Expression of hepatitis A virus poly(U) polymerase in the periplasmic space of Escherichia coli

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A cDNA containing almost all of the hepatitis A virus (HAV) P3 sequences was expressed as a fusion protein with Protein A. A novel poly(U) polymerase activity was detected in the periplasmic space of Escherichia coli cells transformed with this plasmid, and this activity showed many of the expected properties of a picornavirus 3Dpoly. A number of HAV-specific polypeptides were detected in these cells, and it is unclear which of these was responsible for the polymerase activity.

Hepatitis A virus (HAV) is a member of the Picornaviridae with a single-stranded positive-sense RNA genome which is translated into a polyprotein that is then post-translationally cleaved into the mature viral proteins. The genomes of a number of HAV strains have been completely sequenced (Najarian et al., 1985; Cohen et al., 1987b; Paul et al., 1987) and the cleavage sites on the polyprotein have been predicted, along with the sizes and functions of the proteins that would be produced by this proteolytic processing (Diamond et al., 1986). However, these predictions are based on analogies with the known processing pathways of other picornaviruses and there is no direct evidence for the size and function of many of the HAV non-structural proteins. One of the functions which the HAV genome is predicted to encode is an RNA polymerase, 3Dpoly. This enzyme has been well characterized in several picornaviruses, but attempts to detect it in HAV-infected cells have not been successful, even using antiserum raised against a peptide predicted to be part of the HAV 3Dpoly protein (Updike et al., 1991).

This and other non-structural proteins of HAV are of interest because of the slow, non-cytopathic replication of the virus that is observed in tissue culture. When HAV is adapted to growth in cell culture a change in phenotype is associated with a number of changes in the nucleotide sequence of the viral genome. Most of these changes are found in the regions of the genome encoding non-structural polypeptides (Cohen et al., 1987a,b, 1989; Jansen et al., 1988; Ross et al., 1989). One of the more easily assayed functions of the polypeptides encoded by these regions is the RNA- and poly(U)-polymerizing activity of the picornavirus 3Dpoly, and some of the mutations mentioned above have been mapped to the part of the genome predicted to encode the HAV 3Dpoly.

We have therefore attempted to express this polypeptide in vitro, in order to confirm that HAV does encode an RNA polymerase and to compare its properties with the analogous enzymes encoded by other picornaviruses. 3Dpoly proteins of other picornaviruses have been successfully expressed in bacterial cells and have been shown to have properties similar to the activities detected in infected cells. Generally, bacterially expressed picornavirus RNA polymerases are primer-dependent, usually assayed using a poly(A):oligo(U) template, show a requirement for magnesium ions and are inhibited by manganese, with a temperature optimum of about 30°C (Morrow et al., 1987; Richards et al., 1987; Plotch et al., 1989; Neufeld et al., 1991; Sankar & Porter, 1991).

Previous attempts to express the HAV P3D region in Escherichia coli have been unsuccessful, resulting in intracellular accumulation of an insoluble product which showed no activity (A. J. Wolstenholme, unpublished; Updike et al., 1990) although the 3C proteinase has been successfully expressed in E. coli (Gauss-Müller et al., 1991). We decided to attempt to export the HAV-specific sequences into the bacterial periplasmic space as a fusion protein with staphylococcal Protein A, in the hope that this would allow the viral proteins to remain soluble while they were being processed, thus yielding authentic and active products.

HAV (strain LSH/S, passage 7) was grown in MRC-5 cells and purified as described by Garelick et al. (1988). Purified virus was digested with proteinase K and the RNA extracted with phenol:chloroform (1:1). Double-stranded cDNA was synthesized using a cDNA synthesis kit (Amersham) and oligo(dT) primers. The cDNA was cloned into the EcoRI site of pUC19 using linkers, and transformed into competent E. coli cells, strain TG1. The resultant colonies were screened with pHAV207 (Ticehurst et al., 1983) and HAV-specific inserts were
Table 1. Amino acid residues at which the sequence of the P3 region of HAV strain LSH/S differs from strains MBB and HM175

