Transcriptional Induction of the Murine c-*rel* Gene with Serum and Phorbol-12-Myristate-13-Acetate in Fibroblasts

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Transcription of the c-*rel* proto-oncogene was induced transiently when resting mouse NIH 3T3 fibroblasts were stimulated with serum or phorbol-12-myristate-13-acetate. Addition of cycloheximide increased the steady-state levels of c-*rel* mRNA. These results indicate that c-*rel* is another member of the early-response gene family.

Expression of proto-oncogenes is modulated during cell growth, differentiation, and development. On the basis of their putative functions, the products of proto-oncogenes can be classified into the following three categories: (i) growth factors and receptors, (ii) mediators of signal transduction, and (iii) transcriptional regulators. Several investigators have shown that the mRNA levels of nuclear proto-oncogene products, like *fos*, *myc*, and *jun*, also referred to as early-response genes (33), increase in response not only to mitogens but to a wide variety of agents ranging from differentiation factors to pharmacological drugs (1, 4, 12, 19, 22–25, 31). The nuclear proteins *fos* and *jun* have been shown to be regulators of eucaryotic transcription (5, 13, 14, 26, 28). Because p59$^{v\text{-}rel}$, the product of the transforming gene of reticuloendotheliosis virus (10, 11), was detected in the nuclei of infected fibroblasts (16, 17, 36) and has the ability to *trans*-activate the transcription of certain promoters (15), we became interested in determining whether c-*rel*, the cellular homolog of v-*rel* (40), also belongs to the family of early-response genes.

The c-*rel* proto-oncogene product shares extensive amino acid sequence identities with the *Drosophila* dorsal gene product (37), which is essential for establishing dorsal-ventral polarity in developing embryos (reviewed in reference 2). The dorsal protein is specifically localized in the peripheral nuclei of syncytial and cellular blastoderm stage embryos (38). In contrast, the chicken c-*rel* protein has been identified only in the cytosolic fraction (35) and is expressed most abundantly in cells of the lymphoid lineage (7, 9).

