Fusion of SYT to two genes, SSX1 and SSX2, encoding proteins with homology to the Kruppel-associated box in human synovial sarcoma

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We demonstrate that the cytogenetically defined translocation t(X;18)(p11.2;q11.2) found in human synovial sarcoma results in the fusion of the chromosome 18 SYT gene to either of two distinct genes, SSX1 or SSX2, at Xp11.2. The SSX1 and SSX2 genes encode closely related proteins (81% identity) of 188 amino acids that are rich in charged amino acids. The N-terminal portion of each SSX protein exhibits homology to the Kruppel-associated box (KRAB), a transcriptional repressor domain previously found only in Kruppel-type zinc finger proteins. PCR analysis demonstrates the presence of SYT–SSX1 or SYT–SSX2 fusion transcripts in 29 of 32 of the synovial sarcomas examined, indicating that the detection of these hybrid transcripts by PCR may represent a very useful diagnostic method. Sequence analysis has demonstrated heterogeneity in the fusion transcripts with the formation of two distinct SYT–SSX1 fusion junctions and two distinct SYT–SSX2 fusion junctions.

Key words: Kruppel-associated box/SSX oncogene/synovial sarcoma/SYT oncogene/t(X;18) translocation

Introduction

Specific chromosomal translocations have been described in several types of solid tumours. Characterization of these translocations has resulted in the discovery of several types of molecular abnormalities that are thought to contribute to tumorigenesis. For example, the t(12;16) translocation in myxoid liposarcoma results in the fusion of the N-terminal region of TLS/FUS with the dominant-negative, transcriptional regulator CHOP (Aman et al., 1992; Crozat et al., 1993; Rabbitts et al., 1993). In the t(11;22) translocation of Ewing’s sarcoma the N-terminal region of EWS has been found joined to the C-terminal region of FLI1 (Delattre et al., 1992) which contains a DNA binding domain. More recently the t(2;13) translocation in alveolar rhabdomyosarcoma was found to result in the fusion of the N-terminal region of the PAX3 transcriptional regulator to the C-terminal region of FKHR (Barr et al., 1993; Galili et al., 1993).

Synovial sarcoma is an aggressive, soft tissue sarcoma that occurs most commonly in young adults and frequently arises in the limbs adjacent to joints and tendons (Enzinger and Weiss, 1988). Biphasic and monophasic histological subtypes can be distinguished (Enzinger and Weiss, 1988; Fisher, 1994). In biphasic tumours both epithelial-like cells and spindle-shaped cells are observed, while monophasic tumours contain only spindle cells. Cytogenetic analysis of synovial sarcomas has demonstrated the presence of a characteristic chromosomal translocation, t(X;18)(p11.2; q11.2), in both tumour subtypes (Smith et al., 1987; Turc-Carel et al., 1987; Wang-Wuu et al., 1987; Cooper and Stratton, 1991; Limon et al., 1991; Knight et al., 1992b; Cooper, 1993). This translocation is present in a high proportion of synovial sarcomas indicating that it may represent a useful diagnostic marker. Furthermore, in some tumours the t(X;18) is the only detectable cytogenetic abnormality, indicating that its generation may be a key step in tumour development. In previous studies using fluorescence in situ hybridization (FISH) it was demonstrated that a 450 kb yeast artificial chromosome (YAC) containing an ornithine-δ-aminotransferase pseudogene region designated OATL2 spans the breakpoint (Knight et al., 1992a). Use of this YAC in cloning strategies led to the demonstration that t(X;18) results in the fusion of the SYT gene located on chromosome 18 to the SSX gene (now redesignated SSX2) located on the X chromosome (Clark et al., 1994). The resulting chimaeric gene is transcribed to form a 5’-SYT–SSX2-3’ hybrid transcript which is in turn translated to form a SYT–SSX2 fusion protein. Both the normal SYT protein and the C-terminal SSX2 region present in the SYT–SSX2 fusion product failed to exhibit homology to any known protein sequences (Clark et al., 1994).

