Abstract. The human synovial sarcoma-specific translocation t(X;18) results in the fusion of the SYT gene on chromosome 18 with either one of the Krüppel-associated box (KRAB) containing SSX1 or SSX2 genes on the X chromosome, depending on the exact location of the breakpoint within band Xp11.2. Screening of a testis cDNA library yielded several SSX-positive clones. Subsequent sequence analysis revealed that one third of these clones represent an SSX gene that differs from both SSX1 and SSX2. This novel member of the family of KRAB containing SSX genes, which we designated SSX3, is 90% homologous to SSX1 and 95% homologous to SSX2 at the cDNA level. Somatic cell hybrid analysis indicated that SSX3 maps within Xp11.2→p11.1, the region that also harbors the SSX1 and SSX2 genes. However, we conclude from our RT-PCR data and from results reported in the literature that SSX3 does not act as a fusion partner to SYT in any of the 44 independent synovial sarcomas thus far tested.

Materials and methods

Library screening and sequence analysis
The human fibrosarcoma HT1080 (Clonetech) and the human testis 5' stretch (Clonetech) cDNA libraries were used for screening, using essentially the same standard procedures described as before (Leeuw et al., 1993b, 1994a). DNA sequences were analyzed on an automated DNA sequencer (ABI 373A) using a \textit{Taq} dye deoxy terminator cycle sequencing kit (Applied Biosystems).

Patient material and RT-PCR analysis
The patient material used in this study included nine synovial sarcomas that were extensively analyzed before (Leeuw et al., 1995) and six novel tumors. Diagnosis of these latter six tumors as synovial sarcomas was confirmed via a positive SYT-SSX RT-PCR score. RT-PCR and subsequent restriction enzyme analyses were performed as described previously (Leeuw et al., 1995).

Southern blot analysis
DNAs of hybrid and parental cell lines were isolated as described before (Leeuw et al., 1994a); digested to completion with a variety of restriction endonucleases (Life Technologies); and, after agarose gel electrophoresis,
SSX1 ATGAACGGAG ACCACACTTG AGCAAGGAGA CCACAGGATG
SSX2 ATGAAACGGAG ACCACACTTG AGCAAGGAGA CCACAGGATG
SSX3 ATGACACCTT AGCAAGGAGA CCCACGGTTG
SSX1 ATGCTAAAGC ATCAGAGAAG AGAAGCAAGG CCTTTGATGA
SSX2 GTGCTCAAAT ACCAGAGAAG ATCCAAAAGG CCTTCGATGA
SSX3 GTGCTCAAAT ACCAGAGAAG ATACAAAAGG CCTTCGATGA
SSX1 TATTGCCACA TACTTCTCTA AGAAAGAGTG GAAAAAGATG
SSX2 TATTGCCAAA TACTTCTCTA AGGAAGAGTG GGAAAAGATG
SSX3 TATTGCCAAA TACTTCTCTA AGGAAGAGTG GGAAAAGATG
SSX1 AAATACTCAG AGAAAATCAG CTATGTGTAT ATGAAGAGAA
SSX2 AAAGCCTCAG AGAAAATCTT CTATGTGTAT ATGAAGAGAA
SSX3 AAAGTCTCGG AGAAAATCGT CTATGTGTAT ATGAAGAGAA
SSX1 ACTATAAGGC CATGACTAAA CTAGGTTTCA AAGTCACCCT
SSX2 AGTATGAGGC TATGACTAAA CTAGGTTTCA AGGCCACCCT
SSX3 AGTATGAGGC CATGACTAAA CTAGGTTTCA AGGCCATCCT
SSX1 CCCACCTTTC ATGTGTAATA AACAGGCCAC AGACTTCCAG
SSX2 CCCACCTTTC ATGTGTAATA TACGGGCCGA AGACTTCCAG
SSX3 CCCATCTTTC ATGCGTAATA AACGGGTCAC AGACTTCCAG
SSX1 GGGAATGATT TTGATAATGA CCATAACCGC AGGATTCAGG
SSX2 GGGAATGATT TGGATAATGA CCCTAACCGT GGGAATCAGG
SSX3 GGGAATGATT TTGATAATGA CCCTAACCGT GGGAATCAGG
SSX1 TTGAACATCC TCAGATGACT TTCGGCAGGC TCCACAGAAT
SSX2 TTGAACGTCC TCAGATGACT TTCGGCAGGC TCCAGGGAAT
SSX3 TTCTACGTCC TCAGATGACT TTCGGCAGGC TCCAGGGAAT
SSX1 CATCCCGAAG ATCATGCCCA AGAAGCCAGC AGAGGACGAA
SSX2 CTCCCCGAAG ATCATGCCCA AGAAGCCAGC AGAGGAAGGA
SSX3 CTTCCCGAAG ATCATGCCCA AGAAGCCAGC AGAGGAAGGA

**Fig. 1.** The SSX3 cDNA sequence compared to those of SSX1 and SSX2. Differences are printed in boldface type. The locations of the BglII, EcoRV, Smal, and LspI restriction sites are indicated.

**Fig. 2.** Restriction digests of SSX3 testis cDNA clones (BamHI-XbaI inserts into pDR2) using BamHI, BglII, EcoRV, Smal, and LspI restriction sites are indicated.

