Identification and Characterization of a DNA Primase Activity Present in Herpes Simplex Virus Type 1-Infected HeLa Cells

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A novel DNA primase activity has been identified in HeLa cells infected with herpes simplex virus type 1 (HSV-1). Such an activity has not been detected in mock-infected cells. The primase activity coeluted with a portion of HSV-1 DNA polymerase from single-stranded DNA agarose columns loaded with high-salt extracts derived from infected cells. This DNA primase activity could be distinguished from host HeLa cell DNA primase by several criteria. First, the pH optimum of the HSV primase was relatively broad and peaked at 8.2 to 8.7 pH units. In contrast, the pH optimum of the HeLa DNA primase was very sharp and fell between pH 7.9 and 8.2. Second, freshly isolated HSV DNA primase was less salt sensitive than the HeLa primase and was eluted from single-stranded DNA agarose at higher salt concentrations than the host primase. Third, antibodies raised against individual peptides of the calf thymus DNA polymerase:primase complex cross-reacted with the HeLa primase but did not react with the HSV DNA primase. Fourth, freshly prepared HSV DNA primase appeared to be associated with the HSV polymerase, but after storage at 4°C for several weeks, the DNA primase separated from the viral DNA polymerase. Separation or decoupling could also be achieved by gel filtration of the HSV polymerase:primase. This free DNA primase had an apparent molecular size of approximately 40 kilodaltons, whereas free HeLa DNA primase had an apparent molecular size of approximately 110 kilodaltons. On the basis of these data, we believe that the novel DNA primase activity in HSV-infected cells may be virus coded and that this enzyme represents a new and important function involved in the replication of HSV DNA.

The isolation of a virus encoded DNA polymerase from herpes simplex type 1 (HSV-1)-infected cells (21, 22, 35) was an important first step toward understanding viral DNA replication. The DNA polymerases isolated from HSV-1- and HSV-2-infected cells show very similar properties (26) which serve to distinguish the viral enzyme from mammalian DNA polymerases. These properties include salt optimum, inhibition by polyamines (such as spermine), inhibition by phosphonoacetate and phosphonoacetate analogs, column chromatographic behavior, sedimentation rate on sucrose gradients, and inhibition by 9-(2-hydroxyethoxymethyl)GTP and 9-(1,3-dihydroxy-2-propoxymethyl)GTP (10, 11, 26). The presence of a 3' → 5' exonuclease activity (9, 26, 35) also serves to distinguish HSV DNA polymerase from mammalian DNA polymerases α , β , and γ . The size of HSV DNA polymerase was originally estimated to be 180 kilodaltons (kDa) (35), but a polypeptide with a molecular size of approximately 140 kDa has now been assigned to the DNA polymerase activity (26), and the gene for this polypeptide has been cloned and sequenced (28).

Although the properties of HSV DNA polymerase distinguish it from mammalian DNA polymerases, one important similarity exists between HSV DNA polymerase and mammalian DNA polymerase α , the enzyme believed to be responsible for the bulk of the cellular DNA replication (see reference 16 for review). That property is the ability to use a short piece of RNA as a primer for DNA synthesis (35). A DNA primase can be copurified from eucaryotic cells in tight association with DNA polymerase α (6, 14, 20, 34). This primase can synthesize oligoribonucleotides of 8 to 10 bases in length, which the DNA polymerase can then use in DNA synthesis (7, 17, 32). Nascent DNA chains extracted from

mammalian cells contain oligoribonucleotides of this size at their 5' ends (29, 33).

Mature HSV DNA molecules contain alkali-labile sites (12, 13, 31, 36). These may be due to the use of RNA primers for DNA replication, some of which may have persisted during maturation of the DNA, although alternative explanations for alkali lability and the presence of RNA-like material in mature viral DNA are possible (3, 25, 38). Newly synthesized HSV DNA has been demonstrated to contain RNA covalently linked to DNA (2, 15, 24) which is removed over a period of time (24). These RNA segments are estimated to be 35 nucleotides in length and are linked to DNA averaging 36×10^3 nucleotides in length (24). This would indicate a major difference between the way the virus uses RNA to prime DNA synthesis and the way eucaryotic cells replicate. The length of the DNA attached to RNA primers in the eucaryotic cell is usually 200 nucleotides or less (8). These differences may reflect the involvement of virus-coded proteins in the synthesis of the RNA primers as well as in the synthesis of the viral DNA.

