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The Integrity of the Stem Structure of Human Immunodeficiency Virus Type 1 Tat-Responsive Sequence RNA Is Required for Interaction with the Interferon-Induced 68,000-M_r Protein Kinase

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A number of eucaryotic viruses have devised strategies to minimize the deleterious effects on protein synthesis caused by activation of the interferon-induced, double-stranded-RNA-activated protein kinase, P68. In a recent report, we described the down regulation of the P68 protein kinase in cells infected by human immunodeficiency virus type 1 (HIV-1) (S. Roy, M. G. Katze, N. T. Parkin, I. Edery, A. G. Hovanessian, and N. Sonenberg, Science 247:1216–1219, 1990). We now present evidence that such a decrease in amounts of P68 could be essential for HIV-1 replication because of the presence of the Tat-responsive sequence (TAR sequence) present in the 5′ untranslated region of HIV-1 mRNAs, which activates the P68 kinase. We found that poly(A)$^+$ mRNAs prepared from HIV-1-infected cells efficiently activated the protein kinase as did mRNAs from stably transformed cell lines constitutively expressing the TAR region. Furthermore, we found that TAR-containing RNAs complexed with purified P68 protein kinase in vitro by two independent assays and could be cross-linked to P68 kinase present in a HeLa cell extract. Experiments using in vitro-synthesized wild-type and mutant TAR RNAs revealed that both the efficient binding to and the activation of P68 kinase were dependent on the TAR RNA stem structure. The TAR-P68 complex could be competed out by a synthetic RNA that bound to and activated the protein kinase but not by a synthetic RNA that bound with low affinity and did not activate P68. The possible biological consequences of a P68-TAR interaction that may include the switch from latent to active virus replication are discussed.

The double-stranded RNA (dsRNA)-dependent kinase P68 (also called dsI, DAI, or PI/eIF2 kinase) is one of many interferon-inducible genes (28, 30, 36, 40, 48, 60). As a result of activation by dsRNA, P68 kinase undergoes autophosphorylation and catalyzes the phosphorylation of the alpha subunit of eucaryotic initiation factor 2 (eIF-2) (12; for a recent review, see reference 18). This causes trapping of eIF-2 in a complex with guanine nucleotide exchange factor (eIF-2B) and GDP (38, 46). Limitations in functional eIF-2 hinder the initiation step of protein synthesis by preventing the transfer of initiator Met-tRNA (via the ternary complex eIF-2–GTP–Met-tRNA$_i$) to the 40S ribosomal subunit before binding to an mRNA molecule.

Activation of the P68 kinase has been suggested to play a role in the interferon response to viral infection, but no definitive proof has been obtained (for reviews, see references 30 and 36). A number of eucaryotic viruses have devised strategies to minimize the deleterious effects on protein synthesis caused by activation of the kinase (for reviews, see references 51 and 56). Adenovirus encodes an RNA polymerase III product, VA1 RNA, which binds to and sequesters P68 (13, 21, 33, 50), whereas influenza virus blocks P68 activity by activating a cellular inhibitor of P68 (22, 29). Poliovirus induces degradation of the kinase, although what remains is highly phosphorylated (3), while other viruses, such as encephalomyelocarditis virus, vesicular stomatitis virus, and vaccinia virus, sequester the P68 kinase in a detergent-insoluble form (6, 19, 39). Down regulation of the P68 kinase was recently described during productive infection by human immunodeficiency virus type 1 (HIV-1) (44). Decreased amounts of interferon-induced P68 kinase were also observed in established cell lines stably expressing the HIV-1 *trans*-activator protein Tat, suggesting that Tat controls P68 activity during HIV-1 infection (44).

The down regulation of the P68 kinase may be essential for HIV-1 replication to prevent the translational inhibitory effects caused by activation of the kinase. Previous studies have shown that fusion of the Tat-responsive region in the 5′ untranslated region (UTR) of HIV-1 mRNAs (termed TAR; 42) to a heterologous mRNA (chloramphenicol acetyltransferase [CAT]) exhibited *cis*- and *trans*-inhibitory effects on translation in vitro (7, 34). These inhibitory effects, which were dependent on the RNA secondary structure in the TAR region, could be reversed by addition of purified eIF-2B and were prevented by high concentrations of poly(I)-poly(C) that block activation of the P68 kinase (7). Moreover, synthetic RNAs containing TARCAT sequences stimulated phosphorylation of the P68 kinase and the alpha subunit of eIF-2 in reticulocyte lysate and NIH 3T3 cell extracts (7, 54). Taken together, these studies suggested that the TAR region could activate P68 in vitro.

A pertinent question is whether the TAR region activates P68 in vivo. To begin to address this question, we monitored the ability of poly(A)$^+$ RNA from HIV-1-infected cells and from cells constitutively expressing the TAR region to stimulate autophosphorylation of highly purified P68. Our results demonstrate that preparations of TAR containing

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poly(A)$^+$ RNA are activators of the P68 kinase. We also show that TAR RNAs bind to immunoaffinity-purified P68 in vitro and can be cross-linked to P68 present in a HeLa cell extract. Both efficient binding to and activation of the P68 kinase are dependent on the integrity of the RNA stem structure. Possible biological consequences of the interaction between TAR-containing RNAs and P68 kinase are discussed.

