Igf2r and Igf2 gene expression in androgenetic, gynogenetic, and parthenogenetic preimplantation mouse embryos: absence of regulation by genomic imprinting

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Genomic imprinting in mammals is believed to result from modifications to chromosomes during gametogenesis that inactivate the paternal or maternal allele. The genes encoding the insulin-like growth factor type 2 (Igf2) and its receptor (Igf2r) are reciprocally imprinted and expressed from the paternal and maternal genomes, respectively, in the fetal and adult mouse. We find that both genes are expressed in androgenetic, gynogenetic, and parthenogenetic preimplantation mouse embryos. These results indicate that inactivation of imprinted genes occurs postfertilization (most likely postimplantation) and that genomic imprinting and gene inactivation are separate processes. We propose that imprinting marks the chromosome so that regulatory factors expressed in cells at later times can recognize the imprint and selectively inactivate the maternal or paternal allele. For these genes, this finding invalidates models of genomic imprinting that require them to be inactive from the time of fertilization.

[Key Words: Genomic imprinting, preimplantation mouse embryo; Igf2, Igf2r, androgenone, gynogenone]
Results from transgenic studies reveal that both maternal and paternal patterns of transgene methylation are erased in primordial germ cells and become re-established during germ cell maturation (Chaillet et al. 1991; Ueda et al. 1992). Interestingly, whereas the maternal pattern of methylation is completely established during oogenesis, the paternal pattern of methylation of at least one transgene changes between days 3.5 and 6.5 of development (Chaillet et al. 1991), possibly as a consequence of the remodeling of the paternal genome [Howlett and Reik 1991]. For the Igf2r gene, a maternal pattern of methylation in region 2, which is within an intron, is also established during oogenesis, but a paternal pattern of methylation in region 1, which encompasses the promoter and transcription start site, is established well after fertilization [Brandeis et al. 1993; Stoger et al. 1993]. Allele-specific patterns of methylation are not detected for either the Igf2 gene or the H19 gene in gametes and early cleavage-stage embryos [Brandeis et al. 1993]. It has been suggested that the allele-specific patterns of methylation observed for the Igf2, Igf2r, and H19 genes in adult cells are established in a stepwise manner during embryogenesis and that only a select few methylation groups exist in the gamete and early embryo that denote parental identity of imprinted alleles.

It is not yet clear whether differential methylation determines the ability of an allele to be expressed or merely stabilizes an inactive state after it is established. A paradigm for studying methylation effects on gene expression is X chromosome inactivation. Although some X-linked genes become methylated near the time of inactivation [Singer-Sam et al. 1990; Grant et al. 1992], other X-linked genes become methylated after inactivation, indicating that methylation may stabilize the inactive state [Kaslow and Migeon 1987; Lock et al. 1987]. Moreover, selective paternal X inactivation in extraembryonic tissues, which is believed to reflect X chromosome imprinting, apparently does not involve permanent modifications of the DNA itself [Kratzer et al. 1983]. In the midgestation fetus, the imprinted Igf2 gene, which is located on chromosome 7, does not exhibit parent-specific methylation or DNase I insensitivity in or around its promoter, but such methylation differences do exist farther upstream, and despite its potentially open chromatin conformation, only a very low level of expression of the maternal Igf2 gene is detected by reverse transcription polymerase chain reaction [RT–PCR] [Sasaki et al. 1992]. In contrast, the nearby reciprocally imprinted H19 gene exhibits paternal-specific hypermethylation and DNase I insensitivity [Ferguson-Smith et al. 1993; Bartolomei et al. 1993]. Thus, the establishment of parent-specific methylation patterns and altered chromatin states may vary among imprinted genes or their promoters.

The absence of most of the parental-specific differences in methylation in gametes and early embryos, for both certain transgenes and endogenous genes [e.g., Igf2r, Igf2, and H19], raises the question of whether imprinted genes behave as if they are imprinted prior to the establishment of these methylation patterns. With one transgenic line, genetic background effects, as well as parental origin effects, are observed (Surani et al. 1990). The genetic background effects are attributed to modifier factors present in the egg cytoplasm that affect expression from the paternal genome but not the maternal genome [Surani et al. 1990; Engler et al. 1991]. Consistent with this, the developmental potential of androgenetic mouse embryos is also influenced by factors in the egg (Latham and Solter 1991). Taken together, these observations indicate that inactivation of at least some imprinted genes probably involves epigenetic modifications that occur postfertilization as well as those that occur during gametogenesis. Given these observations, there is reason to suspect that the imprint established during gametogenesis may not be manifested as a difference in ability of an allele to be expressed until some time after fertilization.

