

An RNA-Binding Chameleon

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Summary

The arginine-rich RNA binding motif is found in a wide variety of proteins, including several viral regulatory proteins. Although related at the primary sequence level, arginine-rich domains from different proteins adopt different conformations depending on the RNA site recognized, and in some cases fold only in the context of RNA. Here we show that the RNA binding domain of the Jemrana disease virus (JDV) Tat protein is able to recognize two different TAR RNA sites, from human and bovine immunodeficiency viruses (HIV and BIV, respectively), adopting different conformations in the two RNA contexts and using different amino acids for recognition. In addition to the conformational differences, the JDV domain requires the cyclin T1 protein for high-affinity binding to HIV TAR, but not to BIV TAR. The “chameleon-like” behavior of the JDV Tat RNA binding domain reinforces the concept that RNA molecules can provide structural scaffolds for protein folding, and suggests mechanisms for evolving distinct RNA binding specificities from a single multifunctional domain.

Introduction

The diversity of the RNA structure provides a wide array of possible surfaces for protein and ligand recognition. Although chemically less diverse than proteins, RNAs can fold into compact structures with internal and external surfaces, catalytic sites, and binding pockets (Cech and Golden, 1999; Hermann and Patel, 1999); and, like proteins, RNAs can show induced-fit interactions upon binding of proteins or other ligands. Given these features, RNAs, in principle, can provide architectural frameworks to help organize and stabilize the structures of interacting proteins, much as the tertiary framework of a protein can help organize the folding of peptide segments. In some cases, an identical peptide sequence can fold into different secondary structures when placed in different protein contexts or upon interaction with different proteins (Landry et al., 1992; Minor and Kim, 1996; Frankel, 2000), and it seems reasonable that the interaction of a peptide with different RNAs also might cause differences in folding. Indeed, it has been shown recently that an RNA aptamer selected *in vitro* can in-

duce an RNA binding peptide from human immunodeficiency virus (HIV) Rev to adopt an extended conformation, even though it binds to its natural RNA site as an α helix (Ye et al., 1999). Here we show that an arginine-rich RNA binding domain from a viral transcription factor can bind to two different viral RNA sites in two different peptide conformations, further demonstrating how an RNA scaffold can help specify peptide structure, and suggesting how different RNA binding specificities might evolve from an ancestral sequence.

HIV, and the related bovine immunodeficiency virus (BIV), encode transcriptional activators, known as the Tat proteins, which provide good model systems for studying RNA-protein recognition. The Tat proteins are unusual transcription factors that bind to specific RNA sites (transactivating response elements [TARs]) located at the 5' end of the viral mRNA transcripts, rather than to DNA sites. HIV Tat has been shown to increase the efficiency of RNA polymerase II elongation from the HIV promoter, apparently by stimulating phosphorylation of the polymerase C-terminal domain (Reines et al., 1999; Taube et al., 1999). One kinase likely to be involved, the cyclin-dependent kinase cdk9, is recruited to the HIV promoter by cyclin T1. Cyclin T1 binds to the activation domain of Tat and forms a tight ternary complex with TAR RNA (Wei et al., 1998). HIV Tat alone recognizes an internal bulge in the TAR hairpin, whereas the Tat-cyclin T1 complex recognizes the terminal loop in addition to the bulge (Wei et al., 1998). It is not yet clear whether additional contacts to the RNA are made by cyclin T1 or Tat in the context of the ternary complex.

The interaction of Tat with the HIV TAR bulge is mediated by an arginine-rich RNA binding domain in which a single arginine (at position 52 of the protein) makes the key contribution to binding specificity (Calnan et al., 1991). Studies with isolated peptides have shown that additional basic amino acids that surround this arginine help enhance the affinity and kinetic stability of the complex and also improve binding specificity (Weeks and Crothers, 1992; Tao and Frankel, 1993; Long and Crothers, 1995; Long and Crothers, 1999). Nuclear magnetic resonance (NMR) studies indicate that arginine forms hydrogen bonds to G26, and contacts two phosphates positioned by formation of a base triple between U23 in the bulge and an A:U bp above G26 (Puglisi et al., 1992; Long and Crothers, 1999). The arginine-rich domain of HIV Tat does not appear to adopt a discrete structure and is probably in an extended and dynamic conformation in the complex, at least in the absence of cyclin T1 (Calnan et al., 1991; Aboul-ela et al., 1995; Bayer et al., 1995; Tan and Frankel, 1995).

BIV Tat also uses an arginine-rich RNA binding domain to recognize its BIV TAR site but, in marked contrast to the HIV interaction, the BIV Tat domain adopts a β -hairpin conformation upon TAR binding and uses three arginines, three glycines, one threonine, and one isoleucine to make specific contacts to the RNA and to allow the peptide to fold properly (Chen and Frankel, 1995; Puglisi et al., 1995; Ye et al., 1995; Figure 1). The peptide-RNA

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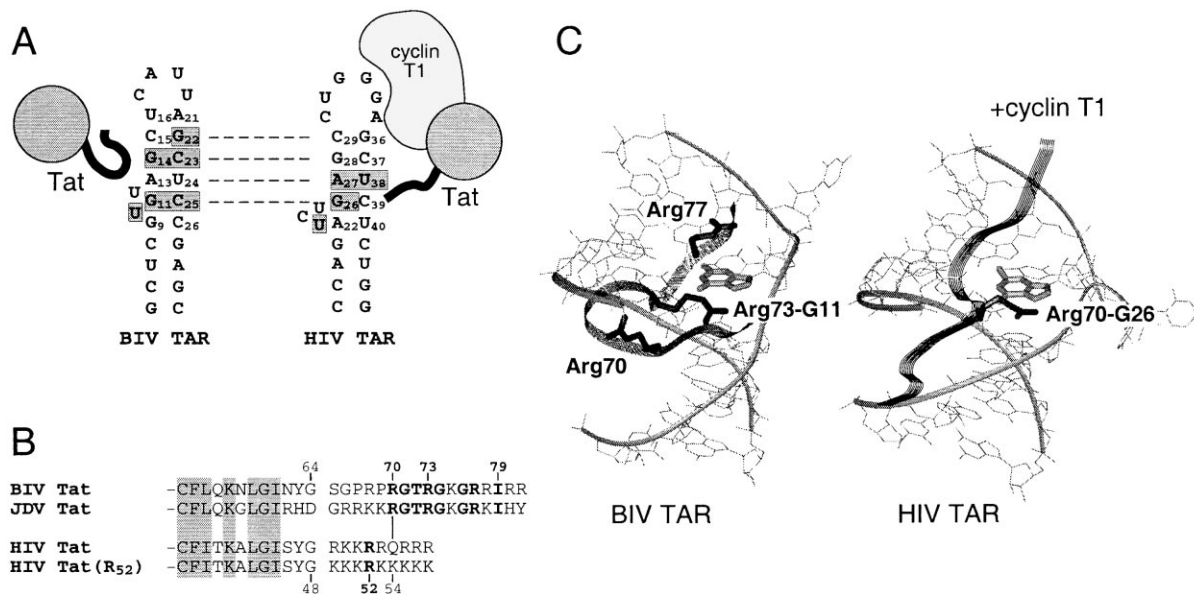


