Use of the Fluorescent Nucleoside Analogue Benzo[*g*]quinazoline 2′-*O*-Methyl-β-D-ribofuranoside to Monitor the Binding of the HIV-1 Tat Protein or of Antisense Oligonucleotides to the TAR RNA Stem-Loop

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Dedicated to Prof. Dr. *Frank Seela* on the occasion of his 60th birthday

The Tat protein is an essential *trans*-activator of HIV gene expression. It interacts with its RNA recognition sequence, the *trans*-activation responsive region TAR, as well as cellular factors. These interactions are potential targets for drug discovery against HIV infection. We have developed a new and sensitive assay for the measurement of Tat binding to TAR in solution under equilibrium conditions based on the change of fluorescence of the base analogue benzo[*g*]quinazoline-2,4(1*H*,3*H*)-dione (BgQ) incorporated into the chemically synthesized model TAR stem-loop **2** to which was added Tat-[37-72] peptide (**3**). The results show that Tat-TAR binding strength is 2–3-fold stronger than has previously been determined by mobility-shift analysis. Changes of fluorescence were used also to measure the binding of antisense 2′-*O*-methyloligonucleotides to TAR **2**.

1. Introduction. – The human immunodeficiency virus type 1 (HIV-1) Tat protein is an essential *trans*-activator required for viral replication (for recent reviews, see [1–3]). Tat interacts with an RNA sequence, the *trans*-activation responsive region TAR, a 59-residue stem-loop that occurs at the 5′-end of all viral RNA transcripts, as well as a Tat-associated kinase complex (TAK) that includes cyclin T1 and the kinase CDK9. The full mechanism of *trans*-activation by Tat is not yet established, but evidence from *in vitro* transcription techniques suggests that, in the absence of Tat, the transcription complex is unstable in the elongation phase leading to a predominance of short viral transcripts. When TAK becomes activated by Tat and TAR, the C-terminal domain of RNA polymerase II becomes hyperphosphorylated, an event that is essential for full-length Tat-dependent transcription [4][5]. The key virus-specific step in *trans*-activation, however, is Tat/TAR recognition.

Tat binds *in vitro* with high affinity near the apex of TAR to a region containing a 3-residue U-rich bulge (see below, *Figs. 1* and *2*). This interaction has been studied in great detail over many years (reviewed in [1]), including much work in our own laboratory on model TAR duplexes together with Tat protein made in *E. coli* or with synthetic Tat peptides [6][7]. More recently, synthetic TAR duplexes have been used for cross-linking studies to Tat [8–11]. Structural models of TAR either free or in the presence of a Tat peptide have also been obtained based on NMR characterization [12–16], but no complete structure of the peptide/RNA complex has been produced.

Models of the interaction are based on location of the arginine-rich region of Tat within the major groove of TAR and formation of a number of contacts with base and phosphate residues in the bulge region.

The TAR stem-loop has been a target for drug discovery and a number of small-molecule inhibitors (reviewed in [17]), and peptidomimetics [18–20] have been shown to bind to TAR and interfere with Tat action either by direct competition or by stabilizing a TAR conformation that cannot be recognized by Tat. Another interesting lead compound is the tripeptide Lys-D-Lys-Asn that was selected from a solid-phase-bound combinatorial library to interfere with Tat-TAR binding by binding the U-rich bulge [21].

An alternative approach to interfere with *trans*-activation is disruption of the TAR structure by complementary antisense oligonucleotides. The apical stem and loop region just above the bulge of TAR is well-conserved among viral strains, and disruption of this region of TAR might be doubly effective by prevention of binding both of Tat and TAK. *Vickers et al.* showed that 18–28-residue phosphodiester and phosphorothioate oligodeoxynucleotides complementary to TAR were able to bind specifically in a gel-mobility-shift assay [22]. Anti-TAR phosphorothioate oligonucleotides showed a dose-dependent activity in cellular *trans*-activation assays, but in antiviral assays, they showed no sequence specificity. Phosphodiester oligonucleotides were inactive in both assays presumably due to degradation by nucleases. We showed recently that nuclease-stabilized 16-mer 2′-*O*-methyloligoribonucleotides are effective agents in steric blocking of Tat-TAR [23][24]. The 2′-*O*-methyloligoribonucleotides targeted to TAR are also strong inhibitors of HIV reverse transcription [25]. Further, DNA aptamers and their 2′-*O*-methyl analogues have been selected to bind strongly to TAR and form kissing complexes [26]. RNA Aptamers have also been selected against the TAR element [27].

For measurement of the strength of the interaction of Tat with TAR *in vitro*, a number of standard assays have been developed involving gel-mobility shift or filter binding [28][29]. We have also used these assays to assess oligonucleotide binding to TAR [23][24]. Unfortunately, neither of these techniques is a measure of the true binding constant in solution under equilibrium conditions. We have now developed a new assay for the measurement of Tat binding to TAR in solution based on the change of fluorescence of the base analogue benzo[*g*]quinazoline-2,4(1*H*,3*H*)-dione (BgQ; see **1**) [30][31] incorporated into the chemically synthesized model TAR stem-loop **2**. Changes of fluorescence could be used also to measure the binding of antisense oligonucleotides to TAR **2**.

2. Results. – *Syntheses and Peptide Binding.* The nucleoside U^{24} in the bulge of TAR is not directly involved in binding Tat protein and may be replaced by any nucleoside [7] or by a 2′-deoxynucleoside [6]. In addition, large conformational changes in this residue are known to occur upon Tat binding [13]. Thus U^{24} is an obvious site for incorporation of a fluorescent base. BgQ is a thymine analogue that has a strong fluorescence-emission intensity which becomes quenched upon stacking within DNA duplexes and triplexes [30a][31]. The excitation spectrum of BgQ as a 2′-deoxynucleoside shows three maxima at 253 (major), 294, and 360 nm and a broad emission spectrum centered between 420 and 440 nm [30a]. The extinction coefficient at 260 nm

is extremely high ($2.6 \cdot 10^5$ M^{-1} cm^{-1}), and the fluorescence quantum yield is also high (0.82) [30a]. This makes it ideal for use in studying protein interactions, since the only two amino acids with absorption in this region, tyrosine (y) and tryptophan (w), would have less than 0.5% fluorescence compared to BgQ (extinction coefficients for Y and W of $1.4 \cdot 10^3$ and $5.6 \cdot 10^3$ M^{-1} cm^{-1}, resp., and quanta yield of 0.12 and 0.20, resp., at their maxima at 270 and 280 nm, resp.). Therefore, we chemically synthesized a 27-mer RNA model, *i.e.* BgQ TAR (**2**) where U^{24} was substituted by the 2′-*O*-methylribonucleoside derivative **1** of this fluorescent base BgQ (*Fig. 1*, *a* and *b*), which has identical fluorescence properties to that of the corresponding 2′-deoxynucleoside. The synthesis was carried out by standard solid-phase RNA-synthesis techniques with a phosphor-

