

Ligation of Double-Stranded and Single-Stranded [Oligo(dT)] DNA by Vaccinia Virus DNA Ligase

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Vaccinia virus DNA ligase has been expressed in *Escherichia coli*, purified, and biochemically characterized. The enzyme ligates double-stranded (ds) DNA substrates with either cohesive or blunt-end termini and the latter reaction is stimulated by PEG. Vaccinia virus DNA ligase can also ligate oligo(dT) when annealed to either a poly(dA) or a poly(rA) backbone and, remarkably, free oligo(dT). This ligation of a single-stranded (ss) substrate is unique among eukaryotic DNA ligases. The enzyme requires high ATP concentrations with a K_m for the overall ligation of a ssDNA substrate of 0.8 mM. The salt, divalent cation, temperature, and pH requirements of the enzyme for the optimal ligation of ss and ds substrate are described.

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INTRODUCTION

DNA ligase enzymes join a 5' phosphoryl group on one nucleotide to a 3' hydroxyl on an adjacent nucleotide in substrates that are generated by DNA replication, repair, or recombination. *Drosophila* expresses two distinct DNA ligases, while four DNA ligases have been identified in mammalian cells (Tomkinson *et al.*, 1991a; Lindahl and Barnes, 1992; Wei *et al.*, 1995). The precise roles for these enzymes are not well defined; however, biochemical characterization of purified mammalian DNA ligases I, II, and III allows them to be distinguished.

DNA ligase I enzymes show immunological cross-reactivity through a conserved 16-amino-acid C-terminal motif (Barnes *et al.*, 1990) and are biochemically distinguished from DNA ligase II by their inability to ligate a synthetic oligo(dT):poly(rA) substrate, ability to ligate blunt-ended DNA substrates in a reaction stimulated by PEG, and by lower K_m for ATP (Arrand *et al.*, 1986; Lindahl and Barnes, 1992). In mammalian cells DNA ligase I functions in DNA replication. In HeLa cells the enzyme purifies with the 21S enzyme complex which can replicate simian virus 40 DNA in the presence of large T antigen (Li *et al.*, 1994). The mutant human cell line 46BR expresses a DNA ligase I with only 3–5% of wild-type ligase I activity and initially displays a normal rate of ligation of Okazaki fragments. Later, however, the sealing of these fragments is delayed. Extracts from this cell line also show abnormal base excision repair, with increased re-

pair patch size and delayed sealing (Prigent *et al.*, 1994). In *Schizosaccharomyces pombe* a DNA ligase I mutant (*cdc17*) is defective in DNA replication and repair and displays abnormal mitotic recombination (Nasmyth, 1979; Sipiczki *et al.*, 1990).

DNA ligase II enzymes were thought not to contain the C-terminal motif identified in DNA ligase I enzymes (Barnes *et al.*, 1990; Tomkinson *et al.*, 1991a), but recently highly purified calf thymus DNA ligase II was recognized by an antiserum raised against this motif (Wang *et al.*, 1994). Moreover, recent sequence data have shown that features of the motif are conserved in mammalian DNA ligases I, II, III, and IV (Wang *et al.*, 1994; Wei *et al.*, 1995). Little is known about the function of DNA ligase II *in vivo*. It is the predominant enzyme isolated from mammalian liver cells (Soderhall and Lindahl, 1976) and its expression is increased during meiotic prophase in mouse testes (Higashitani *et al.*, 1990). Meiotic recombination is unaltered in the yeast DNA ligase I mutant (*cdc17*) (Sipiczki *et al.*, 1990) and may use DNA ligase II, although this enzyme is not well characterized in yeasts (Lindahl and Barnes, 1992).

DNA ligase III is distinguished by having a low K_m for ATP (1–2 μ M) and by being able to ligate oligo(dT) annealed to poly(rA) (Tomkinson *et al.*, 1991a) but is unable to ligate substrates with blunt termini (Elder *et al.*, 1992). The Chinese hamster ovary (CHO) DNA ligase III can interact with the human XRCC1 protein, which complements the base excision repair defect of the CHO mutant cell line EM9 and raises the DNA ligase III expression in these cells to normal levels (Caldecott *et al.*, 1994). DNA ligase III is also thought to be the ligase activity which purifies with RC-1, a high-molecular-weight

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complex from calf thymus, which is capable of recombinational repair of double-stranded (ds) gaps and deletions (Jessberger *et al.*, 1993). Transcription of DNA ligase III is highest in spermatocytes, which suggests that the enzyme may be involved in meiotic recombination (Chen *et al.*, 1995; Husain *et al.*, 1995).

Recently a DNA ligase IV has been described, with an electrophoretic mobility of 100 kDa, similar to DNA ligase III (Wei *et al.*, 1995). Both DNA ligase III and DNA ligase IV possess the C-terminal conserved motif and unusually have extended C-terminal regions when aligned by active site (Wei *et al.*, 1995). Sequence comparisons have shown that DNA ligases I and IV are products of different genes while DNA ligases II and III derive from the same gene (Chen *et al.*, 1995), possibly by alternative splicing.

