CLINICAL AND FUNCTIONAL INVESTIGATIONS OF THE TISSUE SPECIFIC SmN PROTEIN

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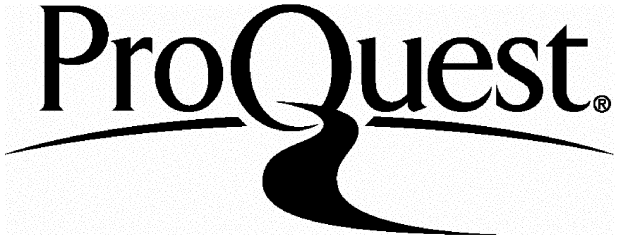

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Declaration

All of the work presented here is the work of J.D. Huntriss except for the ELISA screening with the SmB/B' 174-213 fusion protein, performed by Gladys Fumarino (MTKIR) and the SNRPN transcription data performed by Dr. Breda Twomey (UCLMS) which are both described in chapter 3.

ABSTRACT

The SmN protein is highly homologous to the constitutively expressed small nuclear ribonucleoprotein particle (snRNP)-associated protein SmB but is expressed in brain and heart only. SmN is demonstrated here to associate with snRNPs when expressed naturally or artificially and interestingly, the nature of this association is different to that exhibited by SmB. SnRNPs catalyse RNA splicing of the primary trancripts within the spliceosome to generate mRNAs encoding for proteins. The primary transcripts of some genes however, are processed differently according to the tissue or cell type to generate distinct mRNAs encoding for different proteins. The tissue specific expression of SmN suggests it plays an important RNA splicing role in the tissues expressing it, and hence is a putative regulator of alternative splicing.

The gene encoding SmN in humans, SNRPN has been mapped to the critical region for Prader Willi Syndrome (PWS) in which patients lack a functional paternally inherited copy of the maternally imprinted SNRPN gene. Murine models of PWS that lack expression of the mouse homologue (Snrpn) suffer early postnatal lethality. Data presented here confirms the absence of SmN expression at the protein level in all tissues tested demonstrating that the maternal imprint is not relaxed in these mice. Furthermore, SmB expression was observed to be elevated in the brains of such mice. This suggests that elevated SmB expression cannot compensate for important splicing roles normally performed by SmN in the tissues expressing it.

RNA from the brains of these mice was therefore used to study the proposed alternative splicing role of SmN. Genes proposed to be good candidates for regulation by SmN on the basis that their pattern of splicing is different in tissues expressing SmN were analysed by RT-PCR amplification of endogenous transcripts. Analysis of c-src, NCAM the Oct-2 transcription factor, CGRP and the GS alpha and Go alpha subunits of GTP binding proteins argues against a general role of SmN in alternative splicing, but the fate of these mice suggests SmN has unique functions or properties not assigned to SmB.

This is consistent with a functional analysis by immunoprecipitation whereby SmN demonstrated different affinities for the U1 and U2 snRNPs, showing preferential association with the U2 snRNP at low levels of expression. In addition, ELISA screening using peptides corresponding to the regions of least sequence homology between SmN and SmB/B' revealed that a subset of Systemic Lupus Erythematosus (SLE) patients generate autoantibodies that can discriminate between and SmN and SmB/B' by binding an SmB/B'-specific epitope (SmB/B' 179-190). Therefore the differences in primary structure between SmN and SmB/B' are sufficient to alter both protein conformation and the nature of association with snRNPs, implying that SmN-containing snRNPs may be capable of processing RNA differently.

In view of these findings, further studies have been performed to determine whether a reported significant elevation in gene transcription of SNRPN in certain SLE patients was reflected at the protein level. Data presented in this study suggests a post-transcriptional control mechanism operates to counter this aberrant SNRPN transcription and that elevated SmN protein expression is not a common occurrence in SLE. This is consistent with both immunoprecipitation data and ELISA data in that SmN does not appear to be the antigen stimulating the immune system in SLE.

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Submitted.

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CHAPTER 1
INTRODUCTION

1.1. RNA SPLICING-GENERAL

Comparisons of complementarity between viral DNA and cytoplasmic mRNAs expressed during adenovirus infection established the split gene structure whereby genes (coding units) were separated by intervening sequences (introns) which led to the discovery of RNA splicing (Berget et al.,1977; Chow et al., 1977). This led to the demonstration of intervening sequences in eukaryotic cellular genes. Thus globin genes were found to have two introns (Jeffreys and Flavell 1977; Tilghman et al., 1978), and the immunoglobulin genes to have long and short introns (Tonegawa et al., 1978). Analysis of the intron boundries revealed the presence of conserved sequence motifs that were common to verterbrate, plant and yeast cells and hence evolutionarily conserved (Breathnach and Chambon, 1981; Padgett et al., 1986).

The importance of such sequences and the accuracy of RNA splicing in general is demonstrated by the fact that many human diseases are caused by mutations that affect RNA splicing. Thus for example, a simple AG to GG mutation in the 3' splice site of intron 1 of the cytochrome b5 gene has serious implications and results in pseudohermaphrodism and methemoglobinemia (Giordano et al., 1994). Furthermore, it is recognised that 15% of point mutations that cause genetic disease affect RNA splicing (Krawczak et al., 1992). The complex nature of RNA splicing suggests that mistakes occur during splicing. Indeed it has been suggested that a mechanism exists in the cell to destroy incorrectly spliced RNAs which contain open reading frames interrupted by translational termination signals as they enter the cytoplasm (Pulok and Anderson 1993).

Splicing therefore is an essential process whereby genes are made into functional mRNAs by the removal of introns. The mRNAs are subsequently transported to the cytoplasm and translated into the protein products. By differential use of exons, RNA splicing can also be used as means of post-transcriptional control of gene expression by generating alternative forms of the protein product in a process termed alternative splicing.

1.2. TYPES OF SPLICING

The splicing steps that occur in the three main *cis* -RNA splicing processes- group I, group II and spliceosomal splicing share common features (FIG.1.1). The first step in each is a cleavage reaction at the 5' splice site, this being effected in group I self-splicing intron splicing by a transesterification reaction involving an intronic guanosine that displaces the 5' hydroxyl group on the 5' exon causing cleavage (Cech 1985). This self-splicing proceeds in the absence of proteins (Kruger et al., 1982) and occurs in maize and bean chloroplasts, the ciliated protozoan *Tetrahymena* and yeast mitochondria (Cech, 1986). Group I introns such as the rRNA intron *omega* in yeast mitochondria (Dujon, 1989) can behave as mobile genetic elements. The transposition of these introns is mediated by a DNA endonuclease encoded by an open reading frame in the intron (for review see Lambowitz and Belfort 1993). Alternatively, transposition may occur by reverse splicing that inserts introns containing an internal guide system (IGS) into other RNA molecules that are subsequently reverse transcribed and reinserted into the genome (Woodson and Cech 1989). Group I self-splicing introns are also present in viruses that infect the eukaryotic green algae *Chlorella,* which suggests a mechanism whereby such introns are horizontally transmitted between the protists and the eukaryotic algae (Yamada et al., 1994).

In group II self-splicing mitochondrial introns the, 2' OH at the adenosine branch site is activated to cleave at the 5' splice site which creates a circular lariat RNA structure similar to that produced by the spliceosome, the details of which are discussed later (Peebles et al.,1986; Van der Veen et al., 1986).

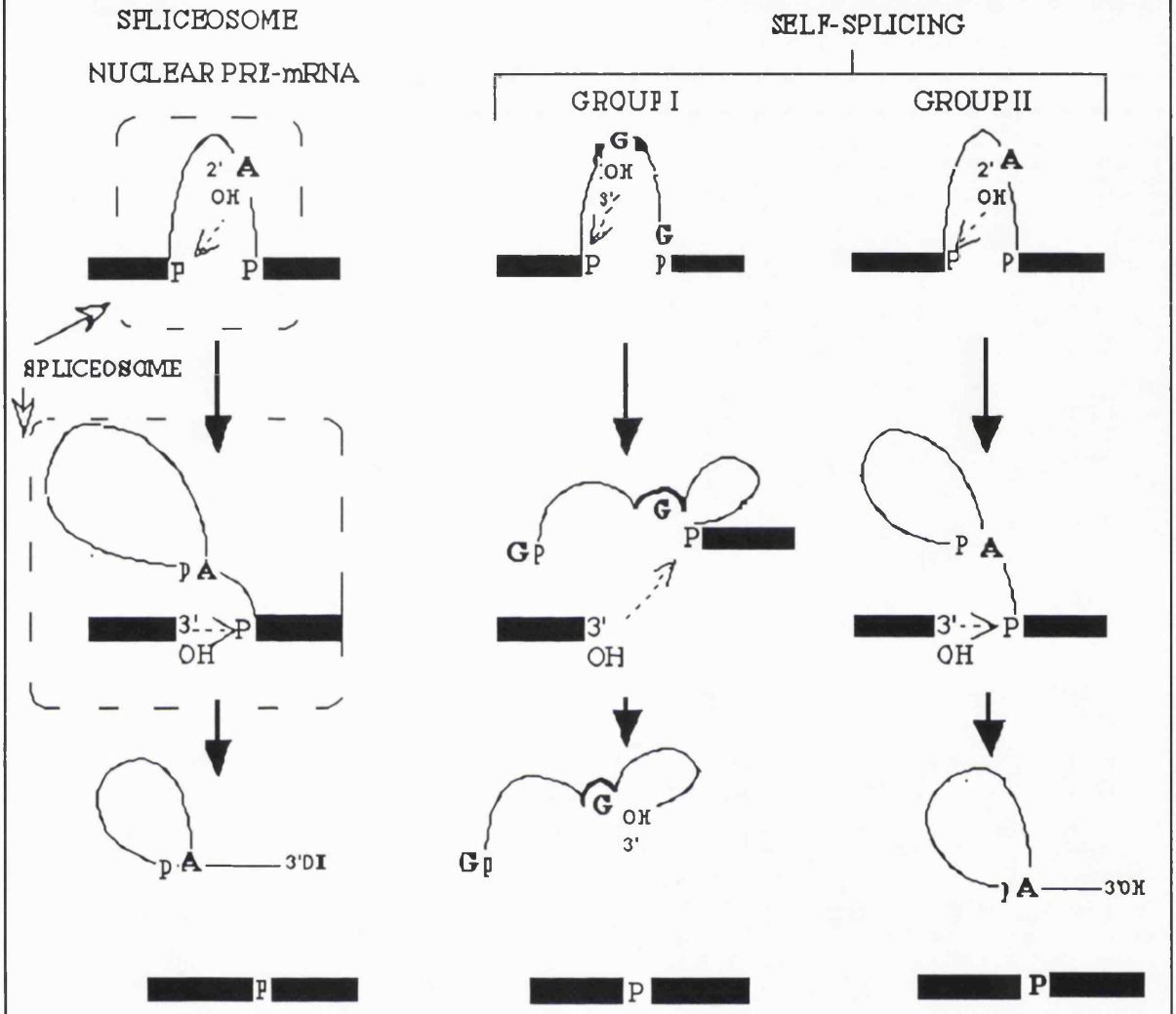
The second step in the three splicing mechanisms is cleavage at the 3' splice site which removes the intron and joins exons. Spliceosomal splicing is proposed to occur using two active catalytic sites and an SN2 nucleophilic displacement reaction that is analogous with group I self-splicing introns (Moore and Sharp 1993; McSwiggen and Cech 1989; Rajagopal et al., 1989). Furthermore, a two-metal-ion mechanism for RNA splicing common to group I, group II and spliceosomal splicing has been proposed in which the role of the RNA is to position the two catalytic metal ions and orientate the substrate using three specific binding sites (Steitz and Steitz,

1993). Such similarities between chemistry and mechanisms, especially between group II introns and spliceosomal splicing suggests these three splicing systems are evolutionarily related (for review see Lamond, 1993).

A further pre-mRNA processing pathway termed splice-leader (SL) *trans*-splicing in which pre-mRNAs obtain the 5' exon from a small SL RNA occurs in the lower eukaryotes including nematodes and the trypanosomes. The SL RNA has features analogous with the snRNAs of the spliceosome and splice donor and acceptor sites used in trans-splicing conform to consensus *cis*-splicing sequences. Moreover, certain *cis*-spliceosomal components such as U2, U4 and U6 snRNPs (see later) are required for trans-splicing in nematodes (Tschudi and Ullu, 1990; Hannon et al., 1991; for review see Steitz, 1992). The *trans* spliceosome is suggested to lack U1 and perhaps U5 snRNPs and thus it is proposed that the U6 snRNP is responsible for interaction with the 5' splice site in trans-splicing (Yu et al., 1993). However, the SL RNA is not an obligatory component in the assembly of the trans-spliceosome and the catalytic activation of these spliceosomes may occur in the absence of an exogenous splice donor.

FIG. 1.1)

COMPARISON OF SELF-SPLICING AND NUCLEAR PRE-mRNA SPLICING MECHANISMS



In nuclear pre-mRNA splicing, (left) splicing occurs within a multicomponent complex, the spliceosome (dashed line). Group I splicing is catalysed by intronic RNA structures (bold semi-circle) that contains a guanosine binding site and utilizes a guanosine (G) factor in the first step. Group II splicing (right) is catalysed by intronic RNA structures and utilizes an adenosine residue (A) within the intron to form a lariat RNA.

SPLICEOSOMAL SPLICING

1.3. COMPONENTS OF THE SPLICEOSOME- THE SMALL NUCLEAR RIBONUCLEOPROTEINS

In spliceosomal splicing, pre-mRNA splicing and 3' end processing is performed by a number of small nuclear RNAs complexed with proteins in particles termed small nuclear ribonucleoprotein particles snRNPs (FIG.1.2.-for reviews see Zieve and Sauterer 1990; Maniatis and Reed 1987). The snRNAs may be likened to the catalytic sites of a group II intron in pieces, whilst the proteins perform a number of proposed roles.

1.4. SMALL NUCLEAR RNAs

SnRNAs are uridine rich RNAs that are highly conserved between species. Thus snRNAs found in yeast are homologous in structure and function to the six abundant major mammalian snRNAs U1-U6 which are present within the snRNPs at 2x10 5-1x10 6 copies per cell (Riedel et al., 1986; Riedel et al., 1987; Reddy, 1986). Plant snRNAs also show high homology with snRNAs from higher animals and the existence of analogous protein components is demonstrated by immunoprecipitation of plant snRNPs with anti-Sm SLE sera (Tollervey and Mattaj 1987; Kiss et al., 1985/ 87/ 87b /88). The mammalian U1 snRNA consists of either 164 nucleotides (nt.) (U1a) or 165 nt. (U1b) whilst U2 snRNA consists of 188 nt. and each exhibits a distinct complex secondary structure of stem and loop structures. Whilst the structures of the snRNAs differ, all except U6 and nucleolar U3 contain a motif near the 3' end termed the Sm binding site which is a single-stranded uridine rich region (PuAUnGPu where n=2-6) flanked by two stem-loop structures (Liautard et al., 1982).

Another common feature (except for U6) is the 5'-terminal trimethylguanosine (m3G) cap that is formed from the co-transcriptionally added m7G cap by hypermethylation in which the presence of the Sm core proteins on the Sm binding site is a pre-requisite (Mattaj, 1986). The m3G cap functions as an important signalling component for the export of newly synthesized U snRNA from the nucleus (Hamm and Mattaj., 1990 ; Terns et al., 1993). The m3G cap is also

part of a bipartite nuclear localisation signal (NLS) that is shared with a region in the Sm core that can bind a transport receptor independently of the m3G cap (Fischer and Luhrmann., 1990; Fischer et al., 1993; Hamm et al., 1990a). Recent *in- vitro* studies demonstrate that the cofactor s-adenosyl methionine and a non-snRNP cytosolic factor proposed to be an snRNA-(guanosine-N2)-methyltransferase are required for hypermethylation. The Sm core alone, without snRNP-specific proteins is sufficient to act as a binding site for this *trans* -active methyltransferase that may be commonly used by all U-snRNPs in hypermethylation. The inhibition of cap hypermethylation by the anti-Sm monoclonal Y12 and other experiments suggest that the core SmB and SmB' proteins may be involved in m3G cap formation. The Sm core is also proposed to provide the binding site for the NLS-recognising factor and other snRNA modification enzymes (Plessel et al., 1994; Fischer et al., 1993). The U6 snRNA however is unique in possessing a gamma-monomethylphosphate cap and no Sm binding site (Singh and Reddy, 1989) and the presence of an m3G cap on U3 snoRNA suggests Sm-core independent methyltransferases exist.

Less abundant snRNAs U7-14 are also present and also possess a 5' trimethylguanosine cap and the common Sm core proteins although the Sm binding sites in U7 and U8 snRNAs differ from consensus. The U7 Sm binding site has been demonstrated to be partly responsible for the low abundance of U7 snRNPs due to inefficient snRNP assembly (Grimm et al., 1993). The gene copy number of U7 and in addition U11 snRNAs also contributes to this low abundance as both are single copy genes in human and mouse (Gruber et al., 1991; Phillips and Turner 1991; Suter-Crazzorola and Keller, 1991) whilst about 30 functional gene copies exist for U1 in mammals (Lund and Dahlberg 1984). U7 functions in histone mRNA 3' end formation (Birnstiel and Schaufele, 1988). U11 is present in the polyadenylation complex involved in 3' end processing. U8, like the more abundant U3 functions in ribosomal RNA processing (Baserga and Steitz 1993). The U3 and U8 species are more correctly termed small nucleolar RNAs (snoRNAs) as they are restricted to the nucleolus. The demonstration that the U14 snoRNA gene maps to an intron within the heat shock cognate protein 70 (Liu and Maxwell ,1990) has revealed the presence of many more (U14-U20) intron-encoded snoRNAs that may function in rRNA

processing (for review see Sollner-Webb 1993). The emergence of examples of intron-encoded genes argues for the retention of introns during evolution.

All snRNA and snoRNA genes are transcribed by RNA polymerase II except for the RNA polymerase III transcribed U6 gene (Kunkel et al., 1986). Transcription for polymerase II is mediated by a conserved proximal sequence element (PSE) which initiates transcription and acts in conjunction with a weak 3' box downstream of the gene to perform correct 3' end formation (Ach and Weiner, 1987; Hernandez and Lucito, 1988). A distal sequence element (DSE) also enhances the initiation of transcription.

1.5. PROTEINS ASSOCIATED WITH MAJOR snRNAs

SnRNAs interact extensively with proteins such that the final functional snRNP particle is comprised of approximately 80% protein. With the exception of the U6 snRNA and U3 snoRNA, snRNAs are complexed with a set of up to nine snRNP proteins which are termed the Sm core complex. This core consists of the 28kDa B, 29kDa B', the 16-18 kDa D 1-3 proteins, 13kDaE, 12kDa F and 11kDa G proteins (FIG.1.2-for review see Luhrman et al., 1990). The B' protein is a primate-specific core component derived by alternative splicing of the universal B protein and is not present in murine tissues (van Dam et al., 1989; Rockeach et al., 1990). The proteins assemble on each snRNA molecule with a stoichiometry of two B proteins, and one each of E,F and G. In humans, one copy of each of B and B' are present in the core in tissues other than the brain and heart in which a tissue specific form of the B/B' proteins termed N is expressed (Schmauss et al., 1989; McAllister et al., 1988; Sharpe et al., 1989). Here the relationships between these highly homologous proteins within the snRNP particles and the subsequent implications are less clear.

The precise stoichiometry concerning the D protein remains unclear due to conflicting literature whereby Andersen et al., 1990 suggests two distinct D proteins exist (D and D' which are distinct gene products) of which two copies of each are present per snRNA whereas others (Luhrmann et al., 1990) describe three D proteins designated D 1-3. The D, E,F and G proteins

protect the Sm binding site from RNAse digestion and these proteins rather than the B proteins remain bound at high ionic strengths. This taken together with the fact that particles containing only B may not be isolated, whereas particles containing D, E and F may be isolated without the B protein suggests D,E,F and G associate with the snRNA whilst B associates with snRNPs via protein-protein interactions (Liautard et al., 1982; Fisher et al., 1985a; Sauterer et al., 1990; Feeney et al., 1989). This is also implied by the lack of any form of RNA binding consensus sequence in SmB (for review of properties of RNA-binding proteins see Burd and Dreyfuss, 1994b). Cross-linking experiments demonstrate protein-protein interaction of D and B with E and G proteins but not with each other (Harris et al., 1988). The F protein is proposed to interact directly with the Sm binding site (Woppmann et al., 1988) and the Sm binding sequence alone when present within single-stranded heterologous RNA is sufficient to initiate Sm core protein binding (Mattaj, 1986). In yeast, certain Sm site mutations are lethal or inhibit growth (Jones and Guthrie 1990). Such mutants demonstrate decreased snRNP stability and increased snRNA decay, suggesting the importance of the Sm site and hence the Sm proteins in confering snRNP stability and function. This stabilising function has also been proposed for U1 (Hamm et al., 1987) and U2 snRNPs (Mattaj and DeRobertis, 1985). More specifically, yeast mutants defective in the smd1 null-allele that show deficiencies in splicing can be compensated for by the human D1 protein (Rymond et al., 1993). This demonstrates that individual core snRNP polypeptides are essential for pre-mRNA splicing and that the functions of such proteins are conserved between species. As previously discussed, the Sm core proteins with particular reference to SmB/ B' and hence probably N are proposed to play a role in hypermethylation to form the m3G cap on the snRNA (Plessel et al., 1994).

Each snRNP also possesses unique proteins in addition to the Sm core. Thus the U1 snRNP is characterised by the presence of three specific proteins termed the 70kDa, 33kDa A, and 22 kDa C proteins demonstrating a stoichiometry of two A and two C proteins per snRNP. The 70 kDa protein possesses an RNA recognition motif (RRM- Query et al., 1989) which contains a nuclear localisation signal (NLS-Romac et al., 1994). Thus the 70kDa protein can be transported

to the nucleus independantly of U1 snRNA and hence shows flexibility in its association with the U1 snRNP (Feeney et al., 1989). The proposed role of this protein is discussed later. The human A protein has been demonstrated to bind a 47 nt. region in the 3' untranslated region of its own pre-mRNA. This causes inhibition of mammalian but not yeast poly (A) polymerase (PAP) by a specific interaction between the A protein and PAP that thus inhibits polyadenylation of its own pre-mRNA (Boelens et al., 1993; Gunderson et al., 1994). The yeast U1A protein is not essential for the selection of most 5' splice sites (Liao et al., 1993), and splicing appears unaffected in yeast mutants that lack the U1A gene (Liao et al., 1994). It has been suggested that the yeast U1A protein maintains the U1 RNA in an active configuration. (Liao et al., 1994).

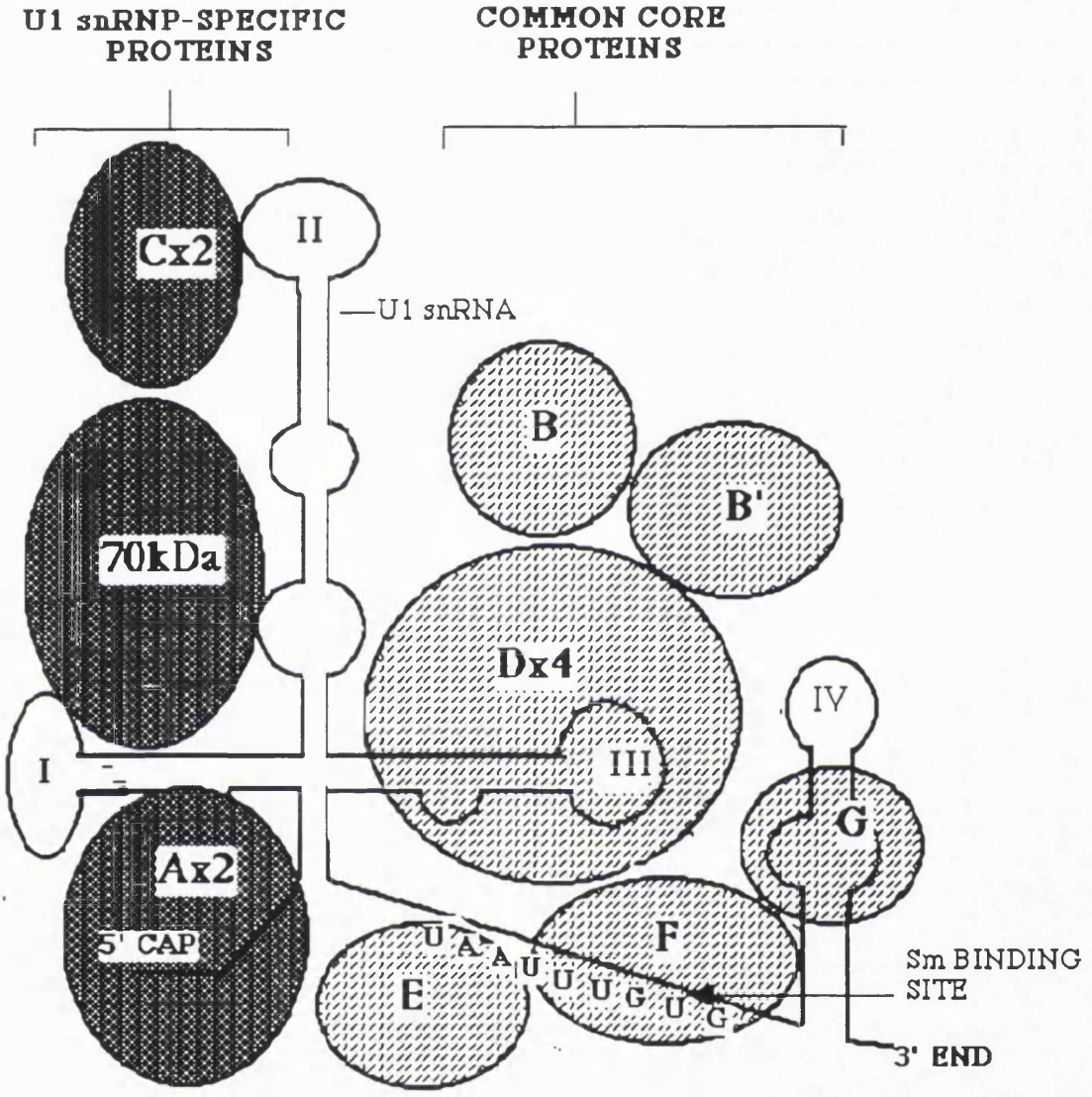
Two major proteins are unique to the U2 snRNP, these being designated the 28 kDa B" and the 30 kDa A' protein which bind to stem-loop IV of U2 snRNA (Habets et al., 1985). The third loop of the RRM of U2 B" is involved in interactions with the U2 A' protein which augments the affinity of RNA binding (Scherly et al., 1990; Bentley and Keene 1991). Recent data demonstrates that the 17S U2 particle which exists under splicing conditions contains up to nine additional U2-specific snRNP associated proteins (SAPs) proteins that are contained within an extra globular domain that gives the functional particle a bipartite appearance (Behrens et al., 1993a). Certain of these proteins are proposed to comprise the essential SF3 a and SF3b splicing factors. Furthermore, six of such U2-specific SAPs have been described which cross-link to the 3' end of the intron near the branch point in the pre-spliceosomal A complex (see later -Staknis and Reed 1994). Four of these SAPs are conserved between *Saccharomyces cerevisiae* and humans. These proteins also cross-link with the RNA in the spliceosomal B complex (see later) along with a 200kDa U5 snRNP-specific protein which cross-links near the 5' splice site. A 100 kDa U5-snRNP-specific Intron Binding Protein (IBP) interactive with the 3' end of introns has also been described (Gerke and Steitz 1986; Tazi et al., 1986).

Two SAP's (SAP 60 and 90) have been recently identified as examples of U4-U6-specific proteins that are present in splicing complex C (see later), suggesting important roles of these proteins during splicing (Gozani et al., 1994). Further U4/U6 snRNP specific proteins (150,

120, 80, 36 and 34 kDa) have been characterised using autoimmune sera from systemic sclerosis and Sjogrens syndrome (Okano and Medsger 1991; Fujii et al., 1982). Scleroderma sera have been used to identify a 65 kDa component of the U11-U12 snRNPs and a 140-kDa U11-specific protein (Gilliam and Steitz 1993).

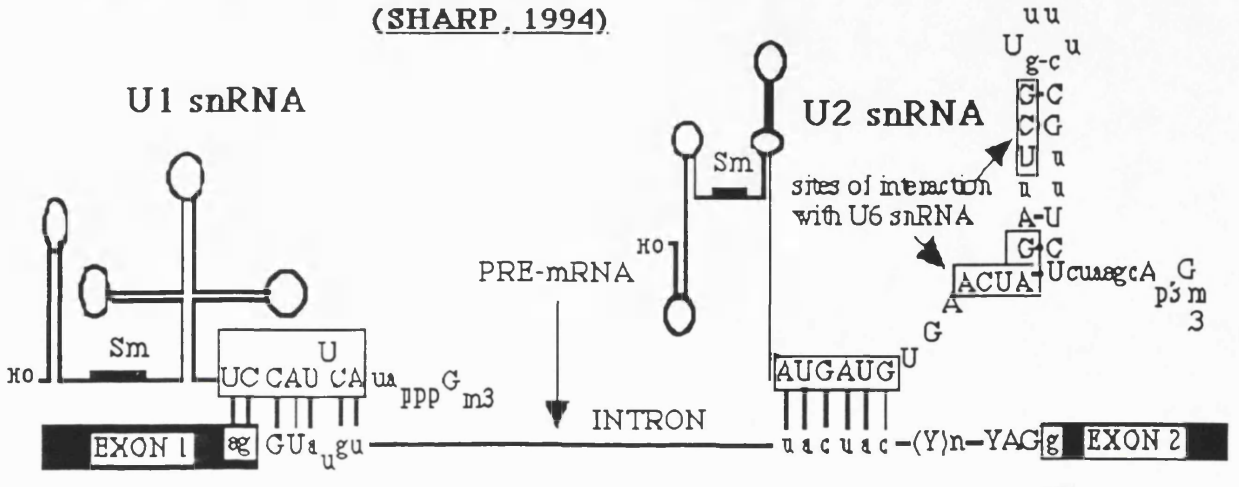
Therefore the snRNP specific proteins described above appear to play a critical role in snRNA-pre-mRNA interactions during splicing.

FIG.1.2.) **STRUCTURE OF THE HUMAN U1 snRNP (ZIEVE AND SAUTERER 1990)**



BASE PAIRING INTERACTIONS BETWEEN U1 AND U2 snRNAs AND THE PRE-mRNA SUBSTRATE IN SACCHAROMYCES CEREVISIAE

(SHARP, 1994)



1.6. snRNP ASSEMBLY

Assembly of snRNPs occurs in the cytoplasm where snRNAs exported from the nucleus associate with core proteins and snRNP specific proteins. The Sm core can bind a transport receptor and thus be transported to the nucleus independently of the m3G cap signal (Fischer et al., 1993). However, the association of Sm proteins with the U1 RNA in the cytoplasm suggests they may also function to transport partially assembled snRNPs to the nucleus (Feeney et al., 1989). Indeed, the cytoplasmic association of Sm core to the snRNAs would seem necessary in order to hypermethylate the m7G cap to the m3G cap (Plessel et al., 1994; Mattaj, 1986). U4 and U5 snRNPs unlike the U1 and U2 snRNPs may enter the nucleus as ApppG-capped particles although this occurs at a slower rate suggesting the requirement of the snRNPs for nuclear transport using the m3G cap varies and that it may only act to enhance the rate of nuclear localisation (Fischer et al., 1991).

The common core proteins are stored in excess in the cytoplasm compared to the snRNAs (Zeller et al., 1983; Zieve and Sauterer 1990). An RNA-free 6S complex composed of core proteins D1 (and D2, D3) and E,F and G initiates the assembly of the snRNP by binding to the Sm binding site on the snRNA (Fischer et al., 1985; Sauterer et al., 1990). The G protein has been demonstrated to cross-link specifically to the Sm-site of isolated snRNP particles (Heinrichs and Luhrmann 1992). The F protein has also been proposed to interact with this site (Wopmann et al., 1988). The B protein is the last to assemble to the Sm core in the form of a dimer (Feeney et al., 1990).

The U1 specific 70 kDa and A proteins are not essential for nuclear import of U1 snRNP particles (Hamm et al., 1990a), this being conferred by the bipartite Sm/ m3G NLS. Thus the U1-A protein and 70 kDa protein both contain nuclear localisation signals. These are located between the two RRMs in the A protein (Kambach and Mattaj, 1992) and within the RRM and also within the arginine-serine-rich region in the 70 kDa protein (Romac et al., 1994). In addition, the U1-specific C protein has been demonstrated to migrate to the nucleus independently where it

associates with preassembled U1-snRNPs (Jantsch and Gall 1992). The presence of the 70kDa protein in the snRNP is proposed to be a pre-requisite for the incorporation of the C protein (Hamm et al., 1990b).

Maturation of snRNPs is completed in the nucleus where specific proteins associate and proteins are proposed to exchange between snRNAs and nuclear pools of unassembled proteins (Feeney and Zieve 1990; Kambach and Mattaj 1992). Here the U1 snRNA precursor U1' is reduced by a few nucleotides in length (Yang et al., 1992) and the U2 snRNA precursor U2' is reduced by 11 nucleotides (Ares et al., 1985).

1.7. THE SPLICEOSOME CYCLE

The process of RNA splicing involves a complex sequential cycle of RNA/protein interactions which ultimately yields a functional mRNA (FIG.1.3.-for reviews see Zieve and Sauterer 1990; Lamm and Lamond 1993; Sharp 1994). The basic cycle involves recognition of the pre-mRNA and assembly of the spliceosome on the intron. The intron is catalytically excised by the spliceosome, joining the exons on the mRNA. The spliceosome components used are subsequently disassembled from the spliceosome and recycled for the next round of splicing.

Recognition of the RNA is ultimately dependant on conserved sequence motifs within the RNA. At the 5' splice site junction is the AG:GUAAGU sequence in which GU motifs are invariant. The CAG:G motif at the 3' splice junction (AG being invariant) and the branch point PyNPyPu(A)Py located 30 nucleotides upstream of the 3' splice site are also critical (PY= pyrimidine, Pu= purine, N= any nucleotide, (A)=Adenosine branch point which the 5' end of the intron ligates to via a 5' to 2' linkage;). In yeast the branch point sequence UACUA(A)C is absolutely conserved (FIG.1.2). Mutating these essential motifs generally inactivates splicing in yeast and activates cryptic splice sites in mammals. Mammalian systems require a further sequence in the form of a polypyrimidine stretch between the branch point and the 3' splice site.

The initial spliceosome complex, termed the commitment complex (CC) is formed when the U1 snRNP recognises the 5' splice site and sequences around the branch site and 3' splice site

are recognised (Seraphin and Rosbash 1991; Michaud and Reed 1991; Jamison and Garcio-Blanco 1992). This is proposed to be achieved through complementarity between the U1 snRNA and the 5' splice site as base pairing between the two can be restored by introducing mutations in U1 snRNA that are compensatory to those in mutant 5' splice sites (Steitz J.A.1992; Zhuang and Weiner, 1986; Seraphin et al., 1988; Siliciano and Guthrie, 1988).

The commitment complex was first identified in the yeast system (Legrain et al., 1988; Seraphin and Rosbash 1989) and is so termed as it is committed to form into a complete spliceosome.

The pyrimidine tract near the branch site and 3' splice site is functionally important in mammalian splicing as it is recognised by the U2AF protein (Ruskin et al., 1988). The binding of U2AF here allows the binding of the U2 snRNP to the pre-mRNA in the form of a stable complex called the A complex. Recent investigations suggest that the U1 snRNP 70K polypeptide and U2AF interact to form a bridge across the intron. This is strongly implied by the demonstration that ASF/SF2 and SC35 specifically interact with both the U1snRNP 70K protein and with the 35 kDa subunit of the U2AF splicing factor U2AF35 (Wu and Maniatis 1993). As the U2AF35-U2AF65 heterodimer binds to the 3' splice site via U2AF65 (for review see Green 1991) and the 5' and 3' splice sites are functionally associated during spliceosome assembly (Michaud and Reed 1993), this suggests an important role for the SR proteins in the interaction of 5' and 3' splice sites.

The U1 snRNP 70K polypeptide is highly phosphorylated in vivo and has been demonstrated to undergo phosphorylation at serine residues within the RS domain by a snRNP-associated kinase (Woppmann et al., 1990, 1993). As this phosphorylation inhibits mRNA splicing (Tazi et al., 1993) this suggests that the U1snRNP 70K polypeptide plays a crucial role during the splicing reaction. Thus the state of phosphorylation of this polypeptide and in addition the SR proteins which are also phosphorylated by this kinase (Woppmann et al., 1993) may determine the nature of protein-protein interactions that occur and hence the nature of splicing.

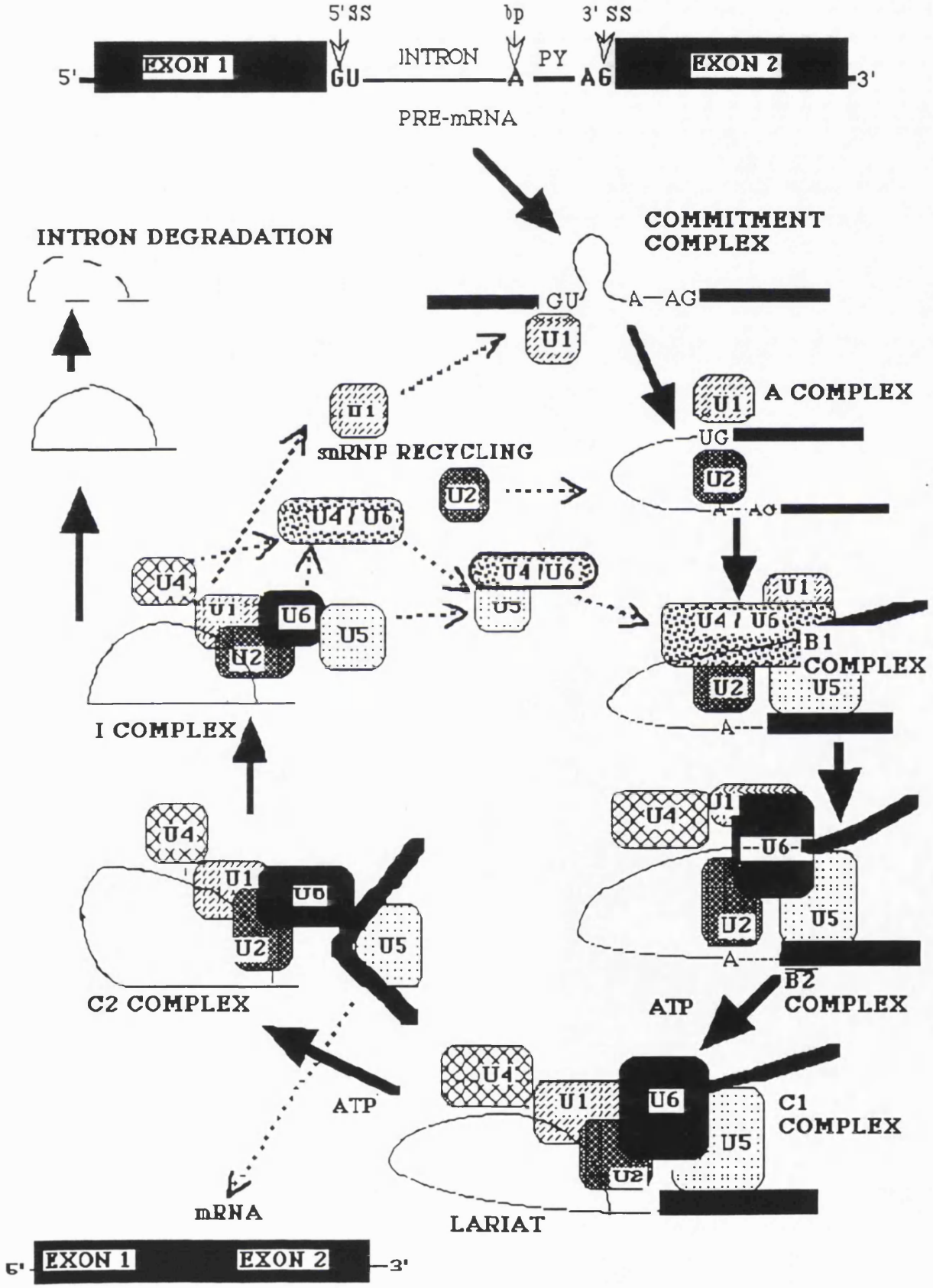
The B1 spliceosome complex is formed as the 25S U4-U6-U5 snRNP complex binds to the A complex in an ATP-dependent reaction. Here U4 plays a crucial role in formation of this

25S particle and in subsequent snRNP interactions to form the 50S five snRNP complex which performs RNA splicing (Kornarska and Sharp, 1987). The U4 snRNA is then proposed to dissociate from the U6 snRNA and the U1 snRNA dissociates from the 5' splice site to form the B2 complex. It is proposed that U1 snRNP and U2AF65 become destabilized at this stage and six U2 snRNP-specific proteins bind the pre-mRNA in the vicinity of the 3' splice site surrounding the branchpoint. (Staknis and Reed 1994). A novel set of 14 SAPs (two of which are U4-U6-specific) together with the essential splicing factor PSF has been observed to stably bind the 3' splice site during splicing complex C, suggesting that PSF and a number of SAPs replace U2AF65 in this complex (Gozani et al., 1994). Indeed, the disruption of base pairing between U1 and the 5' splice site is a pre-requisite for spliceosomal assembly by the interaction of U2-U4-U5-U6 snRNP complex with the 5' splice site (Konforti et al., 1993). The dissociation of U4 snRNA from U6 snRNA allows the U2 and U6 snRNP to interact by complementarity (Datta and Weiner 1991; Wu and Manley 1991; Madhani and Guthrie 1992; McPheeters and Abelson 1992). Another distinct region of U6 snRNA is proposed to interact with the conserved GU dinucleotide of the 5' splice site (Sawa and Abelson 1992; Wasserman and Steitz 1992; Sontheimer and Steitz 1993). U6 and U2 are extensively conserved which suggests these snRNAs possess the essential catalytic functions (Guthrie 1991). Mutations of two conserved U6 sequences that interact with U2 snRNA inhibit the first and second steps of splicing *in-vivo* (Madhani et al., 1990).

The U5 snRNP is proposed to be critical in determing 5' splice site selection in yeast (Newman and Norman 1992), and also in mammalian systems as studies with HeLa extracts suggest the U5 snRNA interacts with the 5' splice site, specifically with the last residue of the upstream exon (Wasserman and Steitz 1992; Wyatt et al., 1992; Sontheimer and Steitz 1993). Further interactions of the U5 snRNP are suggested by identification of a 100 kDa U5-snRNP-specific Intron Binding Protein (IBP) that is reactive with anti-Sm antibodies and interacts with the 3' end of introns (Gerke and Steitz 1986; Tazi et al., 1986; For a review of RNA-RNA interactions within the spliceosome see Nilsen, 1994).

Subsequently, an ATP-dependent transesterification reaction within the C1 complex catalyses the first step of splicing at the 5' splice site. This results in formation of the lariat by the subsequent 5' to 2' ligation of the 5' end of the intron to an adenosine residue within the branch point. This cleavage therefore separates the 5' exon from the intron and 3' exon (Ruskin and Green, 1985; Frendeway and Keller, 1985). The formation of the C2 complex marks further rearrangements between snRNAs which are proposed to form the catalytic site which performs the second splicing reaction (Madhani and Guthrie 1992; McPheeters and Abelson 1992). The I complex which consists of snRNPs and intron forms after the two exons are joined and the mRNA is released and migrates to the cytoplasm. In yeast, the release of mRNA from the spliceosome requires ATP and PRP22 (Company et al., 1991). The I complex then dissociates and snRNPs are re-used for further splicing whilst the intron is degraded.

FIG.1.3. THE ROLE OF THE snRNP PARTICLES IN SPLICEOSOMAL PRE-mRNA SPLICING.



Adapted from Sharp, 1994. SS=splice site; bp=branch point; py=polypyrimidine tract.

1.8. THE FATE OF NEWLY TRANSCRIBED RNA

Following transcription, a 200-300 base poly(A) tail is added to eukaryotic pre-mRNAs by poly(A)polymerase and an additional multisubunit complex following cleavage of the RNA 10-35 bases downstream of the polyadenylation signal (AAUAAA- Takagaki et al., 1990; Proudfoot and Brownlee1976). This sequence is essential both for cleavage and polyadenylation (Fitzgerald and Shenk, 1981; Wickens and Stephenson 1984; Manley et al., 1985; Sheets et al., 1990). A second element (DSE) downstream of the cleavage site is required and the efficiency of 3' end formation is dependent on the distance between the DSE and the polyadenylation signal (Gil and Proudfoot, 1987; Heath et al., 1990; Weiss et al., 1991). The 5' end is modified to form an m7G monomethyl guanosine cap which is necessary for translation (Salditt-Georgieff et al., 1980).

Prior to spliceosomal splicing in the nucleus, the newly transcribed pre-mRNA is rapidly bound by the heterogenous nuclear RNP proteins (hnRNPs-for review see Dreyfuss 1986) which have been proposed to regulate localisation and translation of the mRNA and to confer stabilising effects. The hnRNP proteins hnRNP I (PTB-Guo et al., 1991; Mulligan et al., 1992) and hnRNPA1 (Mayeda and Krainer 1992) have also been demonstrated to affect splice site selection.

The hnRNP A1 protein possesses two RRM's and has been proposed to play a role in facilitating RNA transport out of the nucleus (Pinol-Roma and Dreyfuss 1992). A1 has also been demonstrated to discriminate among pre-mRNAs by differential binding, therefore possibly allowing other splicing factors to discriminate among RNAs (Buvoli et al., 1992). The two RRM's are proposed to play a role as a single component that bind RNAs with different affinities, the highest affinity of binding being recorded for a sequence resembling a duplication of the yeast consensus 5' and 3' splice sites (Burd and Dreyfuss 1994a). Furthermore, A1 binds to the U1, U2 and U4 snRNPs. The binding by A1 to the U2 snRNP and to the branch site may promote their interaction whilst at high concentrations A1 may have a competing effect against the binding of the U1snRNP to the 5' splice site. A further role of hnRNP A1 in RNA strand annealing is

proposed to affect the interaction of *trans*- acting spliceosomal factors with the pre-mRNA (Buvoli et al., 1992; Eperon et al., 1993; Portman and Dreyfuss, 1994).

The hnRNP C1 and C2 proteins have one RRM, are restricted to the nucleus (Pinol-roma and Dreyfuss 1992) and bind poly (U) sequences and the intronic polypyrimidine sequence (Swanson and Dreyfuss 1988; Wilusz and Shenk 1990). The hnRNP C proteins are immunoprecipitable from the spliceosome using a monoclonal specific for them (Choi et al., 1986).

The hnRNP I/ PTB protein is also immunoprecipitable from spliceosomes (Patton et al., 1991) but demonstrates an additional unique location within discrete perinuclear structures (Ghetti et al., 1992). The exact role of hnRNP I/ PTB in pre-RNA splicing is covered later in this account.

1.9. OTHER SPLICING PROTEINS

The arginine-serine rich SR proteins, notably ASF/SF2 (Ge and Manley, 1990; Krainer et al., 1990) and SC35 (Fu and Maniatis, 1990; Fu and Maniatis, 1992a) are essential for splicing. The addition of any one of the SR proteins to a cellular extract competent for splicing but lacking SR proteins restores splicing activity (Krainer et al., 1990; Zahler et al., 1993; Fu et al., 1992). The SF/SF2 and SC35 proteins are required for the formation of splicing complexes early in the splicing pathway (Krainer et al., 1990; Fu, XD, 1993, Fu and Maniatis, 1992b). Recent data suggests that ASF/SF2 bind specifically to pre-mRNAs containing intact 5' splice sites (Zuo and Manley, 1994). It has been proposed that the protein acts to recruit U1 snRNP to the 5' splice site. The SC35 protein is a distinct splicing factor but exhibits equivalent activities and similar structure with ASF/ SF2 (Fu et al., 1992).

Screening for conditional lethal mutants of the yeast *Saccharomyces cerevisiae* identified many genes that affect RNA processing (Vijayraghavan and Abelson 1990; Vijayraghaven et al., 1990). Such mutants are termed pre-mRNA processing (PRP) mutants and 30 of such genes have been identified which are involved in RNA splicing. Some prp gene products (PRP2, PRP16) have been shown to have RNA-dependant ATPase activity, suggesting they function as RNA

helicases and thus modulate RNA base pairing interactions (Kim et al., 1992; Schwer and Guthrie 1991). The PRP5 gene product is essential during spliceosome assembly and is believed to regulate binding of U2 snRNP to the pre-mRNA (Dalbadie and Abelson, 1990). Further evidence of the essential role of prp gene proteins in splicing are evident in the interaction of PRP28 with both U6 and U5 associated proteins (Strauss and Guthrie 1991).

Recent data demonstrate that mammalian systems have counterparts of yeast prp proteins, in the form of snRNP-associated proteins (SAPs). The U2 snRNP within the A splicing complex has at least seven SAPs (Bennet et al., 1992; Behrens et al., 1993a; Staknis and Reed, 1994), three of which (SAP 61, 62, 114) form the essential splicing factor SF3a, whilst SAPs 49,130,145, and 155 are proposed to comprise the SFb splicing factor (Brosi et al., 1993a ; Brosi et al., 1993b; Bennet and Reed 1993). Moreover, the functional activities of PRPs appear to be conserved in SAPs. Thus antibodies to the PRP9 gene product which encodes a 60 kDa protein essential for complex A assembly also bind an essential 60 kDa component of mammalian U2 snRNPs (Behrens et al., 1993b). In addition, PRP9 directed antibodies bind SAP61 due to 30% homology between the proteins. Similar protein-protein activities with other SAPs that reflect those demonstrated in PRPs suggest specifically that the functional activities of PRP9 and PRP11 are retained in SAPs 61 and 114 (Chiara et al., 1994). Interestingly, the mammalian La antigen, an RNA binding protein which is bound by antibodies present in SLE and Sjogrens syndrome sera has been demonstrated to possess dsRNA unwinding activity, being therefore similar to the activity of certain PRP gene products (Xiao et al., 1994). The 50kDa La antigen has also been implicated in the transport of RNA polymerase III transcripts such as the U6 snRNA to the cytoplasm (Bachmann et al., 1989a; 1989b).

1.10. ALTERNATIVE RNA SPLICING

Alternative splicing is a common mechanism whereby different protein isoforms can be generated from the same primary transcript (for review see Latchman 1990,1993; McKeown 1992). This allows the genome to have increased coding capability and also, is a further

mechanism of regulating gene expression This especially important in terminally differentiated cells that have lost the capacity to replicate DNA. Alternative splicing involves the joining of non-consecutive exons or splice sites during the processing of transcripts.

Various patterns of alternative splicing exist (FIG.1.4), the simplest of which is the splice/ don't splice choice observed in the *Drosophila* P-element in which an exonic sequence is involved in the retention of the downstream intron in the somatic tissues (Chain et al., 1991). The choice of 5' splice site determines the splicing of the SV40 T antigen gene to subsequently produce either large T or small t proteins (Noble et al., 1988). Conversely, in *Drosophila* females, the product of the *sex-lethal gene (Sxl)* causes utilisation of the female-specific 3'splice site but not the male 3' splice site in the primary transcript that encodes the Tra protein (Boggs et al., 1987; McKeown et al., 1988). Exon skipping is observed in the splicing of *Drosophila sex-lethal* (Cline, 1984) and the cHras-proto-oncogene (Cohen et al., 1989). Isoforms of the CD45 membrane-bound tyrosine phosphatase possessing different molecular weights are generated by multiple exon skipping involving the inclusion or exclusion of three exons (Streuli and Saito, 1989). Splicing of the β–tropomyosin gene of both rat and chicken involves mutually exclusive exon usage (Helfman et al., 1988; Libri et al., 1990). The pattern of splicing of the CALC-1 gene is a choice of 3' exons whereby splicing of exons 1,2,3,4 yield an mRNA encoding calcitonin in the default pathway whilst splicing exons 1,2,3,5 yields CGRP mRNA (Crenshaw et al., 1987).