We report here on the identification and characterization of an enzyme which can be classified as a DNA primase from HSV-1-infected HeLa cells and whose enzymatic and structural properties distinguish it from the DNA primases isolated from uninfected HeLa cells.

MATERIALS AND METHODS

Chemicals and reagents. Calf thymus DNA and unlabeled deoxynucleoside and nucleoside triphosphates were obtained from Sigma Chemical Co. [8-³H]dATP, [methyl-³H]TTP, and [α -³²P]ATP were obtained from New England Nuclear Corp. *Escherichia coli* DNA polymerase I was obtained from New England BioLabs, Inc. Sepharose 6B was obtained from Pharmacia Fine Chemicals. Single-

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stranded DNA agarose was obtained from Bethesda Research Laboratories. Tissue culture media were obtained from GIBCO Laboratories. Poly(dT)_{3,500} was prepared by using terminal deoxynucleotidyltransferase as described elsewhere (5). Calf thymus DNA polymerase:primase complex and free calf thymus DNA primase were prepared as described previously (6). Antibodies were prepared by immunizing individual rabbits with individual peptides from the calf thymus DNA polymerase:primase complex as described elsewhere (18). They were further purified by chromatography on protein A-Sepharose and then affinity purified by chromatography on a calf thymus DNA polymerase:primase complex Sepharose 4B column. All other chemicals were of reagent grade.

Cells and viruses. HeLa S-600 cells were grown in suspension culture in suspension-modified Eagle medium to a concentration of 1.6×10^6 /ml. Infection was with HSV-1 (mP) grown in Vero cells at a multiplicity of 20 PFU per cell as described by Derse et al. (10). Cells were harvested 8 h after infection, the time viral DNA replication reached its maximum under these conditions. The cells were harvested by centrifugation and stored at -70°C or used immediately for the isolation of HSV DNA polymerase.

Isolation of HSV DNA polymerase:primase. The isolation procedure was based on a procedure described by Powell and Purifoy (27) and Ruyechan and Weir (30). The cells were suspended in extraction buffer (20 mM Tris [pH 7.5], 1.7 M KCl, 5 mM EDTA, 1 mM dithiothreitol [DTT], 0.2% Nonidet P-40, 10% glycerol), sonicated three times for 30 s in a Heat Systems Ultrasonics sonicator, and centrifuged at $500 \times g$, and the supernatant was subjected to a 10% polyethylene glycol phase separation. The precipitated material, including nucleic acids, was spun down. The supernatant was dialyzed against 0.15 M KCl in the buffer described above and chromatographed on a single-stranded DNA agarose column. Enzyme were eluted with a linear 0.15 to 1.0 M KCl gradient. All procedures were carried out at 4°C in a solution containing 20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DTT, and 10% (vol/vol) glycerol. A modified procedure of Allen et al. (1) has also been used to isolate DNA polymerase from HSV-1-infected HeLa cells. In this procedure, the cells were lysed in hypotonic buffer, brought to 0.4 M potassium phosphate, as described above, and passed through DEAE-Tris-acryl. The unbound material was dialyzed against a solution containing 20 mM potassium phosphate (pH 7.5), 1 mM DTT, 20% (vol/vol) glycerol, and 0.2% Nonidet P-40 and rechromatographed on DEAE-Tris-acryl. HSV DNA polymerase bound to the column was eluted with a linear KCl gradient (0 to 0.5 M). Fractions containing DNA polymerase:primase assayable at 0.2 M salt were pooled, dialyzed against the above buffer, and chromatographed on a single-stranded DNA agarose column under identical elution conditions.

In some instances, the DNA polymerase:primase was concentrated by adsorption and batch elution with 0.5 M KCl from a 1-ml single-stranded DNA agarose column and stored at 4°C .

