Cloning and Characterization of the Gene Encoding the Major Protein of the A-type Inclusion Body of Cowpox Virus

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SUMMARY

We have mapped and determined the nucleotide sequence of the cowpox virus (CPV) gene coding for the protein of A-type inclusion bodies (ATI). The ATI gene was mapped to the central part of the largest HindIII restriction fragment of the CPV genome. Vaccinia virus (VV) also contained a similar sequence at the equivalent position in its genome, which even under stringent conditions hybridized with the CPV ATI gene. Nucleotide sequence analysis revealed that the upstream region of the coding frame contained a sequence closely resembling the VV late promoter consensus sequence and that the gene encoded a protein of Mr 150000.

INTRODUCTION

Cowpox virus (CPV) is a member of the orthopoxvirus genus of the family Poxviridae. Its genome is about 220 kb long [40 kb longer than that of vaccinia virus (VV)] and contains approximately 200 genes, the expression of which is temporally regulated. CPV resembles VV in that they have the same morphology and possess common antigens. Their DNAs cross-hybridize, and the central regions of their genomes have essentially the same restriction endonuclease sites (Archard & Mackett, 1979; Dales & Pogo, 1981). In addition, mixed infection with CPV and VV results in the generation of recombinants (our unpublished observation). Thus CPV and VV appear to have similar, if not identical, mechanisms of gene expression (Moss, 1985).

Gene expression of VV is the best studied among the orthopoxviruses. Early genes are transcribed within minutes after infection, whereas late genes are expressed after the onset of viral DNA replication. Structural and functional analyses of viral polypeptides synthesized in infected cells have revealed at least two late gene subsets that can be defined temporally (Pennington, 1974; Ichihashi et al., 1971). The fine structures of several early and late VV genes have been determined (Bertholet et al., 1985, 1986; Cochran et al., 1985; Plucienniczak et al., 1985; Hänggi et al., 1986; Rosel et al., 1986; Weir & Moss, 1987; Weinrich & Hruby, 1986; Niles et al., 1986; Earl et al., 1986), and comparison of nucleotide sequences upstream from these genes has revealed that VV promoters lack both eukaryotic and prokaryotic consensus sequences. Instead they have characteristic signals (Venkatesan et al., 1981, 1982; Weir & Moss, 1983) recognized by VV RNA polymerase but not by the cellular RNA polymerase II (Puckett & Moss, 1983). However, nothing is known about the mechanism dictating the expression of the late gene subsets.

CPV characteristically induces large cytoplasmic inclusions called A-type inclusions (ATIs) (Ichihashi et al., 1971; Shida et al., 1977a, b) in infected cells, a feature commonly used as a morphological marker for differentiation of CPV from vaccinia and smallpox viruses. ATI protein is one of the most abundant viral products, accounting for as much as half of all synthesis during the most rapid phase of ATI formation (Ichihashi & Dales, 1973). Mature viruses are occluded into the ATI, and it has been assumed, by analogy with the inclusions of insect viruses,
that such bodies protect the virus during dissemination from animal to animal (Bergion & Dales, 1971). It has been reported that the ATIs are composed of a single polypeptide of Mr 160000 (160K) (Shida et al., 1977a, b), and that VV also induces an antigenically related polypeptide of 94K despite the morphological absence of ATIs (Patel et al., 1986).

ATIs are synthesized very late in the viral infection cycle, beginning after the commencement of virus maturation (Ichihashi & Dales, 1973). Thus, this has been classified as a late-late function, along with synthesis of VV haemagglutinin (HA) (Ichihashi & Dales, 1973) and the VO factor, which controls virus occlusion into the ATI (Shida et al., 1977a, b). The times at which these products are synthesized are closely related to their various roles in virus dissemination.

VV is now being used as a eukaryotic cloning and expression vector (Mackett & Smith, 1986) and, eventually, it may be used as a polyclonal vaccine (Perkus et al., 1985). The ATI gene may be non-essential for viral replication either in cells or animals injected by scarification, because ATI functions in CPV host-to-host dissemination, and an ATI-related truncated protein exists in VV-infected cells. Thus, in conjunction with its efficient expression, either ATI or the related VV gene may be a good candidate for sites in which foreign genes can be inserted.

