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Genetics

Isolation and characterization of a steroid sulfatase cDNA clone: Genomic deletions in patients with X-chromosome-linked ichthyosis

(human X chromosome/Kallmann syndrome/inactivation)

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ABSTRACT **We have isolated several cDNA clones from a λgt11 expression library by screening with antibodies prepared against the microsomal enzyme steroid sulfatase, which is deficient in classical X-chromosome-linked ichthyosis patients. One of these clones (p422) has been assigned by mapping with a somatic cell hybrid panel and by *in situ* hybridization to Xp22.3. Clone p422 therefore has a coincident localization with the previously ioentified locus for steroid sulfatase expression in the region of the X chromosome escaping from inactivation. Twelve steroid sulfatase-deficient patients, including eight cases of classical ichthyosis, were found to be deleted for genomic sequences detected by the clone.**

The gene for steroid sulfatase (STS) is of particular interest as it is the only well characterized X-chromosome-linked locus in humans that has no functional Y-chromosome-linked equivalent and yet escapes from inactivation. In the last few years, it has been the target of many investigations in relation to steroid metabolism, to the genetics of the X chromosome, and to clinical aspects of *STS* deficiency. The enzyme has essential roles in the placental production of estriol during the last weeks of pregnancy and in the desulfation of cholesterol sulfate, a critical step in skin metabolism (see ref. 1). *STS* deficiency has been identified as the primary defect of X-linked ichthyosis (XLI) (2), a skin disorder often associated with late parturition.

A gene necessary for the expression of human STS has been assigned by somatic cell hybrid studies, deletion mapping, and linkage analysis to the region Xpter–Xp22.3 (3–6) and, in common with other loci in the same region [e.g., the blood group *Xg* gene (7) and the *MXYS1* gene (8)], it has been found to escape X-chromosome inactivation (9). Recent evidence suggests that the *STS* gene on the inactive X chromosome is only partially inactivated (10). Although there is no evidence for a functional Y-linked allele in humans, the *STS* gene in the mouse behaves pseudoautosomally, with an apparently functional homologue on the Y chromosome (11). In humans, the *STS* gene is thought to be proximal to the pseudoautosomal region (see ref. 12), and a homologous, but inactive, gene on the Y chromosome has been hypothesized (13). A deficiency of STS enzyme activity is consistently found in patients affected by two clinical features: (*i*) XLI, a common X-linked disorder, having a frequency in males of 1/6000 (2); and (*ii*) multiple sulfatase deficiency, a very rare autosomal recessive disease in which a deficiency of several sulfatase enzyme activities has been demonstrated (14). Genetic complementation studies, between fibroblasts from patients with XLI and fibroblasts from patients with multiple sulfatase deficiency, demonstrated that at least two genes control the levels of STS activity in humans (15).

The protein has been recently purified from human placenta and polyclonal antibodies have been raised in rabbits (16, 17). We have used anti-STS antibodies to screen a λgt11 expression library prepared from placental mRNA to obtain a steroid sulfatase cDNA clone, p422. The identity of the clone has been confirmed by its localization to Xp22.3 and its isolation has enabled the demonstration that the DNA from 12 patients with *STS* deficiency, including eight cases of classical XLI, were deleted for sequences detected by the cDNA clone.

METHODS

Isolation of cDNA Clones. cDNA sequences were isolated by screening a λgt11 expression library prepared from human placenta with polyclonal anti-STS antibodies (16). The procedure followed for the screening was essentially that described by Young and Davis (18). The *Eco*RI cDNA inserts were isolated from bacteriophage propagated from the positive plaques and subcloned in the vector pUC18 (19).

The inserts, or intact plasmids, were ^{32}P-labeled by nick-translation to a specific activity of $>10^8$ dpm/μg and used as probes on Southern blots by standard techniques (20). Routine washing of filters was carried out in 0.5× standard saline citrate (SSC; 1× SSC = 0.15 M NaCl/15 mM sodium citrate) and 0.15% $NaDodSO_4$.

