

# Members of the SRY family regulate the human LINE retrotransposons

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## ABSTRACT

**LINEs are endogenous mobile genetic elements which have dispersed and accumulated in the genomes of most higher eukaryotes via germline transposition, with up to 100 000 copies for the human LINE-1 (L1H) sequences. Although severely repressed in most normal tissues, L1H is still functional, with evidence for both germline and somatic—essentially in tumors—transpositions. Yet, no transcription factor that could regulate their transcription and be responsible for their transposition has hitherto been described. Here we show that factors belonging to the family of the testis-determining factor gene SRY (the SOX family) can modulate L1H promoter activity over a 10-fold range in a transient transfection assay using a luciferase reporter gene. These effects depend on two functional SRY binding sites which can be identified within the L1H promoter via mobility shift assays. Induction of endogenous L1Hs upon ectopic expression of the SOX11 transcription factor is further demonstrated, thus strengthening the physiological relevance of these new—and highly dispersed—target sites for the otherwise unclassical transcription factors of the SRY family.**

## INTRODUCTION

LINEs are highly reiterated transposable elements (100 000 copies in the human genome), responsible for several genetic diseases through insertional mutagenesis (reviewed in 1). Their transposition requires synthesis of a full-length genomic RNA (gRNA) followed by its reverse transcription and integration by LINE-encoded proteins. Human LINE-1 (L1H) transcription is driven by an internal promoter located within the first 670 bp of the element (2,3). It is severely repressed in most somatic, differentiated cells, but induction can be observed in embryonal carcinoma cells and testicular germline tumors (4–6). DNA methylation (7,8) and RNA polymerase III (9,10) have been tentatively identified as playing a role in L1H promoter activity, but no transcription factor that could regulate transcription of endogenous L1Hs has been described. We show here that the L1H promoter contains two functional

binding sites for transcription factors of the SRY family (11,12), namely the SOX factors (reviewed in 13). SRY, the prototype of the SOX family members, is involved in sex determination (11,12). It is expressed in the urogenital ridge of the embryo and, in the adult, in the testis, hypothalamus and midbrain (reviewed in 13). Although less extensively studied, other SOX factors also appear to be expressed according to restricted and specific patterns, mainly during development and, in some cases, in the adult (13). SOX factors all share a conserved domain of about 80 amino acids, the High Mobility Group (HMG) box (14)—also found in the HMG1 and HMG2 proteins (15). This HMG box is involved in DNA binding and a consensus sequence for SOX binding sites has been derived as A/TA/TCAAA/T (16 and references therein). The two binding sites that we presently identify in the L1H promoter are functional sites, which are required for some of the SOX members to transactivate a luciferase reporter gene driven by the L1H promoter. Ectopic overexpression of one member of the SRY family, SOX11, is further shown to result in a 10-fold transactivation of the endogenous L1Hs in human cells.

## MATERIALS AND METHODS

### Cell culture and transfection

RD and 293 cells (from the European Collection of Cell Cultures) and NTera2/D1 (from the ATCC) were grown in DMEM—in MEM for 293 cells—supplemented with 4.5 g/l glucose, 10% fetal calf serum and antibiotics, at 37°C in 6% CO<sub>2</sub>. Transfections of dispersed cells were carried out with Lipofectamine Plus Reagent (Gibco BRL) or Geneporter (Boehringer Mannheim) according to manufacturers' instructions. Cells were harvested 2 days post-transfection for transient transfection assays. Luciferase activity was measured with a luciferase assay kit (Promega) and  $\beta$ -galactosidase activity with CPRG (Boehringer Mannheim) as a  $\beta$ -galactosidase substrate, using the same cell extract. For stable transfections, cells were cotransfected with the pJ3 $\Omega$ SOX11 vector, or control vector, and the pSVTKneo $\beta$  plasmid, conferring G418 resistance, at a 19:1 molar ratio, and G418R cell clones were isolated using 1 mg/ml G418 (Gibco BRL).

