RNA Binding Assays for Tat-Derived Peptides: Implications for Specificity†

Kevin M. Weeks‡ and Donald M. Crothers*

Department of Chemistry, Yale University, New Haven, Connecticut 06511

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ABSTRACT: RNA recognition by the HIV Tat protein is mediated in part by an arginine- and lysine-rich basic subdomain implicated as a signature element in proteins that bind RNA. Relative RNA binding affinities for a 14-residue peptide derived from Tat that spans the basic region are determined using a competition protocol. Binding specificity is compared with complexation by a 38-residue model for the RNA binding domain of Tat using the same approach. Binding strength for the minimal (14 residue) peptide is correlated with that for the longer peptide: both peptides recognize a short, bulged duplex. However, the shorter peptide dissociates more rapidly from the wild-type site and discriminates less well between nonspecific (double-stranded RNA) and specific sites. Relative dissociation constants for 38-residue peptide determined from direct partition and competition assays differ; the former assay consistently predicts stronger discrimination against RNAs with mutations in the stems flanking the bulge. Differences between the two assays are reconciled in terms of contributions from labile binding which is unstable to native gel electrophoresis. Kinetic stability may constitute a major specificity determinant for basic subdomain-mediated recognition of RNA.

Transactivation of HIV gene expression by the Tat protein follows complex formation with a specific bulged RNA hairpin loop (termed TAR) encoded by the 5′ viral long terminal repeat (Muesing et al., 1987; Feng & Holland, 1988; Berkhout et al., 1989). Transactivation requires at least two activities from Tat: a targeting function (Southgate et al., 1990; Selby & Peterlin, 1990) to direct the protein to its RNA binding site and an activating domain which interacts with the cellular transcriptional machinery [e.g., see Marciniak et al. (1990b), Jeyapaul et al. (1990), and Southgate and Green (1991) and references cited therein]. The Tat RNA-targeting domain includes an arginine- and lysine-rich subdomain found in many RNA binding proteins (Lazinski et al., 1989). Indeed, while biochemical characterization of recombinant Tat protein has proven challenging due to the protein's propensity to aggregate and form nonnative disulfide linkages, short peptides containing the basic subdomain bind TAR RNA.

RNA sequence and structural requirements determined from in vitro binding studies employing Tat-derived peptides containing the basic domain (Weeks et al., 1990; Roy et al., 1990b; Cordingly et al., 1990; Calnan et al., 1991a; Weeks & Crothers, 1991), generalized basic peptides (Calnan et al., 1991b; Delling et al., 1991), or the basic amino acid arginine (Tao & Frankel, 1992; Puglisi et al., 1992) correlate remarkably well with a characteristic subset of the requirements for Tat responsiveness in vivo (Jakobovits et al., 1988; Feng & Holland, 1988; Dingwall et al., 1989, 1990; Roy et al., 1990a,b; Berkhout & Jeang, 1991). Mutations in the loop in TAR have no or modest affects on Tat peptide/protein binding but do modulate transactivation in vivo, implying a role for additional cellular factors (Gatinol et al., 1989, 1991; Gaynor et al., 1989; Marciniak et al., 1990a; Wu et al., 1991; Sheline et al., 1991). Peptide- and arginine-mediated RNA interactions mimic binding by partially active preparations of recombinant Tat protein (Dingwall et al., 1989; Roy et al., 1990a; Cordingly et al., 1990; Sumner-Smith et al., 1991) and relatively highly active refolded protein (Dingwall et al., 1990; Harper & Logsdon, 1991).

Popular methods for determining relative binding affinities for protein/peptide–nucleic acid interactions may be formally divided into two nonequivalent approaches: partition and competition.

In a partition experiment (Fried & Crothers, 1981; Dalma-Weiszhausz et al., 1990; Weeks & Crothers, 1991), one observes the equilibrium:

$$R_1P + R_2 \rightleftarrows R_1 + R_2P \qquad (1)$$

where R_1 and R_2 are the two nucleic acid (RNA) species of interest and P is protein; R_xP represents an RNA–protein complex. If the ratios of free to complexed RNA can be determined directly for each RNA and protein is limiting, the apparent relative dissociation constant is simply

$$K_{rel}^{part} = \frac{[R_1][R_2P]}{[R_1P][R_2]} \qquad (2)$$

Only complexes stable to physical partitioning contribute to apparent binding. Direct determination of absolute binding constants by titration using nonequilibrium methods (including gel electrophoresis and filter binding) also introduces a bias against interactions not stable to complex isolation.

In the more commonly employed competition–titration protocol for determination of relative binding affinities, the fractional binding saturation of only one (radiolabeled) nucleic acid species is followed as a function of unlabeled competitor concentration. Complexes with competitor do not have to be stable to physical separation to be scored.

