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Growth Factor-Induced Delayed Early Response Genes

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Growth factors induce the sequential expression of cellular genes whose products are thought to mediate long-term responses to the growth factors. In mouse 3T3 fibroblastic cells, the first genes to be expressed (immediate-early genes) are activated within minutes after the addition of platelet-derived growth factor, fibroblast growth factor, or serum. By cDNA cloning, we have identified genes that are activated after a delay of a few hours and several hours prior to serum-induced DNA replication. Activation of these delayed early response genes requires new protein synthesis, presumably the synthesis of immediate-early transcription factors described previously. Partial or complete sequencing of 13 different delayed early cDNAs, representing about 40% of the 650 primary cDNA isolates, revealed that 8 were related to known gene sequences and 5 were not. Among the former are cDNAs encoding nonhistone chromosomal proteins [HMGI(Y) and HMGI-C], adenine phosphoribosyltransferase (APRT), a protein related to human macrophage migration inhibitory factor (MIF), a protein of the major intrinsic protein (MIP) family homologous to the integral membrane protein of human erythrocytes, and cyclin CYL1. In 3T3 cells, the delayed early gene response to growth factors appears to be at least as complex as the immediate-early gene response previously described.

The properties of cells of complex organisms appear to be modulated continuously by extracellular signals. Many signaling agents activate specific cell surface receptors, resulting in rapid modification of intracellular proteins, formation of second messenger molecules, and sequential induction of specific genes. These induced genetic programs are thought to mediate long-term cellular responses to signaling ligands.

We and others have been analyzing a prototypic genetic program induced by extracellular signaling agents, namely the program induced in the mouse fibroblastic 3T3 cell line by serum or purified growth factors. When nondividing mouse 3T3 cells are stimulated by serum, platelet-derived growth factor (PDGF), or fibroblast growth factor (FGF), a set of specific genes is activated within minutes, even in the presence of an inhibitor of protein synthesis (1, 9, 25). These primary response genes have been called immediate-early genes, analogous to the immediate-early genes of viruses. Among the proteins they encode are transcription factors, cytokines, membrane proteins, and cytoskeletal proteins (6, 18, 27). After a few hours, but still prior to the onset of DNA synthesis, other genes are activated; in contrast to immediate-early genes, their activation requires protein synthesis (27). Therefore, these delayed early response genes are thought to be activated by immediate-early transcription factors.

So far, a limited number of delayed early response genes have been identified in stimulated cells (27, 31). They include genes that encode biosynthetic enzymes, secreted proteases, potential cytokines, and transcription factors. In an attempt to identify other delayed early genes that are part of the growth factor-induced genetic program of fibroblasts, we have undertaken a search by cDNA cloning for mRNAs that appear in growth-stimulated 3T3 cells with kinetics and dependence on protein synthesis characteristic of the delayed early gene response. We report here that by differential screening of appropriate cDNA libraries, cDNA clones that are derived from delayed early mRNAs have been isolated. Some of these RNAs encode members of known protein families (some not previously associated with the delayed early response to growth factors) including a member of the cyclin family (CYL1) (29), nonhistone chromosomal proteins {HMGI(Y) [22] and HMGI-C [28]}, and proteins related to macrophage migration inhibitory factor (MIF) (40) and to the major intrinsic protein of the lens (MIP) (32). Others encode proteins that have not previously been identified.

MATERIALS AND METHODS

Cell culture. BALB/c 3T3 clone A31 cells were obtained from the American Type Culture Collection and were grown and stimulated as described previously (25). Where indicated, cycloheximide and anisomycin were added to final concentrations of 10 μg/ml and 10 μM, respectively. Mouse Ltk$^-$ L cells were cultured at 37°C either in flasks in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) (DMEM-10) or in roller bottles in DMEM-10 with 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (23). PC12 cells (rat pheochromocytoma) were obtained from M. Bothwell (University of Washington, Seattle, Wash.) and grown at 37°C in RPMI-1640 medium with 10% horse serum and 5% FBS. CTLL cells (3) (nonadherent, interleukin 2 [IL-2]-dependent cytotoxic T-cell line obtained from S. Desiderio) were cultured at 37°C in RPMI-1640 with 10% FBS, 50 μM β-mercaptoethanol, and 100 U of mouse recombinant IL-2 (Genzyme) per ml. BALB/MK cells (BALB/c mouse epidermal keratinocytes) (41) were obtained from S. Aaronson and cultured at 37°C in low-calcium (0.05 mM) Eagle's minimal essential medium (Biofluids) with 10% dialyzed FBS (MEM-10) and 10 ng of recombinant human epidermal growth factor (EGF) per ml.

For stimulation with growth factors, BALB/c 3T3 cells were grown to confluence in MEM-10 and made quiescent by changing the medium to MEM without serum (SF-MEM) supplemented with 5 μg of transferrin (Collaborative Research) per ml, 5 nM sodium selenite, and 500 μg of linoleic acid-bovine serum albumin (Collaborative Research) per ml.

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After 3 days in SF-MEM, cells were stimulated with purified growth factors, and at the indicated times, RNA was harvested and used for Northern (RNA) analysis. The final concentrations of growth factors were, per ml, 50 ng of recombinant human basic FGF (Collaborative Research), and 5 ng of recombinant human BB-PDGF (Amgen). PC12 cells were plated at a density of 6.6×10^6 cells per 15-cm-diameter dish and allowed to grow for 5 days before stimulation. The cultures were fed once with fresh growth medium 3 days prior to stimulation. The subconfluent cells were stimulated by direct addition of concentrated nerve growth factor (NGF; mouse 2.5S from Collaborative Research) into the conditioned medium to give a final concentration of 50 ng/ml. Cells were harvested prior to stimulation and at 1, 5, 9, 13, 17, 24, 48, and 72 h following the addition of NGF, and 10 μg of total RNA from each sample was analyzed by Northern blotting by using various cDNA probes. Following the addition of NGF, RNAs derived from immediate-early genes *zif 268* and *jun-B* were induced, and neurite formation was observed by 72 h. Logarithmically growing CTLL cells were pelleted, washed three times, and maintained for 24 h in growth medium without IL-2, at a density of 10^6 cells per ml. Cells were stimulated by direct addition of concentrated IL-2 to give a final concentration of 100 U/ml. BALB/MK cells were plated at a density of 2×10^6 cells per 15-cm-diameter dish in low-calcium growth medium with 5 ng of EGF per ml. Following an 18-h incubation period, cells were incubated in growth medium without EGF. After 3 days, the subconfluent cultures were stimulated by the addition of concentrated EGF to a final concentration of 20 ng/ml.

cDNA library preparation. Total cellular RNA was isolated by using the procedure of Chirgwin et al. (7), and poly(A)$^+$ RNA was purified by two rounds of selection with oligo(dT)-cellulose chromatography. Two micrograms of poly(A)$^+$ RNA from quiescent cells (Q), cells stimulated for 3 h with 20% FBS in the presence of cycloheximide-anisomycin (3CHX/AN) or cells stimulated for 10 h with 20% FBS (10 h/FBS) were used as template for synthesis of first-strand cDNA by using an *Xba*I-oligo(dT) primer. Second-strand cDNA was prepared by using RNase H/DNA polymerase I (20), and after blunting the ends with T4 DNA polymerase, *Eco*RI-*Not*I adaptors were ligated onto the cDNA. Following phosphorylation with T4 polynucleotide kinase, cDNA was digested with *Xba*I and purified away from free adaptors by using Sephacryl S-400 spin columns. cDNA was cloned directionally into λGem2 (Promega) DNA arms and packaged in vitro. Each library contained $>5 \times 10^6$ recombinants.

