The patterns of protein synthesis during foetal and neonatal organ development in the mouse are remarkably similar

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SUMMARY

The extent to which differential gene expressions can be correlated with organ development was examined at the level of protein synthesis during pre- and postnatal development in the mouse. High resolution, equilibrium, two-dimensional polyacrylamide gel electrophoresis detected, for each of five to ten successive stages for each of seven organ systems, between 850 and 1000 separate newly synthesized proteins. The possibility that the 1000 detectable proteins synthesized at any one time during organ development represent a sampling bias was contra-indicated (a) because a different and larger population of $[^{14}\text{C}]-$amino acid-incorporating protein syntheses gave similar results and (b) because nonequilibrium isoelectric focusing, electrophoresis, isoelectric points between pH 5.5 and 8.7, confirmed the results from yet a different population of protein syntheses. Within limits of the sampling of protein syntheses, the entire period of organ development examined proceeds with altered expression of a small proportion of the total proteins being synthesized. While all protein changes were stage specific, approximately three organ-specific protein syntheses were detected per organ system.

One family of five protein syntheses seen in 16-day foetuses had homologous primary structures and presumably are keratins derived from a single genomic expression. These selected stage-specific protein syntheses examined by electrophoresis of partial proteolytic digests disclosed a programme for post-translational changes in protein syntheses. The current observations indicate that the examined pre- and postnatal organ development of the seven organs occurs in the presence of greater than 99% similarity among proteins synthesized in the same and different organ systems. Functional differentiation during organogenesis, therefore, occurs in the presence of less than 1% change in qualitative or quantitative switch in protein syntheses. Evidence is presented to indicate that even this remarkably small number of changes in protein syntheses during functional organ differentiation may be derived from an even smaller subset of gene expressions. Collectively, the data suggest that explanatory mechanisms for molecular organogenesis must encompass both selective gene expressions along with post-translational programmed events.

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INTRODUCTION

Ontogeny is characterized by an ordered progression of molecular, cellular and extracellular changes (Runner, 1970). Structural complexity during organogenesis increases both by cell differentiation and by acquisition of ability to communicate and to function coordinately. Explanatory mechanisms for morphogenesis and for ontogeny of the phenotype encompass two opposing views. One view argues that programmed expression of genes can account for and predict morphogenetic processes and phenotypic characteristics. The contrasting view is that, within the constraints of the genome, sequentially programmed forces at the level of interactions between molecules, cells and tissues ultimately specify the characteristics of morphogenesis and the ultimate phenotype (Bennett, Boyse & Old, 1971; Caplan, 1981, Lewin, 1981). Although genomic and extragenomic potentials are inextricably coupled, the relative importance of each for organogenesis and for the phenotype remains debatable. The relative role of the genome, however, seems ascertainable because developmentally specific genomic information, manifest by expressions of protein syntheses, should be qualitatively and quantitatively detectable.

Stage-specific protein synthesis and presumed differential gene expression have been reported for mammalian oogenesis (McGaughey & Van Blerkom, 1977; Schultz & Wassarman, 1977; Van Blerkom & McGaughey, 1978a; Richter & McGaughey, 1981; Van Blerkom, 1981a, b), for pre-implantation development (Levinson et al. 1978; Van Blerkom & McGaughey, 1978b; Howe & Solter, 1979; Chen et al. 1980; Cullen, Emigholz & Monahan 1980a; Van Blerkom, 1981a, b) and for postimplantation development (Cullen, et al. 1980b; Johnson & Rossant, 1981). The present study attempted to determine the extent to which changes in protein synthesis can be detected within organ systems during specific embryonic, foetal and neonatal stages. The findings demonstrate that less than 1% of the total population of resolvable proteins can be considered to be stage specific or organ specific, or both. Our findings indicate that throughout foetal and neonatal organ development, progressive development and functional differentiation occurs in the presence of remarkable similarity of protein syntheses among the same and different organ systems and in the absence of a large number of switches in protein expression.

