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The Prion Protein Has RNA Binding and Chaperoning Properties Characteristic of Nucleocapsid Protein NCp7 of HIV-1*

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Transmissible spongiform encephalopathies are fatal neurodegenerative diseases associated with the accumulation of a protease-resistant form of the prion protein (PrP). Although PrP is conserved in vertebrates, its function remains to be identified. *In vitro* PrP binds large nucleic acids causing the formation of nucleoprotein complexes resembling human immunodeficiency virus type 1 (HIV-1) nucleocapsid-RNA complexes and *in vivo* MuLV replication accelerates the scrapie infectious process, suggesting possible interactions between retroviruses and PrP. Retroviruses, including HIV-1 encode a major nucleic acid binding protein (NC protein) found within the virus where 2000 NC protein molecules coat the dimeric genome. NC is required in virus assembly and infection to chaperone RNA dimerization and packaging and in proviral DNA synthesis by reverse transcriptase (RT). In HIV-1, 5'-leader RNA/NC interactions appear to control these viral processes. This prompted us to compare and contrast the interactions of human and ovine PrP and HIV-1 NCp7 with HIV-1 5'-leader RNA. Results show that PrP has properties characteristic of NCp7 with respect to viral RNA dimerization and proviral DNA synthesis by RT. The NC-like properties of huPrP map to the N-terminal region of huPrP. Interestingly, PrP localizes in the membrane and cytoplasm of PrP-expressing cells. These findings suggest that PrP is a multifunctional protein possibly participating in nucleic acid metabolism.

Transmissible spongiform encephalopathies are fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease, kuru, fatal familial insomnia, and Gerstmann-Sträussler-Scheinker (GSS)¹ syndrome in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle (1). A

major characteristic of transmissible spongiform encephalopathy diseases is the accumulation of an abnormal partially protease-resistant prion protein (PrP^{SC}), which is derived from the normal protease-sensitive prion protein (PrP^C). Although accumulation of PrP^{SC} may damage cells of the central nervous system, it is clear that the generation of spongiform encephalopathy requires the presence of both PrP^{SC} and PrP^C (2). The abnormal PrP^{SC} is thought to recruit the normal PrP^C through a proposed trans-conformation process causing the accumulation of PrP^{SC} in the form of plaques and fibrils (3–5).

The cellular prion protein (PrP) is encoded by a single gene corresponding to about 250 amino acids and is highly conserved in vertebrates (4–6). PrP is widely expressed, but it appears to accumulate in the central nervous system and the lymphoreticular system. PrP may be produced via several pathways, because it can be found in the extracellular fluid, on the outer surface of the plasma membrane and Golgi, and as a transmembrane form, designated ctmPrP, where the N-terminal domain is oriented within the cytoplasm (7, 8). In addition, a truncated version of PrP, resulting from a stop codon at position 145 in human PrP (huPrP), is associated with a variant of the GSS syndrome and is found in the cytoplasm (9).

Although PrP is a highly conserved protein in vertebrates, its role remains to be identified. Interestingly, PrP null mice develop normally and appear to be healthy (2). Nonetheless, a number of functions have been proposed for PrP such as superoxide dismutase activity, involvement in copper metabolism (reviewed in Ref. 10), and, very recently, participation in signal transduction during neuronal differentiation (11). In addition, PrP was shown to interact with sulfated glycans (12), RNA aptamers (13), and large nucleic acids (14, 15), causing the formation of nucleoprotein complexes similar to HIV-1 nucleocapsid-RNA complexes formed *in vitro* (16). A recent report shows that MuLV replication accelerates the scrapie infectious process (17), suggesting possible *in vivo* interactions between retroviruses and PrP.

These findings prompted us to compare and contrast the interactions of human PrP and the HIV-1 Gag-encoded nucleocapsid protein with viral nucleic acids. HIV-1, like all retroviruses examined to date (except for the Spumaretroviruses), encodes a major nucleic acid binding protein (NC protein) found in the virion core where about 2000 NC protein molecules completely coat the dimeric RNA genome. The biological roles of NC include the chaperoning of RNA dimerization and packaging during virus assembly and proviral DNA synthesis by

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¹ The abbreviations used are: GSS, Gerstmann-Sträussler-Scheinker syndrome; PrP, prion protein; PrP^{SC}, abnormal partially protease-resistant PrP; PrP^C, normal protease-sensitive PrP; ovPrP, ovine PrP; huPrP, human PrP; HIV-1, human immunodeficiency virus type 1; MuLV, murine leukemia virus; NC protein, nucleic acid binding protein; RT, reverse transcriptase; nt, nucleotide(s); PBS, phosphate-buffered saline; DIS, dimer initiation sequence; DLS, dimer linkage ele-

ment; ss-cDNA, strong stop cDNA; TAR, trans-activation response element; CT, control.

