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Genetics

# Molecular studies of deletions at the human steroid sulfatase locus

**(ichthyosis/mechanism of mutation/X chromosome)**

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**ABSTRACT** **The human steroid sulfatase gene (*STS*) is located on the distal X chromosome short arm close to the pseudoautosomal region but in a segment of DNA that is unique to the X chromosome. In contrast to most X chromosome-encoded genes, *STS* expression is not extinguished during the process of X chromosome inactivation. Deficiency of STS (steryl-sulfatase; steryl-sulfate sulfohydrolase, EC 3.1.6.2) activity produces the syndrome of X chromosome-linked ichthyosis, which is one of the most common inborn errors of metabolism in man. Approximately 90% of STS$^-$ individuals have large deletions at the *STS* locus. We and others have found that the end points of such deletions are heterogeneous in their location. One recently ascertained subject was observed to have a 40-kilobase deletion that is entirely intragenic, permitting the cloning and sequencing of the deletion junction. Studies of this patient and of other X chromosome sequences in other subjects permit some insight into the mechanism(s) responsible for generating frequent deletions on the short arm of the X chromosome.**

Steroid sulfatase (STS; steryl-sulfatase; steryl-sulfate sulfohydrolase, EC 3.1.6.2) deficiency is one of the most prevalent human inborn errors of metabolism. Several studies have estimated the frequency of this condition at between 1/6000 and 1/2000 males (1). In addition, a phenotypic indicator of STS deficiency, X chromosome-linked ichthyosis (XLI), has been recognized with a similar incidence in virtually every social and ethnic group. STS is a microsomal enzyme, expressed in essentially all tissues, that hydrolyzes various 3$\beta$-hydroxysteroid sulfates. The gene that encodes the 62-kDa polypeptide has been cloned and characterized. It contains 10 exons, spread over 146 kilobases (kb) of DNA on the distal short arm of the X chromosome, close to the boundary of the X unique and pseudoautosomal regions (2).

STS deficiency during fetal life results in impaired maternal-fetal estrogen production due to lack of STS activity in the placenta. This endocrinologic situation is sometimes associated with delayed progression of parturition. The clinical abnormalities seen postnatally in STS-deficient patients are usually restricted to abnormal levels of circulating steroid sulfates and ichthyosis. However, some patients have more complex phenotypes, which can include short stature with stippled epiphyses (the Conradi syndrome), hypogonadotropic hypogonadism with anosmia (the Kallmann syndrome), mental retardation, ocular albinism, and neurologic abnormalities. Such individuals are thought to have extensive deletions of their X chromosomes, which could produce contiguous gene loss. In support of this concept, ≈90% of patients with simple deficiency of STS and isolated ichthyosis appear to have molecular evidence of deletion of some or all of their *STS* gene sequences (3–7).

In an effort to understand the basis for the very frequent deletions seen in STS-deficient subjects, we have undertaken a molecular analysis of a number of these patients to attempt to define the heterogeneity of breakpoints in these deletions. We wished to identify any potential sequences that might be prone to undergo either intra- or interchromosomal nonhomologous recombination. Most of the breakpoints occur at a substantial distance from the *STS* gene itself and so far have been difficult to characterize. However, we have found one subject who has an entirely intragenic deletion of a portion of his *STS* locus. Thus, the end points of this deletion can be defined with existing molecular reagents and are amenable to direct analysis.

## MATERIALS AND METHODS

**Patients, Cell Lines, and Probes.** All patients, except nos. 29 and 30, had typical XLI without other phenotypic abnormalities. They derived from a variety of clinical sources and geographic regions and were originally referred to our laboratory for diagnostic purposes. All patient materials were cultured fibroblast cell lines, except nos. 3, 9, 10, 11, 12, in which somatic cell hybrid lines containing the patient's X chromosome had been constructed on a mouse A9 background. Patient 29 had neurologic abnormalities and the Kallmann syndrome in addition to XLI; he was karyotypically normal. He was previously reported by Sunohara *et al.* (8). Patient 30 had a visible deletion of the distal X chromosome short arm and was described by Curry *et al.* (9). Patient J.H. had isolated XLI that was indistinguishable from that seen in other subjects. Probe dic56 (*DXS143*) was kindly provided by Louis Kunkel (Boston), 38j (*DXS283*) was donated by Jean Weissenbach (Paris), and GMGX9 (*DXS237*) was given by Malcolm Ferguson-Smith (Cambridge).

