INTRODUCTION

One goal of developmental biology is to identify mechanisms that regulate the onset of zygotic gene transcription and initiate the developmental program. In the mouse, DNA transcription stops when oocytes undergo meiotic maturation to form eggs and does not begin again until the late 1-cell stage. However, transcription does not appear to be coupled to translation until the 2-cell stage (Nothias et al., 1996) where synthesis of zygotic proteins begins (Latham et al., 1991). This delay in expression of zygotic genes is regulated by a time-dependent mechanism about which little is known (Nothias et al., 1995). One component may be the appearance of an enhancer specific cofactor in 2-cell embryos that is absent in oocytes and 1-cell embryos (Majumder et al., 1997). Another may be the appearance of specific transcription factors that regulate the expression of other zygotic genes. Recent studies revealed that protein synthesis is required for embryonic genome activation, consistent with a need to synthesize essential transcription factors lacking in the oocyte (Wang and Latham, 1997). Only a few transcription factors have so far been identified and extensively studied in mouse oocytes and preimplantation embryos (Schultz, 1993; Majumder and DePamphilis, 1995; Nothias et al., 1995).

One approach to investigating the regulation of gene expression at the beginning of mammalian development has been to inject plasmid encoded reporter genes into the nuclei of mouse oocytes and preimplantation embryos (Majumder et al., 1993; Majumder and DePamphilis, 1995; Nothias et al., 1995). The injected DNA can replicate or express an encoded reporter gene only when specific cis-acting regulatory sequences are present and they are provided with their cognate trans-acting proteins. Moreover, replication and expression of plasmid encoded genes occur only when the embryonic genome executes the same functions during its normal developmental program. These studies indicate that the response of injected plasmids reflects physiological controls that govern expression of cellular genes, thereby revealing the embryo’s capacity for DNA replication and gene expression, as well as its requirements for specific regulatory elements.

This microinjection strategy has been used to search for transcription enhancers that function in cleavage stage mouse...
embryos and during the initial activation of zygotic gene expression (Martinez-Salas et al., 1989; Melin et al., 1993). A survey of polyomavirus mutants that replicate in undifferentiated mouse embryonal carcinoma or embryonic stem cells revealed a single point mutation common to enhancers that also functioned in cleavage stage embryos. This mutation created a binding site for Transcription Enhancer Factor-1 (TEF-1; Xiao et al., 1991). This DNA binding site can stimulate transcription when present in either promoters (Farrance et al., 1992) or enhancers (Melin et al., 1993). The most effective enhancers consist of two tandem TEF-1 DNA binding sites separated by about 53 bp. Stimulation by the enhancer from polyomavirus F101, for example, ranges from 20 to >300-fold, depending on the promoter tested and the amount of DNA injected (Majumder et al., 1993). Since the activity of the F101 enhancer in early embryos can be duplicated by engineering a series of tandem TEF-1 DNA binding sites, a transcription factor must be present at the onset of zygotic gene expression (ZGE) that can utilize this DNA binding site. However, the obvious candidate for this role, mouse TEF-1, does not appear suitable, because embryos homozygous for a disruption in the TEF-1 gene survive past the preimplantation stage (Chen et al., 1994). Therefore, either TEF-1 activity is present in preimplantation embryos but its function is not required, or another related gene can mediate expression of endogenous target genes.

The TEF-1 transcription factor contains a highly conserved 72 amino acid DNA binding domain (TEA domain) that is found in transcription factors from widely divergent species (Burglin, 1991; Blatt and DePamphilis, 1993). TEF-1 mRNA has been detected in many adult mouse tissues (Blatt and DePamphilis, 1993), and TEF-1 protein has been associated with the regulation of a variety of cell-type specific genes (Yockey et al., 1996; ref. therein). More recently, three additional mouse genes have been identified that share the same TEA domain as mouse TEF-1 and bind to the TEF-1 DNA sequence motif in vitro (see Results). Therefore, in order to distinguish this family of genes from unrelated factors that fortuitously bear the same acronym, and to recognize the single common denominator among these proteins, we refer to this gene family as murine TEA Domain genes (mTEAD-1 to 4).

Here we report that mTEAD transcription factor activity was not detected in oocytes, but first appeared at the 2-cell stage in mouse development, concomitant with the onset of zygotic gene expression. mTEAD-2 was the principal member of the mTEAD transcription factor gene family that was expressed from cleavage stage preimplantation embryos up to day-7 embryos. Thus, mTEAD-2 is one of the first transcription factors produced at the beginning of mouse development where its presence most likely accounts for the TEAD-dependent enhancer activity observed at the onset of zygotic gene expression.

MATERIALS AND METHODS

Embryo and oocyte isolation, culture, and microinjection

All procedures were performed with CD-1 mice (Charles River) and have been described previously (DePamphilis et al., 1988; Miranda et al., 1993; Hogan et al., 1994). Germinal vesicle-stage oocytes isolated from 14-day-old mice were used for oocyte microinjection experiments. The day the vaginal plug was observed was designated as day 1. For quantitative RT-PCR analyses, oocytes and embryos from B6D2 F1 mice were used as described by Rambhatla et al. (1995).

