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Analysis of gene expression in the preimplantation mouse embryo: Use of mRNA differential display

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ABSTRACT **The analysis of differential gene expression during preimplantation embryogenesis has been hindered by the paucity of biological material. We report modifications of the recently described mRNA differential display method (Liang, P. & Pardee, A. B. (1992) *Science* 257, 967–971) to analyze differential gene expression during mouse preimplantation development. The method detects the appropriate changes in the temporal pattern of expression of an amplicon that by DNA sequence analysis is the cytokeratin endo A, a gene whose temporal pattern of expression has been previously determined by S1 nuclease digestion. In addition, this method identifies amplicons that likely represent genes (*i*) that encode maternal mRNAs, (*ii*) that are products of early and late zygotic gene activation, (*iii*) whose expression is greatest during the eight-cell stage (i.e., expressed in a stage-specific manner), and (*iv*) whose expression is greatest in the blastocyst. In addition to endo A, sequence analysis of these amplicons reveals that an amplicon that displays a temporal pattern of expression consistent with it being a maternal mRNA is the α subunit of the mitochondrial F_1 ATP synthase.**

Preimplantation development in the mouse is characterized by cell proliferation, differential gene expression, and differentiation. After fertilization, zygotic gene activation is clearly evident by the two-cell stage—paternally encoded isozymes (1) and the synthesis of α-amanitin-sensitive proteins are detected (2)—and is required for further cleavage (3). Some of these zygotically activated genes are expressed in a stage-specific manner [i.e., their synthesis is restricted to the two-cell stage (2, 4)], whereas others are constitutively activated (4). In fact, there is a dramatic reprograming of gene expression after zygotic gene activation (4).

Overt differentiation in the mammalian embryo occurs relatively early with respect to cell number, when compared to embryos of nonmammalian species. For example, although blastomeres of the 16-cell embryo are totipotent (5), by the early blastocyst stage, which consists of about 32 cells, two cell lineages are present (6): the inner cell mass cells, which give rise to the embryo proper and are pluripotent, and the trophectodermal cells, which give rise to extraembryonic tissue and are differentiated. It is tacitly assumed that differential gene expression underlies this developmental event. In support of this conclusion is the observation that the trophectoderm and inner cell mass cells synthesize different proteins, as observed by two-dimensional gel electrophoresis (7), and moreover, these changes in gene expression can be observed between the inner and outer cells present in the morula (8).

What has been problematic in analyzing differential gene expression in the preimplantation mouse embryo (e.g., identifying genes that are expressed in a stage-specific or cell-type-specific manner) has been the paucity of biological material. The generation of high-quality cDNA libraries made from preimplantation mouse embryos at different stages of development (egg, two-cell, eight-cell, and blastocyst) has provided a valuable resource to analyze gene expression in the preimplantation mouse embryo (9). Nevertheless, the use of these cDNA libraries to analyze gene expression is restricted in that they do not allow facile analysis of gene expression during narrower windows of developmental time (e.g., at different times during the two-cell stage).

The recent development of a reverse transcription–PCR (RT–PCR)-based method, termed mRNA differential display (10–12), has opened up a major avenue to study differential gene expression in the preimplantation mouse embryo. The major advantage of this method is that only a modest number of embryos are required and that the amplicons that are obtained can readily be cloned and sequenced. We report here the use and validation of this method to study differential gene expression in the preimplantation mouse embryo. In addition, we find that a putative maternal mRNA encodes for the α subunit of the mitochondrial F_1 ATP synthase.

