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Molecular cloning, sequencing, and mapping of *EGR2*, a human early growth response gene encoding a protein with "zinc-binding finger" structure

(cell growth/transcriptional regulator/multigene family/DNA-binding domain)

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ABSTRACT **Early growth response gene-1 (*Egr-1*) is a mouse gene displaying *fos*-like induction kinetics in diverse cell types following mitogenic stimulation. *Egr-1* encodes a protein with "zinc-binding finger" structure. Zinc fingers are a protein structural motif that serve as DNA-binding domains in several transcriptional regulatory proteins. Using low-stringency hybridization with an Egr-1 cDNA probe, we identified a distinct human cDNA (designated EGR2 for early growth response gene-2), which is coregulated with *EGR1* by fibroblast and lymphocyte mitogens; however, several stimuli that induce Egr-1 mRNA in PC12 (rat pheochromocytoma) cells do not induce Egr-2 mRNA. The cDNA sequence predicts a protein of 406 amino acids, including three tandem zinc fingers of the Cys_2-His_2 class. Strikingly, the deduced amino acid sequences of human EGR2 and mouse Egr-1 are 92% identical in the zinc finger region but show no similarity elsewhere. *EGR2* maps to human chromosome 10 at bands q21–22. Structure–function analysis of EGR2 and EGR1 proteins should provide insight into the mechanisms linking signal transduction and transcriptional regulation of gene expression.**

Genes controlling proliferation or differentiation of eukaryotic cells have been identified by differential screening (1–5) of cDNA libraries. We (6, 7) and others (2, 3) have identified cDNAs the expression of which is upregulated by serum stimulation of quiescent mouse fibroblasts. One cDNA from our initial screening was also induced by epithelial cell and lymphocyte mitogens (7). The cDNA for this mouse early growth response gene (*Egr-1*) encodes a protein that contains three "zinc-binding fingers" of the Cys_2-His_2 subclass (6, 8, 9). *Egr-1* expression is also modulated during neuronal (4, 6) and cardiac differentiation and after cellular depolarization (6). These data suggest a role for *Egr-1* as a nuclear intermediary in signal transduction. We used low-stringency hybridization with an *Egr-1* finger-region probe to isolate several distinct human cDNAs. We report the cDNA sequence, functional characterization, and mapping of one of these clones designated EGR2.‖ Recently, others have used a similar procedure to identify murine clones that cross-hybridize with the Krüppel finger region (10–12). We show that one of these clones, Krox-20, is the murine homologue of *EGR2*. Egr-1 [NGF1-A of Milbrandt (4)], EGR2/Krox-20, and additional EGR cDNAs (L.J.J., V.P.S., unpublished data) encode zinc fingers with remarkable amino acid sequence conservation *throughout* the putative DNA-binding domains, suggesting that they recognize a similar set of target DNA sequences. The differences outside of the finger domains might be important in understanding their other regulatory interactions.

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MATERIALS AND METHODS

Cell Culture. Cell lines 303 and HSWP (human foreskin fibroblasts) are from J. R. Smith (Baylor College of Medicine) and M. Regan (Oak Ridge), respectively. PC12 cells were provided by C. Palfrey (University of Chicago). Cell culture methods were as described (6).

RNA and Southern Hybridizations (13). All blots were done with GeneScreen*Plus* (New England Nuclear–DuPont), except RNA dot blots, for which GeneScreen was used. Hybridizations were at 65°C in 1% $NaDodSO_4$/10% dextran sulfate/1 M NaCl for 16 hr. Filters were washed at room temperature in 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), next at 65°C in 2× SSC/1% $NaDodSO_4$ (low stringency), then at 65°C in 2× SSC/1% $NaDodSO_4$ (moderate stringency), and finally at 65°C in 0.1× SSC (high stringency). Probes were made by random hexamer priming (14). RNA for hybridization analysis was isolated by the method of Chirgwin *et al.* (15), whereas for dot blots the method of Cheley and Anderson (16) was used.

DNA Sequencing. Sequencing was done by the dideoxynucleotide chain-termination method of Sanger *et al.* (17) and the double-stranded method of Zagursky *et al.* (18).

