using a Cytotek centrifuge (Miles Laboratories) at 2K for 10mins and then fixed as before in methanol. Metaphase spreads were prepared using this cell line by incubating a culture passaged the previous day, for 3 - 4 hrs in 0.1µg colcemid (Fluka) per ml of RPMI (in order to arrest the cell cycle in metaphase). The cells were resuspended in RPMI at 3 x 10^5 cells/ml and swollen by the addition of 2 vols of water and incubation at 37°C for 15mins. 500µl of cells, again at 1 x 10^5 cells/ml, were spun onto slides, but in this case, they were not fixed at all.

The slides are washed for 10mins in PBS/KCM prior to staining by indirect immunofluorescence.

2.3 Preparation of Denatured Nuclear Protein from Cells Grown in Culture

Cells from 5 - 10 plates were counted, harvested by pelleting at 1,000 rpm at RT and then gently resuspended in 10ml of freshly prepared 75mM KCl. The cells were swollen at 37°C for 15mins and then cooled on ice for 5mins. The cells were repelleted at 4°C and resuspended in 10ml KCM pH 7 plus 2mM $CaCl_2$. Triton X-100 was added to 0.1% and the cells incubated on ice for 5mins to lyse. Lysis was completed by passing the cells several times through a 20G long needle, using a syringe, and the released nuclei were pelleted at 4°C. The nuclei were then resuspended in 2ml of KCM pH 7 plus 2mM $CaCl_2$, 0.1% Triton X-100 and 1mM phenylmethyl-sulphonylfluoride (PMSF). The nuclei were incubated on ice for 30mins to allow the PMSF to deactivate any proteases and then repelleted. They were washed in KCM pH 7 plus 2mM $CaCl_2$, 0.1% Triton X-100 and 0.1mM PMSF and then resuspended in TBS plus 2mM $CaCl_2$, 0.1% Triton X-100 and 0.1mM PMSF. The nuclei were pelleted and then resuspended in a volume of TBS (as above) such that there are 2.5×10^8 cells/ml. 10µl 0.5M EDTA pH 7.4 is added, then 100µl of 10% SDS, 5% β-mercaptoethanol (βme) per 400µl of TBS and this is then boiled in a waterbath for 3mins and allowed to cool. 100µl (per 400µl TBS) of 50% glycerol, 0.1% bromophenol blue (BPB) is added and the preparation is stored at -20°C.

2.4 Preparation of Fusion Proteins

Recombinant clones of host cells (BL21 DE3 or XL-1 blue) were grown up o/n at 37°C in 10ml LB plus 50µg/ml Amp. The next day 1-2ml of this o/n culture was used to inoculate fresh LB plus Amp, and this was incubated at 37°C for 4 hrs. IPTG was added to a final concentration of 0.5mM and a Time 0 sample taken. After 1 or 2hrs an induced sample was also taken.

1ml of the uninduced and induced sample was spun down in a microcentrifuge, and the supernatant discarded. The bacterial pellet was resuspended in 100µl of TE and 100µl of 1% SDS. The sample was then made up to a final concentration of 10% glycerol and 0.01% BPB. It is now ready to be run on a polyacrylamide gel.

2.5 Separation of Proteins by Electrophoresis

The method used in this project was discontinuous sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), which was originally described by Laemmli, 1970. The following modifications were also applied.

A 20cm x20cm slab gel was used. The stacking gel was 3% acrylamide and the running gel was 15% acrylamide. The running buffer was 192mM glycine, 25mM tris (hydroxymethyl) aminomethan (Tris) and 0.1% SDS. The samples were run at 20mA until they reached the stacking/running gel front and then at 10Watts until the bromophenol blue (BPB) dye was approximately 0.5cm from the bottom of the gel.

The gel was stained overnight in Coomassie Brilliant Blue R250 (Gurrs) at 0.5% w/v in 50% methanol, 10% acetic acid. It was then destained in 10% methanol, 7% acetic acid.

2.6 Transfer of Proteins onto Nitrocellulose Membrane

2.6.1 Western Blotting

This procedure was first described by Towbin *et al.*, 1979 as transfer blotting. The procedure for the purpose of this project was carried out as follows.

The SDS polyacrylamide gel was first equilibrated in transfer buffer (192mM glycine, 25mM Tris, 20% (v/v) methanol) plus 0.1% SDS (w/v) to facilitate the movement of the proteins out of the gel matrix. The proteins were then electrophoretically transferred onto the nitrocellulose membrane (0.45µm pore) (Schleicher and Schuell) at 0.5A for 2 - 3hrs. To check that the transfer has been successful a portion of the nitrocellulose was cut off and stained in Amido black at 0.1% w/v (Hopkin and Williams) in 20% ethanol, 7% acetic acid. It was then destained in 20% ethanol, 7% acetic acid.

2.6.2 Immobilisation of Protein Produced by Expression Vectors

Nitrocellulose discs were soaked in 10mM isopropyl-β-D-thio-galactopyranoside (IPTG) for a few minutes and then dried thoroughly at 42°C. They were then overlaid onto the culture plates, keyed to them with a needle and incubated for 1.5 - 2hrs at 42°C. The filters were then rinsed in TBS pH 8.2.

2.7 Staining Techniques

2.7.1 Indirect Immunofluorescence of Whole Cells and Metaphase Spreads

The fixed (excluding the metaphase spreads) and rinsed cells on slides or coverslips were incubated for 1 hr at room temperature (RT) in a damp environment in the first antibody solution. The serum antibody was diluted to 1% (v/v) in 10% (v/v) whole normal rabbit serum in PBS/KCM. The cells were washed twice in PBS/KCM and incubated under the same conditions for 30mins in the second antibody solution. The second antibody was a fluorescein conjugated rabbit anti-human IgG γ antibody (DAKO) and was diluted to 5% (v/v) in 10% (v/v) whole rabbit serum in PBS/KCM as before. The cells were washed again and fixed in 4% (w/v) paraformaldehyde in PBS/KCM for 30mins. They were then washed in water and air dried.

2.7.2 Counterstaining of Whole Cells and Metaphase Spreads

In order to visualise the nucleus and chromosomes easily the immunofluorescently stained cells were counterstained with a stain which is specific for DNA.

Coverslips were stained with dichlorophenol-indophenol (DAPI) at 0.5µg/ml for 10mins under damp conditions at RT. The coverslips were washed, dried and mounted in glycerol (Merck) and PBS (Citifluor) at 1:1.

Slides were stained with Hoechst 33258 at 0.5µg/ml in 1x sodium saline citrate (SSC). They were washed in 1x SSC for 10mins

then stained for 10mins and then washed in 1x SSC then water for 10mins each. After air drying they were mounted as above.

2.7.3 Immuno Staining of Nitrocellulose Membranes

Nitrocellulose membranes were first blocked in 10% horse serum (HS), 0.05% Tween-20 in TBS pH8.2 (HS/TBS/Tw) for approximately 15mins to reduce any non-specific binding of the serum antibody. The first antibody was diluted 1/300 in HS/TBS/Tw and the nitrocellulose incubated in this for 3hrs at RT. The membrane was washed twice in 0.05% Tween-20 in TBS pH8.2 (TBS/Tw) and then incubated in the second antibody solution overnight (o/n). The second antibody was a peroxidase conjugated rabbit anti-human IgG γ antibody (DAKO) and was diluted 1/200 in HS/TBS/Tw. The membrane was washed as before and then rinsed in 50mM Tris pH 7 to remove the salts deposited by the other washes. The antibody binding sites were visualised using 3mg/ml 4-chloro-1-naphthol in methanol diluted 1:5 in 50mM Tris pH 7 plus hydrogen peroxide (30% w/v) at a 1/3000 dilution and then washed in water and air dried.

2.8 Affinity Purification of Serum Autoantibodies

The method used during the course of this project is that of Olmsted, 1981. The antibody of interest was bound to the protein band on a preparative Western blot for which it showed affinity. Two strips were incubated with the second antibody and stained by the method described earlier. These strips were aligned with the remainder of the blot and the protein band with the bound antibody was excised.

The antibody was eluted from the blot by first washing with Buffer 1 (0.25% gelatin, 0.05M Tris.HCl pH 7.5, 5mM EDTA, 0.15M NaCl, 0.05% Nonidet P-40) minus the the Nonidet P-40 and gelatin. The strips were then cut into small pieces and put into a syringe and washed again with Buffer 1 minus the Nonidet P-40 and gelatin. Then 0.2M glycine.HCl pH 2.8 was added and incubated for 2mins before being forced through the filter twice. It was then neutralised with NaOH and stabilised by the addition of 0.1% BSA. This can be used neat on Western blots and with 10% rabbit serum added for cells instead of first antibody as previously described.

2.9 Bacterial Cell Culture

2.9.1 Media and Additives

All media were sterilised by autoclaving.

L-Broth and Agar

Per litre : 10g tryptone (Difco), 5g yeast extract (Difco), 10g NaCl, 2.46g $MgSO_4$ pH 7.2. The agar contains 15g agar in addition.

SOC Broth

Basic medium per litre : 20g tryptone, 5g yeast extract, 0.59g NaCl, 1.86g KCl. Before use filter sterilised $MgCl_2$ and $MgSO_4$ were added to a final concentration of 10mM and filter sterilised glucose (kept frozen) was added to a final concentration of 20mM.

Top Agar

Per litre : L-broth plus 0.7% agarose (w/v) and re-autoclaved to dissolve.

Bacteriophage Diluting Solution - SM

Per litre : 5.844g NaCl, 6.055g Tris, 2.465g $MgSO_4$, 0.2g gelatin at pH 7.5.

Media Additives

When appropriate, antibiotics were added to the media. Ampicillin was used at 25 - 50µg/ml; tetracycline at 12 - 15µg/ml. 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (X-gal) was stored as a 2% stock solution in dimethylformamide at -20°C. IPTG was also stored as 100mM stock.

2.9.2 Bacterial Strains Used

All strains used are *E. coli*.

XL1-Blue

*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, (rk^-, mk^+), *sup*E44, *rel*A1, λ-, (Δlac), {F', *pro*AB, lacIqZDM15, Tn10(tetR)} (Bullock *et al.*, 1987). This strain is a suitable host for pUC based plasmids.

Y1090

rk, *lac*, *lon*, *hsd*R, *sup*F, Δ*lac*, Δ*lon*, pMC9. Permissive for the growth of λgt11 (Young and Davis, 1983; Jendrisak *et al.*, 1987).

BL21 DE3

*hsd*S, *gal* (λc*Its*857*ind*1 *S*am7 nin5 *lac* UV5-T7 gene 1), *lon*. This strain was used as a host cell for the production of fusion proteins from the vectors pGEX-3X and pGEMEX-1.

2.10 Vectors

2.10.1 Plasmid Vectors

pBluescribe

This is a plasmid/'phage vector of 3.2kb derived from pUC19. It is ampicillin resistant and contains T7 and T3 promoters either side of the pUC19 polylinker and the *lac Z* promoter. It contains a polylinker inserted into the β-galactosidase gene (*lac Z*). Non-recombinant plasmids are able to synthesise this enzyme which breaks down X-gal to release a blue indolyl derivative. In recombinant plasmids the *lac Z* gene is interrupted by foreign DNA, thus the colonies remain colourless.

pBluescript KS(+/-)

This is a phagemid vector of 2,964bp derived from pUC19. It has f1 'phage and *col*E1 origins and T7 and T3 promoters flanking the polylinker. It contains the *lac Z* promoter and has ampicillin resistance.

pGEX-3X

This is a vector of 4,952bp derived from pBR322 which is used to produce fusion proteins. It is ampicillin resistant and contains the glutathione S-transferase gene which is used to select recombinants on a column.

pGEMEX-1

This expression system is based on the pGEM vector. Sequences cloned into the vector are expressed as T7 gene 10 fusion proteins. It is ampicillin resistant and contains three RNA polymerase

promotors, a T7 termination sequence down stream from the multiple cloning region and f1 origin of replication.

2.10.2 Bacteriophage Vectors

λgt11

λlac5, *shn*dIII12-3, *sr*Iλ3°, *cIts*857, *sr*Iλ4°, *nin*5, *sr*Iλ5°, *S*am100.

λgt10

λ*sr*Iλ1°, *b*527, *sr*Iλ3°, *imm*434, (*sr*I434^{+}), *sr*Iλ4°, *sr*Iλ5°.

2.11 Screening of Libraries Constructed in Bacteriophage λ Vectors

2.11.1 Screening of Expression Libraries with Antibodies

For details of all solutions and media used in this protocol refer to 2.9.1.

Bacteriophage λ were grown on lawns of *E. coli* (strain Y1090). (A plating stock of these cells was prepared from an o/n culture of a single colony in 25ml L-broth plus 0.2% maltose.) For the primary round of screening approximately 2 x 10^5 'phage were plated on a 9cm Sterilin dish. 100µl of an appropriate dilution of the library in SM was added to 200µl of plating cells and incubated at 37°C for 15 - 30mins. 3 - 4ml of top agar was added to this, mixed and poured onto an L-agar plate and allowed to set. The plate was inverted and incubated at 42°C for 4hrs after which the plaques should be just about visible on the lawn of bacteria. The plates were overlaid with the nitrocellulose filters soaked in IPTG and incubated for a further period as described earlier. After washing in TBS the

filters were probed using the serum antibodies. After staining the positive plaques were marked and the filters re-aligned with the plate. A plug of agar around the positive signal was taken up with the wide end of a Pasteur pipette and blown into 1ml of SM plus a few drops of chloroform, to lyse the bacterial cells, and incubated at RT for 1hr. This stock should contain approximately 1 x 10^6 pfu (plaque forming units *i.e.* bacteriophage) and was used for the second round of screening. Approximately 1 x 10^4 pfu per plate were plated and screened as before. A noticeable amplification of positive plaques will be observed if the primary plaques were true positives. Again a plug of agar corresponding to a positive signal was blown into SM from the thick end of a Pasteur pipette. A third round of screening was performed with approximately 1,000 pfu per plate. Again a large plug of agar was picked and the final round of screening was performed with only 100 pfu on each plate. Every plaque formed on these plates should give a positive signal with the antibody. This time a single positive plaque was picked with the thin end of the Pasteur pipette into 1ml of SM. This is a suspension of purified bacteriophage which contain the DNA sequence which codes for the protein recognised by the antibody.

2.11.2 Screening of Non-Expression and Expression Libraries with DNA

The principle of this type of screening is the same as above. Again the bacteriophage were grown on lawns of *E. coli* (Y1090) but the 2ml of plating cells infected with 1ml of an appropriate dilution of bacteriophage were plated on large 20 x 20cm plates in

20ml of top agar at 2 x 10^4 pfu per plate. The plates were inverted and incubated at 42°C o/n. Duplicate filters were overlaid and keyed to the plate. The plated bacteriophage were screened by hybridising radiolabelled sequences of DNA to the filters and visualising any binding by autoradiography. Plaques which gave positive signals on both autoradiographs were selected as before and rescreened with approximately 1,000 pfu per plate. If the signal was particularly strong a single plaque may be isolated from the secondary plates. If not a third round at 200 pfu per plate is necessary to ensure that the plaque picked was definitely giving a positive signal.

2.12 Manipulation of Plasmid DNA

2.12.1 Preparation of Frozen Cells for Electroporation

A litre of L-broth was inoculated with $^1/_{100}$ volume of a fresh o/n culture and the cells grown up with vigorous shaking at 37°C until they reached a density of 0.5 to 1 (OD_{600}). The flask was then chilled for 15 - 30mins and the cells harvested by pelleting in a cold rotor at 4,000 x g_{max} for 15 mins. The cells were resuspended in 1 litre of cold water and then repelleted as before. They were again resuspended, this time in 0.5 litre of water and pelleted. The cells were now resuspended in approximately 20ml of 10% glycerol and centrifuged again. The final resuspension was in a final volume of 2 - 3ml in 10% glycerol which gave a cell concentration of 3 x 10^{10} cells/ml. Aliquots of 40µl were frozen on dry ice and stored at -70°C.

2.12.2 Electroporation of XL1-Blue

An aliquot of prepared cells was thawed at RT and put on ice. 1 - 2µl (10 -50ng) of DNA in a low ionic strength buffer was mixed in and left on ice for 1min. The Gene Pulser apparatus is set to 25µF and 2.5kV with the Controller at 200W. The cells and DNA were transferred to a 0.2cm cuvette which is placed in the chilled safety chamber between the contacts. A single pulse was applied and the cells immediately resuspended in 1ml SOC. The suspension was incubated at 37°C for 1hr and then plated on L-agar supplemented with 25µg/ml ampicillin, 2µg/ml X-Gal and 40µl of 100mM stock of IPTG per plate. The plates were incubated at 37°C o/n. The recombinant colonies are white.

2.12.3 Preparation of Frozen Cells for Heat Shock Transformation

400µl of an o/n culture of cells (one colony from a plate inoculated into 10ml of L-broth and grown up o/n at 37°C) was diluted 1/50 into 20ml of L-broth Mg^{2+} and grown up at 37°C to OD_{560} 0.3 - 0.6. 6ml of this culture was then diluted at 1/50 in 294ml L-broth Mg^{2+}, plus 600µl 5M NaCl and 7.5ml 1M KCl (i.e. 10mM NaCl and 25mM KCl). The cells were grown up to OD_{560} 0.48 and then chilled on ice for 15mins. The cells were then pelleted in bottles rinsed in ethanol and DEPC water, for 5mins at 4K at 4°C. The cells were taken up in 100ml of TFB I (1.43g NaOAc, 1.1g $CaCl_2$, 75g glycerol, 6g RbCl and 4.95g $MnCl_2$ per 500ml) at pH 5.8 and stored on ice for a further 10 - 15mins. The cells were repelleted by spinning at 5K for 5mins at 4°C and then resuspended in 12ml of TFB II (0.209g MOPS, 0.12g RbCl, 1.64g $CaCl_2$ and 15g glycerol per 100ml) at pH 6.8.

The suspension was chilled on ice for 20mins before aliquotting into 200µl and snap freezing in liquid N_2. The competent cells are now stored at -70°C.

2.12.4 Heat Shock Transformation of XL1-Blue

10µl (10 - 50ng) of DNA was added to 95µl of XL1-blue cells thawed from -70°C on ice. The two were mixed and incubated on ice for a further 15mins before incubation at 42°C for 75secs. This was then chilled on ice for a further 2mins. 525µl of L-broth was added and the cells grown up for 50mins before plating onto selection plates.

2.12.5 Preparation of Fresh Cells for Heat Shock Transformation

One colony from a streaked plate was grown up o/n in LB. 1ml of this culture was inoculated into fresh LB and grown to an OD_{560} 0.3 - 0.5. The cells were then spun down and resuspended in 0.5 vol of ice cold 50mM $CaCl_2$. The cells were then incubated on ice for 15mins before being spun down again. They were then resuspended in 0.1 vol of ice cold 50mM $CaCl_2$ and are now ready for transformation.

2.12.6 Heat Shock Transformation of BL21 DE3

20µl (10 - 50ng) of DNA was added to 200µl of cells and mixed. The cells were now incubated on ice for 45mins, before heat shocking at 42°C for 80secs. The cells were cooled on ice for 2mins and then grown at 37°C for 45mins in LB, before plating on the appropriate selection media.

2.12.7 Small Scale Plasmid Preparations

The Alkaline Lysis Method

The method used for the purpose of this project was a modification of the methods of Birnboim and Doly, 1979 and Ish-Horowicz and Burke, 1981, as described in Sambrook, Fritsch and Maniatis (2nd edition) Volume 1, p.1.25 - 1.28.

A method involving a further modification of the above method was found to be particularly useful as the preparations could be directly sequenced. 1mg/ml of lysozyme was added to Solution I, 3M CH_3COONa is used instead of Solution III and the DNA precipitated by the addition of 0.7 volumes of isopropanol. The remainder of the method with the omission of the RNase treatment was essentially the same.

2.12.8 Large Scale Plasmid Preparation

The Alkaline Lysis Method

This method is a modification of that of Birnboim and Doly, 1979 and Ish-Horowicz and Burke, 1981, in Sambrook, Fritsch and Maniatis (2nd edition) Volume 1, p. 1.38 - 1.39. The purification procedure was that of equilibrium centrifugation in CsCl-ethidium bromide gradients also in Volume 1, p. 1.42 - 1.43 and the extraction by organic solvent (p. 1.46).

2.13 Manipulation of Bacteriophage λ DNA

2.13.1 Small Scale Bacteriophage λ DNA Preparation

The first step was the production of a plate lysate which is done according to Protocol II in Sambrook, Fritsch and Maniatis (2nd edition) Volume 1, p. 2.66. The DNA was purified by a method involving the use of LambdaSorb (Promega). 100µl of the adsorbent was added per 10ml of lysate and incubated on ice for 30mins. This was centrifuged at 7,500 rpm for 10mins and the supernatant discarded. The pellet was resuspended in 1ml of SM and recentrifuged. This step was repeated and then the pellet resuspended in 500µl of 10mM Tris HCL pH 7.8, 10mM EDTA and heated to 70°C for 5 mins to release the 'phage DNA. The adsorbent was pelleted and the DNA extracted from the supernatant with buffered phenol/chloroform twice and once with chloroform only. The DNA was then precipitated with $^{1}/_{10}$ volume 3M CH_3COONa and an equal volume of iso-propanol on dry ice for 20mins, and then pelleted. The DNA was washed in 70% ethanol, dried and resuspended in 10mM Tris HCl pH 7.8, 0.1mM EDTA (TE).

2.13.2 Large Scale Bacteriophage λ DNA Preparation

A 500ml flask of L-Broth was inoculated with 1ml of a λ infected host suspension and incubated o/n at 37°C with vigorous shaking. 10ml of chloroform was added to the o/n culture which was shaken at 37°C for a further 30mins to ensure complete lysis. The 'phage DNA was purified according to the protocol in Sambrook, Fritsch and Maniatis (2nd edition) Volume 1, p. 2.73 - 2.76 up to

step 11. The protocol was then changed and the next step was equilibrium centrifugation in CsCl at 35,000 rpm at 15°C o/n. The DNA was then dialysed for 2x 1hr in 10mM NaCl, 50mM Tris - HCl pH 8, 10mM $MgCl_2$ to remove the CsCl. EDTA at pH 8 was added to a 20mM final concentration, proteinase K at 50µg/ml and SDS to 0.5% and this was incubated at 65°C for 1hr. The DNA was extracted once each with phenol, phenol/chloroform and chloroform and then precipitated as for the small scale preparation above. It was pelleted, washed and resuspended in TE.

2.14 Manipulation of DNA by Enzymes

2.14.1 Restriction Endonuclease Digestion

Digests were carried out according to the protocols issued by the manufacturers using buffers A, B, L, M and H supplied by Boehringer Mannheim. Genomic DNA was digested o/n with approximately 5 - 10 units of enzyme per µg of DNA. The reactions were stopped by the addition of 1x stop mix from a 10x stock (0.2M EDTA pH 8, 15% Ficoll and Orange G dye).

2.14.2 Ligation of DNA Molecules

Ligase is isolated from *E. coli* infected with bacteriophage T4 and is available commercially from Boehringer Mannheim. It catalyses the formation of a phosphodiester bond between 3'-OH and 5' phosphate groups of DNA (Weiss *et al.*, 1968). It is used to join complementary cohesive ends of ds DNA. These reactions were carried

out o/n at 4°C in 50mM Tris HCl, 10mM $MgCl_2$, 1mM spermidine, 100µg/ml bovine serum albumin (BSA), 1mM ATP, 10mM dithiothreitol (DTT) at pH 7.4.

2.14.3 Nested Deletions of Recombinant Plasmids

Deletions of dsDNA in one direction only are possible due to the observation that blunt ends and 5' overhangs (generated by the reaction of restriction enzymes) are susceptible to exonuclease III, whereas 3' overhangs are not (Henikoff, 1984). The progressive removal of nucleotides from one of the strands by exonuclease III leaves a single strand of DNA which is cleaved off by S1 nuclease. Nested deletions are created by removing aliquots of reaction mix from an ongoing exonuclease III digestion. The molecules of DNA with their progressively longer deletions are recircularised by the ligation protocol above and transformed into XL1-Blues by electroporation. The deletions were performed using a double stranded nested deletion kit from Pharmacia.

2.14.4 *In Vitro* Amplification of Specific Sequences of DNA

This is achieved by means of the polymerase chain reaction using a thermostable DNA polymerase isolated from *Thermus aquaticus* (*Taq*) described by Saiki *et al.*, 1988. The oligonucleotide primers required were prepared in this unit by Doreen Chambers.

2.15 Separation of DNA by Electrophoresis

2.15.1 Agarose Gel Electrophoresis

Conventional horizontal agarose gel electrophoresis was used for the separation of DNA fragments of up to 50kb. The concentration of agarose used was between 0.5 and 2% made up in 1x TAE buffer (per litre of 50x stock : 242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA pH 8). The gels were also run in 1x TAE. To prestain the gels, 0.5µg/ml of ethidium bromide was added to the TAE buffer.

Specific fragments of DNA used for cloning or radiolabelling were also isolated by electrophoresis. However the agarose used was of low gelling temperature (LGT) in order that the DNA fragment required (cut out of the main body of the gel under long wave UV light) could be purified using the method of Burnmeister and Lehrach, 1989. This involves melting the gel slice at 65°C and then adding EDTA at pH 8 to 5mM and NaCl to 100mM. The gel was cooled to 37°C and 2 units of agarase added per 100µl of gel. This was incubated for >1hr and then the DNA was phenol/chloroform extracted, precipitated and then resuspended in TE before use.

2.15.2 Polyacrylamide Gel Electrophoresis

Sequencing reactions performed as described in 2.21 were run on 5% polyacrylamide gels cast and run on Bio-rad apparatus. The plates used were 21 x 50cm and were separated by wedged spacers which graduated from 0.25mm at the top to 0.75mm at the bottom. Casting solutions from Sequagel were used (as described by the

suppliers) and set using 10% ammonium persulphate and NNN'N'-tetramethylethyl ethylenediamine (TEMED). The gel was pre-run to warm it in 1x TBE (per litre of 5x stock : 54g Tris base, 27.5g boric acid and 20ml of 0.5M EDTA pH 8) for approximately 1hr at 60 watts. After the samples were loaded the gel was run for approximately 2.5hrs at 55 watts. It was then cooled, fixed (to remove the urea) in 10% methanol, 10% acetic acid made fresh every time for >30mins and dried under vacuum onto Whatman 3MM paper in preparation for autoradiography.

2.16 Transfer of DNA to Nylon and Nitrocellulose Membranes

2.16.1 Southern Transfer of DNA to Nylon Membranes

DNA separated on agarose gels was transferred onto reusable nylon membrane (Hybond-N) using the method of Southern, 1975. Prior to the transfer the gel was photographed under UV light with a ruler so that the DNA transferred to the membrane could be sized at a later date. The UV illumination introduces thymidine dimers into the DNA which makes the transfer of large DNA molecules easier. The gels were denatured in 1.5M NaCl, 0.5M NaOH for two 30 mins periods at RT, washed in water and then neutralised in 3M NaCl, 0.5M Tris at pH 5 for two further periods of 30mins. The actual transfer of the DNA was achieved by capilliary blotting the DNA with 20x SSC. The gel was placed onto Whatman 3MM wetted with 20x SSC and Hybond-N wetted in 2x SSC was placed on top with no air bubbles trapped in between it and the gel. Two pieces of 3MM wetted in 2x SSC were placed on top of the membrane and then a stack of paper towels put

on top of them. A glass plate is rested on the top and the gel allowed to blot o/n. When the transfer was complete, the membrane was washed in 2x SSC and air dried. The DNA was covalently bound by exposing the membrane DNA-side down to UV light for 5mins.

To reuse the membrane any hybridised probe was removed by washing at 45°C for 30mins in 0.4M NaOH. It is then neutralised by incubation in 0.2M Tris, 0.1x SSC, 0.1% SDS for 30mins at 45°C also.

2.16.2 Immobilisation of DNA from Bacteriophage Colonies onto Nitrocellulose

A piece of nitrocellulose was carefully placed onto the plate from the middle outwards ensuring it was thoroughly wetted by the agar and left for 30 secs whilst it was keyed to the plate with a wide gauge needle. It was then lifted carefully off using Millipore forceps and placed into denaturing solution for 5mins DNA-side uppermost. It was transferred into neutralising solution for a further 5mins and then washed in 2x SSC. A second piece of nitrocellulose was overlaid onto the same plate and left for 2mins whilst it was keyed to the plate using the holes made when keying the first filter. This too was denatured, neutralised and washed. The filters were air dried on 3MM and then baked at 68°C for 2 hrs to bind the DNA to the filter. This was the method used for screening libraries by DNA hybridisation.

2.16.3 Immobilisation of Bacterial Colonies on Nitrocellulose

The method is essentially the same as that of the immobilisation of bacteriophage colonies. An additional incubation

step of 5mins in 1% SDS before denaturation and neutralisation of the DNA ensured that the bacterial cells were broken open to allow the DNA to be released.

2.17 Separation of RNA by Electrophoresis

Horizontal gel electrophoresis was used to separate the RNA samples. The gel was made by melting 1g of agarose in 87ml of DEPC dH_2O. This was then cooled and 10ml of 10x MOPS (0.2M 3-(N-morpholino)propanesulphonic acid, 0.05M CH_3COONa, 10mM EDTA) and 5.1ml of 37% formaldehyde were added, the gel mixed, poured and left to cool. The RNA sample was made up to 10µl with DEPC dH_2O and then 15µl of sample buffer (consisting of 10µl formamide, 2.5µl formaldehyde, 1.25µl 10x MOPS and 1.25µl dH2O) is added and this is then incubated at 55°C for 20mins. $^1/_{10}$ volume of loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol) is then added. In order to prestain the RNA 1µl of 1µg/ml stock of ethidium bromide is added to each sample. The gel is run in 1x MOPS buffer at 20V o/n.

2.18 Transfer of RNA to Nylon Membrane

RNA was blotted o/n onto Hybond-N in 10x SSC using the same method as for the Southern blotting of DNA. The Northern blot was then washed in 2x SSC, air dried and UV crosslinked to the membrane, as described previously.

To reuse the filter after hybridisation, any bound probe was removed by incubating the membrane in 0.2% SDS for 1 hour at 80°C. It was then washed in 2x SSC as before.

2.19 Radiolabelling of DNA

2.19.1 Random Priming

DNA probes were labelled by random priming using the method of Feinberg and Vogelstein, 1983 and 1984. DNA prepared for random priming was in LGT agarose after having been purified by running through a gel. It was boiled for 5mins to denature the DNA before storage at -20°C. Random hexanucleotides anneal to the denatured DNA and act as primers for complementary strand synthesis. This is catalysed by Klenow enzyme from the 3'-OH ends of the primers in the presence of ^{32}P-labelled dCTP and unlabelled dATP, dGTP and dTTP. A commercially available random priming kit from Boehringer Mannheim was used.

25ng of denatured DNA was added to a reaction mixture containing dATP, dGTP and dTTP each at 25µM/litre, random hexanucleotides and buffer. 2 units of Klenow enzyme and 30µCi α-^{32}P dCTP (3,000Ci/mmol, 10mM/ml) were also added. The reaction (in a volume of 20µl) was incubated at 37°C for >1hr. The percentage incorporation of the label into the DNA was estimated by TCA precipitation of counts on a Whatman GF/A filter. (10% TCA quantitavely precipitates oligonucleotides of <20 bases.) Proteins and unincorporated nucleotides were removed from the probe by centrifugation through a Sephadex G-50 column. The DNA is excluded

from the column and passes through first. The probe has to be denatured once more, immediately before use.

2.19.2 End Labelling

T4 polynucleotide kinase (PNK) catalyses the transfer of the γ-phosphate of ATP to a 5'-OH group of DNA or RNA (Sambrook *et al.*, 1989). 20 - 30ng of oligonucleotide was added to a reaction mix containing 1x PNK buffer (50mM Tris.HCl pH 7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA pH 8), 100µCi γ-^{32}P ATP and 20 units of PNK in a total volume of 20µl. This was incubated at 37°C for 30mins and then incorporation was checked by TCA precipitation as before.

2.20 Nucleic Acid Hybridisation

2.20.1 Solutions Used For Hybridisation

Church and Gilbert Buffer

7% SDS, 0.5M $NaPO_4^{2-}$ at pH 7.2. This hybridisation solution is described by Church and Gilbert, 1984. It was used for most random primed DNA probes.

Denhardt's Buffer

0.5% SDS, 0.1% sodium pyrophosphate, 5x SET/Denhardt's solution (Stock solution is 20x : 0.4M Tris pH 7.8, 3M NaCl, 20mMEDTA, 0.4% Ficoll, 0.4% polyvinylpyrolidine (PVP), 0.4% BSA.) Denatured sonicated salmon sperm DNA was added as a competitor at 100µg/ml. This solution was used with certain random primed DNA probes and always when using nitrocellulose membrane.

"Quick Hyb"

0.1% SDS, 0.1% sodium pyrophosphate, 0.05% BSA, 0.05% PVP, 0.05% Ficoll, 5x SSC. This solution was used for hybridisations using end labelled oligonucleotides.

2.20.2 Hybridisation Protocol for Random Primed Probes

All filters were double sealed into plastic bags and pre-hybridised for >30mins at 68°C. All hybridisation incubations were o/n at 68°C in the appropriate solution containing the radiolabelled probe.

After hybridisation, the filters were rinsed twice in 2x SSC, 0.1% SDS at 68°C for 10 mins each and then more stringently washed twice with 1 - 0.1x SSC, 0.1% SDS at 68°C for 30mins each. The filters were then placed in plastic bags in preparation for autoradiography.

2.20.3 Hybridisation Protocol Used for Oligonucleotide Probes

All filters are rinsed in 2x SSC, sealed into plastic bags and prehybridised in Quick Hyb at 68°C for >30mins. The probe is diluted with an aliquot of the solution from the sealed bag before addition and hybridisation o/n at 48°C. After hybridisation the filters are washed 4x for 5mins each with 4x SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 50°C. The filters are then sealed into plastic bags in preparation for autoradiography.

2.21 Sequencing

2.21.1 Sequencing Protocol

The sequencing performed during this project was on denatured double stranded DNA by the dideoxy chain termination method of Sanger *et al.*, 1977. The DNA is denatured by the addition of 1µl 2M NaOH per 4µl DNA (approximately 2µg) in TE. This is incubated at RT for 10mins and then 1.5µl of 3M sodium acetate pH 4.5 and 3.5µl of distilled water are added. 30µl of 100% ethanol is also added and the DNA precipitated on dry ice for 10mins before pelleting. The DNA is then washed in 70% ethanol, dried under vacuum and then resuspended in 7µl of distilled water.

The primer is annealed next. 2µl of Sequenase sequencing buffer and 1µl of primer are added and incubated at 65°C for 2mins. This is then allowed to cool before proceeding.

The sequencing is then carried out using the commercially available Sequenase Version 2.0 kit (USB) according to the protocol supplied with it. This method involves two basic steps; a polymerisation reaction in which limited concentrations of nucleotides (including ^{35}S-dATP) are used to extend the primer previously annealed. The second step is a chain termination reaction in which the radiolabelled chains are rapidly extended and then terminated by the incorporation of a dideoxy nucleotide. These reactions are specific to one particular nucleotide and so are conducted in sets of four, one for each of the bases G, A, T and C.

The samples contain a series of different sized fragments which can be resolved on a polyacylamide gel (Sanger and Coulson, 1978) and as they are radiolabelled, detected by autoradiography.

2.21.2 Sequence Analysis

The sequences were manipulated using the UWGCG programs and homology searches were carried out using the University of Wisconsin Genetics Computer Group's software package. Both of these facilities are available on the Daresbury computer.

2.22 Photography

2.22.1 Photography of Whole Cells and Metaphase Spreads on Slides and Coverslips

Whole cells and metaphase spreads were photographed by epi-illumination on a Leitz Ortholux II fluorescence microscope on black and white Kodak Tri-X and T-Max film.

2.22.2 Photography of Agarose Gels

Agarose gels were placed on a UV transilluminator and photographed using a Polaroid MP4 camera fitted with a red filter using Kodak T-MAX Professional film 4052 with an exposure time of 15 secs. The film was developed in an X1 X-OGRAPH automatic X-ray film processor.

2.23 Autoradiography

2.23.1 Autoradiography of Filters and Gels

The autoradiography in this project was carried out using X-ray film (Fuji Medical or Kodak X-OMAT) in light tight cassettes containing intensifying screens, with the exception of sequencing gel exposures. Filters were first exposed o/n at -70°C and then they were further exposed for different lengths of time and/or temperatures. Sequencing gels were exposed o/n at RT. The X-ray films were developed using an X1 X-OGRAPH automatic X-ray film processor.

CHAPTER THREE

ANALYSIS OF THE HUMAN AUTOIMMUNE SERA

3 ANALYSIS OF THE HUMAN AUTOIMMUNE SERA

3.1 Introduction

Antibodies present in the sera of patients suffering from autoimmune conditions have been shown to bind to specific antigens present in both the nucleus and cytoplasm of cells cultured *in vitro* (as discussed in Chapter 1).

The aim of this project was, if possible to identify and characterise novel nuclear autoantigens recognised by the sera of autoimmune patients. As a first step 24 samples of whole human serum, covering a wide range of systemic rheumatic diseases, were routinely screened in order to analyse the antigenic specificities of the autoantibodies present.

Two methods were employed; first, the antigenic site was localised by the immunocytological staining of whole cells and metaphase spreads of both human lymphoblastoid and Chinese hamster fibroblast cell lines (PARIT and Don K_2 respectively). Whole human cells were used in order to localise the antigen to the nucleus and/or the cytoplasm of the cell. Human metaphase spreads were used as an effective method of displaying any chromosomal associations, whilst the whole hamster cells were used to demonstrate the cross-species conservation of most (if not all) known autoimmune antigens. The cells and serum antibodies were prepared in both PBS and KCM (Chapter 2). As KCM contains a mild non-ionic detergent, soluble antigens within the cell may leach out through the cell membranes during preparation. The nucleoli however are resistant. PBS preparation is less likely to remove soluble antigens from the cell

and hence both methods are employed. However the results obtained by immunocytology are not always easy to interpret thus an additional technique was also employed to obtain further clarification.

The method used was Western blotting. Proteins from the nuclei of the human lymphoblastoid cell line only were isolated and separated according to their size by SDS-PAGE (Chapter 2). The polypeptides are inaccessible to antibodies within the polyacrylamide gel matrix and are therefore electroblotted (Western blotted) onto a nitrocellulose membrane allowing the antibody to bind to its antigen(s). Polyacrylamide gel electrophoresis involves the denaturation of protein samples by treatment with SDS and βme. This may reduce or even abolish the antigenicity of the protein by breaking down tertiary structure and removing disulphide bonds. Any antigenicity dependent upon this will be lost. Serum antibodies which show high levels of activity on immunocytochemistry do so upon antigens present in a more physiological environment, so a negative result on a Western blot does not mean that there are no antigens present, merely that their antigenicity has been lost due to denaturation. In this way known diagnostic antigens can be identified by means of their molecular weights, thus aiding diagnosis of the condition suffered by the patient. Novel nuclear proteins recognised by the serum autoantibodies can also be identified in this way.

The serum autoantibodies were also affinity purified from Western blots. The eluate can be used to check whether the antibodies have single or multiple specificities for nuclear proteins. Whole cells can also be stained and the polypeptide identified on

the blot can be specifically localised within the cell without the presence of other antibodies which produce the characteristically complicated staining pattern.

The information provided by these techniques is essential in order to begin the further characterisation of the serum autoantibodies and their antigens.

3.2 Results

3.2.1 Indirect Immunofluorescence of Whole Cells and Metaphase Spreads Using Sera #1(MJ) to #24(NF)

This method allows the site of the antigen(s) recognised by the serum antibodies to be cytologically localised.

A control serum, JL (obtained from a healthy female volunteer), was used to stain the cells in addition to the test sera, in order to determine the level of background staining achieved with a normal serum.

Figures 3.1, 3.2 and **3.3** show the patterns observed in whole PARIT cells, whole Don K_2 cells and metaphase spreads of PARIT cells respectively. The level of staining in the nuclei of the whole PARIT cells is fairly low and of a granular appearance in both the KCM and PBS preparations. In **(a)** one cell shows a more intense level of staining in both the nucleus and cytoplasm. This effect was observed in several cells in every preparation of whole PARIT cells prepared in KCM and stained with the serum JL. Don K_2 cells again show a generally low level of staining in their nuclei in both preparations. The staining is not quite as granular as that shown in **Figure 3.1** but a occasional cell in the KCM preparations shows

Figure 3.1 Whole PARIT cells stained with the control serum, JL, as the first antibody and fluorescein conjugated rabbit anti-human IgGγ as the second antibody.
(a) and (b) show cells prepared in KCM stained with JL and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with JL and counterstained with DAPI to localise the nucleus, respectively.

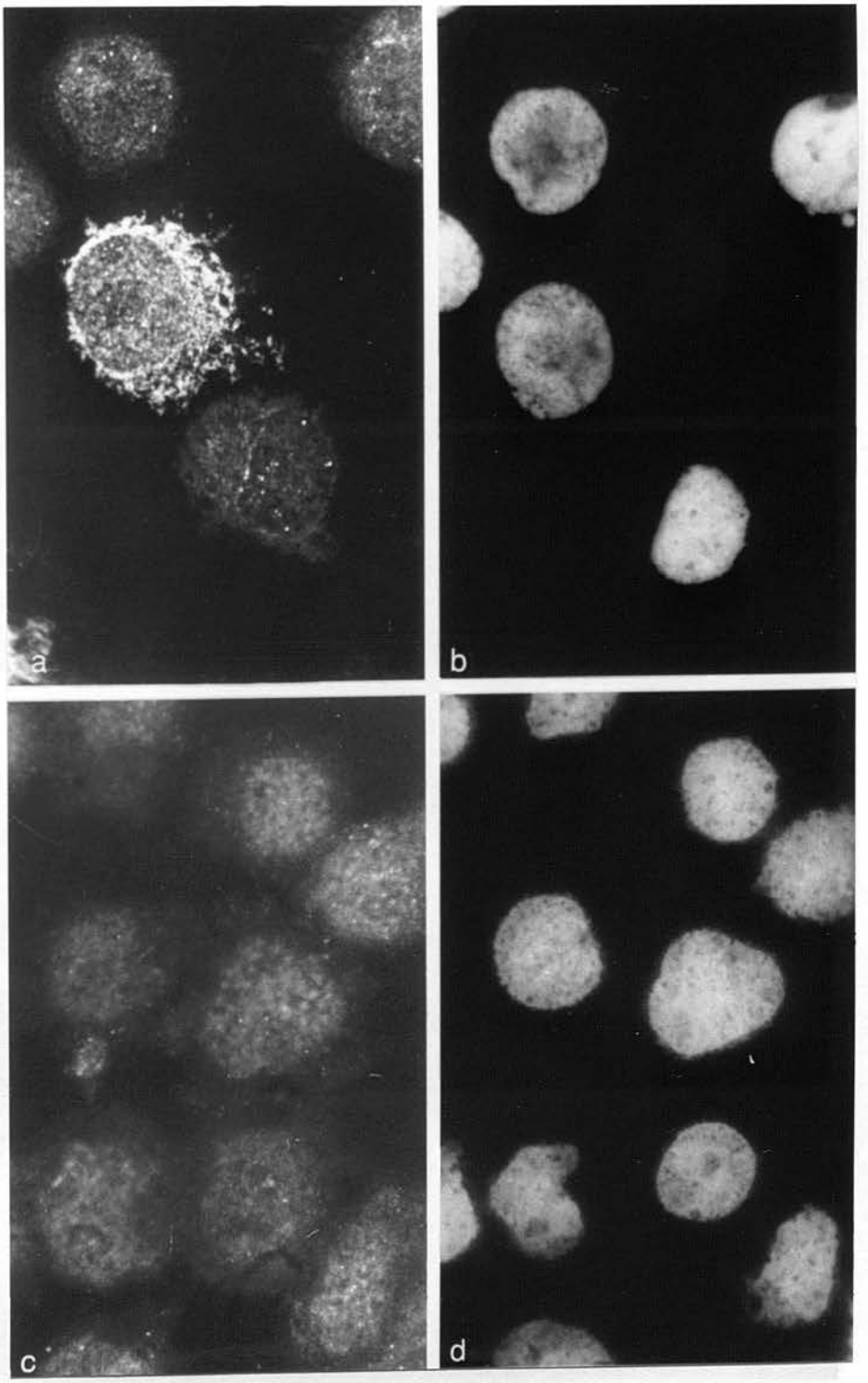


Figure 3.2 Whole Don K_2 cells stained with the control serum, JL, as the first antibody and fluorescein conjugated rabbit anti-human IgGγ as the second antibody.
(a) and (b) show cells prepared in KCM stained with JL and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with JL and counterstained with DAPI to localise the nucleus, respectively.