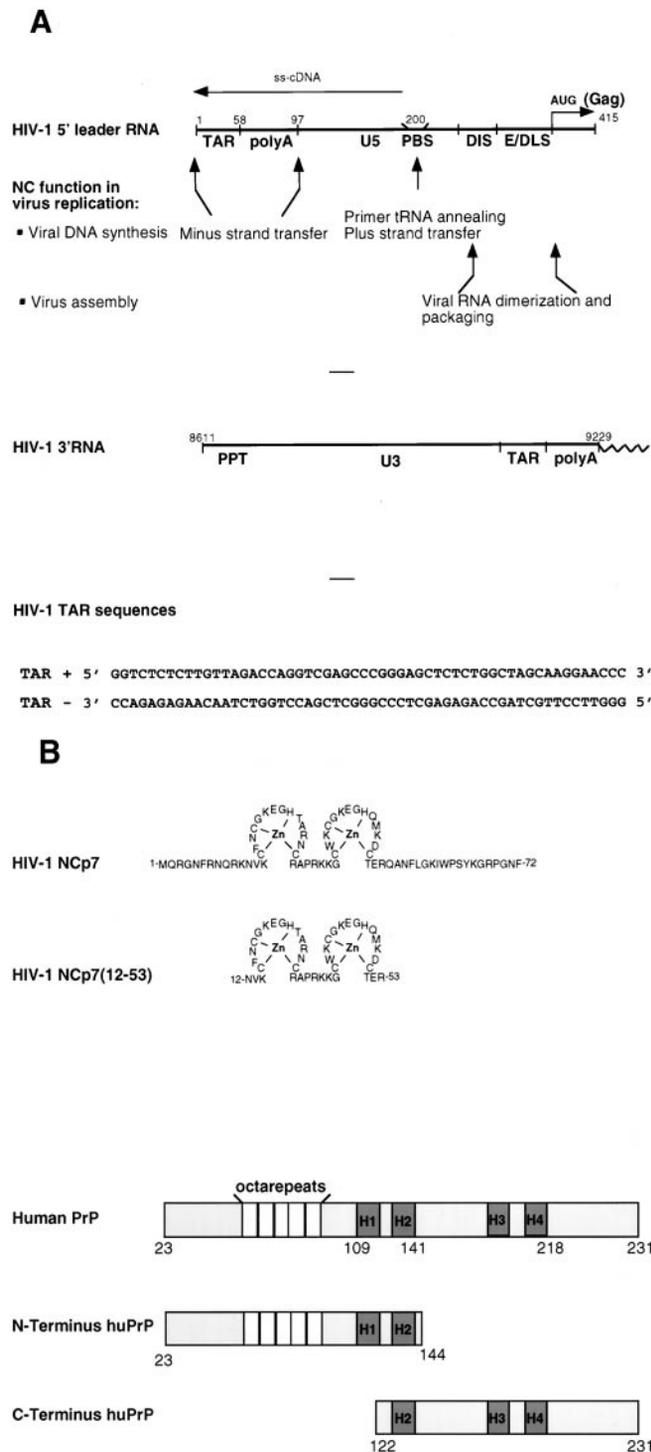


FIG. 1. HIV-1 RNA sequences and functions of NCP7 in virus replication. A, the 5'- and 3'-regions of the HIV-1 genomic RNA and the TAR oligonucleotides are shown. The 5'-leader RNA is made up of several functional domains: TAR and poly(A) stem-loop structures are required for provirus transcription and during reverse transcription for minus strand DNA transfer. Note that the TAR and poly(A) domains form R (Repeat). U5 is the untranslated 5'-sequence. PBS is the primer tRNA^{Lys} binding site and is 18-nt long. PBS is also required for plus strand DNA transfer. The DIS (dimer initiation sequence) and E/DLS (packaging/dimer linkage element) are necessary for viral RNA dimerization and packaging in assembling particles. AUG is the initiator codon for Gag. The 3'-RNA is formed of the PPT (polypurine track), which is the start site for plus strand DNA synthesis, U3 (3'-untranslated sequences), the TAR and poly(A) stem-loop structures, and an tail (broken line). The TAR and poly(A) are required for minus strand transfer during proviral DNA synthesis. Nucleotide positions are indicated above the lines representing the 5'- and 3'-RNAs. B, HIV-1 NCP7 and mutant NCP7(12-53) with the two CCHC zinc fingers (Zn) are

reverse transcriptase (RT) in the course of viral infection. These functions of NC are thought to be largely governed by interactions with the HIV-1 5'-leader RNA (16, 18–20).

Our results show that huPrP largely mimics the chaperone properties of NCP7 with respect to the annealing of complementary nucleic acid strands, viral RNA dimerization, the hybridization of replication primer tRNA^{Lys} to the HIV-1 5'-primer binding site sequence and the initiation of reverse transcription by RT. These NC-like activities of huPrP appear to map to the N-terminal region of huPrP. Such properties are not unique to huPrP, because the ovine PrP was also found to harbor nucleic acid-chaperoning properties similar to those of HIV-1 NCP7 and retroviral nucleocapsid proteins (reviewed in Ref. 16).

EXPERIMENTAL PROCEDURES

Recombinant Proteins, Enzymes, RNA, and Plasmid DNA—The full-length human PrP (huPrP), and the fragments huPrP-(23–144) (N-terminal region) and huPrP-(122–231) (C-terminal region) were produced in *Escherichia coli* and purified as described previously (21). The ovine PrP (ovPrP) (residues 25–234) was prepared using a similar protocol (22). The purity of the proteins was higher than 95% as judged by polyacrylamide gel electrophoresis. HIV-1 nucleocapsid protein NCP7, NCP7-(12–53), and Vpr were synthesized by the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)/opfp chemical method and purified by high pressure liquid chromatography (23). Proteins were dissolved at 1 mg/ml in buffer containing 30 mM Hepes, pH 6.5, 30 mM NaCl, and 0.1 mM ZnCl₂. DNA oligonucleotide corresponding to the Tar(–) 57 nt of HIV-1 sequences (pNL4.3 molecular clone) was from Eurogentec (Belgium). HIV-1 5'-leader RNA corresponding to nucleotides 1–415 was generated *in vitro* as previously described (18) and phenol and chloroform extracted and purified by gel filtration. HIV-1 3'-RNA of 650 nt (positions 8583–9208 of the HIV-1 genome) with a poly(A) tail was prepared by transcription *in vitro* (27).

Natural bovine tRNA^{Lys} from beef liver was a kind gift of Gérard Keith (Strasbourg, France). Synthetic tRNA^{Lys} was generated *in vitro* using T7 RNA polymerase. HIV-1 reverse transcriptase (RT p66/p51), purified from *E. coli*, was provided by S. Le Grice (Frederick, MD). HIV-1 integrase (INp32), purified from *E. coli*, was provided by J.-F. Mouscadet (Villejuif, France).

All plasmid DNAs were amplified in *E. coli* 1035 (RecA–) and purified by affinity chromatography (Qiagen protocol).

Nucleoprotein Complex Formation—Reactions with HIV-1 ³²P-labeled 5'-RNA, and either PrP or HIV-1 NCP7 were performed for 10 min at 37 °C in 10 μl of a buffer containing 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 0.2 mM MgCl₂, 5 mM dithiothreitol, 0.01 mM ZnCl₂, and 1.5 pmol of RNA. PrP or NCP7 was at the indicated molar protein to nucleotide ratios. Reactions were stopped by 5 mM EDTA, and samples were electrophoresed on a 1.3% agarose gel in 50 mM Tris-borate, pH 8.3. Gels were visualized by ethidium bromide staining followed by gel fixation in 5% trichloroacetic acid, drying, and autoradiography. To pellet nucleoprotein complexes, reaction mixtures were centrifuged at 4 °C for 5 min at 10,000 × *g*.

Nucleic Acid Annealing Assays—Reactions with HIV-1 RNA, ³²P-labeled tRNA, or ³²P-labeled minus strand Tar DNA (Tar–) and either PrP or HIV-1 NCP7 were performed for 10 min at 37 °C in 10 μl of a buffer containing 20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 0.2 mM MgCl₂, 5 mM dithiothreitol, 0.01 mM ZnCl₂, 5 units of RNasin (Promega), 1.5 pmol of RNA, 3 pmol of tRNA; or Tar(–) and PrP or NCP7 at the indicated molar protein to nt ratios. Reactions were stopped by SDS/EDTA (0.5%/5 mM), and samples were treated with proteinase K (2 μg) for 10 min at room temperature, phenol-chloroform was extracted, and RNA was analyzed by electrophoresis on 1.3% agarose in 50 mM Tris-borate, pH 8.3. Gels were visualized by ethidium bromide staining

shown. The complete huPrP as well as the N- and C-terminal regions are shown with the octarepeats and the H1 to H4 helices. Amino acid positions are indicated. Functions of NC in HIV-1 replication are mediated by nucleic acid interactions as indicated by arrows. In the course of viral DNA synthesis NC chaperones primer tRNA annealing to the primer binding site, initiation of reverse transcription, and completion of viral DNA synthesis by RT. TAR and poly(A) are required for NC to chaperone minus strand DNA transfer, and primer binding site for plus strand DNA transfer. Also NC chaperones viral RNA dimerization and packaging during virus assembly.