In recent analyses of the t(X;18) it has become apparent that there is heterogeneity in the position of the X chromosome breakpoint, with translocations occurring not only within the OATL2 region but also within a second OAT pseudogene region called OATLI that maps at least 2 Mb telomeric to OATL2 at Xp11.2 (Leeuw et al., 1993, 1994; Shipley et al., 1994). In this study we report the cloning of a gene, designated SSXI, which becomes joined to SYT in t(X;18) variants involving the OATLI breakpoint region. To gain a better understanding of the molecular consequences of the t(X;18) translocation we have, through the sequencing of cDNA clones, investigated the normal products encoded by the SSXI and SSX2 genes. Analysis of 32 synovial sarcomas has provided further details of both the molecular variation that exists for the cytogenetically defined t(X;18) and the frequency of this abnormality in synovial sarcomas.
Results

Cloning of SSX1
Hybridization of a 3’ SSX2 probe from the OATL2 breakpoint to a YAC spanning the OATL1 locus has demonstrated the presence of SSX-related sequences within this region suggesting that t(X;18) translocations with breakpoints within OATL1 may also involve SSX sequences (Clark et al., 1994). In support of this hypothesis Northern analysis demonstrated that SYT (Figure 1A) and SSX2 (Figure 1B) probes each detected a 2.4 kb transcript in the synovial sarcoma cell line HS-SY-II, which contains the breakpoint at the OATL1 locus. This transcript was the same size as the SYT–SSX2 hybrid transcript found in A2243 cells, which contain the breakpoint at the OATL2 locus (Figures 1A and B). In order to characterize the SSX sequence involved in the OATL1 breakpoint, RNA extracted from the HS-SY-II cell line was reverse-transcribed and subjected to PCR using primers which were originally designed to amplify hybrid transcripts encoded by the t(X;18) translocation involving the OATL2 locus (Clark et al., 1994). The observed PCR product was the same size as the product observed for the A2243 cell line (Figure 1C). Cloning and sequencing of the HS-SY-II PCR product revealed that SYT had become fused to a sequence, designated SSX1, that was distinct from but highly homologous to SSX2. The junction sequences of SYT–SSX1 and SYT–SSX2 are presented in Figure 2A and B, respectively. A comparison of the sequences of the SSX1 and SSX2 regions present in these hybrid transcripts is shown in Figure 3. Both the SYT–SSX2 transcript and the newly isolated SYT–SSX1 hybrid transcript encode fusion proteins in which the C-terminal eight amino acids of the normal SYT protein have become replaced by 78 amino acids encoded by an SSX gene (Figure 4). The SSX1 and SSX2 protein sequences present in these two fusion products have 66 of 78 amino acids in common (Figure 3).

Normal SSX1 and SSX2
To learn more about the expression and possible function of SSX1 and SSX2, RNA was extracted from a selection of human tissues and tumour cell lines and examined by Northern analyses using an SSX probe under hybridization conditions that would detect both SSX1 and SSX2 transcripts (Figure 1D). These studies identified discrete transcripts of 1.6 kb in normal testis and in the HT1080 fibrosarcoma cell line (Figure 1D). In addition a more diffuse band in the size range 1.4–1.6 kb was detected in A673 rhabdomyosarcoma cells and at low levels in normal thyroid (Figure 1D).

To characterize the transcripts of the normal SSX1 and SSX2 genes a 3’ SSX2 gene probe was used to screen cDNA libraries prepared from RNA extracted from the HT1080 cell line and from human testis. Sequencing of the clones isolated from the HT1080 cDNA library produced a continuous sequence of 766 bp that contained an open reading frame of 188 amino acids. The 3’ region of this sequence exactly matched the SSX1 sequences identified in the SYT–SSX1 fusion transcript from the HS-SY-II cell line indicating that the HT1080 cDNA clones correspond to transcripts of the normal SSX1 gene. The cDNA clones isolated from the human testis cDNA library corresponded exactly to the SSX2 sequence present in the SYT–SSX2 fusion transcript from the A2243 cell line. Since these clones were not full length, 5’ RACE and PCR amplification were used to isolate the remaining 5’ SSX2 sequences.
from reverse-transcribed testis RNA. The normal SSX2 sequence obtained in these studies has an open reading frame encoding a protein of 188 amino acids which exhibits a high extent of homology (81% identity) to the 188 amino acid, SSX1 protein (Figure 3). Other common features of the SSX1 and SSX2 proteins include the abundance of charged amino acids (40–41%) and the presence of consensus sequences for N-glycosylation and tyrosine phosphorylation. It is also notable that both the SSX1 and SSX2 proteins contain acidic C-terminal tails in common with several nuclear proteins such as the HMG proteins (Shirakawa et al., 1990), TAFII250/CCG1 (Sekiguchi et al., 1991) and yeast SIN1 (Kruger and Herskowitz, 1991).

