not known; determination of an accurate value of λ requires continuous films of different thicknesses. This criterion is not met by the probably discontinuous films formed in this study. Thus, only a qualitative comparison of results from ellipsometry and x-ray photoelectron spectroscopy (XPS) is possible at this time. Using the values of d obtained by ellipsometry and the intensities of the N(1s) peaks, we found that least-squares fitting to the above equation yielded a fit with $r^2 = 0.98$, $n = 11$.

24. The averages and standard deviations of the observed thicknesses of adsorbed protein films on methyl-terminated SAMs were 21 ± 1 Å (RNase), 58 ± 3 Å (fibrinogen), and 38 ± 1 Å (pyruvate kinase). Each value represents an average of 18 measurements. Three measurements were made from different positions on each of six independently prepared samples to derive these values.
25. RNase A (molecular weight ≈13,700) forms monoclinic crystals with one molecule per unit cell. The parameters of the unit cell are a = 30 Å, b = 38 Å, c = 53 Å, β = 106° [A. Wlodawer, L. A. Svensson, L. Sjoelin, G. L. Gilliland, *Biochemistry* **27**, 2705 (1988)].
26. Pyruvate kinase is a tetrameric enzyme of identical subunits; the dimensions of the tetrameric molecule are 75, 95, and 125 Å. The molecular weight of each subunit is 54,600 in yeast and 57,900 in cat muscle. (In this study we used enzyme isolated from rabbit muscle.) The shape of each subunit is approximated by an ellipsoid 75 Å long with a maximum transverse diameter of 45 Å [H. Muirhead, *Biol. Macromol. Assem.* **3**, 143 (1987)]. We do not know whether the molecule retains its tetrameric structure upon adsorption.
27. Human fibrinogen (molecular weight ≈340,000) is a structurally complex protein containing several domains connected by more flexible segments [J. A. Shafer and D. L. Higgins, *Crit. Rev. Clin. Lab. Sci.* **26**, 1 (1988)]. It is, therefore, difficult to provide specific molecular parameters for comparison with our experimental results.
28. F. MacRitchie, *Adv. Protein Chem.* **32**, 283 (1978).
29. R. H. Dettre and R. E. Johnson, *J. Phys. Chem.* **69**, 1507 (1965).
30. Steric stabilization of hydrophobic colloids in aqueous solution occurs when a hydrophilic polymer is adsorbed at the colloid-water interface. This polymer prevents flocculation of two colloid particles in two ways. First, approach of the particles to a distance such that the strength of the attractive hydrophobic interaction rises above kT (where k is the Boltzmann constant and T is temperature) is inhibited enthalpically by changes in the configuration of the polymer and perhaps by its desolvation as it is compressed. Second, for coiled polymers, loss of conformational entropy due to the approach of the two particles to one another creates a repulsive force that helps to oppose the attractive hydrophobic interaction. We refer to the second effect as "entropic repulsion," consistent with D. H. Everett [*Basic Principles of Colloid Science* (Royal Society of Chemistry, London, 1988), pp. 45–50].
31. For a computational treatment of surface-grafted oligo(ethylene glycol) chains, see M. Björling, P. Linse, G. Karlström, *J. Chem. Phys.* **94**, 471 (1990); G. Karlström, *ibid.* **89**, 4962 (1985). For a theoretical consideration of the protein resistance of surface-grafted poly(ethylene glycol) surfaces, see S. I. Jeon, J. H. Lee, J. D. Andrade, P. G. de Gennes, *J. Colloid Interface Sci.* **142**, 149 (1991); S. I. Jeon and J. D. Andrade, *ibid.*, p. 159.
32. H. Matsuura, K. Fukuhara, S. Masatoki, M. Sakakibara, *J. Am. Chem. Soc.* **113**, 1193 (1991); H. Matsuura and K. Fukuhara, *J. Mol. Struct.* **126**, 251 (1985).
33. For several examples, see T. Arnebrant, B. Ivarsson, K. Larsson, I. Lundström, T. Nylander, *Prog. Colloid Polym. Sci.* **70**, 62 (1985); P. A. Cuypers, W. T. Hermens, H. C. Hemker, *Anal. Biochem.* **84**, 56 (1978).
34. Supported by the National Science Foundation (NSF) under the Engineering Research Center Initiative to the Massachusetts Institute of Technology Biotechnology Process Engineering Center (cooperative agreement CDR-88-03014), by the Office of Naval Research, and by the Defense Advanced Research Projects Agency (DARPA). The x-ray photoelectron spectrometer was provided by DARPA through the University Research Initiative and is housed in the Harvard University Materials Research Laboratory, an NSF-funded facility. Initial research in this project was carried out by C. Palegrosdemange. We thank H. Biebuyck, J. Folkers, A. Jain, P. Laibinis, and T. R. Lee for critical readings of the manuscript. K.L.P. was an NSF predoctoral fellow, 1986 to 1989.