MATERIALS AND METHODS

Isolation of poly(A)$^+$ from CEM and HeLa S3 cells. CEM cells were infected with the LAV isolate of HIV-1 as described previously (1, 44). A mock infection was carried out at the same time. Total RNA was extracted by guanidine hydrochloride precipitation and phenol-chloroform extraction (58) 3 days after infection, when over 90% of cells expressed viral proteins, as determined by indirect immunofluorescence. RNA was also extracted from HeLa S3 cell lines stably expressing CAT under the control of the HIV-1 long terminal repeat (LTR) (HeLa/TARCAT) or under the control of the simian virus 40 (SV40) promoter (HeLa/CAT). These cell lines were established by cotransfection of the CAT expression vectors pU3RIII (containing the HIV-1 LTR; 55) or pSV2CAT (containing the SV40 promoter; 14) with pSV2neo (57) by the calcium phosphate precipitation method (59). Clones resistant to G418 (450 μg/ml) were screened for CAT activity (14). Clones expressing similar amounts of CAT activity were expanded for RNA isolation. Polyadenylated RNA was purified from total RNA by oligo(dT)-cellulose chromatography (47).

Site-directed mutagenesis. Mutations were introduced in the TAR region by oligonucleotide-directed mutagenesis in M13 by use of the two-primer method (61). The mutations were confirmed before and after subcloning in pSP64TAR CAT (34) by sequencing with the dideoxy-chain termination method (49). The compensatory mutant TAR I,III,I′,III′ was constructed by subcloning, using the *Sac*I site at position +34 in the HIV-1 TAR sequence.

In vitro transcriptions. The SP64TARCAT constructs (34) were linearized with *Hin*dIII (at nucleotide +80 in the HIV-1 sequence), phenol extracted, passed through a G-50 spun column, and ethanol precipitated.

Uncapped SP6-derived transcripts were generated in the presence of [α-^{32}P]UTP or [5-^{3}H]CTP as described by Green et al. (16). The ^{32}P-labeled RNAs used in the RNA mobility shift assay were purified on 8% polyacrylamide–8 M urea gels, eluted in 0.5 M ammonium acetate–1 mM EDTA–0.1% sodium dodecyl sulfate (SDS) (15), and recovered by two phenol extractions followed by ethanol precipitation.

In vitro kinase assay. The in vitro kinase reaction was performed by mixing ~50 or ~100 ng of immunoaffinity-purified P68 kinase (13) with the indicated amounts of RNA in a buffer containing 17 mM Tris hydrochloride (pH 7.5), 75 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 μM ATP, 0.8 mM dithiothreitol, 0.3 μg of bovine serum albumin per μl, and [γ-^{32}P]ATP (10 μCi; Du Pont Biotechnology Systems) at 30°C for 15 min. The final volume of the reaction mixture was 40 μl. The reaction was stopped by addition of 20 μl of 3× electrophoresis buffer, 10 μg of RNase A, and 1 μl of 1M $MgCl_2$ followed by boiling. The samples were subjected to electrophoresis on an SDS–10% polyacrylamide gel and autoradiography. Quantitations of P68 kinase autophosphorylation were performed by scanning of X-ray films with an LKB laser densitometer. In some experiments, the RNAs were incubated with 1 μl of RNase III (300 μg/ml; generously supplied by Donald Court, NCI-Frederick Cancer Research Facility) in a total reaction volume of 10 μl containing 50 mM Tris hydrochloride (pH 7.5), 130 mM NH_4Cl, and 10 mM $Mg(OAc)_2$ for 30 min at 37°C. After phenol-chloroform extraction and ethanol precipitation, the RNase III-treated RNAs were tested in the in vitro kinase assay.

In vitro binding assays of P68 kinase and TAR RNAs. The RNA mobility shift assay was performed essentially according to Konarska and Sharp (24), with modifications previously described (43). Briefly, ~250 to 500 ng of immunoaffinity-purified P68 kinase (13) was preincubated in a buffer containing 10 mM Tris hydrochloride (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 50 mM NaCl, 0.5 U of RNasin per μl, 0.09 μg of bovine serum albumin per μl, and 0.05% (vol/vol) glycerol at 30°C for 10 min. ^{32}P-labeled RNA (2,000 cpm, 2.94×10^6 cpm/μg; gel purified) was added for a further incubation of 10 min. The final volume of the reaction mixture was 20 μl. Samples were loaded on a 1.5-mm-thick 5% polyacrylamide gel (30:0.8, acrylamide/bisacrylamide) containing 5% glycerol, which had been preelectrophoresed for 30 min at 30 mA. Electrophoresis was carried out in 0.5× Tris-borate-EDTA at a constant current of 30 mA for 2.5 h at 4°C. The gel was dried and exposed to X-ray film with an intensifying screen at −70°C. The second in vitro binding assay used has been described elsewhere (21). Briefly, the P68 kinase was purified from interferon-treated 293 cells, utilizing Sepharose beads coupled to a monoclonal antibody to P68 kinase (MAb-Sepharose). ^{32}P-labeled TAR RNA (150,000 cpm) was added to the immunoprecipitated P68 kinase for a 20-min incubation at 30°C. To control for nonspecific sticking, RNA was added to MAb-Sepharose that had been exposed to an interferon-treated bovine kidney cell extract (the bovine P68 kinase is not retained on MAb-Sepharose since the monoclonal antibody against P68 is specific for the human kinase). The complexes were washed in 10 mM Tris hydrochloride (pH 7.5)–100 mM KCl–0.1 mM EDTA–100 U of aprotinin per ml–20% glycerol and resuspended in a buffer containing 50 mM Tris hydrochloride (pH 7.5), 150 mM NaCl, 0.05% Nonidet P-40, carrier tRNA, and 1% SDS. After phenol-chloroform extraction and ethanol precipitation, TAR RNA specifically bound to P68 was analyzed by electrophoresis on an 8% polyacrylamide–8 M urea gel and then autoradiographed.

