Allele-specific expression and total expression levels of imprinted genes during early mouse development: implications for imprinting mechanisms

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Genomic imprinting determines the monoallelic expression of a small number of genes during at least later stages of development. To obtain information necessary for the elucidation of imprinting mechanisms, we assessed the allele-specific expression and total expression level of four imprinted genes during early stages of development of normal F₁ hybrid mice utilizing quantitative allele-specific reverse transcription-PCR (RT-PCR) single-nucleotide primer extension assays. The *Igf2r* and *Snrpn* genes were activated by the early 4-cell stage and exhibited biallelic and monoallelic expression, respectively, throughout preimplantation development. Thus, with respect to different imprinted genes, epigenetic systems determining monoallelic expression are not uniform in their time of establishment. Biallelic expression of *Igf2r* was observed in single blastomeres, discounting the possibility of random allelic inactivation at this stage. The closely linked *H19* and *Igf2* genes were activated after the blastocyst stage and often exhibited biallelic and monoallelic expression respectively in tissues of pregastrulation postimplantation-stage embryos, rather than reciprocal monoallelic modes as observed at later stages. This raises the possibility that imprinting of *H19* is involved only in the maintenance and not in the initiation of monoallelic expression of *Igf2*. Monoallelic expression of *Snrpn* was observed in each blastomere at the 4-cell stage, demonstrating that the germ line, which exhibits biallelic expression of imprinted genes, must be derived from cells in which imprinting was once manifest.

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Developmentally, genomic imprinting is evident in the abnormal development of diploid zygotes that contain only maternally or paternally derived genomes, termed gynogenetic (also parthenogenetic) and androgenetic, respectively (Barton et al. 1984; McGrath and Solter 1984; Mann and Lovell-Badge 1984; Surani et al. 1984), and of embryos with uniparental duplication of certain autosomal regions (Lyon and Glenister 1977; Cattanach and Kirk 1985; Beechey and Cattanach 1995). Molecularly, imprinting is evident in the monoallelic and parental-specific mode of expression of a small number of genes at least during later stages of development (Beechey and Cattanach 1995). For an understanding of how epigenetic modifications bring about monoallelic expression, and for determining whether there exists a common mechanism for all imprinted genes it is essential that the allele-specific patterns of expression for a number of imprinted genes during normal preimplantation and early postimplantation development be clearly established. Monoallelic expression of an imprinted gene at the late two-cell or early four-cell stage would be indicative of a full establishment of the relevant epigenetic system by the time of embryonic genome activation, whereas an ini-

tially biallelic mode would be indicative of a requirement for additional epigenetic events after this stage. This latter concept of a multistep mechanism was initially formulated in the examination of DNA methylation associated with imprinted transgenes (Allen and Mooslehner 1992; Chaillet 1992) and imprinted endogenous genes (Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993).

With respect to the actual status of allele-specific expression of imprinted genes during early development, it appears that some imprinted genes do exhibit parental-specific expression during early development, whereas others may not (Rappolee et al. 1992; Latham et al. 1994, 1995; Hatada et al. 1995; Tremblay et al. 1995). These studies, however, present one or more of the following difficulties: (1) Total expression levels are unknown or uncertain (It is possible that allele-specific modes of expression may vary according to expression level); (2) parental-specific expression examined in gynogenetic and androgenetic ova may not be representative of that in normal ova [interaction between the maternal and paternal genomes may be required for the normal establishment of monoallelic expression (Latham et al. 1994)];

and (3) rigorously quantitative techniques have not been employed, an important consideration when reverse transcription-PCR (RT-PCR) is used for analyzing very small amounts of total RNA. Furthermore, extrapolation of the parental-specific expression of imprinted genes in embryonic stem cells and embryoid bodies (Allen et al. 1994; Szabó and Mann 1994; Wang et al. 1994) to early development must be cautious in view of the fact that these are cultured cells and are unstable with respect to parental-specific expression and methylation (Szabó and Mann 1994).

The RT-PCR single-nucleotide primer extension, (SNUPE), assay, which is based on a defined nucleotide difference between allelic RNAs, allows for highly sensitive, rigorously quantitative, and allele-specific analysis of expression (Singer-Sam et al. 1992a, b; Singer-Sam and Riggs 1993a). Here, we have used this assay to examine the allele-specific expression of four imprinted genes throughout normal preimplantation development, and at early and later stages of normal postimplantation development [genes examined were *H19* (Bartolomei et al. 1991) and *Igf2r* (Barlow et al. 1991), which exhibit maternal-specific expression, and *Igf2* (DeChiara et al. 1991) and small nuclear ribonucleoprotein N, *Snrpn* (Leff et al. 1992; Cattanach et al. 1992), which exhibit paternal-specific expression]. In addition, at preimplantation and early postimplantation development, total expression levels or RNA concentrations of these genes were quantitated with the SNUPE assay. These data provide implications for imprinting mechanisms and further implications for the relationship between imprinting and cellular potency.

Results

RT-PCR SNUPE assays developed for *Igf2r*, *Igf2*, *H19*, and *Snrpn*, based on exon sequence differences between the two inbred strains C57BL/6J (B6) (*Mus musculus*) and CAST/Ei (CS) (*Mus musculus castaneus*) have been described, and all are rigorously quantitative with respect to the determination of relative proportions of maternal and paternal RNA (Szabó and Mann 1995) and total expression levels (see below, and Singer-Sam and Riggs 1993a). In this study, (B6 female $\times$ CS male) F_1 , B6CS F_1 , and reciprocal CSB6 F_1 normal embryos have been used unless stated otherwise.

