Deletion mapping of the human presenilin-1 (PS1) promoter delineated the most active fragment from −118 to +178 in relation to the transcription start site mapped in this study, in both human neuroblastoma SK-N-SH and hepatoma HepG2 cells. 5’ deletions revealed that a crucial element controlling over 90% of the promoter activity in these cell lines is located between −22 and −6. A mutation altering only two nucleotides of the ETS consensus sequence present at −12 (GGAA to TTAA) has a similar effect. Electrophoretic mobility shift assays showed that a set of specific complexes between nuclear factors and the PS1 promoter are eliminated by this point mutation, as well as by competition with an ETS consensus oligonucleotide. Competition experiments in DNase I footprinting correlated with electrophoretic mobility shift assays and showed that only one of several footprints over the PS1 promoter is eliminated by competition with an ETS consensus oligonucleotide. It extends from −14 to −6 and surrounds the ETS motif present at −12. Thus, a crucial ETS element is present at −12 and binds a protein(s) recognizing specifically the ETS consensus motif. At least one such complex is eliminated by preincubating the nuclear extract with an antibody with broad cross-reactivity with Ets-1 and Ets-2 proteins, thus confirming that an ETS transcription factor(s) recognizes the −12 motif. Several Sp1 binding motifs at positions −70, −55, and +20 surround this ETS element. Competition DNase I footprinting showed that Sp1-like nuclear factors recognize specifically these sites in both cell lines. Furthermore, a combination of 5’ and 3’ deletions indicated the presence of positive promoter elements between −96 and −35 as well as between +6 and +42. Thus, transfection and footprinting assays correlate to suggest that Sp1 transcription factor(s) bind at several sites upstream and downstream from the initiation site and activate the transcription of the PS1 promoter. Sequences downstream from the transcription initiation site also contain major control elements. 3’ deletions from +178 to +107 decreased promoter activity by 80%. However, further deletion to +42 increased promoter activity by 3.4-fold. Collectively, these data indicate that sequences upstream and downstream from the transcription start site each control over 80% of the promoter activity. Hence, this suggests that protein–protein interactions between factors recognizing downstream and upstream sequences are involved.

Mutations within the presenilin (PS)1 genes lead to the most aggressive form of familial Alzheimer’s disease and account for about 25% of early onset cases (1–3). Presenilins are members of a novel family of genes encoding integral membrane proteins (4–6). The function of presenilins and the mechanisms by which mutations in these genes lead to disease are still unclear. They may participate in protein sorting or trafficking, intercellular signaling, or cell death (7). However, the high degree of homology between presenilin-1 (PS1) and presenilin-2 (PS2) and other similar proteins found widely conserved across species has contributed much of the present information on their possible function. Presenilins are homologous to SEL-12, a Caenorhabditis elegans protein that facilitates signaling by the Notch/LIN-12 transmembrane receptor and plays a crucial role in cell fate specification and during development (8, 9). Presenilins and SEL-12 are transmembrane proteins with similar topology (6). They are also functionally similar, since mutations in SEL-12 are efficiently rescued by human PS1 or PS2 (8, 9), while several presenilin mutants identified as leading to disease in humans only result in partial rescue (10, 11). Increasing direct evidence indicates that presenilins also play a crucial role in mammalian embryonic development. Mice bearing a null mutation for PS1 die at birth and display central nervous system defects as well as vertebral skeletal malformations (12, 13). The requirement for PS1 during development implies a crucial importance for the control of PS1 gene expression. PS1 is widely expressed in a variety of tissues (14). Notably, in brain it is primarily found in neurons (15, 16). Both PS1 and PS2 are regulated during development (17), aging (18), brain injury (19), and Alzheimer’s disease (20–22). Thus, regulation of PS1 level may play a role in the pathology of Alzheimer’s disease. This may also be suggested by the recent finding of two familial Alzheimer’s disease-associated mutations causing premature termination of the PS1 protein due to frameshift (23).

Together with the genomic organization of the human PS1 gene, a partial genomic sequence was recently reported including 700 bp of 5’-flanking sequences and the exons 1 and 2 (24). To date, the promoter of the human PS1 gene has not been mapped, and the transcription factors by which it is regulated have not been identified. A recent analysis of the mouse PS1 promoter has indicated that it is expressed only in mouse neuronal cells, suggesting that the expression of the mouse PS1 gene is tissue-specific (25). We have analyzed the promoter activity of the flanking sequences and the first exon by deletion mapping and transient expression assay in human neuroblastoma SK-N-SH cells as well as hepatoma HepG2 cells. We have identified a set of promoter elements, and we have begun to characterize the transcription factors with which they interact.

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The abbreviations used are: PS, PS1, and PS2, presenilin, presenilin type 1, and presenilin type 2, respectively; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; bp, base pair(s); DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay.
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EXPERIMENTAL PROCEDURES

