DNA Helicase and Nucleoside-5′-triphosphatase Activities of Polyoma Virus Large Tumor Antigen†

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ABSTRACT: Polyoma virus large tumor antigen (PyV T antigen) has been purified to near homogeneity by immunoaffinity column chromatography. We have detected DNA helicase and ATPase (nucleoside-5′-triphosphatase) activities in the purified PyV T antigen fraction and characterized these activities. The ATPase activity was stimulated about 2-fold by poly(dT), which was the most effective stimulator among the synthetic polynucleotides tested. Natural nucleic acids, such as calf thymus native and heat-denatured DNA, and single-stranded circular *fd* DNA were also effective, but the degree of stimulation was less than 1.5-fold. The basal and poly(dT)-stimulated ATPase activities showed similar preference for nucleoside 5′-triphosphates, requirement for divalent cations, and pH optima. The preference for nucleoside 5′-triphosphates was ATP, dATP > CTP, UTP ≫ GTP. The only difference observed between the two activities was salt sensitivity. The basal ATPase activity was resistant to KCl up to 300 mM. In contrast, poly(dT)-stimulated activity was reduced to the level of basal activity at 300 mM KCl. DNA helicase activity required divalent cations and was dependent on hydrolysis of ATP. The activity showed similar preference for nucleoside 5′-triphosphates, requirement for divalent cations, and pH optimum as the two ATPase activities, and the salt sensitivity of DNA helicase activity was similar to that of poly(dT)-stimulated ATPase activity. The helicase activity was inhibited competitively by the addition of single-stranded or double-stranded DNA, and a relatively high inhibitory activity was observed with poly[d(A-T)]. The PyV T antigen helicase was found to migrate in the 3′ to 5′ direction along the DNA strand to which the protein bound.

Simian virus 40 (SV40)[1] and polyoma virus (PyV) have been useful models for investigating DNA replication in mammalian cells. The development of a cell-free system capable of replicating SV40 DNA in an origin-dependent manner (Li & Kelly, 1984; Stillman & Gluzman, 1985; Wobbe et al., 1985) has facilitated the analysis of the molecular events involved in DNA replication and enabled the detection and functional characterization of cellular replication proteins (Kelly, 1988).

To understand the molecular mechanism of DNA replication in mammalian cells, we have tried genetic and biochemical approaches using mouse FM3A cells. We have isolated several DNA replication mutants from the mouse cells including the mutant that has temperature-sensitive DNA polymerase α (Murakami et al., 1985). In biochemical approaches, we have purified and characterized DNA replication enzymes and proteins such as two forms of DNA polymerase α (Enomoto et al., 1985), DNA primase (Suzuki et al., 1988), DNA polymerase α stimulating factors (Kawasaki et al., 1984, 1986), DNA helicase B (Seki et al., 1987, 1988), DNA-dependent ATPases C1, C2, and C3 (Tawaragi et al., 1984), DNA topoisomerase II, and RNase H (unpublished data).

To combine these two approaches, we have established a cell-free system that replicates polyoma virus DNA. The system requires extracts prepared from mouse cells, DNA containing the origin of polyoma virus, and PyV T antigen as described (Murakami et al., 1986).

As the initial step of characterization of this system, we tried to characterize biochemical properties of PyV T antigen. PyV T antigen, like SV40 T antigen, has been shown to have several biochemical activities including sequence-specific binding to the PyV origin of replication (Gaudray et al., 1981; Cowie & Kamen, 1984; Scheller & Prives, 1985), nonspecific binding to double-stranded DNA (Gaudray et al., 1980), ATP binding (Clertant & Cuzin, 1982), and ATPase activity (Gaudray et al., 1980). Recently, SV40 T antigen has been shown to possess an intrinsic DNA helicase activity (Stahl et al., 1985, 1986; Dean et al., 1987; Wold et al., 1987; Goetz et al., 1988).

In this study, we have detected DNA helicase activity in PyV T antigen and characterized the helicase activity as well as its DNA-dependent and -independent ATPase activities.

Materials and Methods

Materials. The 21 base long oligonucleotides (21-mer) with the 5′-terminus of OH, 5′-CGACCTGCAG-GCATGCAAGCT-3′, complementary to M13mp19 DNA, was synthesized with a Beckman DNA synthesizer. All chemicals and other DNAs were described previously (Seki et al., 1987, 1988).

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[1] Abbreviations: AMP-PCP, 5′-adenylyl methylenediphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); BSA, bovine serum albumin; CMF-PBS, Ca^{2+}, Mg^{2+}-free phosphate-buffered saline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NP40, Nonidet P-40; NTPase, nucleosidetriphosphatase; PMSF, phenylmethanesulfonyl fluoride; Py, polyoma virus; SDS, sodium dodecyl sulfate; ssc, single-stranded circular; SV40, simian virus 40; T antigen, large tumor antigen; Tris, tris(hydroxymethyl)aminomethane.

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Preparation of DNA Helicase B (DNA-Dependent ATPase B). DNA helicase B (DNA-dependent ATPase B) was purified from mouse FM3A cells to a specific activity of 51 000 units/mg of protein as described previously (Seki et al., 1986). One unit of activity is defined as the amount of enzyme that hydrolyzes 1 nmol of ATP/h at 37 °C.

