A Vector with Transcriptional Terminators Increases Efficiency of Cloning of an RNA Virus by Reverse Transcription Long Polymerase Chain Reaction

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Abstract

Full-length cDNA clones of RNA viruses are advantageous for maintaining the genomic sequence without the generation of diversity by accumulation of sequence mutations during productive virus replication. They permit in vitro manipulation of the genomic clone to test the effect of sequence changes on the phenotype of reactivated virus.

Infectious cDNA clones have been produced by ligation of subgenomic clones but are sometimes difficult to generate in a single cloning operation. We used reverse-transcription to synthesise full-length cDNA from genomic RNA of Coxsackievirus B3 of the Picornavirus family and enzymatically amplified this by long PCR. Five different cloning vectors were used to clone the long PCR product, including the vector Lorist6 which contains transcriptional terminators on either side of the cloning site to prevent transcription of inserts in E. coli. No recombinant colonies were obtained from any of the vectors lacking transcriptional terminators but three full-length clones were obtained using Lorist6. The results suggest that transcriptional terminators increase the recovery of cDNA clones of the 7.4 kb Coxsackie virus genome in this cosmid vector, without resort to phage packaging, representing an advance over previous methods and advantages in the molecular manipulation of these viruses.

Introduction

Group B Coxsackieviruses belong to the genus Enterovirus of the family Picornavirus and are common human pathogens. These viruses cause a variety of syndromes or diseases ranging from mild upper respiratory tract infections to meningitis, myocarditis or neonatal infections. The virus genome is a single molecule of positive-sense RNA of approximately 7.4 kb and has a virus-coded protein, Vpg, linked to the 5’ terminus in lieu of the 5’ cap structure of eukaryotic mRNA molecules. The 5’nontranslated region (5’NTR) of about 740 nucleotides is followed by a monocistronically translated coding region and a variable poly-A sequence at the 3’ terminus. An early strategy for enterovirus genomic cloning was to synthesise cDNA from viral RNA recovered from infected cell cultures and to screen for, and subsequently identify full length cDNA clones, treating the product as a cDNA library. For example, Kandolf and Hofschneider (1985) screened 4,800 clones before identifying a full-length cDNA clone of Coxsackievirus B3 (CVB3) strain Nancy. Recently, sub-genomic cDNA fragments amplified from the viral genome by reverse transcription-polymerase chain reaction (RT-PCR) have been ligated sequentially to construct full-length infectious clones (Blackburn et al., 1991). The more recent description of long PCR (Cheng et al., 1994) applied to RT products (Gritsun and Gould, 1995) provides a more direct route to generating full-length viral cDNA, which can then be cloned.

Reverse transcription-long PCR (RT-LPCR) has been used to clone the genomes of various RNA viruses including Coxsackie viruses (Gow et al., 1996; Lindberg et al., 1997; Martino et al., 1999), tick-borne encephalitis virus (Gritsun and Gould, 1995), hepatitis viruses (Tellier et al., 1996; Shao et al., 1996), HIV-1 (Fang et al., 1996) and simian immunodeficiency virus (Holterman et al., 2000). Complete cDNA clones of positive strand RNA virus genomes cloned can be reactivated to live virus by transfection of susceptible cells, most efficiently with cRNA transcribed from cDNA in vitro. This approach reduces the accumulation of mutations in virus stocks during repeated passage, generated by the infidelity of the viral RNA polymerase, and allows experimental sequence manipulation of virus genomes in vitro. Characterisation of the phenotype of such recombinant viruses after reactivation is an approach to functional gene mapping. Technically, RT-LPCR is routine and amplified partial or complete virus cDNA can be cloned but in some cases, full-length clones

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replicate poorly and are unstable (e.g. Coxsackie virus B3; our unpublished observation). Preferential cDNA cloning of defective viral genomes has been reported: Hepatitis C virus clones unable to encode a full-length polyprotein were selected (Forns et al., 1997) and up to a third of Human Immunodeficiency Virus genomic cDNA clones encoded functionally defective Tat proteins (Meyerhans et al., 1989; Vartanian et al., 1992). The interpretation is that some viral gene products are toxic in E. coli and so viable genomes are unlikely to be cloned unless their expression is constrained.