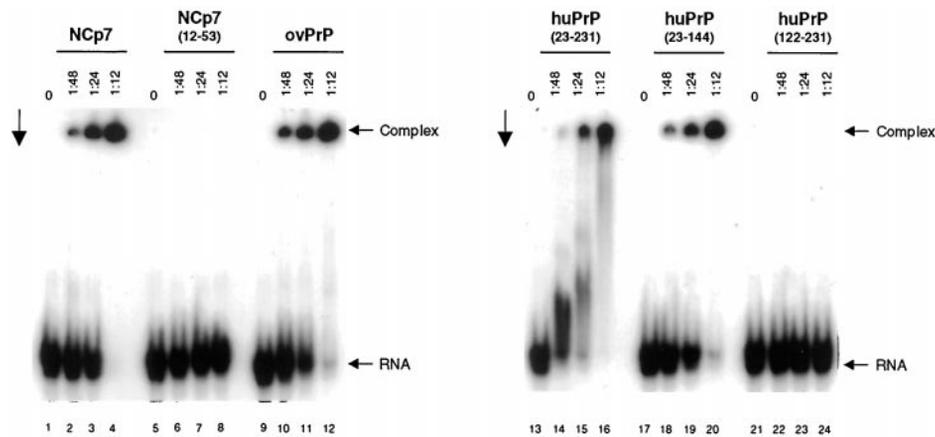


FIG. 2. **Formation of nucleoprotein complexes after binding of PrP to HIV-1 RNA.** 32 P-Labeled HIV-1 5'-leader RNA of 415 nt in length (described in Fig. 1) was incubated at 37 °C for 10 min with or without NCp7 or PrP. Formation of nucleoprotein complexes was analyzed by electrophoresis on a 3% polyacrylamide gel in 50 mM Tris-borate, pH 8.3. One picomole of RNA was used, and the molar ratios of protein to nt are indicated at the top. Lanes 1, 5, 9, 13, 17, and 21, CT without protein; lanes 2–4, NCp7; lanes 6–8, NCp7-(12–53); lanes 10–12, ovine PrP (*ovPrP*); lanes 14–16, human PrP (*huPrP*(23–231)); lanes 18–20, *huPrP*(23–144); lanes 22–24, *huPrP*(122–231). Vertical arrow is direction of electrophoresis. Horizontal arrows are free RNA and nucleoprotein complexes, respectively. Results shown indicate that binding of PrP or NCp7 to HIV-1 RNA is probably cooperative.

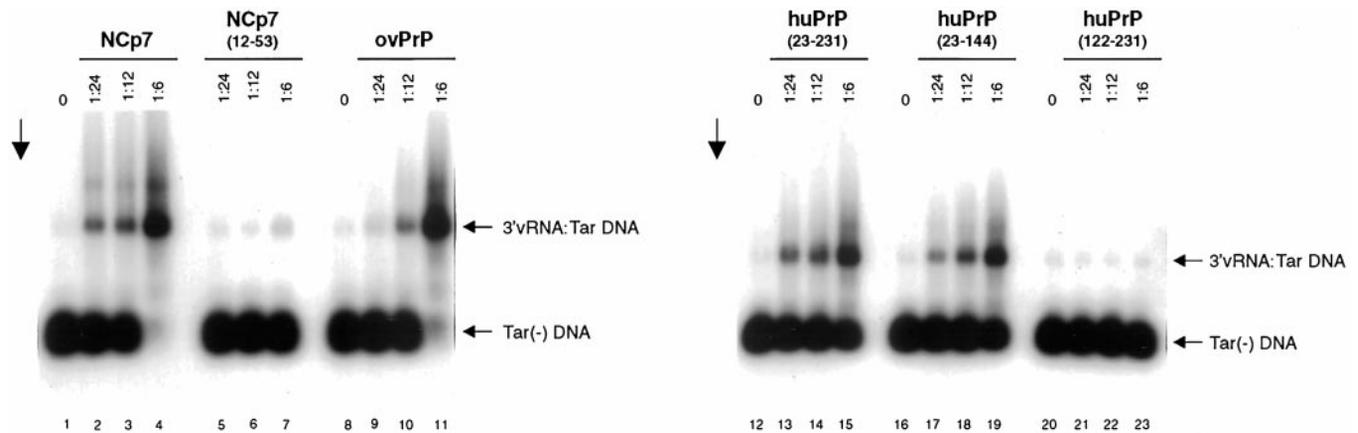


FIG. 3. **Nucleic acid annealing activity of PrP.** Assays were performed as indicated under "Experimental Procedures" with HIV-1 3'-RNA and 32 P-labeled Tar(-) DNA (Fig. 1). At the end of the reaction, protein was removed by proteinase K digestion, followed by phenol extraction. Analysis of 32 P-Tar(-) annealed to HIV-1 3'-RNA was by electrophoresis on 1.3% agarose gels. Gel was fixed with 5% trichloroacetic acid, dried, and autoradiographed. The molar ratios of protein to nt are indicated at the top. The vertical arrow shows direction of electrophoresis and horizontal arrows correspond to Tar(-) DNA (bottom) and HIV-1 3'-RNA:Tar(-) DNA (middle). Lanes 1, 8, 12, 16, 20, control incubation without NCp7 or PrP; lanes 2–4, NCp7; lanes 5–7, NCp7-(12–53); lanes 9–11, ovine PrP (*ovPrP*); lanes 13–15, *huPrP*(23–231); lanes 17–19, *huPrP*(23–144); lanes 21–23, *huPrP*(122–231).

followed by gel fixation in 5% trichloroacetic acid, drying, and autoradiography. A 0.16- to 1.77-kb RNA ladder was used for size determination. The percentage of primer annealed to the HIV-1 RNA was determined by densitometric scanning of the autoradiograph.

Reverse Transcription Assays—First the nucleic acid annealing assay was carried out in 10 μ l for 5 min at 37 °C as above, and next the reaction volume was increased to 25 μ l by addition of 2 pmol of HIV-1 RT, 0.25 mM each of dNTPs, 60 mM NaCl, and 2.5 mM MgCl₂. Samples were incubated for 20 min at 37 °C. The reactions were stopped and processed as for the analysis of nucleic acid annealing, except that after phenol extraction, nucleic acids were ethanol-precipitated, recovered by centrifugation, dissolved in formamide, denatured at 95 °C for 2 min, and analyzed on 8% polyacrylamide gel electrophoresis in 7 M urea and 0.5 \times TBE, pH 8.3. 32 P-Labeled FX174 DNA *Hinf* markers (Promega) were used for size determination (not shown). The levels of cDNA synthesized by RT were quantified by densitometric scanning.

Cell Culture, DNA Transfection, and Immunohistochemistry—293T cells, derived from the HEK cell line (human embryonic kidney cells), were cultured at 37 °C in an atmosphere of 5% CO₂ and in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) complemented with 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μ g/ml), and L-glutamine (2 mM). Transfection of 293T cells were performed using the calcium phosphate method with 3 μ g of pDNA-huPrP for 10⁶ cells. After 10 days under puromycin selection (0.7 μ g/ml), resistant cell colonies were expanded and found to express huPrP.

For immunohistochemistry, cells were grown in eight-well Labtek chamber slides and fixed in ice-cold 2% paraformaldehyde for 30 min at 4 °C. They were then washed twice in PBS and incubated for 30 min in a blocking solution of PBS containing 5% bovine serum albumin, 1% normal goat serum, and 0.2% Tween 20 prior to staining with antibodies. This same solution served to dilute the anti-PrP SAF 37 antibody (IgG2a recognizing amino acids 79–92 of huPrP) used at 1/200 dilution, and the rhodamine-conjugated *Helix pomatia* lectin, which was included with the primary antibody in some experiments at a concentration of 5 μ g/ml (kindly provided by Dr. F. Barde, ENS, Lyon, France). Primary incubation was for 2 h at room temperature. After washing sections 5 \times 10 min in PBS, bound antibodies were revealed with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin at a final dilution of 1/400 in blocking buffer containing bis-benzimide (1 μ g/ml) to stain DNA. Controls included no primary antibody and non-transfected cells. Slides were washed three times in PBS, mounted with moviol and analyzed with a Zeiss axioplan fluorescence microscope, and a Zeiss axiovert inverting microscope equipped with 488- and 514-nm lasers and LSM 510 software for confocal analysis.