Preimplantation development

In early two-cell B6CS F_1 ova, all *Igf2r*, *Igf2*, and *Snrpn* RNA was of the maternal B6 form (Fig. 1A, lanes 2c). As the embryonic genomes are not activated until the late two-cell stage (Hogan et al. 1994), this can be presumed to be the same RNA as that in the cytoplasm of unfertilized oocytes; hereafter referred to as oocyte-specific RNA (Fig. 1A, lanes Oc). *H19* RNA could not be detected in oocytes (Szabó and Mann 1995).

At the early four-cell stage, shortly after the time of embryonic genome activation, *Igf2r* RNA was represented equally by the B6 and CS allelic forms in ova

pools (Fig. 1A, lanes 4c). In comparing the total level of *Igf2r* RNA at this stage and in the oocyte, it is apparent that this RNA represented that derived from expression of the maternal and paternal embryonic genomes (see below). Thus, *Igf2r* was expressed equally from both alleles from the time of embryonic genome activation. Analysis of individual four-cell blastomeres revealed that *Igf2r* expression was biallelic at the single cell level (Fig. 1C). Biallelic expression of *Igf2r* continued throughout preimplantation development, being observed at the eight-cell stage (Fig. 1A, lanes 8c), and in the blastocyst inner cell mass (ICM) and trophectoderm (Fig. 1D, lanes ICM and TRO, respectively).

For *Igf2*, paternal RNA was detected by the early four-cell stage, and this RNA was predominant by the eight-cell stage (Fig. 1A). The total amount of *Igf2* RNA present at these stages was similar to the amount present at the two-cell stage (see below). Thus, pronuclear transplantation experiments were conducted in an attempt to determine what proportion of the maternal or B6-type RNA represented oocyte-specific RNA. The pronuclei of B6 $\times$ B6 zygotes were removed, replaced with the pronuclei from CS $\times$ CS zygotes as depicted in Figure 1B, then these reconstituted zygotes were cultured to the early four-cell stage. In these ova, B6-type RNA represents only oocyte-specific RNA (Fig. 1B, lanes 4c-NT; the asterisk denotes the same sample). In control ova cultured from the zygote to the early 4-cell stage (lanes 4c-C), B6-type RNA represents oocyte-specific RNA plus that derived from maternal embryonic transcription. If the former experimental B6 proportion (65%) is subtracted from the latter control B6 proportion (mean is 72%) to give the proportion of RNA derived from maternal embryonic transcription, it is evident that the B6:CS *Igf2* RNA ratio was strongly paternally biased (7:93) by this stage of development. Similar experiments as just described were also conducted at the eight-cell stage. When the B6 proportion of experimental eight-cell ova that developed from reconstituted zygotes entirely in vitro (mean is 7%) (Fig. 1B, lanes 8c-NT), is subtracted from the B6 proportion of control eight-cell ova that had developed entirely in vivo (mean is 14%) (Fig. 1A, lanes 8c), a very strong paternal bias (7:93) is still evident. The expression of *Igf2* was biallelic in the blastocyst ICM and trophectoderm with only moderate and no paternal bias respectively (Fig. 1D).

For *Snrpn* RNA, the B6/CS ratio was strongly paternally biased by the early four-cell stage (Fig. 1A, lanes 4c). To determine the proportion of the maternal signal contributed by oocyte-specific RNA, the same experiments of zygotic reconstitution as described above for *Igf2* were conducted. When the B6 proportion of experimental four-cell ova that developed entirely in vitro from reconstituted zygotes (mean is 10%) (Fig. 1B, lanes 4c-NT), is subtracted from the B6 proportion of control four-cell ova that had developed entirely in vivo (mean is 16%) (Fig. 1A, lanes 8c), a strong paternal bias (6:94) is evident. For four-cell ova at ~ 5 hr after second cleavage, each of the four blastomeres was examined for allele-specific expression of *Snrpn*. For each of three ova exam-

Figure 1. Allele-specific expression during preimplantation development. (A) Allele-specific expression of each imprinted gene is represented by two rows of autoradiographs, with the *upper* and *lower* representing the presumptive inactive and active alleles respectively, with the parental origin given on the *right*. The number above each band is the amount of RNA of the presumptive inactive allele expressed as a percentage of the total amount, rounded off as follows: 0%, 0.49% or less, and 1, 0.5 to 1.49%, etc. These values were determined after quantitation of bands by a PhosphorImager analysis (Szabó and Mann 1995). The first three lanes (labeled %) are RNA quantitation controls performed at the same time as the experiments: (Lane 1), 0% B6 and 100% CS total RNA; (lane 2), 50% each of B6 and CS RNA; (lane 3), 100% B6 and 0% CS RNA. (13.5 dpc), total RNA isolated from 13.5 dpc B6CSF₁ headless fetuses, unfertilized B6 oocyte, (Oc), two-cell, (2c), early four-cell, (4c), and eight-cell, (8c), B6CSF₁ ova. Number of ova in each pool: (Oc lanes), All genes, 10, 30; (2c lanes), all genes, 10, 10; (4c lanes), *Igf2r*, 4, 9, *Igf2*, 10, 10, *Snrpn*, 5, 10; (8c lanes), *Igf2r*, 1, 5, *Igf2*, 9, 19; *Snrpn*, 1, 5. (B) Determination of contribution of oocyte RNA to maternal-specific signal: Pronuclear transplantation was carried out so that the B6-specific signal could represent only the oocyte RNA (Oc). The CS-specific signal represents RNA derived from paternal CS pronucleus, plus that potentially derived from the maternal CS pronucleus (Em). (4c-NT, 8c-NT); Re-

