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Analysis of Proteins Expressed at the Time of Murine Organogenesis

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ABSTRACT Two-dimensional electrophoretograms were prepared from wild-type C57BL/6J embryos from day 7.5 through day 9.0 of development. This time period encompasses a critical window of development as the embryo traverses from an egg cylinder through major organogenesis. Consequently, we term this resource MOPED (for mouse organogenesis protein electrophoresis database). By resolving and analyzing the behavior of approximately 1,000 polypeptides per time point, we were able to track many of these polypeptides through this time period in development. Of special note was a burst of induced protein synthesis that was observed on day 8.5 of development. Polypeptides observed in mouse embryos that match those identified previously in mouse fibroblasts were noted. Two of them (the intermediate filament-associated protein and tropomyosin-4) were significantly altered in 8.5 day embryos. As more polypeptides are designated, it will be possible to expand the known proteins in the database. MOPED establishes the patterns of synthesis of a large number of polypeptides during a crucial period of development. Thus MOPED is designed to analyze proteins relevant to mouse embryogenesis in the future.

Key Words: Two-dimensional gel electrophoresis, Development, Polypeptides

INTRODUCTION

The development of the mouse embryo from an egg cylinder with a simple form to a recognizable entity with distinct organs occurs during a narrow window of embryogenesis. To distinguish the proteins that are made throughout, are extinguished, and are initiated during this time, we compared two-dimensional gels from 7.5, 8.0, 8.5, and 9.0 day C57BL/6J embryos. In particular, the standardized gel electrophoretic and computerized analysis system (PDQUEST) enabled us to achieve the reproducible, standardized analysis necessary for the construction of a database. Our results establish a MOPED (for mouse organogenesis protein electrophoresis database) for C57BL/6J mice that covers this time period and that can be used as a standard in future investigations. For example, in the accompanying papers we use these databases to examine the determination of laterality in murine embryogenesis and to detect polypeptides made in the developing heart.

MATERIALS AND METHODS

Staging of Mouse Embryos

At the appropriate interval after mice were mated, C57BL/6J pregnant females were sacrificed and embryos were harvested. Embryos were examined in serum-free Eagle's minimum essential medium without methionine under a dark field dissecting microscope. Only embryos that were of the appropriate Theiler (1972) stage were used for experiments. The Theiler stage criteria used were 7.5 day, stage 11 (neural plate and presomite stage); 8.0 day, stage 12 (one to seven somites); 8.5 day, stage 13 (eight to 12 somites); and 9.0 day, stage 14 (13 to 20 somites). Only embryos meeting these two criteria of correct date of conception and morphology were processed for two-dimensional gels.

Embryo Culture

Embryos were cultured for 3 h in Eagle's minimum essential medium (without methionine) supplemented with 0.5 mCi/ml of ^{35}S-l-methionine (>1,000 Ci/mmol; Amersham) without serum or unlabeled l-methionine. This resulted in the incorporation of labeled l-methionine into the >95% of proteins that contain this amino acid. The culture took place in a 37°C incubator with 5% CO_2 and 95% air as a gassing agent. Each embryo was cultured in medium in a sterile Eppendorf tube that was allowed to equilibrate throughout with the gassing mixture. That embryos survived this procedure was confirmed by two criteria: The embryos were larger and the hearts were still beating (in older embryos in whom hearts could be observed) after this incubation. The amounts of liquid were 25 μl for 7.5 day, 50 μl for 8.0 and 8.5 day, and 100 μl for 9 day embryos. The labeled embryos were then spun down for 1 min at 12,000*g*, the supernatant was removed, and the embryo pellet was quick frozen at −80°C.