UV-induced cross-linking assay. Cross-linking was performed essentially as described by Pelletier and Sonenberg (35). Briefly, 30 μg of an interferon-treated HeLa S10 extract (44) was incubated with ^{32}P-labeled TAR RNA (1.5×10^6 cpm, 2.94×10^6 cpm/μg) in a buffer containing 5 mM *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid (HEPES; pH 7.6), 150 mM KCl, 2 mM $MgCl_2$, 3.8% (vol/vol) glycerol, 20 μM dithiothreitol, and 1.5 mM ATP at 30°C for 10 min. The reaction mixture was placed under a germicidal lamp (254 nm) at a distance of 2 cm for 30 min. The UV-irradiated samples were treated with 20 μg of RNase A at 37°C for 30 min. Four-fifths of the sample was subjected to immunoprecipitation using a polyclonal antibody raised in mouse against P68 kinase (27) and protein A-Sepharose that had been prereacted with rabbit anti-mouse immunoglobulin. The immunoprecipitated proteins were analyzed by SDS–10% polyacrylamide gel electrophoresis (PAGE) and autoradiography in parallel with the remaining fifth of the UV-irradiated proteins.

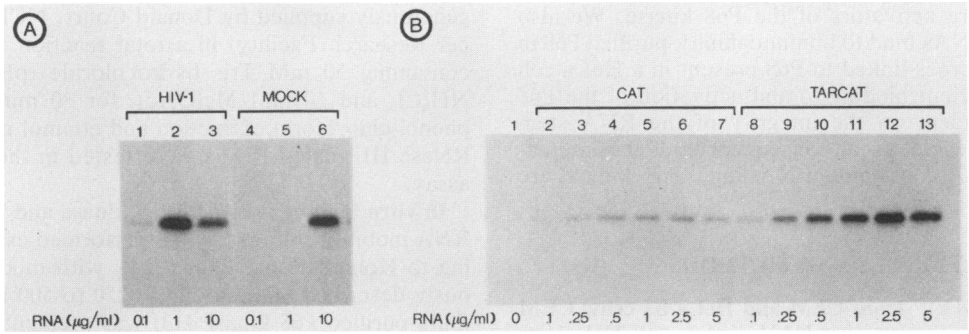

FIG. 1. Activation of the P68 kinase by TAR-containing poly(A)$^+$ RNAs. (A) Poly(A)$^+$ RNAs were isolated from HIV-1-infected (lanes 1 to 3) and mock-infected (lanes 4 to 6) CEM cells 3 days after infection and tested in the in vitro kinase assay, using ~100 ng of immunopurified P68 as described in Materials and Methods. (B) Poly(A)$^+$ RNAs isolated from cell lines stably expressing the CAT gene under the control of the SV40 promoter (CAT; lanes 2 to 7) or the HIV-1 LTR (TARCAT; lanes 8 to 13) were tested in the in vitro kinase assay, using ~ 50 ng of immunoaffinity-purified P68 as described in Materials and Methods. Lane 1, No RNA.

RESULTS

Activation of the P68 kinase by TAR-containing poly(A)$^+$ RNAs. Productive infection by HIV-1 was previously shown to be associated with a decrease in the amount of the P68 kinase (44). This virus-induced control of P68 likely represents a strategy to avoid inhibition of protein synthesis caused by activation of this protein kinase by viral mRNAs. To determine whether HIV-1 mRNAs have the potential to activate the P68 kinase, poly(A)$^+$ RNA was isolated from HIV-1-infected and mock-infected CEM cells 3 days after infection. At this time, over 90% of cells were found to express viral proteins, as determined by indirect immunofluorescence (1, 44). These poly(A)$^+$ RNAs were tested in an in vitro kinase assay that consisted of incubating immunoaffinity-purified P68 kinase with increasing amounts of RNA in the presence of [γ-^{32}P]ATP (Fig. 1A). The activating potential of the mRNAs was measured by the degree of P68 autophosphorylation. Detectable amounts of phosphorylated P68 were seen with as little as 0.1 μg of mRNA per ml from HIV-1-infected cells (lane 1). Kinase activity increased dramatically (~20-fold) with 1 μg/ml (lane 2) and then decreased with 10 μg/ml (lane 3). This result is consistent with the observation that high concentrations of dsRNA inhibit activation of the P68 kinase (9, 20, 23). In contrast, mRNAs from mock-infected cells were poor activators of P68 kinase at 1 μg of RNA per ml (compare lane 5 with lane 2). Significant phosphorylation of P68 kinase was apparent only in the presence of 10 μg of mRNA per ml (lane 6).