Transactivation must reflect the biophysical consequences of protein/peptide–RNA interaction including binding strength, discrimination among folded RNA structures, the lifetime of the activation-competent complex, and interactions with other factors. Relative dissociation constants evaluated by a direct partition assay and dissociation rates determined for a comprehensive set of TAR sequence variants for complexation with a 38 amino acid carboxyl-terminal fragment of Tat, Tfr38, are consistent with the model for the RNA binding site shown in Figure 1 (Weeks & Crothers, 1991).

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‡ Present address: Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309-0215.

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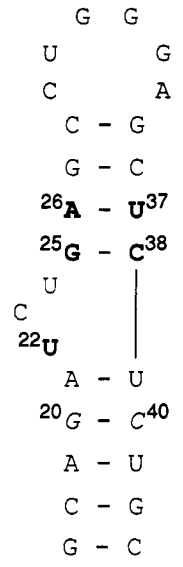

FIGURE 1: ΔTAR RNA. Strong requirements for specific binding by Tfr38 determined by the partition method (Weeks & Crothers, 1991) are indicated by boldface type; modest determinants for specific binding are italicized. Other positions in the stem and loop may be varied as long as base pairing in duplex regions is maintained; specific interaction requires a bulge loop of at least two nucleotides. ΔTAR spans the minimal structures required for Tat responsiveness in vivo (Jakobovits et al., 1988) and peptide binding in vitro (Weeks et al., 1990). Because ΔTAR contains a single bulge, only one complex with the Tat-derived peptides is resolved by native gel electrophoresis.

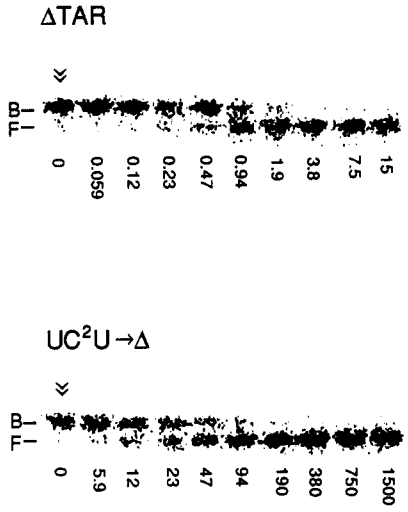

FIGURE 2: Competition by ΔTAR and UC[23]U→Δ for Tfr14. Digitized image shows a native gel in which a Tfr14-radiolabeled ΔTAR complex is dissociated by competition with unlabeled ΔTAR or a bulgeless variant. Positions of the bound (B) and free (F) radiolabeled probe are indicated. Competitor concentrations (in nanomolar) are shown for each lane; arrowheads indicate lanes with no competitor RNA in which 85% of the input radiolabeled probe is bound.

The partition assay requires physical separation of bound and free RNAs by native gel electrophoresis. This assay could not be performed with a minimal (**GRKKRRQRRR**PPQG, basic residues in boldface) peptide, because the relative shift of the bound RNA is very small (Figure 2). Here, relative dissociation constants are determined for this 14-residue peptide (Tfr14) and for Tfr38 by competition: RNA binding specificity for the 2 peptides is correlated. However, specific RNA complexes with Tfr14 have a shorter lifetime than complexes with Tfr38, and the minimal peptide discriminates poorly against double-stranded RNA.

Comparison of relative dissociation constants for Tfr38 determined by partition and competition protocols indicates that the apparent specificity is larger when assayed by the former method for RNAs with mutations in base-paired positions flanking the bulge. The native bulge thus appears to mediate labile interaction which is not stable to native gel electrophoresis. This nonspecific binding is approximately one-fourth as strong as the gel-stable component.

EXPERIMENTAL PROCEDURES

Oligoribonucleotides and Peptides. RNAs were synthesized enzymatically (Milligan et al., 1987) using templates described previously (Weeks & Crothers, 1991). Thus, all RNAs except ΔTAR and the UC[23]U→U and →Δ variants contained a 3′-A_4 tail. Concentrations were determined by assuming an extinction coefficient at 260 nm (ϵ_{260}) of 9.5 mM^{-1} cm^{-1} $residue^{-1}$. This represents an average of the calculated (Fasman, 1975) ϵ_{260} for ΔTAR (Figure 1) with and without an A_4 tail and varies only slightly for different RNAs due to the substantial similarity between sequences. Peptides were synthesized chemically using standard protocols (Weeks & Crothers, 1991).

Equilibrium Dissociation Constants. Binding reactions and native gel mobility shift assays were performed as described below under Competition Assay and by Weeks and Crothers (1991). Absolute apparent dissociation constants (K_d) for Tfr38 and Tfr14 were determined from the fraction of radiolabeled RNA (at 60 pM) bound (θ) as a function of free peptide concentration, [P], from the relationship

$$\theta = \frac{[P]}{[P] + K_d}$$

Dissociation constants for complexes with ΔTAR determined in this way varied from 0.12 to 0.06 nM for both Tfr14 and Tfr38. Competition data were fit using K_d = 0.08 nM for both peptides. The dependence of the absolute dissociation constant on ionic strength was evaluated by performing the binding assay under the standard protocol, but varying the NaCl concentration in the incubation buffer.