Chromosomal Localization. The methods used have been described (19–23).

RESULTS

Isolation of EGR2 cDNA Clones. A 2.1-kb *Apa* I–*Apa* I mouse *Egr-1* fragment that includes the finger region (6) was used at low stringency to screen a lambda ZAP (Stratagene, San Diego, CA) cDNA library constructed from RNA extracted from cells (303 cell line) 3 hr after serum (20%) stimulation and cycloheximide (10 μg/ml) treatment. Of several positive plaques obtained, clones Zap 2, Zap 8, and Zap 32 (Fig. 1) hybridized to a finger-region probe from *Egr-1* but not to probes flanking the finger region and contained common restriction fragments when cut with 4-base cutters. Fig. 2*A* shows an RNA blot of cell line 303 3 hr after stimulation with serum and cycloheximide probed with the

Abbreviations: *EGR1* and *EGR2*, human early growth response genes; *Egr-1* and *Egr-2*, rodent early growth response genes; nt, nucleotide(s); PMA, phorbol 12-myristate 13-acetate.

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‖The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J04076).

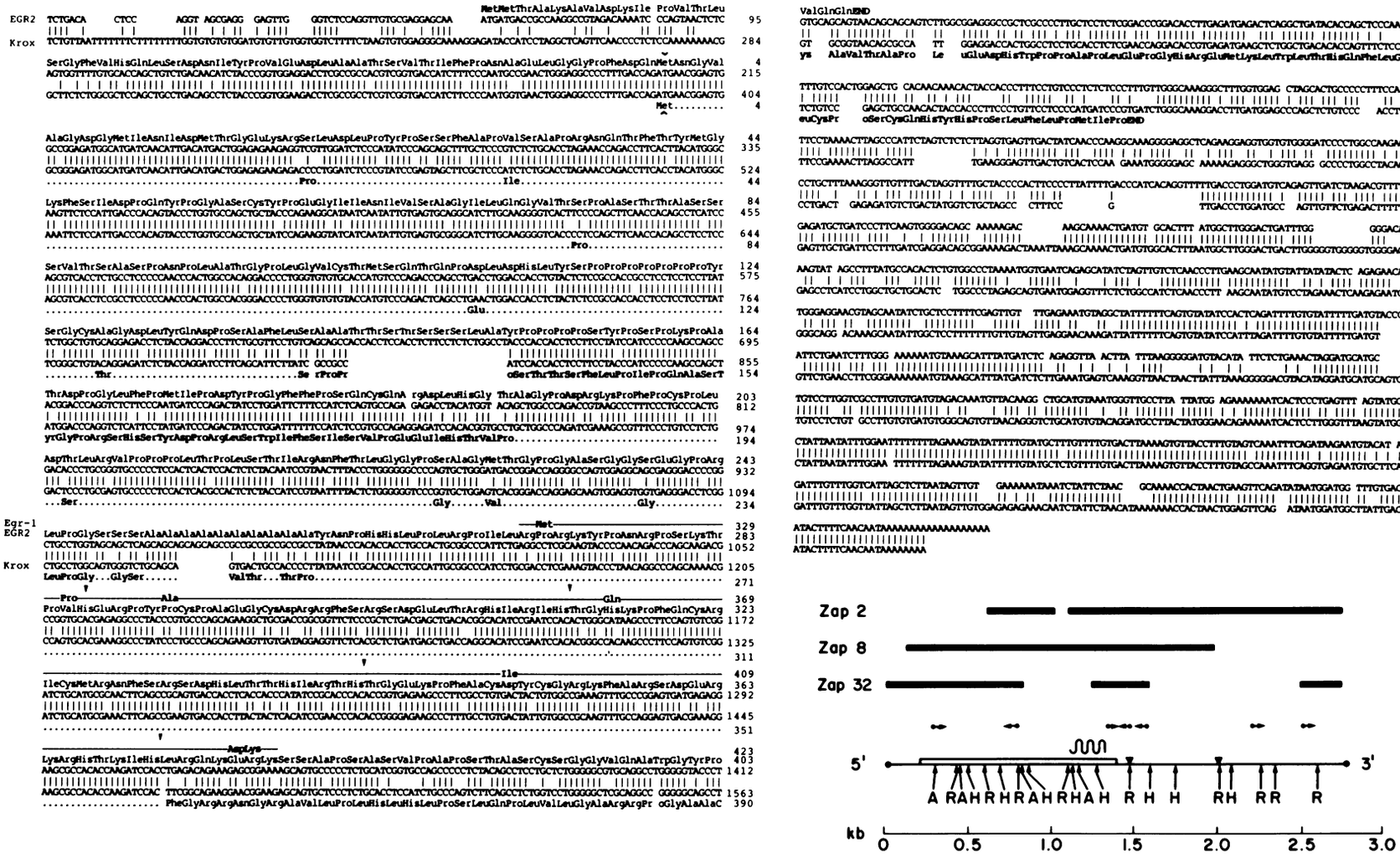

FIG. 1. Restriction map of *EGR2* and sequence comparison with Krox-20 and Egr-1 clones. The open box indicates the coding sequence of EGR2. The regions of each cDNA clone that has been sequenced are darkened. *All Rsa* I (R) restriction endonuclease sites are indicated, *only* those *Hae* III (H) and *Alu* I (A) endonuclease sites used to subclone fragments for sequencing are shown. The location of synthetic oligonucleotide primers and their orientations are shown (●→). Arrowheads (▼) indicate the boundaries of the *Rsa* I–*Rsa* I fragment subcloned into pUC19, designated P80. The zinc fingers are shown schematically. The *EGR2* nucleotide sequence is shown with the deduced amino acid sequence, including a 5′ region extending beyond the putative initiator methionine, which is marked by a carat. The Krox-20 nucleotide and amino acid sequences are shown