For isolating full-length cDNAs, poly(A)$^+$ RNA from cells stimulated with 20% FBS for 2.5, 5, 7.5, 10, and 12.5 h was pooled, and 5 μg of RNA was reverse transcribed and converted to double-stranded cDNA by using the ZAP-cDNA synthesis kit (Stratagene). *Eco*RI-*Not*I adaptors were ligated to the blunt-ended cDNA and phosphorylated by using T4 polynucleotide kinase (New England BioLabs). After restriction with *Xho*I, cDNA was fractionated on a 1.5-ml Bio-Gel A-50 m column to remove free adaptors, and fractions containing cDNA larger than 500 bp were pooled and ligated in *Eco*RI-*Xho*I-digested Uni-ZAP XR (Stratagene). The ligation products were packaged in vitro by using GigapackII Gold (Stratagene) packaging extracts; the resulting library contained $>10^7$ recombinants.

Preparation of biotinylated cRNA for subtraction. Q, 3CHX/AN, and 10 h/FBS cDNA libraries were amplified on 15-cm-diameter NZCYM agarose plates (36) (30 dishes with 10^5 PFU per dish), and phage DNA was prepared from plate lysates. DNA from Q and 3CHX/AN cDNA libraries was linearized at the 3′ end of the cDNA insert by digestion with *Xba*I, and cRNA was prepared from these DNA templates by using T7 RNA polymerase (United States Biochemical). The DNA template was removed by digestion with DNase I, and the cRNA was purified by chromatography on NICK columns (Pharmacia) prior to precipitation with ethanol. cRNA was biotinylated twice with equal amounts of cRNA (1 mg/ml) and Photoprobe (long arm) biotin (1 mg/ml; Vector Laboratories) as described previously (38).

Preparation of a subtracted 10 h/FBS cDNA library. cRNA prepared from the 10 h/FBS cDNA library was used as template to synthesize cDNA by using an *Xba*I-oligo(dT) primer (Promega). cDNA synthesized by using 10 μg of 10-h cRNA was coprecipitated with 50 μg of twice biotinylated cRNA prepared from the Q cDNA library and 50 μg of twice biotinylated cRNA prepared from the 3CHX/ANS library. Solution hybridizations of single-stranded cDNA with biotinylated cRNAs and removal of hybrids with streptavidin-phenol extraction were performed as described previously (38). Following two rounds of subtraction, single-stranded cDNA was converted to double-stranded cDNA by using a T7 spacer primer (Promega) and Sequenase (United States Biochemical). Following digestion with *Eco*RI and *Xba*I, the cDNA was purified by using a Sephacryl S-400 spin column, cloned into λGem2, and packaged in vitro. A library of $>10^6$ PFU was obtained and screened without amplification.

Preparation of subtracted cDNA probes and differential screening of the subtracted cDNA library. Poly(A)$^+$ RNA isolated from Q, 3CHX/AN, or 10 h/FBS cells was used to synthesize high-specific-activity ^{32}P-labelled cDNA. In a typical reaction, 0.3 mCi of [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear) was dried, followed by addition of 2 μg of twice-purified poly(A)$^+$ RNA and 2 μg of oligo (dT_{12-18}) (Pharmacia). After being heated at 70°C and cooled on ice, cDNA was synthesized at 42°C in buffer containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM each of dATP, dGTP, and dTTP, 1 μl of RNAsin (40 U/μl; Promega), and 400 U of Moloney murine leukemia virus RNase H-minus reverse transcriptase. After 15 min, dCTP was added to a final concentration of 1 mM, and the incubation was continued for 60 min at 42°C. Following alkaline hydrolysis of the RNA template, cDNA was purified by chromatography on Sephadex G-50 NICK columns. Each cDNA preparation was coprecipitated with 10 μg of twice biotinylated cRNA from quiescent cells and 10 μg of twice biotinylated cRNA from 3CHX/ANS cells and subtracted twice as described above. The cDNA remaining following two rounds of subtraction was used to probe triplicate nitrocellulose lifts from the subtracted 10 h/FBS cDNA library (600 PFU per 15-cm-diameter dish). The specific activity of cDNA synthesized was 8×10^9 cpm/μg, and two rounds of subtraction removed 80 to 90% of the starting cDNA. After being prehybridized overnight at 65°C in 5× SSPE (pH 7.4) (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1.1 mM EDTA)–5× Denhardt's solution (36)–10% dextran sulfate–0.2% sodium dodecyl sulfate (SDS)–0.1 mg of sonicated salmon sperm DNA per ml, filters were hybridized with 10^6 to 2×10^6 cpm of cDNA per ml in fresh hybridization solution at 65°C for 3 days. Filters were washed twice with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7])–0.2% SDS at room temperature, and twice with 1× SSC–0.2% SDS at 55 to 60°C. Plaques showing stronger hybridization with the 10 h/FBS cDNA probe than with the Q and 3CHX/AN cDNA probes were picked, and DNA was prepared from plate lysates.

DNA inserts were excised by digestion with *Eco*RI-*Xba*I and triplicate Southern blots of the restricted inserts were probed with subtracted cDNA prepared from Q, 3CHX/ANS, or 10 h/FBS poly(A)$^+$ RNA. Restriction fragments showing stronger hybridization to 10 h/FBS cDNA than to Q or 3CHX/ANS cDNA were subcloned into pBluescript (Stratagene) for blotting experiments, nucleic acid sequence analysis, and library probing to select longer cDNAs.

DNA sequencing. The ends of each cDNA isolate were sequenced as double-stranded plasmids by the dideoxynucleotide chain termination method (37), with deoxyadenosine 5′-[α-^{35}S]thiotriphosphate, Sequenase (United States Biochemical), and T3 and T7 primers. The resulting 400- to 600-bp sequence was used to search the GenBank, EMBL, and NBRF data bases. A nearly full-length cDNA (543 bp) for DER6 was isolated from the subtracted 10 h/FBS library, and both strands were sequenced by using oligonucleotide primers derived from the sequence generated. A nearly full-length cDNA for DER2 (2.6 kbp) was isolated by using the original 500-bp cDNA to screen the pooled cDNA library described above. Sets of 5′ and 3′ deletions were prepared by using exonuclease III (Promega), and resulting subclones were sequenced as described above.

Southern blot analysis. Genomic DNA was digested with *Bam*HI, *Eco*RI, or *Hin*dIII (New England Biolabs), fractionated on agarose gels, and transferred to nitrocellulose in 20× SSC. The 543-bp DER6 cDNA and the 2,612-bp DER2 cDNA were labelled with [α-^{32}P]dCTP by nick translation. Prehybridization and hybridization incubations were done in 5× SSC at 60°C, and the final wash was done in 2× SSC at 60°C.

Nuclear run-on transcription assay. Cells were grown to confluence and made quiescent by changing the medium to MEM-0.5 for 3 days. For each time point, four 15-cm-diameter dishes were stimulated with MEM-20, PDGF (50 ng/ml), or FGF (50 ng/ml), and at the indicated time, cells were rinsed three times with ice-cold phosphate-buffered saline and nuclei were isolated as described previously (1). Labelled RNA was prepared and isolated as previously described (15, 16), except that the base hydrolysis step was omitted. Labelled RNA was hybridized to filters containing 5 μg of each cDNA plasmid or pBluescript, as vector control. Filters were prehybridized overnight at 42°C in 5× SSPE (pH 7.4)–0.5% SDS–50% formamide–5× Denhardt's solution–50 μg of sonicated salmon sperm DNA per ml and then hybridized for 3 days at 42°C in the same solution freshly made containing 4×10^6 cpm of labelled RNA per ml. Filters were washed twice with 2× SSC–0.2% SDS for 30 min at room temperature and twice with 0.5× SSC–0.2% SDS at 65°C.

Centrifugal elutriation. Mouse Ltk$^-$ cells were seeded into 850-cm^2 roller bottles at a low density (1.5×10^7 cells per roller bottle) 3 days prior to elutriation, and fresh growth medium was added 16 h prior to elutriation. The logarithmically growing cells were harvested and resuspended in cold spinner MEM-5. Cells were separated into fractions of increasing size in a Beckman JE-6B rotor (standard chamber). Three consecutive elutriations were performed by using the same parameters (3×10^8 cells; 3,000 rpm; flow rates between 20 and 105 ml/min), and like fractions were pooled. From each fraction, 2×10^5 cells were suspended in 0.5 ml of fluorescence-activated cell sorter (FACS) buffer (3.4 mM sodium citrate, 10 mM sodium chloride, 0.1% Nonidet P-40, 75 μM ethidium bromide) and analyzed for cellular DNA content with a Coulter Epics 752 FACS. The fractions were labeled G1, G1/S, early S, mid-S, late S, or G2 on the basis of the sorting pattern. We are indebted to Michael Kauffman and Jim Flook for help with the elutriation and FACS analysis.

