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Tst-1, a Member of the POU Domain Gene Family, Binds the Promoter of the Gene Encoding the Cell Surface Adhesion Molecule Po

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Tst-1, a member of the POU domain gene family, is expressed in specific neurons and in myelinating glia in the mammalian nervous system. Bacterially expressed Tst-1 binds specifically to the promoter of the gene encoding myelin protein Po, a Schwann cell surface adhesion molecule. In cotransfection assays, Tst-1 can specifically repress the Po promoter. The N-terminal part of Tst-1 protein is highly glycine- and alanine-rich, a structural feature shared by the helix-loop-helix protein TFEB.

The study of cell-specific gene expression in mammals and of a developmental mutant in nematodes led to the discovery of POU domain genes (1, 4, 6, 9, 10, 13, 21, 25, 29). A tissue-specific transcriptional activation function of these POU domain genes has been shown for Pit-1 (10, 18) and Oct-2 (21), and a developmental role has been genetically defined for *unc-86* in *Caenorhabditis elegans* (6) and for *pit-1* in mice (17). The identification of a large family of POU domain genes in the mammalian nervous system (8) suggested their possible regulatory function in both neurophysiology and neurogenesis.

Tst-1 is a member of the POU domain gene family expressed in the brain (8). Like all the other known members, Tst-1 is widely expressed in the developing neural tube and is restricted in the mature nervous system to subset of neurons (8) and myelinating glia, where it was named SCIP (20). To initiate a functional analysis of the Tst-1 gene, we screened rat hippocampal (λ ZAP) and hypothalamic (λgt11) cDNA libraries, using a random-primed, [^{32}P]dCTP-labeled probe from DNA fragments corresponding to the Tst-1 POU domain (8). Of 400,000 recombinant bacteriophage from each library, 4 cDNA clones were isolated (2 from each library). Double-stranded DNA sequencing (24) of a clone with a 1.6-kb insert from the hypothalamic library and of a clone with a 2.9-kb insert from the hippocampal library revealed that they shared identical open reading frames, with the former having six more amino acids at the N terminus. The other two clones contained shorter portions of the coding region. The nucleotide sequence of the 1.6-kb cDNA and the predicted open reading frame are shown in Fig. 1A. Structural analysis of the Tst-1 gene showed that the POU domain was located near the C′ terminus and was highly homologous to the gene products of mammalian *Brn-1*, *Brn-2* (8), and *Brn-4* (data not shown), *Drosophila dP-1*/*cf1a* (12, 29a), and nematode *ceh-6* (2), belonging to the POU III class (8) (Fig. 1B). Although the primary amino acid sequence outside the POU domain was diverged, several structural features were observed. A histidine-rich region preceding the POU domain was found in Tst-1. Such a region also existed in Brn-2 (7a) and *Drosophila* POU domain protein dP-1 (29a). The N′ terminus of Tst-1 was highly abundant in glycine and alanine residues (45% of total residues). Similar Gly-Ala-rich sequences have also been observed in the recently cloned human helix-loop-helix protein TFEB, which is related to the immunoglobulin E box-binding proteins (3). The C′ terminus of Tst-1 was also abundant in histidine, glycine, and alanine, as well as proline. While it has been suggested that the proline-rich region can exert a transcriptional activation function (19), the potential functional significance of the histidine- and glycine-alanine-rich sequences is not established. However, the existence of these regions in families of putative DNA-binding proteins across species implies functional roles.

