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Identification and Characterization of the Egr-1 Gene Product, a DNA-Binding Zinc Finger Protein Induced by Differentiation and Growth Signals

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Egr-1 is an immediate-early response gene induced by diverse signals that initiate growth and differentiation. Its cDNA sequence predicts a protein with zinc fingers. We have generated an antiserum to the Egr-1 gene product and identified it as an 80-kilodalton short-lived protein in serum-stimulated mouse fibroblasts. The rat Egr-1 product has also been identified in nerve growth factor-induced PC12 cells. In addition, we show by cell fractionation and immunocytochemistry that the Egr-1 protein is located in the nucleus. We also demonstrate that it is phosphorylated. In vitro-generated Egr-1 protein binds with high affinity to the sequence CGCCCCCGC in a zinc-dependent manner.

The characterization of genes induced by mitogenic signals without a requirement for de novo protein synthesis is a rapidly growing field of research. A subset of these immediate-early response genes encode transcription factors. This list currently includes c-*fos* (12, 19), *fra-1* (6), and *fosB* (39); c-*jun* (20, 31, 32) and *jun-B* (31); Egr-1 (34) (also known as NGFI-A [24]), Krox 24 [22], zif268 [4], and TIS 8 [23]), Egr-2/Krox-20 (3, 17), Egr-3, and Egr-4 (unpublished data); nur 77/NGFI-B (15, 25), and SRF (27). Strikingly, several of these gene products are also induced by signals that initiate differentiation in a variety of model systems, as well as by other alterations in the extracellular environment. Collectively, these data suggest a broad role for these proteins as nuclear intermediates in signal transduction processes.

The deduced amino acid sequence for Egr-1 contains a domain of three tandem repeats (34), each representing an excellent fit to the C_2H_2 type of zinc finger, first noted for TFIIIA (1). Other members of the Egr gene family (e.g., Egr-2, Egr-3, and Egr-4) have finger domains similar to that of Egr-1 but differ in sequence outside the finger regions (17, 34; unpublished data). However, none of the protein products corresponding to this gene family have as yet been identified. To date, Sp1 (16) is the only mammalian C_2H_2 zinc finger structure that has been characterized at the protein level. We report here the generation and characterization of a rabbit antiserum directed against an Egr-1 fusion protein which specifically recognizes the Egr-1 gene product. This antiserum was used to study the kinetics of Egr-1 protein turnover in murine fibroblasts and to show that it is a nuclear phosphoprotein. The rat homolog of Egr-1 has also been identified in PC12 cells. Finally, we show that Egr-1 requires zinc to bind to its target sequence.

MATERIALS AND METHODS

In vitro translation. A 2.1-kilobase (kb) *Apa*I fragment (nucleotides 120 to 2,224 in the Egr-1 cDNA [34]) which includes the entire coding region was blunt-ended and subcloned into the *Sma*I site of Bluescript KS M13(+) (Stratagene, La Jolla, Calif.). This plasmid was called pBS.ApaI. In vitro transcripts were generated by using bacteriophage T3 RNA polymerase, and 1.5 μg of RNA template was translated in a rabbit reticulocyte lysate (25 μl) (Stratagene) in the presence or absence of [^{35}S]methionine. Egr-2 transcripts were generated similarly by using the Egr-2 cDNA clone designated Zap8 (17). The translation products were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The gel was dried and autoradiographed.

Egr-1 expression vectors. Plasmid pMTEA contains a mouse metallothionein-I promoter (10) driving the Egr-1 gene and utilizes a polyadenylation site from the human growth hormone gene (X. Cao, Ph.D. thesis, University of Chicago, 1989). NIH 3T3 cells (10^6) were transfected with 5 μg of test plasmids, 0.5 μg of pSV2-neo (33), and 14.5 μg of carrier DNA (pUC19) by a calcium phosphate transfection procedure (11). G418 (400 μg/ml) was added to cells 48 h after transfection, and resistant colonies were harvested after 2 weeks. Egr-1 expression in a pool of cells transfected by pMTEA and rendered quiescent for 40 h in 0.5% fetal calf serum (FCS) was induced by adding 50 μM zinc acetate 15 h before harvesting for immunoblot analysis.

Preparation of antisera. Egr-1 *Escherichia coli* expression plasmids were constructed from the pCFM vector (2), which possesses a synthetic lambda phage promoter, a ribosome-binding site, a cloning cluster, translation stops in all three amino acid (aa) reading frames, and a transcription stop sequence. The bovine growth hormone (bGH)-Egr-1 fusion protein was expressed in the plasmid V_4, which was constructed by fusing the 192-aa-encoding bGH gene (8) to the 5′ end of the 532-aa-encoding desmet (without the methionine) Egr-1 gene. The pCFM vector was cut with *Xba*I and *Bam*HI, and the following DNA fragments were inserted: a 591-bp recombinant bGH DNA fragment (21) optimized for *E. coli* protein expression (*Xba*I to *Bsm*I), a 46-bp synthetic oligodeoxynucleotide linker (*Bsm*I to *Pst*I), and a 1,655-bp Egr-1 DNA fragment (*Pst*I to *Bgl*II) derived from OC3.1 (34).