VV is an orthopoxvirus with a 192-kb dsDNA genome. This virus replicates in the cytoplasm of mammalian cells and expresses many enzymes required for viral transcription and DNA replication (Moss, 1992). The VV open reading frame A50R encodes a 63-kDa DNA ligase which is expressed early during infection (Kerr and Smith, 1989; Smith *et al.*, 1989). The enzyme shares 30% amino acid identity with the DNA ligase I enzymes from *Saccharomyces cerevisiae* (Barker *et al.*, 1985), *S. pombe* (Barker *et al.*, 1987), and man (Barnes *et al.*, 1990). A much higher identity (65%) was shown with the partial protein sequence of DNA ligase II (Wang *et al.*, 1994) and with the complete sequence of DNA ligase III (50%) (Chen *et al.*, 1995). The VV gene complemented the *S. cerevisiae cdc9* DNA ligase I mutant, suggesting that the enzyme can join Okazaki fragments in yeast (Kerr *et al.*, 1991). This did not, however, establish a similar role in VV DNA replication. The VV linear, dsDNA genome has incompletely base-paired terminal hairpins, making the genome a covalently closed circle. Shortly after infection a nick was reported to be introduced near one of the terminal hairpins (Pogo, 1977) so that, theoretically, VV DNA replication can occur by a unidirectional leading strand synthesis (Moyer and Graves, 1981; Traktman, 1990). This would require only a single ligation event for each progeny genome and this might be carried out by the VV nicking-joining enzyme (Reddy and Bauer, 1989). The VV DNA ligase is nonessential for viral replication and recombination in cell lines (Colinas *et al.*, 1990; Kerr and Smith, 1991) although a DNA ligase deletion mutant is more sensitive to DNA damage and is attenuated *in vivo* (Kerr *et al.*, 1991). It is possible that virus replication and recombination might be complemented by one or more cellular DNA ligases. However, no recruitment of mammalian DNA ligases to virus cytoplasmic factories in cells infected with the VV DNA ligase deletion mutant was detected, using an antiserum raised against the conserved C-terminal motif (Kerr *et al.*, 1991).

Since the biochemical profiles of the three purified mammalian DNA ligase enzymes are distinct, an analysis

of the properties of the VV enzyme might suggest to which cellular ligase the VV enzyme is biochemically most related and this in turn might suggest a possible function(s) of this ligase. To this end we have purified and biochemically characterized the VV DNA ligase expressed in *Escherichia coli*. A similar study of VV DNA ligase was recently published and the biochemical parameters reported are in close agreement with our findings (Shuman and Ru, 1995). The data presented show that the VV DNA ligase has properties characteristic of both DNA ligase I and DNA ligase II enzymes and, remarkably, can efficiently ligate a single-stranded (ss) DNA substrate, oligo(dT).

MATERIALS AND METHODS

Expression of the VV DNA ligase in *E. coli*

Restriction endonuclease digestion, DNA ligations, and plasmid DNA preparations were performed using standard procedures (Sambrook *et al.*, 1989). The VV DNA ligase gene with an *Nde*I site immediately upstream of the ATG codon was excised from pSK17 (Kerr *et al.*, 1991) by digestion with *Nde*I and *Eco*RI and cloned into pGMT7 (Hughes *et al.*, 1991) under the control of the bacteriophage T7 RNA polymerase promoter (Rosenberg *et al.*, 1987). This plasmid, pSK18, was transformed into *E. coli* BL21 lys S cells and expression of the VV DNA ligase was induced by IPTG. Optimal expression of soluble DNA ligase was obtained by induction of mid to late log phase *E. coli* cells in LB medium at 32° for 3 hr with 100 μ M IPTG.

Purification of VV DNA ligase from *E. coli*

Cells containing pSK18 were induced as described above, collected by centrifugation, and resuspended in TGED [50 mM Tris-HCl, pH 7.5, 10% glycerol (v/v), 2 mM EDTA, and 2 mM DTT] supplemented with 2 mM PMSF before being disrupted by sonication. The sonicate was centrifuged for 45 min at 48,000 rpm and 4° in a Beckman Ti70.1 rotor, to pellet cell debris, sheared DNA, and ribosomes. The supernatant was again supplemented with 2 mM PMSF and then subjected to a three-column purification procedure. First, the sample was applied in 0.3 M NaCl TGED to a heparin-agarose (Affinity Chromatography Limited) column equilibrated in the same buffer at 4°. Under these conditions the VV DNA ligase bound to the column and remained bound after extensive washing (at least 10 column volumes) in 0.5 M NaCl TGED. The VV DNA ligase was then eluted in a single step with 1.5 M NaCl. The eluate was concentrated, desalted to 0.3 M, and then applied to a Superdex 75 gel filtration column (Pharmacia) at 15° that had been preequilibrated with 2 volumes of 0.3 M NaCl TGED. The pooled peak fractions containing DNA ligase from this column were concen-

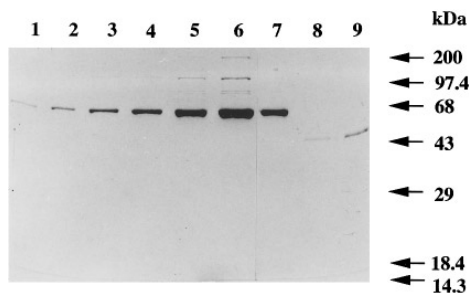


FIG. 1. Polypeptide profile of the purified vaccinia virus DNA ligase. Vaccinia virus DNA ligase, T4 DNA, and RNA ligase were resolved by SDS-PAGE (10%) and detected by staining with Coomassie blue. Lanes 1–6 show 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 μ l of vaccinia virus DNA ligase, respectively; lane 7 shows 4 units of T4 DNA ligase; lanes 8 and 9 show 0.1 and 0.2 μ g of T4 RNA ligase, respectively.