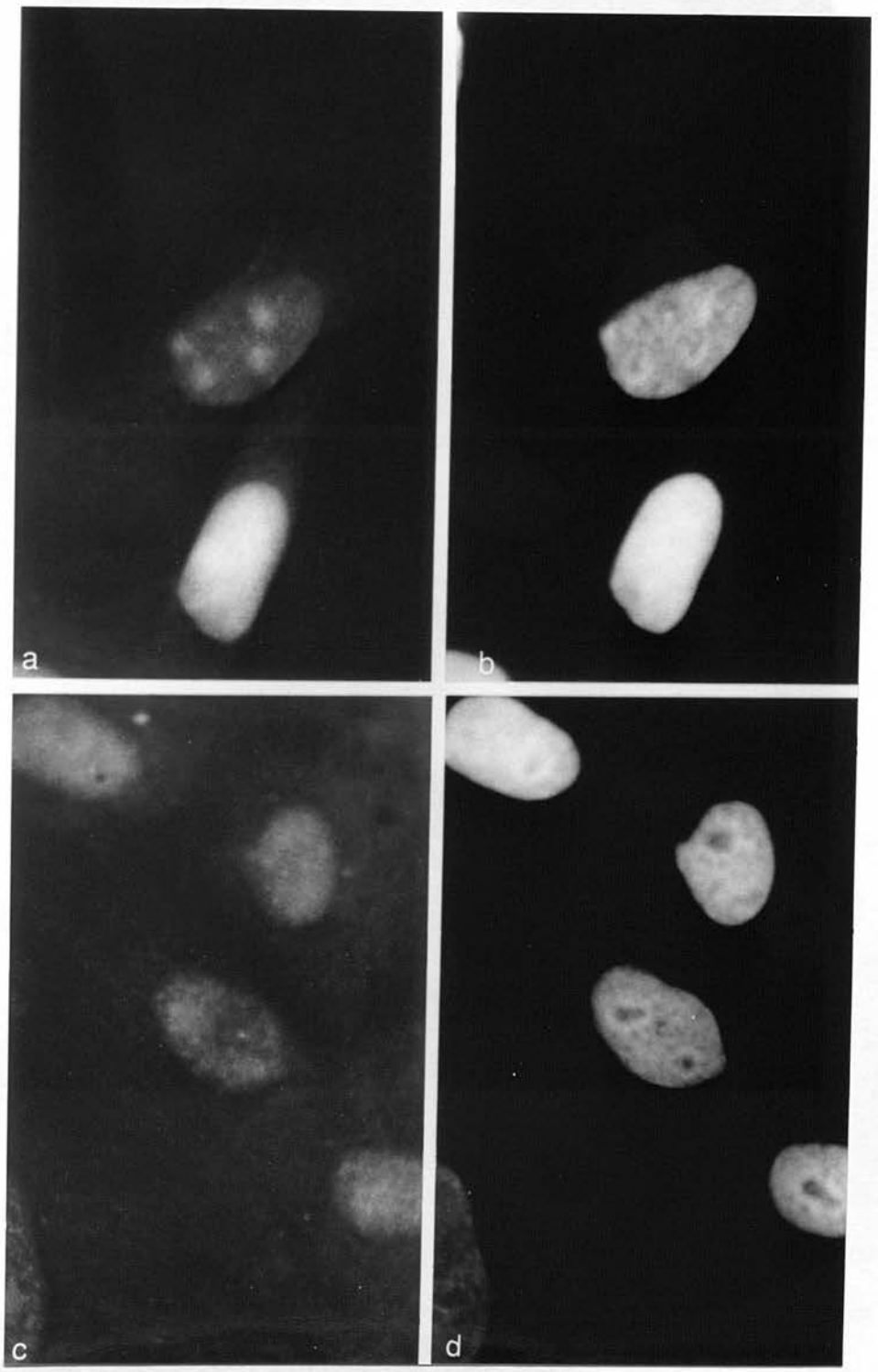
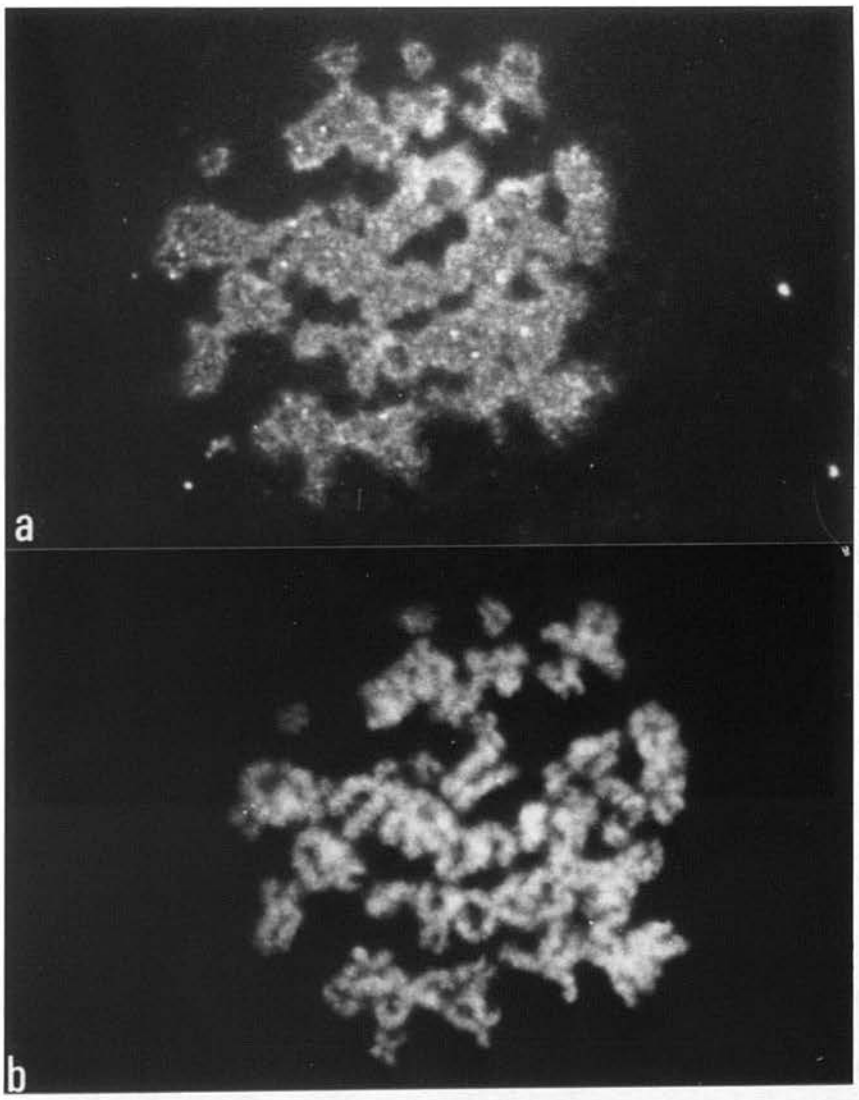


Figure 3.3 Metaphase spreads of PARIT cells stained with the control serum, JL, as the first antibody and fluorescein conjugated rabbit anti-human IgGγ as the second antibody.
(a) and (b) show cells prepared in KCM stained with JL and counterstained with DAPI to localise the chromosomes.



the atypical stronger level of staining observed previously. The metaphase spreads of PARIT show fluorescence localised around the area of the chromosomes themselves, but there is no particularly striking pattern observed.

Figures 3.4, 3.5 and **3.6** show the staining pattern observed when using Serum #7(ML) as the first antibody. PBS and KCM preparations of both the whole PARIT cells and Don K_2 cells show a granular staining pattern with very bright discrete speckles present within the nucleus alone. (Unfortunately the photograph of the PBS preparation of whole PARIT cells is a little underexposed and out of focus to show this as clearly as the photograph of the KCM preparation.) The metaphase spreads show these discrete speckles localised as a doublet at the kinetochore of each chromosome in **Figure 3.6(a)**. This staining pattern is characterisic of the CREST subset of scleroderma patients (as discussed in Chapter 1). Western blotting should show stained bands at the positions of one or more of the centromere antigens (CENP-A at approximately 19kDa, CENP-B at approximately 80kDa, or CENP-C at approximately 140kDa).

Figures 3.7, 3.8 and **3.9** show the staining pattern observed when using Serum #23(BB) as the first antibody. In the KCM preparations of whole PARIT and Don K_2 cells, the staining appears to be localised around the nuclear membrane, whilst the PBS preparations show very strong fluorescence of the whole nucleus, not dissimilar to the DAPI staining pattern. Metaphase spreads of PARIT cells show a high level of fluorescent staining localised to the actual chromosomes themselves. The staining patterns observed when using this serum are characteristic of those given by serum with DNA and/or histone antibodies present. These types of serum are usually

Figure 3.4 Whole PARIT cells stained with serum #7(ML), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #7(ML) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #7(ML) and counterstained with DAPI to localise the nucleus.

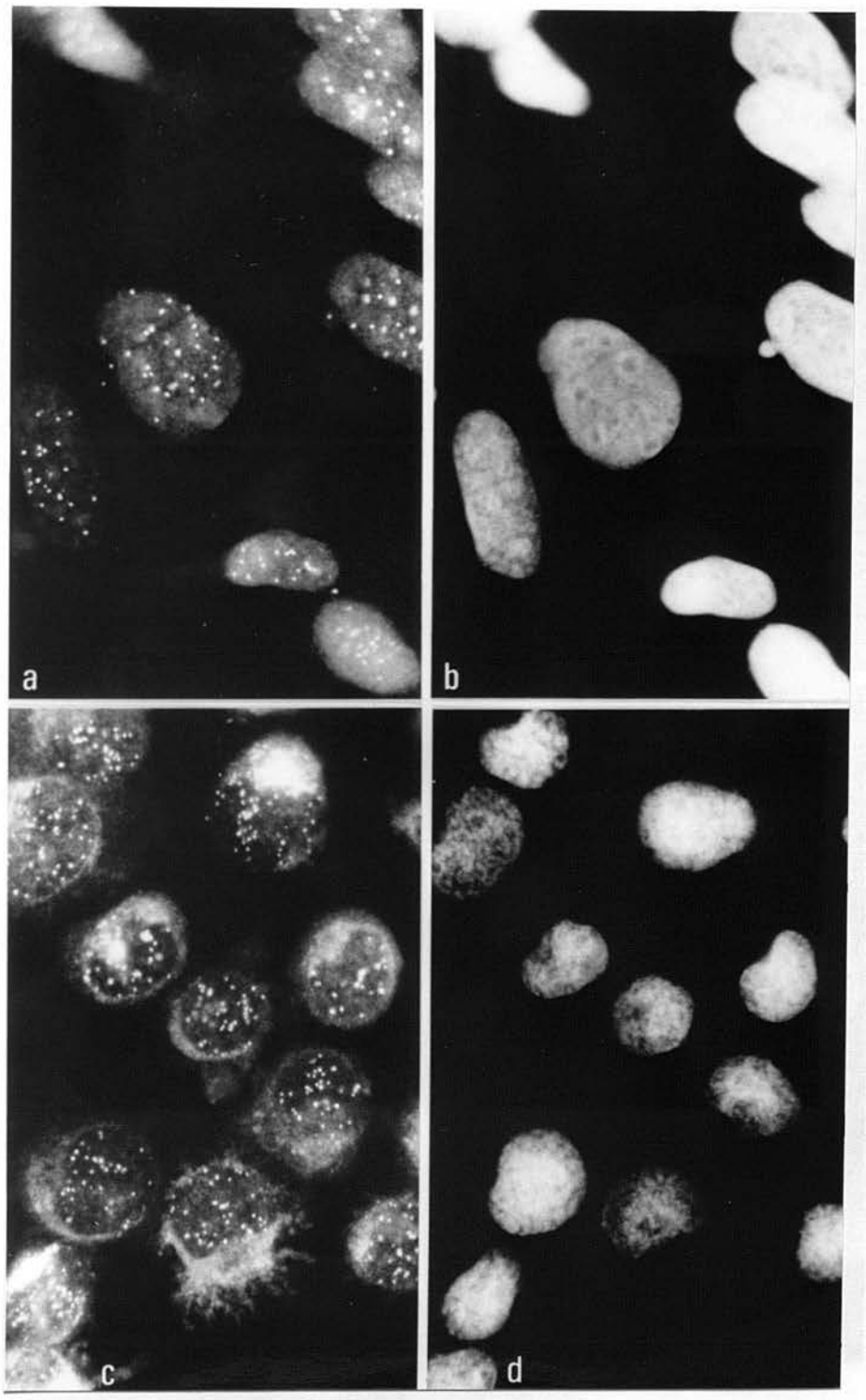


Figure 3.5 Whole Don K_2 cells stained with serum #7(ML), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #7(ML) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #7(ML) and counterstained with DAPI to localise the nucleus.

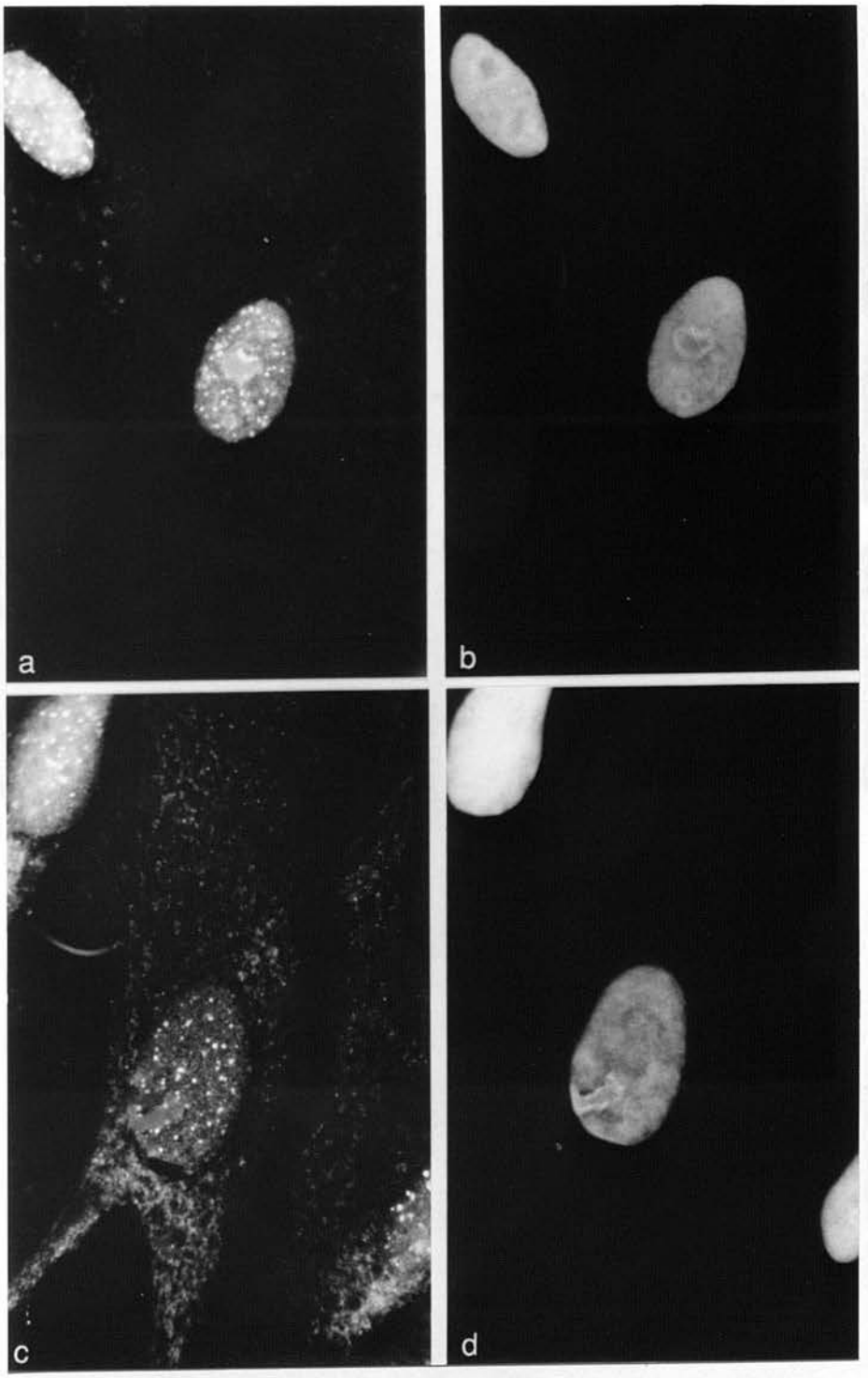


Figure 3.6 Metaphase spreads of PARIT cells stained with serum #7(ML), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #7(ML) and counterstained with DAPI to localise the chromosomes.

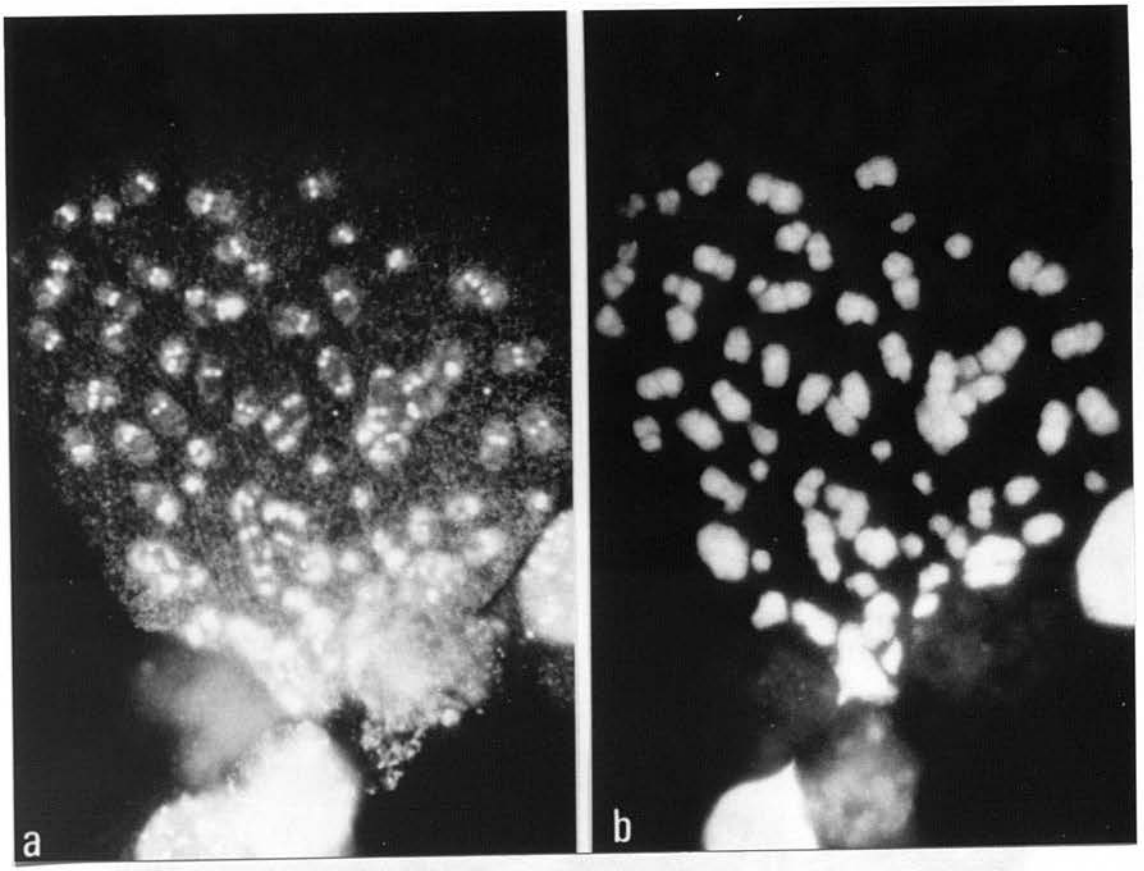


Figure 3.7 Whole PARIT cells stained with serum #23(BB), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #23(BB) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #23(BB) and counterstained with DAPI to localise the nucleus.

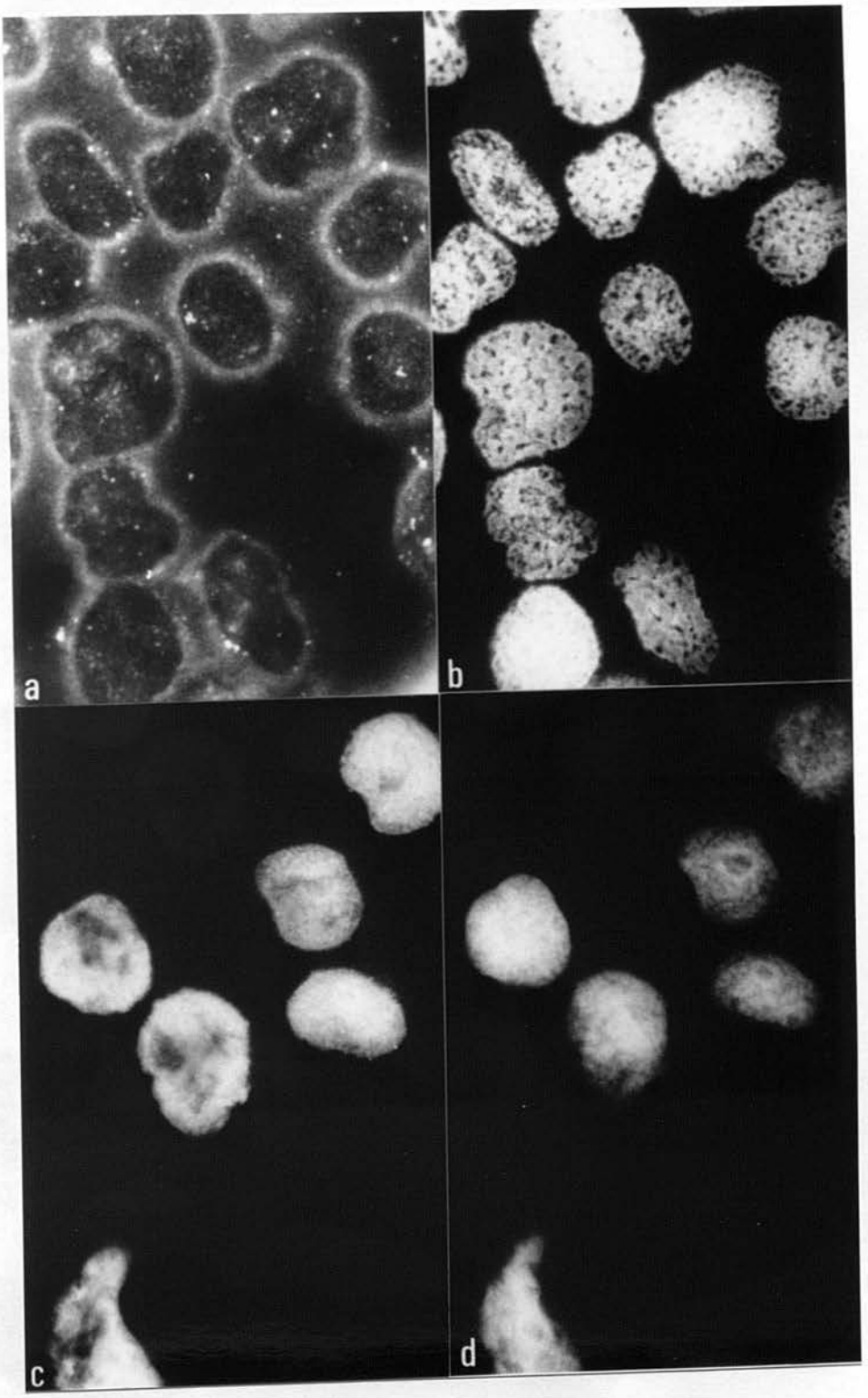


Figure 3.8 Whole Don K_2 cells stained with serum #23(BB), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #23(BB) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #23(BB) and counterstained with DAPI to localise the nucleus.

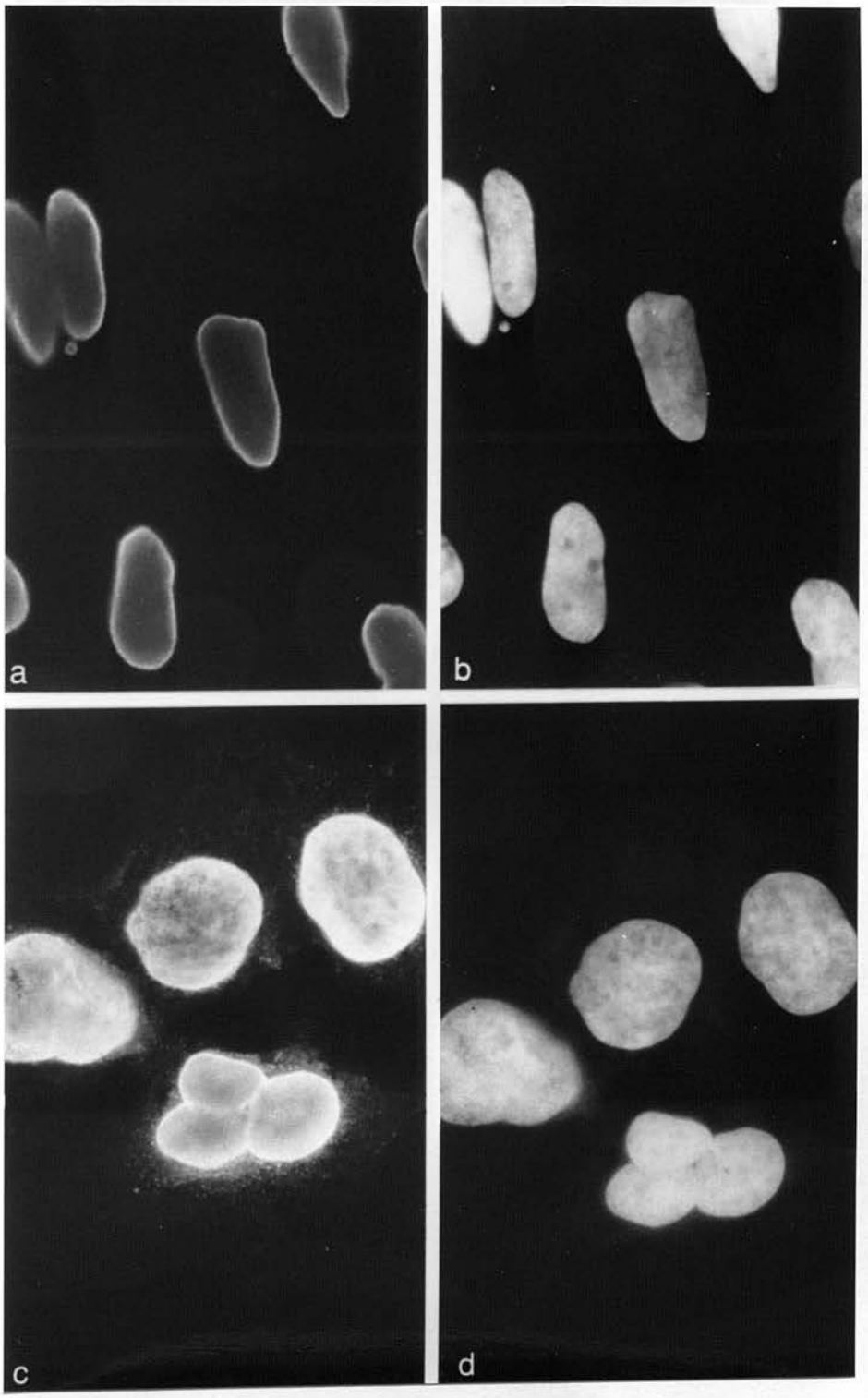
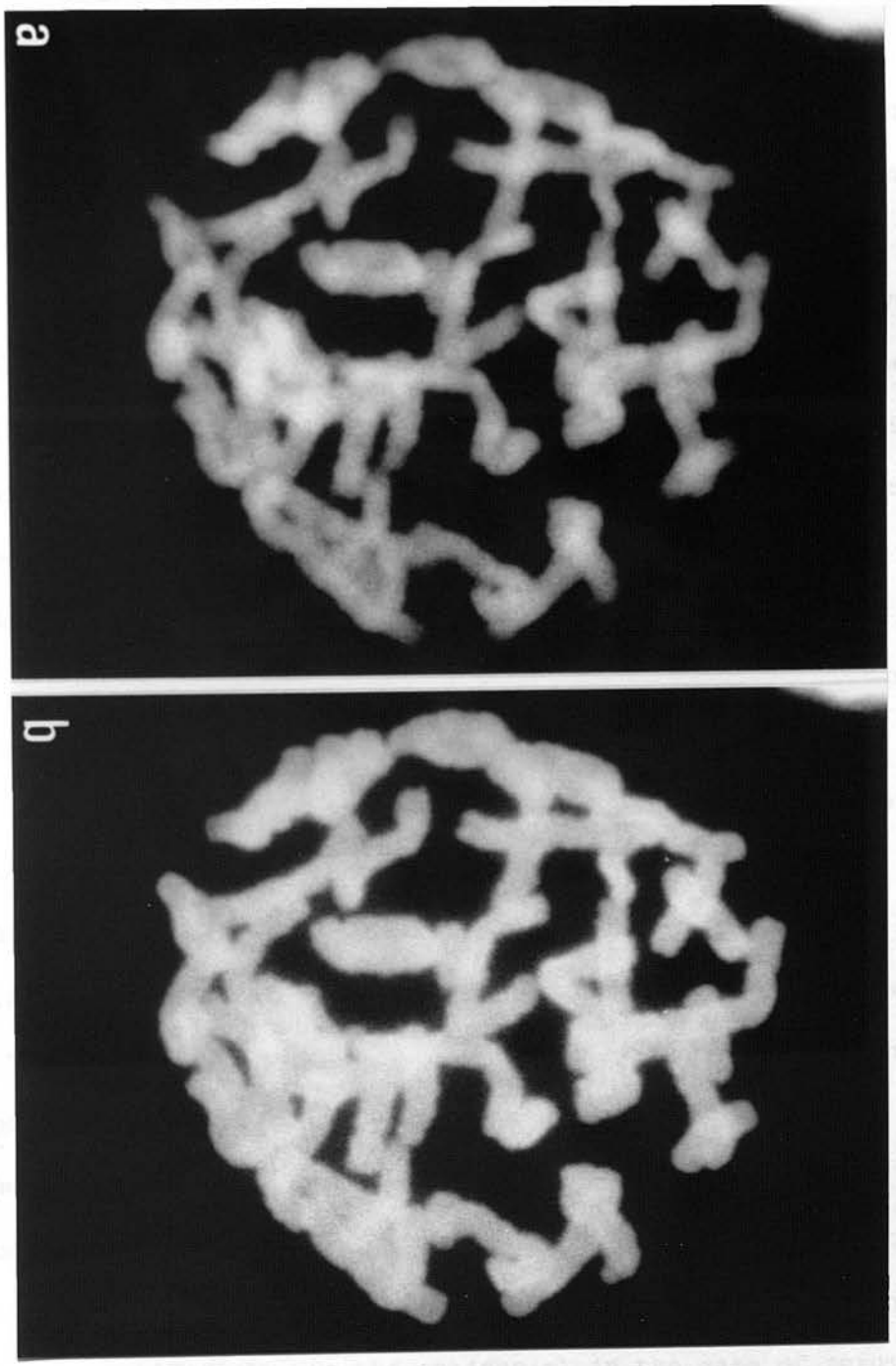


Figure 3.9 Metaphase spreads of PARIT cells stained with serum #23(BB), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #23(BB) and counterstained with DAPI to localise the chromosomes.



from patients with SLE. The Western blotting will identify whether the antigen is in fact DNA alone or whether histone is also recognised.

Figures 3.10, 3.11 and **3.12** show the staining patterns observed when using Serum #24(NF) as the first antibody.

Both types of preparation of the whole cells show a high level of fluorescence throughout the nucleus, again similar to the DAPI staining in **(b)** and **(d)**. The PARIT metaphase spread shown in **Figure 3.12** again has the staining localised to the chromosomes indicating that the antibodies are recognising DNA and/or histone as with #23(BB). There also appears to be some kinetochore recognition with this serum. Most of the chromosomes show brighter fluorescent speckles at their centromeres. Western blotting will shed further light on the actual identity of the antigen(s).

Figures 3.13, 3.14 and **3.15** show the staining patterns observed when #13(H) is used as the first antibody.

Whole PARIT and Don K_2 cells show coarse granular staining of the nucleus with some nucleolar localisation, particularly in the PBS preparation of the Don K_2 cells. The cell in anaphase in **Figure 3.14(c)** shows staining localised to the condensed chromosomes at either end of the newly forming daughter cells. The metaphase spreads show staining localised throughout the chromosome, therefore the antigen must again be a component of the chromosome or its scaffold. The whole cell staining pattern is not like that shown in **Figures 3.7, 3.8, 3.10** and **3.11** (which is typical of DNA and/or histone antibodies), so the antigen(s) in the case of serum #13(H) is(are) unlikely to be the same.

Figure 3.10 Whole PARIT cells stained with serum #24(NF), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #24(NF) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #24(NF) and counterstained with DAPI to localise the nucleus.

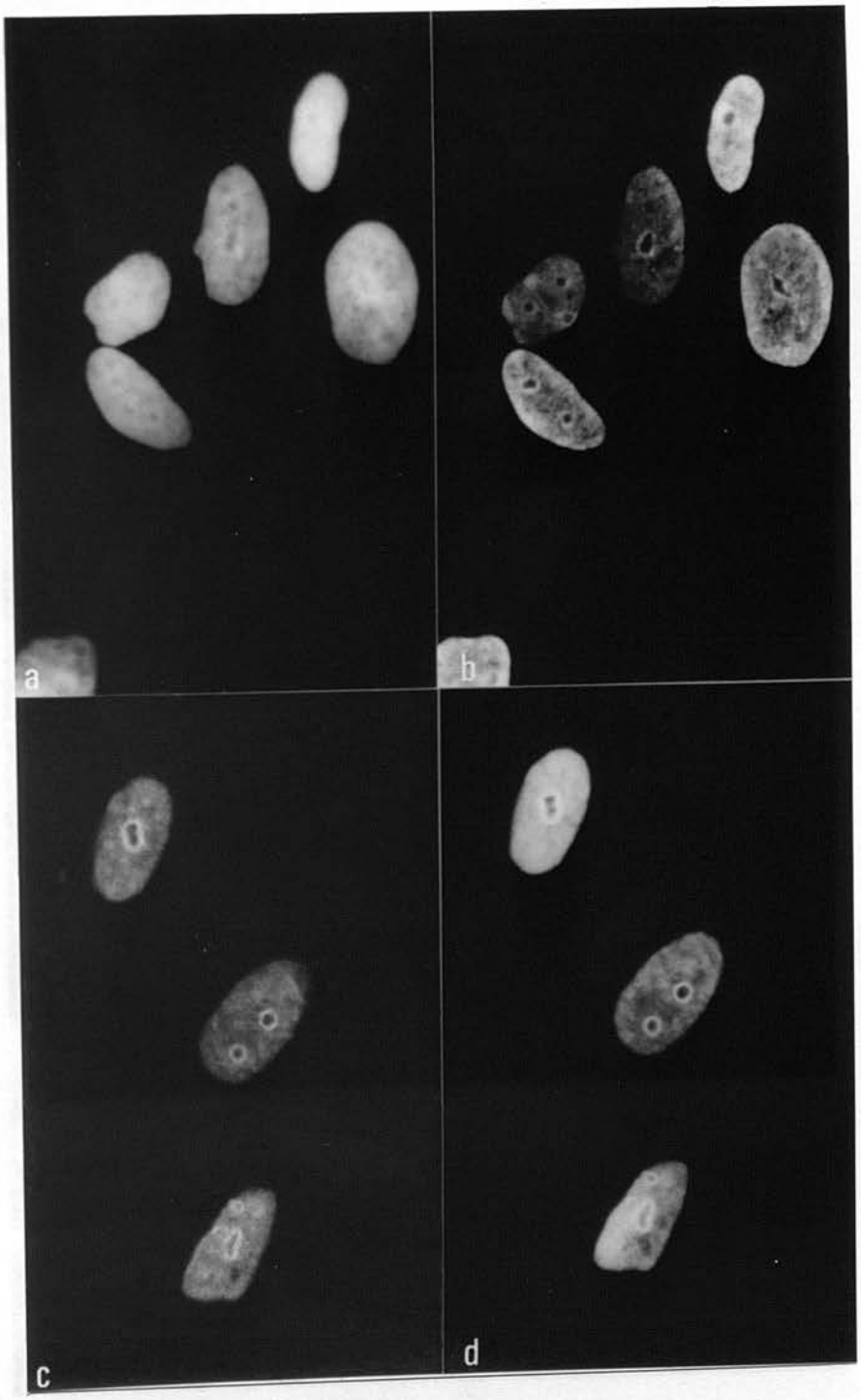


Figure 3.11 Whole Don K_2 cells stained with serum #24(NF), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #24(NF) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #24(NF) and counterstained with DAPI to localise the nucleus.

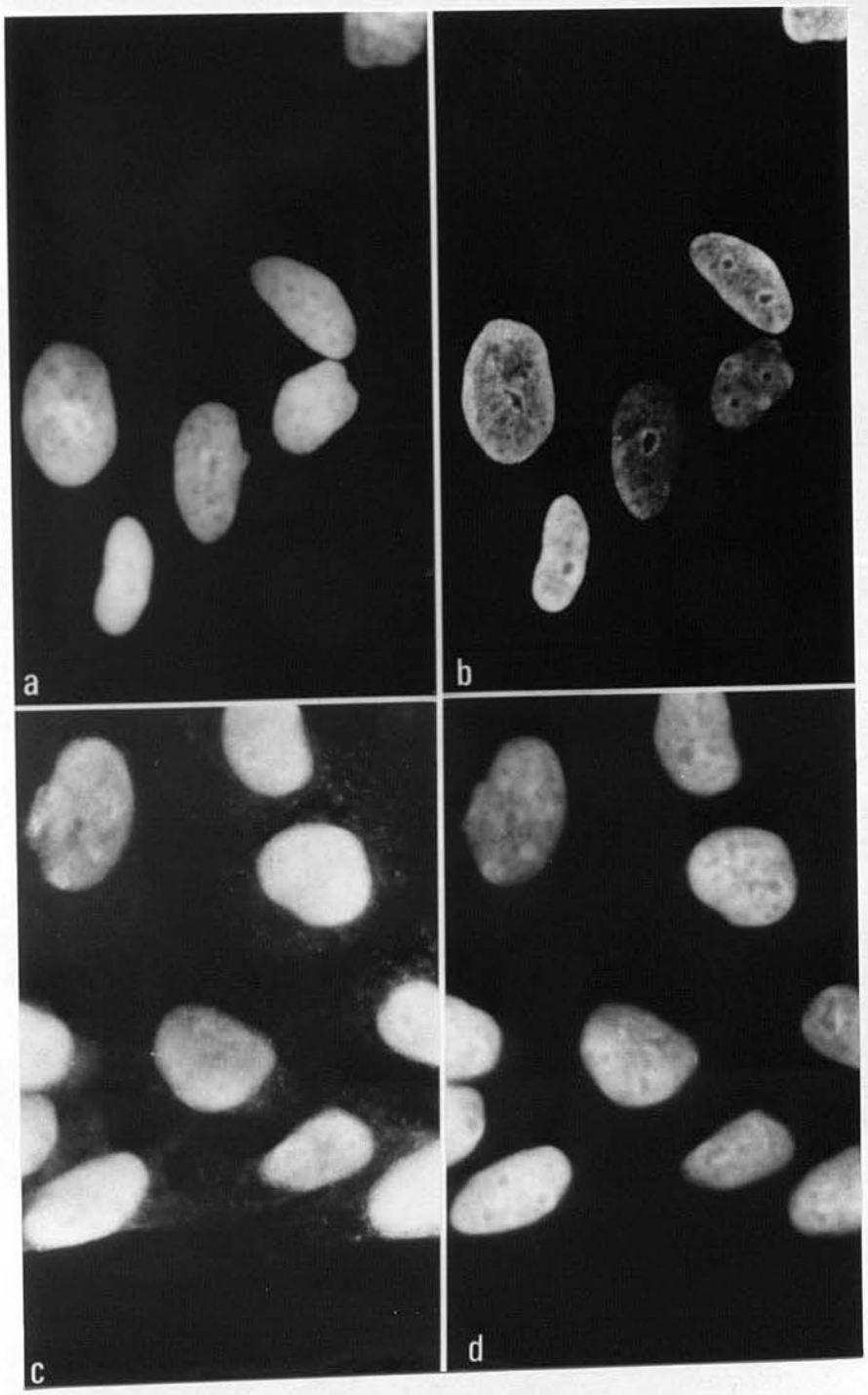


Figure 3.13 Whole PARIT cells stained with serum #13(H), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #13(H) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #13(H) and counterstained with DAPI to localise the nucleus.

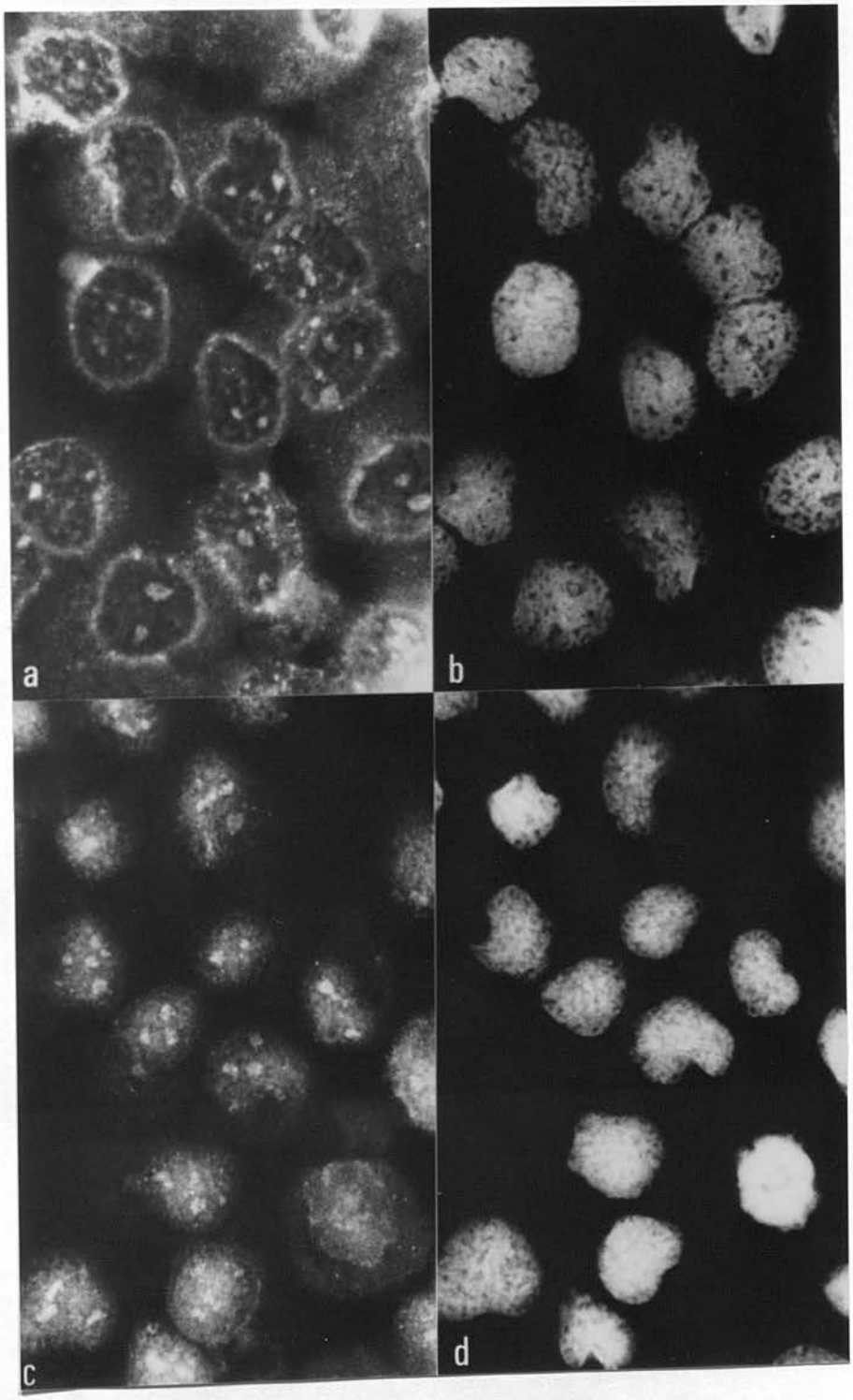


Figure 3.14 Whole Don K_2 cells stained with serum #13(H), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #13(H) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #13(H) and counterstained with DAPI to localise the nucleus.

