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Complexity of sea urchin embryo nuclear proteins that contain basic domains

(nuclear extract/cation-exchange chromatography/image analysis/two-dimensional mapping)

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ABSTRACT **We describe a quantitative two-dimensional gel electrophoretic analysis of nuclear extract from 24-hr sea urchin embryos. The extract was fractionated by using a weak cation-exchange resin, and eight known DNA-binding proteins were shown to be entirely included in a salt eluate that releases proteins containing basic domains. This fraction and a lower-salt fraction containing the majority of the protein species were mapped two-dimensionally by using new algorithms that permit reproducible spot identification, storage of intensity and map-position data, and subtractive comparison of one pattern with respect to another. By reference to a previously characterized DNA-binding factor, spot intensity could be interpreted in terms of the number of molecules per embryo nucleus. A map was constructed displaying all nuclear proteins containing basic domains that are present within the concentration range per nucleus of a set of known DNA-binding factors of the sea urchin embryo. The map includes 265 spots that fulfill both of these criteria, probably representing about 100 different protein species.**

Recent studies suggest that the entire trans-acting regulatory apparatus of the sea urchin embryo may be accessible in easily prepared nuclear extracts. Many specific DNA-binding proteins have already been characterized in such extracts (e.g., refs. 1–8), which can be obtained reproducibly in stable form and in relatively enormous quantities. We describe here an attempt to estimate the complexity of a particularly interesting subfraction of the several thousand protein species included in these nuclear extracts, using a quantitative two-dimensional (2-D) mapping technology and ion-exchange chromatography.

To obtain a subset of proteins that would include DNA-binding factors, we fractionated the extract on a weak cation-exchange resin. This column permits the separation of proteins that contain basic domains (not necessarily basic overall, as measured by the isoelectric point, pI). Basic domains are a canonical feature of specific DNA-binding proteins, which display a general nonspecific affinity for DNA. One-dimensional (1-D) diffusion along the DNA, probably mediated by exchange of basic residues with bound counterions, greatly enhances the rate at which factors at low concentration are able to encounter their specific target sites (9). Of course, many other kinds of proteins in the nuclear extract might be expected to fractionate on an ion-exchange resin together with DNA-binding proteins. Thus, we have focused on those proteins in the fractions of interest that are present in the embryo nuclei within a set prevalence range, which embraces a set of previously characterized sea urchin embryo DNA-binding factors (2). A 2-D map, against polypeptide mass and pI coordinates, has been generated for the selected set of nuclear proteins, and several known DNA-binding factors have been located within it. Though we make no claim that this map includes only DNA-binding regulatory factors, its complexity, $\approx 10^2$ different proteins, is interesting. This map should serve as a valuable data base as additional embryo factors are cloned and different stages of development are investigated.

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MATERIALS AND METHODS

Cation-Exchange Chromatography. The elution profile of each of eight different DNA-binding proteins was initially determined by gel mobility-shift analysis of fractions obtained from a Bio-Rex 70 (Bio-Rad) column eluted with a step gradient of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0 M KCl in column buffer (see below; ref. 2). Nuclear extract obtained (7) from $\approx 5 \times 10^8$ embryos (3.75 ml) was used for the preparation that was subjected to ion-exchange fractionation and 2-D electrophoretic analysis. The extract was brought to 0.1 M KCl and applied to a 5-ml Bio-Rex 70 column (Bio-Rad) that had been equilibrated with 0.1 M KCl in column buffer [20 mM Hepes·KOH (from 1 M stock solution, pH 7.9)/0.1 mM EDTA/1 mM dithiothreitol/0.12% Nonidet P-40/20% (vol/vol) glycerol] at a flow rate of ≈0.07 ml/min. Proteins were first eluted with 0.2 M and then 0.8 M KCl in column buffer [respectively low-salt (LS) and high-salt (HS) column buffer]. Total protein content of each fraction was assayed by the method of Bradford (10), and peak fractions were dialyzed overnight against 0.1 M KCl in column buffer and frozen at −70°C for later analysis by 2-D electrophoresis.