below EGR2. The finger domain of Egr-1 is shown above the corresponding amino acid sequence of EGR2. Amino acids of Krox-20 sequence and of Egr-1 protein that are identical to the corresponding amino acid of EGR2 protein are shown as (· · ·) and (- - -), respectively. Each finger domain is marked by arrowheads. The polyadenylylation consensus signal is denoted by an arrow.

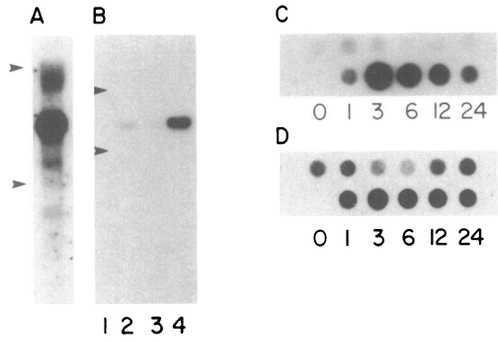

FIG. 2. EGR2 mRNA expression in human fibroblasts. (*A*) RNA blot analysis of EGR2 mRNA expression in confluent serum-deprived cell-line 303 human fibroblasts 3 hr after stimulation with 20% fetal calf serum and cycloheximide. Ten micrograms of total RNA was electrophoresed through a 1% formaldehyde gel, blotted, and probed with the ^{32}P-labeled insert of clone Zap 2. The filter was washed at moderate stringency and exposed for 18 hr without an intensifying screen. Arrowheads indicate the location of the 28S and 18S rRNAs. (*B*) RNA analysis of *EGR2* expression in confluent serum-deprived HSWP cells after stimulation with 20% fetal calf serum. Five micrograms of total RNA were loaded in each lane. Cells were harvested as follows: no treatment (lane 1); 1 hr (lane 2); 3 hr (lane 3); 3 hr after stimulation with serum and cycloheximide (lane 4). The filter was probed with the P80 insert, washed at high stringency, and exposed for 8 days with a single intensifying screen. (*C* and *D*) Extended time course of *EGR2* and *EGR1* induction. HSWP cells treated as in *B*. Replicate dot blots were prepared. Both the top row (serum only) and bottom row (serum and cycloheximide) are from cells harvested at 0, 1, 3, 6, 12, and 24 hr after stimulation. The filter in Fig. 2*C* was probed with the P80 insert. The filter in *D* was probed with an *Msp* I–*Rsa* I 700-base pairs (bp) probe from the region 3′ to the finger domains of *Egr-1* (6). The filters in *C* and *D* were washed to high stringency and exposed overnight with a single intensifying screen.