### Plasmids

L1luc reporter gene and mutated L1A<sup>mluc</sup>, L1B<sup>mluc</sup> and L1A<sup>mb</sup> derivatives (1). The L1H promoter was excised from pL1.2A plasmid [which contains L1.2A L1H element

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(17) with no flanking cellular DNA, subcloned in pGEM5Zf+] as a *Bst*XII (position 902 in L1H) Klenow-treated *Sac*II (in pGEM5Zf+ polylinker) fragment and subcloned in pKS- (Stratagene) opened by *Xba*I Klenow-treated and *Sac*II. L1H promoter was then excised as a *Sac*I–*Bam*HI fragment (both sites in PKS-polylinker) and inserted into pGL3 Basic Vector (Promega) opened by *Sac*I and *Bg*III [both sites in the polylinker, upstream of the luciferase open reading frame (ORF)]. Mutations in the A and B sites were introduced by PCR with oligonucleotide primers carrying the modified sequences. L1H sequences for all the luciferase vectors were verified by DNA sequencing. (2) SOX expression vectors: the *SOX3* sequence was excised from plasmid X2-1 H33 (which contains a *SOX3* genomic fragment cloned at the *Hind*III site of pKS+; gift from P. Hextall, Cambridge, UK) as a *Hind*III (~440 bp upstream of the *SOX3* ORF first AUG codon) Klenow-treated fragment. *SOX11* ORF was excised from the pJ3Ω*SOX11* expression vector (gift from P. Berta, UPR1142, France) as a *Hind*III (located in the polylinker of pJ3Ω and 3' to *SOX11* ORF) Klenow-treated fragment. *SOX* fragments (or no fragment for the CMV-none control expression vector) were cloned into the pCMVβ vector (Clontech) opened by *Not*I (which removes the β-galactosidase ORF) and Klenow-treated. CMV–SRY was a gift from M. Fellous (Pasteur Institute, France).

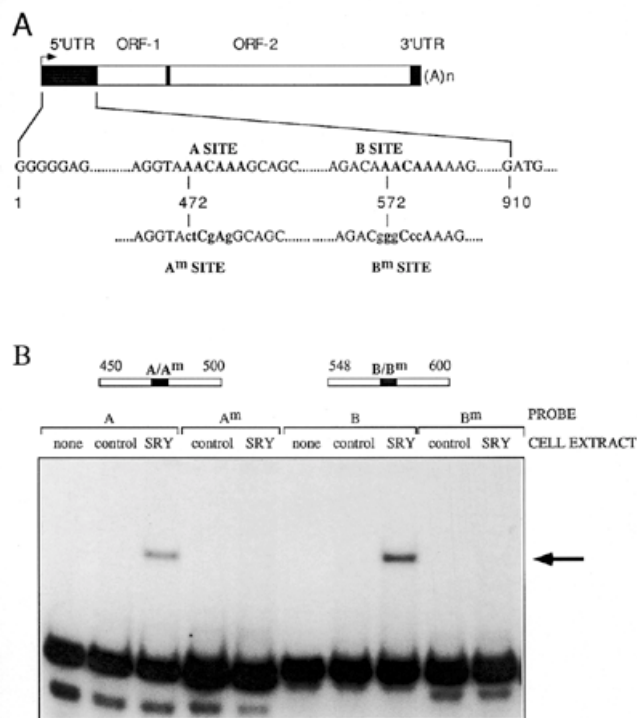
### Northern blot analysis

Cytosolic RNA was extracted using  $10^7$ – $4 \times 10^7$  cells, which were first rinsed twice with isotonic phosphate-buffered (pH 7.5) saline (PBS), and then harvested, after trypsin/EDTA treatment, by soft pipetting in PBS. After centrifugation, the cells were lysed by a 10-min incubation on ice in 1 ml of EBC buffer (0.5% Nonidet P-40, 10 mM Tris–HCl pH 7.9, NaCl 140 mM) supplemented with 30 mM β-mercaptoethanol, 5 mM ribosyl-orthovanadate complexes, 2 mM MgCl<sub>2</sub> and brief vortexing. Supernatants recovered after a 10-min centrifugation at 13 000 r.p.m. were supplemented with 7 ml guanidium-thiocyanate and further processed by the guanidium/cesium chloride procedure (18). RNAs were separated by electrophoresis and blotted onto HybondN<sup>+</sup> filters (Amersham) according to the manufacturer's instructions.