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Arginine-Mediated RNA Recognition: The Arginine Fork

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Short peptides that contain the basic region of the HIV-1 Tat protein bind specifically to a bulged region in TAR RNA. A peptide that contained nine arginines (R_9) also bound specifically to TAR, and a mutant Tat protein that contained R_9 was fully active for transactivation. In contrast, a peptide that contained nine lysines (K_9) bound TAR poorly and the corresponding protein gave only marginal activity. By starting with the K_9 mutant and replacing lysine residues with arginines, a single arginine was identified that is required for specific binding and transactivation. Ethylation interference experiments suggest that this arginine contacts two adjacent phosphates at the RNA bulge. Model building suggests that the arginine η nitrogens and the ε nitrogen can form specific networks of hydrogen bonds with adjacent pairs of phosphates and that these arrangements are likely to occur near RNA loops and bulges and not within double-stranded A-form RNA. Thus, arginine side chains may be commonly used to recognize specific RNA structures.

RNA-PROTEIN INTERACTIONS ARE important for many regulatory processes, but little is known about the details of sequence-specific recognition. From what is known, it appears that both RNA structure and nucleotide sequence function in recognition. The crystal structure of the glutaminyl tRNA synthetase–tRNA complex (*1*) has shown that specific contacts are made between amino acid side chains and bases in non–base paired regions of the RNA, while studies of the R17 coat protein (*2*) have suggested that the overall three-dimensional RNA conformation contributes substantially to recognition. Recently, an arginine-rich RNA-binding motif has been identified in several RNA-binding proteins (*3*), including the human immunodeficiency virus (HIV) Tat protein. Peptides that contain this region of Tat bind specifically to an RNA stem-loop structure named TAR (*4, 5*), which is located in the HIV long terminal repeat, and RNA binding is essential for Tat-dependent transcriptional activation (*5*). The overall charge density of the Tat peptides is important for binding, however, the amino acid sequence requirements are flexible; the sequence can be scrambled and still bind specifically to TAR (*5*).

The basic RNA-binding region of Tat, RKKRRQRRR (residues 49 to 57), is nine amino acids long and contains a glutamine at position 54 that is not essential for binding or activity (*5*). Because it is known that a high positive charge density is important for RNA binding, we synthesized (*6*) two peptides, R_9, which contains a stretch of nine adjacent arginines (with a tyrosine at the NH_2-terminus and an alanine at the COOH-terminus), and K_9, which contains a stretch of nine lysines (and a surrounding tyrosine and alanine), and measured their binding to TAR RNA (*7*). The R_9 peptide bound to TAR RNA with the same affinity as the wild-type Tat peptide and with tenfold higher affinity than K_9 (Fig. 1). The specificity of R_9 binding to TAR was identical to the wild-type peptide, whereas K_9 binding was nonspecific (*7*). Because RNA binding of Tat peptides correlates with Tat's function as a transcriptional activator (*5*), we asked whether R_9 or K_9 could function in the context of the intact protein. The nine– amino acid basic region of Tat was replaced by R_9 or K_9 in a Tat expression vector, and activation of HIV-1 transcription by the chimeric Tat proteins was tested in transient transfection assays (*8*). The R_9-containing protein gave wild-type transactivation activity and was 100-fold more active

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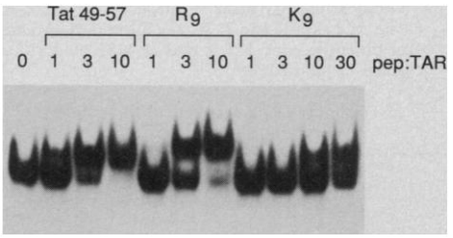


Fig. 1. Specific binding of R_9 and K_9 peptides to TAR RNA. Each peptide was incubated with in vitro–transcribed TAR RNA (2 nM) and binding was measured by electrophoretic mobility shift analysis at the peptide:RNA ratios indicated. The 31-nucleotide unbound TAR RNA is also shown (0). Specificity was confirmed by binding to mutant TAR RNAs (*5*, 7).

than the K_9-containing protein (Fig. 2). These results confirm that transactivation of transcription correlates with peptide RNA binding and suggest that arginine residues are important for specific RNA recognition.

The low level of transactivation achieved by the K_9-containing protein allowed us to systematically replace lysines with arginines in order to identify positions at which arginine side chains are required. Previous results indicated that arginines at positions 55 and 56 could be replaced with lysines without affecting Tat activity (*5*). There were four remaining arginines in the basic region of Tat (positions 49, 52, 53, and 57), and we made every combination of lysine and arginine at these positions (mutating from KKKKKKKKK to RKKRRKKKR) and measured their transactivation ability. A single arginine at either position 52 or 53 restored transactivation to wild-type levels (Fig. 3). Electrophoretic mobility shift experiments with YKKKRKKKKKA (R52) or with YKKKKRKKKKA (R53) peptides showed that RNA-binding affinity and specificity were also restored to wild-type levels by the addition of one arginine at either position 52 or 53 (*10*). To examine the positional dependence of the single arginine within the basic region, we changed each position individually (residues 49 to 57) from lysine to arginine and measured transactivation. The optimal location of the arginine was at position 52 or 53, with activity decreasing as the arginine was moved toward the ends of the basic region (Fig. 4). Thus, a single arginine surrounded by three to four basic amino acids on each side is sufficient for specific recognition of TAR RNA.