E. coli cells containing the bGH-Egr-1 V_4 expression

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vector were grown to an OD_{600} of 20 at 30°C in fed-batch fermentations. Synthesis of the recombinant fusion proteins was induced by shifting the fermentation temperature to 42°C and continuing growth to an OD_{600} of 45. Cells were harvested by centrifugation, washed with buffer A (50 mM Tris chloride [pH 8.0], 2 mM EDTA, 10 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg of pepstatin A per ml, 0.5 μg of leupeptin per ml) and lysed in buffer A with two passes in a model 110Y microfluidizer (Microfluidics, Inc.) at 14,000 lb/in^2. Insoluble inclusion bodies were collected by centrifugation. Recombinant fusion proteins were solubilized with buffer A containing 8.5 M urea and 50 mM DTT. Buffer A was then added to reduce the urea concentration to 5 M, and insoluble material was removed by centrifugation. The soluble fusion proteins were bound to a CM-Sepharose Fast Flow column and eluted with a 0 to 300 mM NaCl gradient made up in buffer A. Fractions containing the V_4 recombinant protein were pooled and concentrated with an Amicon YM30. Guanidine hydrochloride and DTT were added to concentrations of 4 M and 50 mM, respectively. Sephacryl S-200 HR gel filtration column chromatography was performed on the denatured concentrates in buffer B (20 mM Tris chloride [pH 8.0], 1 mM EDTA, 10 mM DTT, 5 M urea). Then 5 mM zinc acetate was added to pooled V_4 S-200 column fractions. The V_4 protein was dialyzed overnight against 10 volumes of 20 mM Tris chloride pH 8.0, and then against several changes of 20 mM sodium phosphate, pH 7.5. The identity of the purified protein was confirmed by N-terminal amino acid sequence analyses with an Applied Biosystems model 477A protein sequencer with an on-line model 120A phenylthiohydantoin (PTH) analyzer. The recombinant fusion proteins and their degradation products constituted 80 to 90% of the total protein in the pooled S-200 fractions, as determined by N-terminal sequence analyses and SDS-PAGE.

To generate rabbit antiserum against V_4, 50 μg of purified protein was emulsified in complete Freund adjuvant and injected at multiple intradermal sites on the shaved back of a rabbit (R-5232). After 6 weeks, an equal quantity of V_4 (without adjuvant) was injected into a peripheral ear vein. The rabbit was bled 1 week later, and the resultant serum was designated R5232-2.

This antiserum was affinity purified as follows. A 700-μg amount of bGH/Egr-1 V_4 fusion protein was mixed with 7 ml of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif.) in 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-sodium (pH 7.5) solution by rotating overnight. The mixture was spun at 2,500 rpm for 5 min, and the supernatant was removed. An equal volume of 0.1 M Tris hydrochloride (pH 7.5 to 8.0) was added to the beads and rotated for 3 h for blocking the unbound sites. The beads were transferred to a column, washed with phosphate-buffered saline (PBS), preeluted with elution buffer (8 M urea in PBS), and washed with PBS again. Then, 15 ml of rabbit antiserum was poured into the column and rotated at 4°C overnight. The serum was drained and saved as the flowthrough fraction. The beads were then washed with PBS. Antibody was eluted with 8 M urea in PBS and collected in 1-ml portions. The protein concentration in each tube was measured at OD_{280}, portions were pooled and dialyzed against ddH_2O, and the final volume was adjusted to 15 ml.

Immunoblotting. PC12 cells were obtained from C. Palfrey (University of Chicago) and maintained in 5% horse serum and 10% FCS. Nerve growth factor (NGF; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), epidermal growth factor (EGF; Biomedical Technologies Inc., Stoughton, Mass.), and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (PMA) (Sigma Chemical Co., St. Louis, Mo.) were added to induce Egr-1 expression. For the serum induction experiments, NIH 3T3 cells were first maintained in 0.75% FCS for 40 h. The medium was then changed to 20% FCS for various times.

Cells were scraped, pelleted, and lysed by addition of an equal volume of RIPA buffer containing protease inhibitors (0.15 M NaCl, 50 mM Tris hydrochloride [pH 7.2], 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS) with 1 mM PMSF, 2 μg of pepstatin per ml, and 2 μg of leupeptin per ml. Protein loading buffer was added, the sample was boiled for 5 min, sheared five times through a 23-gauge needle, and centrifuged for 3 min in a microfuge, and the supernatant was loaded onto a 7.5% polyacrylamide–SDS gel. Typically about 10^6 cells, i.e., 10% of the sample obtained from a T75 flask, were used. Proteins were transferred to a PVDF membrane (Millipore Corp.) in the buffer described by Towbin et al. (36) as modified by Rott and Nelson (30). The membrane was agitated overnight at room temperature with R5232-2 antiserum diluted 1:1,000 in PBS au lait (14). After being washed with PBS au lait solution, the membrane was incubated with 1 μCi of ^{125}I-protein A for 1 h. The membrane was then washed twice with PBS au lait, dried, and exposed to X-ray film for 1 to 3 days.

Immunocytochemistry. BALB/c 3T3 cells were grown in Lab-Tek (Naperville, Ill.) chamber slides, rendered quiescent, and stimulated as described above. Cells were fixed in 4% formaldehyde–PBS (10 min, room temperature), permeabilized with acetone (7 min, −20°C), and incubated with antiserum (1:500 dilution of R5232-2 and flowthrough and 1:250 of affinity purified) for 18 h at 37°C. Reactions with biotinylated anti-rabbit immunoglobulin antibody, avidin-biotin-peroxidase complexes, and visualization with diaminobenzidene-H_2O_2 were done as described in the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, Calif.).

Cell labeling and immunoprecipitation. One million BALB/c 3T3 cells in a 100-mm dish were rendered quiescent in 0.75% FCS for 45 h. Cells were changed to methionine-free, serum-free medium for 1 h. Then, dialyzed 20% FCS was added for 30 min, followed by the addition of 0.5 mCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml for another 30 min. For the $^{32}P_i$ labeling experiments, the medium of the quiescent cells was changed to phosphate-free, 0.75% dialyzed FCS for 3 h. The medium was replaced by the same medium for an additional 1 h. Then, cells were incubated in the same medium with 0.4 mCi of $^{32}P_i$ for 4 h. To effect serum stimulation, dialyzed FCS was added to a final concentration of 20%. At various times, cells were washed with ice-cold PBS and lysed in 4 ml of RIPA buffer. The lysate was spun at 30,000 rpm in an SW41 rotor (Beckman Instruments) for 30 min. Rabbit antiserum (R5232-2) (4 μl) was added and incubated overnight at 4°C. Glutaraldehyde-fixed *Staphylococcus aureus* (Cowan I strain) was added and incubated for 2 h. The pellets were washed with RIPA buffer once, with 1 M urea in RIPA buffer twice, in a high-salt RIPA buffer (1 M NaCl) twice, and finally in RIPA buffer once more. Pellets were finally resuspended in loading buffer and boiled for 3 min, and the supernatants were analyzed on a 7.5% polyacrylamide–SDS gel. The gel was dried, treated with En^{3}Hance solution (Du Pont), and autoradiographed.