Construction of Human PS1 Promoter CAT Reporters—A genomic fragment including the entire first exon, 687 bp of upstream sequences, and 39 bp of the first intron was obtained by PCR amplification of genomic DNA from human B cell line (JY) using primers designed to the previously published sequence of the human PS1 gene (24) (GenBank® accession no. L76518). Primers were designed to incorporate restriction enzyme sites at the ends of the amplified fragment. SacI and XhoI sites were introduced in forward and reverse primers, respectively. A first primer pair, p1-p8, generated a fragment containing 5′-flanking sequences from −687 and the first 91 nucleotides of the first exon (Fig. 1A). A second primer pair, p7-p27, produced an overlapping downstream fragment extending from −245 to the beginning of the first intron (+178). PCR conditions were 30 cycles at 94, 47, and 72 °C for 30, 30, and 1 min, respectively. Amplification products (25 μl) contained 1 μM Tris-HCl, pH 8.3; 50 mM KCl; a 200 μM concentration each of dATP, dGTP, dCTP, and dTTP; 200 ng of each primer; 0.1 μg of genomic DNA; and 2 μl of Taq polymerase (Fisher). MgCl2 was 1 mM for the p7-p27 primer pair and 1.5 mM for p1-p8. The PS1 promoter fragments obtained were then inserted into the promoterless pKT vector (26) upstream from the cat gene. The p1-p8 amplification product was digested with SacI and XhoI, producing fragments because of an internal SacI site at −118 (Fig. 1A). The SacI to XhoI fragment containing sequences from −118 to +91 was inserted in the same sites of pKT, generating the upstream SacI fragment containing sequences from −687 to −118. The SacI site of the pKT fragment was replaced by the SacI to XhoI fragment. Other deletion constructs were obtained by amplification using the primer pairs listed below and insertion into pKT after digestion with SacI and XhoI.

Upstream primers with a 5′-end at −96, −35, −22, −6, and +2 were, respectively, p9, p10, p11, p20, and p11. p30, a mutant primer with the same 5′-end as p19 included an altered GGAA to TTAA ETS motif at −107, and 91 bp of the first intron was obtained by PCR amplification of PS1 sequences in-

boiled for 10 min and incubated at 37 °C for 16–20 h. Nucleic acids were then precipitated with ethanol and dissolved in 20 μl of reverse transcriptase mixture containing 5 units of reverse transcriptase (Promega, Madison, WI). Reactions were incubated at 42 °C for 60 min. They were stopped by the addition of an equal volume of deionized formamide and heated at 100 °C for 3 min. cDNA was visualized by electrophoresis on a 8% polyacrylamide, 7 μm urea sequencing gel.

RNase Protection Assay—A DNA fragment containing sequences from positions −96 to +91 (Fig. 1A) was obtained by PCR amplification and inserted between the SacI and XhoI sites of pGEM4 (Promega). The vector was linearized with EcoRI and used as a template to synthesize a [32P]UTP-labeled RNA probe using T7 RNA polymerase (Promega). The probe was purified by electrophoresis on a 6% polyacrylamide, 7 μm urea gel as described previously (29).

Total RNA was prepared from SK-N-SH and HepG2 cells (30) and added to 1 ng (108 cpm) of antisense RNA probe in 30-μl mixtures containing 60% formamide, 10 m Hepes, pH 7.5, 600 mM NaCl, 2 mM EDTA. Samples were boiled for 10 min, and hybridization was carried out at 55 °C for 16–20 h. Nonhybridized RNA was then digested with RNase A (10–20 μg/ml) for 30 min at 23 °C, and samples were processed as described previously (31). RNAs were analyzed by electrophoresis on an 8% polyacrylamide, 7 μm urea sequencing gel.

Preparation of Nuclear Extracts—The preparation of small scale nuclear extracts is described in a protocol described previously (32).

Nuclease Footprinting—Double-stranded DNA probes labeled at a single end were obtained by PCR amplification of PS1 sequences included in the plasmid pKT(1–27). Primers were labeled with polynucleotide kinase and (γ-32P)ATP. Fragments containing sequences from positions −118 to +91 were generated using as primers p8 and end-labeled p21. The mutant was obtained by annealing 5′-CATAGAAGCTAATGAGTTACTTCCGGTTATGC-3′ and as p21 and labeled p8 for the top and the bottom strand, respectively. Amplification products were purified by electrophoresis on a 6% polyacrylamide gel. DNase I footprinting was carried out as described previously (31). Reaction mixtures (20 μl) included 0.2–0.4 ng of end-labeled fragment, 500 ng of poly(dI-dC) polymerase, 1 μg of nuclear extract from SK-N-SH or HepG2 cells and 12 m Hepes, pH 7.9, 60 m NaCl, 1 mM EDTA, 1 mM MgCl2, 2.5 mM DTT, 15% glycerol. After 30 min at 4 °C, samples were placed at 23 °C, and 100–200 ng of DNase I (Sigma) were added together with 7.5 mM MgCl2. DNase I treatment was stopped after 30 s by the addition of 100 μl of 10 m Tris, pH 7.5, 20 mM EDTA, 5% SDS, and 2 μg PBR22. After digestion with proteinase K, DNAs were extracted repeatedly with phenol, ethanol, and analyzed on an 8% acryl-

amine, 7 μm urea sequencing gel. Fragments used in competition experiments were generated by PCR using primer pair p10-p13 for fragment B and p11-p12 for A. The fragments containing the Elk-1 site have been described previously (33). The wild type fragment was obtained by annealing the entire strand oligonucleotides 5′-CTAGAAGCTAATGAGTTACTTCCGGTTATGC-3′ and 5′-CTAGAAGCTTACCTCGGTGTTATGCAGTCCG-3′.

The mutant was obtained by annealing 5′-CTAGAAGCTTACCTCGGTGTTATGCAGTCCG-3′ with 5′-CTAGAAGCTAATGAGTTACTTCCGGTTATGCAGTCCG-3′. The Sp1 oligonucleotide resulted from annealing 5′-GATCCGGCAAGGGCCCAACTCCCACTCGTTGCCGAGCTTAATGAGTTACTTCCGAGTCC-3′ with 5′-GATCCGGCAAGGGCCCAACTCCCACTCGTTGCCGAGCTTAATGAGTTACTTCCGAGTCC-3′. All DNAs were purified by electrophoresis on 15% polyacrylamide gels.

