Transcriptional activation encoded by the v-fos gene

[fos oncogene/trans-activation/α(III) collagen gene/long terminal repeat of Rous sarcoma virus]

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ABSTRACT We present evidence that the fos oncogene encodes a transcriptional trans-activation function. trans-activation was assayed by cotransfection into NIH 3T3 mouse fibroblasts of v-fos DNA containing plasmids together with a plasmid containing a test promoter. Three v-fos DNA were used: (i) pFB1-1, a plasmid containing the FBR proviral sequences; (ii) pFB2-2, a plasmid harboring the FBJ proviral sequences; and (iii) pFJ-B, a plasmid containing the FBJ fos sequences linked to a mouse metallothionein promoter. Each of these three v-fos DNA plasmids stimulated the expression of a cotransfected chimeric gene consisting of a promoter segment of the mouse α1(III) collagen gene linked to the gene for chloramphenicol transacylase. In similar experiments the v-fos gene also stimulated the long terminal repeat promoter of Rous sarcoma virus (RSV) but neither the early promoter of simian virus 40 nor the β-actin promoter. Evidence that the trans-activation function is specified by the v-fos coding sequence comes from the fact that a frameshift mutation in the v-fos coding sequence inhibits the trans-activation. Two mutations that map around nucleotide −100 in the RSV promoter do not respond to cotransfection with v-fos, whereas other mutations respond like the wild-type RSV promoter. These experiments suggest that the v-fos gene either encodes or induces an activator of transcription that recognizes specific sequences in promoters.

Both the v-fos gene and the c-fos gene, if linked to a strong promoter, are capable of transforming fibroblasts (1). Retroviruses harboring the v-fos genomic sequences as well as cells transformed by the v-fos proviral sequences induce tumors in rodents (2–5). The products of both the c-fos and v-fos genes are localized in the nucleus (6).

Arguments have been presented which favor the view that c-fos may be involved in the differentiation of certain cells. Indeed, both c-fos and its transcript show a rapid induction during differentiation of monocytic cells into macrophages (7, 8). There is also a rapid increase in c-fos after treatment of pheochromocytoma cells with nerve growth factor (9). Furthermore, transfection of a plasmid containing the c-fos gene into embryonal carcinoma cells leads to a certain degree of differentiation of these cells (10). In other experiments, it was shown that the addition of plateau-derived growth factor of serum to quiescent fibroblasts induces a rapid but transient elevation of c-fos and of its transcript (11–14), implying some role in the growth control of these cells.

The mechanisms by which v-fos induces tumors or c-fos may regulate growth and differentiation are unknown. We show here that the v-fos gene encodes a transcriptional trans-activation function. This function was assayed in transient expression experiments of NIH 3T3 cells after cotransfection with both a plasmid containing v-fos and a plasmid containing a test promoter. Two promoters that are stimulated in response to v-fos are the promoters of the mouse α1(III) collagen gene and the long terminal repeat (LTR) promoter of Rous sarcoma virus (RSV). Two RSV promoter mutations that map between nucleotides −98 and −113 do not respond to the trans-activation mediated by v-fos.

MATERIALS AND METHODS

Cell Cultures, DNA Transfections, Chloramphenicol Transacylase (CAT) Assays, and RNA Analysis. NIH 3T3 mouse fibroblasts were cultured in Dulbecco-modified Eagle’s medium supplemented with 10% calf serum. Transfections with recombinant DNAs were carried out by using the calcium phosphate precipitation method. For transient assays, NIH 3T3 cells were seeded at a density of 4–5 × 10⁴ cells per 100-mm dish and 24 hr later were transfected with 5–20 μg of recombinant DNA. After 48 hr, the cells were harvested for assay of CAT or analysis of RNA. CAT assays were performed as described (15); activities were expressed as percent conversion of [14C]-chloramphenicol to the acetylated forms. Total RNAs were isolated as described (16). Primer extension experiments were done with a synthetic oligonucleotide complementary to CAT RNA. Conditions of hybridization and primer extension were as described previously (17).

Plasmids. Plasmid pPrC3-1 (Fig. 1b) is a derivative of pAZ1009 (21) in which the mouse a2(1) collagen promoter fragment has been replaced by a mouse α1(III) collagen gene fragment containing the promoter of this gene between 2.3 kilobases (kb) 5′ of the start site of transcription to position +16 3′ of this site. The α1(III) collagen DNA segment was taken from the recombinant phage PMC3A-5 (22). This plasmid also contains, 3′ to the CAT gene, the SV40 small tumor antigen splicing sites, the early region polyadenylation site, and a segment containing the SV40 enhancer.