**Isolation of DNA and RNA.** High molecular weight DNA was isolated from human leukocytes, fibroblasts, or mouse–human hybrid cells containing a human X chromosome by proteinase K treatment and phenol extraction as described (10). Poly(A)$^+$ RNA was isolated from human placenta or tissue culture cells by guanidinium SCN-CsCl gradient and oligo(dT)-cellulose chromatography (11).

**Restriction Digestion and Hybridization Analysis of DNA and RNA.** Conditions for restriction digestion and Southern blotting of cellular DNA have been described (4). Northern blot analyses of RNA were carried out according to Rozek and Davidson (12) using 3 $\mu$g of poly(A)$^+$ RNA per lane.

**Immunoprecipitation of STS Protein.** Cultured fibroblasts at or near confluence were metabolically labeled with [$^3$H]leucine (Amersham; 1.25 mCi per 100-mm dish; 1 Ci = 37 GBq) for 3 hr and then chased with unlabeled leucine for an additional 3 hr. The cells were lysed in 1.5 ml of immuno-

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Abbreviations: STS, steroid sulfatase; XLT, X chromosome-linked ichthyosis.

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precipitation buffer (10 mM Tris/150 mM NaCl/1% Nonidet P-40, pH 7.5) at 4°C and harvested. The lysate was precleared with goat anti-human fibronectin serum and then with normal rabbit serum. STS protein was then immunoprecipitated at 4°C overnight with rabbit antiserum against human STS protein purified by affinity chromatography (4). Immunoprecipitates were resolved on a 7.5% SDS/PAGE gel followed by fluorography.

**Library Construction and Screening.** About 100 μg of J.H. hybrid DNA was digested to completion with *Bam*HI and size fractionated on a sucrose gradient. The fragments of ≈18 kb in length were ligated to λEMBL3 arms and packaged *in vitro* using a Gigapack Gold kit from Stratagene. About $1 \times 10^6$ recombinant phages from the library were plated and screened according to the plaque-hybridization procedure of Benton and Davis (13).

**Subcloning and DNA Sequencing.** Appropriate *Eco*RI fragments from the λ clones were subcloned in pUC19 for more detailed restriction mapping. DNA sequences of the junction fragment and the corresponding regions from a normal individual were determined by the dideoxy chain-termination method (14) using either the Klenow fragment of *Escherichia coli* DNA polymerase I or Sequenase (United States Biochemical).

## RESULTS

**Characterization of the Molecular Defects in STS-Deficient Patients.** We have studied the molecular defects in 30 unrelated STS-deficient patients (Table 1), including 10 used in our previous study (4). Patients 1–28 have ichthyosis as their only phenotypic abnormality. Patient 29 has the Kallmann syndrome and neurological abnormalities in addition to ichthyosis. Patient 30 (D.R.) has a cytologically visible deletion of the tip of the short arm of his X chromosome. All patients were shown to have undetectable levels of STS enzyme activity.

We have evaluated the defects in these patients in three ways: the *STS* gene structure, the presence of *STS*-encoded mRNA, and the presence of inactive proteins or protein fragments detectable with anti-STS antiserum. For *STS* gene structure studies, DNAs were isolated from the patients, digested with *Eco*RI, and probed with a human *STS* cDNA pSTS331 (Fig. 1). Normal males gave 10 hybridization bands. The sizes of the fragments have been modified from those given in a previous communication (4). The 17-kb and the 1.4-kb fragments detected by pSTS331 contain pseudogene sequences on the Y chromosome, whereas the rest of the fragments correspond to the functional *STS* gene sequences on the X chromosome. Comparison of the Southern hybridization patterns of the patients to that of normal individuals should reveal any gross rearrangements in gene structure.

