# Chemical Cross-Linking of the Human Immunodeficiency Virus Type 1 Tat Protein to Synthetic Models of the RNA Recognition Sequence TAR Containing Site-Specific Trisubstituted Pyrophosphate Analogues†

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ABSTRACT: A chemical ligation procedure has been developed for the synthesis of oligoribonucleotides carrying a trisubstituted pyrophosphate (tsp) linkage in place of a single phosphodiester. Good yields of tsp were obtained when a single 2′-deoxynucleoside 5′ to the tsp was used in the ligation reaction. A tsp linkage was found to be reasonably stable to hydrolysis but cleaved by treatment with ethylenediamine or lysine to give phosphoamidate adducts. A model human immunodeficiency virus type 1 (HIV-1) TAR RNA duplex containing an activated tsp was able to bind to HIV-1 Tat protein with only 3-fold reduced affinity and to a Tat peptide (residues 37−72) with identical affinity compared to that of an unmodified duplex. Tsps incorporated at sites previously identified as being in close proximity to Tat protein were able to cross-link to Tat peptide (37−72) to form a covalent phosphoamidate conjugate. Endopeptidase cleavage followed by MALDI-TOF (matrix-assisted laser desorption ionization time of flight) mass spectrometric analysis provided strong evidence that a TAR duplex containing a tsp replacing the phosphate at 38−39 had reacted specifically with $Lys^{51}$ in the basic region of Tat peptide (37−72). The new chemical cross-linking method may be generally useful for identifying lysines in close proximity to phosphates in basic RNA-binding domains of proteins.

Considerable structural diversity exists amongst the numerous RNA−protein interactions that occur in cellular processes [for reviews see Nagai and Mattaj (1994) and Draper (1995)]. One important paradigm is the interaction between the human immunodeficiency virus type 1 (HIV-1) *trans*-activator protein Tat and its RNA recognition sequence, the *trans*-activation response region TAR, that occurs during viral replication. In the absence of Tat, HIV transcripts are prematurely terminated. As Tat levels rise during gene expression, a considerable boost is obtained in the amount of full-length transcripts. The interaction of Tat with TAR triggers a *trans*-activation process that is believed to involve host cellular factors and which results in enhancement of the elongation properties of the RNA polymerase transcription complex (Karn et al., 1996).

HIV-1 Tat is an 86-amino acid protein which can be subdivided into six regions (Figure 1a). Of these, a basic region which is particularly rich in arginine and which is believed to be a nuclear localization signal is particularly important in binding to TAR RNA. The structure of Tat is not known, but preliminary NMR studies have suggested that the basic region is rather flexible whereas the flanking core and glutamine-rich regions may form more pronounced structural domains that influence the overall folding of Tat (Bayer et al., 1995).

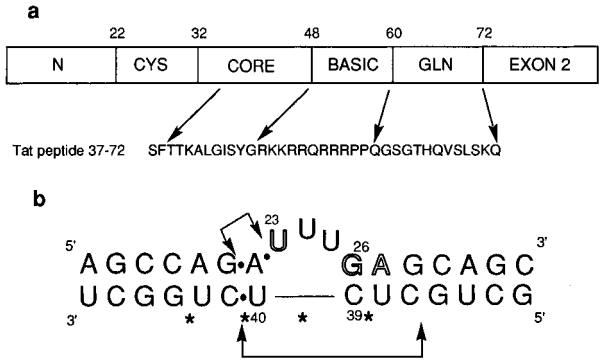


FIGURE 1: (a) Regions of HIV-1 Tat protein and sequence of the Tat peptide (37−72). (b) Structure of the HIV-1 TAR synthetic model duplex containing the U-rich bulge to which Tat binds. Arrows denote the positions of ethylation protection observed (Calnan et al., 1991; Hamy et al., 1993). Filled circles denote phosphates whose substitution by a methylphosphonate ($R_p$ or $S_p$) leads to inhibition of Tat binding (Hamy et al., 1993; Pritchard et al., 1994). Outlined bases are those where there are functional groups on bases crucial to Tat binding (Sumner-Smith et al., 1991; Hamy et al., 1993). Stars denote phosphate sites of incorporation of trisubstituted pyrophosphates (this work).

TAR is a 59-residue RNA stem−loop which is found at the 5′-end of all HIV transcripts. Tat binds to TAR *in vitro* with high affinity (Dingwall et al., 1989, 1990; Müller et al., 1990; Roy et al., 1990). Because of difficulties in obtaining purified and correctly folded Tat protein, many Tat−TAR binding studies have focused on the use of peptides that span the basic region of Tat (Cordingley et al., 1990; Calnan et al., 1991a; Weeks et al., 1990; Weeks & Crothers, 1991; Long & Crothers, 1995). Peptides of this type can bind TAR with high affinity. However to obtain the full specificity of the Tat−TAR interaction, a larger

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region of Tat is required that includes the hydrophobic core of the protein. For example, Tat peptide 37−72 (Figure 1a) mimics in competition binding experiments much more closely the ability of Tat protein to distinguish mutant TARs from wild type than those peptides containing the basic region alone (Kamine et al., 1991; Churcher et al., 1993).

The region of TAR recognized by Tat is near the apex and contains a 3-residue U-rich bulge (Dingwall et al., 1990; Roy et al., 1990). Mutation of one of these residues, $U_{23}$, or either of the two base pairs above the bulge leads to a drastic reduction in Tat binding affinity (Delling et al., 1992; Churcher et al., 1993). Smaller effects were seen for mutation of either of the two base pairs below the bulge (Delling et al., 1992; Churcher et al., 1993). The apical loop is not required for Tat binding and can be dispensed with in TAR models (Sumner-Smith et al., 1991; Hamy et al., 1993). By chemical synthesis of model TAR duplexes (e.g., Figure 1b) containing singly modified base residues and by comparison of their Tat binding abilities with unmodified TAR duplex, we and others have been able to identify a number of essential functional groups on base residues. These are the $N^7$ positions of $G_{26}$ and $A_{27}$ in the two base pairs above the bulge and the $O^4$ and $N^3$-H positions of $U_{23}$ in the bulge (Sumner-Smith et al., 1991; Hamy et al., 1993). All of these functional groups would be expected to be oriented toward the major groove of a presumed A-form RNA duplex structure.

In addition to bases, phosphate residues are also important to Tat binding. Ethylation interference analysis showed two regions of TAR protection, two phosphates on one strand (P21 and P22) and five phosphates on the other strand (P36−P40) (Figure 1b) (Calnan et al., 1991b; Hamy et al., 1993), suggesting that Tat closely approaches these regions. Methylphosphonate substitution analysis using chemically synthesized TAR analogues [where an individual oxygen atom (*R*p or *S*p) on a phosphate is replaced by a methyl group] has led to the identification of three phosphates (P21, P22, and P40) where methylphosphonate substitution in each case is severely harmful to Tat binding (Pritchard et al., 1994). These phosphates are likely candidates for direct interactions with Tat, possibly with basic residues such as arginine or lysine.

Recent NMR studies of free TAR (Aboul-ela et al., 1996) and in the presence of Tat peptide (37−72) (Aboul-ela et al., 1995) have shown that the major groove of TAR in the region of the U-rich bulge is much more accessible than for normal A-type RNA helixes but is narrowed considerably upon peptide binding. A similar conformational change is obtained with a single arginine derivative, argininamide (Puglisi et al., 1992, 1993; Aboul-ela et al., 1995) and with the Tat peptide (Aboul-ela et al., 1995), suggesting that one key arginine residue in the basic region of Tat is sufficient to trigger this change, possibly by interaction with $G_{26}$ and $U_{23}$. All the functional groups on TAR identified by chemical substitution experiments to be important to Tat binding become clustered close to the surface of the duplex on the major-groove side in the NMR structure of the complex and are available for interaction with Tat. Of these, the identified phosphate residues may make essential contributions to the Tat−TAR recognition process via "indirect readout" through their precise positioning away from locations normally found for regular RNA duplexes (Aboul-ela et al., 1996).