Zap 2 insert. Multiple bands were detected after moderately stringent washing, but the band at 3.2 kilobases (kb) was dominant and designated as the *EGR2*-encoded transcript.

EGR2 mRNA Is Induced in Serum-Stimulated G_0 Human Fibroblasts. We asked whether *EGR2* is induced in human fibroblasts by serum in the absence of cycloheximide. HSWP cells were used to exploit the extensive characterization of mitogen-stimulated events in that line (24, 25). Because EGR1 mRNA might cross-hybridize to *EGR2*, a *nonfinger*-encoding *Rsa* I fragment from Zap 8 (Fig. 1) was subcloned into pUC19 and designated P80. Fig. 2*B* shows that the EGR2 mRNA level is elevated in HSWP cells at 1 hr and barely detectable at 3 hr after serum stimulation. Cycloheximide addition results in superinduction. Fig. 2 *C* and *D* show a replicate dot blot comparison of *EGR2* and *EGR1* induction in these cells over 24 hr. The signal intensities suggest that the level of EGR2 mRNA induced was several-fold lower than the level of EGR1 mRNA.

EGR2 Is Induced in Phorbol 12-Myristate 13-Acetate (PMA)-Stimulated Human Mononuclear Cells. To see whether induction of EGR2 mRNA was specific for fibroblasts or a more general phenomenon, we examined human lymphocytes. Fig. 3*A* shows an RNA blot analysis of human peripheral blood mononuclear cells subsequent to PMA stimulation. The P80 probe detects two transcripts at 2.5 and 3.2 kb after high-stringency washing.

Egr-1 But Not Egr-2 mRNA Is Inducible in PC12 Cells. Several stimuli induce Egr-1 mRNA in PC12 cells (4, 6). RNA was prepared from PC12 cells 1 hr after stimulation with the agents indicated in Fig. 3*B–D*. Fig. 3*B* shows the results of hybridization with an *Egr-1* probe that includes the finger-encoding region. Fig. 3*C* shows a replicate filter probed with the Zap 2 insert that includes the finger region of *EGR2*. The filter shown in Fig. 3*D* was probed with P80, a nonfinger

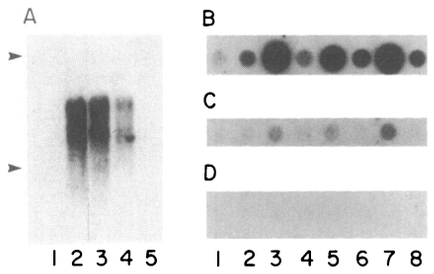

FIG. 3. EGR2 mRNA expression in human lymphocytes and in PC12 cells. (*A*) Peripheral blood was separated on Ficoll/Hypaque. Mononuclear cells (5×10^7) were used for each point. Cells were resuspended in medium containing 10% fetal calf serum and PMA (25 ng/ml). Five micrograms of total RNA was loaded in each lane. Cells were harvested at intervals after stimulation: 0 hr, no treatment (lane 1); 45 min (lane 2); 2.5 hr (lane 3); 5 hr (lane 4); 1 hr after maintenance in autologous serum (L.J.J.) without PMA (lane 5). The filter was probed with the P80 plasmid, washed to high stringency, and exposed overnight with a single intensifying screen. (*B–D*) RNA dot blot comparison of *Egr-1* and *Egr-2* expression in PC12 cells. Cells were harvested at 1 hr after stimulation with the agents indicated. Replicate RNA dot blot filters were made. (*A–D*) 1, no treatment; 2, sham treatment with serum-free medium; 3, PMA (100 nM); 4, nerve growth factor (100 ng/ml); 5, epidermal growth factor (100 ng/ml); 6, calcium ionophore A23187 (5 μg/ml); 7, bradykinin (100 ng/ml); 8, A23187 (5 μg/ml) plus PMA (100 nM). (*B*) This filter was probed with a 2.1-kb *Apa* I–*Apa* I probe, including the finger region, of *Egr-1* (6), washed to high stringency, and exposed overnight with a single intensifying screen. (*C*) This replicate was probed with the Zap 2 insert, washed to moderate stringency, and exposed overnight with a single screen. (*D*) This filter was probed with the insert of P80, washed to moderate stringency, and exposed overnight with a single screen.