Competition Assay. Initially, peptide was titrated against radiolabeled ΔTAR RNA at 60 pM [in 10 mM Tris (pH 7.5), 70 mM NaCl, 0.2 mM EDTA, 5% glycerol, and 0.01% NP40, at 22 °C] to establish a concentration at which 85 ± 3% of the RNA was bound when subjected to native gel electrophoresis [10% w/v acrylamide and 75:1 w/w acrylamide/bis(acrylamide) in 45 mM Tris–borate/1 mM EDTA, 20 °C] for 45 min at 25 V/cm; these conditions were then used for all subsequent competition assays for a given peptide. Competition experiments were performed in 15-μL volumes and included increasing concentrations of serially diluted unlabeled competitor RNA. Peptide (final concentration, 0.65 nM) was added to reactions containing both competitor RNA and radiolabeled probe, and the solutions were incubated 20 min at 22 °C before being loaded onto a running native gel. Gels were dried, and bands corresponding to bound and free radiolabeled probe were quantified directly using a Betascope blot analyzer (Betagan Co., Waltham, MA). From the mass-action equations describing competitive binding of two ligands to a protein/peptide, the fractional saturation (θ) of the radiolabeled species is (Lin & Riggs, 1972)

$$\theta = \frac{P_t(1-\theta)}{K_T(1 + C_t/K_C) + T_t(1-\theta)} \quad (3)$$

where P_t, T_t, C_t, K_T, and K_C are the total concentration of peptide, radiolabeled ΔTAR RNA, and unlabeled competitor RNA, and dissociation constants for ΔTAR– and competitor–peptide complexes, respectively. Solving this quadratic

expression in θ explicitly gives the physically realizable root:

$$\theta = \frac{1}{2T_t}\left[K_T + \frac{K_T}{K_C}C_t + P_t + T_t - \sqrt{\left(K_T + \frac{K_T}{K_C}C_t + P_t + T_t\right)^2 - 4T_tP_t}\right] \quad (4)$$

A competition curve [$\theta = f(C_t)$] may be fit for the best value of K_C using any nonlinear least-squares routine. The apparent relative dissociation constant, K_{rel}, is simply the ratio of competitor to wild-type dissociation constants:

$$K_{rel}^{comp} = K_C/K_T \quad (5)$$

By this definition, a larger K_{rel} implies weaker mutant binding. In practice, P_t was varied ±30% for competition of unlabeled ΔTAR versus labeled probe to achieve a good fit at no added competitor; this value was then used to fit all mutant competition curves for a series of experiments. Competition by wild type for Tfr14- and Tfr38-radiolabeled RNA complexes yielded dissociation constants of 0.12 and 0.084 nM, respectively, in good agreement with the value determined from direct titration ($K_d \approx 0.08$ nM, see above). Absolute magnitudes for K_C determined from the equations given above are sensitive to the values of K_T and P_t; however, because it is the ratio K_C/K_T that determines the shape of the competition curve [see also Lin and Riggs (1972)], K_{rel} determined from eq 5 is relatively insensitive to variation in the parameters K_T, P_t, and T_t.

Partition Assay and Kinetics. All methods and data for the partition and kinetic assays performed with the peptide Tfr38 have been described (Weeks & Crothers, 1991); K_{rel} is given by eq 2. The dissociation rate, k_{off}, for Tfr14 was obtained by challenging a preformed peptide–RNA complex with a final concentration of 5 nM unlabeled ΔTAR and loading aliquots onto a running native gel.

RESULTS

Determination of Relative Binding Constants by Competition. Sequence and structural requirements for high-affinity binding by the peptide Tfr38 to ΔTAR RNA are summarized in Figure 1. This model was derived from pairwise competition of wild-type and mutant RNAs in a direct partition assay, which requires physical separation of bound and free RNA for both probes (Weeks & Crothers, 1991). Tfr14–RNA complexes are barely resolved from free RNA in native gels (Figure 2), preventing analysis of binding specificity by this assay. To analyze the sequence specificity of Tfr14 (and Tfr38), competition assays were performed with these peptides and with the most instructive mutants from the previous study as shown for wild type and a $UC^{23}U \rightarrow \Delta$ (bulgeless) variant in Figure 2.

