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Identification of a phorbol ester-repressible v-*src*-inducible gene

(immediate-early genes/Rous sarcoma virus/protein kinase C/chicken embryo fibroblasts)

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ABSTRACT **Chicken embryo fibroblasts (CEF) infected with a temperature-sensitive Rous sarcoma virus (RSV) mutant, tsNY72-4, express a set of pp60$^{v\text{-}src}$-induced RNAs soon after shift to the permissive temperature. By subtractive and differential screening, we have cloned 12 of these sequences, 2 of which were c-*fos* and *krox-24*. Serum induced all the v-*src*-inducible genes tested, suggesting that these genes serve roles in normal cell division and are not specific to transformation *per se*. Significantly, however, v-*src* produced prolonged, and in some cases kinetically complex, patterns of induction compared to serum. For most of the clones, phorbol 12-tetradecanoate 13-acetate (TPA) induced mRNAs with kinetics similar to that of serum. However, one clone (CEF-4) was expressed in a biphasic manner. Another (CEF-10) was repressed by TPA at 1 hr, after which this mRNA was permanently induced. The pattern of repression-induction of CEF-10 mRNA is the inverse of protein kinase C (PKC) activity in the cell, suggesting that PKC actively represses this gene. *In vivo* expression of CEF-10 mRNA is restricted predominantly to the lung. A full-length CEF-10 cDNA encodes a 41-kDa protein that has an amino-terminal signal peptide for secretion, contains a markedly high number of cysteine residues, and shows no sequence similarity to known proteins.**

Many mitogens and some transforming oncogenes, such as v-*src*, stimulate cell division by transmitting a mitogenic signal from the cell membrane to the nucleus. Recently, several laboratories have cloned genes that may be the nuclear targets for some mitogenic signal transduction pathways. Such "immediate-early" or "early growth response" genes are induced shortly after mitogenic stimulation, causing their cytoplasmic mRNA levels to rise sharply 30 min to 2 hr after stimulation. Furthermore, these genes are induced and, in fact, are often superinduced in the presence of protein translation inhibitors such as cycloheximide. The magnitude and immediacy of the response, combined with the fact that induction does not depend on prior synthesis of other proteins, suggest that these genes are proximate targets of signal transduction and may perform early roles in driving the cell from quiescence (G_0) to the G_1 stage of the cell cycle. A major subset of these genes consists of intranuclear DNA-binding proteins and includes c-*myc* (1), c-*fos* (1–3), c-*jun* (4, 5), *krox-20* (6), and *krox-24*, which is also known as EGR-1 and NGF1-A (7–9). Another group includes secreted proteins, some of which fall into a single family related to low-affinity platelet factor 4 and human connective tissue-activating peptide III (10). Members of this evolutionarily related family that are induced in an immediate-early fashion include *gro* (11), also termed melanoma growth-stimulating activity (MGSA) (12), and CEF-4 (13), which is also called 9E3 by Sugano *et al.* (14). The β-actin gene is also induced in an early manner in the absence of protein synthesis (15). In addition to these known sequences, scores of immediate-early genes have been cloned but have not yet been sequenced or otherwise characterized (16–18).

We have used a temperature-sensitive mutant of Rous sarcoma virus (RSV), tsNY72-4, to isolate immediate-early genes induced by the viral oncogene pp60$^{v\text{-}src}$. Our purpose for doing this was 2-fold: first, we wanted to determine whether this transforming oncogene induced immediate-early genes specific to the transformation process (as opposed to mitogenesis *per se*); second, we wanted to determine whether there was any difference in the induction response of immediate-early genes when pp60$^{v\text{-}src}$ and non-transforming growth factors were compared as mitogens.