Activation of P68 kinase by HIV-1 mRNAs may be due, at least in part, to the presence of the secondary structure in the TAR sequence of the 5′ UTR of viral mRNAs. To examine this possibility, we used a HeLa cell line stably expressing the indicator CAT gene under the control of the HIV-1 LTR (HeLa/TARCAT). The activating potential of mRNAs isolated from this cell line was compared with that from a HeLa cell line stably expressing CAT under the control of the SV40 promoter (HeLa/CAT). Poly(A)$^+$ RNA was isolated from clones expressing similar amounts of CAT activity and tested in the in vitro kinase assay. mRNAs from the HeLa/TARCAT cell line were better activators of P68 than were mRNAs from the HeLa/CAT cell line at all mRNA concentrations tested (Fig. 1B; compare lanes 2 to 7 with lanes 8 to 13). In the presence of 2.5 μg of mRNA per ml from the HeLa/TARCAT cell line, which resulted in maximal kinase activity, the amount of phosphorylated P68 was over 10-fold greater than that observed by the same concentration of mRNA from the HeLa/CAT cell line (compare lane 12 with lane 6). These results are consistent with the notion that the TAR sequence, in the 5′ UTR of mRNAs, activates the interferon-inducible P68 kinase, as defined by the ability of the kinase to undergo autophosphorylation in an in vitro kinase assay. However, these results do not exclude the possibility that other dsRNA structures synthesized during HIV-1 infection also activate P68.

Secondary structure within the TAR region is required for binding to and activation of the P68 kinase. Since earlier reports (7, 34) demonstrated that translational inhibition conferred by the TAR region was dependent on the integrity of the stem-loop structure, we wished to examine the ability of TAR RNAs with decreased secondary structure to stimulate autophosphorylation of P68 kinase in an in vitro kinase assay. We introduced eight nucleotide substitutions on either side of the stem of TAR by site-directed mutagenesis (Fig. 2; 45). The mutations were designed so that when present alone they are predicted to disrupt base pairing, but when combined together they are compensatory and pre-

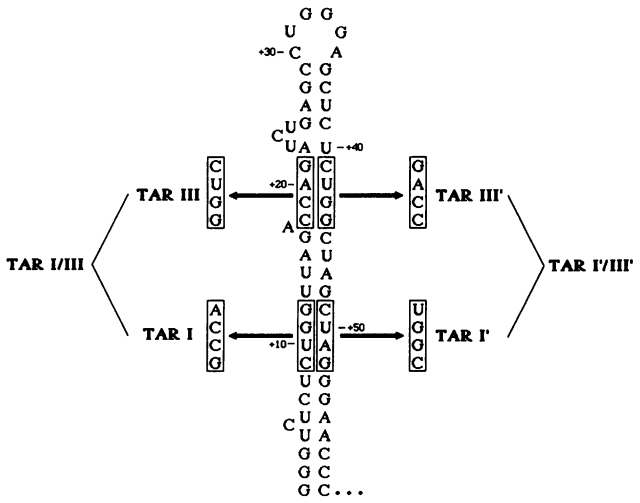

FIG. 2. Stem mutants in the predicted secondary structure of the TAR region. Nucleotides shown in boxes were changed to those indicated by site-directed mutagenesis. The compensatory mutant TAR I,III,I′,III′ was constructed by subcloning, using the *Sac*I site at position +34.

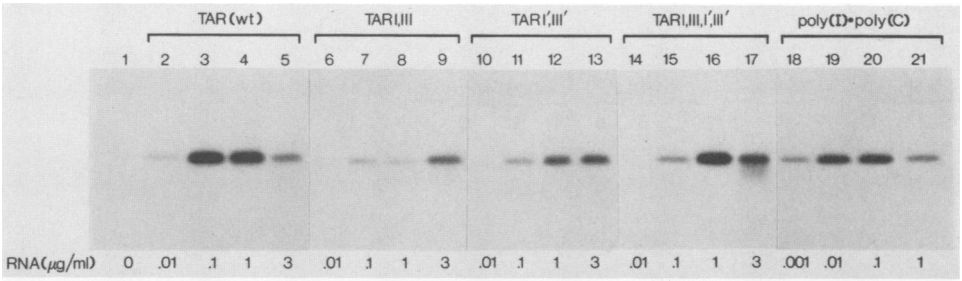

FIG. 3. Activation of the P68 kinase by wild-type (wt) and mutant TAR RNAs. ^{3}H-labeled TAR RNAs comprising nucleotides +1 to +80 of the HIV-1 LTR were synthesized by SP6 polymerase and tested in the in vitro kinase assay, using ~50 ng of immunoaffinity-purified P68 as described in Materials and Methods (lanes 2 to 17). Various concentrations of the synthetic dsRNA poly(I)-poly(C) were tested in parallel (lanes 18 to 21). Lane 1, No RNA.

dicted to restore base pairing. The mutations were subcloned into plasmid pSP64TARCAT. This construct generates RNA which begins at nucleotide +1 of the HIV-1 LTR and thus has the same nucleotide sequence at its 5′ end as do HIV-1 mRNAs synthesized in vivo (34). ^{3}H-labeled RNAs comprising nucleotides +1 to +80 of the HIV-1 TAR sequence and synthesized in vitro by SP6 polymerase were tested (Fig. 3). Phosphorylation of the P68 kinase could be detected in the presence of as little as 0.01 μg of wild-type TAR RNA per ml (lane 2). The extent of phosphorylation was highest with 0.1 and 1 μg/ml (lanes 3 and 4) and then decreased with 3 μg/ml (lane 5). Mutants TAR I,III and TAR I′,III′, predicted to disrupt the integrity of the stem, activated the P68 kinase but to a much lesser degree (lanes 6 to 13). In the presence of 1 μg/ml, the amounts of phosphorylated P68 by TAR I,III and TAR I′,III′ were decreased ~3- and ~17-fold, respectively, compared with that by wild-type TAR RNA at the same concentration (compare lanes 8 and 12 with lane 4). In the presence of 3 μg/ml, the extent of autophosphorylation by TAR I′,III′ was similar to that with 1 μg of TAR I,III per ml but was still less than that with 0.1 and 1 μg of wild-type TAR RNA per ml (compare lanes 9 and 13 with lanes 3 and 4). Activation of the P68 kinase by the double mutant TAR I,III,I′,III′, predicted to restore base pairing in the stem of TAR RNA, was significantly greater than that observed with the mutants TAR I,III and TAR I′,III′ but somewhat less than that with the wild-type RNA (lanes 14 to 17). Although maximal kinase activity occurred with 0.1 μg of wild-type RNA per ml (lane 3), a similar degree of P68 phosphorylation required 1 μg of TAR I,III,I′,III′ per ml (lane 16). The degree of phosphorylation decreased with 3 μg of TAR I,III,I′,III′ per ml (lane 17), as was observed with the wild-type RNA. These results provide evidence that activation of the P68 kinase is dependent on the integrity of the stem structure in TAR RNA. The overall activating potential of wild-type TAR RNA was comparable to that of the synthetic dsRNA poly(I)-poly(C) (lanes 18 to 21) although 10-fold-more TAR RNA was required for maximal kinase activity.