2-D Gel Electrophoresis. 2-D electrophoretic gels were run essentially as described (11) and analyzed by using recently developed software, GALTOOL, that will be described in detail elsewhere (J. E. Solomon, S. Eberlein, and M.G.H., unpublished data). The final 2-D electrophoretic protein pattern was remarkably consistent but deteriorated when the extract was frozen and thawed more than three times, irrespective of the presence of leupeptin, phenylmethylsulfonyl fluoride, and EDTA as protease inhibitors. Silver-stained wet gels were directly digitized on a Molecular Dynamics 300 A densitometer. The usefulness of silver-staining for our purposes was enhanced by the ammoniacal silver stain, which produces a more sensitive staining reaction, and by the incorporation of sodium thiosulfate in the gel matrix, which reduces the silver-stain background (12).

Immunodetection. Polyclonal rabbit antisera were available for regulatory factors P3A1 and P3A2 (7) and were used to stain gel-separated proteins that were electroblotted to poly(vinylidine difluoride) membrane (13). Blots were stained initially with colloidal gold (Bio-Rad 170-6527). The

Abbreviations: HS and LS, high salt and low salt; 2-D, two dimensional.

gold-stained pattern on the blot faithfully reproduced the original silver-stained patterns visualized in the 2-D electrophoretic gels, and gold staining did not interfere with subsequent immunostaining of the same blot. Immunostaining was performed in the conventional manner, with a 1:10,000 dilution of either anti-P3A1 or anti-P3A2 antibody, followed with an alkaline phosphatase-conjugated anti-rabbit antibody that was localized by using the chemiluminescent substrate "AMPPD" (Tropix, Bedford, MA), thus allowing direct visualization of the protein–antibody complex on photographic (Kodak XAR5) film.

RESULTS

Polypeptide Complexity of the Crude Nuclear Extract. Fig. 1*A* shows a silver-stained 2-D electrophoretic gel that includes most of the proteins in the nuclear extract. The spot-finding algorithm of GALTOOL detected 3164 spots distributed broadly over a mass range of 8–250 kDa, a pI range of 4–8, and a relative quantity range of 1000-fold. The reproducibility of these 2-D electrophoretic maps was examined in three ways. (*i*) A sample was split into three, and triplicate gels were run. Less than 0.2% variation in the number of spots was observed between gels. (*ii*) Fractions eluted with HS column buffer (HS fractions) were analyzed from three different cation-exchange columns. Again, <0.2% spot variation was observed. (*iii*) Comparison of blastula nuclear extracts prepared from two different years of embryo harvest revealed <0.5% variation in spots detected.

Qualitative Fractionation of Nuclear Extract by Cation-Exchange Chromatography. In initial studies we found that application of the nuclear extract to a weak cation-exchange column (Bio-Rex 70) provided an impressive separation of known DNA-binding proteins from many other proteins in the extract. The column was eluted in a KCl step gradient, advancing in 0.1 M increments. Each fraction was tested for eight specific DNA-binding activities that were known from previous work to be present in the nuclear extract (2, 3, 8). Target sequences for each of these eight specific DNA-binding activities have been identified in the regulatory domains of the *CyIIIa* and other sea urchin genes (2, 3, 14). These sequences were used as probes for gel mobility-shift measurements carried out on the column eluant fractions (data not shown). The eight factors assayed were P1, P3A2, P3B, P4, P5, P6, P7I, and P8II, for which specific affinity constants had been established (2). Except for P1, all of these have been partially purified and characterized by affinity chromatography or have been cloned, or both (refs. 3 and 7; also unpublished data). All eight of these factors were eluted from the Bio-Rex 70 column with buffer containing between 0.3 and 0.8 M KCl; that is, there were no detectable gel mobility-shift signals in the LS (0.1 and 0.2 M KCl) column fractions. However, these LS fractions contained about one-third to half of the total protein mass in the nuclear extract in each of four independent trials. Bio-Rex 70 cation-exchange chromatography thus affords a means of separating a fraction of the nuclear extract that is likely to include most DNA-binding factors from a fraction in which they are absent.

