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

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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

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around the periphery of the simple, central structure predicted by the IBM PC program. The uncertainties of the energy assumptions used to predict the folding patterns makes it difficult to determine a priori which of a set of structures having predicted folding energies within such a range should predominate (*17*).

We therefore analyzed a set of substitution mutants, CAR-1 through CAR-22 (Figs. 1 and 2) designed to distinguish functionally among the diagrammed alternatives. The mutations were introduced by site-directed mutagenesis directly into the CAR region of the Rev-responsive *gag*-expression plasmid, III-GAG-CAR, which was derived by transferring HXGC-1 (*13*) into PTZ19R (U.S. Biochemicals) for direct mutagenesis. III-GAG-CAR and the mutants derived from it were transfected into COS-1 cells either with or without a co-transfected source of Rev, pSV-CMV-rev. To facilitate expression in the complete absence of the transactivator *tat*, the vectors of both III-GAG-CAR and SV-CMV-rev were constructed to contain SV40 replication origins, permitting amplified expression in the SV40 T antigen–expressing COS cell line. Gag production was measured by p24 enzyme-linked immunosorbent assay (ELISA) (Coulter Diagnostics) of cells 48 hours post-transfection and the results were normalized to protein. In some experiments an SV40-ori–containing expression plasmid was added to III-GAG-CAR to control for the nonspecific effects of a competing plasmid. In other experiments, competing plasmid was omitted to obtain more precise measurements of levels of baseline p24 production in the absence of Rev.

The I/I′ region of CAR appears to adopt the structure diagrammed in the central stem portion of Fig. 1, pairing sequences at the 5′ end of CAR with those near the 3′ end. Deletion of either side of this structure (ΔCAR-A or ΔCAR-I) severely impaired function. The CAR-1 mutation (ACAA) in the stem of the alternate stem/loop I did not inhibit the Rev response, whereas the CAR-2 mutation (TTGT) in the other side of the alternate stem/loop I severely inhibited the Rev response (Table 1). CAR-17 (ACAA), the reciprocal mutation to CAR-2 in the I′ region of the central stem structure, also impaired the Rev response, but the double-reciprocal mutation CAR-2/17 not only restored the Rev-response, but improved it by a factor of 2 (Table 1). Consistent with this improvement, the I/I′ region forms a more perfectly base-paired duplex with the CAR-2/17 double-reciprocal mutation than it does with wild-type sequences. Thus, there is no evidence that the alternate stem/loop I contributes to the Rev response. Furthermore there is probably no sequence-specific information in the bases mutated in the CAR-2/17 double-reciprocal mutant.

Regions II, IV, V, and VI do not adopt the central stem structure depicted in Fig. 1 but adopt the alternate stem/loop structures. Mutations in the stems of all these alternate stem/loops severely impaired CAR function (see CAR-3, CAR-4, CAR9B, CAR10B, CAR-11, CAR-13, CAR-14, and CAR-16; Fig. 1 and Table 1) whereas restoration of the stems by double-reciprocal mutation restored function to between 50 and 168% of wild type (see CAR-3/4, CAR-9B/10B, CAR-11/13, and CAR-14/16, Fig. 1 and Table 1). The formation of stem/loops IV and V was somewhat surprising in that the long central stem/loop IV/V bulging downward from the central structure depicted in Fig. 1 was one of the more constant predictions of Zuker's suboptimal RNA folding program. Although the alternate stem/loop V seems to be a functional structure, the lack of an effect of mutating three bases in the loop (CAR-12) or 6 bp in the stem proximal to the loop (CAR-11/13) suggests that it contains little or no sequence-specific information. The greater efficiency of the CAR-9B/10B mutant as compared to the CAR-9A/10A mutant may derive from sequence-specific protein interactions with stem/loop IV, or may derive from the fact that the larger mutation (9A/10A) displaces a small "bubble" of unpaired material a little further away from the loop.