Electrophoretic Mobility Shift Assay (EMSAs)—Double-stranded oligonucleotide probes were generated by PCR amplification with 32P-end-labeled primers. An oligonucleotide including (−22/6) PS1 sequences was generated using the p19 and p13. The primer pair p9-p13 was designed to generate the (−22/6) fragment including a mutation from GGA to TTA at position −12. EMSAs were carried out by incubating 0.1–2 ng of probe with 2–5 μg of nuclear extracts in the presence of 1–2 μg of poly(dI-dC)poly(dI-dC) in 10 m Hepes, pH 7.9, 50 m NaCl, 0.75 m MgCl2, 0.1 mM EDTA, 1 mM DTT, 10% glycerol for 30 min at 4 °C. DNA-protein complexes formed were then analyzed by electrophoresis on nondenaturing 6% polyacrylamide gels. The electrophoresis
buffer was 0.5× TBE (89 mM Tris, 89 mM boric acid, and 1 mM EDTA). The gels were prerun for 30 min, and sample electrophoresis was for 90 min at 10 V/cm at 4°C. Antibodies used in EMSA supershift or complex inhibition assays are rabbit polyclonal antibodies obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). sc-112X was prepared against a C-terminal peptide of the human Ets-1 protein (amino acids 362-374) and has a broad cross-reactivity with Ets-1 and Ets-2 proteins. sc-111X was raised against the peptide containing amino acids 55–70 of human Ets-1 p54 and is specific for Ets-1. sc-355X recognizes antibodies specific for Elk-1 among Ets proteins and was raised against amino acids 407–426. Antibodies were added to EMSA reactions minus the DNA probe. Mixtures were incubated at 22°C for 45 min; the probe was then added, and reactions were incubated further for 20 min prior to loading on native acrylamide gels as described above.

**Immunoblotting—**Proteins from nuclear extracts from SK-N-SH and HepG2 cells (15 μg) were fractionated on SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Corp.) by standard techniques. Filters were blocked with 1% bovine serum albumin in TBS (10 mM Tris, pH 7.5, 150 mM NaCl) containing 0.1% Tween 20 for 90 min at 22°C. They were then incubated with 1:5000 rabbit polyclonal antibodies specific for Elk-1 or Ets-1 or with a broader specificity for Ets-1/Ets-2 proteins. Blots were washed with TBS containing 0.05% Tween 20 and incubated with anti-rabbit secondary antibody (1:2000) in TBS containing 0.1% Tween 20 for 45 min at 22°C. They were then developed by the ECL detection kit obtained from Amersham Pharmacia Biotech according to the recommended instructions. Exposure to Amersham Pharmacia Biotech Hyperfilms was for 30 s to 5 min.

**RESULTS**

**Mapping of the Transcription Start in SK-N-SH Cells—**An initial localization of the transcription initiation site of the human PS1 gene has been estimated previously from the 5’-end of various cDNAs generated by 5’-rapid amplification of cDNA ends PCR (24) from post-mortem human brain and placenta. These 5’ termini were indicated at t1 to t5 (Fig. 1A). We sought to map the start of transcription of the endogenous gene in human neuroblastoma SK-N-SH cells. We first performed a primer extension experiment using total cellular RNA and a reverse primer with a 5’-end at nucleotides downstream from t1 which corresponds to the longest 5’ extension among the cDNAs described previously (24). We have consistently observed two products extending 14 and 15 nucleotides 5’ from the t1 site. We have assigned the transcription start (+1) to the initial site (Fig. 1B). The cluster of smaller fragments from +41 to +45 appears to vary in intensity relative to the bands at +1 in different assays using the same RNA stock. Thus, they are likely to represent stops in the progression of reverse transcriptase due to sequence or structural features in the mRNA. The weaker stop at position +62 may be consistent with t3. Notably, t3 was observed in two mRNAs obtained independently (24). In our system, no transcription appears to initiate at position t1 or t2. However, other initiation events downstream from t3 cannot be ruled out by this experiment.

Alternatively, we have also performed an RNase mapping experiment in order to compare the level of PS1 mRNA in both of the SK-N-SH and HepG2 cells used for transfection (Fig. 1C). The RNA probe contained sequences from −96 to +91 from the transcription initiation site defined by primer extension. In both cell lines, we have observed two protected fragments, 89 and 90 nucleotides long, consistent with the initial mapping by primer extension and RNA migrating typically slightly faster than the homologous DNA fragment. In both cell lines, PS1 mRNA level was similar, and transcription initiation occurred at the same position. Therefore, our data indicate that the PS1 gene is transcribed with the same efficiency in neuroblastoma and in hepatoma cells. Transcription initiation appears to occur 15 nucleotides further upstream than previously reported for the mRNAs with the longest 5’ extension (24).

**Deletion Analysis of the PS1 Promoter—**When inserted into the pKT vector, PS1 sequences from −687 to +178 promoted efficient CAT expression in both SK-N-SH and HepG2 cell lines, since the CAT activity produced was about 75 and 100% of that observed by transfecting pSVCAT in SK-N-SH and HepG2 cells, respectively (Fig. 2). In SK-N-SH cells, deletion of sequences between −687 and −329 did not have a detectable effect on CAT expression. Deletion of sequences between −329 and −293 increased transcription by 60%. Further deletion from −293 to −22 had little detectable effect. However, deletion of the 15-bp fragment from −22 to −6 reduced promoter activity by 80-fold, to the background level observed with the basic pKT vector. Upon further deletion from −6 to +2, CAT expression remained low but showed a consistent and significant 5-fold increase. Therefore, a minor negative element is present between −329 and −293, and a crucial positive element between −22 and −6 determines over 80% of the expression of the gene. The increased CAT activity following deletion of the start site (+1) may reflect the activity of an alternative start site(s) downstream. Transfection in HepG2 cells showed a minor positive element between −687 and −611 increasing transcription by 20%, and negative elements between −611 and −491 as well as between −245 and −118 affecting transcription by 50% and 20%, respectively. Most significantly, deletions of sequences from −22 to −6 reduced transcription by over 25-fold. Therefore, minor negative elements upstream from the SacI site are detected in both cell lines as well as a minor positive element in HepG2 cells. In both cell types, transcription of the PS1 gene requires sequences between −22 and −6. This region of the promoter contains sequence motifs similar to consensus for the binding sites of several known transcription factors including ETS and p53. Hence, this region is likely to be of crucial importance for the basal as well as the regulated expression of the gene.