By subtractive and differential screening, we have cloned 12 sequences that are induced soon after activation of pp60$^{v\text{-}src}$ in tsNY72-4-infected chicken embryo fibroblasts (CEF). One of these was found to be c-*fos* and another (CEF-5) is homologous to murine *krox-24*. Four of the 12 clones were induced only modestly (2-to 4-fold) by pp60$^{v\text{-}src}$ and were relatively abundant in the cell. These have not been characterized further. In this paper, we describe the responses of three of the six remaining genes (CEF-10, CEF-37, and CEF-147), as well as CEF-5, to induction by pp60$^{v\text{-}src}$, serum, and phorbol 12-tetradecanoate 13-acetate (TPA). One of these, CEF-10, exhibits properties that to our knowledge have not been described previously. It was induced by pp60$^{v\text{-}src}$ in an oscillating manner and was transiently repressed by the protein kinase C (PKC) agonist TPA.

MATERIALS AND METHODS

Cells and Viruses. Cells and viruses studied in this report have been described elsewhere (19–22). Mitogenic stimulation was performed on CEF, which were allowed to grow at 41.5°C to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum and then changed to DMEM containing only 0.5% serum 24 hr prior to stimulation with mitogen. Mitogenic stimulation by pp60$^{v\text{-}src}$ was performed by shifting tsNY72-4 RSV-infected cells from the nonpermissive temperature (41.5°C) to the permissive temperature (35°C). Stimulation by the PKC agonist TPA was done by adding TPA (100 ng/ml) to uninfected CEF cultured as described above. Serum stimulation of uninfected CEF was achieved by adding serum to 10% (vol/vol) final concentration. Superinduction of mRNAs by cycloheximide was performed by simultaneously adding mitogen and cycloheximide (75 μM final concentration) to cells.

RNA Isolation. RNA for gel blot analysis, library construction, and screening was obtained from cells as described (23) and was phenol-chloroform extracted and ethanol precipitated. Poly(A) mRNA used for library construction or synthesis of ^{32}P-labeled cDNA was prepared by two cycles of oligo(dT) chromatography (24). RNA from chicken tissues was prepared by homogenizing whole organs in guanidinium isothiocyanate as described (25).

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Abbreviations: CEF, chicken embryo fibroblasts; TPA, phorbol 12-tetradecanoate 13-acetate; PKC, protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RSV, Rous sarcoma virus.

Library Construction and Screening. Libraries were constructed in λgt10 (26) and λZAP (Stratagene). mRNA for cDNA synthesis was from tsNY72-4-infected CEF held at 35°C for 4-6 hr in the presence of 75 μM cycloheximide, conditions that induce pp60$^{v\text{-}src}$ immediate-early genes. Libraries (>1 × 10^6 recombinants per μg of cDNA) were constructed by the method of Gubler and Hoffman (27).

Libraries were screened at a density of 2–4 × 10^3 recombinants per 150-mm plate. Screening was done in two rounds. In round one, ≈2.5 × 10^4 plaques of the λgt10 library were screened with a subtracted probe. In this procedure, radiolabeled cDNA was prepared from tsNY72-4 RSV-infected CEF cultured at 35°C in cycloheximide for 4–6 hr. This cDNA was then hybridized against control mRNA (from unstimulated tsNY72-4 RSV-infected cells growing at 41.5°C) to a R_0t of 50. The fraction that remained single stranded was eluted from hydroxylapetite and this cDNA was hybridized at 0.5–1 × 10^7 cpm/ml to filters. Three clones were isolated (designated CEF-5, CEF-10, and CEF-57) that hybridized strongly to subtracted cDNA but showed minimal hybridization to radiolabeled control cDNA. Further analysis showed that these clones could be detected readily by simple differential screening. Therefore, a subsequent screen of 7.5 × 10^4 λgt10 plaques was performed by hybridizing one of a triplicate set of filters against radiolabeled cDNA prepared from cycloheximide-superinduced mRNA and hybridizing another of the filters against cDNA prepared against control mRNA. The third filter of each set was hybridized against a mixture of radiolabeled CEF-5, CEF-10, CEF-57, and CEF-4 to prevent recloning these sequences. An additional nine groups of clones were detected by this method. In all, thousands of positive plaques were detected, of which ≈200 were picked and rescreened. These fell into 12 non-crosshybridizing groups that represented ≈4% of the total recombinants in the library.