Serum-inducible c-*rel* expression. The major mouse c-*rel* mRNA has previously been reported as 7.5 kilobases (7). An RNA of this size was barely detectable by Northern (RNA) blotting with a c-*rel* probe in mouse fibroblasts stimulated with serum, but it was clear that Northern analysis would not be sufficiently sensitive for quantitation. Therefore, we turned to an RNase protection assay to examine the kinetics of induction of c-*rel* gene expression on addition of serum to resting NIH 3T3 mouse fibroblast cells (Fig. 1A). Within 30 min of serum addition, a fourfold increase in the steady-state levels of c-*rel* cytoplasmic RNA detected by RNase protection analysis was observed when normalized to β_2-microglobulin expression (lane 2). Maximal levels of induced RNA
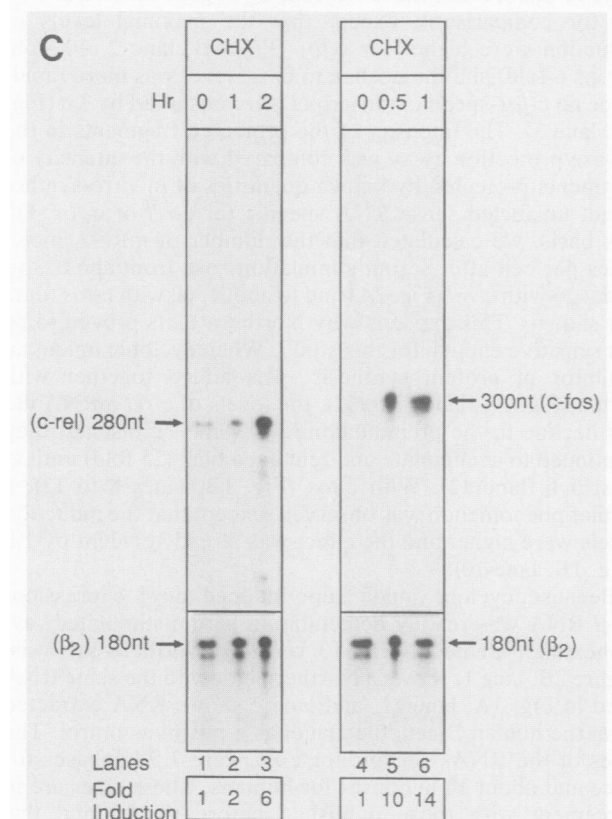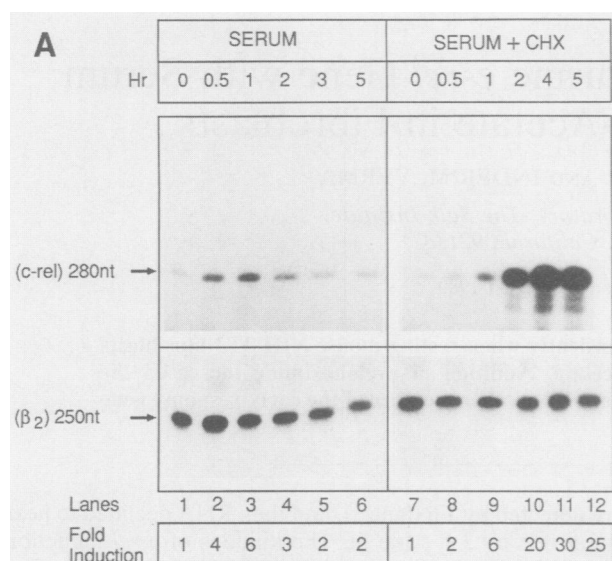
were detected by 1 h (lane 3), and then RNA declined to near basal levels by 3 h (lane 5). The kinetics of c-*rel* induction were reminiscent of those with the c-*fos* gene (shown in Fig. 1B for comparison), except that the maximal levels of induction were higher for c-*fos* (Fig. 1B, lane 2, 100-fold versus 6-fold) and the decline to basal level was more rapid, since no c-*fos*-specific transcripts were observed by 3 h (Fig. 1B, lane 3). The intensity of the protected fragments in the RNase protection assay was compared with the intensity of fragments protected by known quantities of in vitro-synthesized, unlabeled sense RNA specific for c-*rel* or c-*fos*. On this basis, we calculated that the number of mRNA molecules per cell after serum stimulation rose from about 3 to about 20 with c-*rel* (Fig. 2A) and to about 500 with c-*fos* (data not shown). This explains why Northern blots proved to be not sensitive enough for this study. When cycloheximide, an inhibitor of protein synthesis, was added together with serum (Fig. 1A, lanes 7 to 12), the levels of c-*rel* mRNA did not decline to the preinduction level (lane 7); instead, they continued to accumulate and remained high (25-fold) until at least 5 h (lane 12). With c-*fos* (Fig. 1B, lanes 8 to 12), a similar phenomenon was observed, except that the induction levels were higher and the effect was already evident by 1 h (Fig. 1B, lane 10).

Because cycloheximide superinduced c-*rel* expression, c-*rel* RNA was readily detectable in serum-stimulated, cycloheximide-treated NIH 3T3 cells by Northern analysis. Figure 2B, lane 1, shows a Northern blot with the same RNA used in Fig. 1A, lane 11, and lane 2 shows RNA extracted from the human B-cell line Daudi as a positive control. The sizes of the RNAs specific for c-*rel* were 7.5 kilobases for mice and about 10 kilobases for humans. These sizes are in agreement with those published before (7, 9), and this confirms that we studied bona fide c-*rel* mRNA induction. Cycloheximide alone, without serum, induced an increase in the steady-state levels of both c-*rel* and c-*fos* mRNAs, but the increase was lower than with cycloheximide and serum together (Fig. 1C). Nuclear run-on experiments with cycloheximide alone showed no increase in the levels of either c-*rel* or c-*fos* nuclear transcripts (data not shown), thus supporting the notion that cycloheximide acts to enhance the posttranscriptional stability of these mRNAs. This indicates that at least part of c-*rel* activation by serum is independent of protein synthesis, a fact which had previously been demonstrated for c-*fos* and c-*myc* (18, 22).

