Synthesis and RNA polymerase incorporation of the degenerate ribonucleotide analogue rPTP

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ABSTRACT

The synthesis and enzymatic incorporation into RNA of the hydrogen bond degenerate nucleoside analogue 6-[(β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido[4,5-c]-[1,2]oxazin-7-one (P) is described. The 5′-triphosphate of this analogue is readily incorporated by T3, T7 and SP6 RNA polymerases into RNA transcripts, being best incorporated in place of UTP, but also in place of CTP. When all the uridine residues in an HIV-1 TAR RNA transcript are replaced by P the transcript has similar characteristics to the wild-type TAR RNA, as demonstrated by similar melting temperatures and CD spectra. The P-substituted TAR transcript binds to the Tat peptide ADP-1 with only 4-fold lowered efficiency compared with wild-type TAR.

INTRODUCTION

Nucleoside bases differing from the normal purines, adenine and guanine, and the pyrimidines, thymine (uracil) and cytidine, are uncommon in DNA but relatively abundant in RNA, and in particular in transfer RNAs. The vast majority are the result of post-transcriptional modifications of the nucleic acids by specific enzymes. Although a specific role can seldom be assigned to these modifications, their importance can be inferred from their remarkable phylogenetic conservation. Analogues of the natural bases could be incorporated enzymatically in vitro could provide useful functional alterations to synthetic RNA transcripts. Early mutagenesis studies have demonstrated that N4-hydroxycytidine triphosphate (1, Fig. 1) is efficiently incorporated by the DNA-dependent RNA polymerase from Micrococcus luteus (1); the phage Qβ, T2 and Escherichia coli Pol II polymerases also incorporate it into RNA or DNA (2–4). The addition of the electronegative element to the N4-amino group alters the tautomeric ratio of the base; the alternative tautomers can base-pair with either adenine or guanine. The tautomeric constant (K_T) for 1-methyl-N4-hydroxycytosine has been measured giving a ratio of 10:1 in favour of the oximino-form in water (5), and thus correlates qualitatively with the analogue being recognised as either of the natural pyrimidines.

The N4-hydroxyl group can adopt either a syn or an anti conformation; the preferred syn form (6), however, interferes with hydrogen bonding of Watson–Crick base-pairs, and this is more evident in the case of N4-methoxy-derivatives (6). To constrain the hydroxyl group in an anti conformation we have previously synthesised a ribonucleoside containing a 5-membered second ring (2, Fig. 1) (7), but this compound proved too unstable to allow conversion either to its phosphoramidite monomer or its 5′-triphosphate. Strain in the 5-membered ring resulted in its cleavage during further reactions. The 2′-deoxynucleoside (dP) containing a 6-membered second ring proved to be stable. The properties of dP and its 5′-triphosphate have been intensively studied (8–11). The ribonucleoside 6-[(β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido[4,5-c][1,2]oxazin-7-one (rP) (3) has now been synthesised, by a route modified from that described for the deoxynucleoside, and converted to its 5′-triphosphate. Incorporation of the triphosphate by three widely used RNA polymerases, those of the bacteriophages T3, T7 and SP6 has been examined. The properties of HIV-1 TAR RNA transcripts synthesised by T3 RNA polymerase using rPTP in place of UTP or CTP have also been investigated.

MATERIALS AND METHODS

General methods

1H NMR spectra were obtained on Bruker WM-250 and DRX 300, and 31P NMR spectra on a Bruker WM-250 spectrometer. NMR spectra were obtained in d6-DMSP. 31P NMR spectra are referenced to phosphoric acid. Mass spectra were recorded on a Hewlett-Packard G205A Maldi-TOF spectrometer with positive polarity, in a matrix of α-cyano-4-hydroxy-trans-cinnamic acid in MeCN:H2O (1:1) with 3% trifluoroacetic acid. UV spectra were recorded on a Perkin Elmer Lambda 2 spectrophotometer fitted with a Peltier cell and samples were dissolved in 1% aqueous methanol. TLC was carried out on pre-coated F254 silica plates and column chromatography with Merck kieselgel 60. Unless otherwise stated reactions were worked up as follows: after