Plasmids

Plasmids pF101tkluc, pS6luc have been described by Martinez-Salas et al. (1989); Majumder et al. (1993). pG73luc consisted of four tandem copies of the GTIIC site 30-mer containing the TEF-1 DNA binding sites found in the polyomavirus F101 enhancer (Melin et al., 1993). This sequence was constructed by ligating together two oligomers. The first oligomer contained a 5’ restriction site for BglII and a 3’ AGCC overhang, whereas the second oligomer contained a 5’ TCGG overhang and a 3’ restriction site for EcoRI. After annealing and phosphorylating the complementary strands, the two fragments were allowed to anneal and ligated into the EcoRI/BglII sites of p2025 upstream and proximal to a TATA box (Majumder et al., 1993). A fragment containing the 4 TEF-1 sites and a TATA box was amplified by PCR using SP6 and T7 primers with a 5’-SacI restriction site. Since this fragment contained a SacI restriction site next to the SP6 primer, the fragment was digested with SacI and ligated into the SacI site of pluc.

cDNA library screening

A PCC4 cDNA library (Stratagene; Blatt and DePamphilis, 1993) was screened under low stringency conditions (Giguere et al., 1987), using a mouse TEA domain probe from mTEAD-1. This probe (220 nucleotides) was synthesized by PCR using primers A and B (Table 1) which flank the TEA domain of mTEAD-1 cDNA (Blatt and DePamphilis, 1993). Probe was hybridized with nitrocellulose filters of polyoma mutants that replicate in undifferentiated mouse embryonal carcinoma or embryonic stem cells (Burklin, 1991; Blatt and DePamphilis, 1993). Probe was hybridized with nitrocellulose filters of 6xSSPE, 5x Denhardt’s, 0.1% sodium dodecyl sulfate (SDS), 1 mM sodium pyrophosphate, 100 µg/ml sheared salmon sperm DNA, and 25% v/v formaldehyde at 42°C overnight. The filters were washed three times in 2x SSC, 0.1% SDS for 20 minutes at 55°C. The positive phagemids were converted to pBlueScript plasmids (Stratagene). These plasmids were sequenced from both ends by DyeDexoxy Sequencing (Applied Biosystems). The 5’ end of mTEAD-2 and the 3’ end of mTEAD-3 were isolated by rapid amplification of cDNA ends (5’/3’ RACE) method (Frohman et al., 1988) from heart and lung tissues, respectively. The cDNA sequences for both mTEAD-2 and mTEAD-3 were submitted for sequence accession.

Table 1. Oligonucleotides used as primers and probes

<table>
<thead>
<tr>
<th>Name</th>
<th>5’-Primer-3’</th>
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<tbody>
<tr>
<td>A</td>
<td>AAACGACCGCGAGGGGCTTGGAGT</td>
</tr>
<tr>
<td>B</td>
<td>ATACGAGATTTCCTCCCTG</td>
</tr>
<tr>
<td>C</td>
<td>GGGGTATCCCCGAGAAGAATCTCGTGA</td>
</tr>
<tr>
<td>D</td>
<td>ACCCGTCCAGTCCTCGACAGCTTGAAATCGAGATG</td>
</tr>
<tr>
<td>E</td>
<td>GGGGTATCCCCGCGAGAGAATCGAGAGAAGA</td>
</tr>
<tr>
<td>F</td>
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<td>G</td>
<td>GGGGTACCGGAAGAAGGTTGGAGGAATACCCAG</td>
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<td>H</td>
<td>ACGCGTCCGACCTAGTCTTGGCAAAGCTTGAGAC</td>
</tr>
<tr>
<td>I</td>
<td>29-AGAGCCCTGCGGAAACAATCGAGAAA-52</td>
</tr>
<tr>
<td>J</td>
<td>498-CTAGTGGCTATCGCCCTTCTGCCTCAGA-477</td>
</tr>
<tr>
<td>K</td>
<td>190-TGGCTGTCCTTCTGCTTACATC-213</td>
</tr>
<tr>
<td>L</td>
<td>584-CATCCAGCTGGTAACACATCCTAGG-561</td>
</tr>
<tr>
<td>M</td>
<td>77-TGGACAAGGGTCCTGGACAAGATG-99</td>
</tr>
<tr>
<td>N</td>
<td>491-AACCTTGGAGGAGGAGGAGAGACACAGAA-468</td>
</tr>
<tr>
<td>O</td>
<td>ATTACCTTCACGAGTGGGACT</td>
</tr>
<tr>
<td>P</td>
<td>CGTGGCAAGCTCCTGCTGCCAA</td>
</tr>
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<td>Q</td>
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<tr>
<td>T</td>
<td>ATCCTCGGCGGTTCCTTGAGCGCAA</td>
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Numbers refer to the first and last nucleotide in the primer relative to the gene sequences [GenBank accession numbers L06865 (mTEAD-1: I and J), Y10026 (mTEAD-2: K and L), Y10027 (mTEAD-3: L and M), L26343 (mTEAD-4/TEFR1: N and M)].
-3 have been submitted to GenBank (accession numbers, Y10026 and Y10027, respectively).

