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Molecular Cloning of Wild Type and Fusion Genes Associated with the t(X;18) Translocation in Synovial Sarcoma

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A thesis submitted in partial fulfilment of the requirements for the degree of Mphil.

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ABSTRACT

Chromosomal translocations have been well studied in haematopoietic tumours, but with respect to solid tumours, consistent translocations have only been observed in sarcomas. Synovial sarcoma is a soft tissue tumour occuring predominantly in the adolescent age group of 10-20.

A key molecular event in the development of this disease is thought to be the t(X;18) translocation involving the SS18 gene on chromosome 18 and the SSX gene family members 1, 2 and occasionally 4 on chromosome X. The aim of this project was to clone cDNA for the fusion genes produced, SS18/SSX1 and SS18/SSX2, and also the full-length wild type genes SSX1, SSX2, and SS18. This bank of clones would then be used for functional studies into the role these genes play in normal and malignant environments.

To achieve this a PCR based cloning strategy was employed. By amplification of the gene of interest using specific primers located in the 5' and 3' flanking regions of the gene, full-length cDNA could be generated for insertion into plasmid. Fully characterised clones were transfected into cell lines to study transcript and protein expression.

During this project sequencing of a SS18/SSX2 clone led to the discovery of an unidentified exon (exon 8) in the SS18 portion of the fusion gene. Analysis of wild type SS18 gene expression indicated mRNA splicing into at least two transcripts in human tissues and four in mice, with varying expression levels throughout the tissue types tested. In melanoma tumour samples over expression of the SS18 transcript lacking exon 8 was observed indicating a possible role for this particular transcript in the tumourigenesis process.

The molecular function of the genes associated with synovial sarcoma is largely unknown, although recent publications point toward a role in transcription control. The clones produced in this study will provide a platform for *in vitro* studies into the function of the wild type and fusion proteins.

I certify that this thesis which I now submit for examination for the award of __MPhil_____, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

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Signature _____ Date _____

Candidate

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1.0 INTRODUCTION.

1.1 Cancer origin and Progression

Observations of human cancers and animal models argue that tumour development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage leads to the progressive conversion of normal human cells into cancer (Foulds 1954, Nowell 1976).

Somatic cells are regulated by multiple controls, and emancipation of a cell from regulation can allow it to grow into a cancer. As there are multiple controls, therefore deregulation requires multiple steps. The minimum requirements for tumour development are; activation of genes that will aid independent cell growth and proliferation and induce immortalisation, loss of genes that block illegal cell division or promote apoptosis, angiogenesis – the supply of blood to the tumour, insensitivity to anti-growth signals, and activation of tumour invasion and metastasis mechanisms (Klein 2000).

All cancers are genetic in that they involve changes in the genetic material that can be categorised into three main groups: sporadic cancers where gene mutations are acquired, they are many and varied accounting for 90-95% of the total, familial (those that cluster in a family but do not seem to be caused by mutation in one gene) and inherited cancers 5-10% where at least one gene mutation is inherited (Saltus 2004).

Variations in genome sequence underlie differences in our susceptibility to protection from all kinds of disease, for example a single base difference in the APOE gene is associated with Alzheimer's. This knowledge of our own uniqueness will alter all aspects of medicine perceptibly and forever, and will allow better-focused individual treatment strategies via genome data especially in relation to drug targeting and toxicity profiling (Chakravarti 2001).

A finished sequence of the Human Genome will form the framework for a new generation of large scale comparisons between cancer cell and normal cell genomes (Venter *et al.*, 2001). Indeed studies carried out in our laboratory have used array technology to assess synovial sarcoma cell lines before and after blockage of the t(X;18) fusion transcript using antisense directed against the breakpoint. This has resulted in the identification of potential downstream targets of the fusion protein (Xie *et al.*, 2002).

An intensive effort in research at the proteome level is now underway worldwide with mass spectrometry, protein arrays, and large scale protein:protein interaction databases being constructed to complement and verify genomic analysis. As the many facets of technology in current scientific research mature and evolve, so to will the current view of cancer providing movement toward more patient specific care. Cytogenetic analysis of human tumours has led to the recognition that distinct chromosome translocations characterise specific tumour types. Until recently this association was of interest mainly as a diagnostic modality, but recent cloning and characterisation of the many genes disrupted by tumour specific translocations has been carried out for haematopoietic (blood cell formation) and solid tumours. These studies are providing important insights into the neoplastic process, and are allowing new therapies to be designed at the molecular level (Sorenson & Triche 1996).

1.2 Translocations in cancer

The study of chromosomal translocations in tumours has revealed much about the molecular biology of cancer. Chromosomal translocations have been well studied in hematopoetic tumours, but with respect to solid tumours, consistant chromosomal translocations have only been observed in sarcomas (Mitelman *et al.*, 1997).

The creation of fusion molecules through gene translocations is probably an early and necessary event in tumour formation in those tumours where such chromosomal abnormalities are present. If the specific fusion proteins have a prognostic value, it implies that they are functionally important and thus potent targets for therapy. Specific chromosomal translocations have been described in several types of solid tumours, where their presence is often the sole cytogenetic anomaly (Ladanyi 1995, Cooper 1996).

The first consistant chromosomal aberration observed in human neoplasia was that of the Philadelphia chromosome in Chronic Myelogenous Leukemia (CML), created by translocation between the long arms of chromosome 9 and 22 (Nowell & Hungerford 1960). The t(9;22) of CML was cloned on the basis of the assignment of genes to the breakpoint regions of translocation chromosomes. The translocation event causes the juxtaposition of the BCR gene located on chromosome 22 and the Abl gene located on chromosome 9, resulting in the production of an aberrant protein (BCR-ABL) capable of causing a chain reaction of events resulting in the cell affected becoming malignant. CML also proved to be the first disease where a specifically designed molecular therapy proved revolutionary in the treatment of patients. Imatinib or STI-571 is a molecule that specifically targets the BCR-ABL fusion protien blocking its action. This molecule is being used in conjunction with traditional chemotherapy to treat CML resulting in a reduction of patient tumour load (Mauro *et al.*, 2002).

Translocations can pin-point oncogenes, that can have one of two effects,

1. They lead to deregulation caused by over expression of an oncogene by juxtaposing it to an enhancer or promoter sequence that is active, for example parathyroid adenomas, caused by inversion between between the long and short arms of chromosome eleven. The result of this is the juxtapositioning of the parathyoid hormone regulatory elements and PRAD 1 putative oncogene causing over-expression of PRAD1. Also Burkitts lymphoma has been shown to involve a translocation of the proto-oncogene c-myc to the Ig heavy chain, λ or κ enhancer regions, which are the sites of intense transcriptional activity in B lymphocytes (Solomon *et al.*, 1991). This translocation leads to the over-expression of the c-myc gene, and is one of the factors of transformation.

2. Formation of a translocated gene which codes for a chimaeric protein whose transforming abilities are drawn from both partners. For example, in CML the BCR - ABL fusion occurs where BCR normally encodes a GTPase activating protein for P21, and ABL encodes a tyrosine kinase whose activity is unmasked by the BCR sequence.

The involvement of transcription factors at translocation breakpoints is a recurring theme (Rabbitts 1998). For example, in pre-B Acute Lymphocytic Leukaemia (ALL) a fusion protein results from the translocation t(1;19)(q23;p13). This fuses the E2A on chromosome 19, which encodes the Ig enhancer binding proteins E12 and E47, with the homeobox PBX gene on chromosome 1 (Kamps *et al.*,1990). The translocation switches the DNA binding domain of E2A with that of PBX, thus placing those genes usually regulated by PBX under the trans-activational control of E2A. Futhermore, because PBX is not normally transcribed in pre-B cells, the translocation results in ectopic expression of the PBX DNA binding domain.

In relation to solid tumours Ewing's sarcoma is used as a molecular model for oncogenesis. This tumour type is part of the pPNET group of childhood tumours, including Askins tumour and peripheral neuroepithelioma. Ewing's sarcoma is characterised by several translocations all of which involve the EWS gene from chromosome 22 and one of several members of the ETS gene family, namely: ERG, ETV-1, and FLI-1, which are located on chromosomes 21, 7, and 11 respectively. The EWS portion of the chimaeric protein contributes transcription-activating properties, whereas the ETS member maintains a domain with specific DNA binding capacity. EWS-FLI 1 has been shown to transform NIH 3T3 cells efficiently, confirming its oncogenic potential (May *et al.*, 1993). This finding lends significance to the hypothesis that a single translocation may be the key molecular event in development of a tumour type (May *et al.*, 1993, Ohno & Reddy 1993).

The development, maintenance and progression of cancer involves alterations in the expression of a number of genes as well as chromosomal mutations. RNA splicing studies have shown that even a small change in the coding region of mRNA can lead to a substantial switch in protein function (Gilbert 1978). Now that the Human Genome sequence is essentially complete, a greater recognition of the importance of alternative splicing is emerging. As the number of known proteins greatly exceeds the estimated number of genes, it is believed that alternative splicing provides the major mechanism for increasing proteome diversity.

Alternative splicing is also a versatile form of genetic control whereby a common pre-mRNA is processed into multiple mRNA isoforms differing in their precise combinations of exon sequences. Alternative splicing offers signal amplification as a regulatory mechanism (Mercatante & Kole 2000).

For example, a small increase in the concentration of one splice variant, by definition, leads to a concomitant decrease in the concentration of its counterparts and to a major change in the ratio of the products (Xu & Lee 2003). Although the process of alternative splicing is believed to be relatively common, the exact mechanism, which determines the isoform and quantity of the protein product is still relatively unknown.

Many cancer associated genes are alternatively spliced, and there is convincing evidence that in several cancers, the ratio of splice variants is significantly altered. Although the functions of most of these variants are not well defined, many display antagonistic properties related to cell suicide mechanisms. In a number of cancers and cancer cell lines, the ratio of splice variants is frequently shifted so that the anti-apoptotic splice variant predominates. An example is BCL-2 one of the key genes involved in apoptosis, containing 2 exons where the full-length isoform BCL-2 alpha is the predominant isoform and exhibits anti apoptotic properties in a number of cells. Over expression of this isoform, found in many types of cancer such as follicular lymphomas and gastric carcinomas, seems to contribute to chemotherapy resistance and poor prognosis. The truncated isoform, Bcl-2 beta contains only one exon and displays little or no anti apoptotic activity and is associated with better survival rates. Therefore shifts in splice variant ratios for any protein engaged in a process required for tumourigenesis may result in a gain of function for the pre neoplastic cell. (Adams & Cory 2001).

1.3 Synovial Sarcoma

Synovial sarcoma is a soft tissue tumour occuring predominantly in the adolescent age group of 10-20, but can occur at any age (Miser 1997). It accounts for 5-10% of all soft tissue sarcomas and is commonly located in the extremities, in the vicinity of large joints.

Synovial sarcoma is considered a high-grade sarcoma with 5 year and 10 year survival rates of ~50% and 20% respectively (Erzinger & Weiss 1995, Lopes *et al.*, 1998). Tumours typically arise in the lower and upper extremities, particularly in the para-articular regions, often, in close proximity with tendon sheaths, bursae and joint capsules.

The designation of synovial sarcoma is considered a misnomer as these tumours bear no biological or pathological relationship to synovium (Smith *et al.*, 1995). It is generally accepted that synovial sarcomas are derived from as yet unknown multipotent stem cells that are capable of differentiating into mesenchymal and/or epithelial structures. Metastases of synovial sarcoma is primarily to the lungs.

Morphologically synovial sarcoma is characterised by two different cell types, namely epitheloid and spindle cells. These occur in different proportions and therefore there is a subtype classification. The biphasic type, which consists of both epitheloid cells and spindle cells, and monophasic which consists of the spindle cells only.
Poorly differentiated synovial sarcomas contain small spindle shaped cells, which seem to be intermediate in appearance between epithelial and spindle cells.

Cytogenetic studies of synovial sarcomas has revealed a characteristic translocation between chromosome 18 and chromosome X t(X;18) (p11;q11.2) which is present in >90% of both biphasic and monophasic tumours (Turc-Carel *et al.*, 1986, Limon *et al.*, 1986).

The discovery of this translocation which is so far specific to synovial sarcoma, results from the fusion of two novel genes. The presence of this translocation as the sole cytogenetic abnormality in some primary tumours indicated that its formation was a key molecular event in synovial sarcoma development.

Fish analysis using X chromosome specific YAC probes has allowed the identification of 2 mutually exclusive Xp11.2 breakpoints in synovial sarcoma cases. One of the breakpoints maps to within the ornithine aminotransferase (OAT) pseudogene cluster L1, while the second breakpoint maps to the vicinity of the related OAT-L2 cluster (Knight et al., 1992).

These YAC's were used to isolate cDNA clones corresponding to both genes involved in the translocation t(X;18) from a cDNA library prepared using RNA from a synovial sarcoma cell line (Clark *et al.,* 1994).

In different t(X;18) bearing tumours, the breakpoints may occur at distinct sites within the band Xp11.2 giving rise to two forms of the fusion gene namely, SS18-SSX1 and SS18-SSX2 (Clark *et al.,* 1994). The molecular analysis of the t(X;18) breakpoint has shown that the proximal portion of the SS18 gene from chromosome 18 is disrupted and juxtaposed to the distal portion of either SSX1, SSX2, SSX4, genes on the X chromosome, in a mutually exclusive fashion. Also of note is the observation that the der18 portion of the translocation has been lost during oncogenesis, indicating that only the derX portion is necessary for development of synovial sarcoma (Smith *et al.,* 1987, Gilgenkrantz *et al.,* 1990).

It has been suggested that the occurrence of these different breakpoints correlates with a histologic phenotype displayed by the tumours, i.e. monophasic or biphasic, (de Leeuw *et al.,* 1993) but this argument has been refuted also (Crew *et al.,*1995). There is however, strong evidence to support the suggestion for a biological difference between SS18/SSX1 and SS18/SSX2 on a morphological and prognostic basis, where the SS18/SSX1 variant is associated with a high rate of proliferation and poor clinical outcome (Nilsson *et al.,* 1999 Kawai *et al.,* 1998). Although SS18/SSX4 translocations have been discovered they are too rare to assess regarding prognosis to date (Skytting *et al.,*1999).

To try to understand the characteristics and properties of the fusion genes involved in the t(X;18), analysis of the novel wild type genes involved in conjunction with studies on fusion genes was undertaken. The t(X;18) translocation results in the fusion of the SS18 gene from chromosome 18 to either the SSX1 or the SSX2 from chromosome X, which produces a fusion protein product where the 8 amino acids at the C-terminal of the normal SS18 proteins are replaced by 78 C-terminal amino acids of the SSX protein (Clark *et al.,* 1994).

1.4 SS18

The synovial sarcoma translocation gene SS18 is 3071 base pair in length, with an open reading frame of 387 amino acids. The protein encoded by this gene has to date failed to exhibit homology to any other known human protein using data base analysis. (Clark *et al.*, 1994, de Leeuw *et al.*, 1994, Crew *et al.*, 1995).

Examination of the domain structure of SS18 revealed 3 regions within the protein that have putative significance. The first is the SNH domain from amino acids 20-73. This domain is widely conserved and identifies with predicted protein sequences from fish, nematode, and plant. In the C-terminal domain of SS18 lies a transcriptional activation domain. This sequence is composed predominantly of Glutamine (19%), Proline (14%), and Glycine (12%), with Tyrosine residues occurring at variable intervals. This is known as the QPGY domain, and is similar to the N-terminal activating domain of the EWS/FUS/TLS family of proteins which contain (S/G)YQQ(S/Q) repeats, (Theate *et al.*, 1999) and to the Brahma transactivating protein found in Drosophilae (Tamkun *et al.*, 1992).

Two indirect annexin-like repeats were also identified in the SS18 protein sequence, which may help to form beta sheets and have a role in protein:protein interactions (De Bruijn *et al.*, 1996).

The SS18 protein has 2 consensus sequences for protein phosphorylation at amino acids 39-62 and 1405-1413 for tyrosine kinase and protein kinase C respectively.

No known DNA binding domains have been identified in SS18, lending further weight to a role in protein:protein interactions. In accordance with this hypothesis, three SH2 domains and one SH3 domain have been identified. In the SS18/SSX fusion genes the C-terminal SH2 domain of SS18 is lost.

The diagrammatic representation of SS18 below highlights the major domains of SS18 and the Sarc Homology (SH) domains known to interact with other proteins.

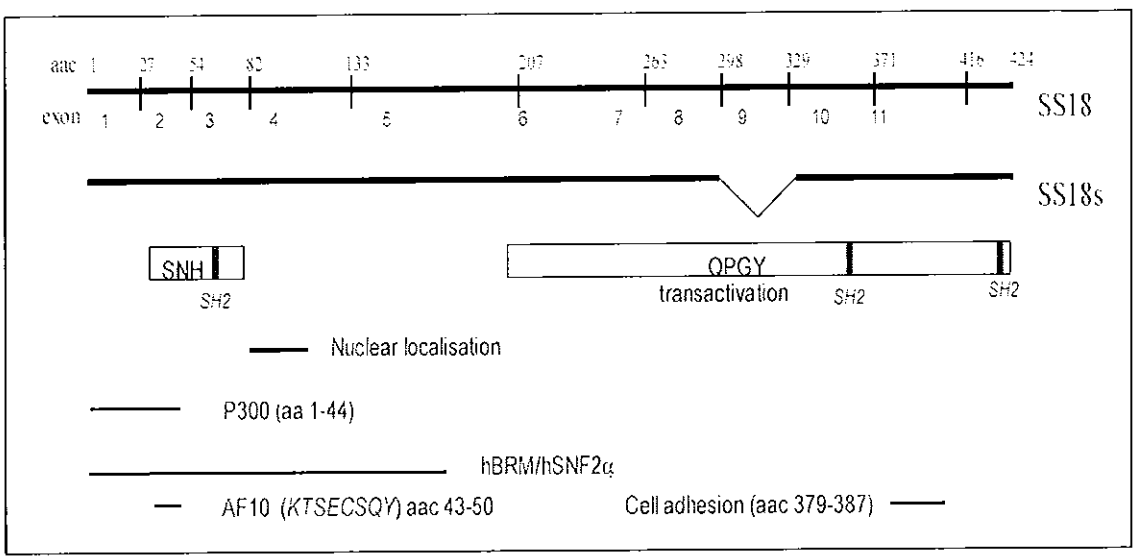


Fig1.1 Diagrammatic representation of SS18.

A role for SS18 in transcriptional activation has been supported by the finding that a seventy fold activation of a heterologous promoter was induced by SS18 in a GAL4 assay. Using deletion constructs, the activating domain was delineated to the C-terminal region of SS18 (QPGY domain). Also removal of the SNH domain alone showed a 30 fold increase in activation over that of full-length SS18. This suggests a putative inhibitory role for the SNH domain. Recently two human SS18 paralogs have also been identified, in humans, which encode proteins with a conserved SNH domain (Theate *et al.*, 1999).

The transcriptional role of SS18 is further supported by immunofluoresecence experiments using polyclonal antibody raised against SS18, which show that SS18 localises to the nucleus in a speckled or punctate distribution pattern (Brett *et al.*, 1997, dos Santos *et al.*, 2000a).