To test this possibility, we examined the expression of the paternally imprinted Igf2r gene and the maternally imprinted Igf2 gene in androgenetic, gynogenetic, and normal preimplantation stage mouse embryos. To do this, we used a semiquantitative RT–PCR assay that allows comparison of the relative amounts of mRNAs present in embryos at either different stages of development or the same stage of development [Temeles et al. 1994]. We find that androgenones at both the eight-cell and blastocyst stages express significant amounts of Igf2r mRNA and protein that are well in excess of what can be attributed to maternally derived Igf2r mRNA or protein. We also find that parthenogenetic blastocysts express significant amounts of Igf2 mRNA. These results indicate that the paternally imprinted Igf2r gene and the maternally imprinted Igf2 gene can be expressed throughout the preimplantation period and that the methylation differences between maternal and paternal genomes observed at fertilization are not sufficient to regulate allelic expression.

Results

Igf2r mRNA expression in normal embryos

Our first objective was to measure changes in the relative amounts of Igf2r mRNA present in egg, two-, and eight-cell stage embryos to provide a quantitative basis to compare relative Igf2r mRNA abundances in eight-cell androgenones, gynogenones, and normal embryos, particularly with regard to how much of this mRNA might be maternally derived by the eight-cell stage. To do this, we used a semiquantitative RT–PCR assay in which exogenously added globin mRNA serves as a control for RNA recovery and the efficiency of the RT–PCR reactions [Temeles et al. 1994]. This assay permits the comparison of relative abundances of a particular mRNA in embryos at different stages of development or different types of embryos [e.g., androgenones vs. gynogenones] at the same stage of development. We have previously documented that the relative changes that we observe in actin mRNA abundance during preimplanta-
tion development with this RT-PCR assay are very similar to those determined by Northern blot analysis [Temeles et al. 1994].

In contrast to an earlier report [Rappolee et al. 1992], we readily detected Igf2r mRNA in the unfertilized egg [Fig. 1A,D]. The Igf2r mRNA abundance declined sharply by the two-cell stage and then increased 15-fold by the eight-cell stage. This trend—a decrease between the egg and two-cell stage, followed by an increase by the eight-cell stage—is characteristic of a number of genes that are expressed following zygotic gene activation, which occurs during the two-cell stage in the mouse [Flach et al. 1982; Bensaude et al. 1983; Latham et al. 1991]. Actin, which is not an imprinted gene, is such an example [Fig. 1B,D]. Thus, the amount of Igf2r mRNA detected at the eight-cell stage reflected zygotic gene transcription and could not be attributed to maternal mRNA.

Igf2r mRNA and protein expression in androgenones and gynogenones

To determine whether parental-specific imprinting mod-

![Figure 1. Changes in the relative amounts of Igf2r and β-actin mRNAs during preimplantation development. (A) Ethidium bromide-stained gel of endogenous 186-bp diagnostic Igf2r fragment. (B) Ethidium bromide-stained gel of endogenous 539-bp diagnostic β-actin fragment. (C) Ethidium bromide-stained gel of exogenous 257-bp α-globin fragment. (D) Relationship between the relative amount of Igf2r mRNA and actin mRNA, and different developmental stages. The ratio of the number of cpm present in the Igf2r or actin diagnostic fragments to that in the globin fragment was calculated for each developmental stage. The value for the two-cell embryo was set at 1 and the values of the other stages were expressed relative to it. (Solid bars) Igf2r; (open bars) actin.](#)

ifications that occur during gametogenesis are sufficient to affect Igf2r gene expression in the preimplantation embryo, we compared the relative levels of Igf2r mRNA in two-cell and eight-cell androgenones, gynogenones, and normal embryos. We chose this approach rather than using interspecies hybrids that permit the expression of maternal and paternal alleles to be distinguished by restriction length polymorphisms because using nuclear transplant embryos eliminates the possibilities of differential modification of parental alleles by egg modifier factors or postfertilization interactions between maternal and paternal genomes.

