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Transcriptional Activation of the CEF-4/9E3 Cytokine Gene by pp60$^{v\text{-}src}$

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The CEF-4/9E3 gene is expressed constitutively in Rous sarcoma virus (RSV)-transformed cells. This expression is largely determined by an increase in transcription of the gene. In this report, we characterize the regulatory elements responsible for the transformation-dependent activation of CEF-4/9E3. Three sequences corresponding to AP-1, PRD II/κB, and TAACGCAATT are involved in the process and therefore define the *src*-responsive unit (SRU) of the CEF-4 promoter. In constructs containing a deletion of the SRU, multiple copies of AP-1 or PRD II/κB, but not TAACGCAATT, led to activation of the promoter. Thus, factors interacting with these elements are constitutively activated in RSV-transformed chicken embryo fibroblasts. In agreement with the results of transient expression assays, proteins binding to AP-1, PRD II/κB, and TAACGCAATT were more abundant in the nuclei of transformed cells. The expression of the CEF-4 promoter was investigated in cells infected by a temperature-sensitive mutant of RSV. No significant increase in CEF-4 promoter activity was detected early after activation of pp60$^{v\text{-}src}$. In contrast, a substantial activation of the CEF-4 promoter was detected late after a temperature shift. Factors interacting with the TAACGCAATT, PRD II/κB, and AP-1 elements accumulated gradually over a period of several hours. Therefore, transcriptional activation plays an important role in the late, constitutive expression of the CEF-4 gene in stably transformed cells.

The stimulation of cell proliferation is characterized by the activation of a complex program of gene expression (2, 18, 30, 39). The induction of genes regulated by growth factors is strictly controlled and often restricted to the G_0/G_1 transition of the cell cycle. Several proto-oncogenes are expressed in response to growth factors. Therefore, precise regulatory mechanisms of gene expression are essential to the ordered proliferation of cells.

Several genes are expressed aberrantly in transformed cells. The proteins encoded by these genes determine some of the fundamental properties of transformed cells. For example, invasiveness is often correlated with the secretion of proteases (43). Increased rates of hexose transport are dependent on the activation the glucose transporter gene in various cell lines (12, 24, 64). The autocrine proliferation of transformed cells requires the continuous synthesis of a growth factor (1, 4, 15, 49). Thus, changes in gene expression are important for the genesis of the transformed cell.

In chicken embryo fibroblasts (CEF) transformed by the Rous sarcoma virus (RSV), the CEF-4/9E3 gene is activated constitutively (8, 59). The expression of CEF-4 is transient in normal cells stimulated by growth factors or inflammatory agents, while continuously proliferating cells show little expression of the gene (7, 28). In contrast, RSV-transformed cells express CEF-4 in a serum- and cell cycle-independent manner. The analysis of several mutants of pp60$^{v\text{-}src}$ indicated that the constitutive expression of CEF-4 is correlated with cell transformation (6, 8, 59). Therefore, the mechanisms controlling the expression of CEF-4 may be critical for the process of cell transformation. CEF-4 encodes a novel cytokine belonging to the interleukin-8 family. Several members of this family are also expressed aberrantly in transformed cells (3, 38, 66, 67). Chemotactic activities specific for various leukocytes or fibroblasts have been ascribed to these cytokines (57).

In this report, we characterize the activity of the CEF-4 promoter in normal and RSV-transformed CEF. Three elements, AP-1, PRD II/κB, and TAACGCAATT, are essential for activation by pp60$^{v\text{-}src}$. Proteins binding to each of these elements were investigated by electrophoretic mobility shift assays (EMSA) and shown to be more abundant in the nuclei of RSV-transformed cells. Thus, the activity of multiple transcription factors interacting with the CEF-4 promoter is regulated by pp60$^{v\text{-}src}$. In CEF infected with a temperature-sensitive (*ts*) mutant of RSV, the accumulation of the CEF-4 mRNA is biphasic (28). In these conditions, an early transient period of CEF-4 mRNA accumulation is followed by a late, prolonged period of expression. To define the molecular basis of this biphasic expression, CEF-4 promoter constructs were transfected in cells expressing a thermosensitive pp60$^{v\text{-}src}$. Transcriptional activation occurred late after the temperature shift, suggesting that the early expression of CEF-4 is not regulated at the transcriptional level. In contrast, the delayed promoter activation was coincident with the late, prolonged period of CEF-4 mRNA accumulation. Therefore, transcriptional activation appears to be important for the constitutive expression of CEF-4 in stably transformed cells. These observations are discussed in relation to results of other investigations on transformation-dependent gene expression.

MATERIALS AND METHODS

Cell culture. Early passages of CEF were grown at 41.5°C in Richter-improved minimal essential medium supple-

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mented with 5% heat-inactivated newborn bovine serum (Bocknek; Rexdale, Ontario, Canada), 5% tryptose phosphate broth, penicillin, and streptomycin. Unless indicated, all experiments were done with actively proliferating cells. Nearly confluent CEF were rendered quiescent after transfer for 24 h to serum-free medium containing 10% tryptose phosphate broth. Cell proliferation was stimulated by the addition of 10% serum or tetradecanoyl phorbol acetate (TPA) at 25 ng/ml. CEF were infected with the wild-type Schmidt-Ruppin A strain of RSV (SR-A RSV), the *ts* mutant NY 72-4 RSV, or the transformation-deficient mutant NY 315 RSV.

Nuclear run-on transcription assays. Run-on transcription assays were performed as described previously (8), with the following modifications. Cells (3×10^7) were suspended in 4 ml of ice-cold lysis buffer (15 mM NaCl, 60 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 14 mM β-mercaptoethanol, 0.5% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine, 10% sucrose, 15 mM Tris [pH 7.5]) and lysed with a Dounce homogenizer (B pestle). Nuclei were pelleted through 6 ml of a 30% sucrose cushion made in lysis buffer by centrifugation at 3,000 rpm in a Sorvall SS34 rotor for 15 min. The nuclei were resuspended in 100 μl of run-on transcription buffer [180 mM Tris-HCl (pH 8), 25 mM NaCl, 0.3 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1.5 mM dithiothreitol, 700 mM $(NH_4)_2SO_4$, 8 mM $MnCl_2$, 20 mM creatine phosphate, 200 μg of creatine phosphokinase per ml, 600 U of RNasine (Promega, Madison, Wis.) per ml, 1 mM each ATP, CTP, and GTP] and incubated in presence of 150 μCi of [α-^{32}P]UTP as described previously (8). Labelled transcripts were hybridized to 2 μg of linearized CEF-4, glutaraldehyde phosphate dehydrogenase, and Bluescript SK$^+$ plasmids blotted on Nytran membranes (Schleicher & Schuell, Keene, N.H.).