trated and desalted to less than 0.1 M NaCl and then applied to an FPLC Mono S column (Pharmacia) at room temperature that had been preequilibrated with TGED. The VV DNA ligase bound to the column and was eluted in the 0.3 to 0.35 M NaCl range of a 0 to 0.5 M NaCl gradient in 50 mM MES, pH 6.0, 10% glycerol (v/v), 2 mM EDTA, and 2 mM DTT. The purest fractions from the Mono-S column, judged by polyacrylamide gel analysis (PAGE), were combined and concentrated by ultrafiltration through a Centricon-30 microconcentrator (Amicon), with a 30-kDa molecular weight cutoff. The final ligase preparation was assessed by optical density and PAGE to be 1 μ g/ μ l with respect to the VV DNA ligase (see Fig. 1). The VV DNA ligase activity was monitored during purification by the formation of a [32 P]AMP–enzyme adduct after incubation with [α - 32 P]ATP.

DNA ligase activity assays

dsDNA fragment ligation. Ligase reactions were performed with 0.5 μ g of DNA fragments in a total reaction volume of 10 μ l for 15 min at 21°. One microgram of ligase was added to the DNA in reaction buffer (60 mM Tris–HCl, pH 7.8, 10 mM MgCl₂, 1 mM ATP, 50 μ g/ml BSA, and 5 mM DTT) preincubated at 21°. Reactions were terminated by the addition of EDTA to 100 mM and then immediately placing on ice. When PEG 6000 was included the reaction was terminated with EDTA, the DNA was precipitated with 100 mM NaCl and 2.5 volumes of ethanol, and then washed with 70% ethanol to remove the PEG 6000. Ligation products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Nicked, ds templates. Oligo(dT) (10 μ g of a 30-mer) was radiolabeled by incubation with 100 μ Ci of [γ - 32 P]-ATP (3000 Ci/mmol, Amersham) and 50 units of T4 polynucleotide kinase (PNK) for 45 min at 37° so that approximately 1 in 10⁵ molecules of oligo(dT) was labeled. Unlabeled ATP was then added to a final concentration of 0.1

mM and the reaction repeated with fresh T4 PNK. Excess ATP was removed by centrifuging the reaction product through a Sephadex G-50 column. Five micrograms of radiolabeled oligo(dT) was then annealed with 5 μ g of either poly(rA) or poly(dA) (obtained from Sigma), by heating to 100° for 5 min and then cooling to room temperature overnight. Following the observation that some oligo(dT) did not anneal under these conditions (Fig. 3A), unannealed oligo(dT) was subsequently removed by centrifugation through a Pharmacia S-300 Micro-spin column. In ligation reactions 10⁴ cpm of the template were incubated with T4 or VV DNA ligase for varying times at 21° in 20- μ l volumes. The reactions were terminated by heating to 90° for 10 min. Reaction products were then incubated with 1 unit of CIP for 1 hr at 37°. Sheared salmon sperm DNA (100 μ g) was added and the samples were precipitated with 8% TCA for 20 min on ice. TCA-precipitable radioactivity was determined by scintillation counting. All reactions were performed in triplicate. Data are expressed as the percentage of cpm recovered compared to a control in which no CIP treatment was included.

ss oligo(dT) template. Radiolabeled oligo(dT) (prepared as for the nicked template) was mixed with a further 30-fold excess of unlabeled oligo(dT), which had been treated with PNK and cold ATP, and preincubated for 20 min in reaction buffer (as described above for DNA fragment ligation) before addition of DNA ligase. Ligations were for variable lengths of time in 20- μ l volumes and the reactions were terminated at 90° for 10 min before treatment as described for nicked, ds templates. All reactions were performed in triplicate. Reaction products were also resolved on a 6% denaturing polyacrylamide gel and detected by autoradiography. Data are expressed as the percentage of cpm recovered compared to a control in which no CIP treatment was included.

Measurement of the K_m for ATP of the VV DNA ligase

The K_m value for the overall ligation of the ssDNA substrate oligo(dT) was determined from experiments as described above. Initially the ligation of the substrate was examined with a range of ATP concentrations (1 μ M to 50 mM). Further experiments were then performed with ATP concentrations from 0.5- to 10-fold the estimated K_m . At each ATP concentration the formation of ligation products was plotted against time and the initial velocities (v) were determined from these graphs. The values of v were then plotted against the concentration of ATP ($-s$) from which they were generated. The intercept of the lines drawn through these points on the x axis indicated the K_m . The v_{max} value was indicated by the intercept on the y axis. Ten mM Mg²⁺ was used throughout and the experiments were performed at 32°. All the reactions were performed in triplicate.

RESULTS

Isolation and preliminary characterization of VV DNA ligase

E. coli lys S cells harboring plasmid pSK18 expressed a novel 63-kDa protein upon induction with IPTG that comigrated with the VV DNA ligase from infected mammalian cells (data not shown). VV DNA ligase was purified from bacterial cell extracts using a three-column procedure as described under Materials and Methods. During the final concentration step the DNA ligase tended to precipitate, as has been reported for purified mammalian DNA ligase II (Wang *et al.*, 1994), but its solubility was increased by maintaining NaCl to at least 90 mM. The purity of the VV DNA ligase preparation was then examined by SDS-PAGE (Fig. 1). The 63-kDa DNA ligase represented about 90% of total protein, but four minor higher molecular weight proteins were also observed (Fig. 1, lane 6). However, the 63-kDa polypeptide was the only protein capable of forming a covalent intermediate with AMP, a reaction diagnostic of DNA ligases. The preparation did not contain *E. coli* DNA ligase (an NAD-dependent enzyme) since all the ligation reactions described below were ATP-dependent and not active in the presence of NAD rather than ATP.

