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Original Article

Analysis of parent-specific gene expression in early mouse embryos and embryonic stem cells using high-resolution two-dimensional electrophoresis of proteins

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ABSTRACT **Genomic imprinting is an important genetic mechanism in mammals whereby certain genes are epigenetically modified and their expression altered according to their parental origin. The most important consequence of this is the requirement for both a maternal and a paternal genome for normal development to proceed to term. Although there are many instances of specific phenotypes (in the mouse) and diseases (in humans) resulting from imbalances in the parental chromosomes, it is only in the past few years that some of the imprinted genes responsible have been identified. It is however unclear what proportion of the genome is imprinted, particularly in the early embryo. To address the question to what extent parent-specific gene expression occurs in the early embryo and with a possible view to identifying new imprinted genes, the protein profiles of parthenogenetic and normal blastocysts were compared using the technique of high-resolution two-dimensional electrophoresis. The protein profiles of parthenogenetic, androgenetic and normal embryonic stem cells were also compared. Hence parent-specific gene expression was examined in embryonic and extraembryonic lineages of the early embryo. Approximately 1000 polypeptides were examined in each of the analyses, however no parent-specific differences were observed for any of these polypeptides. From this result, it is concluded that expression of genes encoding these polypeptides is identical from the parental chromosomes. These findings have important implications for estimates of the number of imprinted genes in the genome and for the interpretation of phenotypes of parthenogenetic and androgenetic embryos.**

KEY WORDS: *two-dimensional electrophoresis, imprinting, embryonic stem cells, preimplantation development, mouse*

Introduction

The inability of monoparental (parthenogenetic (PG), gynogenetic (GG) and androgenetic (AG)) embryos to develop to term is attributed to the actions of a subset of genes whose expression is determined by the parent from which they are inherited. Approximately 15-20 of such imprinted genes have now been identified, which include fetal growth factors such as insulin-like growth factor 2 (DeChiara *et al.*, 1991), one of its receptors *(Igf2r)* (Barlow *et al.*, 1991), the putative proliferation suppressor RNA *H19* (Bartolomei *et al.*, 1991), and other genes as diverse as transcription factors, enzymes, and splicing factors (Efstratiadis, 1994). For some imprinted genes a genetic analysis by mutation has been carried out which has begun to reveal the contributions that the products make to the phenotypes of complete monoparental and monoparentally disomic embryos (DeChiara *et al.*, 1991; Lau *et al.*, 1994; Wang *et al.*, 1994; Leighton *et al.*, 1995). However, for the majority of imprinted genes, their contribution to the 'imprinted' phenotypes are unknown. The assessment of the contribution of imprinted genes to specific phenotypes is particularly important for the developmental analysis of imprinting (Fundele and Surani, 1994; Walsh *et al.*, 1994) and for the verification of evolutionary theories of imprinting that make very specific predictions about the actions of maternally and paternally expressed genes (Moore and Haig, 1991; Haig, 1992).

One important step forward in resolving these questions would be to know or estimate the total number of imprinted genes in the genome and how many of these are active at particular developmental stages, since imprinting can be stage and tissue-specific. If the total number is small in comparison to the number of genes in the genome, then the selection that we already have could be fairly representative. If, on the other hand,

Abbreviations used in this paper: 2DE, two-dimensional electrophoresis; AG, androgenetic; ES, embryonic stem; GG, gynogenetic; PG, parthenogenetic.