**Northern blotting-hybridization assays**

mTEAD-1 probe was identical to the TEA domain probe used for library screening. For mTEAD-2 and 3 probes (approx. 1.8 kb and approx. 2.0 kb, respectively), both cDNAs were released from the pBluescript vector by digesting with EcoRI. Human β-actin cDNA was obtained from Clontech. The probes were radiolabeled with [α-32P]dCTP using a Random Primed DNA labeling kit (Boehringer Mannheim). Hybridizations were carried out at 42°C in solution containing 5x SSPE, 10× Denhardt’s solution, 1 mM sodium pyrophosphate, 50% formamide, 100 µg/ml sheared, denatured salmon sperm DNA, and 2% SDS. The blots were washed at high stringency (50°C, 0.1x SSC), exposed for autoradiography, stripped by-incubating the blots in boiling 1x SSPE, 0.5% SDS and rehybridized.

**In situ hybridization**

In situ hybridizations with digoxigenin-labeled probes were carried out on 10 µm cryostat sections as described previously (DePamphilis et al., 1988), frozen in an ethanol/dry ice bath, and stored at -80°C. After addition of 10 µg tRNA (Boehringer Mannheim), 200 µl of denaturing solution was used to lyse cells and extract RNA using RNAgents Total RNA Isolation System (Promega). RNA was resuspended in 10 µl of DEPC H2O and 1 µl was used to quantify RNA recovery by spectrophotometry. Reverse transcription was performed with the remaining RNA using random primer, RTase inhibitor, and Moloney murine leukemia virus reverse transcriptase (Perkin Elmer). 50 ng of total RNA from mouse lung, F9 and MPC11 cell lines were used as controls. Aliquots (5 µl) of the same RT reaction (20 µl) were used for the four separate PCR reactions. Specific primers used for mTEAD-1, 2, 3, and 4 were I and J, K and L, M and N, and O and P, respectively (Table 1). These primers did not generate a specific product of the correct size when 500 ng of F9 genomic DNA were used as a template, presumably due to presence of introns between the primers. Therefore, any contaminating genomic DNA would not contribute to the specific amplified products observed in the assay. Except for primer K, all primers flank the TEA DNA binding domain. Although primer K is within the TEA domain binding region, it, along with primer L, did not produce specific products from plasmids containing mTEAD-1, 3, and 4 cDNAs. The primers for TEAD-4 were designed so that two alternatively spliced RNAs of different sizes could be amplified simultaneously. PCR reactions (100 µl) were carried out with Taq DNA polymerase (Perkin Elmer) for 40 cycles. PCR products (16 µl) were fractionated by 2% agarose electrophoresis, stained with ethidium bromide, denatured and transferred to Zeta Probe GT membrane (Bio-Rad). The membrane was hybridized with 5'-end labeled 32P-oligonucleotides internal to the amicon. Oligonucleotide probes for mTEAD-1, -2, -3, -4 were Q, R, S, and T, respectively.

Quantitative RT-PCR was done as described by Rambhatla et al. (1995). The mTEAD-2 probe used for hybridization was a 650 bp XhoI/DraI DNA fragment. The probe used for mTEAD-3 was an approx. 220 bp EcoRI fragment from pCRII vector containing the 3'-end of TEAD-3 cDNA obtained by 3' RACE (see above). Both of these probes contained putative polyadenylation signals. The probe used for TEAD-1 was approx. 250 bp HindIII-digested fragment of mTEAD-1 (TEF-1) clone 13-1 (Blatt and DePamphilis, 1993).

**RESULTS**

**mTEAD transcription factor activity first appeared when zygotic gene expression began**

To determine when functional mTEAD transcription factor is produced at the beginning of mouse development, a plasmid encoded reporter gene whose activity depended on mTEAD was microinjected into the nuclei of oocytes, 1-cell embryos, or 2-cell embryos under conditions that produce the greatest amount of transient gene expression (Miranda et al., 1993). To confirm that mTEAD-dependent enhancer activity was present in cleavage-stage embryos, two plasmids (Melin et al., 1993) were microinjected individually into 2-cell embryos. One plasmid contained a mTEAD-dependent enhancer consisting of four tandem copies of the mTEAD binding site found in polyomavirus F9-1 upstream of the polyomavirus early gene promoter linked to the firefly luciferase reporter gene [p[GT30]x-Pyluc]. A second plasmid [p[wt30]x-Pyluc] was identical to the first except for a single base pair change that eliminates binding of mTEAD-1, 2 and 3 proteins in vitro (data not shown; Davidson et al., 1988). This was consistent with the effects of similar mutations in the functionally equivalent SV40 GT1IC site on binding of mTEAD-1, 2, 3 and 4 proteins (Xiao et al., 1991; Yasunami et al., 1995, 1996; Jacquemin et al., 1996; Yasunami et al., 1996). The results (Fig. 1A) showed that a mTEAD-dependent enhancer could strongly (>500-fold) stimulate promoter activity in early cleavage embryos.

