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# Embryo Is Maternally Derived

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**The cDNA encoding the TATA binding protein was isolated from 8- to 16-cell and morula-stage embryonic libraries of two distantly related species of sea urchin, *Strongylocentrotus purpuratus* and *Lytechinus variegatus,* respectively. The two proteins are 96% identical over both the N- and C-terminal domains, suggesting a conservation of transcriptional processes between the two species. The prevalence of SpTBP transcripts at several developmental time points was determined using the tracer excess titration method, and the corresponding number of TBP protein molecules was determined by quantitative Western blot analysis. Our results indicate that the amount of TBP mRNA and protein per embryo remains relatively constant throughout development. An initial large pool of TBP protein ($>10^9$) molecules in the egg becomes diluted as a consequence of cell division and decreases to about $2 \times 10^6$ molecules per cell by the gastrula stage. We found by *in situ* RNA hybridization that the oocyte contains a large amount of TBP mRNA which is depleted late in oogenesis so that the eggs and early embryos have extremely low levels of TBP mRNA. We conclude that the oocyte manufactures nearly all of the TBP protein necessary for embryogenesis.** 

***Key Words:*** **sea urchin; TATA binding protein; maternal supply.**

## INTRODUCTION

The TATA binding protein (TBP) is one of the most extensively studied of the eukaryotic general transcription factors. The TBP of *Saccharomyces cerevisiae* was the first to be cloned by the use of a partial amino acid sequence (Cavallini *et al.,* 1989; Hahn *et al.,* 1989; Horikoshi *et al.,* 1989; Schmidt *et al.,* 1989). Subsequently, TBP was cloned from other organisms following the design of PCR primers from the yeast sequence (Gasch *et al.,* 1990; Hoey *et al.,* 1990; Hoffmann *et al.,* 1990; Horikoshi *et al.,* 1990; Kao *et al.,* 1990). Sequence comparisons of TBP from widely diverged organisms have revealed a bipartite structure with a highly conserved C-terminal domain of 180 amino acids and a variable N-terminus (reviewed in Hernandez, 1993). While the C-terminal domain contains the DNA binding activity and the sequences responsible for interactions with TAFs and general transcription factors, the role of the N-terminal domain is not as clear. The majority of eukaryotes examined contain ≥75% identity in the C-terminal region.

Although it is a relatively small protein, ranging from 27 kDa in yeast to 38 kDa in *Drosophila* and humans, TBP is highly interactive and makes contacts with several general factors within the initiation complexes as well as a number of specific transcription factors. TBP is necessary for the transcription of most nuclear genes and participates in transcription initiation with all three polymerases. However, TBP is present in distinct complexes in each system and functions in different steps of preinitiation complex assembly (reviewed in Zawel and Reinberg, 1995). TBP associates with a number of proteins including TAFs (TBP-associated factors) that form at least four distinct complexes which are essential factors in the preinitiation complexes that function in promoter selectivity. In addition to distinct TAF/TBP interactions, there are several other factors/complexes described which specifically effect the

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expression of protein-coding genes, including SAGA, Mot1, and NC2 (reviewed in Lee and Young, 1998).

Extensive studies of both structure and function have been carried out on TBP from several different species including yeast, plants, fruit flies, and humans (Cavallini *et al.*, 1989; Hahn *et al.*, 1989; Horikoshi *et al.*, 1990; Schmidt *et al.*, 1989; Gasch *et al.*, 1990; Hoey *et al.*, 1990; Hoffmann *et al.*, 1990; Kao *et al.*, 1990 and Muhich *et al.*, 1990). In addition, the gene has been cloned from other organisms such as *Xenopus*, mouse, and maize for the purpose of evolutionary comparisons, promoter analysis, and examination of genomic organization (Tamura *et al.*, 1991; Haass and Feix, 1992; Holdsworth *et al.*, 1992; Hashimoto *et al.*, 1992). We isolated the cDNAs encoding the TBPs of two distinct species of sea urchin, *Strongylocentrotus purpuratus* and *Lytechinus pictus*, to examine this multifaceted transcription factor in a well characterized and easily accessible developmental system. The transcriptional processes occurring during both oogenesis and embryogenesis have been well described in sea urchins (reviewed in Davidson, 1986). In this study we quantitate both the mRNA and protein levels of TBP in the oocyte, egg, and at various developmental stages to understand the strategy the organism uses in supplying TBP for transcription during early embryogenesis.

## MATERIALS AND METHODS

### *Isolation of Sea Urchin TBP cDNA*

An *S. purpuratus TBP* probe was created using nested degenerate primers to the conserved domain of *TBP* (Kao *et al.*, 1990). Twenty-five nanograms each of two upsteam primers, 614 (5′-AAYGCNGARTAYAAYCC) and BK10 (5′-ATAGG-ATCCAAPATGGNGTNACN GGNGCNAA) and two downstream primers, BK11 (5′-ATAGGATCCACD ATYTTNCC-NSWNACPAADAT) and BK 12 (5′-ATCGGATCCCCNGGPAA NAPYYTCNGGYTCPTA), were used in the polymerase chain reaction (PCR) to amplify *TBP* from 100 ng of total egg cDNAs. The resulting PCR products (100 ng) were then used as templates for a final round of PCR amplification with primers BK10 and BK11. The expected 279-bp fragment corresponding to sequences from the C-terminal region was isolated in 1% low melting agarose and random-prime-labeled with 50 $\mu$Ci of [$\alpha$-$^{32}$P]dCTP using the Multiprime System (Amersham). A Lambda ZAP cDNA library, made from poly(A)$^+$ RNA isolated from 8- to 16-cell *S. purpuratus* embryos (kindly provided by Dr. E. Davidson), was used to infect BB4 cells, which were then plated and 400,000 of the resultant plaques were blotted to nitrocellulose and probed as described by Sambrook *et al.* (1989). Two positive clones were obtained; SpTBP.1 contained a portion of the C-terminal domain, but SpTBP.2 corresponded to sequences for the entire coding region.

A phage cDNA library in λZAPII was constructed from poly(A)$^+$ RNA from 4-h (morula stage) *L. variegatus* embryos. A random-prime-labeled 400-nt probe extending from 90 nt before the initiation codon and 310 nt into the coding region of the sea urchin *S. purpuratus* TBP cDNA was used to screen the cDNA library. Two positive clones were isolated from one million plaques, and the recombinant cDNA of each plaque was excised with helper phage R408 (Stratagene) and recovered as a Bluescript plasmid (Short *et al.*, 1988). The plasmid with the largest insert (5.5 kb), referred to as pLvTBP, was subsequently sequenced.

### *DNA Sequencing*

The Sequenase 2.0 kit (U.S. Biochemical) was used to determine the nucleotide sequences from both strands of clone SpTBP.2 (Kraft *et al.*, 1988). All sequencing primers were synthesized by the Albert Einstein College of Medicine DNA Synthesis Facility using an Applied Biosystems automated DNA synthesizer.

