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Virology 331 (2005) 418–428

VIROLOGY

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A PNA-transportan conjugate targeted to the TAR region of the HIV-1 genome exhibits both antiviral and virucidal properties

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Received 7 August 2004; returned to author for revision 3 September 2004; accepted 19 October 2004

Abstract

We have earlier reported that anti-TAR PNA conjugated with the membrane-transducing peptide transportan inhibits transactivation of the HIV-1 LTR resulting in decreased production of HIV-1 virions by chronically infected H9 cells (N., Kaushik, A., Basu, P., Palumbo, R.L., Myers, V.N., Pandey, 2002. Anti-TAR polyamide nucleotide analog conjugated with a membrane permeating peptide inhibits HIV-1 production. J. Virol. 76, 3881–3891). In this study, we have found that the PNA_{TAR}-transportan conjugate is efficiently internalized by cells and kinetics analysis reveals a sigmoidal curve with a cooperativity index of 6, indicating very rapid cellular uptake. Additionally, analysis of uptake at varying temperatures or in the presence of phenylarsine oxide revealed that the mechanism of uptake is neither receptor-dependent nor occurs via endocytosis. We also found that the PNA_{TAR}-transportan conjugate exhibits potent virucidal activity as HIV-1 virions pretreated with the conjugate were rendered noninfectious, suggesting that the conjugate may also permeate the virus envelope. The anti-HIV-1 virucidal activity of the conjugate may be useful either in topical formulations designed to block HIV-1 infection or as a prophylactic agent for inactivation of HIV-1 in the circulating plasma prior to attachment and entry.
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Keywords: PNA-transportan; Virucidal activity; HIV-1 TAR; HIV-1 virions; Cellular uptake; Receptor independent

Introduction

The major route of HIV-1 transmission from infected individuals to non-infected individuals occurs via sexual contacts through mucosal surfaces. Heterosexual transmission accounts for a majority of infections in women worldwide, representing a major portion of all adult infection. The results of a 10-year study has shown that male to female transmission rates are eight times higher than female to male transmission rates (Padian et al., 1997). A number of mechanisms for HIV transmission across genital epithelia have been suggested. These include direct HIV infection of epithelial cells, entry of HIV particles into epithelial cells through transcytosis, transmigration of HIV-infected epithelial cells from infected to non-infected individuals, uptake of HIV virions by intraepithelial Langerhans cells, and HIV entry via damaged epithelial layers (Ibata et al., 1997; Shattock et al., 1996). Prevention of such transmission could be blocked either through vaccination of the non-infected population or through use of virucidal agents that can rapidly inactivate HIV-1 virions in the biological fluids upon contact.

Despite massive efforts during the past two decades toward the development of an anti-HIV vaccine, an effective vaccine is yet to be developed. Another approach for reducing HIV transmission is the use of topical non-toxic virucidal agents prior to sexual intercourse. A sugar binding 11 kDa protein, Cyanovirin-N produced by cyanobacterium, was shown to be a potent virucidal agent against HIV-1 with its virucidal activity mediated through its high-affinity

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doi:10.1016/j.virol.2004.10.032

interaction with the viral coat protein (Botos and Wlodawer, 2003). A 60-kDa cellulose acetate phthalate (CAP), a common pharmaceutical excipient used for enteric film coating of capsules and tablets, was shown to inactivate HIV-1, HSV-1, HSV-2, and also non-viral pathogens which cause sexually transmitted disease (Kawamura et al., 2000; Manson et al., 2000). Recently, a group of tight binding nonnucleoside RT inhibitors was also shown to exhibit virucidal activity upon prolonged incubation with isolated HIV-1 virions (Motakis and Parniak, 2002). Although none of these compounds have yet reached clinical use for combating HIV infection, a novel class of synthetic DNA analogs, peptide nucleic acids (Nielsen, 1999; Nielsen et al., 1991), shows great promise for this application. The antiviral efficacy of PNAs targeted to the HIV-1 RNA genome has been demonstrated in cell culture (Kaushik and Pandey, 2002, Kaushik et al., 2002a, 2002b; Mayhood et al., 2000). We have recently enhanced the biodelivery characteristics and hence, the therapeutic potential of this class of specifically designed compounds by conjugating the anti-HIV PNA with a membrane transducing peptide, transportan, a chimeric peptide derived from the sequences of the neuropeptide galanin and wasp venom toxin mastoparan (Langel et al., 1996; Lindgren et al., 2000). This PNA_{TAR}-transportan conjugate inhibits HIV-1 production by chronically infected H9 cells (Kaushik et al., 2002a) when supplemented in the culture medium. Our previous studies have employed PNA conjugates targeted to two critical sites within the 5′UTR (untranslated region) of the HIV-1 genome. First, we have reported that PNA as well as PNA-DNA chimera complementary to the primer-binding site (PBS) of the HIV genome can completely block priming by $tRNA_3^{Lys}$ (Kaushik and Pandey, 2002; Kaushik et al., 2001, Lee et al., 1998). Secondly, we have shown that PNA targeted to another equally important conserved region, transactivation response region (TAR), inhibits the tat-mediated trans-activation of the HIV-1 LTR transcription by efficient sequestration of TAR (Kaushik et al., 2002a, 2002b; Mayhood et al., 2000). In this communication, we demonstrate that anti-TAR PNA conjugated with transportan peptide is efficiently taken up by cells by a mechanism that is not receptor-mediated or endocytotic and that preincubation with this conjugate protects cells from HIV-1 infection. Furthermore, our results show that the conjugate exhibits potent anti-HIV-1 virucidal activity as it is able to rapidly permeate the virus envelope rendering the HIV-1 virions non-infectious.