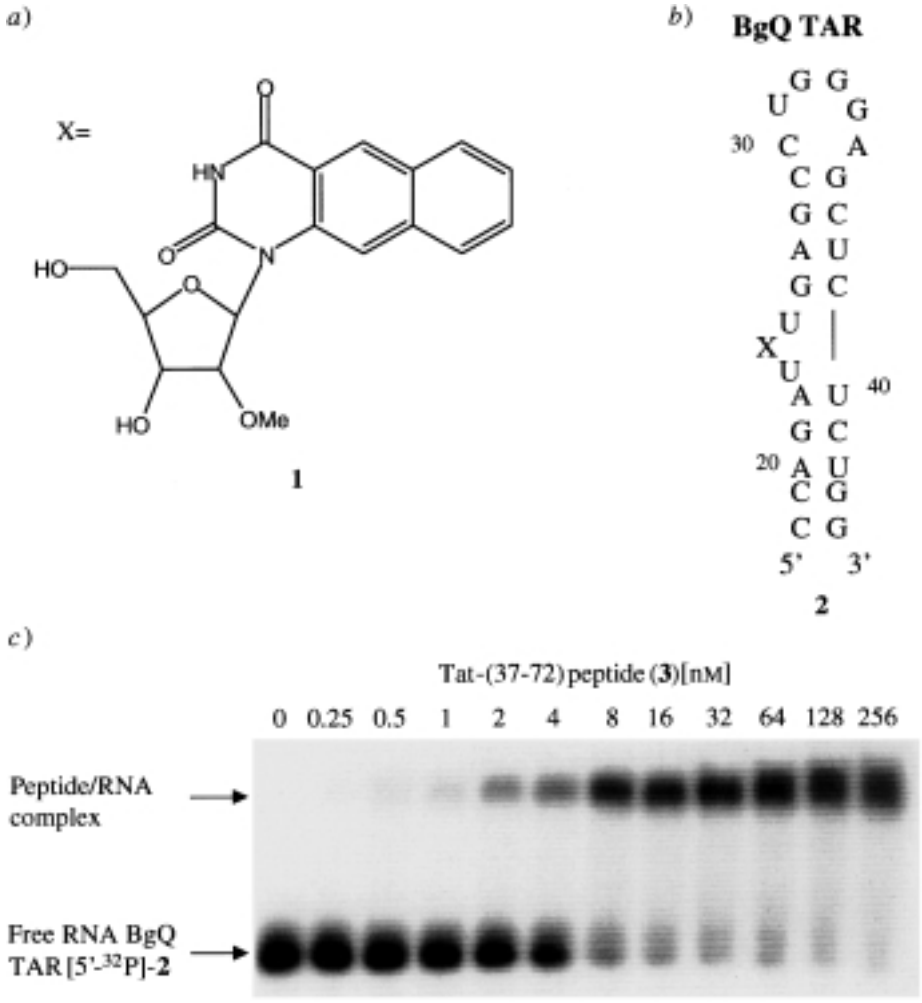


Fig. 1. a) *1-(2′-*O*-Methyl-β-*D*-ribofuranosyl)benzo[*g*]quinazoline-2,4(1*H*,3*H*)-dione* (**1**); b) *synthetic RNA model BgQ TAR* (**2**), *containing the modified nucleoside* (**1**) *in position X;* c) *polyacrylamide-gel-mobility-shift assay of Tat-(37–72) peptide* (**3**) *binding to* ^{32}*P-labelled RNA BgQ TAR [5′-*^{32}*P]-***2**

amidite of **1** [30b] and commercially available ribonucleoside phosphoramidite derivatives.

To check that BgQ TAR (**2**) is a good model for Tat binding, we carried out gel-mobility-shift analysis of synthetic Tat-(37-72) peptide (HIV-1 BRU; **3**) binding to **2** (*Fig. 1,c*; for the sequence of the Tat peptide **3**, see below, *Fig. 2,c*). For the interaction with **3**, an apparent K_d of 16 nM was obtained by gel scanning. In comparison, a longer TAR RNA model that is unmodified, *i.e.*, TAR BRU 39 (**4**), bound the Tat peptide **3** with an apparent K_d of 5 nM, as determined by mobility-shift analysis (*Fig. 2*). The longer TAR BRU 39 (**4**) was expected to bind about twice as strongly as a shorter 27- or 29-mer, based on previous experience [6][32]. Since Tat peptide binding to **2** is *ca.* 3-fold higher in K_d than to **4**, the replacement of U^{24} by BgQ thus results in only a very slight reduction in ability to bind Tat peptide **3**. Therefore BgQ TAR (**2**) is a good model for Tat binding.

*Fluorescence Changes in BgQ TAR (**2**) upon Tat Peptide Binding. Fig. 3* shows the excitation and emission spectra of 5 nM BgQ TAR (**2**) in 50 mM *Tris*·HCl (pH 7.5), 20 mM KCl at 20°. The intensity of both excitation and emission is quite low compared

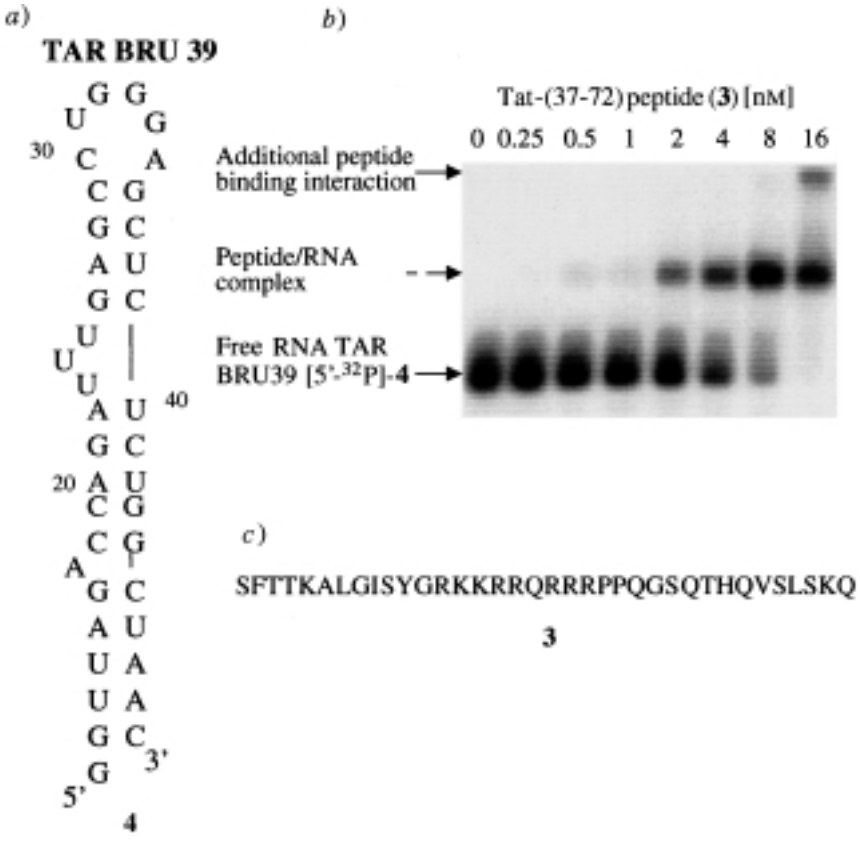


Fig. 2. a) *Synthetic RNA model TAR BRU 39* (**4**); b) *polyacrylamide-gel-mobility-shift assay of Tat-(37–72) peptide* (**3**) *binding to* ^{32}*P-labelled RNA TAR BRU 39 [5′-*^{32}*P]-***4**; c) *sequence of the synthetic Tat-(37–72) peptide* (**3**) *with Cys-37 replaced by Ser*