A wide variety of potential mechanisms operate in alternative splicing as splice site selection can be determined by a number of parameters. Such perameters include the nature of the splice sites themselves (for review see Green, 1991). In general, sequences that show a better match to the consensus splice sites are more tightly bound by splicing factors (Nelson and Green, 1990; Zamore et al., 1992) and such sequences are considered "strong" sites as they are more frequently used (Oshima and Gotoh, 1987; Brunak and Engelbrecht, 1991). The pyrimidine content of 3'splice sites is also implicated in alternative splice site selection (Mullen et al., 1991; Dominski and Kole, 1992).

Exon sequences and relative splice site proximity are involved in splice site selection (Eperon et al., 1986; Mardon et al, 1987; Reed and Maniatis 1986; Streuli and Saito 1989; Libri et al., 1990). Two distinct sequence elements within the calcitonin-specific exon 4 are required for calcitonin-specific splicing of the CALC-1 pre-mRNA (van Oers et al., 1994) and these sequences are bound by proteins that overcome the effect of the weak exon 4 splice acceptor site (Emeson et al.,1989; Adema and Baas, 1991) . Splice site usage is determined by an exon recognition sequence (ERS) in the 5' portion of exon M2 of the mouse immunoglobulin IgM gene that functions as a splicing enhancer, possibly by binding *trans*- acting factors such as the U1 snRNP (Watakabe et al., 1993). A positively-acting purine-rich exon splicing element (ESE) within the developmentally regulated exon 5 of the cardiac troponin T gene is required for splicing to the 3' adjacent splice site and may function in both constitutive and alternative splicing (Xu et al., 1993).

Pre-mRNA secondary structure controls alternative splice-site selection (D'orval et al., 1991; Libri et al., 1991; Solnick, 1985, Eperon et al., 1988). Thus for example, 2 bases located in the vicinity of the exon 3 major splice-acceptor in the human growth hormone gene facilitate the use of a downstream competing alternative acceptor site by effectively stabilising a specific stem-loop structure that encompasses the major acceptor site (Estes et al., 1992)

Exon size also determines splice site selection whereby co-ordinate recognition of splice sites within an exon occurs in a process termed exon definition (Dominski and Kole, 1991; Nasim et al., 1990; Robberson et al., 1990; Hoffman and Grabowski, 1992).

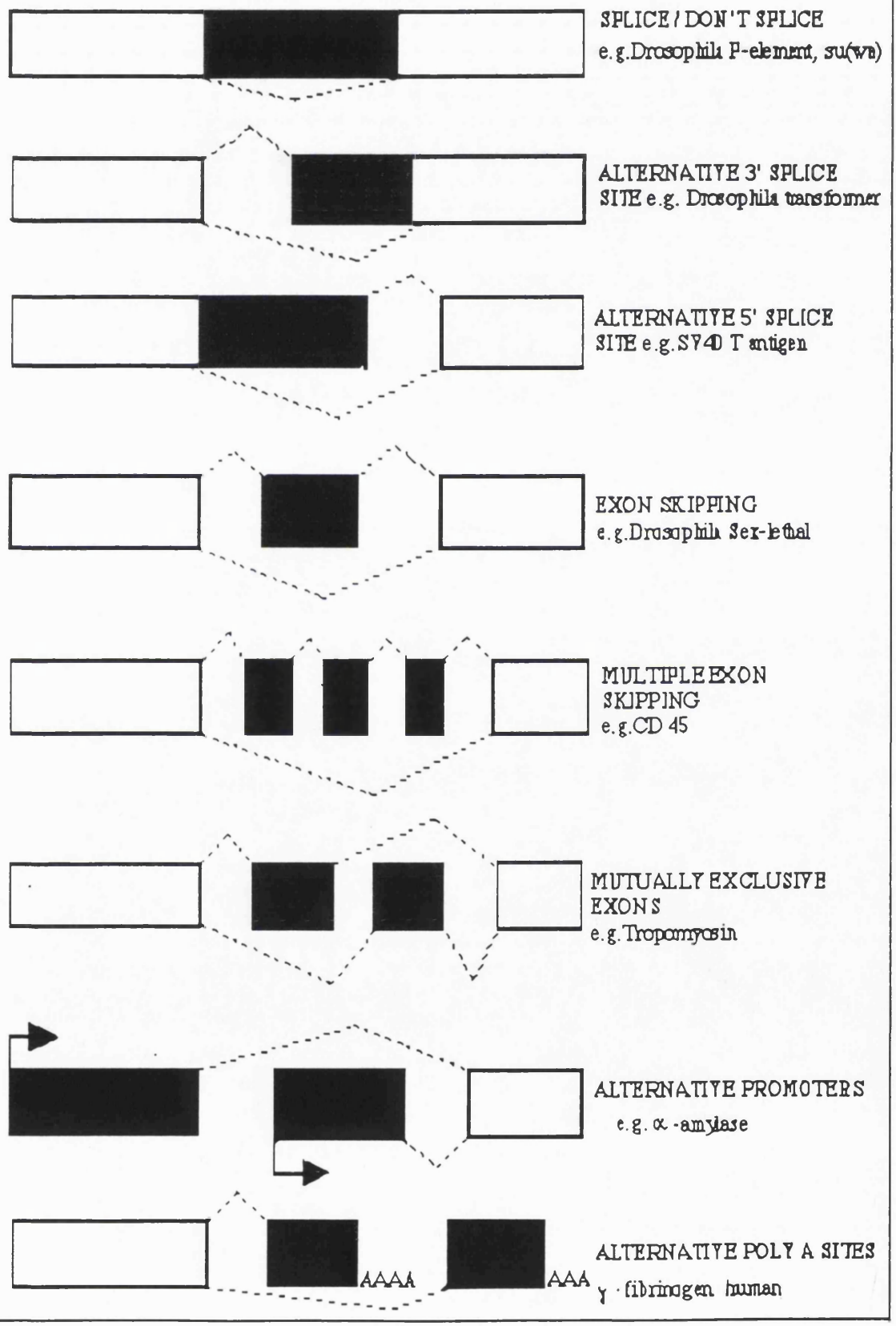
Splice site usage may also be affected by intron sequences (Helfman et al., 1990; Black 1992; Gallego et al., 1992). Thus for example, the cell-type-specific inclusion of the EIIIB exon in rat fibronectin pre-mRNA is regulated by downstream intronic sequences located a long distance (518 nucleotides) from the EIIB exon (Huh and Hines, 1993). Moreover, a short, highly repeated TGCATG motif enhances the usage of the EIIB 5' splice site and can also activate inclusion of the alternatively spliced exon 4 of the rat preprotachykinin pre-mRNA (Huh and Hines,1994). Interestingly, a positively-acting intronic TGCATG sequence is also required for the inclusion of the neural-specific c-src N1 exon (Black, 1992). Furthermore, eight copies of a similar repeated

sequence (GCATG) are clustered around the calcitonin-specific exon 4 splice acceptor in the CALC-1 gene (Huh and Hines, 1994), suggesting such motifs may also play an important role in determining splice-site selection.

A combination of *cis* - acting sequences that interact with cellular factors are proposed to regulate splice site selection in the rat β–tropomyosin gene. Thus elements immediately upstream of exon 6 facilitate the use of the downstream 5'-splice site by exon definition (Tsukahara et al., 1994). In addition, sequences in the skeletal muscle-specific exon 7 and the adjacent upstream intron in addition to multiple-*cis* elements surrounding exon 6 contribute to tissue-specific alternative splice-site selection (Helfman et al., 1990; Guo et al., 1991;Guo and Helfman 1993; Tsukahara et al., 1994).

The generation of distinct proteins from the same primary transcript infers that cell-type / tissue-specific / developmentally regulated protein factors must be present to interact with *cis* - acting sequences on the pre-mRNA and thus affect the way the spliceosome deals with the transcript. This is supported by the fact that the introduction of identical minigene constructs containing alternatively spliced exons leads to different patterns of splicing in different cell types (Barone et al.,1989; Crenshaw et al., 1987). Regulation of alternative splicing therefore involves the use of *cis* -acting sequences in the pre-mRNA in conjunction with *trans* -acting cellular protein factors.

FIG.1.4.) VARIOUS FORMS OF ALTERNATIVE RNA SPLICING



1.11. PROTEINS INVOLVED IN THE REGULATION OF ALTERNATIVE SPLICING

Recent work suggests that the mechanisms underlying the selection of splice sites in alternative splicing frequently utilises the same protein components used in constitutive splicing and such proteins therefore have a dual function and they exhibit similar structural properties (FIG.1.5). In examples investigated thus far, it appears that regulation is brought about by a delicate balance of such proteins that often autoregulate their own expression via feedback loops. Regulatory proteins may act in conjunction with antagonistically acting proteins to determine the choice of 5' splice sites selection.

A feature common to proteins involved in the selection of 5' splice sites is an arginine-serine (RS)-rich sequence. This domain gives rise to the name of the SR protein family which includes SF2/ ASF and the identical SRp20 and SRp30a proteins (Ge et al., 1991; Krainer et al., 1991; Zahler et al., 1992), SC35 and the identical SRp30b protein (Fu and Maniatis, 1992a; Zahler et al., 1992), SRp55 and SRp75 (Zahler et al., 1992), U2AF65 and U2AF35 (Zamore et al., 1992; Zhang et al., 1992). The RS domain is also a feature of the UI-snRNP 70 K protein (Theissen et al., 1986; Spritz et al., 1987) and the *Drosophila* suppressor of white apricot Su(wa) (Chou et al., 1987), suppressor-of-sable (Voelker et al., 1991) and Transformer (Tra) and Transformer 2 (Tra2) proteins (Ge at al.,1991). Therefore the RS domain of Su(wa) is required for function of the protein *in vivo* and this function can be replaced by the RS domain from Tra. The RS domain has also been proposed to direct proteins to the subnuclear compartment (Li and Bingham 1991). Recently the U1-snRNP 70K protein has also been demonstrated to possess a nuclear localisation signal (NLS) within its arginine-serine rich domains (Romac et al., 1994). The RS domain of U2AF65 is essential for splicing activity (Zamore et al.,1992), and fusion of the *Drosophila Sex lethal* (sxl) protein to the RS domain of U2AF65 converts it from a repressor to an activator of splicing (Valcarcel et al., 1993). It has been suggested that as many mammalian and Drosophila splicing factors contain an RS sequence, whereas the splicing factors in yeast that do not perform alternative splicing of RNA do not, then the RS domain may play an important

role in alternative splicing (Lamm and Lamond 1993). In addition, comparison of the ability of several SR factors to alternatively splice different pre-mRNAs demonstrates that they are differentially active in modulating this alternative splicing (Zahler et al., 1993a).

A second feature common to these RNA-binding proteins is the ribonucleoprotein consensus sequence (RNP-CS), which is also termed the RNA-recognition motif (RRM) or RNA-binding domain. This 80-90 amino acid motif has been demonstrated to be necessary for specific RNA binding in vitro (Query et al., 1989; Scherly et al., 1989; Nagai et al., 1990) and is common to many RNA-binding proteins including the general splicing factors that contain RS domains (except U2AF 35) and also the U1A and U2B" snRNP proteins (Sillekens et al.,1987; Habets et al., 1987). The RNP-CS of the U1-snRNP 70 K protein contains one of two nuclear localisation signals (NLS- Romac et al.,1994). The SR protein ASF/SF2 requires both the RS domain and the RNP-CS for performing constitutive splicing, however the RS domain is not essential for splice-site switching (Caceres and Krainer, 1993; Zuo and Manley, 1993).

ASF was originally observed to cause a switch from SV40 large T to small t pre-mRNA splicing in a concentration dependant manner (Ge and Manley, 1990). SF2 was observed to alter β–globin splicing in a similar manner (Reed and Maniatis, 1986; Krainer et al., 1990b) and the two proteins were subsequently proved to be identical (Ge et al., 1991; Krainer et al., 1991). ASF/SF2 contain two RNA-binding domains (Ge et al., 1991; Krainer et al., 1991; Zuo and Manley 1993). In addition to a general splicing role (FIG.1.5), ASF/SF2 also modulates the selection of alternative splice sites in a concentration-dependant manner (Ge and Manley 1990; Krainer et al., 1990b). High concentrations of ASF/SF2 are observed to favour the use of the 5' splice site most proximal (downstream) to the 3' splice site (Harper and Manley 1992). Furthermore, the human pre-mRNA splicing factor hnRNPA1 activates distal 5' splice sites at high concentrations and acts antagonistically against ASF/SF2 and SC35 . Thus the ratio of hnRNPA1 to ASF/SF2 or SC35 affects the ratio of distal to proximal splice site usage (Fu et al., 1992; Mayeda and Krainer 1992; Ben-David, Y. et al.,1992). This suggests that the nature of alternative splicing performed within a tissue may be regulated by the level of expression of SR proteins in

conjunction with proteins acting antagonistically to these SR activities (Zahler et al.,1993a). High concentrations of ASF/SF2 also prevent exon-skipping in vitro, suggesting SR proteins ensure the fidelity of splicing of pre-mRNAs with many exons (Fu, 1993). SR proteins may act to stabilise the base pairing of the U1 snRNP to the 5' splice site (Eperon et al., 1993) and may also regulate interactions between U1 and U2 snRNPs by forming a bridge between 5' and 3' splice sites (Fu and Maniatis 1992b; Wu and Maniatis 1993).

Recently, investigations of insulin-induced genes have demonstrated the hydrophobic HRS protein to be highly insulin-induced and expressed as a delayed early gene in regenerating liver. HRS is highly related to other alternative splicing proteins and different isoforms of HRS appear temporally during growth, suggesting HRS can autoregulate its own pre-mRNA to perform critical roles during the cell cycle (Diamond et al., 1993). A novel 35 kDa essential human splicing factor termed 9G8 has been recently isolated and includes an SR domain and an RRM and hence like other SR proteins may play a role in regulating alternative splicing (Cavaloc et al., 1994).

The polypyrimidine tract binding protein (PTB / hnRNP I) is required for efficient splicing as demonstrated by the resumption of splicing following addition of the protein to extracts depleted by poly (U) affinity chromatography (Patton et al., 1991). PTB is believed to function as part of a complex that recognises the pyrimidine stretch in the 3' splice site and interacts with U1 snRNP to form the earliest commitment complex (Bothwell et al., 1991). More recently, a further function for PTB as a regulator of alternative splicing has been suggested in HeLa cells in which PTB associated with a 100 kDa protein binds an intronic regulatory sequences involved in the repression of exon 7 inclusion in β-tropomyosin pre-mRNA (Mulligan et al., 1992; Guo et al., 1991). Thus non-muscle cells appear to express factors including PTB that repress the use of the skeletal muscle exon. Interestingly, PTB exhibits significant homology to hnRNP L (Pinol-Roma et al., 1988) and *Drosophila* elav proteins (Robinow et al., 1988) and contains RNA binding domains.

Studies in *Drosophila* have led to the discovery of regulatory proteins that repress or promote the use of specific 3' splice sites in pre-mRNAs that are alternatively spliced. The sex-

lethal (*Sxl*) gene in *Drosophila* governs sexual development by controlling pre-mRNA splicing of specific genes (for review see Mattox et al., 1992). The Sxl protein has a female-specific activity that binds its own pre-mRNA in a positive autoregulatory loop that maintains high levels of the protein in females (Cline et al., 1984). The Sxl protein also activates female-specific production of the Transformer protein Tra (Boggs et al., 1987; McKeown et al., 1988). In both cases, regulation is based on Sxl-dependant blockage of a polypyrimidine tract in the default male 3' splice site (Inoue et al., 1990; Sosnowski et al.,1989) which in turn promotes the use of the competing downstream 3' splice site. The Tra and Tra 2 proteins are then required to cause female-specific splicing of transcripts of the *doublesex* (*dsx*) gene, acting in tandem to activate a specific 3' splice site in *doublesex* (*dsx*) pre-mRNA (Nagoshi et al., 1988; McKeown et al., 1988; Hedley and Maniatis, 1991; Hoshijima et al., 1991; Ryner and Baker 1991). Both proteins contain arginine-serine-(RS) rich motifs and Tra 2 contains a ribonucleoprotein consensus sequence (RNP-CS; Boggs et al., 1987; Amrein et al., 1988; Goralski et al., 1989). The human paraneoplastic encephelomyelitis antigen, HuD demonstrates high sequence homology with the Sxl protein and possesses three potential RNA recognition motifs (Szabo et al., 1991). This suggests HuD plays an important role in neuronal-specific splicing events. In addition to Sxl itself, the *snf* gene also regulates *Sxl* splicing (Oliver et al., 1988). Interestingly, the product of the *snf* gene is a U1 snRNP-associated protein that demonstrates high sequence homology to the human U1A and U2B" snRNP proteins (Flickinger and Salz, 1994).

Splicing of *doublesex* (*dsx*) pre-mRNA is regulated positively by the dsx repeat element (dsxRE) which consists of six 13 nucleotide repeats (Burtis and Baker 1989) located 260 nucleotides downstream of the splice site it regulates (Nagoshi and Baker, 1990; Hedley and Maniatis, 1991; Hoshijima et al., 1991; Ryner and Baker , 1989). The repeated sequence is required for the binding of both Tra and Tra 2 to RNA in vitro (Tian and Maniatis, 1992; Inoue et al., 1992). In addition, the binding of Tra to these repeats requires other nuclear proteins and both proteins act to recruit other splicing factors such as SR proteins to the repeat sequence (Tian and Maniatis, 1992,1993). The dsxRE has been demonstrated to function as a Tra and Tra2-

independant constitutive splicing enhancer when located within 150 nucleotides of the female-specific 3' splice site by virtue of its interaction with SR proteins (FIG.1.5.-Tian and Maniatis, 1994). Tra and Tra2 allow the dsxRE to function at a distance from the 3' splice site by stabilising the interaction of SR proteins with the dsxRE and U2AF with the polypyrimidine tract.

The RNP-CS of Tra 2 is essential for male fertility, control of female-specific *dsx* splicing and binding to the doublesex pre-mRNA and its own pre-mRNA. In addition, one of the RS domains of Tra2 (RS2) cooperates with the RNP-CS in RNA binding and is essential for *dsx* splicing and autoregulation. This domain is involved in protein-protein interactions with itself, with Tra and with the SR protein SF2 (Amrein et al., 1994).

Another *Drosophila* gene, the *suppressor of white apricot* (*Su* (*wa*)) gene encodes a protein that influences alternative splicing of its own pre-mRNA (Chou et al., 1987). The protein exerts an autoregulatory effect whereby it prevents the removal of the first intron of *Su* (*wa*) pre-mRNA to produce a non-functional mRNA (Zachar et al., 1987). In addition, it regulates polyadenylation and splicing of *white apricot* transcripts (Zachar et al., 1985).

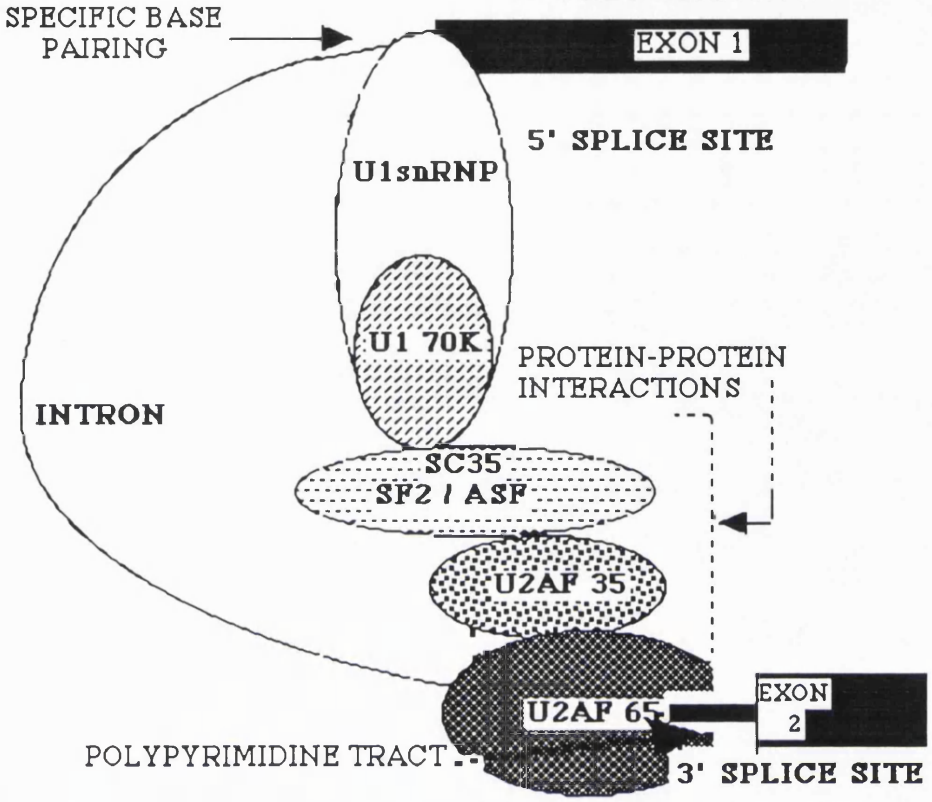
Splicing of the third intron from *Drosophila* P element transposase transcripts is repressed in somatic cells by an activity that is proposed to affect the interaction between the U1 snRNP and the authentic 5 ' splice site by stabilizing binding of the U1 snRNP to a usually inactive splice site (Laski et al., 1986; Siebel and Rio, 1990; Siebel et al., 1992). Recent studies have identified a 97 kDa protein termed the P-element somatic inhibitor (PSI) that interacts with a 5' exon inhibitory element that represses splicing of the third intron (Siebel et al., 1994). In addition, the a 50kDa protein (hrp48) also interacts with the inhibitory element and demonstrates homology with the mammalian hnRNPA1 splicing factor. It is proposed that PSI regulates tissue-specific alternative splicing via interactions with generally expressed splicing factors such as the hrp48 protein.

An example of a cell-type-specific RNA splicing factor is the yeast MER1 protein which is expressed only in meitotic cells and is required for the efficient splicing of the MER2 gene primary transcipt (Nandabalan et al., 1993). The requirement for MER1 in the splicing of MER2 is complemented by mutations in the U1 snRNA gene SNR19 which allows U1 snRNA to base pair

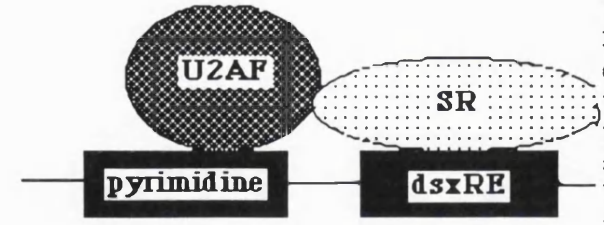
with the an intronic nucleotide in MER2 pre-mRNA that lies outside the 5' splice site. In addition, mutation of the MER2 non-consensus 5' splice site (GUUCGU) back to consensus (GUAYGU) resulting in enhanced base pairing with U1 bypasses the requirement for MER1. This demonstrates that such *trans* -acting splicing factors may affect splicing of pre-mRNAs by stabilising base pairing between U1 snRNP and non-canonical 5' splice sites.

FIG.1.5.) SR PROTEINS IN CONSTITUTIVE AND REGULATED SPLICING

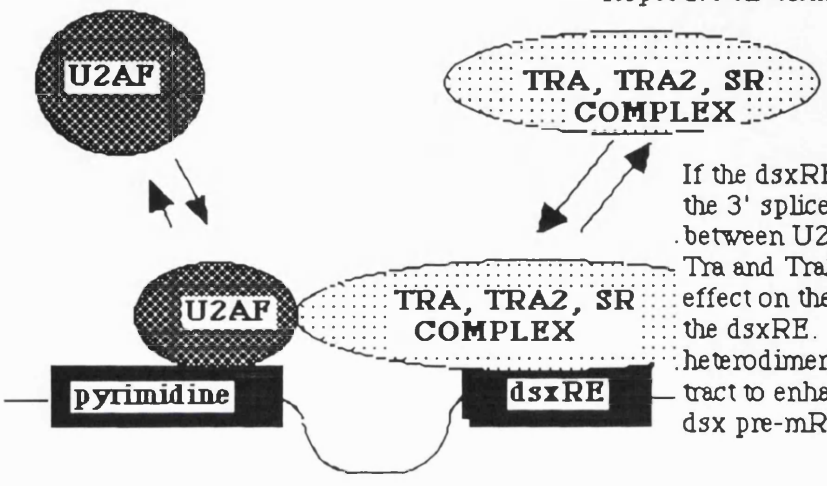
A) MODEL OF 5' AND 3' SPLICE SITE ASSOCIATION MEDIATED BY SR PROTEINS (Wu and Maniatis. 1993)



B) MODEL OF CONSTITUTIVE AND REGULATED ACTIVITIES OF THE dsx REPEAT ELEMENT (dsxRE) SPLICING ENHANCER AND SR PROTEINS IN DOUBLESEX SPLICING (Tian and Maniatis, 1994)



When dsxRE is within 150 nt of 3' splice site it performs as a constitutive splicing enhancer, whereby the close proximity of the pyrimidine tract and the dsxRE allow cooperative interaction between U2AF and SR proteins. This stabilises the association of each protein to the respective cis-element.



If the dsxRE is at a greater distance from the 3' splice site, cooperative interaction between U2AF and SR protein requires Tra and Tra2 which confer a stabilising effect on the binding of SR proteins to the dsxRE. This in turn stabilises U2AF heterodimer binding of the pyrimidine tract to enhance sex-specific splicing of dsx pre-mRNA

1.12. THE HIGHLY HOMOLOGOUS SmB,SmB', AND SmN PROTEINS

Anti-Sm antibodies from patients with the autoimmune disease systemic lupus erythematosus (SLE) patients immunoprecipitate snRNP particles by virtue of their binding of the immunoreactive SmB, SmB', SmD proteins (Lerner and Steitz, 1979; Tan, 1982). Sequence analysis of cDNA clones of the core snRNP SmB protein, and the SmB' proteins from rat (Sm11-Li et al.,1989) and from humans (Ohosone et al.,1989; van Dam et al., 1989; Elkon et al., 1990) together with PCR analysis of genomic DNA from HeLa cells (Chu and Elkon, 1991) demonstrate SmB and B' to be derived from the same gene by alternative splicing. The SmB' protein differs from SmB in that it is not found in rodent tissues (Sharpe et al., 1989a; Schmauss and Lerner, 1990) and SmB' mRNA differs from SmB mRNA in that it lacks a 146 nucleotide insert in the 3' of the open reading frame that contains a termination codon. Thus, the SmB' protein is larger comprising 240 amino acids with a predicted molecular weight of 24.6 kDa (Van Dam et al., 1989; Elkon et al., 1990) whilst 231 amino acids in SmB gives a predicted molecular weight of 23.7 kDa (van Dam et al., 1989). Interestingly, immunoblots and immunoprecipitations studies of human cortex do not detect SmB' whilst SmB is still detectable, suggesting SmB' exhibits tissue specific distribution (McAllister et al., 1989).

Autoantibodies from SLE also identify a 29 kDa Sm protein-SmN that demonstrates tissue-specific expression in brain and to a lesser extent in heart and is virtually undetectable in other tissues (McAllister et al., 1988; McAllister et al.,1989). Sequence comparisons of rodent SmN cDNA clones (rat-McAllister et al., 1989; rat-Sm51 Li et al., 1989; mouse-Gerelli et al., 1991) and human SmN clones (Rockeach et al., 1989; Sharpe et al., 1989b; Schmauss et al., 1989) and between rat and human SmB clones (van Dam et al., 1989) demonstrate 100% conservation between rodents and humans for both SmN and SmB, indicating an important function for these proteins. Comparison with SmB/B' sequences (van Dam et al., 1989; Schmauss et al., 1989) demonstrate SmN to be 81% homologous at the nucleotide level, 93% homologous at the amino acid level and that these highly homologous proteins are derived from distinct genes. SmN, like

SmB' contains 240 amino acids (van Dam et al., 1989) and has a similar pI as the SmB protein, but is more acidic than SmB' as demonstrated by two-dimensional gel analysis (Sharpe et al., 1989a). In contrast, the SmD protein bears little or no homology to SmB/B' or SmN (Rockeach, 1988). Overall there are 17 amino acid substitutions in SmN, mostly in the carboxyl terminal. A common polymorphism is the substitution of a valine or isoleucine in SmN to a methionine residue in SmB/B'. Two regions of least sequence homology between SmN and SmB/B' occur at residues 179-190 where there are five substitutions in SmN and 203-213 where there are four substitutions (van Dam et al., 1989).

Results from *in-situ* hybridisation studies show that SmN mRNA is expressed at different levels in different brain regions, being found at high levels in neurons but being absent from glial or other non-neuronal cells (Schmauss et al., 1992; Horn et al., 1992). SmN is expressed in the rodent neuronal cell lines ND7, but at lower levels to that observed in the brain (Horn et al., 1992). SmN is also expressed in certain HeLa cells (Sharpe et al., 1990b).

Further expression studies reveal SmN is expressed in F9, PCC4, and PCC3 (murine) and tera 2 (human) embryonal carcinoma cells (Sharpe et al., 1989a). Furthermore, SmN expression increases on differentiation of such cells (Sharpe et al., 1990a). The expression of SmN demonstrates developmental regulation in rodent brain, increasing from embryonic day 16 to eventually predominate over SmB expression by the day 2 post-natal stage (Grimaldi et al., 1993). Thereafter, SmB levels in the brain are negligible. In contrast, Western blotting analysis of a human brain developmental series up to 17 weeks of gestation does not demonstrate developmental regulation of SmN (D.Horn-PhD thesis). SmN is not developmentally regulated in rodent heart, but is however identical to the brain and embryonic forms of the protein (Gerelli et al., 1993). Differences may occur in the distribution of SmN between the atria and ventricle (D.Horn-PhD thesis).

The mouse SmN gene, Snrpn is located on chromosome 7 (Leff et al., 1992). The human SmN gene, SNRPN located on chromosome 15 (FIG.1.6.-Ozcelik et al., 1992) contains eight exons, the first of which is non-coding (Schmauss et al., 1992) and is transcribed from centromere

to telomere (Buiting et al., 1993). The high homology between SNRPN and the SmB/B' gene SNRPB suggest that they emerged from a common ancestral gene via a duplication event. The human SmN pseudogene SNRPNP1 is located on chromosome 6 in humans (Ozcelik et al., 1992). The mouse pseudogene is located on chromosome fourteen (Leff et al., 1992), and has been demonstrated to be intronless (Grimaldi et al., 1992; Leff et al., 1992). The mouse pseudogene is processed but contains a stop codon after thirty one amino acids (Grimaldi et al., 1992).

It remains to be resolved why three such highly homologous snRNP proteins (SmN,SmB/B') exist that exhibit different species-specific and tissue-specific expression patterns to each other. The differences in sequences between SmB' and SmB and moreover between SmN and the constitutive SmB suggest that these proteins may possess additional functions in addition to the constitutive splicing function of SmB.

The 17 amino acid substitutions in SmN taken together with the tissue-specific expression pattern of SmN suggests the nature of RNA splicing performed in SmN expressing tissues or cell lines may be different to that performed in those expressing only SmB. On the basis of its expression pattern, SmN has been postulated to play a role in regulating splicing of pre-mRNAs which have alternatively spliced forms in SmN-containing tissues (this is discussed further in the introduction of chapter 5). The replacement of SmB by SmN in the brain and the strong evolutionary conservation would suggest that SmN performs a critical role in tissues expressing it. In agreement with this, SmN is also involved differently in two distinct human diseases. Thus a lack of SmN expression is proposed contribute to the phenotype of a neurogenetic disorder and in addition, SmN is the target of circulating autoantibodies generated in autoimmune disease.

1.13. SmN IS IMPLICATED IN PRADER-WILLI SYNDROME

Prader-Willi syndrome (PWS) is a human neurogenetic disorder (Cassidy, 1984), characterised by infantile hypotonia, physical and mental retardation hypogonadism and hyperphagia with subsequent obesity (Cassidy and Ledbetter 1989). A clinically distinct

syndrome termed Angelman syndrome (AS) is characterised by severe mental retardation, microcephaly, and seizures and like PWS, is caused by a loss of function of closely linked genes on chromosome 15 (Clayton-Smith, 1992-refer to FIG.1.6.). Between 70-80% of PWS patients exhibit a paternal deletion of chromosome 15q11-15q12 (Robinson et al., 1991; Mascari et al., 1992; Nicholls et al., 1989a), whereas 70-80% of Angelman patients exhibit a maternal deletions for this region (Knoll et al., 1991; Magenis, 1987).

Alternatively, maternal uniparental disomy (UPD) for 15q11-12 occurs in 12-35% of PWS patients, whilst paternal UPD is present in 2-3% of AS patients (Nicholls et al., 1989b; Knoll et al., 1991; Mutirangura et al., 1993). These parent-of-origin effects on inheritance suggests symptoms for both syndromes are a legacy of the effects of genomic imprinting in conjunction with the loss of the paternally expressed genes within 15q11-12 for PWS and the loss of the maternally expressed genes within the region for AS (Nicholls 1993-for reviews on imprinting see Varmuza and Mann ,1994; Moore and Haig, 1991; Razin and Cedar 1994). Rare cases for both syndromes have narrowed the critical region to 2 Mb and established the PWS region as being centromeric to AS. Genes mapping within the smallest regions of deletion overlap for PWS include the anonymous DNA marker PW71 (D15S63), which shows parent of origin-specific DNA methylation (Dittrich et al., 1992) and SNRPN which encodes snRNP-associated protein SmN (Ozcelik et al., 1992).

The murine homolog Snrpn maps to a central region of chromosome 7 which is syntenically conserved with the proximal region of human chromosome 15 (FIG.1.6.-Leff et al., 1992). Moreover, murine models of PWS with maternal duplications of central 7 demonstrate that the expression of Snrpn is exclusively from the paternal chromosome as Northern blot analysis shows an absence of Snrpn mRNA expression in the brain (Cattanach et al., 1992). The suckling difficulties observed in these mice reflect the feeding difficulties experienced in PWS. This functional imprinting is also demonstrated for SNRPN in humans (Glenn et al., 1993; Nakao et al., 1994; Reed and Leff 1994). The lack of SNRPN expression observed in cultured skin

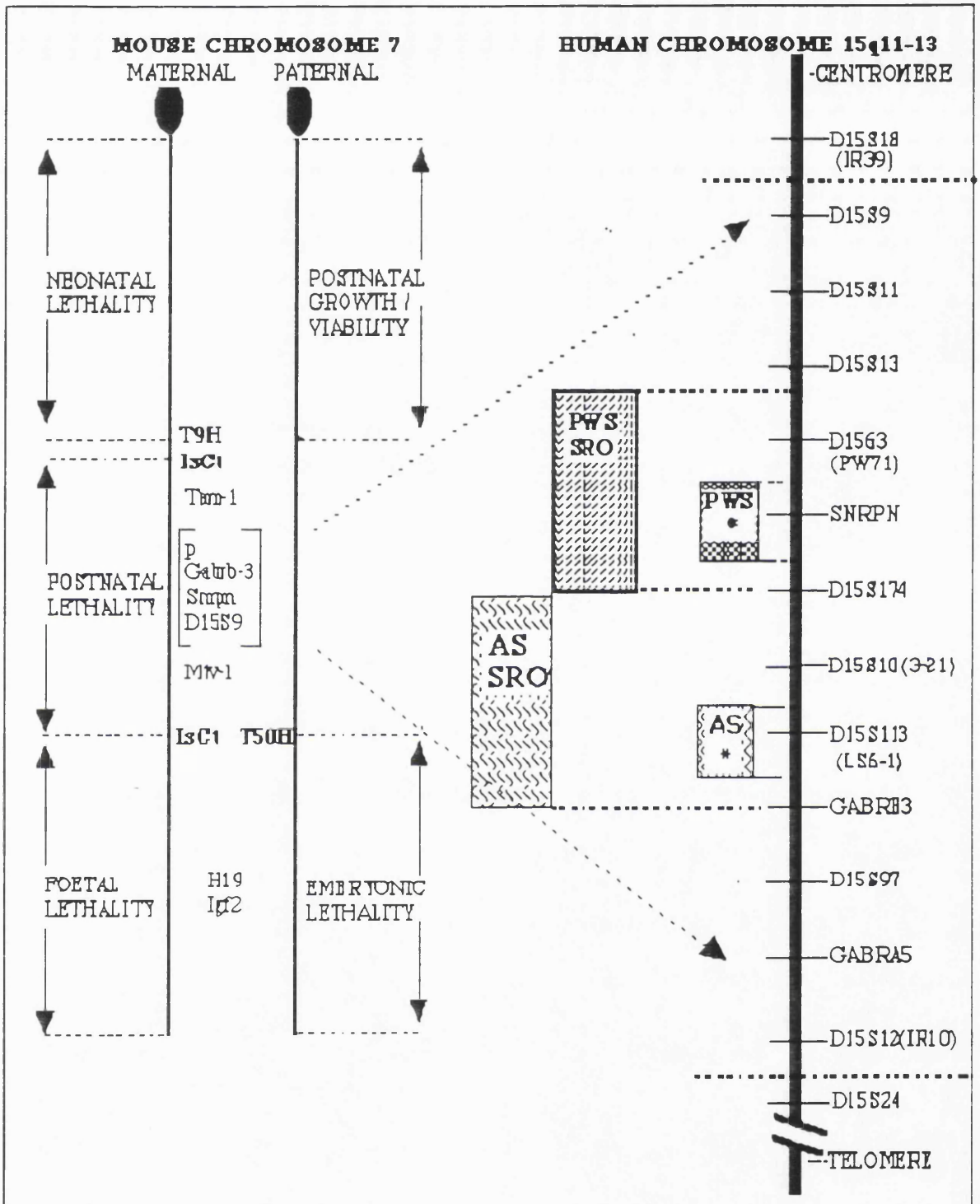
fibroblasts from PWS patients suggests this imprinting effect is not relaxed (Glenn et al., 1993) and this therefore strongly implicates SNRPN in being responsible for the PWS phenotype.

SNRPN exhibits a parent-specific DNA-methylation imprint in intron 5, whereby it is methylated on the paternal allele (Glenn et al., 1993). This is consistent with other imprinted genes H19, insulin-like growth factor 2 (Igf-2) and the Igf-2 receptor (Igf-2r) and further demonstrates that DNA methylation plays a critical role in genomic imprinting (for review see Razin and Cedar, 1994). Recently, two PWS siblings have been reported with a deletion of less than 300 kb that encompasses SNRPN but not any other of the marker loci in the critical region which strongly implicates SNRPN in PWS (FIG.1.6.-Reis et al., 1994). The deletions observed in these patients include sequences that determine the methylation status of adjacent imprinted genes such as PW 71 (Butling et al., 1994), and hence abnormal patterns of DNA methylation may also be responsible for the phenotype observed in these patients.

The use of restriction landmark genomic cloning (RLGS) with methylation-sensitive enzymes identifies a maternally imprinted gene which demonsrates significant homology to the 35 kDa subunit of U2 snRNP auxillary factor (U2AF-Hayashizaki et al., 1994). The U2AF35 gene (U2afbp-rs) maps to the same region as the Gabra-1 locus on proximal chromosome 11 whilst Snrpn maps to the Gabrb-3 locus on chromosome 7. The similarities in imprinting, mapping and the function of SmN and U2AF35 suggest a duplication event during evolution and that the two splicing factors may be evolutionarily related. In addition, uniparental disomy for proximal chromosome 11 in mice results in a similar phenotype to that observed in murine models of PWS (Cattanach and Kirk, 1985; Cattanach et al., 1992). This suggests that U2afbp-rs in humans may map to a region where UPD occurs and thus affects the phenoype as observed for PWS. Furthermore, it is possible that phenotypes caused by UPD in other regions (e.g. Wiedemann-Beckwith syndrome-11p15.5- Henry et al., 1993) may be due to imprinting effects active on other regions that may also include proteins involved in RNA processing.

The putative involvment of SmN in the PWS phenotype suggests that SmN plays a critical role in the tissues expressing it. This putative critical role for SmN is also suggested by the early

post-natal lethality of mice models of PWS at developmental stage when SmN expression begins to predominate over SmB expression (Grimaldi et al., 1993; Kitsberg et al., 1993). This role may be in the alternative splicing of neural genes or may reflect an essential but more constitutive function of SmN that is performed by highly homologous SmB in other tissues. The mild neurological symptons of PWS suggest sufficient levels of SmB may be expressed in the brain to perform some degree of pre-mRNA splicing whilst SmN may possess unique properties that manifest in the phenotype. However, it still remains possible that other imprinted genes within the critical region may also be involved.



Adapted from Cattanach et al., 1992; Glenn et al., 1993; Buitling et al., 1993; Buxton et al., 1994. The shortest regions of deletion overlap (SRO) for Prader-Willi Syndrome and Angelman syndrome (AS) are indicated. The region PWS* corresponds to a deletion in two PWS siblings which encompasses only SNRPN (Reis et al., 1994). The region AS* corresponds to a 200 kb maternal deletion that encompasses only the LS6-1 marker (Buxton et al., 1994). The precise order of genes on the mouse chromosome is not resolved and the markers on the human chromosome are not drawn to scale. The imprinting effects obtained with maternal and paternal duplications for region of the mouse chromosome (vertical arrows) are indicated.

FIG.1.6. SYNTENICALLY CONSERVED REGIONS OF THE HUMAN AND MOUSE CHROMOSOMES CONTAINING THE GENE ENCODING SmN

1.14. SYSTEMIC LUPUS ERYTHEMATOSUS (SLE).

The human autoimmune disease SLE is a multi-symptomatic disease and this diversity of symptons makes diagnosis difficult. In view of this, diagnosis is based on qualification for 4 or more clinical criteria as proposed in the revised criteria for the classification of SLE (Tan et al., 1982). In the U.K. patients are assessed by the British Isles Lupus Assessment Group system (BILAG) which measures disease activity in 8 organ systems.

Such symptons include the distinctive facial butterfly rash of fixed erythema and a photosensitive discoid rash. Non-erosive arthritis, serositis, pericarditis, pleuritis in addition to renal disorder caused by deposition of immune complexes in the kidney are further symptoms. The CNS is involved in 40% of SLE pateints (VanDam, 1990; Hanley et al., 1994) and involvement can be diffuse (generalised seizures, psychosis) or focal (stroke, peripheral neuropathies). Further criteria include hematological disorders such as hemolytic anemia, leukopenia , lymphopenia, and a number of immunologic disorders which are useful diagnostically and are characterised by the presence of circulating autoantibodies against DNA and to protein nuclear antigens.

1.15. AUTOANTIBODIES IN SLE

The earliest diagnostic assays used for lupus were immunofluorescence (Friou, 1957), double diffusion (Ouchterlony, 1949), and counterimmunoelectrophoresis (Kurata and Tan, 1976) techniques which analysed the circulating autoantibodies.

Antibodies against DNA are common in rheumatic diseases, however antibodies against double-stranded DNA occur specifically in SLE with a prevalence of 80% and are therefore used in the specific diagnosis of SLE (Northway and Tan, 1972; Tan, 1982). B-cell hyperactivity appears to play an important role in the origin of such antibodies (Klinman and Steinberg, 1987).

Antibodies to the snRNP-associated Sm antigens (Lerner and Steitz 1979) are used widely as diagnostic markers specific for SLE and are not generated in other autoimmune diseases

(Scopelitis et al., 1980; Barada, 1981). The prevalence of such antibodies is reported as 30% (Notman et al, 1975) and anti-Sm antibodies are frequently found in conjunction with antibodies to the U1-snRNP specific RNP antigens A,C and 70 kDa proteins. Thus anti-Sm positive sera immunoprecipitate all snRNPs with the common core proteins, whilst anti-RNP sera precipitate U1 snRNPs only (Lerner and Steitz, 1979; Hinterburger and Steitz, 1983). Antibodies to SmB/B'/N/D and anti-RNP antibodies have been demonstrated to inhibit splicing and RNA polyadenylation in *in-vitro* systems (Padgett et al., 1983; Moore et al., 1984). Longitudinal studies suggest that antibodies to the Sm proteins do not occur simultaneously and that a sequential pattern of reactivity initially to SmD then B/B' and E occurs (Borg et al., 1988). Levels of antibodies to Sm and RNP antigens fluctuate and temporal shifts in the emphasis of reactivity against Sm or RNP antigens occur in connective tissue diseases (Forman et al., 1985b; Fischer et al., 1985b). The occurence of RNP autoantibodies in the absence of Sm reactivity together with a different genetic background established mixed connective tissue disease MCTD as a separate entity from SLE (Combe, 1989; Sharp et al., 1982). Antibodies against U1-snRNP-specific 70 kDa protein are prevalent in this disease and are used in its diagnosis (Habets et al., 1985; Combe, 1989). Extensive cross-reactivity of autoantibodies occurs between Sm and RNP antigens (Lerner et al., 1981; Wiliams et al., 1986 a/c). These are a result of similar antigenic sequences, for example the PPGM(R/I)PP sequence which occurs twice in SmB three times in SmB' and SmN and once in the U1-A protein (Williams et al., 1990; Habets et al., 1989b). A similar sequence-PAPGMRPP is found in the C protein. However, the isolation of antibodies specific to particular snRNP proteins, from murine models of lupus (SmD-Billings et al., 1985; Williams et al., 1986a) is probably reflected in human SLE. In addition to antibodies against DNA and proteins, autoantibodies against the RNA moiety of snRNPs have been described, specifically against the U1 RNA (Deutscher and Keene, 1988; Venrooij et al., 1990; Hoet et al., 1993). These antibodies are directed against stemloop II and stemloop IV of U1-snRNA (Hoet et al., 1993).

1.16. MURINE MODELS OF SLE

The MRL congenic mouse strains (for review see Cohen and Eisenberg, 1991) closely resemble human SLE in that they produce autoantibodies against Sm antigens (Eisenberg et al., 1978). Thus 25-30% of MRL/MpJ lpr/lpr (lpr) mouse strain develop anti-Sm antibodies and a lupus-like disease by 5 months of age. The lymphoproliferative disorder confered by the *lpr* gene in such mice is a result of defects in apoptosis as mediated by the Fas antigen (Watanabe-Fukunanga 1992). Another MRL strain designated MRL/MpJ-+/+ (+/+) also develop an anti-Sm response but the onset is less rapid however, both strains develop severe glomerulonephritis characterised by infiltration of mononuclear cells and immune complex deposition (Andrews et al., 1978).

Monoclonal antibodies (KSm series) derived from the MRL/lpr strain (Williams et al., 1986a) demonstrate high affinity binding for specific or cross-reactive epitopes on SmB/B'/N and SmD proteins. The epitope bound by the anti-Sm B/B'/N specific KSm5 monoclonal is proline-rich and is also bound by anti-Sm positive human SLE sera, further demonstrating the MRL strain as an effective model of SLE (Williams et al., 1990). Interestingly, in the MRL/lpr and +/+ strain, a high titer anti-Sm response is elicited by administration of the KSm2 anti-Sm D monoclonal antibody but not by the KSm5 antibody (Stocks et al., 1991; Stocks et al., 1987; Eisenberg et al., 1987). The addition of purified Sm antigen to spleen cells from MRL/ lpr mice (Shores et al., 1986) and the treatment of MRL/lpr mice with Sm antigen (Stocks et al., 1991) both significantly increase anti-Sm antibodies, suggesting the anti-Sm response is antigen driven. It has been suggested that administration of anti-Sm antibodies to lpr mice confers a protective effect and increases life-span (Brennan et al., 1986) Antibodies specific for RNP antigens are found in only 3% of MRL/lpr mice (Willliams et al., 1986b).

Recently, a further murine model of SLE, ((SWR x SJL)F1) has been described in which anti-Sm / U1 snRNP antibodies are found as early as 10 weeks of age and increases to 70% by 40

weeks (Vidal et al., 1994). Anti-double stranded DNA antibodies are produced later on and these correlate well with the presence of proteinuria and immunocomplex nephropathy.

Immunisation of normal non-autoimmune mice with human snRNP components supports a model of molecular mimicry in SLE whereby autoantigen presenting B cells are generated by such foreign determinants which in turn elicits an autoimmune T cell response (Mamula et al., 1994).

1.17. AIMS

To date, no specific function has been assigned to the SmN protein. In order to determine whether the amino acid substitutions present in SmN confer unique properties to SmN that subsequently affect the nature of RNA splicing performed in tissues expressing the protein, further investigation is required. Thus investigations of SmN as an autoantigen in SLE would both determine the role of the protein in autoimmune disease and would also reveal any details of the conformation of the protein that are distinct from SmB/B' which would be indicative of a unique function for SmN. In addition, an investigation of the localisation of the SmN protein within snRNPs in different cells and tissues would be valuable to assess whether the protein could affect RNA splicing by nature of its association with snRNPs. The putative role of SmN in alternative splicing and furthermore, the involvement of the protein in human disease may be investigated using a murine model of Prader-Willi syndrome.

CHAPTER 2
MATERIALS AND METHODS

MATERIALS AND METHODS

2.1. PEPTIDES

Peptides representative of the two regions of least amino acid sequence homology between SmN and SmB/B' (179-190 and 203-213, FIG.3.1) were synthesized using FMOC chemistry by Graham Wallace (Department of Medical Parasitology, London School of Hygiene and Tropical medicine, London, UK). Peptides included an additional C-terminal cysteine for coupling to the carrier proteins for the purposes of immunisation and screening.

2.2. PREPARATION OF PEPTIDE-CARRIER CONJUGATES

Peptides were conjugated to immunogenic carrier proteins via the additional cysteine residue included on synthesis. Carrier proteins (20 mg) Keyhole Limpet Haemocyanin (KLH) and Bovine Serum Albumin (BSA), (Imject- PIERCE UK Ltd.) were dissolved in 5 mls of Phosphate Buffered Saline (PBS) and dialysed using a 12-14000 Molecular Weight cut-off membrane against 5 litres of PBS overnight at 4 C with stirring. The amount of dissolved protein of was analysed using a BioRad protein assay kit and reading against a standard BSA curve ranging from 0.05-0.8 mg/ml. Solid Sulfo-SMCC linking reagent (PIERCE UK Ltd.) was added to the carrier protein at 1 mg per 4 mg of carrier, mixed at 30 C for 1 hour and dialysed overnight at 4 C against Tris-HCl 0.1M pH 7.0; 1mM MgCl2; 0.1 mM ZnCl2. Using BioRad protein assay data, the number of moles of carrier protein was calculated, multiplied by the number of reactive sites per carrier (30 for each), and multiplied by the weight of each peptide (1430 or 1320 Da). This calculated amount of solid peptide was added to carrier/ Sulfo-SMCC and mixed at 4 C overnight. Remaining reactive sites were blocked with 0.1 M 2-mercaptoethylamine (10 µl per 8 mg carrier) and excess removed by dialysis against PBS at 4 C overnight. The success of the conjugation was analysed on the basis of an altered molecular weight in comparison to unconjugated carrier protein. Conjugates (30µl) dissolved 1: 2 in non-reducing sample buffer (see Western blotting) were loaded onto a 7.5% gel.

2.3. IMMUNISATION REGIME

The BALB/C mice used were obtained from colonies maintained at the Kennedy Institute Biological Services Unit. For initial immunisations, mice were injected subcutaneously with an emulsion containing 100 µg of peptide-carrier conjugate in 100 µl Freund's complete adjuvant. Subsequent immunisations were performed every 14 days using 100µg antigen and 100 µl Freund's incomplete adjuvant. After 3 immunisations, mice were tail-bled, the resultant blood being kept at room temperature for one hour and microfuged at 13500 rpm to separate the serum.

