Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format

(T7 RNA polymerase/*in vitro* nucleic acid amplification)

D. Y. Kwoh, G. R. Davis, K. M. Whitfield, H. L. Chappelle, L. J. DiMichele, and T. R. Gingeras*

SISKA Diagnostics and The Salk Institute Biotechnology/Industrial Associates, Inc., La Jolla, CA 92037

ABSTRACT   The *in vitro* amplification of biologically important nucleic acids has proceeded principally by a strategy of DNA replication. Polymerase chain reaction was the first such protocol to achieve this goal. In this report, a transcription-based amplification system (TAS) is described. Each cycle of the TAS is composed of two steps. The first is a cDNA synthesis step that produces one copy of a double-stranded DNA template for each copy of RNA or DNA target nucleic acid. During the course of this cDNA synthesis step, a sequence recognized by a DNA-dependent RNA polymerase is inserted into the cDNA copy of the target sequence to be amplified. The second step is the amplification of the target sequence by the transcription of the cDNA template into multiple copies of RNA. This procedure has been applied to the detection of human immunodeficiency virus type 1 (HIV-1)-infected cells. After four cycles of TAS, the amplification of the *vif* region of the HIV-1 RNA genome was measured to be, on the average, 38- to 47-fold per cycle, resulting in a 2–5×10^6-fold increase in the copy number of the original target sequence. This amplification by the TAS protocol allows the detection of fewer than one HIV-1-infected CEM cell in a population of 10^6 uninfected CEM cells. Detection of the TAS-generated RNA from HIV-1-infected cells can easily be accomplished by means of a bead-based sandwich hybridization protocol, which provides additional specificity for the identification of the amplified HIV-1-specific sequence.

The need to detect, identify, and subsequently isolate nucleic acid molecules that are present in very small quantities has long been a difficult problem for clinicians and molecular biologists. An efficient solution to this problem has been developed by the *in vitro* replication of specific segments of the target nucleic acid of interest. Saiki *et al.* (1, 2) were the first to describe a method to accomplish this task by the use of repeated cycles of oligonucleotide primer-directed DNA syntheses. This method (2), called polymerase chain reaction (PCR), has successfully been employed to detect and clone several genetic mutations, in such genes as β-globin (1, 3), factor VIII (4), HLA (5, 6), and dystrophin (7), as well as for the forensic identification of individuals (8) and for detecting specific translocation events characteristic of follicular lymphomas (9). In addition to genetic mutations, the detection and identification of low-copy-number viruses, such as human immunodeficiency virus type 1 (HIV-1) (10) and human T-cell leukemia virus type I (11, 12), have been reported after the use of the PCR amplification protocol.

Enhancements of the PCR method have been reported, including the use of thermal-stable DNA polymerase from *Thermus aquaticus* (13, 14) and the addition of the recognition sequence from the T7 RNA polymerase to the oligonucleotide primers, so that after multiple cycles of PCR, RNA transcripts can be produced from the PCR-amplified DNA (15, 16). In these enhancements of the PCR protocol, the principal method of amplification relies on the use of DNA replication of a defined region of the target sequence.

In this report, a transcription-based amplification system (TAS) is described in which the production of RNA copies of the target sequence provides the principal means of *in vitro* nucleic acid amplification. Using both HIV-1 RNA and proviral DNA targets from HIV-1-infected cells, the efficiency of the steps involved in the TAS protocol is described quantitatively, and the manner in which the RNA products are captured and detected using bead-bound oligonucleotides (17, 18) is also illustrated.

MATERIALS AND METHODS

Materials. Uninfected and HIV-1-infected lymphocyte cells (CEM) and plasmid pARV7A/2 containing a cDNA copy of the HIV-1 genome inserted into the *Eco*RI site of pUC19 (19) were obtained from D. Richman (University of California, San Diego). Extractor columns were purchased from Molecular Biosystems (San Diego, CA). Avian myeloblastosis virus reverse transcriptase (RT) was purchased from Life Sciences (Saint Petersburg, FL). T7 RNA polymerase was purchased from Stratagene, Pharmacia, and New England Biolabs. Oligonucleotides were synthesized by phosphoramidite chemistry by using either an Applied Biosystems model 380A or a Biosearch model 3600 synthesizer. The oligonucleotides used as primers and detection probes are specific for HIV-1 and correspond to the sequences reported by Ratner *et al.* (20) for the *vif* gene (21).

