VIROLOGY 229, 193–200 (1997)
ARTICLE NO. VY968433

A Novel Strategy for Determining Protective Antigens of the Parapoxvirus, Orf Virus

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IRENE POW,† JANICE A. GILRAY,† HUGH W. REID,† and ANTHONY J. ROBINSON*

*Virus Research Unit and Centre for Gene Research, University of Otago, P.O. Box 56, Dunedin, New Zealand; and †Moredun Research Institute, 408 Gilmerton Road, Edinburgh, United Kingdom

Received October 2, 1996; returned to author for revision November 5, 1996; accepted December 30, 1996

We investigated the feasibility of using vaccinia virus (VAC) recombinants containing large multigene fragments of orf virus DNA to identify protective antigens of orf virus (OV). Sixteen OV strain NZ2 DNA fragments with an average size of 11.4 kb were recombined into VAC strain Lister. Each fragment was mapped relative to OV restriction endonuclease maps but was otherwise uncharacterized. Together the recombinants represent 95% of the OV genome in an overlapping manner. Immunofluorescence showed all 16 constructs expressed products recognized by OV antiserum and radioimmune precipitation with the same antiserum allowed the localization of the major antigens of OV to specific recombinants. These data indicated the approximate genomic locations of the genes encoding the OV major antigens and showed that their expression was authentic rather than resulting from read through from VAC sequences adjacent to the site of recombination. Vaccination of OV-naive sheep with the recombinant library provided protection against a subsequent challenge with virulent OV. These data confirm the feasibility of the proposed strategy. © 1997 Academic Press

INTRODUCTION

Orf virus (OV) is the type species of the Parapoxvirus genus in the family Poxviridae (Robinson and Lyttle, 1992). It causes a contagious pustular dermatitis of sheep and goats that is transmissible to humans. The 139-kb genome of OV has been mapped with restriction endonucleases and all but the terminal hairpin loop structures have been cloned (Mercer *et al.,* 1987; Robinson *et al.,* 1982). DNA sequence data of limited regions of the genome have been published (Fleming *et al.,* 1993; Fraser *et al.,* 1990; Mercer *et al.,* 1989, 1996, 1995; Naase *et al.,* 1991; Sullivan *et al.,* 1995a, 1995b, 1994), but in general little is known about OV at the molecular level. This is in contrast to vaccinia virus (VAC), the type species of the Poxviridae, for which the complete DNA sequence has been published and numerous genes have been assigned functions (Goebel *et al.,* 1990).

VAC played a crucial role in the global eradication of smallpox. Despite this distinguished history and our extensive knowledge of VAC, the viral proteins that are the targets of the protective immune response have not been clearly identified for VAC or any poxvirus. This may be in part because it is likely that cell-mediated immune responses play a major role in protection against poxviruses. Antibodies able to neutralize poxviruses have been reported and antibodies directed against antigens such as those derived from the extracellular enveloped form of VAC may restrict the spread of infection and may assist in limiting reinfection but antibody-mediated mechanisms are generally insufficient to protect against infection by a poxvirus (reviewed in Buller and Palumbo, 1991). In the case of poxviruses such as OV for which there is no evidence of systemic spread during infection, it is possible that only the cell-mediated responses are relevant and that antibodies have no significant role in resolving or restricting the infection (reviewed in Robinson and Lyttle, 1992). Identification of viral proteins which stimulate these cell-mediated responses will require the endogenous production of the relevant antigen(s). This could be achieved by using a vector able to express candidate genes in the appropriate animal species. In recent years VAC has been widely used for the expression of foreign genes (Moss, 1991; Tartaglia, Pincus, and Paoletti, 1990). In nearly all cases the sequence of the foreign gene has been determined and the gene placed so as to be transcribed from a strong VAC promoter. The resulting cassette is then recombined into the VAC genome.

We have mapped the transcriptional start points of a limited number of OV genes and identified likely promoter regions 5′ of these points (Fleming *et al.,* 1993, 1991, 1992; Sullivan *et al.,* 1995a, 1995b, 1994). We have also,

[1] To whom correspondence and reprint requests should be addressed. Fax: +64 3 4797744. E-mail: andy.mercer@stonebow.otago.ac.nz.

[2] Present address: The Centre for HIV Research, University of Edinburgh, Edinburgh, U.K.

[3] Present address: CSIRO Division of Wildlife and Ecology, P.O. Box 84, Lyneham, ACT 2602, Australia.