Region III adopts the stem/loop structure at the rightward end of the long central structure depicted in Fig. 1. Stem mutations CAR-5, CAR-8 (see Fig. 2 and Table 1), CAR-6, and CAR-7 (Fig. 1 and Table 1) all eliminated CAR function whereas the double-reciprocal mutation, CAR-6/7 restored CAR function to normal (Table 1). The stem/loop of region III was the most constant feature of the alternate suggested foldings.

Finally, stem/loop VII appears to make no contribution to CAR function. Stem mutation CAR-21 and loop mutation CAR-22 had no effect on the Rev response. Neither did deletion of almost the entire stem loop (ΔCAR-J, see Fig. 2 and Table 1).

The mutation analysis allows us to estimate the 5′ and 3′ borders of CAR. The 3′-most mutation that impaired CAR function was CAR-19 (Fig. 2 and Table 1). CAR-20, just 3′ of this was normal, as was ΔCAR-K, a deletion extending from nt 7602 to 7639. This deletion extended well past the previously delimited 3′ border of CAR, 7627 (*13*) suggesting that the 3′CAR border may be no further downstream than 7559. The suggested base-pairing of the central structure in the I/I′ region would accordingly

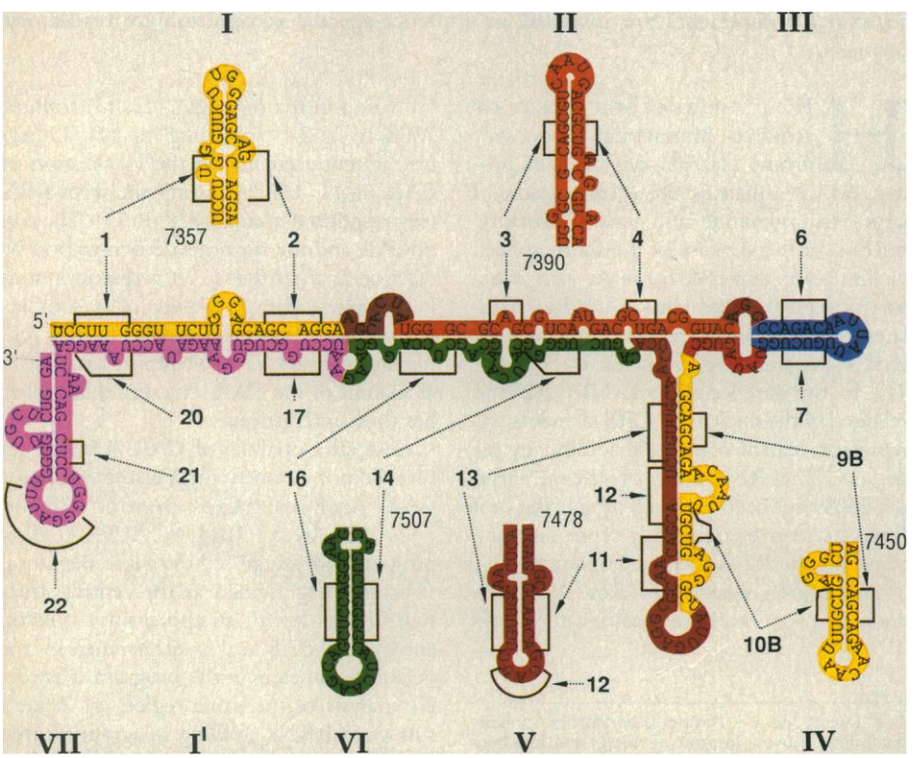

Fig. 1. Alternative potential foldings of CAR and the locations of selected substitution mutations. Zuker's suboptimal RNA folding program (*17*) was run in the "N-BEST" mode with default parameters. User-supplied parameters were ten tracebacks, an energy level within 10%, and a window or distance factor of 1. The location of selected CAR substitution mutations is indicated by brackets and large Arabic numbers. Large Roman numerals refer to the regions which lie nearby.

predict that the 5′ border be no further upstream than 7364.

