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Short communication

Interaction of 68-kDa TAR RNA-binding protein and other cellular proteins with prion protein-RNA stem-loop

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The RNA stem-loop structure of the *trans*-activating region TAR sequence of human immunodeficiency virus-1 mRNA is the binding site for a number of host cell proteins. A virtually identical set of proteins from HeLa nuclear extracts was found to bind to the predicted RNA hairpin element of prion protein (PrP) mRNA, as demonstrated in UV cross-linking/RNase protection and Northwestern assays. We show that the cellular TAR loop-binding protein, p68, is among those proteins which associate with PrP RNA. Competition experiments with various TAR RNA mutants revealed that binding of partially purified p68 to PrP RNA stem-loop occurs sequence-specifically. The 100-kDa 2-5A synthetase which is involved in the cellular antiviral defense was able to bind to PrP mRNA stem-loop in Northwestern blots with cytosolic proteins from HeLa cells treated with interferon. However, the PrP RNA failed to activate this enzyme *in vitro*, in contrast to TAR RNA.

Keywords: prion mRNA stem-loop; HIV-1 TAR RNA; p68; 2', 5'-oligoadenylate synthetase; RNA-binding protein

The scrapie prion protein (PrP^{Sc}), which is the abnormal isoform of the cellular prion protein (PrP^{C}), has been implicated in the pathogenesis of a number of transmissible neurodegenerative diseases in humans (Creutzfeldt-Jakob disease and Gerstmann-Sträussler syndrome) and animals (bovine spongiform encephalopathy and scrapie) (Prusiner, 1991). Although encoded by the same cellular gene (Basler *et al*, 1986) PrP^{Sc} differs from PrP^{C} in many properties, including enhanced protease resistance (Oesch *et al*, 1985), slower turnover rate (Borchelt *et al*, 1990) and abnormal intracellular localization. PrP^{C} is a glycoprotein of the plasma membrane, while PrP^{Sc} is located predominantly in the cytoplasm (Taraboulos *et al*, 1990) and, in smaller amounts, also in the nucleus (Pfeifer *et al*, 1993).

The PrP mRNA may form three separate, virtually identical stem-loop structures within the coding region, at nucleotide positions +170, +194, and +218 (corresponding to nucleotides 219, 243, and 267 in the sequence published by Kretzschmar *et al*, 1986), containing the pentanucleotide CUGGG in the loop, and a uridine bulge and adenine bulge in the stem region (Wills and Hughes, 1990). These features are also characteristic for the TAR (*trans*-acting response element) RNA sequence (+1 to +80) of the human immunodeficiency virus-1 (HIV-1) mRNA (Okamoto and Wong-Staal, 1986). The TAR RNA is the target sequence for the *trans*-activator protein, Tat (Müller *et al*, 1990), which interacts with the uridine bulge in the TAR RNA stem (Dingwall *et al*, 1990). We demonstrated that Tat is also able to bind to the PrP RNA stem-loop (Müller *et al*, 1992). The stem-loop structure (+170 to +220) shown in Figure 1 within the PrP RNA sequence used in the present study has a predicted free energy of folding of −50 to −63 kJ mol^{-1} (Wills and Hughes, 1990).

The TAR element RNA is the binding site for certain host cell proteins that may associate either with the TAR RNA stem (Gatignol *et al*, 1991; Rounseville and Kumar, 1992), the bulge (Sheline *et al*, 1991) or the TAR RNA loop (Sheline *et al*, 1991; Wu *et al*, 1991). A 68-kDa HeLa nuclear protein