Full NMR structure solution of the Tat peptide−TAR RNA complex is still in progress. Meanwhile, an alternative method capable of providing valuable information about possible contacts of specific amino acids to particular RNA residues is cross-linking. Wang and Rana showed that an N-terminal psoralen conjugate of Tat peptide 42−72 bound specifically to TAR and the psoralen could be photochemically cross-linked to $U_{42}$ in the lower stem (Wang & Rana, 1995). Another psoralen conjugate of Tat (38−72) to a unique $Cys^{57}$ residue, introduced synthetically to replace $Arg^{57}$, cross-linked to the TAR apical loop at position $U_{31}$ (Wang et al., 1996). Very recently, Liu et al. (1996) showed that $Tyr^{47}$ in the core region of Tat peptide 42−72 could be specifically UV-cross-linked to $G_{26}$. These experiments have suggested that the orientation of the basic region of Tat lies from N to C in the direction from lower TAR stem to upper TAR stem and loop. A second cross-linking strategy involves the introduction of reactive functionalities on TAR and the determination of acceptor sites on a Tat peptide. For example, a TAR duplex containing 4-thioU at position 23 has been photochemically cross-linked to Tat peptide 38−72, but so far the position(s) of cross-linking on the peptide could not be determined (Wang & Rana, 1996).

Recently a new method has been described for the site-specific incorporation of an activated trisubstituted pyrophosphate (tsp) into synthetic DNA duplexes (Kuznetsova et al., 1990, 1996; Purmal et al., 1992). It was shown that only minor distortion of the double helix occurs upon incorporation of this analogue. A DNA containing the recognition sequence of a restriction nuclease and substituted at the scissile bond by a tsp was found to be reactive toward active-site nucleophiles to form a covalent phosphoamidate bond (presumably with an arginine, histidine, or lysine) when incubated with a restriction enzyme (Purmal et al., 1992; Sheflyan et al., 1995). Formation of the cross-link was limited to specific DNA−enzyme recognition. We now report the successful incorporation of tsps into several sites in TAR RNA model duplexes and their subsequent specific covalent reaction with Tat peptide (37−72) or with Tat protein. Only phosphates in TAR expected to be in close proximity to Tat were sites where tsp reacted readily with Tat peptide. Analysis of the cross-linked product of TAR containing a tsp at site P38 in the upper stem with Tat peptide (37−72) indicated covalent reaction with the $\epsilon$-amino group of $Lys^{51}$ in the basic region of Tat.

## MATERIALS AND METHODS

*Synthesis of Tat Peptide (37−72).* The sequence SFTTKALGISYGRKKRRQRRRPPQGSGTHQVSLSKQ ($Cys^{37}$ was replaced by Ser to avoid problems with disulfide formation) was prepared by continuous-flow Fmoc-polyamide solid-phase peptide synthesis using *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone protection for the addition of $Gly^{44}$ (Johnson et al., 1993; Quibell et al., 1994).

*Preparation of Oligonucleotides.* Oligodeoxyribonucleotides were synthesized on a 1-$\mu$mol scale using controlled pore glass (CPG) by standard phosphoramidite synthesis using an Applied Biosytems 380B or 394 DNA/RNA synthesizer. Oligoribonucleotides were synthesized on a 1-$\mu$mol scale using 2′-*O*-(*t*-butyldimethylsilyl)nucleoside 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoroamidite monomers having phenoxyacetyl amino group protection for A

and G and benzoyl protection for C (Glen Research via Cambio). The syntheses were undertaken using standard RNA synthesis procedures as previously described (Pritchard et al., 1994; Schmidt et al., 1996). Oligonucleotides containing a 3′-phosphate were prepared by use of "3′-phosphate CPG" (Glen Research) or by using the "chemical phosphorylation agent" as the first coupling step on to any deoxynucleotide-substituted CPG support and a deoxy amidite in the second coupling step as appropriate. Thereafter synthesis was carried out by standard synthetic procedures. Oligoribonucleotides were deprotected by suspension of the controlled pore glass in methanolic ammonia overnight, decanting and, evaporation of the resultant solution to dryness. Treatment with triethylamine trihydrofluoride/DMF (3:1) at 50 °C for 1.5 h was used to remove silyl groups. Desalting by Sephadex NAP10 (Pharmacia) filtration or by butanol precipitation was carried out as described previously (Gait et al., 1991) and products were purified by anion-exchange HPLC on NucleoPac PA100 (Dionex) as previously described (Schmidt et al., 1996). Desalting was achieved via extensive dialysis against water. Purities of all oligonucleotides were checked by polyacrylamide gel electrophoresis (PAGE) analysis of $^{32}$P-labeled or unlabeled samples. The presence of a 3′-phosphate was confirmed by treatment of a sample of oligonucleotide with alkaline phosphatase or with ethylenediamine and 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDC) at pH 4.5 and observation of the mobility change by PAGE analysis and UV shadowing. 5′-Phosphorylation of oligoribonucleotides was carried out by treatment with T4 polynucleotide kinase in the presence of ATP as previously described (Slim & Gait, 1991).

*Synthesis of Oligonucleotide 3′-Alkylphosphates. Method 1: Postsynthetic Modification with an Alcohol.* Dry oligonucleotide (1−10 nmol) carrying a 3′-phosphate was dissolved in 30 $\mu$L of MES buffer (0.05 M MES and 0.02 M $MgCl_2$ titrated to pH 4.5 with sodium hydroxide solution). The alcohol (20 $\mu$L, ethanol or methanol) was added and the solution was thoroughly vortexed. After the sample was cooled to 10 °C, 5 mg of EDC was added and the solution was incubated for 6 h at 5 °C. The oligonucleotides were precipitated by adding 200 $\mu$L of 2 M lithium perchlorate solution and 1 mL of acetone, incubating at −20 °C for 2 h, and centrifuging for 5 min at 13 000 rpm. The supernatant was decanted off, and the precipitate was washed with 300 $\mu$L of acetone and dried in air.

*Method 2: Solid-Phase Synthesis.* This involved coupling of 1.5 M 5′-dimethoxytrityl-2′-*O*-(*t*-butyldimethylsilyl)-3′-(*N*,*N*-diisopropylamino)nucleoside ethylphosphite (Koziolkiewicz & Wilk, 1993) to $\beta$-sulfoethyl-controlled pore glass (Volkov et al., 1988) with a nucleoside loading of 40 $\mu$mol $g^{-1}$, followed by oligoribonucleotide synthesis in the usual way. Deprotection of the oligonucleotides, analysis of reaction mixtures, and oligonucleotide isolation were carried out as described previously (Pritchard et al., 1994; Schmidt et al., 1996).

*Small-Scale Synthesis of Oligonucleotides with Trisubstituted Pyrophosphate Internucleotide Linkage.* The oligonucleotide (90 pmol) with [5′-$^{32}$P]phosphate, 100 pmol of the oligonucleotide with 3′-alkylphosphate, and 105 pmol of the DNA template oligonucleotide were dissolved in 20 $\mu$L of MES buffer (pH 6.0) and heated to 90 °C for 2 min. After the sample was slowly cooled to 20 °C, 2 mg of EDC was added, and the solution was thoroughly vortexed and incubated at 4 °C for 48 h. tRNA (5$\mu$g) was added to the reaction mixture and the oligonucleotides were precipitated as described above, dissolved in 10 $\mu$L of gel-loading solution [80% (v/v) formamide, 1 mM EDTA, 0.05% (w/v) bromophenol blue, and xylene cyanol], and isolated on a 20% (w/v) polyacrylamide/7 M urea gel (19:1 acrylamide:bisacrylamide, 0.5 mm thick). The gel was visualized by autoradiography, the band corresponding to the ligation product was excised from the gel, and the oligonucleotide was recovered by passive elution into 200 $\mu$L of 2 M $LiClO_4$. Acetone (1 mL) was added to the eluate and the oligonucleotide was precipitated as described above. Yields of ligation were determined by excision of bands and Cerenkov counting in a Beckman scintillation counter.