MATERIALS AND METHODS

Collection and Culture of Mouse Embryos. Embryos were obtained from superovulated CF-1 female mice (Harlan) mated to B6D2F1/J males (The Jackson Laboratory) as described (13, 14). Two-cell embryos, eight-cell embryos, and blastocyts were collected ≈44 h, ≈68 h, and ≈92 hours after human chorionic gonadotropin, respectively, from either the oviducts or uteri. The embryos were collected in bicarbonate-free minimal essential medium (Earl's salts) supplemented with pyruvate (100 μg/ml), gentamicin (10 μg/ml), polyvinylpyrrolidone (3 mg/ml), and 25 mM Hepes (pH 7.2; MEM/PVP). In all cases embryos were cultured in bicarbonate-containing MEM/PVP (25 mM sodium bicarbonate replacing the 25 mM Hepes) in a humidified atmosphere containing 5% CO_2/95% air at 37°C for 5–6 h after collection, at which time they were selected on the basis of morphology (e.g., compacted eight-cells and expanded blastocysts).

RNA Isolation and Purification. Unless otherwise stated, all solutions were prepared with water that had been treated with 0.1% diethyl pyrocarbonate.

Embryos (usually 100–200) were transferred in <5 μl of MEM/PVP to a 0.5-ml polypropylene tube containing 100 μl of lysis buffer [4 M guanidine thiocyanate/1 M 2-mercaptoethanol/0.1 M Tris·HCl, pH 7.4/20 μg of poly(C) (Boehringer Mannheim)]. The RNA was precipitated by the addition of 8 μl of 1 M acetic acid, 5 μl of 2 M potassium acetate, and 300 μl of 100% ethanol. The samples were precipitated overnight at −20°C.

The nucleic acids were collected by centrifugation at 10,000 × *g* for 15 min at 4°C. The supernatant was removed and the pellets were then washed once at 4°C with 300 μl of ice-cold 75% ethanol. The supernatant was carefully removed with a pipette such that ≈0.5 μl of fluid remained; if

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Abbreviation: RT, reverse transcription.

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the pellets were allowed to dry, they were virtually impossible to solubilize. The pellets were resuspended in 20 μl of resuspension solution (RS; 40 mM Tris·HCl, pH 7.9/10 mM NaCl/6 mM $MgCl_2$). DNA was degraded by incubating the sample with 1 unit of RQ1 DNase (Promega) for 30 min at 37°C. After the addition of 50 μl of diethyl pyrocarbonate-treated water, the samples were extracted with 80 μl of RS-saturated phenol (Amresco, Euclid, OH). The samples were vortex mixed and centrifuged for 8 min at 10,000 × g, and the aqueous phase was transferred to a 0.5-ml microcentrifuge tube. Eight microliters of 3 M potassium acetate (pH 5.2) and 300 μl of 100% ethanol were added and the RNA was precipitated overnight at −20°C. The RNA was collected as described above and the pellet was dissolved in 10 μl of diethyl pyrocarbonate-treated water. The efficiency of RNA recovery, which usually ranged between 50 and 70%, was calculated by removing an aliquot and determining the A_{260}. The RNA was stored at −20°C.

mRNA Differential Display. RT was performed on RNA obtained from an equivalent of 50 two-cell embryos, 25 eight-cell embryos, and 10 blastocysts. In each case, this number of embryos corresponds to about 10 pg of poly(A)$^+$ RNA (15). For RT, the embryo RNA and the 3′ primer (in a quantity to yield a final reaction concentration of 20 μM) were combined in a thin-walled PCR tube (Perkin–Elmer/Cetus) and the volume was brought to 9.5 μl with diethyl pyrocarbonate-treated water; the sequences of the 5′ and 3′ primers that were used in this study are indicated in Fig. 1. The sample was heated to 70°C for 10 min in a model 9600 thermocycler (Perkin–Elmer/Cetus). The temperature was then reduced to 45°C and the stated amounts of the following solutions were then added in the form of a warmed (45°C) mastermixture: 4 μl of 250 mM Tris·HCl, pH 8.3/375 mM KCl/15 mM $MgCl_2$, 2 μl of 100 mM dithiothreitol, 1 μl of RNasin (40 units; Promega), and 2 μl of a solution containing all four dNTPs, each at 200 μM; the final volume was 18.5 μl. The sample was allowed to equilibrate at 45°C for 1 min. Three hundred units of Superscript II reverse transcriptase (GIBCO/BRL) in 1.5 μl was then added (final volume of the reaction mixture was 20 μl) and the sample was incubated at 45°C for 1 h. At the end of this incubation, the temperature was raised to 95°C for 5 min. After chilling the tube on ice for several minutes, any condensate was collected by a brief centrifugation.