RNA Purification. HIV-1 RNA was extracted in total RNA from HIV-1-infected CEM cells by the guanidinium isothiocyanate/cesium chloride gradient procedure (22).

HIV-1-Infected Sample Preparation. Uninfected CEM cells or HIV-1-infected cells were pelleted by low-speed centrifugation. The cell pellet was resuspended in 1 ml of 0.15 M NaCl/10 mM Tris·HCl, pH 7.5/50 mM EDTA/0.2% SDS/proteinase K (1 mg/ml). After the lysate was Vortex mixed vigorously, it was incubated at 50°C for 45 min with occasional Vortex mixing. An Extractor column was used to partially purify the nucleic acids. The lysate was loaded onto a column equilibrated in 20 mM Tris·HCl (pH 7.5), and unbound material was removed by washing the column with 4 ml of 0.3 M NaCl/20 mM Tris·HCl, pH 7.5. Total nucleic acids were eluted from the column with 4 ml of 0.5 M NaCl/20 mM Tris·HCl, pH 7.5. After addition of 0.4 ml of 3 M sodium acetate plus 100 μg of glycogen, the eluted nucleic acid was precipitated with 2.5 vol of ethanol at −20°C for 2 or more hr.

The pelleted nucleic acid was resuspended in 10 mM Tris·HCl, pH 7.4/1 mM EDTA (TE) and further purified by chromatography on a Sephadex G-50 spin column (22). The eluted nucleic acids were precipitated with ethanol and resuspended in TAS amplification buffer.

TAS. HIV-1 RNA or total extracted nucleic acid was resuspended in a total volume of 100 μl of TAS amplification buffer (40 mM Tris·HCl, pH 8.1/8 mM MgCl₂/25 mM NaCl/2 mM spermidine hydrochloride/5 mM dithiothreitol) containing bovine serum albumin at 80 μg/ml with 200 μM dATP, 200 μM dTTP, 200 μM dGTP, 200 μM dCTP, 1 mM ATP, 1 mM GTP, 1 mM CTP, 1 mM UTP, and 0.25 μg of HIV-1-specific oligonucleotides 88-77 (5'-*AATTTAATACGACTC-ACTATAGGGA*CACCTAGGGCTAACTATGTGTCC-TAATAAGG-3') and 25 μg of 86-29 (5'-ACACCTATG-TATGTTTCAGGGAAAGCTA-3'). [The italic portions of oligonucleotides 88-77 and 87-263 refer to the T7 RNA polymerase-binding sequence (PBS) and the preferred transcriptional initiation site (23-26); the remainder of each sequence represents the target-complementary sequence (TCS), which targets each oligonucleotide.] The sample was heated to 65°C for 1 min and annealed at 42°C for 1 min, after which 10 units of avian myeloblastosis virus RT was added. After a further 12-min incubation at 42°C, the sample was heated at 100°C for 1 min and annealed at 42°C for 1 min, and an additional 10 units of RT was added. The sample was incubated for 12 min at 42°C. One hundred units of T7 RNA polymerase was added, followed by a 25-min incubation at 37°C. Multiple cycles of TAS involved repeating the steps above, except that the initial 1-min incubation at 65°C was replaced with a 1-min denaturation step in subsequent cycles. This protocol represents a TAS type I protocol. A modification of this procedure has also been used, designated the TAS type II protocol. The type II protocol differs after the initial cDNA synthesis step (12 min at 42°C with RT); the sample was denatured at 100°C for 1 min and annealed at 37°C for 1 min, and 10 units of RT plus 100 units of T7 RNA polymerase were added simultaneously. Incubation continued at 37°C for 30 min. Subsequent cycles were repeated from the denaturation step. To detect the products of the TAS reaction, aliquots were taken at various times and denatured in 7.4% (vol/vol) formaldehyde/10× SSC (21) at 55°C for 20 min. After chilling on ice, the samples were immobilized onto a nitrocellulose membrane with the use of a slot-blot apparatus (Schleicher & Schuell), and the nucleic acids were fixed to the nitrocellulose by UV irradiation (27). The filters were prehybridized at 55°C for 10 min in 0.5% bovine serum albumin/0.5% polyvinyl pyrrolidone/5× SSPE (22)/1% SDS and then hybridized in the same buffer with oligonucleotide probe 86-31 (5'-GCACACAAGTAGACCCTGAACTAGCA-GACCA-3') or 86-32 (5'-TGGTCTGCTAGTTCAGGGTCT-ACTTGTGTGC-3') at 2-5 × 10⁶ cpm/ml, which are used as detection probes for the negative and positive strands of the amplified product, respectively. The filters were hybridized for 1 hr at 55°C, then washed for three 5-min periods at room temperature using 1× SSPE/1% SDS and washed once for 1 min at 55°C with the same buffer. The filters were autoradiographed overnight at −70°C with one intensifying screen on Kodak XAR film.

