



Synthesis of Long cDNA from Viral RNA Template

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Methods to make long and reliable cDNA from viral RNA template have been optimized. The conditions of the denaturation of the viral RNA template were most critical. For synthesis of the first DNA strand, the concentration of the primer and the presence of an RNase inhibitor were important. During the synthesis of the second strand, the incubation temperature was found to have effect on the length of the transcripts. Application of our optimized conditions on coronaviral genomic RNA as template resulted in cDNA libraries with inserts in the range of 0.5–5 kb without a separate cDNA size selection. Furthermore, a convenient variant of the alcohol precipitation and the analysis of single-stranded DNA on neutral agarose gels are described.

The first step in the study of RNA viruses at the nucleotide sequence level is the synthesis and cloning of cDNA. With established procedures [1], clones with inserts of less than 2 kb, corresponding to the size of most cellular mRNA, may be obtained routinely. However, with long RNA templates, such as viral RNA genomes, longer inserts are essential for manipulation in subsequent steps such as mapping, sequencing, and expression in heterologous systems. Here we describe the testing of a number of conditions that were critical for the length of cDNA. Our optimized procedure was found to be applicable both to the specific priming with a synthetic oligonucleotide and to the random priming by pentanucleotides. Results have been obtained with a number of coronaviruses that compare favorably with results of other published procedures [2–5].

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Materials and Methods

Materials

The growth and purification of coronaviruses as well as the isolation of genomic RNA have been described [6–8]. Reverse transcriptase was supplied by Promega (Madison, WI), DNA polymerase and terminal deoxynucleotidyl transferase by Amersham (Amersham, UK), and RNase H by Boehringer (Mannheim, FRG). Pentanucleotides from DNase-digested calf thymus DNA and dG-tailed pUC9 were from Pharmacia (Uppsala, Sweden). A specific pentadecanucleotide complementary to a sequence in the gene coding for the matrix protein of infectious bronchitis virus was used for the specific priming of cDNA synthesis towards the peplomer gene [6].

cDNA Synthesis

Prior to cDNA synthesis, RNA (0.25 µg/ul) was denatured in 6 mM methylmercuric hydroxide. After 10 minutes at room temperature, 2-mercaptoethanol was added to 56 mM.

Pilot experiments to test different conditions during the first-strand synthesis were carried out with 0.5 µg RNA in 10 µl. Standard reaction conditions were: 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 8 mM MgCl₂, 14 mM 2-mercaptoethanol, 0.5 mM of all four dNTPs, and, per 10 µl 1 µg primer, approximately 1 µCi [α -³²P] dATP, 6 U RNasin, and 5 U reverse transcriptase. Incubation was for 60 minutes at 42°C, with random priming preceded by 10 minutes at 37°C [2].

Preparative single-stranded cDNA synthesis was performed with 2.5 µg RNA in 50 µl under the same conditions but without tracer. After phenol/chloroform extraction and isopropanol precipitation (see below), the second strand was synthesized essentially as described [9]; test samples with [α -³²P]dATP contained cDNA made with 0.15 µg RNA in 10 µl.

Homopolymer tailing with dC residues was carried out essentially as described [1] with 380 pmol [³H]dCTP and 0.3 µg cDNA in 10 µl (a low dCTP concentration was necessary for an accurate measurement of the incorporation above the background signal). Incubation with 4 U terminal deoxynucleotidyl transferase at 37°C for 1 minute was found to be optimal both by calculating the length of the tail [1] and by counting recombinants after different reaction times. Annealing with



pUC9 (Pharmacia) and transformation of JM109 was performed as described [1].

Alcohol Precipitation

Alcohol precipitation was carried out by adding 0.1 volume NaOAc/Dextran (3 M NaOAc, pH 5.6, 100 μ g/ml Dextran 250, Pharmacia), and 0.6 volume isopropanol. After mixing, samples were spun immediately at room temperature (15 minutes, 10,000g). DNA pellets were washed with 70% ethanol, dried, and dissolved in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

Agarose Gel Electrophoresis

Gel electrophoresis of cDNA, denatured either in 0.1 M NaOH for 15 minutes or by glyoxal treatment [10], was carried out in neutral agarose gels (1% in Tris/borate/EDTA) [1]. Lambda DNA/*EcoRI*/*HindIII* digests, endlabeled by Klenow

treatment and denatured in the same way, were used as markers. After drying, gels were subjected to autoradiography.

Results and Discussion

Precipitation and Electrophoresis Procedures

The effects of variations in the conditions of cDNA synthesis were evaluated by measuring the [α - 32 P]dATP incorporation by TCA precipitation, and analysis of DNA by electrophoresis in neutral agarose gels after isopropanol/Dextran 250 precipitation and denaturation by NaOH. Regarding the reliability of the latter procedures, the following observations were made.

