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Isolation and Characterization of Monoclonal Antibodies Directed Against Two Subunits of Rabbit Poxvirus-Associated, DNA-Directed RNA Polymerase

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A library of monoclonal antibodies directed against individual proteins of the rabbit poxvirus (RPV) virion within a complex immunogenic mixture has been generated through the use of in vivo and in vitro immunization regimens. The relative efficacies of the two procedures were compared. Based on immunoblot analysis, the in vitro immunization regimen led both to a wider variety of monoclonal antibodies to different proteins and to a larger number of antibodies directed against proteins of higher molecular weights. Each method, however, has advantages, and the two procedures appear to be complementary. A simple method to recognize antibodies directed against the virion DNA-directed RNA polymerase was developed. Monoclonal antibodies directed against two subunits (137 and 34 kilodaltons [kDa]) of the RNA polymerase were identified and used to study the biogenesis of the enzyme and to map the two corresponding genes within the viral genome by using an RPV DNA library cloned into the λgtll expression vector. Both proteins are synthesized late in the infectious cycle and are restricted totally to the cytoplasm. Preliminary mapping data place the genes encoding the 137-kDa protein within the *Hin*dIII H fragment, whereas the gene for the 34-kDa protein is located within the left most region of the *Hin*dIII A fragment.

As a consequence of a cytoplasmic reproductive cycle, rabbit poxvirus (RPV), like other poxviruses, encodes a number of enzymes normally confined to the cell nucleus. Some of these enzymes, particularly those associated with the transcription of early genes, are packaged within the mature virion (6, 21). The foremost example of such an enzyme is the multisubunit viral DNA-directed RNA polymerase. With the RNA polymerase as a focal point for discussion, the availability of monoclonal antibodies to each of the individual enzyme subunits would facilitate a number of studies, including biogenesis of the enzyme, mapping of the genes encoding the individual subunits, and intracellular localization.

The localization of the RNA polymerase subunits to the nucleus or cytoplasm (or both) of infected cells is of particular interest because there is still a question as to whether a nuclear phase is necessary for successful poxvirus development to occur. Recently, there have been reports not only of viral DNA (1, 18) and RNA (3, 9) synthesis but also of significant levels of viral DNA-directed RNA polymerase activity within the nucleus of infected cells (38). Immunofluorescence studies of intact infected cells with antibodies directed against the viral RNA polymerase would allow us to directly address the question of the possible nuclear localization of this enzyme.

The use of monoclonal antibodies in conjunction with poxvirus DNA libraries contained within a suitable expression vector such as λgtll (39) would allow rapid localization of genes for individual proteins within the viral genome. It should be noted that this approach is particularly suitable for poxviruses because to date, poxvirus genes appear to lack introns (15, 34–37). An expression vector-poxvirus DNA genome library can, therefore, be prepared and used directly for gene mapping without having to first prepare cDNA copies of the viral transcripts.

We present here the results of our studies which were designed initially to generate the widest variety of monoclonal antibodies possible against a semidefined mixture of viral proteins. Next, we present a method which allows screening of the monoclonal antibodies for those directed against the viral RNA polymerase. Finally, we present the characterization of antibodies directed against two different subunits of the viral RNA polymerase. These antibodies have allowed us to map the genes for these two subunits as well as to address the questions of the kinetics of biosynthesis and subcellular localization of the enzyme.

MATERIALS AND METHODS

Cells and virus. Wild-type RPV (Utrecht strain) was obtained from the American Type Culture Collection. Rabbit kidney cells (RK-77) were obtained from J. DeMarchi (Microbiology Department, Vanderbilt University, Nashville, Tenn.) and maintained in modified Eagle medium containing Hanks balanced salt solution (F-12; GIBCO Laboratories) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Viral stocks were prepared and purified as described by Moyer and Rothe (23). Phage λgtll and *Escherichia coli* strains Y1088, Y1089, and Y1090 were obtained from R. Young and R. Davis (39, 40) through K. Rosen and R. Bird of Vanderbilt University.

