Proc. Natl. Acad. Sci. USA
Vol. 75, No. 7, pp. 3332–3336, July 1978
Developmental Biology

Identification of stage-specific polypeptides synthesized during murine preimplantation development

(two-dimensional gel electrophoresis/major histocompatibility complex)

JOHN LEVINSON, PETER GOODFELLOW, MARY VADEBONCOEUR, AND HUGH MCDEVITT

Division of Immunology, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

Contributed by Hugh O. McDevitt, March 20, 1978

ABSTRACT **[^{35}S]Methionine-labeled extracts of mouse ova and preimplantation embryos were analyzed by two dimensional polyacrylamide gel electrophoresis. Of the 400–600 molecular species that have been resolved as distinct spots on autoradiograms of gels for every stage of development from unfertilized eggs to early blastocysts, particular attention has been paid to the identification of 36 of these proteins, each of which is expressed only for a portion of the period under investigation. These molecules are referred to as stage-specific polypeptides and they are biochemical markers of early embryonic development and differentiation.**

The study of early mammalian embryogenesis has long been hampered by the fact that no obvious markers of differentiation are found until the inner cell mass and the trophectoderm have diverged. In order to circumvent this obstacle, several groups have chosen to search for biochemical correlates of early development. It has been well documented that the overall rate of protein synthesis by mouse embryos increases throughout the entire preimplantation period (1). In order to ascertain whether or not these quantitative changes are accompanied by qualitative changes, one- and two-dimensional polyacrylamide gel electrophoresis have been used for the separation of newly synthesized proteins in the mouse and in other animals.

For the preimplantation mouse embryo, it has been shown by conventional one-dimensional sodium dodecyl sulfate/polyacrylamide gel electrophoresis with single or double radiolabels that the major changes in protein synthesis occur between days 1 (two-cell) and 2 (four–eight-cell) of development (2, 3). However, because it was not possible to resolve more than about 50 polypeptides in the gels used, precise changes in the expression of many polypeptides could not be documented.

More recently, various groups have reported the results of high-resolution two-dimensional gel electrophoresis in similar studies. Using this technique, it was demonstrated that the inner cell mass and the trophectoderm synthesize qualitatively distinct sets of proteins (4, 5). In a recent investigation, two-dimensional gels were used to examine the proteins synthesized by preimplantation rabbit embryos (6).

The present study is an analysis of the entire period of murine preimplantation embryogenesis by two-dimensional polyacrylamide gel electrophoresis. From this analysis, a "map" of the proteins synthesized has been obtained. Furthermore, several *H-2* congenic strains have been compared at a variety of stages within this period.

MATERIALS AND METHODS

Mice. All the mice used for this study were taken from our colony at Stanford University Medical School. These strains include C3H/DiSn ($H\text{-}2^k$), C3H.SW ($H\text{-}2^b$), BALB/c ($H\text{-}2^d$), BALB.K ($H\text{-}2^k$), and 129 ($H\text{-}2^{bc}$).

Embryos and Gels. After having developed to the desired stage *in vivo*, embryos were flushed from the oviducts of superovulated, 6-week-old mice. These embryos were then grouped according to how many cells they contained and placed into microdroplets of serum-free medium containing 3–5 mCi of L[^{35}S]methionine per ml with a specific activity of 600–1000 Ci/mmol. After 1.5 hr in a 37° incubator, radiolabeled embryos were washed extensively and then suspended in 20–30 μl of the isoelectric focusing sample buffer. This buffer served to lyse and solubilize the cells. Lysates were stored in this buffer at −70° prior to analysis in the two-dimensional polyacrylamide gel electrophoresis system developed by O'Farrell (7). Kodak NS-2T film was used for autoradiography of the dried gels.

RESULTS

To determine whether or not the procedure used to radiolabel embryos was detrimental to them, a group of [^{35}S]methionine-labeled two-cell embryos was placed in culture alongside a group of unlabeled two-cell embryos. During the following days both groups were scored for viability and finally for the occurrence of the *in vitro* analog of implantation (8). Embryos from the two groups were found to behave identically under these conditions, indicating that the labeling procedure was not excessively traumatic for the embryos.