1. Unincorporated nucleotides as well as pentanucleotide primers were removed completely by the fast isopropanol/Dextran precipitation.
2. The use of Dextran 250 ensured complete re-

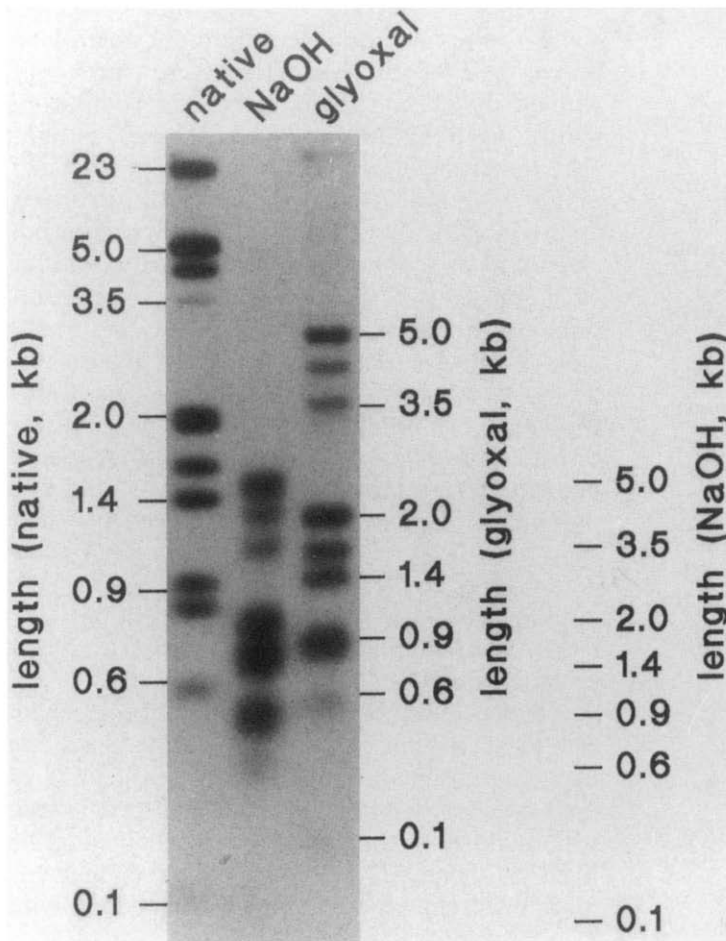
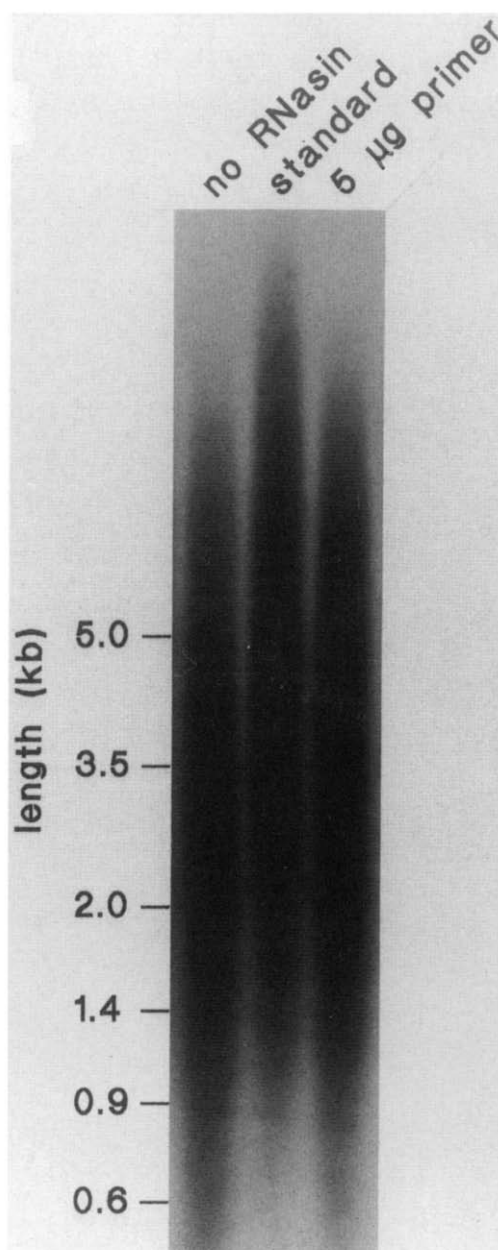


Figure 1. Mobility of double-stranded, NaOH-denatured or glyoxal-denatured DNA fragments from phage lambda on neutral agarose gels (1% in Tris/borate/EDTA, 2.5 V/cm).



covery of double-stranded DNA but obviated lengthy precipitation times recommended for ethanol/NaOAc [1] or ethanol/NH₄OAc precipitations [11]. With cDNA-RNA hybrids, about 50% of the TCA-precipitable material, most probably short transcripts, was not recovered.

