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Human Synaptobrevin-like 1 Gene Basal Transcription Is Regulated through the Interaction of Selenocysteine tRNA Gene Transcription Activating Factor-Zinc Finger 143 Factors with Evolutionary Conserved Cis-elements*

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The synaptobrevin-like 1 (*SYBL1*) gene is ubiquitously expressed and codes for an unusual member of the v-SNAREs molecules implicated in cellular exocytosis. This X-linked gene has the peculiarity of also being present on the Y chromosome in a transcriptional inactive status. Moreover, although ubiquitous, the function of *SYBL1* is prominent in specific tissues, such as brain. As a first insight into the molecular mechanisms controlling *SYBL1* expression, in this report we describe the extent and role of *SYBL1* upstream regions and characterize the binding of trans-acting factors. *In vivo* footprinting experiments identify three protected regions. Band shift and transient reporter gene assays indicate a strong role of two of these evolutionary conserved regions in regulating *SYBL1* transcription. Because one site is the classical CAAT box, we characterized the binding to the other site of the mammalian homologues of the selenocysteine tRNA gene transcription activating factor (Staf) family, zinc-finger transcription factors, and their role in regulating *SYBL1* expression. The results reported here clarify that a Staf-zinc finger family factor, together with the CAAT factor, is the major nuclear protein bound to the *SYBL1* promoter region and is responsible for its regulation in HeLa cells, thus identifying the basic control of *SYBL1* transcription. *In vivo* binding of Staf proteins to the *SYBL1* promoter is confirmed by chromatin immunoprecipitation assays. Our results identify a fourth mRNA promoter stimulated by a member of the Staf-zinc finger family, the function of which on mRNA polymerase II promoters is still very poorly understood.

Synaptobrevin-like 1 (*SYBL1*),[1] a highly conserved gene (1), is ubiquitously expressed and codes for an unusual member of the v-SNAREs molecules (also called synaptobrevins or vesicle-associated membrane proteins, VAMPs) implicated in the cellular exocytosis, *i.e.* the fusion between a donor vesicle and an acceptor membrane, through interaction with target SNAREs molecules.

This gene is localized on the human long arm pseudoautosomal region (XqPAR) (2), a region shared between X and Y chromosomes. Its transcriptional regulation has been largely analyzed as a model for the study of X inactivation maintenance in mammals. This regulation is achieved by epigenetic mechanisms, such as methylation (3, 4), core histone modifications, and chromatin remodeling (5).

An additional level of regulation of this gene could derive from its cellular functions: the SYBL1 protein is also called Ti-VAMP (tetanus-insensitive VAMP) because it is the only v-SNARE insensitive to tetanus and botulinum toxin cleavage (6, 7). Hence, Ti-VAMP is crucial for all cellular transports whose occurrence is not affected by these toxins, such as neurite outgrowth (8, 9) and apical intestinal transport (7).

The tissue-restricted function of an otherwise housekeeping molecule may be dependent upon a plethora of mechanisms, transcriptional, post-transcriptional, or translational. As a first insight into the molecular mechanisms controlling *SYBL1* expression and consequently its function, we describe here the extent and role of *SYBL1* upstream regions and characterize the binding of trans-acting factors possibly regulating *SYBL1* gene expression.

To investigate the regions of the *SYBL1* promoter capable of trans-acting factor binding, we performed *in vivo* footprinting experiments. Three sites were protected; by using bioinformatics analysis we identified two as regions that play an important role in determining the promoter activity of the gene containing, respectively, CAAT and Staf-ZNF motifs. Staf (selenocysteine tRNA gene transcription activating factor) was identified as a transcription factor that binds to the tRNA (Sec) activator element mediating its activation properties. Staf can also stimulate transcription from an mRNA-type pol II promoter (10, 11). To date, only three mRNA pol II promoters, with transcription regulated by Staf, have been identified (12–14). Two mammalian homologues of Staf, ZNF76 and ZNF143, have been

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[1] The abbreviations used are: SYBL1, synaptobrevin-like 1; v-SNARE, vesicle soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor; VAMP, vesicle-associated membrane protein; Staf, selenocysteine tRNA gene transcription activating factor; ZNF, zinc finger factor; pol II, polymerase II; DMS, dimethylsulfate; SL2, *Drosophila* Schneider cells; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; wt, wild type.

isolated (15, 16) that recognize very similar sequences (17–19). ZNF76 and ZNF143 are basically considered to play the same role as Staf, even though the expression levels in various tissues differ between the two homologues. ZNF143 is considered to be the ortholog of Staf, whereas ZNF76 is a DNA-binding protein related to Staf and ZNF143 (18).

