Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 513–517, January 1990
Biochemistry

E1A-dependent trans-activation of the c-*fos* promoter requires the TATAA sequence

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Communicated by Robert G. Roeder, October 16, 1989

ABSTRACT **Previous experiments have demonstrated that transcription of the human c-*fos* oncogene is activated through the action of the 289-amino acid adenovirus E1A gene product. In this study we have utilized a series of c-*fos* promoter deletion and substitution mutants to define regulatory sequences that allow the induction by E1A. Although the deletion of upstream promoter sequences has varying degrees of effect on overall promoter activity, these deletions retain inducibility by E1A. This includes the deletion of the serum response element and two elements that bind the ATF transcription factor. In fact, a c-*fos* promoter deleted to position −53, which leaves the TATA element but no other known functional element, retains inducibility, indicating a role for the TATA element in E1A control. Indeed, substitution of the c-*fos* TATA element (TATAA) with a TATA sequence from the simian virus 40 early promoter (TATTTAT) abolishes E1A inducibility; this promoter does retain responsiveness to cAMP induction, however, demonstrating that this TATTTAT substitution is functional. We conclude that the E1A-dependent activation of c-*fos* transcription is mediated through an effect on a TATA-binding protein that has specificity for the TATAA sequence.**

The mechanisms by which the adenovirus E1A gene products control transcription in virus-infected and transformed cells have been the subject of intense study. A variety of experiments have now shown that the 289-amino acid E1A gene product functions not as a direct transcriptional activator but rather in an indirect manner, influencing the activities of cellular transcription factors that interact with the E1A-regulated promoters (1, 2). In light of this, the influence of E1A trans-activation on cellular gene expression is an obvious possibility. Indeed, E1A can activate numerous cellular genes in transient-transfection assays (3–5), but in addition, examples of endogenous chromosomal cellular genes activated by E1A have been documented. The best studied is the human gene encoding the hsp70 major heat shock protein (6, 7), and recent experiments have demonstrated that the TATA element of the hsp70 promoter is the target for regulation, presumably the result of an E1A-dependent alteration of a TATA-binding factor (8). In addition to the hsp70 promoter, experiments have demonstrated an E1A-dependent activation of transcription of the c-*fos* oncogene, using both transiently transfected genes and the endogenous gene (9). Given the oncogenic properties of c-*fos*, as well as its role in modulating transcription as a component of the Ap1 transcription factor (10–13), the manner in which E1A alters the transcription of the c-*fos* gene is of considerable significance.

Previous experiments suggested that sequences in the c-*fos* promoter necessary for E1A stimulation fell within the region including the serum response element (SRE) (9). However, these experiments did not distinguish between c-*fos* promoter sequences that were important merely for the function of the promoter and those sequences important for specific regulation by E1A. Here we have examined a series of c-*fos* deletion and substitution mutants to attempt to define a target site for E1A-dependent transcriptional activation. Our results indicate that the TATAA sequence of c-*fos* is the primary target for E1A-mediated transcriptional activation.

MATERIALS AND METHODS

Cells and Viruses. HeLa human cervical carcinoma cells (subclone S3, ATCC) and Vero monkey kidney cells were grown in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. PC12 rat pheochromocytoma cells were grown in DMEM containing 10% fetal bovine serum and 5% horse serum. Wild-type and mutant adenovirus type 5 (Ad5) were grown in suspension cultures of the Ad5-transformed human embryonic kidney cell line 293, as described (14). Infectious focus-forming units were determined by the indirect immunofluorescence assay described by Thiel and Smith (15) and modified by Ensinger and Ginsberg (16).

Plasmid DNAs. Plasmids pFC2000, pFC700, pFC225, pFC99, and pFC53 are constructs containing the human c-*fos* gene from positions −2000, −710, −225, −99, and −53, respectively, to position +42 (numbering is relative to the mRNA cap site) linked to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene (17). Plasmids pFCΔ225/99 and pFCΔ94/53 contain internal deletions and were derived from pFC700 as described (17).