The data discussed above strongly support the secondary structure of CAR diagrammed in Fig. 3, which is a modified display from the University of Wisconsin SQUIGGLES output of data from Zuker's suboptimal RNA folding program (*17*) run with forced base-pairing in the regions determined to be base-paired by our analysis. This structure is a subcomponent of a suggested folding of 250 bp of the CAR region, which has a predicted folding energy of −78.1 kcal and is closely similar to a computer-predicted folding suggested by Malim *et al.* (*9*).

Surprisingly, although CAR function can be severely impaired by modification of any of the stem/loops II through VI, it is often only minimally impaired by removal of some of these stem/loops. Table 1 contains the results of the deletion mutations, the extent of some of which are indicated in Fig. 3. Deletion of stem loops III or V (ΔCAR-D or ΔCAR-G, respectively) severely impaired the Rev response, although ΔCAR-G had sustained a 1-base deletion in the neighboring stem of stem/loop VI. Deletion of stem/loop IV (ΔCAR-E, Fig. 3) still left a Rev response of about 43% of normal in contrast to stem disruptions of IV (CAR-9A and CAR-10A) that could completely eliminate the Rev response. Although either of the stem mutations in VI, CAR-14, or CAR-16 eliminated the Rev response, deletion of almost the entire stem/loop (ΔCAR-H, see Table 1 and Fig. 3) had virtually no effect on the Rev response. Deletion of stem/loops IV, V, and VI together (ΔCAR-EGH) left a moiety that still retained 30% normal function. Similarly large amounts of material in stem/loop II could be removed without eliminating the Rev response.

It has been shown (*13*) that replacement of 15 bases of CAR (7393 to 7407) with 6

Table 1. Rev response: p24 ELISA of transfected mutants. COS-1 cells were transfected with 0.5 μg of each of the indicated plasmids according to the DEAE dextran method described by Cullen (*22*). Cultures were incubated for 48 hours and harvested in the lysis buffer provided in the p24 gag antigen capture kit (Coulter). Samples were briefly sonicated and spun in a Dupont microfuge before assay with the p24 antigen capture ELISA kit. Serial twofold dilutions were used to permit sample readings to fall within the linear range of the kit. Results were calculated in arbitrary units normalized to protein and with the background subtracted and are presented as a percentage of the wild type maximally stimulated with Rev for each experiment. "+rev" signifies co-transfection with a rev expression plasmid, pSV-CMV-Rev, generously provided by W. A. Haseltine. Psv2neo was used as a competing plasmid in all experiments with the sole exception of experiment 4 in which pSV2CAT was substituted for pSV2neo. Values below levels considered reliably detectable are marked UD for undetectable.

Construct	Rev response											
	Experiment 1			Experiment 2			Experiment 3			Experiments 4 through 7§		
	−Rev −pSV2neo	−Rev +pSV2neo	+Rev −pSV2neo	−Rev −pSV2neo	−Rev +pSV2neo	+Rev −pSV2neo	−Rev −pSV2neo	−Rev +pSV2neo	+Rev −pSV2neo	−Rev −pSV2 (neo or CAT)	−Rev +pSV2 (neo or CAT)	+Rev −pSV2 (neo or CAT)
III-GAG-CAR*	12	4	100	12	4.5	100	11	1	100	$12^{6};9^{7}$	$8^{4};4^{5}$	100^{4-7}
HXGC-D1†	6.5		7									
CAR-1				8		118		UD	98			
CAR-2	13		24	25		28						
CAR-2/17	15		194				11		>209			
CAR-3	14		23	18		14				11^{6}		10^{6}
CAR-4	13.5		21	21		14.5						
CAR-3/4										12^{6}		53^{6}
HXCD-7‡								UD	UD			
HXCD-9‡								UD	100			
CAR-5				18		18						
CAR-6				13		20						
CAR-7				14		11						
CAR-6/7				14.5		110						
CAR-8				11		13						
CAR-9A				13		15						
CAR-9B							6		26			
CAR-10A				13		10						
CAR-10B							11		19			
CAR-9A/10A				11		47						
CAR-9B/10B							10		130			
CAR-12	7		112	16		178						
CAR-13	14		18	12		12						
CAR-11/13	24		101									
CAR-14	7		16									
CAR-16	15		22									
CAR-14/16	15		168									
CAR-17	14		20									
CAR-18							7		91			
CAR-19							4.5		22			
CAR-20							7		68.5			
CAR-21				0.8		67						
CAR-22				10		85						
ΔCAR-A											UD[5]	3.2[5]
ΔCAR-B				10		11						
ΔCAR-C				16		7.2	15	2	9.8			
ΔCAR-D							19		30			
ΔCAR-E							9.5		43			
ΔCAR-F											UD[4]	136[4]
ΔCAR-G							3		0.9			
ΔCAR-H	7		32				7.5		101		UD[4]	90[4]
ΔCAR-I							9		22			
ΔCAR-J							10		112			
ΔCAR-K							7.9		174			
ΔCAR-EGH										9^{7}		32^{7}