*Large Scale Synthesis of Oligonucleotides with Trisubstituted Pyrophosphate Internucleotide Linkage.* The oligonucleotide (20 nmol) with 5′-phosphate, 20 nmol of the oligonucleotide with 3′-alkylphosphate, and 22 nmol of the DNA template oligonucleotide were dissolved in 50 $\mu$L of MES buffer, pH 6.0, and the annealing was carried out as described above. EDC (5 mg) was added, and the solution was thoroughly vortexed and incubated at 4 °C for 72 h. The oligonucleotides were precipitated as described above, dissolved in 20 $\mu$L of gel-loading solution [80% (v/v) formamide and 1 mM EDTA] and isolated on a 20% polyacrylamide/7 M urea gel (0.5 mm thick), 2 $\mu$L of the solution being loaded into a single 10-mm width well. The gel was visualized by UV shadowing, the bands corresponding to the ligation product were excised from the gel, and the oligonucleotide was recovered by passive elution into 200 $\mu$L of 2 M $LiClO_4$. Acetone (1 mL) was added to the eluate and the oligonucleotide was precipitated as described above. *m*/*z* (MALDI-TOF) for tsp 38−39 (dU) calculated 4466.8, observed 4470.8.

*Stability of Oligonucleotides Containing Trisubstituted Pyrophosphate Internucleotide Linkages.* $^{32}$P-Labeled oligoribonucleotides containing trisubstituted pyrophosphate (100 nM) were treated under various buffer conditions at 10 °C for 16 h in a total volume of 10 $\mu$L in either water, MeImK buffer [50 mM 1-methylimidazole, 20 mM KCl, 10 mM dithiothreitol (DTT), and 0.01% Triton X-100, pH 8.5], or TK buffer (the same as MeImK but containing 50 mM Tris-HCl, pH 8.5, instead of 1-methylimidazole). In some cases ethylenediamine (EDA) (0.5 M, pH 8.5) or lysine (0.5 M, pH 8.5) was used alone or in addition to buffer (see Figure 3). A similar method was used for ethylguanidine, ethanol, and *p*-cresol treatments (data not shown). Samples were subjected to 20% PAGE and visualized by autoradiography. Quantitation of bands was carried out by drying of the gel and then by use of a Molecular Dynamics PhosphorImager.

*Filter Binding Assays.* Binding reactions (250 $\mu$L) contained 2 nM $^{32}$P-labeled duplex TAR RNA (natural or modified), 1 unit of RNasin (Promega), 0.4 $\mu$g $mL^{-1}$ tRNA, 1.0 $\mu$g $mL^{-1}$ calf thymus DNA, 50 mM Tris-HCl (pH 7.4), 20 mM KCl, and 0−2000 nM Tat peptide (37−72). Reactions were incubated on ice for 60 min in individual wells of a prewetted 96-well Multiscreen-HA mixed cellulose ester plate (Millipore) and filtered simultaneously using a manifold (Millipore). Each well was washed once with 200 $\mu$L of ice-cold 50 mM Tris-HCl (pH 7.4), and 20 mM KCl, dried briefly under a lamp, and punched into scintillation vials, and the number of radioactive counts was determined by liquid scintillation.

*Gel Mobility Shift Assays.* To prepare the TAR model duplexes, a solution of 10 pmol of natural or modified r14-mer ($2 \times 10^6$ cpm) and 12 pmol of r17-mer in water was incubated at −20 °C for 1 h and thawed at room temperature. The duplex (0.1−1 pmol) was then added into each of the binding reaction mixtures (10 $\mu$L total volume) containing (i) 50 mM Tris-HCl (pH 8.5), 20 mM KCl, 10 mM DTT, and 0.01% (v/v) Triton X-100 or (ii) 50 mM 1-methylimidazole (pH 8.5), 20 mM KCl, 10 mM DTT, and 0.01% (v/v) Triton X-100. Tat peptide (37−72) was added as indicated and the solution was incubated on ice for 30 min. The mixtures were combined with 5 $\mu$L of gel-loading solution [40% (w/w) sucrose and 0.1% (w/v) bromophenol blue] and applied to a preequilibrated (4 °C) 8% native polyacrylamide gel (1 mm thick). The gel was run in 1× TB (50 mM Tris-HCl and 50 mM boric acid, pH 8.3), 0.01% Triton X-100, and 0.2% glycerol at 4 °C for 1 h, dried in vacuum, and visualized by autoradiography with Fuji RX film at room temperature.

*Covalent Cross-Linking of Chemically Reactive TAR Duplex to Tat Peptide and Protein.* Reaction mixtures (10 $\mu$L total volume) were prepared as described for the gel mobility shift assays and incubated for 1 h on ice to allow Tat−TAR binding and at 10 °C for 18−24 h to allow cross-linking. Either Tat peptide (37−72) or Tat protein (Karn et al., 1995) was used. Then 5 $\mu$L of gel-loading solution [40% (w/w) sucrose, 5% SDS, and 0.1% (w/v) bromophenol blue] was added, and the reaction mixtures were incubated at 90 °C for 5 min and applied to an SDS−8% or 10% polyacrylamide gel (1 mm thick). The gel was run in 1× TB, 0.1% sodium dodecyl sulfate, and 0.2% glycerol for 1 h, dried in vacuum, and visualized by autoradiography with Fuji RX film at room temperature. Very recently we have found that addition of 7 M urea and 0.05% $\beta$-mercaptoethanol to the gel loading buffer leads to sharper bands in most cases (data not shown).

*Large Scale Cross-Linking and Isolation.* Unlabeled r14-mer (tsp 38−39, 5 nmol) and 10 pmol of the same RNA carrying a $^{32}$P label at the phosphorus atom in the disubstituted phosphate of the modified linkage were mixed with 5.5 nmol of r17-mer in a total volume of 50 $\mu$L of water and kept at −20 °C for 2 h to form the modified TAR duplex. MeImK buffer solution [20 mL of 50 mM 1-methylimidazole (pH 8.5) and 20 mM KCl] was cooled in ice and 8 nmol of the Tat peptide (37−72) was added. Finally, the preformed modified TAR duplex was added, and the reaction mixture was kept on ice for 30 min to allow for complex formation and then incubated at 10 °C for 20−24 h. Purification method 1: The solution was lyophilized and salts were removed by gel filtration on a Sephadex NAP-10 column (Pharmacia) in water and lyophilized again. The residue was dissolved in 50 $\mu$L of the gel loading solution (40% sucrose, 0.1% SDS, 7 M urea, and 0.1% bromophenol blue), incubated at 90 °C for 5 min, and applied to an SDS−8% polyacrylamide gel (1 mm thick), 5 $\mu$L being loaded in a single well. The gel was run at 30 mA for 45 min and wrapped with Saran-wrap film on a glass plate, and the position of the cross-linking product was determined by autoradiography at −70 °C. A part of the gel containing the cross-linking product was excised and the material was recovered by elution overnight in 10 mM Tris-HCl, 50 mM NaCl, and 1 mM EDTA (pH 7.5). Supernatant was applied to a Sephadex NAP 10 column to remove salts and then lyophilized. Purification method 2: The residue was dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) and injected immediately on a reversed-phase HPLC column (Spheri-5 RP8, Brownlee Laboratories) and eluted with a linear gradient of 13.5−54% (v/v) acetonitrile in 0.1% trifluoroacetic acid (Figure 9). Collected peak fractions were immediately cooled on dry ice to prevent degradation by acidic cleavage of the phosphoamidate bond between peptide and oligonucleotide fragment.