Preparation of subviral protein fractions used as immunogens. Proteins were solubilized from purified subviral cores by detergent treatment based on procedures described by Baroudy and Moss (2). Purified virus (optical density at 260 nm, 20; equivalent to 1.3 mg of protein) was suspended in 150 μl of 50 mM Tris hydrochloride (pH 8.4) that contained 50 mM dithiothreitol and 0.5% Nonidet P-40 and incubated at 37°C for 60 min to remove the highly imunogenic outer

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membrane. The subviral cores resulting from this treatment were collected by centrifugation for 5 min at 12,800 × *g* at 4°C in a microfuge, suspended in 200 μl of 50 mM Tris hydrochloride (pH 8.4) that contained 0.25 M NaCl, 50 mM dithiothreitol, and 0.2% sodium deoxycholate, and incubated for 30 min at 4°C. The suspension was subsequently centrifugated in a Beckman Microfuge for 15 min at 12,800 × *g* to remove the remaining particulate material (insoluble antigens) from the solubilized material (soluble antigens) and to define the two preparations used as immunogens in this communication. The particulate and soluble fractions contained 600 and 200 μg of protein, respectively.

In vivo immunization regimen. Approximately 20 μg of either soluble or insoluble RPV antigens diluted to a final volume of 0.5 ml in phosphate-buffered saline (PBS; 0.01 M sodium phosphate plus 0.15 M NaCl [pH 7.2]) was mixed with an equal volume of Freund complete adjuvant and injected intraperitoneally into 12-week-old BALB/c mice. Two weeks later, the procedure was repeated except that the antigen was mixed with Freund incomplete adjuvant before injection. Two weeks after the second intraperitoneal injection, approximately 10 μg of antigen in 0.5 ml of PBS was injected into the tail vein of each mouse. Four days later, the animals were sacrificed, and the spleen cells from each mouse (10^8 cells) were fused with 10^7 Sp2/O myeloma cells as described previously (10, 17). After fusion, cells from each individual fusion were distributed into 24-well culture dishes such that each individual well ultimately contained monoclonal antibodies directed against 1 to 10 different antigens when assayed against whole-virus extracts via the immunoblotting procedures described below. Positive hybridoma mixtures resulting from the initial fusions were cloned through soft agar (10). Individual monoclonal antibody-producing cells were recloned at least one more time before expansion and use. Culture supernatant fluid from expanded hybridoma cells was collected and used as the antibody source without further purification.

In vitro immunization regimen. The procedures for the preparation of lymphocyte-conditioned medium and for the in vitro immunization were those described by Luben and Mohler (19). Briefly, spleen cells were prepared from unimmunized 12-week-old BALB/c mice and placed into lymphocyte-conditioned medium at a concentration of 10^7 cells per ml. Either soluble or particulate antigen at a concentration of ~0.5 μg/ml was then added to the medium containing the spleen cells, and the mixture was incubated for 48 h at 37°C. At the end of 48 h, the antigen-containing medium was replaced, and the incubation was continued for an additional 48 h. The spleen cells were checked for viability by trypan blue staining, fused with Sp2/O cells at a spleen cell-to-Sp2/O cell ratio of 10:1 and subsequently grown and cloned as described above.

Screening of hybridoma cells for those producing monoclonal antibodies directed against the viral RNA polymerase. Both purified RNA polymerase and crude preparations of RNA polymerase obtained from deoxycholate extraction of virus cores were prepared as described by Baroudy and Moss (2). Either 5 μl of the crude RNA polymerase (30 μg of total protein) or 2 μl (0.4 μg) of purified RNA polymerase was added to 60 μl of undiluted culture supernatant fluid taken from hybridoma cells (at maximum cell density) and diluted to a final reaction volume of 100 μl with PBS. The mixtures were incubated at 4°C for 2 h to allow for the formation of enzyme-antibody complexes. The complexes were immunoprecipitated at 4°C for 2 h with 50 μl of protein A-Sepharose (Pharmacia Fine Chemicals, Inc.) beads which had been first pretreated and saturated with rabbit anti-mouse immunoglobulin G (IgG) antibody (Cooper-Cappell Laboratories). The beads were collected by centrifugation at 12,800 × *g* for 15 min. The supernatant fluid and, on occasion, the beads were then assayed for RNA polymerase activity as described below.