Competition by representative mutants with wild-type ΔTAR RNA–peptide complexes is illustrated in Figure 3. As expected, the wild-type sequence competes efficiently for binding with the radiolabeled probe. The bulgeless hairpin loop ($UC^{23}U \rightarrow \Delta$) competes poorly, and substitution at the critical U^{22} ($\rightarrow C^{22}$) position decreases binding, as do changes which alter previously identified stem base pairs (A^{26}-$U^{37} \rightarrow$ U-A) whose integrity was shown to be essential for high-affinity binding. Relative dissociation constants (K_{rel}^{comp}) determined by competition are summarized in Table I for both Tfr14 and Tfr38. Larger K_{rel}^{comp} implies weaker mutant binding. Discrimination against RNA structural variants by minimal RNA binding peptides determined here corresponds closely with other quantitative results reported for refolded

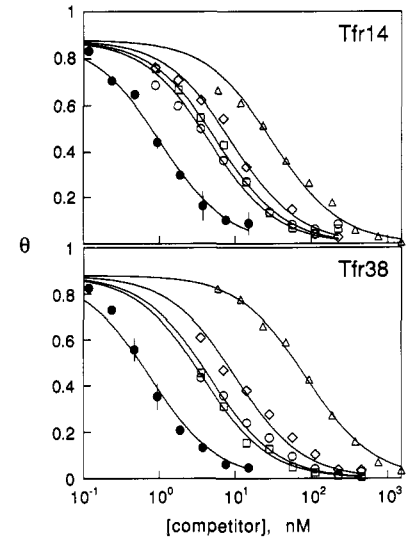

FIGURE 3: Competition titrations for Tfr14 and Tfr38. The fraction of radiolabeled probe RNA bound by peptide (θ) is plotted as a function of unlabeled competitor concentration for the sequence variants $UC^{23}U \rightarrow \Delta$ (bulgeless, △), $UC^{23}U \rightarrow U$ (□), $U^{22} \rightarrow C$ (◇), and A^{26}–$U^{37} \rightarrow$ U-A (○). Error bars shown for competition by wild-type RNA (●) correspond to the standard deviation from two independent experiments. Competition curves (solid lines) are a best fit to eq 4 (see Experimental Procedures).

Table I: Relative RNA Binding Affinities for Tfr14 and Tfr38 Determined by Competition[a]

	RNA	$K_{rel}^{comp:Tfr14}$	$K_{rel}^{comp:Tfr38}$	$K_{rel}^{Tfr38}/K_{rel}^{Tfr14}$ [b]
	ΔTAR	1.0	1.0	1.0
bulge	$UC^{23}U \rightarrow \Delta$	30	120	**4.0**
	$UC^{23}U \rightarrow U$	5.7	4.8	**0.84**
	$UC^{23}U \rightarrow UU$	2.6	1.2	0.46
	$UC^{23}U \rightarrow UUUU$	0.66	0.46	0.70
	$U^{24} \rightarrow G$	0.62	2.1	3.4
	$C^{23} \rightarrow A$	2.4	1.5	0.62
	$U^{22} \rightarrow C$	7.3	14	**1.9**
	$U^{22} \rightarrow A$	8.2	12	**1.5**
stem	A^{26}–$U^{37} \rightarrow$ G-C	3.8	3.0	**0.79**
	A^{26}–$U^{37} \rightarrow$ C-G	2.3	4.9	**2.1**
	A^{26}–$U^{37} \rightarrow$ U-A	4.5	5.9	**1.3**
	G^{25}–$C^{38} \rightarrow$ C-G	2.9	3.8	**1.3**
	G^{25}–$C^{38} \rightarrow$ A-U	6.6	9.9	**1.5**
	G^{25}–$C^{38} \rightarrow$ U-A	4.8	3.3	**0.69**
	A^{2}–$U^{39} \rightarrow$ C-G	0.44	1.1	2.5
	G^{20}–$C^{40} \rightarrow$ C-G	1.1	0.60	0.55
	G^{20}–$C^{40} \rightarrow$ U-A	1.0	0.41	0.41
	A^{19}–$U^{41} \rightarrow$ C-G	0.37	0.42	1.1

[a] Relative binding constants are reproducible to ±50%. [b] Values corresponding to RNAs in which K_{rel} values for both Tfr14 and Tfr38 are greater than 2 (sequences that bind more weakly than wild type) are emphasized in boldface type.

Tat or Tat-derived peptides assayed by analogous competiton approaches (Dingwall et al., 1990; Cordingly et al., 1990).

