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Vaccinia DNA ligase complements *Saccharomyces cerevisiae cdc9*, localizes in cytoplasmic factories and affects virulence and virus sensitivity to DNA damaging agents

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The functional compatability of vaccinia virus DNA ligase with eukaryotic counterparts was demonstrated by its ability to complement *Saccharomyces cerevisiae cdc9*. The vaccinia DNA ligase is a 63 kDa protein expressed early during infection that is non-essential for virus DNA replication and recombination in cultured cells. This implies complementation by a mammalian DNA ligase, yet no obvious recruitment of host DNA ligase I from the nucleus to the cytoplasm was observed during infection. An antiserum raised against a peptide conserved in eukaryotic DNA ligases identified the virus enzyme in discrete cytoplasmic 'factories', the sites of virus DNA synthesis, demonstrating immunological cross-reactivity between host DNA ligase I and the vaccinia enzyme. DNA ligase was not detected in the factories of a mutant virus lacking the ligase gene. Despite this, no difference in growth between wild-type (WT) and mutant virus was detectable even in Bloom's syndrome cells which have reduced DNA ligase I activity. However, DNA ligase negative virus showed an increased sensitivity to UV or bleomycin in cultured cells, and the importance of DNA ligase for virus virulence *in vivo* was demonstrated by the attenuated phenotype of the deletion mutant in intranasally infected mice.

Key words: attenuation/*cdc9* complementation/cytoplasmic factories/DNA ligase/vaccinia virus

Introduction

Vaccinia virus, the prototype poxvirus, replicates in cytoplasmic foci called virosomes or factories and provides a useful model system for DNA replication in mammalian cells. Cytoplasmic DNA replication is possible because the virus encodes many of the enzymes involved in DNA synthesis, such as a DNA topoisomerase, DNA polymerase and DNA ligase. Additionally, the virus expresses enzymes for the production of nucleotide precursors e.g. thymidine kinase, thymidylate kinase and ribonucleotide reductase (for review see Traktman, 1990). Consistent with nuclear independence, the synthesis of virus proteins, DNA and new virus particles can occur in enucleated cells (Prescott *et al.*, 1971; Pennington and Follett, 1974). Nevertheless, reports that the nucleus plays some role in virus replication are supported by the selective recruitment of two host nuclear proteins, the large subunit of RNA polymerase II and a lamin-like protein, into the cytoplasmic factories during infection (Morrison and Moyer, 1986; Bloom *et al.*, 1989).

Vaccinia virus encodes a 63 kDa DNA ligase which is expressed early during infection (Kerr and Smith, 1989; Smith *et al.*, 1989) and which shares 30% amino acid identity with DNA ligase of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Smith *et al.*, 1989) and a similar degree of homology to human DNA ligase I (Barnes *et al.*, 1990). The latter is the major DNA ligase for replication in the mammalian cell nucleus, with the immunologically unrelated DNA ligase I possibly having separate functions. In contrast, the budding yeast has a DNA ligase gene (CDC9) which is responsible for DNA replication, recombination and repair (Johnston and Nasmyth, 1978; Fabre and Roman, 1979) and which can complement the *cdc17* DNA ligase mutant of the fission yeast *S.pombe* (Barker and Johnston, 1983; Barker *et al.*, 1985). Similarly, human DNA ligase I can complement *S.cerevisiae cdc9* (Barnes *et al.*, 1990). To determine whether the vaccinia DNA ligase can complement the yeast enzyme, the virus gene was inserted into *S.cerevisiae cdc9* and the growth of the recombinant yeast examined at the non-permissive temperature.

Surprisingly, the vaccinia DNA ligase is non-essential for virus replication in cultured cell lines (Colinas *et al.*, 1990; Kerr and Smith, 1991) and homologous recombination between virus and plasmid DNA occurs at a normal rate in cells infected with the mutant virus (Colinas *et al.*, 1990). To investigate whether the normal growth of vaccinia virus lacking DNA ligase is attributable to recruitment of a cellular ligase to the cytoplasmic factories, the location of mammalian DNA ligase I was followed during infection by immunofluorescence using two antisera specific for ligase I. As a second approach to assess possible complementation by host DNA ligase, the replication of wild-type (WT) and DNA ligase negative viruses was compared in a cell line derived from a patient with Bloom's syndrome. These cells have a reduced level of DNA ligase I activity (Willis *et al.*, 1987) but still undergo regular cell division, indicating an adequate DNA synthetic capability. In addition to roles in replication and recombination, a DNA ligase is required for the ligation of repaired lesions after excision and DNA polymerization. Although the vaccinia virus endonuclease, DNA ligase and DNA polymerase might function in the repair of DNA damage, there is no evidence for a vaccinia-encoded repair pathway, and mismatch repair was not detected in the cytoplasm of poxvirus infected cells (Parks and Evans, 1991). To determine if vaccinia DNA ligase affects resistance to DNA damaging agents in cultured cells, the sensitivities of WT and ligase negative virus were examined in response to UV and bleomycin, agents known to inhibit vaccinia virus replication (Takeshita *et al.*, 1974; Lambert and Magee, 1977).

The deletion of other vaccinia genes encoding enzymes

involved in DNA synthesis, e.g. thymidine kinase (Buller *et al.*, 1985), ribonucleotide reductase (Child *et al.*, 1990) and thymidylate kinase (G.L.Smith, unpublished data), causes virus attenuation. Therefore, the functional significance of vaccinia virus DNA ligase *in vivo* was assessed by comparing the virulence of the vaccinia virus DNA ligase mutant with WT virus in a murine intranasal model. Decreased virulence would demonstrate the functional importance of the virus enzyme *in vivo* and provide another way of producing attenuated recombinant vaccinia virus vaccines.