Northern Analysis. RNA was electrophoresed on denaturing formaldehyde gels, blotted, and probed (13). cDNA inserts isolated by electrophoresis in low-melt agarose were radiolabeled (28), hybridized at 65°C in Church–Gilbert buffer (29), and washed at 65°C in a solution containing 0.2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate) and 0.5% SDS. Several clones used in these studies had been cloned and characterized previously in this laboratory including CEF-4, c-*fos*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (13).

DNA Sequencing and Computer Analysis. Clones isolated from the λgt10 library were used as probes to screen the λZAP library. Longer, and in some cases full-length, clones were obtained by this method. M13 phage of all λZAP clones were rescued by superinfection with R408 helper virus as described in the protocol supplied by the manufacturer (Stratagene). Infection of *Escherichia coli* (XL1-blue) with these phage stocks produced transformed colonies with the desired inserts cloned into Bluescript plasmid vectors. Both strands of a full-length copy of CEF-10, and part of one strand of full-length CEF-5 were sequenced by the dideoxy chain-termination method of Sanger *et al.* (30).*

Homology searches at the nucleic acid and protein levels of the GenBank and National Biomedical Research Foundation data bases, respectively, were done using both the Intelligenetics (FASTA) and the UWGCG (WORDSEARCH) programs.

RESULTS

We, along with Sugano and coworkers, have reported that pp60$^{v\text{-}src}$ specifically activates the expression of the CEF-4 gene, which encodes a secreted 11-kDa protein (13, 14). The studies presented here report the cloning of 12 additional cDNAs, which detect mRNAs increased by pp60$^{v\text{-}src}$ activation in the presence of 75 μM cycloheximide. One of these, CEF-82, was found by crosshybridization to be c-*fos* (data not shown). Six of the clones, designated CEF-5, CEF-10, CEF-37, CEF-101, CEF-120, and CEF-147, exhibit features similar to CEF-4 and immediate-early genes identified in other systems: (*i*) they are expressed as rare mRNAs in control cells; (*ii*) they increase within 1 hr of temperature shift in tsNY72-4 RSV-infected cells; and (*iii*) they are substantially superinduced by temperature shift in the presence of cycloheximide. Clones CEF-57, CEF-68, CEF-134, CEF-157, and CEF-176 lacked one or more of these features.

Several of the clones—namely, CEF-57, CEF-68, CEF-101, CEF-120, and CEF-147—detect multiple messages. For CEF-57, CEF-101, and CEF-147, only one of the detected messages is induced by pp60$^{v\text{-}src}$.

None of the clones crossreacts with actin, c-*myc*, or CEF-4, genes known to respond to mitogen stimulation (data not shown). However, CEF-5 shows 65–75% sequence identity to the murine *krox-24* gene (7, 8). This gene is induced concomitantly with c-*myc* and c-*fos* in serum-stimulated murine fibroblasts (7) and in rat pheochromocytoma cells stimulated with nerve growth factor (9). Given the evolutionary distance between chicken and mouse and the high degree of similarity of the sequences, it is likely that CEF-5 is the avian homolog of *krox-24*. This hypothesis is strengthened by the fact that CEF-5 is expressed most abundantly in chicken lung, brain, and heart (in that order), a pattern very similar to that observed in the mouse (8).

mRNA Induction by pp60$^{v\text{-}src}$. Fig. 1 shows the induction patterns obtained for four of the pp60$^{v\text{-}src}$ immediate-early genes along with chicken CEF-4 and GAPDH in tsNY72-4 RSV-infected CEF shifted to 35°C. In this and other experiments presented below, GAPDH serves as a control for RNA loading. CEF-5 and CEF-37 were induced with the same kinetic profile (Fig. 1), which was also identical to that of c-*fos* (data not shown). All three mRNAs peaked within 1 hr post-temperature shift and declined to an intermediate level that was maintained to 24 hr.

