The Fos Protein Complex Is Associated with DNA in Isolated Nuclei and Binds to DNA Cellulose

LIDIA C. SAMBUCETTI AND TOM CURRAN*

The properties of the viral and cellular *fos* proteins (Fos) were investigated as a first step toward understanding the function of the *fos* gene. Treatment of nuclei with salt and nonionic detergents solubilized a complex that contained Fos together with several other cellular proteins. The majority of the Fos protein complex was released from isolated nuclei incubated in the presence of deoxyribonuclease I or micrococcal nuclease but not with ribonuclease A, suggesting that Fos is associated with chromatin. This hypothesis is supported by the finding that Fos protein from native or denatured nuclear extracts exhibited DNA-binding activity in vitro. These results suggest that Fos is involved in the regulation of gene expression.

THE RETROVIRAL ONCOGENES v-*fos*, v-*myc*, and v-*myb* encode nuclear proteins that are responsible for inducing neoplasia (*1, 2*). The cellular homologs of these genes are thought to play key roles in the regulation of cell growth and differentiation. However, the mechanism whereby these proteins act is currently obscure. The biochemical properties of the v-*fos* and c-*fos* gene products were investigated to gain insight into the function of the *fos* gene.

The ability of the FBJ and FBR murine sarcoma viruses (MSV) to cause osteogenic sarcomas is encoded by v-*fos* (*3, 4*). The cellular homolog of v-*fos* (c-*fos*) is expressed at high levels in a small number of tissues (*5*). However, c-*fos* can be induced dramatically after treatment of a broad range of cell types with polypeptide growth factors and other agents (*6–10*). Activation of c-*fos* transcription is the earliest known event at the level of gene expression after cells are treated with growth factors (*9*). The c-*fos* protein (Fos) has been proposed to act as a nuclear intermediary in signal transduction systems that couple short-term events, elicited by receptor occupation, to long-term alterations in gene expression (*10*). Recently, Setoyama *et al.* (*11*) presented evidence for a transcriptional *trans*-activation activity associated with v-*fos*.

The v-*fos* and c-*fos* genes encode nuclear phosphoproteins with apparent molecular weights on sodium dodecyl sulfate (SDS) gels of 55 kD (*1, 3*). However, in stimulated cells, the induced c-*fos* product undergoes extensive posttranslational modifications and the major form detected is approximately 62 kD (*6, 7, 12*). The v-*fos* gene has undergone an out-of-frame deletion that results in a gene product with a distinct COOH-terminus (*13, 14*). In fibroblasts, both proteins are bound in a complex with a 39-kD cellular protein (p39) (*14*) and in stimulated pheochromocytoma cells (PC12) the 62-kD protein is bound to a novel 40-kD cellular protein (*15*). The properties of Fos protein complexes solubilized from FBJ-MSV–transformed cells (RS2), from serum-stimulated fibroblasts (208F), and from nerve growth factor– and benzodiazepine (BZD)-treated PC12 cells were compared. In terms of steady-state protein levels, there are comparable amounts of v-*fos* and c-*fos* gene products in all of these cell types as measured by Western transfer analyses (*16*).

Initial attempts to extract Fos protein complexes from isolated nuclei revealed that although treatment with up to 0.5*M* NaCl could release approximately 60% of Fos, the most efficient and reproducible extractions were obtained in the presence of 0.5% nonionic detergent (NP40). Fos protein complexes were readily extracted in buffer containing 0.5% NP40 and 400 m*M* KCl (Fig. 1A). The Fos-related antigen (46K), which is coinduced with c-*fos*, is also present in the nuclear fraction and is also readily extracted with salt and detergent (Fig. 1A). Control sera do not precipitate the Fos protein, the Fos-related antigens, or the proteins complexed with Fos in nuclear or cytoplasmic fractions (*16*). Thus, although Fos is located in the nucleus, it is not tightly associated with any nuclear matrix structure.

To investigate the association of Fos with nucleic acids, nuclei from PC12, 208F, and RS2 cells were treated with nucleases under conditions that completely solubilized nuclear DNA or RNA depending on the enzyme employed. Fos was efficiently released from nuclei incubated with deoxyribonuclease I (DNase I) and micrococcal nuclease but not from ribonuclease A(RNase A)–treated nuclei (Fig. 1B). Treatments that released the c-*fos* and v-*fos* proteins also released the 46K Fos-related antigen and the p39 and p40 proteins complexed with Fos.