To elucidate the expression mechanism of the ATI gene and mode of ATI formation, and to provide a basis for construction of recombinant VV, we cloned the ATI gene and examined its structure. This paper describes the location and nucleotide sequences of the ATI gene and its flanking region.

**METHODS**

**Cells and viruses.** HeLa, RK13, Vero and CV-1 cells and poxviruses were propagated as described previously (Ichihashi et al., 1971). CPV strain CPRO6, which induces ATIs containing no virions (V- phenotype) (Ichihashi & Matsumoto, 1968) and VV strains WR and IHD-J were used. Infection of cells, titration and purification of virus were done as described previously (Joklik, 1962).

**Isolation of A-type inclusions.** To obtain a sufficient amount of ATIs in a highly purified state, a new procedure was devised. Vero cells (2 × 10^8), which had been infected with CPRO6 for 24 h at a multiplicity of 5 p.f.u. per cell, were scraped off the walls of Roux bottles, and pelleted by low speed centrifugation. The cells were washed twice with 50 ml TNC buffer (10 mM-Tris-HCl pH 7.2, 0.15 M-NaCl, 1 mM-CaCl_2) and suspended in 10 ml 10 mM-Tris-HCl pH 7.2 and 1 mM-CaCl_2. The cells were allowed to swell for 10 min at 0°C, lysed by 10 to 15 strokes of a Dounce homogenizer, and was incubated for 30 rain at 0 °C. The material was then centrifuged at 800 g for 10 min for centrifugation, in order to prevent loss of ATIs due to adsorption to the centrifuge tube walls. The pellet, containing the ATIs, was suspended in 25 ml TD buffer (0.15 M-Tris-HCl pH 7.2, 0.1% sodium deoxycholate) with the aid of 15 s sonication, and was incubated for 30 min at 0 °C. The material was then centrifuged at 800 g for 10 min, resuspended by brief sonication in 25 ml 0.15 M-Tris-HCl pH 7.2 and 0.1% Triton X-100, and recentrifuged.

The pellet obtained was resuspended in 2 ml TD buffer and layered onto a discontinuous sucrose gradient composed of 10 ml of 60%, 70% and 85% sucrose in 10 mM-Tris-HCl pH 7.2. After this had been centrifuged for 60 min at 75000 g, the ATIs floated at the interphase between 70% and 85% sucrose. This concentrated ATI fraction was collected from a hole at the bottom of the tube, and after eightfold dilution with TD buffer was centrifuged at 2000 g for 20 min. In some experiments the sucrose density gradient centrifugation was omitted.

**Preparation of anti-ATI serum.** To raise antiserum against the major ATI polypeptide, purified ATIs were dissolved in dissociation buffer and electrophoresed on preparative SDS-PAGE gels. The 160K band was excised and pulsed in a Teflon homogenizer, and gel pieces containing approximately 500 µg of the ATI polypeptide were injected three times with Freund's complete adjuvant at 2 week intervals into a rabbit. The rabbit was bled 1 week after the final injection.

**Preparation of labelled ATI and radioimmunoprecipitation.** HeLa cells (1 × 10^8) grown as subconfluent monolayers were infected with the virus at 10 p.f.u. per cell. After 1 h adsorption at room temperature, the cells were washed three times with phosphate-buffered saline, after which Eagle's MEM supplemented with 5% foetal calf serum (FCS) was added. Four h post-infection (p.i.) the medium was replaced with 1 ml of medium consisting of MEM containing one-tenth the normal amount of unlabelled methionine, 2% FCS and 50 µCi[^35]Smethionine, and incubation was continued overnight. The cells were then subjected to radioimmunoprecipitation, as described elsewhere (Shida & Dales, 1982).

**Cloning protocols for CPV DNA.** DNA was extracted from purified poxvirus as described previously (Shida, 1986) and was digested with either SacI or HindIII restriction endonuclease. The mixture of DNA fragments was
ligated to linearized recipient pUC13 and pUC18 DNA and the resulting chimeric plasmids were used to transform competent Escherichia coli JM103 and JM109. Recombinant plasmids were then extracted by rapid alkaline extraction from individual clones and examined by digestion with restriction endonucleases followed by agarose gel electrophoresis (Maniatis et al., 1982).