*Mass Spectral Analysis.* A Hewlett-Packard G2025A MALDI-TOF mass spectrometer was used to measure the molecular masses of trisubstituted pyrophosphate derivatives of oligonucleotides and of their covalent reaction products with ethylenediamine and lysine, as well as the masses of the HPLC-isolated products from the reaction of Tat peptide (37−72) with the TAR duplex containing tsp 38−39 (Figure 10a). Ionization was in positive mode and the matrix was a 1:1 mixture of diammonium hydrogen citrate (80 mg mL$^{-1}$ in water) and 2,6-dihydroxyacetophenone (60 mg mL$^{-1}$ in methanol). An aliquot of Tat peptide (37−72) or tsp 38−39 RNA product cross-linked to Tat peptide (37−72) (peak 1) was digested with 0.5 $\mu$L of endopeptidase Lys C (Boehringer, 3 units/40 $\mu$L) for 90 min at 37 °C in 0.1 M ammonium bicarbonate solution, mixed 1:1 with matrix, and dried onto the probe tip for mass spectral analysis (Figure 10b,c). An aliquot of peak 1 was also subjected to chymotrypsin (Boehringer, 1 $\mu$L of 10 $\mu$g mL$^{-1}$) digestion for 20 min at room temperature in 0.1 M ammonium bicarbonate solution and dried onto the tip with matrix as before. Calibration was generally by means of an external standard (HP peptide standard). However, for the endopeptidase Lys C experiments, fragments *m*/*z* 964 (Tat 42−50) and 2530 (Tat 51−71 + 52−72) were used as internal standards.

## RESULTS

*Synthesis of Tsp-Modified Oligonucleotides.* It has been shown previously that a tsp could be incorporated at a specific site in a synthetic oligodeoxyribonucleotide (Kuznetsova et al., 1990; Purmal et al., 1992) or in a mixed oligoribo-deoxyribonucleotide (Naryshkin et al., 1996) by template-directed chemical ligation using a water-soluble carbodiimide (EDC). We investigated whether it was possible to incorporate a similar tsp site-specifically into the TAR RNA duplex (Figure 1b). We chose to replace individually three phosphate sites (P38−39, P39−40, and P40−41),[1] all positions thought to be close to the Tat protein in the complex (Hamy et al., 1993; Pritchard et al., 1994). As control we chose P42−43, which was not shown to be protected by ethylnitrosourea interference analysis (Hamy et al., 1993). The method involves ligation of two oligoribonucleotide strands using a template of an oligodeoxyribonucleotide (Figure 2), purification of the joined product, and annealing to a second unmodified oligoribonucleotide strand to form the model TAR duplex containing the single trisubstituted pyrophosphate modification (Figure 1b).

Accordingly, the hexanucleotide GCUGCUpOEt (i.e., carrying a 3′-ethylphosphate) and the octanucleotide [$^{32}$P]pCUCUGGCU were annealed to a 16-mer oligodeoxyribonucle-

[1] For clarity we will refer to individual internucleotide linkages by the nucleoside residues 5′ and 3′ to the linkage, i.e., 38−39, 39−40, and 40−41.

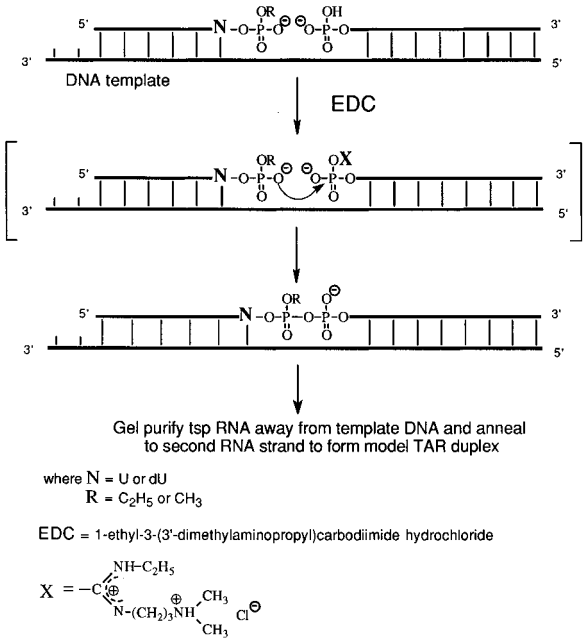


FIGURE 2: Chemical ligation of two oligoribonucleotide strands using an oligodeoxyribonucleotide template to form a trisubstituted pyrophosphate linkage. The intermediate product of EDC-activated oligonucleotide is shown in brackets.

Table 1: Yields of Ligation Products in the TAR (RNA/DNA) Hybrid Duplexes

| type of bond and position in TAR duplex | 3′-p or 3′-pOR acceptor | 5′-p donor | yield (%) |
|---|---|---|---|
| pp 38−39 | GCUGCdUp | pCUCUGGCU | 90 |
| tsp 38−39 (rU) | GCUGCUpOEt | pCUCUGGCU | 1−2 |
| tsp 38−39 (dU) | GCUGCdUpOMe | pCUCUGGCU | 38 |
| tsp 38−39 (dU) | GCUGCdUpOEt | pCUCUGGCU | 35 |
| tsp 39−40 | GCUGCUdCpOEt | pUCUGGCU | 55 |
| tsp 40−41 | GCUGCUCdUpOEt | pCUGGCU | 44 |
| tsp 42−43 | GCUGCUCUCdUpOEt | pGGCUAC | 36 |

otide (dAGCCAGAGAGCAGCAC) of precisely complementary sequence to the aligned oligoribonucleotide strands except for a 2-nucleotide extension at the 3′-end (Figure 2). Surprisingly, addition of EDC and incubation at 4 °C for 48 h led to only a small yield (1−2%) of joined 14-mer [Table 1, tsp 38−39 (rU), and Figure 3, lanes 1 and 2]. By contrast, when the 6-mer GCUGCdUpOEt containing a single 3′-terminal 2′-deoxynucleoside was used in place of the all-ribo 6-mer, the yield increased to 35% [Table 1, tsp 38−39 (dU), and Figure 3, lane 3]. A yield of 38% was obtained for the 6-mer GCUCGdUpOMe (Figure 3, lane 4). It is likely that the proximity of the adjacent 2′-hydroxyl group leads to rapid cleavage of the formed trisubstituted pyrophosphate linkage but that the use of a single 3′-terminal 2′-deoxynucleotide in the ligation site obviates this problem. The presence of a single 2′-deoxynucleotide in model TAR RNA duplexes has no significant effect on Tat binding for a range of positions tested within the duplex regions (Hamy et al., 1993; Pritchard et al., 1994).

Yields of 36−55% were obtained for incorporation of a tsp by chemical ligation into the three other positions (39−40, 40−41, and 42−43) of the same oligonucleotide sequence (Table 1). Interestingly, a 90% yield was obtained for formation of a pyrophosphate linkage in the position 38−39 (Table 1). By contrast, much lower yields were obtained in chemical ligation reactions when the 16-mer oligodeoxyribonucleotide template was replaced by a 16-mer oligoribonucleotide of the same sequence (data not shown). Oligonucleotides containing tsp linkages were separated from oligodeoxyribonucleotide templates by denaturing 20% PAGE (e.g., Figure 3). MALDI-TOF mass spectrometry of tsp 38−39 (dU) isolated on a larger scale showed a molecular mass characteristic of the expected trisubstituted pyrophosphate-containing oligonucleotide.

