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# Quantitative analysis of protein synthesis in mouse embryos. I. Extensive reprogramming at the one- and two-cell stages

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## Summary

**A quantitative, high-resolution, two-dimensional gel protein database has been constructed for the mouse embryo. This database has been used to obtain a detailed accounting of the amount and relative timing of changes in protein synthesis that occur during the 1-, 2-, and 4-cell stages along with a description of the most prevalent developmentally regulated patterns of synthesis. We find that during these early stages of development the pattern of proteins synthesized changes to a much greater extent than previously appreciated. During the 1- and 2-cell stages, the majority (60 % and 85 %, respectively) of the analyzed proteins exhibit twofold or greater changes in their rates of synthesis. The periods of greatest change are the late 1-cell and mid 2-cell stages, during which an extensive remodelling of protein synthetic pattern occurs that is largely complete by 15 h following the first cleavage. Once this reprogramming is complete, very little change is observed during the late 2-cell and 4-cell stages. Cluster analysis of individual protein synthesis patterns reveals a limited number of coordinately regulated protein sets that are responsible for most of the changes observed during the 1- and 2-cell stages. During the 2-cell stage, one third of the proteins increase by an average of fivefold, another third decrease by an average of sevenfold, and 10 % undergo transient changes in rates of synthesis. These patterns reflect the switch from zygotic to maternal mRNA utilization following transcriptional activation. Significantly, a set of proteins exhibiting relatively constant rates of synthesis is not observed during the 2-cell stage, indicating that the rate of synthesis of almost every detectable protein changes significantly during this period. Overall, our data indicate that the transition from maternal to embryonic control of development results in an abrupt and extensive reprogramming of the early embryo with respect to the pattern of proteins synthesized.**

Key words: mouse embryo, protein synthesis, protein database, two-dimensional gel electrophoresis.

## Introduction

Cleavage of the preimplantation mouse embryo is accompanied by specific alterations in gene transcription, messenger RNA content and protein synthesis. Numerous studies have documented translational activation, changes in adenylation state of maternal mRNA, degradation of maternal mRNAs, and *de novo* gene expression (Knowland and Graham, 1972; Levey *et al.* 1978; Braude *et al.* 1979; Sawicki *et al.* 1981; Van Blerkom, 1981; Cascio and Wassarman, 1982; Clegg and Piko, 1982; Clegg and Piko, 1983*a*; Clegg and Piko, 1983*b*; Flach *et al.* 1982; Bensaude *et al.* 1983; Giebelhaus *et al.* 1983; Giebelhaus *et al.* 1985; Graves *et al.* 1985; Paynton *et al.* 1988; Bachvarova *et al.* 1989; Poueymirou and Schultz, 1987). As a result of these changes, maternal mRNAs are largely eliminated by the late 2-cell stage (Bachvarova and De Leon, 1980; Paynton *et al.* 1988) and many new polyadenylated mRNA species appear (Taylor and Piko, 1987), thereby effecting a switch from maternal to embryonic control of development. Both one- and two-dimensional gel electrophoretic analyses have revealed concommitant stage-specific alterations in protein synthesis as well as changes in post-translational modification (Epstein and Smith, 1974; Van Blerkom and Brockway, 1975; Levinson *et al.* 1978; Howe and Solter, 1979; Cullen *et al.* 1980; Johnson and Calarco, 1980; Van Blerkom, 1981; Flach *et al.* 1982; Howlett and Bolton, 1985; Howlett, 1986).

These earlier studies documented the major features of early embryonic protein synthetic patterns and demonstrated the usefulness of two-dimensional gel electrophoresis in the study of early mammalian embryos. For technical reasons, however, the information that has been obtained through this approach has been limited. For all but the most prominent proteins, variability in the electrophoretic conditions

employed and staging of the embryonic material analyzed preclude meaningfull comparisons of data from experiments conducted at different times or in different laboratories. Furthermore, most of these studies did not use high-resolution two-dimensional gel systems and none was coupled to a system for quantitative analysis. Consequently, none of the following (1) the overall amount of change that occurs in the protein synthetic pattern of the early embryo, (2) the precise timing of changes or (3) the developmentally regulated patterns of synthesis have been adequately documented. For example, it has not been clearly shown whether the switch from utilization of maternally encoded mRNA to the use of newly transcribed embryonic mRNA is a conservative event, such that stage specific changes occur on a background of constitutive protein synthesis, or instead results in an extensive reprogramming of protein synthesis.

The ability to construct quantitative protein databases (Garrels and Franza, 1989*a*; Garrels and Franza, 1989*b*; Celis *et al.* 1990*a*; Celis *et al.* 1990*b*; Garrels *et al.* 1990; VanBoelen *et al.* 1990) now provides the means to examine alterations in protein synthesis in much greater detail. A system of standardized, high-resolution two-dimensional gel electrophoresis combined with computerized gel image analysis allows as many as 2000 separate protein spots to be resolved and up to 1600 to be located and accurately quantified (Garrels and Franza, 1989*a*). Additionally, the availability of computer software for matching two-dimensional gel patterns and constructing databases allows quantitative comparison of data from dozens of samples generated in multiple, independent experiments.

To exploit the advantages offered by such a system and to characterize more completely the changes in protein synthesis that occur in the early mouse embryo, we constructed a protein database using the QUEST system (Garrels, 1989). This database was used to examine changes in protein synthesis that occur during normal development from fertilization through the end of the 4-cell stage. A series of 18 gel images representing embryos labeled at 3 h intervals during the 1-, 2- and 4-cell stages were analyzed in detail. The data provide the first quantitative measure of the overall amount of change that occurs in the protein synthetic pattern during this period of development and reveal the precise times during development at which these changes take place. The data also reveal the major patterns of protein synthesis that are exhibited by the developing embryo. We find that the switch from utilization of maternally encoded mRNA to the use of newly synthesized embryonic mRNA results in an extensive reprogramming of the pattern of proteins synthesized during this period of development, such that nearly every detectable protein undergoes significant alteration in its rate of synthesis.

## Materials and methods

### *Embryo isolation and culture*

C57BL/6×DBA/2 $F_1$ hybrid mice (Harlan Sprague Dawley) were used for all experiments. Female mice 4–6 weeks of age were given 5 i.u. of pregnant mare serum gonadotropin (PMSG) followed 46 h later by 5 i.u. of human chorionic gonadotropin (HCG). With the exception of experiments where the 1-cell stage was to be analyzed, superovulated mice were placed with males overnight and checked for copulation plugs the following morning. Embryos were isolated from the ampullae in Hepes-buffered Whitten's medium (Whitten, 1971), treated for 2 min with hyaluronidase to remove cumulus oophorous cells, and washed 4 times in Whitten's medium. Following removal of unfertilized and degenerate eggs, the embryos were incubated at 37 °C in bicarbonate-buffered Whitten's medium supplemented with 100 $\mu$M EDTA (Abramczuk *et al.* 1977) under an atmosphere of 5 % $CO_2$ and 5 % $O_2$. Synchronous cohorts were collected at each cleavage division by collecting all of the embryos that cleaved within 1 h time periods (Bolton *et al.* 1984). The end of each cohorting period was defined as 0 h for that cohort. Cohorts in each experiment were collected over a period of 4–5 h. For experiments where 1-cell embryos were to be analyzed, superovulated mice were placed with male mice for 2 h beginning at 13 h after injection of HCG. At 3 h after mating, the eggs were removed from the ampullae. In this way, embryos were synchronized at fertilization to within 2 h of each other. Fertilized 1-cell embryos, identified initially by the presence of second polar bodies and later by the presence of two pronuclei, were divided into 7 groups and labeled at 3 h intervals beginning at 3.5 h after mating.

