Ligation of double-stranded and single-stranded [oligo(dT)] DNA by vaccinia virus DNA ligase

Citation for published version:

Digital Object Identifier (DOI): 10.1006/viro.1996.0358

Link: Link to publication record in Edinburgh Research Explorer

Document Version: Publisher's PDF, also known as Version of record

Published In: Virology

Publisher Rights Statement: elseviers open archive article

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Ligation of Double-Stranded and Single-Stranded [Oligo(dT)] DNA by Vaccinia Virus DNA Ligase

MARK ODELL, SHONA M. KERR, 1 and GEOFFREY L. SMITH 2

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, United Kingdom

Received January 26, 1996; accepted April 24, 1996

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.

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 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 repair 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 antisera 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 $\mu$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

1 Present address: MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK.
2 To whom correspondence and reprint requests should be addressed. Fax: 44-1865-275521. E-mail: glsmith@molbiol.ox.ac.uk.
complex from calf thymus, which is capable of recombi-
national repair of double-stranded (ds) gaps and dele-
tions (Jessberger et al., 1993). Transcription of DNA li-
gase III is highest in spermatocytes, which suggests that
the enzyme may be involved in meiotic recombination
(Chen et al., 1995; Hussain 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
genres 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 transcrip-
tion 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 Saccharo-
myces 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 se-
quence 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 incom-
pletely base-paired terminal hairpins, making the gen-
ome a covalently closed circle. Shortly after infection a
nick was reported to be introduced near one of the ter-
miial hairpins (Pogo, 1977) so that, theoretically, VV DNA
replication can occur by a unidirectional leading strand
This would require only a single ligation event for each
progeny genome and this might be carried out by the VV
dicking-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 mam-
malian DNA ligases to virus cytoplasmic factories in cells
infected with the VV DNA ligase deletion mutant was
detected, using an antiserum raised against the con-
served 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 pa-
rameters reported are in close agreement with our find-
ings (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, re-
markably, 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 NdeI site immediately upstream
of the ATG codon was excised from pSK17 (Kerr et al.,
1991) by digestion with Ndel and EcoRI 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 solu-
ble DNA ligase was obtained by induction of mid to late
log phase E. coli cells in LB medium at 32°C for 3 hr with
100 μM 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°C in a Beckman
Ti70.1 rotor, to pellet cell debris, sheared DNA, and ribo-
somes. The supernatant was again supplemented with
PMSF and then subjected to a three-column purifi-
cation procedure. First, the sample was applied in 0.3
M NaCl TGED to a heparin–agarose (Affinity Chromatogra-
phy Limited) column equilibrated in the same buffer at
4°C. 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°C 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-
terminated by the addition of EDTA to 100 mM.

Data are expressed as the percentage of cpm recovered in DNA ligase activity assays.

Nicked, ds templates. Oligo(dT) (10 μg of a 30-mer) was radiolabeled by incubation with 100 μCi of [γ-32P]-ATP (3000 Ci/mmol, Amersham) and 50 units of T4 polynucleotide kinase (PNK) for 45 min at 37° so that approximately 1 in 10^5 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 μg 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 10^4 cpm of the template were incubated with T4 or VV DNA ligase for varying times at 21° in 20-μl 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 μg) 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-μl 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 μM 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 MgCl_2 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 HindIII (Materials and Methods). DNA ligation was observed and was dependent on an energy source, ATP, and a divalent cation, Mg2+ (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 (HaeIII-digested øX174) 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 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 oligodeoxyribonucleotides in a reaction whose efficiency is strongly dependent on PEG (Tessier et al., 1986). There is also a report of sequence-dependent oligodeoxyribonucleotide ligation by E. coli and T4 DNA ligases (Barringer et al., 1990). The ligation of oligo(dT) by VV DNA ligase, T4

![Fig. 2. Ligation of blunt-ended DNA fragments by T4 DNA ligase and VV DNA ligase. Ligase reactions were performed with 0.5 μg of HaeIII-digested øX174 and 0.25 μg of T4 DNA ligase (lanes 2–6) or 0.5 μg 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 μg of unligated substrate. Lane 12, kb ladder.](attachment:image.png)
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 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 Mg\textsuperscript{2+} requirements for optimal ligation of each substrate were similar. The enzyme works optimally at 32\degree C, at a pH of 7.8–8.0, and with 10–14 mM Mg\textsuperscript{2+}. These pH and Mg\textsuperscript{2+} optima closely parallel those for other DNA ligases, pH 7.8–8.2 and 15 mM Mg\textsuperscript{2+} for Drosophila DNA ligase I (Rabin and Chase, 1987), pH 7.2–7.8 and 10 mM Mg\textsuperscript{2+} for T4 DNA ligase (Weiss et al., 1968), and pH 7.4–8.0 and 10 mM Mg\textsuperscript{2+} 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 Mg\textsuperscript{2+}, and Mn\textsuperscript{2+} was able to substitute for Mg\textsuperscript{2+} in the ligation of ss and ds substrates, with an efficiency of 40% at 10 mM concentrations (data not shown). In contrast, 10 mM Ca\textsuperscript{2+} or Zn\textsuperscript{2+} did not support ligation of either substrate. Both T4 DNA ligase and mammalian DNA
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. 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.

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 µg 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 >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, \( K_m = 0.8 \text{ mM} \), for a ssDNA substrate, which is most similar to, but still 10-fold higher than, DNA ligase II enzymes. Last, the 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 \( \mu \text{M} \)) (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, \( v_{\text{max}} \), 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 oligonucleotides 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 \( \text{Mg}^{2+} \); however, \( \text{Mn}^{2+} \) was found to substitute with 40% efficiency at the same concentrations. Other DNA ligases can utilize \( \text{Mn}^{2+} \) 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 \( \mu \text{M} \) (Soderhall and Lindahl, 1976; Engler and Richardson, 1982; Lindahl and Barnes, 1992), which contrasts with 0.6 \( \mu \text{M} \) for DNA ligase I (Tomkinson et al., 1990) and 1–2 \( \mu \text{M} \) 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 (Te-raoka 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°C (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.

**ACKNOWLEDGMENTS**

Work in this laboratory was supported by the Medical Research Council and The Wellcome Trust. We are most grateful to Dr. John McCauley for carrying out the pCp RNA ligation experiment and for advice on RNA ligation and to Dr. Dale Wigley for helpful discussions.

**REFERENCES**