Cell fractionation. Subcellular fractions were obtained by the method of Ramsey et al. (29). Briefly, cells were suspended in hypotonic buffer (25 mM Tris hydrochloride [pH

7.4], 1 mM $MgCl_2$, 5 mM KCl, 1 mM PMSF). After incubation on ice for 5 min, an equal volume of hypotonic buffer containing 1% Nonidet P-40 (NP40) was added. Cell lysis was assessed by light microscopy. After an additional 5 min on ice, nuclei were pelleted at 1,000 × *g* for 5 min. The nuclei were washed twice with hypotonic buffer containing 0.5% NP40 and then suspended in lysis buffer (25 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 0.5% NP40, 0.5% sodium deoxycholate, 0.2% SDS). Both nuclear and cytoplasmic fractions were then clarified by centrifugation at 12,000 × *g* for 30 min at 4°C.

Gel retardation assay. Probes for the binding assay were prepared by annealing complementary synthetic oligonucleotides of Egr-1 5′-flanking sequence, end-labeling *Bgl*II or *Xba*I overhangs with [α-^{32}P]dCTP and Klenow fragment, and purification from a 20% acrylamide gel. Two 80-mers, containing nucleotides −695 to −616 or nucleotides −615 to −536 (37) and the oligomer 5′-CGCCCT CGCCCCCGC GCCGGG-3′ (centered at −594), designated EBS-1, were annealed to their respective complementary oligomers as follows. A 60-μg amount of each pair of oligonucleotides was mixed in a total volume of 3 ml, heated to 68°C for 30 min, cooled slowly at room temperature for 1 h, and ethanol precipitated. Three cold competitors, 5′-GTTGGG GCGG GGGCA AGCTGG-3′ (centered at −279), 5′-AGGTGG GCGGGTGAG CCCAGG-3′ (centered at −643), and an unrelated oligonucleotide, 5′-GAATTC GGTACC GCGGCC GCATCG ATGATA TC-3′, were similarly prepared. Egr-1 protein was obtained by translation in a rabbit reticulocyte lysate of capped message from the pBS.ApaI template.

Binding reaction mixes for the gel retardation assay were set up in 10 μl by incubating 0.5 to 2.0 ng of end-labeled probe (10,000 to 35,000 cpm) and 1 μg of poly(dI-dC)-poly(dI-dC) with specified amounts of Egr-1 programmed lysate in a buffer containing 20 mM HEPES (pH 7.5), 70 mM KCl, 5 mM $MgCl_2$, 0.05% NP40, 12% glycerol, 1 mg of bovine serum albumin per ml, 0.5 mM DTT, and 100 μM $ZnCl_2$ (when indicated) for 30 min at room temperature. Each reaction mix contains a total of 3 μl of lysate, consisting of the specified amount of Egr-1 programmed lysate and the remainder being unprogrammed lysate. The complexes were electrophoresed on a 4% polyacrylamide gel (37:1) in 0.5× TBE (1× TBE is 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA) buffer at 200 V at room temperature. For binding assays with the anti-Egr-1 antibody, Egr-1 programmed lysate and probe were incubated for 30 min as described above; subsequently, the indicated dilution of R5232-2 was added for an additional 30 min of incubation at room temperature. For competition experiments, the specified amount of cold oligonucleotide competitor was added to the Egr-1 lysate concurrent with the addition of labeled DNA. For chelation experiments, various concentrations of the indicated chelator were added to the Egr-1 programmed lysate at room temperature 10 min before the binding reaction was begun. Five minutes before the start of the binding reaction, specified concentrations of $ZnCl_2$ or other metal ions were added to the lysate. Finally, probe, poly(dI-dC), and binding buffer (without $ZnCl_2$) were added to the chelated lysate in a total volume of 20 μl, and the reaction mix was treated as described above.

RESULTS

Identification of the Egr-1 gene product in mouse fibroblasts. To characterize the Egr-1 gene product in vivo, we generated a rabbit antiserum directed against a bGH-Egr-1 fusion protein made in *E. coli*. First, we showed that this antiserum reacted with the Egr-1-bGH fusion protein and its degradation products (Fig. 1A, lane 2). Next, we used plasmid pBS.ApaI, which contains the Egr-1 cDNA, to generate an RNA transcript for subsequent translation in a rabbit reticulocyte lysate. The [^{35}S]methionine-labeled product migrated at 80 kilodaltons (kDa) (data not shown) (5). Unlabeled material was used for Western immunoblotting. As shown in Fig. 1B, lane 7, the R5232-2 antiserum reacted predominantly with an 80-kDa band. Faster-migrating bands (probably due to premature termination, alternative usage of start codons, or degradation) were also seen in the ^{35}S-labeled material. These results indicated that the R5232-2 antiserum recognized the Egr-1 protein.