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for some of these OV early genes demonstrated that their promoters are recognized by the VAC transcriptional machinery resulting in faithful transcription of OV early genes from their own promoters both when these genes are carried by VAC recombinants (Fleming *et al.,* 1992) and when they are provided as templates in an *in vitro* assay with transcriptionally active extracts of VAC (Vos *et al.,* 1992). This faithful cross-generic transcription suggests that OV genes could be expressed in VAC recombinants without the need to fuse each OV open reading frame to a VAC promoter. Instead large fragments of OV DNA, carrying multiple, undefined genes could be recombined into VAC and the recombinants then screened for the ability to generate an immune response able to protect against a subsequent challenge with OV. Further analyses of subclones derived from the relevant recombinants would identify the OV genes encoding dominant protective antigens. This approach would circumvent the necessity to first identify and then screen individual OV genes. We have previously shown that infection of sheep with VAC does not provide cross-protection against OV (Mercer *et al.,* 1994; Robinson and Mercer, 1988).

We report here an analysis of the feasibility of the proposed strategy. An OV DNA library whose 16 members cover 95% of the OV genome in an overlapping manner was constructed in VAC. Expression of OV genes was detected and vaccination of sheep with the recombinant library provided protection against challenge with virulent OV.

MATERIALS AND METHODS

Cells and viruses

The OV strain used was NZ2 (Robinson *et al.,* 1982) and the VAC strain, Lister (Robinson and Mercer, 1988). VAC recombinants containing fragments of OV DNA were given the prefix VVOV and a clone number (see below). Primary bovine testis cells were grown in supplemented Eagles minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) (Balassu and Robinson, 1987). TK143B cells were grown in Eagles modified autoclavable MEM plus 10% FBS. African green monkey kidney fibroblasts, CV-1 cells, were grown in Eagle's MEM plus 10% FBS.

DNA manipulations

In vitro DNA manipulations were performed according to standard methods (Sambrook *et al.,* 1989) or have been described previously (Fraser *et al.,* 1990; Mercer *et al.,* 1987). To produce a library of large and overlapping fragments of OV DNA, 4.5 mg of OV DNA was partially digested with *Sau*3AI. Small DNA fragments were removed from the digest by selective precipitation with 0.5% polyethylene glycol 6000 (PEG) and 0.6 *M* NaCl. After 2 hr on ice, the mixture was centrifuged at 9000 *g* for 5 min and the pellet washed with 5% PEG–0.6 *M* NaCl. The pelleted DNA was then resuspended in 10 m*M* Tris, 1 m*M* EDTA, pH 8 (TE), extracted with phenol, and then a 1 to 1 mix of phenol and chloroform, before being precipitated with ethanol, and resuspended in TE. Aliquots of this DNA were ligated to *Bam*HI-digested, calf alkaline phosphatase-treated pVU77 (see below).

Construction of recombinant vaccinia viruses

Insertion vector plasmids containing OV DNA fragments were used to produce VAC recombinants by homologous recombination at the thymidine kinase (TK) locus (Mackett *et al.,* 1985). The vectors used in the construction of the library were pUV1 (Falkner *et al.,* 1987), pGS20 (Mackett *et al.,* 1984), and pVU77, a derivative of pGS20 made in this laboratory. Digestion of pGS20 with *Eco*RI removed a 0.3-kb fragment carrying the P7.5 promoter element and this was replaced with a 42-bp *Eco*RI fragment derived from the cloning cassette of pUC7. Further digestion with *Bam*HI followed by religation reduced the cloning region to a single *Bam*HI site closely flanked by *Eco*RI sites (GAATTCCCCGGATCCGGGGAATTC). This construct was called pVU77.

Plasmid DNA was introduced into VAC-infected TK143B cells by precipitation with calcium phosphate or the use of a liposome preparation (Lipofectin, BRL). Recombinants were selected by plaque assay in the presence of 5-bromodeoxy-uridine (BDUR) and, in the case of vector pUV1, stained with 5-bromo-4-chloro-3indolyl-D-galactopyranoside. In the case of vectors pVU77 and pGS20, BDUR-resistant plaques were individually passaged in 24-well tissue culture plates and recombinants detected by dot-blot hybridization of aliquots of the lysates with OV-specific DNA probes. Four cycles of plaque purification were carried out before virus stocks were prepared. The identity of each recombinant was confirmed by restriction endonuclease and DNA/DNA hybridization analyses of purified viral DNA (Esposito *et al.,* 1981). Each recombinant was given the prefix VVOV and the same number as that of the plasmid used in its construction.