To assess their relative importance in *SYBL1* promoter function, we mutated each of the three sites identified, to disrupt each element separately and yet together in the same promoter. The mutation of the Staf-ZNF motif results in a decrease in promoter activity. On the other hand, co-transfection of the *SYBL1* promoter and Staf-ZNF-expressing plasmids in *Drosophila* Schneider (SL2) cells reveals that Staf-ZNF increases promoter activity. Chromatin immunoprecipitation (ChIP) assays confirmed that endogenous Staf/ZNF is constitutively bound to the endogenous *SYBL1* promoter.

Our results suggest that Staf-ZNF is a nuclear protein bound to the regulatory region of the *SYBL1* promoter and, together with the CAAT factor, can regulate basal promoter activity. These data will provide the platform for understanding the transcriptional control of this peculiar gene.

EXPERIMENTAL PROCEDURES

DMS in Vivo Footprinting—HeLa cells (10^7) grown in 15-cm dishes were incubated with DMS (dimethylsulfate) (1 μl/ml; Sigma) for 5 min. DNA extraction, cleavage with piperidine, and linker-mediated PCR reaction were performed as described (28). The primers used (*a1*, *a2*, *a3* for the upper strand and *b1*, *b2*, *b3* for the lower strand) are shown in Fig. 1. DNA-protein interactions were analyzed on 6% polyacrylamide sequencing gels. As a control, phenol-extracted chromosomal DNA was treated with DMS *in vitro* and analyzed in parallel.

Reporter and Effector Constructs—The reporter gene wt*SYBL1* was generated by subcloning the −217/+37 fragment obtained by PCR amplification of the human *SYBL1* gene 5′-flanking region into XhoI-HindIII-digested pGL3-Basic vector (Promega) containing the firefly luciferase gene. The 5′ and 3′ external vector-targeted primers used (Fw-out, 5′-GGCATCTCGAGGACGCTGTTTACCAC-3′, or Rs-out, 5′-GGACTAAGCTTCAGGGTCGCCAACTG-3′), incorporated a XhoI and a HindIII site, respectively. Nucleotide substitutions were introduced into the wt*SYBL1* (−217/+37) construct by site-directed mutagenesis to obtain single, double, and triple mutants (Fig. 3). Briefly, as a first step, PCRs were carried out using an internal mutagenic primer and one of the external vector-targeted primers (Fw-out or Rs-out). In each mutated site, six nucleotides were substituted with an EcoR1 site (Figs. 1 and 3). The PCR products from each set of first-step PCRs were purified, mixed, and used in the second-step PCR with the external vector-targeted primers. The products from the second-step PCRs were digested with XhoI and HindIII and cloned into a pGL3-basic vector. To construct the double mutants CAAT/Staf-ZNF and SYBL1-X/Staf-ZNF, the −217/+37 SYBL1 fragment was excised from the respective mutated constructs and digested with RsaI. The fragments containing the desired mutations were linked together and with the pGL3-basic vector. For the double mutant CAAT/SYBL1-X the excised fragment from mutant SYBL1-X was digested with TseI, whereas the excised fragment from mutant CAAT was digested with TseI and DdeI. These fragments were ligated together and with pGL3-basic vector. To obtain the triple mutant we digested the mutants SYBL1-X/Staf-ZNF with TseI and the mutant CAAT with TseI and DdeI. The fragments of interest obtained were ligated together and with the pGL3-basic vector. All of the constructs were sequenced using a DNA sequencer (ABI Prism 3100) to confirm the presence of the desired mutations. The *Drosophila* expression vectors pPac-ZNF143, pPac-ZNF76, and pPac-Staf have already been described (18, 20) and were kindly donated by Dr. P. Carbon. The empty control plasmid pPac0 (21), containing only the *Drosophila* actin promoter, was generously provided by Dr. P. De Luca.

Cell Culture and Transfection—HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (streptomycin and penicillin at usual concentrations). For transfection experiments the cells were grown in a 6-well plate at a density of 2×10^5 cells/well. Either 1.0 μg of pGL3-promoter plasmid or an equivalent molar amount of test plasmid was co-transfected into HeLa along with 0.030 μg of pRL-CMV plasmid using FuGENE 6 reagent according to the manufacturer's instructions (Roche Applied Science). The pRL-CMV vector containing the *Renilla* luciferase gene under control of the herpes simplex virus thymidine kinase promoter (Promega) was used as an internal control for differences in transfection efficiency. To ascertain the functional analysis of the promoter region of the *SYBL1* gene, the transfected cells were maintained for 48 h in serum-supplemented medium before harvesting. At the end of the culture period, the transfectants were lysed and the luciferase activity in cell lysates was measured by a dual luciferase reporter assay system (Promega). SL2 were grown at 25 °C in Schneider's medium. When effector constructs were employed, 1.4×10^6 cells were co-transfected with 1.0 μg of the reporter, 0.5 μg of the effector, and 0.020 μg of the internal control. To maintain the ratio between the FuGENE reagent and the amount of transfected DNA as recommended by the manufacturer, 2.0 or 1.5 μg of the inert vector pPac0 were co-transfected in Schneider's cell. Each transfection experiment was undertaken in triplicate, with at least two different plasmid preparations.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from untransfected HeLa cells and from transiently transfected SL2 cells by hypotonic lysis followed by high salt extraction of nuclei as described by Lee *et al*. (22).