Results

Cellular uptake of PNA_{TAR}-transportan conjugate

We have previously shown by fluorescence microscopy that PNA-transportan conjugate labeled with tetramethylrhodamine (TAMRA) fluoroprobe is efficiently taken up by cells. A detectable fluorescence signal in the cells was observed following incubation with 75 nM of the conjugate for 2–12 h. However, it was unclear whether the fluorescence signal in the cells was dependent upon the conjugate concentration. In order to quantitatively evaluate the uptake, we labeled the PNA_{TAR}-transportan conjugate with fluorescein and monitored the cellular uptake by FACscan. The CEM and Jurkat cells were incubated at room temperature with varying concentrations of fluorescein tagged conjugate (50–500 nM) and the fluorescence signal/10,000 cells was obtained by FACscan (Fig. 1A). The uptake of PNA_{TAR}-transportan conjugate occurred rapidly in CEM cells and was concentration dependent. At concentrations ranging from 50 to 500 nM of the conjugate, a rapid increase in fluorescence intensity displaying 20–100% fluorescence-positive cells was observed within 30 s of incubation. Prolonged incubation for 5 min or 1 h did not significantly increase the fluorescence signal in CEM cells. Similar results were obtained with Jurkat and PBMC cells as well as with a phagocytic monocyte human cell line U-937 (Fig. 1B). The uptake in Jurkat cells was slower than that in CEM cells at lower concentrations of the conjugate, as indicated by a 15–20% fluorescence intensity at 100 nM concentration of the conjugate (Fig. 1B). Interestingly, at concentrations above 100 nM, the uptake level in Jurkat cells as judged by fluorescence intensity per 10^4 cells was similar to that in CEM cells. Uptake studies with Flu-tagged unconjugated PNA_{TAR} revealed no significant fluorescent intensity in CEM cells even after 6 h of incubation at 10 μM (data not shown).

The cellular uptake is neither receptor mediated nor endocytotic

It has been shown that cellular uptake via endocytosis is temperature-dependent and occurs at physiological temperature (37 °C), while it is significantly reduced/abolished at lower temperatures (Tomoda et al., 1989). Receptor-mediated uptake has been shown to be sensitive to phenylarsine oxide that cross-links to the thiol groups of membrane surface proteins and blocks both endocytosis and receptor function (Frost and Lane, 1985). In order to examine whether the observed uptake of PNA_{TAR}-transportan occurs via endocytosis or is receptor mediated, we examined the uptake at various temperatures followed by trypsin treatment as well as following pretreatment of cells with phenylarsine oxide. It was noted that the uptake of the conjugate remained unchanged at both 4 and 37 °C (Fig. 2A). A brief trypsin treatment of the cells following incubation with the conjugate did not significantly alter the uptake pattern as only 5–12% reduction in the uptake was noted at both the temperatures (Fig. 2B). Furthermore, a 5-min pretreatment of the CEM cells with 60 μM of phenylarsine oxide, which modifies the membrane surface receptor proteins, did not inhibit the uptake of the conjugate (Fig.

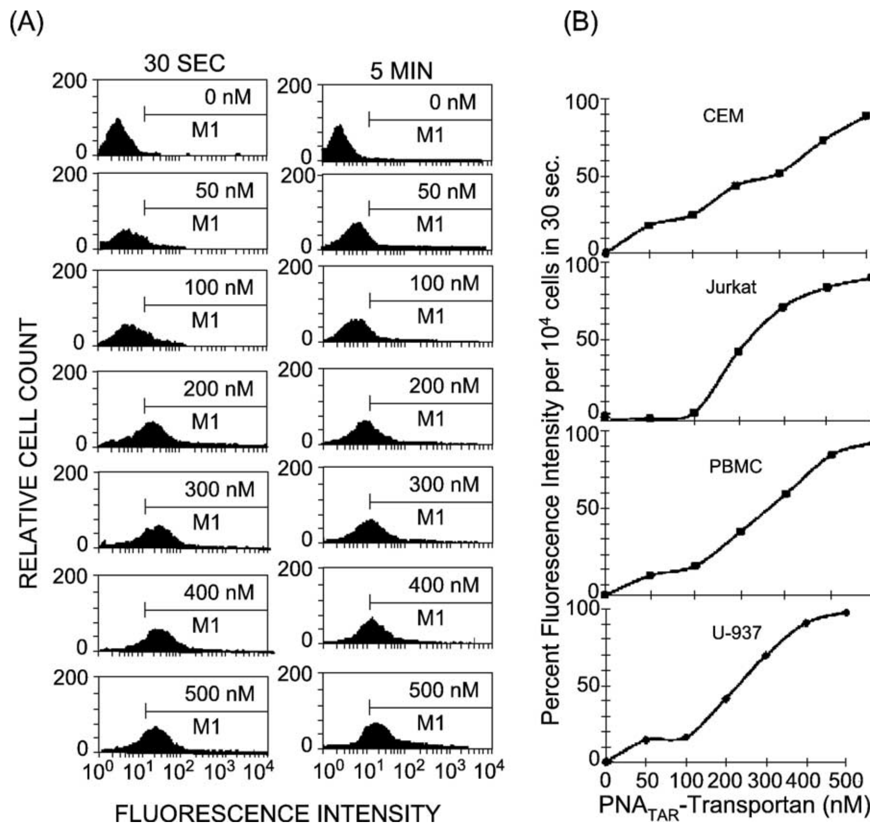

Fig. 1. (A) Uptake of PNA_{TAR}-transportan conjugate by CEM cells. Cells (2×10^6 cells) were incubated with varying concentrations of fluorescein-tagged PNA_{TAR}-transportan conjugate in complete RPMI medium at room temperature. The cells were centrifuged and resuspended in fresh complete RPMI medium and the uptake within 30 s and 5 min was evaluated by FACS analysis. (B) Comparative uptake of the conjugate in various lymphocytes.

2C) indicating that neither endocytosis nor a receptor mediated mechanism is responsible for the uptake. We postulate that the uptake of the conjugate may occur in a fashion similar to the Antennapedia homeodomain peptide penetratin (Derossi et al., 1994, 1996, 1998) via formation of inverted micelles upon interaction of the conjugate with the membrane bilayer. The inverted micelles are proposed to traverse the membrane emptying their contents into the cytoplasm.

