Gene expression during preimplantation mouse development

Jay L. Rothstein, Dabney Johnson, Julie A. DeLoia, Jacek Skowronski,1 Davor Solter,2 and Barbara Knowles

The Wistar Institute, Philadelphia, Pennsylvania 19104 USA; 1Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 USA

To develop a resource for the identification and isolation of genes expressed in the early mammalian embryo, large and representative cDNA libraries were constructed from unfertilized eggs, and two-cell, eight-cell, and blastocyst-stage mouse embryos. Using these libraries, we now report the first stages at which the cytokines interleukin (IL)-6, IL-1β, and interferon (IFN)-γ are transcribed in the developing embryo and the presence of IL-7 transcripts in the unfertilized egg. Transcripts for IL-1α, -2, -3, -4, or -5 were not detected at these stages. To identify novel genes expressed on activation of the embryonic genome, the egg and eight-cell stage-specific cDNA libraries were subtracted from the two-cell library, yielding a specialized cDNA library enriched for transcripts expressed at the two-cell stage. Sequence and Southern blot analysis of several of these cDNAs expressed predominantly at the two-cell stage of embryogenesis revealed them to be from novel genes, thereby providing the first molecular tools with which to approach the study of gene expression in the early mammalian embryo.

[Key Words: cDNA libraries; cytokines; interleukins; IFN-γ; PCR; preimplantation embryos; subtractive hybridization]

Received March 19, 1992; revised version accepted April 22, 1992.

The molecular control of mammalian preimplantation embryogenesis remains largely unexplored, due mainly to the difficulty of obtaining sufficient quantities of timed embryos for experimentation. Nonetheless, knowledge about the changes in gene expression that underlie this period is essential to understanding mammalian development. Several lines of evidence, most notably that inhibition of transcription at the one-cell stage blocks protein synthesis and all subsequent development after the first cleavage division, and that initiation of synthesis of all classes of RNA occurs at the two-cell stage, point to the early activation of the embryonic genome [for review, see Telford et al. 1990a]. No resources existed that allowed investigation of whether activation leads to generalized gene transcription or synthesis of independent stage-specific transcripts. Temporal changes in transcription are also likely to herald the completion of cleavage and the formation of the first differentiated cells, those of the trophoderm [for review, see Schultz 1986], whose origin and fate have been difficult to study without probes to lineage-specific markers. One approach to identifying genes relevant to mammalian development has focused on the sequence homology with genes of developmental importance in other vertebrate or invertebrate organisms. However, considering that early development in mammals results in an implantation-competent embryo, it is likely that a unique combination of genes controlling this process will be utilized in the mammalian embryo. In an effort to identify genes expressed in these early mammalian developmental stages, several investigators have applied the polymerase chain reaction (PCR) technique, thus circumventing the problem of obtaining sufficient embryonic material for study [Rappolee et al. 1988]. However, only transcripts of known genes can be readily identified with this technique. Classically, cDNA libraries have provided a useful resource for identifying novel genes transcribed in specific cell types or tissues. Yet for technical reasons, cDNA libraries prepared from unfertilized eggs or single stages of the preimplantation embryo [Taylor and Pikó 1987; Weng et al. 1989; Ko et al. 1990; Welsh et al. 1990] have not provided reliable sources for the comprehensive analysis of genes differentially expressed during early embryonic development.

Here, we describe the use of large and representative cDNA libraries constructed from poly[A]+ mRNA of preimplantation mouse embryos to demonstrate stage-specific transcription of several cytokines. Subtractive hybridization of these libraries served to identify cDNAs representing novel genes expressed in the two-cell-stage embryo. These libraries provide the first resource for molecular information about genes expressed in the egg and early embryonic stages and a tool to access novel genes.

1Present address: Max-Planck Institut für Immunobiologie, D-7800 Freiburg-Zähringen, Germany.
expressed at the two-cell stage when the mouse embryonic genome is first activated.

