4342–4343 Nucleic Acids Research, 1994, Vol. 22, No. 20 © 1994 Oxford University Press

RT-PCR: 'background priming' during reverse transcription

Barbara Frech* and Ernst Peterhans
Institute of Veterinary Virology, University of Bern, Laenggass-Strasse 122, CH-3012 Bern, Switzerland

Received August 9, 1994; Accepted September 2, 1994

The reverse transcriptase-polymerase chain reaction (RT-PCR) technique is a highly sensitive method for detecting and characterizing RNA molecules in small samples of cells or body fluids. It employs reverse transcription of the target RNA followed by PCR amplification of its cDNA. Widely used primers for cDNA synthesis are oligo (dT), random hexanucleotides or a sequence-specific antisense oligonucleotide primer. In addition it has been shown that low molecular weight cellular RNAs can serve as primers for cDNA synthesis (1).

Here we report that random priming by cellular RNAs also plays an important role in amplifying RNA molecules during RT-PCR. Due to random priming at the level of reverse transcription (RT) a pool of cDNAs of different sequence origin is synthesized, and it has to be considered that it is this pool of cDNAs PCR is accomplished on.

Performing RT-PCR on total RNA of bovine cells infected with bovine viral diarrhea virus (BVDV), a positive-stranded RNA virus, we observed that viral sequences could be amplified independent of whether a primer was added to the RT reaction or not (Figure 1A, lanes 1 and 2). The same RT reaction products could be used to amplify the cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (Figure 1A, lanes 3 and 4). However, RNA did not directly serve as a template for PCR, indicating that the RT step itself and not a RT activity of the Taq polymerase (2) during PCR led to the synthesis of cDNA templates (Figure 1A, lane 5). We conclude that total cellular RNA preparations contain enough small RNA fragments to efficiently prime for the synthesis of diverse cDNAs. Independent of the type of reverse transcriptase used (AMV reverse transcriptase (Figure 1A) and Superscript (RNaseH-) Mu-MLV reverse transcriptase (Figure 1B)) or of the RNA isolation method used (data not shown), the RNA preparations showed self-priming, indicating that this is a general property of the RT technique. Diluting the RT reaction products, we found that, though the addition of the specific antisense primer to the RT reaction led to more sequence-specific cDNAs and thus increased the sensitivity of the RT-PCR, the cDNA 'background' of reverse transcription was quite remarkable (Figure 1B). To evaluate the relative contributions of different primers in priming cDNA synthesis, we used total cellular RNA bound to membrane filters as a template for reverse transcription. RNA immobilized on membranes strongly reduced 'background priming', probably because immobilized RNA fragments were prevented from priming. It also decreased the sensitivity of the RT-PCR (data not shown). However, the RNA was accessible for primers added to the RT reaction. Under conditions where the membrane-bound RNA alone did not give a signal after RT-PCR (Figure 1C, lane 10), we could show that total RNA from uninfected bovine cells and from yeast cells (Figure 1C, lanes 1 to 4) and the specific antisense oligonucleotide primer (Figure 1C, lanes 8 and 9) could prime for cDNA synthesis. The strength of the RT-PCR signal depended on the amount of primer added. As virus-free cellular RNA served as a primer, we could exclude that the 'background priming' depended on viral negative-stranded RNA fragments. Random hexanucleotides which are often described as primers for RT (3), did not prime under these conditions (Figure 1C, lane 7), indicating that priming by random hexanucleotides may simply be priming by cellular RNA fragments present in the RNA preparations. We additionally tested tRNA which is sometimes added to stabilize RNA preparations. It did also not prime (Figure 1C, lanes 5 and 6).

Our data show that RT starting from total cellular RNA leads to cDNA synthesis independent of the addition of primers and that diverse cDNAs may be synthesized under these conditions. Thus it is necessary to reach sequence specificity at the level of PCR. Starting PCR from a pool of cDNA sequences could in some specialized applications of RT-PCR lead to troubling artefacts. One example is an artefact termed polymerase halt-mediated linkage of primers which in the presence of cDNAs with closely related sequences might lead to false positives in recombination studies based on RT-PCR (4). A possibility to make cDNA synthesis more specific might be the use of membrane- or bead-immobilized RNA or gel-purified size fractions of RNA (1) as a template for RT.