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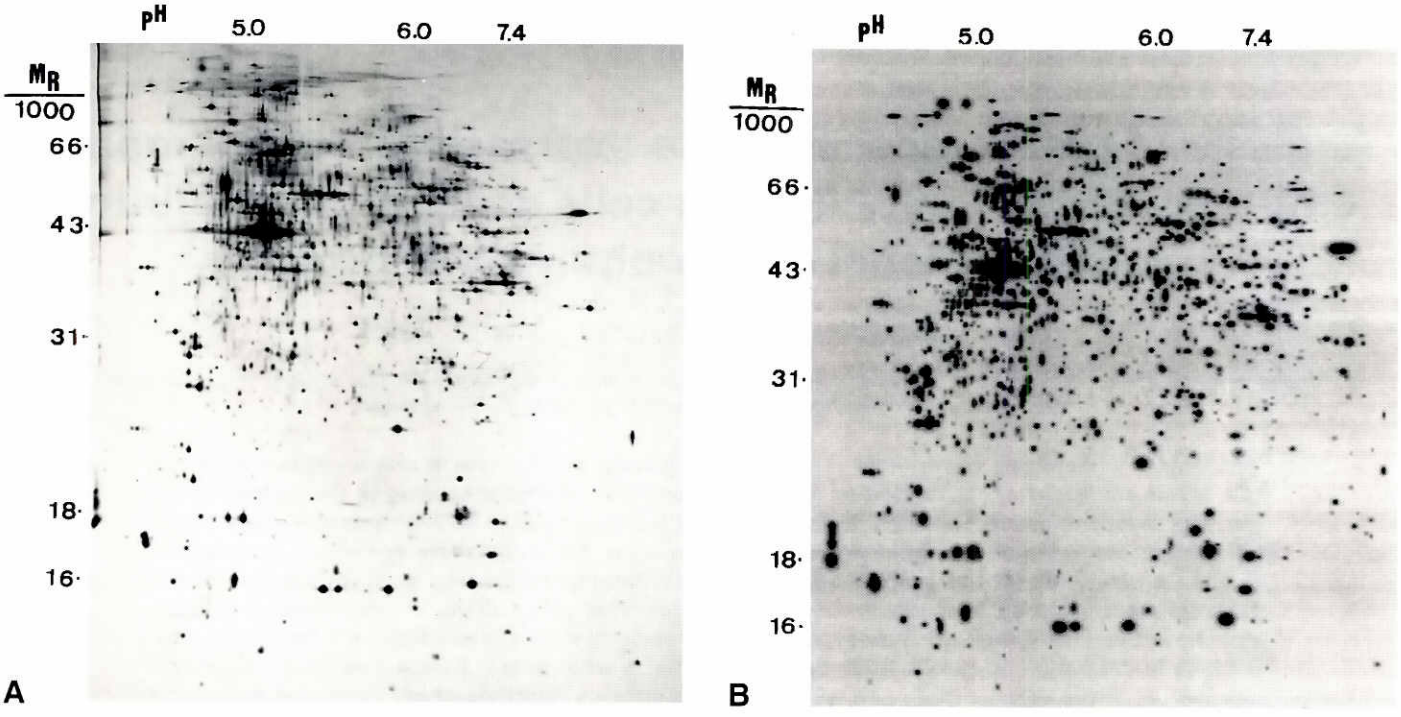

Fig. 1. Protein detection at the blastocyst stage. (A) *Two dimensional gel image of proteins synthesized by 50 normal blastocysts. The proteins were labeled with L-[^{35}S]methionine and separated on gels in the 20-100 kDa range. The gel was processed for fluorography and exposed to autoradiographic film for 10 days. Approximate molecular weights and isoelectric points are shown.* **(B)** *Standard computer image showing all the proteins detected at the blastocyst stage. The image represents a gel after spot extraction and editing to correct discrepancies between the gel and synthetic image. The image comprises 1125 spots. Approximate molecular weights and isoelectric points are shown.*

the total number is quite large, the interpretation of imprinting phenotypes becomes more difficult. One such estimate has already been provided on the basis of the occurrence of differentially methylated CpG rich regions in the genome; that estimate is in the region of 100 differentially methylated sequences in the genome (Hayashizaki *et al.*, 1994). If each of these corresponds to an imprinted gene, and there are 50,000-100,000 genes in the genome, it would suggest that the prevalence of imprinting is 0.1-0.2%. In principle this may be a fairly good estimate since most imprinted genes so far identified do show allelic differences in DNA methylation; however, it might be an underestimate since the type of allelic methylation differences present for example in the *Igf2* gene would preclude detection by this method (Sasaki *et al.*, 1992; Feil *et al.*, 1994). A general estimate of the abundance of imprinted genes at the protein level is not available at present, nor indeed is there evidence for many known gene products of whether they show imprinted expression or not.

One way of approaching this question is through the use of high resolution two-dimensional protein electrophoresis (2DE) to compare monoparental embryos at different stages of development. The preimplantation period of development is particularly interesting for this analysis since there are major epigenetic changes that occur at this stage, and since the precise expression status of the known imprinted genes is not even clear. Both parental genomes undergo major changes in DNA methylation with a globally low level of methylation being reached at the blastocyst stage (Monk *et al.*, 1987; Howlett and Reik, 1991; Kafri *et al.*, 1992). Some but not all of the allele-specific methylation patterns in imprinted genes which are observed later in development are present in the blastocysts (Brandeis *et al.*, 1993). There is some conflicting evidence on the parental expression of imprinted genes. For instance, *Igf2*, which is predominantly expressed from the paternal chromosome at later stages of development (DeChiara *et al.*, 1991; Ferguson-Smith *et al.*, 1991), is expressed in PG embryos (which contain only maternally derived chromosomes) (Latham *et al.*, 1994). We have found biallelic expression of *Igf2* in normal blastocysts (A. Villar personal communication; L.B. unpublished results), but other studies show substantially lower expression of *Igf2* in PG blastocysts than in normal ones (Rappolee *et al.*, 1992; Newman-Smith and Werb, 1995).