Figure 1. Comparison of HIV and BIV TAR RNAs and Tat Arginine-Rich RNA Binding Domains

(A) Nucleotides in each TAR important for binding by the cognate protein (Hamy et al., 1993; Chen and Frankel, 1994) are highlighted, and sequence identity in the upper stems is indicated by dashed lines. The arginine-rich RNA binding domains are shown schematically as a hairpin for BIV Tat and an extended chain for HIV Tat, and the activation domains are shown as balls. The RNA binding domain of BIV Tat binds with high affinity to BIV TAR, whereas HIV Tat requires an interaction between the activation domain and cyclin T1 to generate a ternary complex with HIV TAR. The cyclin still is required for transcriptional activation by BIV Tat, but additional contacts to TAR are not believed to be involved (Chen and Frankel, 1994; Barboric et al., 2000; Bogerd et al., 2000).

(B) The arginine-rich domains of the Tat proteins are aligned based on homology between the N-terminal activation domains (partially shown). Amino acids important for RNA binding specificity in BIV and HIV Tat (Calnan et al., 1991; Chen and Frankel, 1995) are shown in bold. Analogous residues in the JDV Tat domain also are shown in bold.

(C) NMR models of BIV Tat-TAR and argininamide-HIV TAR complexes. For the BIV complex (Puglisi et al., 1995), the three arginines involved in specific RNA recognition are shown, with the Arg73-G11 interaction indicated. The β -hairpin conformation of the peptide backbone is displayed as a ribbon and only the stem regions of TAR are shown. For the HIV complex (Puglisi et al., 1992), the locations of the argininamide side chain (corresponding to Arg70 of the JDV Tat domain, as described in the text) and G26 are shown, and the apparently extended peptide conformation (Aboul-ela et al., 1995; Tan and Frankel, 1995) is shown schematically as a ribbon. Cyclin T1 is presumed to bind to the HIV TAR loop (not shown).

interactions also differ in that additional bases in BIV TAR are contacted in the complex even though the TAR secondary structures, and indeed even their tertiary structures, are closely related (Smith et al., 1998; Figure 1 and Discussion). Furthermore, the BIV TAR loop does not contribute to specific protein recognition, and the interaction of BIV Tat and TAR alone appears sufficient to form a stable RNA-protein complex (Chen and Frankel, 1994; Barboric et al., 2000; Bogerd et al., 2000).

Another lentivirus, Jembrana disease virus (JDV), is closely related to BIV and also encodes a Tat protein whose arginine-rich RNA binding domain is very similar to that of BIV Tat (Chadwick et al., 1995; Chen et al., 1999b). Here we show that this domain can specifically recognize both BIV and HIV TAR RNAs using very different mechanisms and adapting its conformation and interactions to the surrounding RNA context. The JDV domain also binds with high affinity to its own TAR site, whose bulge architecture differs from those of BIV and HIV TARs. The discovery of a short, multifunctional RNA binding domain suggests that new binding specificities can be readily evolved without generating nonfunctional intermediates; a strategy likely to be employed by rapidly evolving viruses.

Results

JDV Tat Binding to BIV and HIV TAR RNAs

JDV Tat is closely related to BIV Tat and its arginine-rich domain contains all of the residues required for BIV TAR recognition (Figure 1B). Thus, we predicted that the JDV Tat domain would bind to BIV TAR with high affinity. In contrast, the JDV domain does not meet the sequence requirements for HIV TAR recognition in which an arginine must be located at the equivalent of position 52 or 53 in the intact Tat protein, flanked on each side by at least three basic residues (Calnan et al., 1991; Tao and Frankel, 1993; Figure 1B). The positioning of the arginine in relation to the Tat activation domain (residues 1–48) is presumed to be important for properly orienting the Tat-cyclin T1 complex for cooperative binding to the TAR bulge and loop (Luo and Peterlin, 1993; Wei et al., 1998). None of the five arginines in the JDV domain is positioned optimally with respect to the activation domain, nor flanked by a sufficient number of basic amino acids. Thus we suspected that the JDV domain would bind HIV TAR with low affinity.

We first examined binding of arginine-rich Tat peptides by *in vitro* gel shift assays and found that, as

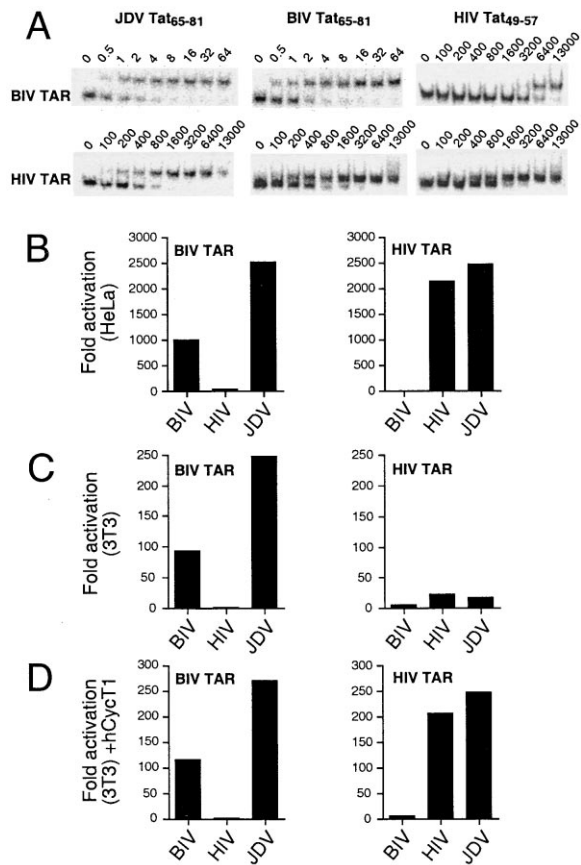


Figure 2. The JDV RNA Binding Domain Recognizes both BIV and HIV TARs

(A) Gel shift assays with JDV, BIV, and HIV Tat peptides and BIV and HIV TAR RNAs were performed with 0.2 nM RNA at the peptide concentrations indicated (nM).

