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# Isolation of virus-neutralizing RNAs from a large pool of random sequences

(*in vitro* selection/nuclease-resistant RNA analogs/antiviral agents)

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**ABSTRACT** **RNA and ribonuclease-resistant RNA analogs that bound and neutralized Rous sarcoma virus (RSV) were isolated from a large pool of random sequences by multiple cycles of *in vitro* selection using infectious viral particles. The selected RNA pool of RSV-binding sequences at a concentration of 0.16 μM completely neutralized the virus. Of 19 sequences cloned from the selected pool, 5 inhibited RSV infection. The selected RNA and RNA analogs were shown to neutralize RSV by interacting with the virus, rather than by adversely affecting the host cells. The selection of the anti-RSV RNA and RNA analogs by intact virions immediately suggests the potential application of this approach to develop RNA and RNA analogs as inhibitors of other viruses such as human immunodeficiency virus.**

In comparison to the large numbers of antimicrobial agents against bacteria, few effective antiviral drugs have been developed (1, 2). The most widely used approach to the discovery of antiviral agents has been the empirical screening of chemically diverse classes of synthetic and natural compounds for the ability to inhibit virus replication (2, 3). However, the empirical screening is usually tedious and inefficient. Other approaches such as rational drug design based on the known structures of viral and cellular proteins are promising but to date have not been largely successful (3).

RNA molecules have a remarkable diversity of structure and function (4, 5). The structural complexity of a combinatorial sequence library of single-stranded RNA oligonucleotides provides the potential to select for molecules that bind defined targets with high affinity and specificity (6–8). Because the selected RNA molecules can be amplified and subjected to further selection, multiple cycles of such selection and amplification can lead to the isolation of the best target-binding RNA molecules from the library. This *in vitro* selection–amplification procedure has been previously used to isolate RNA (and DNA) molecules that bind a variety of small molecules and purified proteins (9–12). We have extended the procedure to a new type of target, an intact virus, to isolate the RNA molecules and the nuclease-resistant RNA analogs that specifically bind and neutralize the virus.

Rous sarcoma virus (RSV), an avian retrovirus, is among the best-studied members of the family Retroviridae, which includes human immunodeficiency virus (HIV). All members of this family are enveloped viruses that display on their surface glycoproteins which are required for binding to a specific receptor on a susceptible cell and for fusion with the cellular membrane (13, 14). Here we use RSV to demonstrate that without knowledge of the structures of viral proteins, antiviral RNAs and RNA analogs can be isolated systematically and effectively from a large pool of random sequences, first by using intact viral particles to select from the sequence pool the RNAs and RNA analogs that bind specifically to the virus, and subsequently by screening the selected molecules for the ability to neutralize the virus. We reason that by binding to virus, some of the selected RNAs and RNA analogs may change the structures of viral surface proteins so that these proteins can no longer function in steps critical for viral infection, such as viral attachment and virus–cell membrane fusion. Alternatively, some of the structural changes may trigger pathways to inhibit the steps which normally occur after virus internalization, such as the uncoating and the expression of the virus genome. Both mechanisms have been previously suggested for many similar virus neutralization reactions induced by the binding of antibodies (15, 16).

## MATERIALS AND METHODS

**Materials.** Nucleoside 5′-triphosphates were purchased from United States Biochemical. T7 RNA polymerase was purified from the overproducing *Escherichia coli* strain BL21/pAR1219 (17). SuperScript II reverse transcriptase was from GIBCO/BRL. *Taq* DNA polymerase was from Promega. Human plasma was provided by David Farrell (Pennsylvania State University). Prague A strain of RSV and anti-RSV serum were prepared as described (18–20).

**Construction of a Large RNA Pool of Random Sequences.** A DNA library containing $\approx 5 \times 10^{16}$ sequences (2.5 mg of DNA) was constructed by automated solid-state synthesis. The sequence diversity was generated by randomizing a central 40-nt region (40N) of the 87-nt oligomer 5′-GCC-GGA-TCC-GGG-CCT-CAT-GTC-GAA-40N-TTG-AGC-GTT-TAT-TCT-GAG-CTC-CC. The 5′-end primer (5′-GCC-GGA-TCC-GGG-CCT-CAT-GTC-GAA-3′) and 3′-end primer (5′-CCG-AAG-CTT-AAT-ACG-ACT-CAC-TAT-AGG-GAG-CTC-AGA-ATA-AAC-GCT-CAA-3′, containing a T7 RNA polymerase promoter) were made for the polymerase chain reaction (PCR) to amplify the 87-mer. Large-scale PCR of the DNA library resulted in double-stranded DNA ($\approx 5 \times 10^{15}$ sequences) which was transcribed by T7 RNA polymerase to generate a pool of multiple copies of $\approx 10^{15}$ RNA sequences (random pool).