2DE of proteins was used by Petzoldt and Hoppe (1980) and Petzoldt *et al.* (1981) to compare protein expression in PG, AG, GG and normal preimplantation embryos. The results of these studies showed that at the protein level, the development of all the embryos was very similar up to the blastocyst stage. Minor quantitative variations were however observed between the PG and normal embryos which became more distinct at the blastocyst stage. The studies were limited by the technology available at the time. Only 150-200 proteins were visible on the gels and there was no possibility to follow up any of their results by characterizing individual spots. Preliminary data from Solter *et al.* (1986) also indicated that differences in protein expression could be detected in preimplantation AG and GG embryos, though again none of these differences were investigated further.

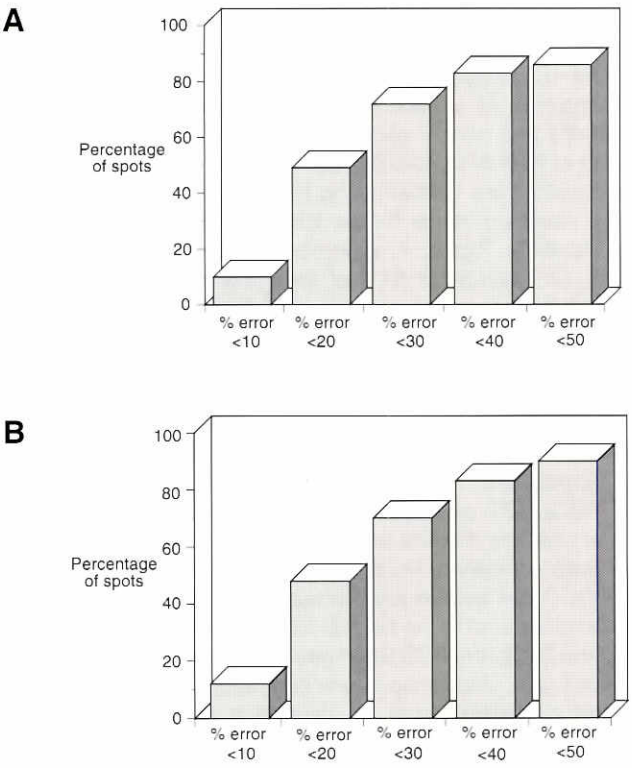

Fig. 2. Reproducibility of detection at the blastocyst stage. (A) *Histogram showing the reproducibility of protein detection in the five gels of normal blastocysts. Of 837 spots, 93% had a percentage error of <50, 86% had a percentage error of <40, 70% had a percentage error of <30, 46% had a percentage error <20 and 12% had a percentage error <10. Only 837 out of the 1125 present in the standard were analyzed. This is because a minimum of two intensity values is required for the calculation of standard deviation.* **(B)** *Histogram showing the reproducibility of protein detection in the five gels of parthenogenetic blastocysts. Of 942 spots, 92% had a percentage error of <50, 85% had a percentage error of <40, 73% had a percentage error of <30, 47% had a percentage error <20 and 12% had a percentage error <10. Only 942 out of the 1125 present in the standard were analyzed.*

Since these studies, the resolution and reproducibility of 2DE has been significantly improved. Under normal circumstances, 1000-2500 protein species can be detected, depending on the biological material investigated and the 2DE-technique used, however under particular conditions, 10,000 polypeptide spots have been resolved from adult mouse tissue (Klose and Kobalz, 1995). Furthermore, it is now possible to blot and sequence individual protein spots allowing the identification and further analysis of the corresponding genes (Patterson, 1994).

High resolution 2DE of proteins has been used to demonstrate parental 'effects' in the mouse (Vogel and Klose, 1992; Römer *et al.*, 1995) and in the investigation of a strain-specific developmental defect in AG embryos (Latham and Solter, 1991). This latter study did suggest that it may be possible to detect differences in the protein expression in AG, GG and normal embryos though in this study it was not possible to distinguish between those differences arising through the parental origin of the chromosomes, and those differences arising through the different strains used for the analysis.