L1H single-stranded DNA probes were synthesised by submitting 100 ng of pL1*luc* plasmid linearised with *Sac*I (site just 5' to the L1H 1–906 promoter sequence) to 20 cycles of PCR using the GLprimer2 (Promega) oligonucleotide (located 65 bp 3' to the L1H promoter) and [ $\alpha$ -<sup>32</sup>P]dCTP. Labelled DNA was purified by elution through a Sephadex G50 column. Control experiments disclosed that this single-strand DNA probe, but not the complementary one (generated as above after linearisation of pL1*luc* and PCR amplification with the Promega RVprimer3 oligonucleotide), specifically hybridised to L1H gRNA from NTera2/D1 cells.

### Electrophoretic mobility shift assay

The indicated DNA probes were synthesised by PCR with linearised pL1*luc* or its mutated derivatives as templates and adequate oligonucleotide primers. Procedures to obtain *Escherichia coli* cytoplasmic cell extracts were as follows: lysis of the washed bacterial cells in 50 mM sodium phosphate buffer pH 7.4, 50 mM NaCl, 10 μg/ml RNase A, 1 mg/ml lysozyme, 1 mM PMSF, 0.2% Tween, brief sonication, centrifugation at 13 000 r.p.m. for 15 min and harvesting of the



**Figure 1.** SRY binding sites in the L1H promoter. (A) Identification of two consensus SRY binding sites in the L1H element. On top, a complete L1H element is schematised with the 5' and 3' untranslated regions (UTRs), the two open reading frames (ORF), and the transcription start site located at the first nucleotide of L1H (indicated with an arrow). The A and B sites in the L1H 5' UTR (at nucleotide positions 472 and 572) are indicated in bold capital letters, and the introduced mutations, in A<sup>m</sup> and B<sup>m</sup>, are indicated below with mutated nucleotides in bold lower case). (B) Electrophoretic mobility shift assay for binding of the SRY HMG box peptide to the A and B sites. The DNA probes encompassing the A or B sites (or the mutated A<sup>m</sup> and B<sup>m</sup> sequences) are shown on top with nucleotide positions indicated. <sup>32</sup>P-labelled probes were incubated with pT7-hSRYbox88-transformed *E.coli* cytoplasmic extracts (SRY), control cell extracts (control) or no extract (none). The shifted bands are indicated with an arrow.

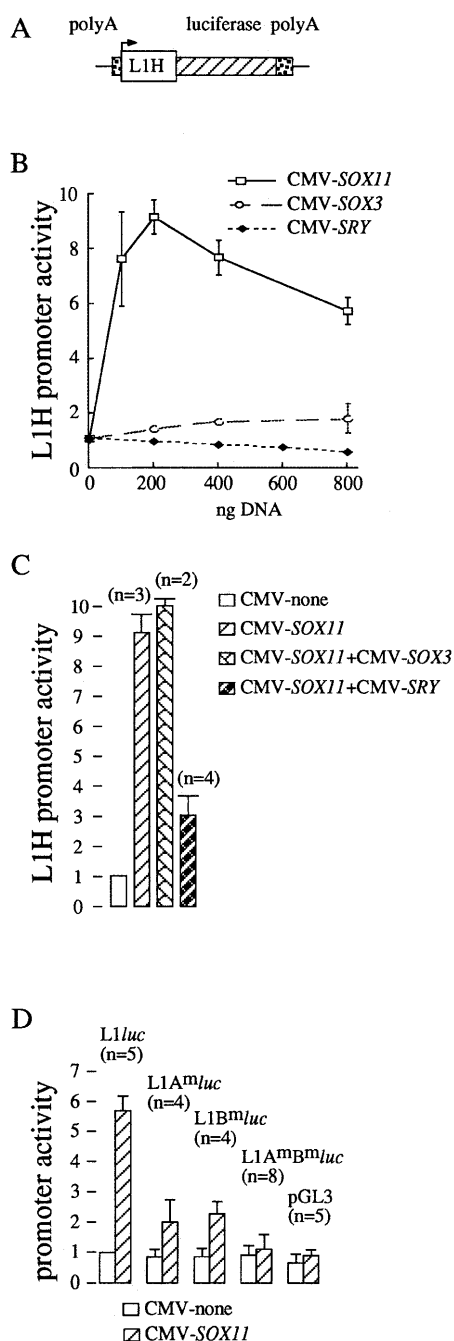
supernatant. pT7-hSRYbox88 bacterial cells contains an IPTG-inducible plasmid encoding the 88 amino acid long HMG box of the human SRY factor (19). In our hand, this peptide was produced in too weak amounts to be detected upon SDS–PAGE electrophoresis and Coomassie blue staining of the bacterial cell extract. Protein–DNA binding reactions were performed in 30 μl, containing 100 mM KCl, 20% glycerol, 20 mM HEPES pH 7.9, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 μg salmon sperm DNA, 6 μg *E.coli* cytoplasmic cell extracts and ~100 000 c.p.m. of the <sup>32</sup>P-labelled DNA probes.