As above, two-cell embryos of all three classes expressed very little Igf2r mRNA [Fig. 2A]. In contrast, eight-cell embryos of all three classes contained a significant amount of Igf2r mRNA [Fig. 2A]. Quantitative analysis of the PCR amplicons revealed that androgenetic eight-cell embryos exhibited an ~40-fold greater amount of Igf2r mRNA/embryo [Igf2r/globin = 4.39] than androgenetic two-cell embryos [Igf2r/globin = 0.11]. Furthermore, the amount of Igf2r mRNA/eight-cell embryo was similar among all three classes [Fig. 3].

Failure to detect Igf2r mRNA in the two-cell embryos of androgenones and gynogenones, and the reduced amount detected in normal two-cell embryos could not be attributed to inadequate RT-PCR conditions, as both actin and exogenously added globin mRNAs were detected in all three classes of two-cell embryos [Fig. 2C,E]. As a control for correct pronuclear identification, we also assayed expression of Xist RNA in androgenones, gynogenones, and normal embryos. Xist is expressed exclusively from the paternal X chromosome beginning at the eight-cell stage [Kay et al. 1993] and so should not be expressed in gynogenones. As expected, androgenones and control embryos expressed Xist at the eight-cell stage, but not the two-cell stage, whereas very little Xist mRNA was detected in either two-cell or eight-cell gynogenones [Figs. 2D and 3]. The >20-fold lower Xist/globin ratio in gynogenones as compared with androgenones or controls indicated a maximum error of 5% in pronuclear identification or, more likely, a low but detectable level of expression of Xist in eight-cell gynogenones. Thus, incorrect pronuclear identification could not account for the observed Igf2r expression in androgenones.

To determine whether the mRNA detected in androgenones at the eight-cell stage could support the expression of IGF2R, we examined androgenetic, gynogenetic, and normal embryos at the eight-cell stage for the presence of IGF2R using a specific rabbit polyclonal antiserum [C-1; Hartshorn et al. 1989]. As with the RT-PCR analysis, immunocytochemical analysis of normal eggs, two-cell, and eight-cell stage embryos revealed detectable expression of the IGF2R in the egg, reduced expression in the two-cell embryo, and elevated IGF2R expression at the eight-cell stage [Fig. 4A]. Our finding that IGF2R was present in the egg differed from a previous report [Harvey and Kaye 1991], and this difference was most likely attributable to our use of permeabilized embryos; nonpermeabilized embryos were used by Harvey.
Expression of imprinted genes in mouse embryos

It has been observed that the expression of imprinted genes is regulated in a tissue-specific manner. Figure 2 illustrates the expression of Igf2r, Igf2, actin, and Xist RNAs in androgenetic, gynogenetic, and normal two-cell and eight-cell embryos. (A) Igf2r; (B) Igf2; (C) actin; (D) Xist; (E) exogenously added globin. (Lanes 1-3) two-cell embryos; (lanes 4-6) eight-cell embryos. (Lanes 1,4) Androgenones; (lanes 2,5) gynogenones; (lanes 3,6) normal embryos. The two outermost lanes show a 100-bp ladder. The arrowhead points to the 200-bp marker.

Androgones also expressed the receptor at the blastocyst stage (Fig. 4E).

To verify further that IGF2R expression in androgenones was attributable to expression of the paternally derived alleles, androgenetic embryos from C57BL/6 eggs fertilized by T<sup>br</sup> heterozygous mutant males (McGrath and Solter 1984b) were analyzed for IGF2R expression by immunofluorescence. Approximately one-fourth of such embryos should be homozygous for the T<sup>br</sup> deletion and so lack a gene encoding the IGF2R. Of 36 such androgenones subjected to immunofluorescent staining, 29 showed significant staining and 7 showed only very low, background fluorescence (data not shown). This additional evidence is consistent with the interpretation that the expression of the Igf2r gene in androgenones was mediated by the paternally derived genes.

Taken together, these results indicate that the Igf2r gene is transcribed, the mRNA accumulates in androgenetic eight-cell embryos, and the receptor is expressed at both the eight-cell and blastocyst stages. Because androgenones contain only paternally derived chromosomes, these results indicate that paternal Igf2r genes can be...
Figure 4. Immunocytochemical analysis of IGF2R in androgenones, gynogenones, and control embryos. (A) Field of unfertilized eggs, two-cell, and eight-cell control embryos stained with the IGF2R antiserum. Note that a reduced intensity of staining is observed between the unfertilized egg and two-cell stages and that this intensity increases by the eight-cell stage. The arrowheads point to a representative egg, two-cell embryos (2C), and eight-cell embryo (8C). (B) Field of unfertilized eggs, two-cell, and eight-cell control embryos stained with the immunodepleted IGF2R antiserum. Note the reduction in the intensity of staining, when compared with the embryos shown in A. The arrowheads point to a representative egg, two-cell embryo (2C), and eight-cell embryo (8C). The intense staining material adjacent to the 8C embryo is attributable to nonspecific staining of debris. (C) Eight-cell androgenone. The arrows point to punctate regions of immunofluorescence characteristic of IGF2R staining. (D) Eight-cell gynogenone. (E) Androgenetic blastocyst.