Since activators of P68 kinase such as poly(I)-poly(C) were previously shown to bind to P68 (13), we next examined the binding capacity of wild-type and mutant TAR RNAs by a variety of methods. We first performed an RNA mobility shift assay in which ^{32}P-labeled wild-type or mutant TAR RNAs were incubated with immunoaffinity-purified P68 kinase. Ribonucleoprotein complexes were resolved on a nondenaturing polyacrylamide gel and then autoradiographed (Fig. 4A). Wild-type TAR RNA formed a discrete complex with 0.25 μg of P68 (lane 2). In the presence of 0.5 μg of P68, the amount of protein-bound RNA increased although the complex was more diffused (lane 3), suggesting that more than one kinase molecule bound to the RNA. Mutants TAR I,III (lanes 5 and 6) and TAR I′,III′ (lanes 8 and 9), which were poor activators of the kinase, failed to form stable complexes with P68, although upon longer exposure faint nucleoprotein complexes could be seen in the presence of 0.5 μg of P68 (data not shown). The double mutant TAR I,III,I′,III′ formed a discrete complex in the presence of 0.25 μg of P68 (lane 11) which was more prominent but more diffuse in the presence of 0.5 μg of P68 (lane 12). Complex formation was slightly less efficient than that observed with the wild-type RNA (compare lane 3 with lane 12). Lanes 4 and 10 also contained a slower-migrating RNA. This minor band, which appeared occasionally, is presumably due to the formation of an alternative RNA structure.

Further evidence that binding of TAR RNA to P68 kinase is dependent on the integrity of the RNA secondary structure was obtained by using an assay previously described by Katze et al. (21). P68-specific MAb-Sepharose (26) was incubated with an extract from interferon-treated 293 cells (an adenovirus type 5 human embryonic kidney cell line). After removal of unbound proteins, ^{32}P-labeled wild-type and mutant TAR RNAs were added. The RNAs were extracted from the column with phenol-chloroform, ethanol precipitated, analyzed on a denaturing urea-polyacrylamide gel, and autoradiographed. To control for nonspecific binding, the RNAs were added to MAb-Sepharose preincubated with an interferon-treated cell extract of bovine origin. Since the monoclonal antibody is species specific and recognizes only the human P68 kinase, the RNAs are not expected to be retained on this column lacking P68. Specific binding to immunoprecipitated P68 could be detected only with the wild-type and double-mutant TAR I,III/I′,III′ RNAs (Fig. 4B, lanes 1 and 7). Although the mutant TAR I,III and TAR I′,III′ RNAs did bind weakly to immunoprecipitated P68, the interaction was nonspecific since these RNAs bound with a similar efficiency to the column lacking P68 (compare lanes 3 and 4 and lanes 5 and 6). These results demonstrate that TAR RNA binding to the P68 kinase is dependent on the integrity of the stem-loop structure and correlates with activation of the kinase.

Previous studies have demonstrated that the P68 kinase contains high- and low-affinity RNA binding sites (13). High-affinity binding was correlated with activation of the kinase, whereas low-affinity binding was correlated with inhibition of the kinase activity (13). In light of these results, TAR RNAs which activate P68 should bind to P68 with high