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dimethylsilyl) derivative (18H, m, 6H\(^{′}\)), 0.79–0.89 (27H, m, 3×C(CH\(_3\))\(_3\)), 2.61–2.71 (2H, m, CH\(_2\)CH\(_2\)OH), 3.36–3.43 (2H, m, CH\(_2\)CH\(_2\)OH), 3.69–3.79 (2H, m, H\(_5\)', H\(_5\)'\(^\prime\)), 3.91 (1H, br s, H2'), 4.02–4.04 (1H, m, H3'), 4.18–4.22 (1H, m, H4'), 4.61 (1H, t, OH), 5.86 (1H, d, J = 7.1 Hz, H1'), 7.40 (1H, s, H6), 11.40 (1H, s, NH). UV \(\lambda_{\text{max}} 267, 209\); \(\lambda_{\text{min}} 230\). M/z 654.487 (M+Na\(^+\)), 671.172 (M+K\(^+\)).

2',3',5'-Tri-(tert-butyldimethylsilyl)-5-(2-hydroxyethyl)-uridine (7). The above diol (5) (8.3 g, 12.6 mmol) was dissolved in dioxane (125 ml, 10 mmol/ml) and water (25 ml, 2 mmol/ml) added. To this was then added a solution of sodium periodate (8.0 g, 37 mmol) in water (25 ml, 2 mmol/ml) and the solution stirred at room temperature for 2.5 h. The solution was concentrated then worked up as usual to give the crude aldehyde, 6. This was dissolved in THF (200 ml) and sodium borohydride (0.5 g, 13 mmol) added followed by water (1 ml) and the solution stirred at room temperature for 1 h. The reaction was quenched with acetic acid, the solution evaporated and the product worked up as usual and chromatographed (CHCl\(_3\)/3% MeOH) to give an off-white foam. Yield 4.42 g, 56%, remainder (3.06 g) unreacted diol 5. \(^1\)H NMR \(\delta\) (p.p.m.) –0.10–0.10 (18H, m, 6×C(CH\(_3\))\(_3\)), 0.79–0.89 (27H, m, 3×C(CH\(_3\))\(_3\)), 2.33–2.37 (2H, m, CH\(_2\)CH\(_2\)OH), 3.36–3.43 (2H, m, CH\(_2\)CH\(_2\)OH), 3.69–3.79 (2H, m, H\(_5\)', H\(_5\)'\(^\prime\)), 3.91 (1H, br s, H2'), 4.02–4.04 (1H, m, H3'), 4.18–4.22 (1H, m, H4'), 4.61 (1H, t, OH), 5.86 (1H, d, J = 7.1 Hz, H1'), 7.40 (1H, s, H6), 11.40 (1H, s, NH). UV \(\lambda_{\text{max}} 267, 209\); \(\lambda_{\text{min}} 230\). M/z 654.487 (M+Na\(^+\)), 671.172 (M+K\(^+\)).

2',3',5'-Tri-(tert-butyldimethylsilyl)-5-(2-phthalimidooxyethyl)-uridine (8). To a solution of the alcohol (7) (3.1 g, 5 mmol) in THF (50 ml) was added triphenyl phosphine (2.6 g, 1 mmol), N-hydroxypthalimide (1.6 g, 1 mmol) and then diisopropylazidocarboxylate (DIAD) (2 g, 1 mmol) and the solution stirred at room temperature overnight. The solution was then evaporated, worked up as described and then chromatographed (twice, CHCl\(_3\)/1% MeOH) to give a pale yellow foam. Yield 3.65 g, 96%. \(^1\)H NMR \(\delta\) (p.p.m.) –0.11–0.10 (18H, m, 6×C(CH\(_3\))\(_3\)), 0.58–0.59 (27H, m, 3×C(CH\(_3\))\(_3\)), 2.61–2.71 (2H, m, CH\(_2\)CH\(_2\)ON), 3.60–3.69 (2H, m, CH\(_2\)CH\(_2\)ON), 3.72–3.90 (3H, m, H2', H5', H5'\(^\prime\)), 4.04 (1H, br s, H3'), 4.14–4.27 (1H, m, H4'), 5.87 (1H, d, J = 7 Hz, H1'), 7.56 (1H, s, H6), 7.54–7.64 (4H, m, Ph), 11.54 (1H, s, NH). UV \(\lambda_{\text{max}} 265, 220\); \(\lambda_{\text{min}} 245\). M/z 798.201 (M+Na\(^+\)), 814.482 (M+K\(^+\)).