**Transcription of RNAs and 2′-Fluoropyrimidine-Containing RNAs (2′-F-RNAs).** Unmodified RNAs were transcribed *in vitro* by T7 RNA polymerase at 37°C for 2–3 hr in a buffer containing 1 mM each ATP, CTP, GTP, and UTP; 60 mM Tris·HCl (pH 8.0), 12 mM $MgCl_2$, 1 mM spermidine, 5 mM dithiothreitol, and 0.001% Triton X-100. 2′-F-RNAs were transcribed at 35°C for 8 hr in the same buffer except that 1

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Abbreviations: RSV, Rous sarcoma virus; HIV, human immunodeficiency virus; 2′-F-RNA, 2′-fluoropyrimidine-containing RNA.

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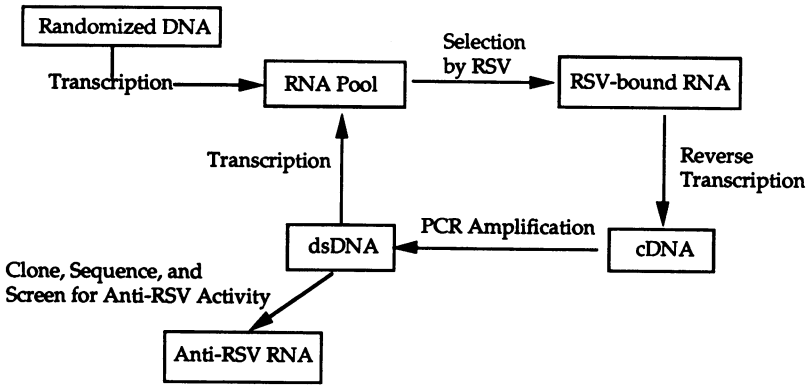


FIG. 1. *In vitro* selection–amplification procedure for isolating RSV-binding and -neutralizing RNAs and RNA analogs from a combinatorial sequence library. dsDNA, double-stranded DNA.

mM 2′-F-CTP and 2′-F-UTP replaced CTP and UTP. The full-length transcripts were purified by polyacrylamide gel electrophoresis.

***In Vitro* RNA Selection by RSV Particles.** The RNA (or 2′-F-RNA) sequence pool was incubated with RSV (Prague A strain) for 10 min at 37°C in 2.5 mM $MgCl_2$/100 mM NaCl/20 mM Tris·HCl, pH 7.5. The mixture was then filtered through a prewetted nitrocellulose membrane. The RNA–virus complexes were recovered from the nitrocellulose membrane after the unbound RNAs were washed away. The RNA molecules which bound anything else but RSV (e.g., the nitrocellulose membrane, the cellular proteins contaminating the virus solution) were removed from the RNA pool by a background selection either prior to or after the selection by virus. The RNA–RSV complexes were denatured and viral proteins were removed by phenol/chloroform extraction. The RNAs were then recovered by ethanol precipitation and reverse-transcribed to their cDNAs by SuperScript II reverse transcriptase. The cDNAs were PCR-amplified and subsequently transcribed to give a new pool of RNA which was enriched for better RSV-binding sequences. This new pool of RNA was used for selection by intact RSV to begin the next cycle. The selection stringency was increased by lowering the RSV concentration as the number of selection cycles increased. The RNA–virus complexes were also separated from the unbound RNA by high-speed centrifugation rather than filtering during cycles 4 and 9; the RNA–virus complexes formed a pellet along with any unbound virus while the unbound RNA remained in the supernatant. After 12 cycles, the selected RNA pool was tested for the ability to bind and neutralize RSV.

**Binding of the Selected RNAs to RSV.** The $^{32}P$-labeled RNA pool (or a cloned RNA sequence) obtained after 12 cycles of selection was incubated at 0.2–0.5 nM with various concentrations of RSV for 15 min at 37°C in 2.5 mM $MgCl_2$/100 mM NaCl/20 mM Tris·HCl, pH 7.5. The RNA–virus mixtures were filtered on prewetted nitrocellulose membranes; >95 of the RNA–RSV complexes were retained on the membrane, and the unbound RNAs were washed away by the binding buffer. The radioactivity retained on the membranes was measured by liquid scintillation counting and corrected for any background due to the binding of RNAs to nitrocellulose membrane.

