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The cellular factor TRP-185 regulates RNA polymerase II binding to HIV-1 TAR RNA

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Activation of HIV-1 gene expression by the transactivator Tat is dependent on an RNA regulatory element located downstream of the transcription initiation site known as TAR. To characterize cellular factors that bind to TAR RNA and are involved in the regulation of HIV-1 transcription, HeLa nuclear extract was fractionated and RNA gel-retardation analysis was performed. This analysis indicated that only two cellular factors, RNA polymerase II and the previously characterized TAR RNA loop binding protein TRP-185, were capable of binding specifically to TAR RNA. To elucidate the function of TRP-185, it was purified from HeLa nuclear extract, amino acid microsequence analysis was performed and a cDNA encoding TRP-185 was isolated. TRP-185 is a novel protein of 1621 amino acids which contains a leucine zipper and potentially a novel RNA binding motif. In gel-retardation assays, the binding of both recombinant TRP-185 and RNA polymerase II was dependent on the presence of an additional group of proteins designated cellular cofactors. Both the TAR RNA loop and bulge sequences were critical for RNA polymerase II binding, while TRP-185 binding was dependent only on TAR RNA loop sequences. Since binding of TRP-185 and RNA polymerase II to TAR RNA was found to be mutually exclusive, our results suggest that TRP-185 may function either alone or in conjunction with Tat to disengage RNA polymerase II which is stalled upon binding to nascently synthesized TAR RNA during transcriptional elongation.

Keywords: gene expression/HIV-1/RNA polymerase II/TAR RNA/TRP-185

Introduction

Regulation of HIV-1 gene expression is dependent on *cis*-acting regulatory elements in the LTR (Gaynor, 1992). Upstream regulatory elements including three SP1 binding sites (Jones *et al.*, 1986; Harrich *et al.*, 1989) and the TATA element (Berkhout and Jeang, 1992; Olsen and Rosen, 1992; Lu *et al.*, 1993; Ou *et al.*, 1994) are critical for both basal and Tat-induced gene expression. In addition to these upstream elements, a region located downstream of the HIV-1 transcriptional initiation site, known as TAR, is critical for Tat-induced gene expression (Rosen *et al.*, 1985). In the absence of Tat, the TAR element generates short or non-processive transcripts which likely reflect the result of paused or abortive transcription complexes (Kao *et al.*, 1987; Ratnasabapathy *et al.*, 1990; Feinberg *et al.*, 1991; Marciniak and Sharp, 1991). The mechanism by which TAR generates short transcripts is not known, although both a TAR DNA element in combination with TAR RNA seems likely (Ratnasabapathy *et al.*, 1990; Sheldon *et al.*, 1993).

The TAR element which extends from +1 to +60 encodes a double-stranded RNA structure whose maintenance is essential for Tat activitation (Muesing *et al.*, 1987; Feng and Holland, 1988; Berkhout and Jeang, 1989; Berkhout *et al.*, 1989; Garcia *et al.*, 1989; Selby *et al.*, 1989). TAR functions optimally when located near the transcription initiation site and its ability to stimulate Tat activation markedly decreases when moved further downstream (Selby *et al.*, 1989). However, TAR is able to function, though to a decreased extent in response to Tat, when moved downstream from the HIV-1 transcription initiation site (Churcher *et al.*, 1995). The importance of TAR has also been demonstrated by inserting TAR downstream of the transcription initiation site in heterologous promoter constructs where the presence of TAR is able to confer induction in response to Tat (Peterlin *et al.*, 1986; Muesing *et al.*, 1987; Ratnasabapathy *et al.*, 1990; Berkhout and Jeang, 1992).

Disruption of the TAR RNA stem structure greatly reduces Tat activation (Feng and Holland, 1988; Berkhout and Jeang, 1989; Garcia *et al.*, 1989; Selby *et al.*, 1989; Harrich *et al.*, 1994; Klaver and Berkhout, 1994). Mutagenesis has indicated that two elements within the TAR RNA, including a 3 bp bulge between nucleotides +23 and +25 (Berkhout and Jeang, 1989; Cordingley *et al.*, 1990; Dingwall *et al.*, 1990; Calnan *et al.*, 1991) and a 6 bp loop between +30 and +35 (Feng and Holland, 1988; Berkhout and Jeang, 1989; Garcia *et al.*, 1989; Roy *et al.*, 1990; Wu *et al.*, 1991), are also critical for Tat activation. Although constructs containing point mutations in the bulge are not extremely defective for activation by Tat in transient expression assays, these mutations have dramatic effects on HIV-1 gene expression in the context of infectious proviral constructs (Harrich *et al.*, 1994). HIV-1 constructs with mutations in the TAR RNA loop do not alter the basal HIV-1 gene expression, but markedly reduce the level of Tat activation (Feng and Holland, 1988; Berkhout and Jeang, 1989; Garcia *et al.*, 1989; Roy *et al.*, 1990; Wu *et al.*, 1991; Churcher *et al.*, 1995). Mutations in both the loop and bulge sequences essentially destroy TAR function, suggesting that these elements may have independent but related roles in activation by Tat (Churcher *et al.*, 1995). HIV-1 templates containing TAR RNA loop mutations have been demonstrated to be defec-

tive for Tat activation using a variety of assay systems including *in vitro* transcription, transient expression, nuclear run-on and viral replication (Feng and Holland, 1988; Berkhout and Jeang, 1989; Garcia *et al.*, 1989; Laspia *et al.*, 1989, 1990; Roy *et al.*, 1990; Wu *et al.*, 1991; Kato *et al.*, 1992; Harrich *et al.*, 1994; Churcher *et al.*, 1995).

Since TAR plays an essential role in Tat activation, a number of studies have investigated the viral and cellular factors that bind to TAR RNA. The Tat protein binds via its basic domain to the bulge region in TAR RNA (Dingwall *et al.*, 1989, 1990; Cordingley *et al.*, 1990; Roy *et al.*, 1990; Calnan *et al.*, 1991; Weeks and Crothers, 1991). A number of studies indicate that this binding is important for Tat activation. The TAR RNA loop sequences specifically bind a 185 kDa cellular protein known as TRP-1 TRP-185 (Sheline *et al.*, 1991; Wu *et al.*, 1991). The binding of TRP-185 to the TAR RNA loop sequences requires the presence of a group of cellular cofactor proteins which themselves do not stably bind to TAR RNA, but yet are essential for TRP-185 binding (Sheline *et al.*, 1991; Wu *et al.*, 1991). TRP-185 has been demonstrated to increase the level of HIV-1 gene expression using *in vitro* transcription assays in both the presence and absence of Tat (Sheline *et al.*, 1991; Wu *et al.*, 1991). However, the mechanism by which factors like Tat and TRP-185 are able to modulate HIV-1 gene expression remains to be determined.

Several models exist to explain how TAR RNA functions to modulate Tat activation. One model suggests that TAR acts as an RNA enhancer to increase the transcription from the HIV-1 promoter (Marciniak *et al.*, 1990). A second model suggests that *cis*-acting regulatory sequences present in the HIV-1 LTR specify the binding of a particular class of non-processive transcription complexes that are uniquely sensitive to the action of the elongation properties of Tat (Olsen and Rosen, 1992; Lu *et al.*, 1993; Zhou and Sharp, 1995). A third model suggests that Tat may act to overcome the transcriptional attenuation properties of TAR RNA by directly modifying the elongation properties of RNA polymerase II (Kao *et al.*, 1987; Bengal and Aloni, 1991; Graeble *et al.*, 1993). To address these different models for the regulation of HIV-1 gene expression, we wished to further characterize cellular factors that bind to TAR RNA. In this paper, we demonstrate that both RNA polymerase II and TRP-185 bind specifically to TAR RNA. Furthermore, we purified and isolated the gene encoding TRP-185 and characterized its binding properties to TAR RNA. These studies further define the role of cellular factors that bind to TAR RNA and suggest potential mechanisms by which these factors may regulate HIV-1 gene expression.

Results

Purification of TRP-185

Previously, we described a cellular factor TRP-185 that bound specifically to the HIV-1 TAR RNA loop sequences (Wu *et al.*, 1991). TRP-185 binding to TAR RNA required the presence of both the TRP-185 protein and a separate set of factors designated the cofactor fraction. The mechanism by which these cofactors stimulate TRP-185 binding to TAR RNA has not been elucidated, although either direct binding to TAR RNA with subsequent dissociation of these factors during gel electrophoresis or post-translational modification of TRP-185 seem possible. To further characterize the potential role of TRP-185 in regulating HIV-1 gene expression, we developed a purification scheme for this factor from HeLa nuclear extract (Figure 1).

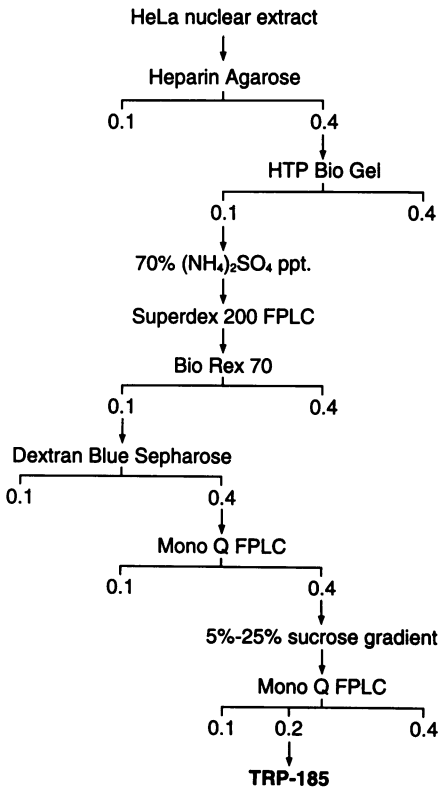


Fig. 1. Purification scheme for TRP-185. The protocol for the fractionation of HeLa cell nuclear extract to purify TRP-185 is shown with the molarity of the buffers used in the column elution indicated.

Protein fractions isolated from different column chromatographic steps were assayed for TRP-185 binding using gel-retardation analysis with wild-type and mutant TAR RNAs. Cofactors purified from HeLa cells, which by themselves exhibited no binding to wild-type TAR RNA, were added to these assays to stimulate TRP-185 binding. The purification and cloning of the genes encoding the cofactors will be presented elsewhere (Wu-Baer *et al.*, submitted). HeLa nuclear extract prepared from 60 l of cells was applied sequentially to columns containing heparin agarose, hydroxyapatite, Superdex 200 FPLC, Bio-Rex, dextran blue and Mono Q. Following these chromatographic steps, the fractions containing TRP-185 were applied to a preparative sucrose gradient and concentrated on a Mono Q FPLC. A silver-stained polyacrylamide gel of the chromatographic fractions containing TRP-185 eluted from the final Mono Q column and a corresponding gel-retardation analysis of these fractions with wild-type TAR RNA is shown (Figure 2). This analysis indicated that the presence of a 185 kDa species (Figure 2A, lanes 2 and 3) correlated with binding activity to TAR RNA (Figure 2B, lanes 2 and 3).