For mRNA studies, poly(A)$^+$ RNA was isolated from cultured fibroblasts of the patients or from mouse–human hybrid cells containing patients' X chromosomes as in the case of J.H. The RNAs were separated on gels, transferred to nitrocellulose membranes, and probed with pSTS331 (Fig. 2). Three major species of *STS* mRNA (7.2 kb, 5.2 kb, and 2.7 kb) have been identified in various human tissues. This variation is produced by alternative sites of poly(A) addition. Normal fibroblasts and mouse–human hybrids with normal human X chromosomes contain mainly the 7.2-kb and the 5.2-kb mRNA forms.

For protein studies, normal or patients' fibroblasts were grown in the presence of [$^3$H]leucine, and the cell extracts were treated with anti-STS antiserum. The immunoprecipitated fractions were then analyzed on SDS/PAGE gels (Fig. 3). As positive controls, a female fibroblast cell line, IMR90, and A9(hSTS) cells were used. A9(hSTS) are mouse A9 cells transfected with a human STS expression vector pMSG: STS331 and thus express high level of STS enzymatic activity (4). Anti-STS antiserum precipitated a 62-kDa protein from the cell extracts of IMR90 and A9(hSTS) cells.

Table 1. Molecular defects in STS-deficient patients

| Patient | 38j (*DXS283*) | STS | dic56 (*DXS143*) | mRNA | Protein |
|---|---|---|---|---|---|
| mRNA-positive patients | | | | | |
| 1. T.A. | | + | | + | − |
| 2. C | | + | | + | − |
| 3. J.H. | | Partial | | Truncated | − |
| mRNA-negative patients | | | | | |
| 4. R.F. | + | − | + | − | − |
| 5. V | + | − | + | − | − |
| 6. D.B. | + | − | + | | |
| 7. M.E. | + | − | + | | |
| 8. T.J. | + | − | + | | |
| 9. CF92 | + | − | + | | |
| 10. CF93 | + | − | + | | |
| 11. CF102 | + | − | + | | |
| 12. CF103 | + | − | + | | |
| 13. A | + | − | + | | |
| 14. EE | + | − | + | | |
| 15. F | + | − | + | | |
| 16. G | + | − | + | | |
| 17. L | + | − | + | | |
| 18. N | + | − | + | | |
| 19. Q | + | − | + | | |
| 20. T | + | − | + | | |
| 21. WG780 | + | − | + | | |
| 22. C.K. | + | − | + | | |
| 23. J.An | + | − | + | | |
| 24. S | + | − | + | | |
| 25. W | + | − | + | | |
| 26. B | + | − | + | | |
| 27. Mc | + | − | + | | |
| 28. O | + | − | + | | |
| STS deficiency and Kallmann syndrome | | | | | |
| 29. Kall | + | − | − | | |
| STS deficiency with terminal deletion of Xp | | | | | |
| 30. D.R. | − | − | + | | |

The results of all of the studies are summarized in Table 1. Three different kinds of defects were observed in these patients. The first is a total absence of STS-X sequences. Twenty-seven of the 30 patients have total gene deletions. This is an extension of our previous finding that 8 of 10

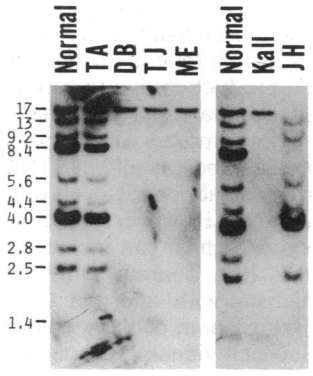


FIG. 1. Southern blot analysis of *STS* genes in unrelated STS-deficient patients. DNAs were isolated from cultured fibroblasts of normal males and patients except for J.H., in which case DNA was isolated from a mouse–human hybrid cell line containing the X chromosome of J.H. The DNAs were digested with *Eco*RI and probed with the *STS* cDNA clone pSTS331. The 17-kb and the 1.4-kb fragments contain the pseudogene sequence on the Y chromosome, whereas the rest of the fragments correspond to the functional *STS* gene on the X chromosome. Sizes are given in kb.