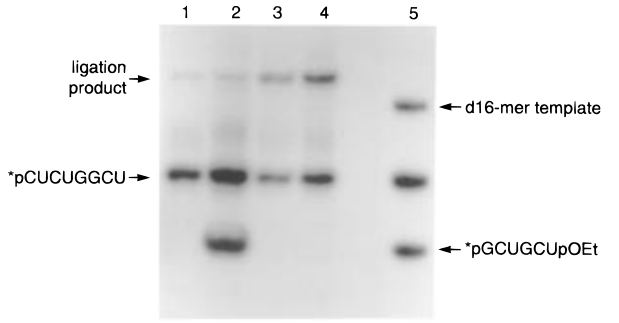


FIGURE 3: Polyacrylamide gel electrophoresis showing formation of r14-mer tsp 38−39 by EDC-induced chemical ligation. d16-mer is the oligodeoxyribonucleotide template used for the ligation (dAGCCAGAGAGCAGCAC). Lane 1, ligation of r6-mer (OEt) with $^{32}$Pr8-mer; lane 2, ligation of $^{32}$Pr6-mer(POEt) with $^{32}$Pr8-mer; lane 3, ligation of r5-mer dU(OEt) with $^{32}$Pr8-mer; lane 4, r5-mer dU(OMe) with $^{32}$Pr8-mer; lane 5, marker $^{32}$P-labeled r6-mer, r8-mer, and d16-mer. *p denotes $^{32}$P.

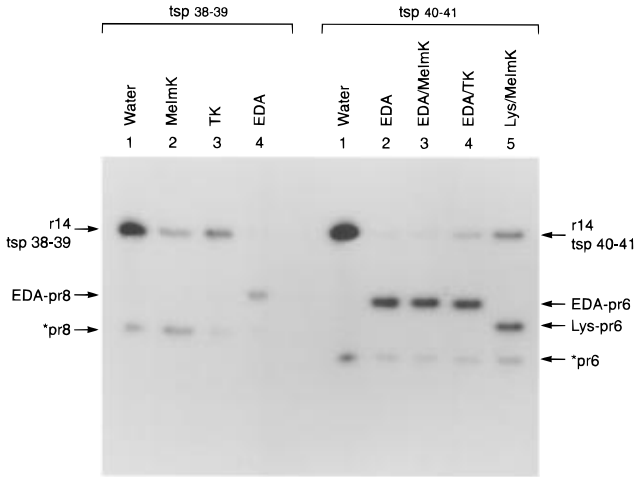


FIGURE 4: Polyacrylamide gel electrophoresis showing the stability of r14-mers tsp 38−39 and tsp 40−41 toward hydrolysis in water and in various buffers (see Materials and Methods) and reaction of the chemically active pyrophosphate linkage with ethylenediamine (EDA) or lysine.

The stability of the tsp linkage to hydrolytic cleavage was tested. Less than 5% cleavage was observed in water (pH ca. 5−6) for 16 h at 10 °C (Figure 4). Approximately 40% cleavage took place in MeImK buffer (pH 8.5) but less than 5% in TK buffer (pH 8.5). The tsp linkage was much more prone to nucleophilic attack by certain amines. Thus complete reaction to give an EDA−oligonucleotide conjugate, which has slower electrophoretic mobility than the oligonucleotide hydrolysis product, was obtained with 0.5 M ethylenediamine (EDA, pH 8.5) in water or in MeImK buffer (Figure 4). In TK buffer, EDA reacted more than 90%. About 60% reaction with lysine in MeImK buffer was achieved in the same time period. However, no reaction took place with *N*-ethylguanidine, *p*-cresol, or ethanol under the same conditions (data not shown). Very similar results were obtained for hydrolytic cleavage and nucleophilic attack

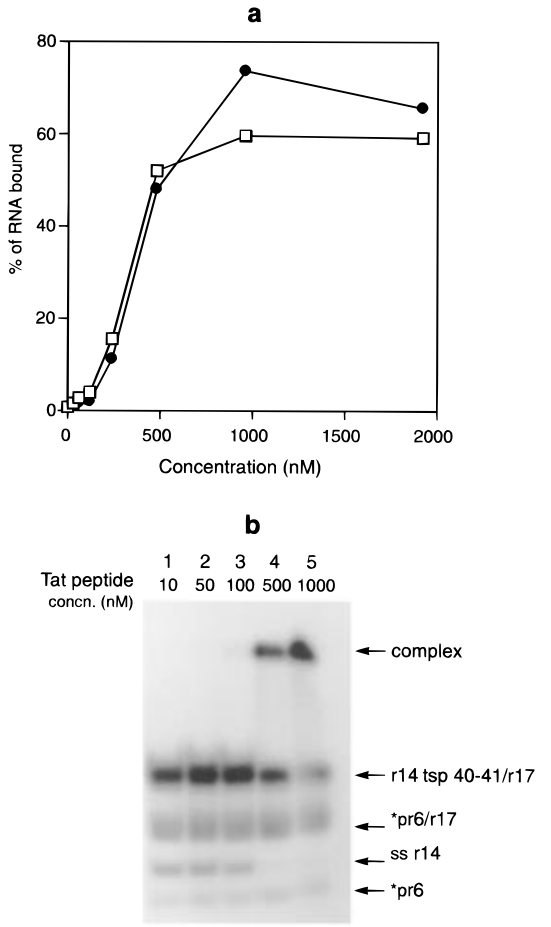


FIGURE 5: (a) Filter binding assay showing the amount of complex formation of the duplex of r17-mer and r14 tsp 40−41 (filled circles) and unmodified duplex r17/r14 (open squares) with increasing concentrations of Tat peptide (37−72); (b) polyacrylamide gel mobility shift assay showing complex formation of the duplex of r17-mer with r14 tsp 40−41 with increasing concentrations of Tat peptide (37−72).

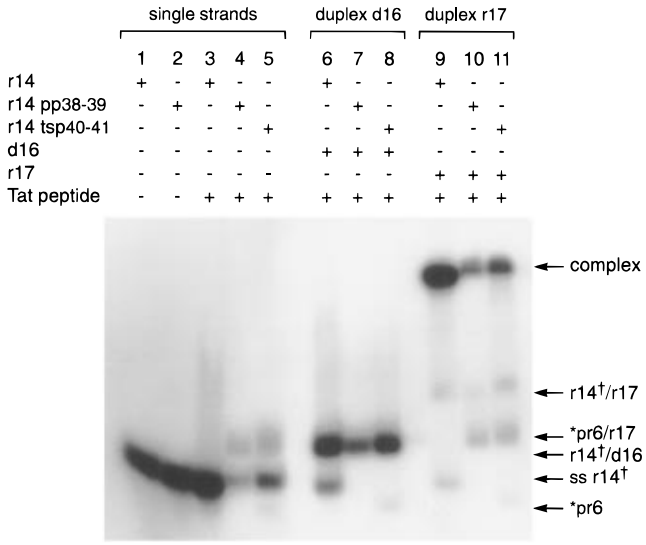


FIGURE 6: Polyacrylamide gel mobility shift assays showing the specificity of complex formation between 500 nM Tat peptide (37−72) and unmodified or modified TAR single stranded r14-mers (lanes 3−5), their duplexes with complementary d16-mer (lanes 6−8), or their duplexes with TAR r17-mer (lanes 9−11). Lanes 1 and 2 show unmodified and modified TAR 14-mers alone. †The type of r14-mer arrowed depends on the substitution type shown in the key (top left); *pr6 is the 1-methylimidazole-catalyzed hydrolysis product of r14-mer tsp.

when the oligonucleotide was annealed to its complementary RNA to form the model TAR duplex (data not shown). These results suggest that tsp linkages may be susceptible to attack by lysine residues in proteins but the likelihood of reaction is much less with hydroxylic amino acid side-chain nucleophiles or with arginine side chains in proteins, at least at this pH (8.5), presumably because of protonation.