Preparation of Anti-PyV T Antigen Conjugated Sepharose CL-4B Beads. Monoclonal anti-PyV T antigen antibody (F4) was prepared as follows. F4 hybridoma cells (Scheller & Prives, 1985; Pallas et al., 1986) ($1-2 \times 10^7$ cells) were inoculated into the peritoneal cavity of nude mice to which pristane had been injected more than 2 weeks before. The mice were bred for 2–3 weeks, and ascites fluid was collected. Proteins in the ascites fluid were precipitated with ammonium sulfate at 50% saturation. The pellet was dissolved with 3 mL of buffer (0.1 M $NaHCO_3$, pH 8.3, and 0.5 M NaCl and dialyzed against 1 L of the same buffer. Cyanogen bromide activated Sepharose CL-4B was added to the dialysate, and the mixture stood at room temperature for 2 h with rocking. After being precipitated by centrifugation, beads were suspended in 1 M ethanolamine (pH 8.0) at room temperature for 2 h. The beads were washed three times with buffer containing 0.1 M $NaHCO_3$, pH 8.3, and 0.5 M NaCl and soaked in buffer containing 20 mM Tris-HCl, pH 8.5, 1 mM EDTA, 10% glycerol, 0.5 M NaCl, and 50% ethylene glycol for 30 min. The beads were precipitated by centrifugation and washed four times with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (CMF-PBS). The antibody-conjugated Sepharose CL-4B beads thus prepared adsorbed approximately 10 mg of protein/mL of resin.

Purification of PyV T Antigen. PyV T antigen was purified from *Spodoptera frugiperda* (Sf27) cells infected with a recombinant baculovirus vector vEV51LT (Rice et al., 1987), which expressed PyV T antigen. Cells were infected with vEV51LT (a gift from L. K. Miller, University of Georgia) as described (Miller et al., 1986). At 42 h postinfection, infected cells cultured in 50 150-mm plastic dishes were collected and centrifuged at 1200 rpm for 5 min at 4 °C and then washed twice with ice-cold CMF-PBS. All operations described below were carried out at 0–4 °C. The pellet (approximately 10 mL) was suspended in 60 mL of pH 9.0 buffer [20 mM Tris-HCl, pH 9.0, 0.3 M NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol (v/v), 1% NP40, and 0.25 mM PMSF] and stood at 0 °C for 15 min with stirring every 5 min to extract PyV T antigen. The cell extract was centrifuged at 20000*g* for 40 min, and the supernatant was mixed with a half-volume of pH 6.8 buffer (100 mM Tris-HCl, pH 6.8, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% NP40, and 0.25 mM PMSF) to adjust the pH to 8.0 and then mixed with 30 mL of Sepharose CL-4B equilibrated with loading buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.15 M NaCl, 10% glycerol, 1% NP40, and 0.25 mM PMSF) to remove proteins which bound nonspecifically to the Sepharose. The mixture was rocked for 60 min at 4 °C and then centrifuged. The supernatant was mixed with 4 mL of anti-PyV T antigen antibody-conjugated Sepharose CL-4B equilibrated with loading buffer, rocked for 12 h at 4 °C, and centrifuged. The beads were washed with 40 mL of loading buffer without NP40 and packed into a column. The immunoaffinity column was washed with loading buffer without NP40 and then with buffer B (20 mM Tris-HCl, pH 8.5, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.25 mM PMSF) containing 0.5 M NaCl. PyV T antigen was eluted from the column with buffer B containing 0.5 M NaCl and 50% ethylene glycol, and the protein concentration of each fraction was determined by the method of Bradford (1976). The peak fractions were pooled and dialyzed against 1 L of buffer (10 mM Hepes–KOH, pH 7.5, 5 mM NaCl, 0.1 mM EDTA, 0.25 mM PMSF, 50% glycerol). The dialysate was used as purified PyV T antigen. This fraction (0.8 mL) contained 0.2 mg of protein/mL and on SDS–polyacrylamide gel electrophoresis showed one nearly homogeneous band around 100 kDa, the monomeric size of PyV T antigen (Figure 1). The broad bands of PyV T antigen and the molecular weight standards may be due to the overloading of the samples. The specific activities of the purified PyV T antigen as ATPase in the presence or absence of poly(dT) were 14 000 and 7000 units/mg of protein, respectively. One unit of activity is defined as the amount of enzyme that hydrolyzes 1 nmol of ATP/h at 37 °C. This fraction was stored at −20 °C after the addition of BSA at a final concentration of 0.5 mg/mL. The ATPase and DNA helicase activities of this fraction were stable at least for 3 months under these conditions.

Preparation of Helicase Substrates. DNA concentrations are expressed in molarity of molecules. To prepare labeled 21-mer, the 21-mer was 5′ end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The short duplex (21 base pairs) was prepared by annealing 20 pmol of the labeled 21-mer to 30 pmol of single-stranded circular (ssc) M13mp19 DNA as described previously (Seki et al., 1988). The substrate thus prepared contains an excess single-stranded region. Helicase substrates used to determine the direction of translocation were also prepared as described (Seki et al., 1988).