Enzyme assays. DNA polymerase activities were assayed by using three different protocols. DNA polymerase eluted from single-stranded DNA agarose columns to which high-salt extracts had been applied were carried out in a solution containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0), 8 mM MgCl₂, 300 µg of DNase I-treated DNA per ml, 0.2 mM each dATP, dCTP, and dGTP, 0.04 mM [³H]TTP at 372 dpm/pmol of 2 mM 2-mercaptoethanol, and 200 mM KCl. Aliquots (10 µl) of

individual column fractions were added to 40 µl of the reaction mixture and incubated at 35°C for 20 min. Aliquots (40 µl) were spotted on glass fiber squares and placed in 5% trichloroacetic acid–1% sodium pyrophosphate. The squares were washed twice with 1 N HCl and once with 95% ethanol, air dried, and counted in a liquid scintillation counter. One unit of DNA polymerase activity in this assay is defined as the incorporation of 1 pmol of [³H]TTP into insoluble material in 1 min at 35°C . DNA polymerase isolated by the modified procedure of Allen et al. (1) was assayed in 40 mM phosphate with or without 200 mM KCl. All other conditions were exactly as described above. After the initial isolation of HSV DNA polymerase activity by these two methods, all additional polymerase assays were carried out in a solution containing 40 mM potassium phosphate (pH 7.0), 8 mM MgCl₂, 100 µg of bovine serum albumin (BSA) per ml, 1 mM DTT, 100 µg of DNase I-treated DNA per ml, 0.1 mM each dATP, dCTP, and dGTP, and 0.1 mM [³H]TTP at 40 to 50 cpm/pmol, with or without 0.15 M NaCl. Assays were incubated at 35°C , and at the times indicated, samples were spotted on glass fiber squares and placed in the 5% trichloroacetic acid–1% sodium pyrophosphate. One unit of DNA polymerase activity is defined as the incorporation of 1 nmol of [³H]TTP into acid-insoluble material in 60 min at 35°C in this assay.

DNA primase was assayed directly in a solution containing 50 mM Tris (pH 8.0), 8 mM MgCl₂, 100 µg of BSA per ml, 1 mM DTT, 10 µM poly(dT)_{3,500}, and 0.1 mM [α -³²P]ATP (100 to 200 cpm/pmol). Assays were done at 35°C , and aliquots were spotted on Whatman DE-81 papers previously spotted with an equal volume of 5 mM unlabeled ATP. This technique reduces contamination by the [³²P]ATP. The papers were washed four times with 0.2 M dipotassium hydrogen phosphate and twice with glass-distilled water and counted in glass-distilled water in a liquid scintillation counter set on the ³H-labeled channel. The counts remaining on the paper give a direct measurement of oligoribonucleotide synthesis. One unit of enzyme activity is defined as the incorporation of 1 nmol of [³²P]AMP into oligoribonucleotide in 60 min at 35°C .

DNA primase was routinely assayed by an indirect assay. The assay conditions were as described above, except that 2 mM unlabeled ATP replaced the 0.1 mM [α -³²P]ATP and 0.1 mM [³H]dATP at 40 to 50 cpm/pmol and 20 U of *E. coli* DNA polymerase I per ml was usually present. Assays were carried out at 35°C , and aliquots were spotted on glass fiber paper and processed as for DNA polymerase assays. This coupled assay procedure measures the extension of primers synthesized by the DNA primase, and the rate of incorporation of [³H]dAMP into acid-insoluble product is dependent on the rate of primer synthesis. In the absence of ATP or DNA primase, no incorporation of [³H]dAMP is observed. This is an amplification of DNA primase activity. One unit of enzyme activity in this instance is defined as the incorporation of 1 nmol of [³H]dAMP into acid-insoluble material in 60 min at 35°C . Exonuclease activity was measured in a solution containing 50 mM Tris (pH 8.0), 8 mM MgCl₂, 1 mM DTT, 100 µg of BSA per ml, and 10 µg of single-stranded ¹⁴C-labeled T7 DNA at 30×10^3 cpm/µg. Assays were incubated at 35°C , and at the times indicated, aliquots were placed on glass fiber squares and the squares were processed as for DNA polymerase assays.

Sepharose 6B chromatography. Sepharose 6B chromatography was carried out on an 85-by-1.4-cm column in a solution containing 0.25 M NaCl, 50 mM Tris (pH 8.0), 100 µg of BSA per ml, 1 mM DTT, and 20% (vol/vol) glycerol.

Fractions (50 drops, or 1.7 ml, each) were collected. The column was calibrated with calf thymus DNA, beef liver catalase, rabbit IgG, *E. coli* DNA polymerase I, ovalbumin, cytochrome *c*, and DTT.

