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DNA–protein binding assays from a single sea urchin egg: A high-sensitivity capillary electrophoresis method

(mobility-shift assay/laser-induced fluorescence/transcription factor/nuclear extract)

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ABSTRACT **A capillary electrophoresis method has been developed to study DNA–protein complexes by mobility-shift assay. This method is at least 100 times more sensitive than conventional gel mobility-shift procedures. Key features of the technique include the use of a neutral coated capillary, a small amount of linear polymer in the separation medium, and use of covalently dye-labeled DNA probes that can be detected with a commercially available laser-induced fluorescence monitor. The capillary method provides quantitative data in runs requiring <20 min, from which dissociation constants are readily determined. As a test case we studied interactions of a developmentally important sea urchin embryo transcription factor, SpP3A2. As little as 2–10 × 10^6 molecules of specific SpP3A2–oligonucleotide complex were reproducibly detected, using recombinant SpP3A2, crude nuclear extract, egg lysates, and even a single sea urchin egg lysed within the capillary column.**

Measurement of sequence-specific DNA–protein interactions is a key experimental procedure in the molecular biology of gene regulation. The most commonly used method is the electrophoretic gel mobility-shift assay (EMSA), in which a radioactively labeled DNA probe is mixed with a solution containing the protein(s) of interest and, after a brief reaction period, loaded on an electrophoretic gel. The complex migrates more slowly than does the free probe, and despite the limited stability of such complexes their recovery is greatly facilitated by the "caging" effect of the gel, which essentially retains the protein in the vicinity of the probe during the rather slow process of electrophoresis (1). While EMSA is used extensively (2–4), a limitation is the amount of material required per assay, at least in standard practice. A typical protocol requires $\geq 10^{10}$ molecules of kinase-labeled probe, and at least 1–10% of this number of active DNA-binding protein molecules. Since transcription factors are often present in the range of only 10^3–10^4 molecules per nucleus, the application of EMSA for extracts of only a few hundred, or thousand, cells is precluded. For certain problems this is a severe limitation: for example, in studies of embryonic development, it is often possible to dissect out by hand a biologically important element of an embryo containing a few hundred cells, or to collect certain cells from a hundred embryos or so. Invaluable information regarding the spatial activity of key regulatory factors could be obtained from such preparations were it possible to measure interactions with specific DNA probes on this scale.

There are several prior studies in which capillary electrophoresis was applied to the study of DNA–protein complexes by means of a mobility-shift assay (CEMSA) (5, 6, *). By use of a laser-induced fluorescence detection system, we have developed rapid and quantitative procedures that permit accurate assessment of specific DNA–protein interactions on a scale more than 100-fold below the minimum usually necessary for EMSAs. This procedure can be used for a variety of purposes, quantitative and qualitative, including studies involving the effects of antibodies on DNA–protein complexes. The experiments we present were carried out with a sea urchin embryo transcription factor, SpP3A2, that had been cloned and extensively characterized in earlier studies (3, 4, 7). SpP3A2 is the initial member of a small family of transcription factors that now includes the *Drosophila* erect wing gene product and human nuclear respiratory factor 1 (NRF-1) (8).

MATERIALS AND METHODS

Proteins. Specific SpP3A2–DNA binding was assayed with purified recombinant protein (9) from a 300 nM stock solution (rSpP3A2), with nuclear extract of blastula-stage sea urchin (*Strongylocentrotus purpuratus*) embryos (3) or with cytoplasmic extracts of sea urchin eggs; see below.