region probe from *EGR2*: no hybridization is detected after a 3-day exposure. These results indicate that the weak signal seen in Fig. 3*C* is from cross-hybridization due to the finger region and sets an upper limit on the level of induction of other zinc finger-encoding transcripts.

cDNA Sequence. Fig. 1 shows the restriction map of the EGR2 cDNA and its complete nucleotide sequence. The sequence is 2719 nucleotides (nt) long and terminates in a poly(A) tract. There are in-frame termination codons at nt 3 and 15. Following these are several methionine codons; however, none fulfill the Kozak (26) criterion for an initiator codon: RNNATGG, where R represents adenine or guanine. The most 5′ ATG is usually the functionally important initiator; a common exception is for protooncogenes (26). The methionine designated as amino acid number 1 (nt 204) for EGR2 corresponds to the most 5′ methionine reported in the Krox-20 protein. We made this choice based on the fact that the nucleotide comparison (see below) of *EGR2* and Krox-20 sequences suggests that they are homologues. The two nucleotide sequences diverge before this methionine. This ATG initiates an open reading frame of 1218 nt, terminating at the stop codon at nt 1422. A polyadenylylation signal consensus sequence, AATAAA (27), is located at nt 2681, 14 nt before the poly(A) tract.

Structural Features of the Deduced Amino Acid Sequence. The cDNA sequence predicts a protein of 406 amino acids with a M_r of 43,307. Amino acids 286–370 form three tandem zinc fingers of the form Thr-Gly-Xaa$_3$-(Tyr/Phe)-Xaa-Cys-Xaa$_{2-4}$-Cys-Xaa$_3$-Phe-Xaa$_5$-Leu-Xaa$_2$-His-Xaa$_3$-His described as a consensus sequence for members of the Cys$_2$-His$_2$ class (6, 28, 29). The fingers are connected by H–C links [Thr-Gly-Glu-(Arg/Lys)-Pro-(Phe/Tyr)-Xaa], a highly conserved motif described by Schuh *et al.* (30) and found in the Egr-1 protein and other, but not all, members of the Cys$_2$-His$_2$ family. EGR2, like Egr-1, is rich in proline (15%), serine (11%), alanine (8%), and threonine (7%) residues. There is a

Table 1. Concordancy analysis of somatic cell hybrid panel

	Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Concordant	(+/+)	7	10	16	10	16	9	12	15	8	25	11	19	10	18	11	9	17	16	6	18	18	6	11
hybrids, no.	(−/−)	7	6	6	6	6	7	3	4	7	7	6	5	4	3	7	7	3	6	6	5	3	5	4
Discordant	(+/−)	16	16	7	16	10	17	13	11	16	0	13	7	16	8	15	17	7	10	20	8	8	19	8
hybrids, no.	(−/+)	0	1	1	1	1	0	3	3	0	0	1	2	3	4	0	0	4	1	1	2	4	2	3
	% Discordancy	53	52	27	52	33	52	52	42	52	0	45	27	58	36	45	52	35	33	64	30	36	66	42

The table is compiled from 33 cell hybrids involving 15 unrelated human cell lines and 4 mouse cell lines (21–23). The P80 *EGR2* probe was hybridized to Southern blots of *Eco*RI-digested DNA from human–mouse hybrids. The *EGR2* gene localization was determined by scoring the presence or absence of a human band in the hybrids. Concordant hybrids have either retained or lost the human *EGR2* band together with a specific chromosome or the reverse. These concordances are designated (+/+) and (−/−), respectively, where the first symbol denotes the presence or absence of the human *EGR2* band and the second symbol denotes the presence or absence of the specific human chromosome. Percent discordancy indicates the degree of discordant segregation for a marker and a chromosome ([−/+] or [+/−]). A 0% discordancy is the basis for chromosome assignment. The *EGR2* gene mapped to human chromosome 10. DNA from hybrid XTR-3BSAgB with no intact chromosome 10 but retaining 10pter → 10q23 showed hybridization with the P80 probe indicating that *EGR2* is located 10pter → 10q23.