Gel Mobility Shift Assays—Gel shifts were performed using standard methods (23). The double-stranded oligonucleotide probes (Staf-ZNFwt 5′-actactactcccagaatgcagtacggct-3′ and Staf-ZNFmut 5′-actactagaattcgaatgcagtacgcct-3′), end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase, were incubated with nuclear extracts for 20 min at room temperature. To carry out supershift assays, antibodies were added to the binding assay mixtures and incubated for 2 h on ice before the radiolabeled probe was added. Rabbit anti-serum against ZNF143 (12) was kindly provided by Dr. H. Kubota. IgG fraction was purified with this rabbit serum by protein A-Sepharose (Amersham Biosciences) column chromatography. DNA-protein complexes were separated on 5% polyacrylamide gels.

Chromatin Immunoprecipitation—ChIP procedure was performed as described previously (5). Briefly, we incubated 3×10^6 HeLa cells/immunoprecipitate with 1% formaldehyde for 10 min at 37 °C. Shearing of chromatin was performed to an average size of 200–1000 bp; after a spinning step to reduce debris, the soluble chromatin was diluted with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, and protease inhibitors). Immunoprecipitates of cross-linked complexes were prepared using 1.5 μg of anti-ZNF143 antibody. A "no antibody" sample (No Ab) was carried out as a control for nonspecific interactions. After an overnight incubation at 4 °C, the bound antibody-protein-DNA complexes (immunoprecipitates) were brought down with protein A-Sepharose beads. The supernatant fraction from the No-Ab sample was kept as the "total input chromatin." After cross-linking reversal and proteinase K digestion, each individual immunoprecipitate was purified by phenol/chloroform extraction and UV quantified.

The following primers were used for gene-specific PCR on the *SYBL1* promoter (Fig. 1) and on *SYBL1* exon 3 (negative control): *SYBL1* promoter, Syb14 GCGACACGGCTAGGCCTG and a2 TATGCCGCCTACTCATTGCTGGTAGA; Exon 3-specific primers, es3telF GTAGAATTCAGAAGTAAAAGTGATGTTG and es3telR GTCGGATCCAAGAACTGGCTGTATTAACA. Amplification was performed using Gold Taq polymerase (PerkinElmer Life Sciences) at thermocycler settings of 1 cycle at 95° × 10′ followed by 33 cycles of 95° × 30″, 62° × 30″, 72° × 30″ with a final extension of 72° × 5′. PCR products were separated by electrophoresis through 1.7% agarose gels and visualized by ethidium bromide intercalation.

RESULTS

In Vivo Footprinting Indicates Three Protected Sites in the SYBL1 Promoter Region—To identify regions of DNA-protein interactions in the *SYBL1* promoter, we performed *in vivo* genomic footprinting in the region from −319 to +102, which includes the two conserved regions (human-mouse) CR1 and CR2 and the three previously identified start sites (24, 3) (Fig. 1). Footprinting was carried out using DMS as a modifying agent, which methylates freely accessible guanosine residues of DNA in living cells unless they are protected by DNA-bound factors.

In HeLa cells, three protected sites were detected (Fig. 2). The profiles *in vivo* compared with the control DNA (*in vitro*) indicate, in fact, a protection of residues around −67/−68, around −100/−103,and around −144/−146 with respect to the principal transcriptional start site (3). A computer analysis

