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# Isolation and Characterization of an RNA-Dependent RNA Polymerase from Black Beetle Virus-Infected *Drosophila melanogaster* Cells

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Crude lysates of black beetle virus (BBV)-infected cells of *Drosophila melanogaster* contain an RNA-dependent RNA polymerase activity not detectable in uninfected cells. The activity (designated BBV polymerase) sedimented at 20,000 × *g*, indicating an association with particulate material. It was solubilized from the pellet by sonication in a magnesium-deficient buffer. Differential centrifugation resulted in a 43-fold purification with 84% recovery of polymerase activity. The effects of divalent and monovalent cations, time, temperature, and pH on the activity of the partially purified polymerase were examined. RNA synthesis was not stimulated by the addition of exogenous BBV RNA, suggesting that an enzyme-template complex existed. Analysis of the RNA products of the RNA polymerase reaction indicated that full-length "negative" strand BBV RNAs were synthesized.

Black beetle virus (BBV) is a member of a group of small riboviruses which have divided genomes (nodaviruses) (8). BBV can be grown readily in tissue culture (7) and provides a very favorable experimental system for studying the molecular biology of such viruses. BBV contains two RNAs with approximate molecular weights of $1 \times 10^6$ (RNA 1) and $0.5 \times 10^6$ (RNA 2). BBV RNA is translated efficiently in a number of cell-free extracts, including a homologous system derived from *Drosophila melanogaster* (12). BBV RNA 1 codes for a protein of molecular weight 120,000 (protein A), and BBV RNA 2 specifies the synthesis of BBV coat protein precursor (molecular weight, 47,000).

Viral-specific protein synthesis has also been examined in vivo (3, 8). BBV-infected *D. melanogaster* cells synthesize large amounts of coat protein and A protein and, in addition, large amounts of a protein of molecular weight 10,000 (protein B). The function of proteins A and B are unknown, although it has been proposed (8, 17) that the protein A comprises all or part of an RNA-dependent RNA polymerase which is necessary for BBV replication. To examine this possibility, we have isolated BBV replication complexes from infected cells. We here report the partial purification and characterization of a BBV-specific RNA-dependent RNA polymerase activity.

## MATERIALS AND METHODS

**Preparation of infected cell lysates.** Exponential-phase cells ($8 \times 10^6$ cells per ml) of *D. melanogaster* line 1 were infected with BBV at a multiplicity of 1,000 particles per cell as has been described (7, 12). A 50-ml amount of infected cells was harvested 8 h postinfection and washed by centrifugation three times in ice-cold HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (146 mM sodium chloride-35 mM HEPES [pH 7.6]). The final pellet was resuspended in 5 ml of 10 mM HEPES (pH 7.6). The cells were allowed to swell on ice for 5 min and were then broken by 10 strokes of a Dounce homogenizer. The nuclei were pelleted by centrifugation at 600 × *g* for 10 min in a Sorvall SS-34 rotor. The supernatant is referred to as the cell lysate (fraction 1).

**Isolation and solubilization of membrane-bound replicase.** The particulate fraction was pelleted by centrifugation of the cell lysate at 20,000 × *g* for 20 min in a Sorvall SS-34 rotor. The pellet (fraction 2) was suspended in 1 ml of 10 mM HEPES (pH 7.6)-1 mM EDTA and sonicated in a Bransonic-B3 cleaner for 5 min. The suspension was clarified as above. The supernatant (fraction 3) was removed and centrifuged at 100,000 × *g* for 2 h in a Ti50 rotor at 4°C. The resulting pellet was suspended in 10 mM HEPES (pH 7.6)-1 mM dithiothreitol-50% glycerol and frozen in 100-μl volumes at −70°C (fraction 4).