Preparation of cytoplasmic extracts from infected cells for immunoblots. Rabbit kidney cells (2.4 × 10^8 cells) were infected at a multiplicity of 10 PFU per cell. On occasion, the inhibitors cytosine arabinoside (40 μg/ml) or rifampin (100 μg/ml) were added as described for individual experiments. Twelve hours after infection at 37°C, the medium was replaced with leucine-free medium and incubation was continued for an additional 20 min. Newly synthesized proteins were briefly labeled by the addition of 500 μCi of [4,5-^{3}H]leucine (45 Ci/mmol) and further incubation for 30 min. The cells were then scraped into the medium, collected by centrifugation, suspended in 0.5 to 1.0 ml of swelling buffer (10 mM Tris hydrochloride [pH 8.0], 5 mM EDTA, 10 mM KCl) that contained 0.1% Nonidet P-40, and ruptured in a Dounce homogenizer. The nuclei were removed by centrifugation for 5 min at 800 × *g*. Before the separation of the proteins of the cytoplasmic extracts on polyacrylamide gels and the preparation of immunoblots, the extract was diluted with an equal volume of lysis buffer (60 mM Tris hydrochloride [pH 6.8], 4% [wt/vol] sodium dodecyl sulfate [SDS], 200 mM dithiothreitol, 40% glycerol, 0.12% bromophenol blue), heated to 100°C for 2 min, and incubated at 37°C for 30 min.

Resolution of cytoplasmic proteins by polyacrylamide gel electrophoresis and the preparation and use of immunoblots. Cytoplasmic proteins (60 μg per well) were separated by electrophoresis either on gradient (7 to 17%) SDS-polyacrylamide gels (10 cm) for immunoblots or on 13% gels for routine analytical purposes. Electrophoresis was carried out at room temperature in Tris-glycine buffer (6.1 g of Tris base per liter, 28.8 g of glycine per liter; final pH, 8.4) that contained 0.1% SDS at 28 V for 18 h as described by Moyer and Graves (25). When necessary, gels were processed for autoradiography as described by Bonner and Laskey (4). Transfer of the proteins from the gel to 0.1-μm nitrocellulose paper was achieved essentially as described by Towbin et al. (33). Electrotransfer was carried out at 60 V for 6 h and then at 40 V for 10 to 16 h at 4°C in 25 mM Tris–192 mM glycine (pH 8.6) in the presence of 20% methanol and in the absence of SDS. Immunoblots were blocked with a solution of PBS containing 5% bovine serum albumin and 10% fetal calf serum, at 42°C for 90 min. The immunoblots were then washed twice in washing solution (PBS containing 0.1% Tween 20) for 20 min per wash at room temperature. Blots were then air dried and stored at 4°C. Individual strips were then incubated overnight at room temperature with undiluted culture supernatant fluid from hybridoma cells. The antibody was removed, and the strips were washed three times in washing solution for 15 min per wash. The strips were then incubated with affinity-purified sheep anti-mouse IgG conjugated to horseradish peroxidase (Cooper-Cappell Laboratories) diluted 1:200 in PBS containing 0.05% Tween 20 and 5% fetal calf serum for 90 min at room temperature. The strips were then washed three times for 15 min each at room temperature with washing solution. After removal of the final wash, immunopositive bands were then detected by the addition of a saturated solution of DAB to the moist strips. The DAB solution was prepared by first adding 50 mg of 3-3′-diaminobenzidine tetrahydrochloride to 100 ml of 50 mM phosphate buffer (pH 7.4). The solution was filtered, and 3 ml of a 1% cobalt chloride–1% nickel ammonium

sulfate solution was added. Finally, 10 μl of 30% hydrogen peroxide was added just before use (7).