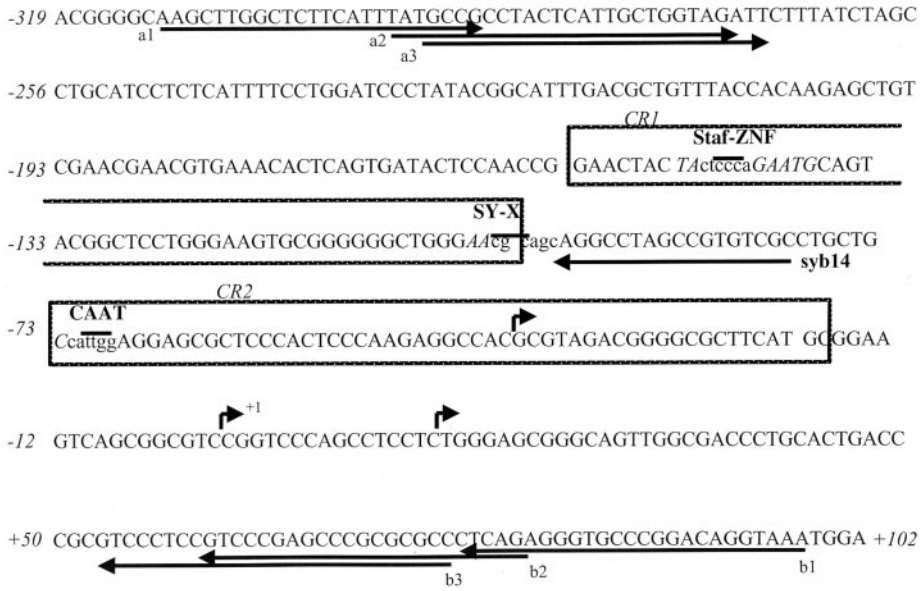

FIG. 1. **Nucleotide sequence of the 5′ flanking genomic DNA of the human *SYBL1* gene.** Consensus sites for potential DNA-protein binding sites, *top lines*. Transcription initiation sites are denoted by the *arrows above* the bases. *+1* indicates the principal start site (3). *Arrows under* the sequence indicate the positions of primers used for *in vivo* footprinting (*a1*, *a2*, *a3*, *b1*, *b2*, *b3*) and for the chromatin immunoprecipitation assays (*a2*, *syb14*). *Small letters* indicate mutated bases. The two conserved regions, CR1 and CR2, are *boxed*.

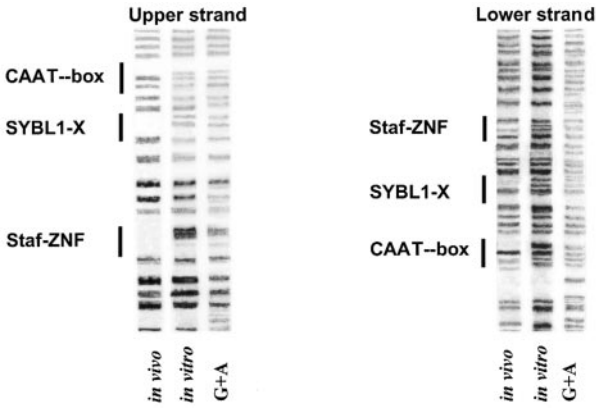

FIG. 2. **DMS *in vivo* footprinting across the human *SYBL1* promoter in HeLa cells.** The three protected sites identified are reported corresponding to CAAT, Staf-ZNF, and an unknown protein consensus binding site, SYBL1-X. Both the *upper* and *lower strands* were examined. The cleavage patterns obtained from genomic DNA methylated *in vivo* and the control DNA methylated *in vitro* in the absence of cellular protein are shown. *G+A* chemical cleavage is indicated.

(MatInspector 6.1; www.genomatix.de/cgi-bin/./eldorado/main.pl) to search for possible trans-acting factors recognizing these three regions indicated the possibility that the site around −67/−68 is a CAAT box binding site, whereas for the second site, around −100/−103 (SYBL1-X), no factors are presently indicated in trans-factor databases. For the third site, however, around −144/−146, the possible binding for a Staf-ZNF protein is also indicated.

Analysis of SYBL1 Mutants in HeLa Cells—To assess the relative importance of the three transcription factor binding sites in the *SYBL1* promoter function, mutations were introduced into each of the sites identified to disrupt each element separately in the same molecule (single, double, and triple mutants: see Fig. 3). Mutated constructs were then transfected into HeLa cells.

Promoter activities were determined by quantification of luciferase reporter gene levels (Fig. 3). Disruption of the CAAT element resulted in a decrease of the activity to 67% of wt (Fig. 3, *CAATmut*), disruption of the SYBL1-X element resulted in a slight decrease of activity to 81% of wt (Fig. 3, *SYBL1-Xmut*), and disruption of the Staf-ZNF element resulted in a decrease of activity to 39% of wt (Fig. 3, *Staf-ZNFmut*), indicating that this site is of major importance in the *SYBL1* promoter. With the double mutant CAAT/SYBL1-Xmut, the promoter activity was reduced almost to the same extent as the CAAT-mut (Fig. 3, *CAATmut/SYBL1-Xmut*). With the double mutant CAAT/Staf-ZNFmut, the promoter activity was reduced to 19% of wt (Fig. 3, *CAAT/Staf-ZNFmut*), and this activity is further reduced with the triple mutant to 8% of wt (Fig. 3, *CAAT/SYBL1-X/Staf-ZNFmut*). Surprisingly, in the double mutant SYBL1-X/Staf-ZNF, the activity is only slightly reduced to 71% of wt (Fig. 3, *SYBL1-X/Staf-ZNFmut*).