MATERIALS AND METHODS

In vitro radiolabelling of tissues

Organs were dissected from random-bred, HS mice. The day of detection of a vaginal plug was designated as day 0 of pregnancy. Brain, liver, kidney, forelimb, hindlimb, lung and yolk sac were obtained from foetal mice at days 10 through 18 of gestation. Foetal tail (somites) samples were obtained at days 10 through 13 of gestation. Brain, liver, kidney and lung samples were dissected
Protein synthesis during organ development from newborn mice up to day-30 postnatal. Organs and tissues were cut into small fragments and washed several times in serum-free, HEPES-buffered Waymouth's 751/1 medium (Waymouth, 1972). Fragments of washed tissues and organs were gently teased apart with forceps, placed into 500 μl of medium previously gassed with a mixture of 95% O₂, 5% CO₂ and pre-incubated at 37 °C for at least 30 min to remove cellular debris and blood cells. Incubation occurred in stoppered, round-bottomed glass culture tubes placed on a tube rotator which rotated at 10 r.p.m. The incubation medium was replaced with 300 μl serum-free medium containing 200 μCi/ml of [³⁵S]L-methionine (1000 Ci/m mole, Amersham). Radiolabelling lasted for 2 h. The following procedures were evaluated to determine the most appropriate method of sample preparation: (1) radiolabelled tissue fragments were treated with lysis buffer (O'Farrell, 1975) and solubilized proteins applied immediately to the first dimension gel; (2) tissues were sonicated, lyophilized, reconstituted with lysis buffer and subjected to electrophoresis; and (3) sonicated, lyophilized samples were stored at −80 °C and reconstituted with lysis buffer immediately prior to electrophoresis. The three methods provided identical protein synthetic patterns for representative samples of each tissue studied. Therefore, changes in protein synthesis detected during organ development could not be attributed to artificial alterations induced during sample preparation. For routine analysis, radiolabelled tissue fragments were washed several times in unlabelled medium and placed into 100 μl of sonication buffer containing 10 mM-Tris (pH 7.2), 5 mM-MgCl₂ and 60 μg/ml RNase, and sonicated briefly at 4 °C. Samples were lyophilized and stored at −80 °C for no more than 4 days.

Two-dimensional polyacrylamide gel electrophoresis (PAGE)

Each of the lyophilized samples was reconstituted with 40 μl of lysis buffer, from which 2 μl were withdrawn to determine the amount of radioactivity incorporated into TCA-precipitable material (Van Blerkom, 1978). The remainder was layered onto equilibrium isoelectric focusing gels prepared and prerun as described by O'Farrell (1975). Gels were electrophoresed for 14 h at 340 V with a final hour at 800 V. Nonequilibrium isoelectric focusing in acidic and basic pH ranges was accomplished according to the procedure of O'Farrell, Goodman & O'Farrell (1977) for a total of 1500 V h. Electrophoresis in the second dimension involved 8–15% linear gradient SDS slab gels. Slab gels were fixed and stained in a solution of 12% acetic acid, 25% methanol and 0.1% Coomassie brilliant blue. Gels were dried under heat and vacuum and subsequently exposed to Kodak NS 5T X-ray film. In order to normalize spot intensities among samples containing differing amounts of incorporated radioactivity, the following equation was used to determine duration of exposure: acid-precipitable c.p.m. × days of exposure = 16 × 10⁶ c.p.m.-days. The average duration of exposure was 15 days. A total of 386 autoradiographic patterns were compared in this study. Patterns of protein synthesis were evaluated by
superimposing the X-ray films on a fluorescent lightbox. Approximately 85% of the autoradiographic patterns were entirely superimposable making comparisons relatively straightforward. For approximately 15% of the patterns, variation inherent in the electrophoretic process resulted in slight positional shifts for groups of proteins, especially at the acidic end of the first-dimensional separation. Thirty-five quantitatively major proteins common to all tissues examined and representing the entire pH and molecular weight range were used as reference markers to align the patterns for comparison. Completely reproducible patterns were obtained either from multiple two-dimensional separations of the same tissue preparation or from different radiolabellings of the same tissue. At no time were randomly variable proteins detected.

**Polypeptide mapping by partial proteolysis**

Individual spots were obtained from dried slab gels and prepared for polypeptide mapping as described previously (Van Blerkom, 1981b). The procedure of Cleveland, Fischer, Kirschner & Laemmli (1977) was used to obtain polypeptide profiles of individual proteins. Each well of a 15% polyacrylamide SDS slab gel contained $1 \times 10^{-3}$ g of *Staphylococcus aureus* V-8 protease (Miles). Electrophoresis through the stacking gel occurred at a constant current of 20 mA. When the bromphenol tracking dye was 4 mm from the surface of the running gel, the current was turned off for 30 min. Electrophoresis was resumed at a constant current of 30 mA in a chamber precooled and maintained at 15 °C. Slab gels were infiltrated with Enhance (New England Nuclear) and exposed to Kodak X-AR5 X-ray film.

