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Analysis of the human TATA binding protein promoter and identification of an Ets site critical for activity

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ABSTRACT

The TATA binding protein (TBP) is a general transcription factor required for initiation by all three eukaryotic nuclear RNA polymerases. Little is known about how TBP gene expression is regulated. To identify sequence elements and proteins contributing to human TBP (hTBP) gene transcription, we have characterized the promoter in two human cell lines. Multiple 5′-ends of TBP mRNA mapped throughout a 111 bp region immediately upstream of a previously reported hTBP cDNA. Upon transient transfection into cells, the hTBP 5′-flanking region was shown to contain a fairly active promoter. The *cis*-acting elements responsible for this promoter activity in Namalwa and HeLa cells were localized *in vivo* by deletion analysis. The minimal promoter defined from these experiments was a 54 bp region that encompassed all but one minor start site and contained a functional Ets protein consensus binding site, which was shown to be required for promoter activity in both cell lines. The importance of other potential elements to promoter activity was found to differ between the two cell lines. Consistent with that finding, different complexes were formed on promoter-containing DNA fragments upon incubation with nuclear extracts prepared from the different cells.

INTRODUCTION

Transcription in the eukaryotic cell nucleus is carried out by three different DNA-dependent RNA polymerases (pol I, II and III), which synthesize ribosomal, messenger and transfer RNAs respectively. The process of transcription initiation is dependent on the interactions of a set of basal transcription factors with the appropriate RNA polymerase and the core promoter sequence. One such transcription factor, the TATA binding protein (TBP), is required for initiation by all three RNA polymerases both *in vivo* and *in vitro* (reviewed in 1,2) and, thus, is central to expression of all eukaryotic nuclear-encoded genes.

In the cell, TBP interacts with other polypeptides (TBP-associated factors or TAFs) to form distinct multiprotein complexes that act selectively at different classes of promoters (reviewed in 2,3). Despite the large number of genes that rely on TBP for transcription and the fact that TBP assembles into a number of different protein complexes, TBP mRNA does not appear to accumulate to high levels in many cells and tissues that have been analyzed (4–8). Experimental support that TBP is limiting for transcription of some cellular genes has been provided by overexpression of TBP *in vivo* from a heterologous promoter (9–11). Such studies have indicated that increased TBP levels may stimulate transcription of some protein-encoding genes but not others (9,10). TBP may also be limiting for synthesis of stable RNAs (11). Thus, changes in the level of TBP gene expression might be expected to have important consequences for cellular growth rates and gene expression.

Several groups have reported results suggesting that mammalian TBP mRNAs and/or protein levels are regulated. For example, low to moderate levels of human TBP (hTBP) mRNA were detected in a variety of normal tissues, whereas much higher levels were observed in lung and breast carcinomas (5). It was also reported that the hTBP transcript accumulated rapidly after addition of serum to starved cells (5). In addition, several groups have observed higher levels of TBP mRNA in rodent testis and spermatids than in other tissues (7,8), suggesting that there might be developmental and/or cell type-specific regulation of TBP expression. Berk and colleagues (12) have suggested that hTBP expression might be negatively autoregulated, because they observed a decrease in the accumulation of endogenous TBP in several stably transformed HeLa cell lines expressing an epitope-tagged hTBP protein. The effect of increasing TBP concentrations on hTBP gene transcription has not been measured directly, however.

TBP genomic clones from a number of species have been isolated and sequenced (13–17 and references therein), but only two TBP promoters have been analyzed in detail to identify functional sequence elements. Those TBP promoters, from *Acanthamoeba castellanii* (an amoeba) and *Saccharomyces cerevisiae*, both contain consensus TATA boxes as well as other positive and negative *cis*-acting elements (6,18–19).

Very little is known about TBP gene expression in higher organisms, although the 5′-flanking regions of three vertebrate TBP genes (snake, mouse and human) have been isolated and sequenced (20–22). None of these genes contains a TATA box in

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the region immediately upstream of the mapped or deduced positions of transcription start sites and the sequence elements that are important for promoter activity have not been identified.

As a first step in the study of human TBP expression, we have cloned the hTBP gene 5′-flanking region and identified sequences important for promoter function in two human cell lines. We present evidence that an Ets protein binding site is a critical element in both cell lines and that the contributions of other sequence elements may differ between the two cell types.

MATERIALS AND METHODS

Cell culture

Namalwa (Nam) cells were cultured in Biorich1 medium (ICN) as described (23). HeLa S3 cells (ATCC CCL-2.2) were cultured as monolayers at 37°C under 5% CO_2 in Biorich1 supplemented with 10% iron-enriched bovine calf serum (Summit).

RNA isolation and analysis

Total RNA was isolated by guanidinium isothiocyanate cell lysis and centrifugation through 5.7 M CsCl (24) and enriched for poly(A)$^+$ RNA by oligo(dT)–cellulose chromatography (24). Clones representing the 5′-termini of TBP mRNA were obtained by the 5′-RACE method (25) using 1 µg poly(A)$^+$ RNA. Synthesis of the cDNA and the complement strand were primed using 5′-RT (5′-GGAGTCATGGCACCCTGAGG-3′) and dT17Adaptor [5′-GCCGCTCGAGGCGCCGC(T)$_{17}$-3′] respectively. DNA was amplified by 40 PCR cycles using 5′-AMPL (5′-GGCCGGTAC-CGTCGACACCCTTGCGCTGGAACTCG-3′) and the Adaptor primer lacking the poly(dT) sequence. PCR products were cloned into pBluescript-KS+ (Stratagene).