constituted ova cultured to the four-cell and eight-cell stages, respectively. The same sample marked with an asterisk under 4c-NT was assayed again at the same time as four-cell control ova also cultured from the zygote stage (4c-C). The numbers of ova in each pool were 15, 15, (4c-C lanes); *Igf2*, 15, 12, *Snrpn*, 7, 8, (4c-NT lanes); 10, 16, (8c-NT lanes). (C) Allele-specific expression of *Igf2r* and *Snrpn* in four-cell single blastomeres: (*Igf2r*); Each lane represents a single blastomere isolated at random. (*Snrpn*); Each lane also represents a single blastomere, and each blastomere of three individual ova (EI, EII, and EIII) were analyzed. (D) (ICM, TRO); Blastocyst inner cell mass and mural trophoderm, respectively. Other details are described as in A. The numbers of blastocyst ICMs and trophoderm used in each pool were *Igf2r*, 1, 4, *Igf2*, 14, 19, *Snrpn*, 4, 4, (ICM lanes); *Igf2r*, 10, 10, *Igf2*, 24, 24, *Snrpn*, 10, 10, (TRO lanes).

ined, all four blastomeres exhibited strong paternal bias (Fig. 1C). Expression was clearly monoallelic by the eight-cell stage (Fig. 1A) and remained as such in the blastocyst ICM and trophoderm (Fig. 1D).

With respect to the pronuclear transplantation experiments described above, it appears likely that the maternal *Igf2* and *Snrpn* alleles were completely inactive at the outset of embryonic transcription when it is taken into account that for *Igf2* at the eight-cell stage, and *Snrpn* at the four-cell stage, experimental ova were cultured, whereas control ova, to which they were compared, had developed entirely in vivo. Culture results in a relatively lower proportion of oocyte-specific RNA, perhaps related to developmental retardation; compare the proportion of oocyte-specific *Igf2* RNA in in vivo four-cell ova (Fig. 1A, lanes 4c) with that in in vitro four-cell ova (Fig. 1B, lanes 4c-C).

Early postimplantation development

Studies at postimplantation stages were performed to determine the time of establishment and stability of monoallelic expression. B6CSF₁ embryos at 6.5 days post coitum (dpc), prior to primitive streak and mesoderm formation, were dissected into embryonic ectoderm (epiblast), embryonic visceral endoderm, extraembryonic ectoderm, extraembryonic visceral endoderm, parietal endoderm, and trophoblast. For each tissue type, two samples were isolated from two different embryos, and each sample was analyzed for all four genes. A striking variability in the degree of bias of the B6:CS RNA ratio, dependent on gene and tissue, was apparent (Fig. 2). For *Igf2r*, a strong maternal bias was observed only in the visceral endoderm components. For *H19*, no strong maternal bias in any tissue was evident. For *Igf2*, strong

Figure 2. Allele-specific expression in tissues of the 6.5-dpc egg cylinder. Tissues: (EEC), Embryonic ectoderm; (EXEC), extraembryonic ectoderm; (EEN), embryonic visceral endoderm; (EXEN), extraembryonic visceral endoderm; (PE), parietal endoderm; (TR), ectoplacental cone or trophoblast. Each lane represents one individual tissue sample. Other details are as described in the legend to Fig. 1 (A). The embryos analyzed were derived from the asynchronous transfer of 3.5-dpc blastocysts, derived from the culture of morulae for one day in vitro, to the uteri of 2.5-dpc recipients. For all genes, results were similar in all tissues in embryos derived from the synchronous transfer of zygotes to the oviducts of 0.5-dpc recipients (data not shown).

paternal bias was observed in the visceral endoderm components, but not in the ectoderm components. Some, or all of the maternal-specific expression of *Igf2* in the parietal endoderm and trophoblast could be accounted for by maternal decidua or blood contamination (see below). For *Snrpn*, paternal-specific monoallelic expression was evident in all tissues. Again, some, or all of the maternal-specific expression in the parietal endoderm and trophoblast could be accounted for by maternal decidua or blood contamination (see below).

Allele-specific expression in relation to RNA concentration

The above results demonstrate a considerable degree of variability in the degree of bias of allele-specific expression during early development, dependent on gene and tissue examined. We wished to determine if any of this variability could be related to the total level of expression or RNA concentration. Such information is indispensable for the development of mechanistic models to explain the acquisition of monoallelic expression. For example, the mode of expression may be monoallelic and biallelic at high and low levels of expression respectively. As the SNUPE assay can be used for quantitation of absolute amounts of RNA (Singer-Sam et al. 1993a),

we developed our existing assays accordingly (Fig. 3). Briefly, the quantitation was carried out as follows: The concentration of CS-type *Igf2r*, *H19*, *Igf2*, and *Snrpn* RNA molecules in total RNA isolated from 13.5-dpc embryos was predetermined as described in Figure 3. Then, a known number of B6 ova or cells was combined with a known amount of 13.5-dpc CS total RNA, and the RT-PCR SNUPE assays conducted. This provided a B6:CS RNA ratio from which the number of RNA molecules/ovum or cell could be calculated.