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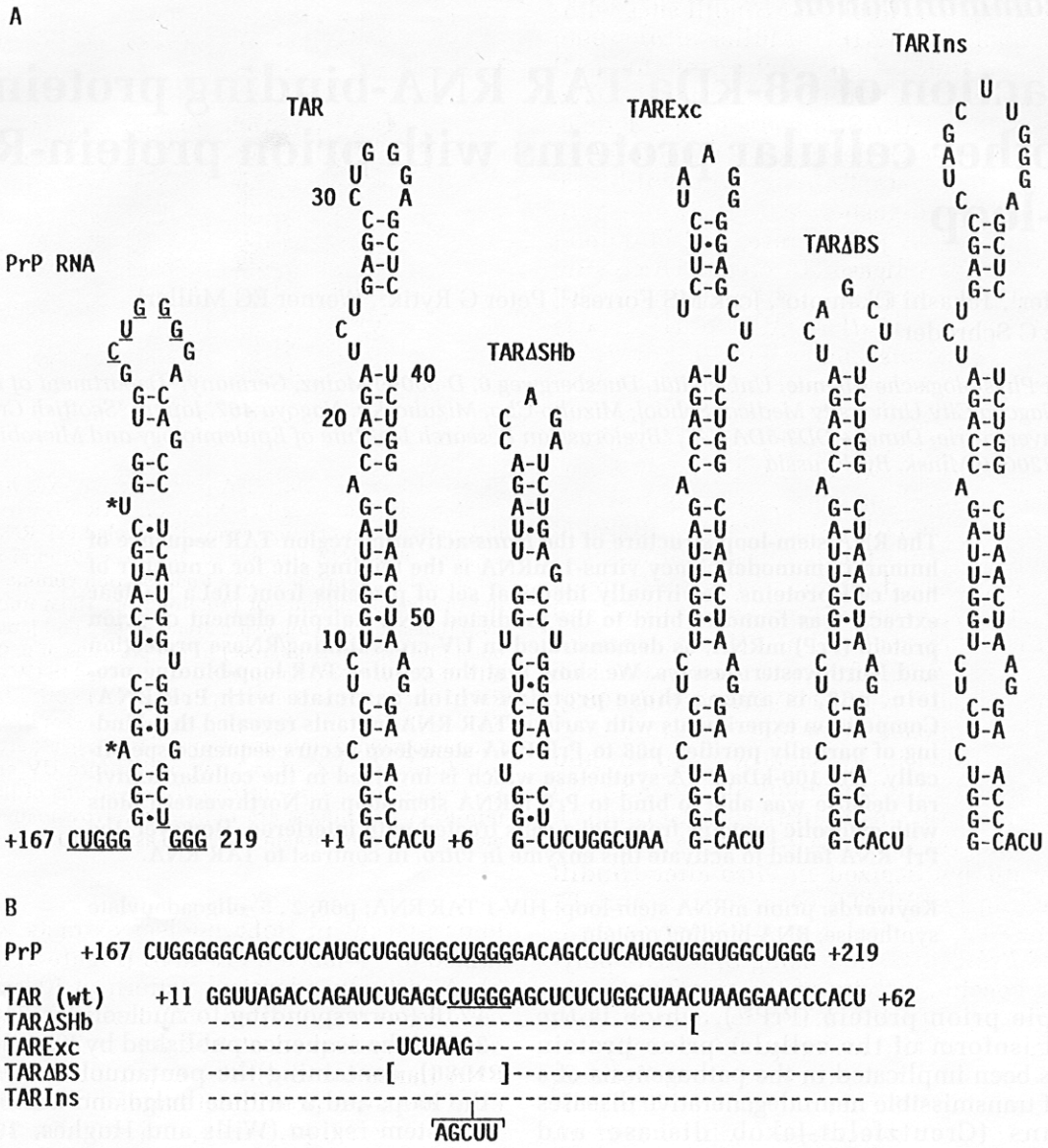

Figure 1 Sequence and computer-predicted secondary structure of PrP RNA, wild-type 'TAR RNA and TAR RNA mutants. (A) Predicted stem-loop structures of human PrP mRNA (coding region, +167 to +219), TAR RNA (+1 to +62) and TAR RNA mutants. The structures were determined using the RNAFOLD programme (PC/Gene, 1991). The characteristic CUGGG motif in the PrP and TAR loop is underlined and the uridine bulge and the adenine bulge in the stem are labeled with asterisks. (B) Sequence of the RNAs. The TAR sequence from +11 to +62 and identical bases (–) in the TAR mutants are shown.

investigated in the present study has been shown to bind to the TAR RNA loop in a sequence-specific manner (Marciniak *et al*, 1990). Recently we reported that the β-galactoside-specific lectin, CBP35, which has been identified in nuclear RNP complexes (Wang *et al*, 1991), is present in the complexes formed between PrP RNA and nuclear protein (Schröder *et al*, 1994). CBP35 has also been found in purified prions (Schröder *et al*, 1994), in addition to other nucleic acid binding proteins (Sklaviadis *et al*, 1993).