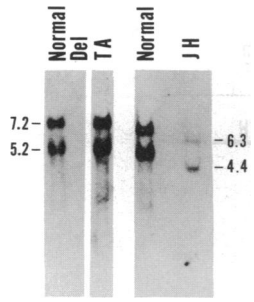


FIG. 2. Northern blot analyses of STS transcripts in STS-deficient patients. Poly(A)$^+$ RNA from fibroblasts of normal individuals, patient V with a complete deletion (Del), and patient T.A. or from J.H. hybrid cells was fractionated on formaldehyde gels and probed with pSTS331. Sizes are given in kb.

patients have complete gene deletions and is consistent with previous observations (3–7). Southern hybridization patterns of some of these deletion patients' DNA with *STS* cDNA are shown in Fig. 1. D.B., T.J., M.E., and Kall DNA all have only the Y chromosome-specific fragments. Protein and mRNA studies were carried out on 2 of these patients (R.F. and V), and Figs. 2 and 3 show the results of one of them. As expected, neither mRNA nor STS immunoreactive protein can be detected in these patients. To further study the extent of the deletions in these patients, we reprobed the DNA blots with the genomic fragment p126SRA/H3D, which is 3 kb 5′ of STS exon 1, and genomic fragment p154A/H3+R1F, which is 20 kb 3′ of the last STS exon (exon 10). Neither of these probes hybridizes with DNA from any of the total deletion patients. In addition, we used two other probes derived from outside the *STS-X* locus. Probe 38j (*DXS283*), which maps distal to STS but proximal to the pseudoautosomal marker MIC2 (32), and probe dic56 (*DXS143*), which is located 5 centimorgans proximal to STS (15). Patients 4–28 contain both sequences. The Kallmann syndrome patient lacks dic56 sequences, whereas D.R. lacks 38j sequences. Since the physical distances between 38j, STS, and dic56 are unknown, we cannot use this information to determine the physical size of these deletions.

T.A. and C are the only two patients we studied with normal DNA hybridization pattern with pSTS331 (results for T.A. are shown in Figs. 1 and 2). Additional enzymes, such as *Hin*dIII, *Msp* I, or *Taq* I, failed to detect any abnormality in these patients' DNA (data not shown). The defects of T.A. and C do not seem to affect the transcription of the gene, since both T.A. and C have normal amounts of STS mRNAs of normal sizes (Fig. 2). Immunoprecipitation of T.A. and C cell extracts failed to detect any protein that binds specifically to anti-STS antiserum (Fig. 3). It thus appears that T.A. and C carry missense mutations or small deletions or insertions that result in the production of truncated or unstable proteins.

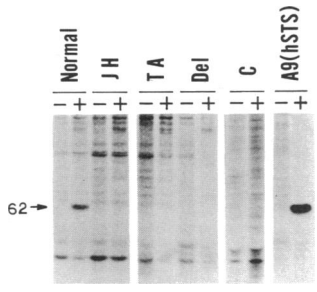


FIG. 3. Immunoprecipitation of STS protein in STS-deficient patients. Cell lysates were incubated with either normal rabbit serum (−) or rabbit anti-human STS antiserum (+). Normal fibroblasts and A9(hSTS) cells (mouse A9 cells containing a human STS cDNA expression construct) showed the STS protein at the expected position corresponding to 62 kDa (arrow). No STS was immunoprecipitated from either J.H., who has an intragenic deletion, or Del, patient V who has a complete deletion. T.A. and C, both of whom showed normal Southern and Northern blot patterns, have no immunoprecipitable STS protein.

Patient J.H. is the only patient with a partial deletion of *STS-X*. We have constructed a mouse–human hybrid cell line containing the X chromosome but not the Y chromosome of J.H. As shown in Fig. 1, DNA isolated from a J.H. hybrid lacks the 8.4-kb and the 2.8-kb fragments, indicating partial deletion of the gene. These two *Eco*RI fragments contain exons 3–5 and exon 2, respectively (2). There are a total of 810 base pairs (bp) encoded by these four missing exons. One would thus expect truncated mRNAs in the J.H. hybrid cells. As expected, Northern blot analysis of mRNA isolated from J.H. hybrids (Fig. 2) shows two truncated mRNAs of 6.3 kb and 4.4 kb as compared to the normal sizes, 7.2 kb and 5.2 kb. The truncated mRNAs are present in reduced amount, suggesting that either they are less stable in the cells or the intragenic deletion of J.H. affects the transcription of the gene, or both. The deletion of 810 bases in the mRNA should not cause a frameshift of translation. However it eliminates 270 of 584 amino acid residues of the STS precursor protein. This includes 18 of the 22 amino acid residues in the signal sequence in addition to 252 residues from the N terminus of the mature protein. One would expect such an alteration to have a dramatic effect on the processing and the stability of the protein. In addition, the antigenic determinants detected by the STS antiserum might have been lost in such a protein. It is thus not surprising that we could not detect any immunoprecipitable protein in the cells of J.H. (Fig. 3).

