Magnetic Bead Technology Aids Sequence Determinations for the 3’ and 5’ Ends of Viral RNAs

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We have recently obtained extensive sequence information for the genomes of single-stranded (ss)RNA plant viruses that cannot be purified properly because they are too fragile or occur in too low concentrations (5,6). Randomly primed cDNAs were obtained for plus-strand RNA from minute amounts of virus particles that were immunocaptured from crude plant sap and for minus-strand RNA from denatured preparations of the viral double-stranded (ds)RNA present in infected plants. If a short stretch of the nucleotide (nt) sequence of a viral RNA is known, most of the remaining parts except for the 5’ and 3’ ends can be amplified by polymerase chain reaction (PCR) using the individual random primers that had been used for cDNA synthesis in combination with specific primers that are derived from the known part of the sequence (6). Anchored or one-sided PCR (10,11) with specifically primed cDNAs on which a homopolymer would have to be generated was considered as a possibility for also obtaining sequence information for the 5’ and 3’ ends of these RNAs. A prerequisite for the generation of a homopolymer tail on a cDNA is the complete removal of the four dNTPs that are left in the cDNA preparations after reverse transcription (RT). Unfortunately, conventional purification by phenol extractions and precipitations with ethanol and salt are not feasible with the minute amounts of cDNA obtained with RNA released from immuno-captured virus particles. For polyadenylated RNAs, cDNAs have been generated directly on oligo(dT)-coated paramagnetic beads, where they can easily be washed and tailed (9). However, this method is not applicable to non-polyadenylated RNAs; e.g., the plus-strand RNAs of many plant viruses and the minus-strand RNAs that can be obtained from denatured preparations of dsRNAs.

To also be able to use magnetic bead technology for tailing minute amounts of cDNAs to non-polyadenylated RNAs, we have developed a method that is outlined in Figure 1. For obtaining the 5’-terminal sequences of viral RNAs, plus-strand RNA from immuno-captured virus particles was used as a template for cDNA synthesis, whereas denatured preparations of dsRNA served as a template when the 3’-terminal sequences of viral RNAs were to be amplified from minus-strand RNA. cDNA synthesis was started with a specific 20- to 25-mer oligonucleotide primer (Pa) that was complementary to the respective plus- or minus-strand RNA in a region about 100 nt upstream of the end of the known part of its sequence. This primer Pa was annealed to the template RNA under highly stringent conditions to avoid the formation of additional misprimed cDNAs complementary to other parts of the RNA template. Using the computer program Oligo (National Biosciences, Plymouth, MN, USA), 25-mer 3’-biotinylated trapping oligonucleotides were designed that were complementary to the cDNAs slightly downstream of their 5’ ends that correspond to primer Pa. Care was taken that these trapping oligonucleotides showed minimal internal base-pairing and that dimer formation was negligible. The trapping oligonucleotides were bound to streptavidin-coated magnetic beads by their 3’-biotin residues, and cDNAs were bound to the beads by these oligonucleotides. Contaminating dNTPs were washed off, and terminal deoxynucleotidyl transferase (TdT) was used for generating dG tails on the trapped cDNAs. After tailing, the cDNAs were removed from the beads by boiling and subsequent rapid cooling on ice, and PCR was done with oligo(dC) and a second primer (Pb), which corresponded to a region close to the end of the known part of the cDNA sequence downstream of the region where the trapping oligonucleotide binds (Figure 1).

