Identification and characterization of the Tupaia herpesvirus DNA polymerase gene

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Tupaia herpesviruses (THVs) have been isolated from malignant lymphomas and from degenerating lung or spleen cell cultures of tree shrews (Tupaia spp.), but because of a lack of genetic information the final classification of THVs is still open. In the present work the viral DNA polymerase (DPOL) gene was mapped within the genome of the different THV strains using PCR and degenerate oligonucleotide primers. Nucleotide sequences of the DPOL genes of THV strains 1 to 5 were determined and used for comparative analyses. The transcriptional activity of the THV-2 DPOL gene was confirmed by RT–PCR. It was found that the different THV strains are very closely related to each other. When compared to other herpesviruses the highest amino acid sequence identities detected were with DPOLs of the murine and human cytomegaloviruses. These results justify the conclusion that THVs are members of the subfamily Betaherpesvirinae.

Herpesviruses are large DNA viruses that infect a wide range of vertebrate species ranging from fish to primates (Roizman, 1996). Five different strains of Tupaia herpesvirus (THV-1 to -5) have been isolated from different organs of tree shrews (Tupaia spp.) (Mircovic et al., 1970; Darai et al., 1979, 1982). Isolates THV-2 and -3 were isolated from malignant lymphomas, whereas the other strains were isolated from degenerating tissue cultures from lung (THV-1) or spleen (THV-4 and -5) of apparently healthy animals. The genome of THVs is a linear double-stranded DNA molecule of about 200 kbp. Physical maps of the THV-2 genome for the restriction endonucleases EcoRI and HindIII have been constructed, and a defined genomic library has been established (Koch et al., 1985). Until now, THVs have not been classified within one of the herpesvirus subfamilies (Alpha-, Beta- or Gammaherpesvirinae).

The DNA polymerase (DPOL) genes of herpesviruses are well suited for the investigation of their evolutionary relationships (McGeoch et al., 1995). Alignments of the amino acid sequences of different herpesvirus genes, e.g. DPOL, glycoprotein B or the major capsid protein, result in equivalent phylogenetic trees and in general confirm the classification of individual herpesviruses into different subfamilies as based originally on a variety of biological properties (Roizman et al., 1992; McGeoch et al., 1995). Therefore, we analysed the DPOL genes of different THV strains in order to gain sufficient genetic information for classification of THVs into one of the herpesvirus subfamilies.

Several highly conserved regions within the amino acid sequences of herpesvirus DPOLas were identified by comparative amino acid sequence analysis of the DPOLas of herpes simplex virus (Quinn & McGeoch, 1985), varicella-zoster virus (Davison & Scott, 1986), Epstein–Barr virus (Baer et al., 1984), human (Kouzarides et al., 1987) and murine cytomegalovirus (Elliott et al., 1991), human herpesvirus-6 (Teo et al., 1995), pseudorabies virus (Berthommé et al., 1995) and herpesvirus saimiri (Albert et al., 1992). Six oligonucleotide primers were synthesized corresponding to two highly conserved regions (CFDIEC and TGYNIINF D) (Table 1a). For subsequent cloning of the PCR products the oligonucleotide primers were flanked with recognition sites for the restriction endonucleases EcoRI and BamHI. The six oligonucleotide primers were used in PCR experiments in different combinations with purified genomic DNA of THV-1 and -2 as a template. The PCRs were carried out using 0.5 fmol of the template DNA in 100 µl volumes containing 1.5 mM MgCl₂, 12.5 nmol of each dNTP, 50 pmol of each primer and 2.5 units of Tag DNA polymerase (Applied Biosystems). Thirty-five cycles were run in an automated temperature cycling reactor (Genius, Techne) under cycling conditions of 96 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min per cycle. The oligonucleotide primer HHV4DP3, in combination with either oligonucleotide primer HHV1DP4 (Fig. 1a, lanes 1 and 2), HHV4DP4 or MCMVDP4, was able to amplify a specific DNA fragment of the expected size (309 bp).
Table 1. Properties of oligonucleotide primers used for identification of the DPOL genes of THVs

Abbreviations: NP, nucleotide position within the DPOL coding region according to the virus species from which the sequence was derived; HSV-1, herpes simplex virus type 1; EBV, Epstein–Barr virus; HHV-6, human herpesvirus-6; MCMV, murine cytomegalovirus.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence</th>
<th>Virus</th>
<th>NP</th>
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<td>(a)</td>
<td></td>
<td></td>
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<tr>
<td>HHV1DP3</td>
<td>Forward</td>
<td>5’ GGAGATTCTGCTTTGATATCGAATGC 3’</td>
<td>HSV-1</td>
<td>1096–1113</td>
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<td>HHV4DP3</td>
<td>Forward</td>
<td>5’ GGAGATTCCCTTCGATATCGAATGC 3’</td>
<td>EBV</td>
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<td>Reverse</td>
<td>5’ GGGATCCGTCAAGAGTTGATGTTGATACCCGGT 3’</td>
<td>HSV-1</td>
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</tr>
<tr>
<td>HHV4DP4</td>
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<td>1152–1126</td>
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<td>MCMVDP4</td>
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<td>(b)</td>
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<td>THV-2</td>
<td>3648–3626</td>
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</table>

Fig. 1. (a)–(c) PAGE of PCR products obtained using different oligonucleotide primers and DNA of different THV strains as a template. Lanes M, 100 bp ladder; lanes 1 to 5, PCR products using DNA of THV-1 to -5, respectively. (a) Primers HHV4DP3 and HHV1DP4; (b) primers THVDP1 and THVDP2; (c) primers THVDP3 and THVDP4. (d) PAGE of RT–PCR products obtained using primers HHV4DP3 and HHV1DP4 and total RNA of THV-2 infected TBF cells. Lane M, 100 bp ladder; lane 1, RT–PCR; lane 2, control PCR without reverse transcription step; lane 3, RT–PCR positive control.

when DNA of THV-1 or -2 was present in the reaction. The specificity of the PCR products was confirmed by nucleotide sequence analysis.