PCR amplification of a given RT reaction was carried out in triplicate. For each 20-μl PCR mixture, 2 μl of the well-mixed RT reaction mixture (this corresponded, for example, to 1 blastocyst equivalent) was added to 18 μl of a solution such that the final concentrations were 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.5 μM 5′ primer, 2.5 μM 3′ primer, and all four dNTPs (each at 2 μM); the solution also contained 10 μCi of ^{35}S-labeled dATP (specific activity, >1000 Ci/mmol; 1 Ci = 37 GBq; Amersham) and 2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus). The sample was then overlaid with 50 μl of ice-cold mineral oil and subjected to 40 cycles of PCR on a model 9600 thermocycler using the following parameters: 94°C for 30 sec, 42°C for 60 sec, and 72°C for 30 sec; the last cycle was followed by a 5-min extension at 72°C. In some cases, the samples were stored at −20°C at this point.

The radiolabeled amplicons were resolved on a 6% DNA sequencing gel; 5 μl of the reaction mixture was applied. Gels were dried without fixation onto filter paper and then subjected to autoradiography using Kodak XAR-5 x-ray film at −80°C. The typical length of exposure was 1–5 days.

Recovery and Reamplification of Amplicons. Dots of phosphorescent ink were triangulated in the area of a band of interest on the dried gel. The band was then cut from the resulting autoradiogram, and this was used as a template to cut the corresponding section from the dried gel. (The gel was then reexposed to x-ray film to ensure that the appropriate band had been excised.) This gel fragment and its associated filter paper were transferred to a 0.5-ml polypropylene tube. The gel slice was first soaked for 10 min in 100 μl of diethyl pyrocarbonate-treated water at room temperature and then incubated in boiling water for 15 min. After centrifugation for 2 min at 17,000 × g to pellet any solid debris, the supernatant was removed and 8 μl of 3 M potassium acetate and 2.5 μl of glycogen (20 mg/ml) were added. After the addition of 300 μl of 100% ethanol, the DNA was precipitated overnight at −20°C. The DNA was collected by centrifugation, and the pellet was washed with 200 μl of ice-cold 85% ethanol and resuspended in 10 μl of diethyl pyrocarbonate-treated water.

For reamplification, 5 μl of this solution was used. PCR conditions were as described above except that the reaction volume was 40 μl, all four dNTPs (each at 20 μM) were used, and no ^{35}S-labeled dATP was present. The amplified products of the appropriate sizes were clearly evident after electrophoresis in a 4% agarose gel containing ethidium bromide (16).

Cloning of Amplicons and DNA Sequencing. The reamplified amplicons were cloned using the Invitrogen TA cloning kit according to the manufacturer's instructions except that undiluted PCR product and one-tenth to one-fifth the recommended amount of cloning vector were used for the DNA ligation.

Double-stranded DNA was sequenced using an ABI Taq Dyedeoxy Terminator Cycle Sequencing (Applied Biosystems), kit and the sequences were analyzed using the BLAST program as described (16).

RESULTS

Optimization of Conditions for mRNA Differential Display. Several modifications of the method of Liang and Pardee (10) were necessitated due to (*i*) the limitations on the number of preimplantation embryos that are readily isolated, (*ii*) the small amount of mRNA that is present in these embryos (i.e., picogram amounts) (15), and (*iii*) the technical difficulties in isolating picogram amounts of poly(A)$^+$ RNA. The use of *Escherichia coli* rRNA as a carrier for embryo RNA isolation and use for quantification of recovery was not permissible because the pattern of amplicons after gel electrophoresis was the same whether or not embryo RNA was present (data not shown). Presumably the conditions for the differential display RT–PCR were so permissive that the resulting mispriming and amplification from the carrier rRNA completely masked the priming and amplification of the embryonic mRNAs, which were present in drastically lower concentrations.