Immunofluorescence. Immunofluorescence was carried out by using the Zeiss photomicroscope III on either uninfected or RPV-infected cells cultured on glass cover slips in the presence or absence of inhibitors at 4 or 12 h after infection. The cells were first fixed with methanol for 5 min at −20°C followed by incubation for 5 min in acetone at room temperature. Cover slips were then washed twice for 5 min in PBS. Culture supernatant fluids were used directly as immunological probes and incubated with the fixed cells for 90 min at 37°C in a humidified chamber. Cells were washed and incubated with fluorescein isothiocyanate-conjugated, affinity-purified goat anti-mouse IgG (Cooper-Cappell Laboratories) used at a dilution of 1:400 in PBS. Fluorescent images were preserved by adding *p*-phenylenediamine to the mounting buffer before fluorescence excitation (16).

Southern blot analysis of RPV-cloned DNA. Blots were prepared from restriction digests of total RPV DNA that had been electrophoretically separated on agarose gels and transferred to nitrocellulose as described by Moyer and Graves (24). Radiolabeled probes were prepared from either RPV DNA or DNA derived from λgtll clones nick translated by the procedure of Rigby et al. (29). Hybridizations were carried out for 24 to 48 h at 42°C in the presence of 30% formamide as described by Southern (31).

Construction and screening of a λgtll library containing random 3- to 5-kb fragments of RPV DNA. Unless otherwise noted, all enzymes were purchased from New England Biolabs. A library prepared from randomly generated RPV DNA fragments was constructed essentially as described by Young and Davis (39, 40). RPV DNA (180 kilobases [kb]) was methylated with *Eco*RI methyltransferase (11) and sheared by sonication. Fragments of 3 to 5 kb (average size) were isolated from sucrose gradients, treated with DNA polymerase I (Klenow fragment) (20) to generate flush ends, and blunt end ligated to *Eco*RI linkers (Collaborative Research, Inc.). The fragments were then digested with *Eco*RI and ligated to *Eco*RI-digested λgtll which had been dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim Chemicals). The resulting mixture was packaged in vitro as described by Hohn (13). After packaging, the library was titered on *E. coli* Y1088 in the presence of 0.3 mM isopropyl β-D-thiogalactopyranoside and 0.03% 5-bromo-4-chloro-3-indolyl-β-D-galactoside. The resulting library, which had $>10^6$ total inserts, was amplified on *E. coli* Y1088 (39, 40).

Phage for screening were plated in 0.7% soft agarose on a lawn of *E. coli* Y1090 and incubated at 42°C for 3 h. The plates were then overlaid with a dry nitrocellulose filter (pore size, 0.1 μm; Schleicher & Schuell, Inc.) that had been presoaked in 10 mM isopropyl-β-D-thiogalactopyranoside and incubated at 37°C for 4 h. The filters were marked, removed from the plates, and washed for 10 min in PBS at room temperature. All subsequent manipulations of the filters were at room temperature on a rotary shaker. The filters were blocked in a solution of PBS containing 5% bovine serum albumin and 10% fetal calf serum for 1 h. After blocking, the filters were washed twice for 10 min with a solution of 0.05% Tween 20 in PBS and then incubated overnight with undiluted supernatant from hybridoma cell cultures. The filters were then processed as described for immunoblots to detect immunopositive plaques. Positive phage plaques were picked, cloned, and rescreened until all plaques were immunopositive. DNA was isolated from the phage as described by Maniatis et al. (20).

Fusion protein production by λgtll clones. Fusion proteins were produced in *E. coli* Y1089 from either lysogens or freshly prepared infected-cell cultures. Lysogenic bacteria were grown with aeration at 30°C in L broth containing 0.25% maltose to an optical density of 0.3 at 600 nm before induction. To prepare infected cells, uninfected *E. coli* Y1089 cells were grown as above to an optical density of 0.3 at 600 nm. The cells were then collected by centrifugation and suspended in 1% of the original volume of 10 mM $MgSO_4$. Phage were added to a multiplicity of infection of 5 to 10 and allowed to adsorb at 32°C for 10 min. The cells were then diluted to their original volume with L broth and induced.