### *Embryo labeling and lysis*

Synchronous cohorts of 25–50 embryos were labeled for 3 h at 3 h intervals following cohorting, thereby providing a series of contiguous samples spanning the approximately 57 h of *in vitro* preimplantation development from fertilization to the end of the 4-cell stage. The embryos were incubated in 40 $\mu$l droplets of Whitten's medium containing 1 mCi ml$^{-1}$ of L-[$^{35}$S]methionine (Amersham, >1100 Ci mmol$^{-1}$) under oil. Consistent with previous observations (Van Blerkom and Brockway, 1975), the relative rates of L-[$^{35}$S]methionine incorporation were very similar for the 1-, 2- and 4- cell stages (mean disints min$^{-1}$/embryo 29900, 23100, 27400, respectively). Phosphoproteins were labeled by incubation in phosphate-free Whitten's medium containing 2 mCi ml$^{-1}$ orthophosphate (carrier free, Amersham). At the end of the labeling period, the embryos were washed once in phosphate-buffered saline and then lysed in a small volume (10–30 $\mu$l) of hot (100 °C) SDS buffer and processed for electrophoresis as described (Garrels and Franza, 1989*a*).

### *Two-dimensional gel image analysis*

Electrophoresis using pH 4–8 ampholines (British Drug House) in the first dimension and 10 % polyacrylamide in the second dimension was performed as described (Garrels, 1983). Following electrophoresis, gels were processed for fluorography and multiple exposures obtained. The use of calibration strips containing known amounts of radioactivity during the exposure of films allowed for accurate quantification of the amount of radioactivty contained in each protein spot. Aliquots (1–2 $\mu$l) of each sample were used for determination of trichloroacetic acid precipitable radioactivity. Typically, 10 $\mu$l of sample were applied per gel, containing $2\times10^5$–$5\times10^5$ disints min$^{-1}$ of TCA precipitable material.

The QUEST system of two-dimensional gel image analysis was used to analyze the gel autoradiographic images and to construct the database. Details of the procedures used in creating and analyzing gel images and for matching protein

patterns have been described (Garrels, 1989). A few gels were of low intensity or damaged and therefore not usable. Such gels were omitted from experimental sets and, where necessary, replaced with gels from duplicate experiments. For the series of gels analyzed here, duplicate gels representing embryos labeled at 15.5 h of the 1-cell stage and 6 h of the 2-cell stage were substituted with gels from duplicate experiments. The gel representing embryos labeled at 0 h of the 4-cell stage was omitted. No substitution was made since the last time point of the 2-cell stage encompassed part of this time period.

A standard protein map was created using the coordinate system of a standard gel (Fig. 1). The gel representing the 18 h 2-cell-stage embryo was chosen as the standard since it was most easily matched to each of the other gels. Spots not detected on the standard gel were assigned standard coordinates by the automatic matching program that interpolates spots from one gel pattern into the coordinate system of another. All spots on the standard map were assigned standard spot (SSP) numbers for identification and standard coordinates (in mm). Apparent isoelectric point and relative molecular mass were calculated for each protein based on spot position relative to proteins of known isoelectric point and relative molecular mass, using the REF52 (rat) standard map as a reference scale (Garrels and Franza, 1989*a*).

For each detected protein, the radioactivity (in disints $min^{-1}$) incorporated during the labeling period was compared to total protein radioactivity in the applied sample, and the result was expressed as parts per million (ppm) of total incorporation. Changes in the relative rate of synthesis could therefore be measured for each detected protein.

A total of 1100 high quality spots were detected and followed through the series of 18 gels (3 h time intervals) spanning these stages. These were classified as high quality spots (global quality >50; see Garrels, 1989) based on their intensities, shapes and amounts of overlap, if any, with neighboring spots and corresponded to those spots of good quantitative reliability. All of the analyses discussed below were restricted to this set of high quality spots.

Gel patterns were compared by three methods to quantify the degree to which the patterns of proteins synthesized during different 3 h intervals resembled one another. First, the percentage of detected proteins that increased or decreased by a specified amount (usually twofold or more) was determined. Second, the intensity ratios of spots on matched gel pairs was plotted as a histogram on the logarithmic scale (log2) and the standard deviation of the distribution calculated. In both of these methods, only spots detected on both gels being compared were scored; spots with low quality in either gel and spots with low intensity on both gels were excluded (Garrels, 1989; Garrels and Franza, 1989*a*). The standard deviation (S.D.) value provides a statistical measure of relatedness that accounts for both the number and magnitude of differences between samples (Garrels and Franza, 1989*a*). In earlier studies of REF52 fibroblasts, the S.D. ranged from 0.3–0.4 for duplicate samples to 1.18 for comparisons between proliferating and quiescent fibroblasts. The percentage of proteins exhibiting twofold or greater differences ranged from 2 % for comparisons between duplicate samples to nearly 30 % for comparisons between proliferating and quiescent fibroblasts (Garrels and Franza, 1989*b*). As a third means of comparison, we calculated the average rates of synthesis (in ppm) during 6 h intervals of each stage. This was done by computing the average of the ppm values for gels from two consecutive 3 h time intervals. We then determined the fraction of proteins for which the maximum of these averages was at least 2 or 4 times greater than the minimum of these averages. By using the average ppm values for 6 h intervals, we minimized the effect of any single aberrantly high or low values that might have arisen. Since this method is not limited to comparisons between pairs of gel images, it at once accounts for very rapid changes, for more long-term, progressive changes, and for changes that occur at different times during each stage, and provides an estimate of the number of analyzed proteins that change over the entire course of each stage.

To identify sets of coordinately regulated proteins, profiles of synthesis for individual proteins were compared by cluster analysis as described (Garrels *et al.* 1990). Briefly, the clustering process begins by comparing the expression profile of every spot to that of every other spot to generate similarity scores. Clustering is then a process of grouping spots that have similar expression profiles. The clustering algorithm is hierarchical; that is, at each step the two most similar clusters are joined to form a larger cluster, generating a tree structure. The clustering process terminates when all clusters differ by more than a predetermined similarity score. To speed the algorithm, clustering begins not from individual proteins but from 'preclusters' of proteins with highly similar expression profiles. Because a protein can be a member of more than one precluster, there is a small amount of overlap among the members of some of the final clusters. Clustering is an automatic process, but the QUEST system allows the investigator to adjust the final set of clusters by manual combining or splitting, i.e. moving up or down the tree of clusters. Manual adjustment of the final clusters was not performed except where noted. For each cluster generated, an expression profile is obtained that shows the logarithmic average of the individual ppm values for the spots in that cluster at the times indicated.

