We have expressed the putative RNA-dependent RNA polymerase encoded by the potyvirus tobacco vein mottling virus (TVMV) in Escherichia coli as a glutathione S-transferase fusion protein. As prepared, the fusion protein possessed the poly(U) polymerase activity that is a hallmark of other picornavirus-encoded polymerases. In addition, this protein was able to utilize full-length TVMV RNA as a template for RNA synthesis. A fusion protein containing a mutation in the highly conserved GDD motif of the polymerase (GDD → ADD) possessed 7% of the activity of the wild type. Our results confirm that the presumed polymerase encoded by TVMV is in fact an RNA-dependent RNA polymerase and that the GDD motif so widely seen in viral polymerases has an important function in the TVMV protein.

INTRODUCTION

Potyviruses constitute the largest family of plant viruses and are members of the picorna superfamily of (+)-sense single-stranded RNA viruses. They are monopartite viruses with a ca. 10-kb RNA genome that encodes a single polyprotein (Riechmann et al., 1992). This polyprotein is processed, through the action of three virus-encoded proteinases (Dougherty and Carrington, 1988; Carrington et al., 1989; Verchot et al., 1991), to yield the battery of gene products observed in infected cells. These gene products include the three proteinases, a putative helicase (Lain et al., 1990; Eagles et al., 1994), a protein (one of the proteinases) necessary for transmission of the virus by insects (Pirone and Thornbury, 1984; Hellmann et al., 1985), the genome-linked protein (also one of the proteinases; Shahabuddin et al., 1988; Murphy et al., 1990, 1991), the coat protein, and a putative RNA-dependent RNA polymerase.

The potyvirus RNA-dependent RNA polymerases have received scant experimental attention. The so-called Nib proteins have been assigned as the virus-encoded polymerases based on the homology of these proteins with other viral RNA-dependent RNA polymerases (Domier et al., 1987). This assignment has been supported by the observation that a potyvirus Nib protein, as well as fusion proteins containing the Nib and other potyvirus-encoded proteins, copurifies with virus-specific polymerase activity in extracts prepared from infected plants (Martin et al., 1995). However, a direct demonstration of polymerase activity associated with Nib proteins has not been made. Here, we describe the production of glutathione S-transferase (GST)-potyvirus Nib fusion proteins in Escherichia coli and the demonstration of RNA-dependent RNA polymerase activity associated with these proteins. Our results demonstrate that potyvirus Nib proteins are in fact polymerases and that the GDD motif found in the Nib studied here is important for the polymerase activity of the protein.

METHODS

Recombinant DNA manipulations

Recombinant DNA manipulations and DNA sequencing were carried out using standard protocols (Sanger et al., 1977; Sambrook et al., 1989). Plasmids were grown and maintained in E. coli strain TB1 (ara, Δlac-proAB, rpsL, hsdR [rk, mk], ϕ80lacZΔM15).

The tobacco vein mottling virus (TVMV) Nib gene and the G2575A mutant (Hong et al., 1995) were cloned into a modified version of pGEX2T (Pharmacia) that had a multiple cloning site suited for accepting TVMV coding regions with the reading frame used for cloning into the two-hybrid plasmids used by Hong et al. (1995). The coding regions for the Nib protein and the G2575A mutant were excised as BglII fragments from pBluescript clones as described (Hong et al., 1995) and subcloned into the modified pGEX2T plasmid, yielding recombinants with the structure around the Nib coding region that is shown in Fig. 1.

Production of fusion proteins

Overnight cultures of E. coli cells carrying the appropriate plasmid were diluted 1000-fold into LB media and
grown for 3 hr. IPTG was added to 0.1 mM and the culture grown for an additional 4 hr. The cells were collected by sedimentation and suspended in 10 ml of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.3) per 200 ml of culture. Cells were disrupted by sonication and Triton X-100, ATP, MgSO4, and Tris – HCl (pH 7.4) were added to final concentrations of 1%, 2 mM, 10 mM, and 50 mM, respectively. (ATP was added to minimize the copurification of a 70-kDa putative chaperonin with the GST fusion proteins.) Cellular debris was removed by centrifugation (12,000 g for 10 min). Two hundred microliters of a slurry (50% w/v of matrix in PBS) of glutathione – Sepharose (Pharmacia) was added to the supernatant and the mixture incubated at room temperature for 30 min with gentle agitation. The matrix was collected by sedimentation (500 g for 5 min) and washed three times with 10 bed volumes per wash of PBS. Fusion proteins were eluted by suspending the washed matrix with 1 ml per milliliter of bed volume of PBS + 1 mM glutathione; the matrix was removed by sedimentation as above. This elution procedure was repeated a total of three times, and the three eluates were pooled and dialyzed against Buffer D (50 mM Tris – HCl, pH 8.5, 20 mM NaCl, 0.1% Nonident-P40, and 1 mM phenylmethylsulfonyl fluoride). Aliquots of these elutions were analyzed by SDS – PAGE. In addition, other aliquots were treated with 100 units/ml of thrombin (2 hr at 37°) before SDS – PAGE. Typical yields after elution with glutathione were 20–40 μg protein for each 200 ml of cells.