RNA blot analyses were performed (10) with poly(A)$^+$ RNA from adult rat brain and nerve tissue. A major 3.2-kb mRNA was detected with a ^{32}P-labeled, random-primed DNA probe for Tst-1 (Fig. 2A). In vitro translation in reticulocyte lysate produced a [^{35}S]methionine-labeled polypeptide of about 42 kDa (Fig. 2B), the same size as predicted from the first ATG codon in the open reading frame in the Tst-1 cDNA (Fig. 1A). Antibody staining experiments (28) revealed a single protein band of approximately 48 kDa in the adult sciatic nerve (homogeneous Schwann cell population) nuclear extract (14) (Fig. 2B), but not in the nuclear extract from some other tissues tested such as liver, kidney, muscle, and spleen (data not shown), in which Tst-1 mRNA is also not detectable (8, 20). We estimated that the Tst-1 cDNA clone may lack the coding sequence for about 30 amino acids on the N′ terminal of the open reading frame.

Previous in situ hybridization analysis in embryonic and adult rat brain revealed Tst-1 expression in a subset of neurons (8), and RNA blot analysis showed Tst-1 expression in myelinating glial populations (20). In situ hybridization (26) was therefore performed in sections of 2-day-old (p2) rat heads, when nerves are being myelinated. Dense hybridization to the myelinating trigeminal nerve (Fig. 3, 5N) was observed, as well as to specific neurons, as previously

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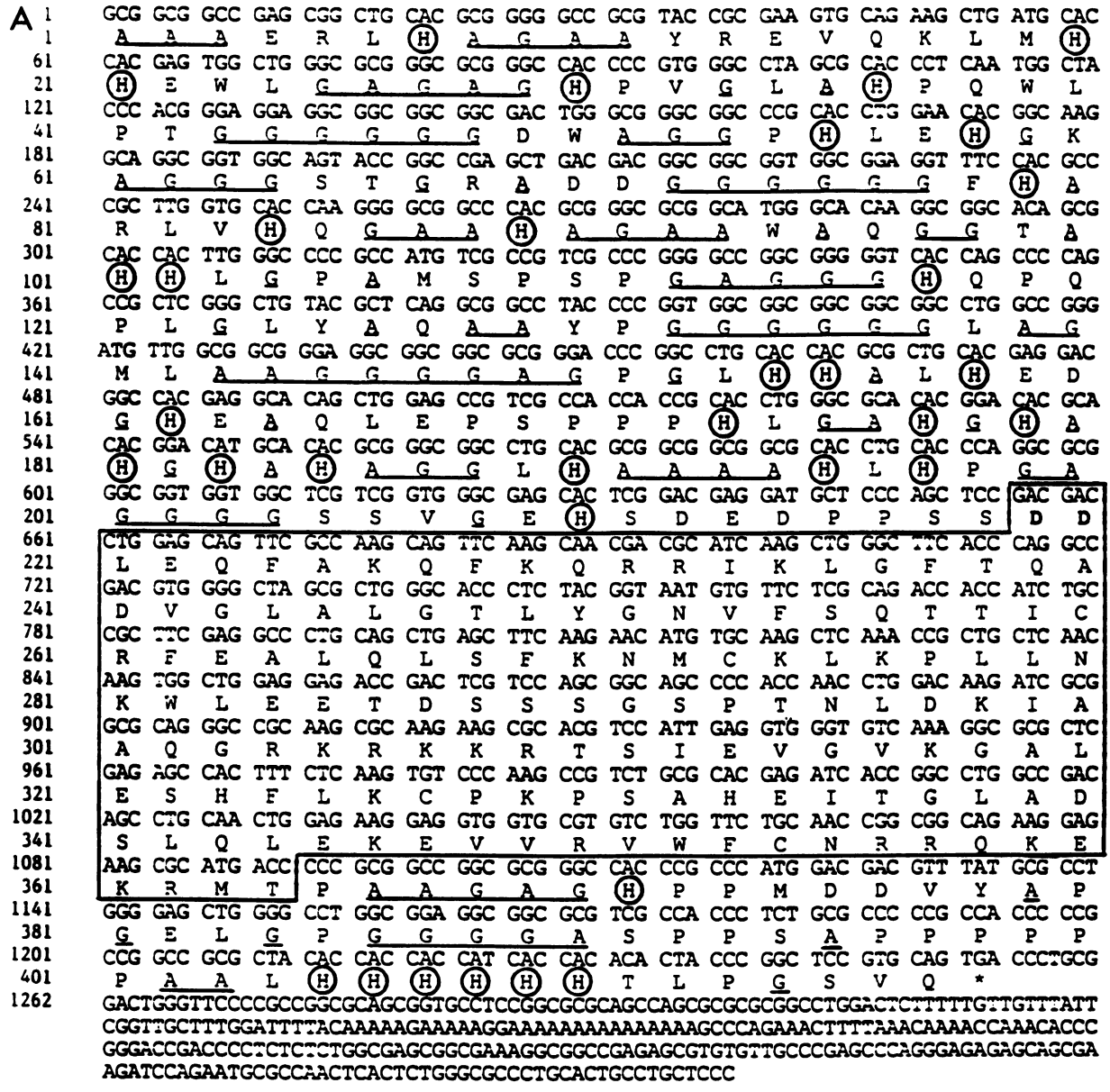
A
B
POU-SPECIFIC DOMAIN
Tst-1 DDLEQFAKQFKQRRIKLGFTQADVGLALGTLYGNVFSQTTICRFEALQLSFKNMCKLKPLLNKWLEETD
Brn-1
Brn-2
dP-1
Ceh-6
LINKER
POU-HOMEODOMAIN
Tst-1 SSSGSPTNL DKIAAQ GRKRKKRTSIEVGVKGALESHFLKCPKPSAHEITGLADSLQLEKEVVRVWFCNRRQKEKR
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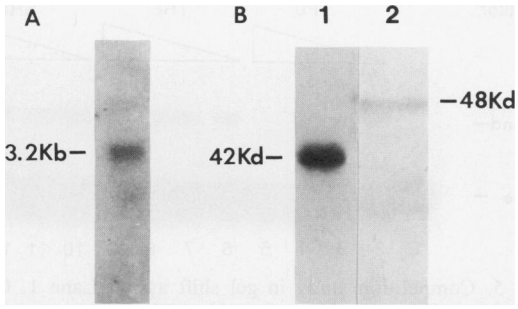