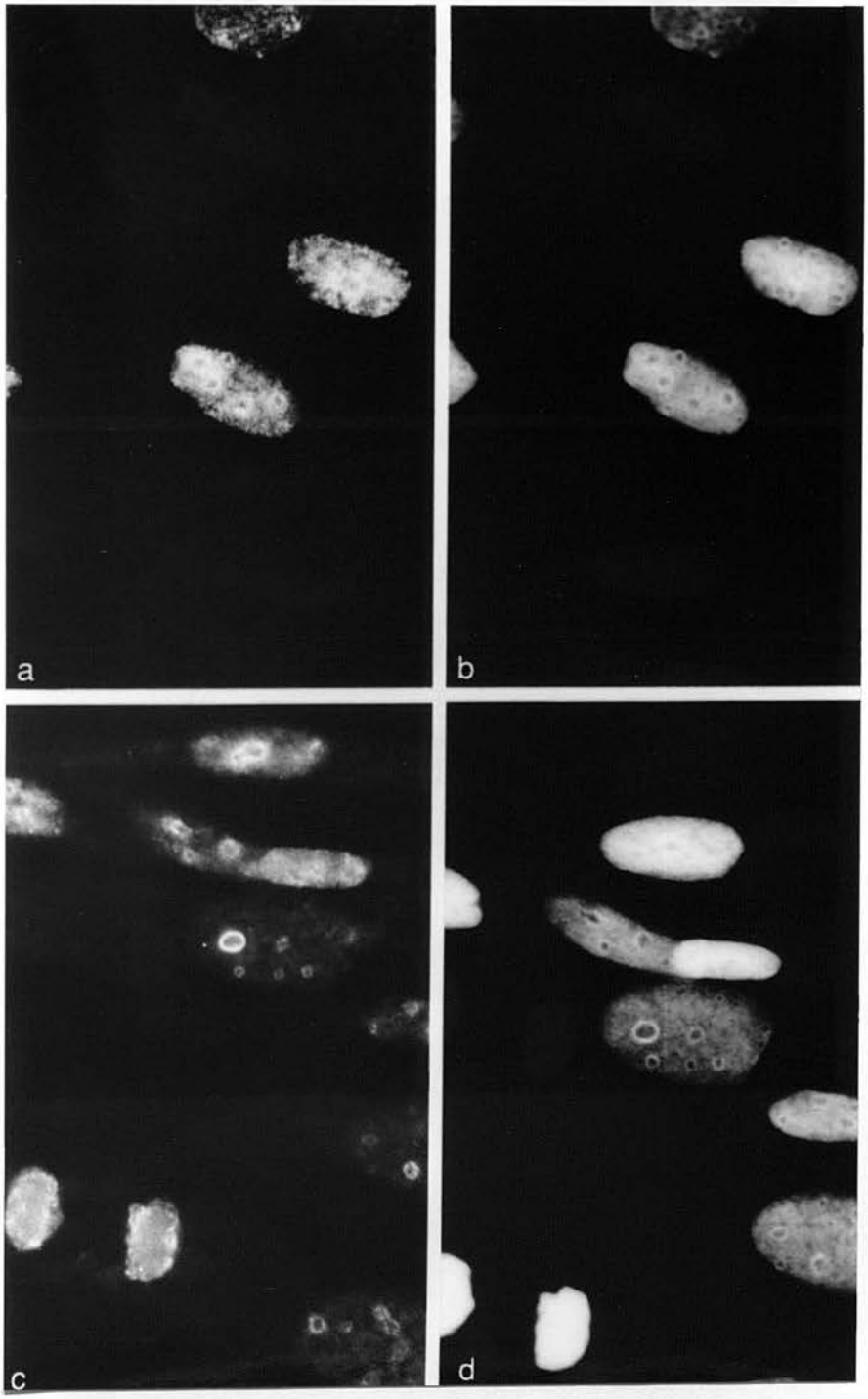
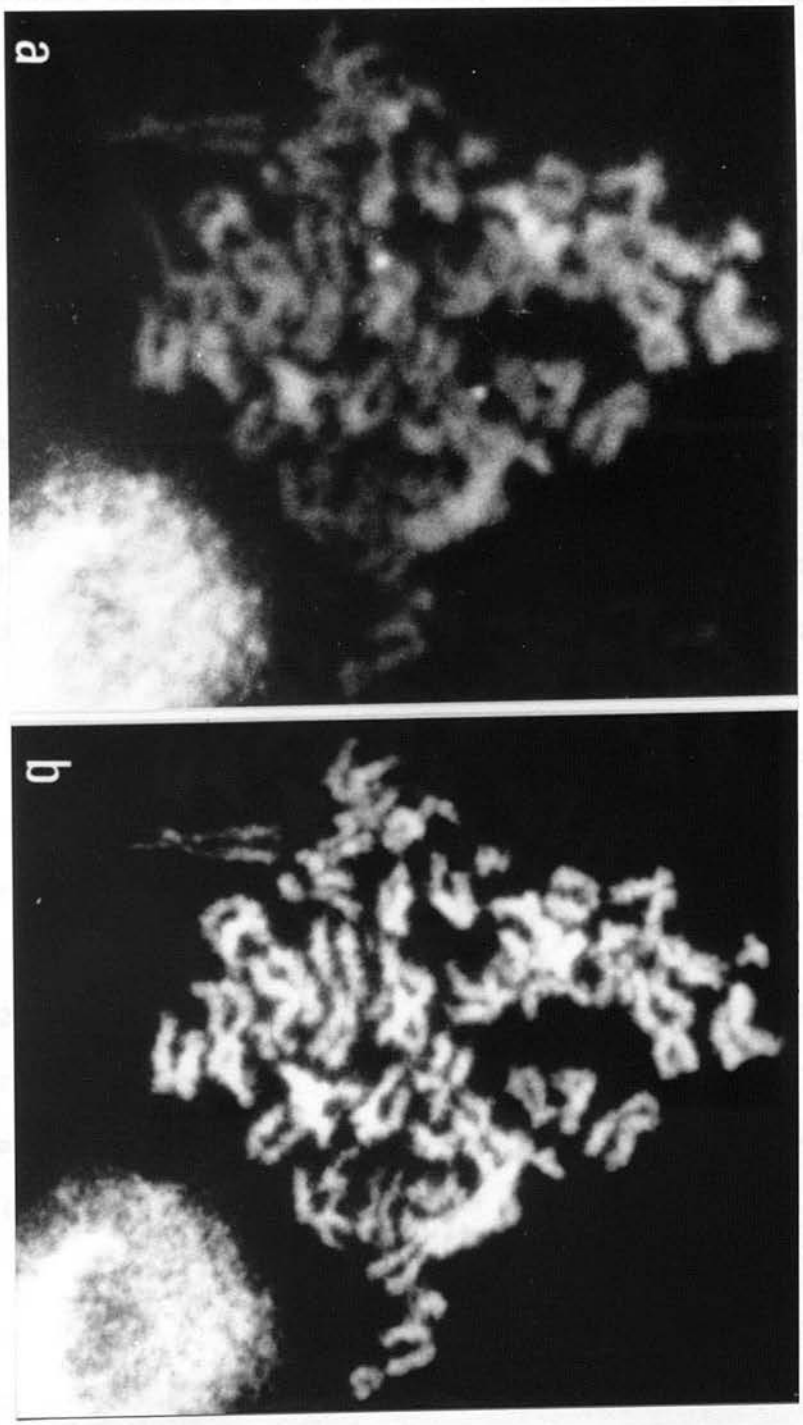


Figure 3.15 Metaphase spreads of PARIT cells stained with serum #13(H), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #13(H) and counterstained with DAPI to localise the chromosomes.



Figures 3.16, 3.17 and 3.18 show the staining patterns observed when #14(R) is used as the first antibody.

The cytospin preparations of whole PARIT cells show coarse granular staining of the nucleus. The cells prepared in KCM show a high level of staining at the nuclear membrane also. The Don K_2 cells show very different staining patterns depending on the buffer used in preparation. The cells prepared in KCM show a high level of granular staining within the nucleus, whereas the cells prepared in PBS show a very low level of staining of the nucleus, with one or two more densely stained areas within, which are probably nucleoli. The metaphase spreads show the periphery of the chromosomes lit up with a lower level of staining within. The patterns do not correspond to any disease in particular, but the Western blots may indicate particular antigens which are markers for some types of disease.

The other sera screened also gave nuclear staining patterns (some showed additional cytoplasmic staining), but the level of staining was not appreciably greater than the background level shown by the control serum, JL, with the notable exception of #21(EP) and #22(SC). These serum samples were taken from CREST patients and included in the trial batch as positive controls. They showed staining patterns like those shown in **Figures** 3.4, 3.5 and 3.6, when #7(ML) was used as the first antibody.

The observations described here show that although some staining patterns, such as kinetochore recognition, provide valuable information as to the identity of some diseases such as CREST, most of the observations do not provide much aid alone. However with the

Figure 3.16 Whole PARIT cells stained with serum #14(R), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #14(R) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #14(R) and counterstained with DAPI to localise the nucleus.

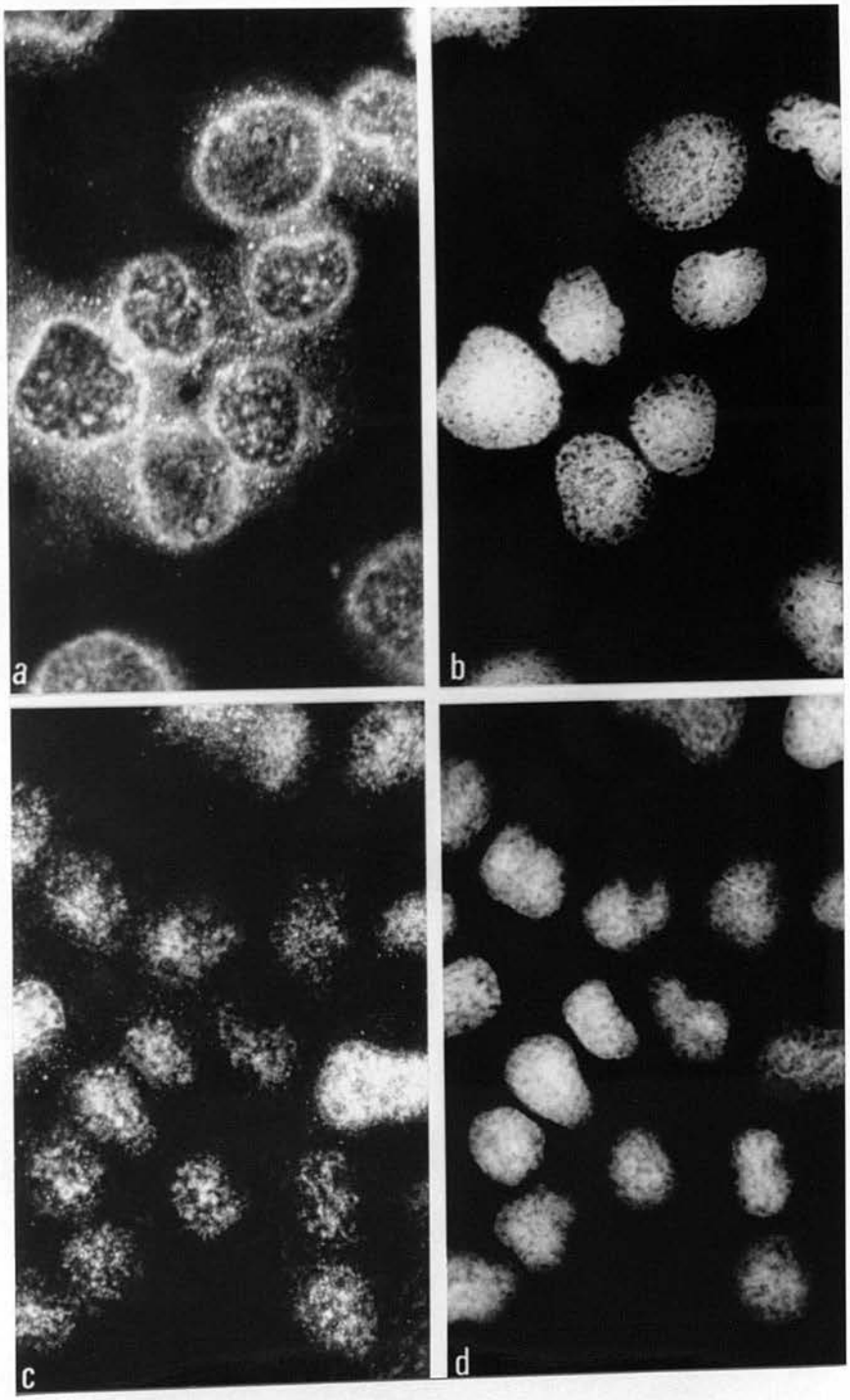


Figure 3.17 Whole Don K_2 cells stained with serum #14(R), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #14(R) and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with #14(R) and counterstained with DAPI to localise the nucleus.

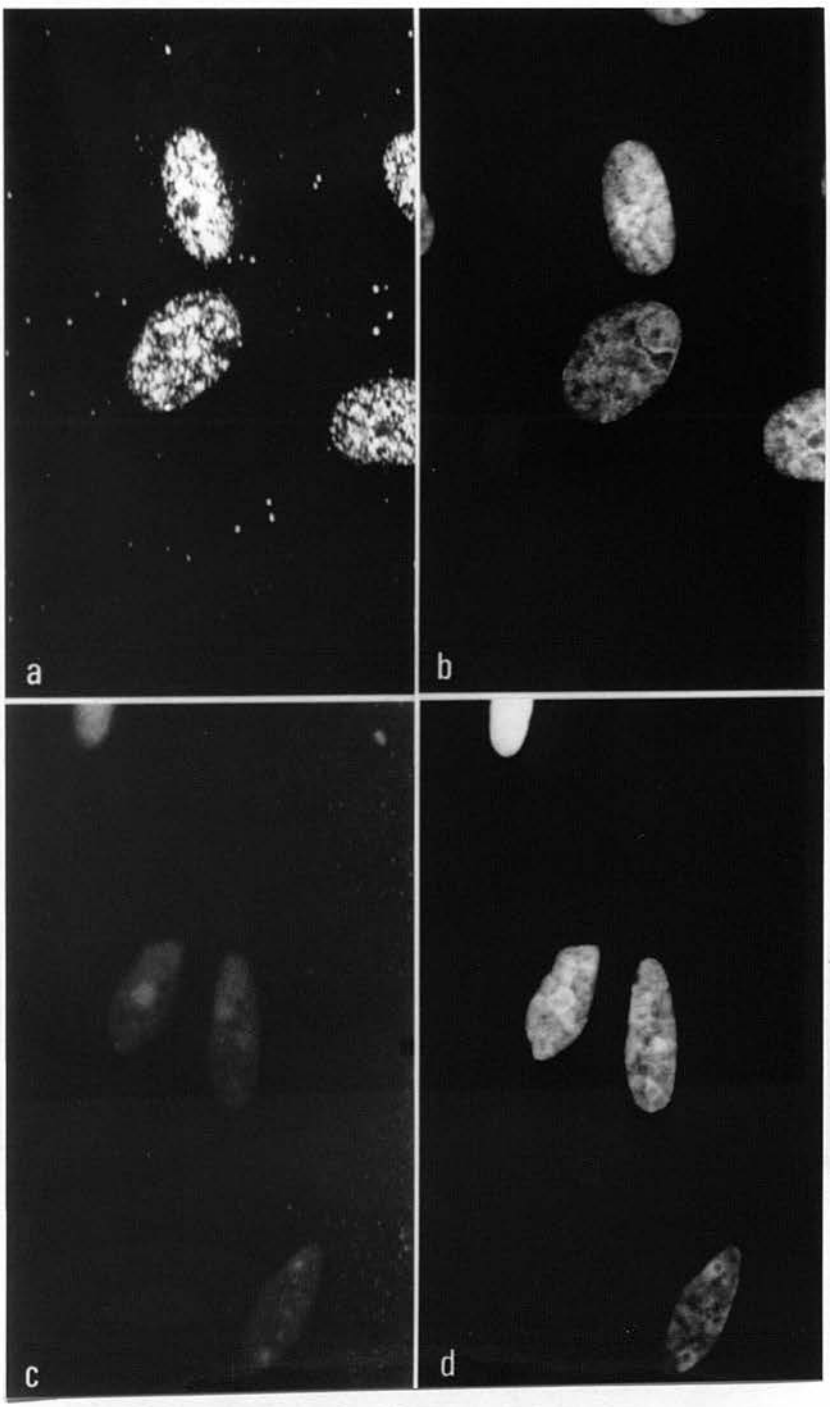
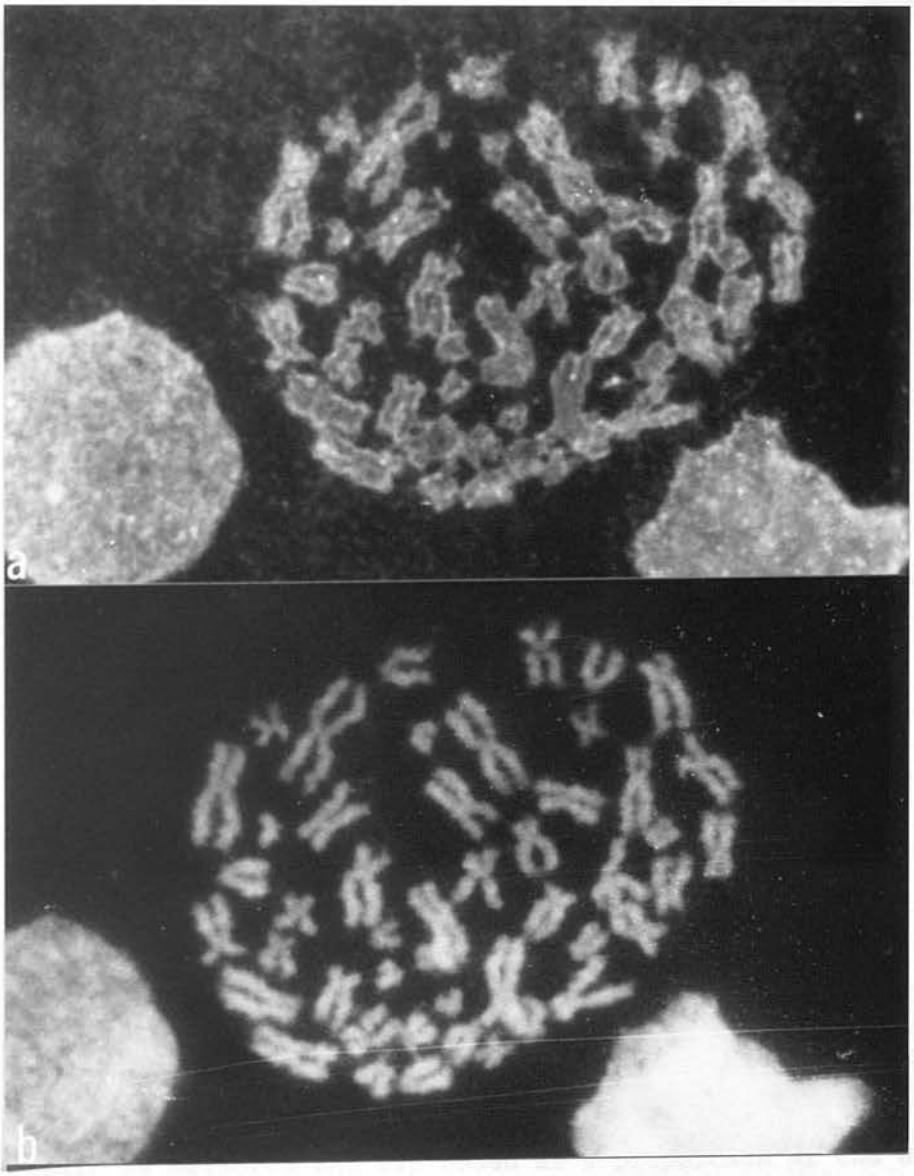


Figure 3.18 Metaphase spreads of PARIT cells stained with serum #14(R), as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with #14(R) and counterstained with DAPI to localise the chromosomes.



additional information provided by Western blotting, the situation can be further clarified.

3.2.2 Immunoblotting Using Sera #1(MJ) to #24(NF)

This method allows the polypeptide specificity of the autoantibodies present in the sera to be investigated.

Figure 3.19 shows the banding patterns produced by sera #1(MJ) to #12(JG) plus the normal control serum, JL, whilst **Figure 3.21** shows those produced by sera #13(H) to #24(NF). From the number of bands present on each strip it can be seen that this method is indeed sensitive. Many of the bands appear to be present in two or more of the sera. These may be proteins of a particularly "sticky" nature to which the antibodies bind in a non-specific manner. However if that were the case these bands would be observed on all the stained strips. It is also possible that the bands may represent diagnostic antigens. In order to determine whether these are antigens which have been previously identified as diagnostic for the disease, their molecular weights must be calculated.

This is achieved by using the calibration curves displayed in **Figures 3.20** and **3.22**. Calibration curves are created by running markers of a known molecular weight on the gel with the sample to be studied. The distance of migration of each marker band from the origin is measured and expressed as a proportion of the distance from the origin to the solvent front, known as the Rf, which corresponds to a particular molecular weight. Calibration curves result from plotting the molecular weights of the markers against their Rf values. By measuring the distance of migration of the

Figure 3.19 Banding patterns produced by sera #1(MJ) to #12(JG), plus the control serum, JL, on PARIT nuclear protein extracts, when used as the first antibodies. The second antibody was peroxidase conjugated rabbit anti-human IgG γ, visualised with chloronaphthol plus hydrogen peroxide.
Molecular weight is indicated in kDa..

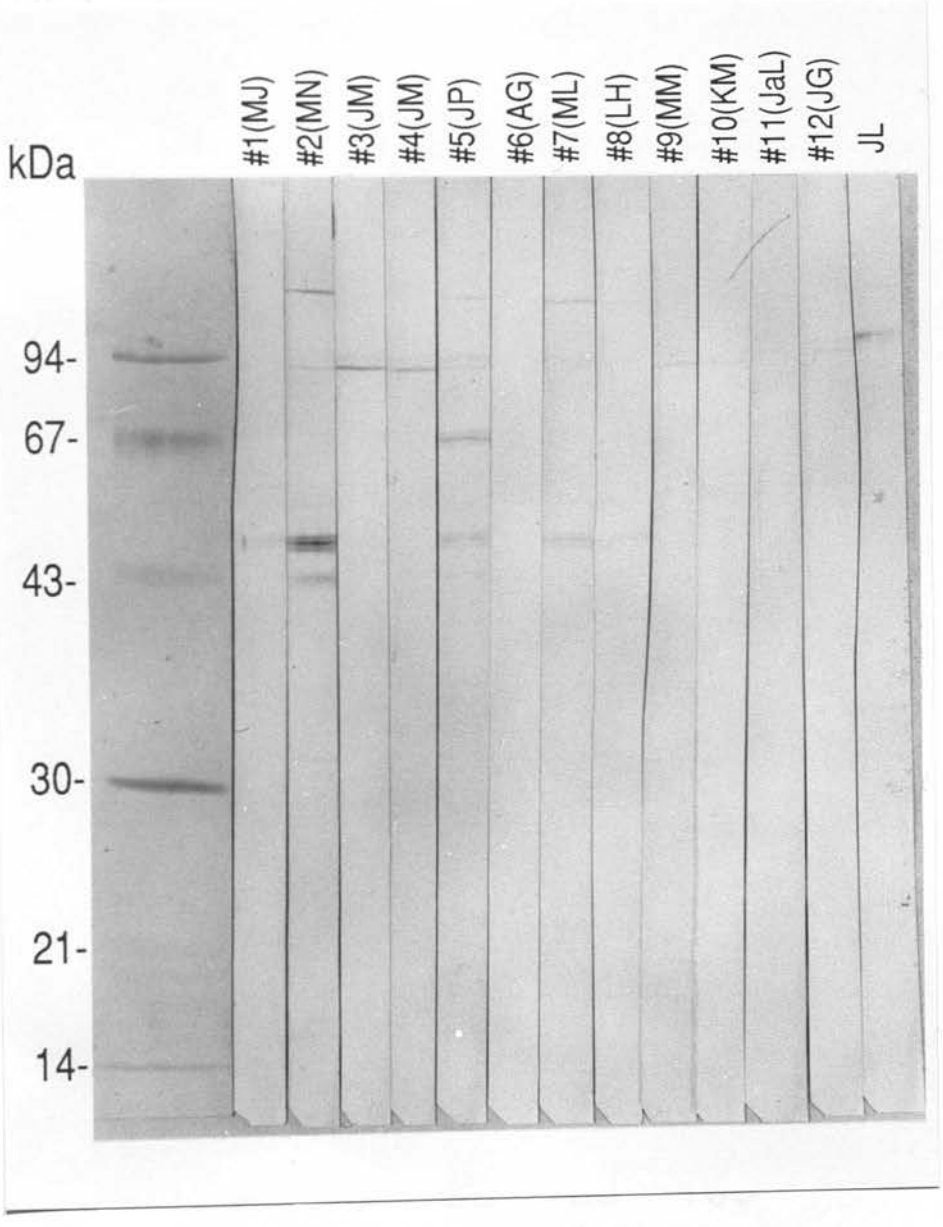


Figure 3.20 Calibration curve for the Western blot shown in **Figure** 3.19

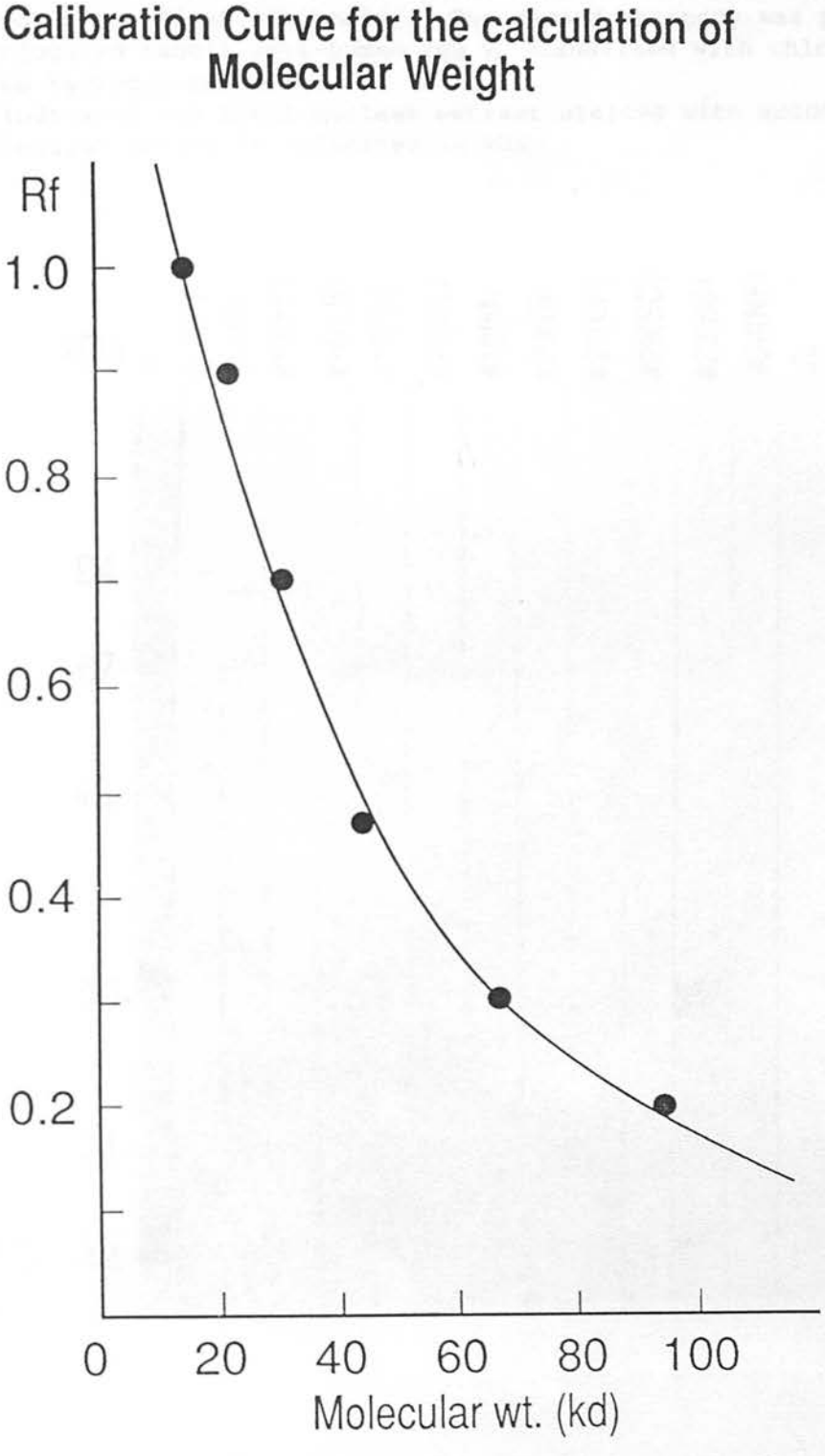


Figure 3.21 Banding patterns produced by sera #13(H) to #24(NF), plus the control serum, JL, on PARIT nuclear protein extracts, when used as the first antibodies. The second antibody was peroxidase conjugated rabbit anti-human IgG γ, visualised with chloronaphthol plus hydrogen peroxide.
A indicates the total nuclear extract stained with amido black.
Molecular weight is indicated in kDa.

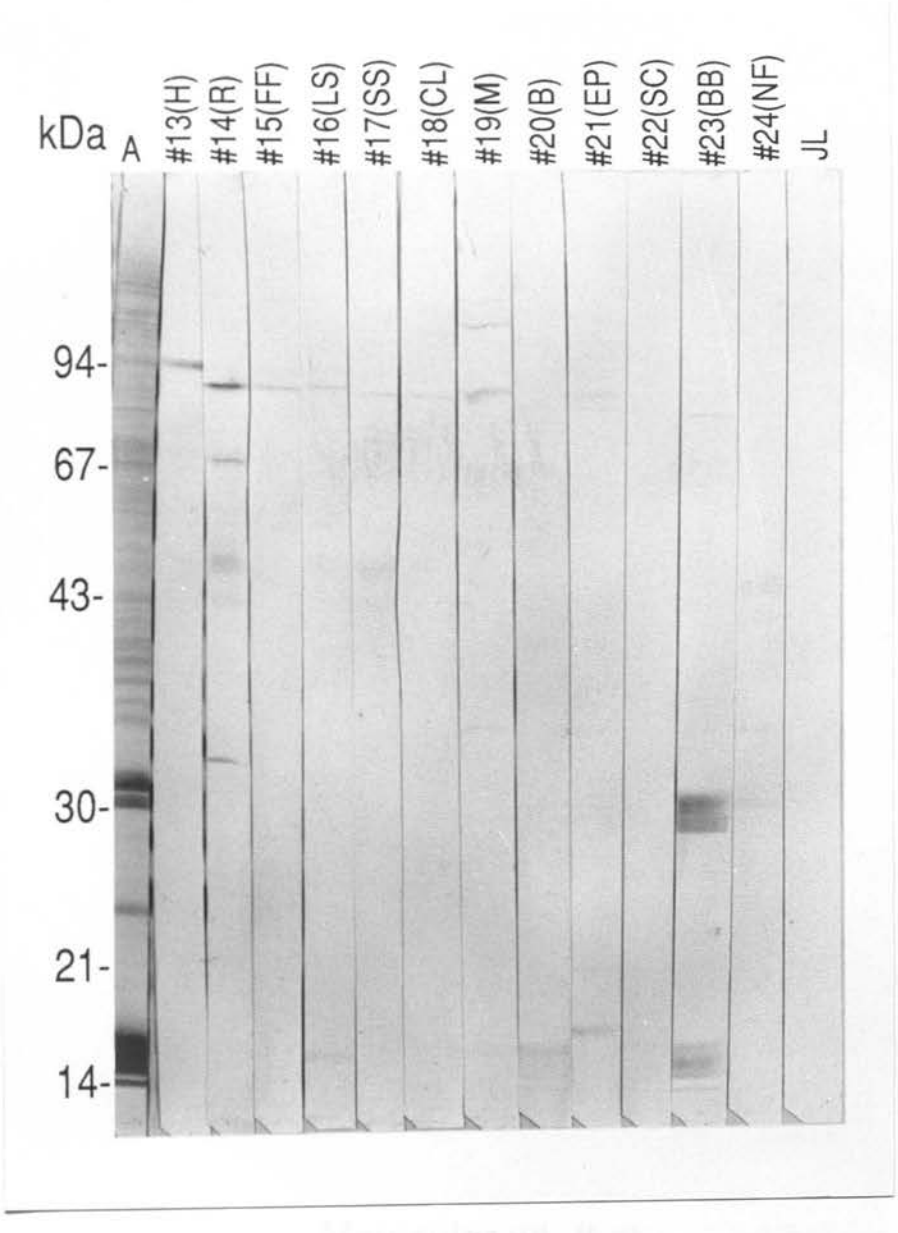
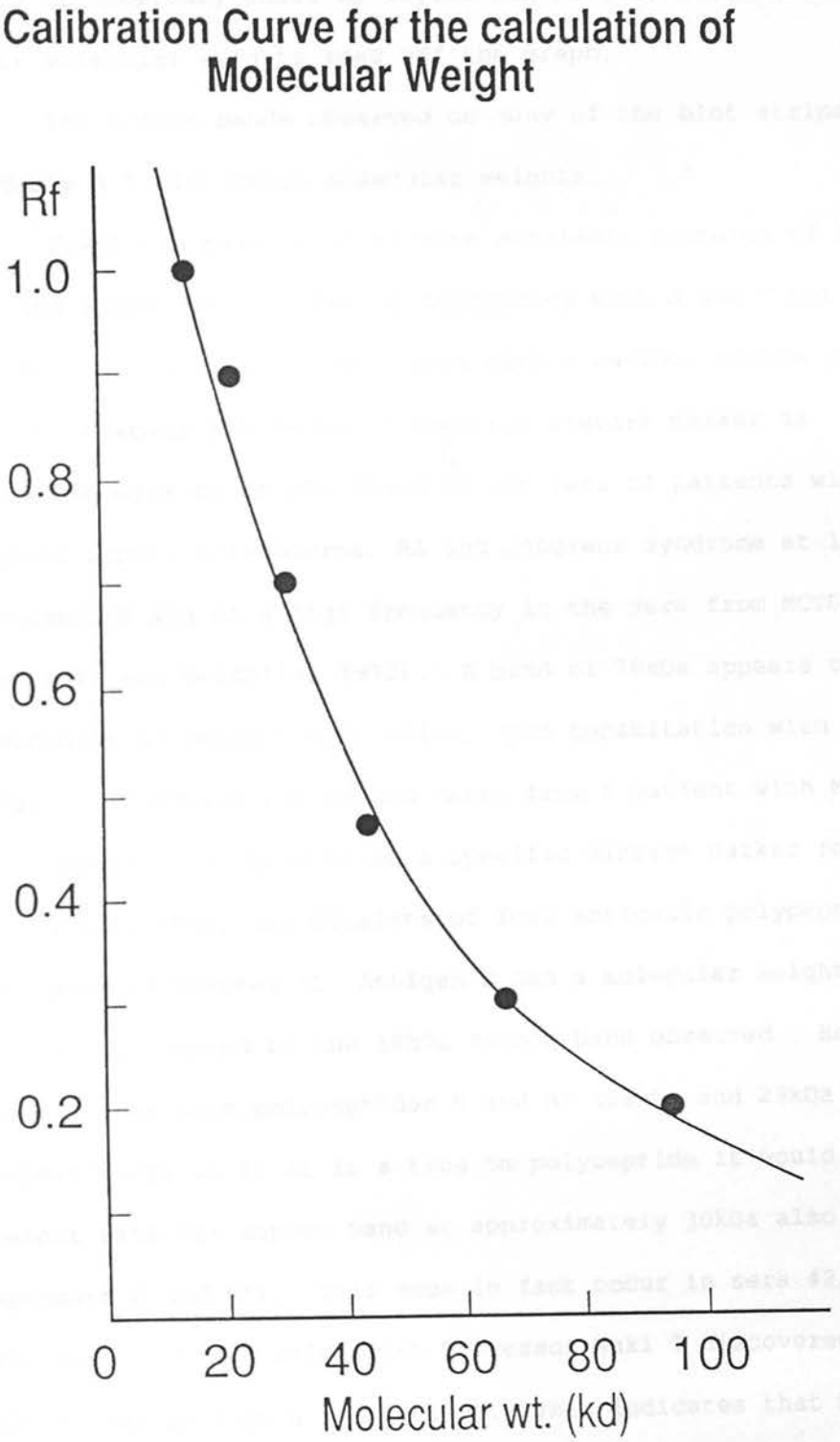


Figure 3.22 Calibration curve for the Western blot shown in **Figure 3.21**



bands of interest, their Rf values can also be calculated and hence their molecular weights read off the graph.

The common bands observed on many of the blot strips are shown in **Table 3.1** with their molecular weights.

SnRNP has been shown to have antigenic proteins of sizes 33kDa (A) and 22kDa (C). U1 RNP is associated with A and C but to be antigenic it has to be associated with a nuclear matrix protein of 70kDa. However RNP is not a specific disease marker as autoantibodies to it are found in the sera of patients with SLE, discoid lupus, scleroderma, RA and Sjogrens syndrome at low frequencies and at a high frequency in the sera from MCTD patients (Mattioli and Reichlin, 1973). A band of 70kDa appears on the immunoblot of serum #14(R) which, upon consultation with Professor Nuki, I discovered was indeed taken from a patient with MCTD.

Sm is considered to be a specific disease marker for SLE (Tan and Kunkel, 1966) and consists of four antigenic polypeptides as discussed in Chapter 1. Antigen D has a molecular weight of 16kDa and may correspond to the 18kDa common band observed. However D cross-reacts with polypeptides B and B' (28kDa and 29kDa respectively) so if it is a true Sm polypeptide it would have to be present with the common band at approximately 30kDa also (which may represent B and B'). This does in fact occur in sera #2, #7, #9 and #23, but on consultation with Professor Nuki I discovered that only #23(BB) was in fact a SLE patient. This indicates that blind immunoblotting may not be particularly useful alone for diagnostic purposes. (However if the indirect immunofluorescence information and the presence of the more specific histone bands is taken into account then the correct diagnosis would be made.)

Table 3.1 To show the presence of common bands on the Western blots stained with each of the 24 sample sera, #1(MJ) to #24(NF) plus JL. + indicates the presence of the band.

Molecular weights in kDa

Test Serum	110	89	86	66	47	43	36	30	18
#1(MJ)					+				
#2(MN)	+		+		+	+	+	+	+
#3(JM)		+	+						
#4(JM)		+	+						
#5(JP)	+	+	+	+	+	+	+		+
#6(AG)									
#7(ML)	+		+		+	+	+	+	+
#8(LH)	+		+	+	+	+	+		
#9(MM)			+	+				+	+
#10(KM)		+	+						
#11(JaL)									
#12(JG)		+	+	+				+	
#13(H)							+		
#14(R)			+	+	+	+			
#15(FF)			+						
#16(LS)			+						+
#17(SS)			+		+				+
#18(CL)	+		+		+	+			
#19(M)	+		+				+		+
#20(B)		+	+						+
#21(EP)		+	+				+		
#22(SC)									
#23(BB)		+	+					+	+
#24(NF)					+		+	+	

Ro(SS-A) is in fact two antigens with the molecular weights of 52kDa and 60kDa but these have been shown to be associated with cytoplasmic RNAs (Wolin and Steitz, 1984). As the blots only have nuclear material present the Ro antigens are not likely to be present. La(SS-B) has a molecular weight of 48kDa and there is a band present of 47kDa. However La is never observed without Ro accompanying it, therefore neither will be present on these immunoblots. Cytoplasmic material would be required if immunoblotting were to be used as a diagnostic method for Ro and La associated diseases.

Ku antigen consists of a pair of antigens 60-66kDa and 80-86kDa which are DNA binding. These weights correspond to the common bands of 66kDa and 86kDa which are observed to be present together in sera #5, #8, #9, #10, #12 and #14. This antigen is associated with 55% of scleroderma/polymyositis cases and 33% of SLE/scleroderma/polymyositis cases. However these are overlap conditions so a specific diagnosis would not be possible.

Scleroderma is a disease of which small percentages of patients' sera have been shown to bind to extremely wide ranges of antigens with molecular weights from 12.5kDa up to 210kDa (as discussed in Chapter 1). This range includes all the common bands observed; a phosphoprotein recognised by 4% of scleroderma patients of 18kDa is one of these which may be the common band at 18kDa. (However this band may also be the D antigen of Sm). Again this is a demonstration of the complexity of autoimmunity and the difficulty of diagnosis. 8% of patients recognise fibrillarin which has a molecular weight of 34kDa (Parker and Steitz, 1987). This corresponds to the 36kDa common band. Scleroderma does however have

a specific marker, Scl-70, originally claimed to have a weight of 70kDa (Douvas *et al.*, 1979) but subsequently discovered to be DNA topoisomerase I with a molecular weight nearer 100kDa (Shero *et al.*, 1986; Guldner *et al.*, 1986). It can be easily degraded accounting for the documented weight range. However there does not appear to be a common band within this range of weights.

PCNA has been shown to have a molecular weight of 35-36kDa which corresponds with the 36kDa common band as does fibrillarin. PCNA is associated with 3% of SLE patients but is also found in other conditions and thus is not useful as a disease marker.

The common bands are not the only bands observed on the immunoblots. More specific bands occurring in only one or two of the sera may be more useful for the diagnosis of particular diseases.