These and other results (*4, 5, 11*) clearly suggested that RNA structure is important in Tat-TAR recognition. It seemed plausible that the RNA backbone might be adopting a highly defined conformation and that a specific configuration of phosphates was being recognized by arginine. To identify phosphates involved in recognition, we performed ethylation interference experiments (*12*) with the R52, R53, K_9, and wild-type peptides. Modification of two particular phosphates located at the 5′ end of the three-nucleotide bulge (between A22 and U23, and U23 and C24) interfered with specific binding of R52 (Fig. 5). An identical pattern was seen with the wild-type peptide (Fig. 5) and with R53 (*10*), while no interference was seen with K_9 (Fig. 5). At higher R52 concentrations, where nonspecific binding occurred, no interference was observed (*10*). These results suggest that a single specific arginine simultaneously contacts two adjacent phosphates (even in the wild-type peptide, which contains multiple arginines), although we cannot rule out the possibility that ethylation alters the RNA structure and indirectly interferes with binding. Ethylation of some phosphates, particularly the phosphate between G26 and A27, seems to enhance binding (Fig. 5), possibly by stabilizing the RNA structure. The minimal interference observed with lysines or with nonspecific arginines suggests that these residues may make weaker contacts with the RNA or that alternative phosphates may be contacted when one is modified.

How does a single arginine recognize TAR RNA? Arginine contains two terminal amino (NH_2) groups at the η position and a secondary amine (NH) at the ε position, each of which can donate hydrogen bonds to appropriate acceptor groups. The positions at which acceptor atoms would be located in order to form hydrogen bonds with ideal distances and geometries are shown in Fig. 6A. Clearly, an arginine side chain can form many possible hydrogen bonds with appropriately positioned acceptor groups on the RNA. These acceptors can include phosphate oxygens, the ribose 2′ OH, and groups on the bases (for example, O-6 and N-7 on guanine or O-4 on uridine in the major groove, or N-3 on guanine or O-2 on uridine in the minor groove). In contrast, lysine, which contains a single terminal amino group, cannot form such an extensive network of hydrogen bonds; this amino group also has tetrahedral geometry rather than the planar geometry of the arginine amino groups. Our ethylation interference data indicate that a single arginine in Tat contacts two adjacent phosphates at the TAR bulge, suggesting that the phosphate backbone adopts a defined conformation that can be bridged by arginine in a fork-like arrangement. To determine a plausible con-

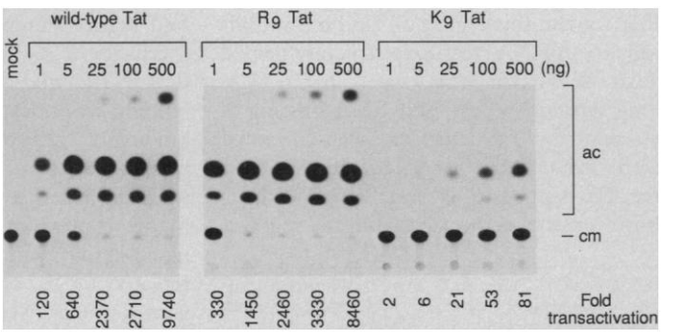


Fig. 2. Transactivation by R_9- and K_9-containing Tat proteins. The nine– amino acid basic region of Tat was replaced by nine arginines or nine lysines in the mammalian Tat expression vector pSV2tat72 (*9*). HeLa cells that contain an HIV–LTR CAT reporter were transfected with the amounts of plasmid indicated above each lane (ng) and CAT activity was determined after 48 hours with a fixed amount of cell extract. Acetylated (ac) and unacetylated (cm) ^{14}C-chloramphenicol were separated by thin-layer chromatography. Transactivation (the ratio of CAT activity from each protein to mock-transfected cells) was quantitated by assaying an appropriate amount of extract and counting the chromatograms (*8*).

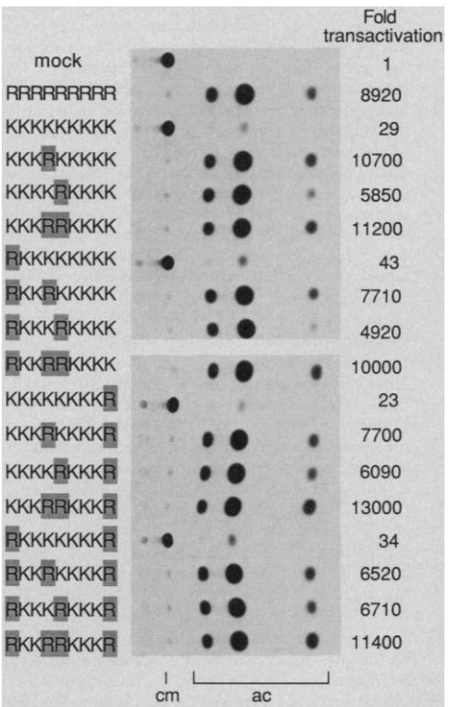


Fig. 3. Activity of arginine-substituted mutants. The basic region of Tat was replaced by the sequences indicated. Each mutant plasmid (25 ng) was transfected into HeLa cells and CAT activity was assayed and quantitated as in Fig. 2. Assays were repeated at least two times.