Oligonucleotides. The probe we used in this study contains two adjacent SpP3A2 sites of differing affinity for SpP3A2 protein. The relative equilibrium constant (K_r) for this probe (i.e., K_{eq} for binding to the probe containing the two sites/K_{eq} for binding to any DNA sequence) is $\approx 10^6$. For probes containing single sites, K_r values for this factor range from 2 × 10^4 to 2 × 10^5 (4). In addition to the wild-type (wt) probes we also constructed a probe on which the strong site was destroyed by mutating its sequence. The wt 5′-GATCTTTTCGGCTTCTGCGCACACCCCACGCGCATGGGGC-3′ (sense) and mutated 5′-GATCTTTTCGGCTTCTGCGCACACCCCACATATATGGGC-3′ (sense) oligonucleotides (sense and antisense) were synthesized and labeled with the fluorescent dye 5′-carboxyfluorescein phosphoramidite (6-FAM) at the 5′ end, following the protocol provided by Applied Biosystems, in our Core Facility. After column purification, the labeled, single-stranded oligonucleotides were vacuum dried and stored in the dark at −20°C. Equimolar sense and antisense DNAs were annealed in 0.1 M NaCl at 93°C for 5 min and allowed to cool at room temperature overnight. Double-stranded DNA was purified from single-stranded DNA on a nondenaturing 8% polyacrylamide gel, from which the fluorescent bands were excised, electroeluted, purified on Sep-Pak C_{18} columns (Millipore), vacuum dried, and stored at −20°C. Prior to use, the probes were dissolved in 10 mM Tris·HCl, pH 7.9/10 mM KCl, and their concentrations were determined on a photon counting spectrofluorometer, SLM 8000TMC (SLM Aminco, Urbana, IL). Stock solutions of 60 nM wt 6-FAM-labeled DNA and 50 nM mutated 6-FAM-labeled DNA were used.

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Abbreviations: EMSA, electrophoretic gel mobility-shift assay; CEMSA, capillary electrophoresis mobility-shift assay; wt, wild-type; 6-FAM, 5′-carboxyfluorescein phosphoramidite.

*Maschke, H. E., Frenz, J., Williams, M. & Hancock, W. S., Fifth International Symposium on High Performance Capillary Electrophoresis, Jan. 25–28, 1993, Orlando, FL, Poster T121.

Antibodies. Two monoclonal antibodies (1 μM stock) were made against rSpP3A2: 7B12/1H7 inhibits the formation of SpP3A2–DNA complexes; 6F1/2E9 supershifts (shifts forming a slower trimolecular complex) this DNA–protein complex.

Gel Electrophoresis. For the conventional EMSA experiment, electrophoresis was carried out in an 8% polyacrylamide gel in 1× TBE buffer (89 mM Tris base/89 mM boric acid/2 mM EDTA, pH 8.3) for 2 hr at 200 V. The probes were labeled with ^{32}P by kinase reaction. The gel was dried, placed under Kodak XAR5 film, and exposed for 4 hr.

Capillary Electrophoresis. Separation was performed in a 50 μm × 37 cm long (30 cm effective length) neutral coated capillary (eCAP neutral, Beckman) with the P/ACE System 2000 (Beckman) upgraded with GOLD (version 8.1) software and laser-induced fluorescence (LIF) detector (488 nm excitation). The capillary was filled with 1× TBE buffer and 0.2% linear polyacrylamide (750,000–1,000,000 Da, from Polyscience). Electrophoresis was at reversed polarity—i.e., the anode at the detector end—at 18 kV and 18°C. Between each run, the capillary was rinsed with 1× TBE buffer for 2 min and then in running buffer for 5 min. Therefore, a complete run lasts 17 min. The sample was introduced by high-pressure injection (10-sec injection corresponds to ≈10 nl of sample) followed by a second injection of running buffer for 5 sec.

Reactions with Recombinant SpP3A2 Protein. The reaction mixture, including buffer C [20 mM Hepes, pH 7.9/40 mM KCl/0.1 mM EDTA/1 mM dithiothreitol/20% (vol/vol) glycerol], 5× binding buffer (100 mM Hepes, pH 7.9/375 mM KCl/25 mM $MgCl_2$/2.5 mM dithiothreitol), 6-FAM-labeled DNA, 300 fmol of total SpP3A2 recombinant protein (rSpP3A2), and poly(dA-dT)·poly(dA-dT) (Pharmacia) was incubated on ice for 10–30 min before injection. The total mass of rSpP3A2 overestimates the amount of the recombinant protein active in DNA binding, as only 1–2% renatures successfully after extraction from bacteria (10).

Reaction with Nuclear Extract. The reaction mixture was similar to that for recombinant protein, except for the use of 5 μg of 24-hr sea urchin nuclear extract and 60 fmol of probe together with 5 μg of poly(dA-dT).