A mouse ortholog for the SS18 protein has been indentified which has a sequence homology of approx 90% identity at DNA level. This group extracted a full-length clone of 3.1kb and found the presence of a 93 base pair insert located within the QPGY region when comparing the sequence with that of the known SS18 (de Bruijn et al., 1996).

Upon comparison of the mouse and human cDNA sequences the putative start of the human SS18 coincides exactly with the first ATG present in the mouse, at position 179. Before this point homology is 70%. Mouse SS18, like the human form was found to be rich in glutamine, proline, and glycine. The C-terminal tyrosines in both human and mouse SS18 homologues are totally conserved as are most of the glutamines. Also it has been shown that the SS18 protein, in both human and mouse possesses putative SH2 and SH3 domains which are well conserved over evolution and strengthen the hypothesis for protein:protein interactions involving the human SS18. Murine SS18 was also found on chromosome 18B1- syntenic with the human chromosome 18 region q11-q12.

Research carried out in this lab and by others recently reported the presence of two distinct splice variants of SS18 (Tamborini *et al.*, 2001, Brodin *et al.*, 2001). Both groups detected a 93 base pair insertion into the SS18 open reading frame at position 896, which is located in the QPGY domain between exons 8 & 9. This insert proved to be identical to the insert found in the mouse ortholog of SS18 previously mentioned. The corresponding 31 amino acid residues which the insert codes for is consistent with its surroundings in that it is rich in proline, glutamine, glycine and is heavy with tyrosine at 21%. As this region (QPGY) has been shown to be important in the transactivating properties of SS18, the presence of the insert is likely to be of functional significance also.

Considering that the insert was in-frame, identical to the mouse insert identified, contained the intron/exon donor and acceptor splice site consensus sequences, and has been shown to be present on chromosome 18 at q11.2 at the site of the SS18 human genomic sequence the insert appears to be a true exon (de Bruijn et al., 2001). Thus SS18 mRNA appears to undergo two alternative splicing events.

Research into the expression of wild type SS18 across a wide variety of mouse and human tissues revealed that SS18 appears to be ubiquitously expressed in all tissues and in both spliced and un-spliced form (Tamborini *et al.*, 2001, Brodin *et al.*, 2001). At the genomic level the SS18 gene promoter resembles those of housekeeping genes in that they are TATA-less and embedded in canonical CpG islands, which may account for its ubiquitous expression. Also upon checking for the presence of the insert in the t(X;18) translocated SS18, we found that the alternate splicing mechanism was maintained and that two forms of the SS18/SSX fusion transcripts could be identified.

Research into the tumourigenic aspects of synovial sarcoma development has moved from cytogenetic techniques in order to identify the genes involved in the t(X;18) translocation, to more molecular and biochemistry based techniques in order to establish the functional aspects of these proteins. As a result of this there has been a number of recent publications where several molecules have been identified as associating with SS18. The detailed study of Eid *et al.*, (2000) identified SS18 via its complex with P300 at G1 in confluent cells but not in sparse or S phase cells. Also the P300/SS18 complex was found to be preserved among mammals and therefore throughout evolution.

Below is a diagrammatic representation of the P300 protein structure.

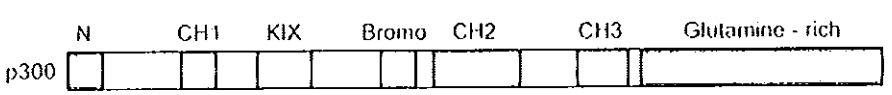


Fig 1.2 Schematic highlighting P300 protein structure

Adhesion of a cell to its extra cellular matrix and its ability to communicate with its environment are fundamental properties integral to proliferation control, and cellular homeostasis (Keely et al., 1998, Rouslahti 1999) A deletion construct with the last 8 amino acids of SS18 removed resulted in a 79% decrease in adhesion while still preserving P300 association.

As this same portion of SS18 contains a SH2 domain and is lost in the t(X;18) translocation in synovial sarcoma, this result may shed some light on the cause of oncogenicity in the fusion protein and suggests a possible dominant negative effect of the fusion gene on cell adhesion.

P300 is known to bind to many proteins involved in transcriptional activation and cell cycle processes including nuclear hormone receptors, basal transcriptional activity machinery, and SS18. Although it is generally thought that P300/CBP are transcriptional activators P300 has been shown to act as a repressor as well, through its reversible association with yy1 (Yao *et al.*, 2001). Also of interest is that several factors compete for binding sites on P300, for example transcription factors STAT2 and RelA both recognise the same structural motif of P300 at CH1 (cysteine/histidine rich zinc finger motifs) (Giles *et al.* 1998). SS18 partially binds to P300 at this region and also at CH3 as discussed above. Thus, SS18 may be competitively vying for access to target bind sites, a function that would be disrupted by the translocation event.
As SS18 is known to localise in distinct speckles in the nucleus, studies were performed in order to determine whether it co-localised with other transcription factors with a known speckled distribution (Theate *et al.*, 1999) It was found that SS18 and SS18/SSX co-localise and associate with BRM a member of large protein multimers known as SWI/SNF complexes (Theate *et al.*, 1999, Nagai *et al.*, 2001). These complexes function by disrupting nucleosome structure at a promoter and facilitating the binding of transcription factors to their cognate sites. Therefore SS18 and SSX have been shown to be indirectly associated with transcription activators in the cell.

1.5 SSX.

The SSX genes are a multigene family with 9 different members, SSX1- SSX9, (Gure *et al.*, 1997, & 2002) all of which map to the X chromosome at band p11.2. To date, only SSX1, SSX2 and very recently SSX4 have been shown to take part in the t(X;18) translocation of synovial sarcoma. SSX has a very restricted expression pattern and has only been found expressed in testis and in very low amounts in thyroid tissue (Crew *et al.*, 1995, Gure *et al.*, 1997, Tureci *et al.*, 1998) in contrast to SS18 which appears to be

ubiquitously expressed. SSX genes consist of 6 exons, which make up a 1.6kb transcript coding for 188 amino acids. Homology between individual SSX genes ranges between 88-95%. Approximatey 40% of the amino acids are charged, with the C-terminal tail containing predominantly acidic amino acids.

Little is known about the biological function of SSX gene products, but some characteristics of the proteins have been uncovered. For example they have been shown to possess repressional activity in two separate domains, the Kruppel Associated Box like domain (KRAB) and the SSX repressional domain (SSXRD) (Crew *et al.*, 1995, Lim *et al* 1998). KRAB domains are known protein domains capable of transcription repression (Witzgall et al., 1994). SSX1 and SSX2 encode closely related proteins of 188 amino acids in length, which share 83% homology at that level. The predicted amino acid sequence is rich in amino acids arginine and lysine, and both SSX1 and SSX2 contain consensus sequences for N-glycosylation and tyrosine phosphorylation (Clark et al.,1994). Three putative nuclear localisation sequences (NLS) have been also been reported. SSX proteins have been observed to localise to the nucleus where they appear to be evenly distributed in a diffuse pattern, which contrasts the speckled distribution of SS18 (Brett *et al.*1997, dos Santos *et al.*, 2000a).

The main region of divergence between SSX genes is located toward the C-terminal portion of the genes in exon 5. This is known as the divergent domain (DD), and it has been speculated that this domain may be of significance tumourigenically, in that it may account for the difference in prognosis and aggression between SS18/SSX1 and SS18/SSX2 carrying synovial sarcoma tumours (Skytting *et al.*,1999). Diagrammatic representation of SSX gene illustrating major domains is shown in Figure 1.3

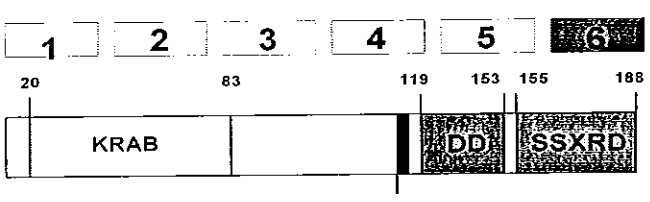


Fig 1.3 Diagrammatic representation of the SSX gene, showing the major domains

A central goal of tumour immunology has been the identification of tumour antigens that can elicit a tumour rejection in the autologous host. Identification of SSX2 (HOM-MEL-40) as a tumour antigen that can elicit an Ig G antibody response in tumour patients has resulted in its classification as a CT or cancer testis antigen. I.e. This gene is only expressed in tumour tissues and normal testis. SSX gene expression in human neoplasms showed SSX3 is least expressed whereas SSX1 is the most frequently expressed (Ayyoub *et al.*, 2003a).

The restricted expression of SSX genes has suggested them as promising agents for immuno and gene therapies with Ayyoub *et al.*, (2003b) showing CD8+ T cell recognition of HLA-A2+ SSX-2+ sarcoma cells that were efficiently recognized and lysed by SSX-2-specific CTLs. This indicates SSX antigens are relevant targets for the development of vaccine-based immunotherapy of sarcoma. Aberrant CT expression is found in a range of different tumour types and in various proportions. (Boel *et al.*, 1995) The significance of this selective expression of CT antigens is unknown. It should be noted that proof of SSX genes as CT antigens has so far only been shown for SSX2 (Tureci *et al.*,1998)

The methylation of DNA is an epigenetic modification that can play an important role in the control of gene expression in mammalian cells. In different types of tumours, aberrant or accidental methylation of CpG islands in the promoter region has been observed for many cancer related genes resulting in the silencing of their expression. In contrast, hypomethylation of the promoter region can result in the ectopic expression of otherwise silent genes (Momparler & Bovenzi 2000).
The aberrant expression of SSX genes in a variety of human tumours suggests that these genes are privy to a mechanism of escaping silencing in the cells. In synovial sarcoma, it is the translocation which allows the SSX genes to be expressed, and for cells in which the SSX genes are expressed by means other than translocation, demethylation has been suggested as a possible candidate for the mechanism of action (Tureci *et al.*,1998).

Recently, using co-localisation experiments Soulez *et al.*, (1999) showed that SSX and SS18/SSX but not SS18 co-localised with Polycomb group proteins Ring-1 and BMI-1 near

centromeres in interphase nuclei. PcG complexes are pericentromeric heterochromatin associated multiprotein complexes, involved in the stable repression of homeotic gene expression in Drosophila (Saurin *et al.*,1998) Thus, there appears to be gathering data suggesting that both SSX and SS18 proteins associate with several multimeric complexes involved in transcription.

1.6 SS18/SSX

Characterisation of translocations has resulted in the discovery of several types of molecular abnormalities thought to contribute to tumourigenesis. Analysis of the t(X;18) has shown that there is heterogeneity in the position of the chromosome translocation point, resulting in the different forms of the fusion protein i.e. SS18-SSX1, SS18-SSX2, (Clark *et al.*, 1994 Crew *et al.*, 1995) and more recently in some rare cases SS18-SSX4 (Skytting et al., 1999).

Both SS18-SSX1 and SS18-SSX2 encode fusion proteins where the C-terminal 8 amino acids of normal SS18 are replaced by 78 amino acids encoded by the SSX genes.
In genomic terms this corresponds to exon 11 (coding for only the last eight amino acids) of SS18 being lost and replaced in-frame by exons 5 and 6 of SSX. Thus, the actual breakpoint itself, unique for each individual clone, is located within intron 4 of SSX between exons 4 & 5.

As the ratio of SS18/SSX1 fusions to that of SS18/SSX2 is roughly 2:1, analysis of SSX1 and SSX2 intron 4 sequence for an insight into differences in ratio of fusion genes was carried out but yielded no significant data. Both introns were 1985 and 1983 base pair respectively with no differences in repetitive elements, ruling out a simple stochastic model of breakpoint occurrence (Ladanyi 2001).

This general rule holds true for the vast majority of SS18/SSX fusions detected, but several variants have been described which contain deletions or insertions of SS18 or SSX sequence (dos Santos *et al.*, 2001).

The SS18-SSX transcripts are encoded by the (X) chromosome formed in the t(X;18) and not chromosome 18 as 5'-SSX-SS18-3' transcripts in synovial sarcomas could not be detected. This suggests that the derX fusion in synovial sarcoma cells is the key molecular event in the development of synovial sarcoma as it is the only derivative which persists throughout development of the disease.

To try to determine functions of the SS18, SSX, and SS18-SSX fusion protein, nuclear localisation studies were undertaken by several groups. Using immunofluorescence techniques, upon cells transiently transfected with SS18-SSX/green flourescent protein (GFP) fusions, FLAG-tagged or untagged SS18, SSX, and the SS18-SSX fusion proteins it was shown, that SS18-SSX proteins localised in a punctate pattern throughout the nucleoplasm excluding the nucleoli much the same way as SS18 proteins did, whereas SSX proteins stained diffusely throughout the nucleus also excluding the nucleoli (Brett *et al.*, 1997, Theate *et al.*, 1999, Soulez *et al.*, 1999, dos Santos *et al.*, 2000a).

Double immunofluorescence assays using monoclonal antibodies (mAb) against known nuclear bodies for example SC35 splicing factor and PML bodies, showed no consistant co-localisation with these factors. Assessment of SS18 co-localisation against BRM, a human homologue of yeast SNF2 protein which has the same type of distribution pattern as SS18. provided evidence that GFP-SS18 and GFP-SS18/SSX proteins co-localise with BRM (Theate *et al.*, 1999).

Using deletion constructs the methionine rich (16%) central region of the SS18 protein between the SNH and the C-terminal was found to play a strong role in co-localisation with BRM suggesting that this area, which has 96% homology with mouse SS18, is the area where the BRM associates with the protein. This observation was later confirmed at the protein level by immunoprecipitation of hBRM using an anti-SSX antibody in 3Y1 rat fibroblast cells transfected with SS18/SSX1 and also in an established synovial sarcoma cell line HS-SY-II. (Nagai *et al.*, 2001). Taken together these results suggest that the binding region of SS18 to hBRM can be delineated to amino acids 73-181.

Also observed was that SS18/SSX1 stably transfected 3Y1 cells had a promoted growth rate in culture, were anchorage independent, formed colonies in soft agar, and tumours in nude mice. The transforming ability of the transfected cells was low compared to ras or src as 50 times higher concentrations of SS18/SSX expressing cells were required for transformation than ras or src.

In contrast to published reports (Brett *et al.*, 1997, Theate *et al.*, 1999, dos Santos *et al.*, 2000a), Soulez *et al.*, (1999) through their own co-localisational studies put forward the model that it is not the SS18, but the SSX portion of the t(X;18) chimaeric protein that is responsible for its location in the nucleus.

Through use of 2C4 cells which had previously been characterised for nuclear antigens by the same group, Saurin *et al.*,. (1998) showed immunolabelling of two components of polycomb group bodies namely, BMI-1 and RING-1 showed that the signal they produced overlaps with that of the SS18-SSX2 protein. This suggested that SS18-SSX2 proteins accumulate with polycomb group proteins (PcG's) in the vicinity of centromere at interphase. No association of SS18 and BMI-1 was found but SSX1 and SSX2 were seen to co-localise with BMI-1. Also as PcG distribution is heterogeneous in different cell lines it may explain the diffuse staining for SSX2 which has been reported in other studies (Soulez *et al.*, 1999).

The prognosis for metastasis free survival in synovial sarcoma cases carrying the SS18-SSX1 gene is less than that of those cases carrying the SS18/SSX2 translocation, with the overall rate at 5 years being ~ 55% (Kawai *et al.*, 1998, Nilsson *et al.*, 1999). This suggests that base pair differences between the different fusion gene products is biologically significant.

Chromosomal translocations are in tumours at presentation because they contribute to the tumour phenotype and therefore because there is a selective advantage to the cell which acquires the aberrant chromosome (Rabbitts TH. 1999).

In synovial sarcoma the frequency of aberrations per tumour are higher for the biphasic than the monophasic form. Some variants of the standard t(X;18) have been observed which are indicative of clonal evolution. Typical in recurrent or metastatic tumours is the development of an increase in secondary aberrations compared to primary tumours. The appearance of secondary changes may therefore reflect the genetic instability of sarcoma cells rather than exposure to clastogenic agents (Mandahl 1995).

Significantly over represented in large tumours are gains to the whole, or part of the long arm of chromosome eight, suggesting that tumours with this genetic abnormality have an increased growth rate. It is known that tumour size reflects a more severe prognosis in synovial sarcoma and is the most significant influence on stage and hence survival. In accordance with this, secondary aberrations are also associated with tumour size (Skytting *et al.*, 1999, Bergh *et al.*, 1999, Spillane *et al.*, 2000).

1.7 Conclusion.

From the information available on SS18, SSX and the SS18/SSX fusions to date, there seems to be an emerging picture with regard to the role of these genes in the normal and tumourigenic cell. Their association with several different large proteins and complexes involved in regulation of gene expression via nucleosome manipulation lends weight to the hypothesis for SS18 and SSX activity to be centralised around transcription co-activation and co-repression respectively.

Several models for SS18/SSX function have been proposed to date.
Dos Santos *et al* 2000a., have provided evidence that the SSXRD contains the dominant targeting signal over that of the SS18 amino terminal portion, and also suggest that PcG repression is therefore most likely dominant over that of SWI/SNF activation. Therefore the primary sub-nuclear compartment affected by the SS18/SSX fusion may be that normally targeted by native SSX in germ cells. In tumour cells then, we would have PcG mediated repression orchestrated through the SSXRD and other as yet unknown protein:protein interactions between SSX and other DNA binding transcription factors.

Thus, the normal precursor cell to synovial sarcoma may lack SSX expression (as it appears to be very restricted) and could result in the unknown target genes of SSX being expressed. After the translocation event, these genes may then be repressed by the SS18/SSX fusion.

Another model also put forward by dos Santos suggests that the SS18/SSX re-directs the dominant SSXRD to repress genes recognised and activated by the SS18 protein:protein:DNA interaction. Nagai et al have suggested that the SS18/SSX may disturb the function of BRM, which may be bound to SS18 thus leading to a transforming phenotype, based on the evidence that BRM binds to the SS18/SSX1 fusion gene.

There are no definitive answers yet for the oncogenic mechanism underlying development of synovial sarcoma, but as research into this field continues, the functional picture will continue to sharpen leading to clearer knowledge of the roles played by SS18/SSX and SS18/SSX in both normal and tumourigenic cells. It is hoped that this knowledge will clear a path for therapeutic applications and better patient care.

The aim of this project was to recombinantly clone the cDNA of genes involved in synovial sarcoma for future use in characterisation and functional studies A key molecular event in the development of this disease is thought to be the t(X;18) translocation involving the SS18 gene on chromosome 18 and the SSX gene family members 1, 2 and occasionally 4 on chromosome X.

The fusion genes produced, SS18/SSX1, SS18/SSX2 and rarely SS18/SSX4, and wild type forms of the genes involved namely SS18, SSX1 and SSX2 were the targets of this project.

The initial scope of the project was extended after identification of a splice variant of the SS18 gene to include preliminary analysis of variant wild type expression in normal and malignant tissues and incorporation of the variant transcript into the fusion gene mRNA.