The extraction conditions used to release the Fos protein complex from nuclei are compatible with the buffers used here for column chromatography. All extracts were diluted so that proteins were applied to columns in buffer containing 100 m*M* KCl. The results of cellulose and phosphocellulose chromatography with Fos extracted from PC12 cells (Fig. 2) were also obtained with the v-*fos* protein complex and with the Fos protein complex isolated from serum-stimulated rat fibroblasts. Virtually all of the Fos protein applied to the cellulose column was collected in the flow-through fractions

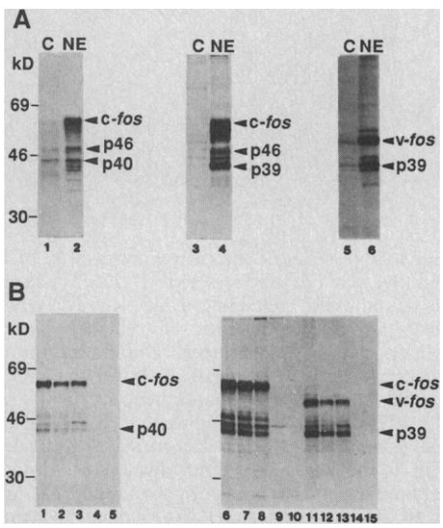

Fig. 1. Preparation of nuclear extracts and release of Fos protein complex. (A) Immunoprecipitation of Fos with M-*fos* peptide antibodies (*14*) was performed on subcellular fractions prepared as described (*20*). C, Cytoplasmic extract; NE, nuclear extract PC12, lanes 1 and 2; 208F, lanes 3 and 4; RS2, lanes 5 and 6. PC12 cells (*21*) were cultured and induced as described (*12*) and labeled with [^{35}S]methionine at 0.15 mCi/ml (Amersham International, 800 Ci/mmol) for 30 minutes before harvesting. The 208F fibroblasts (*22*) and FBJ-MSV–transformed cells (RS2) (*23*) were maintained and labeled as described (*1*). (B) Nuclei were treated with nucleases to release the Fos protein complex. The immunoprecipitated supernatants from approximately 10^6 PC12 nuclei were extracted with SDS and salt-containing buffer (RIPA) (*25*) (lane 1), or treated with DNase I at 50 unit/ml (Cooper Biomedical) (lane 2), micrococcal nuclease S7 (MCN) (Boehringer Mannheim Biochemicals) at 150 unit/ml (lane 3), boiled RNase A at 20 μg/ml (Sigma Chemical Company) (lane 4), or no enzyme (lane 5) for 60 minutes on ice. Supernatants were also immunoprecipitated from RIPA-extracted 208F nuclei (lane 6) or 208F nuclei treated with DNase I (lane 7), MCN (lane 8), RNase A (lane 9), or no enzyme (lane 10). Similarly, the immunoprecipitated supernatants are shown from RIPA-extracted RS2 nuclei (lane 11) or RS2 nuclei treated with DNase I (lane 12), MCN (lane 13), RNase A (lane 14), or no enzyme (lane 15). Nuclei were then collected by centrifugation for 10 minutes in a microfuge and supernatants were adjusted to RIPA buffer conditions and immunoprecipitated as described above. All samples were analyzed by polyacrylamide gel electrophoresis (PAGE) (*25*) and autoradiography (*26*).

Department of Molecular Oncology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.

* To whom correspondence should be addressed.

and the column buffer washes (Fig. 2A, lanes 1 to 5). A significant amount of the Fos complex did bind to phosphocellulose; however, it eluted at a low concentration of KCl (0.2 to 0.5*M* KCl) (Fig. 2B). These results indicate that Fos and its associated proteins do not bind to cellulose alone, but that they do have an affinity for phosphocellulose. This resin has been employed frequently in the purification of DNA-binding proteins.