to that of the free nucleoside [30a], suggesting that the BgQ fluorescence is quenched considerably, presumably by stacking interactions within the TAR RNA. A stacking interaction of U^{24} has been established by NMR experiments within free TAR RNA [14]. In the presence of excess Tat peptide **3** (20 nM), a dramatic increase of 2–3 fold in the fluorescence was observed (*Fig. 3*). The maximum of emission was shifted upfield slightly from 424 to 429 nm.

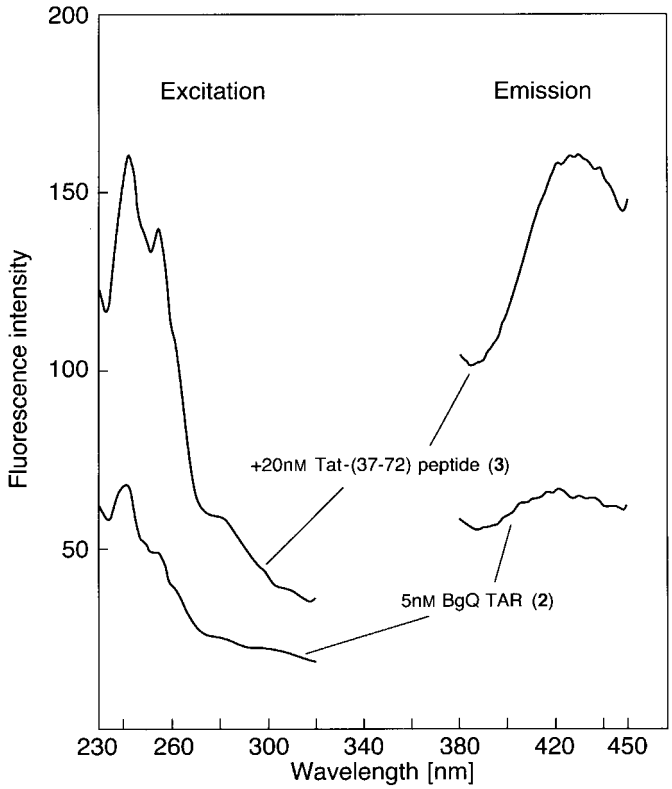


Fig. 3. *Excitation and emission spectra of BgQ TAR* (**2**) *alone and in the presence of Tat-(37-72) peptide* (**3**) *carried out in 50 mM* Tris·HCl (pH 7.4) *and 20 mM KCl at 20°*. The excitation fluorescence spectrum was recorded for emission wavelength set at 430 nm, and the emission spectrum recorded at 240 nm excitation wavelength

Upon titration of 20 nM BgQ TAR (**2**) with increasing concentration of Tat peptide **3** under the same buffer and relatively low salt conditions, a fluorescence-intensity curve of the emission spectra that reached a plateau at *ca.* 40 nM of Tat peptide and a *ca.* 3-fold intensity increase (*Fig. 4*) was observed. Curve fitting using a simple 1:1 binding isotherm gave an apparent K_d of 6 ± 3 nM. However, the slight deviations from this simple binding isotherm suggest a small amount of binding of a second molecule of Tat peptide **3** to BgQ TAR (**2**). Such second Tat peptide binding was not observed in the mobility-shift assay with **2** (*Fig. 1*) but can be seen at higher Tat concentrations with the longer TAR BRU 39 ((**4**); *Fig. 2*).

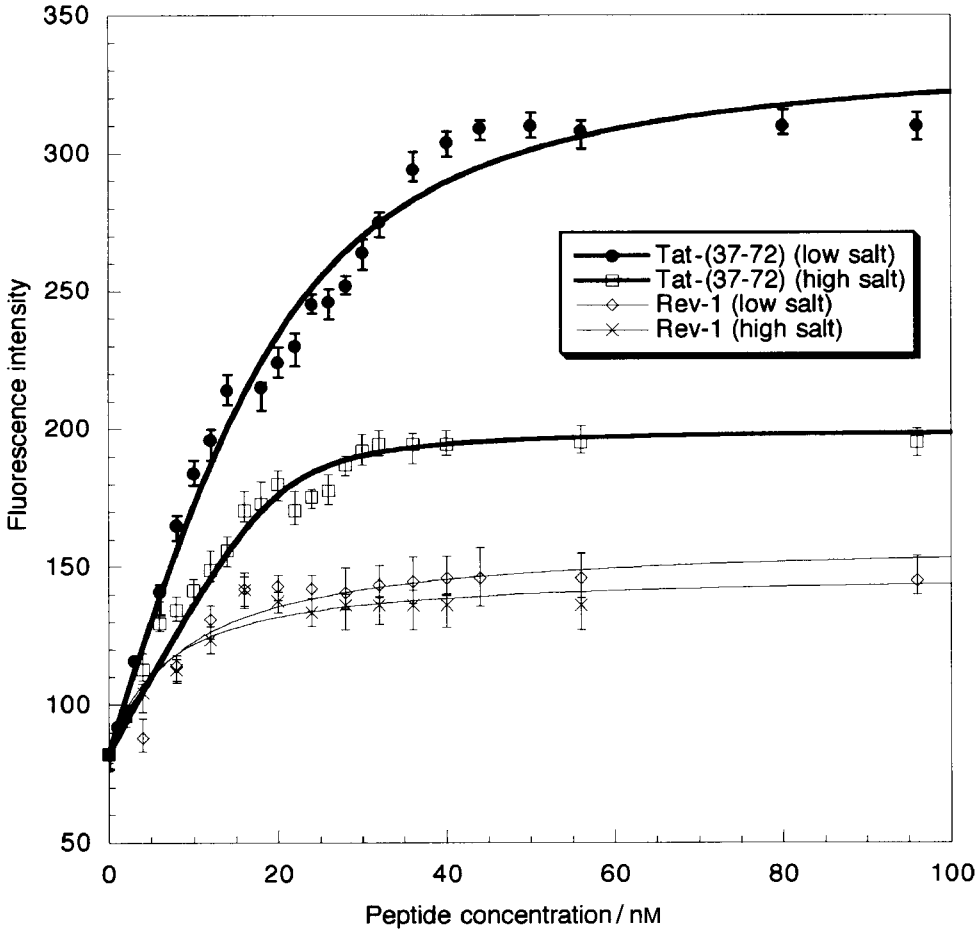


Fig. 4. *Fluorimetric emission titration curves of 20 nM BgQ TAR* (**2**) *by Tat-(37–72) peptide* (**3**) *and Rev-(34–51) peptide in low* (20 mM KCl) *and high* (80 mM KCl) *salt buffers.* Curves in bold are fitted to *Eqn. 1* to determine K_d. Note that a perfect fit to a single binding isotherm was only obtained for Tat peptide under the higher salt conditions.