In order to examine the question to what extent parent-specific gene expression occurs in the early embryo and with a view to possibly identifying new imprinted genes, we have examined the protein expression in PG and normal blastocysts. Two electrophoresis systems were used for the analysis of protein expression in blastocysts allowing examination of proteins in the ranges 20-100 kDa and 5-25 kDa. Ideally it would have been useful to include AG blastocysts in the comparison, however, the difficulties in obtaining AG blastocysts in suitable numbers and the developmental variability observed for AG embryos are prohibitive of such an analysis. As an alternative source of material which represented an additional developmental lineage, the protein expression in embryonic stem (ES) cells derived from AG, PG and normal blastocysts was examined. These cells are derived from the inner cell mass outgrowths of explanted blastocysts and allow the generation of large quantities of experimental material. For the analysis of ES cells, proteins in the range 20-100 kDa were examined. The two-dimensional gels were analyzed both visually, and also using the PDQUEST computer analysis package.

Results

Analysis of protein expression in PG and normal blastocysts

Metabolically labeled proteins from five replicate batches of normal blastocysts and five replicate batches of PG blastocysts were analyzed on gels which separate proteins in the range 20-100 kDa (Fig. 1A). The two groups of gels were initially compared by visual analysis, however no significant differences could be detected at this level of analysis. The gels were then analyzed using the PDQUEST computer analysis system. The standard image to which all other images were compared contained 1125 spots (Fig. 1B). Using both automatic and manual matching, an average of 87% of the spots on each gel were matched to the standard.

Before a comparison of protein expression in PG and normal blastocysts could be made, it was necessary to establish the levels of variation observed for individual spots within the sample groups, i.e. between replicate samples of PG blastocysts and between replicate samples of normal blastocysts. This was achieved by calculating the standard error of the mean for each of the spots. The analysis showed good reproducibility of quantitated spots in both the PG and normal blastocyst gel images (Fig. 2A and B).

Having assessed the levels of variability within sample groups, the PG blastocysts were compared to the normal blastocysts using both quantitative and qualitative tests to identify any differences between the two groups. Quantitative analysis involved analyzing the two groups to identify any protein spots which showed differences in intensity. Qualitative analysis involved analyzing the two groups to identify any protein spots which were present in one group and absent in the other group. The analysis was performed first on the two groups taking values from each individual gel image, and second by looking at the average values of the spots on each set of gel images. In addition, spot values for the two groups were compared and Student's *t* test performed in order to look for any statistically significant differences between the two groups. The differences

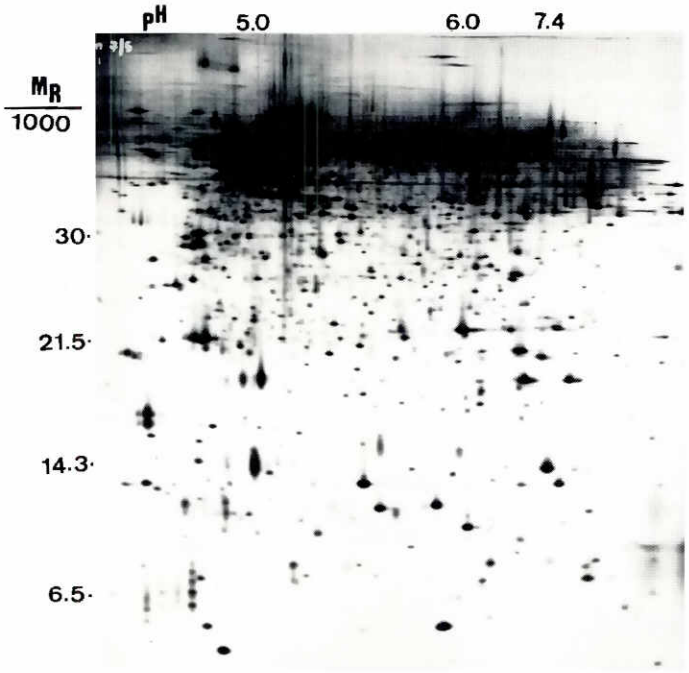

Fig. 3. Two dimensional gel image of proteins synthesized by 50 normal blastocysts. *The proteins were labeled with L-[^{35}S]methionine and separated on gels in the 5-25 kDa range. The gel was processed for fluorography and exposed to autoradiographic film for ~14 days.*

should include both quantitative and qualitative differences. Comparison of the normal blastocyst samples to the PG blastocyst samples revealed no consistent differences either in the intensity or in the presence or absence of any spots with any of the tests described above.

Analysis of proteins in the range 5-25 kDa

Although proteins <20 kDa can be seen on the 20-100 kDa separation system, the resolution of these spots is not optimal. Since this region could contain potentially interesting proteins such as peptide growth factors, we adapted the large (20-100 kDa) gel system to allow better resolution of smaller proteins. The system used allowed resolution of proteins in the range 5-25 kDa (Fig. 3). For the analysis, an additional four experimental replicates were made of both the PG and the normal embryos. The gels were analyzed visually. Of the 204 protein spots which were examined, none were reproducibly different between the two groups.