Results

The vaccinia DNA ligase gene complements S.cerevisiae cdc9

To determine if the vaccinia DNA ligase is functionally compatible with eukaryotic DNA ligases, the gene was introduced under a galactose-inducible promoter into *S.cerevisiae cdc9* (Materials and methods), a strain temperature sensitive for the DNA ligase required for DNA replication, repair and recombination in yeast (Johnston and Nasmyth, 1978; Fabre and Roman, 1979). If the vaccinia virus enzyme is functionally equivalent, its expression in the presence of galactose should allow growth of the mutant at the restrictive temperature of 37°C.

Cells of the *cdc9* mutant, L94-4D, containing the parent vector pEMBLyex4 (Murray *et al.*, 1987) were unable to grow at 37°C on any carbon source. However, cells carrying the vaccinia DNA ligase gene inserted into this vector showed substantial growth at 37°C in medium containing the inducing carbon sources raffinose and galactose (Figure 1). In contrast, with the repressing carbon source (glucose) there was only limited growth at 37°C (Figure 1).

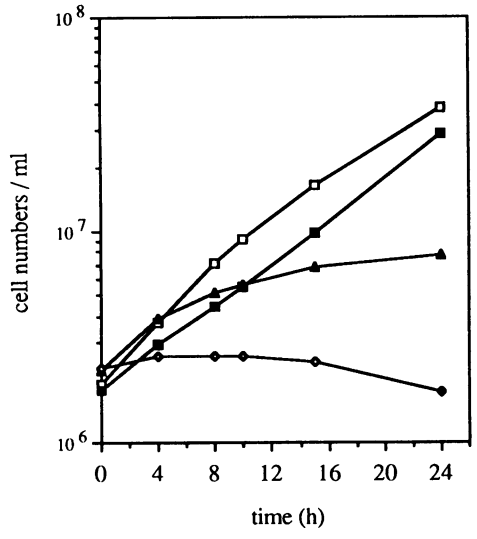

Fig. 1. Growth of *S.cerevisiae* DNA ligase mutant *cdc9* transformed by plasmids with or without the vaccinia DNA ligase ORF. Cultures were grown at 25°C in Yeast Nitrogen Base containing the appropriate carbon sources and at time zero were either switched to 37°C or maintained at 25°C and cell numbers monitored. Open diamonds, *cdc9* containing pEMBLyex4 grown in the presence of 2% galactose and 2% raffinose at 37°C; closed triangles, *cdc9* containing pSK19 grown in 2% glucose at 37°C; closed squares, *cdc9* containing pSK19 (including the vaccinia virus DNA ligase ORF) in 2% galactose and 2% raffinose at 37°C; open squares, *cdc9* containing pSK19 in 2% galactose and 2% raffinose at 25°C.

Moreover, by 24 h in this culture, over 80% of the population were large swollen pairs of cells, the abnormal morphology associated with a defect in DNA ligase (Johnston and Nasmyth, 1978), whereas cells grown in the presence of galactose at 37°C were of normal size and morphology. The vaccinia enzyme thus clearly complements the DNA ligase deficiency in *cdc9* mutants.

Surprisingly, in view of this complementation, the vaccinia polypeptide was not detected in crude extracts of transformed yeast cells on Western blots probed with either antiserum to a bacterial fusion protein (Kerr and Smith, 1989) or a conserved peptide (Lasko *et al.*, 1990). Possibly this is due to degradation before or during extraction by one or more of the abundant yeast proteases or to low level expression.

Characterization of DNA ligase mutant viruses

A recombinant vaccinia virus (vSK14) lacking the vaccinia DNA ligase gene (renamed from SalF15R (Smith *et al.*, 1989) to SalF13R (Smith *et al.*, 1991)) is able to replicate in cultured cells (Kerr and Smith, 1991). This virus contains the *Escherichia coli gpt* gene at the DNA ligase locus replacing 1 kb of the ligase ORF. For the present studies the *gpt* gene was removed, forming virus vSK20, to eliminate any possible contribution of that gene to virus growth or pathogenesis (Materials and methods). A further recombinant virus, vSK21, has the DNA ligase gene re-inserted into the thymidine kinase locus of vSK20 to create a 'pseudo-WT' virus (Materials and methods and Figure 2A). The generation of vSK20 confirmed previous observations (Colinas *et al.*, 1990) that a virus which does not encode DNA ligase remains competent for homologous recombination between plasmid and viral DNA within infected cells. The structures of the virus genomes were confirmed by Southern blotting (Figure 2). Digestion of WT DNA with *Bam*HI and probing with DNA specific for the region flanking the ligase ORF (Figure 2B) detected a 3 kb band, which in vSK14 increased to 4 kb due to the inclusion of the 2 kb *gpt* cassette and loss of 1 kb of DNA ligase ORF. In vSK20 and vSK21 the *gpt* cassette is removed and as expected the fragment was reduced to 2 kb. Digestion with *Hin*dIII and probing with the vaccinia *Hin*dIII J fragment (Figure 2C) showed that the WT 5 kb is increased to 7 kb in vSK21 due to the insertion of the DNA ligase gene at the TK locus. These data were confirmed by PCR analysis (data not shown).