Bead-Based Sandwich Hybridization. Sephacryl beads containing oligonucleotide 87-83 (5'-ATGCTAGATTGGTAA-TAACAACATATT-3') were prepared as described (18). This bead-bound oligonucleotide was used to capture the TAS-amplified HIV-1 sequences after these targets had hybridized to ³²P-labeled detection oligonucleotide 86-31.

In a typical bead-based sandwich hybridization experiment, the TAS-amplified target and ³²P-labeled oligonucleotide 86-31 were denatured in 10 μl of TE containing 0.2% SDS at 65°C for 5 min in an Eppendorf tube. To this, 10 μl of a 2× solution hybridization mixture [10× SSPE/10% (wt/vol)

dextran sulfate] was added. The solution was mixed and incubated at 42°C for 2 hr.

Approximately 50 mg (wet weight) of Sephacryl beads was prehybridized in 250 μl of hybridization solution (5× SSPE/10% dextran sulfate/0.1% SDS) for 30 min at 37°C. Immediately prior to the capture step, the beads were centrifuged for 10 sec, the prehybridization solution was removed, and 80 μl of fresh hybridization solution was added, after which the hybridization reaction mixture was transferred to the beads. The beads were then incubated at 37°C for 1 hr with occasional mixing.

After the bead hybridization step, the beads were centrifuged and the hybridization solution was transferred to a scintillation counter vial. The beads were then washed five times with 2× SSC at 37°C. The first three washes were rapid; 1 ml of 2× SSC was added, and the beads were mixed well and centrifuged 10 sec. For the final two washes, 1 ml of 2× SSC was added, and the beads were mixed and incubated at 37°C for 5 min before being centrifuged. Radioactivity of the hybridization solution, each of the five washes, and beads was measured by Cerenkov counting for 5-10 min. Scintillation counter background was subtracted from all samples (20-40 cpm). The percent target detected was calculated as follows: [(cpm on beads/total cpm) × fmol of oligonucleotide/fmol of target] × 100. The total cpm was the sum of the cpm for the hybridization solution, five washes, and beads.

RESULTS

TAS Strategy and Efficiency of Amplification. Two cycles of the TAS protocol are illustrated in Fig. 1. With either RNA or DNA as the target sequences to be amplified, each cycle is composed of a DNA synthesis and RNA transcription step. During the cDNA synthesis part of a TAS cycle, short nucleotide sequences recognized by a DNA-dependent RNA polymerase (the PBSs) are positioned on the 3' side of the region of the target sequence that is to be amplified. This is accomplished through the use of an oligonucleotide primer containing two domains. A target-complementary sequence positions the PBS in the desired location prior to amplification. This oligonucleotide (primer A) is employed in a primer-extension reaction using RT or DNA polymerase, depending upon the target sequences to be amplified. A second oligonucleotide (primer B) is used in a similar primer-extension reaction to make a double-stranded PBS-containing cDNA copy of the target sequence (Fig. 1). Sequences recognized by the RNA polymerases T7, T3, and SP6 can be employed in the PBS domain, although the experiments described will be limited to the use of the T7 RNA polymerase.