RESULTS

The Human and Ovine PrP Bind HIV-1 RNA in Vitro—HIV-1 nucleocapsid protein NCp7 is a small Gag-encoded protein. During the infectious process NCp7 is required to chap-

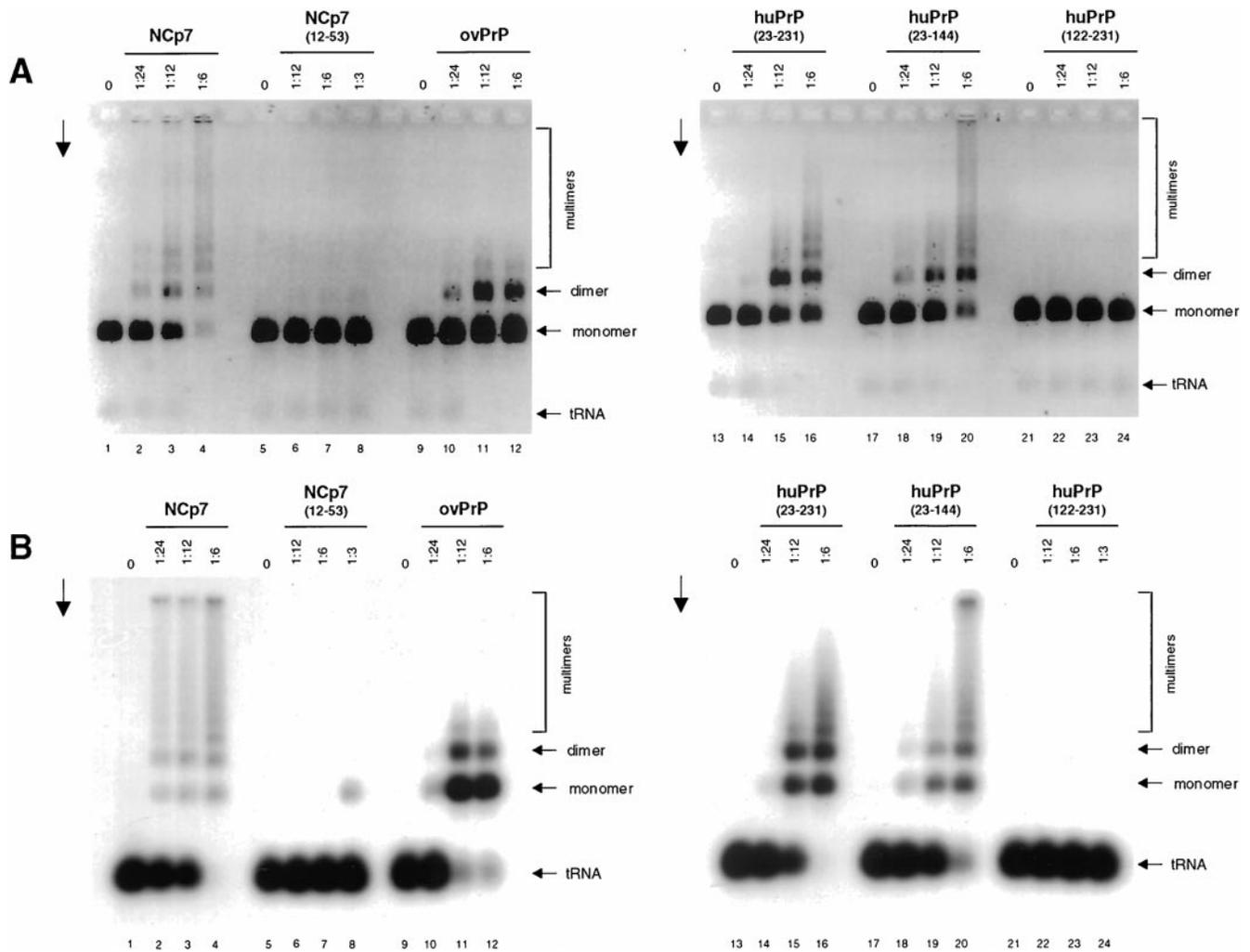


FIG. 4. PrP directs HIV-1 RNA dimerization and annealing of primer $tRNA_3^{Lys}$ to the primer binding site. *A*, ethidium bromide staining of the 1.3% agarose gel. *B*, autoradiography to show primer ^{32}P - $tRNA_3^{Lys}$ annealing to HIV-1 primer binding site. The HIV-1 5'-leader RNA and ^{32}P -labeled $tRNA_3^{Lys}$ were incubated at 37 °C with or without NCp7 or PrP and reactions were processed. The protein-to-RNA molar ratios are indicated at the top. The arrow shows direction of electrophoresis and markers (in nt) are indicated on the left. HIV-1 monomer, dimer and multimer RNAs, and $tRNA_3^{Lys}$ are indicated on the right. Lanes 1 and 7, CT without protein. Lanes 2–4, with NCp7; lanes 5 and 6, with NCp7-(12–53); lanes 8–10, with huPrP; lanes 11–13, with huPrP-(23–144); lanes 14 and 15, with huPrP-(122–231). Note that huPrP-(122–231) did not promote HIV RNA dimerization or tRNA annealing to the primer binding site (lanes 14 and 15), whereas huPrP and huPrP-(23–144) were very active.

erone proviral DNA synthesis by reverse transcriptase (RT) (Fig. 1A) (16, 18, 19). In addition, NCp7 is pivotal in virus assembly, because it recruits the genomic RNA and chaperones its dimerization and packaging into assembling particles (Fig. 1A). Interactions between the HIV-1 5'-leader RNA and NCp7 appear to govern these viral processes (Fig. 1A). NCp7 with the two zinc fingers and an NCp7 mutant, designated NCp7-(12–53), without the basic N- and C-terminal domains necessary for RNA dimerization *in vitro* and virus assembly *in vivo* (16, 24, 25), were generated by peptide synthesis (Fig. 1B and data not shown) (23). Recombinant huPrP-(23–231) and the N (23–145)- and C (122–231)-terminal regions were produced in *E. coli* and purified to homogeneity (21). To compare the nucleic acid binding abilities of PrP and HIV-1 NCp7, we used the multifunctional 5'-leader of HIV-1 (Fig. 1), because it is formed of contiguous functional domains that are required for viral DNA transcription (TAR and poly(A) stem-loops), minus strand DNA synthesis by RT (5'-primer binding site for initiation and Tar for strand transfer), viral RNA dimerization and packaging (DIS and DLS), and translation (the AUG of Gag) (Fig. 1). NCp7 interacts with the DIS-DLS domains to chaperone viral RNA dimerization and packaging during virus assembly and with the 5'-primer binding site and TAR sequences during minus strand

DNA synthesis by RT in the course of infection (16, 19).

