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Vaccinia virus–bacteriophage T7 expression vector for complementation analysis of late gene processes

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A vaccinia virus–bacteriophage T7 RNA polymerase hybrid transient expression vector has been developed for complementation analysis of late gene functions in vaccinia virus. The conditionally defective virus *ts21* was modified to express the bacteriophage T7 RNA polymerase. The derived virus, vtsT7, was conditionally defective in viral late gene expression but produced high levels of a target protein under the control of a T7 promoter at non-permissive temperatures. The level of *β*-galactosidase expression under the control of a T7 promoter was slightly lower in vtsT7 infections than those with the prototypical T7 RNA polymerase vector vTF7.3. However, the levels of expression for the human immunodeficiency virus envelope gene, a protein which undergoes post-translational modification, was slightly higher in vtsT7 infections, suggesting that some proteins may be expressed better in the absence of vaccinia virus late gene expression. Infections using vtsT7 at a low m.o.i. at 39 °C resulted in the accumulation of high molecular mass, non-linear replicative intermediates of vaccinia virus DNA replication and high levels of expression of a transfected gene proximal to a T7 promoter. The virus vtsT7 provides a means for the analysis of potential *trans*-acting factors participating in vaccinia virus late processes such as resolution of DNA replicative intermediates.

Introduction

The replication of large DNA viruses proceeds via a programmed path of gene expression with the early phase of viral gene expression dedicated to the establishment and enhancement of virus replication and the late phase of viral gene expression involved in the production of the structural proteins and enzymes required for formation and maturation of infectious progeny. The prototypical member of the orthopoxvirus family, vaccinia virus, is a large double-stranded DNA virus encoding approximately 200 proteins during its replicative cycle (Moss, 1996). Early proteins participate in DNA synthesis and the generation of nucleotide precursors, the modification of the transcriptional or translational machinery for selective viral expression, and the enhancement of cell growth. After the onset of DNA replication, previously silent viral genes, the intermediate and late gene families, are expressed. The late genes include those for components of the virion capsid, the factors needed to initiate replication in the next round of replication, and the enzymes required for processing of the DNA replicative intermediates. A series of conditionally lethal viruses (Condit & Motyczka, 1981; Condit *et al.*, 1983) has been used to demonstrate that the temporal classes of genes encode discrete sets of functional activities. The constituents and activity of RNA polymerase change between utilization of early or late gene products (Hooda-Dhingra *et al.*, 1989; Kane & Shuman, 1992). Also, DNA replication in vaccinia virus has been shown to consist of two discrete phases, a synthesis phase which relies on early gene expression during which input DNA is converted into replicative intermediates, followed by a processing or resolution stage, which relies on late gene products (DeLange, 1989; Merchlinsky & Moss, 1989).

Analysis of the replicative intermediates of DNA replication by pulse-field gel electrophoresis has shown that high molecular mass, non-linear, replicative intermediates are transiently formed during the course of a wild-type infection and accumulate in cells infected by viruses blocked in late gene synthesis (DeLange, 1989; Merchlinsky & Moss, 1989).

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However, little is known about the details of the DNA structure of these replicative intermediates or the protein products which participate in their processing. We decided to utilize the block in the resolution of DNA replicative intermediates exhibited by the late defective conditionally lethal mutants to design a complementation system which can be used to identify the gene(s) which participate in resolution. The bacteriophage T7 RNA polymerase was incorporated into a conditionally lethal vaccinia virus generating a hybrid virus that expresses high levels of proteins from genes adjacent to a T7 promoter in the absence of normal vaccinia virus late gene production. Transfection of genes proximal to a T7 promoter into cells infected with vtsT7 allows one to measure the effects of a specific, exogenously added protein(s) on vaccinia virus late functions including the processing of the DNA replicative intermediates.

Methods

Cells and viruses. BSC-1 cells (ATCC CCL6) and thymidine kinase-deficient cells, 143B (ATCC CRL8303), were grown in Eagle's minimal essential medium (Gibco or Cellgro) supplemented with 10% foetal bovine serum (Hyclone). The medium for 143B cells was also supplemented with bromodeoxyuridine (Earl & Moss, 1991). CV-1 cells (ATCC CCL70) were propagated in Dulbecco's modified Eagle's medium (Gibco or Cellgro) supplemented with 10% foetal bovine serum (Hyclone). Wild-type recombinant vaccinia virus expressing T7 RNA polymerase, vTF7.3 (Fuerst *et al.*, 1986), was derived from the WR strain (ATCC Vr119). To prepare stocks of virus, monolayers of BSC-1 cells were infected with 1 p.f.u. of virus per cell at 37 °C for vaccinia virus WR or vTF7.3 and 31 °C for *ts21* and vtsT7. Cells were harvested 72 h post-infection and virus stocks prepared by three freeze–thaw cycles.