Antibody-binding experiments. Antibody-binding experiments were carried out as follows. A total of 20 μ l of a given enzyme preparation and 20 μ l of rabbit immunoglobulin G (IgG) raised against a specific calf thymus DNA primase peptide (68 kDa, 55 kDa, or 48 kDa) in TBS buffer (100 mM Tris, 0.15M NaCl, 2 mg of BSA per ml) were mixed and incubated for 18 h at 4°C. Samples (20 μ l each) were removed, and to each sample was added 20 μ l of 50% (vol/vol) protein A-Sepharose in TBS buffer. Incubation continued for an additional 18 h. The samples were then spun down in an Eppendorf centrifuge, and 5- μ l aliquots of the resulting supernatants were assayed for primase or polymerase activity as described above.

RESULTS

Association of DNA primase with HSV DNA polymerase.

When extracts of HSV-1-infected HeLa cells were chromatographed on single-stranded DNA agarose, the HSV DNA polymerase activity eluted between 0.3 and 0.35 M KCl. With the indirect, or coupled, DNA primase assay, a peak of DNA primase activity was detected toward the back of the HSV DNA polymerase peak (Fig. 1). The direct DNA primase assay also detected some DNA primase activity in this area of the elution profile (data not shown). It was difficult to quantitate the DNA primase present in these fractions by the direct procedure, since to observe sufficient [³²-P]AMP incorporation, it was necessary to extend the assay time far beyond that shown in Fig. 1 and the presence of high levels of exonuclease activity destroyed the template at later times. The direct assay was also complicated by the presence of a DNA-dependent ATPase activity (and RNase H activity in this area of the elution profile (data not shown). The direct DNA primase assay contained only 0.1 mM ATP, whereas the indirect, or coupled, assay contained 2 mM ATP, helping to negate the effects of this contaminating ATPase. Further, the coupled DNA primase assay amplified the DNA primase activity to such an extent that assay time and the amount of the DNA agarose fraction used in the

FIG. 1. Chromatography of HSV DNA polymerase on single-stranded DNA agarose. HSV DNA polymerase was isolated from infected cells by the modified Powell and Purifoy procedure (27, 30). Assays were done as described in Materials and Methods. Symbols: $\square$, DNA polymerase in the presence of 0.2 M NaCl; $\circ$, DNA primase in the coupled assay in the presence of *E. coli* DNA polymerase I; $\triangle$, exonuclease; $\cdots$, KCl gradient.

assay could be reduced so that neither exonuclease nor ATPase affected the assay. The incorporation of [³H]dAMP shown in Fig. 1 was taken from time courses of assays of individual fractions in which the incorporation was still linear with time. The result shown in Fig. 1 indicates that the position of elution of the DNA primase was not affected by the contaminating enzyme activities. That is, the DNA primase elution position was toward the rear of the DNA polymerase elution position. The fractions rich in DNA primase activity were pooled, and the pooled fractions were referred to as HSV DNA primase.

Uninfected HeLa cells were subjected to the same extraction and chromatography procedures. DNA polymerase activity present in control extracts did not bind to the DNA agarose column. This DNA polymerase activity was inhibited by 0.15 M NaCl in the assay and could only be assayed in the absence of salt. This salt-inhibited polymerase activity was also present in the DNA agarose column breakthrough of the infected cell extracts. The properties of this DNA polymerase are consistent with those of the HeLa DNA polymerase α . On application of the KCl gradient to this control (uninfected) DNA agarose column, a small amount of DNA primase began to elute from the column and continued to elute up to about 0.4 M KCl (data not shown). This material, comprising less than 10% of the activity eluting from the infected cell DNA agarose column, was pooled for enzyme comparison purposes and is referred to as control DNA primase. This relatively small amount of DNA primase activity identified in uninfected cell extracts would suggest that the much greater amount of DNA primase observed in this area of the elution profile when extracts of infected cells were subjected to the same procedures is a consequence of the viral infection, although it does not mean that it is necessarily virally encoded.

By using the modified procedure of Allen et al. (1) to isolate DNA polymerase from infected cells, partial separation was achieved between the HeLa DNA polymerase and the HSV DNA polymerase by the KCl elution from the second DEAE-Tris-acryl column. The peak of HSV DNA polymerase was pooled, dialyzed, and applied to single-stranded DNA agarose as indicated in Materials and Methods. The elution profile from this DNA agarose column is shown in Fig. 2. The salt-sensitive HeLa DNA polymerase α eluted between 0.1 and 0.15 M KCl. The salt-activated HSV DNA polymerase again eluted between 0.3 and 0.35 M KCl. Two peaks of DNA primase activity were observed; the major peak was associated with the HeLa DNA polymerase α and the minor peak eluted at the rear of the HSV DNA polymerase peak. The first peak of DNA primase activity was pooled for enzyme comparison studies and is referred to as the HeLa DNA primase.