FIG. 2. Analysis of Tst-1 transcripts and protein. (A) Size of Tst-1 transcript determined by Northern analysis. Poly(A)$^+$ RNA from the trigeminal ganglion and the brain (data not shown) of adult rats was probed with randomly primed Tst-1 probe. While the POU domain probe hybridized to 3.2-, 4.4-, and 2.4-kb mRNAs in the brain (data not shown), the N′-terminal probe (nucleotides 1 to 360) only hybridized with the 3.2-kb band. (B) In vitro translation of Tst-1 cDNA and immunoblot of Tst-1 protein in sciatic nerve tissue (Schwann cells). Lane 1, In vitro-translated Tst-1 protein is approximately 42 kDa. Lane 2, Immunoblot of Tst-1 protein with rabbit anti-Tst-1 antiserum and I^{125}-protein A. Antiserum was raised against a peptide corresponding to amino acids 288 to 302 of Tst-1 protein. Only one band of about 48 kDa was detected in the sciatic nerve. Such a band is not detected in liver, kidney, skeleton muscle, and spleen (data not shown).

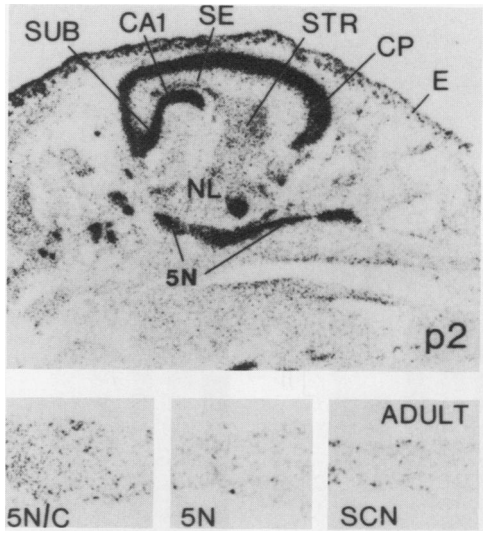


FIG. 3. In situ hybridization of Tst-1 in a sagittal section through the head of a 2-day-old (p2) rat pup (top) and sections of the adult trigeminal (5N) and sciatic (SCN) nerves (bottom). An antisense RNA probe corresponding to nucleotide 960 to the end of the cDNA (Fig. 1A) was used. Note the dense hybridization to the myelinating trigeminal nerve in the neonate and the absence of detectable hybridization in the adult nerves. As a control, note the level of hybridization to the adult trigeminal nerve (5N/C) when a sense-strand probe was used. As reported previously (8), many neuronal populations also express Tst-1 mRNA in the developing brain (CA_1, hippocampal field CA_1; CP, isocortical cortical plate; NL, nucleus of the lateral olfactory tract; SE, subependymal zone; STR, striatum; SUB, subiculum). Also, note intense hybridization in the ectoderm (E) of the developing epidermis and hair follicles, as well as in many other nerves of the head (unlabeled oval structure). Photographs of X-ray films; p2, ×6; nerves, ×20.

reported (8). Hybridization to the myelinating sciatic nerve was also observed (data not shown). In the adult, Tst-1 expression in peripheral nerves was hardly detectable (Fig. 3). Thus, the Tst-1 expression pattern in myelinating glia observed by in situ hybridization was consistent with the Northern (RNA) analysis, i.e., Tst-1 transcripts peaked at the onset of myelination in glial cells in vivo. This temporal correlation, together with data showing that in cultured Schwann cells Tst-1 gene expression is induced dramatically by cyclic AMP (20), an agent known to trigger Schwann cell proliferation and differentiation in vitro (15), suggests that Tst-1 is involved in certain aspects of the myelinating process.

By homology with the known POU domain transcription factors Pit-1 (10), Oct-2 (21), and Brn-2 (7a), Tst-1 is proposed to function as a sequence-specific DNA-binding transcription regulator that regulates the expression of myelin genes in glial cells. In fact, the Tst-1 gene and several myelin genes are induced by cyclic AMP, with induction of the Tst-1 gene preceding the induction of the myelin genes (20). We particularly investigated the Po gene, a major component of the Schwann cell myelin sheath and a cell surface adhesion molecule (5, 16). Previous transfection assays have shown that the region sufficient to confer Schwann cell-specific expression and cyclic AMP induction of the Po gene is within −915 to +49 bp of the cap site (16). Using a bacterial expression system (28), Tst-1 protein was produced from the cDNA and was used to perform DNase I footprinting (18) on the Po gene promoter. Double-stranded DNA probes were generated by polymerase chain reaction (23) with a plasmid containing the Po promoter (−915 to +49 bp) as a template, with one of the two primers kinased with T4 kinase and [γ-^{32}P]ATP. Tst-1 bound to five sites in the Po promoter. Site III (−708 to −676 bp) exhibited high affinity, site II (−630 to −596 bp) and site IV (−804 to −785 bp) showed moderate affinity, and site I (−35 to +1 bp) and site V (−894 to −867 bp) had low affinity (Fig. 4; see Fig. 6A). It is noteworthy that site I overlapped the TATA box. Since *Drosophila* homeodomain proteins can bind TATA elements and inhibit transcription (22), we speculate that this binding site serves as a negative element for Tst-1 regulation of Po gene transcription.