Kinetics of cellular uptake of PNA_{TAR}-transportan conjugate

In order to determine the kinetics of uptake, we radiolabeled PNA_{TAR}-transportan conjugate with ^{125}Iodine. Jurkat cells (2×10^6) were incubated with varying concentrations of the radiolabeled conjugate at room temperature for 30 s followed by rapid filtration on glass fiber filters (GF-B). The filters were washed with PBS to remove free radiolabeled conjugate as well as conjugate

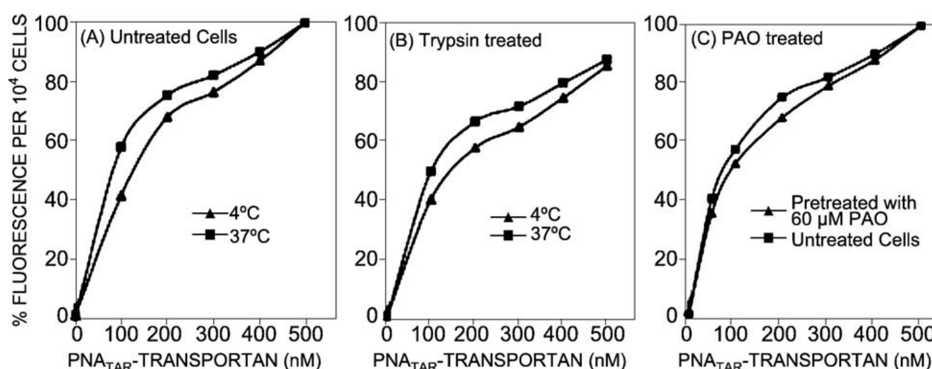

Fig. 2. Effect of temperature and phenylarsine on the cellular uptake of PNA_{TAR}-transportan conjugate. CEM cells (2×10^6 cells) were incubated with varying concentrations of fluorescein-tagged PNA_{TAR}-transportan conjugate in complete RPMI medium at indicated temperatures for 30 s. The cells were centrifuged, washed twice with PBS solution without trypsin (Panel A) or once with PBS containing 100 µg trypsin/ml and another wash with PBS alone (Panel B). In another set of experiment, cells were preincubated in the absence and presence of 60 µM phenylarsine oxide for 5 min at 25 °C (Panel C). The cells were centrifuged and resuspended in fresh complete RPMI medium and examined by FACS analysis.

adhering externally to the cell membrane. The labeled conjugate taken up by the cells was determined by counting the radioactivity retained on the filter by gamma counting. Results shown in Fig. 3A illustrate the sigmoidal nature of the uptake curve, suggesting a cooperative interaction between the conjugate and the cellular membrane. The $[S]_{0.5}$ value and cooperativity index (ratio of $[S]_{0.9}/[S]_{0.1}$) determined from the sigmoidal plot were 1.5 and 6 μM, respectively (Fig. 3B). The $[S]_{0.5}$ value indicates the concentration of the conjugate at which uptake velocity is equal to 0.5 V_{max}, while the cooperativity index is the ratio of substrate (conjugate) concentration at 0.9 V_{max} and 0.1 V_{max}. A Hill coefficient (nH) of 0.53 was obtained from the slope of the Hill plot suggesting that the observed cooperativity is not due to multiple conjugate binding sites on the membrane.

Inhibition of HIV-1 replication

Earlier, we have shown that PNA_{TAR}-transportan conjugate inhibits HIV-1 production by chronically infected H9 cells when supplemented in the culture medium (Kaushik et al., 2002a). We have further shown that the mechanism of inhibition is at the transcription level via down regulation of Tat-mediated transactivation of the HIV-1 LTR. In the present study, we examined whether PNA_{TAR}-transportan conjugate, designed primarily to inhibit the Tat-mediated transactivation of HIV-1 transcription, is also able to block infection of the cells by disrupting the synthesis of proviral DNA. The CEM cells (0.5×10^6) were infected with VSV-G pseudotyped HIV-1 virions at MOI of 350 in the presence of varying concentrations of PNA_{TAR}-transportan conjugate. Scrambled PNA-transportan (random PNA sequence) was also included as a control. The infected cells were pelleted, washed with PBS, and resuspended in the fresh medium without conjugate. After 48 h of incubation at 37 °C, the cells were harvested, lysed, and an aliquot of each lysate containing equal amounts of protein was assayed for firefly luciferase activity. Results shown in Fig. 4 indicate that inhibition of luciferase expression was observed at all concentrations of PNA_{TAR}-transportan conjugate. Infection of CEM cells by HIV-1 virions was performed in the presence of varying concentration of conjugates. Subsequent to infection, cells were pelleted and resuspended in the culture medium in the absence of the conjugate. This resulted in reduced levels of luciferase expression measured 48 h post infection. Approximately 75–99% inhibition was noted at concentrations ranging from 50 nM to 2 μM of the conjugate. It may be noted that during this period following post-infection, inhibitor was not present in the medium and the reduced level of luciferase expression may not be due to inhibition of Tat mediated transactivation. This could be due to reduced level of infection either due to inhibition of proviral DNA synthesis upon viral entry in the cell or inactivation of virions infectivity by disrupting the viral membrane. These possibilities were further addressed in the following experiments shown in Fig. 5A and B. Scrambled PNA-transportan conjugate even at 5 μM did not exhibit any antiviral activity.

Infectivity of HIV-1 virions pre-treated with the PNA_{TAR}-transportan conjugate

A significant reduction of HIV-1 infection by PNA_{TAR}-transportan conjugate as judged by reduced level of luciferase expression in CEM cells (Fig. 4) suggests that the conjugate may also be effective in blocking proviral DNA synthesis, a step in the HIV-1 life cycle critical for establishing persistent infection. The interaction of the conjugate with the viral RNA genome may occur both in extracellular virions and in the cytosol following fusion and entry. To examine the first proposed interaction, HIV-1 virions were pretreated with the varying concentrations of the PNA_{TAR}-transportan conjugate or with scrambled PNA-transportan conjugate in complete RPMI medium and ultracentrifuged through a 20% sucrose cushion to separate the virion particles from the conjugate. The pre-treated virions were then used to infect CEM cells at MOI of 350. The infection was monitored by determining the level of