Primary results of these analyses are presented in Figure 4(A,B), and are also depicted graphically in Figure 5. For direct comparison, also depicted in Figure 5 are the primary results of allele-specific expression shown in Figures 1(A–D) and 2. During preimplantation development, it was evident that *Igf2* and *H19* were expressed at exceedingly low or basal levels up to the 3.5-dpc blastocyst stage. *Igf2* RNA reached a maximum concentration in the ICM, being present at only 100 copies/ICM. Although we could not obtain a signal for *H19* by RT-PCR, the quantitations show that the maximum number of *H19* RNA molecules present was 20/ovum. Some *H19* RNA must be present in 3.5-dpc blastocysts, as monoallelic expression has been reported in a sample size of 167 blastocysts (Tremblay et al. 1995). In 4.5-dpc blastocysts, in situ hybridization studies show that *H19* RNA is localized to the trophectoderm (Poirier et al. 1991). Although the modes of allele-specific expression of *Igf2r* and *Snrpn* were opposite, being equally biallelic and strictly monoallelic respectively, both were expressed at high levels. *Igf2r* RNA was at 60,000 copies/early four-cell ovum, and at 25,000 copies/ICM, and *Snrpn* RNA was at 5,000 copies/early four-cell ovum, and at 20,000 copies/ICM.

By 6.5 dpc, the expression levels of *H19* and *Igf2* were considerably higher than during preimplantation development, although *Igf2* RNA levels were always lower than those of *H19* in the identical tissue samples. The tissue specificity of expression of these two genes was correlated; RNA levels were relatively low in the embryonic ectoderm and were at their highest in the extraembryonic components as would be expected from in situ hybridization studies (Lee et al. 1990; Poirier et al. 1991). In all tissues, *H19* expression was biallelic with only moderate maternal bias. With the identical tissue samples, *Igf2* expression was monoallelic or strongly paternally biased in the visceral endoderm components, and moderately paternally biased in the ectoderm components. Thus, in general, *Igf2* expression exhibited a strong paternal bias in those 6.5-dpc tissues in which expression was activated to high levels. This must also have been the case in mesoderm, which arises from the embryonic ectoderm and begins to appear at 6.5 dpc, as monoallelic expression of *Igf2* was exhibited by the embryo proper at 8.5 dpc (see below). Levels of *Igf2r* and *Snrpn* RNA in 6.5-dpc tissues were lower than during preimplantation development. Some tissue specificity in the acquisition of monoallelic expression of *Igf2r* was apparent, being attained in the visceral endoderm components, but not in other tissues. The expression of

Expression of imprinted genes

Figure 3. RT-PCR SNUPE assays in the determination of RNA concentration. For each gene, the bottom row of the autoradiograph represents a fixed amount of total RNA isolated from 13.5-dpc CS embryos (*Igf2r*, 10 ng; *H19*, 1 ng; *Igf2*, 1 ng; *Snrpn*, 10 ng). The top row represents varying numbers of synthetic B6 molecules, 10^1 – 10^{10} (labeled 1–10), which were mixed with the fixed amount of total CS RNA. Bands obtained after RT-PCR SNUPE were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Where the adjusted B6/CS signal ratio (see Szabó and Mann 1995) was 1.0 ($\log 10^0$, point of intersection of y axis on graph) the number of synthetic B6 molecules equaled the number of CS RNA molecules. The graphs demonstrate that, for the known fixed amount of CS RNA added, the quantitation was linear over five orders of magnitude of input B6 RNA. The range of linearity was extended for lesser amounts of input B6 RNA by decreasing the fixed quantity of CS total RNA used (data not shown; also see Szabó and Mann 1995). These experiments demonstrate the assays to be quantitative for determining absolute amounts of RNA provided the B6/CS ratios obtained are not extreme. For theoretical considerations, see Gilliland et al. (1990). The absolute number of *Igf2r*, *H19*, *Igf2*, and *Snrpn* molecules in a given amount of whole 13.5-dpc CS embryo total RNA was thereby quantitated. Mean of three experiments: *Igf2r*, 6.6×10^4 molecules/ng; *H19*, 2.6×10^5 molecules/ng; *Igf2*, 7.4×10^4 molecules/ng; *Snrpn*, 4.0×10^3 molecules/ng. Known amounts of CS total RNA were subsequently mixed with RNA isolated from a known number of B6 ova or cells, for determination of the number of molecules/ova or cell from the B6/CS ratio obtained.

Snrpn was monoallelic in all tissues, although the total level was very low in the extraembryonic endoderm (~ 30 molecules/cell).

Later postimplantation development

Further analyses of B6CSF₁ embryos were conducted at 8.5, 10.5, and 16.5 dpc. Results are shown in Figure 6(A–D). For *Igf2r*, biallelic expression in the embryo proper persisted throughout development, although was strongly maternally biased. Analysis at 16.5 dpc shows this biallelic expression to be localized predominantly to the head (Fig. 6D). The amnion, yolk sac, parietal endoderm, and trophoblast exhibited monoallelic expression by 8.5 dpc (Fig. 6A,B). For *H19*, strict monoallelic expres-

sion was not observed in the embryo proper and the amnion until 10.5 dpc (Fig. 6C), whereas strong maternal bias in expression persisted up to 16.5 dpc in the remaining extraembryonic tissues. At these later stages, the difference in the mode of allele-specific expression in the embryo-proper and amnion, evident for *Igf2r* and *H19*, indicates that cell lineage is not necessarily predictive of the finally attained mode, as both components are derived from the embryonic ectoderm. For *Igf2* and *Snrpn*, monoallelic expression was exhibited by all tissues at 8.5 dpc and at later stages [the primers for the *Igf2* assay detect all promoter-specific transcripts, being derived from exons 5 and 6 (Rotwein and Hall 1990)]. For these two genes, the maternal-specific RNA in the parietal endoderm and trophoblast at 8.5 dpc (Fig. 6A), and probably