Immunofluorescence

CV-1 cells were grown to confluence on coverslips. After washing, the cells were inoculated at a multiplicity of infection (m.o.i.) of 0.01 with one of the 16 VVOV recombinants under test. Other cells were inoculated with VAC or a VAC recombinant (VVBGAL) containing the *Escherichia coli* β-galactosidase gene under the direction of the VAC P11 promoter (Falkner *et al.,* 1987) as negative controls or OV as positive controls. Eighteen hours later the coverslips were washed in PBS, air-dried, fixed in

cold acetone for 10 min, and stained for presence of OV protein in an indirect immunofluorescence test. The first stage serum was a 1/200 dilution of a sheep hyperimmune serum preadsorbed with VAC-infected CV-1 cell lysate (McKeever *et al.,* 1987) and the 2nd stage serum a 1/80 dilution of donkey anti-sheep IgG conjugated to fluorescein isothiocyanate (Scottish Antibody Production Unit, Carluke). The intensity of fluorescence was judged relative to cells infected with OV and the results expressed on a scale of + to +++++.

Immune precipitation

Bovine testis cells were infected with virus at an m.o.i. of 10. At 6 hr postinfection for VAC and VVOV and at 19 hr postinfection for OV, the medium was changed to methionine-free RPMI-1640 medium (Sigma) containing [^{35}S]methionine (25 μCi per 4×10^5 cells). After 3 hr the cells were washed twice with PBS and lysed in 175 μl RIPA buffer (0.05 *M* Tris, pH 7.5, 0.15 *M* NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg per ml aprotinin, 100 μg per ml phenylmethylsulfonyl fluoride) before centrifugation in a microfuge at 4°. Cell extract (150 μl) was mixed with 2 μl of the same preadsorbed hyperimmune anti-OV sheep serum that had been used for the immunofluorescence studies and incubated at 4° overnight. Immune complexes were reacted with protein G-agarose (Sigma) at 4° for 1.5 hr, collected by centrifugation, and washed three times with RIPA buffer. The precipitated proteins were resuspended in gel-loading buffer, boiled for 2 min, electrophoresed on a 12.5% SDS–polyacrylamide gel, and visualized by autoradiography.

Immunization and challenge

Four hysterectomy-derived, colostrum-deprived gnotobiotic lambs obtained according to published methods (Hart *et al.,* 1971) were reared in pairs in sterile plastic isolators. When 24 hr old, two lambs were each vaccinated with 16 VVOV recombinants applied at separate sites on the hairless skin of the inguinal and axillary region. Vaccination was done by lightly scarifying a discrete 1-cm^2 area with a 16-g needle and applying approximately 0.1 ml of VVOV recombinant with an infectivity titer of approximately 1×10^6 TCID50 per milliliter. The two control lambs were vaccinated in the same way except that each of the 16 scarified sites received 0.1 ml of VVBGAL with an infectivity titer of 1×10^6 TCID50 per milliliter. At 21 days old all four lambs were challenged with virulent OV and the resulting lesions scored on a daily basis for 3 weeks as previously described (Nettleton *et al.,* 1996). All lambs were bled at weekly intervals and serum antibody levels to OV were measured using an ELISA assay (Nettleton *et al.,* 1996).

TABLE 1

OV Insertion Library

Number	Vector	Origin	Coordinates
pVU216	pUV1	*Kpn*I G	1.3–10.4
pVU80	pGS20 *Eco*RI	*Bam*HI B/*Hin*dIII C*	8.2–16.8
pVU215	pUV1	*Kpn*I F	10.4–20.3
pVU213	pUV1	*Hin*dIII A/*Kpn*I MSO*	16.8–26.4
pVU97	pVU77	*Sau*3A	24.3–34.0
pVU96	pVU77	*Sau*3A	30.3–46.3
pVU212	pUV1	*Kpn*I D	41.9–53.1
pVU245	pUV1	*Sau*3A	48.3–66.4
pVU247	pUV1	*Sau*3A	56.3–68.8
pVU86	pVU77	*Sau*3A	64.6–81.4
pVU285	pUV1	*Sau*3A	75.4–89.4
pVU243	pUV1	*Bam*HI A/*Hin*dIII A	82.9–93.5
pVU283	pUV1	*Sau*3A	89.4–104.6
pVU330	pUV1	*Bgl*II D	103.3–108.6
pVU85	pVU77	*Eco*RI D	107.4–118.1
pVU82	pVU77	*Sau*3A	123.1–137.6