The ability of a mTEAD-dependent enhancer to stimulate promoter activity could not be used to assay mTEAD transcription factor activity in oocytes or 1-cell embryos, because enhancers cannot be utilized in mouse cells until formation of a 2-cell embryo (Majumder et al., 1997). However, the same transcription factors can be assayed prior to formation of a 2-cell embryo by placing their sequence-specific DNA binding sites proximal, rather than distal, to the mRNA start site (Majumder et al., 1993, 1997). Previous studies (Majumder et al., 1993) showed that promoter activity depended strictly on the presence of both specific transcription factors and their cognate DNA binding sites. These genes are most active when injected into the paternal pronucleus of S-phase arrested 1-cell embryos where promoters are not repressed (Martinez-Salas et al., 1989; Wiekowski et al., 1991, 1993; Majumder et al., 1993, 1997), and least active in oocytes and 2-cell to 4-cell embryos where chromatin structure mediates repression of promoter activity (Henery et al., 1995; Wiekowski et al., 1997). Furthermore, in fertilized mouse eggs, the onset of transcription and translation of both the zygotic genome as well as injected plasmids is delayed by a time dependent mechanism until the 2-cell stage in development (Nothias et al., 1995, 1996). Thus, even when morphological development stops as 1-cell embryos are arrested at the beginning of S-phase, ZGE still begins at the normal time (2-cell stage) after fertilization. Therefore, the activity of promoters injected into the paternal pronucleus of S-phase arrested 1-cell embryos reflected the amount of
Sp1-dependent promoters (Fig. 1D). However, by the time 2-cell embryos (chronologically at 4/8-cell stage), since Sp1 and mTEAD transcription factor activities were already present, because ZGE, the paternal pronucleus of 1-cell embryos, and one of the two zygotic nuclei of 2-cell embryos as described by DePamphilis et al. (1988). Oocytes were cultured in the presence of dibutyryl cAMP to prevent germinal vesicle breakdown and cessation of transcription. Early 1-cell embryos were cultured in the presence of aphidicolin (a specific inhibitor of replicative DNA polymerases) in order to arrest their development as they entered S-phase and prevent repression of transcription during formation of a 2-cell embryo. Two-cell embryos were isolated from pregnant females, and then injected and cultured under the same conditions as 1-cell embryos. Since most of these embryos had already completed S-phase, they soon cleaved into 4-cell embryos where they arrested their development as they entered S-phase. Plasmid DNA concentrations were 0.5 mg/ml (oocytes), and 0.15 mg/ml (1-cell and 2-cell embryos). Injected embryos were assayed for luciferase activity at 24 to 44 hours post-injection. Each data point is the mean from 44 to 167 injected oocytes or embryos. These levels of activity have been corrected for background levels produced by a promoterless plasmid (pluc). Error bars indicate the standard error of the mean. The ratio of activities produced by the indicated plasmids are shown in D.

These data demonstrated that mTEAD activity first appears at the onset of ZGE. In S-phase arrested 1-cell embryos, ZGE begins about 12 hours after injection, concurrent with the beginning of a shutdown of maternal mRNA translation (Wiekowski et al., 1991; Nothias et al., 1995). Thus, less time was available for assembly of an active mTEAD-dependent promoter than for an active Sp1-dependent promoter, because Sp1 protein was inherited from the oocyte and therefore was present when the plasmid gene was injected. However, when the same plasmids were injected into late 2-cell embryos isolated from pregnant females, both mTEAD and Sp1 transcription factor activities were already present, because ZGE begins immediately after 1-cell embryos cleave into 2-cell embryos (Nothias et al., 1996), approximately 12 hours prior to injection. The data obtained from both mTEAD-dependent enhancer/promoter constructs show that mTEAD activity first appears at a time chronologically equivalent to the 2-cell stage, as reflected in the S-phase arrested embryos, and then continues to be expressed into the 4-cell stage.

**Only mTEAD-2 RNA was detected during preimplantation development**

The above results demonstrated that mTEAD transcription factor activity first appeared at the 2-cell stage in mouse development. However, mouse embryos disrupted in the mTEAD-1 cognate transcription factor activity present at the onset of ZGE, in the absence of chromatin mediated repression.