CEF-147 detected a family of low-abundance mRNAs, of which only one species of ≈5 kilobases (kb) (Fig. 1, arrowhead) was induced by temperatures shift. Long exposures of autoradiographs demonstrate that this mRNA was induced biphasically—with an initial plateau being reached by 1 hr

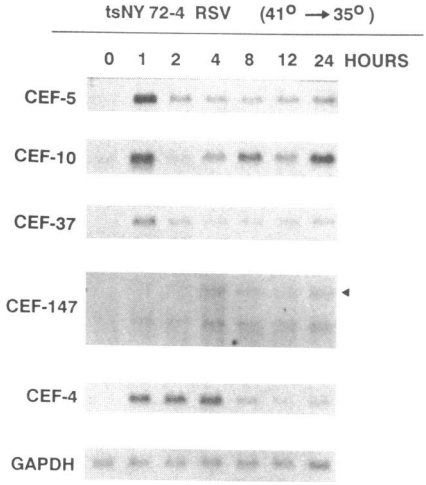

FIG. 1. mRNA induction by pp60$^{v\text{-}src}$ in tsNY72-4 RSV-infected CEF cells. Cells were seeded on 150-mm dishes at density of 2 × 10^7 cells per plate. At time 0, cells were shifted from the nonpermissive (41.5°C) to the permissive (35°C) temperature. Total RNA isolated at 0, 1, 2, 4, 8, 12, and 24 hr post-temperature shift was electrophoresed on formaldehyde denaturing gels, blotted to nylon membranes, and hybridized to the probes indicated. Arrowhead indicates 5-kb band.

*The CEF-10 sequence is being deposited in the EMBL/GenBank data base (accession no. J04496).

and a second, 10-fold higher, plateau occurring at 4 hr and remaining elevated to 24 hr. The exposure used in Fig. 1 shows only the second plateau.

Activation of $pp60^{v\text{-}src}$ by temperature shift produced an unusual induction pattern for CEF-10, in which the mRNA reproducibly exhibited three peaks during the 24-hr time course. CEF-10 mRNA increased 9-fold at 1 hr postshift, decreased to control or subcontrol levels by 2 hr, peaked again at 8 hr, fell 40% at 12 hr, and increased back to maximal levels at 24 hr.

The shift of cells from one temperature to another in these experiments raised the possibility that the genes described above were induced by changing the ambient temperature from 41.5°C to 35°C and not by activation of $pp60^{v\text{-}src}$. To address this question, temperature-shift experiments were performed on CEF infected with the nontransforming myristoylation-defective virus NY315 RSV. In these experiments, CEF-5, CEF-37, and GAPDH revealed no change in mRNA levels following temperature shift (data not shown). However, CEF-10 showed nearly a 5-fold increase 24 hr following temperature shift, and CEF-147 showed a similar, but weaker, induction (Fig. 2).

Serum Stimulation of $pp60^{v\text{-}src}$ Immediate-Early Genes. We have reported that serum induces CEF-4 with kinetics quite different from those invoked by temperature shift of tsNY72-4 RSV-infected cells (13). Activation of the viral oncogene produces a long-term induction, whereas serum treatment produces a transient increase in CEF-4 mRNA that rapidly declines within 2 hr of treatment to control levels (Fig. 3). Indeed, comparison of the results obtained with serum (Fig. 3) and tsNY72-4 RSV (Fig. 1) shows that all $pp60^{v\text{-}src}$ immediate-early genes thus far cloned are inducible by serum and that induction by serum in all cases is early, brief, and monophasic. Activation of $pp60^{v\text{-}src}$, on the other hand, generates a persistent induction of all the immediate-early mRNAs to 24 hr. For CEF-10 and CEF-147, this induction is multiphasic (Fig. 1).