Figs. 1–4 are reproductions of selected two-dimensional gels for extracts of embryos at various stages of preimplantation development. The complete series of gels from which these figures were selected is described in Table 1. In the most detailed of the gels shown, a total of about 600 different spots can be resolved.

By comparing gels representing embryos from any two successive stages of development it is possible to identify spots that corresponded to proteins whose synthesis was about to be turned off. Such spots were decreasing in intensity with time of development and are marked in the figures with arrows pointing downward. Similarly, spots can be identified as increasing in intensity. These spots correspond to newly synthesized proteins, and they have been marked in the figures with arrows pointing up. Because technical variation between successive gels is occasionally significant, only those changes which have been reproducibly found in several independent gels are noted in the figures.

After all changes had been catalogued at the highest level of resolution possible, it was then a simple matter to follow forward in time the pattern of expression of each new spot and to follow backward the pattern of expression of each disappearing spot. In this way a "map" of the synthesis of stage-specific poly-

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: SSP, stage-specific polypeptide.

FIG. 1. Pattern obtained from autoradiogram of high resolution, two-dimensional gel electrophoretic separation of a whole-embryo lysate. The isoelectric focusing dimension is horizontal with the basic end at the left (pH 7) and the acidic end at the right (pH 4.5). Sodium dodecyl sulfate gel electrophoresis in 10% acrylamide is the vertical dimension with the origin at the top. Upward pointing arrows identify stage-specific proteins that are not found in gels of earlier embryos. Downward pointing arrows identify stage-specific proteins that are decreasing according to the key in Fig. 5. The box labeled 21 and the parentheses labeled 23 and 24 enclose regions where the SSPs that were first detected in two-cell embryos would migrate if they were present. This photograph represents a 40-day autoradiographic exposure of a gel that contained proteins from 119 [^{35}S]methionine-labeled C3H/DiSn single-cell zygotes and about 150,000 trichloroacetic acid-precipitable cpm.

peptides (SSPs) in murine preimplantation development has been prepared. This map for C3H/DiSn mice appears as Fig. 5. In the cases of certain SSPs it has not been possible to determine exactly the first or last embryonic stage at which the polypeptide was expressed. This has been indicated in Fig. 5 with the dotted bars. Space limitations necessitated the omission of several gels; therefore no figures are included to show the definition of the last 6 of the 36 SSPs.

The first four figures are taken from the chronological sequence of gels for C3H/DiSn embryos. It is from this sequence that the SSPs in Fig. 5 were defined. Fig. 1 shows the autoradiographic pattern obtained for the single-cell zygote stage. Most apparent is the first group of 18 polypeptides (SSPs 1–18), the majority of which were quantitatively significant contributors to the total cell protein. Of these 18 spots, 12 were definitely present in gels of unfertilized eggs and so were presumably synthesized by using maternally derived genetic information. It is possible that the other six of these SSPs were also expressed in unfertilized eggs and that we have failed to detect them for technical reasons.

The synthesis of each of these first 18 SSPs decreased in single-cell embryos and, as indicated in Fig. 5, ceased by the four-cell stage. Interestingly, however, they were not all shut off at the same time. This finding has served to define three protein synthetic stages for two-cell embryos. Fig. 2 represents a gel pattern for early two-cell embryos. In that gel several of the first 18 SSPs, such as numbers 6, 9, 10, 11, 12, 13, and 14, either were not present or were too faint for certain identification. All the rest were significantly reduced. While it is very likely that most of the unmarked differences between Figs. 1

