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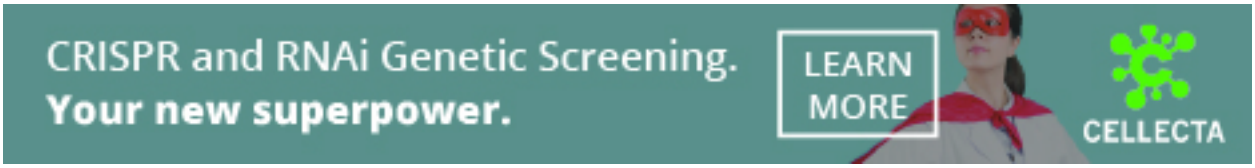
Genome Res. 1994 4: 62-64



Reverse Transcriptase Can Inhibit PCR and Stimulate Primer–dimer Formation

Konstantin M. Chumakov

Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20852-1448

Presence of residual reverse transcriptase (RT) activity in cDNA used for PCR results in accumulation of primer–dimers and in inhibition of amplification of a specific DNA product. Inactivation of RT by heat or some other method solves both these problems. We suggest that RT is capable of adding nucleotides at the 3′ end of primers before PCR amplification begins, thereby extending short complementary regions in primers facilitating their interaction. RT should be inactivated in all protocols involving PCR amplification of cDNA.

PCR amplification of cDNA synthesized in vitro by reverse transcriptase (RT–PCR) is used widely in studies of both cellular and viral RNAs, enabling the powerful tools of DNA research to be used for studies of RNA.[1] One of the applications of RT–PCR is to detect and quantitate neurovirulent revertants in oral poliovirus vaccine (OPV).[2,3] Mutant analysis by PCR and restriction enzyme cleavage (MAPREC), developed in our laboratory for this purpose, involves synthesis of poliovirus cDNA by RT, and amplification of the DNA segment under study by PCR with simultaneous creation of restriction sites that are affected by the mutation of interest. While using this method for quantitation of numerous mutations in three types of OPV, we found that some crude cDNA preparations (i.e., products of RT reactions without any further treatment) contained inhibitor(s) that blocked PCR amplification of DNA. Dilution of such cDNA samples restored PCR amplification. Another problem with crude cDNA samples was the apparent enhancement of the formation of artifactual DNA products, presumably primer–dimers. In this short communication, we report that both of these problems are caused by RT itself and not by any other component of the RT reaction mixture or the RT storage buffer. Phenol extraction or heating of newly synthesized cDNA solved both of these problems. Incubation of primers with RT and dNTPs resulted in the incorporation of [α-^{32}P]dATP into primer molecules, suggesting that enzymatic extension of primer molecules may be responsible for their increased tendency to form dimers and inhibition of amplification of specific DNA products.

MATERIALS AND METHODS

cDNA of Sabin 3 poliovirus was synthesized by Moloney murine leukemia virus (M-MuLV) RT (GIBCO–BRL) with random hexanucleotide primers on a template of RNA extracted from the virus. Conditions of this reaction were described previously in detail.[2,3] Two segments of type 3 OPV cDNA spanning nucleotides 431–513 (segment 1) and 2463–2603 (segment 2) were PCR amplified in this study using primers listed in Table 1. In both cases, sense primers were taken in a 10-fold excess over antisense primers (final concentrations of 3 μg/ml and 0.3 μg/ml, respectively) to ensure accumulation of predominantly single-stranded DNA. [4] Forty PCR cycles were done (15 sec at 94°C, 15 sec at 55°C, and 1 min at 72°C). The second strand was synthesized by adding ^{32}P-labeled antisense primer and extending it with *Taq* DNA polymerase at 72°C. Radioactively labeled DNA was analyzed in 7.5% polyacrylamide gel electrophoresis (PAGE).

RESULTS AND DISCUSSION

Inhibition of PCR

If the PCR reaction is primed with a freshly made cDNA, the amplification is often poor, as evidenced by both the absence or lower quantities of DNA product, and by large amounts of residual unused primers. A 5- to 10-fold dilution of cDNA prior to its addition to PCR restored the amplification. Phenol extraction/alcohol precipitation of cDNA also yielded cDNA that did not inhibit amplification. To discover whether this phenomenon is caused by RT or some component present in cDNA preparation, PCR mixtures were seeded with a DNA segment spanning nucleotides 431–513

TABLE 1 Primers Used in This Study

pS-3/470.1	431	TGAGCTACAT GAGAGTGCTC CGGCCCCTGA ATGCGGCTGA	470
pA-3/484	513	CAGGCTGGCT GCTGGGTTGC AGCTGCCTGC	484
pS-3/2491	2463	CTGCGCTTCC ACAGGGTATT GAAGAATCG	2491
pA-3/2574	2603	TGAGTGCAGG TACCTCCTTG GAATGCGCCG	2574

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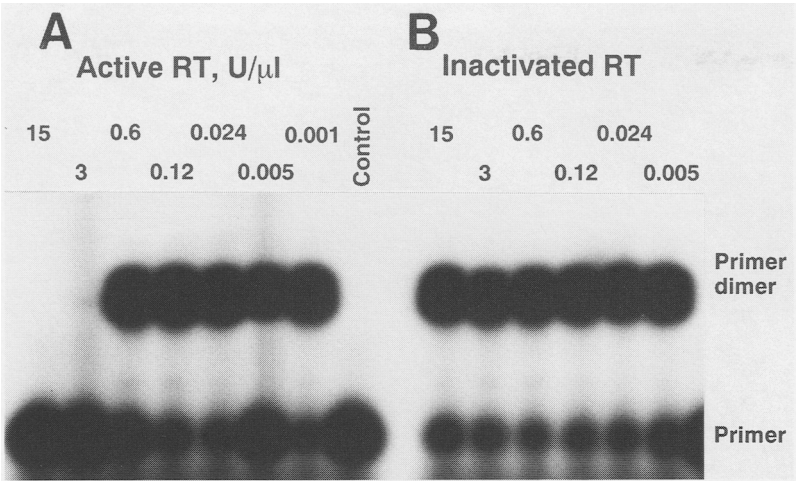