Point mutants in the c-*fos* TATA region were made by oligonucleotide-directed site-specific mutagenesis of double-stranded plasmid DNA (18). Gapped heteroduplexes were prepared by mixing *Xmn* I-digested pFC700 DNA with *Bss*HII (position −99)/*Xba* I (position +42)-digested pF700 DNA after heat denaturation. Renaturation was carried out in the presence of an oligodeoxynucleotide (5′-CGCTTGTTATTTATGCAGTGGCT-3′), and gaps were repaired with Klenow DNA polymerase and DNA ligase (Boehringer Mannheim). *Escherichia coli* MC1061 was transformed with these DNA preparations and positive colonies were identified by hybridization to oligonucleotides containing the desired base substitutions (19). Positive clones were confirmed by dideoxy sequencing of double-stranded plasmid minipreps (20).

Transfections. Cells were subcultured (1:5) 24 hr prior to infection with wild-type Ad5 or mutant dl312 (20 focus-forming units per cell) and maintained in the presence of cytosine arabinonucleoside (40 μg/ml culture medium). Cal-

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Abbreviations: Ad5, adenovirus type 5; CAT, chloramphenicol acetyltransferase; hsp, heat shock protein; SRE, serum response element.

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cium phosphate precipitates of plasmid DNA were prepared and added to cells 8 hr after infection. Twelve hours later, fresh medium was added to the transfected cells. After an additional 12-hr incubation, cell extracts were prepared and assayed for CAT activity (21). For cAMP induction assays, PC12 cells were transfected with pFC700 and pFCpm1 (5 μg of each). After 12 hr, the medium was removed and the cells were fed with complete medium containing 1 mM dibutyryl cAMP. Cells were harvested 24 hr later and extracts were prepared. Cytoplasmic RNA was isolated from transfected cells as described (14) and analyzed by S1 nuclease mapping (22). Double-stranded DNA probes were made by end-labeling a 400-base-pair (bp) *Bss*HII–*Eco*RI restriction fragment derived from pFC700 that included sequences from −99 to +42 in c-*fos* and the first 250 bp of the CAT gene coding sequence (17). Cotransfection experiments not involving virus infection were carried out as described (23).

RESULTS

Upstream *c-fos* Promoter Sequences Are Not Required for E1A Trans-Activation. Previous experiments have defined sequences in the c-*fos* promoter that are important for transcription (Fig. 1). There is a SRE between −317 and −298 that mediates the ability of the promoter to respond to serum stimulation, epidermal growth factor, phorbol 12-myristate 13-acetate, and insulin (17, 24–27). Immediately downstream, between −303 and −283, is a sequence with homology to an AP1 as well as an ATF binding site (28). An additional ATF recognition site is located between −63 and −57 (17), in the cAMP response element (CRE). In addition to these elements, there are two direct repeats (DR) between −97 and −76 that are important for c-*fos* promoter activity and that bind nuclear proteins (17). Finally, there is a TATA sequence at −30. Considering sequence elements that might represent targets for E1A control, there are two obvious possibilities. The ATF sites at −300 and −60 are candidates because of the postulated role of this sequence in the control of adenovirus early genes (29). In addition, the sequence of the c-*fos* TATA element (TATAA) is the same as that in the hsp70 promoter and in the E1B promoter, both of which have been shown to be targets for E1A trans-activation (8, 30).

To assess the role of the various sequence elements in the c-*fos* promoter in allowing E1A-dependent activation, we employed a variety of c-*fos* promoter constructs fused to the CAT gene to assay in transient transfections. The protocol was similar to one we used previously whereby cells are infected with the E1A mutant dl312 or wild-type Ad5 and then subsequently transfected with the given plasmid (8). Extracts are prepared and then CAT assays are performed. These