2.0 MATERIALS AND METHODS.

METHODOLOGY FLOW CHART

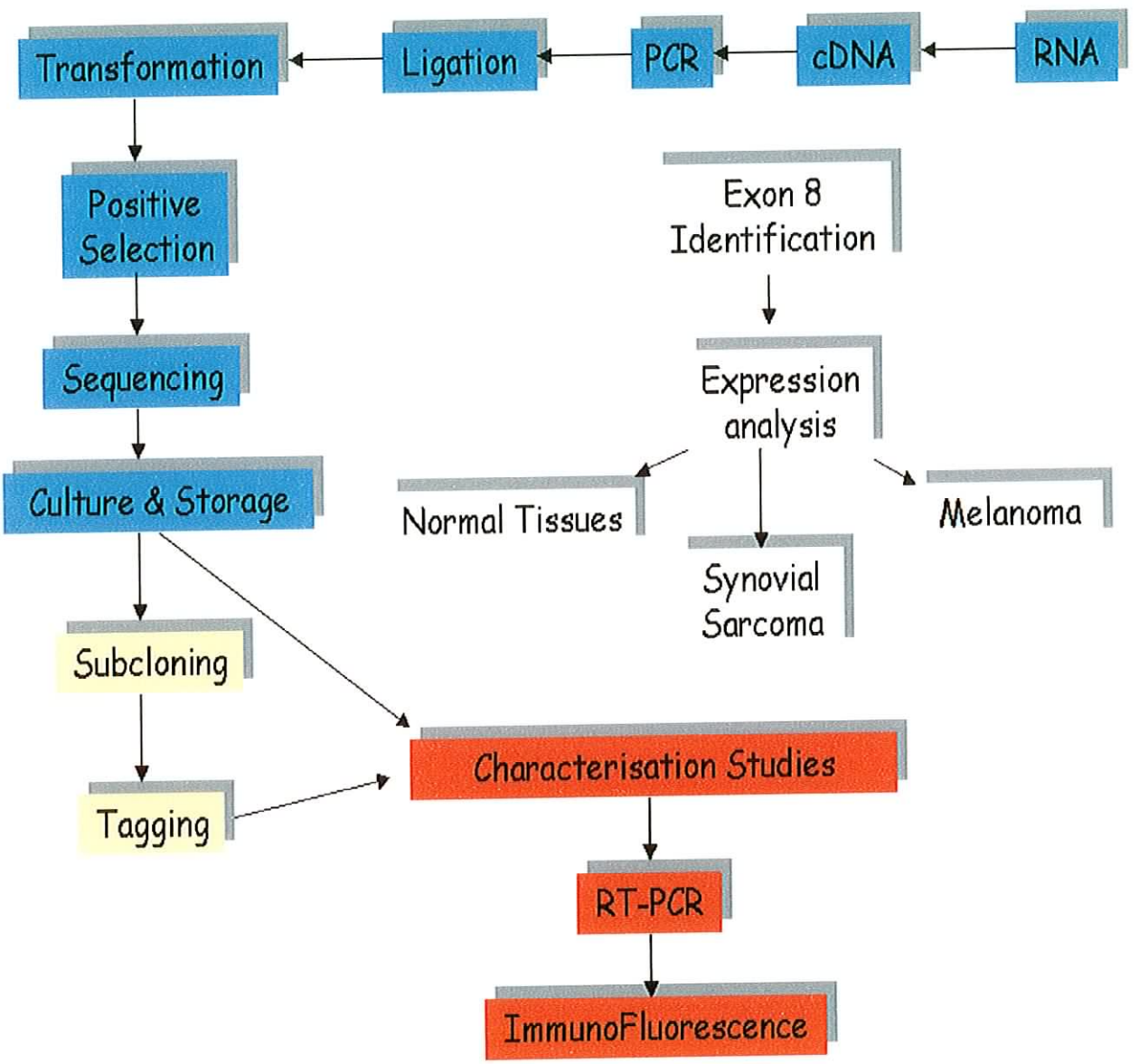


Fig 1.4 Flowchart highlighting overall methodology used throughout project

2.1 Qualitative agarose gel preparation for RNA integrity.

A 1% w/v agarose gel, (Gibco BRL lot# 3024474) buffered to 1X with TAE and stained with ethidium bromide at a concentration of 0.5µg/ml was prepared using RNase free water. 4µl of total RNA in 2µl of loading buffer was electrophoresed on the gel at 120 volts for 15 mins, and the gel result was visualised on an auto analyser (Biorad Multi Analyst).

2.1.1 RNA extraction Protocol.

For the tumour samples a sterile petri dish and scalpel was used to slice the tumour ($0.3cm^2$) to a fine mixture in the presence of 600µl RLT lysis buffer. The entire mixture was then placed into the Qiashredder column and the protocol followed as normal.

For cell lines 600µl RLT buffer was added to a pellet containing $1x10^7$ cells and protocol followed as normal.

The entire volume was pipetted onto the lilac QIAshredder column sitting in a 2ml collection tube. Samples were centrifuged at maximum speed and 4°C, for 2 minutes

Column was discarded and 1 volume of chilled 70% ethanol was added to the homogenised lysate and mixed by pipetting.

700µl of each sample was applied to the pink RNeasy column sitting in a 2ml collection tube. Samples were centrifuged at maximum speed and 4°C, for 15 seconds. The remainder of the sample was loaded and the centrifuge step repeated Flow through was discarded and tubes blotted on paper.

350µl of buffer RW1 was added onto the RNeasy column and centrifuged at maximum speed for 15 seconds at 4°C.

The flow-through was discarded and tubes blotted on paper towels.

80µl of DNase/Buffer RDD mix was pipetted onto the membrane of each column, and incubated at room temperature for 15mins.

350µl of buffer RW1 was pipetted onto the RNeasy column and centrifuged at maximum speed and 4°C, for 15 seconds. RNeasy column was transferred to a new 2ml collection tube. 500µl of buffer RPE was added onto the column, and samples were centrifuged at maximum speed and 15 seconds at 4°C.

The flow-through was again discarded into the waste bottle and tubes blotted on paper towels. 500µl of buffer RPE was added onto the RNeasy column, and centrifuged at maximum speed and 4°C, for 2 minutes to dry the membrane.
RNeasy column was transferred to a 1.5ml collection tube and 60µl of chilled RNase-free water was pipetted directly onto the column membrane.
Centrifugation at maximum speed and 4°C, for 1 minute caused the RNA to elute and samples were stored at -70°C.

2.2 Amplification of full-length cDNA.

2.2.1 Reverse Transcription

The RT reaction was carried out using the Enhanced Avian RT-PCR kit supplied by Sigma (prod. No. RT-PCR-20). The two-step protocol was used to maximise high yield and high fidelity. To a 200µl micro centrifuge tube the following was added; 5µl of RNA template at ~ 0.25 µg/µl, 1µl dNTP's at 500µm each dNTP, 2µl Primer (SSX-A) at 10pmol/µl to a total volume of 16.5µl. The contents are briefly centrifuged before being placed in a thermal cycler at 70°C for 10mins. The tubes are then removed, placed on ice, and re-centrifuged before the remaining components of the reaction are added. To complete the reaction mix, 2µl 10X buffer for AMV-RT at 1X final concentration is added along with 1µl Enhanced Avian RT at 1U/µl, and 0.5µl RNase inhibitor at 1U/µl bringing the total reaction volume to 20µl. The tubes are incubated at 42°C for 50 minutes.

2.2.2 Polymerase Chain Reaction.

To a 500µl microcentrifuge tube the following reagents were added; 5µl 10X Accu Taq buffer to final concentration of 1X, 1µl DMSO at 2% v/v,1µl dNTP's at 200µm each dNTP, 5µl of template cDNA 1.25µg/5µl, 1µl primers at 10pm/µl, 0.5µl Accu Taq LA DNA polymerase at 0.05U/µl, 0.25µl Taq Gold at 0,025U/µl, water to a final volume of 50µl. The contents were centrifuged for 20 seconds at 2000rpm and the following cycling parameters are followed, Initial denaturation at 95°C for 2mins, cycling denaturation at 98°C for 20secs, annealing/extension at 68°C for 15mins. Steps 2 and 3 for 1 repeat cycle.

Then: denaturation at 98°C for 20 secs, annealing at 50°C for 1 min, extension at 68°C for 5 mins, cycle steps 5-7 25 times. Final extension at 68°C for 7 mins.
The above reaction and all others were run on a MJ research PCR machine.

2.2.3 Gel electrophoresis for visualisation of PCR/column-prep product.

A 1% agarose gel was prepared with Millipore quality water. 4µl of the PCR/prep product was loaded onto the gel in 2µl of loading buffer and run at 100 Volts for 30-40 minutes. Viewing of the stained gel was the same procedure as in methods 2.1.

2.2.4 Purification of PCR product.

Purification was carried out using Promega PCR Preps DNA Purification System (Cat.# A7170). For each completed PCR amplification the aqueous phase was transferred to a clean microcentrifuge tube. 100µl of Direct Purification Buffer was aliquoted into a 1.5ml tube, and 35µl of PCR product was added. The tubes contents were briefly vortexed before the addition of 1ml of resin. The mixture was then vortexed 3 times over a 1minute period before being added to a prepared Multiple Wizard Prep minicolumn (Cat.# A7211). A vacuum was applied and the resin/DNA mix drawn over the minicolumn. The column was washed by drawing 1ml of 80% isopropanol through the column, then dried by vacuum for 30 seconds after the isopropanol had passed.

The minicolumn was then centrifuged at 10,000g for 2 mins to remove any residual isopropanol. The DNA was eluted into a new microcentrifuge tube by addition of 30µl of TE buffer to the minicolumn and centrifugation at 10,000g for 20 seconds, after a 1 minute wait. The concentration of the purified product was calculated by optical visualisation on a 1% agarose gel.

2.3 Cloning of the full-length cDNA into vector.

Ligation of cDNA insert into vector.

Prior to ligation of the amplified cDNA into a suitable vector, the insert:vector molar ratio was calculated using the following recommended equation. (Promega pTarget expression vector kit).

$$\frac{\text{Ng of vector} \times \text{kb size of insert}}{\text{Kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}.$$

2.3.1 Ligation reaction.

Ligation reactions were set up as shown in table 2.1 using a 3:1 insert:vector ratio, and the reactions were incubated overnight at 4°C.

Table 2.1 Ligation reaction mix.

Reagents:	*Standard rxn.*	*Pos. control*	*Neg. control*
T4 DNA Ligase 10X Buffer	1µl	1µl	1µl
pTarget Vector 60ng	1µl	1µl	1µl
PCR Product 50ng	1µl	-----	-----
Control insert DNA 8ng	-----	2µl	-----
T4 DNA Ligase (3 units/µl)	1µl	1µl	1µl
d-H20 to final volume of 10µl	10µl	10µl	10µl

2.3.2 Choice of vector.

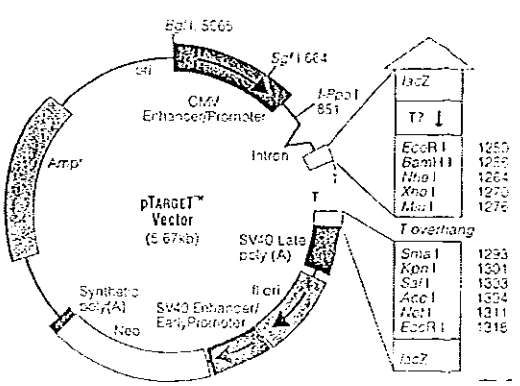


Fig 2.1 pTarget™ Vector circle map.(www.promega .com)

The pTarget ™ mammalian expression vector (Promega) fig.2.1 was chosen as the cloning vector. This vector is provided with single 3' T overhangs in the insertion site, which allows direct ligation of the PCR products containing a single dATP at the 3' end added by certain polymerases, for example Accu Taq LA DNA polymerase or Taq Gold (A/T cloning). In addition, the vector contains the ß-galactosidase gene, which is disrupted upon insert ligation. This allows the selection of positive clones by growing transformed bacteria in agar plates containing the appropriate substrates (blue/white screening).

2.4 Transformation of E.Coli JM109

2.4.1 Transformation using pTarget vector.

Duplicate plates of Tryptose Blood Agar (TBA) from Oxoid (Lot.# 208279) containing IPTG 0.1M (Promega Cat.# V3951) at a concentration of 1:200, X-Gal (Promega Cat.# V3941) at 100µg /100ml, and Ampicillin at 100µg/ml, were prepared for each ligation reaction.

The tubes containing the ligation reactions (methods 2.3.2) were centrifuged for 20 seconds at 2000rpm to collect the contents at the bottom of the tube. 2µl of each ligation reaction was then added to a microcentrifuge tube on ice. JM109 high efficiency competent cells were placed on ice until just thawed. After gentle mixing 50µl of the cells were transferred into the tubes containing 2µl of ligation reaction. The tubes were gently mixed and placed on ice for 1 hour, after which they were heat shocked for 45secs at 42°C exactly. The tubes were placed directly on ice for 2 mins. 450µl of Tryptic Soy Broth (TSB) (SIGMA Lot.# 68H1077) at room temperature was added to the tubes with the bacteria and ligation reactions.

The samples were incubated at 37°C for 1.5 hours in a shaker (~ 250rpm) after which 300µl of each transformation was plated out onto the duplicate antibiotic plates. The plates were incubated overnight at 37°C, following which they were placed in a 4°C refrigerator to facilitate blue/white screening.

2.5 Selection of bacteria transformed with positive clones.

2.5.1 Screening of positive clones for insert.

20 positive clones were individually selected from the transformation plates using a small, sterile, wooden stick and transferred firstly to a clean antibiotic plate (methods: 2.4.1) which had been marked out with a numbered grid for reference (this plate was subsequently cultured at 37°C over night and stored at +4°C in the refrigerator), and then on to a correspondingly numbered well of a 24 microtitre well plate containing 50µl of T.E. buffer per well. All samples in the microtitre plate were subjected to a short freeze-thaw cycle by warming in a microwave for 1min at 750W followed by freezing in a -20 freezer for 20 minutes to disrupt the bacterial cells.

2.5.2 Primary PCR for selected transformed samples.

A PCR using primers annealing the insert site flanking regions of the vector was carried out on all 20 selected samples to identify clones with the correct sized insert (1.6kb). Microcentrifuge tubes containing 2µl of template from the appropriate well and 48µl of the following 1ml master-mix were added, 100µl 10X PCR Buffer, 100µl dNTP's 200µM, 100µl $MgCl_2$ 1.5mM, 20µl Forward primer 10pM/rxn, 20µl Reverse primer 10pM/rxn, 6µl TAQ Polymerase 5U/µl, H_20 to a final volume of 1ml.

The above reaction tubes were run under the following conditions, initial denaturation at 94°C for 5mins, denauration at 94°C for 30secs, annealing at 54°C for 45secs, extension at 70°C for 1min, cycling 30 times of denaturation, annealing, and extension steps, before a final extension at 70°C for 7mins.

PCR products were visualised by agarose gel electrophoresis.

2.6 Characterisation of selected positive clones

2.6.1 Sequence analysis of positive clones.

2.6.1.1 Primer design.

The majority of primers required for sequencing analysis were present at hand in the laboratory with the exception of one primer which was required for overlapping sequencing, and thus had to be designed. As the product we were looking to amplify was < 500 base pairs, a primer of 16-18 nucleotides would be adequate. Location upstream of the area for sequencing a region with greater than 50% G-C content was required (as this would increase the primer-template binding specificity), with no 3' terminal complementarity with other primers so as to eliminate primer dimer formation and no complementarity within itself to prohibit hairpin loop formation.

2.6.1.2 The Cycle Sequencing Reaction.

Cycled sequencing reactions were performed using the BIG DYE kit supplied by PE Biosystems. For sample preparation, a reaction mixture composed of the following reagent concentrations (per reaction) was prepared; 5µl 1X CS Buffer, 3µl 'Big Dye' terminator ready reaction mix, (composed of A-Dye terminator labelled with dichloro[R6G], C-Dye terminator labelled with dichloro[ROX], G-Dye terminator labelled with dichloro[R110], T-Dye terminator labelled with dichloro[TAMRA], deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, with thermally stable pyrophosphatase, $MgCl_2$, Tris-HCL buffer, pH9.0), 0.2µl of required primer at 10pM/µl, 1µl of primary PCR product ~30-90 ng, water to a final volume of 20µl. The cycling reaction was as follows: denaturation at 96°C for 10secs, annealing at 55°C for 10secs, extension at 60°C for 4mins, cycle 25 times, 4°C until required.

2.6.2 DNA precipitation for purification of extension products.

A sodium acetate/ethanol precipitation step was performed to purify the extension products, thus reducing background from unincorporated dye-labelled terminators that can obscure data at the beginning of the sequence.

The standard protocol for this procedure was as follows; the complete sequencing reaction product was added to an Eppendorf 1.5ml tube containing 2µl (1/10 the volume) of 5M sodium-acetate and mixed. 55µl (2.5X the volume) of 95% cold ethanol was added and the mixture was left to stand in a -20°C freezer for 30mins to help aid precipitation of the DNA. The samples were centrifuged in the cold room at 14,000g for 15mins.The precipitated DNA was washed in 70% ethanol and centrifuged for a further 14,000g for 15mins. All liquid was decanted off and the Eppendorf tubes were allowed to dry. Precipitated DNA was resuspended in 2µl loading buffer for sequencing analysis.

2.6.3 Sequencing.

Sequencing of all samples was carried out on an ABI Prism 310 genetic analyser. This is a single capillary based system running one sample per hour. BIG DYE chemistry is used and the method is based on the Sanger technique with the exception that it is possible to run one sample per well due to the different fluorochromes attached to the relevant di-deoxynucleotides. A brief overview of the procedure is shown below in Figure 2.

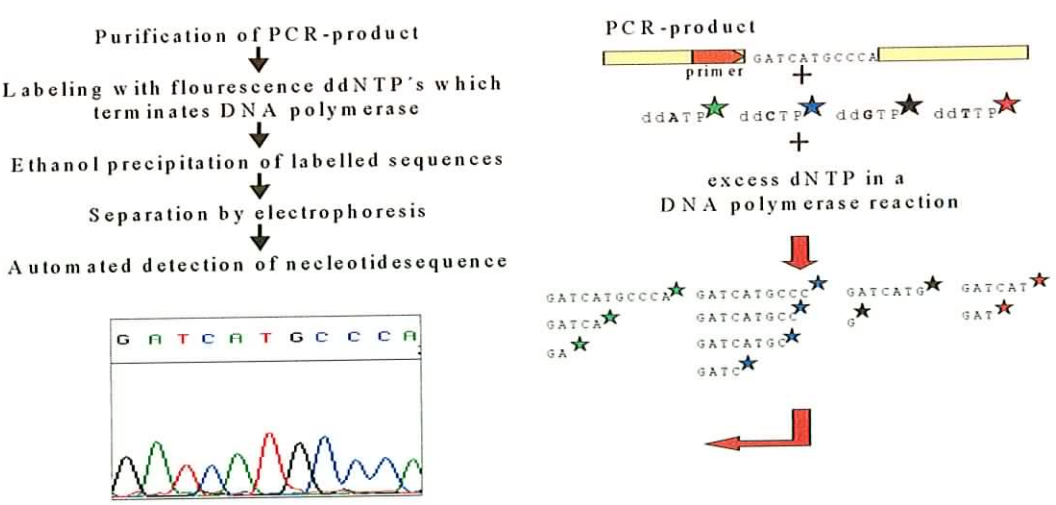


Fig 2.1 Overview of sequencing

2.6.4 Post sequencing selection of positive clones.

Samples identified as correctly oriented full-length cDNA clones of the fusion gene being sought, were traced back to the master plate produced in methods: 2.5.2. A loopful of these colonies was selected aseptically and the bacteria were distributed into an Erhlen-Meyer 200ml flask containing 100ml Tryptic Soy Broth (TSB) containing 100µg/ml Ampicillin. The clones were cultured overnight at 37°C in a shaker incubator at 150rpm. At log phase (O.D. ~1) the bacteria were centrifuged at 2000rpm for 15mins in the cold room (~ 4°C), the supernatant was poured off and the cells were resuspended in 3ml TSB plus 10% glycerol. 1ml doses were aliquoted into cryotubes for storage at -70°C. These cultures would then serve as a source of clones for the respective fusion gene.