## RESULTS

Using a computer search program (TFSEARCH version 1.3), we identified two potential binding sites for SOX transcription factors within the first 670 nt of the L1H sequence (9,17): one between nucleotides 472 and 477 (site A) and another between nucleotides 572 and 577 (site B), both with the sequence AACAAA (Fig. 1A). Electrophoretic mobility shift experiments

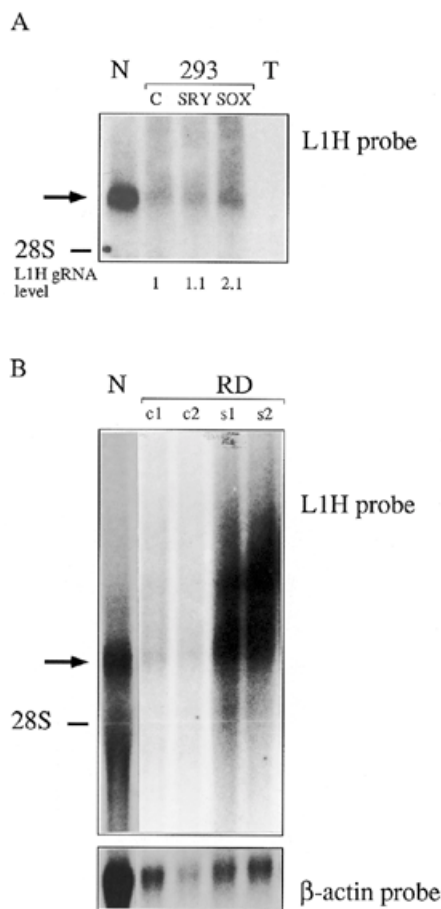
(Fig. 1B) confirmed that these A and B sites can specifically interact with the DNA binding domain of SRY—the prototype of the SOX family members (11,12). Indeed, cytoplasmic extracts from *E.coli* cells expressing the SRY HMG box domain (hSRYbox88) (19), but not cytoplasmic extracts from control cells, alter the mobility of DNA probes encompassing the A or B sites. Moreover, mutations introduced into the A ( $A^m$ ) or B ( $B^m$ ) sites abolished the observed SRY-dependent mobility shift (Fig. 1B).

These observations led us to examine whether L1H promoter-driven transcription is modulated by SOX factors. A luciferase reporter gene (*L1luc*) was therefore constructed by



inserting the first 906 nt of L1H [corresponding to the promoter-containing 5' UTR of the L1.2A clone (2,17), Fig. 1A] into a commercially available luciferase vector (Fig. 2A and Materials and Methods). The effects of various SOX proteins on L1H promoter activity was then analysed by transient co-transfection of cultured cells with the *L1luc* reporter together with different SOX expression vectors and a  $\beta$ -galactosidase expressing gene (pCMV $\beta$ , see legend to Fig. 2) to normalise for transfection efficiency. Cells from the human RD cell line—derived from a human embryo rhabdomyosarcoma—were used as recipients because they display a very low basal level of endogenous L1H gRNA (T.Tchénio, unpublished). As illustrated in Figure 2B, the SOX11 factor (20,21) transactivates the *L1luc* reporter gene up to a maximum of 9-fold (at 200 ng DNA), followed by a small decrease at higher DNA concentrations. The SOX3 factor (22,23) also transactivates *L1luc*, but only to a limited extent. Conversely, the human SRY factor reduces *L1luc* expression by ~2-fold, under conditions where no effect of SRY on a control luciferase reporter gene driven by the minimal SV40 promoter (pGL3promoter vector; Promega) was observed (data not shown). This antagonistic effect of SRY on L1H promoter is also manifested by the observed reduction of the SOX11-mediated *L1luc* transactivation in the presence of SRY, not observed with SOX3 (Fig. 2C). These results are in agreement with the absence of an activation domain in the human SRY factor, and with the reported inhibitory effects of SRY on a recombinant thymidine kinase promoter flanked by SRY binding sites (24). Finally, to demonstrate the role of the A and B sites in the SOX-mediated regulation of L1H promoter activity, the  $A^m$  and/or  $B^m$  mutations (Fig. 1A) were introduced into the *L1luc* vector. The data in Figure 2D show that both sites are required for full transactivation by the SOX11 protein and that mutation of both sites completely abolishes the SOX11 stimulatory effect.