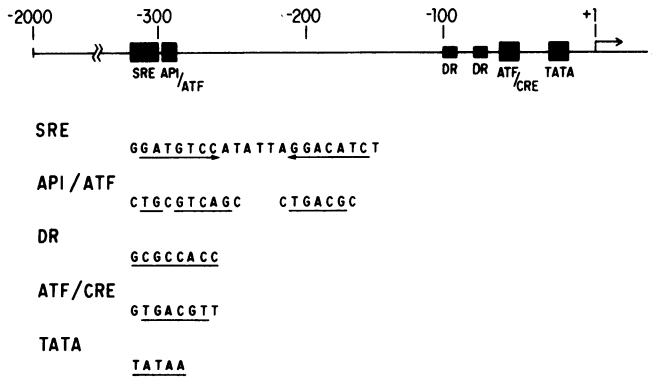

FIG. 1. Regulatory elements of the human c-*fos* promoter. A schematic depicting the various elements in the c-*fos* promoter is shown above the DNA sequences at these sites. See text for further explanation.

conditions thus provide an E1A$^-$ condition (dl312) versus an E1A$^+$ condition (Ad5). Of course, the lack of E1A expression is not the only difference between the two infections, since in the absence of E1A there is little or no expression of the other early genes. Nevertheless, the c-*fos* promoter is clearly induced by E1A as evidenced by cotransfection with an E1A-expressing plasmid (9) and as shown in assays depicted in Figs. 2 and 3. Indeed, we have not detected any differences in the two types of assay systems. As shown in Fig. 2*A*, an analysis of 5′ promoter deletions in such an assay indicated that induced expression was not affected by a deletion to −225 but that there was a reduction upon further deletion to −99 or −53. This would therefore indicate that the SRE and the AP1/ATF site at −300 are not uniquely required for the stimulated expression. Although a previous study reported a sharp reduction in E1A-stimulated c-*fos* promoter activity when sequences between −402 and −240 were deleted (9), there was also a drop in basal expression; thus, the deletion of the SRE did not eliminate E1A inducibility but rather affected overall promoter activity. We doubt that this difference represents a significant discrepancy; rather, we believe that it reflects the variation in relative activity of this deletion due to cell-specific differences or alternatively the somewhat different assay procedures used in the two studies.

In addition to the fact that the −225 promoter deletion mutant was functional in E1A induction as well as overall activity compared to the full c-*fos* promoter, it was also clear from the results in Fig. 2*A* that deletion to −99 or to −53 did not abolish E1A inducibility. The induced level of expression of these promoters was significantly reduced compared to the −225 promoter, but both were responsive to E1A stimulation. Thus, there must be functional promoter elements within this interval (−225 to −53) but there was no clear evidence of an E1A-responsive element in this region. Since the −53 deletion removes the cAMP regulatory site at −60, the ATF binding site, it appears that induction of c-*fos* by E1A does not require CREB/ATF. The lack of requirement of this site in the E1A stimulation of c-*fos* was further demonstrated by the experiment in Fig. 2*B* in which internal deletions were assayed for E1A inducibility. Once again, the −225 promoter was significantly increased in activity in Ad5-infected cells compared to dl312-infected cells. The two internal deletion mutants of the c-*fos* promoter, which contain sequences up to 700 bp upstream of the transcription initiation site but have sequences between −225 and −99 deleted or sequences between −94 and −53 removed, are only slightly less active than the −225 promoter. Thus, removal of the CRE/ATF site located at −60, leaving the rest of the promoter intact, had very little effect on the activity of c-*fos* promoter in response to E1A. Furthermore, the activation of the −53 deletion promoter was indeed E1A-dependent as demonstrated by a cotransfection with an E1A-expressing plasmid (Fig. 2*C*). Thus, although other early genes could play a role in the trans-activation process, it is clear that the c-*fos* activation observed in these experiments is E1A-dependent.

A Specific TATA Sequence Is Necessary for E1A Stimulation of c-*fos*. The results described above and presented in Fig. 2 indicate that although upstream promoter elements contribute to the overall level of activity, they do not define an E1A-regulated site since all of the deletions were still clearly inducible by E1A. This left the possibility that the target for regulation could be redundant, with multiple elements affording such a response, or that the TATA box was the target for control. That the −53 promoter deletion mutant, which lacks virtually everything upstream of the TATA, was still clearly E1A-inducible suggests a role for the TATA element in the activation process. Whether the TATA box is the sole target for control, however, is tentative since the promoter is clearly impaired when compared to the full c-*fos* promoter. A