In this study formation of RNA-protein complexes was detected in UV cross-linking/RNase protection assays and Northwestern assays, using *in vitro* synthesized PrP RNA, TAR RNA and TAR RNA mutants. The *Mst*II-*Nla*IV fragment (nucleotides 200 to 329; corresponding to nucleotides +151 to +280 of the coding region) of the human PrP cDNA (Kretzschmar *et al*, 1986) containing the proposed stem-loop structure was ligated, after creation of blunt ends, into the *Sma*I site of the polylinker of the plasmid pTZ18U (Pharmacia, Uppsala, Sweden). The wild type TAR-containing HIV-1 fragment (–17 to +80) and the TAR mutants (Figure 1)

were inserted into the *Hinc*II and *Hin*dIII sites within the polylinker of pTZ18U (Müller *et al*, 1990; Okamoto *et al*, 1990). Construction of the TAR mutants, TARΔBS containing a deletion in TAR loop (+25/+34) and TARΔSHb containing a 3' deletion of the stem, was as described (Okamoto *et al*, 1990). Briefly, to create TARΔBS, the wild-type TAR sequence was digested with *Bgl*II and *Sst*I, filled in with T4 DNA polymerase, and blunt-end ligated with T4 DNA ligase. To create TARΔSHb, the wild-type TAR sequence was digested with *Sst*I and *Hin*dIII and the *Sst*I-*Hin*dIII portion was replaced by a synthesized oligonucleotide of sequences from +39 to +48. Construction of TARExc which has a substitution of the two 5' terminal bases of the loop and the following four bases of the stem (+26/+31) and of TARIns containing an insertion (AGCTT) in the loop was carried out as follows. The wild-type TAR sequence was digested with *Bgl*II and *Sst*I and the *Bgl*II-*Sst*I portion was replaced by synthesized oligonucleotides to create GAGCCT → TCTAAG substitution for TARExc and insert AGCTT sequence after GAGCCT sequence for TARIns. The *Xho*I-*Bst*EII fragment (nucleotides 66 to 866) of the human c-*myc* cDNA (Watt *et al*, 1983), containing the proposed stem-loop structure of c-*myc* RNA, was filled-in by Klenow fragment and blunt-end ligated into the *Sma*I site of pSPT18 (Boehringer Mannheim, Mannheim, Germany). RNAs of the cloned PrP-, TAR- and TAR mutant fragments were synthesized *in vitro* after *Hin*dIII digestion using [γ-^{32}P]UTP (Amersham) and T7 RNA polymerase. Control c-*myc* RNA was transcribed after *Bst*EII digestion using SP6 RNA polymerase. The specific activity of the transcripts was 1.5×10^8 cpm μg^{-1} of RNA. RNAs for competitions were labeled at a 10^4-fold lower specific activity.

Nuclear extracts were prepared from HeLa S_3 cells as described by Dignam *et al* (1983). The cells were maintained as described (Schröder *et al*, 1994). UV cross-linking/RNase protection assays were performed, if not mentioned otherwise, by incubating (10 min, 30°C) the ^{32}P-labeled RNA (4×10^5 cpm assay^{-1}) with the indicated amount of HeLa protein in 20 mM HEPES buffer (pH 7.5, containing 6% v/v glycerol, 62 mM KCl, 2 mM $MgCl_2$, and 150 μM dithiothreitol), supplemented with 0.1 mg ml^{-1} of *E. coli* tRNA and 5 mg ml^{-1} of heparin (final volume, 25 μl) (Schröder *et al*, 1994). The samples were then irradiated for 10 min on ice with short-wave UV light at 1,800 J m^{-2} min^{-1} using an UV Stratalinker 1800 (Stratagene, La Jolla, CA, USA), digested with RNase A (final concentration, 1 mg ml^{-1}; 15 min, 37°C), boiled in SDS sample buffer (5 min) and subjected to electrophoresis on 12.5% SDS-polyacrylamide gels. Northwestern assays were carried out as described previously (Schröder *et al*, 1994).