Vaccinia DNA ligase is localized to cytoplasmic factories

The ability of the DNA ligase mutant virus to replicate with WT kinetics in cultured cells was unexpected. To investigate whether this was due to complementation of the DNA ligase deletion mutant by host DNA ligase I, the location of the mammalian DNA ligase I during infection was determined by immunofluorescence using two antisera which recognize this enzyme. One antiserum, raised against purified bovine DNA ligase I, identifies this enzyme in the nucleus of uninfected MDBK cells (Lasko *et al.*, 1990) and MDBK cells infected with either WT or DNA ligase-mutant vaccinia vSK20 (data not shown). A second antiserum was raised against a synthetic peptide representing a conserved basic region near the C-terminus of the mammalian, yeast and vaccinia DNA ligases (Lasko *et al.*, 1990) (Figure 3). This antiserum recognizes the vaccinia DNA ligase by Western

blotting of extracts of cells infected with vSK21 but not vSK20 or mock infected cells (Figure 3). The blot shows a weak, high molecular weight band in all lanes which may represent cellular DNA ligase I. The anti-peptide serum was also used to locate DNA ligases by immunofluorescence (Figure 4). Nuclear staining was visible in uninfected cells as previously demonstrated (Figure 4B), and in cells infected with the mutant virus vSK20 (Figure 4D), but there was no obvious recruitment of DNA ligase I into the cytoplasm upon vaccinia virus infection. No staining was seen with pre-immune serum. In cells infected with vSK21 in which the DNA ligase gene has been re-inserted into the TK locus of the mutant virus, strong fluorescence in discrete cytoplasmic foci was visible, in addition to nuclear staining (Figure 4F). WT infected cells showed a similar pattern. Staining of the same cells with the DNA-binding fluorochrome Hoechst 33258 (Figure 4A, C and E) revealed that the cytoplasmic foci contain DNA and therefore correspond to virus factories. These factories were clearly present in vSK20 infected cells but were not recognized by the antiserum due to the absence of the viral ligase gene. The integrity of nuclei during vaccinia virus infection was independently assessed with a recombinant vaccinia virus expressing influenza nucleoprotein (NP) (Smith *et al.*, 1987). Anti-NP antiserum showed good localized nuclear staining up to 9 h post-infection (p.i.) (data not shown). At the high multiplicity of infection used in these experiments, vaccinia DNA synthesis is maximal between 2-5 h p.i. therefore time points beyond 9 h p.i. were not analysed.

The DNA ligase is expressed early in infection, prior to the onset of DNA replication. However, if virus DNA replication is blocked by cytosine arabinoside (ara C), neither factories nor obvious pre-replicative sites are detectable using the anti-peptide antiserum (data not shown). The cytoplasmic factories form between 2 and 4 h p.i., and their appearance is unaffected by the absence of the vaccinia DNA ligase gene (Figure 3D). Collectively, these data demonstrate immunological cross-reactivity between host DNA ligase I and the vaccinia virus enzyme, show that the latter is normally localized in sites of viral DNA synthesis and provide no evidence for recruitment of cellular DNA ligase I into the cytoplasmic structures to complement the missing virus enzyme.

MDBK cells are non-permissive for growth of vaccinia virus, although synthesis of early virus proteins and DNA and virus-induced cytopathic effect are normal (Hruby *et al.*, 1980). The MDBK cells used here were also found to be non-productive (data not shown) but cytoplasmic factories were formed showing that the block of virus growth occurs at a stage subsequent to assembly of these structures. To check that the pattern of immunofluorescence observed in MDBK cells using the anti-peptide antibody was not aberrant

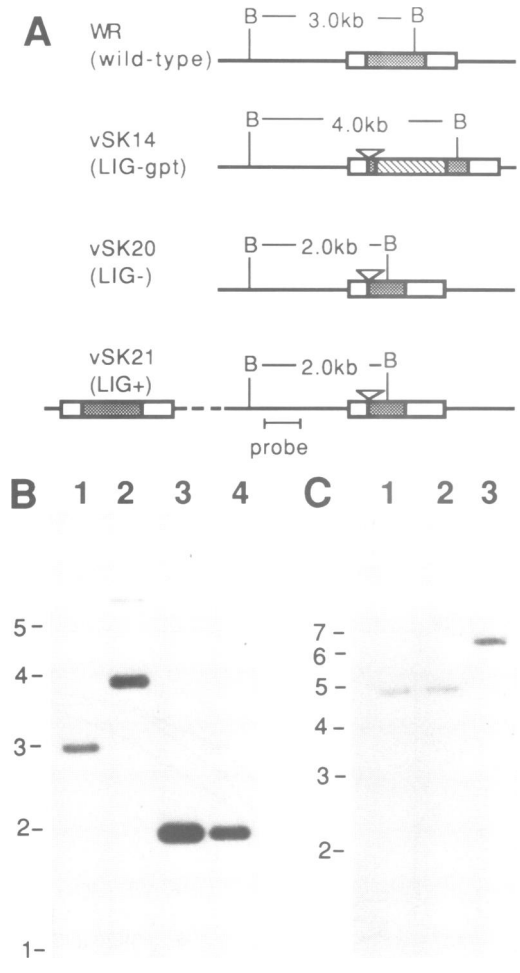

Fig. 2. Southern blot analysis of recombinant vaccinia virus genomes. **A**. Structure of selected regions of virus genomes. The DNA ligase gene is indicated by a stippled box and the *gpt* cassette by a hatched box. B = *Bam*HI restriction site. **B** and **C**. DNA extracted from virus cores was digested with *Bam*HI (panel B) or *Hin*dIII (panel C), separated by electrophoresis through a 1% agarose gel and transferred to nitrocellulose. Filters were probed with either radiolabelled DNA from the vaccinia virus TmpK gene (B) or with the *Hin*dIII J fragment (C). Panel A lane order: 1, WR; 2, vSK14; 3, vSK20; 4, vSK21. Panel C lane order: 1, WR; 2, vSK20; 3, vSK21. Positions of molecular weight markers in kb are indicated. Autoradiographs are shown.