To circumvent this problem, poly(C) was used as a carrier. Poly(C) was chosen since the 3′ primers used for the RT reaction were essentially poly(T) and hence mispriming should be virtually absent. Although a few bands were observed after RT–PCR of poly(C) alone, the banding pattern was not reproducible; i.e., similar banding patterns were not observed from lane to lane and different patterns were also observed from experiment to experiment (data not shown). To minimize the likelihood of detecting and cloning spurious bands due to the poly(C), we adopted the policy of analyzing each set of primers in triplicate for each developmental stage; i.e., after the RT reaction, three aliquots were removed and subjected to separate PCRs. Only bands that appeared in each lane and were of similar intensity were used for analysis of gene expression.

The effect of the temperature used for the RT reaction and PCR on the number of bands detected and the reproducibility of the band pattern was also examined. The RT was conducted at 35°C, 42°C, and 45°C, followed by PCR with an annealing temperature of either 42°C or 45°C for each RT

reaction. We observed that RT at 45°C followed by PCR with an annealing temperature of 42°C gave the best results with respect to the number of bands and band reproducibility (data not shown).

Validation of mRNA Differential Display for Mouse Preimplantation Embryos. Even though the samples were treated with DNase prior to RT, two additional lines of evidence suggested that DNA contamination, which could result in the generation of amplicons, was minimal. (*i*) The banding pattern showed dramatic and reproducible changes with respect to both the number of bands detected and their intensity during the stages of preimplantation development that were analyzed (Fig. 1). This would not be anticipated if the bands were due to amplification of DNA. (*ii*) When the RT reaction was not conducted, essentially no bands were observed (data not shown).

To validate further this method for preimplantation embryos, we designed a set of primers that would detect the cytokeratin endo A whose temporal pattern of expression during preimplantation development has been determined by S1 nuclease digestion (17). Endo A is virtually undetectable in the two-cell embryo, a low level can be detected in the eight-cell embryo, and its expression significantly increases by the blastocyst stage. In addition, the sequence of the murine gene is known, and therefore, we could confirm the identity of a putative endo A amplicon by DNA sequencing.

Although primers were synthesized that would generate an endo A-specific amplicon of 249 bp and the other amplicons that would be produced during the PCR portion of the reaction, we did not detect a band of this size that displayed the appropriate developmental changes in intensity (data not shown). Nevertheless, when the 5′ endo A-specific primer was used in conjunction with the 3′ primer $T_{11}GA$, there was a prominent amplicon of about 250 bp with the correct temporal pattern of expression (Fig. 1*A*, band 2). It was virtually absent in two-cell embryos, could be detected in the eight-cell embryo, and was quite apparent in the blastocyst; quantification of these changes is shown in Fig. 2*A*. Sequence analysis of this amplicon revealed that it was identical to that of endo A (see the *Discussion* for an explanation as to how this situation arose).