CEF-4 promoter constructs. The CEF-4 gene and flanking regions were isolated from a chicken genomic library (20). The nucleotide sequence was determined by the chain termination method, using a series of progressively and directionally deleted DNA subclones (generated by digestion with exonuclease III). A 3′ deletion beginning at nucleotide +36 from the transcription start site (59) was chosen to generate all CEF-4 promoter constructs. DNA fragments from the 5′ flanking region of the CEF-4 gene were isolated by restriction enzyme digestion and inserted into the unique *Sma*I site of pUMSVOCAT (51). The inactive, internally deleted S-N/CAT construct was generated by digesting plasmid Xho/CAT (nucleotides −1312 to +36 of CEF-4; see Fig. 4) with *Spe*I and *Nhe*I, followed by ligation of the resulting cohesive ends. Digestion with *Spe*I and *Nhe*I removes a DNA fragment corresponding to nucleotides −64 to −214 of the CEF-4 promoter (see Fig. 2). The APC/CAT, PC/CAT, 4XAP1/CAT, 4XPRD/CAT, and 6XCAAT/CAT plasmids were generated by inserting synthetic double-stranded DNA molecules in the internally deleted S-N/CAT construct at the site of deletion (*Spe*I-*Nhe*I digestion). DNA fragments corresponding to nucleotides −119 to −64 and nucleotides −95 to −64 of the CEF-4 promoter were synthesized to generate APC/CAT and PC/CAT, respectively. The following oligonucleotides were synthesized to obtain 4XAP1/CAT, 4XPRD/CAT, 6XCAAT/CAT, and other plasmids containing one or several copies of AP-1, PRD II/κB, or TAACGCAATT:

AP-1: 5′AGCTTGTGACTCATTT3′
3′ACACTGAGTAAACTAG5′

PRD II/κB: 5′AGCTTCTGGGAAATTCCTG3′
3′AGACCCTTTAAGGACCTAG5′

↓ ↓ ↓
TAACGCAATT: 5′GAGCTAACGCAATTACATG3′
3′ATTGCGTTAATGTACTAGG5′

Underlined nucleotides were substituted in the mutant form of the oligonucleotide as depicted in Fig. 4. Nucleotides were added to generate 5′ protruding ends. Complementary oligonucleotides were annealed, filled in with Klenow enzyme, and inserted into S-N/CAT by blunt-end ligation as described above. The orientation and copy number of the inserted element(s) were determined by sequencing analysis. All oligonucleotides were synthesized on a Gene Assembler machine (Pharmacia, Baie d'Urfe, Quebec, Canada). Site-directed mutagenesis was done according to the method of Taylor et al. (60), using commercially available enzymes and reagents (Amersham Canada, Ltd). The following oligonucleotides were synthesized for site-directed mutagenesis:

μCAAT/CAT: 5′-CATGTACTTACGCTAGAGGAA-3′
μPRD/CAT: 5′-AGAGGAATTTGAGAGATGTAG-3′
μAP1/CAT: 5′-GTAAAAATGCTGCACATGACTG-3′

Transfections and CAT assays. CEF were seeded at a density of 2×10^6 cells per 100-mm dish a day prior to transfection. Ten micrograms of test plasmid and 2.5 μg of the *lacZ* gene-containing plasmid pRSV-βgal (23) were co-transfected by the DEAE-dextran method (42). Cells were lysed 44 h after transfection and assayed for β-galactosidase activity to correct for differences in transfection efficiency. Lysates containing equal levels of β-galactosidase activity were then assayed for chloramphenicol acetyltransferase (CAT) activity (50). [^{14}C]chloramphenicol and its acetylated derivatives were separated by thin-layer chromatography and detected by autoradiography. Conversion levels were determined by scintillation counting. All test plasmids were analyzed in triplicate cultures in at least three separate experiments. In these conditions, the results varied by 20% or less.

EMSA. Buffers A, C, and D used for preparation of nuclear extracts were described by Dignam et al. (19). All steps were done on ice, and proteolysis was minimized by addition of 0.5 mM phenylmethylsulfonyl fluoride and 0.3 μg each of antipain and leupeptin per ml to all buffers. Aprotinin (0.5 μg/μl) was also added to buffers A and C. Cells were washed twice with ice-cold phosphate-buffered saline and collected with a rubber policeman. The cells were then resuspended in an equal volume of buffer A and homogenized with a glass Dounce homogenizer (B pestle). Cell lysis was monitored by phase-contrast microscopy. The cell lysate was centrifuged at 12,000 × *g* for 20 min at 4°C, and the nuclear pellet was extracted with 2 volumes of buffer C for 20 min at 4°C. The nuclear lysate was centrifuged at 12,000 × *g* for 20 min at 4°C; the supernatant was dialyzed against 500 ml of buffer D at 4°C for 5 h and centrifuged for 5 min in a microfuge at 4°C. The extract was removed, quickly frozen, and stored in liquid nitrogen. Protein concentration was determined by the method of Bradford at 545 nm, using bovine serum albumin as a standard. Binding reactions were performed essentially as described by Lenardo et al. (40). Briefly, 5-μg samples of nuclear extracts were incubated with approximately 0.25 ng (40,000 cpm) of end-labelled oligonucleotide probes in the presence of 50 ng of Bluescript SK$^+$ plasmid DNA (Stratagene, San Diego, Calif.) and 2 μg of poly(dI-dC) as a nonspecific DNA competitor. Reactions were done in a final volume of 20 μl containing 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM $MgCl_2$, and 5% glycerol at room temperature for 25 min;

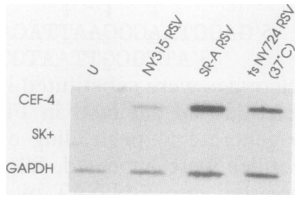

FIG. 1. Characterization of CEF-4 gene expression by transcription run-on analysis. Nuclei were isolated from uninfected CEF (U), CEF infected by NY 315 RSV, and CEF transformed by SR-A RSV or *ts* NY 72-4 RSV at the permissive temperature of 37°C. The Bluescript SK^+ and glutaraldehyde phosphate dehydrogenase (GAPDH)-containing plasmid DNAs were included as negative and positive controls, respectively.