A preparative scale fractionation of the nuclear extract (≈45 mg of total protein) was carried out as described by loading the column with column buffer containing 0.1 M KCl, first eluting with 0.2 M KCl-containing column buffer (LS fraction), and then eluting with 0.8 M KCl-containing column buffer (HS fraction). The eluates were dialyzed against the 0.1 M KCl-containing column buffer and run on 2-D electrophoretic gels. Fig. 1*B* (LS fraction) and 1*C* (HS fraction) illustrate the separation. Note that there is no obvious difference in the 2-D electrophoretic maps of the LS and HS fractions in overall pI distribution, as would have occurred if the column had responded to total molecular charge rather than to regional positive charge. Yet the patterns shown in Fig. 1 *B* and *C* include very few protein species that are prominent in both fractions.

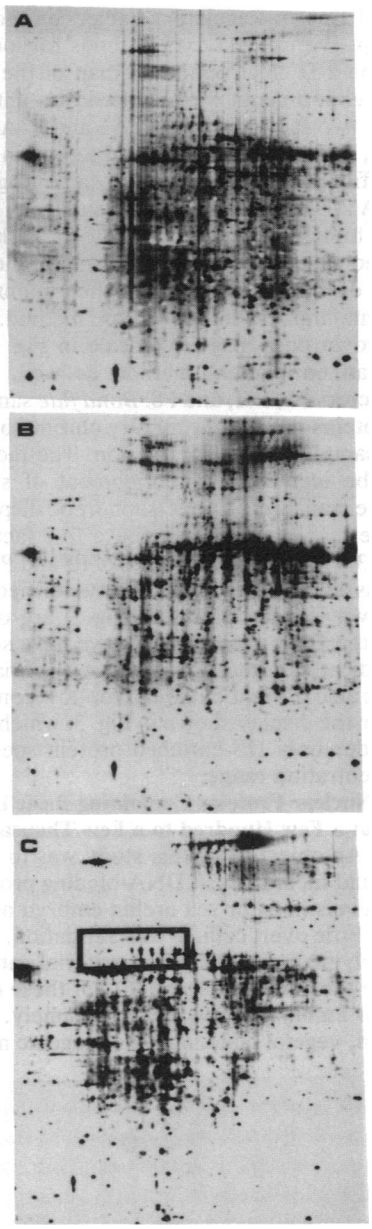

FIG. 1. 2-D electrophoretic maps for Bio-Rex 70 column fractions of nucleic extracts from 24-hr-old sea urchin embryos. The starting extract was fractionated into LS and HS eluates as described in text. (*A*) Silver-stained 2-D electrophoretic map of a reconstituted sample consisting of 30 μg of the HS fraction and 15 μg of the LS fraction. The 2:1 ratio was used to improve visibility of minor HS proteins. This pattern is indistinguishable from that of the unfractionated nuclear extract. Therefore, there is no significant loss of protein species either in the flow-through (0.1 M KCl loading buffer) fraction from the Bio-Rex 70 column or in the fraction remaining bound after 0.8 M KCl-containing column buffer elution. A total of 3164 individual spots were detected and catalogued. (*B*) 2-D electrophoretic map of LS fraction proteins run under identical conditions as in *A*. Thirty micrograms of protein was loaded on the gel, and 2382 individual spots were detected and catalogued. (*C*) 2-D electrophoretic map of HS fraction proteins run and displayed as in *A*. Thirty micrograms of protein was loaded, and 1545 spots were detected. The patterns in *B* and *C* are so dissimilar that it was necessary to use the map of the mixture of HS and LS fractions shown in *A* to line them up in register.