**Replicase assays.** The standard assay mixture (25 μl) contained the following: 0.05 M HEPES (pH 8.2), 10 mM magnesium acetate, 80 mM potassium acetate, 1 mM ATP, 0.25 mM GTP and 0.25 mM CTP, 10 μg

of actinomycin D per ml, 10 μCi of [5-$^3$H]UTP (specific activity, 20.8 Ci/mmol) (New England Nuclear), and 5 μg of fraction 4 enzyme. The reaction mixtures were incubated at 29°C for 2 h and assayed for trichloroacetic acid-insoluble radioactivity by spotting 10 μl onto Whatman GF/A filter disks. The filters were immersed in cold 10% trichloroacetic acid-containing 10 mM sodium pyrophosphate washed twice in 5% trichloroacetic acid and twice in ethanol, dried, and counted by liquid scintillation at a counting efficiency of 15.7%. Except where otherwise noted, the results are expressed as nanomoles of [$^3$H]UMP incorporated per milligram of protein. The values given are corrected for background incorporation which was determined by spotting μl from a complete reaction mix before incubation. Protein concentration was measured by the procedure of Lowry et al. (15) with human gamma globulin as a standard.

**Analysis of the polymerase reaction products.** To analyze the RNA products of the polymerase reactions, the volume of reaction mixture was increased 10-fold. After incubation at 29°C for 3 h, the reaction was stopped by treatment with 50 μg of proteinase K per ml at 29°C for 30 min. The RNA was then extracted with phenol, and the aqueous phase was precipitated with ethanol. Double-stranded RNA was isolated by digesting the phenol-extracted RNA with a mixture of RNase A (10 μg/ml) and RNase T1 (25 U/ml) in 0.3 M NaCl-0.03 M sodium citrate (2× SSC) for 30 min at 29°C. RNases were inactivated by treatment with 50 μg of proteinase K per ml for 30 min at 29°C. The double-stranded RNA was extracted with phenol and precipitated with ethanol as above. The RNA was analyzed by electrophoresis on 2% agarose gels (Seakem) with 89 mM Tris-borate (pH 8.3) buffer (18). Before electrophoresis, the RNA was heated to 65°C for 2 min in Tris-borate (pH 8.3). Where indicated, the RNA was denatured by boiling for 1 min in 80% formamide.

**Hybridization analysis of BBV polymerase products.** Solutions of double-stranded RNA were divided into 20-μl volumes; 20 μg (100-fold excess) of BBV or brome mosaic virus RNA was added where indicated. Each tube was heated at 100°C for 5 min to denature the double-stranded RNA. After quick cooling, the samples were adjusted to 2× SSC, and one sample was treated with RNase (as above) to verify that thermal denaturation was complete. The remaining samples were allowed to reanneal at 70°C for 2 h and then were treated with RNase. The acid-insoluble radioactivity was determined as described above.

## RESULTS

**Appearance of BBV polymerase activity in infected cells.** *D. melanogaster* cells were infected with BBV at an input multiplicity of 1,000 particles per cell. It had been previously determined that this multiplicity of infection resulted in optimal induction of polymerase activity 10 h postinfection (data not shown). At the indicated times postinfection, 5-ml volumes were removed from infected and uninfected cells, and cell lysates were prepared and assayed for the ability to incorporate [$^3$H]UTP into acid-insoluble material (Fig. 1). In the infected cells, polymerase activity was detectable 3 h postinfection. UTP incorporation per cell increased rapidly 3 to 10 h postinfection and increased at a slower rate until 48 h postinfection. By 72 h, polymerase activity had decreased markedly. The insert in Fig. 1 shows that the specific activity, expressed as nanomoles of UTP incorporation per milligram of protein of crude lysate, is maximal at 16 h. The UTP incorporation was unaffected by the presence of 40 μg of exogenous BBV RNA per ml. Lysates made from mock-infected cells did not contain a detectable polymerase activity when assayed either in the presence or absence of BBV RNA (data not shown).

The infected cell lysates were centrifuged at 20,000 × *g*, and the resulting particulate and cytoplasmic fraction were assayed for polymerase activity. Throughout the time course, the majority (92 to 98%) of the polymerase activity in the cell lysate sedimented at 20,000 × *g*. Sedimentation under these conditions indicates that BBV RNA polymerase is bound to particulate material. It has been shown in many other systems (1, 2, 5, 7, 20, 23, 25) that replication complexes which sediment under these conditions are bound specifically to membranes.