When this second purification procedure was used on uninfected HeLa cells, a salt-sensitive peak of DNA polymerase activity was observed again eluting between 0.1 and 0.15 M KCl. Salt-activated DNA polymerase activity was not observed in this elution profile (data not shown). One peak of DNA primase activity was observed, in the same elution position as the DNA polymerase, but a small amount of DNA primase smeared off the column up to a salt concentration of about 0.4 M KCl. Again, this was less than 10% of the amount of enzyme activity observed when infected cell extracts were subjected to these procedures.

RNA polymerase was not detected with either purification procedure.

Initial characterization of the HSV DNA primase. The relationships between the different DNA primases present in

FIG. 2. Chromatography of HSV DNA polymerase on single-stranded DNA agarose. HSV DNA polymerase was isolated from infected cells by the modified procedure of Allen et al. (1). Assays were done as described in Materials and Methods. □, DNA polymerase in the presence of 0.2 M NaCl; ○, DNA polymerase in the absence of NaCl; ▲, DNA primase in the coupled assay in the presence of *E. coli* DNA polymerase I; ---, KCl gradient.

infected and uninfected cell extracts were explored. The effect of increasing concentrations of NaCl on the HSV and HeLa DNA primases was determined by using the coupled assay in the presence of *E. coli* DNA polymerase I (Fig. 3). It is important to note that these primase samples also contained HSV and HeLa DNA polymerases, respectively. Freshly prepared HSV primase was less sensitive than the HeLa DNA primase, which showed the same salt sensitivity as the DNA primase activity of the calf thymus DNA polymerase:primase complex. *E. coli* DNA polymerase I was active throughout the range of salt concentrations used. Since HSV DNA polymerase is a salt-activated enzyme (26, 35), however, the relative salt resistance of the HSV DNA primase could have been due to the HSV polymerase beginning to function at high salt concentrations to efficiently elongate a reduced number of primers synthesized by a salt-sensitive DNA primase. Accordingly, a comparison was made of DNA primase activities in the presence and absence of 0.15 M NaCl and with and without the addition of *E. coli*

FIG. 3. Effects of increasing NaCl concentration on DNA primase activity. DNA primase was assayed in the coupled assay system in the presence of *E. coli* DNA polymerase I. □, HSV DNA primase; △, HeLa DNA primase; ○, DNA primase of the calf thymus DNA polymerase:primase complex.

TABLE 1. Effects of the addition of 0.15 M NaCl on the activities of HeLa DNA primase and freshly prepared HSV DNA primase

DNA primase	Picomoles of [³ H]dAMP incorporated per 5 μl per 60 min			
	With <i>E. coli</i> DNA polymerase I		Without <i>E. coli</i> DNA polymerase I	
	No NaCl	0.15 M NaCl	No NaCl	0.15 M NaCl
HeLa	55	1	25	0
Freshly prepared HSV	180	72	105	33

DNA polymerase (Table 1). In the absence of both *E. coli* DNA polymerase I and NaCl, the elongation of primers synthesized by both the HeLa and HSV DNA primases by the respective DNA polymerases was relatively efficient compared with the elongation of the primers in the presence of *E. coli* DNA polymerase I (Table 1). At 0.15 M NaCl, there was no incorporation of [³H]dAMP with the HeLa DNA primase either in the presence or absence of *E. coli* DNA polymerase I, whereas there was still incorporation in both instances with the HSV DNA primase. Table 2 shows the activities of the DNA polymerase at the two salt concentrations.

These results were obtained by using freshly prepared HSV DNA primase. The salt profile shown in Fig. 3 was obtained with HSV DNA primase which had been isolated a few days before. HSV DNA primase stored at 4°C for several weeks was almost as salt sensitive as the HeLa and calf DNA primases were (Table 3). Furthermore, although both the DNA polymerase and DNA primase activities of this stored enzyme sample had not declined significantly (the DNA primase activity being measured in the presence of *E. coli* DNA polymerase I by the indirect assay), the HSV DNA polymerase could now not elongate the primers produced by the DNA primase in either the presence or absence of 0.15 M NaCl, indicating a major change in the relationship of the DNA polymerase and DNA primase in this sample. This change could be the decoupling of the two enzyme activities or the loss of another factor which mediates the two activities. This change in the salt sensitivity and the inability of the HSV DNA polymerase to function in the coupled DNA primase assay has been observed on long-term storage of the enzyme at 4°C or after dialysis of the enzyme against 50% (vol/vol) glycerol and storage of the enzyme at -20°C. Thus, these data indicate that the relative insensitivity to salt displayed by freshly prepared HSV DNA primase is not an intrinsic property of the primase but may also indicate its participation in a complex. The uncoupled HSV DNA polymerase is still capable of incorporating [³H]dAMP into product by using a poly(dT) template when given an