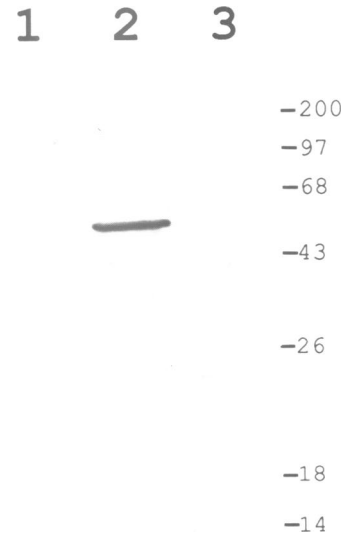

Fig. 3. Detection of vaccinia DNA ligase by Western blot with anti-peptide serum. Human TK$^-$143 cells were infected at 10 p.f.u./cell with vSK20 (lane 1) or vSK21 (lane 2) or mock infected (lane 3). Cells were harvested 8 h p.i. and blots incubated with anti-peptide serum (Materials and methods). The positions of protein molecular weight markers are indicated.

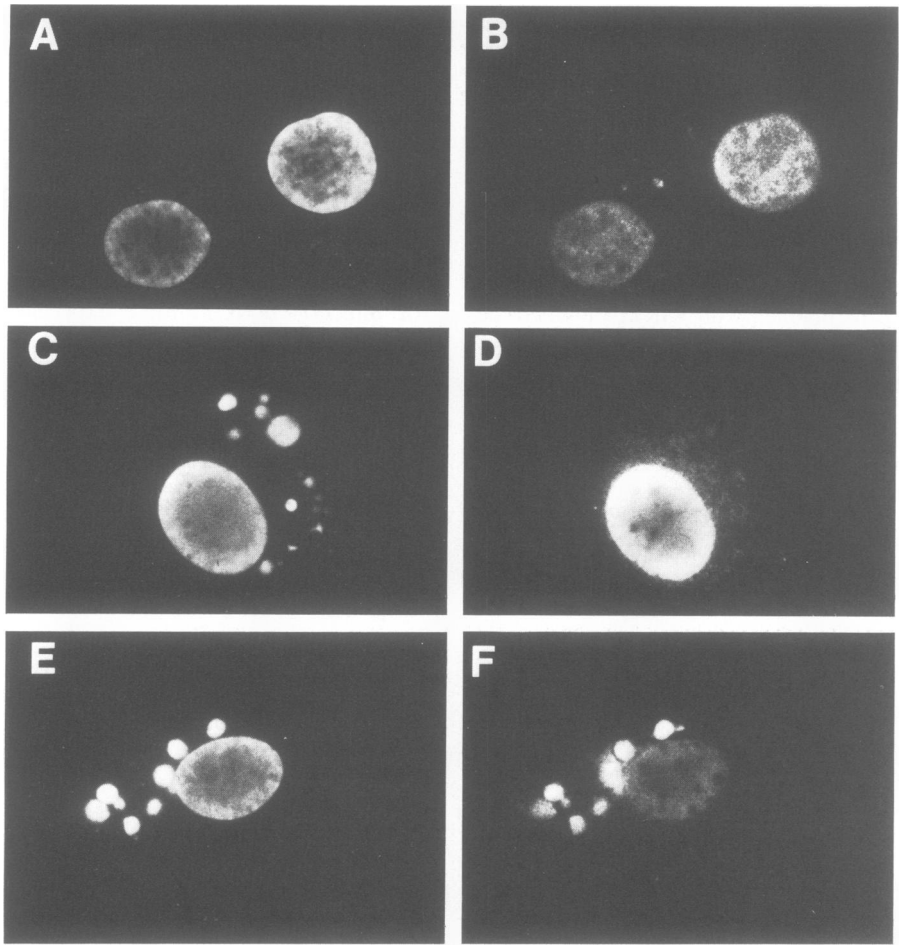

Fig. 4. Localization of vaccinia DNA ligase in infected cells. MDBK cells were either mock infected or infected with vSK20 or vSK21 at 10 p.f.u./cell. 8 h p.i. the cells were stained with Hoechst 33258 dye and anti-peptide serum to DNA ligase and then the same cells photographed under two different wavelengths as described in Materials and methods. Panels **A**, **C** and **E** are photographed for Hoechst 33258 staining of DNA and panels **B**, **D** and **F** for antibody staining. A and B are mock infected, C and D infected with vSK20, and E and F infected with vSK21.

due to the abortive infection, similar experiments were performed in B-SC-1 cells which are permissive for virus replication. The vaccinia DNA ligase was also localized to cytoplasmic factories during a fully productive infection (data not shown).