**Solubilization and partial purification of BBV RNA polymerase.** BBV polymerase was dissociated from the particulate material by sonication in the presence of 1 mM EDTA. The addition of EDTA, resulting in cation depletion, has been shown to solubilize two plant virus replicases which are membrane bound (2, 24). Cation depletion, alone, solubilized only 20% of the activity in 60 min at 4°C. Sonication of the polymerase for 1 min in the presence of EDTA solubilized 80 to 100% of the activity. Sonication in the presence of EDTA did not release polymerase activity. Solubilization resulted in a two-fold purification of the enzyme (15.4-fold relative to the crude lysate). The use of ionic and non-ionic detergents, which have been useful in solubilizing several membrane-bound replicase complexes (1, 5, 20), resulted in inhibition of BBV polymerase activity.

The solubilized BBV polymerase was further purified by sedimentation at 100,000 × *g* for 2 h. The pellet was suspended in 10 mM HEPES (pH 7.6)–1 mM dithiothreitol–50% glycerol. The pellet contained most of the enzymatic activity and represented a 43-fold purification with an 84% yield relative to that of the starting lysate. The enzyme activity was stable for several months at least when stored at −70°C (data not

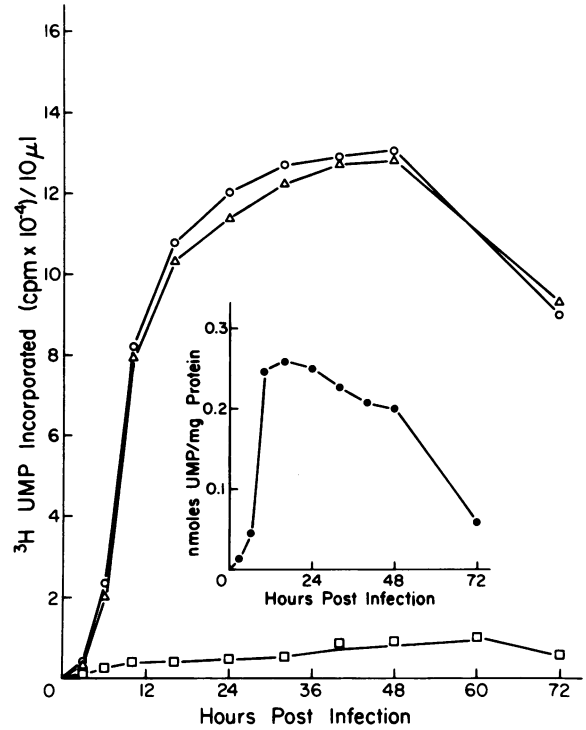


FIG. 1. *Time course of appearance of BBV RNA polymerase activity in D. melanogaster cells infected with BBV. Exponential-phase cells (4 × $10^8$) were infected in suspension culture at 26°C. At the indicated times, aliquots were removed and cell lysates were prepared as described in the text. RNA polymerase activity in the cell lysates* (○), *the particulate fraction* (△), *and the cytoplasmic fraction* (□) *were assayed. (Insert) The polymerase activity in the cell lysates per milligram of protein is shown.*

TABLE 1. *Purification of BBV RNA-dependent RNA polymerase*

| Fraction no. | Fraction description | Total protein (mg) | Total activity (U)[a] | Sp act (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| 1 | Lysate | 51.7 | 5.7 | 0.11 | | 100 |
| 2 | Particulate fraction | 6.6 | 6.3 | 0.95 | 8.6 | 110 |
| 3 | Solubilized enzyme | 2.9 | 4.9 | 1.7 | 15.4 | 86 |
| 4 | 100,000 × *g* pellet | 1.0 | 4.8 | 4.8 | 43.6 | 84 |

[a] One unit of RNA replicase activity is defined as the amount of enzyme capable of catalyzing the incorporation of 1 pmol of [$^3$H]UTP into acid-insoluble material in 30 min at 29°C.

shown). A summary of the purification scheme is presented in Table 1.