DNA Helicase Assay. The standard reaction mixture (20 μL) consisted of 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mg/mL BSA, and 0.017 pmol (5 μM) of ^{32}P-labeled helicase substrate. The reaction was started by the addition of purified PyV T antigen and terminated by chilling the reaction mixture to 0 °C followed by the addition of 5 μL of the solution containing 75 mM Na_3EDTA, 5% (v/v) Sarkosyl, 0.1% bromophenol blue, and 30% (v/v) glycerol. After being kept at 0 °C for 20 min, a 25-μL aliquot was loaded on a 12% polyacrylamide gel in TBE buffer (89 mM Tris–borate, pH 8.2, and 2 mM EDTA) and subjected to electrophoresis. Autoradiography was performed at −80 °C. Helicase activity was determined on the basis of densitometric tracings of autoradiograms. The activity was normalized by the formula $X = P/(P + S)$, where P (products) is the value for displaced oligonucleotides and S (substrates) is the value for nondisplaced substrates. The helicase activity is expressed as a percentage of control value, i.e., $100[(X_{sample} - X_n)/(X_p - X_n)]$, where X_n is the negative control assayed at 37 °C without enzyme and X_p is the positive control where the reaction mixture containing no enzyme was heated in boiling water for 2 min.

ATPase Assay. The standard reaction mixture (50 μL) consisted of 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 mM ATP, and 0.5 mg/mL BSA. The reaction was started by the addition of T antigen and terminated by chilling the reaction mixture to 0 °C. The amount of produced ADP was determined by the method of Korn and Yanofsky (1976).

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out essentially according to the procedure of Laemmli (1970). After electrophoresis, the gel was stained with silver as described by Oakley et al. (1980).

Results

Characterization of Nucleoside-5′-triphosphatase (ATPase) Activity of PyV T Antigen. PyV T antigen has been purified

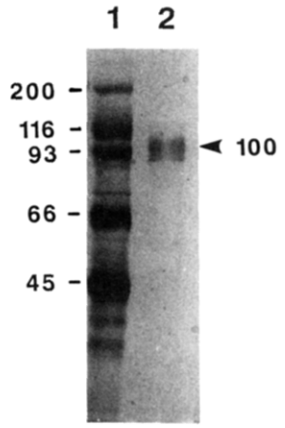

FIGURE 1: SDS–polyacrylamide gel electrophoresis of immunopurified PyV T antigen. (Lane 1) Molecular weight markers (myosin, 200000; β-galactosidase, 116000; phosphorylase B, 93000; bovine serum albumin, 66000; ovalbumin, 45000). (Lane 2) Immunoaffinity-purified PyV T antigen (1 μg) was electrophoresed on a 10% SDS–polyacrylamide gel according to the method by Laemmli (1970). The protein bands in the gel were visualized by the silver staining procedure of Oakley et al. (1980).

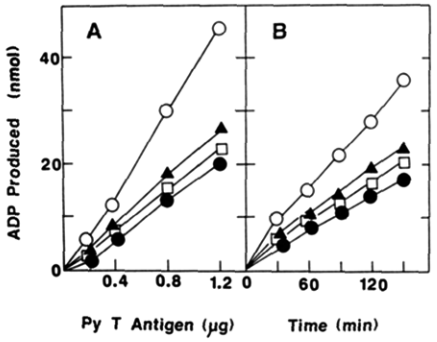

FIGURE 2: ATPase activity of PyV T antigen. (A) ATPase activity as a function of the amount of purified PyV T antigen. Increasing amounts of PyV T antigen were incubated at 37 °C for 120 min under the standard conditions in the presence of 30 μM poly(dT) (○), ssc *fd* DNA (▲), or heat-denatured calf thymus DNA (□) or in the absence of DNA (●). (B) Time course of ATPase activity of PyV T antigen. PyV T antigen (1 μg) was incubated at 37 °C for various times under the standard conditions in the presence of 30 μM of poly(dT) (○), ssc *fd* DNA (▲), or heat-denatured calf thymus DNA (□) or in the absence of DNA (●).

from the extracts of Sf27 cells infected with a recombinant baculovirus that expresses PyV T antigen by immunoaffinity chromatography. The purified T antigen was almost free from contaminating proteins as judged by silver staining of polyacrylamide gels after electrophoresis in the presence of SDS (Figure 1). The purified T antigen supported the replication of DNA containing the polyoma virus replication origin in the previously reported cell-free system (Murakami et al., 1986) (data not shown).

Because it was reported that PyV T antigen had an intrinsic ATPase activity (Gaudray et al., 1980), we first characterized the ATPase activity of PyV T antigen. As shown in Figure 2, the hydrolysis of ATP was linear with the amount of T antigen up to 1.2 μg and with time up to 150 min in the presence or absence of DNA. The ATPase activity was stimulated about 2-fold by poly(dT) and slightly by ssc *fd* DNA or heat-denatured calf thymus DNA. The K_m and V_{max} of PyV T antigen for ATP were 0.64 mM and 240 pmol min^{-1} μg^{-1}, respectively, in the presence of poly(dT) and 1.0 mM and 130 pmol min^{-1} μg^{-1}, respectively, in its absence.