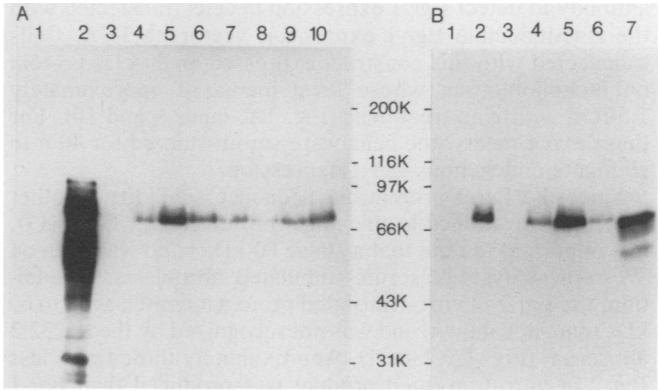

FIG. 1. (A) Immunoblot analysis of time course of Egr-1 protein induction by serum. BALB/c 3T3 cells were serum-deprived for 48 h and then induced by 20% FCS for various times. Cells were harvested and lysed as described in Materials and Methods. Extracts from about 10^6 cells were loaded onto a 7.5% polyacrylamide–SDS gel and transferred to a PVDF membrane. The membrane was incubated with R5232-2 antiserum (1:1,000 dilution) and counterdecorated with ^{125}I-protein A. Lane 1, R5232-2 antiserum reacted with 10 μl of in vitro-translated Egr-2; lane 2, 100 ng of bGH-Egr-1 fusion protein V_4; lane 3, uninduced cells; lanes 4, 5, 6, 7, and 8 represent 0.5, 1, 2, 5, and 8 h of serum induction, respectively; lane 9, R5232-2 antiserum reacted with cells transfected with pMTEA without Zn^{2+}; lane 10, cells transfected with pMTEA induced by 50 μM of Zn^{2+} for 15 h. (B) Immunoblot of rat PC12 cells induced by different agents and detection of in vitro-translated mouse Egr-1 protein. Lane 1, Uninduced BALB/c 3T3 cells; lane 2, 1-h-serum-induced BALB/c 3T3 cells; lane 3, unstimulated PC12 cells; lane 4, PC12 cells stimulated by NGF (100 ng/ml) for 2 h; lane 5, PC12 cells stimulated by PMA (100 ng/ml) for 2 h; lane 6, PC12 cells stimulated by EGF (100 ng/ml) for 2 h; lane 7, R5232-2 antiserum reacted with 10 μl of in vitro-translated Egr-1.

Next, this antiserum was used to identify the Egr-1 protein in vivo. In quiescent mouse fibroblasts, a very faint band was detected at 80 kDa (visible after prolonged exposure). The intensity of this signal increased rapidly following serum stimulation, reached a peak at 1 h, and then gradually decreased to very low levels by 8 h (Fig. 1A, lanes 3 to 8). The size of this band was in agreement with that of the in vitro-translated Egr-1 gene product (Fig. 1B, lanes 2 and 7). Nonimmune rabbit serum did not react with any cellular protein or the V_4 fusion protein in a duplicate blot exposed twice as long as the blot in Fig. 1A (data not shown). Affinity-purified antiserum gave the same results as those seen with R5232-2, whereas the flowthrough fraction showed no reactivity with any cellular protein (data not shown).

To provide definitive evidence that the 80-kDa band corresponds to the Egr-1 protein, we used the R5232-2

antibody to detect Egr-1 expression in cells transfected with the zinc-inducible Egr-1 expression vector pMTEA. Cells transfected with this construct expressed an 80-kDa protein on immunoblotting whose level increased approximately 1.5-fold with zinc treatment (Fig. 1A, lanes 9 and 10). For these experiments, the cells were serum-starved for 40 h to eliminate endogenous Egr-1 expression.

Since the Egr-1 protein has sequence similarity to other growth factor-inducible zinc finger proteins, e.g., Egr-2 (17), it is important to note that a single 80-kDa band was seen on Western analysis of serum-stimulated fibroblasts. In addition, the Egr-2 in vitro-translated protein migrated at 55 to 60 kDa (data not shown) and was not recognized by the R5232-2 antiserum (Fig. 1A, lane 1). Approximately three times less Egr-2 in vitro-translated product was produced than Egr-1 protein. However, a 2-week exposure of Fig. 1A showed no detectable band in lane 1. These results establish the specificity of the R5232-2 antiserum.

Next we asked whether the R5232-2 antiserum could detect the Egr-1 protein by immunoprecipitation (see Fig. 3A). BALB/c 3T3 cells were labeled with [^{35}S]methionine for 30 min in uninduced or serum-induced conditions. The labeled cell lysate was incubated with the R5232-2 rabbit antiserum, and the immune complex was precipitated by glutaraldehyde-fixed *S. aureus*. An 80-kDa protein in the lysate from serum-induced cells was immunoprecipitated by the R5232-2 rabbit antiserum but not by nonimmune serum. Under the wash conditions used (1 M urea followed by 1 M NaCl), only this prominent diffuse band was seen.

Egr-1 is expressed in PMA-, NGF-, and EGF-stimulated PC12 cells. The PC12 cell line, derived from a rat pheochromocytoma, is a model system for neuronal differentiation. It responds to NGF by extending neurites and increasing the transcription of several genes, including Egr-1 (24, 34). We have shown previously that several other stimuli also induce Egr-1 mRNA in PC12 cells (17). Thus, we were interested in examining whether the R5232-2 antiserum could detect the Egr-1 gene product in PC12 cells and whether the protein was the same size as in murine fibroblasts. PC12 cells were induced by PMA, NGF, or EGF. The cell lysates were analyzed by Western blotting (Fig. 1B, lanes 3 to 6). An 80-kDa polypeptide comigrating with mouse Egr-1 (Fig. 1B, lane 2) reacted with the R5232-2 antiserum in all cases but was undetectable in unstimulated rat PC12 cells.

Egr-1 is a nuclear protein. Two approaches were used to determine the subcellular location of the Egr-1 protein. One method used indirect immunoperoxidase staining of mouse BALB/c 3T3 cells (Fig. 2A). A 1:250 dilution of the affinity-purified R5232-2 antiserum showed striking nuclear staining in serum-induced cells. The pattern was homogeneous with the exception of several punctate areas in each nucleus that showed decreased staining. Most uninduced cells were negative, although a few cells did show some nuclear staining, probably because not all cells were completely quiescent under low-serum conditions. The nuclear staining increased dramatically within 30 min, reached a peak at 1 to 2 h, and then decreased gradually over the next 4 h. The time course seen by immunostaining was similar to that detected by Western blotting. Similar results were noted with the crude antiserum (R5232-2) (Fig. 2B). The flowthrough fraction served as a negative control and showed no inducible nuclear staining (Fig. 2B).

As an independent means of determining the subcellular location of Egr-1, we fractionated NIH 3T3 cells into nuclear and cytoplasmic components and reacted these with R5232-2 antiserum in immunoblots (Fig. 2C). The 80-kDa protein was observed in the serum-induced nuclear portion, with stronger intensity than in serum-stimulated total-cell lysates. A similar band was barely observable in the serum-induced cytoplasmic portion of the same blot. These findings indicate that the Egr-1 protein is localized to the nucleus shortly after synthesis and remains in the nucleus thereafter.