**Characterization of BBV polymerase activity.** Figure 2 illustrates the effects of various reaction conditions on the activity of BBV polymerase. The enzyme has an absolute requirement for divalent cation (Fig. 2A). Optimal activity was observed in the range of 10 to 20 mM magnesium acetate. When 10 mM magnesium chloride was added, RNA synthesis was somewhat lower, suggesting an inhibitory effect of chloride ions. The $Mg^{2+}$ requirement could be partially replaced by $Mn^{2+}$, although the optimum concentration for magnesium acetate was much lower (1.6 mM) and the curve was much sharper. Excess $Mn^{2+}$ (greater than 2 mM) completely inhibited activity.

Some monovalent cations slightly stimulated BBV polymerase activity at low concentrations (Fig. 2B). Optimal activity was achieved with 60 mM potassium acetate. Addition of potassium, as the chloride salt, resulted in less stimulation, again suggesting an inhibitory effect of chloride ions on RNA synthesis. Ammonium chloride did not significantly stimulate activity, nor did ammonium sulfate.

BBV polymerase activity exhibited a rather broad temperature optimum, with the peak of

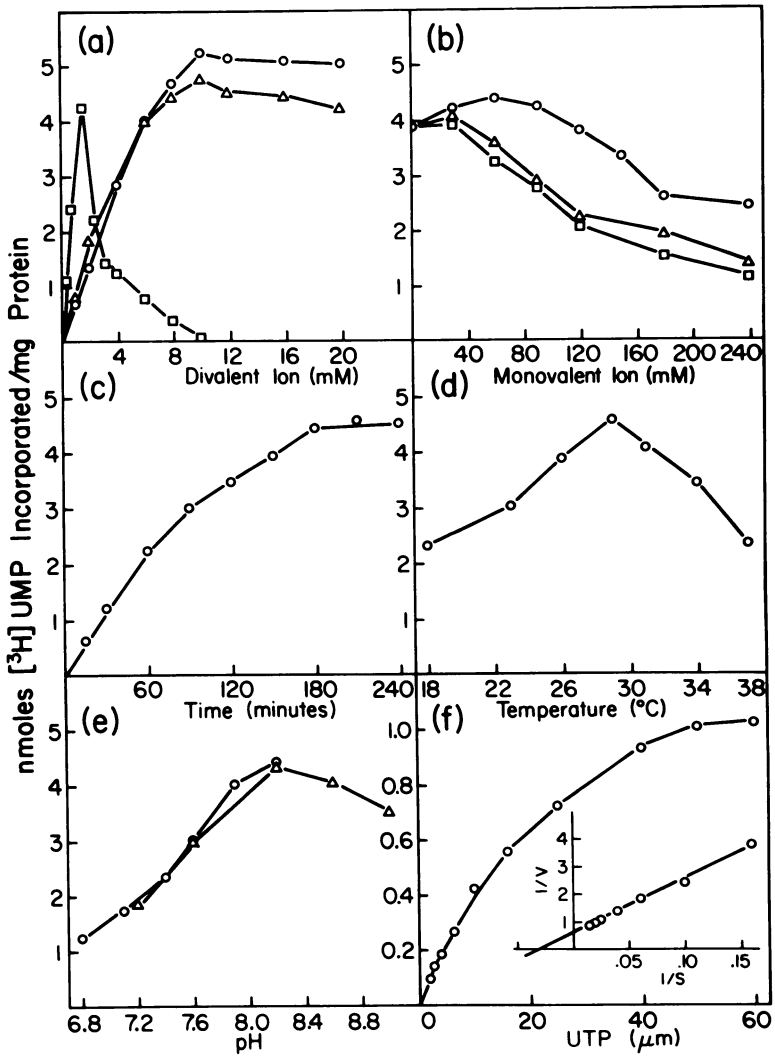


FIG. 2. *Characteristics of the BBV replicase reaction. Reaction mixtures were as described in the text, except that components were varied as indicated. Unless otherwise indicated, the reactions were incubated at 29°C for 2 h. (A) Effect of $Mg(CH_3CO_2)_2$ (○), $MgCl_2$ (△), and $Mn(CH_3CO_2)_2$ (□); (B) effect of $K(CH_3CO_2)$ (○), KCl (△), and $NH_4Cl$ (□); (C) time course of the BBV replicase reaction at 29°C; (D) effect of incubation of BBV replicase at various temperatures. All samples were assayed after 4 h of incubation. (E) Effect of pH using HEPES-KOH (○) or Tris-acetate (△); (F) $K_m$ of UTP. Polymerase reactions (25 μl) were prepared, and 5-μl aliquots were removed at 0, 10, and 20 min at 29°C to ensure that the reaction was linear. The results are plotted as nanomoles of UMP incorporated per milligram of protein per 20 min in F only. (Insert) The reciprocal plot from which the $K_m$ of UTP was interpolated.*

activity at 29°C (Fig. 2D). At this temperature, [$^3$H]UTP was incorporated linearly for 60 min (Fig. 2C). The rate of incorporation decreased slightly over the following 120 min and ceased after 180 min of incubation.

The optimal pH for RNA synthesis was determined using HEPES titrated with KOH in the range of pH 6.8 to 8.2 and Tris-acetate in the range of pH 7.2 to 9.0 (Fig. 2E). The optional pH of replicase activity was pH 8.2, with essentially no difference between HEPES and Tris buffers.