1-(2',3',5'-Tri-(tert-butyldimethylsilyl)-β-D-ribofuranosyl)-4-triazolo-5-(2-phthalimidooxyethyl)-1H-pyrimidin-2-one (9). To a solution of 1,2,4-triazole (4.8 g, 69.5 mmol) in dry acetonitrile (75 ml) at 0°C was added phosphorus oxychloride (1.3 ml, 14 mmol) and the solution stirred at 0°C for 15 min. To this was then added triethylamine (11.6 ml, 8.3 mmol) and the solution stirred for a further 15 min at 0°C. The solution was then added a solution of 2',3',5'-tri-(tert-butyldimethylsilyl)-5-(2-phthalimidooxyethyl)-uridine (8) (3.6 g, 4.6 mmol) in acetonitrile (25 ml) and the solution stirred at room temperature overnight (product has same R\(_f\) as starting material). The solution was evaporated and worked up as described and then chromatographed (CHCl\(_3\)/1% MeOH) to give an off-white foam. Yield 2.44 g, 64%. \(^1\)H NMR \(\delta\) (p.p.m.) –0.11–0.12 (18H, m, 6×C(CH\(_3\))\(_3\)), 0.83–0.91 (27H, m, 3×C(CH\(_3\))\(_3\)), 3.23–3.30 (2H, m, CH\(_2\)CH\(_2\)ON), 3.79–3.83 (2H, m, CH\(_2\)CH\(_2\)ON), 3.93–4.02 (1H, m, H2'), 4.03–4.08 (1H, br s, H3'), 4.21–4.38 (2H, m, H5', H5'\(^\prime\)), 4.38–4.40 (1H, m, H4'), 5.87 (1H, d, J = 4 Hz, H1'), 7.51–7.64 (5H, m, H6, Ph), 8.19 (1H, s, triazole CH), 9.36 (1H, s, triazole CH). UV \(\lambda_{\text{max}}\) (nm) (10% removal of the solvent, the product was dissolved in chloroform and washed with aqueous sodium bicarbonate solution. The combined organic fractions were dried over sodium sulphate and evaporated.

**Synthesis**

5-Alllyluridine was prepared according to literature procedures (12). This was then converted to the 2',3',5'-tris-(tert-butyldimethylsilyl) derivative (4) as previously described (13).

2',3',5'-Tri-(tert-butyldimethylsilyl)-5-(2,3-dihydroxypropyl)-uridine (5). To a solution of 2',3',5'-tri-(tert-butyldimethylsilyl)-5-allyluridine (4) (9.5 g, 15 mmol) in acetone (250 ml) was added N-methylmorpholine-N-oxide (5.4 g, 46 mmol) followed by potassium osmate dihydrate (70 mg) in water (10 ml) over 5 min. The solution was then stirred at room temperature overnight, and the solvent was removed. The product was worked up as described and then chromatographed (CHCl\(_3\)/5% MeOH) to give a white foam. Yield 9.84 g, 98%. \(^1\)H NMR \(\delta\) (p.p.m.) –0.11–0.09 (18H, m, 6×SiCH\(_3\)), 0.79–0.89 (27H, m, 3×C(CH\(_3\))\(_3\)), 2.30–3.31 (2H, m, CH\(_2\)CH\(_3\)), 3.52–3.55 (3H, m, CH\(_2\)CH\(_2\)OH), 3.68–3.77 (2H, m, H5', H5'\(^\prime\)), 3.91–3.92 (1H, m, H2'), 4.03 (1H, t, H3'), 4.19–4.21 (1H, m, H4'), 4.49 (1H, t, OH), 4.56 (1H, d, OH), 5.85 (1H, t, J = 7.4 Hz, H1'), 7.36 (1H, d, J = 5.3 Hz, H6), 11.40 (1H, s, NH). UV \(\lambda_{\text{max}} 268, 208\); \(\lambda_{\text{min}} 230\). M/z 684.306 (M+Na\(^+\)), 700.809 (M+K\(^+\)).
MeOH/H2O) 331, 264. pH 12 λmax 267. M/z 828.869 (M+H)+, 849.629 (M+Na)+, 865.969 (M+K)+.