3’ deletion of sequences between +178 and +107 reduced transcription by about 10-fold, to 8–10% of the maximum level observed with the pKT(S-27) construct in both cell lines, whether 5’ sequences extended to −687 or were truncated to −118, −96, or −35. Hence, a strong positive element(s) is present downstream from the transcription start site within the distal 30 bp of the first exon or/and the first 40 bp of the first intron. This region contains several sequence elements with homology to the binding sites of c-Ets-2, c-Fos, Sp1, AP-1, and AP-2. Further delineation by deletion analysis should help identify the relevant signals. Deletion of sequences between +107 and +42 increased transcription by 3–4-fold in both cell types, indicating the presence of a negative element in the context of the remaining promoter fragment. Further 3’ deletions between +42 and +6 decreased transcription by 25–30% in constructs with a 5’-end at −118 or −96. However, the same deletion had a more pronounced 50% effect in shorter promoter fragments with a 5’-end at −35. Conversely, a 5’ deletion of sequences from −96 to −35 had no effect in constructs with a 3’-end extending to +178 or +107. This 5’ deletion decreased transcription by 30% with a 3’-end at +42, but it reduced promoter activity by 2-fold when the 3’-end was truncated to +6. Similar results were obtained in both cell types. Several Sp1 consensus binding sites are located between −96 and −35 as well as between +42 and +6 (Fig. 1A). Perhaps several Sp1 sites are functional both upstream and downstream from the transcription initiation site and have partially redundant functions. Deletion of one or more sites may enhance the requirement for the remaining sites, which appear to function as positive elements.

**Identification of Nuclear Factor Binding Sites on the PS1 Promoter by DNase I Footprinting—**We sought to visualize the interaction of nuclear factors with DNA sequences that affect transcription in transfection assays by DNase I footprinting. Fig. 3 displays footprints using a probe including sequences
Fig. 1. Nucleotide sequence of the human PS1 promoter and localization of a main transcription initiation site. A, the sequence of the PS1 promoter was essentially as described previously (24) with minor differences. The transcription start site identified in this report is shown as +1. The positions of the 5'-end of the mRNAs described previously and designated as t1, t2, t3, t4, and t5 are indicated by vertical lines. The end points of the 5’ and 3’ deletions analyzed by transfection assays in this study are shown by arrows. The first exon-intron junction is indicated ( ). B, mapping of the 5'-end of the human PS1 gene by primer extension. A reverse primer with a 5'-end at 33 nucleotides upstream from the 3'-end of the first intron was end-labeled and hybridized with 15 μg of total RNA from SK-N-SH cells (N) or 15 μg of tRNA and extended with avian myeloblastosis virus reverse transcriptase. The same primer was used to generate a DNA sequencing ladder by the chain termination technique (lanes A, T, G, and C). The arrowheads mark the positions of reverse transcriptase stop sites. The filled arrows indicate major stop sites. +1 marks the mRNA 5'-end. C, comparison of PS1 mRNA level and initiation site in SK-N-SH and HepG2 cells by RNase protection assay. Total RNA from SK-N-SH cells (20 μg, lanes 1 and 2; 10 μg, lanes 4 and 5), HepG2 cells (10 μg, lane 3), or tRNA (10 μg, lane 6) were analyzed by RNase protection assay using an antisense RNA probe including PS1 sequences from -96 to +91. RNA-RNA hybrids were digested with 5 μg/ml (lanes 2, 5, and 6) or 10 μg/ml (lanes 1, 3, and 4) RNase A. The protected RNA fragment is indicated by an arrow. Molecular weight markers (M) indicate the size in nucleotides of fragments of the homologous DNA strand of the same polarity generated by a Maxam and Gilbert cleavage reaction.
from −118 to +91. On the top strand (Fig. 3A), incubation of the probe with nuclear extract from SK-N-SH cells prior to digestion with DNase I (lanes 2 and 3) resulted in a large area of protection from −82 to −21, leaving only unaltered cuts at −40 and −59, as compared with the digestion pattern of naked DNA (lane 1). A hypersensitive site also appeared at −17. In addition, a shorter footprint was present between +11 and +28 with a hypersensitive site at +9. On the bottom strand (Fig. 3B) the area protected in the presence of nuclear extract (lanes 2 and 3) appeared even larger, extending from +33 to −82 with hypersensitive sites at −14 and −85. The very large footprints observed probably result from the binding of several proteins.