In this communication, we describe cloning cDNA of a variant Coxsackievirus B3 genome by RT-LPCR. We found that use of a vector with transcription terminators flanking the cloning site to insulate inserts increased the efficiency of cloning full-length cDNA.

Results and Discussion

The genomic RNA of an attenuated CVB3 (Zhang et al., 1993) was amplified by RT-LPCR and the full-length PCR product of ~7.4 kb separated in a 0.85% agarose gel (Figure 1) and its identity confirmed by partial nucleotide sequencing. Only ligation to Lorist6 generated recombinant colonies upon transformation of E. coli. Ligation to the other four vectors failed to produce colonies despite repeated attempts, although a subgenomic cDNA fragment (511 bp) ampliﬁed from the 5’NTR of virus RNA was cloned successfully as a positive control in each (data not shown). A total of 204 Lorist6 colonies were screened with a hybridisation probe corresponding to nucleotides 3089 to 3773 of the viral genome. Twelve positive clones were found and these contained long cDNA inserts on the basis of plasmid size determined by agarose gel electrophoresis. Linearization of recombinant plasmids with Sal I or Hind III indicated that six of these inserts had intact full-length ends, representing 3% of 204 colonies screened. All were in the 5’ to 3’ orientation downstream of the SP6 promoter and were confirmed by PCR amplification of 3′- and 5′-end sequences of the insert. These 6 clones replicated normally and were stable in E. coli. In contrast, a previously derived full-length cDNA clone of CVB3 in the cloning vector pGEM4Z-2 (Zhang et al., 1993), which does not have transcription terminators, replicates poorly or is not stable in E. coli (unpublished data). These results suggest that expression from the full-length CVB3 insert may be toxic to E. coli.

To confirm the effect of transcriptional terminators in the cloning vector on expression from CVB3 inserts in E. coli, RT-PCR and Western blotting were employed to detect CVB3 RNA and proteins respectively. 5′NTR sequences of CVB3 could be ampliﬁed by RT-PCR from RNA extracted from E. coli containing CVB3 constructs without transcriptional terminators and from CVB3-infected Vero cells as a positive control, but not from RNA from a Lorist6 clone (Lorist6/p14V1-8) containing the transcriptional terminators. However, transcription was not detected from any construct when RT-PCR was carried out with primers specific for sequences coding for the viral capsid protein VP1 (Figure 2), or if the reverse transcription step was omitted (data not shown). Consequently, viral capsid proteins were not detected by Western blot analysis (Figure 3). These results suggest that viral cDNA inserts were transcribed partially in vectors without transcriptional terminators and these transcripts may be toxic to E. coli cells, demonstrating the advantage of transcriptional terminators in cloning full-length cDNA of an RNA virus. The potential capacity of a cosmid vector to accommodate large inserts did not contribute to the cloning efficiency because this feature of Lorist6 was not exploited here by in vitro lambda phage packaging and so this vector has constraints on size of insert similar to the other four.

Partial nucleotide sequencing confirmed that three of the six full-length inserts had intact 5′ and 3′ ends. Further sequencing of a total of 8232 nucleotides (approximately 40% of these three clones) revealed 7 apparently random mutations,
Figure 2. Agarose gel electrophoresis of RT-PCR products of RNA transcripts from CVB3 inserts. RNA was extracted with Tri-reagent from E. coli cells transformed with pGEM4Z-1 (lane 1), pGEM4Z-2 (lane 2), pGEM vector (lane 4), Lorist6/p14V1-8 (lane 5), Lorist6 vector (lane 6), or from CVB3-infected Vero cells (lane 3). Lane 7 is water and reagent control (without template). pGEM4Z-1, pGEM4Z-2 and Lorist6/p14V1-8 all contain a full-length insert of CVB3 genome. RT-PCR was performed with the primer set P2/P3 amplifying a 198 bp fragment within the 5' nontranslated region (panel A) or with the primer set 011/012 amplifying a 447 bp fragment within the VP1 region (panel B) as described previously (Li et al., 1995; Peng et al., 2000).