Figure 2. Length distributions of the first cDNA strand. Genomic RNA (0.5 µg) from infectious bronchitis virus served as template. Standard conditions included denaturation by methylmercuric hydroxide, 6 U RNasin, and 1 µg random primer. Samples were precipitated by isopropanol/Dextran, denatured by NaOH, and analyzed by gel electrophoresis.



3. DNA denatured by NaOH has a much higher mobility than double-stranded DNA (Figure 1), indicating that there is no reassociation during neutral electrophoresis.
4. NaOH-denatured DNA has also a higher mobility than DNA denatured by glyoxal (Figure 1). This difference is too large to be explained solely by an increase in molecular weight by glyoxylation. Presumably, intramolecular base pairing in NaOH-denatured DNA, leading to a more compact shape, increases mobility. However, the same effect of NaOH denaturation has been observed with denatured cDNA. Therefore, denaturation by NaOH and electrophoresis in normal gels provides a fast and reliable way to estimate the length of cDNA strands, allowing the convenient use of the same gels as used normally for double-stranded DNA.

Synthesis of the First cDNA Strand

The first-strand cDNA synthesis has been tested with genomic RNA (27kb) from infectious bronchitis virus, strain M41, and randomly primed by pentanucleotides. Figure 2 shows a typical gel electrophoretic analysis of the lengths of the transcripts; relative effects on length and yield are summarized in Table 1.

Complete RNA denaturation by methylmercuric hydroxide increases the length, but not the yield, of the cDNA (Table 2). Inhibition of RNase activity by RNasin had a similar effect. Although these effects will vary from sample to sample, it is clear that the use of both methylmercuric hydroxide and RNasin may be generally recommended.

A third important factor is the concentration of the primer. Apparently, a high concentration (5

Table 1. Relative Length Distributions and Yields of the First cDNA Strand Made Under Different Conditions

	Length range (kb)	Yield (µg)
Standard conditions	1.0-15	0.08
RNA denaturation by heating (3 min, 56°C):	0.6-4.5	0.08
Omitting RNasin:	0.4-7	0.09
5 µg random primers/10 µl:	0.6-10	0.30

Relative length ranges have been estimated by agarose gel electrophoresis after NaOH treatment (Figure 1); yields reflect TCA-precipitable material.



Table 2. Coronavirus cDNA Libraries

Virus	RNA	Primer	Insert length (kb)	Mean length (kb)
Infectious bronchitis virus	Genomic	Specific	0.3-4.0	1.3
Infectious bronchitis virus	Genomic	Random	0.6-3.0	1.1
Feline infectious peritonitis virus	Genomic	Random	0.4-5.5	2.1
Porcine transmissible gastroenteritis virus	RNA3	Random	0.6-5.0	2.1

Coronaviral genomic RNA has a length of about 27 kb; RNA3 is about 9 kb long. Plasmid DNA from clones hybridizing to viral RNA fragments was isolated by alkaline lysis [1] and analyzed by *Pst*I cleavage and agarose gel electrophoresis.

$\mu\text{g}/0.5 \mu\text{g}$ RNA) stimulates the reaction but has a negative effect on the length of the transcript (Figure 2, Table 1). Concentrations lower than our optimal concentration of $1 \mu\text{g}/0.5 \mu\text{g}$ RNA gave the same length distribution, but a lower yield (not shown). This size distribution was also obtained with a synthetic oligonucleotide de-

signed to start synthesis from one position and tested at the same concentration of $1 \mu\text{g}/0.5 \mu\text{g}$ RNA (Figure 3, first lane).

