Journal of Cellular Biochemistry 76:615–624 (2000)

Identification of a Cell Type-Specific Silencer in the First Exon of the *His-1* Gene

Fan Xu,[1] **Alice J. Paquette,**[3] **David J. Anderson,**[2,3] **Andreas Charalambous,**[1] **and David S. Askew**[1]*

[1]Department of Pathology and Laboratory Medicine, University of Cincinnati, Cincinnati, Ohio 45267-0529

[2]Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California 91125

[3]Division of Biology, California Institute of Technology, Pasadena, California 91125

Abstract The *His-1* gene is developmentally expressed in the murine choroid plexus but is silenced in the adult brain. To test the hypothesis that the gene contains *cis*-acting elements that contribute to this repression, we have analyzed segments of the proximal promoter for negative regulatory sequences by transient transfection analysis. The activity of the proximal promoter was moderately influenced by positively and negatively acting sequences located from −335 to −168 and −617 to −335, respectively. A strong *His-1*-positive regulatory element (HPRE, +18 to +29) was essential for maximal promoter activity and could also enhance the activity of the heterologous SV40 promoter in an orientation-dependent manner. The HPRE contains homology to the neuronal restrictive silencer element (NRSE) but interacted with nuclear proteins that were distinct from the NRSE-binding factor (NRSF). By contrast, a potent negative regulatory sequence (HNRE) was identified in the first exon that repressed either the *His-1* or SV40 promoters by greater than 80%. This negative regulatory sequence interacted with nuclear proteins from cells that contain a silent *His-1* gene but showed no interaction with nuclear proteins from cells that actively transcribe the endogenous gene. HNRE-mediated repression was orientation independent; most of this activity was mapped to a minimal 26-bp sequence. These findings suggest that the first exon of the *His-1* gene contains a cell type-specific silencer that contributes to the regulation of *His-1* transcription. J. Cell. Biochem. 76:615–624, 2000.

Key words: oncogene; HIS-1; promoter; silencer; gene regulation; choroid plexus

Examples of biologically functional RNAs that do not code for proteins are increasingly reported in diverse species, including mammals [Brockdorff et al., 1992; Brown et al., 1992a; Rastinejad et al., 1993; Tam et al., 1997; Velleca et al., 1994; Wevrick et al., 1994; Brannan et al., 1990], *Drosophila* [Lakhotia and Sharma, 1996; Nakamura et al., 1996; Meller et al., 1997], *Xenopus* [Kloc and Etkin, 1994], *Caenorhabditis elegans* [Goodwin et al., 1993; Lee et al., 1993], fission yeast [Watanabe and Yamamoto, 1994], and bacteria [Altuvia et al., 1997]. The function of these untranslated RNAs are diverse, including gene regulation [Rastinejad and Blau, 1993; Goodwin et al., 1993; Lee et al., 1993], X-chromosome inactivation [Penny et al., 1996], genomic imprinting [Leighton et al., 1995], stress responses [Lakhotia and Sharma, 1996; Altuvia et al., 1997], cell cycle regulation [Watanabe and Yamamoto, 1994], and differentiation [Nakamura et al., 1996; Kloc and Etkin, 1994; Lee et al., 1993; for reviews, see Askew and Xu, 1999; Lakhotia, 1996].

The *His-1* gene specifies an untranslated RNA of unknown function that has been implicated in oncogenesis by its common activation in retrovirus-induced mouse leukemias [Askew et al., 1991, 1994]. The highest levels of *His-1* expression are normally found in the developing choroid plexus of the brain, at a time when the choroid plexus is just beginning to form [Li et

Abbreviations used: EMSA, electrophoretic mobility shift assay; HNRE, *His-1*-negative regulatory element; HPRE, *His-1*-positive regulatory element; NRSE, neuronal restrictive silencer element; NRSF, neuronal restrictive silencer factor; LTR, long terminal repeat; tsp, transcription start point; UTR, untranslated region; CSF, cerebrospinal fluid.

Grant sponsor: National Institutes of Health; Grant number: CA61909; Grant number: NS23476; Grant number: 5T32GM07737; Grant sponsor: American Cancer Society; Grant number: JFRA-527.

*Correspondence to: David S. Askew, Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, P.O. Box 670529, Cincinnati, Ohio 45267-0529. E-mail: david.askew@uc.edu

Received 21 July 1999; Accepted 2 September 1999

Print compilation © 2000 Wiley-Liss, Inc.
This article published online in Wiley InterScience, January 2000.

al., 1997b]. The RNA is restricted to the epithelial cells that line this developing organ but is absent from the ependymal epithelial cells that are continuous with the choroid plexus epithelium. The normal function of the mature choroid plexus is to secrete cerebrospinal fluid (CSF) and to form a barrier between the blood and the CSF. However, since the *His-1* gene is silenced in the adult choroid plexus, and in most other adult tissues, we have hypothesized that negative regulation will play a prominent role in the regulation of *His-1* expression, and that the RNA is likely to function during development. Negative regulation is increasingly recognized as an important mechanism for limiting gene expression to specific cell types, particularly during embryogenesis [reviewed in Gray and Levine, 1996]. Known mechanisms of negative regulation involve either position-dependent steric interference with positive regulatory factors, or an active suppression effect that is similar to that of enhancers [reviewed in Hanna-Rose and Hansen, 1996]. Active suppression is position and orientation independent, and by analogy to enhancer elements the regulatory sequences that mediate this repression are termed silencers.

To identify *cis*-acting regulatory elements that contribute to *His-1* gene silencing, we have analyzed segments of the mouse *His-1* gene for their ability to repress transcription of a luciferase reporter gene in transient transfection assays. Our results define the proximal *His-1* promoter, and describe two important regulatory regions within the first exon. The first contains a positive regulatory element located 18 bp downstream of the transcription start point (tsp) that is required for optimal promoter activity, and the second contains a potent silencer element that represses transcription from the normal promoter. These data show that the *His-1* promoter is governed by a combination of both positive and negative regulatory elements and provide the first evidence that the *His-1* gene is under the control of a silencer element in the first exon that may contribute to tissue-specific expression of the *His-1* gene.