(B) Activation of HIV LTR CAT reporters containing BIV or HIV TAR RNA sites by the HIV Tat(1-49)-BIV Tat(65-81) fusion protein, HIV Tat(1-72), or the the HIV Tat(1-49)-JDV Tat(65-81) fusion protein. Tat-expressing and reporter plasmids were cotransfected into HeLa cells and CAT activity was measured after 44 hr. Fold activation is the level of activity normalized to the activity of each reporter plasmid alone.

(C) Same experiment as in (B) performed in NIH 3T3 cells.

(D) Same experiment as in (C), but also including a human cyclin T1-expressing plasmid (Wei et al., 1998) in the transfection.

expected, both the JDV and BIV peptides bound to BIV TAR with high affinity (apparent K_d s = 0.7 nM and 1.3 nM, respectively) and to HIV TAR with low affinity (K_d s = 360 nM and 310 nM, respectively; Figure 2A). It is interesting that the JDV peptide binds to BIV TAR with slightly higher affinity than does the cognate BIV Tat peptide (see below). The HIV Tat peptide binds to HIV TAR with higher affinity than to BIV TAR, but the interactions are relatively weak in the absence of cyclin T1, as observed previously (Chen and Frankel, 1994).

To examine TAR binding specificity in the presence of cyclin T1, we fused the RNA binding domains of JDV, BIV, and HIV to the activation domain of HIV Tat and measured transcriptional activation of HIV long terminal repeat chloramphenicol acetyltransferase (LTR CAT) reporter plasmids containing BIV or HIV TAR sites located

at the 5' ends of the transcripts (Smith et al., 1998). Unexpectedly, the JDV fusion protein strongly activated transcription through both BIV and HIV TARs, whereas HIV Tat activated only through HIV TAR, and a fusion between the HIV activation domain and the BIV RNA binding domain activated only through BIV TAR (Figure 2B). The results indicate that a single RNA binding domain is able to recognize two different TAR sites and are consistent with experiments showing that the intact JDV Tat protein can activate the HIV LTR and support viral replication (Chen et al., 1999b; Chen et al., 2000).

Given the high degree of sequence conservation, it seemed likely that the JDV domain bound BIV TAR in a β -hairpin conformation, as does the BIV domain, but the mode of binding to HIV TAR was less clear. In principle, the JDV domain also might adopt a β -hairpin conformation upon binding to HIV TAR (although the BIV domain apparently does not; Smith et al., 1998), or the JDV domain might bind HIV TAR in a cyclin T1-dependent manner, and most likely in an extended conformation, as does HIV Tat. To distinguish between these possibilities, we measured activation of the HIV LTR by the hybrid proteins in mouse (3T3) cells, which contain a cyclin T1 variant that cannot be used to recognize the HIV TAR loop (Bieniasz et al., 1998; Garber et al., 1998; Chen et al., 1999a; Fujinaga et al., 1999; Kwak et al., 1999; Ramanathan et al., 1999). Both the JDV and BIV domains mediated efficient activation through BIV TAR, even in the absence of human cyclin T1, whereas neither could mediate activation through HIV TAR (Figure 2C). This suggests that binding of the JDV domain to HIV TAR is weak without the appropriate cyclin. To confirm that binding to HIV TAR is cyclin dependent, we cotransfected the human cyclin T1, known to complement the mouse cyclin (Bieniasz et al., 1998; Garber et al., 1998; Chen et al., 1999a; Fujinaga et al., 1999; Kwak et al., 1999; Ramanathan et al., 1999), into 3T3 cells along with the Tat fusion proteins. In this case, we found that both the JDV and HIV Tat RNA binding domains mediated efficient activation through HIV TAR (Figure 2D). Under no circumstance could the BIV domain support activation through HIV TAR (Figures 2B-2D). Thus it appears that the JDV domain binds to BIV TAR in a high-affinity conformation, while its binding to HIV TAR requires formation of a complex with human cyclin T1.

Amino Acid Requirements for BIV and HIV TAR Recognition

The fundamentally different RNA binding modes of the JDV domain suggested that it might adopt different conformations when bound to BIV and HIV TAR, and use different amino acids for RNA recognition. BIV Tat requires three arginines (corresponding to positions 70, 73, and 77 in the BIV or JDV sequence; Figure 3A) to recognize BIV TAR, whereas HIV Tat requires only one arginine (at position 52 in HIV Tat) to recognize HIV TAR. If, indeed, the JDV domain recognizes BIV and HIV TAR in different ways, one also might expect different amino acid requirements. To determine which arginines in the JDV domain are important for BIV or HIV TAR recognition, we mutated each arginine to lysine and measured activation of BIV and HIV TAR reporters (Figure 3A). Strikingly, arginines at positions 70, 73, and 77 all are

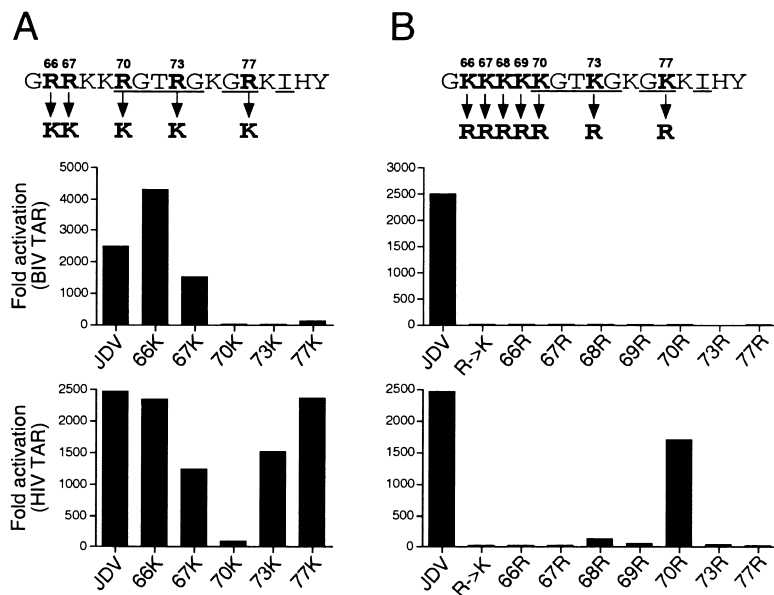


Figure 3. Activation of BIV and HIV TAR Reporters by JDV Tat Mutants

(A) Activation of BIV TAR (upper graph) and HIV TAR (lower graph) reporters by JDV Tat mutants in which the individual arginines indicated were replaced with lysines.