As observed previously (18), NCp7 binds in a cooperative manner to the multifunctional 5'-leader of HIV-1 RNA and forms nucleoprotein complexes (Fig. 2, lanes 2–4), whereas the NCp7 mutant (12–53) does not form stable complexes (lanes 6–8). huPrP binds to the 5'-leader RNA as evidenced by the formation of nucleoprotein complexes (lanes 14–16). The N-terminal fragment (23–144) of huPrP was also able to bind to the 5'-leader RNA (lanes 18–20) in a manner similar to NCp7 (lanes 2–4), whereas the C-terminal fragment (122–231) did not show any RNA binding affinity (lanes 22–24). The ovine PrP was also expressed in *E. coli* as a recombinant protein and purified to homogeneity (not shown). The ovine PrP also formed nucleoprotein complexes in a dose-dependent manner similar to NCp7 (lanes 10–12). These NC- and PrP-nucleoprotein complexes are of high molecular mass, because they can be recovered by 5-min centrifugation at $10,000 \times g$ (data not shown) (26).

Human and Ovine PrP Have Nucleic Acid Chaperoning Properties—The interactions of PrP with nucleic acids suggested that this cellular protein might have RNA and DNA annealing activities similar to those of HIV-1 NCp7 or the human p53 protein (27). This was examined using HIV-1 sequences corresponding to TAR and the 3'-region of the HIV-1

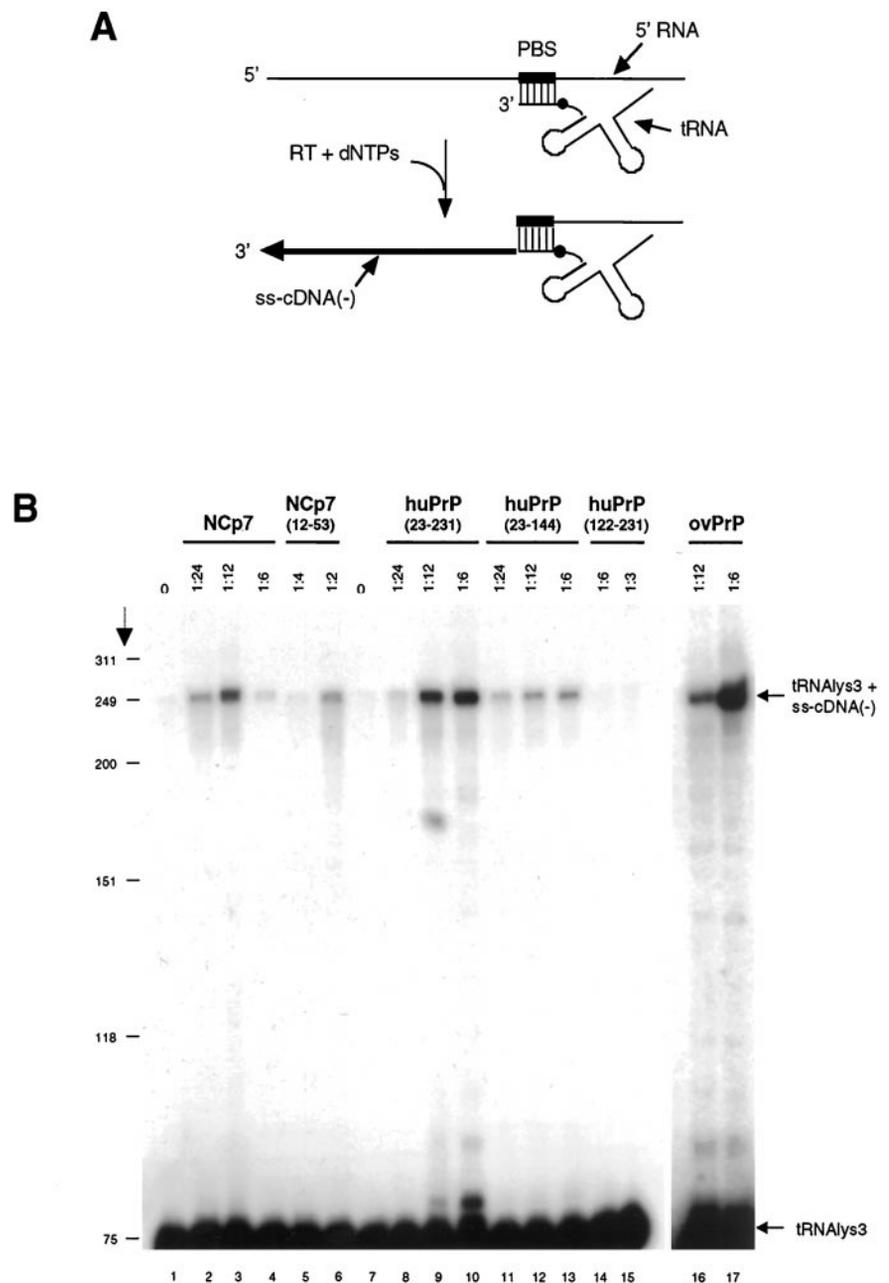


FIG. 5. PrP chaperones initiation of reverse transcription of HIV-1 RNA. A, a scheme to show reverse transcription initiation of HIV-1 RNA and synthesis of minus strand strong stop cDNA, ss-cDNA(-), where PBS stands for primer binding site. B, HIV-1 5'-RNA and ^{32}P -labeled tRNA $^{\text{Lys}}$ were incubated with or without NCp7 or PrP. HIV-1 RTp66-p51 was added together with the dNTPs to allow reverse transcription initiation. Reactions were processed, and ss-cDNA(-) was analyzed. The protein-to-nt molar ratios are indicated above the figure. The arrow shows the direction of electrophoresis, and markers (in nt) are indicated on the left. The ss-cDNA-tRNA and primer tRNA $^{\text{Lys}}$ are indicated on the right. Lanes 1 and 7, CT with RT but without NCp7 or huPrP. Lanes 2-4, with NCp7; lanes 5 and 6, with NCp7-(12-53); lanes 8-10, with huPrP; lanes 11-13, with huPrP-(23-144); lanes 14 and 15, with huPrP-(122-231); lanes 16 and 17, with ovine PrP (ovPrP). Note that even at a high molar ratio of huPrP-(122-231) ss-cDNA was not synthesized (lane 15).

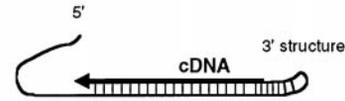
RNA genome corresponding to U3, TAR, and poly(A) sequences (Fig. 1A). The minus strand TAR sequence (Tar-) represents the 3'-end of the so-called strong stop cDNA (ss-cDNA(-)), the initial product of reverse transcription. Under physiological conditions, NCp7 directed annealing of Tar- to Tar+ present within the 3'-RNA (Fig. 3, lanes 2-4). Interestingly, huPrP was also able to hybridize Tar- to the 3'-RNA in a dose-dependent manner (lanes 13-15). A similar level of hybridization was obtained with huPrP-(23-144) (lanes 17-19). On the other hand, NCp7-(12-53) and huPrP-(122-231) did not direct annealing of Tar- to HIV-1 3'-RNA (lanes 5-7 and 21-23, respectively). The ovine PrP (ovPrP) was found to be as effective as huPrP and HIV-1 NCp7 in directing the specific annealing reaction (lanes 9-11). These data demonstrate that PrP of human or ovine origin has nucleic acid annealing activity characteristic of HIV-1 NCp7 and retroviral NC proteins in general (16, 18, 19, 27-30).