Protein Extraction

Protein samples were prepared using the methods of Garrels (1979, 1983). For lysis, the frozen embryos were

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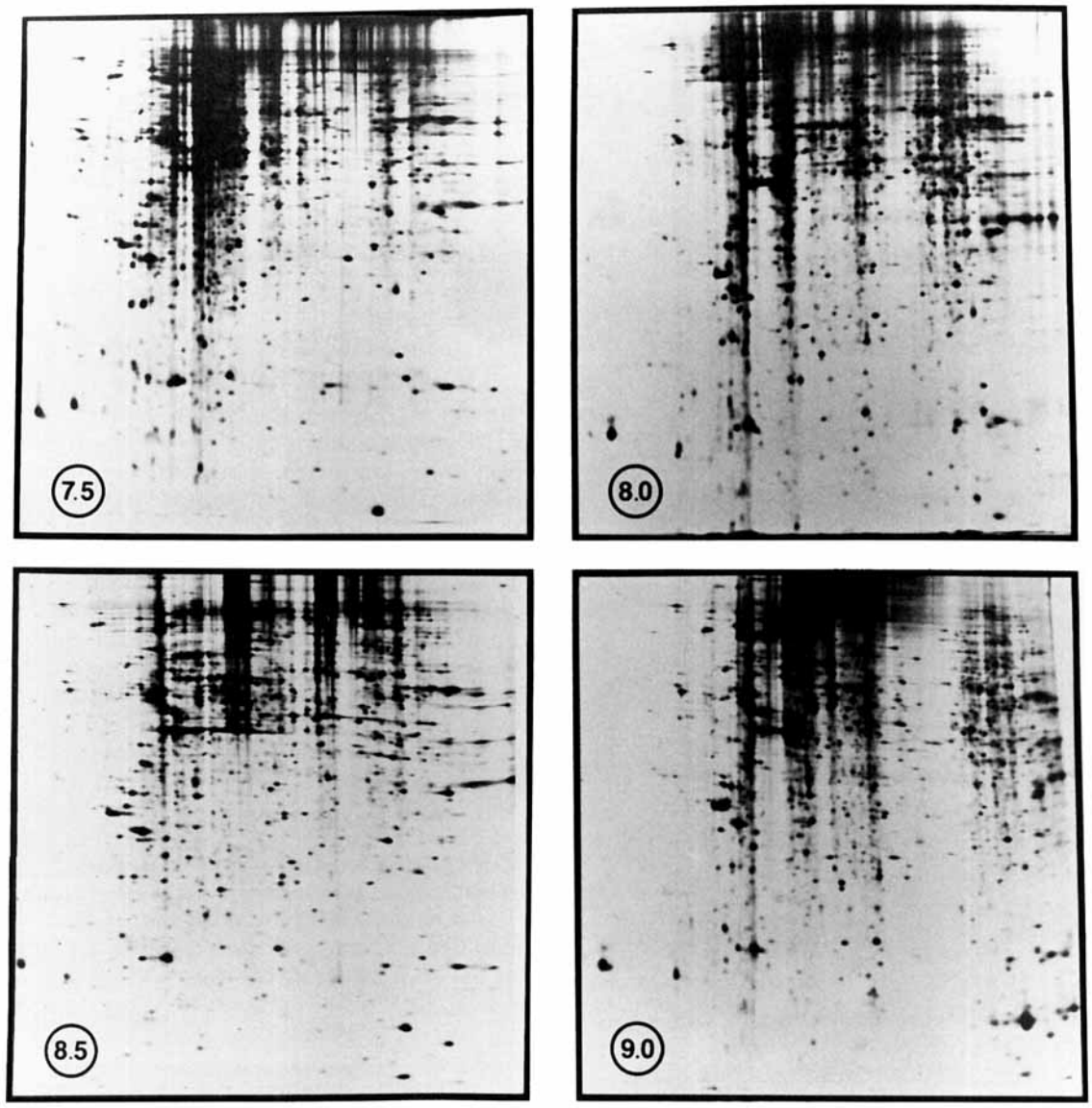

Fig. 1. Autoradiograms of representative broad-range pH gradient gels (pl 4.0–7.8) of proteins isolated from 7.5, 8.0, 8.5, and 9.0 day embryos.