Figure 3 shows the ATPase activity of PyV T antigen in the presence of various concentrations of homopolymers (A) and nucleic acids (B). The most effective stimulator among

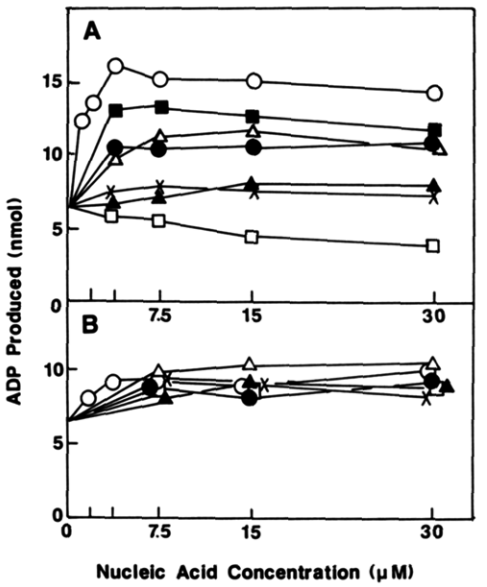

FIGURE 3: Effect of various nucleic acids on ATPase activity of PyV T antigen. (A) PyV T antigen (0.5 μg) was incubated for 120 min under the standard conditions in the presence of various concentrations of poly(dT) (○), poly(rU) (■), poly[d(A-T)] (●), poly(dC) (△), oligo$(dT)_{12-18}$ (▲), poly(dA) (×), or poly(dG) (□). (B) PyV T antigen (0.5 μg) was incubated for 120 min under the standard conditions in the presence of various concentrations of native calf thymus DNA (△), ssc *fd* DNA (○), activated calf thymus DNA (●), yeast tRNA (▲), pBR322 RFI DNA (□), or heat-denatured calf thymus DNA (×).

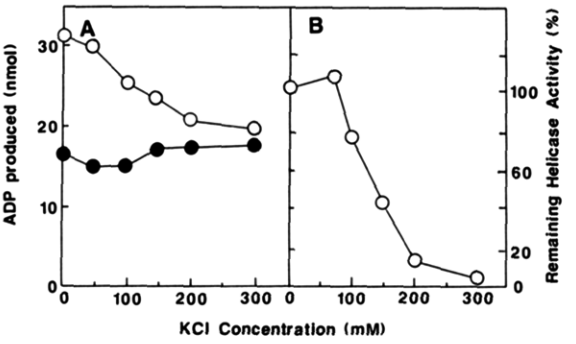

FIGURE 4: Effect of KCl on DNA helicase and ATPase activities of PyV T antigen. (A) ATPase activity. Reactions were carried out with 1 μg of PyV T antigen for 120 min at 37 °C in the presence of 30 μM poly(dT) (○) or in the absence of poly(dT) (●). (B) DNA helicase activity. Helicase reactions were performed with 0.3 μg of PyV T antigen at 37 °C for 30 min as described under Materials and Methods. The 100% value corresponds to 40% oligonucleotides released per 30 min.

the polynucleotides tested was poly(dT). The relative efficiency of the synthetic polymers for stimulation was poly(dT) > poly(rU) > poly[d(A-T)] > poly(dC). Essentially no stimulation was observed with poly(dA) or oligo$(dT)_{12-18}$. A slight inhibition was observed with poly(dG). All of the natural nucleic acids tested were slightly effective, but the degree of stimulation was less than 1.5-fold.

The basal and poly(dT)-stimulated ATPase activities showed similar requirement for divalent cations. In the presence of 5 mM ATP, the optimal concentrations of $MgCl_2$ and $MnCl_2$ were 2.5–10 and 2.5–5 mM, respectively. The maximum activity obtained with $MgCl_2$ was almost the same as that with $MnCl_2$. Both ATPase activities had a broad pH optimum from pH 6.5 to 8.0.

Figure 4A shows the effects of KCl concentration on the basal and poly(dT)-stimulated ATPase activities. The basal activity was not inhibited by KCl at concentrations up to 300

Table I: Nucleotide Specificity for Nucleoside-5′-triphosphatase Activity and DNA Helicase Activity of PyV T Antigen[a]

NTPase (%)				helicase (%)			
ATP	100[b] (100)[b]	dATP	68 (72)	ATP	100[b]	dATP	91
CTP	32 (33)			CTP	25	dCTP	33
GTP	7 (13)			GTP	0	dGTP	7
UTP	56 (69)			UTP	38	dTTP	21

[a] NTPase activity was assayed with 1 μg of T antigen in the absence of DNA or in the presence of 30 μM poly(dT) (in parentheses). Reactions were carried out at 37 °C for 120 min in the presence of a 5 mM sample of the indicated nucleotide. DNA helicase activity was assayed with 0.4 μg of T antigen in the presence of a 5 mM sample of the indicated nucleotide. Reactions were carried out at 37 °C for 30 min. [b] The values indicate the percentage of activity in the presence of ATP as 100%. The 100% values correspond to 16 nmol of ADP produced/120 min, 32 nmol of ADP produced/120 min, and 45% of annealed oligonucleotides released/30 min for NTPase activity, NTPase activity in the presence of poly(dT), and DNA helicase activity, respectively.