Polymerase assays

Polymerase activity was assayed using a modification of the poly(A) polymersases assay described by Das Gupta et al. (1995). Unless otherwise noted, reactions contained 1 μg of purified GST-Nib (or, in some instances, fusion protein that had been cleaved with thrombin) in 60-μl reactions containing 25 mM Tris – HCl, pH 8.0, 40 mM KCl, 0.5 mM MgCl2, 0.5 mM DTT, 0.05 mM EDTA, 33 μg/ml poly(A) (average length of about 300–400 nts; ICN Biochemicals), 5 μg/ml dT20 (or oligo(U)), and 0.5 mM [α-32P]UTP (100–200 mCi/mmoll). Reactions were incubated at 30° for the 90 min and terminated by extraction with 50 μl phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v), and duplicate 20-μl aliquots of the aqueous phase were spotted onto 1-cm2 pieces of DE81 paper (Whatman). These were washed four times with 5% Na2HPO4 and washed twice with water, and the retained radioactivity was determined by liquid scintillation spectrometry.

In some instances, poly(A) was replaced with poly(dA); this was obtained from Sigma Chemical Co. and used as provided. In other cases, poly(A) was replaced with full-length (polyadenylated) TVMV RNA. This RNA was prepared by in vitro transcription (see Li et al., 1996) using pXBS7 (Domier et al., 1989) as a template. When TVMV RNA was used as a template, the labeled UTP concentration was decreased to 0.05 mM and was supplemented with 0.5 mM each ATP, GTP, and CTP, unless where noted; in addition, 50 units of RNAsin (5Prime3-Prime) was added for each reaction. Under these conditions, the specific activity of the [α-32P]UTP was between 1000 and 2000 mCi/mmol.

In some experiments, the products of the polymerase reaction were treated with nuclease S1. In these cases, the labeled products were recovered from reactions by extraction with phenol:chloroform as above and precipitated with ethanol. The recovered nucleic acids were treated with nuclease S1 (GIBCo-BRL) using the conditions described by Hunt (1988); for this, each sample was digested in a total volume of 20 μl. After digestion, each sample was spotted onto a 1 cm square of DE-81 paper (Whatman) and processed as described above. For comparison, poly(A) that had been 3’-labeled with a pea poly(A) polymerase (as described by Das Gupta et al., 1995) was treated in an identical manner. As controls for these treatments, labeled products were treated identically, except S1 nuclease was omitted.

RESULTS

We set out to develop assays similar to those described for the poliovirus 3Dpol polymerase for use in studying the TVMV-encoded Nib protein. Unlike the Nib protein encoded by the potyvirus tobacco etch virus (TEV), the TVMV Nib protein does not accumulate in easily purified nuclear inclusions in infected plants. Thus, we decided to prepare the TVMV Nib from appropriately programmed E. coli. To facilitate purification, we cloned a portion of the TVMV genome corresponding to amino acids 2225 to 2740 of the TVMV polyprotein (residues 1 to 516 of the Nib protein) into pGEX2T (Pharmacia) so that GST – Nib fusion proteins would be produced in E. coli (see Fig. 1). We also subcloned the identical region from an Nib mutant in which the GDD domain was changed to ADD (mNib; Hong et al., 1995).

FIG. 1. Structures of the TVMV Nib genes as present in the pGEX plasmid. The amino acid and nucleotide sequences at the N- and C-termini of each gene are shown; non-TVMV nucleotides and amino acids derived from the oligonucleotides used for cloning and from the vector are shown in lowercase letters, and the TVMV amino acid coordinates of the N- and C-termini are shown above the appropriate amino acid. The thrombin cleavage site is shown with a double slash (//).
two polypeptides of ca. 56 and 30 kDa (Fig. 2, lanes 3 and 4), confirming that the 86-kDa polypeptides were in fact GST-NIb fusion proteins. Typical yields for these preparations were 200 - 300 μg of fusion protein per liter of culture.