MATERIALS AND METHODS

Plasmid Constructions

A series of *His-1* promoter fragments were generated by restriction fragment digests or PCR amplification of *His-1* genomic subclones, and these were inserted upstream of the luciferase gene in the reporter plasmid pGL2-basic (Promega, Madison, WI). The pGL2-basic plasmid carries the luciferase gene (LUC), followed by the SV40 T antigen intron and polyadenylation signals. This plasmid lacks eukaryotic promoter and enhancer sequences and has a second copy of the SV40 polyadenylation sequence upstream of the multiple cloning site to reduce background transcription from spurious plasmid promoters.

The *His-1* gene contains three major transcription start points located within 6 base pairs (bp) of each other [Askew et al., 1994], the first of which is indicated as +1 (the +1 transcription start point corresponds to position 641 in the mouse genomic sequence, Genbank Accession no. U56439). The ends of each promoter fragment are shown relative to the +1 transcription start point, and this is indicated in the names of each plasmid. Plasmids pB−617/+29, pB−135/+29, and pB−168/+29 contain a common 3′ end (+29) and progressively deleted 5′ ends. The upstream primers used to generate these plasmids by polymerase chain reaction (PCR) amplification were: 5′-acagatctAACCCTTGATGAACC-3′(pB−617/+29), 5′-gaagatctTCAGTCCTTGCCCTAATG-3′(pB−135/+29), 5′-gaagatctCCTCTCCCCTTTATG-3′ (pB−168/+29) The common downstream primer used was: 5′-gaagatctGGTGCTTAGAGAAGAAGAAG-3′.

All these primers contained an additional *Bgl*II restriction site (lowercase) to facilitate cloning into the pGL2-basic vector. The remaining reporter constructs shown in Figure 1 contain a common 5′ end at −335, with different 3′ boundaries. The common upstream primer used to amplify the promoter fragments by PCR was: 5′-gaagatctTCAGTCCTTGCCCTAATG-3′. The downstream primers used were: 5′-tcagatctACCTGATGCTGCTCACTG-3′ (pB−135/+150), 5′-gaaagcttGTGCTCCTGTCATGGTGCTTAGA-3′(pB−135/+40), and 5′-gtaagcttAGAAGAAGAAGAATTAC-3′ (pB−135/+18).

All these primers contained an additional *Bgl*II or *Hin*dIII restriction site (lowercase) to facilitate cloning into the reporter vector. The construct pB−135/+60 was generating by isolating the largest *Bgl*II-*Kpn*I fragment from pB−135/+150 and inserting it into the pGL2-basic plasmid at the corresponding sites. The plasmid pB−135/-21 was constructed by isolating the *Bgl*II-*Pst*I digestion fragment from pB−135/+150 and ligating it into the pGL2-basic vector at the corresponding sites. The

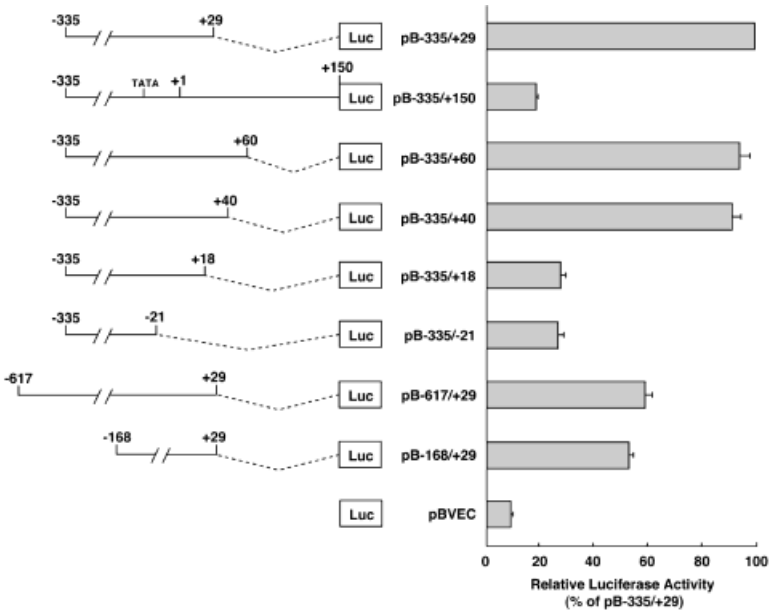

Fig. 1. Luciferase reporter gene analysis of the *His-1* promoter region. Indicated fragments of the *His-1* gene were examined for promoter activity in transient transfections of NIH3T3 cells. The luciferase activity of each construct, relative to the activity of the most active fragment (pB−135/+29), is indicated in the graph. The ends of each fragment are shown relative to the tsp at +1 [Li et al., 1997a]. Exon 1 spans the region from +1 to +150; deletions from the 3′ end of the exon are indicated by the dashed lines. Data shown are the mean of triplicate transfections ± SE. Comparable results were obtained in two additional experiments. All experiments were normalized for transfection efficiency by co-transfection with an SV40-β-galactosidase reporter plasmid (the luciferase activity of the −335/+29 fragment was approximately 20% of the activity of the control SV40-luciferase plasmid in this experiment). The activity of the luciferase reporter gene in the empty pGL2-basic vector is indicated in the lane marked pBVEC.

constructs shown in Figure 3 were made by PCR amplification, using the common 5′ primer 5′-gaagatctTCAGTCCTTGCCCTAATG-3′, together with each of the following 3′ primers: 5′-agaagcttGAAGGCCACCTTGAACTTAG-3′ (pB−135/+83), 5′-ccaagcttgGAGCACCTGGTTATAG-3′ (pB−135/+108), 5′-tgaagcttaTCCCCCTCTTCTGG-3′ (pB−35/+122), 5′-tcaagcttTAATGATGCTCATCCCCCTC-3′ (pB−135/+131), 5′-ccgagcttTGCTCACTGCTTAATG-3′ (pB−135/+142).