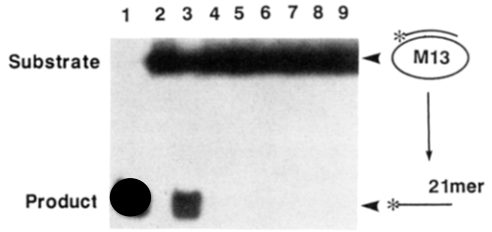

FIGURE 5: Detection of DNA helicase activity of PyV T antigen. The helicase substrate was incubated for 30 min in the standard reaction mixture with or without 0.4 μg of immunopurified PyV T antigen. Electrophoresis and autoradiography were performed as described under Materials and Methods. Assay conditions are as follows: (lane 1) 100 °C for 2 min without T antigen; (lane 2) 37 °C without T antigen; (lane 3) 37 °C with T antigen; (lane 4) 37 °C with heat-inactivated T antigen (at 100 °C for 2 min); (lane 5) 0 °C with T antigen; (lane 6) 37 °C with T antigen in the absence of ATP; (lane 7) 37 °C with T antigen in the absence of $MgCl_2$; (lanes 8 and 9) same as lane 3 except that ATP was replaced by ADP and ATPγS, respectively.

mM, while in the case of poly(dT)-stimulated ATPase activity, inhibition was observed at 100 mM, and the activity was reduced close to the level of basal activity at 300 mM.

Table I shows the preference of the basal and poly(dT)-stimulated activities for nucleoside 5′-triphosphates. ATP was the most preferred substrate among the ribo- and deoxyribonucleoside 5′-triphosphates tested. The relative values of hydrolysis of dATP, UTP, CTP, and GTP were 68, 56, 32, and 7% of the value of ATP, respectively. Poly(dT)-stimulated activity showed preference for nucleoside 5′-triphosphates similar to that of the basal activity.

Detection of DNA Helicase Activity of PyV T Antigen. Single-stranded circular M13mp19 DNA, carrying an annealed ^{32}P-labeled 21-mer, was used as a substrate for DNA helicase. PyV T antigen displaced the 21-mer when the reaction mixture was incubated at 37 °C in the presence of ATP (Figure 5, lane 3), but was ineffective when ATP was omitted (lane 6). No helicase activity was observed at 0 °C (lane 5), in the absence of Mg^{2+} (lane 7), or with the T antigen that had been heated in boiling water for 2 min (lane 4).

The amount of 21-mer released in the helicase assay was dependent on the amount of T antigen added to the reaction mixture (Figure 6A). With 1.2 μg of T antigen, about 75% of all hydrogen-bonded 21-mer was released from M13mp19 DNA after an incubation for 30 min. The amount of released 21-mer increased almost linearly for up to 20 min and then gradually up to 80 min (Figure 6B).

Characterization of Helicase Activity of PyV T Antigen. The DNA helicase activity of PyV T antigen required a divalent cation. The optimal concentrations of $MgCl_2$ and $MnCl_2$ were 2.5–10 and 2.5 mM, respectively. The requirement of divalent cations for the helicase activity was similar to that for the basal and poly(dT)-stimulated ATPase activities of T antigen. The optimal pH was pH 7.5, and the helicase activity was effectively supported from pH 6.5 to 8.5.

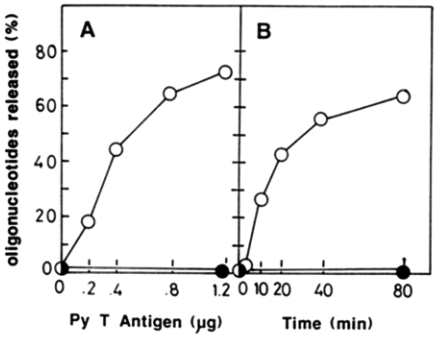

FIGURE 6: DNA helicase activity of PyV T antigen. (A) DNA helicase activity as a function of the amount of PyV T antigen. Increasing amounts of PyV T antigen was incubated at 37 °C for 30 min in the presence of 5 mM ATP (○) or in the absence of ATP (●). (B) Time course of DNA helicase activity of PyV T antigen. PyV T antigen (0.4 μg) was incubated at 37 °C for various times in the presence of 5 mM ATP (○) or in the absence of ATP (●).

The helicase activity was relatively sensitive to ionic strength. In the presence of 100, 150, and 300 mM KCl, the displacement activity was inhibited 23, 56, and 94%, respectively (Figure 4B).