Construction of recombinant virus. To construct vtsT7 plasmid pTF7.3, which contains the T7 RNA polymerase gene behind the p7.5 promoter and flanked by DNA sequences corresponding to the vaccinia virus thymidine kinase gene (Fuerst *et al.*, 1986), was recombined into *ts21* (Condit & Motyczka,1981). Approximately 5 μg of pTF7·3 was transfected into about 2×10^6 CV-1 cells infected 2 h earlier with 1×10^5 p.f.u. of *ts21* using calcium phosphate (Merchlinsky, 1989). The cells were incubated at 31 °C with 5% CO_2 for 3 days, harvested and analysed by plaque assay on 143B cells in the presence of bromodeoxyuridine (Earl & Moss, 1991). Plaques arising in the presence of bromodeoxyuridine were used to infect secondary monolayers of 143B cells and the resulting virus stocks were tested by plaque assay on 143B cells at 31 and 40 °C to ensure the stocks were conditionally viable. Viral DNA was isolated and analysed for T7 RNA polymerase DNA sequences by Southern blot hybridization (Earl & Moss, 1990). One virus, designated vtsT7, which contained the T7 RNA polymerase gene and produced plaques only at low temperatures, was further plaque-purified by three passages on 143B cells and expanded by infecting monolayers of 143B cells.

Determination of β-galactosidase (β-Gal) activity. Plasmids were transfected into BSC-1 cells infected with vtsT7 or vTF7.3 at an m.o.i. of one using PerFect Lipid number 6 (Invitrogen) as suggested by the manufacturer. The cells were harvested 24 h post-infection, resuspended in 0·5 ml PBS, and disrupted by three cycles of freeze–thawing. The extract was clarified by a short microfuge spin (1 min, 14000 r.p.m.) and the supernatant was analysed for β-Gal units as described by Miller (1972). The A_{405} values were determined on a microplate reader (Dynatech MR3000) and the β-Gal activity was determined by comparison to β-Gal (Clontech) standards analysed in the same assay.

Western blot analysis. Plasmids were transfected into BSC-1 cells infected with vtsT7 or vTF7.3 at an m.o.i. of one using PerFect Lipid number 6 (Invitrogen) as suggested by the manufacturer. The cells were incubated at 39 °C for 24 h, harvested, incubated in RIP lysis buffer (100 mM Tris–HCl pH 8·0, 100 mM NaCl, 0·5% NP-40), and, after freezing and thawing, the debris was removed by a short microfuge spin. Proteins were separated by SDS–PAGE (Novex), transferred to Protran (Schleicher & Schuell), and blocked with 5% nonfat dry milk in PBS. The filters were probed with specific antibodies to vaccinia virus proteins, and visualized by subsequent incubations with an anti-rabbit IgG conjugated with alkaline phosphatase and Western Blue reagent (Promega).

Analysis of viral DNA. Monolayers of BSC-1 cells were infected with vtsT7. After 24 h the cells were harvested and resuspended in cell suspension buffer (10 mM Tris–HCl pH 7·2, 20 mM NaCl, 50 mM EDTA) at 10^7 cells/ml. An equal volume of 2% CleanCut agarose (Bio-Rad), preincubated at 50 °C, was added, and the suspension was formed into 100 μl plugs. After solidification at 4 °C, the plugs were treated with Proteinase K as previously described (Merchlinsky & Moss, 1989). The DNA from the equilibrated agarose plugs was electrophoresed through a 1% agarose gel on a Bio-Rad CHEF DRII apparatus for 22 h at 6 V/cm with a switching time of 70 s. The DNA was transferred to Nytran (Schleicher & Schuell) and analysed by hybridization to fluorescein-labelled vaccinia virus DNA and visualized by incubation with ECL detection reagents (Amersham) followed by exposure to Kodak Bio-Max film.