In RNase protection assays, formation of multiple RNA-protein complexes was observed when proteins present in HeLa nuclear extracts were cross-linked to ^{32}P-labeled PrP RNA (Figure 2, lanes a and b). A virtually identical pattern of RNase-resistant complexes was created with HeLa nuclear proteins covalently linked at 254 nm to ^{32}P-labeled TAR RNA (lanes c and d). The apparent molecular masses of the most prominent bands obtained with both RNAs were 37, 42, 54, 68, and ~90 kDa. The intensities of some of the labeled RNA-protein complexes were diminished by addition of heparin (5 mg ml^{-1}) as a non-specific competitor (lanes e, f, h, and i), allowing the identification of additional bands not detectable in the absence of this additive. Under these conditions (presence of both competitor tRNA and heparin) only a weak p68 band was detected in the autoradiograms (lanes e and h); the intensity of this band further decreased when the concentration of tRNA was increased to 0.5 mg ml^{-1} (lanes f and i). However, when adding heparin to assays without tRNA as described by Marciniak *et al* (1990), p68 could be clearly identified, especially after previous binding of HeLa proteins to heparin-Sepharose (lanes g and j).

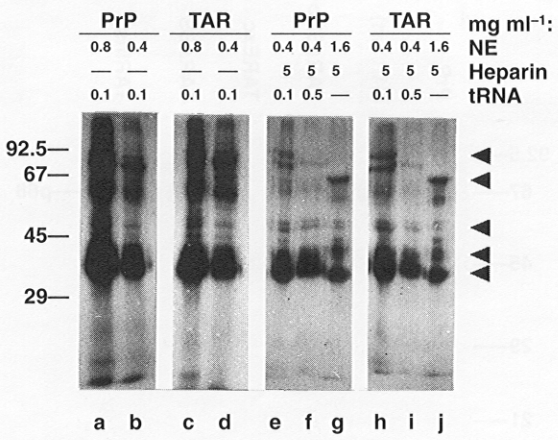

Figure 2 Formation of RNA-protein complexes between HeLa nuclear extract and PrP RNA or TAR RNA in UV cross-linking/RNase protection assay. ^{32}P-labeled PrP RNA (lanes a, b, e-g) and TAR RNA (lanes c, d, h-j) (4×10^5 cpm assay^{-1}) were incubated (10 min, 30°C) in a final reaction volume of 25 μl with the indicated amounts of proteins from HeLa nuclear extracts (NE) (lanes a-f, h, i: nuclear extract, lanes g and j: nuclear extract bound to heparin-Sepharose) in the presence of 0, 0.1, or 0.5 mg ml^{-1} of *E. coli* tRNA. Samples were subjected to UV cross-linking, RNase digestion and electrophoresis in 12.5% SDS-polyacrylamide gels. After electrophoresis, the gels were fixed, dried, and autoradiographed. In some assays (lanes e-j) heparin was added to a final concentration 5 mg ml^{-1} before UV cross-linking. Shown are the autoradiograms. The positions of the major protein-RNA complexes are indicated (arrowheads). Molecular masses of marker proteins are given as $M_r \times 10^{-3}$.

It should be noted that the secondary structure of PrP RNA stem-loop shown in Figure 1A is not the most stable form (Wills and Hughes, 1990). In view of the great similarity of the protein binding patterns of PrP RNA and TAR RNA we suppose that

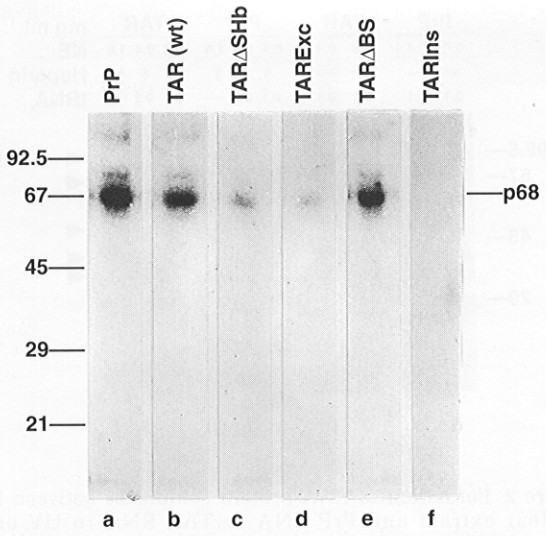

Figure 3 Formation of RNA-protein complexes between partially purified p68 and PrP RNA, TAR RNA, and TAR RNA mutants in UV cross-linking/RNase protection assay. PrP RNA (lane a), wild-type TAR RNA (lane b), TARΔSHb RNA (lane c), TARExc RNA (lane d), TARΔBS RNA (lane e), and TARIns RNA (lane f) (4×10^5 cpm assay^{-1}) were incubated with partially purified p68 (20 µg protein assay^{-1}) and RNA-protein complexes were analyzed as described in legend to Figure 2.