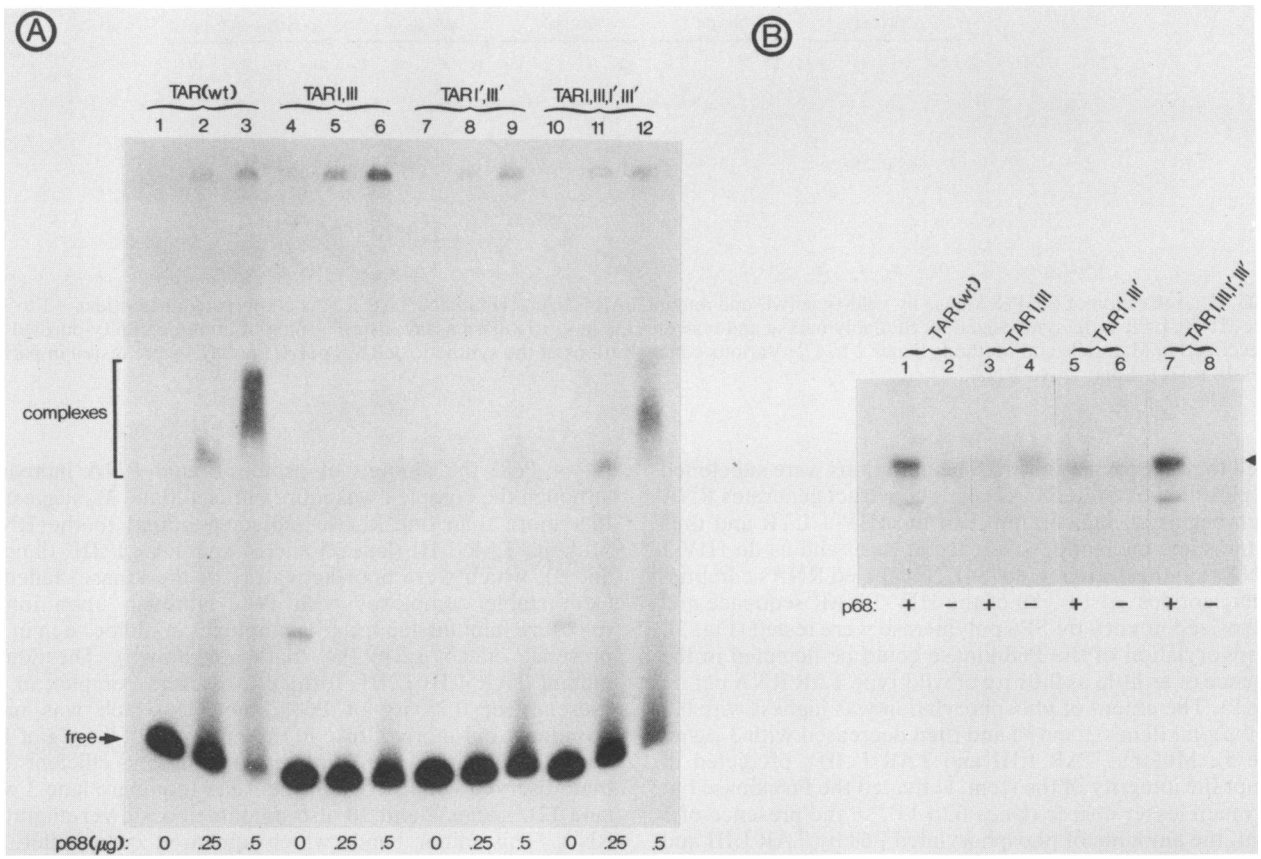

FIG. 4. Binding of wild-type (wt) and mutant TAR RNAs to immunoaffinity-purified P68 kinase. (A) ^{32}P-labeled TAR RNAs synthesized by SP6 polymerase were incubated in the absence or presence of immunoaffinity-purified P68, and complexes were resolved on a nondenaturing polyacrylamide gel as described in Materials and Methods. (B) ^{32}P-labeled TAR RNAs synthesized by SP6 polymerase were added to Sepharose beads coupled to a monoclonal antibody against P68 kinase, which had been prereacted with an interferon-treated 293 cell extract (+) or an interferon-treated bovine kidney cell extract (−) (the P68 kinase of bovine origin is not retained on the column since the monoclonal antibody is specific for the human P68). The bound RNAs were recovered by phenol-chloroform extraction and ethanol precipitation and were analyzed on a polyacrylamide-urea gel as described in Materials and Methods. The arrowhead on the right indicates the full-length TAR RNA (80-mer).

affinity. To obtain evidence for a high-affinity interaction between P68 kinase and TAR RNAs, we performed the RNA mobility shift assay in the presence of competitor RNAs which bind to P68 with different affinities (Fig. 5). Addition of as little as 1 μg of poly(I)-poly(C) per ml, which binds to and activates P68, abrogated TAR RNA binding to the kinase (lane 3). In contrast, poly(C), which binds with low affinity and fails to activate P68 (13), was a poor competitor. Addition of 1 and 10 μg of poly(C) per ml did not prevent P68 binding to TAR RNA; complex formation was reduced by less than fourfold in the presence of 100 μg/ml (lane 8). These results suggest a high-affinity interaction between the P68 kinase and TAR RNA.

Covalent cross-linking of TAR RNAs to P68 kinase in a cell extract. To provide further evidence for the specificity of the interaction between TAR RNA and the P68 kinase, we performed UV-induced covalent cross-linking studies in cytosolic extracts from interferon-treated HeLa cells, using ^{32}P-labeled TAR RNA as a probe. Following RNase digestion, cross-linked proteins were analyzed by SDS-PAGE and autoradiography (Fig. 6). Although several radiolabeled proteins were observed, proteins of approximately 40 and 70 kDa cross-linked most efficiently. To determine whether the ~70-kDa protein was the P68 kinase, the UV-cross-linked cell extract was subjected to immunoprecipitation, using a polyclonal antibody to the kinase (27), prior to analysis by SDS-PAGE and autoradiography. This antibody immunoprecipitated a radiolabeled protein of ~70 kDa; no other radiolabeled proteins were apparent after immunoprecipitation. A monoclonal antibody against the kinase (26) did not immunoprecipitate radiolabeled proteins, suggesting that the epitope recognized by the antibody was not accessible because of the presence of the RNA. These results demonstrate that TAR RNA binds to P68 kinase in a cell extract and further supports the specificity of this interaction.