FIGURE 1 Effect of RT on PCR amplification of DNA. (*A*) PCR was seeded with DNA segment corresponding to nucleotides 431–513 of type 3 OPV (segment 1), and RT was added at the indicated concentrations. (*B*) Heat-inactivated RT (5 min at 94°C) was added to samples. Control is a sample with no template DNA or RT added. ^{32}P-Labeled PCR product was separated from the primers by PAGE.

of type 3 poliovirus genome (segment 1), and decreasing amounts of RT were added to a row of eight tubes. Figure 1A shows that no amplification occurred in the first two tubes containing 15 and 3 U/μl of RT. The inhibition was caused by RT and not by components of the enzyme storage buffer, because RT heated for 5 min at 94°C had no inhibitory effect (Fig. 1B). Heat inactivation of cDNA samples that otherwise inhibited PCR also restored their activity. Therefore, the active RT rather than any components of the storage buffer inhibits PCR.

Enhancement of Primer–dimer Formation

In an experiment with amplification of DNA segment 2 (nucleotides 2463–2603) of type 3 poliovirus gene, another type of problem caused by cDNA was observed. In this case, primer–dimer was the predominant PCR product in a sample to which no DNA template had been added. Figure 2 shows that adding as little as 0.001 U/μl of RT strongly stimulated primer–dimer formation. Adding RT heated to 94°C had no effect.

The above results demonstrate that the presence of active RT in a PCR mixture can inhibit specific amplification of DNA and stimulate accumulation of primer–dimers. It should be noted that the latter process occurs only with selected primer pairs that are prone to dimer formation and that it requires much lower amounts of RT than the former one. To explain these effects of RT, it was suggested that it adds nucleotides to the 3′ end of primers, changing their sequence and specificity. Apparently, this occurred within a few minutes after all of the PCR components were combined and before amplification began, as the first step in PCR is heating to 94°C. To determine whether RT can add nucleotides to primers, primers used for amplification of segment 2 were incubated with RT and [α-^{32}P]dATP at room temperature. Analysis of reaction products in PAGE clearly demonstrated that dATP was incorporated into primers and that this incorporation was dependent on the presence of active RT (results not shown).

Inhibition of PCR and stimulation of primer–dimer formation described in this communication are likely to result from enzymatic modification of primers by RT. At room temperature, primers can form unstable short-lived complexes with each other by the annealing of short complementary nucleotide stretches. Because RT readily uses DNA as a template[(5)] and is known to use very short oligonucleotide primers to initiate DNA strand synthesis, it is possible that it can add nucleotides to the 3′ end of primers in these unstable complexes. Hence, it may extend complementary stretches and facilitate further primer–dimer formation during PCR. The same mechanism may lead to inhibition of PCR if a substantial fraction of primers becomes modified and therefore fails to bind the template at specific sites. The latter effect should require much higher activity of RT. It is not known whether the PCR inhibition and stimulation of primer–dimer formation can be caused by RT of any source or whether these effects are specific to the Moloney RT that I have used.

The practical implication of these findings is clear. When cDNA is used to prime PCR, RT should be inactivated either by phenol extraction or heating.

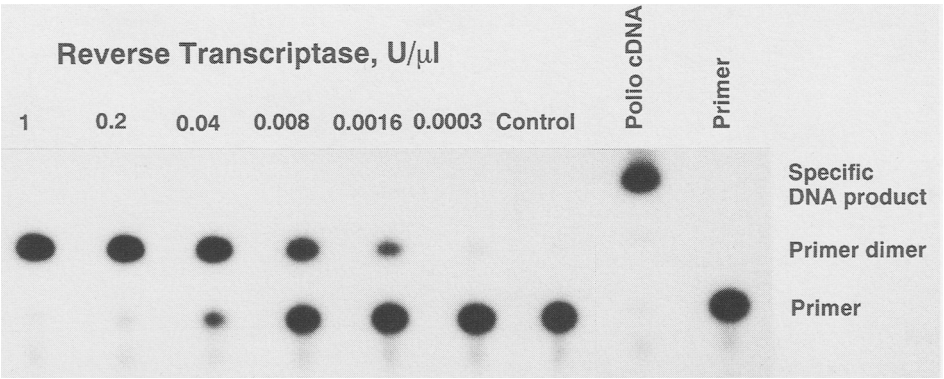

FIGURE 2 Stimulation of primer–dimer formation by RT. Varying amounts of RT were added to PCR containing no template DNA. Primers used for amplification of DNA segment 2 (nucleotides 2463–2603 of type 3 OPV) were used in this reaction. (Control) A sample with no template DNA or RT added. ^{32}P-Labeled PCR product was analyzed by PAGE.

ACKNOWLEDGMENTS

This work was supported in part by grant 9102A01 from the National Vaccine Program Office.

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Received April 19, 1994; accepted in revised form June 24, 1994.