Staf-ZNF Binds to the SYBL1 Gene Promoter—To establish whether endogenous proteins from HeLa cells are able to bind to the Staf-ZNF site in the *SYBL1* promoter, nuclear extracts were incubated in the presence of a labeled probe containing the Staf-ZNF sequence (Fig. 4). A specific retarded protein-DNA complex was observed (Fig. 4, *lane 2*). This complex was abrogated with a 100-fold molar excess of unlabeled homologous oligonucleotide (Fig. 4, *lane 3*), establishing binding specificity. No competition of the specific complex was observed using an unlabeled oligonucleotide with mutation of the consensus Staf-ZNF binding sequence (data not shown). When this mutated oligonucleotide was used in gel mobility shift assays as labeled probe, complex formation was completely abolished (Fig. 4, *lane 6*). When protein-DNA complex was incubated with antibodies against ZNF143, a supershift was observed, suggesting that ZNF143 binds to the *SYBL1* promoter *in vitro* in the Staf-ZNF region (Fig. 4, *lane 4*).

ChIP (25) was performed to demonstrate the *in vivo* binding of ZNF143 to the *SYBL1* promoter in HeLa cells. This high-resolution technique is a powerful method successfully employed to verify the *in vivo* protein-DNA interactions of DNA-binding proteins (26, 27). To immunoprecipitate the ZNF143-DNA consensus site complexes, we used the same antibody described above (see Fig. 4). ZNF143 antibody enriched the *SYBL1* promoter sequences relative to the no antibody control sample, indicating the specific binding of ZNF143 on its consensus sequence of this promoter (Fig. 5). As a negative control, a region in the body of the *SYBL1* gene, which does not contain any binding site, was analyzed. As expected, this region was not enriched by the antibody to ZNF143.

Activation of the SYBL1 Promoter in Drosophila Schneider Cells—To further confirm a role for Staf-ZNF in the *SYBL1* promoter *in vivo* and determine whether members of the Staf-ZNF family of transcription factors functionally interact with

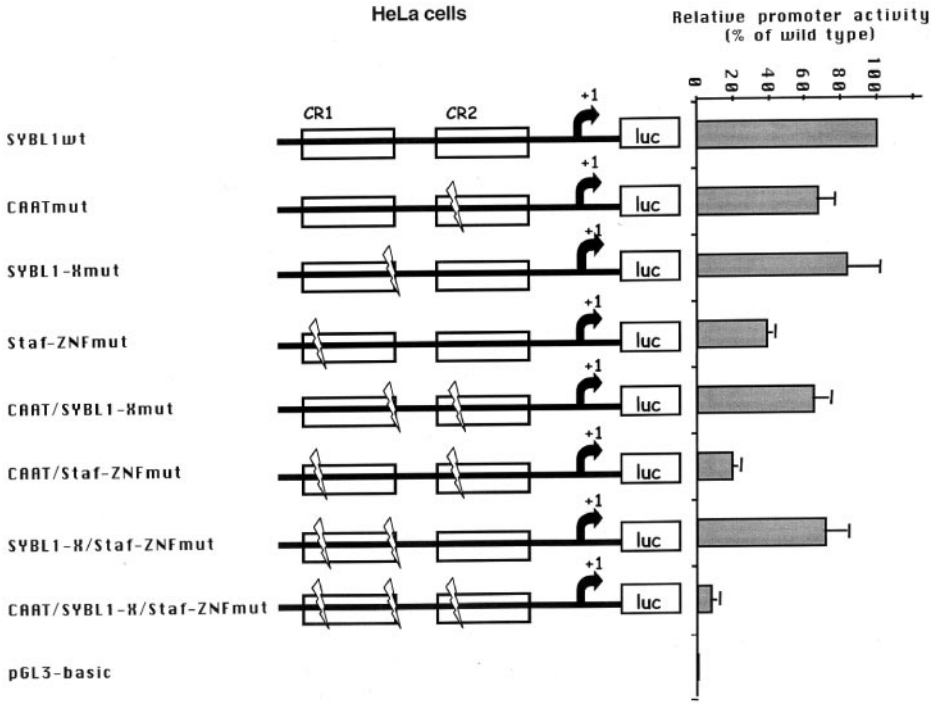

FIG. 3. **Reporter gene assay of the human *SYBL1* gene promoter region.** Luciferase constructs containing the wild type −217 to +37 or derivative thereof, mutated as indicated by Thunderbolt, were transfected with pRL-CMV vector into HeLa cells as described. The major start site (*+1*) is indicated by the *arrow*. The two conserved regions (*CR1* and *CR2*) are indicated by *boxes*. Corrected luciferase activity was calculated, and promoter activity was expressed as a percentage of that of the wild type promoter. The data are mean ± S.D. of at least six independent transfection experiments.