RNA Blot Hybridization. Poly(A)$^+$ RNA from human fibroblasts (1 μg) was electrophoresed through a 1.5% agarose gel containing formaldehyde (21) and transferred to a nitrocellulose filter. Hybridization with a nick-translated probe was carried out at 65°C for 15 hr in 5× SSPE (1× SSPE = 180 mM NaCl/10 mM $NaPO_4$/1 mM EDTA/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/1% $NaDodSO_4$/yeast RNA (100 μg/ml). The final stringency of washing was at 0.2× SSC at 65°C.

***In Situ* Hybridization.** The cDNA plasmid (p422) was labeled with ^{125}I-labeled dCTP to a specific activity of 5×10^8 dpm/μg and hybridized *in situ* to replication-banded metaphase chromosome spreads from lymphocytes of a normal male as described (22).

Mapping with Somatic Cell Hybrid Panel. The procedures for DNA isolation from cells and hybrids (Table 1) and for the screening of somatic cell hybrids have been described (23).

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

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Table 1. Hybridization of STS clone p422 to cell lines and hybrid cell panel

Cell line	Description	Human autosomes	Human sex chromosomes	Hybridization to p422
RAG	Mouse	None	None	−
817/175	Hybrid	Several	Xp22.3–Xqter Yq11–Yqter	+
UCLAB2	Hybrid	Several	Xp22.3–Xqter	−
FRAG A13R3	Hybrid	Several	Xpter–Xp21.2	+
WAG 8	Hybrid	Several	Xp21.1–Xqter	−
MOG T	Hybrid	None	X	+
3E7	Hybrid	None	Y	−
GM1416	Human	All	48,XXXX	++++
OXEN	Human	All	49,XYYYY	+
Normal female	Human	All	XX	++
Normal male	Human	All	XY	+

Hybrid 817/175 (27) is derived from a human cell line 46,X,t(X;Y)(Xqter–Xp22.3::Yq11–Yqter) (see ref. 25). UCLAB2 retains the chromosome associated with chondrodysplasia punctata (26). See references 23 and 31 for details of other cell lines and hybrids.

RESULTS

Anti-STS polyclonal antibodies (16) were used for the immunoscreening of a λgt11 expression library prepared from placental mRNA. Among 10^5 plaque-forming units screened, 6 positive cDNA clones were isolated and purified. Preliminary analysis indicated that only one of these, p422, was localized to the X chromosome short arm. This clone was therefore selected for subsequent investigations. It contains a 560-base-pair cDNA insert, the restriction map for which is shown in Fig. 1*b*. In RNA blots of human fibroblast poly(A)$^+$ mRNA (Fig. 1*a*), the probe recognizes two high molecular weight species, both of which migrate slower than 28S ribosomal RNA marker. Presumptive mRNA species of similar size together with a third, smaller, component were also observed in choriocarcinoma and placental preparations; however, the high molecular weight component was extensively degraded in the latter (not shown).

The localization of the genomic sequences recognized by the cDNA clone p422 was established by its hybridization to restriction digests of DNAs from somatic cell hybrids with a variable representation of human sex chromosomes and from human cell lines with 1, 2, and 4 X chromosomes (Table 1; Fig. 2). In the dosage experiments, the decreasing signal, observed in lanes loaded with the same amount of DNA from 48,XXXX, 46,XX, and 46,XY cell lines indicated a clear localization to the X chromosome. There was no evidence for a Y-localized sequence, even under conditions of reduced stringency washing of filters (see legend to Fig. 2). The analysis of hybrids retaining various portions of the human X chromosome refined this localization to the region Xp22.3.

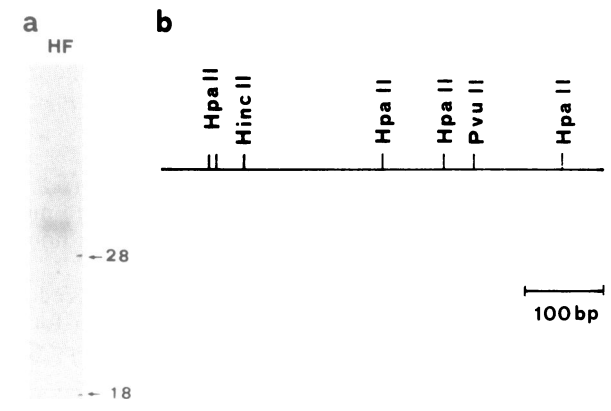

FIG. 1. (*a*) RNA blot analysis of STS mRNA in human fibroblasts (HF). Positions of 18S and 28S rRNA markers are indicated. (*b*) Restriction map of the cDNA clone p422. bp, Base pairs.