Figure 4. Concentration of RNA in B6 ova and tissues of B6 6.5-dpc egg cylinders. (A) Ova; The number (N) of B6 ova or ICMs (emb) used in the analyses is given above the autoradiographs. The amount of total 13.5-dpc CS RNA used as standard (st) was 20 ng for *Igf2r* and *Snrpn*. Other details are as described in Fig. 1 (A). (B) 6.5-dpc tissues; Numbers above the autoradiographs represent the estimated number of cells analyzed, based on estimates of total cell number in these tissues (Snow 1977). The amounts of total CS RNA used as standards were *H19*, 0.5 ng (except lanes marked ○, where 0.1 ng was used) and *Igf2*, 0.1 ng (except lane □, where 0.5 ng was used). Other details are as described in A, and Fig. 2.

at 10.5 dpc (Fig. 6C), was derived from maternal decidua or blood contamination. This is demonstrated by the fact that monoallelic expression was observed in these components of 9.0-dpc 18-somite CSB6F₁ embryos (Fig. 6B) that had developed in B6-type recipients. In this case, the tissue of the mother was of a different allelic form to the maternal allele of the embryo.

Discussion

This study provides quantitative information, in terms of allele specificity and total expression level, of imprinted genes during normal early mouse development. Also, it provides important information as to the allele

specificity of expression at the level of single blastomeres.

Stage- and tissue-specific variation in acquisition of monoallelic expression

Significant stage- and tissue-specific variation between imprinted genes in the acquisition of monoallelic expression has been described. It is now clear that for at least one imprinted gene (*Snrpn*), the epigenetic system that

Figure 5. RNA concentrations in B6 ova and 6.5-dpc tissues, and allele-specific expression in B6CSF₁ ova and tissues at equivalent stages. Vertical bars show number of RNA molecules $\times 10^3$ /ovum or cell. Above each bar, allele-specific expression is depicted by the circles with vertical lines (derived from Figs. 1 and 2, average of the two observations). Each circle represents 100% expression as contributed by the maternal and paternal alleles, with the shaded portion representing the proportion contributed by the presumptive inactive allele. (F) Fertilization stage. Before F, the parental-specific expression indicated for unfertilized oocytes (Oc) is from Szabó and Mann (1995) and refers to the parents of the B6CSF₁ females from which the oocytes were obtained. After F, allele-specific expression of the embryonic genome is depicted, except for (1) the two-cell stage—in early two-cell ova, we detected only B6 or oocyte-specific RNA—and (2) the parietal endoderm (PE) and ectoplacental cone or trophoblast (TR) for *Igf2* and *Snrpn*—some, or all of the maternal-specific contribution may have been derived from maternal decidua or blood contamination. (<) Less than 20 RNA molecules; (nq) not quantitated. Other details are as described in Figs. 1 (A) and 2.

Expression of imprinted genes

Figure 6. Allele-specific expression in later postimplantation stage embryos. (A) 8.5-dpc 12-somite embryos; (EM) embryo proper; (AM) amnion; (YS) whole yolk sac; (PE) parietal endoderm; (TR) developing placenta (predominantly trophoblast). (B) 9.0-dpc 18-somite reciprocal CSB6F₁ embryos (method of production is in Materials and methods). (C) 10.5-dpc embryos. (D) 16.5-dpc embryos. (HE) Whole head; (BO) whole of embryo minus the head; (AM) amnion; (PL) whole placenta. (N.D.) Not done. Other details are as described in the legend to Fig. 1 (A).

determines monoallelic expression is fully established by the time of activation of the embryonic genome, whereas for other imprinted genes, this is not the case. Furthermore, we have demonstrated that the mode of expression of imprinted genes during these early stages is not in any general way related to the total expression level, that is, monoallelic and biallelic modes can exist once genes have been activated to high levels.

The finding that the maternally derived *Snrpn* allele is transcriptionally silent by the time of embryonic genome activation is of particular interest in view of the fact that this same allele is active in oocytes prior to fertilization; the concentration of *Snrpn* RNA in ovulated unfertilized oocytes is equivalent to that in four-cell ova (see Fig. 5). Although *Snrpn* RNA in unfertilized oocytes may be stored product derived from transcription during oocyte growth (Szabó and Mann 1995), these findings indicate the existence of a fundamental switch in relation to the activity of the maternally derived *Snrpn* allele that takes place sometime between probably the later stages of oocyte growth and the time of embry-