**Transient expression of ATI protein.** Subconfluent CV-1 cells infected with VV strain WR at 30 p.f.u. per cell were transfected with 5 to 10 μg of plasmids containing CPV DNA fragments as described previously (Shida, 1986). The plasmids used had been extracted by rapid alkaline extraction (Maniatis et al., 1982) without further purification. The cells were incubated until 24 h p.i. and were then scraped off the dishes and lysed with dissociation buffer. Cell lysates were analysed for ATI synthesis by SDS–PAGE, followed by Western blotting (Towbin et al., 1979) using anti-ATI serum produced as described above.

**DNA sequencing.** The chimeric plasmids containing ATI genomic DNA were linearized by digestion with appropriate restriction enzymes. The fragments were trimmed by digestion with exonuclease III in a controlled manner, and were then digested with nuclease S1. After Klenow polymerase treatment and recircularization with T4 DNA ligase, the plasmids were used to transform JM109. The deleted plasmids were then extracted from individual clones, and used to determine the nucleotide sequences directly by the dideoxy chain termination method of Sanger et al. (1980) and Yanisch-Perron et al. (1985).

**Southern blotting.** The DNA fragments separated by agarose gel electrophoresis were electrophoretically transferred to Zeta-Probe membranes, as described in the manufacturer's instructions (Bio-Rad). The membranes were baked, prehybridized, and then hybridized overnight at 42 °C in a solution consisting of 50% formamide, 5 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-sodium citrate), 0.1% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone, 0.1% SDS, 250 μg/ml sheared denatured salmon sperm DNA, 15 mM-sodium phosphate buffer pH 6.5 and denatured 32P-labelled probe. The DNA fragments shown in Fig. 5 were nick-translated using an Amersham kit and used as probes. After hybridization, the membranes were washed four times with 2 × SSC–0.1% SDS at room temperature, and in some cases twice with 0.1 × SSC–0.1% SDS at 65 °C. The membranes were then dried and autoradiographed at −70 °C.

**RESULTS**

**Purification of ATIs**

In the new ATI purification procedure sodium deoxycholate, Triton X-100 and sonication treatments, to which ATIs are resistant, were used to disrupt large cellular structures. After low speed centrifugation in detergent solution, the resultant pellet contained primarily ATIs, which were then further purified by sucrose density gradient centrifugation. The purity of isolated ATIs was evaluated by phase contrast microscopy (Fig. 1). SDS–PAGE of ATIs and vaccinia virion proteins analysed in parallel revealed that the former were composed of only one polypeptide, with an $M_r$ of 160K (Fig. 2). ATIs purified by this method were very sticky and tended to aggregate, as already described by Patel et al. (1986).

To obtain monospecific anti-ATI antibodies, the 160K polypeptide, further purified by preparative SDS–PAGE, was injected into a rabbit. The antiserum elicited was examined by
Fig. 2. Comparative electrophoretograms of ATI and of vaccinia virion proteins separated by SDS-PAGE. ATIs were isolated from CPRO6-infected cells as described in Methods. The gel was photographed after electrophoresis, staining and destaining. Lane 1, purified ATIs; lane 2, purified VV.

Fig. 3. Autoradiogram of PAGE-separated material immunoprecipitated by anti-ATI serum. HeLa cells infected with CPV were labelled with [35S]methionine and subjected to immunoprecipitation. The immunoprecipitated materials were separated on a 10% polyacrylamide gel. Lane 1, immunoprecipitate with anti-ATI serum; lane 2, extract from cells infected with CPV.

Fig. 4. Immunoblotting analyses of extracts from cells infected with VV and then transfected with various plasmids. CV-1 cells were infected with either VV (lanes 1 and 4 to 10), CPV (lane 3), or VV plus CPV (lane 2). After adsorption of virus, plasmids were transfected (lanes 4 to 10), and the next day cell extracts were prepared and subjected to Western blotting. The plasmids transferred were (lane 4) pUC13, (lane 5) p0804, (lane 6) pB6, (lane 7) pB20, (lane 8) pB23, (lane 9) pC3 and (lane 10) pC6. See Fig. 5(a) for the description of the plasmids used here.

immunoprecipitation followed by SDS-PAGE, using CPV-infected cells labelled with [35S]methionine as antigen. Fig. 3 shows that the antiserum obtained was highly specific for the ATI polypeptide (lane 1) and that this polypeptide was abundantly synthesized in CPV-infected cells (lane 2).