**Characterization of the Intragenic Deletion in J.H.** As stated above, the *STS* gene in J.H. lacks exons 2–5. The breakpoints of the deletion thus lie within intron 1 (35 kb in length) and intron 5 (17 kb in length). Due to the large sizes of these two introns, the breakpoints were further localized by Southern hybridization of cloned single-copy fragments from various regions of the introns to J.H. DNA. As shown in Fig. 4, the breakpoint at the 5′ end of the deletion lies between probe II and probe III since the sequence detected by probe II is present, whereas that detected by probe III is absent in J.H. At the 3′ end of the deletion, the breakpoint lies between the 3′ ends of probe V and probe VI since sequences detected by probe V were missing, whereas probe VI detects a fragment of altered size in J.H. DNA. Additional Southern blotting using various restriction enzymes identified an altered 18-kb *Bam*HI fragment in J.H. that hybridized to probe II and probe VI (data not shown). This fragment presumably contains sequences from either side of the deletion. The presence of such a junction fragment suggests that no gross rearrangement or large insertions occurred in the process of generating this deletion.

A recombinant phage library was constructed in λEMBL3 using DNA from a sucrose gradient fraction containing 18-kb *Bam*HI fragments of J.H. DNA. The library was screened with probe II and probe VI. One clone, λJH, was identified that hybridized with both probes. *Bam*HI digestion of DNA isolated from this clone showed that it contains a single 18-kb insert. We had therefore cloned an 18-kb fragment from J.H. containing sequences that are separated by >40 kb in normal individuals. Comparison of *Eco*RI digests of λJH and normal phage DNAs containing sequences from the 5′ end (λ126) and the 3′ end (λ116) of the deletion identified a 2.7-kb fragment that contains the breakpoint. This 2.7-kb *Eco*RI fragment was then subcloned in pUC19 to give pJ2.7R. Comparison of the restriction map of pJ2.7R with that of the normal 5′ fragment of p126SRB and the normal 3′ fragment p116B further narrowed down the breakpoint in pJ2.7R to a 0.6-kb *Xba* I/*Rsa* I fragment (Fig. 5 *Upper*). DNA sequences of this junction fragment and the corresponding areas in normal individuals were then determined (Fig. 5 *Lower*). Sequence analysis showed that the two end points of the deletion were juxtaposed with the generation of an 8-bp direct repeat of the sequence

