which is particularly short in TSH and LH-CG receptors and which has been implicated in the interaction with the α subunit of G_s ($G_s\alpha$) (*8, 9*), is identical in both receptor types. This pattern of similarities gives support to the view that the extracellular domain functions in ligand recognition (*4*), whereas the membrane-inserted domain is responsible for the activation of $G_s\alpha$ (*16, 17*). The partial clone HGMP09 also appears 70% identical to corresponding regions of TSHR and the LH-CG receptors (Fig. 1). The fact that HGMP09 obviously belongs to the same G protein–coupled receptor subfamily, together with the tissue distribution of its transcript (Fig. 2A), makes HGMP09 a possible candidate for the FSH receptor.

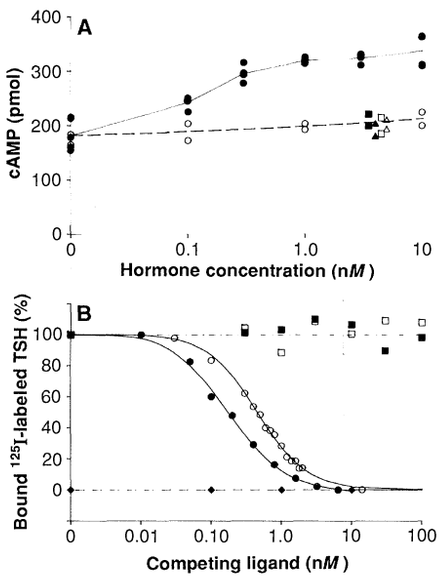

Fig. 4. (**A**) TSH-induced cAMP accumulation in *Xenopus* oocytes that had been microinjected with TSH receptor mRNA. *Xenopus* oocytes were handled as described (*23*) and injected with water (open symbols) or recombinant TSHR mRNA (*14*) (50 nl at a concentration of 0.1 μg/μl) (filled symbols). After 3 days in control medium, batches of 35 oocytes were incubated for 90 min in medium supplemented with various concentrations of TSH (circles), LH (squares), or FSH (triangles). cAMP was determined as described (*15*). RO 201724 and isobutylmethylxanthine (10^{-6}*M* each) were present in all incubations. Results were concordant in three independent experiments. Incubation of control oocytes with 10^{-4}*M* forskolin resulted in a doubling of the cAMP concentration (M. Parmentier *et al.*, unpublished data). (**B**) Displacement of ^{125}I-labeled TSH from TSH receptors expressed in COS7 cells. COS7 cells were transfected with TSHR cDNA subcloned in pSVL (*24*). After 72 hours, cells were harvested and a membrane fraction was prepared (*25*). Membranes were similarly prepared from wild-type COS7 cells and from dog thyrocytes in primary culture (*22*). Binding of ^{125}I-labeled TSH (Trak, Henning Gmbh, Berlin, FRG) was performed at 0°C for 120 min in the presence of various concentrations of competitors (TSH; Armour, Chicago, IL. FSH and LH; UCB Bioproducts, Brussels, Belgium). Bound radioactivity was separated by centrifugation and measured. Results are expressed as a percentage of the ^{125}I-labeled TSH bound by transfected cells in the absence of competitor (3000 cpm) after correcting for nonspecific binding (radioactivity bound in the presence of 100 n*M* unlabeled TSH, 800 cpm). Open and filled circles, displacement by unlabeled TSH from COS7 cells and thyrocyte membranes, respectively. Open and filled squares, displacement for COS7 cells by LH and FSH, respectively. Diamonds, displacement by unlabeled TSH from untransfected COS7 cells. Similar displacement curves were obtained in three independent experiments.