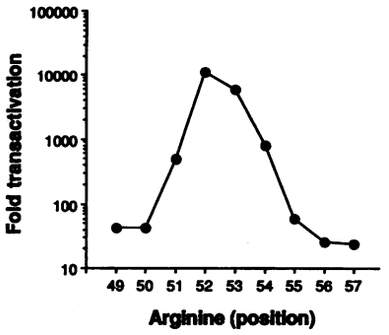


Fig. 4. Positional dependence of arginine. The basic region of Tat was replaced by eight lysines and a single arginine located at the amino acid positions indicated. Plasmids (25 ng) were transfected into HeLa cells and transactivation was determined as in Fig. 2.

formation for this interaction, molecular modeling (*13*) was used to locate the most favorable positions of two phosphates with hydrogen (H)-bonds to arginine. The best arrangement (Fig. 6B) has a pair of H-bonds between a phosphate and two N_η's, and another pair of H-bonds between the second phosphate and $N_{\eta 1}$ and N_ϵ. Each phosphate is shared by a pair of nitrogens,

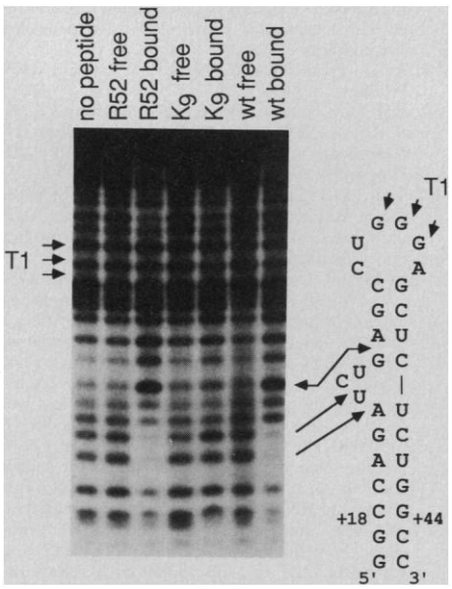


Fig. 5. Ethylation interference of R52, K_9, and wild-type Tat peptides. Peptides were bound to ethylated TAR RNA under gel-shift conditions that gave <50% binding (*12*), the peptide-RNA complexes (bound) and the unbound RNA (free) were separated on polyacrylamide gels, and RNAs were cleaved at the modified sites and run on a sequencing gel. An alkali-cleaved RNA ladder and a ribonuclease T1 digestion were used as markers (not shown). Ethylation of phosphates that interfere with binding results in the absence of bands at those positions. The sequence of TAR is shown, with arrows indicating positions of T1 cleavage, two modified phosphate positions that interfere with binding, and one position that shows enhanced binding.

with a distance between phosphates of 7.1 Å (center to center distance between phosphorus atoms). We define the arginine fork as an interaction between a single arginine and a pair of adjacent phosphates, which mediates specific recognition of RNA structure. Other arginine-phosphate arrangements are possible (for example, see legend to Fig. 6B), and arginine forks with additional H-bonds are possible (for example, with a specific base or a 2′ OH).

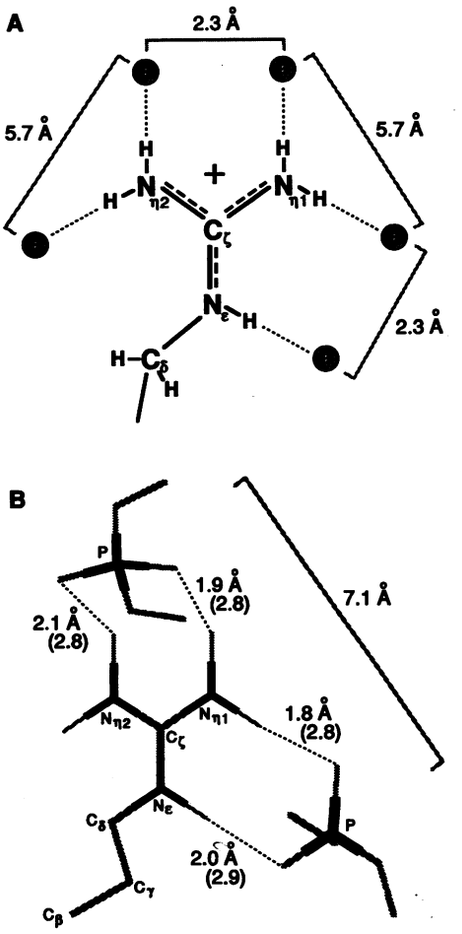
To determine whether such phosphate arrangements are found in RNA structures, the modeled phosphate coordinates from Fig. 6B were superimposed on all phosphate pairs in tRNA crystal structures (*14*). The results indicate that double-stranded A-form RNA cannot readily accommodate this arrangement; the P-P distance in the model (7.1 Å) is longer than the P-P distance in A-form RNA (5.6 Å), and the phosphate oxygens in A-form RNA are not properly oriented to form H-bonds between a single arginine and a pair of adjacent phosphates. Reasonable H-bonding arrangements are much more likely to be found at discontinuous regions of RNA, for example, at junctions between double-stranded A-form RNA and a bulge or loop. The two critical phosphates in TAR are located precisely at the junction of the double-stranded stem and the 3-nucleotide bulge.