Reaction of Extract from Multiple Eggs. A known number of fresh *S. purpuratus* eggs suspended in sea water were pipetted into a 1.7-ml microcentrifuge tube. After centrifuging at 13,000 rpm for 5 min in an Eppendorf centrifuge, excess sea water was discarded. Then 1 μl of buffer A [10 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM EGTA/1 mM spermidine tris(hydrochloride)/1 mM dithiothreitol] was added and the sample was stored at −70°C. Immediately before assay, the eggs were thawed at room temperature for 5 min to break the cell membrane, and

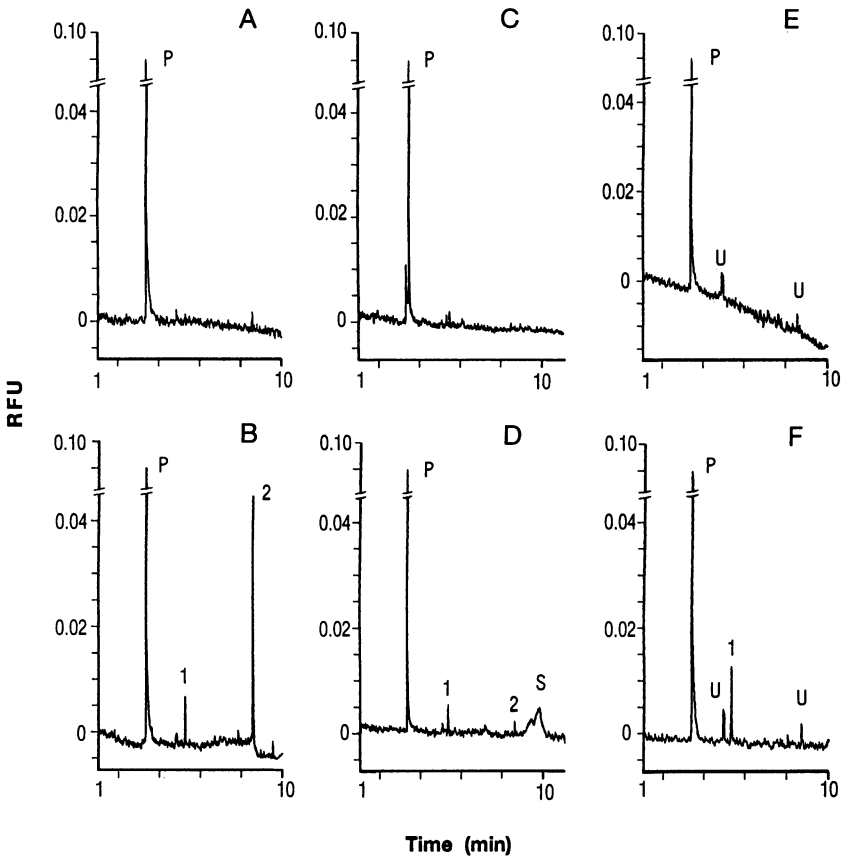

FIG. 1. Resolution of recombinant SpP3A2-DNA complexes by CEMSAs. The reaction mixtures contained the following: (*A*) 5 μl of buffer C, 2 μl of 5× binding buffer, 1 μl of wt DNA probe, 1 μl of poly(dA-dT), and 1 μl of water; (*B*) 5 μl of buffer C, 2 μl of 5× binding buffer, 1 μl of wt DNA probe, 1 μl of poly(dA-dT), and 1 μl of rSpP3A2; (*C*) 4 μl of buffer C, 2 μl of 5× binding buffer, 1 μl of wt DNA probe, 1 μl of poly(dA-dT), 1 μl of rSpP3A2, and 1 μl of antibody 7B12/1H7; (*D*) 4 μl of buffer C, 2 μl of 5× binding buffer, 1 μl of wt DNA probe, 1 μl of poly(dA-dT), 1 μl of rSpP3A2, and 1 μl of antibody 6F1/2E9; (*E*) 5 μl of buffer C, 2 μl of 5× binding buffer, 1 μl of mutated DNA probe, 1 μl of poly(dA-dT), and 1 μl of water; (*F*) 5 μl of buffer C, 2 μl of 5× binding buffer, 1 μl of mutated DNA probe, 1 μl of poly(dA-dT), and 1 μl of *rSpP*3A2. In the experiment of *B*, the probe (D_0) was present at 6×10^{-9} M; the bimolecular complexes of peak 1 (P_1D), at 1.45×10^{-10} M; the trimolecular complex of peak 2 (P_2D), at 1.35×10^{-9} M, and renatured rSpP3A2 protein (P), at 3×10^{-8} M. In the experiment of *F*, the bimolecular complex formed at the weak site was present at 2.85×10^{-10} M, and total probe and rSpP3A2 were as in the experiment