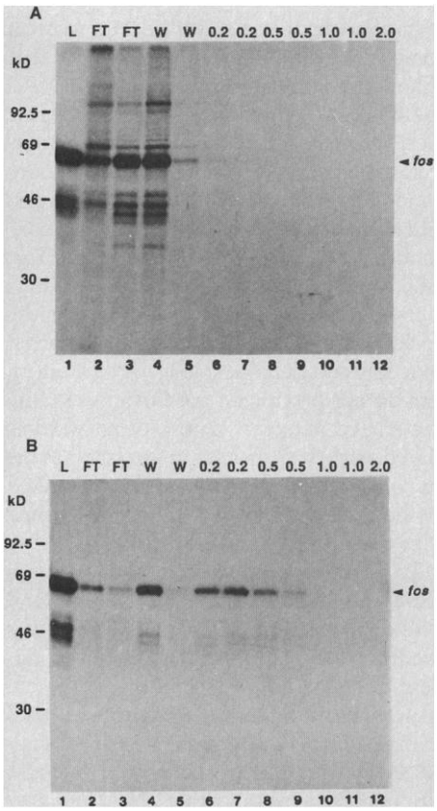

Fig. 2. Cellulose and phosphocellulose chromatography of PC12 nuclear extracts. (A) PC12 nuclear extracts were prepared by extracting nuclei with an equal volume of DNA column buffer [20 m*M* KPO_4 (*p*H 7.0), 1 m*M* EDTA, 10% glycerol, 0.5% NP40, leupeptin (2 μg/ml), aprotinin (3 μg/ml), 0.1 m*M* phenylmethyl sulfonyl fluoride (PMSF)] with 800 m*M* KCl. This suspension was incubated for 30 minutes on ice during which time it remained nonviscous. After centrifugation at 80,000*g* for 30 minutes, the clear supernatant was removed, diluted to 100 m*M* KCl and applied to a 1.2-ml Whatman CF cellulose column (Pharmacia). Immunoprecipitation was performed on a portion of PC12 nuclear extract equivalent to that loaded on the column (L; lane 1), the protein that flowed through the column (FT; lanes 2 and 3) and sequential washes of one column volume each of DNA cellulose column buffer without salt (W; lanes 4 and 5) and elutions with 0.2, 0.5, 1.0, and 2.0*M* KCl in DNA column buffer (lanes 6 to 12) were immunoprecipitated with M-*fos* peptide antibodies as in Fig. 1. (B) PC12 nuclear extracts were applied to a 1.2-ml cellulose phosphate (Medium mesh, Sigma) column. Immunoprecipitation and lane designations are as described in (A).

The ability of the Fos protein complex to bind to double-stranded DNA was assayed by retention on native calf thymus DNA cellulose. Extracts of [^{35}S]methionine-labeled nuclei were prepared, diluted to 0.1*M* KCl, and applied to native DNA cellulose columns (Fig. 3). The Fos protein complexes isolated from PC12, RS2, or 208F extracts exhibited similar DNA-binding properties. Approximately 85% of Fos extracted from PC12 nuclei, 80% of Fos extracted from RS2 nuclei, and 62% of Fos extracted from 208F nuclei was retained on double-stranded DNA cellulose and eluted with 0.5 to 1.0*M* KCl. Although the difference was small, the v-*fos* protein complex routinely bound to DNA cellulose more efficiently than the 208F c-*fos* complex. Fos protein complexes were also retained on single-stranded DNA cellulose (*16*). One-dimensional gel analysis of the Fos protein complex that binds to DNA did not reveal any labeled proteins unique to the active complex. The associated p39 and p40 proteins were found both in the fractions of bound and unbound proteins (Fig. 3).

To determine whether any of the Fos-associated proteins contributed to the DNA-binding property of the protein complex, we examined denatured nuclear extracts. After denaturation by treatment with 4*M* guanidine hydrochloride, a 208F nuclear extract was diluted to 0.1*M* guanidine hydrochloride and applied to a DNA cellulose column. This treatment disrupted the protein complex with the result that p39 was no longer co-immunoprecipitated with Fos (Fig. 3D, lanes 1 and 2). The absence of associated p39 did not affect the ability of Fos to bind to DNA cellulose (Fig. 3D). Similar results were obtained with denatured extracts of PC12 and RS2 nuclei. These data suggest that DNA binding is an intrinsic property of Fos and is not dependent on associated proteins. However, it is formally possible that DNA binding is me-