Results

Library and insert size of egg and embryonic cDNA libraries

A single mouse egg or mouse embryo at any stage of preimplantation development contains no more than 50 pg of poly(A)+ mRNA (Clegg and Pikó 1983). We therefore optimized a cDNA cloning strategy to permit efficient library construction using 10–100 ng mRNA (J.L. Rothstein, D. Johnson, J. Jessee, J. Skowronski, D. Solter, and B. Knowles, in prep.). Plasmid vectors, which can accommodate the directional cloning of cDNA, were employed so that T7 and T3 RNA polymerase promoter sequences could be used to generate sense and antisense transcripts for subtractive hybridization. Libraries containing 1 × 10⁶ to 2 × 10⁹ independent cDNA clones were constructed from the 50–175 ng of poly(A)+ RNA isolated from unfertilized eggs, and two-cell, eight-cell, and blastocyst-stage embryos (Table 1). Because a library of 10⁹ clones has a >99% probability of including rare transcripts (<10 copies per cell) at a detectable frequency (Sambrook et al. 1989), these egg- and embryonic-stage libraries are not only likely to contain representatives of abundant but also of medium- and low-abundance transcripts in the egg or embryonic stage. Each of these four libraries contains at least 10⁶ independent cDNA clones (Table 1). The insert size of 25–50 randomly picked independent clones per library was determined by PCR, and blastocyst-stage embryos (Table 1). The insert size of 25-50 randomly picked independent clones per library was determined by PCR, and the four libraries contained clones representative of genes known to be expressed at these stages of development, they were probed with a mouse β-actin cDNA. Between 200 and 355 of the 250,000 clones screened in each library hybridized with the β-actin probe (Table 2). These values, when converted [see Materials and methods], suggest that 18,700 actin mRNA molecules are present in the mouse egg and 5600 are present in the late two-cell stage. We find that the levels of actin correspond well to those reported previously for the egg and two-cell stages, that is, 21,000 copies of actin mRNA in the egg and 3700 in the two-cell stage (Taylor and Pikó 1990), and are close to those of Bachvarova et al. (1989). We observe an increase in the number of actin transcripts in the eight-cell stage (18,460) and blastocyst stages (41,480), a pattern similar to previous reports (Taylor and Pikó 1990). The levels of actin in the libraries corroborate those of Taylor and Pikó and Bachvarova et al. and are substantially lower than the figures from comparable stages reported previously (Giebelhausen et al. 1983, 1985).

Transcripts of tissue-type plasminogen activator (t-PA) previously have been shown to decrease in maturing oocytes until they become nearly undetectable at ovulation (Huarte et al. 1985, 1987; Strickland et al. 1988). Expression of t-PA in the oocyte has been estimated at <0.05% of the total mRNA (Huarte et al. 1987). To determine the representation of t-PA in the unfertilized ovulated egg library, we hybridized a mouse t-PA cDNA clone (Rickles et al. 1988) to replica filters containing 250,000 clones and found that 60, or 0.024%, of the clones in the egg library were positive (Table 3). Three representative t-PA clones were partially sequenced from the 3' end and found to be homologous to the 3'-untranslated region of the mouse t-PA gene cloned from the F9 teratocarcinoma-derived cell line (Rickles et al. 1988; data not shown). As expected, no t-PA cDNAs were detected in the two-cell, eight-cell, or blastocyst-

<table>
<thead>
<tr>
<th>Embryonic stage</th>
<th>Number of females</th>
<th>Number of embryos</th>
<th>RNA [ng]</th>
<th>Library size [cfu]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized egg</td>
<td>200</td>
<td>5,000</td>
<td>1750⁶</td>
<td>175</td>
</tr>
<tr>
<td>Two-cell</td>
<td>665</td>
<td>13,500</td>
<td>910⁶</td>
<td>46</td>
</tr>
<tr>
<td>Eight-cell</td>
<td>300</td>
<td>2,778</td>
<td>1740⁶</td>
<td>87</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>100</td>
<td>600</td>
<td>900⁶</td>
<td>45</td>
</tr>
</tbody>
</table>

*Estimate based on 5% poly[A]+ RNA.
†Total number of independent cDNA clones (colony forming units) plated on primary filters.
‡Amount of RNA calculated based on values of Pikó and Clegg (1982).
§Values determined by use of Northern analysis of 18S/28S rRNA in the embryonic samples compared with rRNA from a standard amount of cellular RNA.
Rothstein et al.

Table 2. Analysis of gene expression in cDNA libraries

<table>
<thead>
<tr>
<th>cDNA library</th>
<th>28S/18S rRNA</th>
<th>cytochrome-c oxidase</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized egg</td>
<td>0</td>
<td>275 (0.110)</td>
<td></td>
</tr>
<tr>
<td>Two-cell</td>
<td>0</td>
<td>200 (0.080)</td>
<td></td>
</tr>
<tr>
<td>Eight-cell</td>
<td>1 (0.0004)</td>
<td>355 (0.142)</td>
<td></td>
</tr>
<tr>
<td>Blastocyst</td>
<td>0</td>
<td>305 (0.122)</td>
<td></td>
</tr>
</tbody>
</table>

*aProbes used for hybridization are pA-28S/18S rRNA; cytochrome-c oxidase I and II, and mouse β-actin isolated from a chicken embryo (Pikó et al. 1984) and mouse blastocyst (Pikó et al. 1984), which would not be represented in the blastocyst cDNA library.

*bNumber of positive clones of 250,000 independent cDNA clones screened [%].

stage library (0/250,000 clones screened); initiation of embryonic transcription of t-PA has been reported previously to occur in the trophoblast at implantation (Hart et al. 1985). Thus, the limited amount of t-PA cDNAs in these libraries is qualitatively and quantitatively consistent with previous information on transcription of this gene product.