Note. Members of the library are recombinant plasmids made in the vectors pUV1, pVU77, or pGS20. Each OV DNA fragment was derived by either partial digestion with *Sau*3AI or was a defined restriction fragment described in published maps of OV (Mercer *et al.,* 1987). *Bam*HI A/*Hin*dIII A and *Bam*HI B/*Hin*dIII C represent the region of overlap between each pair of fragments. Those fragments marked with an asterisk were cloned into *Eco*RI-digested vectors after the fragment had been made blunt with T4 DNA polymerase and ligated to *Eco*RI linkers. The location of each OV DNA fragment on the OV restriction map is given in kb from the left end of the genome.

RESULTS

OV DNA was partially digested with *Sau*3AI and size-selected by precipitation with 5% PEG–0.6 *M* NaCl. Agarose gel electrophoresis of a portion of this DNA revealed that it ranged in size from greater than 20 kb to no smaller than 4 kb. It was ligated to the insertion vector, pVU77, and then transformed into *E. coli* HB101. Recombinant plasmids were recovered either by direct screening of transformants or after colony hybridization with an OV DNA probe. A total of 80 recombinant plasmids were analyzed. These contained OV DNA fragments ranging in size from 4 to 18 kb with an average size of 12 kb.

Restriction endonuclease digestion and hybridization with mapped fragments of OV DNA provided information on the genomic location of inserts carried by the recombinant plasmids. Eight of the plasmids were included in the library (Table 1). The OV DNA contained in these eight plasmids totalled 116.4 kb in length (average 14.6 kb) and when the overlap between clones was allowed for they covered 92.4 kb of the 139 kb genome. Advantage was taken of the *Eco*RI sites flanking the point of insertion into pVU77 to subclone the inserts of four plasmids to pUV1 (pVU245, 247, 86, and 285). Gaps in the collection were filled by cloning mapped restriction fragments into the insertion vectors pVU77, pGS20, or pUV1.

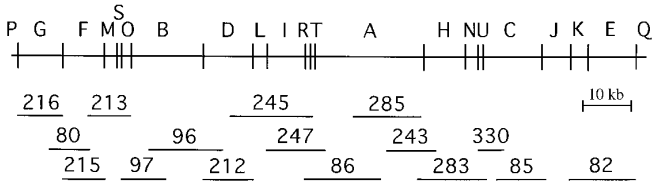

FIG. 1. Genomic locations of the DNA fragments constituting the insertion library. The position of each DNA fragment is shown against the *Kpn*I map of OV strain NZ2. Note that *Kpn*I map has been inverted from that published previously (Mercer *et al.*, 1987).

The resulting library of 16 clones is shown in Fig. 1 and details of each clone are provided in Table 1. The average overlap between clones is 4.3 kb with the smallest being 1.2 kb. The only regions of the OV genome not included in the library are sections of approximately 1.3 kb at each terminus and a 5-kb gap between clones pVU82 and pVU85. Repeated attempts to construct a clone spanning this latter region have been unsuccessful.

Each plasmid of the library was used to make a VAC recombinant. Plasmid DNA was introduced into VAC-infected cells and TK-deficient recombinants were selected from the progeny by growth in the presence of BDUR. *Hin*dIII digests of DNA purified from each recombinant gave the predicted fragmentation pattern. The identity of each recombinant was further confirmed by hybridization with radiolabeled OV DNA (not shown). The largest recombinant genome is that of VVOV245 in which a total of 22.3 kb has been recombined into VAC (18.1 kb of OV DNA plus 4.2 kb derived from pUV1).

In order to test that the recombinants were able to express at least some of the OV genes they carried, cells infected with each of the recombinants were examined by immunofluorescence with preadsorbed hyperimmune anti-orf virus antiserum. All VVOV recombinants gave clear specific immunofluorescence. The intensity varied between recombinants (see Table 2) with recombinants 96, 245, 247, 285, and 330 giving the strongest fluorescence and 82, 97, 215, and 216 the weakest fluorescence. Cells infected with VVBGAL did not react.