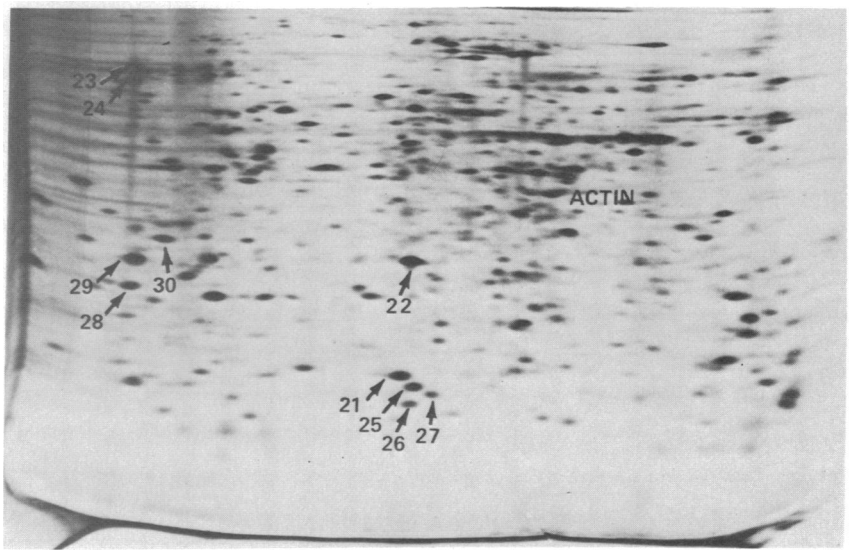

FIG. 2. Pattern obtained as in Fig. 1 from a 40-day exposure of a gel that contained proteins from 55 C3H/DiSn two-cell embryos and about 100,000 cpm.

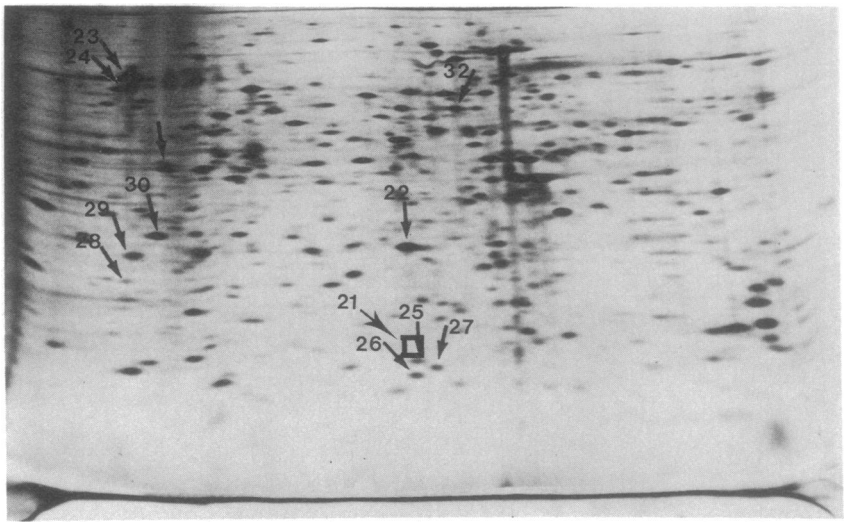

FIG. 3. Pattern obtained as in Fig. 1 from a 40-day exposure of a gel that contained proteins from 55 C3H/DiSn eight-cell embryos and about 100,000 cpm. As in Fig. 1, the box labeled 21 indicates the region to which that SSP would migrate if present.

and 2 also represent true SSPs, they have not been indicated as such because we do not have sufficient proof of their reproducibility.

Also of interest in Fig. 1 is the pair of acidic spots labeled 19 and 20. These spots were present neither in unfertilized eggs nor in two-cell embryos, and so they are specific markers for single-cell zygotes.

Fig. 2 represents the electrophoretic pattern of early two-cell embryos. Each of the 10 SSPs labeled in this pattern was undetectable in gels of earlier embryos or of ova. To better illustrate the appearance of these new proteins, a box in the gel of single-cell embryos (Fig. 1) encloses the area where SSP 21 would have run had it been present in the lysate. Similarly, a box in the gel for eight-cell embryos (Fig. 3) shows where SSP 21 would run had it been present in that lysate. In an analogous way, the pair of SSPs labeled 23 and 24 has been followed in Figs. 1–4. The parentheses in Fig. 1 mark the area in which these SSPs would have appeared were they present. In Figs. 2 and 3, the pair of spots is clearly detectable and has been marked as usual with arrows. For the gel of blastocysts, Fig. 4, parentheses once again indicate the position where SSPs 23 and 24 would be found if present.