The levels of highly expressed transcripts such as those of the intracisternal A-type particles (IAP; Luers et al. 1980; Mietz et al. 1988) and B1/B2 repeat sequences (Kramerov et al. 1979; Krayev et al. 1980) have been analyzed previously in total embryonic RNA (Pikó et al. 1984; Taylor and Pikó 1987, Mietz et al. 1988). We find that 0.035% of the clones in the egg library hybridized with an IAP probe (Pikó et al. 1984) or, by calculation, 5950 transcripts in the unfertilized egg are IAP. Similarly, 0.11% of the clones in the two-cell library or an estimated 7700 transcripts in the two-cell-stage embryo, 0.021% of those in the eight-cell library or 2730 transcripts in the eight-cell-stage embryo, and 0.001% of those in the blastocyst library or 272 transcripts in the blastocyst, are IAP. These results are quantitatively comparable at the two-cell and similar at the eight-cell stage to those reported, previously, that is, 7100 IAP mRNA molecules in the two-cell-stage embryo and 9700 IAP mRNA molecules in the 8-cell-stage embryo (Pikó et al. 1984). IAP levels appear higher in the egg and lower in the blastocyst libraries than those reported, that is, 1300 mRNA molecules in the mouse egg and 37,900 mRNA molecules in the early blastocyst were estimated to be IAP (Pikó et al. 1984). The differences between the library data and that reported previously may reflect the known variation in IAP expression among mouse strains (Kuff and Fewell 1985) and/or the high percentage of nonadenylated IAP mRNA in the mouse blastocyst (Pikó et al. 1984), which would not be represented in the blastocyst cDNA library.

B1 and B2 repeat sequences are expressed abundantly in the preimplantation embryo (Taylor and Pikó 1986). Repeat sequences are found in the 5'-untranslated regions of RNA polymerase II-generated transcripts and as small (~<500 bp) poly(A)+ RNA polymerase III-dependent transcripts of unknown function (Kramerov et al. 1979; Krayev et al. 1980; Murphy et al. 1983). We find that the abundance of these transcripts increases dramatically in these libraries at the two-cell stage (~0.1–0.2% of the clones detected in the unfertilized egg vs. 2–4% of the clones detected in the two-cell stage, Table 3)—values that are quantitatively similar to those reported previously (Vasseur et al. 1986; Taylor and Pikó 1987). Following this initial increase, B1 and B2 transcript levels decrease in the eight-cell and blastocyst libraries, a result at odds with those in the literature. However, the cDNAs used for library construction are derived from poly(A)+ RNA and are also size selected. Size selection would exclude the smaller polymerase III-dependent B1 and B2 (~<500 bp) transcripts from these libraries; additional B1 sequences were found in uncloned cDNAs smaller than 500 bp (data not shown).

From this analysis of β-actin, t-PA, IAP, and B1/B2 repeats, genes known to be expressed in the egg and preimplantation embryonic stages, we conclude that these egg and embryonic cDNA libraries represent the transcripts present in the corresponding stages in vivo. Thus, the libraries provide an in vitro source of genes transcribed at these stages of development.

Detection of cytokines in the cDNA libraries

As a first approach to identifying genes expressed during early embryogenesis that may have regulatory functions, we investigated the representation of several cytokines in the libraries. Studies of polypeptide growth factors in early mammalian development have focused on expres-

Table 3. Representativeness of gene expression in cDNA libraries

<table>
<thead>
<tr>
<th>cDNA library</th>
<th>t-PA</th>
<th>IAP</th>
<th>B1</th>
<th>B2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized egg</td>
<td>60 (0.024)</td>
<td>88 (0.035)</td>
<td>26 (0.130)</td>
<td>30 (0.150)</td>
</tr>
<tr>
<td>Two-cell</td>
<td>0</td>
<td>275 (0.110)</td>
<td>404 (0.202)</td>
<td>725 (3.625)</td>
</tr>
<tr>
<td>Eight-cell</td>
<td>0</td>
<td>53 (0.021)</td>
<td>150 (0.750)</td>
<td>100 (0.500)</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>0</td>
<td>2 (0.001)</td>
<td>4 (0.020)</td>
<td>50 (0.250)</td>
</tr>
</tbody>
</table>

*aProbes used were pTAM, t-PA, clone 11, IAP, and pB1/B2 (B1 and B2 repeats).

*bNumber of positive clones when 250,000 colonies from each stage were screened with pTAM and clone 11, or 20,000 independent cDNA clones were screened with pB1/B2 [%].
sion of epidermal growth factor (EGF), transformation growth factor (TGF-α), platelet-derived growth factor (PDGF), TGF-β1, insulin-like growth factor (IGF-I), and IGF-II at specific times during preimplantation development (Rappolee et al. 1988; Lee et al. 1990; Telford et al. 1990b). Although some of these growth factors are known to play a role in differentiation, their major function appears to involve regulation of the cell cycle. On the other hand, the cytokines not only regulate proliferation but also appear to induce differentiated functions.

