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Self-sustained Sequence Replication (3SR): An Isothermal Transcription-based Amplification System Alternative to PCR

E. Fahy,¹ D.Y. Kwoh,¹ and T.R. Gingeras²

¹Salk Institute Biotechnology/Industrial Associates, San Diego, California 92186;

²Life Science Research Laboratory, Baxter Diagnostics, Inc., San Diego, California 92186

The processes of DNA replication, DNA ligation, and RNA transcription have provided the basis of distinct and separate in vitro nucleic acid amplification strategies. The first described and the most widely utilized amplification protocol is the polymerase chain reaction (PCR).^(1,2) The PCR method consists of multiple cycles of oligonucleotide primer-directed DNA replication that are punctuated by short periods of elevated temperature (92–97°C) to permit strand separation of newly synthesized DNA. The modifications, adaptations, and enhancements of the basic PCR protocol now number in the hundreds (reviewed partially in refs. 3 and 4) and have arisen primarily to meet the challenges of the numerous biological questions to which this valuable technology has been applied. More recently, the description of an amplification method based on cycles of oligonucleotide-targeted ligation, called the ligase chain reaction (LCR) (see review by Barany in this issue), has offered users the ability to amplify short (oligonucleotide-length) segments of DNA and simultaneously to monitor these amplified segments for the presence of mutations.^(5–7) Similar to the PCR protocol, the LCR method includes thermocycling steps to permit the denaturing of newly ligated oligonucleotide duplexes so that these products can serve as templates for subsequent cycles of amplification.

The requirement for multiple heat-denaturation steps by both the PCR and LCR amplification methods has prompted the rapid development of programmable thermocycling instruments and has hastened the isolation

and purification of thermostable forms of DNA polymerase^(8,9) and ligase.^(7,10,11) These thermostable enzymes allow the amplification reactions to be carried out over many cycles of heating and cooling without debilitating loss of catalytic activities. The necessity of employing thermocycling as an essential part of an in vitro amplification strategy has been obviated by changing the product of the amplification process from double-stranded DNA to single-stranded RNA, thereby eliminating the heat denaturation step from the procedure.

RNA transcription is a process employed by all cellular and viral systems to copy discrete segments of nucleotide sequences into multiple single-stranded RNA molecules. In exploring modifications of the previously described in vitro transcription-based amplification system (TAS),^(12,13) it was discovered that isothermal replication of a targeted nucleic acid is possible using a concerted three-enzyme, in vitro reaction. This amplification strategy has been termed a self-sustained sequence replication (3SR) reaction, and was modeled after the general scheme employed during retroviral replication.⁽¹⁴⁾ In this reaction, activities of avian myeloblastosis virus (AMV) reverse transcriptase (RT), *Escherichia coli* RNase H, and T7 RNA polymerase produce an average 10-fold amplification every 2.5 min (for the first 15 min), leading to 10⁷-fold amplification in 60 min.⁽¹⁴⁾ Figure 1 provides a description of the steps in the 3SR amplification reaction.