*Determined in duplicate for each experiment. †HXCD-D1 is a gag expression plasmid constructed similarly to III-GAG-CAR (see *13*) except that it has a complete deletion of the entire CAR fragment. ‡HXCD-7 and HXCD-9 are proviral constructs having a small deletion in the *tat* gene in addition to the mutations in CAR (see *13*). Results for these two plasmids were normalized to HXCD-9 maximally stimulated with Rev. §Data from individual experiments 4 through 7 are indicated as superscripts.

bases of linker material left CAR function intact (mutant HXCD-9). Consequently, it was unexpected that a slightly larger deletion of 7391 through 7408 (with no interposition of linker material—see ΔCAR-B, Table 2) or a small disruptive deletion of one side of the base of the stem, ΔCAR-C, would abolish the Rev response. Although further studies will be needed to resolve this apparent discrepancy, we reexamined the phenotype of mutant HXCD-9 with the assay employed here and confirmed our previous result (see Table 1). Possibly CAR structure is influenced by flanking regions that differ in the two series of constructs or possibly something peculiar to the material inserted in HXCD-9 prevented the disruption from impairing CAR function. Whatever the case, it is clear that stem disruptions in CAR are frequently more deleterious than stem/loop deletions.

To the extent that the stem/loops form independently of one another, the "clean excision" of a single stem/loop from a set of multiple independent stem/loops at equilibrium should not alter the equilibrium structure of the remaining stem/loops. Disruption of a stem/loop, however, releases sequences from confinement in the local structure allowing them to interact with entirely different sequences, possibly in neighboring stem/loops. Alternatively the unaltered stem/loops may retain their structure while the disrupted stem/loop sterically hinders access of binding proteins to other targets.

That CAR may function in the absence of stem/loops II, V, VI, and possibly IV suggests that these structures neither directly bind critical regulatory proteins involved in Rev regulation nor maintain other structures in a conformation critical for CAR function. Although their role remains unclear it is possible that they are involved in other regulatory pathways or that they serve merely to confine required protein coding information in structures that do not interfere with CAR function. CAR is located in a highly conserved and functionally important region of *env* at the NH_2-terminus of gp41 (*18*). A third, though less likely alternative is that some or all of the "unnecessary" stem/loops (II, IV, and VI) are actually involved in a cooperative binding of Rev and that our assays are only minimally affected by their absence because of the abnormally high, possibly supersaturating levels of Rev supported by the highly active CMV promoter of pSV-CMV-rev.