### *RNA Gel Blot Analysis*

Twenty micrograms of poly(A)$^+$ *S. purpuratus* RNA from egg and blastula stage embryos was resolved by agarose gel electrophoresis (Thomas, 1980), blotted to Z-probe (Amersham), and prehybridized in 5× Denhardt's (1×: 0.02% Ficoll, 0.02% polyvinyl pyrollidone, 0.02% BSA), 5× SSPE (1×: 0.18 M NaCl, 10 mM $NaH_2PO_4$ (pH 7.7), 1 mM EDTA), 0.1% SDS, 200 $\mu$g/ml of denatured salmon sperm DNA at 37°C for 4 h. The SpTBP.2 clone was PCR amplified either with primers GC267 and GC276 to produce a 279-bp C-terminal fragment or with primers T7 and GC279 to produce a 500-bp fragment corresponding to the 5′UTR. Fragments were isolated in low melting agarose, and 25 ng was used in the labeling reactions. Probes were labeled with 50 $\mu$Ci of [$\alpha$-$^{32}$P]dCTP using the Multiprime System (Amersham) and purified on P6 spin columns (Bio-Rad). Approximately $5 \times 10^6$ cpm/ml were used per hybridization on the Northern and Southern blots. Hybridizations were carried out in 50% formamide, 2× Denhardt's, 5× SSC (1×: 150 mM NaCl, 15 mM sodium citrate), 5% dextran sulfate, 0.1% SDS, 200 $\mu$g/ml denatured salmon sperm DNA at 42°C for approximately 18 h. Blots were washed four to five times with 0.5× SSC, 1% SDS, 1% sodium pyrophosphate at 65°C and exposed to X-ray film.

### *Genomic DNA Analysis*

Ten micrograms of *S. purpuratus* genomic DNA prepared from the sperm of a single animal was digested with the appropriate restriction enzyme. Restriction fragments were resolved on an agarose gel (Southern, 1975) and blotted to nitrocellulose. Blots were prehybridized in 5× SSC, 50% formamide, 5× Denhardt's, 200 $\mu$g/ml of denatured salmon sperm DNA at 37°C for 2 h, and hybridizations were carried out in 5× SSC, 50% formamide, 5× Denhardt's, 10% dextran sulfate, 200 $\mu$g/ml of denatured salmon sperm DNA at 37°C for 18 h using random-prime-labeled probe from SpTBP.2. Blots were washed four to five times with 0.5× SSC, 1% SDS, 1% sodium pyrophosphate at 65°C and exposed to X-ray film.

### *RNA Analysis in Situ*

For *in situ* RNA hybridization, ovaries, eggs, and embryos from *L. variegatus* and *S. purpuratus* were fixed in 2% glutaraldehyde and were prepared for whole mount *in situ* RNA hybridization as described (Ransick *et al.*, 1993). Digoxygenin-labeled antisense transcripts were synthesized by first linearizing the plasmid pSpTBP with *Xho*I and then transcribing the template using T3 RNA polymerase. A sense probe was synthesized by linearizing the plasmid with *Xba*I and then transcribing the template using T7 RNA polymerase (Megascript Kit, Ambion).

### *Primer Extensions*

The sequencing primer GC279 (5′-TCCTGGTCAGTACCTAT) was end-labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase. Approximately 50,000 cpm of labeled primer was hybridized to 15 $\mu$g of *S. purpuratus* poly(A)$^+$ RNA, isolated from blastula-stage embryos, in 30 ml of hybridization solution (1 M NaCl, 0.167 M Hepes, 0.33 M EDTA) for 18 h at 25°C. Primer extensions were carried out with 40U of AMV reverse transcriptase at 42°C for 90 min (McKnight and Kingsbury, 1982). Reactions were stopped by the addition of EDTA to 1 mM and treatment with 0.05 $\mu$g/ml of RNase A for 30 min at 37°C. Reaction products were analyzed on 6% acrylamide, 7 M urea gels.

### *Measurement of Transcript Prevalence*

All RNA was prepared exactly as described previously (Lieber *et al.,* 1986). The methods and materials for preparation and gel purification of RNA probes, hybridization conditions, RNase digestion, and analysis of protected fragments were as described for the T3/Sp6 Maxiscript and RPAII kits (Ambion). RNA probes were labeled with [$\alpha$-$^{32}$P]GTP and purified by electrophoresis.

The template for the RNA probe was synthesized using sequencing primer GC276 and the T7 primer to PCR amplify the 5′-UTR and 500 nt of the coding region of *SpTBP* from SpTBP.2. The 1-kb PCR product was digested with *Xho*I and *Bgl*II to produce an 800-bp fragment which was ligated into the polylinker of pBluescript II (SK$^+$) (Stratagene). The resulting plasmid, pGC476, was digested with *Bse*RI and a 275-base antisense RNA probe was transcribed from the T3 promoter.

The tracer excess titration method was performed as described by Lee *et al.* (1986). RNA probes were synthesized with 500 $\mu$Ci of [$\alpha$-$^{32}$P]GTP, 400 Ci/mmol. (Amersham). Approximately 5000 to 10,000 cpm of probe with a specific activity of either 740 or 840 cpm/pg was used per hybridization reaction. The amount of input embryo RNA was between 10- and 600 $\mu$g per reaction and the total amount of RNA was kept constant by the addition of yeast RNA. After RNase digestion the protected fragments were precipitated with 5% (w/v) trichloroacetic acid and collected on GF/C Whatman filters. The radioactivity bound to the filters was measured by scintillation counting. Background levels of radioactivity were subtracted from all samples and typically were between 0.5 and 5% of the total input radioactivity.

### *Expression and Purification of Recombinant SpTBP*

Full-length SpTBP protein was produced in *Escherichia coli* using the pET expression system (Studier *et al.,* 1990). Primers GC 301 (5′-GGCGGCTCGAGGGAAGTCT TTTTGAAGGA) and GC 302 (GGGGGGGATCCTACAATCCTAGTCAAGCA) were used to PCR amplify SpTBP.2 from codon 2 to codon 265 and also to introduce a *Bam*HI site upstream of codon 2 and an *Xho*I site downstream of codon 265. The 800-bp PCR product, digested with *Bam*HI and *Xho*I and ligated into the same sites in the polylinker of pET 23a(+), yielded plasmid pGC477 with full-length SpTBP in frame including 6 histidine residues downstream of the polylinker and a 12 amino acid insertion at the N-terminus which serves as a T7 antigen tag. Recombinant SpTBP (bSpTBP) from pGC477 was expressed in *E. coli* BL21(DE3) by induction of a 100-ml log-phase culture with a final concentration of 1 mM IPTG (isopropylthio-$\beta$-D-galactoside) for 2 h. bSpTBP was isolated from the insoluble fraction by denaturation in 2 M urea and purification on a 2.5-ml, $Ni^{2+}$ charged Sepharose column using His-bind resin (Novagen). Recombinant SpTBP was recovered by gradual removal of urea by dialysis against buffer Z (1 M KCl, 250 mM Hepes (pH 7.9), 0.1% Nonidet P-40, 20% glycerol, 1 mM DTT, 1 mM PMSF, 5 $\mu$g/ml pepstatin A, 5 $\mu$g/ml aprotinin) with 2 M urea, 1 M urea, and 0M urea, respectively. After final dialysis 0.5 ml aliquots were stored at −80°C. Thawed fractions were adjusted to 50% glycerol and stored at −20°C. Alternatively, IPTG-induced bacterial cultures were denatured in 8 M urea, directly applied to a metal affinity spin column (Talon), and eluted in pH 6.0 buffer.