(B) Activation of BIV and HIV TAR reporters by JDV Tat mutants in which all arginines were replaced by lysine (R→K) and the individual lysines indicated were replaced with arginines. Tat-expressing and reporter plasmids were cotransfected into HeLa cells and activities were determined as in Figure 2.

essential for activation through BIV TAR, whereas just one arginine (position 70 in the BIV or JDV sequence, corresponding to position 54 in the HIV sequence; Figure 1B) is essential for activation through HIV TAR. As discussed below, the positioning of Arg70 most closely meets the HIV binding requirements; Arg70 is nearest to position 52 in the HIV sequence and also is flanked on each side by three basic residues.

To further confirm the distinct arginine requirements, we engineered a JDV variant in which all arginines were replaced by lysines (JDV R→K) and then reintroduced single arginines at each of seven lysine positions (Figure 3B). It is known that a single arginine embedded within a stretch of lysines [HIV Tat (R₅₂) in Figure 1B] supports full activation through HIV TAR (Calnan et al., 1991). Indeed, while the all-lysine variant of the JDV domain was inactive, introducing a single arginine at position 70 was sufficient to fully restore activity through HIV but not BIV TAR (Figure 3B). Thus, binding of the JDV domain to BIV TAR requires three arginines, as does BIV Tat; whereas binding to HIV TAR requires a single correctly positioned arginine, as does HIV Tat. As discussed below and shown in Figure 1C, structural studies of the BIV Tat peptide-BIV TAR and argininamide-HIV TAR complexes (Puglisi et al., 1992; Aboul-ela et al., 1995; Puglisi et al., 1995; Ye et al., 1995; Long and Crothers, 1999) have shown that Arg73 in BIV Tat and Arg52 in HIV Tat bind to structurally analogous guanines and phosphates at the bulges (G11 in BIV TAR and G26 in HIV TAR). Assuming that the JDV domain also makes the same contacts, it appears that Arg73 contacts G11 in BIV TAR, whereas Arg70 makes the analogous contact to G26 in HIV TAR. Thus, not only does the JDV domain appear to adopt different conformations and use different amino acids for recognition of the two TAR sites, it also appears to bind in two different registers.

JDV Tat Binding to a Hybrid TAR

We previously described a hybrid TAR having the determinants of both HIV and BIV TAR that can be recognized

by both HIV and BIV Tat (Figure 4), and wished to determine if the JDV domain could bind to this “bifunctional” TAR in both binding modes. To test this possibility, we measured the activities of the JDV domain and a JDV mutant on a hybrid TAR reporter, reasoning that (1) activity of the JDV domain would be observed in 3T3 cells, which lack the human cyclin T1, only if the peptide could bind in the BIV β-hairpin mode; and (2) activity of the Arg73Lys mutant, which removes an essential BIV binding residue, would be observed in HeLa cells only if the peptide could bind together with cyclin T1 in the HIV mode. Indeed, strong activation was observed in both cases (Figure 4). These results suggest that the JDV Tat domain may recognize a single RNA in two different ways, although we cannot determine which binding mode dominates when both are possible. It seems reasonable that the presence or absence of the human cyclin may dictate which binding mode is used.

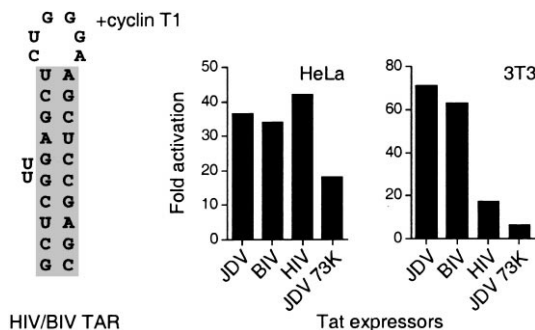


Figure 4. Activation of a BIV/HIV TAR Hybrid Reporter by JDV Tat

The Tat-expressing and hybrid reporter plasmids were cotransfected into HeLa and 3T3 cells, and activities were determined as in Figure 2. The shaded region of the RNA secondary structure indicates the BIV TAR portion, and the bulge and loop are those of HIV TAR. The two-nucleotide, rather than three-nucleotide, bulge creates a more active TAR (Weeks and Crothers, 1991; Smith et al., 1998).

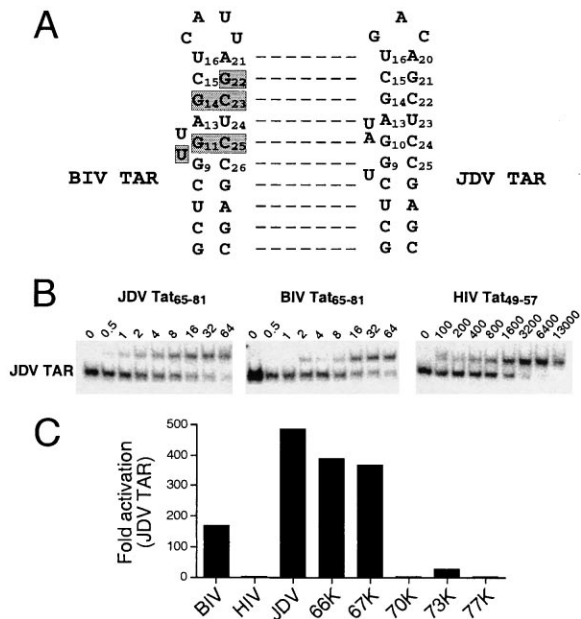


Figure 5. RNA Binding to JDV TAR In Vitro and In Vivo
 (A) Comparison of the BIV and JDV TAR secondary structures. Nucleotides in BIV TAR important for binding are highlighted, and sequence identity is indicated by dashed lines.
 (B) Gel shift assays with JDV, BIV, and HIV Tat peptides were performed with 0.2 nM RNA at the peptide concentrations indicated (nM).
 (C) Activation of a JDV TAR reporter by JDV Tat mutants. Tat-expressing and reporter plasmids were cotransfected into HeLa cells, and activities were determined as in Figure 2.

JDV Tat Binding to JDV TAR

We next examined how the JDV domain recognizes its own TAR site. Given that the amino acids required for BIV TAR binding are completely conserved in JDV Tat, it seemed likely that the JDV domain would recognize its own RNA in a manner similar to BIV. A comparison of the RNA sites revealed that while the stem regions of JDV TAR are identical to those in BIV TAR (Figure 5A), the bulge architecture is somewhat different: BIV TAR contains two single nucleotide bulges separated by a single bp, whereas JDV TAR contains a single nucleotide and two-nucleotide bulge separated by two bps. To test whether the JDV domain bound as a β -hairpin, we measured JDV TAR binding affinities of JDV, BIV, and HIV peptides in vitro (Figure 5B) and observed high-affinity interactions with the JDV and BIV peptides (K_d s = 3.3 nM and 15 nM, respectively), consistent with a BIV mode of binding. We next measured the activities of Arg-to-Lys mutants on a JDV TAR reporter and found that, like BIV, three arginines (at positions 70, 73, and 77) were required for binding (Figure 5C); further suggesting that the JDV domain recognizes its own TAR site in a β -hairpin conformation.