Analysis of protein expression in AG, PG and normal ES cells

Analysis of protein expression in undifferentiated stem cells was carried out on four samples of PG, four samples of AG and four samples of normal ES cells. No differences in protein expression could be detected by visual analysis of the AG and normal gels. ~4% of spots varied on the gels of PGES cells when compared to the AG and normal ES cells. This variation was attributed to the difference in strain used to generate the PGES cells. The AG and normal cells were of 129 genotype whereas the PG cells were of F1(C57BL/6xCBA) genotype. A similar figure (5%) was observed by Klose *et al.* (1991) for differences observed between different strains of mice detectable by high resolution 2DE. The supposition that the variation was due to strain differences was further confirmed by the observation that for the majority of variant spots, no variation was observed between PG and normal samples on the blastocyst gels which were also of F1(C57BL/6xCBA) genotype.

The results were verified using the PDQUEST analysis system. The standard image for the ES cells contained 983 spots and is shown in Figure 4. Using both automatic and manual matching, an average of 87% of the spots on each gels were matched to the standard. As described for the blastocyst samples, the ES gels were initially analyzed to assess the reproducibility of the system. Since no differences were found in the visual comparison of the AG and normal ES cells, and since they were of the same genotype, the two groups were analyzed together. The analysis showed the gels of the ES cells to be highly reproducible. These results are shown in Figure 5.

The PDQUEST analysis allows the comparison of only two groups at one time. For the analysis of ES cells, all combinations of gels were compared, i.e. the AGES cell gels were compared to the PGES cell gels in one comparison, the AGES cell gels were then compared to the normal ES cell gels in a second comparison and finally the PGES cell gels were compared to the normal ES cell gels. The groups were compared using both quantitative and qualitative tests as described for the analysis of blastocysts. The comparison of AG and normal ES cells revealed no significant differences between the two groups. ~5% of spots were observed to differ on comparison of the PG and normal and AGES cells. The slight increase in differences detected by computer analysis compared to those detected by visual analysis is a reflection of the increased sensitivity of computer analysis over visual analysis.

Discussion

We have examined early embryos and ES cells for parent-specific gene expression using the technique of high resolution 2DE of proteins. In the samples analyzed, no proteins were identified which showed significant differences in expression according to the parental origin of the chromosomes.

Overall, the gels examined for this study indicate that the 2DE system is both sensitive and reproducible. It is however notable that the reproducibility of protein detection in the ES cell gels using the PDQUEST computer analysis system was considerably higher than for the blastocyst gels. Whereas only 46% of spots on the blastocyst gels had a percentage error of <20, 76% of spots on the ES cell gels had a percentage error of <20 (compare Figs. 2 and 5). The high level of reproducibility observed is no doubt a consequence of the ES cells being in an undifferentiated state. The blastocysts, in contrast, were undergoing dynamic changes reflected in their protein profiles. The difference in reproducibility between ES cell proteins and blastocyst proteins demonstrates the necessity of examining the reproducibility of each type of sample analyzed. An estimate of the reproducibility is of paramount importance in deciding the number of samples required to distinguish meaningful results from those arising from inherent biological variability.

Poor reproducibility may have contributed to the results reported by Petzoldt and Hoppe, (1980), who observed differ-