**ATI polypeptide gene cloning**

In order to clone the gene encoding the ATI polypeptide, CPV DNA was digested with either HindIII or SalI and the mixture of DNA fragments was ligated with linearized plasmids. To
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(a)

\[
\begin{align*}
&\text{D C O G F M J NNK E A I B L H} \\
&\text{HindIII} \\
&\text{H S S S} \\
&\text{S X K K Sa Sa P P S} \\
&\text{B Sp Sp}
\end{align*}
\]

1 kb

\[2 \text{kb}\]

\[10 \text{kb}\]

Probe 1

Induction of ATI protein

<table>
<thead>
<tr>
<th>Plasmid</th>
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<tr>
<td>p0804</td>
<td>+</td>
</tr>
<tr>
<td>pK2</td>
<td>+</td>
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<tr>
<td>pS6</td>
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<td>pC3</td>
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<td>pC6</td>
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(b)

\[
\begin{align*}
&C \text{ NMK F E O I G LJ H D A B} \\
&\text{HindIII} \\
&\text{H S S E S S S H} \\
&\text{X X X}
\end{align*}
\]

\[2 \text{kb}\]

Probe 2

Probe 3

Fig. 5. Restriction maps of CPV (a) and VV (b) DNAs. Selected restriction sites (H, HindIII; S, SalI; X, XhoI; B, BamHI; K, KpnI; Sp, SphI; Sa, SacI; P, PstI; E, EcoRI) are indicated. The plasmids used for transfection and induction of ATI protein synthesis are illustrated. Probes used for mapping the ATI gene are also indicated.

To identify plasmids carrying the ATI gene, plasmid DNA was transfected into VV-infected cells; 160K protein synthesis was examined by SDS–PAGE and immunoblotting using the anti-ATI serum. Fig. 4 shows the results of such an analysis. It was confirmed that VV induced synthesis of a 94K polypeptide with the same antigenicity as the CPV-induced 160K ATI polypeptide (Fig. 4, lane 1). Out of a number of plasmids harbouring CPV DNA fragments, only one, named p0804 and carrying a 22 kb fragment generated by SalI digestion, induced synthesis of the 160K polypeptide (Fig. 4, lane 5); pUC13 induced no synthesis (Fig. 4, lane 4).

Various restriction sites were mapped in the 22 kb fragment cloned in p0804 (Fig. 5a). It was found that this fragment did not have a HindIII-sensitive site, suggesting that it was included in the longest fragment generated from CPV DNA by HindIII digestion.
To determine the position of the ATI gene in p0804, the plasmid was digested with appropriate restriction endonucleases to delete various parts of the 22 kb fragment and the products were subcloned into either pUC18 or pUC13 (Fig. 5a). For example, pB6 lacked the central part between the left KpnI and the middle SphI sites; pB20 contained the region between the right KpnI and the middle SphI sites, pB23 contained the region between the right KpnI and the rightmost SphI sites, pC3 contained sequences to the right of the left SacI site and pC6 contained the region to the right of the right SacI site (Fig. 5a). Analysis of these plasmids indicated that pB23 and pC3 contained the whole ATI gene but that neither pB6 nor pB20 did (Fig. 4 and 5a). Interestingly, plasmid pC6 induced synthesis of an antigenically ATI-related polypeptide with an Mr of 110K (Fig. 4, lane 10), suggesting that this plasmid carried the 5' portion of the ATI gene and its promoter region. These results taken together indicated that the ATI gene was located in the right-hand one-third of the 22 kb fragment and was transcribed from right to left (Fig. 5a).

**Location of the gene**

To locate the ATI gene in CPV DNA, the fragments generated from CPV DNA by digestion with either SalI or HindIII were separated by agarose gel electrophoresis and analysed by Southern blotting using as the probe a fragment released from plasmid pB20 by digestion with PstI (probe 1, Fig. 5a). The approximately 45 kb HindIII A and the 22 kb SalI A fragments hybridized with the probe (data not shown), indicating that the 22 kb carried by plasmid p0804 was derived from the HindIII A fragment.

To map the position of the 22 kb fragment within the HindIII A fragment, the latter was eluted from agarose gels, digested with SalI and analysed by Southern blotting using as probes two VV DNAs, the leftmost fragment (probe 2, Fig. 5b) and the middle portion (probe 3, Fig. 5b) of the VV HindIII A fragment, previously mapped (Schümperli et al., 1980) and cloned by us (Shida, 1986). The rationale for this approach relied on the assumption that the gene organizations of CPV and VV were quite similar. The results showed that the 22 kb fragment was located in the central part of the HindIII A fragment (Fig. 5a).