This report discusses additional developments in the originally described

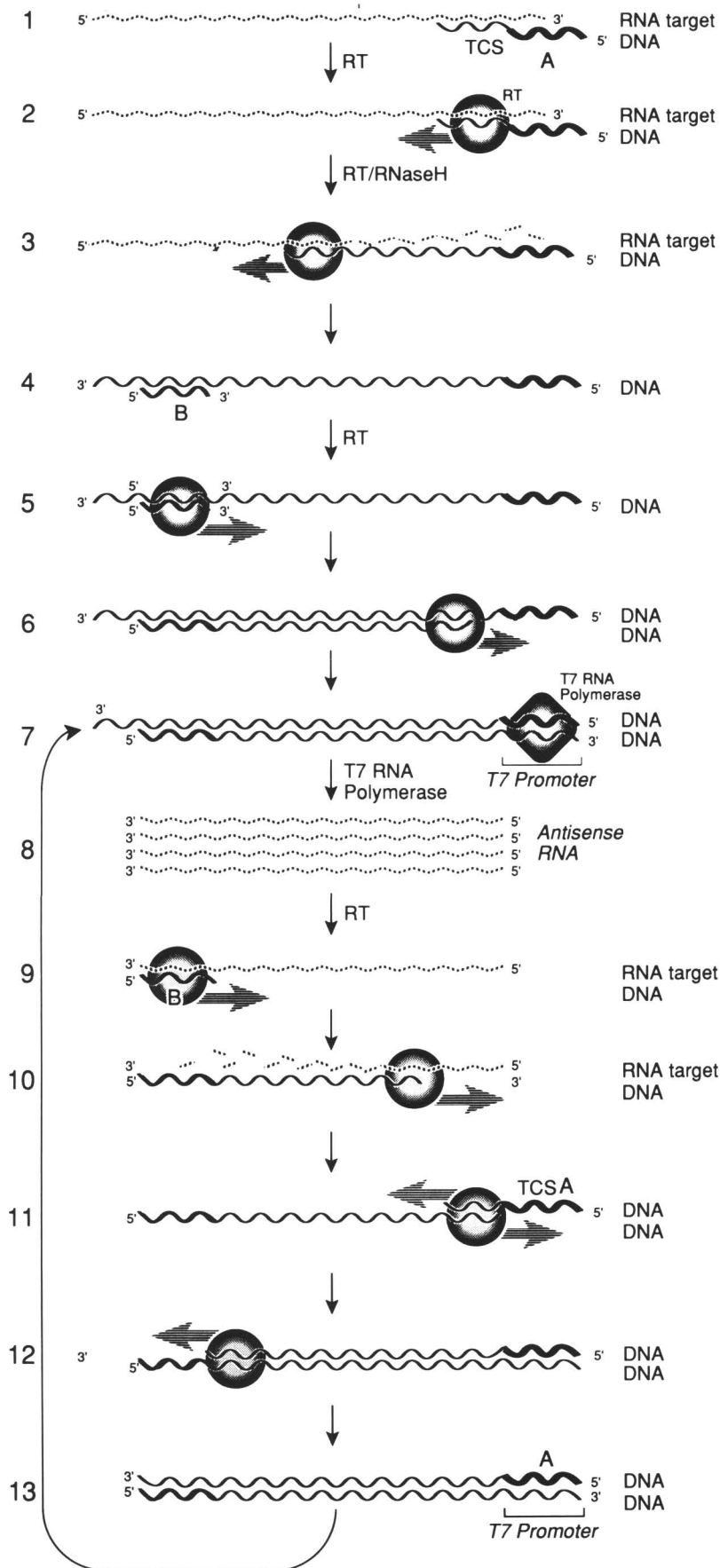
3SR reaction.⁽¹⁴⁾ Such developments include: (1) optimization of substrate concentrations, (2) the effect of temperature, pH, and ionic strength on amplification productivity, and (3) the reaction conditions required that enable the 3SR reaction to be carried out with only AMV RT and T7 RNA polymerase (two-enzyme 3SR reaction).

OPTIMIZATION OF 3SR REACTION

Since the original description of the protocol for 3SR amplification,⁽¹⁴⁾ the reaction conditions have been analyzed and modified to achieve more productive and quantitative amplifications. The production of 10⁸ copies of each target molecule in 30 min (see below for data) is well within the capability of the modified 3SR reaction (Fig. 2). To accomplish this level of amplification, all three enzyme activities (RT, RNase H, and RNA polymerase) must operate in an efficient and concerted manner. The 3SR reaction conditions presented in Figure 2 are the result of experiments designed to investigate the optimal concentrations of nucleotide triphosphates, magnesium, oligonucleotide primers, and enzymes, as well as to select optimal pH and temperature conditions.

Nucleotide Triphosphates and Monovalent Cations

Because the deoxynucleotide (dNTP) and ribonucleotide (rNTP) triphosphates chelate Mg²⁺, optimization of the nucleotide triphosphate concentrations was initiated with a higher MgCl₂ concentration (40 mM) in place of the 20 mM concentrations reported



earlier⁽¹⁴⁾ to ensure that sufficient quantities of Mg^{2+} were present to meet the requirements of the 3SR enzymes. The dNTP concentrations were varied from 1 to 6 mM in reactions with the rNTP concentrations fixed at 4 mM. Conversely, the rNTP concentrations were varied from 3 to 7 mM in reactions with the dNTP concentrations fixed at 4 mM (different ratios of rNTPs to dNTPs were explored in other experiments; see below). The maximum level of amplification of a 214-base region of the envelope (*env*) gene of the human immunodeficiency virus type 1 (HIV-1) was achieved with concentrations of 4 mM rNTPs, as shown in Figure 3A. The optimal concentration of Mg^{2+} in 3SR reactions with 4 mM dNTPs and rNTPs was observed to be 30 mM (Fig. 3B). The ability of T7 RNA polymerase to make multiple transcripts from each DNA template has been exploited previously to give yields of 400 moles of RNA per mole of template⁽¹⁵⁾ under reaction conditions similar to those used in 3SR. In addition, the ratio of RNA to DNA products in the 3SR reaction was previously observed to be approximately 100:1,⁽¹⁴⁾ so it was suspected that a dNTP-to-rNTP ratio should be established that reflected this bias toward RNA product.