Growth in Bloom's syndrome cells

A second approach to determine whether cellular DNA ligase I complements growth of the vaccinia DNA ligase mutant used a cell line, GM8505, derived from a patient with Bloom's syndrome. These cells have DNA ligase I activity reduced to approximately one-third the level of normal cell lines whereas ligase II is normal (Willis *et al.*, 1987). A comparison of the rate of growth of WT virus, DNA ligase mutant vSK20 and the pseudo-WT virus vSK21 in GM8505 cells showed that loss of DNA ligase (or TK) did not restrict virus replication (Figure 5). Similarly, the plaquing efficiency of each virus was indistinguishable on these cells.

Sensitivity to DNA damaging agents

To assess the sensitivity of vSK20, vSK21 and WT to DNA damaging agents, cells infected with these viruses were treated with various doses of UV 2 h p.i. or with bleomycin 2 h before and throughout infection, and the titre of virus from the infected cells 30 h later was determined. All viruses

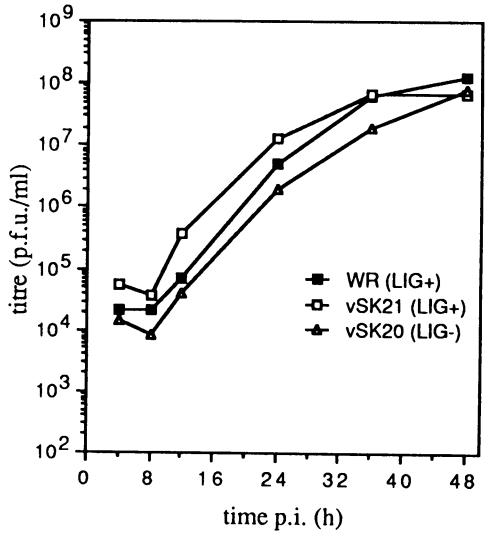

Fig. 5. Virus growth curves in Bloom's cells. Cultures of GM8505 Bloom's cells were infected with WT, vSK20 or vSK21 at a multiplicity of 0.1 p.f.u./cell. Cells were harvested at the indicated times post-infection by scraping and collected by low speed centrifugation. Virus within infected cells was released by three cycles of freeze–thawing and briefly sonicated before being measured by plaque assay on fresh monolayers of B-SC-1 cells.

showed a dose-dependent sensitivity to both agents but this was highest with vSK20 (Figure 6); for instance, a UV dose of 180 J/m^2 or bleomycin at 1.0 U/ml inhibited vSK20 replication 16- or 20-fold more than WT virus, respectively (Figure 6). Re-insertion of the DNA ligase gene into the TK locus of vSK20 reduced the UV and bleomycin sensitivity although not fully to WT levels, indicating that the loss of DNA ligase is the major factor responsible for the enhanced sensitivity of vSK20 to damage by these agents.

Virulence of vaccinia virus lacking DNA ligase

Some vaccinia virus genes are non-essential for virus replication in cultured cells but influence virus virulence *in vivo*. To test the virulence of vaccinia viruses lacking the DNA ligase gene, mice were infected intranasally with dilutions of either WT virus or recombinants vSK14 or vSK20. The latter viruses differ only in the presence (vSK14) or absence (vSK20) of the *gpt* gene within the deleted DNA ligase locus. Mice were monitored daily for signs of illness and any deaths were recorded. These data (Table I) showed that while doses of WT virus of 5×10^7 and 1×10^7 plaque forming units (p.f.u.) killed all of the mice and 1×10^6 p.f.u. killed two out of five animals, mice inoculated with any dose of either virus lacking the ligase gene survived. Indeed none of these animals showed any sign of illness for three weeks after inoculation, by which time all animals surviving infection with WT virus had recovered from initial illness. This high degree of attenuation was also observed with a TK$^-$ recombinant virus. In contrast, deletion of some other vaccinia virus genes, such as serpin K2L, had no effect on virulence in the same mouse model (Law and Smith,1992). These data showed that although the DNA ligase gene is non-essential for virus replication *in vitro*, it is important for virus virulence *in vivo*.

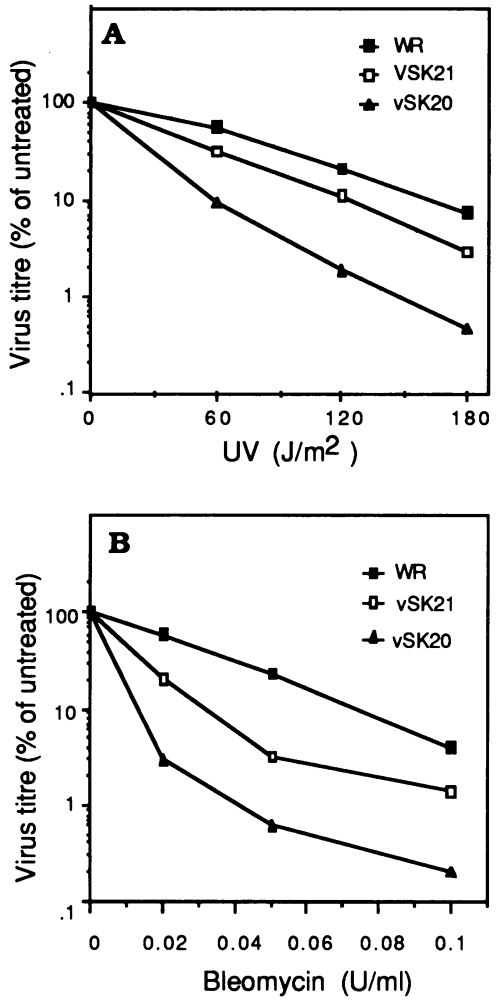

Fig. 6. Virus sensitivity of DNA damaging agents. **A** CV-1 cells infected with WT, vSK20 or vSK21 as described in Materials and methods were treated with indicated doses of UV 2 h p.i. an virus recovered from infected cells 30 h later titrated in B-SC-1 cells as described in Figure 5. These data are mean values from four experiments expressed as the percent reduction compared with non-irradiated cells. **B.** Sensitivity to bleomycin. CV-1 cells were treated with indicated doses of bleomycin for 2 h before infection with viruses as in A and maintained in the presence of the drug for 30 h. Virus from cells was titrated by plaque assay on B-SC-1 cells as described in A. Mean data are shown from four experiments expressed as the percent reduction compared with control without bleomycin.