To determine the orientation of the 22 kb fragment within the CPV genome, plasmid p0804 was digested with BamHI, KpnI, SphI or SacI and the fragments were analysed by Southern blotting using probes 2 and 3 (data not shown). The results obtained are illustrated in Fig. 5 (a). The data combined with those of Fig. 4 indicated that the ATI gene was transcribed from right to left in the CPV genome (Fig. 5a).

Since VV also induced synthesis of an ATI-related polypeptide (see Fig. 4, lane 1), VV DNA was examined by Southern blotting using probe 1 (Fig. 5b). Even under stringent hybridization conditions, VV HindIII A and SalI A fragments hybridized with this probe (data not shown). We did not map the VV gene more precisely, but by referring to our previous report and those of others showing the locations of these fragments (Schümperli et al., 1980; Wittek et al., 1984), it was possible to conclude that the gene encoding VV's ATI-related protein was probably located in the same position as CPV's ATI gene (Fig. 5a).

**Sequencing**

To clarify the organization of the ATI gene, we determined the DNA sequence of plasmid p0804 between the left SacI and right SphI sites (see Fig. 5a). ATI DNA was unidirectionally deleted by exonuclease III from plasmids pB20, pB23 and pC6, and both strands were then completely sequenced by the Sanger method (Fig. 6). The DNA sequence revealed one open reading frame composed of 1284 amino acids and the M_r of ATI protein calculated from the codon sequence to be 150328, close to that estimated by SDS–PAGE. The results provide further evidence that the DNA cloned here was actually the ATI gene.

The deduced amino acid composition, shown in Table 1, suggested high hydrophilicity, with charged amino acids making up about 40% of the total: 9.6% arginine, 7.7% lysine, 0.9% histidine, 8.6% aspartic acid and 12.5% glutamic acid. Computer-aided analysis revealed that there were 10 slightly variable repeats between amino acids 611 and 912, each composed of about 30 residues (see Fig. 7). The existence of cysteine residues located towards the downstream
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Fig. 6. Nucleotide sequences of AT1 gene and flanking regions, and the deduced amino acid sequences. The underline indicates novel GAT repeats. The boxed nucleotides at −3 to 3, TAATG, are in the VV late promoter consensus sequence.
end of each repeat suggested that they played an important role in ATI's secondary structure. Computer searches detected no strong homologies with protein sequences already registered in the National Biomedical Research Foundation library.

A significant feature of the gene's upstream sequence was the existence of 29 GAT repeats between -525 and -439 and an additional three repeats between -429 and -421 (underlined in Fig. 6). At the start of the ATI gene was a 23 bp long A/T stretch, which included the VV late gene promoter consensus sequence TAAATG (Hänggi et al., 1986; Rosel et al., 1986).

**DISCUSSION**

Since CPV and VV share many properties (Moss, 1985), we assumed that CPV's gene expression regulatory signals would be recognized by VV's intracellular transcription machinery. This was proved to be so by the finding that the transfected ATI gene could be efficiently expressed in VV-infected cells. It was recently reported that an early gene of fowlpox virus, encoding thymidine kinase (TK), can also be expressed in VV-infected cells (Boyle & Coupar, 1986). We used procedures involving transfection and Western blotting to clone and locate the ATI gene. This method has several advantages over hybrid-selected and -arrested translation. First, plasmids extracted by the conventional rapid alkaline method can be used without further purification. Second, there is no need to handle labile mRNA, an especially important advantage with ATI since it has not been possible efficiently to translate the long mRNA coding for ATI in vitro. Third, the direction of transcription, as shown with plasmid pC6, can be elucidated (see Fig. 4). These analyses, in combination with Southern blotting, localized CPV's ATI gene and VV's related gene to the central portion of the HindIII A fragment. It may be noteworthy that this position is close to that encoding other proteins, P4a.
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and P4b, which are abundantly synthesized during the late period (Wittek et al., 1984; Rosel & Moss, 1985).