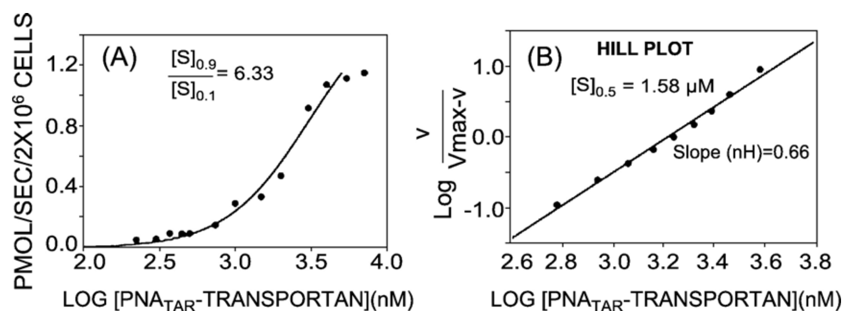

Fig. 3. Uptake of ^{125}I-labeled PNA_{TAR}-transportan conjugate by Jurkat cells. The ^{125}I-labeled conjugate was incubated with CEM cells and the amount of conjugate taken up by the cells was determined from specific radioactivity of the conjugate as described in Materials and methods. The uptake of the conjugate was plotted against the log of conjugate concentration and the cooperative index ($[S]_{0.9}/[S]_{0.1}$) was determined. Hill coefficient (nH) was determined from the slope of the Hill plot of the uptake data shown in the left panel. Cooperative index represents the ratio of the substrate at 0.9 V_{max} and 0.1 V_{max}, while Hill coefficient (nH) represents the concentration of the conjugate at which the velocity of the uptake is equal to 0.5 V_{max}.

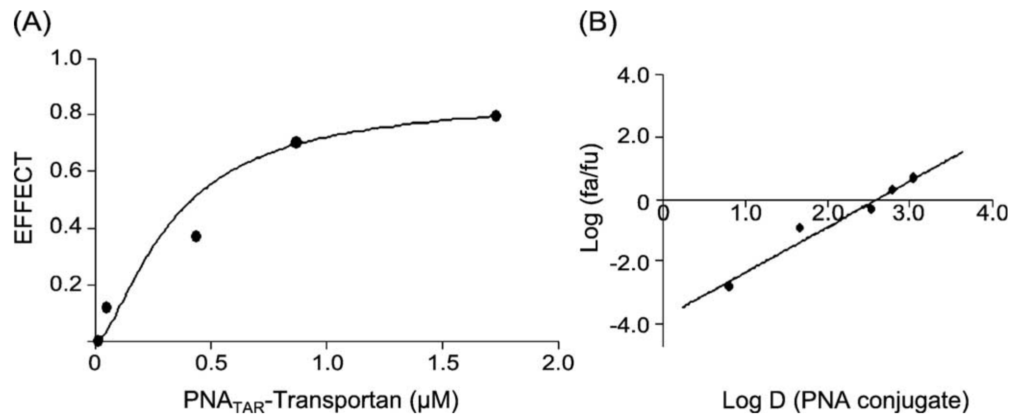

Fig. 4. (A) Dose effect curve of inhibition of viral infection/replication as a function of PNA_{TAR}-transportan conjugate. CEM cells (0.5×10^6 cells) were infected with pseudo HIV virions, pNL4-3.Luc.R.E (equivalent to 14 ng p24) in the presence of varying concentrations of PNA_{TAR}-transportan or scrambled PNA-transportan conjugate as described in Materials and methods. The infected cells were grown in complete RPMI medium at 37 °C in CO_2 incubator for 48 h. Cells were harvested, lysed, and an aliquot of the lysate was assayed for luciferase activity. The percent inhibition of luciferase expression is expressed as 'EFFECT' with respect to untreated virions (control). (B) Median effect plot and dose–response curve for the effect of PNA_{TAR}-transportan on viral replication. The dose median for the PNA_{TAR}-transportan conjugate was determined from the dose effect curve using the CalcuSyn software (Biosoft) program. D = dose of the PNA_{TAR}-transportan conjugate, fa = fraction of inactivation, fu = infectivity of control (untreated). Dose median for PNA_{TAR}-transportan conjugate as estimated from the above graph is 382 nM and regression coefficient is $r = 0.98$ and $m = 1.4$. The scrambled PNA-transportan had no effect on viral infection/replication as the levels of luciferase expression were similar to the control in the absence of PNA_{TAR}-transportan conjugate. As scrambled PNA-transportan conjugate displayed no inhibitory effect, no value could be put in the dose effect curve plot of CalcuSyn software.

firefly luciferase expression after 48 h of cell growth. The results shown in Fig. 5 demonstrate that virion particles pre-treated with PNA_{TAR}-transportan conjugate were severely impaired in their ability to infect CEM cells. Since expression of firefly luciferase is a direct consequence of conversion of viral RNA into proviral DNA and its subsequent integration into the host genome, our results indicate that PNA_{TAR}-transportan conjugate permeates the virion particles rendering them non-infectious while scrambled PNA-transportan conjugate had no effect on viral infectivity. This observation suggests that the conjugate is able to permeate virion particles and tightly bind to its target sequence on the viral genome. The tightly bound conjugate would abort reverse transcription thus preventing integration and expression of the luciferase-encoding proviral DNA.

Uptake of the conjugate by HIV-1 virions

The loss of infectivity of HIV-1 virions pretreated with PNA_{TAR}-transportan conjugate implies that the conjugate, besides being rapidly taken up by the cells, may either permeate the viral envelope which is also derived from the cell membrane or may affect virion integrity by