<table>
<thead>
<tr>
<th>Residue number</th>
<th>Region</th>
<th>LSH/S</th>
<th>MBB</th>
<th>HM175</th>
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<tbody>
<tr>
<td>1448</td>
<td>3A</td>
<td>Ser</td>
<td>Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>1471</td>
<td>3A</td>
<td>Val</td>
<td>Ile</td>
<td>Ile</td>
</tr>
<tr>
<td>1495</td>
<td>3A</td>
<td>Thr</td>
<td>Ala</td>
<td>Ala</td>
</tr>
<tr>
<td>1661</td>
<td>3C</td>
<td>Ala</td>
<td>Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>1744</td>
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<td>Asp</td>
<td>Glu</td>
<td>Glu</td>
</tr>
<tr>
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<td>3D</td>
<td>His</td>
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<td>Thr</td>
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<td>Ser</td>
</tr>
<tr>
<td>1930</td>
<td>3D</td>
<td>Lys</td>
<td>Cys</td>
<td>Arg</td>
</tr>
<tr>
<td>2017</td>
<td>3D</td>
<td>Ile</td>
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<td>Ile</td>
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<tr>
<td>2071</td>
<td>3D</td>
<td>Ser</td>
<td>Phe</td>
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Fig. 1. Poly(U) polymerase activity in periplasmic extracts from E. coli cells transformed with pRITPOL (○), or untransformed (■). Activity was measured by monitoring the incorporation of [3H]UTP into acid-insoluble material during the incubation. All measurements are the average of triplicate samples.

subcloned into M13 and sequenced by the chain-termination method (Sanger et al., 1977).

The sequence of the P3 region of the LSH/S strain of HAV is very similar to that reported for other strains of the virus. Jansen et al. (1990) reported that, based on amplification and sequencing of two small regions of the genome, the LSH/S strain is very closely related to the MBB strain, the complete sequence of which was reported by Paul et al. (1987). Variations in the amino acid sequence of the P3 region of the LSH/S strain from that reported for the MBB and wild-type HM175 strains are shown in Table 1.

A cDNA insert corresponding to the sequence from residue 5064 (an EcoRI site) to the 3' terminus of the HAV genome was ligated into the plasmid pRIT5 (Pharmacia) which had been digested with EcoRI and Sall, forming an open reading frame consisting of the Protein A sequence followed by the C-terminal 780 amino acids of the HAV polyprotein, i.e. all the P3 residues except for the first 11 predicted amino acids of p3A. Recombinant bacteria containing this plasmid, pRITPOL, were grown at 37 °C until they reached early stationary phase. The cells were then pelleted, washed with 20% sucrose, 0.3 M-tris·HCl pH 8.1, 1 mM-EDTA, 0.5 mM-MgCl₂ and repelleted. Periplasmic extracts were prepared by osmotic shock in ice-cold 0.5 mM-MgCl₂ for 10 min on ice, followed by centrifugation at 10000 g for 10 min. Supernatants were assayed for poly(U) polymerase activity essentially as described by Flanagan & Baltimore (1977), except that rifampicin (20 µg/ml) replaced the actinomycin D. Typically, 50 µl of periplasmic extract was assayed in a total volume of 125 µl. Oligo(U) was prepared as described by Plotch et al. (1989). Assays were carried out at 31 °C unless otherwise stated. Samples were taken at various times and the TCA-insoluble radioactivity was collected on GF/C filters (Whatman), which were solubilized in scintillant and counted on a LKB 1217 Rackbeta liquid scintillation counter.

As shown in Fig. 1, an increase in the incorporation of [3H]uridine into TCA-insoluble material was observed for 45 min in reactions containing periplasmic extracts from pRITPOL-transformed cells, but not from pRIT5-transformed or untransformed cells. Incubation for longer than 45 min usually resulted in reduced incorporation, presumably due to the presence of nucleases in the periplasmic extracts. We then examined the divalent cation and temperature requirements of this poly(U) polymerase activity (Fig. 2). It showed a broad optimum for Mg²⁺ concentration around 6 to 8 mM, was inhibited by Mn²⁺ and had a temperature optimum of 30 °C.