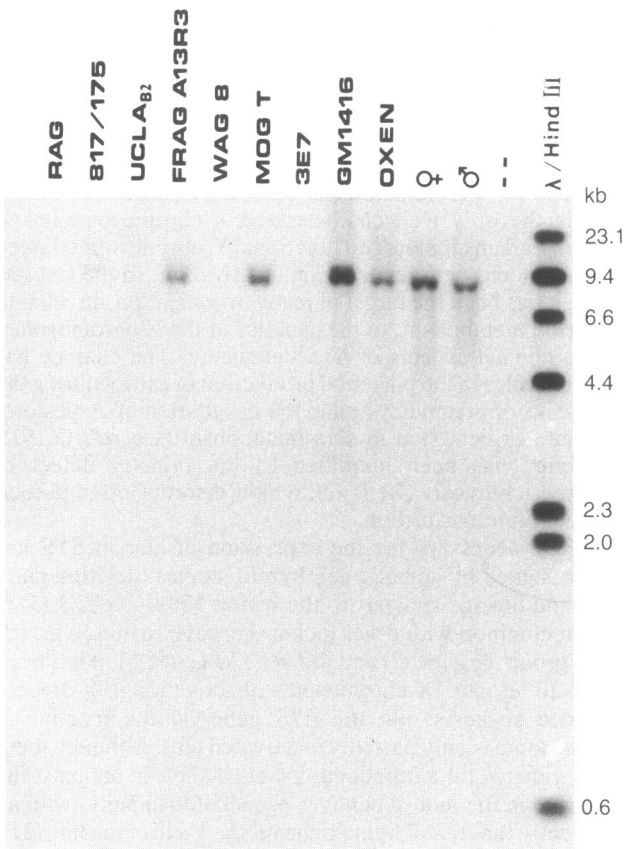

FIG. 2. Localization and dosage analysis in human cell lines and human–mouse somatic cell hybrids. The type of cell line and content of human sex chromosomes are indicated in parentheses. The *Bam*HI digests were as follows: lane 1, RAG (mouse cell line); lane 2, 817/175 (hybrid, Xp22.3–Xqter::Yq11–Yqter); lane 3, UCLAB2 (hybrid, Xp22.3–Xqter); lane 4, FRAG A13R3 (hybrid, Xp21–Xpter); lane 5, WAG 8 (hybrid, Xp21–Xqter); lane 6, MOG T (hybrid, X only); lane 7, 3E7 (hybrid, Y only); lane 8, GM1416 (human, 48,XXXX); lane 9, OXEN (human, 49,XYYYY); lane 10, female control (human, 46,XX); lane 11, male control (human, 46,XY). The decreasing signal, observed for lanes loaded with the same quantity of DNA from 48,XXXX to 46,XY cell lines, indicates a clear assignment to the X chromosome. The lack of a significant increase in signal in the 4Y line compared to that of a normal male and the absence of a signal in the Y-only hybrid exclude the presence of an extensively homologous Y-linked copy. kb, Kilobases.