In both cases the fractions corresponding to purified recombinant SpTBP were electrophoresed on a 15% SDS–PAGE gel and stained with Coomassie brilliant blue dye reagent (Bio-Rad). The percentage of SpTBP in the column sample was determined using an LKB densitometer. Total protein concentrations were measured using protein assay reagents (Bio-Rad).

### *Growth of Sea Urchin Embryos and Preparation of SDS-Lysed Cultures*

*S. purpuratus* adult sea urchins were purchased from Marinus, Inc. (Long Beach, CA). Intracoelomic injection of 0.55 M KCl was used to induce adult animals to shed gametes. Eggs were collected in synthetic seawater (0.48 M NaCl, 0.01 M KCl, 0.03 M $MgCl_2$, 0.03 M $MgSO_4$, 0.01 M $CaCl_2$, 0.2% $NaHCO_3$, adjusted to pH 8.0), filtered through gauze mesh, and washed once with seawater and twice with seawater plus 1 mM *para*-aminobenzoic acid (PABA) to prevent hardening of the fertilization membrane. Eggs were then fertilized *in vitro* with sperm diluted in seawater and grown in a 17°C shaker bath at a concentration density of 1% (1 packed ml of eggs(one million eggs)/100 ml seawater).

One-milliliter aliquots of unfertilized eggs, 4 h, 11 h, 24 h, and 43 h embryos were removed from a 1% culture and microfuged at 4°C for 30 s. Embryo pellets were washed once with $Ca^+$, $Mg^{2+}$ free seawater (0.5 M NaCl, 0.009 M KCl, 0.03 M $NaSO_4$, 0.002 M $NaHCO_3$, pH 8.0) and twice with STE buffer (150 mM NaCl, 10 mM Tris · HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA). One milliliter of boiling SDS buffer (0.5% SDS, 50 mM Tris · HCl (pH 7.4), 1 mM PMSF, 5 $\mu$g/ml pepstatin A, 5 $\mu$g/ml aprotinin) was added to the final pellet and the solution was boiled for 10 min or until the solution cleared. The boiled samples were stored at −80°C.

### *Affinity Purification of Antibodies*

The methods used for affinity purification of anti-*S. cerivisiae* TBP antisera were adapted from Weinburger *et al.* (1985). Nitrocellulose filters were soaked with 1 ml (≈100$\mu$g) of purified recombinant SpTBP and were dried completely. The filters were initially blocked in TBST (TBS + 0.5% Triton X-100) containing 3% BSA followed by overnight incubation at 4°C with a 1:20 dilution of crude rabbit anti yeast TBP antisera in TBST with 3% BSA. Antibodies were eluted with a 5-ml wash in 5 mM glycine (pH 2.3), 150 mM NaCl, 0.5% Triton X-100, and 100 $\mu$g/ml BSA and were immediately neutralized with 5 ml of 100 mM Tris · HCl buffer (pH 7.4).

### *Western Blots*

For Western blots, samples of bacterially expressed SpTBP from BL21(DE3) cultures and SDS-lysed sea urchin cultures were separated on a 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel and were electrophoretically transferred onto Immobilon-P mem-

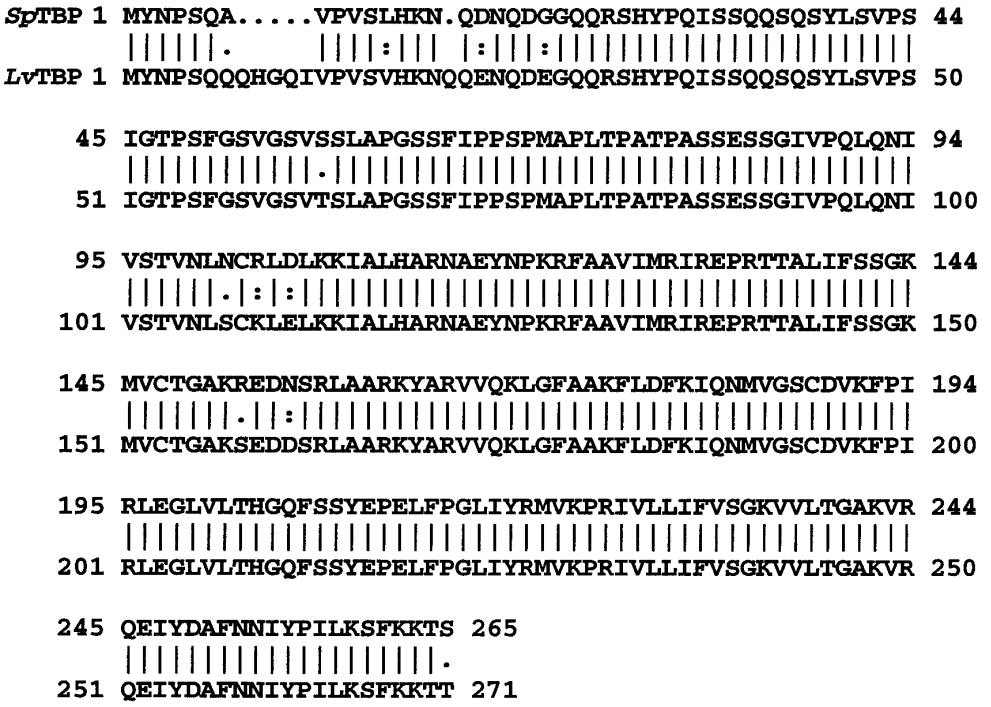


**FIG. 1.** The amino acid sequences of the *L. variegatus* (bottom) and *S. purpuratus* (top). TBPs are aligned for comparison. Conservative amino acid changes are indicated by a colon between the sequences and periods within a sequence represent amino acid gaps in the SpTBP.

brane (Millipore). Blots were washed three times in TBSN (10 mM Tris · HCl (pH 7.0), 0.9% NaCl, 0.05% Nonidet P-40) for 5 min at room temperature (RT) and incubated in 5% nonfat dry milk in TBSN for 1 h at RT. Blots were then washed three times in TBSN and incubated with affinity-purified rabbit anti-yeast TBP antibodies (Moir *et al.*, 1997) diluted 1:10 with 5% nonfat dry milk in TBSN, for 1 h at RT. Blots were washed five times in TBHSN (TBSN + 1.8% NaCl) and once in TBSN. The secondary antibodies, HRP-conjugated donkey anti-rabbit IgG (Amersham), were diluted 1:10,000 in TBSN and blots were incubated for 1 h at RT. Blots were washed five times in TBHSN, three times in TBSN, and once in TBS (10 mM Tris · HCl (pH 7.0), 0.9% NaCl). Blots were visualized by chemiluminescence using the ECL Reagent Kit (Amersham).

Autoradiographs generated from Western blots were analyzed using an LKB densitometer. A standard curve was generated for each of the experiments from known concentrations of the recombinant SpTBP and was used to determine the amount of SpTBP in sea urchin SDS-lysed cultures. X-ray film was preflashed to ensure linear response of the film within the exposure ranges used to quantitate TBP protein.