Inspection of Table I indicates that both peptides bind least strongly to the bulgeless mutant and are sensitive to other disruptions either in the size of the bulge (~5-fold) or in the identity of U^{22} (~10-fold), elements whose importance is well established (Dingwall et al., 1990; Weeks et al., 1990; Roy et al., 1990b; Weeks & Crothers, 1991; Sumner-Smith et al., 1991). In addition, mutation of the two base pairs above the bulge to any other Watson–Crick pair weakens binding by both peptides 3–9-fold. In contrast, changes at positions 23 and 24 in the bulge or at base pairs A^{21}-U^{39} and A^{19}-U^{41} (Figure 1) have a modest or no effect on binding affinity ($K_{rel} \leq 2$, Table I), consistent with the model for structural

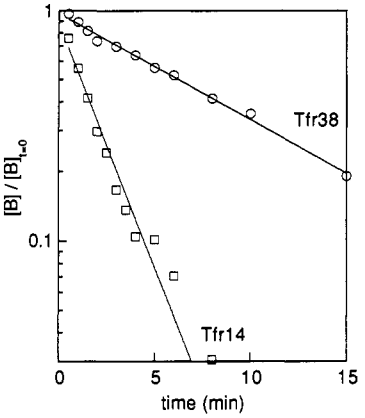

FIGURE 4: Dissociation rates (k_{off}) for Tfr14– and Tfr38–ΔTAR complexes. Pseudo-first-order dissociation of Tfr14 and Tfr38 complexes ([B]) with ΔTAR RNA was determined by challenging a preformed complex with 5 nM unlabeled RNA. k_{off} values for Tfr14– and Tfr38–ΔTAR complexes are 0.11 and 0.49 min^{-1}, respectively. Data for Tfr38 have been described previously (Weeks & Crothers, 1991).

requirements for peptide binding established by partition in Figure 1.

Correlation of RNA Binding by Tfr14 and Tfr38. Competition experiments are consistent with the trimodal binding motifs proposed for the Tfr38–RNA interaction on the basis of partition experiments (Weeks & Crothers, 1991). Both peptides interact specifically ($K_{rel} \approx 1$) with RNAs presenting the nucleotide constellation emphasized in boldface in Figure 1. Tfr14 and Tfr38 both bind with lower affinity (K_{rel} = 3–15) to many other bulged RNAs and bind nonspecifically to bulgeless RNA (K_{rel} is 30 and 120, respectively) (Table I).

Dissociation rates (k_{off}) for both peptides were determined under pseudo-first-order conditions. The half-life of the Tfr38–RNA complex is 6.3 min, while that for the Tfr14–RNA complex is 1.4 min, 4.5 times shorter (Figure 4). The two peptides have similar equilibrium dissociation constants (see Experimental Procedures), which implies that Tfr14 may bind the RNA more rapidly. Given values of 0.08 nM (see Experimental Procedures) and 0.11 min^{-1} (Figure 4) for the dissociation constant and rate, respectively, the association rate constant for the Tfr38–ΔTAR complex is approximately 2×10^7 M^{-1} s^{-1}. This value is consistent with observed and expected rates for protein–ligand and protein–nucleic acid association (Fersht, 1985). The circular dichroism signature of both isolated peptides is consistent with a predominant (random) coil conformation; specific RNA binding introduces a structural change in both peptides (C. Ampe and T. Steitz, personal communication). Folding of Tfr38 may be more extensive, requiring a longer (rate-limiting) organization time.

The ratios of relative dissociation constants from competition for Tfr14 versus Tfr38 provide one measure of the relative discrimination of the two peptides and are given in the final column of Table I. Relative dissociation constant ratios for mutants whose affinity is significantly weaker than that of ΔTAR are emphasized in boldface type. Due to the small differences observed and the modest precision of the competition-type assays, any obvious trend is obscured. However, Tfr38 may discriminate somewhat more strongly between wild-type and noncognate RNAs than Tfr14: 7 of the 10 boldface values in Table I are greater than 1.

The Specificity of the Tfr38–RNA Interaction Determined by Partition Is Larger than for Competition. Relative dissociation constants for Tfr38–RNA complexes have been determined by a direct partition assay and by competition and may be estimated as a ratio of dissocation rates using

Table II: Relative RNA Binding Affinities for Tfr38 Estimated from Partition, Competition, and Kinetic Methods

	RNA	*a* K_{rel}^{part}	*b* K_{rel}^{comp}	*c* $k_{off}^{mut}/k_{off}^{\Delta TAR}$	*d* $\sum/K_1^{\Delta TAR}$
	ΔTAR		1.0	1.0	
bulge	$UC^{23}U \rightarrow \Delta$	*e*	120	*e*	
	$UC^{23}U \rightarrow U$	8	4.8	≥18	
	$UC^{23}U \rightarrow UU$	0.6	1.2		
	$UC^{23}U \rightarrow UUUU$	1.6	0.46		
	$U^{24} \rightarrow G$	0.9	2.1		
	$C^{23} \rightarrow A$	1.1	1.5	2.3	
	$U^{22} \rightarrow C$	8	14	≥13	
	$U^{22} \rightarrow A$	16	12		
stem	A^{26}–$U^{37} \rightarrow$ G-C	7	3.0		0.29
	A^{26}–$U^{39} \rightarrow$ C-G	≥40	4.9	≥18	0.22
	A^{26}–$U^{37} \rightarrow$ U-A	18	5.9		0.14
	G^{25}–$C^{38} \rightarrow$ C-G	20	3.8		0.29
	G^{25}–$C^{38} \rightarrow$ A-U	15	9.9	≥10	0.04
	G^{25}–$C^{38} \rightarrow$ U-A	30	3.3	≥18	0.39
	A^{21}–$U^{39} \rightarrow$ C-G	0.48	1.1	1.3	
	G^{20}–$C^{40} \rightarrow$ C-G	4	0.60	9.0	
	G^{20}–$C^{40} \rightarrow$ U-A	2.9	0.41		
	A^{19}–$U^{41} \rightarrow$ C-G	0.62	0.42	1.2	