From the time embryos reached the four-cell stage, changes occurred much less frequently and were relatively minor. Thus, Fig. 3, which shows an autoradiographic pattern from a gel of C3H/DiSn eight-cell embryos, represents a reasonably mature protein synthetic profile that will not change in any great degree for the remainder of preimplantation development. This can be seen by comparing Figs. 3 and 4, which represent eight-cell embryos and early blastocysts, respectively.

To determine whether or not several inbred mouse strains differed either in their developmental profiles of protein synthesis or in their expression of SSPs, the gels listed in Table 1 were run for comparison. Fig. 6 *a* and *b* shows corresponding regions from gels of C3H/DiSn and BALB/c single-cell zygotes, respectively. Careful comparisons of the entire autoradiograms from which the panels had been taken similarly revealed no significant reproducible differences between these two strains

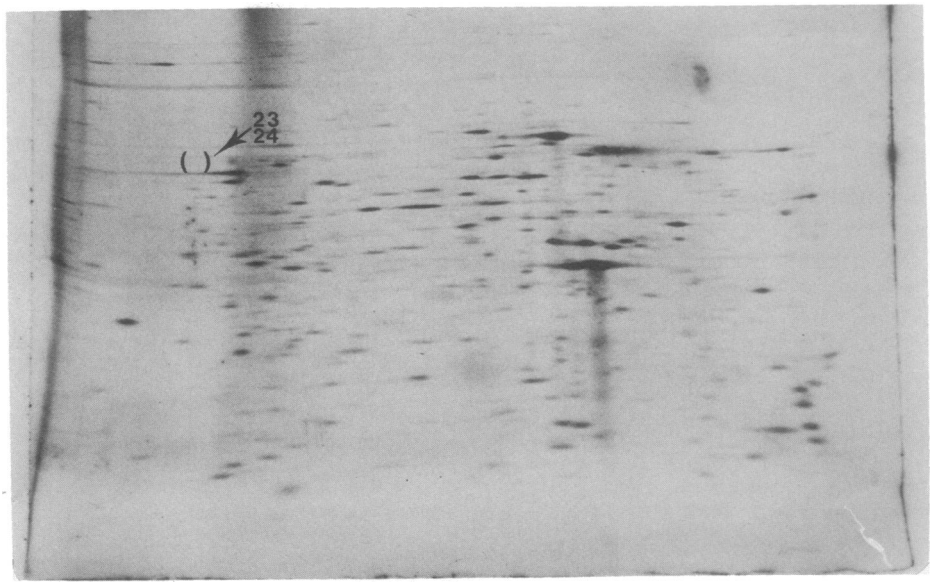

FIG. 4. Pattern obtained as in Fig. 1 from a 44-day exposure of a gel that contained proteins from 15 C3H/DiSn blastocysts and about 70,000 cpm. The parentheses labeled 23 and 24 indicate the region to which that pair of SSPs would migrate if present.

Table 1. Stages and strains examined by two-dimensional gel electrophoresis.

Embryo Genotype	Unfert.	Sc	2-c			4-c	8-c	16-c	Mor	Blast
			A	B	C					
C3H/DiSn	+	+	+	+	+	+	+	+	+	+
C3H/DiSn*	+	+		+		+	+	+		
BALB/c	+	+		+		+	+	+		
BALB.K						+	+	+		
C3H/DiSn × BALB/c		+		+		+		+		
(BALB/c × 129)F_1 × BALB/c	+	+		+	+	+		+	+	+

A + indicates those stages of development for which we have obtained two-dimensional gels of the proteins synthesized. See Fig. 5 legend for explanation of abbreviations.