Regulation of $pp60^{v\text{-}src}$ Immediate-Early Genes by TPA. Treatment of CEF with TPA (100 ng/ml) produced a response similar to that of serum for CEF-5, CEF-37, CEF-147, c-*myc*, and c-*fos* (Fig. 4). However, the duration of the induction of these mRNAs appeared to be slightly longer in TPA- versus serum-treated cells.

In contrast, the response of CEF-4 was very different from serum in that it demonstrated two distinct peaks of induction—one at 1–2 hr, and the other at 8–12 hr (Fig. 4).

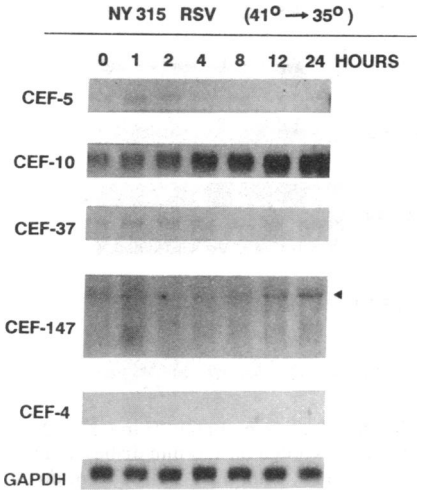

FIG. 2. Effects of temperature shift on mRNA levels in NY315 RSV-infected CEF cells. CEF cells infected with the myristoylation-defective mutant NY315 RSV were treated as described in Fig. 1.

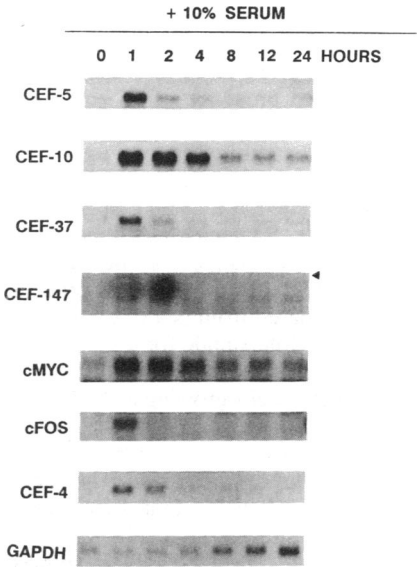

FIG. 3. Serum induction of $pp60^{v\text{-}src}$ immediate-early genes. CEF cells were cultured as described in Fig. 1, with the exception that cells were seeded at 1×10^7 cells per 150-mm plate. At time 0, cells were exposed to 10% calf serum and RNA was isolated and analyzed as described in Fig. 1. As can be discerned by hybridization to the GAPDH probe, increased amounts of mRNA appear to have been loaded for the last three time points, which has to be taken into consideration in evaluating kinetics of induction.

CEF-10 expression exhibited kinetics, which to our knowledge have not been described previously for a TPA-regulated gene. It was repressed by TPA to <20% of basal levels at 1 hr, after which it gradually increased to ≈10-fold above the basal level at 24 hr (Fig. 4). The autoradiogram exposure in Fig. 4 is longer than in Fig. 1 to demonstrate this decline in mRNA at 1 hr posttreatment.

Sequence Analysis of CEF-10. Seven independent isolates were cloned that span the entire coding region of CEF-10. One of these full-length clones, isolate 26, was sequenced in its entirety. The predicted mRNA is 1.8 kb long [neglecting poly(A) addition] and contains a single long open reading frame, extending from nucleotide 53 to 1179, that encodes a protein of 40,651 Da (Fig. 5). The 3′ untranslated region of