ADP and AMP were not effective for the helicase activity. The nonhydrolyzable ATP analogues AMP-PCP, AMP-PNP, and ATPγS were not capable of serving as cofactor for the helicase activity, suggesting that hydrolysis of nucleoside 5′-triphosphates is essential for T antigen to exhibit DNA helicase activity. ATP was the most effective cofactor among ribo- and deoxyribonucleoside 5′-triphosphates tested. The efficiency of a nucleoside 5′-triphosphate to serve as cofactor for the helicase activity correlated with the capacity of the nucleotide to serve as substrate for the basal and poly(dT)-stimulated ATPase activities (Table I).

Inhibition of Helicase Activity of PyV T Antigen by Coaddition of Nucleic Acids. Previous studies have shown that PyV T antigen binds nonspecifically to double-stranded DNA (Gaudray et al., 1980) and specifically to the origin of replication in PyV DNA (Scheller & Prives, 1985). Thus, the binding of T antigen to substrate DNA may be antagonized by various DNAs.

As shown in Figure 7, the apparent helicase activity was inhibited by the addition of DNA. Single-stranded circular *fd* DNA (5 μM), which is equivalent to M13mp19 DNA, a constituent of the helicase substrate, inhibited helicase activity by 50% in the presence of 5 μM helicase substrate. The order of potency of added DNAs to inhibit helicase activity was ssc *fd* DNA > poly[d(A-T)] > activated calf thymus DNA > heat-denatured calf thymus DNA > poly(dT) > native calf thymus DNA. Only a slight inhibition was observed with poly(dC) and poly(rU). Essentially no inhibition was caused by poly(dA) and oligo$(dT)_{12-18}$.

Determination of the Direction of Translocation of PyV T Antigen. To determine the direction of translocation of PyV T antigen, a pair of linearized helicase substrates (substrates A′ and B′) were prepared by digesting substrates A and B, respectively, with *Sma*I endonuclease (Figure 8A). If T antigen initially binds to the single-stranded region of these DNA molecules and then moves unidirectionally to the duplex region, translocation in a 3′ to 5′ direction along the single-

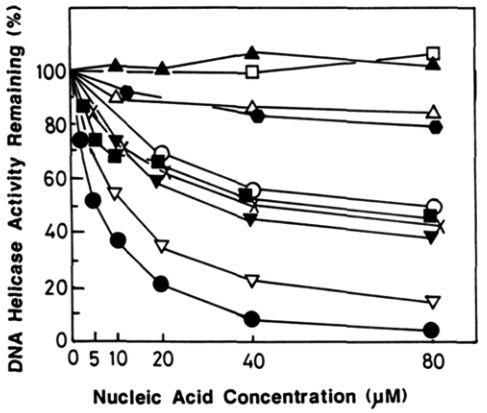

FIGURE 7: Inhibition of PyV T antigen helicase activity by the addition of various nucleic acids. The effect of various nucleic acids on the DNA helicase activity was examined with 0.6 μg of PyV T antigen. Helicase reactions were carried out for 30 min at 37 °C in the presence of various concentrations of nucleic acids. The values were determined by densitometric tracing of the autoradiograms and normalized as described under Materials and Methods. The values indicate the percentage of the activity in the absence of nucleic acids as 100%, which corresponds to 58% oligonucleotides released without addition of nucleic acids. (□) Oligo(dT)$_{12-18}$; (▲) poly(dA); (△) poly(rU); (●) poly(dC); (○) native calf thymus DNA; (■) poly(dT); (×) heat-denatured calf thymus DNA (▼) activated calf thymus DNA; (▽) poly[d(A-T)]; or (●) ssc *fd* DNA.

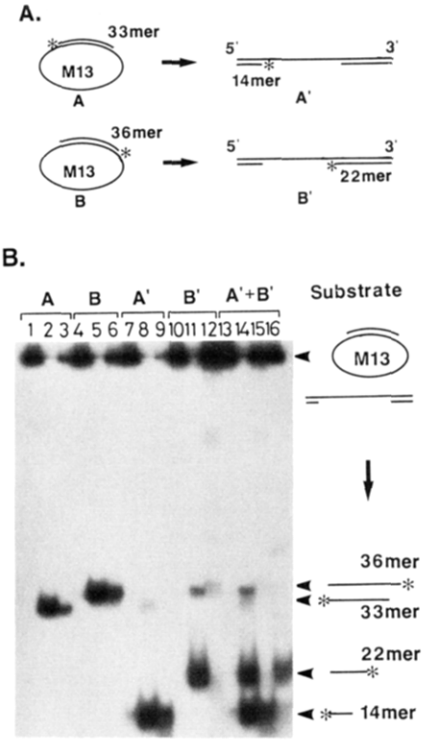

FIGURE 8: (A) DNA helicase substrate designed to determine the direction of translocation of DNA helicase. The 5′-labeled helicase substrate (substrate A) and the 3′-labeled helicase substrate (substrate B) were digested with *Sma*I endonuclease as described previously (Seki et al., 1988) to construct the linear substrates (A′ and B′). These substrates were used in the experiments shown in part B. (B) Determination of the direction of translocation of PyV T antigen helicase. Substrate A, B, A′, or B′ (0.017 pmol), or the mixture of A′ and B′ (0.017 pmol of each), shown on the top, was incubated with or without 0.8 μg of PyV T antigen for 60 min. (Lanes 1, 4, 7, 10, and 13) Incubated at 33 °C without T antigen; (lanes 2, 5, 8, 11, and 14) heated at 100 °C for 2 min without T antigen; (lanes 3, 6, 9, 12, and 15) incubated at 33 °C with T antigen; (lane 16) incubated at 33 °C with 28 units of DNA-dependent ATPase B (DNA helicase B).

stranded DNA segment should cause the displacement of the labeled 14-mer, while translocation in a 5′ to 3′ direction should cause the displacement of the labeled 22-mer.