The protocol of the method is as follows. Sixty microliters of a suspension of streptavidin-coated paramagnetic beads (PolyATtract; Promega, Madison, WI, USA) were washed 3 times with 50 µL 0.5x standard saline citrate (SSC), and the beads were then immediately incubated for 10 min at room temperature with a mixture of 19.5 µL of the stock solution of the 3’-biotinylated trapping oligonucleotide (synthesized at a 0.2-µmol scale by Pharmacia Biotech, Uppsala, Sweden) and 0.5 µL 20x SSC (the final SSC concentration in the mixture was therefore 0.5x). Unbound trapping oligonucleotide was removed by three washes with 50 µL 0.5x SSC each. cDNAs were obtained for plus-strand RNA by immuno-capture RT-PCR (8) or for minus-strand RNA from denatured dsRNA (4) essentially as described in previous papers. The only difference was that the annealing of the primers Pa to the template RNAs was done under more stringent conditions, starting at 80°C for ssRNAs or 90°C for dsRNAs, with slow cooling until the Tm of the respective primer (obtained from the computer program Oligo) was reached. To complete the annealing, the samples were kept for 10 min at this temperature (60°–70°C depending on the primer) and then chilled on ice. RT (8) was done for 1 h at 42°C, and 48.75 µL of the resulting cDNA solution were heated for 3 min at 94°C, chilled rapidly on ice and then mixed with 1.75 µL 20x SSC. This mixture, containing 0.5x SSC, was added to the pretreated paramagnetic beads and then gently shaken for 10 min at 32°C to anneal the cDNA to the trapping oligonucleotide, thus binding it to the beads. The dNTPs...
and other remnants of the RT reaction were removed by three washes with 50 µL 0.5× SSC each. For tailing, the cDNAs trapped on the beads were incubated for 15 min at 37°C with a mixture of 1 µL (20 U) TdT, 4 µL 10× TdT buffer (both from Pharmacia Biotech), 0.6 µL of a 100 µM dGTP solution and 34.4 µL water. The reaction was stopped by adding 1 µL 500 µM EDTA (pH 8.0), and the beads were washed twice with 50 µL 0.5× SSC and once with 50 µL 0.1× SSC. To remove the tailed cDNAs from the beads, 20 µL distilled water were added, and the reaction tubes were placed for 3 min in a boiling water bath and then cooled immediately on ice. Ten to fifteen microliters of the supernatant were used for PCR. The PCR products were purified from agarose gels using the QIAquick™ Gel Extraction Kit (Qia-gen, Hilden, Germany) and cloned into the pT7Blue T-vector (Novagen, Madi-

Figure 1. Schematic representation of the amplification of cDNA ends after dG-tailing on magnetic beads coated with streptavidin and 3'-biotinylated trapping oligonucleotides.
Their nucleotide sequences were determined with the Sequenase® DNA Sequencing Kit Version 2 (Amersham, Cleveland OH, USA) using [α-35S]dATP in the labeling reaction.

Using this method, PCR products were obtained for the 5′ and 3′ ends of the non-polyadenylated RNAs 2 (6) and 3 (5) of beet soil-borne virus (BSBV) and for the 5′ end of the polyadenylated RNA 5 of the Pithiviers strain of beet necrotic yellow vein virus (BNYVV) (7). For these viral RNAs, we had determined almost all of the nt sequences, except for the 5′ and 3′ ends, using the methods reviewed in the introductory paragraph of this paper. Analyses of several clones of the PCR products obtained for the 5′ and 3′ ends of the various RNAs revealed that the sequences of the specific primers (Pb) were extended into flanking known parts and, later, unknown parts of the cDNAs until the homopolymer sequence of the tails was reached. Usually, three clones were sequenced. Occasionally, it was found that the sequence in one of these clones reached the homopolymer tail earlier than in the others. This was presumably due to a premature termination of cDNA synthesis by the reverse transcriptase. Various clones of PCR products obtained for the 5′ ends of viral RNAs contained an extra C residue just upstream of the G tail that was not present in others. This extra C residue was apparently incorporated in response to a cap structure at the 5′ end of the RNA (1,2). Ideally, different homopolymer tails should be generated on each cDNA to determine which nucleotides belong to the genuine viral sequence and which ones are part of the tail. However, attempts to obtain visibly stained PCR products after dC or dA tailing failed, thus confirming observations made by others with conventional methods that dGTP is the most suitable dNTP for homopolymer tailing (3).

Using the method described here, we were able to extend the known sequences of the 5′ ends of RNAs 2 and 3 of BSBV and RNA 5 of BNYVV by 64, 282 and 680 nt, respectively, and of the 3′ ends of BSBV RNAs 2 and 3 by 28 and 29 nt, respectively (5,6). All of the nucleotide sequences of BSBV RNAs 2 and 3 and of two sources of BNYVV RNA 5 have been deposited with data libraries under the Accession Nos. Z66493 (EMBL), U64512, U78292 and U78293 (GenBank®), respectively.

We suggest that this method should be of general use for the amplification of minute amounts of cDNAs to the 5′ and 3′ ends of viral RNAs and of unknown flanking nucleic acid sequences in general.

REFERENCES


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