Under more stringent conditions (higher salt concentration, 80 mM KCl), the fluorescence increase during titration of **2** with **3** fitted very well to a 1:1 binding isotherm (*Fig. 4*). There was a smaller fluorescence-intensity increase (2-fold), but the K_d from curve fitting showed a slightly stronger interaction with an apparent K_d of 1.5 ± 0.5 nM. We also studied the binding of a related peptide derived from the highly arginine-rich region of HIV-1 Rev, residues 34–51, which interacts with the high-affinity site of the Rev-responsive element RNA [33]. Rev-(34-51) peptide is known to bind also to TAR at low affinity and specificity [34]. Titration of BgQ TAR (**2**) with increasing Rev peptide resulted in only a very small increase in fluorescence, both at low salt and at higher salt concentration (*Fig. 4*). Further, a 1:1 binding isotherm could not be fitted to either of these curves, suggesting a complex, nonspecific binding pattern

for the Rev-(34–51) peptide. This demonstrates that specific TAR binding in the bulge region by Tat peptide **3** can be distinguished from the less specific Rev-peptide binding at higher salt concentration by the higher fluorescence increase and the better fit to a 1:1 binding isotherm.

We also measured the effect of addition of Mg^{2+} ions to the binding equilibrium (*Fig. 5*). At low salt concentration (20 mM KCl), the fluorescence intensity of a mixture of 20 nM BgQ TAR (**2**) and 50 nM Tat peptide **3** was gradually quenched upon Mg^{2+} addition. The half-point of loss of fluorescence was observed at *ca.* 3 mM Mg^{2+} concentration, and full loss at 7 mM. This result suggests that the Tat-TAR interaction is not stable at moderately high Mg^{2+} concentration (10 mM), conditions quite commonly used in protein-nucleic acid interaction studies, but is relatively unaffected by concentrations of 1 mM Mg^{2+} ion or less, conditions expected to be closer to physiological. At higher salt concentration (80 mM KCl), the point of half-inhibition

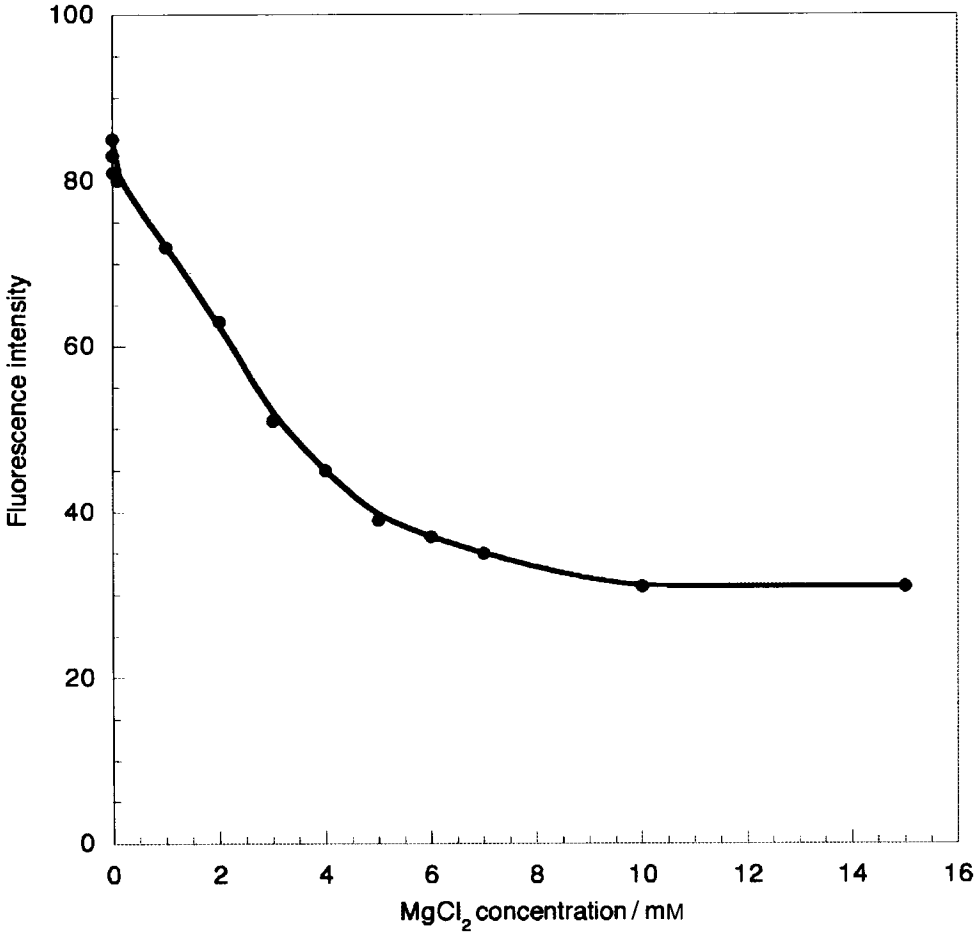


Fig. 5. *Fluorimetric emission titration curve of BgQ TAR* (**2**)*/Tat-(37–72) peptide* (**3**) *complex with increasing concentrations of $MgCl_2$ under lower salt conditions*

was observed at *ca.* 1 mM Mg^{2+} concentration (data not shown). This experiment shows how fluorescence changes of **2** can be used to measure the stability of Tat interaction under a variety of buffer and additive conditions.