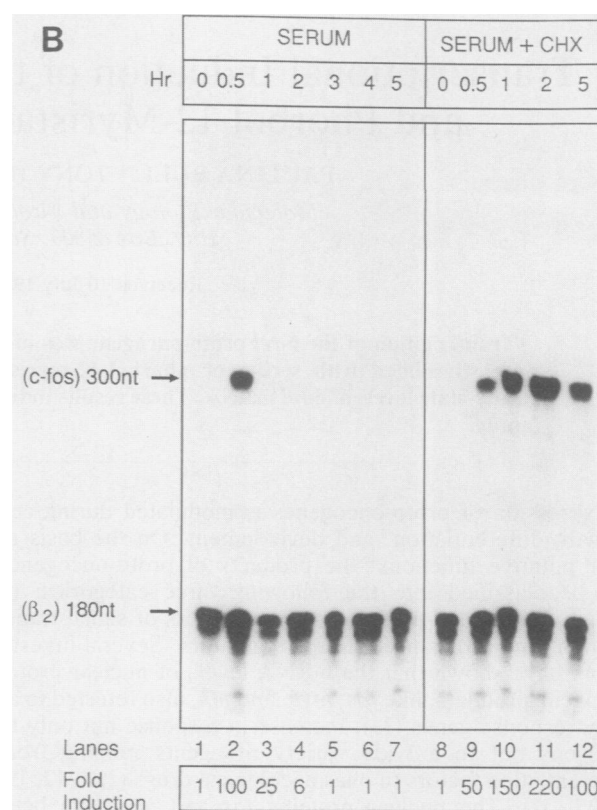Expression of c-*rel* was also induced when NIH 3T3 cells

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mRNA induced by serum were due to increased transcription, nuclear run-on experiments were performed with nuclei isolated from serum-starved and 20% fetal bovine serum-treated cells and used for RNA transcription in vitro. A fourfold increase in c-*rel*-specific transcripts was detected at 1 h; this then declined to the basal level by 4 h (Fig. 4). As controls, similar assays for c-*fos* and β_2-microglobulin showed an 11-fold increase in c-*fos* gene transcription, whereas the levels of β_2-microglobulin transcripts were unchanged (Fig. 4). Since the time course of increase in nuclear run-on is consistent with the time course of accumulation of mRNA (Fig. 1), we conclude that, as with c-*fos*, the increase in c-*rel* mRNA following addition of serum occurs at the level of transcription.

Our results indicate that c-*rel* is a member of the growing family of early-response genes. This finding is in agreement with that of Bravo et al. (6) that one of the many serum-inducible genes in mouse fibroblasts has homology with the chicken c-*rel* gene. The premise that c-*rel* is a transcriptional regulator has been partially confirmed by the finding that v-*rel* can induce the transcription of a number of promoters (15). Furthermore, chimeric constructs containing a *lexA* DNA-binding domain linked to the chicken c-*rel* proto-oncogene has been shown to *trans*-activate the transcription of β-galactosidase downstream of a *lexA* operator and the *Saccharomyces cerevisiae GAL1* promoter in *S. cerevisiae* cells (T. Gilmore, personal communication). Although we have shown that c-*rel* is inducible in fibroblasts in response to serum or TPA, it is constitutive in lymphoid cells (7, 9; P. Bull, unpublished data). Interestingly, lymphoid cells are the only cells in which constitutive expression of c-*rel* has been observed. The dual localization of v-*rel* protein in either the cytoplasm or nucleus (16, 17) is reminiscent of another lymphoid transcription factor, NF-κB, which is found in the

were treated with the phorbol ester phorbol-12-myristate-13-acetate (TPA; Fig. 3). A fivefold increase in steady-state levels was observed when cells were treated with 80 ng of TPA per ml for 60 min (lane 3). The kinetics of induction with TPA essentially paralleled those of serum induction (Fig. 1A). Again, the effect was greater with c-*fos* (shown in parallel for comparison [30]). The induction with TPA suggests that c-*rel* expression can be modulated by protein kinase C.