**Figure 2.** Modulation of L1H promoter activity by SOX proteins in transient transfection assays using human RD cells. (A) Structure of the *L1luc* luciferase reporter gene. The L1H promoter sequence (L1H) from nucleotide 1 to 906 was inserted into the polylinker of pGL3 basic vector (Promega), upstream of the luciferase ORF. PolyA, polyadenylation sites. (B) Effects of varying amounts of transfected SOX expression vectors on *L1luc* reporter gene activity. About  $10^6$  human RD cells were cotransfected with 20 ng of luciferase reporter gene, the indicated amount of CMV-SOX expression vector, 200 ng of pCMV $\beta$  plasmid, complemented to a total of 1  $\mu$ g of DNA with CMV-none. Luciferase activity was normalised to  $\beta$ -galactosidase activity measured in the same cell extracts. L1H promoter activity is expressed relative to that measured upon transfection with CMV-none set as 1 [and similarly for *L1luc* in (C) and (D)]. Luciferase activity for a control *luc* vector lacking the L1H promoter was <3% that of *L1luc*. Standard deviations are indicated by error bars. (C) Interactions between SOX proteins. RD cells were cotransfected with *L1luc* as in (B) with 800 ng of control plasmid (CMV-none) or with 200 ng CMV-SOX11 plus 600 ng of one of the three following plasmids: CMV-none, CMV-SOX3 or CMV-SRY; n indicates the number of independent experiments, and errors bars are standard deviations. (D) Effect of mutations within the A and B sites on SOX11-mediated L1H promoter activation. RD cells were cotransfected as in (B) with the *L1luc* reporter gene or one of its mutated  $A^m$  and/or  $B^m$  derivative, and 800 ng of either CMV-none or CMV-SOX11. pGL3 is a luciferase reporter gene under the control of the minimal SV40 promoter (Promega); n indicates the number of independent experiments, and errors bars are standard deviations.



**Figure 3.** Sox11-mediated transactivation of endogenous L1Hs. **(A)** Northern blot analysis of transiently transfected 293 cells. RNA (15  $\mu$ g per lane) extracted from cells transiently transfected with either pCMV $\beta$  (C), CMV-SRY (SRY) or CMV-SOX11 (SOX) were electrophoresed together with Ntera2/D1 (N) and NIH 3T3 (T) RNA samples, and hybridised with an antisense (nucleotide 906 to 1) L1H single-stranded DNA probe. Arrows indicate the position of L1H gRNA. The numbers indicate the relative level of L1H gRNA as normalised to the amount of 3-glyceraldehyde phosphate dehydrogenase transcripts. Exposure time was 3 days. In this experiment, the percentage of transfected cells was close to 20% as deduced from the percentage of  $\beta$ -galactosidase-positive cells in the transfection with pCMV $\beta$ . **(B)** Northern blot analysis of total RNA (15  $\mu$ g per lane) from independent RD cell clones stably transfected with control (c1 and c2) or pJ3 $\Omega$ SOX11 expression vector (s1, s2), using the same antisense single-stranded DNA probe as in (A). Exposure time was 1 and 3 days for Ntera2/D1 and RD cells, respectively. Control hybridisation with a  $\beta$ -actin probe is shown (exposure time 16 h).