The ethylenediamine and lysine adducts were characterized by MALDI-TOF mass spectrometry (data not shown) and the molecular masses agreed with expectations that nucleophilic attack takes place on the less substituted of the two phosphates in the trisubstituted pyrophosphate linkage to yield a covalent phosphoamidate adduct of the amine with the original ligation oligonucleotide component that contained the 5′-phosphate (Kuznetsova et al., 1990, 1996; Purmal et al., 1992).

*Binding of tsp-Modified TAR Duplexes to Tat Peptide (37−72).* The purified oligonucleotides containing tsp linkages were each annealed to a complementary 17-mer oligoribonucleotide to form a series of singly modified TAR RNA model duplexes, and their affinities for Tat peptide (37−72) were measured. For example, the TAR duplex of 17-mer strand annealed to the 14-mer tsp 40−41 [one of the sites thought by methylphosphonate substitution analysis most likely to be forming a direct contact to Tat (Pritchard et al., 1994)] bound to Tat peptide (37−72) indistinguishably from the binding of unmodified TAR duplex, as shown by a stringent filter binding assay in the presence of competitor DNA and tRNA (Figure 5a). The binding affinity of tsp 40−41 duplex to Tat protein was only slightly reduced compared to the unmodified TAR duplex (data not shown). It should be noted that the time scale of these assays is short and the pH is 1 unit lower compared to cross-linking experiments (see below) and therefore the majority of this binding is expected to be noncovalent under these conditions.

Complex formation with Tat peptide (37−72) was also evaluated by polyacrylamide gel mobility shift analysis. Titration of 10 nM tsp 40−41 duplex RNA showed that about 50% complex formation was obtained with 500 nM Tat peptide (37−72) (Figure 5b). Complex formation was also seen in each case (Figure 6) when 500 nM Tat peptide was added to 50 nM TAR duplexes containing the ribo-17-mer annealed to unmodified ribo-14-mer (lane 9), pyrophosphate 38−39 (lane 10), or tsp 40−41 (lane 11). By contrast, no complex formation was seen for Tat peptide with single-stranded 14-mer RNAs (lanes 3−5) or with duplexes formed from ribo-14-mers and the 16-mer oligodeoxynucleotide template (lanes 6−8). Complexes of Tat peptide with TAR RNA duplexes containing tsp 38−39, tsp 39−40, or tsp 42−43 were also formed and had similar affinities (data not shown). These results show that the TAR RNA model duplexes containing tsp linkages form complexes with Tat peptide (37−72) which are indistinguishable from the complex formed with unmodified TAR RNA duplex.

*Chemical Cross-Linking of TAR Duplexes to Tat Peptide (37−72) or Tat Protein.* We next investigated the ability of TAR duplexes containing tsp linkages to form covalent cross-links to Tat peptide (37−72) or to Tat protein. When 100 nM duplex tsp 38−39 (OMe)/r17 was incubated with 400 nM Tat peptide (37−72) for 18−24 h at 10 °C, there was approximately 50% cross-linking to give predominantly

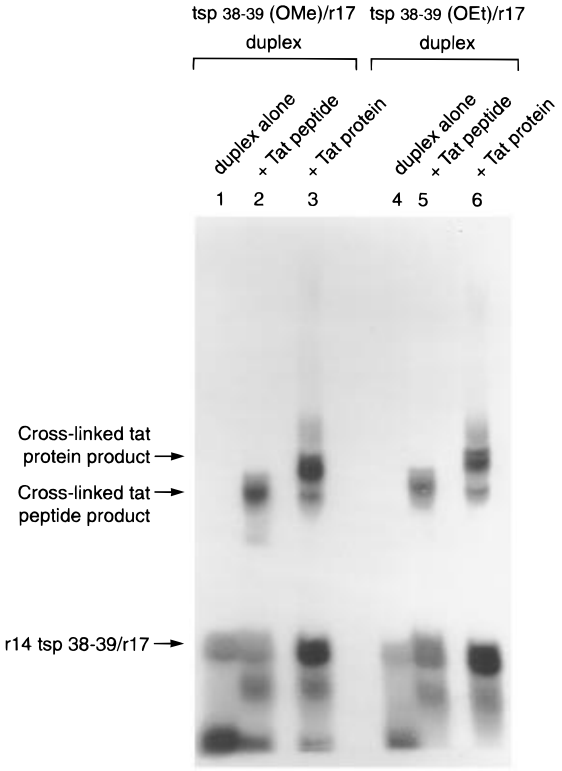


FIGURE 7: SDS−polyacrylamide gel electrophoresis showing cross-linking between Tat peptide (37−72) or Tat protein and TAR RNA duplexes of r17-mer with tsp 38−39 (OMe) (lanes 1−3) or tsp 38−39 (OEt) (lanes 4−6).

a single band upon SDS−polyacrylamide gel electrophoresis (Figure 7, lane 2). Tat protein (60 nM) reaction with tsp 38−39 (OMe)/r17 (15 nM) produced a lower mobility cross-linked product under the same conditions (lane 3). By contrast, in the absence of Tat peptide or protein, the main product was the hydrolytically cleaved oligonucleotide (lane 1). Similar results were found for cross-linking of the tsp 38−39 (OEt)/r17 duplex (lanes 4−6). No cross-linking was seen under these conditions of tsp 38−39 single strand, unmodified duplex, or duplex with an unsubstituted pyrophosphate linkage to Tat protein or Tat peptide (data not shown).

We now wished to compare the ability of TAR duplexes of r17 with tsp 38−39, 39−40, 40−41, or 42−43 to each form cross-links to Tat peptide (37−72) at 500 or 300 nM concentrations. For the first three tsp positions there was good cross-linking (Figure 8, lanes 1−3 and 7−9). By contrast, with the duplex of r17 with tsp 42−43, where the tsp lies outside the region of previously defined ethylation protection, only a trace of cross-linking was seen (lanes 4 and 10). Cross-linking could be completely suppressed by competition with a 7-fold excess of unlabeled and unmodified r14/r17 TAR duplex but only 20% suppressed by competition with a 7-fold excess of a bulgeless heteroduplex of r14 with 16-mer template oligodeoxynucleotide (data not shown). Thus only in those complexes where a nucleophilic residue in the Tat peptide comes into close proximity with the tsp in the TAR RNA was significant cross-linking obtained.