**SSX1** and **SSX2** PCR products generated from reverse-transcribed RNA can be distinguished by digestion with the restriction enzymes *LspI* and *SmaI*. *LspI* only digests **SSX1** while *SmaI* only digests **SSX2** (Figure 3). Using this approach we demonstrated that both **SSX1** and **SSX2** were expressed in the thyroid (Figure 1E). By comparison the PCR product observed for testis was derived predominantly from **SSX2** transcripts while that obtained from HT1080 cells was derived mainly from **SSX1** transcripts (Figure 1E).

**Homology to the Kruppel-associated box**
A search of the EMBL and SWISS PROT databases using the program BLASTP revealed that the N-terminal ends of **SSX1** and **SSX2** exhibit homology to an evolutionary conserved domain called the Kruppel-associated box (KRAB). The KRAB was identified as a region of ~75 amino acids which has been found exclusively at the N-terminal end of approximately one third of zinc finger proteins (Bellefroid et al., 1991; Margolin et al., 1994; Witzgall et al., 1994). This conserved box can be subdivided into two domains: the KRAB-A box which has been implicated in transcriptional repression (Licht et al., 1993; Margolin et al., 1994; Witzgall et al., 1994) and a shorter box called KRAB-B. The **SSX1** and **SSX2** proteins show greatest homology (39–49%) to KRAB-A and contain a high proportion of the amino acids which are usually conserved between the KRAB-A regions of different zinc finger proteins (Figure 5). In common with the KRAB, **SSX1** and **SSX2** are rich in charged amino acids but notably both of the **SSX** proteins lack the C-terminal Kruppel-class zinc finger motifs which are invariably found in other proteins containing the KRAB domain.

**Different types of SYT-SSX transcripts**
To investigate the degree of variability in the t(X;18) breakpoint, RNA from a series of 32 synovial sarcomas and cell lines was reverse-transcribed and subjected to PCR with **SYT** and **SSX** primers. PCR products were detected in 29 of the tumours. In the three tumours which did not yield PCR products we failed to detect either abnormal **SSX** transcripts by Northern analysis or rearrangement of the **SYT** gene by Southern analysis (Table I). Sequencing of the PCR products revealed that in 26 of the tumours, the junction between **SYT** and **SSX** occurred at the same position as the **SYT-SSX1** and

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**Fig. 2.** Schematic representation of the SYT-SSX1 and SYT-SSX protein junctions together with nucleic acid and protein sequences. (A) The SYT-SSX1 junction originally identified in the HS-SY-II cell line. (B) The SYT-SSX2 junctions originally identified in the A2243 cell line. (C) The SYT-SSX1 junction present in STS498. (D) Representation of a complex fusion that is found in tumours STS159 and STS253. In these two tumours an 87 bp sequence that was shown by PCR and Southern hybridization to map to the X chromosome was found between previously identified **SYT** and **SSX2** sequences. Hatched boxes denote **SYT** protein and the filled boxes represent **SSX1** and **SSX2** sequences. Numbers above the boxes indicate the amino acids at the position of the junction. The number of tumours which contain each type of junction are listed.
Fig. 3. The human SSX1 and SSX2 cDNA nucleotide and predicted amino acid sequences. The SSX1 nucleotide sequence was obtained from clones isolated from an HT1080 human fibrosarcoma cDNA library. Normal SSX2 nucleotide sequence was obtained from cDNA clones isolated from a human testis cDNA library and from clones isolated in PCR and 5' RACE experiments from reverse-transcribed testis RNA. The SSX2 sequence presented extends to the 5' end of clones isolated by 5' RACE. The amino acid sequence is numbered from the methionine predicted to act as the site of translational initiation (Kozak, 1991). The position of the SSX1 and SSX2 breakpoint found in the majority of synovial sarcomas is indicated. The sequences 3' to this breakpoint, which are present in the SYT–SSX1 and SYT–SSX2 hybrid transcript are shown in bold type. Additional 3' untranslated SSX2 sequence obtained during the analysis of SYT–SSX2 cDNA clones was shown to be present in an earlier study (Clark et al., 1994). The regions of the SSX1 and SSX2 proteins exhibiting homology to the Kruppel-associated box A are underlined. A polymorphism (T→C) is found at position 634 in ~50% of the sarcomas with translocations involving SSX1. The SSX1 and SSX2 sequences can be distinguished by digestion with Smal and Lspl. Smal only digests SSX2 while Lspl only digests SSX2. The Smal and Lspl restriction sites are shown. Accession numbers have been obtained for SSX1 (X86174) and SSX2 (X86175).