Single-stranded DNA probes for S1 nuclease mapping, which contained 66 nt identical to the HeLa cDNA and either 387 or 736 nt of 5′-flanking region, were synthesized by primer extension, cleavage at the unique *Sty*I or *Sac*I sites and gel purification (24). The probes (5×10^4 c.p.m.) were mixed with various RNA samples, heated to 85°C for 10 min and incubated overnight at 50°C in 20 µl S1 hybridization solution (24). S1 digestion proceeded for 1 h at 37°C after addition of 300 µl of a solution containing 300 mM NaCl, 2 mM zinc acetate, 60 mM sodium acetate, pH 4.5, and ~710–850 U/ml (final) S1 nuclease (Sigma). Reaction products were analyzed on denaturing 5% polyacrylamide gels.

RNase protection assays were performed as described (24). pTZSmSac, containing TBP sequences from –736 to +66 (numbered as in Fig. 2) in pTZ18U (US Biochemicals), was linearized with *Sty*I and transcribed to generate a ^{32}P-labeled 467 nt riboprobe containing 14 nt of vector sequence at its 5′-end. Riboprobe (5×10^4 c.p.m.) was combined with RNA in 30 µl hybridization buffer, heated to 85°C for 10 min and incubated overnight at 50°C. RNase digestion buffer (24) containing RNase T1 (Gibco BRL) at ~20 U/µl (final volume 350 µl) was added to each hybridization and incubated for 1 h at 30°C. The protected fragments were resolved on a 5% denaturing polyacrylamide gel.

Transient transfections

Human TBP promoter–luciferase constructs were made by inserting hTBP restriction fragments or PCR products into pGL2-basic (Promega). pSV40luc, pHSVtkluc and pRSVluc were made by cloning the simian virus 40 (SV40) early promoter/enhancer from pSVβgal (Promega), the herpes simplex virus thymidine kinase (HSVtk) promoter from ptkC1 (a gift of David Peterson, Texas A&M University) or the Rous sarcoma virus (RSV) LTR promoter from pRSVβgal (26) respectively into pGL2-basic. Plasmids used for transfection were purified on Qiagen-tip 500 columns.

One day before transfection, Nam and HeLa S3 cells were seeded at ~1×10^6 cells/ml and ~4×10^5 cells/35 mm well respectively. Transfections were by lipofection according to Gibco BRL. For Nam cell transfections, 2.25 µg luciferase (luc) reporter plasmid and 0.75 µg pSVβgal were incubated with 10 µg lipofectamine or dmrie-c (Gibco BRL) in 1 ml medium prior to the addition of ~4×10^6 washed cells. The lipid/DNA/cell mix was incubated at 37°C, 5% CO_2 for 4 h, then 2 ml medium containing 15% serum were added. Cells were harvested after ~40 h. For HeLa cell transfections, the luc and pSVβgal plasmids (1.5 µg each) were incubated with 12 µg lipofectamine and added to washed cells.

Measurement of promoter activity

Whole cell extracts (300 µl in 2× reporter lysis buffer) were made as suggested by Promega. Extract (20 µl) was added to 100 µl Luciferase Assay Reagent (Promega) and the relative light units emitted were read on a luminometer. Luciferase activities were corrected by subtracting the light units measured for untransfected cells. β-Galactosidase (β-gal) activities were determined by combining 150 µl extract with 150 µl 2× β-Gal Assay Reagent (Promega), incubating at 37°C overnight and measuring OD_{420}. β-Gal activities were corrected by subtracting the background measured for untransfected cells. Corrected luc activities were normalized by dividing by corrected β-gal activities. To normalize among different Nam cell transfections, each assay included a transfection with pSV40luc and hTBP promoter activities were compared with the activity observed for that construct.

Gel shift assays

Nam (23) and HeLa (27) cell nuclear extracts were prepared as described, except that 20% glycerol was included in buffer C (27). Standard gel shift assays (20 µl) contained ~7000–9000 c.p.m. labeled probe, 2 µg poly(dI·dC), 60 mM KCl, 12 mM Tris–HCl, pH 7.9 at 4°C, 6 mM β-mercaptoethanol, 0.12 mM EDTA, 12% glycerol, 225 µg/ml BSA and 1.4 µg (HeLa) or 1.5 µg (Nam) nuclear extract. Mouse Ets-1 protein, a gift of Barbara Graves (University of Utah), was included at 3 nM in binding reactions lacking poly(dI·dC). Proteins were incubated with probes for 20 min at room temperature. For competition assays, proteins and competitor DNA were incubated for 10 min before probe addition. Reactions were loaded onto 5% polyacrylamide gels containing 2.5% glycerol and 0.25× TBE buffer. Gels were run at room temperature for ~2 h at 30 mA. Top strand sequences of oligos used as competitor DNAs are shown below, with the protein binding sites indicated in bold (mutations underlined): Ets WT, 5′-AGCT-CAGAACT**GGCGGAAGTG**ACAT-3′; Ets MUT, 5′-AGCTC-AGAACT**GGC<u>A</u>AGAAGTG**ACAT-3′; Myb WT, 5′-GATCTG-CACGTC**TAACTG**CGACTGGCAGGTGCGCACGG-3′; AP-2 WT, 5′-AGCTGAACTGACC**GCCCGCGGC**CCGTGTGCAG-AG-3′; AP-2 MUT, 5′-AGCTGAACTGACC**<u>TTT</u>CGCGGC**C-CGTGTGCAGAG-3′; Sp1 WT, 5′-ATTCGATC**GGGGCGGG-G**CGAGC-3′; Sp1 MUT, 5′-ATTCGATC**GG<u>TT</u>CGGGG**CGA-GC-3′. Double-stranded oligos contained 4 nt overhangs on each