Bulge Requirements for JDV Tat Binding

Because the bulge architectures of JDV and BIV TARs are rather different, we wished to determine the bulge nucleotide requirements for recognition of JDV TAR. Typically, bulge nucleotides are important for widening

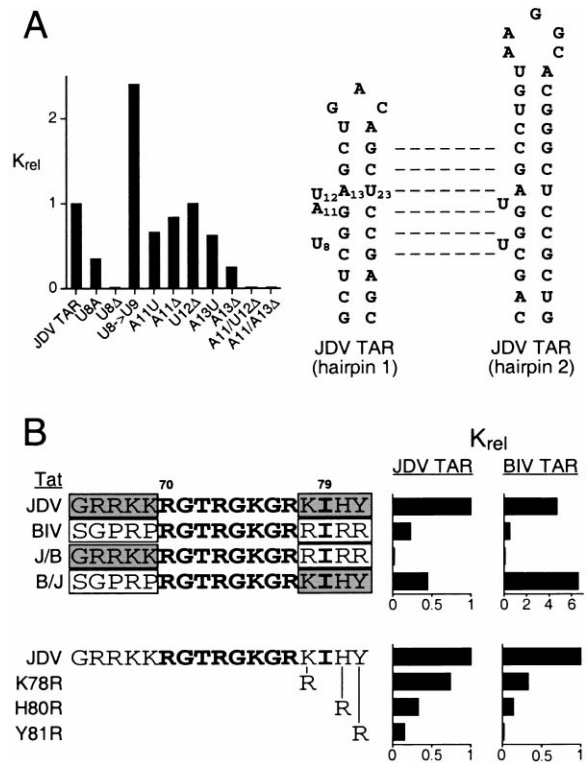


Figure 6. Nucleotides in JDV TAR and Amino Acids in JDV Tat Important for Binding
 (A) JDV Tat peptide binding to JDV TAR mutants in vitro. Gel shift assays were performed with the JDV Tat peptide and 0.2 nM of each RNA indicated. Association constants were calculated as described in Materials and Methods, and K_{rel} values were calculated as the ratio of apparent binding constants between mutant TARs and wild-type JDV TAR. Secondary structures of the two putative JDV TAR hairpins are shown, and sequence identity is indicated by dashed lines.
 (B) Binding of JDV Tat peptide variants to JDV and BIV TARs in vitro. The sequences of the peptide variants are shown, with the N-terminal and C-terminal regions flanking the core boxed. Shaded boxes correspond to JDV sequences and unshaded boxes correspond to BIV sequences. K_{rel} values for the upper set of peptides were calculated as the ratio of apparent binding constants normalized to the wild-type JDV Tat-JDV TAR interaction. K_{rel} values for the lower set of peptides show values normalized to JDV Tat peptide complexes with JDV TAR or BIV TAR.

the RNA major groove and facilitating access to a protein (Weeks and Crothers, 1993). In BIV TAR, a bulge nucleotide at the U10 position (Figure 5A) is essential for binding, but its identity is not (Chen and Frankel, 1994). In the NMR structure of the BIV peptide-TAR complex, U10 is observed to form a base triple with the A13:U24 bp (Ye et al., 1995), but given that the analogous U bulge in JDV TAR (U8) is separated from the equivalent A:U pair by an additional bp (see Figure 5A), it seems unlikely that a base triple will be stably formed in this case. The upper bulge nucleotide in BIV TAR (U12) (Figure 5A) can be deleted with little effect on binding (Chen and Frankel, 1994).

We generated a series of mutations of the U8 and A11/U12 bulges of JDV TAR and measured binding of the JDV peptide to the mutant RNAs in vitro (Figure 6A).

As with the BIV Tat-TAR interaction, the lower U8 bulge is essential for binding, but its identity is not. Interestingly, shifting U8 up the stem by one bp (U8→U9), to the position equivalent to U10 in BIV TAR, enhanced binding by more than 2-fold, indicating that the BIV bulge configuration generates a better binding site, possibly due to formation of the base triple or to a more widened major groove in the region of peptide binding. This is consistent with the observed higher affinity of the JDV peptide for BIV TAR than for its own TAR ($K_{d,s} = 0.7$ nM and 3.3 nM, respectively; Figure 2). Unlike the BIV Tat-TAR interaction, the second bulge in JDV TAR also is essential for binding, as deleting both A11 and U12 abolished binding. In the case of the upper bulge, a single nucleotide bulge is sufficient for binding; and, as with the lower bulge, the identity of the nucleotide is not important (see mutants A11U, A11 Δ , and U12 Δ). Interestingly, the JDV LTR contains a second hairpin adjacent to JDV TAR with two single nucleotide bulges in the same locations as in the first hairpin (Figure 6A), perhaps suggesting a second JDV Tat binding site (see Discussion). It seems likely that the requirement for the second upper bulge results from the suboptimal location of the lower bulge. It also is interesting that the JDV peptide binds relatively well to the A13 Δ mutant, where A11 presumably is forced to pair with U23, thereby shifting the U12 bulge one bp further up the stem. The importance of the second bulge is further supported by the observation that deleting both A11 and A13, which presumably replaces the A13:U23 bp with a U:U mispair and also removes the bulge, eliminates binding.

C-Terminal Residues in the JDV Domain Enhance RNA Binding Affinity

As mentioned earlier, the JDV peptide was observed to bind BIV TAR with about 2-fold higher affinity than the BIV peptide ($K_{d,s} = 0.7$ nM and 1.3 nM, respectively; Figure 2). Similarly, the JDV peptide also bound JDV TAR with 4- to 5-fold higher affinity than the BIV peptide ($K_{d,s} = 3.3$ nM and 15 nM, respectively; Figure 5). We suspected that the enhanced affinity of the JDV peptide might result from the three additional basic residues located at its N terminus (Figure 6B). To identify the amino acids that enhance binding affinity, we first synthesized two hybrid peptides containing the conserved "core" sequence with either the N-terminal residues of the JDV domain and C-terminal residues of the BIV domain (J/B Tat) or the converse arrangement (B/J Tat) (Figure 6B); and measured their affinities for JDV and BIV TARs *in vitro*. Surprisingly, changing the C-terminal residues of the JDV domain to BIV residues markedly reduced affinity for both RNAs (Figure 6B). Having the N-terminal residues of JDV together with the C-terminal residues of BIV actually decreased affinity even further (Figure 6B). Thus amino acids at the C terminus of JDV are responsible for the enhanced affinity, while the additional basic residues at the N terminus can even be detrimental.