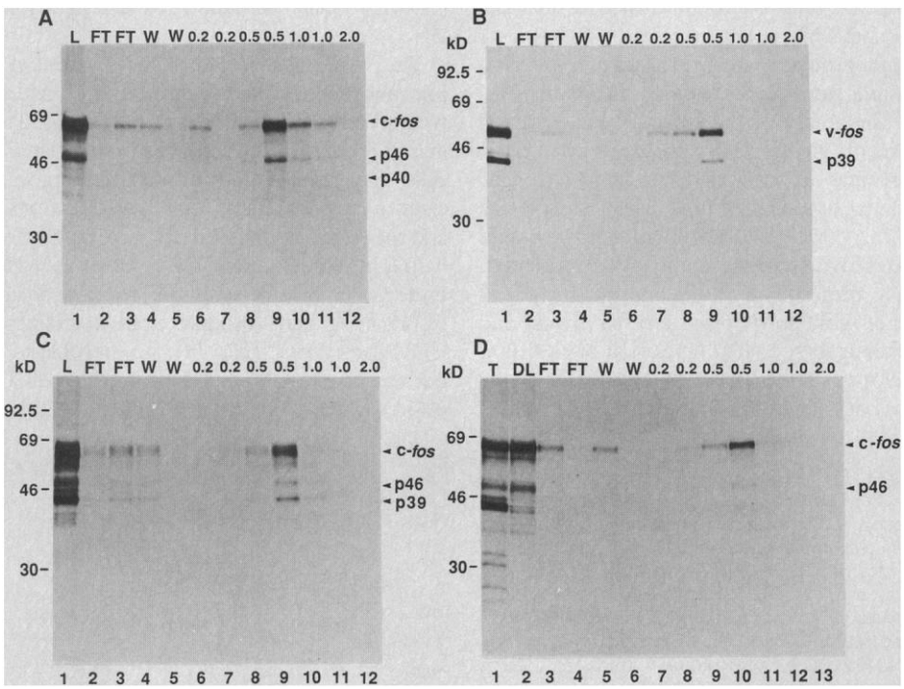

Fig. 3. DNA cellulose chromatography in (A) PC12, (B) RS2, and (C) PC12 nuclear extracts. Extracts of nuclei were prepared as described (Fig. 1) and applied to a 1.2-ml bed volume DNA cellulose column in column buffer containing 0.1*M* KCl. The DNA cellulose contained 1.14 mg of native calf thymus DNA per mole of Whatman CF cellulose (Pharmacia). A portion equivalent to that applied to the column (L; lane 1); the protein that flowed through the column (FT; lanes 2 and 3); washes of one column volume each with column buffer containing no salt (W; lanes 4 and 5), and step elutions of one column volume each containing sequentially 0.2, 0.5, 1.0 and 2.0*M* KCl in DNA column buffer (lanes 6 to 12). All samples were adjusted to RIPA buffer conditions and immunoprecipitated prior to PAGE and autoradiography. (D) Denatured 208F nuclear extracts. 208F nuclei were prepared as described (Fig. 1) and collected by centrifugation. For denaturation, the nuclear pellet was resuspended in 0.1 ml of buffer A (*20*) containing 4*M* guanidine-HCl. After 15 minutes on ice the extract was diluted with 4.9 ml of DNA column buffer and clarified by centrifugation at 80,000*g* for 30 minutes. A sample of nondenatured nuclei was extracted with RIPA buffer as described in the legend to Fig. 1 for comparison (T; lane 1). The denatured lysate containing 0.1*M* guanidine-HCl was applied to a DNA cellulose column as described above. A sample equivalent to that applied to the column (DL; lane 2); the proteins that flowed through (FT; lanes 3 and 4); washes of one column volume each with DNA cellulose column buffer containing no salt (W; lanes 5 and 6), and step elutions of one column volume each containing sequentially 0.2, 0.5, 1.0, and 2.0*M* KCl in DNA column buffer (lanes 7 to 13). All samples were adjusted to RIPA buffer conditions, immunoprecipitated, and analyzed by PAGE and autoradiography.

diated by another factor that reassociates with Fos after guanidine denaturation but is not detectable by [^{35}S]methionine labeling.

The early appearance of Fos after cell-surface stimulation and its nuclear location imply a role in signal transduction systems, perhaps at the level of gene expression. This hypothesis is strengthened by the recent description of transcriptional *trans*-activation in the presence of v-*fos* (*11*) and the evidence presented here for a DNA-binding activity. Although protein binding to DNA cellulose can often result from nonspecific interactions, the release of Fos from isolated nuclei treated with DNase and micrococcal nuclease (Fig. 1B) suggest that this in vitro property reflects a real association. In contrast, recent data obtained from immunofluorescence studies indicate that the protein product of the *myc* oncogene can be released from nuclei by treatment of cells with RNase but not with DNase (*17*).