DISCUSSION

In this study, we demonstrated that mRNAs isolated from HIV-1-infected cells and from an established cell line stably expressing a heterologous gene under the control of the HIV-1 LTR activate the P68 kinase. These results point to the TAR sequence in the 5′ UTR as a strong activator of the kinase. Thus, HIV-1 resembles other viruses which have been shown to express RNAs that can activate P68 (2, 3, 31). We also showed that TAR RNAs bind to P68, as do other activators (13). Our results suggest a high-affinity interaction between the P68 kinase and TAR RNA, since ribonucleo-

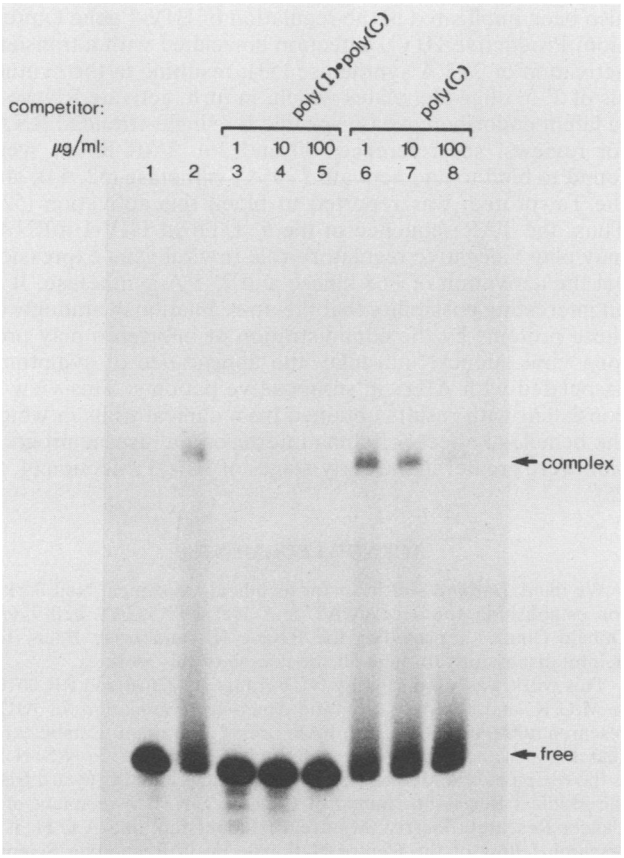

FIG. 5. Mobility gel electrophoresis analysis of P68 kinase binding to TAR RNA: effect of competitors. Approximately 0.3 μg of immunoaffinity-purified P68 was preincubated in the absence (lane 2) or presence of various concentrations of the synthetic RNA poly(I)-poly(C) (lanes 3 to 5) or poly(C) (lanes 6 to 8) prior to the addition of ^{32}P-labeled TAR RNA synthesized in vitro by SP6 polymerase. Ribonucleoprotein complexes were resolved on a nondenaturing polyacrylamide gel as described in Materials and Methods. Lane 1, Free DNA.

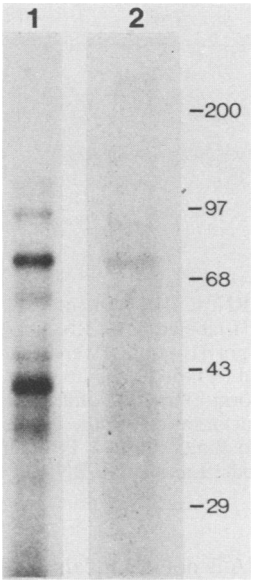

FIG. 6. UV-induced cross-linking of TAR RNA to P68 kinase present in a cell extract. ^{32}P-labeled TAR RNA synthesized by SP6 polymerase was incubated with 30 μg of an interferon-treated HeLa cell lysate and then UV irradiated as described in Materials and Methods. Samples were resolved by SDS-PAGE and autoradiography (lane 1) or were subjected to immunoprecipitation using a polyclonal antibody against the P68 kinase prior to SDS-PAGE and autoradiography (lane 2). The molecular masses of standard proteins are indicated in kilodaltons on the right.

protein complex formation was abolished in the presence of an RNA competitor that binds to P68 with high affinity but was not affected by an RNA competitor that binds to P68 with low affinity (Fig. 5). Furthermore, we found that intact base pairing in the stem of TAR RNAs is a critical requirement for binding to the kinase (Fig. 4). It is noteworthy that Marciniak et al. (32) recently identified and characterized a HeLa nuclear protein of 68 kDa which bound specifically to TAR RNA. However, this protein is unlikely to be the P68 kinase because it showed specificity for the loop sequence. In agreement with this view, the binding capacity and activation potential of a TAR RNA mutant with a four-nucleotide substitution in the loop (+31 to +34; 45) were indistinguishable from those of the wild-type RNA (data not shown). This finding indicates that binding to and activation of the P68 kinase is dependent solely on intact base pairing in the TAR RNA stem structure.