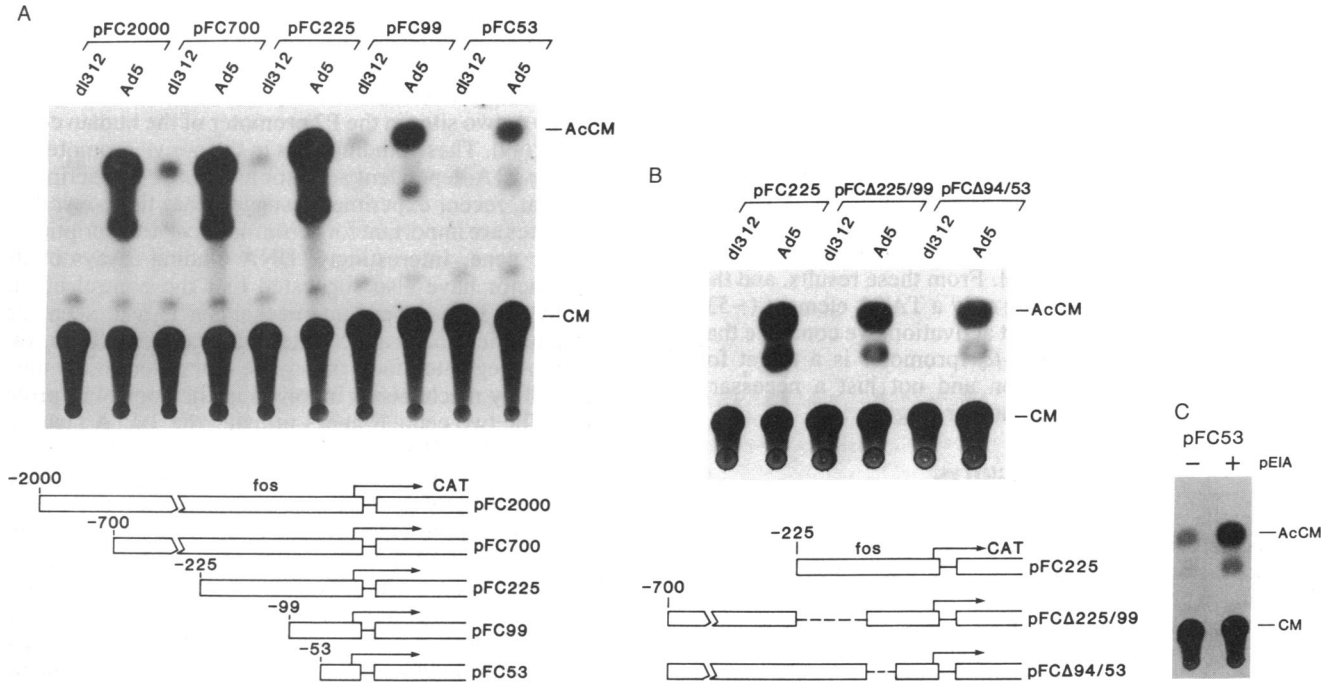

FIG. 2. c-*fos* upstream promoter sequences are not essential for E1A-dependent trans-activation. (*A*) c-*fos* 5′ promoter deletion mutants, as depicted schematically below the autoradiogram, were assayed by transfection into HeLa cells infected with either dl312 (E1A$^-$) or Ad5 (E1A$^+$). Cell extracts were assayed for CAT activity. (*B*) c-*fos* internal promoter deletion mutants were assayed as in *A*. (*C*) Cotransfection of the pFC53 plasmid with the E1A-expressing plasmid pE1A was performed and cell extracts were prepared and assayed for CAT activity. CM, chloramphenicol; AcCM, acetylated chloramphenicol.