The binding specificity of Tst-1 to the Po gene was demonstrated in the gel shift assay. A double-stranded DNA fragment corresponding to the footprint III sequence 5′-GTA GAAAGAACTGAATTACCATTCTAATACGAG-3′ was end labeled with [γ-^{32}P]ATP. This DNA probe was shifted in

FIG. 1. Sequence of Tst-1. (A) Sequence of Tst-1 cDNA from the rat hypothalamic cDNA library (λgtII) and the predicted amino acid sequence. The POU domain is shown in the boxed region. The high content of glycine and alanine residues in the N′ and C′ termini is underlined. The histidine residues are circled. Note that the Tst-1 cDNA is highly GC-rich (70% G+C). (B) Comparison of Tst-1 POU domain with that of the other genes in the POU III class. Amino acids identical to those of Tst-1 protein are represented by dashes. Note that the POU-specific domains are more conserved than the homeodomains.

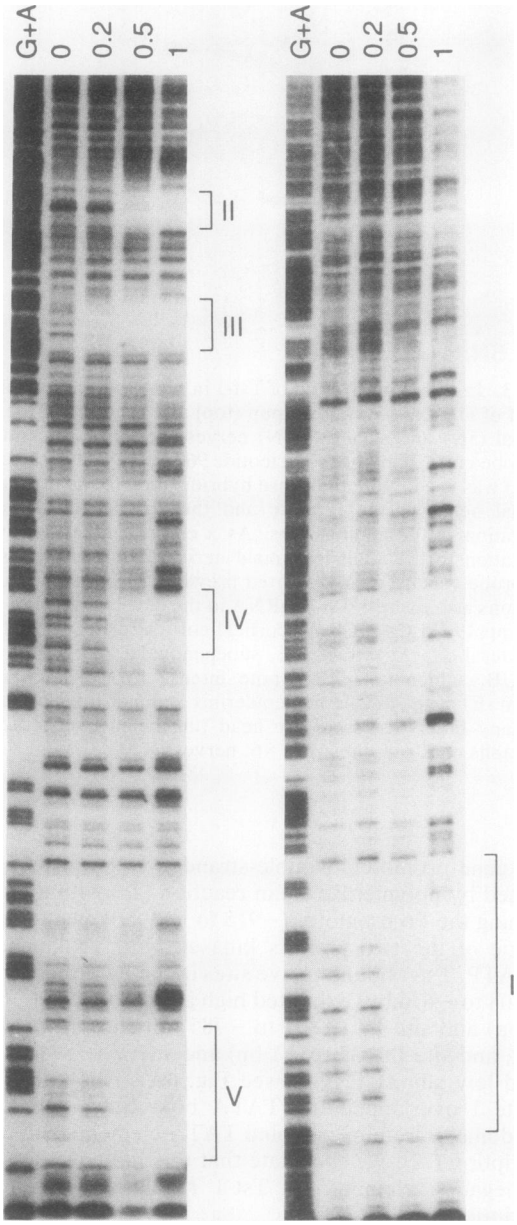


FIG. 4. DNase I footprinting analysis of Po promoter (−915 to +49 bp) by bacterially expressed Tst-1 protein. Five binding sites were observed and are marked I to V. Sites II to V are shown on the sense strand (left); site I is shown on the antisense strand (right). G/A and 0, 0.2, 0.5, and 1 represent G+A sequencing ladder and 0, 0.2, 0.5, and 1 μg of bacterial Tst-1, respectively. Crude bacterial extract was prepared (18) and used.

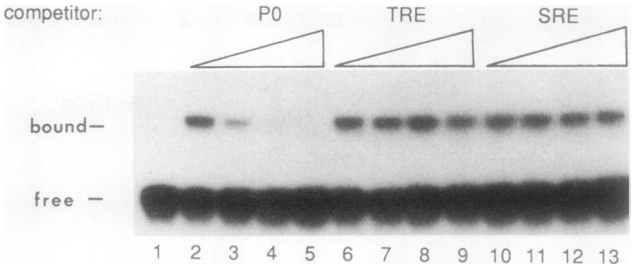
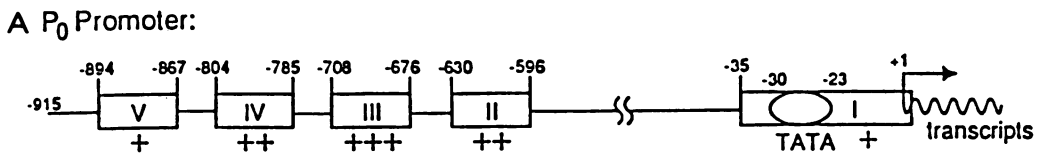