Protein identifications were tentatively assigned to twelve spots either by immunoprecipitation or by alignment with gels from cultured rat or mouse cell lines in which these proteins have been previously identified (Garrels and Franza, 1989*b*). Phosphoproteins were identified by alignment of gels containing $^{32}P$-labeled proteins with gels containing L-[$^{35}S$]methionine-labeled proteins.

## Results

### *A standard protein map for the early mouse embryo*

A standard protein map defining a standard coordinate system and a standard numbering system was created for the series of gels analyzed (Fig. 1). This map is based on the coordinate system of the gel representing the 18 h 2-cell stage embryo that is typical of the quality and resolution of the gels in the mouse embryo database. Spot position can be expressed either in millimeters or in apparent isoelectric point and relative molecular mass. The map is divided into 14 vertical and 12 horizontal zones. Standard spot numbers are assigned to each detected protein. These consist of two letters that denote the vertical and horizontal zones in which the spot is located followed by a number that is assigned so that the largest spot in that region receives the lowest number. Since each of the 18 gel images analyzed are matched directly or indirectly to this gel image, all spots can be assigned a standard spot number even if they do not appear in the 18 h 2-cell stage gel image. The standard spot numbers provide a convenient means for locating and referencing each of the

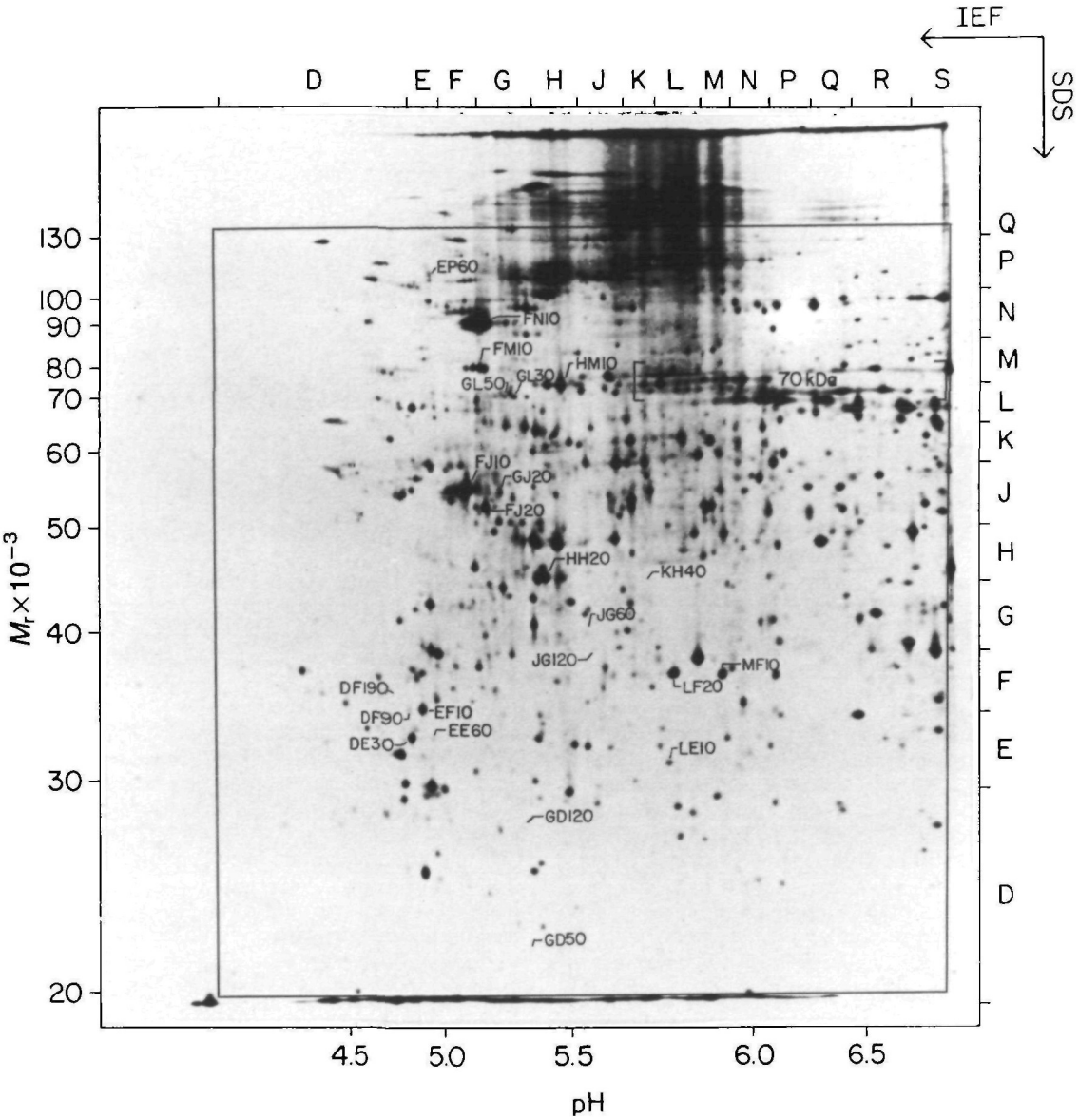



**Fig. 1.** Standard protein map for early preimplantation stage mouse embryos. A coordinate system of vertical and horizontal zones was imposed upon the gel image from 18 h 2-cell stage embryos to generate the standard gel map. The positions of all of the proteins detected in gels from the 1-cell, 2-cell and 4-cell stages were entered into the gel map. $M_r$ and pI were estimated according to the positions of proteins observed in the REF52 database. Proteins were assigned standard numbers consisting of 2 letters (vertical and horizontal zones) and a number where the most intense spot in that zone was assigned the lowest number. The region bordered by the inner box denotes the gel region analyzed. Characteristics of the proteins labeled in the figure, including the twelve known proteins, are given in Table 1.

detected proteins. The location of 24 proteins are indicated and characteristics of these 24 proteins are given in Table 1. These include the 12 known proteins and an additional 12 unidentified proteins that exhibit developmentally regulated patterns of synthesis.