**Virus-Neutralizing Activity of the Selected RNAs.** Fresh RSV suspension (Prague A strain, $\approx 10^5$ focus-forming units/ml) was supplemented with 2.5 mM $MgCl_2$, mixed with the selected RNA pool or a specific RNA sequence, and incubated at 37°C for 15 min. The RNA–virus mixture was placed onto $10^6$ quail fibrosarcoma cells (QT6 cell line), incubated for 1 hr at 37°C in a 5% $CO_2$ atmosphere, and then replaced with complete growth medium (19). At 18 hr postinfection, the cells were labeled with L-[$^{35}S$]methionine for 2 hr. Viral proteins in cell lysates and media were recovered by immunoprecipitation with an anti-RSV serum and analyzed on 12% polyacrylamide/0.1% SDS gels (19, 20). The yields of viral proteins on the gels were estimated by densitometry.

## RESULTS

***In Vitro* Selection of RSV-Binding RNA Sequences.** An RNA pool of $\approx 10^{15}$ different sequences, each 87 nt long, was subjected to selection by sucrose-gradient purified RSV particles to isolate the RNA molecules that bound the virus. The RSV-binding RNA sequences were then amplified and subjected to the next cycle of selection at a lower RSV concentration to increase the selection stringency (Fig. 1). Multiple cycles of selection and amplification resulted in an exponential increase of the best RSV-binding RNA molecules. After 12 cycles, the RSV concentration required to bind 50% of ≈0.5 nM selected RNA pool (dissociation constant, $K_d$) decreased to ≈3 μg of viral protein per ml, which was at least 1000 times lower than that required to bind 50% of the pool of random sequences (Fig. 2). The RSV concentration was expressed in terms of total viral protein concentration because the molar number of viral particles could not be accurately measured. Sequencing of the selected RNA pool revealed that the RNA sequences were no longer random and particular sequences appeared to dominate the population. The selected RNA pool was then tested for its ability to neutralize RSV.

**Neutralization of RSV by the Selected RNA Pool.** In the absence of any added RNA, avian cells (QT6) were efficiently infected by RSV as shown by the large amount of newly

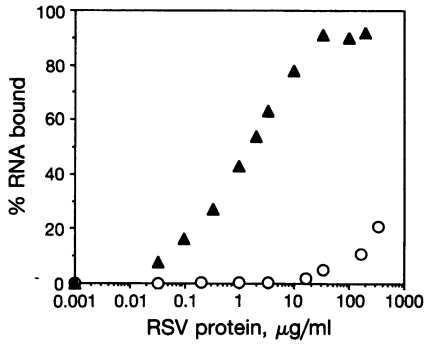


FIG. 2. Binding of 0.2–0.5 nM $^{32}P$-labeled RNA to RSV. ▲, Selected RNA pool obtained after 12 selection–amplification cycles; ○, random RNA pool. Because the detection limit for the $^{32}P$-labeled RNA was about 0.05 nM and the affinity between the selected RNA pool and RSV was high, the measured percentage of the bound selected RNA at low concentrations of RSV may be limited by the available binding sites on the virus rather than by the dissociation constant ($K_d$). Thus, the $K_d$ of 3 μg of viral protein per ml derived from such measurements may be an estimate of its upper limit.