Cells infected with OV were rounded and fluorescence was distributed widely in the cytoplasm (Fig. 2a). In cells containing VVOV recombinants fluorescence was more commonly patchy and restricted to discrete sites (Fig. 2b). At higher magnification, recombinants generally gave a patchy granular distribution of fluorescence (Fig. 2c) although areas where the whole cytoplasm was fluorescing were also obvious in those recombinants giving the strongest fluorescence (Fig. 2d).

To further examine the expression of OV proteins by the library, the recombinants were tested by immune precipitation with antiserum raised against OV. Recombinant-infected cells were pulse labeled with [S^{35}]-methionine at a late stage of infection and the labeled proteins were precipitated with hyperimmune anti-OV sheep antiserum in conjunction with protein G agarose. Precipitated proteins were electrophoresed on a SDS–polyacrylamide gel in parallel with proteins similarly precipitated from OV-infected cells labeled at an equivalent time postinfection. The results are shown in Fig. 3. Eight strong bands were seen in the sample derived from OV-infected cells (lane 3). The molecular weights of these bands were calculated as 108, 85, 72, 69, 47, 43, 28, and 27 kDa. Bands comigrating with five of these bands were also seen in samples derived from members of the recombinant library, suggesting that the 108-kDa OV antigen is encoded by VVOV283 and VVOV243, the 85- and 72-kDa OV antigens are encoded by VVOV285, the 43-kDa OV antigen is encoded by VVOV245, and the 27-kDa antigen is encoded by VVOV283. Additional faint bands of 38 and 34 kDa were seen in the sample derived from OV-infected cells and these two antigens appear to be encoded by VVOV96 and VVOV283, respectively.

Production by recombinants of the other OV antigens may be concealed by their comigration with VAC-derived proteins which form a significant background in this gel. Serological cross-reactivity between OV and VAC has been reported previously (Webster, 1958). Proteins of less than 20 kDa were not resolved in this gel. The remaining five members of the library were also screened

TABLE 2

Detection by Immunofluorescence of OV Proteins Expressed from VVOV Recombinants

VVOV	Intensity	VVOV	Intensity	VVOV	Intensity	Virus	Intensity
80	+++	212	+++	247	+++++	VVBGAL	Negative
82	+	213	+++	283	++++		
85	+++	215	++	285	+++++	OV	+++++
86	+++	216	++	330	+++++		
96	+++++	243	+++				
97	+	245	+++++				

Note. Cells infected with the indicated virus were reacted with serum from sheep hyperimmunized with OV. Bound antibody was detected with anti-sheep IgG conjugated to fluorescein isothiocyanate. The intensity of fluorescence was judged relative to cells infected with OV and the results expressed on a scale of + to +++++.

