Detection of a protein encoded by the vaccinia virus C7L open reading frame and study of its effect on virus multiplication in different cell lines

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Vaccinia virus encodes several proteins, the activity of which is essential for multiplication in different cell types. Both the C7L and K1L open reading frames (ORFs) have been characterized as viral determinants for multiplication in human cells. To confirm and extend these findings we inserted the C7L ORF into the genome of a mutant virus unable to multiply in human cells and showed that this virus recovered its ability to replicate. Deletion of C7L from a wild-type viral genome did not adversely affect virus multiplication in human cells but it did reduce replication in hamster Dede cells. When both C7L and K1L were deleted from the vaccinia virus genome only poor or no viral yields were obtained from various human cell lines. Recombinant viruses were also constructed to facilitate the study of C7L protein synthesis during infection. One virus in which the *lacZ* ORF was fused downstream and in-frame with the C7L ORF enabled us to characterize the C7L protein as an early gene product. Another recombinant virus was constructed so that the carboxy terminus of the C7L ORF product contained an additional 28 amino acids from the carboxy terminus of K1L. Tagging of C7L in this way allowed us to detect the fusion protein by immunoprecipitation with antibodies against the K1L protein. Furthermore, the hybrid protein retained its biological properties. The recombinant viruses constructed in this work should be useful for studies of the molecular basis of the activity of viral host range proteins.

Vaccinia virus (VV), the prototype member of the Orthopoxvirus genus within the Poxvirus family, possesses a 190 kbp DNA genome that encodes many of the enzymes necessary for DNA replication and transcription and which thus endows the virus with considerable autonomy with respect to cellular functions (Moss, 1990). A notable feature of VV is its ability to multiply in a large number of cell types from various species. The isolation of VV host range mutants which are unable to multiply in some cell types has suggested that viral functions are involved in determining such a wide host range. One mutant isolated in our laboratory from the Copenhagen strain, designated VV hr, has 18 kbp deleted near the left end of the genome (Drillien *et al.*, 1981). Similar mutants have been isolated by multiple passages of the CVA strain on chick embryo fibroblasts (Mayr *et al.*, 1975; Altenburger *et al.*, 1989; Meyer *et al.*, 1991) or by directed deletion of VV sequences using recombinant plasmids (Perkus *et al.*, 1991). The VV hr mutant we isolated could not multiply on a number of human cell lines nor on the rabbit kidney cell line RK13. In previous work, the original host range phenotype was restored by inserting a single gene from wild-type (wt) VV into the thymidine kinase (tk) locus of VV hr (Gillard *et al.*, 1985, 1986). This gene, designated K1L according to the nomenclature of Rosel *et al.* (1986) for open reading frames (ORFs) on the *Hin*dIII restriction map of VV, was shown to encode an early protein of M_r 30K (Gillard *et al.*, 1986). Unexpectedly, when the K1L gene was deleted from wt VV, the virus retained the ability to multiply in human cells but lost the capacity to multiply in RK13 cells (Perkus *et al.*, 1990; Wild *et al.*, 1992). This suggested that another gene functionally equivalent to K1L for multiplication in human cells was present in the VV genome. Indeed, Perkus *et al.* (1990) discovered that the C7L ORF on the *Hin*dIII restriction map of VV displays the properties predicted for a functional homologue of the K1L ORF. In an independent search for a VV host range gene with similar properties to K1L we have confirmed the host range function of the C7L ORF. We have also studied the influence of this gene on VV multiplication in a variety of cell types. Furthermore, we investigated the expression of the C7L ORF by assaying

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Table 1. *Viral multiplication on HEp2 cells*

Virus	Viral titre*		
	T^0	T^{48}	T^{48}/T^0
VV wt	$4{\cdot}7 \times 10^4$	$3{\cdot}0 \times 10^8$	6383
VV hr	$2{\cdot}9 \times 10^4$	$6{\cdot}6 \times 10^4$	2
VVIV038	$3{\cdot}1 \times 10^4$	$4{\cdot}1 \times 10^7$	1322
VVIV047	$3{\cdot}5 \times 10^4$	$4{\cdot}1 \times 10^7$	1171

* Viral titres were determined by plaque titration on BHK21 cells from samples frozen immediately after the adsorption period (T^0) or 48 h after infection. The ratios of the titres determined after 48 h over the titres at the beginning of infection are shown in the right hand column.

for β-galactosidase activity of a C7L–*lacZ* fusion gene or by immunoprecipitating the C7L protein tagged at its carboxy terminus with an immunogenic peptide.