*Extensive changes in protein synthesis occur during the 1- and early 2-cell stages*

The availability of quantitative data describing the relative rates of synthesis of individual proteins during consecutive 3 h intervals of the 1–, 2-, and 4-cell stages provides an opportunity to measure the overall amount of change in the protein synthesis pattern that occurs during these stages and to determine the time at which major changes occur. As described in Methods, gels can be compared pairwise by either scoring the number of matched spots that differ in intensity by at least twofold, or by calculating the standard deviation (S.D.) of the distribution of spot intensity ratios plotted on a log2 scale. Additionally, the average rates of synthesis over different 6 h intervals can be compared to determine the fraction of analyzed proteins that change quantitatively over the entire course of each cleavage stage. The first two methods provide a quantitative measure of the

**Table 1.** *Known proteins and some developmentally regulated proteins in the mouse embryo database*

| SSP[1] | Identification | Cluster | pI | $M_r$ | max ppm[2] |
|---|---|---|---|---|---|
| 1. DE30 | Tropomyosin 5[a] | 2A | 4.80 | 31800 | 325 |
| 2. DF90 | PCNA Satellite[b] | | 4.78 | 33900 | 51 |
| 3. DF190 | | 2D | 4.69 | 34912 | 96 |
| 4. EE60 | | 2D | 4.94 | 31700 | 647 |
| 5. EF10 | PCNA[b] | | 4.88 | 33800 | 674 |
| 6. EP60 | | 2D | 5.05 | 112005 | 82 |
| 7. FJ10 | β-Tubulin[c] | 2A | 5.17 | 53900 | 4358 |
| 8. FJ20 | β-F1-ATPase[c] | 2A[d] | 5.23 | 51500 | 1049 |
| 9. FM10 | HSP80[b] | 2D | 5.23 | 77500 | 1634 |
| 10. FN10 | HSP90[b] | 2A | 5.25 | 92000 | 13881 |
| 11. GD50 | | 2B | 5.29 | 20300 | 1052 |
| 12. GD120 | | 2B | 5.29 | 26600 | 696 |
| 13. GJ20 | α-Tubulin[b] | 2D | 5.27 | 54800 | 965 |
| 14. GL30 | Lamin B[b] | 2A | 5.31 | 69700 | 92 |
| 15. GL50 | Lamin B (phos)[b,c] | 2A | 5.29 | 69800 | 78 |
| 16. HH20 | Actin[b] | | 5.41 | 44700 | 1234 |
| 17. HM10 | HSP73[b] | 2A | 5.50 | 71600 | 4575 |
| 18. JG60 | | 2B | 5.55 | 39400 | 241 |
| 19. JG120 | | 2B | 5 57 | 37900 | 233 |
| 20. KH40 | | 2B | 5.69 | 43745 | 214 |
| 21. LE10 | | 2D | 5.73 | 29500 | 422 |
| 22. LF20 | | 2A | 5.76 | 35600 | 747 |
| 23. MF10 | | 2A | 5.93 | 35300 | 587 |
| 24. $70\times10^3$ $M_r$ complex[c] | | 2D | 5.99–6.6 | 65000–71000 | 40770 |

[1] SSP denotes the standard spot number derived from the standard gel map shown in Fig. 1.
[2] Maximum spot intensity observed from fertilization through the end of the 4-cell stage expressed as parts per million.
[a] Identified by immunoprecipitation with anti-tropomyosin serum.
[b] Identified by alignment with gels of mouse 3T3 and rat REF52 cells.
[c] Phosphoproteins.
[d] Increased by only 30% during the 2-cell stage.

degree of similarity between two gel images and have been used extensively in the construction of the REF52 database (Garrels and Franza, 1989*a*; Garrels and Franza, 1989*b*). As a basis for comparison to the data presented below, values ranged from about 2% twofold differences and a S.D. of 0.3–0.4 for comparisons of duplicate REF52 samples to nearly 30% twofold differences and a S.D. of 1.18 as proliferating REF52 fibroblasts become quiescent. The third method compares data from more than two samples and at once accounts for very rapid changes, for long-term, progressive changes, and for changes that occur at different times during a given stage.

During the 1-cell stage, approximately 60% of the analyzed proteins exhibit twofold or greater changes in synthesis and 27% change by at least fourfold when the average rates of synthesis during different 6 h intervals are compared. Throughout the 1-cell stage, S.D. values are high, ranging from 0.89 to 1.13 over 3 h intervals (Fig. 2A) and up to 1.7 between embryos labeled at 3.5 and 15.5 h after mating. Examinination of the amount of change that occurs during sequential 3 h intervals reveals a period of time (12.5–18.5 h post-mating) during which the rate of change is especially great (Fig. 3) with a peak rate of change between 12.5 and 15.5 h post-mating. The majority (80%) of the proteins that change by at least twofold during this period (12.5 and 18.5 h post-mating) decrease in synthesis. The generally high S.D. values and the prevalence of relative decreases over increases indicate a continuously changing pattern of protein synthesis during the 1-cell stage that is dominated by the loss or inactivation of numerous mRNA species. One additional feature of the 1-cell stage is that the early 1-cell stage gels exhibit slightly more resemblance to the 18.5 h gel than the 15.5 h gel (Fig. 2A) indicating that the pattern of proteins synthesized at 18.5 h post-mating shares some feature(s) in common with the pattern seen for the early 1-cell embryo.

During the 2-cell stage, the amount of change observed is even greater than that seen during the 1-cell stage. Approximately 85% of the detected proteins change by at least twofold and 55% change by at least fourfold. A second major period of change is observed between 3 and 15 h post-cleavage (Fig. 3). This second period of change ends abruptly at 15 h post-cleavage (Fig. 2B and C, Fig. 3). The S.D. values for comparisons between early (0, 3, 6 h) 2-cell stage gels and progressively later gels reach a maximum by 12 h and remain relatively constant thereafter (Fig. 2B and C) and S.D. values at the end of the 2-cell stage are quite low (0.52 and 0.42, Fig. 2B). Relative to the value of 0.4 expected for duplicate samples, these later values indicate that relatively little change in the protein synthesis pattern occurs between 15 and 21 h post-cleavage.

Values as high as 85% twofold or greater changes and 2.4 S.D. are far in excess of the maximum differences observed between proliferating and quiescent REF52 cells, indicating that the amount of change occuring during the 1- and 2-cell stages is much greater than the changes observed in REF52 cell cultures. In

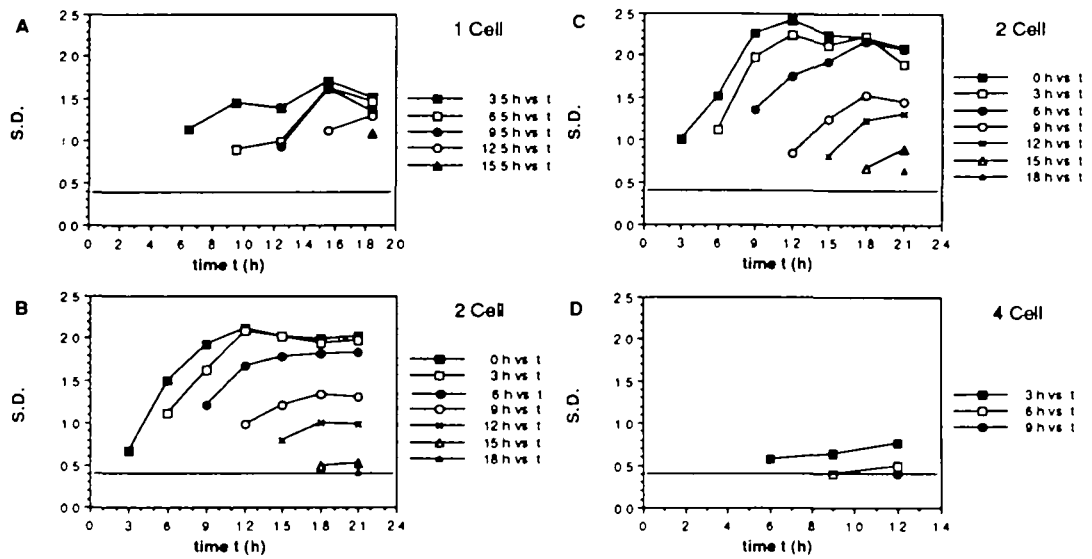



**Fig. 2.** Quantitative comparisons of gel patterns from each stage. The four graphs depict the standard deviation (S.D.) values for all comparisons between pairs of gels. (A) 1-cell stage; (B–C) two independent 2-cell stage series; and (D) 4-cell stage. Each curve depicts the results of comparing the gel for one 3 h time interval with gels for every subsequent time interval within that stage. The line drawn at 0.4 S.D. denotes the baseline corresponding to the degree of difference observed between gels of duplicate samples.