*Characterization of Cross-Linked Products*. The cross-linked products were characterized to assess which nucleophilic residue might be in the closest position to react with the tsp linkage. For an initial evaluation we chose to cross-link Tat peptide (37−72) to tsp 38−39 TAR RNA duplex

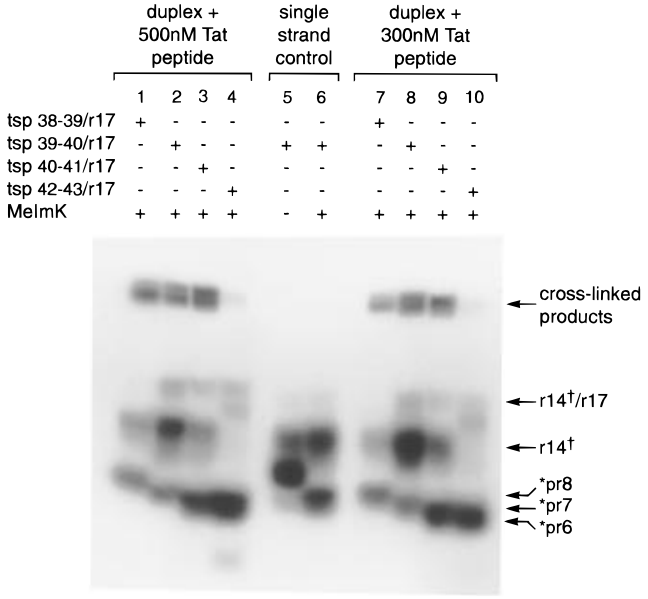


FIGURE 8: SDS−polyacrylamide gel electrophoresis showing cross-linking between Tat peptide (37−72) and duplexes of r17-mer with tsp 38−39, 39−40, or 40−41 (lanes 1−3 and 7−9) but not with tsp 42−43 (lane 4 and 10); lanes 5 and 6, tsp 39−40 duplex with r17-mer in the absence or presence of MeImK buffer only. †For tsp 42−43, the oligonucleotide containing the tsp modification is a 16-mer. *pr6, *pr7, and *pr8 are the 1-methylimidazole-catalyzed hydrolysis products of the respective tsp oligonucleotides.

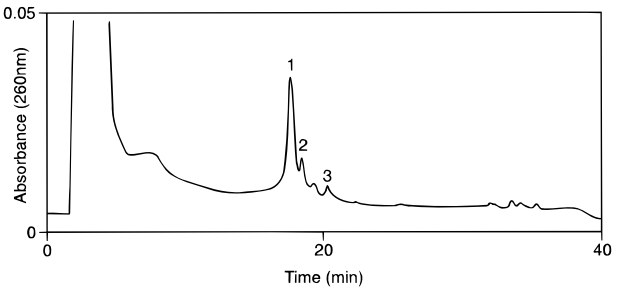


FIGURE 9: Chromatogram showing reversed-phase HPLC purification of cross-linked product of Tat peptide (37−72) with tsp 38−39 TAR RNA duplex (for conditions see Materials and Methods).

on a preparative scale. When the products were separated by SDS−polyacrylamide gel electrophoresis, a pattern was seen similar to that obtained analytically (Figure 8, lane 1). The most retarded radioactively labeled band expected to contain the cross-linked product was eluted and desalted. As expected for a conjugate containing a phosphoamidate linkage, treatment with 4 M hydroxylamine (pH 5) (Rjabova et al., 1965; Shabarova, 1970) completely cleaved it to give a nucleic acid product of faster electrophoretic mobility (data not shown). Since preliminary mass spectral analysis of the gel-eluted product showed that there was still considerable contamination with unreacted Tat peptide (37−72), an alternative method of purification by reversed-phase HPLC was used (Figure 9). Most (90%) of the cross-linked material that was UV-absorbing at 260 nm was eluted in a single peak (peak 1). Peak 1 was also strongly absorbing at 218 nm (data not shown). MALDI-TOF mass spectrometry showed that it corresponded in mass to that of the expected phosphoamidate conjugate (*m*/*z* found 6704, calculated 6701; Figure 10, panel a) of Tat (37−72) with the fragment of TAR RNA (pr8-mer) produced by attack of an amino nucleophile at the tsp 38−39 linkage. The minor product

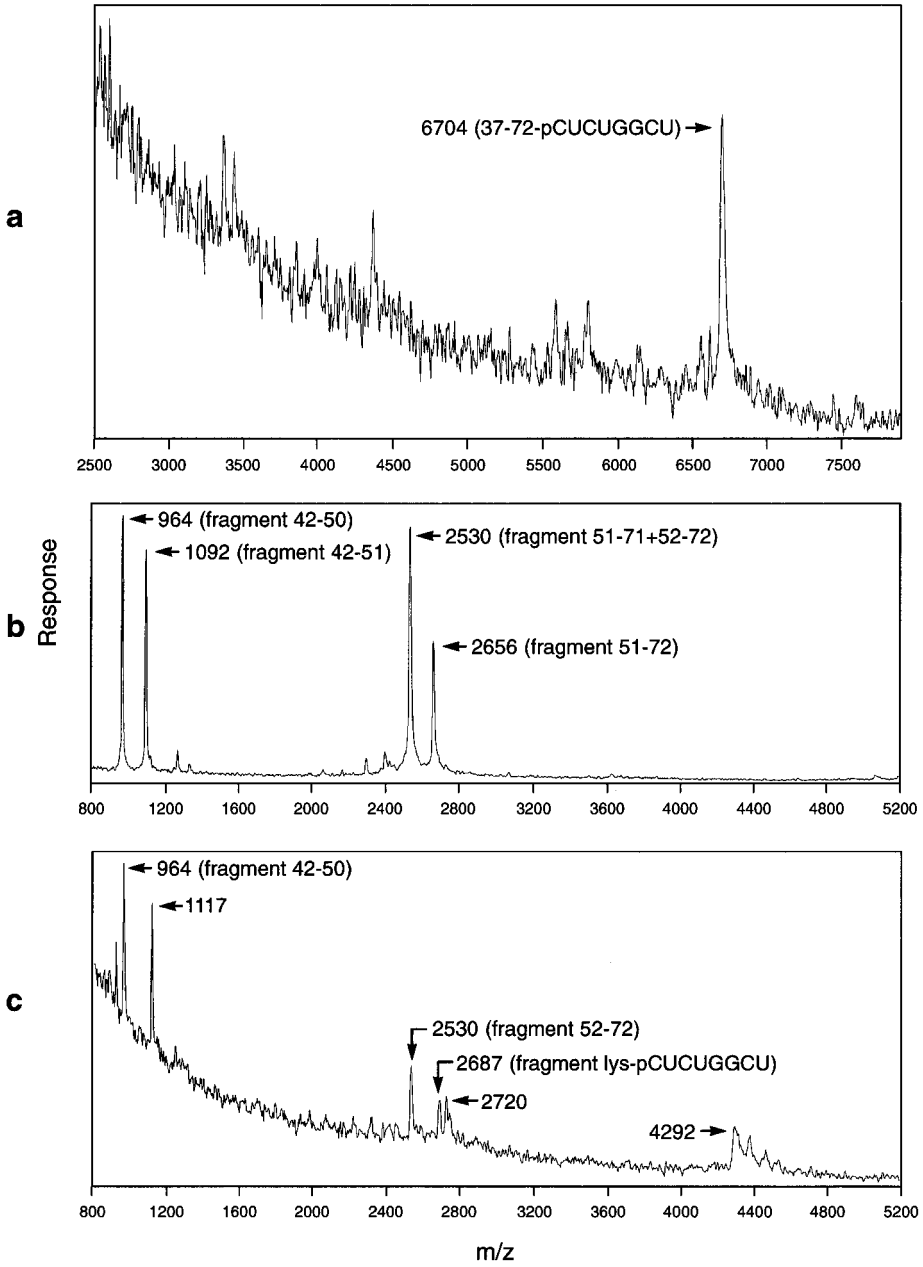


FIGURE 10: MALDI-TOF mass spectra: (a) peak 1 of HPLC-purified product of Tat peptide (37−72) reaction with tsp 38−39 TAR RNA duplex; (b) digestion of Tat peptide (37−72) with endopeptidase Lys C; (c) digestion of peak 1 product with endopeptidase Lys C.

(Figure 9, peak 2) had a mass of 6711 and was not identified further. A second minor product (Figure 9, peak 3) had much higher absorption at 218 nm and was identified as free Tat peptide (37−72) by its elution position compared to a standard and its mass (*m*/*z* found 4170, calculated 4170)(data not shown).