2.7 Plasmid Purification.

For purification of the plasmid from the cells a Promega corp. Wizard® DNA plasmid midi prep system (Cat.# A7640) was used. Prior to the purification step itself a cleared lysate must first be produced.

2.7.1 Production of a cleared lysate.

Cultured bacterial cells were pelleted by centrifugation at 10,000g for 15mins at 4°C. The supernatant was decanted and the cell pellet was resuspended in 3ml of Cell Resuspension Solution (containing 50mM Tris-HCL, (pH 7.5), 10mM EDTA, 100µg/ml RNase A). 3ml of Cell Lysis Solution (0.2M NaOH and 1% SDS) was added, and the tube mixed by inversion. 3ml of neutralization solution (1.32M potassium acetate pH4.8) was then added and the tube was again mixed by inversion. The lysate was centrifuged at 10,000rpm for 20 mins at 4°C. The supernatant containing the DNA was carefully decanted into a new centrifuge tube by filtering through filter paper (Whatman. # 1).

2.7.2 Plasmid purification.

10ml of DNA purification resin was added to the DNA solution produced in methods: 2.7.1. The resin/DNA mix was then transferred into a previously prepared Midicolumn (Promega Cat.# A7651). A vacuum was applied only until the resin/DNA mix was drawn into the midicolumn. 15ml of column wash solution (containing 80mM potassium acetate, 8.3mM Tris-HCL pH 7.5, 40μM EDTA.) was then applied and drawn through the midicolumn using the vacuum. Continuing to draw on the midicolumn for 30 seconds after the column wash had passed through dried the resin. The midicolumn was removed from the vacuum and separated from it's reservoir by cutting with a scissors, before being transferred to a microcentrifuge tube and spun at 10,000g for 2mins to remove any residual column wash solution. The midicolumn was then transferred to a new microcentrifuge tube and 300μl of TE buffer preheated to 45°C was applied. This was allowed to soak through the column for one minute, after which the midicolumn was spun at 10,000g for 20 seconds to elute the plasmid DNA.

The eluted DNA was centrifuged at 10,000g for 5mins to remove any residual resin fines and the DNA containing supernatant was transferred to a new microcentrifuge tube. For increased cleanliness of the plasmid DNA, a precipitation was performed as in methods:2.6.2 and the resuspended DNA was stored at +4°C in the refrigerator.

2.8 SS18-SSX1 retrieval from agarose.

2.8.1 Low Melting point Agar (LMP) preparation.

1% LMP agar was prepared using Sea Plaque® GTG® agarose (FMC Bio products Cat.# 50111). 1g was dissolved in 98ml of Millipore quality water and buffered to 1X TAE by the addition of 2ml 50X TAE.

The gel was poured and allowed set at 4°C in the cold room, before being electrophoresed slowly at 80 Volts.

2.8.2 Purification of bands from LMP agarose using the Promega PCR preps DNA purification system.

A 300mg (300µl) slice of agarose containing the band required, was cut from the gel and placed in a 1.5ml microcentrifuge tube. The tube was then incubated at 70°C until the agarose was completely melted. 1ml of resin was added to the melted agarose slice, and mixed for 20 seconds by inversion before being added to a prepared Multiple Wizard Prep minicolumn (Cat.# A7211). A vacuum was applied and the resin/DNA mix drawn into the minicoloumn. The column was washed by drawing 1ml of 80% isopropanol through the column, then dried for 30 seconds after the isopropanol had passed.

The minicoloumn was then centrifuged at 10,000g for 2 mins to remove any residual isopropanol. The DNA was then eluted into a new microcentrifuge tube by addition of 30µl of TE buffer to the minicolumn and centrifugation at 10,000g for 20 seconds, after a 1 minute wait.

The concentration of the purified product was calculated by optical visualisation in a 1% agarose gel .

2.9 SSX1 & 2 cloning.

The cloning of SSX1 and SSX2 was carried out using cDNA generated from melanoma samples, which were reported to express SSX genes. Initially a $MgCl_2$ titration was carried out using a human diploid fibroblast cell line and melanoma samples for optimal detection of SSX genes. RNA isolation and Reverse Transcription was carried out as before with the exception that RT was carried out using a consensus primer located in the 3' UTR of the SSX genes (SSX-A).

2.9.1 Amplification of SSX genes

PCR was carried out using 5µl of 10x PCR buffer, 5µl of dNTP at 200µM each, 1µl of each primer at 10pM/µl, 5µl cDNA, 0.3µl Taq Gold Polymerase, MgCl2 at concentrations of (1.5mM, 2mM, & 2.5mM) dH20 to 50µl.

Cycling parameters: 95°C for 10mins, 30cycles of 95°C for 30sec, 55°C for 30 sec, 70°C for 45sec, 70°C final extension for 7mins.

2.10 Tagged Vector notes:

The epitope tagging technique involves fusion of a peptide epitope (recognised by an available antibody) to a target sequence. Using this technique, expression of the fusion protein is monitored using a tag-specific antibody. Epitope tagging can be used for many different functions, including localisation of gene products in living cells, or identification of associated proteins. Two different vectors were used in this project to generate the tagged proteins required. The first epitope tagged gene produced was FLAG-tagged SS18 full-length (SS18-FL including exon 8). FLAG is a synthetic epitope composed of eight amino acid residues (DYKDDDDK).

Flag tagged vectors were provided by Stratagene in three different reading frames A, B, and C. These vectors differed only the addition of 1 or 2 bases into the multiple coding sequences. Thus, each pCMV-Tag vector has a reading frame that will allow cloning of a gene of interest so that it is fused correctly with the epitope tag at the N-terminus. Tagged constructs can then be transfected into mammalian cells and the fusion protein can be characterised using commercially available antibodies. The other vector used was pCMV-HA, which uses an antibody directed against the HA(hemaglutinin epitope) the sequence of which is positioned 5' to inserted sequence. The CMV promoter allows constitutive expression of the cloned DNA in a wide variety of mammalian cell lines. The Neomycin resistance gene is under the control of both beta lactamase gene for kanamycin resistance in bacteria and the SV40 early promoter to provide G418 resistance in mammalian cells. Below is a diagram showing restriction enzyme recognition sequences in relation to HA tag position at the pCMV-HA multiple cloning site.

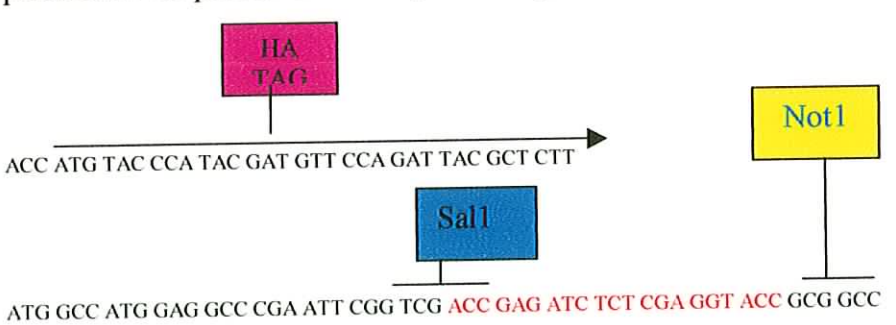


Fig 2.2 Schematic of two cut enzyme strategy at plasmid multiple cloning site. Red sequence is removed by double cut and replaced by sequence of interest.

Amplification of wild type SS18-FL and wild type SS18-S for insertion into tagged vectors was carried out using primer pairs that had been designed to have a Not1, Sal1 or Pst1 cut site at the 5' prime end of the oligonucleotide.

SS18-FL and SS18-S recombinant cloning vectors were used for PCR amplification of the respective genes. For insertion of SS18-FL into the pCMV FLAG vector the Pst1 cut site was used to insert the gene in frame, whereas SS18-S was inserted into the pCMV-HA vector using Not1 and Sal1 one digested PCR product.

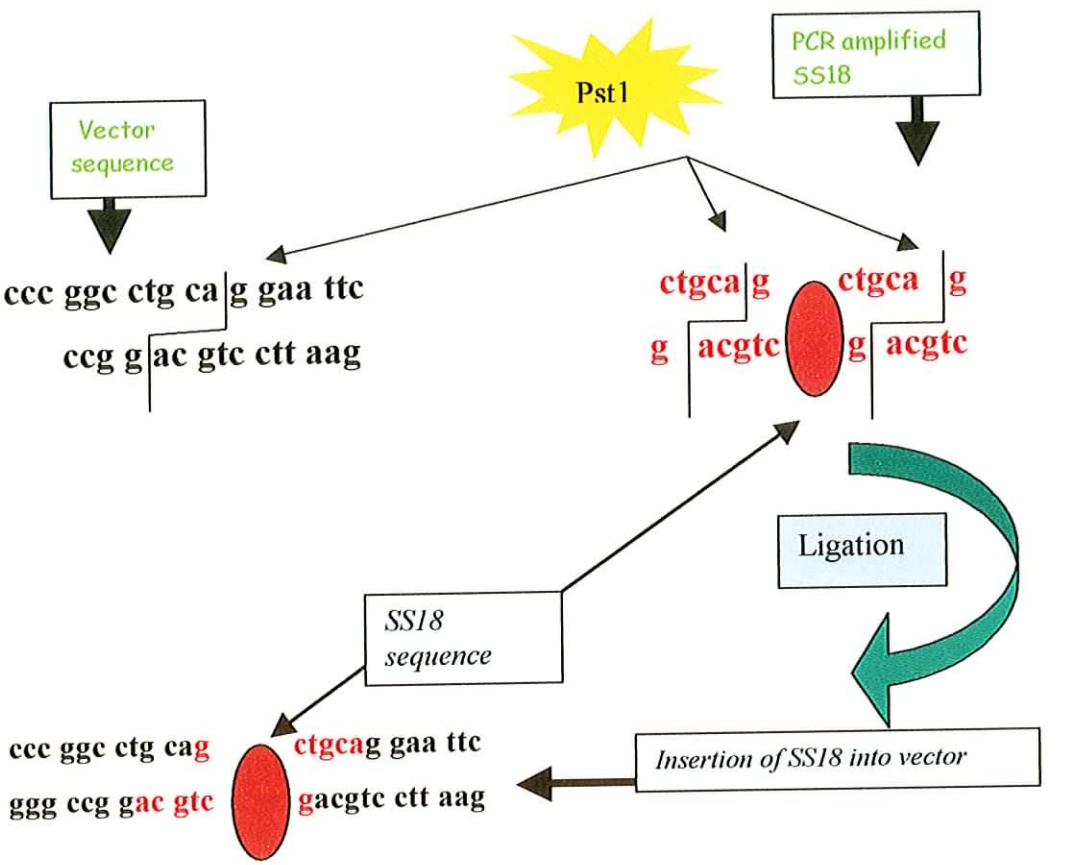


Fig 2.3. Schematic of one cut enzyme strategy to produce FLAG tagged SS18-FL.

The diagram above schematically shows the recognition sequence and shape of cleavage by Pst1. Both vector and PCR product are subjected to cleavage by the enzyme, before they are ligated together to produce an in-frame recombinant clone. With the one cut enzymatic protocol insertion can be bi-directional.

Before insertion of the desired gene sequence into the appropriate vector, preparation of both insert and vector was required to allow in-frame insertion.

The pCMV vector that was supplied by the appropriate company was transformed into bacteria and cultured. The amplified Plasmid was purified by a mini-prep protocol kit. Aliquots of vector were quantified using the GeneQuant spectrophometer.
The relevant gene sequence for insertion into the linearised tagged vectors was PCR amplified under stringent conditions using Accu Taq LA (a high specificity polymerase with proof reading capabilities) with primers that were designed to carry restriction endonuclease recognition sequences at their 5' ends.

2.10.1 Preparation of vector and PCR Product for Restriction enzyme digestion.
For amplification of the appropriate gene from cloning plasmid using Accu Taq LA Polymerase the following reaction conditions were employed. In a 50µl reaction 5µl of 10x Accu Taq LA buffer, 5µl dNTP at 200µm each, DMSO 1µl, SS18-A and SS18-B primers at 10pM each / reaction, 0.5µl plasmid, 0.5µl Accu Taq La enzyme at 0.05U/µl & 36µl dH20 to final volume of 50µl were added to a PCR tube. The contents were spun down briefly and the following cycling parameters were followed; 94°C for 5mins initial denaturation, followed by 25cycles of denaturation for 30 secs, annealing for 1min, and extension for 1min at 94°C, 55°C, & 70°C respectively, before a final extension of 70°C for 7 minutes.
10-15µg of PCR product was restriction digested in a 20µl reaction in order to remove the end terminal sequence of nucleotides, leaving an overhang at either end compatible with that of the digested and linearised vector.

2.10.2 Protocol for subIconing DNA fragments with homologous ends using a single restriction enzyme digest.

10μg of plasmid DNA was added to a reaction mixture along with 2μl 10x Buffer for the relevant enzyme, 15units of restriction enzyme and water to 20μl for each digestion. The sample was incubated at 37°C for one hour.

After one hour 0.5μl of product were checked in a 1% agarose gel to observe whether the plasmid was linearised. If complete the reaction was stopped by the addition of 1μl 0.5M EDTA. 10-15μg PCR Product was also run at the same time in a 20μl reaction to remove end terminal sequence of nucleotides to produce an overhang at either end of the sequence compatible with the vector. The linearised vector was then purified using Qiagen gel extraction kit. PCR products were purified using Promega PCR preps.

2.10.3 Dephosphorylation.

In the case of a single cut enzymic restriction digest, vector dephosphorylation is a necessary step to prevent the vector from re-circularising and thus creating false positive results at the ligation/transformation stage. This was achieved by using Shrimp Alkaline Phosphatase (SAP), an enzyme with the capability to work in a broad range of buffering conditions. To 10μl of restriction digested vector reaction SAP enzyme at 10U/μg plasmid was added. The mixture was then buffered to 1x with 10x SAP buffer and water in a total volume of 50μl. The reaction was incubated at 37°C for 15 minutes and then at 75°C for a further 15minutes to deactivate the enzymes present. The vector was then purified using the Qiagen gel extraction protocol.

2.10.3.1QIAquick Gel Extraction Kit Protocol.

This protocol is designed to extract and purify DNA of 70bp to 10kb from standard agarose gels in TAE buffer. This procedure can also be used for DNA cleanup after enzymatic reactions.

After electrophoresis of the DNA the fragment in 1% agarose was excised from the gel with a clean sharp scalpel. The size of the gel piece was minimised by removing excess agarose.

The gel slice was then weighed in an eppendorf tube and 3 volumes of buffer QG were added to 1 volume of gel (100mg~ 100µl). The tubes were incubated at 50°C for 10 minutes with occasional vortexing to dissolve the gel. Once dissolved 1 gel volume of isopropanol was added to the tube and mixed. The sample was then applied to a Qiaquick column, which was placed into a 2ml tube. The DNA was bound to the column by centrifugation at >10,000g for 1 minute. The flow through was discarded and the column placed back into the 2ml tube. 750µl of buffer PE was added to the column and the tubes were spun at >10,000g for 1 minute. The Qiaquick column was placed into a 1.5ml eppendorf and 50µl of dH20 was applied to the centre of the column for 1 minute before the sample was centrifuged at maximum speed for 1minute to elute the DNA. Retrieved DNA was stored at -20°C.

2.10.4 Protocol for directional cloning using fragments with heterologous ends using 2 restriction enzymes.

With a two cut system self-ligation is theoretically eliminated, thus producing very low background. All recombinant molecules will contain the insert in the desired direction with respect to the vector. The major problem with this approach is incomplete digestion of the vector or insert by the restriction enzymes, which is why careful primer design is necessary when preparing the insert by way of PCR. Usually the gene of interest is cleaved from a cloning vector at its multiple cloning site with 2 different restriction enzymes that will preserve orientation when inserted into another vector, which has been linearised with the same two enzymes. In this way the sequence of the gene remains undisturbed and allows it to be placed in-frame in the secondary vector. The PCR in contrast, is used when the

multiple cloning sites of the vectors are incompatible with each other. This method requires a double check of the entire gene sequence once it has been placed into the secondary vector.

In this case as the restriction enzymes required different salt concentrations to work effectively, restriction was carried out using the enzyme, which preferred the lower salt concentration first. Once the first enzyme linearised the plasmid (which was visualised on the gel), the salt concentration was adjusted to that of the second enzyme using 1µl of 1M NaCl (which corresponds to an increase in salt concentration of 50mM) and the restriction was completed with the second enzyme.

The efficiency of the second enzyme in the adjusted buffer was monitored in a control tube, where plasmid was digested in the adjusted buffer with the second enzyme alone and visualised in the gel.

No control was established directly for the PCR digestion as the product of digestion is too small to differentiate between cut and uncut, instead the PCR was run in parallel with the Vector reactions.

10µg of plasmid and half of the PCR reaction were digested in a reaction volume of 20µl for 1 hour with the appropriate buffer and enzyme. The set up of the tubes was as follows.

DNA	*10x Buffer*	*NaCl*	*Enzyme(s)*
Control 1. Vector	React 3	---------	Not 1
Vector	React 3	1µ 1M NaCl	Not 1 & Sal 1
PCR prod	React 3	1µl 1M NaCl	Not 1 & Sal 1
Control 2. Vector	React 3	1µ 1M NaCl	Sal 1
Uncut Vector	----------	---------	---------

Table 2.2 Tube set up for restriction enzyme digestion reactions

2.10.5 Ligation of purified vector and cDNA.

10x ligation buffer was used for ligation and the reaction was left at 4°C overnight to complete before transformation. There is also a rapid 2x ligation buffer available, which allows ligation on the bench top within a couple of hours. This was not chosen on this occasion because 50% of the available volume for the reaction is required by the buffer, thus leaving a maximum volume of 3µl for insert in the reaction. This would decrease the likelihood of successful incorporation of the insert into the vector as it significantly reduces the insert:vector ratio. Experience of ligation reactions has shown that the maximum insert:vector ratio possible produced the best results in all cDNA's clone. The tube sett up for ligation is shown below.

Reagents	*Standard rxn.1*	*Standard rxn. 2*	*Standard rxn. 3*	*Neg. control*
T4 DNA ligase 10X buffer	1µl	1µl	1µl	1µl
pCMV-HA / pCMV-Flag	1µl	1µl	1µl	1µl
PCR Product	4µl	7µl	2µl	0µl
T4 DNA Ligase	1µl	1µl	1µl	1µl
dH20 to final volume of 10µl	3µl	0µl	5µl	7µl

Table 2.3. Description of tube contents for ligation.