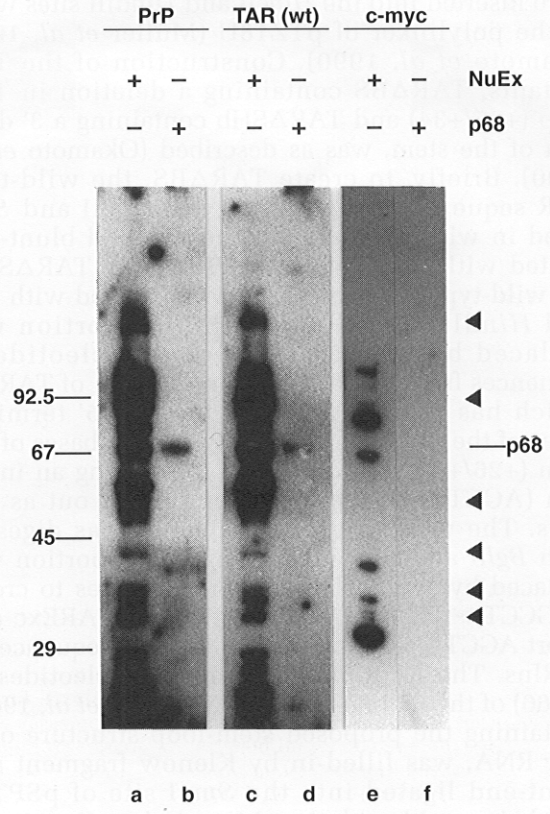

Figure 4 Detection of proteins in HeLa cell extracts binding to PrP RNA and TAR RNA in Northwestern assay. Proteins present in HeLa nuclear extracts (NuEx) (20 µg protein assay^{-1}; lanes a, c, and e) or partially purified p68 (1 µg protein assay^{-1}; lanes b, d, and f) were separated by 12.5% SDS-PAGE, transferred to Immobilon membrane and probed with ^{32}P-labeled PrP RNA (lanes a and b), wild-type TAR RNA (lanes c and d), or control c-*myc* RNA (lanes e and f) (about 4×10^5 cpm lane^{-1}). Each assay contained 50 µg ml^{-1} of *E. coli* tRNA. The membranes were dried and autoradiographed. Positions of major protein-RNA complexes formed with PrP RNA and TAR RNA are indicated (arrowheads).

this secondary structure may be stabilized by protein binding. However, recognition of the alternative structure by some of the bound proteins cannot be excluded.

In the following we were interested in the interaction of one specific TAR RNA-binding protein with the PrP RNA, the p68 (Marciniak *et al*, 1990). This protein was selected because its binding site, ie the TAR RNA loop containing the CUGGG motif, is that element within the TAR RNA sequence, which is most similar to the PrP RNA stem-loop structure. p68 was partially purified from HeLa nuclear extract by chromatography on DEAE-Servacel (Serva) and heparin-Sepharose essentially as described (Marciniak *et al*, 1990). As shown in Figure 3, the p68 preparation yielded one major UV cross-linked product of 68 kDa when analyzed with either PrP RNA (lane a) or wild-type TAR RNA transcript (lane b).

Binding of p68 and other nuclear proteins to PrP RNA and TAR RNA was also demonstrated in Northwestern assays. As shown in Figure 4, HeLa nuclear proteins and partially purified p68, separated on a polyacrylamide gel and then immobilized to Immobilon membrane, were recognized by both the ^{32}P-labeled PrP RNA (lanes a and b) and the TAR RNA (lanes c and d). However, the patterns of RNA-binding proteins in Northwestern blots displayed only partial similarity to those found in UV cross-linking/RNase protection assays. The approximate molecular masses of the major PrP RNA- and TAR RNA-binding polypeptides in Figure 4 were 32, 35, 42, 52, 68, 89, and ~150 kDa. Possible reasons for the observed differences in the RNA-binding protein patterns might be changes in the migration of the proteins caused by the covalently bound RNA (UV cross-linking/RNase protection assay) or inability of the proteins to bind to the RNA in the absence of distinct factors which are required for complex formation (Northwestern blot). Control experiments using ^{32}P-labeled c-*myc* RNA (lane e) yielded a RNA-binding polypeptide pattern which was different from that obtained with PrP and TAR RNA, showing that the binding was specific. No binding of the c-*myc* RNA probe to p68 was found (lane f).