Each of these primers contained a *Hin*dIII restriction site (lowercase) to facilitate cloning into the reporter vector. All plasmid constructions were sequenced before use in transient transfections. The pBSV40 plasmid described in Figure 2 is structurally identical to pGL2-basic, with the exception of an insertion of the SV40 promoter upstream of the luciferase gene.

Transient Transfections and Luciferase Assays

Tissue culture media and supplements were obtained from Life Technologies (BRL), and NIH3T3 cells were obtained from the American Type Culture Collection (ATCC). The Bac-1 and C10 cells were obtained from Dr. Jim Ihle (St. Jude Children's Research Hospital, Memphis, TN). NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The CSF-1-dependent macrophage cell line, Bac-1.2F5, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and supplemented with 20% L-cell conditioned medium as a source of CSF-1 [Askew et al., 1994]. Although Bac-1 cells transcribe the *His-1* RNA from the normal genomic *His-1* promoter under study, its resistance to multiple transfection procedures, including electroporation, SuperFect (Qiagen), Lipofectin (Gibco-BRL), LipoTAXI (Stratagene), dextran sulfate, and calcium phosphate have prevented its use in transient transfection analysis. All transfections were therefore performed in NIH3T3 cells using Lipofectin, an activated dendrimer transfection reagent.

Before transfection, the cells were seeded into 6-well plates at 5×10^5 cells/ml. The next day, the cultures were washed twice with phosphate buffered saline (PBS), pH 7.4, and plasmid transfections were performed using SuperFect. A control construct, containing an SV40-driven β-galactosidase gene (pSV40βgal) was used to control for transfection efficiency. For cell transfection, 2 µg of each luciferase construct and 0.5 µg of pSV40βgal were mixed with DMEM containing no serum, proteins, or antibiotics to a final volume of 100 µl. Ten µl of SuperFect reagent was added, incubated at room temperature for 8 min and transferred to the 6-well culture dish containing the cells to be transfected. After the cells were incubated at 37°C for 2.5 h in an atmosphere of 5% CO_2, the cells were washed in phosphate-buffered saline (PBS), and the medium was replaced with fresh

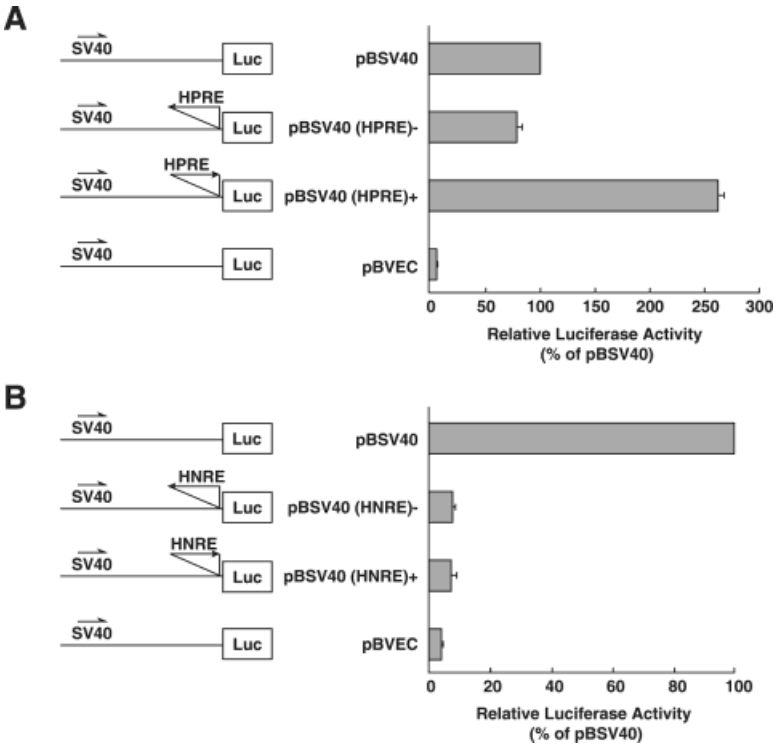

Fig. 2. Analysis of *His-1*-positive regulatory element (HPRE) and *His-1*-negative regulatory element (HNRE) activity on a heterologous viral promoter. The ability of the HPRE (**A**) or HNRE sequence (**B**) to regulate the activity of the heterologous SV40 promoter was determined by inserting single copies of the 22-bp HPRE oligonucleotide (+18 to +40), or the HNRE fragment (+60 to +150), into the pBSV40 promoter-luciferase construct as indicated. Solid bars, mean luciferase activity of each plasmid normalized to β-galactosidase activity (mean ± SE) and are shown as relative values of the pB-SV40 promoter-luciferase plasmid. Arrows, orientation of the HPRE oligonucleotide in each construct (+ in the name of the plasmid if it is in the sense orientation, − if antisense). pBVEC represents the activity of the promoterless reporter plasmid, pGL2-basic. Data shown represent the results of a single experiment performed in triplicate.

medium containing 10% FBS. The transfected cells were cultured for 48 h at 37°C, then washed in PBS, and lysed in 0.5 ml of cell lysis buffer (Promega). The cell extracts were cleared of debris by microcentrifuging for 2 min, and assayed for luciferase activity using the Luciferase Assay System (Promega). An aliquot of the same extract was analyzed in parallel for β-galactosidase activity using the β-Galactosidase Assay System (Promega). For the luciferase analysis, 20 µl of the cell extract was mixed with 100 µl of the luciferase assay reagent according to the manufacturer's specifications, and luciferase activity was measured in a luminometer (model LB9501, Berthold Biolumat, Berthold Analytical Instruments). For the β-galactosidase assays, 100 µl of cell extracts was mixed with equal amounts of 2X β-galactosidase assay buffer according to the manufacturer's procedure (Promega). The reaction was incubated at 37°C, and the absorbance was measured at 420 nm after stopping the reaction with the addition of 500 µl of 1 M sodium carbonate.