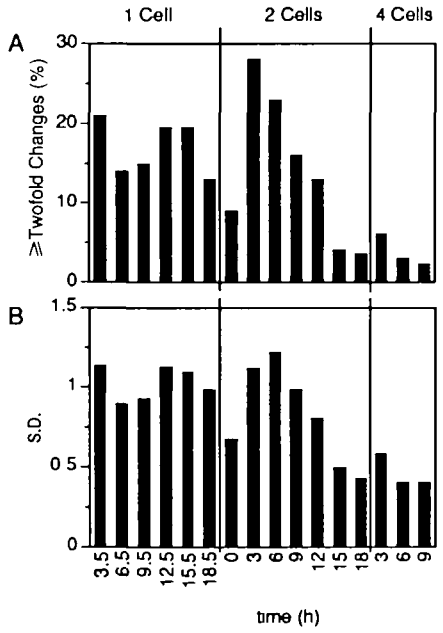



**Fig. 3.** Rate of change in protein synthetic patterns of early preimplantation mouse embryos. The two graphs illustrate the amount of difference arising during consecutive 3 h intervals from fertilization to the end of the 4-cell stage: (A) the percentage of the detected proteins exhibiting two-fold or greater changes in spot intensities and (B) the standard deviation (S.D.) of average spot intensity ratios. Each bar shows the amount of difference between the gel pattern of embryos labeled at the times indicated and that of embryos labeled during the following 3 h interval. Data were not obtained for the 0 h time point of the 4-cell stage, hence no comparison between the 0 and 3 h of the 4-cell stage is shown.

fact, even the change that occurs during certain 3 h intervals is greater than the change that occurs as proliferating REF52 cells become quiescent (Fig. 3). Both the amount and timing of the changes in the 2-cell stage protein synthesis pattern were observed in two independent experiments (Fig. 2B and C).

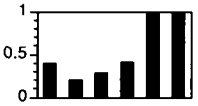
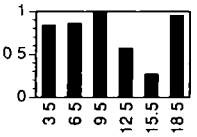
The low rate of change observed at the end of the 2-cell stage continues into the 4-cell stage when only 8 % twofold changes and 0.3 % fourfold changes occur. Over any 3 h interval, the S.D. ranges from only 0.40 to 0.58 and as few as 2.6 % of the proteins change by twofold or more. Thus, the 4-cell stage exhibits many fewer quantitative changes than the 1- and 2-cell stages, with the protein synthesis patterns for embryos labeled 3 h apart being as closely related as duplicate samples in the REF52 database.

### *Cluster analysis reveals developmentally regulated patterns of synthesis*

To determine whether the dramatic changes in protein synthesis that occur during the 1- and 2-cell stages could be explained by a small number of regulatory processes, we used cluster analysis to examine individual protein synthesis profiles. Cluster analysis, as detailed in Methods, is a procedure for finding sets of proteins that exhibit similar patterns of induction or repression. This procedure reveals the patterns of synthesis that exist in a series of gel images as well as the number of proteins that exhibit each pattern.

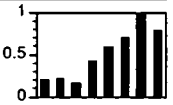
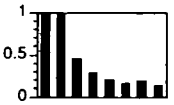
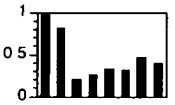
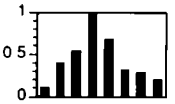
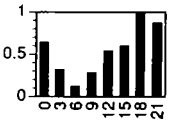
Cluster analysis of the 1-cell stage revealed 4 major patterns of synthesis (Table 2). One pattern, exhibited by cluster 1A, depicts 41 proteins that increase in synthesis over the course of the 1-cell stage, especially between 12.5 and 15.5 h post-mating. A reciprocal pattern, exhibited by cluster 1B, includes 49 proteins

Table 2. Major clusters of the 1-cell stage.

| cluster | description | no in cluster | fraction of analyzed (%) | profile[1] |
|---|---|---|---|---|
| 1A | increase | 41 | 14 |  |
| 1B | decrease | 49 | 16 |  |
| 1C | high mid 1-cell | 19 | 6 |  |
| 1D | decrease, high 18 5 h | 40 | 13 |  |
| | Total[2] | 143 | 48 | |

[1] Profiles show the average intensities at the times indicated Each bar represents the average intensity for the members of the cluster (averaged as the loganthm of ppm values) normalized to the maximum value for each profile.
[2] Because the clustering algorithm allows some spots to be members of more than one cluster, the total number of spots in all clusters combined is less than the sum of the cluster members

Table 3 Major clusters of the 2-cell stage

| cluster | description | no. in cluster | fraction of analyzed (%) | no. phos. | profile[1] |
|---|---|---|---|---|---|
| 2A | increases | 244 | 37 | 12 |  |
| 2B | decreases | 133 | 20 | 14[2] |  |
| 2C | transient decreases, high early | 105 | 16 | |  |
| 2D | transient increases | 38 | 6 | 1[3] |  |
| 2E | transient decreases, low early | 28 | 4 | |  |
| | Total[1] | 502 | 77 | | |

[1] As in Table 2.
[2] Six phosphoproteins are components of the $35\times10^3$ $M_r$ protein complex
[3] The $70\times10^3$ $M_r$ protein complex.

that decline, on average, approximately threefold between 12.5 and 15.5 h post-mating. Other proteins (cluster 1C) are synthesized at elevated rates during the mid 1-cell stage. Cluster 1D, generated by combining three similar clusters, contains 40 proteins that decline by an average of fourfold betwen 12.5 and 18.5 h and then increase again to an elevated rate of synthesis at the very end of the 1-cell stage. Included in this cluster are the acidic components of the $35\times10^3$ $M_r$ protein complex shown previously to exhibit this pattern of synthesis (Howlett, 1986). This cluster likely accounts for the observation above that the 18.5 h gel resembled gels from earlier times (i.e 3.5–9.5 h) more than the gel from 15.5 h did. Clusters 1A–1C contain approximately one third of the analyzed proteins, and share the property of being either induced or repressed between 12.5 and 18.5 h post mating. This is consistent with the timing of the first major period of change shown above (Fig. 3).