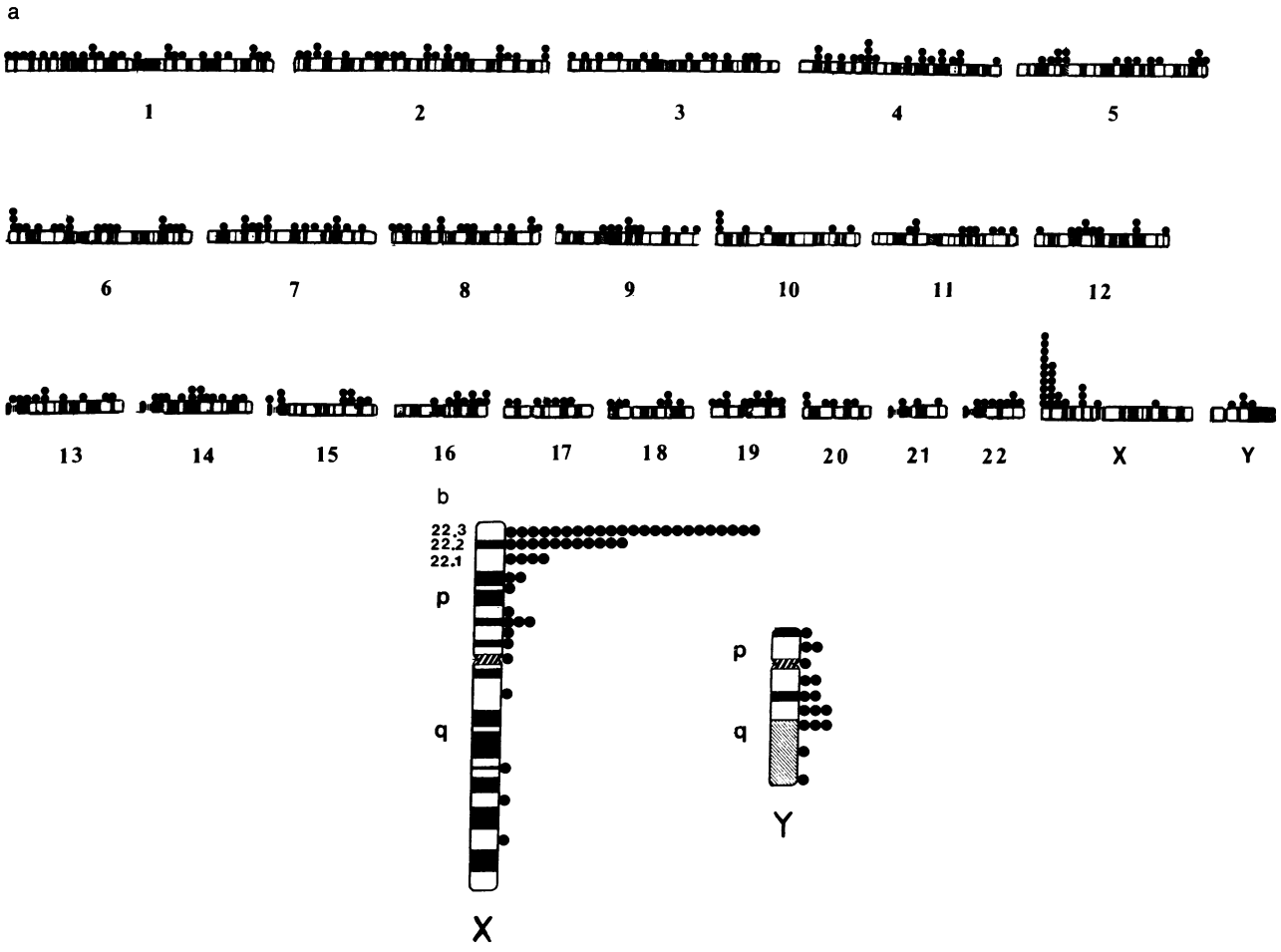

FIG. 3. (*a*) *In situ* hybridization of probe p422 to normal male chromosomes. The p422 probe (plasmid) was labeled with ^{125}I-labeled dCTP to a specific activity of 5×10^8 dpm/μg and hybridized *in situ* to replication-banded metaphase chromosome spreads from lymphocytes of a normal male. 55 cells analyzed (*b*). Grain distribution on the sex chromosomes. The method used for the *in situ* hybridization has been described (22).

The most precise information is provided by comparison of the hybridization to the DNA of two hybrids that retain human X chromosomes with breakpoints in Xp22.3 (Fig. 2, lanes 2 and 3). One hybrid expresses human levels of STS activity but lacks MXYS1 (MIC2), which has been assigned to Xp22.3, and the other lacks both STS activity and the MXYS1 locus (26). The observation of a positive signal in the former hybrid (lane 2) and not the latter (lane 3) provides a clear localization of the sequences recognized by p422 to Xp22.3.