As indicated in Fig. 1, steps D–F, the RNA produced during the first TAS cycle can serve as target sequences for additional TAS cycles. Since DNA-dependent RNA transcription can produce from 10 to 1000 RNA copies per DNA template (24), only a few cycles of TAS are required to achieve large-scale amplification of target nucleic acid sequences. Fig. 2A shows the amplification detected after one cycle of TAS in which a 50-fold increase in the copy number of the target RNA was achieved. This result emphasizes the point that the amplification was achieved during the T7 RNA polymerase transcription step and not through the cDNA synthesis step. Interestingly, while the T7 RNA polymerase is transcribing RNA copies of the target, the residual RT and oligonucleotide primer B (Fig. 1, step D) are synthesizing a cDNA copy of the amplified RNA transcript (Fig. 2A). It is for this reason that oligonucleotide 86-32 can detect products from the amplification, even though it is not complementary to the RNA product produced by the T7 RNA polymerase. If the RT was inactivated (by heating to 65°C for 5 min) prior to the transcription step in a TAS cycle, only the hybridization to the original target RNA and first-strand cDNA was observed with oligonucleotide probe 86-32 (Fig. 2B); how-
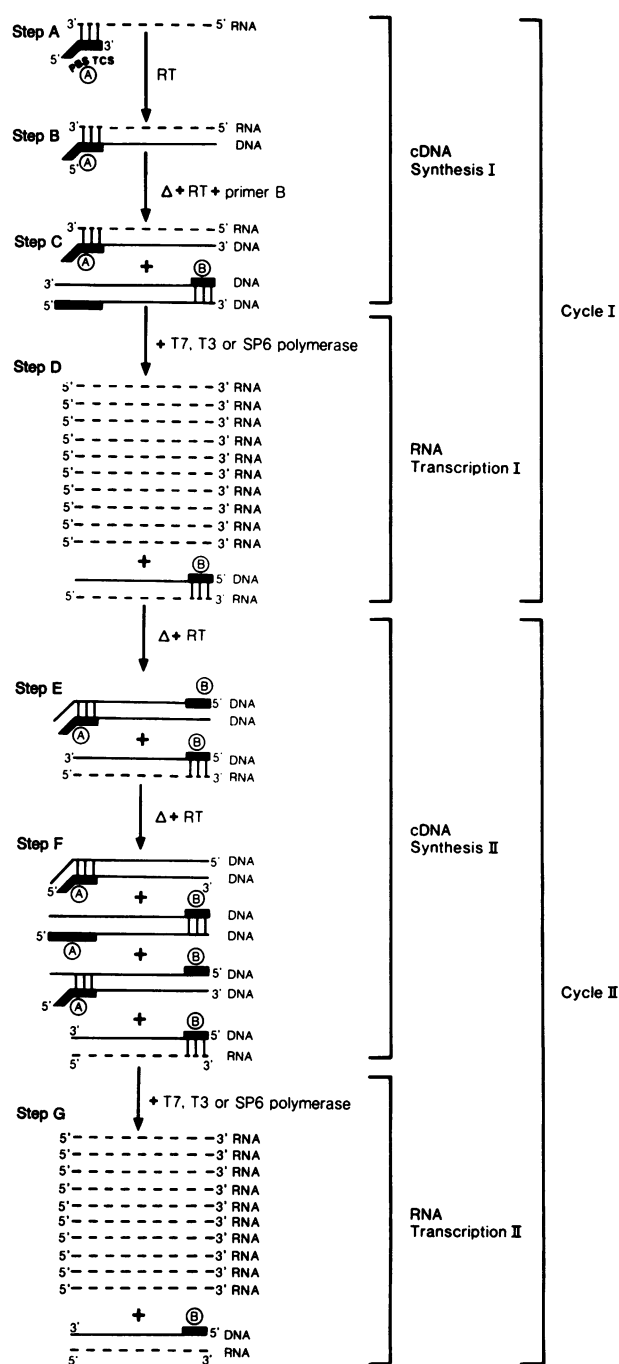
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FIG. 1. TAS. A two-cycle scheme for amplifying an RNA target sequence (– – –) using sequential cDNA synthesis and RNA transcription is displayed as seven steps. A target nucleic acid molecule RNA (or denatured DNA) is hybridized to a primer oligonucleotide (primer A) that contains a PBS (for T7, T3, or SP6 polymerase) and a target-complementary sequence (TCS) (step A). RT elongates primer A to yield a newly synthesized DNA strand complementary to the target RNA (step B). The RNA·DNA heteroduplex is denatured by heat (Δ) and oligonucleotide B is annealed to the newly synthesized DNA strand containing the PBS. RT is added to produce a double-stranded cDNA and a new DNA·RNA heteroduplex (step C). Incubation of the double-stranded cDNA with T7 (T3 or SP6) RNA polymerase results in the synthesis of multiple RNA transcripts from the PBS-containing double-stranded DNA template (step D). Some of this RNA is immediately converted to RNA·DNA heteroduplex by RT (still in the reaction mixture from step C) using oligonucleotide B as a primer. Further amplification of target sequences can be obtained by a second cycle of cDNA synthesis (steps E and F) and RNA transcription (step G).