Location of Known Regulatory Factors on the 2-D Electrophoretic Map. We utilized two methods for locating known factors on the 2-D electrophoretic map of the HS fraction. When these experiments were carried out, antibodies were available for two factors called P3A1 and P3A2. These are, respectively, a Zn-finger protein and a protein of no known structural affiliation that bind to the same target regulatory site (7, 15). A 2-D electrophoretic gel similar to that shown in Fig. 1*C* was blotted to the poly(vinylidene difluoride) membrane, stained with colloidal gold to visualize the total protein spot pattern on the membrane, and then immunostained by using a chemiluminescent detection method. Results for P3A2 are shown on a magnified scale in Fig. 2. P3A2 was located by an enrichment method as well, as were the additional factors P5, P7I, and P8. *Bona fide* samples of these proteins (proteins purified by affinity chromatography) were added to a sample of the HS fraction. The factor locations could then be identified by enrichment of specific spots relative to the standard 2-D electrophoretic display of the HS fraction. The map positions for these five factors are indicated in Fig. 3. Note that in the cases of P8 and P7I, more than one spot was enriched by the affinity-purified factor. The variants shown in parentheses in Fig. 3 are otherwise unknown, and the DNA-binding forms we have so far isolated are those indicated without parentheses. Variants of P3A2 and P3A1 are also present but are of too-low concentration to be included in the display shown in Fig. 3, which as described below, includes only HS-enriched protein species within a certain concentration range.

A Map of Nuclear Proteins Containing Basic Domains That Are Present at a Few Hundred to a Few Thousand Molecules per Nucleus. An objective of this study was to set up a data base that would include most DNA-binding proteins present at effective levels in 24-hr sea urchin embryo nuclei. At this stage there is little overt cellular differentiation, and there are as yet probably no more than five territorial patterns of gene expression in the entire embryo (16). Of these only four are significant for embryogenesis *per se*—namely, oral and aboral ectoderm, vegetal plate, and skeletogenic mesenchyme.

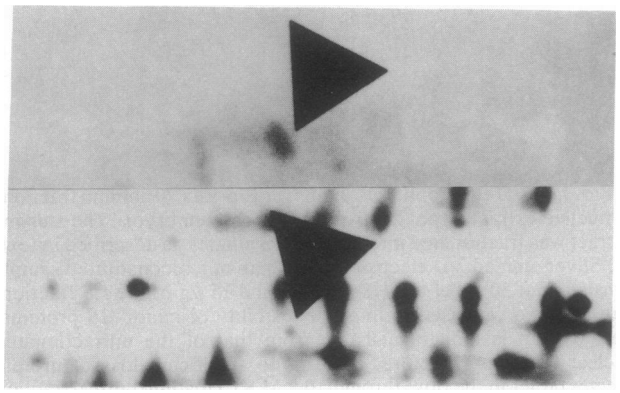

FIG. 2. Location of the P3A2 factor in a region of the HS 2-D electrophoretic map (rectangle in Fig. 1*C*) by immunostaining. A gel loaded with 30 μg of HS fraction proteins, run exactly as in Fig. 1*C*, was blotted to a membrane, stained with colloidal gold to visualize the total protein spot pattern, and then allowed to react with the anti-P3A2 antibody, whose specific binding was located by using a chemiluminescent detection method. The arrowhead points to the immunostained spot identified in the upper image as P3A2, which is also shown in the lower image in the context of a blow-up of the surrounding region of the silver-stained gel, identified by reference to the gold-labeled spots on the same membrane that was used for the immunological reaction. The antibody detection procedure is much more sensitive than silver or gold staining and revealed a number of minor P3A2 charge variants. These variants appear to be the consequence of posttranslational modifications (to be described elsewhere).

Even if a given regulatory factor is confined to only one of these territories, it will be present in an appreciable fraction of the cells ($\geq 10^{-1}$). Thus at 24 hr or earlier, we could not expect to encounter factors that are, say, only 1% as prevalent as are other factors, solely because they are confined to a very few cells out of the entire embryo (later in development this might not be at all unlikely).

