Enzymatic Amplification of RNA Purified from Crude Cell Lysate by Reversible Target Capture

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For increased clinical applications of nucleic acid probes to gene diagnosis, current procedures must be modified to become more amenable to the rapid processing of many samples with as few manipulations as possible. Here we summarize progress in the development of a strategy for performing molecular hybridization directly in lysate of biological samples dissolved in solutions containing the chaotropic agent, guanidine thiocyanate. Hybrids are purified by a process referred to as "reversible target capture," in which specific nucleic acid sequences are rapidly purified from crude lysate. We illustrate the use of this strategy to assay HIV-1 RNA and to rapidly purify HIV-1 RNA before enzymatic amplification by the polymerase chain reaction method.

Molecular hybridization is the specific interaction of complementary nucleic acids, resulting in the formation of highly stable duplexes. Advances in molecular cloning technology, combined with the development of chemical means to produce synthetic oligodeoxyribonucleic acids of known sequence, have made it routine to generate nucleic acid probes that can be used in a clinical setting to perform gene diagnosis (1).

The requirements for performing molecular hybridization in a research setting differ from those of a clinical setting. In the research laboratory, methods to prepare nucleic acids are generally time consuming, and can require extraction with organic solvents and high-speed centrifugation. These methods are not easily adapted to a clinical setting, in which many samples, from a wide variety of biological sources, must be processed in a single day.

In the strategy we developed to overcome these difficulties (2-5), biological samples were first dissolved in a solution containing guanidine thiocyanate, a chaotropic agent, which facilitated lysis of cells and dissolution of macromolecular complexes. Guanidine thiocyanate supports molecular hybridization (2), and is a potent inhibitor of cellular nucleases (6). Nucleic acid probes were then added, and hybridization was accomplished at 37 °C directly in the lysate. The advantage of this strategy was that nucleic acids did not need to be extracted and purified before hybridization. Also, molecular hybridization was driven in solution with excess probe—a more rapid and efficient reaction than the more-conventional blot hybridization strategy, in which hybridization occurs between probe in solution and target nucleic acids immobilized on membranes. In addition, this approach obviated uncertainties arising from loss of nucleic acids during purification and binding to membranes.

We also adopted a strategy referred to as "reversible target capture" (3), to selectively purify hybrids from the crude cell lysate after hybridization. In this method, two types of probes were hybridized, a "labelprobe," which provided signal, and a dA-tailed synthetic oligonucleotide "captureprobe," which allowed for selective purification of the target nucleic acid. After hybridization, ternary hybrids—consisting of the target nucleic acid, the labelprobe, and the captureprobe—were captured onto paramagnetic beads coated with oligo-dT (dT-beads), which bound to the poly(dA) portion of the captureprobe (3). In this sandwich hybridization strategy, labelprobe cannot bind to beads unless it is bound to target, and the target is bound by a captureprobe. The advantage of this capture strategy is the reversible nature of the captureprobe:bead interaction. Ternary hybrids can be eluted from dT-beads simply by raising the concentration of chaotrope, then recaptured onto fresh dT-beads by lowering the chaotrope concentration. Repeated cycles of capture effectively eliminated background associated with the nonspecific interaction of labelprobe with material in the lysate, and dT-beads, thereby increasing the range of sensitivity (3-5). In this strategy, the specificity of hybridization can be adjusted via both the labelprobe and the captureprobe, thereby increasing the confidence that signals result from true probe:target interactions.

Reversible target capture can also potentially be used to purify target nucleic acids, which can then be used for further manipulation. Nucleic acids can be purified by performing hybridization with captureprobe alone, capturing the hybrid onto dT-beads as described above, then eluting the target nucleic acid from the last set of dT-beads in any convenient buffer. In the following, we demonstrate that reversible target capture can be used to purify HIV-1 RNA from crude cell lysate and then amplified by use of the polymerase chain reaction method (7-9).

Materials and Methods

Solutions and Probes

Sources of reagents and a description of the devices used in reversible target capture have appeared elsewhere (4,5). Lysis buffer consisted of 5 mol of guanidine thiocyanate, 0.1 mol of EDTA (pH 8.0), and 100 g of dextran sulfate per liter. SSC buffer was 150 mmol of NaCl and 15 mmol of sodium citrate per liter. Binding buffer was 0.75 mol/L sodium phosphate buffer (pH 6.8), containing 5 g of sodium laurylsarcosine, 2 g of acetylated bovine serum albumin, and 10 mg of degraded herring sperm DNA per liter. Wash buffer was a 30/70 mixture by volume of lysis buffer (without dextran sulfate) and binding buffer. Release buffer was 3.25 mol of guanidine thiocyanate and 65 mmol of EDTA (pH 8.0) per liter.