**Immunoprecipitation of alphafetoprotein**

Samples of liver, yolk-sac and brain sonicates (foetal) were diluted to 100 μl in phosphate-buffered saline (pH 7.2) containing 1 mM methionine and 1% Triton X-100. Immunoprecipitation was accomplished by the addition of antibodies specific for alphafoetoprotein (AFP) (Muir, Law, Nishi & Tamaoki, 1979). Immunoprecipitates were collected by centrifugation, dissolved in SDS sample buffer (Laemmli, 1970) and analyzed on 10% polyacrylamide SDS slab gels. Gels were infiltrated with Enhance and prepared for fluorography with Kodak X-AR5 X-ray film.

**RESULTS**

High resolution, two-dimensional PAGE was used to compare proteins synthesized by forelimb, hindlimb, tail, liver, lung, kidney, brain and yolk sac at various days (stages) of gestation and, where appropriate, during the neonatal period. Electrophoresis in the first dimension was done both by equilibrium and nonequilibrium isoelectric focusing. A total of 850–1000 proteins were compared for each of the tissues examined. Radiolabelling of each developmental stage labelled was repeated a minimum of three times. Proteins indicated below underwent consistent and reproducible stage-specific
Fig. 1. Portion of the autoradiographic patterns of limbs at prenatal days 12 (A), 14 (B) and 16 (C) obtained by routine, high-resolution 2D-PAGE. Proteins indicated by arrows with asterisk are reference markers for comparison of patterns. Numbered proteins undergo reproducible qualitative or quantitative change during limb development. (D) Polypeptide pattern after proteolytic digestion of proteins 8–10 (C). First dimension separation involved equilibrium isoelectric focusing (IEF). The second dimension was accomplished on polyacrylamide slab gels containing sodium dodecyl sulphate (SDS). Approximate molecular weights are given on right ($\times 10^3$). Arrow A = actin.
Table 1. Changes in protein synthesis observed during prenatal and postnatal development in the mouse

<table>
<thead>
<tr>
<th>Tissue and day studied</th>
<th>Observed change in synthesis</th>
<th>Type of change*</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Limb</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–14, 16, 18</td>
<td>14 13 (1B)</td>
<td>S</td>
<td>Synthesized concurrently in kidney and lung</td>
</tr>
<tr>
<td></td>
<td>14 1 (1B)</td>
<td>O</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 2 (1B)</td>
<td>S</td>
<td>Probably protein 8 (1C)</td>
</tr>
<tr>
<td></td>
<td>16 3a–c (1B)</td>
<td>S</td>
<td>Synthesized concurrently in kidney and lung</td>
</tr>
<tr>
<td></td>
<td>4a–c (1B)</td>
<td>S</td>
<td>Synthesized concurrently in kidney and lung</td>
</tr>
<tr>
<td></td>
<td>5a–c (1B)</td>
<td>S</td>
<td>Synthesized concurrently in kidney and lung</td>
</tr>
<tr>
<td></td>
<td>16 6, 7 (1C)</td>
<td>O</td>
<td>8–10 have homologous primary sequences (Fig. 1D) – probably skin keratins</td>
</tr>
<tr>
<td></td>
<td>16 8–12 (1C)</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td><strong>Tail</strong></td>
<td>12 1–3, (2B)</td>
<td>O</td>
<td>Resolved by non-equilibrium isoelectric focusing in first dimension</td>
</tr>
<tr>
<td></td>
<td>4 (2B)</td>
<td>?</td>
<td>May be present at low intensity in limb (2A)</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>12-(1) AFP (3A, B)</td>
<td>S</td>
<td>Progressive increase in intensity (see yolk sac)</td>
</tr>
<tr>
<td>12-(1)</td>
<td>9 (3A, B)</td>
<td>S</td>
<td>Progressive increase in intensity</td>
</tr>
<tr>
<td>12-(10)</td>
<td>10-13 (3B)</td>
<td>S</td>
<td>Progressive decrease in intensity</td>
</tr>
<tr>
<td>12-(10)</td>
<td>14</td>
<td>S</td>
<td>Present from day 12 on, also in yolk sac (Fig. 7)</td>
</tr>
<tr>
<td>12</td>
<td>1, 1a, 2, 12 (3A, B)</td>
<td>S</td>
<td>Not seen after day 12</td>
</tr>
<tr>
<td>13</td>
<td>4, 5, 8 (3B)</td>
<td>S</td>
<td>Protein 8 increased in intensity after day 12</td>
</tr>
<tr>
<td>16</td>
<td>3 (3B)</td>
<td>O</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>6, 7 (3B)</td>
<td>O</td>
<td>Proteins 3–8 seen at day (1)</td>
</tr>
</tbody>
</table>

change during the foetal and neonatal periods examined; some of the stage-specific proteins are also organ specific. The results are summarized in Table 1.