FIGURE 1 Strategy of the 3SR amplification scheme. The 3SR reaction consists of continuous cycles of reverse transcription and RNA transcription designed to replicate a nucleic acid (RNA-target in figure) using a double-stranded cDNA intermediate. Oligonucleotides A and B prime DNA synthesis producing a double-stranded cDNA containing a functional T7 promoter (steps 1–6). Complete cDNA synthesis is dependent upon digestion of RNA in the intermediate RNA·DNA hybrid by RNase H (step 3). Transcription-competent cDNAs are used to produce multiple (50–1000) copies of antisense RNA transcript of the original target (steps 7–8). These antisense transcripts are immediately converted to T7 promoter-containing double-stranded cDNA copies (steps 9–12) and used again as transcription templates. This process continues in a self-sustained cyclic fashion under isothermal conditions (42°C) until components in the reaction become limiting or inactivated (enzymes). (Dotted lines) RNA; (thin lines) DNA; (thick lines) T7 promoter sequence (see Fig. 4); (circles) reverse transcriptase; (diamonds) T7 RNA polymerase; (TCS) target complementary sequence.

The results in Figure 3C clearly indicate this greater need for rNTPs in the 3SR reaction when the ratio of dNTP to rNTP is varied.

It should be noted that the optimized nucleotide triphosphate concentrations used in the 3SR amplification reactions are much higher than the reported K_m values of 25 μM for dNTPs in AMV RT assays⁽¹⁶⁾ and of 47–160 μM for rNTPs in T7 RNA polymerase assays.⁽¹⁷⁾ Preliminary experiments have shown that, whereas the use of 50 μM dNTP results in approximately equivalent amounts of 3SR product as use of 1 mM dNTPs in 1- to 2-hr reactions, reduction of the rNTP levels below 4 mM results in a significant decrease in 3SR amplification levels (data not shown). Furthermore, the inability to obtain detectable 3SR amplification at rNTP concentrations of 0.2–1 mM could not be offset by altering the MgCl_2 or KCl concentrations in the reaction. These data suggest that the concerted, multienzyme 3SR reaction has a set of substrate requirements that are different from those observed for the individual enzyme reactions.

Experiments designed to study the influence of monovalent salts on the 3SR amplification indicated that use of 20 mM KCl rather than 25 mM NaCl, as recommended in the previously described 3SR protocol,⁽¹⁴⁾ resulted in a slight improvement in overall amplification. The remaining buffer components were maintained at 40 mM Tris-HCl (pH 8.1), 10 mM dithiothreitol (DTT), and 4 mM spermidine.

During the course of these optimization studies, it was noticed that the level of 3SR amplification is significantly affected by the total ionic strength of the reaction. Using the Debye-Hückel formula⁽¹⁸⁾:

$$I = \frac{1}{2} \sum C_i Z_i^2$$

(where I is the ionic strength, C_i is the initial molar concentration of each ionic species, and Z is the net charge of each ionic species), an approximation of the total ionic strength of the 3SR reaction was calculated as each of the ionic species in the reaction was varied. Ionic strengths exceeding 550 mM were observed to cause significant inhibition of amplification. Because

3SR AMPLIFICATION PROTOCOL

- To an autoclaved 1.5-ml eppendorf tube add:

20 μl	5x buffer (200 mM Tris•HCl (pH 8.1), 150 mM MgCl_2 , 100 mM KCl, 50 mM DTT, 20 mM spermidine)
5 μl	each primer (0.1 μM each, final)
4 μl	dNTP mix (1 mM each, final)
24 μl	rNTP mix (6 mM each, final)
5 μl	HIV-1 RNA (0.1 attomoles)
37 μl	H_2O (total volume = 100 μl)

Use H_2O instead of RNA for water control reactions.
- Denature at 65°C for 1 min and transfer to 42°C.
- Add enzymes as a mix: 30 units AMV RT, 4 units RNase H, and 100 units T7 RNA polymerase. In the case of the two-enzyme reaction, 10% (vol/vol) DMSO and 15% (wt/vol) sorbitol are included and the enzyme concentrations are 10 units AMV RT and 20 units of T7 RNA polymerase. This mix can be stored on ice for up to 1 hr.
- After enzyme addition, mix by flicking the tube and incubate at 42°C for 1 hr.
- Stop the reaction by freezing the tube on dry ice. Reactions may be stored at -20°C.

All reagents were prepared with 0.1% diethylpyrocarbonate (DEPC)-treated water to suppress nuclease activity. AMV reverse transcriptase was obtained from Life Sciences Inc., *E. coli* RNase H was purchased from Bethesda Research Laboratories, and T7 RNA polymerase was obtained from Stratagene. Oligonucleotides were synthesized by phosphoramidite chemistry using an Applied Biosystems 380 A synthesizer. The amount of enzyme required in the 3SR reaction might vary depending on the supplier and lot of enzyme.

FIGURE 2 Description of the current amplification protocol using either the two-enzyme (AMV RT and T7 RNA polymerase) or three-enzyme formats (AMV RT, T7 RNA polymerase, and *E. coli* RNase H).

the ionic strength is proportional to the square of the formal charge of each molecule, the concentrations of nucleotides (containing four negative charges at pH 8.1) have a profound effect on the total ionic strength of the 3SR reaction. This effect is noticeable in Figure 3A where amplification decreases rapidly at high dNTP or rNTP concentrations. The same trend, though less pronounced, was observed with titrations of divalent (MgCl_2 , Fig. 3B) and monovalent (KCl, NaCl) salts (data not shown), but not seen in Figure 3C because here overall ionic strength remained constant.