Igf2 mRNA expression in gynogenones and parthenogenones

To test whether absence of regulation by imprinting in preimplantation embryos was either a specific property of the Igf2r gene or restricted to paternally imprinted genes, we examined expression of the maternally imprinted Igf2 gene in gynogenones (Figs. 2B and 3). Gynogenones appeared to have less Igf2 mRNA than either androgenones or control embryos at the eight-cell stage. This would be consistent with lack of expression of maternal Igf2 alleles due to imprinting, as apparently occurred with Xist gene expression (Figs. 2D and 3). In contrast to the situation with Xist, for which no RNA was detected in the egg or two-cell stage embryo, and in contrast to a previous report (Rappolee et al. 1992), we detected a substantial amount of Igf2 mRNA in both eggs (data not shown) and androgenetic, gynogenetic, and normal two-cell embryos (Fig. 2B). Moreover, for androgenones, gynogenones, and control embryos, more Igf2 mRNA was present at the two-cell stage than at the eight-cell stage (Fig. 2B). For example, two-cell androgenones contained about four times more Igf2 mRNA than eight-cell androgenones. Thus, the differences in the amount of Igf2 mRNA observed between gynogenetic, androgenetic, and normal eight-cell embryos may have arisen from differences in rates of development or rates of maternal Igf2 mRNA degradation. Consequently, we could not conclude that the lower Igf2 mRNA abundance in gynogenones reflected imprinting.

The aforementioned results indicated that we could not assess the effect of imprinting on Igf2 gene expression at the eight-cell stage or before. Since we could conclude, however, that most of the maternal Igf2 mRNA for the gynogenone was degraded by the end of the eight-cell stage, we examined the effect of imprinting on Igf2 gene expression at the later blastocyst stage. For these experiments, we used parthenogenetically activated eggs in order to obtain readily sufficient numbers of two-, eight-cell, and blastocyst stage embryos (Figs. 5 and 6). As with the gynogenones, we observed more (~43%) Igf2 mRNA at the two-cell stage than at the eight-cell stage (Figs. 5B and 6). In addition, there was, however, about 2.3-fold more Igf2 mRNA expressed in parthenogenetic blastocysts than parthenogenetic eight-cell embryos (Figs. 5B and 6). The amounts of Igf2r and actin mRNA also increased between the two-cell and blastocyst stages as expected (Figs. 5 A,C and 6). Immunosurgically isolated parthenogenetic inner cell masses also expressed Igf2 mRNA (data not shown). Thus, the absence of regulation by genomic imprinting observed with the Igf2r gene was not a specific feature of that gene.

Taken together, these results indicate that the Igf2 gene is transcribed and the mRNA accumulates in parthenogenetic blastocysts. Because parthenogenones contain only maternally derived chromosomes, these results indicate that maternal Igf2 genes can be expressed during preimplantation mouse development and therefore are not inactivated as a consequence of the maternal imprint.

Discussion

The Igf2r and Igf2 genes are reciprocally imprinted and expressed exclusively from the maternal and paternal ge-
Expression of imprinted genes in mouse embryos

We report here an important and previously undocumented feature of regulation of imprinted genes—namely, that for both of these reciprocally imprinted genes, both maternal and paternal alleles can be expressed during preimplantation development in the mouse. This is revealed as an increase in expression at the mRNA level for both genes, as well as the protein level for the IGF2R gene in developing preimplantation embryos that contain exclusively either paternal or maternal chromosomes. The observation that the appropriate stage-specific increases in expression of both genes occur in both classes of embryos is indicative of a bona fide developmentally regulated activation and expression of both alleles rather than unregulated “leaky” expression. Thus, although these genes are endowed with a genomic imprint during gametogenesis that regulates allelic expression during fetal and adult life, this imprint does not by itself regulate allelic expression during preimplantation development. Furthermore, our observation indicates that the differences in methylation observed between maternal and paternal genomes at the time of fertilization are not sufficient to regulate allelic expression.