role for the TATA element as the target for E1A control is supported by past experiments with the hsp70 promoter in which the TATA element was identified as the regulatory target for E1A control (8). Furthermore, the hsp70 promoter analysis suggested that multiple TATA sequences could be distinguished based on their responsiveness to E1A control. The TATA sequence found in the hsp70 promoter (TATAA) allowed a response to E1A, whereas a TATA sequence found in the simian virus 40 (SV40) early promoter (TATTTAT) was unresponsive to E1A. To test this possibility for the c-*fos* promoter, we generated a promoter in which the c-*fos* TATAA sequence was replaced with the SV40 TATTTAT sequence, leaving the remainder of the promoter intact. As shown in Fig. 3*A*, transfection of the pFC700 plasmid, containing the normal TATAA sequence, into cells cotransfected with pE1A resulted in a large stimulation in CAT activity. In contrast, pFCpm1, in which the TATAA sequence has been converted to TATTTAT, leaving the re-

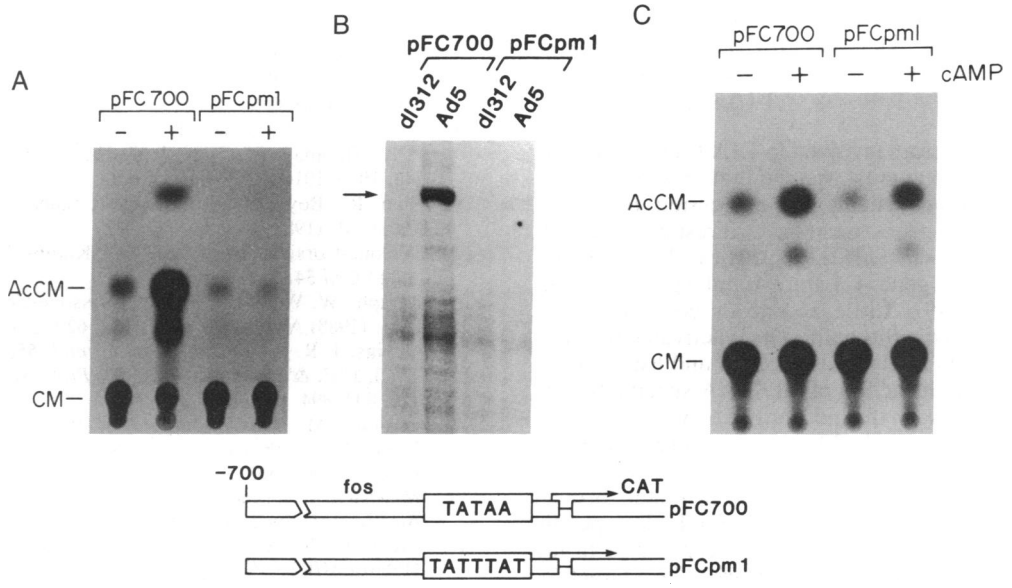

FIG. 3. c-*fos* TATAA sequence is essential for E1A-dependent trans-activation. Shown below the autoradiograms are the sequences at the TATA site for plasmids pFC700 and pFCpm1. (*A*) The pFC700 plasmid and the pFCpm1 TATA substitution mutant were transfected into Vero cells with (+) and without (−) the E1A-expressing plasmid pE1A. Extracts were prepared and assayed for CAT activity. (*B*) pFC700 and pFCpm1 were assayed by transfection into dl312- or Ad5-infected cells. RNA was prepared and assayed for c-*fos*–CAT transcripts by 5′ S1 analysis. (*C*) pFC700 and pFCpm1 were transfected into PC12 cells with (+) and without (−) the addition of 1 mM dibutyryl cAMP. Extracts were prepared and assayed for CAT activity.

mainder of the promoter intact, was unresponsive to E1A. In addition, S1 analysis of transcripts produced in transfections with pFC700 and pFCpm1 indicated that the loss of CAT activity in the TATTTAT mutant was reflected in reduced RNA levels rather than a change in initiation site as the result of a TATA change (Fig. 3*B*). Finally, using these same two plasmids in a transfection assay with or without cAMP demonstrated that the c-*fos* TATTTAT substitution mutant (pm1) retained responsiveness to cAMP equivalent to that of the wild-type promoter (Fig. 3*C*), confirming that the TATA-altered promoter was functional. From these results, and the fact that a promoter containing only a TATA element (−53) is responsive to E1A-dependent activation, we conclude that the TATAA element in the c-*fos* promoter is a target for E1A-dependent trans-activation and not just a necessary element in conjunction with another target.