2.4. GENERATION OF SmB/B' 174-213-GST FUSION PROTEIN

The regions of least sequence homology between SmN and SmB occurring between residues 179-190 and 203-213 were chosen as inclusive sequences from which short fusion proteins for SmN and SmB could be made for the production of polyclonal antibodies specific for SmN or SmB and for ELISA screening of SLE sera. Primers for PCR were designed on a DNA-star sequence analyser to encompass the amino acid residues 174-213 which contains 10 amino acid replacements between SmN and SmB. Only the SmB 174-213 fusion protein was generated and was used in a diagnostic ELISA for SLE.

FORWARD PRIMER SEQUENCES.

	BamH1 site
SmB	CCTGGC|GGATCC|GGTCCTCCCCCACCTATG
SmN	CCAGGA|GGATCC|ACTCCGCCCCCACCCCTC

BACKWARD PRIMER SEQUENCES:

	EcoR1 site
SmB	AGGGGG|GAATTC|CATTGGAGTCCCTCTTCC
SmN	AGGGGG|GAATTC|TATTGGCGTCCCTCGAGC

2.5. POLYMERASE CHAIN REACTION

Separate reactions were performed for SmB and SmN using 100ng of each primer and 10ng of the respective cDNAs. For SmB, cDNA was a kind gift of A. P.van Dam (van Dam et al., 1989). For SmN, cDNA clone PP1 was used (Sharpe, N.G., et al., 1989). The reaction was carried out under silicone oil (B.P.) for 30 cycles at 94 C for 2 minutes, annealed at 42 C for 1.6 minutes and extended at 72 C for 2 minutes using 0.5 units of *Amplitaq*. DNA polymerase (Perkin Elmer Cetus), 1X reaction buffer (10mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% w/v gelatin) and 200 μM of each deoxynucleotidetriphosphate. PCR products (10 μL in 2 μL PCR sample buffer) were then electrophoresed on a 1 % agarose gel containing ethidium bromide with reference to 1kb DNA markers (Gibco BRL) and control PCR reactions in which the cDNA template was replaced with water . Gel fragments containing bands of the appropriate size were cut out of the gel with a sterile scalpel and placed in a Millipore Minicolumn. The columns were then placed in liquid nitrogen for 10 minutes and then spun at 13500 rpm for 5 minutes. The DNA was precipitated on ice for 1 hour using 2 volumes of ice cold ethanol, 1/10 volume of 3M Sodium acetate before spinning at 13500 for 12 minutes at 0C, removing excess ethanol with a vacuum pump and vacuum dessication. DNA was resuspended in 15 μL Tris-EDTA buffer pH 8.3 (9mM Tris, 9 mM orthoboric acid, 0.2 mM EDTA, pH 8.3).

2.6. RESTRICTION ENZYME DIGESTS

Purified PCR fragments were digested overnight at 37 C under silicone oil in a 15 μL total reaction volume comprised of 11 μL purified PCR fragment, 3 μL 10X One- Phor-All buffer, and 1 unit each of BamH1 and EcoR1 (Boehringer Mannheim).

2.7. LIGATION

Digested fragments were gel purified as above, resuspended in 10μLTris-EDTA buffer and ligated for 4 hours at 16 C into BamH1/EcoR1 digested pGUC2 vector (a kind gift of Declan McEever, I.L.R.A.D., Nairobi, Kenya) using 0.4 units of T4 DNA ligase (Boehringer Maneim), 2 μL 5X Focus buffer or the appropriate Gibco BRL REact buffer, and 2 μL of digested fragment in a 10 μL reaction volume.

2.8. TRANSFORMATION OF LIGATIONS INTO E.COLI TG1

Glycerol preserved TG1 E.coli (B. Edwards) were innoculated into 30 mls of LB (Luria-Bertani medium-1% (w/v) bactotryptone (Difco laboratories), 0.5% (w/v) bacto yeast extract, (Difco) 170 mM sodium chloride pH7) and cultured with shaking at 37 C until early log-phase. Cells were then diluted 1/20 in LB and incubated at 37 C with shaking for a further 1.5 hours. Cells were harvested in 50 ml Falcon tubes by centrifugation at 1000g for 10 minutes at 4 C, the media discarded and the tubes drained by inversion. Pellets were resuspended in 25 mls ice-cold 100mM calcium chloride, then harvested at 1000 g for 5 minutes at 4 C. The cells were resuspended by mixing with 2.5 mls of 100mM calcium chloride and either diluted with 0.5 volumes of glycerol for storage at -70 C or competent cells (200 µL per reaction) were transformed on ice for 45 minutes using 2µL of the ligation mixture, after which cells were heat shocked for 90 seconds at 42 C and chilled on ice for 2 minutes. Cells were diluted in 800µL of LB, left at 37 C for a further 45 minutes, pelleted and resuspended in 200 µL of LB, and then plated out and incubated at 37 C overnight on 1% agarose LB plates containing 100 µg/ml Ampicillin.

The success of the transformation was analysed with reference to controls by simultaneous transformations with p-hen supercoiled plasmid, undigested pGUC2 vector, and restricted but unligated pGUC2. Positive colonies were innoculated into 30 mls LB, incubated at 37 C overnight and then pelleted for analysis by Western blotting of total protein.

2.9. ENZYME -LINKED IMMUNOSORBANT ASSAY (ELISA)

PREPARATION OF ELISA PLATES AND ANTI-PEPTIDE ANTIBODY ELISA PROTOCOL FOR SCREENING HUMAN AND HYPERIMMUNE MOUSE SERA

Peptide-Bovine Serum Albumin conjugates (50 µl) at 10 µg/ ml in PBS were incubated for a minimum of 24 hours at 4 C on 96-well polystyrene microtitre plates (IMMULON 2, Dynatech), sealed with plastic plate sealers (Dynatech, U.K.). Prior to use, plates were washed 5 times with Tween 20 (0.5 % in PBS) followed by one wash with PBS. This washing procedure was repeated between all incubations, which were performed in duplicate for one hour at 37 C at a

volume of 50 µl per well. Plates were blocked with casein (2 % w/v in PBS), followed by incubation with sera (1: 100 in Tween 20 (0.05 % in PBS)) and finally with anti-mouse or anti-human IgG alkaline phosphatase conjugate (Sigma, Poole, U.K.) diluted 1:1000 in PBS. Antibody binding was detected at O.D. 405 nm by measuring colour development following an overnight incubation at room temperature using p-nitrophenyl phosphate (Sigma 104), diluted to 1 mg/ml in 0.1M glycine, 1mM MgCl2, 0.1 mM ZnSO4, pH 10.4. The O.D. 405 was read on an Olivetti M24 computer and EL309 microplate reader (BIO-TEK Instruments Inc., U.S.A.). Background binding of all sera to the free carrier protein (BSA) was also measured and subtracted from the peptide-carrier binding readings. For the screening of human sera, the level of significance of binding was determined as being greater than the mean +2 s.d (0.494) of the binding recorded for the healthy group.

2.10. COMPETITIVE INHIBITION ELISA

Competitive inhibition assays were performed with human sera to determine the peptide specificity of anti-peptide antibodies by incubation of two representative SLE sera (1:50 in PBS) with an equal volume of fluid-phase unconjugated peptide over a range of concentrations up to 1000 µg/ml (800 mM) in PBS for one hour at 37 C on casein blocked flexible microwell plates (Falcon 3912 Micro Test III). The supernatants were then assayed in the anti-peptide antibody ELISA as previously described.

2.11. PATIENTS AND CONTROLS

Whole blood was taken from patients (age range 23-66 years) with SLE, each of whom met the revised criteria of the American Rheumatism Association for the classification of the disease (Tan et al., 1982). The patients were all bled at morning out-patient clinics at the Bloomsbury Rheumatology unit. Control samples were taken from age and sex matched normal healthy laboratory workers.

2.12. ANTI-Sm ANTIBODY ELISA.

Anti-Sm SLE sera were detected initially by counterimmunoelectrophoresis (CIE) using rabbit thymus extract (Williams et al., 1986c) and confirmed by quantitation using an anti-Sm ELISA

(Shield Diagnostics Ltd. Dundee U.K.) and the associated protocol. Positive anti-Sm sera were defined using the supplied positive and negative reference sera.

2.13. PREPARATION OF NUCLEI FROM BLOOD MONONUCLEAR CELLS

Blood samples were treated with 200 µL of heparin (1000 units / ml), diluted 1:1 with RPMI 1640 (Gibco), layered on to a Ficoll-Hypaque (Pharmacia) gradient and centrifuged at 1900 rpm for 20 minutes at room temperature. The buffy layer of cells were counted and washed once in RPMI and once in PBS by centrifugation at 200 g for 10 minutes at 4 C. Pellets containing 1 x 10 6 cells were resuspended in 50 µL SDS sample buffer and loaded onto SDS polyacrylamide gels.

2.14. IMMUNOPRECIPITATION

The basic immunoprecipitation protocol of Steitz. J.A. (1989) was used in all experiments. Protein A Sepharose (PAS) beads (CL-4B, Bioprocessing Ltd. CO.Durham) were washed, diluted 1:5 in NET-2 buffer (50 mM Tris, 150 mM NaCl, 0.1 % NP40 V/V) and incubated overnight at 4C with monoclonal antibody ascites or SLE serum (0.2 mls bead suspension + 40 µl antibody / sera). The beads were microfuged for 10 seconds at 13000 r.p.m. and washed 3 times with NET-2.

For immunoprecipitations performed using rat tissue, the basic protocol was modified in order to detect specifically SmN and SmB by probing the blots with the KSm5 monoclonal antibody. The KSm5 antibody was covalently cross-linked to the PAS beads by incubation with 0.25 mls 0.5% glutaraldehyde in PBS at 4 C for 30 minutes. This treatment minimised the appearance of mouse IgG on the nitrocellulose blot which allowed an anti-mouse IgG Horse Radish Peroxidase (HRP) conjugate (ICN 67-428-1) could be applied as a second layer and hence non-specific binding was significantly reduced. The beads were then washed 5 times in 0.5 ml volumes of NET-2 buffer.

The 4G3 anti-B" monoclonal did not bind PAS beads and hence required the pre-incubation of the beads with 20 µl of rabbit anti-mouse IgG (Sigma UK) for one hour followed by 5 PBS washes and then normal incubation with 4G3.

F9 cells (1x 10 5) were labelled for 24 hours with 100 Ci L-[35S] methionine (SJ 1015 in-vivo cell labelling grade, Amersham U.K. supplemented with 10% foetal calf serum FCS (Gibco BRL) and 0.11 g/L socium pyruvate. Both cell lines (F9 and ND7-grown in Leibowitz L15 medium-Gibco BRL 10% FCS 0.35% w/v sodium bicarbonate, 0.35% w/v glucose) were pelleted, washed and diluted in 2 mls of NET-2 buffer, sonicated on ice (3x 30 seconds with an MSE soniprep 150) and then centrifuged at 16000g for 30 minutes at 4 C. Whole rat tissue samples (2g / 5 mls NET-2) were homogenised on ice an then treated as the cell lysates. In order to preclear nonspecific background, the sonicates were incubated for 30 minutes at 4 C with 100 µl of a 20% suspension of PAS beads in NET-2. The sonicates were then microfuged at 13000 rpm and the beads discarded. The resulting precleared cell / /tissue supernatants were used in immunoprecipitations.

Eighty microlitres of precleared cell / tissue supernatant were added to the antibody-coated PAS beads , diluted with 0.5 mls NET-2 buffer and mixed for 1 hour at room temperature. The mixture was microfuged at 13000 r.p.m. and the supernatant discarded. The beads were then washed 5 times with NET-2 and then extracted by boiling for 3 minutes in 0.2 mls SDS sample buffer. The beads were separated by microfuging for 2 minutes at 13500 r.p.m and 25 µl of the sample solution loaded onto SDS-polyacrylamide gels.

2.15. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein samples were resolved on 12.5 % or 15% polyacrylamide gels according to the general protocol of Laemmli (1973). Gels were prepared using 140 x 120 mm glass plates with 1.5 mm spacers from the following solutions :

RESOLVING GELS

	12.5 % GEL	15 % GEL
30:1 Acrylamide: Bisacrylamide solution (National Diagnostics)	15.15 mls	18 mls
Resolving Gel Buffer	9 mls	9 mls
Distilled Water	11.85 mls	9 mls
10 % Ammonium Persulphate	200 µl	200 µl SIGMA
10 % TEMED (N,N,N',N'-tetramethylethylenediamine)	200 µl	200 µl SIGMA

Resolving gels were buffered with 1.5 M Tris, 0.4% SDS at pH 8.8.

The TEMED was added last to initiate polymerisationof the gel solution which was then mixed thoroughly, poured and then overlayed carefully with a layer of distilled water to provide a smooth interface between resolving and stacking gels. The gels were then allowed to set at room temperature for a minimum of 3 hours. The overlaying water was then removed and a common stacking gel recipe was used for all gels in association with a 15 or 20 well comb inserted horizontally between the top of the gel plates.

STACKING GEL RECIPE (QUANTITIES PER GEL)

30:1 Acrylamide: Bisacrylamide solution	2 mls
Stacking Gel Buffer	3 mls
Distilled Water	7 mls
Ammonium Persulphate	100 µl
TEMED	100 µl

Stacking gels were buffered with 0.5 M Tris , 0.4 % SDS at pH 6.8.

Fully polymerised gels were then assembled within the gel tanks and running buffer (0.05 M Tris, 0.384 M Glycine, 0.1 % w/v SDS) was added to cover the electrodes. Protein samples dissolved in SDS sample buffer (10 % glycerol, 70mM SDS, 250mM Tris, and 200 mM dithioreitol, pH 6.8 containing Bromophenol Blue tracking dye) were then loaded into wells under the running buffer, using a Hamilton 100 µl syringe. SDS-Page gels were run at 200 volts and 30 mA per gel for approximately 4 hours or until the pre-stained molecular weight markers included in one well on every gel had resolved sufficiently.

COOMASSIE STAINING OF GELS FOR TOTAL PROTEIN.

To analyse total protein configurations of resolved samples, gels were stained with 0.1 % Coomassie Brilliant Blue R in methanol / acetic acid / ditilled water (5:1:5) with agitation on a horizontal shaker. The excess staining was removed form the gels by washing in methanol / acetic acid / distilled water (3:1:6) until a clear background was obtained.

2.16. WESTERN BLOTTING

Western blotting was performed according to the general protocol of Towbin (1979). SDS-PAGE gels were removed from the glass plates and placed onto a nitrocellulose filter (Hybond-C) cut to the appropriate size, and pre-soaked in the electroblotting buffer (0.0248M Tris, 0.193M glycine, 20% methanol). Air bubbles were removed and the gel and filter were placed between two sheets of filter paper (Whatman No.1, Whatman, UK) presoaked in electroblotting buffer and the entire gel / filter / paper sandwhich was placed in the blotting casettes. Casettes were orientated in the electroblotting tanks with the nitrocellulose towards the anode, and 3 litres of electroblotting buffer were added to cover the casettes. Gels were electroblotted overnight at 210 mA, 30 volts the gels then discarded and the nitrocellulose sheet stored at 4 C in PBS with 0.01 % NaN3.

The Nitrocellulose sheet was then incubated in blocking buffer (PBS with 5% milk powder (Marvel) and 0.2% Tween 20) for one hour at room temperature. The sheet was then washed for

5 minutes in 2 changes of blocking buffer, and then incubated for one hour at room temperature with the primary antibody using a 1: 2000 dilution in blocking buffer for mouse monoclonals or a 1: 100 dilution for anti-Sm positive human SLE sera. After three washes the sheet was then incubated for one hour with a secondary horseradish peroxidase conjugated antibody at 1: 1000 dilution (anti-mouse IgG (DAKO) or anti-human IgG (H+L), ICN 67-416-1). Bound immunoglobulin was then visualised after 3 washes in PBS using either 0.5 mg/ml diaminobenzidine in PBS with 1% CoCl2, 1% ammonium nickel sulphate and 0.1 % v/v 1:60 diluted hydrogen peroxide (BDH) or by incubation with enhanced chemiluminescence reagent (ECL, Amersham, UK) followed by exposure to Hyperfilm ECL film.

2.17. MONOCLONAL ANTIBODIES USED FOR WESTERN BLOTTING AND IMMUNOPRECIPITATION

The monoclonal antibodies KSm2, KSm5, and K8.43 and KSm4 were derived from MRL mouse spleen cell hybridomas (Williams et al., 1986). KSm2 binds an epitope restricted to the 16 kDa SmD snRNP core polypeptide. KSm5 binds to a proline rich epitope restricted to the 29 kDa SmB' and SmN polypeptides and the 28 kDa SmB polypeptide. The KSm4 monoclonal binds SmB and B' but demonstrates no binding to SmN. The K8.43 monoclonal used in immunoprecipitations is specific for the U-1 restricted 70 kDa polypeptide (Williams et.al., 1986 unpublished) whilst the non anti-Sm OX-12 is an isotype matched BALB/c derived anti-rat light chain monoclonal used as a negative control. The 4G3 monoclonal, specific for the U2 snRNP restricted 28kDa B" polypeptide was a kind gift of Dr.W.J. Van Venrooij, University of Nijmegen (Habets et al., 1989).

2.18. RESOLVING SmN, SmB, and B" POLYPEPTIDES

In order to ensure that the anti-Sm positive SLE serum used to probe blots of immunoprecipitations did not blot the U2 restricted B" polypeptide and hence confuse interperatation, it was necessary to resolve the B" from SmB and SmN using five different 12.5% gels with varying ratios of to acrylamide and bisacrylamide. The acrylamide: bisacrylamide compositions (w/v) of the gels used were 1.) 30 % : 0.5% , 2.) 30% : 1 % , 3.) 30 % : 2 % , 4.)

34% : 1 %, 5.) 24 : 1% respectively. An ND7 total cell extract was resolved on the gels which were then each Western blotted in a multi-channel immunoblotting casette with the 4G3 anti B" monoclonal, the KSm5 monoclonal, and the same SLE serum used to probe immunoprecipitations.

2.19. 2-DIMENSIONAL GEL ELECTROPHORESIS

In order to study the effect on total cellular protein in cell lines expressing different levels of SmN, the Pharmacia Immobline dry strip two-dimensional gel electrophoresis system was used. Cells (1X107)were labelled with L-[35 S] methionine as in the immunoprecipitation protocol, pelleted, washed in PBS and dissolved in 50 µL lysis buffer (2% Triton X-100, 2 % 2-Mercaptoethanol, 2% ampholines pH range 3.5-10 (Pharmacia), 24.0g Urea, 70mg Phenylmethylsulfonyl fluoride per 50ml), and 50 µL sample buffer (2% 2-Mercaptoethanol, 2% Ampholine pH 3.5-10, 0.5 % Triton X-100, 24g Urea , 0.05g Bromophenol Blue per 50ml). The sample (25 µL) was loaded onto Immobiline strips (pH 3.5-10), electrophoresed under silicone oil (20: 10 centistokes) for at a total of 22650 Vh. and could then be stored at -20 C or run immediately on the second dimension 12.5% SDS-PAGE gel. This involved equilibrating each Immobline strip in 10 mls of 0.05 M Tris HCl pH 6.8, containing 1 % SDS w/v, 36% Urea w/v , 30% v/v Glycerol and 10mg dithiothreitol laying it horizontally on a specially engineered stacking gel mould and fixing it into place with molten agarose. The gel was then run as a normal SDS-PAGE gel, electroblotted onto a nitrocellulose which was then dried and exposed to Kodak β–max film.

2.20. RNA PURIFICATION

An adaptation of the single step RNA isolation method of Chomczynski and Saachi (1987) was used to prepare RNA from PBS washed cell pellets. Initially, 1X107 cells were lysed in 1 ml of denaturing solution (4M guanidinium isothiocyanate, 20mM sodium acetate , pH 5.2, 0.1 mm dithioreitol, 0.5 % N-lauroylsarcosine), and mixed by inversion with 50 µL of 2M sodium acetate, pH4 in autoclaved Eppendorf tubes. The homogenate was then mixed and incubated for 15 minutes at 4 C with 500 µL of water-saturated phenol, and 100 µL of 49:1 chloroform / isoamyl

alchohol. Following centrifugation at 9000 rpm for 20 mins at 4 C, the upper aqueous phase was transferred to a fresh tube and the RNA precipitated by incubation for 30 minutes at -20 C in 1 ml of 100% isopropanol and centrifugation at 9000 rpm for 10 minutes at 4 C. The residual pellet was washed once in 70 % ethanol, centrifuged at 9000 rpm for 5 minutes, the residual pellet dissolved in 0.3 ml of denaturing solution, and reprecipitated in 0.3 ml 0f 100% isopropanol. Contaminating guanidinium isothyocyanate was removed by washing in 70 % ethanol and the RNA harvested by centrifugation at 9000 rpm for 5 minutes. The RNA pellet was dissolved in 100 µL DEPC (SIGMA) treated water (0.1% DEPC, shaken for 3 hours and autoclaved for 1 hour). RNA concentrations were equalised at O.D. 260 nm on a Shimadzu UV-150U2 double beam spectrophotometer.

2.21. REVERSE TRANSCRIPTION PCR (RT-PCR)

Complementary DNA (cDNA) was prepared for 40 minutes at 37 C using 1 ng of RNA and 0.25 units of random hexamer primers (Pharmacia) in a 20 µL total reaction volume comtaining 10mM each of dATP, dCTP, dGTP, dTTP, 1X Taq DNA polymerase buffer (Amersham), 20 units of RNAsin and 200 units of Moloney murine leukaemia virus reverse transcriptase. RNA samples were preheated to 65 C for 5 mins on a PCR block prior to addition of the random hexamers and cooled on ice before addition of reverse transcriptase. Oligonucleotide PCR primers were stored at -20 C as 5 µM stock solutions. Reactions were performed according to the general method of Kawasaki (1990) under liquid paraffin (B.P.) in a 50 µL reaction volume containing 30 picomoles of each primer, 0.1 mM each dATP, dCTP, dGTP, dTTP, 1X Taq DNA polymerase buffer and 2 units of Taq DNA polymerase (Amersham). Initial experiments were performed with different amounts of cDNA and different numbers of cycles to establish the conditions in which the amount of product was linearly relatedto the amount of input cDNA used. Analysis of products was carried out on 1-1.5% agarose TBE, ethidium bromide gels. Preliminary amplifications were carried out with different cDNA dilutions and

different numbers of cycles to establish conditions where the PCR product signal was linearly related to cDNA input.

PRIMER SEQUENCES AND PCR PERAMETERS

r-NCAM7 sense AGG AGC AAG TCA CTC TGA CT

r-NCAM8 antisense TTC AAG GTA CAT GGA CTG GG

30 cycles at 94 C for 30 seconds, 60 C for 30 seconds, 72 C for 30 seconds.

m-src3 sense CTG TCC TTC AAG AAA GGG GAG C

m-src-4 antisense TGG ATG GAG TCG GAG GGC GC

35 cycles at 94 C for 30 seconds 65 C for 30 seconds and 72 for 30 seconds.

GSα , U2 sense ATG GGC TGC CTC GGC AAC

GSα D1 antisense TGT AGC CAT CAT CTA GTG GGG

GSα , EX4. antisense TTC CGT TGG TTT CAC GT

30 cycles at 94 C for 1 minute, 55 C for 1 minute and 72 C for 2 minutes.

m-Go6 sense TCT TCT GTG TCG CAC TCA GC

m-Go8 antisense CTG TAG ACT TCC TTG TGA GC

m-Go9 anti-sense CAG CAA AGA GTG CAT GAA GC

35 cycles at 94 C for 45 seconds, 56 C for 45 seconds and 72 C for 45 seconds.

Oct-2 RACE PCR

5' RACE TGA TGC GGC GTT GCT TGA

3' RACE GAC TCG AGT CGA CAT CGA (T)17

Initial treatments: 94 C for 5 minutes, 72 C for 1 minute, 55 for 5 minutes, 72 C for 10 minutes. 9 cycles at 94 C for 40 seconds, 55 C for 5 minutes, 72 C for 3 minutes.

OCT 2.4/5 PCR

3(ub) GAT CGA ATT CGG GAC CTT ACC ATT GTC CCA

2.4 / 2.5 D GAT CGA ATT CGG CTC CAC CAG AGG CCA GGG

Initially, 5 cycles at 94 C for 49 seconds, 52 C for 40 seconds 72 C for 40 seconds, then 25 cycles at 94 C for 40 seconds and 72 C for 75 seconds.

CGRP

RATCAL-A GAG GCA TCA TGG GCT TTC TG

RATCAL-B TCA CGC AGG TGG CAG TGT T

CAL-C EX4 CCT GAA CCT CTG TTT GGT GG

35 cycles at 94 C for 1 minute, 62 C for 1 minute, 72 C for 1 minute.

2.22. SOUTHERN BLOTTING

Agarose gels were washed in 0.4 M NaOH for 20 minutes and then overlayed with nitrocellulose sheet (Hybond N) and several layers of filter paper and blotted overnight by capillary action in 20X SSC buffer (150 mM sodium chloride, 15 mM sodium citrate pH 8). DNA was cross linked to the nitrocellulose by UV irradiation for 1 minute and blots were stored at - 20 C. Prior to hybridisation with a specific probe, blots were prehybridised at 65 C for a minimum of 2 hours in 6X SSC, 10 % w/v dextran sulphate (Pharmacia), 0.1 % w/v SDS, 5X Denhardts solution (X100 stock- 0.02% w/v ficoll type 400 (Sigma). 0.02% w/v PVP (Sigma), 0.02% w/v BSA (Sigma)) and 100 μg/ml herring sperm DNA (Sigma). Initially, the appropriate fragments were isolated from gels and approximately 30 ng was denatured for 3 minutes at 100 C and labelled for 3 hours with 2 μCi [α32] dCTP in oligo labelling buffer of final concentration of (50mM Tris pH 8, 5 mN MgCl2, 0.1% v/v β–mercaptoethanol, 200 μM dATP, dCTP, dTTP (Pharmacia), 200mM Hepes pH 6.6, 100μM random hexamers (Pharmacia) and 1unit of Klenow.

The labelled DNA was retrieved by centrifugation at 2000 rpm for 5 minutes through a Sephadex G50 column (Pharmacia), and then added to the prehybridisation solution for overnight incubation at 65 C. The blots were washed 3 times in 1X SSC, 0.5% SDS, dried and exposed to X-ray film.

CHAPTER 3
STUDIES OF SmN AND SmB PROTEINS AS AUTOANTIGENS IN SYSTEMIC LUPUS ERYTHEMATOSUS

3.1. INTRODUCTION

The high sequence homology between tissue-specific SmN and ubiquitous SmB/B' polypeptides accounts for the cross-reactivity of monoclonal and polyclonal anti-Sm autoantibodies on both Western blots and ELISA (Williams et al., 1990; Schmauss and Lerner, 1990; Mc.Allister et al., 1989). Indeed, autoantibodies and monoclonal antibodies binding to an SmN fusion protein and peptides derived from the carboxyl-terminal of SmN cross react with SmB/B' and also the U1-snRNP-specific A protein (McAllister et al., 1989; Habets et al., 1989). Anti-Sm monoclonal antibodies reactive with SmB/B' probably recognise SmN by virtue of shared linear epitopes present in the primary structure. The KSm5 monoclonal for example, is cross reactive due its specifity for the proline rich PPGMRPP epitope (Williams et al., 1990),which is repeated three times in SmN/SmB' and twice in SmB. This epitope is immunodominant in SLE and hence SLE sera contain autoantibodies cross reactive with both SmN and SmB/B'. Extensive epitope mapping reveals that most of the autoimmune epitopes map to the carboxyl-terminal half of SmB/B' (Rokeach et al ., 1990) and hence also of SmN.

Despite the presence of cross reactive autoantibodies, it remains possible that antibodies specific for SmN may occur in SLE. These may occur due to unique linear epitopes as determined by the difference in primary structure due to the 17 amino acid substitutions in SmN. In addition, conformational epitopes that are due to the effect of this difference in primary structure on the final protein conformation may be unique to SmN. It has been demonstrated that certain autoantibodies are unique for the COOH terminus of SmB, probably due to the presence of two terminal leucines not found in SmB' or SmN (Elkon et al., 1990). Moreover, antigenic differences between SmN and SmB/B' have been previously demonstrated with the monoclonal Y-12 which binds to SmB with greater avidity than SmN (Schmauss and Lerner, 1990). In contrast, the N216-238 polyclonal antibodies recognize SmN to a greater extent than SmB, this specificity being probably due to the extra proline rich PPPGMRPP epitope present in SmN.

The precise biological effect of autoantibodies reactive with various snRNP polypeptides remains unclear. However, anti-U1 RNP autoimmune sera and anti-Sm autoimmune sera and the

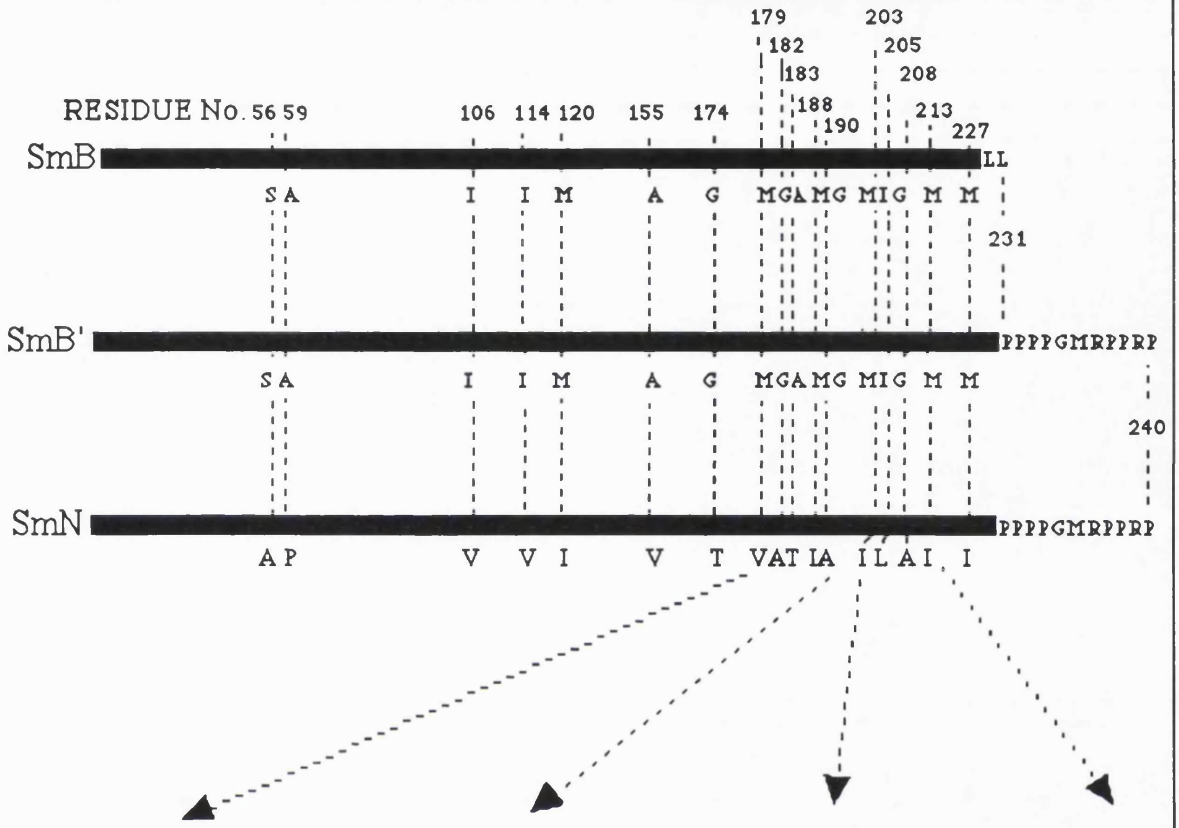
Y-12 anti-SmB/B'/N monoclonal have been demonstrated to significantly inhibit splicing in *in-vitro* systems (Padgett et al., 1983). This inhibition is probably due to the formation of antibody-snRNP complexes that sequesters snRNPs thus making them unavailable for efficient splicing. In addition anti-Sm and anti-(U1) RNP autoantibodies and monoclonals have been implicated in inhibition of polyadenylation of RNA (Moore et al., 1984), this probably being due to inhibition of the prior cleavage reaction (Hashimoto and Steitz, 1986).

It may be proposed that if autoantibodies exist that are reactive specifically with SmN, they may affect the proposed role of SmN as a regulator of alternative splicing and/or the constitutive splicing duties of SmN in the brain where it replaces SmB. The presence of such autoantibodies would be important in deducing a role for the protein, for example in *in-vitro* splicing assays, and the observation of any clinical manifestations that would seem to be associated with the presence of these antibodies would be very useful in this respect. Previously, the presence of anti-Sm antibodies has been correlated significantly with late onset lupus nephritis (Homma et al., 1987). More specifically, IgG anti-SmB/B' antibodies have been associated with renal disorder and pleuritis/ pericarditis (Takeda et al., 1989) and serositis (Lundberg et al., 1992), whilst IgM antiB/B' antibodies are associated with the presence of arthralgias, Raynauds phenomenon, and arthritis (Lundberg et al., 1992). The reduction of calcitonin gene related peptide (CGRP)-containing neurons in the digital skin of patients with primary Reynaud's phenomenon could be indicative of the effect of autoantibodies on splicing (Bunker et al., 1990). This is of particular relevance to SmN which has been proposed to be a regulator of the alternative splicing of the calcitonin / CGRP (CALC1) gene (McAllister et al., 1988/ 89; Sharpe et al., 1989; Rokeach e al., 1989; Amara et al., 1982; Crenshaw et al., 1987). In addition, anti-Sm activity has been reported in 71 % of SLE patients with isolated CNS lupus which may possibly reflect involvement of the tissue-specific SmN autoantigen (Winfield et al., 1978).

In order to determine whether autoantibodies that discriminate between SmN and SmB are generated in SLE, and to further characterise the role of Sm epitopes in SLE, sera were screened by ELISA against peptides corresponding to the regions of least sequence homology that occur

between SmB/B' and SmN. This was performed with reference to normal controls and autoimmune disease control sera. The regions used for screening correspond to amino acid residues 179-190 in which there are five substitutions for SmN and residues 203-213 in which there are four substitutions (FIG.1).

FIG.1 COMPARISON OF AMINO ACID SEQUENCES OF SmN, SmB AND SmB' AND DERIVATION OF PEPTIDE AND FUSION PROTEIN SEQUENCES USED IN ELISAs AND IMMUNISATIONS



VGRATPPPGIMA©
SmN 179-190

IGLPPARGTPI©
SmN 203-213

SmB/B' 179-190
MGRGAPPPGMMG©

SmB/B' 203-213
MGIPPGRGTPM©

GPPPPMGRGAPPPGMMGPPPGMRPPMGPPMGIPPGRGTPM
SmB/B' 174-213 FUSION PROTEIN

Derivation of peptides used in ELISA screening of SLE sera and for immunisation of BALB/C mice. Sequences correspond to regions of least sequence homology between SmN and SmB/B' (179-190 and 203-213). A C-terminal cysteine, © was added for coupling peptides to carrier proteins BSA and KLH. Also included is the sequence of the SmB/B' 174-213 fusion protein used in a diagnostic ELISA for SLE.

3.2. RESULTS

SLE SERUM IgG BINDING TO PEPTIDES CONSISTING OF RESIDUES 179-190 AND 203-213 OF SmB/B' AND SmN.

Initial screening of 19 SLE sera revealed significant specific binding to the peptide consisting of residues 179-190 of SmB/B' (FIG.2). The long time of development required for visualisation (overnight at room temperature) is indicative of low affinity or low titre autoantibodies. Binding was not observed to the corresponding peptide derived from residues 179-190 of SmN or to either peptides consisting of residues 203-213 of SmB/B' or SmN.

FIG.2a. Preliminary screening of SLE sera on peptides -SLE serum binding of N or B/B' 179-190 peptides.

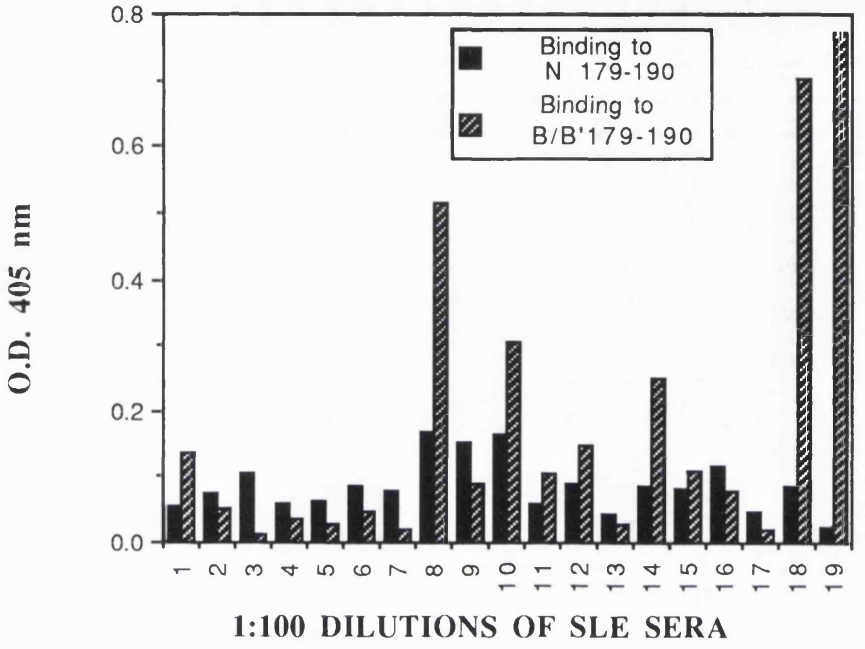


FIG.2.b. SLE serum binding of N or B/B' 203-213 peptide

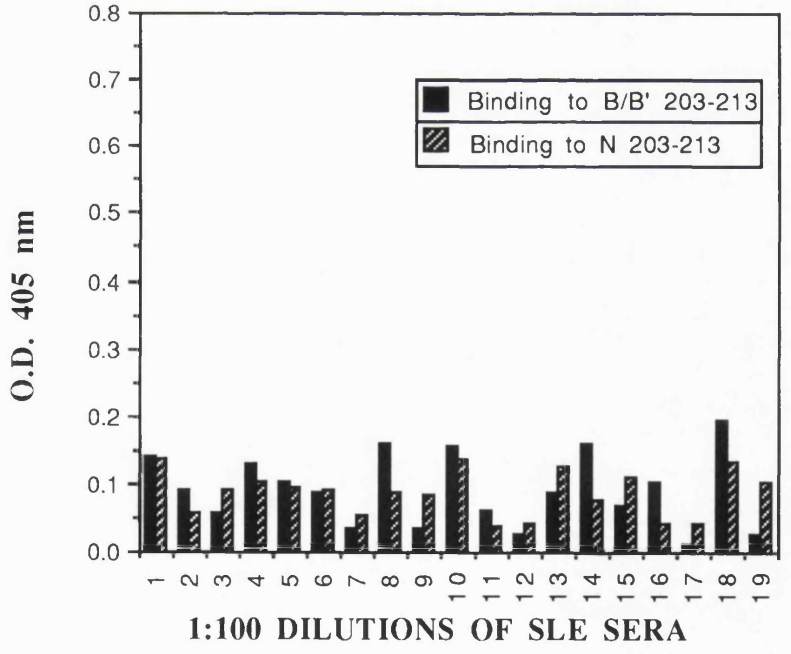


FIG.2. IgG binding of 19 SLE sera to SmN and SmB/B' 179-190 and 203-213 peptides. (a) SLE serum binding of SmN or SmB/B' 179-190 sequences. (b) SLE serum binding of SmN or SmB/B' 203-213 sequences

3.3. SCREENING OF 60 SLE SERA AND CONTROLS

In light of the preliminary screening results, a more extensive study involving 60 SLE sera was performed (FIG.3). Sera had been previously assayed for anti-Sm reactivity by counterimmunoelectrophoresis (CIE) using rabbit thymus extract (Williams et al., 1986c). This was further confirmed using an anti-Sm antigen ELISA (Shield Diagnostics) with reference to positive and negative reference sera. IgG binding to the B/B' 179-190 peptide only was observed and was restricted to the anti-Sm positive sera (with or without anti-RNP specificity). Thus no significant binding was observed in any of the the 17 anti-Sm negative SLE sera or the 18 healthy non-SLE sera Of the anti-Sm positive sera, six out of 43 (14 %) demonstrated significant specific IgG binding to B/B' 179-190. The level of significance was set as being greater than the mean plus 2 standard deviations (0.494) of the binding recorded for the non-SLE control group. The apparently negative values in FIG.3 are probably a result of the primary amine coupling reagent, sulpho-SMCC (Pierce) binding bovine serum albumin and thus blocking sites of non-specific antibody binding. The control ELISA was performed using carrier protein alone (BSA). These results were subtracted from the peptide ELISA results which used conjugated carrier in order to account for background binding to free carrier protein.

Fig.3 Binding of sera to the B/B' 179-190 peptide

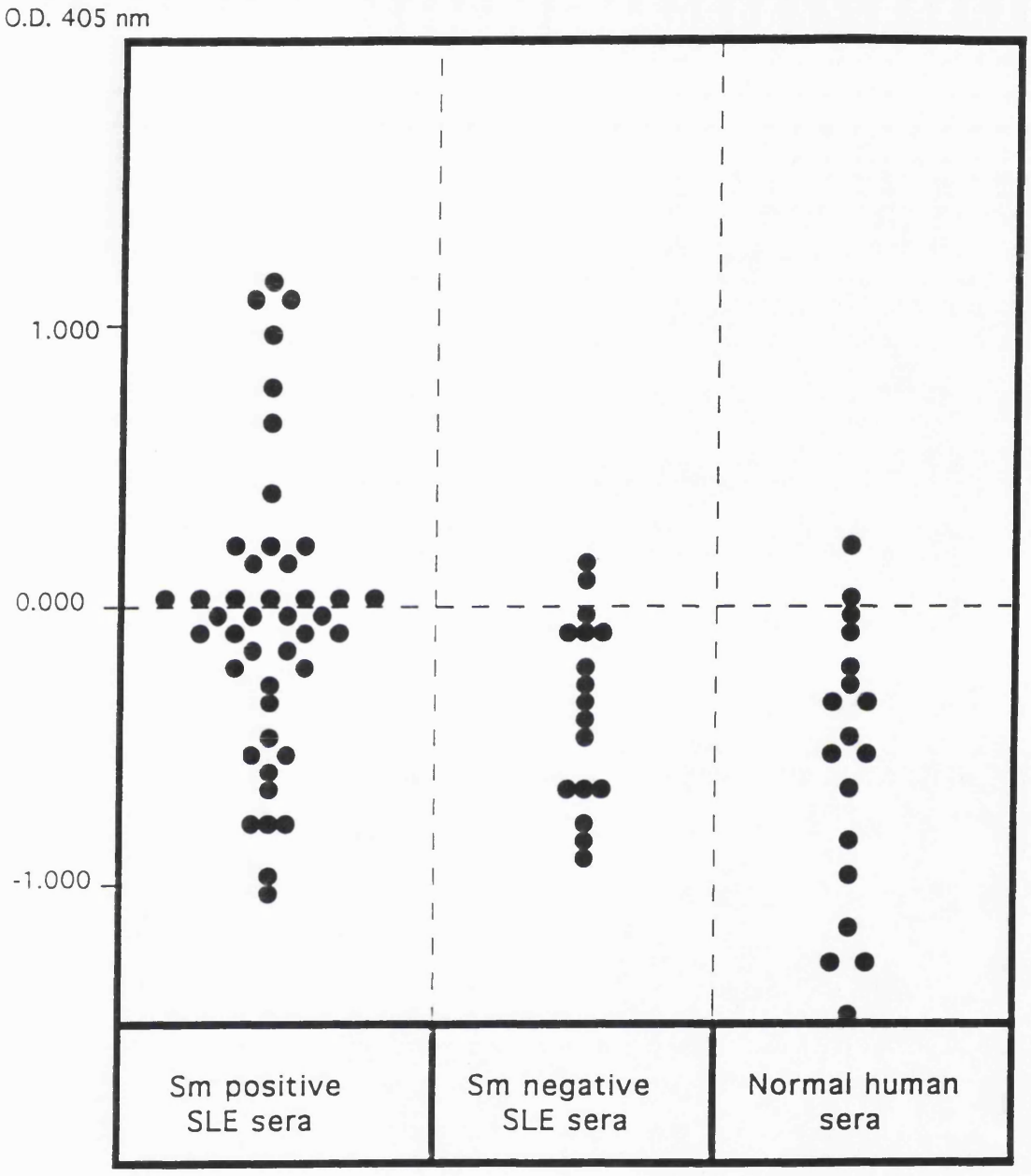
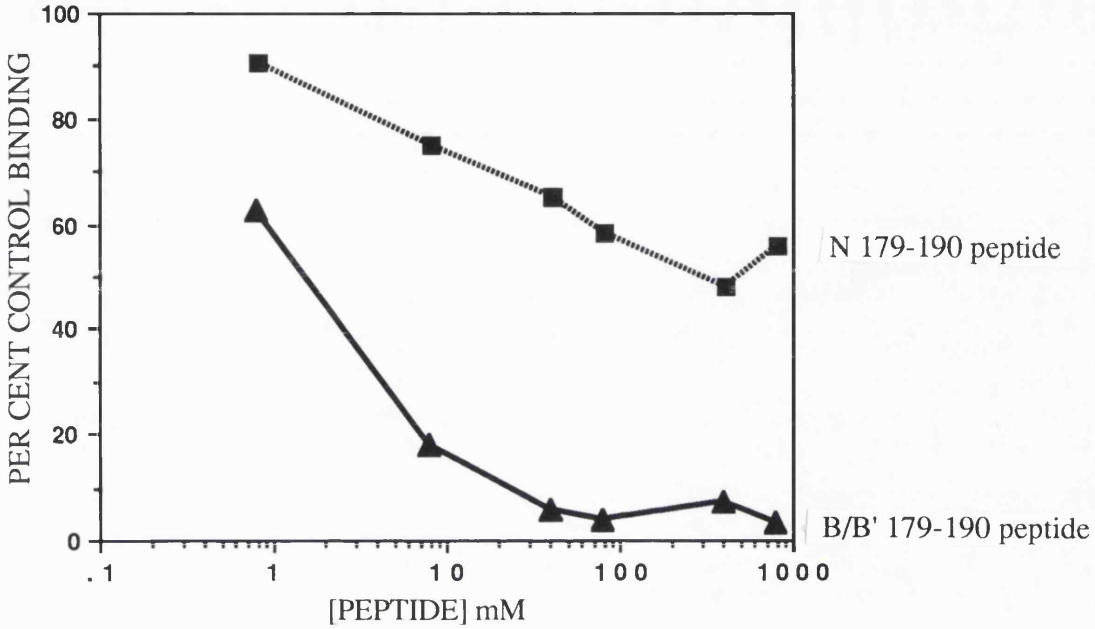


FIG.3. Enzyme immunoassay of 60 SLE patients and 18 normal non-SLE sera controls to demonstrate serum IgG binding to the B/B' 179-190 peptide. The optical density due to binding to the carrier protein alone was subtracted from the optical density due to binding to the peptide-carrier conjugate to measure true peptide binding. The SLE sera were designated anti-Sm positive or anti-Sm negative by anti-Sm ELISA (Shield Diagnostics Ltd.) and by counterimmunoelectrophoresis (Williams et al., 1986c).

3.4. PEPTIDE SPECIFICITY OF B/B' 179-190 REACTIVE SERA

To further confirm the specificity of the anti-Sm positive sera for the SmB B/B'179-190 sequence, unconjugated peptides for both SmN and SmB 179-190 sequences were used at a range of concentrations to competitively inhibit autoantibody binding to the solid-phase conjugated B/B' peptide (FIG.4). Two patients were analysed in this immunoasssay which shows the degree of binding to the B/B' 179-190-BSA conjugate over a range of competing peptide concentrations up to 1000 mg/ml (800mM). As expected, the B/B' 179-190 peptide gave the greatest level of inhibition of autoantibody binding to solid-phase B/B' 179-190 peptide by competition in preincubation reactions. This was observed for both patients and the maximum inhibition caused by the highest applied concentration of competing B/B' peptide was 96 % (patient 1) and 80 % (patient 2), with 50% inhibition occurring at 1-3 mM. In comparison, the N 179-190 peptide produced a maximum of 52 % inhibition (patient 1) and 62 % inhibition (patient 2). This competition by the N peptide was interpreted as the result of two B/B' 179-190 binding antibody populations within these sera, only one of these populations being capable of binding the SmN version of this sequence.

FIG. 4.A. COMPETITIVE INHIBITION IMMUNOASSAY-PATIENT 1



COMPETITIVE INHIBITION IMMUNOASSAY -PATIENT 2

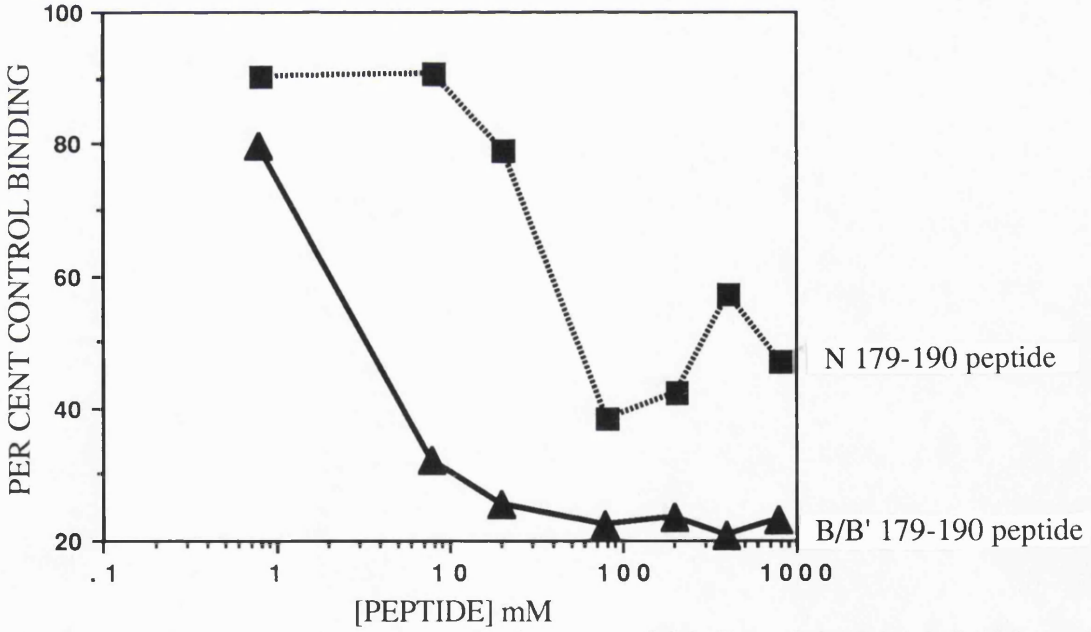


FIG.4. Competitive enzyme immunoassay of serum IgG binding of the B/B' 179-190 peptide by two representative anti-Sm positive sera, data being expressed as a percentage of the normal uncompeted binding by these sera. Competitive inhibition of anti-peptide antibodies was performed using a range of competing fluid-phase N or B/B' 179-190 peptide concentrations up to 1000mg/ml (800mM). (a) Patient 1 (b) Patient 2.

3.5. PARALLEL LONGITUDINAL STUDIES OF SERUM IgG BINDING TO TOTAL Sm ANTIGEN AND THE SmB/B' 179-190 PEPTIDE IN TWO REPRESENTATIVE SLE PATIENTS.

Two patients were analysed in a parallel longitudinal study of B/B' 179-190 and Sm antibody binding by ELISA to elucidate whether the two antibody specificities were co-ordinately regulated in vivo (FIG.5). Both patients were studied over a 5 year period.