Oligonucleotide Primers

Both the concentration and structure of the oligonucleotide primers can have a significant influence on the specificity and productivity of the 3SR reaction. Although both 3SR primers contained the T7 promoter sequence in the optimization reactions described

above, the 3SR reaction is effectively carried out with only one primer (primer A, Fig. 1) containing the promoter sequence (T3 and SP6 promoter sequences can be substituted for the T7 promoter).^(19,20) In the optimization studies on primer concentrations in the 3SR reaction (Fig. 3D), only the antisense primer contained the T7 promoter sequence. The optimal primer concentration range is from 0.1 to 0.2 μM . Not surprisingly, as observed with PCR reactions,^(3,4) there is a decrease in amplification when the ratio of sense to antisense primer is varied from 1:1 (Fig. 3D). Increasing the concentration of antisense oligonucleotide does not increase the overall amplification levels.

The structure of the T7 promoter-containing primer is considered to be composed of four distinct regions (Fig. 4). The inclusion of extra nucleotides (1–7 nucleotides) at the 5' end of T7-promoter-containing primers was

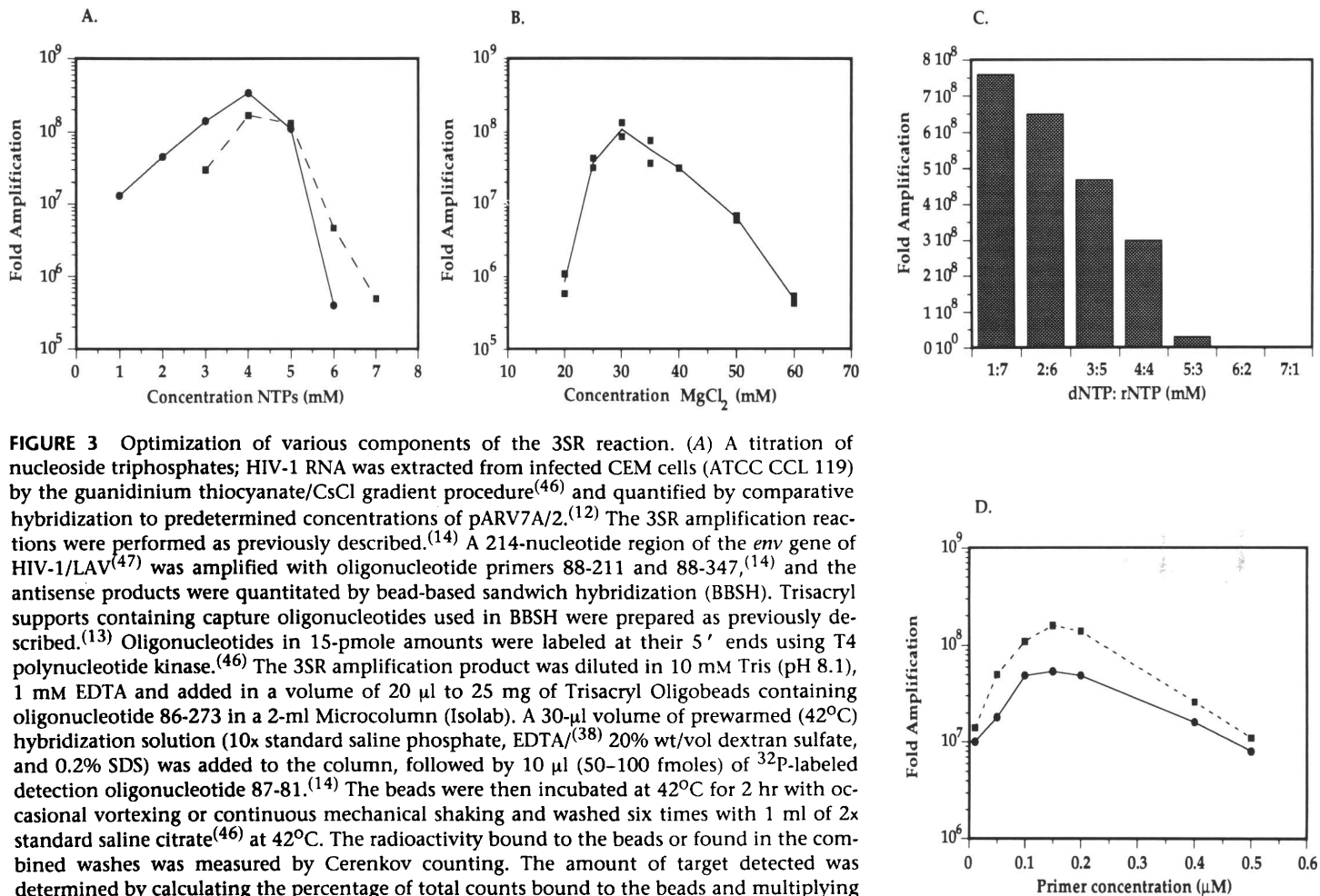


FIGURE 3 Optimization of various components of the 3SR reaction. (A) A titration of nucleoside triphosphates; HIV-1 RNA was extracted from infected CEM cells (ATCC CCL 119) by the guanidinium thiocyanate/CsCl gradient procedure⁽⁴⁶⁾ and quantified by comparative hybridization to predetermined concentrations of pARV7A/2.⁽¹²⁾ The 3SR amplification reactions were performed as previously described.⁽¹⁴⁾ A 214-nucleotide region of the *env* gene of HIV-1/LAV⁽⁴⁷⁾ was amplified with oligonucleotide primers 88-211 and 88-347,⁽¹⁴⁾ and the antisense products were quantitated by bead-based sandwich hybridization (BBSH). Trisacryl supports containing capture oligonucleotides used in BBSH were prepared as previously described.⁽¹³⁾ Oligonucleotides in 15-pmole amounts were labeled at their 5' ends using T4 polynucleotide kinase.⁽⁴⁶⁾ The 3SR amplification product was diluted in 10 mM Tris (pH 8.1), 1 mM EDTA and added in a volume of 20 μ l to 25 mg of Trisacryl Oligobeads containing oligonucleotide 86-273 in a 2-ml Microcolumn (Isolab). A 30- μ l volume of prewarmed (42°C) hybridization solution (10 \times standard saline phosphate, EDTA/⁽³⁸⁾ 20% wt/vol dextran sulfate, and 0.2% SDS) was added to the column, followed by 10 μ l (50–100 fmoles) of ³²P-labeled detection oligonucleotide 87-81.⁽¹⁴⁾ The beads were then incubated at 42°C for 2 hr with occasional vortexing or continuous mechanical shaking and washed six times with 1 ml of 2 \times standard saline citrate⁽⁴⁶⁾ at 42°C. The radioactivity bound to the beads or found in the combined washes was measured by Cerenkov counting. The amount of target detected was determined by calculating the percentage of total counts bound to the beads and multiplying this quantity by the femtomole amount