To address whether mTEAD activity can be detected prior to the formation of 2-cell embryos, a mTEAD-dependent promoter consisting of four tandem mTEAD binding sites was placed 10 bp upstream of a TATA box (pGT4 Tluc). For comparison, two Sp1-dependent promoters were also examined: ptkluc contained the herpes simplex virus thymidine kinase promoter, consisting of two Sp1 sites, a CTF site, and a TATA box; pS6Tluc contained a tandem series of six Sp1 DNA binding sites and a TATA box. As expected, oocytes and 1-cell embryos were capable of transcribing control reporter genes driven by Sp1-dependent promoters (Fig. 1C) since both Sp1 and TATA binding proteins have been shown to be present in oocytes, fertilized eggs and early cleavage stage embryos (Majumder et al., 1993; Worrad et al., 1994).

In contrast, mTEAD-dependent promoter (pGT4Tluc; Fig. 1B) was inactive in oocytes and became active in S-phase arrested 1-cell embryos (chronologically at 2-cell stage) and in 2/4-cell embryos (chronologically at 4/8-cell stage). Since oocytes were capable of utilizing the Sp1-dependent promoters (Fig. 1C) but not the mTEAD-dependent promoter (Fig. 1B), mTEAD transcription factor activity must be absent in oocytes. mTEAD-dependent promoter activity was detected in S-phase arrested 1-cell embryos but was only about 10% as active as Sp1-dependent promoters (Fig. 1D). However, by the time 2-
(TEF-1) locus can develop past the preimplantation stage (Chen et al., 1994), suggesting that another gene, related to mTEAD-1, mediates the mTEAD-dependent enhancer/promoter function in preimplantation embryos. Since this putative mTEAD-1 like protein must be able to bind to the mTEAD DNA binding sequence, it presumably contains a TEA DNA binding domain. Therefore, we screened an embryonic carcinoma cell line with the TEA DNA binding domain probe from mTEAD-1 under low stringency conditions to identify additional mTEAD genes. In this way, mTEAD-2 and 3 cDNAs were isolated (GenBank accession numbers, Y10026 and Y10027, respectively). mTEAD-2 is identical to ETF (Yasunami et al., 1995) and TEF-4 (Jacquemin et al., 1996), while mTEAD-3 is identical to ETFR-1 (Yasunami et al., 1996). A fourth member of this mouse gene family (designated mTEAD-4) has recently been isolated (TEFR-1; Yockey et al., 1996). All four proteins have nearly identical DNA binding domains and can bind specifically to the SV40 GTIIC and polyomavirus F9-1 sequences (see above). Southern analyses of genomic DNA restriction endonuclease fragments under low stringency conditions were consistent with the presence of only four mTEAD genes (data not shown).

To determine whether mTEAD genes were expressed as maternal mRNAs, mTEAD-1, 2, and 3 RNAs were assayed in oocytes using in situ hybridization. Frozen tissue sections through adult mouse ovaries were hybridized with digoxigenin-labeled RNA probes complementary to the C-terminal portion of the mTEAD-1, 2, and 3 cDNA. A specific mTEAD mRNA was considered present in cells only if the antisense probe produced a signal significantly greater than that produced by the sense probe. These analyses showed that mTEAD-2 RNA was not detected in oocytes using an antisense probe, even though the signal was clearly present in the granulosa cells within the follicles (Fig. 2A,C). Neither the antisense probes for mTEAD-1 and 3, nor the three sense probes hybridized with any of the cells in the ovary (Fig. 2B,D; data not shown). mTEAD-4 RNA was not assayed in this and subsequent studies, because it was not detected in oocytes or preimplantation embryos by RT-PCR (Fig. 3). Therefore, mTEAD-1, 2 and 3 were not expressed in oocytes at levels detectable by in situ hybridization.

To determine which mTEAD gene might be responsible for TEAD activity in preimplantation embryos, a more sensitive assay was employed using reverse transcriptase coupled with the polymerase chain reaction (RT-PCR). mTEAD RNAs were assayed in oocytes, 1-cell embryos, 2-cell embryos, morula and blastocysts. Mouse lung and teratocarcinoma F9 cells provided positive controls, while mouse lymphoma cell line MPC11, in which TEAD-1 RNA has been reported absent (Xiao et al., 1991), provided a negative control. cDNA was first synthesized using reverse transcriptase (RT) and random primers. Aliquots of each RT reaction were then amplified using primers specific for one of the four mTEAD genes (see Materials and methods). The resulting amplified DNA sequences were visualized by ethidium bromide staining and by Southern blotting hybridization using probes internal to the two PCR primers (Fig. 3).

All four mTEAD RNAs were detected in lung and F9 cells, while only low levels of mTEAD-3 and 4, and no mTEAD-1 or 2 RNA was detected in MPC11 cells. In each case, amplified DNA products of the expected sizes were produced, and these products were present in some cells but not in others, confirming the specificity and the validity of the RT-PCR assay. Only transcripts from mTEAD-1, 2 and 3 were detected in oocytes. Following fertilization, mTEAD-1 and 3 RNA disappeared, whereas the amount of mTEAD-2 RNA steadily increased during preimplantation development, reaching levels in morula and blastocysts great enough to be detected by ethidium bromide staining. These results suggest that mTEAD-2 is the principal mTEAD gene family member expressed during preimplantation development.