The cocrystal structure of glutaminyl tRNA synthetase–tRNA shows a similar interaction of arginine with the acceptor strand of tRNA (*1*). Arg^{133} forms H-bonds with two adjacent phosphates and an additional H-bond with a ribose 2′ OH. It is

Fig. 6. Model of an arginine fork. (**A**) Possible hydrogen bonding configurations for arginine. The end of an arginine side chain is shown with ideal positions of potential hydrogen bond acceptor atoms (a). There are five hydrogen bond donors on arginine: four from the two terminal η nitrogens and one from the ϵ nitrogen, and hydrogen bonds are assumed to be linear. Distances between pairs of acceptor positions are indicated. (**B**) One possible configuration of an arginine fork. Energy minimization was used to calculate the most favorable orientation of two phosphate groups hydrogen-bonded to a single arginine side chain. One phosphate is shared by the two terminal η nitrogens, the other is shared by the η1 nitrogen and the ϵ nitrogen. All hydrogen bonds are near the ideal linear geometry, and the distances are indicated [both oxygen to hydrogen and oxygen to nitrogen (parentheses) distances are shown]. The upper H-bonds appear bent only because they are slightly out of the plane. This orientation is favored because it maximizes both hydrogen bonding and electrostatic interaction. Two other favorable orientations were found: in one, the plane of the arginine bisects the pair of phosphates, but no H-bonds are formed; in the other, one phosphate forms two H-bonds with N_ϵ and $N_{\eta 1}$ and the other forms two H-bonds with $N_{\eta 2}$.

plausible that the arginine in Tat also interacts with a 2′ OH, thus discriminating between RNA and DNA (*4*). Although we cannot rule out base-specific contacts, for example, with an essential uridine in the bulge (*4, 5, 11*), it seems reasonable that contacts between one arginine and a highly oriented pair of phosphates can account for the modest 10- to 20-fold specificity of Tat binding to TAR (*4, 5, 11*). The interactions that stabilize the overall folding of TAR and the orientation of these particular phosphates remain to be determined. In addition to the specific arginine contact, the charge density of the basic region of Tat is important for binding (*5*) and may provide a nonspecific electrostatic scaffold to help orient the arginine. Tat is perhaps the simplest example of RNA recognition in that a single amino acid interacts with a single feature of the RNA; other proteins may achieve higher specificity through multiple arginine-RNA or other interactions. In the case of Tat, although RNA binding is essential for transactivation, the modest specificity for TAR is insufficient to account for the high specificity of Tat function. Other interactions of Tat, perhaps with cellular proteins, are likely to be required.

The recognition of TAR by Tat highlights fundamental differences between RNA recognition and DNA recognition. It



is clear from the structures of protein-DNA complexes that sequence-specific discrimination derives primarily from direct base-specific contacts, most commonly made in the DNA major groove (*15*). In most cases, DNA tertiary structure does not seem to be of major importance in recognition. That RNA recognition often seems to rely on RNA tertiary structure is emphasized by the finding that the Tat-TAR interaction uses only a single arginine side chain in the midst of an apparently unstructured segment of basic amino acids (*5*) to recognize a specific backbone conformation of TAR. The unstructured nature of the unbound polypeptide is supported by the fact that the sequence of the Tat basic region can be simplified to a single arginine embedded in a set of eight lysines. The peptide conformation when bound to TAR remains to be determined. Specific recognition of TAR appears to occur by indirect readout of the base sequence and direct contact with the phosphate backbone, and may not involve any base-specific contacts. Differences between DNA and RNA recognition are also apparent in studies of TFIIIA, a zinc finger–containing protein that binds to the same site on both DNA and RNA. DNA recognition seems to occur through base-specific contacts in the major groove; RNA recognition appears to be primarily backbone structure–specific (*16*).

Other RNA hairpins and bulges form stable, ordered tertiary structures (*17*), further emphasizing that RNA structure can provide information for protein-RNA complex formation. The structure of an RNA pseudoknot reveals that phosphates can be arrayed in unusual, electrostatically unfavorable geometries through tertiary RNA interactions (*18*); binding of basic amino acids to these phosphates might help to stabilize a more favorable RNA conformation that, in turn, would provide favorable energy for the protein-RNA interaction. This could explain why interaction of HIV Tat or Rev with RNA causes a change in RNA conformation upon binding (*5, 19*).

Other RNA-protein interactions will likely follow some of the principles outlined here. Arginine-rich motifs similar to the basic region of Tat are found in several RNA-binding proteins, including bacterial antiterminators, ribosomal proteins, and HIV Rev (*3*). Other RNA-binding proteins may bring basic amino acids together through protein tertiary structure rather than primary sequence and may position specific arginines to interact with defined RNA structures. For example, the U1 A protein, which contains a ribonuclear protein (RNP) RNA-binding motif, has a highly defined structure with a cluster of basic amino acids at one end and at least one arginine that is essential for specific recognition (*20*). It is not yet known if this arginine makes a base-specific or structure-specific contact. For TFIIIA, the strongest interactions with 5*S* RNA are localized to junctions between stems and loops (*21*), similar to the stem-bulge junction in TAR; perhaps arginines participate in some of these interactions. Arginine has also been shown to bind to the guanosine binding site of a group I intron (*22*); however, this interaction involves H-bonding to a guanine base in the RNA and is distinct from the arginine fork proposed here. Many RNA-binding proteins, including heterogeneous nuclear RNA-binding proteins and nucleolar proteins, contain clusters of methylated arginines, most commonly N^G,N^G-dimethylarginine (*23*). Because methylation would block H-bonding but would not alter the charge of the side chain, arginine methylation could provide a mechanism to regulate RNA binding between specific and nonspecific modes. While it is clear that RNA recognition will involve more than just arginine forks, it seems reasonable to suggest that arginine-mediated recognition of RNA structure may be an important part of many RNA-protein complexes.