of labeled probe used in the assay. NTP titrations were performed as previously described⁽¹⁴⁾ except that the MgCl₂ concentration was raised to 40 mM. The dNTP (solid line) titration was carried out using a fixed concentration of 4 mM rNTPs and the corresponding rNTP titration (dashed line) was produced using a fixed concentration of 4 mM dNTPs. The values plotted are the average of duplicate experiments. (B) Optimization of the MgCl₂ concentration for the 3SR amplification; the optimal MgCl₂ concentration was determined using previously described reaction conditions,⁽¹⁴⁾ except that 4 mM dNTP concentrations were used. (C) Effect of the dNTP:rNTP ratio on 3SR amplification; varying ratios of rNTP:dNTP were used in 3SR amplifications that were performed under the reaction conditions described previously,⁽¹⁴⁾ except that 30 mM MgCl₂, 10 mM DTT, 20 mM KCl, and 4 mM spermidine were used and NaCl was omitted. (D) Optimization of primer concentrations for 3SR amplification; titration of the 15-base *env* sense primer 90-517 (5'-GGTTTTGCGATTCTA-3') was performed under the optimized conditions described in Fig. 2. Reactions were carried out for 40 min (solid line) and 60 min (dashed line). The concentration of the 40-base T7 promoter-containing antisense primer 90-514 (5'-AATTTAATACGACTCACTATAGGGATAGCATTGTCTGTGA-3') was kept at 0.1 μ M.

prompted by the results of experiments detailed in Table 1. A significant increase in amplification was noted as the length of sequence at the 5' end of these primers was increased to 7 nucleotides. This increase may likely reflect the previously observed requirement to include all of the 17 nucleotides of the T7 promoter sequence as a double-stranded structure.⁽²¹⁾ The previously observed fail-

ure of AMV reverse transcriptase to terminate polymerization at the last nucleotide of a template is consistent with the necessity of positioning extra nucleotides at the 5' end of the T7 promoter-containing primer.⁽²²⁾ Although exhaustive experiments have not been conducted, it appears that the sequence of these extra bases does not affect amplification levels.

The tetranucleotide sequence

+¹GGGA⁺⁴ is a preferred site for T7 RNA polymerase transcription initiation.^(23,24) Alterations in the length⁽²⁵⁾ and composition⁽²⁶⁾ of this sequence have been studied. Interestingly, while the primer containing the preferred GGGA sequence does not yield the highest levels of 3SR amplification after 60 min, the reactions using the GGGA sequence-containing primers are more productive earlier in the 3SR reaction (Table 2). Martin et al.⁽²⁵⁾ noted increased abortive cycling by T7 RNA polymerase immediately following the incorporation of UMP into the RNA within the initial 8–10 bases. Although we have not noted an overall negative effect on the total 3SR amplifications achieved, an increase in the level of aborted transcripts in 3SR reactions in which incorporation of UMP occurs within the first 8–10 bases cannot be ruled out.