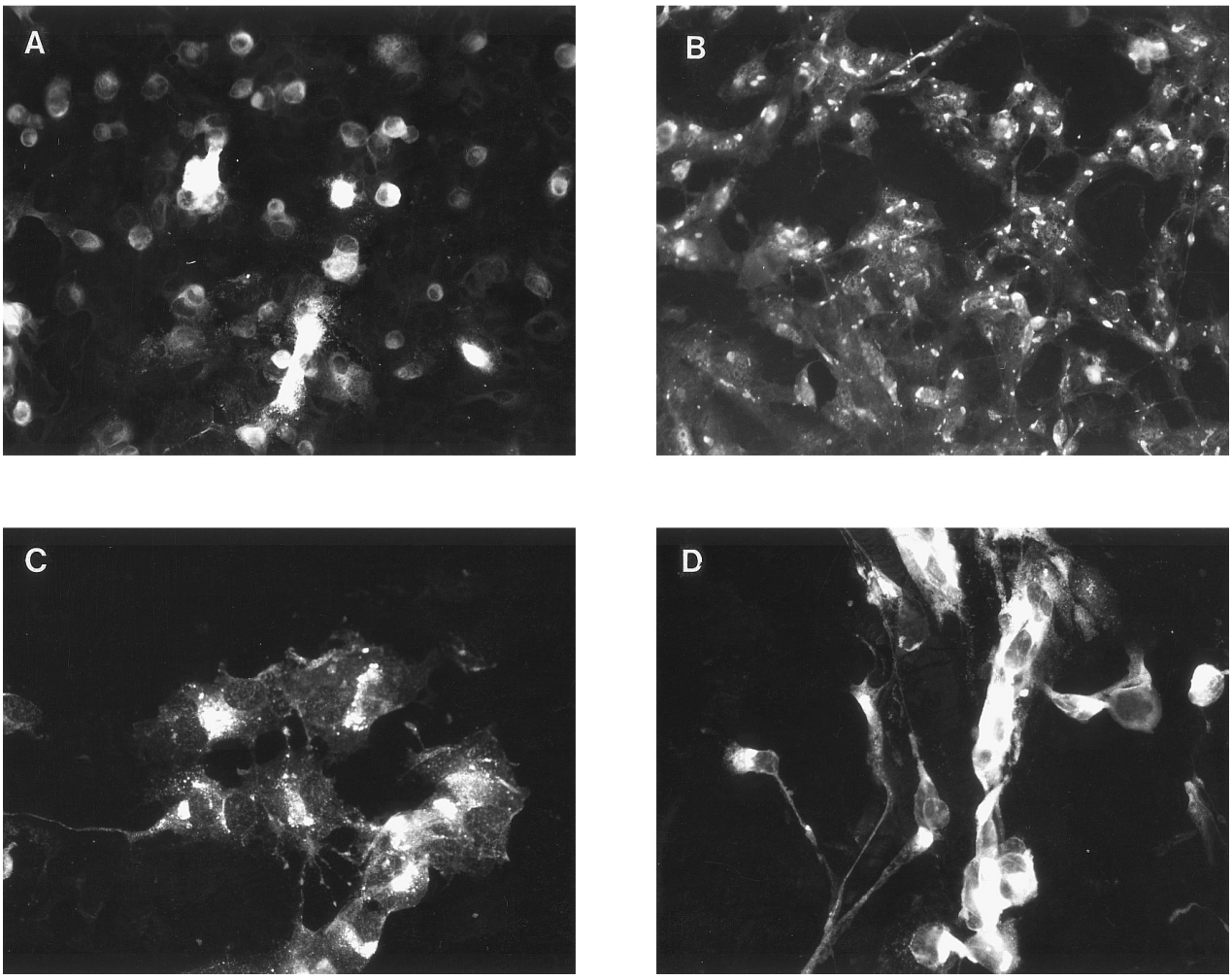

FIG. 2. Expression of OV proteins by VVOV recombinants visualized by immunofluorescence. (A) CV-1 cells infected with OV, ×200; (B) CV-1 cells infected with VVOV recombinant 330, ×200; (C) CV-1 cells infected with VVOV recombinant 96, ×400; (D) CV-1 cells infected with VVOV recombinant 245, ×400.

by immune precipitation and a strong band of 43 kDa was shown to be produced by VVOV80 (results not shown). The gene encoding this antigen has since been sequenced and shown to be a homologue of the VAC 37-kDa envelope (Sullivan *et al.,* 1994).

There was general agreement between the results obtained by immunofluorescence and immune precipitation. For example VVOV243, 245, 283, and 285 gave clear indications of the production of dominant OV antigens with both techniques, whereas VVOV82 and 97 scored low with both techniques.

The above results indicated that all 16 VVOV recombinants expressed OV-specific antigen. We wished to determine if the recombinant library was able to immunize OV-naive lambs against a challenge with OV. In order to exclude the possibility of an adventitious infection with OV which might have compromised the experiment, lambs were derived by hysterectomy from date-mated ewes and held in sterile isolators. Lambs were vaccinated with either each of the VVOV recombinants or with VVBGAL. All sites on all four lambs showed typical, discrete VAC-induced lesions which had resolved and detached by 2 weeks postscarification. At 3 weeks of age all lambs were challenged with OV. The clinical response to challenge of the four lambs is summarized in Table 3. In both lambs vaccinated with VVBGAL the OV lesions were typical of a primary OV lesion, progressing from erythema through vesicle and pustule formation to scab over 3 weeks. By contrast, both lambs receiving VVOV recombinants had a much milder and shorter course of disease with scabs detaching by 13 days after challenge. The protective effect of prior immunization with the VVOV recombinants is clearly apparent when the total combined clinical scores at 21 days postchallenge are compared: control animals scored 236, whereas animals vaccinated with the recombinant library scored 52 (Table 3). The mean results for the two sets of animals were significantly different from Day 7 postchallenge. Second-