in *B*. From the experiment of *F*, for the weak site $K_{WD} = 1.9 \times 10^{-7}$ M, given that for the nonspecific reaction of rSpP3A2 with poly(dA-dT) present, $K_D = 1.8 \times 10^{-3}$ M (4) [about 20% of the protein is engaged in the complex with the poly(dA-dT)]. From $Y = (K_WP + K_SP + K_WK_SP^2)/(1 + K_WP + K_SP + K_WK_SP^2)$, where $Y = (P_1D + P_2D)/D_0$, $K_W = K_{WD}^{-1}$, and $K_S = K_{SD}^{-1}$, $K_{SD} = 3.9 \times 10^{-8}$ M. RFU, relative fluorescence unit; P, free probe peak; U, background impurity from the probe; peaks 1 and 2, 1:1 and 2:1 protein–DNA complexes; S, DNA–protein–antibody complex.

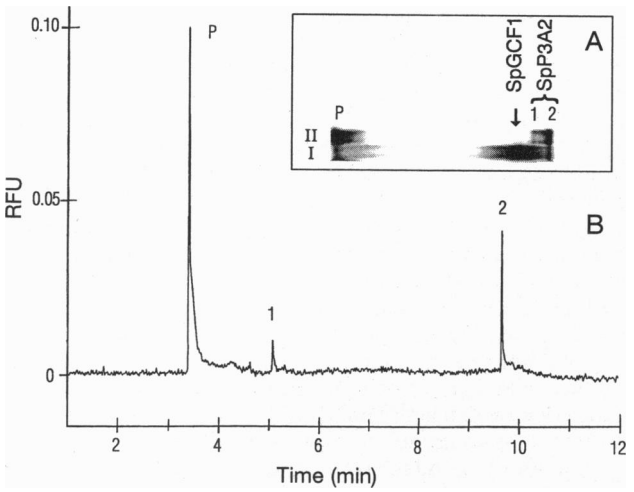

FIG. 2. Identification of SpP3A2–DNA complexes in nuclear extract by using conventional and capillary electrophoretic separation procedures. Separation conditions for the CEMSA experiment are as in Fig. 1. (*A*) Conventional EMSA. Lane I, 10 μg of nuclear extract of 24-hr sea urchin embryos allowed to react with 40 fmol of wt probe together with 5 μg of poly(dA-dT); lane II, 300 fmol of recombinant SpP3A2 protein allowed to react with 40 fmol of wt probe with 0.5 μg of poly(dA-dT). (*B*) CEMSA of 5 ng of 24-hr embryo nuclear extract, allowed to react with 60 amol of wt probe, together with 5 ng of poly(dA-dT). RFU, relative fluorescence unit; P, free probe peak; 1 and 2, 1:1 and 2:1 DNA–protein complexes of SpP3A2; DNA–protein complexes of SpGCF1 are indicated by arrow.

the reaction mixture was added. For the experiments illustrated in Fig. 3 *A* and *B*, the contents of 1000 sea urchin eggs were released into a total of 10 μl of reaction mixture, containing 3 μl of buffer C, 2 μL of 5× binding buffer, 1 μl of buffer A, 1000 sea urchin eggs, 1 μl of wt probe (about 60 fmol), 1 μl (5 μg) of poly(dA-dT), and 1 μl of distilled water. The reaction mixture was incubated for 30 min on ice.