* Analysis by nonequilibrium pH gradient gel electrophoresis instead of isoelectric focusing for resolution of even the most basic polypeptides.

at the single-cell stage. Fig. 6 *c* and *d* was taken from gels of C3H/DiSn and (C3H/DiSn × BALB/c)F_1 two-cell embryos, respectively. These panels, which contained the same region as that shown in the first two panels, are from gels run in a single experiment, and are clearly identical.

It should be noted that Fig. 6 *a*, *c*, and *d* all come from gels run in a single experiment. As seen by comparing Fig. 6 *c* and *d*, when similar or identical samples are electrophoresed side by side, a high degree of reproducibility can be achieved. It is for this reason that we have noted above that most of the many differences detected between single-cell zygotes (Figs. 1 and 6*a*) and two-cell embryos (Figs. 2 and 6*b*) from C3H/DiSn mice are probably real and only need independent confirmation. Comparisons of gels for all the inbred strains listed in Table 1 show that no genetic differences in protein synthesis have been detected.

DISCUSSION

In this study we have used two-dimensional polyacrylamide gel electrophoresis to define 36 SSPs. Each of these SSPs is expressed only for a short period during the preimplantation development of mouse embryos.

The interpretation of results obtained by this method is not always straightforward. For example, there is a limit to the exact reproducibility from experiment to experiment of protein synthetic patterns. Despite this difficulty, however, precise comparisons are possible, especially for gels which are run together in a single experiment. SSPs defined in this study have all been confirmed by several independent, conservatively interpreted gel experiments.

Three major limitations must also be considered with the data in these experiments. To obtain sufficient amounts of radiolabeled protein it was necessary to use pools of 10 to 119 embryos for each gel. With so many embryos comprising the lysate for each gel, it is inevitable that some of the lysates contained either parthenogenetically activated or dying embryos. Also, with such large groups, it is probable that the exact ages of embryos in any group formed a distribution over several hours. In order to circumvent this general problem of sample heterogeneity, we have recently combined the use of very thin slab gels (1.25 mm) with fluorography. These modifications have increased the sensitivity of the gel system so that we have been able to detect over 50 polypeptides in a 20-day exposure of a two-dimensional gel containing the proteins from only a single one-cell zygote. By analyzing electrophoretic patterns obtained from individual embryos, it will be possible to control for abnormal proteins, and to use developmental mutants for genetic studies.

A second constraint of the gel analysis system used involves the limits of resolution of each dimension. In the isoelectric focusing dimension, only those polypeptides with isoelectric points of pH 4.5–7 are included in the gel. To determine if this limitation is significant, a single experiment was completed for C3H/DiSn unfertilized eggs, single-cell, 2-cell, 4-cell, 8-cell, and 8–16-cell embryos using nonequilibrium pH gradient gel electrophoresis (9) which allows resolution of all the proteins. The results of this experiment indicate that in 4-cell and 8–16-cell embryos as many as 5–10 major spots are too basic to be resolved by the isoelectric focusing gels usually employed.

The third constraint involves the sensitivity of detection. According to O'Farrell (7), the faintest detectable spot in 20-day autoradiographic exposure corresponds to a polypeptide that emits about 1 cpm. Because we used a 40-day exposure, comparable spots in these gels probably contain somewhat less than 1 cpm. For a gel on which 100,000 cpm have been loaded, such barely visible spots should represent polypeptides that constitute 1/1000 of 1% or less of the total cell protein. Though this sensitivity is considerable, it is possible that many functionally