Plasmid PrC3-104 was obtained from pPrC3-1 by deleting a BamHI fragment containing the SV40 enhancer sequences.

Plasmid MF-J (Fig. 1a) was constructed by using plasmid pMMTneo, a gift of P. M. Howley (23). This plasmid contains the promoter region of the mouse metallothionein I gene. A 2.0-kb Bgl II/Hpa I fragment containing the neo gene and SV40 DNA sequences was replaced by a 1.5-kb FnuIII–Pvu II fragment isolated from pFB2-2 DNA, a generous gift of T. Curran (19). This fragment contains sequences encoding the p55 fos protein. The noncomplementary ends were converted to blunt ends by using the Escherichia coli

Abbreviations: LTR, long terminal repeat; RSV, Rous sarcoma virus; CAT, chloramphenicol transacylase; SV40, simian virus 40; kb, kilobase(s); bp, base pair(s).

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RESULTS

We wanted to determine whether the fos protein has a trans-activation function that might help explain its role in differentiation, transformation, or both. Since we had observed a considerable increase in the expression of the type III collagen gene in v-fos-transformed cells (C.S., unpublished data), plasmid pPrC3-1 was constructed in which the 5' flanking sequences of the mouse α1(III) collagen gene (22) were fused to the bacterial CAT gene (Fig. 1b). This plasmid was used in cotransfection experiments with three different plasmids containing v-fos sequences: (i) pFBR-1, a plasmid containing the FBR proviral sequences (18); (ii) pFBJ-2, a plasmid containing the FBJ proviral sequences (19); (iii) pMF-J, a plasmid containing the FBJ fos sequences linked to a mouse metallothionein promoter. These plasmids are schematically shown in Fig. 1a. Cotransfection of NIH 3T3 cells with the DNA of pPrC3-1 and the plasmid containing the FBR genomic sequences (pFBR-1) results in a 5- to 10-fold increase in the levels of CAT 48 hr after transfection (Fig. 2a, lane 2). In the control experiment pPrC3-1 was cotransfected with calf thymus DNA (Fig. 2a, lane 1). To exclude the possibility that calf thymus DNA might titrate factors that might be needed for activation of the α1(III) collagen promoter, the levels of expression of this promoter were compared after cotransfection with equal quantities of DNA from calf thymus, pSV2-gpt [a plasmid in which the early promoter of SV40 is fused to the bacterial guanine phosphoribosyltransferase gene (gpt)], or pRSV-gpt (a plasmid in which the LTR of the RSV genome is fused to the gpt gene). Both promoters contain enhancer sequences that exert a strong cis-acting stimulation on homologous and heterologous promoters in NIH 3T3 cells (19). Figure 2b shows that the level of CAT is the same in each of these three cotransfection experiments, implying that the enhancer present on these

![Fig. 1. Schematic representations of plasmids. (a) v-fos-containing plasmids. Plasmid pFBR-1 represents a mouse DNA segment containing the FBR proviral sequences in the HindIII site of pBR322 (18). Plasmid pFBJ-2 represents a mouse DNA segment containing the FBJ proviral sequences similarly inserted into the HindIII site of pBR322 (19). pMF-J is a plasmid in which the mouse metallothionein I promoter is linked to the FBJ-fos sequence. It is composed of pML2 sequences (pML2 is a derivative of pBR322 containing the ampicillin gene and the replication origin of pBR322); a 1.6-kb EcoRI-Bgl II fragment containing the mouse metallothionein I promoter (MMT pro); a 1.5-kb FnuDI-Pvu II fragment containing the v-fos coding sequences isolated from pFBJ-2 DNA, and a simian virus 40 (SV40) segment that spans the early region polyadenylation site. (b) pPrC3-1 is a plasmid in which the mouse α1(III) collagen promoter is fused to the gene for CAT. The SV40 segment to the right of the CAT gene contains the splicing sites of the small tumor antigen gene and the early region polyadenylation sites; SV-e is a short SV40 fragment containing one intact copy and part of one copy of the 72-base-pair (bp) repeat sequence. (c) pRSVCAT-1 is a derivative of pSV2-CAT (15) in which a 650-bp Pst I fragment of pSR1 (20) replaces the SV40 promoter fragment. pSR1 is a plasmid that contains the 3' LTR of the Schmidt–Ruppin strain of RSV inserted into the Pst I site of pBR322. The magnified region is the segment between the SpI site at -138 preceding the start of transcription to the HindIII site. End points of deletions (C3, C723, H114, H121) are indicated for each mutant as well as the substituting sequence. Construction of pRSVCAT-1 and the deletion-substitution mutants will be described in detail elsewhere.