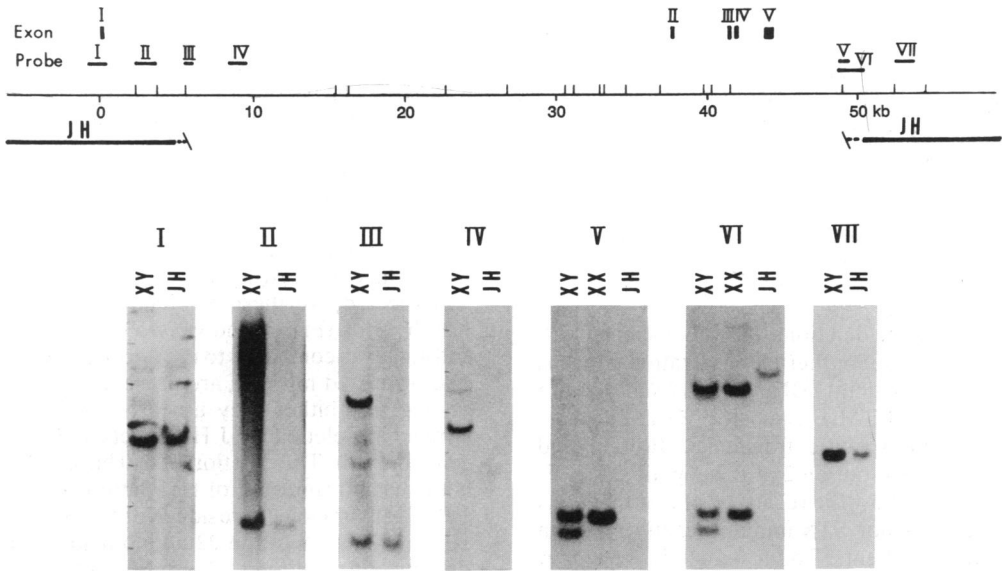


FIG. 4. Localization of the deletion end points in J.H. (*Upper*) Organization of the 5′ end of human X chromosome-encoded *STS* gene. The restriction map shows only *Eco*RI sites. The end points of the deletion in J.H. are indicated. (*Lower*) Southern blot hybridizations of various probes from the 5′ end of *STS* gene (as shown in *Upper*) to DNAs from a normal male (XY) and a normal female (XX) and from a mouse–human hybrid containing the X chromosome of patient J.H. The DNAs were digested with various restriction enzymes. The probes are as follows: probe I, p126SRA/Pst C; probe II, p126B; probe III, p126SRB/H3+R1 C; probe IV, p126S/Pst+H3 C; probe V, p116B/H3+R1 C; probe VI, p116B/DraI+R1 A; and probe VII, p116D/Pst C. Some of the probes detected sequences outside the region shown in *Upper*.

AATAATAG, which is 8 bp 5′ of the breakpoint. p126SRB and p116B contain only single-copy sequences. Computer searches could not identify any significant homology between the 5′ and the 3′ flanking regions of the deletion, nor could any extensive predicted secondary structures be found.

## DISCUSSION

Several groups have studied the molecular defects in STS-deficient patients using either human *STS* cDNA clones (3–7) or probe GMGX9, an anonymous DNA fragment tightly linked to the *STS* locus (16). All found a high incidence of deletions. A total of 102 STS-deficient patients has been studied collectively and only 13 patients' DNA contains all or part of the *STS* X chromosome-encoded sequences. The results in 57 of these patients were inconclusive because their DNAs were studied with incomplete cDNA fragments (3, 5, 7), which would score a partial deletion either positive or negative depending on the sizes and locations of the deletions. In the two studies using nearly full-length cDNA (refs. 4, 6 and present studies), 41 of 45 patients (91%) have complete deletions of the *STS-X* locus. This renders STS deficiency the human genetic defect with the highest incidence of complete gene deletion so far reported (17). We and others have suggested that this high incidence of deletions might be caused by aberrant X–Y interchange. In fact, several lines of evidence now argue against such events. All of the deletion patients we have studied retain sequences detected by 38j (18), an X chromosome-specific marker distal to *STS* which would have been lost if the deletion was caused by X–Y interchange. Furthermore, using a number of distal Yp probes, we were unable to detect any Y sequences on the X chromosomes of four STS patients (unpublished observation). Finally, in a recent study of seven XX males, we found that all of them retain the *STS-X* locus on their X chromosomes, which have acquired Y DNA sequences through a documented X–Y interchange process.

The magnitude of the deletion at the *STS-X* locus is not known. There appears to be heterogeneity among patients. Cooke *et al.* (19) were able to detect the deletions in some but not all STS-deficient patients using flow cytometry. They suggested deletions ranging from 1.2% to 3.4% of the length