PrP Chaperones HIV-1 RNA Dimerization and Annealing of Primer tRNA to the Primer Binding Site—In the viral particle, the retroviral genome is dimeric and has replication primer

tRNA annealed to the primer binding site, which constitutes the start site for reverse transcription (see Fig. 1A). As demonstrated before, genomic RNA dimerization and tRNA annealing to the primer binding site are promoted by NC protein. In control experiments we used well characterized nucleic acid binding proteins of viral origin such as retroviral reverse transcriptases (RT), HIV-1 Vpr and integrase, T4 gp32 or nucleic acid binding proteins of cellular origin like *E. coli* RecA and human p53. All these proteins, some of which have nucleic acid chaperone properties (40), were found to be inactive in these viral processes (see Refs 18 and 23 for RTs and *E. coli* RecA; Ref. 27 for human p53 or RT). Using the HIV-1 system with the multifunctional 5'-leader RNA and primer tRNA $^{\text{Lys}}$ (Fig. 1B) NCp7 was indeed critical for HIV-1 RNA dimerization and primer tRNA $^{\text{Lys}}$ annealing to the primer binding site (Fig. 4, A and B, lanes 2-4) (18, 23). Interestingly, we found for the first time that a cellular protein, namely PrP, was capable of promoting HIV-1 RNA dimer formation and tRNA $^{\text{Lys}}$ annealing to the primer binding site in a dose-dependent manner (lanes 14-16 for huPrP). Very similar data were obtained with the N

A

(1) cDNA synthesis by self-priming



(2) Inhibition by NCp7 or PrP

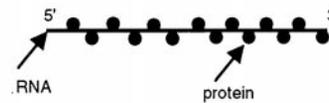
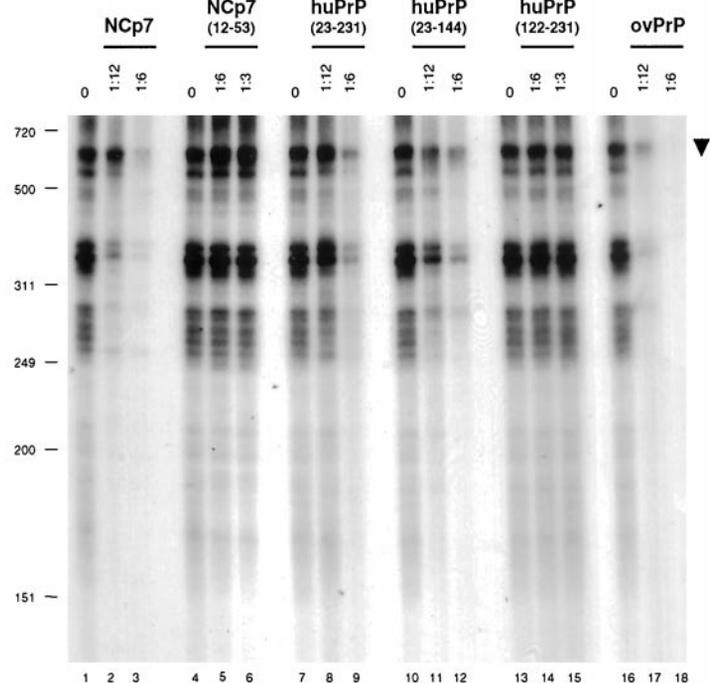


FIG. 6. PrP inhibits self-priming of reverse transcription. A, self-priming of reverse transcription is shown in Ref. 1. Coating of the free RNA by NCp7 or PrP, which results in the inhibition of self-priming is schematized in Ref. 2. B, self-priming of reverse transcription. Assays with HIV-1 3'-RNA were performed as described under "Experimental Procedures" except that [³²P]dCTP was used. The protein-to-nt molar ratios are indicated at the top. The vertical arrow on the right shows direction of electrophoresis and DNA markers (in nt) are indicated. Lanes 1, 4, 7, 10, 13, and 16 are CT without added protein; lanes 2 and 3, NCp7; lanes 5 and 6, NCp7-(12-53); lanes 8 and 9, huPrP-(23-231); lanes 11 and 12, huPrP-(23-144); lanes 14 and 15, huPrP-(122-231); lanes 17 and 18, ovine PrP (ovPrP).

B



terminus (23-144) of huPrP (lanes 18-20). NCp7-(12-53) retained little activity (lanes 6-8) (16) and the C-terminal fragment (122-231) of huPrP was completely inactive (lanes 22-24). The ovine PrP (ovPrP) was also found to show an NC-like activity, because it directed HIV-1 RNA dimerization and primer tRNA₃^{Lys} annealing to the primer binding site (lanes 10-12).

Reverse transcription of the viral RNA is initiated by RT-mediated extension of the primer tRNA hybridized to the primer binding site by NCp7 (Figs. 1A and 5A). NCp7 was indeed critical for the initiation of minus strand cDNA synthesis (Fig. 5B, compare lanes 1 and 2-4) (1-3, 16). Once again, huPrP was found to duplicate the properties of NCp7 by actively promoting initiation of reverse transcription in a dose-dependent fashion (Fig. 5B, lanes 8-10). The N-terminal fragment (23-144) of huPrP exhibited part of this activity (lanes 11-13). NCp7-(12-53) retained some activity, but only at a high protein to nt ratio (lanes 5 and 6, protein to nt molar ratio of 1:3) (16). The C-terminal fragment (122-231) of huPrP was completely inactive (lanes 14 and 15). Similarly to huPrP, the full-length ovine PrP was also very active in chaperoning the initiation of reverse transcription (lanes 16 and 17).

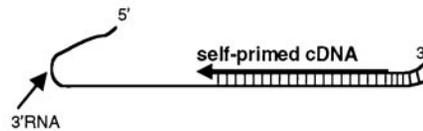
PrP Promotes Specific Proviral DNA Synthesis—A succes-

sion of specific events is required for the conversion of the genomic RNA into a complete proviral DNA with the two LTRs. Nevertheless, nonspecific replicative events can take place, such as self-initiation of reverse transcription, which is enhanced by template interactions, nicks in the genome or folding back of the template (31-33). Retroviral NC proteins behave as nucleic acid chaperones, which can destabilize RNA secondary structures and thus inhibit self priming of reverse transcription (see scheme in Fig. 6A) (31-33).

A 650-nt RNA, with a 25-nt poly(A) tail, corresponding to the 3'-region of the HIV-1 genomic RNA was used to mimic resumption of minus strand cDNA synthesis after minus strand transfer (Fig. 1B) (31-34). However, a possibility exists that RT transcribes the genomic RNA by virtue of self-priming at the 3'-end and thus in the absence of minus strand transfer after ss-cDNA synthesis (Fig. 6A). As shown in Fig. 6B and in agreement with published data (31, 32) NCp7 strongly inhibited self-primed cDNA synthesis in a dose-dependent manner (lanes 2 and 3), whereas the zinc finger domain (NCp7-(12-53)) was completely inactive in this process (lanes 5 and 6). Interestingly, PrP of both human and ovine origins strongly inhibited self-priming of cDNA synthesis (lanes 8-9 and 17-18, respec-

A

(1) Free 3' RNA



(2) Addition of NCp7 or PrP : hybridization of Tar(-) and specific cDNA synthesis

