High level expression of DNA polymerases from herpesviruses

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The DNA polymerase genes of human cytomegalovirus (HCMV) and varicella-zoster virus (VZV) were inserted separately into the polyhedrin gene of Autographa californica nuclear polyhedrosis virus (AcNPV) by cotransfection of Spodoptera frugiperda (SF9) cells with baculovirus transfer vectors carrying the genes and AcNPV infectious DNA. Infection of SF9 cells with the recombinant viruses resulted in expression from the polyhedrin promoter of proteins of the expected Mr. These proteins possessed DNA polymerase activities similar to that of the enzymes induced by the respective herpesvirus in infected cells, and were identified as HCMV and VZV DNA polymerase using inhibitors and specific antisera reactive with each enzyme.

Human cytomegalovirus (HCMV) is a major pathogen (Weller, 1981; Glenn, 1981; Griffiths, 1987) which currently is poorly managed by chemotherapy using e.g. gancyclovir (Field et al., 1983; Freitas et al., 1985). Similarly, varicella-zoster virus (VZV) is a cause of significant morbidity which can be treated with acyclovir (McKendrick et al., 1986), although there is a clear need for more effective chemotherapy. Since the herpesvirus DNA polymerase is important in the action of existing antiviral agents, studies of this enzyme are of interest in the development of new therapeutic agents. The HCMV and VZV DNA polymerases have not been characterized as fully as that of herpes simplex virus (HSV), but they are known to possess a number of common characteristics. All three enzymes are stimulated by high levels of salt and are sensitive to certain drugs, including phosphonoformic acid (PFA) (Knopf, 1979; Miller & Rapp, 1977; Nishiyama et al., 1983). The HSV DNA polymerase catalytic subunit copurifies with a DNA-binding protein, producing a two subunit enzyme consisting of polypeptides of 150K, and 65K (HSV-1) or 54K (HSV-2) (Powell & Purifoy, 1977; Vaughan et al., 1985). Purified HCMV DNA polymerase also consists of two proteins, of 140K and 58K, suggesting that the larger protein has the polymerase active site (Mar et al., 1985; Huang, 1975). VZV DNA polymerase from infected cells is relatively unstable and has not been purified (Mar et al., 1978; Miller & Rapp, 1977), but probably also interacts with a DNA-binding protein because the VZV gene 16 protein shows considerable sequence similarity to the HSV DNA polymerase-associated DNA-binding protein and may have a similar role. The gene location and sequence of both polymerases have been identified by homology studies, comparing them with the DNA polymerases of HSV-1 and Epstein-Barr virus, as well as other DNA polymerases (Hodgman, 1986; Kouzarides et al., 1987; Wang et al., 1988; Davison & Scott, 1986).

We report here the subcloning of these genes into baculovirus transfer vectors, and the subsequent selection of recombinant baculoviruses expressing the HCMV and VZV DNA polymerases from the polyhedrin promoter at high levels.

Transfer vectors containing the HCMV or VZV DNA polymerase gene were constructed as shown in Fig. 1. Briefly, the HindIII F fragment of HCMV strain AD169 cloned into pAT153 was obtained from Dr Bryan Rogers, Wellcome Diagnostics, a 48 kb EcoRI–SacI fragment containing the entire polymerase gene was isolated and this was cloned between the EcoRI and SacI sites of the baculovirus transfer vectors pUC19. This construct was cut at the unique RsrII site just upstream of the initiating ATG and a synthetic oligonucleotide linker was inserted to introduce KpnI and EcoRI sites, at the same time removing the RsrII site. The entire polymerase gene was then excised as an EcoRI–KpnI fragment and cloned between the BamHI and KpnI sites of the baculovirus transfer vectors pAc373 (Smith et al., 1983) or p36C (Page, 1989) using a synthetic oligonucleotide linker to link the BamHI and EcoRI ends. The deletion mutant, Del-67, was made by inserting the BclI–KpnI fragment of the polymerase gene into BamHI- and KpnI-digested p36C. The BamHI A
Fig. 1. Construction of transfer vectors for HCMV and VZV DNA polymerases. The diagram shows a schematic representation of vector construction (see Methods). The BamHI–EcoRI linker used to insert the HCMV polymerase gene into the transfer vectors contained an additional sequence made up of the 15 bases immediately preceding the start codon of the wild-type polyhedrin gene to optimize expression.