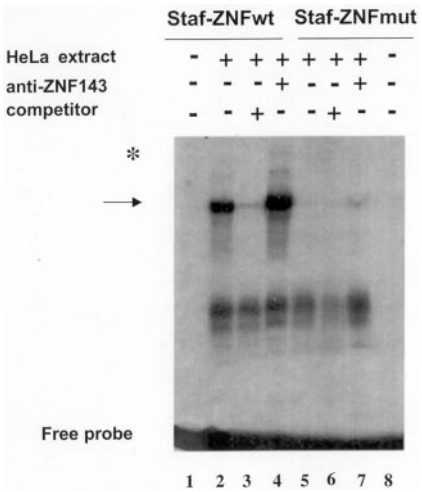

FIG. 4. **EMSA analysis on human *SYBL1* Staf-ZNF motif with HeLa cell nuclear extracts.** Bandshift analysis was performed with a probe containing the wt Staf-ZNF (*lanes 1–4*) or mutant version Staf-ZNFmut (*lanes 5–8*) of the human SYBL1 binding site. Probes were incubated in the absence of protein (*lanes 1*, *8*) or with HeLa extract. Incubation was in the absence (*lanes 1*, *2*, *4*, *5*, *7*, *8*) or presence of a 100-fold molar excess of unlabeled wt (*lane 3*) or mutant (*lane 6*) Staf-ZNF oligo or ZNF143 antibody (*lanes 4*, *7*; 5 μg). The position of the shifted complex is indicated by the *arrow*. The position of the supershift is shown with an *asterisk*.

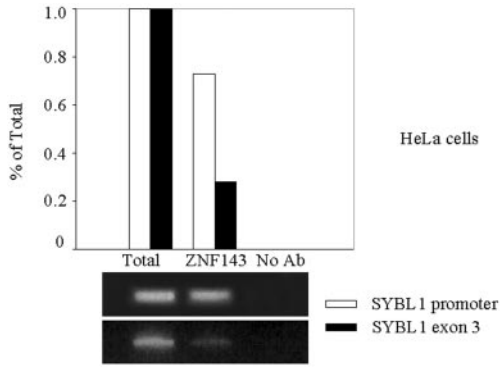

FIG. 5. **The *SYBL1* promoter is bound *in vivo* by ZNF143 in HeLa cells.** ChIP analysis was performed with ZNF143 antibody using 3×10^6 HeLa cells/antibody. All the immunoprecipitates and the input sample were examined by PCR with primers specific for the *SYBL1* promoter and exon 3. The sample no antibody (*No Ab*) was amplified to control for nonspecific sequence enrichment. Each image was quantitated with ImageQuant software and shown as a fraction of the input to compare PCR fragments. Here we show the two independent ChIP experiments performed, which gave similar results.

the promoter of *SYBL1*, transient transfection experiments were performed with SL2 cells, which lack many mammalian transcription factors such as ZNF143 and ZNF76 (18, 20). Expression constructs under the control of an SL2-specific promoter for ZNF143 (pPac-ZNF143), ZNF76 (pPac-ZNF76), Staf (pPac-Staf), and empty pPac0 were co-transfected along with reporter vectors (pGL3) under the control of different *SYBL1* promoters, the wild type promoter (Fig. 6, *wtSYBL1*), and the Staf-ZNFmut construct (Fig. 6, *Staf-ZNFmut*). Normalized luciferase activities for the pGL3 reporter constructs were compared with those obtained with the empty SL2 expression vector pPac0. Co-transfection of SYBL1wt with the expressing vectors pPac-Staf, pPacZNF76, or pPacZNF143 induced a notable increase (54,1%; 51,5%; and 101,2%, respectively) in relative luciferase activity over co-transfection with pPac0 (Fig. 6, *wtSYBL1*). In contrast, highly reduced values were observed after co-transfection of SL2 cells with Staf-ZNFmut and the expression constructs pPac-Staf, pPacZNF143, and pPac-ZNF76 (5,5%; 11,8%; and 10,6%, respectively) (Fig. 6, *Staf-ZNFmut*). These experiments demonstrated that Staf, ZNF76, and ZNF143 can specifically mediate the transcriptional activation of wild type *SYBL1* promoter in *Drosophila* SL2 cells.

Mobility Shift Assay in SL2 Cells—Expression in SL2 cells after transient transfection with the different pPac expression plasmids (pPac-Staf, pPacZNF76, or pPacZNF143) was confirmed using EMSA (electrophoretic mobility shift assay) (Fig. 7). In fact, the binding of expressed Staf, ZNF76, and ZNF143 proteins with the consensus oligonucleotides for Staf-ZNF was seen in gel shift assays using nuclear extracts from SL2 cells transiently transfected with the different expression plasmids (Fig. 7, *lanes 2*, *4*, *6*). No binding was observed with untransfected SL2 cells (Fig. 7, *lane 1*). Only a light supershift was

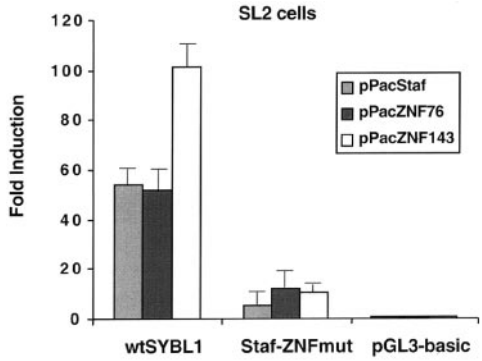

FIG. 6. **Staf-ZNF factors trans-activate the human *SYBL1* promoter in *Drosophila* Schneider cells.** SL2 cells were co-transfected with the indicated *SYBL1* promoter/pGL3-luciferase reporter gene constructs and pPac-Staf, pPacZNF76, pPacZNF143, or the empty pPac0 expression vector as described under "Experimental Procedures." Luciferase activity was assayed 48 h after transfection. Data are expressed as fold-induction of normalized luciferase activity relative to that obtained following co-transfection of the pGL3 reporter plasmids with empty pPac0, which does not express Staf-ZNF proteins. The data are mean ± S.D. of at least six independent transfection experiments.