We have attempted to discriminate by competition footprint experiments as described further. The same region of the PS1 promoter is also recognized similarly by several nuclear factors from HepG2 cells (Fig. 4). Areas of protection appeared on the top strand (Fig. 4A) from −81 to −61, and −55 to −45 with an adjacent hypersensitive site at −40, as well as approximately from −18 to +1 and from +11 to +24 with enhanced cleavages at −20, from +1 to +11, and at +25. On the bottom strand (Fig. 4B) protection extended from −80 to −40 approximately and −13 to −3 with enhanced cleavages at −81, −20, and −14. The weak footprint that was observed in SK-N-SH cells between +13 and +27 on the bottom strand was not detectable with HepG2 extracts. However, it did appear on the top strand in both cell types. Therefore, the pattern of protection from DNase I appears mostly similar in both cell lines. Among the minor differences are the absence of a detectable footprint in HepG2 cells between −40 and −20 on either strand and a more intense cluster of hypersensitive sites from +1 to +10 on the top strand in HepG2 cells.

A footprint was also apparent around +90 with nuclear extract from both cell types from +82 to +102 on the top strand (Fig. 5A) and from +99 to +78 on the bottom strand (Fig. 5B). Notably, a sequence homology to ETS transcription factor binding sites is present at the center of the protected area. Therefore, data from promoter analysis by deletion mapping and the localization of nuclear protein binding sites on the PS1 promoter reveal notable similarities in both cell lines. In particular, a series of promoter elements span the −96/−42 region. The same area (−80/−40) is recognized by a virtually continuous array of nuclear factor binding sites including ETS and Sp1 elements.

**Competition of DNase I Footprints Identifies Sp1 and Elk-1 Binding Sites around the Transcription Initiation Site of the PS1 Promoter**—In order to delineate individual footprints within the large area of protection from DNase I extending from −80 to +30 and in an initial attempt to identify the transcription factors involved, we performed competition ex-

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**Fig. 2.** Mapping of sequence determinants required for activity of the human PS1 promoter by deletion analysis. The positions of the 5′- and 3′-ends of each deletion fragment are indicated on the left (5′ Δ) and on the right (3′ Δ). Promoter activity was expressed as the ratio of CAT to β-galactosidase activity for each transfected plate. The mean values for each construct (n = 3 or 4) are indicated. S.D. values were 10–20% in all cases. All constructs were tested in at least three different experiments. In all experiments, the relative activity of different constructs was consistent within statistical variations. The construct with the highest activity was expressed as 100%.

**Fig. 3.** Analysis of nuclear factor binding sites on the PS1 promoter by DNase I footprinting with SK-N-SH cell nuclear extracts. A PS1 promoter fragment from −118 to +91 was labeled on the top strand (A) or the bottom strand (B) and incubated without (lanes 1) or with 9 µg (lanes 2) or 12 µg (lanes 3) of nuclear extract from SK-N-SH cells. Areas protected from digestion by DNase I are indicated by brackets, and enhanced cleavage sites are shown by arrowheads. A Maxam and Gilbert sequencing ladder (G + A) was run beside the gel. The numbers on the left indicate the nucleotide positions in relation to the transcription start site.
proteins recognize areas A, D, and E. Furthermore, within the large area of protection from −40 to −80 regions D and E are competed with different efficiency by the same oligonucleotide A. This indicates that the −40/−80 region actually includes two protein-binding sites. Results on the bottom strand (Fig. 6B) correlated well with those of the top strand. The Sp1 oligonucleotide competed footprints from +26 to +14 as well as from −48 to −80 (region D + E). The PS1 fragment containing sequences from +2 to +42 competed the footprint A and D clearly. Competition at site E was not detectable. Therefore, sites at +15 to +30 (region A), −40 to −60 (region D) and −60 to −80 (region E) appear to represent three binding sites of protein factor(s) with binding specificity similar to Sp1. Regions B and C appear not to interact with Sp1-like proteins.

An oligonucleotide containing sequences from +6 to −35 eliminated only the short footprint present in the homologous region of the bottom strand, between −4 and −14 (region B). This site includes at its center a sequence GGAA similar to the core motif present at the binding sites of the ETS family of transcription factors. Furthermore, the sequence immediately surrounding the motif is most similar to binding sites for Elk-1.2

Fig. 4. DNase I footprinting of the PS1 promoter region −118/+91 with HepG2 cell nuclear extract. DNase I footprinting was carried out as described in Fig. 3 for the top strand (A) and the bottom strand (B). No extract was included in minus lanes (−). Plus lanes (+) show the pattern of protection from DNase I digestion in the presence of 12 μg of nuclear extract from HepG2 cells. Protected areas are indicated by brackets. The arrows mark enhanced cleavage sites. The numbers on the left indicate the nucleotide positions in relation to the transcription start site.

Fig. 5. DNase I footprinting analysis of the +2/+178 region of the PS1 promoter. Nuclear extract (10 μg) from SK-N-SH cells (lanes 2) or HepG2 cells (lanes 3) was incubated with a promoter probe including sequences from +2 to +178 labeled on the top strand (A) or the bottom strand (B). No extract was added in lanes 1. Brackets mark the +90 protected area. G + A sequence ladders were run beside the gel, and the numbers on the left indicate the nucleotide position in relation to the transcription start site.