RNA from rat liver and hippocampus. For regenerating liver, adult male Sprague-Dawley rats were subjected to midventral laparotomy and approximately 70% liver resection as described previously (30). Livers were isolated from animals before and at 2, 4, 12, 48, 72, and 96 h following resection. Total RNA was prepared from each sample, and 10 μg was analyzed by Northern blotting by using various cDNA probes. For some delayed early cDNAs (DER1, DER2, DER4, DER5, and DER7) the results obtained by using rats were confirmed by using regenerating livers from BALB/c mice. We are indebted to Raymond DuBois for these RNA samples. For hippocampal stimulation, seizures were induced in adult Sprague-Dawley rats by multiple electroconvulsive shocks (10). Hippocampal tissue was isolated at 4, 8, or 24 h following induction and from control animals. Total RNA was prepared from each sample, and 10 μg was analyzed by Northern blotting by using various cDNA probes. We are indebted to Paul Worley for these tissue samples.

Determination of RNA stability. BALB/c 3T3 cells were grown to confluence in MEM-10 and made quiescent by changing the medium to MEM-0.5 for 3 days. Cells were stimulated by addition of MEM-20 for 8 or 12 h prior to the addition of actinomycin D, and RNA was isolated at various times following the addition of actinomycin D. Cells were also stimulated with MEM-20 in the presence of actinomycin D. Total RNA (10 μg) from each sample was analyzed by Northern blotting by using various delayed early cDNA probes. All gels contained 0.25 μg of ethidium bromide per ml to assess the loadings of different samples.

RESULTS

Isolation of delayed early cDNAs. To isolate cDNAs corresponding to delayed early response genes, a subtracted directional cDNA library was prepared in λ coliphage from poly(A)$^+$ RNA of resting BALB/c 3T3 cells stimulated with 20% serum-containing medium for 10 h, as detailed in Materials and Methods. Under these conditions of stimulation, the cells enter S phase synchronously at approximately 14 h after addition of serum, as assessed by FACS analysis of cellular DNA content and by [^{3}H]thymidine autoradiography. Therefore, the time chosen for preparation of mRNA corresponds to pre-S phase of the induced cycle. To enrich the library and probe for delayed early cDNAs, sequences derived from mRNAs also present in quiescent cells and in cells stimulated in the presence of protein synthesis inhibitors were selectively reduced by subtractive procedures (Fig. 1). For differential screening of the library we prepared two probes, one derived from poly(A)$^+$ RNA of cells stimulated with serum for 10 h, and the second derived from poly(A)$^+$ RNA of resting 3T3 cells and cells stimulated with serum for 3 h in the presence of cycloheximide and anisomycin. Approximately 10% of recombinant phage plaques showed greater hybridization to the first probe; 650 plaques were picked for further analysis.

Analysis of delayed early cDNAs. The first step in the analysis of delayed early cDNAs was to determine approximately how many different sequences were in our collection by cross-hybridization tests. So far we have found that the first 13 confirmed delayed early cDNA clones that fail to cross-hybridize with each other represent 233 of the 650 initial phage isolates. Since the initial 13 cDNAs are likely to

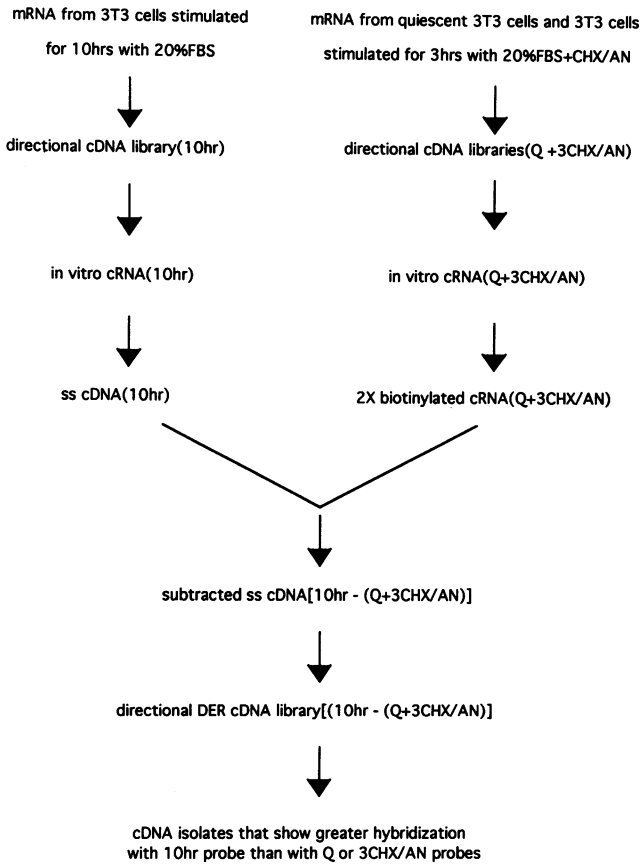

FIG. 1. Isolation of delayed early response cDNAs. For details, see Materials and Methods.

include most of the abundant species, we infer that a large number of different delayed early cDNAs remain unidentified. Sequences from the 5′ and 3′ ends of each of the 13 different clones were compared with sequences in the GenBank and EMBL data banks. Eight were related to known gene sequences and five were not (Table 1). Of the former, some were not previously recognized as DER genes (HMGI-C, MIF, CHIP28, and APRT).

DER gene expression and response to purified growth factors. To determine the timing of delayed early gene expression, each of the 13 cDNA clones was used to detect specific mRNA by Northern blotting and to detect gene transcripts in nuclei from stimulated cells. Examples of these analyses are shown in Fig. 2 and 3. The mRNAs were low or absent in quiescent cells and appeared between approximately 2 and 4 or 5 h after the addition of serum, PDGF, or FGF, generally reaching a maximum level by 7 to 10 h. In the presence of the protein synthesis inhibitors cycloheximide and anisomycin, the mRNAs increased little, if at all. In contrast, a typical immediate-early mRNA (Zif 268 [8]) appeared within 30 min, peaked by 1 1/2 h, and was super induced in the presence of inhibitors of protein synthesis (26) (Fig. 2). As shown in Fig. 2, PDGF and FGF led to a time of appearance of the mRNAs similar to that seen for serum, but the mRNA levels were more sustained.

To determine whether the delayed early genes are transcriptionally activated, measurements of nuclear transcripts by run-on assays were carried out. Each of the 13 delayed early genes was transcriptionally activated within approximately 2 h following serum, PDGF, or FGF addition, with some variation in the time of appearance of different transcripts. An example of these results is shown in Fig. 3. With the exception of DER3 (VL30 provirus), all the delayed early genes showed a level of activation, as measured by nuclear run-on assays, considerably lower than that of a typical immediate-early gene *zif 268* (Fig. 3). In contrast to activation of immediate-early genes (9), activation of delayed early genes was inhibited by cycloheximide and anisomycin (Fig. 3). There is also a difference in the stability of immediate-early and delayed early mRNAs. Many of the former have short half-lives (approximately 20 min. [26]) whereas the latter have half-lives of over 3 h, as determined in cells treated with actinomycin D after 8 or 12 h of serum stimulation (data not shown). We conclude that the delayed early genes identified are transcriptionally activated within 2 h or so after immediate-early genes and prior to the appearance of their respective mRNAs and that activation depends on new protein synthesis. This pattern is consistent with the supposition that these genes are activated by immediate-early transcription factors.