REFERENCES AND NOTES

1. M. A. Rould, J. J. Perone, D. Soll, T. A. Steitz, *Science* **246**, 1135 (1989).
2. P. J. Romaniuk, P. Lowary, H.-N. Wu, G. Stormo, O. C. Uhlenbeck, *Biochemistry* **26**, 1563 (1987).
3. D. Lazinski, E. Grzadzielska, A. Das, *Cell* **59**, 207 (1989).
4. K. M. Weeks, C. Ampe, S. C. Schultz, T. A. Steitz, D. M. Crothers, *Science* **249**, 1281 (1990); M. G. Cordingley *et al. Proc. Natl. Acad. Sci. U.S.A.* **87**, 8995 (1990).
5. B. J. Calnan, S. Biancalana, D. Hudson, A. D. Frankel, *Genes Dev.* **5**, 201 (1991).
6. Syntheses were performed with the use of Fmoc chemistry on a MilliGen/Biosearch model 9600 peptide synthesizer as described (*5*) except that a polyethylene glycol–polystyrene graft copolymer (PEG-PS) was used for the support (G. Barany and D. Hudson, unpublished data). All peptides were synthesized as their COOH-terminal amides with acetylated NH_2 termini. Amino acid composition was determined by hydrolysis at 110°C in HCl (6 M) that contained phenol (0.5%) and analysis on an LKB 4151 Alpha Plus analyzer. Peptides were purified on a C_4 reversed-phase high-performance liquid chromatography (HPLC) column (Vydac) with an acetonitrile gradient (0.25% per minute in 0.1% trifluoroacetic acid). Peptide absorption spectra were recorded and concentrations were determined by tyrosine absorbance at 278 nm [ϵ = 1420 M^{-1} cm^{-1}; T. E. Creighton, in *Proteins: Structures and Molecular Principles* (Freeman, New York, 1984), p. 17]. Peptide masses were confirmed by fast atom bombardment mass spectrometry (University of California, Berkeley), and purity and concentrations were confirmed by native polyacrylamide (20%) gel electrophoresis in sodium acetate, pH 4.5 (30 mM).
7. TAR RNAs were transcribed by T7 RNA polymerase in vitro with synthetic oligonucleotide templates (*5*) [J. F. Milligan and O. C. Uhlenbeck, *Methods Enzymol.* **180**, 51 (1989)]. All RNAs contained GG at their 5′ end, which increased the efficiency of transcription, and CC at the 3′ end to base pair with GG. Wild-type TAR RNA contained sequences +18 to +44 of the HIV long terminal repeat TAR site. Mutant TARs were also synthesized and specificity of binding of each peptide was determined (*5*). All RNAs were purified on 10% polyacrylamide/8 M urea gels, eluted from gels in ammonium acetate (0.5 M), magnesium acetate (10 mM), EDTA (1 mM), and SDS (0.1%), extracted twice with phenol, and ethanol-precipitated. Purified RNA was resuspended in sterile deionized water. The concentrations of labeled RNAs were determined from the specific activity of [^{32}P]CTP incorporated into the transcripts. Unlabeled RNA was quantitated by spectrophotometry. RNA electrophoretic mobility shift assays were performed as described (*5*). Briefly, peptide and RNA were incubated together for 10 min on ice in binding reactions (10 μl) that contained tris-HCl pH 7.5 (10 mM), NaCl (70 mM), EDTA (0.2 mM), and glycerol (5%). Peptide-RNA complexes were resolved on polyacrylamide (10%) gels in tris-borate-EDTA [0.5× TBE; in *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, Eds. (Wiley, New York, 1987)] gels that had been pre-electrophoresed for 1 hour and allowed to cool to 4°C. Gels were subjected to electrophoresis at 200 V for 3 hours at 4°C, dried, and autoradiographed.
8. Oligonucleotide cassettes that contained the basic region mutants were synthesized and cloned into the *tat* gene of pSV2tat72 (*9*). Mutations were confirmed by dideoxynucleotide sequencing (Sequenase; U.S. Biochemicals, Cleveland, OH). Various amounts of plasmid were transfected into HL3T1 HeLa cells [B. K. Felber and G. N. Pavlakis, *Science* **239**, 184 (1988)] (50% confluent in 25 mm wells) by lipofection [P. L. Felgner *et al., Proc. Natl. Acad. Sci. U.S.A.* **84**, 7413 (1987)], cells were harvested 48 hours after transfection, and CAT activity was assayed and quantitated as described [A. D. Frankel, S. Biancalana, D. Hudson, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7397 (1989)]. In all cases, plasmid concentrations were adjusted to 1 μg of total DNA with nonspecific pBR322 DNA. Cells were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%) and penicillin/streptomycin.
9. A. D. Frankel and C. O. Pabo, *Cell* **55**, 1189 (1988).
10. B. J. Calnan and A. D. Frankel, unpublished data.
11. S. Roy, U. Delling, C.-H. Chen, C. A. Rosen, N. Sonenberg, *Genes Dev.* **4**, 1365 (1990); C. Dingwall *et al., EMBO J.* **9**, 4145 (1990).
12. TAR RNA (31 nucleotides) was labeled with ^{32}P at the 5′ end with T4 polynucleotide kinase. RNA phosphates were ethylated with ethylnitrosourea under denaturing conditions as described [V. V. Vlassov, R. Giege, J.-P. Ebel, *Eur. J. Biochem.* **119**, 51 (1981)], except that the reactions contained ethylnitrosourea (20% final concentration, using a saturated solution of ethylnitrosourea in ethanol) and tRNA (2 μg), and were incubated at 80° C for 5 min. After modification, the RNA was ethanol -precipitated, washed with 100% ethanol and lyophilized. Peptides were bound to the modified RNA (500,000 cpm) at concentrations that gave <50% binding (specific) by gel shift (7) or at higher concentrations that gave nonspecific binding. Free and bound RNAs were visualized by autoradiography. The bands were excised and the RNA was eluted from the gel (7), ethanol precipitated with yeast tRNA (20 μg), and lyophilized. To cleave the phosphotriester bonds (at the modified phosphates), samples were resuspended in 10 μl of 100 mM triethylammonium bicarbonate, pH 9.0, and heated at 50° C for 5 min. Samples were then lyophilized and subjected to electrophoresis on 10% polyacrylamide/8 M urea sequencing gels.
13. Local energy minima for the interaction of a single arginine side chain with two phosphate groups were determined with the use of the molecular modeling program CHARMM [B. R. Brooks *et al., J. Comput. Chem.* **4**, 187 (1983)] with standard parameters (param19) for the polar hydrogen model. The arginine side chain was fixed in the center of an 11 Å sphere and surrounded by 70 pairs of randomly placed dimethylphosphate molecules. The potential function was modified so that each dimethylphosphate in the pair interacted with its partner and with the arginine but not with other dimethylphosphates. A constant dielectric of one was used with nonbonded