3 mM GTP was also added in the reaction mixture with the PRD II/κB probe. In competition analyses, an excess of unlabelled oligonucleotides was included in the binding mixture 15 min prior to addition of the labelled probe. After incubation, the samples were subjected to electrophoresis on a native 4% polyacrylamide gel in 7.1 mM Tris-HCl (pH 7.9)–3.3 mM sodium acetate (pH 7.9)–1 mM EDTA at 80 V for 2.5 h. All gels were prerun at 125 V for 1 h and run at room temperature with circulating buffer. After electrophoresis, the gels were dried under vacuum and exposed on X-AR films (Kodak, Rochester, N.Y.) with intensifying screens at −70°C. Synthetic double-stranded oligonucleotides, described above, were used to generate specific probes. The 5′ protruding ends were filled in with Klenow enzyme, using a mixture of unlabelled nucleotides and [^{32}P]dCTP.

Nucleotide sequence accession number. The sequence of the CEF-4 promoter can be found in GenBank under accession number M83361.

RESULTS

pp60$^{v\text{-}src}$ activates CEF-4 at the transcriptional level. The CEF-4/9E3 mRNA is 20 to 30 times more abundant in RSV-transformed CEF than it is in their normal counterparts (8, 59). The level of CEF-4 mRNA is significantly lower (by 10-fold) in cells infected by NY 315 RSV, a transformation-deficient mutant of RSV encoding a myristylation-minus form of pp60$^{v\text{-}src}$. These differences in the expression of the CEF-4 mRNA are mirrored by the activity of the gene in run-on transcription assays (Fig. 1). Transcriptional activation was particularly obvious in cells stably transformed by a wild-type strain of RSV, such as SR-A RSV. A 10-fold difference in the activity of the CEF-4 gene was found between nuclei isolated from SR-A-transformed cells and nuclei isolated from normal cells (Fig. 1). High levels of CEF-4 expression were also observed with nuclei isolated from cells transformed by *ts* NY 72-4 RSV at the permissive temperature. However, the transcriptional activity of the CEF-4 gene in CEF transformed by the *ts* mutant did not represent more than 50% of the activity measured in CEF stably transformed by SR-A RSV (Fig. 1) (8). Thus, the constitutive expression of CEF-4 in RSV-transformed fibroblasts is largely dependent on transcriptional activation.

Isolation and characterization of the CEF-4 promoter in transient expression assays. Southern blotting analysis indicated that CEF-4 is encoded by a single-copy gene (data not shown). Recombinant phages harboring sequences hybridizing to the CEF-4 cDNA were isolated from a chicken genomic DNA library. Sequencing analysis indicated that

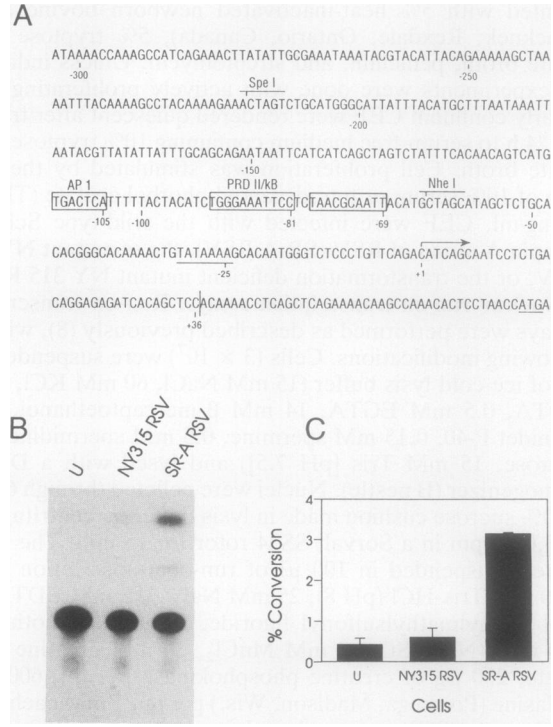

FIG. 2. (A) Nucleotide sequence of the 5′ flanking region of the CEF-4 gene. The site of transcription initiation (59) is indicated by the arrow. The TATAAAA box and initiating ATG are underlined. Restriction sites described in the text are indicated. Regulatory elements of the promoter are boxed, and the position of the 3′ deletion used in CEF-4 promoter constructs is indicated at position +36. (B and C) Analysis of the Spe/CAT construct in transient expression assays. A DNA fragment beginning at nucleotide −214 and ending at nucleotide +36 of the CEF-4 promoter and gene was inserted in proximity of the CAT reporter gene and transfected in uninfected (U) CEF, CEF infected by NY 315 RSV, and CEF transformed by SR-A RSV. The results of CAT assays are shown in panel B and expressed as percent conversion of [^{14}C]chloramphenicol into its acetylated forms in panel C.

the CEF-4 gene is composed of four exons and three introns (our unpublished results). A 2.8-kb segment of DNA located upstream of the site of transcription initiation was also sequenced (Fig. 2A and data not shown) and analyzed in transient expression assays. To this end, DNA fragments containing 5′ flanking sequences and 36 bp of the CEF-4 gene were inserted in the unique *Sma*I site of pUMSVOCAT (51) and transfected into normal uninfected CEF, CEF infected by NY 315 RSV, and CEF transformed by wild-type SR-A RSV. As shown in Fig. 2B, a fragment containing as few as 214 bp of 5′ flanking DNA was capable of conferring a transformation-dependent expression to the CAT reporter gene (Spe/CAT construct). The activity of the CEF-4 promoter was elevated 4- to 12-fold in SR-A RSV-transformed cells (Fig. 2 to 4 and data not shown), in agreement with the results of run-on transcription assays. A plasmid consisting of the RSV long terminal repeat (LTR) and the *lacZ* gene (23) was cotransfected in all experiments to control for differences in transfection efficiency. The RSV LTR is not regulated by transformation when cells are cultured in presence of serum (i.e., when the cells are actively proliferating [8, 21]). In our experimental conditions, the activity of a CAT reporter gene under the control of the RSV LTR was

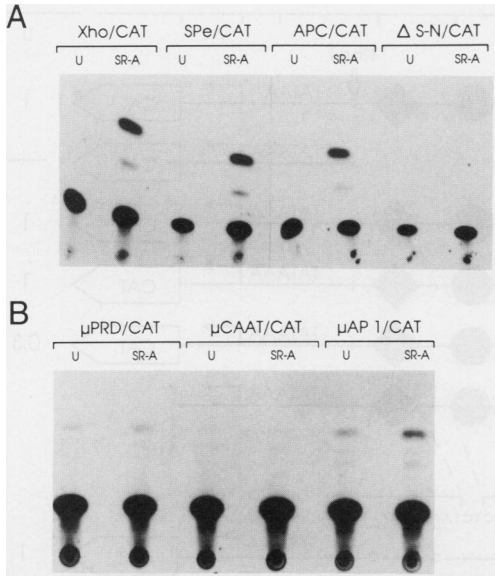

FIG. 3. Characterization of the CEF-4 promoter. (A) CEF-4 promoter constructs depicted in Fig. 4 were analyzed in transient expression assays. The results of CAT assays are shown for constructs transfected in normal, uninfected (U), or SR-A RSV-transformed CEF. (B) The PRD II/κB, CAAT, and AP-1 elements were modified separately by site-directed mutagenesis of the Spe/CAT construct and analyzed in normal and SR-A RSV-transformed CEF. The autoradiograph shown in panel B was overexposed to reveal the basal activity of CEF-4 promoter constructs.