TABLE 1. Summary of results for delayed early response cDNAs[a]

Delayed early response cDNA	Abundance (%)	RNA (kb)	Identity
DER1	0.10	3.4	HMGI-C
DER2	0.07	3.0	MIP related (CHIP-28)
DER3	0.35	5.2	VL30 provirus
DER4	0.17	2.7	T1 (CEA family)
DER5	0.30	1.8	HMGI(Y)
DER6	0.23	0.5	MIF related
DER7	0.04	4.0	CYL1
DER8	0.008	3.0	Novel
DER9	0.008	1.8, 2.8, 4.0	Novel
DER10	0.13	1.4	APRT
DER11	0.003	3.0	Novel
DER12	0.01	2.5	Novel
DER13	0.27	1.3, 2.2, 3.0	Novel

[a] Abundance was determined by using the initial cDNA isolate to screen $>10^5$ recombinants from an unsubtracted cDNA library prepared by using RNA from cells stimulated with serum for 10 h. mRNA size was based on electrophoretic mobility of hybridizing species compared with those of 18S and 28S rRNA. The identity of the clones indicated was determined by comparison of partial nucleotide sequences with the GenBank, EMBL, and NBRF data bases. For DER7, a partial sequence in the 3′ noncoding region was similar to the corresponding segment of *bcl-1* cDNA (42), the human homolog of CYL1 (29). A genomic clone hybridizing to DER7 had a coding sequence identical to that of CYL1. References to known proteins: MIP related (32, 33), VL30 (19), T1 (24), HMGI (22), MIF related (40), APRT (12), CYL1 (29), and HMGI-C (28).

Expression of delayed early genes in cycling cells. So far we have shown that delayed early genes are expressed in the pre-S phase following stimulation of resting cells by serum or purified growth factors. To determine whether mRNA levels change similarly during exponential cell growth, proliferating mouse L cells were separated into G1, S, and G2 cells by centrifugal elutriation, and the levels of specific mRNAs in each cell fraction were compared by Northern blotting. L cells were used instead of BALB/c 3T3 cells because the L-cell elutriated fractions were more uniform, as monitored by DNA content. All DER mRNAs except DER4 were detectable in cycling L cells. Figure 4 shows examples of the general findings. Whereas delayed early mRNAs increased after stimulation of quiescent L cells, in cycling cells there was little or no change in RNA levels over the cycle. As expected, histone H4 mRNA (17) increased coincident with

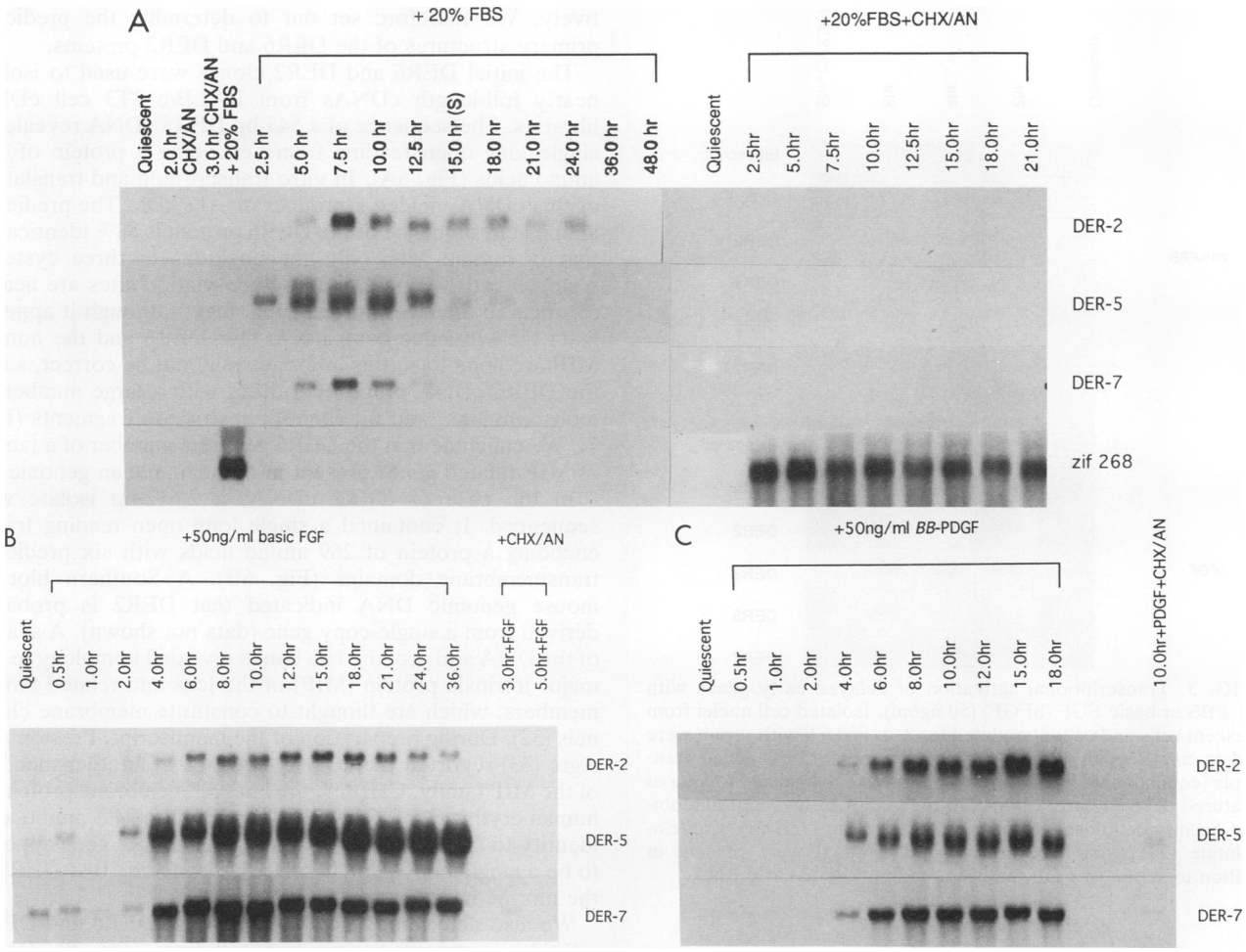

FIG. 2. (A) Time course of delayed early RNA induction with 20% FBS and inhibition of induction by inhibitors of protein synthesis. Confluent monolayers of BALB/c 3T3 fibroblasts were maintained in MEM-0.5 for 3 days and then stimulated with 20% FBS in the presence or absence of cycloheximide (10 μg/ml) and anisomycin (10 μM). Total RNA (10 μg) was harvested at the indicated times following stimulation, fractionated by formaldehyde-agarose electrophoresis, blotted, and hybridized with various ^{32}P-cDNAs. 2.0 hr CHX/AN, quiescent cells treated with cycloheximide and anisomysin alone. Zif 268 RNA is an immediate-early gene product. The rRNA content of each sample was similar, as assessed by ethidium staining. (B and C) Response of delayed early RNAs to purified growth factors. Confluent monolayers of BALB/c 3T3 fibroblasts were maintained in SF-MEM for 3 days and then stimulated by addition of either recombinant FGF (B) or PDGF (C), each to a final concentration of 50 ng/ml; where indicated, cycloheximide (10 μg/ml) and anisomycin (10 μM) were included. Total RNA (10 μg) harvested at the indicated times was resolved by formaldehyde-agarose electrophoresis, transferred to nitrocellulose filters, and hybridized to ^{32}P-labelled cDNA. The rRNA content of each sample was similar, as assessed by ethidium staining.

the onset of DNA synthesis (determined by FACS analysis of DNA content) and was higher in S and G2 cells than in G1 cells. We conclude that none of the delayed early mRNAs assayed changes appreciably during the L-cell division cycle.