As shown in Figure 8B, the T antigen helicase displaced the radioactive 14-mer from substrate A′ (lanes 9 and 15), but did not displace the 22-mer from substrate B′ (lanes 12 and 15), indicating that the helicase translocates unidirectionally in the 3′ to 5′ direction.

In contrast, the mouse DNA helicase B, with which we had shown translocation in a 5′ to 3′ direction (Seki et al., 1988), displaced the labeled 22-mer (lane 16).

Discussion

PyV T antigen is a multifunctional protein (Tooze, 1981). Previous studies have shown that the T antigen has an intrinsic ATPase activity, and the activity is essential for the replication of PyV DNA (Gaudray et al., 1980). However, the biochemical function of the ATPase activity has remained obscure. An analogous protein of SV40, SV40 T antigen has been shown to have DNA helicase activity for which the hydrolysis of ATP is essential (Stahl et al., 1986; Goetz et al., 1988). In this study, we have detected DNA helicase activity in the immunoaffinity-purified PyV T antigen and characterized the helicase activity as well as its ATPase activity, and several important properties of this protein have been found as follows.

First, the hydrolysis of ATP is a prerequisite for the helicase to exhibit its activity (Figure 5). The nucleoside-5′-triphosphatase activity of PyV T antigen showed almost identical specificity for the nucleoside 5′-triphosphates that support helicase reaction (Table I). ATP was the most effective nucleotide for the helicase activity, while other nucleoside 5′-triphosphates, except for GTP and dGTP, supported the helicase activity considerably. The nucleotides GTP and dGTP were virtually inactive. In this context, it is interesting that the helicase activity of SV40 T antigen is also inactive with GTP, dGTP, and CTP (Goetz et al., 1988).

Second, in contrast to the observation that the ATPase activities of the DNA helicases so far reported are highly stimulated by or completely dependent on DNA, PyV T antigen exhibited a relatively high level of ATPase activity in the absence of DNA (Figures 2 and 3). This fact is consistent with the report by Gaudray et al. (1980) that PyV T antigen was purified as a DNA-independent ATPase. The ATPase activity of PyV T antigen was stimulated about 2-fold by poly(dT) and slightly by single-stranded circular *fd* DNA. Among the various DNAs and synthetic polynucleotides tested, poly(dT) was the best stimulator (Figure 3). In the case of SV40 T antigen, poly(dT) is also the best stimulator, although the degree of stimulation of the activity of SV40 T antigen is much greater than that of PyV T antigen (Giacherio & Hager, 1979). The basal and poly(dT)-stimulated ATPase activities showed similar specificities for nucleoside 5′-triphosphates, requirement for divalent cations, and pH optima. The only difference observed between the two activities was the sensitivity to salt concentration. The basal activity was resistant to KCl up to 300 mM. In contrast, poly(dT)-stimulated ATPase activity reduced to the level of basal activity at 300 mM KCl (Figure 4A). This sensitivity may be explained by the inhibition of binding of T antigen to poly(dT). The same explanation may be also applied to the sensitivity of the helicase activity to KCl, which is very similar to that of poly(dT)-stimulated ATPase activity (Figure 4B). At present, it is not clear what biochemical function is related to the basal ATPase activity. It is reported that SV40 T antigen exists as monomeric, dimeric, and multimeric forms. Thus, it can be speculated from the similarity in the properties between PyV T antigen and SV40 T antigen that the basal

ATPase activity is related to the interconversion between these forms (Mastrangelo et al., 1989).

Third, PyV T antigen binds nonspecifically to single-stranded and double-stranded DNAs. The experimental support for this conclusion is found in Figure 7, which shows that the apparent helicase activity is inhibited by the addition of single-stranded and double-stranded DNAs because of the competition of added DNA with the helicase substrate for helicase binding. It must be noted that the difference in binding affinity does not simply correlate with the difference in ability to stimulate the ATPase activity of PyV T antigen (compare Figure 3 with Figure 7).

Fourth, PyV T antigen helicase translocates unidirectionally in the 3′ to 5′ direction along the DNA to which the enzyme binds (Figure 8). This is the opposite direction exhibited by the mouse DNA helicase B (Seki et al., 1988) and the same direction exhibited by SV40 T antigen (Goetz et al., 1988; Wiekowski et al., 1988), *Escherichia coli* rep protein (Yarranton & Gefter, 1979), *uvrD* protein (Matson, 1986), and *E. coli* 75-kDa helicase (Wood & Matson, 1987). However, the latter helicases, except for SV40 T antigen, have not been shown to be the primary helicase involved in DNA replication. On the contrary, prokaryotic DNA helicases involved in DNA replication such as the T7 gene 4 protein (Tabor & Richardson, 1983), the T4 gene 41 protein (Venkatesan et al., 1982), and the *E. coli dnaB* protein (LeBowitz & McMacken, 1986) translocate in the 5′ to 3′ direction. These prokaryotic helicases exhibit DNA primase activity or interact with DNA primase.