identical when transfected in normal and RSV-transformed CEF (our unpublished results). Hence, activation of the CEF-4 promoter in RSV-transformed cells did not reflect differences in transfection efficiency, nor was it due to an increased rate of cell proliferation. Moreover, CEF-4 promoter constructs were more active in SR-A RSV-transformed CEF than in CEF infected by NY 315 RSV (Fig. 2). Hence, the increased activity of the promoter was transformation dependent. This conclusion was also supported by results obtained with CEF infected by a *ts* mutant of RSV (see below [Fig. 6]). With all constructs investigated, we did not observe any significant difference in the transient expression of the CEF-4 promoter in uninfected and NY 315 RSV-infected cells. Hence, elements responsible for increased activity of the CEF-4 promoter in NY 315 RSV-infected cells may lie outside the region investigated (Fig. 1). Alternatively, it is possible that our assay was not accurate enough to detect small differences in the expression of the reporter gene.

The activities of constructs containing as few as 214 bp (Spe/CAT) or as many as 1,312 bp (Xho/CAT) of 5′ flanking DNA were indistinguishable (Fig. 3 and 4). These constructs had identical basal and enhanced activity in uninfected and RSV-transformed cells, respectively. Deletion of nucleotides −64 to −214 (fragment *Spe*I-*Nhe*I; S-N/CAT construct) abolished the activity of the CEF-4 promoter in normal and transformed cells, suggesting that critical regulatory elements were located in this region of the promoter. In constructs containing a deletion of the *Spe*I-*Nhe*I fragment, the activity of the promoter was restored by inserting a synthetic double-stranded DNA molecule corresponding to nucleotides −64 to −119 of the CEF-4 promoter (APC/CAT construct; Fig. 3 and 4). Therefore, the −64 to −119 region contains one or several regulatory elements responsible for transformation-dependent activation by pp60$^{v\text{-}src}$. Identical results were obtained when NY 315 RSV-infected cells were transfected in place of normal CEF (data not shown). Constructs containing the −64 to −119 region were as active or nearly as active as undeleted promoter constructs (Fig. 4 and data not shown). Other regions of the promoter may share similar properties with the −64 to −119 proximal region. However, their contribution, if any, appears to be redundant and nearly dispensable for the transformation-dependent activation of the CEF-4 promoter. For this reason, we focused our attention on the −64 to −119 region of the promoter.

Multiple elements of the CEF-4 promoter are required for activation by pp60$^{v\text{-}src}$. The sequence of the CEF-4 promoter is shown in Fig. 2A. The −64 to −119 region consists of several potential regulatory elements, including AP-1, PRD II/κB, and TAACGCAATT. The AP-1 complex, composed of c-Jun and c-Fos (or related proteins), is activated by a wide variety of oncoproteins, growth factors, and mitogens (14). PRD II was first characterized in the promoter of the beta interferon gene (29). Several viral enhancers and genes encoding proteins involved in the inflammatory process and immune response contain a similar element. Members of the NF-κB family of transcription factors interact with the PRD II or κB sequence (40). The activity of numerous promoters is dependent on a CAAT-like sequence (45).

To determine the role of AP-1, PRD II/κB, and TAACG CAATT in the activity of the promoter, each element was modified separately by site-directed mutagenesis (Fig. 3 and 4). Transient expression assays indicated that each mutation severely impairs the activity of the CEF-4 promoter in SR-A RSV-transformed CEF (Fig. 3 and 4). Therefore, all three elements are essential to the activation by pp60$^{v\text{-}src}$. Since the transformation-dependent expression was nearly suppressed with all three mutants, AP-1, PRD II/κB, and TAACGCAATT appear to cooperate in the activation process. These elements define the *src*-responsive unit (SRU) of the CEF-4 promoter.

The enhanson properties of each element were investigated in the context of the CEF-4 promoter. To this end, single or multiple copies of a single element were inserted in the *Spe*I-*Nhe*I deletion of CEF-4 promoter constructs and transfected into normal and SR-A RSV-transformed CEF. A single copy of PRD II/κB or TAACGCAATT had no effect on the activity of the deleted promoter, while a single AP-1-binding site conferred a weak activation (data not shown). In contrast, constructs containing four copies of AP-1 (4XAP1/CAT) or PRD II/κB (4XPRD/CAT) were as efficient in promoter activation as was the intact −64 to −119 region of the CEF-4 promoter (Fig. 4). Therefore, the specific combination of AP-1, PRD II/κB, and TAACG CAATT was not required, nor was it particularly sensitive to the activation by pp60$^{v\text{-}src}$. The property of regulatory elements such as AP-1 or PRD II/κB to function as multimers or in cooperation with distinct elements has been described previously (25, 32). Multiple copies of a mutant PRD II/κB or mutant AP-1 element did not increase the activity of the deleted promoter (data not shown). Thus, we conclude that factors interacting with the AP-1 and PRD II/κB elements are constitutively activated in RSV-transformed fibroblasts. Multiple copies of TAACGCAATT had no effect on the promoter (6XCAAT/CAT construct; Fig. 4), suggesting that this element functions only when located in proximity of a distinct element such as AP-1 or PRD II/κB. Therefore, it behaved as a class B enhanson (25) and not simply as a