In an initial series of experiments we undertook a search for a host range gene equivalent to K1L by constructing viral recombinants derived from VV hr into which DNA fragments from wt virus had been inserted. We reasoned that the gene in question should overlap the two contiguous *Eco*RI fragments initially used to isolate the K1L gene (Gillard *et al.*, 1985) and that failure to identify a second host range gene in previous work could have been due to its inactivation by an *Eco*RI cut. The *Sal*I G fragment of wt VV previously cloned in the plasmid vector pAT153 (Drillien & Spehner, 1983) was a particularly useful starting point because this fragment, deleted in VV hr, overlaps both *Eco*RI sites used in the previous studies. In order to determine which of the *Eco*RI sites was situated in the hypothetical gene, we subcloned two separate fragments which each overlapped one of the *Eco*RI sites of the *Sal*I G fragment to the tk locus of a VV transplacement vector, pTG186 (Kieny *et al.*, 1984). The two plasmids obtained were then used to generate tk^- viruses from VV hr using a procedure previously described to identify the K1L ORF (Gillard *et al.*, 1985, 1986). Of the two viral recombinants isolated only the one spanning the *Eco*RI site furthest to the left was capable of multiplication in HEp2 cells (data not shown). This result led us to construct an additional plasmid containing a 2·1 kbp *Bam*HI fragment surrounding the left-hand *Eco*RI site which was then used to generate a tk^- recombinant virus from the hr virus and which was designated VVIV038. This virus multiplied successfully in HEp2 cells (Table 1) indicating that a host range gene had been successfully transferred into its genome. The 2·1 kbp *Bam*HI fragment spanned three intact ORFs, C5L, C6L and C7L, on the *Hin*d III map of VV (Kotwal & Moss, 1988; Goebel *et al.*, 1990). The C7L ORF was the only one to contain an *Eco*RI site and the only one which was expected according to Perkus *et al.* (1990) to encode a host range gene.