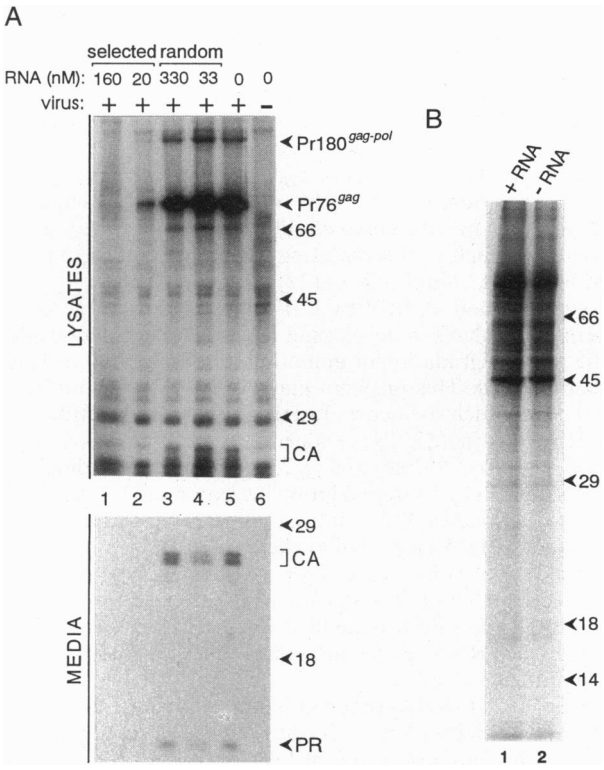


FIG. 3. Effect of the selected RNA pool on RSV infection of QT6 quail cells and on host cell protein synthesis. (*A*) Inhibition of RSV production. Viral proteins in cell lysates and media were recovered by immunoprecipitation with an anti-RSV serum and analyzed on 12% polyacrylamide/0.1% SDS gels. $Pr76^{gag}$, full-length Gag protein; $Pr180^{gag\text{-}pol}$, Gag-Pol fusion protein; CA (capsid proteins) and PR (protease), mature cleavage products derived from $Pr76^{gag}$. The RNA concentration in each virus sample is shown above each lane. However, the effective RNA concentration in each sample should be lower than the concentration shown here and in Fig. 4*A*, because up to 70% of the initially added RNA was found to be degraded within 75 min by nucleases in the RSV suspension. Unlike the sucrose gradient-purified RSV used for the selection, the RSV suspension used for the infection assay was freshly prepared and not purified, and thereby was more likely to be contaminated with ribonucleases from cell culture. (*B*) Effect of the selected RNA pool on the host cell protein synthesis. QT6 cells were treated in the absence of RSV with (lane 1) and without (lane 2) 500 nM selected RNA pool under the same conditions as used in *A*. After radiolabeling, total cellular proteins were analyzed by electrophoresis and autoradiography.

synthesized viral Gag ($Pr76^{gag}$) and Gag-Pol ($Pr180^{gag\text{-}pol}$) proteins in cell lysates, and capsid proteins (CA) and viral protease (PR) of the Gag cleavage products in growth media (Fig. 3*A*, lane 5) (19, 20). The presence of random-sequence RNA molecules at 330 nM had no effect, within experimental fluctuation, on the production of either the viral proteins or the background cellular proteins such as the 29-kDa band (Fig. 3*A*, lanes 3 and 4). In contrast, the selected RNA pool at 20 nM reduced the yield of viral proteins by 85–92% (Fig. 3*A*, lane 2, estimated by densitometry). At 160 nM, the selected RNA pool completely blocked RSV production in this assay (Fig. 3*A*, lane 1). The selected RNA pool did not affect the yield of the cellular proteins to a detectable extent (Fig. 3*A*, compare the background bands in lanes 1 and 2 with those in lane 5). Thus the RNA pool of selected sequences appeared to block RSV production by neutralizing the virus, rather than by adversely affecting the host cells. Such specific inhibition of RSV infection by the selected RNA pool and the cloned individual RNA sequences described below was reproduced in five and four independent sets of experiments, respectively.

The effect of the selected RNA pool on the host cell's growth and susceptibility to viral infection was further tested.