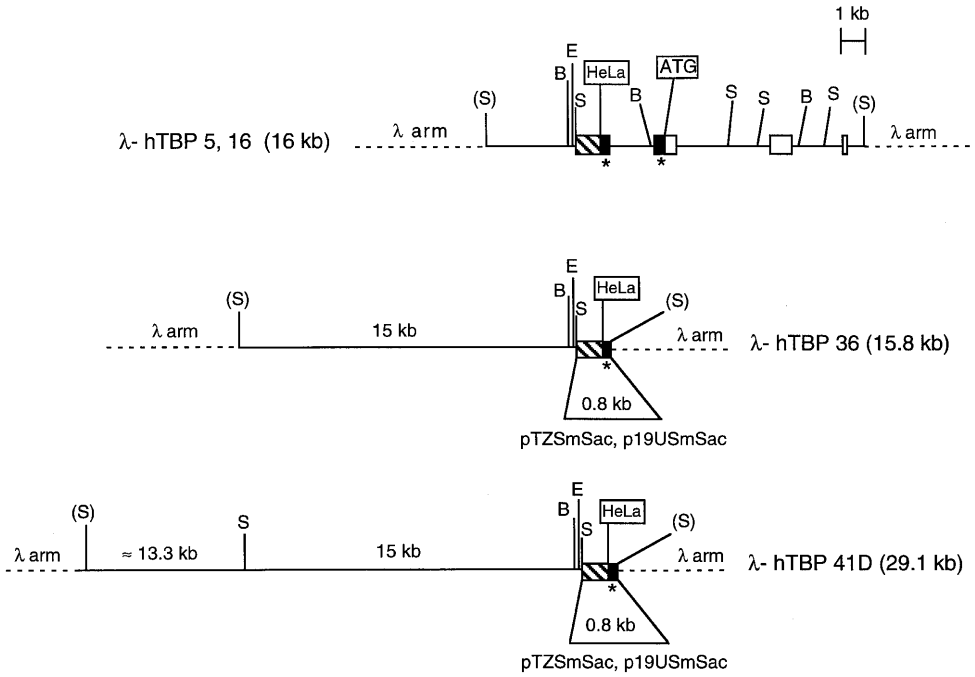


Figure 1. Restriction enzyme map of human TBP genomic clones showing area of overlap in the 5′-flanking region. The untranslated and translated hTBP exons are depicted as black and white boxes respectively and the promoter region as a striped box. Exons are not drawn to scale. Regions that hybridized to the probe used to screen the genomic library are shown by an asterisk. Positions of the translation start codon and the 5′-end of the HeLa TBP cDNA (30) are listed as ATG and HeLa respectively. Positions of *Eco*RI (E), *Sac*I (S) and *Bam*HI (B) restriction sites are shown. *Sac*I sites in parentheses were derived from the phage vector.

end, except the Sp1 oligos, which were blunt ended. The AP-2 and Sp1 oligos were derived from human metallothionein IIa promoter sequences (28,29).

RESULTS

Cloning and sequence analysis of the hTBP 5′-flanking region

We used a 5′-untranslated region probe from a HeLa cell TBP cDNA (30) to isolate four hybridizing clones from a human genomic DNA library (Fig. 1). Analysis by restriction enzyme mapping, Southern blotting and DNA sequencing showed that two of the clones (λ-hTBP 5 and 16) were identical in structure and contained the first four exons and ~4.5 kb of 5′-flanking sequence of the hTBP gene, whereas the other two clones contained only 66 bp of previously identified exon 1 sequence and ≥15 kb of 5′-flanking sequence (Fig. 1). The structure of our clones agrees with the complete exon/intron structure of the hTBP gene published by Chalut *et al.* (22).

We determined the DNA sequence of each of our clones between 736 bp upstream and 95 bp downstream of the HeLa cDNA 5′-end, which we have designated +1 (Fig. 2). This sequence contains many putative transcription factor binding sites; the only TATA-like sequence occurs around –560. We found only one sequence difference among our clones in the 5′-flanking region. Two clones had a T at –97, matching the published sequence (22), whereas the other clones had CG nucleotides at that position (Fig. 2).

hTBP transcription initiation sites

To define the transcriptional start sites of the hTBP gene, we mapped the 5′-ends of mRNAs isolated from two human cell lines: Namalwa (Nam), a B cell (Burkitt lymphoma) line, and HeLa S3, an epithelial (cervical carcinoma) line. First, we used a 5′-RACE procedure to subclone and sequence cDNAs derived from the 5′-end(s) of Nam cell hTBP mRNA (Fig. 3A). We analyzed 21 independent 5′-RACE clones, all of which matched the genomic sequence; no intron 1 sequence was detected in any clone. Four of the 5′-ends mapped to sites within the HeLa cDNA sequence (Fig. 3B), while 16 were clustered within a 41 bp region upstream of the HeLa cDNA 5′-end. One RACE clone (Nam 2-2) contained sequence extending further upstream, to position –108. Thus, the 5′-RACE assay suggested that transcription of the hTBP gene initiates at multiple positions within an ~110 bp region upstream of the HeLa cDNA 5′-end.

We used nuclease protection assays to confirm that the hTBP gene 5′-flanking region generates various transcripts with different 5′-ends. Figure 4A shows a schematic diagram of the DNA probe used for the S1 assay and the riboprobe used in the RNase T1 assay. Both had 66 nt complementary to the HeLa cDNA and 387 nt of 5′-flanking sequence. We observed six distinct S1 nuclease-protected probe fragments larger than that seen with the HeLa cDNA control RNA when Nam cell poly(A)$^+$-enriched RNA was assayed (Fig. 4B). However, only the two most prominent bands could be easily detected when Nam cell total RNA was assayed. The most upstream 5′-end detected in this assay was located within a few nucleotides of the 5′-most sequence observed among our RACE clones (Fig. 3B). We also assayed both Nam and HeLa poly(A)$^+$ RNAs with another S1