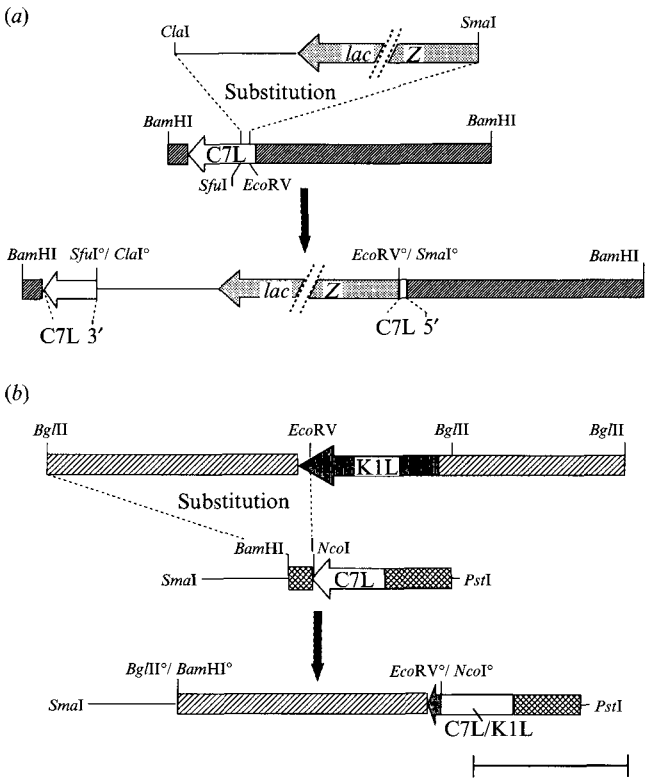

Fig. 1. Schematic representation of plasmid constructions used to inactivate or add a tag sequence to the C7L ORF. (*a*) A *Cla*I–*Sma*I fragment harbouring the *lacZ* gene replaced a *Sfu*I–*Eco*RV fragment within the C7L ORF. Note that the bacterial replicons outside the regions of interest are not represented. (*b*) A *Bgl*II–*Eco*RV fragment containing the 3′ end of the K1L ORF replaced the *Bam*HI–*Nco*I fragment overlapping the 3′ end of the C7L ORF. Genes of interest (*lacZ*, C7L, K1L) are represented with arrows. VV regions are shown as boxes hatched differently to indicate their origins. Thin lines symbolize bacterial plasmids which were recombined with the VV fragments. Restriction sites labelled with ° were modified by the cloning procedure. The bar marker represents 1000 bp on all except the *lacZ* ORF.

To confirm these findings we sought to inactivate the C7L gene in wt VV. For this purpose, a *lacZ* gene lacking a methionine initiation codon was isolated from pMC1871 (Shapira *et al.*, 1983) and was cloned 14 codons downstream and in-frame with the initiation codon of the C7L ORF in a plasmid vector harbouring the 2·1 kbp *Bam*HI fragment used previously. This was carried out by inserting the *lacZ* ORF, as a *Sma*I–*Cla*I fragment, into the C7L gene cut with *Eco*RV and *Sfu*I (Fig. 1*a*). In the process, a total of 22 codons were deleted from C7L. The construct, pIV043, placed the *lacZ* gene under the control of the presumed transcription and translation signals of the C7L ORF. pIV043 was transfected into cells previously infected with wt VV and the progeny were screened by the blue plaque method (Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986) for expression of β-galactosidase activity. Blue

Table 2. *Ratio of viral yields on various cell types**

Virus	Phenotype	Cell type†						
		RK13	Dede	BRL	NRK	HEp2	MRC_5	HeLa
VV wt	$K1L^+$ $C7L^+$	298	151	1743	540	3934	856	4098
VVTG2131	$K1L^-$ $C7L^+$	0·4	192	1546	0·7	7438	747	2312
VVIV043	$K1L^+$ $C7L^-$	464	4	585	0·1	7380	1259	8725
VVIV043-3128	$K1L^-$ $C7L^-$	0·8	18	196	0·2	0·5	26	4
VV hr	$K1L^-$ $C7L^-$	0·5	4	187	0·1	1·4	29	2

* Cells grown in monolayers were infected with the indicated viruses at about 1 p.f.u./cell and frozen either after 1 h of adsorption (input virus) or after 48 h of infection (yield). Viral input and yield titres were determined by plaque formation on BHK21 cells and the ratio of yield to input was calculated.

† The species of origin of the cells is as follows: rabbit (RK13), hamster (Dede), rat (BRL, NRK), human (HEp2, MRC_5 and HeLa).

plaques were isolated purified to homogeneity by several rounds of plaque formation and one, designated VVIV043, was studied further. Analysis of the DNA restriction profiles of the recombinant virus, by hybridization of selected probes to Southern blots, revealed the expected pattern for a double crossover event which would inactivate the C7L ORF (results not shown). This virus, however, was shown to multiply as efficiently as wt virus in HEp2 cells (Table 2) suggesting that C7L is not required for multiplication in this cell line.

If, as previous work indicated, the C7L gene is a functional equivalent of the K1L gene, then deletion of both the K1L and C7L genes should disable VV multiplication in HEp2 cells. To construct a recombinant virus lacking both C7L and K1L chick embryo fibroblasts were co-infected with VVIV043 and VV-NP. K1L is deleted in the latter, giving rise to plaques on permissive cells in which cell aggregation is considerably less extensive than in wt VV plaques (Wild *et al.*, 1992). Recombinant virus that retained both the modifications introduced into the parental viruses could be recognized, therefore, by visual screening for the plaque morphology typical of a K1L-defective virus and for the blue plaque phenotype. Thus, a recombinant virus of this kind, VVIV043-3128, was isolated. This virus, in contrast to the two parental viruses from which it was derived, was unable to multiply in HEp2 cells (Table 2), confirming the requirement for either of C7L or K1L.