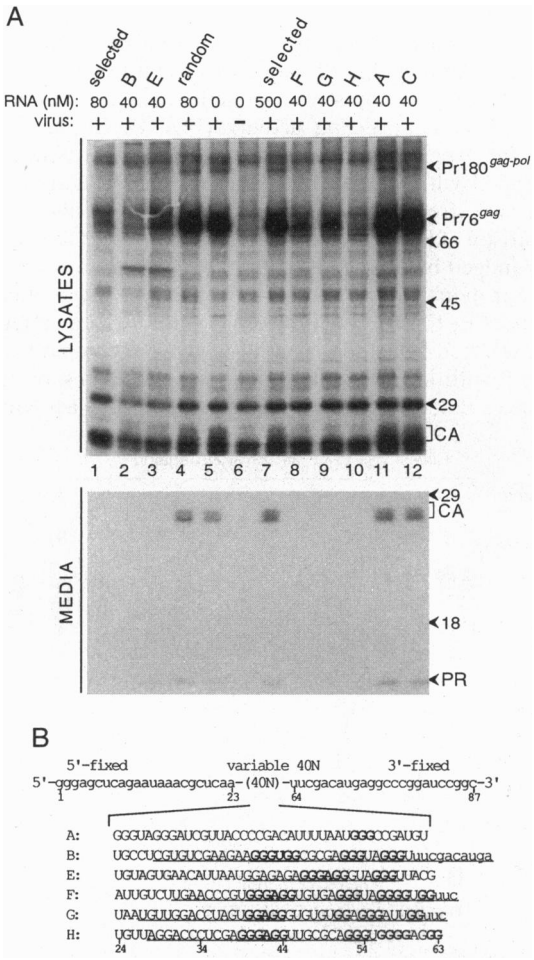


FIG. 4. (*A*) Inhibition of RSV by individual RNA sequences. The infections were performed in the same way as for the selected RNA pool (Fig. 3). In this particular experiment, viral protein $Pr76^{gag}$ overlapped with some of the background cellular proteins (comparison with lane 6). For lane 7, the QT6 cells were treated with the selected RNA pool (500 nM) for 1 hr and washed with medium prior to infection by RSV. (*B*) Nucleotide sequences of the RNAs that exhibited neutralizing activity (B, E, F, G, and H). Sequence A bound both RSV and nitrocellulose membrane and was the most abundant sequence in the selected pool but did not inhibit RSV. Each sequence consists of a 5′ fixed region, the variable 40 nt (40N) region (uppercase letters), and a 3′ fixed region. Bold letters show the groups of guanine nucleotides that appear at similar positions in at least two sequences. The underlined regions are the minimum sequences required for specific binding to RSV, as determined by the partial alkaline hydrolysis method (21).

Exposure of uninfected QT6 cells to the selected RNA pool at 500 nM did not affect cellular protein synthesis (Fig. 3*B*). Furthermore, pretreatment of cells with the selected RNA pool at 500 nM for 1 hr prior to infection by RSV did not change the yield of viral proteins (Fig. 4*A*, compare lane 7 with lane 5), indicating that the selected RNA pool had no effect on the susceptibility of the cells to RSV infection. In addition, the selected RNA pool showed no higher affinity for the host cells than did the random RNA pool (unpublished data), indicating no specific binding to the cells. All these results were consistent with the conclusion that the antiviral activity of the selected RNAs was due to their specific interactions with RSV.

**Specific RNA Sequences That Neutralize RSV.** To identify specific RSV-neutralizing sequences in the selected RNA pool, we cloned the DNA templates for the selected RNA pool into pUC19 vectors and sequenced 36 clones. The most prevalent sequence (sequence A) appeared in 9 clones, followed by

sequences B, C, D, and E, which were found in 4, 4, 3, and 2 clones, respectively. Each of the other 14 clones had a unique sequence. Among the 19 sequences identified from the 36 clones, RNA transcripts of 5 sequences (B, E, F, G, and H) showed RSV-neutralizing activity (Fig. 4). Because in the particular experiment shown in Fig. 4*A* viral protein $Pr76^{gag}$ overlapped with some of the background cellular proteins, which vary from one experiment to another (lane 6), the comparison of RSV production between different lanes may be better judged by the yields of $Pr180^{gag\text{-}pol}$ (in the lysates) and capsid proteins and protease (in the media). It was clear that sequences E, F, and G at 40 nM inhibited RSV as effectively as the selected RNA pool at 80 nM (Fig. 4*A*, compare lanes 3, 8, and 9 with lane 1). Side-by-side experiments showed that sequences B and H were 2- to 3-fold as effective as sequences