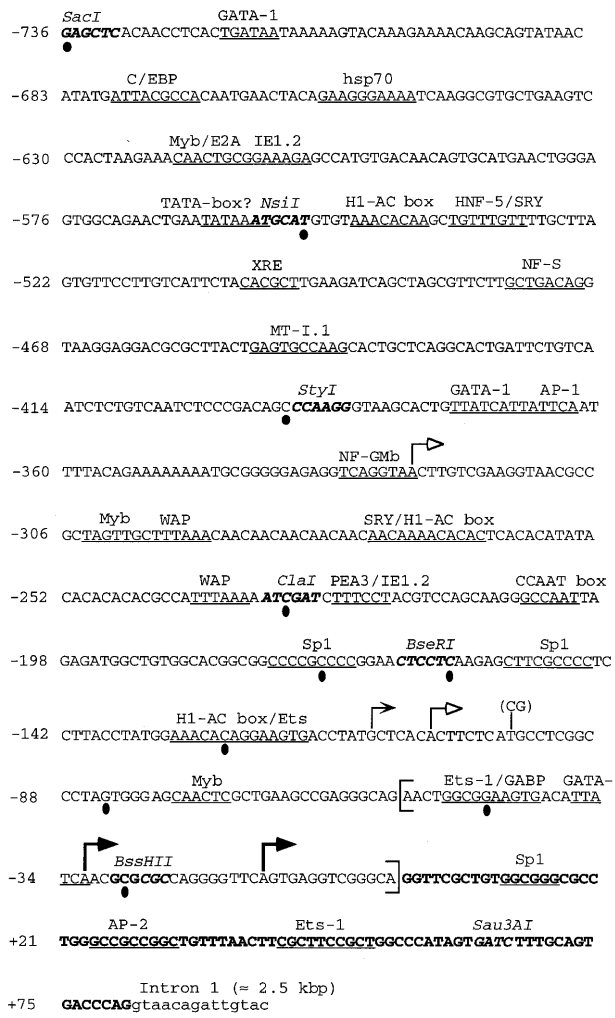


Figure 2. Nucleotide sequence of the human TBP 5′-flanking region. The sequence shown contains the 5′-flanking region, the first exon and the beginning of intron 1 (lower case letters). Nucleotides in bold represent sequence identical to the HeLa TBP cDNA (30); all numbering is relative to the 5′-end of this cDNA (+1). Restriction sites referred to in the text are indicated in bold italics. Dark ovals designate the 5′-boundaries of some of the deletion constructs described in Figure 5A. Bases in parentheses indicate a sequence polymorphism. Putative regulatory elements identified by computer searches using MatInspector 1.0 (46) and Transcription Factors Database 7.5 (1996) (47) are underlined. Open arrows indicate the positions of transcription start sites mapped by Chalut *et al.* (22); thick dark arrows show major start sites mapped in this work. The thin dark arrow shows the position of our most upstream start site. The –54/–1 minimal promoter region is enclosed in brackets.

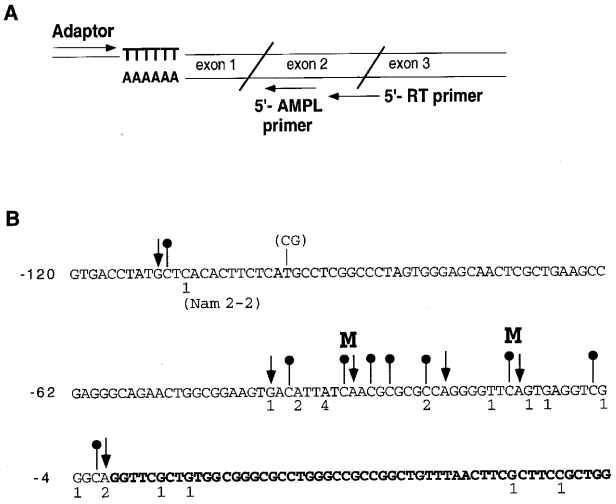


Figure 3. Mapping of human TBP mRNA 5′-ends. (**A**) Description of the 5′-RACE protocol, showing where the primers hybridized. (**B**) Summary of the 5′-ends of Nam cell TBP mRNA detected in three separate assays. The DNA sequence of some of the hTBP 5′-flanking region and the beginning of exon 1 are shown. The numbers below the sequence indicate how many independent RACE clones were obtained that contained hTBP sequence extending as far as the designated position, with one clone (Nam 2-2) containing the most upstream sequence. Numbering of nucleotides is as in Figure 2, with bold letters representing sequence identical to the HeLa cDNA. The dark arrows and dark circles refer to the positions of the hTBP mRNA 5′-ends mapped by S1 nuclease and RNase T1 assays respectively. The two putative major transcription start sites are denoted by a large letter M.

probe containing 736 nt of 5′-flanking region, so that we could detect larger transcripts, if they existed. The sizes of protected probe fragments were the same as observed in the experiment of Figure 4B and were identical for both cell lines (data not shown). However, the relative intensities of the various protected fragments suggested that the most downstream start site is used more in HeLa than in Nam cells (data not shown).

In the RNase T1 assay, approximately nine nuclease-protected riboprobe fragments were observed with Nam cell RNAs (Fig. 4C). The positions of the 5′-ends represented by the two most prominent bands in this experiment and in the S1 assays corresponded closely, suggesting that these 5′-ends represent two major transcription initiation sites.

The TBP mRNA 5′-ends identified by the three assays are summarized in Figure 3B. All of the 5′-ends mapping upstream of the HeLa cDNA sequence were located within 4 nt of a 5′-end identified by at least one other method, suggesting that they corresponded to (or mapped near) true transcription start sites. These start sites are consistent with the size of hTBP mRNA (~1.8–2.2 kb) from Northern blot analyses (30,31) and would produce mRNAs with untranslated leaders between 230 and 345 nt. Based on primer extension analysis of HeLa cell total RNA, Chalut *et al.* (22) previously reported a potential start site at –325 (in our numbering scheme), although the data that led to this conclusion were not shown. We did not detect a transcript of this size in either cell line.