Patient 1 initially showed high antibody levels for both parameters at the time of admission in 1977 which decreased to a negligible level with time. A flare in anti-Sm binding in 1982 was not accompanied by a parallel rise in B/B' 179-190 binding.

Patient 2 demonstrated a general decrease in both anti-Sm autoantibodies and anti-B/B' reactivity over the time course after initially high levels of binding for both perameters at the time of admission. In this patient, the levels of anti-B/B' 179-190 antibodies were observed to follow the anti-Sm levels very closely. Thus each flare (21/10 81) and decline (9/11/81) in anti-Sm reactivity is accompanied by a similar change in anti-B/B' reactivity.

3.6. MRL / lpr MOUSE SERUM IgG BINDING TO B/B' 179-190

In a parallel study to further characterise the nature of this autoimmune epitope, 5 MRL / lpr and 8 MRL / (+/+) sera (all anti-Sm positive) were assayed for binding to the peptides consisting of residues 179-190 and 203-213 of both SmN and SmB/B' (FIG.6). Results were corrected for background binding to unconjugated BSA. Despite the similar levels of binding observed for B/B' 179-190 peptides and both 203-213 sequences, MRL sera do appear to reflect data from SLE patients. Thus MRL mice sera discriminate between the 179-190 sequences (FIG.6.a.) which is in agreement with SLE data in that most MRL sera demonstrated appreciable binding to the SmB/B' 179-190 peptide whilst binding to the SmN 179-190 peptide was negligible. In contrast, MRL sera did not discriminate between the 203-213 sequences (FIG.6.b.) and binding was similar for both SmN and SmB/B' 203-213 sequences, especially for MRL/ lpr mice. This was consistent with data for anti-Sm positive SLE patients.

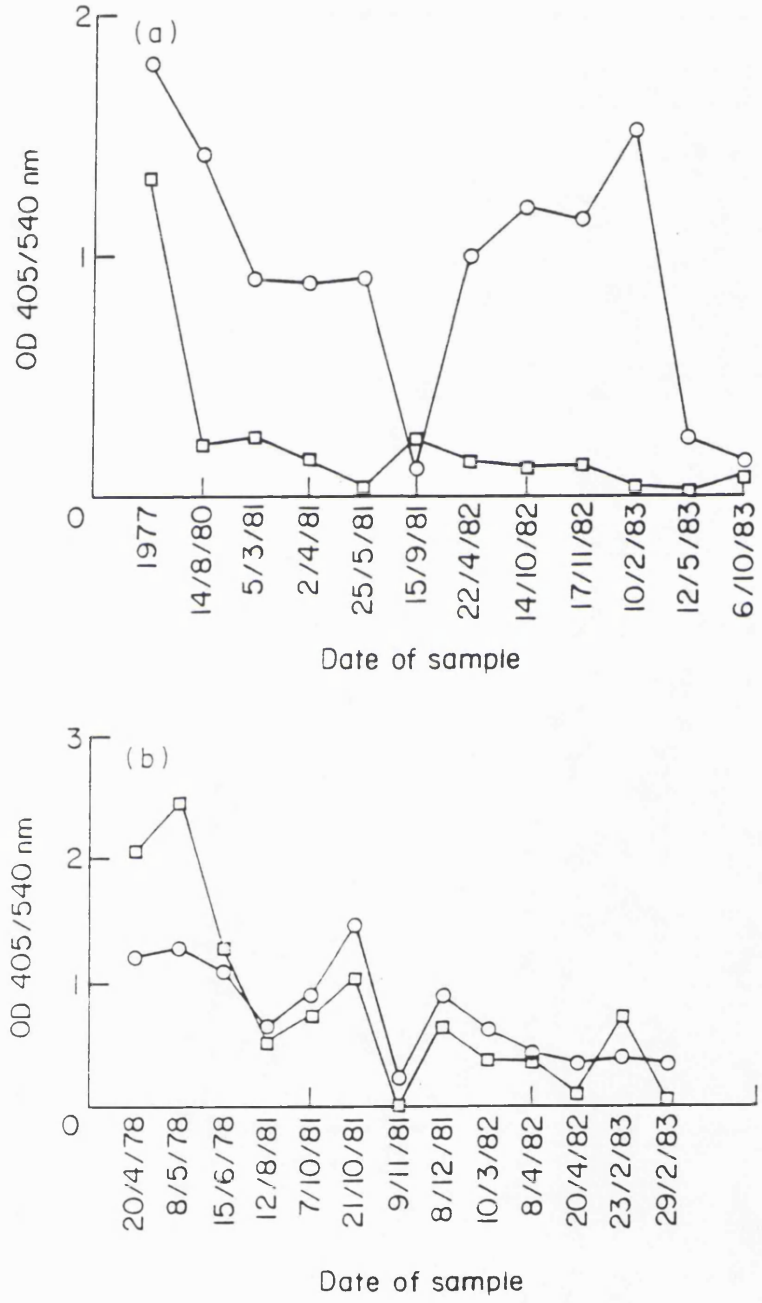


FIG.5. Enzyme immunoassay of sera from two anti-Sm positive systemic lupus erythematosus (SLE) sera in a comparative longitudinal study of serum IgG binding to the SmB/B' 179-190 peptide and that to total Sm. (a) Patient 1 (b) Patient 3. Circles represent binding to total Sm; boxes represent binding to B/B' 179-190

FIG. 6.a. ENZYME IMMUNOASSAY OF ANTI-Sm POSITIVE MRL MOUSE SERA BINDING TO SmN OR SmB/B' 179-190 SEQUENCES

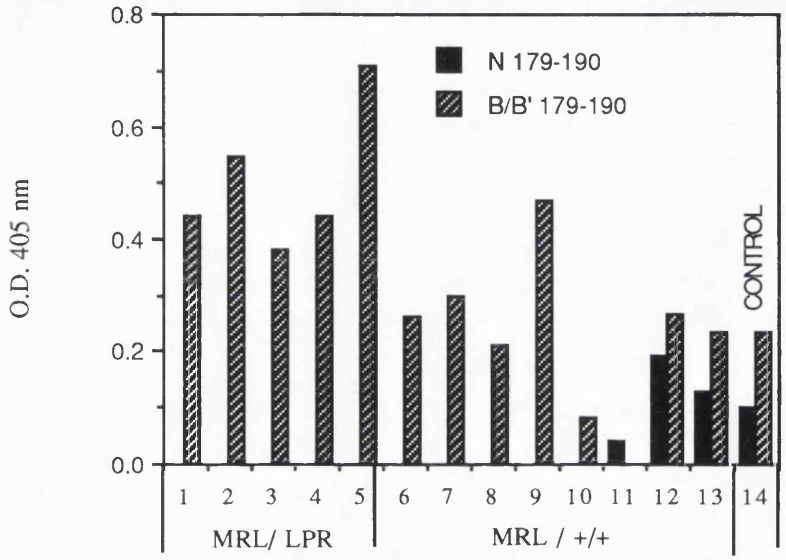


FIG. 6.b. ENZYME IMMUNOASSAY OF ANTI-Sm POSITIVE MRL MOUSE SERA TO SmN OR SmB/B' 203-213 SEQUENCES

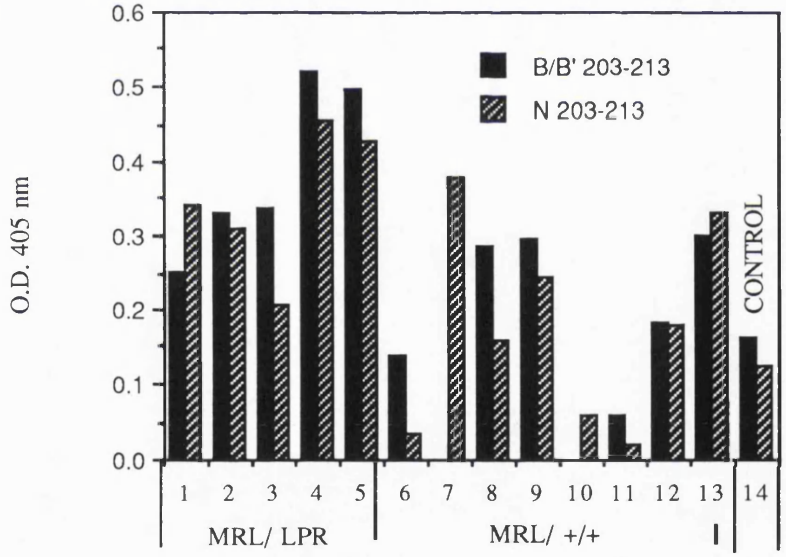


FIG.6. Enzyme immunoassay demonstrating MRL/lpr and MRL/(+/+) serum IgG binding of SmN or SmB/B' 179-190 or 203-213 peptides. True peptide binding analysed by subtracting binding to BSA alone from peptide-BSA conjugate binding.

3.7. OBSERVATIONS OF IMMUNE RESPONSE ELICITED ON IMMMUNISATION OF MICE WITH PEPTIDES REPRESENTATIVE OF REGIONS OF LEAST SEQUENCE HOMOLOGY BETWEEN SmN AND SmB/B'

In an attempt to generate antibodies specific for SmN for use in subsequent expression studies, BALB/c mice were subjected to 3 immunisations with one of the peptides representing the most divergent amino acid sequences between SmN and SmB. Mice were immunised with either uncoupled peptide or peptide coupled to the carrier protein keyhole limpet haemocyanin (KLH). Mice were then tail-bled and immune response generated to the peptides analysed by ELISA (FIG.7). A significant immune response was only evident when using coupled peptides and control mouse sera demonstrated no binding of peptides.

Results for the 179-190 sequence peptides reveal that the B/B' 179-190 peptide generated the largest response and the epitope presented by this peptide in solid-phase was bound with appreciably less avidity by sera hyperimmune for the N 179-190 peptide or either of the 203-213 sequence peptides or control sera. This reflects data from anti-Sm positive SLE patients in that the B/B' 179-190 peptide appears to contain a unique epitope that on immunisation generates a population of B/B' specific antibodies. This epitope does not appear to be present in the other peptides tested. Administering the N 179-190 peptide generated a response to the N 179-190 peptide in solid-phase that was similar to binding of this SmN peptide observed by sera hyperimmune for the BB' 179-190 peptide (FIG.7a). This suggests that the N 179-190 peptide shares an epitope with the Sm B/B' version of the sequence. This is in agreement with data from competitive inhibition analysis in SLE sera (FIG.4) whereby two populations of autoantibody that bind 179-190 sequences are present, only one of which is capable of binding the SmN version of the sequence. Thus the large immune response observed on administering B/B' 179-190 peptide would appear to be a sum of these populations. However, the latter interpretation must be treated with caution as sera hyperimmune for B/B' 179-190 also bound the 203-213 sequence peptides in solid-phase with similar avidity, suggesting a non-specific antibody activity is present. It would appear that the primary amino acid sequence presented to the immune system by the N 179-190

peptide is potentially antigenic and does appear to generate a fairly specific response (mouse 1, FIG. 7a). Administering either of the 203-213 sequence peptides generated less specific responses.

Attempts to generate monoclonal antibodies specific for SmN and SmB were not successful despite the isolation of many hybridomas.

FIG.7.a. ELISA DEMONSTRATING IMMMUNE RESPONSE ELICITED ON IMMUNISATION WITH SmN OR SmB/B' 179-190 PEPTIDES

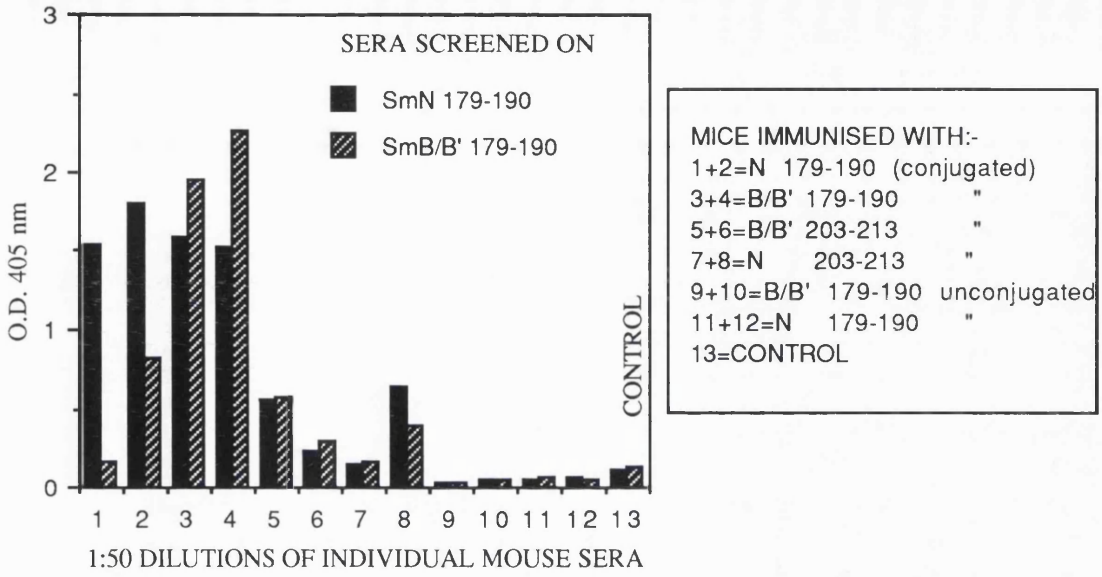


FIG.7.b. ELISA DEMONSTRATING IMMUNE RESPONSE ELICITED ON IMMUNISATION OF BALB/C MICE WITH SmN OR SmB/B' 203-213 PEPTIDES

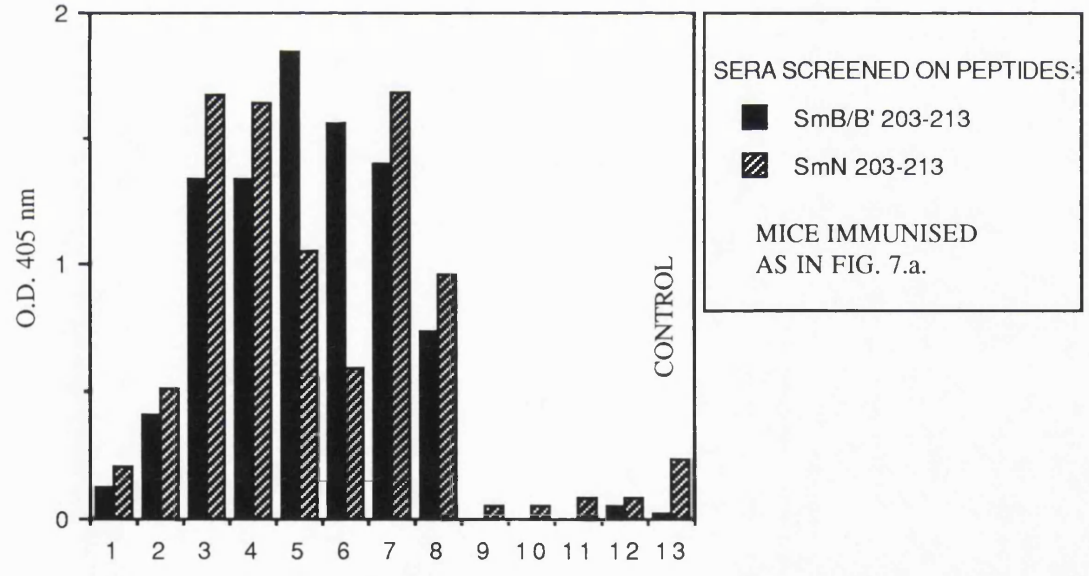


FIG.7. Enzyme immunoassay of sera from BALB/C mice immunised with SmN or SmB/B' 1790-190 or 203-213 peptides. True anti-peptide binding was obtained by subtracting binding to the carrier peptide alone from the binding to the peptide-carrier conjugates.

3.8. POTENTIAL USE OF AN SmB FUSION PROTEIN ENCOMPASSING RESIDUES 174-213 AS A DIAGNOSTIC ELISA FOR SLE

In view of the specific binding of the B/B' 179-190 peptide by only anti-Sm positive sera, a fusion protein encompassing residues 174-213 of SmB (FIG.8) generated by PCR from a cDNA of SmB (van Dam et al., 1989) was used to screen autoimmune sera in an attempt to develop a diagnostic assay specific for SLE. The purified protein, expressed as a fusion protein with glutathione-S-transferase was applied to ELISA plates and used to screen sera from SLE, mixed connective tissue disease (MCTD) and Sjogrens syndrome (SS) disease groups. The Sm antigen used in the conventional diagnostic assay (Shield), contains both SmB and SmD polypeptides. This may lead to unintentional binding of anti-(U1)-RNP directed antibodies commonly found in MCTD patients due to similar epitope sequences being present in U1-snRNP specific polypeptides A and C and the SmB polypeptide. Thus the incorporation of the SmB 174-213 fusion protein into an ELISA could provide a more specific assay for anti-Sm activity in SLE.

The results shown graphically in FIG.9a reveal that the fusion protein did accurately distinguish between disease groups tested. Thus 36.5% (23 out of 63) of SLE sera bound the B 174-213 fusion protein at levels greater than the mean + 3 standard deviations (S.D.) of the normals (120 Units). This figure is in agreement with the 28% prevalence of anti-Sm antibodies in SLE sera. No reactivity with the fusion protein was observed for MCTD or SS disease groups or normals. In comparison, the conventional anti-Sm diagnostic assay detected a 32% prevalence of anti-Sm activity in these patients (20/63 demonstrated binding greater than mean + 3 S.D. normals =11.79 units), and this was also restricted to SLE patients (Fig.9b). Therefore the SmB 174-213 assay is as sensitive as the anti-Sm ELISA. The use of a bacterially expressed protein in this assay indicates that the immunoreactivity observed is not a result of post translational modification. A study of anti-(U1)-RNP binding in the disease groups (FIG.9C) reveals the presence of such autoantibodies as a common occurence in SLE and MCTD (binding greater than mean + 3 S.D. normals = 9.22 units), but not in Sjogrens syndrome.

Further analysis revealed that 7 of the SLE sera which significantly bound the SmB 174-213 fusion protein but did not demonstrate significant binding of total Sm antigen, suggesting that some of the epitopes presented by the SmB 174-213 peptide were not exposed as epitopes in the total Sm ELISA antigen. However, four of these sera had high anti-(U1)-RNP reactivity as demonstrated in a comparison of anti-SmB 174-213 binding in parallel with anti-(U1)-RNP reactivity in these sera (FIG 9D). This suggest either a cross reaction with the glutathione-S-transferase motif of the fusion protein or a cross reaction of anti-(U1)-RNP directed antibodies with an SmB 174-213 epitope. The most probable linear epitope responsible for the latter cross reaction is the PPPGMRPP motif (residues 191-198 in SmB/B') that is similar to the PPPGMIPP motif in U1-snRNP-specific A polypeptide (residues 165-172) and the PAPGMRPP / PAPAMIPP sequences in the C polypeptide (residues 93-100 and 48-55 respectively). Chou and Fasman algorithm (Chou and Fasman 1978) predicts that proline rich sequences such as those common to SmB/B' SmN, A and C are both structurally dynamic and on the surface of protein molecules which may account for their immunoreactive nature. Cross-reactive antibodies to proline rich regions in other proteins (A and C) may be falsely interpreted as anti-Sm antibodies. These autoantibodies would also be expected to bind abundant proline-rich epitopes present in the Sm ELISA to give positive results. As this is not the case with these four anti-RNP sera that demonstrate binding of SmB 174-213 but not the total Sm antigen, it suggests that the apparently high anti-SmB 174-213 activity observed is directed to the glutathione-S-transferase motif. In agreement with this, the anti-(U1)-RNP reactivity observed in MCTD sera did not result in any significant binding of SmB174-213 or the total Sm ELISA by these sera.

The three remaining sera demonstrating significant binding of the anti-SmB 174-213 fusion protein but not the total Sm antigen ELISA did not demonstrate significant anti-(U1)-RNP binding (FIG. 9d.). Thus it remains possible that the conformational nature of the SmB 174-213 fusion protein presents auto-epitopes that are not exposed in the total Sm antigen ELISA. Such an epitope may be the linear SmB/B'-specific 179-190 epitope.

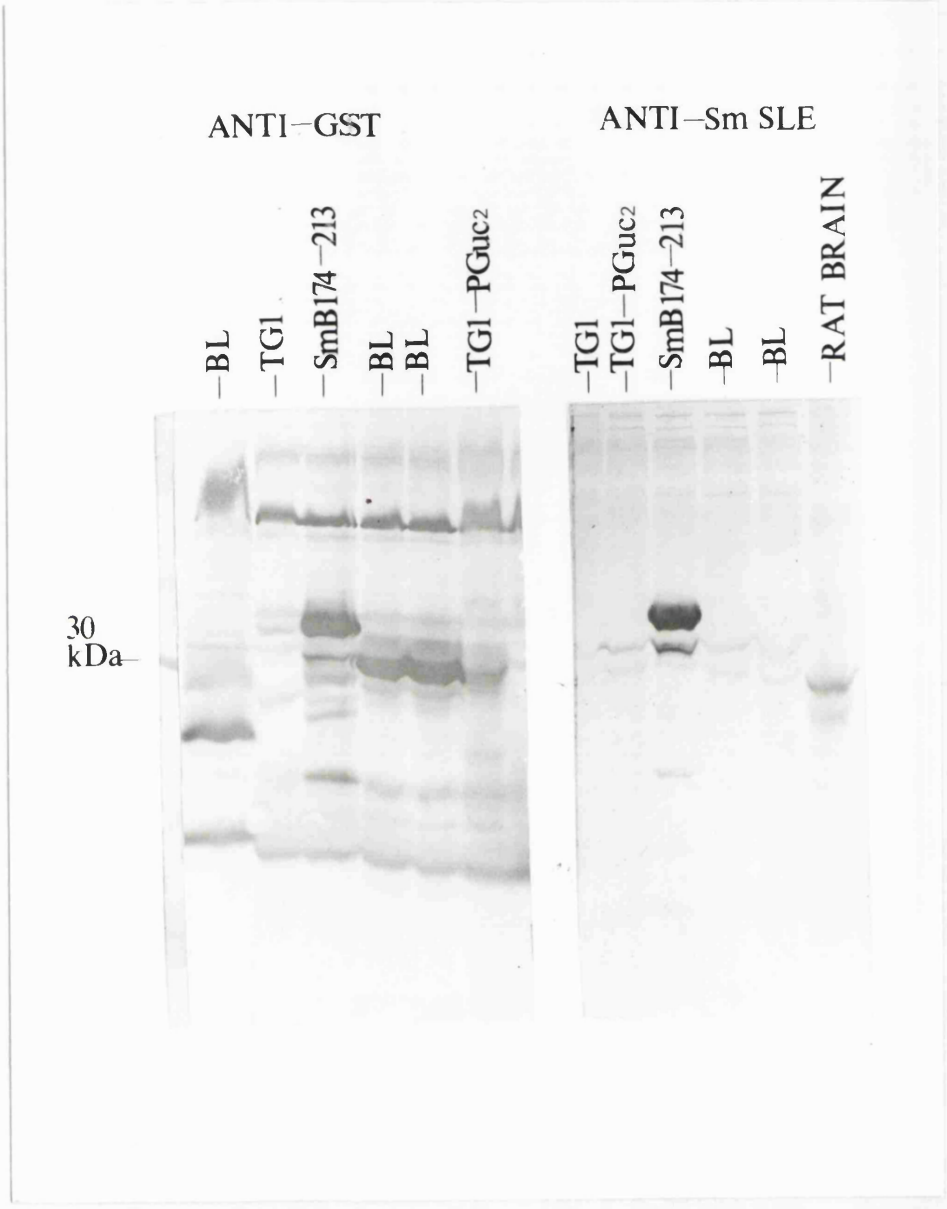


FIG.8. Western blot of total protein samples from bacterial lysates (BL) probed with either Anti-Sm positive SLE sera or anti-glutathione-S-transferase (anti-GST) sera in reference to TG1 E.coli strain TG1 and pGuc2 (no insert) transformed TG1 E.coli. The parallel blotting demonstrates generation of a recombinant fusion protein for residues 174-213 of SmB/B' which is bound by anti-Sm positive SLE sera and anti-GST antibodies.

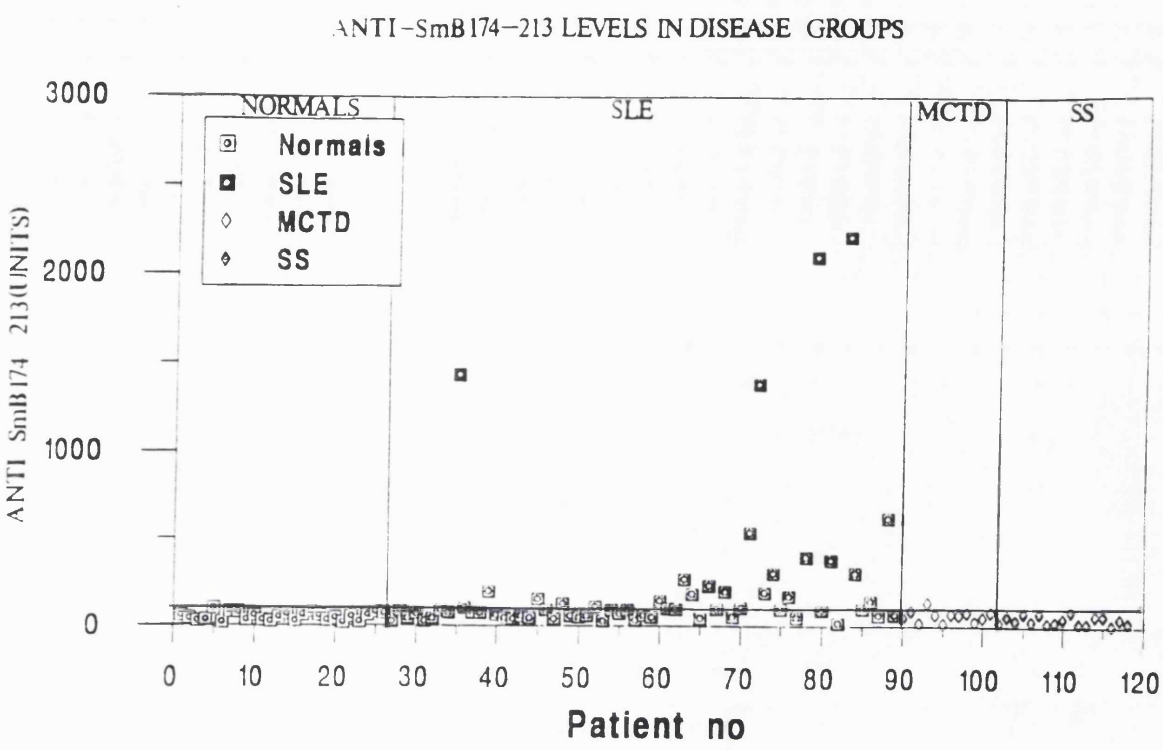


FIG.9.a. Enzyme immunoassay of serum IgG binding of SmB 174-213 fusion protein by systemic lupus erythemtosus (SLE), mixed connective tissue disease (MCTD) and Sjogrens syndrome (SS) sera with reference to normals. The mean plus two standard deviations of the results for the normals is indicated.

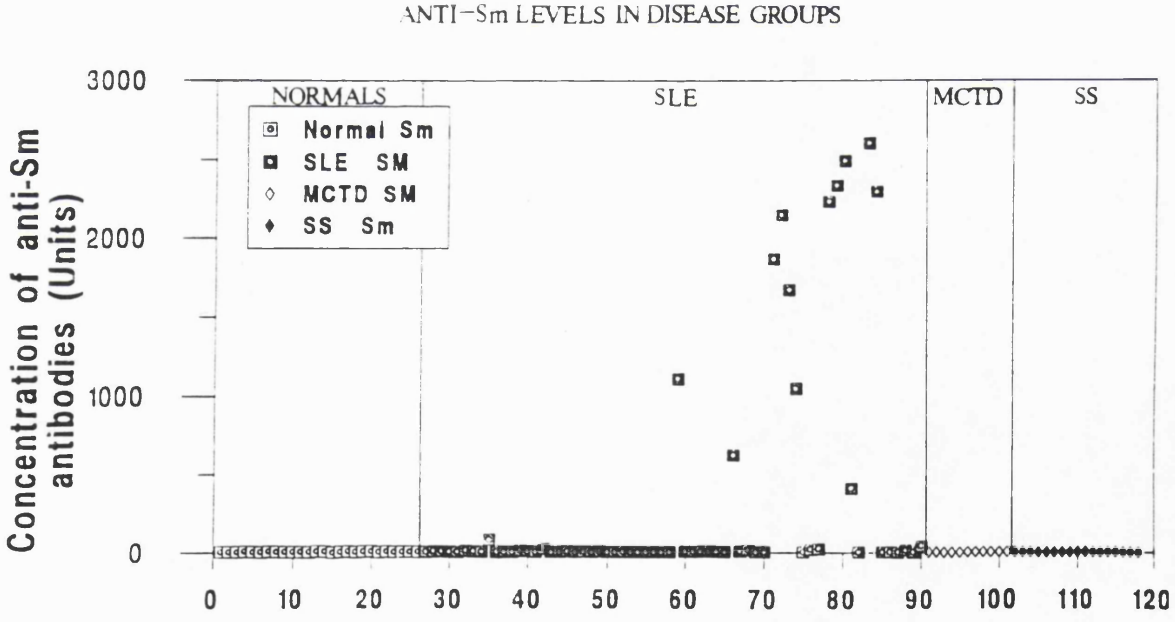


FIG.9.b. Enzyme immunoassay of serum IgG binding of disease groups and normals to total Sm antigen (Shield Diagnostics Ltd.)

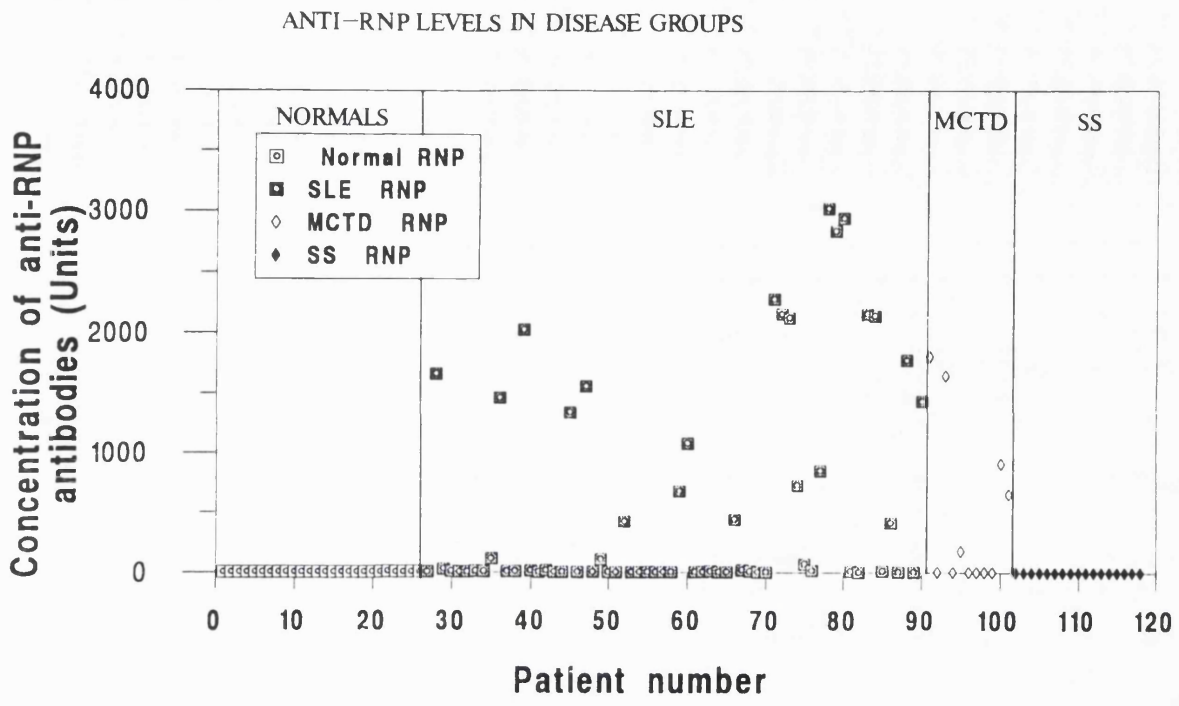


FIG.9.c. Enzyme immunoassay of serum IgG binding by disease groups and normals to the (U1)-RNP antigen (Shield Diagnostics Ltd.)

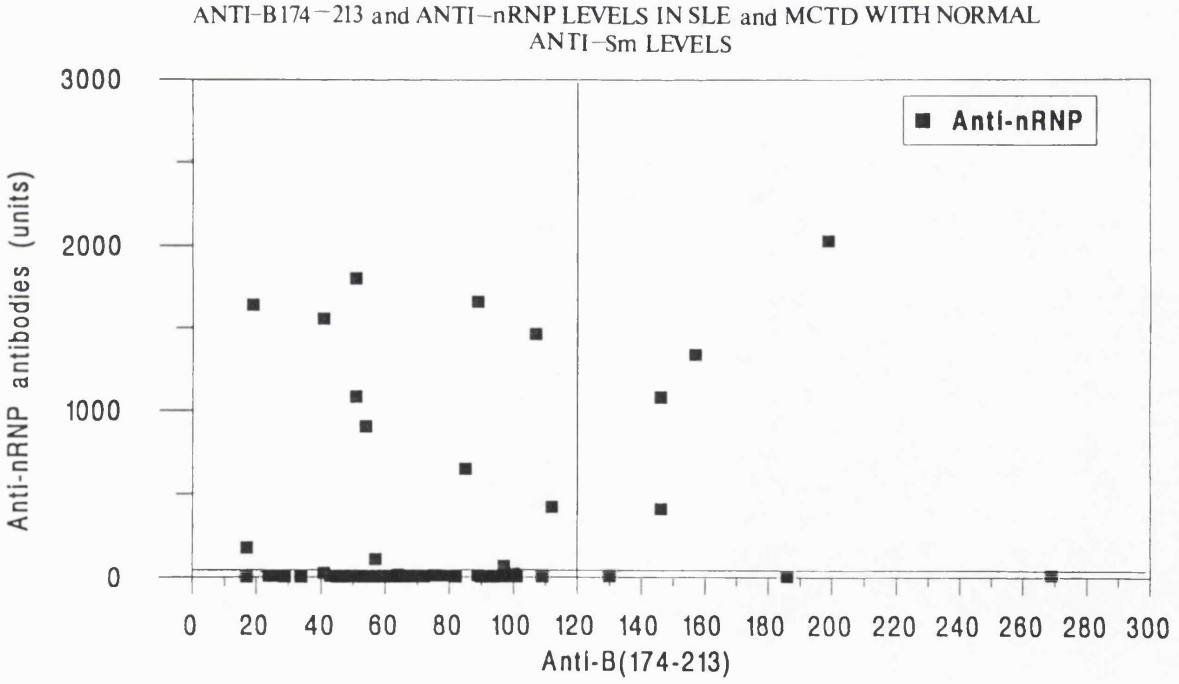


FIG. 9.d. Comparitive study of serum IgG (U1)-RNP antigen binding and anti-SmB 174-213 fusion protein binding in sera that demonstrate no significant binding of the total Sm antigen.

3.9. ANALYSIS OF ECTOPIC EXPRESSION OF SmN IN SLE

INTRODUCTION

Interesting observations from previous studies of gene transcription in peripheral blood mononuclear cells (PBMCs) from SLE patients (B.Twomey) warranted further investigation into the ectopic SmN expression in SLE. In the course of experiments with heat shock protein genes, genes encoding other autoantigens were included in nuclear run-on assays, in particular SmN and SmB. Thus in such studies it was observed that overexpression of the hsp90 protein occured in SLE (Norton et al., 1988), this being a result of enhanced hsp90β transcription (Twomey et al., 1993) and this was reflected by the presence of autoantibodies (Dhillon et al., 1993).

It was subsequently observed that SmN gene transcription was significantly elevated in SLE patients (0.275 +/- 0.050) over that of normal controls (0.102 / +/- 0.022) to a significant degree (p=0.0163) and that in eight out of nineteen patients SmN gene transcription was above the normal range. In contrast, SmB/B' gene transcription although elevated, was not significant (mean=0.203 +/- 0.041 compared to controls mean = 0.132 +/- 0.022 where p=0.167). Furthermore, transcription of the U1 snRNP specific 70 kDa polypeptide was lower in SLE patients (0.32 +/- 0.06) compared to controls (0.42 +/- 0.07).

This significant elevation in SNRPN transcription in SLE is an interesting observation as levels of SmN expression in PBMCs are usually low, high level expression being normally limited to brain and heart. This therefore suggested that the elevated SmN gene transcription may result in elevated SmN protein expression. This could potentially have consequences in terms of autoantigen presentation in the subset of SLE patients demonstrating such an elevation as previously demonstrated for the hsp90 protein (Norton et al., 1988; Twomey et al., 1993; Dhillon et al., 1993). Thus elevated SmN expression could potentially lead to the stimulation of the immune system and the subsequent generation of anti-SmN-specific antibodies or anti-Sm antibodies cross reactive with SmB/B'. In addition, if SmN is involved in alternative splicing or

possesses unique splicing properties, then elevated levels of SmN may affect the nature of RNA splicing performed in such cells, leading to changes in immune function.

Therefore to determine whether the observed elevation in SmN gene transcription was reflected at the protein level, and to further elucidate the role of SmN as an autoantigen in SLE, levels of SmN expression in SLE PBMCs were quantitated with reference to normal controls. To address this, a novel Western blotting assay was developed which allows quantitation of levels of SmN expression despite the presence of SmB' which co-migrates with SmN. The assay is based on the difference in affinity exhibited by the KSm4 monoclonal antibody for SmB/B' and SmN, whereby KSm4 recognises an epitope present on SmB/B' but does not demonstrate any appreciable binding with SmN. This is clearly shown in FIG.10 in which PBMCs from SLE patients and controls were Western blotted with the two antibodies. The KSm5 monoclonal antibody demonstrates the presence of SmB, Sm B' and SmN in both SLE and control PBMCs whilst KSm4 only binds SmB and SmB'. The expression of SmN in PBMCs as demonstrated using KSm5, and the subsequent non-recognition by KSm4 is an important demonstration of the validity and specificity of this antibody assay in quantitating SmN expression.

Complete resolution of SmN from SmB' was observed in only very rare instances and co-migration of the proteins is the usual occurrence. Hence SmN expression was quantitated by measuring differences in the relative intensities of the upper 29 kDa band which represents both SmN and SmB' in parallel KSm4 and KSm5 studies (FIG.11). Obtaining accurate values for levels of actin for subsequent equalisation were unsuccessful and hence values were equalised for levels of SmB obtained with the two antibodies. Therefore the relative levels of SmN in each sample were obtained by subtracting the SmB' value (obtained with the KSm4 antibody) from the SmB' plus SmN value (obtained with the KSm5 antibody) after equalising the SmB values obtained in each case to control for any differences in the efficiency of binding by the two antibodies in a particular experiment. The demonstration that transcription of the SmB gene is not significantly elevated in these SLE patients justifies equalising with SmB rather than with actin.

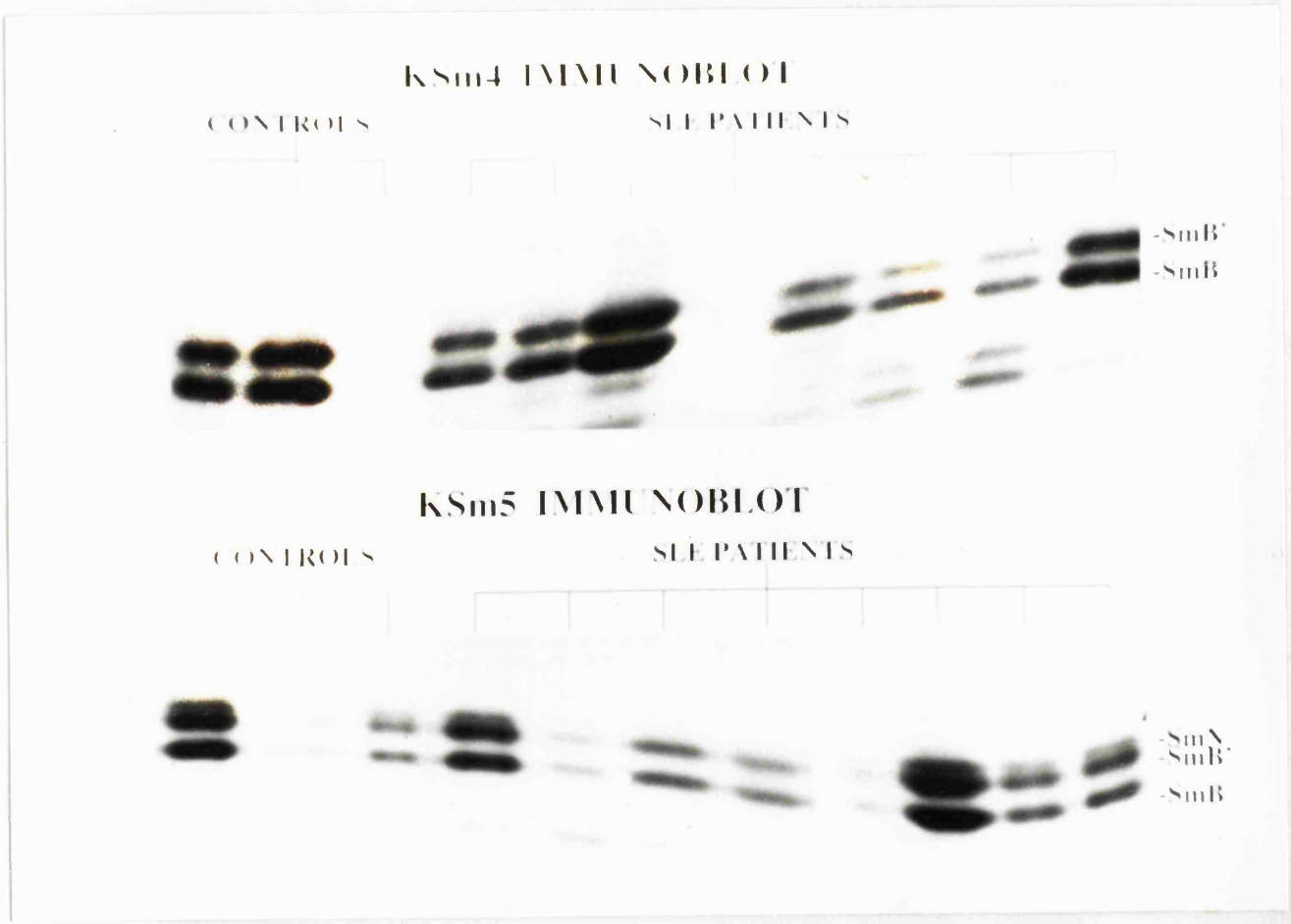


FIG.10. Parallel Western blot analysis of PBMCs from SLE patients and healthy control in which SmN is resolved from SmB', demonstrating SmN expression in PBMCs (KSm5 antibody probe-lower panel). The lack of reactivity of the KSm4 monoclonal antibody with SmN (KSm4 antibody probe-upper panel) demonstrates the basis of the subtractive antibody assay for determining SmN expression. SmB and SmB' are bound by both KSm4 and KSm5 monoclonal antibodies.

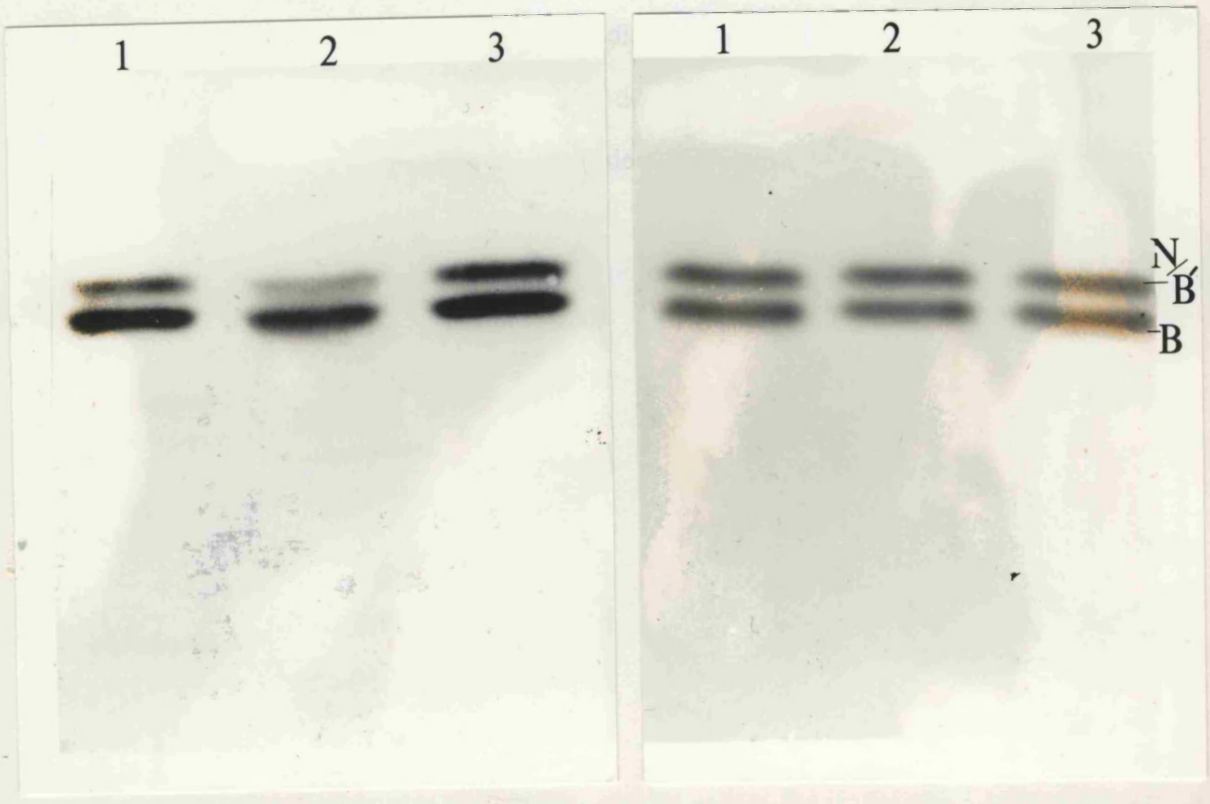


FIG.11. Parallel Western blot analysis of SmN expression in PBMCs from three SLE patients. Here, SmN is not resolved from SmB' which allows more accurate densitometric analysis of SmN expression as revealed by the KSm4 monoclonal antibody (left-hand panel) and KSm5 monoclonal antibody (right hand panel). The upper band from sample 2 demonstrates weak activity with the KSm 4 antibody, and a stronger reactivity with the KSm5 antibody whereas samples 1 and 3 demonstrate similar upper band reactivities with both antibodies. This indicates low or undetectable levels of SmN expression in samples 1 and 3 compared to a significant elevation of SmN expression in sample 2.

3.10. RESULTS FROM A STUDY OF ECTOPIC EXPRESSION OF SmN IN SLE

The mean level of SmN protein expression in SLE PBMCs was not elevated above that observed in rheumatoid arthritis (RA) or other autoimmune disease control (ADC) or normal control samples (FIG.12). Thus, only two SLE patients demonstrated SmN expression greater than the mean plus two standard deviations of levels in the normals (164425 units). SmN expression greater than this mean was also observed in one RA patient and one normal, suggesting it is a common effect of inter-individual variation.

In addition, a parallel comparison of SNRPN transcription against SmN expression is consistent with the above data (FIG.13). Thus, enhanced SNRPN transcription was not observed to result in enhanced SmN expression, and the SLE patient demonstrating the highest level of SNRPN transcription in this comparison (0.575) did not demonstrate significant SmN expression (161606 units). Moreover, non of the SLE sera demonstrating SNRPN transcription above the normal range (0.102 +/- 0.022) were observed to demonstrate significant SmN expression.

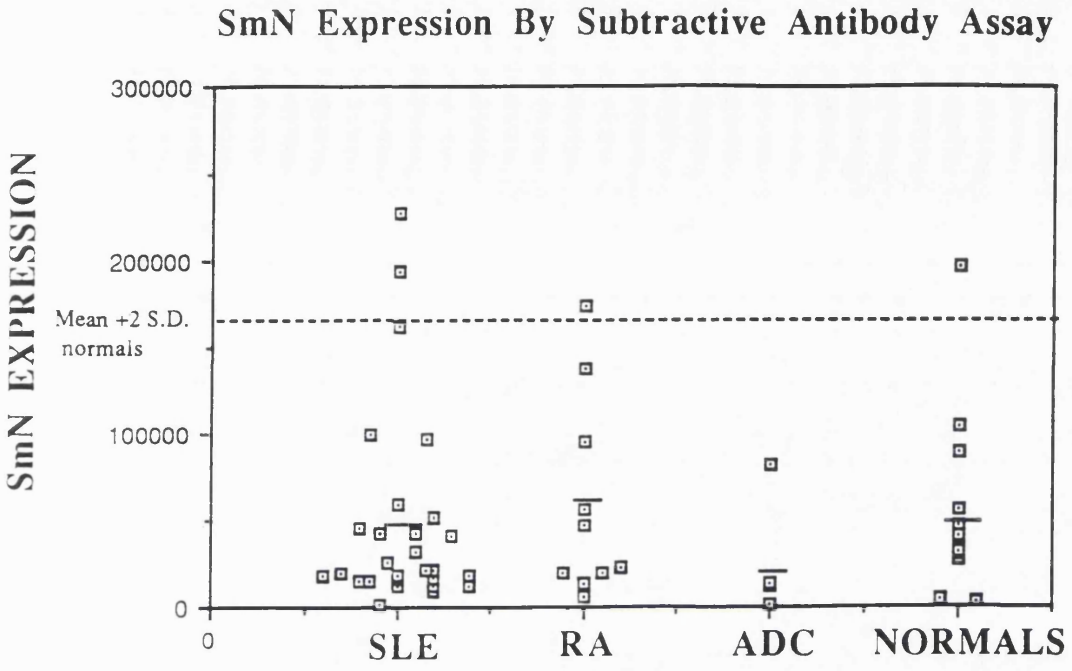


FIG. 12. Data from densitometric analysis of ectopic expression of SmN in PBMCs from Systemic Lupus Erythematosus (SLE) patients, rheumatoid arthritis (RA) patients, autoimmune disease controls (ADC) and normal control samples from a subtractive KSm4 / Ksm5 antibody assay. The mean plus two standard deviations of SmN expression in PBMCs of normals (164425 units) and the mean value of SmN expression in each sample group (horizontal bar) are indicated.