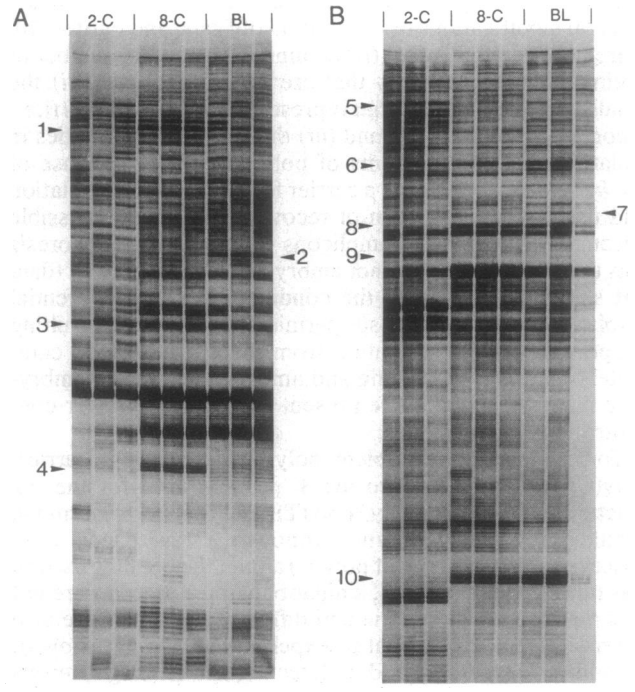

FIG. 1. Portion of an autoradiogram of amplicons obtained from mRNA differential display using preimplantation mouse embryos. The primers used in *A* were 5′-AGTGAATGGC-3′ (5′ primer) and 5′-T_{11}GA-3′ (3′ primer), and those in *B* were 5′-GAGGATCAGC-3′ (5′ primer) and 5′-T_{11}GC-3′ (3′ primer). For each developmental stage, the isolated RNA was subjected to RT using a specific 3′ primer and then triplicate portions were subjected to PCR. 2-C, two-cell stage; 8-C, eight-cell stage; BL, blastocyst. The numbered chevrons point to specific amplicons that are discussed in the text and that were sequenced. The overall lower intensity of the last lane in *B* was likely due to less efficient PCR after the RT reaction.

Use of mRNA Differential Display to Analyze Gene Expression in the Preimplantation Mouse Embryo. A series of primer combinations were utilized to generate a series of expression profiles for two-cell, eight-cell, and blastocyst-stage embryos, and the results of two sets of primers are shown in Fig. 1. Examination of these gels revealed many developmental changes in gene expression and some of these are indicated. Some amplicons (e.g., amplicons 5 and 9) were most prominent in the two-cell embryo and then were virtually absent by the eight-cell stage; these may represent maternal mRNAs, the identity and function of which is poorly understood in the mouse embryo. The expression of another class of amplicons was essentially constant at each of the stages analyzed (e.g., amplicons 3 and 6), and these may represent genes that are constitutively activated at the two-cell stage. An increase in expression of another class of amplicons increases between the two-cell and eight-cell stages (e.g., amplicons 8 and 10), and these likely represent genes whose activation is delayed relative to the initial onset of zygotic transcription that is clearly evident at the two-cell stage (3). The expression of several amplicons (e.g., amplicons 1 and 4) was greatest at the eight-cell stage, when compared to the expression at the two-cell and blastocyst stages, and these may represent genes expressed in a stage-specific manner. Last, the expression of some amplicons [e.g., amplicons 2 (endo A) and 7] was greatest in the blastocyst, and these may represent genes that are markers for blastocyst development. The relative changes in band intensity are summarized in Fig. 2.

The bands marked in Fig. 1 were eluted, amplified, cloned, and sequenced. As stated above, amplicon 2 corresponded to endo A. Amplicon 9 was identical to a region of the nuclear-encoded α subunit of the mouse F_1 ATP synthase (nt 1584–1833) (19). This gene contains two polyadenylylation consensus sequences that yield transcripts of two distinct lengths (19); amplicon 9 corresponds to the shorter transcript. The remaining amplicons did not reveal any significant homology to any sequence present in the databanks scanned by the BLAST program.

DISCUSSION

We describe here modifications and the use of the mRNA differential display method to examine changes in gene expression during preimplantation embryogenesis in the mouse. The method detects the known changes in the temporal pattern of expression of endo A and anticipated patterns of gene expression—e.g., loss of maternal mRNAs, onset of constitutively activated genes, and genes expressed in a stage-specific manner.