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from NIH3T3 fibroblasts or Bac-1 macrophages according to the procedure established by Dignam et al. [1983]. The nuclear pellets from this procedure were quick-frozen and aliquots were stored at −80°C. Electrophoretic mobility shift assays (EMSA) were performed as described [Brown et al., 1992b], with the following modifications. Nuclear extracts (approximately 3.5 µg of total cellular protein) were incubated with 0.1 ng (about 10,000 cpm) of labeled probe per reaction. Unlabeled competing DNA was used at a 200-fold molar excess. The HPRE probe was synthesized as a 23-bp oligonucleotide with the following sequence:5′-TCTAAGCACCATGAC AGGAGCAC-3′. The NRSE probe was synthesized as a 34-bp oligonucleotide based upon the NRSE sequence that is present in the SCG10 gene [Mori et al., 1992]. The HPRE probe that was used as positive control in Figure 6 is a 37-bp oligonucleotide composed of the sequence: 5′-agCTTCTTCTCTAAGCACCATGACAGGAG CACAGCCA-3. All oligonucleotide probes were prepared by annealing sense and antisense strands of complementary oligonucleotides, followed by labeling with T4 polynucleotide kinase and [γ-^{32}P]dCTP. The HNRE probe consisted of a 90-bp *Kpn*I-*Bgl*II fragment from plasmid pB−135/+150 and this was labeled by Klenow DNA polymerase and [α-^{32}P]dCTP. DNA-protein complexes were resolved on nondenaturing 6% polyacrylamide gels and visual-

ized with a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA), using ImageQuant software. EMSA that incorporated recombinant human NRSF or mouse monoclonal antibody 12C11 [Chen et al., 1998] to human NRSF in the binding reaction were performed under the same conditions, except that the protein or antibody was preincubated with the indicated proteins for 30 min at room temperature before the addition of the radiolabeled probe. The in vitro-translated human NRSF protein is a truncated protein encoded by the λH11 clone [Chen et al., 1998] and has been previously used to demonstrate NRSF binding to functional NRSEs [Schoenherr et al., 1996].

RESULTS

A Positive Regulatory Element (HPRE) in the First Exon Is Required for *His-1* Promoter Activity

We have previously identified the transcription start point (tsp) for the mouse *His-1* gene and have shown that segments of the 5′ upstream promoter region are highly conserved among vertebrate homologues [Li et al., 1997a]. To identify *cis*-acting sequences in this region that are important for *His-1* transcriptional regulation, various lengths of 5′-flanking *His-1* sequences were fused to the luciferase gene in the promoterless/enhancerless reporter vector pGL2-basic (pBVEC). The activity of each construct was tested in transient transfection experiments. Luciferase activity was normalized for transfection efficiency by cotransfection with the SV40-driven β-galactosidase reporter plasmid (pBSV40βgal). Figure 1 shows the mean stimulation of luciferase reporter gene expression by the various promoter deletion fragments. The shortest segment with sufficient promoter activity to drive expression of the luciferase reporter gene was pB−168/+29 indicating that the minimal *His-1* promoter is located in this region (Fig. 1, bottom). However, since the −135/+29 construct consistently directed the highest levels of luciferase activity (10-fold above the activity of the empty vector, pBVEC), the activity of each promoter fragment is shown as a percent of the activity of this one plasmid (Fig. 1 top). For comparison, a strong promoter such as SV40 generated 50-fold higher luciferase activity than was exhibited by the promoterless vector plasmid in these assays (not shown).

To identify sequences required for the activity of the pB−135/+29 construct, various deletions were made at the 3′ end of this construct and the promoter activity of each fragment measured after transient transfection into NIH3T3 cells. The removal of 50 bp from the 3′ end of the −335/+29 fragment reduced promoter activity by more than fourfold (pB−135/−21). No further increase in activity was observed when the 3′ boundary at −21 was ext ended to +18 (pB−135/+18), indicating that the activity of the −335/+29 proximal promoter fragment required the presence of a *His-1* positive response element (HPRE) located between +18 and +29. To determine whether this HPRE could enhance the activity of the heterologous SV40 promoter, a 23-bp oligonucleotide containing the HPRE from positions +18 to +40 was inserted in both orientations into the pBSV40 promoter-luciferase construct. The luciferase gene is driven by the SV40 viral promoter in this plasmid, allowing putative enhancer sequences to be assayed for their ability to influence the activity of the heterologous promoter. Since the normal location of the HPRE is downstream of the *His-1* tsp, the HPRE sequence was inserted between the SV40 promoter and the luciferase reporter gene. The luciferase activity of each construct, relative to the activity of the pBSV40 promoter alone, is shown in Figure 2A. In the correct orientation, the HPRE enhanced the activity of the SV40 promoter (Fig. 2A). No enhancement was observed from the insertion of the HPRE in the opposite orientation, however, indicating that the HPRE has position-dependent enhancer activity.