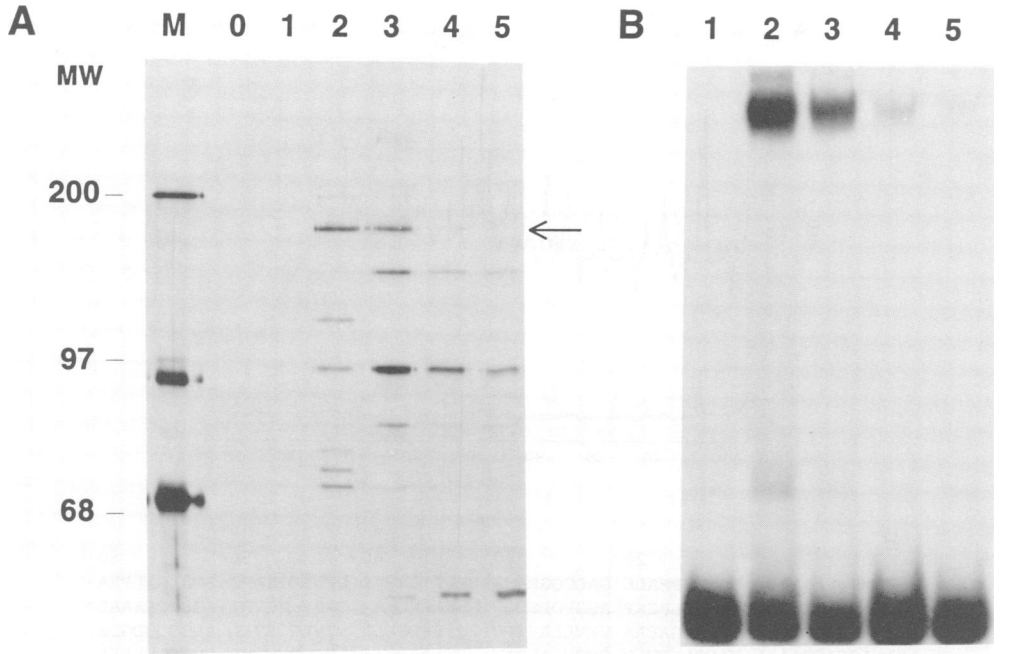


Fig. 2. Binding of purified TRP-185 to HIV-1 TAR RNA. (**A**) The binding of purified cellular cofactors and (0.4 μg) and TRP-185 (50 ng) contained in fractions eluted from the final Mono Q column to wild-type TAR RNA is shown in lanes 1–6. (**B**) A silver-stained SDS–polyacrylamide gel of the TRP-185 corresponding to the above fractions is also shown.

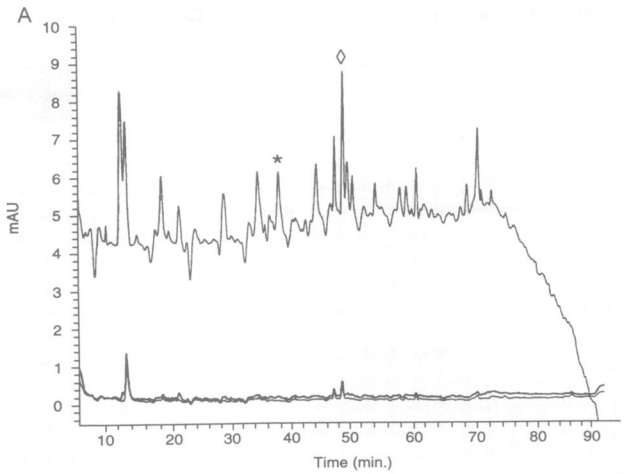
Cloning of the gene encoding TRP-185

Twenty-six separate preparations of TRP-185 were isolated from HeLa nuclear extract. The active fractions were pooled, subjected to SDS–PAGE electrophoresis and transferred to nitrocellulose for microsequence analysis. Approximately 60 μg of TRP-185 were digested with the endoprotease Lys C and the peptides generated were separated by HPLC as described (Figure 3A) (Ha *et al.*, 1991; Lane, 1991). Two peptides of 24 amino acids and 14 amino acids, which contained the amino acid sequences LKPGDWSQQDIGTNLVEADNQAEW and TEGYTIIGVEQTAK, respectively, were obtained. Degenerate oligonucleotide primers were then synthesized which corresponded to the 24 amino acid peptide and these primers were used in polymerase chain reaction (PCR) analysis with HeLa cDNA to generate a 72 nucleotide fragment. This PCR-generated fragment was cloned into pUC19 and DNA sequence analysis was then performed which confirmed that the nucleic acid sequences encoded the TRP-185 peptide sequence. Oligonucleotides corresponding to this peptide sequence were then used in PCR analysis with degenerate oligonucleotide primers corresponding to the predicted amino acid sequence of the 14 amino acid TRP-185 peptide and these primers were used to generate a 435 bp PCR fragment from HeLa cDNA. This fragment was cloned into pUC19 and the DNA sequence analysis contained an open reading frame with the amino acid sequence of both previously isolated peptides. This cDNA fragment was then used as a probe to screen a HeLa cDNA library and resulted in the isolation of a 5.6 kb cDNA encoding the full-length TRP-185 protein. Multiple stop codons were noted in both the 5′ and 3′ portions of the cDNA indicating that it contained the full-length TRP-185 protein.

The full-length 1621 amino acid sequence of the TRP-185 protein is shown in Figure 3B. TRP-185 is a novel protein with no classical RNA binding motifs such as zinc fingers or ribonucleoprotein binding domains. However, a leucine zipper domain consisting of leucine residues at every seventh position between amino acids 535 and 556 was identified (Landschulz *et al.*, 1988). In addition, a number of so-called lysine-helix repeats, which are a novel structural element consisting of lysine or arginine residues spaced at seven amino acid intervals, were noted throughout the TRP-185 (Meisterernst *et al.*, 1989). Two of the 19 sets of these repeats found in TRP-185 are indicated in Figure 3B. These repeats were previously noted in the transcription factor nuclear factor 1 and they have been postulated to be involved in its DNA binding properties (Meisterernst *et al.*, 1989). Finally, a variety of potential nucleotide binding sites (Moller and Amons, 1985) were noted in the C-terminal portion of the TRP-185 protein, as were potential sites for post-translational modification such as phosphorylation and glycosylation. The significance of these potential modifications on TRP-185 function will require site-directed mutagenesis and subsequent assays of TRP-185 function.

Immunoreactivity of cloned and purified TRP-185

We next cloned the cDNA that we isolated downstream of the T7 promoter in the expression vector pTM1 to conclusively demonstrate that it encoded the full-length TRP-185 protein (Elroy-Stein *et al.*, 1989). We placed six histidine residues at the C-terminus of the TRP-185 cDNA to facilitate purification of the TRP-185 protein. In addition, the 12 amino acid influenza hemagglutinin sequences were also placed at the C-terminus of TRP-185 to facilitate the detection of this protein with the monoclonal antibody (mAb) 12CA5 (Field *et al.*, 1988). The pTM1 vector has been used to overexpress a variety of cDNAs when transfected into HeLa cells followed by



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        10         20         30         40         50         60
 1 MEWVLAEALL SQSRDPRALL GALCQGEASA ERVETLRFLL QRLEDEEARG SGGAGALPEA
61 AREVAAGYLV PLLRSLRGRP AGGPDPSLQP RHRRRVLRAA GAALRSCVRL AGRPQLAAAL
121 AEEALRDLLA GWRAPGAEAA VEVLAAVGPC LRPREDGPLL ERVAGTAVAL ALGGGGDGDE
181 AGPAEDAAAL VAGRLLPVLV QCGGAALRAV WGGLAAPGAS LGSGRVEEKL LVLSALAEKL
241 LPEPGGDRAR GARREAGPDAR RCWRFWRTVQ AGLGQADALT RKRARYLLQR AVEVSAELGA
301 DCTCGPQEGN GPSLFWWSER KKDELLKFWE NYILIMETLE GNQIHVIKPV LPKLNNLFEY
361 AVSEENGCWL FHPSWHMCIY KRMFESENKI LSKEGVIHFL ELYETKILPF SPEFSEFIIG
421 PLMDALSESS LYSRSPGQPI GSCSPLGLKL QKFLVTYISL LPEEIKSSFL LKFIRKMTSR
481 HWCAVPILFL SKALANVPRH KALGIDGLLA LRDVIHCTMI THQILLRGAA QCYLLQTAMN
541 LLDVEKVSLS DVSTFL SLR QEESLGRGTS LWTELCDWLR VNESYFKPSP TCSSIGLHKT
601 SLNAYVKSIV QEYVKSSAWE TGENCFMPDW FEAKLVSLMV LLAVDVEGMK TQYSGKQRTE
661 NVLRIFLDPL LDVLMKFSTN AYMPLLKTDR CLQLLLKLLN TCRLKGSSAQ DDEVSTVLQN
721 FFMSTTESIS EFILRRLTMN ELNSVSDLDR CHLYLMVLTE LINLHLKVGW KRGNPIWRVI
781 SLLKNASIQH LQEMDSGQEP TVGSQIQRVV SMAALAMVCE AIDQKPELQL DSLHAGPLES
841 FLSSLQLNQT LQKPHAEEQS SYAHPLECSS VLEESSSSQG WGKIVAQYIH DQWVCLSFLL
901 KKYHTLIPTT GSEILEPFLP AVQMPIRTLQ SALEALTVLS SDQVLPVFHC LKVLVPKLLT
961 SSESLCIESF DMAWKIISSL SNTQLIFWAN LKAFVQFVFD NKVLTIAAKI KGQAYFKIKE
1021 IMYKIIEMSA IKTGVFNTLI SYCCQSWIVS ASNVSQGSLS SAKNYSELIL EACIFGTVFR
1081 RDQRLVQDVQ TFIENLGHDC AANIVMENTK REDHYVRICA VKFLCLLDGS NMSHKLFIED
1141 LAIKLLDKDE LVSKSKKRYY VNSLQHRVKN RVWQTLLVLF PRLDQNFLNG IIDRIFQAGF
1201 TNNQASIKYF IEWIIILILH KFPQFLPKFW DCFSYGEENL KTSICTFLAV LSHLDIITQN
1261 IPEKKLILKQ ALIVVLQWCF NHNFSVRLYA LVALKKLWTV CKVLSVEEFD ALTPVIESSL
1321 HQVESMHGAG NAKKNWQRIQ EHFFFATFHP LKDYCLETIF YILPRLSGLI EDEWITIDKF
1381 TRFTDVPLAA GFQWYLSQTQ LSKLKPGDWS QQDIGTNLVE ADNQAEWTDV QKKIIPWNSR
1441 VSDLDLELLF QDRAARLGKS ISRLIVVASL IDKPTNLGGL CRTCEVFGAS VLVVGSLQCI
1501 SDKQFQHLSV SAEQWLPLVE VKPPQLIDYL QQKKTEGYTI IGVEQTAKSL DLTQYCFPEK
1561 SLLLLGNERE GIPANLIQQL DVCVEIPQQG IIRSLNVHVS GALLIWEYTR QQLLSHGDTK
1621 P
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Fig. 3. Amino acid sequence of the TRP-185 protein. (**A**) The HPLC profile of peptides released after Lys C protease digestion of TRP-185 is shown and the position of the 14mer and 24mer TRP-185 peptides is marked with * and ◇, respectively. (**B**) The sequence of the 1621 amino acid TRP-185 protein is shown and the position of the peptide sequences obtained from amino acid sequencing of TRP-185 are underlined, the leucine residues in the leucine zipper are shaded, and the arginine residues in two of the potential lysine helices are circled. The GenBank accession number of TRP-185 is U38847.

infection with a recombinant vaccinia virus that is capable of expressing T7 polymerase (Janknecht *et al.*, 1991). At 48 h post-transfection, the recombinant TRP-185 was purified by binding and elution of the histidine-tagged TRP-185 protein from Sepharose nickel beads using increasing concentrations of imidazole as described previously (Tanog, 1995). Western blot analysis was then performed with equivalent amounts of both vaccinia-expressed TRP-185 and native TRP-185 purified from HeLa nuclear extract (Figure 4A, lanes 1 and 2) using a mAb raised to a portion of TRP-185 extending from amino acids 1409 to 1541. This analysis demonstrated that both the recombinant and the native TRP-185 proteins had the same molecular weight (Figure 4A).