resuspended in TS-SDS sample buffer (Protein Databases, Inc., Huntington Station, NY), using 25 μl for 7.5 day, 50 μl for 8.0 and 8.5 day, and 100 μl for 9 day embryos, followed by heating in a boiling water bath and incubation at 100°C for 3 min. The samples were vortexed and reincubated at 100°C for an additional 3 min. The samples were then quick cooled on ice and spun for 10 min at 12,000*g*. The supernatant was incubated on ice for 2 min with an equal volume of DNAse/RNAse (Protein Databases, Inc.) to reduce viscosity. The mixture was then snap frozen in liquid nitrogen and sent to Protein Databases, Inc. on dry ice for electrophoretic analysis. Following determination of trichloroacetic acid (TCA)-precipitable counts, samples were lyophilized and dissolved in isoelectric focusing buffer (9.5 M urea, 2% NP-40, 100 mM dithiothreitol [DTT], and 2% basic ampholines) at 37°C for 30 min.

Two-Dimensional Gel Analysis of Labeled Mouse Embryos

Two-dimensional gel electrophoresis was performed at Protein Databases, Inc. on an equilibrium two-dimensional gel in a standardized manner as described elsewhere (Garrels, 1979, 1983). Two hundred fifty thousand disintegrations per min of TCA-precipitable material was applied to each gel. This study was restricted to the more informative and reproducible equilibrium format, which uses broad range ampholytes (pH 3–10) in the first dimension, yielding a pH

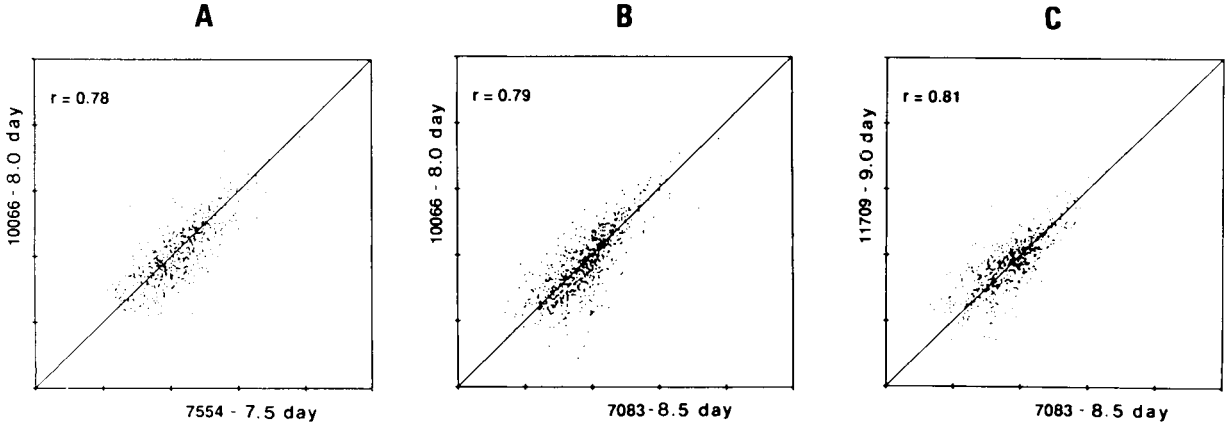

Fig. 2. Representative scatterplots from each comparison showing relatedness of two member gels. Each matched spot is plotted as a point according to its quantitation in the first gel (x axis) and its quantitation in the second gel (y axis). The correlation coefficient is r.

gradient of 4.0–7.8. The second-dimension gel electrophoresis was performed with 12.5% acrylamide, which resolves a useful range of 12,000–200,000 M_r. Gels with radioactive calibration strips containing known amounts of radioactive protein were processed for fluorography, and multiple autoradiographic exposures of each gel were made as described previously (Garrels, 1979, 1983; Garrels et al., 1984).