Identification of Silencer Activity in the First Exon of the *His-1* Gene

No strong negative regulatory elements were detected in 600 bp of the *His-1* 5′ flanking region, although sequences between −617 and −335 were moderately repressive (cf. pB−617/+29 with pB−335/+29). Because gene silencing elements are sometimes located within intragenic regions [Schoenherr et al., 1996], we therefore examined sequences in the first exon for their ability to repress the activity of the −335/+29 fragment. When the 3′ boundary of the −335/+29 proximal promoter fragment was extended to include the entire length of exon 1 (pB−335/+150), luciferase activity was reduced by 80% (Fig. 1). Fragments that ended at position +40 or +60 did not show this suppressive effect (pB−335/+40 and pB−335/+60), im-

plying the existence of a negative regulatory element (HNRE) between bp +60 and +150 (Fig. 1). To determine whether this negative activity was promoter context-specific, a single copy of the HNRE sequence (from position +60 to +150) was inserted in both orientations into the pBSV40 promoter-luciferase construct. The insertion was placed downstream of the SV40 promoter to correspond to its natural location in the *His-1* gene. The plasmids were transfected into NIH3T3 cells and the luciferase activity of each construct, relative to the activity of the SV40 promoter alone is shown in Figure 2B. A single copy of the HNRE sequence in either orientation reduced the luciferase activity of the SV40 promoter construct by over 90%, indicating that the repressive effects of the HNRE region are also operative on a heterologous promoter. These results suggest that exon 1 silencer activity is mediated by an active repression mechanism.

To identify the minimal transcriptional repressor element in the first exon, the plasmid that terminates at the end of exon 1(pB−335/+150) was progressively deleted from the 3′ end, and the promoter activity of each deletion was assayed in transient transfections of NIH3T3 cells. The most active construct contained a deletion of the region from +83 to +150. The activity of all other deletion fragments is shown relative to the activity of this construct in Figure 3 (pB−135/+83). The region between +83 and +108 was the most repressive, causing an 80% reduction in promoter activity relative to the activity of pB−617/+83. An additional repressive sequence of moderate but significant activity was also identified between +131 and +150 (cf. pB−135/+131 and pB−135/+142 with pB−135/+150).

Nuclear Protein Binding to the HPRE

Binding site analysis of the HPRE and its flanking regions suggested that it was part of a 22-bp sequence with homology to the neuronal restrictive silencer element (NRSE). A multiple alignment between the HPRE and several known NRSEs is shown in Figure 4. Although most known examples of the NRSE function as silencer elements that repress neuronal-specific gene expression in non-neuronal cell types [Schoenherr et al., 1996], examples of NRSE-like elements with enhancer activity have also been reported [Bessis et al., 1997; Kallunki et al., 1998]. EMSAs were therefore performed to determine whether the HPRE interacts with the NRSE-binding factor, NRSF. The same double-stranded HPRE oligonucleotide that enhanced SV40 promoter activity (Fig. 2A) was incubated with nuclear extracts from both NIH3T3 cells (which contain a silent *His-1* gene), and Bac-1 cells (which actively transcribe the endogenous *His-1* gene from the normal promoter). The mobility of the labeled probe was retarded by interactions with nuclear extracts from both cell types, resulting in the appearance of two differently migrating complexes (Fig. 5) in both cell types. To verify the specificity of the two complexes, the labeled HPRE oligonucleotide was incubated with nuclear extracts in the presence of an excess of either the unlabeled HPRE oligonucleotide, or an unlabeled oligonucleotide containing the NRSF binding element (NRSE) from the SCG10 gene [Mori et al., 1992]. The unlabeled HPRE competitor specifically blocked both complexes, but an unlabeled NRSE oligonucleotide was unable to compete for HPRE-nuclear protein

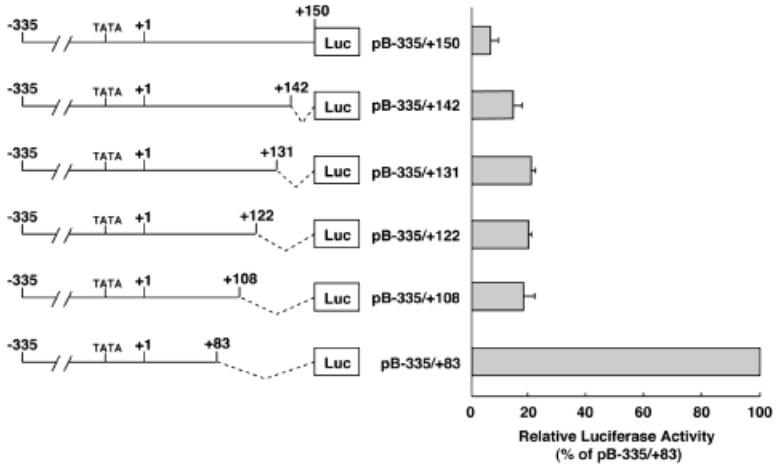

Fig. 3. Identification of the minimal silencer element in exon 1 The plasmid containing the entire repressive sequence in exon 1(pB−135/+150) was progressively deleted from the 3′ end, and the promoter activity of each deletion was assayed in transient transfections of NIH3T3 cells. Solid bars, mean luciferase activity of each plasmid normalized to β-galactosidase activity (mean ± SE), shown as relative values of the most active construct (pB−135/+83).

```
consensus          TTCAGCACCACGGACAGCGCC
hGlycineR          ..............G....T.
hP450-11beta       .........TA.......AAG.
mCalbindin         AG.......G..........
rNMDAR1            .........T........AT.
rSCG10             ..............G..T...
rSynaptophysin     .C........GT......A...
rBDNF              .........TT......A...
rAPRT              G........C.......G...
mHis1              C.A.......T-.....GAG.
```

Fig. 4. Sequence homology between the *His-1*-positive regulatory element (HPRE) and the neuronal restrictive silencer element (NRSE). Multiple alignment is shown between the region containing the *His-1* HPRE (+19 to +38) and known NRSE sequences that have been previously demonstrated to bind to NRSF (Schoenherr et al., 1996). Human glycine receptor; hGlycineR, Human P-450–11β; hP-450–11β, mouse calbindin; mCalbindin, rat N-methyl-D-aspartate receptor-1; rNMDAR1, rat SCG10; rSCG10, rat synaptophysin, rSynaptophysin. The consensus sequence is shown at the top, and identical nucleotides are shown by the periods.