FIG. 13. COMPARISON OF SNRPN TRANSCRIPTION AND SmN TRANSLATION IN PBMCs FROM SLE PATIENTS

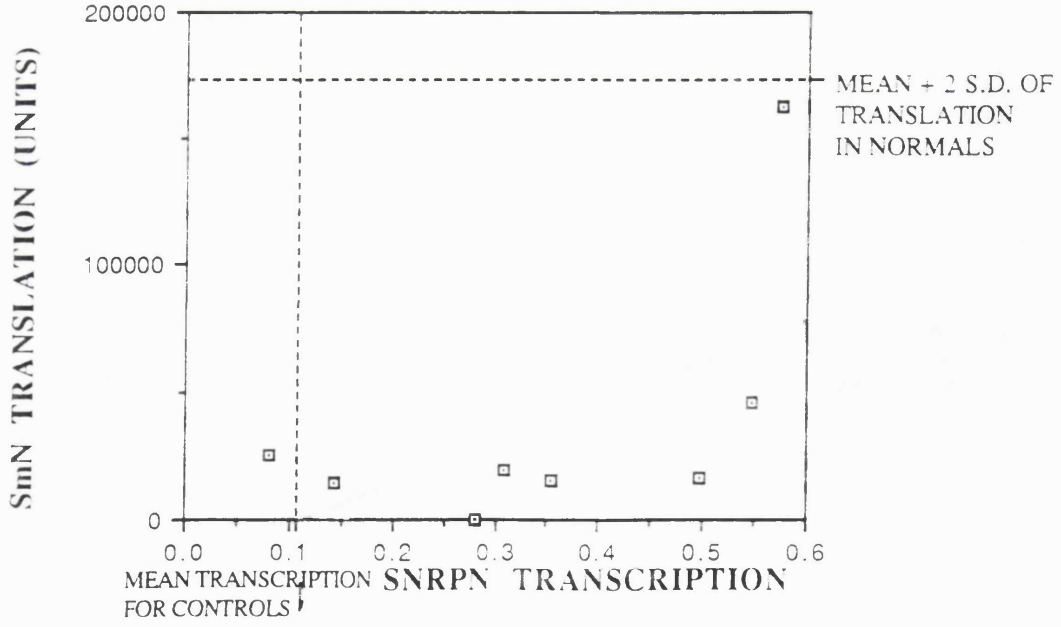


FIG.13. Comparison of SNRPN transcription (nuclear run-on assay) and SmN expression (KSm4/ KSm5 subtractive assay) in SLE patients.

3.11. CHAPTER DISCUSSION

Peptides corresponding to the regions of least sequence homology between SmN and SmB/B' have been used to establish whether the differences in primary structure between the proteins is sufficient to alter protein conformation and hence ultimately suggest whether the function of the proteins could be different. By screening SLE sera on these peptides, the sequence corresponding to residues 179-190 of SmB/B' has been shown to be immunoreactive. In contrast, the polymorphisms within the equivalant SmN sequence are sufficient to stop any significant binding by the SmB/B' 179-190 specific antibodies. The prevalence of autoantibodies to this epitiope in SLE are 14% , this activity being restricted to the anti-Sm positive SLE sera. The presence of antibodies that discriminate between residues 179-190 of SmN and SmB/B' has also been demonstrated in the MRL/lpr and MRL (+/+) murine models of SLE. The second region of least sequence homology (203-213) in both SmN andSmB/B' does not bind SLE autoantibodies.

The competitive inhibition data suggest that the B/B' 179-190 reactive antibodies have up to a 100 times lower affinity for the SmN sequence, thus demonstrating significant specificity for SmB/B'. The half maximum binding at about 1mM (patient 1) shows that the ELISA is detecting low affinity antibodies. This low affinity may be due to the peptide representing only part of the epitope that may extend beyond residues 179-190 or it may be part of a conformational epitope in the 3-dimensional structure of SmB.

Longitudinal studies suggest that the anti-SmB/B' activity is part of the general anti-Sm response. Thus levels of antibody to B/B' 179-190 and Sm show close co-modulation in patient 3, suggesting common regulatory mechanisms. These activities were observed to fluctuate semi-independently in patient 1 such that anti B/B' activity became an insignificant part of the total Sm response with time. This reflects data from screening SLE sera with the SmB 174-213 fusion protein whereby it was observed that 21% of sera tested as being anti-Sm positive sera were negative on the SmB 174-213 ELISA, the assay therefore being 79% sensitive for detecting anti-Sm positive sera. These observations can be interpreted as being due to the autoantibody profiles

against distinct SmB epitopes varying with time and also between patients. This also demonstrates that important SmB epitopes are present outside of the cloned region.

Antibody responses to various Sm polypeptides may also vary with time (E.J. ter Borg et al., 1988) and hence as the assay does not include SmD specific epitopes, it will not be capable of detecting anti-SmD directed activities which may at certain time points be prevalent. In summary therefore, the SmB 174-213 assay is both sensitive and highly specific for the disease group. However it does not account for the entire anti-Sm antibody repertoire present in SLE patients. Thus anti-Sm sera without anti-PPGMRPP activity or the SmB/B' 179-190 specific activity detected using peptides will appear negative. As the conventional anti-Sm assay also proved to be highly specific for SLE, then SmB 174-213 does not offer any obvious advantages as a diagnostic marker for SLE.

It has previously been shown that SmB/B' proteins possess additional epitopes that are unique to these proteins as components of the U1 snRNP only (Ohosone et al., 1992). Data presented here has demonstrated the presence of a further SmB/B'-specific eptitope that is not found in SmN. SmB specific autoantibodies have previously been demonstrated (Elkon et al., 1990) that do not bind SmB' and hence are unlikely to bind SmN as they are directed to carboxyl-terminal leucines present only in SmB. These SmB specific antibody activities taken together with the observation that KSm4 also discriminates between SmN and SmB/B' such that it does not bind SmN appreciably (this chapter) suggest that the amino acid replacements that occur between SmN and SmB/B' are sufficient to alter protein antigenicity.

Many different Sm epitopes are recognised by both human and murine anti-Sm antibodies (Williams et al., 1986) inferring that the Sm antigen itself is driving the autoimmune response. In agreement with this, Sm antigen when administered to MRL mice stimulates the autoimmune

response to Sm proteins (Stocks et al., 1991). The presence of four SmB specific autoantibody activities (outlined above) and the lack of any reported SmN specific antibody activities strongly suggest that the nuclear antigen stimulating the immune system in SLE does not derive from the tissues expressing SmN, namely the brain or the heart. In agreement with data presented here which demonstrates that SLE autoantibodies do not specifically bind the regions containing the greatest number of sequence polymorphisms for SmN (179-190 and 203-213), there are no other reports of SmN-specific autoantibodies against other regions containing polymorphisms for SmN. Thus peptides representitive of residues 49-65 of SmN which contains two polymorphic residues and 223-232 which contains one polymorphic residue were not bound by anti-Sm positive SLE sera (Habets et al., 1989). Therefore, these polymorphic regions in SmN are either non-immunogenic or alternatively, the lack of any reported SmN-specific autoantibodies suggests that SmN is not the antigen stimulating the immune system in SLE. The observation that the immunisation of BALB/c mice with the SmN 179-190 peptide did generate a fairly specific immune response to this peptide (FIG.7a) would seem to support the latter proposal.

Further evidence that SmN is not the antigen stimulating the immune system in SLE is suggested by the demonstration that levels of SmN protein in SLE PBMCs are not elevated despite a significant elevation of SNRPN gene expression in a subset of SLE patients. An elevation of ectopic SmN expression in cells normally expressing very low levels such as PBMCs would suggest a mechanism whereby the immune system could be stimulated to generate autoantibodies to the protein as in the case with the hsp90 gene (Dhillon 1993). However, in agreement with the lack of any SmN-specific antibody activities, an elevation of SmN expression was not observed. Therefore it would appear that antibody reactivities to SmN are a result of cross reaction due to the high sequence homology with SmB. The apparently greater specificity of the N216-238 antibody for SmN (Schmauss and Lerner 1990) is due to a further repeat of the PPPGMRPP epitope and hence this antibody will also bind SmB' with greater avidity than SmB. The lack of any SmN-specific autoepitopes suggest it is not neccessary to include the tissue specific SmN antigen in diagnostic ELISA assays.

The lack of SmN specific autoantibodies occuring in SLE, suggests that it would not be SmN alone that is affected by autoantibodies binding and sequestering it. Rather, antibodies originally reactive with SmB will cross react with SmN and if splicing is affected it would be due to immune complex formation involving many distinct snRNP polypeptides bound by a wide spectrum of autoantibodies. Thus, provided autoantibodies are capable of entering cells, and furthermore the nucleus, all constitutive or SmN-specific functions of SmN and indeed the function of other snRNP polypeptides are likely be affected in SLE. If autoantibodies remain outside of the cell, they are unlikely to affect splicing.

Enhanced SNRPN transcription in a subset of SLE patients is indicative of aberrant function of transcription factors in PBMCs, however as this enhancement is not reflected at the protein level, it suggests that a post-transcriptional process acts to counter this. This is consistent with similar studies in SLE patients with the hsp72 gene (Twomey et al., 1992) whereby elevated gene transcription did not result in elevated protein expression. However an enhanced transcription of the hsp90 gene (Twomey et al., 1993) did result in elevated protein expression and the subsequent generation of autoantibodies (Norton et al., 1988; Dhillon et al., 1993). This suggests that elevated transcription of certain genes due to aberrant function of transcription factors is a common feature of SLE and that post-transcriptional mechanisms may or may not act to control this. SmN expression levels in SLE patients are consistent with those in normals as indicated in this study. This suggests that if the nature of RNA splicing occuring in PBMCs and perhaps other cells in SLE patients does differ from that in normals, it cannot be attributed to altered levels of SmN expression, and is thus more likely to be an effect of cross-reactive autoantibodies.

CHAPTER 4
ANALYSIS OF SmN AND SmB WITHIN snRNP PARTICLES

4.1. INTRODUCTION

In order to study the role of SmN in RNA splicing it is essential to determine the nature of its association within the snRNP particle. It may be postulated that any unique properties of SmN could potentially affect the activity of the spliceosome and therefore how RNA transcripts are processed. Previous studies have demonstrated that SmN, like SmB does in fact associate with snRNAs, as demonstrated by immunoprecipitaing with antibodies against the 5' CAP structure of snRNAs (Mc.Allister et al ., 1988). Anti-Sm antibodies also precipitate SmN but this does not necessarily demonstrate that SmN is snRNP-associated. This may only be proved by indirect methods such as using monoclonal antibodies against other snRNP proteins, for example SmD. Evidence of protein-protein interactions with other snRNP polypeptides is suggested by the fact that SmN may be immunoprecipitated indirectly using anti-(U1) RNP antibodies (Mc.Allister et al., 1988; McAllister et al.,1989).

An understanding of the nature of association of SmN with particular snRNP particles would be valuable in understanding the function of the protein. Thus if SmN was to associate with particular snRNPs in a way that was different to the closely related SmB protein, then RNA splicing performed by SmN-containing snRNPs may also be different. To address this, a study of the localisation of SmN within the snRNP particles was performed using antibodies against components specific to the U1 and U2 snRNPs. This localisation was investigated using extracts from adult rat tissues and F9 (embryonal carcinoma) and ND7 (neuronal) cell lines. In addition, the association SmN with snRNPs in 3T3 mouse fibroblasts artificially expressing SmN and the subsequent effect on total protein composition in such cells was investigated.

Antibodies to components specific to the U2-snRNP occur naturally in SLE, scleroderma and polymyositis (Mimori et al., 1984). Indeed, such autoantibodies have been of great importance in deducing the polypeptide composition of the U2 snRNP (Mimori et al 1984; Habets et al ., 1987). However, extensive cross reactivity of such sera occurs such that antibodies that bind the U2 specific B" polypeptide also bind the U1-snRNP associated A polypeptide and vice-versa due to common epitopes (Craft et al., 1988; Habets et al., 1989a). Hence such antibodies

are not satisfactory for immunoprecipitating specific snRNP particles. The 4G3 monoclonal however, is specific for an epitope unique to the U2- associated B" protein (Habets et al., 1989b). This therefore represents a highly specific means of isolating the U2 snRNP-associated polypeptides.

Autoantibodies to the U1-snRNP-specific 70K, A and C polypeptides are common in mixed connective tissue disease (Sharp et al., 1972; Tan ,1982). Anti-(U1) RNP sera have been used in previous investigations of the the association of SmN with U1-snRNPs in PC-12 cells and human cortex (Mc.Allister et al., 1988; McAllister et al., 1989). In addition to using anti-(U1) RNP sera, data presented in this report was obtained using the K8.43 monoclonal which is specific for the U1 snRNP specific 70K polypeptide (Williams and Stocks et al., 1986, unpublished).

Other antibodies used in this study are reactive with various snRNP polypeptides. Thus the KSm 1 and 2 monoclonals are specific for two different epitopes on the 16 kDa SmD polypeptide (Williams et al., 1986). The KSm5 monoclonal is specific for a proline rich carboxyl terminal epitope (PPGMRPP) common to SmB/B' and SmN (Williams et al 1990).

The KSm4 monoclonal demonstrates reactivity to SmD and SmB/B' (Wiliams et al., 1986). Interestingly despite the high homology of SmB/B' with SmN, the KSm4 monoclonal does not demonstrate appreciable reactivity with SmN. Thus KSm4 did not bind the PP1 SmN cDNA clone that lacks only the 23 N-terminal amino acids of SmN (Williams et al., 1990), and does not bind SmN in expression studies on rodent tissue (D. Horn PhD. thesis). This is also demonstrated in results for F9 immunoprecipitaions presented here and in the analysis of SmN expression in SLE PBMCs also presented here. These observations taken together with the lack of sequence homology between SmD and SmB/B' suggests that the epitope for KSm4 is a conformational one or a result of post-translational modification. Thus, further evidence of a difference in protein conformation between SmN and SmB/B' is indicative of SmN possessing unique properties.

4.2. ND7 NEURONAL CELL LINE

The association of SmN with snRNPs was analysed in the ND7 cell line (Wood et al ., 1990) which express SmN (Horn et al., 1992). Due to the lack of availability of a suitable methionine free media for the growth of these cells, immunoprecipitations were performed using unlabelled ND7 extracts and proteins visualised by Western blotting with an anti-Sm/ RNP positive SLE serum (FIG.1). As expected, the KSm2, KSm4 and KSm5 precipitates contain both SmB and SmN, verifying the association of SmN with snRNPs in this neuronal cell line. In addition, the U1-snRNP associated 33 kDa A polypeptide is present in these precipitates. The U1-snRNP specific K8.43 monoclonal and the anti-(U1)RNP sera do not immunoprecipitate SmN. As expected, the U1-specific A polypeptide was present in these samples however strangely, SmB did not appear to be present. This may however be a result of the apparent higher affinity of the particular serum used in visualisation for SmN and the A polypeptide than for SmB, as reflected by its performance in this immunoprecipitation. In contrast to the U1-snRNP results, SmN was clearly precipitated using U2-snRNP specific 4G3 monoclonal, whereas SmB was not. The band corresponding to the A polypeptide is negligible in this precipitate again showing the specificity of 4G3 for the U2-snRNP. Therefore, in ND7 cells the nature of the association of SmN with the U2 snRNP is different to its association with the U1-snRNP.

To correctly verify the bands observed in the ND7 immunoprecipitates as being SmN rather than the U2-snRNP associated B" polypeptide which has a similar molecular weight (28.5 kDa, Habets et al., 1985), further experiments were performed. ND7 total cell protein samples were run on five different 12.5% gels incorporating different ratios of acrylamide and bisacrylamide. Of these, a gel with an acrylamide to bisacrylamide ratio of 30:0.5 % was found to sufficiently resolve SmN and SmB from B" however resolution between SmN and SmB was reduced (FIG.2). The anti-Sm sera used in the ND7 immunoprecipitations clearly did not demonstrate any reactivity with the B" polypeptide, which was specifically visualised with 4G3 monoclonal antibody as a band migrating slower than SmB/N in a seperate track of a multi-channel

cassette blotting apparatus. Furthermore the occurrence of anti-(U2) antibodies in autoimmune diseases is reported as being infrequent (Mimori et al., 1984). The SLE sera used demonstrated a reactivity identical to that observed by the KSm5 SmN/B specific monoclonal on this gel. Thus the 29kDa band in ND7 immunoprecipitaions is verified as being SmN.

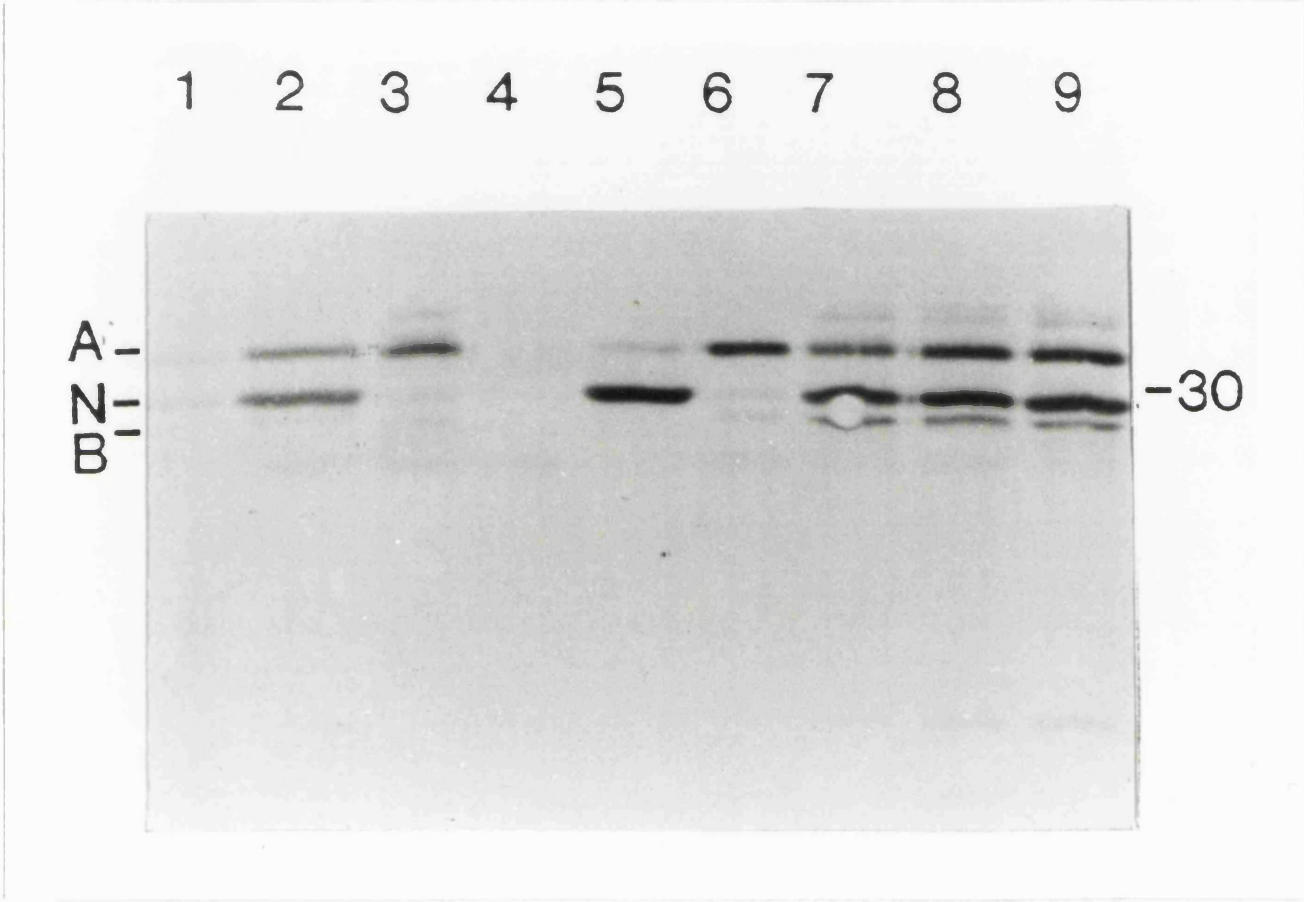


FIGURE 1. Immunoblot with anti-Sm positive SLE sera of snRNP polypeptides immunoprecipitated from ND7 cells using the indicated antisera. Lanes: 1, ND7 total cell extract; 2, anti-Sm positive SLE sera; 3, anti-(U1)RNP SLE sera; 4, OX-12 non anti-Sm monoclonal antibody; 5, 4G3 anti-U2B"monoclonal antibody; 6, K8.43 anti- U1-70kDa monoclonal antibody; 7, KSm5 anti-SmB/B'/N monoclonal antibody; 8, KSm4 anti-B/B'monoclonal antibody; 9, KSm2 anti-SmD monclonal antibody.

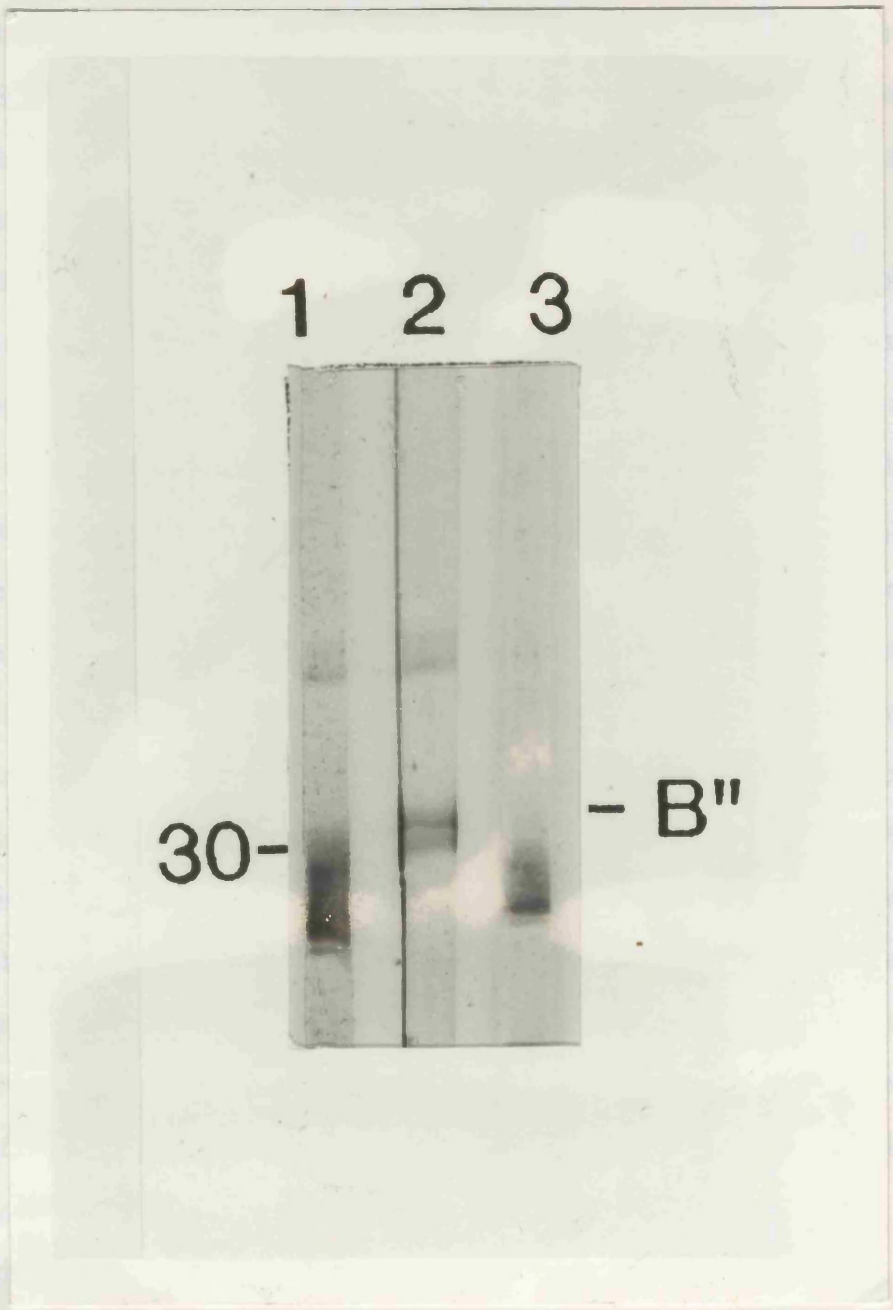


FIGURE 2. Immunoblot of ND7 total cell extract. Lanes: 1, probed with KSm5; 2, probed with 4G3 anti-U2B"; 3, probed with anti-Sm positive sera used as the probe in FIG.1

4.3. F9 EMBRYONAL CARCINOMA (EC) CELL LINE

Previous studies have shown that the embryonal carcinoma (EC) cell lines F9, PCC3, PCC4 and the embryonic stem cell (ES) line CCE cells demonstrate SmN expression (Sharpe et al., 1989; Sharpe et al., 1990). Therefore, immunoprecipitations were performed using [L-35S-Met]-labelled rodent embryonal carcinoma cell line F9 extracts in order to examine the association of SmN with the U1 and U2 snRNPs.

Preliminary experiments in F9 cells were performed using a battery of anti-Sm antibodies in order to confirm the association of SmN with snRNPs by using indirect immunoprecipitation with antibodies directed at other components of snRNPs (FIG.3). The KSm1 and KSm2 antibodies which are specific for SmD (16 kDa) immunoprecipitated the 33 kDa A polypeptide, a 29 kDa band representing SmN, a 28 kDa band representing SmB the 22kDa C polypeptide and SmD. The KSm4 monoclonal precipitated an identical pattern of polypeptides as the anti-SmD monoclonals (although the band corresponding to SmN was slightly weaker). Taken together, the results for these monoclonals show that SmN is indeed associated with snRNPs via protein-protein interactions with other snRNP components. This indirect method verifies the existence of such interactions that are not fully confirmed in previous reports (MC.Allister et al., 1989; 1990). Thus in this case either autoimmune sera are used which may contain cross reactive antibodies or the monoclonal antibody Y-12 was used which was specific for SmN. The control non-anti-Sm isotype-matched antibody OX-12 (IgG2a subclass) did not precipitate any of the bands obtained using the other antibodies thus confirming the specificity of the latter for snRNP components. Three out of five anti-Sm sera precipitated the same polypeptides as the anti-Sm monoclonals. An interesting observation in such preliminary experiments was the lack of a 29 kDa band corresponding to SmN in the U1-snRNP specific K 8.43 immunoprecipitates.

This observation was examined in more detail using anti-(U1) RNP sera and the 4G3 monoclonal specific for the U2-associated B" polypeptide (FIG.4). Results for the anti-(U1) RNP sera used are consistent with the K8.43 monoclonal results in that the 29 kDa band corresponding to SmN is absent whereas other snRNP components (SmB, SmD, A and C) are

clearly present. In contrast, both SmB and SmN are clearly detectable in immunoprecipitates with the 4G3 monoclonal antibody. The specificity of this monoclonal for the U2-snRNP is demonstrated by the lack of U1-snRNP specific A and C polypeptides in such immunoprecipitates. Therefore SmN is detectable in the U2-snRNP but not the U1snRNP in F9 cells.

Further conformation that the 29 kDa band observed in autoradiographs corresponds to SmN is demonstrated by immunoprecipitation of unlabelled F9 extract and subsequent Western blotting using the KSm5 and KSm4 antibodies (Fig.5). Here the protocol was modified slightly to minimise the appearance of mouse IgG on the blot, thus allowing the use of the anti-mouse IgG Horse-Raddish Peroxidase conjugate for visualisation (materials and methods). Results from Western blotting with KSm5 (left-hand panel) clearly show the presence of the 29 kDa SmN and 28 kDa SmB in immunoprecipitations using KSm2 and 4G3 monoclonal antibodies. The identity of the 29 kDa band as being SmN is further verified by Western blotting with the KSm4 monoclonal (right-hand panel). Here, the 29 kDa band corresponding to SmN is not seen in identical immunoprecipitates or indeed the total protein sample due to the non-reactivity of KSm4 with SmN (see above). This therefore proves that the 29 kDa band observed in the 4G3, KSm1-5 and anti-Sm positive SLE sera immunoprecipitates from [L-35S-Met]-labelled F9 cells (Fig.3) corresponds at least in part to SmN. Unfortunately, the K8.43 immunoprecipitation in Fig.5 was of insufficient quality to determine the presence of SmB only in this precipitate as suggested by data from the labelled F9 extracts.

Therefore, data from immunoprecipitations of F9 EC cells appears consistent with observations in ND7 cells in the respect that affinity of SmN for the U2 snRNP was higher than that for the U1snRNP relative to that observed for SmB.

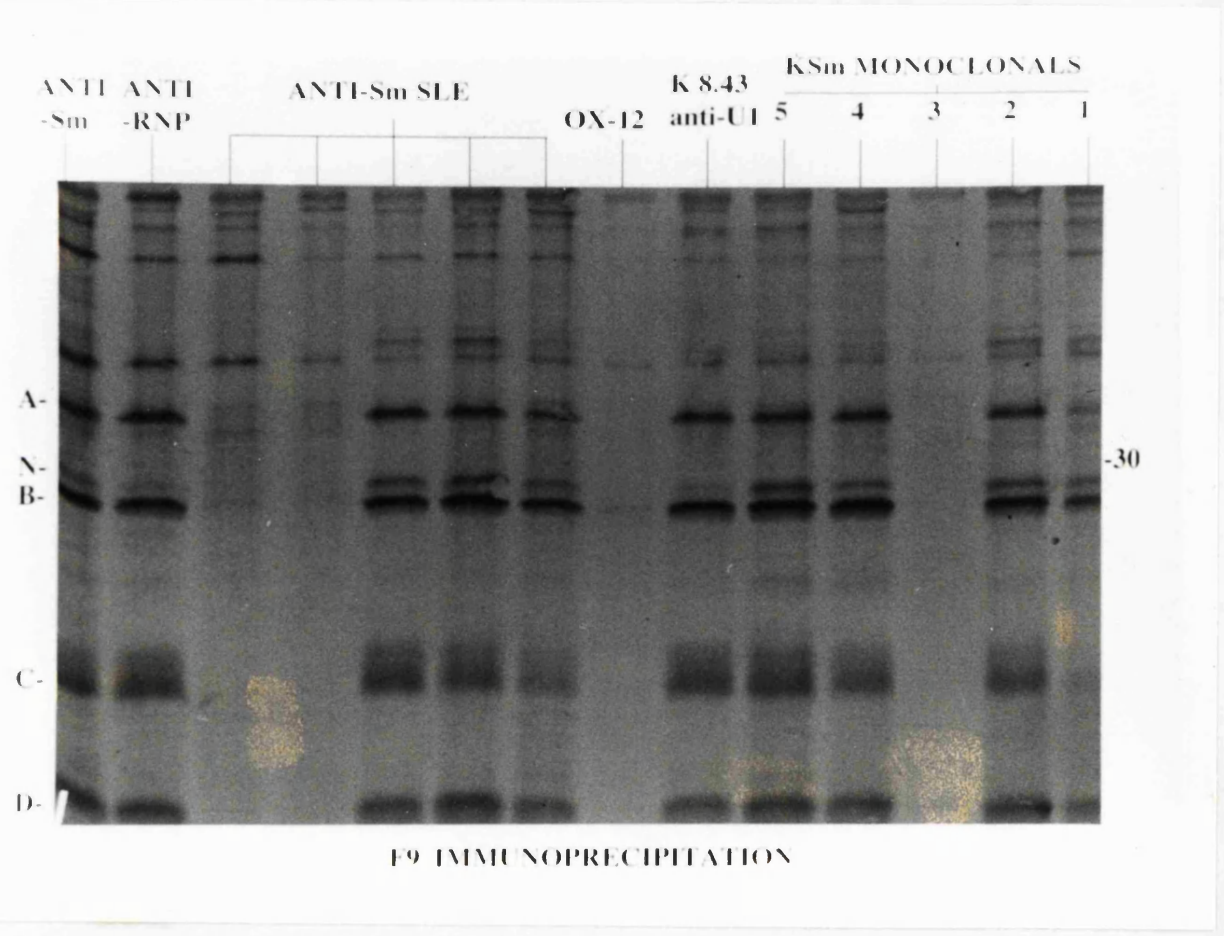


FIGURE. 3. Autoradiograph of L-[35S]-methionine-labelled F9 embryonal carcinoma cell line immunoprecipitations: Lanes as indicated.

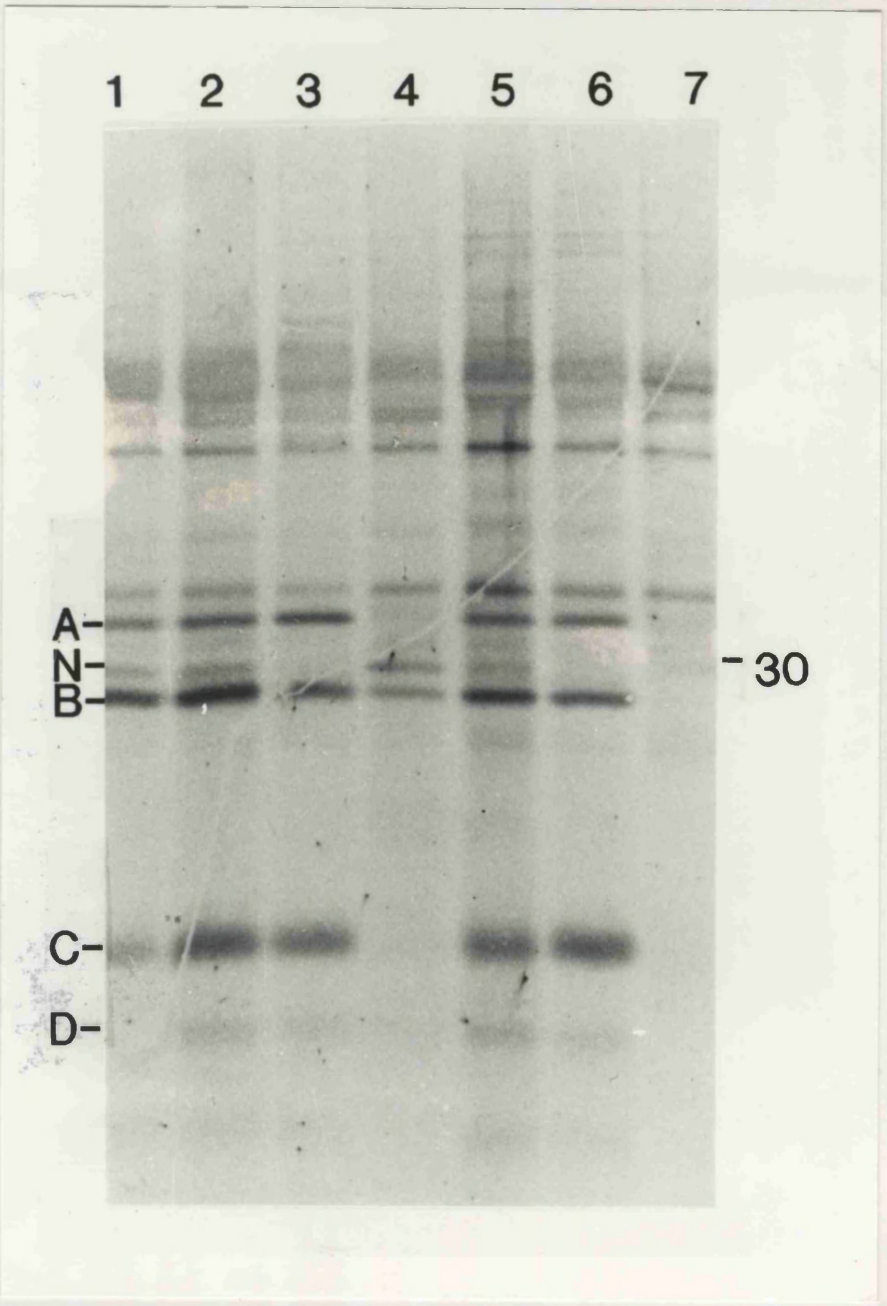
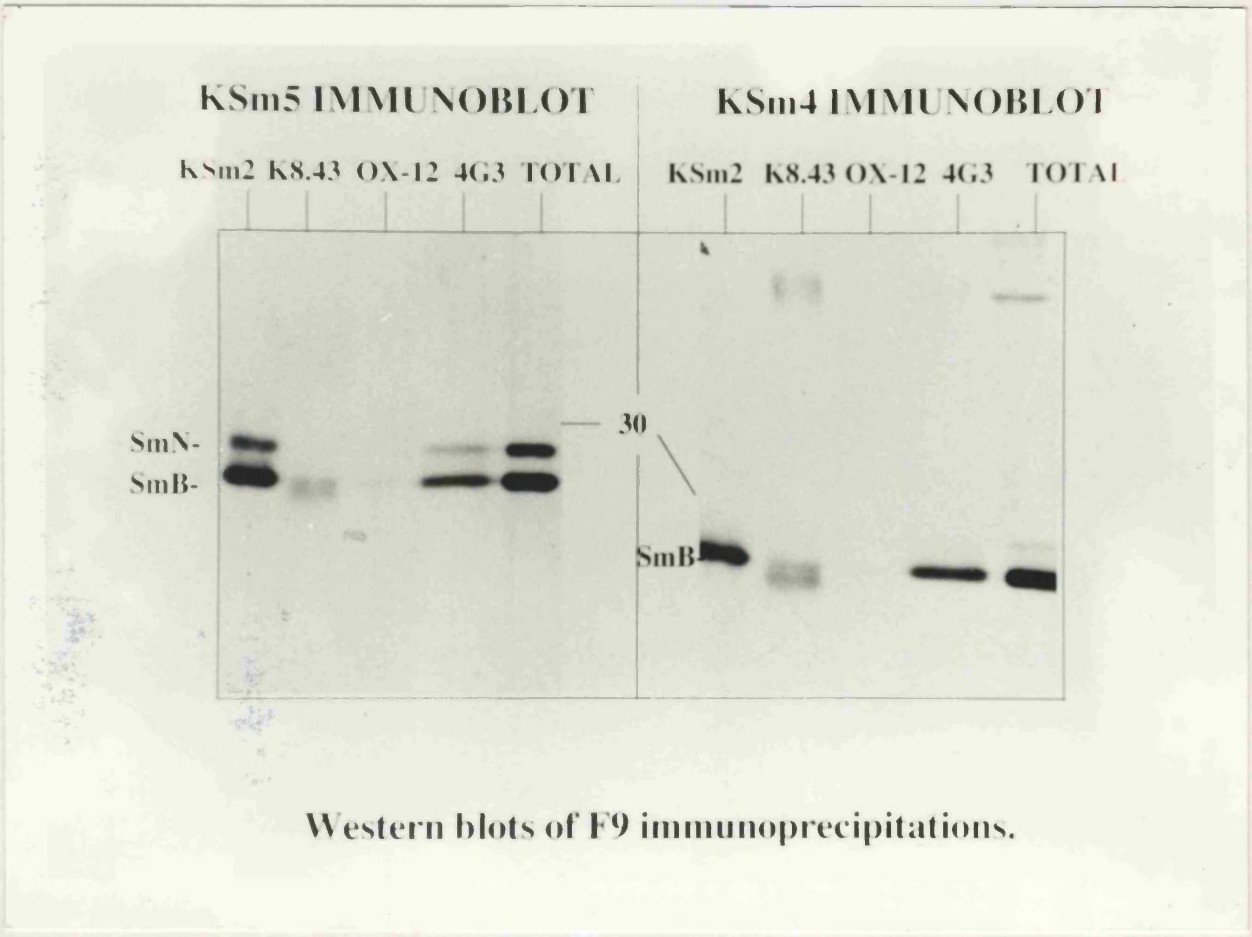


FIGURE 4: Autoradiograph of L-[35S]-methionine labelled F9 embryonal carcinoma cell line immunoprecipitations. Lanes 1, KSm4; 2, KSm5; 3, K8.43; 4, 4G3 anti-U2B"; 5, anti-Sm positive SLE sera; 6, anti-(U1)RNP SLE sera; 7,OX-12.



Western blots of F9 immunoprecipitations.

FIGURE 5. Immunoblot of F9 embryonal carcinoma cell immunoprecipitations probed with KSm4 or KSm5-lanes as indicated

snRNP LOCALISATION OF SmN IN RODENT TISSUE.

4.4. RAT BRAIN

The interesting results obtained from the cell lines prompted an investigation into the association of SmN with snRNPs in rodent tissue. Here the protocol was modified as in the F9 experiment (Fig.5) to detect SmN and SmB specifically with KSm5. The immunoblot (FIG.6) shows strong association of SmN with snRNPs in rat brain by indirect precipitation with KSm2, reflecting the high level of SmN expression in the brain relative to SmB. In fact, SmB was undetectable in these initial precipitates (FIG.6) and was just detectable in KSm2 and KSm5 immunoprecipitates from a separate repeat of the experiment which was visualised with anti-Sm SLE sera (FIG.7). In contrast to results observed in the cell lines, SmN was found to localise in both the U2 and U1-snRNPs in rat brain. The presence of SmN in the U1-snRNP in brain was further verified with the anti-(U1) RNP sera. As expected, no reactivity with KSm5 or the SLE sera was observed when immunoprecipitating with the OX-12 control monoclonal antibody.

4.5. RAT LIVER

In contrast to brain snRNPs, immunoprecipitates from rat liver show no evidence of SmN association with snRNPs reflecting the undetectable levels of SmN expression in this tissue (FIG.7). As expected, the SmB core snRNP polypeptide was readily detectable in the KSm2 and KSm5 precipitates and more specifically in the U1-snRNP precipitates.

4.6. QUANTITATIVE ANALYSIS OF RELATIVE EXPRESSION OF SmN AND SmB.

Data obtained from the observed discrepancy in U1-snRNP localisation between brain and cell lines prompted us to question whether there was insufficient SmB expression in brain to produce functional U1-snRNPs, thus necessitating the production of SmN-containing U1-snRNPs. This was addressed by performing densitometric analysis on 5 separate immunoblots of total protein from ND7, F9 cells and rat brain probed with KSm5. The averages obtained from

this analysis reveal levels of SmN relative to SmB respectively are: F9=54%:46%; ND7= 37%: 63%; and brain 85%:15 % (FIG.8). The implications of this are addressed in the chapter discussion.

FIGURE 6. Immunoblot of immunoprecipitations from rat brain probed with KSm5 monoclonal antibody. Lanes: 1, Brain total protein; 2, Blank; 3, 4G3 anti-U2B"; 4, OX-12; 5, Anti-(U1)RNP SLE sera; 6, K8.43 anti-U1 70kDa; 7, KSm2.

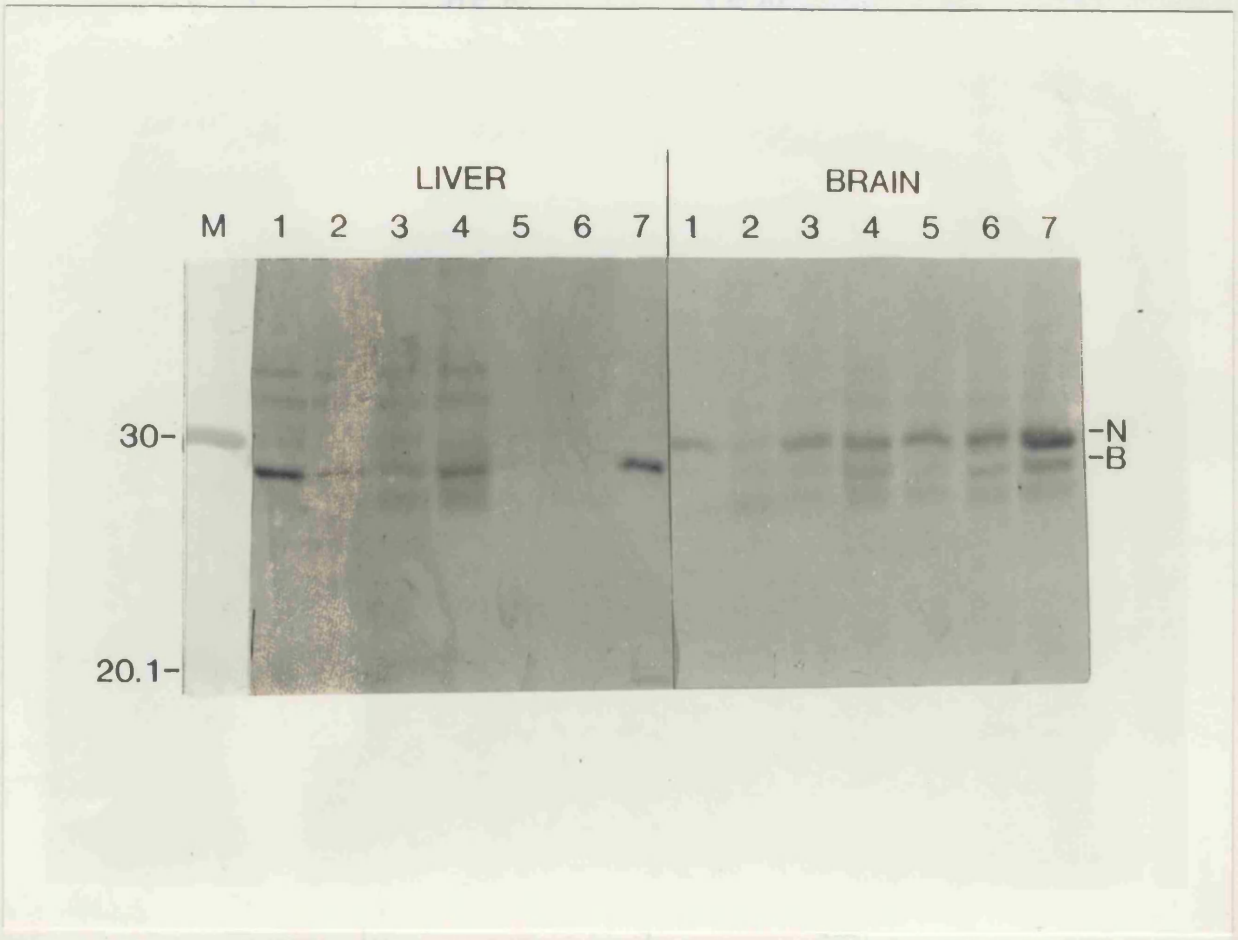


FIGURE 7. Immunoblot of immunoprecipitations from rat liver and brain. Liver lanes: 1, KSm2; 2, KSm5; 3, K8.43 anti-U1 70 kDa; 4, anti-(U1)RNP sera; 5, 4G3 anti-U2B"; 6,OX-12; 7, Total protein extract.
Brain lanes: 1, Total protein extract; 2, OX-12; 3, 4G3 anti-U2B"; 4, Anti-(U1)RNP SLE sera; 5, K8.43 anti-U1 70kDa; 6, KSm5; 7, KSm2.

a

	SmN	SmB
BRAIN	85 %	15 %
F9	54 %	46 %
ND7	37 %	63 %

b

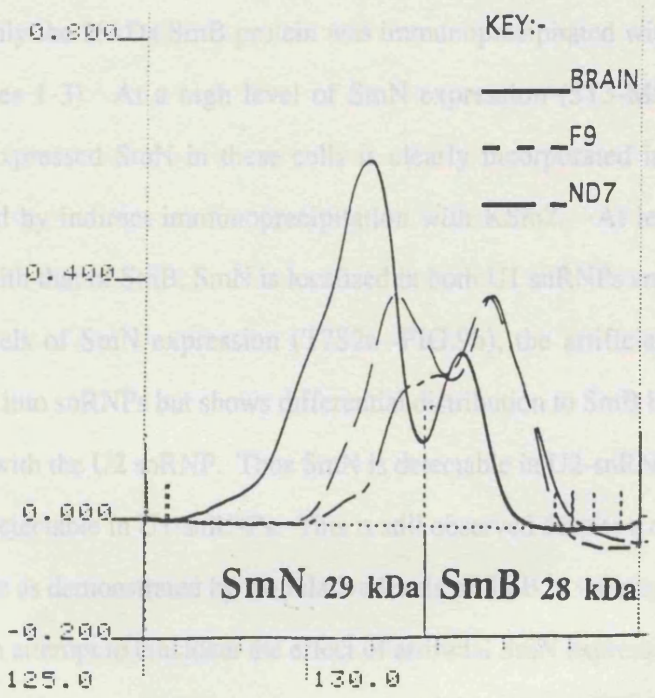


FIG.8. a.) Quantitative analysis of the relative expression of SmN and SmB in rat brain, F9 and ND7 cell lines. Densitometer data obtained from averages of 5 scans of KSm5-probed Western blots of total tissue/cell extract covering three different sample concentrations. b.) Output from typical single densitometer scan of KSm5 Western blot of total cell/ tissue extracts.

4.7. snRNP DISTRIBUTION OF SmN IN 3T3 CELLS ARTIFICIALLY EXPRESSING SmN AND THE EFFECT ON TOTAL PROTEIN EXPRESSION

To further examine the nature of association of SmN with snRNP particles, immunoprecipitations were performed on mouse 3T3 fibroblasts which lack endogenous SmN expression (Horn et al., 1992), and in 3T3 cells artificially expressing SmN. These cell lines were generated by stable transfection of 3T3 cells with the mouse SmN cDNA clone 201 introduced into the M5Gneo or pJ7 vectors as an EcoR1 fragment in the sense orientation, and containing the complete coding region for SmN (Horn and Latchman., 1993). This generated two different stably transfected cell lines expressing different levels of SmN. Thus the 3T3-MSN cell line expressed high levels of SmN and the 3T3-T7S2a expressed relatively low levels of SmN.

Immunoprecipitations were performed using unlabelled cell extracts and by probing blots with KSm5. The parental 3T3 cell line clearly demonstrates no endogenous expression of SmN and hence only the 28kDa SmB protein was immunoprecipitated with both U1 and U2-snRNPs (FIG.9a, lanes 1-3). At a high level of SmN expression (3T3-MSN-FIG.9a, lanes 4-7), the artificially expressed SmN in these cells is clearly incorporated into the snRNP particles as demonstrated by indirect immunoprecipitation with KSm2. At levels of expression roughly equivalent with that of SmB, SmN is localised in both U1 snRNPs and U2-snRNPs. Conversely, at lower levels of SmN expression (T7S2a -FIG.9b), the artificially expressed SmN is also incorporated into snRNPs but shows differential distribution to SmB by demonstrating a preferred localisation with the U2 snRNP. Thus SmN is detectable in U2-snRNP immunoprecipitates but is virtually undetectable in U1-snRNPs. This is still observed despite a deliberate overloading of the K8.43 sample as demonstrated by the relative levels of SmB in the respective lanes.

In an attempt to elucidate the effect of artificial SmN expression in the 3T3 cell line, total [35S-Met]-labelled protein samples from parental 3T3 cells and 3T3-MSN cells were subjected to 2-dimensional gel analysis under identical conditions (FIG.10). This allowed the visualisation of a number of protein spots that were unique to either parental cells or 3T3-MSN cells. The most noticeable consistent differences were the disappearance in 3T3-MSN samples of a protein (A) which

was usually observed as part of a horizontal triplet pattern of protein spots in parental cells. Likewise, the appearance of protein designated C in MSN cells appears to be an effect of SmN expression in these cells. In addition, a number of proteins between 30-45 kDa were observed to consistently appear in 3T3-MSN samples only (boxed region). Proof that these differences in total protein expression are attributable to the nature of SmN expression is provided by the inclusion of the antisense sample (FIG.11b). This acts as a control for the effect on total protein expression on transfection with a plasmid. The protein designated A in parental / MSN samples is still present on analysis of the antisense control sample, suggesting its disappearance in MSN samples is due to the expression of SmN. Conversely, expression of the protein designated B appears to be upregulated in both MSN and antisense samples whilst being negligible in parental cells. Hence this is probably an effect on protein expression caused by plasmid transfection. The differences in boxed region proteins including protein C in the parental/MSN comparison is better demonstrated in the MSN/ antisense comparison, these differences appearing to be due to the effect of artificial SmN expression. The observed differences in protein expression may possibly reflect a change in the nature of RNA splicing performed on induced expression of SmN, for example the appearance of different protein isoforms. They may also be due to an effect on constitutive splicing or some other putative function of SmN. Unfortunately it was not possible to perform sequencing of these proteins and hence their identification was not resolved.

FIGURE 9a. KSm5 probed immunoblot of immunoprecipitations from 3T3 parental cells (lanes 1-3) and 3T3-MSN cells stably transfected with the mouse SmN cDNA clone 201, artificially expressing high levels of SmN (lanes 4-7). Lanes: 1, 3T3 4G3 anti-U2B"; 2, 3T3 K8.43 anti-U1 70 kDa; 3, 3T3 total cell extract; 4, 3T3-MSN 4G3 anti-U2B"; 5, 3T3-MSN K8.43 anti-U1 70kDa; 6, 3T3-MSN KSm2; 7, 3T3-MSN total cell extract.