run of 7 consecutive prolines (amino acids 117–123) and of 13 serines and alanines (amino acids 247–259). The high content of threonines and serines suggests that EGR2 could be phosphorylated, a potentially important means of regulation.

Comparison of the Amino Acid Sequence of the Zinc Finger Regions. The amino acid sequence of the finger domains of EGR2 is 100% identical with that of Krox-20 sequence (Fig. 1). The finger region sequence of EGR2/Krox-20 shows an average identity of 37% with the Krüppel fingers (11, 12, 30), due primarily to the conserved H–C link sequence. The loop of the first finger of EGR2/Krox-20 sequence matches the loop of the second finger of the transcription factor Sp1 at 8 of 12 amino acids and at 10 of 12 amino acids when one includes conservative changes (12, 29). More striking is the 92% identity between the amino acid sequence of the EGR2/Krox-20 zinc finger region and the corresponding region in Egr-1 (nucleotide identity in region is 78%). Notably, none of the four amino acid differences between Egr-1 and EGR2 are located at the "finger tips," which are thought to contact the target DNA sequence (31). There is marked sequence similarity among Egr-1, EGR2, and Krox-20 sequence immediately 5′ of the finger region and for Egr-1 and EGR2 immediately 3′ of the finger region (Fig. 1). There is no significant similarity elsewhere between Egr-1 and EGR2.

***EGR2* (Human) and Krox-20 (Murine) Sequences Are Homologues.** There is extensive nucleotide similarity between EGR2 and Krox-20 cDNAs (Fig. 1), suggesting that these two cDNAs are homologues. The overall nucleotide identity is 75% (87% in the coding region and 89% in the finger region). The amino acid identity is 84% from the initiator methionine to the last amino acid of the finger domains (after which the two deduced sequences diverge markedly because of a single nucleotide difference at position 1314 in *EGR2*—see *Discussion*).

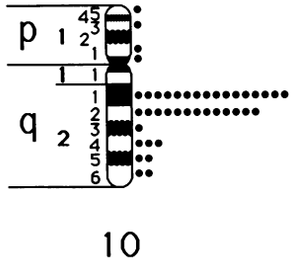

FIG. 4. Distribution of labeled sites on chromosome 10. The figure summarizes the analysis of 100 normal human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes that were hybridized with the EGR2 Zap 32 cDNA probe. Each dot indicates one labeled site observed in the corresponding band. Seventy percent (29/41) of the labeled sites on chromosome 10 were located at q21–22; this cluster represented 18.1% of all labeled sites (29/160).

Chromosomal Localization. The results of Southern blot analysis of genomic DNA from mouse × human hybrids probed with the P80 (nonfinger region) plasmid are shown in Table 1. The discordancy scores localize the *EGR2* gene to chromosome 10. To determine the chromosomal sublocalization of *EGR2*, we hybridized the Zap 32 plasmid to normal human metaphase chromosomes. Of 100 metaphase cells examined from this hybridization, 31 (31%) were labeled on region q2 of one or both chromosome 10 homologues. The distribution of labeled sites on chromosome 10 is illustrated in Fig. 4; of 160 total labeled sites observed, 41 (25.6%) were located on this chromosome. These sites were clustered at bands q21–22, and this cluster represented 18.1% (29/160) of all labeled sites (cumulative probability for the Poisson distribution is $<<0.0005$). Thus, these results indicate that the *EGR2* gene is localized to chromosome 10 at bands q21–22. However, 20 grains, representing 12.5% ($P < 0.0005$) of all labeled sites were seen at 3p24–26. Similar results were obtained in three additional hybridization experiments with this probe. The observation of specific labeling on both chromosomes 3 and 10 in hybridizations using the Zap 32 cDNA probe, which contains the *EGR2*-encoded finger domain sequence, raised the possibility that this probe was hybridizing to another finger domain-containing gene located on the short arm of chromosome 3. Use of the P80 probe, which does not contain finger domain sequences, resulted in specific labeling of the proximal long arm of chromosome 10. Of 147 labeled sites seen in 100 metaphase cells, 21 (14.3%, $P << 0.0005$) were located at 10q21–22. Hybridization of this probe resulted in a substantial reduction of labeling on 3p; however, a few grains of unknown significance were noted at 3p24–26. Two additional experiments resulted in specific labeling only of the long arm of chromosome 10.