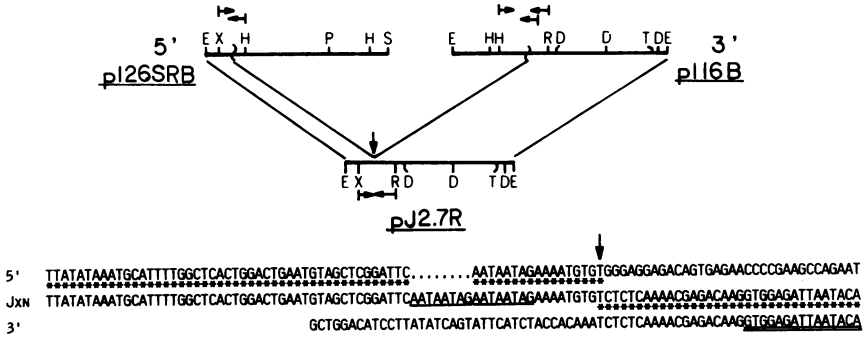


FIG. 5. Characterization of the deletion breakpoint in STS⁻ patient J.H. (*Upper*) Restriction maps of the junction fragment in J.H. (pJ2.7R) and fragments from normal individuals containing the 5′ (p126SRB) and the 3′ (p116B) breakpoints. The restriction sites are as follows: D, *Dra* I; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; R, *Rsa* I; S, *Sal* I; T, *Taq* I; and X, *Xba* I. The horizontal arrows above and below the maps indicate the directions of DNA sequencing. The vertical arrow points to the breakpoint. (*Lower*) DNA sequence of the deletion junction in J.H. as compared to that of the normal 5′ and 3′ sequences. Asterisks between the sequences indicate identical bases. The 8-bp direct repeats generated near the breakpoint of J.H. are underlined. A possible topoisomerase II recognition sequence is indicated by double underlining.

of the X chromosome (equivalent to 1.9–5.2 million bp) in these patients. This probably represents the upper limit of the size of the deletion in patients with XLI as the only phenotypic defect. Among the patients whose deletions cannot be detected by flow cytometry, some have DNA sequences detected by the probe GMGX9, whereas others do not (7). It is not known precisely how far GMGX9 is physically from *STS* but preliminary pulsed-field gel electrophoresis studies place GMGX9 about 500 kb 3′ of *STS* (X.-M. Li and L.J.S., unpublished). We have cloned a continuous stretch of 180-kb DNA of *STS-X* locus. In addition, we have recently isolated from a "jumping library" (20) a fragment that is about 100 kb 5′ of *STS* gene. None of the STS-deficient patients we have studied to date has DNA that cross-hybridizes with this jump clone. Thus, we estimate the lower limit of the size of most of the deletions at the *STS* locus as being about 750–800 kb in length.

Two patients with partial deletions of *STS* genes have been identified. Ballabio *et al.* (7) reported a patient with a deletion of the 3′ end of the gene. We describe here a patient with a 45-kb intragenic deletion. The molecular mechanisms that generate deletions are not known. Some deletions appear to be produced by recombination between homologous sequences. Deletions generated by unequal crossing-over between homologous chromosomes have been defined in the human globin gene clusters (21) and the growth hormone cluster (22). Other deletions seem to involve recombination between repetitive sequences, often *Alu* sequences, as in the case of low density lipoprotein (LDL) receptor gene (23–25) and α-globin gene cluster (26). There are also others that do not seem to involve any repetitive sequences (27, 28). It has been suggested that such deletions can be caused by nonhomologous DNA exchanges between stretches of DNAs that were in the proximity of each other through their attachment to some kind of cellular structure, possibly scaffolding proteins. In *Drosophila* scaffolding-associated DNA fragments were found to contain sequences homologous to topoisomerase II cleavage sites (29). The deletion in J.H. fell in regions containing only single-copy sequence. There is no obvious secondary structure around the breakpoints that could facilitate the recombination. It is noted that the sequence (GTGGAGATTAATACA) that matches 13/15 of the consensus sequences of the topoisomerase II cleavage site [GTNA/TATCATTNATNNG/A (30)] is present 18 bp 3′ of the breakpoint. An 8-bp repeat was generated during the creation of this deletion. A similar situation was found in a deletion in a LDL receptor gene where an 11-bp sequence was duplicated at the deletion junction (24).

Although X–Y interchange does not appear to be a likely mechanism for producing *STS* deletions, unequal X–X recombination in female germ cells cannot be excluded nor can intrachromosomal recombinations and/or sister chromatid exchanges during male or female meiosis. Finally, gene loss during mitotic division of spermatogenia or oogonia is a possibility. It is of note that at least 70% of patients with Duchenne muscular dystrophy have sustained deletions of their dystrophin genes, which are also situated on the X chromosome short arm (31). From the evidence presented here and elsewhere, it is clear that there is some heterogeneity in the position of breakpoints in *STS* deletions. Further studies to determine the precise location and sequences of such deletion junctions will be required to determine the precise molecular mechanisms that produce these frequent rearrangements.

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