Image Analysis

Autoradiograms were scanned with an Eikonix 78/79 camera system at 200 μm resolution. Images were processed using the PDQUEST computer analysis programs based on the initial design of the Quest program of Garrels et al. (1984). For each gel, films corresponding to four autoradiographic exposure time points were developed to ensure that each spot was quantitated using an appropriate film on which the spot was present in the linear range for the film. The three most appropriate exposures were scanned and merged to form a composite image of a given embryo's protein synthesis. This provided a dynamic range of detection and allowed all proteins to be quantitated accurately. Images of embryo proteins from each time point (7.5, 8.0, 8.5, and 9.0 days) were assembled into matchsets and analyzed on a MassComp 68020 minicomputer. One image from each matchset was selected to be the standard (reference) image against which protein spots in the other images of the matchset were matched. The PDQUEST program assigned identification numbers to each protein spot in the standard image. Thus identical proteins in images in different matchsets will not necessarily have the same identifying number. It was then possible to compare polypeptides synthesized at different times during development. This analysis was performed by comparing two different matchsets, each containing images from samples of one time point. The comparisons included 7.5 day postcoitum vs. 8.0 day, 8.0 day vs. 8.5 day, and 8.5 day vs. 9.0 day. This method increased the reliability of the comparison; it required the analysis of multiple gels for each time point during embryogenesis. All data in each comparison were normalized between samples. The t test was performed at the two-tailed 99% confidence level ($P = 0.01$) (Spurr and Bonini, 1973). In addition, the PDQUEST program performed the Mann-Whitney rank-sum test (Sokal and Rohlf, 1969) and the log t test (Spurr and Bonini, 1973) at the two-tailed 95% confidence level ($P = 0.05$). Only spots satisfying all three tests are presented here.

TABLE 1. Day 7.5 vs. Day 8.0 (four gels each; 23 significant spots)*

Spot No.	Fold change	M_r (kD)	pI
6204	1.95	27.1	6.92
7305	1.58	35.0	7.10
4421	1.54	49.0	6.29
2216	1.44	26.6	5.58
1509	1.37	53.3	5.06
2105	−1.47	23.6	5.77
7405	−1.87	42.7	7.19
3511	−1.92	51.7	6.02
3621	−1.97	77.0	6.10
3615	−2.02	73.5	6.14
3215	−2.15	25.7	6.00
3415	−2.16	46.6	6.03
4513	−2.28	58.0	6.28
3224	−2.32	30.0	5.92
3419	−2.34	48.3	6.07
3608	−3.07	77.2	6.16
3221	−3.12	31.9	5.97
3624	−3.93	80.1	6.07
3310	−5.61	37.8	6.09
3325	−7.39	36.8	6.02
602	7.5 day only	83.7	4.74
1102	7.5 day only	17.4	5.25
4321	7.5 day only	35.1	6.25

*The standard image contains 1,014 spots. Fold change is average quantitation value of the spot in (8.0 day/7.5 day) if 8.0 day is more than 7.5 day or −(7.5 day/8.0 day) if 7.5 day is more than 8.0 day.

TABLE 2. Day 8.0 vs. Day 8.5 (four gels and nine gels, respectively; 71 significant spots)*