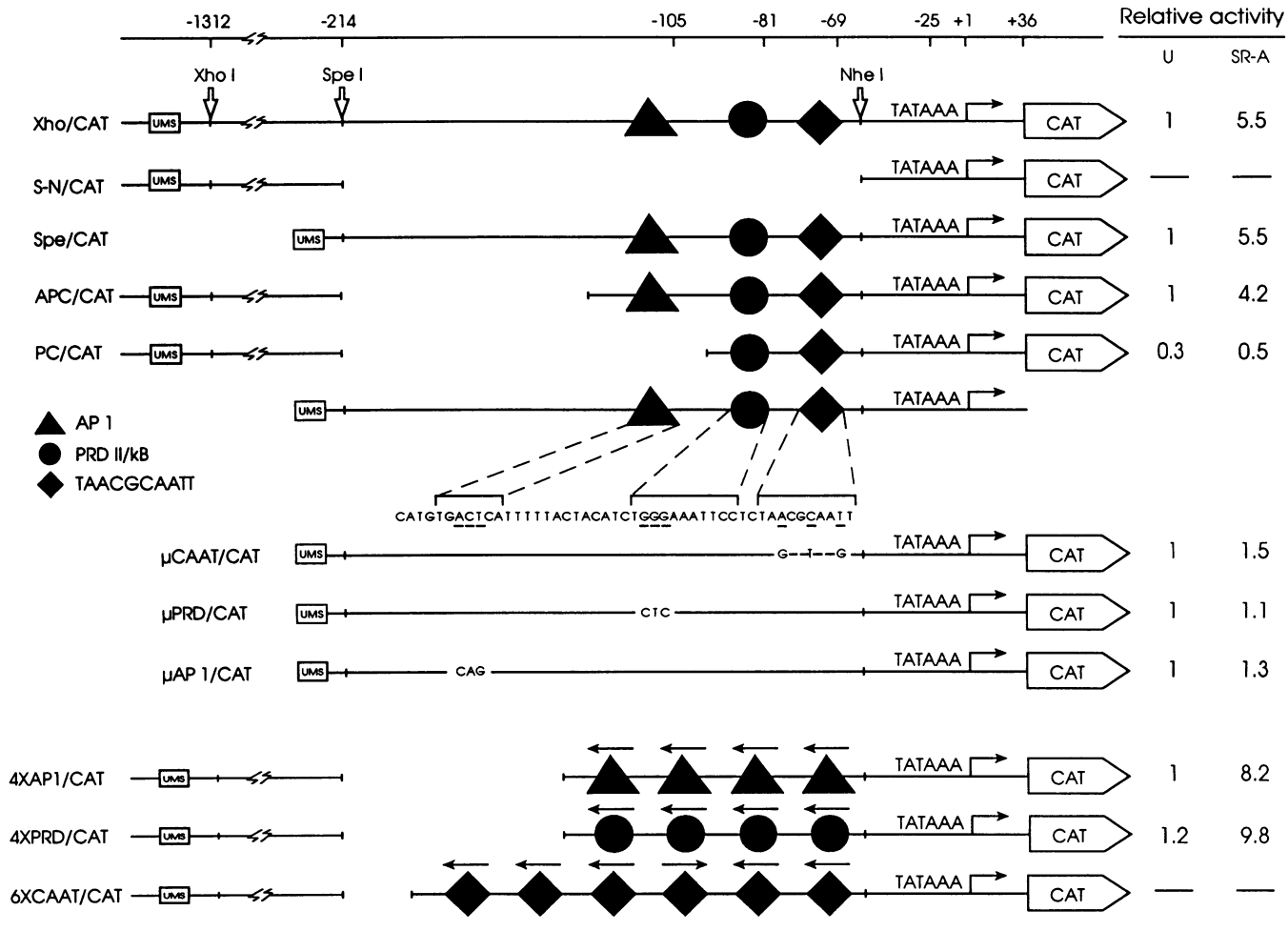

FIG. 4. Description and activity of CEF-4 promoter constructs. DNA fragments containing various regions of the CEF-4 promoter were inserted in pUMSVOCAT (51) and analyzed in transient expression assays. The position of the upstream mouse sequence, a transcription terminator inserted upstream of the CEF-4 promoter fragments (65), is indicated by the UMS box. Major restriction sites and regulatory elements of the promoter are described. The transcription start site is indicated by nucleotide +1, and the site of insertion of the CAT reporter gene is indicated by nucleotide +36. Nucleotides mutagenized in μCAAT/CAT, μPRD/CAT, and μAP1/CAT are underlined. Arrows in the 4XAP1/CAT, 4XPRD/CAT, and 6XCAAT/CAT constructs indicate the orientation of the element inserted. The activity of each construct is expressed in relation to that of an intact CEF-4 promoter construct (Xho/CAT or Spe/CAT) transfected in uninfected CEF (defined as 1). Lines indicate undetectable levels of CAT activity. Values of CAT activity represent the average of triplicate samples. For any given construct, the results did not vary by more than 20% of the indicated value.

constitutive promoter element. A synthetic DNA fragment consisting essentially of the PRD II/κB and TAACGCAATT elements (−64 to −95 region) was also inserted into the *Spe*I-*Nhe*I deletion. Transfection of this construct resulted in a weak but reproducible activation of the promoter in RSV-transformed cells (PC/CAT in Fig. 4). To a limited extent, the TAACGCAATT element functions in association with PRD II/κB. The activity of deletion constructs containing the −64 to −95 region was also diminished in normal, uninfected CEF. Therefore, sequences located between −95 and −119 appear to be important for basal activity of the CEF-4 promoter.

RSV-transformed cells contain elevated levels of several DNA-binding proteins. DNA-binding proteins interacting with AP-1, PRD II/κB, and TAACGCAATT were investigated by EMSA. Synthetic double-stranded oligonucleotide probes were end labelled and incubated with nuclear extracts prepared essentially as described by Dignam et al. (19). Nucleoprotein complexes, resolved on native polyacrylamide gels, are shown in Fig. 5. Proteins binding to AP-1, PRD II/κB, and TAACGCAATT were all more abundant in the nuclei of SR-A RSV-transformed CEF. The formation of labelled DNA-protein complexes was abolished when an excess of cold homologous oligonucleotides was preincubated with the nuclear extracts. In contrast, a vast excess of mutant oligonucleotides did not compete or competed poorly for formation of the same complexes (see below). Therefore, we conclude that $pp60^{v\text{-}src}$ regulates the activity of specific factors interacting with AP-1, PRD II/κB, and TAACGCAATT.