6-(2′,3′,5′-Tri-(tert-butyldimethylsilyl)-β-d-ribofuranosyl)-3,4-dihydro-8H-pyrimido[4,5-c][1,2]oxazin-7-one (10). The above triazole (9) (2.0 g, 2.4 mmol) was dissolved in dioxane saturated ammonium fluoride (25 ml) and the solution stirred at room temperature overnight. The solution was evaporated and the product chromatographed (CHCl3/2% MeOH) to give an off-white foam. Yield 0.88 g, 58%. 1H NMR (d, p.p.m.) –0.06 –0.09 (18H, m, 6x SiCH3), 0.81 –0.89 (27H, m, 3× C(CH3)3), 3.31 –3.34 (2H, m, CH2ON), 3.66 –3.74 (2H, m, H5′, H5″), 3.77 –3.87 (3H, m, H2′, H2′′, CH2CH2ON), 3.99 –4.01 (1H, m, H3′), 4.09 –4.13 (1H, m, H4′), 5.83 (1H, d, J = 7.5 Hz, H1′), 6.79 (1H, s, H6), 10.63 (1H, s, NH). UV λmax (nm) (MeOH) 298 (ε = 7400); λmin (nm) 262. pH 1 λmax 304 (ε = 12 400); pH 12 λmax 303 (ε = 7700). M/z 651.697 (M + Na)+.

6-(β-d-Ribofuranosyl)-3,4-dihydro-8H-pyrimido[4,5-c][1,2]oxazin-7-one (3). The above product (10, 0.85 g, 1.35 mmol) was dissolved in methanol (25 ml) and ammonium fluoride (0.3 g, 8.1 mmol) added and then the solution heated at 50°C overnight. The solvent was removed and the product chromatographed (CHCl3/20% MeOH) to give a white solid. Yield 0.31 g, 80%. 1H NMR δ (p.p.m.) 3.01 –3.04 (2H, m, H6), 10.50 (1H, s, NH). UV λmax (nm) (H2O) 295 (ε = 6100), 301 (ε = 7800). M/z 286.727 (M+H)+, 308.711 (M+Na)+.

Circular dichroism (CD) measurements

CD measurements were carried out on a Jobin-Yvon Dichrograph CD6 spectrometer. Data was collected at 0.25 nm intervals, and measurements were carried out between 190 and 330 nm at 20°C. Five such runs were averaged, calculated net of buffer and factor 3 smoothed. Samples were prepared with an oligonucleotide concentration of A260 = 0.5 in 10 mM sodium phosphate (pH 7) buffer (15), with a path length of 1 mm.

Polymerase reactions

Polymerase incorporation assays were carried out using the Riboprobe® System (Promega) using SP6, T3 and T7 RNA polymerases, and using pGEM® Express Positive Control Template (Promega). Reactions were carried out according to the manufacturers instructions. rPTP was used in place of either CTP or UTP, and at both 1× and 10× NTP concentrations. Products were electrophoretically separated on 1% agarose gels containing ethidium bromide and visualised under UV light.

TAR RNA synthesis

Wild-type TAR and the TAR mutant G26:C39 to C:G RNAs were transcribed from the plasmids BTO and BT76, respectively (16). In these plasmids, the TAR sequence abuts directly the T3 promoter. The plasmids were digested with EcoRI prior to the transcription reactions. Transcripts of 60 nucleotides were generated using either the Riboprobe® System (Promega), or for large scale synthesis Ribomax® system (Promega). Small scale reaction mixtures for TAR RNA synthesis (typically containing 40 mM Tris–HCl, pH 7.9, 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 2 mM DTT, 40 U RNasin, 400 µM NTPs, 1 µg template DNA, 40 U T3 RNA polymerase, and 20 µCi [α-32P]GTP in 50 µl solution) were incubated for 1 h at 37°C. When rPTP was used instead of UTP and/or CTP, the concentration of rPTP in the reaction was 400 µM (1× concentration) or 4 mM. The products were electrophoretically separated using 12% polyacrylamide gels (165 × 200 × 1 mm) containing 7 M urea at 35 W for 1 h.