Spot No.	Fold change	M_r (kD)	pl	Spot No.	Fold change	M_r (kD)	pl
6309	4.33	38.6	6.59	5210	1.21	30.3	6.21
7212	3.38	28.6	6.96	1605	−1.36	72.4	5.17
8008	2.61	18.6	7.15	4402	−1.41	47.2	6.04
1118	2.36	23.8	5.07	4605	−1.51	79.9	6.04
501	2.22	53.6	4.50	5408	−1.51	44.0	6.28
5209	2.21	29.8	6.21	3501	−1.54	57.8	5.82
7109	2.17	25.6	6.86	7408	−1.69	41.0	7.00
2210	2.13	32.5	5.51	3314	−1.74	38.3	5.66
7413	2.10	40.7	6.89	5505	−1.75	55.4	6.27
210	2.04	31.4	4.78	5009	−1.80	17.4	6.19
1308	1.95	36.9	5.20	2701	−1.81	95.0	5.41
1311	1.82	39.8	5.15	7402	−1.87	40.7	7.11
8007	1.79	17.2	7.15	8208	−1.87	32.7	7.35
2601	1.78	72.1	5.55	2312	−1.88	35.4	5.45
6401	1.73	46.8	6.78	2702	−1.93	94.7	5.44
6107	1.73	27.4	6.60	2615	−2.04	63.6	5.58
7220	1.67	32.7	6.81	8308	−2.08	38.1	7.15
5309	1.67	37.0	6.29	2208	−2.11	30.6	5.47
2405	1.64	44.9	5.50	5212	−2.14	30.3	6.35
5503	1.64	52.6	6.28	5212	−2.14	30.3	6.35
309	1.62	35.1	4.37	3410	−2.15	41.3	5.71
1209	1.60	28.5	5.14	4210	−2.18	32.4	6.02
4403	1.59	45.5	6.06	3310	−2.27	35.0	5.72
2302	1.59	36.0	5.60	5311	−2.30	35.8	6.25
503	1.57	52.0	5.03	7708	−2.42	83.6	7.09
7104	1.55	27.1	6.92	5214	−2.44	31.5	6.18
5413	1.53	45.6	6.28	7707	−2.50	84.5	7.03
3315	1.51	37.9	5.73	3215	−2.63	28.9	5.72
6102	1.49	23.0	6.80	3517	−3.27	49.2	5.79
601	1.49	68.0	5.05	5414	−3.65	45.7	6.37
203	1.48	31.3	5.01	7706	−3.97	84.6	7.00
3117	1.47	25.1	5.71	4203	−4.27	29.4	6.14
4002	1.45	17.8	6.16	4005	−4.30	14.9	6.05
103	1.43	27.8	4.94	2605	−4.71	59.0	5.47
5107	1.31	25.1	6.26	3431	8.0 day only	43.9	5.79
				6509	8.0 day only	50.8	6.52

*The standard contains 1,103 spots. Fold change is average quantitation value of the spot in (8.5 day/8.0 day) if 8.5 day is more than 8.0 day and −(8.0 day/8.5 day) if 8.0 day is more than 8.5 day.

TABLE 3. Day 8.5 vs. Day 9.0 (nine and seven gels, respectively; 44 significant spots)*

Spot No.	Fold change	M_r (kD)	pl	Spot No.	Fold change	M_r (kD)	pl
303	4.89	34.7	4.98	7707	−1.46	84.5	7.03
306	4.82	37.9	4.84	1304	−1.61	35.1	5.35
8102	4.01	26.8	7.30	7609	−1.65	60.0	6.90
401	3.03	44.2	4.90	1106	−1.71	23.4	5.35
1313	2.75	35.9	5.10	3501	−1.77	57.8	5.82
4008	2.21	14.7	6.00	4504	−1.92	55.9	6.12
6101	2.19	26.5	6.77	2504	−1.98	58.7	5.63
502	2.03	52.6	4.78	7321	−2.02	40.1	7.02
5214	2.01	31.5	6.18	5506	−2.17	49.7	6.39
4005	1.99	14.9	6.05	2502	−2.20	54.7	5.64
6310	1.89	38.2	6.52	8308	−2.21	38.1	7.15
407	1.88	47.6	4.31	5505	−2.28	55.4	6.27
3208	1.78	30.8	5.78	7405	−2.38	48.1	7.03
7109	1.76	25.6	6.86	5313	−2.47	38.1	6.18
2305	1.55	34.8	5.60	2605	−2.47	59.0	5.47
210	1.52	31.4	4.78	5301	−2.52	40.0	6.48
2119	1.42	24.8	5.46	6203	−2.54	34.3	6.79
7201	1.42	28.4	7.09	7404	−3.45	47.1	7.03
4001	1.32	16.5	6.17	4502	−3.96	53.0	6.12
2302	−1.33	36.0	5.60	7410	−4.21	48.2	6.87
3603	−1.37	60.5	5.84	13	−6.04	15.4	4.69
5404	−1.44	46.9	6.38	7202	8.5 day only	34.1	7.04

*The standard image contains 1,006 spots. Fold change is average quantitation value of the spot in (9.0 day/8.5 day) if 9.0 day is more than 8.5 day or −(8.5 day/9.0 day) if 8.5 day is more than 9.0 day.