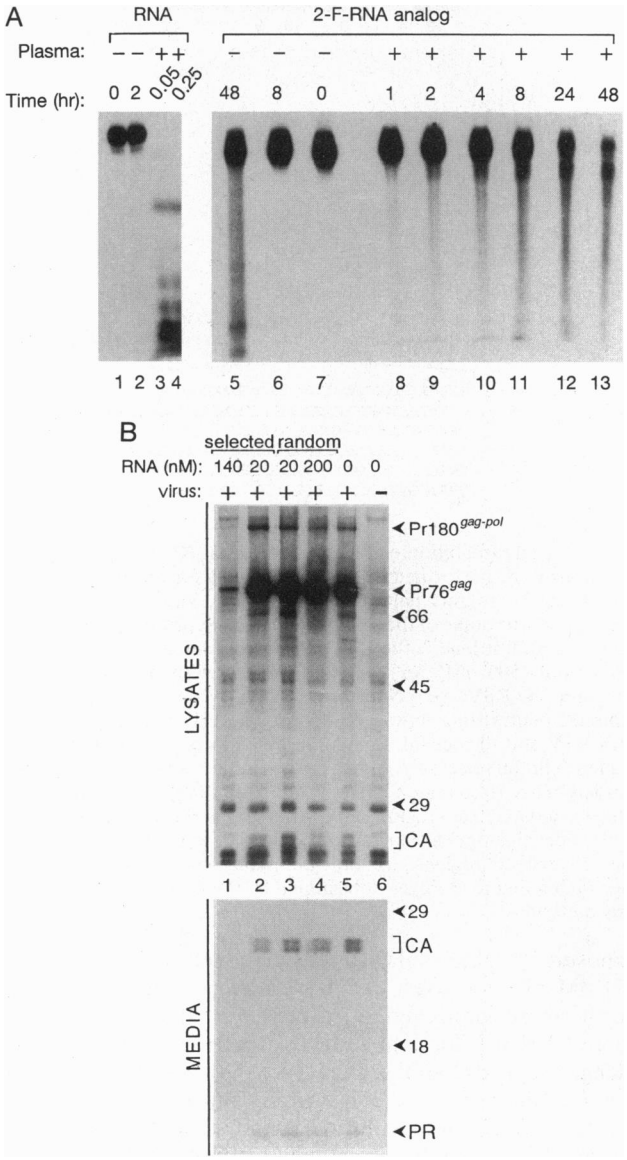


FIG. 5. (*A*) Stability of RNA and 2′-F-RNA of sequence H against degradation by nucleases in human plasma. The RNA and the RNA analog were incubated in human plasma supplemented with 2.5 mM $MgCl_2$ at 37°C for the times shown. Lane 5 shows the $Mg^{2+}$-enhanced background hydrolysis in 48 hr. The half-life of the RNA analog in human plasma (22 ± 4 hr) was about the same as those of the analog pools of the selected and random sequences. (*B*) Inhibition of RSV by the analog pool of the selected sequences. The analog pool was transcribed from the pool of sequences that was generated after 12 cycles of selection with an unmodified RNA pool.

E, F, and G in neutralizing the virus (unpublished data). Sequence A bound to RSV with an affinity comparable to that of sequences B and H ($K_d \approx 2$–3 μg of viral protein per ml) but had at least 4 times higher affinity for nitrocellulose membrane, which might explain why it was the most frequent sequence among the clones. Comparison of the cloned sequences revealed that groups of guanine nucleotides appeared at similar positions in all the RSV-neutralizing RNA sequences (Fig. 4*B*), but no consensus could be discovered among their secondary structure (base-pairing) diagrams that were generated by energy minimization (22).

**Neutralization of RSV by 2′-F-RNA Analogs.** One of the potential problems in developing RNA as a therapeutic agent is the rapid degradation of unmodified RNA by ribonucleases present *in vivo*. This problem may be solved by a number of approaches, such as incorporating modified nucleotides into the RNA chain (23, 24). Indeed, the incorporation of 2′-fluoro-2′-deoxycytidine and 2′-fluoro-2′-deoxyuridine into the RNA chain by T7 transcription increased the RNA stability against the nuclease digestion by more than 2 orders of magnitude (Fig. 5*A*). A similar increase in RNA stability was observed when either the RNA pool of random sequences or the selected RNA pool or sequence H was tested (unpublished data), indicating little dependence on RNA sequence (presumably each RNA chain contained about the same number of pyrimidines).

A pool of 2′-F-RNAs of the same selected sequences as used in Fig. 3*A* inhibited RSV infection by ≈85% (comparison of lane 1 with lanes 3, 4, and 5 of Fig. 5*B* by densitometry) and ≈75% (unpublished data) when used at 140 nM and 100 nM, respectively. In contrast, the level of 85% inhibition was achieved by only 20 nM unmodified RNA pool (Fig. 3*A*, lane 2), indicating that the analog pool was 7–10 times less effective in neutralizing RSV. The RNA analogs of random sequences at 200 nM had no effect on RSV infection (Fig. 5*B*, lanes 3 and 4). Neither selected nor random sequences in the analog pool at up to 200 nM affected host cell protein synthesis (Fig. 5*B*, lanes 1 and 4). Because each of the 12 cycles of selection was performed with unmodified RNAs, it was likely that the lower anti-RSV activity of the RNA analogs was due to the RNA structural changes induced by the 2′-fluoro modification in pyrimidines. Nevertheless, the neutralizing activity of the analogs at high concentrations implied that some structural features of the unmodified RNA important for antiviral activity remained.