To determine which particular C-terminal amino acids in the JDV domain enhance RNA binding affinity, we measured the affinities of three additional peptides in which Lys78, His80, or Tyr81 were replaced by corresponding arginines from the BIV domain. Substitution

of tyrosine markedly reduced affinity, substitution of histidine had a modest effect, and substitution of lysine had a relatively small effect (Figure 6B). Thus, the C-terminal tyrosine and histidine residues of JDV contribute to RNA binding affinity, perhaps by making additional RNA contacts not present in the BIV complex or stabilizing the peptide structure.

NMR Spectroscopy of the JDV Peptide Bound to BIV TAR

To confirm directly that the JDV domain adopts a β -hairpin conformation in the context of BIV TAR, we acquired NMR spectra of the peptide-RNA complex and searched for nuclear Overhauser effects (NOEs) indicative of the β -hairpin structure. In the BIV Tat-TAR complex, several NOEs are observed between side chain protons of Thr72 and Ile79 located in close proximity across the two strands of the hairpin (Figure 7A; Puglisi et al., 1995; Ye et al., 1995). We observed a similar set of NOEs for the JDV peptide bound to BIV TAR, with slightly displaced chemical shifts due to sequence differences between the two peptides (Figure 7A). In addition, the two complexes displayed a similar set of NOEs between side chain protons of Ile79 and protons on the bulged U10 base (Figure 7A), further suggesting that the two peptides bind to BIV TAR in similar ways. Although the spectra of the JDV Tat-BIV TAR complex are only partially assigned, we also found several strong NOEs between Tyr81 and Ile79 protons (Figure 7B), indicating that the C-terminal tyrosine is well ordered, unlike the corresponding Arg81 in the BIV peptide complex (Puglisi et al., 1995; Ye et al., 1995). The close proximity of Tyr81, Ile79, and the bulged U10 base in the JDV peptide-BIV TAR complex suggests that additional hydrophobic and/or stacking interactions of the tyrosine may, in part, account for the enhanced affinity of the JDV peptide described above.

Discussion

Chameleon-Like Properties of the JDV Tat Domain

Here we have shown that a single arginine-rich RNA binding domain, found in JDV Tat, can specifically recognize four different RNA sites using two different binding strategies. The mutagenesis and NMR data provide strong evidence that the JDV domain binds to BIV and JDV TARs in a β -hairpin conformation, while it binds to HIV TAR in a different, extended conformation together with cyclin T1. The structures of the BIV Tat peptide bound to BIV TAR and of HIV Tat peptides, or arginamide, bound to HIV TAR, have been solved by NMR (Figure 1C), and in both cases the RNAs adopt very similar tertiary structures (Smith et al., 1998). In the HIV complex, the peptide does not adopt a discrete conformation and is thought to be relatively extended, although the structure of the intact HIV Tat protein complexed to cyclin T1 and TAR remains to be determined. Nevertheless, we can reasonably infer that JDV peptide binding to BIV and HIV TARs involves distinct peptide conformations, because different arginines of JDV Tat are used to bind analogous guanines in the two TARs (Figure 1C). G11 of BIV TAR and G26 of HIV TAR occupy similar positions in their respective secondary (Figure 1A) and

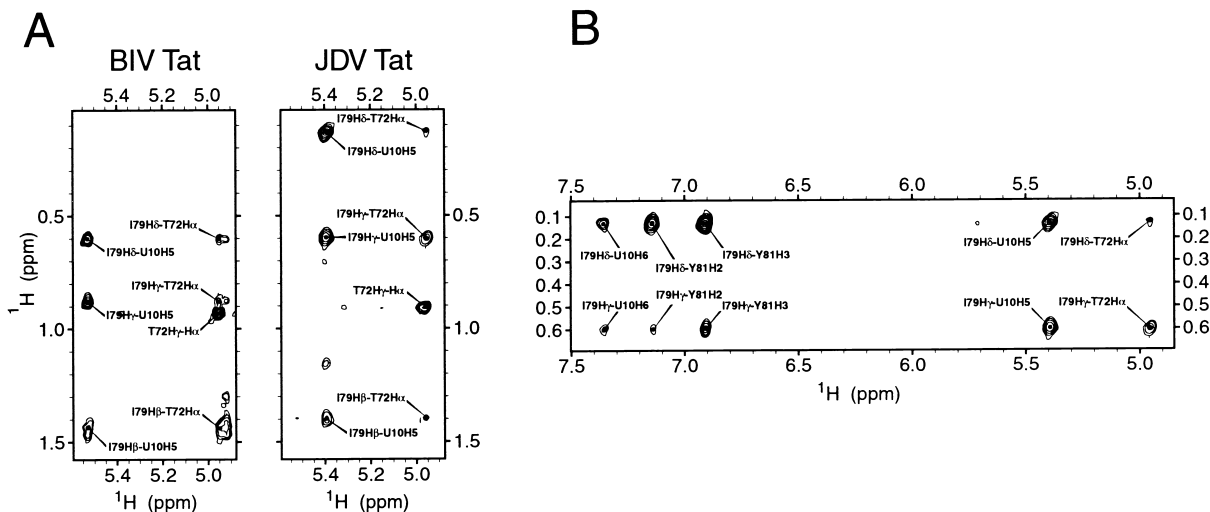


Figure 7. NMR Spectra of the JDV Tat Peptide-BIV TAR Complex

(A) A region of the 2D ^1H - ^1H NOESY spectrum of the BIV Tat-peptide-TAR complex (left; Puglisi et al., 1995) compared to the same region of the JDV Tat peptide complex (right). NOEs between Ile79 and U10 protons and between Ile79 and Thr72 protons are indicated. An identical NOE between Thr72 γ and H α in the two complexes also is indicated. The NOESY spectrum of the JDV peptide complex was collected using a 300 ms mixing time at 15°C and the spectrum of the BIV peptide complex was collected using a 400 ms mixing time at 25°C (Puglisi et al., 1995). Assignments for some of the JDV cross-peaks were aided by the previous BIV assignments (Puglisi et al., 1995). (B) Another region of the same NOESY spectrum of the JDV complex showing additional NOEs between Ile79 and Tyr81 protons, and between Ile79 and U10 protons.

tertiary (Figure 1C) structures, and are contacted by Arg73 of BIV Tat and Arg52 of HIV Tat (Puglisi et al., 1992; Aboul-ela et al., 1995; Puglisi et al., 1995; Ye et al., 1995; Long and Crothers, 1999). Assuming that the analogous arginines identified in JDV domain make similar contacts, we infer that Arg73 contacts G11 in BIV TAR, whereas Arg70 contacts G26 in HIV TAR. Thus, the JDV peptide binds in entirely different registers to these two RNAs. In binding to its own TAR site, the JDV peptide requires the same three arginines as for binding to BIV TAR, strongly suggesting that similar contacts are made. However differences in the TAR bulge architectures and the involvement of additional amino acids at the JDV C terminus suggest that the structures of the JDV and BIV complexes will differ in structural detail. The “chameleon-like” behavior of the JDV Tat domain is further highlighted by the observation that it can recognize a single, hybrid HIV/BIV TAR in either binding mode, depending on the availability of human cyclin T1.