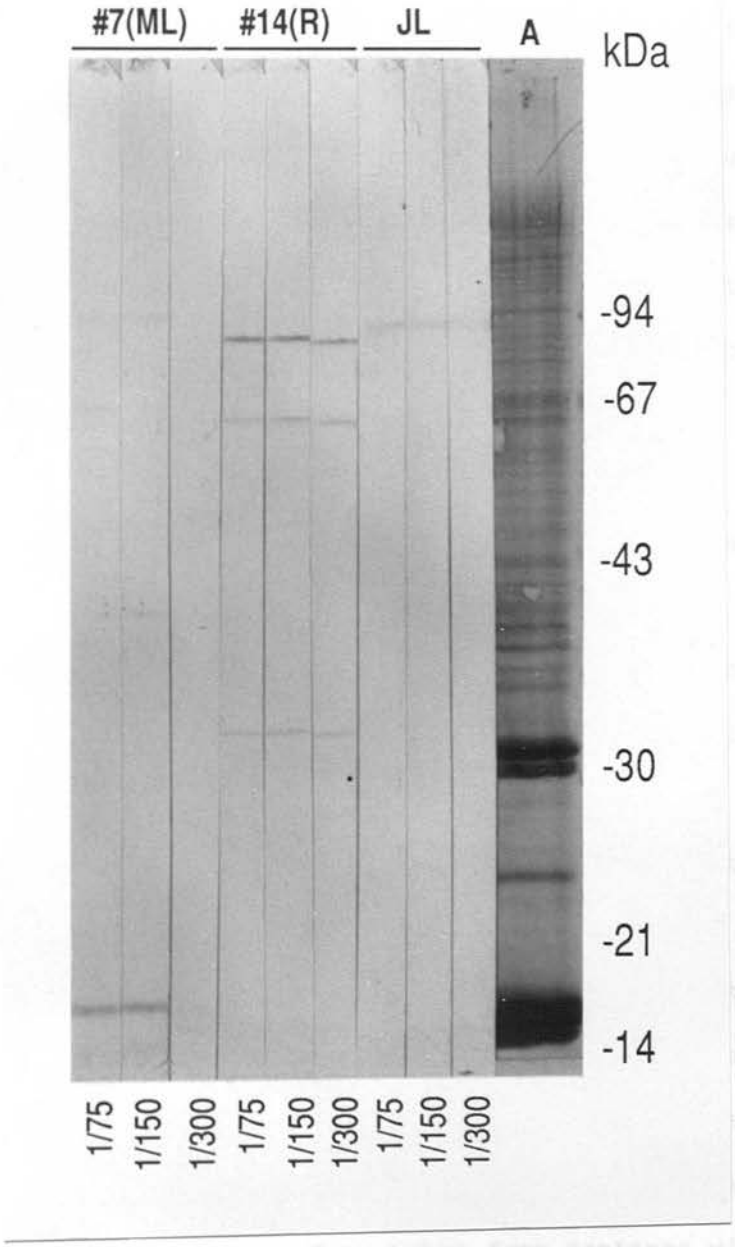
Serum #7(ML) has autoantibodies which recognise a band of 19kDa, which is one of the kinetochore antigens (CENP-A) (Earnshaw *et al.*, 1986). These antibodies indicate that the serum is from a patient with CREST, a subset of scleroderma. (This band is much more clearly visible in **Figure 3.23.**) Serum #8(LH) also shows this 19kDa band and therefore may also be a CREST patient, although the immunofluorescent staining pattern did not give any indication that this might be the case. The band may be that of a co-migrating protein which is not specific for any particular disease. Sera #21(EP) and #22(SC) are from CREST patients provided as a positive controls. However only #21(EP) recognises the 19kDa band. None of these sera appear to have antibodies to any of the other classes of kinetochore antigens (CENP-B and CENP-C).

Serum #23(BB) shows a very strong reactivity towards histones, particularly class H1. This is characteristic of SLE patients. Sera #23(BB) and #24(NF) are from SLE patients which were provided as positive controls also. However only #23(BB) shows anti-histone activity.

Two sera show bands which are specific to that serum only. Serum #13(H), from a scleroderma patient has a band at 95kDa and serum #14(R), from a MCTD patient has a band at 38kDa (in addition to the band at approximately 70kDa), both of which do not appear to correspond to any of the antigens previously discussed. (As mentioned earlier, the 70kDa band most probably corresponds to the U1-RNP antigen. The discovery of this antigen caused the identification of MCTD as a disease in its own right and although it is not a specific disease marker, its presence is easily accounted for with the knowledge of the identity of the disease from which the patient is suffering.)

In order to confirm that the bands observed on the immunoblots of these 24 sera are due to the autoantibodies in the sera and not to the second antibody binding to the human nuclear proteins, the concentration of the second antibody was increased from $^{1}/300$ to $^{1}/150$ and $^{1}/75$. If the bands were an effect of the second antibody, more bands and/or the same bands at a higher intensity would be observed. **Figure 3.23** shows the results of that experiment. The three immunoblots of JL and #14(R) show the same bands at the same intensity with all three concentrations of second antibody. Serum #7(ML) shows the same bands at the same intensity with the higher two concentrations, but the immunoblot with the $^{1}/300$ dilution of

Figure 3.23 Western blotted PARIT nuclear extracts stained with #7(ML), #14(R) and the control serum, JL, as first antibodies. The second antibody was peroxidase conjugated rabbit anti-human IgG γ, visualised with chloronaphthol and hydrogen peroxide, at differing concentrations in HS/TBS/Tw. The concentration is indicated below each individual strip.
A indicates the total nuclear extract stained with amido black.
Molecular weight is indicated in kDa.



second antibody applied shows no bands at all. This strip of nitrocellulose may have been allowed to dry out, or the first or second antibody may not have been added to the HS/TBS/Tw in error. This would give the observed result. The other two panels prove that the bands are not an effect of the second antibody or its concentration.

The two sera #13(H) and #14(R) which display reactivity towards the two previously unidentified polypeptides were chosen for further study. In order to determine whether the autoantibodies binding to these bands were disease specific, 24 further sera were supplied. 12 of these came from scleroderma patients and the other 12 were from MCTD patients. These sera were immunoblotted on nuclear proteins also.

3.2.3 Further Immunoblotting and Immunofluorescence With The Second Batch of Sera From Scleroderma and MCTD Patients

First, the sera were immunoblotted in order to determine whether these sera also contained antibodies recognising the same antigens.

Figure 3.24 shows the immunoblots of the scleroderma sera 1 to 12 and **Figure 3.25** shows the MCTD sera 13 to 24.

Serum 11 shows an extremely strong band at 95kDa with several minor bands beneath it. These are likely to be breakdown products of the first band. This is the only serum in the second batch which recognises the 95kDa band but it proves that this band may be related to the disease. Sera taken from patients with autoimmune disease do not all contain the same autoantibodies as discussed in Chapter 1.

Figure 3.24 Western blots of PARIT nuclear protein extracts stained with sera from additional scleroderma patients 2 to 12, plus the control serum JL, as first antibodies. The second antibody was peroxidase conjugated rabbit anti-human IgG γ visualised with chloronaphthol plus hydrogen peroxide.

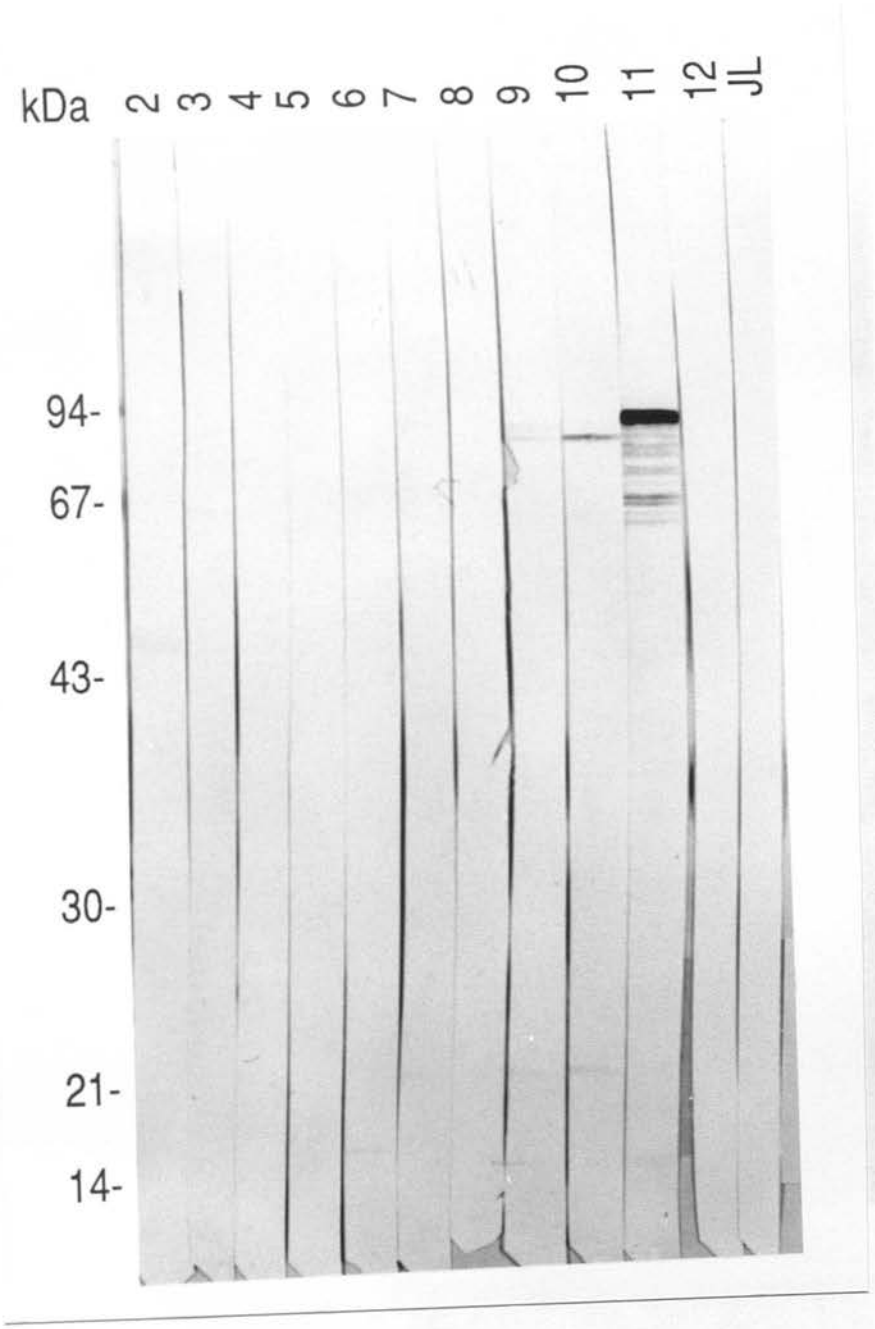
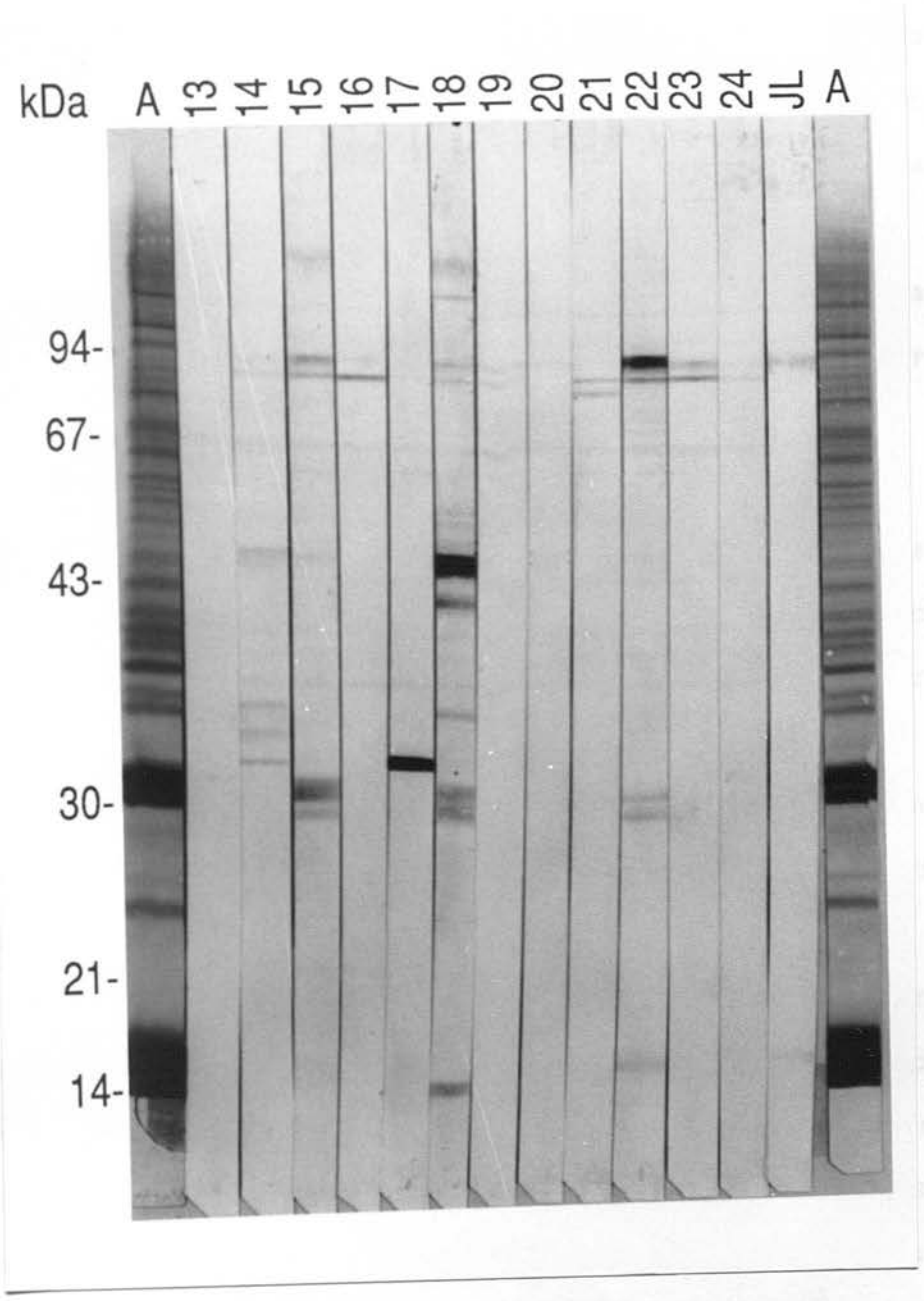


Figure 3.25 Western blots of PARIT nuclear protein extracts stained with sera from additional MCTD patients 13 to 24, plus the control serum JL, as first antibodies. The second antibody was peroxidase conjugated rabbit anti-human IgG γ visualised with chloronaphthol plus hydrogen peroxide.
A indicates the total nuclear extract stained with amido black.



The immunoblots of the second batch of MCTD sera show two sera with activity to the same band, serum 14, at approximately the same level as #14(R) and serum 17, which shows a much stronger reaction. Many of the common bands are present on these blots as with the first 24 sera, some being stained particularly strongly. However, as with the scleroderma immunoblots, the presence of autoantibodies in the second batch of sera which show activity to the 38kDa band suggests that this band is linked to the disease. Serum 11 with activity towards the 95kDa protein will now be referred to as 13A and the sera 14 and 17 with 38kDa peptide activity will be referred to as 14B and 14A respectively.

The sera #13(H) and #14(R) were used for indirect immunofluorescence as part of the routine screening procedure of the sera. The sera 13A and 14A may give an indication of the actual locality of the antigen within the cell if they are used for the same experiment.

Figures 3.26, 3.27 and 3.28 show the results of 13A on PARIT cytospins, Don K_2 monolayers and metaphase spreads, whilst **Figures 3.29, 3.30** and **3.31** show the results for 14A.

PARIT cytospin preparations stained with 13A show coarse granular staining with brighter,dense patches of fluorescence indicating the nucleoli within the nucleus. The Don K_2 cells show the same pattern in those cells prepared in KCM, but the PBS preparation shows denser, uniform staining throughout the nucleus. The metaphase spread again shows the localisation of staining to the chromosomes as with #13(H).

Whole cells stained with 14A show bright granular fluorescence throughout the nucleus. PARIT cells prepared in KCM again show

Figure 3.26 Whole PARIT cells stained with serum 13A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with 13A and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with 13A and counterstained with DAPI to localise the nucleus.

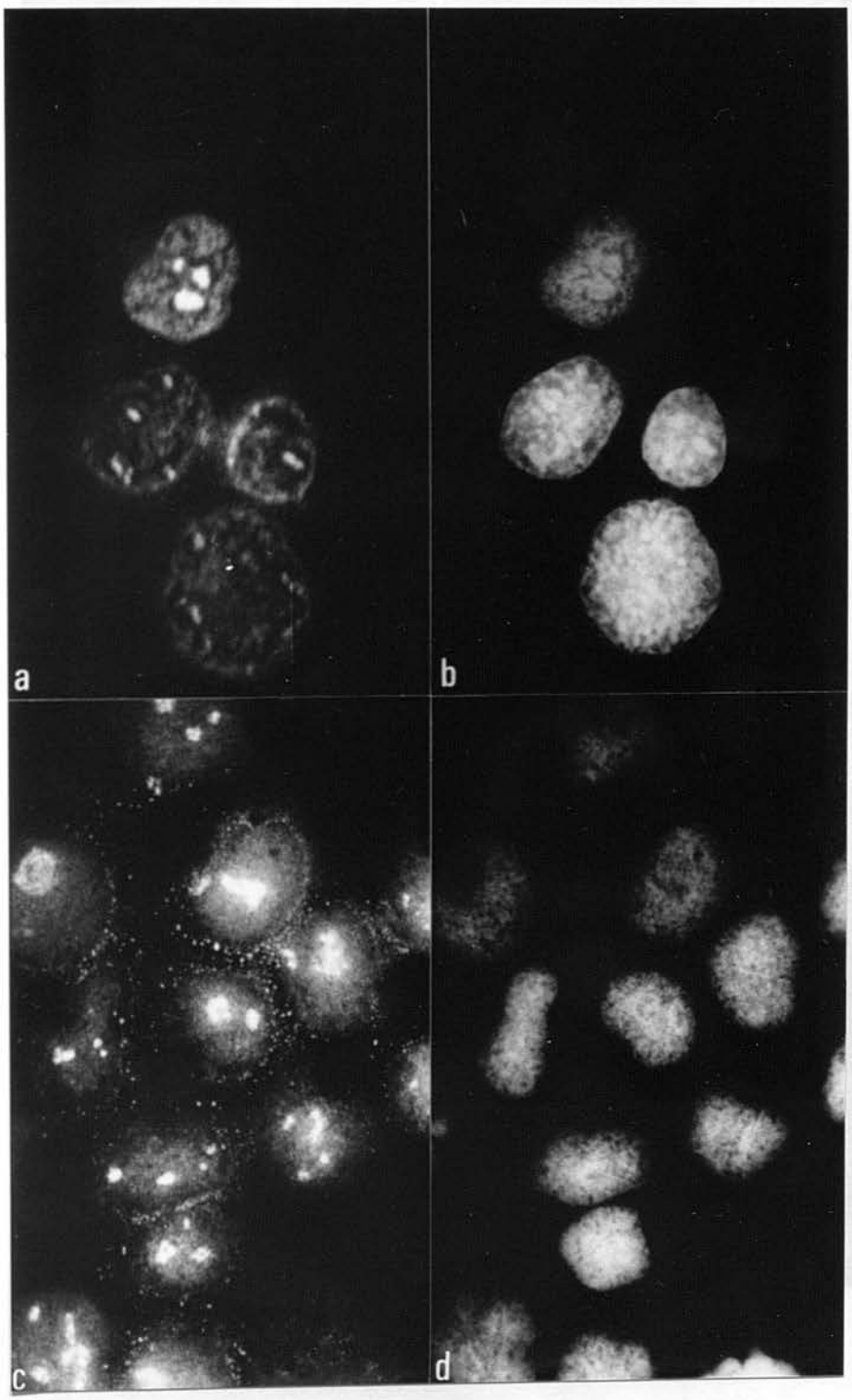


Figure 3.27 Whole Don K_2 cells stained with serum 13A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with 13A and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with 13A and counterstained with DAPI to localise the nucleus.

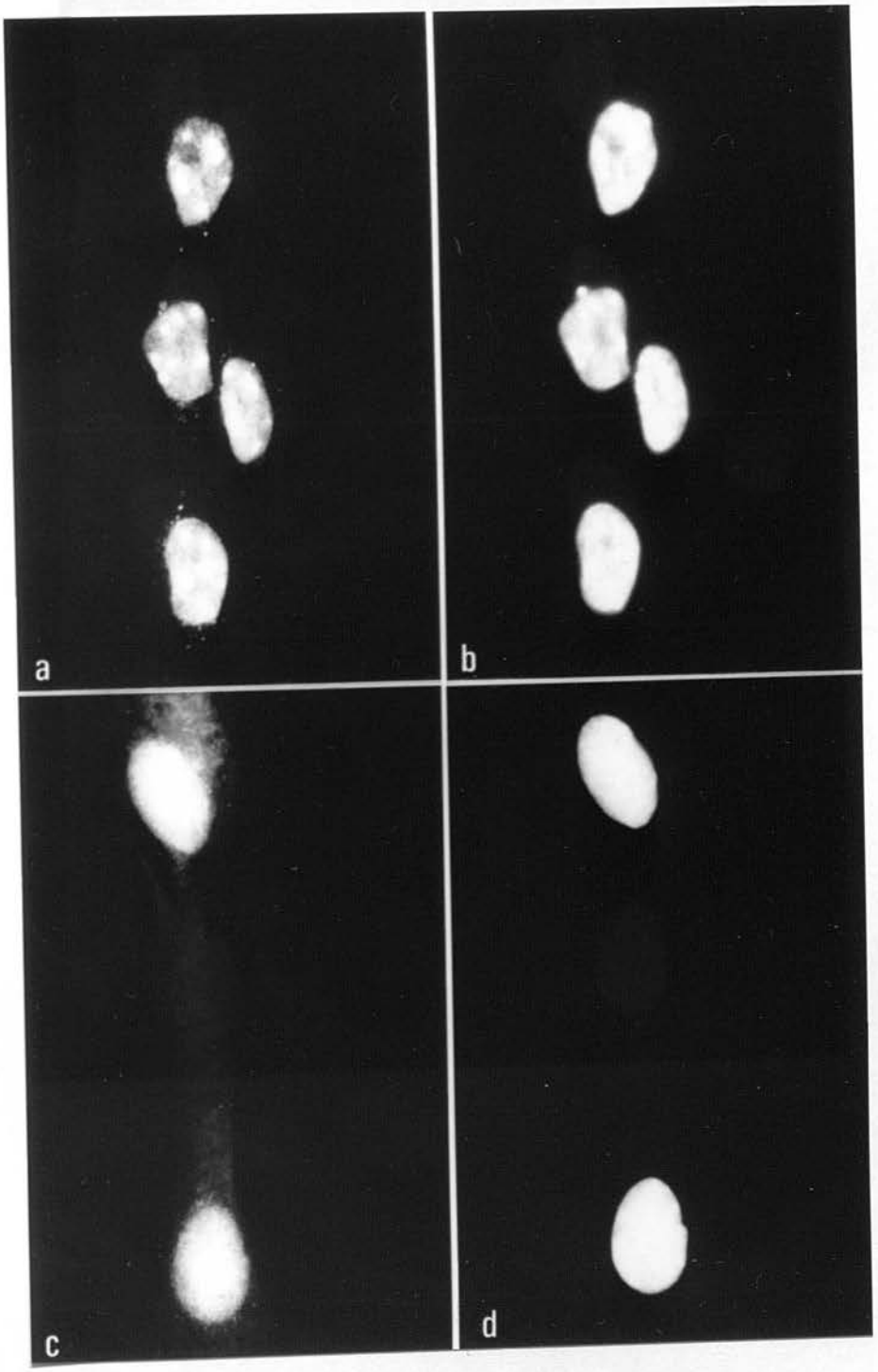


Figure 3.28 Metaphase spreads of PARIT cells stained with serum 13A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with 13A and counterstained with DAPI to localise the chromosomes.

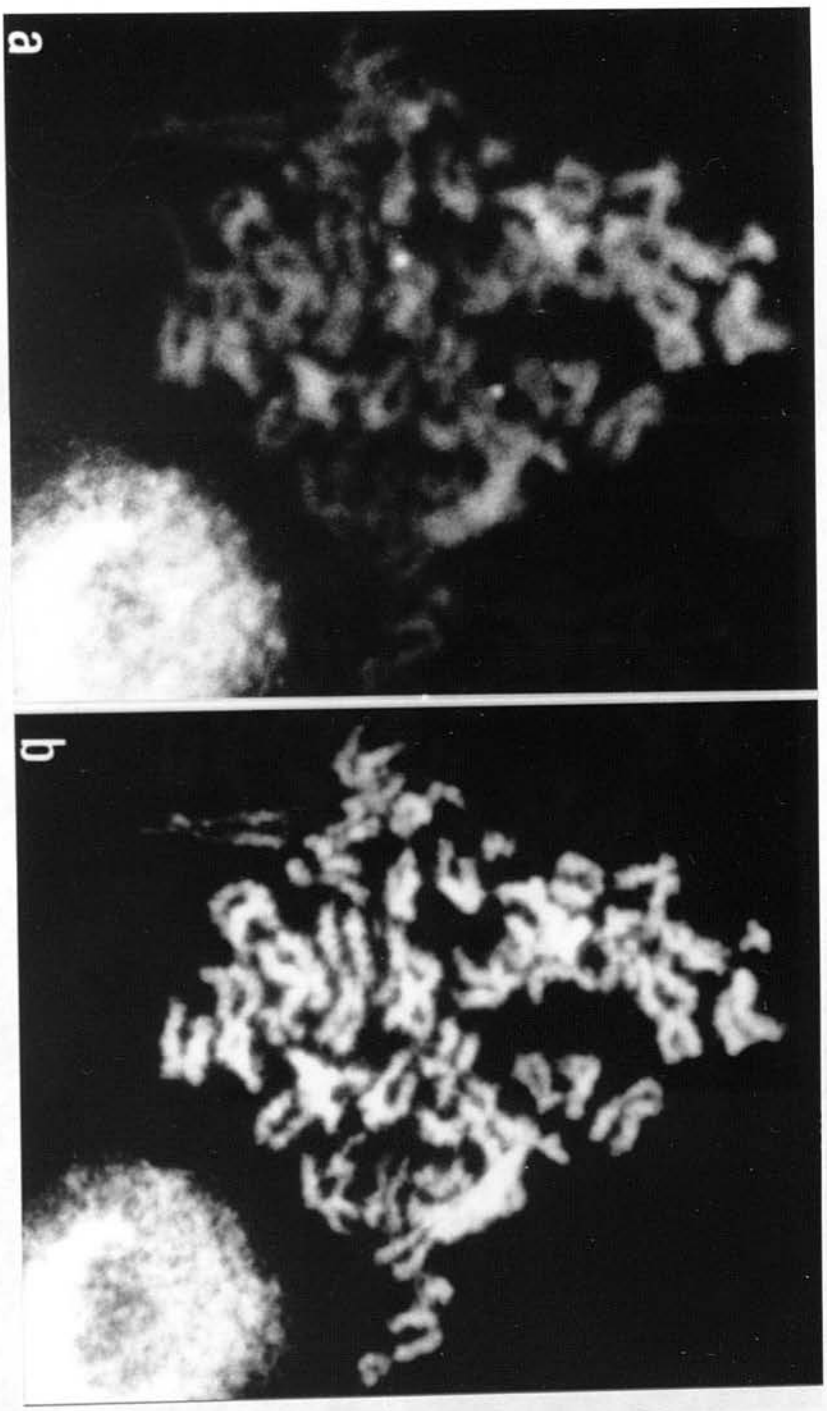


Figure 3.29 Whole PARIT cells stained with serum 14A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with 14A and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with 14A and counterstained with DAPI to localise the nucleus.

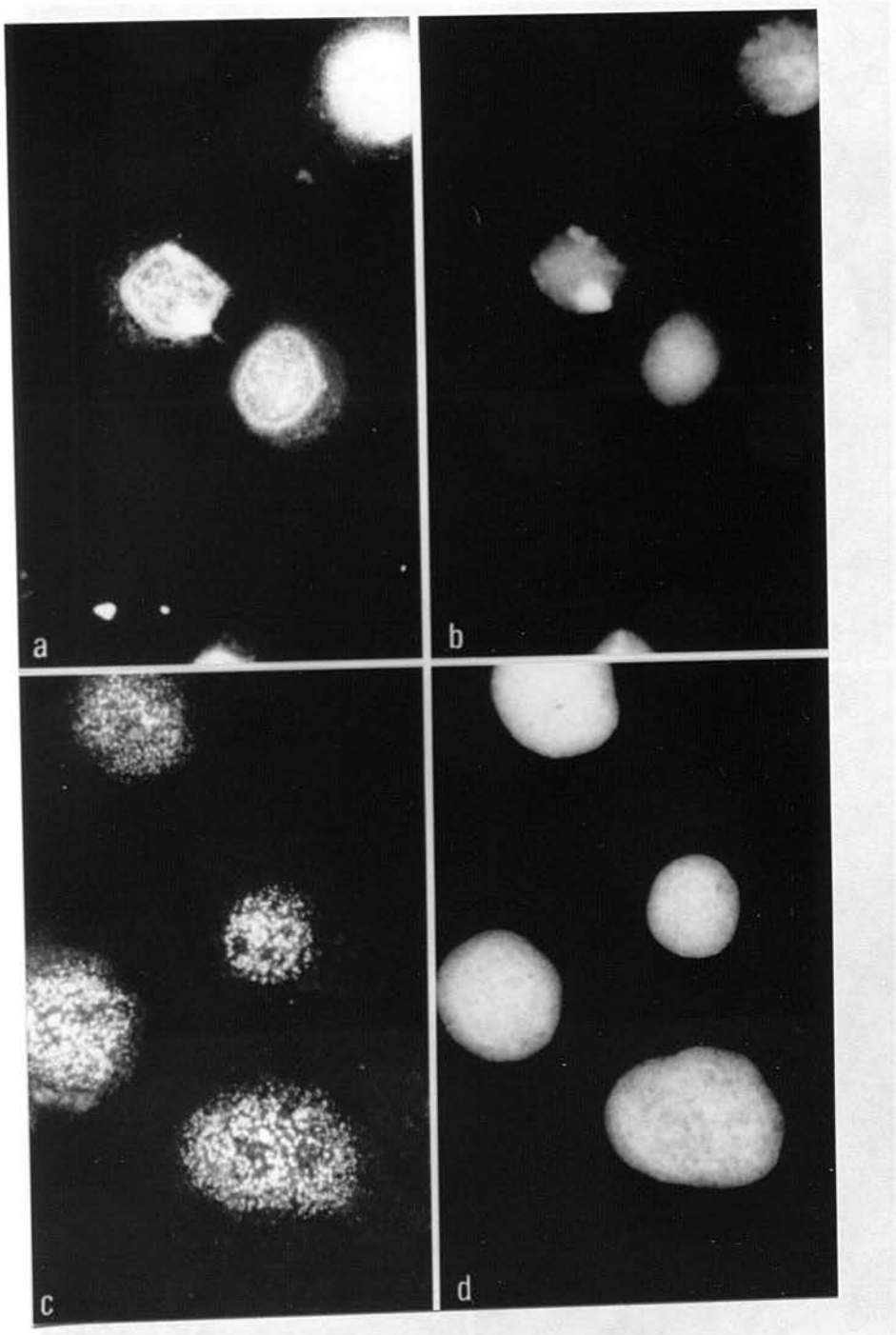


Figure 3.30 Whole Don K_2 cells stained with serum 14A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with 14A and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with 14A and counterstained with DAPI to localise the nucleus.

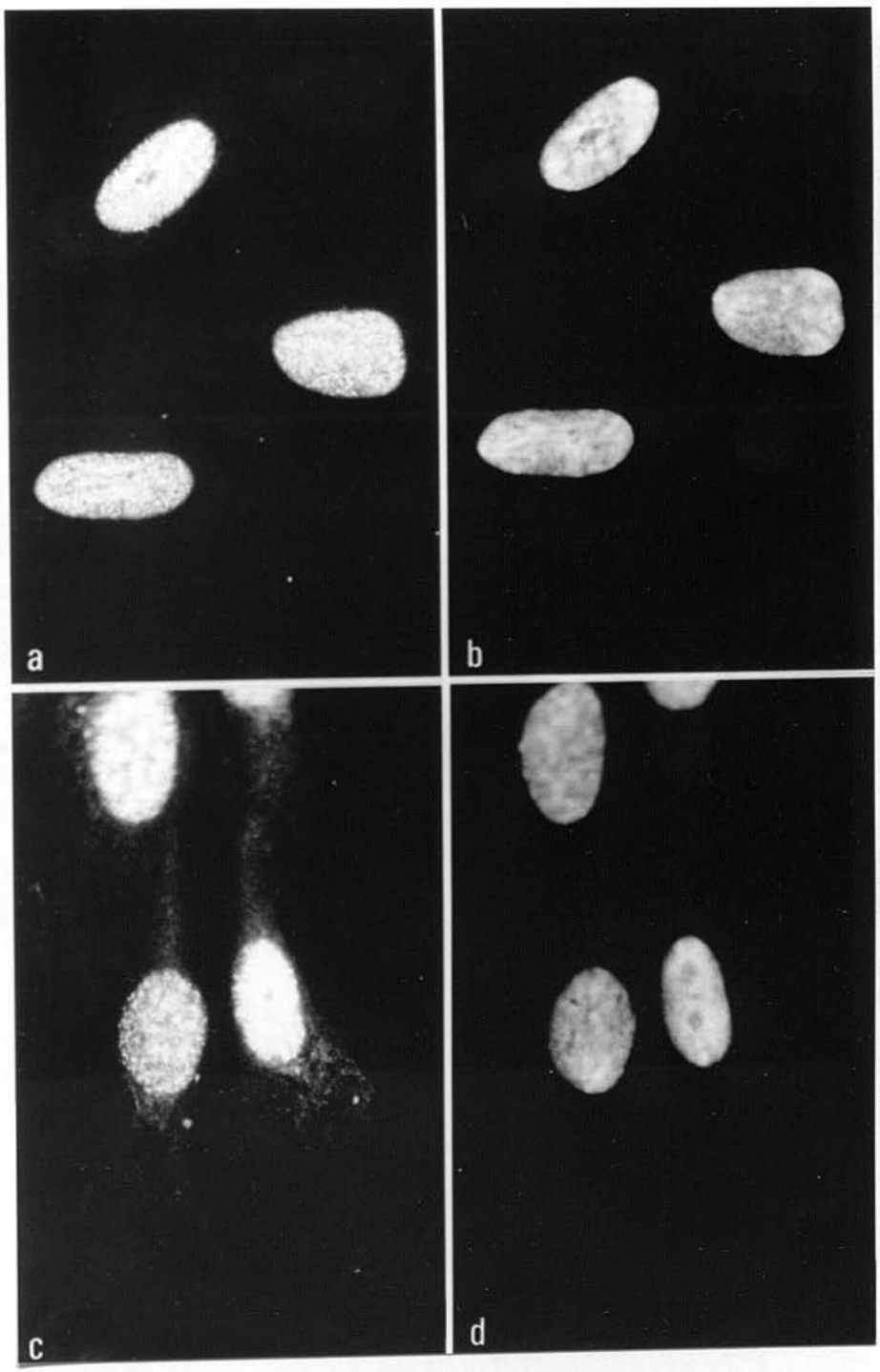
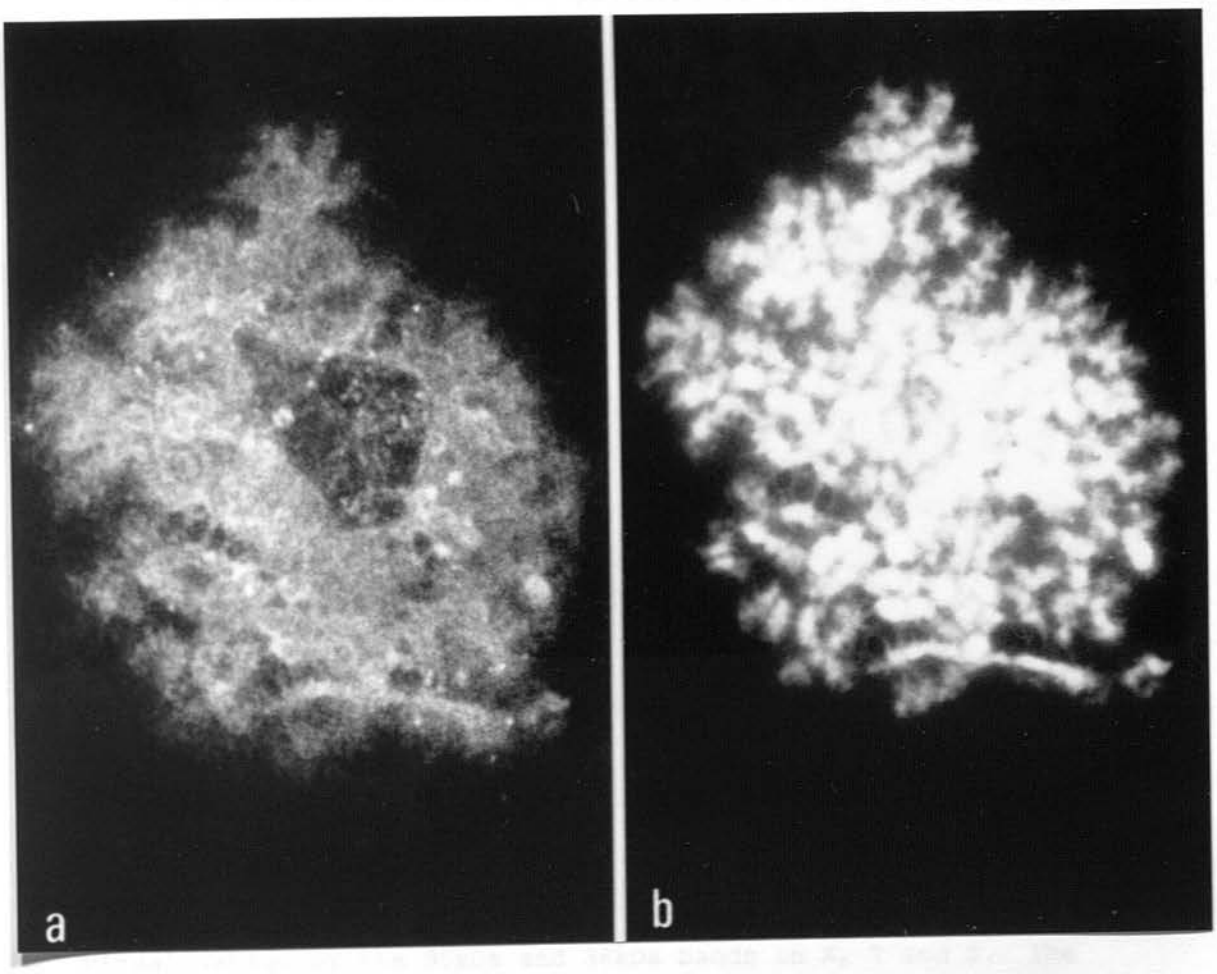


Figure 3.31 Metaphase spreads of PARIT cells stained with serum 14A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with 14A and counterstained with DAPI to localise the chromosomes.



localisation of staining to the nuclear membrane, as with #14(R). Metaphase spreads show staining of the whole chromosome to a moderate level of intensity without the recognition of the periphery as before.

Essentially the staining patterns of #13(H) and 13A, and #14(R) and 14A are the same, with minor variations. As 13A and 14A only show single peptide reactivity it is quite possible that the variation seen is due to the absence of the antibodies which bound to the other bands on the immunoblots, particularly in the case of #14(R).

The sera 13A and 14A have strongly reacting, high affinity antibodies for the bands of 95kDa and 38kDa respectively and therefore could be affinity purified quite easily. In this way the affinity of the antibodies can be checked and the purified antibodies used for further cell staining procedures.

3.2.4 Affinity Purified Antibodies From Sera 13A and 14A

The purification was carried out according to the protocol of Olmsted as explained in Chapter 2. **Figures 3.32** and **3.33** show the visualisation of the 95kDa and 38kDa bands in X, Y and Z. The eluted antibody is then reapplied neat (in its elution buffer) to a Western blot in order to check the specificity ((**a**) and (**b**)). The strips (**c**) and (**d**) have had only second antibody applied. This shows that the bands other than the 95kDa or 38kDa ones are due to second antibody and not to any cross-reactivity of the purified antibody with other nuclear proteins.

Figure 3.32 To show 13A before and after affinity purification.
A indicates the total nuclear extract stained with amido black.
X, Y and Z show the strips, from either side and the middle of a Western blot, stained with 13A (as the first antibody) in order to visualise the position of the protein band bound.
a and b show affinity purified 13A used as the first antibody on Western blots of PARIT nuclear extracts.
c and d show strips from the same Western blot stained with the second antibody only.(The second antibody was peroxidase conjugated rabbit anti-human IgG γ.)
Molecular weight is indicated in kDa.

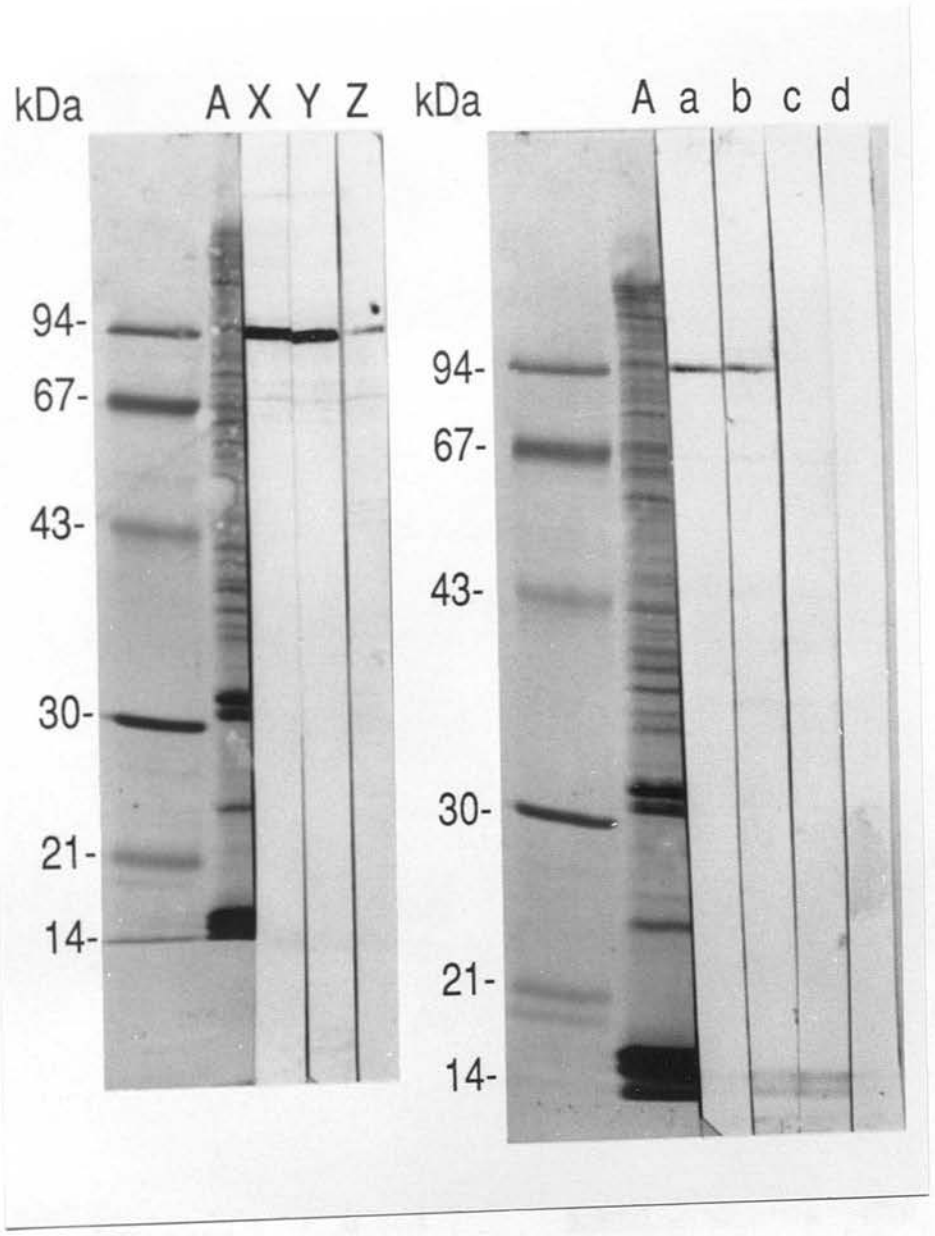


Figure 3.33 To show 14A before and after affinity purification.
A indicates the total nuclear extract stained with amido black.
X, Y and Z show the strips, from either side and the middle of a Western blot, stained with 14A (as the first antibody) in order to visualise the position of the protein band bound.
a and b show affinity purified 14A used as the first antibody on Western blots of PARIT nuclear extracts.
c and d show strips from the same Western blot stained with the second antibody only.(The second antibody was peroxidase conjugated rabbit anti-human IgG γ.)
Molecular weight is indicated in kDa.