Independent confirmation of this localization is provided by the results of *in situ* hybridization of this probe to replication-banded metaphase chromosome spreads derived from lymphocytes of a normal male. For the chromosomal assignment, a total of 55 cells were analyzed. Of 298 silver grains scored, 16 (5%) were localized at Xp22.2–ter. This was the only major site of hybridization observed. Calculated on the basis of a haploid genome, as only one X chromosome is present per cell, this region corresponds in length to <0.5% of the total, but it is recognized by 10% of the grains assigned. A more detailed examination of the distribution of grains on sex chromosomes was examined in 56 cells in which both the X and Y were clearly analyzable. No strong subregionally localized signal was observed on the Y chromosome and although the majority of grains scored was on the euchromatic region of the Y long arm (Fig. 3*b*), this region did not appear to be significantly more labeled than some autosomal regions (Fig. 3*a*). Of 52 grains scored on the X chromosome, 34 (66%) were in the region Xp22.2–ter, and 23 (44%) of these were clearly localized to the distal band Xp22.3. These results clearly assign the probe to the X chromosome and provide a subregional localization to the distal band Xp22.3 (Fig. 3). The assignment of the probe p422 to the Xp22.3 band is coincident with the previously reported localization of a gene necessary for expression of STS and is therefore entirely consistent with its identification as a cDNA corresponding to the STS structural gene. It is of interest that neither Southern blotting studies on somatic cell hybrids, even under washing conditions of reduced stringency (2× SSC/0.1% $NaDodSO_4$), nor *in situ* hybridization detected any strongly homologous sequence on the Y chromosome.

The STS cDNA clone, p422, has been subsequently used to investigate the molecular defect involved in different patients with *STS* deficiency. Fig. 4*a* shows the hybridization of probe p422 to the DNA from a family in which the proband is *STS* deficient and has an X/Y translocation with a deletion of the Xp22–Xpter region. The chromosome analysis of this patient revealed a 46,Y,t(X;Y) (p22;q11) karyotype (data not shown). His mother and sister carry the same chromosomal abnormality. As expected for a STS cDNA sequence and in agreement with the observed localization (see above), the p422 probe did not hybridize to the patient's DNA. Fig. 4*b* shows the result of the hybridization of probe p422 to a Southern blot containing the DNA from three patients with classical XLI–*STS* deficiency syndrome (lanes 2, 3, and 5) and a patient with XLI associated with Kallmann syndrome (28) (lane 4). DNA preparations from all these patients lacked the normal hybridization band (at ≈9 kilobases) observed in