c-*rel* induction at the level of transcription. The data in Fig. 1 show augmented steady-state levels of c-*rel* mRNA. To determine whether the augmented steady-state levels of c-*rel*

FIG. 1. Analysis of c-*rel* mRNA following induction. Confluent 150-mm-diameter dishes of NIH 3T3 cells were serum starved (0.5% serum) for 24 h before stimulation. The starved cells were induced with 20% fetal bovine serum (A and B), 20% fetal bovine serum and 10 μg of cycloheximide (CHX) per ml (A and B), or 10 μg of cycloheximide per ml alone (C). Cytoplasmic RNA was isolated at the indicated times as described by Maniatis et al. (29), and c-*rel*, c-*fos*, and β$_2$-microglobulin mRNA levels were quantitated by an RNase protection assay by hybridizing in solution 20 μg of RNA and an anti-sense ^{32}P-labeled RNA probe for c-*rel*, c-*fos*, or β$_2$-microglobulin. Digestion of hybridized products by single-strand-specific RNase A and RNase T1 yielded protected c-*rel*, c-*fos*, and β$_2$-microglobulin species that were separated by electrophoresis on 4% polyacrylamide–6 M urea gels. The gels were exposed with an intensifying screen at −80°C for 1 h to detect β$_2$-microglobulin and for 12 h to detect c-*rel*. For c-*fos* detection, the exposure time was 1 (B) or 12 (C) h. The bands were quantified by densitometry of the X-ray film (A and C) or by Cerenkov counting (B). The ratios of the densitometric readings for c-*rel* or c-*fos* to that of the β$_2$-microglobulin band at each time interval were calculated, and the fold induction (compared with zero time) is shown at the bottom of each panel. Each series of experiments was performed at least twice with similar results. The c-*rel*-specific probe was obtained as follows. A mouse partial cDNA fragment encompassing nucleotides (nt) 941 to 1221 (numbered as described by Grumont and Gerondakis [21]) was obtained from a mouse methylcholanthrene-transformed C3H 10T 1/2 Okayama-Berg cDNA expression library by amplification through polymerase chain reaction (PCR), using the following oligonucleotides as primers for the PCR reaction: 5'CCAAATACTGCAGAATTAAGG AT3' and 5'TCACTAACTTCCTGGTCAGAAGG3'. The PCR fragment obtained (280 base pairs long) was cloned by blunt-end ligation into the *Sma*I site of the Bluescript SK(−) vector (Stratagene, La Jolla, Calif.) and sequenced as single-stranded DNA with the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio), which uses the chain termination method of Sanger et al. (34). The sequence obtained was identical to the reported mouse c-*rel* cDNA sequence (21), except that bases 966 (T), 967 (A), and 970 (C) were missing in our clone. It was named rel-PCR. This partial cDNA clone was used as a template to prepare a single-stranded antisense RNA probe using T3 RNA polymerase in a transcription reaction assay containing [α-^{32}P]UTP as described in the Promega Biotec catalog. The mouse c-*fos*-specific probe encompasses part of exon 4, from nucleotides 2,504 to 2,800 (39). The β$_2$-microglobulin-specific probes were obtained by using plasmid pSPT672 (which contains β$_2$-microglobulin cDNA) as a template for SP6 RNA polymerase cut with *Acc*I (for the 180-nucleotide probe) or *Hin*dIII (for the 250-nucleotide probe) (32).
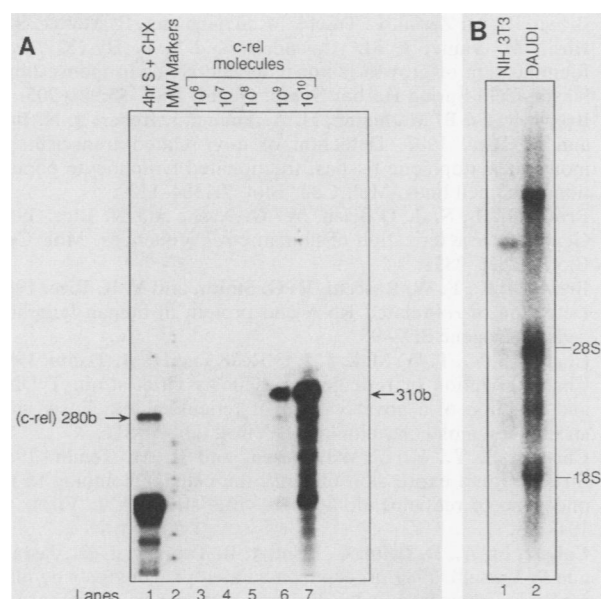