Posttranslational modification. Phosphorylation plays an important role in regulating protein activity. We therefore examined whether the Egr-1 protein underwent phosphorylation. The uninduced BALB/c 3T3 cells were labeled by $^{32}P_i$ for 3 h and then induced with 20% serum for 1 or 3 h. The cell lysates were immunoprecipitated with the R5232-2 antiserum as described for the [^{35}S]methionine-labeled cells. A broad band at about 78 to 80 kDa was detected in the 1-h-serum-induced samples but was barely detectable in samples from both uninduced and 3-h-induced cells (Fig. 3B). These data suggest that the Egr-1 protein is phosphorylated following serum stimulation and that the turnover of phosphoryl groups in vivo is rapid, since by 3 h the phosphorylation signal was weak compared with the amount of protein detected by Western blot or immunocytochemistry. However, further experiments will be required for accurate measurements of the turnover rate. Besides the induced band, two other bands also appeared in lysates from induced and uninduced cells. One of them migrated at 200 kDa. We considered it a nonspecific band because it was also detected with nonimmune rabbit serum. The other band appeared at 60 kDa. This phosphorylated protein, present in both serum-induced and uninduced cells in approximately equal amounts, was not detected by nonimmune rabbit serum. It could represent a highly phosphorylated species which has antigenic similarity to Egr-1.

Zinc is required for Egr-1 binding to its target sequence. Preliminary data derived from transient cotransfection assays in fibroblasts showed that the Egr-1 protein autoregulates its own expression (D. Gius, X. Cao, and V. P. Sukhatme, unpublished). Because Egr-1 encodes a putative zinc finger DNA-binding motif, we asked whether this process is directly mediated by the binding of Egr-1 protein to its upstream regulatory region. A gel mobility shift assay with several upstream fragments revealed that in vitro-generated Egr-1 protein bounds to oligomers spanning nucleotides 695 to −616 and −615 to −536. Comparison of the nucleotide sequences from these regions showed that a core sequence of nine nucleotides was highly conserved between these oligomers. A recent report demonstrates that these regions are footprinted by Egr-1/Zif268 protein generated in *E. coli* (5). In addition, we noted that a third sequence at −279 in the Egr-1 promoter had close sequence similarity to these two sites.

A complex unique to the Egr-1 programmed lysate was formed with probes spanning nucleotides −695 to −616 and −615 to −536 (Fig. 4). Although some nonspecific binding due to the reticulocyte lysate was also observed, a more slowly migrating complex was definitely attributed to Egr-1 based on the correlation of this complex with increasing amounts of Egr-1 programmed lyste and on the ability of anti-Egr-1 antisera to block stable formation of this complex (Fig. 5B). Furthermore, Egr-1 binding to the synthetic probe 5′-CGCCCT CGCCCCCGC GCCGGG-3′ (designated EBS-1) was specific, with a 100-fold excess of cold competitor almost entirely abolishing complex formation (Fig. 5A). In order to compare the affinity of Egr-1 for related sequences in the 5′ region of the Egr-1 gene, we used two other competitors differing from EBS-1 by one or two nucleotides in the nonamer core. From the experiments shown in Fig.