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Regulation of Proenkephalin by Fos and Jun

JUNE L. SONNENBERG, FRANK J. RAUSCHER III, JAMES I. MORGAN, TOM CURRAN

Fos and Jun form a heterodimeric complex that associates with the nucleotide sequence motif known as the AP-1 binding site. Although this complex has been proposed to function as a transcriptional regulator in neurons, no specific target gene has yet been identified. Proenkephalin mRNA increased in the hippocampus during seizure just after an increase in c-*fos* and c-*jun* expression was detected. Fos-Jun complexes bound specifically to a regulatory sequence in the 5′ control region of the proenkephalin gene. Furthermore, c-*fos* and c-*jun* stimulated transcription from this control region synergistically in transactivation assays. These data suggest that the proenkephalin gene may be a physiological target for Fos and Jun in the hippocampus and indicate that these proto-oncogene transcription factors may play a role in neuronal responses to stimulation.

THE PROTO-ONCOGENES, c-*fos* AND c-*jun*, are members of the class of inducible genes termed cellular immediate-early genes. Their protein products, Fos and Jun, have been proposed to function as transcriptional regulators that couple extracellular signals to alterations in cellular

J. L. Sonnenberg, Departments of Molecular Oncology and Virology and Neurosciences, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.
F. J. Rauscher III and T. Curran, Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.
J. I. Morgan, Department of Neurosciences, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.

phenotype by regulating expression of specific target genes (*1*). This hypothesis has been strengthened by the discovery that the Fos-associated protein p39 is the product of c-*jun* (*2*) and that Fos and Jun interact cooperatively with the DNA regulatory element known as the AP-1 binding site (*3, 4*).

Induction of cellular immediate-early gene expression occurs in response to a wide range of stimuli in many cell types (*5*). In neuronal cells in culture, nerve growth factor, neurotransmitters, and agents that provoke a voltage-gated calcium influx are potent inducing agents of c-*fos* and c-*jun* (*6*). We have utilized a seizure model system (pentylenetetrazole) to demonstrate that increases in the expression of c-*fos*, c-*jun*, *egr*-1, and several Fos-related antigens (Fra) occur in certain subsets of neurons in vivo, particularly in the dentate gyrus of the hippocampus, after induction of seizure (*7–9*). While immediate-early gene products, such as Fos and Jun, may contribute to some of the long-term consequences of seizure activity (*7*), it is not possible to ascribe a specific role to these proteins until target genes have been identified. Although in vitro transactivation studies have shown that Fos and Jun may stimulate expression of reporter genes linked to reiterated AP-1 sites (*10*), a physiologically relevant target gene has not yet been described. We chose to investigate the proenkephalin gene (*enk*) as a potential target for the action of Fos and Jun in the hippocampus for two reasons. Proenkephalin mRNA levels increase in granule cells of the dentate gyrus of the hippocampus in several seizure models (*11*), and the 5′ region of the proenkephalin genes has an AP-1 binding site (*12*). This binding site contributes to the regulation of proenkephalin by adenosine 3′,5′-monophosphate (cAMP) and phorbol esters in C6 glioma cells (*13*).

We compared the time course of induction of several cellular immediate-early gene mRNAs and proenkephalin mRNA in whole brain and hippocampal extracts (Fig. 1). The probes were hybridized to mRNA isolated from whole brain or hippocampal tissue at the indicated times after intraperitoneal injection of pentylenetetrazole. Seizures occurred within 3 min and continued for up to 30 min. Both *jun*B and *egr*-1 showed similar temporal patterns of induction as c-*fos*, with maximal stimulation occurring in the hippocampus at 30 to 60 min after treatment and returning to basal levels by 2 to 4 hours. The time course of proenkephalin mRNA induction was slightly delayed compared to the immediate-early gene mRNAs (Fig. 1). One hour after injection of pentylenetetrazole, proenkephalin mRNA in the hippocampus was increased compared to control animals and remained elevated for at least 6 hours, consistent with the long half-life of this mRNA. Thus, the level of proenkephalin mRNA increased in the hippocampus after elevation of c-*fos* and c-*jun* mRNAs. The basal level of proenkephalin mRNA in whole brain samples was high and no increase was observed after seizure (Fig. 1).