To determine whether both the recombinant and the native TRP-185 proteins were present in a gel-retarded complex bound to TAR RNA, gel-retardation assays were performed with TRP-185 in the presence of either monoclonal or polyclonal antibodies directed against TRP-185. There was no binding of either native or recombinant preparations of TRP-185 to wild-type TAR RNA in the absence of added cofactors (Figure 4B, lanes 2 and 4). However, in the presence of the cofactor fraction, there was binding of both of these TRP-185 preparations to the wild-type TAR RNA (Figure 4B, lanes 3 and 5). There was no binding of the cofactors to the wild-type TAR RNA alone (Figure 4B, lane 6). Addition of pre-immune rabbit sera did not alter the mobility of the TRP-185 gel-retarded complex (Figure 4B, lanes 8 and 12). However, the addition of either polyclonal (Figure 4B, lanes 9 and

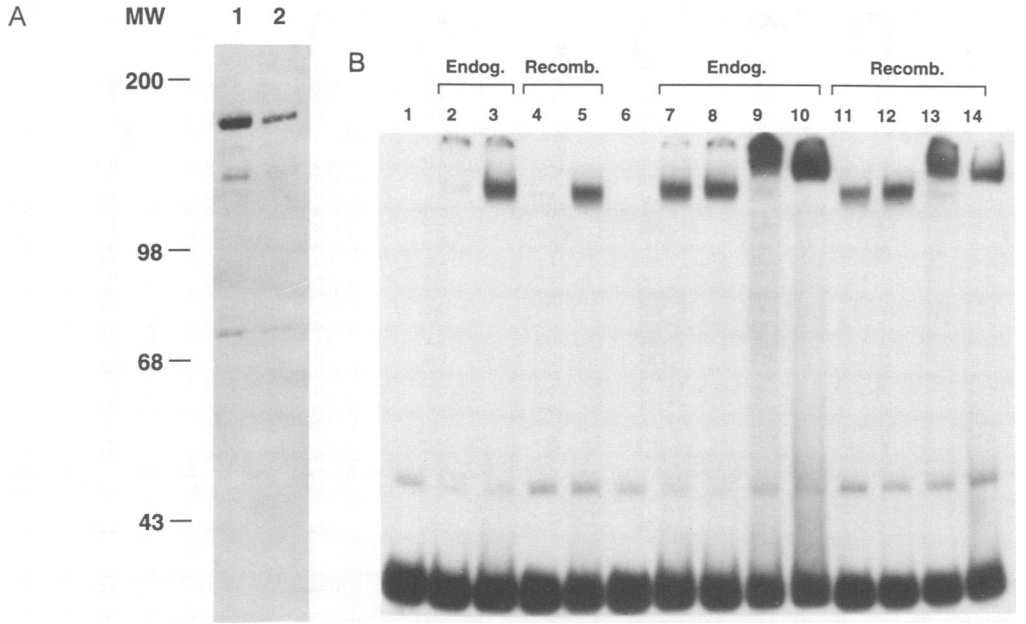


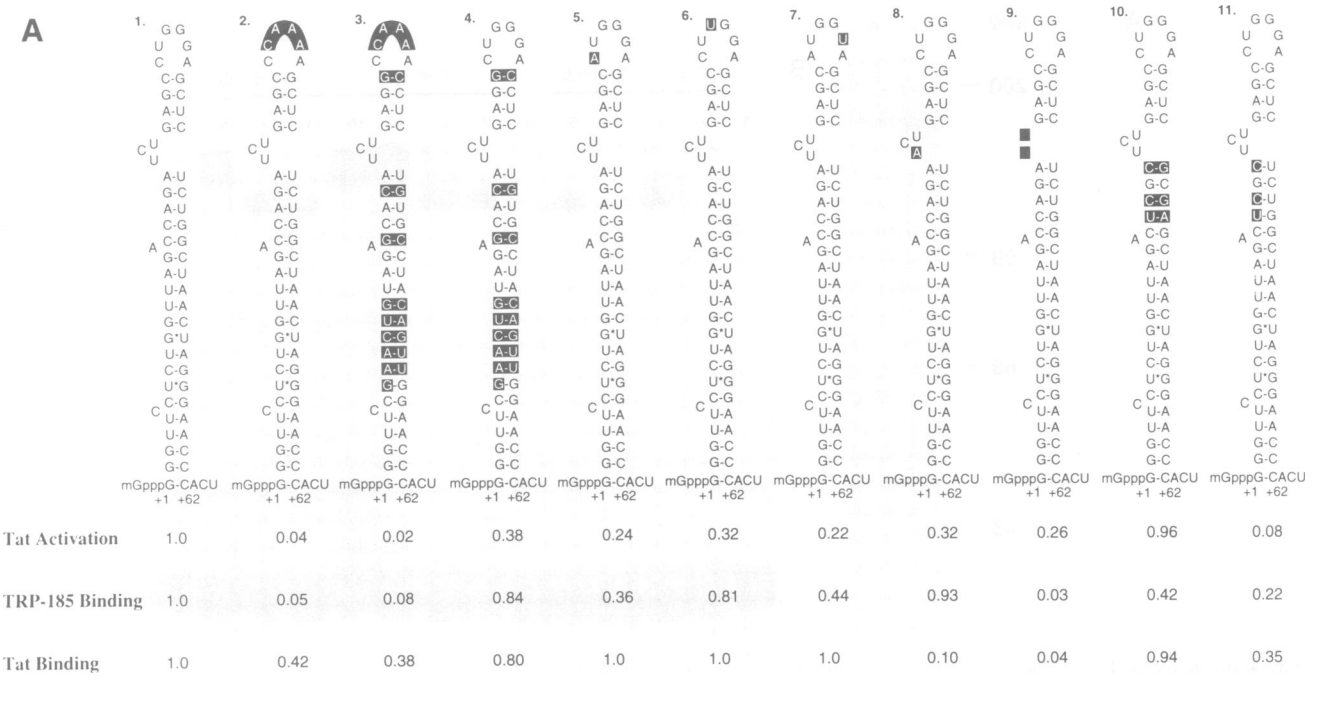
Fig. 4. Immunological cross-reactivity of purified endogenous and recombinant TRP-185. (**A**) Western blot analysis was performed with 160 ng of recombinant (lane 1) and 80 ng of endogenous TRP-185 (lane 2) using the NK 5.18 TRP-185 mAb. (**B**) The response of either endogenous or recombinant TRP-185 to added cofactors is shown in gel-retardation assays with labeled HIV-1 TAR RNA (lane 1), TRP-185 (40 ng) purified from HeLa nuclear extract (lane 2), the same amounts of TRP-185 with 0.4 μg of cofactors (lane 3), recombinant TRP-185 (40 ng) (lane 4), recombinant TRP-185 with 0.4 μg of cofactors (lane 5) and 0.4 μg of cofactor alone (lane 6). The binding of endogenous and recombinant TRP-185 to wild-type TAR RNA (lanes 7 and 11) is shown in the presence of 1 μg of protein A–Sepharose column-purified pre-immune rabbit sera (lanes 8 and 12), polyclonal rabbit sera directed against TRP-185 (lanes 9 and 13), or a mAb directed against TRP-185 (NK 5.18) (lanes 10 and 14).

13) or mAb (Figure 4B, lanes 10 and 14) directed against TRP-185 resulted in a supershifted complex. Thus, both the recombinant and the endogenous TRP-185 proteins require the presence of cofactors for binding to TAR RNA, and the TRP-185 protein was present in the gel-retarded complex bound to TAR RNA.

Recombinant TRP-185 binds to TAR RNA loop sequences

Next we wished to analyze the binding properties of recombinant TRP-185 to wild-type and mutant TAR RNAs. A diagram of the mutant RNAs which were used in competition assays is shown in Figure 5A. It was important to correlate the binding properties of both TRP-185 and Tat to these different RNAs, and compare this binding with the activity of these TAR structures when inserted into HIV-1 LTR CAT constructs and analyzed in transient expression assays in both the presence and absence of Tat. The activity of these mutant TAR RNAs relative to wild-type TAR RNA were compared in transient expression assays (Tat activation), Tat binding and binding to recombinant TRP-185 is shown in Figure 5A. A variety of different TAR RNAs were assayed including wild-type TAR RNA (Figure 5A, lane 1), two mutants in the TAR RNA loop sequences which were very defective for Tat activation (Figure 5A, lanes 2 and 3), a mutant in the primary sequence of TAR RNA which maintains stem–base pairing and was only mildly defective for Tat activation (Figure 5A, lane 4), three point mutants in the TAR RNA loop sequences which exhibited moderate defects in Tat activation (Figure 5A, lanes 5–7), a point mutant and a deletion mutant in the TAR RNA bulge sequences which failed to bind Tat and exhibited moderate defects in Tat activation (Figure 5A, lanes 8 and 9), and either a TAR RNA stem mutation which restored stem–base pairing and gave wild-type Tat activation or a stem disruption mutant that was defective for Tat activation (Figure 5A, lanes 10 and 11). Recombinant TRP-185 in the presence of cellular cofactors bound to wild-type TAR RNA (Figure 5B, lane 3). There was a 20-fold reduction in the amount of the TRP-185 gel-retarded complex using a 50-fold molar excess of unlabeled wild-type TAR RNA competitor (Figure 5B, lane 4), but little competition with a similar excess of two TAR RNAs which contained mutations of the loop sequences between +31 and +34 (Figure 5B, lanes 5 and 6). A TAR RNA containing mutations in its primary sequence, but that maintained both stem–base pairing and the bulge and loop sequences, competed as well as wild-type TAR RNA for TRP-185 binding (Figure 5B, lane 7). Competition with TAR RNAs containing point mutations in the loop sequences was then performed. TAR RNAs containing mutations of individual nucleotides in the loop sequences at +30 and +34 were very defective for competition of TRP-185 binding (Figure 5B, lanes 8 and 10). Although a TAR RNA mutant at nucleotide +32 was not defective for competition of TRP-185 binding (Figure 5B, lane 9), this mutant was still defective for competition of TRP-185 relative to wild-type TAR RNA using a 5-fold lower molar excess of competitor RNA (data not shown). The results with both single and multiple mutations in the TAR RNA loop indicated that the primary sequence of the loop was critical for TRP-185 binding.