To investigate whether interleukins IL-1α, IL-1β, IL-2-7, or γ-interferon (IFN-γ) are transcribed during preimplantation embryogenesis, the cDNA libraries from the unfertilized egg, eight-cell, and blastocyst stages were screened. Cytokine expression, identified initially by PCR analysis of pooled inserts from each cDNA library, was confirmed by direct screening of the cDNA libraries with authentic probes. PCR analysis of the libraries revealed expression of IL-1β, IL-6, IL-7, and IFN-γ but not IL-1α, or IL-2–IL-5 (Table 4). As expected, β2 microglobulin (β2M) was present at all stages tested (Sawicki et al. 1981). Southern hybridization of the PCR gels using probes to IL-1β, IL-6, IL-7, IFN-γ, and β2M verified the presence of these transcripts in the libraries (Fig. 1).

To quantify cytokine expression in the embryonic libraries, we screened each stage with a PCR-generated gene-specific probe. Screening 250,000 clones of each library with an IL-7 probe indicated 8 positive clones in the unfertilized egg library, whereas no colony hybridization was seen with the same number of clones from eight-cell and blastocyst libraries. Thus, IL-7 transcripts appear to be rare in the mouse egg (0.003% of the independent cDNA clones in the library) and undetectable in the early embryo. Sequence analysis of two of the IL-7 hybridizing clones from the unfertilized egg library confirmed these to be the mouse IL-7 gene (Namen et al. 1988; data not shown). IL-6, a cytokine with effects on many cell types (Hirano et al. 1990; Sehgal 1990), was shown previously to be expressed at the blastocyst stage (Murray et al. 1990). Here, we report that IL-6 is transcribed as early as the eight-cell stage, persisting into the blastocyst stage (Table 4; Fig. 1). IL-1β, a pleiotropic cytokine expressed by multiple cell types with an important role in the inflammatory response (Oppenheim et al. 1986; Dinarello 1989), is expressed by mammalian placental tissue and cultured trophoblast-derived cell lines (Taniguchi et al. 1991). The function of IL-1β in the developing embryo is not known, and there have been no reports of its synthesis during early embryonic development. Although IL-1β expression at the blastocyst stage was indicated by our PCR analysis of the libraries (Fig. 1) and by direct analysis of freshly isolated blastocysts by
reverse transcriptase [RT]–PCR [data not shown], no hybridizing colonies were detected in the $5 \times 10^5$ colonies screened using a probe homologous to the 5′ end of IL-1β. However, 20-fold more cDNA was screened by PCR than by filter hybridization. These data suggest that IL-1β is either expressed as a rare message in each cell or by a small number of specialized cells in the mouse blastocyst, or is actually present in the blastocyst library at a higher level but not detectable using the 5′ IL-1β probe. Analysis of IL-1β expression in the inner cell mass and the trophectoderm, as well as screening the blastocyst library by use of a 3′ IL-1β probe, will likely resolve this issue. IFN-γ is also expressed in the mouse blastocyst [Table 4; Fig. 1], consistent with the observation that the mouse blastocyst secretes a factor with IFN-like antiviral activity in vitro [Cross et al. 1990; Nieder 1990]. A member of the IFN-α gene family was identified previously as one of the major proteins expressed by bovine, ovine, caprine, and porcine blastocysts [Imakawa et al. 1987, 1989; Hansen et al. 1988, Cross and Roberts 1989, Roberts et al. 1989; Baumbach et al. 1990]. Our data provide the first evidence for expression of any interferon in the murine blastocyst. Thus, the screening of these libraries with probes of known cytokines demonstrates the transcription of genes whose products are often expressed in differentiated cell types and mediate changes in gene expression.

Isolation of novel stage-specific genes by subtractive hybridization

To identify genes whose expression changes during preimplantation development, we generated specialized libraries by subtractive techniques. Directional cloning in the Bluescript vector allowed us to use a modification of the biotin–streptavidin method [Sive and St. John 1988; J.L. Rothstein, D. Johnson, J. Jessee, J. Skowronski, D. Solter, and B. Knowles, in prep.] to obtain unique mRNA molecules. T3-initiated, biotinylated, antisense single-stranded, and hybrid RNA molecules were separated from T7-initiated, sense single-stranded molecules after binding to streptavidin [Fig. 2]. Using this approach, we generated a two-cell-specific subtraction library by hybridizing a fivefold excess of biotinylated RNA from the egg library to that of the two-cell library. The resulting two-cell-specific single-stranded RNA was sexaplated from biotinylated RNA bound to streptavidin, and hybridized to a 10-fold excess of biotinylated RNA from the eight-cell library. Following a second streptavidin treatment, the remaining single-stranded RNA was reverse-transcribed and cloned into plasmid vectors. The average insert size of the cDNAs in the two-cell subtraction library [2CSL-I], which contains $2 \times 10^6$ clones, was 1.0 kb [data not shown]. Repeating the procedure schematized in Figure 2 with the 2CSL-I library as starting material resulted in a second two-cell-specific subtraction library [2CSL-II] of $2 \times 10^7$ clones with an average insert size of 300–400 bp [data not shown]. The smaller size of the cDNAs in the 2CSL-II library is consistent with RNA degradation during the multiple and long incubation periods of double-stranded RNA hybrids at high temperatures.