### *Nucleotide Sequence Accession Number*

The nucleotide sequences *of SpTBP and LvTBP* have been deposited in GenBank and can be found under Accession Nos. U86586 and AF085685, respectively.

## RESULTS

### *TBP from S. purpuratus and L. variegatus Is Highly Conserved*

To obtain the *S. purpuratus TBP* cDNA clone we used a strategy which was successful in the isolation of *TBP* cDNA clones from several other species (Gasch *et al.*, 1990; Hoey *et al.*, 1990; Kao *et al.*, 1990). Nested, degenerate oligonucleotide primers 614, BK10, BK11, and BK12 were used in the polymerase chain reaction (PCR) to amplify *TBP* from *S. purpuratus* egg cDNAs resulting in the synthesis of a 279-bp fragment from the highly conserved C-terminal domain (reviewed in Hernandez, 1993). This C-terminal PCR product was then used to isolate full-length cDNA clones by plaque hybridization from an 8- to 16-cell Lambda ZAP *S. purpuratus* library. Initially 400,000 plaques were screened, and two independent positive clones were identified. SpTBP.1 (sequence not shown) contained a portion of the C-terminal domain, but SpTBP.2 corresponded to sequences for the entire coding region including 5′ and 3′ untranslated regions.

The nucleotide sequence of 1524 bp of SpTBP.2 was obtained. The initiator methionine codon is at nucleotide 467 and the open reading frame encodes a protein of 265 amino acids with a molecular weight of 29 kDa. A TBP cDNA clone containing the entire coding region was also

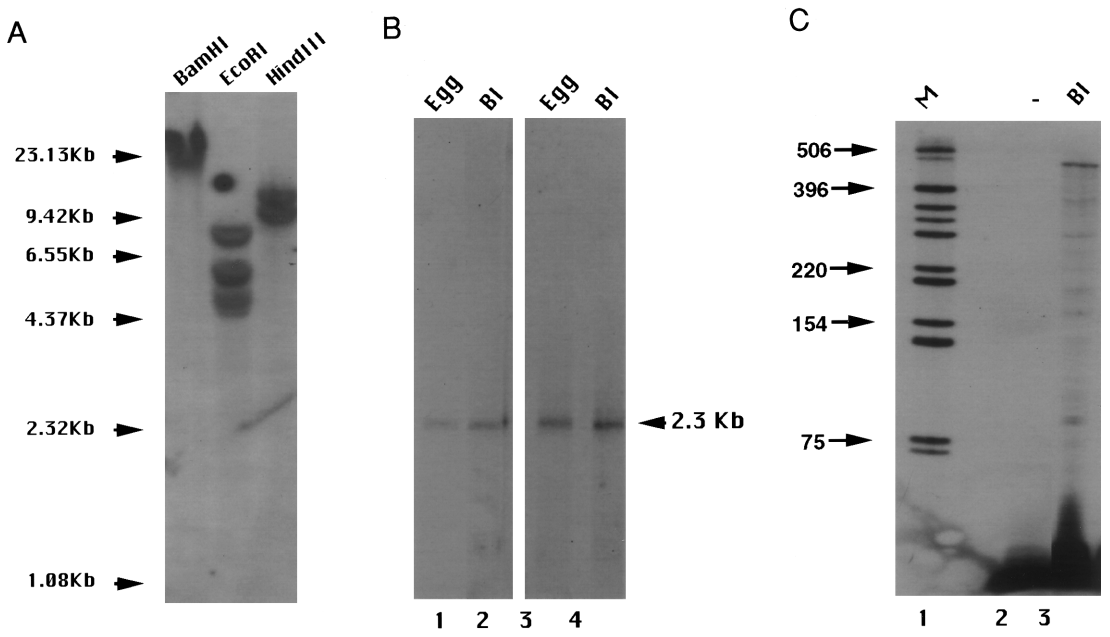


**FIG. 2.** (A) Genomic Southern blot analysis of *SpTBP*. 10 $\mu$g of sperm DNA from a single adult male sea urchin was digested with the restriction enzymes, *Bam*HI, *Eco*RI, *Hin*dIII as indicated and resolved by agarose gel electrophoresis. The result of a Southern blot probed with a random-primed [$\alpha$-$^{32}$P]dCTP-labeled, 279-bp PCR product from clone TBP.2 corresponding to the conserved region between primers BK10 and BK11 is shown. Molecular weight of the hybridized fragments is indicated at the left. (B) Northern blot analysis of *SpTBP*. 20 $\mu$g of *S. purpuratus* poly(A)$^+$ RNA from egg and blastula-stage embryos were resolved by denaturing agarose gel electrophoresis, transferred to Z-probe membrane (Amersham) and probed with two different *SpTBP* probes. A 495-bp PCR fragment, synthesized with primers GC279 and T7, corresponding to the entire 5′-untranslated region was used as probe in lanes 1 and 2. A 279-bp PCR fragment to the C-terminal region was the probe used in lanes 3 and 4. The transcript size is indicated at the right as 2.3 kb. (C) Primer extension of *SpTBP* 5′-untranslated region. 15 $\mu$g of *S. purpuratus* poly(A)$^+$ RNA from blastula (Bl)-stage embryos was used as template with the primer GC279, which hybridizes to the 3′ end, nt 460, of the 5′-untranslated region. Reaction products were resolved on a 5% acrylamide, 7 M urea gel. Lane M, [$\gamma$-$^{32}$P]DTP end-labeled pBR322, *Hin*dIII digested marker (sizes of bands indicated at the left); lane −, control without RNA. The size of the full-length major product of the primer extension reactions is indicated at the right with an arrow.

isolated from the distantly related sea urchin species *L. variegatus*. Its amino acid sequence is 96% identical to the *S. purpuratus* TBP with conservation extending throughout the N- and C-terminal domains (Fig. 1); however, there is no significant homology between the sea urchin N-termini and the N-termini of TBPs from other phyla.

### *Sea Urchin TBP Is a Single Copy Gene That Encodes One mRNA with a Long 5′-UTR*

The genome of *S. purpuratus* was examined by probing sperm chromosomal DNA with a labeled, 279-bp PCR product corresponding to the conserved C-terminal domain of *SpTBP*. The DNA from a single adult, digested with several different restriction enzymes, was blotted and probed as shown in Fig. 2A. In the *Bam*HI digest, a single restriction fragment was detected of greater than 23 kb, and in the *Hin*dIII digest there were two fragments of approximately 9.5 and 13 kb. Since the genome of *S. purpuratus* is highly polymorphic between individuals (Britten *et al.*, 1978), it is most likely that the sea urchin has a single *TBP* gene.

The long 5′ untranslated region (UTR), 466 bp in length, is an interesting feature of the cDNA clone, SpTBP.2. To examine *TBP* transcripts in the embryo and confirm the presence of the 5′-UTR in the mature transcripts, 20 $\mu$g of *S. purpuratus* poly(A)$^+$ RNA from egg and blastula stage embryos was used in Northern analysis. In Fig. 2B the results with parallel Northern blots hybridized with two different probes, one to the conserved C-terminal domain (lanes 1 and 2) and the other to the 5′-UTR (lanes 3 and 4), indicate that a single transcript, 2.3 kb in size, is recognized by both probes. The 5′-UTR in the cDNA clone is an authentic part of mature *SpTBP* transcripts.