onic genome activation. This switch could constitute imprinting itself, or represent a cessation in bypass of imprints already established. The findings for *Igf2* provide some indication that the epigenetic system necessary for its monoallelic expression is largely in place by the time of its activation, as during early cleavage, it was expressed monoallelically. Intriguingly, it was then expressed biallelically in normal blastocysts as has been suggested from studies of parthenogenetic, gynogenetic, and androgenetic blastocysts (Latham et al. 1994). In terms of the status of imprinting, however, the fact that these observations are of basal levels of expression of *Igf2* renders the significance of all these findings unclear. The findings at 6.5 dpc are therefore more informative. Of three tissues that possessed high levels of *Igf2* expression, two had essentially monoallelic expression, and one moderately paternally biased expression. It remains possible that high levels of biallelic expression of *Igf2* may exist in tissues at 5.5 dpc, which we have not examined. In addition to *Igf2*, the U2 auxiliary factor-related sequence-1 *U2afbp1* gene is also expressed mono-

allelically from the outset of embryonic transcription, as its exclusive paternal expression has been observed in normal F₁ two-cell stage ova (Hatada et al. 1995). Its total expression level at this stage, however, is unknown. In contrast, *U2afbp1* expression has been detected in both gynogenetic and androgenetic ova, although the total level appears to be less in the former (Latham et al. 1995).

Similar to *Snrpn*, *Igf2r* expression was activated to high levels during preimplantation development, but its mode was exactly opposite, being equally biallelic at all stages. This result has been suggested in a previous study that detected the presence of *Igf2r* RNA by RT-PCR in both gynogenetic and androgenetic ova (Latham et al. 1994), and in another study that examined RNA levels in embryonic stem cell clones targeted for either the maternal or paternal *Igf2r* allele (Wang et al. 1994). Thus, unlike *Snrpn* and possibly *Igf2*, the earliest time of expression of *Igf2r* is not subject to imprinting. The possibility that *Igf2r* expression is in fact monoallelic at the single cell level, with allelic inactivity present at random with respect to parental derivation as is the case for somatic X chromosome inactivity in XX embryos (Chapman 1986), was ruled out by our observations of biallelic expression in single blastomeres of four-cell ova. Thus, the earliest expression of *Igf2r* is not subject to a counting mechanism which requires one allele to be inactive. The finding of biallelic expression of *H19* in isolated 6.5-dpc tissues shows that this gene is also not subject to imprinting at the initial stage of high level expression.

Implications for imprinting mechanisms

Although these findings demonstrate that epigenetic systems determining monoallelic expression vary between genes in the time and tissue specificity of establishment, it is not necessarily true that the imprinting mechanism of genes per se also varies. It is probably most convenient to think of imprinting as only those epigenetic modifications imparted in the germ line and/or zygote. After karyogamy, there is no possibility for de novo parental-specific chromosome marking, presuming that an effect of oocyte-derived cytoplasm during cleavage must be imparted to both alleles indiscriminately. Thus, imprinting of all genes could involve a similar mechanism, but the variation between genes in their early patterns of allele-specific expression could depend on differences in the status of certain postzygotic events that must occur before monoallelic expression is attained. For example, the events could involve (1) further epigenetic modifications imparted in *cis* (Allen and Mooslehner 1992; Chaillat 1992; Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993; Feil et al. 1994; Latham et al. 1994; Szabó and Mann 1994), (2) the expression of *trans*-acting factors that interact with imprints (Latham et al. 1994; Barlow 1994), (3) the use of a regulatory system that is subject to imprinting, that is, an alternative regulatory system may be used early in development which is not subject to imprinting [on this point, not all promoters of the human *IGF2* gene are

subject to imprinting (Ekström et al. 1995; Taniguchi et al. 1995)], and (4) maturation of chromatin structure inherited from the germ line, which might occur at different rates in different genome regions: It has been postulated that some unique aspect of germ line chromatin structure bypasses imprint-dependent regulatory elements, for example, enhancers, resulting in the persistent biallelic expression of imprinted genes throughout germ-cell development (Szabó and Mann 1995). For this to explain the biallelic expression of *Igf2r* and *H19* in early development, strong promoters for these genes would need to be postulated. Biallelic and monoallelic modes of expression at gene activation could also be explained by differences in imprinting mechanisms per se. On this point, it is relevant to note certain differences between paternally and maternally expressed genes which might be indicative of different imprinting mechanisms: For the former (*Snrpn* and *Igf2*, also possibly *U2afbp1*) the predominant initial mode of expression was monoallelic, and these exhibited monoallelic expression in all tissues of the conceptus by 8.5 dpc, whereas the latter (*H19* and *Igf2r*), the predominant initial mode was biallelic, and these did not attain strict monoallelic expression in all tissues even by 16.5 dpc. Also, imprinting of the former two is apparently controlled at least in part by long-range *cis*-acting mechanisms (Sutcliffe et al. 1994; Buiting et al. 1995; Leighton et al. 1995b). Additional imprinted genes must be examined to establish if these correlations are a general rule.

DNA methylation can influence gene expression, be clonally inherited, and be reset in the germ line, and therefore could be an imprinting mechanism (for reviews, see Jost and Saluz 1993). It is likely to be at least auxiliary to imprinting in the attainment or stabilization of monoallelic expression, as many parental-specific methylation patterns of imprinted genes have been described (Nicholls 1994; Razin and Cedar 1994). In addition, somite-stage mouse embryos deficient in methylation exhibit biallelic expression of imprinted genes (Li et al. 1993). Aside from a loss of imprinting or postzygotic epigenetic modifications, however, it has been suggested that this biallelic expression could be a secondary effect of genome-wide alterations in chromatin structure caused by methylation deficiency (Szabó and Mann 1995). A similar explanation could be offered for the biallelic expression of imprinted genes in tumors (Ogawa et al. 1993; Rainier et al. 1993) as these in general are methylation-deficient (Lapeyre et al. 1981; Feinberg and Vogelstein 1983; Lee-Jane et al. 1983). Genome-wide demethylation occurs during preimplantation development such that blastocysts are essentially nonmethylated, and remethylation of the genome occurs rapidly at early postimplantation stages (Monk et al. 1987; Kafri et al. 1992). From the present studies, it is now apparent that in many instances the stage of attainment of monoallelic expression essentially coincides with the stage of genome-wide remethylation, and this provides additional circumstantial evidence that imprint-dependent methylation does help to promote or stabilize monoallelic expression. On the other hand, the fact that