Inactivation of paternally imprinted genes postfertilization may be indicative of an ongoing process that initiates at fertilization and continues thereafter. This process may begin with modifications mediated by egg modifier factors, which affect both transgene expression (Surani et al. 1990; Engler et al. 1991) and development of androgenones (Latham and Solter 1991), and then continue during later stages as the paternal patterns of chromatin structure and DNA methylation become established. It is also possible that interactions between maternal and paternal genomes during early embryogenesis or the presence of maternally derived X chromosomes might contribute to such postfertilization modifications. Such interactions would not occur in androgenones, gynogenones, or parthenogenones. The paternal patterns of methylation of certain transgenes and of the Igf2r locus itself (region 1) are not present at fertilization but, instead, arise following implantation (Chaillet et al. 1991, Stoger et al. 1993). Maternal patterns of methylation of certain transgenes, and of the Igf2r gene itself, are present in the egg and at fertilization (Chaillet et al.)

**Figure 5.** Patterns of expression of Igf2r, Igf2, and actin in parthenogenetic embryos. (Lanes 1–3) Two-cell, eight-cell, and blastocyst stage embryos, respectively. (A) Igf2r; (B) Igf2; (C) actin; (D) exogenously added globin. The molecular weight markers are as in Fig. 2. The diagnostic amplicon corresponds to the major, slower migrating band; only this band was used for quantification.

**Figure 6.** Histogram summarizing Igf2r, Igf2, and actin expression in parthenogenetic embryos. (Solid bars) Two-cell embryos; (open bars) eight-cell embryos; (stippled bars) blastocysts. The data, which are derived from the gels shown in Fig. 5, are expressed as the ratios of the number of cpm in the amplicon of each mRNA divided by the number of cpm in the globin amplicon.
The allele-specific patterns of methylation of the IGF2 and H19 genes seen in adult cells are not present in gametes and early cleavage-stage embryos [Brandeis et al. 1993]. Thus, the allele-specific patterns of methylation of all three of these endogenously imprinted genes are acquired progressively during embryogenesis. Our data indicate that the acquisition of these methylation patterns may be essential to achieve selective allelic expression.

It has been suggested [Stoger et al. 1993] that the maternal-specific methylation in region 2 of the IGF2r gene promoter is necessary for expression, as methylation at this locus is present at fertilization. Our data, however, suggest that the maternal methylation pattern (region 2) of the IGF2r gene is not essential for gene expression in the preimplantation embryo, as paternal alleles, which lack both the region 2 and region 1 methylation modifications, are expressed in androgenotes. Because our data indicate that functional inactivation of the IGF2r gene occurs postfertilization, it is more likely that the maternal methylation in region 2 either enhances expression or prevents inactivation of this allele while paternal-specific methylation of region 1 accompanies or follows overt inactivation of the paternal allele.

Given the genetic data that demonstrate that imprinting occurs during gametogenesis [Forejt and Gregorova 1992], the simplest interpretation of our data is that imprinting and subsequent inactivation of a gene comprise two distinct events that are separable both spatially and temporally. This would be consistent with the original definition of chromosomal imprinting put forth by Crouse (1960) as a mark that the cell can use to distinguish maternal and paternal alleles. Although imprinted genes in mammals have been historically defined in a functional sense, our data suggest that the functional state of an imprinted gene depends upon the cellular context within which gene expression is assayed. We propose that the genomic imprint, when defined as a chromosomal mark that distinguishes maternal and paternal alleles, does not by itself determine the functional state of a gene. Rather, a genomic imprint may provide a bias for inactivation of one allele by other processes during later development, and this inactivation is likely to involve the establishment of the complete allele-specific patterns of methylation [Brandeis et al. 1993]. The low level of maternal IGF2 allelic expression [Sasaki et al. 1992] indicates that when this inactivation occurs, it need not be absolute. Because the genomic imprint that is present in the gamete is apparently not sufficient to inactivate a gene, we suggest that specific regulatory factors that recognize the imprint are required to inactivate the imprinted allele. Such factors could be expressed in a stage-specific or tissue-specific manner and this could account for the delayed inactivation of the maternal IGF2 allele and paternal IGF2r allele shown here, as well as the contribution of egg modifiers [Surani et al. 1990; Engler et al. 1991; Latham and Solter 1991], expression of the maternal IGF2 gene in select tissues [DeChiara et al. 1991], expression of the H19 gene in human androgenetic trophoblasts [Mutter et al. 1993], differences in DNA methylation and chromatin state among imprinted genes [Sasaki et al. 1992; Ferguson-Smith et al. 1993], and selective paternal X chromosome inactivation in extraembryonic cells and early somatic cells in the absence of hypermethylation and heterochromatization [Kaslow et al. 1987; Lock et al. 1987]. Lack of expression of such factors could also contribute to loss of imprinting [Ogawa et al. 1993; Rainier et al. 1993].