The arrangement of amino acids in the JDV domain shows how a diverse set of RNA contacts can be contained within a short stretch of polypeptide, and can be used to recognize multiple RNA elements. Amino acids 70–79 comprise the core of the β -hairpin and contain all of the residues essential for BIV TAR binding (Figures 1B and 1C). Recognition of JDV TAR likely requires the same core amino acids and also appears to use C-terminal tyrosine and histidine residues for binding (Figure 6B). These aromatic residues also enhance affinity for BIV TAR, and conceivably might form additional stacking or hydrogen bonding interactions not seen in the wild-type BIV complex, or might help stabilize the peptide β -hairpin structure. Recognition of HIV TAR requires one arginine residue flanked on each side by three basic amino acids, with the arginine optimally located at

position 52 or 53 in the full-length protein (Figure 1B; Calnan et al., 1991; Tao and Frankel, 1993). We presume that the positioning requirement reflects a discrete spatial orientation between Tat, cyclin T1, and the bulge and loop of TAR in the ternary complex. In the JDV domain, Arg70 provides the critical arginine for HIV TAR recognition, and corresponds to position 54 of HIV Tat (Figure 1B). It is interesting that placing arginine at JDV position 69, corresponding to position 53 in HIV Tat, gives much lower activity (Figure 3), despite the fact that position 53 in HIV Tat is much preferred (Calnan et al., 1991). Thus, the sequence requirements for recognition of HIV TAR, in the context of the cyclin T1 ternary complex, appear to be even more flexible than previously thought. The additional basic residues at the JDV N terminus relative to BIV Tat (Figure 1B) presumably provide the flanking charged side chains required for HIV TAR recognition, and explain why the JDV, but not the BIV, domain can be used to bind HIV TAR. A more detailed understanding of how JDV Tat recognizes HIV TAR, including the conformation of the arginine-rich domain, will require high-resolution structures of Tat-cyclin T1-TAR ternary complexes. Given that the JDV Tat domain can recognize several different TAR sites, it is interesting to speculate that other TAR-like sites might exist in cellular mRNAs and might be additional targets for Tat-mediated regulation.

Evolution of RNA Binding Specificity

The Tat proteins from HIV-1, HIV-2, SIV, BIV, and JDV all contain arginine-rich RNA binding domains, highly related TAR elements, and highly conserved activation domains that mediate interactions with cyclin T1 (Wei et al., 1998; Bieniasz et al., 1999; Barboric et al., 2000; Bogerd et al., 2000). While interactions between Tat and

cyclin T1 appear to be important for transcriptional activation in all cases, the cyclin also enhances the RNA binding affinities of the HIV-1, HIV-2, and SIV Tat proteins by mediating cooperative interactions to their respective TAR bulges and loops. In contrast, the BIV and JDV Tat proteins bind to their TAR sites with high affinities and specificities in the absence of the cyclin (Chen and Frankel, 1994; Barboric et al., 2000; Bogerd et al., 2000). The JDV arginine-rich domain is especially notable in that it can use both binding strategies, adopting different conformations in each case. The multifunctional behavior of the JDV domain, as well as the previous finding of a bifunctional TAR that can be recognized in both binding modes (Smith et al., 1998), suggests plausible mutational routes in which stepwise changes in the protein or RNA can lead to the evolution of different binding specificities or modes of recognition without necessarily generating loss-of-function intermediates. Rapidly evolving viruses, such as JDV, BIV, and HIV, may be particularly apt to generate multifunctional intermediates, which can even involve different requirements for host proteins such as cyclin T1.

It is interesting that the highest affinity Tat-TAR interactions were not necessarily those between the cognate partners, and, indeed, no cognate interaction was the strongest. In particular, the JDV peptide bound BIV TAR with severalfold higher affinity than the BIV Tat-BIV TAR or JDV Tat-JDV TAR interactions, and also bound both BIV TAR and HIV TAR with higher affinity than the cognate domains *in vivo*, as monitored by transcriptional activation. These results suggest that the natural interactions have not necessarily evolved to bind as tightly as possible, and that improvements in affinity can occur with relatively minor mutations in either the protein or RNA element. For example, positioning the U8 bulge in JDV TAR one bp further up the stem creates a BIV-like bulge configuration and enhances binding affinity. SIV and HIV-2 Tat-TAR interactions also display suboptimal binding, and it is interesting that SIV, HIV-2, JDV, and BIV all contain two adjacent TAR elements (Carpenter et al., 1993; Tao and Frankel, 1993; Tong-Starksen et al., 1993; Fong et al., 1997; Bieniasz et al., 1999; Chen et al., 1999b). In these cases it seems possible that the viruses utilize two Tat binding sites, each having suboptimal affinity, as a means to achieve sufficiently high RNA binding affinity. Given these observations, it is possible to envision several strategies for evolving new RNA-protein interactions in which functional binding intermediates are maintained: new RNA binding sites may evolve from a duplicated element, as in SIV, HIV-2, BIV, or JDV, or from a multifunctional RNA, such as the hybrid BIV/HIV TAR; new protein specificities may evolve from a gene duplication, from a single domain of a multidomain protein, or from a single multifunctional domain, as in JDV Tat.

The potential benefits of structural plasticity for evolving new folds or functions have been described in other cases. A ribozyme recently was found that can adopt two different conformations and catalyze two separate reactions, albeit with low activities; and mutational pathways were defined in which both activities could be evolved from the other without generating loss-of-function intermediates (Schultes and Bartel, 2000). In addition to allowing the evolution of new ribozyme activities,

the ability of RNAs to adopt alternative structures, each of which can be relatively resistant to mutation, may facilitate the evolution of new RNA-protein interactions by allowing recognition of one of several folded states. It also is known that peptides placed in different structural frameworks can adopt different conformations (Landry et al., 1992; Minor and Kim, 1996; Ye et al., 1999; Frankel, 2000) and small changes in sequence can be sufficient to cause peptides to fold into very different structures with relatively low energy barriers between folded and unfolded states (Cordes et al., 1999). Thus, the flexibility inherent to peptide-RNA interactions and the arginine-rich motif seems to provide an ideal context in which to evolve new RNA binding specificities. Indeed, it has been possible to isolate and evolve peptides from relatively simple combinatorial libraries that recognize the HIV Rev response element RNA in different binding modes (Harada et al., 1997; Tan and Frankel, 1998). Finally, it is tempting to speculate that RNA-protein complexes that undergo conformational rearrangements, such as those assembled during pre-mRNA splicing, might utilize peptides that fold differentially as a means to allow the same protein to switch between different RNA binding sites or undergo conformational changes while bound to the same site as needed.