Large scale reaction mixtures contained 80 mM HEPES–KOH, pH 7.5, 24 mM MgCl2, 2 mM spermidine, 40 mM DTT, 7.5 mM NTPs, 5 µg template DNA, and 10 µl of T3 enzyme mix in 100 µl solution. The synthesis of TAR was also carried out using 10 mM rFTP instead of UTP. The reaction mixtures were incubated at 37°C for 4 h. The products were electrophoretically separated using 6% polyacrylamide gels (350 × 200 × 1 mm) containing 7 M urea at 35 W for 2.5 h. The transcripts were visualised by autoradiography or UV shadowing for small and large scale syntheses respectively. The correct band was cut out and the RNA was extracted with 0.5 M ammonium acetate/1 mM EDTA. The extracts were then desalted using NAP-10 columns (Pharmacia).

Digestion of TAR RNAs

Digests were carried out as described (17), and the nucleosides separated by HPLC on a Waters µBondapak™ RP-C18 (3.9 × 300 mm) column.

RNA bandshifts

RNA bandshifts were carried out according to the methods described previously (16). In summary, 10 000 c.p.m. (∼2 nM) of...
[α-32P]GTP labelled TAR RNA (prepared as described above, but using 5-fold excess of rPTP, i.e. 2 mM) was incubated at room temperature for 5 min with various concentrations of ADP-1 peptide (50–1000 nM) in TK buffer (50 mM Tris, pH 7.5, 20 mM KCl) containing 0.1% Triton X-100 and 100 mM DTT. The mixtures were electrophoretically separated (10 W, 1 h) on 8% native polyacrylamide gels (165 × 200 × 1 mm) containing 0.1% Triton X-100. The bands were visualised by overnight exposure to autoradiography film. The autoradiograph was scanned using a Molecular Dynamics Scanning Imager 300A-T.

RESULTS

The synthesis of the ribosyl analogue, rP, is shown in Figure 2. 5-Allyluridine (12), was silylated with t-butyldimethylsilyl chloride to give (13). This was then converted to the 5-hydroxyethyluridine (7) by dihydroxylation of the olefinic bond with potassium osmate/N-methylmorpholine-N-oxide followed by periodate cleavage to the aldehyde (6). Curiously, the periodate reaction was not effective using THF/water or acetone/water, but reaction was obtained using dioxane/water as solvent. The aldehyde 6 was not isolated but converted immediately to the alcohol, 7, by reduction with sodium borohydride. This was then converted to the bicyclic P analogue in a manner similar to that described for the deoxynucleoside (8) by a Mitsunobu reaction of the alcohol (7) with N-hydroxyphthalimide, triazolylation and then ring closure using ammonia in dioxane. Finally, the silylated derivative (10) was deprotected using ammonium fluoride in methanol to give the free nucleoside. This was then converted to its 5′-triphosphate derivative.

To screen for the incorporation of the analogue into RNA by the RNA polymerases of the bacteriophages SP6, T3 and T7 the positive control template from the pGEM Express Positive kit (Promega) was used. UTP or CTP were replaced by rPTP in the polymerase transcription reactions. The 2′-deoxynucleoside triphosphate, dPTP, is incorporated opposite dA or dG by Taq polymerase in PCR reactions. However, neither TTP or dCTP could be entirely replaced by dPTP (10). Both as a substrate triphosphate and as a template for Taq polymerase, dP resembled T more than dC (18). It had also been demonstrated that, in terms of hybridisation, P:A base pairs are equivalent to T:A base pairs, whilst P:G pairs are slightly destabilising when compared with C:G pairs in deoxyribo-oligomers (8). Using T7 polymerase, full length products were formed: when used to replace CTP a distinct, but different, product was obtained. Product yields were lower than the controls using the four NTPs (Fig. 3), but when the rPTP concentration was increased 10-fold compared with the other triphosphates the yield of products was substantially improved. In this instance T3 RNA polymerase incorporated rPTP better in place of CTP rather than in place of UTP (but see below P-TAR transcripts, Fig. 4 and refs 1,3). T7 and SP6 showed a marked preference for replacing UTP rather than CTP with rPTP.