The 5′ control region of the preproenkephalin gene contains binding sites for several different transcription factors, including AP-1 (*13*) (Fig. 2). The AP-1 site is located in a regulatory element that is essential for basal and induced levels of transcription (*13*). Gel-shift assays were performed with the ^{32}P-labeled oligonucleotides ENK-2 and AP-1 (Fig. 2) and with Fos and Jun polypeptides that had been translated in vitro (*3, 14*). Fos and Jun interacted cooperatively with the proenkephalin AP-1 site (ENK-2); however, the apparent affinity of this interaction was lower than that with the human

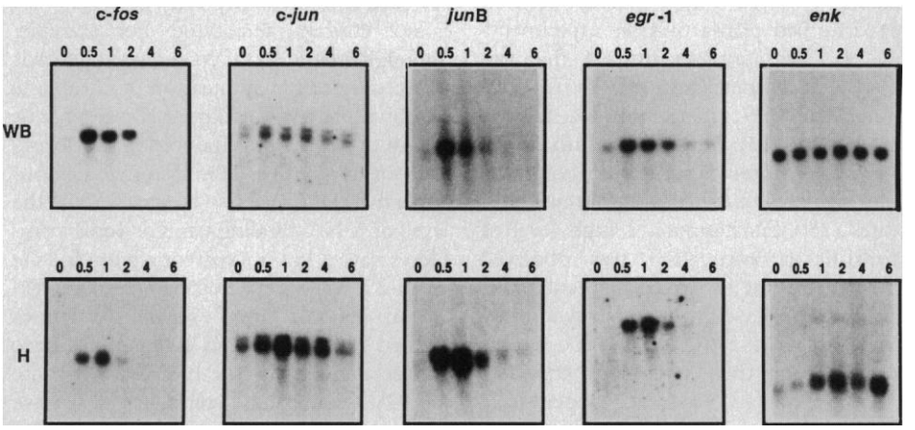

Fig. 1. Time course of immediate-early gene and *enk* mRNA expression after induction of seizure with pentylenetetrazole. Total cellular RNA was extracted from whole mouse brain (WB) and dissected rat hippocampus (H) as described (*7, 8*) at the indicated times (in hours) after induction of seizure by intraperitoneal injection of pentylenetetrazole (45 mg kg^{-1}). Two micrograms of each RNA was analyzed by electrophoresis on 0.8% agarose gels in the presence of 2.2*M* formaldehyde. The total RNA content of each lane was monitored by staining with ethidium bromide. RNAs were transferred onto nitrocellulose and hybridized with c-*fos*, c-*jun*, *jun*B, *egr*-1 (*8*), and *enk* (*23*) probes. The nitrocellulose membrane was washed with 2× standard sodium citrate plus 0.1% SDS for 90 min at 55°C prior to autoradiography.