The labelprobe we used was a 32P-labeled anti-sense RNA probe (specific activity = 1.2 × 10⁶ counts/min per microgram) complementary to the pol region of the HIV-1 genome, and was synthesized from the clone pGAP as described (10). Oligonucleotide primers "A" and "B" used
for enzymatic amplification, captureprobes 839 and 1197, dT-beads, and devices for performing reversible target capture were supplied by GENE-TRAK Systems, Framingham, MA.

Cell Cultures

The cell line, H9, and the HIV-1 chronically-infected cell line, H9im, were grown and maintained at 37 °C in CO2-enriched (50 mM/L) air in a humidified incubator. Growth medium consisted of RPMI 1640 (Mediatech, Washington, DC) supplemented with, per liter, 100 mL of heat-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, NY), 50 USP units of penicillin, 50 mg of streptomycin (Hazleton Research Products, Denver, PA), and 2 mmol of L-glutamine (Mediatech).

Ampligen treatment. H9 cells (5 × 10⁶ cells) were pelleted and resuspended in 5 mL of either growth medium alone, or growth medium supplemented with poly(I):poly(C₃₃⁵U) (Ampligen, 50 mg/L; HEM Research Inc., Rockville, MD), then cultured for 24 h.

Infections. The source of HIV-1 virus for infections was undiluted H9im culture fluid that had been filtered through 0.45-μm (pore-size) membranes just before use. Cells grown for 24 h in the presence or absence of Ampligen were pelleted and resuspended in 1 mL of virus-containing H9im culture fluid, incubated for 5 h at 37 °C, washed twice with growth medium, then cultured at a density of 6 × 10⁶ cells/mL in either medium alone or medium supplemented with Ampligen (50 mg/L). Cells were removed on subsequent days, pelleted, and dissolved in lysis buffer to give a concentration of 10⁷ cells/mL. Fresh medium (containing no drug) was added to all cultures on days that cells were removed to maintain cell densities at 0.25–1 × 10⁶ cells/mL.

Molecular Hybridization

Hybridizations were performed essentially as described elsewhere (4). In brief, 20 μL of cell lysate (2 × 10⁶ cells) in lysis buffer was combined with 5 μL of 2 × SSC containing 10⁶ counts/min of labelprobe and 10 ng each of captureprobes 839 and 1197 (5), then incubated at 37 °C for 16 h.

Reversible Target Capture

dT-beads were prehybridized in binding buffer overnight at 37 °C (4). Hybridization reactions were diluted with 50 μL of dT-bead suspension and incubated at 37 °C for 10 min. The dT-beads were magnetically separated from solution and washed twice with 400 μL of wash buffer. The hybrids were eluted from the dT-beads in 50 μL of release buffer by incubating at 37 °C for 10 min. Release buffer containing the hybrids was transferred to a fresh tube, and the hybrids were recaptured onto fresh dT-beads by adding 50 μL of dT-beads suspended in binding buffer and incubating the suspensions at 37 °C for 10 min.

Quantification of Hybridization

Hybrids were eluted from the third set of dT-beads in 100 μL of TE (10 mmol of Tris HCl, pH 8.0, and 1 mmol of EDTA per liter) at 65 °C for 5 min. The dT-beads were magnetically separated, and the fluid containing the hybrids was applied to Ready Caps (Beckman Instruments, Fullerton, CA) and dried; the radioactivity was counted in a liquid scintillation counter (11).

Enzymatic Amplification of Purified RNA

H9im lysate was diluted into lysate from the noninfected myeloid blood-cell line K562 to maintain the total concentration of cell lysate at 10⁷ cells/mL; 20 μL of lysate was combined with 5 μL of 2 × SSC containing 10 ng each of captureprobes 839 and 1197 (5), then heated at 37 °C for 20 min. Hybrids were purified by use of three cycles of capture as described above. After the third capture, dT-beads were washed three times with KTE (500 mmol of KCl, 10 mmol of Tris HCl, pH 8.0, and 1 mmol of EDTA per liter), and the hybrids were eluted in 50 μL of TE (see above) at 65 °C for 10 min. Oligonucleotide primers A and B (0.1 nmol each in 2 μL of water) were added, and the tubes were heated to 65 °C for 10 min, then allowed to cool to room temperature for 10 min. The reactions were diluted with 50 μL of 2 × RT/Taq reaction buffer (per liter: 20 mmol of Tris HCl, pH 8.3, 100 mmol of KCl, 3 mmol of MgCl₂, 2 g of gelatin, and 0.4 mmol each of dATP, dCTP, dGTP, and TTP) containing 200 units of MuMLV reverse transcriptase (BRL, Gaithersburg, MD) and 2.5 units of Taq DNA polymerase (Perkin-Elmer Cetus Corp., Emeryville, CA). The reactions were overlaid with 100 μL of mineral oil to prevent evaporation, heated at 37 °C for 1 h, then subjected to 30 cycles of amplification. The temperatures and times of each cycle were as follows: 1 min at 95 °C, 1.5 min at 37 °C, and 3 min at 68 °C. For temperature cycling we used an automatic thermocycler (ERICOMP Inc., San Diego, CA).