Blotted onto Genescreen Plus membranes (Dupont) using standard protocols. Blots were hybridized overnight in 0.5 mM PBS, 1 mM Na2EDTA, and 7% (v/v) SDS at 65 °C; washed once in 40 mM phosphate, 0.1% SDS and then in 10 mM phosphate, 0.1% SDS at 65 °C; and, exposed to X-ray film (Kodak) at -80 °C for 1–3 d, using intensifying screens.

**Results and discussion**

Screening of a human fibrosarcoma cDNA library with a synovial sarcoma-derived SYT-SSX RT-PCR product (Leeuw et al., 1995) yielded several partial SSX2 fragments. One of these fragments was used as a probe to screen a human testis cDNA library, which resulted in 15 positive clones. Five of these full-length clones (pDR2-30, pDR2-33, pDR2-34, pDR2-36, and pDR2-42) were found to lack a BglII site that is normally present in both the SSX1 and SSX2 cDNAs (Leeuw et al., 1995). Sequencing of these five clones yielded identical results and revealed a novel SSX transcript that was significantly different from SSX1 and SSX2 (for comparison, see Fig. 1). Overall, this novel sequence, which we designated SSX3, is 90% homologous to SSX1 and 95% homologous to SSX2 at the cDNA level. At the position of the BglII site in SSX1 and SSX2...
synovial sarcomas revealed a BglII site and either an Smal or Lapl site, whereas none showed an EcoRV site or both Smal and Lapl sites (Fig. 3). From these results we conclude that the SSX3 gene is not fused to SYT in any of our 15 independent synovial sarcomas.

Crew et al. (1995) published a series of 29 synovial sarcomas which showed either a Smal site or Lapl site within the SYT-SSX RT-PCR products, but never both sites. They also mentioned that only SSX1 or SSX2 sequences were encountered. Therefore, we conclude that SSX3 is not included in the fusion with SYT in any of the 44 independent synovial sarcomas thus far tested. In addition, three synovial sarcomas without any evidence for the presence of SYT-SSX1 or SYT-SSX2 fusion products were reported (Crew et al., 1995). Whether in these cases SSX3 is involved cannot, at present, be excluded with certainty, but it should be expected that aberrant SSX transcripts would have been detected by the investigators using the highly homologous (95%) SSX2 as a probe.

Figure 4 compares the amino acid sequences of SSX1, SSX2, and SSX3 (marked 1, 2, and 3, respectively). Differences as compared to the SSX2 sequence are indicated by asterisks. The KRAB boxes are marked with the conserved amino acids in boldface type, and the semiconserved amino acids are underlined (modified after Crew et al., 1995).

Fig. 3. Restriction digest of SYT-SSX RT-PCR products from 12 different synovial sarcomas with EcoRV (A), BglII (B), Lspl (C), and Smal (D). The PCR was performed on 1 μl of RT material with SYT (5′-CAACAGCACA-GATGCATACCA-3′) and SSX (5′-CAGTTGATGACACGTGAT-3′) primers (Leeuw et al., 1995). 1 min at 92 °C, 1 min at 48 °C, and 3 min at 72 °C for 35 cycles. Lanes 1–12 contain RT-PCR material from tumors 94-50418, 89-52115, 2374/90, CATC, 89-50654, 243090, H1ss, KN, PTN SS1, 4873/92, 950501XC, and 2214-287, respectively (Leeuw et al., 1993a, 1993b, 1994a, 1994b, 1995; Janz et al., 1995; and unpublished cases). Lane M: size marker (100-bp ladder, Life Technologies). The 600-bp marker band is indicated by an arrow.

(Figs. 1 and 2) an EcoRV site is found in SSX3 that is not present in SSX1 and SSX2. Aside from these differences, SSX3 shows the same Smal site as SSX2 (absent from SSX1) and the same Lspl site as SSX1 (absent from SSX2). As a consequence, digestion of SYT-SSX RT-PCR products must reveal BglII and Lspl sites in case SSX1 is involved. BglII and Smal sites in case SSX2 is involved, and Smal, Lspl, and EcoRV sites in case SSX3 is involved. Previous sequencing and restriction enzyme digestion of SYT-SSX RT-PCR products from nine different synovial sarcomas revealed fusion products that contained only SSX1- or SSX2-derived sequences (Leeuw et al., 1995). Digestion of RT-PCR products from six additional independent
A have arisen by reversed transcription. In this respect, they are different from, for example, the interspersed OAT-like sequences, some of which have been reported to be processed pseudogenes (Lafreniere et al., 1991; Geraghty et al., 1993). It has been hypothesized before that the OATL1 and OATL2 clusters may have evolved via a duplication event of the entire region (Shipley et al., 1994). Our present results indicate that during the course of evolution, several of these duplication events must have occurred within this particular genomic segment.

In addition to the SSX3 gene reported here, we have evidence for the existence of at least two other SSX-like genes. The first one, designated SSX4, was found after RT-PCR with two SSX internal primers on RNA extracted from a primary human fibrosarcoma. Preliminary sequence data indicate that this gene may give rise to a protein truncated just after the KRAB A box. The second one, SSX5, was detected via the presence of an exon in one of our OATL1 YAC-derived cosmids (Leeuw et al., 1993b). This exon shows between 80% and 90% base-pair homology to the corresponding exons in SSX1, SSX2, SSX3, and SSX4. Whether SSX4 and SSX5 actually represent functional genes still remains to be established. The possible involvement of any of these novel SSX sequences in the development of other neoplastic disorders carrying X-autosome translocations, such as renal cell carcinomas (Sinke et al., 1993; Dijkstra et al., 1995), is currently under investigation.

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