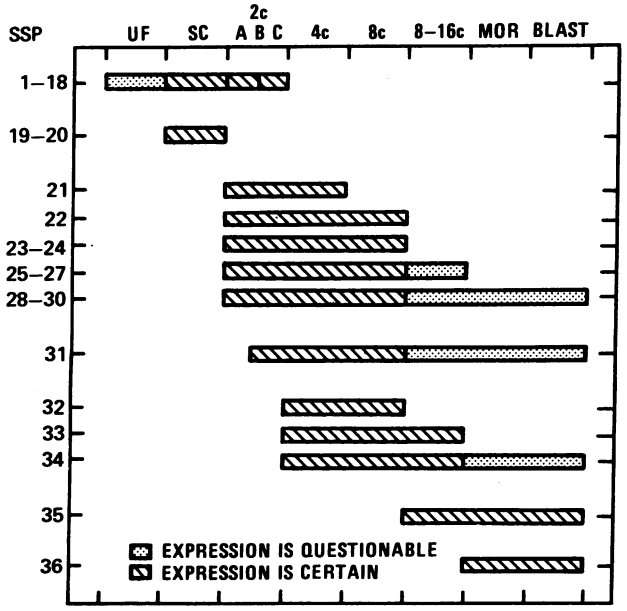

FIG. 5. Map of the expression of SSPs defined in two-dimensional gels of C3H/DiSn preimplantation embryos. The abbreviations across the top of the figures (UF–Blast) refer to unfertilized eggs, single cell, 2-cell, 4-cell, 8-cell, and 8–16-cell embryos, morulae, and blastocysts, respectively. The two-cell stage has been split into three protein synthetic periods labeled A, B, and C, during which the first 18 SSPs were differentially decreasing in their rates of synthesis. Similarly, the initial appearance of SSP 31 appears to subdivide the two-cell stage.

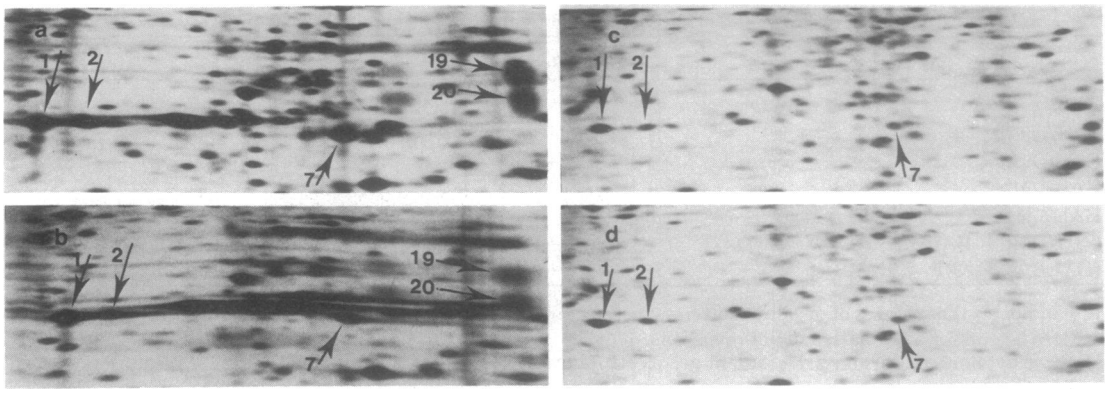

FIG. 6. Corresponding regions from autoradiograms of gels of (*a*) C3H/DiSn single-cell embryos, (*b*) BALB/c single-cell embryos, (*c*) C3H/DiSn two-cell embryos, and (*d*) (C3H/DiSn × BALB/c)F_1 two-cell embryos. Several SSPs have been indicated with arrows.

important molecules are present in much smaller quantities, and so are not detected.

Despite the foregoing limitations, a map of protein synthesis in murine preimplantation development has been obtained. The most important conclusions to be drawn from this map are that (*i*) changes in protein synthesis by the embryo as a whole occur in development and begin long before the onset of gross morphological differentiation, (*ii*) most of the changes in preimplantation protein synthesis occur by the eight-cell stage, and (*iii*) the most rapidly synthesized proteins in both single-cell zygotes and two-cell embryos are almost all electrophoretically identical to certain oocyte proteins. These findings are consistent with previous one-dimensional electrophoretic analyses of mouse embryos and also with more recent two-dimensional studies of early embryos from sea urchins (10) and rabbits (6). It is especially interesting to compare gels from rabbit embryos (6) with gels from mouse embryos. Though the overall trends are very similar with respect to shut-down of maternally determined gene products and to stabilization of synthetic patterns early in development, the actual proteins in question have quite different mobilities in both dimensions of these gels.