Results

Using probes, reagents, and devices for quantifying concentrations of HIV-1 RNA as described elsewhere (5, 6), we examined the effect of the antiviral drug, poly(I):poly(C₃₃⁵U), or Ampligen, which has previously been shown to inhibit HIV-1 replication in vitro (12), on HIV-1 RNA concentrations during acute infection in vitro. Figure 1 compares the hybridization signals obtained with HIV-1 RNA in lysate of 2 × 10⁵ H9 cells that were grown for 24 h in the presence or absence of Ampligen before infection. Both the rate of appearance, and the magnitude, of the

![Fig 1. Effect of Ampligen on the accumulation of HIV-1 RNA in H9 cells during lytic infection in vitro](image-url)
hybridization signals were lower in lysate of cells grown in the presence of Ampligen than in lysate of cells grown in medium alone. These results indicate that hybridization to HIV-1 RNA in crude cell lysate combined with reversible target capture can be used to determine the effect of antiviral agents on replication and gene expression of HIV-1.

HIV-1 viral antigens in serum of infected individuals often are undetectable between the period of seroconversion and onset of clinical symptoms (13). Instead, HIV-1 RNA could serve as an alternative viral product for determining virus load, and changes in the number of HIV-1 RNA molecules in peripheral blood cells could potentially be used to determine the efficacy of antiviral drug therapies. Using the above technique, we have quantified HIV-1 RNA in peripheral blood cells of 70 patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC) (14). With this assay, 15 of 29 AIDS patients' samples, and 20 of 41 ARC patients' samples, were found to contain detectable amounts of HIV-1 RNA (>3 × 10^6 RNA molecules per 2 × 10^9 peripheral blood mononuclear cells). However, for the remaining patients, the assay lacked the sensitivity to detect HIV RNA.

One means of overcoming the difficulty of detecting rare mRNA sequences is to amplify the sequences by using the polymerase chain reaction amplification procedure (9). To see if HIV-1 RNA purified by reversible target capture could serve as a template for enzymatic amplification, we diluted H9-infected lysate into lysate of the noninfected leukemic blood-cell line, K562, and purified HIV-1 RNA from the lysates by three cycles of capture. The purified RNA was eluted from the third set of d-T-beads, reverse-transcribed into a DNA copy, then enzymatically amplified (7). The reaction products were analyzed by Southern blot hybridization (Figure 2). Amplification product of the expected length (220 bp) was observed with lysate in quantities equivalent to as few as 20 H9-infected cells in a background of 2 × 10^6 noninfected K562 lysate (lane 6). This ratio of infected to noninfected cells corresponds to that found in the peripheral blood of infected individuals (15). No signal was obtained in the reaction that did not first include the reverse transcription step (lane 2), confirming that signal arose from RNA sequences. No signal was obtained with K562 lysate alone (lane 7), indicating that signal was dependent upon the presence of H9-infected lysate in the samples.

**Discussion**

In the strategy described, molecular hybridization was performed directly in crude cell lysate. The advantage is that nucleic acids did not need to be purified before hybridization. Hybrids were purified from cell lysate and unreacted probe by the reversible target capture method (3), which utilizes a bipartite probe, the captureprobe, capable of binding to both the target nucleic acid and to paramagnetic beads. Probes designed to measure HIV-1 sequences were used to determine the effect of the antiviral drug, Ampligen, on synthesis of HIV-1 RNA during lytic infection in vitro.

Because this purification scheme relies on the ability of the captureprobes to hybridize with their intended target, reversible target capture is also particularly suited for the isolation of specific nucleic acid sequences from crude cell lysate. We demonstrated that RNA purified by this technique can serve as template for enzymatic amplification. The potential to adjust the specificity of purification by adjusting the stringency of the captureprobe hybridization could reduce problems associated with amplification of sequences to which many related sequences exist. A set of captureprobes could be designed either to cross-react with a variety of related sequences or to react with a single species.

It is apparent from Figure 2 that the conditions used for amplification did not result in the production of signal that was proportional to the amount of target present; e.g., the sample containing lysate from 2000 infected cells (lane 4) generated a signal greater than the sample containing 20 000 infected cells (lane 3). We are currently investigating amplification conditions that will produce quantitative results.

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**References**