To demonstrate further that the fluorescence-intensity changes are related to specific Tat-TAR binding, we carried out a competition assay by addition of increasing amounts of the unmodified RNA TAR BRU 39 (**4**) to a mixture of Tat peptide **3** and BgQ TAR (**2**). The fluorescence intensity decreased as the concentration of TAR **4** increased (*Fig. 6*). The concentration of **4** required to reduce fluorescence intensity by half was 14 nM, which is similar to that found by mobility-shift analysis using a competition assay (16 nM, data not shown). By curve fitting, a K_d of binding of the TAR 39-mer **4** to Tat peptide **3** was calculated to be 1.6 nM. This is a slightly stronger binding than that found for direct binding of **2** to **3** by fluorescence measurements under the same conditions (6.3 nM, *Fig. 3*) and is in line with the slightly stronger binding of **4**

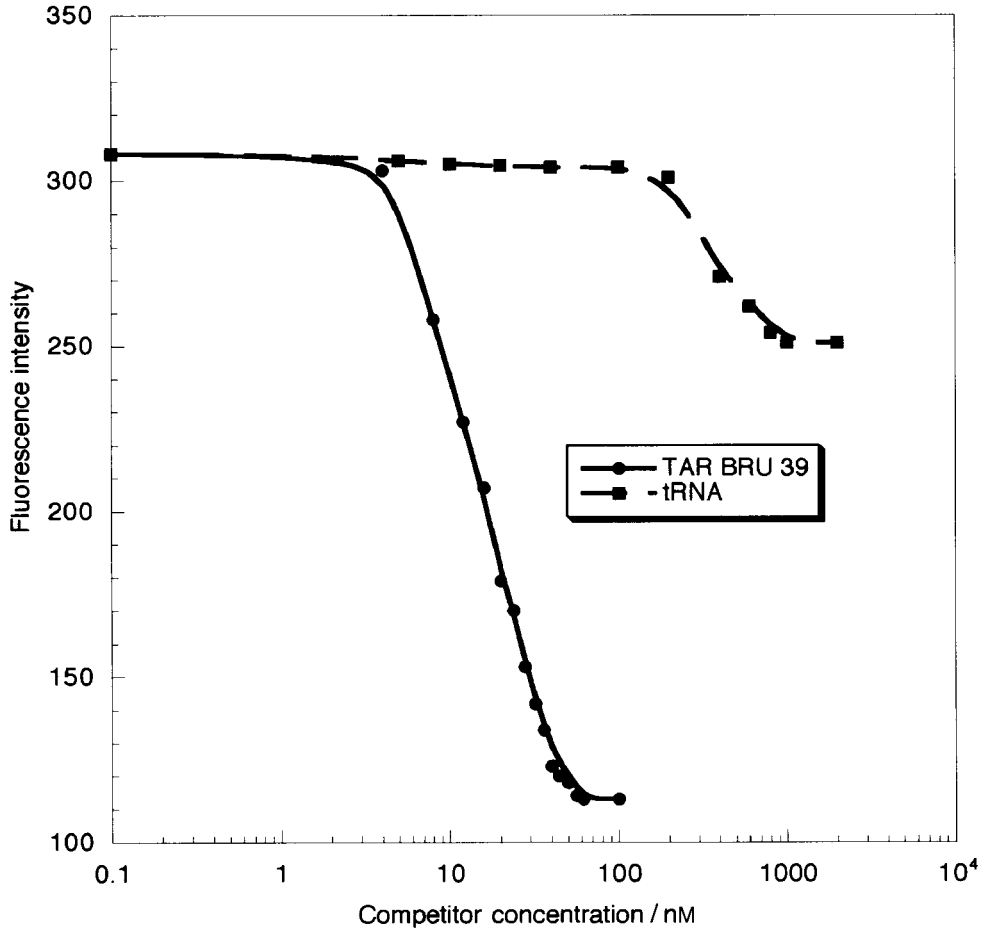


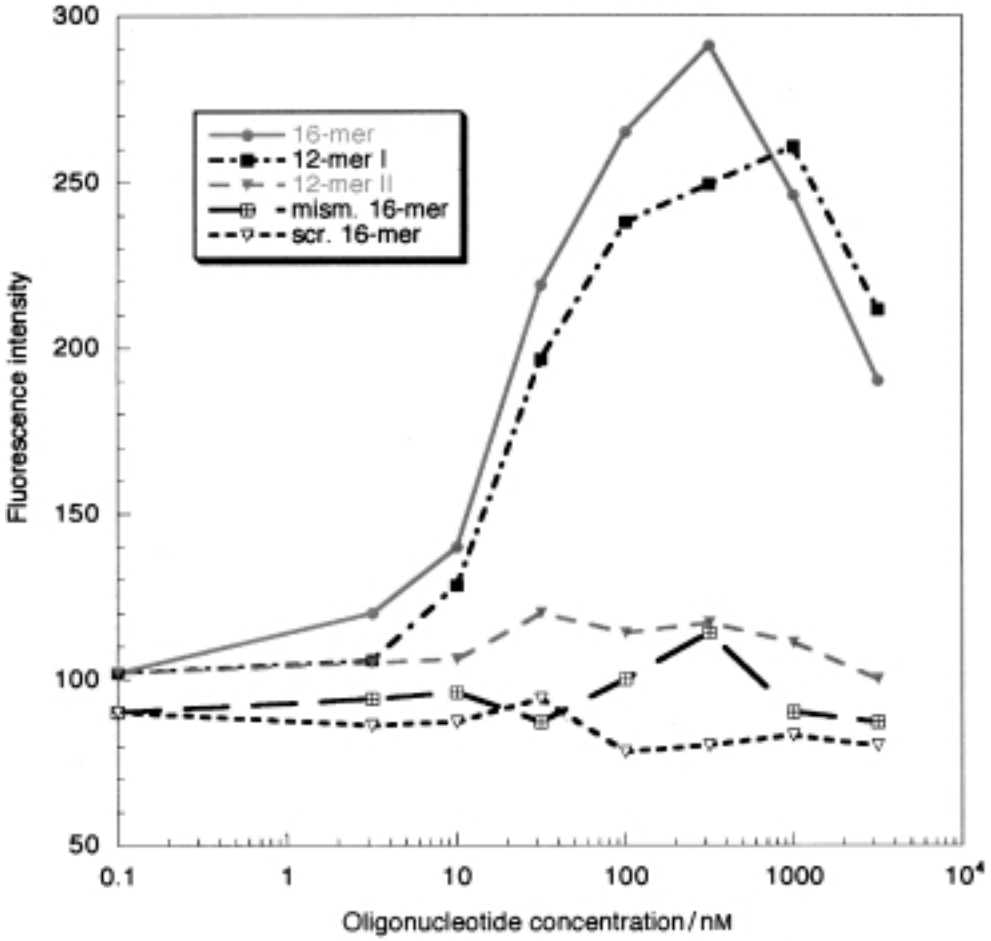
Fig. 6. *Fluorimetric emission titration curves of TAR BRU 39* (**4**) *and nonspecific* (tRNA) *competitors with the BgQ TAR* (**2**)*/Tat-(37–72) peptide* (**3**) *complex under lower salt conditions*

than **2** as observed by mobility-shift analysis (*Figs. 1* and *2*). By contrast, addition of increasing amounts of a nonspecific competitor (tRNA) to **2**/**3** gave rise to a much poorer competition curve. Curve fitting showed an approximate K_d of 760 nM for tRNA binding to **2**.