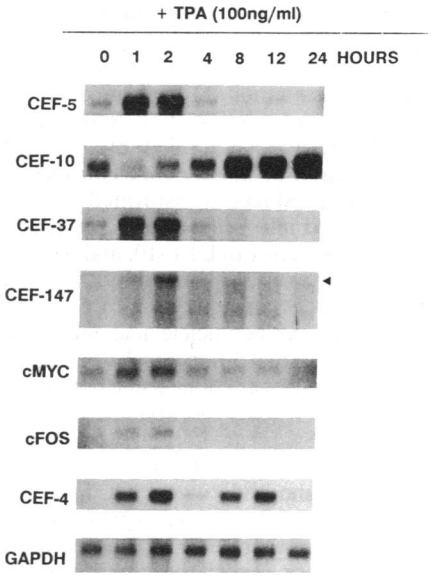

FIG. 4. Regulation of $pp60^{v\text{-}src}$ immediate-early genes by TPA. Cells were cultured as described in Fig. 3. At time 0, cells were exposed to TPA (100 ng/ml).

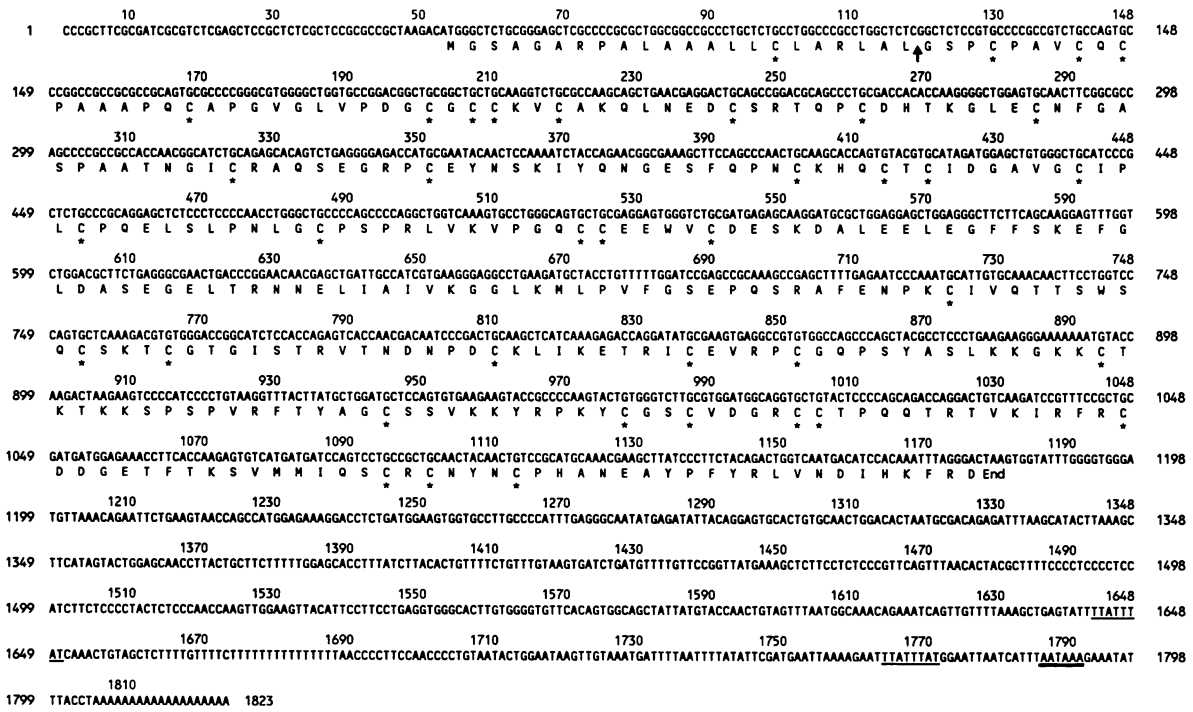

FIG. 5. Nucleotide and predicted amino acid sequence of CEF-10. Underlined sequences are copies of a conserved motif shown by Shaw and Kamen (31) to confer message instability. Arrow represents the predicted site of cleavage for the signal peptide. Cysteines are highlighted with an asterisk underneath the amino acid symbol (single-letter code). A consensus polyadenylylation signal is underlined in bold face.