SYT–SSX2 breakpoints described above for the HS-SY-II and A2243 cell lines respectively. The uniform structure of these transcripts agrees with the breakpoint being present in specific introns of the SYT, SSX1 and SSX2 genes. Three tumours containing variant transcripts were detected. In tumour STS498 the SYT–SSX1 transcript contained an additional 144 bp of normal SSX1 sequence but had lost 132 bp of SYT sequence, indicating that heterogeneity in the position of the breakpoint can occur within both the SYT and SSX1 genes. Tumours STS159 and STS255 contained a second variant transcript in which there was an insert of 87 bp between previously identified SYT and SSX2 sequences. Mapping by PCR and Southern analyses using OATL1 and OATL2 YACs and somatic cell hybrid lines containing individual human chromosomes demonstrated that this sequence is derived from the X
chromosome (data not shown). The 87 bp sequence did not, however, match the sequences present in the normal SSX2 transcripts and could not be detected by RT-PCR in cell lines and tissues that expressed normal SSX1 and SSX2 transcripts suggesting that it may represent a cryptic exon that is derived from SSX2 intronic sequences. The generation of a cryptic exon from intronic sequences has also been documented for the EWS–FLI1 gene rearrangement observed in Ewing's sarcoma (Zucman et al., 1993).

Further examination of the 19 tumours with breakpoints involving SSX1, showed that four had a biphasic histology and 15 were monophasic. Considering the 10 tumours with breakpoints involving SSX2, two were biphasic and eight were monophasic. Thus for this series of 29 tumours containing SYT–SSX fusions (Table I) there was no correlation between the particular SSX gene involved in the translocation and the tumour subtype.

The SYT–SSX hybrid transcripts are encoded by the der(X) chromosome formed by t(X;18). Using PCR and Northern analyses we failed to detect transcripts consisting of 5′ SSX and 3′ SYT which would be encoded by the der(18) in synovial sarcomas. This observation highlights the importance of the der(X) chromosome and its encoded 5′-SYT–SSX-3′ transcript in the development of synovial sarcoma.

Discussion

We have shown that the t(X;18)(p11.2;q11.2) synovial sarcoma translocation results in the fusion of SYT to either of two closely related genes, SSX1 or SSX2. Fusion of a single gene to different partners has been observed for translocations found in solid tumours and haematopoietic malignancies (Rabbitts, 1994). In the solid tumour, Ewing's sarcoma, 5′ regions of the EWS gene on chromosome 22 can become fused to either FLI1 or ERG, two members of the ETS transcription factor family of genes located, respectively, on chromosomes 11 and 21 (Delattre et al., 1992; Zucman et al., 1993; Sorensen et al., 1994). In alveolar rhabdomyosarcoma 3′ FKHR sequences on chromosome 13 usually become joined to 5′ PAX3 sequences from chromosome 2 but in some tumours the FKHR gene becomes joined to the PAX7 gene located in chromosome 1 (Barr et al., 1993; Galili et al., 1993; Davis et al., 1994). For these and other malignancies the alternative translocation partners are usually located on distinct chromosomes and the degree of homology between the different partners is considerably less than that observed between SSX1 and SSX2 (Rabbitts, 1994). Thus

Fig. 4. Schematic representation of wild type SYT and SSX proteins and of the SYT–SSX chimaeric protein. Both SSX1 and SSX2 proteins are predicted to be 188 amino acids in length and contain an N-terminal region (KB) that exhibits homology to the Kruppel-associated box (KRAB) domain commonly found in zinc finger proteins. Isolation of the 5′ end of the normal SYT transcript by 5′ RACE has allowed us to predict that translational initiation occurs at the first methionine of the SYT protein sequence presented in Clark et al., (1994). The normal SYT protein is therefore predicted to be 387 amino acids in length. Formation of SYT–SSX1 and SYT–SSX2 in most tumours involves the C-terminal eight amino acids at SYT and being replaced with the C-terminal 78 amino acids of SSX1 or SSX2. The arrows show the positions of the variant fusion found in tumour STS498.