FIG. 2. (A) Quantification of the number of c-*rel* mRNA molecules. rel-PCR was linearized with *Bam*HI and transcribed in vitro with T7 RNA polymerase. RNA was purified by phenol extraction and column chromatography through a Sephadex G50 column and quantified by A_{260}. Tenfold dilutions were processed by an RNase protection experiment (lanes 3 to 7) together with 20 μg of RNA extracted from NIH 3T3 fibroblasts stimulated with 20% fetal bovine serum and 10 μg of cycloheximide (CHX) per ml (lane 1). The gel was exposed for 2 h. b, Bases. (B) Northern blot analysis of c-*rel* expression. A 50-μg sample of cytoplasmic RNA extracted from NIH 3T3 cells stimulated with 20% serum and 10 μg of cycloheximide per ml for 4 h (lane 1) or a sample of exponentially growing Daudi cells (lane 2) was fractionated by formaldehyde gel electrophoresis (29), blotted onto nitrocellulose, and hybridized with a nick-translated human c-*rel* probe (8). The specific activity of the probe was about 10^8 cpm/μg, and it was used at a concentration of 10^6 cpm/ml. Filters were hybridized overnight, and washes were performed at 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) at 55°C. The filters was exposed to X-ray film with an intensifying screen for 1 week.

cytoplasm and upon activation with TPA is translocated in the nucleus (3). It has been shown that in the cytoplasm NF-κB is bound to an inhibitor, which is removed upon TPA induction so that the protein can migrate to the nucleus to act as a transcription factor. The relatively restricted distribution of c-*rel* protein to lymphoid tissue suggests that it is a member of an NF-κB-like family of transcriptional regulators which can be inducibly shuttled into the nucleus. We are studying the role of c-*rel* protein in the transcriptional regulation of other cellular genes. In addition, we have preliminary evidence of detectable serum induction of a 80-kilodalton c-*rel* protein.
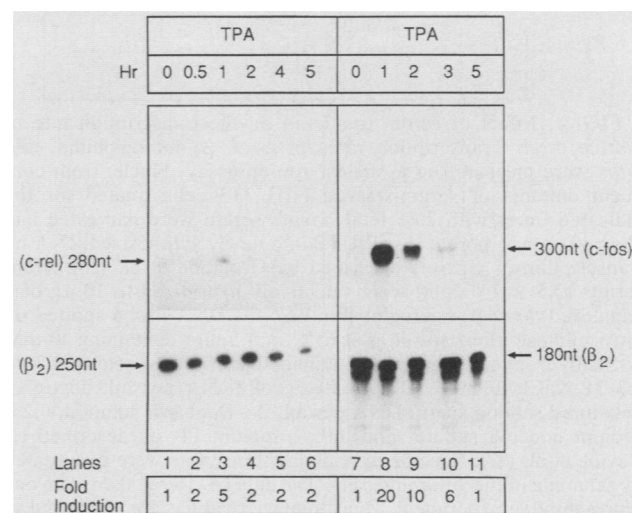