2.11 Characterisation studies

2.11.1 Transient transfection of pTarget SS18/SSX2 vector into HIH 3T3 cells.

NIH 3T3 mouse fibroblast cell line were transfected with 10μg of SS18/SSX2 plasmid DNA using a gene pulser apparatus from BioRad, (Hercules, California, USA) using cuvettes with a gap of 0.4cm.

This was achieved by transferring cells to an eppendorf tube and centrifuging at 500g for 5mins. Cells were washed in 1x PBS and spun down again. Supernatant was aspirated, and cell pellet was resuspended in 10μg plasmid DNA solution. The mixture was transferred to a metal cuvette and brought to 500μl with Iscoves IMDM without FCS. Mixture was shocked @ 960μF and 220 Volts.

Cuvettes were placed on ice for 5 minutes before the transfected cells were transferred to a 10cm culture dish containing 10ml IMDM plus 10% FCS. 48 hours post transfection cells were harvested and analysed by immunofluorescence for the expression of the SS18/SSX fusion gene using a rabbit polyclonal antibody raised against a peptide spanning the SS18/SSX breakpoint (RPYGYDQIMPKKPAEC).

2.11.2 Immunofloursecence

Cell spots from SS18/SSX2 transfected and untranfected NIH 3T3 cells were prepared and fixed in 4% paraformaldehyde in phosphate buffered saline for 30 minutes. Slides were washed twice in PBS plus 0.05% Tween 20 and incubated overnight with a 1:1000 dilution of the polyclonal antibody that was directed against the fusion gene breakpoint. The cells were washed three times with PBS plus 0.05% Tween 20 and incubated with a FITC-conjugated anti-rabbit antibody (Dakopatts, Denmark) for 1 hour, followed by washing three times in PBS plus 0.05% Tween 20 for 10 minutes per time.

Preparations were mounted in Vectashield mounting solution containing DAPI (Vector Laboratories Inc.), and visualised using a fluorescence microscope using a Zeiss Plan-Neofluor 40x or 65x oil immersion lens. The images were captured by a cooled charged-coupled device camera and the data was analysed with the Delta vision System (Applied Precision Inc.).

2.12 Analysis of SS18 splice variants.

Preliminary experiments were carried out to assess the presence of SS18 splicing in a variety of tissues. As SS18 expression has been reported as ubiquitous a wide variety of tissues were assayed namely melanoma, mouse and human tissue. This would enable a broad overview of SS18 splicing and would permit identification of tissue specific splicing if present. Reverse Transcription was carried out as before using the SS18-2 primer located in the 3'UTR of the SS18 gene. This detects wild type SS18 only and interference from the SS18/SSX2 sequence can be avoided (applies to synovial sarcoma sample only).

2.12.1 Outer PCR.

To detect wild type SS18 in tumour samples a nested PCR protocol was employed to achieve detection.

For the outer PCR 5µl of cDNA produced by reverse transcription was added to microfuge tubes containing 45µl of the following 1ml mastermix; 100µl 10xPCR buffer Gold, 100µl dNTP's at 200µM each, 100µl $MgCl_2$ at 2.5mM, 20µl of each primer (SS18-B & SS18+1) at 10pM /µl, 6µl Taq Gold polymerase enzyme at 0.025U/µl, and 664µl of dH_2O to make a total volume of 1ml. The cycling parameters were as follows: 95°C initial denaturing for 10mins, which is required to activate Taq Gold enzyme and permits a hot start to the reaction which increases the specificity of the reaction. 30 cycles of the following sequence was then carried out: 95°C for 30seconds denaturing, 50°C for 1minute annealing, 70°C for 1 minute extension. Final extension was for 7 minutes at 70°C.

2.12.2 Nested PCR.

1µl of PCR product from the outer reaction was carried forward as template into the nested PCR and was added to a microfuge tube containing 49µl of the following 1ml master mix: 100µl of 10x PCR buffer, dNTP and $MgCl_2$ at final concentrations of 1x, 200µM each, and 2.5mM respectively. 10µl of each primer (SS18 343+ and SS18 935-) at a concentration of 10pM/µl each. 6µl of Taq polymerase at 0.025U/µl. 660µl of dH20 was added to make a final volume of 1ml. The cycling parameters for the reaction were as follows: 95°C for mins initial denaturing, followed by 25 cycles of 95°C denaturing for 30secs, 55°C for

1min to anneal primers, and 70°C for 1 min extension. Final extension was for 7 mins at 70°C.

2.13 Transient transfection of pCMV-SS18 vectors using LipofectAMINE reagent.

LipofectAMINE reagent is a 3:1 w/w liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE in membrane filtered water. It is suitable for the transfection of DNA into cultured eukaryotic cells.

In a six well plate 1-3x10^5 NIH3T3 cells / well were seeded in 2ml IMDM + 10% FCS. Cells were incubated at 37°C in a CO_2 incubator overnight / until the cells were 80% confluent. The following solutions were then prepared, solution A containing 2µg of DNA diluted in 100µl of Opti-MEM (Gibco BRL cat.# 31985)serum free medium per transfection. Solution B containing 30µl of LipofectAMINE in 100µl Opti-MEM per transfection. The two solutions were then combined and gently mixed before incubation at room temperature for 15-45 mins. This allowed DNA-liposome complexes to form. Cells to be transfected were then rinsed with serum free medium.

For each transfection, 0.8ml of serum free medium was added to the tube containing the complexes, bringing the total volume per transfection to 1ml. The tubes were mixed and the solution was layered gently over the cells in the appropriate well. Cells were incubated at 37°C in a CO_2 incubator for 5 hours.

The transfection mixture was then removed completely and the cells were washed with 1xPBS once, before 2ml of complete growth medium was added (IMDM +10% FCS). Cells were harvested after 48 hours and used for RT-PCR analysis and/or slide preparation for immunofluorescence.

3.0 RESULTS.

3.1 SS18/SSX 2 RNA integrity check.

Figure 3.1 below shows the presence of undegraded total RNA extracted from a tumour previously diagnosed as synovial sarcoma by resident pathologists within the Karolinska hospital. Total RNA was used to facilitate RT-PCR and was the source of mRNA for the synovial sarcoma fusion gene SS18-SSX2.

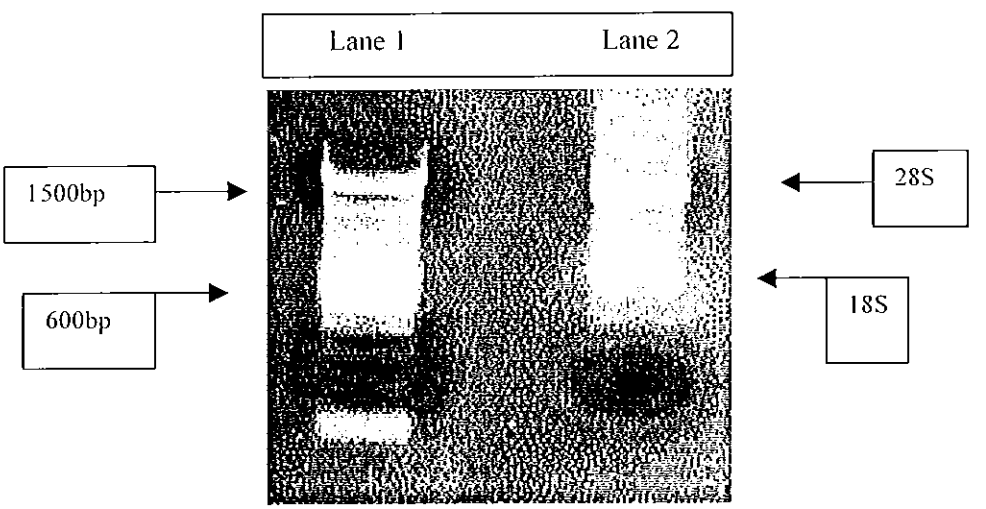


Fig. 3.1 RNA integrity assessment

Lane 1 shows 100base pair DNA ladder, lane 2 shows high integrity RNA extracted from sample bearing SS18/SSX2 translocation.

3.1.1 SS18/SSX2 PCR

Figure 3.2 shows the product generated by RT-PCR carried out as described in method: 2.2. for the amplification of SS18-SSX2 full-length cDNA.

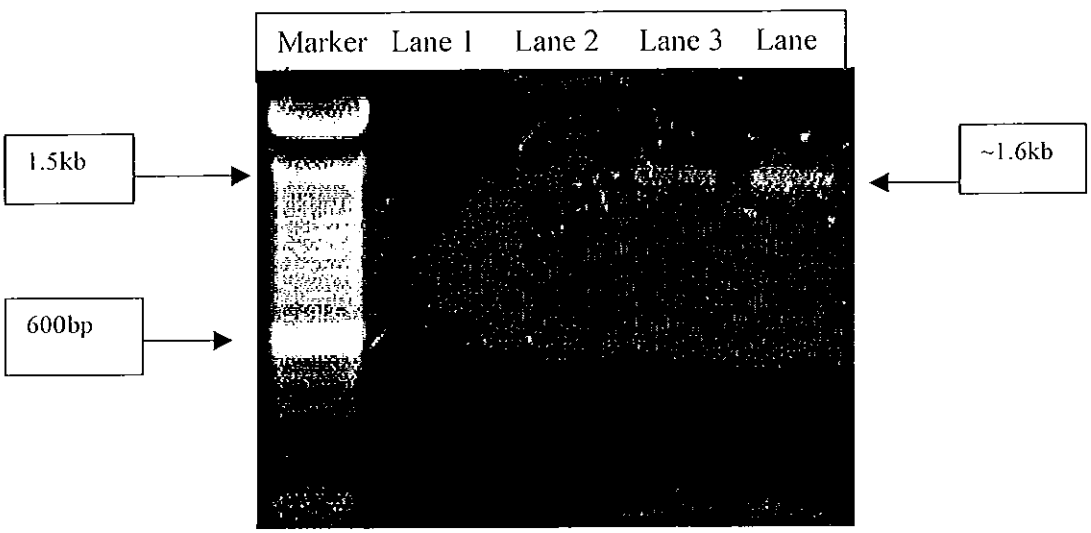


Figure 3.2 SS18 /SSX2 RT-PCR

100 base pair DNA ladder for sizing of PCR product is on left of image. Lanes 1 and 2 contain negative controls, lanes 3 and 4 show full-length SS18/SSX2 specific PCR product at 1.6kb. Samples were run in duplicate.

3.1.2 Transformation of SS18/SSX2 clones

Following purification of the cDNA, a ligation reaction was performed and the resultant plasmids containing insert were transformed into competent bacterial cells. A PCR screening reaction was carried out on 20 positive clones selected with blue/white screening, using primers that flank either side of the insert to determine if ligation was successful, and whether the required full cDNA length (1.6kb) was present. (Method:2.3 / 2.4)

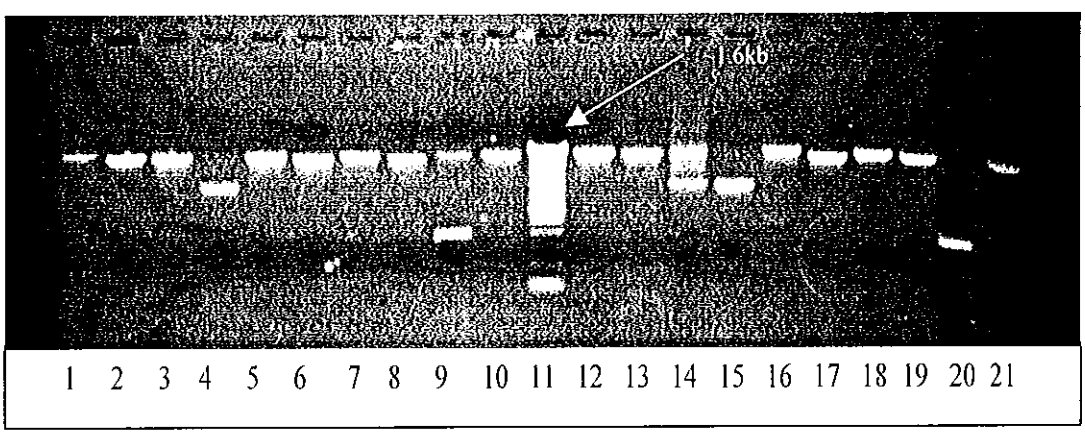


Fig 3.3 Transformation /Screening of E.Coli JM109

Lane 11; molecular weight marker, all other lanes contain product of insert PCR on individual bacterial clones. Clones corresponding to lanes 4, 9, 14, 15 and 20 contain inserts which are not full-length cDNA's for the SS18-SSX2 gene (<1.6kb).
A selction of approximately 10 clones which proved to contain sequences of correct length were chosen for full-length cDNA sequencing analysis. This was achieved by using primers which were located approximately 300 bases apart in the BIG DYE sequencing reaction. As the read length for the sequences were between 350-450 bases long, the sequence data generated by one primer would overlap with that of the next, thus allowing the entire length of the cDNA sequence present in the clone to be checked. Reactions were carried out in triplicate to ensure true base calling.

3.1.3 Sequencing results on positive selected clones.

The results of the sequencing analysis on one of samples selected is shown below. An overview of the sequencing process can be found in methods: 2.6.3. As it is not feasible to show the entire chromatagrams for the sequences produced, a sample screen shot of one was taken to display the results observed when using Sequencher 3.0 software for analysis of sequencing data (fig 3.5). Also shown is the alignment of a primer and sequences from analysed samples which can be used to define orientation of insert and length of sequence produced in the cycling reaction (see methods: 2.6.1.2).

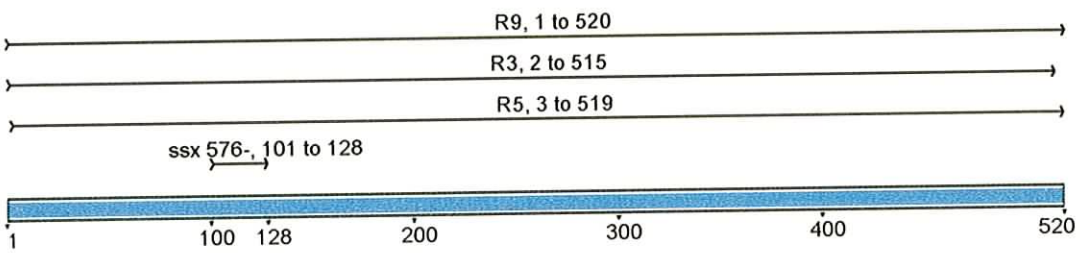


Fig 3.4 Diagrammatic alignment of sequences.

Below is the base call chromatogram for three aligned sequences as interpreted by sequencher. Triplicate analysis aids in distinguishing bases where background is high. See A/N for example:

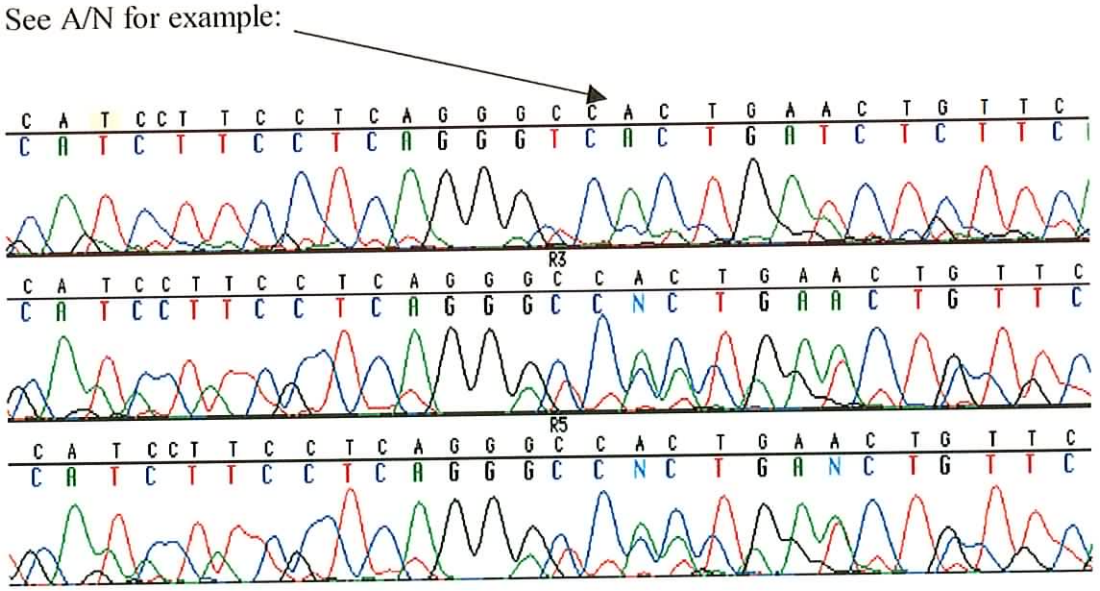


Fig 3.5 Chromatogram showing peaks/location of bases.

3.1.4 BLAST alignment

The result in Fig 3.8 shows the sequence alignment hit achieved when the sequence obtained from a cloned cDNA insert was checked against all known sequences of DNA located in the Genbank database using the BLAST alogorithm.

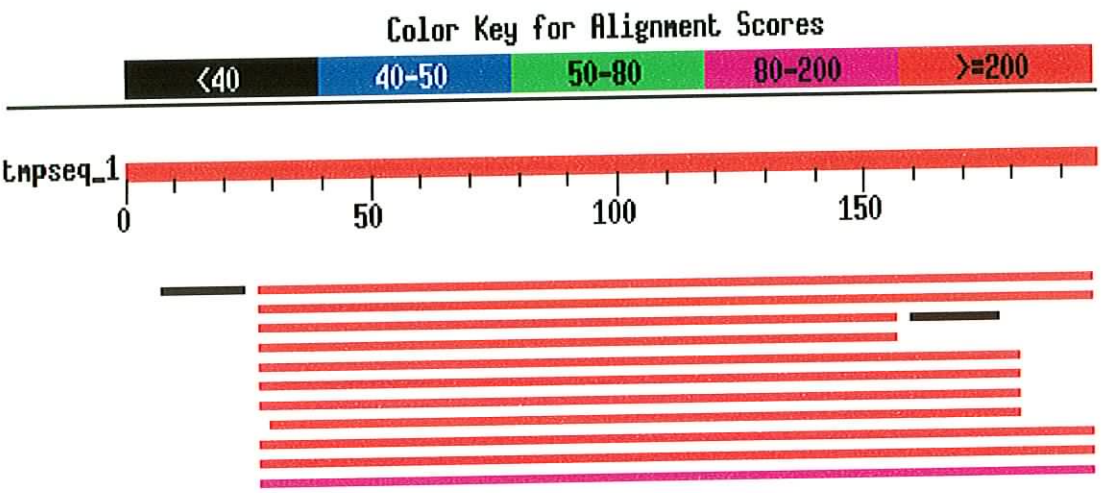


Fig 3.6 Diagrammatic result of a BLAST search.

The top line shows 'best hit' in schematic fashion. The red line shows sequence homology of the cDNA clone to SS18/SSX2 sequence. The black line shows homology of cDNA clone sequence to pCMV vector.