Discussion

The vaccinia virus DNA ligase gene complements the *cdc9* mutant of the budding yeast *S.cerevisiae*, thus confirming earlier data that it encodes an active DNA ligase (Kerr and Smith, 1989; Colinas *et al.*, 1990) and demonstrating functional compatibility with the yeast enzyme despite being cytoplasmic in mammalian cells and 21.5 kDa smaller. Vaccinia DNA ligase is present within cytoplasmic virus factories, the sites of virus DNA synthesis, but deletion of the gene does not affect factory formation or virus replication or recombination in cultured cells. This presumably reflects complementation by a mammalian ligase, although replication *in vitro* is unaltered in cells containing reduced levels of DNA ligase I. Two phenotypic differences were observed between the WT and ligase negative viruses. In cultured cells the replication of vSK20 was more sensitive to UV and bleomycin than WT virus, and in intranasally infected mice the ligase deletion mutant had reduced virulence, demonstrating the functional importance of DNA ligase *in vivo*.

The DNA ligase is the second vaccinia gene which complements a cell division control mutant of *S.cerevisiae*; the vaccinia thymidylate kinase gene functionally complements *cdc8* cells (Hughes *et al.*, 1991). This underlines the

Table I. Virulence of DNA ligase-negative vaccinia viruses in mice

Virus strain	Dose (p.f.u.)	Illness[a]	Mortality
WR (wild-type)	5×10^7	5/5	5/5
	1×10^7	5/5	5/5
	1×10^6	5/5	2/5
	1×10^5	5/5	0/5
vSK14 (LIG$^-$)	5×10^7	0/5	0/5
	1×10^7	0/5	0/5
	1×10^6	0/5	0/5
	1×10^5	0/5	0/5
vSK20 (LIG$^-$)	5×10^7	0/5	0/5
	1×10^7	0/5	0/5
	1×10^6	0/5	0/5
	1×10^5	0/5	0/5

[a]After intranasal infection with the dose of virus shown, mice were examined daily for symptoms of illness (ruffled fur, arched backs and huddled behaviour) or death.

fundamental conservation of DNA replication in eukaryotic cells. Since *S.cerevisiae* encodes a DNA ligase gene for replication, repair and recombination (Johnston and Nasmyth, 1978; Fabre and Roman, 1979), the vaccinia enzyme may be able to perform all these functions within the yeast cell. Certainly the vaccinia ligase can join Okazaki fragments produced during discontinuous replication of yeast DNA. However, this does not necessarily mean that vaccinia employs this mode of DNA replication as a model for poxvirus DNA replication that does not require a DNA ligase is possible. This involves an initial nicking event near one terminal hairpin followed by unfolding, copying from the free 3′ end and refolding of the terminal hairpin. DNA synthesis could then occur exclusively by unidirectional, leading strand synthesis to generate the concatemeric molecules observed during replication. Resolution of the palindromic junction fragments from within these concatemers to form unit length daughter molecules need not involve a DNA ligase because a virion-associated nicking–joining enzyme can resolve and reseal such structures into covalently closed telomeres (Bauer *et al.*, 1977; Lakritz *et al.*, 1985; Reddy and Bauer, 1989). A more conventional mechanism of leading and discontinuous lagging strand synthesis with Okazaki fragment formation requires a DNA ligase. In the absence of the vaccinia DNA ligase, Okazaki fragments would, if they exist during virus DNA replication, have to be sealed by a cellular ligase.

A peptide antibody raised against a conserved basic region near the C-termini of the vaccinia, yeast and mammalian DNA ligases recognizes both the vaccinia virus and host type I enzymes. This is the second example of a vaccinia enzyme sharing an epitope with a mammalian counterpart. Previously one of the large subunits of the vaccinia DNA-dependent RNA polymerase and the corresponding host cell subunit were shown to be recognized by the same monoclonal antibody (Morrison and Moyer, 1986). The immunological cross-reactivity of the conserved region of the DNA ligases was predicted (Smith *et al.*, 1989) and was useful during the cloning of the human DNA ligase I gene (Barnes *et al.*, 1990). The function of the conserved region is unknown although it is noteworthy that its location near the C-terminus is a constant distance from the deduced or predicted active site lysine of all ATP-dependent DNA ligases (Tomkinson *et al.*, 1991). Cellular proteins targeted to the nucleus contain a nuclear localization sequence but such a signal in DNA ligase I has not been identified. Although the vaccinia DNA ligase would not be expected to contain a nuclear targeting signal, sufficient enzyme must reach the nucleus in yeast cells to allow functional complementation. Many vaccinia virus proteins may share a virosome localization signal, as all virus proteins directly involved in the synthesis of DNA are likely to be present in cytoplasmic factories. Poxvirus-encoded proteins known to be localized in these factories include subunits of the viral RNA polymerase (Morrison *et al.*, 1985) and the essential rifampicin-sensitive protein L65 (Miner and Hruby, 1989). Three abundant DNA-binding proteins also co-purify with these structures (for review see Traktman, 1990).