Further virus yield experiments were carried out to determine whether the C7L gene might be essential for multiplication in a particular cell line. For these studies we compared virus yields obtained with wt VV, viral mutants defective for K1L (VVTG2131) or C7L (VVIV043), viral mutants defective for both K1L and C7L (VVIV043-3128) and VV hr with a deletion of 18 kbp overlapping K1L and C7L (Table 2). The increase in titre of wt VV over a 48 h infection period varied considerably from one host cell to another. Hamster Dede cells were the least successful host cells, and HEp2 and HeLa were the best. The mutant lacking K1L failed to multiply in rabbit RK13 and rat NRK cells, but multiplied in the other cell lines tested. The mutant lacking C7L multiplied at only a low level in Dede and NRK cells. Deletion of both C7L and K1L completely abolished or considerably reduced multiplication in all of the cell types tested (Table 2). This behaviour was identical for the VV hr mutant. In contrast, all of the viruses multiplied equally well in BHK21 or chick embryo fibroblasts (results not shown).

We took advantage of the VV recombinant that contained a *lacZ* ORF downstream and in-frame with the C7L ORF to study the time of expression of this gene. BHK21 cells were infected with VVIV043 and β-galactosidase activity was determined at various intervals after infection (Sambrook *et al.*, 1989). β-Galactosidase activity increased as early as 2 h post-infection (p.i.) until 5 h p.i., and was not inhibited by the addition of 1 mM-cytosine arabinoside hydrochloride (results not shown), indicating that C7L is expressed early in infection.

To enable more direct detection of the product of the C7L ORF this gene was tagged at its 3′ end with the carboxy-terminal codons from K1L. In previous work, a rabbit serum had been raised against the 19 carboxy-terminal amino acids of K1L (Gillard *et al.*, 1989) that could potentially be used to identify any protein containing the same amino acid sequence. Construction of a fusion gene was carried out by inserting an *Eco*RV–*Bgl*II fragment overlapping the 3′ end of the K1L ORF into the *Nco*I–*Bam*HI-cut end of the C7L ORF (Fig. 1*b*). The *Nco*I site of C7L was filled in with nucleotides, using the DNA polymerase I Klenow fragment to enable ligation to the blunt-ended *Eco*RV site of K1L. This procedure deleted the last codon of C7L and added 28 codons from K1L. The fusion gene was then inserted as a *Sma*I–*Pst*I fragment into the tk locus of the plasmid vector pTG186 cut with the same

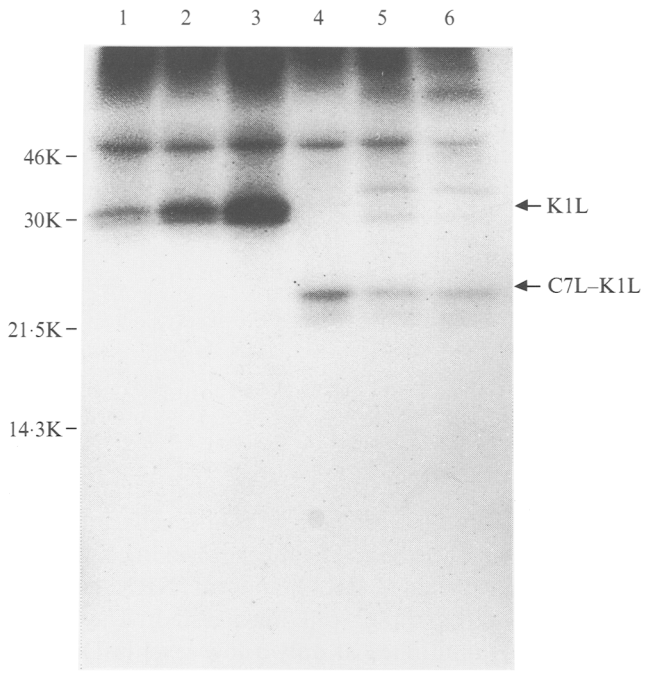

Fig. 2. Immunoprecipitation of the C7L tagged protein from infected cells. BHK21 cells were infected with wt VV (lanes 1, 2 and 3) or VVIV047 (lanes 4, 5 and 6). After 1 h (lanes 1 and 4), 3 h (lanes 2 and 5) or 5 h (lanes 3 and 6) of infection cells were labelled with [^{35}S]methionine for 2 h. Cell lysates were then prepared and immunoprecipitated with an anti-K1L rabbit antibody. Immunoprecipitated proteins were separated on a 15% SDS–polyacrylamide gel and the gel was treated for fluorography and dried. The position of the M_r standards is given on the left.