The software and data bases used to search for homologies to known transcription factor binding sites are available on the World Wide Web as TESS: “Transcription Element Search System on the www” by Jonathan Schug and G. Christian Overton, Technical Report CBIL-TR-1997-1001-v0.0 of the Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, 1997.
FIG. 6. Competition of the DNA binding activities over the −118 to +91 region of the PS1 promoter. Nuclear extract from SK-N-SH cells (9 µg) was preincubated for 20 min with various competitor DNAs. The labeled probe (0.3 ng) containing sequences from −118 to +91 was then added, and reactions were further incubated for 20 min prior to digestion with DNase I. Samples were analyzed as described in Fig. 3. A, analysis with the top strand. B, analysis of the bottom strand. Lanes 1 contained no extract. No competitor was added in lanes 2. Competitor DNAs were HaeIII fragments of pBR322 (10 ng in lane 3, 30 ng in lane 4), Sp1 oligonucleotide (10 ng in lane 5, 30 ng in lane 6), PS1 promoter fragment B (−35 to +6) (10 ng in lane 7, 30 ng in lane 8), PS1 fragment A (+2 to +42) (10 ng in lane 9, 30 ng in lane 10). C, competition of the footprints on the bottom strand by the Elk-1 oligonucleotide wild type (wt) (lanes 5–7) or mutant (m) (lanes 8–10). Nuclear extract (9 µg) was preincubated with 5 ng (lanes 5 and 8), 10 ng (lanes 6 and 9), or 20 ng (lanes 7 and 10) of competitor DNA prior to adding 0.2 ng of probe. Lanes 1 and 2 contained no extract. Lanes 3 and 4 contained no competitor. Protected areas are indicated by brackets. The numbers on the left indicate nucleotide positions in relation to the start of transcription (+1).
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The −22/+178 promoter fragments containing the wild type (wt) GGAA or the mutated (m) TTAA ETS motif were inserted into the pKTCAT basic vector, and their activity was measured by transfection into SK-N-SH and HepG2 cells as described in the legend to Fig. 2. The mean values indicated are for n = 3 or 4. S.D. values were 5–20%.

| pKTCAT construct | Promoter Activity |
|------------------|------------------|
| SK-N-SH          | HepG2            |
| −22/+178 wt      | 100              | 100              |
| −22/+178 m       | 4                | 1.2              |
| −6/+178          | 1                | 0.8              |
| pKT (basic vector)| 0.5             | 0.1              |

**TABLE I**

A GGAA to TTAA mutation in the ETS consensus motif at −12 from the transcription start site abolishes PS1 transcription.

Drosophila E74 ETS site (33) (Fig. 6C, lanes 5–7), which has been shown to bind Elk-1 (33) and Ets-1, eliminated selectively a very narrow area of the footprint observed with nuclear extract (lanes 2 and 3). Protection between −5 and −14 was abolished as well as the hypersensitive site at −14, and the enhanced cleavage sites on naked DNA at −5 and −19 are restored as compared with the pattern on naked DNA (lanes 1 and 2). Strikingly, the same oligonucleotide with a single base alteration in the ETS consensus core (33) had no effect on the footprint profile (lanes 8–10). Altogether, the data strongly suggest that the short footprint B (Fig. 6C) represents the binding of a protein belonging to the ETS family of regulated transcription factors. An analysis of the homology of the sequence within B to ETS sites suggests that among ETS factors, Elk-1 and Ets-1 may show more affinity for this area of the PS1 promoter.

Figs. 7. Summary of the nuclear factor binding sites identified by DNase I footprinting with neuroblastoma cell extracts on the PS1 promoter. The positions of the nuclear factor binding sites identified in Figs. 3–6 are indicated by brackets on both the top and the bottom strand of the promoter region −118/+178. The arrowheads show enhanced cleavage sites induced by the binding of nuclear proteins. Sequence homologies to consensus motifs for known transcription factor binding sites are underlined. +1 indicates the 5′ transcription initiation site identified in this report. The positions of 5′ and 3′ deletions tested by transfection assay are indicated by arrows. The nucleotide positions in relation to the start site of transcription are marked above the sequence.
An Upstream ETS Binding Site Is Crucial for PS1 Transcription

species detected by α-Ets-1 (lanes 5 and 6) are not detected by α-Ets-1/2 (lanes 1 and 2) in similar amounts. The identity of the 48- and 58-kDa proteins in lane 5 is not known. The major band in lanes 1 and 2 is likely to represent Ets-2, which could indeed be detected as a faint band in lanes 5 and 6, since a sequence related to the α-Ets-1 immunizing peptide is present in Ets-2. Thus, among Ets proteins at least Ets-2 appears to be present in the cell lines examined, with a minor amount of Elk-1 in SK-N-SH cells.

The α-Ets-1/2 was then added to EMSAs in attempt to observe a supershift in complex mobility or alternatively an inhibition of complex formation (Fig. 8D). No mobility supershift could be observed; however, the lower half of band E decreased consistently (lanes 4, 5, 8, and 9), indicating that E indeed includes two different complexes and that the lower complex involves an Ets-1/2 protein. Any alterations in complexes A and B could not be reliably observed. Perhaps protein-protein interactions reduce the accessibility of the epitope to the antibody. Thus, at least one of the DNA-protein complexes forming with nuclear extracts from SK-N-SH cells appears to include an ETS protein. No inhibition or supershift of protein-DNA complexes was observed using Elk-1 antibody (lanes 10 and 11).

**DISCUSSION**

The promoter fragment extending from −118 to +178 from the initiation start site mapped in this report confers maximum promoter activity, comparable with that of SV40. Our data from primer extension and RNase protection, together with a previous report (24), indicate that we have identified the transcription start site most upstream. We cannot rule out other downstream sites (24). However, no mRNA with 5′-end downstream from +102 (t5) (24) has been identified. Furthermore, 3′ deletions of sequences from +107 to +91 and +91 to +42 each increase promoter activity by 1.5–2-fold (data not shown). Thus transcription initiation downstream from +42 is unlikely. In addition, we did not detect any start site between +2 and +42 by primer extension and RNase protection (Fig. 1, B and C).