BBV polymerase activity was dependent on the concentration of UTP (Fig. 2F). The $K_m$ for UTP-dependent activity was determined to be approximately 30 μM.

Other characteristics of the BBV replicase reaction are shown in Table 2. The enzyme

requires ATP, GTP, and CTP. The reaction is sensitive to the addition of pancreatic and T1 RNases. The residual activity observed (20% of the control) in the presence of RNase could either be due to the tight interaction of enzyme with its template or the double-stranded nature of the template and product. The addition of phosphoenolpyruvate and phosphoenolpyruvate kinase as an ATP-regenerating system did not significantly affect reaction. Exogenous BBV RNA did not greatly affect the incorporation of UTP. Omission of actinomycin D, either in the presence or absence of DNase, had essentially no effect, indicating that the incorporation of [$^3$H]UTP was due to an RNA-dependent RNA polymerase activity.

TABLE 2. *Characteristics of the BBV polymerase reaction*

| Reaction conditions | % of control |
|---|---|
| Complete[a] | 100[b] |
| − ATP | 3.3 |
| − GTP | 14.9 |
| − CTP | 6.1 |
| + RNase A (10 μg/ml) + RNase T1 (25 U/ml) | 20.1 |
| + Phosphoenolpyruvate (1 mM) + phosphoenolpyruvate kinase (5 μg/ml) | 94.2 |
| + BBV RNA 1+2 (80 μg/ml) | 105.0 |
| − Actinomycin D | 102.0 |
| − Actinomycin D + DNase (30 μg/ml) | 98.6 |

[a] The complete reaction mixture was as described in the text.

[b] 100% = 5.45 nmol of [$^3$H]UMP incorporated per mg of protein (fraction 4).

**Sedimentation analysis of BBV replicase complex.** To determine the size of the BBV replication complex, fraction 3 material was layered onto a 10 to 40% sucrose gradient and centrifuged at 36,000 rpm in an SW41 rotor for 4 h. The gradient was fractionated from the top, and then polymerase activity was assayed across the gradient. The peak replicase activity comigrated with reticulocyte disomes (Fig. 3), indicating the replication complex has a sedimentation value of approximately 120S (10). This is a considerably lower value than 250S, which has been reported for the picornaviruses (1, 11).

**Characterization of the product of BBV polymerase.** The size of the product synthesized in vitro by BBV replication complex was analyzed by electrophoresis of $^3$H-labeled product on 2% agarose gels (Fig. 4). Undenatured [$^3$H]RNA product (lane 1) migrates half as far