TABLE 2. Effects of the addition of 0.15 M NaCl on the DNA polymerase activities of HeLa DNA primase and freshly prepared HSV DNA primase

Origin of DNA polymerase	Picomoles of [³ H]dTTP incorporated per 5 μl per 60 min	
	No NaCl	0.15 M NaCl
HeLa DNA primase	73	10
Freshly prepared HSV DNA primase	92	720

TABLE 3. Effects of addition of 0.15 M NaCl on the activities of freshly prepared HSV DNA primase and concentrated HSV DNA primase stored at 4°C for 35 days

HSV DNA primase	% Activity			
	With <i>E. coli</i> DNA polymerase I		Without <i>E. coli</i> DNA polymerase I	
	No NaCl	0.15 M NaCl	No NaCl	0.15 M NaCl
Freshly prepared	100 ^a	64	76	60
Concentrated and stored	100 ^b	3	5	3

^a An activity of 100% for freshly prepared HSV DNA primase was 590 pmol of [³H]dAMP incorporated per 20 µl per 60 min.

^b An activity of 100% for concentrated stored HSV DNA primase was 152 pmol of [³H]dAMP incorporated per 1 µl per 60 min.

oligo(A) initiator (data not shown), indicating that this loss of coupling of DNA polymerase and primase activities is not due to the DNA polymerase losing the capability of using an oligoriboadenylate initiator.

The pH optima of both freshly prepared and stored HSV DNA primase were the same, pH 8.2 to 8.7 (Fig. 4A and B). Those of the HeLa DNA primase and the DNA primase of the calf thymus DNA polymerase:primase complex and free DNA primase from calf thymus were all pH 7.9 to 8.2 (Fig. 5A and B). The control DNA primase (the DNA primase from the DNA agarose column chromatography of extracts of uninfected HeLa cells) did not show a defined pH optimum over the pH range studied but rather indicated a gradual increase in activity with increasing pH (Fig. 4B).

Size of the HSV DNA primase. The results of the chromatography of a stored (uncoupled) sample of HSV DNA primase on a calibrated Sepharose 6B column are shown in Fig. 6. The HSV DNA polymerase activity eluted in advance of beef liver catalase with an apparent molecular size in excess of 250 kDa, compared with the molecular size of 140 kDa obtained from sedimentation studies and predicted from the DNA sequence (28). Allen et al. (1) have previously made a similar observation with the equine herpesvirus DNA polymerase. This discrepancy in the molecular size estimates is explicable on the basis that the molecule is asymmetric in shape (19).

The HSV DNA primase activity eluted between the positions of elution of ovalbumin and cytochrome *c* with an

FIG. 4. The pH optima of HSV DNA primase. DNA primase was assayed in the coupled assay system in the presence of *E. coli* DNA polymerase I in constant-molarity Tris buffers. Symbols: Stored HSV DNA primase (□); freshly prepared HSV DNA primase (○); control DNA primase (▲).

FIG. 5. The pH optima of mammalian DNA primases. DNA primase was assayed in the coupled assay in the presence of *E. coli* DNA polymerase I in constant-molarity Tris buffers. Symbols: HeLa DNA primase (□); calf thymus DNA polymerase primase complex (○); free calf thymus DNA primase (Δ).

apparent molecular size of approximately 40 kDa (Fig. 6). The material eluting between fractions 40 and 50 was not present in chromatography of other samples of HSV DNA primase on the Sepharose 6B column and may represent aggregated material. Most of the DNA primase activity of freshly prepared HSV DNA primase also eluted from Sepharose 6B with an apparent molecular size of 40 kDa. These data indicated that (i) either the primase activity was uncoupled from the HSV DNA polymerase activity on long-term storage or uncoupling occurred due to dilution during chromatography or (ii) it was not part of a tight complex to begin with. Most of the HeLa DNA primase eluted at the same volume as *E. coli* DNA polymerase I, with an apparent molecular size of 110 kDa (Fig. 6). This result seems to indicate that most of this DNA primase is also not tightly