*Interaction of Antisense 2′-*O*-Methyloligonucleotides with BgQ TAR* (**2**). A large increase of fluorescence intensity as well as a shift in the fluorescence-emission maximum was observed when a 16-mer 2′-*O*-methyloligonucleotide complementary to the RNA BgQ TAR (**2**) [23][24] was added (*Fig. 7*). A maximum value of fluorescence intensity was reached at *ca.* 300 nM of 16-mer oligonucleotide and the half-intensity at *ca.* 20 nM. This compares with *ca.* 70–80 nM for 50% complex formation by mobility-shift analysis for 16-mer binding to **2** (data not shown) and *ca.* 50–60 nM for TAR BRU 39 (**4**) [24]. At very high oligonucleotide concentration, the intensity of fluorescence was reduced again. No increase of fluorescence intensity was observed on addition of a mismatched or scrambled oligonucleotide to **2** (*Fig. 7*). Shorter 12-mer 2′-*O*-methyloligonucleotides gave different results depending on their precise alignment compared to the position of the BgQ-containing residue **1** in the bulge. The 12-mer I, which is antisense to residues 23–34 (*Fig. 1*) and would be expected to form a duplex that includes the BgQ residue **1** at position 24, showed a fluorescence increase somewhat similar to the 16-mer. By contrast, 12-mer II, which is complementary to residues 25–36 and which does not reach to the BgQ residue **1** at position 24, does not give a fluorescence shift, even though it is known to bind TAR 39 **4** with a *ca.* 50 nM apparent K_d by mobility-shift analysis [24].

3. Discussion. – The fluorescence emission or excitation spectrum of BgQ-TAR (**2**) is a sensitive measure of the binding of Tat peptide **3** to TAR RNA and gives an apparent K_d by curve fitting that is 2–3 fold lower than that determined by mobility-shift analysis. This suggests that mobility-shift analysis results in underestimation of the binding strength of the Tat-TAR interaction by a factor of 2–3. An explanation for this is that during the polyacrylamide-gel electrophoresis, there is time for some dissociation of the Tat-TAR complex to take place, whereas the fluorescence assay measures the true equilibrium binding strength. The results also show that a large fluorescence-intensity increase is observed only in binding of the cognate Tat peptide, and show that the best fit to a 1:1 interaction is obtained under higher salt (80 mM KCl) conditions. By contrast, titrations of BgQ TAR (**2**) with the less specific Rev-(34-51) peptide led to only a very small fluorescence increase and curves which could not be fitted to a simple binding isotherm under either salt concentration. The assay is also useful in its competition mode to assess the strength of binding of Tat peptide **3** to other unlabelled RNA constructs, by curve fitting to the fluorescence-intensity decrease as the BgQ TAR (**2**) is displaced when the competitive RNA concentration is increased.

Fluorescence increases were also used to assess the strength of binding of antisense oligonucleotides to the TAR RNA stem-loop. But this was only possible for those oligonucleotides that formed hybrids with the RNA that included the BgQ residue **1** at position 24. Again the strength of the interaction was found to be 2–3 fold stronger in the fluorescence assay as compared to mobility-shift analysis. At higher oligonucleotide concentration, a general reduction in fluorescence intensity was seen. This may be due to fluorescence quenching because of nonspecific interaction of the oligonucleotide



16-mer	5'-(CUC CCA GGC UCA AAU C)-3'
12-mer I	5'-(CCC AGG CUC AAA)-3'
12-mer II	5'-(CUC CCA GGC UCA)-3'
mism. 16-mer	5'-(GCU CCC **G**GG CUC **GA**C **C**)-3'
scr. 16-mer	5'-(CUC CGC GCC UGC GCA G)-3'

Fig. 7. *Fluorescence emission intensity of 20 nM BgQ TAR* (**2**) *at 430 nm* (excitation at 240 nm) *at different 2'-O-methyloligonucleotide concentrations.* The sequences of the oligonucleotides studied are shown below the graph. Nucleotides in bold represent mismatches to the TAR RNA sequence.

with the fluorophore or, more likely, to a filter effect due to the increased absorption of the oligonucleotide at the excitation wavelength (240 nm). Nevertheless, the assay could still be used up to 1 μM oligonucleotide and the binding strength measured. One way to get around a filter effect is to carry out excitation at the secondary absorbance maxima (294 or 360 nm), an approach we are currently investigating.

The fluorescence-intensity curves for BgQ TAR (**2**) are complex when both peptide and oligonucleotide are simultaneously present (data not shown), and we found that

the intensity changes cannot be simply related to concentrations of the TAR binding species when arranged in this competition format. Probably, the fluorescence intensity of BgQ is dependent on to what extent it is stacked into the free TAR RNA structure, or whether it is fully or partially unstacked when the Tat peptide or an oligonucleotide is bound. Similarly, complex fluorescence changes were found when small-molecule TAR-binding molecules competed against Tat peptide (data not shown). Therefore, it seems unlikely that the assay can be used for a competition screen of potential inhibitors of the Tat-TAR interaction.

However, we have shown that changes in the fluorescence intensity can be used to assess the direct binding of molecules (peptides, oligonucleotides) to the bulge of TAR. Since the fluorescence extinction coefficient and quantum yield of BgQ is very high, small molecules, even those containing aromatic groups, should be able to be assessed for direct TAR binding in the region of the bulge, and studies of this nature are in progress. Our results suggest that the fluorescent base BgQ should be generally useful also in studying protein interactions with synthetic RNA binding sites.

We thank *Justine Michel* (INSERM) for the synthesis of BgQ TAR (**2**) and *David Owen* for the synthesis of the Tat and Rev peptides.

Experimental Part

Syntheses. The HIV-1 Tat-(37-72) peptide (**3**) with sequence SFTTKALGISYGRKKRRQRRRPPQG-SQTHQVSLSKQ (Cys[37] was replaced by Ser to avoid problems with disulfide formation) and HIV-1 Rev-(34-51) peptide (Succ-TRQARRNRRRRWRERQRK-OH) were prepared by continuous-flow Fmoc-polyamide solid-phase synthesis as previously described [35–37]. The synthesis of TAR BRU 39 (**4**) was previously reported [23]. BgQ TAR (**2**) was synthesized on a 1-μmol scale on an *Expedite 8909*-DNA/RNA synthesizer (*PE Biosystems*) using RNA monomers having (*tert*-butyl)phenoxyacetyl protecting groups at the exocyclic NH_2 groups and (*tert*-butyl)dimethylsilyl protection at the 2′-*O*-position. The phosphoramidite of the BgQ-containing 2′-*O*-methylribonucleoside **1** was dissolved in anh. MeCN (0.1M final concentration) and the coupling time for this monomer was increased up to 15 min. The resulting protected BgQ TAR was deprotected by standard techniques and purified by prep. polyacrylamide-gel electrophoresis visualized by UV shadowing.