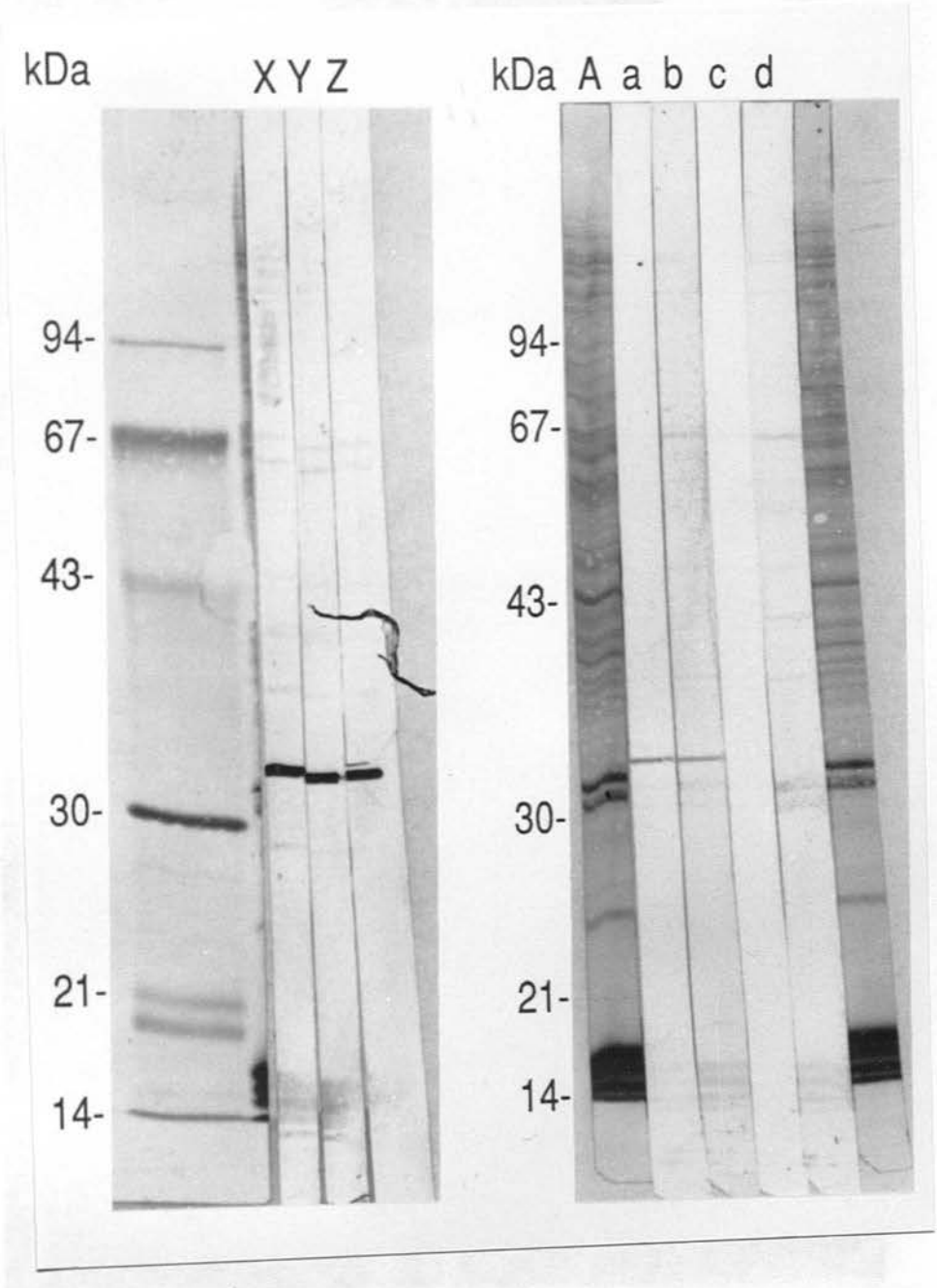


Figure 3.34 Whole PARIT cells stained with affinity purified 13A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with affinity purified 13A and counterstained with DAPI to localise the nucleus, (c) and (d) show cells prepared in PBS stained with affinity purified 13A and counterstained with DAPI to localise the nucleus.

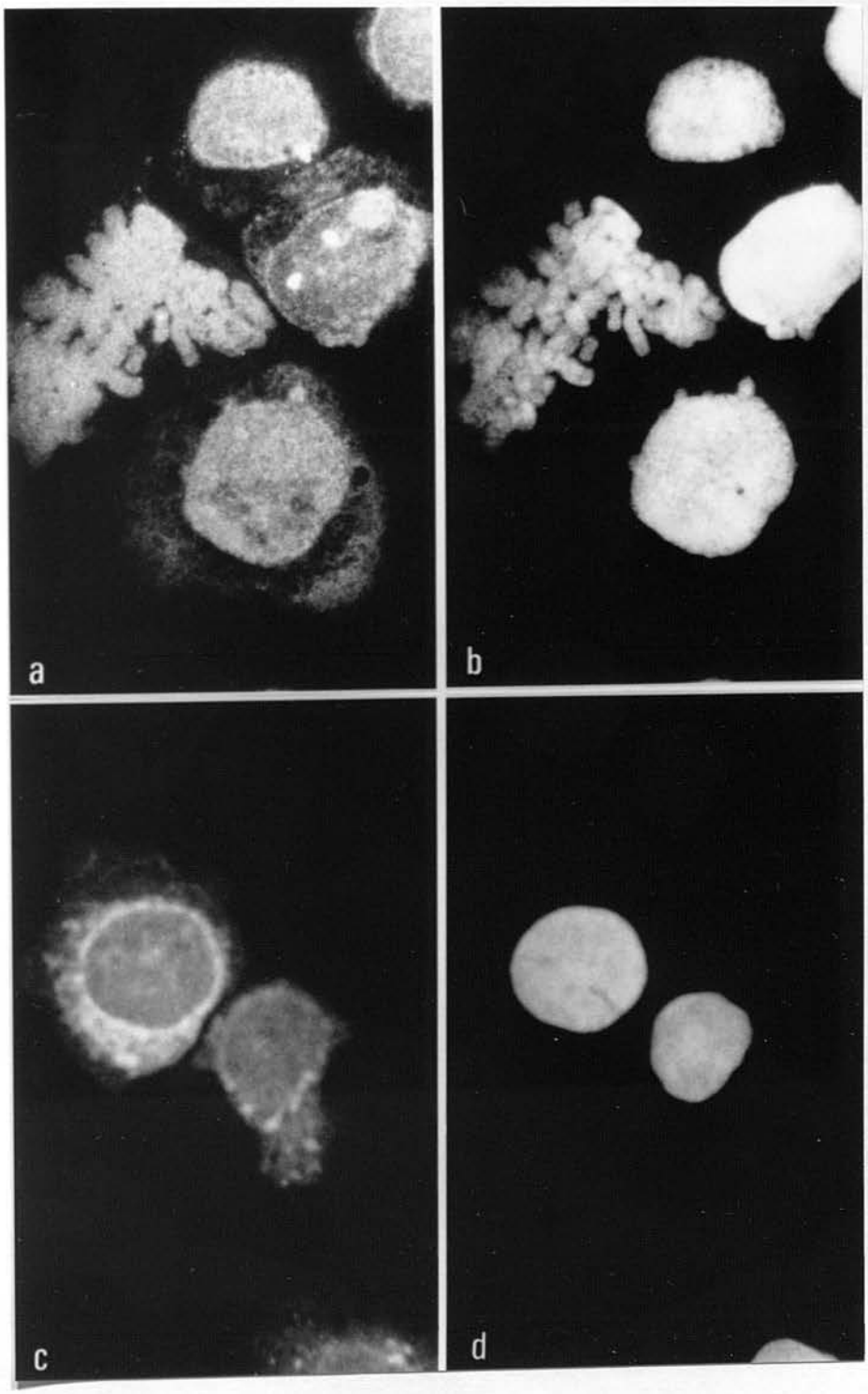


Figure 3.35 Whole Don K_2 cells stained with affinity purified 13A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with affinity purified 13A and counterstained with DAPI to localise the nucleus, (c) and (d) show cells prepared in PBS stained with affinity purified 13A and counterstained with DAPI to localise the nucleus.

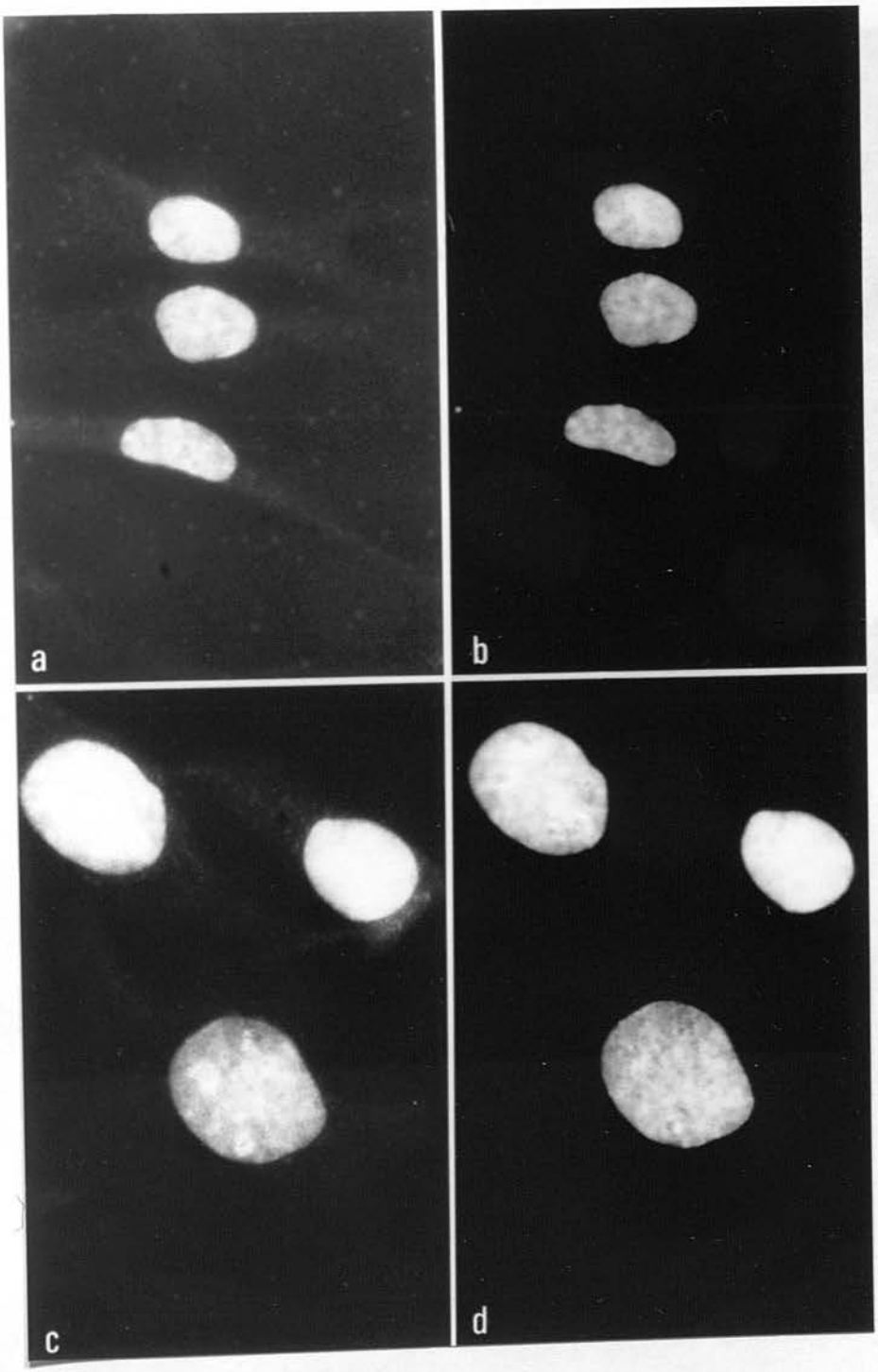
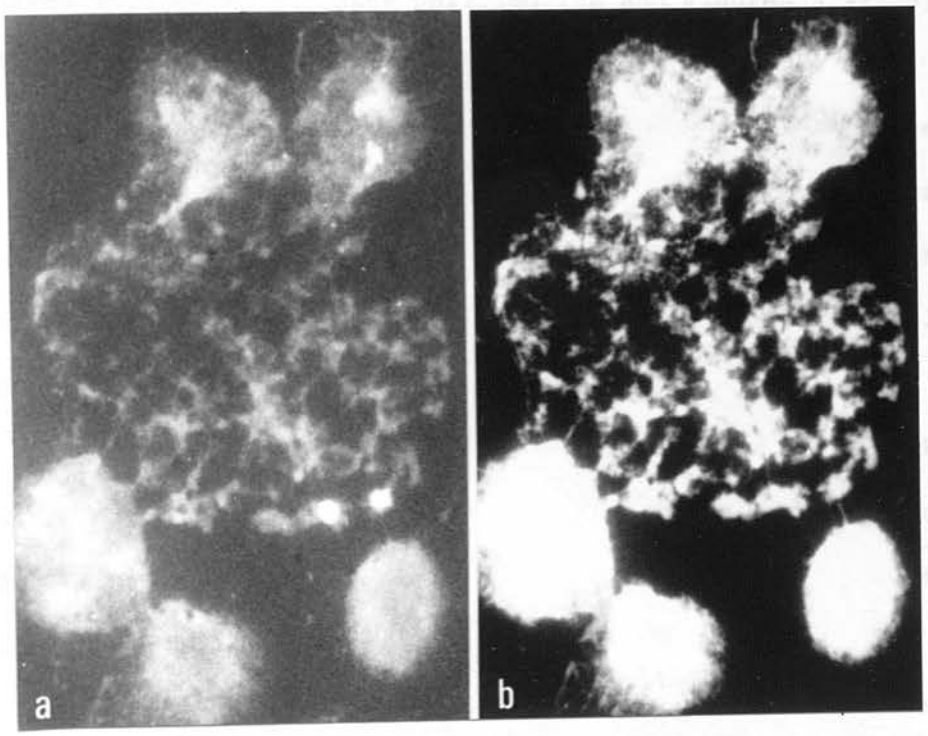


Figure 3.36 Metaphase spreads of PARIT cells stained with affinity purified 13A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with affinity purified 13A and counterstained with DAPI to localise the chromosomes.



The eluted antibody supplemented with 10% rabbit serum was also applied undiluted to cells and spreads to localise the antigen. Any staining pattern observed will be due to the purified antibody.

Figures 3.34, 3.35 and **3.36** show the staining patterns observed when using affinity purified 13A and **Figures 3.37, 3.38** and **3.39** show those when 14A was used as the first antibody.

Affinity purified 13A shows nuclear staining in whole cells and chromosomal staining on metaphase spreads. Whole PARIT cells show some degree of cytoplasmic staining also whilst the Don K_2 cells have a patchy appearance which may indicate some nucleolar recognition.

Affinity purified 14A shows granular staining of whole PARIT cells, with the nuclear membrane also stained. Whole Don K2 cells show uniform nuclear staining, and metaphase spreads show a moderate level of staining localised to the chromosomes.

These staining patterns are less well defined than those observed with the unpurified sera. This may be due to the purification procedure itself and/or the storage of the eluted antibodies in neutralised HCl, despite having been dialysed and the antibodies stabilised by the addition of 1% bovine serum albumin (BSA). In this instance the use of affinity purified antibodies does not appear to have added to the information deduced from using whole sera.

Figure 3.37 Whole PARIT cells stained with affinity purified 14A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with affinity purified 14A and counterstained with DAPI to localise the nucleus, (c) and (d) show cells prepared in PBS stained with affinity purified 14A and counterstained with DAPI to localise the nucleus.

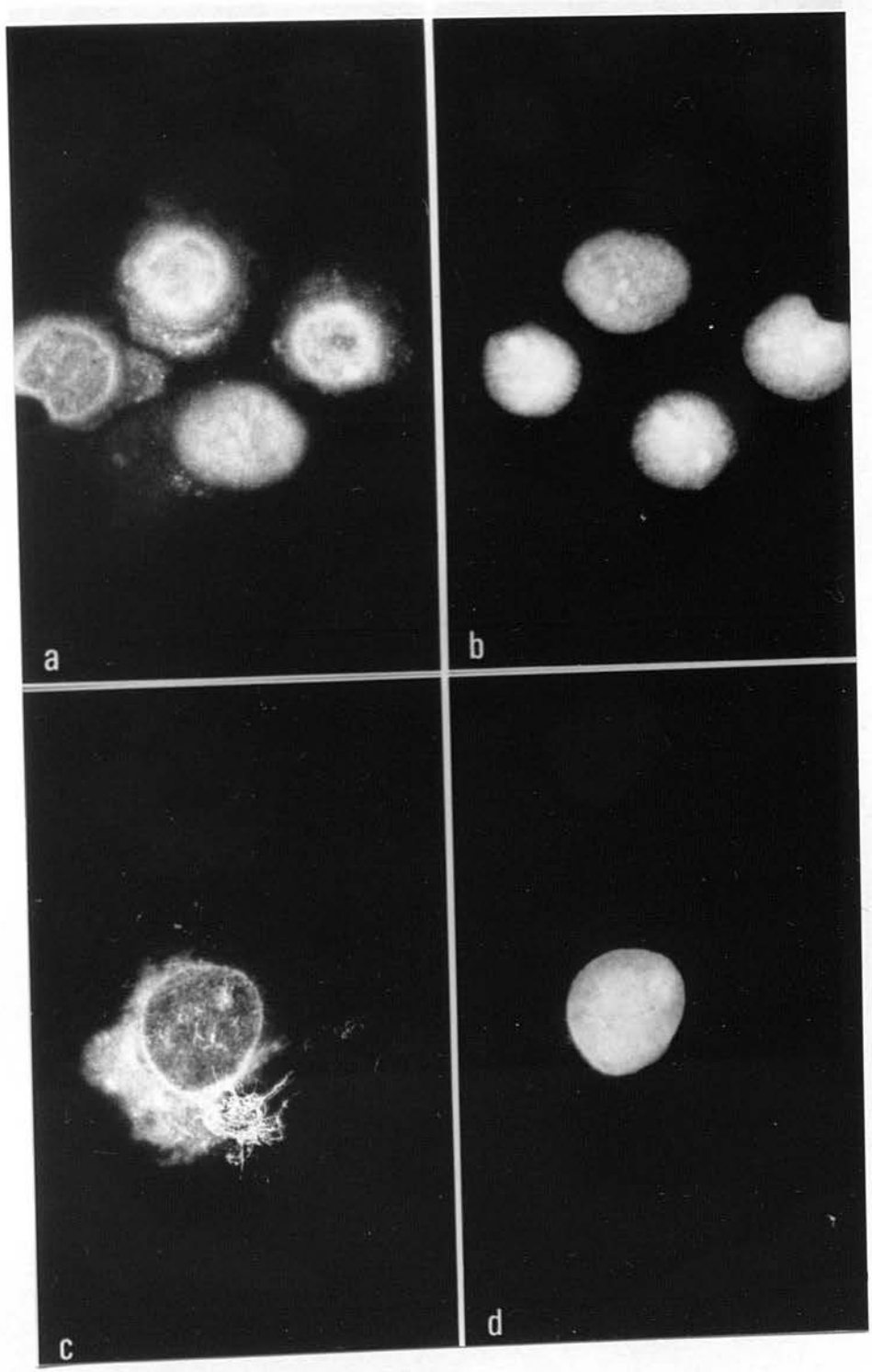


Figure 3.38 Whole Don K_2 cells stained with affinity purified 14A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with affinity purified 14A and counterstained with DAPI to localise the nucleus, (c) and (d) show cells prepared in PBS stained with affinity purified 14A and counterstained with DAPI to localise the nucleus.

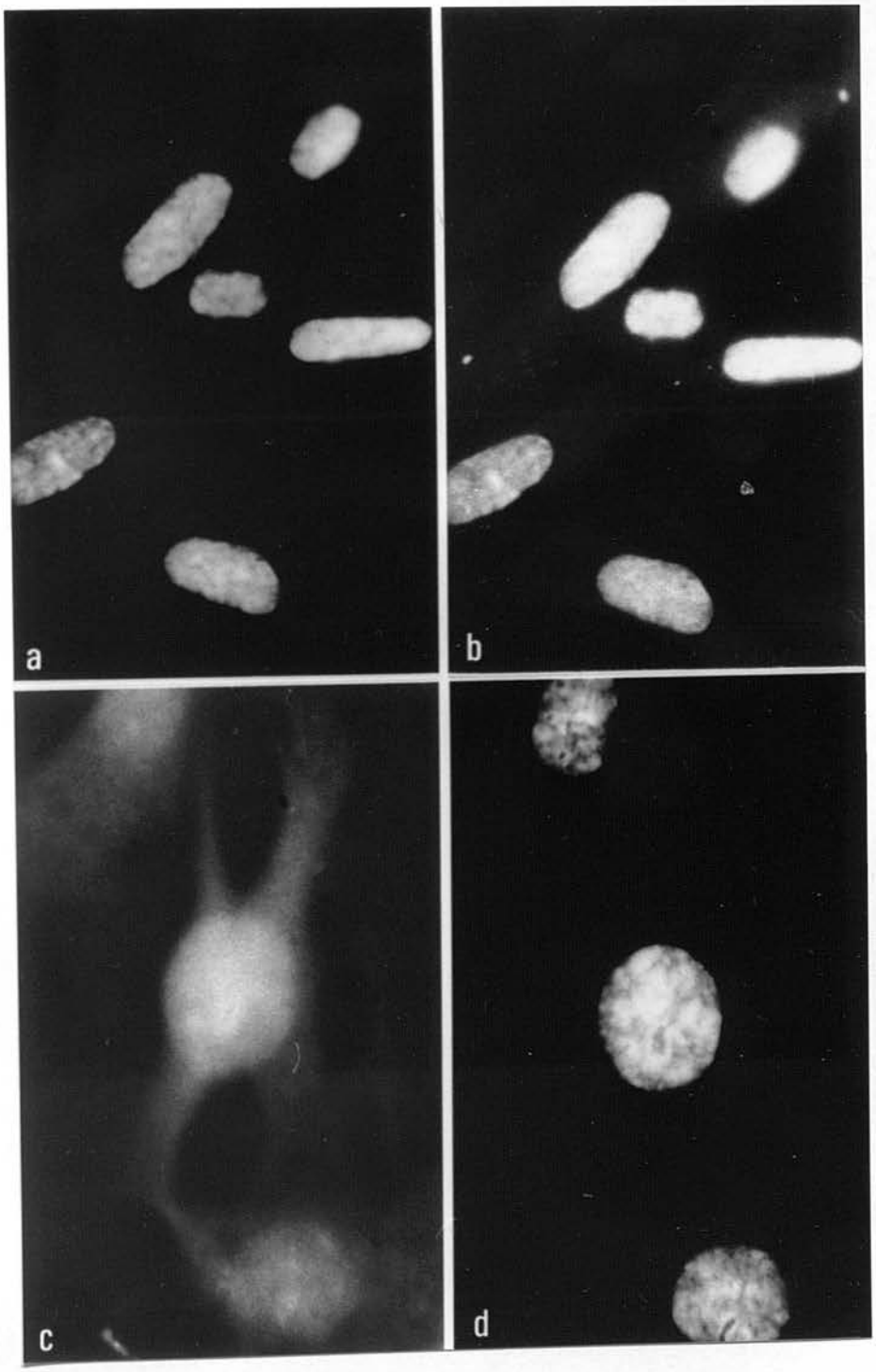
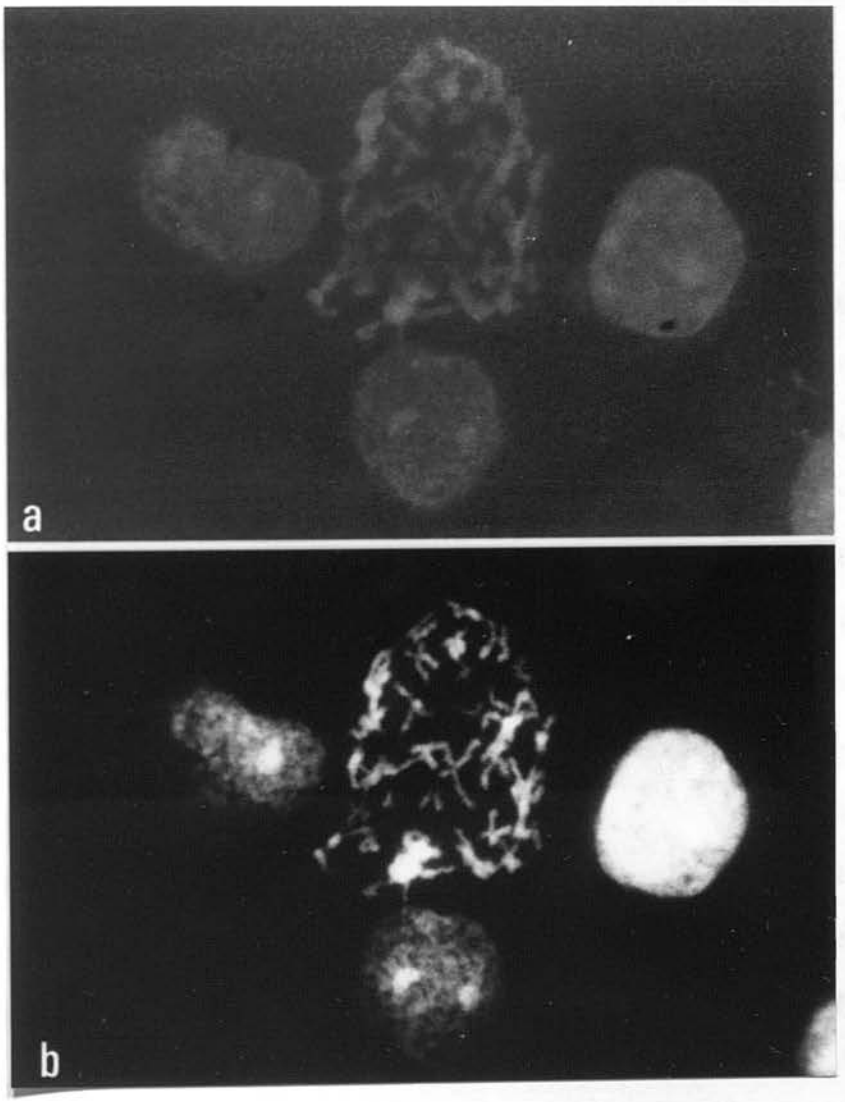


Figure 3.39 Metaphase spreads of PARIT cells stained with affinity purified 14A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with affinity purified 14A and counterstained with DAPI to localise the chromosomes.



3.3 Discussion

Both the immunocytochemistry and immunoblotting techniques employed for the purpose of initial characterisation of the antigenic specificities of the serum samples provided are extremely sensitive.

The preparation of samples for immunocytochemical analysis employed two different buffers, PBS and KCM. KCM contains detergent and this causes some of the soluble antigens within the nucleus to leach out, whereas PBS does not and therefore provides a more natural representation of the cells. (The nucleoli are resistant to this phenomenon.) This accounts for the differing staining patterns which are sometimes observed.

In addition to the differing preparation procedures used, different cell types were also employed. The use of whole cells from a human cell line and a hamster one demonstrates the cross species specificity of many of the autoantibodies in the serum samples screened. Although many of the sera discussed do not show identical staining patterns in both cell types, similarities are easily observed. This justifies the use of mouse models of autoimmunity as one avenue for the study of this type of disease in humans.

The use of metaphase spreads, as well as whole cell preparations ensures that any striking chromosomal associations of the autoantibodies present within the sera are clearly demonstrated.

JL, the normal control serum, shows quite a high level of activity on some preparations, which may not necessarily have been expected. However as discussed in Chapter 1, autoantibodies are

present in apparently healthy individuals in increasing titres with the advancing age of the individual (Tomer and Shoenfeld, 1988). Serum JL was taken from a middle aged female and the staining patterns show very clearly that autoimmune activity can and does occur in normal individuals with no adverse consequences. The application of serum antibodies using immunocytochemistry revealed the identity of two SLE patients and one CREST patient, due to the distinctive staining patterns observed. In the case of the CREST patient, the individual concerned was recalled to the clinic and was rediagnosed after further clinical tests. These results were confirmed in two cases by Western blotting information which demonstrated the antigenic specificities of #7(ML) and #23(BB) to be CENP-A and histone (in particular H1). Serum #24(NF) showed no peptide recognition on Western blotting, so it is highly likely that the antigen recognised on immunocytochemistry was DNA, also a characteristic antigen of SLE. In addition to demonstrating, in a blind study, that these techniques can be gainfully employed to aid diagnosis, they provide positive controls to prove that the technique is giving reliable results, due to the antigenic specificities of the serum antibodies used in the staining procedures.

The 95kDa peptide initially identified by #13(H) has been shown to be scleroderma related by the Western blotting of more serum samples from different patients with the same disease. The discovery of a serum with an increased level of activity as shown by 13A has allowed the staining pattern to be clarified a little further; it is nuclear, with a higher specificity for nucleoli in whole cells, and is associated chromosomally upon condensation at

metaphase. The affinity purification of the antibody(ies) which recognises that particular polypeptide has allowed me to prove that the serum is specific for that polypeptide, but unfortunately has not provided any further information as to the identity of the antigen(s) by immunocytochemistry. The affinity of this serum antibody for its antigen will prove invaluable when screening an expression library in order to determine the antigen.

The 38kDa antigen, initially identified by serum #14(R) has also been shown to be disease related, in this case to MCTD, by the identification of two other sera from MCTD patients which show the same polypeptide reactivity. Western blotting also enabled me to identify serum #14(R) as that from a patient with MCTD due to the presence of antibodies to the marker antigen U1-RNP. As found previously, one of the additional sera screened showed a much increased level of activity to the 38kDa peptide on Western blots and this was used to stain whole cells and metaphase spreads in order to try to clarify the cellular localisation of the antigen. It appears to be nuclear, sometimes present at or in the nuclear membrane in whole cells, whilst the staining of metaphase spreads indicates an association to some degree with condensed chromosomes. Affinity purification again proved the single antigenic specificity of the serum antibodies, but provided no further information on the cellular nature of the antigen. As with 13A, 14A will also be used to screen an expression library in order to determine the true identity of the antigen.

CHAPTER FOUR

THE CLONING OF DNA TOPOISOMERASE I

4 THE CLONING OF DNA TOPOISOMERASE I

4.1 Introduction

Sera #13(H) and 13A display reactivity with the 95kDa band on immunoblots, appear to stain the nucleoli in whole cells and immunofluorescence is localised to chromosomes in metaphase spreads as discussed in Chapter 3. These observations provide us with much useful information about the antigen, but in order to determine its actual identity serum 13A was used to screen a λgt11 expression library. 13A was chosen as it shows much higher reactivity to the antigen than #13(H).

The library used in this case was a gift from the ICRF unit of Edinburgh University and was commercially prepared by Clontech. It was a human testis cDNA library and the cDNAs were cloned into a λgt11 vector at the Eco RI site. **Figure 4.1** briefly explains the principles of screening an expression library. The mRNA source was a normal human testis biopsy from a healthy 50 year old. The insert sizes ranged from 0.7kb to 3.3kb, with an average of 1.2kb.

4.2 Results

4.2.1 Isolation of Positive Clones Using Serum 13A

The library was first titred to ensure that the correct number of plaques were plated for the primary round of screening. Five plates

Figure 4.1 Schematic representation of the construction and screening of a λgt11 expression library.

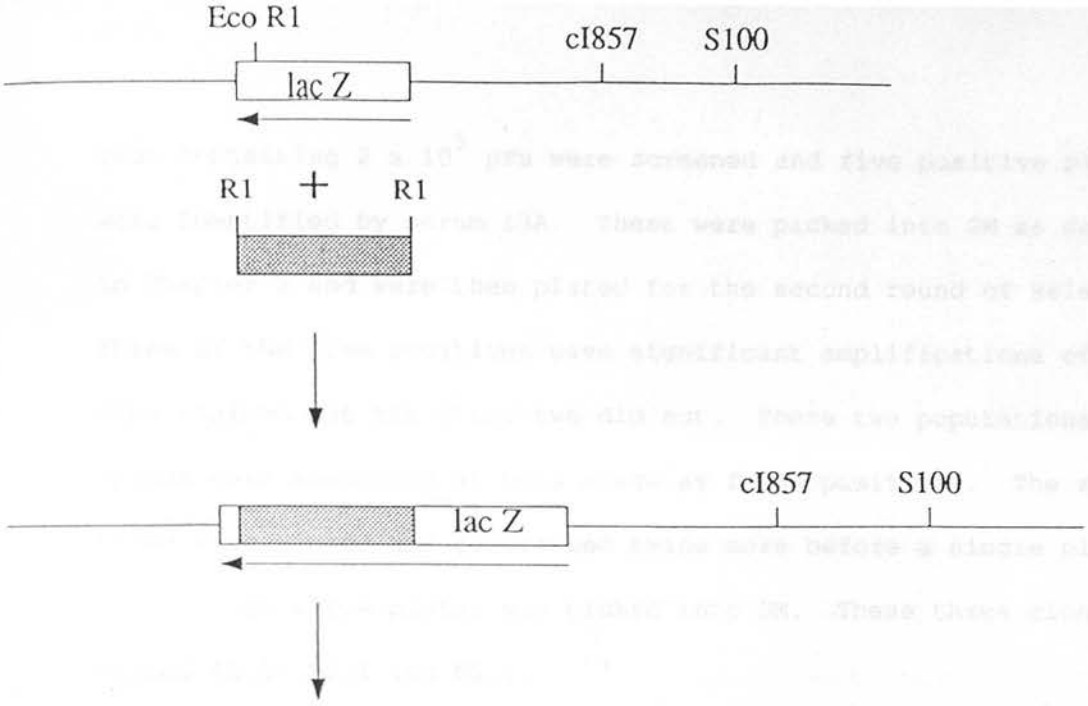
A cDNA copy of mRNA isolated from the testis biopsy is synthesised enzymatically. The first strand is produced from an oligo-dT primer annealed to the poly-A tail of the 3' end of the mRNA. The RNA template is removed and the second strand is synthesised using the hairpin structures formed at the end of the first strand, as primers. The hairpin is removed with S1 nuclease and the double stranded cDNA has linker sequences added, which contain restriction enzyme sites. The linkers are cut using the restriction enzyme and the cDNA ligated into the *lac Z* gene of the bacteriophage vector λgt11.

The library is amplified in *E. coli* Y1088 cells.

The library is plated out by infection of *E. coli* Y1090 cells and grown up until plaques are formed. The plate is overlaid with a nitrocellulose filter soaked in IPTG, which stimulates the bacteriophage vector to produce the β-galactosidase gene (encoded by *lac Z*) fused to a peptide encoded by the inserted piece of synthesised cDNA, by reading through the restriction site, in this case Eco RI.

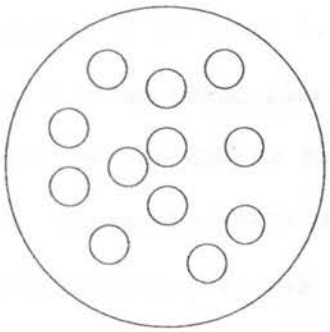
The filter is removed and the first antibody is applied. This will bind to a fusion protein containing the correct epitope. The position of any bound first antibody is localised with a peroxidase conjugated second antibody, which gives a colour reaction with chloronaphthol and hydrogen peroxide. The position of plaques containing bacteriophage expressing a fusion protein with the first antibody epitope is marked on the filter by a small, dark blue circle or doughnut-like stain.

The plaque expressing the recognised protein is picked and rescreened.

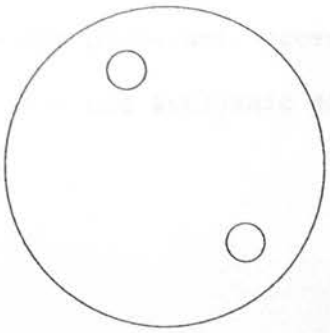


Amplify library: E.coli: Y1088 ($hsdR^-$ supF $lacI^+$)

Plate library: E. coli: Y1090 ($lon\triangle$ supF $lacI^+$)



1. Transfer antigen to IPTG- soaked nitrocellulose
2. Probe nitrocellulose filter with antibody
3. Probe antibody with peroxidase-conjugated 2nd antibody



Chloronaphthol stained filter

each containing 2 x 10^5 pfu were screened and five positive plaques were identified by serum 13A. These were picked into SM as described in Chapter 2 and were then plated for the second round of selection. Three of the five positives gave significant amplifications of signal when stained but the other two did not. These two populations of 'phage were abandoned at this stage as false positives. The remaining three were picked and rescreened twice more before a single plaque from each of the three plates was picked into SM. These three clones were called A2.1, D1.1 and D2.1.

The insert cDNA of bacteriophage λ DNA was isolated by LGT agarose electrophoresis after digestion of the vector with the restriction enzyme Eco RI. These inserts were then cloned into the Eco RI site of Bluescribe, a pUC based vector.

The sizes of the inserts were estimated by gel electrophoresis; A2.1 was estimated to be approximately 1.7kb and D1.1 and D2.1 were both estimated to be about 2.0kb in length.

The isolated cDNAs cloned into the plasmid vectors should all contain information coding for the antigenic site(s) recognised by serum 13A. Since the 95kDa band was by far, the major reacting species on the Western blots, it was likely that at least one of the clones coded for this polypeptide. To test whether the three clones shared the same DNA sequences, or contained dissimilar ones (indicating that they represented different antigenic determinants, either from the same or different proteins), cross hybridisation was carried out. There may be more than one antigenic site within the protein as autoantibodies

found in the serum of autoimmune patients are polyclonal and therefore recognise different epitopes of the same protein. The results of the cross hybridisation are shown in **Figure 4.2.**

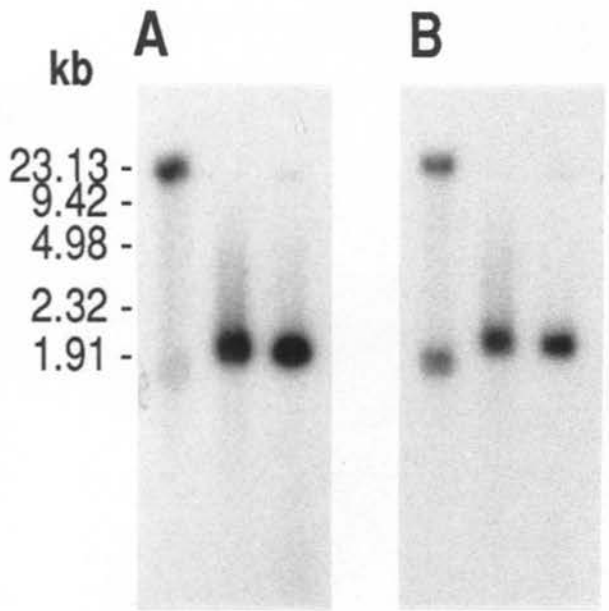
λ DNA of each clone was digested with Eco RI and the samples run on an agarose gel. The DNA was then Southern blotted onto Hybond-N as described in Chapter 2 and probed with the isolated inserts cut from the plasmid vectors. This ensures that any signal is due to the binding of the inserts, not the vectors. Panel A shows λA2.1, D1.1 and D2.1 probed with the insert from pD1.1. A positive signal is observed with A2.1, but at about 23kb. This is due to the incomplete digestion of λA2.1, so the insert has not been released from the vector. It therefore does not run at its estimated position (approximately 1.7kb) within the gel. Positive signals are observed also for D1.1 and D2.1; D1.1 is acting as a positive control for the reaction and a signal is expected, but the presence of signal at the position of D2.1 insert shows that the DNA of the probe and the sample is the same. Panel B shows the three digests probed with insert cut from pD2.1 and gives the same results. This time the positive control is pD2.1 onto λD2.1 and the digestion of λA2.1 was again incomplete. The labelled filters were washed at 0.1x SSC, 0.1% SDS, a stringency at which only very similar sequences of DNA remain bound to each other. Therefore it can be concluded that the three inserts A2.1, D1.1 and D2.1 all contain the same sequence of DNA, albeit of different lengths.

To discover whether these sequences were part of a known protein

Figure 4.2 Autoradiograph of the isolated λ clones hybridised with the inserts subcloned into plasmids.

Panel A shows Eco RI restriction digested λA2.1, D1.1 and D2.1 probed with the insert from pD1.1.

Panel B shows the same three digests probed with the insert from pD2.1.



or motifs of a group of proteins with a particular function, they were sequenced.

4.2.2 The Sequencing of Clones A2.1, D1.1 and D2.1

The sequencing method used was as described in Chapter 2. The first clone to be sequenced was D1.1 as it was one of the two longest isolated. The Sequenase forward primer was used and the sequence read from the gel was used to search the Genbank/EMBL database at Daresbury. A perfect match was found as shown in **Figure 4.3**. The sequence of D1.1 is shown on the top numbered 1 to 242 and it matched nucleotides 1556 to 1797 of the human DNA topoisomerase I gene. The other two clones were sequenced in the same way using the forward primer and then all three were sequenced with a reverse primer (prepared by D. Chambers in the Unit) to disclose the sequence of the other end of the clones. All these sequences also matched up with the DNA topoisomerase I gene, and the positions of each clone are shown in **Figure 4.4**. As predicted by the cross-hybridisation experiment the clones do indeed contain basically the same sequence of DNA towards the 3' end of the gene.

4.3 Discussion

The discovery that the antigen recognised by #13(H) and 13A was DNA topoisomerase was not, in retrospect, surprising. These two sera were samples taken from two scleroderma patients and DNA

Figure 4.3 Sequence read from the forward primed pD1.1 matched to nucleotides 1556 to 1797 of the human DNA topoisomerase I gene.