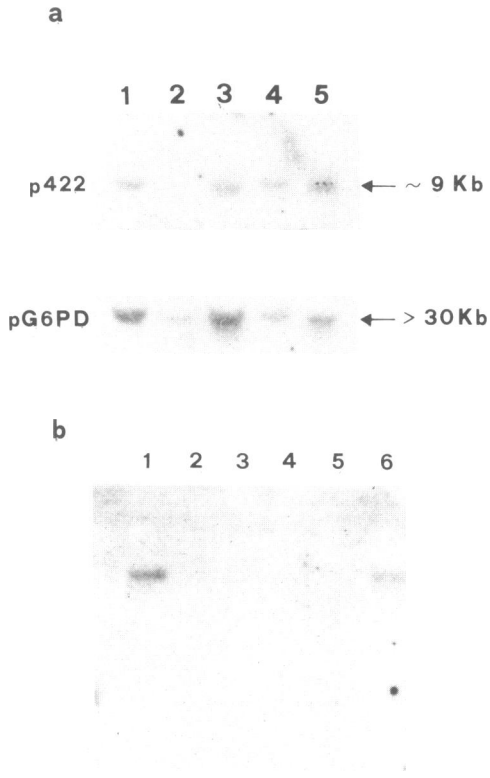

FIG. 4. (*a*) Southern blotting analysis of *Bam*HI digests of DNA from the family of a *STS*-deficient patient with an X/Y translocation. Lanes: 1, sister, karyotype 46,X,t(X;Y)(p22;q11); 2, proband, karyotype 46,Y,t(X;Y)(p22;q11); 3, mother, karyotype 46,X,t(X;Y)-(p22;q11); 4, father, karyotype 46,XY; 5, female control, karyotype 46,XX. No hybridization to the proband's DNA is observed using p422 as a probe, whereas normal hybridization bands are present in all the lanes using a glucose-6-phosphate dehydrogenase cDNA probe as a control. Kb, kilobases. (*b*) Southern blotting analysis of *Bam*HI digests of DNA from patients with different types of *STS* deficiency. Lanes: 1, female control; 2, patient M.C. (XLI); 3, patient A.F. (XLI); 4, patient D.S. (XLI + Kallmann syndrome); 5, patient F.P. (XLI); 6, male control. The STS cDNA probe p422 hybridized only to the DNA of the controls (band at ≈9 kilobases), thus revealing a deletion of the *STS* gene in all the patients. When the same filter was also hybridized using the glucose-6-phosphate dehydrogenase cDNA probe, the normal hybridization bands were visible in all the lanes (data not shown).

the controls (lanes 1 and 6). A normal hybridization pattern was observed in all lanes when a glucose-6-phosphate dehydrogenase cDNA clone (29) was used as a probe on the same filter (data not shown). In all cases, karyotype analysis has not revealed any chromosomal abnormality. Control experiments have not revealed any alterations to the typical hybridization signal with p422 for >10 normal males. Further cases examined subsequently have shown similar features. We have extended these studies to include a total of 12 unrelated Italian patients including 8 cases of classical XLI; all *STS*-deficient individuals have been found to be deleted for the fragment detected by p422. The detailed characterization of these will be published elsewhere.

DISCUSSION

The isolation of a cDNA clone by immunological screening with anti-STS antibodies, its assignment to Xp22.3, and the observation that the corresponding sequences are deleted in patients with classical ichthyosis provide strong evidence for its provenance as a cDNA representing the *STS* structural gene. Final confirmation of the identity of clone p422 will require comparison of its nucleotide sequence with the amino acid sequence of the enzyme. The availability of the clone presents an opportunity to investigate several significant problems in contemporary molecular genetics.

These include the nature of processes underlying chromosomal inactivation, the mechanisms of X/Y exchange in the etiology of XX males, and the nature of the genetic defects in *STS*-deficient patients.

Earlier studies on preparations of placentae or fibroblasts from *STS*-deficient patients, including both classical XLI and XLI/Kallmann syndrome, have failed to detect the presence of any polypeptides antigenically related to STS (16, 17, 30). Our results demonstrate a deletion of the *STS* gene in patients with classical XLI and normal karyotype. Although deletion may not be an absolute finding in cases of classical XLI, our observations suggest that a gene deletion may be the basic defect of many cases of *STS* deficiency. In comparison with other X-linked disorders studied at the molecular level, the high frequency of deletions observed for *STS* sequences is of considerable interest and suggests that this region of the chromosome may be particularly susceptible to rearrangements that result in deletions. The close proximity of the pseudoautosomal region that undergoes regular exchange with the Y chromosome and the absence of the *STS* gene on the Y short arm may be significant in this context. The association of *STS* deficiency in combination with other X-linked disorders, such as chondrodysplasia punctata (26) and Kallmann syndrome (28), has suggested that deletions occurring in the distal part of the Xp region, and that are undetectable by cytogenetic analysis, may involve contiguous genes. The finding of a deletion of the *STS* gene in a patient with the complex phenotype, resulting from the association of XLI with Kallmann syndrome, reinforces the impression that this association may represent a "contiguous gene syndrome" (24), in which a deletion of both the *STS* and the X-linked Kallmann syndrome loci is the basic defect. This allows us a tentative mapping of the X-linked Kallmann syndrome locus to the Xp22.3 region.

The absence of a significantly homologous Y chromosomal sequence to p422 excludes the presence of an intact, but nonexpressed, Y chromosome-linked allele in humans; a situation that differs significantly from the putative pseudoautosomal localization of *STS* alleles in mice (see ref. 12). This provides additional evidence for substantial differences in the molecular organization of the Y chromosome in the two species. Lack of significant hybridization to any autosomal sites indicates that the genes for STS (arylsulfatase C) and the arylsulfatases A and B are not strongly conserved at the sequence level.

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