All cultures were induced by incubation at 42°C for 15 min followed by growth at 37°C for 2 h in the presence of 5 mM isopropyl-β-D-thiogalactopyranoside. The cells were then collected by centrifugation and suspended in 1% of the original volume of DNase buffer (50 mM Tris hydrochloride [pH 8.0], 0.2 mM EDTA, 10% [vol/vol] glycerol, 2.5 mM $MgCl_2$, 0.15 M NaCl) containing 4 mM phenylmethylsulfonyl fluoride. The cells were disrupted by either brief sonication or repeated freezing and thawing before treatment with 1 μg of pancreatic DNase per ml for 10 min at 37°C. Samples (40 to 60 μg) of the bacterial extracts were then subjected to immunoblot analysis as described above.

Assay of RNA polymerase. RNA polymerase was purified, and activity was assayed essentially as described by Baroudy and Moss (2). Reactions in a volume of 100 μl contained 50 mM Tris hydrochloride (pH 7.9), 2 mM dithiothreitol, 4 mM manganese chloride, 90 mM potassium chloride, 1 mM ATP, 1 mM CTP, 1 mM GTP, 4 μCi of [5, 6-^{3}H]UTP (35 to 50 Ci/mmol; New England Nuclear Corp.), 5 μg of denatured calf thymus DNA, and 5 to 15 μg of viral protein. Incubations were carried out at 30°C for 30 min and terminated by the addition of 2 ml of cold 20% trichloroacetic acid that contained 0.05 M sodium phosphate and 0.05 M sodium pyrophosphate. Bovine serum albumin was then added (500 μg), and the acid-insoluble nucleic acid was collected on glass fiber filters, which were dried and counted in a liquid scintillation counter.

Protein determinations. Protein concentrations were determined by the colorimetric Coomassie blue assay of Bradford (5).

RESULTS

Preparation of the RPV antigens for immunization. One of our primary goals was to generate a library of monoclonal antibodies directed against the large number of proteins contained within the RPV virion and to determine which immunological procedure best suited that purpose. The subclasses of viral proteins that interested us most were: (i) the readily detergent-solubilized enzymes of the core and (ii) the proteins of the nucleoid which are resistant to solubilization by detergents and bind to DNA with high avidity. To improve our chances of generating antibodies against these diverse proteins, we first removed the outer membrane from the virus, which contains highly immunogenic surface glycoproteins or glycolipids or both. Beginning with purified virus, the fractionation strategy which we used is summarized in Fig. 1. The first step involves the removal of the surface glycoproteins together with 41% of the total protein of the virus by treatment of the virions with 0.5% Nonidet P-40–50 mM dithiothreitol (8). Only a small amount of the virus-associated RNA polymerase (<5%) is lost during this treatment.

The subviral cores that remain are then treated with 0.2%

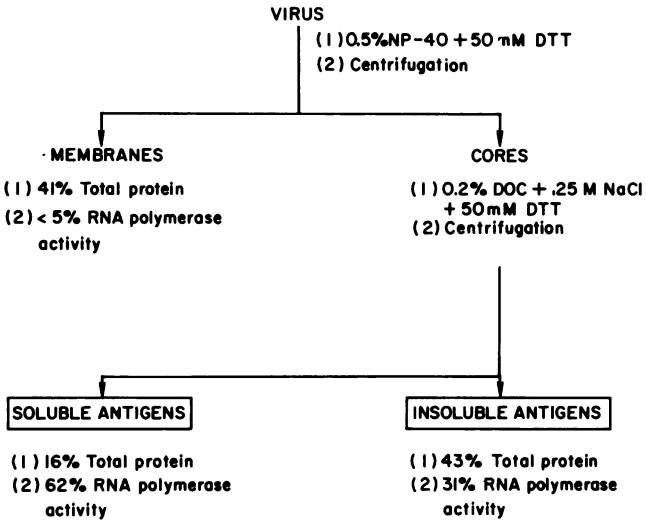

FIG. 1. Schematic depiction of the fractionation of purified RPV into the mixtures of soluble and insoluble RPV antigens used for immunization. DDT, Dithiothreitol; NP-40, Nonidet P-40.

sodium deoxycholate in the presence of 0.25 M NaCl–50 mM dithiothreitol. Many of the core-associated enzymes have been shown to be solubilized by this treatment (8). For example, 62% of the total virion-associated RNA polymerase activity is released from the core together with 16% of the total viral protein. We will refer to the mixture of proteins solubilized by deoxycholate as the soluble antigens. An examination of the insoluble core material of the virus shows that 31% of the total RNA polymerase activity remains within the core, together with 43% of the total viral protein. We will refer to the core proteins not solubilized by deoxycholate as the insoluble antigens. The proteins of the purified virus and the soluble and insoluble proteins derived from the detergent-treated cores are shown after resolution by SDS-polyacrylamide gels in Fig. 2. Included is a gel profile of extensively purified RPV RNA polymerase for comparison.