FIG. 3. Effect of TPA on c-*rel* mRNA levels. Confluent 150-mm-diameter dishes of NIH 3T3 cells were serum starved (0.5% serum) for 24 h before stimulation. The starved cells were treated with 80 ng of TPA per ml. Cytoplasmic RNA was isolated and steady-state c-*rel*, c-*fos*, and β$_2$-microglobulin mRNA levels were determined as described in the legend to Fig. 1. The gels were exposed for 12 h to detect c-*rel* and c-*fos* and for 1 h to detect β$_2$-microglobulin. Each experiment was done twice, with the same result. nt, Nucleotides.
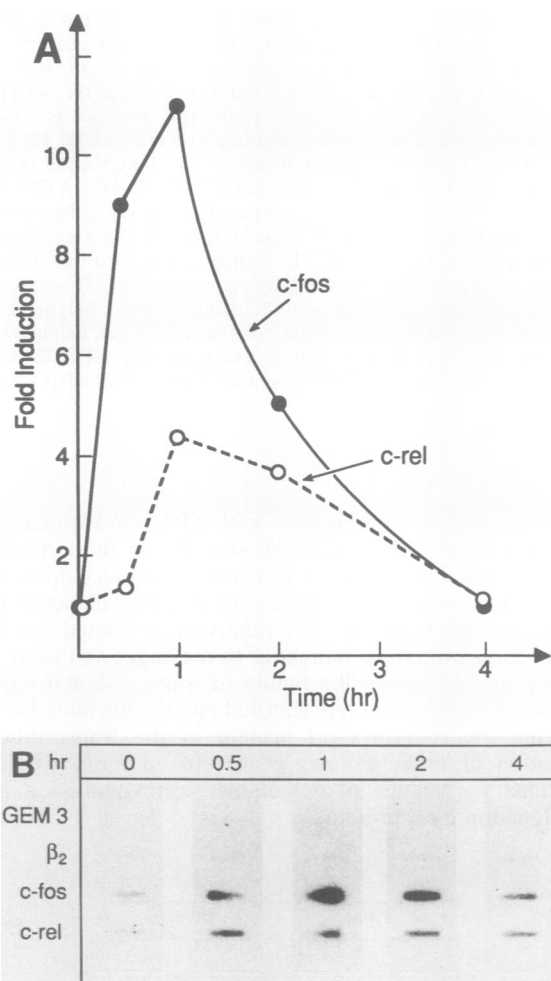
LITERATURE CITED

1. **Almendral, J. M., D. Sommer, H. MacDonald-Bravo, J. Burck-hardt, J. Perera, and R. Bravo.** 1988. Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. **8:**2140–2148.

2. **Anderson, K. V.** 1987. Dorsal-ventral embryonic pattern gene of Drosophila. Trends Genet. **3:**91–97.

3. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53:**211–217.

4. **Billestrup, N., R. L. Mitchell, W. Vale, and I. M. Verma.** 1987. Growth hormone-releasing factor induces c-fos expression in cultured primary pituitary cells. Mol. Endocrinol. **1:**300–305.

5. **Bohmann, D., T. J. Bos, A. Admon, T. Nishimura, P. K. Vogt, and R. Tjian.** 1987. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science **238:**1386–1392.

6. **Bravo, R., M. Zerial, L. Toschi, M. Schurmann, R. Muller, S. I. Hirai, M. Yaniv, J. M. Almendral, and R.-P. Ryseck.** 1988. Identification of growth factor-inducible genes in mouse fibroblasts. Cold Spring Harbor Symp. Quant. Biol. **53:**901–905.

7. **Brownell, E., B. Mathieson, H. A. Young, J. Keller, J. N. Ihle, and N. Rice.** 1987. Detection of c-rel-related transcripts in mouse hematopoietic tissues, fractionated lymphocyte populations, and cell lines. Mol. Cell. Biol. **7:**1304–1309.