enzymes. Employing the method described previously (Gillard *et al.*, 1985) the plasmid carrying the fusion gene was used to generate a tk$^-$ virus from VV hr. A tk$^-$ virus, VVIV047, was isolated on Ltk cells in the presence of 100 μg/ml 5′ bromodeoxyuridine. This virus was able to multiply in HEp2 cells (Table 1), in contrast to the parental VV hr, demonstrating that addition of the K1L tag sequence did not alter the biological properties of the C7L gene. To visualize synthesis of the C7L tagged protein, BHK21 cells were infected with 10 p.f.u./cell of VVIV047 or wt VV, and proteins were labelled with 50 μCi/ml [^{35}S]methionine in methionine-free MEM medium. Cell lysates were prepared, immunoprecipitated with rabbit serum directed against the peptide tag, and proteins were separated on a 15% polyacrylamide gel (Fig. 2). Cells infected with VVIV047 yielded a protein of M_r nearly 22K, which corresponds closely to the value expected for the C7L fusion protein (18K from the C7L ORF and 4K from the K1L ORF). This protein, which was not found in wt VV-infected cells, was synthesized as early as the first 2 h labelling period. Immunoprecipitates prepared from cells infected with wt VV contained a protein of M_r 30K corresponding to the product of the K1L ORF.

This investigation clearly demonstrated that the VV C7L ORF is involved in the determination of VV host range in tissue culture. In some cell lines (HEp2, MRC_5 and HeLa) C7L and K1L behaved as equivalent genes despite the fact that they display no similarity at amino acid level. Thus, deletion of one gene could be compensated for by the presence of the other. In other cell lines, such as RK13 and Dede, either the K1L (RK13) or the C7L (Dede) gene alone was critical for viral multiplication. In NRK cells, in contrast, VV required both C7L and K1L for efficient multiplication. Previous work has identified a third host range gene in cowpox virus (Spehner *et al.*, 1988) that displayed no amino acid similarity to the K1L and C7L ORFs but which can substitute for either one of these in a number of host cells (Perkus *et al.*, 1990). An ORF identified by Takahashi-Nishimaki *et al.* (1991) also appears to be involved in VV host range but cannot substitute for the other known host range genes. These findings can be interpreted in several ways. K1L, C7L and the cowpox virus host range gene products could have different targets in an infected cell but which are part of the same pathway. Then, in specific cell lines one or several of the targets might not be operative. On the other hand, the targets of the host range genes could be identical in all cell types but species variation could affect the amino acid sequence of the targets and therefore their ability to interact with the host range proteins. Further understanding of this requires the identification of the target molecules.

In previous work, Belle-Isle *et al.* (1981) mapped, by cell-free translation of VV hybrid-selected mRNA, a protein (of M_r 19K) to a region at the left-hand end of the viral genome which corresponded approximately to the position of the C7L ORF. Wittek *et al.* (1981) went on to map the 5′ and 3′ ends of a 760 nucleotide early mRNA that coincided precisely with the predicted mRNA product of the C7L ORF. They also identified a polypeptide of 18K as the product of the 760 nucleotide mRNA, by using cell-free translation of hybrid-selected early mRNA. Sequencing of the VV genome by Kotwal & Moss (1988) and Goebel *et al.* (1990) has established the nucleotide sequence of the C7L ORF and its deduced amino acid sequence. Knowledge of these sequences enabled us to construct recombinant viruses in which the C7L ORF was disrupted by an in-frame *lacZ* ORF or was modified by addition of a heterologous carboxy-terminal peptide. These recombinant viruses were used to confirm that C7L is an early gene that encodes the expected polypeptide during infection. Interestingly, tagging C7L with an additional 28 amino acids did not affect its biological activity. This tag has also been added to a number of other VV proteins with similar results,

establishing it as a useful tool for identifying viral gene products from known sequences.

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