The effects of several mutations in other portions of TAR RNA were also tested for their ability to compete for TRP-185 binding. Addition of a TAR RNA bulge mutant at nucleotide +23, which prevents the binding of



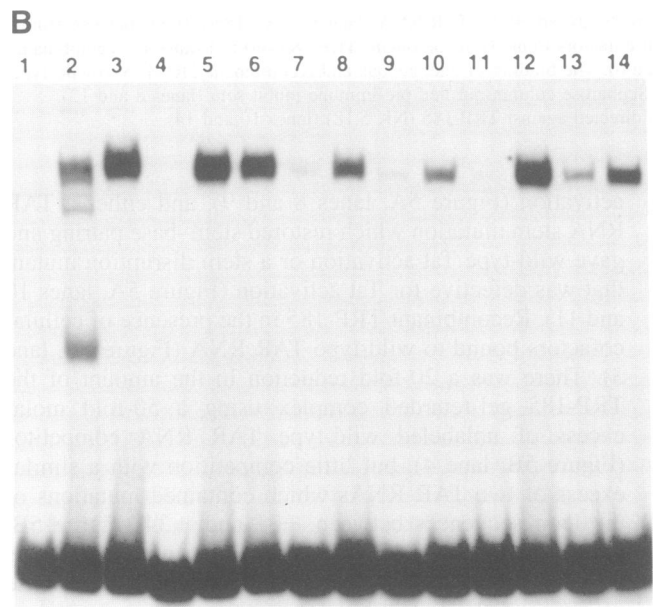


Fig. 5. Binding specificity of recombinant TRP-185 to HIV TAR RNA. (**A**) The stem–loop structures of HIV-1 TAR RNAs extending from +1 to +62 are shown for a series of constructs that were synthesized following *in vitro* transcription with T7 polymerase. The shaded areas indicate the nucleotides substituted and/or deleted in each construct. The constructs include (1) wild-type, (2) +31/+34, (3) TAR-sense /(+31/+34), (4) TAR sense, (5) +30, (6) +32, (7) +34, (8) +23, (9) a deletion of bulge nucleotides (+23/+25), (10) (+19/+22)/(+40/+43) and (11) (+19/+22). The effects of these TAR RNA mutants relative to wild-type TAR RNA on *in vivo* Tat activation, Tat binding and TRP-185 competition using a 50-fold molar excess of each RNA were averaged for three different experiments. (**B**) Gel-retardation assays with labeled wild-type HIV-1 TAR RNA (lane 1), recombinant TRP-185 prior to purification (lane 2), purified recombinant TRP-185 (40 ng) and cofactors (0.4 μg) (lane 3) or with competition with 50 ng of unlabeled TAR RNAs wild-type (lane 4), +31/34 (lane 5), TAR sense/(+31/+34) (lane 6), TAR sense (lane 7), +30 (lane 8), +32 (lane 9), +34 (lane 10), +23 (lane 11), a deletion of bulge nucleotides (+23/+25) (lane 12), (+19/+22)/(+40/+43) (lane 13) and +19/+22 (lane 14).

Tat to TAR RNA (Dingwall *et al.*, 1989, 1990; Cordingley *et al.*, 1990; Roy *et al.*, 1990; Calnan *et al.*, 1991), did not alter TRP-185 binding (Figure 5B, lane 11). However, a TAR RNA containing a deletion of the entire bulge structure did not compete for TRP-185 binding (Figure 5B, lane 12). Addition of a TAR RNA with a three nucleotide disruption of the stem structure resulted in decreased competition for TRP-185 (Figure 5B, lane 14) as compared with a TAR RNA which restored TAR RNA stem–base pairing by a compensatory mutation (Figure 5B, lane 13). In several other experiments, the level of competition with this stem restoration mutant was nearly equivalent to that of wild-type TAR RNA (data not shown). These results indicated that the binding of TRP-185 was dependent on the primary sequence of the loop in addition to the maintenance of the overall TAR RNA stem and bulge structures. Furthermore, the binding of TRP-185 to TAR RNA mutants can be differentiated from Tat binding to TAR RNA by the TAR RNA mutant +23 which was severely defective for Tat binding, but bound TRP-185 to nearly wild-type levels. Thus, TRP-185 bound efficiently to a variety of TAR templates which had wild-type activity in response to Tat, but it did not bind efficiently to a variety of TAR RNA mutants which were defective for Tat activation.

RNA polymerase II also binds directly to TAR RNA

Since we were able to demonstrate that both native and recombinant TRP-185 bound specifically to TAR RNA, we wished to investigate whether complexes comprised of TRP-185 and other transcription factors may exist in HeLa nuclear extract and be capable of binding to TAR RNA. This analysis was facilitated by the presence of specific antibodies which we generated against TRP-185

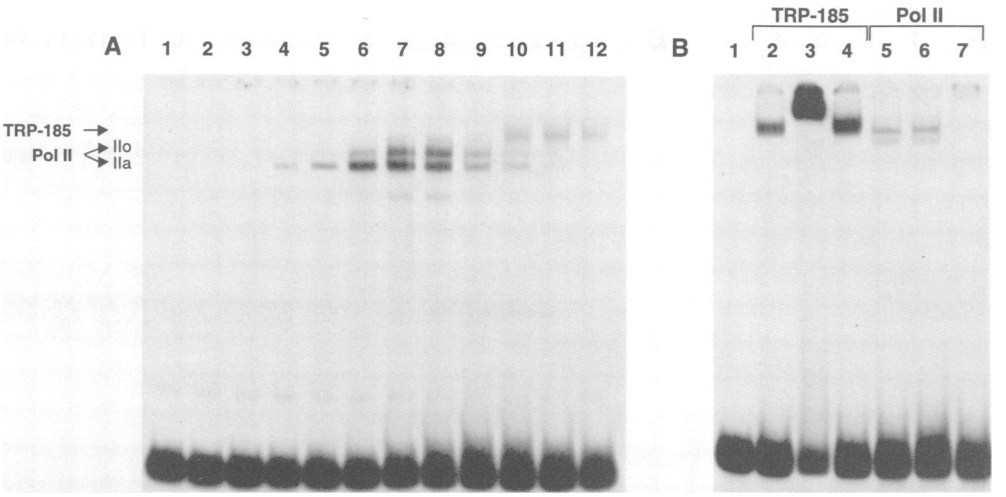


Fig. 6. Both RNA polymerase II and TRP-185 bind to TAR RNA. (**A**) HeLa nuclear extract was fractionated on heparin agarose, hydroxyapatite and Superdex 200 FPLC, and then assayed in gel-retardation assays with wild-type TAR RNA (lanes 1–12). The binding of different column fractions to a labeled TAR RNA probe and the positions of RNA polymerase (II_a and II_o) (lanes 4–9) and TRP-185 (lanes 10–12) species are indicated. (**B**) Gel-retardation analysis was performed with a TAR RNA probe (lane 1) and the Superdex 200 FPLC fraction used in (A) lane 11 alone (lane 2), or in the presence of either 1 μg of TRP-185 mAb (lane 3) or the RNA polymerase II CTD mAb (lane 4). The Superdex 200 FPLC fraction used in (A) lane 8 is shown alone (lane 5) or with TRP-185 mAb (lane 6) or mAb directed against the RNA polymerase II CTD (lane 7).

that could be used to analyze the components of the gel-retarded complexes bound to TAR RNA. HeLa nuclear extract, chromatographed on heparin agarose and hydroxyapatite columns, was analyzed following chromatography on a Superdex 200 FPLC column as shown in Figure 1. This purification scheme allowed the detection of TRP-185 and also removed a variety of non-specific double-stranded RNA binding proteins which were present in HeLa nuclear extract (data not shown).

Gel retardation was performed with wild-type TAR RNA on different fractions eluted from the Superdex 200 column. The binding of two closely migrating species in early fractions (Figure 6A, lanes 4–9) and a slower migrating species in later fractions was detected (Figure 6A, lanes 10–12). Gel retardation with these fractions was performed without the addition of cellular cofactors since they co-purified during the initial stages of the chromatography. The proteins that were responsible for these gel-retarded species were purified as described and then further characterized. The proteins which comprised the slower mobility complex that bound to TAR RNA corresponded to TRP-185 (Figure 6A, lanes 10–12). The proteins which gave rise to the two faster mobility gel-retarded species contained peptide sequences which corresponded to the largest subunit of RNA polymerase II (Figure 6A, lanes 4–9).

To further demonstrate the identity of these gel-retarded species, antibody supershift experiments were performed with chromatographic fractions which gave rise to either the slower (Figure 6A, lane 11) or faster mobility (Figure 6A, lane 8) complexes bound to TAR RNA. The protein which generated the slower mobility complex (Figure 6B, lane 2) was supershifted by a mAb directed against the TRP-185 (Figure 6B, lane 3). However, there was no change in the mobility of this species upon the addition of a mAb 8WG16 directed against the C-terminal domain of RNA polymerase II (Thompson *et al.*, 1989) (Figure 6B, lane 4). In contrast to these results, the addition of mAb directed against TRP-185 did not alter the mobility of the two faster mobility species bound to TAR RNA (Figure 6B, lanes 5 and 6) which were previously detected in Figure 6A. However, the addition of the 8WG16 mAb directed against the RNA polymerase II CTD prevented the binding of these two species to TAR RNA (Figure 6B, lane 7). Alkaline phosphatase treatment indicated that these two species were the hypophosphorylated (II_a) and hyperphosphorylated (II_o) forms of RNA polymerase II (Cisek and Corden, 1989; Young, 1991) (data not shown). Owing to the fact that native gels were used in the gel-retardation analysis, the TAR RNA complex containing RNA polymerase II exhibited a faster mobility than TRP-185, even though the polymerase complex is of higher molecular weight than that of TRP-185 (Young, 1991). UV cross-linking confirmed that the largest subunit of RNA polymerase II (210 kDa) was present in the complex bound to TAR RNA (data not shown). These data indicate that following initial chromatography to remove a variety of non-specific double-stranded RNA binding proteins, that two proteins, RNA polymerase II and TRP-185, were the only two species that bound specifically to wild-type TAR RNA.