Transfection assays detect promoter activity in the 5′-flanking region

We fused DNA sequences from 736 bp upstream to 66 bp downstream of the 5′-end of the HeLa cDNA to a firefly luciferase reporter gene (p–736/+66TBPluc; Fig. 5A). Nam cells were transiently transfected with this plasmid and, to control for transfection efficiency, co-transfected with an SV40-driven β-gal reporter plasmid. We compared the expression of luciferase from the hTBP 5′-flanking region with three well-characterized eukaryotic promoters and observed promoter activity comparable with that of the HSVtk promoter, ~33% that of the RSV promoter (data not shown) and ~20% that of the SV40 early promoter/enhancer (Fig. 5B). In contrast, promoter activity of the same

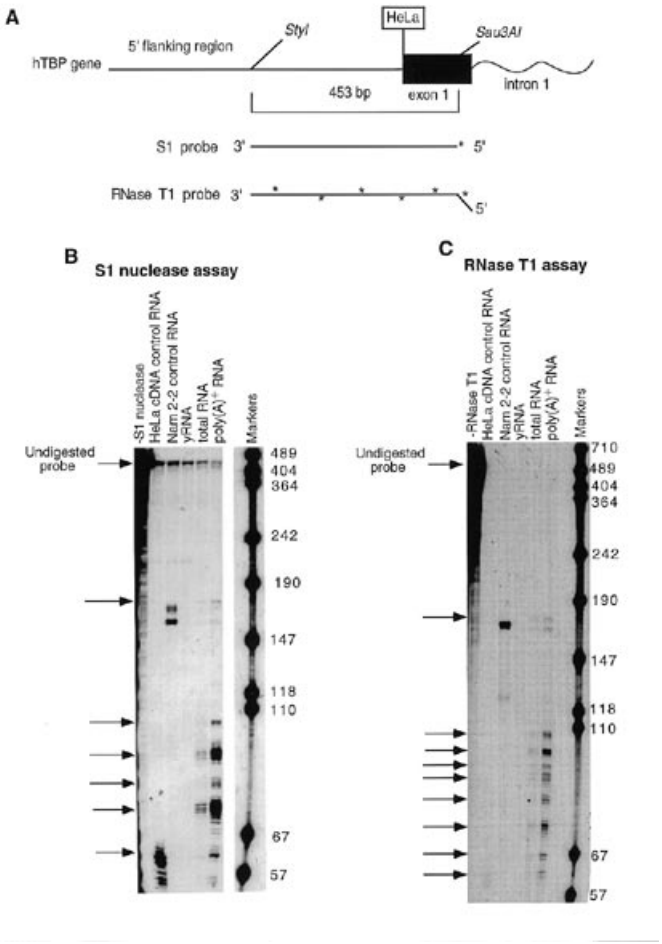


Figure 4. Nuclease protection assays map the 5′-ends of Nam cell TBP mRNA. (**A**) Diagram showing the single-stranded probes used in each assay, with asterisks indicating the radiolabeling patterns. The probes were complementary to a 453 bp *Sty*I–*Sau*3AI fragment, containing 66 bp of known 5′-untranslated exon 1 (black box). The riboprobe had additional unrelated sequence at its 5′-end (illustrated as a bent line). The position of the HeLa TBP cDNA 5′-end is listed as HeLa. (**B**) S1 nuclease protection assay, with unlabeled arrows showing the S1-protected probe fragments. The following amounts of RNA were hybridized: *in vitro* transcribed control RNAs, 130 pg with 40 µg yeast RNA (Sigma); yeast RNA (yRNA), 40 µg; total Nam cell RNA, 40 µg; Nam cell poly(A)$^+$-enriched RNA, 40 µg. (**C**) RNase T1 protection assay of the RNAs described in (B).

hTBP sequence when inverted relative to the reporter was not reproducibly above the background level observed for the promoterless plasmid, pGL2-basic (data not shown). Thus, the hTBP gene 5′-flanking region contains a fairly active promoter in Nam cells.

We also compared the promoter activities of two constructs differing at –97 in the 5′-flanking region (T or CG; Fig. 2). We found that the two promoter alleles had comparable activity (data not shown; but see Fig. 7). All but one of the deletion constructs described below were made from the CG-containing promoter allele (see Fig. 5A).

Localization of sequences contributing to promoter activity

To localize the DNA elements that contribute to promoter activity, we constructed the series of plasmids shown in Figure 5A. The relative promoter activities in Nam cells observed for the series of constructs containing different amounts of 5′-flanking region are shown in Figure 5B. Deletion of sequences from –4.5 to –1120 kb resulted in a minor increase in promoter activity. No dramatic loss of promoter activity was observed until the 5′-flanking sequence was deleted to –46, which severely crippled promoter activity, to ~2% of that measured for the –84/+66 vector.

We made additional constructs to test the effect of removing various amounts of sequence downstream from the mapped transcription start sites (Fig. 5C). The –736(T)/+2.6 kbTBPluc construct (Fig. 5A) gave approximately two times lower luciferase expression in Nam cells than the corresponding vector containing only the first 66 bp of HeLa cDNA sequence (–736/+66TBPluc). Deletion of the remaining 66 bp resulted in a minor loss of promoter activity (i.e. compare the –172/+66 and –172/–1 constructs). Deletion of another 23 bp (–172/–24 vector), which removed two minor and one major transcription start sites, resulted in an additional small decrease in promoter activity. We also made a –84/–1 construct, to test whether the downstream sequences are more important when combined with the most extensive 5′-deletion that still retained high level activity. Comparison of the promoter activities of the –84/+66 and –84/–1 (Fig. 5C) constructs indicated that the downstream sequence did not contribute to promoter activity in this context in Nam cells.