We generated a separate map of those proteins that met the following two criteria: (*i*) they were at least 10-fold enriched in the basic domain fraction shown in Fig. 1*C* with respect to the same proteins, if they were present at all, in the LS fractions shown in Fig. 1*B*; and (*ii*) their prevalence was in the range that would provide a minimum average frequency of $\approx$250 molecules per nucleus and a maximum average frequency of $\approx$7000 molecules per nucleus. Both criteria could be regarded as conservative (i.e., admitting more than DNA-binding proteins, but including most of these). Thus, with respect to the first criterion, the gel mobility-shift analyses that we carried out on the Bio-Rex 70 fractions, as described above, showed that no activity could be detected at all in the LS fraction for eight known DNA-binding factors; this perhaps could be true of all *bona fide* DNA-binding factors in the sample. About 50% of the spots recorded in Fig. 1*B* were passed by the criterion of $\geq$10-fold enrichment. The second criterion was based on quantitative DNA-binding factor prevalence data obtained earlier by Calzone *et al.* (2), including the eight factors utilized in our experiments to test the Bio-Rex 70 fractionation. Almost all of these prevalences fell within the limits we set for this analysis. To apply criterion *ii* to the map shown in Fig. 1*B*, we utilized P3A2 as a quantitative standard. This factor is present at 700 molecules per nucleus in 24-hr embryos (2), which is toward the low end of the observed prevalence range. P3A2 is probably functional earlier in development, in late cleavage, when it is several-fold more concentrated on a per-nucleus basis (2, 17). Having identified the P3A2 spot (Fig. 2) in the silver-stained 2-D electrophoretic gel of the HS fraction (Fig. 1*C*), we scored all proteins that were present between one-third of the intensity of P3A2 and 10 times its intensity. The lower limit is about 3 times above the background silver stain on the gel. It follows that DNA-binding proteins that fall in the normal range of affinities for their target sites (see ref. 2) will produce spots that are within the detection range. Few DNA-binding proteins that exist at 24 hr at functional concentrations will have been lost in the background haze that underlies the 2-D electrophoretic patterns unless they stain anomalously.

The result of this computational analysis is shown in Fig. 3. The number of spots in Fig. 1*C* that pass both criteria is 265. However, 265 is almost certainly an overestimate of the number of different polypeptide species. Modifications such as phosphorylation, glycosylation, and proteolytic processes contribute to the generation of a pattern that has greater complexity than does the primary population of polypeptide sequences. This has been shown explicitly in studies of several different 2-D electrophoretic data bases (18). Thus, for example, the ratio of spots to polypeptides for 39 known proteins within a plasma protein data base is 3.6 (excluding immunoglobulins and some other unusually polymorphic proteins); for 22 liver proteins, 3.4; for 371 amnion cell proteins, 2.0; for 143 keratinocyte proteins, 1.85. A conservative identification of variants that are probably the same protein is shown by connecting lines in Fig. 3, which identify proteins of the same mass that differ by integral numbers of charges, and by circles in which two spots are shown where the density patterns actually overlap and are probably generated by single species. Counting each spot group as one putative polypeptide decreases the complexity from 265 to 184. However, judging from the ratios from other sets of proteins cited (18), the most likely true complexity of the pattern shown in Fig. 3 is likely to be half to one-third of the