FIG. 6. Chromatography of HSV DNA primase on an 86-by-1.4-cm Sepharose 6B column. Stored HSV DNA primase was chromatographed on Sepharose 6B as described in Materials and Methods. Assays were done as described in Materials and Methods. Symbols: □, DNA polymerase activity in the presence of 0.15 M NaCl; ○, DNA primase activity in the coupled assay in the presence of *E. coli* DNA polymerase I. The numbers refer to standards run on the column under the same conditions: 1, Calf thymus DNA; 2, beef liver catalase; 3, rabbit IgG; 4, *E. coli* DNA polymerase I; 5, ovalbumin; 6, cytochrome *c*; 7, DTT. The arrow marks the peak of elution of the free HeLa DNA primase run under the same conditions.

associated with DNA polymerase under these elution conditions. It is important to note that free calf thymus DNA primase elutes at the same position and sediments with the same *S* value as *E. coli* DNA polymerase I, indicating that it is a globular molecule with an approximate molecular size of 110 kDa (18). This does not necessarily indicate that the apparent molecular size of 40 kDa observed for the HSV DNA primase is the true molecular size of this enzyme (see Discussion).

Reactivity with anti-calf DNA primase IgGs. Rabbit IgGs raised against individual peptides of the calf thymus DNA polymerase:primase complex and previously used to assign functions to these individual peptides (18) were used in antibody-binding experiments to ascertain the relationship of the HSV, HeLa, and calf DNA primases. Previously, by using both an immunoblotting procedure and enzyme neutralization, peptides of 55 and 48 kDa had been assigned to the free DNA primase of calf thymus and peptides of 68 kDa, 55 kDa, and 48 kDa had been assigned to the DNA primase of the calf thymus DNA polymerase:primase complex, with the 48-kDa peptide being derived, perhaps proteolytically, from the 68-kDa peptide (18). The results of the antibody-binding experiments are presented in Table 4. All three IgG samples reacted with the HeLa DNA primase, but none of them reacted with the HSV DNA primase. Three times the amount of calf thymus DNA primase showed the same reactivity with these antibodies as the HeLa enzyme did. Thus, although cross-reactivity of these antibodies with the HeLa DNA primase was not 100%, the results show a significant difference between the HeLa and HSV DNA primases. Immunoblots with the anti-calf DNA primase antibodies showed cross-reactivity between these antibodies and the HeLa DNA primase and indicated that the HeLa DNA primase has a peptide composition similar to that of the calf DNA primase. No cross-reactivity with the HSV DNA primase was observed (data not shown).

DISCUSSION

A new DNA primase activity has been identified in extracts derived from HSV-1-infected HeLa cells. This activity appears to be coupled with the HSV DNA polymerase when freshly isolated, but it appears to decouple from the polymerase activity upon long storage and is separable by gel filtration chromatography. Several properties of this primase activity distinguish it from the host HeLa cell primase. First, freshly prepared HSV primase retains approximately 50% activity at salt concentrations up to 125 to 150 mM, although this salt resistance is lost when the primase is uncoupled

from the polymerase. In contrast, HeLa primase is extremely salt sensitive both when it is coupled to the host polymerase and when it is free. Second, the pH optimum for the HSV primase is higher and much broader than that determined for the HeLa primase. Third, the apparent molecular weight of the HSV primase as determined by gel filtration is approximately one third that determined for the HeLa primase. Fourth, antibodies raised against subunits of the calf thymus DNA primase complex which bind to the HeLa primase activity do not show any cross-reactivity with the HSV primase.