DISCUSSION

Many details of the E1A-mediated trans-activation pathway have been elucidated. For instance, it is now evident that the trans-activation process must involve the utilization of cellular transcription factors and that more than one transcription factor must be involved in the actual regulatory process (1, 2). Previous studies have provided strong evidence for the role of the E2F promoter-specific factor in E1A-dependent trans-activation of E2 transcription (31–34). The VA gene and other genes transcribed by polymerase III appear to be stimulated by the TFIIIC factor (35–38). An element of the E4 promoter required for trans-activation is the binding site for several cellular factors including E4F (39), ATF (29), and EivF (40). Although such complexity clouds the issue of which factor is actually responsible for induction of E4 transcription, it is nevertheless true that the binding activity of the E4F factor is induced during a viral infection, dependent upon E1A action, and with kinetics coincident with the activation of E4 transcription (39). Finally, our previous studies of the hsp70 promoter have demonstrated a role for a specific TATA-binding protein in the trans-activation of hsp70 transcription (8), consistent with studies of the adenovirus E1B promoter, which also implicated the TATA element as a target for regulation (30). The analysis of the c-*fos* promoter described here suggests once again that the TATAA sequence can be the target for E1A control. Thus, it appears that at least four promoter-specific transcription factors are targets in the pathway of E1A-dependent trans-activation.

Since the various factors involved in E1A trans-activation are of cellular origin, one must assume that they play a role normally in the control of cellular gene expression. Examples of endogenous cellular genes activated as a result of the E1A trans-activation process include the hsp70 heat shock gene (6, 7), an hsp89 heat shock gene (41), the β-tubulin gene (42), and the c-*fos* oncogene (9). Little is known concerning the mechanisms by which the β-tubulin gene is activated by E1A. In contrast, it appears likely that the c-*fos* and hsp70 genes are activated through the action of a TATAA-specific factor, apparently coincident with the mechanism by which the viral E1B gene is trans-activated (30). Furthermore, the E1A-dependent activation of the long terminal repeat of human immunodeficiency virus appears also to be mediated through the TATA element (43). Thus, the role of a TATA-specific factor in E1A-dependent trans-activation seems to be firmly established. The TATA factor also appears to be a target for the pseudorabiesvirus immediate early protein (44, 45) and the TATA element appears to be the essential regulatory site for herpes simplex virus late genes activated by infected-cell protein 4 (ICP4) (46, 47). A mechanism for TATA factor control has not been elucidated, but there is an increase in TFIID activity in extracts of adenovirus-infected cells (48).

One additional factor involved in the E1A trans-activation pathway has recently been shown to be utilized by a cellular gene. The E2F factor, which binds to two sites in the E2 promoter important for E1A-dependent E2 transcription, also binds to two sites in the P2 promoter of the human c-*myc* oncogene (49). These binding sites in the c-*myc* promoter are critical for E1A-dependent activation of c-*myc* transcription. In addition, recent experiments suggest that the c-*myc* E2F binding sites are important for serum-induced transcription of the c-*myc* gene. Interestingly, DNA binding assays of the TFIIIC factor have also suggested that the control of this factor by an E1A-dependent mechanism is also seen, depending on the proliferative state of the cell (50). Thus, two of the E1A-regulated factors may be controlled in the uninfected cell by mechanisms involved in the control of proliferation. The two cellular genes utilizing the TATA factor as a mechanism for E1A control, c-*fos* and the hsp70 gene, are also regulated by proliferation, although this growth factor regulation is largely due to the activation of factors that bind to upstream response elements. However, we wonder if the control of the TATA factor may also contribute to this growth-regulatory event. One could imagine that in circumstances where a large number of genes are activated, such as a growth response or a viral infection, the increase of a factor used by a large number of these genes would further enhance the stimulation.

This work was supported by grants from the National Institutes of Health to J.R.N. (GM26765) and N.H. (GM32544) and a Pew Scholars Award to N.H. M.C.S. and R.J.R. were supported by National Institutes of Health postdoctoral fellowships, and T.M.F. was a Medical Scientist Training Program Fellow.

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