Using the blast search results, clones that proved to have the correctly oriented and full-length cDNA inserts were selected from the master plate produced in methods: 2.5 and cultured for storage.

3.2 SS18/SSX1 cloning.

3.2.1 SS18/SSX1 High Resolution electrophoresis.

SS18/SSX1 specific RT-PCR resulted in a smeared product, although a faint band in the region of 1.6kb was observed. A comparative gel in 1% low melting point agar was run, using the total RT-PCR sample product. This allowed us to see if the majority of PCR product corresponded to the full-length cDNA for SS18/SSX1. An image of the gel is presented in Fig.3.14. The strong band observed at the 1.6kb region, was subsequently cut out and purified.

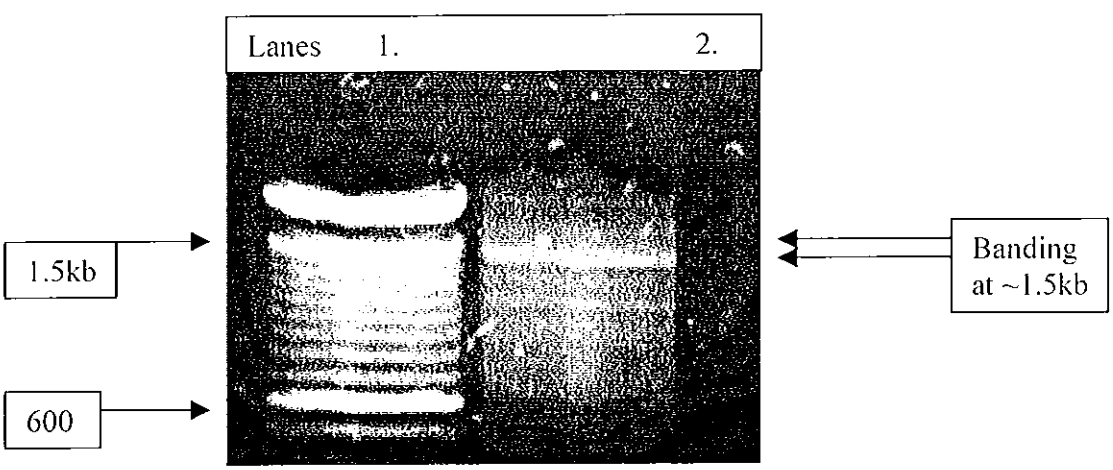


Fig 3.7 Comparative gel in LMP agar, showing base pair ladder in lane 1 and in lane 2, banding observed in RT-PCR product for SS18-SSX1 cDNA.

Following purification of the PCR product from the agarose, a ligation reaction was performed as for SS18/SSX2 and the resultant plasmids containing insert were transformed into competent bacterial cells. A PCR reaction was carried out on 20 positive clones selected with blue/white screening, using primers that span either side of the insert to determine insert length (~1.6kb).

3.2.2 Screening of SS18/SSX1 transformed bacterial clones.

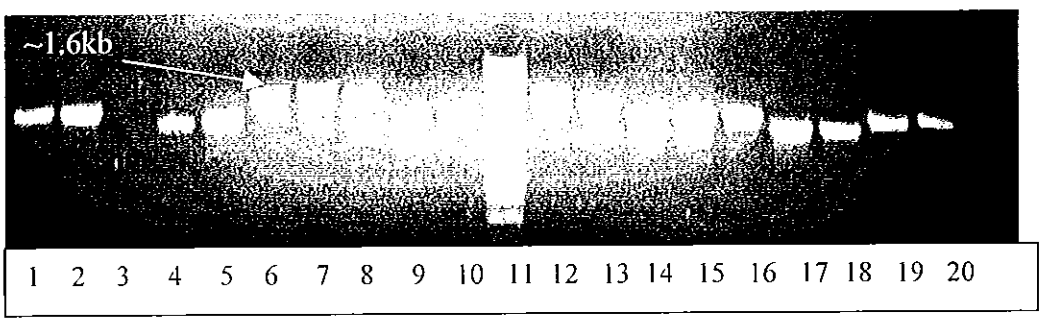


Fig 3.8 Primary PCR on purified bands from RT-PCR above (Fig 3.13.).

Lane 11; molecular weight marker. Samples 6, 7, 8 and 12 only possess the required insert length (~1.6kb). This was to be expected as the PCR product electrophoresed on LMP agar and used in ligation showed non specific banding. The products of correct length were sequenced as before and correctly oriented SS18/SSX1 full-length cDNA was retrieved.

3.3 SSX1 and SSX2 cloning.

Cloning of SSX genes was carried out after a screen using DFW cell line and melanoma tumour samples. Pan SSX primers were used to assess SSX gene expression and PCR efficiency.

SSX genes are cancer testis (cT) antigens and the literature cites expression of SSX in testis and tumour samples. Tureci *et al.*, (1998) Using pan SSX primers located in the 5' and 3' regions of the SSX genes it was initially hoped that the PCR products generated would contain both SSX1 and SSX2 full-length cDNA, and that upon ligation both genes would be present in the clones produced. SSX2 gene retrieval proved successful by this method but screening of over 50 clones for SSX1 proved unfruitful. To circumvent this problem, SSX1 and SSX2 specific primers Tureci *et al.*,(1998) were used to screen 12 melanoma tumour samples for expression of SSX1 and SSX2 tumour RNA. One tumour expressing SSX1 but not SSX2 was chosen to clone the SSX1 gene using the original pan SSX primer pair.

3.3.1 Optimisation of magnesium concentration for SSX.

Low stringency conditions were used for this PCR to allow amplification of as many SSX genes as possible. RT was carried out using SSX-A primer, located in the 3' untranslated region of the SSX gene. This would generate cDNA for all SSX genes.

Three different Mg++ concentrations were used in this titration PCR. 4 sample RNA's were used for template. 3 were RNA derived from Melanoma tumours, and 1 sample was RNA derived from DFW cell line.

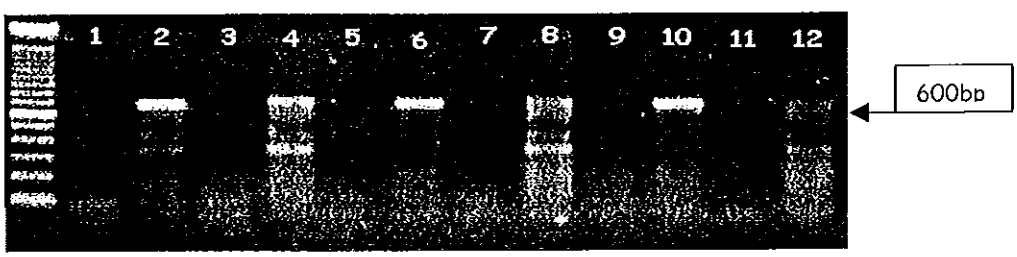


Fig 3.9. Mg++ conc optimisation for SSX gene PCR.

MgCl concentrations used; Lanes 1-4; 1.5mM, lanes 5-8; 2mM, lanes 9-12; 2.5mM.
Lanes 4,8, & 12 contain DFW PCR product, whereas all other lanes contain Product from the melanoma cell lines.

From the results it is clear that the sample producing the most specific SSX gene product is melanoma sample 2, as seen in lanes 2,6 & 10.
Product derived from the 2mM MgCl titration using sample 2 RNA as template was purified and used for transformation.

3.3.2 Screening of bacterial clones transformed with pan SSX RT-PCR product

Below is an image of transformed bacterial clones screened for full-length SSX cDNA. Lanes 2,3,4,9 & 12 contain clones of the correct length for full-length SSX2 cDNA insert. The clones bearing these cDNA's were sequenced as before (see SS18/SSX2 protocol) using primers located at the plasmid ends and within the SSX sequence itself. All clones were SSX2 positive.

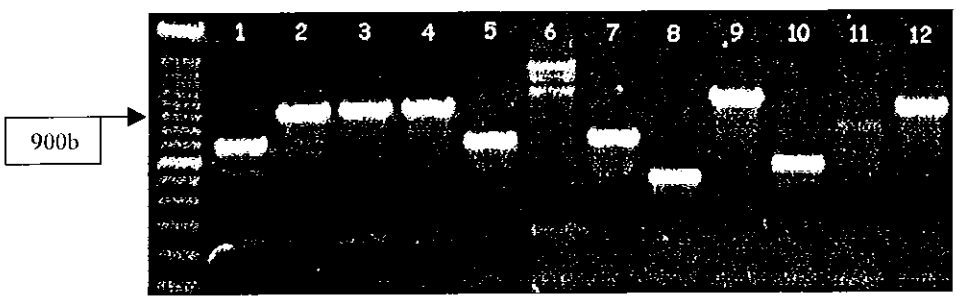


Fig 3.10. Screen for SSX insertion post ligation.

3.3.3 Screening of melanoma samples for differential expression of SSX1 and SSX2 RNA.

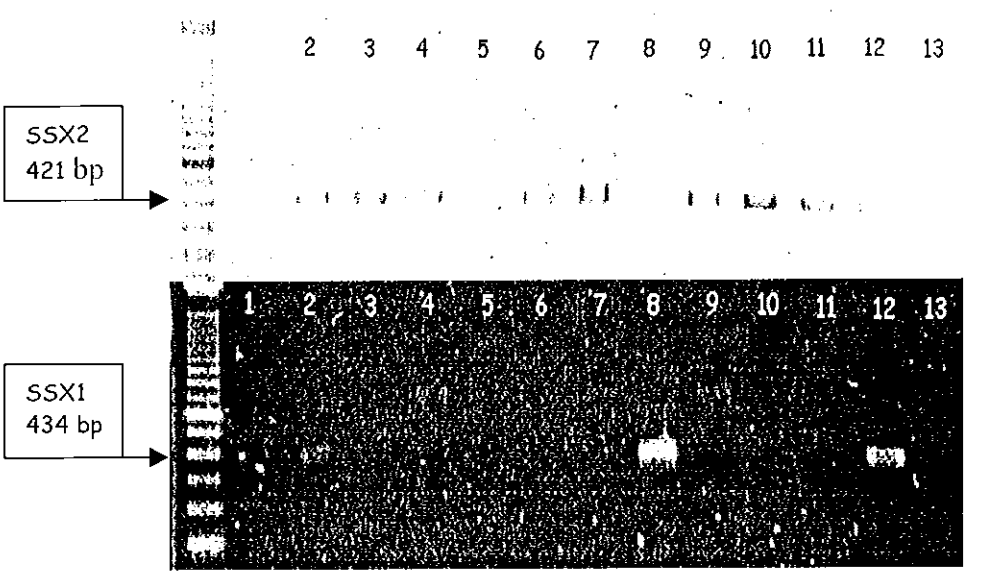


Fig. 3.11. SSX1 and SSX2 expression screening of 12 melanoma samples.

Lanes 2-13 contain melanoma samples, lane 1 contains negative control. It can be seen that sample 8 appears to express SSX1 but not SSX2 RNA. The RNA from this melanoma sample was subsequently chosen for cloning of the SSX1 gene cDNA.

Melanoma 8 RNA was used for cloning by the same method as described for SS18/SSX2 using SSX primers.

3.3.4 Plasmid PCR for SSX1 positive clones.

The result below shows plasmid PCR products of clones from melanoma sample 8 that proved to contain correctly oriented full-length cDNA for SSX1.

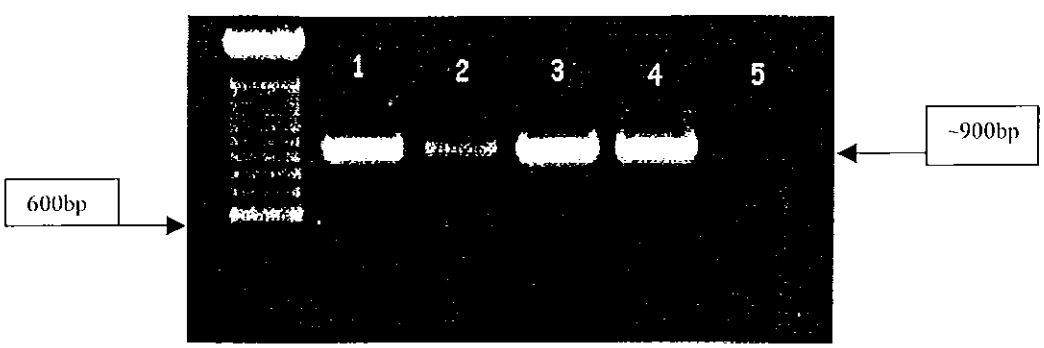


Fig 3.12. Plasmid PCR product for SSX1 full-length cDNA positive clones.

Lanes 1-4; plasmid PCR product for SSX1 gene clones. Lane 5 ; negative control.

3.4 Tagged Project.

The motive behind production of tagged vectors during this project was to enable the group to overcome the unavailability of a monoclonal or reliable polyclonal antibody for SS18. No commercially available antibodies to SS18 are available, and although attempts had been made by a visiting researcher to generate a specific monoclonal antibody to SS18, efforts to validate the specificity of these reagents at the western blot level were unresolved. With two SS18 splice variants identified, a model was devised to distinguish between SS18-S and SS18-FL by differentially tagging the two genes with epitopes FLAG and HA to allow identification of SS18 at the protein level. This required sub cloning SS18-S and

SS18-FL into vectors possessing tags in the 5' sequence prior to the insertion site. A discussion of the subcloning strategy is outlined in methods 2.10. In doing this, functional studies such as double immunofluoescence and immunoprecipitation experiments would be possible and may give insight into a functional difference between SS18-S and SS18-FL.

3.4.1 Enzymatic digestion of vector product by restriction enzymes Not1 and Sal1.

Below is the image of a gel used as an indicator to determine whether the enzymes used in the two cut strategy had worked sufficiently before the sample was taken onto purification and ligation stages.

The two enzymes used in this strategy required different buffer salt concentrations to work. Thus, the enzyme requiring the lower salt concentration was used to cut at its recognition sequence first, the buffer salt was adjusted and the second enzyme was added to the mix to cut at the second site required before ligation. The enzymes used were Not1 and Sal1, with Not1 requiring a lower salt concentration.

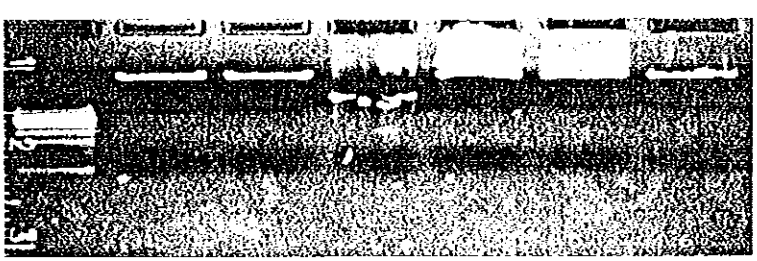


Fig 3.13. Assessment of Restriction enzyme digests.

Lane 1: Plasmid after 1 hour with Not1 - low salt buffer.

Lane 2: Plasmid with Not1 1 hour after Sal1 addition and buffer adjustment.

Lane 3: PCR product with Not1 and Sal1 in adjusted buffer 2 hour total.

Lane 4: Uncut Plasmid.

Lane 5: Plasmid with Sal1 in adjusted buffer after 15mins.

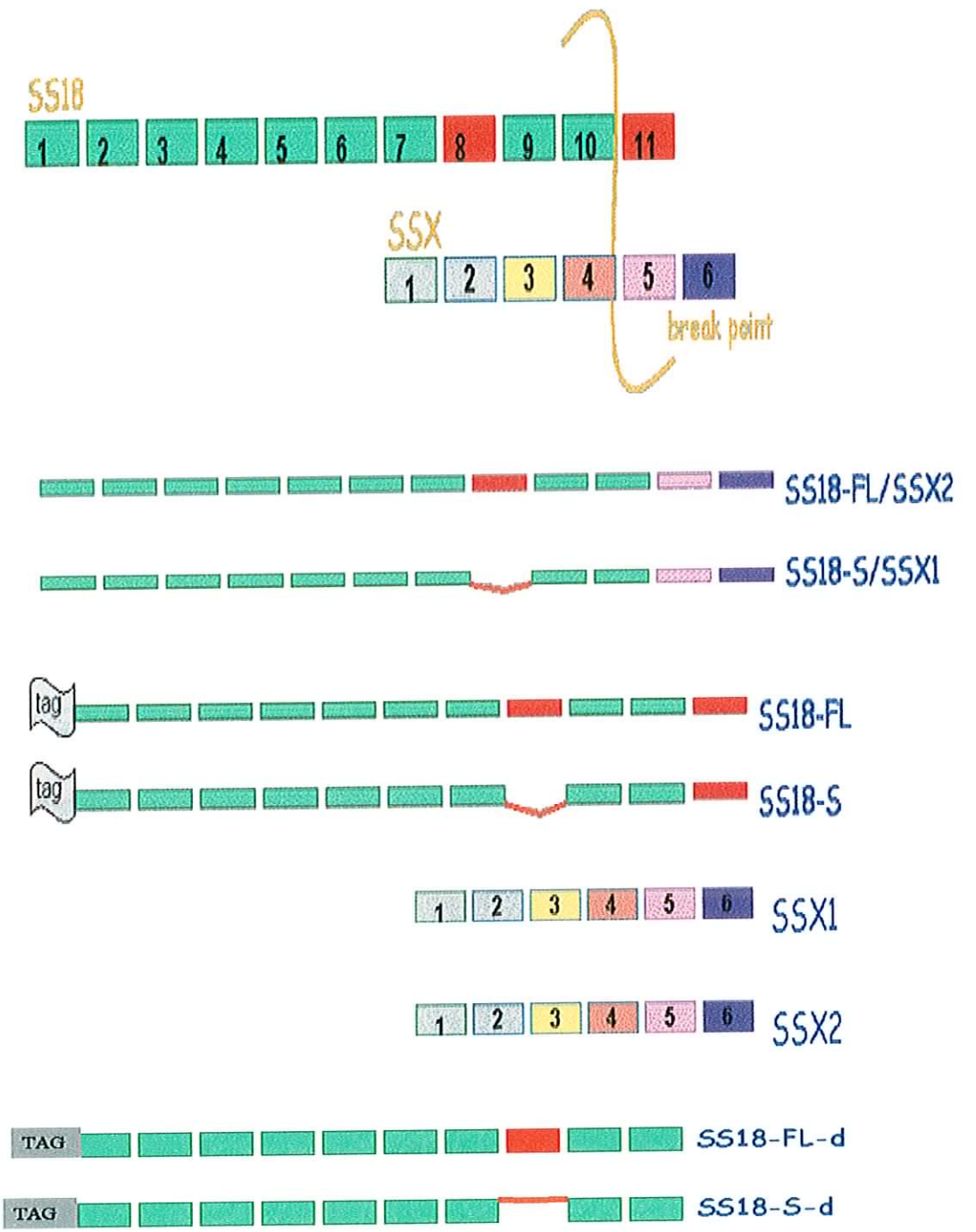
Lane 6: Plasmid with Sal1 in adjusted buffer after 1 hour.

The plasmid from lane 2 was excised and purified for the ligation with the PCR product from lane 3. (Note: Poor well shape caused ripple effect in banding at Lane 3.

3.4.2 Schematic representation of cDNA clones produced over the course of project.

The following diagrams show the clones produced, and their general structure.