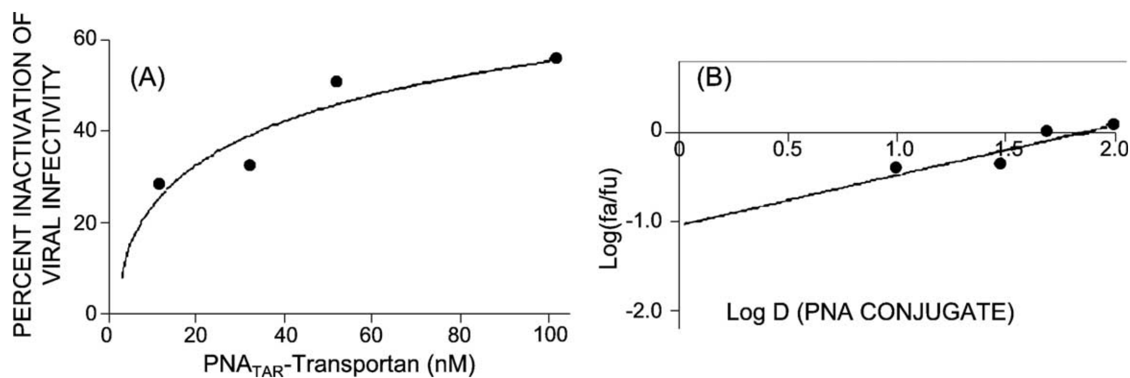

Fig. 5. (A) Dose effect curve of inactivation of viral infectivity following pretreatment of HIV-1 virions as a function of PNA_{TAR}-transportan conjugate. HIV-1 virions (15 ng p24) pretreated with the PNA_{TAR}-transportan conjugate were used to infect the CEM cells as described in Materials and methods. The infected cells were grown in complete RPMI medium at 37 °C in CO_2 incubator for 48 h. Cells were harvested, lysed and an aliquot of the lysate was assayed for luciferase activity. The percent inactivation of viral infectivity is expressed with respect to the untreated virions (control). (B) Median effect plot for the log inactivation of infectivity for HIV-1 virions pretreated with various concentrations of PNA_{TAR}-transportan. The Dose median for PNA_{TAR}-transportan conjugate was determined from the dose effect curve using the CalcuSyn software (Biosoft) program. D = dose of the PNA_{TAR}-transportan conjugate, fa = fraction of inactivation, fu = infectivity of control (untreated). Dose median for PNA_{TAR}-transportan conjugate as estimated from the above graph is 66 nM and regression coefficient is $r = 0.9243$. The scrambled PNA-transportan had no effect on viral infectivity as the levels of luciferase expression were similar to the control (untreated virions). As scrambled PNA-transportan conjugate displayed no inhibitory effect, no value could be put in the dose effect curve plot of CalcuSyn software.

disrupting the viral envelope. To investigate these possibilities, we incubated the purified HIV-1 virions with I^{125}-labeled PNA_{TAR}-transportan conjugate (2×10^4 CPM/pmol) in complete RPMI medium for 20 min and then ultracentrifuged through a discontinuous sucrose density gradient. The complete RPMI medium and mock virus sample prepared from culture supernatant of uninfected cells were used as negative controls. Fractions were collected from the bottom and analyzed for radioactivity and p24 antigen levels. It was observed that a significant portion of the radiolabeled conjugate sedimented with the HIV-1 virions, as judged by the analysis of p24 antigen peak (Fig. 6B). In contrast, the entire radiolabeled conjugate remained at the top of the gradient in the RPMI medium and mock virus controls (Figs. 6A and C). These results imply that the conjugate is able to permeate or attach to the viral envelope without disrupting the virion integrity.

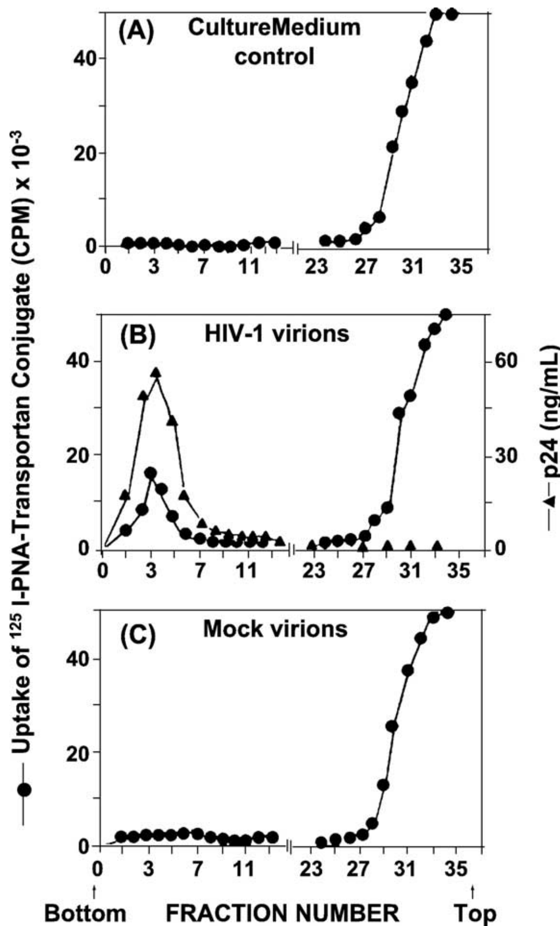

Fig. 6. Internalization of PNA_{TAR}-transportan in HIV-1 virions. Radiolabeled PNA_{TAR}-transportan incubated in RPMI culture medium with or without HIV-1 virions was ultracentrifuged through a discontinuous sucrose density gradient. Fractions were collected from the bottom and analyzed for radioactivity and p24 antigen. Panel A: culture medium control without HIV-1 virion. Panel B: with HIV-1 virion. Panel C: mock virion sample processed from uninfected cells.

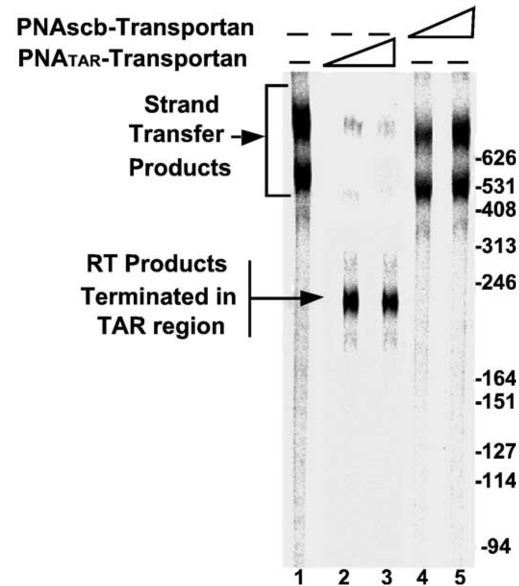

Fig. 7. Abortive endogenous reverse transcription in HIV-1 virions pretreated with PNA_{TAR}-transportan conjugate. An aliquot of HIV-1 virions pretreated with 500 nM or 1 μM of PNA_{TAR}-transportan conjugate or scrambled PNA-transportan conjugate were ultracentrifuged through a 20% sucrose cushion to remove external PNA-transportan conjugate. The virion pellet was resuspended in 0.1% NP-40 containing 10 mM DTT. An aliquot of the disrupted virions were assayed for endogenous reverse transcription. Lane 1, untreated HIV-1 virions; lanes 2 and 3, pre-treated with 0.5 μM and 1.0 μM of PNA_{TAR}-transportan conjugate, respectively; lanes 4 and 5, pre-treated with scrambled PNA_{TAR}-transportan conjugate at 0.5 and 1 μM concentrations, respectively.