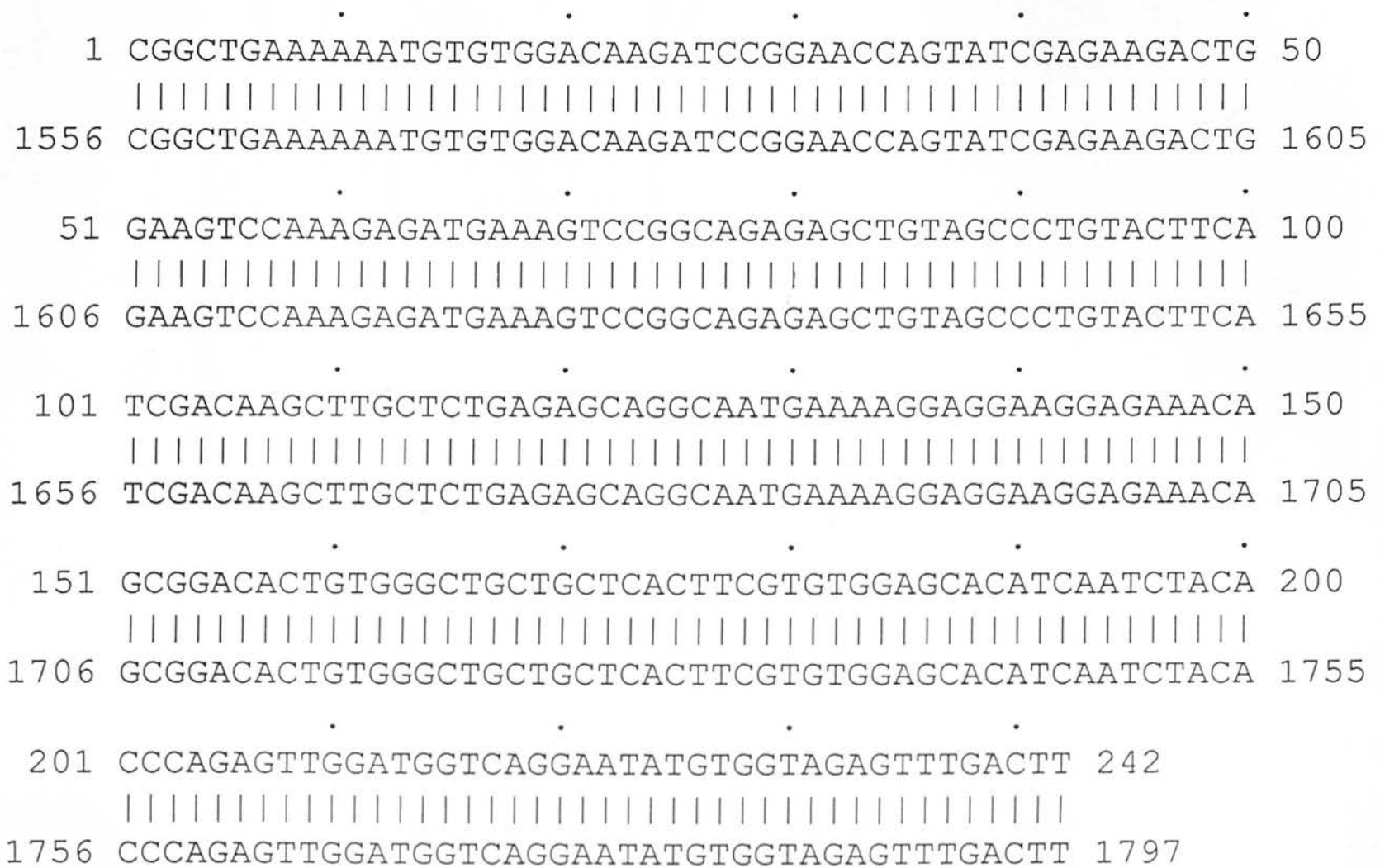
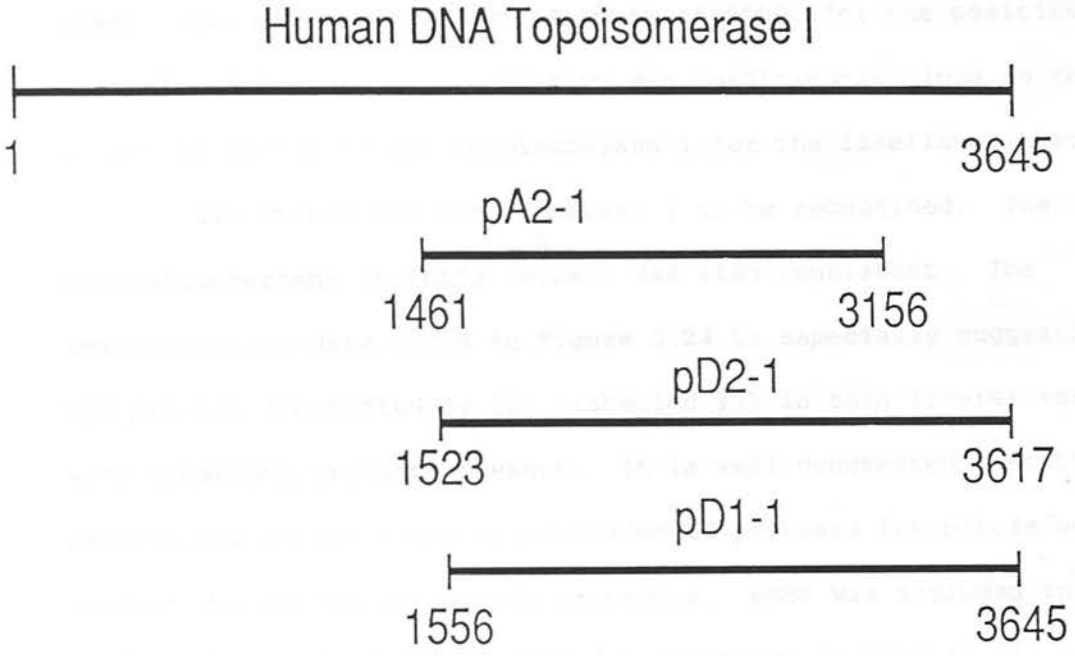

1 CGGCTGAAAAAATGTGTGGACAAGATCCGGAACCAGTATCGAGAAGACTG 50
1556 CGGCTGAAAAAATGTGTGGACAAGATCCGGAACCAGTATCGAGAAGACTG 1605
51 GAAGTCCAAAGAGATGAAAGTCCGGCAGAGAGCTGTAGCCCTGTACTTCA 100
1606 GAAGTCCAAAGAGATGAAAGTCCGGCAGAGAGCTGTAGCCCTGTACTTCA 1655
101 TCGACAAGCTTGCTCTGAGAGCAGGCAATGAAAAGGAGGAAGGAGAAACA 150
1656 TCGACAAGCTTGCTCTGAGAGCAGGCAATGAAAAGGAGGAAGGAGAAACA 1705
151 GCGGACACTGTGGGCTGCTGCTCACTTCGTGTGGAGCACATCAATCTACA 200
1706 GCGGACACTGTGGGCTGCTGCTCACTTCGTGTGGAGCACATCAATCTACA 1755
201 CCCAGAGTTGGATGGTCAGGAATATGTGGTAGAGTTTGACTT 242
1756 CCCAGAGTTGGATGGTCAGGAATATGTGGTAGAGTTTGACTT 1797

Figure 4.4 To show the positions of clones A2.1, D1.1 and D2.1 within the human DNA topoisomerase gene.

A2.1 spans nucleotides 1416 to 3156, D1.1, nucleotides 1556 to 3645 and D2.1, nucleotides 1523 to 3617.


Human DNA Topoisomerase I
1
3645
pA2-1
1461
3156
pD2-1
1523
3617
pD1-1
1556
3645

topoisomerase I is a marker antigen for this disease, as discussed in Chapter 1. It was originally called Scl-70 as the molecular weight of it was estimated at 70kDa (Douvas *et al.*, 1979). It was subsequently revised to approximately 100kDa (Shero *et al.*, 1986; Guldner *et al.*, 1986). The molecular weight estimate of 95kDa for the positive band in the initial Western blot screening was sufficiently close to the molecular weight of DNA topoisomerase I for the likelihood that the antigen was indeed DNA topoisomerase I to be recognised. The immunofluorescent staining pattern was also consistent. The immunoblotting data shown in **Figure 3.24** is especially suggestive. The protein identified by 13A (labelled #17 in this figure) was shown with breakdown products present. It is well documented that the 100kDa protein can be spontaneously degraded if protease inhibitors were not present during the extraction procedure. PMSF was included in the buffers in the procedure I used (as described in Chapter 2), but had obviously not been effective in that particular preparation. The initial immunoblot using #13(H) did not show the antibody binding to any breakdown products. Although the 95kDa polypeptide did not turn out to be a novel antigen, the results obtained prove the validity of the cloning approach for the identification of autoimmune antigens.

The nucleolar and diffuse granular staining in whole cells (Douvas *et al.*, 1979) and the apparent localisation of staining with metaphase chromosomes shown in Chapter 3 correlates well with the documented intracellular distribution of DNA topoisomerase I (Muller *et al.*, 1985). This demonstrates the use of indirect

immunofluorescence as a tool for the intracellular localisation of autoimmune antigens, but as discussed in Chapter 3.3 it is not of great assistance alone in a blind study.

The actual sizes of the inserts can be calculated as we now know where within the DNA topoisomerase I gene they start and finish. A2.1 was estimated to be approximately 1.7kb and is actually 1,695bp. D1.1 was estimated at 2.0kb and is actually 2,089bp and D2.1 also estimated at 2.0kb is actually 2,094bp. The estimations of size of the three clones were in fact quite close.

All three clones were located towards the 3' end of the gene. When the library was prepared the first cDNA strand was transcribed from the mRNA from an oligo-dT primer bound to the poly-A tail of the mature RNA using reverse transcriptase which produces complementary DNA from 5' to 3'. This means that the beginning of the first cDNA strand is in fact the end of the mRNA. The reverse transcriptase rarely produces a full length sequence and the complementary RNA is removed by digestion. The second DNA strand is produced by a DNA polymerase resulting in a double stranded copy of the 3' end of the mRNA. The length of the cDNA and how far it extends into the coding region from the 3' end depends on how much of the mRNA was transcribed by the reverse transcriptase. The 3' end points of the three clones are also different. This is due to the 5' to 3' exonuclease activity of the DNA polymerase I used to produce the second strand. This exonuclease removes non-base-paired nucleotides from the 5' end of double stranded molecules of DNA. If the polymerase did not end at the very terminus

of the molecule, the unmatched bases would be removed, as in clones A2.1 and D2.1.

Approximately 1 x 10^6 pfus were screened (five plates each containing 2 x 10^5 pfu) and three positive clones were isolated from these. The library used was in fact an amplified library so all sequences transcribed into DNA from the mRNA by reverse transcriptase should be multiplied, but some λ clones will amplify better than others, due to their size, constituent nucleotides etc..

Other studies have shown that DNA topoisomerase I is a single copy gene located on chromosome 20 q12-13.2 and gives rise to an mRNA of 4,000 nucleotides (Juan *et al.*, 1988).

D'Arpa *et al.* (1988) found that the length of the mRNA was 4,100 nucleotides, but their published sequence is 3,645 nucleotides in length. There is a discrepancy of approximately 500 nucleotides between the two. Although the length of the mRNA is only estimated the difference is too great to be explained by experimental error. However on closer examination of the published sequence it is observed that the 3' end terminates in a natural Eco RI site instead of the cDNA equivalent of the poly-A tail of a mature mRNA. This "tail" may be 150 to 200 bp in length which accounts for some of the missing nucleotides but it is quite possible that there is a portion of sequence in the order of 200 nucleotides missing from the 3' end of the sequence. One of my clones, D1.1, also ends at that natural Eco RI site.

When a library is constructed the transcribed lengths of cDNA have linkers ligated to their ends which have the restiction site required for insertion into the vector within them. The cDNAs are then digested with the enzyme in question (Eco RI in this case) and ligated into the vector. However the cDNA itself may contain that particular restriction site. It is, therefore, first modified with Eco RI methylase to prevent cleavage. As the sequence of DNA topoisomerase I ends at a natural Eco RI site an explanation for this may be that the methylase modification step was either not included or was inefficiently carried out in the libraries used. An alternative solution is that the library was successfully methylated and my clone, D1.1, terminating with the restriction site, originally contained the missing sequence. However when it was digested to liberate the insert for sub-cloning, two fragments were produced; one of approximately 2.0kb and another of 200bp or so. This tiny fragment would be lost on a 1% agarose gel. In order to sequence the end of this gene, the digest of the original clone would have to be run under conditions in which the small fragment could be visualised and cloned individually. It too could then be sequenced. The deduction of the remaining few hundred base pairs of the sequence of DNA topoisomerase I has not yet been done.

Two sera out of a batch of thirteen from scleroderma patients possessed autoantibodies to this protein, yet DNA topoisomerase I is the diagnostic marker for this disease. Although the number of sera studied in this instance was too small to be of real statistical value,

it can be used to estimate the proportion of patients in this batch who have autoantibodies to the antigen present in their sera. $^{2}/13$ sera with the autoantibodies gives us a figure of approximately 16%. Guldner *et al.* (1986) have reported that out of approximately 80 scleroderma sera studied, 20% of them possessed these autoantibodies, so considering the number of sera studied in this project, my calculated proportion is quite a reasonable estimate. Shero *et al.* (1986) concluded that sera containing autoantibodies to DNA topoisomerase I (or Scl-70, as they called it) seemed to be taken from patients with the more severe, diffuse states of the disease. The number of sera containing autoantibodies against DNA topoisomerase I will vary according to the number of patients with the more severe condition with serum samples in the batch provided for analysis. This explains why 11 of the sera I analysed did not contain autoantibodies to DNA topoisomerase I. Perhaps the term "diagnostic antigen" should be qualified in this case to refer only to the severe states of the disease.

These results demonstrate that the presence of a diagnostic antigen can aid the diagnosis of an autoimmune condition as previously shown by the immunoblotting of serum #7(ML). (This patient was diagnosed with scleroderma, but the discovery of autoantibodies to CENP-A resulted in revision of the original diagnosis to CREST.) However, the lack of a particular autoantibody does not necessarily mean that the patient does not have the disease for which its reactivity is a marker. These diseases are progressive in their

development, so all the autoantibodies which characterise the disease are not necessarily present at initial diagnosis.

It is not known why autoantibodies are produced to nuclear antigens in any of the known autoimmune diseases, and so the production of antibodies to DNA topoisomerase I in 20% of scleroderma patients alone is just as much of a mystery. If cellular components become exposed to the immune system by some mechanism of the disease autoantibodies will be produced. Autoimmunity can be thought to be the breakdown of tolerance, or the presentation of previously sequestered antigens, possibly due to the inefficient degradation of cellular debris.

DNA topoisomerase I is a highly active and abundant enzyme which is involved in relaxing positive and negative supercoils by nicking and reclosing single strands of DNA (Fink, 1989). However it is not crucial for the survival of the cell as discovered by mutation experiments in *Saccharomyces cerevisiae* (Brill and Sternglanz, 1988). Supercoils can be relaxed by DNA topoisomerase II (an enzyme which cuts and rejoins double strands of DNA) but the process takes much longer. Wallis *et al.* have isolated a third structural gene in yeast which codes for another type I topoisomerase (Wallis *et al.*, 1989) which may also assist with the relaxation of supercoiled DNA in the absence of DNA topoisomerase I.

Therefore this abundant enzyme may stimulate the production of autoantibodies against it. However it is far from the only abundant protein within the nucleus, and it is not exclusively selected for

presentation to the immune system, so this does not explain why this protein in particular is the antigenic target of autoantibodies in a proportion of sera from patients with scleroderma. The phenomenon of auto-reactions whether they are disease specific or not, are not well understood. As discussed in Chapter 1 it is not known whether they are a cause or an effect of the disease. Even though our knowledge of autoimmune diseases and their aetiology is improved with every antigen discovered, there is still a long way to go before we can begin to understand their full complexity and find a cure.

CHAPTER FIVE

THE CLONING OF THE PUTATIVE HUMAN *ENHANCER OF SPLIT* GENE

5 THE CLONING OF THE PUTATIVE HUMAN *ENHANCER OF SPLIT* GENE

5.1 Introduction

Sera #14(R) and 14A have been shown to recognise a nuclear antigen by indirect immunofluorescence, and a protein of 38kDa on Western blots of nuclear proteins. Chapter 4, describing the cloning of DNA topoisomerase I, has shown how effective the screening of an expression library can be for identifying autoantigens, so 14A, which reacts more strongly, was selected to screen the normal human testis expression library used previously.

5.2 Results

5.2.1 The Isolation of a Single Positive Clone Using Serum 14A

As in Chapter 4, five plates each with 2 x 10^5 pfu were screened with the serum. On two occasions serum 14A did not identify any plaques on the primary round of screening. At the third attempt, having plated some 3 million bacteriophage, two positive plaques were identified. They were picked and rescreened but only one gave the expected amplification of signal. This clone was picked and rescreened as before; the other was discarded as a false positive. From the positive bacteriophage population isolated in the primary round of screening, a single positive plaque was picked into SM after four rounds of purification. (In retrospect it would have been safer to

have picked more than one plaque, in order to check that a pure population was present on the final plate, by cross hybridisation of the isolated bacteriophage.) Preparations of bacteriophage were made and then the insert was cut out of the vector using the restriction enzyme Eco RI and isolated by running on an agarose gel. The insert, with an estimated size of 1.9kb was subcloned into the Eco RI site of the phagemid vector, Bluescribe. I called this positive clone V1.1.

As with the DNA topoisomerase clones the insert was sequenced in an attempt to identify the protein antigen of serum 14A.

5.2.2 The Sequencing of Clone V1.1

V1.1 was sequenced using the dideoxy nucleotide method with the forward primer from the Sequenase kit as described in Chapter 2. The sequence read from the gel was used to search the Genbank/EMBL database at Daresbury. No similar sequence was found. The clone was then sequenced in the same manner from the reverse oligo-primer (prepared by D. Chambers) and this sequence was also run through the database. This time a matching sequence was found and the alignment of the two is shown in **Figure 5.1**. The sequence of V1.1 is shown along the top numbered 4 to 166 and is matched with nucleotides 2171 to 2333 of the *Drosophila* gene, *Enhancer of Split (E(spl))*. This gene is involved in the development of the central nervous system in the fly.

The central nervous system in *Drosophila* is derived from a population of cells known as the neurogenic region, after invagination of the syncytial blastoderm to produce the mesoderm. Precursor cells

Figure 5.1 The sequence of reverse primed pV1.1 matched to nucleotides 2171 to 2333 of the *Drosophila* developmental gene *Enhancer of split* (*E(spl)*).

```
   4 gtgccgtgaccatcagcaaccccacgaggcacgtctacacaggtggcaag 53
     |||| || ||||||   || ||||| | | |||| ||||| || ||||||
2171 GTGCGGTAACCATCTCTAATCCCACAAAGTACGTGTACACGGGCGGCAAG 2220
               .         .         .         .         .
  54 ggctgcgtgaagatctgggacatcagccagccaggcagcaagagccccat 103
     |||||||| ||| | |||||||||   || || |||| |||||  ||  |
2221 GGCTGCGTCAAGGTATGGGACATCTCGCAACCGGGCAACAAGAATCCAGT 2270
               .         .         .         .         .
 104 ctcccagctggactgcctgaacagggacaattacatgcgctcctgcaagc 153
        |||||||||  || |||  |  | |||| |||||| |||||    ||||
2271 TAGCCAGCTGGATTGTCTGCAGCGCGACAACTACATCCGCTCGGTAAAGC 2320
               .
 154 tgcaccctgatgg 166
     |||  || || ||
2321 TGCTGCCCGACGG 2333
```

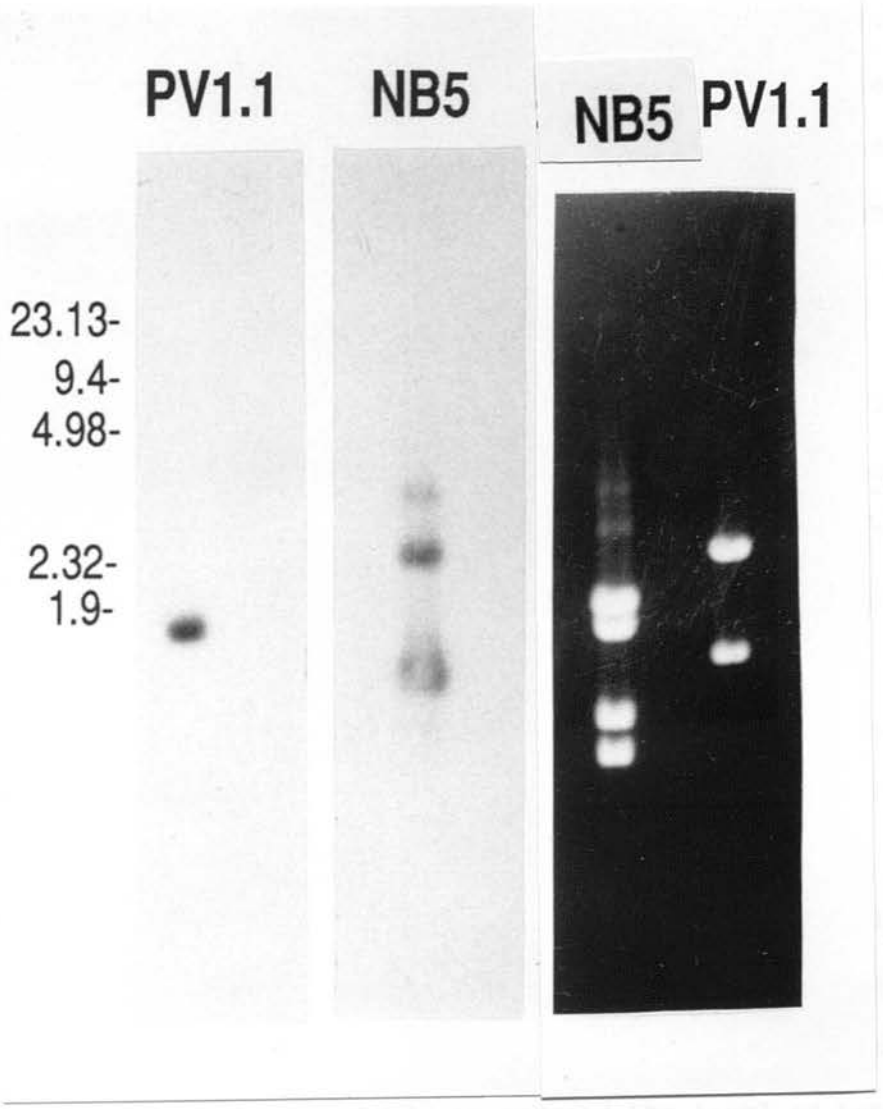
delaminate from this layer and these cells, known as neuroblasts, go on to form the nervous system, whilst the remaining cells develop into the epidermis of the fly (Poulson, 1950). The determination of cell fate from this single population of cells has been shown to be due to cellular interactions (Technau and Campos-Ortega, 1986) and under genetic control, by studying mutant phenotypes defined by the neurogenic genes (Poulson, 1937; Lehmann *et al.*, 1983). The mutant neurogenic phenotype (consisting of hypertrophy of the nervous system and absence of epidermis) is the result of a loss of function of any one of the six zygotic gene loci; *Notch*, *big brain*, *mastermind*, *Delta*, *neuralized*, and *Enhancer of split*.

Notch (Wharton *et al.*, 1985) and *Delta* (Vassin *et al.*, 1987) have been shown to code for transmembrane proteins supporting the theory that cellular interactions between the neurogenic genes takes place at the product level.

The *Drosophila melanogaster E(spl)* is a multi-gene complex consisting of 11 transcripts, m1 to m11. m9 was cloned by Hartley *et al* (1988) and it is this *E(spl)* transcript to which V1.1 shows homology. I was given a full length cDNA, NB5, a construct, BXB25, which rescues otherwise lethal mutations at the locus, and a mouse serum antibody, IC2, raised to a fusion protein of *E(spl)*, by D. Hartley.

In order to show the relationship between the human gene and the *Drosophila E(spl)* gene, V1.1 was cross hybridised to NB5 and BXB25. **Figure 5.2** shows the results of these experiments. NB5 was digested with Eco RI giving rise to four bands, plus two from partial digestion

Figure 5.2 The left hand panels show the autoradiographs of V1.1 hybridised to NB5 and pV1.1 (as a positive control), when both are digested with Eco RI. The right hand panels show the ethidium bromide stained gels before Southern blotting.



of the cDNA as shown by the ethidium bromide stained agarose gel. V1.1 cross hybridises with three of those bands, one a partial digest band at ~4.3kb, and bands at ~2kb and ~1.3kb. The band at ~1.3kb correlates closely with an expected band of 1216 bases from NB5, as there are Eco R1 restriction sites at bases 2117 and 3333. The band at ~2.3kb is Bluescript plasmid and the hybridisation is caused by labelled plasmid contaminating the insert probe, V1.1. The partially digested band at 4.3kb may represent the insert alone and is therefore recognised by the probe as it contains the region from bases 2171 to 3021, which is extremely homologous to the human probe.

V1.1 was also shown to hybridise to the construct BXB25. **Figure 5.3** shows V1.1 hybridised to itself as a positive control and also to a band of ~1.3kb produced by the restriction digestion of BXB25 with Eco R1. (The upper band at ~3kb in the pV1.1 lane which also shows hybridisation to the insert is due to plasmid contaminating the labelled probe.) The hybridisation to this construct shows that the V1.1 bears homology to the region of the gene which is required for the normal neurogenic development of *D. melanogaster*.

Figure 5.4 shows the results of cross hybridising the 1.3kb band of NB5 to pV1.1 and total NB5 (as a positive control), digested with Eco RI as previously, and then washed with differing concentrations of SSC. As the concentration of SSC is reduced the level of hybridisation signal with the 1.9kb band of pV1.1 is reduced also, until at 0.1x SSC the level of hybridisation has almost disappeared. This shows that although the two sequences are extremely homologous, they are not

Figure 5.3 The left hand panels show the autoradiographs of V1.1 hybridised to BXB25 and pV1.1 (as a positive control), when both are digested with Eco RI. The right hand panels show the ethidium bromide stained gels before Southern blotting.

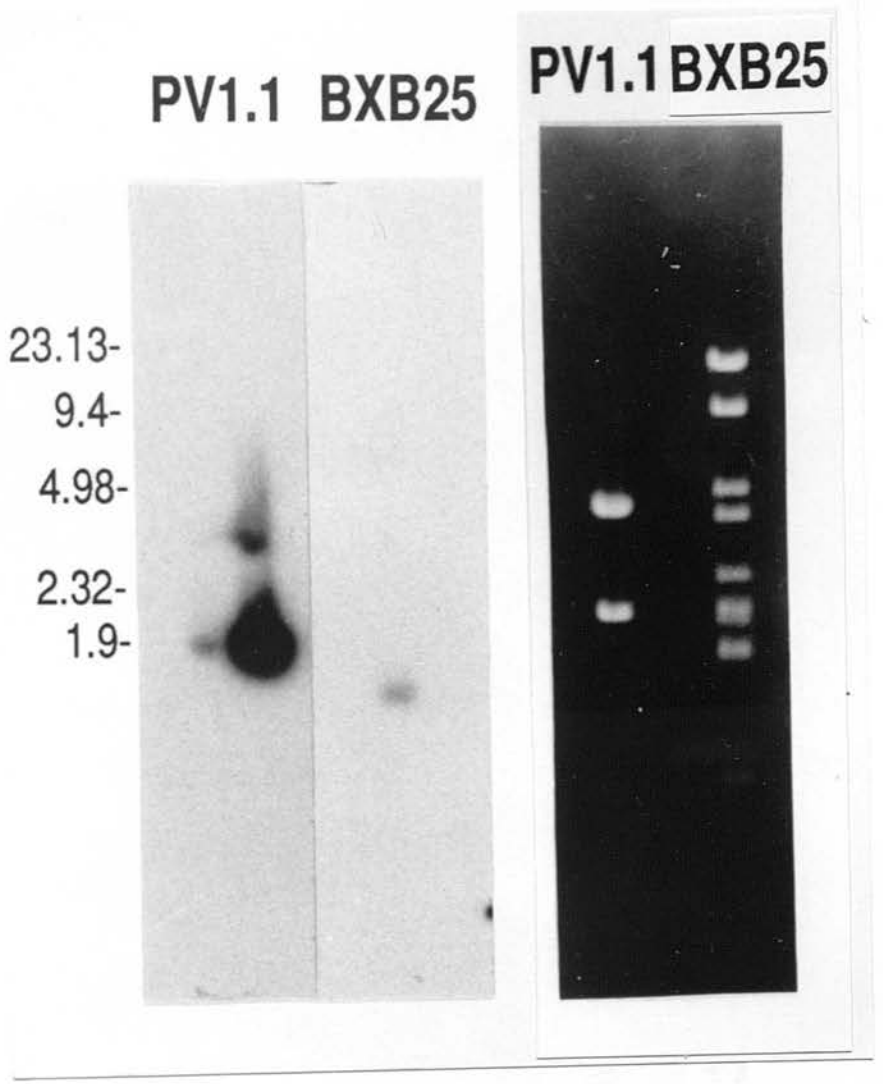
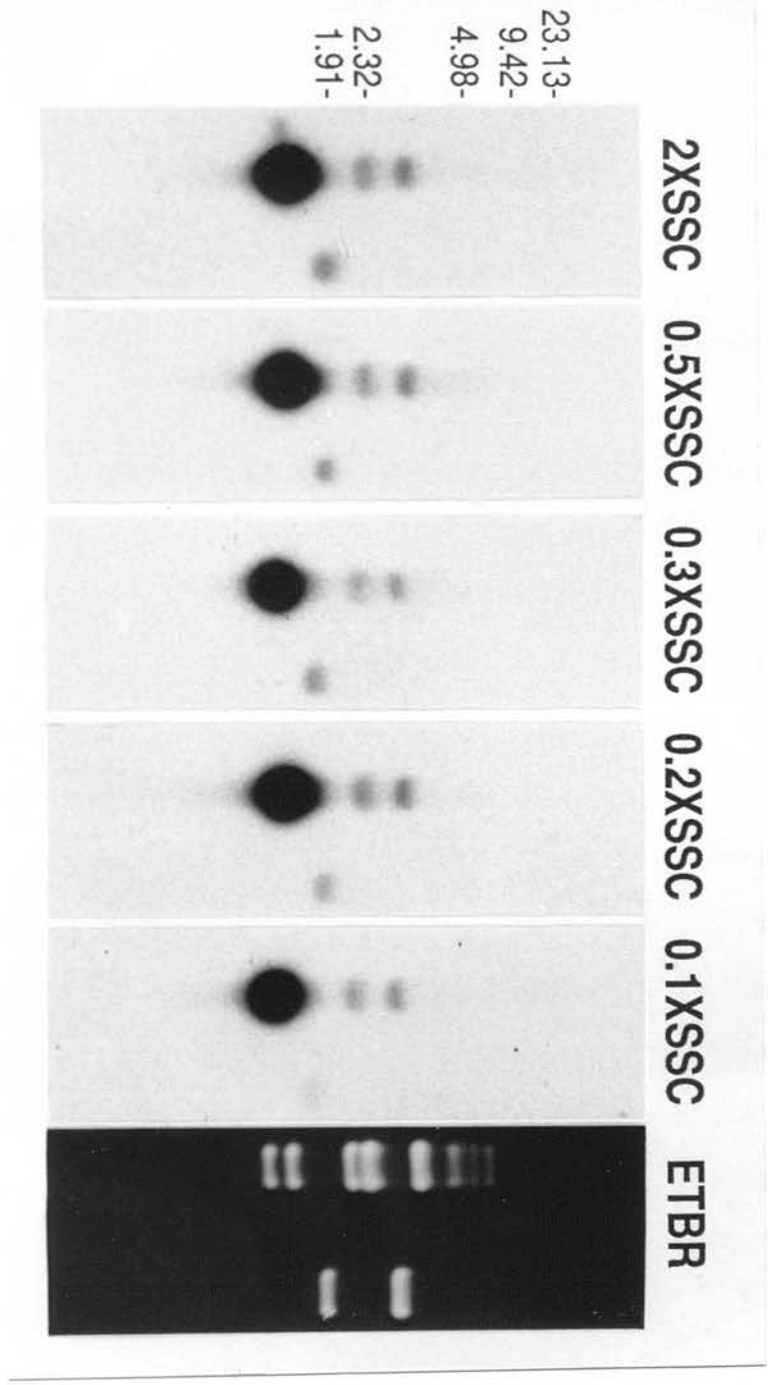


Figure 5.4 shows autoradiographs of pV1.1 and NB5 digested with Eco RI hybridised with the 1.3kb band of NB5. Each panel has been washed with a different concentration of SSC. The concentration of SDS in the wash is maintained at 0.1%. Panel ETBR shows the ethidium bromide stained gel before Southern blotting.



identical, as washing with 0.1x SSC has reduced the amount of DNA hybridised. If they were identical the level of signal would not be affected by washing more stringently. Hybridisation to the two upper bands of NB5 by the 1.3kb fragment is due to contamination of the probe with remnants of these pieces of DNA. These experiments showed that V1.1 is related to the *Drosophila E(spl)* gene.

The human clone pV1.1 was further characterised by completing the sequencing of the open reading frame by using double stranded nested deletions of the clone (as described in Chapter 2) and 18-mer oligo-primers prepared by D. Chambers. **Figure 5.5** shows the position of the deletion clones, the primers used and the designated 5' and 3' ends of the clone. **Figure 5.6** shows the comparison of the open reading frames of the *Drosophila* gene and V1.1. The two sequences have the termination codon, TAA, in the same position and after this point show no homology to each other. However, within the reading frame, the sequences show ~76.5% identity.

In the longest open reading frame, the human clone encodes a peptide which shows ~88% identity with the *Drosophila E(spl)* protein as shown in **Figure 5.7**. However this open reading frame is out of frame with *lac Z* and so should not produce a fusion protein containing *E(spl)* sequences. If the longest open reading frame is not capable of producing a protein, the question arises as to whether the insert is transcribed. The simplest test was to determine the orientation of the insert within the λgt11 expression library. Therefore λV1.1 was mapped using restriction enzymes.

Figure 5.5 To show the forward and reverse primed sequences of pV1.1, the position of deletion clones and the position of the oligo-primers used to complete the sequencing of the open reading frame.

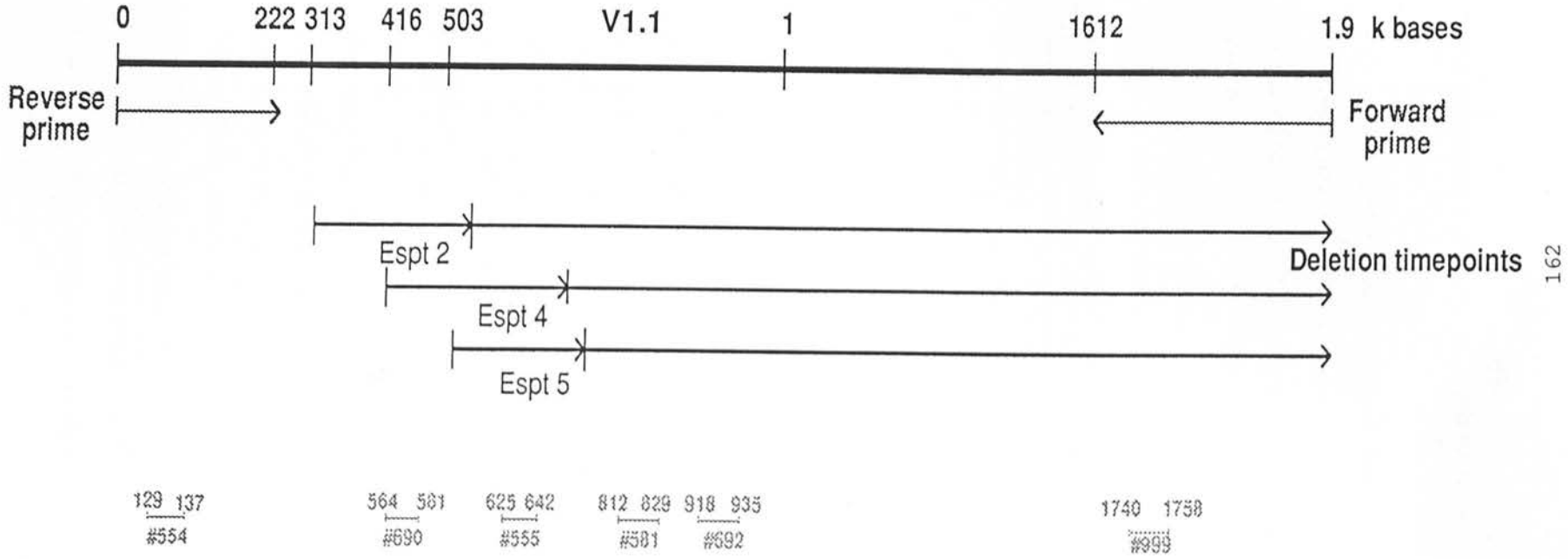

0
222 313
416 503
V1.1
1
1612
1.9 k bases
Reverse prime
Forward prime
Espt 2
Espt 4
Espt 5
Deletion timepoints
129 137
#554
564 581
#690
625 642
#555
812 829
#581
918 935
#692
1740 1758
#999

Figure 5.6 To show the entire open reading frame of V1.1 matched to nucleotides 2171 to 3021 of *Drosophila E(spl)*.

```
   4 gtgccgtgaccatcagcaaccccagcaggcacgtctacacaggtggcaag 53
2171 GTGCGGTAACCATCTCTAATCCCACAAAGTACGTGTACACGGGCGGCAAG 2220

  54 ggctgcgtgaagatctgggacatcagccagccaggcagcaagagccccat 103
2221 GGCTGCGTCAAGGTATGGGACATCTCGCAACCGGGCAACAAGAATCCAGT 2270

 104 ctcccagctggactgcctgaacagggacaattacatgcgctcctgcaagc 153
2271 TAGCCAGCTGGATTGTCTGCAGCGCGACAACTACATCCGCTCGGTAAAGC 2320

 154 tgcaccctgatgggcgcacgctcatcgtgggcggcgagggcagcacgctc 203
2321 TGCTGCCCGACGGCCGTACGCTGATCGTGGGCGGTGAGGCGTCCAACCTG 2370

 204 accatctgggacctggcctcgcccacgccccgcatcaaggccgagctgac 253
2371 TCCATCTGGGATCTGGCCAGTCCGACGCCTCGCATAAAGGCGGAACTAAC 2420

 254 gtcctcggctcccgcctgttatgccctggccattagccctgacgccaaag 303
2421 ATCGGCGGCGCCCGCCTGCTACGCTCTGGCCATTAGCCCTGACTCGAAGG 2470

 304 tctgcttctcctgctgcagcgatgggaacattgctgtctgggacctgcac 353
2471 TGTGCTTCTCGTGCTGCAGCGACGGCAATATCGCTGTGTGGGACCTGCAC 2520

 354 aaccagaccctggtcaggcagttccagggccacacagatggggccagctg 403
2521 AACGAGATCCTGGTGCGCCAGTTCCAGGGCCATACCGACGGCGCTTCATG 2570

 404 catagacatctcccatgatggcaccaaactgtggacaggggggcctggaca 453
2571 CATCGACATCAGTCCGGATGGCTCCAGGCTGTGGACGGGCGGCTTAGACA 2620

 454 acacggtgcgctcctgggacctgcgggagggccgacagctacagcagcat 503
2621 ACACGGTGCGCTCCTGGGATCTGCGCGAGGGTCGCCAGCTGCAACAGCAC 2670

 504 gacttcacttcccagatcttctcgctgggctactgccccactggggagtg 553
2671 GACTTCAGCTCTCAAATATTCTCGCTCGGCTACTGTCCCACAGGCGACTG 2720

 554 gctggctgtgggcatggagagcagcaacgtggaggtgctgcaccacacca 603
2721 GCTGGCTGTGGGTATGGAGAACTCGCATGTGGAGGTGCTGCACGCATCGA 2770

 604 agcctcacaagtaccagctgcacctgcacgagagctgcgtgctctccctc 653
2771 AACCGGACAAGTATCAACTGCATCTGCACGAGAGCTGCGTTCTGTCGCTG 2820

 654 aagttcgcctactgcggcaagtggttcgtgagcactgggaaagataacct 703
2821 CGCTTTGCCGCCTGCGGCAAATGGTTCGTTTCCACCGGCAAAGACAACCT 2870

 704 tctcaacgcctggaggacgccttatggagccagcatatcccagtctaaag 753
2871 GCTTAACGCATGGCGAACACCTTACGGTGCCAGCATATTCCAGTCGAAGG 2920

 754 aatcctcgtctgtcttgagttgtgacatttcagcggatgacaaatacatt 803
2921 AAACATCATCCGTACTTAGCTGCGACATATCAACTGACGACAAATACATT 2970

 804 gtaacaggctctggtgacaagaaggccacagtttatgaggtcatctacta 853
2971 GTGACGGGTTCGGGCGATAAGAAGGCTACTGTCTACGAAGTTATTTATTA 3020

 854 a 854
3021 A 3021
```

Figure 5.7 To show the peptide encoded by V1.1 matched to the protein product of *Drosophila E(spl)*.

```
                .         .         .         .         .
  1 AVTISNPSRHVYTGGKGCVKIWDISQPGSKSPISQLDCLNRDNYMRSCKL 50
    |||||||.:.||||||||||:|||||||.|.|:||||||.||||:||.||
438 AVTISNPTKYVYTGGKGCVKVWDISQPGNKNPVSQLDCLQRDNYIRSVKL 487
                .         .         .         .         .
 51 HPDGRTLIVGGEGSTLTIWDLASPTPRIKAELTSSAPACYALAISPDAKV 100
     ||||||||||:|.|.||||||||||||||||.||||||||||||.||
488 LPDGRTLIVGGEASNLSIWDLASPTPRIKAELTSAAPACYALAISPDSKV 537
                .         .         .         .         .
101 CFSCCSDGNIAVWDLHNQTLVRQFQGHTDGASCIDISHDGTKLWTGGLDN 150
    ||||||||||||||||:.||||||||||||||||.||.:||||||||
538 CFSCCSDGNIAVWDLHNEILVRQFQGHTDGASCIDISPDGSRLWTGGLDN 587
                .         .         .         .         .
151 TVRSWDLREGRQLQQHDFTSQIFSLGYCPTGEWLAVGMESSNVEVLHHTK 200
    ||||||||||||||||.||||||||||||:|||||||.|:||||| .|
588 TVRSWDLREGRQLQQHDFSSQIFSLGYCPTGDWLAVGMENSHVEVLHASK 637
                .         .         .         .         .
201 PHKYQLHLHESCVLSLKFAYCGKWFVSTGKDNLLNAWRTPYGASISQSKE 250
    |.||||||||||||||:|| |||||||||||||||||||||||| ||||
638 PDKYQLHLHESCVLSLRFAACGKWFVSTGKDNLLNAWRTPYGASIFQSKE 687
                .         .         .
251 SSSVLSCDISADDKYIVTGSGDKKATVYEVIY 282
    .|||||||||.||||||||||||||||||||
688 TSSVLSCDISTDDKYIVTGSGDKKATVYEVIY 719
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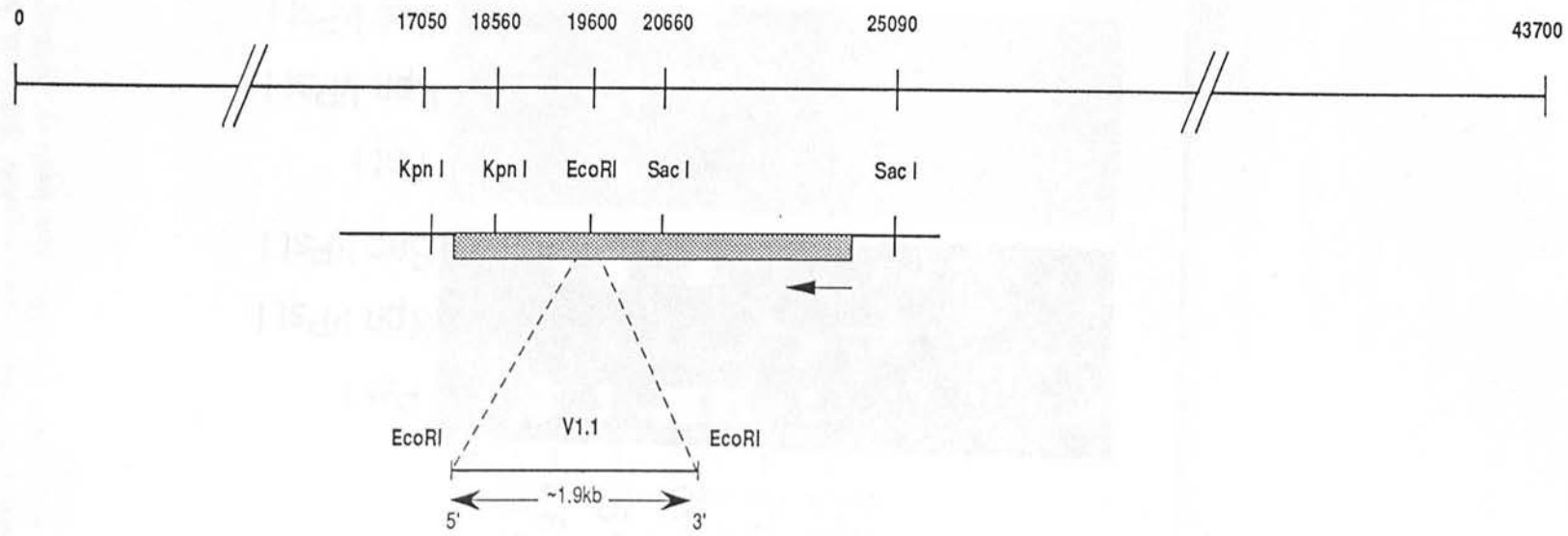
5.2.3 The Mapping of Clone V1.1 Within the λ Bacteriophage Vector

The "MAPSort" programme of the UWGCG software at Daresbury analyses any given sequence for restriction enzyme sites. Informative sites were identified for V1.1 in this manner in order to map the orientation of the clone within the λgt11 expression vector. V1.1 has a PstI site 317bp from the predicted 5' Eco RI site. λV1.1 was cut with PstI alone and with PstI and enzymes flanking the Eco RI cloning site within the bacteriphage vector; in this case KpnI and SacI. (These enzymes have no restriction sites within V1.1.) **Figure 5.8** shows a map of the λgt11 expression vector, indicating the restriction sites of the flanking enzymes chosen and the position of the *lac Z* gene. The restriction digested λV1.1 was analysed by Southern blotting.

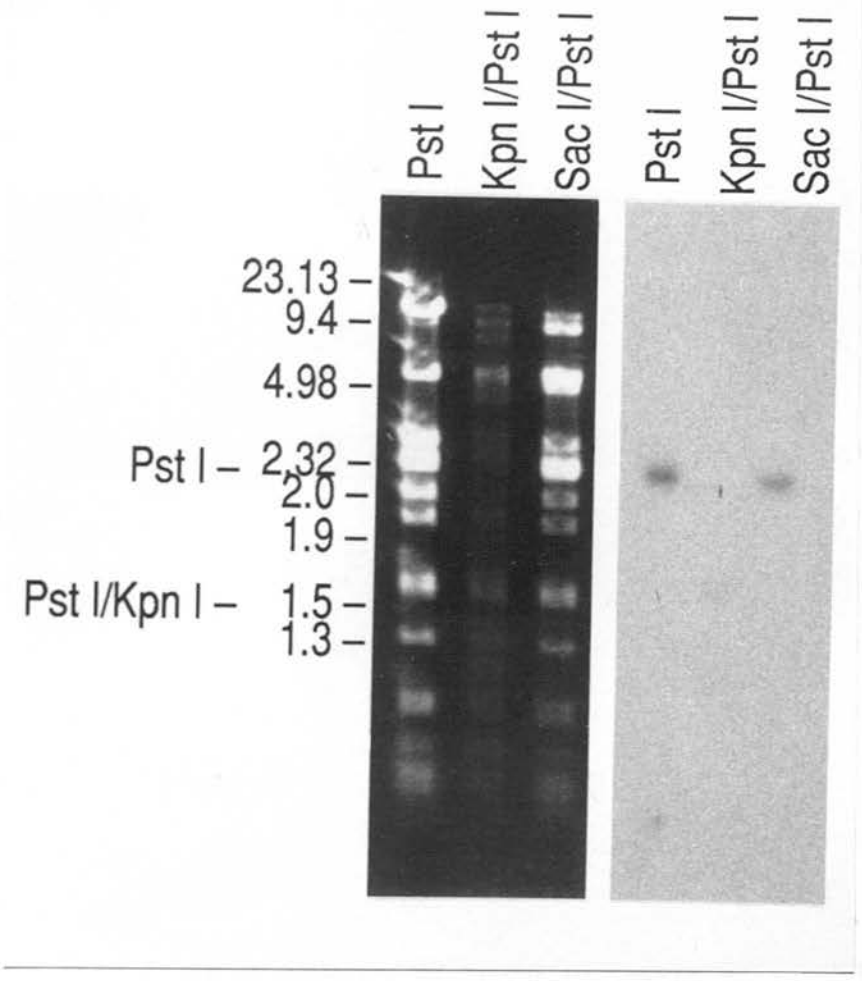
The oligo primer #554 (used to complete the sequencing of DNA within the reading frame of the clone) begins 129 bases from the 5' end of V1.1 and was used to probe for restriction fragments at the 5' side of the PstI site. The Eco RI cloning site is 1040bp from the flanking KpnI site and 1060bp from SacI,and the PstI site is 317bp from the Eco RI site at the 5' end of the clone. If the KpnI restriction site is 5' to the PstI site, a double digest of λV1.1 with PstI and KpnI will yield a fragment of 1357bp, which will hybridise with the labelled oligomer, #554. However, if the SacI site is 5' to the PstI site, a double digest with PstI and SacI will produce a fragment of 1397bp which will also hybridise with #554. **Figure 5.9** shows the results of this experiment.

Figure 5.8 Map of the λgt11 expression vector, indicating some restriction sites, the position of the *lac Z* gene and the insert V1.1.



indicates Lac Z gene
indicates direction of transcription

Figure 5.9 Autoradiograph of λV1.1 digested with PstI, PstI and KpnI, and PstI and SacI, probed with the oligo-primer #554 is shown in the right hand panel. The ethidium stained gel before Southern blotting is shown in the left hand panel.



V1.1 digested with PstI alone shows a band of ~2.3kb which hybridises with labelled #554. The length of this fragment is the distance from the internal PstI site to the nearest external PstI site at the 5' end of the clone. In the KpnI/PstI digest a band of ~1.5kb is clearly visible. This is approximately the predicted distance for KpnI to be at the 5' end of the clone. The larger band of ~2.2kb in the SacI/PstI digested DNA is not consistent with the SacI site being 5' of the PstI site. It is due to the fragment produced by the internal PstI site and a SacI site beyond KpnI at the 5' end of the clone.

The λgt11 expression vector produces protein encoded by the inserted piece of DNA as a fusion with β-gal. The above results show that the 5' end of the human clone is not in the correct orientation to produce a fusion protein. This means that the protein produced by the λ vector is a portion of β-gal fused to a peptide encoded by the complement of the 3' end of the clone and not the human protein equivalent to the *Drosophila E(spl)* m9 gene.

The potential protein encoded by the complement of the 3' untranslated end of V1.1 has an open reading frame of only 93 bases before a stop codon is reached as shown in **Figure 5.10**. Therefore, the induced λgt11 vector should produce a portion of the β-gal protein fused to 31 amino acids encoded by the complement of the 3' end of V1.1. There are four possible reasons for the apparent recognition of this clone by serum 14A. First, this short peptide alone may be related to the real epitope of the antibody. Second, the peptide

Figure 5.10 The sequence of forward primed V1.1 and the translated peptide, Protein X.