Cluster analysis of the 2-cell stage revealed 5 major patterns of synthesis (Table 3). The largest cluster (2A) contains 244 proteins that increase in synthesis by an average of fivefold over the course of the 2-cell stage. The average expression profile for this cluster exhibits a smooth rise from 9 to 18 h post-cleavage. The proteins in this cluster are distributed uniformly throughout the entire gel, ranging in pI from 4.45 to 6.63 and apparent relative molecular mass from 21.6 to $131.6\times10^3$ (Fig. 4). This cluster includes several known proteins, namely the heat-shock proteins HSP90 and HSP73, $\beta$-tubulin, Lamin B, and tropomyosin-5 (TM-5). HSP73, TM-5, and proteins LF20 and MF10 (Fig. 1) are examples of a subset of more than 100 proteins within this cluster that are synthesized for the first time during the 2-cell stage. Twelve of the proteins in this cluster, including HSP90, are phosphoproteins.

The next largest cluster identified for the 2-cell stage (2B) contains 133 proteins that decrease in synthesis by an average of sevenfold over the course of the 2-cell stage, with more than 100 falling below the level of detection by the end of the 2-cell stage (e.g. GD50, GD120, JG60, JG120, KH40 Fig. 1). Most of this decrease occurs during the first 9 h post-cleavage. Proteins in this cluster are also distributed uniformly throughout the gel, ranging in pI from 4.63 to 6.45 and in apparent relative molecular mass from 20 to $126.8\times10^3$ (Fig. 4A). The most prominent members of this cluster are the basic members of the complex of approximately $35\times10^3$ $M_r$ that first appear during the 1-cell stage. We identified 14 spots in this cluster as phosphoproteins, eight of which are members of the $35\times10^3$ $M_r$ complex.

The third largest cluster (2C) contains 105 proteins that also decrease during the early 2-cell stage (fourfold on average) but, unlike cluster 2B, the members of this cluster increase by an average of twofold during the second half of the 2-cell stage. Taking clusters 2B and 2C together (216 members), it is apparent that more than one third of the proteins analyzed decline dramatically in rate of synthesis between 6 and 9 h post-cleavage.

One group of 38 proteins (cluster 2D) is transiently induced by an average of tenfold at the mid 2-cell stage. This cluster includes HSP80, $\alpha$-tubulin, the prominent complex at $70\times10^3$ $M_r$ (Fig. 4B), and a number of other

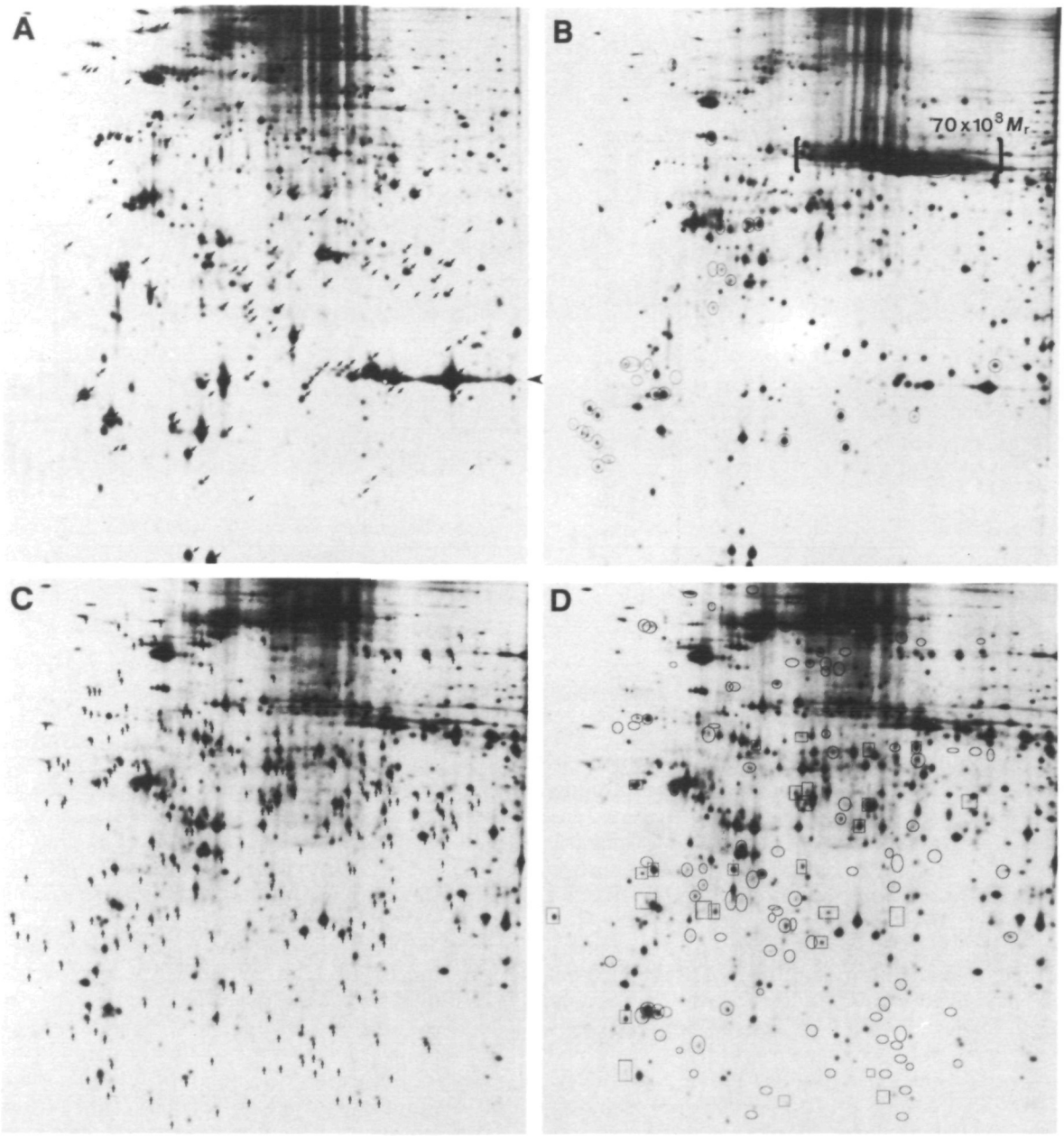
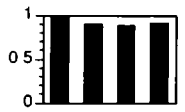
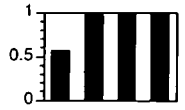
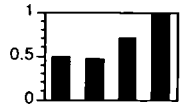
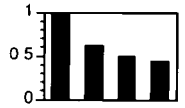
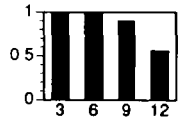



**Fig. 4.** Members of major 2-cell stage clusters. (A) Gel for the early (0 h) 2-cell stage embryo showing proteins that decrease in synthesis (cluster 2B) marked with downward pointing arrows (chevron marks the $35\times10^3$ $M_r$ complex); (B) gel for the mid (9 h) 2-cell stage embryo showing proteins that increase transiently during the 2-cell stage (cluster 2D) marked with ovals; (C–D) gel for the late (18 h) 2-cell stage showing proteins that (C) increase during the 2-cell stage (cluster 2A) marked with upward pointing arrows and (D) proteins that decrease transiently (clusters 2C and E, marked with ovals and boxes, respectively).

unknown proteins (e.g. DF190, EE60, EP60, LE10 Fig. 1). Four of the proteins in this cluster appear to be phosphorylated. Many of the proteins in this cluster are located in the acidic portion of the gel with 26 exhibiting a pI of 5.3 or less (Fig. 4B). Synthesis of this group of proteins was maximal at 9 h post-cleavage. The most prominent member of this cluster, the $70\times10^3$ $M_r$ complex, accounts for approximately 4.1 % of the total incorporation at 9 h post-cleavage. These transiently induced proteins increase by an average of fourfold between 0 and 3 h, and so are induced slightly before many of those in cluster 2A for which the average expression profile shows a sharp increase between 6 and 9 h.