To test whether selection of RNA analogs would lead to a new pool with improved neutralizing activity, we transcribed a pool of 2′-F-RNAs from the sequences that were generated after 9 cycles of selection by RSV from an unmodified RNA pool, and then continued the selection with the analog pool for another 3 cycles. The resulting pool of 2′-F-RNAs inhibited RSV production by 75% when used at 22 nM (unpublished data), whereas the same level of inhibition was achieved at 100 nM for the analog pool transcribed after 12 cycles of selection with an unmodified RNA pool. Thus, the selection with 2′-F-RNAs for only 3 cycles improved the anti-RSV activity by 4.5-fold. An even more potent anti-RSV analog pool would have likely been obtained if all the 12 cycles of selection had been done with analog pools. Nevertheless, it is clear that ribonuclease-resistant antiviral RNA analogs can be obtained either by selecting from an unmodified RNA pool and then transcribing the selected sequences with modified nucleotides or by direct selection from an RNA analog pool.

## DISCUSSION

Because of the permeation problem imposed by the RSV membrane, the selected RNAs are expected to inhibit RSV infection by binding to the virus surface and not by entering the virus. However, the identification of the RNA binding sites on

RSV and of the roles of these viral components in the virus infection has not yet been completed.

**Selection for RNAs Inhibiting Multiple Strains of Virus.** Although the selections were performed with the Prague A strain of RSV, preliminary results indicated that the selected RNAs also inhibited the Prague C strain of RSV. The RNA concentration required to inhibit the Prague C strain was about 15–20 times higher than that needed to achieve the same level of inhibition of the Prague A strain (unpublished data). The surface glycoprotein (gp85) of these two strains contains conserved regions (≈95% homology) as well as three or four small variable regions (subgroup-determining regions) (25, 26). If the selected RNAs and analogs neutralize RSV by interacting with the surface protein, it is likely that a small number (e.g., ≈5%) of the Prague A-neutralizing sequences in the selected pool bind to the conserved regions of gp85 and thus also neutralize the Prague C strain.

The selection may also be performed by alternating between several strains of a virus during the different cycles of selection to specifically isolate the RNAs and RNA analogs that would bind to the viral conserved structural motifs and thereby inhibit diverse strains of the virus (S. Dallabrida, J. C. Sanders, E. M. Eyster, and J.-F.W., unpublished data). The isolation of RNAs and RNA analogs that are effective against diverse viral strains will be particularly necessary for inhibiting viruses such as HIV, which has a high mutation rate and readily develops drug-resistant strains. Such selection is possible and practical because a very large structurally diverse library is used and the selection is a rapid process.

**Selection by Intact Biological Entities.** Although an isolated viral protein can be used in the *in vitro* selection for the protein-binding RNAs and some of the selected sequences may inhibit viral infection, there are at least three advantages to the use of intact viral particles in the selection. (*i*) The selection by intact virus does not require a full understanding of the usually very complex mechanism of viral infection, whereas the selection by an isolated viral protein is limited to only a few cases in which the proteins responsible for the viral infection have been identified and isolated. (*ii*) Because of structural differences in the protein (27, 28), the RNA selected to bind a viral protein in its purified form may not interact with the protein complexed on a virion. (*iii*) The selection using intact virus may lead to the identification of viral components that have not previously been known for their critical roles in viral infection. The success of isolating numerous anti-RSV RNAs and RNA analogs from a large pool of random sequences immediately suggests the potential application of this approach to isolate RNA and nuclease-resistant RNA analogs against other viruses such as HIV.

The selection of RNAs (or DNAs) and their analogs may also be performed by other biological entities such as bacteria, yeast, and tumor cells (S.G., W.P., and J.-F.W., unpublished data). The selected nucleic acids and their analogs would bind to these cells and may change their biological functions by affecting cellular interactions such as signal transduction pathways. Therefore, the selection of RNAs and RNA analogs by intact viruses or cells may provide valuable tools for studying virus–cell and intercellular interactions. The selected RNAs and analogs may be used to control such interactions so that the infection by virus, bacterium, or yeast or the proliferation or death of cells may be inhibited.

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