Typically, highly purified picornaviral polymerases show an absolute requirement for the addition of exogenous template and primer. We examined the template and primer requirements of our crude extracts (Table 2), and found that the activity was absolutely dependent on the addition of exogenous template. In the absence of added primer we observed 50% of total activity. This is not unusual for non-purified polymerases and we presume that some factor in the periplasmic extracts can replace the oligo(U) primer in this assay. We were not able to demonstrate any poly(U) polymerase activity in cytoplasmic extracts from E. coli cells containing pRITPOL.

At present there is no experimental evidence for the 3C:3D cleavage site on the HAV polyprotein and hence only indirect estimates of the predicted size of the HAV 3Dpol are available. When whole cell extracts and periplasmic extracts of recombinant E. coli cells were subjected to PAGE and staining with Coomassie blue,
Fig. 2. Properties of the HAV-specific poly(U) polymerase. Periplasmic extracts from E. coli cells transformed with pRITPOL or pRIT5 were incubated for 30 min in duplicate, then each sample was split in half and the incorporation of [3H]UTP into acid-insoluble material measured. The counts incorporated by the pRIT5 extracts were subtracted from the pRITPOL results and the resultant HAV-specific incorporation was plotted against the temperature (a) or [Mg<sup>2+</sup>] (b) of the reaction. Reactions in (a) were performed in the presence of 8 mM-magnesium acetate; reactions in (b) and (c) were at 31 °C.

Table 2. Template and primer requirements of the HAV poly(U) polymerase

<table>
<thead>
<tr>
<th>Template</th>
<th>Primer</th>
<th>UTP incorporated into TCA-insoluble material after 30 min at 31 °C (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>0.0</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>None</td>
<td>4.4</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>Oligo(U)</td>
<td>8.5</td>
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<tr>
<td>None</td>
<td>Oligo(U)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Fig. 3. Western blotting of E. coli extracts. Whole-cell extracts were separated on a 12% SDS-polyacrylamide gel and electroblotted onto nitrocellulose. The filter was probed with a convalescent human serum and the bands were visualized by incubation with a monoclonal anti-human IgG antibody conjugated to alkaline phosphatase. The positions of the Mr markers were determined by staining the blot with Ponceau S. Lane 1, untransformed E. coli; lane 2, E. coli transformed with pRIT5; lane 3, E. coli transformed with pRITPOL.

no virus-specific bands could be observed (data not shown). We therefore attempted to detect HAV-specific polypeptides by Western blotting, probing the filters with human convalescent serum. Extracts were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and the proteins electroblotted onto nitrocellulose membranes. Fig. 3 shows the products that react with the convalescent serum: the major band has an Mr of 43000, with a minor band of Mr, 80000 and very faint bands of Mr, 62000, 58000, 48000, 41000 and 23000. The predicted sizes of the HAV-specific polypeptides are 53000 (3D), 24000 (3C), 2500 (3B) and 36000 (Protein A–3A). It is possible to correlate the sizes of some of the immunoreactive products with some of the predicted HAV proteins e.g. the 23000 band could represent 3C and the 80000 band is about the size predicted for 3CD, but there is no direct evidence for any such assignment. In particular the major reactive species of Mr, 43000 does not correspond to any predicted HAV product.

Expression of the P3 region of HAV fused to Protein A sequences thus gives rise to a number of HAV-specific polypeptides, one or more of which possess a poly(U) polymerase activity very similar to those described for other picornaviruses. It seems likely that this activity represents the HAV equivalent of 3D<sub>pol</sub> from other members of the family. This is the first report of such an activity for HAV, whether in infected cells or from cloned cDNA, and demonstrates the possibility of using such clones to study the properties of the HAV enzymes. However, the system described here is less than satisfactory for several reasons. The activity detected is very low which could reflect the inefficiency of the expression system or a low intrinsic activity of the HAV polymerase.
It has been suggested that the slow replication of HAV in cell culture may be due to inefficient replication of viral RNA (Anderson et al., 1988) although this could also be explained by an inefficient viral RNA polymerase. Secondly, the number of HAV-specific polypeptides produced makes it difficult to assign the activity to any particular species. We are currently attempting to purify the polypeptide responsible for poly(U) polymerase activity from the periplasmic space of bacteria transformed with pRITPOL and are investigating alternative systems for the expression of the HAV RNA polymerase.

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References


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