Cleavage assay for vaccinia virus topoisomerase activity. Oligonucleotides MM487 (AGTATGATTCAACATATCCGTGTCG-CCCTTATTCCGATAGTGACTACAGCGGCATGAGTG), containing the cleavage sequence CCCTT recognized by vaccinia virus topoisomerase (Shuman, 1991 *b*), and MM488 (GTGCACTCATGCCGC-TGTAGTCACTATCGGAATAAGGGCGACACGGATATGTTGAA-TCATACT), were annealed, and the double-stranded form was purified and labelled with [α-^{32}P]dCTP using DNA polymerase, Klenow fragment. Samples were incubated with the labelled substrate in 16 μl reactions for 30 min at 37 °C, treated with 1 μl 20% SDS and 1 μl 20 mg/ml Proteinase K for 1 h at 37 °C, electrophoresed through a 20% acrylamide–7·5 M urea gel, and visualized by exposure to Kodak XAR film.

Results

Construction of vtsT7

The vaccinia virus–bacteriophage T7 hybrid gene expression system has been developed for high-level gene expression in mammalian cells (Elroy-Stein *et al.*, 1989; Fuerst *et al.*, 1986). In its most common application, plasmid(s) containing a target gene placed behind the T7 promoter are transfected into cells infected with a recombinant vaccinia virus expressing the bacterial T7 RNA polymerase. In order to adapt the T7 expression protocol to serve as a complementation system for analysis of vaccinia virus late gene functions, the conditionally lethal vaccinia virus *ts21*, a member of the 'late defective' class of viral, mutants was used (Condit & Motyczka, 1981; Condit *et al.*, 1983). This viral genome contains a lesion in the vaccinia virus D7 gene, a small subunit of the viral RNA

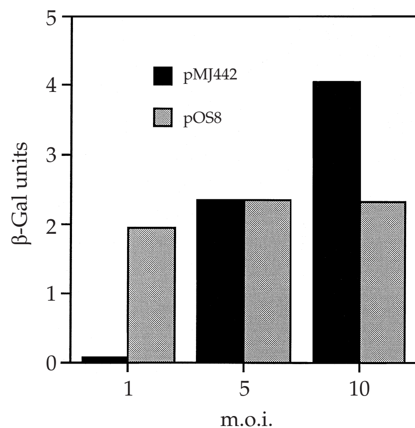

Fig. 1. Reporter gene expression depends on m.o.i. BSC-1 cells were infected with vtsT7 and transfected at 39 °C with the reporter plasmids containing the *β*-Gal gene adjacent to a strong synthetic vaccinia late (pMJ442) (Davison & Moss, 1989) or T7 promoter (pOS8) (Wyatt *et al.*, 1995). Samples were assayed for *β*-Gal on 96-well plates with 8 mM chlorophenol red (CPRG, Boehringer Mannheim) as substrate. The A_{570} values were determined on a microplate reader (Dynatech MR3000) and the *β*-Gal activity was determined by comparison to *β*-Gal (Gibco) standards analysed in the same assay.

polymerase. Infection at 39 °C leads to greatly reduced levels of late gene products and results in the accumulation of replicative intermediates of DNA replication (Merchlinsky & Moss, 1989).

The late complementation conditional expression system was constructed by recombining plasmid pTF7.3, containing the T7 RNA polymerase gene behind the p7.5 promoter and flanked with sequences derived from the vaccinia virus thymidine kinase gene (Fuerst *et al.*, 1986), into the thymidine kinase gene of *ts21* by homologous recombination. One plaque-purified virus resistant to bromodeoxyuridine, containing the T7 RNA polymerase gene and producing plaques only at low temperatures, was designated vtsT7 and used to prepare virus stocks by infecting monolayers of 143B cells.