Since we expected the phosphoamidate bond between peptide and oligonucleotide to be cleaved under the harsh acidic conditions of Edman peptide sequencing, we tried to find an alternative analytical procedure that avoided the use of strong acids to determine the site on the Tat peptide bonded to the oligonucleotide. We have found that a combination of endopeptidase treatment followed by mass spectral fragment analysis was very effective in this regard. Treatment with chymotrypsin of peak 1 product from the HPLC purification, which would be expected to cleave after the single tyrosine at position 47, gave a single large fragment of *m*/*z* 5540 (spectrum not shown). This agrees well with the expected mass for fragment 48−72 covalently attached to pCUCUGGCU (*m*/*z* 5533) and shows that the oligonucleotide could not have been attached to the other smaller fragment containing Lys[41]. Peak 1 product from the HPLC purification was also treated with endopeptidase Lys C, expected to cleave only after lysines, and the product was subjected to mass spectroscopy. Whereas Tat peptide (37−72) alone after 90 min of digestion with endopeptidase Lys C gave four main peaks consistent with the expected cleavages or partial cleavages at all the four lysine sites (Figure 10, panel b), the similarly treated product of peak 1 showed a different pattern (panel c). The peaks at *m*/*z* 964 (fragment 42−50) and 2530 (fragment 52−72) were still present but the peaks at *m*/*z* 1092 (fragment 42−51) and 2656 (fragment 51−72) were absent. In their place were fragments at *m*/*z* 1117, 2687, and 2720 and a series of peaks beginning at *m*/*z* 4292. We tentatively assign the peak at *m*/*z* 2687 as the conjugate Lys−pCUCUGGCU (calculated *m*/*z* 2679). As yet we are unable to assign the other new peaks with confidence. Nevertheless, the presence of the

peaks at *m*/*z* 964, 2530, and 2687 as well as the absence of the peaks at 1092 and 2656 can only be explained if the conjugate peak 1 has been produced from attack of the $\epsilon$-amino group of Lys[51] in the basic region of the Tat peptide (37−72) on the tsp 38−39 duplex RNA at the position equivalent to P38−39 to give an oligonucleotide 5′-phosphoamidate product.

## DISCUSSION

We have shown that a tsp linkage may be introduced readily into a defined position in an oligoribonucleotide by means of a chemical ligation procedure involving a DNA template. Good ligation yields depended on the use of a single 3′-terminal 2′-deoxynucleoside in the ligation site. As had been suggested from some previous studies on incorporation of activated tsps into various types of DNA or mixed RNA/DNA duplex (Kuznetsova et al., 1990, 1996; Naryshkin et al., 1996), we found that tsp linkages incorporated into oligoribonucleotides or RNA duplexes (containing the requisite single 2′-deoxynucleoside) are relatively stable to hydroxylic nucleophiles but could be cleaved readily by primary amino nucleophiles. However, the tsp-containing oligonucleotide failed to react with *N*-ethylguanidine at pH 8.5. We then showed that a tsp incorporated into a model TAR RNA duplex reacted uniquely with a lysine residue in Tat peptide (37−72) even though there are neighboring arginine residues present. These results suggest that tsp linkages are useful as unique probes of the proximity of lysine residues to phosphates in RNA−protein interactions. The results also explain why a tsp linkage incorporated in place of a suitable phosphate in a model HIV-1 Rev-responsive element (RRE) high-affinity site (Pritchard et al., 1994) failed to show cross-linking to Rev protein (data not shown), since there are no lysines in the basic RNA-binding region of Rev. The basic region of Tat enters the major groove of TAR, which in the absence of ligands is unusually accessible due to the presence of the three bulged pyrimidine residues, and induces a pronounced conformational change in TAR. The conformational change can be mimicked by a single argininamide (Puglisi et al., 1992, 1993; Aboul-ela et al., 1995). It seems clear that when such a basic ligand binds to TAR, the bulged residues become looped out of the helix, creating a binding pocket that places the guanidinium and $\delta$-NH groups of the arginine within hydrogen-bonding distance of functional groups on $G_{26}$ and $U_{23}$.

From RNA binding and *trans*-activation analysis of Tat proteins containing mutated basic regions, it was proposed that Arg[52] is crucial to this recognition process (Tao & Frankel, 1992, 1993). It was further proposed that an arginine in Tat forms contacts with phosphates 21−22 and 22−23 (Calnan et al., 1991b). It is possible for a single arginine to carry out both of these roles (Puglisi et al., 1993) and there is a similar proposal in the case of the BIV (bovine immunodeficiency virus) Tat−TAR interaction (Puglisi et al., 1995; Ye et al., 1995). However, it seems structurally more likely that in the HIV Tat−TAR system two neighboring arginines are responsible for these interactions. From some preliminary model building (Aboul-ela et al., 1996; F. Aboul-ela, J. Karn, and G. Varani, personal communication), it is proposed that Arg[52] could interact with P21−22 and P22−23 while Arg[53] could interact with $G_{26}$. If this is the case, the proximal Lys[50] and Lys[51] residues would be located in the major groove close to the series of phosphates on the opposite strand (between residues 36 and 40). The finding that tsp 38−39 reacted specifically with Lys[51] in Tat peptide (37−72) is completely consistent with the placement of the two arginines in this model. Further work will be required to determine if Lys[51] is in direct hydrogen-bonding contact with P38−39 or whether it is just the nearest lysine residue lying in the groove. We are currently studying the other two positions (P39−40 and P40−41) to see if they have been uniquely cross-linked to lysine residues or not.

Tat peptide (37−72) does not take up a defined structural motif in the absence of RNA (Aboul-ela et al., 1995). Spectroscopic analysis of the binding of Tat peptide (32−72) to TAR has led to a proposal of a more extended structure which may have some $\beta$ character (Metzger et al., 1996). Alternatively, as in the analogous BIV Tat−TAR interaction (Puglisi et al., 1995; Ye et al., 1995), the Tat peptide may adopt a $\beta$-turn in the basic domain. Thus the TAR domain could wrap around the peptide in various possible ways and models of the Tat−TAR interaction will need to take all of these into consideration.

Previous cross-linking studies have helped to orient the Tat peptide in the major groove of TAR (Wang & Rana, 1996; Wang et al., 1996), but not all the data have been fully consistent with model building studies. For example, since it now seems clear that the basic region of Tat enters the major groove of TAR and causes it to close significantly (Aboul-ela et al., 1995, 1996), it is difficult to position Tyr[47] in the core region close to $G_{26}$ as suggested from a specific UV cross-link obtained (Liu et al., 1996). Similarly, a proposal from photochemical cross-linking studies that Lys[41] in the core region is close to $U_{42}$ in the lower stem (Wang & Rana, 1995) is hard to reconcile with the fact that in our experiments the tsp 42−43 TAR duplex failed to form a cross-link with Lys[41], as might have been expected if the core domain was positioned close to this region. Some caution is needed, therefore, when interpreting cross-linking data, since absence of a cross-link could be due to unfavorable lysine orientation, and it will be necessary to obtain a range of evidence with different cross-linking methods before the results can be fully confirmed. The extremely mild and short-range chemical cross-linking procedure we have described here is a promising new technique for identifying sites of interactions between proteins and nucleic acids. Although there have been several reports of identification of sites on TAR cross-linked to predefined modifications on Tat peptides, we are the first to identify a precise amino acid in a Tat peptide which has cross-linked to a predefined location on a model TAR RNA. In addition, the use of a combination of endopeptidase cleavage and MALDI-TOF mass spectroscopy provides a rapid and accurate method for identifying protein−nucleic acid cross-linking sites.

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