To analyse whether ectopic expression of SOX proteins can modulate the level of endogenous L1H gRNA, a transient transfection assay was performed, using the easily transfectable human kidney-derived 293 cells. Endogenous L1H RNAs were then analysed by northern blot, upon hybridisation with an antisense L1H single-stranded DNA probe (Materials and Methods). Ntera2/D1 embryonal carcinoma cells—one of the only few human cell lines in which high level expression of a discrete 6 kb L1H gRNA can be detected by northern blot analysis (6)—as well as murine NIH 3T3 cells (devoid of L1H genetic elements) were used as controls (Fig. 3A). Although

endogenous L1H transcripts are not highly expressed in the 293 cell line, transient transfection of the cells with the CMV-SOX11, but not the CMV-SRY, vector resulted in an increase in the accumulation level of the 6 kb L1H-hybridising transcript (2–3-fold, Fig. 3A and data not shown). To further confirm these results, stably transfected SOX-expressing cells were established. A vector expressing SOX11 under the control of the SV40 early promoter (pJ3 $\Omega$ SOX11 vector) was introduced by transfection into RD cells together with a vector for the selectable neomycin gene. G418-resistant clones were then isolated, and those expressing stably integrated SOX11 vectors (about one-third of the transfected clones) were characterised by Southern and northern blot analyses (data not shown). As illustrated for independent clones by the northern blot shown in Figure 3B, L1H gRNAs—as detected using the antisense L1H probe—were induced in the SOX11 expressing cells, ~10-fold as compared to control transfected cells. A heterogeneous population of L1H-hybridising transcripts of larger size, most probably originating from inefficient transcription termination of endogenous L1Hs (25), was also induced upon SOX11 expression, resulting in the observed smear of high molecular weight RNAs. No antisense transcripts were detected in these clones using, under identical experimental conditions, a sense L1H single-stranded DNA probe (data not shown), thus indicating that the transcripts observed above do originate from L1Hs.

## DISCUSSION

The present results therefore show that the human L1H promoter not only contains SRY binding sites, *sensu stricto*, but that these sites are indeed ‘functional’, being responsible for the efficient transactivation of the L1H promoter by a SOX family member. Although one would expect the rather degenerate consensus SOX binding sequence (i.e. A/TA/TCAAA/T) to be frequently encountered, and despite the limited sequence specificity for SOX binding (26), functional SOX sites have been identified in only a limited number of genes (including the Müllerian Inhibiting Substance, the FGF4, the collagen II and the TCR $\alpha$  genes; reviewed in 13). It has been suggested that the much more stringent conditions required for a SOX ‘effect’ versus ‘binding’ is due in part to a recognition sequence that extends beyond the consensus sequence and/or the ability of SOX to make protein–protein contacts with other DNA binding proteins (13,16). In fact, it has been proposed that the mode of action of the SRY factor and related proteins is to organise the local chromatin structure—in part *via* their ability to bend DNA in a sequence-dependent manner—and assemble other DNA-bound factors into biologically active, sterically defined multiprotein complexes (27,28). In the case of L1H, no conspicuous binding site for other known transcription factors has yet been identified in the promoter sequence, except those previously characterised for the YY1 protein (29,30). Being ubiquitously expressed, it is very unlikely that YY1 accounts for the restricted pattern of L1H expression as observed *in vivo*, but it remains plausible that it cooperates with SOX factors to assemble the transcription machinery at the L1H promoter, all the more as YY1 is itself an ‘architectural’ factor which can promote DNA binding to the nuclear matrix (31). Whatever the underlying mechanisms for the SOX-mediated transactivation, a noteworthy consequence of the

present investigation is that the high copy number of full-length L1H transposable elements results *de facto* in the dispersion of thousands of functional SOX binding sites in the genome. Targeting of SOX factors to these sites may therefore result not only in the activation of the LINE elements themselves, but also in that of nearby genes, possibly via SOX-mediated 'architectural' effects. This would constitute an unexpected and important additional consequence of transposon mobility on genome structure and gene regulation. Finally, an important issue will be to determine whether SOX factors, such as SOX11, are expressed *in vivo* in the human germ cell lineage, that could activate L1H elements. In fact, several SOX family members are likely to be expressed in these cells, as suggested by the demonstrated expression of various SOX factors in germline-derived tumours (32 and references therein). Such germline-expressed SOX factors would be serious candidates as essential determinants of L1H activity *in vivo* under both normal and pathological conditions.

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