Analysis of gene expression in cells infected with vtsT7

The utility of vtsT7 as a complementation vector for vaccinia virus late gene functions is only realized when a high level of expression of the target gene behind the T7 promoter is coupled with low levels of expression of endogenous vaccinia virus late genes. In order to establish the optimum conditions for use of vtsT7 as a complementation vector, the expression of *β*-Gal from the *lacZ* gene behind the vaccinia virus late and bacteriophage T7 promoters was compared. The constructs pMJ442, a plasmid containing the bacterial *lacZ* gene behind a synthetic strong vaccinia virus late promoter (Davison & Moss, 1989) and pOS8, a plasmid containing the same gene adjacent to a T7 promoter (Wyatt *et al.*, 1995), were used to transfect BSC-1 cells and the level of *β*-Gal activity was measured (Baldick *et al.*, 1992) (Fig. 1). High levels of *β*-Gal activity were always noted regardless of the m.o.i. for transfections with the plasmid pOS8 containing *lacZ* behind the T7 promoter. In contrast, the expression of *β*-Gal with *lacZ* adjacent to the strong vaccinia virus late promoter was directly proportional to the m.o.i. This is consistent with the observation that conditionally lethal viruses have 'leaky' phenotypes, especially at high m.o.i. (Merchlinsky & Moss, 1989). A 300 bp intermediate promoter–*lacZ* construct provided by Carl Baldick (Baldick *et al.*, 1992) was also active in cells infected with vtsT7 at 39 °C, in concurrence with previous observations (Carpenter & DeLange, 1992). Thus, vtsT7 is applicable as a complementation vector to study vaccinia virus late gene functions at an m.o.i. of approximately one.

The high levels of *β*-Gal expression by vtsT7 led us to investigate its applicability as an expression vector. The prototypical T7 expression vector vTF7.3 was constructed in the same manner as vtsT7 except that the T7 RNA polymerase gene was inserted into the vaccinia virus WR genome (Fuerst *et al.*, 1986). Plasmids pMJ442 or pOS8 were used to transfect BSC-1 cells infected with vtsT7 or vTF7.3 at an m.o.i. of one and then *β*-Gal activity was measured (Baldick *et al.*, 1992). Much lower levels of *β*-Gal expression were observed in cells infected with vtsT7 as compared to vTF7.3 at 39 °C using pMJ442, which contains *lacZ* under the control of a vaccinia virus late promoter (data not shown). This result is consistent with the defect in late gene expression seen at 39 °C for vtsT7 (see Fig. 1). Transfections with pOS8, which contain the *lacZ* gene proximal to the T7 promoter, resulted in high levels of *β*-Gal expression in both viral infections, although slightly higher levels of *β*-Gal activity were reproducibly observed in vTF7.3 infections at 39 °C (data not shown).

Expression of the T7 RNA polymerase is under the control of the vaccinia virus p7.5 promoter for both vtsT7 and vTF7.3. This promoter is normally active both early and late after infection. The difference in expression of *β*-Gal at 39 °C may simply reflect a lower level of T7 RNA polymerase production in cells infected by vtsT7 relative to vTF7.3 as the expression of late genes in vtsT7 is reduced at 39 °C. Since the expression of late genes in vaccinia virus infection depends on DNA replication, only early class genes are expressed in the presence of inhibitors of DNA replication. To determine the type of expression for the T7 polymerase in each virus, the level of *β*-Gal activity was measured for pOS8 transfections in cells infected with vtsT7 or vTF7.3 at 39 °C in the presence of cytosine arabinoside, an inhibitor of DNA replication. In cells infected with vtsT7 the level of *β*-Gal expression was much higher in the presence of cytosine arabinoside than its absence, consistent with early promoter expression of the T7 RNA polymerase in vtsT7. In contrast, cells infected with vTF7.3 demonstrated measurable but lower levels of *β*-Gal expression in the presence of cytosine arabinoside, an observation consistent with the constitutive expression of T7 RNA polymerase from this virus. The time-course of reporter gene synthesis was also measured by incubation for extended times before harvesting and analysis. For both vtsT7 and vTF7.3 the

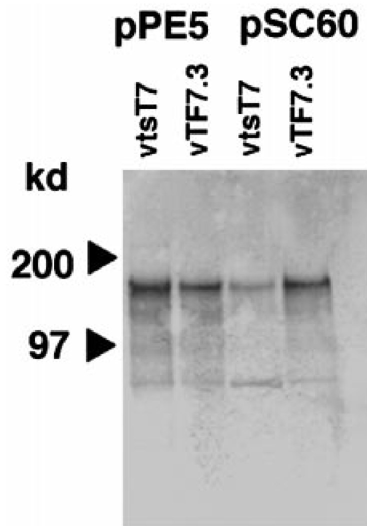

Fig. 2. Western blot analysis of HIV envelope gene expression. Plasmid pPE5 (Fuerst *et al*., 1987), which contains the envelope gene of HIV behind a T7 promoter, and pSC60 (S. Chakrabarti, unpublished), a plasmid containing the HIV envelope gene behind a vaccinia virus early/late promoter, were transfected into BSC-1 cells infected with vtsT7 or vTF7.3 at an m.o.i. of one. Samples were analysed by Western blot using a rabbit antibody against HIV envelope and visualized by incubation with Western Blue reagent (Promega) subsequent to incubation with an anti-rabbit IgG conjugated to alkaline phosphatase (Promega).