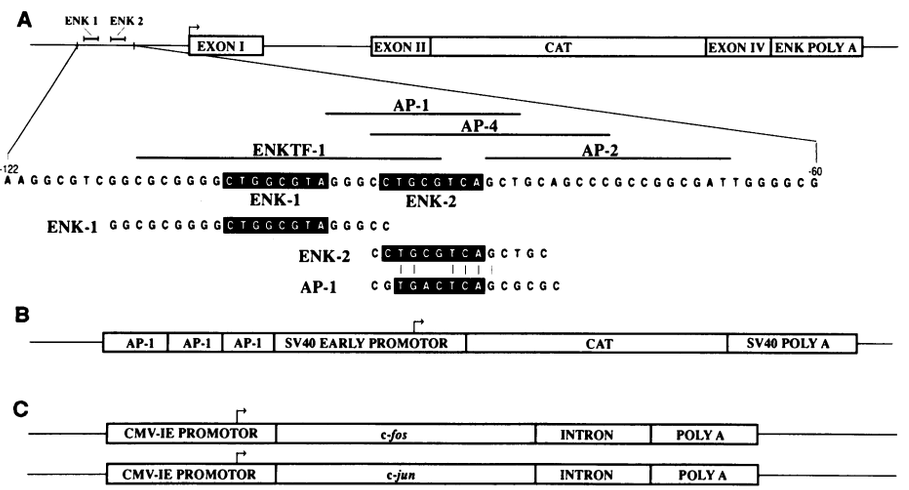

Fig. 2. Structure of the plasmids and oligonucleotides used for these studies. (**A**) The structure of the proenkephalin-CAT reporter plasmid (pENKAT-12) (*13*). The 5′ flanking region from base −122 to −60 (relative to the CAP site) is expanded to show the binding sites for transcription factors AP-1, AP-2, AP-4, and ENKTF-1 (solid bars above nucleotide sequence) as defined by footprinting analyses in (*13*). The boxed sequences (ENK-1 and ENK-2) have been defined genetically by Comb *et al.* (*13*) to be necessary for basal and inducible transcription. The oligonucleotides containing the ENK-1 and ENK-2 sequences (used in gel retardation assays) are shown below the expanded 5′ region. The AP-1 oligonucleotide is from the promotor of the hMTII$_A$ gene (*3*). Each oligonucleotide contained Sal I (5′) and Xho I (3′) overhangs (not shown) to facilitate labeling with [^{32}P]deoxynucleotide triphosphates. (**B**) The (AP-1)$_3$-CAT reporter plasmid contains three concatamerized AP-1 sites from the hMTII$_A$ gene fused to the SV40 early promoter. The plasmid is equivalent to plasmid X$_3$CAT as described in (*24*). (**C**) For transactivation assays, c-*fos* and c-*jun* cDNAs were expressed from vectors containing the CMV immediate-early promoter (CMV-IE). These plasmids included rat preproinsulin intron sequences and SV40 polyadenylation signals.

metallothioneinII$_A$ (hMTII$_A$) AP-1 site (Fig. 3). Although Fos and JunB formed a complex that associated efficiently with the hMTII$_A$ AP-1 site, only very low levels of Fos-JunB binding activity were detected with the ENK-2 site. This demonstrates that the various AP-1 complexes formed between Fos- and Jun-related proteins may have differential specificities for DNA binding.

To determine if the DNA-binding properties of Fos and Jun on the proenkephalin enhancer reflected a functional interaction, we performed transactivation experiments (Fig. 4). These studies utilized chloramphenicol acetyltransferase (CAT) reporter plasmids and F9 teratocarcinoma cells as described (*10, 15*). The data confirm that c-*fos* and c-*jun* stimulate expression from promoter elements fused to three synthetic AP-1 sites (*16*). Furthermore, a high level of synergistic transactivation was observed with the natural proenkephalin enhancer-promoter. We have not detected synergistic transactivation of ENK-CAT with c-*fos* plus *jun*B, although these two genes stimulate expression of (AP-1)$_3$-CAT cooperatively (*16*). This lack of activity appears to correlate with the much reduced binding of Fos-JunB complexes to the ENK-2 site.