Although the direction of translocation is opposite for T antigen and prokaryotic DNA helicases involved in DNA replication, T antigen may interact with primase because an interaction between SV40 T antigen and DNA polymerase α, which usually exists as a complex with DNA primase, has been detected (Smale & Tjian, 1986). In addition, it has been observed that the host-cell source of DNA polymerase α–primase complex plays an important role in discriminating between SV40 T antigen dependent and PyV T antigen dependent replication of their homologous DNA (Murakami et al., 1986). Our preliminary results with cell-free PyV DNA replication system have indicated that DNA primase rather than DNA polymerase α plays the major role in the discrimination. Further studies with this cell-free system should clarify this issue.

Registry No. ATPase, 9000-83-3.

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Alanine Dehydrogenases from Two *Bacillus* Species with Distinct Thermostabilities: Molecular Cloning, DNA and Protein Sequence Determination, and Structural Comparison with Other NAD(P)$^+$-Dependent Dehydrogenases†

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ABSTRACT: The gene encoding alanine dehydrogenase (EC 1.4.1.1) from a mesophile, *Bacillus sphaericus*, was cloned, and its complete DNA sequence was determined. In addition, the same gene from a moderate thermophile, *B. stearothermophilus*, was analyzed in a similar manner. Large parts of the two translated amino acid sequences were confirmed by automated Edman degradation of tryptic peptide fragments. Each alanine dehydrogenase gene consists of a 1116-bp open reading frame and encodes 372 amino acid residues corresponding to the subunit (M_r = 39 500–40 000) of the hexameric enzyme. The similarity of amino acid sequence between the two alanine dehydrogenases with distinct thermostabilities is very high (>70%). The nonidentical residues are clustered in a few regions with relatively short length, which may correlate with the difference in thermal stability of the enzymes. Homology search of the primary structures of both alanine dehydrogenases with those of other pyridine nucleotide-dependent oxidoreductases revealed significant sequence similarity in the regions containing the coenzyme binding domain. Interestingly, several catalytically important residues in lactate and malate dehydrogenases are conserved in the primary structure of alanine dehydrogenases at matched positions with similar mutual distances.

Alanine dehydrogenase (L-alanine:NAD$^+$ oxidoreductase, deaminating, EC 1.4.1.1) catalyzes the reversible deamination of L-alanine to pyruvate and is found in vegetative cells (Hong et al., 1959; Zink & Sanwal, 1962; McCowen & Phibbs, 1974) and spores (O'Conner & Halvorson, 1961; Nitta et al., 1974) of various bacilli and in some other bacteria (Germano & Anderson, 1968; Holmes et al., 1965; Ohshima & Soda, 1979). This enzyme is a key factor in the assimilation of L-alanine as an energy source through the tricarboxylic acid cycle during sporulation (McCowen & Phibbs, 1974). Alanine dehydrogenase has been purified to homogeneity from *Bacillus subtilis*, *B. sphaericus*, and *B. cereus*, and its enzymological properties have been elucidated (Yoshida & Freese, 1965; Ohshima & Soda, 1979; Porumb et al., 1987). The kinetic and chemical mechanisms of the enzyme reaction have also been extensively studied (Grimshaw & Cleland, 1981; Grimshaw et al., 1981). It has been shown that the enzyme has different mechanisms for coenzyme binding (A stereospecificity) and substrate binding from those of glutamate dehydrogenase.

To date, a large number of NAD(P)$^+$-dependent dehydrogenases have been sequenced, and several of these have been structurally analyzed in atomic detail. For the dehydrogenases that act on amino acids, however, structural data are available only for primary structures of B-stereospecific dehydrogenases, such as glutamate (Smith et al., 1975), leucine (Nagata et al., 1988), and phenylalanine (Okazaki et al., 1988) dehydrogenases. In this work, we report the cloning and sequencing of the alanine dehydrogenase gene from a mesophile, *B. sphaericus*, and also the complete sequence of the thermostable alanine dehydrogenase from a moderate thermophile, *B. stearothermophilus*, the gene for which has been cloned recently in this laboratory (Nagata et al., unpublished results). The difference in thermostability between the two alanine dehydrogenases is discussed on the basis of their primary structures. Sequence comparison with other NAD(P)$^+$-dependent dehydrogenases has suggested possible locations of the coenzyme binding region and several catalytically important residues in the primary structure of alanine dehydrogenases.

Experimental Procedures

Strains and Media. *Escherichia coli* C600 $r_k^-m_k^-$ *thi thr leu* was used as a host strain for cloning and plasmid construction. A mesophile, *B. sphaericus* IFO3525, was used as the source for chromosomal DNA. Transformants were grown

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