Experimental Procedures

Peptide and RNA Synthesis and Purification

Tat peptides (residues 65–81 from JDV or BIV Tat and residues 49–57 from HIV Tat) were synthesized on an Applied Biosystems Model 432A peptide synthesizer using Fmoc chemistry and standard Applied Biosystems resin (25 μ mol) and protecting groups. The N-termini were acetylated using acetic acid (75 μ mol) with HBTU activation. Following cleavage and deprotection in the presence of reagent R, peptides were purified on a C4 reverse-phase high-performance liquid chromatography column (Vydac) using an acetonitrile gradient of 0.2%/min in 0.1% trifluoroacetic acid. The identity of the peptides was confirmed by laser desorption mass spectrometry and peptide concentrations were determined by quantitative amino acid analysis (University of Michigan Protein and Carbohydrate Structure Facility).

TAR RNAs were transcribed by T7 RNA polymerase using synthetic oligonucleotide templates (Milligan and Uhlenbeck, 1989). All RNA hairpins contained an additional GG at their 5' ends, which increases the efficiency of transcription, and CC at their 3' ends to base pair with the Gs. For randomly labeled RNAs, [α - 32 P]CTP (3,000 Ci/mmol) was included in the transcription reaction. RNAs were purified on 15% polyacrylamide/8 M urea gels, eluted from the gels in 0.6 M NaOAc (pH 6.0), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.01% sodium dodecyl sulfate, and ethanol precipitated twice. Purified RNA was resuspended in sterile deionized water. The concentrations of radiolabeled RNAs were determined from the specific activity of the [32 P]CTP incorporated into the transcripts. RNAs were renatured by incubation in renaturation buffer (20 mM Tris-HCl [pH 7.5], and 100 mM NaCl) for 2 min at 85°C, followed by slow cooling to room temperature.

RNA Binding Gel Shift Assays

Gel shift assays were performed at 4°C. Peptide and RNA were incubated together for 10–30 min on ice in 10 μ l binding reaction mixtures containing 10 mM HEPES-KOH (pH 7.5), 100 mM KCl, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 50 μ g/ml *E. coli* tRNA as a competitor. To determine relative binding affinities, 0.2 nM radiolabeled TAR RNA was titrated with peptide. Peptide-RNA complexes were resolved on 10% polyacrylamide/0.5X Tris-borate-EDTA gels prerun for 1 h at 4°C. Gels were electrophoresed at 200 V for 3–4 hr at 4°C, dried, and autoradiographed. Two methods were used to estimate binding constants. In the first,

the fraction of bound RNA was determined by measuring the disappearance of the unbound band; and in the second, bound bands were directly quantitated (D. Campisi, V. C., and A. D. F., submitted). In both cases, binding curves were fit to the data using Kaleidagraph software (Synergy Software, Reading, PA), and the two methods produced similar K_d values.

Plasmids, Transient Transfection, and CAT Assays

The TAR reporter plasmids were constructed by cloning synthetic oligonucleotide cassettes into an HIV LTR-CAT reporter plasmid constructed from pU3R-III CAT (Rosen et al., 1985). The sequence between +1 and the AUG of CAT was modified to place restriction sites on both sides of the TAR site (Smith et al., 1998). All TARs contained identical lower stems cloned into the TAR cassette. Tat fusion proteins were expressed from pcDNA3-derived vectors (S. G. Landt and A. D. F., unpublished results). Reporter plasmids and Tat plasmids were transfected into HeLa or NIH 3T3 cells, and CAT activities were assayed after 44 hr. For HeLa cell transfections, 100 ng of reporter plasmid and 50 ng Tat-expressing plasmid were cotransfected with carrier plasmid (2 μ g total DNA) using 5 μ l Lipofectin (Life Technologies) in 3.8 cm² wells for 4 hr. For NIH 3T3 cell transfections, 100 ng reporter plasmid and 50 ng Tat-expressing plasmid were cotransfected with carrier plasmid (1 μ g total DNA) using 4 μ l Lipofectamine for 5 hr. For some experiments in 3T3 cells, 100 ng of a plasmid expressing human cyclin T1 (residues 1–272) (Wei et al., 1998) was also transfected. Cell extracts were assayed for CAT activity as described (Chen and Frankel, 1994).

NMR Spectroscopy

The 28-nucleotide BIV TAR RNA was synthesized using large-scale T7 *in vitro* transcription reactions and purified by electrophoresis on 20% (w/v) polyacrylamide gels, electroelution, and ethanol precipitation. Samples were dialyzed for 48 hr, first against 10 mM sodium phosphate buffer (pH 6.5), 100 mM NaCl, 0.1 mM EDTA; then against 10 mM sodium phosphate buffer (pH 6.5), 50 mM NaCl; and finally against water. The RNA concentration was adjusted to 2 mM in 10 mM sodium phosphate buffer (pH 6.5), 50 mM NaCl and was renatured by heating to 85°C for 10 min, followed by slow cooling to room temperature. A 1:1 peptide:RNA complex was formed by incrementally adding JDV Tat peptide to the RNA until no further changes were observed in the imino proton spectrum. For NOESY spectroscopy experiments in D₂O, the peptide-RNA complex was repeatedly lyophilized and resuspended in D₂O-containing buffer. Spectra were acquired at 600 MHz on a Varian spectrometer, processed with NMRPipe (Delaglio et al., 1995), and analyzed with SPARKY (Goddard and Kneller, 1998). Partial assignments for the JDV Tat peptide-BIV TAR complex were made in part based on previous assignments of the BIV peptide complex (Puglisi et al., 1995).

Acknowledgments

We thank Cynthia Honchell, Ralph Peteranderl, Damian McColl, and Steve Landt for help with oligonucleotide and peptide synthesis and characterization; Kathy Jones for cyclin T1 expression vectors; members of the Frankel laboratory for helpful discussions; and Raul Andino, Peter Walter, Donna Campisi, Alan Cheng, Chandreyee Das, Steve Landt, Rob Nakamura, Hadas Peled-Zehavi, and Ralph Peteranderl for comments on the manuscript. This work was supported by an American Cancer Society postdoctoral fellowship (to C. A. S.) and by grants from the National Institutes of Health.

Received July 25, 2000; revised September 11, 2000.

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