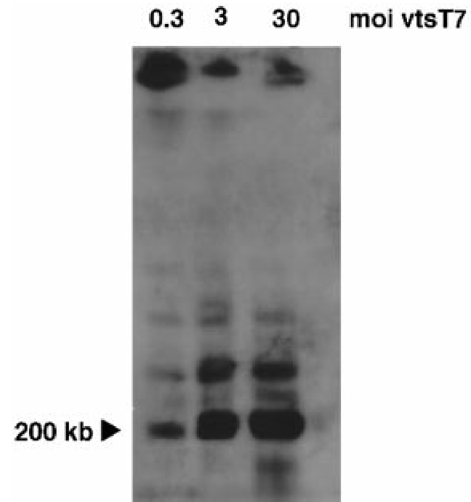

Fig. 3. Accumulation of DNA replication intermediates depends on m.o.i. for vtsT7. BSC-1 cells were infected with vtsT7 at 0·3, 3 or 30 p.f.u. per cell and after 24 h at 39 °C the cells were harvested and DNA prepared in agarose plugs (Merchlinsky *et al*., 1997). The samples were electrophoresed through 1 % agarose and analysed by Southern blotting by hybridization to ECL-labelled vaccinia virus DNA.

amount of *β*-Gal activity increased rapidly through 24 h, increased only modestly between 24 and 48 h and decreased after 48 h (data not shown).

Expression of the HIV envelope gene in cells infected with vtsT7

The expression of *β*-Gal and many other proteins proximal to a T7 promoter is generally higher than the expression of such gene products behind vaccinia virus late promoters. However, some genes, such as HIV envelope, are not efficiently expressed under control of T7 promoters (P. Earl, personal communication). The comparatively reduced expression of the HIV envelope using the T7 expression system may reflect competition, possibly with post-translational activities such as glycosylation, with the coincident expression of vaccinia virus late genes. Since the vtsT7 expression system expresses foreign genes in the absence of vaccinia virus late gene expression, we decided to measure the expression of HIV envelope gene in this system. Plasmid pPE5 (Fuerst *et al*., 1987), which contains the envelope gene of HIV behind a T7 promoter, and pSC60, containing the HIV envelope gene behind a synthetic vaccinia virus early/late promoter (S. Chakrabarti, unpublished), were transfected into BSC-1 cells infected with vtsT7 or vTF7.3 at an m.o.i. of one and the level of HIV envelope expression measured by Western blot (Fig. 2). In cells infected with vTF7.3, the expression of the HIV envelope gene from the plasmids was similar using the T7 promoter or the vaccinia virus early/late promoter (second and fourth lanes from left). In cells infected with vtsT7, the expression of HIV envelope was higher from plasmids using the T7 promoter than the vaccinia virus early/late promoter (compare first and third lanes from the left). Also, the expression of HIV envelope is slightly higher in the vtsT7-infected cells for pPE5 than for cells infected with vTF7.3 and transfected with pPE5 or pSC60 (first lane compared to the second and fourth lanes). This suggests that the vtsT7 vector may serve as an alternative to the vTF7.3 vector in cases where vaccinia virus late gene synthesis interferes with optimal foreign gene expression.

Analysis of viral DNA in vtsT7 infection

Progeny viral genomes are generated by a two-step process during a productive vaccinia virus infection. First, in a step requiring early viral gene expression, the input DNA is converted into high molecular mass non-linear molecules, the concatemeric intermediates (DeLange, 1989; Merchlinsky & Moss, 1989). The DNA replicative intermediates are converted, by a mechanism(s) requiring late vaccinia virus genes, into unit length progeny genomes (DeLange, 1989; Merchlinsky & Moss, 1989). One of the anticipated applications for vtsT7 is the complementation of late gene activities needed for vaccinia virus replication, and, in particular, those used in the processing of vaccinia virus DNA replicative intermediates. The characteristics of the vaccinia virus replicated DNA with regard to m.o.i. was investigated by infecting BSC-1 cells with varying multiplicities of vtsT7, incubating at 39 °C for 24 h, harvesting the cells and analysing the viral DNA by Southern blotting after pulse-field gel electrophoresis. The majority of the viral specific DNA produced during vtsT7 infection at a low m.o.i. (0·3) was retained in the well of the agarose gel (Fig. 3). This material is composed of the high