[a] Data are from Figure 3; Weeks & Crothers (1991). [b] Reproduced from Table I. [c] Dissociation rates are from Table II; Weeks & Crothers (1991). This calculation is provided for comparison with column *a* and yields a correct K_{rel} only if (unmeasured) values for k_{on} ($\sim 2 \times 10^7$ M^{-1} s^{-1}) are unchanged for the mutants. [d] $\sum/K_1^{\Delta TAR}$ is given for only those stem mutants for which it is well-defined: K_{rel}^{comp} is greater than 1.5 and less than K_{rel}^{part} (see text). [e] This mutant does not form a gel-resolvable complex with Tfr38; k_{off} is expected to be fast.

native gel electrophoresis. The assays give comparable results for RNAs in which the bulge is disrupted. In contrast, inspection of Table II (compare columns *a* and *b*) reveals that the partition assay consistently predicts stronger discrimination than does the competition assay for RNAs in which base pairing in the helices flanking the bulge is altered. The trend is general but is particularly striking at position G^{20}–C^{40}, where the small but reproducible decrease in binding strength observed for mutants by partition is completely obscured in the competition assay.

We have also estimated relative dissociation constants as a ratio of dissociation rates, relying on the untested assumption that association rates for Tfr38 with wild-type and mutant RNAs are similar. This approach has the advantage that it is free of potential contributions from dissociation of peptide–RNA complexes in the gel or during gel entry. These values are given in Table II (column *c*); relative binding energies determined from the partition assay and approximated from dissociation rates agree well and predict differences in binding strengths significantly greater than seen in the competition assay for *stem* mutants.

That the assays consistently yield different results implies they measure different phenomena. In the partition assay, only complexes thermodynamically and kinetically stable to native gel electrophoresis contribute to the partition equilibrium. In the competition assay, all complexes that occupy a (specific or nonspecific) site on the RNA contribute to observed binding.

The apparent binding constant (K_{app}) may be formally considered as the net contribution of multiple binding events (K_i):

$$K_{app} = \sum K_i$$

$$= K_1 + \sum\nolimits_2 K_i$$

where K_1 is operationally defined as the (sum of the)

contribution from gel-stable binding. [Here, binding (association) constants are used; elsewhere, the results are reported as dissociation constants (K_d's).] In the partition experiment, only the ratio of K_1's is observed:

$$K_{rel}^{part} = K_1^{\Delta TAR}/K_1^{mut} \quad (6)$$

the superscripts ΔTAR and mut identify wild-type and mutant primary binding events, respectively. However, in the competition experiment, all binding events count:

$$K_{rel}^{comp} = \frac{K_1^{\Delta TAR} + \sum}{K_1^{mut} + \sum} \quad (7)$$

where $\sum = \sum_2 K_i$ and it is assumed for simplicity that nonspecific binding is independent of the RNA substrate (i.e., $\sum \approx \sum^{\Delta TAR} \approx \sum^{mut}$). The assumption that labile interaction is independent of the RNA is made only for competition of ΔTAR with the stem mutants, all of which contain an intact bulge (see below). For large $\sum$, the relative dissociation constant determined by competition approaches 1, while if there is no significant contribution from non-gel-stable interaction ($\sum = 0$), eq 7 reduces to the expression for the partition assay (eq 6).

Solving eq 6 for K_1^{mut} and substituting this expression into eq 7 yield the relationship:

$$\frac{\sum}{K_1^{\Delta TAR}} = \frac{1 - K_{rel}^{comp}/K_{rel}^{part}}{K_{rel}^{comp} - 1} \quad (8)$$

defined for $K_{rel}^{comp} > 1$. The left-hand side of eq 8 is simply the net contribution of labile binding normalized to wild-type binding affinity.

Values for net non-gel-stable binding for stem mutants calculated from eq 8 are given in Table II (column *d*); the average $\sum/K_1^{\Delta TAR}$ for stem mutants is 0.23 ± 0.12. Nonspecific complexes of double-stranded (bulgeless) RNA compete for binding by Tfr14 and Tfr38 (Table I); however, complexes with this RNA are unstable to electrophoresis in native gels (Weeks & Crothers, 1991). Relative dissociation constants for RNAs with variations in the bulge generally agree within experimental error, suggesting no bias for this class of mutants is introduced by partitioning in native gels. Thus, the quality of non-gel-stable binding varies depending on the RNA substrate.