Primer extension reactions were also performed to map the 5′ end of the TBP transcripts and determine whether all 466 nt were within this region. The primer, pGC279, which hybridized to the sequences near the initiation codon, was used in the reverse transcription reactions (McKnight and Kingsbury, 1982). The results of the reactions with substrate poly(A)$^+$ RNA from early blastula-stage embryos are shown in Fig. 2C. Primer extension reactions using early blastula-stage RNA result in a major product of approximately 450 nt. Together

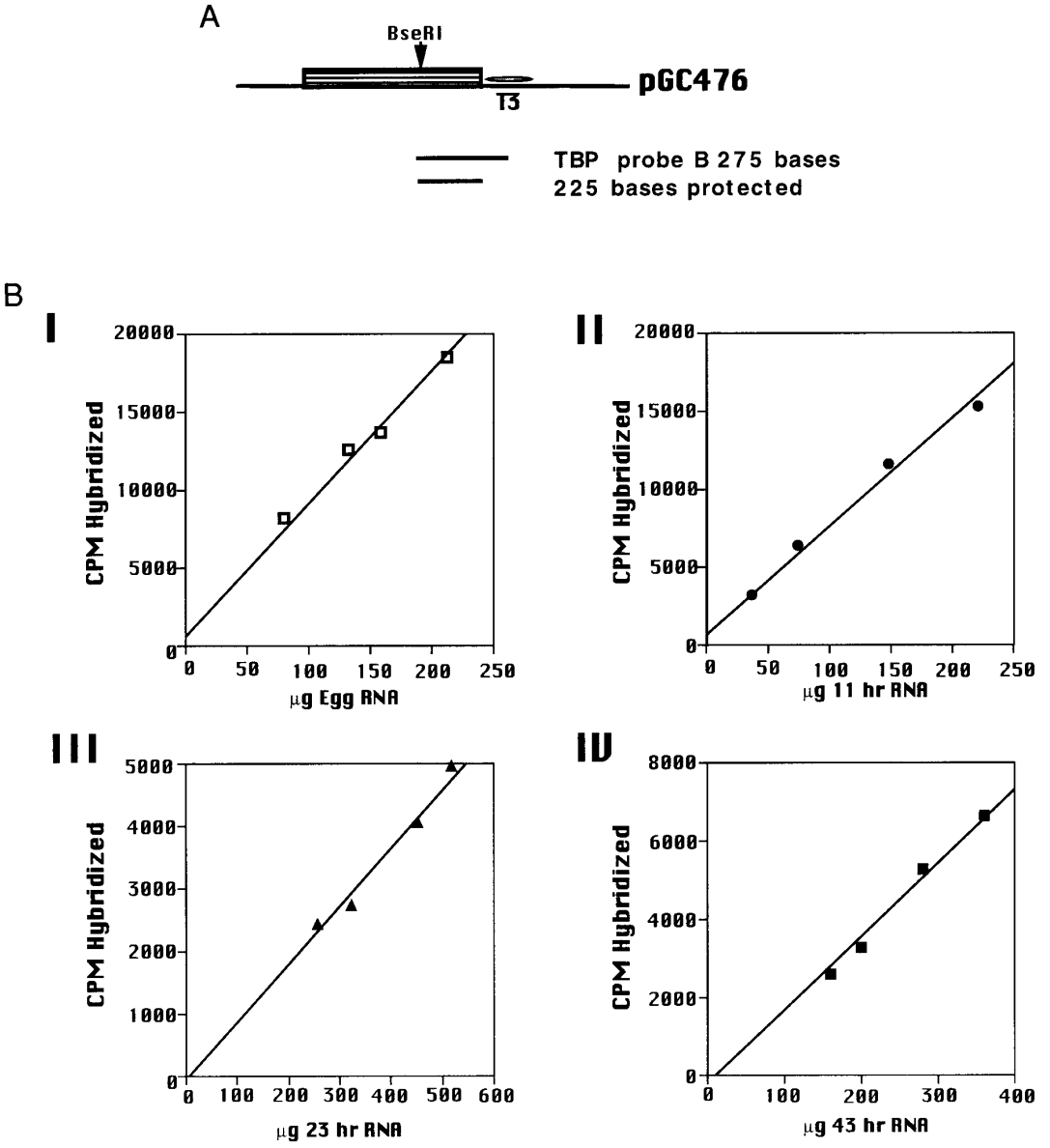


**FIG. 3.** Probe excess titration of *SpTBP* mRNA. (A) Schematic of the template plasmid, pGC476, which was linearized with *Bse*RI as indicated to synthesize a 275-base RNA probe from the T3 promoter. The probe protects 225 bases of TBP message after RNase digestion. (B) Titration curves plotted from probe excess titration experiments. The amount of total RNA ($\mu$g) is plotted against the cpm hybridized of [$\alpha$-$^{32}$P]GTP-labeled RNA probe. The slope of the line generated from least-squares analysis of the data points is used to calculate the amount of TBP mRNA (values are shown in Table 1). I, egg RNA; II, 11-h RNA; III, 24-h RNA; IV, 43-h RNA.

with the Northern blot, these results suggest that the Sp cDNA clone contains most if not all of the 5′-UTR.

### *TBP Transcripts Are Extremely Rare in Eggs and throughout Embryogenesis*

The abundance of TBP transcripts at various stages of development was initially examined by RNase protection assay. Using this method, TBP message was slightly more abundant in the egg than the other developmental time points that were examined, including 9-h early blastula, 11-h blastula, 24-h mesenchyme blastula, 43-h gastrula, and 72-h pluteus-stage embryos (Rybacki, 1996). To more carefully quantify the absolute numbers of TBP transcripts present at several of the developmental time points examined by RNase protection, the probe excess titration method was used (Lee *et*

**TABLE 1**
Prevalence of SpTBP mRNA during Development

| Stage | Probe hybridized per μg of total RNA, cpm[a] | mRNA per embryo, pg[b] | Transcripts, No. per embryo[c] |
|---|---|---|---|
| Egg | 38.038 | $1.57 \times 10^{-3}$ | $1.24 \times 10^{3}$ |
| 11 h | 17.386 | $6.08 \times 10^{-4}$ | $4.82 \times 10^{2}$ |
| 24 h | 9.282 | $3.69 \times 10^{-4}$ | $2.93 \times 10^{2}$ |
| 43 h | 18.740 | $7.45 \times 10^{-4}$ | $5.91 \times 10^{2}$ |

[a] Slopes of the titration curves from Fig. 4 were determined by a linear least-squares analysis of data points.

[b] Mass of mRNA (pg/embryo) = $XY/\alpha\beta\delta$, where $X$ is the cpm of probe/mg of RNA, $Y$ is the mass of total RNA/embryo (2.8 ng), $\alpha$ is the efficiency of scintillation counting (97.3%), $\beta$ is the specific activity of the probe (dpm/pg), and $\delta$ is the fraction of mature mRNA represented on the probe (225 nucleotides/2300 = 0.098). The size of the transcript was taken from Northern blot in Fig. 2.