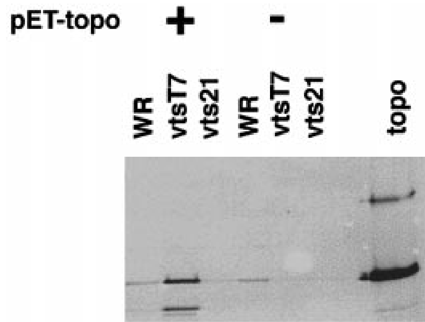

Fig. 4. Expression of vaccinia virus topoisomerase. Plasmid pET-topo, containing the vaccinia virus topoisomerase under the control of the bacteriophage T7 promoter (Shuman *et al*., 1988), was transfected into cells infected with WR, *ts21* or vtsT7. Samples were analysed by Western blotting using a rabbit antibody against vaccinia virus topoisomerase followed by incubation with an anti-rabbit alkaline phosphatase conjugate (Promega) and visualized using Western Blue stabilized substrate for alkaline phosphatase (Promega).

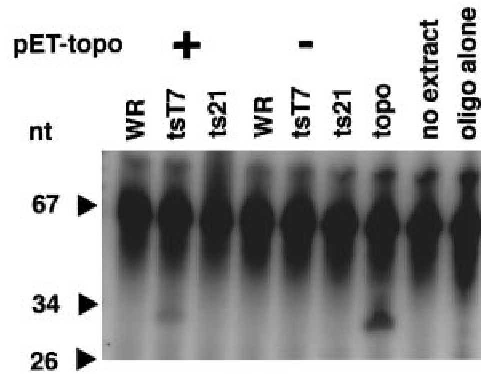

Fig. 5. Cleavage assay for vaccinia virus topoisomerase activity. Oligonucleotides MM487 (AGTATGATTCAACATATCCGTGTCGCCCTTATT-CCGATAGTGACTACAGCGGCATGAGTG), containing the cleavage sequence CCCTT recognized by vaccinia virus topoisomerase, and MM488 (GTGCACTCATGCCGCTGTAGTCACTATCGGAATAAGGGCGACAC-GGATATGTTGAATCATACT), were annealed, and the double-stranded form was purified, labelled with [α-^{32}P]dCTP and incubated with cell extracts, purified topoisomerase supplied by Stewart Shuman or nothing. The samples were electrophoresed through a 20% acrylamide–7·5 M urea gel. The expected cleavage fragment is 31 nucleotides.

molecular mass non-linear DNA replicative intermediates. At higher multiplicities a larger percentage of the material is converted to unit length DNA. The blot demonstrates that infections with vtsT7 at relatively low m.o.i. generate the DNA replicative intermediates in sufficient quantities for complementation studies.

Expression and activity of vaccinia virus topoisomerase in cells infected with vtsT7

One application of the vtsT7 virus is measurement of the activity of specific candidate proteins in the absence of vaccinia virus late genes to determine their effect on the distribution of the DNA replicative intermediates. First, we wanted to test the vtsT7 vector by measuring the expression and activity of a candidate resolvase vaccinia virus late gene. The gene product of H7R, a virally encoded topoisomerase (Shuman & Moss, 1987), has been demonstrated to mediate recombination in prokaryotes and has been proposed to participate in genome resolution in vaccinia virus (Shuman, 1991*a*). The plasmid pET-topo, containing the vaccinia virus topoisomerase under the control of the bacteriophage T7 promoter (Shuman *et al*., 1988), was transfected into BSC-1 cells infected with vaccinia virus WR, *ts21* or vtsT7. The quantity of vaccinia virus topoisomerase protein was determined by Western blotting of infected cells using a rabbit antibody against the topoisomerase (Fig. 4). In cells infected with WR the same level of topoisomerase was noted in both transfected and non-transfected samples (first and fourth lanes from left). Very little, if any, topoisomerase was detected for cells infected with *ts21*. Infection with vtsT7 resulted in levels of topoisomerase equivalent to infection with *ts21* (fifth lane from left) whereas addition of pET-topo resulted in large amounts of topo-isomerase (second lane from left). The cross-reacting material with a more rapid mobility is of unknown origin.