Klenow fragment of DNA polymerase I, before circularization with T4 DNA ligase.

Frameshift mutant MF-J-B was constructed by cleaving MF-J DNA with Bgl II, filling out the resulting staggered ends with Klenow fragment, and religating the blunt ends with T4 DNA ligase.

A detailed description of pRSVCAT-1 and mutants C3, C723, H114, and H121 will be reported elsewhere (R.F., unpublished data). Plasmid pSE-90 contains the 87-bp SpI–EcoRI fragment of the RSV (Schmidt–Ruppin strain) LTR inserted into the EcoRI and SpI sites of pBR322.

![Fig. 2. trans-activation by v-fos. (a) Transfections were carried out with 5 × 10^6 NIH 3T3 cells per 100-cm^2 dish. Ten micrograms of pPrC3-1 DNA was cotransfected with either 10 μg of calf thymus DNA (lane 1) or 10 μg of pFBR-1 DNA (lane 2). After 48 hr, the levels of CAT were measured as described by Gorman et al. (15). An autoradiograph of the CAT reaction products after chromatography is shown. (b) Ten micrograms of pPrC3-1 DNA was cotransfected with 10 μg of calf thymus DNA (lane 1), 10 μg of pSV2-gpt DNA (lane 2), or 10 μg of pRSV-gpt DNA (lane 3). (c) Three micrograms of pPrC3-1 was cotransfected with increasing amounts of pFBJ2 DNA. The total amount of transfected DNA was kept constant at 20 μg by addition of calf thymus DNA. CAT was assayed as shown in a and b. (d) Ten micrograms of pPrC3-104 DNA (a derivative of pPrC3-1 without SV40 enhancer element) was cotransfected with 10 μg of calf thymus (C.T.) DNA or 10 μg of pFBJ2 DNA. (e) Ten micrograms of pPrC3-1 was cotransfected with 10 μg of calf thymus (C.T.) DNA, 10 μg of MF-J DNA, or 10 μg of MF-J-B DNA plasmid carrying a frameshift mutation in the fos sequences. This experiment was done four times with very similar results.
plasmids has no effect on the expression of a gene present on the cotransfected plasmid. A similar cotransfection was performed with pFBJ-2, a plasmid in which the fos sequences represent the only significant open reading that is common between the FBR and FBJ proviral sequences (18). Increasing concentrations of FBJ-2 DNA were used, whereas the concentration of pPrC3-1 DNA was constant and the total DNA concentration was kept equal by varying the concentrations of calf thymus DNA (Fig. 2c). In this particular concentration-dependent experiment the final stimulation was 4-fold. In three other experiments using the same plasmids, the stimulation was between 5- and 10-fold. These experiments are consistent with the notion that the trans-activation observed with both pFBR-1 and pFBJ-2 is encoded in the fos sequence. Fig. 2d shows that cotransfection of pFBJ-2 with a derivative of pFBR-1 from which the SV40 enhancer sequences have been deleted results in a 15-fold stimulation of CAT activity. In the absence of the SV40 enhancer the unstimulated level of expression of the α(III) collagen promoter is much lower than when the enhancer is present on the plasmid. The SV40 enhancer sequence is, therefore, not required to mediate the transactivation encoded in plasmid pFBJ-2.

To rule out that the trans-activating function is encoded by a non-fos segment common to the DNA of pFBR-1 and pFBJ-2, plasmid pMF-J was constructed. In this plasmid a segment of pFBJ-2 DNA containing 81 bp preceding the AUG of v-fos, the v-fos coding segment, and 304 bp 3' to the v-fos coding sequence was fused to the promoter segment of the mouse metallothionein I gene (19) (see Fig. 1). In addition, a derivative of pMF-J was made in which a frameshift mutation was introduced in the v-fos coding sequence. This was done by cleavage of pMF-J with Bgl II, which cleaves at a unique site in the fos coding sequence, followed by filling the staggered ends with the Klenow fragment of DNA polymerase and circularization of the plasmid by blunt-end ligation. In this plasmid, designated pMF-J-B, the first 60 amino acids of the fos polypeptide are intact but the subsequent sequences of fos are placed in a different translational frame as a result of a 4 bp insertion. This was confirmed by DNA sequence analysis. Fig. 2e shows the results of cotransfection experiments comparing the effects of calf thymus DNA, pMF-I-J DNA, and pMF-J-B DNA on the activity of the cotransfected α(III) collagen promoter. In this experiment the levels of CAT are about 4 times higher when the collagen promoter plasmid is cotransfected with the wild-type fos DNA than with the mutated fos DNA. Although the mutation inhibits the trans-activation activity, it did not suppress it completely to the level observed in cotransfection of pPrC3-1 DNA and calf thymus DNA. This experiment was done four times, with very similar results each time. The mutation appears to exhibit some leakiness, which could be due either to the presence of frameshift suppressors in the cells or to reinitiation of translation in the fos RNA or, alternatively, to the fact that the trans-activational effects of trans-activation activity is located in the amino-terminal portion of the v-fos protein.