DISCUSSION

A major goal of cell biology is to analyze the molecular mechanisms controlling gene expression. An important component of this network are DNA-binding proteins with transcriptional activity. The importance of such proteins in cell growth is suggested by the finding that *c-jun* (the cellular homologue of the oncogene *v-jun*) encodes the transcription factor AP1 (32, 33), the discovery of *c-jun*-related genes (34) that are growth-factor regulated, and the identification of a zinc-finger-encoding gene (*Egr-1*) coregulated with *c-fos* (6). This paper reports the isolation and characterization of a second mitogen-inducible cDNA (designated EGR2), which also encodes a protein with zinc fingers. Furthermore, its levels are elevated after growth stimulation in fibroblasts as well as in lymphocytes; thus, like *Egr-1*, the expression of *EGR2* is not restricted to one cell type. *EGR2* is therefore probably involved in the network of genes controlling the proliferative response. Whether *EGR2* acts to transmit, am-

plify, or limit responses to such stimuli is unknown. Whether *EGR2* expression is specific to the G_o–G_1 transition remains to be determined. However, unlike *Egr-1*, *Egr-2* induction is not seen in PC12 cells after stimulation by various agents, suggesting that differences exist in the 5′ regulatory regions of these genes.

Several reports show that the zinc-finger region alone confers sequence specificity of binding (35–37). The amino acids at the tips of the Cys_2-His_2 loops are thought to be responsible for DNA contact (31). The surprisingly high degree of amino acid similarity of Egr-1 and EGR2 throughout the finger region and dissimilarity elsewhere offers a rare example among the Cys_2-His_2 zinc-finger proteins for comparing structure and function. As a working hypothesis it seems reasonable that Egr-1 and EGR2 might recognize the same DNA target sequences through their zinc fingers but that interactions with other transcriptional regulatory elements could differ greatly.

The high level of nucleotide similarity throughout EGR2 and Krox-20 sequence suggests they are homologues. Although some differences in amino acid sequence could represent alternative splicing of small exons or evolutionary divergence, this possibility is unlikely to explain the extensive amino acid dissimilarity 3′ to the finger domains resulting from the single nucleotide frameshift at position 1314 in the EGR2 sequence. We sequenced this area on three independently selected clones (Fig. 1). In addition, in our predicted sequence the four amino acids immediately after the last histidine of the third zinc finger match perfectly the corresponding four amino acids in Egr-1 thereby extending the region of their amino acid identity.

The results of *in situ* chromosomal hybridization and the Southern blot analysis of somatic cell hybrids demonstrate that *EGR2* maps to 10q21–22. Although few structural rearrangements involving the long arm of chromosome 10 have been seen in human tumors, a loss of an entire chromosome 10 has been reported as a recurring abnormality in gliomas in adults (38). Relatively few genes have been mapped to 10q21–22; of these, only the gene(s) implicated in multiple endocrine neoplasia type 2A and the gene for lipocortin IIc are potentially involved in cell activation or growth (39, 40). As a result of the putative regulatory activity of *EGR2*, loss of this gene could lead to deregulated cell growth.

Other EGR cDNAs exist that encode proteins with highly related zinc fingers to those in Egr-1 and EGR2 (L.J.J., V.P.S., unpublished data). This multigene family offers a rich opportunity to investigate the relationship of signal transduction to gene expression in normal and transformed cells.

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