CEF-10 mRNA from nucleotides 1600–1805 is composed 77% of A and T residues. Similar A+T-rich sequences have been found in numerous immediate-early genes isolated in other systems (e.g., c-*fos*, CEF-4, *Gro*, *Krox-20*, *Krox-24*). At positions 1643–1650 and 1764–1771 are copies of the octanucleotide consensus sequence, TTATTTAT, shown by Shaw and Kamen (31) to confer instability to transcripts.

The protein predicted from the cDNA sequence shows several distinguishing features (Fig. 5). The N terminus is hydrophobic and contains the three features of a signal sequence: (*i*) a basic region (amino acids 1–8); (*ii*) a hydrophobic "core" (amino acids 9–16); and (*iii*) a more polar region (amino acids 17–24). A cleavage site is predicted between leucine-22 and glycine-23 by von Heijne's weighted matrix method, which is reported to predict eukaryotic signal peptide cleavage sites with 75–85% accuracy (32). With the exception of the hydrophobic N terminus, CEF-10 protein lacks significant hydrophobic regions. Hence, it is likely that this protein is not membrane bound.

CEF-10 protein contains an unusually high number of cysteines (39 residues) that are not clustered (Fig. 5); however, a short segment in the center of the protein from amino acid 164–223 contains no cysteine. Two hydrophilic charged regions are also present: one acidic region at residues 159–174 and a basic region at residues 276–286. The protein contains 29 proline residues that are not clustered and prevent α-helix formation throughout much of the molecule. Overall, the protein has a positive charge (+11) and contains no consensus sites for N-linked glycosylation.

Hybridization of CEF to mRNA isolated from a selection of chicken tissues (Fig. 6) demonstrates that CEF-10 is expressed almost exclusively in lung. Many other tissues show barely detectable levels of CEF-10 transcripts that are present <1/10th the level of this organ.

DISCUSSION

The clones identified here are probably only a subset of pp60$^{v\text{-}src}$ immediate-early genes. That this set is not complete is evident from the fact that we did not clone c-*myc*, which we know is superinduced in tsNY72-4 RSV-infected CEF cultured at the permissive temperature in 75 μM cycloheximide (D. Alcorta, personal communication). Furthermore, Almendral and coworkers (16) recently have reported isolating 82 independent murine sequences that are induced by serum in the presence of cycloheximide. All the clones we have tested thus far are inducible by serum, suggesting that they likely form a subset of Almendral's clones. Therefore, we expect more pp60$^{v\text{-}src}$ immediate-early genes remain to be cloned.

Our studies thus far have failed to identify any transformation-specific immediate-early genes for pp60$^{v\text{-}src}$, since all the genes we have tested were also induced during normal serum-stimulated cell division. However, pp60$^{v\text{-}src}$ clearly

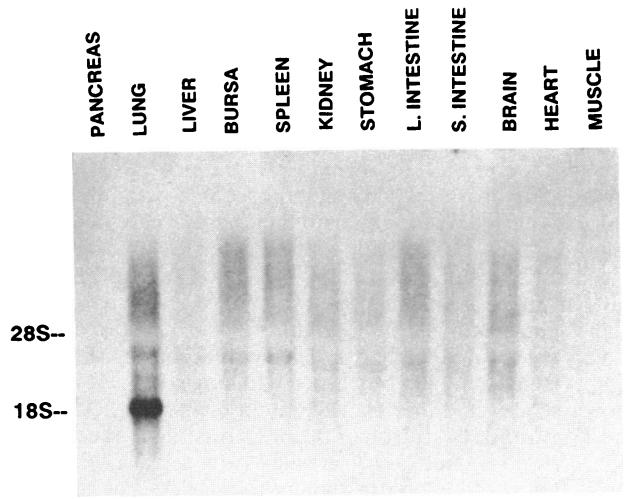

FIG. 6. Tissue distribution of the CEF-10 transcript. Tissues from an adult White Leghorn chicken were used for RNA isolation. Five micrograms of total RNA was assayed from each tissue. The hybridized blot was exposed to Kodak XAR-5 film for 12 hr with an intensifying screen.

produced different kinetics of induction than serum. The single most important difference is that pp60$^{v\text{-}src}$ persistently elevated expression of its immediate-early genes, whereas serum, and in most cases TPA, produced transient increases that returned to baseline levels within hours of stimulation.