RNA polymerase II binding to TAR RNA requires TRP-185 associated cofactors

It was important to determine whether there was any specificity for RNA polymerase II binding to TAR RNA and whether the same cofactors required for TRP-185 binding to TAR RNA were also required for the binding of RNA polymerase II. To address these questions, we proceeded to further purify the RNA polymerase II using column chromatography described in a previously published purification scheme (Reinberg and Roeder, 1987). This RNA polymerase II preparation was active in reconstituted *in vitro* transcription assays with the HIV-1 LTR (data not shown). Gel retardation was then performed with wild-type TAR RNA and with quantities of the RNA polymerase II necessary to initiate *in vitro* transcription from the HIV-1 LTR. Different fractions from the final step of the RNA polymerase II purification were used in Figure 7A and B so that a non-specific binding species

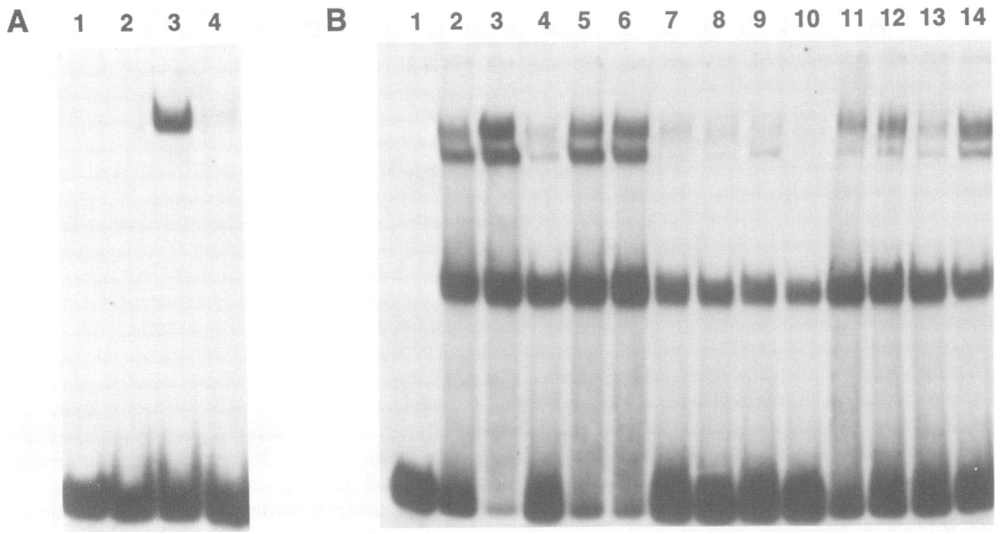


Fig. 7. RNA polymerase II binding specificity to HIV-1 TAR RNA. (**A**) Gel-retardation analysis was performed with labeled TAR RNA (lane 1) and RNA polymerase II (50 ng) either alone (lane 2) or in the presence of 0.4 μg of cofactors (lane 3) or with cofactors alone (lane 4). (**B**) To determine the specificity of RNA polymerase II binding to TAR RNA, the gel-retardation reactions were performed with labeled TAR RNA (lane 1), partially purified RNA polymerase II 20 ng and 50 ng and cofactors (0.4 μg) (lanes 2 and 3), 50 ng of polymerase and cofactors or with 50 ng of unlabeled TAR RNA competitor including wild-type (lane 4), +31/34 (lane 5), TAR sense/(+31/+34) (lane 6), TAR sense (lane 7), +30 (lane 8), +32 (lane 9), +34 (lane 10), +23 (lane 11), a deletion of bulge nucleotides (+23/+25) (lane 12), (+19/+22)/(+40/+43) (lane 13) and (+19/+22) (lane 14).

was present in the gel-retardation assays to serve as an internal control in the competition analysis (Figure 7B). RNA polymerase II alone bound poorly to the TAR RNA (Figure 7A, lane 2), but its binding increased dramatically upon the addition of the purified cofactors (Figure 7A, lane 3). There was not a similar increase in the binding of RNA polymerase II upon the addition of albumin (Figure 7A, lane 4) or other proteins (data not shown). Thus, RNA polymerase II, like TRP-185, exhibited an increase in its binding to TAR RNA upon the addition of purified cellular cofactors.

Next we determined whether there was any specificity for RNA polymerase II binding to TAR RNA. Gel-retardation analysis was performed with wild-type TAR RNA and competition analysis was performed with a 50-fold molar excess of unlabeled wild-type and mutant TAR RNAs previously used to determine the binding specificity of TRP-185 (see Figure 5A). Cellular cofactors were added to enhance RNA polymerase II binding to TAR RNA (Figure 7B, lanes 2 and 3). A 50-fold molar excess of unlabeled wild-type TAR RNA was able to significantly compete for the binding of RNA polymerase II (Figure 7B, lane 4), while mutation of four nucleotides in the loop sequences (Figure 7B, lane 5) or in the context of additional changes in the TAR RNA primary sequence with preservation of stem–base pairing resulted in little competition (Figure 7B, lane 6). A TAR RNA which maintained stem–base pairing, but changed the primary sequence of TAR (Figure 7B, lane 7), competed for RNA polymerase II binding similar to that of wild-type TAR RNA, as did a variety of point mutants at +30, +32 and +34 in the TAR RNA loop sequences (Figure 7B, lanes 8–10). TAR RNAs which deleted the bulge sequences or contained a point mutation in the bulge at +23 both resulted in markly decreased competition for RNA polymerase II binding (Figure 7B, lanes 11 and 12). Finally, a TAR RNA which disrupted stem–base pairing resulted in decreased competition for RNA polymerase II binding (Figure 7B, lane 14), while a TAR RNA which restored stem–base pairing by the addition of compensatory mutations resulted in increased competition (Figure 7B, lane 13). A non-specific complex which bound to TAR RNA was not significantly changed by the addition of any of the competitor RNAs (Figure 7B). These results indicated that the binding of RNA polymerase II to TAR RNA was dependent to a lesser degree than TRP-185 on the primary sequence of the loop, but was more dependent on specific nucleotide sequences in the TAR RNA bulge.

TRP-185 and RNA polymerase II compete for binding to TAR RNA

It was possible that both TRP-185 and RNA polymerase II could simultaneously bind to TAR RNA. To test this possibility, we performed gel-retardation analysis with wild-type TAR RNA and purified preparations of RNA polymerase II, recombinant TRP-185 and cofactors. When the amount of RNA polymerase II was kept constant (Figure 8, lane 2) and increasing amounts of TRP-185 were added (Figure 8, lanes 3–5), a slower mobility species became predominant which was consistent with that of TRP-185 alone (Figure 8, lane 6). When the amount of TRP-185 was kept constant and increasing amounts of RNA polymerase II were added (Figure 8, lanes 7–10), the faster mobility species which became predominant was consistent with that of RNA polymerase II (Figure 8, lane 2). No slower migrating complexes were detected in gel-retardation assays, which would be consistent with a complex comprised of TRP-185 and RNA polymerase II. These results suggested that RNA polymerase II and TRP-185 mutually excluded the binding of each other to TAR RNA.

Since changes in the state of phosphorylation have been noted to markedly alter the function and interactions of RNA polymerase II with the basal transcriptional machinery, we wished to determine whether changes in the phosphorylation state of either TRP-185 or RNA

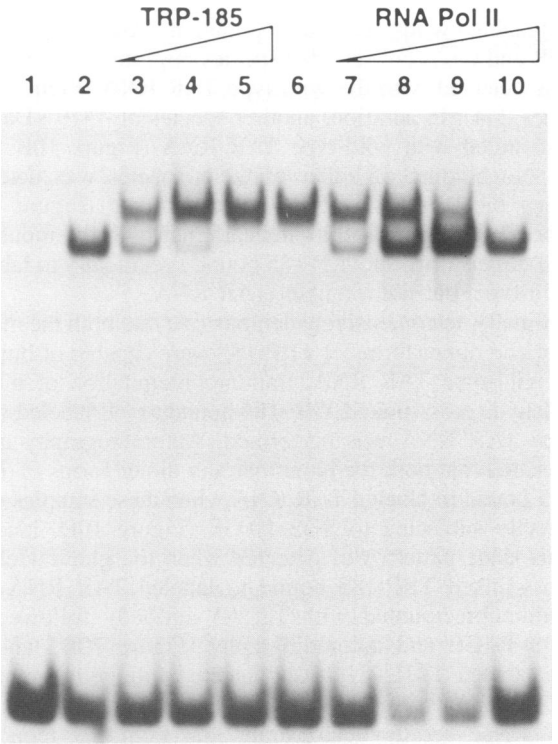


Fig. 8. RNA polymerase II and TRP-185 compete for binding to HIV-1 TAR RNA. Gel retardation was performed with labeled HIV-1 TAR RNA (lane 1), RNA polymerase II (50 ng) with 0.4 μg of cofactors alone (lanes 2 and 10) or with 25 ng (lane 3), 50 ng (lane 4) or 70 ng of TRP-185 (lane 5). Gel retardation analysis with wild-type TAR RNA and TRP-185 and cofactors alone (25 ng) (lane 6) or with increasing amounts of RNA polymerase II (50 ng) (lane 7), (100 ng) (lane 8), (160 ng) (lane 9) are shown.

polymerase II were able to alter their binding to TAR RNA. Both TRP-185 and RNA polymerase II were treated with alkaline phosphatase, and their ability to bind to TAR RNA before and after treatment was tested (Figure 9). Alkaline phosphatase treatment of TRP-185 markedly decreased its binding to TAR RNA (Figure 9A, lanes 3 and 4). In contrast, treatment of RNA polymerase II with alkaline phosphatase increased its ability to bind to TAR RNA (Figure 9A, lanes 6 and 7). There was no effect of alkaline phosphatase treatment on the amount of the TRP-185 protein, as determined by Western blot analysis (Figure 9B, lanes 3 and 4), or that of RNA polymerase II (data not shown). The effect of the alkaline phosphatase was likely due to dephosphorylation of RNA polymerase II and TRP-185 rather than on the cofactor fraction since dephosphorylation had opposite effects on the binding of these proteins to TAR RNA. The role that specific kinases may have in either enhancing or otherwise modulating the binding activity of TRP-185 and RNA polymerase II to TAR RNA remains to be determined.

TRP-185 can bind to TAR RNA as both a monomer and a dimer

In addition to the effects of changes in phosphorylation on the binding properties of a protein, changes in the ability to homodimerize or heterodimerize can also alter their binding properties. Since TRP-185 contains a putative leucine zipper structure (Landschulz *et al.*, 1988), we wished to determine whether it was capable of binding to TAR RNA as either a heterodimer or a homodimer using glutaraldelyde cross-linking. Glutaraldelyde cross-linking has been used in a number of studies to detect the dimerization of leucine zipper-containing proteins

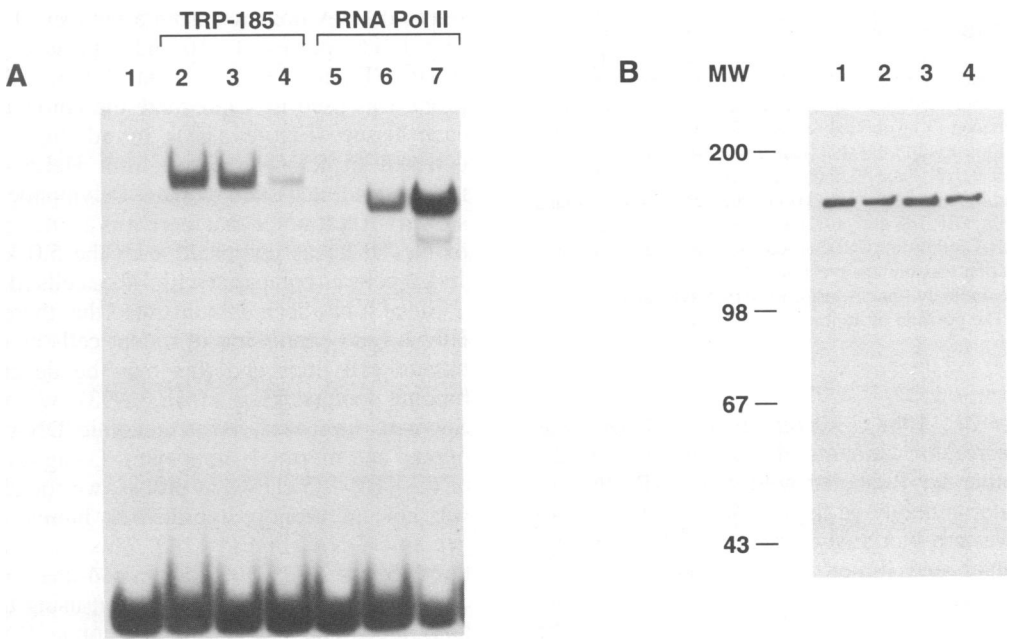


Fig. 9. Dephosphorylation of TRP-185 inhibits binding to TAR RNA. (**A**) Gel-retardation analysis was performed with TAR RNA (lane 1) with TRP-185 (50 ng) and 0.4 μg of cofactors (lane 2) in the presence of either 1 mM ATP (lane 3) or 20 U of alkaline phosphatase (lane 4). The TAR RNA probe was incubated with alkaline phosphatase alone (lane 5), with purified RNA polymerase II (50 ng) and 0.4 μg of cofactors (lane 6) alone or with 20 U of alkaline phosphatase (lane 7). (**B**) Western blot analysis of TRP-185 was performed with the 12CA5 mAb which recognizes the influenza hemagglutinin sequences attached to the C-terminus of TRP-185 using TRP-185 alone (150 ng) (lane 1) or following incubation in the presence of cofactors (0.4 μg) (lane 2) with either 1 mM ATP (lane 3) or 20 U of alkaline phosphatase (lane 4).