To determine whether these subtraction libraries are reduced in complexity, we hybridized both subtracted libraries with probes to IAP, β-actin, and B1/B2 repeat sequences; no positive hybridization was detected in either subtraction library [250,000 clones screened; data not shown]. Because cDNAs in the 2CSL-I and -II libraries should be highly enriched for transcripts expressed in greatest abundance at the two-cell stage of embryogenesis, we randomly selected clones for further analysis. Twenty such clones with inserts >500 bp in length were partially sequenced and compared with those listed in the GenBank/EMBL data bases. Of these 20 clones, 14 did not match any sequence listed in the data bases. The other six clones proved to be bacterial cDNAs, most likely from the Escherichia coli tRNA used as a carrier in the preparation of the subtraction libraries. Following sequence analysis, the 14 unique cDNAs were hybridized to the original two-cell cDNA library and any clones that showed specific hybridization were analyzed further for stage-specific expression by hybridization to the egg and eight-cell libraries. In this manner, four stage-specific cDNAs were identified that were expressed predominantly or exclusively at the two-cell stage of preimplantation development [Table 5]. One clone, stage-specific embryonic clone-3 (SSEC-3), appears to be expressed predominantly at the two-cell stage, with a low number of hybridizing clones [3 hybridizing clones/250,000 colonies screened] in the egg library. SSEC-D is a highly expressed message; 0.16% of the clones in the two-cell library hybridized with this cDNA, approximately fivefold more than in the egg library. SSEC-C is two-cell specific, but only 0.002% of the colonies screened hybridized with this cDNA. There are more colonies hybridizing with the SSEC-P probe in the two-cell-stage library [0.02%] than in the egg [0.01%], suggesting that this gene is either newly transcribed at the two-cell stage or that its message is somehow protected during the generalized RNA degradation that occurs after fertilization [Clegg and Pikó 1983]. Each of these clones represents an authentic single-copy mouse gene as determined by Southern analysis [data not shown]. In addition, expression of SSEC-C, SSEC-D, and SSEC-P was confirmed by direct RT–PCR analysis of freshly isolated two-cell embryos using SSEC-specific primers [data not shown]. Since SSEC-3, SSEC-C, SSEC-D, and SSEC-P are small cDNAs without apparent open reading frames, isolation of full-length cDNAs corresponding to these clones is a necessary and an ongoing effort. All of the remaining 14 cDNAs were confirmed by Southern blot hybridization to be of mouse origin, but were not detected in the two-cell library after screening 250,000 clones and, therefore, are likely to represent extremely rare transcripts.

Discussion

The unfertilized egg and embryonic stage-specific cDNA libraries we have described provide a unique resource to
study genes expressed in the early mammalian embryo. The results obtained by probing these libraries with single-copy genes suggest that they are representative of the genes transcribed at these stages. One aspect of this analysis is that our estimate of actin levels will serve to resolve the controversy over the quantity of actin message in the preimplantation embryo. Previous estimates of actin mRNA abundance were made by comparing the level of embryonic actin mRNA to that in mRNA from a nonembryonic standard source, a technique subject to variation. Quantitation of independent actin clones in cDNA libraries overcomes this limitation. Another interesting aspect arises from the difference between B1 and B2 transcript levels in the unfertilized egg and two-cell-stage libraries on the one hand, and the eight-cell- and blastocyst-stage libraries on the other. Previous studies of total unfractionated RNA revealed an increase in B1 and B2 repeat-containing transcripts throughout preimplantation development [Taylor and Piko 1987; Pozanski and Calarco 1991], whereas the frequency of B1 and B2 repeat-containing cDNAs decreases in the libraries after the two-cell stage. These data suggest that there may be changes in the RNA polymerase II and III activity in the embryo after the activation of the embryonic genome at the two-cell stage. Previous studies have suggested that changes occur in the relative amounts of RNA polymerase II and III activity between the eight-cell stage and blastocyst, the earliest embryonic stages investigated [Warner 1977].

The expression of polypeptide growth factors was investigated because the interactions of these factors with their receptors mediate changes in gene expression, re-
The capacity to generate specialized subtractive cDNA libraries provides access to mammalian genes expressed at a predetermined temporal or spatial coordinate. These embryonic cDNA libraries will now serve as the starting point for the generation of a series of subtraction libraries enabling identification of stage-specific genes. The first of these libraries, enriched for genes expressed at the two-cell stage of embryogenesis, has been successfully constructed, of the first 20 cDNA clones investigated, 14 novel mammalian genes were identified. Ten of these clones represent rare transcripts, and the other 4, or 20%, are expressed predominantly at the two-cell stage. The results obtained using these subtraction libraries demonstrate the feasibility of this approach in identifying novel cDNA probes for genes whose transcription changes during development, and in isolating novel cDNA clones of relatively rare transcripts from a specific embryonic stage.