interactions beyond 8.5 Å smoothed to zero by use of a shifting function for electrostatic terms and a switching function for van der Waals terms. A boundary potential was used to keep the dimethylphosphates within the sphere. Langevin molecular dynamics simulated annealing was performed on this potential surface for 4.75 ps, starting at 1000 K and cooling slowly to 10 K. The final configuration was further optimized by performing 100 steps of ABNR (adopted-basis Newton-Raphson) minimization. The entire procedure was repeated five times starting from different random configurations and the interaction energy of each dimethylphosphate pair with the arginine was calculated. The configuration with the most favorable interaction energy is shown (Fig. 6B).

14. Coordinates of two tRNA crystal structures (yeast Phe and Asp tRNAs) in the Brookhaven Protein Data Bank [F. C. Bernstein *et al., J. Mol. Biol.* **112**, 535 (1977)] were searched to determine the location of phosphate pairs oriented in a manner similar to the phosphates in Fig. 6B. We required the P-P distance to be between 6.5 and 7.5 Å and the free oxygens (O1P and O2P) to point toward each other. Although more stringent criteria are possible (for example, requiring oxygen-oxygen distances to match the modeling results more closely), a broader range of phosphate orientations was accepted to accommodate other possible conformations indicated by the modeling (see legend to Fig. 6B) or conformational changes that might occur upon arginine binding. Three types of phosphate pairs were found: phosphates adjacent in the sequence (i, $i+1$), those one away from each other (i, $i+2$), and those distant in primary sequence but near each other in the tertiary structure. In no case did phosphate pairs in double-stranded RNA match the template. The (i, $i+1$) pattern frequently appeared surrounding the first or last unpaired base in a bulge or loop, and the (i, $i+2$) pattern frequently appeared in a bulge or loop that bound a hydrated magnesium ion. In fact, water molecules coordinated to Mg^{2+} produce an array of hydrogen-bond donors similar to that of an arginine side chain (Fig. 6A) and bind to a phosphate pair in a manner similar to Fig. 6B.
15. T. A. Steitz, *Quart. Rev. Biophys.* **23**, 205 (1990).
16. Q. You, N. Veldhoen, F. Baudin, P. J. Romaniuk, *Biochemistry* **30**, 2495 (1991).
17. For examples see J. D. Puglisi, J. R. Wyatt, I. Tinoco, Jr., *Biochemistry* **29**, 4215 (1990); A. Bhattacharyya, A. I. H. Murchie, D. M. J. Lilley, *Nature* **343**, 484 (1990); C. Cheong, G. Varani, I. Tinoco, Jr., *ibid.* **346**, 680 (1990).
18. J. D. Puglisi, J. R. Wyatt, I. Tinoco, Jr., *J. Mol. Biol.* **214**, 437 (1990).
19. T. J. Daly, J. R. Rusche, T. E. Maione, A. D. Frankel, *Biochemistry* **29**, 9791 (1990).
20. K. Nagai, C. Oubridge, T. H. Jesse, J. Li, P. R. Evans, *Nature* **348**, 515 (1990).
21. J. Christiansen, R. S. Brown, B. S. Sproat, R. A. Garrett, *EMBO J.* **6**, 453 (1987).
22. M. Yarus, *Science* **240**, 1751 (1988); F. Michel, M. Hanna, R. Green, D. P. Bartel, J. W. Szostak, *Nature* **342**, 391 (1989).
23. M. A. Lischwe, R. G. Cook, Y. S. Ahn, L. C. Yeoman, H. Busch, *Biochemistry* **24**, 6025 (1985); M. E. Christensen and K. P. Fuxa, *Biochem. Biophys. Res. Comm.* **155**, 1278 (1988).
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Requirement of GTP Hydrolysis for Dissociation of the Signal Recognition Particle from Its Receptor