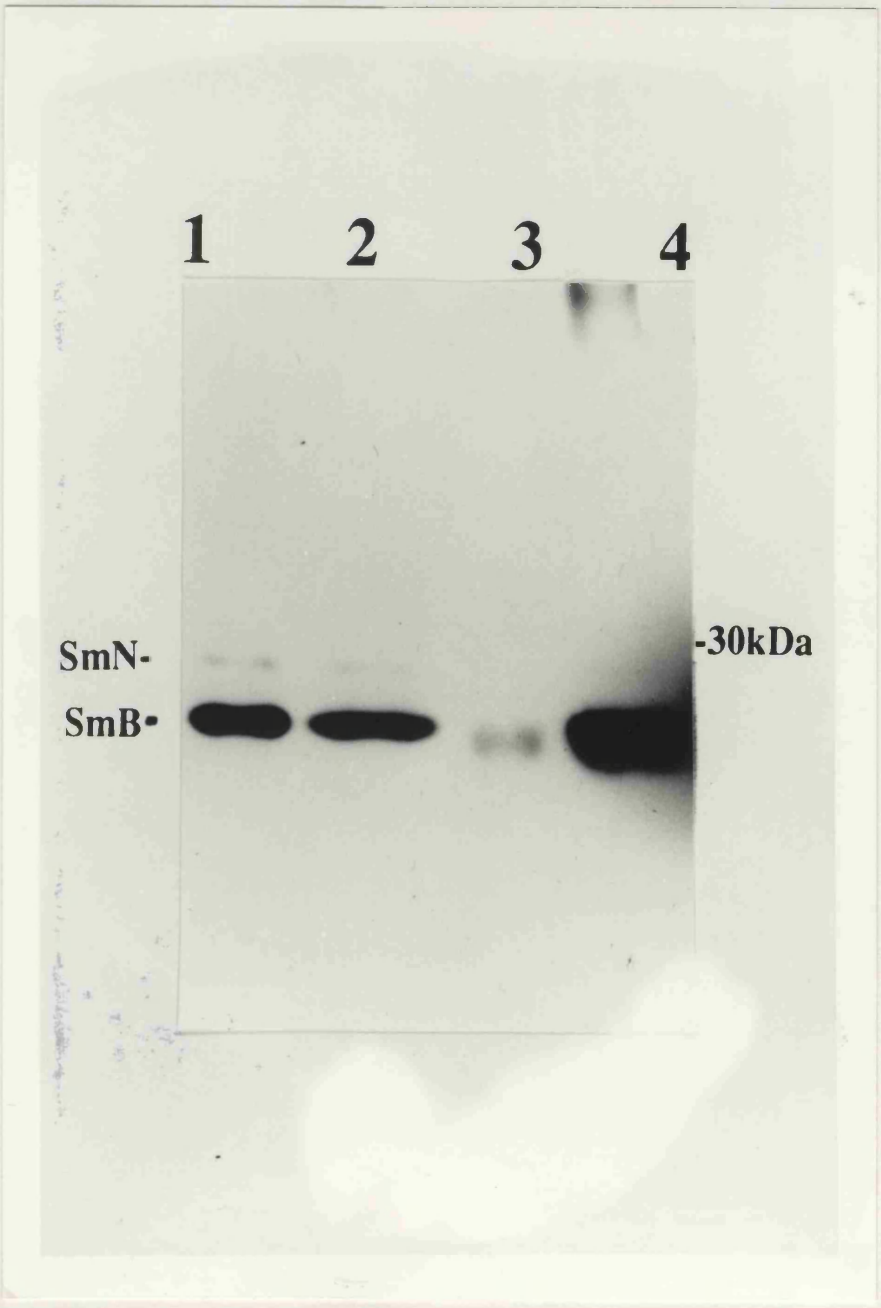


FIGURE 9b. KSm5 probed immunoblot of immunoprecipitations from 3T3-T7S2a cells stably transfected with mouse SmN cDNA clone 201, stably expressing low levels of SmN. Lanes: 1, 3T3-T7S2a total cell extract; 2, 4G3 anti-U2B"; 3, OX-12; 4, K8.43 anti-U1 70kDa.

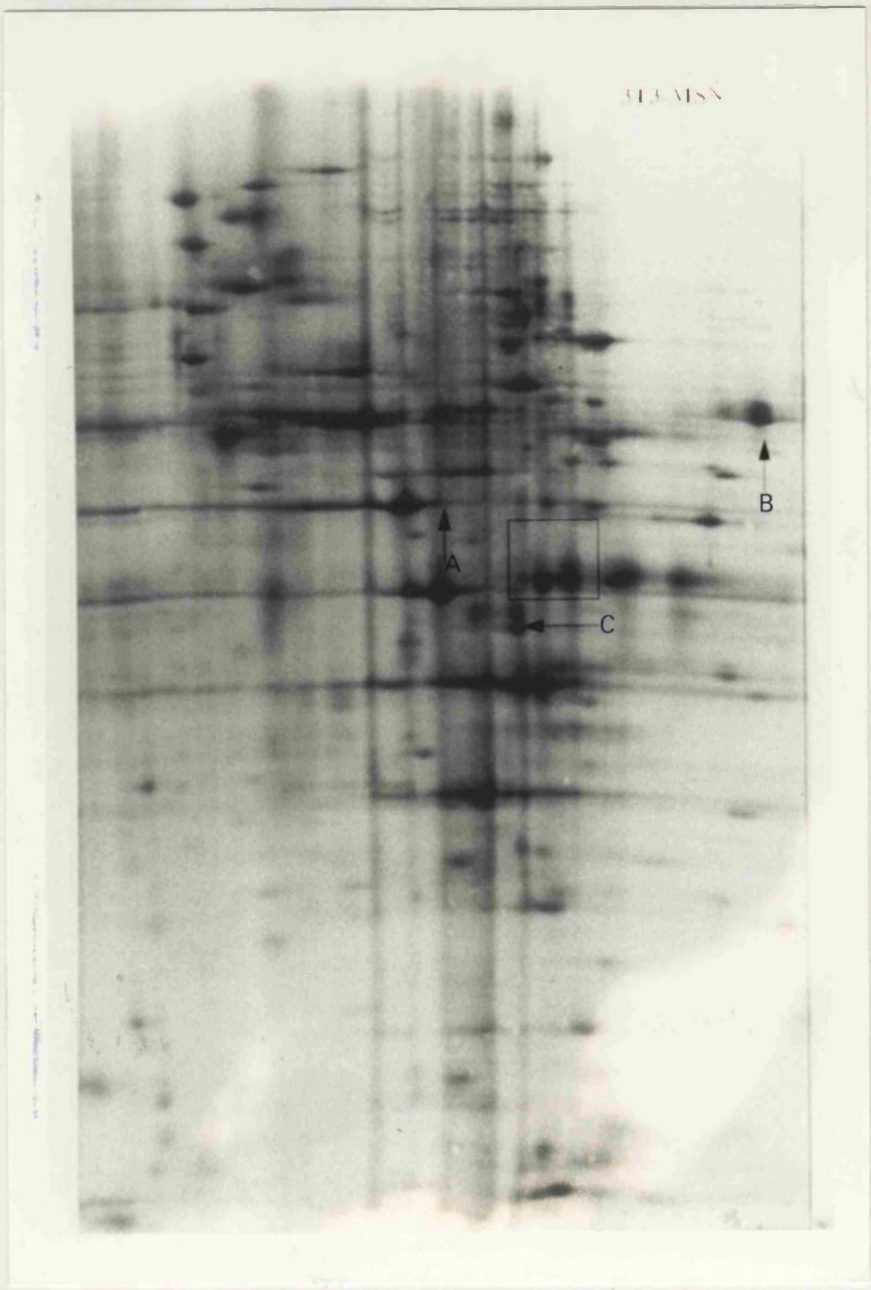


FIGURE 10.a. 2-dimensional gel analysis of L-[35S-Met]-labelled total cellular proteins from 3T3-MSN cells. First dimension separation by pH 3.5-10 Immobiline strip, second dimension separation by 12.5 % SDS-PAGE.

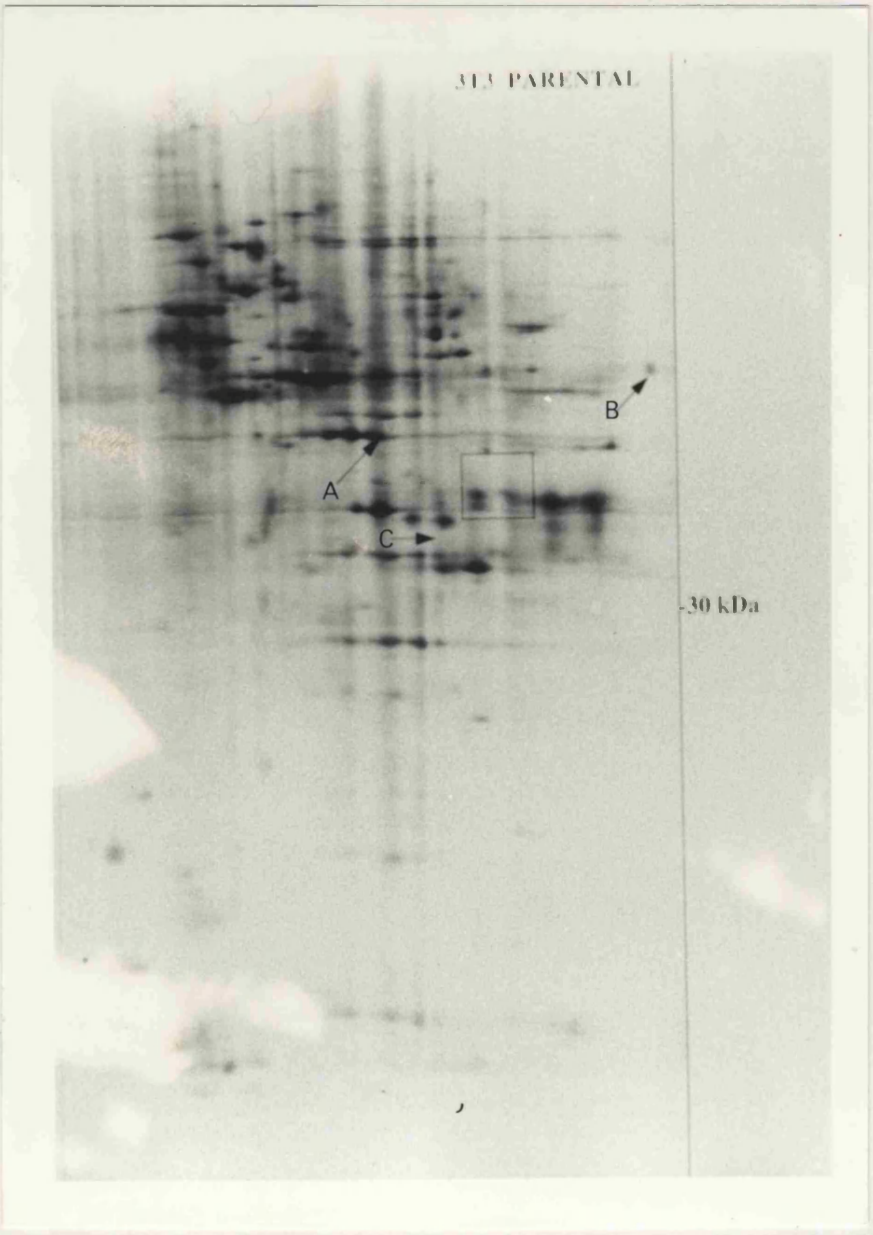


FIGURE 10.b. 2-dimensional gel analysis of L-[35S-Met]-labelled total cellular proteins from 3T3 parental cells. First dimension separation by pH 3.5-10 Immobiline strip, second dimension separation by 12.5 % SDS-PAGE.

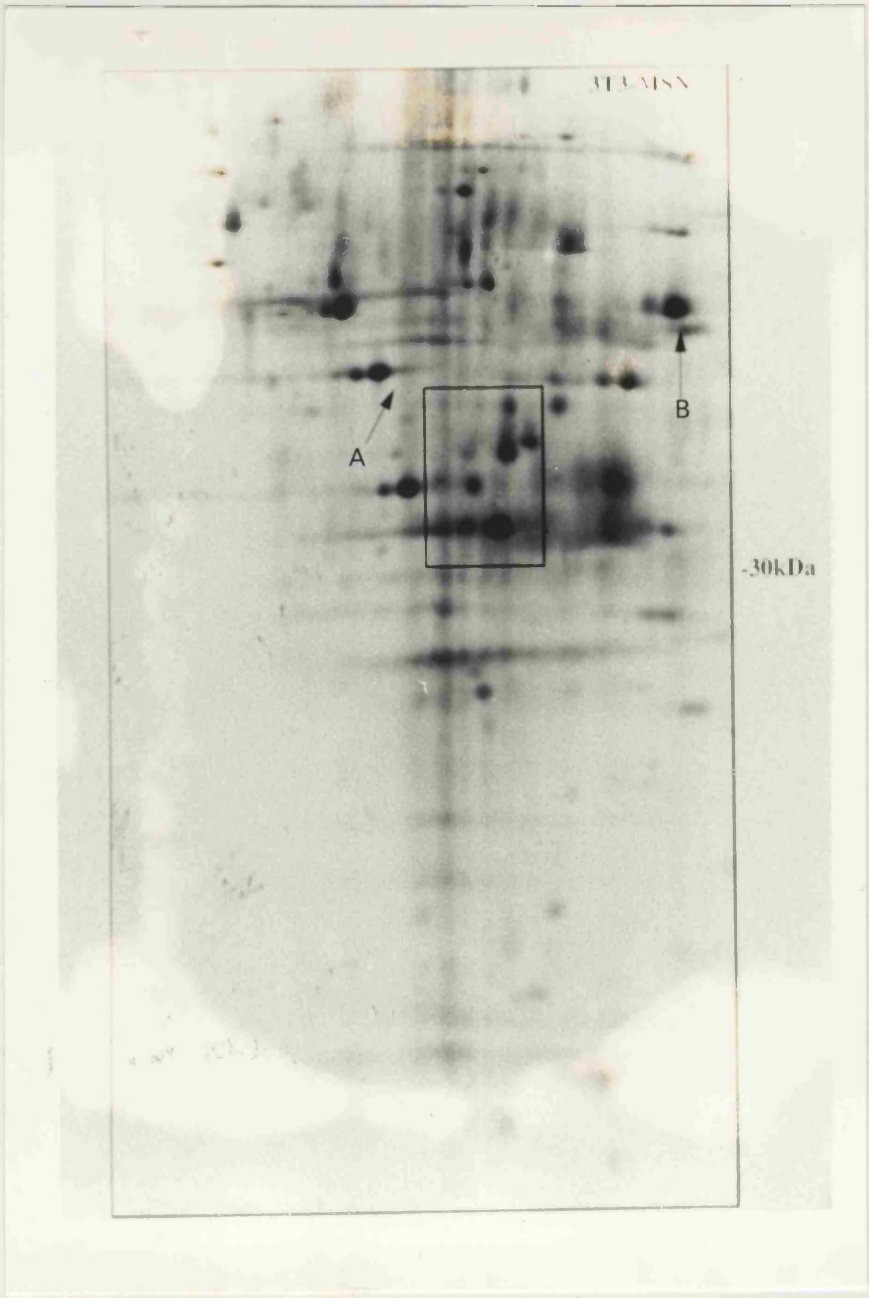


FIGURE 11.a. Two-dimensional gel analysis of L-[35S-Met]-labelled total cellular protein from 3T3-MSN cells. First dimension separation by pH 3.5-10 Immobiline strip, second dimension separation by 12.5% SDS-PAGE.

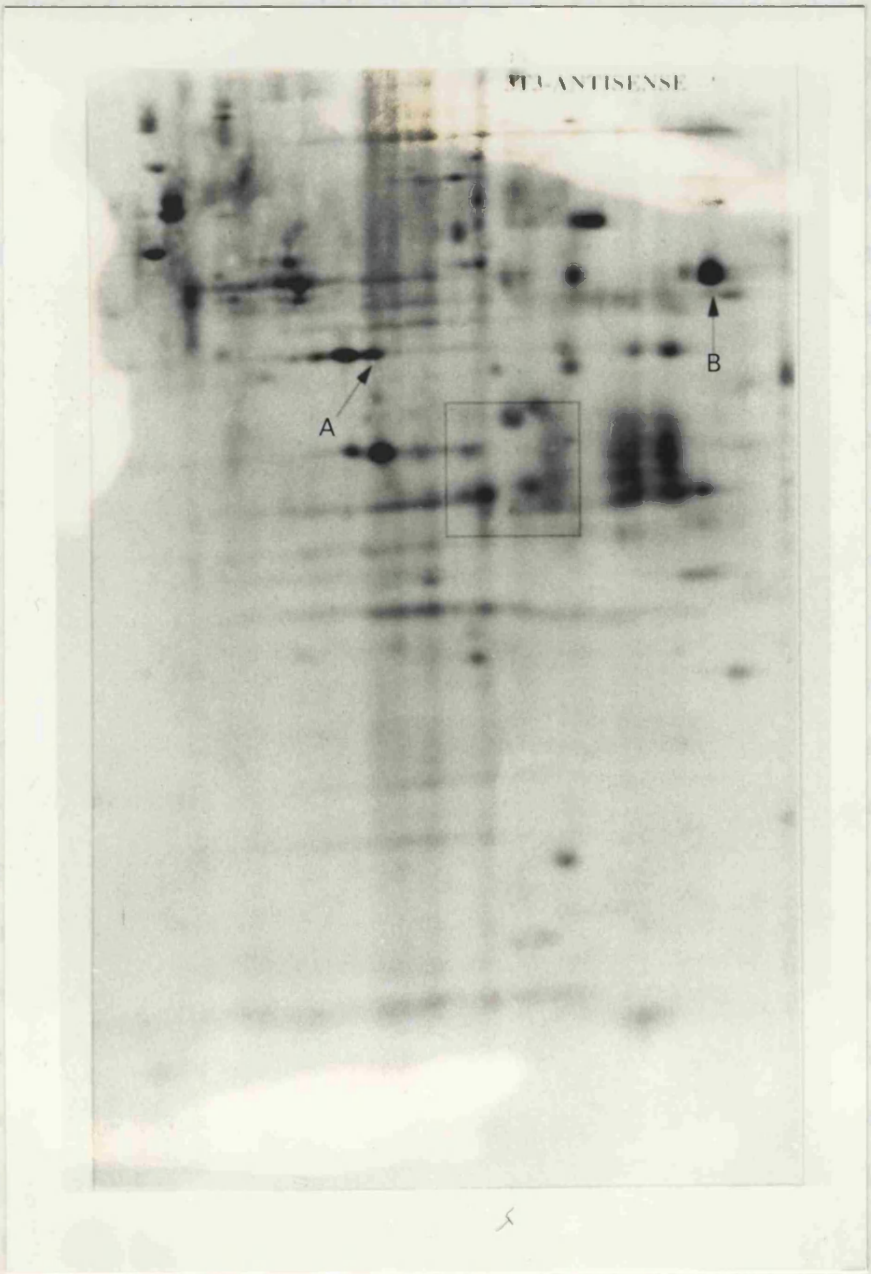


FIGURE 11.b. Two-dimensional gel analysis of L-[35S-Met]-labelled total cellular protein from 3T3-Antisense cells. First dimension separation by pH 3.5-10 Immobiline strip, second dimension separation by 12.5% SDS-PAGE.

4.8. CHAPTER DISCUSSION

Quantitative densitometry revealed a six-fold excess of SmN expression in brain relative to SmB expression compared with a two-fold excess of SmB relative to SmN in ND7 cells and roughly equal proportions of each in F9 cells (FIG.8). These differences in levels of SmN expression may provide an explanation for the difference in U1-snRNP association of SmN between cell lines and brain. Thus a model is proposed whereby at low to intermediate levels of SmN expression (ND7, F9, 3T3-7SNa cells), SmN which demonstrates higher affinity for the U2 snRNP will preferentially incorporate into such particles. However, at high levels of SmN expression (brain and 3T3-MSN cells), the U2 snRNPs become saturated with SmN and the remaining SmN therefore locates in the U1 snRNP. The precise threshold at which this occurs has not been determined but will rely ultimately on the availability of U2-snRNPs in a particular cell line or tissue. It is proposed that brain and 3T3-MSN cells express SmN at a level above this threshold. In addition, similar experiments in PC-12 cells (Mc.Allister et al., 1988) support this model. Here immunoprecipitates using anti-(U1) RNP sera contained considerably less SmN than that observed using the Y-12 anti-Sm monoclonal, whereas the levels of SmB, and A and C polypeptides are equivelant between the two samples. This may be the result of a level of SmN expression marginally above the threshold whereby all U2-snRNPs and perhaps other snRNPs are SmN-saturated, accounting for the relatively low levels of SmN-containing U1-snRNPs observed.

The incorporation of artificially expressed SmN into snRNPs in 3T3-MSN/ T7S2a cells is an important observation. These cells may be used for subsequent assays on the effect of SmN expression in isolation (e.g. in alternative splicing assays) with the reassurance that the SmN expressed in them is a seemingly functional snRNP polypeptide component. Analysis by 2-dimensional SDS-PAGE suggests that the artificial expression of SmN and the subsequent incorporation into the snRNP particles does significantly affect the nature of total protein expression. Furthermore, preferential localisation of artificially expressed SmN with the U2 rather than the U1-snRNP at low levels of expression (T7S2A) is consistent with natural expression of

SmN in F9 and ND7 cell lines and likewise demonstrates that SmN has unique properties not assigned to SmB.

The observed preferential association of SmN with the U2 snRNP is of interest as the U2-snRNP associates with the branch point region of the pre-mRNA close to the 3' splice acceptor. Thus it may be postulated that in tissues expressing SmN, the preferential association of SmN with U2 snRNPs may affect the choice of 3' splice acceptor, thus allowing the pre-mRNA to be spliced alternatively. This suggests a means of how SmN could possibly act as an alternative splicing factor, by virtue of its different affinities for particular snRNPs. At high levels of SmN expression, data presented here demonstrates that SmN associates with both U1 and U2-snRNP particles and hence the association of the U1-snRNP with the 5' splice site may also be affected as SmB is replaced by SmN. Alternatively, the association of SmN with U1 snRNPs may be neccessary due to low levels of SmB expression such that SmN must incorporate to perform constitutive splicing roles. The precise mechanism involved remains unclear, however it is plausible that a delicate balance exists between levels of SmN expression and thus its association with particular snRNPs and hence ultimately the nature of RNA splicing performed.

It is tempting here to draw parallels with data for the SF2 splicing factor that has been demonstrated at high concentrations to activate proximal 5' splice sites (Krainer et al.,1990). A similar effect was observed for the alternative splicing of SV40 pre-mRNA by human alternative splicing factor (ASF) (Ge and Manley 1990) which has since been proved to be identical to SF2 (Ge et al.,1991; Krainer et al., 1991). In addition, the human pre-mRNA splicing factor SF5/ hnRNPA1 at high concentrations activates distal 5' splice sites, and acts antagonistically against SF2/ASF and SC35 (Fu et al., 1992; Mayeda and Krainer 1992; for review-Horowitz and Krainer 1994). Therefore the ratio of hnRNPA1 to SF2/ASF determines the use of proximal or distal splice sites and the levels of these proteins control the nature of alternative splicing observed. In agreement with the model proposed for SmN, the levels of expression of hnRNPA1 and SR proteins vary in different tissues (Zahler et al., 1993; Horowitz and Krainer, 1994).

The predominant expression of SmN over SmB within brain snRNPs demonstrated here however, suggests that SmN must also be capable of performing the constitutive splicing duties of SmB. Furthermore, *in-situ* hybridisation experiments reveal that neurons are predominantly the only brain cells expressing SmN (D.Horn et al., 1992; Schmauss et al,. 1992). This observation taken together with the very low levels of SmB expression in the brain suggests that in other non-neuronal brain cells the nature of RNA splicing performed must be different and may occur at a comparatively minimal level as they express little in the way of total snRNP Sm polypeptides.

The possibility of SmN exhibiting different affinities for other snRNPs requires further investigation and furthermore, whether SmN and SmB co-exist in the same snRNP particle remains unclear. However, use of antibodies specific for SmN or SmB would resolve this question (e.g. affinity-purified autoantibodies from anti-Sm positive SLE that are reactive specifically with residues 179-190 of theSmB/B' sequence-Huntriss et al., 1993a). The appearance of SmN in precipitates using an antibody reactive with SmB (KSm4) is indicative of protein-protein interactions within the snRNP and hence SmN and SmB may be considered to co-exist in the same particle. Alternatively, the reported reactivity of KSm4 with SmD (Williams et al., 1986- not personally observed) may be responsible for the precipitation of SmN from only SmN-containing snRNPs. A slight reduction of SmN levels is evident in KSm4 immunoprecipitates from F9 cells relative to the amount of SmN in KSm5 and KSm2 precipitates whilst other snRNP proteins (SmB, A ,C, SmD) were constant between these three samples (FIG.3). This may be interpreted as an effect of a greater specificity of KSm4 for SmB than SmD thus meaning fewer of the snRNPs containing only SmN are precipitated via SmD.

A further point of interest is presented by immunoprecipitation of F9 extract with the KSm1 anti-SmD monoclonal. In such precipitates, SmN, SmB and SmD are clearly visible whereas relatively, the U1-snRNP-specific A and C polypeptides appear reduced. This may possibly reflect a binding property of KSm1 such that it does not bind to or have an appreciable affinity for SmD in the U1-snRNP configuration. The existence of antigenic differences in snRNP core proteins according to which snRNP they are associated with has been previously proposed

for SmB in the U1 snRNP (Petterson et al., 1984; Lelay-Taha et al., 1986; Ohosone et al., 1992). If KSm1 does bind an SmD epitope not present in the U1 snRNP configuration, then the appearance of SmN in these precipitates adds further weight to the proposal of the preferential localisation of SmN with non-U1 snRNPs at low levels of expression.

CHAPTER 5
ANALYSIS OF snRNP PROTEINS AND RNA SPLICING IN MICE LACKING Snrpn EXPRESSION

5.1. INTRODUCTION

In order to assign a function to SmN and determine the nature of its proposed causative role in determining the PWS phenotype, tissue was obtained from mice models of PWS for analysis of SmN expression in various organs. In addition, RNA derived from the brains of such mice has been used to explore the putative role of SmN in neuronal-specific alternative splicing.

The mice models used in these studies possess two maternally derived copies of the Snrpn gene (Cattanach et al., 1992). This is due to maternal duplication of the central region of chromosome 7 produced by crossing males carrying the balanced form of Is(In7;X) 1Ct X-autosome translocation with females which carry the unbalanced form and have 2 normal copies of chromosome 7 as well as the extra region of central 7 within X (see FIG.1.6). Northern blot analysis reveals an absence of Snrpn expression in the brains of mice with two maternal copies of central chromosome 7 (Cattanach, et al., 1992). This is consistent with other reports (Leff, et al., 1992) in that Snrpn is subject to imprinting and only the paternal copy is expressed. The early postnatal lethality of these mice (within 2-8 days) indicate that one or more maternally imprinted genes within this translocation region play an important role and thus determine the observed phenotype. A paternal duplication for central chromosome 7 generated by reciprocal translocations leads to an increase in Snrpn mRNA expression on Northern blot analysis (Cattanach et al., 1992). This suggests monoallelic paternal expression of Snrpn and that this overexpression is suggested to be regulated as mice appear phenotypically unaffected.

Therefore, to confirm whether the imprinting effect observed in the brain is effective at the protein level in brain and other tissues, Western blots of various organs using the KSm 5 monoclonal and anti-Sm positive SLE sera were performed. In addition, mice with paternal duplications for the central region of chromosome 7 were analysed to determine whether the observed increase in Snrpn gene expression is reflected at the protein level.

The association of SmN with the snRNPs as shown by immunoprecipitation (McAllister et al.,1988; Huntriss et al., 1993b) suggests that SmN may influence the nature of RNA splicing in tissues expressing it. As SmN, like SmB/B' does not possess an RNA binding sequence, it is

unlikely that it would act to bind to *cis*- acting sequences to promote or inhibit the selection of a particular splice site. Rather, it is more plausible that if SmN is to act as an alternative splicing factor then the association of SmN with a particular snRNP would affect the conformation of the snRNP in such a way as to change splice site selection of the transcript. The difference in primary structure between SmN and SmB/B' (17 amino acids in total -Van Dam et al. 1989) and the discovery that such regions act as SmB/B' specific autoantibody epitopes but not SmN specific autoepitopes (Huntriss et al., 1993a), suggests that the polymorphic regions in SmN are sufficient to generate conformational differences in SmN that therefore might affect its function in the snRNPs, thus affecting splicing. In addition, it is possible that the two main polymorphic regions in SmN are involved in interactions with other snRNP proteins and hence are unavailable for presentation as potential autoantigens.

A number of alternative splicing events which had been proposed to be candidates for regulation by SmN were investigated. The genes were chosen on the basis that they exhibit a different pattern of splicing in tissues expressing SmN (brain and / or heart) compared to tissues not expressing SmN, suggesting SmN could play a role in regulating the nature of the splicing of such transcripts. In addition the splicing of these transcripts was observed to alter as the expression of SmN increased during brain development. Whilst correlations can be drawn between SmN expression patterns and alternative splicing patterns, more detailed analysis reveals discrepancies in these correlations. Previously, PCR assays have been used to investigate the splicing patterns of the endogenous transcripts of such genes in cells and tissues expressing different amounts of SmN (Horn and Latchman 1993a/b). This can be analysed by designing primers that flank the alternatively spliced regions and observing the molecular weight of the RT-PCR products generated on non-denaturing gels. On the basis of this previous data, it appears that SmN may not be responsible for the regulation of certain specific alternative splicing events such as the inclusion in brain NCAM transcripts of the VASE exon and in brain c-src transcripts of N I and NII exons (Horn and Latchman 1993a). However conclusions were partly reliant on data from the artificial expression of SmN into 3T3 mouse fibroblasts which does not account for the

possibility that SmN may require co-factors for performing alternative splicing. Attempts to analyse the effect of reducing SmN expression on splicing in neuronal cells using anti-sense plasmids were unsuccessful. Therefore to determine whether SmN performs a role in alternative splicing, using tissue from mice that lack SmN expression (Cattanach et al., 1992) provides an excellent opportunity to study this putative role. Therefore, identical RT-PCR experiments with candidate genes have been performed using brain RNA isolated from mice lacking SmN expression. Previous quantitative analysis of these PCR reactions has demonstrated that the amount of PCR product generated reflects the levels of mRNA present in the reaction (Horn and Latchman., 1993 a/b). Furthermore, all PCR assays were performed using the same cDNA pool and any differences in amplification efficiency between samples will also be evident in the level of constitutive PCR products obtained in certain reactions.

The subsequent experiments were executed using brain tissue from 1 and 4 day old mice with reference to age and sex matched controls. The early postnatal lethality of mice lacking SmN expression in the brain (Cattanach et al., 1992) due to maternal duplication of the region containing the maternally imprinted Snrpn gene suggests SmN may play a critical splicing role in tissues expressing it. The nature of this role, whether it is a regulator of alternative splicing or whether it performs a constitutive splicing role requires further investigation. The normal replacement of SmB by SmN in the brain would seem to argue against a purely constitutive role. Assays performed on mouse brain tissue will provide a better insight into the nature of splicing performed in the presence or absence of SmN within the true neuronal splicing environment. It remains possible however that the fate of the mice lacking SmN expression may be due to other genes within the maternally duplicated region. In addition, to provide a clearer picture of the role of SmN in alternative splicing, two further candidate genes not previously tested, the GSα guanine nucleotide binding protein and the Oct-2 transcription factor were analysed. These novel assays included data from the 3T3 cell lines artificially expressing different levels of SmN with reference to parental 3T3 cells.

5.2. WESTERN BLOTTING ANALYSIS OF TISSUES FROM MICE WITH TWO MATERNALLY DERIVED COPIES OF THE Snrpn GENE.

RESULTS

Analysis of Western blots performed on 9 day old mice using KSm2 (anti-SmD) and KSm5 monoclonals (anti-SmN/B-FIG.1) clearly demonstrates that the imprinting effect on the maternally derived Snrpn genes is effective throughout all the organs tested. Thus the 29 kDa band corresponding to SmN is not observed in brain and heart samples from these mice in clear contrast to controls. In addition, SmN was not expressed in the tissues normally observed to express negligible levels of SmN (liver, spleen, kidney, lung). The levels of SmB expression generally reflect that of SmD in all tissues except the brain and are consistent with controls. The brain sample from mice lacking SmN expression demonstrates a marked increase of SmB expression occurs relative to SmD, and this level of SmB expression far exceeds that normally observed in the brain. This interesting finding was examined in more detail by blotting with an anti-Sm / RNP SLE sera (FIG.2). This reveals that this increase in SmB expression is unique to the brain of mice lacking SmN expression and is not reflected by an increase in other snRNP proteins (C and SmD), the levels of which were identical to controls. Quantitative analysis of the levels of expression of SmB compared to SmD from this Western blot reveals a ratio of 5.333 (SmB/SmD) in mice with a maternal duplication that encompasses the Snrpn gene compared to a value of 5.05 for control samples.

5.3. WESTERN BLOT ANALYSIS OF MICE WITH A PATERNAL DUPLICATION OF THE CENTRAL REGION OF CHROMOSOME 7

The apparently normal phenotype of mice demonstrating an increase in Snrpn mRNA by Northern blot prompted an investigation at the protein level (FIG.3). Results suggest that this increase in gene expression is subject to regulation as no evidence for increased SmN expression relative to SmB was observed from mice with paternal duplications for central 7 as levels reflected

those in controls in all tissues tested. Quantitative assessment of the expression of SmN and SmB in mice with a paternal duplication of the central region of chromosome 7 reveals no increase in SmN expression relative to SmB. Thus the average value of SmN:SmB ratios obtained from scanning four different Western blots of control brain samples was 2.2298 compared to 2.1899 for brain samples from mice with the paternal duplication of the central region of central chromosome 7 that encompasses Snrpn.

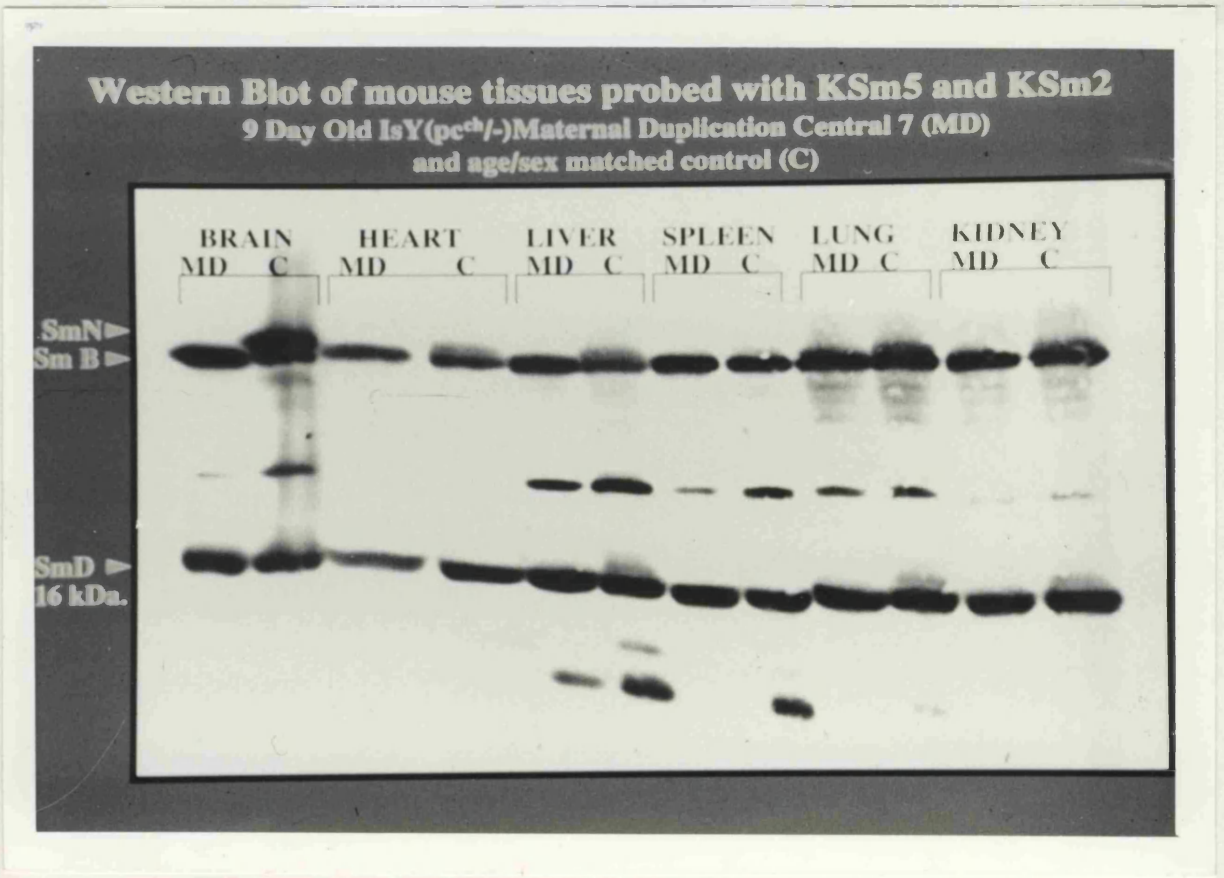


FIGURE 1 Western blot analysis of tissue from mice lacking Snrpn expression with reference to age and sex matched controls.

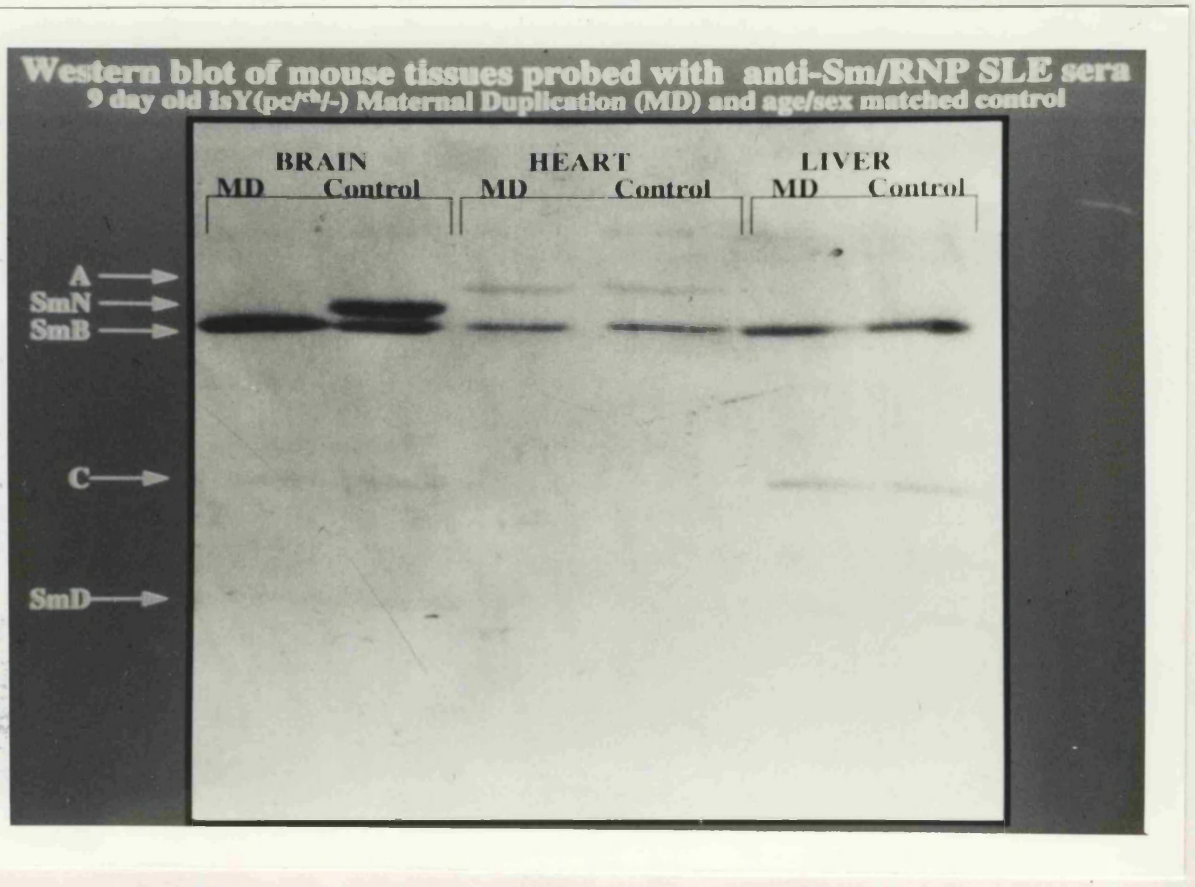


FIGURE 2 Western blot analysis of tissue from mice lacking Snrpn expression with reference to age and sex matched controls.

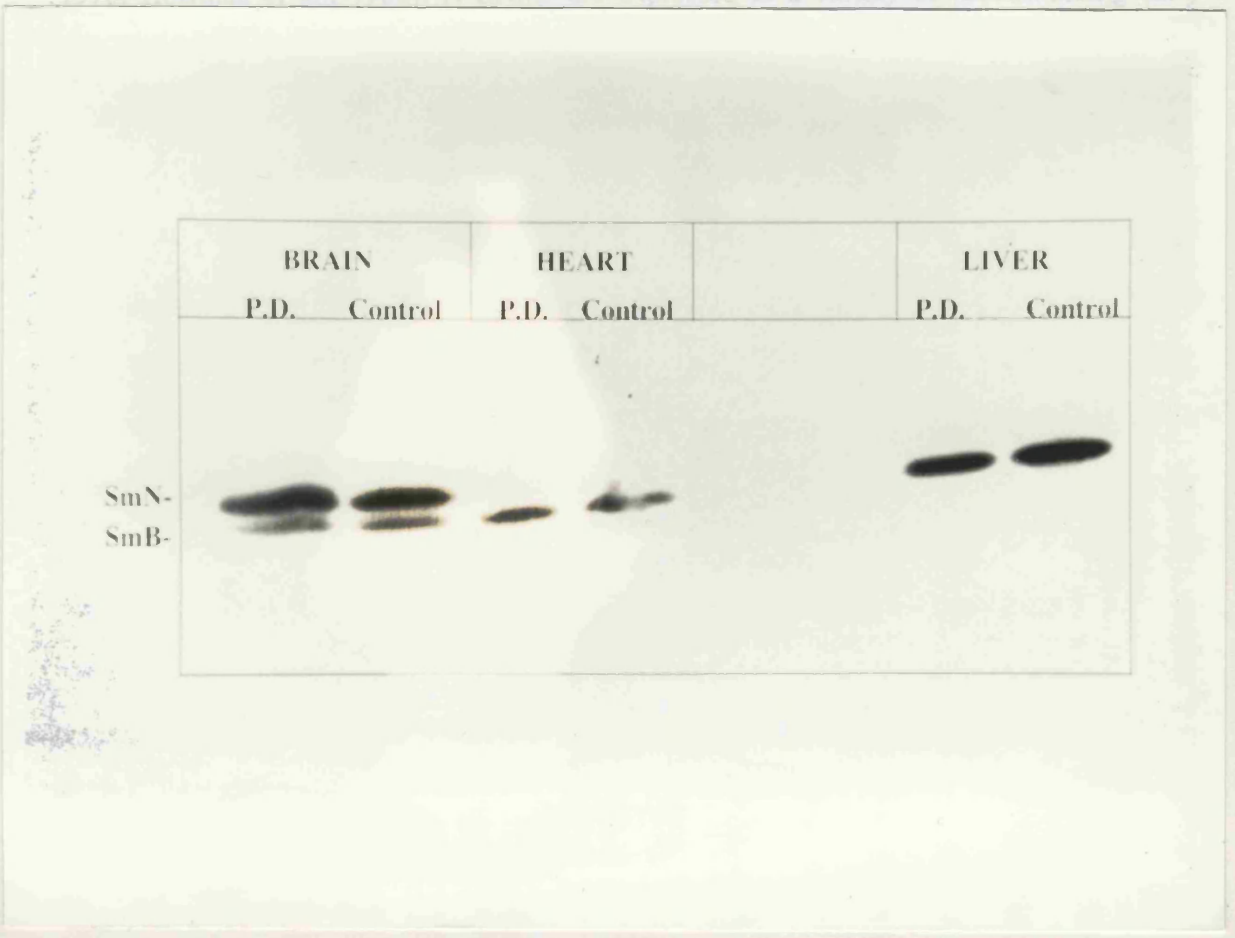


FIGURE 3. KSm5 probed Western blot of tissue from mice with paternal duplication (P.D.) of the central region of chromosome 7 with reference to age and sex matched controls.

5.4. ALTERNATIVE SPLICING OF NCAM

The Neuronal cell adhesion molecules (N-CAMs) are cell surface sialoglycoproteins that are responsible for the adhesion of N-CAM expressing cells to each other (Rutishauser et al., 1976; Hoffman et al., 1982). N-CAMs are expressed in a variety of tissues during early developmental stages (Thiery et al., 1982) and are later expressed in the nervous system (Brackenbury et al.,1977), skeletal muscle (Rutishauser et al., 1983), smooth muscle (Akeson et al., 1988) and glial cells (Keilhauer et al., 1985). N-CAMs play critical roles in development of neural systems such as neural crest migration (Thiery et al., 1982) and otic development (Richardson et al., 1987). The N-CAM gene is one of the first genes expressed when a neural phenotype is induced (Kinter and Melton, 1987). A number of N-CAM isoforms exist which differ structurally, this diversity being a result of alternative splicing of a primary transcript derived from the single N-CAM gene (D'Eustachio et al., 1985; Nguyen et al., 1986). The N-CAM gene consists of 20 exons in mammals and birds (Barbas et al., 1988; Owens et al., 1987), the alternative transcripts being generated by differences in 3' exon usage (Barthels et al., 1987) to yield 3 major isoforms. The 120 kDa isoform is membrane linked through phophatidylinositol, whilst those with masses of 140 and 180 kDa are transmembrane isoforms with large and small cytoplasmic domains respectively (Barbas et al., 1988; Owens et al., 1987). The identification of at least 27 alternative transcripts in rat heart reveals the complex nature of N-CAM splicing (Reyes et al., 1991).

Further regions of alternative splicing exist within the N-CAM gene. Thus a 108bp sequence called the MSD1 (muscle specific domain) between exons 12 and 13 comprises of 4 different exons MSD 1a-c and a triplet AAG (Santoni et al., 1989; Thompson et al., 1989), and the murine homologue of exon MSD1a and the AAG triplet have been shown to be regulated in a tissue specific and developmental manner (Hamshere et al., 1991). A very similar exon arrangement is present in embryonic chicken heart (Prediger et al.,1988).

In human skeletal muscle NCAM, a further alternatively spliced exon called SEC lies between exons 12 and 13, use of which yields a secreted polypeptide isoform due to the lack of inclusion of a transmembrane or membrane linked domain (Gower et al., 1988).

A 30 bp exon termed VASE (Immunoglobulin Variable domain-like Alternatively Spliced Exon) between exons 7 and 8 is present in N-CAM cDNAs derived from rodent brain (Reyes et al., 1988; Small et al., 1987). The inclusion of the VASE exon is developmentally regulated, increasing from an inclusion in 3 % of N-CAM transcripts in embryonic brain to around 45 % of transcripts in adult brain (Small et al., 1988) and from around 10 % in day 15 embryonic heart to 50 % in adult heart (Reyes et al .,1991). Developmental inclusion of the VASE exon is regulated independently between the brain and heart (Small and Akeson, 1990) and the inclusion in nervous system transcripts differs between specific regions. Thus whilst regions such as the olfactory bulb and spinal chord express little or no VASE containing N-CAM transcripts, the general level of inclusion in the CNS was higher than that in PNS regions such as the dorsal root ganglia. The addition of the VASE exon yields an isoform with an altered configuration, containing a 10 amino acid insertion in the fourth immunoglobulin-like loop (Small et al., 1988). This terminology being due to homology with similar units in immunoglobulins. In total, five of such folds make up the extracellular amino-terminal portion of N-CAMs and are responsible for the binding of other cells and heparin. This suggests that isoforms with the 10 amino acids encoded by the VASE exon may have a unique properties, possibly in that of binding affinities. The inclusion of the VASE exon has been demonstrated to reduce the neurite outgrowth-promoting activity of N-CAM and is suggested to be responsible for the general stability and therefore poor regenerative capacity of the CNS (Doherty et el., 1992).

The tissue specific and developmental regulation of VASE inclusion in N-CAM transcripts makes it a good candidate for the regulation of its splicing to be performed by SmN. However, the artificial expression of SmN in 3T3 fibroblasts does not alter the pattern of N-CAM splicing in terms of the inclusion of the VASE exon (Horn and Latchman, 1993a). Although this suggests that SmN is not sufficient to alter splicing in the case of the VASE exon, it is possible that a co-

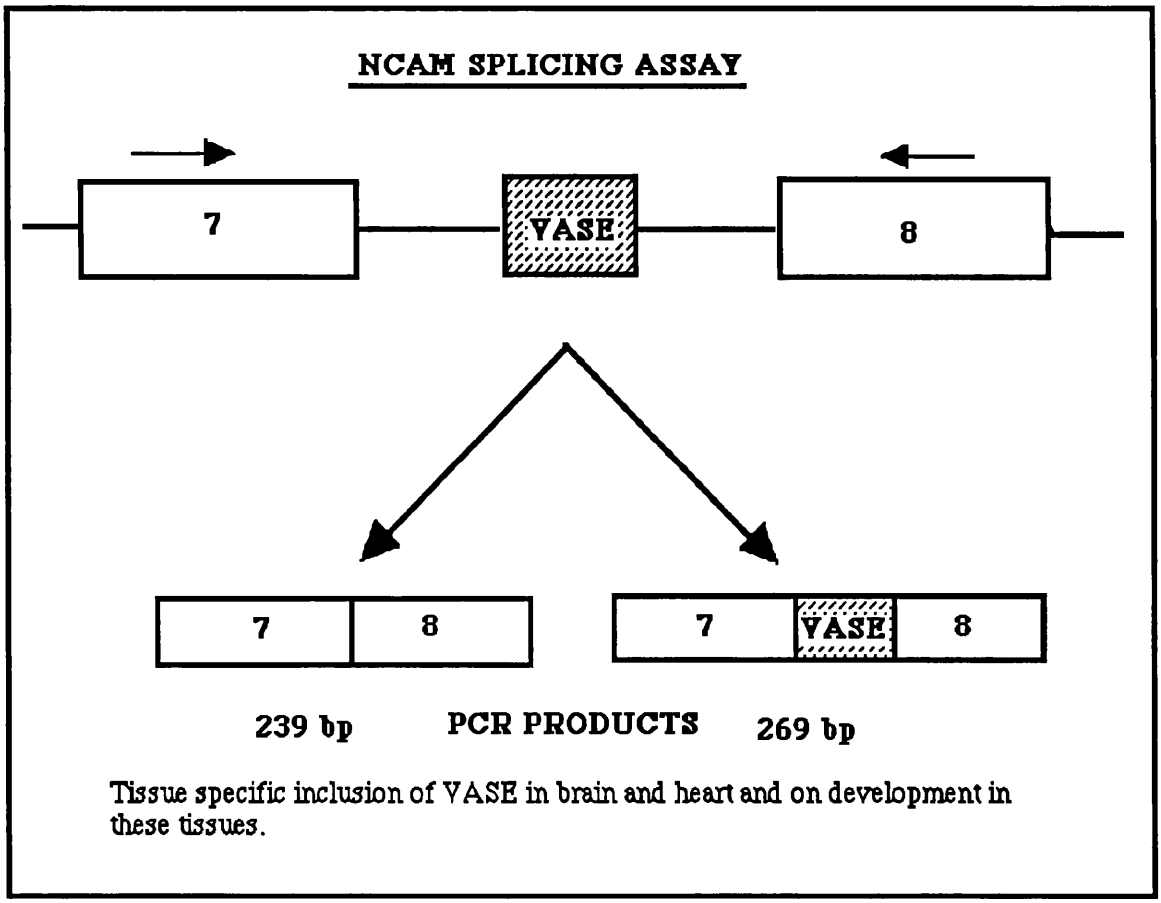
factor not present in 3T3 cells is required. A further discrepancy is that parental 3T3 cells that lack SmN expression are capable of including the VASE exon, suggesting that SmN is not necessary for such splicing to occur. In order to further investigate the putative role of SmN in VASE inclusion, PCR analysis of such N-CAM transcripts from the brains of mice lacking SmN expression was performed.