Our data can also be interpreted within the broader context of the overall function that imprinting serves in development. It has been suggested that imprinting in mammals permits a necessary incremental reduction in the expression of certain genes that might not be otherwise achievable because of an inability of certain genes to be transcriptionally modulated with a sufficient degree of control [Solter 1988]. By analogy with paternal X inactivation in extraembryonic tissues, this need might in part relate to demands of placenta tion, although a similar need might also exist in somatic tissues [Solter 1988]. Imprinting provides a means by which the cell can consistently reduce its expression of certain genes by one-half. If imprinting evolved to allow such an incremental regulation of gene expression in specific tissues, then those tissues would by necessity express specific factors that recognize an imprint and then promote allelic inactivation. The prediction from such a system would be that if a gene that is normally regulated in this way is also expressed in the preimplantation embryo, before expression of the factors that promote allelic inactivation, then both alleles should be expressed.

Materials and methods

Mouse embryo collection and culture

Mice were obtained from Harlan Sprague-Dawley. Embryos were isolated at the one-cell stage from C57BL/6 females 6-8 weeks of age that had been mated to C57BL/6 males and cultured as described [Latham and Solter 1991]. Nuclear transplantation was performed as described [McGrath and Solter 1983; Latham and Solter 1991]. Embryos were cultured until ~48 hr (two-cell) or 76 hr (eight-cell) post-hCG injection when they were lysed for PCR analysis. For the C57BL/6 strain, the culture conditions employed allow a high efficiency of development of both androgenotes and gynogenotes to the compacted eight-cell stage, typically 80–100% for androgenotes and 95–100% for gynogenotes. Embryos that blocked in development were excluded from analyses. Thus, possible effects of differences in developmental potentials of the different classes of embryos were largely avoided. Parthenogenetic embryos were obtained following ethanol activation of B6D2 eggs [Hogan et al. 1986]. Two-cell, eight-cell, and blastocyst stage embryos were collected 27, 52, and 93 hr, respectively, postactivation. Immuno- surgery of blastocysts was performed as described previously [Solter and Knowles 1975].

RT-PCR

Unless stated otherwise, all solutions were prepared with water that had been treated with 0.1% diethylpyrocarbonate. For lysis, embryos were transferred in a minimal volume to a chilled 0.6-ml microcentrifuge tube on ice containing 20–100 μl of lysis buffer (100 μl of 4 M guanidine thiocyanate, 0.1 M Tris-HCl [pH
30 min at 37°C. Following the addition of a second volume of pH 7.9, 10 mM NaCl, 6 mM MgCl₂. DNA was degraded by incubating the sample with 1 unit of RQ1 DNase (Promega) for 1 hr at 42°C. The samples were precipitated overnight at -20°C.

The nucleic acid was collected by centrifugation at 10,000g for 15 min at 4°C. The large off-white pellets were then washed once at 4°C with cold 75% ethanol. The pellets were resuspended in 20 μl of resuspension solution (RS: 40 mM Tris-HCl at pH 8.3, 75 mM KCl, and 3 mM MgCl₂). DNA was degraded by incubating the sample with 1 unit of RQ1 DNase (Promega) for 30 min at 37°C. Following the addition of a second volume of RS, the samples were extracted with 40 μl of RS saturated phenol (Amresco). The samples were vortexed, centrifuged for 3 min at 10,000g, and the aqueous phase was transferred to a 0.5-ml microcentrifuge tube. Five microliters of 3M potassium acetate, and 60 μl of 100% ethanol. The samples were precipitated overnight at -20°C.

The recovery of carrier RNA, and by inference, of embryo RNA, usually ranged between 85 and 100%.