The small amount of poly(A)$^+$ RNA present in the preimplantation mouse embryo coupled with the severe limitations in readily obtaining large numbers of embryos and in the ability to quantify recovery of poly(A)$^+$ RNA prohibited us from using purified poly(A)$^+$ RNA in these studies. Consequently, total embryo RNA was used. Two lines of evidence suggest that the observed bands are not attributable to endogenous rRNA. (*i*) The pattern of banding changes during preimplantation development, and such should not be the case if the bands are derived from endogenous rRNA. (*ii*) None of the bands that have been reamplified and sequenced

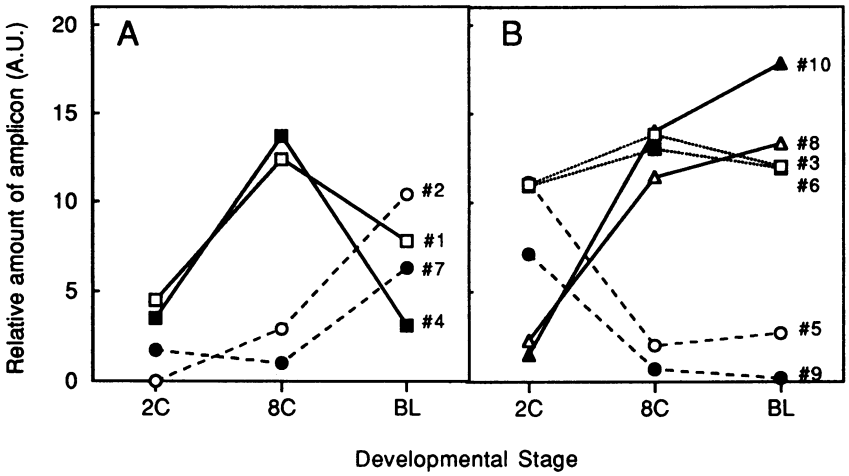

FIG. 2. Relative changes in amplicon expression during preimplantation embryogenesis. (*A*) Amplicons whose expression pattern is consistent with stage-specific expression or markers for blastocyst formation. (*B*) Amplicons whose expression pattern is consistent with being a maternal mRNA or genes that are zygotically activated at the two-cell stage or eight-cell stage. The number refers to the corresponding amplicon shown in Fig. 1. Quantification of changes in the expression of the amplicons indicated in Fig. 1 was performed with an Image I/AT image processor (Interactive Video Systems, Concord, MA.) as described (18). For each band, an area of the same size in a region of the gel that did not have any bands was used to determine the background and this value was subtracted from the value obtained for the band being analyzed. The data are presented as the mean number of arbitrary units (A.U.) and the range was usually <5% of the mean. 2C, two-cell embryo; 8C, eight-cell embryo; BL, blastocyst.

reveals any homology to rRNA. The ability to use total RNA for the mRNA differential display method is in agreement with a recent report that total RNA obtained from somatic cells is suitable (12).

Three lines of evidence suggest that the changes detected in gene expression during preimplantation development by the mRNA differential display method are likely to reflect real differences, as opposed to being due to some intrinsic variability of the method. (*i*) The changes are reproducible. (*ii*) The method detects the appropriate temporal changes in endo A expression, and moreover, the magnitude of these changes is similar to that detected by S1 nuclease digestion (17). (*iii*) Although amplification of different amounts of RNA can result in the change in intensity of some bands (11), similar amounts of poly(A)$^+$ RNA were subjected to RT–PCR; i.e., we typically subjected about 10 pg of poly(A)$^+$ RNA at each developmental stage to RT–PCR.

As reported (11–13), we also observe that amplicons smaller than 300 bp frequently appear as doublets or triplets; these doublets/triplets behave as a functional unit in that developmental changes in their intensity occur in a coordinate fashion. These multiple bands have been shown to be due to the additional deoxyadenosine that is added by *Taq* polymerase and the small differences in the electrophoretic mobility of the two complementary DNA strands in the DNA sequencing gel (13).