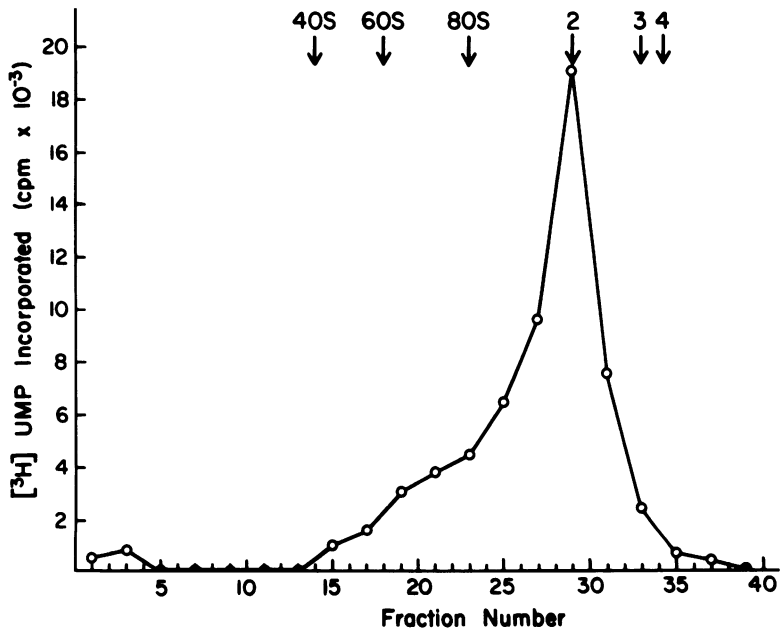


FIG. 3. *Sucrose gradient centrifugation of solubilized BBV replicase. Solubilized replicase was prepared as described (fraction 3) and layered on a 12-ml, 10 to 40% sucrose gradient in 20 mM Tris (pH 7.6)-80 mM KAc[K(CH$_3$CO$_2$)]-4 mM MgAc[Mg(CH$_3$CO$_2$)$_2$]. The gradient was centrifuged in an SW41 rotor for 4 h at 36,000 rpm. Fractions of 0.4 ml were collected from the top of the gradient and assayed for replicase activity at 29°C for 2 h as described in the text. The positions of rabbit reticulocyte ribosomal subunits, monosomes, disomes, and trisomes in a parallel gradient are indicated.*

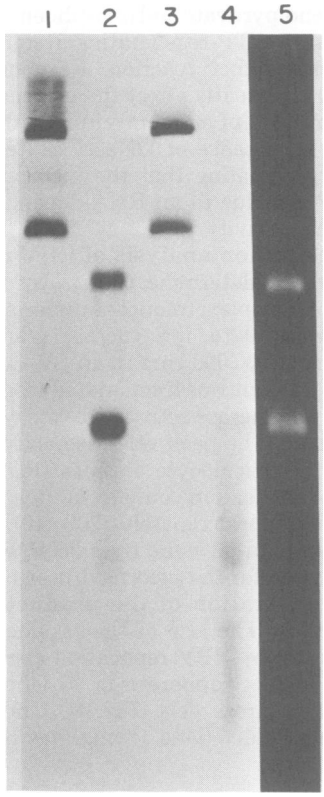


FIG. 4. *Agarose gel analysis of BBV replicase products. [$^3$H]RNA synthesized in vitro by the BBV replicase complex as described in the text was phenol extracted and electrophoresed on 2% agarose gels in Tris-borate buffer (pH 8.3) containing 1 μg of ethidium bromide per ml. Before electrophoresis, the samples were heated to 65°C for 2 min (lanes 1, 3, and 5) or boiled for 1 min in 80% formamide (lanes 2 and 4). Lanes 1 and 2 contain untreated reaction products. Lanes 3 and 4 contain RNA treated with pancreatic and T1 RNases in 2× SSC. Lane 5 shows the migration of genomic BBV RNA as visualized with UV light.*

as viral RNA (lane 5). When the labeled RNA was denatured by boiling for 1 min in 80% formamide before electrophoresis (lane 2), the RNAs comigrated with viral RNA. The undenatured RNA product was resistant to treatment with a mixture of pancreatic and T1 RNases in 2× SSC (lane 3) indicating that it is double stranded. After denaturation (lane 4), the [$^3$H]RNA became sensitive to treatment with RNase in 2× SSC.