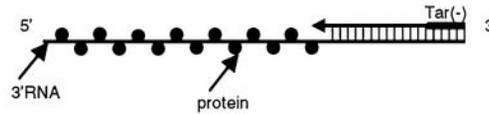
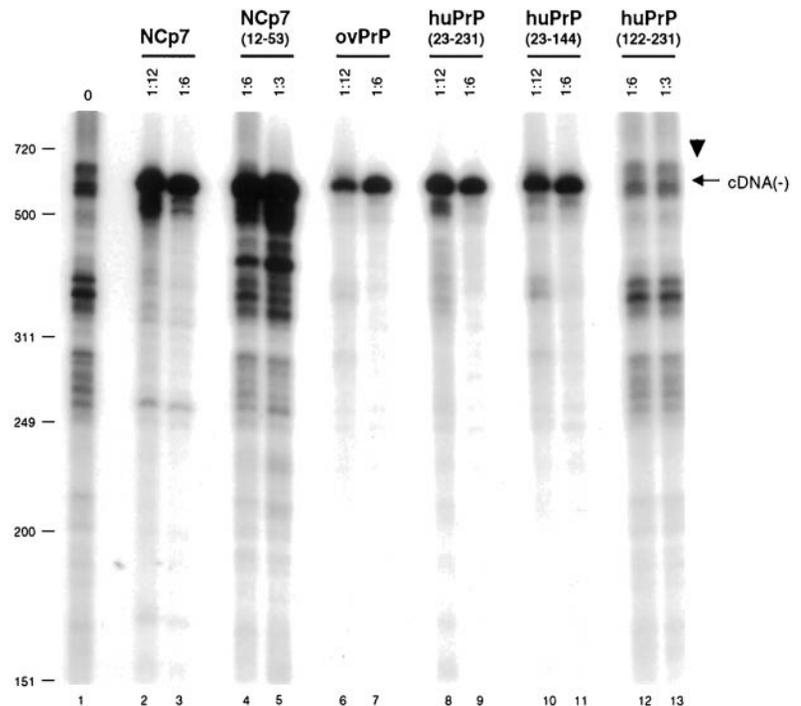


FIG. 7. PrP chaperones-specific cDNA synthesis by RT. A, schemes showing reverse transcription by self-priming (lane 1 in B) and primer-directed cDNA synthesis (lanes 3, 7, 9, and 11 in B). B, primer-directed reverse transcription of HIV-1 RNA. Assays were performed in the presence of HIV-1 3'-RNA and primer Tar(-) DNA (see Fig. 2) and in conditions described under "Experimental Procedures" except that [³²P]dCTP was used (31). The protein-to-nt molar ratios are indicated at the top. The vertical arrow shows the direction of electrophoresis and DNA markers (in nt) are indicated. The horizontal arrow shows the full-length cDNA. Lanes 1, CT without added protein; lanes 2 and 3, NCp7; lanes 4 and 5, NCp7-(12-53); lanes 6 and 7, ovine PrP; lanes 8 and 9, huPrP-(23-231); lanes 10 and 11, huPrP-(23-144); lanes 12 and 13, huPrP-(122-231).

B

tively), and this activity mapped to the N terminus (positions 23-144) and not the C terminus (positions 122-231) (lanes 11-12 and 14-15, respectively).

To examine the continuation of cDNA synthesis by reverse transcription of HIV-1 3'-RNA after minus strand transfer, the Tar- DNA representing the 3'-region of ss-cDNA(-) was included in the assays (Fig. 7A). Under these physiological conditions (see "Experimental Procedures") both NCp7 and PrP were able to hybridize Tar- to HIV-1 3'-RNA (Fig. 3). Extension of Tar- by reverse transcription of the 3'-RNA was clearly specific in the presence of HIV-1 NCp7 (Fig. 7B, compare lane 1 CT with lane 3 where the NCp7:nt ratio was 1:6; see also Fig. 6B) in agreement with published data (30, 31). PrP of either ovine or human origin was found to completely replace NCp7, because in both cases specific cDNA(-) synthesis was observed (Fig. 7B; compare CT lane 1, with lane 7 (ovPrP-to-nt ratio of 1:6) and lane 9 (huPrP-to-nt ratio of 1:6). In addition, the N terminus of huPrP was also functional in these assays (see lane 10 in which the huPrP-(23-144)-to-nt ratio was 1:6). Mutant NCp7-(12-53) started to be partially effective at a protein-to-nt molar ratio of 1:3 (lane 5). However, huPrP-(122-231) was

completely inactive in promoting specific cDNA synthesis (lanes 12 and 13).

Subcellular Localization of PrP—The above results clearly show that PrP has the ability not only to interact with nucleic acids (Fig. 2) but also to modify their conformation (Fig. 5) and chaperone the hybridization of nucleic acids with complementary sequences (Fig. 3) that are critical for HIV-1 replication (Figs. 3-6). PrP is thought to localize mainly at the cell surface of PrP-expressing cells (4, 6). This would render interactions between PrP and cellular or viral nucleic acids unlikely. However, PrP has also been reported to localize to the Golgi and the nucleus (8, 35). To examine the subcellular localization of PrP, we used the 293T cells derived from a human embryonic kidney cell line. Endogenous expression of PrP in these cells is below the threshold of detection by immunocytochemistry, and no staining was observed in control 293T cells (Fig. 8, A and B). This allowed us to be confident that staining of transfected PrP in 293T cells was selective, giving an accurate picture of its subcellular distribution other than being nonspecific reactivity against other cellular proteins. 293T cells were transfected with a huPrP plasmid construct, and a 293T cell population