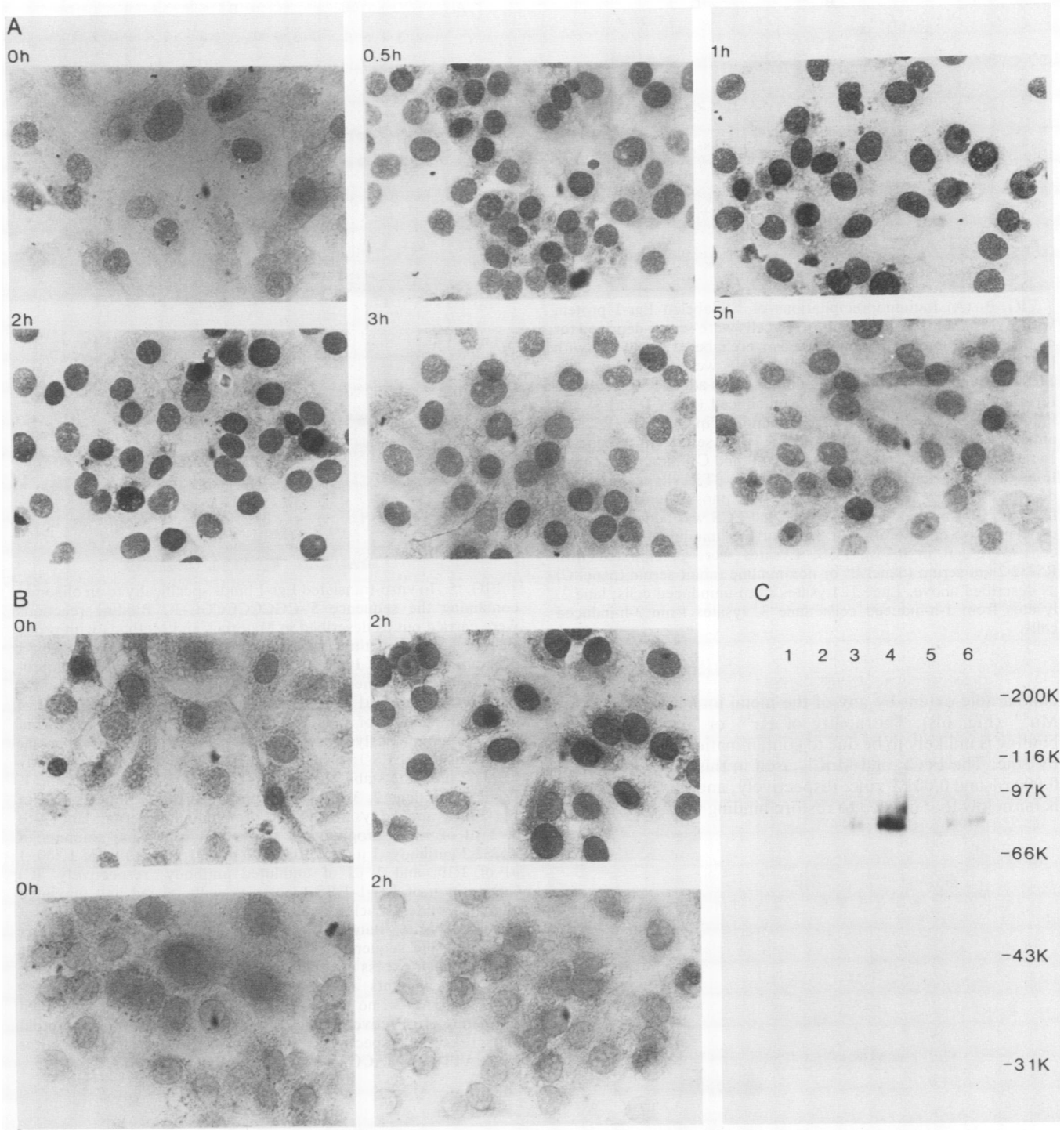

FIG. 2. (A) Time course of Egr-1 protein induction in BALB/c 3T3 cells analyzed with immunoperoxidase staining. BALB/c 3T3 cells were induced by 20% FCS. Cultures were prepared for immunoperoxidase staining with the affinity-purified R5232-2 antiserum at a 1:250 dilution at 0, 0.5, 1, 2, 3, and 5 h. (B) The upper panels show uninduced cells or 2-h-serum-induced cells stained with R5232-2 antiserum (not affinity purified). The lower panels show the same cells stained with the flowthrough fraction obtained during the affinity purification of the R5232-2 antiserum. (C) Immunoblot of cell-fractionated BALB/c 3T3 fibroblasts. BALB/c 3T3 cells were serum-deprived and then induced for 2 h. Cytoplasmic and nuclear portions were analyzed in a 7.5% polyacrylamide–SDS gel. Lanes 1 and 2, Protein sample of uninduced and induced, respectively, cytoplasmic fraction of BALB/c 3T3; lanes 3 and 4, nuclear fraction of uninduced and induced cells, respectively; lanes 5 and 6, uninduced and induced whole-cell lysate, respectively.

5C, we conclude that 5′-CGCCCCCGC-3′ is a higher-affinity binding site than either 5′-GCGGGGGCA-3′ or 5′-GCGG GTGAG-3′, though an effect of differing flanking sequence cannot be excluded.

We further characterized the Egr-1–EBS-1 protein-DNA complex, establishing that it was sensitive to metal chelators, specifically EDTA and 1,10-phenanthroline (Fig. 6A). Moreover, Egr-1 binding activity could be restored to a

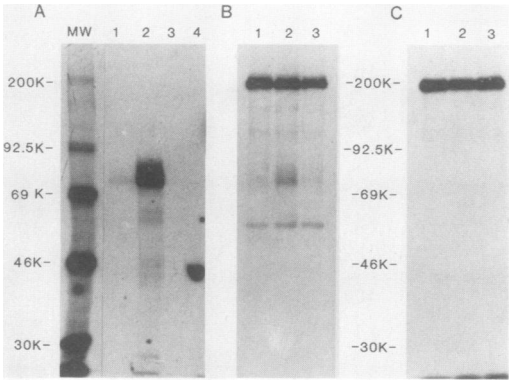

FIG. 3. (A) Immunoprecipitations of ^{35}S-labeled Egr-1 protein with R5232-2 antiserum. BALB/c 3T3 cells were serum-deprived for 45 h. For the uninduced sample, cells were labeled for 30 min with [^{35}S]methionine. For induced samples, cells were induced by 20% FCS for 30 min, and then [^{35}S]methionine was added and incubated for another 30 min. Lysates from uninduced (lanes 1 and 3) and induced (lanes 2 and 4) cells were immunoprecipitated with R5232-2 antiserum (lanes 1 and 2) or nonimmune rabbit serum (lanes 3 and 4). Lane MW, Molecular weight markers. (B and C) Immunoprecipitations of ^{32}P-labeled Egr-1 protein. BALB/c 3T3 cells were serum-deprived for 45 h and then incubated with phosphate-free medium for 4 h. $^{32}P_i$ was then added for 4 h, and FCS was added to a final concentration of 20% for 1 or 3 h. For the uninduced sample, the cells were left in 0.75% FCS. Lysates were precipitated with R5232-2 antiserum (panel B) or nonimmune rabbit serum (panel C) as described above. Lane 1, Lysates from uninduced cells; lane 2, lysates from 1-h-induced cells; lane 3, lysates from 3-h-induced cells.

comparable extent by any of the metal ions Zn^{2+}, Fe^{2+}, or Mn^{2+} (Fig. 6B). The ability of Fe^{2+} or Mn^{2+} to restore binding is unlikely to be due to contamination of these metals by zinc. The $FeCl_2$ and $MnCl_2$ used in this assay contained 0.0005% and 0.001% zinc, respectively, and this level of zinc is far below that needed to restore binding (data not shown).

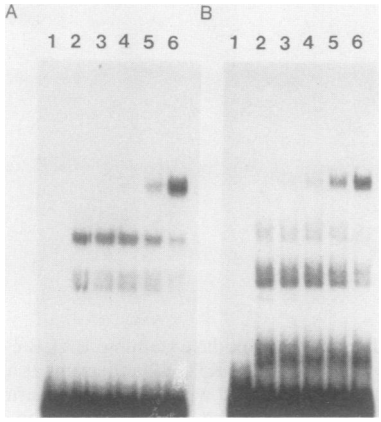

FIG. 4. In vitro-translated Egr-1 binds to its own 5′-flanking region. DNA-binding reactions were performed as described in Materials and Methods. (A) Binding to an Egr-1 5′ region probe containing nucleotides −695 to −616. Lane 1, Probe alone; lane 2, 3 μl of unprogrammed lysate; lanes 3 to 6, increasing amounts of Egr-1 programmed lysate: 1 μl of 1:10 dilution, 1 μl of 1:3 dilution, 1 μl of undiluted, and 3 μl of undiluted, respectively. (B) Binding to an Egr-1 5′ region probe containing nucleotides −615 to −536. Lanes are as in panel A.