Reaction with a Single Egg. A single sea urchin egg was placed in a drop of a 3:1 mixture of filtered sea water and buffer C. After removing the cartridge from the machine, the egg was introduced by capillarity at the inlet, under the microscope, by manually closing/opening the outlet of the capillary, and held at the inlet, as the diameter of the egg, about 80 μm, is larger than the internal diameter of the capillary, 50 μm. After the cartridge had been placed back into the machine, the egg contents were taken up into the tube by a 10-sec high-pressure injection of the reaction mixture, including 3 μl of buffer C, 2 μl of 5× binding buffer, 4 μl of wt probe (about 0.24 pmol), and 1 μl containing 5 μg of poly(dA-dT), and the mixture was allowed to react with the DNA probe at the inlet end for 10 min at room temperature. After a second injection of buffer solution—i.e., 1× TBE with 2% linear polyacrylamide polymer (750,000–1,000,000 Da)—to ensure that no sample was lost during the reaction, electrophoresis was commenced at 18 kV.

RESULTS AND DISCUSSION

CEMSA with Recombinant SpP3A2. Fig. 1 shows various experiments carried out with wt and mutant probes that had been allowed to react with renatured recombinant SpP3A2 protein preparations, in the presence of a large excess of poly(dA-dT) (see *Materials and Methods* and Fig. 1 legend for details). Migration of the probe alone is shown in Fig. 1*A*. The bimolecular complex formed at one of the two sites (peak 1) and the trimolecular complex formed when both sites are occupied (peak 2) are shown in Fig. 1*B*. With the field reversed (anode at detection end) the free DNA migrates faster than the DNA–protein complex, as in conventional EMSA. The mobility of the DNA–protein complexes depends directly on their charge-to-mass ratio. The separation achieved was completed in this case within 10 min (see abscissa). The samples were loaded in 5–10 nl, and in Fig. 1*A* the peak shown represents only about 45 amol of probe. We utilized these data to calculate an equilibrium dissociation constant (K_D) for the weak and strong sites on the probe—namely, 1.9×10^{-7} M and 3.9×10^{-8} M, respectively. These values are within the range of equilibrium constants for the reaction of SpP3A2 with single sites found in various sea urchin genes, as described earlier (4).

Fig. 1*C* shows that when the reaction mixture includes a monoclonal antibody against SpP3A2 protein which specifically prevents DNA–protein complex formation, peaks 1 and 2 are absent. Fig. 1*D* shows that a different monoclonal antibody, that does not prevent, but rather supershifts SpP3A2-DNA