Sequence analysis of the endo A amplicon suggests a mechanism by which the 3′ $T_{11}GA$ primer could serve as a primer for this mRNA, when the appropriate 3′ primer, which was based on the endo A sequence, should have been $T_{11}AG$. The nucleotide sequence of endo A 5′ to the poly(A) tail is TCAACT (20). Thus, the low temperatures used for the annealing of the 3′ primer results in a situation in which the AACT sequence that precedes the poly(A) tail acts as a continuation of the poly(A) tail via tolerated mismatches at the C and T residues. A consequence is that the $T_{11}GA$ primer now efficiently serves as a primer for endo A.

Two of the 10 amplicons that were sequenced reveal homology to previously identified genes, namely, endo A (amplicon 2) and the α subunit of the mitochondrial F_1 ATP synthase (amplicon 9); for each of these genes the sequence of the 5′ region of the amplicon extends into the coding region. None of the other sequences reveal any significant homology to known genes and, therefore, may represent uncharacterized genes. The temporal pattern of expression of the putative α subunit of the F_1 ATP synthase is consistent with it being a maternal mRNA that is essentially degraded by the eight-cell stage. The number of maternally inherited mitochondria remains constant at about 92,500 between the egg and blastocyst stages (21, 22); this number is likely to increase with the onset of trophoblastic giant cell transformation, which coincides with the onset of embryo growth. Thus, for this nuclear-encoded mitochondrial gene (and perhaps for other nuclear-encoded mitochondrial genes), there is no apparent demand for zygotic transcription during preimplantation development to replace the maternally derived and degraded transcripts. Reactivation of transcription of this gene, however, must presumably occur later in development when there is net embryo growth. Interestingly, although the transcripts of this nuclear-encoded gene decrease during preimplantation development, transcripts of mitochondrial-encoded genes (e.g., subunits I and II of cytochrome *c* oxidase) increase (22).

The major advantage of the mRNA differential display method to analyze gene expression as opposed to the use of the cDNA libraries generated to preimplantation embryos at different stages of development is that the mRNA differential display method requires readily obtainable numbers of embryos and permits analysis of gene expression during very narrow developmental windows. For example, results of recent experiments using nuclear transfer (18) or a luciferase reporter gene (23) suggest that transcription may start during the late one-cell stage. Analyzing highly synchronized one-cell embryos (23) that are in G_2 phase by the differential display method should afford a means to ascertain whether endogenous genes are expressed at this time. Results of experiments that employed two-dimensional gel electrophoresis to analyze gene expression during the two-cell stage reveal a class of proteins whose synthesis is inhibited by α-amanitin and is restricted to the two-cell stage (2). The mRNA differential display method is aptly suited to detect the expression of such genes that are expressed in a stage-specific manner, since it is quite feasible to isolate RNA from two-cell embryos every 3 h after harvest of cohorts that cleave to the two-cell stage within 30 min. In each of these instances, the production of cDNA libraries to embryos at

each of these rather restricted periods in preimplantation development would be most difficult, if not impossible. Because the mRNA differential display method is optimized to generate cDNAs that are relatively small (i.e., 100–500 bp), the marriage of this method with the cDNA libraries generated from preimplantation embryos (9) should permit the isolation and characterization of longer or full-length cDNAs.

In summary, the results described here demonstrate the suitability of the mRNA differential display method to analyze gene expression in the mouse preimplantation embryo. To date, we have used 12 combinations of 5′ and 3′ primers and have noted numerous changes in the pattern of gene expression during preimplantation development. The approach can readily be expanded to include the use of enough primer combinations that should display the entire spectrum of transcripts at each developmental stage, since 20–25 different 5′ primers in combination with four degenerate 3′ primers are required to cover the total pattern of gene expression (11, 12). This type of analysis should provide a most accurate and detailed description of gene expression during preimplantation embryogenesis and open up many avenues of investigation of gene function during this stage of embryogenesis that were previously either not feasible or technically very difficult and laborious.

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