Normal CEF contained significant levels of the PRD II/κB-binding protein(s). This level was roughly four- to sixfold higher in the nuclei of RSV-transformed cells. An excess of mutant PRD II/κB oligonucleotides competed partially with formation of this complex (complex B in Fig. 5B). Thus, distinct factors may be present in complex B. Moreover, a slower-migrating complex (labelled A in Fig. 5B) was formed with nuclear extracts prepared from CEF

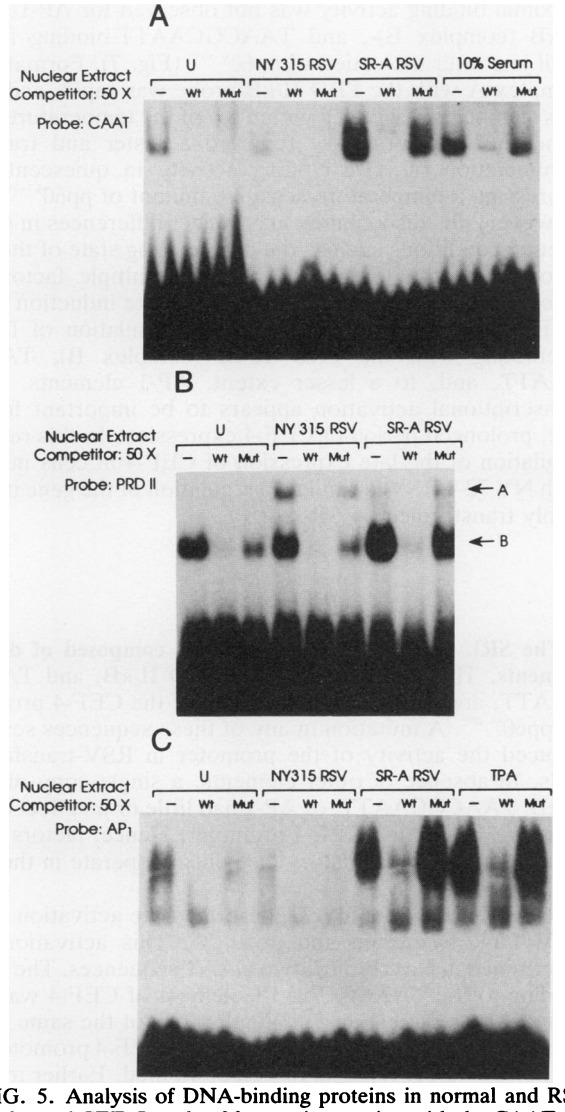

FIG. 5. Analysis of DNA-binding proteins in normal and RSV-transformed CEF. Levels of factors interacting with the CAAT (A), PRD II/κB (B), and AP-1 (C) elements were examined by EMSA. Nuclear extracts were prepared from uninfected CEF (U), CEF infected by NY 315 RSV, or CEF transformed by SR-A RSV. In panel A, the level of the CAAT-binding factor was also examined in quiescent CEF stimulated with 10% serum for 30 min. Serum was replaced by TPA at 25 ng/ml in panel C (AP-1 probe). An excess of unlabelled homologous (Wt) or mutant (Mut) oligonucleotides was added in competition assays. Complexes A and B formed with the PRD II/κB probe (B) are described in the text.

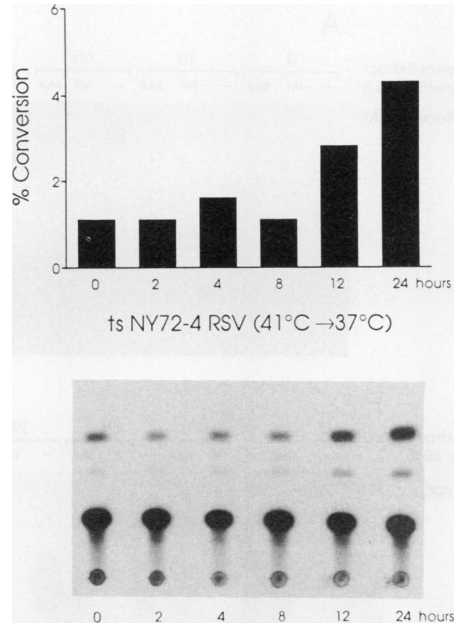

FIG. 6. Kinetics of CEF-4 promoter activation. CEF infected by *ts* NY 72-4 RSV were transfected with the Spe/CAT CEF-4 promoter construct at the nonpermissive temperature of 41.5°C. CAT activity was determined in parallel cultures transferred to the permissive temperature of 37°C for increasing period of times. Values are expressed as percent conversion of [^{14}C]chloramphenicol into its acetylated forms.

infected by NY 315 RSV and, to a lesser extent, from CEF transformed by SR-A RSV. Although this complex appears to be specific, its significance remains unclear.

A considerable increase in AP-1-binding activity was detected in extracts of SR-A RSV-transformed CEF. These levels of AP-1 were significant since they were comparable to levels observed after stimulation by TPA (maximal at 30 min; data not shown). Bands formed with the AP-1 probe were always very broad and may also include distinct nucleoprotein complexes.

Levels of the TAACGCAATT-binding proteins increased after transformation by RSV. The activity of this factor was also regulated by growth factors. Indeed, greater amounts of the TAACGCAATT-binding activity was detected in the nuclei of quiescent CEF stimulated by the addition of serum for 30 min (Fig. 5A).

Transcriptional activation is important for the late, constitutive expression of CEF-4 in stably transformed CEF. Upon transfer to the permissive temperature, the CEF-4 mRNA accumulates rapidly in CEF infected by the *ts* mutant, NY 72-4 RSV (6, 8, 59). Under these conditions, however, the expression of CEF-4 is biphasic (28). An early transient period of CEF-4 mRNA accumulation, maximal within 1 h of the temperature shift, is followed by a decline in the abundance of the mRNA and, at about +8 h, by a late and prolonged period of CEF-4 mRNA expression. The results of run-on transcription assays indicated that the rapid accumulation of the CEF-4 mRNA is regulated predominantly by posttranscriptional mechanisms (8, 13). More significant increases in run-on transcription activity of CEF-4 were observed several hours after activation of pp60$^{v\text{-}src}$. To determine the role of transcriptional activation in the expression of the CEF-4 gene, promoter constructs were transfected in NY 72-4 RSV-infected CEF at the nonpermissive temperature of 41.5°C. The activity of the reporter gene was then quantitated in parallel cultures transferred to the permissive temperature of 37°C for increasing periods of time. Results shown in Fig. 6 indicate that significant increases in the activity of the CEF-4 promoter were not detected until 8 to 12 h after activation of pp60$^{v\text{-}src}$. In the same conditions, the expression of the CAT reporter gene in normal, uninfected CEF transfected with the same construct was identical at 41.5 and 37°C (data not shown). Hence, the activation of the CEF-4 promoter in NY 72-4 RSV-infected cells was dependent on the temperature-sensitive pp60$^{v\text{-}src}$ (data not shown). Increases in CAT activity were observed at a time