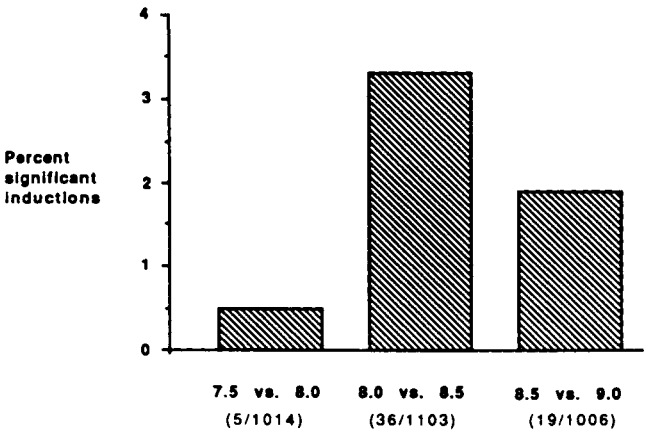

Fig. 3. Number of induced protein spots as a percent of spots resolved in the standard image for each of the three comparisons.

The 95% level test of significance for these three tests is performed automatically by the PDQUEST program. We increased stringency to 99% for the t test. These levels are comparable to levels used in studies employing two-dimensional gel electrophoresis to determine significant protein changes under various conditions. For example, Merril et al. (1981) used both 95% and 99% levels of the t test to determine significant quantitative alterations in labeled phytohemaglutinin-stimulated lymphocytes from Lesch-Nyhan syndrome patients and controls; the 95% level of the t test was also used to study cerebrospinal fluid proteins in neurological disease patients by Harrington et al. (1984); and a study of human brain proteins from cortical sections obtained under different conditions used the t test at 95%, 97.5%, and 99% levels (Narayan et al., 1984).

This computer analysis distinguished proteins that differed significantly in quantity between the two groups in each comparison as well as qualitative differences (specific to one or the other group). These putative significant protein spots were each examined critically in all autoradiograms for authenticity. Spots that were obscured in a streak or smudge, were very faint, displayed an inconsistent pattern, or were otherwise questionable were excluded from the analyses.

RESULTS

Following examination of images, we accepted only autoradiograms of sufficient reproducibility to permit standardization of time points during embryogenesis. An example of these autoradiograms for each time point is shown in Figure 1. Four time points with four gels per time point were used for this study, except for 8.5 day embryos, for which nine gels were used. To ensure reproducibility, each time point was described by multiple gels, as indicated in Tables 1–4. A representative scatterplot from each comparison, demonstrating the relatedness of two images, is shown in Figure 2.

The first comparison made was between 7.5 and 8.0 day embryos. As is shown in Table 1, using three different tests of significance, there were five spots that were increased on day 8.0 and 15 spots that were increased on day 7.5. In addition, there were three spots in 7.5 day embryos that were not seen in 8.0 day embryos.

The second comparison was between 8.0 and 8.5 day embryos, which coincides with the time when organogenesis is beginning. As is shown in Table 2, 36 spots increased significantly on day 8.5 and 33 spots increased significantly on day 8.0. In addition, there were two spots unique to 8.0 day embryos.

The third comparison was between day 8.5 and 9.0 embryos. As is shown in Table 3, 19 spots increased significantly on day 9.0 and 24 spots increased significantly on day 8.5. There was a single spot that was unique to 8.5 day embryos.

The frequency of these qualitative protein changes that appeared to be induced in each comparison is depicted in Figure 3. It is apparent from Figure 3 that the embryo undergoes a significant alteration in protein synthesis on day 8.5 of development. The increased number of proteins induced (or that had not been expressed previously in sufficient quantity to be detected) in the 8.0–8.5 day embryo comparison vs. the 7.5–8.0 day embryo comparison indicates that a considerable shift in protein synthesis is occurring on day 8.5.