ever, the TAS-generated RNA product was observed with oligonucleotide probe 86-31.
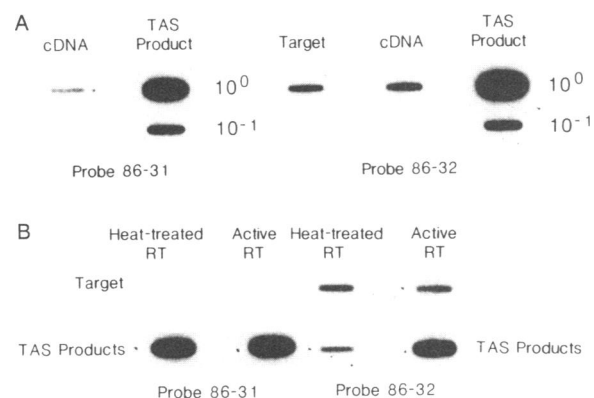

FIG. 2. (*A*) Detection of products in a single-cycle TAS reaction. HIV-1 RNA (10 fmol) was amplified in a single cycle of a TAS type I reaction. Oligonucleotides 88-77 and 86-29 were used as primers. Two samples (each 5% of the total reaction mixture) were taken prior to amplification (target), after the cDNA synthesis step (Fig. 1, step C) (cDNA), or after the transcription step (Fig. 1, step D) (TAS product). The nitrocellulose-immobilized samples were hybridized to ^{32}P-labeled oligonucleotide 86-32 or 86-31. Oligonucleotide 86-31 detects the first-strand cDNA product as well as the amplified RNA transcript made by the T7 polymerase. Oligonucleotide 86-32 detects the target HIV-1 RNA, the second-strand cDNA product, and the first-strand cDNA product made from the RNA transcript synthesized by T7 RNA polymerase. In this instance, the specific activity of probe 86-31 is 2 times lower than the specific activity of probe 86-32, as determined using pARV7A/2 as a control on the filters (data not shown). (*B*) Detection of DNA synthesis by RT during the transcription step in a TAS protocol. Two reactions using HIV-1 RNA (10 fmol) as target were amplified in one cycle of a TAS type I protocol using oligonucleotides 88-77 and 86-29 as primers. Prior to the addition of the T7 RNA polymerase (i.e., prior to Fig. 1, step D), one reaction was heated to 65°C for 5 min to inactivate the RT. Samples (5% of the total volume) of each reaction mixture were taken prior to amplification (target) or after amplification (TAS products).