Reverse transcription was conducted on 50 egg or embryo equivalents. The reactions were carried out in 20 μl of 25 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂, containing 1 μM each of dATP, dCTP, dGTP, dTTP, 10 mM dithiothreitol, 1 U/μl of RNasin, and 0.4 μg of oligo(dT)₁₂₋₁₈ (Pharmacia). The tubes (Gene Amp thin-walled tubes) were incubated at 37°C for 2 min, 200 units of reverse transcriptase (SuperScript, BRL) were added, and the tubes were transferred to a 9600 PCR thermocycler (Cetus-Perkin Elmer). Reverse transcription was conducted for 1 hr at 42°C. The samples were then heated for 5 min at 99°C and placed on ice. At this point the samples were either used immediately for PCR or stored at -20°C.

The PCR was performed in 100 μl of 10 mM Tris-HCl (pH 8.3), containing 50 mM KCl, 1.45 mM MgCl₂, 0.2 mM each of the four dNTPs, 5 μCi of [α-³²P]dCTP (sp. act. 3000 Ci/m mole, Amer sham), 2.5 units of AmpliTaq polymerase (Perkin-Elmer Cetus), 20 pmol (0.4 μM) each of the appropriate 3′ and 5′ primers, and a volume of the reverse transcription reaction. When Igf2r, Igf2, Xist, β-actin, or β-globin cDNAs were amplified, this volume was equivalent to 10, 10, 5, 5, or 5 embryos, respectively. PCR primers used and the size of the diagnostic amplicons are shown in Table 1.

The basic PCR program used was 95°C for 1 min, followed by a cycle program of 95°C for 10 sec and 60°C for 15 sec. The last cycle was concluded with a 6-min extension at 60°C. For each set of primers, semilog plots of the amount of radioactively labeled amplicon product as a function of cycle number was used to determine the range of cycle number over which amplicon production was linear on such plots (data not shown). This region of linearity varied for each set of primers due to differences in efficiency of amplification. The number of cycles was 50, 35, 33, and 29 for Igf2r, Igf2, actin, Xist, and globin, respectively. It should be noted that because the absolute efficiency of amplification for each set of primers during each cycle is not known, one cannot compare the abundance of one mRNA to that of another mRNA. The assay can be used, however, to compare relative abundances of one mRNA among different samples [Temeles et al. 1993].

Following PCR, the tubes were chilled briefly on ice and centrifuged to collect any condensation. Then 25 μl was removed and treated with 2 μl of a 20 μg/ml solution of RNase A (Worthington) for 10 min at room temperature to digest any remaining carrier RNA. After the addition of 5 μl of 6× loading buffer (0.25% bromphenol blue in 40% sucrose), 13 μl of each sample was subjected to electrophoresis on a 4% agarose gel [Manejwala et al. 1991]. The gels were photographed under UV light, the bands were excised with a clean razor blade, and the incorporation of radioactively labeled dCTP in each gel slice was determined by Cerenkov counting. Generation of the diagnostic fragment was strictly dependent on the presence of RNA in the reverse transcription reaction, as when the reverse transcription step was omitted, no amplified fragments were observed (data not shown). In addition, amplicon identities were confirmed by appropriate restriction digests of PCR products (data not shown).

<table>
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<tr>
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*The references for the α-globin (rabbit) and β-actin primer sequences are Cheng et al. [1986] and Clonetech. References for the sequences of the Igf2 and Igf2r primers can be found in Rappolee et al. [1992]. The reference for the sequences of the Xist primers is Kay et al. [1993].
Immunofluorescence staining of eggs and embryos

Zonae pellucidae were removed with acid Tyrode’s buffer in agarose-coated dishes, and the eggs or embryos were fixed for 45 min in 2% paraformaldehyde in phosphate-buffered saline (PBS). After fixation, the embryos were treated for 15 min with 50 mM NH₄Cl in PBS, permeabilized for 10 min at room temperature in 0.1% Triton X-100, and washed in M2 medium. They were incubated sequentially in C-1 antiserum diluted 1:1000 in M2 for 30 min at 37°C, biotinylated donkey anti-rabbit immunoglobulin (Amersham) diluted 1:200 in M2 for 30 min at 37°C, and streptavidin-conjugated with Texas Red (Amersham) diluted 1:200 in M2 for 30 min at 37°C. After staining, the embryos were mounted in M2 medium under oil for fluorescence visualization of antigen.

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Expression of imprinted genes in mouse embryos


Igf2r and Igf2 gene expression in androgenetic, gynogenetic, and parthenogenetic preimplantation mouse embryos: absence of regulation by genomic imprinting.

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