Fig 3.13.1 **Schematic representation of cDNA clones produced over the course of project.**



3.6 Characterisation Studies.

The images presented below in Fig 3.14 represent immunostaining using a polyclonal antibody against transfected SS18-SSX fusion gene. Photograph 1 shows nuclear specific staining observed in NIH 3T3 cells when transfected with SS18/SSX2 fusion gene recombinant cDNA. Photograph 2 shows the background staining observed in non transfected NIH 3T3.

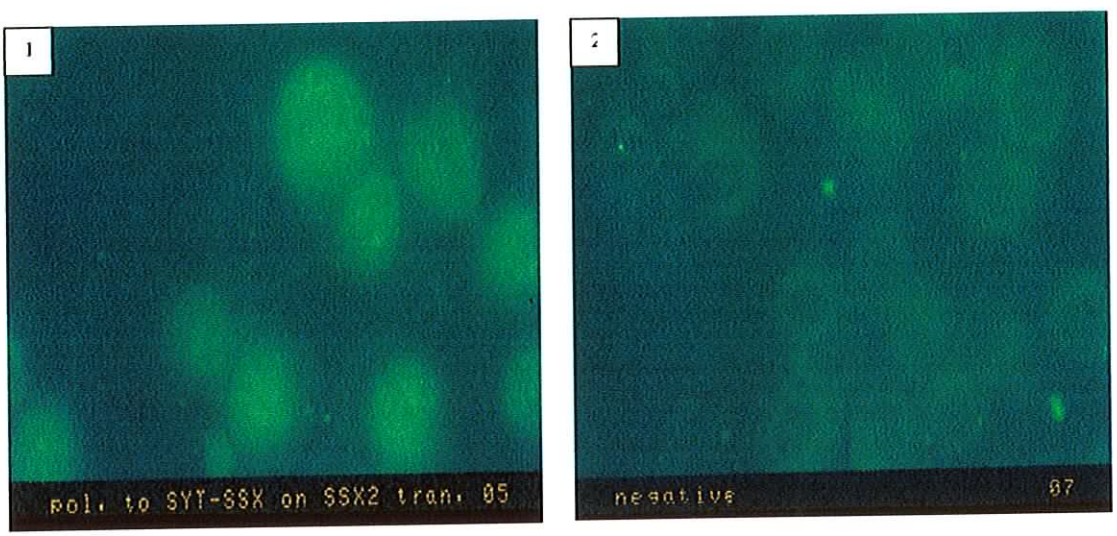


Fig 3.14 Immunoflourescence using polyclonal antibody raised against Breakpoint portion of SS18/SSX

3.7 Screening of Synovial Sarcoma tumour sample RNA for wild type SS18 gene transcripts expressing exon 8.

This RT-PCR assay was run to assess whether synovial sarcoma tumours expressed both forms of wild type SS18 in the fusion gene.

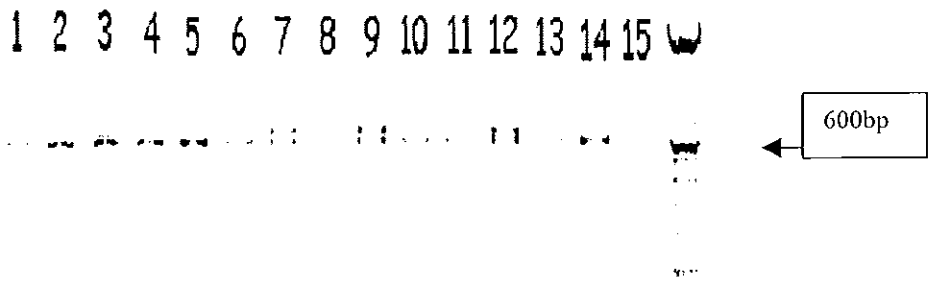


Fig 3.15 PCR analysis of 14 random synovial sarcoma tumours for SS18-S and SS18-FL presence.

Lanes 1-14 contain RT-PCR product amplified from synovial sarcoma tumour sample total RNA. Lane 15 contains negative control. From these results it is apparent that both SS18/SSX1 and SS18/SSX2 expressing tumours carry both forms of wild type SS18 RNA. Although this is a semi quantitative method only, it may be suggested that the concentration of each SS18 form in the samples examined vary from tumour to tumour. Although there appears to be a predominance of the spliced form in the majority of tumours sampled. Too few samples have been analysed above to have any statistical significance, a large evaluation is required for reliable analysis of splicing in synovial sarcoma.

3.8 RT-PCR detection of wild type SS18 in 12 melanoma samples.

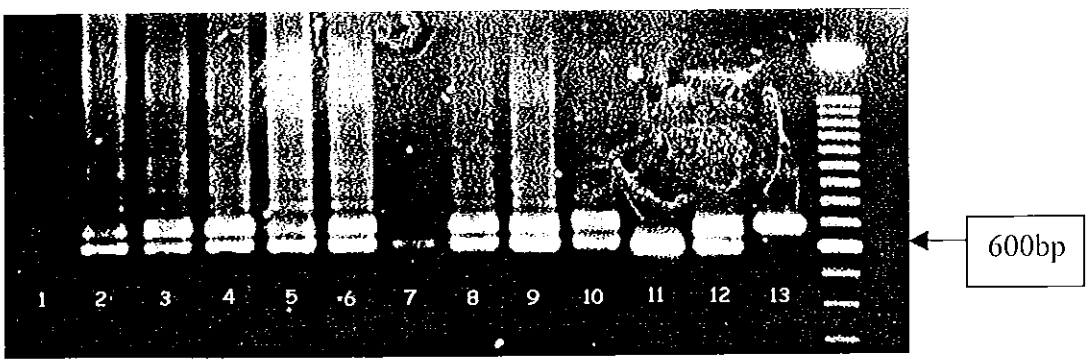
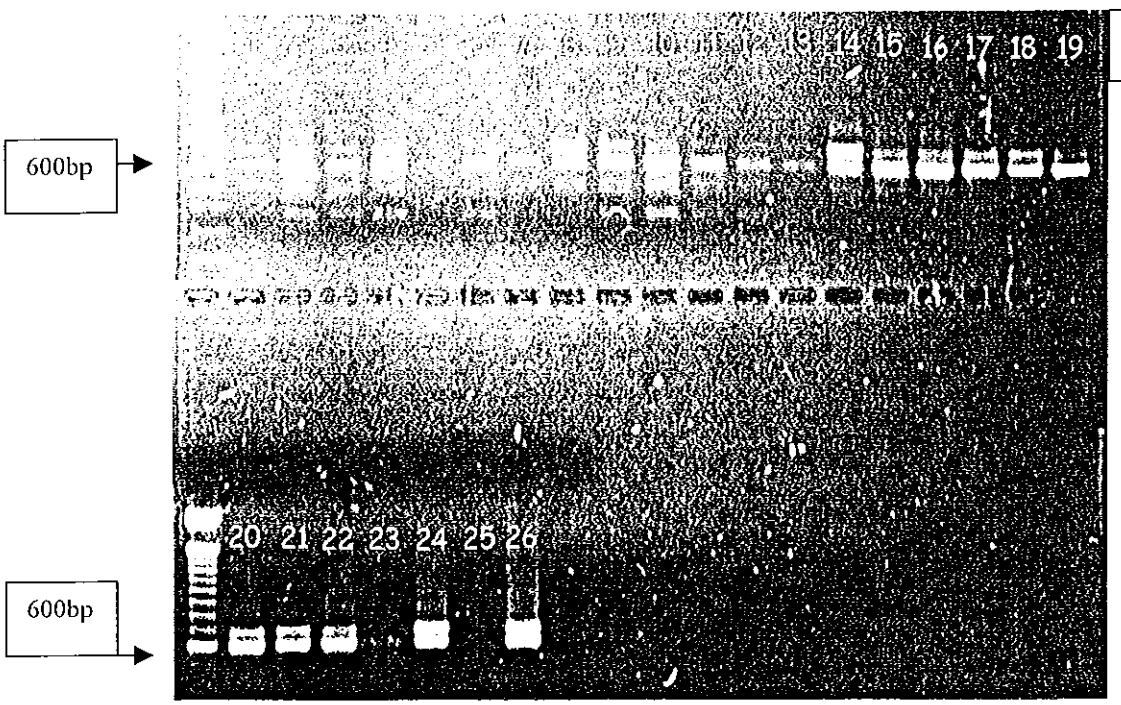


Fig 3.16 PCR Assessment of 12 melanoma tumour samples for wild type SS18 variants.

Lane 1; negative control, lanes 2-13 contain RT-PCR product amplified from malignant melanoma RNA. The results show presence of wild type SS18 in all samples tested. 9 samples show SS18-S and SS18-FL to be present in approximately equivalent amounts, whereas two samples (7 & 11) show a predominance of the spliced variant, and sample 13 shows the opposite with the unspliced form predominating.

3.9 Screening of mouse and human cDNA panels for wild type SS18 expression.

This experiment was carried out to assess levels of wild type SS18-S and SS18-FL expression in a cDNA panel from mouse and human fetal tissue.



B

Lane No.	Tissue Type	*Lane No.*	Tissue Type	*Lane No.*	Tissue Type
1	Mouse Heart	11	Mouse 15 day embryo	21	Human fetal SK muscle
2	Mouse brain	12	Mouse 17 day embryo	22	Positve control human cDNA
3	Mouse spleen	13	Mouse Positive cDNA control	23	Pos control SS tumour
4	Mouse lung	14	Human fetal brain	24	Positive control NIH cell line
5	Mouse liver	15	Human fetal lung	25	Negative control
6	Mouse SK muscle	16	Human fetal liver	26	Pos control SS tumour
7	Mouse Kidney	17	Human fetal kidney		
8	Mouse Testis	18	Human fetal heart		
9	Mouse 7-day embryo	19	Human fetal spleen		
10	Mouse 11 day embryo	20	Human fetal thymus		

Fig 3.17 Screening of mouse and human cDNA panels for SS18 expression. The first well on the left-hand side of both rows in (A) carry a molecular weight marker. The tissues tested and corresponding lanes are outlined in (B).

From the image above, the mouse tissues appear to express several splice variants of SS18. All tissues were assessed for control gene G3PDH expression and 1μg cDNA was used in each sample RT-PCR.

3.10 Lipofectamine Transfection of FLAG-SS18 into NIH 3T3 cells.

The results below are of a preliminary transfection experiment using the newly constructed FLAG-SS18-FL construct. The plasmid was transfected into NIH 3T3 cells in duplicate using lipofectamine for 5 hours. After 48 hours growth, cells were harvested. RNA was extracted and analysed by RT-PCR for expression of construct RNA using a 5' upstream primer located in the tag sequence in order to distinguish product from wild type SS18.

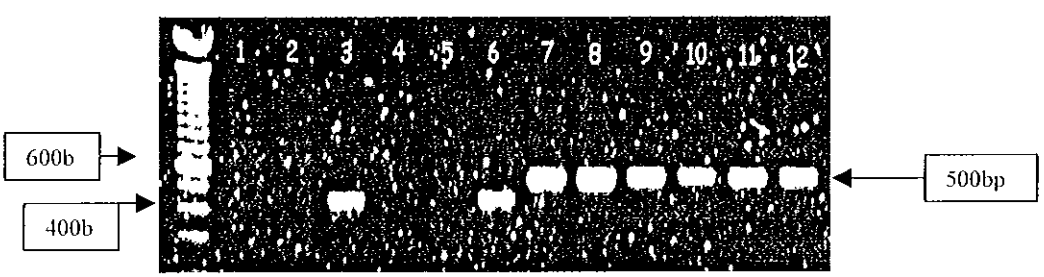


Fig 3.18 Transfection of FLAG-SS18-FL into NIH 3T3 cells.

Lanes 1&4: Untransfected NIH 3T3 cells.

Lanes 2&5: Empty Vector.

Lanes 3&6: FLAG-SS18-FL Vector.

Lanes 7-12: Actin internal control PCR for RNA integrity.

From the image above it can be seen that the FLAG-SS18-FL vector is transcribed in NIH 3T3 cells, indicating that the construct is viable in vivo.

3.11 Immunoflourescent detection of flag-tagged SS18 using Anti-FLAG antibody.

The following experiment was carried out on cells that had been transfected with FLAG-SS18-FL vector or empty FLAG vector. This was to assess if the FLAG SS18-FL RNA shown to be transcribed in the above experiment, could be translated into protein that would localise in the nucleus as wild type SS18-FL has been shown to do.

Below are immunofluorescent images of NIH 3T3 cells transfected with FLAG-SS18-FL as detected by anti-FLAG antibody.

Image A: FLAG detection in the nucleus of NIH3T3 cells.

Image B: Mock transfected cells showing diffuse background staining.

Image C: Untransfected cells showing low background staining.

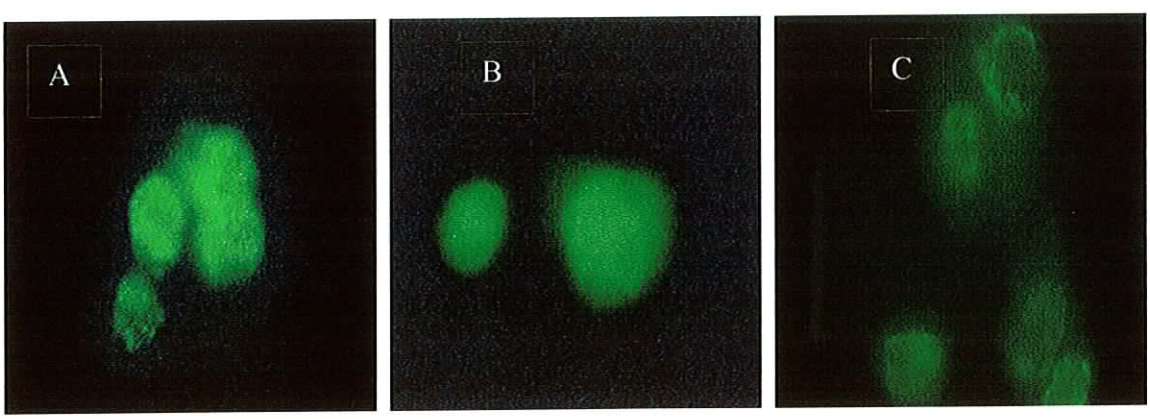


Fig 3.19 Immunofluorescent analysis of FLAG-SS18-FL transfected NIH 3T3 cells.

4.0 DISCUSSION.

Modern molecular medicine encompasses the utilization of many molecular biological techniques in the analysis of disease genes and their function. One tool by which the study of these genes has been possible is the development of cloning techniques. Cloning refers to the process of preparing multiple copies of an individual recombinant DNA molecule. The mechanism for producing these recombinant molecules involves the insertion of exogenous fragments of DNA into bacterially derived plasmid.

The aim of this project was to clone cDNA for synovial sarcoma associated fusion genes SS18/SSX1 and SS18/SSX2, and also the full-length wild type genes SSX1, SSX2, and SS18. This bank of clones would then be used for functional studies into the role these genes play in normal and malignant environments.

To achieve this a PCR based cloning strategy was employed. By amplification of the gene of interest using specific primers located in the 5' and 3' flanking regions of the gene, full-length cDNA could be generated for insertion into plasmid. This method was considered advantageous given that the full-length sequence of the fusion genes was published and primers therefore easily designed. Also the ability of plasmids to produce high cloning efficiency and their use in both prokaryotic and eukaryotic systems was attractive as it meant a single vector format could be used to generate multiple copies of the target gene and deliver it into the nucleus of the target cells.

Several vectors were used throughout the project to clone the required genes, each having distinct features necessary for specific use. For cloning of the SS18/SSX fusion genes the pTarget Mammalian Expression Vector System was chosen as it allowed use in both prokaryotic and eukaryotic systems. Thus one vector could be used to produce and assay the target gene. In the case of the wild type genes pGemt-Easy vectors were used, as they allowed simple insertion of the target sequence by T-A cloning, therefore these genes could be rapidly cloned and stored for future use. Also they contained large multiple cloning sites with many restriction enzyme recognition sites present to allow sub-cloning of the inserted gene into more specific vectors for functional analysis. The final type of vector chosen was pCMV-Tag vectors.

These allowed high level constitutive expression in mammalian cells. The tagged portion of the vector is transcribed adjacent to the target sequence and allows assay of the gene product using antibodies directed against the tag.

The procedure for cloning of SS18/SSX2 has been presented in a step-wise format in the results section and started with extraction of RNA from a histologically diagnosed fresh frozen synovial sarcoma tumour known to express SS18/SSX2 RNA. The RNA integrity was assessed, with good quality RNA denoted by clear banding of the 28s and 18s Ribosomal RNA being present. This was necessary to ensure maximum potential for full-length SS18/SSX2 cDNA to be generated.

Using a robust RT-PCR protocol, full-length cDNA of 1.6kb was generated *(Fig 3.2)*. This PCR product was purified and used in a ligation reaction with the pTarget plasmid before being transformed into competent E.Coli cells. Blue and white screening was used to distinguish between colonies of bacteria harbouring plasmid.
Upon gene insertion into the lacZ gene site, the plasmids lose b-galactosidase activity, and when stained on X-Gal containing agar plates, appear as white colonies. Blue colonies will contain plasmids without the insert. Twenty positive colonies were selected and assayed for presence of full-length SS18/SSX2 sequence (*Fig3.3*) The majority of the colonies contained full-length sequence and approximately ten were chosen at random for initial sequence analysis. This utilised a forward and reverse primer located at the 5' and 3' ends of the plasmid. BLAST analysis of the resultant sequence determined whether the clones contained correctly oriented SS18/SSX2 gene. A disadvantage of the T/A cloning system was that insertion could be in either direction thus reducing efficiency for the target by fifty percent. Correctly oriented clones were fully sequenced using primers where the sequence product of one primer overlapped that of the next. Samples were run in triplicate to ensure correct calling of the bases (refer to information presented in Fig 3.5) and from this analysis a full-length correctly oriented cDNA clone for SS18/SSX2 was retrieved.

All cDNA cloning exercises did not produce such consistent results as those obtained for SS18/SSX2. Below are discussed the cases where deviation from the SS18/SSX2 protocol was necessary to overcome problems.

The cloning of SS18/SSX1 from tumour samples carrying this t(X;18) breakpoint RT-PCR produced a smear at 1.2-1.6kb. The PCR reaction did not appear as efficient for SS18/SSX1 but after running the entire sample on a gel in low melting point agarose for increased separation, a dense region of banding was observed at 1.6kb (*Fig 3.7*). Although the chance of full-length SS18/SSX1 retrieval was low the band was excised and purified before ligation. Screening of the resultant colonies produced clones with a range of insertion size (*Fig 3.8*), but a small number of colonies produced held full-length clones. After sequencing of these clones, full-length SS18/SSX1 was retrieved. As the cloning procedure is robustly effective, optimised PCR reactions, although preferable are not necessarily required for retrieval of the full-length target sequence.