DNA ligase activity was first examined on DNA fragments with cohesive ends, generated by the digestion of phage λ DNA with *Hind*III (Materials and Methods). DNA ligation was observed and was dependent on an energy source, ATP, and a divalent cation, Mg^{2+} (data not shown). The VV enzyme was also dependent upon DTT for maximal activity, presumably due to the presence of reactive thiol groups, in common with the DNA ligase enzymes of T4 (Weiss *et al.*, 1968), *Drosophila* (Rabin *et al.*, 1986), and *Crithidia* (Brown and Ray, 1992).

The ligation of blunt-end DNA fragments (*Hae*III-digested ϕ X174) was examined under these same conditions and compared to ligation by T4 DNA ligase (Fig. 2). The VV enzyme ligated this substrate inefficiently compared to T4 DNA ligase, but the inclusion of 5–10% (v/v) PEG 6000 stimulated the reaction considerably. Stimulation of blunt-end ligation by macromolecular crowding has been observed for DNA ligase I but not for DNA ligase II (Lindahl and Barnes, 1992). The VV enzyme, however, was more sensitive to the PEG concentration and was inhibited by 15% PEG. In contrast, this concentration stimulated ligation by both *Crithidia* (Brown and Ray, 1992) and *Drosophila* (Rabin and Chase, 1987) DNA ligases and 17.5% PEG was optimal for stimulation of bovine DNA ligase I (Arrand *et al.*, 1986).

The ligation of a blunt-ended substrate in a PEG-stimulated reaction suggested that the VV DNA ligase was a DNA ligase I-like enzyme. This was further investigated using a substrate, oligo(dT) annealed to poly(rA), that can be ligated by the mammalian DNA ligase II but not the

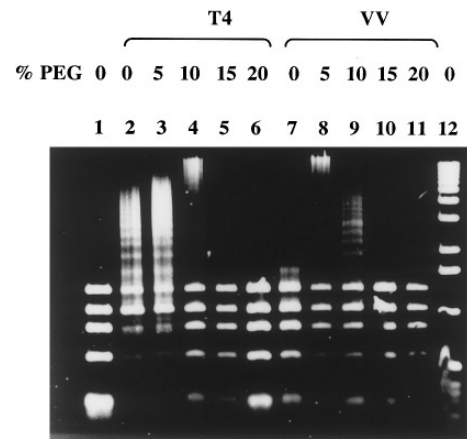


FIG. 2. Ligation of blunt-ended DNA fragments by T4 DNA ligase and VV DNA ligase. Ligase reactions were performed with 0.5 μ g of *Hae*III-digested ϕ X174 and 0.25 μ g of T4 DNA ligase (lanes 2–6) or 0.5 μ g of VV DNA ligase (lanes 7–11). PEG 6000 was preincubated at 5% v/v (lanes 3 and 8), 10% (lanes 4 and 9), 15% (lanes 5 and 10), and 20% (lanes 6 and 11). Lane 1, 0.5 μ g of unligated substrate. Lane 12, kb ladder.

DNA ligase I enzymes (Fig. 3A). Surprisingly, the VV DNA ligase ligated this substrate. Furthermore, at saturating enzyme levels the VV DNA ligase protected 100% of the oligo(dT) molecules from phosphatase digestion whereas T4 DNA ligase activity plateaued after protection of 50% of the substrate (Fig. 3A).

VV DNA ligase ligates ssDNA [oligo(dT)]

A possible explanation for the protection of all of the oligo(dT) by the VV DNA ligase was that the oligo(dT) was in excess over the poly(rA) and that VV DNA ligase was ligating free oligo(dT). This was examined first by ligating substrates containing a constant amount of poly(rA) and radiolabeled oligo(dT) but increasing amounts of cold oligo(dT). At low ratios of oligo(dT) relative to poly(rA) the oligo(dT) was ligated equally well by both T4 and VV DNA ligases. However, when oligo(dT) was in excess, the proportion of oligo(dT) ligated by the T4 DNA ligase decreased, while the VV enzyme continued to ligate all the substrate (data not shown). These data indicated that both enzymes can ligate oligo(dT) annealed to poly(rA) and again suggested that the VV enzyme could ligate free oligo(dT). This was confirmed using 5'-labeled oligo(dT) alone as substrate (Fig. 3B). Remarkably, the VV enzyme ligated the oligo(dT), while, as expected, the T4 DNA ligase had no activity on this substrate. No other eukaryotic DNA ligase has been reported to ligate ssDNA. T4 RNA ligase can ligate oligodeoxynucleotides in a reaction whose efficiency is strongly dependent on PEG (Tessier *et al.*, 1986). There is also a report of sequence-dependent oligodeoxynucleotide ligation by *E. coli* and T4 DNA ligases (Barringer *et al.*, 1990). The ligation of oligo(dT) by VV DNA ligase, T4

DNA ligase, and T4 RNA ligases was therefore compared and the reaction products analyzed by PAGE (Fig. 4). The VV enzyme ligated free oligo(dT) to multimers of at least 300 bp (Fig. 4, lanes 1–3). In contrast, under the conditions used T4 RNA ligase achieved the formation of only a phosphatase-resistant labeled group (Fig. 4, lanes 5–7), probably by the transfer of AMP from the ligase to a covalent bond with the 5' phosphate, but no ligated product was detected. T4 DNA ligase could not ligate this substrate (Fig. 4, lane 4). The VV DNA ligase was found not to ligate other homopolymers (dA, dC, and dG) or mixed-base oligonucleotides (data not shown) and thus the ligation of ssDNA appears to be specific for thymine.