Example of Partition Assay with Tfr38. An unusually auspicious application of the direct partition approach for determining relative dissociation constants is shown in Figure 5, in which full-length TAR (58 nucleotides; Weeks et al., 1990) and a $C^{28} \rightarrow G$ mutant (64 nucleotides, which disrupts the 4 base pair stem above the bulge, to form a 3 base pair stem and an 8-nucleotide loop) compete for binding by Tfr38. Relative dissociation constants calculated according to eq 2 are given below each gel lane and emphasize the reproducibility of the method. The first binding event for the wild-type RNA is 4-fold stronger than for the mutant; however, binding of a second peptide equivalent (to one or both of the single-nucleotide bulges in TAR) is comparable for both RNAs. At saturating concentrations of peptide, Tfr38 binds 3 times to TAR as visualized in native gels [see Figure 1C of Weeks et al. (1990)]. These data are consistent with a model (Weeks & Crothers, 1991) in which Tfr38 discriminates accurately between wild-type and mutant RNAs at a specific bulged binding site (Figure 1) and forms weaker gel-resolvable complexes at alternative bulged structures. Deconvolution of multiple binding events whose affinities vary by only 4-fold would be impossible by the alternative competition protocol.

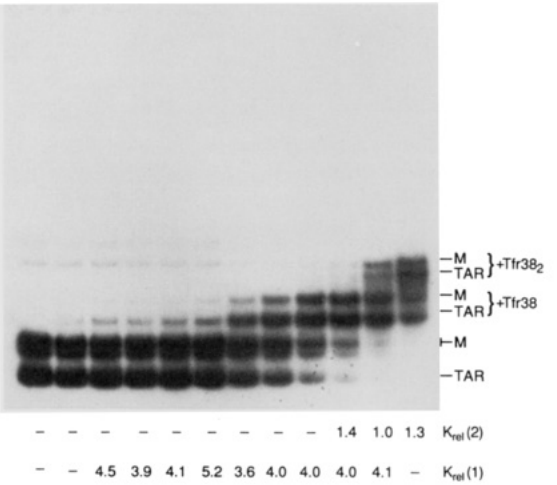

FIGURE 5: K_{rel} determination by partition assay. Full-length TAR RNA (58 nucleotides) and a $C^{28} \rightarrow G$ point mutant (M, 64 nucleotides) compete for binding by Tfr38 (titrated in steps of $2^{1/2}$). The assignment and stoichiometry of complexes are shown. The relative binding constants (eq 2) for the first and second binding events are given below each lane; the mean and standard deviations for K_{rel} for binding 1 and 2 equiv of peptide are 4.2 ± 0.5 and 1.2 ± 0.2, respectively. The free mutant runs as two closely spaced bands due to 3′-end or structural heterogeneity.

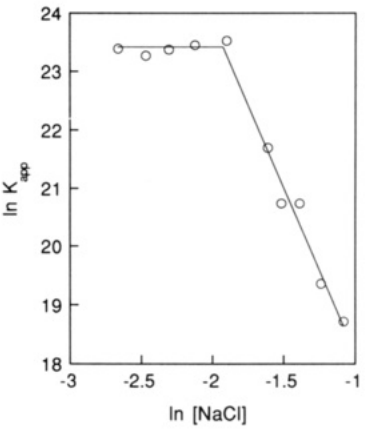

FIGURE 6: Ionic strength dependence of the apparent binding constant. The logarithm of the association constant (M^{-1}) for the Tfr38–ΔTAR interaction as a function of the logarithm of the ionic strength (M) is linear for NaCl concentration above 120 mM.

Electrostatic Contributions to Binding. The ionic strength dependence of the Tfr38–ΔTAR interaction was evaluated by varying the NaCl concentration in the incubation buffer prior to complex resolution by native gel electrophoresis. Binding of a peptide (P) to RNA (R) releases cations (M^+) condensed on the charged RNA helix (Record et al., 1976; Lohman et al., 1980):

$$P + R \rightleftarrows RP + Z\psi M^+$$

where Z is the number of ion pairs formed and ψ is the fractional thermodynamic association of counterions per phosphate. From standard definitions (Lohman et al., 1980)

$$\ln K_{app} = -Z\psi \ln [M^+] + \ln K^0$$

Thus, a plot of $\ln K_{app}$ versus $\ln [M^+]$ should be linear with the slope corresponding to minus the number of cations released upon peptide binding; this plot is linear for the Tfr38–ΔTAR interaction for NaCl concentrations greater than 120 mM and yields a slope of 5.5 ± 0.7 (Figure 6). Assuming a value of 0.89 for ψ (Record et al., 1976) and neglecting anion effects, approximately six phosphates are involved in ion pairs with peptide cationic groups.