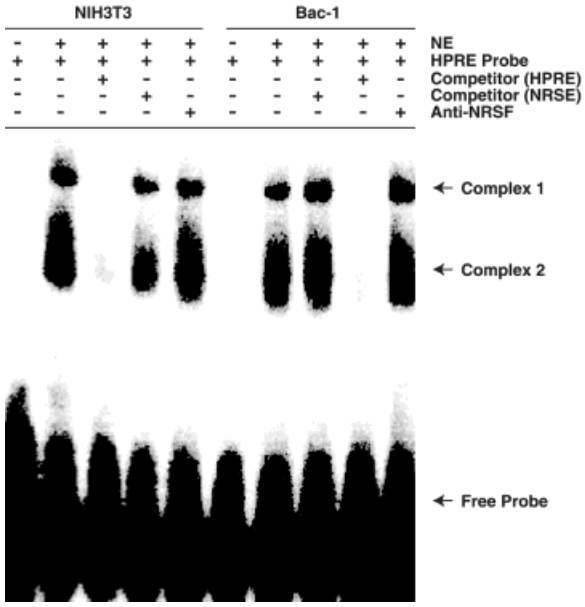

Fig. 5. Nuclear protein binding to the *His-1*-positive regulatory element (HPRE) by electrophoretic mobility-shift assay (EMSA). The radiolabeled double-stranded HPRE oligonucleotide (bp +18 to +40) was incubated with nuclear extracts from NIH3T3 fibroblasts or Bac-1 macrophages in the presence or absence of a 50-fold excess of the unlabeled double-stranded HPRE oligonucleotide or an unlabeled NRSE oligonucleotide, as described under Materials and Methods. An antibody against the neuronal restrictive silencer element (NRSE)-binding protein (anti-NRSF) was incorporated into the binding reactions as indicated. Two major bands, representing protein-DNA complexes, are shown by arrows. NE, nuclear extract. All lanes are from a single gel. Data were obtained with a STORM phosphorimager (Molecular Dynamics) using ImageQuant software.

binding, suggesting that these oligonucleotides are not competing for interactions with the same protein(s). Furthermore, the inclusion of an antibody against NRSF into the binding reaction [Schoenherr and Anderson, 1995; Chong et al., 1995], failed to supershift either of the two complexes, suggesting that NRSF is not a component of the HPRE-nuclear protein complex. To verify that the HPRE is binding to a protein that is distinct from NRSF, the ability of recombinant NRSF protein to directly bind to the HPRE was tested. Although recombinant NRSF was able to retard the NRSE control oligonucleotide, it was unable to influence the migration of the HPRE (data not shown). These results demonstrate that both NIH3T3 and Bac-1 cells express nuclear proteins that bind to the HPRE sequence and establish that this interaction involves a protein distinct from the NRSE-binding protein, NRSF.

Nuclear Protein Binding to the HNRE Is Cell Type-Specific

We were unable to test the silencer activity of the HNRE in cells that express the *His-1* gene because there are only two established cell lines that use the endogenous *His-1* promoter, and both are extremely refractory to transfection procedures. We therefore used gel-shift analysis to compare HNRE-nuclear protein binding in cells that have a silent *His-1* gene (NIH3T3 fibroblasts) with cells that actively transcribe the endogenous *His-1* gene (Bac-1 macrophages). A probe consisting of the entire repressive sequence in exon 1 (+60 to +15) formed a single prominent band upon incubation with nuclear extracts from NIH3T3 cells, and this band was competed by an excess of the unlabeled probe (Fig. 6). By contrast, the same probe did not form any complex with nuclear proteins isolated from Bac-1 cells. The integrity of the Bac-1 nuclear extracts in this experiment was verified by demonstrating nuclear protein binding to a control oligonucleotide containing the *His-1* HPRE sequence. This HPRE probe is longer than the one used in Figure 5 and therefore interacts with additional proteins. These results suggest that a cell type-specific nuclear protein(s), present in NIH3T3 cells but not in Bac-1 cells, mediates the silencing activity of the HNRE.

DISCUSSION

We have previously demonstrated that the *His-1* gene specifies an untranslated RNA that is expressed in the developing choroid plexus

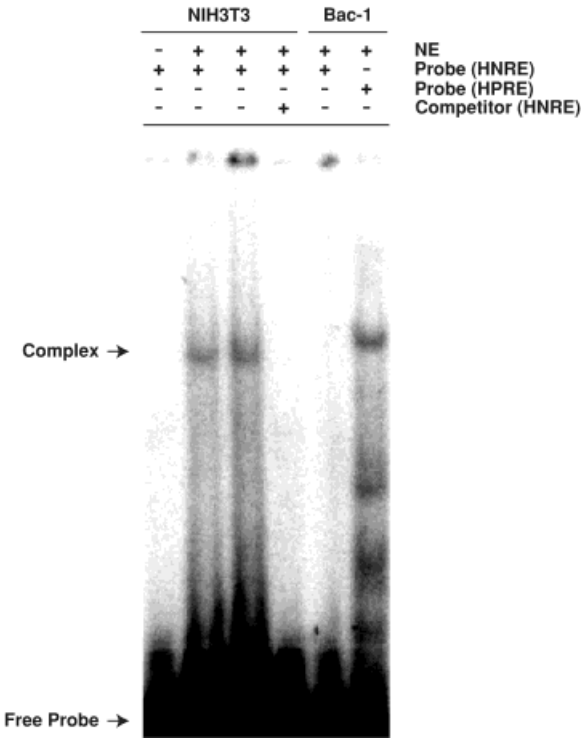

Fig. 6. Nuclear protein binding to the *His-1*-negative regulatory element (HNRE) by electrophoretic mobility-shift assay (EMSA). The radiolabeled HNRE fragment (from +60 to +150) was incubated with nuclear extracts from NIH3T3 fibroblasts or Bac-1 macrophages in the presence or absence of a 50-fold excess of the unlabeled *His-1*-positive regulatory element (HPRE) oligonucleotide as described under Materials and Methods. The integrity of the Bac-1 nuclear extracts was confirmed by demonstrating that Bac-1 nuclear proteins could interact with an oligonucleotide containing the HPRE sequence. NE, nuclear extract. All lanes are from a single gel; lanes 2 and 3 are duplicates. Data were obtained with a STORM phosphorimager (Molecular Dynamics) using ImageQuant software.