Competition experiments with PrP RNA, TAR RNA, and TAR RNA mutants were performed in order to determine the specificity of binding of p68 to PrP RNA (Figure 5). As summarized in Table 1, PrP RNA competed almost as efficiently as wild-type TAR RNA. Both RNAs are more effective com-

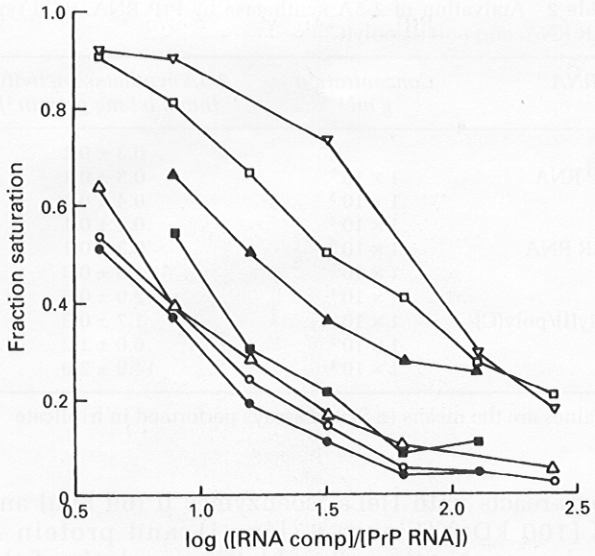

Figure 5 Competition analysis of specificity of binding of p68 to PrP RNA. Partially purified p68 (3 µg protein) was added to reaction mixtures containing a constant amount of ^{32}P-labeled PrP RNA (specific activity 2900 Ci mmol^{-1}, 8 pmol) and increasing amounts of competitor RNA. RNA-protein complexes formed were analyzed by UV cross-linking/RNase protection assay. Each assay (25 µl) contained 20 µg ml heparin^{-1}. Fraction saturation is defined as described (Marciniak *et al*, 1990). ○, PrP; ●, wild-type TAR; □, TARΔSHb; ■, TARExc; △, TARΔBS; ▲, TARIns; ▽, poly(I)/poly(C). Concentration ratios between competitor RNAs and PrP RNA are the molar concentration ratios except for poly(I)/poly(C) as competitor where the base ratio to the PrP RNA was determined.

petitors than TARΔSHb, TARExc and TARIns RNA. The TARΔSHb RNA which contains a 3' deletion of the TAR stem is expected to form a TAR unrelated secondary structure with a predicted free energy of folding of –51.0 kJ mol^{-1}, compared to –91.6 kJ mol^{-1} for the wild-type TAR sequence (RNAFOLD programme; PC/Gene, 1991). This mutant competed no better than poly(I)/poly(C) (Pharmacia) for p68 binding. In the TARExc RNA six bases of TAR sequence are deleted comprising the upper part of the stem and the first two 5' terminal bases of the loop and are substituted by a TAR-unrelated sequence; this RNA has a predicted free energy of folding of –89.1 kJ mol^{-1} TARIns RNA contains an insertion in the single-stranded region of the TAR loop and has approximately the same predicted free energy of folding (–90.4 kJ mol^{-1}) as the wild-type TAR RNA. The RARIns RNA competed less effectively than wild-type TAR RNA but was a more efficient competitor than poly(I)/poly(C). On the other hand, the TARΔBS RNA (predicted free energy of folding, –79.5 kJ mol^{-1}) exhibited a high efficiency to compete with PrP RNA for p68 binding even though five bases of the upper part of the TAR stem and five bases of the TAR loop are deleted. However, the predicted secondary structure of this TAR mutant RNA contains a C residue and a G

Table 1 Competition of TAR RNA and TAR RNA mutants with binding of PrP RNA to p68