The complete synthesis of the phosphoramidite of **1** will be published elsewere [30b]; the key step relies on the highly efficient procedure developed by *Ross et al.* for the synthesis of 2′-*O*-methyluridine, which is based on the opening of the 2-*O*,2′-anhydrouridine by trimethyl borate in hot MeOH [38]. We successfully extended this procedure to the benzo[*g*]quinazoline-2,4(1*H*,3*H*)-dione ribonucleoside [30a]. The ribonucleoside was obtained from the glycosylation of α-D-ribofuranose 1-acetate 2,3,5-tribenzoate by the silylated derivative of the benzo[*g*]quinazoline-2,4(1*H*,3*H*)-dione with trimethylsilyl triflate as a catalyst. The phosphoramidite was then obtained by classical dimethoxytritylation and phosphitylation procedures.

The 2′-*O*-methyloligoribonucleotides were prepared by standard solid-phase chemical synthesis from phosphoramidite reagents obtained from *Cruachem* or *Glen Research via Cambio*. The mass of each oligonucleotide was checked by MALDI-TOF mass spectrometry on a *Perseptive Biosystems Voyager DE* mass spectrometer as previously described [10]. Sequences of oligonucleotides: 16-mer 5′-(CUC CCA GGC UCA AAU C)-3′, 12-mer I 5′-(CCC AGG CUC AAA)-3′, 12 mer II 5′-(CUC CCA GGC UCA)-3′, mismatched 16-mer 5′-(GCU CCC **G**GG CUC **GACC**)-3′ (bold units show positions of mismatch), scrambled 16-mer 5′-(CUC CGC GCC UGC GCAG)-3′.

Folding of TAR RNA. For gel-mobility-shift experiments, 28.6 nM ^{32}P-labelled TAR BRU 39 [5′-^{32}P]-**4** or 35.7 nM ^{32}P-labelled BgQ TAR [5′-^{32}P]-**2** in 70 μl of 50 mM *Tris*·HCl (pH 7.4) and 20 mM KCl was incubated for 10 min at 75° and slowly cooled to 37°. Then the stock solns. (125 μl) of the corresponding TAR RNA (16 or 20 nM) in 200 mM *Tris*·HCl (pH 7.4), 80 mM KCl, 20 mM DTT, and 0.2% *Triton X-100* containing 40 units of RNase inhibitor RNasin (*Promega*) were made and kept on ice.

For fluorescence measurements, 50 or 200 nM BgQ TAR (**2**) in 50 mM *Tris*·HCl (pH 7.4) and 20 or 80 mM KCl was incubated for 10 min at 75°, slowly cooled to 37°, and then put on ice. The final portions (400 μl) of BgQ TAR **2** (5 or 20 nM) in 50 mM *Tris*·HCl (pH 7.4) and 20 or 80 mM KCl were prepared and stored on ice before

use. TAR BRU 39 (**4**) (800 nM) for competitive binding assays was refolded similarly in 50 mM *Tris*·HCl (pH 7.4) and 20 mM KCl and kept on ice.

Gel-Mobility-Shift Analysis: 1) *TAR RNA with Tat-(37–72) peptide* (**3**). The 4–5 nM [5′-^{32}P]-**4** or [5′-^{32}P]-**2** was incubated with increasing amounts of Tat peptide **3** in 50 mM *Tris*·HCl (pH 7.4), 20 mM KCl, 5 mM DTT, 0.05% *Triton X-100*, and 0.08 unit/μl RNasin at 37° for 15 min. To each sample, loading buffer was added to give 0.025% bromophenol blue and 13% sucrose, and electrophoresis was carried out on 6% native polyacrylamide gel and run in 44.6 mM *Tris* borate (pH 8.3), 1 mM EDTA, 0.05% *Triton X-100*, and 0.2% glycerol at r.t. for 1 h. The gel was dried and visualized by autoradiography. The dried gels were also exposed to a phosphor storage screen (*Molecular Dynamics*) and scanned by a model *425S PhosphorImager*™ (*Molecular Dynamics*). The resulting digitized images were analyzed by *Geltrak* on a *DEC/Alpha 2100*™ (*Digital Equipment Corporation*, Maynard, MA, USA) through an X-terminal [39] and apparent K_d values estimated as the concentration required for 50% complex formation.

2) *TAR RNA with 2′-*O*-Methyloligonucleotides.* The 4–5 nM [5′-^{32}P]-**4** or [5′-^{32}P]-**2** was incubated with increasing amounts of 16-mer 2′-*O*-methyloligonucleotide in 50 mM *Tris*·HCl (pH 7.4), 20 mM KCl, 1 mM DTT, and 0.08 unit/μl RNasin at 37° for 15 min. To each sample, loading buffer was added to give 0.025% bromophenol blue and 13% sucrose, and electrophoresis was carried out on 8% native polyacrylamide gel and run in 20 mM *Tris* acetate (pH 8.3), 1 mM EDTA, and 0.2% glycerol at r.t. for 1 h. The gel was analyzed as described above.

Fluorescence Measurements. Fluorescence measurements were carried out on a *Perkin-Elmer LS-50B* luminescence spectrometer with thermostat control accurate to ±0.1°. The soln. of BgQ TAR (**2**) was excited at 240 nm and monitored at 430 nm. The integration time was 2 s. For calculations of K_d, the average values of three measurements were used. Measurements were carried out at 20° in buffer soln. containing 50 mM *Tris*·HCl and 20 or 80 mM KCl (pH 7.4). The solns. of **2** with increasing amounts of oligonucleotides were preincubated on ice for 1 h before recording the fluorescent spectra.

Determination of Dissociation Constants. Eqn. 1 [40] was used for the determination of the dissociation constant K_d for the interaction between BgQ TAR (**2**) and Tat-(37–72) peptide (**3**)

$$F = F_o + \Delta F\{([RNA]_o + [P]_o + K_d) - (([RNA]_o + [P]_o + K_d)^2 - 4[RNA]_o[P]_o)^{1/2}\}/2[RNA]_o \quad (1)$$

where F and F_o are the fluorescence intensity of **2** in the presence and absence of **3**, resp. ΔF is the fluorescence coefficient of the **2**/**3** complex per nmol of complex. $[RNA]_o$ and $[P]_o$ are the initial concentrations of **2** and **3**, resp.

In the competitive binding assay, *Eqn. 2* generated from the equation for fractional saturation of labelled probe [41] was used for the calculation of the dissociation constant K_c for competitor RNA/Tat peptide complex:

$$F = F_o + \Delta F\{[RNA]_o + [P]_o + K_d + (K_d/K_c)[C] - (([RNA]_o + [P]_o + K_d + (K_d/K_c)[C])^2 - 4[RNA]_o[P]_o)^{1/2}\}/2[RNA]_o \quad (2)$$

where F is the fluorescence intensity in the presence of competitor RNA, F_o the fluorescence intensity in the absence of both peptide and competitor RNA, and ΔF, $[RNA]_o$, $[P]_o$, and K_d are the same as in *Eqn. 1*. [C] is the concentration of competitor RNA. A competition curve, $F = f([C])$, may be fitted for the best value of K_c by the nonlinear least-squares method with *KaleidaGraph* software (*Abelbeck Software*).