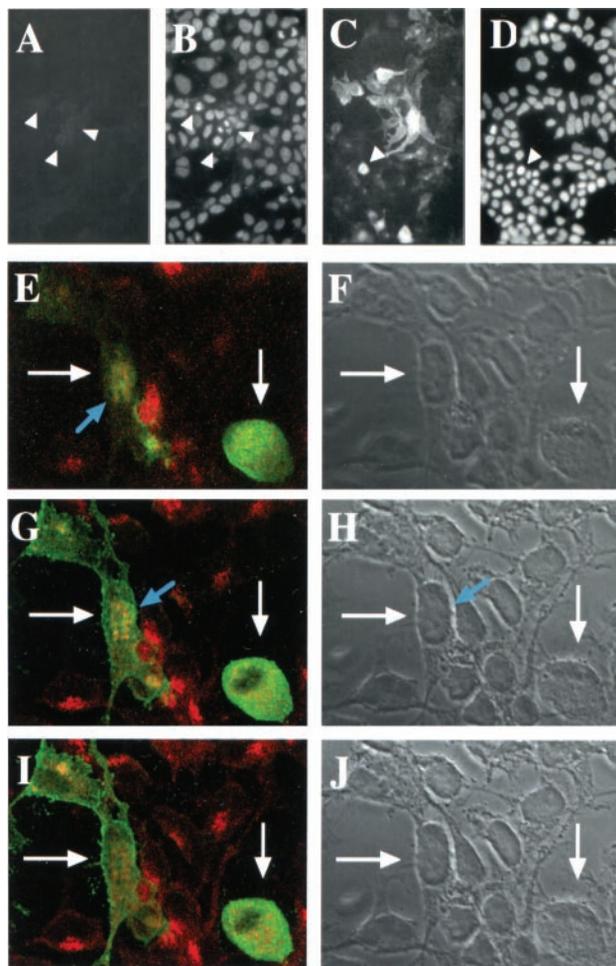


FIG. 8. Localization of PrP in 293T cells. 293T cells expressing human PrP^C (C–J) were analyzed by immunocytochemistry for the expression of PrP. In control 293T cells (A and B) no staining was observed and background was very low. In 293T cells stably expressing huPrP, we observed strong staining of PrP in the membrane and cell body (see arrowheads in C and D). Cytoplasmic staining was intense throughout the cytoplasm of cells determined to be in metaphase by DNA staining with bis-benzimide (B and D). This was not simply a cell cycle-related artifact, because no staining occurred in metaphase cells that were not transfected with the huPrP plasmid (compare cells indicated by arrowheads in A and B with those in C and D). Confocal microscopy was performed to analyze the subcellular distribution of PrP in stained cells (E–J). The same cells are shown at three different depths in the Z axis (going progressively deeper from the cell membrane to the nucleus). Green fluorescence shows anti-PrP staining, and red fluorescence shows binding of the *H. pomatia* lectin to show the Golgi (E, G, and I), close co-localization appears as yellow labeling. Phase contrast in F, G, and H corresponds to the immunofluorescent staining in E, G, and I, respectively. The vertical downward pointing arrow indicates a cell that was established to be in metaphase by DNA labeling (not shown). Note the round morphology and absence of a defined nuclear compartment under phase contrast. The cell indicated by the horizontal arrow was apparently not in metaphase, because it contains a well defined nucleus and typical fusiform morphology with a short process. In cells that were not undergoing mitosis, huPrP was localized primarily in the membrane and also in a cellular compartment depicted by the Golgi label (red fluorescence). Co-localization, indicated by yellow staining was not perfect, and often patches and points of green anti-PrP staining occurred in the midst of a patch of red Golgi staining (blue arrows in E and G). In the metaphase cell the Golgi stain was dispersed quite evenly throughout the majority of the cell body, although it was excluded from the region occupied by the chromatin (unstained region of the metaphase cell in G and I). PrP staining was also diffuse and cytoplasmic; however, intense points and patches of labeling occurred in the cytoplasm with a distribution distinct from that of the more evenly distributed Golgi label. Occasionally, PrP staining appeared to occur in the nucleus, as indicated by the blue arrows in G and H, however, the Golgi label also stained and co-localized in the same region suggesting that the apparent nuclear labeling may well be in a distinct overlying compartment.

constitutively expressing PrP was derived upon puromycin selection. Both 293T and 293T-huPrP cells were examined by immunocytochemistry using an antibody recognizing amino acids 79–92 of huPrP (SAF 37, J. Grassi, Commissariat à l’Energie Atomique Saclay, France). As reported in Fig. 8, intense punctate staining of huPrP occurred in the plasma membrane of many cells, as was expected, but also in a cytoplasmic compartment and more rarely in what appeared to be the nucleus (Fig. 8, C and D). In cells undergoing mitosis, as determined by the organization of chromatin revealed by DNA staining, a quite different pattern of expression was seen. In such cells there was an intense staining throughout the cytoplasm. This was clearly not an artifact, because metaphase control cells showed no staining (compare A with C). Confocal analysis with double labeling of PrP and a Golgi marker (rhodamine-conjugated *H. pomatia* lectin) showed that, in cells not undergoing mitosis, PrP was selectively localized in the plasma membrane and an intracellular compartment, including the Golgi, although cytoplasmic PrP staining did not co-localize very well with the Golgi marker (Fig. 8, E, G, and I, from the plasma membrane to the nucleus). When nuclear staining of PrP appeared to occur, the Golgi marker was also present, indicating that in such cells the Golgi compartment was superimposed on the nucleus. In cells undergoing mitosis, the cytoplasmic localization of PrP was confirmed. Indeed the Golgi is dispersed during mitosis, and it was apparent that PrP was not so uniformly localized as the Golgi marker, indicating that PrP is probably present in both the Golgi and a distinct cytoplasmic compartment (vertical arrows in E–J). In conclusion, these data strongly suggest that huPrP can accumulate in several cell compartments where it might interact with nucleic acids.

DISCUSSION

The findings that PrP of human or ovine origin binds RNA confirm and extend previous data showing that the human prion protein exhibits nucleic acid binding properties (13–15). Furthermore, we have found that binding of PrP to HIV-1 RNA causes the formation of condensed nucleoprotein structures similar to those obtained with retroviral NC proteins. Interestingly, the interactions between PrP and the viral RNA in these nucleoprotein structures appear to share many of the specific functional characteristics of HIV-1 NCp7 in the viral context (see below) (16, 20). PrP differs from other nucleic acid binding proteins and nucleic acid chaperone proteins, because it mimics the functions of NCp7 in HIV-1 replication such as HIV-1 RNA dimerization and primer tRNA₃^{lys} annealing to the 5′-primer binding site during virus assembly and the chaperoning of proviral DNA synthesis by RT during virus infection (see Figs. 1, 4, and 5) (reviewed in Ref. 16). The latter properties are not shared by nucleic acid binding proteins such as HIV-1 Vpr and RT, or human p53 (see Figs. 5–7 and Refs. 18, 26, 27, 32, 33, and data not shown). Many cellular proteins with nucleic acid chaperoning properties are essential to nucleic acid maintenance and metabolism (36), such as DNA repair for p53 (37), pre-mRNA processing for hnRNP proteins (38), and telomere elongation for hnRNP A1 (39). This suggests that PrP may exert one of its functions at the level of nucleic acid metabolism (36, 40).

The length and precise location of the functional domain responsible for the nucleic acid binding and chaperoning activities of PrP is at present unknown. However, our data clearly show that this domain is localized within the N-terminal region encompassing residues 23–144 (Figs. 3–7) (41) analogous to a mutant PrP associated with a variant of the GSS syndrome (9).

The capacity of PrP to interact with nucleic acids (RNA, DNA, and oligonucleotides) and to modify their conformation and degree of compactness as well as to chaperone nucleic acids interactions in a manner characteristic of retroviral NC pro-

teins (Figs. 5–7) (16, 42, 43) raises a number of questions. PrP is found in several cell compartments. A major fraction is usually attached to the plasma membrane via the GPI anchor. However, a transmembrane form with the N terminus in the cytosol, designated ctmPrP, has also been described (44). In addition to accumulation in the plasma membrane, huPrP was also found in the cytosol and probably in the nucleus, where it might interact with cellular and/or viral nucleic acids (Fig. 8). PrP may thus belong to a growing class of proteins exhibiting numerous activities and functions, for example the 37-kDa laminin receptor precursor (45). The latter protein is present on the cell surface as well as in a form associated with ribosomes as a component of the mRNA translational machinery (46, 47). Interestingly, PrP interacts with this 37-kDa laminin receptor precursor (45). Another example of a protein with both membrane- and nucleic acid-associated functions is C17. This protein is either membrane-associated, functioning in G-protein-mediated signal transduction related to the calcitonin gene-related receptor (48) or associated with RNA polymerase III for tRNA gene transcription (49). The possibility that PrP is associated with ribosomes or other cellular nucleoprotein components is presently under investigation.

In cells infected by HIV-1, the retroviral Gag polyprotein encoding NC is membrane-anchored via its N terminus during virion formation and budding at the cell surface (16, 25, 43, 50). Therefore, it is tempting to speculate that Gag and PrP interact at the plasma membrane in infected cells. This might well influence virus replication and accelerate the scrapie infectious process as suggested by Carp *et al.* (17) in the MuLV/mouse system. In that respect, the impact of huPrP expression on HIV-1 formation and replication is currently being investigated, and preliminary results indicate that PrP interactions with NC and the viral RNA can strongly influence HIV-1 virion formation and replication.

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The Prion Protein Has RNA Binding and Chaperoning Properties Characteristic of Nucleocapsid Protein NCp7 of HIV-1

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