	Relative competition efficiency[a]
PrP	1.00
Wild-type TAR`	1.14
TARΔSHb	0.15
TARExc	0.54
TARΔBS	0.81
TARIns	0.30
Poly(I)/poly(C)	0.07

[a] The relative competition efficiency (ie the ratio of the dissociation binding constant for the PrP RNA to the dissociation binding constant for the competitor RNA) was determined by the competition curves shown in Figure 5

residue at the second and the fourth position of the loop, respectively, like PrP RNA. It has been shown that point mutations in the TAR loop at C30 (eg C30-G) are highly detrimental to *trans*-activation (Jones and Peterlin, 1994). This nucleotide is not present in the loop of the predicted secondary structure of the TARExc RNA, which displays a low binding efficiency. Insertion of an additional AGCUU sequence between C30 and U31 in the CUGGG motif of TAR RNA, resulting in a large loop was even more detrimental for p68 binding, as shown for TARIns RNA. These results indicate that binding of p68 to PrP RNA occurs sequence-specifically, like binding to TAR RNA.

The same conclusions can be drawn from the results of direct RNA-protein cross-linking assays. As shown in Figure 3, the truncated TAR RNA, TARΔSHb RNA (lane c), and the TARExc RNA (lane d) labeled the 68-kDa protein at a significantly lower intensity compared to PrP RNA (lane a) and wild-type TAR RNA (lane b). On the other hand, the TARΔBS RNA efficiently labeled p68 (lane e). The TARIns RNA gave only a very weak signal (lane f).

The functional consequences of binding of p68 to PrP mRNA stem loop are not yet known. This protein has been proposed to stabilize Tat binding to TAR *in vivo* (Marciniak *et al*, 1990). Tat is able to bind also to PrP RNA (Müller *et al*, 1992). Recently, a cellular 38-kDa protein has been identified that competes with Tat binding to TAR (Baker *et al*, 1994). It would be interesting to know whether this protein which specifically interacts with the same motif that is recognised by Tat (the bulge and upper part of the stem) is modulated by p68. Preliminary results indicate that the amount of p68 increases after UV treatment (unpublished result).

In a previous study we demonstrated that the TAR RNA sequence of HIV-1 mRNA is able to bind and activate the 2',5'-oligoadenylate (2-5A) synthetase (Schröder *et al*, 1990). This enzyme is involved in the host cell defense of virus infection (Hovanessian, 1991; Schröder *et al*, 1992). The 2-5A produced by the synthetase is known to act as an allosteric activator of RNase L, which cleaves viral

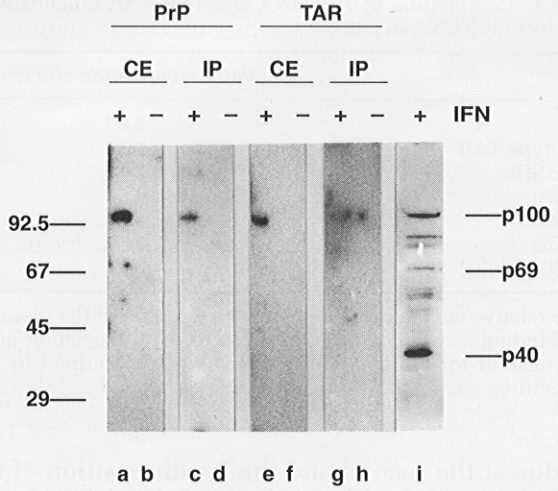

Figure 6 Binding of *in vitro* transcribed PrP and TAR RNA stem-loop sequence to IFN-induced 100-kDa 2-5A synthetase in Northwestern blots. Cytosolic extracts (CE) (30 μg protein assay^{-1}) were prepared from HeLa cells treated with (lanes a and e) or without IFN (lanes b and f) and analyzed by SDS-PAGE and Northwestern blotting procedure using ^{32}P-labeled PrP RNA (lanes a and b) or TAR RNA (lanes e and f) as a probe. In a further experiment cytosolic proteins from IFN-treated (lanes c and g) and untreated HeLa cells (lanes d and h) were subjected to immunoprecipitation (IP) using polyclonal anti 2-5A synthetase I antibody and protein A Sepharose. The immunoprecipitated material was subsequently separated by SDS-PAGE and analyzed for binding of ^{32}P-labeled PrP RNA (lanes c and d) or TAR RNA (lanes g and h) as above. Shown are the autoradiograms. Lane i, Western-blot detection of 2-5A synthetases present in extracts from IFN-treated HeLa cells. *p40, p69,* and *p100,* positions of migration of the 40-kDa, 69-kDa, and 100-kDa forms of 2-5A synthetase. Molecular masses are given as $M_r \times 10^{-3}$ on the left.