Changes in delayed early gene expression in nonfibroblastic cells. To determine whether induction of the delayed early genes identified in 3T3 cells is a general response to growth factors, we tested nonfibroblastic mouse and rat cell lines for the induction of these genes by specific growth factors, as assessed by Northern blotting. We also tested the response in the livers of rats subjected to partial hepatectomy and in the hippocampi of rats following electroshock, conditions that induce a number of the same immediate-early transcription factors found in growth factor-stimulated 3T3 cells (10, 30, 43). Examples of the experimental findings are shown in Fig. 5, and the results are summarized in Table 2. As seen in Table 2, some of the delayed early mRNAs induced in 3T3 cells (DER3, DER5, DER6, DER8, DER9, DER10, DER12, and DER13) were also induced or constitutively expressed in a mouse epidermal cell line (BALB/MK) stimulated with EGF and in a mouse T-cell line (CTLL) stimulated with IL-2. Some of these (and others) were also found in regenerating rat liver and in a rat pheochromocytoma cell line (PC12) stimulated with NGF. However, none increased in PC12 cells after addition of NGF nor in the hippocampi of rats after electroshock. These results suggest (i) that several of the delayed early genes that we identified in 3T3 cells are not part of a general proliferation program and (ii) that compared with the immediate-early transcription factor response, the delayed early response is more diverse in different cell types.

Proteins encoded by DER6 and DER2 cDNAs. As indicated in Table 1, partial nucleotide sequences of the original DER6 and DER2 isolates indicated that they encode proteins related to human macrophage migration inhibitory factor MIF (40) and a family of transmembrane proteins related to the major intrinsic protein of the lens, MIP (14, 32), respec-

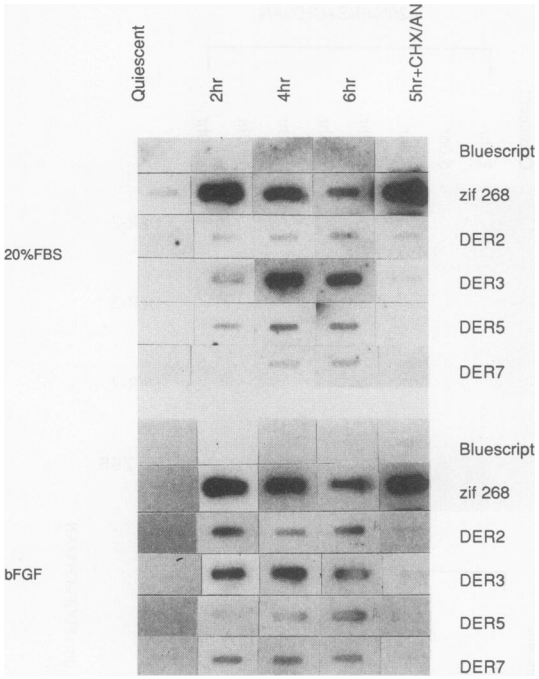

FIG. 3. Transcriptional activation of delayed early genes with 20% FBS or basic FGF (bFGF) (50 ng/ml). Isolated cell nuclei from quiescent cells and cells stimulated for 2, 4, and 6 h with serum were used in nuclear run-on transcription reactions. ^{32}P-labelled transcripts (equal counts from each reaction) were hybridized to 5 μg of denatured delayed early cDNAs in the vector pBluescript immobilized on nitrocellulose. In the lane labelled 5hr+CHX/AN, cycloheximide (10 μg/ml) and anisomycin (10 μM) were present in addition to serum or FGF. zif 268 is an immediate-early RNA.

tively. We therefore set out to determine the predicted primary structures of the DER6 and DER2 proteins.

The initial DER6 and DER2 clones were used to isolate nearly full-length cDNAs from BALB/c 3T3 cell cDNA libraries. The sequence of a 543-bp DER6 cDNA revealed a single long open reading frame encoding a protein of 115 amino acids (Fig. 6A). In vitro transcription and translation of this cDNA yielded a product of ~12 kDa. The predicted amino acid sequence of the DER6 protein is 88% identical to that of human MIF (40); the positions of three cysteine residues and two potential N glycosylation sites are nearly identical in the two proteins (Fig. 6A). Although it appears from the sequence comparison that DER6 and the human MIF are homologs, this inference may not be correct, since the DER6 cDNA probe hybridized with a large number of mouse, human, and rat genomic restriction fragments (Fig. 7). We conclude that the DER6 gene is a member of a family of MIF-related genes present in the mammalian genome.

In the case of DER2 cDNA, a 2,612-bp isolate was sequenced. It contained a single long open reading frame encoding a protein of 269 amino acids with six predicted transmembrane domains (Fig. 6B). A Southern blot of mouse genomic DNA indicated that DER2 is probably derived from a single-copy gene (data not shown). A search of the DNA and protein data banks revealed homology to the major intrinsic protein (MIP) of the lens and related family members, which are thought to constitute membrane channels (32). During preparation of the manuscript, Preston and Agre (33) reported the cDNA sequence of another member of the MIP family, CHIP28, the integral membrane protein of human erythrocytes. The DER2 protein has 94% amino acid identity to CHIP28 (Fig. 6B). Since the DER2 gene appears to be a single-copy gene in mice, we conclude that DER2 is the murine homolog of CHIP28.

We also determined the distribution of DER6 and DER2 mRNAs in mouse tissues and cell lines (Fig. 8). DER6

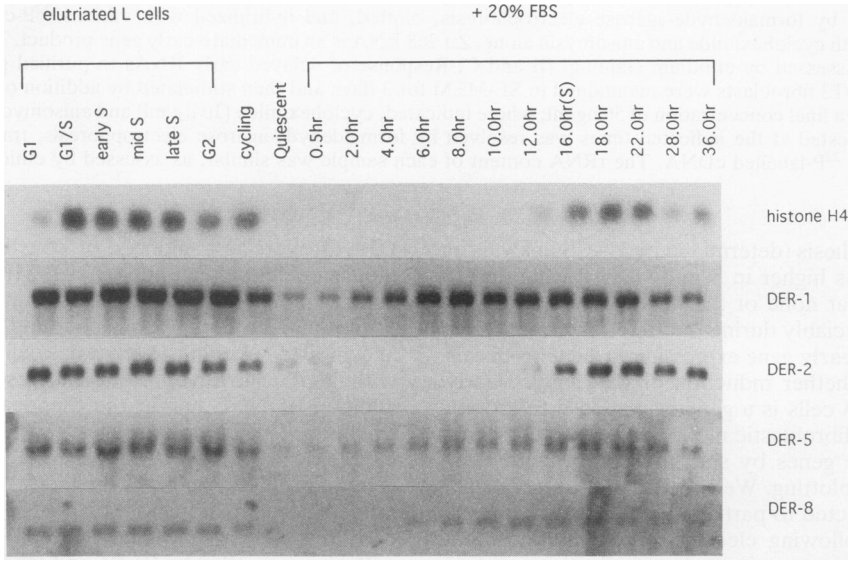

FIG. 4. Delayed early RNAs in elutriated mouse L cells and L cells stimulated with 20% FBS. Logarithmically growing mouse L cells were separated by centrifugal elutriation into different size classes that were analyzed for DNA content by cell sorting. Confluent cultures of mouse L cells were incubated for 3 days in DMEM-0.1 and stimulated by addition of fresh DMEM-20. In each case, 10 μg of total RNA was harvested at the indicated times or 20 μg from the indicated cell fraction was resolved by formaldehyde-agarose electrophoresis, transferred to nitrocellulose filters, and hybridized to various ^{32}P-labelled cDNAs. H4 is a human histone H4 cDNA probe. The rRNA content of each sample was similar, as assessed by ethidium staining.