The increased sensitivity of vSK20 to DNA damaging agents is mostly attributable to the loss of DNA ligase since re-insertion of the ligase gene into the TK locus increases virus resistance to these agents. However, the pseudo-WT virus consistently had a greater sensitivity to DNA damaging agents than WT virus. This had several possible explanations. First, insertion into the TK locus could alter the expression of DNA ligase or adjacent genes. Second, there could be other mutations in the vSK21 genome acquired during selection of vSK20, or more likely during selection of TK^- vSK21 in the mutagenic compound BUdR. Consistent with this, a second isolate of vSK21 had acquired a fusogenic phenotype. Third, it is possible that loss of the TK gene contributes to increased virus sensitivity to DNA damaging agents. In this regard the HSV 2 TK gene is reported to be important for repair of UV-induced damage to virus DNA, although this property was not mapped to the TK locus by marker rescue (Rainbow, 1989).

The mechanism by which loss of DNA ligase contributes to an increased virus sensitivity to DNA damaging agents is unclear. Perhaps the quantity of cytoplasmic cellular ligase(s) is usually sufficient to enable virus replication and recombination but not repair in response to extensive DNA damage and could vary according to metabolic state of the cells. Indeed, some variability in the virus sensitivities in infected CV-1 cells to UV treatment was noted, so that on occasions only small differences between WT, vSK20 and vSK21 were observed. Possibly when differences between viruses were not observed in CV-1 cells, sufficient cellular DNA ligase(s) was present in the cytoplasm to overcome the loss of the virus enzyme. Lastly, it is unclear whether the altered sensitivity to DNA damaging agents is repair mediated by excision, re-polymerization and ligation, or by recombination, although a ligase is required for either event.

It is possible that if DNA ligase is required for either replication or recombination, one or more of the cellular DNA ligases complement the defect of the virus deletion mutant *in vitro*. Herpes simplex virus DNA replication takes place in discrete nuclear foci to which several cellular proteins, including DNA ligase I, are recruited (Wilcock and Lane, 1991). However, using antisera specific for mammalian DNA ligase I, no recruitment of this enzyme to vaccinia virus cytoplasmic factories was detected, although this does not eliminate recruitment at a level undetectable with the reagents used. Complementation by a mammalian DNA ligase in cultured cells possibly occurs because of the artificially high levels of DNA synthetic enzymes in cell lines selected for rapid division. However, the reduced virus virulence *in vivo* implies that adequate recruitment of a host ligase does not take place to allow complementation *in vivo*. Even without specific recruitment in cultured cells there could be sufficient host DNA ligase in the cytoplasm to complement the vaccinia virus DNA ligase mutant. There is a small amount of staining for DNA ligase I in the cytoplasm of the cells examined in the present study, which possibly represents newly synthesized protein awaiting transport to the nucleus. Mitotic cells show some cytoplasmic staining for DNA ligase I (Lasko *et al.*, 1990) and mitochondrial DNA is presumably ligated by a cellular enzyme. The immunologically unrelated enzymes DNA ligase II (reviewed by Soderhall and Lindahl, 1976), or the recently identified DNA ligase III (Tomkinson *et al.*, 1991) could possibly complement due to endogenous cytoplasmic activity or specific recruitment upon infection, but investigation of such possibilities will require the development of additional reagents.

Materials and methods

DNA cloning for expression in yeast cells

Restriction endonuclease digestions, DNA ligations and plasmid DNA preparation were performed using standard procedures (Sambrook *et al.*, 1989). A DNA fragment containing the entire vaccinia DNA ligase open reading frame (ORF) with convenient restriction enzyme sites at each end was constructed by a combination of subcloning and polymerase chain reaction (PCR). A 2 kb *ClaI*–*MluI* DNA fragment containing the whole DNA ligase ORF and 249 5′ and 79 3′ nucleotides was excised from the vaccinia virus (strain WR) *SalI* F fragment and inserted into *SmaI*-cut pUC13. The resulting plasmid, pSK16, was grown in dam$^-$ *E.coli* and then digested with *BclI*, which cuts 176 nucleotides downstream from the 5′ end of the DNA ligase ORF (Smith *et al.*, 1989), and *SalI*, to remove the 5′ region of the ligase ORF and upstream sequences. Next, a *SalI* site was introduced 5′ of the ligase ORF by PCR using oligonucleotides 5′ CCCGTCGACA-T(ATG)ACGTCGCTTCGCG 3′ and 5′ CCGGGAAGCAATAGCTT-AATG 3′ on a plasmid template containing the DNA ligase gene. The *SalI* site is underlined and the first codon of the vaccinia DNA ligase ORF is bracketed. The resulting PCR product of 148 nucleotides was digested with *BclI* and *SalI*, inserted into *SalI* and *BclI*-cut pSK16 to form pSK17 and sequenced to check the fidelity of the *Taq* polymerase. For expression in yeast cells, the ORF was released from pSK17 by digestion with *SalI* and *Eco*RI, the ends filled in with Klenow and the gene inserted into the *SmaI* site of pEMBLyex4 (Murray *et al.*, 1987), yielding pSK19.