Table 1. Comparison of partial sequence of three full-length clones to the parental virus

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>p14V1</th>
<th>Lorist6/14V1–2</th>
<th>Lorist6/14V1–8</th>
<th>Lorist6/14V1–12</th>
<th>Amino acid position</th>
<th>Amino acid change</th>
<th>Region of virus</th>
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<tr>
<td>294</td>
<td>T</td>
<td>C*</td>
<td>T</td>
<td>T</td>
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<td>A</td>
<td>G</td>
<td>G</td>
<td>51</td>
<td>Gly to Ser</td>
<td>VP4</td>
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<tr>
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<td>T</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>68</td>
<td>Leu to Pro</td>
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<tr>
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<td>T</td>
<td>C</td>
<td>G</td>
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<tr>
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<td>A</td>
<td>10</td>
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<td>2B</td>
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<tr>
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<td>A</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>62</td>
<td>Asp to Gly</td>
<td>2B</td>
</tr>
<tr>
<td>4099</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>19</td>
<td>Glu to Gly</td>
<td>2C</td>
</tr>
</tbody>
</table>

* Mutations observed are highlighted in bold and underlined.

Figure 3. Western blot analysis of CVB3 capsid proteins. Cell lysate of E. coli transformed with different constructs were analyzed by 12% SDS-PAGE and then immunoblotting with antibodies directed against virus capsid protein VP1 (panel A) or VP2 (panel B). Lane 1–6 are: CVB3-infected Vero cells, E. coli transformed with pGEM4Z-1, pGEM4Z-2, pGEM vector, Lorist6/p14V1-8 or Lorist6 vector respectively.

Cloning of RNA Virus with Transcriptional Terminators

The poliovirus RNA-dependent RNA polymerase has an error rate of 10^{-3} to 10^{-4} during DNA replication. The error rate observed in this study is within the range for virus RNA replication and RT-LPCR and the errors are likely to have been introduced during these steps, rather than in propagation of cloned cDNA. Error rates have not been assessed in previous reports on cloning enterovirus cDNA by RT-LPCR (Gow et al., 1996; Lindberg et al., 1997; Martino et al., 1999), though they are likely to have occurred.

It is recommended that a vector containing transcriptional terminators be used to clone full-length cDNA of RNA virus genomes. A further advantage of Lorist6 is the presence of T7 or SP6 promoters on either side of the cloning site allowing in vitro transcription of full-length cRNA of either polarity. Genomic sense RNA transcribed from modified cDNA clones in vitro is used to reovactivate infectious virus by transfection of cultured cells and so to investigate the phenotype of recombinant virus as an approach to functional mapping.

Experimental Procedures

Extraction of Enteroviral RNA from Infected Cell Culture or Transformed E. Coli Clones

Vero cell monolayers were infected with an attenuated CVB3 strain and harvested as described previously (Zhang et al., 1993). The supernatant was clarified by low speed centrifugation and virus pelleted by ultracentrifugation at 100,000 g for two hours at 4°C in a Sorvall TTT505 rotor. The virus pellet was resuspended in diethylpyrocarbonate (DEPC)-treated dH2O, extracted with phenol and viral RNA precipitated with ethanol and resuspended in DEPC-treated dH2O. RNA for RT-PCR was extracted from 1ml log-phase cultures of transformed E. coli cells using Tri-reagent (Sigma-Aldrich Company Ltd., UK) and dissolved in 50 μl of DEPC-treated dH2O.