Snrpn is monoallelically expressed during preimplantation development demonstrates that the epigenetic system that determines monoallelic expression of this gene is stable throughout the genome-wide demethylation events. As suggested by Monk (1988), some parental-specific methylation is inherited from gametes and is retained during preimplantation development, and therefore could constitute imprints (Brandeis et al. 1993; Razin and Cedar 1994; Hatada et al. 1995; Tremblay et al. 1995). Thus, although there are no reports of the parental-specific methylation status of *Snrpn* during preimplantation development, it could be hypothesized that a limited amount of differential methylation at specific sites within or adjacent to this gene may be sufficient to invoke monoallelic expression. An imprinting control element apparently determines at a distance the monoallelic expression of a set of genes in the human Prader-Willi syndrome region, including the homolog of *Snrpn* (Sutcliffe et al. 1994; Buiting et al. 1995). This then may be a region which harbors differential methylation necessary for determining monoallelic expression. On this basis, one could predict that other genes in the mouse Prader-Willi syntenic region, for example, *Znf127* (Beechey and Cattanaach 1995), would also display monoallelic expression at initial activation.

From observations of preimplantation development, it has been suggested that paternal-specific methylation of a region located 5' of *H19* and inherited from gametes is sufficient for determining monoallelic expression of this gene (Tremblay et al. 1995). Our finding of biallelic expression of *H19* during high level expression at 6.5 dpc, however, suggests that at least some of the additional paternal-specific methylation observed at later stages (Bartolomei et al. 1993; Ferguson-Smith et al. 1993), and which is possibly laid down in response to the inherited methylation (Tremblay et al. 1995), is also necessary for the attainment of monoallelic expression. Further, at 6.5 dpc, we saw little evidence for a coupling of the allele-specific expression of *H19* and *Igf2* in accordance with an enhancer competition model: This states that these two genes compete in cis for a common set of enhancer elements, located 3' of *H19*, such that their expression is mutually exclusive in cis. Further, imprinting of *H19* governs the choice, and is identified as a methylation of the 5' region of the paternal *H19* allele, which acts to inhibit binding of the enhancer elements (Leighton et al. 1995a, 1995b; Tremblay et al. 1995). Thus, in the extraembryonic ectoderm, we observed biallelic expression of *H19* and *Igf2*, and in the embryonic and extraembryonic endoderm, biallelic expression of *H19* and monoallelic or strongly biased expression of *Igf2*. Similar deviations from expectation based on the model have been observed in populations of embryonic stem cells (Szabó and Mann 1994) and in tissues (Mutter et al. 1993; Jinno et al. 1995; Li et al. 1995). These findings could be made compatible with the model if, in addition to the operation of the enhancers shared between *H19* and *Igf2*, and which are subject to imprinting, it is postulated that at the same time other unidentified *H19*-specific enhancers stimulate *H19* expression regardless of imprinting. Other-

wise the findings raise the possibility that the initiation, but not the later maintenance of monoallelic expression of *Igf2* may be determined by an additional imprinting mechanism which is independent of the shared mechanism. If so, in embryos which possess a deletion of the enhancers 3' of *H19* (Leighton et al. 1995b), monoallelic expression of *Igf2* in the extraembryonic and embryonic endoderm at 6.5 dpc should be unaffected. An example of an epigenetic mechanism maintaining but not initiating an inactivated state is seen in the relationship between methylation and the inactive X chromosome (Singer-Sam and Riggs 1993b).

Relation between imprinting and cellular potency

A generalized neutralization of imprinting is apparent in the germ line, such that imprinted genes are expressed biallelically in female and male germ cells at all stages up to late gametogenesis (the term neutralization includes the possibility that imprints may be present or absent). Thus, there is a correlation between imprinting neutralization and the totipotent state (Szabó and Mann 1995). However, the present study demonstrates that the correlation is not absolute: Blastomeres of the cleavage-stage ovum are totipotent (Kelly 1977), yet at the same time they exhibit monoallelic expression of *Snrpn*. It is likely that additional imprinted genes are expressed strictly monoallelically during cleavage, and such findings are more compatible with biallelic expression of imprinted genes being more a consequence rather than a cause of totipotency (Szabó and Mann 1995). That genomic imprinting, or functional differences between the maternal and paternal genomes, might be involved in cellular differentiation (Monk 1988; Surani et al. 1990; Holliday 1990) is not consistent with the many examples of biallelic expression we observed during preimplantation and early postimplantation development. On the other hand, our observation that monoallelic or strongly biased allele-specific expression of imprinted genes was often in place by 8.5 dpc is consistent with the possibility that monoallelic expression may be involved in later developmental decisions. Evidences which can be viewed as being against a role for imprinting in most events of cellular differentiation, however, are that significant parthenogenetic or androgenetic contributions to many cell lineages can be obtained in fetuses and neonates (Fundele et al. 1990; Mann et al. 1990), and maternal and paternal duplication of imprinted autosome regions can often support development to or beyond term (Beechey and Cattanaach 1995). Although lack of expression of an imprinted gene might affect cellular differentiation, the only example to date being murine achaete-scute homolog 2, *Mash2*, which is required for the development of placental spongiotrophoblast (Guillemot et al. 1995), it is yet to be shown that biallelic modes of expression of imprinted genes adversely affect cellular differentiation. Observations of this latter type would provide evidence that the monoallelic or imprinted mode of expression per se was important in differentiation.