With four cycles of TAS, following a type I protocol, a 2–5 × 10^6 amplification was obtained, with an average increase of 38- to 47-fold per TAS cycle (Fig. 3). However, modifications of the type I TAS protocol were made based on the observations that the RT remained active during the T7 RNA polymerase transcription step (Fig. 1, step D) and that the cDNA primed from oligonucleotide B could efficiently be used to prime a fill-in reaction of the promoter binding site on primer A (Fig. 1, step E). The protocol modification, described as type II TAS, eliminates one of the RT synthesis steps after the initial RT step (in a multiple-cycle reaction); RT and T7 RNA polymerase are added simultaneously after the initial RT step. Fig. 3*B* shows the efficiency of the type II protocol per cycle over a total of six cycles. After six cycles, a 2 × 10^6-fold amplification was obtained, giving an average amplification of 11- to 13-fold per cycle. Measured on a per-cycle basis, the amount of amplification decreased as the number of cycles increased (by the sixth cycle, only a 2-fold amplification was observed). Although these modifications lower the efficiency of TAS per cycle, the number of manipulations and time required per cycle are reduced, thereby allowing more cycles to be done in a shorter time. Four cycles of the type I TAS protocol require 4 hr, whereas six cycles of the type II protocol require only 3.25 hr.

Sensitivity in Detecting HIV-1-Infected Cells. Harper *et al.* (28) have reported that only 1 in 10^4 or 10^5 peripheral blood lymphocytes appears to be infected with HIV-1. This implies that in a 1-ml sample of whole blood, only 1–100 HIV-1-infected cells are present in the 10^6 lymphocytes isolated. The detection with TAS of the *vif* region of HIV-1 present in fewer than one HIV-1-infected cell equivalent in a total population of 10^6 uninfected lymphocytes is demonstrated in Fig. 4. Only 25% of the total nucleic acid extracted from each
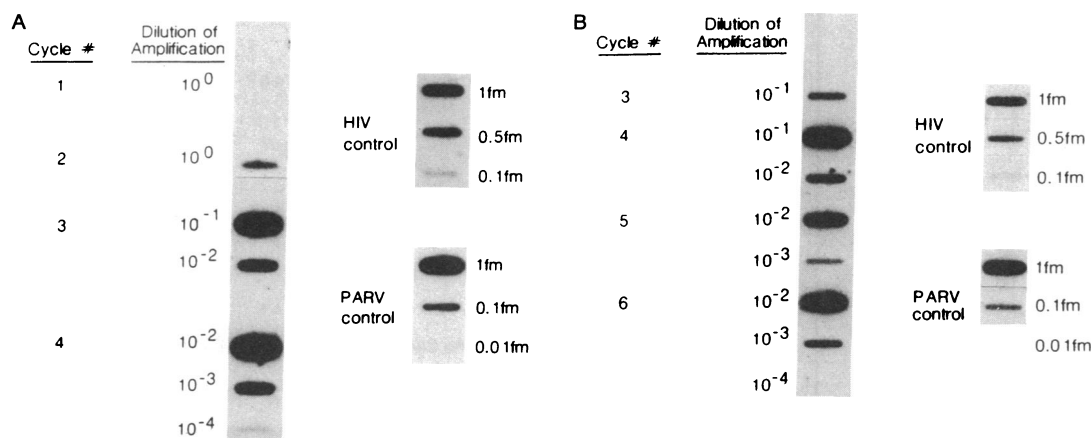
FIG. 3.   (A) Efficiency of amplification per cycle in four cycles of a type I TAS protocol. HIV-1 RNA (10 amol) was used as target in a four-cycle TAS type I amplification reaction using priming oligonucleotides 88-77 and 86-29. Samples (5% of the total reaction mixture) were taken after each cycle of TAS, diluted as necessary, denatured, and immobilized onto nitrocellulose. The filter was hybridized to ^{32}P-labeled oligonucleotide 86-31, which detects the amplified product RNA made from the T7 promoter. As controls, both pARV7A/2 plasmid DNA (full-length HIV-1 cDNA inserted into the *Eco*RI site of the pUC19 vector) and HIV-1 RNA were blotted and hybridized to probes 86-31 and 86-32, respectively, to allow calculation of the overall amplification. (B) Efficiency of amplification per cycle in six cycles of a type II TAS protocol. Purified HIV-1 RNA (10 amol) was used as target in a six-cycle TAS type II amplification reaction using priming oligonucleotides 88-77 and 86-29. Samples (5% of the total reaction mixture) were taken after each cycle of TAS, diluted as necessary, denatured, and slot-blotted to nitrocellulose. The filter was hybridized to ^{32}P-labeled oligonucleotide 86-31, which detects the amplified product RNA made from the T7 promoter. As controls, both pARV7A/2 plasmid DNA and HIV-1 RNA were blotted and hybridized to probes 86-31 and 86-32, respectively, to allow calculation of the overall amplification.

dilution was employed for amplification, and only 5% of the amplification reaction was used for the hybridization analysis. With a simple slot-blot assay, it was possible to detect above background the HIV-1 sequence from less than one HIV-1-infected CEM cell equivalent.