To provide a more quantitative assessment of mTEAD-2 expression, the number of copies of mTEAD-2 mRNA was estimated using a RT-PCR assay based on uniform amplification of the 3′-terminal region of all poly(A)+ mRNAs (Rambhatla et al., 1995). The amplified products could then be quantified by hybridization with 32P-labeled DNA probes specific for each mTEAD gene. The data were expressed as cpm bound per ovum or embryo (Fig. 4A) and used to calculate the number of mRNA copies per ovum or embryo (Fig. 4B; Rambhatla et al., 1995).

Fig. 2. Distribution of mTEAD-2 mRNA in the adult mouse ovary. In situ hybridization was carried out as described in Materials and methods. Adjacent serial sections through the ovary were hybridized with antisense (A,C) or sense (B,D) mTEAD-2 probes. C and D show higher magnifications (6.25×) of one of the follicles from A and B, respectively. No specific staining was detected in adjacent sections when hybridized with either mTEAD-1 or mTEAD-3 specific probes (data not shown). Some follicles (Fc) are indicated in A, whereas the oocyte (Oc), granulosa cells (Gc), and theca cells (Tc) are indicated in C. Bar represents 50 μm.
mTEAD-2 poly(A)+ mRNA was present in oocytes and unfertilized eggs at 4000 to 5000 copies per cell. Following fertilization, the level of mTEAD-2 mRNA decreased until the late 2/4-cell stage and then increased rapidly (Fig. 4C). Since mTEAD-2 mRNA levels in 1-cell and 2-cell embryos were insensitive to α-amanitin (a specific inhibitor of RNA polymerase II), most of this mRNA was inherited from the egg. From the 8-cell to the blastocyst stage, mTEAD-2 mRNA accumulated dramatically, consistent with its expression from zygotic genes. In blastocysts, the level of mTEAD-2 mRNA was about 100,000 copies per embryo, or about 15% the level of β-actin mRNA in blastocysts (Rambhatla et al., 1995). Thus, the level of mTEAD-2 in blastocysts was about 20-fold greater than in oocytes or about 50-fold greater than in 2-cell and 4-cell embryos.

In striking contrast to mTEAD-2 mRNA, the level of mTEAD-3 poly(A)+ mRNA in ova and preimplantation embryos was essentially indistinguishable from background (Fig. 5). A similar result was obtained with mTEAD-1.

However, since the 3′-ends of all mTEAD-1 clones reported do not contain a recognizable polyadenylation signal (Blatt and DePamphilis, 1993; Shimizu et al., 1993), our 3′-probe may not have been close enough to the polyA tail to register in this assay. Nevertheless, taken together, the results of these three independent analyses revealed that mTEAD-2 was selectively expressed during preimplantation development.

Only mTEAD-2 RNA was detected in embryos up to day 7
To determine whether other members of the mTEAD gene
family were expressed later in development, mTEAD-1, 2, and 3 RNAs were assayed by in situ hybridization in day-7 embryos, which were sectioned within the decidua. In situ hybridization of serial sections revealed that both mTEAD-1 and mTEAD-3 were expressed only in the decidual cells; not in the embryo (Fig. 6D-I). Antisense probe for mTEAD-3 stained the anti-mesometrial portion of the decidua, but not the embryo (Fig. 6G,I). mTEAD-1 mRNA was detected throughout the decidua, but not in the embryo (Fig. 6D,F). None of the three sense mTEAD probes stained any portion of either decidua or embryo (Fig. 6B,E,H). In contrast, mTEAD-2 was expressed in the embryo, as well as in the mesometrial portion of the decidua (Fig. 6A-C). The mTEAD-2 antisense probe stained cells uniformly throughout the embryo (Fig. 7), while the mTEAD-2 sense probe failed to stain any of the sections (data not shown). Interestingly, staining appears to be reduced in the extraembryonic regions (Fig. 6C). Thus, only mTEAD-2 RNA was detected in the embryo at day 7 of gestation.

Because previous studies showed that mTEAD-1 gene function is required by around day 10 (Chen et al., 1994), northern blotting hybridization was used to examine mTEAD gene expression at day 9 and 10 of development (Fig. 8). These results revealed that although mTEAD-2 RNA was expressed more abundantly, mTEAD-1 RNA also was expressed at least by day 9. mTEAD-3 RNA also appeared to be expressed at low but detectable levels. Thus, mTEAD-1 RNA appeared concurrent with the requirement for its function during mouse development.

mTEAD-2 was also expressed in some adult tissues

The above results raised the possibility that mTEAD-2 expression is confined to early embryos, while other members of this transcription factor gene family are expressed in later embryos and adult tissues. Therefore, RNA expression patterns of mTEAD genes were examined in adult mouse tissues using northern blotting hybridization analysis of poly(A)⁺ RNA (Fig. 8). Each mTEAD mRNA was identified by hybridization to its cognate probe under conditions in which only the indicated gene mRNA was recognized. In contrast to mTEAD-4 (TEFR-1), whose expression was reported to be restricted to certain tissues (Yockey et al., 1996), mTEAD-1, 2, and 3 genes were ubiquitously expressed in adult tissues, with marked differences existing among their relative levels of expression.