[c] The number of transcripts per embryo is derived from the quotient: pg of RNA per embryo/pg of RNA per transcript (molecular weight of the transcript).

*al.,* 1986). The experiments were performed by using total RNA at a range of different concentrations from egg, 11-, 24-, and 43-h *S. purpuratus* embryos hybridized to the excess radiolabeled RNA probe. The results from each of the stages are displayed by plotting the amount of input RNA against the amount of hybridized probe as shown in Fig. 3. The slope of the line generated from the individual titration curves was used to calculate the mass of TBP mRNA and number of transcripts present on a per embryo basis (Table 1). TBP mRNA is a relatively rare transcript. There are about 1250 TBP transcripts stored in the egg. However, by the mesenchyme blastula stage (24 h postfertilization), which is about 400 cells, this number drops to about one to two transcripts per cell or about 800 transcripts per embryo. The absolute number of transcripts per embryo remains relatively constant during the early developmental stages examined (Table 1). These results indicate that TBP is an extremely low abundance mRNA in the embryo.

### *TBP Protein Is Relatively Abundant in Eggs and Embryos*

The abundance of the *Sp*TBP in the egg and in 4-, 11-, 24-h blastula- and 43-h gastrula-stage embryos was examined by quantitative Western blot analysis using affinity purified, anti-*S. cerevisiae*-TBP antisera (Moir *et al.*, 1997). Following fertilization the rate of translation in the embryo increases by 100-fold compared to the mature egg. However, the total protein content of the embryo remains relatively constant throughout development (Berg and Mertes, 1970; Regier and Kafatos, 1977; reviewed in Davidson, 1986). To compare embryonic stages, extracts were prepared from $10^4$ sea urchin embryos lysed in SDS buffer and the protein from the aliquots was resolved by SDS–PAGE. The results of an experiment performed with embryo cultures from a single animal are shown in the Western blot (Fig. 4A). The authentic *S. purpuratus* TBP (29 kDa) runs slightly faster on SDS–PAGE gel than the recombinant SpTBP (33 kDa) due to the histidine tag at the C-terminus and T7 antigen tag at the N-terminus. The levels of TBP in the egg, 4-h morula-stage (≈16 cell), 11-h blastula-stage (≈128 cells), 24-h mesenchyme blastula-stage (≈400 cells), and 43-h gastrula-stage (≈700 cells) embryos (lanes 1–5), were measured by performing laser scanning densitometry on the Western blots. The results indicated that the amount of TBP per embryo was relatively constant throughout embryogenesis.

To determine the number of TBP molecules present in the egg and embryo extracts, known quantities of recombinant TBP were included in the same SDS–PAGE gel along with lysed embryo cultures (lanes 6, 7, and 8). At the 11-h time point and subsequent time points there is a closely spaced doublet of TBP recognized by the antibodies, which may reflect posttranslational modification of TBP molecules, such as phosphorylation (White *et al.*, 1995). Both species were included in the analysis. In Table 2 the results of two separate experiments using independently isolated and quantitated recombinant TBP preparations are averaged, indicating the concentration and corresponding number of TBP molecules per cell at different stages of embryogenesis. The results, graphed in Fig. 4B, demonstrate that there is an exponential decrease in the number of TBP molecules present per cell as embryogenesis progresses. The relationship between TBP and cell number is also plotted in Fig. 4B. The large pool of TBP protein ($>10^9$) molecules in the egg remains constant in the embryo, but is greatly reduced as a consequence of cell division to approximately $10^6$ molecules per cell by the gastrula stage. These results also reveal that TBP protein, unlike TBP mRNA, is relatively abundant in the embryo. Given the low amount of TBP mRNA present in all stages of embryogenesis, it is not likely that a significant fraction of the TBP protein is synthesized during embryogenesis, but rather that TBP synthesized during oogenesis is stable throughout embryogenesis.

### *TBP Is Maternally Derived*

To test the hypothesis that TBP is maternally derived, *in situ* mRNA analysis was performed on embryos, eggs, and oocytes of both *L. variegatus* and *S. purpuratus.* Although we cannot isolate large quantities of pure oocytes at different developmental stages to perform the same RNA and protein prevalence analysis as we performed on eggs and embryos, we were able to document that the TBP mRNA is selectively accumulated in oocytes of both species (Fig. 5). Oocytes of all developmental stages accumulate increasing levels of TBP (on a per cell basis) based on the density of label seen, whereas in eggs and embryos, only background levels of hybridization were detected. Thus, the rather abundant TBP mRNA must be turned over, probably late in oogenesis or during maturation,

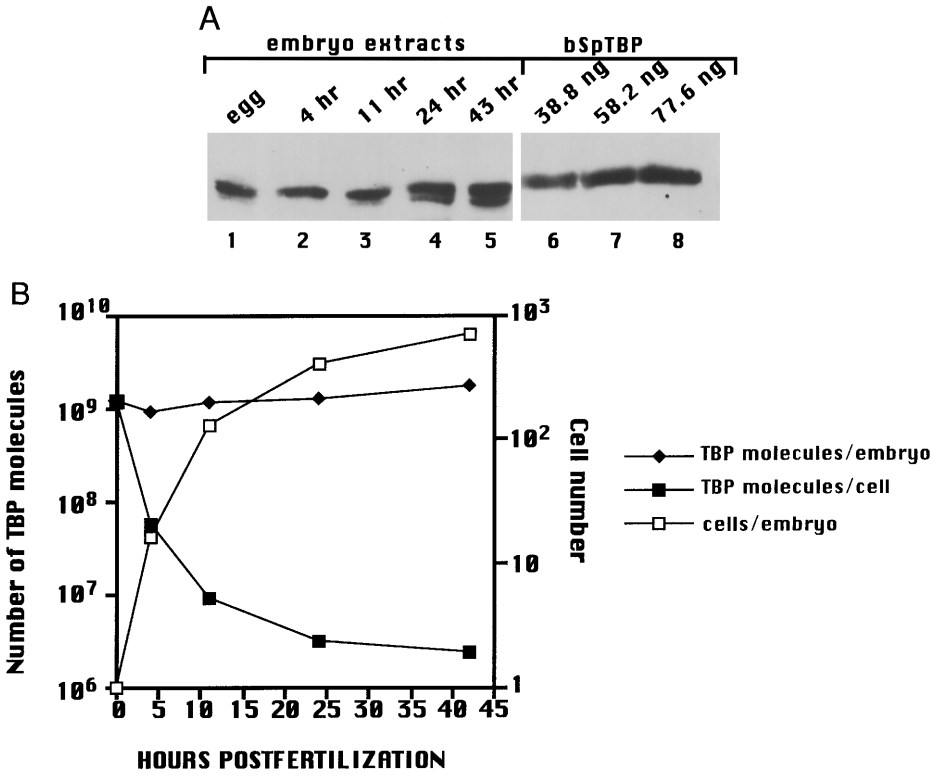


**FIG. 4.** (A) Developmental Western blot of extracts prepared from whole embryo lysates and recombinant SpTBP. 733 embryo equivalents were loaded in lane 1, egg; lane 2, 4 h; lane 3, 11 h; lane 4, 24 h; lane 5, 43 h; lanes 6, 7, and 8, 38.8, 58.2, and 77.6 ng of recombinant SpTBP, respectively. (B) Graph of data for hours postfertilization vs TBP protein molecules/embryo (closed diamond). Values for number of molecules/cell are shown in Table 2, TBP protein molecules/cell (closed square) and cells/embryo (open square); Egg, 1 cell; 4 h, 16 cells; 11 h, 128 cells; 24 h, 400 cells; 43 h, 700 cells. *Y* axes are plotted on a log scale.

since only in some of the largest (most mature) oocytes does the density of label decrease.