Fig. 3 illustrates the results of transfection experiments in which the trans-activation effect of fos was tested on other promoters. In these experiments plasmid pFBJ-2 was cotransfected with plasmids in which the CAT gene is fused to the chicken β-actin promoter (pAZ1037) (21), the early promoter of SV40 (pSV2CAT), or the LTR promoter of RSV (pRSV CAT). Cotransfection with v-fos stimulates the activity of the RSV promoter (3.3 times) but not the activity of the actin or the early SV40 promoter. We conclude that not all promoters are equally trans-activated by v-fos. We also asked whether the stimulation of the RSV LTR promoter by v-fos was due to an increase in correctly initiated RNA. Fig. 3d shows the results of a primer extension experiment in which a primer was used that is complementary to a segment of CAT RNA. If CAT RNA starts at the correct place in the RSV promoter, the extended primer should be 234 nucleotides long. There is an intense doublet at 118 nucleotides that is probably due to a strong stop caused by a stretch of 17 G+C base pairs that are present in the DNA at this location as a result of the cloning procedure. In cells cotransfected with v-fos there is a clear increase in the intensity of the 234-nucleotide band, indicating that v-fos stimulates specific and correct initiation at the RSV promoter.

We have also examined the effects of v-fos in cotransfection experiments using a series of deletion-substitution mutations in the RSV promoter. The mutations are cotransfected into 5 × 10⁶ NIH 3T3 cells with 10 μg of calf thymus DNA (lane 1) or with 10 μg of pFBJ-2 DNA (lane 2). CAT reaction products are shown. (a) pRSVCAT-1. This plasmid contains the RSV LTR fused to the CAT gene. (b) pAZ1037. This plasmid contains a fragment of the chicken β-actin gene, which spans 350 bp upstream of the transcription start site and 1 kb downstream of this site (21). (c) pSV2CAT. This plasmid contains the early SV40 promoter segment, including the SV40 enhancer sequences, fused to the CAT gene (15). The ratios of CAT activity found in v-fos-transfected cells over that found in control cells are 3.4 for pRSVCAT-1, 1.3 for pAZ1037, and 0.7 for pSV2CAT. (d) Levels of CAT RNA in untransfected NIH 3T3 cells, cells cotransfected with 6 μg of pRSVCAT-1 and 20 μg of pBR322 DNA (pRSVCAT lane), or cells cotransfected with 6 μg of pRSVCAT-1 and 20 μg of PMJ DNA (pRSVCAT + v-fos lane). Transfections were carried out in 150-cm² dishes. Forty-eight hours after transfection, total RNAs were isolated (16) and used for primer extensions, utilizing a 24-mer synthetic probe, labeled at its 5' end, that is complementary to a segment proximal to the 5' end of CAT mRNA (see map below the gel autoradiograph). The expected size for a cDNA extending to the 5' end of the mRNA correctly initiated at the LTR promoter is 234 nucleotides (n). Lane M, size markers (in nucleotides); lane t-RNA, products of a reverse transcription reaction using excess yeast t-RNA.
deletion substitutions that remove bases between −57 and 
−32 and between −57 and +8, substituting the same 
sequence as in C3 and C723. However, two mutants, H114
and H121, are not responsive to v-fos, suggesting that a specific
site in the LTR promoter may be the target of trans-activation
by v-fos. Both mutants are short deletion-substitutions that
map in a small area of the promoter between −98 and −113.
In other experiments, it was shown that the promoter
strength of these mutants is 40% of wild-type for H114 and
65% for H121 (R.F., unpublished results).