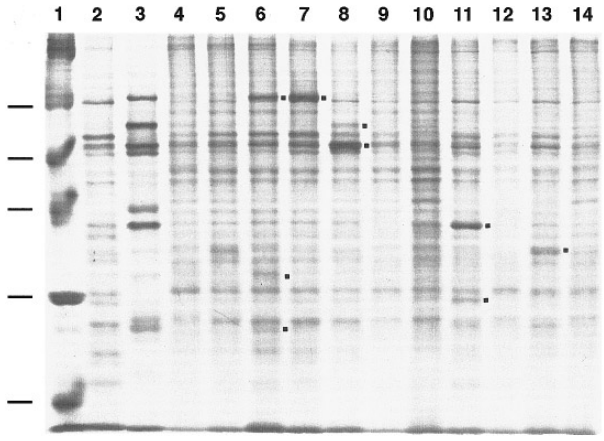

FIG. 3. Analysis of proteins immunoprecipitated from the expression library by anti-OV serum. Infected cell extracts labeled with [^{35}S]-methionine were precipitated with hyperimmune sheep anti-OV serum and protein G. Precipitated proteins were detected by autoradiography after separation on a 12.5% SDS–polyacrylamide gel. Lanes 2 to 14 show proteins precipitated from cells infected with VAC, OV, VVOV82, VVOV85, VVOV283, VVOV243, VVOV285, VVOV86, VVOV247, VVOV245, VVOV212, VVOV96, and VVOV97, respectively. Lane 1 contains molecular weight markers and the bars at the left indicate the bands of 97.4, 69, 46, 30, and 21.5 kDa. Filled squares mark the location of bands discussed in the text.

ary OV lesions developed on the lips of both control lambs, whereas no secondary lesions were observed in the two lambs receiving VVOV recombinants.

Evidence of priming of the immune system by the VVOV recombinants was provided by the ELISA results. The two lambs receiving the VVOV recombinants seroconverted to OV within 1 week of challenge and before any antibody was detected in the control lambs (Table 4).

DISCUSSION

The use of VAC to express foreign genes has become commonplace. In most studies the sequence of the inserted gene has been determined and the gene placed so it is transcribed from a strong VAC promoter. In contrast to this, we have constructed an OV expression library composed of VAC recombinants each carrying an uncharacterized multigene fragment of OV DNA and have relied on the VAC transcriptional complex recognizing the OV promoters for expression of the genes. The immunofluorescence and immune precipitation studies demonstrated that OV antigens are indeed expressed from these constructs and suggest that a high proportion, perhaps all, OV genes are expressed by the library. We have previously shown that specific OV early (Fleming *et al.,* 1992; Vos *et al.,* 1992) and late (Sullivan *et al.,* 1994) promoters resemble their VAC counterparts and that OV early prompters are recognized by VAC. The results presented here support and extend those observations.

Cross-recognition between a promoter and the RNA polymerase complex of different genera of poxviruses has been exploited previously to identify the fowlpoxvirus TK gene (Boyle and Coupar, 1986). In an approach similar to that used here, random fragments of fowlpoxvirus DNA were recombined into a TK^- VAC and TK^+ recombinants recovered. Although it was not directly demonstrated, it is reasonable to assume that the promoter sequence of the fowlpoxvirus TK gene was recognized by VAC. It is significant that in the studies reported here all recombinants were made by selecting TK^- recombinants from a TK^+ parent. This, in combination with the evidence that OV promoters are recognized by the VAC transcriptional machinery, suggests that OV lacks a functional TK gene. This suggestion is supported by our inability to find a TK gene in the NZ2 strain of OV by hybridization with redundant sets of oligonucleotides (unpublished data). There remains, however, the possibility that an OV TK gene is located in the region not represented in the library (5 kb between VVOV85 and VVOV82).

As the DNA fragments used to construct the library were not characterized in terms of their gene content, it was recognized that some aberrant transcriptional products might arise from OV sequences at the ends of the inserted fragments. These could result from read-through into adjacent VAC sequences of an OV open reading frame interrupted at the 3′ end or transcripts might originate from adjacent VAC promoters and extend into the terminal regions of the OV DNA fragment. In the case of VVOV80 and those recombinants made with plasmids based on pVU77 the only VAC promoter located such that it could potentially direct transcription into the OV sequences is the relatively weak early promoter of the TK gene. Recombinants based on the insertion vector, pUV1, have a strong late promoter adjacent to one end of the inserted DNA. The decision to construct an overlapping library was aimed at reducing problems resulting from such events.