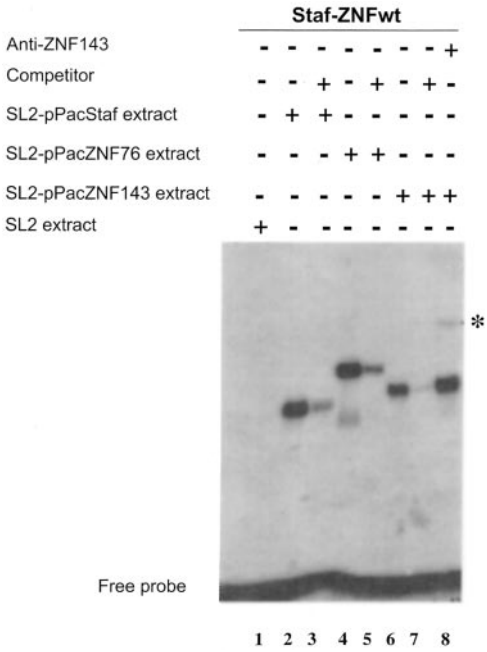

FIG. 7. **Expression of Staf-ZNF factors in transiently transfected SL2 cells.** Expression in SL2 cells after transient transfection with different pPac expression plasmids (pPacStaf, pPacZNF76, or pPacZNF143) was confirmed using EMSA. The wt Staf-ZNF probe was incubated with untransfected SL2 extract (*lane 1*) or with SL2 transfected with pPac-Staf (*lanes 2*, *3*), pPacZNF76 (*lanes 4*, *5*), or pPacZNF143 (*lanes 6*, *8*). Incubation was in the absence (*lanes 1*, *2*, *4*, *6* and *8*) or presence (*lanes 3*, *5*, *7*) of a 100-fold molar excess of unlabeled wtStaf-ZNF motif or 5 μg of ZNF143 antibody (*lane 8*). The supershift is indicated by the *asterisk*.

observed in incubating protein-DNA complexes obtained from *Drosophila* extracts after co-transfection with ZNF143 with an antibody against ZNF143 (Fig. 7, *lane 8*), as already observed in HeLa cells.

DISCUSSION

To provide insight into the regulation of the human *SYBL1* gene and better clarify its role in cellular mechanisms, we have characterized cis- and trans-acting elements regulating the basal transcription of the human *SYBL1* promoter. By *in vivo* footprinting we have identified three cis-acting elements, a Staf-ZNF site, a canonical CAAT box, and a third unknown binding site that we named SYBL1-X (Figs. 1 and 2) because at the moment the data present in the international transcription factor databases do not suggest which element can bind to this site.

Transfection experiments in the human cell line HeLa (Fig. 3), using mutant constructs, have shown that the first two sites (Staf-ZNF and CAAT) have a fundamental role in directing high level expression of the human *SYBL1* gene promoter, whereas the third element (SYBL1-X) does not seem to have a relevant role in driving *SYBL1* gene promoter expression, at least in HeLa cells. In fact, though *SYBL1* is a ubiquitous gene, it has specific cellular functions, and it cannot be excluded that the SYBL1-X factors may need a tissue-specific co-factor, not present in HeLa cells, to function in a more efficient manner. To test which factors are important for the tissue-specific regulation of the *SYBL1* gene it should be useful to undertake experiments in other cellular lines and in inducible systems where the role of the SYBL1 protein has already been shown to be important, such as PC12-treated cells or differentiated colon carcinoma cells (7, 8).