Finally, both the length and structure of the target complementary sequence (TCS) have been studied as fac-

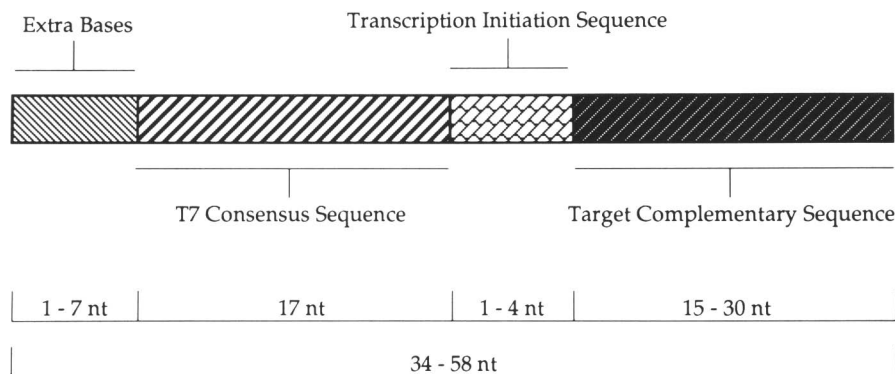


FIGURE 4 Diagram of a T7 promoter-containing primer, outlining the four functional regions discussed in the text. The lengths of these regions, expressed in nucleotides, are given below the diagram.

tors influencing the 3SR reaction. Primers with lengths of TCS ranging from 15 to 30 nucleotides have routinely been employed in the 3SR reaction. The use of shorter-length TCS enables more stringent hybridization of the primers. Rules governing the choice of length and composition of the TCS for 3SR primers are similar to those previously suggested for selection of PCR primers.⁽²⁷⁻³²⁾ These rules include use of primers with 40-60% GC content and similar melting temperatures. Kwok et al.⁽³²⁾ reported that primers having TCS with T:T, T:G, T:C, G:G, and A:C mismatches at the 3' termini amplify as efficiently as the correctly matched base pairs. Consequently, primers can be tailored to amplify heterogeneous populations of sequences, or to be highly specific by judicious selection of terminal nucleotides.

3SR Enzymes

The enzyme activities required for maximal amplification have been determined for the 3SR reaction. Under the optimized buffer and primer conditions (Fig. 2), maximal 3SR amplification resulted when 30 units of AMV RT, 100 units of T7 RNA polymerase, and 4 units of *E. coli* RNase H were used (data not shown). The use of these enzymes to carry out the steps of the 3SR amplification places restrictions on the temperature and pH conditions. The pH optima for *E. coli* RNase H (7.5-9.1), T7 RNA polymerase (7.7-8.3), and AMV RT (7.8-8.5) have previously been defined.^(17,33,34) Consequently, it is not surprising to note

that in a 3SR reaction, these three enzymes operate efficiently over a range of pH from 7.7 to 8.6 (data not shown). Similarly, the selection of a temperature for the 3SR reaction is also guided by the optimal temperature ranges for each of the enzymes. However, the choice of temperature is also influenced by the need to establish a relatively high stringency for the primer-mediated cDNA synthesis steps of the 3SR reaction. Comparison of the 3SR reaction kinetics at 37°C and 42°C indicates that 10⁸-fold amplification of

0.1 attomoles of a 214-base region of the *env* gene of HIV-1 RNA was achieved after 30 min at 42°C, whereas at 37°C the 3SR reaction proceeded more slowly with a final yield of approximately one-third the product obtained at 42°C (Fig. 5). Increasing the temperature to 45°C and 47°C results in decreases of 10- and 1000-fold, respectively, in the total amount of 3SR amplified product after 120 min (data not shown).

TWO-ENZYME 3SR REACTIONS

The addition of 10% dimethyl sulfoxide (DMSO) in a *Thermus aquaticus* (*Taq*) DNA polymerase-catalyzed PCR reaction has been reported to be inhibitory.^(3,4) However, the use of DMSO in a RT-catalyzed nucleotide sequencing reaction has been observed to be helpful in assisting to resolve problematic RNA secondary structure.^(35,36) Since 3SR amplification relies on RNA as both intermediary and final products in the amplification reaction, the utility of DMSO in the reaction was investigated. The use of 10% DMSO in the three-enzyme 3SR reaction resulted in only a modest increase (two- to fourfold) in the amplification of the 214-nucleotide *env*

TABLE 1 Effect on 3SR Amplification of Variations in the Length of Nucleotide Sequence at 5' Ends of Oligonucleotide Primers Containing T7 Promoter Sequences