Total cellular RNA was isolated from non-infected or BVDV-infected embryonal calf nose epithelial cells using three different methods: SDS-acid phenol/chloroform extraction (5), acid guanidinium thiocyanate–phenol–chloroform extraction (6) and guanidinium thiocyanate extraction combined with centrifugation through a $CsCl_2$-cushion (7). Oligonucleotide primers specific for the BVDV strain NADL (1) correspond to nucleotide positions 6810–6829 (5′-TGGACACGGTTATAGACACG-3′, sense primer) and 7367–7348 (5′-CAGTAGCGTAGATATACA-CG-3′, antisense and cDNA primer). GAPDH-specific (8) oligonucleotides correspond to nucleotide positions 4–22 (5′-GTGAAGGTCGGAGTCAACG-3′, sense primer) and 340–359 (5′-GAGATGATGACCCTTTTTGGC-3′, antisense and cDNA primer). First strand cDNAs were synthesized by incubating 1 μg of total RNA from virus-infected cells with 0.8 μM of the appropriate antisense primer in a 50 μl reaction volume containing 50 mM Tris–HCl pH 8.3, 1 mM $MgCl_2$, 75 mM KCl, 10 mM DTT, 40 U Rnasin (Promega, Madison, WI, USA)

* To whom correspondence should be addressed

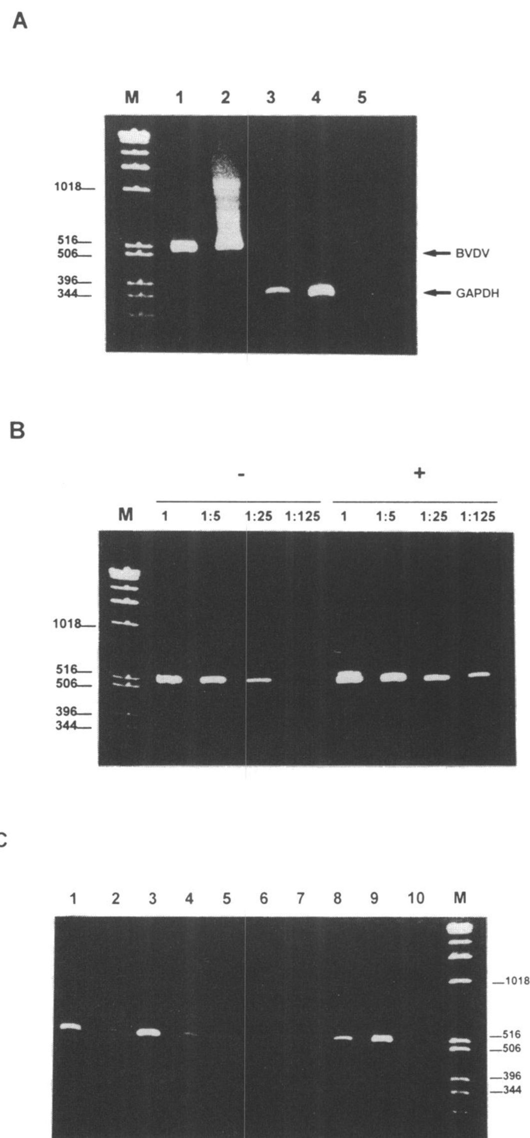

Figure 1. Ethidium bromide stained 4% polyacrylamide gels **A:** RT-PCR on total cellular RNA from BVDV-infected bovine cells using AMV reverse transcriptase. BVDV-specific (lanes 1 and 2) and GAPDH-specific (lanes 3 and 4) RT-PCR with (lanes 2 and 4) or without (lanes 1 and 3) addition of the specific antisense primer during RT. Lane 5: BVDV-specific PCR directly performed on 1 μg RNA. **B:** RT-PCR on total cellular RNA from BVDV-infected bovine cells using Superscript Mu-MLV reverse transcriptase. Comparison of cDNA priming in the absence (−) or presence (+) of the BVDV-specific downstream primer. Serial 5-fold diluted cDNAs were used as templates for PCR. **C:** Test of different primers on membrane-bound RNA as a template for cDNA synthesis. cDNAs were primed by total RNA from bovine cells (lanes 1 and 2, 10 and 1 μg, respectively), total RNA from yeast (lanes 3 and 4, 10 and 1 μg, respectively), tRNA (lanes 5 and 6, 10 and 1 μg, respectively), 150 pmoles random hexanucleotides (lane 7), 0.4 and 40 pmoles of the BVDV-specific antisense primer (lanes 8 and 9, respectively) or no primer (lane 10). RT was performed using AMV reverse transcriptase and cDNAs were amplified by BVDV-specific PCR. **A−C:** In every lane 1/10 of the PCR reaction was loaded. M: Molecular weight standards (1 kb ladder).