and repressed in the adult [Askew et al., 1997b]. Although the gene is not normally expressed in hematopoietic tissues, high levels of the *His-1* RNA were identified in mouse retrovirus-induced leukemias that harbor an integrated provirus in the first intron [Askew et al., 1994]. The strong promoters located in the viral LTRs were able to overwhelm the mechanisms that normally silence the gene in these leukemias, resulting in the activation of *His-1* gene expression. This observation suggests that untranslated RNAs can be oncogenic when inappropriately expressed in the hematopoietic lineage, a view that is supported by the recent identification of a second example of a retrovirally activated untranslated RNA in B-cell lymphomas [Tam et al., 1997].

As a first step to identify sequences that influence the tissue-specific expression of the *His-1* gene, we have evaluated the *His-1* promoter for its ability to control the expression of a luciferase reporter gene in a heterologous system. *His-1* promoter activity required a short positive regulatory element, the HPRE, located at the beginning of exon 1. The ability of this short element to act as a positive regulator in *cis* was also demonstrated on a heterologous viral promoter and was dependent on orientation.

Orientation dependence is atypical for most enhancers but has been reported for other genes [Gemel et al., 1999; Surinya et al., 1999]. This finding suggests that the HPRE can function as a transcriptional enhancer, although an alternative mechanism of post-transcriptional regulation is also possible. Analysis of the HPRE by EMSA suggested that the stimulatory activity of the HPRE is mediated by specific interactions with nuclear proteins. Although binding site analysis of the HPRE revealed homology to the NRSE, the lack of NRSF in the HPRE-nuclear protein complexes indicated that the HPRE is binding to a different factor(s). Experiments to reveal the identity of these factors are in progress.

Of particular interest to this study was the identification of potent silencer activity in the first exon. In addition to repressing the *His-1* proximal promoter by more than 80%, this region could also repress the activity of the heterologous SV40 promoter, regardless of orientation. This suggests that the HNRE can either actively repress the general transcriptional machinery or influence gene expression at the posttranscriptional level, and experiments to distinguish between these possibilities are in progress. The significance of the intragenic location of the HNRE to mechanisms of gene silencing remains unknown, but it is worth noting that a similar intragenic location has also been reported for several examples of the NRSE silencer [Schoenherr et al., 1996]. NRSE-binding factors were identified in NIH3T3 cells that do not express *His-1* RNA, but were not present in Bac-1 cells that express the *His-1* RNA from the normal promoter. These results suggest that a cell type-specific nuclear protein is responsible for the silencing activity of the HNRE.

Binding site analysis of the minimally active HNRE (+83 to +108) identified two candidate transcription factor-binding sites: a GATA-1 site and an E2-box. The GATA-1 transcription factor is a member of a family of zinc-finger pro-

teins, which are most often associated with transcriptional activation rather than repression, particularly for genes in the erythroid, megakaryocytic, and mast cell lineages [Whyatt et al., 1993]. The E2-box (CANNTG) is the binding site for various basic helix-loop-helix proteins, several of which have been previously implicated in transcriptional repression [Sekido et al., 1994; Grbavec et al., 1998]. Site-directed mutagenesis of a 26-bp oligonucleotide containing the HNRE E2-box (CAGGTG to AAAATG) did not affect its nuclear protein binding capacity however, suggesting that it is unlikely to be a functional target for nuclear factors from these cells (not shown). Collectively, our results argue that the HNRE is a novel silencer element that represses gene expression by an active silencing mechanism, and experiments to delineate the identity of the factors that bind to the core silencer element are in progress. Further characterization of the *His-1* silencer may contribute to a broader understanding of tissue-specific gene repression and its derangement during oncogenesis.

ACKNOWLEDGMENTS

We thank Molly McFarland for excellent technical assistance and Dr. Phil Howles for critical review of the manuscript. These studies were supported by National Institutes of Health grant CA61909 and by an American Cancer Society Junior Faculty Research Award (to D.S.A.), National Institutes of Health grant NS23476 (to D.J.A.), and a National Institutes of Health predoctoral training grant (to A.J.P.).

REFERENCES

Altuvia S, Weinstein-Fischer D, Zhang A, Postow L, Storz G. 1997. A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. Cell 90:43–53.

Askew DS, Xu F. 1999. New insights into the function of noncoding RNA and its potential role in disease pathogenesis. Histol Histopathol 14:235–241.

Askew DS, Bartholomew C, Buchberg AM, Valentine MB, Jenkins NA, Copeland NG, Ihle JN. 1991. *His-1* and *His-2*: identification and chromosomal mapping of two commonly rearranged sites of viral integration in a myeloid leukemia. Oncogene 6: 2041–2047.

Askew DS, Li, Ihle JN. 1994. Retroviral insertions in the murine *His-1* locus activate the expression of a novel RNA that lacks an extensive open reading frame. Mol Cell Biol 14:1743–1751.

Bessis A, Champtiaux N, Chatelin L, Changeux J-P. 1997. The neuron-restrictive silencer element: a dual enhancer/silencer crucial for patterned expression of a nicotinic receptor gene in the brain. Proc Natl Acad Sci USA 94:5906–591.

Brannan CI, Dees EC, Ingram RS, Tilghman SM. 1990. The product of the H19 gene may function as an RNA. Mol Cell Biol 10:28–36.