No extract was added in lanes 1 and 5. Lanes 2 and 6 contain 5 μg of SK-N-SH nuclear extract. HepG2 extract was added in lane 3 (5 μg) and lanes 4 and 7 (7.5 μg). The arrows indicate the position of specific complexes. B, the specific binding of nuclear factors to the PS1 promoter (−22/+6) region is abolished by competition with an ETS/Ets-1 consensus oligonucleotide. The oligonucleotide probe containing (−22/+6) PS1 sequences was incubated in the absence of extract (lane 1) or with 5 μg of SK-N-SH nuclear extract (lanes 2–8). In lanes 3–5, the extract was preincubated with an unlabeled heterologous oligonucleotide containing a known binding site for Elk-1/ETS-1. In lanes 6–8 the extract was preincubated with the same oligonucleotide competitor containing a mutation (GGAA to TTAA) in the ETS motif. Lanes 3 and 6 included a 30-fold molar excess of competitor over probe; in lanes 4 and 7 competitor was added to a 90-fold molar excess; and it was added to a 300-fold excess in lanes 5 and 8. The arrows indicate the positions of complexes.

C, analysis of nuclear proteins by immunoblotting with antibodies to Ets-1/Ets-2, Elk-1, and Ets-1. Nuclear extract (20 μg) from SK-N-SH cells (lanes 1, 3, and 5) and HepG2 cells (lanes 2, 4, and 6) were fractionated by SDS-polyacrylamide gel electrophoresis, and the same gel was simultaneously analyzed by immunoblotting with antibodies to broad cross-reactivity with Ets proteins (lanes 1 and 2) or specific for Elk-1 (lanes 3 and 4) or specific for Ets-1 (lanes 5 and 6). The positions of proteins recognized specifically by the antibodies are indicated by arrows. The size in kDa of molecular mass standards is marked beside the gel. D, inhibition of complex formation by α-Ets. EMSA mixtures including 0.5% Nonidet P-40 and excluding the oligonucleotide probe were preincubated with 2 μl of antibody for 45 min at 22 °C and further incubated for 30 min after the addition of the probe. Phosphate-buffered saline containing 0.1 mg/ml bovine serum albumin (2 μl) was added to control lanes 1, 3, 5, and 7 instead of antibody. No extract was included in lane 1; 5 μg of extract from SK-N-SH cells was included in lanes 2–5; and 2.5 μg was included in lanes 6–11. An antibody (2 μl) with broad cross-reactivity to Ets-1 and Ets-2 (α-Ets) was included in lanes 4, 5, 8, and 9. An antibody specific for Elk-1 was included in lanes 10 and 11.

**FIG. 8.** EMSA analysis of the protein complexes interacting with the (−22/+6) region. A, a GGAA to TTAA mutation abolishes the specific DNA binding of nuclear factor(s) to the PS1 promoter. The binding of nuclear factors recognizing specifically the (−22/+6) region of the PS1 promoter was visualized by EMSA. Lanes 1–4 include a −22/+6 wild type (wt) probe. Lanes 5–7 include a (−22/+6) mutant where the GGAA of the ETS motif present at −12 is altered to TTAA.

No extract was added in lanes 1 and 5. Lanes 2 and 6 contain 5 μg of SK-N-SH nuclear extract. HepG2 extract was added in lane 3 (5 μg) and lanes 4 and 7 (7.5 μg). The arrows indicate the position of specific complexes. B, the specific binding of nuclear factors to the PS1 promoter (−22/+6) region is abolished by competition with an ETS/Ets-1 consensus oligonucleotide. The oligonucleotide probe containing (−22/+6) PS1 sequences was incubated in the absence of extract (lane 1) or with 5 μg of SK-N-SH nuclear extract (lanes 2–8). In lanes 3–5, the extract was preincubated with an unlabeled heterologous oligonucleotide containing a known binding site for Elk-1/ETS-1. In lanes 6–8 the extract was preincubated with the same oligonucleotide competitor containing a mutation (GGAA to TTAA) in the ETS motif. Lanes 3 and 6 included a 30-fold molar excess of competitor over probe; in lanes 4 and 7 competitor was added to a 90-fold molar excess; and it was added to a 300-fold excess in lanes 5 and 8. The arrows indicate the positions of complexes.

C, analysis of nuclear proteins by immunoblotting with antibodies to Ets-1/Ets-2, Elk-1, and Ets-1. Nuclear extract (20 μg) from SK-N-SH cells (lanes 1, 3, and 5) and HepG2 cells (lanes 2, 4, and 6) were fractionated by SDS-polyacrylamide gel electrophoresis, and the same gel was simultaneously analyzed by immunoblotting with antibodies to broad cross-reactivity with Ets proteins (lanes 1 and 2) or specific for Elk-1 (lanes 3 and 4) or specific for Ets-1 (lanes 5 and 6). The positions of proteins recognized specifically by the antibodies are indicated by arrows. The size in kDa of molecular mass standards is marked beside the gel. D, inhibition of complex formation by α-Ets. EMSA mixtures including 0.5% Nonidet P-40 and excluding the oligonucleotide probe were preincubated with 2 μl of antibody for 45 min at 22 °C and further incubated for 30 min after the addition of the probe. Phosphate-buffered saline containing 0.1 mg/ml bovine serum albumin (2 μl) was added to control lanes 1, 3, 5, and 7 instead of antibody. No extract was included in lane 1; 5 μg of extract from SK-N-SH cells was included in lanes 2–5; and 2.5 μg was included in lanes 6–11. An antibody (2 μl) with broad cross-reactivity to Ets-1 and Ets-2 (α-Ets) was included in lanes 4, 5, 8, and 9. An antibody specific for Elk-1 was included in lanes 10 and 11.
Collectively, the data indicate that the +1 site identified in this study represents the major transcription start site for the human PS1 gene. The sequence over the (~118/+178) region contains 70% GC, and similarly to other GC-rich promoters (34, 35), PS1 does not contain a TATA box. Rather, it contains several GC boxes at positions −70, −40, and +20, which we have shown to bind Sp1-like proteins. Footprint competition assays have suggested that the most distal at −70 appears to constitute a stronger binding site, whereas sites at −40 and +20 are relatively weaker or bind a different Sp1-like factor. Indeed, the −70 GC box matches perfectly the consensus (G/T)GGCCGGRRY originally described (36, 37), whereas the proximal box at −40 (GGGGAGGAGC) and that at +20 (CGGGCGGGG) deviate from the consensus by one base (underlined). Notably, the most critical residues appear to be conserved within these two elements. Position 1, which deviates in the +20 element, is the least critical of the 10 residues. At the −40 site, A instead of C is present in position 5. This is likely to be a functional substitution, since A is found at this position in natural Sp1 element (37). Thus, comparison of sequences of these three elements with the consensus is consistent with the −70 site being a stronger binding site as compared with sites at −40 and +20. This is also consistent with our footprint. We have begun to assess the function of these sites as discussed above. Single point mutations in each site and their combinations will test the function of individual sites and their interdependence or redundancy.