The binding free energy for the Tfr38–ΔTAR interaction is independent of ionic strength below 120 mM, which is not predicted for this (polycationic) peptide basic domain–(polyanionic) RNA interaction, suggesting there may be compensating favorable contributions from increasing salt at low ionic strength. The required folding of Tfr38 on the RNA may be stabilized by ionic screening which facilitates close approach of charged moieties in the bound peptide structure. Alternatively, the charge density at the bulge may be high: proper folding of the RNA may require cation screening. We cannot rule out contributions from decreasing peptide activity (e.g., due to aggregation) at low ionic strength.

DISCUSSION

Our results emphasize the contribution that kinetic stability may make to specificity for Tat peptide binding to TAR. The *dosage* of Tat recruited effectively to the vicinity of cellular transactivation-responsive elements is a function of multiple factors: absolute binding affinity, discrimination between correct and noncognate sites, complex lifetime, and cooperative interactions. Tfr14 and Tfr38 bind ΔTAR RNA with comparable affinities, and the specificity of the two peptides for interaction with TAR sequence variants in a bulged structural context is correlated. Tfr14 harbors a ubiquitous RNA binding motif and interacts specifically with TAR RNA (Table I) at the nucleotide constellation emphasized in boldface in Figure 1. However, this peptide may mediate a smaller transactivation dosage than Tfr38: it discriminates only modestly (30-fold) between the specific site and featureless double-stranded RNA and mediates specific interaction for a relatively short lifetime. Thus, this 14-residue peptide and, probably, other generalized basic peptides only partially model the RNA binding activity of Tfr38. It remains unresolved how well Tfr38 reflects specific RNA binding by the intact Tat protein.

The requirement for base pairs flanking the bulge in TAR reflects the complex interplay of sequence-specific interaction (Table I), kinetic stability (Table II), destabilization of competing structures, and steric accessibility [see Weeks and Crothers (1991), Figures 4B and 9].

RNA recognition by Tat-derived peptides involves several specificity-mitigating features. Binding energy (driven in part by a significant electrostatic component involving the formation of approximately six ion pairs, Figure 6) is high, intermediate affinity binding is structurally degenerate, and there is only modest binding discrimination between the best and worst sites (Figure 7). These characteristics are shared by another basic subdomain containing protein, HIV-1 Rev. Rev binds semispecifically to a family of related sites (Kjems et al., 1991), but like Tat interacts at a specific site spanning a region of helical irregularity (Bartel et al., 1991; Tiley et al., 1992; Kjems et al., 1992). While the basic subdomain clearly mediates RNA interactions for both proteins (Weeks et al., 1990; Cordingly et al., 1990; Calnan et al., 1991a; Weeks & Crothers, 1991; Kjems et al., 1992), RNA targeting requires additional information contained in flanking protein sequences (this work; Kjems et al., 1992) and higher order protein–protein interactions [for discussion, see Weeks and Crothers (1991), Malim and Cullen (1991), and Tiley et al. (1992)].

Relative binding strengths determined by competition will be a function of the detailed contribution of labile binding (eq 7 and 8) and thus of RNA probe size. The native gel-based partition approach imposes a kinetic threshold and offers a complementary method for evaluating individual contributions to binding at specific sites. Free energy consequences of sequence and structural changes are deconvoluted from the obscuring sea of nonspecific events (Figure 7), and multiple (gel stable) binding events are visualized directly (Figure 5).

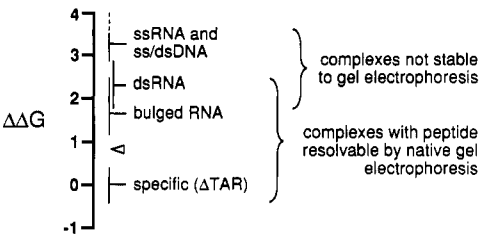

FIGURE 7: Discrimination between cognate and noncognate RNAs by Tfr38. Relative binding constants are converted to free energies ($\Delta\Delta G = -RT \ln K_{rel}$, $T = 295$ K) as determined from the partition assay for complexes with specific and bulged RNAs and from competition data for other RNAs. Tfr38 discriminates weakly between cognate sites and any bulged RNA (~1–2.5 kcal/mol) or double-stranded RNA (2–3 kcal/mol). Values for ssRNA, ssDNA, and dsDNA are from competition experiments with homopolymer competitors (Weeks et al., 1990). The arrowhead indicates the average net contribution from *gel-labile* binding in a wild-type bulge context (Table II). Binding free energies for nucleic acids that bind nonspecifically are a function of polymer size (i.e., $\sum$; see eq 7 and 8). Thus, the free energy for binding double-stranded RNA is shown spanning values corresponding to $K_{rel} = 20$–120. There is significant overlap between binding free energies of complexes stable and unstable to native gel electrophoresis.

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