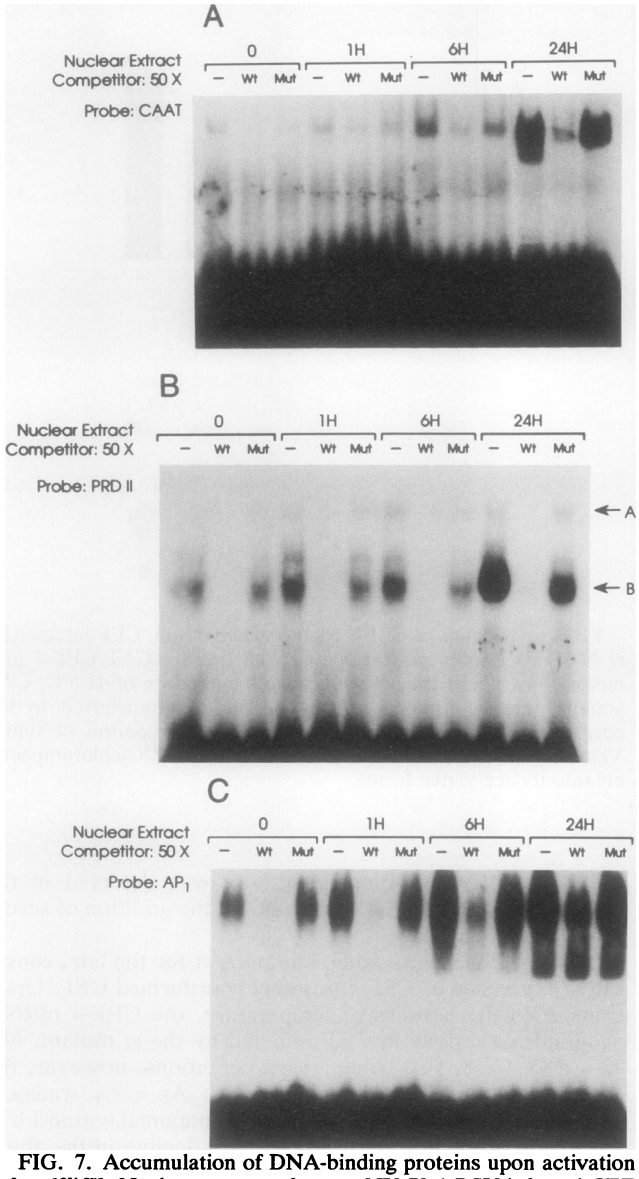

FIG. 7. Accumulation of DNA-binding proteins upon activation of pp60$^{v\text{-}src}$. Nuclear extracts from *ts* NY 72-4 RSV-infected CEF were prepared at different times after transfer to the permissive temperature of 37°C (0, 1, 6, or 24 h). Nucleoprotein complexes formed with the CAAT (A), PRD II/κB (B), and AP-1 (C) probes were analyzed by EMSA. Excesses of unlabelled homologous (Wt) or mutant (Mut) oligonucleotides were used as competitors. Complexes A and B formed with the PRD II/κB probe are described in the text.

corresponding to the late accumulation of the CEF-4 mRNA (28). Thus, transcriptional activation of the CEF-4 promoter appears to be a delayed event of transformation by pp60$^{v\text{-}src}$.

A prediction based on the results of transient expression assays would be that DNA-binding proteins interacting with regulatory elements of the CEF-4 SRU will also accumulate with a delayed kinetics in NY 72-4 RSV-infected cells. This prediction is largely supported by results shown in Fig. 7. In this analysis, levels of proteins interacting with the TAACG CAATT, PRD II/κB, and AP-1 elements were investigated by EMSA at different times after activation of pp60$^{v\text{-}src}$. Although each factor accumulated with a distinct kinetics, maximal binding activity was not observed for AP-1-, PRD II/κB (complex B)-, and TAACGCAATT-binding factors until late after activation of pp60$^{v\text{-}src}$ (Fig. 7). Formation of complex A with the PRD II/κB probe was rapid, and AP-1 was significantly induced within 6 h of the temperature shift. Other investigators have reported a faster and transient accumulation of AP-1-binding activity in quiescent cells expressing a temperature-sensitive mutant of pp60$^{v\text{-}src}$ (63). However, this discrepancy may reflect differences in experimental conditions such as the proliferating state of the cells before activation of pp60$^{v\text{-}src}$. Since multiple factors are essential to the activation process, the late induction of the CEF-4 promoter may reflect the accumulation of factors interacting with the PRD II/κB (complex B), TAACG CAATT, and, to a lesser extent, AP-1 elements. Thus, transcriptional activation appears to be important for the late, prolonged period of CEF-4 expression. In this respect, regulation of the late expression of CEF-4 in cells infected with NY 72-4 RSV is similar to regulation of the gene in cells stably transformed by SR-A RSV.

DISCUSSION

The SRU of the CEF-4 promoter is composed of distinct elements. Three elements, AP-1, PRD II/κB, and TAACG CAATT, are required for activation of the CEF-4 promoter by pp60$^{v\text{-}src}$. A mutation in any of these sequences severely reduced the activity of the promoter in RSV-transformed cells. In absence of other elements, a single copy of PRD II/κB, TAACGCAATT, or AP-1 had little or no effect on the activity of a deleted CEF-4 promoter. Hence, factors interacting with these regulatory elements cooperate in the activation process.

Recently, Dutta et al. (22) described the activation of the RSV LTR by serum and pp60$^{v\text{-}src}$. This activation was determined in part through two CAAT sequences. The factor binding to the TAACGCAATT element of CEF-4 was also induced by serum (Fig. 5). Whether or not the same factor interacts with the CAAT element of the CEF-4 promoter and of the RSV LTR remains to be determined. Earlier investigations described the activation of AP-1 by *src* and a variety of other oncogenes (46, 52, 62). We now present data indicating that AP-1 is important for expression of the CEF-4 promoter in RSV-transformed cells. The same conclusion applies to PRD II/κB. In the absence of other elements, multiple copies of PRD II/κB conferred a transformation-dependent activation to a deleted CEF-4 promoter (Fig. 4). Thus, several genes regulated through PRD II/κB may be activated by pp60$^{v\text{-}src}$.

AP-1, PRD II/κB, and TAACGCAATT define the SRU of the CEF-4 promoter. In this respect, the organization of this promoter is similar to that of other promoters and viral enhancers regulated by the product of oncogenes (32). A cluster of serum response elements is necessary for activation of the *egr-1* promoter by pp60$^{v\text{-}src}$ (47). Several regions of the c-*fos* promoter, including the serum response element, respond to the action of Raf and other oncoproteins (26, 27, 33, 37, 52). The polyomavirus enhancer and the collagenase and stromelysin promoters require the presence of AP-1- and c-Ets-binding sites for activation by numerous oncogenes (31, 52, 61). AP-1 and other uncharacterized elements are also necessary for the activation of the transforming growth factor β1 and T64 genes by pp60$^{v\text{-}src}$ (11, 34). Hence, a variety of transcription factors are constitutively activated by the products of various oncogenes. Unique patterns of

gene activation in transformed cells are likely to arise from specific combinations of promoter elements.

