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Journal of Virological Methods, 11 (1985) 265–269
Elsevier
JVM 00422

SHORT COMMUNICATION

THE USE OF A RANDOM PRIMING PROCEDURE TO GENERATE cDNA LIBRARIES OF INFECTIOUS BRONCHITIS VIRUS, A LARGE RNA VIRUS

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(Accepted 1 April 1985)

An efficient method for the generation of gene banks from large RNA viruses is described using infectious bronchitis virus as an example. Randomly primed clones have been characterized and found to be representative of the viral genome including 5′ leader sequences.

cDNA cloning coronavirus IBV oligonucleotide primers

The cloning of large RNA molecules presents practical problems due to template degradation and premature termination during the reverse transcription reactions. The presence of strong stops, probably associated with the formation of RNA secondary structures, results in a decreasing probability of extension from the 3′ terminus. While attempting to clone the structural genes of infectious bronchitis virus (IBV), a 20 kilobase (kb), single-stranded RNA virus, no clones extending more than 3.3 kb from 3′ poly A region could be obtained using oligo-dT primed cDNA synthesis despite repeated attempts (Boursnell et al., 1984).

Two approaches towards overcoming the problem were employed. Firstly, a specific oligonucleotide was synthesised with sequence complementary to a region close to the 5′ end of the 3.3 kb of clones which we had obtained. This was used to specifically prime cDNA synthesis from this region and resulted in the isolation of clones extending approximately 8 kb from the priming site towards the 5′ end of the virus (Binns et al., 1985). This approach, although successful, is relatively expensive, requires knowledge of specific sequences and requires that a new oligonucleotide be synthesised for each extension of clones required.

Although calf thymus oligonucleotides have been used extensively to generate hybridisation probes, they have only infrequently been used for cDNA cloning of randomly primed material (Dowling et al., 1983). Our second approach involves the use of calf thymus oligonucleotides to randomly prime cDNA synthesis. It is cheap, requires no sequence information and the same oligonucleotide preparation can be used for all cloning attempts. It also has the advantage that it will effectively generate

clones representing the entire RNA sequence required with the possible exception of a small number of bases at the 3′ end.

The detailed procedures used were as follows. Oligodeoxynucleotides were prepared from calf thymus DNA (Sigma) by treatment with pancreatic DNase and size fractionation on DEAE-cellulose (Maniatis et al., 1982). IBV genomic RNA was prepared for strains M41 and Beaudette as described previously (Brown and Boursnell, 1984). cDNA synthesis was carried out using the method of Gubler and Hoffman (1983). cDNA synthesis with approximately 20 μg virion RNA and 100 μg calf thymus oligonucleotide primers was carried out in a reaction volume of 50 μl (50 mM Tris–HCl pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 4 mM sodium pyrophosphate, 1.25 mM dNTPs and 160 U reverse transcriptase [Anglian Biotechnology]) at 43°C for 30 min. After stopping the reaction with 20 mM EDTA followed by phenol extraction, the products were precipitated with ethanol and ammonium acetate. For second-strand synthesis the products were resuspended in 100 μl of 20 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 100 mM KCl, 0.15 mM β-NAD, 50 μg/ml BSA, and 40 μm dNTPs. 22.5 U DNA Polymerase 1 (Biolabs), 2.5 U RNaseH (BRL), and 5 U *E. coli* DNA ligase (Biolabs) were added to the reaction which was incubated at 12°C for 60 min and then at 22°C for 60 min. The products were phenol extracted twice and precipitated with ethanol and ammonium acetate. Double-stranded cDNA was tailed with dC residues, size fractionated on CL Sepharose 4B and cloned into dG tailed Pst1-cleaved pBR322. This material was used to transform *E. coli* LE392 by the method of Hanahan (1983) and selection made for tetracycline-resistant colonies. Between 2 and 4×10^4 tetracycline-resistant clones were obtained in each experiment of which approximately 5% were derived from uncut vector molecules. Clones were screened for the presence of viral inserts by colony hybridisation (Grunstein and Hogness, 1975) using ^{32}P-labelled, alkali-treated IBV genomic RNA as a probe.