The induction of cellular immediate-early genes is a rather stereotypic response to several distinct extracellular stimuli. Many cellular immediate-early genes encode known or putative transcriptional regulators. In particular, several Fos-related and Jun-related proteins that can form heteromeric and homomeric protein complexes capable of association with the AP-1 binding site (*17, 18*) are induced with overlapping time courses after seizure (*8, 19*). These proteins have been proposed to fulfill a role in stimulus-response coupling by regulating target gene expression in response to environmental cues (*1*). However, the ubiquity of the cellular immediate-early gene responses raises the question of how specific target genes are regulated in different cell types. One partial answer to this question is that the cellular immediately-early gene response is not entirely stereotypic. For example, although a large set of c-*fos*–related genes is induced by serum stimulation of fibroblasts and by seizure in the hippocampus, at least one of these (*fra*-1) is induced in fibroblasts but not in neurons (*20*). Here, we demonstrate that there may also be specificity at the level of DNA binding, as Fos-JunB complexes have a lower apparent affinity for the ENK-2 AP-1 site than do Fos-Jun complexes. In contrast, most of the complexes formed by the Fos and Jun families have similar affinities for the hMTII$_A$ AP-1 site (*17, 18*). Comb and colleagues (*13*) have shown that proenkephalin transcription is controlled by the cooperative interaction of at least two regulatory elements and that multiple factors can bind to the 5′ control region. It is possible that any one of the regulatory factors could be rate-limiting in specific cell types. Therefore, it is likely that the target genes of the cellular immediate-early response are controlled by a combinatorial interaction of resident and induced transcription factors.

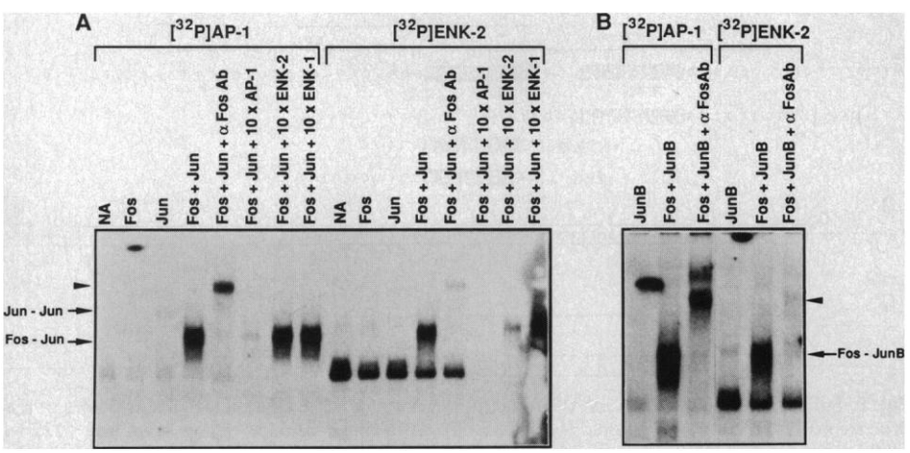

Fig. 3. Fos and Jun complexes bind to ENK-2. (**A**) Fos and Jun were synthesized in vitro in reticulocyte lysates (*14*) and used in DNA-binding assays with [^{32}P]AP-1 (the hMTII$_A$ AP-1 site) or [^{32}P]ENK-2 (the *enk* AP-1 site) oligonucleotides. In addition to the [^{32}P]-labeled oligonucleotide, antibody to Fos (raised to a Fos peptide comprising amino acids 127 to 152) or a tenfold molar excess of unlabeled competitor oligonucleotide was added to the DNA-binding reaction as indicated in each lane. The position of the Jun-Jun and the Fos-Jun complexes with DNA are indicated with arrows. An arrowhead indicates the position of a "super-retarded" band representing a Fos-Jun-DNA-antibody complex. NA, reticulocyte lysate that was incubated without added RNA. (**B**) The ability of Fos-JunB complexes to bind AP-1 and ENK-2 oligonucleotides was determined as in (A). No specific DNA-protein complexes were detected with the ENK-1 probe in lysates containing Fos-Jun or Fos-JunB complexes (not shown).

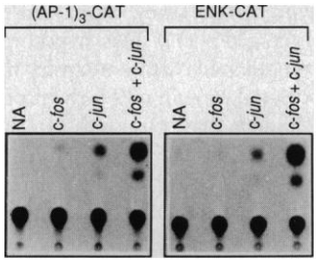

Fig. 4. Synergistic transactivation of the proenkephalin enhancer-promoter by c-*fos* and c-*jun*. F9 teratocarcinoma cells were transfected (*15*) with 10 μg of CAT reporter plasmids (Fig. 2) and 5 μg of each CMV plasmid expressing c-*fos* or c-*jun*. The total amount of CMV expression vector per dish was kept constant at 10 μg by adding CMV-plasmid containing no cDNA inserts. Forty-eight hours after transfection cell extracts were prepared, and CAT enzyme activity was determined (*16*).