Because each cDNA library described in this report is representative, it should contain at least one cDNA clone of most of the genes transcribed in the corresponding stage in the mouse. Thus, by using probes derived from known genes and new probes isolated by such techniques as subtraction, the libraries provide the much needed instrument to determine whether the genes transcribed at the two-cell stage are activated independently to perform a stage-specific function or whether most of the embryonic genome is transcriptionally activated at the two-cell stage and, on differentiation, enhanced expression or specific repression of specific gene subsets occurs. Although few studies have directly addressed either notion, the generalized decrease in methylation during preimplantation mouse development [Monk et al. 1987] suggests a global activation of the embryonic genome. Similarly, the low-level constitutive expression of cell-lineage-specific genes, such as myoD and other mesoderm-associated genes [Rupp and Weintraub 1991], at the time when the frog embryonic genome is first activated, also supports the generalized activation hypothesis. The availability of cDNA libraries representing serial stages of early mouse development may now allow this basic issue to be experimentally addressed in the earliest embryonic stages.

**Materials and methods**

**Mice and embryo recovery**

Unfertilized eggs, two-cell, eight-cell, and blastocyst embryos were collected from immature B6D2F1 mice [Jackson Laboratories, Bar Harbor, ME or Harlan-Spague Dawley, Indianapolis, IN] after superovulation [Hogan et al. 1986] and mating to B6D2F1, males, where appropriate. Unfertilized eggs were treated with hyaluronidase and subsequently with Pronase, whereas cleavage-stage embryos and blastocysts were treated with Pronase alone [Hogan et al. 1986]. Embryos from all stages were washed repeatedly in modified Whitten's medium [Abramczuk et al. 1977], and pools of 500–1000 were

---

**Table 5. cDNA clones obtained from two-cell subtraction library**

<table>
<thead>
<tr>
<th>Clone</th>
<th>cDNA insert size [bp]</th>
<th>egg [ % expression ]</th>
<th>two-cell [ % expression ]</th>
<th>eight-cell [ % expression ]</th>
<th>Sequence information [bp]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSEC-3</td>
<td>500</td>
<td>3 [0.001]</td>
<td>10 [0.004]</td>
<td>0</td>
<td>320</td>
</tr>
<tr>
<td>SSEC-C</td>
<td>600</td>
<td>0</td>
<td>5 [0.020]</td>
<td>0</td>
<td>300</td>
</tr>
<tr>
<td>SSEC-D</td>
<td>600</td>
<td>75 [0.030]</td>
<td>400 [0.160]</td>
<td>10 [0.004]</td>
<td>382</td>
</tr>
<tr>
<td>SSEC-P</td>
<td>900</td>
<td>25 [0.010]</td>
<td>50 [0.020]</td>
<td>0</td>
<td>172</td>
</tr>
</tbody>
</table>

*Approximate size based on agarose gel (pX174 standard).

bNumber of positive colonies detected of 250,000 independent cDNA clones screened.

Obtained from combining partial 3' and 5' sequence of each clone. Nucleotide sequences were found to be novel when compared with those listed in GenBank/EMBL by use of WORDSEARCH and FASTA commands of the GCG software program (Devereux et al. 1984).
placed in 200 μl of embryo lysis buffer [ELB: 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% SDS, 5 μg of E. coli tRNA, [Boehringer Mannheim]], which had been initially incubated with 0.5 mg/ml of proteinase K [Boehringer Mannheim] for 30 min at 37°C to remove any contaminating RNase.