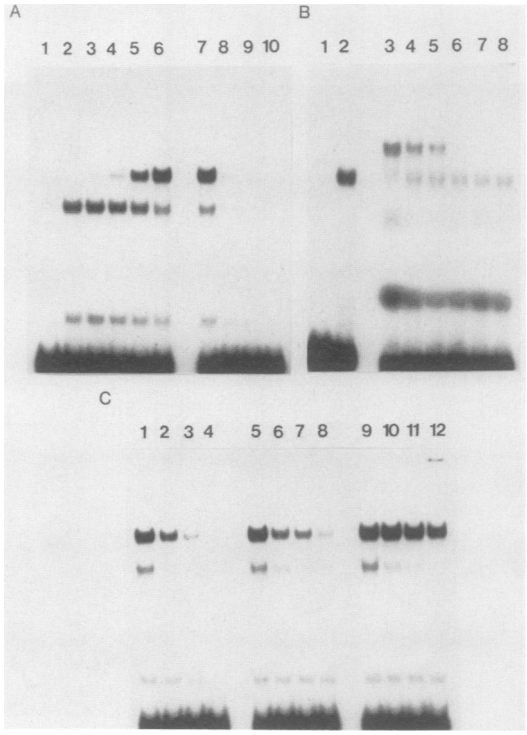

FIG. 5. In vitro-translated Egr-1 binds specifically to an oligomer containing the sequence 5′-CGCCCCCGC-3′. Binding reactions were carried out as described in Materials and Methods with probe EBS-1. (A) Competition of a complex that is specific to Egr-1 programmed lysate. Lane 1, Probe alone; lane 2, 3 μl of unprogrammed lysate; lanes 3 to 6, increasing amounts of Egr-1 programmed lysate: 1 μl of 1:10 dilution, 1 μl of 1:3 dilution, 1 μl of undiluted, and 3 μl of undiluted, respectively; lanes 7 to 10, 3 μl of Egr-1 programmed lysate with no or 10-, 30-, or 100-fold molar excess of cold EBS-1, respectively. (B) Egr-1 polyclonal antibody R5232-2 prevents stable Egr-1–EBS-1 complex formation. Lane 1, Probe alone; lane 2, 3 μl of unprogrammed lysate; lane 3, 3 μl of Egr-1 programmed lysate and 1 μl of nonimmune serum; lanes 4 to 8, 3 μl of Egr-1 programmed lysate and increasing amounts of R5232-2 antibody: 1 μl of 1:10,000, 1 μl of 1:1,000, 1 μl of 1:100, 1 μl of 1:10, and 1 μl of undiluted antibody, respectively. (C) Competition of Egr-1–EBS-1 complex with related and unrelated oligonucleotides. Each reaction mix contained 3 μl of Egr-1 programmed lysate. Lanes 1 to 4, Increasing amounts of oligomer containing the sequence 5′-GCGGGGGCA-3′: no or 10-, 30-, or 100-fold molar excess of cold competitor, respectively; lanes 5 to 8, increasing amounts of oligomer containing the sequence 5′-GCGGGTGAG-3′: no or 10-, 30-, or 100-fold molar excess of cold competitor, respectively; lanes 9 to 12, no or 10-, 30-, or 100-fold molar excess, respectively, of the cold nonspecific competitor 5′-GAATTC GGTACC GCGGCC GCATCG ATGATA TC-3′.

DISCUSSION

Understanding Egr-1 gene expression is important for two reasons. First, the deduced Egr-1 amino acid sequence contains a zinc finger structure of the C_2H_2 class, suggesting a transcriptional regulatory function. Second, the gene is induced by diverse signals that initiate growth and differentiation even in the presence of cycloheximide. Thus, like c-*fos* and c-*jun*, Egr-1 is an immediate-early gene likely to be involved in a regulatory cascade of gene interactions.

In this study, we identified and characterized the mouse and rat Egr-1 gene products both in vitro and in vivo by using immunoblotting, immunoprecipitation, and immunocytochemistry. First, we raised a rabbit antiserum (R5232-2)

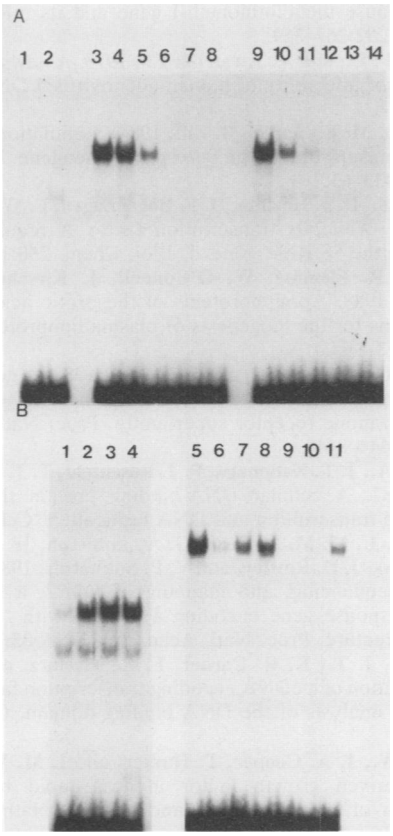

FIG. 6. Egr-1 binding is metal dependent. Binding reactions were performed with probe EBS-1 as described in Materials and Methods. (A) Pretreatment of Egr-1 programmed lysate with either of the chelators EDTA or 1,10-phenanthroline (Sigma) prevents Egr-1–EBS-1 complex formation. Lane 1, Probe alone; lane 2, 3 μl of unprogrammed lysate; lanes 3 to 7, 3 μl of Egr-1 programmed lysate and 0, 2.5, 5, 7.5, and 10 mM EDTA, respectively; lane 8, no lysate and 10 mM EDTA; lanes 9 to 13, 3 μl of Egr-1 programmed lysate and 0, 0.25, 0.5, 1, and 2 mM 1,10-phenanthroline; lane 14, no lysate and 2 mM 1,10-phenanthroline. (B) Zn^{2+} and certain other metal ions restore Egr-1 DNA-binding activity after chelation with 1,10-phenanthroline. Lanes 1 to 4, Each reaction mix contained 3 μl of Egr-1 programmed lysate pretreated with 0.5 mM 1,10-phenanthroline and 0, 50, 100, and 500 μM $ZnCl_2$, respectively; lane 5, 3 μl of Egr-1 untreated lysate; lane 6, 3 μl of Egr-1 lysate treated with 0.5 mM 1,10-phenanthroline; lanes 7 to 11, 3 μl of Egr-1 programmed lysate treated with 0.5 mM 1,10-phenanthroline and 0.5 mM $ZnCl_2$, $FeCl_2$, $MgCl_2$, $CaCl_2$, and $MnCl_2$, respectively, as described in Materials and Methods.