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The signal recognition particle (SRP) directs signal sequence specific targeting of ribosomes to the rough endoplasmic reticulum. Displacement of the SRP from the signal sequence of a nascent polypeptide is a guanosine triphosphate (GTP)–dependent reaction mediated by the membrane-bound SRP receptor. A nonhydrolyzable GTP analog can replace GTP in the signal sequence displacement reaction, but the SRP then fails to dissociate from the membrane. Complexes of the SRP with its receptor containing the nonhydrolyzable analog are incompetent for subsequent rounds of protein translocation. Thus, vectorial targeting of ribosomes to the endoplasmic reticulum is controlled by a GTP hydrolysis cycle that regulates the affinity between the SRP, signal sequences, and the SRP receptor.

RIBOSOMES SYNTHESIZING PROTEINS with rough endoplasmic reticulum (RER)–specific signal sequences are cotranslationally recognized by SRPs and then delivered to the RER membrane via interaction between the SRP and the SRP receptor or docking protein (*1–4*). The SRP receptor–mediated displacement of the SRP from the signal sequence of the nascent polypeptide is a GTP-dependent reaction (*5–7*). One protein subunit from both the SRP receptor (SRα) (7) and the SRP (SRP54) (*8, 9*) contains protein sequence motifs that are similar to those in GTP binding proteins (*10*). We examined the role of GTP hydrolysis in SRP receptor function by replacing GTP with the nonhydrolyzable analog β-γ-imidoguanosine 5′-triphosphate [Gpp(NH)p] during the targeting and insertion steps of a protein translocation reaction.

A truncated mRNA encoding the NH_2-terminal 90 residues of the G protein of vesicular stomatitis virus was translated in vitro in the presence of ^{125}I-labeled SRP to prepare complexes containing SRP, ribosomes, and a nascent polypeptide. After translation, ribonucleotides were removed by gel filtration chromatography, and the SRP-ribosome complexes were incubated in the absence or presence of Gpp(NH)p and microsomal membranes that were depleted of SRP (K-RM) (Fig. 1, A and B). The SRP-ribosome complexes were then separated from free SRPs by sedimentation on sucrose density gradients that were underlayered with a 2 M sucrose cushion. Under these conditions, membrane vesicles sediment at the interface between the sucrose layers. Addition of K-RM and GTP to the complexes increased the amount of unbound SRP recovered after centrifugation while the amount of SRP bound to ribosomes decreased (Fig. 1, A and C), indicating that the SRP enters a soluble pool. In

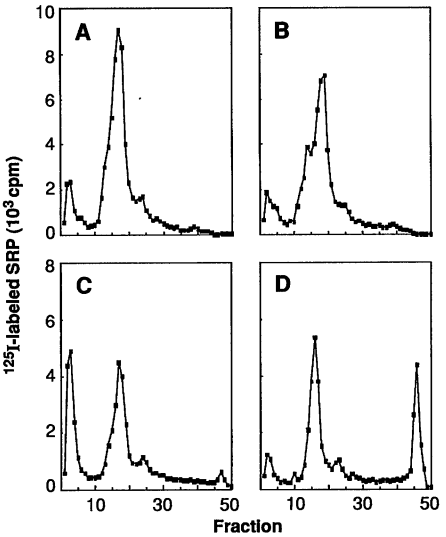

Fig. 1. Recycling of SRP after GTP hydrolysis. A truncated mRNA transcript (*7, 16*) was incubated for 20 min in a wheat germ system containing 6.5 nM SRP (including ^{125}I-labeled SRP) (*3, 7, 19*). SRP-ribosome complexes were separated from ribonucleotides (*5*) and incubated in 50 mM triethanolamine-acetate, pH 7.5, 150 mM potassium acetate, 2.5 mM magnesium acetate, and 1 mM dithiothreitol for 5 min at 25°C as follows. (**A**) No additions, (**B**) K-RM [5 equivalents, as defined (*3*)], (**C**) K-RM (5 equivalents) and 100 μM GTP, and (**D**) K-RM (5 equivalents) and 100 μM Gpp(NH)p. The abbreviation K-RM refers to rough microsomal membranes depleted of SRPs by extraction with 0.5 M potassium acetate (*3*). Samples were applied to sucrose density gradients (10 to 30%) underlain with 0.5 ml of 2 M sucrose. The gradients contained 50 mM triethanolamine-acetate, pH 7.5, 150 mM potassium acetate, 5 mM magnesium acetate, and 1 mM dithiothreitol. Centrifugation, fractionation, and quantitation of gradients were as described (*3, 7*). The top and bottom of the gradient were in fractions 1 and 50, respectively. The interface between the sucrose layers was in fraction 45. The sedimentation position of 80*S* ribosomes (fractions 14 to 20) was determined from the ultraviolet-absorbence profile as recorded with a continuous flow cell. Free SRPs sedimented in fractions 1 to 5 in gradients lacking ribosomes. Similar results were obtained in three separate experiments.

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