8. **Brownell, E., S. J. O'Brien, W. G. Nash, and N. Rice.** 1985. Genetic characterization of human c-rel sequences. Mol. Cell. Biol. **5:**2826–2831.

9. **Brownell, E., F. W. Ruscetti, R. G. Smith, and N. R. Rice.** 1988. Detection of rel-related RNA and protein in human lymphoid cells. Oncogene **3:**93–98.

10. **Chen, I. S. Y., T. W. Mak, J. J. O'Rear, and H. M. Temin.** 1981. Characterization of reticuloendotheliosis virus strain T DNA and isolation of a novel variant of reticuloendotheliosis virus strain T by molecular cloning. J. Virol. **40:**800–811.

11. **Chen, I. S. Y., K. C. Wilhelmsen, and H. M. Temin.** 1983. Structure and expression of c-rel, the cellular homolog to the oncogene of reticuloendotheliosis virus strain T. J. Virol. **45:** 104–113.

12. **Collart, M. A., D. Belin, C. Briottet, B. Thorens, J.-D. Vassalli, and P. Vassalli.** 1989. Receptor-mediated phagocytosis by macrophages induces a calcium-dependent transient increase in c-fos transcription. Oncogene **4:**237–241.

13. **Distel, R. J., H.-S. Ro, B. S. Rosen, D. L. Groves, and B. M. Spiegelman.** 1987. Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct participation of c-fos. Cell **49:**835–844.

14. **Franza, B. R., Jr., F. J. Rauscher III, S. F. Josephs, and T. Curran.** 1988. The fos complex and fos-related antigens recognize sequence elements that contain AP-1 binding sites. Science **239:**1150–1153.

15. **Gelinas, C., and H. M. Temin.** 1988. The v-rel oncogene encodes a cell-specific transcriptional activator of certain promoters. Oncogene **3:**349–355.

16. **Gilmore, T. D., and H. M. Temin.** 1986. Different localization of the product of the v-rel oncogene in chicken fibroblasts and spleen cells correlates with transformation by REV-T. Cell **44:**791–800.

17. **Gilmore, T. D., and H. M. Temin.** 1988. v-rel oncoproteins in the nucleus and in the cytoplasm transform chicken spleen cells. J. Virol. **62:**703–714.

18. **Greenberg, M. E., A. L. Hermanowski, and E. B. Ziff.** 1986.

FIG. 4. Effect of serum treatment on the transcription rate of murine c-rel. Transcription rates of c-rel, β₂-microglobulin, and c-fos were measured in a nuclear run-on assay. Nuclei from confluent cultures of serum-starved NIH 3T3 cells treated for the indicated times with 20% fetal bovine serum were harvested and allowed to incorporate [α-³²P]UTP into newly synthesized RNA by transcription in vitro as described by Groudine et al. (20). Transcripts (2.5 × 10⁶ cpm) were selectively hybridized to 10 μg of a denatured c-rel, β₂-microglobulin, or c-fos DNA probe spotted on nitrocellulose filters for 40 h at 65°C in a buffer containing 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.4), 0.3 M NaCl, 10 mM EDTA, 50 μg of tRNA per ml, 50 μg of denatured salmon sperm DNA per ml, 2× Denhardt solution, 0.2% sodium dodecyl sulfate, and 0.05% sodium PPᵢ as described by Levine et al. (27). Specific hybridizing transcripts were quantitated by scanning of the autoradiogram. The data (A) were taken from one representative experiment. Densitometry readings are expressed as ratios of c-rel-to-β₂-microglobulin band intensities obtained after 1, 2, or 4 h of treatment as fold induction with respect to time zero. (B) Autoradiograph of hybridized transcripts on which the quantification was based. GEM 3, Plasmid pGEM3 (control).