against a bacterially generated bGH-Egr-1 fusion protein. This immunogen was sufficiently pure to allow N-terminal amino acid sequencing. This antiserum reacted with an Egr-1 in vitro-generated protein and with a comigrating band in fibroblasts transfected with an Egr-1 expression vector. These results indicated that the R5232-2 antiserum recognized the Egr-1 protein and identified it as an 80-kDa product in mouse fibroblasts. The specificity of this antiserum was demonstrated by the presence of a single band on Western analysis of serum-stimulated fibroblasts and in NGF-induced PC12 cells. In addition, the antiserum did not react with in vitro-translated Egr-2 protein.

We then used the antiserum to examine the detailed time course of Egr-1 protein expression following serum stimulation. Using Western analysis and immunostaining, we found that the protein starts to accumulate within 30 min, reaches a peak level of expression at about 1 to 2 h, and decreases gradually thereafter over the next few hours. The overall time course of protein expression is similar to that seen for Egr-1 mRNA levels, except that the RNA levels peak at about 0.5 to 1 h and decay rapidly over the next 1 to 2 h (35). These kinetic data are very similar to those observed for c-*fos*. Interestingly, Egr-1 protein expression persists longer (8 to 12 h) in murine B lymphocytes following immunoglobulin stimulation (V. Seyfert, J. Monroe, X. Cao, and V. P. Sukhatme, unpublished observations).

An 80-kDa protein was also detected by the R5232-2 antiserum in rat PC12 cells stimulated by three different agents, PMA, NGF, and EGF. It was not present in unstimulated cells. Since we have previously shown that Egr-1 mRNA is induced by these agents in PC12 cells, these data are further supporting evidence that the 80-kDa protein is indeed the Egr-1 gene product. It is not surprising that the R5232-2 antiserum reacts with the rat Egr-1 protein, since mouse and rat Egr-1 are highly homologous (98% at the amino acid level).

Both immunocytochemical and cell fractionation-immunoblot analysis of mouse fibroblasts show that the Egr-1 protein is localized within the nuclei of these cells. These data are consistent with the predicted function of this protein as a transcriptional regulator in view of its C_2H_2 zinc finger structure.

The immunoprecipitation assay allowed us to ask whether the Egr-1 protein is phosphorylated in serum-stimulated fibroblasts. That this is indeed the case was verified by the $^{32}P_i$ in vivo labeling data. Interestingly, the peak ^{32}P label occurred at 1 h, but there was relatively little labeling at 3 h following serum stimulation, a time at which a significant amount of Egr-1 protein is still present by Western blot and immunocytochemical analysis. It is therefore possible that modulation of Egr-1 activity can be fine-tuned by its phosphorylation state. Similar results have been noted for c-*fos*, though the latter is more highly phosphorylated.

We used gel shift assays to show that in vitro-translated Egr-1 protein binds to three sites in its own promoter region centered at positions −643, −594, and −279. The nonamer core sequences differ by one or two bases. Among these oligomers, we found that the highest-affinity site, as defined through cold competition experiments, has the sequence CGCCCCCGC.

Several transcription factors with conserved C_2H_2 structures have been shown to bind DNA in a zinc-dependent manner. Treatment of *Xenopus laevis* transcription factor IIIA with EDTA or 1,10-phenanthroline destroyed its ability to footprint the 5S control region (13). Similarly, footprinting with the mammalian factor Sp1 is dependent on zinc. Zn^{2+} but not Co^{2+} or Ni^{2+} partially restored DNA binding after chelation with EDTA. The chelator 1,10-phenanthroline, however, did not affect binding (18). An independent study with a gel shift assay showed that EDTA-chelated Sp1 binding can be restored by zinc but not Cd^{2+}, Cu^{2+}, or Mg^{2+} (39). The yeast activator SWI5 also required zinc for binding in a DNase I footprinting assay (26). Finally, gel shift assays with the yeast activator ADR1 demonstrate that Zn^{2+} but not Cd^{2+}, Co^{2+}, Ca^{2+}, or Mg^{2+} (at the concentrations tested) restores binding after chelation with 1,10-phenanthroline (9). We have established that Egr-1 binding is metal dependent, since two chelators, EDTA and 1,10-phenanthroline, abolished binding in a dose-dependent manner. We extended these studies by showing that binding can be restored by readdition of Zn^{2+}, as well as by Fe^{2+} and Mn^{2+}. These results, along with the deduced amino acid

sequence of Egr-1, suggest that it is a zinc-dependent finger protein.

The identification of the Egr-1 protein and the availability of antisera directed against it will facilitate a variety of studies aimed at understanding its role in regulating cellular proliferation and differentiation. Egr-1 mRNA is modulated in diverse systems containing mixed cell populations, for example, in embryonal carcinoma cell differentiation (34), as a consequence of renal ischemia, and by alpha-adrenergic agents in cardiac cells in vitro and in vivo (unpublished data). Immunohistochemistry will allow the localization of Egr-1 protein expression to individual cells within tissue sections. Given the human chromosomal localization of Egr-1 to the long arm of chromosome 5, in a region that is often deleted in patients with therapy-related acute nonlymphocyte leukemia (34 and references therein), it will be of particular interest to define Egr-1 expression in leukemic as well as normal cells from these patients. Chromosome 5q deletions are also common in colorectal carcinomas (38). We are currently comparing Egr-1 levels in human tumor specimens with Egr-1 expression in adjacent normal tissue. The anti-Egr-1 antiserum will also be useful for microinjection experiments to help establish a function for Egr-1.

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