In agreement with previously published results (Blatt and DePamphilis, 1993; Shimizu et al., 1993), mTEAD-1 mRNA was expressed in most mouse tissues, ranging from very high levels in lung, muscle, kidney and heart to low levels in brain, spleen, liver and testes (Fig. 8). However, while all three mTEAD genes were expressed at high levels in lung, and at lower levels in heart and spleen, mTEAD-1 and 3 were expressed strongly in kidney and liver, whereas mTEAD-1 and 2 were preferentially expressed in brain. Furthermore, these and other results show that mTEAD-2 was not confined to embryonic tissues, since it was strongly expressed in testes (Fig. 8) and ovarian follicle cells (Fig. 2). A previous report that mTEAD-2 (ETF) is expressed only in embryonic tissues (Yasunami et al., 1995) may have resulted from their analysis of total RNA instead of poly(A)⁺ mRNA. Thus, while at least one member of the mTEAD gene family was expressed in most, perhaps all, adult mouse tissues, distinct patterns of expression existed for individual family members, suggesting that each of the four genes had a distinct function.

**DISCUSSION**

**TEAD transcription factor activity first appears at the onset of zygotic gene expression**

The most critical event to occur following fertilization is the activation and precise timing of zygotic transcription. This event is essential for viability of the embryo, and its timing must be precisely controlled. Recent studies indicate that the stage-specific production of key transcription factors likely plays a central role in controlling and mediating this genome activation (Wang and Latham, 1997). The experiments presented here identify one of the first transcription factors (mTEAD-2) expressed specifically when zygotic genes are activated in mouse embryos. Enhancers whose activity depends on the presence of one or more mTEAD DNA binding sites (GTIIC sequence; Davidson et al., 1988) are the most powerful transcription enhancers so far identified when plasmid encoded genes are injected into 2-cell mouse embryos (Martinez-Salas et al., 1989; Melin et al., 1993). Similar studies in the absence of aphidicolin have shown that mTEAD-dependent enhancer activity is present at least up to the morula stage in development, and transfection studies have identified this activity in ES cells, suggesting that it is present in the inner cell mass of blastocysts (Martinez-Salas et al., 1989; Melin et al., 1993). Therefore, it is not surprising that a promoter constructed from a tandem series of mTEAD binding sites was as active in

![Fig. 5. Quantitation of mTEAD-1 and mTEAD-3 mRNA from oocytes to blastocysts. The experiment described in Fig. 4 was repeated using 32P-labeled DNA probes specific for either mTEAD-1 or mTEAD-3. These data were then compared with those for mTEAD-2 (shaded area is from Fig. 3C). cpm for mTEAD-1 were 37±4 in oocytes, 58±7 in 2-cell embryos and 67±4 in blastocysts. cpm for mTEAD-3 were 181±14 in oocytes, 89±28 in 2-cell embryos and 149±17 in blastocysts.](image-url)
cleavage stage mouse embryos as promoters that depended on Sp1 (Fig. 1), a ubiquitous transcription factor active in mouse oocytes and early embryos (Majumder et al., 1993; Worrad et al., 1994). The level of luciferase activity in these experiments depends on the number and composition of the transcription factor binding sites present, and on the presence of transcription factors that bind to these sites (Majumder et al., 1993, 1997). The TATA box element does not contribute to the level of promoter activity in cleavage stage embryos (Majumder and DePamphilis, 1994), although a TATA box alone is about 10% as active as a complete promoter (data not shown; Majumder et al., 1993).

What is surprising is that mTEAD activity was reduced in arrested 1-cell embryos and undetectable in oocytes; both of which yield easily detected levels of Sp1-dependent promoter activity (Fig. 1). The level of luciferase produced from paternal pronuclei in S-phase arrested 1-cell embryos reflects the level of promoter activity at the onset of zygotic gene expression at the 2-cell stage in the absence of chromatin mediated repression (Majumder and DePamphilis, 1995; Nothias et al., 1995). Therefore, we conclude that the appearance of TEAD transcription factor occurs concomitant with the onset of zygotic gene expression.

mTEAD-2 appears to be the only member of the TEAD transcription factor family expressed in early mouse embryos