RNA (Schröder *et al*, 1989). Three isoenzymes of 2-5A synthetase (40-46, 69, and 100 kDa) have been described (Chebath *et al*, 1987; Müller and Schröder, 1994).

For preparation of 2-5A synthetase enriched cell extracts, HeLa S_3 semiconfluent monolayer cultures maintained in Dulbecco's modified Eagle's medium supplemented with 5% bovine serum were treated with 300 IU/ml of human recombinant interferon (IFN) α2a (Hoffmann-La Roche) for 20 h. Cell extracts were prepared as described (Ushijima *et al*, 1993).

Northwestern blotting experiments revealed no appreciable binding of proteins to *in vitro* synthesized PrP RNA or TAR RNA when cytosolic extracts from HeLa cells not treated with IFN were tested (Figure 6, lanes b and f), in contrast to nuclear extracts from the same cells (see Figure 4). However, treatment of the cells with IFN resulted in the appearance of one major PrP RNA-binding and TAR RNA-binding polypeptide band of 100 kDa (Figure 6, lanes a and e). The 100-kDa polypeptide could be immunoprecipitated using an antibody to 2-5A synthetase I (40 kDa; Kelve *et al*, 1994) that cross-reacts with HeLa isoenzymes II (69 kDa) and III (100 kDa) (Figure 6, lane i), and protein A Sepharose. Northwestern blotting analysis of the immunoprecipitated material again revealed the presence of the 100-kDa band, comigrating with 2-5A synthetase III in IFN-treated HeLa cell extract (lanes c and g). Addition of the antibody to cytosolic protein from untreated HeLa cells did not result in immunoprecipitation of any PrP RNA-binding or TAR RNA-binding protein (lanes d and h).

Table 2 Activation of 2-5A synthetase by PrP RNA, wild-type TAR RNA, and poly(I)/poly(C)

dsRNA	*Concentration g ml^{-1}*	*2-5A synthetase activity[a] (nmol h^{-1} mg protein^{-1})*
—		0.3 ± 0.1
PrP RNA	1×10^{-6}	0.3 ± 0.1
	1×10^{-5}	0.4 ± 0.1
	1×10^{-4}	0.4 ± 0.1
TAR RNA	1×10^{-6}	0.3 ± 0.1
	1×10^{-5}	1.0 ± 0.3
	1×10^{-4}	2.9 ± 0.8
Poly(I)/poly(C)	1×10^{-6}	1.7 ± 0.3
	1×10^{-5}	6.0 ± 1.2
	1×10^{-4}	14.9 ± 2.0

[a] Values are the means (± SD) of assays performed in triplicate

Next we determined whether PrP RNA is able to activate the 2-5A synthetase, like HIV-1 TAR RNA (Schröder *et al*, 1990). The 2-5A synthetase activity was determined in IFN-treated HeLa cell extracts in the absence or presence of exogenous RNA as described (Ushijima *et al*, 1993). As shown in Table 2 the enzyme was activated by poly(I)/poly(C) and TAR RNA in the range 1×10^{-6} to 1×10^{-4} g ml^{-1}. TAR RNA reached, at a concentration of 1×10^{-4} g ml^{-1}, about 20% of the efficiency of poly(I)/poly(C). By contrast, no significant stimulation of enzyme activity was observed with PrP RNA compared to control (Table 2). From this result we conclude that PrP RNA stem-loop is able to bind but not to activate 2-5A synthetase.

It should be noted that binding but not activation by certain double-stranded RNAs has been reported also for the second enzyme involved in the double-stranded RNA-dependent intracellular immunity system, the p68 kinase (Mellits *et al*, 1990). This enzyme is not identical with the p68 investigated in the present study (Marciniak *et al*, 1990). The latter protein was also not recognized by 2-5A synthetase antibodies (results not shown). The finding that PrP RNA stem-loop is unable to activate 2-5A synthetase might be important also in view of the fact that there are no hints that PrP^{Sc} infection is associated with an activation of the immune system.

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