Cluster 2E contains 28 proteins that are transiently repressed by an average of sixfold during the early 2-cell stage. Because of its overall higher rate of synthesis at the end than at the beginning of the 2-cell stage, many

Table 4. Major clusters of the 4-cell stage.

| cluster | description | no. in cluster | fraction of analyzed (%) | profile[1] |
|---|---|---|---|---|
| 4A | constant | 314 | 63 |  |
| 4B | increase early | 92 | 17 |  |
| 4C | increase late | 63 | 12 |  |
| 4D | decrease early | 79 | 15 |  |
| 4E | decrease late | 52 | 10 |  |
| | Total[1] | 508 | 94 | |

[1] As in Table 2

(20) of the members of this cluster are also members of cluster 2A. The members of this cluster are also distributed uniformly throughout the gel (Fig. 4D).

These five clusters contain a total of 502 proteins, or 77 % of those analyzed. Thus, the majority of detectable proteins are included within these clusters, indicating that, although the total amount of change is great, much of it can be accounted for by five patterns of coordinate regulation. More than a third of the analyzed proteins are induced, another third are repressed, and many of the remaining proteins increase or decrease transiently. Significantly, all five of these clusters exhibit patterns of synthesis that change most during the first half of the 2-cell stage, consistent with the completion of the reprogramming in synthesis by 12–15 h post-cleavage (Figs 2 and 3). Although a few proteins including β-F1-ATPase and actin (data not shown) exhibit only slight changes in their rates of synthesis during the 2-cell stage, none of the major 2-cell stage clusters represents a sizeable set of proteins that are synthesized at a constant rate. Cluster anlaysis of a second 2-cell stage series produced a similar set of clusters (data not shown), with no cluster of proteins synthesized at a constant rate being found, even accounting for clusters containing as few as five members.

Cluster analysis of the 4-cell stage produced results very different from those obtained with the 2-cell stage. A total of 542 spots were analyzed and 94 % of these were grouped into five clusters (Table 4). The single largest cluster (4A) contains 314 (63 % of total) proteins synthesized at essentially constant rates, consistent with the rather small differences revealed by the statistical comparisons of the gel patterns described above. Two clusters (4B and 4C) contain a total of 149 proteins that increased during the 4-cell stage whereas two other clusters (4D and 4E) contain a total of 124 proteins that decreased. The proteins in clusters 4B and 4D, for which synthesis at 3 h post-cleavage differed from later time points, are likely responsible for the slightly greater amount of difference in S.D. and percent twofold changes observed between the 3 and 6 h gel images (Fig. 3). None of the clusters 4B-E exhibit the dramatic increases or decreases displayed by clusters 2A-E and the proteins in these clusters tend to be relatively minor spots or exhibit very modest changes.

*Overall patterns of synthesis from fertilization to third cleavage*

The clusters identified for the 2-cell stage should encompass most of the proteins analyzed from all three stages. Consequently, the patterns of synthesis revealed by these clusters should reflect the major developmental modulations in protein synthesis that occur in the early cleavage stage embryo, particularly those related to the switch from maternal to zygotic gene expression. To characterize these patterns of synthesis more fully, we examined the behavior of the 2-cell stage protein clusters, as well as individual members within each cluster, over the entire period from fertilization to the end of the 4-cell stage (Fig. 5). The proteins of cluster 2A displayed, on average, a low rate of synthesis during the 1-cell stage followed by a steady increase during the 2-cell stage to a high rate that persisted through the end of the 4-cell stage. Of the 244 proteins in this cluster, 145 (77 %) either persisted at a constant rate of synthesis or actually increased further during the 4-cell stage and only 23 % declined. The protein synthesis profile for cluster 2B shows a high rate of synthesis during the 1- and early 2-cell stages that falls to a basal rate by the mid 2-cell stage and remains repressed, as expected for the products of maternally derived mRNAs that become degraded or inactivated. Proteins in cluster 2C were synthesized predominantly during the 1-cell stage, declined during the 2-cell stage, and then increased in synthesis during the late 2-cell and 4-cell stages to a rate that was less than that observed during the 1-cell stage. Among those proteins that were synthesized at elevated rates at the mid 2-cell stage (cluster 2D), some, such as HSP80 and α-tubulin, were also synthesized during the 1- and 4-cell stages, while the synthesis of others, such as the $70\times10^3$ $M_r$ complex, was essentiallty stage specific. The remaining cluster (2E) contains proteins that were synthesized at a lower rate during the 1-cell stage than during the 2- and 4-cell stages.

## Discussion

The most striking result of the studies shown here is the enormous amount of synchronous change that occurs during the 1- and 2-cell stages. Although previous studies demonstrated many qualitative changes during

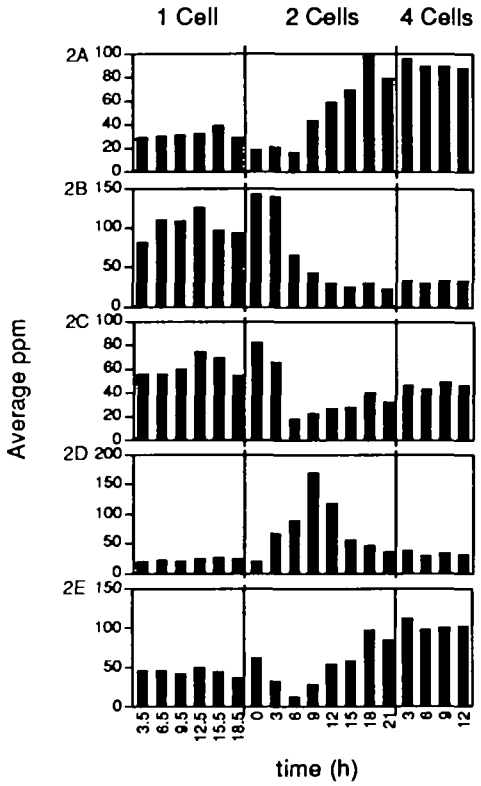



**Fig. 5.** Average protein synthesis profiles for the major 2-cell stage clusters. Each graph depicts the logarithmic average of the rates of synthesis (in ppm) of the proteins in clusters 2A-E (Table 3) for consecutive (left to right) 3 h time intervals beginning at the times indicated for the 1-, 2-, and 4-cell stages. Rates of synthesis of individual proteins as low as 20 ppm can be reliably measured.