Synthesis of the Second cDNA Strand

The second-strand synthesis was tested via the

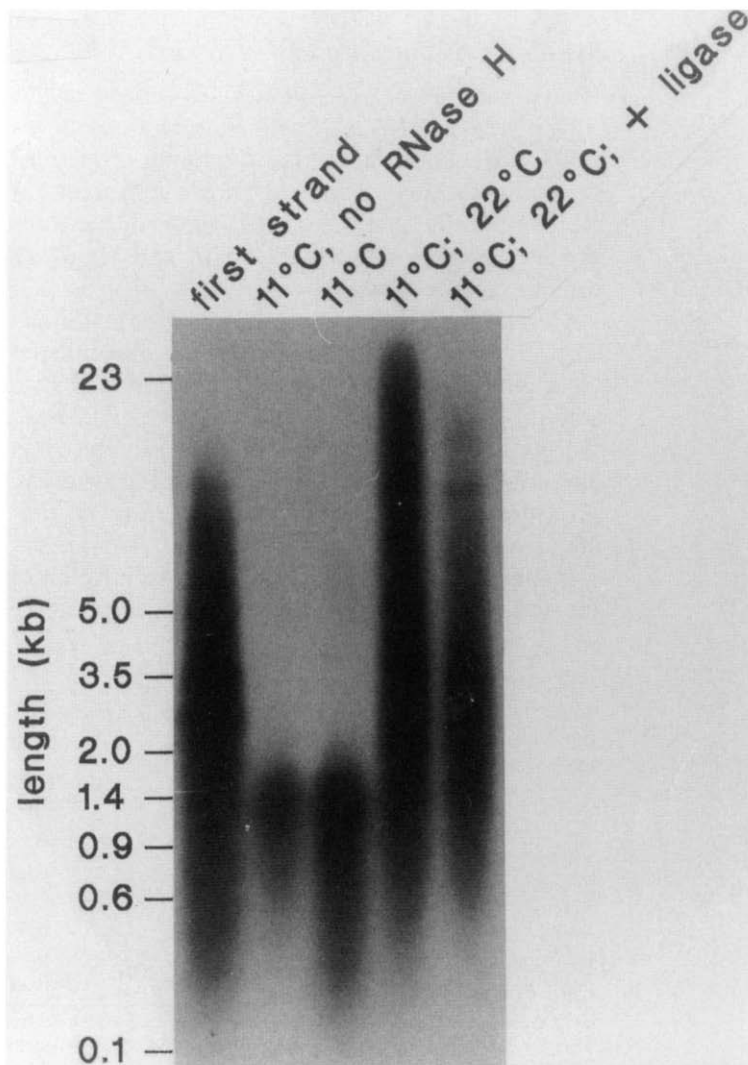


Figure 3. Length distributions of the second cDNA strand. After synthesizing the first strand with genomic RNA from infectious bronchitis virus as template (first lane), the RNA-DNA hybrids were converted to double-stranded cDNA by the combined action of RNase H and DNA polymerase [9, 11]. The second and third lane show the length distributions after the first 11°C incubation without and with RNase H, respectively. The fourth and fifth lane show the length distributions after the second incubation (at 22°C) without and with T4 DNA ligase. Identical results have been obtained with RNA-cDNA hybrids generated by random priming.



same procedures as used for the first strand, with the [³²P]dATP tracer added after the first-stranded synthesis. Carrying out the reaction as described [9], we obtained the same size distribution observed for the first-strand synthesis, with a yield approaching 100%. Apparently, there is no need to optimize this reaction further. However, regarding the mechanism of the reaction, we made the following observations (Figure 3).

1. Cleavage of the RNA associated with the first cDNA strand is only partially needed as omitting RNase H only decreased the yield about 50%.
2. At 11°C, DNA polymerase made transcripts of 1.5 kb or less.
3. During the second incubation (at 22°C [9]), these short transcripts were converted to full-length transcripts with the same length distribution as observed after the first-strand synthesis.
4. This conversion was due to the action of DNA polymerase because, in accordance with a previous study [9], DNA ligase had no influence on length or yield.

Cloning of Double-Stranded cDNA

The double-stranded DNA thus synthesized may be cloned by any of several procedures [1]: by linker addition or homopolymer tailing, by CaCl₂ or Hanahan transformation, and in plasmid or phage vectors. We used the conventional system of dC homopolymer tailing and annealing with dG-tailed pUC9, after which JM109 was transformed by either the CaCl₂ or the Hanahan procedure [1]. Our procedure has been used for the construction of cDNA libraries for several coronavirus strains and types. Typically, about 80% of the clones hybridized to labeled viral RNA. Table 2 shows the results of the positive clones of four representative libraries. Apparently, our results compare favorably with other published procedures [2-5]. The abundance of several clones with inserts of several kilobases obviated the need for a separate cDNA size selection by electropho-

resis or chromatography. Furthermore, the fidelity of the cloning procedure has been demonstrated by the complete absence of deletions or shuffling in the many clones sequenced until now [6-8].

Conclusion

A fast and convenient optimization of the cDNA synthesis has been developed. Our optimized conditions have been used for the construction of coronavirus cDNA libraries that contain several clones of up to 5 kb. This has been essential for the sequencing and expression of the 3.5-5-kb peplomer genes [6-8, unpublished results].

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