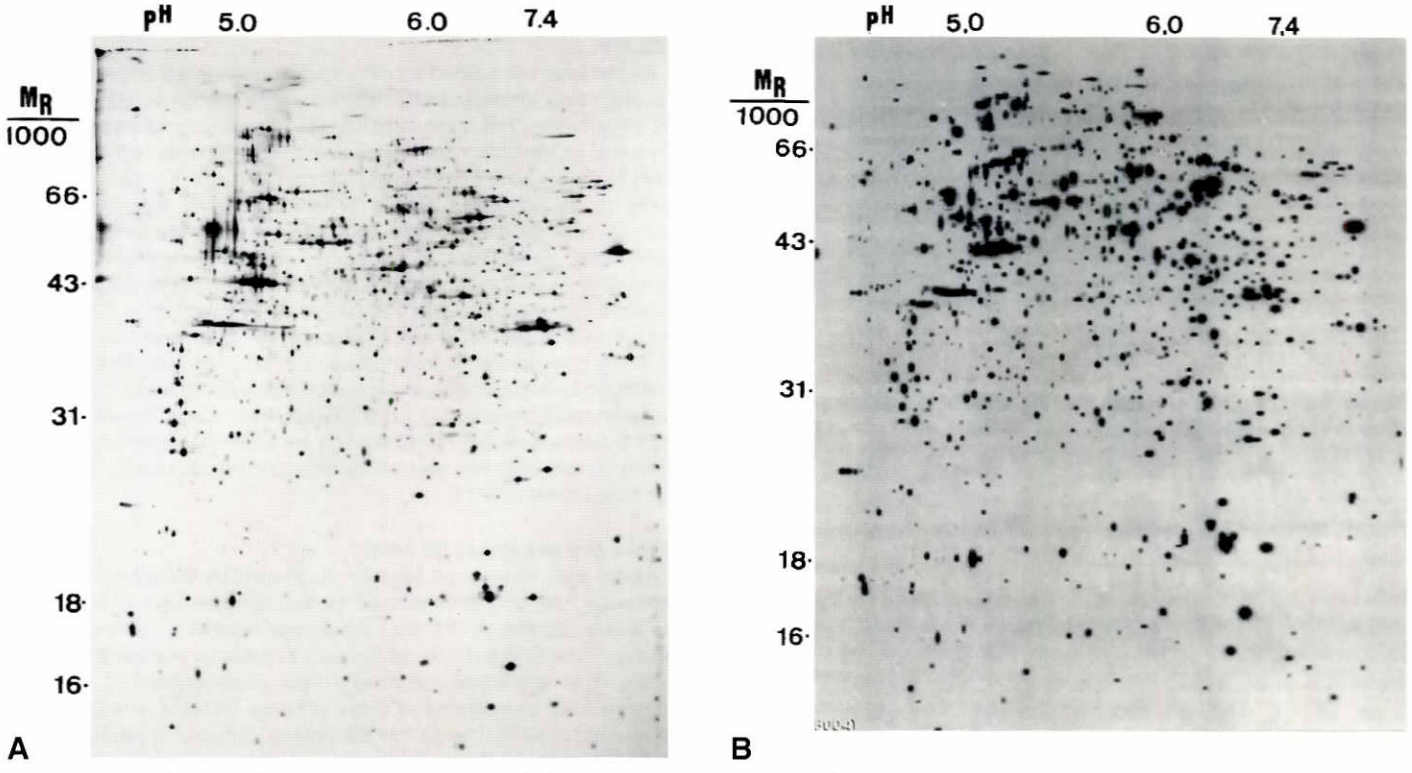

Fig. 4. Protein detection in embryonic stem cells. (A) *Two dimensional gel image of proteins synthesized in embryonic stem cells. The proteins were labeled with L-[^{35}S]methionine and separated on gels in the 20-100 kDa range. The gels were processed for fluorography and exposed to autoradiographic film for ~10 days. Approximate molecular weights and isoelectric points are shown.* **(B)** *Standard computer image showing all the proteins detected in the embryonic stem cells. The image represents a gel after spot extraction and editing to correct discrepancies between the gel and synthetic image. The gel contains 983 spots. Approximate molecular weights and isoelectric points are shown.*

ences in protein expression of PG and normal embryos on analysis of only 150-200 protein spots. Given the great increase (~10x) in resolving power, it might be reasonably assumed that these differences and more, would have been identifiable on our gels. Petzoldt and Hoppe analyzed between two and five samples from each developmental stage, but gave no mention as to how many blastocyst samples were analyzed. Were they to have analyzed only two samples of blastocyst-stage embryos, it would not have allowed them to distinguish between the biological variability of the system and meaningful results. This may also be true for the results obtained by Latham and Solter (1991) who only analyzed embryo samples in duplicate.

In order to interpret our results in the context of whether imprinting is a general regulatory mechanism or whether it is a more specialized phenomenon restricted to a limited number of genes, we must consider the nature of the proteins that are visualized by our separation system. Although to our knowledge there has not been a comprehensive analysis of what classes of proteins are visualized on 2D protein gels, an indication can be gained from those proteins whose identity has been ascertained. Proteins identified by immunoblotting or microsequencing include cytoskeletal proteins such as tropomyosin, components of the blood such as hemoglobin, heat shock proteins and enzymes such as alkaline phosphatase (databases are found in Celis *et al.*, 1993 and Corbett *et al.*, 1994). From our results we conclude that imprinting is not abundant in this class of proteins. Although the list is by no means exhaustive, notably absent are regulatory proteins such as transcription factors, growth factors and their receptors. These may be among the thousands of proteins that are seen in the gels but have not yet been identified, however, it is more likely that their low abundance precludes visualization in separations of total cell protein mixtures. From our results we can not say that imprinting is not widespread amongst these less abundant proteins However, our results show that monoparental embryos and cells are not generally compromised in their development and physiology, but rather that their phenotypes result from very specific defects, some of which are being defined (Newman-Smith and Werb, 1995).