To further characterize the possible interactions between
this area of the RSV promoter and either v-fos itself or
proteins induced by v-fos, an 87-bp fragment located between
the Sph I site at −138 and the EcoRI site at −51 was
subcloned in pBR322. This plasmid, pSE-90, was used in
competition experiments to determine whether the stimulation
by v-fos could be inhibited by adding increasing concen-
trations of this plasmid in cotransfection experiments. As
shown in Fig. 5c, the 87-bp fragment itself does not inhibit
the activity of either the RSV or the α3(III) collagen promoter.
However, when the RSV promoter–CAT chimeric gene is
cotransfected with the v-fos-containing plasmid pMF-J
and increasing concentrations of pSE-90, the plasmid containing
the 87-bp RSV sequence inhibits the stimulation that is
produced by v-fos. In this experiment the total DNA con-
centration was kept constant by varying the concentrations of
pBR322 DNA (Fig. 5a), and the 87-bp RSV fragment does not
inhibit v-fos RNA synthesis (not shown). This experiment
suggests that an activator of transcription is titrated by
increasing concentrations of the 87-bp DNA fragment. The
inhibition occurs only in these experiments in which the v-fos
DNA plasmid is used. The inhibition by pSE-90 also occurs
when the test promoter is the α3(III) collagen promoter (Fig.
5b). Taken together with the absence of response to v-fos of
two closely linked mutations in the RSV promoter, the results
of the competition experiment illustrated in Fig. 5 are
consistent with the view that the 87-bp fragment of the RSV
promoter is the target site either for fos itself or for a factor
that is induced by v-fos.

DISCUSSION

Our data show that the product of the v-fos oncogene has a
transcriptional trans-activation activity. This activity was
assayed by cotransfection of NIH 3T3 cells in transient
expression experiments. The trans-activation was observed
with three different v-fos-containing plasmids. The most
convincing evidence for assigning a trans-activation function
to v-fos comes from the observation that a frameshift muta-
tion in the v-fos gene inhibits the trans-activation function.
The two test promoters that exhibit a stimulation are the
promoters for the α3(III) collagen gene and the LTR promoter
of RSV. Since the test promoters that we used in our
cotransfection experiments were fused to the CAT gene, we
have measured increases in CAT activity, assuming that the
levels of CAT activity reflect the activities of the linked
promoters (21, 24). With the RSV LTR promoter–CAT chimeric gene lacking the SV40 enhancer in the plasmid was, in general, more strongly stimulated by v-fos than the RSV LTR–CAT gene and the α(III) collagen promoter–CAT gene containing the SV40 enhancer. CAT gene expression with the latter plasmids has a much higher basal level than with the former plasmid. Hence, trans-activation by v-fos may be more easily detectable with promoters that do not have a high level of constitutive expression in NIH 3T3 cells.

The sequence around the RSV promoter mutations was compared with the sequence in the α(III) collagen promoter between −359 and +1. No obvious similarities were found except for a short sequence 5′-ACAGACA-3′, which is present between −89 and −83 in the RSV promoter and is also found either intact (once) or with one or two base changes (twice) in the α(III) promoter sequence. It is possible that the two mutations that do not respond to fos and that map at a short distance 5′ to this sequence cover only a portion of the recognition site and that the above heptanucleotide is another part of the interaction site. A deletion analysis of the α(III) collagen promoter should help determine which sequences are needed in this promoter to observe the effects of v-fos.

A similar trans-activation function has been demonstrated for the protein products of other “oncogenes” such as the EIA protein of adenovirus (25–28), the large tumor proteins of the SV40 and polyoma viruses (29, 30), and the product of the c-myc gene (31). The genomes of human T lymphotropic viruses HTLV-I, HTLV-II, and HTLV-III and the genome of the bovine leukemia virus encode a gene product that can trans-activate the LTR of the homologous viruses (32–35). All these oncogenic products have a common nuclear localization. The genome of RSV also codes for a trans-activation function, but this function is not encoded by the src gene (36).

Our experiments do not allow us to determine whether the v-fos product itself stimulates the α(III) collagen promoter and the RSV promoter or whether v-fos induces an activator of these promoters. DNA binding experiments using purified preparation of fos should help elucidate this point.

In NIH 3T3 cells that are transformed by the plasmid containing the FBR proviral sequences, there is a considerable increase in both the steady-state levels and synthesis of α(III) collagen RNA (C.S., unpublished). This increase could be caused by the same trans-activating function that is found associated with v-fos in transient expression experiments. This hypothesis is supported by the finding that transfection of NIH 3T3 cells with v-fos results within 48 hr in an increase in the levels of endogenous type III collagen RNA. We suspect that the type III collagen gene is one among a series of cellular genes whose expression is influenced by v-fos. Type III collagen is one of the first collagens to be made in mouse embryos, and its appearance can first be detected at a pre-implantation stage well before the appearance of type I collagen (37).