The deletion of sequences from −22 to −6 affects promoter activity most significantly. This region contains a homology to binding motifs for ETS transcription factors (38, 39), and altering only the ETS consensus motif present at −12 from GGA to TTA results in a similar drastic decrease in promoter activity. This mutation also eliminates the formation of specific DNA-protein complexes detected by EMSA. The same complexes are abolished by competition with an ETS consensus oligonucleotide, and DNase I footprinting has shown that competition with the same oligonucleotide eliminates the binding of a protein to the −14 to −5 region. Collectively, these data indicate that an ETS protein(s) indeed recognizes the −12 motif and is required for the transcription of the PS1 gene. It will be interesting to determine which among ETS proteins are able to activate the PS1 gene in different cell types and in vivo. Comparison of the sequence at this site with that of different ETS sites reveals that it is most similar to that of Elk-1 binding sites and somewhat less similar to that of Ets-1. The oligonucleotide competitor used in Fig. 6 has been shown to bind Elk-1 and Ets-1 (33). Typically, the promoter context, including sequences flanking the consensus motif as well as protein-protein interactions with factors binding to adjacent sites, plays an essential part in determining the specificity of ETS factors for their natural target sites (38, 39). In SK-N-SK cells, at least one of the specific protein complexes appearing in EMSAs is affected by an antibody reacting with Ets-1/2, indicating that in SK-N-SH cells, the PS1 promoter binds an Ets-1/2 protein, probably Ets-2, which is present in larger amounts in the cell types examined. Whether Elk-1, which is present in relatively more reduced amounts, also interacts with the PS1 −12 element remains to be determined. Cotransfections of the PS1 gene with expression vectors encoding various ETS proteins should help to determine their relative ability to activate its transcription in different cell types. Recent studies in the adult brain in vivo have shown that Elk-1 is expressed throughout the brain and that it is localized exclusively to neurons (40). This is consistent with the primary localization of PS1 in brain to neurons as well (20–22). Ets-1 and Ets-2 are widely expressed in different tissues and differentially regulated (41, 42). Ets-2 is present in high levels in adult brain, including in postmitotic neurons. Ets-1 is particularly abundant in the nervous system during specific developmental stages (42). Thus in vivo, several ETS proteins could potentially be involved in the regulation of PS1 during central nervous system development or in the adult brain.

The −12 motif is required to control over 90% of the expression of the PS1 gene. Notably, together with the +20 GC box, it is strictly conserved between the human and mouse sequences (25). Furthermore, sequences from −22 to +178, which confer 80% of promoter activity, coincide with the region of high homology with the mouse promoter, and the human +1 site is located only 6 nucleotides downstream from that of the mouse gene (25). This points out further the likely importance of this region for the function of the promoter in both species. By contrast, the two upstream GC boxes present in the human gene are not conserved in the mouse, and they do not appear to be active in the context of the entire promoter, including downstream sequences to +178.

A recognition motif for p53 is present immediately downstream from the −12 Ets site. p53 has recently been shown to reduce the level of PS1 mRNA (43). This down-regulation appears to be a primary effect of p53 expression, because it occurs within 2 h of p53 down-regulation. Considering the overlapping position of p53 and ETS sites and the requirement of ETS for PS1 transcription, it is plausible that p53 binding may simply compete ETS binding near the initiation site and inhibit transcription. This mechanism would be consistent with a primary effect. It would also constitute a new example of a TATA-less gene repressed by p53. Indeed, contrary to TATA-containing promoters, very few TATA-less promoters are inhibited by p53. Interestingly, Ets-1 and Ets-2 promoters are also repressed by p53 (44). In these cases, although p53 does not bind to DNA, it can be detected by antibodies within the protein-DNA complexes, indicating protein-protein interactions as a mechanism. If Ets-1 and Ets-2 indeed act on PS1, this would provide a secondary mechanism for the repression of PS1 by p53. p53 overexpression in normal neoplastic cells induces either cell cycle arrest or apoptosis, depending on the cellular context (45). Notably, PS1 is also down-regulated by p21 and other cellular conditions, leading to apoptosis (43). Collectively, these data have suggested that PS1 may have an antiapoptotic role. Indeed, PS mutations may predispose neurons to apoptosis (46–49). Considering the increasing evidence for neuronal apoptosis in Alzheimer’s disease (50–54), the transcriptional control of PS1 and its down-regulation by p53 may relate directly to the pathology of the disease. It may also represent a key in pathways leading to cell differentiation or to cancer.

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