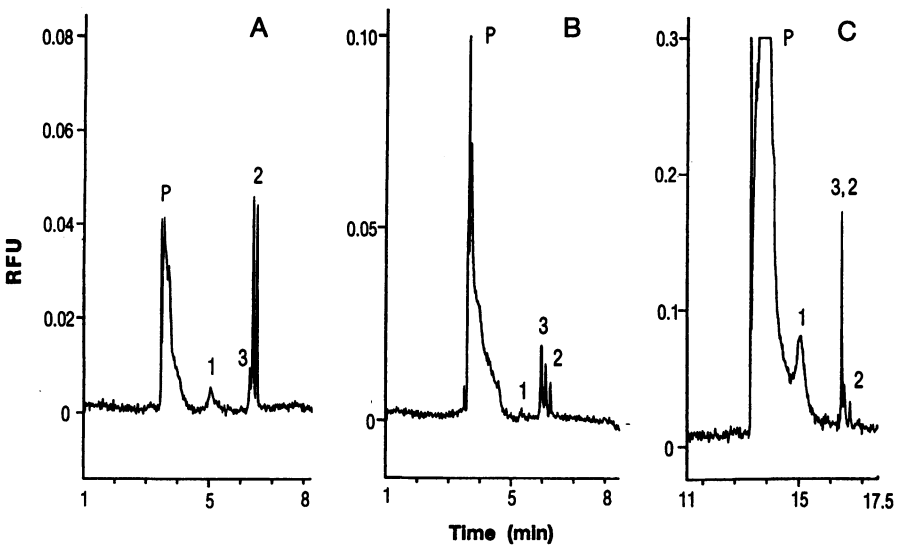

FIG. 3. Complexes formed with sea urchin egg cytoplasm, detected by CEMSA. The CEMSA separation conditions were as in Fig. 1. (*A*) Capillary electropherogram of sea urchin egg cytoplasm with wt probe. (*B*) Complexes formed in the presence of the monoclonal antibody 7B12/1H7, which inhibits the formation of SpP3A2–DNA complexes. (*C*) CEMSA carried out on the contents of a single sea urchin egg. The mobilities of the complexes are greater in this experiment than with nuclear extract and pure rSPP3A2; we believe this is a consequence of change in the overall ion content in the sample containing lysed cell contents. RFU, relative fluorescence unit; P, free probe peak; 1 and 2, 1:1 and 2:1 DNA–protein complex of wt probe–SpP3A2; 3, DNA–protein complex of wt probe–SpP3A1.

complexes, generates a broad, more slowly moving peak (S), at the expense of peaks 1 and 2 (compare Fig. 1*B*). Fig. 1*E* shows the mutated probe alone, which contains some minor contaminants labeled U, and Fig. 1*F* displays the reaction of this probe with SpP3A2 protein. As expected, only the bimolecular complex is formed (peak 1). In addition (not shown) we carried out competition experiments, which demonstrated that addition of excess unlabeled wt probe in the presence of poly(dA-dT) quantitatively abolishes the fluorescent peaks 1 and 2.

CEMSA with Nuclear Extract. The superior resolution available in the CEMSA system is illustrated in Fig. 2. These experiments were carried out with unfractionated nuclear extract. SpP3A2 is present in 24-hr embryo nuclei at about 10^3 molecules per nucleus (9). The extract was allowed to react with the wt probe, and the complexes were analyzed by conventional EMSA and by CEMSA. Both methods revealed the same DNA–protein complexes, but the conventional method took over 6 hr, as opposed to 12 min, and in this case consumed about 1000 times more sample than did the CEMSA. Conventional EMSA does not resolve the SpP3A2 complexes clearly because of the presence in 24-hr embryo extract of another DNA-binding factor, SpGCF1, which is relatively prevalent, and which interacts weakly at a CCCC site on the wt probe that we used (10). SpGCF1 complexes account for the broad set of bands that extend below the SpP3A2 complexes in the EMSA shown in lane I of Fig. 2*A* (compare the complexes formed with recombinant SpP3A2 in lane II). In the absence of the EMSA "caging effect" the SpGCF1 complexes do not survive in the CEMSA experiment with nuclear extract shown in Fig. 2*B*. However, the CEMSA in Fig. 2*B* clearly reveals the same two SpP3A2 complexes seen in Fig. 1*B* (peaks 1 and 2). There is a small difference in the retention times of the complex peaks formed in the nuclear extract reaction, as shown in Fig. 2*B*, compared to those of the recombinant protein–DNA complexes shown in Fig. 1*B*. This is not surprising, given the high protein content of the nuclear extract present in the capillary. Note that in the CEMSA the bimolecular and the trimolecular complexes are widely separated, while in this particular EMSA system they are much more difficult to separate. The CEMSA peaks obtained in the nuclear extract are also efficiently eliminated by excess unlabeled wt probe (not shown), and as in Fig. 1*C*, the inhibiting monoclonal antibody again eliminates both complex peaks when added to the nuclear extract (not shown).