Interestingly, the two more important cis-acting elements are both present in their entirety in the two previously identified strictly human-mouse conserved regions, CR1 and CR2 (24), which argues for an important, biologically conserved function of these transcriptional control elements (Fig. 1). Single mutant constructs indicate a principal role for the Staf-ZNF site (Fig. 3, *Staf-ZNFmut*) and for the CAAT site (Fig. 3, *CAAT-mut*); mutation of the SYBL1-X site had little effect on the activity of the promoter (Fig. 3, *SYBL1-Xmut*). The level of activity obtained with the double mutant construct CAAT/SYBL1-Xmut (Fig. 3, *CAAT/SYBL1-Xmut*) confirms the role of both CAAT and SYBL1-X sites and indicates that Staf alone can efficiently drive the promoter activity. When both Staf-ZNF and CAAT sites were mutated, the level of promoter activity was strongly reduced (Fig. 3, *CAAT/Staf-ZNFmut*), indicating that these two sites together have a fundamental importance for *SYBL1* gene promoter activity. This result is further confirmed when using the triple mutants CAAT/SYBL1-X/Staf-ZNFmut (Fig. 3). The level of promoter activity obtained by the construct SYBL1-X/Staf-ZNFmut (Fig. 3) is surprisingly higher than that obtained with the single mutant Staf-ZNFmut. This result could be explained by considering that the low activity obtained with the construct Staf-ZNFmut is not only due to the absence of the important factor Staf-ZNF but also to an inhibitory effect such as steric hindrance by the factor SYBL1-X on the CAAT factor. In the absence of the Staf-ZNF factor, the SYBL1-X factor may reduce the capacity of the CAAT factor to function. EMSA experiments, using HeLa nuclear extracts, identified a protein-DNA complex, indicating that endogenous proteins from HeLa cells are able to bind to the Staf-ZNF site in the *SYBL1* promoter. Supershift analysis indicated ZNF143 as the factor bound to the Staf-ZNF element of the *SYBL1* promoter gene (Fig. 4, *lane 4*). In this case, the intensity of the specific protein-DNA complex is higher than that obtained without the use of the antibody (Fig. 4, *lane 2*), indicating that the antibody can stabilize the DNA-protein interaction. The slightness of the supershift band obtained can be due to low efficiency of the antibodies. In fact, the same faint supershift band (Fig. 7, *lane 8*) is also obtained in EMSA experiments with transfected *Drosophila* SL2 extracts, where there are no other proteins that can bind to the Staf-ZNF site. Here, the retarded complexes obtained (Fig. 7, *lanes 2*, *4*, *6*) can only be because of Staf-ZNF factors. In fact, when using nuclear extracts from SL2 cells not transfected with the effector constructs (pPacStaf, pPacZNF143, or pPacZNF76), no any protein-DNA complex is observed (Fig. 7, *lane 1*).

In vivo evidence of specific recruitment of ZNF143 to the Staf/ZNF site is provided in the chromatin immunoprecipitation experiments (Fig. 5). This technique is able to overcome the limitations present in other methods by taking a picture or freezing the DNA-protein interaction *in situ* through fixation with formaldehyde. This method has been largely used to identify binding sites for transcription factors and characterize their *in vivo* interaction with target DNA (26) as well as to analyze regulatory proteins associated with chromatin (27).

The co-transfection of Staf, ZNF76, or ZNF143 in *Drosophila* SL2 cell lines, which are deprived of any Staf-ZNF transcription factors, resulted in a strong induction of the human *SYBL1* promoter (Fig. 6, *wtSYBL1*), otherwise completely inactive in these cells (data not shown). In particular, the mammalian homologue of Staf, ZNF143 (15, 16), appears to be the major trans-acting factor up-regulating *SYBL1* transcription through the Staf-ZNF element. Co-transfection of Staf, ZNF76, or ZNF143 with the Staf-ZNF mutant results instead in a very low level of induction (Fig. 6, *Staf-ZNFmut*), confirming the importance of the Staf-ZNF element in the *SYBL1* gene promoter.

Overall, we have demonstrated that Staf-ZNF family transcription factors, first characterized for their role as snRNA gene promoters, are very important stimulatory factors for the human *SYBL1* gene promoter in HeLa cells. Our results are consistent with the previous finding that Staf-ZNF can stimulate not only transcription by RNA polymerase III but also transcription by RNA polymerase II (10, 11). To date, only three mRNA pol II promoters whose transcription is regulated by Staf have been identified: the mouse cytosolic chaperonin containing t-complex polypeptide 1α subunit (*Ccta*) gene (12), the human interferon regulatory factor-3 (*IRF-3*) gene (13), and the human neuronal nitric oxide synthase (*nNOS*) gene (14). At present, very little is known about the role of Staf-ZNF transcription factor family members in the trans-activation of mRNA promoters and, given the limited dataset of mRNA promoters containing STAF-ZNF elements, it is very difficult to establish whether a correlation exists between other features of these promoters. The present study extends the role of Staf-ZNF-binding proteins to the transcription of a fourth mRNA promoter. Moreover, this is the first time that the role for Staf-ZNF family factors on a TATA-less promoter containing a CAAT box has been demonstrated. Further studies are needed to obtain more details concerning *SYBL1* transcriptional regulation and, in particular, its tissue-restricted expression.

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Human Synaptobrevin-like 1 Gene Basal Transcription Is Regulated through the Interaction of Selenocysteine tRNA Gene Transcription Activating Factor-Zinc Finger 143 Factors with Evolutionary Conserved Cis-elements

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