Finally, the finding that the *Snrpn* gene displayed monoallelic expression in all four blastomeres of the four-cell ovum is fundamental in that it precludes the formal possibility that one blastomere, or more, escapes the manifestations of imprinting and develops as a discrete cell lineage giving rise to primordial germ cells. Thus, an imprinting neutralization event must occur with respect to germ cell development. If this occurs prior to, or after primordial germ cell emergence at the late gastrulation stage remains to be determined.

Materials and methods

Quantitative RT-PCR SNUPE assays

The assays for *Igf2r*, *H19*, *Igf2*, and *Snrpn* have been described in detail (Szabó and Mann 1995). Synthetic B6 RNA was made by in vitro transcription from linearized pCR II plasmid (Invitrogen, San Diego, CA) templates which contained the same B6 fragments amplified by RT-PCR used in the SNUPE assays. Templates, restriction enzyme for linearization, and RNA polymerases were *Igf2r*, TP30/3, *HindIII*, T7; *H19*, TP19/3, *XbaI*, SP6; *Igf2*, TP28/2, *BamHI*, T7; and *Snrpn*, TP22/2, *XbaI*, SP6. Synthetic RNA stock solutions were stored at 10^{11} molecules/ μ l in 75% ethanol at -80°C .

Production and collection of preimplantation stage ova

Prepubertal B6 female mice (Jackson Laboratory, Bar Harbor, ME) were superovulated by standard procedures (Hogan et al. 1994). Unfertilized B6 oocytes were removed from the oviduct ampullae of B6 females 16 hr after injection of human chorionic gonadotrophin (HCG), and adhering follicle cells were released by hyaluronidase treatment (Hogan et al. 1994). Fertilized ova were obtained from superovulated B6 females mated to CS or B6 males (Jackson Laboratory). Ova were handled in medium M2 (Wood et al. 1987) and were cultured in medium CZB (Chatot et al. 1989) plus glucose minus EDTA at 37°C in 6% CO_2 in air. Early two- and eight-cell ova were flushed from oviducts at ~32 hr and 68 hr after HCG injection respectively. To obtain early four-cell ova, two-cell ova were flushed from oviducts at ~46 hr after HCG injection, then were cultured for no longer than 1.5 hr after both blastomeres had cleaved. After removal from the reproductive tract, oocytes and ova were rinsed until all contaminating somatic cells were absent. For all ova, zona pellucidae were removed by acid Tyrode's treatment (Hogan et al. 1994), then were pipetted into 0.1-ml aliquots of RNAzol (Tel-Test Inc. Friendswood, TX) in microfuge tubes, and stored over liquid nitrogen until analysis. Individual blastomeres of four-cell ova were isolated according to Hogan et al. (1994). Blastocysts for ICM and trophectoderm isolation were obtained by transferring 0.5-dpc zygotes to the oviducts of 0.5 dpc pseudopregnant recipients, then flushing uteri at 3.5 dpc. ICMs were isolated by immunosurgery (Hogan et al. 1994). Trophectoderm was isolated by hand with the aid of a stereomicroscope: The zona pellucida was removed as above, the blastocyst immobilized at the position of the ICM utilizing a mouth-controlled 50 μm outer diam. holding pipette, and the trophectoderm cut off above the ICM with a glass needle perpendicular to the holding pipette. Isolated blastomeres and blastocyst components were collected and stored as described above.

Production and collection of postimplantation stage tissues

B6CSF₁ embryos were produced as previously described (Szabó and Mann 1995). Reciprocal CSB6F₁ embryos were produced by

naturally mating CS females (Jackson Laboratory) to B6 males, flushing blastocysts from the uterus at 3.5 dpc, then immediately transferring them to 2.5-dpc pseudopregnant recipients. B6 ova and embryos for quantitation of absolute amounts of RNA were obtained by natural mating. Tissue layers of 6.5-dpc egg cylinders were isolated by incubation of the cylinders in trypsin/pancreatin followed by microdissection (Hogan et al. 1994), then placed into 0.1-ml aliquots of RNAzol and stored as described above. In obtaining of RNA samples, at all developmental stages the whole of the conceptus component was homogenized to ensure representative samples.

Pronuclear transplantation

Pronuclei were transplanted according to Hogan et al. (1994). In these experiments only, "CS" mice used were from a B6.CS (N2) stock bred to homozygosity for the CS alleles of *H19*, *Igf2*, *Igf2r*, and *Snrpn* with the aid of Mouse Map Pairs D7Mit70, D7Nds4, and D17Mit48 (Research Genetics Inc., Huntsville, AL), and homozygosity confirmed by DNA SNUPE analysis utilizing exon sequence differences already described (Szabó and Mann 1995).

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Allele-specific expression and total expression levels of imprinted genes during early mouse development: implications for imprinting mechanisms.

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