The remaining structures, stem/loop III and the I/I′ central stem structure, seem the most likely candidates for sequence-specific binding of the most critical components of the Rev axis and may only have to lie in close proximity to one another to function properly. With respect to the paradigm of a nonspecific stem "presenting" a specific sequence in the loop, however, mutation of one side of the prominent "bubble" in the I/I′ region, CAR-18, had no effect on CAR function. It is possible that there is no sequence-specific recognition of CAR at all

```
                7357                                                                7416
w.t.            TCCTTGGGTT CTTGGGAGCA GCAGGAAGCA CTATGGGCGC AGCGTCAATG ACGCTGACGG
CAR-1           --ACAA---- ---------- ---------- ---------- ---------- ----------
CAR-2           ---------- ---------- TTGT------ ---------- ---------- ----------
ΔCAR-A          ---------- ---------[           ] ---------- ---------- ----------
CAR-3           ---------- ---------- ---------- ---------A -A-------- ----------
CAR-4           ---------- ---------- ---------- ---------- ---------- ---T-T----
ΔCAR-B          ---------- ---------- ---------- ----[                    ]--------
HXCD-9          ---------- ---------- ---------- ------[    ..AATTCC.. ]---------
ΔCAR-C          ---------- ---------- ---------- ---------- ---------- ---------[

                7417                                                                7476
w.t.            TACAGGCCAG ACAATTATTG TCTGGTATAG TGCAGCAGCA GAACAATTTG CTGAGGGCTA
ΔCAR-C cont.         ]---- ---------- ---------- ---------- ---------- ----------
CAR-5           ------AAT- ---------- ---------- ---------- ---------- ----------
CAR-6           ---------C CGC------- ---------- ---------- ---------- ----------
CAR-7           ---------- --------GC GG-------- ---------- ---------- ----------
CAR-8           ---------- ---------- --ATT----- ---------- ---------- ----------
ΔCAR-D          ------[                  ]----- ---------- ---------- ----------
CAR-9A          ---------- ---------- ---------- -----ATAGT ---------- ----------
CAR-9B          ---------- ---------- ---------- ------GTG- ---------- ----------
CAR-10A         ---------- ---------- ---------- ---------- --------ACTATC-------
CAR-10B         ---------- ---------- ---------- ---------- ---------C AC--------
ΔCAR-EGH        ---------- ---------- ---------- ---[
ΔCAR-E          ---------- ---------- ---------- ----[
ΔCAR-F          ---------- ---------- ---------- ---------- ---------- -------[

                7477                                                                7536
w.t.            TTGAGGCGCA ACAGCATCTG TTGCAACTCA CAGTCTGGGG CATCAAGCAG CTCCAGGCAA
ΔCAR-EGH cont.
ΔCAR-E cont.    ]--------- ---------- ---------- ---------- ---------- ----------
ΔCAR-F cont.    ]--------- ---------- ---------- ---------- ---------- ----------
CAR-11          --------AT AGT------- ---------- ---------- ---------- ----------
CAR-12          ---------- -----AGC--- ---------- ---------- ---------- ----------
CAR-13          ---------- --------AC TAT------- ---------- ---------- ----------
ΔCAR-G          ---[                     ]------ ----*----- ---------- ----------
CAR-14          ---------- ---------- ---------- -----GTACT ---------- ----------
CAR-16          ---------- ---------- ---------- ---------- ---------- AGTAC-----
ΔCAR-H          ---------- ---------- ---------- --[        ..AA..          ]----
ΔCAR-I          ---------- ---------- ---------- ---------- ---------- ------A---

                7537                                                                7596
w.t.            GAATCCTGGC TGTGGAAAGA TACCTAAAGG ATCAACAGCT CCTGGGGATT TGGGGTTGCT
ΔCAR-EGH cont.     ]------ ---------- ---------- ---------- ---------- ----------
ΔCAR-I cont.    ----[                           ]--------- ---------- ----------
CAR-17          -----ACAA* ---------- ---------- ---------- ---------- ----------
CAR-18          ---------- ---AAG---- ---------- ---------- ---------- ----------
CAR-19          ---------- -------TCT CTG------- ---------- ---------- ----------
CAR-20          ---------- ---------- ----TTGT*- ---------- ---------- ----------
CAR-21          ---------- ---------- ---------- ---------G A--------- ----------
CAR-22          ---------- ---------- ---------- ---------- -----ACCGC ----------
ΔCAR-J          ---------- ---------- ---------- -------[                  ]-----

                7597                                                                7656
w.t.            CTGGAAAACT CATTTGCACC ACTGCTGTGC CTTGGAATGC TAGTTGGAGT AATAAATCTC
ΔCAR-K          -----[                                        ]------- ----------
```