For SSX gene cloning, melanoma samples available in the laboratory were used to retrieve the desired sequences. As SSX gene expression has been observed in melanoma samples (Tureci *et al.*, 1998, dos Santos *et al.*, 2000, Ayouub *et al.*, 2003) a set of consensus primers flanking the 5' and 3' end of the SSX genes were used to screen three melanoma samples and a DFW cell line in a magnesium titration experiment (*Fig 3.9*). Melanoma sample two appeared to produce the most specific reaction and was chosen at 2mM MgCl for excision and purification before ligation. The transformation of full-length sequence was visible as was lower sized product in some clones, possibly due to carry over of non-specific product from the PCR reaction (*Fig 3.10.*)

All positive clones were shown to carry SSX2 sequence. Several attempts to clone the SSX1 gene from melanoma failed with all clones screened bearing SSX2 sequence exclusively. A single correctly oriented full-length SSX2 clone was selected and stored. To try to avoid SSX2 and isolate SSX1 carrying tumours, thirteen melanoma samples were screened using SSX1 and SSX2 specific primers (see Tureci *et al.*, 1998).

From this result melanoma sample 8 was chosen for cloning as it appeared to express high levels of SSX1 and undetectable levels of SSX2. Full-length SSX1 clones were subsequently retrieved from this melanoma sample and stored (*Fig 3.12*).

In the case of the tagged vector project, the lack of a commercially available or sufficiently reliable antibody against the SS18 gene product led to the devising of a method of detection for SS18 protein by tagging with an epitope. As two splice forms of SS18 were now known, having been discovered initially during SS18/SSX2 clone sequencing, tagging of SS18FL and SS18-S sequences with different epitopes would enable distinction between both variants. This would allow experiments such as double immunoflourescence to be carried out to assess if different nuclear localisation patterns between the variants occurred. Also western Blot and immunoprecipitation experiments could be performed in the future, and could discern whether the variants associated with similar or distinct proteins and protein complexes.

Both SS18-S and SS18FL were cloned into pGEMT Easy vectors from a melanoma tissue sample using the protocol developed for SS18/SSX2 . For addition of the tags, it was initially hoped that sub-cloning using restriction enzymes to digest out the gene of interest from the cloning vectors followed by insertion of the target sequence into a tagged plasmid (cut by the same enzyme) would be the most convenient method. It was found that there was no enzyme sequences compatible to both vectors that would not also cut the gene of interest. Therefore the gene sequences were amplified by PCR using a highly specific Taq polymerase: Accu Taq LA to minimise incorporation of incorrect nucleotides. Upon insertion, the full-length of the genes were fully sequenced again to ensure that the coding was correct.

The primers used contained restriction sites at their 5'end to allow digestion with restriction enzymes. An enzyme was then used to cut open the plasmid and create overhangs for gene insertion. Two methods were used to achieve this. The first method used a single enzyme to open the plasmid followed by dephosphorylation to prevent re-ligation.

The gene sequence for insertion contained the same restriction enzyme recognition sequence at either end and the primers used to amplify the gene sequence carried the required restriction enzyme sequence at the 5' end. Once plasmid and PCR product were digested and purified they were ligated together. This method was successful for FLAG-SS18-FL. In the case of HA-SS18-S several attempts at a single enzyme format proved unfruitful. To overcome this, a double digest was carried out using two different enzymes. As both vector and gene sequence would possess two different overhangs at their 5' and 3' ends this prevented re-ligation without dephosphorylation and would ensure insertion of the gene sequence in the correct orientation. Although transformation was lower than with the single enzyme cut method this was more than compensated for, as all clones possessed the full-length sequence in the correct orientation. In hindsight the two enzyme digest method would have been a preferable route to take in the first place, as it would save both time and resources on screening of clones and repeated ligations and transformations.

4.1 Characterisation Studies.

Once all clones had been produced and banked for use, the next phase of investigation could begin. A polyclonal antibody raised against the breakpoint of the SS18/SSX2 fusion gene was used to determine if nuclear staining for SS18/SSX2 could be detected in NIH3T3 cells by immunoflourescence. From results shown in *Fig 3.14* it was clear that those cells transfected with the construct showed much greater fluorescence than the untransfected cells. This supports the theory that the construct can be transcribed and the gene sequence translated into protein within the cell. Attempts to produce a clear signal for the construct using Western Blot were unsuccessful. This was somewhat unsurprising as detection of the fusion gene in synovial sarcoma cell lines using an antibody against the break-point region of the fusion protein by others in our group proved unsuccessful also. This may be due to the efficiency of the antibodies for their target given that they are polyclonal in nature, or may be due to inaccessibility of the antibody to the necessary epitope in the cell nucleus, given that the emerging picture places the SS18/SSX proteins in association with large multi-subunit complexes.

For testing of the tagged vectors a similar experiment was carried out. Cells were transfected with constructs and expression was checked at the transcription level by RT-PCR (*Fig 3.18*) and at the protein level by immunofluoresence (*Fig 3.19*). Data shown represents the FLAG-SS18-FL construct. For immunoflourescent analysis, the cells transfected with the construct fluoresced brighter green than the untransfected and empty vector carrying cells, with a distribution pattern appearing speckled against a diffuse background, whereas the untransfected and empty vector carrying cells appear diffuse only. In accordance with this SS18 expression in the nucleus has been reported as punctate or speckled (Brett *et al.*, 1997, Theate *et al.*, 1999, dos Santos *et al.*, 2000a) Considering that the base sequence and orientation of the SS18ins insert in the vector were correct, and that RT-PCR results were positive at the transcription level, expression of the FLAG tagged SS18-FL protein appears possible from this construct, however detection by western blot was unresolved at the end of this project. Several attempts to produce a clear signal from the constructs in this regard proved unsuccessful. Non-specific background interference was present and may be due to the association of SS18 with different nuclear bodies and complexes. More optimisation was required for this type of experiment. Due to time constraint further characterisation of cloned genes was not possible, but from the preliminary results above, those tested appeared to work and could be used for functional studies.

However transfection studies using the above SS18 constructs have since been carried out in our group. The result of this experiment is located in the appendix 1 of the thesis. From the fluorescent images it is apparent that those deletion constructs lacking exon11 show poor nuclear staining, with the SS18-FL-d construct appearing to fluoresce outside the nucleus in the cytoplasm of the cell. In contrst to this the constructs containing exon11 have strong signals emanating from the nucleus only. This suggests that exon 11 has a role in the localisation and/or retention of the SS18 protein within the nucleus. Exon 11 contains a SH2 domain, is lost in the t(X;18) translocation, and is known to be involved with cellular adhesion. Loss of this region of the protein may lead to SS18 deregulation through the inability of SS18 to interact with other proteins in the correct manner. Further experiments using the SS18 deletion constructs may help answer these questions.

The SS18/SSX2 construct produced during this project has also been used functionally in our group to demonstrate that transfection of mouse fibroblasts with SS18-SSX cDNA increased the stability of cyclin D1. SS18-SSX was shown to be critical for the protein level of cyclin D1 in synovial sarcoma cells and use of anti-sense against the fusion transcript resulted in a drastically decreased Cyclin D1 level. The construct was used to correlate these results in the NIH 3T3 cell line system (Xie *et al.*, 2002). This study suggested SS18-SSX-dependent expression of cyclin D1 may be an important mechanism in the development and progression of synovial sarcoma.

4.2 Analysis of splice variant expression in tumours and fusion transcripts.

During full-length sequencing of the SS18/SSX2 gene a 93 base pair in-frame insertion in the SS18 portion of the fusion gene within the QPGY domain, one that had not been previously reported in the human SS18 gene was identified. When this portion of the SS18 gene was run against the BLAST database, the sequence had 100% homology with a murine SS18 sequence previously reported in the literature (de Bruijn *et al.*, 1996) This suggested that the insert was not a cloning artefact but may be an alternatively spliced exon unseen to date. The reason for this may have been the common use of published primers for detection of the SS18/SSX fusion genes located downstream of the new exon. The first publication of the SS18/SSX fusion sequence was of a clone lacking exon 8. (Clark *et al.*, 1994). Cloning of a novel fusion transcript (SS18/SSX4) in our group from a synovial sarcoma tumour was found to contain insertion of the 93 base pair exon also. (Brodin *et al.*, 2001) This provided more evidence that the 93 base pairs were not a random insertion.

Subsequently the full genomic sequence of both human and murine SS18 was published showing that the human SS18 composed of 11 exons over 70kb with an alternately spliced 8^{th} exon. The murine SS18 was shown to possess 4 splice variants: namely mSs18-α the unspliced form, mSs18-β minus exon 8, mSs18-χ minus exons 7 and 8, and mSs18-δ minus exons 5 and 8 (De Bruijn *et al.*, 2001). We made preliminary analyses on various normal tissues of murine and human origin, as well as malignant melanoma and synovial sarcoma samples for expression of alternate splicing in wild type SS18.

At the same time Tamborini *et al.*, (2001) discovered the same insertion in SS18 and analysed its expression in human tissues and within the SS18/SSX fusion transcripts noting differing amounts of both forms of SS18 expression in tissues and consistent over expression of SS18-FL in synovial sarcoma tumour samples suggesting a role in pathogenesis. Our analyses in melanoma samples also showed a predominant over-expression of SS18-FL in the tumour samples, but in synoival sarcoma tumour samples the expression levels of wild type SS18 varied. (*Fig 3.15 & 3.16.*) These results were a preliminary investigation into the expression patterns of splice variants and show that both forms of SS18 are expressed at different levels in different tumour samples.

As the sample number used was small, any further conclusions cannot be drawn. A large scale statistically significant study would be necessary before any firm conclusions regarding the contribution of SS18 splicing ratios to tumourigenic progression could be drawn, in synovial sarcoma or melanoma.

Analysis of SS18 splice variants in normal mouse and human tissues showed varied expression. *Fig 3.17a* shows 4 splice variants present in the mouse in correlation with genomic analysis by de Bruijn *et al.*, (2001). The human form of SS18 appears to possess only one splice variant, although a faint 3rd band appears to be present in human fetal brain, lung and liver the size of which is approximate to the mouse splice variant lacking exon 7 & 8 (mSs-γ). A recent scan of the myScience database for SS18 has shown that although only two splice variants are known in the public databases, the Celera discovery system (CDS) database counts six human SS18 splice variants. The location and sequence of these variants is available only upon subscription to the CDS system.

Considering that the QPGY domain has been shown to possess transcriptional activating properties prompts the question whether the splicing of this region has a functional significance. This was recently addressed (Morimoto *et al.*, 2003) where the NIH 3T3 cell line was transfected with SS18-FL or SS18-S and the growth rate of the two transfects analysed. The result showed SS18-FL transfected cells grow faster and may confer a gain

of function to the tumour. In our group recent preliminary analysis of SS18 transcript type incorporation into the fusion gene has shown that there is a correlation between SS18/SSX1 containing tumours and the spliced fusion transcript SS18-S/SSX1 where the spliced transcript is predominant. (P= 0.006). As SS18/SSX1 containing synovial sarcomas carry a poorer prognosis, these results are being assessed in a larger cohort through a multi-centre study to investigate whether there is a correlation between transcript type and clinical outcome. (B. Brodin personal communication).

Exons 5-11 of SS18 code for the QPGY domain in human and mouse genomes. All murine splice variants and the known human splice variant lack exon 8 and are all located within the QPGY domain. As this region has been observed to have a functional role (Theate *et al.,* 1999, Nagai *et al.*, 2001) it highlights a possible regulatory function for this exon which may prove crucial to understanding the interactions and role of SS18 in the cell. Ongoing research into the functional significance of Wilms Tumour-1 gene splicing events have shown that different splice variants can have distinct biological effects (Scharnhorst *et al.*, 2001).

4.3 Future prospectives:

From the bank of cloned genes assembled during this project several questions regarding the role of the SS18, SSX, and SS18/SSX genes in both normal and tumourigenic cell environments can be approached.

The nuclear localisation of tagged SS18 splice variants using double immunoflourescence techniques and differentially labelled antibodies against the two tags (FLAG & HA) could be addressed. If the pattern observed appeared different, follow on experiments using antibodies against known associating proteins such as BRM BRG1 (Theate et al., 1999), SWI (Kato et al., 2002) P300 (Eid *et al.*, 2000) etc and other nuclear proteins may show a preference between SS18 variants for particular protein complexes. This may shed light on the functional impact a shift in the ratio of splice variants may have in tumourigenic cells.

The SS18/SSX2 fusion gene generated in this project has been used to confirm experimental data obtained in synovial sarcoma cell line models (Xie *et al.*, 2002). Over-expression of transfected SS18/SSX fusion gene constructs can also be used to assess the transforming potential of the fusion proteins in various cell line systems, and the high expression level of the fusion protein achieved in this way would enable better protein based studies such as immunoprecipitation to be carried out.

On the molecular level with regard to SS18 splice variants, real time PCR based analysis on a large number of tumour samples would produce valuable information. Combined with patient clinical data the extent of SS18 splicing ratios could be assessed with regard to prognosis.

There are no definitive answers yet for the oncogenic mechanism underlying development of synovial sarcoma, but as research into this field continues, the functional picture will continue to sharpen leading to clearer knowledge of the roles played by SS18, SSX and SS18/SSX in both normal and tumourigenic cells. Currently the data being generated points toward a role for these proteins in complexes involved in transcription regulation. The future use of clones generated during this project will hopefully help answer some of these questions.

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6.0 APPENDICES

Appendix 1.

Transfection of NIH 3T3 cell line with SS18 constructs.

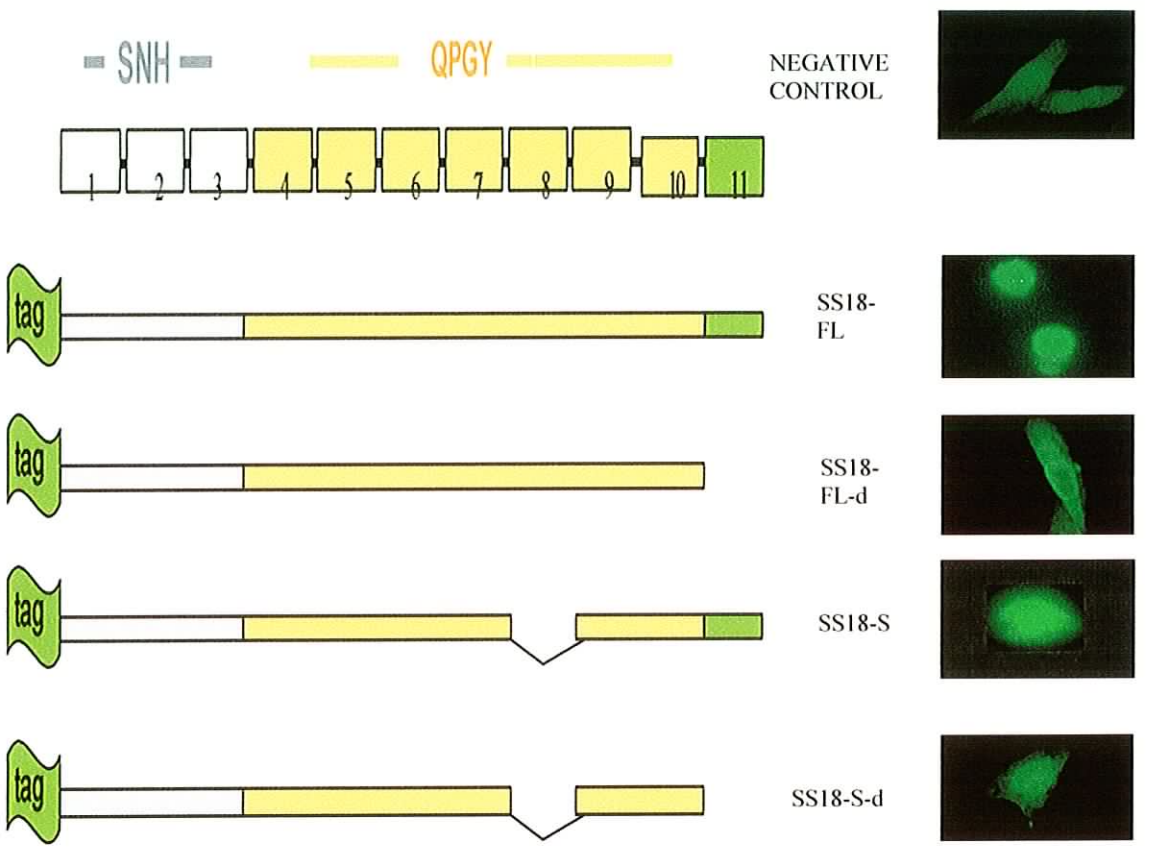

Fig6.1 Immunofluorescent analysis of NIH 3T3 transfected with SS18 deletion constructs.

Appendix 2.

Primers used in RT-PCR or sequencing reactions.

pTarget 5'	tta cgc caa gtt att tag gtg aca
SYT+1	atg ggc ggc aac atg tct gtg
RSP	tcc agt cac gac gtt gta aaa cg
SYT 343+	ggt ggg ggt cct cct gca ccg
SSX 576-	att cga gct cga att cct agg gga gtt act cgt ca
SYT 913+	att ccc gcg gct cga gca aca gca aga tgc ata cca
SYT 935-	ccc tgg tat gca tct tgc tg
SSX-A	cac ttg cta tgc acc tga tg
SSX 2-4	tct cgt gaa tct tct cag agg
SSX 1-4	ggt gca gtt gtt tcc cat cg
SSX 2-3	tgc tat gca cct gat gac ga
SYT B	ttc act gct ggt aat ttc c
Mel40a	atg aac gga gac gac acc t
Mel40b	cat gaa cgg aga cga cac ct
SYT 3'UTR	
FLAG 5'	caa gga tga cga cga taa g
Actin 1	cat gcc atc ctg cgtctg gac
Actin 2	tag aag cat ttg cgg tgg acg
Pgemt F	agg gat ttg gcc cga cgt cga tg
Pgemt R	gag agc tcc gaa cgc gtt gg
Pst1 SYT+1	nnnnCTGCAG atg ggc ggc aac atg tct gtg
Sal1 SYT+1	nnnnGTCGACatg ggc ggc aac atg tct gtg
Pst1 SYT B	nnnnCTGCAG ttc act gct ggt aat ttc c
Not1 SYT B	nnnnGCGGCCGC ttc act gct ggt aat ttc c

PCR Product	*RT Primer*	*Forward Primer*	*Reverse Primer*
SS18/SSX	SSX-A	SYT +1	SSX 2-3
Vector PCR SS18/SSX		pTarg	Rsp
SSX Full length	SSX-A	Mel40a	Mel40b
Vector PCR for pGemt Vectors		PGemt-F	PGemt-R
SSX1 and 2 Screening	See Tureci O. etal Int.JCancer:77, 19-23 1998.		
Wild Type SS18 Detection	SYT 3'UTR	1st Round SYT+1	SYT-B
		2nd Round SYT 343+	SYT 935-
FLAG Tagged Vector Constructs		FLAG 5'	SYT 343-
SS18 Full-length		SYT+1	SYT-B

Table 6.1 Primer pairs used during project.

LIST OF PUBLICATIONS

Brodin B, Haslam K, Yang K, Bartolazzi A, Xie Y, Starborg M, Lundeberg J, Larsson O. (2001) Cloning and characterization of spliced fusion transcript variants of synovial sarcoma: SYT/SSX4, SYT/SSX4v, and SYT/SSX2v. Possible regulatory role of the fusion gene product in wild type SYT expression.*Gene*. 268(1-2) p.173-82.

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