```
  1   ggg|ggt|tat|c ag|tcc|gga|tg c|cca|gac|ccg| tgt|gga|ggc|a ca|cgc|att|cc
 51   a|gca|ggc|acc| tta|cgg|cca|a cc|tgg|ttg|cc a|ttt|cca|cac| tct|taa|gact
101   tttctcctct ttcttcaatt ggttaccctc cctgtctcct cttcccaggt
151   gagagcatct gtttatttaa aaaaaaaaaa aaaaaaagcc accacgagtg
201   attacaaata cagcaggcga aatacacact cagagtttcc ggtgggaagg
251   ggcctagc

  1   GGYQSGCPDP CGGTRIPAGT LRPTWLPFPH S*
```

coupled with β-gal could have created a false epitope causing the serum antibody to bind to what appears to be its true binding site. Thirdly, the clone picked may have been a negative clone on the quarternary plate which had not been purified out in the previous three rounds of screening. Lastly, it may be that a weak bacterial promoter (or some sequence of DNA behaving as such) present as part of the inserted piece of DNA, was transcribing the human *E(spl)* protein in frame and that the antibody was recognising that protein.

In order to distinguish some of these possibilities, the *Drosophila* antibody, IC2, was applied to Western blots of total nuclear extract, to see if it recognised the 38kDa protein identified by sera #14(R) and 14A. It did not. It was also applied to whole cells, and the staining pattern compared to that of 14A. 14A gave bright granular staining of PARIT cell nuclei prepared in KCM, with a brighter, denser level of staining around the periphery of the nuclei. PARIT nuclei prepared in PBS showed coarser granular staining. These results are shown in **Figure 5.11**. IC2, when applied to PARIT cells prepared in KCM, gave bright granular staining of the nuclei with a very dense bright level of staining of the periphery. It also showed granular staining of PARIT cell nuclei prepared in PBS. In both cases, IC2 showed additional cytoplasmic staining. These results are shown in **Figure 5.12**. Staining patterns given by the normal serum, JL, are shown in Chapter 3. The nuclear staining patterns of 14A and IC2 are strikingly similar. The antibodies were also applied to mouse cells, to see if the staining pattern observed with IC2 was due to a

Figure 5.11 Whole PARIT cells stained with serum 14A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with 14A and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with 14A and counterstained with DAPI to localise the nucleus.

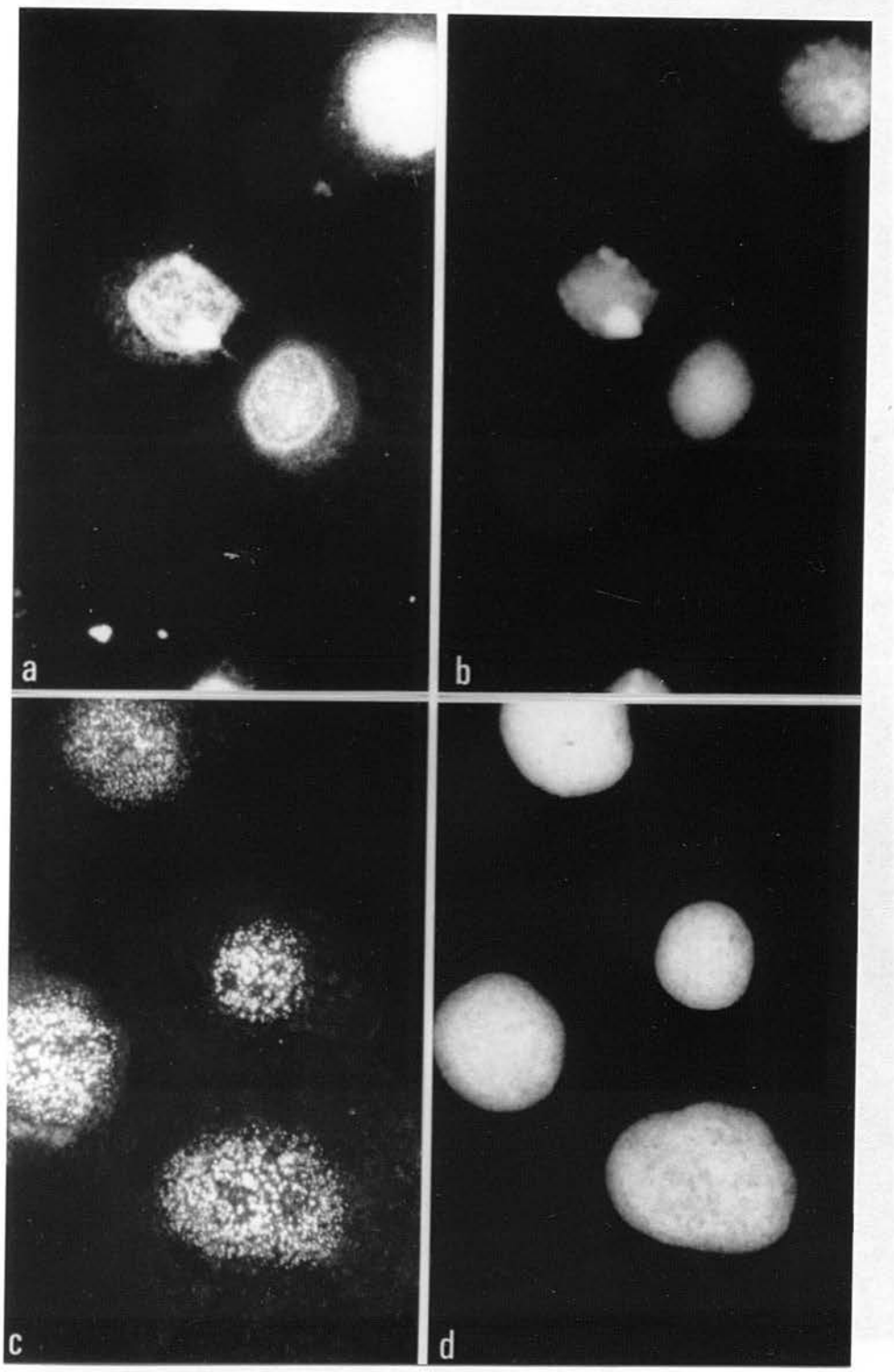
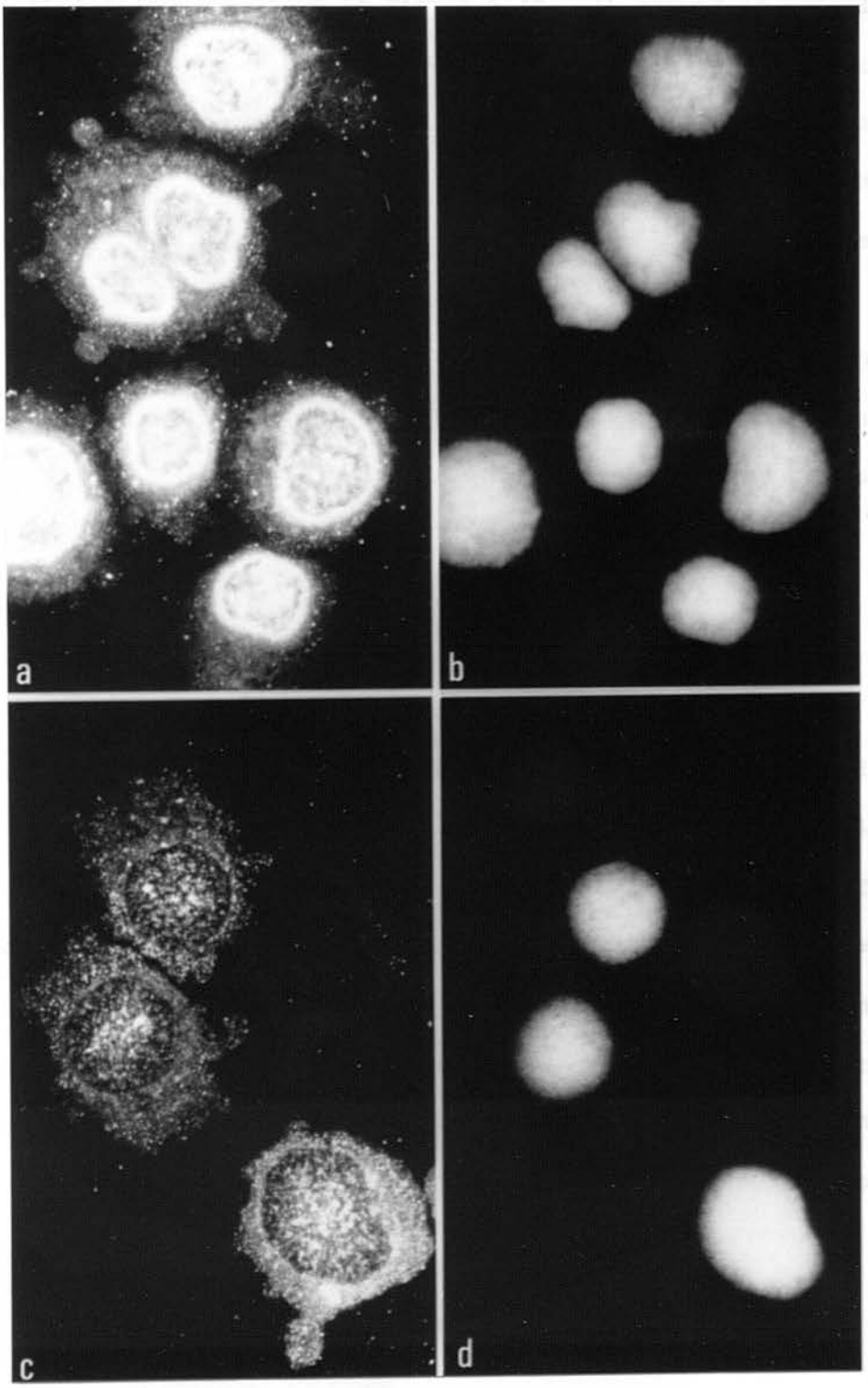


Figure 5.12 Whole PARIT cells stained with IC2, as the first antibody and fluorescein conjugated rabbit anti-mouse IgG as the second antibody.
(a) and (b) show cells prepared in KCM stained with IC2 and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with IC2 and counterstained with DAPI to localise the nucleus.



conserved antigen(s). 14A showed coarse granular staining of the L cells when prepared in KCM and PBS, with a little cytoplasmic staining of those in PBS, as shown in **Figure 5.13.** IC2 showed a uniform level of staining throughout the nuclei with brighter patches in both types of preparation, as shown in **Figure 5.14.** It showed no cytoplasmic staining in either preparation. The two antibodies show quite similar staining in mouse cells also, although not the same as the patterns observed in PARIT cells. It seemed possible, therefore by this assay, that the autoimmune serum 14A was recognising the human and mouse *E(spl)* homologue.

In order to determine what protein product the serum antibody was recognising in the screen, fusion proteins of human *E(spl)*, in and out of frame, and of the 31 amino acids from the 3' end of V1.1 and referred to as Protein X, were produced.

5.2.4 Fusion Proteins of V1.1

Fusion proteins can be produced using bacteriophage in a lysogenic growth phase. *E. coli* Y1089 cells were infected with the bacteriophage λV1.1, and cultured, with the bacteriophage multiplying within their hosts as an integral part of the host genome. The bacteriophage was then induced to begin transcribing the β-gal fusion protein. After a suitable time interval the cells were lysed and the liberated proteins isolated. The induced product should be clearly visible on SDS-PAGE, at a molecular weight a little higher than β-gal (as the inserted piece of DNA produces a fusion protein). However this

Figure 5.13 Whole mouse L cells stained with serum 14A, as the first antibody and fluorescein conjugated rabbit anti-human IgG γ as the second antibody.
(a) and (b) show cells prepared in KCM stained with 14A and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with 14A and counterstained with DAPI to localise the nucleus.

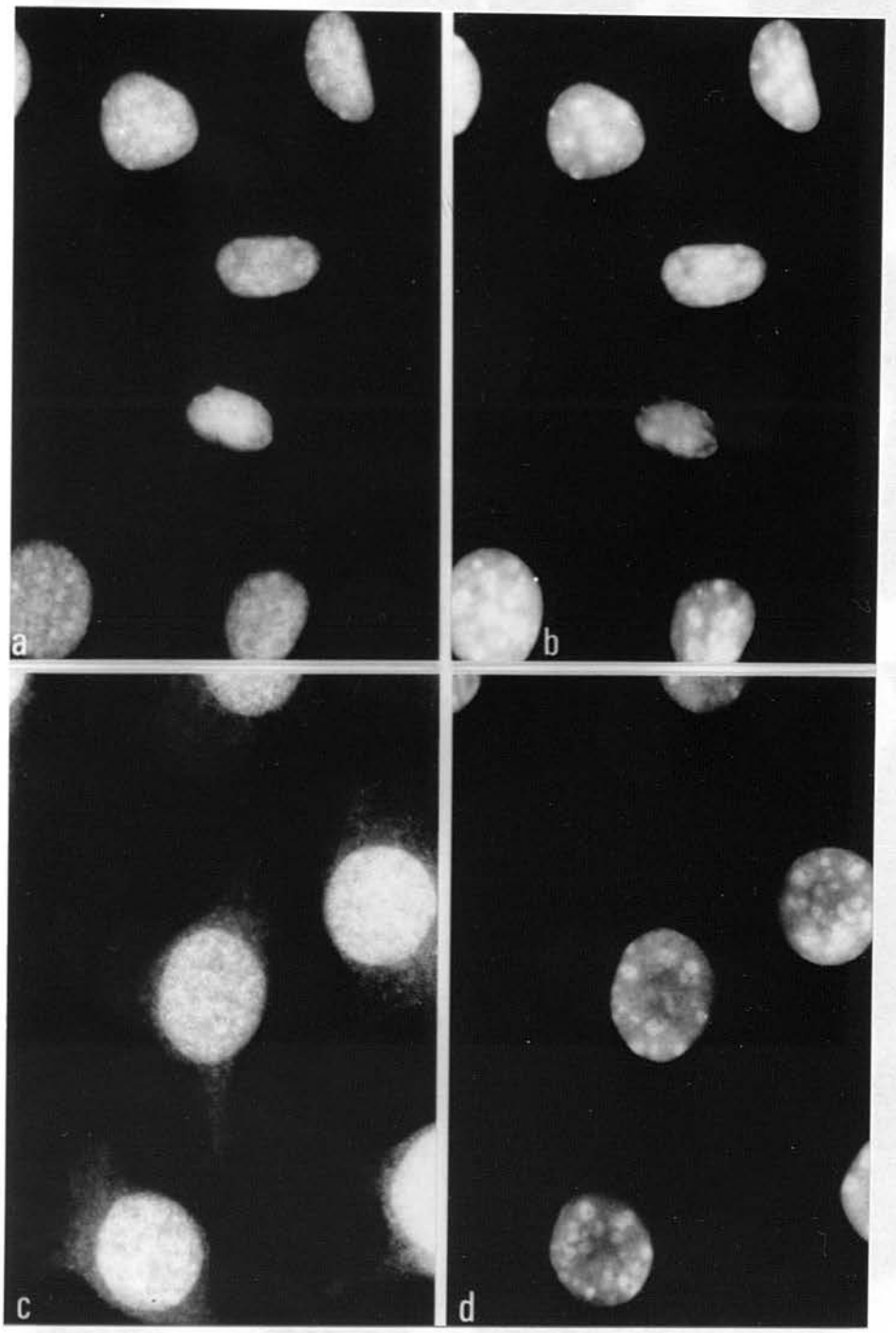
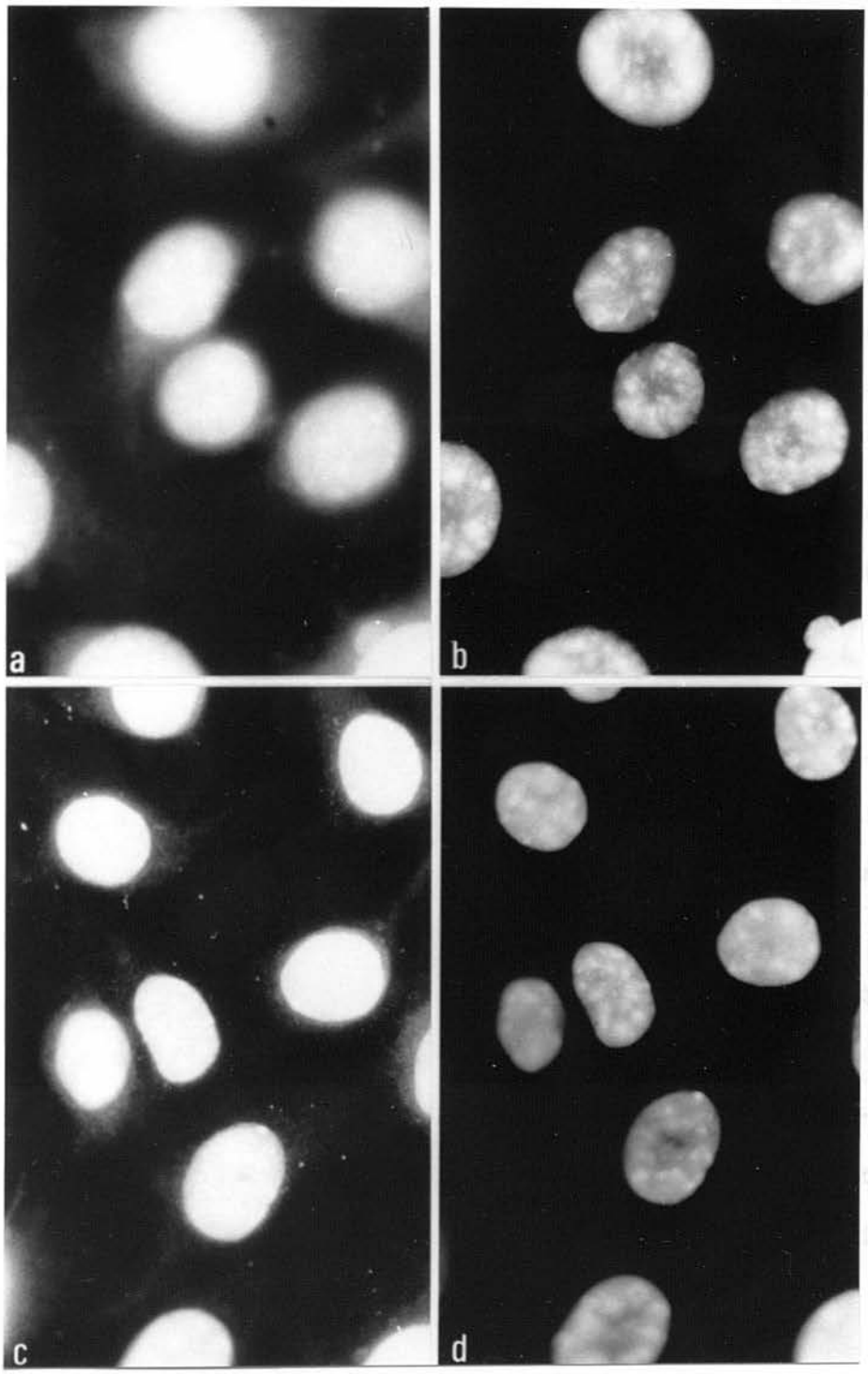


Figure 5.14 Whole mouse L cells stained with IC2, as the first antibody and fluorescein conjugated rabbit anti-mouse IgG as the second antibody.
(a) and (b) show cells prepared in KCM stained with IC2 and counterstained with DAPI to localise the nucleus,
(c) and (d) show cells prepared in PBS stained with IC2 and counterstained with DAPI to localise the nucleus.



was not the case: there was no new protein band visible at the correct molecular weight, after induction. There was also no non-fusion β-gal product visible in the vector-only control extract. There are three possible explanations for these results. Firstly, the lysogenic bacteriophage may have been failing to produce any fusion protein at all upon induction with IPTG. Secondly, the fusion protein may have been produced but subsequently degraded by the host cell, before the cells were lysed and protein extracts run on the gel, or lastly, as there is only one copy of the gene within each host cell, the protein may have been at too low a level to detect. Fusion proteins were produced upon induction when cultured in Y1090 cells, in order for the library to have been screened, but the lytic growth cycle allows amplification of the bacteriophage, and therefore the number of copies of the *Lac Z* gene, and the quantity of protein required was very small. Although there appeared to be no fusion proteins present, I Western blotted the gels and probed them with the antibody 14A. There was no reaction of the antibody with protein bands of the correct molecular weight, just non-specific staining due to second antibody activity. I made several attempts to produce fusion proteins by this method without success, and eventually used a different strategy.

The insert, V1.1, was cloned into another fusion protein production vector, pGEX-3X, by R. Hill. pGEX-3X was used to produce the human *E(spl)* in frame, as a fusion with glutathione S-transferase, by cloning the V1.1 insert into a Bam HI/SmaI site. The fusion protein construct was transformed into *E. coli* strain BL21 DE3 which is *lon*$^-$

and is deficient of some cellular proteases. D. Hartley kindly provided a gift of a *D. melanogaster* fusion protein construct which contains the ~1.2kb Eco RI fragment in pGEX-1N. This vector will produce the C-terminal half of the *Drosophila* protein in frame fused to glutathione S-transferase in the host cell XL-1 blue. The fusion proteins were produced by induction of the vectors with IPTG, denatured and size separated by SDS-PAGE. The gels were then Western blotted onto nitrocellulose and the filters probed with the serum 14A, plus a polyclonal mouse serum, IC2, (precipitated with ammonium sulphate) to the *Drosophila* fusion protein.

Figure 5.15 shows the protein produced by the induction of the fusion vectors in *E. coli* strains BL21 DE3 and XL-1 blue, run on SDS-PAGE and stained with Coomassie Blue. **Figure 5.16** shows the results of the antibody probing of these protein extracts.

Panel A shows the Western blot probed with the *Drosophila* antibody IC2. IC2 stains a band at ~20kDa in the extract of pGEX vector only after induction for one hour. This is glutathione S-transferase alone. Bands at ~55 kDa in the extracts of fusions of in frame human *Enhancer of split* and of *Drosophila*, after one and two hours of induction, are also stained with IC2. As IC2 is a polyclonal mouse serum raised against the fusion protein in pGEX, the antibodies present are against *E(spl)* and glutathione S-transferase fusion protein. This explains why binding to the glutathione S-transferase alone is observed. However, the staining of the ~55kDa proteins in the induced fusion protein vector lanes may also be due to anti-glutathione

Figure 5.15 Total protein extracts of fusion vectors pGEX-1N and 3X in *E.coli* strains BL21 DE3 and XL-1 blue, before and after induction with IPTG. Lane 1 shows the total protein extract of vector, pGEX-3X, before induction, lane 2 shows it after induction. Lane 3 shows total protein extract of *Em9* plus the fusion protein vector, pGEX-3X, before induction, lanes 4 and 5 show total protein extracts after 1 and 2 hours of induction. Lane 6 shows total protein extracts of *D. melanogaster* NB5 plus the fusion protein vector pGEX-1N, before induction, lanes 7 and 8 show total protein extracts after 1 and 2 hours of induction.

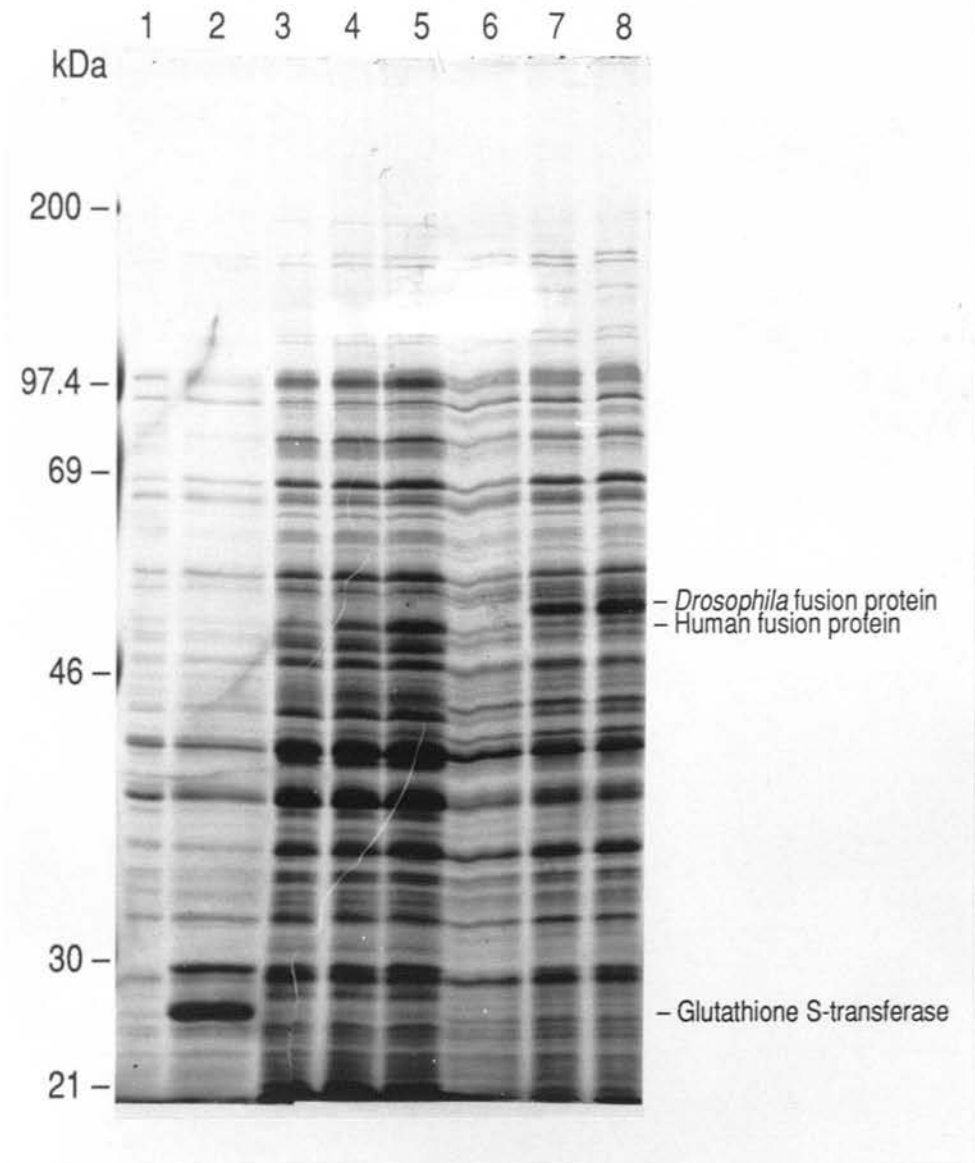
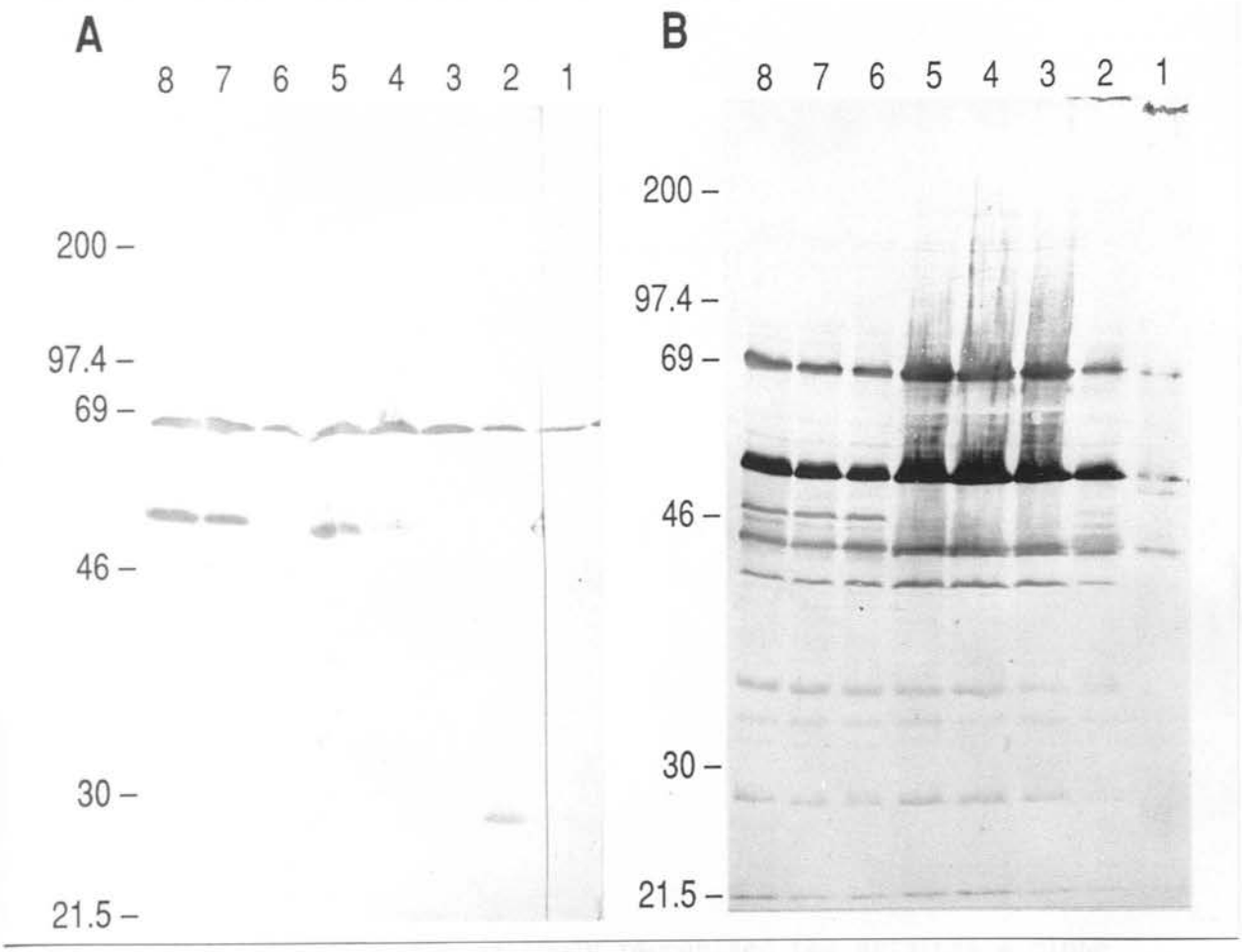


Figure 5.16 Western blots of duplicate gel to the one shown in **Figure 5.15**, probed with the first antibodies IC2 and 14A. IC2 is localised with peroxidase conjugated rabbit anti-mouse IgG and 14A is localised with peroxidase conjugated rabbit anti-human IgG γ.
Panel A shows the Western blot probed with IC2. Lane 1 shows the total protein extract of vector, pGEX-3X, before induction, lane 2 shows it after induction. Lane 3 shows total protein extract of V1.1 in the fusion protein vector, pGEX-3X, before induction, lanes 4 and 5 show total protein extracts after 1 and 2 hours of induction. Lane 6 shows total protein extract of *D. melanogaster* NB5 in the fusion protein vector, pGEX-1N, before induction, lanes 7 and 8 show total protein extracts after 1 and 2 hours of induction.
Panel B shows an identical Western blot probed with 14A. The lanes are loaded as above.
Molecular weight markers are shown in kDa.



S-transferase activity, and not to antibody specificity for the fusion proteins encoded by V1.1 and the Eco RI fragment of the *Drosophila* clone. However the binding of IC2 does prove that the peptides recognised are indeed fusion proteins, and therefore the induction was successful.

Panel B shows the staining pattern observed with 14A as the first antibody. There is no binding to any of the induced products at all, just background staining probably due to second antibody activity or non-specific binding due to the effects of applying a human serum onto extracts of *E. coli* cells. Therefore, it can be concluded that serum 14A does not bind to the product of the open reading frame of the sequence of human *E(spl)* or the *Drosophila* fusion protein.

Human *E(spl)* out of frame and the 31 amino acid peptide from the 3' end (further referred to as Protein X) were cloned in pGEMEX-1 and produced as fusions with 260 amino acids of the T7 gene 10 leader peptide. However, no induction could be seen on the stained polyacrylamide gel when the extracts were run out and size separated. This may be due to problems with the vector, as the induction conditions were known to be successful, as pGEX-3X and 1N proved. Without the fusion proteins of the 31 amino acids of Protein X or the out of frame human *E(spl)*, the possibility cannot be excluded that either of these fused with β-gal in the λ vector is the true antigen. It is unlikely that either are of biological significance and leaves the possibilities that the antibody recognised the original λ clone artefactually, or that V1.1 was a spurious clone on the quarternary

plate which had not been purified out in the rounds of screening. However, the human *Enhancer of split* gene has been isolated. The 38kDa protein recognised by sera #14(R) and 14A on Western blots is still to be identified and cloned.

As this clone, V1.1, is part of a previously unidentified gene in humans, it was necessary to determine that the gene was present within the normal human genome. This was done by cross hybridising V1.1 to digested human genomic DNA. **Figure 5.17** shows the results of this experiment. Human kidney DNA was digested with Eco RI, Bam HI and Hind III, and all three digests show bands of hybridisation with V1.1 showing that this gene is a component of the normal human genome.

Thus it would appear that 14A has fortuitously allowed the isolation of a human gene, homologous to the *Drosophila E(spl)* gene, which had not been previously identified. The gene will now be referred to as *Em9*. However, further studies in human are difficult, therefore mouse clones of *Em9* were identified.

5.2.5 The Identification of the Mouse Equivalent of *Em9*

A mouse 8.5 day embryonic λgt10 cDNA library was screened with the human clone V1.1 by Robert Hill and Christine Sime. Several positive clones were identified and these were prepared on a large scale in the λ vector and the inserts were subcloned into the Eco RI site of the phagemid vector, Bluescript. Three of these clones were thought to represent the entire gene; Esp4.1, Esp3.1 and Esp2.3. **Figure 5.18** shows the positions of these clones within the *Drosophila* gene.

Figure 5.17 The right hand panel shows the autoradiograph of human genomic kidney DNA digested with Eco RI, Bam HI and Hind III, hybridised with V1.1. The left hand panel shows the ethidium bromide stained gel before Southern blotting.

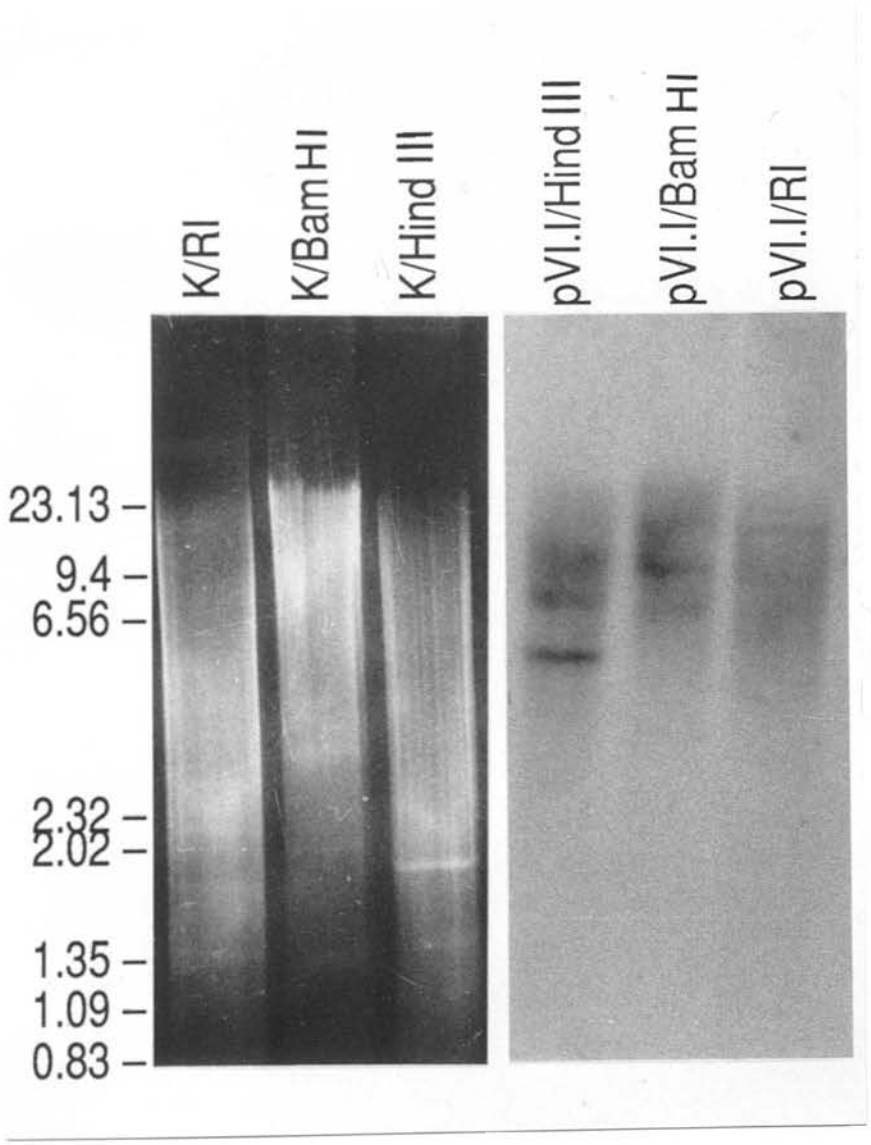


Figure 5.18 To show the positions of mouse clones Esp4.1, Esp3.1 and Esp2.3 within the *Drosophila E(spl)* gene.

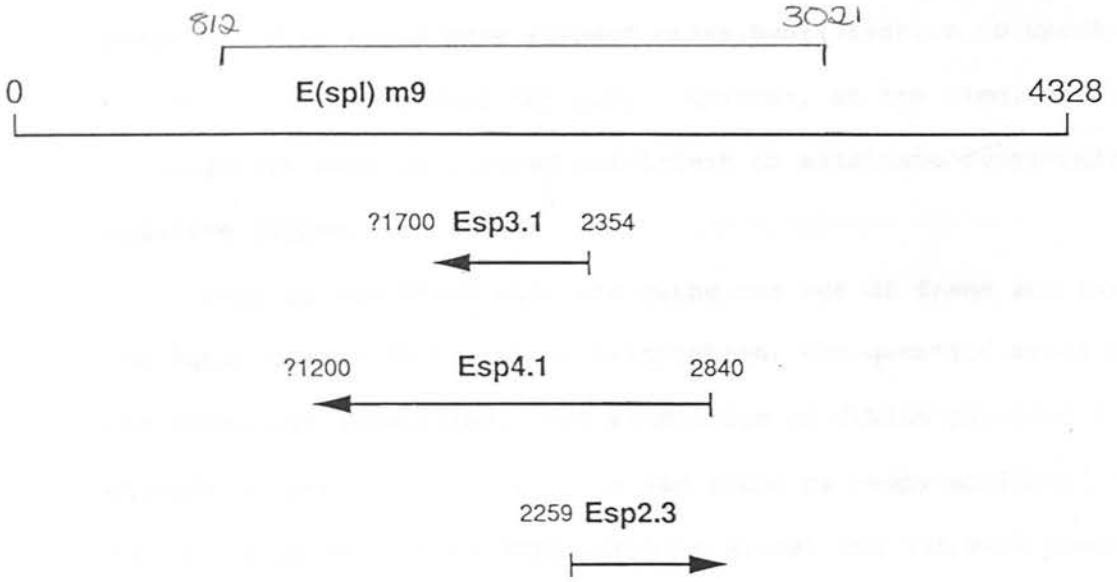
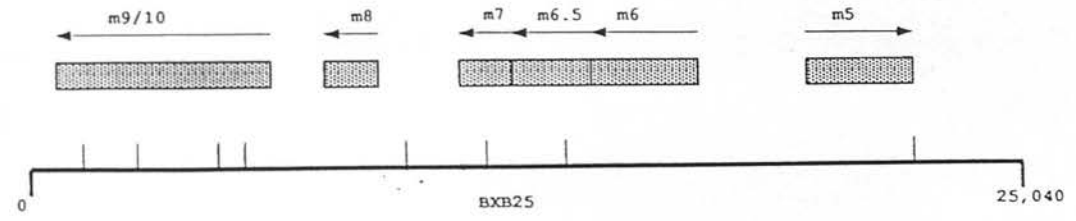


Figure 5.19 A diagrammatic representation of the *Drosophila melanogaster* construct, BXB25. The Eco RI sites are indicated by the vertical lines, whilst the essential transcripts of the *E(spl)* complex are indicated by the shaded boxes labelled m9/10, m8, m7, m6.6, m6 and m5.



5.3 Discussion

V1.1 was purified from an adult human testis cDNA expression library. In retrospect, as previously mentioned, it would have been useful to have picked more than one positive clone from the final plate. This would have allowed cross hybridisation to check that all clones on the plate were the same. However, at the time, four rounds of selection were considered sufficient to eliminate contaminating negative clones.

When it was shown that the clone was out of frame and present in the λgt11 vector in the wrong orientation, the question arose as to how the clone was identified. The production of fusion proteins allowed clarification; whether the clone was found by happy accident, or that the serum antibodies of MCTD patients #14(R) and 14A were specific for the putative product of the partial *Em9* clone. 14A did not bind to *E(spl)* in frame, showing that a weak bacterial promoter transcribing the gene was not present. The possibilities remaining are those of there being a false epitope created by the fusion of Protein X to β-gal or that Protein X alone is an antigen. The remaining possibility is that V1.1 was picked by mistake. This means that the clone is unrelated to the 38kDa antigen and explains why the *Drosophila* antibody did not bind to the 38kDa antigen on Western blots of PARIT nuclear extracts. Problems of repeating the positive signal from the purified clone infected into Y1090 cells, which were dismissed as trivial

problems with library screening (experienced by others in the Unit at that time), are also explained by this hypothesis.

The results of cross hybridisation of the *Drosophila* clone, NB5, and the construct, BXB25, to V1.1, and the sequence comparisons prove that V1.1 is indeed a partial clone of the human homologue to the *E(spl)* gene. I attempted to isolate further cDNA clones of *Em9* from the human testis library, plus an adult human liver library and an adult lung library, using V1.1 as a probe. However after screening another 3 million or so plaques from each library, none hybridised with V1.1. The absence of clones in the other two libraries is perhaps an indication that the *Em9* gene is not expressed in these tissues. Unfortunately a full length cDNA of *Em9* has yet to be isolated. Possibly it would be easier to find in a human foetal library, if the gene is expressed developmentally in humans as it is in *Drosophila*.

The hybridisation of V1.1 to human genomic kidney DNA shows that the gene is present in the human genome. In order to determine an expression pattern in both adult and foetal tissues, I ran several Northern blots with RNA given to me by Paula Monaghan and Kathy Pritchard-Jones, and probed them with V1.1. There was no hybridisation at any detectable level with adult or foetal samples. In adult tissues, the gene may not be expressed at all so this result was expected, but no expression in foetal material was surprising. However it may be that the expression of this gene occurs at a stage when human foetal samples are not available, or that *Em9* is expressed within a short time span, or/and at a low level, so it is difficult to detect.

In situ hybridisation is a much more sensitive technique and foetal expression may be detected by this method. As human samples are extremely difficult to come by, and mouse clones of the gene have been identified, this approach was tried using mouse samples and will be discussed in Appendix I.

The mouse antibody, IC2, to a fusion protein of the 3' half of the *Drosophila* gene gave very similar staining patterns to that of 14A. As it is now known that V1.1 is in no way related to 14A, the similar staining must be viewed with extreme caution. As discussed in Chapter 3, indirect immunofluorescence gives many ill defined staining patterns, which are difficult to correlate with particular antigens unless these are to distinct regions such as kinetochore recognition by #7(ML). #14(R) and 14A show staining that probably relates to the 38kDa antigen and to U1-RNP also. The fact that staining of 14A appears similar to the staining of IC2 is most likely to be an artefact. The apparent staining of the nuclear membrane by 14A in cells prepared in KCM may be the effect of soluble antigens leaching out of the cell being caught on the membrane, thus giving a false antigenic localisation. This staining pattern is not observed in the staining with 14A of cells prepared in PBS. However cells prepared in both types of buffer show staining at the nuclear membrane with IC2. Is this the true cellular position of the *Em9* protein? IC2 stains the nuclei of embyronic *Drosophila* cells (D. Hartley, pers. comm.). RNA from adult tissues probed with V1.1 has not shown expression of *Em9*. In order to be confident that this staining pattern was real, RNA from

PARIT cells would have to be isolated and probed with V1.1 to check for the expression of *Em9*. Without this additional information, the staining pattern cannot be taken as a true positive result.

V1.1 was used as a probe to search for other mammalian gene equivalents. An 8.5 day mouse embryonic cDNA library contained several mouse clones which were subsequently isolated. As the gene is developmentally expressed in *Drosophila*, the presence of these clones within a library made from developing mouse is not surprising. The absence of clones in adult libraries indicates that the mouse homologue of *E(spl)*, may well be performing a developmental role. The relative abundance of clones compared with the single clone found in the adult human library indicates that the gene is being expressed at a much higher level in the mouse embryo. If the mouse gene is developmental in function, it should follow, therefore, that the human gene is also performing a developmental role. However the human library that contained V1.1 was a normal adult cDNA expression library, made from RNA derived from a testis biopsy. Therefore the gene must have been transcribed in the testis for the RNA to have been present, but the tissue was essentially adult and not from a developing embryo as with the mouse library. However the testis is not a typical somatic tissue, as the process of spermatogenesis takes place within it. It is documented that normally inactive genes may be transcribed at low levels in the testis due to spermatogenesis (Willison and Ashworth, 1987), which may account for the presence of a developmentally expressed gene in an adult cDNA library.

The discovery of a mouse equivalent opens many avenues for the further study of this gene, which would be much more difficult to perform in the human. These are discussed in Chapter 6.

CHAPTER SIX

GENERAL DISCUSSION

6 GENERAL DISCUSSION

6.1 Summary of Results

The use of autoimmune patients' sera containing anti-nuclear antibodies as probes for nuclear substructure has been well documented and is discussed in the Introduction. The aim of this project was to use autoimmune sera to aid the identification of novel nuclear antigens and, if possible, to characterise them further.

Two techniques were used for the initial analysis of patient sera provided. These were immunocytochemistry and Western blotting, as discussed in Chapter 3. Immunocytochemistry alone allowed the identification of a patient with CREST syndrome, a subclass of scleroderma (progressive systemic sclerosis). It has been shown that a high proportion of CREST patients possess serum autoantibodies which recognise the centromeres of metaphase chromosomes (Spowart *et al.*, 1985; Moroi *et al.*, 1980). This gives rise to a very distinctive immunofluorescence pattern. which was detected using this patient's serum. The immunocytochemistry was confirmed by Western blotting, which allowed the reacting antigen to be identified as the 19kDa polypeptide which has been called CENP-A (Earnshaw *et al.*, 1986). The patient in question had not been previously classified as CREST, and therefore the detection of anti-centromere antibodies in her serum was of help to the clinicians in managing her condition.

Two SLE patients were similarly identified by their sera. The autoantibodies showed characteristic intense staining of the whole nucleus and of metaphase chromosomes by indirect immunofluorescence, indicating an affinity for DNA and/or histone, as previously described (Tan, 1982). When the sera were applied to proteins which had been size separated and Western blotted, #23(BB) stained histone H1 very clearly, whilst #24(NF) did not appear to recognise a protein antigen at all. It can therefore be concluded that serum #23(BB) had autoantibodies which stained histone H1 and possibly DNA also. Serum #24(NF) probably contains autoantibodies which bind to only DNA.

The staining patterns produced by autoantibodies in the sera of patients with SLE and CREST are very distinctive, whereas the patterns produced by other types of autoantibody related to other diseases tend to be less well defined. Serum #13(H), from a scleroderma patient, was shown to stain nucleoli of whole cells with background granular staining and appeared to be localised to the chromosomes in metaphase spreads. The Western blots with this serum showed autoantibodies present bound to an antigen of 95kDa. The molecular weight of the antigen did not appear to correspond that of any known antigen, so the antigen did appear to be novel. However, with hindsight, this assumption was incorrect. Serum #14(R) showed granular staining of whole nuclei also. On Western blots the serum antibodies bound to two bands, one of 70kDa and the other of 38kDa. The 70kDa band corresponded to the size documented for one of the antigenic proteins associated with snRNP (Mimori *et al.*, 1984; Habets *et al.*, 1985). When

the patient from whom the serum sample had been taken was revealed to be suffering from MCTD, this correlation with nRNP was further strengthened, as U1 RNP is frequently associated with this condition (Mattioli and Reichlin, 1973). The band of 38kDa did not appear to correspond with any known nuclear antigen.

It therefore seemed that the combination of these two techniques had allowed the identification of two novel nuclear antigens, one of 95kDa and the other of 38kDa, which was the initial aim of the project. The next goal was to further characterise and hopefully identify these antigens. In order to facilitate this, further serum samples from more patients suffering from the same diseases were obtained to see if the antigens were disease related. The 95kDa antigen was found to be recognised by autoantibodies from one more serum sample, while the 38kDa antigen was identified by two further samples. In both cases a serum with autoantibodies at a greater titre or with higher affinity for the antigen in question was discovered. These sera appeared to show the same staining patterns by immunocytochemistry and it was these sera, called 13A and 14A, which were used in the next stages of the project, the screening of a λgt11 expression library.

A human testis expression library was used for the purposes of this project as I was not looking for tissue specific genes, but ubiquitously expressed genes coding for nuclear proteins possibly of a structural nature. 13A was used to screen the library first, and three positive clones were isolated. These were found to cross hybridise with each other and upon sequencing, they were found to encode human

DNA topoisomerase I. This is not a novel protein, and is in fact the diagnostic antigen (Scl-70) for scleroderma, the disease suffered by the patients from whom the serum samples were taken. The identity of the antigen had not been realised earlier, despite the staining of the 95kDa band on Western blots and the pattern of staining shown in whole cells and metaphase spreads which, with hindsight, show characteristic staining for Scl-70. However, the recloning of this gene proved quite conclusively that this approach for the isolation and identification of novel nuclear antigens is a feasible one and, in theory should allow the characterisation of truly novel proteins.