## DISCUSSION

The embryos of many animals rely on rapid development to the larval stage to enhance the chance of survival and these embryos rely on maternal pools of mRNA and protein resources. For example, sea urchins and frogs have sufficient ribosomes and mRNA to develop to blastula stages in the absence of new transcription (reviewed in Davidson, 1986). In these animals, oocytes must accumulate not only yolk, other energy reserves, and the machinery required for fertilization, but also many other molecules important for the development and physiology of the embryo. Here we show that oocytes stockpile nearly all the TBP necessary for embryogenesis. Since TBP is required for the transcription of most nuclear genes and is abundant in oocytes that are actively transcribing many genes using all three RNA polymerases, it is not surprising that the oocyte has high levels of this mRNA. What is surprising, however, is that the late oocyte destroys the mRNA so completely prior to fertilization. This pattern is very similar to what has been reported for the mRNAs encoding the corticle granule protein, which are extremely abundant in oocytes and then effectively turned over during oocyte maturation (Laidlaw and Wessel, 1994). With the low but constant levels of TBP mRNA present throughout embryogenesis, the TBP protein accumulated in the mature egg must be very stable.

### *Sequence Comparisons of SpTBP*

The predicted amino acid sequence of the *S. purpuratus* TBP is 91% identical to the human protein within the conserved 180 amino acids of the C-terminal domain. More than half of the differences are conservative changes that do not alter the charge of the protein (data not shown). A GenBank Blastp search of the protein database indicated that SpTBP (conserved domain) was most related to the TBP protein of the moth, *Spodoptera frugiperda* (Rassmussen and Rohrman, 1994). Analysis of the N-terminus of SpTBP revealed no significant homology with any other proteins, including TBP from other phyla.

**TABLE 2**
SpTBP Protein Molecules in the Embryo

| Stage | Amount of protein per embryo (ng)[a] | Amount of protein per cell (ng)[b] | No. of Sp TBP molecules per cell[c] |
|---|---|---|---|
| Egg | $5.71 \times 10^{-2}$ ($\pm 3.57 \times 10^{-2}$) | $5.71 \times 10^{-2}$ ($\pm 3.57 \times 10^{-2}$) | $1.19 \times 10^{9}$ ($\pm 7.40 \times 10^{8}$) |
| 4 h | $4.33 \times 10^{-2}$ ($\pm 5.50 \times 10^{-3}$) | $2.73 \times 10^{-3}$ ($\pm 3.25 \times 10^{-4}$) | $5.66 \times 10^{7}$ ($\pm 6.75 \times 10^{6}$) |
| 1 h | $5.47 \times 10^{-2}$ ($\pm 2.47 \times 10^{-2}$) | $4.25 \times 10^{-4}$ ($\pm 1.95 \times 10^{-4}$) | $8.79 \times 10^{7}$ ($\pm 4.02 \times 10^{6}$) |
| 24 h | $5.89 \times 10^{-2}$ ($\pm 1.99 \times 10^{-2}$) | $1.47 \times 10^{-4}$ ($\pm 4.98 \times 10^{-5}$) | $3.05 \times 10^{6}$ ($\pm 1.03 \times 10^{6}$) |
| 43 h | $8.15 \times 10^{-2}$ ($\pm 3.65 \times 10^{-2}$) | $1.19 \times 10^{-4}$ ($\pm 4.97 \times 10^{-5}$) | $2.39 \times 10^{6}$ ($\pm 1.13 \times 10^{6}$) |

[a] The amount of protein in ng/embryo is determined from the quantitative Western blot as shown in Fig. 4A, where known quantities of recombinant SpTBP within a linear range are used to determine the concentration of protein from a known quantity of embryos. The values presented are averaged from two experiments using two separate batches of recombinant SpTBP.

[b] The amount of protein from column a divided by the quantity of cells in the embryo; egg, 1 cell; 4 h, 16 cells; 11 h, 128 cells; 24 h, 400 cells; and 43 h, 700 cells.

[c] The number of TBP molecules per cell = concentration of SpTBP per cell (g) divided by the molecular wt of SpTBP (kDa = 29,000g/mol) multiplied by $N_A$ (Avogadro's No.).

The amino acid sequences of the TBPs from the two sea urchin species we examined are compared in Fig. 1. There is 96% amino acid identity between the two species over the entire molecule. The N-terminal domains of the two proteins have been extremely well conserved (89% identity) over the 40 million years that these species last shared a common ancestor (Smith, 1988). The most notable difference between them is a 5-amino-acid insertion containing a glutamine triplet near the N-terminus in *L. variegatus*. The extensive homology between these two TBPs most likely signifies the conservation of the transcriptional processes in the organism even between these widely diverged species.

## *Significance of the Long 5′-UTR of SpTBP mRNA*

Sequencing of the *SpTBP* cDNA revealed a large 5′-untranslated region of approximately 466 nt in length. Long untranslated regions are not a conserved feature of TBP mRNAs, but are common to many sea urchin mRNAs (W.M., unpublished observation). In yeast there are 190 nt upstream of the initiator Met, but in human and *Drosophila* there are less than 90 nt (Horikoshi *et al.*, 1989; Kao *et al.*, 1990; Muhich *et al.*, 1990). Long sequence stretches in the 5′-UTR of messenger RNAs have been associated with posttranscriptional control of mRNAs by competition of upstream Met codons (Hinnebusch, 1988). This could also be the case for *SpTBP*, since there are two short ORFs present in the 466 nt of the untranslated region. The relevance of these sequences *in vivo* are unknown, but they could pose a potential translational block or provide a binding site for a factor involved in the sequestration or stability of *TBP* transcripts (reviewed in Hentze, 1995).

## *TBP mRNA and Protein during Development*

*SpTBP* transcripts are equally abundant in the egg and in the embryo. The tracer excess titration experiments indicated that the highest levels per cell, 1250 transcripts, occur in the mature egg (Table 1), and that the number of transcripts per embryo remain relatively constant. The presence of TBP mRNA in the egg is not surprising because the sea urchin oocyte produces a store of maternally encoded transcripts and 90% of embryo mRNA sequence of all classes are represented in the mature egg (reviewed in Davidson, 1986). The prevalence of *TBP* transcripts in the egg is in agreement with measurements reported for the majority of maternal sequences (Hough-Evans *et al.*, 1977).