To test the purified GST-NIb protein for RNA polymerase activity, we adapted the assay described by van Dyke and Flanagan (1980) to evaluate polymerase activity of the poliovirus 3Dpol. In this assay, the incorporation of label from [α-32P]UTP into high-molecular-weight polynucleotides (determined here by retention on ion-exchange paper) is measured; for the poliovirus 3Dpol, activity is usually measured using poly(A) as a template, and either oligo(U) or oligo(dt) as a primer. We also elected to use oligo(dt) as a primer and poly(A) as a template. However, we chose to perform our assays using conditions similar to those in which plant poly(A) polymerase activity is measured (Das Gupta et al., 1995). When this was done, significant incorporation of label into polynucleotides could be detected (Fig. 3A). This incorporation was dependent on the presence of primer and template (Fig. 3A). Oligo(dt) (Fig. 2A) and oligo(U) (data not shown) could serve equally well as primers. Activity could be seen with intact GST-NIb fusion proteins (Fig. 3) or with NIb that had been freed from GST by treatment with thrombin and purification on glutathione-Sepharose (not shown). Purified GST displayed no activity under the conditions used (Fig. 3B). The intact fusion protein displayed a $K_m$ for UTP of about 220 μM, a salt optimum of 40 - 80 mM KCl, a pH optimum of about 8.0, and a temperature optimum between 30 and 37°C. Activity in the presence of actinomycin D was 84% of that observed in the absence of this inhibitor.

To further evaluate the observed polymerase activity,

![FIG. 2. SDS-PAGE analysis of GST fusion proteins purified from appropriately-programmed E. coli. Each lane contained 10% of the protein from a typical preparation starting with 200 ml of cells. Lane 1, purified GST-NIb. Lane 2, purified GST-mNIb. Lane 3, thrombin-treated GST-NIb. Lane 4, thrombin-treated GST-mNIb. Lane 5, purified GST (this was prepared from the same host as that used here, and using the same affinity purification protocol). The positions of molecular weight size standards is shown to the right of the gel, and the positions of GST, NIb, and the GST-NIb fusion proteins are shown on the left.](image)

![FIG. 3. Polymerase activity of NIb proteins. (A) Dependence of polymerase activity on primer and template. Reactions containing poly(A) and oligo(dt), poly(A), or oligo(dt) were incubated for the indicated times, terminated, and analyzed. (B) Polymerase activity is diminished by the G2575A mutation. Increasing quantities of purified GST-NIb, GST-mNIb, or GST (provided by Jaydip Das Gupta) were assayed for 90 min and the results plotted as shown. The specific activities of the GST-NIb and GST-mNIb proteins under these conditions were 0.450 and 0.032 nmol UTP incorporated/min-mg fusion protein, respectively. No activity could be detected with GST alone.](image)
TABLE 1
Template Preference of the GST-NIb Protein

<table>
<thead>
<tr>
<th>Template</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(A)</td>
<td>100</td>
</tr>
<tr>
<td>Poly(dA)</td>
<td>8.0</td>
</tr>
<tr>
<td>TVMV RNA</td>
<td>55c</td>
</tr>
</tbody>
</table>

* Templates were added at concentrations of 33 mg/ml for poly(A) and 167 µg/ml for TVMV RNA and poly(dA).
* Activity was determined as cpm incorporated into polynucleotide in 90 min and was normalized with the activity with poly(A) being set as 100.
* Activity using TVMV RNA as a template was corrected for differences in specific activity of the [α-32P]UTP and for the difference in approximate U content of the products, compared to poly(U) polymerase reactions using poly(A) as a template.

we compared the ability of the GST-NIb fusion protein to use polymers other than poly(A) as templates. Thus, we replaced poly(A) with poly(dA) and with full-length, polyadenylated TVMV obtained by in vitro transcription of infectious clones (Domier et al., 1989). Replacement of poly(A) with poly(dA) diminished activity by greater than 90% (Table 1), indicating a preference for RNA over single-stranded DNA as a template. In contrast, TVMV RNA proved to be an effective template (Table 1), thus showing that activity is possible with RNAs other than homopolymers.

The observation that polymerase activity with poly(A) is dependent on the presence of a primer (Fig. 3A) suggested that the observed activity was not due to a terminal transferase-like activity in our preparations. To further rule out this possibility, the effects of omission of one of the four NTP substrates on activity using the full-length TVMV RNA as a template were evaluated. The results of this study (Table 2) indicate that omission of ATP, CTP, or GTP from reactions eliminated polymerase activity. This result is consistent with the activity of a templated RNA polymerase activity and not with a terminal transferase-like reaction.

TABLE 2
Polymerase Activity with TVMV RNA as Template Requires All Four NTPs

<table>
<thead>
<tr>
<th>NTP substrate</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>– ATP</td>
<td>n.d.</td>
</tr>
<tr>
<td>– CTP</td>
<td>n.d.</td>
</tr>
<tr>
<td>– GTP</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* For reactions with TVMV RNA as a template, ATP, GTP, CTP, were present at 500 µM and UTP at 50 µM. The NTP omitted is so indicated.
* Activity was determined as cpm incorporated into polynucleotide in 90 min and was normalized with the activity with the complete set of NTPs being set as 100. n.d., no activity could be detected (~5% of the control).