Other candidate target genes for Fos and Jun in the hippocampus are those encoding secreted polypeptides, such as nerve growth factor (NGF), dynorphin, cholecystokinin, and somatostatin (*11, 21*), that mediate neuronal responses. In particular, the increase in NGF expression has been proposed to promote elaboration of axonal branches and somatic spines in dentate gyrus granule cells, changes that occur after seizure (*22*).

Induction of seizure by electrical and pharmacological stimuli or by lesion leads to increased levels of enkephalin peptides and proenkephalin mRNA (*11*). The physiological consequences of these increases in opiate peptide expression are unclear, although they have been suggested to mediate some of the behavioral alterations associated with seizure (*11*). Here, we show that Fos and Jun can bind to the 5′ regulatory region of the proenkephalin gene in vitro and that c-*fos* and c-*jun* stimulate expression from this region cooperatively in transactivation assays. This implies, but does not prove, that Fos and Jun contribute to the regulation of proenkephalin expression in the hippocampus.

Under conditions in which peptide secretion exceeds biosynthesis, there could be a compensatory mechanism that involves a recruitment of the immediate-early genes with a subsequent activation of peptide precursor gene transcription. In this context, stimulus-secretion and stimulus-transcription coupling represent integrated components of a general homeostatic process. Under certain circumstances, this may result in a protracted elevation of neuropeptide levels, as occurs with enkephalin after seizure (*11*). Cellular immediate-early genes may underpin a general trophic response in neurons to stimulation that is mediated by neuropeptides and that may include alterations in synapse structure and function.

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14. Detailed methods for measuring in vitro association of Fos and Jun and DNA-binding assays can be found in (*3*). Briefly, plasmids encoding Fos or Jun proteins were used to generate positive sense RNA with the Sp6 in vitro transcription system. RNAs were used to program messenger-dependent rabbit reticulocyte lysates (Promega Biotech). In all cases, in vitro translation reactions were performed in parallel in the presence and absence of [^{35}S]methionine to generate labeled proteins (for quantitation) and unlabeled proteins (for DNA-binding assays). Prior to use, the [^{35}S]methionine-labeled proteins were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and fluorography to normalize for differences in translation efficiency. In vitro association of Fos and Jun was accomplished by incubating lysates containing the appropriate amount of Fos and Jun proteins at 37°C for 60 min. For measurement of DNA-binding activity, reaction mixtures were assembled by combining 3 μl of lysate containing Fos-Jun complexes with 5 μl of binding buffer and 1 μg of poly (dIdC). After 15 min at room temperature, 1 μl of ^{32}P-labeled oligonucleotide probe (40 to 60,000 cpm; 0.2 to 1.0×10^6 cpm/pmol) was added, and the incubation was continued at room temperature for 15 min. DNA-protein complexes were separated from unbound oligonucleotide by nondenaturing PAGE. The oligonucleotide probes were synthesized, annealed, and labeled with [^{32}P]deoxynucleotide triphosphates as described (*3*).
15. F9 teratocarcinoma cells were grown on gelatin-coated dishes in Dulbecco-Vogt modified Eagle medium containing 10% heat-inactivated fetal calf serum for 24 hours prior to transfection. Cells (2×10^6) were transfected by calcium phosphate precipitation and precipitates were left in contact with cells for 12 to 18 hours. In addition to the CAT reporter and the cytomegalovirus (CMV) immediate-early promoter expression plasmids (Fig. 2), each transfection mix contained 2 μg of an internal control plasmid containing the CMV promoter driving expression of the β-galactosidase gene (pON260). Forty-eight hours after removal of the precipitate, cell extracts were prepared and β-galactosidase activity was determined. Appropriate amounts of cell extract, normalized for β-galactosidase activity, were utilized for determination of CAT activity. Incubation was carried out for 1 hour, and the reaction products were analyzed by thin-layer chromatography.
16. Although these experiments were repeated several times with different preparations of reporter and expression plasmids, a significant degree of interexperiment variability was encountered. The data reported here have been obtained in at least three independent experiments; however, on several occasions no transactivation was detected. Furthermore, although the transactivations were observed in F9 teratocarcinoma cells, as reported by others (*10*), we have not been able to demonstrate a similar response in fibroblasts. The basis of this variability and cell specificity is presently unclear.
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25. We thank S. Udenfriend and C. Rosen for helpful comments on this manuscript; B. Cullen for CMV vectors; P. Mitchell for pX_3CAT; M. K. Stachowiak for the rat proenkephalin cDNA probe pRPE2; D. Nathans for the JunB probe; and M. Comb for the pENKAT-12 plasmid and for helpful discussions.