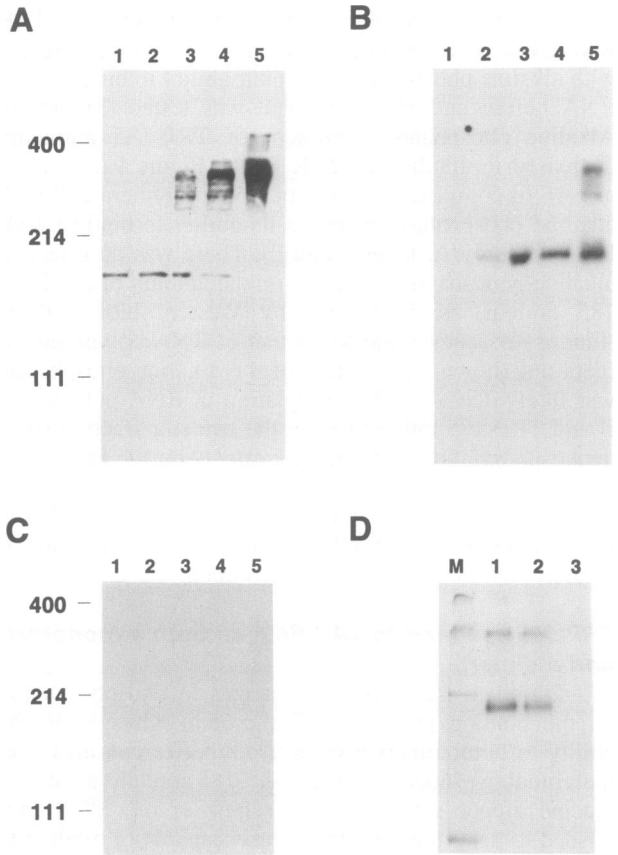


Fig. 10. Glutaraldehyde cross-linking of TRP-185. (**A**) Western blot analysis was performed with recombinant TRP-185 (200 ng), cofactors (0.4 μg) and 3 ng of labeled wild-type TAR RNA either alone (lane 1) or cross-linked with the following final concentrations of glutaraldehyde: 0.0004% (lane 2), 0.001% (lane 3), 0.002% (lane 4) or 0.01% (lane 5), followed by SDS–PAGE and probing with 12CA5 antibody. Markers indicate the position of the monomer and dimer forms of TRP-185. (**B**) An autoradiogram of the gel in (A) is shown with increasing concentrations of glutaraldehyde (lanes 1–5) and the position of the ^{32}P-labeled TAR RNA bound to monomer and dimer forms of TRP-185 indicated. (**C**) An autoradiogram of a ^{32}P-labeled TAR RNA loop mutant (3 ng) bound to TRP-185 and cofactors alone (lane 1) or cross-linked with 0.0004% (lane 2), 0.001% (lane 3), 0.002% (lane 4) or 0.01% (lane 5) glutaraldehyde followed by SDS–PAGE and autoradiography is shown. (**D**) Glutaraldehyde cross-linking was performed with TRP-185 and cofactors bound to a ^{32}P-labeled wild-type TAR RNA followed by SDS–PAGE directly (lane 1) or this same binding reaction was immunoprecipitated with 12CA5 (lane 2) or β-galactosidase antibody (lane 3) prior to SDS–PAGE and autoradiography. The position of molecular weight markers is also indicated.

(Nakabeppu *et al.*, 1988). Recombinant TRP-185 was treated with increasing amounts of glutaraldehyde in the presence of either a ^{32}P-labeled wild-type TAR RNA or a TAR RNA loop mutant and then subject to SDS–PAGE followed by Western blot analysis with 12CA5 antibody.

Both monomer and dimer forms of TRP-185 were detected in Western blot analysis in the presence of either the wild-type TAR RNA (Figure 10A, lanes 3–5) or the TAR RNA loop mutant (data not shown). Thus, TRP-185 could dimerize in the presence of either wild-type or a mutant TAR RNA. Next we wanted to determine whether both the monomer or dimer forms of TRP-185 bound directly to TAR RNA. Autoradiography of these same gels was then performed to detect the position of the ^{32}P-labeled wild-type and loop mutant TAR RNAs (Figure 10B and C). A gel-retarded species migrating at ~200 kDa was detected with the wild-type TAR RNA (Figure 10B, lanes 2–4). In addition, another species of ~370 kDa was also noted with wild-type TAR RNA (Figure 10B, lane 5). No binding of either of these species was detected using the labeled TAR RNA loop mutant (Figure 10C, lanes 2–5). These results indicate that both the monomer and dimer forms of TRP-185 bound specifically to labeled wild-type, but not a mutant TAR RNA.

Finally, to conclusively demonstrate that both the monomer and dimer forms of TRP-185 were capable of binding to wild-type TAR RNA, immunoprecipitation of glutaraldehyde cross-linked TRP-185 bound to ^{32}P-labeled wild-type TAR RNA was performed. Autoradiography again revealed that both the monomer and dimer forms of TRP-185 bound to labeled TAR RNA when these samples were directly subjected to SDS–PAGE (Figure 10D, lane 1). This same pattern was detected when the glutaraldehyde cross-linked TRP-185 bound to labeled TAR RNA was immunoprecipitated with 12CA5 antibody followed by SDS–PAGE and autoradiography (Figure 10B, lane 2). No labeled TAR RNA was detected following immunoprecipitation with β-galactosidase mAb (Figure 10D, lane 3). These results demonstrate that both the monomer and dimer forms of TRP-185 were capable of binding specifically to a labeled wild-type, but not a TAR RNA loop mutant. Whether cellular factors are present which catalyze the dimerization of TRP-185 under physiological conditions remains to be determined.

TRP-185 gene structure and expression in human and rodent cells

To determine the patterns of expression of TRP-185 RNA, we probed a Northern blot containing 2 μg of poly(A) selected RNA prepared from a number of human tissues. Two RNA species of ~10 and 5 kb were detected in all tissues (Figure 11A). A control blot using a GAPDH probe was used to standardize the amount of RNA from each tissue (Figure 11B). In addition, TRP-185 was detected in RNA prepared from HeLa cells and both resting and activated Jurkat T lymphocytes (data not shown). It was noted that there was an increased abundance of the 10 kb as compared with the 5.0 kb transcript in Jurkat cells as compared with HeLa cells (data not shown).

Since it has been demonstrated that there was restricted HIV-1 gene expression in rodent cells as compared with human cell lines and this may be dependent on loop binding factors (Hart *et al.*, 1993), we also performed Southern blot analysis on genomic DNA isolated from human and murine lymphocytes. Using different portions of the TRP-185 cDNA as probes, we found that TRP-185 was able to strongly hybridize to human but not mouse genomic DNA (Figure 12A). This was true for each of three fragments which comprised the entire TRP-185 cDNA (data not shown). However, using this same filter, both the human and mouse genomic DNA hybridized similarly to a probe consisting of a portion of the largest subunit of RNA polymerase II (Figure 12B). Thus, the TRP-185 gene appeared to diverge significantly between human and mouse. To further characterize the ability of TRP-185 to hybridize to DNA isolated from various species, the TRP-185 cDNA was used to probe genomic

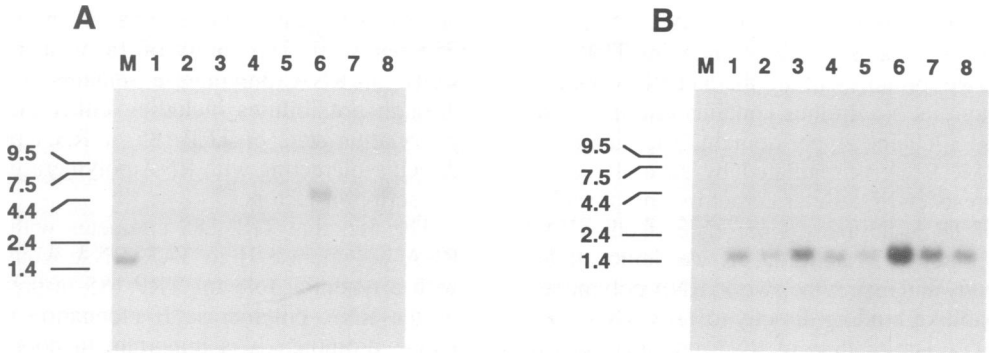


Fig. 11. Northern analysis of TRP-185. (**A**) Northern analysis of a human multiple tissue blot is shown using a portion of the TRP-185 cDNA encoding amino acids extending from 392 to 817 and 2 μg of poly(A) selected RNA isolated from heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), pancreas (lane 8), followed by autoradiography. (**B**) This blot was probed with a full-length GAPDH cDNA as a control for the amount of poly(A) selected RNA in each sample.

DNA isolated from several different species. Again the TRP-185 cDNA was able to strongly hybridize to human DNA and also hybridized to DNA isolated from the monkey, dog and cow, but there was little detectable hybridization to DNA isolated from the rat, mouse, chicken and yeast (data not shown). Furthermore, using reverse transcriptase (RT)-PCR we did not readily detect TRP-185 transcripts in a variety of rodent cell lines (data not shown). These results indicate that TRP-185 has diverged significantly from human to rodent. Chromosomal mapping demonstrated that TRP-185 was present on human chromosome 1p, indicating that TRP-185 alone was not the gene on human chromosome 12 that complemented HIV-1 gene expression in human mouse cell hybrids (Hart *et al.*, 1993).