The Ets motif from –50 to –41 is required for a functional promoter

The results with the 5′- and 3′-deletion constructs suggested that the minimal hTBP promoter capable of supporting transcription in Nam cells was located between –84 and –1. This region contains a sequence, from –50 to –41, that matches the consensus recognition site for the Ets family of DNA binding proteins (32). To test whether disruption of this sequence was responsible for the loss of promoter activity upon deletion to –46, we made and tested a –54/–1 construct, which contains all of the putative site. When transfected into Nam cells, this plasmid showed only a minor loss of promoter activity relative to the –84/–1 construct (Fig. 5C). Thus, the sequences containing the Ets site were critical to promoter activity, whereas sequences upstream of –54 were not absolutely required, although they appeared to contribute.

Taken together, these results suggested that the minimal hTBP promoter capable of supporting transcription in Nam cells is contained within a 54 bp region encompassing all of the RNA 5′-ends that were mapped by more than one method, except for the minor site around –110 (Fig. 3B). To further examine the possibility that the Ets site is the critical sequence element within this region, we altered the site by introducing a single point mutation (G→A at –47) that would be expected to severely reduce binding of all but one of the characterized Ets family members (32,33). In the context of the –84/–1 TBP construct this mutation nearly eliminated promoter activity (Fig. 6). Thus, our results are consistent with the hypothesis that binding of an Ets protein is required for hTBP promoter activity in Nam cells.

Sequences required for promoter activity in HeLa S3 cells

The Ets protein family contains both ubiquitous and cell type-specific members, including several lymphoid-specific Ets proteins that might be expressed in Nam cells (34,35). Therefore, we wanted to determine whether similar or different regions of the

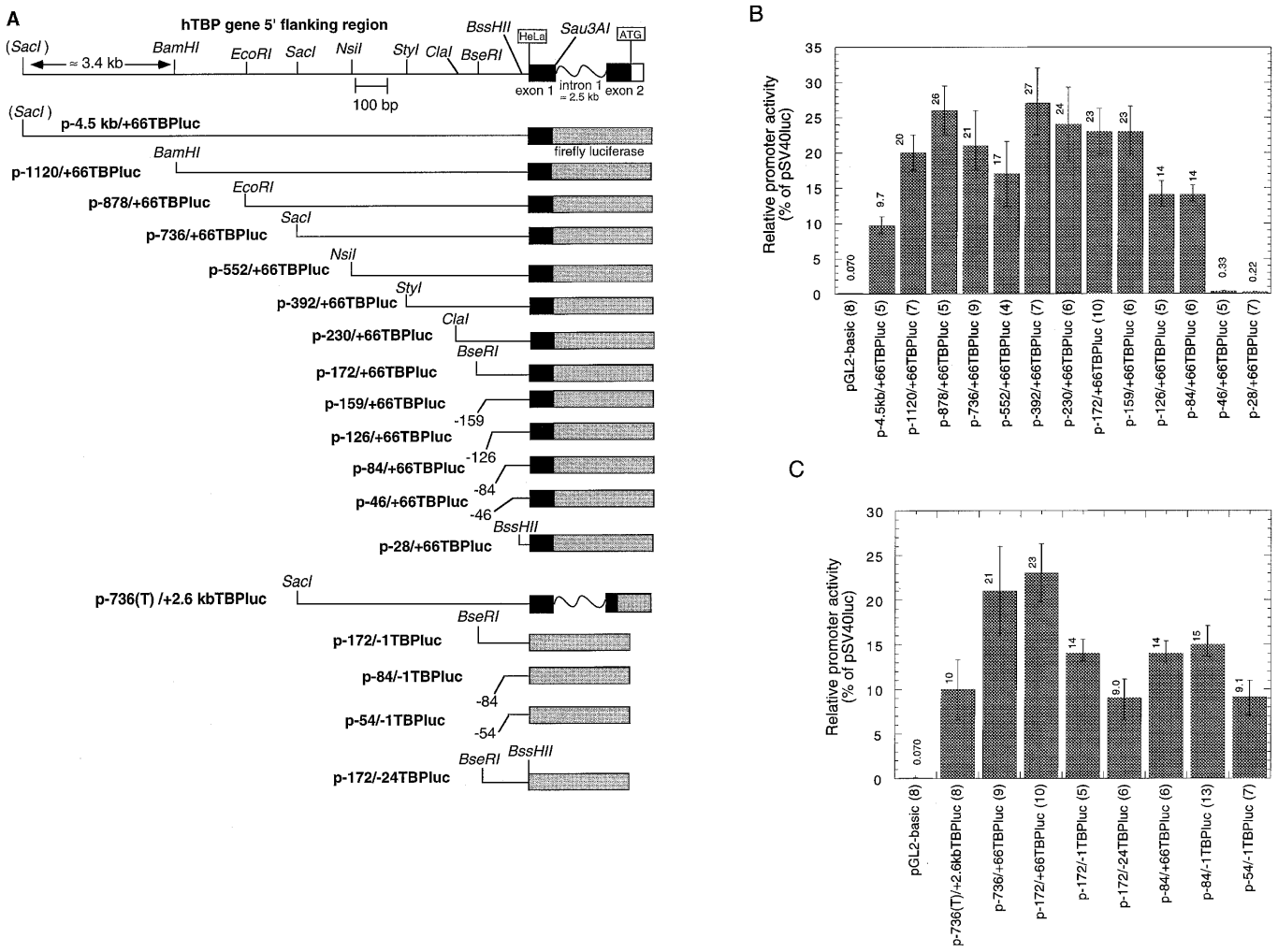


Figure 5. Transient transfection assays of hTBP promoter constructs in Nam cells. (**A**) Diagram comparing various promoter constructs and the hTBP gene. Each TBP vector is numbered to indicate the amount of DNA upstream and downstream of the HeLa TBP cDNA 5′-end (+1). The firefly luciferase gene is shown as a grey box. Intron 1 and the ~3.4 kb *Sac*I–*Bam*HI fragment were not drawn to scale. (**B**) Promoter activities of the various 5′-deletion constructs. Data for each construct were plotted as a percent of pSV40luc promoter activity. For each construct, the mean value and standard error calculated for a number of transfections (indicated in parentheses) are shown. (**C**) Promoter activities of the various 3′-deletion constructs. The data were analyzed and displayed as in (B).