There is evidence from other experimental systems that suggests that even among the less abundant proteins, imprinting is not widespread. Firstly, although the regions defined by analysis of monoparental disomies suggest that 10% of the genome may contain imprinted genes (Cattanach and Beechey, 1990), it is becoming apparent that imprinted genes are clustered into very small regions within the larger domains defined by Cattanach and Beechey. Of the imprinted genes so far identified, four (*Igf2, H19, Ins2* and *Mash2*) are very closely linked on the distal region of chromosome 7. Furthermore, some of the phenotypes associated with uniparental disomy for particular chromosomal regions can be explained by the actions of a very limited number of genes. The reduced growth observed in embryos maternally disomic for the distal region of chromosome 7 can essentially be explained by the imprinting of the *Igf2* gene (DeChiara *et al.*, 1991; Ferguson-Smith *et al.*, 1991).

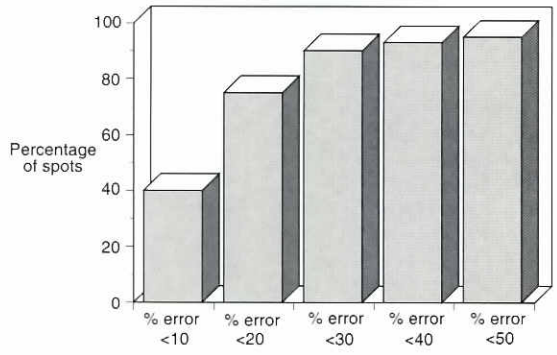

Fig. 5. Histogram showing the reproducibility of protein detection of embryonic stem cell gels. *In total, six gels were analyzed. These included three androgenetic samples of 129 genotype and three normal samples of 129 genotype. Of 918 spots, 99% had a percentage error of <50, 97% had a percentage error of <40, 91% had a percentage error of <30, 76% had a percentage error <20 and 39% had a percentage error <10.*

A second factor to consider is that, of the imprinted genes so far identified, three (*H19*, *Xist* and *IPW* [Brannan *et al.*, 1990; Brockdorff *et al.*, 1992; Wevrick *et al.*, 1994]) are putative functional RNA's with no protein product. The parent-specific expression of such genes would not be detectable using 2DE. Finally, the temporal and spatial specificity of imprinting should be taken into account when assessing the merits of any system used to identify new imprinted genes. As the expression patterns of more imprinted genes are analyzed, it is evident that there is no definitive on/off state for the parental alleles, rather the control of expression of the individual alleles is more subtle. Only the paternal allele of *Igf2* is expressed in the majority of fetal tissues, however in the choroid plexus and leptomeninges, both alleles are expressed. The *Mas* protooncogene is expressed from the paternal allele in early stages of fetal development but the maternal allele then becomes expressed in certain restricted tissues after e13.5 (Villar and Pedersen, 1994).

Systems that may result in the identification of new imprinted genes include the Restriction Landmark Genome Scanning system (Hatada *et al.*, 1993; Hayashizaki *et al.*, 1994) and that of differential display (Liang and Pardee, 1992), though these would also be limited by some of the features of imprinted genes outlined above. Ultimately we must wait until the signals in the DNA sequence which determine monoparental expression are identified before we can definitively identify imprinted genes and in turn, form a clearer picture of the extent of the phenomenon.

Materials and Methods

Embryo culture and parthenogenetic activation

Fertilized eggs were obtained from female F_1(C57BL/6 femalesx CBA/CA males) (henceforth abbreviated to F_1) x male F_1 matings and cultured in T6 medium according to Howlett and Reik (1991). The day on which the vaginal plug was found was taken as day 1 of gestation. Fertilization was confirmed by the presence of both male and female pronuclei.

For PG embryos, eggs were activated 18.5-19 h post-hCG by exposure to 7% ethanol (Cuthbertson, 1983) for 4 min, washed and transferred to T6 plus BSA containing 5 μg/ml cytochalasin B (Sigma) for 4 h. The embryos were then washed and subsequently cultured in T6 until collection.