and 0.5 mM deoxynucleotide triphosphates. Either 8 U AMV reverse transcriptase (Promega, Madison, WI, USA) or 200 U Superscript (RNase H^-) Mu-MLV reverse transcriptase (BRL, Gaitersburg, MD, USA) were added and the mixes incubated for 1.5 h at 42°C or 37°C, respectively. PCR reactions contained 10 mM Tris−HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatine, 1% (v/v) Triton X-100, 0.25 mM deoxynucleotide triphosphates, 0.2 μM sense and antisense primer, 1 U Taq polymerase (Stehelin, Basel, CH) and 10 μl RT reaction in a 100 μl volume. A temperature profile of 30 sec at 94°C, 1 min at 56°C and 1 min at 74°C was used for 30 cycles. For amplification of RNA supported on membranes the following modification was used (9): 2 μg of total RNA were dissolved in 6×SSC containing 7.4% formaldehyde, dotted onto 9 mm^2 nitrocellulose filters (Millipore nitrocellulose filter type HA, pore size 0.45 mm; Millipore, Bedford, MA, USA) and UV cross-linked. The filters were added to the RT reaction mix, overlaid with mineral oil and incubated for lh at 42°C, for 6 min at 96°C and then put on ice. For priming the cDNA reaction either the antisense oligonucleotide primer, random hexanucleotides, total RNA from yeast (Boehringer, Mannheim, FRG), tRNA (Sigma Chemicals, St. Louis, MO, USA) or total RNA from non-infected embryonal calf nose epithelial cells were added. PCR was performed as described above.

ACKNOWLEDGEMENTS

The authors wish to thank Drs G.Bertoni and U.Pauli for critically reading the manuscript. The work was supported by Swiss National Science Foundation, grant no. 31-39733.93.

REFERENCES

1. Collett,M.S., Larson,R., Gold,C., Strick,D., Anderson,D.K. and Purchio,A.F. (1988) *Virology* **165**, 191−199.
2. Tse,W.T. and Forget,B.G. (1990) *Gene* **88**, 293−296.
3. Kawasaki,E.S. (1990) In Innis,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (eds), *PCR protocols*. Academic Press, Inc., San Diego, pp. 21−27.
4. Frohman,M.A. and Martin,G.R. (1990) In Innis,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (eds), *PCR protocols*. Academic Press, Inc., San Diego, pp. 228−236.
5. Stallcup,M.R. and Washington,L.D. (1983) *J. Biol. Chem.* **258**, 2802−2807.
6. Chomcszynski,K. and Sacchi,N. (1987) *Anal. Biochem.* **162**, 156−159.
7. Glisin,V., Crkvenjakov,R. and Byus,C. (1973) *Biochemistry* **12**, 2633−2637.
8. Tso,J.Y., Sun,X-H., Kao,T-H., Reece,K.S. and Wu,R. (1985) *Nucl. Acids Res.* **13**, 2485−2502.
9. Ruiz,A. and Bok,D. (1993) *BioTechniques* **15**, 882−886.