Protein synthesis by limb and tail

Patterns of protein synthesis from fore and hindlimbs were obtained on gestation days 10–14, 16 and 18. On day 14, at least ten proteins indicated by arrows 3, 4, 5 and 13 (Fig. 1B) are examples of qualitative changes in syntheses that are stage specific. They cannot be classified as organ specific because they
<table>
<thead>
<tr>
<th>Tissue and day studied</th>
<th>Observed changes in synthesis</th>
<th>Type of change*</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prenatal (postnatal) Day Protein (figure no.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>12–16, 18 (1), (5), (10), (30)</td>
<td>16</td>
<td>5–9 (4B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1–4 (4B)</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10–13 (4B)</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Doublet (4A, B)</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L and 4 (4A, B)</td>
<td>S</td>
</tr>
<tr>
<td>Kidney</td>
<td>6, 18 (1), (5), (10), (30) (1)–(10)</td>
<td>18–(10)</td>
<td>1, 3 (5A, B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1)–(10)</td>
<td>2, 4 (5B, C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1)–(10)</td>
<td>5, 6, 7 (5B, C)</td>
</tr>
<tr>
<td>Brain</td>
<td>10–14, 16, 18 (1), (5), (10)</td>
<td>10</td>
<td>1 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (6)</td>
<td>S</td>
</tr>
<tr>
<td>Yolk sac</td>
<td>10, 12, 14, 16, 18</td>
<td>10</td>
<td>AFP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>1–5 (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6, 7 (7)</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 (7)</td>
<td>S</td>
</tr>
</tbody>
</table>

* S: Stage not organ-specific; O: stage and organ-specific.

were seen as concomitant changes in protein synthesis in both lung and kidney (Figs. 4B and 5A). Proteins 1, 6 and 7, (Fig. 1C), are qualitative changes that were seen to be limb-bud specific.

Proteins 8–12 (Fig. 1C) represent appearance of new proteins that are stage specific but not limb specific. The radioautographs for proteins 8–12 showed that they were among the most abundant proteins detectably synthesized by the limb on day 16. The appearance of protein 2 on day 14 (Fig. 1B), may represent the earliest detected synthesis of protein 8 seen on day 16 (Fig. 1C). Electrophoresis of partial proteolytic digests for detection of primary sequence for proteins 8, 9 and 10 (Fig. 1D), showed that they have nearly identical primary structures; two variations in polypeptide band positions were seen for protein 10 (Fig. 1D, arrows). Electrophoretic positions and similarities of primary structure suggest that proteins 8–12 are keratins (Laskin et al. 1981). Their positions and electrophoretic mobilities suggest that they represent a family of electrophoretic variants derived from the post-translational modification of a common protein.
Fig. 2. Autoradiographic patterns of 2D-PAGE separations of the more basic proteins from a day-12 forelimb bud (A) and tailbud (B). Electrophoresis in the first dimension involved nonequilibrium isoelectric focusing between approximately pH 5 and pH 9. Proteins 1–4 may be tailbud specific. (B).
Protein synthesis during organ development

The possibility that protein syntheses, not resolved by equilibrium isoelectric focusing in the first dimension, might change during limb and tail development was tested by nonequilibrium isoelectric focusing electrophoresis. Resolutions of nonequilibrated basic proteins, pH 5.5 to 8.7, are shown for 12-day limb (Fig. 2A) and 12-day tail (Fig. 2B). Although equilibrium isoelectric focusing electrophoresis showed that limb and tail buds from day 10, 25-somite stage, through day 13, 58-somite stage, were indistinguishable (data not shown), nonequilibrium isoelectric focusing showed that proteins 1–3, and possibly 4, were synthesized by day-12 tailbud (Fig. 2B) and could be classified as tail-specific protein syntheses.