We also provide direct evidence for the activation of the P68 kinase by TAR RNAs synthesized in vitro by SP6 polymerase. Spurious transcription by SP6 polymerase in the reverse orientation could give rise to small amounts of dsRNA species (47), which might be responsible for the activation of the kinase. Recently, it was claimed that contaminating dsRNAs were present in preparations of in vitro-transcribed RNAs containing the TAR sequence (17). However, we consider this unlikely in view of the observation that the activation of P68 by SP6-derived TAR RNAs was dependent on the template transcribed. Indeed, nucleotide substitutions predicted to disrupt base pairing in the TAR secondary structure were poor activators of the kinase compared with RNAs with the wild-type TAR sequence or with compensatory mutations (Fig. 3). Nonetheless, these results do not rigorously exclude the presence of a dsRNA contaminant in our preparations, and it was of utmost importance to rule out the possibility that such contaminants were responsible for the activation of the kinase. Our first attempt to eliminate potential dsRNA contaminants included gel purification of the TAR RNAs. For reasons that are unclear to us, the gel-purified RNAs did activate the kinase but with reduced efficiency. We therefore performed additional experiments to distinguish the P68 kinase activation by TAR RNAs from that by potential dsRNA contaminants. First, we examined the chromatographic profile of SP6-derived TAR transcripts on CF11 cellulose. The eluate fraction enriched for TAR RNAs in 15% ethanol–85% STE (0.1 M NaCl, 0.05 M Tris [pH 7.0], 0.001 M EDTA) at room temperature coeluted with the fraction that activated the kinase (data not shown). The 100% STE eluate fraction, previously shown to be enriched for dsRNAs synthesized as viral replicative intermediates and expected to contain contaminating dsRNA species (11), exhibited no detectable activating activity (data not shown). Second and most convincingly, we examined the effect of TAR RNA digestion by RNase III on activation of the P68 kinase (Fig. 7). RNase III, under high-salt conditions, is highly selective for dsRNA structures which include synthetic and viral dsRNAs (41). In

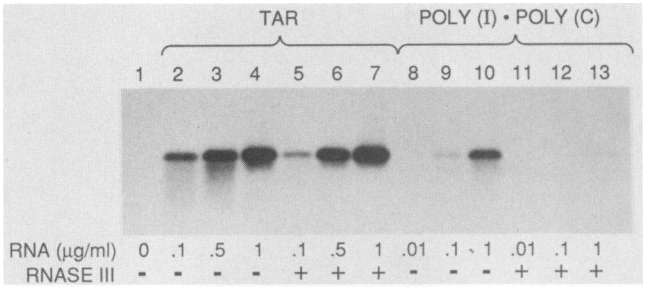

FIG. 7. Effect of RNase III treatment of TAR RNA on activation of the P68 kinase. ^{3}H-labeled TAR RNAs synthesized in vitro by SP6 polymerase and poly(I)-poly(C) were treated with RNase III (+) or were mock treated (−) for 30 min at 37°C. The RNAs recovered after phenol-chloroform extraction and ethanol precipitation were tested at various concentrations in the in vitro kinase assay, using ~100 ng of immunoaffinity-purified P68 kinase, as described in Materials and Methods. Lane 1, No RNA.

contrast, TAR RNA is not significantly cleaved by RNase III (less than 10%), possibly because of its imperfect dsRNA structure (data not shown). RNase III treatment of TAR RNAs did not result in a significant reduction in P68 autophosphorylation (Fig. 7; compare lanes 2 to 4 with lanes 5 to 7). To ensure that the RNase III preparation was active, poly(I)-poly(C) was treated in a similar fashion and lost its activation potential (compare lanes 8 to 10 with lanes 11 to 13). Taken together, these results strongly suggest that the activation of the kinase by TAR RNA is due to the integrity of the RNA stem-loop structure and not to the presence of a dsRNA contaminant. It could be argued that the formation of TAR-TAR homodimers may be responsible for the observed activation of P68. We consider this possibility less likely, since heating and quick cooling of the RNAs did not affect their ability to activate the P68 kinase (42a). Such a treatment is expected to disrupt intermolecular but not intramolecular base pairing.

Activation of the P68 kinase by the TAR region in the 5′ UTR of HIV-1 mRNAs may bear relevance to the mechanism of latency characteristic of HIV-1 infections. It is tempting to speculate that such activation in cells harboring low amounts of viral mRNAs may prevent premature translation of viral proteins. This would allow latently infected cells to escape immune surveillance and act as viral reservoirs. However, the switch from latent to active replication would necessitate a mechanism to depress P68 kinase activity and thus permit viral gene expression. HIV-1 appears to have evolved such a mechanism, since active viral replication was shown to be associated with a decrease in the expression of P68, and the *trans*-activator protein Tat was suggested to mediate this reduction (44). The Tat protein required for HIV-1 replication may be synthesized upon antigenic stimulation of latently infected cells or secreted from productively infected cells (8, 10). This molecular model of latency is consistent with recent results by Pomerantz et al. (37). They demonstrated that cell lines nonproductively infected with HIV-1 expressed only very low levels of singly and multiply spliced viral mRNAs which encode the regulatory proteins such as Tat and Rev. It is possible that the translation of these regulatory proteins was inhibited as a result of activation of the P68 kinase, thus preventing the switch to a productively infected state.

It is relevant to note that the activity of another dsRNA-dependent interferon-inducible gene, 2′-5′A synthetase, has also been implicated in the regulation of HIV-1 gene expression. Productive HIV-1 infection correlated with a transient activation of 2′-5′A synthetase (53), resulting in the synthesis of 2′-5′ oligoadenylates which, in turn, activate RNase L (a latent endoribonuclease specific for single-stranded RNA; for reviews, see references 30 and 36). TAR RNAs were found to bind to and activate 2′-5′A synthetase (52, 54), and the Tat protein was reported to block this activation (52). Thus, the TAR sequence in the 5′ UTR of HIV-1 mRNAs may play a negative regulatory role in viral gene expression via the activation of P68 kinase and 2′-5′A synthetase. It is an interesting possibility that the up-regulation in amounts of these proteins by the administration of interferon may prolong viral latency and delay the appearance of symptoms associated with AIDS in seropositive patients. This view is consistent with results obtained from clinical trials in which the beneficial effects of alpha interferon on disease progression were greater in the early stages of HIV-1 infection (4, 5, 25).

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