FIG. 5. Competition study in gel shift assay. Lane 1, Control *Escherichia coli* extract. Lanes 2 to 13, 0.07 μg of bacterial Tst-1, footprint III, was radiolabeled and used as the DNA probe. Competition was observed in the presence of a 0-, 20-, 100-, or 500-fold molar excess of nonradiolabeled DNA fragment corresponding to footprint III (Po, lanes 3 to 5), but not in the presence of the same amount of excess thyroid hormone response element (TRE, lanes 6 to 9), and serum response element (SRE, lanes 10 to 13), respectively. Gel shift assay was done by the method described in reference 27.

the presence of Tst-1 protein (Fig. 5, compare lanes 1 and 2), indicating Tst-1 binding. The binding was specifically blocked in the presence of excess nonradiolabeled Po fragment (lanes 3 to 5). Excess DNA fragments corresponding to a 30-bp sequence encompassing either the palindromic thyroid hormone response element (TRE, Fig. 5, lanes 6 to 9) (see reference 7) or the serum-response element (SRE, Fig. 5, lanes 10 to 13) from the c-*fos* gene (see reference 30) had no effect. Thus, DNA binding by Tst-1 protein was proved to be specific.

A schematic diagram of the Po promoter and Tst-1 binding sites is shown in Fig. 6A. The Tst-1 protein DNA-binding consensus derived from those sites is an AT-rich sequence, $GA^{A}_{T}\ T^{T}_{A}$ ANA (Fig. 6B). To further determine the transcriptional activity of Tst-1, we subcloned the cDNA shown in Fig. 1A into an expression vector driven by a cytomegalovirus promoter in both sense and antisense orientations and cotransfected it into CV-1 cells with luciferase reporter plasmids controlled by the Po promoter and other promoters. Surprisingly, while it was neutral or slightly active on the rat oxytocin promoter and herpes simplex virus thymidine kinase promoter, Tst-1 specifically repressed the Po promoter (Fig. 6C). As Pit-1 had no such effect on the Po promoter (data not shown), the repressor activity was specific for Tst-1. Thus, Tst-1 was confirmed to be a sequence-specific DNA-binding protein and a transcription regulator. Its expression during myelination and its ability to bind and repress the promoter of the Po gene suggests a role in regulation of the Po gene. Tst-1 is likely to have a function in both neurons and in myelinating glia, potentially analogous to Oct-2, which is expressed both in neurons (8) and lymphoid B cells (4, 13, 21, 25).

Myelin protein Po is a member of the immunoglobulin superfamily. It is known that many members of this family are expressed in the nervous system and participate in growth cone guidance and neuronal targeting (11). As Oct-2 activates immunoglobulin gene transcription, and Tst-1 binds and can repress the Po promoter and potentially regulates Po gene transcription, it becomes of particular interest to determine whether Tst-1 and other POU domain proteins in the brain physiologically regulate the expression of members of the large immunoglobulin gene superfamily.



B Tst-1 Binding Sites in P0 Promoter (-915 to +49)