The question then arises as to the origin of this primase activity. Several possibilities must be considered. First, the putative HSV primase could simply be HeLa primase which has become associated with the HSV DNA polymerase and is used in viral DNA replication. Such an association could alter the pH optimum and salt sensitivity of the HeLa primase. The fact that the pH of uncoupled HSV primase is the same as that of freshly prepared (coupled) HSV DNA primase would argue that this activity is not the HeLa primase. This fact, in conjunction with the different apparent molecular weights determined for the two primase activities by gel filtration, appears to eliminate this possibility. Second, the HSV primase could be a modified (proteolytic fragment) of the HeLa primase. In this case, the salt and pH optima of the enzyme could again be altered significantly and would not necessarily change upon decoupling of the primase from the polymerase. The evidence we have gathered argues against this possibility. The cross-reactivity of antibodies to the peptides of the calf DNA primase with the HeLa DNA primase and the elution position of the HeLa primase from Sepharose 6B suggest a structural similarity of the DNA primases from these two sources. The free calf DNA primase has been demonstrated to contain peptides of 55 and 48 kDa (18). If the size of the HSV DNA primase is indeed 40 kDa, it is possible that it is derived from the catalytic subunit of the HeLa primase. In this case, however, it would be expected that some epitopes recognized by these polyclonal antibodies raised against the calf DNA primase would be retained and that some reactivity with these antibodies would have been observed with the HSV primase. No such reactivity was observed. Thus, while we cannot absolutely rule out this second possibility, we believe it to be unlikely on the basis of the above line of reasoning.

A third possible source of the HSV primase could be mitochondrial DNA primase, which is solubilized and becomes fortuitously associated with HSV DNA polymerase during the extraction procedure. This possibility also does not seem likely. The rat liver mitochondrial DNA polymerase has a pH optimum of 9.3, with very little activity below pH 8.5 (23). Further, the human mitochondrial DNA primase has been shown to be bound to RNA, which, when removed, leaves an enzyme that sediments at the same rate as the *E. coli* polymerase I large fragment (37). If the protein is spherical, it would correspond to a molecular size of about 70 kDa. In the light of these considerations, the control DNA primase we have observed may represent a mixture of mitochondrial DNA primase and HeLa DNA primase on the basis of the lack of a pH optimum (Fig. 4B) and a small amount of reactivity with the antibodies raised against the calf DNA primase peptides (data not shown).

The final possibility to be considered is that the HSV DNA primase activity is in fact a virus-coded enzyme. The coupling of the primase with the viral DNA polymerase in fresh extracts, the unique pH profile of its activity, and its lack of cross-reactivity with antibodies against the calf primase all

TABLE 4. Percentage of DNA primase activity remaining in solution after treatment with anti-calf peptide IgGs and after binding of the antibody-antigen complex to protein A-Sepharose^a

Enzyme	% Activity with antibodies against the following calf peptides:		
	68 kDa	55 kDa	48 kDa
Calf thymus DNA polymerase: primase complex	41	74	25
HeLa DNA primase	54	73	31
HSV DNA primase	96	96	118

^a An activity of 100% was that obtained after treatment of enzymes with control IgG and was 34, 13, and 14 pmol of [³H]dAMP incorporation per 5 μ l per 60 min for the calf thymus DNA polymerase:primase complex, the HeLa DNA primase, and HSV DNA primase, respectively, with *E. coli* DNA polymerase I present in the assays.

argue that this may be the case. Challberg (4; personal communication) has recently demonstrated that there are at least seven open reading frames present on the HSV genome whose products are essential for the replication of HSV DNA. The gene products potentially encoded by these open reading frames range in size from 140 to 51 kDa. The two largest open reading frames correspond to the genes for the viral DNA polymerase and the HSV major DNA-binding protein, ICP8. The gene products from the other five open reading frames are all larger than the apparent molecular size of 40 kDa which we have determined for the HSV primase. This would imply that if the HSV primase is encoded by one of these open reading frames, the activity which we detected represents a posttranslationally modified protein. Whether this modification is required for the activity of the HSV primase or represents nonspecific proteolytic degradation of the primase during extraction and partial purification of the enzyme is not known.

Further experiments need to be carried out to purify the HSV DNA primase to homogeneity, to characterize its enzymatic activity in depth, and to demonstrate that the primase can synthesize RNA primers of the size seen in newly replicating HSV DNA, both in the presence and absence of HSV DNA polymerase and deoxynucleoside triphosphates. These last experiments would demonstrate the effects of the DNA polymerase on primer synthesis and the use of such primers by the DNA polymerase. However, such product characterization is meaningless with the HSV DNA primase at its present level of purification because of the presence of other activities, such as RNase H, which affect the size of the product or the ability of the DNA polymerase to use these products. Additional work also needs to be done to definitively show that the DNA primase is part of an HSV DNA polymerase:primase complex and to discover other proteins which may be involved in holding enzymatically active peptides together or in coupling the DNA primase activity with the DNA polymerase activity. These experiments are under way.

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