Equilibrated and nonequilibrated electrophoresis disclosed, during limb and tail development, 23 proteins from seven different days of development (Fig. 1A, B and C) that consistently showed qualitative or quantitative stage-specific change. Three changes in protein synthesis (1, 6 and 7) were considered to be specific to limb and three or four were specific to tail bud. Ten shifts in protein synthesis (indicated by arrows 3–5 and 13) were classified as stage-specific changes not restricted to the limb and tail bud. Five spots (8–12) as judged by position in the two-dimensional array appear to be stage specific but not limb specific post-translational modifications derived from a single protein species.

Protein synthesis by developing liver

Liver tissue was studied for patterns of protein synthesis on prenatal days 12–14, 16 and 18; and postnatal days 1, 5 and 10. Liver stage-specific protein 3 (Fig. 3) was first detected on prenatal day 16 (Fig. 3B), while proteins 6 and 7 were first observed on day 18 (Fig. 3B). Liver proteins, 4, 5 and 8 appeared after prenatal day 12, and were stage specific but not liver specific. Proteins 3 through 8 were being synthesized at postnatal day 1 (Fig. 3B). Proteins 1, 1a and 2, by contrast, did not appear in the autoradiographic patterns after day 12. Proteins 10–13, although continually detected after prenatal day 12, progressively decreased in intensity. Postnatally minor quantitative changes in protein synthesis were seen. Protein 9, barely detectable in the other tissues examined, significantly increased in intensity after day 12 (Fig. 3A) up to and including postnatal day 1 (Fig. 3B).

A major protein product of the liver during the last third of the prenatal period is alphafoetoprotein (AFP). Immunoprecipitation showed that low levels of AFP synthesis were detectable in the liver at 10 days of gestation (data not shown). Liver AFP on day 12 (Fig. 3A and 3C, lane 1) was calculated to constitute approximately 3% of the total methionine incorporated (data not shown, see results for yolk sac) and increased in intensity at subsequent stages studied (see neonate day 1, Fig. 3B).

Fourteen liver protein syntheses studied at nine different stages over a period
Fig. 3. Autoradiographic patterns of [35S]L-methionine labelled proteins synthesized by day-12 prenatal liver (A) and by day 1 neonatal liver (B) obtained by routine, high resolution 2D-PAGE. Arrows with asterisks are positional markers for comparison. Numbered arrows indicate stage- and tissue-specific proteins and are discussed in text. AFP denotes alphafetoprotein. (C) Pattern of immunoprecipitation of AFP from (1) 12-day prenatal liver; (2) 10-day yolk sac; and (3) 12-day yolk sac. Equilibrium isoelectric focusing in the first dimension (IEF) occurred between approximately pH 4-5 and pH 7.5. Approximate molecular weights for the second dimension (SDS) are given on far right.
of 18 days of development showed reproducible quantitative or qualitative stage-specific changes. Three of the 14 proteins were observed to be liver specific.

Protein synthesis by developing lung

Patterns of protein synthesis by developing lung were examined on days 12–16 and 18 of gestation and on days 1, 5, 10 and 30 neonatal. Thirteen changes in protein synthesis were readily observed during lung development, between day 12 of gestation (Fig. 4A) and day 1 postnatal (Fig. 4B). Lung-specific proteins 5–9 were first detected on day 16 of gestation and increased markedly in relative intensity at day 1 postnatal. Proteins 1–4 and 10–13 were stage specific but not lung specific. These particular proteins also showed a progressive increase in relative intensity from day 12 of gestation through the early neonatal period. The encircled doublet in Fig. 4A and B, like proteins 1–4 and 10–13, is noted as a possible example of a change in relative rate of syntheses. On day 12 of gestation the two proteins were of equal intensity (Fig. 4A). However, by day 1 postnatal (Fig. 4B), the lower protein became quite intense while the upper protein had diminished significantly in intensity. Proteins indicated by arrows L and number 4 are the same proteins observed in the limb (proteins 3–5 and 13, Figs. 1B, C) and kidney (labelled L and unmarked arrow in Fig. 5A). These proteins appear to be synthesized concomitantly although in the lung protein 4 was not detected prior to day 16 of gestation. A few minor quantitative changes were visible when day 1 through day 30 postnatal lung patterns were compared. In summary, at ten prenatal and early neonatal periods, five proteins (5–9), are lung-specific and fifteen proteins undergo quantitatively detectable shifts.