Fig. 2. Sequence of CAR mutations. CAR substitution and deletion mutations were introduced directly into III-GAG-CAR by means of the Bio-Rad "Muta-Gen Phagemid" in vitro mutagenesis kit according to the manufacturer's directions. Each plasmid "maxi-prep" was sequenced to ensure the identity and purity of the actual DNA samples used for transfection. CAR-6 and the double-reciprocal mutant CAR-6/7 both contain the ΔCAR-J deletion. HXCD-9, as previously reported (*13*) is a proviral clone with a deletion in *tat* in addition to the deletion in the CAR element displayed here.

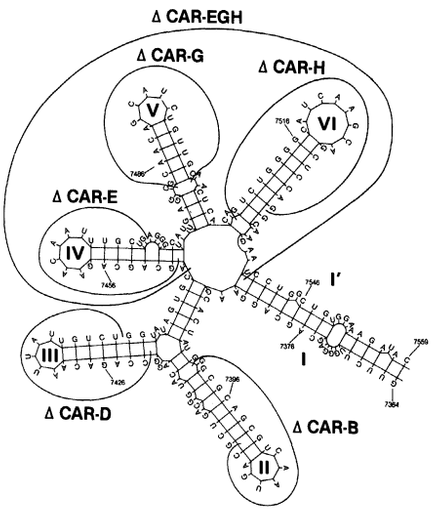

Fig. 3. Structure of CAR and selected deletion mutations. The structure depicted is almost identical to the seventh most stable predicted folding (out of ten predicted by Zuker's suboptimal RNA folding program run without forced base-pairing information). Regions 5′ and 3′ of CAR according to the data presented have been deleted. The full 250-bp fragment folded had a folding energy of −78.1 kcal versus an energy of −81.3 for the most stable structure.

and that the only requirement for function is a properly oriented or clustered set of stem structures. In this context regions of RNA containing the Rex-responsive sequences of HTLV-I and HTLV-II (*19*) also may be folded into multiple stem/loop structures with "per base" folding energies within 10% of the structures predicted for CAR, yet they lack obvious sequence homology with CAR (*7, 20*). A requirement only for clustered stem loops may underlie the observation that Rex of HTLV-I can substitute for Rev of HIV-1 (*21*).

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Differential Effects of *nef* on HIV Replication: Implications for Viral Pathogenesis in the Host

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Stable lymphoid cell lines expressing the human immunodeficiency virus type 1 (HIV-1) *nef* gene product, p27, were established. The presence of p27 in the lymphoid cells suppressed replication of some strains of both HIV-1 and HIV-2. This observation indicates that *nef* could be important in the establishment of HIV latency. In contrast, fast replicating and highly cytopathic HIV-1 isolates recovered from patients with advanced disease states were not affected by the negative effect of *nef* present in these lymphoid cell lines. This lack of response to *nef* appears to constitute another viral feature that correlates with disease progression. Thus, manipulating expression of the *nef* gene in vivo might influence pathogenesis in the host.

THE GENOMES OF THE TWO SUBtypes of the human immunodeficiency virus, HIV-1 and HIV-2, contain genes coding for structural proteins (*gag*, *pol*, and *env*), regulatory sequences in the long terminal repeat (LTR), as well as at least six additional open reading frames. Three of these latter genes code for regulatory proteins (*tat*, *rev*, and *nef*). The *tat* and *rev* genes are positive regulators of HIV replication; in contrast, *nef* appears to act as a negative regulator of HIV replication (*1*). The *nef* gene encodes a protein, p27, which is myristylated and phosphorylated (*2*). The protein has biochemical properties similar to that of a G-binding protein, that is, guanosine triphosphate (GTP)–binding activity, GTPase activity, and autophosphorylation (*2, 3*). Furthermore, expression of *nef* via a vaccinia vector in the CEM cell line downmodulates the CD4 receptor molecule (*2*).