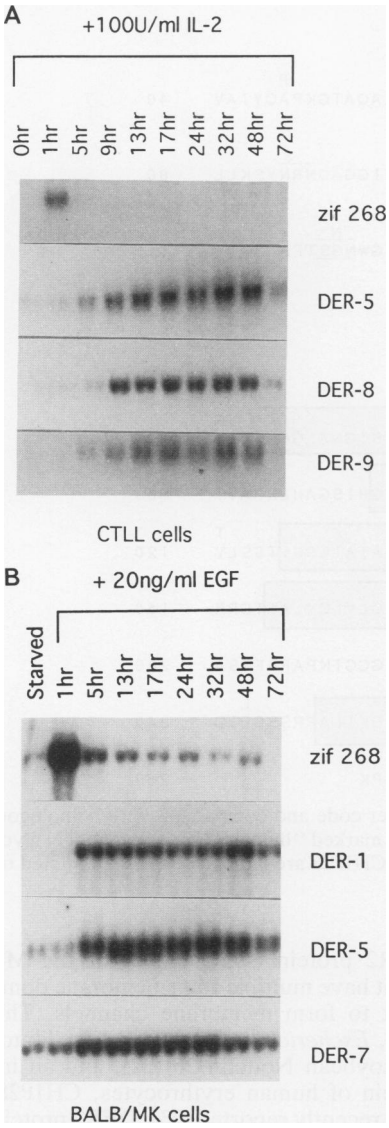

FIG. 5. (A) Response of delayed early RNAs to IL-2 in mouse T cells. IL-2-dependent CTLL cells were maintained in growth medium without IL-2 for 24 h and then stimulated by direct addition of IL-2 to a concentration of 100 U/ml. Total RNA (10 μg) harvested at the indicated times was analyzed by Northern blotting with various ^{32}P-labelled cDNA probes. zif 268 is an immediate-early RNA. (B) Response of delayed early RNAs to EGF in BALB/MK epithelial cells. Subconfluent cultures of BALB/MK cells were incubated for 3 days in growth medium without EGF and then stimulated by direct addition of EGF to a concentration of 20 ng/ml. Total RNA (10 μg) harvested at the indicated times was analyzed by Northern blotting with various ^{32}P-labelled cDNA probes.

mRNA (or a related RNA with a similar size) was present in every tissue examined and was most abundant in brain, ovary, and kidney tissues. DER2 mRNA was most abundant in the kidney, spleen, lung and heart and was undetectable in the brain and pancreas.

DISCUSSION

In this report, we describe the isolation of cDNA clones corresponding to delayed early response genes of BALB/c 3T3 cells stimulated by serum, PDGF, or FGF. From this collection we have so far identified 13 different species that constitute approximately 40% of the 650 isolates. On the assumption that most of the more abundant species have already been detected, we estimate that several dozen different delayed early cDNAs in our collection remain to be characterized.

Of the 13 clones that have been partially or completely sequenced, 5 of the cDNA clones which we isolated correspond to genes not previously identified, i.e., not entered in the DNA sequence data banks. However, for some of these, we do not yet have complete sequences. Like a number of immediate-early genes identified by cDNA cloning, some may turn out to encode proteins with recognizable sequence motifs that will provide clues to function. Eight of the cDNAs encode proteins that have been described previously or are related to known proteins. Among the known proteins are HMGI(Y) and HMGI-C, nonhistone chromosomal proteins that bind to A/T rich sequences in DNA (28, 35, 39). The physiological functions of these proteins are not known. Although they are elevated in proliferating cells, including neoplastic cells of spontaneous or induced tumors (13), a regulatory role in cell proliferation is not established. The regulation of HMGI and HMGI-C gene expression in stimulated 3T3 cells is clearly different from that of histone genes, whose expression begins later, coincident with the onset of the S phase (17, 21). In view of the appearance of these mRNAs in the pre-S phase of the induced cell cycle, the question of whether the proteins play a role in the G0-to-S transition arises. Another gene in this category is that encoding adenine phosphoribosyltransferase (APRT), a key enzyme in the salvage pathway of adenine nucleotide biosynthesis. An increase in this protein would presumably increase the pool of dATP needed for DNA replication.

TABLE 2. Induction of delayed early RNAs in different cell types[a]

Delayed early response gene[b]	Results of induction in cell type or source[c]:				
	BALB/MK (EGF stimulated)	CTLL (IL-2 stimulated)	Regenerating liver	PC12 (NGF stimulated)	Hippocampus (with MES)[d]
DER1	+	−	−	−	−
DER2	−	−	+	−	−
DER3	+	+	+[e]	−	−
DER4	−	−	−	−	−
DER5	+	+	−	c	−
DER6	c	+	+	c	c
DER7	+	−	+	c	−
DER8	c	+	−	c	−
DER9	c	+	+	c	c
DER10	c	+	+	c	c
DER11	−	+	−	−	−
DER12	+	+	−	−	−
DER13	+	+	c	c	c

[a] BALB/MK and CTLL cells were stimulated as described in the legend to Fig. 5, and total RNA was analyzed by Northern blotting with various cDNA probes. For regenerating rat liver, NGF-stimulated PC12 cells, and hippocampal RNA from electroshocked rats, conditions were as described in Materials and Methods. In each case, timed RNA samples were analyzed by Northern blotting.

[b] Gene expressed in BALB/c 3T3 fibroblastic cells.

[c] +, induced; −, not detected; c, constitutive.

[d] MES, multiple electroconvulsive shock.

[e] Determined for mouse liver because the DER3 gene is not detectable in the rat genome.

A

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HUMAN MIF                 D                   P
DER6      MPMFIVNTNVPRASVPEGFLSELTQQLAQATGKPAQYIAV    40

HUMAN MIF        A G SSE                    S
DER6      HVVPDQLMTFSGTNDPCALCSLHSIGKIGGAQNRNYSKLL    80

HUMAN MIF     AE  R                 S   N
DER6      CGLLSDRLHISPDRVYINYYDMNAANVGWNGSTFA         115
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B

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CHIP28        F                   T                K  VG
DER2      MASEIKKKLFWRAVVAEFLAMTLFVFISIGSALGFNYPLE    40

CHIP28    N   A
DER2      RNOTLVQDNVKVSLAFGLSIATLAQSVGHISGAHLNPAVT    80

CHIP28                F  L                        T
DER2      LGLLLSCQISILRAVMYIIAQCVGAIVATAILSGITSSLV    120

CHIP28    G         D
DER2      DNSLGRNDLAHGVNSGQGLGIEIIGTLQLVLCVLATTDRR    160

CHIP28
DER2      RRDLGGSAPLAIGLSVALGHLLAIDYTGCGINPARSFGSA    200

CHIP28     I H                                    L
DER2      VLTRNFSNHWIFWVGPFIGGALAVLIYDFILAPRSSDFTD    240

CHIP28    V
DER2      RMKVWTSGQVEEYDLDADDINSRVEMKPK               269
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FIG. 6. Predicted amino acid sequences of DER6 (A) and DER2 (B) in single-letter code and comparison with homologous proteins. In panel A, only amino acid differences between DER6 and MIF are indicated in the lines marked "human MIF." Potential N glycosylation sites in each protein are underlined. In panel B, amino acid differences between DER2 and CHIP28 are indicated, as are predicted transmembrane domains (boxed and shaded) and potential N glycosylation sites (underlined).

A third DER cDNA, which has been only partially sequenced, corresponds to a cDNA encoding a previously described member of the murine cyclin family CYL1 (29). CYL1 was discovered through cDNA cloning from mRNAs that are induced in mouse macrophages by colony-stimulating factor 1 with kinetics typical of a delayed early mRNA (29). Its human homolog was discovered in three separate contexts: (i) via a cDNA (CYC D1) that can correct the growth defect of *Saccharomyces cerevisiae* CLN mutants (44), (ii) as the product of the *bcl-1* gene activated in certain B-lymphocyte malignancies (42), and (iii) as the PRAD1 gene product implicated in the genesis of parathyroid adenomas (2). CYL1 has been shown to associate with a $p34^{cdc2}$-related protein (29) and is a prime candidate for a key regulator of the G1-to-S transition in mammalian cells.

Two of the cDNAs we have sequenced encode proteins related to MIF and MIP, respectively. The DER6-encoded protein has 88% sequence identity with human MIF. A DER6 probe hybridizes with a large number of human and rodent genomic DNA restriction fragments. This finding suggests that there is a family of related MIF-like genes; therefore it is uncertain whether DER6 is the murine homolog of human MIF or a closely related family member. Although MIF was initially detected as a macrophage MIF produced by T cells (5, 11), we have found that DER6 mRNA (or closely related RNAs) is present in a wide variety of mouse tissues and cell lines. We suspect that DER6 and other members of the MIF family have a broad range of cytokine activities other than inhibition of macrophage motility, some of which are related to cell proliferation.