As a further test to rule out the involvement of a terminal transferase-like activity, the labeled products produced by the GST-NIb protein using TVMV RNA as a template were recovered (along with the unlabeled template) by extraction with phenol:chloroform and treated with S1 nuclease. This experiment showed that the polymerase products were resistant to S1 nuclease (Fig. 4). In contrast, labeled poly(A) that had been produced by purified pea poly(A) polymerase (which is a terminal transferase) was susceptible to a similar treatment (Fig. 4). The latter sample indicates that end-labeled, single-stranded RNA was susceptible to S1 nuclease under the conditions used here. This experiment indicates that the labeled RNAs produced by the GST-NIb protein were protected from S1 nuclease digestion, presumably because they were hybridized with the unlabeled template RNA. This result, along with the primer dependence and requirement for all four NTPs for activity with a “natural” RNA template, rules out the possibility that the observed activity is due to a terminal transferase-like reaction.

A mutant NIb protein in which the conserved GDD motif was changed to ADD (G2575A; see Hong et al., 1995) was also tested for polymerase activity. In contrast to the results obtained with the wild-type NIb protein, comparable quantities of the G2575A mutant possessed very low polymerase activity (Fig. 2B). This result was obtained with several different preparations of the mutant protein and indicates that a mutation in the conserved GDD motif suffices to diminish the polymerase activity of the NIb protein. The specific activity of the mutant protein was determined to be about 7% of that of the
wild-type NIb. Importantly, since the chromatographic properties of the wild-type and mutant proteins are indistinguishable, this result indicates that the activity seen with the wild-type protein is not due to undetected traces of one or more E. coli proteins.

DISCUSSION

Our results indicate that the TVMV NIb protein, the putative virus-encoded RNA-dependent RNA polymerase, is able to catalyze template and primer-dependent poly(U) formation. In this respect, the TVMV NIb resembles the poliovirus 3Dpol. Thus, the similarities in gene order, gene expression strategy, and amino acid sequence of individual polypeptides that potyviruses and picornaviruses share (Domier et al., 1987) can be extended to the enzymatic activity of the RNA-dependent RNA polymerase. This in turn supports the model, proposed by several groups, that potyviruses and picornaviruses share a common strategy for genome replication.

One hallmark of viral RNA-dependent RNA polymerases, putative or otherwise, is the presence of a highly conserved “GDD” motif. Alteration of this motif in the poliovirus 3Dpol has dramatic effects on polymerase activity (Jablonski et al., 1991; Jablonski and Morrow, 1995). In particular, changing the glycine in this motif to cysteine, methionine, proline, or valine abolished the activity of the polymerase, whereas changing this residue to alanine or serine yielded polymerases with 5–20% of the activity of the wild-type enzyme. A change of the glycine in the GDD motif of the TVMV NIb to ADD yielded an enzyme with about 7% of the activity of the wild-type NIb (Fig. 3B). This effect is quite similar to that of the corresponding G→A alteration in the poliovirus polymerase and corroborates a wealth of sequence comparison data (e.g., Domier et al., 1987) suggesting an evolutionarily conserved function for this motif in polymerase function.

It has been reported that the putative RNA-dependent RNA polymerase of a plant comovirus differs from the poliovirus 3DPol in that it lacks the poly(U) polymerase seen with purified 3Dpol (van Bokhoven et al., 1991). In this respect, the comovirus polymerase is also different from the TVMV protein, even though comoviral and potyviral polymerases share many features such as genome structure, gene organization, gene expression strategy, and amino acid sequence of specific proteins. The reasons for the lack of activity reported for the comovirus protein are not known, but may be trivial. We have consistently found that NIb-catalyzed poly(U) polymerase activity is greater in buffers that we have developed for the study of plant poly(A) polymerases than in buffers routinely used for the assay of 3Dpol. van Bokhoven et al. studied the polymerase activity of the comovirus polymerase in conditions identical to those used for 3Dpol, conditions that would have yielded marginal activity with the TVMV NIb protein (unpublished observations). Further studies with polymerases related to potyvirus NIb proteins may help to clarify this issue.

We have previously described interactions between the TVMV NIb protein and the TVMV NIa and coat proteins (Hong et al., 1995). Although these interactions involved domains of the NIa and NIb proteins known to be important for functioning in infected cells, the specific roles of these interactions in the infection process remain to be elucidated. Martin et al. (1995) have noted that the plum pox potyvirus NIa, coat, and CI proteins copurify with the NIb protein in membrane-associated RNA polymerase complexes. The availability of an in vitro assay for polymerase activity provides a tool with which to further study these (and other) interactions and associations. This assay may also be of use in identifying possible host factors involved in the replication process.

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