hTBP promoter are important for activity in a different human cell line. We chose HeLa S3, because we had already mapped TBP RNA 5′-ends from this cell line. We transfected HeLa cells with a variety of TBP promoter constructs, along with negative (pGL2-basic) and positive (pHSVtkluc) control vectors (Fig. 7). As observed for Nam cells, the –54/–1 construct represented a minimal construct with promoter activity in HeLa cells. In addition, the –50/–41 Ets site was important for promoter activity in HeLa cells, since both deletion into (–46/+66 vector) and a point mutation within (–84/–1etsmut) the element severely reduced promoter activity (compared with –84/+66 and –84/–1 respectively). However, the magnitude of the decrease in promoter activity when the Ets site was mutated differed between Nam and HeLa cells (e.g. ~62- and ~16-fold respectively when comparing –84/–1 with –84/–1etsmut). We also observed an ~3-fold loss of promoter activity in HeLa cells when sequences from +1 to +66 were deleted in the context of the –84 construct (compare –84/+66 and –84/–1). In contrast, no loss of activity was observed with the same deletion in Nam cells, although this downstream region appeared to have a minor (<2-fold) effect on promoter activity when the construct contained sequences to –172 (Fig. 5C). These results suggested that there are one or more sequence elements in this downstream region that modulate TBP promoter activity differently in HeLa than in Nam cells.

Distinct Nam and HeLa nuclear proteins bind the critical Ets site at –50 to –41

Since a sequence matching a consensus Ets site was shown to be critical for hTBP promoter activity in the context of the –84/–1 construct in both cell lines, we did gel shift assays to demonstrate that the site could be bound by an Ets protein. We radiolabeled the –84/–1 wild-type hTBP promoter fragment and incubated it with purified mouse Ets-1 protein. The resultant complexes were separated by electrophoresis on a native polyacrylamide gel (data not shown). Several complexes formed specifically on the critical Ets site, as shown by elimination of binding by competition with an oligo containing the wild-type Ets site. In addition, these

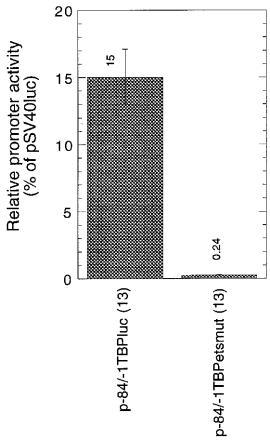


Figure 6. Comparison of the activity of a minimal hTBP promoter and a mutant derivative in Nam cells. Transfections were done and promoter activity determined as for the assays of Figure 5B to compare the promoter activities of the p–84/–1TBP wild-type and point mutant Ets site constructs.

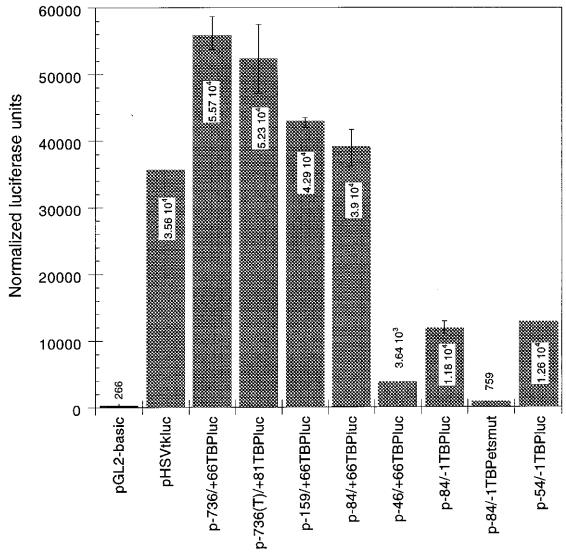


Figure 7. Functional analysis of the hTBP promoter in HeLa S3 cells. Transfections were as described in Materials and Methods. Data were plotted as the ratio of corrected luciferase to β-galactosidase activities (normalized luciferase units) obtained from three transfections of each construct. HeLa cells transfected in parallel with pRSVluc yielded 1.06×10^6 luciferase units in this experiment (data not shown).

complexes failed to form on a labeled probe bearing the mutant Ets site (data not shown). Thus, the critical hTBP promoter element is an authentic Ets binding site.

We then tested whether protein(s) in Nam and HeLa cell nuclear extracts bound specifically to this Ets site, thereby representing candidates for positively acting factors contributing to hTBP promoter activity. We incubated the –84/–1 wild-type promoter probe with nuclear extract and resolved the resulting complexes on native polyacrylamide gels (Fig. 8). Multiple complexes were formed on the –84/–1 probe with Nam nuclear extract (Fig. 8A). To determine which (if any) complexes represented protein(s) bound to the Ets site, we used competition assays, pre-incubating nuclear extracts with increasing amounts of unlabeled Ets WT, Ets MUT and Myb WT oligos. Only the Ets WT oligo competed well for the C1 complex; this oligo also competed for formation of the C2 complex, but to a lesser extent (Fig. 8A). The Ets MUT oligo, which has the same single point mutation (–47 G→A) as the –84/–1TBPetsmut vector, did not compete for C1 and C2 at the same oligo concentrations. The Myb WT oligo also failed to compete for either complex. These data suggest that C1 and C2 complexes contained Nam nuclear proteins interacting specifically with the critical Ets site.