PCR ASSAY

Primers were derived from exons 7 and 8 that flank the alternatively spliced VASE exon to generate a PCR product of 269 bp for transcripts containing the VASE exon and 239 bp for transcripts without this exon.

RESULTS

A normal pattern of inclusion of the VASE exon was observed in both 1 and 4 day old mice lacking SmN expression at the same level as in the controls (FIG.4). Thus the amplification of the central 269 bp PCR band corresponding to N-CAM transcripts with VASE inclusion is consistent with levels in the controls, as are the levels of the 239bp PCR band corresponding to N-CAM trancripts without the VASE exon. The larger band is reported to be a heteroduplex of the 269 and 239bp bands caused by the nondenaturing gel. This data is therefore consistent with previous observations in 3T3 fibroblasts (Horn and Latchman, 1993) in that SmN does not appear to be required for inclusion of the VASE exon. Hence artificially increasing levels of SmN in 3T3 fibroblasts is unlikely to cause increased VASE inclusion as previously observed. Additional discrepancies are evident from *in-situ* hybridisation studies, whereby regions of the PNS such as the dorsal root ganglia are not capable of expressing N-CAM transcripts containing VASE (Small and Akeson, 1990) despite the reported presence of high levels of SmN mRNA in this region (Horn and Latchman 1993). Although this may be the result of post-translational control mechanism whereby SmN mRNA is not translated into functional protein in such regions, this study provides strong evidence for the non-involvment of SmN in regulating N-CAM VASE splicing.



Tissue specific inclusion of VASE in brain and heart and on development in these tissues.

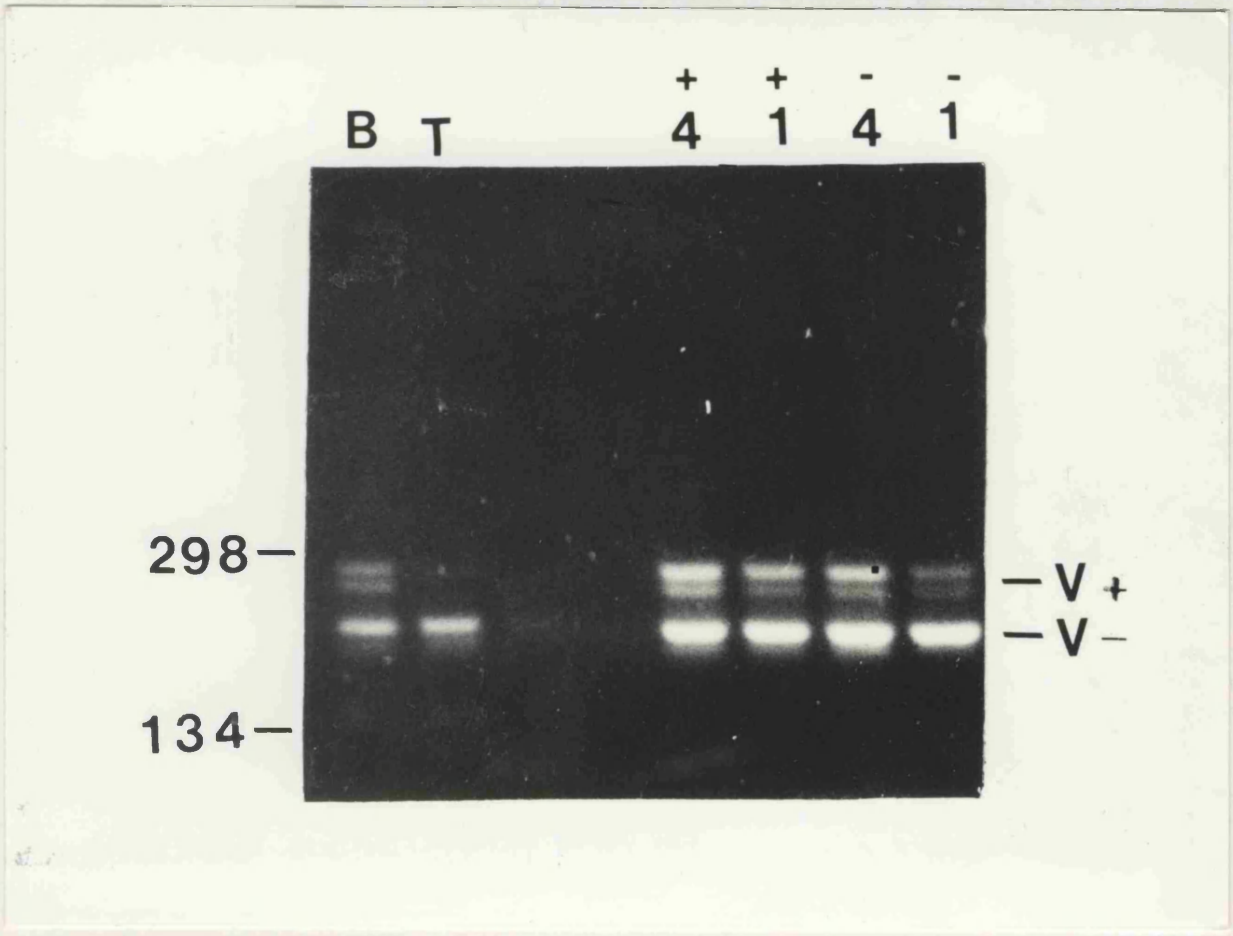


FIGURE 4. PCR of brain derived NCAM cDNAs from 1 day (1-) and 4 day (4-) old mice lacking Snrpn expression or from 1 and 4 day old age and sex matched controls (1+,4+). Results from adult mouse brain (B) and 3T3 fibroblasts (T) included for comparison. V+/V- indicates presence/ absence of VASE exon in PCR products of NCAM mRNAs.

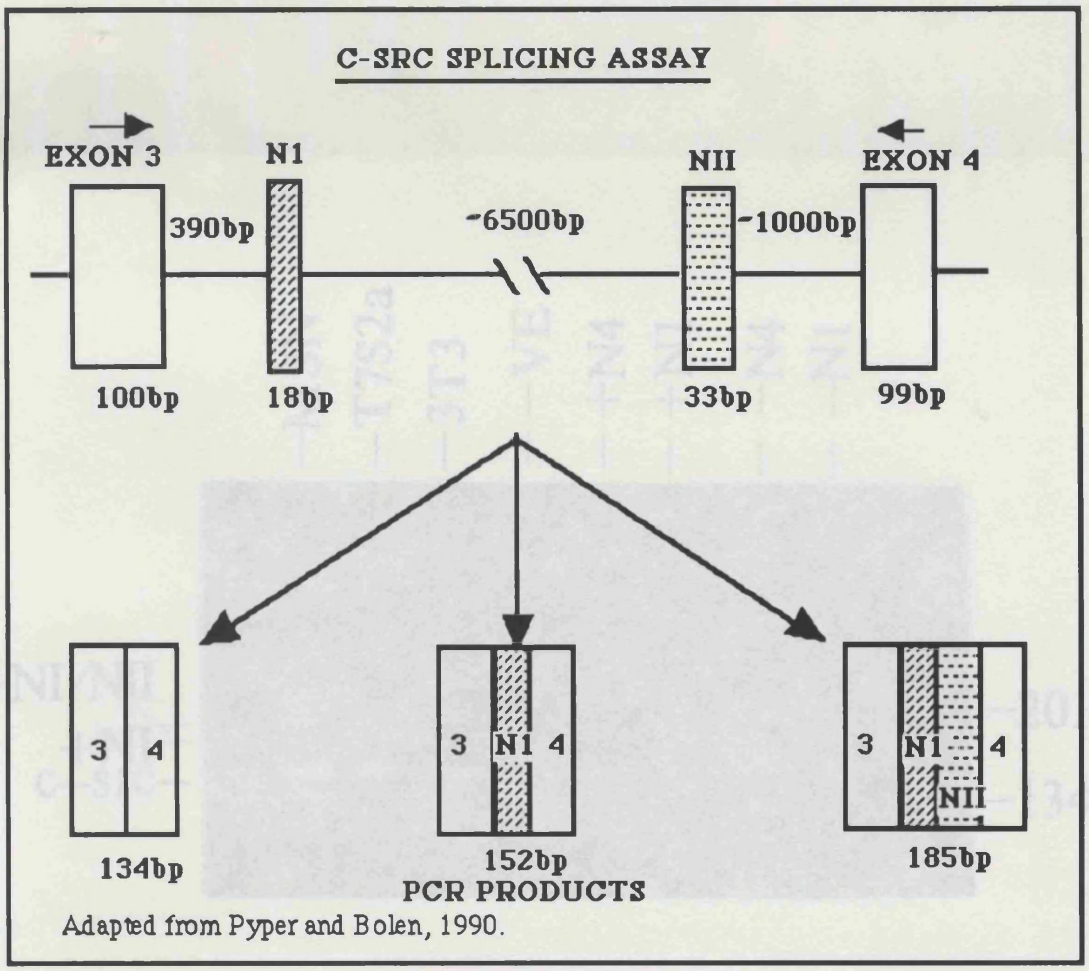
5.5. ALTERNATIVE SPLICING OF PP60 c-src PHOSPHOPROTEIN.

The cellular src proto-oncogene (c-src), encodes a 60 kDa phosphoprotein termed pp60 c-src which possesses tyrosine kinase activity (Hunter 1987). The term oncogene is applied to c-src as mutational analysis generates an altered protein product with transforming potential (Kato et al 1986). The normal function of pp60 c-src is proposed to be in neuronal differentiation and maintenance (Mannes et al., 1986) and is consistent with the isolation of a structurally distinct isoform of pp60 c-src expressed at high levels in neurones (Brugge et al., 1985 and 1987). This novel neuronal isoform (pp60 c-src+), is encoded by a unique mRNA (Levy et al., 1987), generated by alternative splicing whereby an 18-nucleotide exon termed NI is included between exons 3 and 4 (Martinez et al., 1987). Chick embryo fibroblasts overexpress pp60 c-src+ that demonstrates increased tyrosine kinase activity and altered transforming activity relative to nonneuronal pp60 c-src (Levy et al., 1987).

In addition to the N1 exon, a second neuron-specific exon (NII) that encodes 11 amino acids was reported between exons 3 and 4 in human brain transcripts (Pyper and Bolen, 1990). This exon is used in conjunction with the NI exon to generate transcripts capable of encoding a c-src protein with a novel phosphorylation site. The relative expression of NI-plus NII transcripts is equivelant in adult and foetal brain tissue. PCR analysis of transcripts during brain development demonstrate a progressive increase in transcripts containing the NI exon from the day 16 embryonic stage (Horn and Latchman,1993a). This, together with the neuronal pattern of N1 inclusion suggest that its inclusion may be regulated by SmN.

PCR ASSAY

To directly test the proposed involvment of SmN in NI exon inclusion, endogenous transcripts from mouse brain lacking SmN expression were analysed by PCR using primers flanking the alternatively spliced regions derived from exon 3 and exon 4. A PCR product of 158bp represents NI containing transcripts, whilst a 191 bp product is derived from transcripts containing both NI and NII exons.



RESULTS

The inclusion of the NI exon in c-src transcripts from mouse brain lacking SmN expression was equivelant with that observed in age and sex matched controls (FIG.5). The inclusion of the NII exon in conjunction with NI and the presence of the non-neuronal transcript were also equivelant with levels in controls. The 3T3 fibroblasts and stable SmN transfectants reflect the expected non-neuronal splicing pattern of c-src transcripts (Horn and Latchman, 1993a). Therefore, in addition to the observation that SmN in isolation is not sufficient to generate a neuronal splicing pattern of c-src transcripts, data presented here directly proves that SmN is not necessary for NI or NI-plus-NII inclusion to occur.

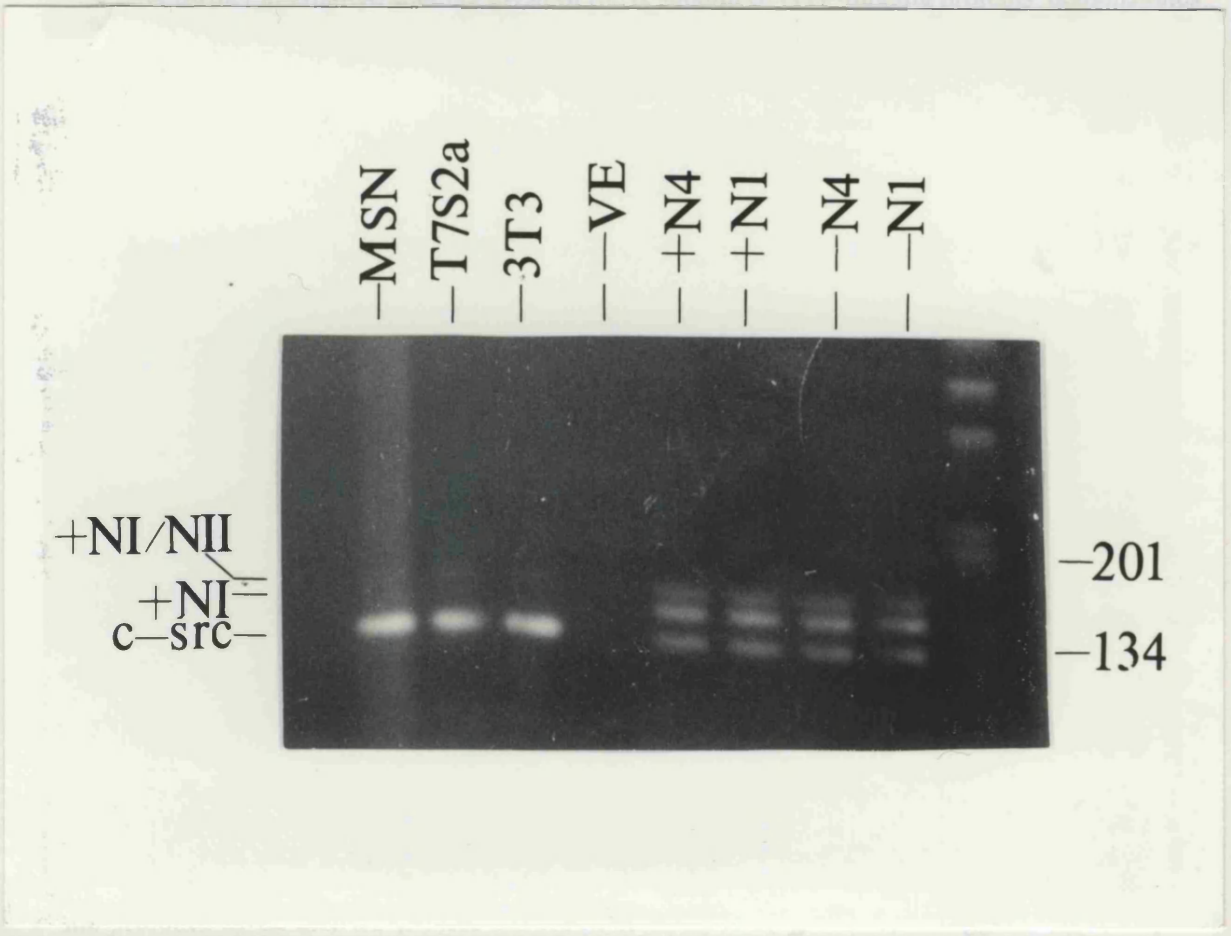


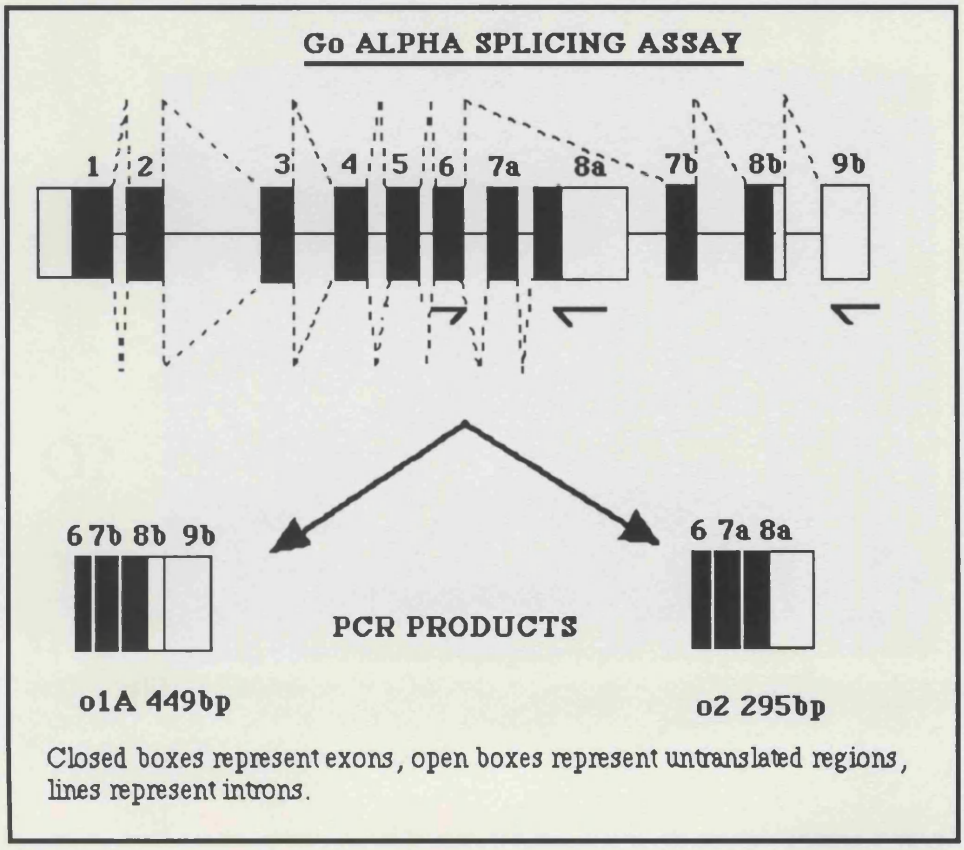
FIGURE 5. PCR of brain derived c-src cDNAs from 1 day (-N1) and 4 day old (-N4) mice lacking Snrpn expression or from 1 and 4 day old age and sex matched controls (+N1/+N4). Also included are results from 3T3 fibroblasts and transfected 3T3 cells expressing low (T7S2a) and high (MSN) levels of SmN. Inclusion of 18 base N1 exon and 33 base N2 exons in transcripts indicated.

5.6. ALTERNATIVE SPLICING OF THE α SUBUNIT OF THE Go-GTP BINDING PROTEIN

A further alternative splicing event of the α subunit of GTP-binding proteins demonstrates a degree of correlation with SmN expression. The Go protein (" o" for other), is a heterotrimeric membrane associated G protein expressed at high levels in the brain (Sternweis and Robishaw, 1984; Neer et al., 1984) that is believed to be responsible for inhibition of neuronal Ca2+ channels (Hescheler et al., 1987), stimulation of neuronal K+ channels (VanDongen et al., 1988) and phospholipase-C (Kikuchi et al.,1986; Moriarty et al., 1990) and mediating α2-adrenergic and muscarinic receptors (Cerione et al., 1986; Florio and Sternweis, 1989). The Goα subunit is reported as being sensitive to pertussis toxin inhibition via ADP-ribosylation of a C-terminal cysteine residue (Casey and Gilman, 1988; Lochrie and Simon, 1988). At least 10 exons are described in the human Goα subunit gene which covers >90 kbp (Kaziro et al 1990). Alternative splicing events generate three distinct forms of mRNA that encode for two isoforms of the α subunit (Bertrand et al., 1990). The 354 amino acid αo1 subunit is encoded by two mRNAs, αo1a and αo1b which differ only in their non-coding regions. Another larger mRNA encodes the αo2 subunit which also contains 354 amino acids but differs from αo1 the subunit in 26 carboxyl terminal amino acids (Hsu et al., 1990). The presence of o1A mRNA in both brain and heart and the presence of o2 mRNA in these tissues in addition to lung and testis suggests SmN may be involved in regulating splicing of the former. Hence this putative role for SmN was examined further using brain tissue from mice lacking SmN expression.

PCR ASSAY.

The presence of distinct 3'exons in the o1A and o2 transcripts required the simultaneous use of 3 primers. Thus a common upstream primer derived from exon 6 was used in conjunction with an exon 9b derived primer for analysis of o1A transcripts and an exon 8a derived primer for o2 analysis. This generates a PCR product of 459 bp for o1A transcripts and a 281 bp band representative of o2 transcripts.



Closed boxes represent exons, open boxes represent untranslated regions, lines represent introns.

RESULTS

Endogenous o1A and o2 transcripts from mouse brain tissue lacking SmN expression are present at equivelant levels with those in age and sex matched controls (FIG.6). Hence the normal pattern of Goα transcript splicing occurs in the brain in the absence of SmN suggesting it is not required to regulate such alternative splicing. This is consistent with previous investigations using 3T3 fibroblasts and 3T3 cells transfected with the SmN expression plasmid (Horn and Latchman,

1993b). Here induced SmN expression was not sufficient to generate o1A transcripts in addition to the o2 transcripts normally present in parental 3T3 cells. In addition, the levels of o1A transcripts did not change during brain development as would be expected if SmN mediated Goα transcript processing.

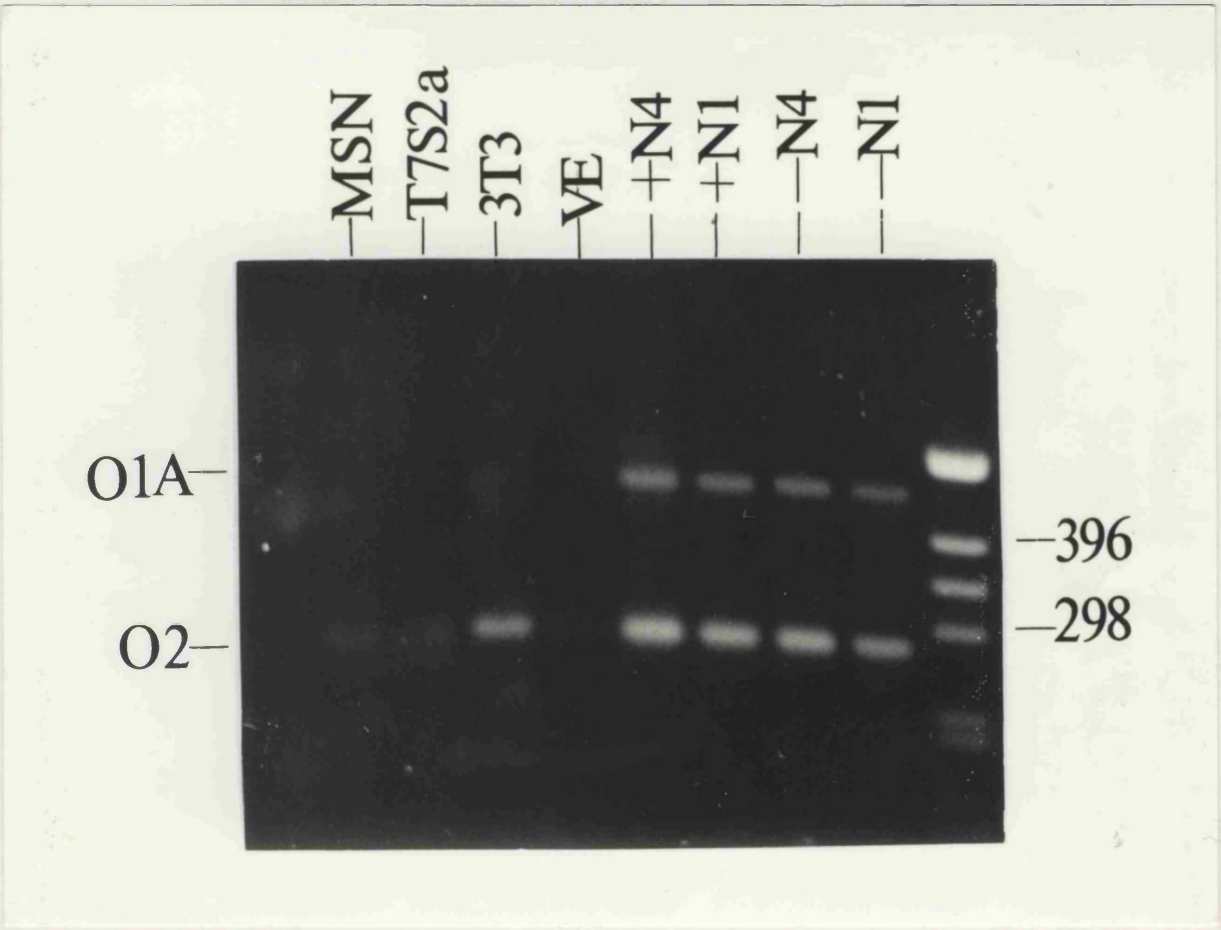


FIGURE 6. PCR of brain-derived Goα cDNAs from 1 (-N1) and 4 day (-N4) old mice lacking Snrpn expression or from age and sex matched controls (+N1, +N4). Also included are results from 3T3 fibroblasts and transfected 3T3 cells expressing low (T7S2a) or high (MSN) levels of SmN. The positions of PCR products of O1A and O2 transcripts are indicated.

5.7. ALTERNATIVE SPLICING OF THE CALCITONIN / CGRP GENE

The calcitonin / CGRP gene was one of the first examples of tissue specific alternative splicing to be described (Rosenfeld et al., 1984; Amara et al., 1984). The human gene family in humans is comprised of three genes- CALC-I, CALC-II, and CALC III (Lips et al 1989). Only the CALC-I gene is capable of producing the serum calcium-regulating hormone calcitonin, which is encoded by exon 4. The CALC-I and CALC-II genes both have 6 exons however, CALC-II transcripts have not been observed to include exon 4 in the tissues expressing the gene (Lips et al., 1989; Hoppener et al., 1987), and the CALC-III gene does not contain exon 4. The inclusion or exclusion of exon 4 in CALC-I transcripts occurs in a tissue specific manner. Thus in thyroid C-cells, 98% of CALC-1 transcripts include exon 4 which is spliced to the first three exons to produce mRNA encoding calcitonin (Sabate et al., 1985). In neuronal cells however, 99% of CALC-I transcripts lack exon 4 and the first three exons are spliced together with exons 5 and 6 to yield an mRNA encoding the calcitonin gene related peptide (CGRP) (Amara et al., 1982). CGRP functions as a neuropeptide (Marshall et al ., 1986), a vasodilator (Brain et al., 1985) and has an anti-inflammatory activity (Raud et al., 1991).

The precise mechanism regulating CALC-I splicing however remains unresolved. Deletion of 21 nucleotides from the third intron, just upstream of exon 4 increases calcitonin production in cells normally making CGRP, suggesting that this site interacts with a negative regulator of calcitonin-specific splicing that is expressed in CGRP producing cells (Emeson et al., 1989). This altered splicing pattern may however be a result of removing the branch site sequences from the calcitonin-specific splice acceptor. Another possible site of interaction lies within the 5' region of exon 4 and is important for efficient calcitonin mRNA production in HeLa cells. Mutation of this site results in increased CGRP-specific splicing. This is consistent with an observed increase in CGRP mRNA production in HeLa cells when supplemented with nuclear extracts from F9 cells which predominately make CGRP mRNA (Emeson et al., 1989). In addition, a 66 kDa RNA-binding protein has been isolated that interacts with this sequence and is proposed to enhance recognition of the calcitonin-specific 3' splice site (Cote et al., 1992). In-vitro analysis in HeLa

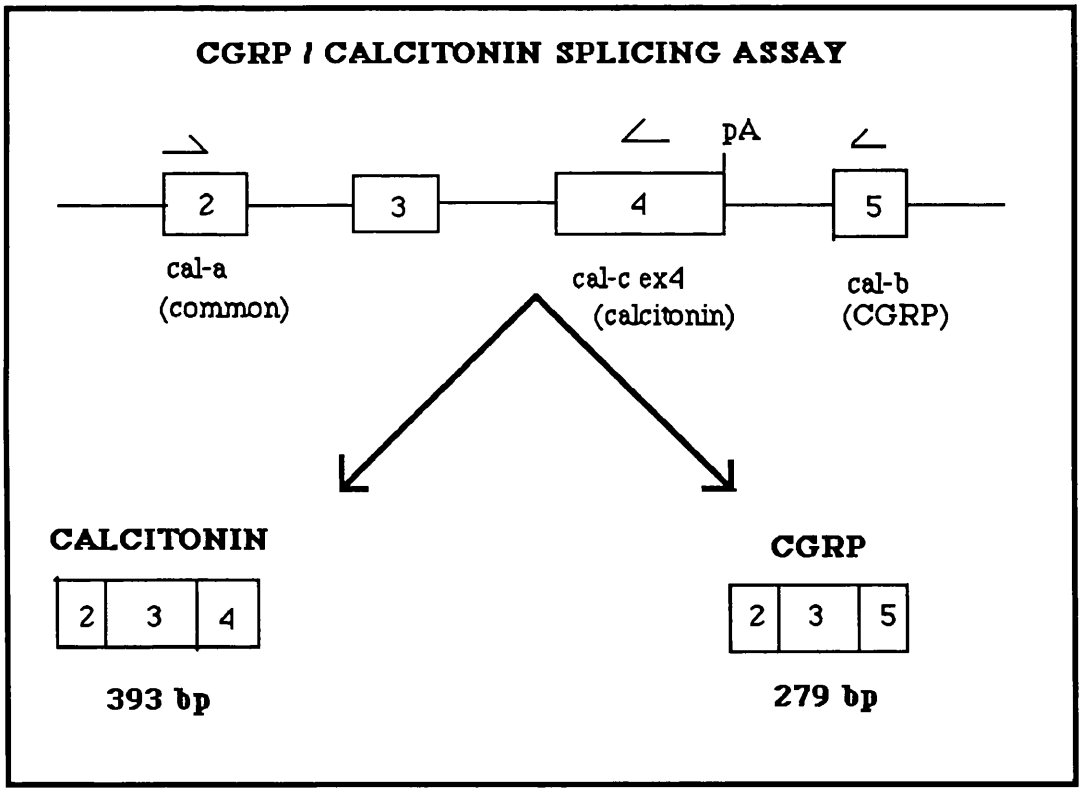
cells demonstrate inhibition of RNA-binding of this protein when supplementing with F9 nuclear extracts. It is proposed that a factor/ factors expressed in F9 cells inhibits binding of the 66 kDa protein to the critical exon 4 sequences, thus favouring exon 4 exclusion. Regulation may involve this site in addition to the 21 nucleotide regulatory region upstream of exon 4 (Emeson et al., 1989). Inefficient usage of the calcitonin specific splice acceptor for exon 4 is observed in F9 cells (Roesser et al., 1993). The addition of nuclear extracts from rat brain or F9 cells to HeLa nuclear extracts inhibits calcitonin-specific splicing in *in-vitro* splicing reactions in HeLa cells. Moreover, two polypeptides (43 and 41 kDa), isolated from rat brain specifically bind the calcitonin-specific splice acceptor and demonstrate an inhibition of calcitonin-specific splicing in HeLa cells. These proteins may be responsible for the observed inhibition of binding by the 66 kDa protein to exon 4 sequences (Emeson et al., 1989). Two distinct sequence elements termed A and B within the 5' region of exon 4 are both required for calcitonin-specific splicing in nonneural cells (van Oers et al., 1994). Mutation of the weak calcitonin-specific uridine branch acceptor to the more commonly preferred adenosine residue can overcome the effect of deletions within these regulatory exon sequences. This mutation also overcomes the regulatory effect of sequences A and B, leading to a switch to calcitonin-specific processing in F9 cells (Adema and Baas., 1991). Thus, a model for CALC-1 alternative splicing drawn from such data implies that inclusion of exon 4 requires the binding of factors to such exon 4 sequences due to the weak nature of the exon 4 splice acceptor site. A low level of expression of such factors or the presence of inhibitory factors present in neural cells may lead to exon skipping and hence CGRP processing.

The extensive correlations between CGRP-specific splicing and SmN expression suggest its involvment in regulating CALC-1 splicing (McAllister et al., 1988 / 1989; Sharpe et al., 1989; Li et al., 1989; Rockeach et al., 1989; Amara et al 1982, Crenshaw et al., 1987). Most striking of such correlations is the observation that the only tissues to produce CGRP rather than calcitonin mRNA in transgenic mice expressing a metallothionein-CALC-1 fusion gene are the brain and to a lesser extent the heart (Crenshaw et al., 1987). Therefore in order to determine this putative role

for SmN and whether it plays any role in the above model, endogenous calcitonin / CGRP transcripts from mice brains lacking SmN expression were analysed by RT-PCR.

PCR ASSAY

The presence of distinct 3' exons in CGRP and calcitonin transcripts required the use of a common exon 2 derived primer and primers derived from exon 4 or exon 5 for calcitonin or CGRP analysis respectively. This gives a 279 bp PCR product from CGRP trancripts and a product of 393 bp from calcitonin transcripts.



RESULTS

The levels of PCR product corresponding to CGRP transcripts in mice lacking SmN expression are similar to those observed in controls, confirming that SmN is not necessary for CGRP-specific splicing to occur (FIG.7.). This proposed non-involvement of SmN in CALC-1 splicing is consistent with similar studies with ND3, 3T3 and HeLa ICRF cells (D.Horn PhD.thesis, 1993) and 293 cells (Delsert and Rosenfeld, 1992)which do not express SmN but demonstrate CGRP-specific splicing on transfection with minigene constructs. Furthermore, discrepancies occur in the correlation between SmN mRNA levels and CGRP-specific splicing such that high levels of SmN expression are observed in regions of the brain performing calcitonin-specific splicing (Purkinje layer, lateral cerebellar nuclei)- (Horn and Latchman 1992; Li et al., 1989; Crenshaw et al., 1987). In addition, transfection of SmN in isolation into HeLa cells does not induce CGRP-specific splicing, suggesting SmN alone is not sufficient to influence CALC-1 splicing. Therefore, it would appear that SmN is not a necessary component of the proposed model (see above) for the mechanisms determining CALC-1 splicing that has been suggested from data obtained thus far.

In addition, the levels of calcitonin transcripts in mice brain lacking SmN expression are similar to the low levels observed in controls. Southern blotting was required for sufficient visualisaton in this case, using the weak 390 bp calcitonin PCR band as a probe (FIG.8). The validity of the products generated for both CGRP and calcitonin assays is proved by the observed weak hybridisation of the calcitonin probe with the CGRP PCR product (FIG.9). Results reveal that calcitonin-specific splicing can occur normally in the absence of SmN. This would seem to suggest that SmN plays no general splicing role in the non-neural processing of the CALC-1 gene.

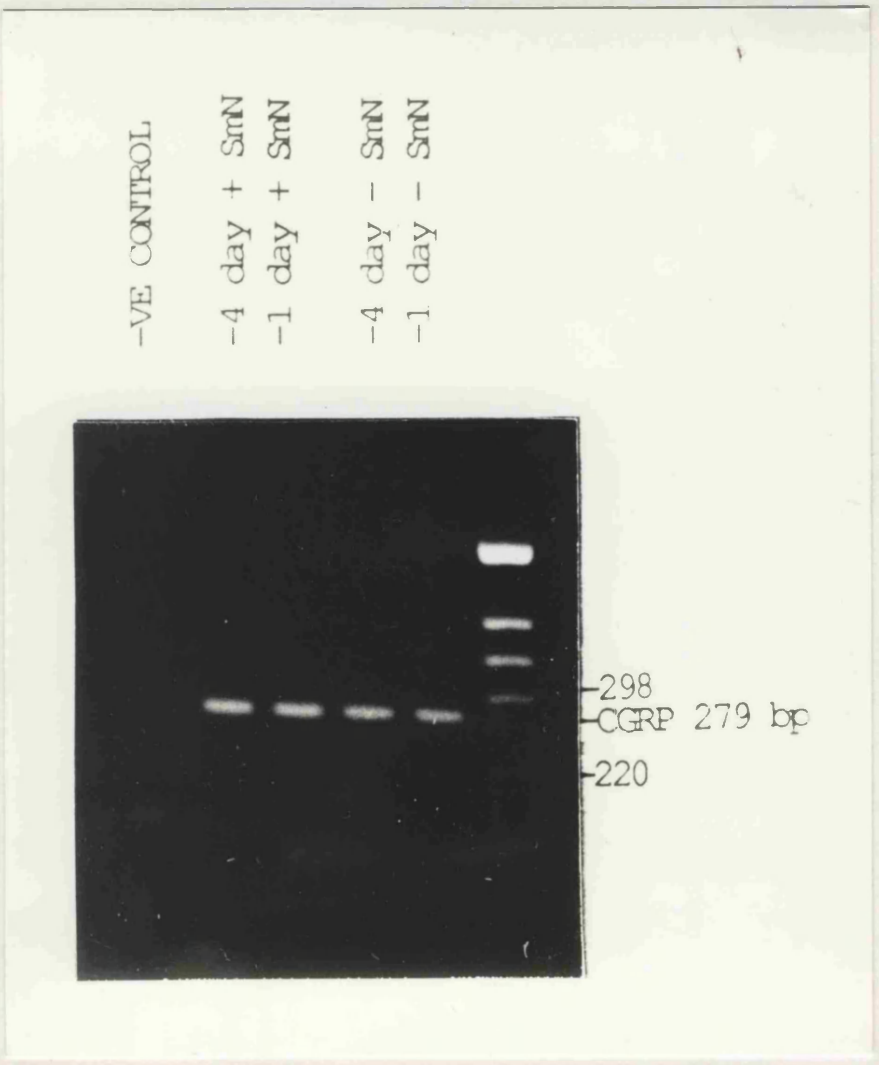


FIGURE 7. PCR of brain-derived CGRP cDNAs from 1 and 4 day old mice lacking Snrpn expression or from age and sex matched controls.

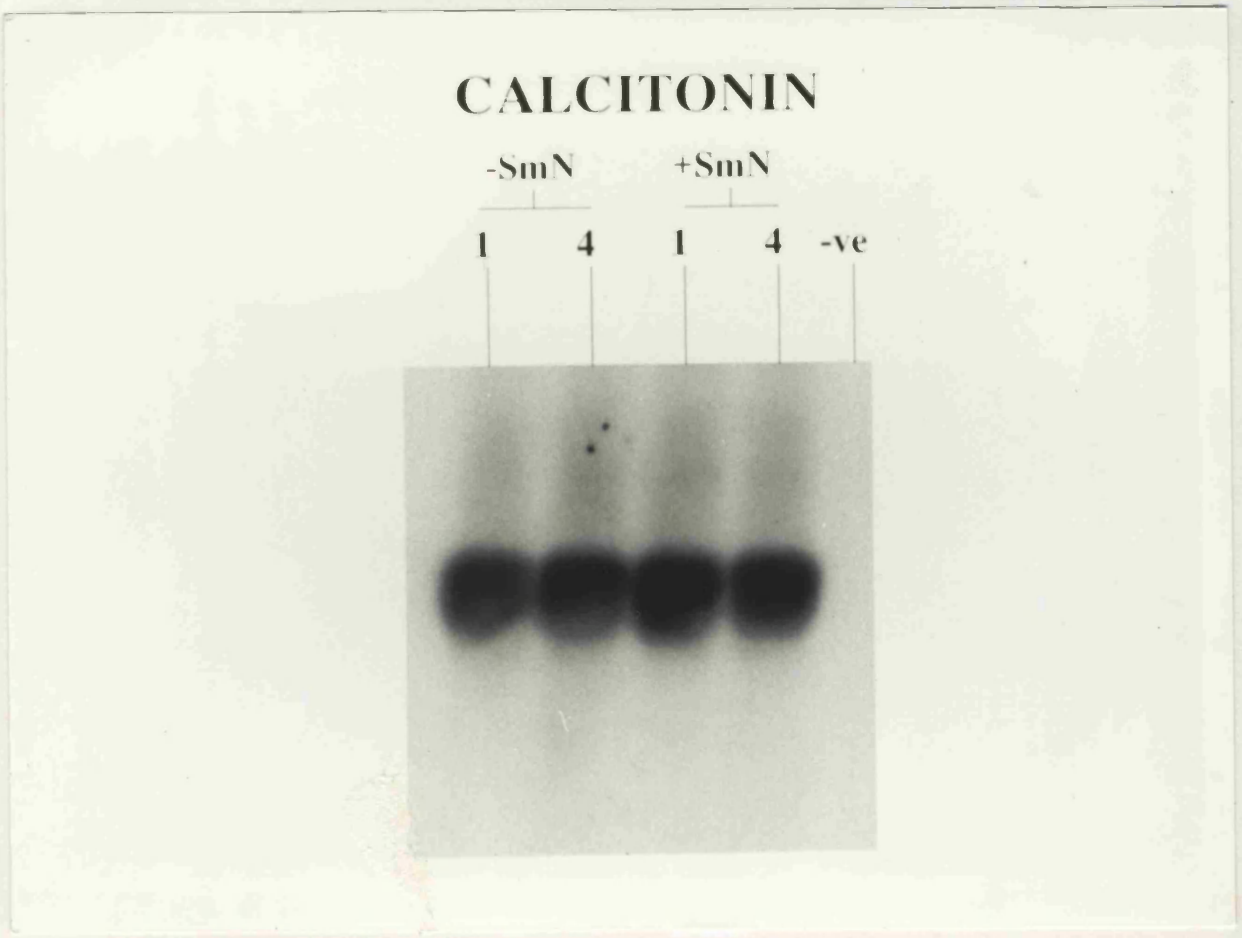


FIGURE 8. Southern blot of PCR products from brain-derived calcitonin cDNAs from 1 and 4 day old mice lacking Snrpn expression (-SmN) or age and sex matched controls (+SmN) probed with a calcitonin PCR product probe for enhanced visualisation.

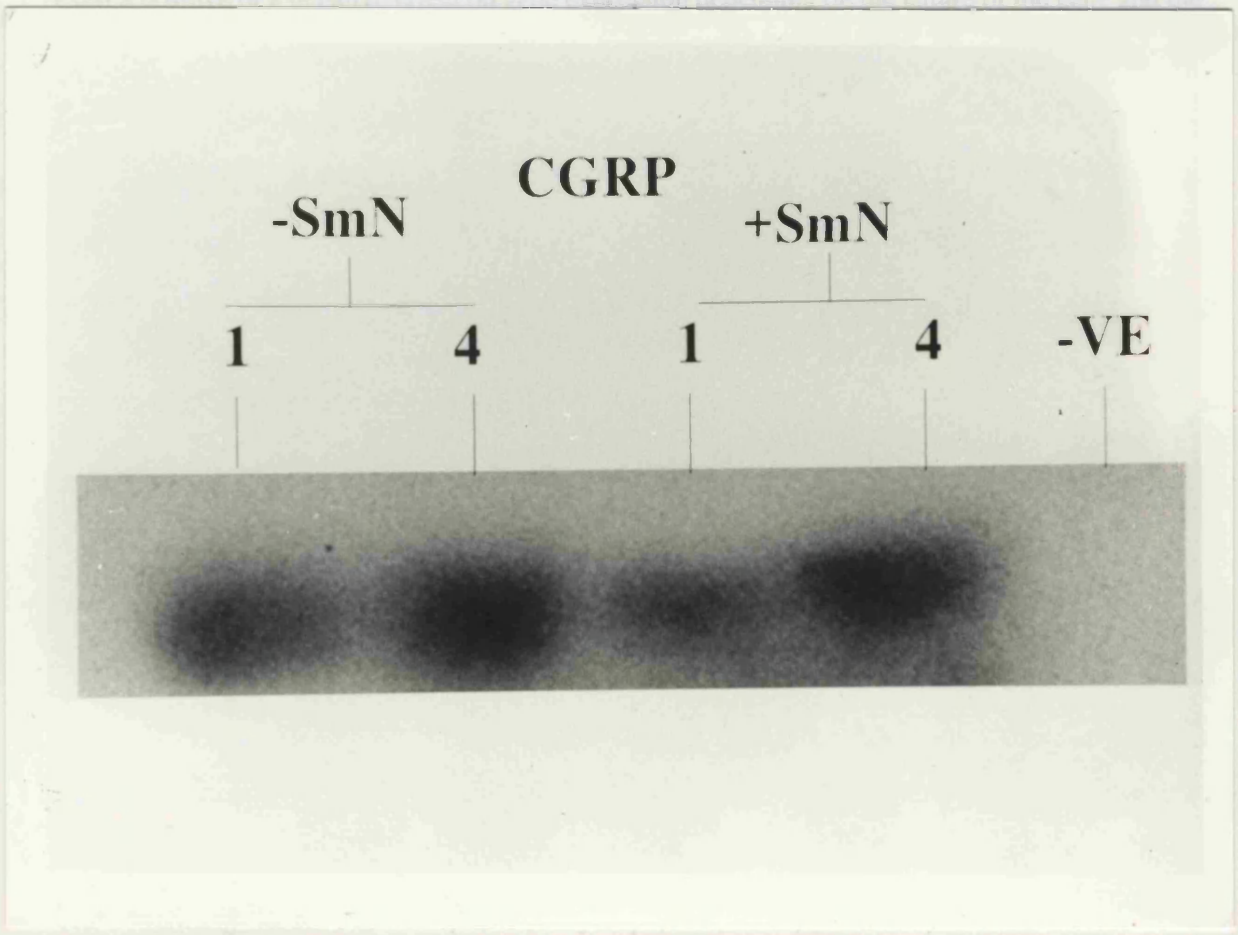


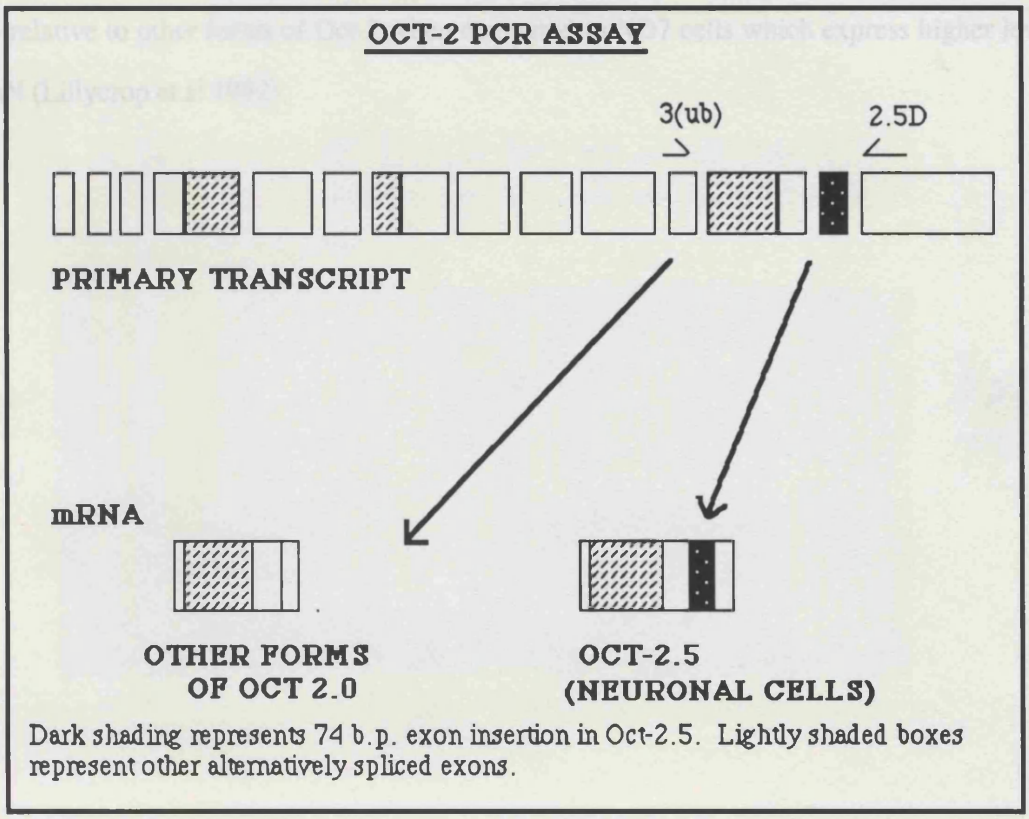
FIGURE 9. Southern blot of CGRP PCR products from 1 and 4 day old mice lacking Snrpn expression (-SmN) or age and sex matched controls (+SmN) probed with calcitonin PCR product probe.

5.8. ALTERNATIVE SPLICING OF THE OCT-2 TRANSCRIPTION FACTOR

The promoter regions of certain genes contain an octamer motif (ATGCAAAT) which can exert a positive or a negative effect on gene expression depending on the nature of the gene and the cell type. Thus the octamer motif acts positively by promoting expression of the immunoglobin genes in B-cells (Lenardo et al ., 1987; Mason et al 1985) and negatively by repressing expression of immunoglobin genes in embryonal carcinoma cell lines (Lenardo et al., 1989). Likewise the octamer motif plays an important positive role in Histone H2B expression (Sive et al., 1986 ; Sive and Roeder, 1986) and can repress immediate-early gene expression of herpes simplex virus (Kemp et el., 1990, Wheatley et al., 1991). The mechanism whereby octamer motifs can repress or activate promoter regions in different cell types is a result of the presence of octamer binding proteins exhibiting different activities. The Oct-2 protein is such an octamer-binding transcription factor that demonstrates stimulation of expression of certain octamer-containing genes in B-cells and repression in neuronal cells (Dent et al., 1991; Lillycrop et al., 1991). This difference in activity is mediated by tissue-specific alternative splicing of the Oct-2 transcript to yield the isoforms Oct 2.1 through to 2.5 (Wirth et al., 1991). The expression of the Oct- 2.4 and 2.5 isoforms is enhanced in neuronal cells typified by the ND7 cell line (Wood et al., 1990) and in brain tissue relative to the observed expression of Oct-2.1which predomimates in B-cells and B-cell containing tissues such as spleen (Lillycrop et al., 1992). The Oct- 2.4 and 2.5 isoforms differ from other isoforms by having an altered C-terminus. Thus Oct-2.4 is truncated and lacks the C- terminus whilst the reading frame for Oct 2.5 is altered due to a 74 base pair insertion that generates a novel C- terminus. Hence promoters which require a C-terminal activation domain present in Oct 2.1, 2.2 and 2.3 will not be activated by Oct 2.4 or 2.5. Therefore promoters that require both N-terminal and C-terminal activation domains may be repressed by Oct-2.4 or 2.5 or other C-terminally altered neuronal Oct-2 isoforms (Stoykova et al ., 1992) due to inhibition effects.

OCT 2.5 PCR ASSAY

The presidence of Oct 2.4 and 2.5 in neuronal cells such as ND7 and within the brain made it feasible that the alternative splicing that generates these isoforms could be regulated by SmN. Therefore PCR primers were designed to flank the alternatively spliced exons of the Oct 2.4 and 2.5 isoforms following an initial race PCR reaction that amplifies endogenous Oct-2 transcripts by using a POU-domain specific primer and a primer for the poly A tail of transcripts.



Dark shading represents 74 b.p. exon insertion in Oct-2.5. Lightly shaded boxes represent other alternatively spliced exons.