The standard images were each compared with published images in which the identities of some polypeptide spots were identified as known mouse proteins (Blose, 1986). Those that could be located on our images are indicated in Table 4. It appeared that one of the proteins (No. 7202), which was significantly less in 8.5 day embryos than 8.0 day embryos, is the intermediate filament-associated protein. Another one (No. 203) that was significantly greater in 8.5 day embryos than 8.0 day embryos appeared to be tropomyosin-4.

DISCUSSION

Previous investigations have focused on two-dimensional protein patterns of mouse cells and tissue (Fey et al., 1984; Jungblut et al., 1989; Pluschke and Lefkovits, 1984), mouse embryos (Klose, 1983), and early mouse embryos (Magnuson and Epstein, 1981; Van Blerkom, 1981; Van Blerkom et al., 1982). However, differences in gel running conditions between individuals laboratories can make published gel patterns very difficult to compare with one's own images. This was a major reason for selecting Protein Databases, Inc. as the place for all electrophoresis to be performed: Gels that resolved many polypeptides were run according to a standard format with a high degree of reproducibility. Our experiments involved comparing matchsets of gels run at different times. There were distortions, mainly in the pH gradient of gels run at different times, that prevented accurate matching of all spots. Those polypeptides displaying significant changes as reported here were ascertained carefully and were in reproduc-

TABLE 4. Location of Mouse Proteins Identified Previously (Blose, 1986)

Protein	Spot number in 7.5 vs. 8.0 day matchset	Spot number in 8.0 vs. 8.5 day and 8.5 vs. 9.0 day matchsets
Heat shock protein 73	3616	2603
Heat shock protein 80	2606	1601
Heat shock protein 90	2704	1701
Heat shock protein 100	1703	1801
Intermediate filament-associated protein	2707	2702[a]
α-Actinin	1703	2802
Vimentin	2509	1502
α-Tubulin	2517	2511
β-Tubulin	1511	1509
Actin isoforms	2417, 2415, 2416, 2419, 3420	1405, 1406, 2417, 2412, 2421
Cyclin	1313	1212
Tropomyosin-4	1210	203[b]
Tropomyosin-5	1216	1217

[a]Significantly less in 8.5 day embryos than in 8.0 day embryos (Table 2).
[b]Significantly greater in 8.5 day embryos than in 8.0 day embryos (Table 2).

ible areas of the gels. Other changes may have occurred, but they could not be ascertained because they were inconsistent, crowded, and/or of poor quality.

The protein patterns were compared with published patterns in which the identities of some polypeptide spots were identified as known mouse proteins (Blose, 1986). Two identifiable ones were altered significantly in the 8.0 day vs. 8.5 day comparison. The intermediate filament-associated protein, a component of the intermediate filament system of cultured cells (Lin and Feramisco, 1981), appeared to be decreased significantly (by 1.93-fold) in the 8.5 day samples. Tropomyosin-4 was increased significantly (by 1.48-fold) in the same comparison. Tropomyosin is a ubiquitous protein, which is a component of the contractile apparatus of all cells. Several isoforms have been described, including five distinguishable by monoclonal antibodies in cultured rat cells (Matsumura et al., 1983). Their positions on two-dimensional protein gels has been determined in rat (Garrels and Franza, 1989) and mouse (Blose, 1986) cells.

In conclusion, we have created a database of proteins expressed in murine development that serves two functions. First, it serves as a road map to development, documenting how multiple polypeptide spots behave during the critical period of organogenesis. As such, it will be a resource to study both normal development and perturbations of this process. Second, it reveals findings unknown previously: 1) There is an abundance of quantitative protein synthesis changes coinciding with the onset of organogenesis on day 8.5 of murine development; 2) most changes are quantitative, involving the up- or down-regulation of existing polypeptides; 3) few consistent changes are qualitative, involving polypeptides with novel or extinguished synthesis at given times of development. However, these polypeptides are particularly useful in that they can seve as anchors for particular time points in embryogenesis.

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