All embryos were typed morphologically during their growth *in vitro*. The last easily characterizable morphological change to take place is that of cavitation. This begins late on day 3 for the normal embryos, and very early on day 4 for the PG embryos. The development of the PG embryos was ~6-10 h behind that of normal embryos. This was due mainly to the difference in time of fertilization and activation of the embryos. The delay was taken into account when the embryos were labeled with L-[^{35}S]methionine on day 5. Labeling was achieved by incubating batches of 70-100 embryos in 10 μl of T6+1 mg/ml polyvinylalcohol (PVA; Sigma) containing 100 μCi/ml L-[^{35}S]methionine (1100-1300 Ci/mmol; Translabel ICN) for 3 h. 50 of the best embryos, selected according to morphology, were collected in <1 μl medium. The embryos were lysed by addition of 5 μl lysis buffer (9 M urea, 70 mM DTT, 2% carrier ampholytes Servalyt 2-4, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). The samples were stirred for 20 min at RT to ensure complete lysis and solubilization of all proteins. The samples were stored at -70°C.

Culture and labeling of ES cells

All ES cells were a gift from Dr. N. Allen (The Babraham Institute, Cambridge, UK) and were derived as described in Allen *et al.* (1994). The AGES cell line (AK12) was at passage number 6 and was of 129 genotype. The PGES (PK9) cell line was at passage number 9 and was derived from ethanol-activated eggs from superovulated F_1 females. Since the main components of these cells are C57BL/6 and CBA, they are referred to as F_1. The normal ES cell line (D3) was at passage number 30 and was of 129 genotype.

Confluent dishes of cells were labeled by incubation with Eagles methionine free medium (Gibco) containing 1 mg/ml PVA and 100 μCi/ml L-[^{35}S]methionine (1100-1300 Ci/mMol; Translabel ICN) for 4 h. The cells were then lysed by addition of 1 ml lysis buffer (9 M urea, 70 mM DTT, 2% carrier ampholytes Servalyt 2-4 (Serva), 2% CHAPS). Cell scrapers were used to aid detachment of cells from the surface of the dish. The samples were transferred to eppendorfs using drawn out glass pipettes and stirred for 20 min at RT to ensure solubilization of all proteins. An aliquot was removed for determination of radioactive incorporation into the trichloroacetic acid-insoluble fraction. The samples for analysis by 2DE were stored at -70°C.

Two-dimensional electrophoresis and gel analysis

2DE of proteins in the range 20-100 kDa was performed as described by Klose and Kobalz (1995). For separation in the range 5-25 kDa, the same technique was used for isoelectric focusing but combined with the SDS-PAGE technique described in Schägger and Von Jagow (1987). In both instances, the size of the gels used for separating the proteins in the second dimension was 30 cm (running direction) x 23 cm x 0.75 cm.

Following fixation of the gels for 2 h in 10% acetic acid and 50% ethanol, they were dehydrated in concentrated acetic acid for 5 min. The gels were then impregnated with diphenyloxazole (PPO) (25% w/v PPO in acetic acid) for 2 h (Bonner and Laskey, 1974, modified by Pulleyblank and Booth, 1981), rinsed for 1 min in concentrated acetic acid and transferred to 2 l distilled water. The gels were shrunk to their original size in concentrated acetone. Gels were dried between two sheets of cellophane under constant vacuum driven by a standard water pump for 2.5 h at 60°C. Gels were exposed to X-ray film (Kodak X-Omat AR 5, Eastman Kodak Company, Rochester, UK) at -70°C, for 14-28 days.

The gels were analyzed both visually, and using the PDQUEST system running on a Sun IPX workstation (Protein Databases Inc., Huntingdon Station, New York, USA) described in detail by Garrels (1989). Briefly, gels were digitized at 176 μm resolution using a molecular Dynamics 300A laser densitometer (Sunnyvale, CA, USA). These images were processed by the computer software to remove image noise, background and vertical streaks. Spots were then detected and

their individual parameters entered into a 'gel spots' file. Manual editing was performed to correct any discrepancies between the gel and synthetic images. The gel images were then collected into a matchset and a standard protein map created for the set. The standard was chosen as the image within the matchset which contained the greatest number of detected spots. All gels were then matched to the standard using a combination of automatic and manual matching. For each spot detected, the intensity was divided by the total intensity of the gel and the result multiplied by 10^6. This 'normalization' evens out differences in intensity due to slightly different film exposures or different amounts of radioactivity loaded.

To calculate the reproducibility of the system, the percentage error of intensity values was calculated for each spot.

$$\text{Percentage error} = \frac{\text{Standard error of the mean}}{\text{Average}}$$

$$\text{Standard error of the mean} = \frac{\text{Standard deviation}}{\sqrt{n}}$$

Where n is the number of samples analyzed.

Acknowledgments

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