The ligation of RNA was also examined. However, no appreciable RNA ligase activity was observed with the oligonucleotide rA₍₁₅₎, nor was it possible to transfer the mononucleoside pCp onto a T7-generated RNA transcript (J. McCauley, I. A. H. Compton, personal communication). Therefore, the enzyme does not exhibit RNA ligase activity, although it remains formally possible (albeit

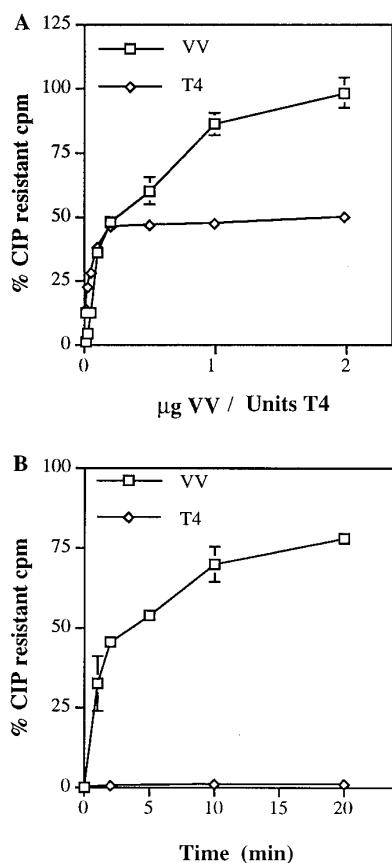


FIG. 3. Comparison of ligation reactions catalyzed by VV and T4 DNA ligases. (A) Ligation of oligo(dT):poly(rA) by T4 and VV DNA ligases. The substrates and reaction conditions are as described under Materials and Methods. Reactions were performed with increasing amounts of DNA ligase, as indicated. (B) Ligation of oligo(dT) by VV and T4 DNA ligases. A time course of oligo(dT) ligation catalyzed by 0.1 μg of VV DNA ligase and 1 μg of T4 DNA ligase.

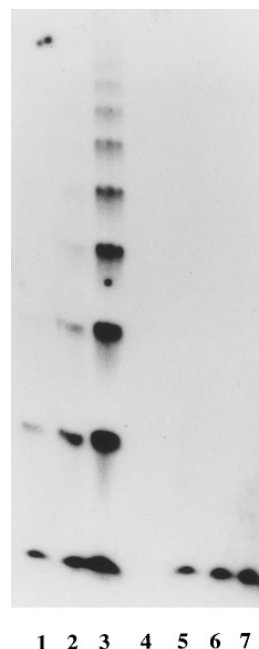


FIG. 4. Ligation of oligo(dT) by VV, T4 DNA, and T4 RNA ligases. Labeled oligo(dT) was incubated for 5 min with 0.1 μg (0.1 μl) of VV DNA ligase for 1, 2, and 5 min (lanes 1–3, respectively), with 2.0 μg (4 units) of T4 DNA ligase (lane 4), or with 1.16 μg (31 units), 2.32 μg (62 units), and 6.64 μg (124 units) of T4 RNA ligase (lanes 5–7, respectively), under conditions described in Materials and Methods. Reaction products were resolved on a 6% denaturing polyacrylamide gel and detected by autoradiography.

unlikely) that it has RNA ligase activity on a different substrate.

Biochemical parameters for ss and dsDNA ligation

The biochemical parameters for optimal oligo(dT) ligation were determined and compared with those for oligo(dT) annealed to poly(dA), a ds substrate (Figs. 5 and 6). The temperature, pH, and Mg²⁺ requirements for optimal ligation of each substrate were similar. The enzyme works optimally at 32°, at a pH of 7.8–8.0, and with 10–14 mM Mg²⁺. These pH and Mg²⁺ optima closely parallel those for other DNA ligases, pH 7.8–8.2 and 15 mM Mg²⁺ for *Drosophila* DNA ligase I (Rabin and Chase, 1987), pH 7.2–7.8 and 10 mM Mg²⁺ for T4 DNA ligase (Weiss *et al.*, 1968), and pH 7.4–8.0 and 10 mM Mg²⁺ for mammalian DNA ligase I (Soderhall and Lindahl, 1973). However, the VV DNA ligase was unusually tolerant of high pH, exhibiting ≥80% of maximal activity at pH 9.0 (Figs. 5B and 6B).

The divalent cation requirement of VV DNA ligase was not specific for Mg²⁺, and Mn²⁺ was able to substitute for Mg²⁺ in the ligation of ss and ds substrates, with an efficiency of 40% at 10 mM concentrations (data not shown). In contrast, 10 mM Ca²⁺ or Zn²⁺ did not support ligation of either substrate. Both T4 DNA ligase and mammalian DNA