Fragment of VZV strain G31 in the Bluescribe plasmid was obtained from Professor Ian Hay, Uniformed Services of the Health Sciences, Bethesda, Md., U.S.A., and the polymerase gene was excised as two sequential fragments, HpaI–PstI (1.0 kb) and PstI–SphI (3.0 kb) (Fig. 1). These fragments were sequentially inserted into a modified pUC19 vector in which the HindIII site had been converted into a BamHI site using a synthetic linker. From this construct the entire VZV polymerase gene could be excised as a BamHI fragment and this fragment was inserted into BamHI-digested pAc373 and p36C. The structure of both constructs was checked by DNA sequencing. The transfer vectors were cotransfected with Autographa californica nuclear polyhedrosis virus (AcNPV) infectious DNA into Spodoptera frugiperda (SF9) cells and recombinant baculoviruses were isolated by standard methods (Kang, 1988) or by a blue/white selection method (P. Ertl, unpublished data).

The expression of HCMV DNA polymerase by two baculovirus recombinants is shown in Fig. 2. It can clearly be seen that the enzyme is expressed as a late protein with expression reaching maximum levels 48 h post-infection (p.i.) (Fig. 2a), and that DNA polymerase activity was induced in line with the appearance of the novel polypeptides (Fig. 2b). The polypeptide was identified as the HCMV DNA polymerase by Western blots using a synthetic peptide-induced antibody directed to the N terminus of the DNA polymerase.
Fig. 3. Characteristics of total (72 h p.i.) cell extracts (as in Fig. 2) of (△) AcNPV-, (□) HCMV Pol 414- or (○) VZV Pol 1121-infected cells. (a) Effect of KCl concentration on polymerase activity; (b) inhibition of polymerase activity by PFA.

sequence (M. Thomas, unpublished results). This antibody also reacted with recombinant virus-infected cell extracts, as detected by ELISA, immunoprecipitation and immunofluorescence. Similar recombinant viruses expressing VZV DNA polymerase were obtained which produced similar levels of enzyme, however no reagents are available to confirm its identity. The M₆s of the polypeptides obtained were 140K (HCMV) and 130K (VZV).

Analysis of polymerase activity is complicated by the presence of cellular, baculovirus (Kelly, 1980) and herpesvirus enzymes in a single infected cell. However, it can be seen that (i) the induced enzyme has a salt optimum of about 100 mM-KCl (Fig. 3a) and (ii) the induced enzyme is very sensitive to PFA in comparison to the host polymerases (Fig. 3b). The yield of polymerase 48 h p.i. reaches as much as 3 mg/10⁸ infected cells, but the induced enzyme is, unfortunately, around 99% insoluble and was precipitated by low speed centrifugation, whereas most of the activity was associated with the small soluble enzyme fraction (Fig. 4 and

Fig. 4. Examination of recombinant polymerases, the products of solubility fractionation in extraction buffer containing 1.7 M-KCl (Powell & Purifoy, 1977), on 8% polyacrylamide gels. The 130K VZV polymerase and 140K HCMV polymerase are concentrated in the high salt pellet (lanes 1), with only traces present in the high salt supernatants (lanes 2) and low salt supernatants (lanes 3). The positions of M₆ markers are shown.

Fig. 5. Glycerol gradient ultracentrifugation of high salt pellet extracts of HCMV recombinant Pol 414- (□) or VZV recombinant Pol 1121-infected (○) SF9 cells. Samples were loaded after sonication of high salt pellets in 10 mM-Tris-HCl pH 7.5, 0.5 mM-dithiothreitol and 0.5 M-NaCl. Fraction numbers run from the bottom to the top of the gradient, Two distinct peaks of polymerase activity were detected with both recombinant viruses, corresponding to soluble and high M₆ forms of the polymerase, although the VZV Pol 1121 activity had mainly disappeared during centrifugation. The recovery of enzyme activity was 35-18% for HCMV and 14-25% for VZV.
Table 1. Sensitivity of purified recombinant DNA polymerases to various reagents