Protein synthetic patterns by kidney

Patterns of protein synthesis by kidney were examined on prenatal days 16 and 18 and on neonatal days 1, 5, 10 and 30. At the level of resolution used in this study no particular set of proteins was found to be kidney specific for the six stages observed. However, significant quantitative changes in protein synthesis did occur between days 18 of gestation (Fig. 5B) and day 10 postnatal (Fig. 5C). For example, protein 3 was barely detectable by day 1 postnatal (Fig. 5A), while protein 1 (Figs. 5A, B) was no longer visible at postnatal day 10 (Fig. 5C). Proteins 2 and 4 also decreased markedly in intensity between days 1 and 10 postnatal. Three obvious examples of proteins that increased in relative intensity from day 1 to day 10 postnatal were proteins 5, 6 and 7.

Protein synthesis by developing brain

Patterns of protein synthesis of brain tissues were obtained on prenatal days 10–14, 16 and 18 and on postnatal days 1, 5 and 10. The characteristic protein synthetic pattern of a day-10 embryonic brain is shown in Fig. 6. This pattern was representative of ten different days of brain tissue studied. Only one
Fig. 4. Autoradiographic patterns of newly synthesized proteins at two stages of lung development obtained by routine high-resolution 2D-PAGE: (A) 12-day prenatal lung; (B) day-1 neonatal lung. Numbered and lettered arrows indicate quantitative and/or qualitative changes and are discussed in the text. Proteins indicated by an asterisk are position markers for comparison of the patterns. Equilibrium isoelectric focusing occurred in the first dimension (IEF) and electrophoresis in the presence of sodium dodecyl sulphate (SDS) occurred in the second. Approximate molecular weights (×10³) on far right.
Fig. 5. Routine, high-resolution 2D-PAGE separation of newly synthesized, \([^{35}\text{S}]\)-methionine labelled proteins from day-1 neonatal kidney (A), Day-18 prenatal kidney (B) and day-10 neonatal kidney (C). Proteins which change quantitatively and/or qualitatively are indicated by numbered and lettered arrows. Proteins indicated by an arrow with an asterisk are positional markers. Equilibrium isoelectric focusing (IEF) in the first dimension occurred between approximately pH 4.5 and 7.5. Electrophoresis in the second dimension occurred in the presence of sodium dodecyl sulphate (SDS). Approximate molecular weights are given on the far right for (A).
Fig. 6. Routine, high-resolution, 2D-PAGE autoradiographic patterns of [35S]methionine labelled proteins of a day-10 embryonic brain. Isoelectric focusing (equilibrium) in the first dimension (IEF) occurred between approximately pH 4.5 and 7.5. Proteins were separated in the second dimension on slab gels containing sodium dodecyl sulphate (SDS). Approximate molecular weights are given on the far right. Numbered arrows indicate proteins that underwent quantitative or qualitative change during brain development.

relatively major protein could be considered brain-specific (protein 1). Less than 20 minor quantitative changes were apparent during brain development. For example, protein 2 in Fig. 6 was present in all other tissues examined but at very low intensity. In contrast to other tissues examined, protein synthetic patterns in embryonic, foetal and neonatal brain remained relatively unchanged.

Protein synthesis by yolk sac

The visceral yolk sac, vascularized by the vitelline circulation, encloses the entire foetus and has as a major protein synthetic product alphafetoprotein (Wilson & Zimmermann, 1976). Patterns of protein synthesis were examined on days 10, 12, 14, 16 and 18 of gestation. Immunoprecipitation of yolk sac sonicates (Fig. 3C, lanes 2 and 3) enabled calculation that AFP constituted 7% and 20% of the total methionine incorporation on days 10 and 12 of gestation, respectively. As was observed for liver (Figs. 3A, B), microheterogeneity of yolk sac AFP increased progressively during gestation. Proteins 1-5 (Fig. 7) were yolk sac specific and appeared in the electrophoretic patterns by day 12. Proteins 6 and 7 may also be synthesized by the liver (Fig. 3B,
Protein synthesis during organ development

**DISCUSSION**

The extent to which detectable stage- and tissue-specific protein synthesis occurs in foetal and neonatal mice was examined by high resolution, equilibrium 2D-PAGE. Between 850 and 1000 separate, newly synthesized proteins have been compared for each of 5–10 successive days for each of seven organ systems (Table 1). Approximately three of these protein syntheses per organ system have been attributable to organ-specific shift or switch in protein expression. This small proportion of detectable tissue-specific protein synthesis (less than 1%) demonstrated a remarkable similarity among the same and different organ systems throughout pre- and postnatal development.