Fig. 5. Comparison of regions of the SSX1 and SSX2 proteins with the Kruppel-associated box (KRAB) domains of a selection of zinc finger proteins. The KRAB domain is subdivided into two boxes designated KRAB-A and KRAB-B (Bellefroid et al., 1991; Witzgall et al., 1994). In consensus sequences presented at the bottom of the alignment the capital letters indicate that there is at least 70% identity between the previously identified members of this family. Where highly conserved substitutions (i.e. S or T or I or V) are present, in at least 70% of cases, two letters are shown. The boxes highlight the amino acids in the SSX1 and SSX2 proteins which exhibit identity (shaded) or homology (open) to the conserved positions of the KRAB-A and B boxes. The source of the sequences of these KRAB domains represented here can be found in Bellefroid et al. (1991) and Witzgall et al. (1994).
Table 1. Molecular analysis of synovial sarcomas

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<sup>a</sup>Rearrangement was detected by Southern analysis using a SYT gene probe as described (Clark et al., 1994).

<sup>b</sup>Abnormal SSX transcripts of 2.3–2.4 kb were detected using an SSX2 gene probe as described (Clark et al., 1994).

<sup>c</sup>The identity of the SYT partner, either SSX1 or SSX2, was determined by sequencing of PCR products. The assignments were checked by digestion with Smal and Lspl restriction enzymes.

<sup>d</sup>M, Monophasic synovial sarcoma; B, Biphasic synovial sarcoma. For cell lines the assignments were those made for the tumour specimen from which the cell line was derived.

<sup>e</sup>Cytogenetic data demonstrating the presence of the t(X;18)(p11.2;11.2) translocation is available for a limited number of tumours (Knight et al., 1992b; Shipley et al., 1994).

<sup>f</sup>For selected tumours and cell lines FISH has been used to map the X chromosome breakpoint either to the OATLI region or to the OATL2 region, two OAT pseudogene loci at Xp11.2 that are separated by at least 2 Mb (Shipley et al., 1994).

<sup>g</sup>Cell line.

<sup>h</sup>Variant SYT-SSX fusions (see Figure 2).

<sup>i</sup>NS, no sample available.

The situation found for synovial sarcoma is unique in that a single cytogenetically defined translocation results in the fusion of the 5' region of one gene to the 3' domains of two highly related genes at the same cytogenetic location. We have shown that in the cell lines A2243, SS255 and FUJI, which contain t(X;18) with a breakpoint in the OATL2 region, the SYT gene becomes joined to the SSX2 gene. By comparison the HS-SY-II cell line and tumour STS416, which contain a t(X;18) with the breakpoint in the OATLI region, the SYT gene becomes joined to the SSX1 gene (Table I). These results imply that the SSX2 gene is located in the OATL2 region and that the SSX1 gene is located in the OATLI region, a conclusion that is supported by the detection of SSX sequences in YACs spanning both the OATLI and OATL2 regions (Clark et al., 1994).

Two distinct SYT-SSX1 transcripts and two distinct SYT-SSX2 transcripts have been identified. Similar variations in the structure of hybrid transcripts have also been observed in other solid tumours. The t(11;22) in Ewing's sarcoma, for example, can result in the formation of nine different EWS-FLI1 chimaeric transcripts (Zucman et al., 1993). Despite this heterogeneity a single set of PCR primers could be used to detect SYT-SSX transcripts in 90% of synovial sarcomas. Since this level of detection is higher than the level of detection of the t(X;18) in cytogenetic studies (70%) (Turc-Carel et al., 1987; Wang-Wuu et al., 1987) it is likely that the PCR detection method will prove very useful in the diagnosis of synovial sarcoma.

It has been suggested that there is a correlation between the locus involved in the t(X;18) translocation and tumour subtype (Leeuw et al., 1994). Using YAC probes in FISH studies it was found that all six tumours containing breakpoints in the OATL2 region were monophasic while five out of seven tumours with breakpoints in the OATLI region were biphasic. It should be noted, however, that
this apparent correlation was not absolute since two of the tumours containing breakpoints in the OATL1 region were monophasic. In the present study on a larger series of well documented tumours we have failed to confirm this correlation. Indeed the ratio of biphasic:monophasic tumours for translocations involving the SSX1 gene (4:15) was similar to that found in the translocations involving the SSX2 gene (2:8). In three of the 32 tumours examined we did not detect SYT–SSX1 and SYT–SSX2 hybrid transcripts and failed to observe either abnormal SSX transcripts by Northern analysis or rearrangement of the SYT gene by Southern analysis. Each of the three tumours exhibited the morphological, ultrastructural and immunohistochemical features used to define synovial sarcoma (Fisher, 1986, 1994). For all tumour specimens care was taken to ensure that the majority of clinical sample collected was tumour tissue. It is therefore possible that a small proportion of histologically defined synovial sarcoma do not contain abnormalities of either the SYT or SSX genes.