RESULTS

Results were analysed on agarose gels (FIG.10) and on Southern blots hybridised with a probe prepared form the PCR product of other forms of Oct-2 (FIG.11). No differences in the relative proportions of Oct 2.5 PCR product (larger by 74 base pairs) compared to the other forms were observed in mouse brain tissues lacking SmN expression when compared to age and sex matched controls. This suggests that SmN is not required for the neural pattern of alternative splicing of Oct-2 transcripts. The PCR did not yield any bands corresponding to that of Oct 2.4 in

any tissues, possibly reflecting the previously reported lower level of expression relative to Oct 2.5 (Lillycrop et al., 1992). The levels of Oct 2.5 relative to the other forms did not appear to alter between parental 3T3 fibroblasts and those expressing intermediate (T7S2A) and high levels of SmN (MSN), suggesting that SmN alone is not sufficient to affect the pattern of splicing of this gene. These results are consistent with the observation that neuronal cell lines expressing very little or no SmN (N18,ND3 respectively) are capable of expressing higher or similar levels of Oct 2.5 relative to other forms of Oct-2 when compared to ND7 cells which express higher levels of SmN (Lillycrop et al 1992).

FIGURE 10. PCR products of Oct-2 cDNAs from the brains of 1 and 4 day old mice lacking Snrpn expression (-N1,-N4) or from age and sex matched controls (+N1,+N4). Results of the effect of artificial SmN expression in isolation on Oct-2 alternative splicing are included with 3T3 fibroblasts and 3T3 cells expressing low (T7S2a) and high (MSN) levels of SmN. Also included are results from 2 and 7 day post-natal mouse brain samples. The positions of products of the Oct 2.5 (2.5) transcripts and other Oct-2 (2.0) transcripts are indicated.

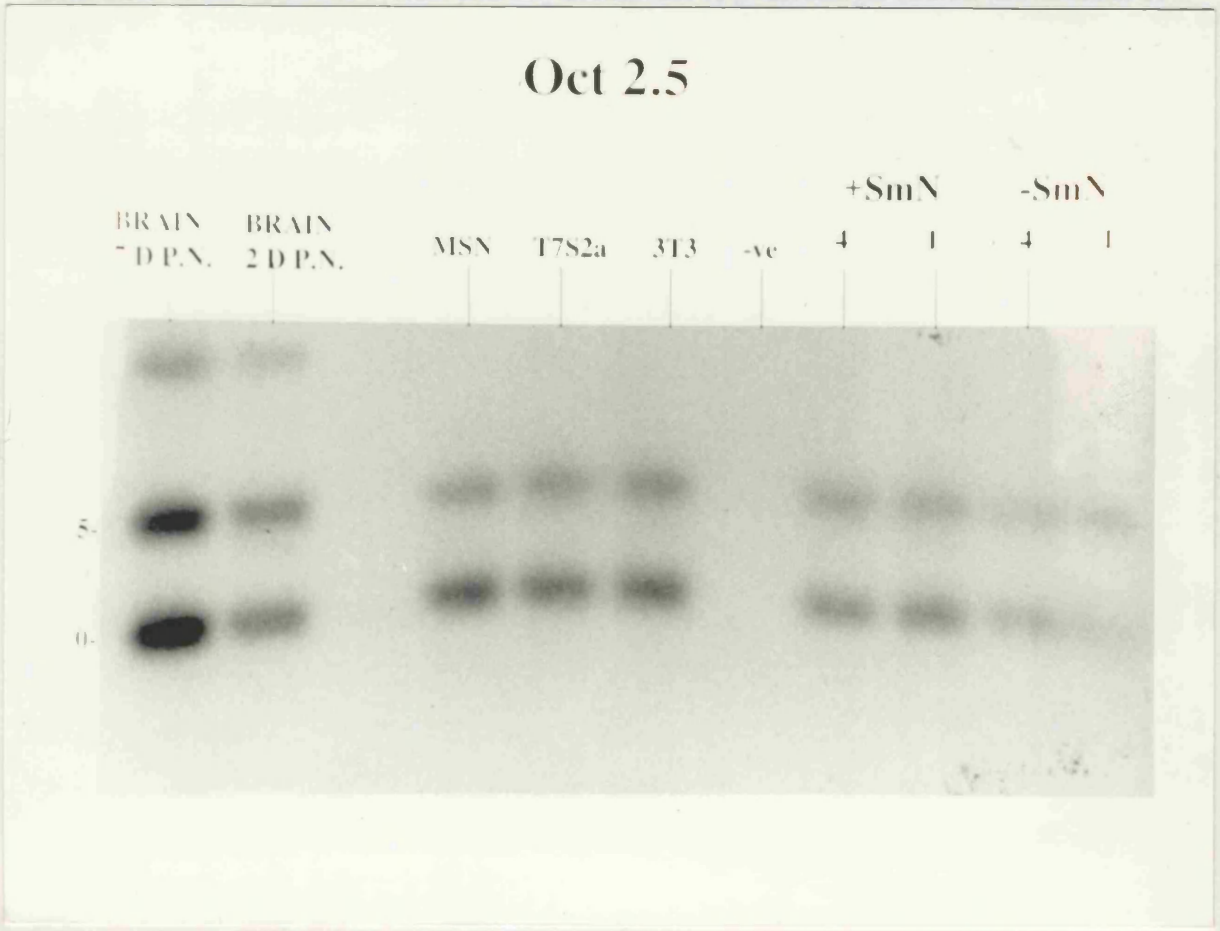


FIGURE 11. Southern blot of above (FIG.10) Oct-2 PCR products probed with a probe derived from Oct 2.0 transcripts for enhanced visualisation.

5.9. GSα GUANINE NUCLEOTIDE BINDING PROTEIN

The GSα protein is a member of the heterotrimeric guanine nucleotide binding protein family involved in signal transduction from transmembrane receptors and the subsequent stimulation of the adenylate cyclase pathway in response to β–adrenergic stimuli (Birnbaumer et al., 1990). The G proteins are composed of α, β and γ subunits, the α subunit being unique to each G protein and responsible for binding of the guanine nucleotide. Structural analysis of the human GS gene demonstrates four species of GSα mRNA may be generated by alternative splicing by the alternate inclusion or exclusion of exon 3 and use of two 3' splice sites in intron 3. Two cDNA species (GS α-1 and GSα-4) have been isolated from bovine adrenal tissue (Robishaw et al., 1986) and human liver (Mattera et al 1986) and generate a long (52-kDa) and short (45-kDa) isoforms demonstrate similar activities (Mattera et al., 1989) but differences in tissue and developmental expression patterns (Mumby et al., 1986, Cooper et al 1990., Granneman ansd Brannon, 1991). Further isoforms are generated by alternative promoter usage and alternative splicing involving exon 2 (Ishikawa et al., 1990 ; Swaroop et al., 1991).

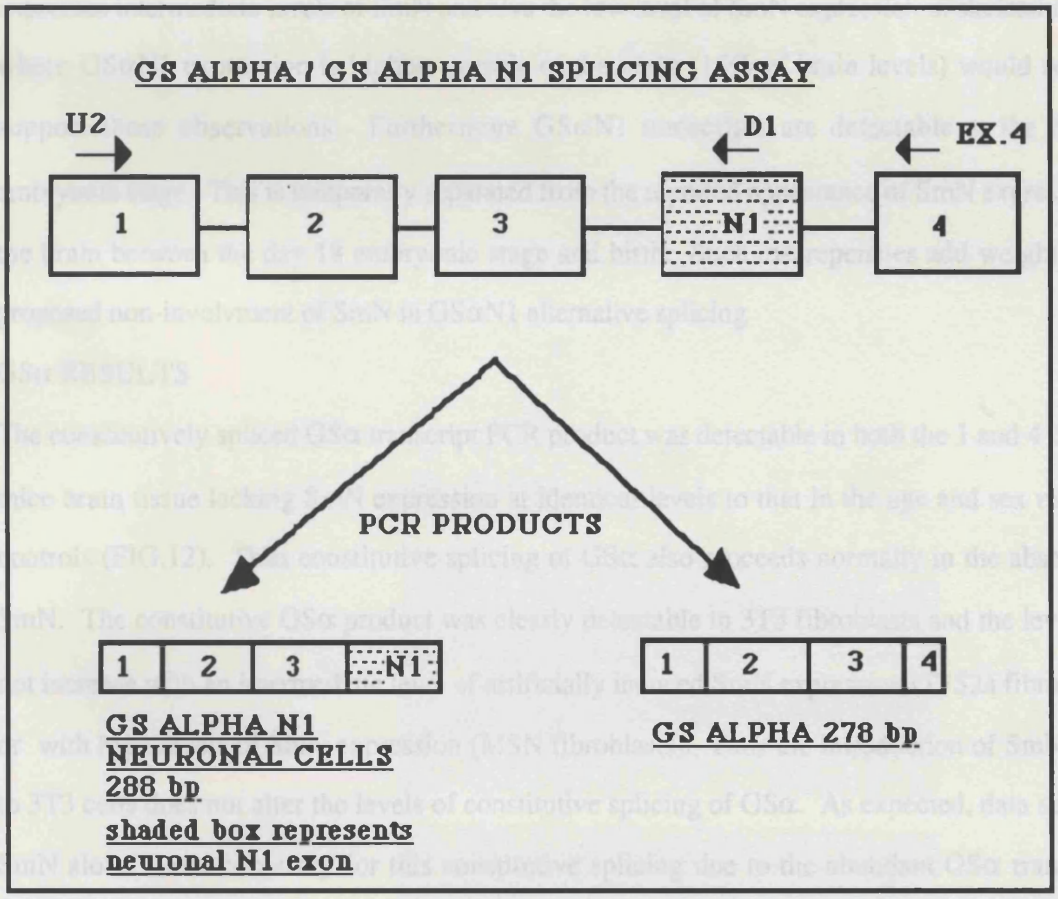
A novel alternative transcript of the rat GSα gene has recently been isolated from neuronal-like CA77 rat thyroid C-cells (Crawford et al., 1993) which differs structually from other isofoms, being generated by the alternative splicing of exon 3 to an internal terminal exon termed N1 situated 800 bp downstream. The mRNA encodes the 86 NH2-terminal amino acids of GSα and then diverges at the position of the exon-intron splice site of exon 3 with 5 novel amino acids encoded by the N1 exon. The structure of GSαN1 suggests that it is not capable of signal transduction due to the lack of receptor / effector and GTP binding domains which are located in the COOH-terminal region of other GSα isoforms (Johnson and Dhanaskaran., 1989; Birnbaumer et al., 1990) and its precise function remains unclear. The expression pattern of GSαN1 mRNA differs from the widely expressed GSα mRNA (Mumby et al., 1986; Jones and Reed 1987), demonstrating tissue specific expression throughout the brain and to a lesser extent in skeletal muscle. Within the brain, the expression of GSαN1 is greatest in the hypothalamus and regions

of the brainstem, a pattern which follows that of GSα expression (Brann et al., 1987, and Largent et al., 1987) which is roughly 10-fold greater. Developmentally, GSα N1 expression can be detected at day 14 compared to day 13 for GSα mRNA and is restricted to the head region which consists mainly of brain tissue and not to the craniofacial region.

PCR ASSAY ANALYSIS OF GSα SPLICING IN CELLS AND TISSUES.

The tissue restricted expression of the GSαN1 transcript in the brain and its wide pattern of distribution within make it a suitable candidate for processing by SmN. High levels of GSαN1 transcript are found in the neurons and specific areas of the brain found to have high levels of SmN mRNA (brainstem, hypothalamus). Thus the neuronal-specific alternative splicing of GSα merited investigation using a PCR assay on endogenous GSα transcripts.

Due to the presence of distinct 3' exons, a 3' primer specific to exon N1 (D1 -3' untranslated region of GSαN1) and a 5' primer derived from exon 1 (U2-GSα NH-2 terminal coding) were required to give a PCR product of approximately 300 base pairs. The levels of expression of the constitutive GSα were analysed in a separate reaction using the common 5' primer and a primer derived from the constitutive exon 4 (Ex.4 - GSα exon 4 coding) to yield a marginally smaller PCR product.



GSαN1 PCR RESULTS

PCR products of the expected size for GSαN1 were generated using mouse brain tissue lacking SmN expression at similar levels to that observed in the age and sex matched controls in both the 1 and the 4 day old animals (FIG.12/13). It would therefore appear conclusive that SmN is not required for the alternative processing of the primary transcript to generate the GSαN1 transcript. The GSαN1 transcript was undetectable in the 3T3 fibroblasts, reflecting the low levels of expression in tissues other than the brain. However, the PCR product was not detectable in the T7S2a fibroblasts which artificially express intermediate levels of SmN and did not show any increase in the MSN fibroblasts which express high levels of SmN. Thus, SmN alone does not appear to be sufficient for the observed neural processing pattern of the GSα primary transcript to produce GSαN1-encoding mRNA. The lack of detectable GSαN1 transcripts in the heart which

expresses intermediate levels of SmN and also the low level of SmN expression in skeletal muscle where GSαN1 expression is highest outside of the brain (15% of brain levels) would seem to support these observations. Furthermore GSαN1 transcripts are detectable at the day 14 embryonic stage. This is temporally separated from the reported appearance of SmN expression in the brain between the day 18 embryonic stage and birth. Such discrepencies add weight to the proposed non-involvment of SmN in GSαN1 alternative splicing

GSα RESULTS

The constitutively spliced GSα transcript PCR product was detectable in both the 1 and 4 day old mice brain tissue lacking SmN expression at identical levels to that in the age and sex matched controls (FIG.12). Thus constitutive splicing of GSα also proceeds normally in the absence of SmN. The constitutive GSα product was clearly detectable in 3T3 fibroblasts and the levels did not increase with an intermediate level of artificially induced SmN expression (T7S2a fibroblasts) or with high levels of SmN expression (MSN fibroblasts). Thus the introduction of SmN alone to 3T3 cells does not alter the levels of constitutive splicing of GSα. As expected, data suggests SmN alone is not necessary for this constitutive splicing due to the abundant GSα transcripts found in non-SmN expressing tissues. The validity of the PCR products obtained in both the GSα N1 and the GSα assays is demonstrated by southern blotting and the subsequent hybridisation by GSα N1 PCR products with a probe derived from a mouse brain GSα PCR product due to shared NH-2 terminal sequences (FIG.13, lower panel).

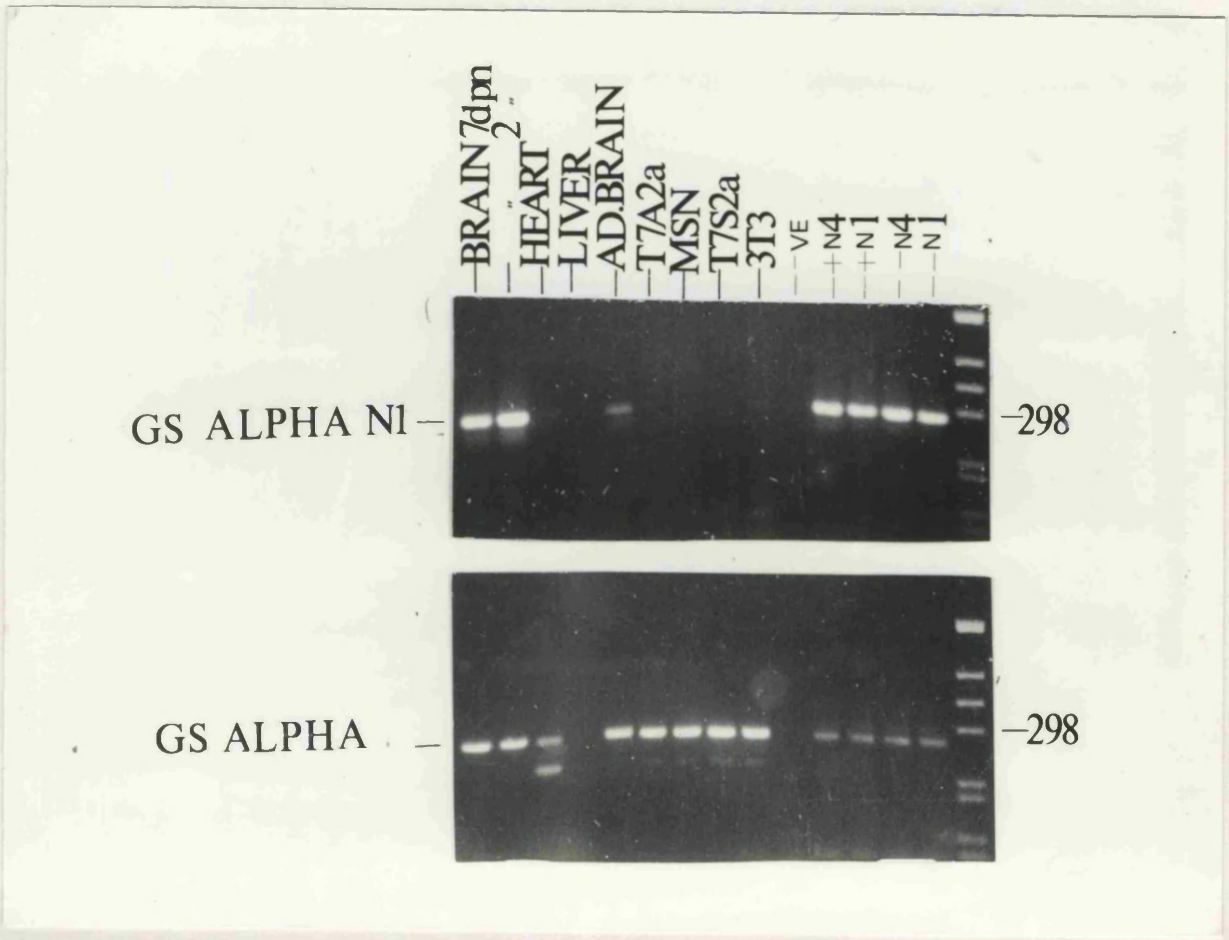


FIGURE 12. PCR products of GSα and GSαN1 cDNAs derived from the brains of 1 and 4 day old mice lacking Snrpn expression (-N1,-N4) or from age and sex matched controls (+N1,+N4). Also included are results of the effect of artificial expression of SmN in isolation on GSα / GSαN1 splicing as demonstrated by 3T3 fibroblasts and 3T3 cells artificially expressing low (T7S2a) and high levels (MSN) levels of SmN and 3T3-antisense (T7A2a) cells. Results from various tissues of normal control mice also included as indicated.

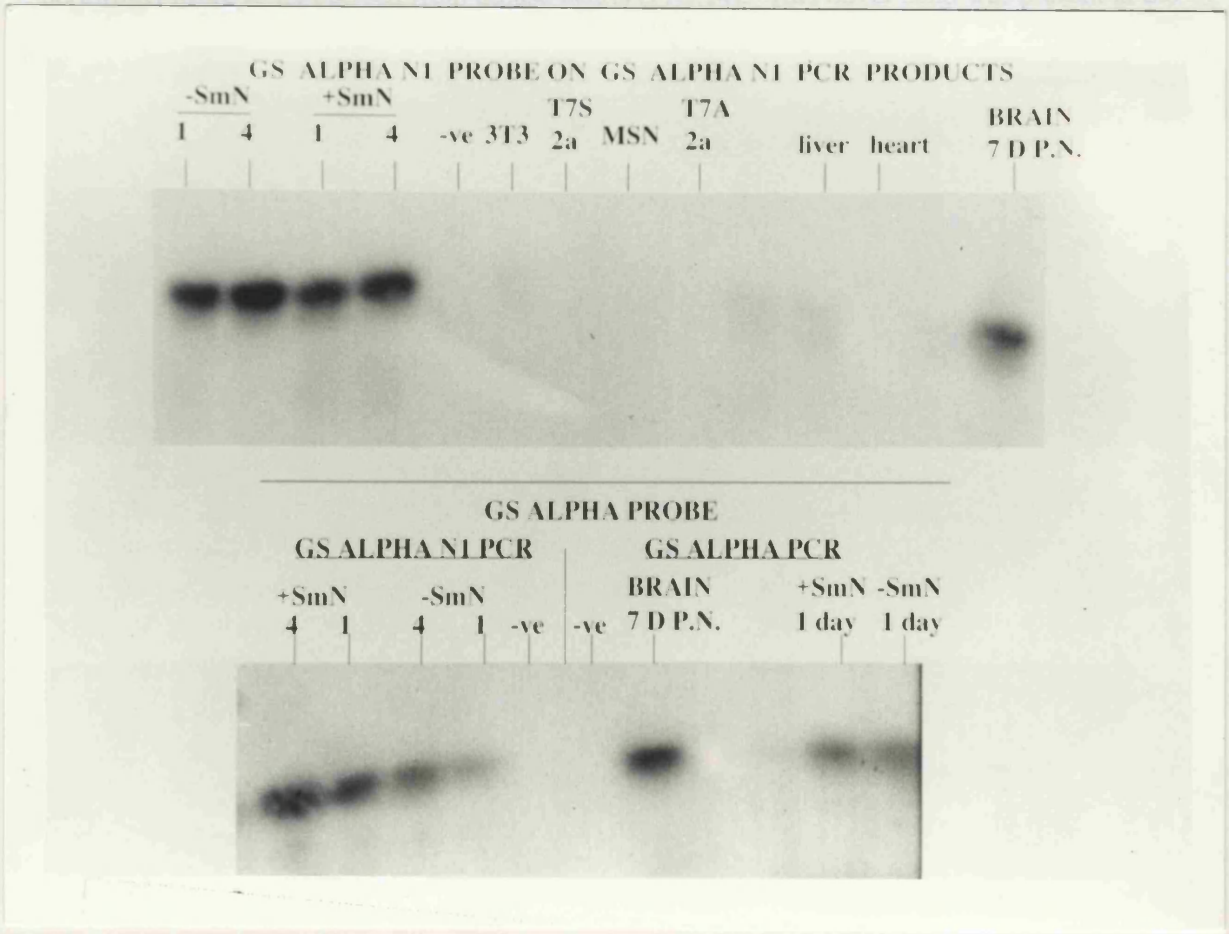


FIGURE 13. Upper panel-Southern blot of GSαN1 PCR products derived from brains of 1 and 4 day old mice lacking Snrpn expression (-SmN 1,4) and age and sex matched controls (+SmN 1,4). Also included are results for 3T3 fibroblasts, 3T3 cells expressing SmN (T7A2a, MSN), 3T3-antisense cells and liver, heart and 7 day post-natal brain samples from normal control mice.

Blot probed with a probe derived from the GSαN1 PCR product.

Lower panel-GSαN1 and GSα PCR products as indicated, probed with GSα PCR-derived probe for product validification.

5.10. HEART DEVELOPMENTAL ASSAYS.

Whilst performing constitutive splicing assays of GSα in mouse tissues, an additional unique band was observed in adult heart tissue. To investigate this further, a developmental study was performed using RNA derived from mouse hearts (FIG.14). This novel band was present at the 16 day embryonic stage and all subsequent developmental stages at similar levels. Southern blotting revealed this band to hybridise to a probe prepared from the expected GSα PCR product which had been prepared from brain (FIG.15). The exact nature of this band has not been determined but the positive hybridisation suggests it may possibly correspond to a novel heart specific isoform of GSα. This band also appeared in the GSα PCR from all 3T3 fibroblast samples (FIG.12) but was significantly weaker than that observed in the heart relative to the expected GSα PCR product as demonstrated by Southern blotting (FIG.15).

FIGURE 14. PCR products of GSα cDNAs derived from developmental series of normal mouse heart samples. Lanes: 2=2 day post-natal; 7=7days post-natal; 16=16 days embryonic.

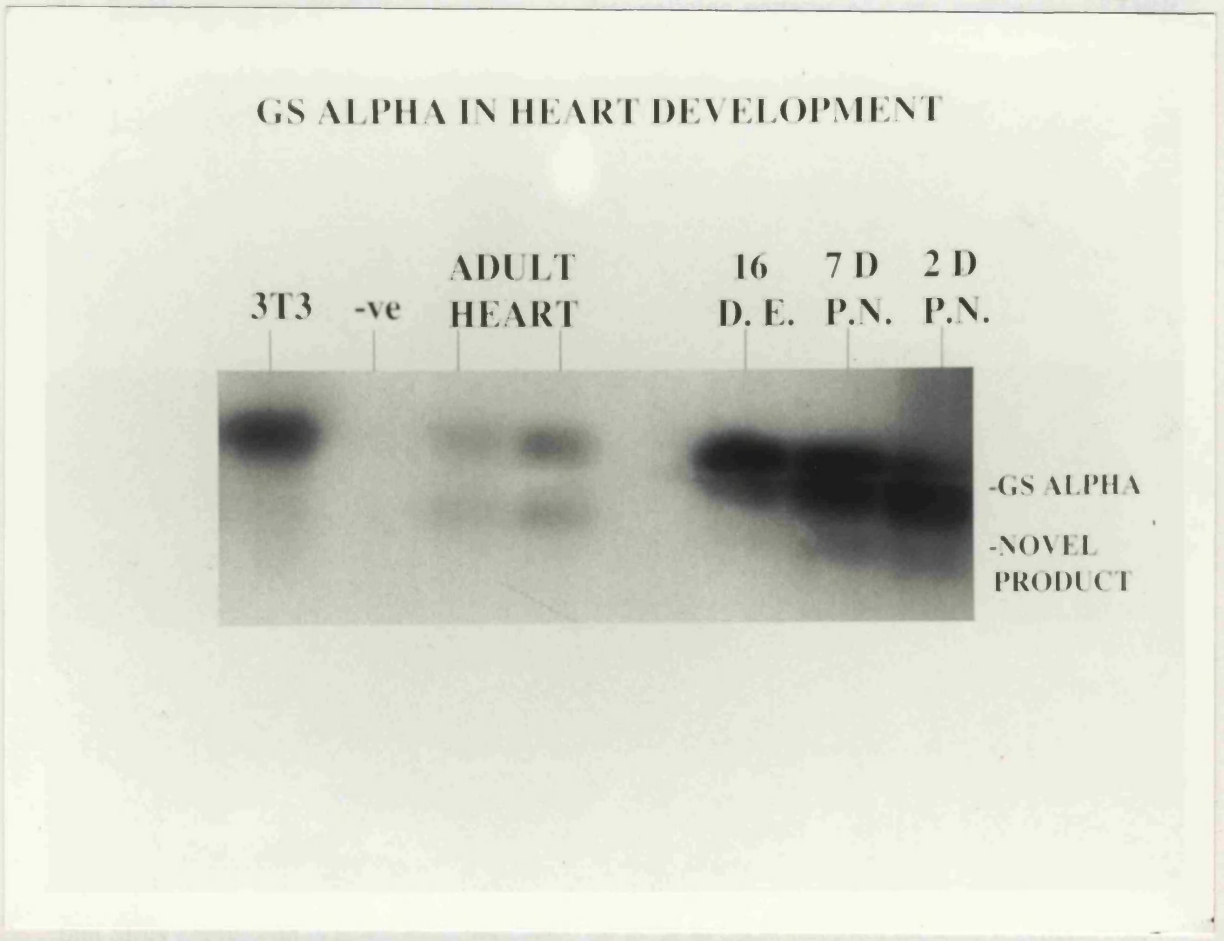


FIGURE 15. Southern blot of PCR products of GSα cDNAs derived from normal heart developmental series visualised with a probe derived from the expected GSα PCR product. Also included is a 3T3 fibroblast sample.

5.11. CHAPTER DISCUSSION.

Previous alternative splicing assays using 3T3 cells stably transfected with SmN suggested that SmN was not sufficient in isolation to alter splicing patterns of c-src, calcitonin / CGRP, NCAM, and Goα (Horn and Latchman, 1993 a/b). The artificially expressed SmN in these 3T3 cell lines has been confirmed to associate with snRNP particles in a pattern that reflects that observed in cell lines naturally expressing SmN-ND7, F9 (Huntriss et al., 1993b-preceeding chapter). This strongly suggests that although the SmN in the transfected cells is seemingly performing as an integral snRNP component, its presence within the snRNPs does not affect the patterns of splicing of these transcripts. The proposal that SmN, when expressed in isolation is not sufficient to affect splicing patterns is further confirmed with data presented here for Oct-2 and GSα splicing. Thus whilst SmN may normally be necessary in the brain for performing a general RNA processing role of the genes tested here, it is not sufficient in isolation to regulate the pattern of their splicing.

Data presented here further confirms that SmN is not involved in regulating these splicing decisions as the six neuronal-specific alternative splicing events tested proceed normally in the complete absence of SmN protein expression, as determined here by Western blotting. The six splicing decisions tested encompass a wide cross-section of alternative splicing events. The fact that SmN expression is is not even necessary for them to occur does not indicate a general role for SmN in alternative splicing as previously proposed.

The replacement of SmB by SmN in snRNPs in the brains of normal mice implies SmN does performs general constitutive splicing roles normally performed by SmB in other tissues. The enhanced SmB expression in the brains of mice lacking SmN expression may allow normal constitutive splicing to continue due to a common function of SmN and SmB. The normal proceeding of constitutive splicing events in the brain in the absence of SmN is demonstrated here with the GSα gene. All splicing functions of SmN for the genes tested here appear to be fully replaced by SmB.

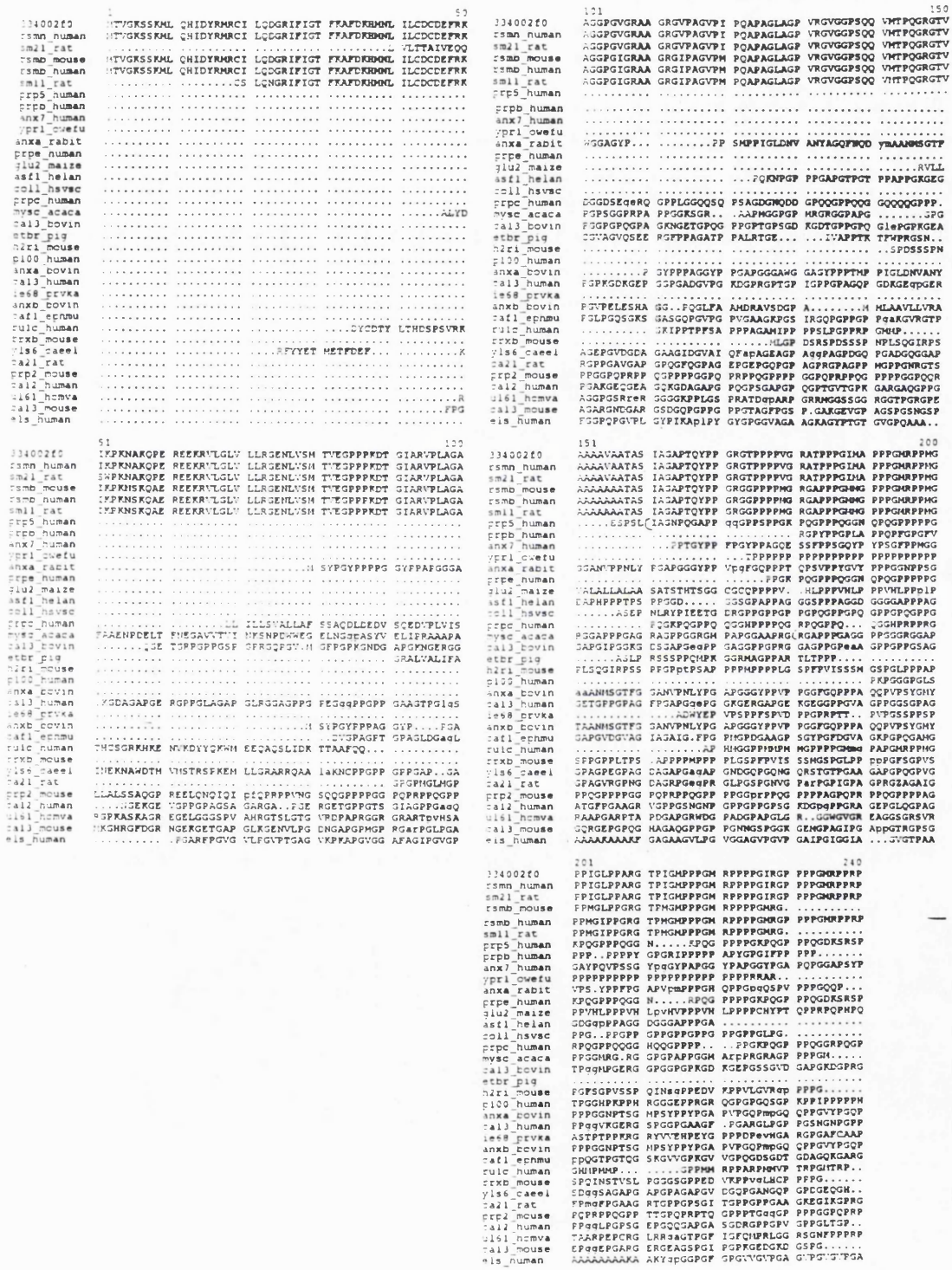
The early post-natal lethality of mice lacking SmN expression however suggests that whilst the elevation of SmB in such mice may have been sufficient to compensate for the constitutive roles normally performed by SmN in the brain, it could not compensate for as yet undefined specific activities of SmN. It must be taken into account that other imprinted genes may be present within the translocated region, and that the fate of the mice may be a combination of this and the demonstrated lack of SmN expression.

The lack of SmN expression throughout the mouse tissues tested demonstrates that the imprinting effect on Snrpn on the maternal chromosome is effective throughout the entire body. Thus it follows that as SNRPN is imprinted in humans, then an analysis of SmN expression of any cell or tissue would give a representative picture of the status of SmN expression throughout the human body. The availability of PBMCs from PWS patients therefore provides a suitable means to assess SmN expression in such patients in an attempt to attribute a critical role for SmN that is suggested by the fate of the murine models of PWS.

CHAPTER 6
GENERAL DISCUSSION

In an attempt to assign a putative function to the SmN protein, and to establish whether any of the 17 amino acid substitutions in SmN produce any recognisable structural or functional motifs, analysis of the full amino-acid sequence of SmN was performed utilising the HSSP/ MaxHom programme from the PHD EMBL -Heidelberg. DE facility. Results include data from alignments with 29 non-SmN or SmB/B'derived polypetides that demonstrate maximum homology to the SmN sequence derived from Schmauss et al., 1992. From such alignments, details of secondary structure of SmN include a predicted α–helix from residues 9 to 19 and also from residues 31 to 36. Extended β-sheet regions are predicted from residues 26 to 29, from residues 40 to 43 and from residues 70 to 71. The remainder of the amino acid residues, and hence the vast majority of the protein is predicted to consist of structurally less-well defined loop region structure. The predicted α–helix and β-sheet regions will also be common to SmB/B' as no polymorphisms occur in these regions, and thus any differences between SmN and SmB/B' occur in the loop regions. From the aligned sequences, non of the regions of least sequence homology between SmN and SmB/B'(179-190 and 203-213) appeared to be consistently present as distinct motifs in the 29 aligned polypeptides. This would therefore seem to suggest that such SmN or SmB/B' specific regions as primary amino acid sequences do not represent common functional motifs. It remains possible, however that differences in secondary structure that arise from these regions may be functionally important.

FIG. 1. Polypeptides aligned with the SmN protein sequence using the HSSP/ MaxHom. program from the PHD EMBL-Heidelberg . DE facility. Details of polypeptides are outlined in the text.



Fom the 29 aligned polypeptides, a number were chosen for closer analysis and to determine their precise functions, with the aim of assigning functions to SmN. Data suggests that none of these polypeptides were involved in the role of RNA splicing and the alignments with SmN appear to be a result of proline-richness that occurs in these polypeptides. Thus, the proline-rich human and bovine CAL3 polypeptides that form the collagen alpha I (III) chain (Ala-Kokko, et al., 1989;) were amongst the aligned sequences. Other aligned sequences correspond to the proline-rich proteins (PRP) including human PRP5 (Kauffmann et al., 1986), human PRPB (Isemura et al., 1979) and murine PRP2 (Ann and Carlson, 1985) that are derived from saliva and have no defined function. A further alignment is human P100, a proline-rich nuclear DNA-binding protein associated with cell proliferation (Zhang al., 1993). The only aligned polypeptide with a motif identical to one of the regions of least sequence homology between SmN and SmB/B' is the RGAPPP motif of the myosin IC heavy chain polypeptide from amoeba (Hamer et al., 1986) which is identical to residues 181-186 in SmB/B'. The significance of this if any, remains unclear.

A region of particular interest is the alanine-rich AAAAKAAA motif present in the aligned sequence of the human elastin precursor (Fazio et al., 1988). Similar motifs are present in SmN (AAAAVAAT) and SmB (AAAAAAAT) from residues 151-157 and this motif has been previously compared with the ATAAAAAAA motif from residues 422-430 in yeast poly (A) binding protein (Rockeach et al., 1989). Poly-alanine sequences are not included in the motifs considered to have RNA-binding potential (Burd and Dreyfuss, 1994) and a literature search did not suggest any particular function of the sequence in human elastin. However, the amphiphillic nature of such a motif has been suggested to potentially confer the ability to mediate protein-protein interactions (Rockeach et al., 1989) and in this respect it is interesting that one of the amino acid substitutions in SmN occurs within the motif.

Thus SmN sequence does not appear to be homologous with non-core RNA splicing factors or alternative splicing factors from other species which again does not suggest a general role for the SmN protein in alternative RNA splicing. This is in contrast to the mammalian U1A and U2B" snRNP-specific proteins that demonstrate high homology with the product of the

Drosophila snf gene. This protein, SNF regulates splice site selection in *Sex-lethal* splicing and is U1 snRNP-associated, suggesting a mechanism whereby the SNF protein could influence splicing regulation by determining recognition of 5' splice sites (Flickinger and Salz, 1994). It remains possible that some of the properties of SNF in terms of recognising regulated splice sites are retained in the mammalian U1A and U2B" proteins. Data from analysis of various alternatively spliced genes in mice devoid of SmN expression due to maternal duplication for the central region of chromosome seven (chapter 5) again does not suggest a general role for SmN in alternative splicing.

Recent discoveries have established the dual functions of components of the spliceosome, namely the SR proteins such as ASF/SF2 in both constitutive splicing (Wu and Maniatis, 1993) and alternative splicing (SV40 T/t antigen splicing-Ge and Manley, 1990; β–globin splicing-Krainer et al., 1990). SR proteins are differentially active with different pre-mRNAs (Zahler et al., 1993) and the concentration-dependant splice-site activation of other splicing factors such as hnRNPA1 taken together with the antagonistic effects produced by this protein against ASF/SF2 and SC35 functions (Fu, XD, 1992; Mayeda and Krainer, 1992; Ben-David, et al., 1992) suggests how a complex interaction of these proteins with each other and with *cis* -acting sequences on pre-mRNAs could determine splice site selection. It remains possible that other unidentified factors may be present in mammalian systems that mimic the action of factors that affect splice site selection in well characterised organisms such as Drosophila. For example, the tissue-specific PSI factor involved in Drosophila P element splicing (Siebel et al., 1994) is proposed to interact with the generally expressed hrp48 protein that demonstrates homology with mammalian hnRNPA1 to affect the splice site selection of the U1 snRNP. The tissue-specific expression in mammalian sytems of a factor that mimics the action of PSI by interacting with general splicing factors such as hnRNPA1 or SR proteins suggests how tissue-specific alternative splicing could be achieved. In agreement with this model, the isolation of tissue-specific RNA-binding proteins such as the neural-specific activities that inhibit calcitonin-specific splicing have been reported (Roesser et al., 1993).

The involvement of SmN within such a model is less clear. Analysis of SmN protein sequence in relation to recognised RNA-binding motifs (Burd and Dreyfuss, 1994) does not identify any of such motifs, this also being true for SmB/B'. Thus the predicted secondary structure of SmN described here does not constitute the secondary structural requirements ($\beta\alpha\beta\beta\alpha\beta$) of the RNP-motif. In addition, other RNA-binding motifs namely RGG boxes, arginine-rich motifs, KH motifs, zinc-finger knuckle motifs or double-stranded RNA-binding motifs are not a feature of either SmN or SmB/B' sequences. In light of this, the only mechanism whereby SmN could potentially affect splice site recognition appears to be by virtue of protein-protein interactions with the core snRNP proteins. To date, the only suggestion of a potential mechanism whereby SmN could affect splice site selection is provided by its apparent difference in affinity for the U1 snRNP and U2 snRNP particles than that which is exhibited by SmB (chapter 4). Whether SmB/B' and SmN co-exist in the same snRNP particles remains unclear. It is possible that different species of snRNP particles exist in the context of SmB/B' and SmN status. Thus homodimers consisting of two SmB particles may occur in rodent tissues apart from brain and heart where heterodimers of SmN plus SmB or homodimers of only SmN may be present. In humans, the presence of SmB' adds further variation to this model. The difference in size between SmN, SmB' and SmB proteins and furthermore the conformational differences that exist between these proteins as suggested by variations in their antigenic nature (chapter 3) may be sufficient to alter the configuration of the RNA species within the snRNP thus affecting splice site selection. However, as analysis of alternatively spliced genes did not suggest a role for SmN in alternative splicing it is more feasible to suggest that differences occurring in SmN are associated with other functions of this protein. In agreement with this, the Sm core of the U1 snRNP is proposed to specifically bind the guanosine-N2 methyltransferase that is essential for hypermethylation of the m7G cap of the U1 snRNA to the m3G cap structure (Plessel et al., 1994). This methyltransferase activity is also proposed to be shared by other snRNPs and preliminary data suggests that the binding site for the methyltransferase is presented by the SmB/B' proteins. Therefore it follows that substitutions in SmN and/ or the SmN/B/B' status of a snRNP may confer differences in the

affinity of binding of the methyltransferase. This in turn may affect the ability of snRNP particles to be transported back to the nucleus, as the m3G cap forms part of a bipartite nuclear localisation signal in conjunction with a domain within the Sm core (Fischer and Luhrmann, 1990; Fischer et al., 1993; Hamm et al., 1990). Thus the presence of SmN within snRNPs may affect snRNP localisation and furthermore the difference in affinity demonstrated by SmN for particular snRNP particles may reflect the requirements of cells in which it is expressed for a particular snRNP particle. In addition, Sm proteins have been proposed to confer stability to U1 (Hamm et al. 1987) U2 (Mattaj and DeRobertis, 1985) and thus the balance of SmN/B/B' within snRNPs may reflect different requirements for snRNP stability in different tissues.

Two lines of evidence presented here suggest the SmN gene in both human and murine tissues is subject to post-transcriptional control. Firstly, the elevated SNRPN transcription in certain SLE patients was not reflected at the protein level and levels of SmN expression in SLE patients were equivalent with controls. Secondly, whilst the reported enhanced levels of Snrpn mRNA in mice with a paternal duplication of central chromosome 7 (Cattanach et al., 1992) suggests that both copies of the gene are expressed, results from densitometric analysis of Western blots did not reveal enhanced SmN expression in such mice. This is also reflected by apparently unaffected development of these mice. The precise mechanism of regulation however, remains unclear. It is possible that even if excess levels of SmN protein expression occured, the limiting amount of RNA-free 6S core protein complex (Zieve and Sauterer, 1990) available for any excess SmN protein to bind to would imply that much of the SmN would be free in the cytoplasm and perhaps would be subject to rapid degradation.

In agreement with the normal levels of SmN protein expression in SLE, no SmN-specific autoantibodies have been described to date in SLE. Conversely, studies with the hsp90 gene indicated elevated hsp90 protein expression which was paralleled by the presence of autoantibodies (Norton et al., 1988; Twomey et al., 1993; Dhillon et al., 1993). The study of ectopic SmN expression in SLE taken together with data from ELISA screening of SLE sera whereby data was consistent with previous literature in that no anti-SmN-specific antibody activity

has been described, suggest that SmN is not the antigen stimulating the immune system in SLE. Rather, it would appear that the immune system is stimulated by antigens expressed elsewhere rather than in the brain and heart. It has been suggested that the U1 snRNP is the major immunogenic focus in SLE (Fatenejad et al., 1994). This is conceivable given the high copy number of the U1 snRNP (Zieve and Sauterer, 1990) and is suggested by experimental evidence from immunisation of mice with purified U1 snRNPs (Reuter and Luhrmann, 1986). In addition, analysis of sequential development of antibodies in MRL/lpr mice demonstrate that the U1 A protein is the first protein against which autoantibodies are generated (Fatenejad et al., 1994). The discovery of autoantibodies against stemloop structures of U1 snRNA (Hoet et al., 1993) and evidence from a new murine model of SLE (Vidal et al., 1994) also suggest that the U1 snRNP is the primary focus of the immune response in SLE. Data from immunoprecipitations performed here whereby SmN demonstrated preferential association with the U2 snRNP at low levels of expression appears to conform to this model in terms of the potential antigenicity of SmN. Thus the low level of SmN expression outside of the brain and heart suggest it will occupy the snRNPs other than the U1 snRNP and hence is less likely to stimulate the immune system.

Mice with a maternalduplication for the central region of chromosome 7 suffer early post-natal lethality (Cattanach et al., 1992). The elevation of SmB protein expression in the brains of these mice is demonstrated by densitometric analysis of Western blots, values being equalised for SmD (SmB value for mice lacking SmN expression=3.2 ; SmB value for age/sex matched control= 3.03; SmN value for control =3.23). This elevation of SmB expression may represent an attempt to compensate for the constitutive RNA splicing functions normally performed in the brain by SmN and the level of expression of SmB in the affected mice is similar to the level of SmN expression in controls. The total amount of SmN plus SmB in normals (6.26) far exceeds the elevated SmB expression in mice with maternal duplications of the central region of chromosome 7 (3.2-values equalised for SmD between samples). This suggests that this compensatory elevation in SmB expression is quantitatively insufficient to cover the total brain requirement for constitutive functions common to SmN and SmB. Furthermore, at the time that the mice show signs of failure,

the normal trend of a reduction in SmB expression during rodent brain development may offset the compensatory elevation of SmB expression to levels below the threshold required for efficient constitutive RNA processing. Alternatively, the fate of these mice may indicate that SmN does indeed possess unique functions and that these functions cannot be replaced by an elevation in SmB expression which occurs to fulfill the constitutive roles of the absent SmN protein.

Analysis of SNRPN expression in cultured skin fibroblasts and lymphoblasts isolated from PWS patients demonstrate that SNRPN is expressed exclusively from the paternal chromosome and that hence, in both UPD and deletion cases of PWS, SNRPN expression is absent (Glenn et al., 1993). The lack of SNRPN expression in deletion cases demonstrates that the imprint that is effective on the SNRPN gene on the maternal allele is not relaxed. Data from analysis of SmN protein expression in the tissues of mice with maternal duplications for the central region of chromosome 7 are consistent with these findings in that they demonstrate that the imprint is still effective and that this also occurs throughout the body as in PWS patients. Therefore the lack of SNRPN expression in PWS suggests that a general deficiency may occur in neuronal mRNA processing. The mild impairment of the nervous system in PWS suggests that the expression of SmB may be elevated in the brains of PWS patients as in murine models of PWS in order to fulfill constitutive mRNA processing functions. In addition, it remains possible that the expression of SmB' in human cells may also compensate for the lack of SmN protein in PWS. Thus the normally low level of SmB' expression in the brain (McAllister et al., 1989; Schmauss et al., 1992) may be elevated in PWS brain tissue. SmB' may fulfill roles normally performed by SmN that cannot be achieved even by elevated SmB expression, and this may at least in part explain why PWS patients suffer impairment whereas murine models of PWS die. However, differences in other genes affected in murine models of PWS and in PWS patients must also be taken into account. Recently, a small deletion (<25kb) just upstream of SNRPN displayed by PWS siblings and a deletion that encompasses part of SNRPN in another PWS patient have been demonstrated to affect expression of both SNRPN and the PAR-1 and PAR-5 genes which are mapped just distal to SNRPN (Sutcliffe et al., 1994; for review see Lalande, 1994). A newly identified 5' α exon in

SNRPN located within the deleted region of the former patients, in addition to other elements within both of these deleted regions are proposed to be important in regulating the expression of the SNRPN, PAR-1, and PAR-5 genes. Thus SNRPN appears to be part of an imprinted transcriptional domain and the transcriptional activity of all three genes (and perhaps other genes in the region) is controlled by elements located within a common region. The maintenance of this imprinted transcriptional domain is likely to require a normal paternal copy of the common control region. Therefore it would appear that other genes are likely to contribute to the PWS phenotype even with small deletions that encompass only SNRPN. If a similar imprinted transcriptional domain is also present in mice, then other genes within the region will not be expressed in PWS mice which may also contribute to the phenotype. It therefore follows that the observed significant increase in SNRPN transcription in certain SLE patients described here (chapter 3), is a result of an aberrant transcriptional mechanism affecting this control region and it remains possible that the transcription of other genes within the region may also be affected in such patients.

FUTURE WORK

To continue this work and to determine a function for the SmN protein, a number of studies could be undertaken. Perhaps the most pressing investigation would be the introduction of an SmN transgene into murine models of PWS with a maternal duplication of the central region of chromosome 7 (Cattanach et al., 1992). If these mice are rescued from their normal fate of early post-natal lethality by the transgene, it would place a critical function on SmN in neural pre-mRNA processing that is not exhibited by SmB and would establish SmN as the causative gene in murine PWS

The differential display technique would be useful to analyse the differences in mRNAs present in brain tissue from mice with maternal duplications of central chromosome 7 compared to sibling controls. Any differences in patterns of mRNA expression between the two may indicate that such mRNAs require SmN for efficient processing. The mRNAs could be identified and the genes studied in greater detail in splicing assays in relationship to SmN expression.

Labelled peptides such as those used in ELISA screening in this study, or fusion proteins which correspond to the regions of least sequence homology between SmN and B/B' could be used to in Far-Western blots to establish whether these regions are important in forming protein-protein interactions with other components of the spliceosome.

In-situ hybridisation studies of SNRPB expression in the brain of mice lacking SmN expression would be useful to deduce whether the elevation in SmB expression in these mice occurs in areas where SmN expression is usually high. This may provide a further insight into putative common constitutive functions of these proteins. A study of the expression pattern of SmB' and SmB in the brain tissue from PWS patients would also be interesting in this respect.

A more extensive study of the association of SmN with snRNP particles other than U1 and U2 and in other cell lines or tissues to those investigated here in addition to a developmental immunoprecipitation study in the brain or on differentiation of EC cells may provide further evidence of the unique snRNP-association properties of SmN. A developmental series of immunoprecipitations from primate brain tissue may give greater insight into the functional relationships of SmN, SmB and SmB' within snRNPs.

The 3T3 parental cells, together with 3T3-MSN and T7S2a cell lines that demonstrate negative, high and intermediate levels of SmN expression respectively provide an excellent basis for the study of the effect of SmN expression on the nuclear localisation of snRNPs as mediated by nuclear localisation signals (NLS) in the Sm core and the m3G cap structure. Thus the effect SmN expression has on nuclear localisation of snRNPs may be studied by microinjection of snRNPs isolated from these cells into Xenopus oocytes. Furthermore, Hypermethylation of the m3G cap could be investigated by chemical modification of reconstituted snRNPs immunoaffinity purified from these cell lines. This may reveal differences between SmN containing snRNPs with respect to SmB/B' containing snRNPs in the context of the putative role of these proteins in providing the binding site for the methyltransferase required for m3G cap formation (Plessel et al., 1994).

Other studies to further the understanding of the role of SmN and SmB/B' proteins as autoantigens in SLE could also be performed. Thus a competitive inhibition ELISA in which total

Sm antigen in liquid-phase is used to compete the SmB/B' 179-190 binding observed in certain SLE patients would determine whether or not this antigen is exposed as a surface epitope on the native SmB/B' proteins. If this region is revealed to be a surface antigen by this assay, this taken together with the lack of autoantibody binding of the same region in SmN would further suggest that SmN is not stimulating the immune system in SLE and that the 179-190 region in SmN is also exposed and hence possibly important in interactions with other proteins. In addition to these studies, FACS analysis of PBMCs using the KSm4 and KSm5 monoclonals would be useful in verifying whether SmN or SmB/B' proteins are ectopically expressed in PBMCs from SLE patients and to which cell populations this expression (if any) is restricted.

CHAPTER 7
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