Effect of protein synthesis inhibitors on growth factor activation of c-*fos*, c-*myc*, and actin gene transcription. Mol. Cell. Biol. 6:1050–1057.

19. 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.

20. Groudine, M., M. Peretz, and H. Weintraub. 1981. Transcriptional regulation of hemoglobin switching in chicken embryos. Mol. Cell. Biol. 1:281–288.

21. Grumont, R. J., and S. Gerondakis. 1989. Structure of a mammalian c-rel protein deduced from the nucleotide sequence of murine cDNA clones. Oncogene Res. 4:1–8.

22. Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder. 1983. Cell specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35:603–610.

23. Kruijer, W., D. Schubert, and I. M. Verma. 1985. Induction of the proto-oncogene *fos* by nerve growth factor. Proc. Natl. Acad. Sci. USA 82:7330–7334.

24. Lamph, W. W., P. Wamsley, P. Sassone-Corsi, and I. M. Verma. 1988. Induction of proto-oncogene JUN/AP-1 by serum and TPA. Nature (London) 334:629–631.

25. Lau, L. F., and D. Nathans. 1987. Expression of a set of growth-related immediate early genes in BALB/c3T3 cells: coordinate regulation with c-*fos* or c-*myc*. Proc. Natl. Acad. Sci. USA 84:1182–1186.

26. Lee, W., P. Mitchell, and R. Tjian. 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49:741–752.

27. Levine, R. A., J. E. McCormack, A. Buckler, and G. E. Sonenshein. 1986. Transcriptional and posttranscriptional control of c-*myc* gene expression in WEHI 231 cells. Mol. Cell. Biol. 6:4112–4116.

28. Lucibello, F. C., M. Neuberg, J. B. Hunter, T. Jenuwein, M. Schuermann, R. Wallich, B. Stein, A. Schonthal, P. Herrlich, and R. Muller. 1988. Transactivation of gene expression by *fos* protein: involvement of a binding site for the transcription factor AP-1. Oncogene 3:43–51.

29. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

30. Mitchell, R. L., C. Henning-Chubb, E. Huberman, and I. M. Verma. 1986. c-*fos* expression is neither sufficient nor obligatory for differentiation of monocytes to macrophages. Cell 45:497–504.

31. Muller, R., R. Bravo, J. Burckhardt, and T. Curran. 1984. Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. Nature (London) 312:716–720.

32. Parnes, J. R., and J. G. Seidman. 1982. Structure of wild-type and mutant mouse β₂-microglobulin gene. Cell 29:661–669.

33. Pledger, W. J., C. D. Stiles, H. N. Antoniades, and C. D. Scher. 1978. An ordered sequence of events is required before BALB/c-3T3 cells become committed to DNA synthesis. Proc. Natl. Acad. Sci. USA 75:2839–2843.

34. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.

35. Simek, S., and N. R. Rice. 1988. Detection and characterization of the protein encoded by the chicken c-rel proto-oncogene. Oncogene Res. 2:103–119.

36. Simek, S. L., R. M. Stephens, and N. R. Rice. 1986. Localization of the v-rel protein in reticuloendotheliosis virus strain T-transformed lymphoid cells. J. Virol. 59:120–126.

37. Stewart, R. 1987. *dorsal*, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, c-rel. Science 238:692–694.

38. Stewart, R., S. B. Zusman, L. H. Huang, and P. Schedl. 1988. The *dorsal* protein is distributed in a gradient in early *Drosophila* embryo. Cell 55:487–495.

39. Van Beveren, C., F. van Straaten, T. Curran, R. Muller, and I. M. Verma. 1983. Analysis of FBJ-MuSV provirus and c-*fos* (mouse) gene reveals that viral and cellular *fos* gene products have different carboxy termini. Cell 32:1241–1255.

40. Wilhelmsen, K. C., K. Eggleton, and H. M. Temin. 1984. Nucleic acid sequences of the oncogene v-rel in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-rel. J. Virol. 52:172–182.