Molecular characterization of the chromosomal translocations found in solid tumours has revealed that many of the genes which become rearranged encode transcription factors. Examples are the CHOP, FLI1, ERL, PAX3, PAX7 and FKHR genes, involved in the liposarcoma, Ewing’s sarcoma and rhabdomyosarcoma translocations described above. The observed homology to the KRAB transcriptional repressor domain provides an indication that the SSX protein could also act as a modulator of transcription. Since transcriptional repression by the KRAB domain is believed to be mediated by protein–protein interactions (Licht et al., 1993) it is possible that the SSX proteins, which lack the zinc fingers required for DNA binding, may mediate their effects by interacting with other proteins involved in transcriptional control. Indeed it is interesting to speculate that SSX may act in a manner similar to that documented for transcription factors such as CHOP (Ron and Habener, 1992) and Id (Benezra et al., 1990). Both of these proteins lacks the DNA binding function present in related family members, but control transcription through their ability to participate in protein–protein interactions.

As observed for other solid tumour translocations the t(X;18) found in synovial sarcoma results in the formation of hybrid transcripts. For the t(11;22) found in Ewing’s sarcoma the EWS–FLI1 hybrid transcript has been shown to be oncogenic (May et al., 1993), and it is probable that the SYT–SSX1 and SYT–SSX2 hybrid transcripts are also responsible for transforming activity. However, the sequences of the encoded SYT–SSX1 and SYT–SSX2 fusion proteins, that have lost the N-terminal regions of SSX1 and SSX2 which showed homology to the KRAB domain, provide few clues to the mechanism of action. It is likely, therefore, that the elucidation of the normal function of the SYT and SSX proteins and of the mechanism of cell transformation by the SYT–SSX proteins will provide a fascinating area for future study.

Materials and methods

Cell lines and tumours

The origin and karyotype of two synovial sarcoma cell lines, STS255 and Fuji have been described previously (Reeves et al., 1989; Nojima et al., 1990). The A2243 line was provided by S.A.Aaronson. All three of these lines contain a t(X;18) translocation breakpoint within the OATL2 region. The HS-SY-II cell line (Sonobe et al., 1992) contains a t(X;18) translocation breakpoint in the OATL1 region. Tumour samples were collected from the Royal Marsden Hospital, and St Thomas’ Hospital, London and the Royal Orthopaedic Hospital, Birmingham. For each sample, care was taken to ensure that the majority of the material collected was tumour tissue. To confirm tumour diagnosis and assignment of synovial sarcomas to biphasic and monophasic subtypes we collected formalin-fixed material from all of the synovial sarcomas, with the exception of STS105 the A2243, HS-SY-II and Fuji cell lines. The formalin-fixed specimens were examined in immunohistochemical studies using antibodies which detected cytookeratin, epithelial membrane antigen, S100 protein, desmin and smooth muscle actin. Synovial sarcoma is defined, as described by Fisher (1986), as a soft-tissue sarcoma which displays epithelial differentiation either immunohistochemically or ultrastructurally. Biphasic tumours have a variable proportion of well-defined, glandular or solid, epithelial structures in a spindle cell stroma, whilst monophasic tumours have only the spindle cell component.

Analysis of DNA and RNA

Extraction of genomic DNA and total cellular RNA and the respective Southern and Northern analyses were performed as described previously (Knight et al., 1992b; Mitchell and Cooper, 1992b). Equal loading of the samples was verified by staining the DNA and RNA with ethidium bromide and visualising under UV light. Radiolabelling of DNA probes was performed by random priming (Feinberg and Vogelstein, 1983) using [α-32P]dCTP (3000 Ci/mM, Amersham) and hybridization was carried out as described (Reeves et al., 1989; Knight et al., 1992b; Sonobe et al., 1992). Autoradiography was carried out at −70°C between 1 and 4 weeks using Fuji Ra film and an intensifying screen. Size markers for Southern blots were BRL kilobase ladder. The BRL 0.24–9.5 kb ladder was used as RNA size markers.