A novel property of vaccinia virus topoisomerase is its ability to cleave duplex DNA at the conserved sequence element 5′ (C/T)CCTT (Shuman, 1991*b*). In order to demonstrate that the presence of topoisomerase as shown by Western blot in Fig. 4 corresponds to the expression of enzymatically active vaccinia virus topoisomerase, each extract was incubated with a double-stranded oligomer containing the cleavage sequence for the topoisomerase (Fig. 5). Sufficient activity for cleavage was observed only when the oligomer was incubated with purified vaccinia virus topoisomerase (seventh lane from left) or the extract derived following transfection of pET-topo into cells infected with vtsT7 (second lane from left). The relative amount of topoisomerase activity, as determined by reduction of superhelicity (Shuman *et al*., 1988), was also measured for each extract and shown to correspond to the protein level detected by Western blot in Fig. 4. The activity for pET-topo in extracts from vtsT7 infections was approximately tenfold higher than derived from extracts of cells infected with WR. Southern blot analysis of the vtsT7 infections using the procedure described for Fig. 3 did not show a difference in the distribution of DNA replicative intermediates in the presence or absence of transfected pET-topo (data not shown), demonstrating that expression of vaccinia virus topoisomerase alone is not sufficient for resolution of the DNA replicative intermediates.

Discussion

The vector vtsT7 provides a complementation system for the analysis of late gene functions in vaccinia virus. The activity of specific candidate proteins can be checked in the absence of other late genes by transfection of the open reading

frame proximal to a T7 promoter into cells infected with vtsT7 at an m.o.i. of one. Vaccinia virus late processes such as telomere and branch resolution, protein processing and the assembly of the intermediates for the morphogenesis pathway can be investigated.

In this paper we have described the construction of vtsT7 and its initial application in elucidating genes that participate in telomere resolution. The data demonstrate that a plasmid encoding the vaccinia virus topoisomerase is efficiently expressed in cells infected with vtsT7, although the expression of that gene alone is not sufficient for telomere resolution. Plasmids containing candidate genes proximal to the T7 promoter can be transfected individually or in combination to screen for the components needed for resolution. A gene implicated in resolution of bacteriophage T4 replicative intermediates, the T4 endo VII gene, was enzymatically active when a plasmid containing the gene behind a T7 promoter was transfected into cells infected with vtsT7 (data not shown). Present efforts are directed towards determining if T4 endo VII alone, batteries of candidate vaccinia virus proteins, or combinations of T4 endo VII and vaccinia virus candidate proteins will provide the activities necessary for resolution.

The activity of a transfected protein on the components of a viral infection will only be manifested if the transfection is efficient. The fraction of cells in a vtsT7 infection transfected with pOS8 was measured by staining treated cells with X-Gal (Wyatt *et al*., 1995). Approximately 60% of the cells were shown to express β-Gal (data not shown). Since at an m.o.i. of one not all cells are infected the actual percentage of transfected cells is probably higher. Presently, transfections using different protocols and cell lines with β-Gal reporter genes are being examined to determine which combination will generate the optimum signal and highest percentage of transfected cells.

The accumulation of the non-linear, high molecular mass DNA molecules in the conditionally lethal late defective viruses provides an opportunity for the purification and determination of the structure of the vaccinia virus DNA replicative intermediates. The protocols originally developed for the purification of the replicating factories or 'virosomes' (Moyer & Graves, 1981) may be applicable to the isolation of the DNA replication intermediates from *ts21* or vtsT7 infections. Therefore, studies are under way to isolate the resulting structures to allow determination of the physical nature of the branch-point and the design of an *in vitro* system for the analysis of candidate genes involved in resolution.

Although the virus vtsT7 was originally designed to provide a tool for a complementation assay to study vaccinia virus DNA resolution, the absolute level of expression using vtsT7 was comparable to that for the vaccinia virus WR T7 expression vector, vTF7.3, for the β-Gal reporter gene. Also, the expression of the HIV envelope, a protein which undergoes extensive post-translational modification, was slightly higher in cells infected with vtsT7 than vTF7.3, implying this vector may be a useful alternative when the expression of vaccinia virus late genes interferes with optimum expression of the target gene. Another advantage to the use of vtsT7 is the higher level of safety for replication defective T7 expression vectors (Wyatt *et al*., 1995) as vtsT7 does not grow at 37 °C (data not shown). Therefore, this virus provides a means to unravel the steps, by the selective expression of potential proteins required for each process, of vaccinia virus late gene processes. We plan to utilize this vector for the elucidation and characterization of the products required for the resolution of the DNA replicative intermediates during vaccinia virus replication.