The DER2 protein is a member of the MIP family of proteins that have multiple transmembrane domains (14) and are thought to form membrane channels. This family includes MIP, *Escherichia coli* glycerol facilitator, *Drosphila* big brain, soybean Nodulin 26 (32), and an integral membrane protein of human erythrocytes, CHIP28, whose sequence was recently reported (33). DER2 protein shows 94% amino acid sequence identity to CHIP28. Since the DER2 gene appears to be a single-copy gene, we conclude that the DER2 protein is the murine homolog of CHIP28. CHIP28 has been reported to form water channels in *Xenopus* oocytes microinjected with CHIP28 cDNA (34). Our results suggest that such channels are induced in 3T3 cells in response to growth factors.

As shown by nuclear run-on assays, all the delayed early response genes we have identified are transcriptionally activated within a few hours of serum, FGF, or PDGF stimulation of resting cells, and all require new protein synthesis for expression, suggesting that immediate-early transcription factors are required to activate these genes. The availability of a number of delayed early response genes, including those described previously (27), will make it possible to study regulation of the induced genetic program in some detail, including the involvement of immediate-early transcription factors in the activation of given genes. Of particular interest is whether a specific member of a transcription factor family (e.g., a specific Fos-Jun heterodimer) activates a given target gene. Another interesting aspect of the regulation of delayed early genes is its cell and stimulus specificity. Our survey indicates that some of the DER genes activated by PDGF or

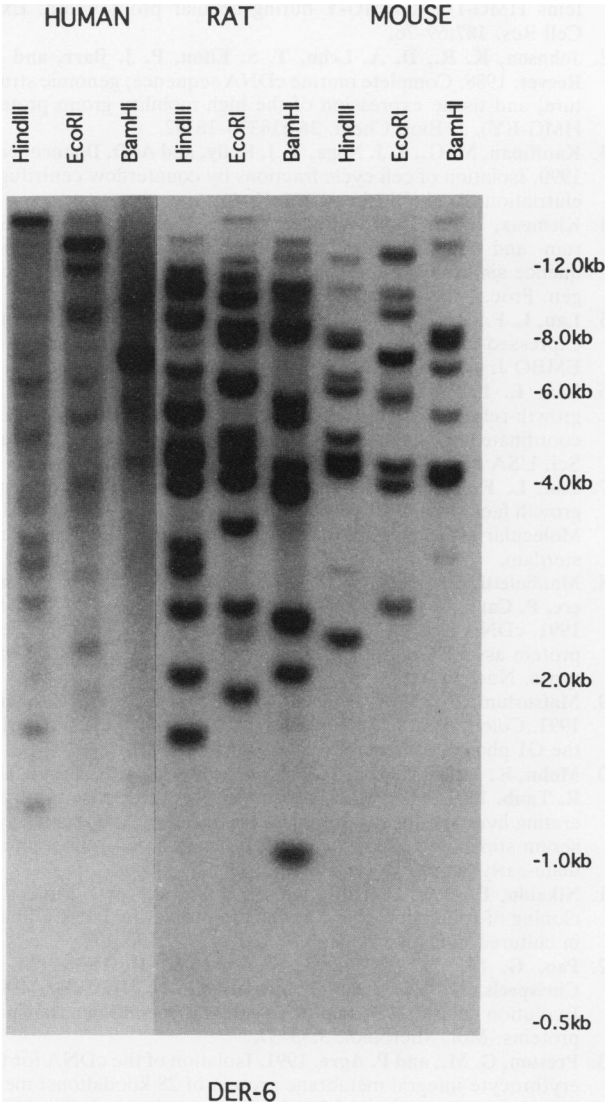

FIG. 7. Analysis of human, rat, and mouse genomic DNA for DER6-related sequences. DNA was prepared from BALB/c mouse liver, Sprague-Dawley rat liver, and human blood. DNA was digested with the restriction enzymes indicated, fractionated on agarose gels, transferred to nitrocellulose, and hybridized with the 543-bp DER6 cDNA probe. The lanes labelled mouse and human contained 10 μg of DNA, and the lanes labelled rat contained 15 μg of DNA. The panel showing mouse and rat sequences was exposed for 1 day, and the panel of human samples was exposed for 5 days.

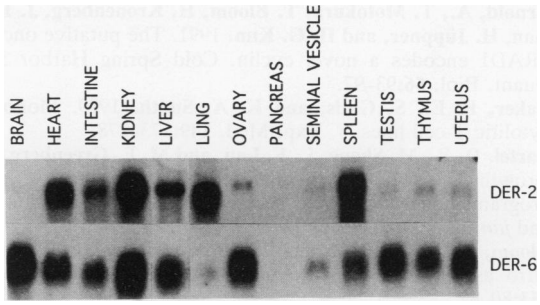

FIG. 8. Tissue distribution of DER6 and DER2 RNAs. Total RNA (20 or 10 μg [ovary, liver, and lung]) was isolated from adult mouse tissues and fractionated on agarose-formaldehyde gels. After the RNA was transferred to nitrocellulose sheets, duplicate blots were hybridized with either a 543-bp DER6 or a 2,612-bp DER2 cDNA probe.

FGF in fibroblastic 3T3 cells are also activated or expressed constitutively in rat liver after partial hepatectomy, by EGF in an epidermal cell line, and by IL-2 in a T-cell line. These genes are the best candidates for direct involvement in the proliferative response. On the other hand, none of the genes is activated in the hippocampi of rats following electroshock or in PC12 cells stimulated with NGF (although some are constitutively expressed). Since these stimuli rapidly induce many of the immediate-early transcription factors identified in 3T3 cells (4, 10), the results suggest that a different set of delayed early genes is likely to be activated in the neuronal cells, as anticipated from the different physiologic responses. Activation of these genes may be dependent on additional cell-specific transcription factors that act in combination with the common factors, on cell-specific modification of immediate-early transcription factors, or on structural accessibility of particular transcriptional regulatory elements.

In contrast to the observations of serum-stimulated cells, exponentially growing cells showed little or no change in the levels of delayed early mRNAs during the cell cycle. This difference in transcriptional response of stimulated quiescent cells and cycling cells suggests that growth factor-induced genes play a special role during the transition of cells from G0 to S phase, perhaps to replenish proteins that are degraded during prolonged cellular quiescence. It should be noted, however, that we have not directly measured transcriptional activities of DER genes in cycling cells, leaving open the possibility that changes in transcription are masked by high levels of stable mRNAs.

Finally, it is apparent that the genetic program induced by a single polypeptide growth factor in quiescent cultured fibroblasts is quite complex. A large number of immediate-early genes and a large but undetermined number of delayed early genes are part of the program. Since growth factors often have multiple effects on cells, it is likely that two or more genetic programs, not all of which are related to cell proliferation, are induced simultaneously. Determining whether a given delayed early gene plays a role in a specific cell response will require demonstration of the effects of underexpression or overexpression of the gene or inactivation of the encoded protein. Clues may also come from protein structural homology to known regulators of the cellular response, such as the finding of cyclin-related delayed early genes (29).

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REFERENCES

1. **Almendral, J. M., D. Sommers, H. Macdonald-Bravo, J. Burckhardt, J. Perea, and R. Bravo.** 1988. Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. **8**:2140–2148.