I	sense	(-33)	GGG	GATTTTAA	GCAG (-19)
II	sense	(-625)	GAT	CATTTAAA	CTGC (-4)
IIIa	sense	(-699)	ACT	GAATTACC	ATTC (-611)
IIIb	antisense	(-676)	CTC	GTATTAGA	ATGG (-685)
IV	antisense	(-786)	TAG	GAATACCA	CTTA (-800)
V	antisense	(-882)	GGG	AAATAAGG	GGAT (-896)

consensus GA(A/T)T(T/A)ANA

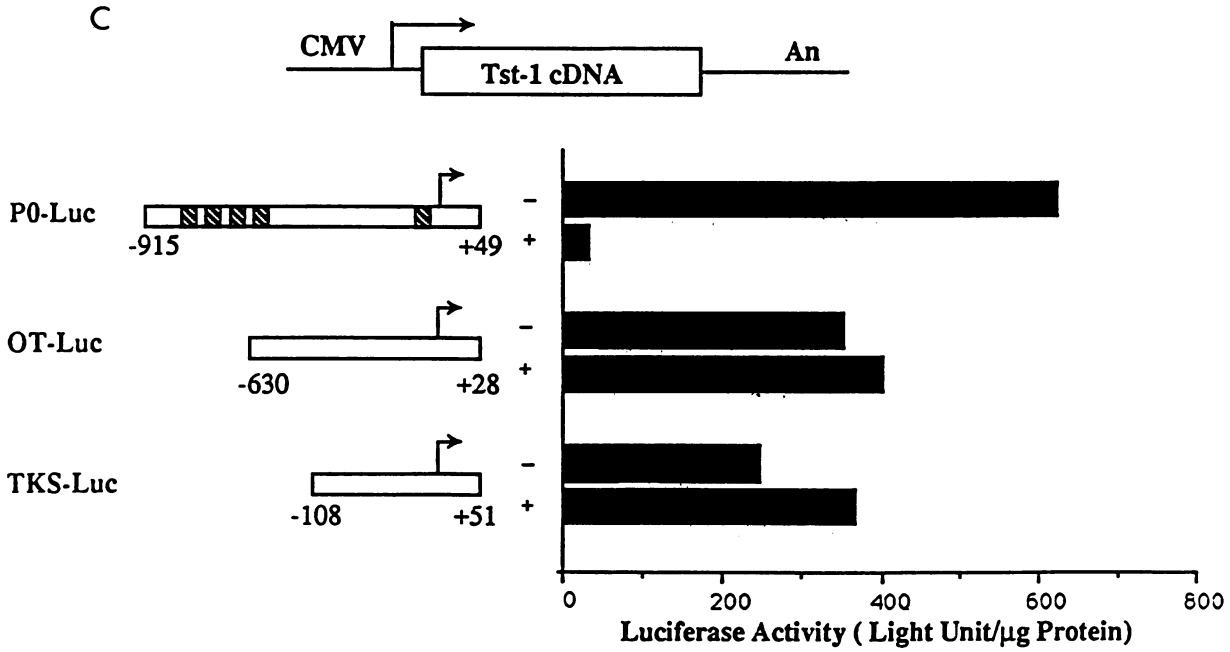


FIG. 6. (A) Schematic diagram of Po promoter (−915 to +49 bp). Tst-1 protein-binding sites are boxed. The relative affinity of each site in the DNase I footprinting analysis is indicated by a + symbol, with +++ being the highest. (B) Nucleotide sequence of Tst-1-binding sites and derived Tst-1-binding consensus sequence. (C) Cotransfection in CV-1 cells. Tst-1 cDNA (Fig. 1A) was cloned in cytomegalovirus plasmids in both sense (+) and antisense (−) orientations. Reporter plasmids were the luciferase gene driven by the promoters shown in the figure. A 3-μg portion of expression plasmid and reporter plasmid each was cotransfected into CV-1 cells by the calcium phosphate precipitation method as described in reference 10. The luciferase assay was done as described in reference 10. OT, Oxytocin; TKS, thymidine kinase.

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ADDENDUM IN PROOF

Two related papers were published recently (E. S. Monuki, R. Kuhn, G. Weinmaster, B. D. Trapp, and G.

Lemke, Science **249**:1300–1303, 1990, and N. Suzuki, H. Rohdewohld, T. Neuman, P. Gruss, and H. R. Schöler, EMBO J. **9**:3723–3732, 1990).

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