Primer	Length (bases)	Sequence ^a	Fold amplification ^b
90-425	59	5' - <i>AGTAA</i> <u>TTAATACGACTCACTATAGGGA</u> -3'	3.1 × 10 ⁸
88-347	56	<i>AATTA</i> <u>AATACGACTCACTATAGGGA</u>	1.9 × 10 ⁸
90-427	55	<i>ATTTA</i> <u>AATACGACTCACTATAGGGA</u>	1.2 × 10 ⁸
90-428	54	<i>TTTA</i> <u>AATACGACTCACTATAGGGA</u>	5.5 × 10 ⁷
90-429	53	<i>TTA</i> <u>AATACGACTCACTATAGGGA</u>	1.1 × 10 ⁷
90-205	52	<u>TAATACGACTCACTATAGGGA</u> ...	<10 ⁵
90-206	51	<u>AATACGACTCACTATAGGGA</u> ...	<10 ⁵

^aThe sequence of the antisense primer is shown with extra bases at the 5' end indicated in italics, the underlined bases correspond to the T7 promoter sequence and the initiation of transcription is denoted with an asterisk (*); the target complementary sequence is the same for each of the primers (5' -TTATTGTCCTCGGCTGGTTTTCGATTCTA-3').

^bReactions were performed in 50 μl containing 0.05 attomoles of HIV-1 RNA under the conditions described in Fig. 2; the same sense primer, 89-255, was used in all reactions. The lower limit of detection for the BBSH in this experiment was 5 fmoles/50 μl, which is equivalent to 10⁵-fold amplification.

TABLE 2 Nucleotide Alterations in the Transcription Initiation Sequence and the Effect of 3SR Amplification

Primer	Length	Sequence ^a	Fold amplification ^b	
			15 min	60 min
88-347	56	5' -AATTTAATACGACTCACTATAGGGA-3'	3.3×10^6	2.0×10^8
90-426	56	AATTTAATACGACTCACTATAGAAA....	5.6×10^5	2.4×10^9
90-202	55	AATTTAATACGACTCACTATAGGAT....	2.4×10^6	1.5×10^9
90-203	54	AATTTAATACGACTCACTATAGATG....	5.7×10^5	3.7×10^9
90-204	53	AATTTAATACGACTCACTATAGTGT....	6.3×10^5	1.9×10^9
90-430	52	AATTTAATACGACTCACTATATGTA....	3.0×10^5	1.5×10^9

^aThe transcription initiation site of the antisense primer is represented by underlined bases; the bases with double underline represent part of the target-complementary sequence, and the complete target-complementary sequence for each of the primers is (5'-TGTACTATTATGGTTTATGACATTGTCTGTGA-3').

^bReactions were performed with 0.05 attomoles of HIV-1 RNA for 1 hr at 42°C using 89-255 (5'-TTATTGTCCCCGGCTGGTTTTCGATTCTA-3') as the sense primer under the same conditions described in Fig. 2.

region of HIV-1 RNA target (data not shown). Interestingly, the use of 10% DMSO supplemented with a neutral polyalcohol such as glycerol (10%) or sorbitol (15%) increased the efficiency of the 3SR amplification from 10- to 1000-fold, especially in cases where longer regions were targeted for amplification (Table 3, reactions 7 and 8).

Perhaps the most interesting effect these additives have on the 3SR reaction is their apparent ability to enhance the endogenous RNase H activity of AMV RT.^(37,38) When only AMV RT and T7 RNA polymerase are used in a 3SR reaction under optimized conditions without additives, no amplification is detected for the polymerase (*pol*) region of the HIV-1 RNA genome (Table 3, reaction 4). However, the two-enzyme 3SR reaction when supplemented with 10% DMSO and 15% sorbitol yields amplification levels in excess of 10⁶-fold, showing that the RNase H activity of AMV RT in the presence of additives can replace the need for *E. coli* RNase H activity.

The number of units of AMV RT and T7 RNA polymerase required in the two-enzyme reaction with additives is less than those required for a three-enzyme reaction without additives (Table 3). Twenty units of T7 RNA polymerase is optimal for the two-enzyme 3SR reactions with no increased amplification noted at higher concentrations. In the two-enzyme 3SR

reaction, when AMV RT concentrations are increased beyond 10 units/100- μ l reaction, there is a measurable (twofold relative to reactions with 30 units/100 μ l) decline in the overall amplification consistent with an overabundance of RNase H activity in these reactions. At this time, the mechanism of the effect of additives on either the RNA-DNA template or 3SR enzymes cannot be differentiated. However, the observation that somewhat longer segments of target nucleic acids can more efficiently be amplified using additives in the 3SR reaction clearly suggests that analysis of the mechanism underlying these results will prove to be valuable.