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References

Baldick, C. J., Jr, Keck, J. G. & Moss, B. (1992). Mutational analysis of the core, spacer, and initiator regions of vaccinia virus intermediate-class promoters. *Journal of Virology* **66**, 4710–4719.

Carpenter, M. S. & DeLange, A. M. (1992). Identification of a temperature-sensitive mutant of vaccinia virus defective in late but not intermediate gene expression. *Virology* **188**, 233–244.

Condit, R. C. & Motyczka, A. (1981). Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. *Virology* **113**, 224–241.

Condit, R. C., Motyczka, A. & Spizz, G. (1983). Isolation, characterization and physical mapping of temperature-sensitive mutants of vaccinia virus. *Virology* **128**, 429–443.

Davison, A. J. & Moss, B. (1989). Structure of vaccinia virus late promoters. *Journal of Molecular Biology* **210**, 771–784.

DeLange, A. M. (1989). Identification of temperature-sensitive mutants of vaccinia virus that are defective in conversion of concatemeric replicative intermediates to the mature linear DNA genome. *Journal of Virology* **63**, 2437–2444.

Earl, P. L. & Moss, B. (1990). Expression of proteins in mammalian cells using vaccinia. In *Current Protocols in Molecular Biology*, pp. 16.15.1–16.18.10. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: John Wiley.

Earl, P. L. & Moss, B. (1991). Generation of recombinant vaccinia viruses. In *Current Protocols in Molecular Biology*, pp. 16.17.1–16.17.16. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Greene Publishing Associates/Wiley Interscience.

Elroy-Stein, O., Fuerst, T. R. & Moss, B. (1989). Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5′ sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. *Proceedings of the National Academy of Sciences, USA* **86**, 6126–6130.

Fuerst, T. R., Niles, E. G., Studier, F. W. & Moss, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proceedings of the National Academy of Sciences, USA* **83**, 8122–8126.

Fuerst, T. R., Earl, P. L. & Moss, B. (1987). Use of a hybrid vaccinia virus–T7 RNA polymerase system for expression of target genes. *Molecular and Cellular Biology* **7**, 2538–2544.

Hooda-Dhingra, U., Thompson, C. L. & Condit, R. C. (1989). Detailed phenotypic characterization of five temperature-sensitive mutants in the

22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. *Journal of Virology* **63**, 714–729.

Kane, E. M. & Shuman, S. (1992). Temperature-sensitive mutations in the vaccinia virus H4 gene encoding a component of the virion RNA polymerase. *Journal of Virology* **66**, 5752–5762.

Merchlinsky, M. (1989). Intramolecular homologous recombination in cells infected with temperature-sensitive mutants of vaccinia virus. *Journal of Virology* **63**, 2030–2035.

Merchlinsky, M. & Moss, B. (1989). Resolution of vaccinia virus DNA concatemer junctions requires late gene expression. *Journal of Virology* **63**, 1595–1603.

Merchlinsky, M., Eckert, D., Smith, E. & Zauderer, M. (1997). Construction and characterization of vaccinia direct ligation vectors. *Virology* **238**, 444–451.

Miller, J. (1972). *Experiments in Molecular Genetics*, pp. 352–355. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Moss, B. (1996). *Poxviridae*: the viruses and their replication. In *Virology*, 3rd edn, pp. 2637–2672. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott–Raven.

Moyer, R. W. & Graves, R. L. (1981). The method of cytoplasmic orthopox replication. *Cell* **27**, 391–401.

Shuman, S. (1991*a*). Recombination mediated by vaccinia virus DNA topoisomerase I in *Escherichia coli* is sequence specific. *Proceedings of the National Academy of Sciences, USA* **88**, 10104–10108.

Shuman, S. (1991*b*). Site-specific DNA cleavage by vaccinia virus DNA topoisomerase I. *Journal of Biological Chemistry* **266**, 1796–1803.

Shuman, S. & Moss, B. (1987). Identification of a vaccinia virus gene encoding a type I DNA topoisomerase. *Proceedings of the National Academy of Sciences, USA* **84**, 7478–7482.

Shuman, S., Golder, M. & Moss, B. (1988). Characterization of vaccinia virus DNA topoisomerase I expressed in *Escherichia coli*. *Journal of Biological Chemistry* **263**, 16401–16407.

Wyatt, L. S., Moss, B. & Rozenblatt, S. (1995). Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* **210**, 202–205.

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