The viral inserts present in a number of clones which were strongly positive in the colony hybridisation assay were characterised further. Firstly the size of the viral inserts in 24 Beaudette and 24 M41 clones was examined by making 'mini-preparations' of plasmid DNA (Holmes and Quigley, 1981) and digesting the DNA with Pst1. Analysis revealed that these strongly positive clones contained inserts ranging from approximately 600 bp to 3,500 bp with an average size of approximately 1,000 bp. Table 1 shows the details of the M41 clones, the results for the Beaudette clones being similar. Secondly the distribution of the clones along the viral genome was studied by Southern blots (Southern, 1975) using as probes 4 cDNA clones derived from Beaudette genomic RNA, C5-322, C5-136, MB179, and MB182, which extended from the 3′ polyA region to approximately 11.3 kb on the genome (see Fig. 1). With each probe one or more of the randomly primed cDNA clones gave a positive signal (see Table 1). About half of the viral positive clones failed to hybridise to any of the probes (which covered 11.3 kb), consistent with estimates of 20 kb for the genomic size of IBV. Clones which failed to hybridise were digested with a range of restriction endonucleases and many were found to possess different restriction sites. Furthermore, results of

TABLE 1

Characterisation of M41 CT-primer clones

Clone	Size of insert (bp)	Hybridisation[a]			
		C5-322	C5-136	MB179	MB182
165	900	-	+	+	-
168	1,100	-	-	-	-
169	1,500	-	-	-	-
170	1,150	-	-	+	+
173	500	-	+	-	-
183	800	-	-	-	-
185	650	-	-	-	-
186	700	-	-	-	-
187	1,200	-	-	-	-
188	850	-	-	-	-
189	1,000	-	-	+	+
190	2,100	-	-	-	-
191	1,300	-	-	+	+
192	950	-	-	-	+
193	700	-	+	+	-
194	800	-	-	+	+
195	800	-	-	+	+
196	900	-	-	-	-
197	900	-	-	-	-
198	1,300	-	+	+	-
199	1,800	-	-	-	+
200	1,500	+	+	-	-
201	1,100	-	-	-	-
202	1,200	-	-	+	+

[a] Clones showing positive hybridisation in Southern blots (+), no hybridisation (-).

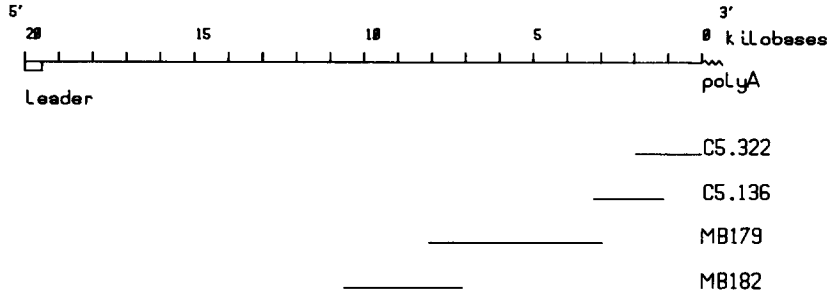

Fig. 1. Organisation of the clones used to determine the locations of randomly primed clones. Approximately 8.3 kb of the genome is not represented by the 4 clones.

Southern blots indicated that approximately 7 kb of the 8.3 kb of viral sequences not present in C5-322, C5-136, MB179 and MB182 were represented in 3 large recombinants. These results indicate that the clones were well distributed on the viral genome.

In one experiment 2,000 M41 clones were screened with a ^{32}P-labelled oligonucleotide with a sequence complementary to part of the leader sequence present on the 5′ ends of the genome and mRNAs, and which we had previously determined from mRNA A (Brown et al., 1984). Eight positive clones were identified. Four were found to have been derived from mRNA A as they contained nucleocapsid gene sequences in addition to leader sequences. This indicates that some mRNA A must copurify with genomic RNA in the virus isolation procedures we use. The other 4 clones appear to be genuine genomic leader clones as they do not contain any sequences present in the bodies of IBV mRNAs A, B, C, D, or E as determined by Southern blots. Preliminary sequence data for 2 of the clones shows that 1 clone extends to the 5′ end of the virus and the other to within 29 bases of the 5′ end (Brown, Boursnell, Tomley and Binns, in preparation).

In conclusion this paper describes a cDNA cloning protocol that is particularly useful for generating gene banks from large RNA viruses. We had previously used calf thymus primers with 'traditional' cDNA cloning methods employing S1 nuclease but find that the method of cDNA synthesis used here generates significantly larger clones. The clones obtained have been shown to have a random distribution on the genome and in the case of the leader clones at least 4 clones from our gene bank contained the specific sequence we searched for. The method is to be recommended for its ease, speed, and cheapness.

ACKNOWLEDGEMENTS

We thank Bridgette Britton and Penny Gatter for excellent technical assistance. This work was supported by Research Contract No. GBI-2-011-UK of the Biomolecular Engineering Programme of the Commission of the European Communities.

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