COMPARISON OF IN VITRO NUCLEIC ACID AMPLIFICATION SYSTEMS

The basic mechanisms underlying the 3SR, PCR, and LCR amplification methods are different.^(39,40) The differences have been used to address particular diagnostic, molecular biological, or clinical problems in a manner that exploits the strengths of the amplification methods. These varied applications exemplify the useful characteristics of these amplification protocols. Some of the advantages and disadvantages inherent in the 3SR, PCR, and LCR amplification protocols are summarized in Table 4. The advantages of the PCR protocol are well-known and many have been discussed in previous reviews.^(3,4) The 3SR amplification protocol has been shown to be effective in the specific amplification of RNA target molecules, which permits the independent amplification of mRNA and not the genomic copies present in the same sample.⁽⁴¹⁾ In addition, the 3SR amplification protocol coupled with a bead-based sandwich hybridization method^(13,42,43) has been used to detect and characterize mutations responsible for HIV-1 zidovudine (AZT) resistance,⁽⁴⁴⁾ as well as provide an in vitro selection method to evolve ribozymes that can specifically cleave single-stranded DNA.⁽⁴⁵⁾ The LCR protocol also brings the specific advantage of combining both amplification and mutation detection that is not immediately found with PCR or 3SR.^(6,7) The disadvantages of the LCR and 3SR reactions, such as a current

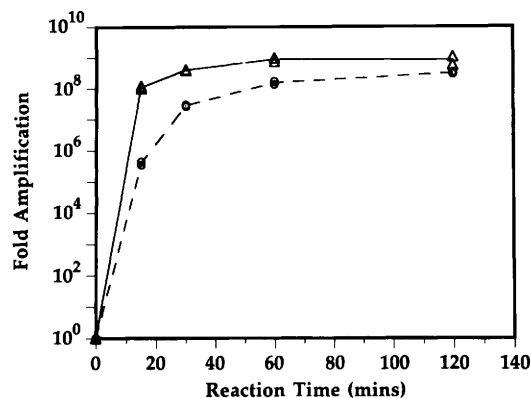


FIGURE 5 Effect of reaction temperature on the efficiency of 3SR amplification. The reaction conditions used were those described in Fig. 3C, using 1 mM dNTPs and 7 mM rNTPs and omitting bovine serum albumin (BSA). The detection of the 3SR antisense products was carried out as described for Fig. 3. The dashed and solid lines refer to reactions conducted at 37°C and 42°C, respectively.

TABLE 3 Effect of Additives on Two- and Three-Enzyme 3SR Reactions^a

Reaction number	Enzymes (units/100 μ l)			Additives			Amplification product size (nucleotides)	Fold amplification ^b
	AMV RT	T7 RNA Pol	RNase H	DMSO (%vol/vol)	glycerol (%vol/vol)	sorbitol (%wt/vol)		
1	30	100	4	—	—	—	347	1×10^7
2	30	100	4	10	10	—	347	5×10^7
3	30	100	4	10	—	15	347	4×10^7
4	10	20	—	—	—	—	347	$<10^4$
5	10	20	—	10	10	—	347	1×10^6
6	10	20	—	10	—	15	347	5×10^6
7	30	100	4	—	—	—	709	8×10^4
8	30	100	4	10	—	15	709	7×10^6
9	10	20	—	10	—	15	709	1×10^6

^aThe primer pairs 90-47/89-391 and 90-249/89-391 were used to amplify 342-base and 704-base regions, respectively of the *pol* gene of HIV-1. Only the 89-391 (antisense) primer contained a T7 promoter sequence. Products of the 90-47/89-391 reaction were detected by BBSH using 89-534 probe and 89-535 bead sequences, and those of the 90-249/89-391 reaction using 89-534 probe and 89-419 beads. The *pol* sequences described in this experiment are given in ref. 36.

^bDetection limitation of BBSH in these experiments was approximately 10^4 -fold amplification.

TABLE 4 Comparison of In Vitro Amplification Systems

PCR	3SR	LCR
<i>A. Advantages</i>		
Single-step addition of enzyme	Single-step addition of enzyme	Single-step addition of enzyme
High specificity and processivity because of thermostable polymerase	Rapid kinetics	High specificity because of thermostable ligase
Automated thermocycler available	Isothermal reaction; no instrumentation required	Can combine amplification with mutation detection—even single single-point mutations
Many adaptations of PCR reaction developed	Can differentiate RNA from DNA target sequences	Can be used in combination with PCR or 3SR
Carryover contamination control strategies available	RNA products are single-stranded; amenable for direct sequencing and quantitation	Automated instrument with only two steps/cycle needed
Long sequences can be amplified (100– 10^4 bases)	Can be coupled directly to in vitro translation systems	
DNA products are easily characterized by agarose gels		
<i>B. Disadvantages</i>		
Kinetics of amplification slower	Three enzyme activities required	Kinetics of amplification slower
More steps to differentiate RNA from DNA targets	Length limitation of amplification	Amplification efficiency is lower
Control of time and temperature for each amplification cycle	RNA products difficult to size	Smaller length sequence amplified
	Currently no contamination control system	Currently no contamination control system
	Currently no thermostable enzymes; lower specificity	

lack of carry-over contamination control or length limitations for amplification, will most likely disappear as these technologies mature.

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