We found novel repetitive sequences upstream of the ATI gene, consisting of the trinucleotide GAT tandemly arranged. Recently, Wildeman et al. (1986) reported a new 'CAT' family of repetitive sequences in yeast and suggested that similar repetitive sequences occur in both prokaryotes and higher eukaryotes. Our findings imply that the repeat sequences in VV belong to the CAT family, since the complement to ---GATGATGAT--- is ---ATCATCATC---. However, the function of these repeats is unclear since our preliminary results suggested that they are not involved in expression of the ATI gene (data not shown).

ATI is abundantly synthesized at a very late stage of the virus infection cycle (Moss, 1985; Patel et al., 1986). The nucleotide sequence upstream of the ATI coding region has a sequence close to the consensus sequence for the VV late gene promoters, including TAAATG and a T cluster (Hänggi et al., 1986; Bertholet et al., 1986; Rosel et al., 1986). However, the ATI promoter is more A/T-rich than the already well known VV late promoter sequences, perhaps explaining why ATI is so strongly expressed. Indeed, a gene coding for the 11K VV protein and carrying an A/T-rich promoter has been reported to be expressed strongly in VV-infected cells (Bertholet et al., 1985). Furthermore, expression of a gene coding for the 28K protein was greatly reduced following a point mutation from A to G in a run of eight As between -15 and -7 upstream of the structural gene (Weir & Moss, 1987). This strongly suggests that a T cluster in the ATI promoter, complementary to an A cluster at the same position, could have an important function in ATI gene expression. Our preliminary results have also shown that the chloramphenicol acetyltransferase gene when linked to the putative promoter region is expressed in transfected cells approximately six- to tenfold more abundantly than when linked to the more commonly used p7.5 promoter (Venkatesan et al., 1981) (details to be published elsewhere). These data explain to some extent the mode of ATI gene transcription, and confirm that CPV and VV have either the same or at least very similar transcriptional controls.

We have already reported the fine structure of the HA gene and its putative promoter region (Shida, 1986). Knowledge of the ATI gene structure should help to understand the expression mechanisms of the late-late genes by comparison with that of the HA gene.

A characteristic feature of ATI is the formation of large masses with no surrounding membranes in the cytoplasm of infected cells (Shida et al., 1977a,b; Ichihashi & Dales, 1973; Ichihashi & Matsumoto, 1968), perhaps due to the protein's tendency to aggregate (Patel et al., 1986). Its predicted amino acid composition showed an abundance of both positively charged basic (arginine, lysine and histidine) and negatively charged acidic (glutamic and aspartic acids) amino acids, suggesting that the protein's large mass formation is mediated at least partly by electrostatic interactions between oppositely charged amino acid side-chains. The fact that ATI dissolves in alkaline and acidic solutions (Ichihashi & Dales, 1973; Bergion & Dales, 1971) and remains intact in the presence of the detergents Triton X-100 and sodium deoxycholate, as well as its overall hydrophilicity, as calculated by computer-aided analysis (data not shown), provide further data illustrating ATI's highly charged nature.

Several sites in VV DNA suitable for inserting foreign genes have been reported (Perkus et al., 1985, 1986) and already recombinant VVs based on the WR strain have successfully protected vaccinated animals from challenge with pathogenic viruses (Mackett & Smith, 1986; Mackett et al., 1985; Paoletti et al., 1984; Blancou et al., 1986). The WR strain has been used in molecular biological studies but has never been licensed for use as a vaccine against smallpox, and all recombinant viruses studied so far have been attenuated in comparison with the parental WR strain by disruption of the original VV gene, such as that encoding TK (Buller et al., 1985). In the application of vaccines to human beings, recombinant VV derived from the strain widely used for smallpox eradication ought to be better and safer than those derived from the WR strain. However, a recombinant virus based on the attenuated VV strain may not be sufficiently antigenic to elicit protective immunity, and in some cases it would not be desirable to involve gene disruption leading to further attenuation. In this sense, the ATI gene may be a better site for insertion of foreign genes, since in CPV-infected cells the inclusion seems to be involved only in host-to-host virus dissemination, and we think that in VV-infected cells the equivalent gene
merely induces synthesis of a non-functional truncated form, although there is no direct evidence for this assumption. The data presented in this paper should provide a basis to enable construction of a recombinant VV harbouring foreign genes at the ATI gene sites and should allow examination of the function(s) of the ATI-related protein.

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