Two pp60$^{v\text{-}src}$ immediate-early genes, CEF-10 and CEF-147, were induced by temperature shift of transformation-defective NY315 RSV-infected cells. CEF-10 exhibited the largest increase (5-fold at 24 hr). It must be noted that, although NY315 RSV is myristoylation defective and non-transforming, neuroretinal cells infected with this virus gain the ability to proliferate during multiple passages (uninfected cells divide only through two passages) and grow more rapidly and to a greater density than normal cells (20). Furthermore, pp60$^{v\text{-}src}$ encoded by this virus has tyrosine kinase activity and phosphorylates numerous cellular proteins (33). Changing the ambient temperature of the cell may enhance the weak mitogenic activity of NY315 RSV producing an increase in CEF-10 and CEF-147 mRNAs.

Of the clones isolated, CEF-10 exhibited properties that distinguish it from other immediate-early genes thus far cloned by us or others. It was induced in a multiphasic pattern following temperature shift (Fig. 1). Although the complex kinetics of CEF-10 mRNA induction by pp60$^{v\text{-}src}$ could be the result of more than one closely related 1.8-kb CEF-10 mRNA increasing and decreasing over the time course, it is equally likely that they reflect fluctuations in expression of a single gene. In fact, 30 independent isolates of CEF-10 were found to be identical for the *Eco*RI site at nucleotide position 1211, and 7 independent full-length clones analyzed for 12 restriction sites were identical (unpublished data), favoring the second alternative.

However, the most remarkable feature of CEF-10 is that its expression was transiently repressed within 1 hr of TPA treatment, after which mRNA levels rebounded, surpassed the baseline level by 10-fold, and remained elevated out to 24 hr (Fig. 4). Repression of CEF-10 mRNA was found to be coordinate with induction of CEF-5, CEF-37, CEF-147, CEF-4, c-*myc*, and c-*fos* (Fig. 4). Allowing time for transcription to occur, this pattern closely parallels the known time course of translocation–activation–deactivation of PKC at cellular membranes. That is, within 15–30 min of exposure to TPA, PKC associates with cellular membranes where it is transiently activated and then degraded (34, 35). Because of this degradation, cells given long-term treatment with TPA possess <10% of normal PKC levels (35). The pattern observed in Fig. 4 for CEF-10 suggests that following TPA addition, CEF-10 expression is repressed at 1 hr when PKC is transiently activated and derepressed as PKC is degraded. In support of this hypothesis, identical kinetics have been observed for other PKC-mediated effects, the most striking of which is the increased sensitivity of WB (liver epithelial) cells to angiotensin II following TPA treatment, which shows kinetics that are essentially superimposable with our results (36). It is intriguing to speculate that the decrease in activity seen at 2 hr in temperature-shifted tsNY72-4-infected cells (Fig. 1) may result from activation of PKC or the PKC signal-transduction pathway by pp60$^{v\text{-}src}$.

The sequence of CEF-10 predicts a 40.5-kDa protein that is likely secreted from the cell. This protein shows a distinctly high cysteine content but lacks homology with any other known sequence either at the protein or nucleic acid level. The 3′ untranslated region of the mRNA contains 2 copies of the Shaw and Kamen (31) consensus sequence, TTATTTAT, that is thought to confer instability to mRNAs and has been shown to be present in mRNAs encoding numerous secreted growth factors, including human tumor necrosis factor, lymphotoxin, colony-stimulating factor, interleukin 1, and most interferons (37).

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