The description of SSPs in the mouse is important for many reasons. First, these polypeptides may be the biochemical markers which have been sought for use in studies of very young embryos. Second, some of the SSPs may actually be mediators of development, functioning to regulate early cell–cell interactions and differentiation. In addition, the pattern of their synthesis may provide clues to the nature of gene activity in embryos. For example, the finding that at least 12 of the first 18 SSPs are expressed in oocytes supports the idea that maternally derived genetic information is crucial in early development. The observations that a pair of "new" proteins is switched on in single-cell zygotes and that many additional "new" proteins appear during the two-cell and four-cell stages are consistent with the possibility that, in this period, the embryonic genome is beginning to exert control. Previous studies have shown that early cleavage in the mouse and the rabbit can occur in the presence of either actinomycin D (11) or α-amanitin (12, 13). It is therefore important to determine whether the appearance of any of these SSPs is transcription dependent.

No differences were discerned among the patterns obtained for any of the inbred or congenic strains used in this study. This observation suggests that theories that postulate that embryonically active, H-2-linked genes are important in the control of preimplantation development (14–16) would require that such genes either are not detectably polymorphic or produce very small quantities of gene product.

Special thanks are due to Dr. Patricia Jones for her many conceptual contributions to this work and for her critical assistance in the preparation of the manuscript. This research was supported by National Institutes of Health Grant HD 09410. J.L. is a predoctoral fellow of the Medical Scientist Training Program, National Institutes of Health, GM 1922. P.G. is a postdoctoral fellow of the Jane Coffin Childs Memorial Fund.

1. Epstein, C. J. & Smith, S. A. (1973) *Dev. Biol.* **33**, 171–184.
2. Epstein, C. J. & Smith, S. A. (1974) *Dev. Biol.* **40**, 233–244.
3. Van Blerkom, J. & Brockway, G. L. (1975) *Dev. Biol.* **44**, 148–157.
4. Van Blerkom, J., Barton, S. C. & Johnson, M. H. (1976) *Nature* **259**, 319–321.
5. Handyside, A. H. & Barton, S. C. (1977) *J. Embryol. Exp. Morph.* **37**, 217–226.
6. Van Blerkom, J. & McGaughey, R. W. (1978) *Dev. Biol.* **63**, 151–164.
7. O'Farrell, P. H. (1975) *Proc. Natl. Acad. Sci. USA* **250**, 4007–4021.
8. Spindle, A. J. & Pedersen, R. A. (1973) *J. Exp. Zool.* **186**, 305–318.
9. O'Farrell, P. Z., Goodman, H. M. & O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142.
10. Brandhorst, B. P. (1976) *Dev. Biol.* **52**, 310–317.
11. Tasca, R. J. & Hillman, N. (1970) *Nature* **225**, 1022–1075.
12. Manes, C. (1973) *Dev. Biol.* **32**, 453–459.
13. Golbus, M., Calarco, P. G. & Epstein, C. G. (1973) *J. Exp. Zool.* **186**, 207–216.
14. Bennett, D. (1975) in *T-Locus Mutants: Suggestions for the Control of Early Embryonic Organization through Cell Surface Components in the Early Development of Mammals*, eds. Balls, M. & Wild, A. E. (Cambridge University Press, Cambridge, England), pp. 207–218.
15. Bennett, D., Boyse, E. A. & Old, L. J. (1972) in *Cell Interaction Third Le Petet Colloquium*, ed. Silvestri, L. G., pp. 247–263.
16. Edelman, G. M. (1975) in *Perspective in the Cell Surface, Immunological and Chemical Approaches*, eds. Kahan, B. & Reisfeld, R. (Plenum, New York), pp. 260–266.