In studies on c-*fos* induction, we have frequently detected Fos-related antigens with Fos-peptide antibodies (*7, 10, 12, 18*). One of these, the 46K antigen, displays similar biochemical traits to Fos. It is extracted from nuclei by DNase I and micrococcal nuclease but not by RNase A (Fig. 1B), and it binds to DNA cellulose (Fig. 3, A and C). This protein is detected in serum-stimulated fibroblasts, in PC12 cells treated with nerve growth factor and benzodiazepines, and in many other situations in which c-*fos* is induced. The time course of induction of 46K appears to be slightly delayed with respect to Fos (*18*). Thus, Fos may be considered a marker for a family of genes, perhaps functionally and structurally related, that are induced with similar kinetics in response to extracellular stimuli. The ability of these proteins to bind to DNA and the *trans*-activation function associated with Fos suggest that this gene family is analogous to the immediate-early genes of many viruses (*19*). Thus, Fos, and its related antigen, 46K, are candidate regulatory proteins that function in the long-term transcriptional response of cells to growth factors or other external stimuli.

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Tumor Necrosis Factor Reduces c-*myc* Expression and Cooperates with Interferon-γ in HeLa Cells

ANAT YARDEN AND ADI KIMCHI*

The suppression of the c-*myc* nuclear oncogene is associated with growth arrest and may therefore be directly controlled by naturally occurring growth inhibitors. The effect of tumor necrosis factor (TNF) and of interferon-γ (IFN-γ) on c-*myc* expression was investigated in HeLa cells, which respond to these cytokines by a specific arrest in the G_0/G_1 phase of the cell cycle. Northern blot and nuclear transcription analyses indicated that each cytokine reduced within 1 to 3 hours the c-*myc* messenger RNA levels as a result of transcriptional inhibition. Adding the two cytokines together at saturating levels resulted in enhanced inhibition of c-*myc* transcription and of the c-*myc* messenger RNA steady-state levels. While the reduction of c-*myc* messenger RNA by IFN-γ was dependent on new protein synthesis, the inhibitory effect of TNF on c-*myc* messenger RNA was direct and was not abrogated by cycloheximide. The differential effect of the protein synthesis inhibitor and the cooperative inhibitory effects of the two cytokines when added together suggest that IFN-γ and TNF reduce c-*myc* transcription through different molecular mechanisms.

THE ACTIVE EXPRESSION OF C-*myc* IS associated with competence for cell division and is switched off when cells enter the quiescent nonproliferative state during terminal differentiation (*1*). The current concept concerning the activation of c-*myc* in tumorigenesis suggests that it is associated with the loss or disruption of the control elements that enable the normal gene to be switched off when cells stop dividing. Thus, to analyze the molecular basis of abnormal activation of c-*myc* in tumor cells it is important to identify the mechanisms that normally turn off the gene. We recently demonstrated in vitro that an autocrine factor related to interferon-β (IFN-β) that is produced by differentiating hematopoietic cells is part of the mechanism that reduces c-*myc* expression (*2–4*). We based this conclusion on the finding that an antiserum to IFN-β partially abrogates the typical reduction of c-*myc* messenger RNA (mRNA) during differentiation of M1 myeloid cells (*4*). In addition, exposure of certain actively growing hematopoietic cells to purified preparations of exogenous IFN (α and β) selectively reduces the steady-state levels of the c-*myc* mRNA transcripts (*5, 6*).

Together, these data demonstrate that some IFN's may function as natural growth inhibitors that autoregulate c-*myc* expression during terminal differentiation. However, it is clear that the effect of IFN is partial and not sufficient to account for the entire reduction of c-*myc* mRNA during differentiation (*4*). It was therefore interesting to test the possibility that cooperativity between IFN and other potential growth inhibitors might be essential in achieving the complete shutoff of c-*myc*. Two other growth inhibitory substances that have been characterized biochemically and shown to exert antimitogenic functions on certain target cells are transforming growth factor-β (TGF-β) (*7*) and tumor necrosis factor (TNF) (*8, 9*). In this report we describe studies of the possi-

Department of Virology, Weizmann Institute of Science, Rehovot 76100, Israel.

*To whom correspondence should be addressed.