Detection of TAS-Generated, HIV-1-Specific RNA by Sandwich Hybridization Using OligoBeads. The problems associated with the use of nitrocellulose and nylon membranes as supports for hybridizations have been studied (17). The ability to carry out quasi-homogeneous hybridizations (i.e., near solution-like hybridization conditions) by using bead-bound oligonucleotides as a hybridization matrix has permitted the rapid detection of the TAS-amplified HIV-1 RNA product. RNA produced by TAS amplification from a dilution of HIV-1-infected cells was captured and detected with a straightforward sandwich hybridization format (Fig. 5 and Table 1). Each sandwich hybridization was conducted by first hybridizing the amplified RNA to an excess of ^{32}P-labeled detection oligonucleotide (86-31). This RNA·DNA hybrid

was captured by a second oligonucleotide probe (86-83) that was tethered by its 5' end to the surface of a Sephacryl bead (OligoBead). The percent of the ^{32}P-label associated with the beads allows a calculation of the number of fmol of target detected by sandwich hybridization. As indicated in Fig. 5 and Table 1, this hybridization format is capable of quantitatively detecting the presence of HIV-1 sequences in infected cells over two orders of magnitude by using only a small portion of the TAS amplification reaction mixture. Considering the results of this experiment, several interesting features of this method of detection merit notice. (*i*) The results from duplicate experiments indicate close reproducibility of the assay. (*ii*) The levels of sensitivity of this detection method are underscored by the need to use only a small quantity (i.e., >1 μl of the 140-μl volume) of the amplification reaction mixture. (*iii*) It is possible to distinguish HIV-1 sequences present in 25% of a sample derived from 1 infected cell in a population of 10^6 uninfected cells.

DISCUSSION

The utility of *in vitro* target amplification, accomplished principally through cycles of DNA replication, has been demonstrated by the application of PCR to a variety of cloning and diagnostic uses. This report focuses on TAS as a means of achieving the same goals. This alternative method provides several distinct features not associated with the PCR protocol. (*i*) Because of the inherent property of RNA polymerases to produce 10–10^3 copies of RNA per copy of DNA template, fewer cycles of amplification are required to achieve large increases in the copy number (10^6 copies in four cycles). As a consequence, fewer thermal denaturation steps are required, thus simplifying the overall protocol. (*ii*) Since the product of the TAS protocol is in part single-stranded RNA, it can be detected without the need for denaturation prior to detection by hybridization. The utility of such RNA products as templates for sequencing using the dideoxy chain-termination method has been demonstrated (16, 29).

Detection and positive identification of the TAS-generated RNA by OligoBeads with a sandwich hybridization protocol provide an added level of specificity to the amplification reactions. As described, the detection of PCR-amplified
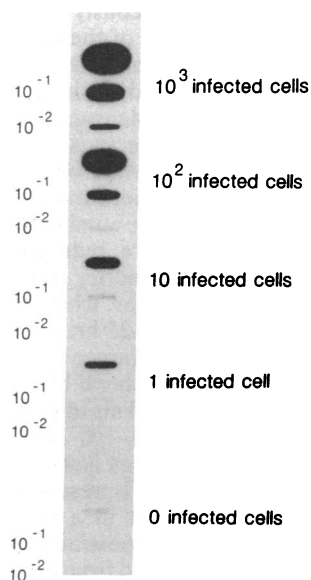

FIG. 4.   Amplification of HIV-1 sequences in infected CEM cells after six cycles of TAS (type II). HIV-1-infected CEM cells in the number indicated to the right were placed in a background of 10^6 uninfected CEM cells and the nucleic acid was extracted. Only 25% of each extracted nucleic acid preparation was used as target for six cycles of a TAS type II reaction protocol using primers 88-77 and 86-29. Aliquots of 5%, 0.5%, 0.05% of the reaction mixture were denatured and slot-blotted to nitrocellulose. The filters were hybridized to the ^{32}P-labeled HIV-1 *vif* detection oligonucleotide 86-32. The signals for each infected cell sample shown represent 1.25% of the total extracted nucleic acid [i.e., 1.25% of 10^3, 10^2, 10, and 1 HIV-1-infected CEM cell(s)].