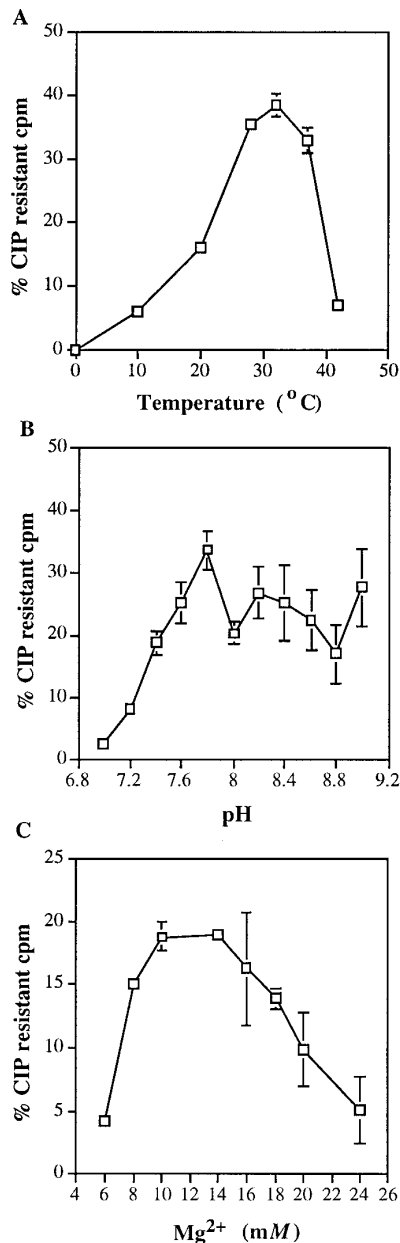


FIG. 5. The effect of varying reaction conditions on the ligation of oligo(dT) by VV DNA ligase. The reactions were performed as described under Materials and Methods, and data are expressed as the mean CIP-resistant radioactivity from triplicate samples. A, B, and C show the effect of temperature, pH, and magnesium concentration, respectively, on the reaction.

ligase I can use Mn^{2+} , but less efficiently than Mg^{2+} (Weiss *et al.*, 1968; Soderhall and Lindahl, 1973). At its optimal concentration with T4 DNA ligase Mn^{2+} was only 25% as efficient as Mg^{2+} (Weiss *et al.*, 1968).

The VV ligation of the two substrates displayed differential sensitivity to salt concentration (Fig. 7). Both reactions were sensitive to salt; however, the ss ligation reaction was 50% inhibited by 80 mM KCl, whereas 160 mM was required to inhibit the ds ligation to the same extent.

Measurement of K_m for ATP

The K_m value of the VV DNA ligase for the overall ligation of a ssDNA substrate, oligo(dT), was determined and found to be 0.8 mM (Fig. 8). The K_m value for a nicked ds substrate, poly(dA):oligo(dT), was found to be within twofold of that for oligo(dT) (data not shown). These K_m values are the highest reported for any DNA ligase. While the K_m for ATP for the ligation of ss or dsDNA were similar, the V_{max} values for the two substrates showed

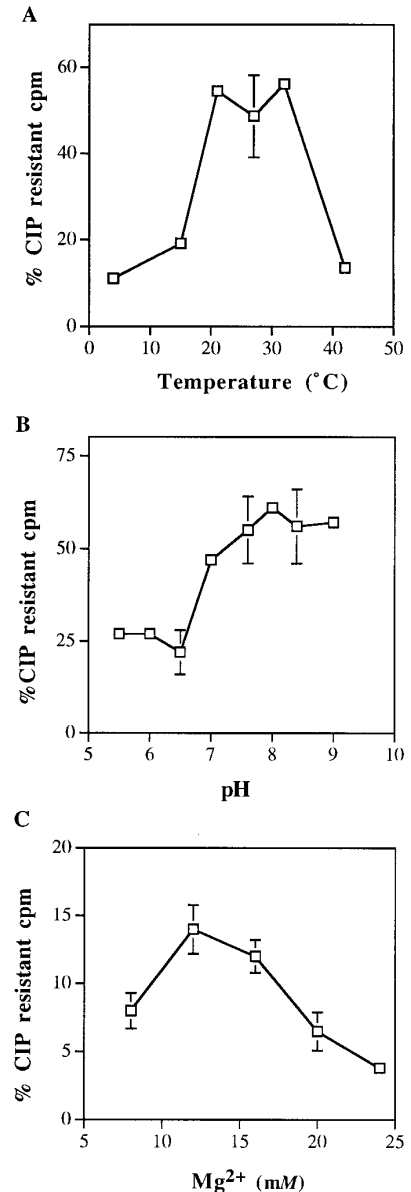


FIG. 6. The effect of varying reaction conditions on the ligation of oligo(dT):poly(dA) by VV DNA ligase. The reactions were performed as described under Materials and Methods except that 0.001 μ g of VV enzyme was added and the reactions were incubated for 8 min. Data are expressed as the mean CIP-resistant radioactivity from triplicate samples. A, B, and C show the effect of temperature, pH, and magnesium concentration, respectively, on the reaction.

that the VV enzyme sealed the nicked dsDNA substrate seven times faster than oligo(dT). This was not unexpected as the sealing takes place upon a template strand and does not require the ligase to bring the two molecules together.