Pretreatment of HIV-1 virions with PNA_{TAR}-transportan impairs endogenous reverse transcription

Following our observation that suggested the possibility of PNA_{TAR}-transportan conjugate permeating the virion particles, it was reasonable to infer that the conjugate may either penetrate the nucleocapsid and bind to the TAR region of the viral genome or may have simply attached to the viral envelope. In the first scenario, the synthesis of minus (−) strand strong stop DNA should be severely impaired since we have earlier demonstrated that PNA bound to the viral RNA could not be displaced by HIV-1 RT (Lee et al., 1998). To examine this possibility, HIV-1 virions pretreated with 500 nM or 1 μM of PNA_{TAR}-transportan conjugate were ultracentrifuged through a 20% sucrose cushion to remove external PNA_{TAR}-transportan conjugate. The virion pellet was resuspended in 0.1% NP-40 containing 10 mM DTT. An aliquot of the disrupted virions were assayed for endogenous reverse transcription. Results shown in Fig. 7 indicate that endogenous reverse transcription in HIV-1 virions pretreated with PNA_{TAR}-transportan conjugate is predominantly blocked in the TAR region (lanes 2 and 3). In the control having no conjugate or pretreated with scrambled PNA-transportan conjugate, the reverse transcription products were larger than 400 nucleotides, which indicates that strand transfer has taken place after the completion of the (−) strand strong stop DNA

synthesis (lanes 1, 4, and 5). Although these results suggest that the conjugate permeates the virions and binds to the TAR region of the viral RNA, it is also possible that the conjugate may have tightly attached to the viral envelope externally and may have bound to the target only after the virion was disrupted. However, the FACScan analysis in which cells treated with trypsin to degrade the PNA conjugate revealed a similar pattern of conjugate uptake to untreated cells, suggesting that uptake by virions having cell membrane-derived envelope would likely occur by a similar mechanism. Regardless of the mechanism, the infectivity of the virion particles is significantly impaired indicating the potential of this class of compounds as virucidal agents against HIV-1.

Discussion

A number of potential drug candidates fail because of associated cellular uptake problems. This problem is often addressed by optimizing their biophysical properties to enhance cellular uptake to acceptable levels. The therapeutic potential of PNAs as gene-specific, nontoxic, non-immunogenic compounds has been limited due to their poor cellular uptake (Derossi et al., 1998). We have used a highly effective strategy to overcome this obstacle and improve their biodelivery by conjugating the PNA with a membrane-transducing peptide, transportan, a chimeric peptide derived from the neuropeptide galanin and wasp venom toxin mastoparan (Langel et al., 1996; Lindgren et al., 2000). Using PNA_{TAR}-transportan targeting the TAR region of the HIV-1 genome as the model conjugate, we observed that it is efficiently taken up by cells. Unlike other membrane transducing peptides such as those derived from HIV-1 Tat protein (Fawell et al., 1994; Frankel and Pabo, 1988; Schwarze and Dowdy, 1999; Silhol et al., 2002) or HSV VP22 (Dilber et al., 1999; Elliott and O'Hare, 1997, 1999; Phelan et al., 1998), which are highly basic, transportan is comparatively hydrophobic in character containing only four lysine residues. The cellular uptake of this peptide alone has been shown to be nonsaturable and resistant to treatment with phenylarsine oxide or hyperosmolar sucrose solution, suggesting that the mechanism of uptake is not endocytotic- or receptor-mediated (Pooga et al., 1998). Similar results were obtained with PNA_{TAR}-transportan conjugate except that the uptake shows a sigmoidal curve indicating cooperative interaction between the cellular membrane and the conjugate. The observed cooperativity of uptake may be due to micelle formation, which requires a concentration threshold of components. A cooperativity index of 6 suggests that in order to increase the uptake velocity from 0.1 to 0.9 V_{max}, only 6-fold increase in the concentration of conjugate is required. Any drug/compound displaying a sigmoidal pattern of uptake kinetics is expected to be more effective than that displaying a hyperbolic pattern since theoretically the latter would require a 90-fold increase in the substrate concentration to achieve an uptake velocity of 0.9 V_{max} from 0.1 V_{max}. We have earlier shown that supplementing PNA_{TAR}-transportan conjugate in the culture medium significantly inhibited HIV-1 production by chronically infected H9 cells (Kaushik et al., 2002a). This inhibition of virus production was shown to be due to down regulation of Tat-mediated transactivation of HIV-1 transcription. In the present study, we found that infection of CEM cells by HIV-1 virions was also drastically reduced in the presence of PNA_{TAR}-transportan conjugate. This result was surprising since PNA targeting the TAR region of the viral genome was predicted to function at the transcriptional level after infection has taken place. The PNA_{TAR}-transportan conjugate may also interfere with the synthesis of proviral DNA, a crucial step for establishing the viral infection. We made this inference following our observation that infectivity of HIV-1 virions was drastically reduced when pretreated with PNA_{TAR}-transportan conjugate. The EC_{50} was found to be as low as 50 nM. These results suggest that the conjugate may either permeate the virus envelope and bind to its target sequence on the viral genome or alternatively it may affect viral entry by binding to viral membrane and disrupting its integrity. If the first scenario holds true, when pretreated virions enter the cell, reverse transcription is likely to be aborted in the TAR region since the PNA conjugate bound to the TAR region cannot be displaced by reverse transcriptase. The fact that the radio-iodinated PNA_{TAR}-transportan conjugate co-sediments along with virion particles in discontinuous sucrose density gradient suggest that conjugate may have been taken up by the virion particles or strongly attached to the viral membrane. When such virions were separated from the free conjugate and examined for endogenous reverse transcription activity, all reverse transcription products were found to be aborted exclusively in the TAR region.