Brockdorff N, Ashworth A, Kay GF, McCabe VM, Norris DP, Cooper PJ, Swift S, Rastan S. 1992. The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell 71:515–526.

Brown CJ, Hendrich BD, Rupert JL, Lafreniere RG, Xing Y, Lawrence J, Willard HF. 1992a. The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. Cell 71:527–542.

Brown CJ, Baltz KA, Edenberg HJ. 1992b. Expression of the human ADH2 gene: an unusual Sp1-binding site in the promoter of a gene expressed at high levels in liver. Gene 121:313–320.

Chen Z-F, Paquette AJ, Anderson DJ. 1998. NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. Nature Genet 20:136–142.

Chong JA, Tapia-Ramirez J, Kim S, Toledo-Aral JJ, Zheng Y, Boutros MC, Altshuller YM, Frohman MA, Kraner SD, Mandel G. 1995. REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell 80:949–957.

Dignam JD, Lebovitz RM, Roder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

Gemel J, Jacobsen C, MacArthur CA. 1999. Fibroblast growth factor-8 expression is regulated by intronic engrailed and Pbx1-binding sites. J Biol Chem 274:6020–6026.

Goodwin EB, Okkema PG, Evans TC, Kimble J. 1993. Translational regulation of tra-2 by its 3′ untranslated region controls sexual identity in *C. elegans*. Cell 75:329–339.

Gray S, Levine M. 1996. Transcriptional repression in development. Curr Opin Cell Biol 8:358–364.

Grbavec D, Lo R, Liu Y, Stifani S. 1998. Transducin-like Enhancer of split 2, a mammalian homologue of Drosophila Groucho, acts as a transcriptional repressor, interacts with Hairy/Enhancer of split proteins, and is expressed during neuronal development. Eur J Biochem 258:339–349.

Hanna-Rose W, Hansen U. 1996. Active repression mechanisms of eukaryotic transcription repressors. Trends Genet 12:229–234.

Kallunki P, Edelman GM, Jones FS. 1998. The neural restrictive silencer element can act as both a repressor and enhancer of L1 cell adhesion molecule gene expression during postnatal development. Proc Natl Acad Sci USA 95:3233–3238.

Kloc M, Etkin LD. 1994. Delocalization of Vg1 mRNA from the vegetal cortex in *Xenopus* oocytes after destruction of Xlsirt RNA. Science 265:1101–1103.

Lakhotia SC. 1996. RNA polymerase II dependent genes that do not code for protein. Ind J Biochem Biophys 33:93–102.

Lakhotia SC, Sharma A. 1996. The 93D (hsr-omega) locus of *Drosophila*: non-coding gene with house-keeping functions. Genetica 97:339–348.

Lee RC, Feinbaum RL, Ambros V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854.

Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM. 1995. Disruption of imprinting caused by deletion of the H19 gene region in mice. Nature 375: 34–39.

Li J, Rhodes JC, Askew DS. 1997a. Evolutionary conservation of putative functional domains in the human homolog of the murine *His-1* gene. Gene 184:169–176.

Li J, Witte DP, Van Dyke T, Askew DS. 1997b. Expression of the putative proto-oncogene *His-1* in normal and neoplastic tissues. Am J Pathol 150:1297–1305.

Meller VH, Wu KH, Roman G, Kuroda MI, Davis RL. 1997. roX1 RNA paints the × chromosome of male *Drosophila* and is regulated by the dosage compensation system. Cell 88:445–457.

Mori N, Schoenherr C, Vandenbergh DJ, Anderson DJ. 1992. A common silencer element in the SCG10 and type II Na^+ channel genes binds a factor present in nonneuronal cells but not in neuronal cells. Neuron 9:45–54.

Nakamura A, Amikura R, Mukai M, Kobayashi S, Lasko PF. 1996. Requirement for a noncoding RNA in *Drosophila* polar granules for germ cell establishment. Science 274:2075–2079.

Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N. 1996. Requirement for Xist in × chromosome inactivation. Nature 379:131–137.

Rastinejad F, Blau HM. 1993. Genetic complementation reveals a novel regulatory role for 3′ untranslated regions in growth and differentiation. Cell 72:903–917.

Schoenherr CJ, Anderson DJ. 1995. The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. Science 267:1360–1363.

Schoenherr CJ, Paquette AJ, Anderson DJ. 1996. Identification of potential target genes for the neuron-restrictive silencer factor. Proc Natl Acad Sci USA 93:9881–9886.

Sekido R, Murai K, Funahashi J-I, Kamachi Y, Fujisawa-Sehara A, Nabeshima Y-I, Kondoh H. 1994. The delta-crystallin enhancer-binding protein delta EF1 is a repressor of E2-box-mediated gene activation. Mol Cell Biol 14:5692–5700.

Surinya KH, Cox TC, May BK. 1999. Identification and characterization of a conserved erythroid-specific enhancer located in intron 8 of the human 5-aminolevulinate synthase 2 gene. J Biol Chem 273:16798–16809.

Tam W, Ben-Yehuda D, Hayward WS. 1997. bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. Mol Cell Biol 17:1490–1502.

Velleca MA, Wallace MC, Merlie JP. 1994. A novel synapse-associated noncoding RNA. Mol Cell Biol 14:7095–7104.

Watanabe Y, Yamamoto M. 1994. *S. pombe* mei2$^+$ encodes an RNA-binding protein essential for premeiotic DNA synthesis and meiosis I, which cooperates with a novel RNA species meiRNA. Cell 78:487–498.

Wevrick R, Kerns JA, Francke U. 1994. Identification of a novel paternally expressed gene in the Prader-Willi syndrome region. Hum Mol Genet 3:1877–1882.

Whyatt DJ, deBoer E, Grosveld G. 1993. The two zinc finger-like domains of GATA-1 have different DNA binding specificities. EMBO J 12:4993–5005.