**Embryo RNA isolation**

The embryo/ELB solution was incubated for 1 hr at 37°C and extracted twice with phenol–chloroform, and nucleic acids were collected by ethanol precipitation and stored at −70°C in absolute ethanol [Sambrook et al. 1989]. Aliquots were removed and microcentrifuged for 60 min. The 70% ethanol-washed pellet was air-dried, resuspended in 80 μl of RNA-seq-water free and 20 μl of 5× DNase buffer [250 mM Tris-HCl (pH 7.5), 1 mM NaCl, 50 mM MgCl₂, 25 mM CaCl₂], and 1.5 μg of DNase I [Worthington Biochemicals], which was initially incubated with 0.5 mg/ml of proteinase K [Boehringer Mannheim] at 37°C for 30 min to remove contaminating RNase, and was added to the sample which was incubated at 37°C for 30 min. DNase digestion was terminated by adding 10 μl of 0.25 M trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetate acid monohydrate (CDTA), 5 μl of 10% SDS, and 2 μl of proteinase K [20 mg/ml] and incubated for 15 min at 56°C. The solution was extracted with twice phenol–chloroform, and total embryonic RNA was ethanol precipitated. Poly(A)⁺ mRNA was selected using poly(dU)-Sephadex according to the manufacturer’s protocol [GIBCO/BRL]. Briefly, 20–50 μg of poly(dU)-Sephadex beads were resuspended in 1 ml of NTS [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% SDS, 0.4 M NaCl] in a 1.5-ml microcentrifuge tube, swollen, and spun lightly for 10–20 min. Unbound RNA was removed by three washes with 1 ml of NTS and a single wash with low-salt NTS. Bound poly[A]+ RNA was eluted from the beads by adding 20 mM Tris-HCl (pH 8.3), followed by phenol extraction and ethanol precipitation. Nucleate-treated cDNA was end repaired by resuspension in 11 μl of nuclelease-free H₂O, 4 μl of 5× T4 polymerase buffer [0.2 mM CDTA, 5 μl of glycerol [1 mg/ml], 0.37 M NaCl, 1 unit of T4 polymerase] and incubated at 37°C for 60 min. The amount of RNA converted to cDNA was consistently 30 ± 8%. Second-strand synthesis was performed in the same tube in a total volume of 200 μl. Briefly, 30 μl of first-strand reaction, 20 μl of 10× second-strand buffer [Sambrook et al. 1989], 2 units of RNase H [Pharmacia], 70 units of DNA polymerase I, 0.25 M MgCl₂, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% SDS, and 200 units of S1 nuclease [Boehringer Mannheim], 20 μl of dNTPs (10 mM each), and sterile, nuclease-free H₂O were incubated for 1 hr at 15°C, followed by 1 hr at room temperature. The reaction was terminated by the addition of 2.5 μl of 0.25 M CDTA, 5 μl of glycerol [1 mg/ml], 0.37 M NaCl, and 200 units of S1 nuclease [Boehringer Mannheim] in 1× S1 buffer [0.1 M NaAc, 0.8 μM NaCl, 2 mM ZnCl₂], for 20 min at 37°C. Nucleate reactions were terminated by adding 20 μl of Tris-HCl (pH 8.3), followed by phenol extraction and ethanol precipitation. Nucleate-treated cDNA was end repaired by resuspension in 11 μl of nuclease-free H₂O, 4 μl of 5× T4 polymerase buffer [0.2 mM CDTA, 5 μl of glycerol [1 mg/ml], 0.37 M NaCl, 1 unit of T4 polymerase] and incubated at 37°C for 15 min [Sambrook et al. 1989]. End-repaired cDNA was phenol/chloroform-extracted and ethanol-precipitated. 5'-Phosphorylated MluI linkers [3 μg, Pharmacia LKB Biotechnology] were ligated to blunt-ended cDNA by use of 1 Weiss unit of T4 ligase in 30 μl for 16–18 hr at 15°C [Sambrook et al. 1989]. Ligase was inactivated [65°C for 10 min] and the cDNA was double-digested with the restriction enzymes Sali and MluI [New England Biolabs] in a total volume of 400 μl for 5–6 hr at 37°C, under conditions suggested by the manufacturer. Digestion reactions were terminated, phenol/chloroform-extracted, and ethanol-precipitated as described above. Digested cDNA was resuspended in 15 μl of nuclease-free H₂O, 10 μl of saturated urea, and 1 μl of bromophenol blue tracer dye [1 mg/ml] and loaded onto a 1-ml Sepharose CL4B column [Pharmacia] initially washed in column buffer [20 mM Tris-HCl (pH 7.5), 0.2 mM NaAc, 4 mM EDTA, 0.1% SDS]. Fractions of 100–200 μl were collected; cDNA >500 bp eluted in the first radioactive peaks. After counting, the peak fractions were pooled and cDNA was precipitated with ethanol using 15 μg of glycerol as carrier. Precipitated cDNA was resuspended in water to a concentration of 0.25–1 ng/μl and ligated intron excess of linearized PBS vector for 18 hr at 15°C (Stratagene, modified so that the EcoRI site was converted to an MluI site, and the HindIII site was converted to a Sali site). Ligation reactions were phenol/chloroform-extracted, ethanol-precipitated, and resuspended in 10 μl of TE [10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA] prior to bacterial electroporation.

**Bacterial electroporation, plating, and composition of cDNA libraries**

E. coli strain DH10B was kindly provided by Joel Jessee.
number of polylA + RNA molecules previously estimated to be present in a single egg or embryo, that is, $1.7 \times 10^7$ poly(A) + gene in a single egg or embryo, the frequency of its occurrence in the early blastocyst (Clegg and Pikó 1983). Therefore, from mRNA molecules in the mouse egg, $7 \times 10^7$ in the eight-cell-stage embryo, and $3.4 \times 10^7$ colonies per total cDNA colonies screened. For example, library was determined by calculating the number of positive clones for sequencing. The frequency of β-actin transcripts in the egg library is 0.0011 with 10 pg of pUC19. Electroporation of eDNA libraries resulted in transformation efficiencies of $2 \times 10^8$ to $3 \times 10^8$ cfu/g of plasmid were routinely obtained with 10 pg of pUC19. Electroporation of cDNA libraries resulted in transformation efficiencies of $2 \times 10^8$ to $3 \times 10^7$ cfu/μg of cDNA. cDNA was electroporated in independent aliquots containing 1 μl of cDNA and 25 μl of electrocompetent bacteria. Aliquots were electroporated, grown in 1 ml of SOC medium (Hananah et al. 1991) at 37°C for 60 min, pooled, and spun at 400g for 10 min. Cell pellets were resuspended in 1 ml of SOC for every 250,000 estimated transformants, and each milliliter was spread onto 8.5 × 8.5-inch MS1 nylon membranes that were placed on top of LB/Amp agar plates (Sambrook et al. 1989) and incubated overnight at 37°C. The following day, filters were replica-plated and prepared for hybridization, and master filters were stored at −70°C (Rothstein et al. 1992). For all libraries, additional plates were scraped with 20 ml of LB/Amp, and 0.1- to 0.5-ml aliquots were stored at −70°C.