The PNA_{TAR}-transportan conjugate contains potential anti-HIV virucidal activity which is able to inactivate virions before they can infect healthy cells. Currently available drugs targeting viral RT and protease operate by inhibiting replication of HIV only after it has infected target cells. Entry inhibitors have recently been shown to block HIV entry into cells but do not inactivate virions circulating in the plasma (Derdeyn et al., 2000; Nagashima et al., 2001; Wild et al., 1993). Our approach is unique in the sense that HIV-1 virions in the plasma may be rapidly inactivated prior to their entry into cells upon exposure to PNA conjugates targeting critical regions of the HIV-1 genome. Future development of PNA technology may continue to improve biodelivery of these antisense agents which, due to their resistance to degradation by nucleases, have great advantages over other antisense molecules. In addition to their potential value as therapeutic agents, these conjugates may also have great potential as prophylactic agents to block HIV-1 infection following accidental exposure to the virus or as virucidal agents used to prevent infection during sexual contact.

Materials and methods

PNA oligomers

The anti-TAR PNA was synthesized and fluorenyl methoxycarbonyl cysteine (Fmoc-Cys) was coupled directly to the PNA at the N-terminal. For fluorescein labeling, Fmoc-O-Cys PNA was synthesized with 'O' spacer known as egl linker at the N-terminus while for ^{125}I radiolabeling, a Tyr residue was placed at the C-terminus. All the PNA samples were purified on C_{18} reverse phase HPLC columns which was pre-equilibrated with solvent A (0.1% TFA in water) at 45 °C. The elution profile was developed by increasing the concentration of solvent B (0.1% TFA in 100% acetonitrile) as follows: 0–5 min (0% B), 5–50 min (60% B).

Fluorescein labeling of PNA

The Fmoc-O-Cys-PNA targeting TAR region of HIV-1 genome was labeled with fluorescein while still immobilized on the resin. The Fmoc group protecting the amino group was removed by treating with 1 ml of 20% piperidine in dimethyl formamide (DMF) for 5 min followed by washing the column with equal volume of DMF three times. The 5-carboxy fluorescein succinimidyl ester (5 mg) dissolved in 325 μL of DMF containing 7.5% diisopropyl ethylamine (v/v) and added to the resin. The labeling reaction was carried out at room temperature for 1 h with occasional shaking by moving the solution back and forth using two syringes attached at each end of the column. The reaction was stopped by flushing out the labeling solution from the column followed by washing the resin three times with DMF and with DCM (1 ml each washing). The resin was treated with 100 μl of deprotecting solution containing 10% TFA and 2% m-cresol (v/v) in DCM to remove the trityl group from the terminal cysteine and Bhoc (benzhydrylcarbonyl) group from PNA. After 5 min, the deprotecting solution was replaced with the fresh one and further incubated for 5 min. The resin was then treated with 250 μl of TFA/m-cresol (4:1 v/v) at room temperature for 2 h to release the PNA. The TFA/m-cresol solution containing PNA was collected and the resin was washed twice with 100 μl of TFA. The fluoresceinated PNA was precipitated by 5-fold excess of dry cold ether followed by incubation on dry ice for 10 min. The precipitate was collected by centrifugation at 5,000 rpm for 5 min. The precipitate was washed once with the cold dry ether and air-dried. The fluorescein-labeled PNA was quantified spectrophotometerically by measuring the absorbance of a diluted sample at 260 and 490 nm.

Synthesis of PNA-transportan conjugate

All the solvents used were degassed prior to use by bubbling with helium gas for 10 min. PNA (1 μmol, 1 eq.) was dissolved in NMP (N-methyl pyrrolidinone)-H_2O (1:1, v/v) to a final concentration of 1 mM. The NPYS-peptide (1.3 eq) dissolved in NMP with a final concentration of 10 mM was added to the PNA solution. The solution was supplemented with 100 eq. of 1 M sodium acetate buffer (pH 5.0), mixed by vortexing and incubated at 40 °C. After 3 h of incubation, the coupling reaction was quenched by addition of 5% TFA to a final conc. 650 mM. The purification of the conjugate was carried out by HPLC on YMC guardpak ODS-AQ C_{18} column (S-3 μm, 12 nm, 4 × 50 mm) using water: acetonitrile gradient (containing 0.5% acetic acid v/v) with column temperature maintained at 45 °C. The desired fractions were lyophilized and stored at −20 °C. Quantitation of the PNA-transportan conjugate was carried out by measuring absorbance at 260 nm and the OD value was divided by the molar extinction coefficient. Mass spectrometry of the conjugate was performed on a Perspective Biosystems Voyager DE MALDI-TOF system using sinapinic acid matrix.

Proviral clones and Plasmid

An env (−) HIV-1 proviral clone, pNL4-3.Luc.R.E., encoding a luciferase reporter gene, was obtained from the NIH-AIDS Reference and Reagent Program, while pVSV-G plasmid expressing the G glycoprotein of vesicular stomatitis virus was purchased from CLONETECH.

Production of HIV-1 virions

The 293T cells were transfected with 10 μg of pNL4-3.Luc.R.E and 10 μg of pVSV-G DNA (Akkina et al., 1996) by calcium phosphate method (Chen and Okayama, 1987) and grown in Dulbecco's modified Eagle's medium supplemented with FCS (10%) and antibiotics. After 12 h, the transfected cells were washed with PBS and grown for another 48 h in complete medium. The culture supernatant was collected and passed through 0.45 μm cellulose acetate filters. The p24 antigen level in the filtered supernatant was determined by ELISA. The viral stock was aliquoted and stored at −80 °C.

Purification of HIV-1 virions

The filtered culture supernatant (500 ml) containing HIV-1 virions was centrifuged at 70,000 × g for 45 min. The virion pellet was resuspended in 2 ml of PBS and recentrifuged through 5 ml of 20% sucrose/PBS cushion for 30 min at 100,000 × g. The virion pellet was resuspended in complete Dulbecco's medium and stored at −80 °C.