Mutational analysis has shown that mutants defective in expression of the *nef* gene product replicate faster (*4, 5*) and to several-fold higher titers than wild-type virus (*5, 6*). Recent studies indicate that *nef* suppresses HIV transcription by acting on the LTR, probably via an interaction with cellular factors (*6, 7*). We have previously proposed that *nef*, by suppressing HIV expression, could be involved in HIV latency (*5*). To further investigate this activity of the *nef* gene product on HIV replication and to evaluate its potential role in HIV pathogenesis, we constructed two plasmids for the expression of *nef* from HIV-1_{SF2} [formerly designated AIDS (acquired immunodeficiency syndrome)-associated retrovirus, ARV-2] (*8*). Its gene product has been shown in vitro to have GTP-binding and kinase activities (*3*). Expression of *nef* in plasmid p3R is under the control of the HIV-1–LTR, which contains sequences responsive to both viral (for example, *tat*) and cellular transcriptional activators (*1*). In plasmid pAM3, the expression of *nef* is under the control of the SV40 early promoter (*9–14*). In this latter plasmid, in contrast to the p3R construct that is autoregulated, the *nef* protein is made constitutively. Hu-

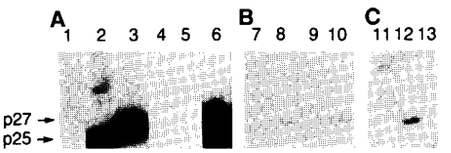

Fig. 1. Identification of cells expressing *nef*: 4×10^6 cells each of control HUT 78 and Jurkat cells, HUT 78 and Jurkat cells expressing *nef*, and a cell line chronically infected with HIV-1_{SF2} were lysed in virus disruption buffer (50 m*M* tris-Cl, *p*H 7.5, 0.1% Triton X-100, and 0.15 g of dithiothreitol per milliliter) and subjected to immunoblot analysis as described (*17*). Fractionated proteins were reacted with a pool of sera from HIV-1–positive individuals that had been screened for reactivity to the p27 protein or with a polyclonal rabbit antibody to p27 (anti-p27) antiserum. (**A**) The specificity of the sera used for screening p27 expression. Lanes 1 and 4, control HUT 78 *neo* cell lysate; lanes 2 and 5, HUT 78 chronically infected with HIV-1_{SF2}; lanes 3 and 6, p27 protein expressed in yeast (1 μg of protein per lane). Lanes 1 to 3 were reacted with HIV-1 positive human sera at a 1:50 dilution; lanes 4 to 6 with a polyclonal rabbit anti-p27 antiserum also at 1:50 dilution. (**B** and **C**) P27 reactivity in HUT and Jurkat *nef*-expressing cell lines as detected by the same pool of HIV-1 positive human sera. In (B): lane 7, control HUT 78 *neo* cell lysate; lane 8, HUTpAM3 *neo* cell lysate; lane 9, HUTp3R *neo* cell lysate, lane 10, lysate prepared from HUTp3R *neo* cells infected with HIV-1_{SF2}. In (C): lane 11, control Jurkat *neo* cell lysate; lane 12, JURpAM3 *neo* cell lysate; lane 13, JURp3R *neo* cell lysate. The p27 reactivity was absent in HUT 78 cells chronically infected with HIV-1_{SF2} (lanes 2 and 5). In lane 2, only reactivities to the *gag* p25 and *pol* p31 proteins were detected. The polyclonal rabbit anti-p27 showed a much stronger reactivity to the yeast p27 protein (lane 6) as compared to the positive HIV-1 human sera (lane 3).

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