Mechanisms of transcriptional activation by pp60$^{v\text{-}src}$. pp60$^{v\text{-}src}$ acts upon the signal transduction circuitry to modulate the activity of the transcriptional machinery. In RSV-transformed cells, the inhibitors of protein kinase C interfere with the expression of CEF-4/9E3 (55). In agreement with this result, we found that factors interacting with the AP-1, PRD II/κB, and TAACGCAATT elements were all activated, to various degrees, in response to TPA (our unpublished results; Fig. 5C). CEF-4 promoter constructs were also stimulated by TPA in transient expression assays, but this activation was modest in comparison with the response to pp60$^{v\text{-}src}$. Thus, activation of the protein kinase C pathway may be critical yet not sufficient for the constitutive expression of CEF-4 in RSV-transformed cells. Interfering with a pathway leading to the activation of a single factor of the SRU would severely impair the activation of the CEF-4 promoter, as discussed above. Differences in the activation kinetics of AP-1-, PRD II/κB-, and TAACGCAATT-binding proteins argue in favor of multiple pathways of activation (Fig. 7). It is worth noting that recent reports described the regulation of gene expression by pp60$^{v\text{-}src}$ through protein kinase C-dependent and -independent pathways (36, 48).

The activation of transcription factors in transformed cells is still poorly understood. We and others have determined that the expression of c-*fos*, c-*jun*, *junB*, and *junD* is not significantly altered in RSV-transformed cells (35, 63; our unpublished results). Hence, pp60$^{v\text{-}src}$ appears to activate AP-1 by a posttranscriptional mechanism. The dephosphorylation of amino acid residues located at the C terminus enhanced the DNA-binding capacity of c-Jun and AP-1 in TPA-stimulated cells (16). These dephosphorylation events have also been described in cells expressing Ha-*ras* (10, 54). Moreover, transformation by Ha-*ras* results in phosphorylation of the activation domain of c-Jun and potentiation of its transactivation function (10, 54). In both cases, the effect of these changes on gene expression is to increase transcription through the TPA-responsive element. Thus, similar phosphorylation/dephosphorylation events may account for the increased DNA-binding activity and function of AP-1 in RSV-transformed CEF (Fig. 3 to 5). Results described by Baichwal et al. (5) also suggested that in CEF, the activation of c-Jun by *src* and *ras* is mediated through the negative regulatory domain (δ) of the protein. Hence, the interaction of c-Jun with its inhibitor could be disrupted by pp60$^{v\text{-}src}$. Certain events such as formation of complex A with the PRD II/κB probe appears to be exquisitely sensitive to the action of pp60$^{v\text{-}src}$ (Fig. 7B). On the other hand, genes encoding transcription factors may themselves be induced by pp60$^{v\text{-}src}$ and lead to a more delayed pattern of activation. Thus, pp60$^{v\text{-}src}$ may regulate the activity of transcription factors through a variety of mechanisms, including potentiation of the activation function, increase of DNA-binding capacity, modification of inhibitory molecules, and changes in gene expression.

Delayed activation of the CEF-4 promoter by pp60$^{v\text{-}src}$. The accumulation of the CEF-4 mRNA in response to the activation of a thermosensitive pp60$^{v\text{-}src}$ is biphasic (28). The early phase of CEF-4 expression is rapid, independent of protein synthesis and transient. The second phase, detectable several hours after pp60$^{v\text{-}src}$ activation, is prolonged. Hence, different levels of CEF-4 gene expression appear to be regulated by pp60$^{v\text{-}src}$. The results of run-on transcription analyses, transient expression, and mobility shift assays indicated that the early, rapid accumulation of the CEF-4 mRNA is not regulated at the transcriptional level (8) (Fig. 6 and 7). Differences in the stability of the CEF-4 mRNA have been reported and may account for the early phase of CEF-4 expression (8, 58). Recently, Blobel and Hanafusa described the activation of CEF-4/9E3 through elements located in the promoter and in the 3′ untranslated region of the CEF-4 gene (13). The authors showed that the addition of the untranslated region of CEF-4/9E3 to the 3′ end of the CAT gene (under the control of the CEF-4 promoter) resulted in enhanced gene expression in RSV-transformed cells. Although the mechanism was not investigated, it is probable that the 3′ untranslated region of CEF-4/9E3 acted at the posttranscriptional level (13). This region of the gene includes two A+U-rich domains also found in a number of unstable transcripts (8, 17, 58). The addition of such A+U-rich repeats is sufficient to destabilize the globin mRNA (53). Therefore, factors responsible for the decay of A+U-containing transcripts may be regulated by the action of pp60$^{v\text{-}src}$. Such factors appear to be particularly important for the early phase of CEF-4/9E3 expression in cells infected by NY 72-4 RSV (28) and for expression of the gene in serum- or TPA-stimulated cells (13, 58).

Several results indicate, however, that transcriptional activation is important for the second phase of CEF-4 expression. CEF-4 promoter constructs were induced several hours after activation of pp60$^{v\text{-}src}$ (Fig. 6). Factors interacting with the PRD II/κB, TAACGCAATT, and, to a lesser extent, AP-1 elements accumulated gradually in cells infected by *ts* NY 72-4 RSV (Fig. 7). Increases in run-on transcription activity were also more significant late after activation of pp60$^{v\text{-}src}$ (8). Thus, CEF-4 appears to be regulated by similar mechanisms in cells stably transformed by a thermosensitive or a wild-type pp60$^{v\text{-}src}$. Hence, transcriptional activation is important for the constitutive expression of CEF-4.

The existence of multiple regulatory mechanisms responsible for the expression of a single gene illustrates the complex and pleiotropic action of pp60$^{v\text{-}src}$. On the other hand, the existence of early and delayed mechanisms of gene expression suggests that a subset of early-immediate genes, such as CEF-4, and genes normally expressed later in the cell cycle may be regulated by the same mechanisms. Genes such as those encoding collagenase, stromelysin, urokinase-type plasminogen activator, or GRP78 are activated late after activation of pp60$^{v\text{-}src}$ and/or require protein synthesis for expression (9, 44, 52, 56). Their activation by pp60$^{v\text{-}src}$ depends primarily on transcriptional activation and often involves common regulatory elements recognized by AP-1, c-Ets-1, and c-Est-2 (31, 32). Thus, although several transcription factors appear to be activated by pp60$^{v\text{-}src}$, a subset of these factors may be critical for cell transformation. The recent demonstration that a dominant negative mutant of c-*jun* is capable of interfering with the transforming activity of *ras* argues in favor of this hypothesis (41). Accordingly, the identification of such critical factors and the investigation of their mode of activation are crucial for our understanding of cell transformation.

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