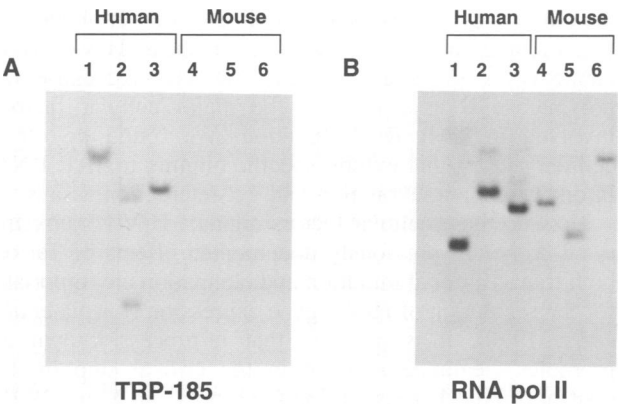


Fig. 12. Southern blot analysis of human and rodent genomic DNA with TRP-185 and RNA polymerase II cDNA fragments. (**A**) Southern blots were probed with a portion of the TRP-185 cDNA extending from amino acid position 817 to 1162. Approximately 10 μg of either human genomic DNA (lanes 1–3) or mouse genomic DNA (lanes 4–6) were restricted with *Bam*HI, *Pst* and *Eco*RI corresponding to the lane as shown in the figure above. The positions of the 12.0, 8.0 and 5.0 and 10 kb species, respectively, are seen. (**B**) The same blots above were probed with human RNA polymerase II cDNA encoding amino acid positions 1290–1640. The positions of the 3.0, 6.0 and 4.0 kb species in human DNA, and the 4.5, 3.5 and 10 kb species in mouse DNA, are seen.

Discussion

Tat activation of HIV-1 is dependent on a downstream RNA regulatory element designated TAR (Rosen *et al.*, 1985; Muesing *et al.*, 1987; Feng and Holland, 1988; Garcia *et al.*, 1989; Selby *et al.*, 1989). The mechanism by which TAR functions in conjunction with Tat to increase the transcriptional elongation properties of RNA polymerase II remains to be determined. Several potential models for TAR function include its ability to serve as an attenuator element which pauses elongating transcription complexes, to bind Tat and subsequently activate a class of transcription complexes that are uniquely responsive to the action of Tat, or to act as an RNA enhancer which delivers Tat and cellular factors to the transcriptional initiation complex. Evidence that supports each of these models has been presented (Kao *et al.*, 1987; Marciniak *et al.*, 1990; Bengal and Aloni, 1991; Olsen and Rosen, 1992; Graeble *et al.*, 1993; Lu *et al.*, 1993).

It is clear from a number of studies that TAR does not function as a classical RNA terminator sequence, in that deletion of this element does not markedly increase HIV-1 gene expression (Rosen *et al.*, 1985; Peterlin *et al.*, 1986; Garcia *et al.*, 1987, 1989; Muesing *et al.*, 1987; Feng and Holland, 1988; Selby *et al.*, 1989). However, in the absence of Tat, HIV-1 transcription complexes pause downstream of TAR, suggesting that TAR may have an attenuator function (Kao *et al.*, 1987; Selby *et al.*, 1989; Marciniak *et al.*, 1990; Ratnasabapathy *et al.*, 1990; Bengal and Aloni, 1991; Feinberg *et al.*, 1991; Marciniak *et al.*, 1991; Kato *et al.*, 1992; Graeble *et al.*, 1993). If TAR RNA does in fact have such a function, then its effects on pausing the HIV-1 transcription complex are likely critical for the subsequent action of Tat to increase the transcriptional elongation properties of RNA polymerase II. Since both the loop and bulge sequences are critical for TAR function (Feng and Holland, 1988; Berkhout and Jeang, 1989; Garcia *et al.*, 1989; Cordingley *et al.*, 1990; Dingwall *et al.*, 1990; Roy *et al.*, 1990; Calnan *et al.*, 1991; Sheline *et al.*, 1991; Wu *et al.*, 1991), it is possible that two signals are required for subsequent effects on transcriptional elongation from the HIV-1 promoter. One of these signals is likely Tat, while the other is likely a cellular factor which binds to the TAR RNA loop sequences.

In the current study, we used a biochemical approach to attempt to characterize cellular factors that are involved in TAR RNA function. We purified a cellular factor

TRP-185 that bound specifically to the TAR RNA loop sequences and then isolated the cDNA encoding TRP-185. TRP-185 is a novel protein with no distinct RNA binding domains, although its N-terminus contains multiple basic amino acids and a leucine zipper domain. The binding of TRP-185 to TAR RNA is regulated by several factors, including the presence of cellular cofactors, the phosphorylation state of TRP-185 and the presence of other TAR RNA binding proteins. In addition, as we demonstrated in the current study and in previous work, RNA polymerase II was also capable of binding directly to TAR RNA (Wu-Baer *et al.*, 1995). The binding of RNA polymerase II to TAR RNA, like that of TRP-185, is dependent on the addition of the same set of cellular cofactors that stimulated the binding of TRP-185 to TAR RNA. Furthermore, the binding of RNA polymerase II is relatively specific in that it binds well to wild-type TAR RNA templates, but binds poorly to TAR RNAs containing mutations in either the bulge and loop. Thus, the binding of RNA polymerase II correlates well with the role of these HIV-1 TAR elements for *in vivo* activation by Tat. TRP-185 and RNA polymerase II appear to be the major cellular factors involved in TAR RNA function. We found no other cellular factors that exhibit specific binding to TAR RNA in our cellular fractionation and gel-retardation assays.

How do these cellular factors regulate HIV-1 transcription? Although previously documented effects of Tat on both transcriptional initiation and elongation are important for the regulation of HIV-1 gene expression (Laspia *et al.*, 1989, 1990), it is possible that a process known as promoter clearance may be in the critical step in the control of HIV-1 transcription (Kerppola and Kane, 1991; Lee *et al.*, 1992; Zawel and Reinberg, 1993; Goodrich and Tjian, 1994; Maxon *et al.*, 1994; O'Brian *et al.*, 1994). This process involves the ability to regulate the natural stalling of RNA polymerase II downstream of the transcription initiation site of viral and cellular promoters. The stimulation of promoter clearance can appear as changes in both the levels of transcriptional initiation and elongation (Goodrich and Tjian, 1994). RNA polymerase II naturally pauses downstream of the transcription initiation site due to its binding to both promoter DNA and nascently synthesized RNA (Johnson *et al.*, 1994; Nudler *et al.*, 1994, 1995; Chamberlin, 1995; Wang *et al.*, 1995). Such a model, which has been validated in a number of studies using both eukaryotic and prokaryotic promoters, indicates that RNA polymerase II can bind and elongate in both a continuous and a discontinuous manner, leading to pausing (Johnson *et al.*, 1994; Nudler *et al.*, 1994, 1995; Wang *et al.*, 1995). The discontinuous movement occurs when the downstream edge of the footprint of RNA polymerase on the DNA remains fixed for up to 10 rounds of nucleotide addition while the upstream edge of the footprint slides forward. This appears to be accompanied by the extension of the RNA chain within the transcription complex and increases the likelihood of transcriptional arrest as the active site slips backward along the RNA chain. It is possible that the structure of TAR RNA acts as a strong binding site for RNA polymerase II, thus acting as an efficient pause site for the elongating RNA polymerase II (Bengal and Aloni, 1991). The function of TRP-185 or Tat either alone or in combination may be to first disrupt the paused RNA polymerase II and subsequently directly or indirectly alter the elongation properties of RNA polymerase II. The ability of Tat to increase the processivity of RNA polymerase remains to be determined through possibilities including either altering the phosphorylation state of TRP-185 or RNA polymerase II or directly interacting with RNA polymerase II or associated transcription factors.

The fact that TRP-185 competes with the binding of RNA polymerase II to TAR RNA would be consistent with a potential role for TRP-185 in preventing pausing of the RNA polymerase II elongation complex on the HIV-1 promoter. It is important to note that both TRP-185 and RNA polymerase II binding to TAR RNA are extremely dependent on the TAR RNA loop sequences. This is expected given the fact that single-stranded RNA in stem–loop structures is the binding site for many RNA binding proteins (Das, 1993). Whether the ultimate function of TRP-185 is found to be only a steric effect which prevents stable RNA polymerase II binding to TAR RNA or whether it may catalyze a process such as TFIIS cleavage to overcome RNA polymerase II pause sites (Reines *et al.*, 1989; Izban and Luse, 1992), or directly associate with the RNA polymerase II, remains to be determined. Kinetic analyses of the binding properties of RNA polymerase II and TRP-185 to TAR RNA are under way to compare their binding affinities with TAR RNA.

Finally, it appears that TRP-185 is ubiquitously expressed in a variety of human tissues. The fact that the TRP-185 cDNA hybridized to two RNAs suggests that there may be multiple forms of TRP-185. We noted that although TRP-185 was widely expressed in a variety of human tissues, it was not present in significant levels in RNAs extracted from rodent cells. We then proceeded to determine whether the TRP-185 gene was present in the mouse genome. Southern blot analysis was performed to determine whether the TRP-185 gene could be detected in mouse DNA. Using different fragments which spanned the entire TRP-185 cDNA, we detected only weak hybridization to mouse DNA as compared with human DNA. In comparison, there was strong hybridization to both human and mouse DNA with the RNA polymerase II cDNA. These results suggest that the TRP-185 gene has significantly diverged between mouse and human DNAs. Since it has been demonstrated that the presence of human chromosome 12 will increase the degree of Tat activation in rodent cells and that this may be dependent on cellular factors binding to the TAR RNA loop, it is possible that other cellular factors present on human chromosome 12 could serve as potential cofactors with the rodent form of TRP-185 to confer Tat activity on human rodent cell hybrids (Hart *et al.*, 1993). Reconstituted *in vitro* transcription assays with the HIV-1 LTR, RNA polymerase II, TRP-185 and Tat will be required to determine how these factors alter the properties of the HIV-1 transcriptional elongation complex.

Materials and methods

Purification of TRP-185

All protein purification procedures were performed at 4°C. Nuclear extract was prepared from 60 l of HeLa cells as previously described (Dignam *et al.*, 1983) and applied to a heparin agarose column (2.5× 9 cm) equilibrated with buffer A [20 mM Tris–Cl (pH 7.9), 20% glycerol

(v/v), 0.2 mM EDTA] containing 0.1 M KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM dithiothreitol (DTT). The column was washed with the same buffer until A_{280} was almost zero, and the bound proteins were eluted with buffer A containing 0.4 M KCl, 0.5 mM PMSF and 0.5 mM DTT. The buffer A fractions were pooled and dialyzed against buffer with 0.1 M KCl, 0.5 mM PMSF and 0.5 mM DTT. The dialyzed fraction was then applied to a HTP Bio Gel (2.5×7 cm) column equilibrated with the same buffer. The column was washed with the same buffer above and eluted with buffer containing 0.1 M potassium phosphate (pH 7.0), 0.5 mM PMSF and 0.5 mM DTT. The active fractions were pooled and precipitated with 70% ammonium sulfate for 20 min at 4°C and then centrifuged at 12 000 r.p.m. for 20 min. The pellet was resuspended in 6 ml of buffer with 0.1 M KCl and 1 mM DTT and then applied to a Superdex 200 FPLC (HiLoad 26/60 prep grade) column equilibrated with this buffer. The active fractions were pooled and applied to a Bio Rex 70 (1.5×3 cm) column. The active flow-through fractions were pooled and applied to a Dextran Blue Sepharose (1×2.5 cm) column equilibrated in the same buffer. The column was washed and eluted with buffer containing 0.4 M KCl and 1 mM DTT. The active fractions were pooled and dialyzed against buffer containing 0.1 M KCl and 1 mM DTT. The pooled and dialyzed fraction was then applied to a 1 ml Mono Q FPLC column equilibrated in the same buffer. The column was washed and then eluted with buffer containing 0.4 M KCl and 1 mM DTT. The active fraction was dialyzed against 20 mM Tris–Cl (pH 7.9), 5% glycerol and 0.2 mM EDTA, 0.1 M KCl and 1 mM DTT, and loaded onto centrifuge tubes (1.4×8.9 cm) containing 10 ml of a 5–25% continuous sucrose gradient. A preparative sucrose gradient was then performed using a Beckman SW40 Ti rotor centrifuged at 30 000 r.p.m. for 40 h at 4°C. The sucrose gradient was fractionated from the bottom of the tube and assayed. The active fractions were pooled, diluted 1:1 (v/v) with buffer containing 0.1 M KCl and 1 mM DTT, and loaded onto a 1 ml Mono Q FPLC column. The column was washed with the same buffer and eluted with buffer containing 0.2 M KCl and 1 mM DTT. The active fractions were stored at −70°C. This purification scheme yielded TRP-185 with >95% purity, as visualized by silver staining of a polyacrylamide gel.