<table>
<thead>
<tr>
<th>Template†</th>
<th>Purified* enzyme</th>
<th>pdAdT10</th>
<th>pdCdG10</th>
<th>act.calf Thy</th>
<th>ssDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl‡</td>
<td>HCMV</td>
<td>50§</td>
<td>80</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>VZV</td>
<td>100</td>
<td>150</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄‡</td>
<td>HCMV</td>
<td>0</td>
<td>25</td>
<td>75</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>VZV</td>
<td>25</td>
<td>125</td>
<td>125</td>
<td>100</td>
</tr>
<tr>
<td>IC₅₀ PFAI</td>
<td>HCMV</td>
<td>0·55¶</td>
<td>0·47</td>
<td>0·70</td>
<td>3·6</td>
</tr>
<tr>
<td></td>
<td>VZV</td>
<td>0·05</td>
<td>0·06</td>
<td>0·7–1·0</td>
<td>1·0</td>
</tr>
<tr>
<td>IC₅₀ ACV-TP**</td>
<td>HCMV</td>
<td>&gt;10</td>
<td>0·07</td>
<td>0·22</td>
<td>0·10</td>
</tr>
<tr>
<td></td>
<td>VZV</td>
<td>ND††</td>
<td>0·005</td>
<td>1·0</td>
<td>1·0</td>
</tr>
</tbody>
</table>

* Purified from host polymerase by solubility.
† The templates used were pdAdT₁₀, poly(dA)-oligo(dT)₁₀; pdCdG₁₀, poly(dC)-oligo(dG)₁₀; act.calf Thy, activated calf thymus DNA; ssDNA, single-stranded salmon sperm DNA.
‡ Optimum concentration (μM).
§ Represent the optimum concentration for the activity of the polymerase.
¶ Values for PFA and ACV-TP represent the median inhibitory concentration (IC₅₀).
** μM values.
†† ND, Not determined.

The recombinant baculoviruses described in this paper represent a first step in the process of assembly of the HCMV and VZV DNA replication proteins for study in vitro. Previous attempts in our laboratory and elsewhere to express herpesvirus DNA polymerases in prokaryotic systems have met with only limited success (Haffey et al., 1988; Dorsky & Crumpacker, 1988; B. Larder, personal communication). The baculovirus system has enabled us to obtain very efficient expression of both HCMV and VZV DNA polymerases, has given similar success with the HSV enzyme (Marcy et al., 1990) and could allow large scale production of these enzymes (Maiorella et al., 1988). There are still considerable problems with the system in terms of the quantity of the active enzyme that we can obtain in a soluble form, but the VZV- and HCMV-infected cell systems yield far smaller amounts.

The expression and characterization of the HCMV and VZV DNA polymerases in this system have confirmed that the open reading frames predicted to encode the polymerases by studies of their homology with the HSV DNA polymerase sequence (Davison & Scott, 1986; Kouzarides et al., 1987), do indeed encode the HCMV and VZV DNA polymerases and enable the synthesis of active enzyme.

The prokaryotic nature of the baculovirus system enables mutagenesis to be performed, and the altered proteins to be expressed and examined. Initially, this technique was used in an attempt to improve the solubility of the HCMV polymerase by deleting 67 amino acids from the amino terminus of the polymerase. This did not improve the solubility of the polymerase and, in
contrast to a similar 67 amino acid deletion in HSV-1 DNA polymerase (Dorsky & Crumpacker, 1988), removed the polymerase activity.

The increased sensitivity to PFA of VZV polymerase in its purified state is an example of the advantage of this system. In earlier work on this enzyme from VZV-infected cells, Miller & Rapp (1977) and Mar et al. (1978) obtained quite different results from each other and from our results in investigating sensitivity to PFA. One plausible explanation of this difference is the much greater purity of our enzyme.

It has been difficult to purify the large HSV DNA polymerase subunit from the small one (ICSP 34, 35 or Vm65) (Powell & Purifoy, 1977; Vaughan et al., 1985). The ability to express the large subunit of both VZV and our results in investigating sensitivity to PFA. One obtained quite different results from each other and from the process of DNA synthesis. This will be aided further by the expression of the small subunits (HCMV ICP 36 and VZV gene 16 product) in the baculovirus system. The expressed polymerases have been obtained free from the polymerase accessory proteins with which they are normally associated. In HSV-1 this protein has been shown to be important for maximal polymerase activity and may operate by increasing the ability of the polymerase to progress along a template (Gallo et al., 1989; Gottlieb et al., 1990; Hernandez & Lehman, 1990).

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References


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