Preparation of subtilisin-treated HIV-1 virions

Purified HIV-1 virion preparations have been shown to be contaminated with cellular microvesicles of 50–500 nm

size (Gluschankof et al., 1997; Ott et al., 1996). To remove these microvesicles, viral pellets were resuspended in 500 μL of Dulbecco's phosphate buffered saline containing 1 mM $CaCl_2$ and treated with subtilisin (1 mg/ml) at 37 °C for 18 h (Ott et al., 1996). The subtilisin digestion was stopped by PMSF (5 μg/ml) and virion particles were centrifuged through 1 ml of 20% sucrose. The virion pellet was resuspended in Dulbecco's phosphate-buffered saline without $CaCl_2$ or $MgCl_2$.

Determination of HIV-1 virion number in the purified sample

HIV-1 virions were quantified by determination of the RNA copy number in the sample using Nuclesins HIV-1-QT Amplication Kit (Organon Teknika, Durham, NC). The virion number was also determined from the p24 concentration, as excellent correlation between HIV-1 RNA copy number and p24 concentration has been demonstrated (Vass, 2002). Considering that 2000 copies of p24 are present per virion particle, the virion number estimated from the RNA copy number were in agreement with the number determined by p24 quantification (1 pg p24/12500 virions).

FACscan analysis of cellular uptake

CEM and Jurkat cells grown in complete RPMI 1640 medium containing 10% FCS were harvested, washed with PBS and resuspended in the same medium at a cell density of 4 × 10^6 cells/ml. Cells were aliquoted in 12-well microtiter plate at 2 × 10^6 cells per well and incubated at room temperature with varying concentrations of fluorescein tagged PNA_{TAR}-transportan conjugate (50–500 nM). At varying time points, the cells were centrifuged and resuspended in the same medium without the conjugate and fluorescent signal/10,000 cells were obtained by FACscan.

Uptake kinetics

The PNA_{TAR}-transportan conjugate was labeled with ^{125}I using the radioisotope and the chloramine-T labeling kit from ICN. For radiolabeling, 0.5 nmol of the conjugate and 0.28 nmol of ^{125}-I (0.5 mCi) were reacted in the presence of 2.8 nmol of chloramine-T for 1 min according to the manufacturer's protocol and quenched by the addition of 62 nmol of sodium metabisulfite. The labeled conjugate was purified by NAP-10 gel filtration (Amersham), followed by C_{18} disposable cartridges. The final purified product was lyophilized, dissolved in water, and quantified by absorption at 260 nm. The specific radioactivity was adjusted to desired concentration by adding unlabeled conjugate. For uptake experiments, 2 × 10^6 Jurkat cells were incubated with varying concentrations of the radiolabeled conjugate at room temperature. Following a 30-s incubation, each individual sample was filtered on glass fiber filter (GF-B), washed extensively with phosphate buffered saline solution and the amount of radiolabel internalized into the cells was determined by gamma counting. The $[S]_{0.9}/[S]_{0.1}$ ratio (cooperativity index) of the substrate at 0.9 V_{max} and 0.1 V_{max} was determined by plotting the uptake versus substrate concentration. Hill coefficient (nH) was determined from the Hill plot of uptake data.

Uptake of PNA_{TAR}-transportan conjugate by virion particles

The purified HIV-1 virion particles were incubated with 50 nM of I^{125}-labeled PNA_{TAR}-transportan conjugate (1.8 × 10^5 CPM/pmol) in complete RPMI medium (250 μl) for 20 min. The RPMI medium and mock-virus sample served as negative controls. The incubation mixture was then subjected to ultracentrifugation through a discontinuous sucrose density gradient (1 ml of 40% sucrose/PBS + 2 mL of 20% sucrose/PBS + 2 ml of 10% sucrose/PBS) for 1 h. Fractions were collected from the bottom and analyzed for p24 and presence of radioactivity.

Infectivity of HIV-1 virions pre-treated with PNA_{TAR}-transportan conjugate

The HIV-1 virions (equivalent to 15 ng p24) containing firefly luciferase reporter were incubated with varying concentrations of PNA_{TAR}-transportan conjugate for 1 h in complete RPMI medium in a final volume of 250 μl. Control experiments without the conjugate or with scrambled PNA-transportan conjugate were also performed simultaneously. The virion particles were separated from the conjugate by centrifuging through a 20% sucrose/PBS cushion for 30 min at 100,000 × *g* and the virion pellets were resuspended in 500 μl of complete RPMI medium. Infection was performed by incubating the pretreated virions with CEM cells (0.5 × 10^6 cells) at 37 °C for 2 h in a final volume of 1.0 ml RPMI medium containing 10 μg/ml polybrene. Infected CEM cells were washed with PBS and resuspended in 1 ml of complete RPMI medium and incubated in a 37 °C, CO_2 incubator. After 48 h, cells were harvested, washed and lysed in 250 μl of Passive Lysis Buffer (Luciferase assay kit, Promega) for 15 min on a rocking shaker. The lysates were centrifuged at 13,000 rpm for 10 min and the supernatants were collected. An aliquot of each supernatant containing equivalent protein was analyzed for firefly luciferase activity in a 96-well fluorotrac plate using a Packard Top Count Luminescent counter as described before (Nadal et al., 1999).

Endogenous reverse transcription in HIV-1 virions pretreated with PNA_{TAR}-transportan

HIV-1 virions (5 × 10^{11}/mL) were incubated with 0.5–1.0 μM concentrations of PNA_{TAR}-transportan or scrambled PNA-transportan in complete RPMI medium (200 μl) for 30 min at 4 °C. The incubation mixture was then layered on 3

ml of 20% sucrose and centrifuged at 100,000 × *g* for 30 min. The virions pellet free from external PNA_{TAR}-transportan conjugate was resuspended in Tris buffer containing 0.1% NP-40 and 10 mM DTT to disrupt the viral membrane. An aliquot of the disrupted virions was examined for endogenous RT activity by supplementing with 2 mM $MgCl_2$ and 5 μM of each dNTPs containing 5 μCi each of [α-^{32}P]dCTP and [α-^{32}P]dATP. Following 1-h reaction at 37 °C, the mixture was extracted with phenol-chloroform, precipitated with alcohol and lyophilized. The lyophilized material was dissolved in TE buffer and resolved on denaturing polyacrylamide-urea gel.

Acknowledgment

This research was supported by a grant from the NIAID (AI42520).

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