Purification of RNA polymerase II

RNA polymerase II was prepared using a previously described purification scheme (Reinberg and Roeder, 1987). Calf thymus DNA at a concentration of 100 μg/ml was used as the template for measuring the RNA polymerase II activity. Using 2×10^{10} HeLa cells, we obtained ~0.5 mg of RNA polymerase II at >80% purity. The amount of RNA polymerase II required to obtain detectable binding to TAR RNA is the amount of polymerase II activity present in 1.5 μl of nuclear extract. The nuclear extract was tested in *in vitro* transcription analysis with the HIV-1 LTR and was sensitive to 2 μg/ml of α-amanitin.

Amino acid sequence analysis of TRP-185

Active fractions of TRP-185, as judged by their ability to bind to TAR RNA, were isolated from 26×60 l HeLa cell preparations and purified as described. These fractions were pooled and concentrated on a Centricon 30 membrane which was previous blocked with bovine serum albumin (BSA) and washed. The concentrated sample (150 μl) was loaded into three wells of a 8% polyacrylamide–0.1% SDS protein gel and was subject to electrophoresis in Tris–glycine buffer. The gel was then blotted overnight onto a nitrocellulose membrane (0.45 μm) and the protein bands were visualized by Ponceau's stain and excised as described (Aebersold, 1987). The NaOH destaining step was omitted. The amount of TRP-185 used for digestion with the endoprotease Lys C for this analysis was ~50 μg, and this was followed by separation by HPLC and N-terminal sequencing of the peptides by automated Edman degradation on an ABI model 477A protein sequencer (Ha *et al.*, 1991; Lane, 1991). Two peptides with the amino acid sequence LKPGDWSQQDIGTNLVEADNQAEW and TEGYTIIGVEQTAK were obtained.

Cloning the cDNA encoding TRP-185

Degenerate oligonucleotide primers were made to the 5′ and 3′ ends of the 24mer peptide sequence obtained from Lys C digestion of TRP-185, and PCR analysis was performed to generate a 72 bp fragment of the TRP-185 cDNA. PCR primers were then made according to the actual nucleic sequences in this fragment and PCR analysis was performed with the degenerate oligonucleotide primers which corresponded to the 14 amino acid peptide obtained from amino acid sequence analysis. A 435 bp fragment of cDNA encoding TRP-185 was obtained. This fragment was then used as the probe to screen a HeLa cDNA library in lambda zap (ClonTech). A 5.6 kb cDNA encoding a 1621 amino acid open reading frame encoding the full-length TRP-185 protein was obtained.

Expression and purification of the recombinant TRP-185

PCR primers were used to modify the 5′ ATG and 3′ end of the TRP-185 cDNA into *Nco*I and *Bam*HI restriction sites, respectively. The TRP-185 cDNA was then cloned downstream of the T7 promoter in a modified pTM1 vaccinia expression vector (Elroy-Stein *et al.*, 1989) with the 12 amino acid influenza hemagglutinin amino acid sequence (Field *et al.*, 1988) and six histidine residues at the 3′ end (Tanog, 1995). This construct was transfected by phosphate precipitation onto HeLa cells infected with a vaccinia recombinant virus that produced T7 polymerase (Janknecht *et al.*, 1991). The cells were harvested at 40 h post-transfection and nuclear extract was prepared as described previously, except that 1 μg/ml of leupeptin and aprotinin were included in the buffers. The nuclear extract was prepared from 30 plates (150 mM) of HeLa cells and then subjected to chromatography on a 2 ml Q-Sepharose column (1.5×2 cm) equilibrated with buffer A containing 0.1 M KCl, 0.5 mM PMSF, 1 μg/ml of leupeptin and aprotinin, and 10 mM β-mercaptoethanol. The column was washed and eluted with this same buffer containing 0.3 M KCl. The eluted fractions were pooled and then loaded onto a 1 ml Ni-NTA agarose (Qiagen) column equilibrated with this same buffer. The flow through was reloaded onto this column a second time and the column was washed with (i) 20 ml of the same buffer, (ii) 20 ml of this buffer containing 1.0 M KCl and (iii) 20 ml of this buffer containing 0.1 M KCl. The column was then eluted with this same buffer containing 0.1 M KCl and 60 mM imidazole. The eluted fractions were then dialyzed against buffer containing 0.1 M KCl and 1 mM DTT, and stored at −70°C. A typical yield of recombinant TRP-185 from 30 plates of HeLa cells is 60–70 μg with >90% purity, as judged by silver staining following PAGE.

Gel-retardation analysis of TRP-185 and RNA polymerase II to HIV-1 TAR RNA

Wild-type and mutant HIV mRNAs were constructed by fusing a synthetic linker containing a T7 RNA polymerase promoter to DNA fragments of the indicated TAR constructs from +1 to +80 (Wu *et al.*, 1991). Transcription of these constructs was performed after they were linearized with *Hin*dIII (+80) by using T7 RNA polymerase, resulting in transcripts consisting of nucleotides +1 to +80 of the HIV LTR. RNA synthesis, labeling and purification were performed by using the reagents and procedures of the Riboprobe System II (Promega).

Approximately 1.5 ng of TAR RNA probe were mixed with RNA polymerase II (0.04–0.4 μg), poly(I)-poly(C) (0.2 μg), and a final concentration of 10 mM Tris–Cl (pH 7.4), 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, 0.5 mM PMSF, 1.5 mM Pefabloc 5C (AEBSF) and 10% glycerol in 30 μl total volume (Wu-Baer *et al.*, 1995). For competition analysis, 50 ng of each unlabeled *in vitro* transcribed competitor TAR PNAS were mixed with probe and binding was performed. Binding was carried out at room temperature for 20 min, and the samples were loaded onto a 5% polyacrylamide gel containing 1× Tris–borate–EDTA (TBE) with 2% glycerol, and subject to gel electrophoresis at 180 V in 1× TBE at room temperature.

The binding assays with recombinant TRP-185 were performed as described for endogenous TRP-185 (Wu *et al.*, 1991). The amounts of protein used in these assays were 50 ng of recombinant TRP-185 and 0.4 μg of cellular cofactors. Dephosphorylation of TRP-185 and RNA polymerase II was performed by incubating 50 ng of either TRP-185 or RNA polymerase II with 20 U of alkaline phosphatase (BMB) in binding reaction conditions for 20 min at room temperature, then cofactors and TAR RNA probe were added and incubated for an additional 15 min at room temperature. ATP was included at a final concentration of 1 mM in the binding reaction when necessary. For the antibody supershift studies, 1 μg of each of the antibodies was purified following chromatography on protein A–Sepharose and added to the binding reactions 10 min after the start of reaction and continued for an additional 20 min at room temperature. The TRP-185 polyclonal and monoclonal antibodies used in this study were raised against a portion of TRP-185 corresponding to amino acids 1409–1541 fused in-frame to GST. The antibody directed against the C-terminal domain of RNA polymerase II 8WG16 was described previously (Thompson *et al.*, 1989).

Glutaraldehyde cross-linking and immunoprecipitation

The cross-linking reactions were performed under identical conditions to the gel-retardation binding reactions described above except that glutaraldehyde was added at final concentrations ranging from 0.0004 to 0.01% (v/v) and then incubated at room temperature for 30 min

(Nakabeppu *et al.*, 1988). For cross-linking in the presence of HIV-1 TAR RNA, TRP-185 was first bound to the labeled RNA probe for 20 min at room temperature prior to the addition of glutaraldehyde. The reactions were then incubated at room temperature for 25 min. For immunoprecipitation, the glutaraldehyde cross-labeled TRP-185 was immunoprecipitated with either 3 μg of NK 5.18 or 12CA5 antibody overnight at 4°C. All reactions above were stopped by the addition of SDS–β-mercaptoethanol and subject to electrophoresis on a 7% polyacrylamide gel with 0.1% SDS, followed by either Western blot analysis and/or autoradiography as needed.

Southern, Northern and Western analysis

For Southern analysis (Sambrook *et al.*, 1989), 6 μg of human and mouse genomic DNA isolated from human and mouse lymphocytes (ClonTech) were digested with either *Bam*HI, *Pst*I or *Eco*RI and then subject to electrophoresis on a 1% agarose gel in 1× Tris–acetate EDTA buffer at 150 V. The gel was then treated, blotted and the DNA was fixed to Hybon-N membrane as described in the manufacturer's protocol (Amersham). The membrane was then pre-hybridized at 65°C for 3 h in 5× SSPE, 10× Denhardt's, 100 μg/ml salmon sperm DNA and 2% SDS, and hybridized overnight in the same buffer containing 1×10^6 c.p.m./ml of labeled DNA probe. The probes were made from nick translation of portions of the TRP-185 cDNA encoding amino acid positions 392–817, 817–1162 and 1162–1572, respectively. The same blots were also probed with a portion of the largest subunit of an RNA polymerase II cDNA encoding amino acid positions 1290–1640. Southern blot analysis of the zoo blot used in this study (Clontech) and the somatic cell hybrid panel used for chromosome mapping were purchased from Oncor Inc. and probed with the same portions of the TRP-185 cDNA used above as described in the manufacturer's protocols. Northern analysis of TRP-185 on poly(A) selected RNA isolated from HeLa cells was performed as described in the rapid hybridization protocol from Amersham (Sambrook *et al.*, 1989). Northern analysis of the human multiple tissue (MTN) blot from Clontech was carried out as described in the product protocol. The probe used in these analysis was a portion of the TRP-185 cDNA encoding amino acids between 392 and 817 prepared by nick translation. A full-length GAPDH probe was used as a control for these Northern blots.

Western analyses were performed using either 12CA5 mAb (Field *et al.*, 1988) which is directed against the 12 amino acid influenza virus hemagglutinin sequence (HA1) or a mAb (NK 5.18) raised against a portion of the TRP-185 amino acids extending from position 1409 to 1541. ECL reagents and protocols from Amersham were used in this analysis.

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