Towards this end, the same library was screened with serum 14A, but this time only one positive clone was identified after some 3 million plaques had been plated. This clone, V1.1, was sequenced also and was found to show homology to the m9 transcript of a *Drosophila melanogaster* multi-gene complex called *Enhancer of split (E(spl))*. This gene is involved in the development of the nervous system of the fly as briefly discussed in Chapter 5. The question then arose as to how human autoimmune disease was related to a gene showing homology with a fruit fly neural development gene. It has since been shown that serum 14A does not recognise the protein encoded by V1.1 when expressed as a fusion product, and that the clone was in fact contained in the λ vector in the opposite orientation to the direction of transcription. There was a short open reading frame in the direction of transcription for 31 amino acids, but these alone are highly unlikely to be of any biological importance. In conclusion, the clone isolated from the

library is most likely to be a spurious negative clone remaining on the quarternary plate, and was picked by mistake instead of a true positive plaque. The method, having been successful in the cloning of DNA topoisomerase I, is therefore not flawless. The 38kDa protein stained by #14(R) and 14A on Western blots is yet to be identified.

However, serendipity has allowed the isolation of the human homologue of the *Drosophila E(spl)* gene. The human clone, V1.1, has been shown to cross hybridise with the *Drosophila* cDNA, NB5, and with a construct, BXB25, which rescues otherwise lethal mutations. It has also been shown to hybridise to human genomic DNA, proving that the gene is present within the human genome. The remainder of the reading frame of V1.1 has been sequenced and shows ~76.5% identity at the nucleic acid level and ~88% similarity at the amino acid level to *Drosophila E(spl)*. A full length clone of the gene could not be detected in the testis library or two other libraries screened with V1.1.

Human genes are difficult to study due to the lack of human material available and the ethical problems of experimentation upon a human system, therefore V1.1 was used as a probe to screen an 8.5 day mouse embryonic library, with the intention of isolating a mouse homologue of the gene which would be very much easier to study. Three clones were isolated. It is thought that these clones contain the full length sequence of the mouse gene within them. The degree of homology of the mouse clones, Esp4.1, Esp3.1 and Esp2.3, to the *Drosophila* gene is similar to that shown with the human sequence. The isolation of

mouse clones opens many more avenues for the further study of this gene.

6.2 Future Work

6.2.1 Autoimmunity

There is much work to be done in the field of autoimmunity generally. During the course of this project a 38kDa antigen was identified on Western blots by #14(R) and 14A. This has yet to be further characterised. The strategy I employed during the course of this project would seem to be a suitable way forward. This will hopefully allow the true identity of the antigen to be determined. Although the aim of the project was to identify novel nuclear antigens associated with autoimmune disease, the true identity of this antigen was not pursued in this particular case, as the gene isolated by accident, the previously unidentified human *Enhancer of split*, is an interesting gene in its own right.

However further antigens important both in nuclear structure and autoimmune diseases may also be detected and identified in this manner. In order to fully understand the problems which face us in the study of autoimmune disease, we require as much basic information and background knowledge about the antigens and autoantibodies involved, as possible. This method therefore, may be providing a way forward in the study of autoimmunity.

6.2.2 Mammalian *Enhancer of split*

The three mouse clones of *E(spl)* have yet to be fully sequenced. Some preliminary work indicates that the mouse gene may well be shorter than that in *Drosophila*. Forward and reverse primed sequences of all of the clones have been compared to the *Drosophila* sequence. The 5' end of Esp2.3 has been matched to a stretch of nucleotides beginning at base 2259. The 3' ends of Esp 3.1 and 4.1 have also been matched to stretches of nucleotides ending with bases 2354 and 2840 respectively. The 3' end of Esp2.3 does not show any homology to the *Drosophila* sequence, presumably because it is untranslated as was observed with the 3' end of V1.1. The 5' ends of Esp3.1 and 4.1 also do not show any homology. The 5' end of Esp4.1 does not contain an open reading frame within the few hundred bases that have been sequenced, so this may be 5' untranslated, but the forward primed sequence of Esp3.1 does contain an open reading frame. This stretch of the sequence does not show any homology to the *Drosophila* gene so it is quite probable that the level of homology at the 5' end of the gene is lost. The mouse gene is thought to be shorter as the length of the clones has been used to estimate the possible length of the gene. The *Drosophila* protein begins at nucleotide 862 and finishes at nucleotide 3021 giving a protein of some 720 amino acids. However Esp4.1 only extends to approximately base 1200, and has no open reading frame at its 5' end. This means that with ~340 fewer bases at the 5' end, the protein will be over 100 amino acids smaller. This calculation is made with the assumption that the mouse gene will end at the same point as the

Drosophila gene as seen with the human clone. (The position of the mouse clones within the *Drosophila* gene is shown in **Figure 5.18.**)

A polymorphism in the mouse gene has been identified (data not shown) and this will be used to map the gene to a particular chromosome in the mouse. There are several known neurological mutations in the mouse and it may be that this gene will map to the same area as one of these mutations.

As already mentioned, the *E(spl)* gene in *Drosophila* is in fact a multi-gene complex and we have been supplied with full length cDNAs of two of the other transcripts, m5 and m8. Work is underway at the present time in order to try and identify mouse equivalents for these two genes also. It is not known whether the localisation of the 11 or more genes making up the *E(spl)* complex in the same area of the same chromosome is fortuitous or whether there is some fundamental reason behind it. If the mouse has equivalent genes corresponding to these other transcripts and they too are localised within the same area of a particular chromosome, it will argue for the latter.

The *Drosophila* protein has been localised to the nuclei of cells within the embryo. Preliminary work done with the *Drosophila* antibody, IC2, showed staining within the nucleus and around the nuclear membrane of cells from a human lymphoblastoid cell line. However it is not known whether the cells were expressing the protein, so these results are in no way confirmed. They may well be artefactual, so further work is required in order to determine the cellular localisation of the m9 protein (and the m5 and m8 proteins if they exist in mammals).

The *Drosophila* gene might code for a protein involved in signal transduction. The outcome of the signal is that some cells from a single population are restricted to an epidermal lineage whilst others delaminate and follow a neural pathway. It is not possible to say that the gene will have the same function in mouse as in *Drosophila* merely because they are homologous, as the development of the nervous system in a fruit fly is vastly different to that of a mouse,

In the mouse the precursor of the CNS, the neural tube, is derived from the primitive ectoderm of the developing embryo (Snow, 1977) due to the delamination of cells from the epithelium to form a mesodermal layer. This process forms the primitive streak and at the anterior end of this structure a swelling known as Hensen's node forms. Primitive ectoderm cells are laid down along the midline of the mesodermal cells forming the notochord, whilst the overlying ectoderm is induced to form a neural plate which folds forming the hollow neural tube. The neural tube dissociates from the ectoderm of the embryo and chains of ectodermal cells align with it in chains. These chains are known as the neural crests and these cells are the precursors of the peripheral parts of the sensory nervous system, the dorsal root ganglia of the spinal nerves and sensory ganglia of the brain amongst other structures.

In *Drosophila*, the nervous system develops from the ventral ectoderm which is a population of cells which lie either side of cells which will form the mesoderm. These flanking cells are known as the ventral ectoderm. From this population of cells the neuroblasts

segregate and move towards the inside of the embryo. The remaining cells form the ectoderm. The control of this delamination is due to the neurogenic genes as discussed in Chapter 5.

The differences in the development of the CNS in mouse and *Drosophila* are obvious, however in both systems, a delamination process is fundamental. In the mouse it gives rise to the neural tube from which the nervous system forms, whilst in *Drosophila* it gives rise to neuroblasts which then divide to give neurons of the ventral cord. The similarity of these processes in this respect may well indicate that the two genes are performing essentially the same function in two very different systems. In fact, the degree of similarity in the two genes may well be indicating that this is the case.

Much of the work to determine the effects of *Drosophila* genes is done by complementation. The gene of interest often has mutants existing in the fly. A construct containing all or part of the gene can be introduced and the resulting phenotypes recorded. In this way, the function of the gene can be studied and the essential parts of that gene determined. This approach was used to determine the function of the m9 transcript of the *E(spl)* complex. We want to attempt the same type of experiment. We aim to build a construct which contains the mouse gene instead of the *Drosophila* one and to introduce this into *Drosophila* embryos homozygous for the mutant gene. This will enable us to determine whether the mouse protein is indeed capable of performing the same function as that of *Drosophila*.

A human fusion protein has been produced, as discussed in Chapter 5, and I am in the process of purifying enough of this protein to inject rabbits in order to produce polyclonal serum antibodies. Mice may also be injected in order to produce monoclonal antibodies.

In situ work in order to determine the pattern of expression in mouse embryos has been undertaken. 9 and 11 day embryos have been studied, but the initial impressions are that the gene is expressed more or less everywhere in the 9 day embryo with some differences in the density of staining in the 11 day embryo. More sections and more ages of embryos will have to be studied before a definite expression pattern can be determined. Some of the preliminary results are shown in Appendix I. The way forward with *Em9* depends very much on the outcome of the experiments discussed here, those underway and those not yet begun. The prospects are undeniably exciting and the work may shed light on the mysteries of mammalian development unsolved to date.

APPENDIX

APPENDIX

Introduction

Three clones of *Em9* were isolated from a λgt10 mouse 8.5 day embryonic cDNA library as previously described. The isolation of these clones is advantageous, as material is more readily available when studying mouse as opposed to human. We know that *Em9* was expressed in the 8.5 day embryo as a cDNA library is prepared from RNA isolated from the tissue of interest. *In situ* hybridisation was the method chosen to detect the pattern of this expression.

Of the three clones Esp4.1 was the longest and therefore was selected to be used to make the RNA probe required for the *in situ* technique. As the clones were detected in the mouse 8.5 day embryonic library, mice at 9 days of development were used. Younger mice may not be expressing the gene and are also difficult to handle experimentally. 11 day mice were also used to see if the expression of this gene was extended beyond 8.5/9 days, and, whether the pattern of expression was restricted by organogenesis.

Materials and Methods

In Situ Hybridisation

The method used during the course of this project was that modified by Duncan Davidson and Elizabeth Graham in this unit from Wilkinson *et al.*, 1987.

Preparation of Embryos

C57BL/6 (inbred) and Swiss (outbred) mice were used. Females of each strain were placed in a cage with a single male and were examined for the presence of a sperm plug. Positive females were separated from the males. The day following the presence of a sperm plug was considered "day zero". On designated days, females were sacrificed by cervical dislocation and the foetuses removed into ice cold PBS. The foetuses were then staged according to morphological criteria and transferred into ice cold paraformaldehyde (PFA) pH 7.2 and left to fix at 4°C o/n. PFA is a weak cross linking fixative which, while preserving the tissue, permits access of the probe.

Wax Processing of Staged Embryos

The fixed embyos wre rinsed in PBS for 30 mins and then transferred into 0.85% NaCl for 30 mins at 4°C. The embryos were dehydrated in the following steps and all washes were carried out at RT. They were washed twice for 15 mins each in 1:1 0.85% NaCl:ethanol, 15 mins in 70% ethanol, 30 mins in 70% ethanol, 30 -60 mins in 85% ethanol, 1 hr in 95% ethanol and finally for 3x 1hr in 100% ethanol. The embryos were then incubated in a clearing solution "Histoclear" for 30 mins twice and then again o/n. The following morning the embyos were incubated for 3x 60 mins at 60°C in molten wax. Glass blocks were used to embed the embryos after careful orientation. The wax was then allowed to solidify under water at 4°C. The blocks can now be stored at RT indefinitely.

Wax Sectioning of the Embedded Embryos

Histology slides were pretreated to prevent the sections from washing off during processing. They were washed in 10% HCl in 70% ethanol for 10secs and then rinsed in distilled water. They were then washed in 95% ethanol for 10secs and dried in the oven at 150°C for 5mins. Cooled slides were dipped into 2% TESPA in acetone for 10secs, then rinsed for 2x 10secs in 100% acetone, once in distilled water and baked at 42°C until dry.

During the prehybridisation of the sections, coverslips were prepared, having been previously soaked in 100% ethanol o/n. They were dipped in 100% ethanol, then silane for 20secs and air dried. They were then rinsed again in 100% ethanol, air dried and cut to size.

Ribbons of sections 6µm thick, were cut on a microtome and then groups of three were lowered onto a floating bath of sterile distilled water at 55°C to stretch and remove any creases. The sections were then carefully oriented and removed onto the pretreated slides. The sections were sealed onto slides at 60°C o/n, desiccated and stored at RT.

Pretreatment of the Sections on Slides

The sections were desiccated o/n and then treated for 2x 10mins in xylene to remove the wax. They were then rehydrated through an ethanol series for 2mins each, beginning with 100% ethanol. They were then passed through 0.85% NaCl and PBS for 5mins each. The sections were then treated in 4% PFA in PBS (pH 7.4) for 20mins to further fix the sections to the slides before washing for 2x 5mins in PBS. The slides were drained and treated with 20µg/ml

proteinase K in 50mM Tris, 5mM EDTA pH 7.2 for 7.5mins before washing for 5mins in PBS. The proteinase K treatment facilitates the access of the probe to the mRNA. The slides were again treated with 4% PFA in PBS for 5mins and then briefly washed in sterile distilled water for 10secs. The sections were then acetylated by washing for 30secs in 0.1M triethanolamine (TEA) pH 8 before incubating in TEA plus 625µl acetic anhydride for 2x 10mins each. The acetylation step blocks basic groups, which would otherwise contribute to the background by nonspecific binding of the nucleic acid probe. The sections were then washed in PBS and 0.85% NaCl for 5mins each before dehydrating through an ethanol series for 2mins each, ending with 100% ethanol and then air drying.

Radiolabelling of Probes For *In Situ* Hybridisation

The mouse clone, Esp4.1 was subcloned out of the λ vector, in which it was isolated from the library, into Bluescript. In this vector the insert was situated between opposing T7 and T3 promotors, from which it can be transcribed. The probe to be used has first to be linearised in order that a sense strand can be transcribed from one promoter and an anti-sense strand transcribed from the other. This was achieved by restriction endonuclease digestion with NotI after which the DNA was phenol/chloroform extracted, precipitated, washed and dried under vacuum before being resuspended in TE.

The labelling transcription reaction involves the addition of 6µl of labelling buffer, 1µl of each of 10mM rCTP, 10mM rATP, 10mM rGTP, 1M DTT, 3µl of water, 12µl of ^{35}S rUTP (>1mCi/100ul), 1µg of prepared DNA in 5µl of TE, 1.2µl of RNase block and 0.8µl T7/T3 polymerase. This was incubated at 37°C for 25mins. A further 0.8µl

of polymerase was added and the incubation continued for another 25mins. The labelling efficiency was checked by comparing TCA precipitated and non-precipitated samples in 10ml of Aquasol, using a scintillation counter. 2µl of carrier yeast RNA (10mg/ml) was added and the labelling mix was then incubated with 1µl of DNase I at 37°C for 10mins in order to digest the template DNA. 2µl of 0.1M EDTA and 67.5µl of TE plus 50mM DTT were then added to stop the reaction and the probe was phenol extracted twice and then phenol/chloroform extracted once before precipitation o/n at -20°C with $^{1}/_{10}$ vol 3M sodium acetate and 3vols cold ethanol. The probe was pelleted and washed twice in 80% ethanol, 50mM DTT in TE and once in 100% ethanol. It was then dried and resuspended in 100µl DEPC water. In order to reduce the size of the probe to ~150 bases, to facilitate penetration into the tissue during hybridisation, the probe was digested under mild alkaline conditions: 90µl of sodium bicarbonate and 5µl of 1M DTT were added and this was incubated at 60°C for 1hr. The probe was then precipitated with $^{1}/_{10}$ vol 6M ammonium acetate pH 5.2 and 3vols cold ethanol with 1µl yeast RNA as a carrier o/n at -20°C. The probe was pelleted and washed as above plus a further wash in 80% ethanol in TE. The pellet was dried again, dissolved in approximately 50µl of TE, 50mM DTT and incubated on ice for 20mins. 1µl of this was counted as before and the probe dissolved in TE:hybridisation mix, 50mM DTT (1:9) so that the final concentration was 1.2 x 10^{5} dpm/ml.

Hybridisation Mix for *In Situs*

50% formamide, 10% dextran sulphate, 1x Denhardt's solution, 20mM Tris pH 8, 0.3M NaCl, 5mM EDTA, 10mM sodium phosphate, 0.5µg/ml

yeast RNA. This can be stored at -70°C. 50mM DTT is added immediately before use.

Hybridisation

The probe was added to the hybridisation mix and heated to 80°C for 2mins to denature it. The probe was then cooled on ice and then ~ 5µl was pipetted onto each row of sections. The sections and probe are then covered with the prepared coverslips and the slides were put into a slide box with a tissue soaked in 50% formamide, 5x SSC. The box is sealed into a plastic bag and submerged in a waterbath at 55°C o/n.

After hybridisation the slides are washed for 15 - 30mins in 5x SSC, 10mM DTT at 55°C to remove the coverslips, then for 20mins in 50% formamide, 2x SSC, 0.1M DTT at 65°C. They are then washed 3x in 0.5M NaCl, 10mM Tris, 5mM EDTA at pH 7.5 at 37°C for 10mins each before treatment with RNase A (40µg/ml) in NTE buffer pH 7.5 at 37°C for 30mins. The RNase A removes single strand, nonspecifically bound RNA probe and this reduces the background labelling. The slides were then washed for 15mins in NTE buffer alone at 37°C then in 50% formamide, 2x SSC, 0.1M DTT for 20mins at 65°C. The remainder of the washes were at RT. The slides were washed 3x in 2x SSC for 10mins each and then 3x in 0.1x SSC again for 10mins each. The slides are then incubated for 1min each in 30%, 50%, 70% and 90% ethanol, each made up in 0.3M ammonium acetate. The slides are finally washed in 100% ethanol for 2x 5mins before air drying prior to autoradiography.

Autoradiography of Slides

This was carried out under safe light conditions, using an S902 light pointed towards the wall. Ilford K5 emulsion at 40°C was mixed 1:1 with water at the same temperature. The slides were dipped twice into this and then left to drain. They were then loaded into the light tight slide box containing silica gel and left to dry for a minimum of 3hrs. The box was then wrapped in foil and put in the cold room to expose for 6 weeks.

Development and Fixation of *In Situ* Hybridised Sections

All solutions are stored at 4°C, but warmed to RT before use. The slides at RT were incubated in developer D19 for 4mins and then rinsed in distilled water. They were fixed in 1:3 Kodafix:distilled water for 4mins before washing for 2x 10mins in distilled water. The sections were stained in methyl green for a few secs before rinsing once more in distilled water and drying in air o/n. The slides were mounted in DPX mountant the following day.

Photography of *In Situ* Hybridised Sections

In situ hybridised sections were photographed by Duncan Davidson on a Wild Photomakroscop M400 with a Wild Leitz MPS46 photoautomat on colour Kodak Ektar 25 film. High power photographs were taken by Norman Davidson on a Leitz Orthoplan microscope, with the same type of film.

Results and Discussion

In an 11 day mouse embryo a high level of staining is apparent in **Figure 1** in the dark field photograph (**B**), and it appears that the probe has hybridised to all parts of the embryo. this raises the question of whether the grains are present due to specific hybridisation or whether they are representing a non-specific high level of background.

Figure 2 shows a section through a blood vessel from the same section in both light and dark field. The arrow (on the dark field photograph) indicates the position of the red blood cells within the vessel and the figure quite clearly shows the high level of staining of the vessel walls but a much reduced amount in the area of the red blood cells. The cell density is comparable, so this effect is not due to differing concentrations of cells. This clearly indicates that the grains are not due to to nonspecific binding, but to the specific labelling of the probe.

The evidence shown here indicates that there is a high level of expression of Em9 throughout the mouse embryo at 11 days. A similar observation in the embryo of *Drosophila* has also been noted (D. Hartley, pers. comm.). However on closer examination of the sections differences in the level of expression in some areas were observed.

Figure 3 shows a posterior section of the neural tube of an 11 day mouse. In both light and dark field, the neural tube shows a slightly higher level of expression than the flanking tissues, with a denser region lying within. The inner edge also appears to show

Figure 1 shows a longitudinal section through an 11 day C57BL/6 mouse embryo.

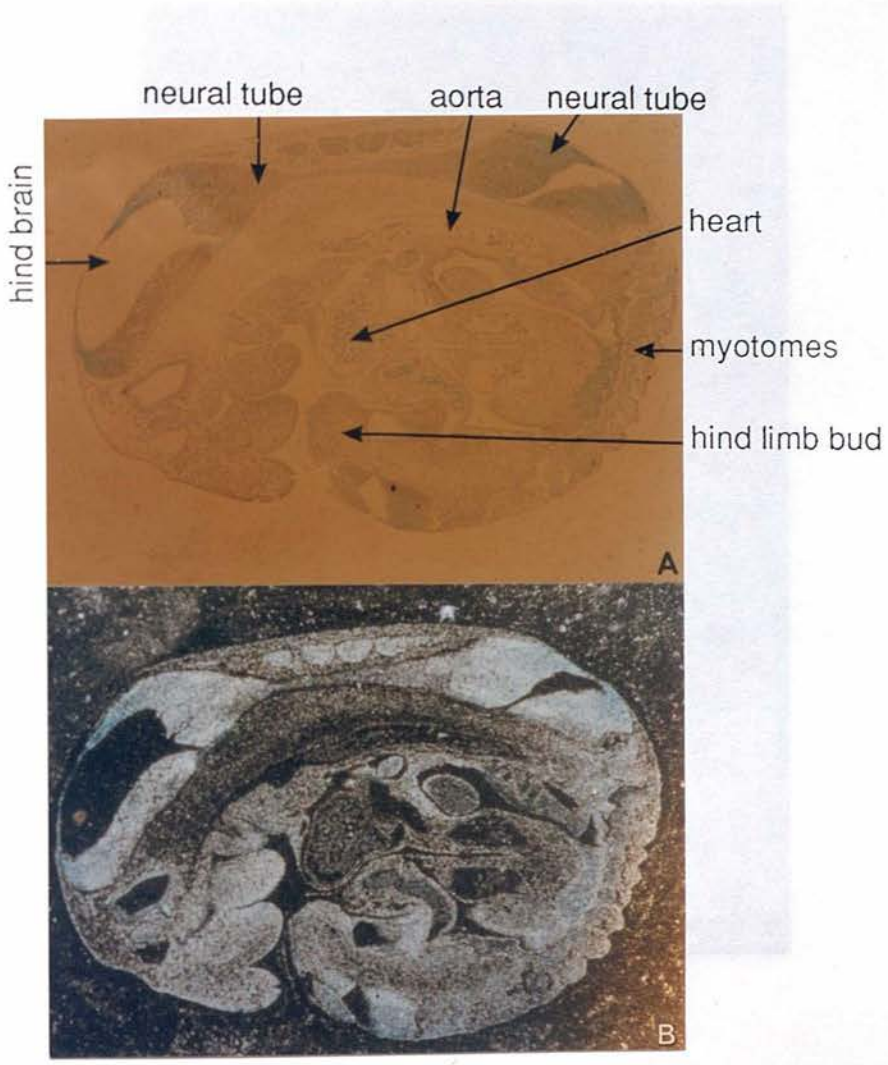


Figure 2 shows a section through a blood vessel of an 11 day C57BL/6 embryo.

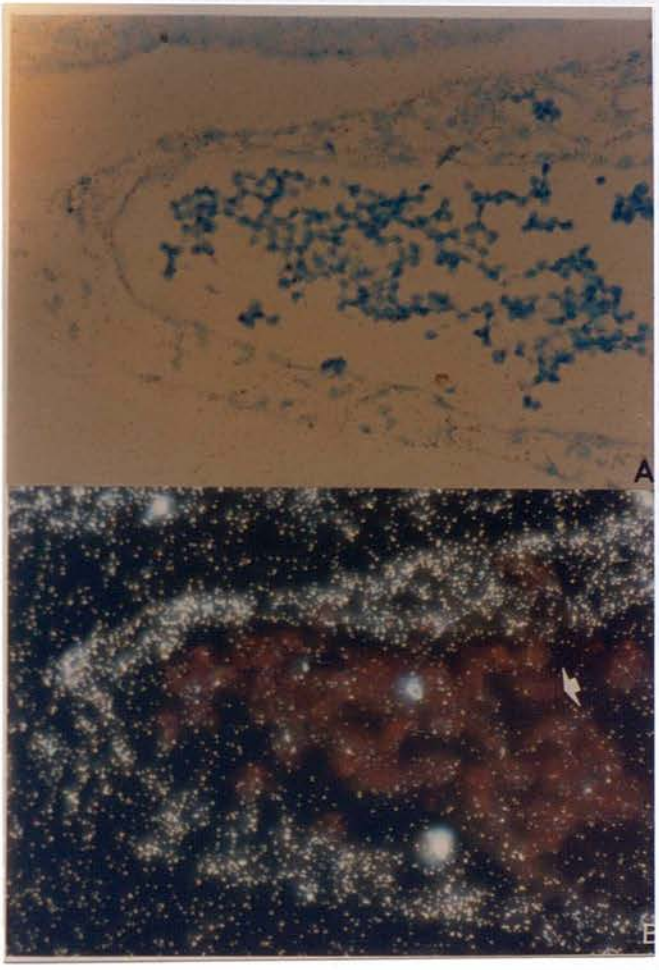
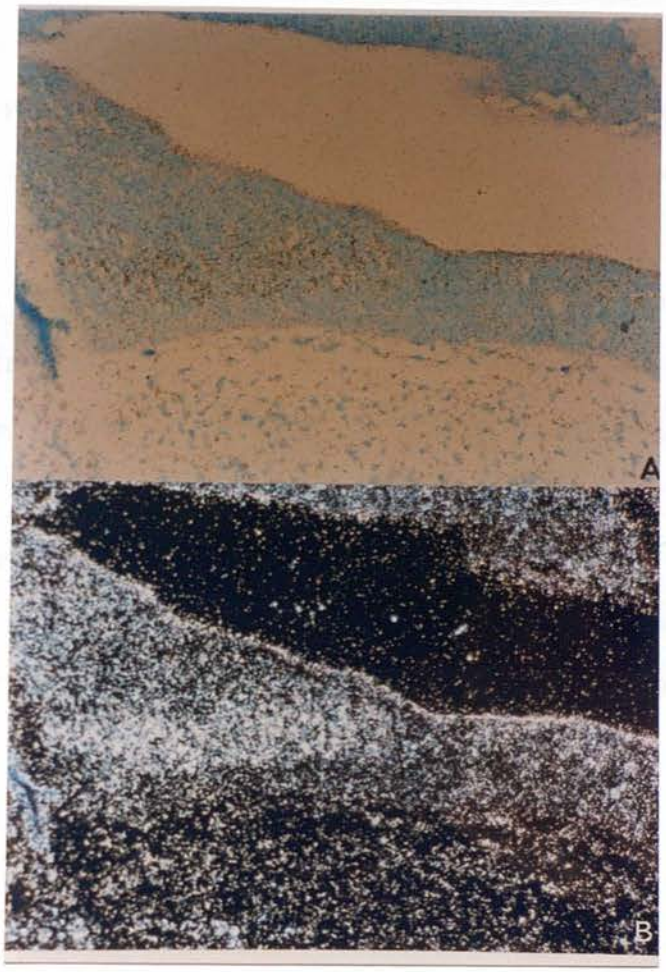


Figure 3 shows a section through the posterior end of the neural tube of an 11 day C57BL/6 mouse embryo.



an elevated level of expression when compared to that in the rest of the neural tube.

Figure 4 shows a section through the fore brain and Rathke's pouch in an 11 day embryo. Again the level of staining in the fore brain appears to be elevated when compared to that of the surrounding tissues. In the dark field photograph the neck of Rathke's pouch seems to be showing a higher level of staining than the rest of the structure as indicated by the lower arrow. Also the floor plate of the brain seems to show a reduced level of expression when compared to the rest of the brain (as indicated by the upper arrow). The reduced level of expression in the floor plate is shown more clearly in a section through the hind brain in **Figure 5** in both light and dark field. The heart was also observed to show a reduction in the level of expression (data not shown).

In the 9 day embryos studied, a general high level of expression was observed. Sections through these regions in 9 day embryos showed no reduction in the level of expression in the heart and floor plate, or any increase in the level in the neck of Rathke's pouch and within the posterior of the neural tube. This may be because the expression pattern is more uniform at 9 days and that the pattern changes and is restricted by 11 days. The tissues which show the reduced level of expression do not seem to have anything in common with each other in terms of their derivation or eventual function in the mouse. More sections at different stages of development in the mouse will have to be studied, before a complete picture of the embryonic expression of *Em9* can be produced.

The evidence here shows that the gene is indeed expressed in

Figure 4 shows a section through the fore brain and Rathke's pouch of an 11 day C57BL/6 mouse embryo.

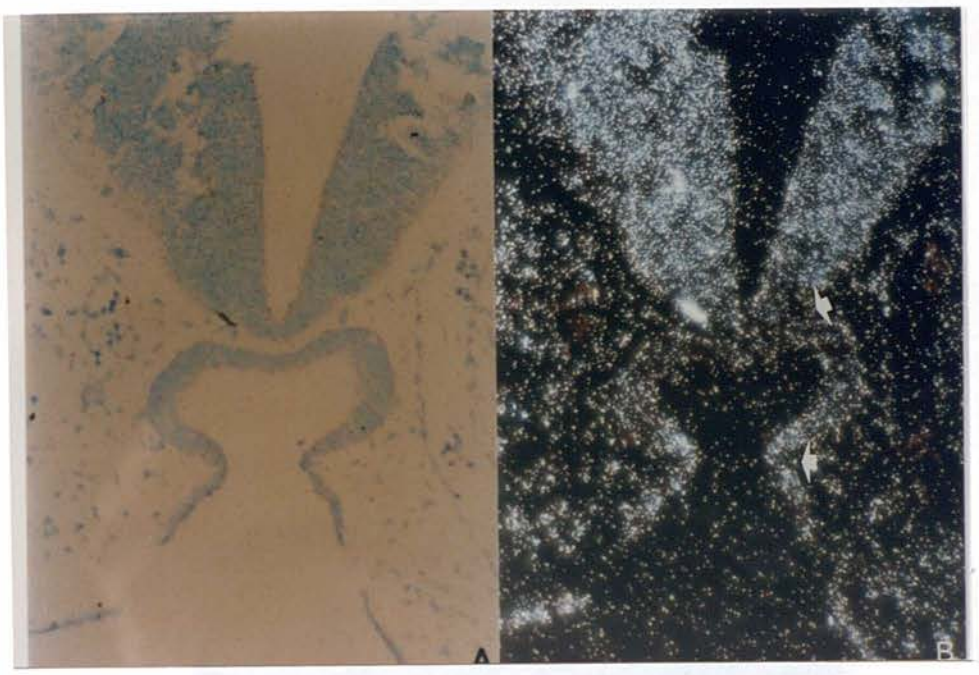
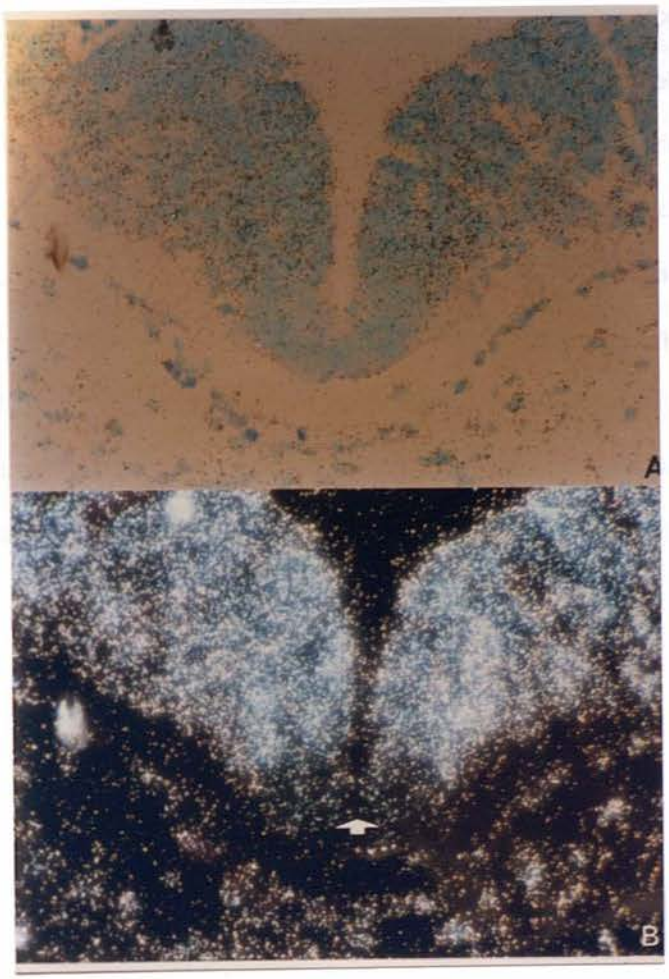


Figure 5 shows a section through the hind brain of an 11 day C57BL/6 mouse embryo.



the developing mouse embryo as expected from its isolation from a cDNA library. The more or less uniform pattern of this expression is unexpected, as the gene has a very specific role to play in a specific region in the *Drosophila* embryo. It may be that the spatial arrangement of the mRNA is particularly specific but that translational control restricts the functional protein. It is also possible that the control of the specific delamination of neuroblasts within the neurogenic ectoderm requires the interaction of a further gene in order to define the area in which this function occurs. If this is the case, the gene may not yet have been identified. Further work in this area will hopefully allow a better understanding of the complex process of development.

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