CEMSA with Cytoplasm from Multiple Eggs. SpP3A2 is also present in unfertilized egg cytoplasm, at about 2×10^6 molecules per egg (9). The egg contains in addition a second factor that interacts with the same target site as does SpP3A2—namely, a zinc finger protein, SpZ2-1 (formerly denoted P3A1; this protein is also present in 24-hr embryo nuclear extract, but only at very low concentration; ref. 11). Fig. 3*A* depicts a CEMSA of the complexes formed with a crude egg cytoplasmic extract. An extract of 1000 *S. purpuratus* eggs in 10 μl of medium was allowed to react with the wt probe, and 10 nl, or the amount equivalent to one egg, was injected into the apparatus. The results were somewhat different from those obtained with nuclear extract or recombinant protein. First, the free probe and the bimolecular DNA–protein complex peaks became broader, which we think is probably due to the presence of nucleic acids and cell membrane fragments in the crude extract. Second, a triple peak migrated out at 7 min, which is different from the single 2:1 protein–DNA complex peak shown in Figs. 1*B* and 2*B* (i.e., peak 2 in these figures). All three peaks of the triplet can be eliminated by excess wt probe DNA in the presence of the nonspecific poly(dA-dT) competitor, demonstrating that they are specific DNA–protein complexes. In Fig. 3*B* the complexes formed in the presence of the inhibitory anti-SpP3A2 monoclonal antibody (7B12/1H7) are shown. Addition of this antibody severely depressed the formation of the two peaks labeled 2, while the free probe correspondingly increased. However, peak 3 was not altered (compare Fig. 3*A*). Peak 3 is probably due to the SpZ2-1 transcription factor, while the twin peaks (2) are due to SpP3A2. However, we do not know why two closely migrating SpP3A2 peaks, rather than one peak, form with the egg cytoplasmic extract. This could of course be of biological interest if it results from a covalent difference in a maternal fraction of the P3A2 factor (12).

Table 1. Sensitivity of CEMSA

Method	Sample	Molecules of DNA probe used per run	Molecules of SpP3A2 detected	Number of *S. purpuratus* egg equivalents	Data source
EMSA	Recombinant SpP3A2	$\approx 10^{10}$	$\approx 5 \times 10^9$	2300	Fig. 2*A*
CEMSA	Recombinant SpP3A2	3.6×10^7	1.68×10^7	9	Fig. 1*B*
	Nuclear extract	3.6×10^7	1.1×10^7	9	Fig. 2*B*
	Egg cytoplasm extract	3.6×10^7	2×10^6	1	Fig. 3*A*
	Single egg	1.4×10^8	2×10^6	1	Fig. 3*C*

CEMSA from a Single Cell. Having achieved a detection capability sufficient for assay of SpP3A2 activity from the *equivalent* of one egg, we undertook to measure complexes formed by the molecules present within a single egg that binds this probe specifically. The egg was lysed at the mouth of the electrophoresis capillary by the external pressure applied when the sample is injected, and the contents of the egg were mixed within the capillary with the probe and other constituents. Fig. 3*C* illustrates the result, which was reproducibly obtained. The retention times for all the peaks are similar to those observed in the 1000-egg extract, except that the SpZ2-1 and SpP3A2 peaks (i.e., peaks 2 and 3) comigrated as one peak. In multiple analyses, the only difference observed was that the quantity of SpP3A2 was slightly different from egg to egg.

Quantitation. Since the binding of protein with the DNA probe does not affect the quantum yield of the fluorescent dye in this assay, it is possible to obtain the DNA and protein quantities in the CEMSA directly from the peak areas, and these can be used for any quantitative measurement that can be carried out with EMSA. Table 1 summarizes the salient quantitative aspects of these experiments. The capillary electrophoresis system with laser-induced fluorescence is at least 100 times more sensitive than conventional EMSA utilizing radioactive probes. As Fig. 3*C* shows dramatically, we can now see DNA–protein interactions in a single egg cell. In general no more than about 10^6 molecules of a given transcription factor should be required for quantitative measurement by CEMSA, so long as the relative equilibrium constant for the reaction of a factor with its DNA target site is not greatly lower than that of SpP3A2. That is, since reactions at the weak SpP3A2 site alone can be detected (Fig. 1*F*), we should expect that factors displaying a $K_D \leq 4.7 \times 10^{-7}$ M, a relatively modest value, or $K_r \geq 2 \times 10^4$ M are detectable by CEMSA. Furthermore, the limits of detection of this method are well below the actual quantities listed in Table 1. This means that such assays can now be performed comfortably on extracts from 10^2–10^3 ordinary somatic cells. Perhaps surprisingly, even very crude extracts such as the whole lysed eggs used in the experiments of Fig. 3 work well. We expect that this method will enable many new experimental explorations of transcription factor activity, in specific cell types or embryonic regions that can be separated out only by hand dissection, or in cell populations separated by fluorescence-activated cell sorting.

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