RT-PCR analyses

Total RNA (0.5 μg) was reverse-transcribed using Superscript II reverse transcriptase (GIBCO BRL) and random primers (Geners, Pharmacia) according to the recommended conditions. The resulting cDNA was subject to amplification using a variety of primer sets. The amplification conditions were 93°C for 1 min, 55°C for 1 min and 72°C for 1 min for 30 cycles in a final volume of 25 μl containing 2.25 mM MgCl2, 60 mM KC1, 15 mM Tris–HCl, pH 8.8. To amplify SYT–SSX junction fragments from synovial sarcoma derived samples, PCR was carried out with the SYT primer 5′-CAACAGCAAGATGCATACCA-3′ and the SSX2 primer 5′-TGATATGCACCTGTAGCAGA-3′. PCR products corresponding to the 5′ coding region of the normal SSX2 transcript were isolated by subjecting reverse-transcribed testis RNA to amplification using the primer 5′-TGAAGTGTAGACTCTGCTCTG-3′ corresponding to the 5′ region of the normal SSX1 cDNA clone (position 68–88, Figure 3) and nested primers 5′-TCTCCTGAAAATTTCTCAAGG-3′ (primer C, positions 551–531, Figure 3) and 5′-GGCACAGAACCTTTCCACTA (primer D, positions 512–493, Figure 3) corresponding to 3′ SSX2 sequence. The 5′ untranslated end of the normal SSX2 transcripts was isolated by 5′ RACE using a BRL Life Technologies 5′ RACE system with SSX2 primer D. Human testis and thyroid and the HT1080 human fibrosarcoma cell line were screened for the presence of normal SSX1 and SSX2 transcripts by subjecting reverse-transcribed RNA to amplification using a single set of primers corresponding to sequences that were nearly identical in SSX1 and SSX2. The primers are 5′-AGGTGTGACACATCTCAGATG-3′ (position 369–388, Figure 3) and 5′-CTCGTACATCTTCCAGGRTC-3′ (position 655–635, Figure 3). These primers yield PCR products of 287 bp from reverse-transcribed RNA but failed to give products in control experiments with genomic DNA. The PCR products derived from SSX1 and SSX2 sequences and from SYT–SSX1 and SYT–SSX2 sequences can be distinguished by digesting the products with LspI and Smal restriction enzymes. LspI only digests SSX1 while Smal only digests SSX2. As a positive control to confirm that each RNA sample could yield products after RT-PCR, amplification was carried out with two actin primers: 5′-AGGCGGGAATCTGTGTCGACATT-3′ and 5′-GCTAGGACATTTGCTGGT-3′. These priming conditions were performed from separate exons and should amplify a 234 bp cDNA actin sequence. In these analyses all the reverse-transcribed samples give actin PCR products of the expected size.

Screening of cDNA libraries

A random, primed cDNA library was made from poly(A) RNA from the HT1080 fibrosarcoma cell line in the λ ZAP II (Stratagene) vector.
as described (Mitchell and Cooper, 1992a,b). The human testis cDNA library, made using ψgt11, was obtained from Promega. The libraries were plated out at a density of 30,000 plaques per 22 cm² plate. Plaque lifts were carried out using Zeta probe membrane (Bio-rad) according to the manufacturer’s protocol.

**DNA sequencing**

For sequence analysis, PCR products were either subcloned with the T A Cloning kit (Invitrogen), following the manufacturer’s instructions or sequenced directly from the PCR product which was purified using the Geneclean II (BIO101) kit. Both PCR products and cDNA inserts were sequenced by the dideoxy method (Sanger et al., 1977) using the Sequenase version 2 sequencing kit (Amersham) after subcloning of selected restriction fragments in the phagemids KS⁺, KS⁻, SK⁺ and SK⁻ (Stratagene) and resucing single-stranded templates as described by the supplier. Sequencing of both strands of SSX1, SSX2 and the different variants of SYT-SSX1 and SYT-SSX2 was completed using this method.

**Acknowledgements**

We thank Christine Bell for typing the manuscript. This work was funded by the Cancer Research Campaign.

**References**