**Hybridization analysis of the BBV polymerase product.** To determine whether BBV replication complex synthesized "plus" or "minus" strands of BBV RNA, the $^3$H-labeled reaction product was subjected to hybridization analysis (Table 3). The newly synthesized [$^3$H]RNA was treated with RNase in 2× SSC to remove single strands which interfere with the analysis. The RNase-resistant material was heat denatured by boiling for 5 min in 0.1× SSC. After denaturation, the RNA is sensitive to RNase in 2× SSC, indicating a disruption of double-stranded structures. When the RNA is self annealed, 52% of the [$^3$H]RNA was again engaged in double-stranded structure. Reannealing in the presence of BBV RNA 1 or RNA 2 separately increased the RNase resistance 28 and 35%, respectively, whereas reannealing in the presence of both RNAs protected all of the [$^3$H]RNA from RNase. This result indicates that the [$^3$H]RNA synthesized in vitro was complementary to viral RNA, i.e., minus strand. Addition of a heterologous brome mosaic virus RNA had no effect on reannealing.

## DISCUSSION

Infection of *D. melanogaster* cells with BBV results in the appearance of an RNA polymerase activity. This is apparently an RNA-dependent RNA polymerase because activity is inhibited by RNase but not DNase. The polymerase is bound to particulate material and appears to be in a complex with its template, BBV RNA. Such membrane-enzyme-template complexes have been described for the picornaviruses (1, 15, 23) and several plant viruses (2, 7, 25). Solubilization of the enzyme either in a template-dependent or -independent form is a crucial step in replicase purification. Most complexes have been solubilized by the use of ionic or nonionic detergents (1, 5, 20, 25). Attempts to solubilize BBV replicase using detergents resulted in inactivation of the enzyme. Some membrane-bound replicases have been solubilized by washing the particulate fraction in a $Mg^{2+}$-deficient buffer (2, 24). Diva-

TABLE 3. *Hybridization of polymerase products*[a]

| Reaction conditions | % Hybrid yield |
|---|---|
| Control | 100 |
| RNase | 5 |
| Selfanneal, RNase | 52 |
| Anneal + BBV RNA 1, RNase | 80 |
| Anneal + BBV RNA 2, RNase | 87 |
| Anneal + BBV RNA 1+2, RNase | 98 |
| Anneal + BMV RNA, RNase | 50 |

[a] RNase-resistant [$^3$H]RNA (16,000 cpm in 20 μl of 0.1× SSC) was denatured by boiling 5 min and quickly cooled in an ice-water bath. The samples were adjusted to 2× SSC. Where indicated, they were allowed to anneal at 70°C for 2 h and then treated with a mixture of pancreatic and T1 RNases (10 μg/ml and 25 U/ml, respectively), before determining the acid-insoluble radioactivity.

lent cation depletion of bound BBV replicase resulted in a slow release of enzyme. The rate of solubilization was greatly increased by sonication of the membranes in the presence of 1 mM EDTA. It has been reported that, for poliovirus RNA polymerase, most of the active enzyme is in the soluble form in the cytoplasm (4, 6). However, this does not appear to be the case for BBV.

Agarose gel analysis of the [$^3$H]RNA synthesized by partially purified BBV replication complex indicated that the product was double-stranded BBV RNA. Denaturation of the double-stranded product resulted in RNA which comigrated with viral RNA. Hybridization analysis of the double-stranded [$^3$H]RNA synthesized by BBV replicase indicated that it was predominantly minus-strand BBV RNA. This result is surprising in view of the fact that most replicase complexes contain minus-strand template and synthesize plus-strand RNA (2, 16, 22), although some negative strand synthesis has been reported (13), as well as synthesis of both plus and minus strands (26). We cannot presently account for the predilection of BBV replication complex toward minus-strand synthesis. Perhaps a protein or other factor lost during the purification scheme is required for plus-strand synthesis.

It has been suggested that BBV A protein is, or forms part of, the BBV replicase (8, 17). Verification of A protein as a component of the replicase awaits complete purification of the enzyme. However, analysis of the protein composition at various stages of the purification scheme indicated that A protein copurifies with the replication complex, BBV, whereas most of the coat protein and B protein in the cell were found in the cytoplasm.

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