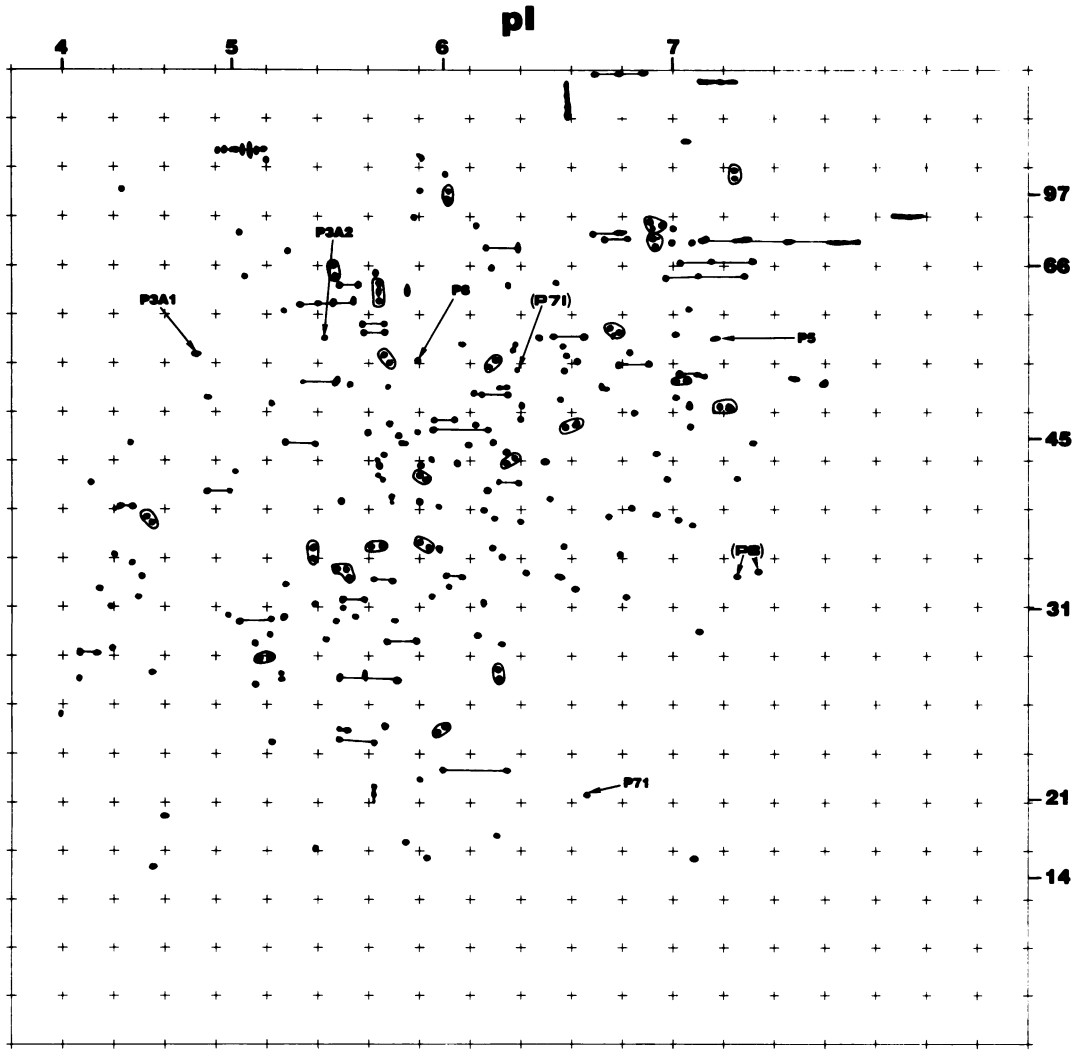

FIG. 3. Map of proteins that contain basic domains and that are present at between ≈250 and ≈7,000 molecules per nucleus (molecular mass shown in kDa). Proteins were selected from those shown in the HS 2-D electrophoretic map of Fig. 1*C* on the bases that (*i*) they are ≥10-fold enriched with respect to the LS fraction and (*ii*) they are between one-third and 10 times as prevalent as the known factor P3A2 (700 molecules per nucleus; ref. 2). The positions of five known DNA-binding proteins (P3A1, P3A2, P5, P7I, and P8; refs. 2, 3, 7, 8, and 15) are indicated. Spots linked by horizontal lines are of the same mass but differ by integral numbers of charges; encircled spots are derived from contiguous and overlapping silver-stain density distributions. In both cases the multiple spots within each group are likely to represent only a single primary polypeptide species.

total of 265, or perhaps $\approx 10^2$. Thus, in sum, the starting nuclear extract contained 3164 spots detectable in our 2-D electrophoretic system; the basic domain fraction contained a total of 1545 spots; those that were enriched 10 times or more in this fraction and are present in the allowed concentration range produced 265 spots, possibly representing about 100 different protein species.