RNase protection analysis also indicated that *TBP* transcript levels in adult tissues were highest in ovary and testis (data not shown). These results are consistent with a study of *TBP* mRNA accumulation in rodent tissues (Schmidt and Schibler, 1995). In rodents, the adult spleen and liver contained between 0.7 and 2.3 molecules of mRNA per haploid genome-equivalent, whereas the testis contained 80–200 molecules. Higher levels of both *TBP* mRNA and protein also correlated with increased expression of the other components of the RNA Pol II machinery. These results offer an explanation of how early spermatids accumulate the mRNA necessary for the final stage of spermatogenesis. The situation may be analogous to the store of mRNAs accumulated in the oocytes of sea urchins in preparation for the demands of early development. This scenario is particularly evident with respect to the transcription of the ribosomal *RNA* genes. Measurements taken from the sea urchin, *Tripneustes gratilla*, indicate that the rate of rRNA synthesis, which is operating at maximum efficiency, is 28-fold higher in the growing oocyte than it is during embryogenesis (Griffith and Humphreys, 1979; Griffith *et al.*, 1981). Therefore, most of the rRNA utilized during development, $>10^8$ ribosome equivalents, is synthesized during oogenesis (Kleene and Humphreys 1977). It should be noted that sea urchin oocytes lack lampbrush chromosomes so that the transcriptional apparatus does not appear to be loaded with nascent transcripts, which may be

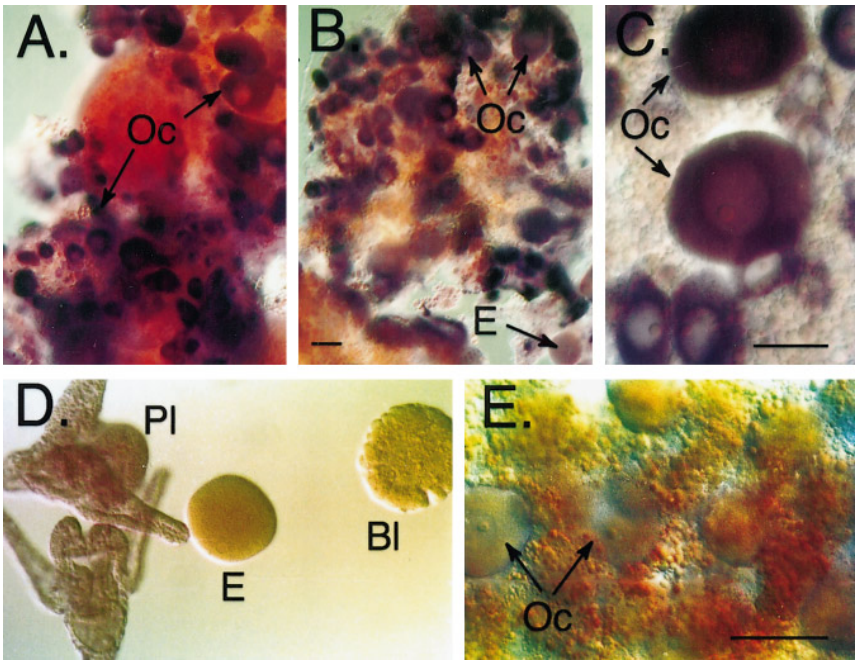


**FIG. 5.** TBP mRNA localization *in situ:* (A) A piece of an ovary from *S. purpuratus* hybridized with antisense TBP probe showing intense labeling in oocytes of all stages, though somewhat decreased intensity in the largest of oocytes (top right arrow). Somatic cells of the ovary (surrounding all oocytes) and mature eggs (below the plane of focus) have no detectable TBP mRNA. (B) A piece of an ovary from *L. variegatus* hybridized to an antisense TBP probe. As in A, oocytes of all developmental stages contain significant levels of TBP hybridization, but mature eggs do not. (C) High magnification of oocytes within an ovary of *L. variegatus* showing oocyte specificity of TBP mRNA accumulation. Note the large germinal vesicle and nucleolus, characteristic of these cells. (D) Egg and embryos hybridized to TBP antisense probe showing no significant mRNA accumulation relative to oocytes (as in A–C). (E) Oocytes within an ovary of *L. variegatus* hybridized to sense strand probe show no significant label. Oc, oocytes; E, mature egg; Bl, blastula; Pl, pluteus; scale bar in B for A and B, 100 $\mu$m, scale bar in C, 50 $\mu$m, scale bar in E for D and E, 100 $\mu$m.

indicative of a low rate of RNA Pol II transcription (reviewed in Davidson, 1986). Consequently, the sea urchin uses the period of oocyte growth and maturation, which can take several months, to stockpile sufficient *TBP* mRNA to allow for the synthesis of enough TBP protein to supply the needs of rapidly dividing embryonic cells. Our steady-state measurements at several developmental stages clearly demonstrate that there is no significant accumulation of TBP mRNA that could account for a significant fraction of the large pool of TBP protein. Therefore, our results strongly suggest that the TBP in the embryo is largely maternally derived. In addition the maternal stockpiling of common factors points out the possibility that embryonic libraries may not be the best source of material to clone cDNAs encoding a variety of embryonic proteins. In many cases, oocyte derived libraries might be more appropriate to clone cDNAs encoding proteins whose mRNAs are destroyed prior to egg maturation.

Quantitative Western blots were used to determine the number of TBP molecules present at different developmental stages for comparison with the estimates of transcript prevalence. The $10^9$ molecules provided by the egg must be synthesized during oogenesis and must also supply the embryo with essentially all of its TBP protein, since the number of TBP transcripts present/cell during embryogenesis at the measured translation rate of 1.8 codons/s (Goustin and Wilt, 1981) could not possibly provide the amount of TBP protein present, $2–3 \times 10^6$ molecules/cell. Figure 4B illustrates that the decrease in number of TBP molecules/cell over time correlates extremely well with the increase in cell number, but that the total amount of TBP protein remains relatively constant throughout development.

The sea urchin embryo offers the advantage of being able to make quantitative measurements in a developmental system on a per cell basis. It was reported that there are approximately 8500 different RNA species per cell represented on the polysomes of gastrula-stage embryos (reviewed in Davidson, 1986). The ≈200 *rDNA* genes (diploid) are transcribed at extremely low rates in gastrula embryos by RNA polymerase I (reviewed by Davidson, 1986) and the assorted RNA polymerase III transcription units constitute a relatively small fraction of the total number of genes utilizing TBP. The number of TBP molecules at that stage as determined by quantitative Western blots is about 2 million/cell. Therefore, from these measurements the ratio of TBP molecules for each active gene is on the order of

about 100:1 in gastrula-stage embryos. This result raises interesting questions about the storage and distribution of TBP protein during early development. Is all of this TBP protein nuclear and is it distributed into multiprotein complexes with TAFs? Preliminary results (Fei, 1993) suggest that in addition to TBP contained within high-molecular-weight complexes, there is a large pool of TBP that behaves as a small complex on Superose column chromatography perhaps as a TBP dimer (Taggart and Pugh, 1996). These results should also allow us to address the question of how the embryo assembles different TBP containing complexes to supply the different needs for the three RNA polymerase gene products. We conclude that whatever the location and nature of the large store of TBP protein present in the egg, it provides the embryo with essentially all the TBP it needs during development.

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