2. **Arnold, A., T. Motokura, T. Bloom, H. Kronenberg, J. Ruderman, H. Jüppner, and H. G. Kim.** 1991. The putative oncogene PRAD1 encodes a novel cyclin. Cold Spring Harbor Symp. Quant. Biol. **56:**93–97.
3. **Baker, P. E., S. Gillis, and K. A. Smith.** 1979. Monoclonal cytolitic T-cell lines. J. Exp. Med. **149:**273–278.
4. **Bartel, D. P., M. Sheng, L. F. Lau, and M. E. Greenberg.** 1989. Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of *fos* and *jun* induction. Genes Dev. **3:**304–313.
5. **Bloom, B. R., and B. Bennett.** 1966. Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. Science **153:**80–82.
6. **Bravo, R.** 1989. Growth factor inducible genes in fibroblasts. *In* A. Habenich (ed.), Growth factors, differentiation factors and cytokines. Springer-Verlag, Berlin.
7. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
8. **Christy, B. A., L. F. Lau, and D. Nathans.** 1988. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. USA **85:**7857–7861.
9. **Cochran, B. H., A. C. Reffel, and C. D. Stiles.** 1983. Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell **33:**939–947.
10. **Cole, A. J., S. Abu-Shakra, D. W. Saffen, J. M. Baraban, and P. Worley.** 1990. Rapid rise in transcription factor mRNAs in rat brain after electroshock-induced seizures. J. Neurochem. **55:**1920–1927.
11. **David, J. R.** 1966. Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. Proc. Natl. Acad. Sci. USA **56:**72–77.
12. **Dush, M. K., J. M. Sikela, S. A. Khan, J. A. Tischfield, and P. J. Stambrook.** 1985. Nucleotide sequence and organization of the mouse adenine phosphoribosyltransferase gene: presence of a coding region common to animal and bacterial phosphoribosyltransferases that has a variable intron/exon arrangement. Proc. Natl. Acad. Sci. USA **82:**2731–2735.
13. **Giancotti, V., E. Buratti, L. Perissin, S. Zorzet, A. Balmain, G. Portella, A. Fusco, and G. H. Goodwin.** 1989. Analysis of the HMGI mouse nuclear proteins in mouse neoplastic cells induced by different procedures. Exp. Cell Res. **184:**538–545.
14. **Gorin, M. B., S. B. Yancy, J. Cline, J. P. Ravel, and J. Horowitz.** 1984. The major intrinsic protein of the bovine lens fiber membrane: characterization and structure based on cDNA cloning. Cell **39:**49–59.
15. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature (London) **311:**433–438.
16. **Groudine, M., M. Peretz, and H. Weintraub.** 1981. Transcriptional regulation of hemoglobin switching in chicken embryos. Mol. Cell. Biol. **1:**281–288.
17. **Heintz, N., H. L. Sive, and R. G. Roeder.** 1983. Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. Mol. Cell. Biol. **3:**539–550.
18. **Herschman, H. R.** 1991. Primary response genes induced by growth factors and tumor promoters. Annu. Rev. Biochem. **60:**281–319.
19. **Hodgson, C. P., P. K. Elder, T. Ono, D. N. Foster, and M. J. Getz.** 1983. Structure and expression of mouse VL30 genes. Mol. Cell. Biol. **3:**2221–2231.
20. **Huynh, T. V., R. A. Young, and R. W. Davis.** 1985. Constructing and screening cDNA libraries in λgt10 and λgt11, p. 49. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press, Oxford.
21. **Johnson, K. R., J. E. Disney, C. R. Wyatt, and R. Reeves.** 1990. Expression of mRNAs encoding mammalian chromosomal proteins HMG-I and HMG-Y during cellular proliferation. Exp. Cell Res. **187:**69–76.
22. **Johnson, K. R., D. A. Lehn, T. S. Elton, P. J. Barr, and R. Reeves.** 1988. Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMG-I(Y). J. Biol. Chem. **263:**18338–18342.
23. **Kauffman, M. G., S. J. Noga, T. J. Kelly, and A. D. Donnenberg.** 1990. Isolation of cell cycle fractions by counterflow centrifugal elutriation. Anal. Biochem. **191:**41–46.
24. **Klemenz, R., S. Hoffmann, and A.-K. Werenskiold.** 1989. Serum- and oncoprotein-mediated induction of a gene with sequence similarity to the gene encoding carcinoembryonic antigen. Proc. Natl. Acad. Sci. USA **86:**5708–5712.
25. **Lau, L. F., and D. Nathans.** 1985. Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. EMBO J. **4:**3145–3151.
26. **Lau, L. F., and D. Nathans.** 1987. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-*fos* or c-*myc*. Proc. Natl. Acad. Sci. USA **84:**1182–1186.
27. **Lau, L. F., and D. Nathans.** 1991. Genes induced by serum growth factors, p. 257–293. *In* P. Cohen and J. G. Foulkes (ed.), Molecular aspects of cellular regulation, vol. 6. Elsevier, Amsterdam.
28. **Manfioletti, G., V. Giancotti, A. Bandiera, E. Buratti, P. Sautiere, P. Cary, C. Crane-Robinson, B. Coles, and G. H. Goodwin.** 1991. cDNA cloning of the HMGI-C phosphoprotein, a nuclear protein associated with neoplastic and undifferentiated phenotypes. Nucleic Acids Res. **19:**6793–6797.
29. **Matsushime, H., M. F. Roussel, R. A. Ashmun, and C. J. Sherr.** 1991. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell **65:**701–713.
30. **Mohn, K. L., T. M. Laz, J.-C. Hsu, A. E. Melby, R. Bravo, and R. Taub.** 1991. The immediate-early growth response in regenerating liver and insulin-stimulated H-35 cells: comparison with serum-stimulated 3T3 cells and identification of 41 novel immediate-early genes. Mol. Cell. Biol. **11:**381–390.
31. **Nikaido, T., D. W. Bradley, and A. B. Pardee.** 1991. Molecular cloning of transcripts that accumulate during the late G1 phase in cultured mouse cells. Exp. Cell Res. **192:**102–109.
32. **Pao, G. M., L. F. Wu, K. D. Johnson, H. Höfte, M. J. Chrispeels, G. Sweet, N. N. Sandal, and M. H. Saier.** 1991. Evolution of the MIP family of integral membrane transport proteins. Mol. Microbiol. **5:**33–37.
33. **Preston, G. M., and P. Agre.** 1991. Isolation of the cDNA for the erythrocyte integral membrane protein of 28 kilodaltons: member of an ancient channel family. Proc. Natl. Acad. Sci. USA. **88:**11110–11114.
34. **Preston, G. M., T. P. Carroll, W. B. Guggino, and P. Agre.** 1992. Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. Science **256:**385–387.
35. **Reeves, R., and M. S. Nissen.** 1990. The A-T DNA-binding domain of the mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. J. Biol. Chem. **265:**8573–8582.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
38. **Sive, H. L., and T. St. John.** 1988. A simple subtractive hybridization technique employing photoactivatable biotin and phenol extraction. Nucleic Acids Res. **16:**10937–10938.
39. **Solomon, M. J., F. Strauss, and A. Varshavsky.** 1986. A mammalian high mobility group protein recognizes any stretch of six A · T base pairs in duplex DNA. Proc. Natl. Acad. Sci. USA **83:**1276–1280.
40. **Weiser, W. Y., P. A. Temple, J. S. Witek-Giannotti, H. G. Remold, S. C. Clark, and J. R. David.** 1989. Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor. Proc. Natl. Acad. Sci. USA **86:**7522–7526.
41. **Weissman, B. E., and S. A. Aaronson.** 1983. BALB and Kirsten

murine sarcoma viruses alter growth and differentiation of EGF-dependent BALB/c mouse epidermal keratinocyte lines. Cell **32**:599–606.

42. **Withers, D. A., R. C. Harvey, J. B. Faust, O. Melnyk, K. Carey, and T. C. Meeker.** 1991. Characterization of a candidate *bcl-1* gene. Mol. Cell. Biol. **11**:4846–4853.
43. **Worley, P. F., A. J. Cole, T. H. Murphy, B. A. Christy, Y. Nakabeppu, and J. M. Baraban.** 1990. Synaptic regulation of immediate early genes in brain. Cold Spring Harbor Symp. Quant. Biol. **55**:213–223.
44. **Xiong, Y., T. Connolly, B. Futcher, and D. Beach.** 1991. Human D-type cyclin. Cell **65**:691–699.