REFERENCES

[1] J. Karn, *J. Mol. Biol.* **1999**, *293*, 235.
[2] R. Taube, K. Fujinaga, J. Wimmer, M. Barboric, B. M. Peterlin, *Virology* **1999**, *264*, 245.
[3] T. M. Rana, K.-T. Jeang, *Arch. Biochem. Biophys.* **1999**, *365*, 175.
[4] N. J. Keen, M. J. Churcher, J. Karn, *EMBO J.* **1997**, *16*, 5260.
[5] C. Isel, J. Karn, *J. Mol. Biol.* **1999**, *290*, 929.
[6] F. Hamy, U. Asseline, J. A. Grasby, S. Iwai, C. E. Pritchard, G. Slim, P. J. G. Butler, J. Karn, M. J. Gait, *J. Mol. Biol.* **1993**, *230*, 111.
[7] M. J. Churcher, C. Lamont, F. Hamy, C. Dingwall, S. M. Green, A. D. Lowe, P. J. G. Butler, M. J. Gait, J. Karn, *J. Mol. Biol.* **1993**, *230*, 90.
[8] Z. Wang, T. M. Rana, *Biochemistry* **1996**, *35*, 6491.

[9] N. A. Naryshkin, M. A. Farrow, M. G. Ivanovskaya, T. S. Oretskaya, Z. A. Shabarova, M. J. Gait, *Biochemistry* **1997**, *36*, 3496.
[10] M. A. Farrow, F. Aboul-ela, D. Owen, A. Karpeisky, L. Beigelman, M. J. Gait, *Biochemistry* **1998**, *37*, 3096.
[11] Z. Wang, T. M. Rana, *Biochemistry* **1998**, *37*, 4235.
[12] J. D. Puglisi, R. Tan, B. J. Calnan, A. D. Frankel, J. R. Williamson, *Science (Washington, D.C.)* **1992**, *257*, 76.
[13] F. Aboul-ela, J. Karn, G. Varani, *J. Mol. Biol.* **1995**, *253*, 313.
[14] F. Aboul-ela, J. Karn, G. Varani, *Nucleic Acids Res.* **1996**, *24*, 3974.
[15] M. J. Seewald, A. U. Metzger, D. Willbold, P. Rösch, H. Sticht, *J. Biomol. Struct. Dynamics* **1998**, *16*, 683.
[16] K. S. Long, D. M. Crothers, *Biochemistry* **1999**, *38*, 10059.
[17] D. Daelemans, A.-M. Vandamme, E. De Clercq, *Antiviral Chem. Chemotherapy* **1999**, *10*, 1.
[18] F. Hamy, E. R. Felder, G. Heizmann, J. Lazdins, F. Aboul-ela, G. Varani, J. Karn, T. Klimkait, *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 3548.
[19] I. Huq, X. Wang, T. M. Rana, *Nat. Struct. Biol.* **1997**, *4*, 881.
[20] N. Tamilarisu, I. Huq, T. M. Rana, *J. Am. Chem. Soc.* **1999**, *121*, 1597.
[21] S. Hwang, N. Tamilarisu, K. Ryan, I. Huq, S. Richter, W. Still, T. M. Rana, *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12997.
[22] T. Vickers, B. F. Baker, P. D. Cook, M. Zounes, R. W. J. Buckheit, J. Germany, D. J. Ecker, *Nucleic Acids Res.* **1991**, *19*, 3359.
[23] B. Mestre, A. Arzumanov, M. Singh, F. Boulmé, S. Litvak, M. J. Gait, *Biochim. Biophys. Acta* **1999**, *1445*, 86.
[24] A. Arzumanov, M. J. Gait, in 'Collection Symposium Series', Eds. A. Holy and M. Hocek, Academy of Sciences of the Czech Republic, 1999, p. 168.
[25] F. Boulmé, F. Freund, S. Moreau, P. Nielsen, S. Gryaznov, J.-J. Toulmé, S. Litvak, *Nucleic Acids Res.* **1998**, *26*, 5492.
[26] C. Boiziau, E. Dausse, L. Yurchenko, J.-J. Toulmé, *J. Biol. Chem.* **1999**, *274*, 12730.
[27] F. Ducongé, J.-J. Toulmé, *RNA* **1999**, *5*, 1605.
[28] J. Karn, M. J. Churcher, K. Rittner, A. Kelley, P. J. G. Butler, D. A. Mann, M. J. Gait, in ' HIV. A Practical Approach', Ed. J. Karn, Oxford University Press, Oxford, 1995, p. 147.
[29] M. J. Gait, D. J. Earnshaw, M. A. Farrow, J. H. Fogg, R. L. Grenfell, N. A. Naryshkin, T. V. Smith, in 'RNA-Protein Interactions: A Practical Approach', Ed. C. Smith, OUP, Oxford, UK, 1998, p. 1.
[30] a) F. Godde, S. Moreau, J.-J. Toulmé, *Antisense and Nucleic Acid Drug Develop.* **1998**, *8*, 1; b) F. Godde, J. J. Toulmé, S. Moreau, submitted to *Nucleic Acids Res.*
[31] F. Godde, J.-J. Toulmé, S. Moreau, *Biochemistry* **1998**, *37*, 13765.
[32] G. Slim, C. E. Pritchard, E. Biala, U. Asseline, M. J. Gait, *Nucleic Acids Res. Symp. Ser.* **1991**, *24*, 55.
[33] J. L. Battiste, R. Tan, A. D. Frankel, J. R. Williamson, *Biochemistry* **1994**, *33*, 2742.
[34] C. T. Rigl, D. H. Lloyd, D. S. Tsou, S. M. Gryaznov, W. D. Wilson, *Biochemistry* **1997**, *36*, 650.
[35] T. Johnson, M. Quibell, D. Owen, R. C. Sheppard, *J. Chem. Soc., Chem. Commun.* **1993**, 369.
[36] M. Quibell, W. G. Turnell, T. Johnson, *J. Org. Chem.* **1994**, *59*, 1745.
[37] M. Quibell, L. C. Packman, T. Johnson, *J. Chem. Soc., Perkin Trans. 1* **1996**, 1227.
[38] B. S. Ross, R. H. Springer, Z. Tortorici, S. Dimock, *Nucleosides Nucleotides* **1997**, *16*, 1641.
[39] J. Smith, M. Singh, *Biotechniques* **1996**, *20*, 1082.
[40] J. Cho, K. Hamasaki, R. R. Rando, *Biochemistry* **1998**, *37*, 4985.
[41] S.-Y. Linn, A. D. Riggs, *J. Mol. Biol.* **1972**, *72*, 671.

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