this period (Van Blerkom and Brockway, 1975; Levinson *et al.* 1978; Braude *et al.* 1979; Howe and Solter, 1979; Cullen *et al.* 1980; Van Blerkom, 1981; Howlett and Bolton, 1985; Howlett, 1986), the overall amount of quantitative change has never been measured. Our data demonstrate that the qualitative changes documented previously occur within the context of a generalized and extensive reprogramming of embryonic protein synthesis. The rates of synthesis of approximately 60% and 85% of the proteins synthesized during the 1- and 2-cell stages, respectively, change by at least twofold, and most change by considerably more. During the 2-cell stage, more than half of the proteins analyzed undergo fourfold or greater changes in their rates of synthesis, and approximately one fourth of the detected proteins either cease to be synthesized or are synthesized *de novo*. Using the REF52 database as a comparison, we find that the amount of change occurring within as little as 3 h during the 1- and 2-cell stages exceeds even the differences between proliferating and quiescent REF52 cells or between normal and transformed REF52 cells (Garrels and Franza, 1989*b*). Furthermore, our cluster analysis fails to detect a group of proteins that are synthesized at a constant rate during the 2-cell stage, indicating that the rate of synthesis of almost every detectable protein is modulated quantitatively during this stage of development. These data indicate that the transition from reliance upon maternally derived mRNAs to the utilization of newly synthesized mRNAs leads to an extensive reprogramming of embryonic protein synthesis that includes both widespread changes in the array of proteins synthesized combined with additional quantitative changes in rates of synthesis of other proteins. Thus, the switch from maternal to embryonic control of development exerts a much greater effect on protein synthesis than previously realized.

This reprogramming of the embryonic protein synthesis pattern is essentially complete by 15 h after the first cleavage division. Very few proteins change during the late 2-cell and 4-cell stages. Overall, the amount of change that occurs during each 3 h interval at the end of the 2-cell stage and during the 4-cell stage approaches the difference seen either between duplicate samples or between two subclones of REF52 cells (Garrels and Franza, 1989*b*). This is consistent with previous observations that relatively few qualitative changes occur during later stages of preimplantation development (Van Blerkom and Brockway, 1975; Levinson *et al.* 1978; Howe and Solter, 1979; Flach *et al.* 1982).

Most of the change that is observed during the 1- and 2-cell stages can be attributed to four developmentally regulated patterns of synthesis: (1) synthesis during the 1-cell stage followed by repression during the early 2-cell stage, (2) induction at the 2-cell stage with continued synthesis during the 4-cell stage, (3) transient induction during the 2-cell stage, or (4) transient repression during the 2-cell stage. These patterns reflect the effects of translational activation followed by deadenylation or degradation of maternal mRNA combined with the appearance of new zygotic mRNAs following onset of transcription. More than a third (37%) of the proteins analyzed increase in synthesis throughout the 2-cell stage while another 6% undergo transient increases at the mid 2-cell stage. These proteins most likely reflect the products of newly transcribed genes. The transiently induced set includes the $70\times10^3$ $M_r$ protein complex that is known to be transcription-dependent (Flach *et al.* 1982; Poueymirou and Schultz, 1987), and the appearance of so many proteins *de novo* is consistent with the active utilization of the large set of new mRNA species that appears at the 2-cell stage (Taylor and Piko, 1987). Another 40% exhibit a sharp decline at the start of the 2-cell stage. These proteins are most likely encoded by maternally derived mRNAs that become degraded or inactivated during the course of the 1- and early 2-cell stages (Bachvarova and De Leon, 1980; Piko and Clegg, 1982; Clegg and Piko, 1983*a*; Giebelhaus *et al.* 1983; Giebelhaus *et al.* 1985; Graves *et al.* 1985; Paynton *et al.* 1988). The proteins that decrease in synthesis transiently may represent proteins for which maternal mRNAs become degraded and then replaced with zygotic transcripts.

Experiments employing $\alpha$-amanitin demonstrated the occurrence of two bursts of transcription during the

2-cell stage (Flach *et al.* 1982; Bolton *et al.* 1984). Although we have not yet tested the sensitivity of the changes that we observe to $\alpha$-amanitin treatment at these different times, our data are at least consistent with the existence of two bursts of transcription. The class of proteins that are transiently synthesized during the mid 2-cell stage increase initially between 0 and 3 h post-cleavage, including the $70\times10^3$ $M_r$ complex shown previously to be encoded by mRNA transcribed during the first period of transcription (Flach *et al.* 1982). By contrast, most the proteins that are induced and continue to be synthesized become induced slightly later between 6 and 9 h post-cleavage, most likely as a consequence of the second period of transcription.

That induction of the transiently synthesized proteins precedes the induction in synthesis of many other proteins is consistent with the possible fulfillment of some regulatory function by some of these proteins. It is possible that one function of the first period of transcription is to direct the synthesis of these mid 2-cell stage proteins, which could then either promote the transcription of other genes, leading to the second burst of transcription, or possibly direct the elimination of many maternally derived mRNAs. Consistent with this notion, the $70\times10^3$ $M_r$ protein exhibits a nuclear localization (Conover *et al.* 1991).

Some of the changes that we observe are known to be due to alterations in post-translational modifications. Post-translational modifications have been shown to contribute to many previously observed changes (Van Blerkom, 1981; Howlett and Bolton, 1985; Howlett, 1986), including the prominent $35\times10^3$ $M_r$ complex that contains structurally related proteins that are phosphorylated and/or glycosylated (Van Blerkom, 1981; Howlett and Bolton, 1985; Howlett, 1986). Indeed, we find that the distribution of newly synthesized material among the 11 component spots of this complex can change dramatically while the rate of synthesis of the complex as a whole changes very little (data not shown). The extent to which such modifications contribute to the reprogramming that we observe has yet to be determined. Based on our own labelings to detect phosphoproteins, however, it appears unlikely that phosphorylation alone can account for more than a small fraction of the changes that we observe.

The embryonic mouse protein database described here will facilitate the analysis of mammalian development in two important ways. First, we have detected and monitored the rates of synthesis of many more individual spots than in any previous study. The precise quantitation of the relative rates of synthesis of developmentally regulated proteins at 3 h intervals using synchronous cohorts of embryos permits a much more definitive appraisal of similarities and differences in patterns of synthesis of 'stage-specific' proteins than has been possible previously. This allows the identification of groups of proteins for which synthesis is truly coordinately regulated as well as periods of development that are especially significant. Data such as those described here could only be obtained using a quantitative database such as this one. Second, the database is cumulative and employs an integrated system of electrophoresis and gel image analysis that allows reproducible spot detection and matching between samples obtained in multiple experiments. These features will allow it to be applied to the study of a variety of developmental problems and to serve as a cumulative resource for accumulating a variety of data pertinent to early mouse development. The extensive reprogramming and dynamic nature of embryonic protein synthetic patterns revealed by the present analysis demonstrate the potential difficulty in distinguishing between temporal variations in protein synthesis, possibly related to some developmental delay or acceleration, and more specific qualitative or quantitative differences in the expression of one or more particular genes. Such distinctions are essential in studies seeking to exploit two-dimensional gel electrophoresis to evaluate the impact of genetic alteration or experimental manipulation on gene expression. Where such a goal exists, it may be necessary to examine comparably staged embryos sampled at multiple time intervals during the critical developmental stage. Such analyses will be facilitated by the availability of a cumulative and quantitative protein database such as this one.

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