Regulation of HIV-1 transcription is controlled by a specialised RNA/protein interaction (for review see 19). The trans-activation responsive region (TAR) of HIV-1 is located immediately downstream of the HIV-1 transcription start site from positions +1
Figure 3. Agarose gel electrophoresis of the products of RNA transcription reactions using the positive control template in the pGEM Express kit (Promega). The products obtained using three separate bacteriophage RNA polymerases T7, T3 and SP6 are shown. Above each lane, the omission of either CTP or UTP (-C or -U) or the addition of rPTP at either the same or 10× the concentration of the other NTPs (+ or +10xP) is recorded.

Figure 4. TAR transcripts from wild-type DNA using rPTP to replace UTP (left), CTP or both pyrimidine triphosphates. The figure shows the gel after exposure to film for 30 min and after 2 h where it can be seen that rPTP can be used to entirely replace the pyrimidine triphosphates.

to +59, and is therefore transcribed immediately upon initiation of transcription from the HIV promoter (20). TAR is known to form a highly stable stem–loop and it has a tripyrimidine bulge near the apex of the structure (Fig. 5) (21). TAR RNA is bound by the virally-specified trans-activator protein Tat (22). This leads to a dramatic increase in HIV gene expression. The Tat/TAR interaction is very specific and mutations in and around the tripyrimidine bulge abolish both Tat binding and trans-activation of HIV transcription (16,23). The binding of Tat to TAR is decreased by the mutation G26:C39 to C:G (mGC). A shorter Tat-derived peptide (ADP-1), which contains the core and basic regions of Tat (residues 37–72), has been shown to exhibit similar specificity to the wild-type protein (16,23).

Both wild-type and mGC TAR transcripts were made using the bacteriophage T3 polymerase to assess the efficiency of incorporation of rPTP in place of either UTP or CTP. When transcription was carried out using rPTP to replace UTP, full length RNA transcripts were readily obtained. When replacing CTP, the yield of transcript was markedly reduced (Fig. 4), but full length transcripts could still be visualised on gels even when both CTP and UTP were replaced by rPTP. Although the reason for this low efficiency of incorporation into a smaller transcription product is unclear, the fact that TAR RNA is a highly ordered structure may provide a possible explanation.

In order to characterise the transcripts, large scale reactions were carried out. TAR RNA itself has been shown to have a high melting temperature, 65°C in 10 mM potassium phosphate, 50 mM sodium chloride (24) consequent on its hairpin structure, and this correlated with our findings ($T_{m}$ in 100 mM sodium phosphate, 78°C). The transcripts containing rPTP in place of UTP also had a high melting temperature (76°C). This was unexpected in that P:G base-pairs in DNA duplexes are ∼2°C less stable per modification than a C:G base-pair (8); there are also two U:G base-pairs in the native TAR RNA which will be replaced by P:G base-pairs in the analogous P-containing TAR (hereafter called P-TAR). However, the derived stacking enthalpy was lower than that of the native TAR; these results are shown in Figure 5. Similar findings were obtained with the mutant (G26:C39 to C:G) TAR transcription products. The two P-TAR transcripts also have the same melting temperature and very similar stacking enthalpies. The TAR transcripts were also digested with snake venom phosphodiesterase and alkaline phosphatase to determine their nucleoside composition. The P-TAR transcript was shown to contain only C, G, A and P, though the exact composition could not be accurately determined (data not shown), and therefore it was not possible to show unequivocally that rPTP had also been incorporated in place of CTP, although it might be expected that it would be, albeit at a low frequency and randomly.
Circular dichroism measurements were carried out on the transcripts. The native TAR RNA had a CD spectrum similar to that previously described (15, 24); the P-TAR produced a rather similar spectrum (Fig. 6), although there was a decrease in intensity of the 265 nm band accompanied by a red shift in the crossover and a decrease in intensity of the far UV region bands present in the native structure. Spectra for the mGC mutant TAR and its P-derivative were similar (data not shown).