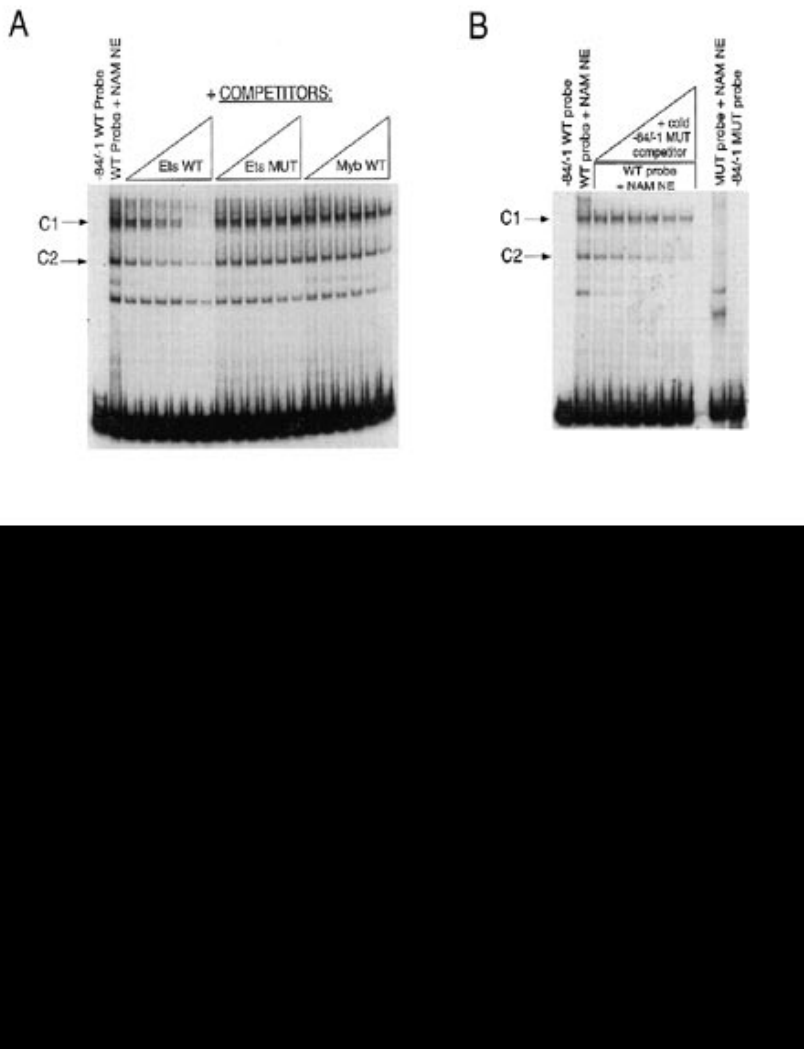
Further support that the two Nam cell complexes bound the Ets site was obtained with the demonstration that the –84/–1 MUT probe, containing the –47 G→A mutation in the Ets site, was reduced relative to the WT probe in its ability to form the complexes (Fig. 8B). In addition, we observed that the –84/–1 MUT unlabeled fragment could not compete well with labeled –84/–1 WT probe for binding of the C1 complex, but did compete (at higher concentrations) for formation of the C2 complex (Fig. 8B). Thus, the two complexes that were specifically bound to the Ets site appeared to have different properties.

Figure 8C shows the results of a gel shift assay using HeLa S3 nuclear extract with the –84/–1 WT and MUT probes. Three complexes (C3, C4 and C5) were formed on the WT but not MUT probe; all of these HeLa nuclear complexes have different mobilities than the Nam C1 and C2 complexes. Oligo competition assays tested whether the C3, C4 and C5 complexes were specific to the Ets site. All three complexes were competed by the Ets WT oligo, but not the Ets MUT or Myb WT oligos at the same concentrations (data not shown). However, unlike the case with the Nam C1 and C2 complexes, each HeLa complex was completely competed by 0.32 pmol Ets WT oligo (data not shown).

Distinct HeLa nuclear proteins interact with the downstream 66 bp region

The transfection assays had shown that the sequences between +1 and +66 apparently modulated promoter activity differently in Nam and HeLa cells. We used gel shift assays to determine whether a different protein(s) in Nam and HeLa cell nuclear extracts bound this region, which contains a potential Sp1 site and consensus AP-2 (36) and Ets binding sites (Fig. 2). Two distinct HeLa protein complexes were formed on the +1/+66 probe, along with a non-specific complex (Fig. 9A). The slower migrating specific complex was competed for binding to this probe by an oligo containing a well-characterized AP-2 site (AP-2 WT) but not a mutant derivative (AP-2 MUT). Since AP-2 has been shown to be expressed in HeLa cells (28), this complex may contain AP-2. The faster migrating specific complex represented protein(s) bound to the consensus Ets site, since the Ets WT oligo but not the Ets MUT or Sp1 WT oligos competed effectively for its binding to the probe.

When Nam nuclear extract was included in the binding reaction with the +1/+66 probe, several complexes were observed (Fig. 9B). However, only the complex running as a doublet exhibited some degree of sequence specificity. This complex did not migrate to the same positions in the gel as the faster migrating HeLa-specific complexes (data not shown). Neither the Ets WT nor Sp1 WT oligo competed better than the corresponding mutant oligo for formation of this complex. However, the AP-2 WT oligo was more effective at reducing the doublet complex than the AP-2


A
-84/-1 WT Probe
WT Probe + NAM NE
+ COMPETITORS:
Ets WT
Ets MUT
Myb WT
C1
C2
B
-84/-1 WT probe
WT probe + NAM NE
+ cold -84/-1 MUT competitor
WT probe + NAM NE
MUT probe + NAM NE
-84/-1 MUT probe
C1
C2