DISCUSSION

By late cleavage, the various lineage elements of the sea urchin embryo have instituted territorial patterns of differential gene expression (17, 19). Considering only the primary tier of regulatory interactions, those mediated by sequence-specific DNA-binding proteins, it would be extremely valuable to have an estimate of the regulatory complexity required to generate and maintain this territorial gene expression. There is of course no method at hand by which to identify categorically all DNA-binding proteins, and only DNA-binding proteins, given their great structural variety. To approach this problem we separated from nuclear extract polypeptides containing basic domains, as these are required for the characteristic nonspecific affinity of these proteins for DNA. The nonspecific affinity is typically less than the affinity for specific target sites by factors of 10^3 to 10^6 (e.g., see refs. 2 and 20). Use of the Bio-Rex 70 cation-exchange column is essentially equivalent to using DNA-conjugated cellulose (except that it is more convenient because of its much higher capacity), since nonspecific DNA–protein interaction is mainly electrostatic (9). However, even if this resin efficiently segregates most specific DNA-binding proteins, as suggested by the observation that it bound all of a set of eight known factors tested, it will trap other proteins with basic domains as well. A prominent class of these, that should be represented in the nuclear extract, is the heterogeneous nuclear RNA (hnRNA)-binding proteins. Enzymes that interact with DNA and general chromatin proteins might also be included [histones are, however, not present (2, 7)]. For this reason we superimposed a rather narrow (30-fold) window of protein concentrations on the initial criterion that the selected protein population be significantly enriched by binding to the cation exchanger. General chromatin proteins, hnRNPs and other classes of RNA-binding proteins are expected to be present in much higher concentrations than

250–7000 molecules per cell. By taking hnRNP as a reasonably well-studied example, the protein/RNA mass ratio is about 4 (in HeLa cells), and the average mass of these RNA-binding proteins is about 40 kDa (21). The mass of hnRNA per nucleus in sea urchin embryos at the relevant stage is 2.9×10^8 nucleotides per cell (22), and if one assumes 50–100 hnRNP species (23), there would be $1–2 \times 10^5$ molecules of each species per nucleus. At the other end of the scale, it could be argued that we might have missed some *bona fide* DNA-binding proteins. Although most known DNA-binding factors have been obtained from extracts prepared by similar protocols, there could be exceptions. Furthermore, the lower limit of our allowed concentration window is only 3 times above the background staining intensity, and certain protein species might fail to stain as intensely as the average. On the other hand, this lower level is already one-third of the level of the relatively rare P3A2 factor, at a stage when its prevalence per nucleus has already fallen several fold below its earlier peak concentration. Nonetheless, the sensitivity limit is too close to our lower threshold of allowed concentration for comfort, and it will remain to be seen if any purified factors represent moieties that were indeed lost in the background and are not included in Fig. 3.

From the considerations given in the last section of *Results*, we interpret the complexity of the map shown in Fig. 3 to indicate a hundred or so different polypeptides, and some of these are probably not DNA-binding proteins. This is not on the face of it an unreasonable estimate for trans-regulatory-system complexity at this early stage of sea urchin development. About half this number of factors has been genetically implicated in the construction of a far more complicated spatial pattern of expression in the precellular *Drosophila* embryo (24, 25), and, as detailed molecular dissections of regions of the *ftz* (26) and *Ubx* (27) promoters illustrate, a significant number of additional factors specifically not revealed by pattern formation mutations are also required.

The map shown in Fig. 3 visualizes a stored data set to which any subsequently obtained embryo nuclear extract can be compared, both quantitatively (i.e., intensity of each spot) and qualitatively (i.e., in respect to the appearance or disappearance of given spots). The map will thus serve as a basis for measurement of changes in the developmental profile of this interesting set of nuclear proteins as they are obtained from earlier and later embryos or from different embryo fractions that can be prepared *en masse*, such as ectoderm and endoderm–mesenchyme. Furthermore, many new DNA-binding factors are now being isolated from these embryos by affinity chromatography, with target sites from a variety of genes expressed in the four main territories of the embryo. These factors can now be identified by map position as each is obtained. By this means, and by direct sequencing of spots isolated from the 2-D electrophoretic gels, the uncertainties considered here should eventually be resolved. Since all of the spot-location and intensity data from this analysis are stored and accessible, the data base could easily be reformulated were further experiments to reveal a better factor concentration window than that we chose *a priori*. Thus, this approach should ultimately provide a useful estimate of regulatory DNA-binding-factor complexity for the early sea urchin embryo.

Note Added in Proof. Two additional antibodies against cloned factors have been tested since the manuscript has been prepared. Both react with proteins in the map shown in Fig. 3. These are factors P6 (2), 1 spot; and USF (28), 2 spots.

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