Binding of the Tat fragment, ADP-1, a fragment of the tat protein (residues 37–72), to the modified TAR transcript was studied. Wild-type TAR RNA, labelled with $[^\alpha{}-32P]GTP$ was synthesised using either UTP or rPTP. The mutant mGC TAR was prepared as a control. RNA bandshift assays were performed in the presence of increasing concentrations of the tat fragment ADP-1. The modified P-TAR ran with a slightly lower mobility due to the PMP residues; this altered mobility has also been observed with oligodeoxyribonucleotides containing dP. The mutant mGC TAR is known to have a 9-fold lower affinity for ADP-1 than the wild-type (16). Figure 7 shows that the affinity of P-TAR for ADP-1 is lower than that of the wild-type TAR, but, remarkably, is higher than that of the mGC TAR mutant, and this was confirmed using densitometry measurements. The artefactual second shifted band, known to occur at high concentrations of ADP-1 peptide (16), is also seen with the P-TAR transcript.

**DISCUSSION**

The ribo-P-5′-triphosphate analogue was incorporated into RNA by all three RNA polymerases T3 and T7, and to a lesser extent for SP6. In the control system (pGEM Express Positive control template) rPTP was successfully used to replace entirely one of the two natural pyrimidine triphosphates to obtain full length transcripts (1.5 kb). In the TAR system using T3 polymerase it proved to be more difficult to obtain a high yield of product when using rPTP in place of CTP. Nevertheless, it was possible to obtain transcripts when both pyrimidine triphosphates were replaced by rPTP if the rPTP concentration was raised 10-fold. When rPTP was used to replace UTP there was a decrease in net synthesis between this and RNA derived by using the four natural triphosphates, but the yield was acceptable.

The replacement of UTP in TAR RNA by rPTP affects a significant number of base-pairs. There are eight U:A base-pairs in addition to the three uridine residues involved in the bulge and the single uridine in the hairpin. It has been shown that of these U23, the first base in the bulge, is essential for recognition by either Tat protein or the peptide ADP-1 (25). C5-Substituents are tolerated, and therefore it might be expected that the analogue P could be accommodated in this position. Remarkably, binding in the band-shift assay was only reduced by a factor of four compared with the wild-type TAR. In contrast, the biologically inactive mutant (mGC) showed 15-fold reduction in binding (Fig. 7), consistent with the earlier finding that mGC binding was reduced 9-fold (16). It is therefore clear that the P-TAR does bind to ADP-1, demonstrating that P can be tolerated not only throughout the TAR structure, but more importantly in replacing the essential uridine at position 23. The observations provide a prima facie case for assuming that the proposed U23:arginine interaction in the TAR:ADP-1 complex (26) can be mimicked by a P23:arginine contact. Overall the experiments indicate that P-TAR has a very similar structure to the wild-type TAR transcript and strongly suggests that it can undergo the large conformational change that occurs on binding (27).

In addition to the nine U:A base-pairs there are also two U:G base-pairs in the native TAR RNA stem. In DNA duplexes the P:G base-pair is in rapid chemical exchange between Watson–Crick and wobble configurations with a very low free energy difference between them (28, 29). Evidently the U:G base-pairs in TAR are correspondingly replaced by P:G with very little effect on structure.

The similarity between the wild-type structure and P-TAR was further demonstrated by the fact that they have similar melting temperatures, and CD spectra. They also showed virtual identity in $T_m$, stacking enthalpy and CD spectra.
Earlier work on the 2′-deoxynucleotide analogue of P (dPTP) demonstrated that it had very similar kinetic parameters of incorporation to those of TTP (10). Further work is in progress to investigate the kinetics of incorporation of the ribo-P triphosphate, and the hybridisation properties of rP-containing oligomers as well as their templating properties.

Work during recent years has shown that interactions in RNA secondary and tertiary structures are much richer than those in DNA and that correspondingly RNA–protein interactions are likewise complex and only now beginning to be understood. We have used the Tat (ADP-1)/TAR system purely as a model to examine the incorporation of rPTP into a biologically active RNA oligomer, without necessarily expecting to learn anything new about the Tat/TAR interaction. Nevertheless, our findings have shown that incorporation of rPTP into TAR has had very little effect on its physical characteristics and biological activity. We are therefore in the process of examining the effect of site specific incorporation of P by chemical synthesis into TAR to examine its effects more rigorously. We believe that these aspects of RNA chemistry can be modulated by the introduction of hydrogen bond degenerate base residues which, in turn, should lead to a variety of novel outcomes and applications.

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