The protein syntheses that have been studied by routine high-resolution 2D-PAGE methods are obviously among the 1000 methionine-containing proteins synthesized in the greatest quantities. This sample of protein syntheses, although an impressively large number, possibly represents those constitutive proteins 5 and 14). Protein 8 is also present in the kidney at postnatal day 10 (Fig. 5C, protein 7). Proteins 5–7, as well as AFP, are secreted into the surrounding medium by mouse yolk sac (Janzen, Andrews & Tamaoki, 1982).
proteins ubiquitously required during organogenesis for cell maintenance, growth and replication. The possibility that the 1000 protein syntheses observed represent a potential sampling bias, invoked by the routine 2D-PAGE, was addressed in two ways: (1) Alternatively, 10- to 13-day mouse limb buds were labelled with [14C]amino acids and their two-dimensional autoradiographs were exposed to photosensitive emulsion for 18 months. This procedure increased by more than 50% the number of protein syntheses detected and provided observations on a larger and different population than seen with the routine two-dimensional [35S]methionine method. About 10 of the 1500 detectable protein syntheses resolved could be attributed to stage- or organ-specific syntheses, or both (Van Blerkom, 1981a). (2) Another procedure, nonequilibrium isoelectric focusing, by sampling populations of basic proteins, extended the range of detectability. This procedure enabled detection of three or four organ-specific proteins out of more than 500 that were currently being synthesized. Neither of the two alternative sampling methods detected proportions of organ-specific synthesis that meaningfully differed from the routine equilibrium, 2D-PAGE procedure.

The proteins common to different organs may be a reflection of (1) different cell types synthesizing similar proteins and/or (2) synthesis from cell types common among different organs, such as fibroblasts. In both cases these proteins would be expected to be part of the background synthetic pattern. The extent to which proteins from common cell types contribute to the overall pattern is unknown but would be anticipated to be in proportion to their representation in a particular organ. Passenger cells, such as blood cells, are also a potential source of commonality of protein synthesis among different organs. Patterns of protein synthesis obtained from foetal blood cells at various stages of development consistently showed a small number of quantitatively major proteins. These 'marker' proteins were not detected in any of the tissues examined, thus demonstrating that the method of tissue preparation for radiolabelling and electrophoresis reduced or eliminated potential for blood cell contamination.

The three sampling methods suggest that each of the subpopulations examined sampled the total population of ongoing protein syntheses, so the 850 to 1000 protein syntheses seen at anyone stage during routine equilibrium electrophoresis has provided unbiased samples of the entire population of proteins being synthesized. The infrequently detected stage-specific shifts in protein syntheses, both restricted and nonrestricted to specific organs, probably do not represent changed components needed for maintenance and growth, but more likely indicate functional changes during organ development and differentiation. Having detected shifts in a relatively small proportion of protein synthesis with or without restriction to specific organs, the nature of these protein changes becomes relevant.

The frequencies with which developmentally altered protein syntheses, either constitutive or special, are structurally similar and/or are post-translationally
modified proteins, is unknown. Precedence suggests that an appreciable number of stage-specific proteins occur as post-translational modifications derived from from a significantly smaller number of genomic expressions. Certain proteins such as keratin and AFP, for example, exhibit stage-dependent changes in microheterogeneities which, as a consequence of post-translational modification, constitute families of structurally related protein syntheses. Another example is that approximately half of the detectable stage-specific proteins synthesized by early pre-implantation mouse embryos are post-translationally modified and have either very similar or identical primary structures (Van Blerkom, 1981b). We show in the present study that three out of five newly synthesized limb proteins characteristic of the 16-day foetus (Fig. 1C, 8–12) had homologous patterns after protein digestion (Fig. 1D). These particular proteins apparently represent keratins (Laskin et al. 1981) accompanying functional differentiation in the overlying epidermis. These presumed keratins having homology of primary structure possibly demonstrate a family of peptides derived from a single genomic expression. The results indicate that stage-specific protein syntheses need to be further characterized in order to be able to designate those seen as programmed changes in genomic expressions as distinct from those that undergo structurally related post-translational modifications.