DISCUSSION

The VV DNA ligase has been expressed in *E. coli*, purified to $\geq 90\%$ homogeneity, and biochemically characterized. The properties of the enzyme do not enable it to be classified as any of the three characterized eukaryotic DNA ligases. The enzyme resembles eukaryotic DNA ligase I enzymes in that it ligates blunt-ended DNA fragments, in a PEG-stimulated reaction. However, it resembles DNA ligase II and III enzymes in its ligation of oligo(dT) annealed to a poly(rA) backbone. T4 DNA ligase is the only other DNA ligase which exhibits PEG-stimulated blunt-end ligation and activity on oligo(dT):poly(rA). The VV DNA ligase has a high requirement for ATP, K_m 0.8 mM, for a ssDNA substrate, which is most similar to, but still 10-fold higher than, DNA ligase II enzymes. Last, the

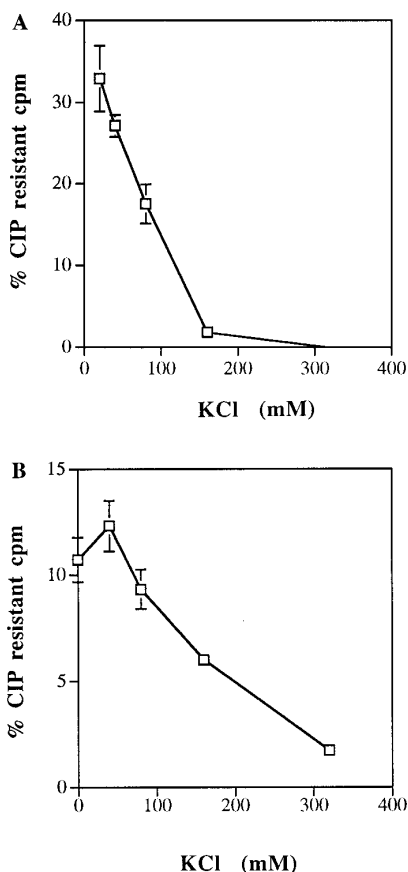


FIG. 7. Effect of KCl concentration on the ss and ds ligation reactions of VV DNA ligase. KCl was supplemented into the reaction buffer at the levels indicated. A shows the affect of KCl concentration on VV ss ligation. B shows the affect on ds ligation.

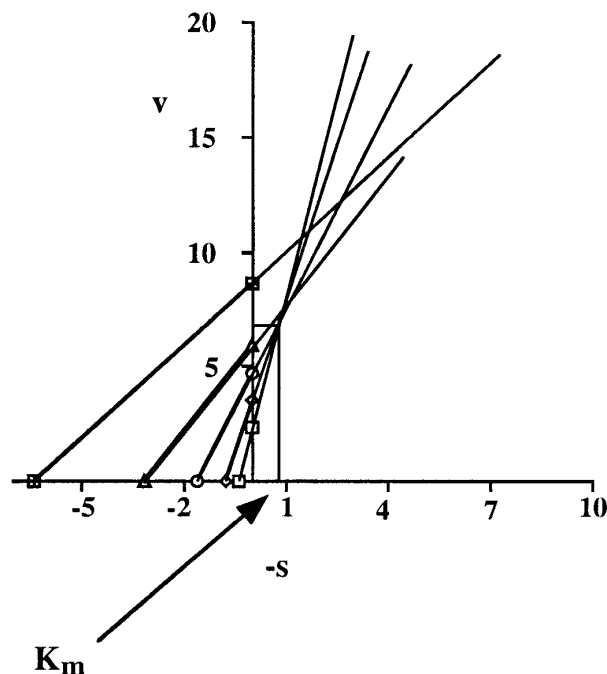


FIG. 8. Determination of the K_m for the overall ligation reaction of VV DNA ligase with the ssDNA substrate, oligo(dT). The values plotted were obtained as described under Materials and Methods.

efficient ligation of ssDNA, oligo(dT), by the VV enzyme is unique among eukaryotic DNA ligases.

Overall the VV DNA ligase showed closest functional similarity to the calf thymus 70-kDa DNA ligase II with which it also shares closest amino acid similarity [65% identity with the partial protein sequence (Wang *et al.*, 1994)]. While the VV DNA ligase also showed 50% identity with DNA ligase III [which apparently derives from the same gene as DNA ligase II (Chen *et al.*, 1995)], DNA ligase III is larger, 100 kDa, has a much lower K_m for ATP (1–2 μM) (Tomkinson *et al.*, 1991a), and is unable to perform blunt-end ligation (Elder *et al.*, 1992). The DNA ligase II peptides which have been aligned with VV DNA ligase (Wang *et al.*, 1994) are in the presumed catalytic C-terminal domain, a region with remarkable size conservation from the active site lysine to the carboxyl terminus throughout eukaryotic and prokaryotic DNA ligases (Tomkinson *et al.*, 1991b), with the exception of mammalian DNA ligases III and IV (Wei *et al.*, 1995). Given this conservation it is interesting that the VV enzyme can perform blunt-end ligation, like mammalian DNA ligase I, whereas the DNA ligase II enzyme cannot. The significance of these differences in *in vitro* substrate specificities for DNA ligase function in the cell are unclear.

The role of VV DNA ligase in the viral life cycle is not yet defined. Deletion of this enzyme renders VV more sensitive to DNA damage *in vitro* and less virulent *in vivo* (Kerr *et al.*, 1991). Based on these data it was proposed (Wang *et al.*, 1994) that the closely related mammalian

DNA ligase II was involved in DNA repair. However, it is possible that the VV DNA ligase fulfilled this role by virtue of being the only DNA ligase encoded by the virus and extrapolation of the function of DNA ligase II from these data is uncertain. Equally, complementation of the *S. cerevisiae cdc9* DNA ligase I mutation by VV DNA ligase (Kerr *et al.*, 1991) did not provide evidence that VV enzyme has a replicative function for the virus. Recently, the VV DNA ligase has been implicated in telomere resolution since the inhibition of DNA ligase action caused the accumulation of DNA forms which were suggested to be branched replication or recombination intermediates (DeLange *et al.*, 1995). Similarly, mammalian DNA ligase II activity increases during meiotic prophase and may function in the resolution of meiotic recombination intermediates (Higashitani *et al.*, 1990). The transcription of DNA ligase III, which appears to derive from the same gene as DNA ligase II, also increases in spermatocytes undergoing recombination (Chen *et al.*, 1995). There is, however, no evidence that the mammalian DNA ligase II or III directly compensate for the DNA ligase deficiency in a VV DNA ligase-negative virus. Indeed, no recruitment of a cellular DNA ligase to cytoplasmic virus factories in cells infected with a DNA ligase-negative VV was observed by immunofluorescence using an antiserum directed against the conserved C-terminal 16-amino-acid motif, which recognizes DNA ligases I, II, and III (Wang *et al.*, 1994; Husain *et al.*, 1995).