Although not considered useful for identifying protective antigens, the immune precipitation studies demonstrated the utility of the library for locating OV genes encoding immunodominant antigens. For example, a dominant OV antigen was expressed by recombinant VVOV285 (72 kDa, Fig. 3) but not by the two overlapping recombinants, VVOV86 or VVOV243, suggesting this antigen is encoded by a gene which lies at least partly in the 1.5-kb gap between VVOV86 and 243. Similar deductions are possible for the other antigens detected. The location and DNA sequence of only one known immunogenic gene of a parapoxvirus has been reported (Sullivan *et al.,* 1994). The results reported here will assist in the characterization of other dominant antigens.

A similar approach has been used to map the location of genes encoding a limited number of antigens of African swine fever virus (ASFV) (Hammond and Dixon, 1991). That study used a transient assay in which ASFV

TABLE 3

Effect of Immunizing Lambs with VVOV Recombinants on Subsequent Challenge with OV

	Controls							Vaccinates						
	Lamb 103			Lamb 104				Lamb 109			Lamb 110			
Day	E	V/P	S	E	V/P	S	CCS	E	V/P	S	E	V/P	S	CCS
1	1			1			2	1			1			2
2	1			1			2	1			1			2
3	2			2			4	2			2			4
4	2			2			4	2			2			4
5	2			2			4	2			2			4
6	2	2		2		2	8	2	2		2	2		8
7	2	2		2		2	8	1		2	1		2	6
8	2	2	2	2	2	2	12	1		2	1		2	6
9	2	2	2	2	2	2	12			2			2	4
10	3	2	2	3	2	2	14			2			2	4
11	3	2	2	3	2	2	14			2			2	4
12	3	2	2	3	2	2	14			2			2	4
13	2	2	3	3	3	3	16							
14	2	3	3	3	3	3	17							
15	2	3	3	3	3	3	17							
16	2	3	3	3	3	3	17							
17	2	1	3	3	3	3	15							
18	2		3	3	3	3	14							
19	2		3	3	3	3	14							
20	2		3	3	3	3	14							
21	2		3	3	3	3	14							
Total CCS							236							52

Note. Twenty-four hour-old lambs were infected with either all 16 VVOV recombinants (vaccinates) or with VVBGAL (controls). At 3 weeks of age all lambs were challenged with OV. The resulting lesions were examined daily and scored on a scale of 0 to 3 for erythema (E), vesicle/pustule formation (V/P), and scab formation (S) (Nettleton *et al.,* 1996). Scores of zero have been omitted from the table. The scores were summed each day to give a combined clinical score (CCS) for controls and vaccinates. At the end of the experiment all scores for each pair of animals were combined to give a total CCS.

DNA fragments representing more than 90% of the genome were introduced into VAC-infected cells and ASFV-specific proteins detected by immune precipitation. The anti-ASFV serum used was able to detect at least 30 proteins from ASFV-infected cells but detected synthesis of only eight ASFV proteins in the VAC-infected cells, suggesting that only a minority of ASFV promoters are recognized by the appropriate VAC elements.

The animal studies included in this report demonstrate the validity of the approach we have proposed; they demonstrate that the genes necessary and sufficient for effective immunization are expressed by the library. The de-

TABLE 4

Titers of Anti-OV Antibody in Serum Samples Taken from Gnotobiotic Lambs

		Age					
	Lamb No.	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
Controls	103	—	—	—	—	1/100	1/400
	104	—	—	—	—	1/400	>1/12800
Vaccinates	109	—	—	—	1/400	1/6400	1/6400
	110	—	—	—	1/100	1/400	1/3200

Note. Twenty-four-hour-old lambs were infected with either 16 VVOV recombinants (vaccinates) or with VVBGAL (controls). At 3 weeks of age all lambs were challenged with OV. Titers of less than 1/100 are shown as a dash.

gree of protection, as measured by the combined clinical scores, provided by the VVOV recombinant library was comparable to that recorded in similar experiments in which lambs were immunized with OV (Nettleton *et al.,* 1996). These studies are also a first step in identifying those OV proteins which induce a protective immune response in sheep. Further experiments using subsets of the library are planned. It is hoped that this will enable us to identify individual recombinants and thereafter individual genes whose products are able to induce protection. The identification of epitopes able to induce a protective immune response might lead to their incorporation into a vaccine able to deliver these relevant epitopes in the absence of infectious OV.

ACKNOWLEDGMENTS

This work was supported by funds received from the Health Research Council of New Zealand and the Scottish Office Agriculture, Environment and Fisheries Department. We gratefully acknowledge the assistance of J. Sullivan and A. Ramsay in early stages of this work.

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