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Functional Analysis of CAR, the Target Sequence for the Rev Protein of HIV-1

ELAHE T. DAYTON, DOUGLAS M. POWELL, ANDREW I. DAYTON

Expression of high levels of the structural proteins of the human immunodeficiency virus type 1 (HIV-1) requires the presence of the protein encoded by the *rev* open reading frame (Rev) and its associated target sequence CAR (cis anti-repression sequence) which is present in the env region of viral RNA. Extensive mutagenesis demonstrated that CAR has a complex secondary structure consisting of a central stem and five stem/loops. Disruption of any of these structures severely impaired the Rev response, but many of the stem/loops contain material that was unnecessary for Rev regulation and must be retained in these structures to avoid disturbing adjacent structures critical for CAR function. Probably no more than two of the described structural components are involved in sequence-specific recognition by regulatory proteins.

HIV-1, THE ETIOLOGIC AGENT OF acquired immunodeficiency syndrome (*1*), encodes the Rev protein, which enhances the accumulation of large virus genomic and virion structural mRNAs at the expense of the short, spliced viral mRNAs that code for many viral accessory or regulatory proteins (*2–6*). Rev regulates viral protein expression by regulating viral RNA transport from the nucleus (*7–11*). In the absence of Rev, HIV RNA is retained in the nucleus by CRS elements [cis repressor sequences (*8–12*)] located in *gag* and *env*. Viral RNA only exits the nucleus if the CRS have been removed by splicing or if Rev acts to overcome their repression and transport the RNA to the cytoplasm.

The target sequence for Rev, CAR (cis anti-repressor) has been localized to a 269-bp region in the *env* RNA (*12, 13*) from nt 7358 to 7627 (according to *14*). Others have recently confirmed the localization of CAR (*8–11, 15*) suggesting the term RRE (rev-responsive element) (*8, 9, 15*). The size of CAR and the previous demonstration by deletion analysis that CAR is discontinuous (*13*) suggest the possibility of a complex interaction between CAR and multiple regulatory proteins. Here we report a functional analysis of the CAR structure that underlies these interactions.

Computer analysis of CAR RNA structure did not uniquely suggest a structure for CAR. Analysis of CAR from nt 7357 to 7601 (*14*) by an IBM PC RNA folding program (*16*) suggested the triple stem/loop structure diagrammed as the central structure in Fig. 1 with an approximate folding energy of −67.8 kcal as determined by the default parameters in the program. However, analysis of the same region by Zuker's suboptimal RNA folding program written for the VAX (*17*) suggested a variety of alternative structures for the CAR RNA with folding energies in the range of −81.1 to −77.7 kcal. Figure 1 diagrams the salient characteristics of these alternative foldings

E. T. Dayton and A. I. Dayton, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD 20892.
D. M. Powell, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD 20892 and Genetics Department, George Washington University, Washington, DC 20052.