Reverse Transcription

Full-length cDNA was synthesised by reverse transcription of CVB3 RNA at 42°C for 3h. The 50 μl reaction mix contained 100u Superscript II reverse transcriptase, 20–40 u RNase inhibitor (Gibco-BRL, USA), 2 mM dNTPs, 1 mM virus-specific primer 3’ (CCG CAC CGA ATG CGG AGA AT; nucleotides 7400–7381) or OL40 (GAA TTT ACC CCT ACT GTA CCG TTA;
nucleotides 7384-7361), 10 µl of 5 x Superscript buffer, 10 mM dithiothreitol (DTT), 1–2 µg of template RNA in DEPC-treated dH₂O.

Long PCR and Characterisation of Product

Full-length cDNA was amplified using the XL-PCR kit (PE- Applied Biosystems, USA). The 100 µl reaction mix contained 0.8 mM forward primer F16 (TTA AAA CAG CCT GTG GTT TG; nucleotides 1–20), 0.8 mM reverse primer EVGD1 (GCA CGG GTT AAC ACG GTT TTT TTT TTT TTT TTT TTT CCG CAC CGA ATG CGG AGA ATT; nucleotides 7440–7380), 4U rTth DNA polymerase, 30 µl 3 x buffer, 0.8 mM dNTPs, 1.0 to 2.0 mM MgAc₂ 10 µl of RT product in distilled H₂O. PCR was performed on a PE Applied Biosystems Thermal Cycler 4000 by 40 cycles of 94°C, 40 seconds; 60°C, 30 seconds and 72°C, 10 minutes. RT-LPCR products were analysed by agarose gel electrophoresis and purified by electrophoretic elution from diethylaminoacetate paper (Schleicher and Schuell, Germany). The identity of the cDNA was confirmed by partial nucleotide sequencing: semi-automated cycle sequencing on an ABI model 373 or 377 sequencer and sequence analysis were as described previously (Zhang et al., 1997; Cameron-Wilson et al., 1998).

Vectors and Host

Vectors pUC9 (Gibco-BRL, USA), Bluescript (Pharmacia, UK), the Novagen T vector (Novagen, The Netherlands), a T vector made according to the method of Khan et al. (1994) and Lorist6 (Gibson et al., 1987a) were used to clone purified RT-PCR products. The phage-based cosmid vector Lorist6, a later version of Lorist2 (Gibson et al., 1987b), contains transcriptional terminators flanking the multiple cloning site and downstream of the ned⁴ gene to prevent transcription into inserts. It also features a T7 or SP6 promoter on either side of the insert DNA to allow in vitro transcription of cRNA for use as strand-specific riboprobes or for transfection. Purified cDNA was cloned by phosphorylation and ligation to either of the two T vectors or by removal of the terminal 'A' residue by pfu DNA polymerase (Stratagene, UK) in the presence of 1 mM dNTPs and blunt-end ligation to the Smal I site in pUC9 or Bluescript or to the Sca I site in Lorist6, followed by transformation of E. coli XL-1.

Western Blot Analysis

Cell lysates were prepared from E. coli cultures transformed with recombinant constructs containing enteroviral cDNA inserts or with vector alone. Putative virus proteins were separated by SDS-PAGE, electro-transferred to nitrocellulose membranes and immunostained with antibodies specific for enterovirus capsid proteins by methods described previously (Ouyang et al. 1996). A monoclonal antibody (5-DB/1) specific for the enterovirus capsid protein VP1 was purchased from Dako Ltd. (UK) and a rabbit antiserum directed against a peptide antigen corresponding to the amino acids 150–168 (GDTAKEFKPVSAGSNKL) of capsid protein VP2 of Coxsackievirus B3 was designed in our laboratory and prepared by Cymbus Biotechnology Ltd. UK (Aasa-Chapman, PhD thesis of University of London, 2001). A chemiluminescence detection system (ECL; Amersham International, UK) was used to visualize protein bands via X-ray film.

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