Developmental shifts in quantitative and qualitative patterns of protein synthesis are readily observed (Table 1). Distinctions between quantitative and qualitative changes, however, may not always be dependable because some quantitative shifts may express thresholds of technical detectability that would be seen as qualitative changes. Although the distinctions at times may be equivocal, use of specified procedures enables significant interpretations to be derived from distinctions between detectable vs. non-detectable syntheses. Quantitative shifts seen as relative changes in intensity, are straightforward changes in rates of production of protein relative to a very large reference base, the entire population of 1000 proteins being detectably synthesized. Those quantitative and presumed qualitative shifts in protein syntheses, occurring with developmental stage specificity, demonstrate that stage specificity can occur synchronously in several organs and tissues or can be restricted to organ stage-specific changes. Both the general stage-specific and the tissue stage-specific protein syntheses signify developmental events at the molecular level. Understanding of the causal mechanisms in development will progress when we can distinguish between those programmed changes in protein syntheses attributable to genomic expression and those resulting from post-transcriptional programmes. Both of the molecular mechanisms appear to account for each of general stage-specific and tissue stage-specific changes in detectable protein syntheses during organogenesis.

Relatively small numbers of qualitative shifts in protein expression have been reported for specific developmental events: namely in *Dictyostelium discoideum* (Alton & Lodish, 1977a, b), neuroblastoma (Prashad et al. 1977),
Friend erythroleukemic cells (Affara & Daubas, 1979), myoblasts (Blau & Epstein, 1979), and trophectoderm (Van Blerkom, Barton & Johnson 1976; Handyside & Johnson, 1978). These examples are supported by our current observations that synthesis of AFP by liver and yolk sac and synthesis of keratins by epidermis exhibit a relatively small set of changes in protein synthesis at the time of development of differentiative functions.

Qualitative shifts in protein syntheses have been reported in embryonic and foetal tissues. Again, the numbers of changes in protein expressions during development are relatively small. The developmental period from resumed meiosis in the mouse oocyte through fertilization and cleavage to the 4- to 8-cell stage has been shown to exhibit approximately 40 qualitative changes of protein syntheses (Levinson et al. 1978; Chen et al. 1980; Cullen et al. 1980a; Richter & McGaughey, 1981; Van Blerkom, 1981a, b). The 7-5-day mouse embryo, in specialized extraembryonic tissues as in cells destined to become embryo, showed 50 proteins differentially distributed among these tissues (Johnson & Rossant, 1981). Day-14 foetal mouse liver, brain and forelimb were reported to have 22, 15 and 2 organ-specific proteins respectively (Klose & Von Wallenberg-Pachaly, 1976). Van Nest, MacDonald, Raman & Rutter (1980) reported tissue-specific proteins from the foetal rat that ranged from 3 in ‘mesenchyme’ to 27 in the gut. These findings, confirmed by our data, show that a relatively small proportion of total protein syntheses, less than 1% of the total detectable population can be classified as qualitative or quantitative, or both, shifts in protein synthesis that are stage specific or organ specific. The period of organ development studied, therefore, occurs with a synthetic background of a large set of constitutive proteins necessary for maintenance and growth but with relatively few detectable changes in protein synthesis.

Studies emphasizing the qualitative shifts in protein synthesis during development have invariably sorted these changing syntheses from a large array of nonchanging protein syntheses. Dewey, Filler & Mintz (1978), for example, concluded that development occurs in the absence of changing protein syntheses. They suggested that the varying syntheses seen in their material were ‘physiological’ in nature and therefore neither relevant nor significant for developmental events. Van Nest et al. (1980) noted ‘remarkable degree of similarity’ in the protein synthetic patterns for the foetal rat at 14 to 18 days. Our data and the results of others indicate that, within the current limits of sampling protein syntheses, the entire period of organ development studied proceeds with altered expression of a small proportion, less than 1%, of total proteins being synthesized.

The relative contributions of genomic and extragenomic influences for organogenesis and for the phenotype are still debated. Extensive phenotypic variability in the laboratory mouse has long been known to occur in the presence of identical genetic programmes (Runner, 1954). These observations indicate that programmed expression of genetic information alone may incompletely
Protein synthesis during organ development

account for organogenesis and for evolution of the phenotype. However, to exclude genomic influences as a major factor in organogenesis would be premature because the total number of proteins required for cell differentiation as well as the total number of proteins synthesized by a particular cell are unknown. The findings do indicate, however, that transcription of the genome must be coordinated with translational and post-translational events during organogenesis.

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REFERENCES


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