The *S.cerevisiae* strain L94-4D (MATa *cdc-9-7 ura3 trp1*) was used throughout. The medium was 0.67% Difco Yeast Nitrogen Base with either 2% glucose or 2% galactose and 2% raffinose as carbon sources. The appropriate nutritional requirements were added at 40 μg/ml. Cell numbers were determined using a Coulter counter (Coulter Electronics, England).

Growth of cells and viruses

WT vaccinia virus (strain WR) and recombinants derived from it were grown in CV-1 or B-SC-1 cells which were passed in minimal essential medium (Gibco). MDBK cells and GM8505 Bloom's cells were grown in Ham's F12 medium and Dulbecco's medium (Gibco), respectively. All culture media were supplemented with 10% fetal bovine serum (FBS). Virus stocks were prepared by sedimentation through a sucrose cushion (Mackett *et al.*, 1985), titrated by plaque assay on B-SC-1 cells and stored in aliquots at −70°C.

Construction of DNA ligase recombinant viruses

vSK20. Plasmid pSK20, containing a deletion in the DNA ligase gene, was constructed by digesting pSK13 (Kerr and Smith, 1991) with *NruI* and *BglII*, gel-purifying the largest DNA fragment, end-filling with Klenow and self-ligating. This removes 993 bp from the coding sequence of the DNA ligase gene. This plasmid pSK20 was transfected into cells infected with vSK14, a recombinant vaccinia virus containing a deleted DNA ligase gene and the *E.coli* guanine phosphoribosyltransferase (*gpt*) gene at this locus (Kerr and Smith, 1991). A recombinant virus lacking the *gpt* gene was selected from extracts of these cells by plaque purification on HGPRT$^-$ D98R cells in medium containing 6-thioguanine (6-TG) at 1 μg/ml in a procedure similar to that described (Isaacs *et al.*, 1990). Purified virus stocks were prepared following three plaque purifications under selection.

vSK21. A 2 kb fragment containing the DNA ligase gene and 249 bp 5′ and 79 bp 3′ was obtained by digestion of pSK13 with *MluI*, treatment with Klenow and digestion with *ClaI*. This fragment was inserted into *Eco*RI-cut, Klenow-treated and *ClaI*-cut pGS50 producing a plasmid, pSK15, which contains the DNA ligase gene flanked by thymidine kinase (TK) DNA. pGS50 was formed by treatment of pUC13 with *Eco*RI, S1 nuclease, Klenow and then *Hin*dIII. The large fragment was then ligated with a 1.8 kb *Hin*dIII–*Pvu*II DNA fragment excised from the *Hin*dIII J fragment of the Wyeth strain of vaccinia virus. pSK15 was transfected into cells infected with vSK20 and recombinant virus selected by plaque purification on TK$^-$ cells with BUdR (Mackett *et al.*, 1985). The resulting virus, vSK21, contains an intact copy of the DNA ligase gene inserted into the TK locus and a deleted version at the WT DNA ligase locus.

Analysis of virus DNA

Southern blotting, preparation of radiolabelled probes and polymerase chain reaction (PCR) were performed as described previously (Kerr and Smith, 1991).

Western blotting and immunofluorescence

Western blot analysis were performed as previously described (Rodriguez and Smith, 1990) except that the second antibody was biotinylated anti-rabbit immunoglobulin and the immune complexes were detected by incubation with diaminobenzidine (Harlow and Lane, 1988). Immunofluorescence was carried out essentially as described (Lasko *et al.*, 1990). At appropriate times post-infection, MDBK cells on glass coverslips were fixed with freshly prepared 4% paraformaldehyde in PBS for 15 min at 4°C. Cells were rinsed twice with PBS and permeabilized with 90% ethanol in Tris-buffered saline (TBS) for 2 min at 4°C. Antibodies were diluted in 20% FBS in TBS and incubated overnight with the cells. The second antibody, fluorescein-conjugated donkey anti-rabbit (Amersham), was applied at a 1:100 dilution for 1 h. The cells were then washed in TBS plus 0.05% Tween 20 followed by PBS and incubated for 8 min with 50 ng/ml Hoechst 33258 (Sigma) in PBS. The coverslips were washed in PBS and mounted in Citifluor (Amersham). The cells were photographed under oil immersion using a Zeiss Axiophot microscope with a mercury lamp and filters of 450–490 nm for fluorescein and 395–440 nm for Hoechst.

Analysis of virus sensitivity to DNA damaging agents

UV light. CV-1 cells were infected at 0.1–0.25 p.f.u./cell with either WT, vSK20 or vSK21 for 1 h after which unbound virus was removed by washing with PBS and cells incubated in MEM with 2.5% FBS for a further 1 h. Virus dilutions used for infections were titrated and found to vary less than 2.5-fold. Medium was then removed and infected cells irradiated with a germicidal UV lamp (254 nm). Fresh MEM containing 2.5% FBS was then added and the cells were incubated for a further 30 h. Cells were harvested and the infectious virus titrated on fresh monolayers of B-SC-1 cells in duplicate.

Bleomycin. Monolayers of CV-1 cells were treated with bleomycin sulphate (Sigma) for 2 h and then infected as above (i) in the presence of bleomycin and maintained with drug for 30 h. Virus recovered from infected cells was titrated on fresh monolayers of B-SC-1 cells in duplicate.

Animals

Five to six-week old female Balb/c mice were anaesthetized and infected intranasally with 20 μl of diluted virus in 1 mM Tris–HCl (pH 9.0). Mice were monitored daily for signals of illness.

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