The VV DNA ligase demonstrated a completely novel activity for a eukaryotic DNA ligase, the ligation of oligo(dT), a ssDNA substrate. Despite the A:T-rich nature of the vaccinia genome (68%) there is no obvious requirement for the ligation of thymine homopolymers during virus DNA replication and the enzyme did not ligate other ss substrates, including homopolymers of dA, dG, and dC and ss heteropolymers or RNA substrates (data not shown). The biochemical optima for oligo(dT) ligation closely matched those for a nicked dsDNA substrate [oligo(dT):poly(dA)]. Ligation of oligo(dT) by VV DNA ligase was efficient and the rate of oligo(dT) ligation, v_{\max} , was only sevenfold slower than the ligation of a nicked dsDNA substrate where the oligo(dT) was already aligned to a DNA template. Three prokaryotic ligases have been reported to ligate ss substrates: T4 RNA ligase, T4 DNA ligase, and *E. coli* DNA ligase. However, neither T4 RNA nor DNA ligase ligated oligo(dT) under conditions that worked efficiently for the VV DNA ligase, and the ligation of ssDNA by T4 RNA ligase is strongly promoted by PEG (Tessier *et al.*, 1986), which is not required by VV DNA ligase. Ligation of ssDNA by the *E. coli* and T4 DNA ligases was sequence dependent and the reaction products were limited to dimers (Barringer *et al.*, 1990). However, it is conceivable that in part this ligation resulted from the formation of partially ds substrates by self-annealing and looping back of the oligonu-

cleotides used, allowing a pseudotemplated ligation event to occur. This has been described for T4 DNA ligase (Western and Rose, 1991).

The biochemical characteristics of the purified VV DNA ligase were similar in some respects to those of other purified ATP-dependent DNA ligases. The need for DTT for optimal activity suggested the presence of reactive thiol groups, as for many other DNA ligases (Weiss *et al.*, 1968; Rabin *et al.*, 1986; Brown and Ray, 1992). The enzyme required a divalent cation, primarily Mg^{2+} ; however, Mn^{2+} was found to substitute with 40% efficiency at the same concentrations. Other DNA ligases can utilize Mn^{2+} with slightly lower efficiency (Weiss *et al.*, 1968; Soderhall and Lindahl, 1973). However, the VV enzyme differed from other DNA ligases in that it was more tolerant of high pH and required a very high concentration of ATP. For comparison, the K_m for ATP of eukaryotic DNA ligase II enzymes is 10–100 μM (Soderhall and Lindahl, 1976; Engler and Richardson, 1982; Lindahl and Barnes, 1992), which contrasts with 0.6 μM for DNA ligase I (Tomkinson *et al.*, 1990) and 1–2 μM for DNA ligase III (Tomkinson *et al.*, 1991a). As the DNA ligase II and III enzymes derive from the same gene it is interesting that the smaller enzyme (70 kDa) has such a high ATP requirement, similar to the VV enzyme, which is also a small eukaryotic DNA ligase (63 kDa).

There are a number of factors which may explain the high ATP requirement. First, the VV enzyme was purified from *E. coli* and may have an ATP requirement different from that of the native enzyme in mammalian cells. However, a full-length human DNA ligase I enzyme expressed in *E. coli* has been shown to have a K_m for ATP in close agreement with data obtained with native proteins (Teiraoka *et al.*, 1993). Second, the stability of the enzyme-adenylate intermediate may also affect the ATP requirement. Many DNA ligase enzymes are purified as a doublet with some of the enzyme in the ATP-activated form. The VV DNA ligase preparation was inactive in the absence of ATP, despite the presence of ATP-independent ligase activity (not stimulated by NAD) in earlier stages of the purification. The ligase appears to lose the AMP moiety during the gel filtration stage at 15° (data not shown). Third, the high ATP requirement may be a general feature of some VV enzymes. For instance, a VV DNA-dependent ATPase activity has a K_m for ATP of 2.4 mM (Bayliss and Condit, 1995) and the efficient transcriptional termination by vaccinia RNA polymerase requires at least 0.5 mM ATP (Hagler *et al.*, 1994). The cellular levels of ATP are reported to be 2.5 ± 1.2 mM in normal cells and 3.2 ± 1.7 mM in tumor cells (Traut, 1994).

In summary, the VV DNA ligase has been expressed, purified, and biochemically characterized. The properties of the VV DNA ligase exhibit the closest relationship to the mammalian DNA ligase II enzyme, to which it is closely related at the amino acid level. The VV enzyme

displays a novel characteristic for eukaryotic DNA ligases, the efficient ligation of oligo(dT) a ssDNA substrate. It remains to be determined whether mammalian DNA ligase II can also ligate this substrate.

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