Results presented here support the conclusion that mTEAD-dependent transcription factor activity in preimplantation embryos most likely results from zygotic expression of mTEAD-2 genes. mTEAD transcription factor activity was not detected in oocytes, suggesting that maternally inherited transcription factors do not contribute to mTEAD activity in early cleavage stage embryos. mTEAD-4 RNA was not detected in either oocytes or preimplantation embryos, and the low levels of maternal mTEAD-1 and 3 RNA that were detected in oocytes were essentially eliminated by the 2-cell stage (Figs 3, 4, 5), consistent with previous studies on degradation of mouse maternal mRNAs (Ebert et al., 1984). mTEAD transcription factor activity was detected at low levels relative to constitutive transcription factor Sp1 in arrested 1-cell embryos and to a much higher level in 2-cell/4-cell embryos (Fig. 1D), consistent with expression of a zygotic gene. While it is possible that transient expression of maternally inherited mTEAD-1 or 3 RNA contributes to the initial mTEAD transcription factor activity seen in early cleavage embryos, it seems more likely that this activity results from expression of zygotic mTEAD-2 genes. Only mTEAD-2 mRNA accumulated during subsequent preimplantation development, reaching levels at least 100-fold greater than those for mTEAD-3 RNA. Moreover, mTEAD-2 was the only mTEAD gene expressed at detectable levels in day-7 embryos. The sharp rise in mTEAD-2 mRNA that was observed with 8-cell rather than 2-cell embryos can be explained as follows. First, maternal mTEAD-2 RNA was likely being degraded while zygotic RNA was being produced. Second, assuming that TEAD-2 is expressed uniformly throughout preimplantation embryos (as it is in embryos at day 7; Fig. 7), the amount of mTEAD-2 RNA per embryo will increase exponentially as cell cleavage occurs. Thus, the early increase in mTEAD activity at the 2-cell stage and later, observed with injected reporter genes, can be accounted for by the temporal pattern of mTEAD-2 expression.

Nevertheless, the initial phase of mTEAD transcription factor activity seen in early cleavage embryos could come from maternal RNA or proteins. The apparent absence of mTEAD activity in oocytes was not due to an inability of oocytes to...
utilize injected plasmid encoded promoters, because activity from Sp1-dependent promoters was easily detected. Neither was it due to insufficient levels of oocyte mTEAD-2 mRNA, because the levels of mTEAD-2 mRNA in oocytes and 1-cell embryos were at least twice the levels in 2/4-cell embryos (Fig. 4C). Therefore, mTEAD mRNAs detected in oocytes may not participate until later in development. Since one mechanism of regulating ZGE may involve the delayed translation of maternal mRNAs that encode transcription factors (Wang and Latham, 1997), it is possible that mTEAD-1, 2 or 3 maternal RNA might be translated prior to or concomitant with ZGE. Alternatively, translated mTEAD transcription factors may not become active until a specific cofactor is expressed at ZGA (Majumder et al., 1997).

The levels of mTEAD-2 mRNA in oocytes and early cleavage embryos [2000 to 6000 copies per ovum or embryo (Fig. 4C)] were substantially lower than the levels observed at later times during development. In fact, in situ hybridization analysis failed to detect any of the three mTEAD mRNAs in oocytes, although mTEAD-2 was readily apparent in the granulosa cells surrounding the oocytes (Fig. 2). Therefore, approx. 5000 copies of mRNA per cell was below the level of detection for our in situ hybridization protocol. Using in situ hybridization analysis, only mTEAD-2 mRNA was detected in day-7 embryos (Fig. 6) and it was distributed throughout the embryo (Fig. 7), suggesting that mTEAD-2 was the only member of this gene family required for early embryonic development. While these data also suggested that mTEAD-2 was not expressed in extraembryonic tissues, the frozen sections used in this study lacked sufficient morphological detail to allow definitive conclusions on mTEAD-2 expression in the individual tissues of the gastrulating embryo.

Jacquemin et al. (1996) also found that mTEAD-2 RNA was expressed abundantly throughout a day 6.5 d.p.c. conceptus (equivalent to our day-7 decidual), and that mTEAD-2 expression in the extraembryonic tissues declined between 6.5 and 8.5 d.p.c., consistent with the observations reported here. However, they also reported that mTEAD-1 was expressed at 6.5 d.p.c., but this expression was barely detectable and highly localized, while mTEAD-2 expression was strong and uniformly distributed throughout their embryos. While it is possible that the TEAD-1 promoter is active prior to day 9, as suggested by the expression of a reporter gene downstream of the mTEAD-1 promoter (Chen et al., 1994), both RT-PCR (Figs 3, 4) and in situ hybridization (Figs 6, 7) analyses show that the level of mTEAD-1 promoter activity is quite low relative to that of the mTEAD-2 gene up to day 7 of development. Therefore, we conclude that mTEAD-2 is expressed predominantly, if not exclusively, in developing embryos up to day 7, while mTEAD-1 (Fig. 8; Jacquemin et al., 1996), mTEAD-
factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. Genes. Dev. 8, 2293-2301.


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