Genetic probes and reagents

Probes used in this study were: pTAM (full-length t-PA cDNA), a kind gift from S. Strickland (State University of New York at Stony Brook), clone 11 (genomic clone containing the 5′ LTR and coding regions of a mouse IAP gene), and mitochondrial cytochrome-c oxidase I and II cDNA clone (Pikó and Taylor 1981), kindly provided by L. Pikó (Veterans Administration Hospital, Sepulveda, CA); murine β1/β2 cDNA probe (Larin et al. 1986), a kind gift from M. Bucán (University of Pennsylvania, Philadelphia, PA). We isolated the murine β-actin cDNA clone from a mouse blastocyst library using a chicken β-actin cDNA (Alonso et al. 1986). The full-length murine IL-7 cDNA probe was kindly provided by S. Gillis and L. Park (Immunex, Seattle, WA). PCR primer sets specific for the central and 5′ regions of mouse IL-1 and IL-7 and IFN-γ genes were purchased from Clontech Laboratories. Radioactive probes were obtained by isolating inserts from plasmids by appropriate restriction enzyme digestion, agarose gel purification, and 32P-labeling by use of the random primer method (Feinberg and Vogelstein et al. 1983). Probes were hybridized to library filters (1 × 106 to 2 × 106 cpm/ml) in Church buffer (7% SDS, 1 mM EDTA, 0.5 M sodium phosphate buffer [pH 7.2]) for 18–20 hr at 65°C.

cDNA library screening

Only colonies hybridizing with a given probe on two replicated library filters were considered positive. Subsequent secondary screening was performed to verify positive signals and to isolate clones for sequencing. The frequency of a given transcript in a library filters was considered positive. Subsequent secondary screening was performed to verify positive signals and to isolate clones for sequencing. The frequency of a given transcript in a library filters was considered positive. Subsequent secondary screening was performed to verify positive signals and to isolate clones for sequencing. The frequency of a given transcript in a library filters was considered positive. Subsequent secondary screening was performed to verify positive signals and to isolate clones for sequencing.
times, ethanol-precipitated, and washed. The two-cell library-derived sense RNA remaining after hybridization with egg library-derived RNA was hybridized to a 10-fold excess of eight-cell antisense RNA and treated as described above. The two-cell library-derived sense RNA remaining after hybridization and phenol–chloroform subtraction was reverse-transcribed and cloned into the pBS cloning vector as described above.

Seqencing
All cDNA clones were sequenced by use of the Sequenase kit [U.S. Biochemical] from the 5′ end with the T7 primer and the 3′ end with the T3 primer with [3S]dATP as described by the manufacturer. Sequencing reactions were run on a 10% polyacrylamide/6% urea gel at 2000 V for 6–8 hr and exposed to X-ray film (X-Omat, Kodak) overnight at room temperature. All sequences were compared with those listed in the GenBank/EMBL data bases by use of the WORDSEARCH and FASTA commands of the GCG sequence analysis program [Devereux et al. 1984].

Acknowledgments
We gratefully acknowledge J. Jessee [BRL] for the DH10B bacteria and expert assistance in electroporation, Susan Johnson for her discussion and critical reading of this manuscript, and we thank Emma DeJesus and Geoffrey Doerre for their technical help. D.S. and B.B.K. thank James Watson for his hospitality and the members of the James Laboratory at Cold Spring Harbor Laboratory, in particular Douglas Hanahan and his group, for their guidance and encouragement in the early phases of this study. This work was supported by U.S. Public Health Service from the National Institutes of Health grants [CA-10815, CA-1734 solely to indicate this fact.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

References


Vasseur, M., H. Condamine, and P. Duprey. 1985. RNAs con-
Preimplantation mouse development


Gene expression during preimplantation mouse development.

J L Rothstein, D Johnson, J A DeLoia, et al.

*Genes Dev.* 1992, 6:
Access the most recent version at doi:10.1101/gad.6.7.1190

References

This article cites 61 articles, 21 of which can be accessed free at:
http://genesdev.cshlp.org/content/6/7/1190.full.html#ref-list-1

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.