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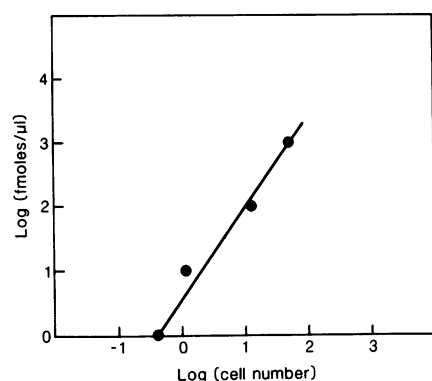*Proc. Natl. Acad. Sci. USA 86 (1989)* 1177

FIG. 5. Bead-based sandwich hybridization protocol used to detect TAS-amplified HIV-1 sequences. Sandwich hybridization reactions were carried out by using 50 mg (wet weight) of OligoBeads containing end-attached capture oligonucleotide 87-83 and 20 fmol of ^{32}P-labeled detection oligonucleotide per reaction mixture. The HIV-1 sequences present in each dilution [from 10^3 to 1 HIV-1 CEM infected cell(s) per 10^6 cells] were amplified by the TAS protocol. Volumes of the amplification reaction mixture from 0.016 to 0.3 μl (from a total 140 μl) were used in the sandwich hybridization reaction mixture. The logarithms of the number of HIV-1 sequences detected (fmol/μl) and the number of HIV-1-infected cells are plotted.

DNA fragments has most routinely been accomplished by either standard Southern hybridization methods (9, 13) or by the solution hybridization with a ^{32}P-labeled oligonucleotide, followed by gel electrophoresis (10). In the use of the TAS and bead-based sandwich hybridization approaches, positive identification of the presence of the target sequence of interest depends upon (*i*) oligonucleotide primer-directed transcription of a specific region of interest, (*ii*) hybridization of TAS-generated RNA by a specific detection oligonucleotide, and (*iii*) a capture hybridization of the amplified RNA·detection oligonucleotide complex. This combination of the TAS and OligoBead detection protocols decreases the probability of detecting partially homologous but nonspecific target sequences in a complex biological sample.

The time required to carry out each cDNA and transcription step in a single cycle of TAS is 24 and 25 min, respectively. Consequently, four cycles of TAS can be accomplished in <4 hr. The detection of TAS-generated RNA by sandwich hybridization requires a subsequent 4 hr. This mode of detection eliminates the need for autoradiography.

The ability to amplify and quantitate biologically important but rare nucleic acid molecules provides the tools to carry out studies hitherto unapproachable. Natural history studies focusing on the levels of HIV-1 present during various clinical stages of the disease process or drug efficacy studies

Table 1. Bead-based sandwich hybridization detection of TAS-amplified HIV-1 sequences

HIV-1 target sequences, no.	Vol. of reaction mixture amplified, μl	Total cpm ($\times 10^{-2}$)	Bead-bound cpm ($\times 10^{-2}$)	% of detection probe on beads	HIV-1 sequences detected fmol/μl
10^3	0.016	857.3	41.1	4.8	53
		869.9	40.1	4.7	
10^2	0.033	876.4	23.9	2.7	13.6
		761.9	21.6	2.8	
10	0.33	770.1	19.1	2.5	1.2
		776.4	18.3	2.4	
1	0.33	790.1	9.2	1.2	0.4
		898.7	10.1	1.1	
0	0.33	939.0	4.9	0.5	—
		913.4	5.0	0.5	

that require quantitative measurements of levels of HIV-1 before and after therapy are only two of the areas that can be investigated using straightforward amplification and hybridization technologies.

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