The THV-2 gene library (Koch et al., 1985) was screened by PCR using the oligonucleotide primers HHV4DP3 and HHV1DP4, for which specificity had been demonstrated. A specific DNA fragment was amplified using the recombinant plasmid pTH2-H-G, which harbours the THV-2 HindIII DNA fragment G (6±2 kbp). The complete primary structure of the THV-2 DPOL gene (4120 bp) including the DPOL open reading frame (3513 bp) was determined by nucleotide sequence analysis of the regions adjacent to the PCR product within fragment G using a primer walking strategy as described previously (Tidona & Darai, 1997). The deduced amino acid sequence was compared to the amino acid sequences of known DPOL proteins of herpesviruses using the CLUSTAL program (Higgins & Sharp, 1988) included in PC/Gene program release 6.85 (Intelligenetics Inc.). These analyses revealed the presence of the motifs and signatures typical of the B-family of DPOls (Wong et al., 1988) and that the DPOL of THV-2 shows highest identity to the DPOLs of murine and human cytomegaloviruses (MCMV, HCMV; amino acid sequence identity 48±5% and 43±5%, respectively). An alignment of the THV-2 DPOL with the corresponding gene products of MCMV and HCMV as well as the locations of the conserved DPOL motifs and signatures is shown in Fig. 2.

Owing to the lack of a defined genomic library of THV-1 the viral DNA fragments were isolated after cleavage with the restriction endonuclease HindIII from 0±8% low melting point agarose gels as described previously (Koch et al., 1985). PCR screening of the individual THV-1 HindIII DNA fragments with oligonucleotide primers HHV4DP3 and HHV1DP4 led to identification of the DPOL gene of THV-1 within HindIII DNA fragment G (6±2 kbp). This fragment was molecularly
Fig. 2. Multiple alignment of the amino acid sequences of the DPOL genes of THV-2, human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV). Identical amino acids are denoted by dots. Below the aligned sequences, well-conserved and identical amino acid positions are indicated by plus and asterisk symbols, respectively. Dashes indicate gaps introduced to achieve maximal amino acid matching. Selected conserved regions typical of the B-family of DPOLs (Wong et al., 1988) are boxed.
cloned in plasmid vector pUC19. DNA of the resulting plasmid clone (pUC19-THV1-H-G) was used in determining the complete DNA nucleotide sequence of the DPOL gene of THV-1 (4117 bp). Comparison of the DNA sequences of the DPOL genes of THV-1 and -2 revealed the presence of only seven nucleotide differences resulting in two amino acid exchanges at amino acid positions 549 (threonine to proline) and 699 (valine to alanine).

In order to gain genetic information on the DPOL genes of THV-3 to -5, two additional sets of primers (THVDP1 to -4, Table 1 b), corresponding to the DNA sequences of the DPOL genes that had been determined for THV-1 and -2, were synthesized. These primer pairs together with primers HHV4DP3 and HHV1DP4 led to the amplification of three specific DNA fragments (380, 578 and 309 bp, respectively) within the DPOL genes of THV-3 to -5 (Fig. 1 a, b, c). The nucleotide sequences of the resulting PCR products were determined and compared to the sequences of THV-1 and -2. The three different PCR products that were obtained from each THV strain together span 1010 bp of the 41 bp of the complete THV DPOL gene. The sequences obtained from THV-3 to -5 were almost identical to the sequences of THV-1 and -2; the few nucleotide differences detected did not result in any amino acid exchanges.

The transcriptional activity of the THV-2 DPOL gene was investigated by RT–PCR using oligonucleotide primers HHV4DP3 and HHV1DP4. For RT–PCR, total cellular RNA was extracted [using the guanidinium–cesium chloride procedure as described previously (Rösen-Wolf et al., 1988)] from Tupaia baby fibroblast (TBF) cells that had been infected with THV-2 for 14 h. The reverse transcription step was carried out using the RNA LA PCR kit version 1.1 (TaKaRa Shuzo Co.). The RNA preparation was treated with DNase I prior to reverse transcription. As expected, the RT–PCR resulted in the amplification of a specific PCR product of 309 bp (Fig. 1d). The specificity of the PCR product was confirmed by nucleotide sequence analysis. No differences were detected between the amplification products of viral DNA and the mRNA transcript. To exclude DNA contamination in the RNA preparation a control PCR without reverse transcription treatment was performed. As expected, no specific amplimer was detectable.

In summary, the complete nucleotide sequences of the DPOL genes of THV-1 and -2 and PCR products from three different regions of the DPOL genes of THV-3, -4 and -5 were determined. Comparison of the different sequences showed only two amino acid exchanges between the DPOL genes of THV-1 and -2 and a few nucleotide differences in comparison with the PCR products of THV-3, -4 and -5. It seems therefore that the different THV strains are more closely related than judged previously by comparison of their restriction patterns. From the genetic data presented here it is evident that THVs show a close relationship to the human and murine cytomegaloviruses; therefore they can be finally classified as members of the subfamily Betaherpesvirinae.

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References


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