Comparison of in vivo and in vitro methods of immunization. Hybridoma cells secreting monoclonal antibodies may be generated by either in vivo or in vitro immunization regimens. One of the goals of these studies was to determine which procedure is best for preparing a library of monoclonal antibodies against antigenic mixtures of RPV proteins. The in vivo method of immunization originally described by Kohler and Milstein (17) is the procedure most widely used. The in vitro method, a more recent innovation, was originally developed by Luben and Mohler (19) and has several technical advantages over the more classical procedure. First, it is much more rapid, with a total sensitization time for spleen cells of 4 days as opposed to 4 weeks for in vivo immunization. Second, far less antigen (1 ng compared with 1 μg per 10^7 spleen cells) is required for in vitro sensitization. One disadvantage with the in vitro immunization procedure, however, is that there is no way to measure the response of the spleen cells to the antigen before fusion and selection of antibody-producing cells. On the other hand, the response of the animal to an antigen administered in vivo is quite simple to ascertain by assay of the serum.

We have used the two RPV antigen preparations (derived as shown in Fig. 1 and visualized in Fig. 2, lanes A and B) to compare the diversities of monoclonal antibody-producing

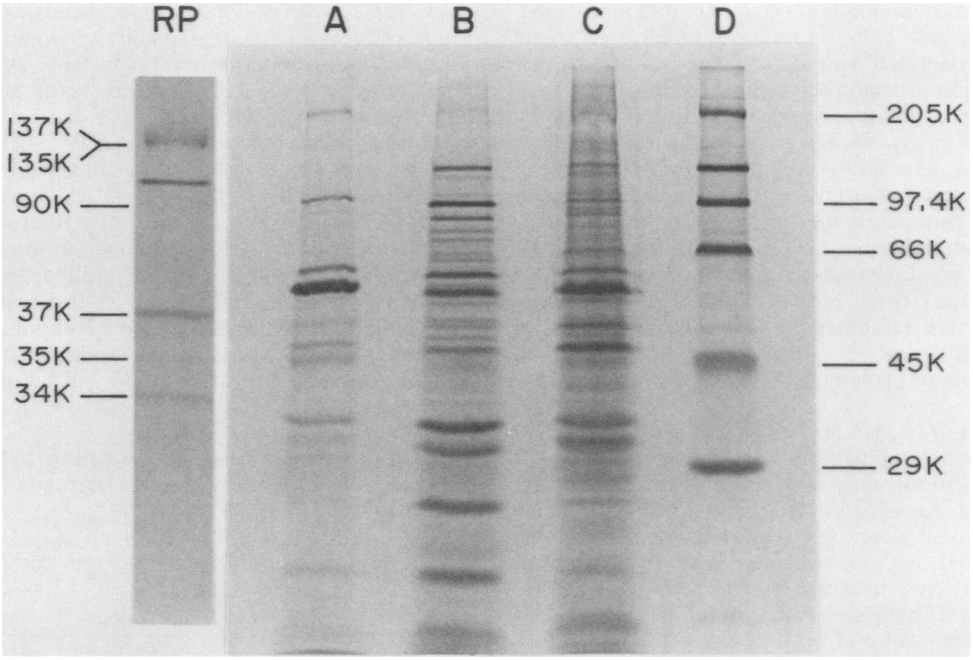

FIG. 2. Comparative protein profiles of purified RPV and several subviral fractions. Samples (10 to 20 μg) of protein from the deoxycholate-insoluble (lane A) and -soluble (lane B) fractions derived from viral cores (Fig. 1), the total protein of purified RPV (lane C), molecular weight standards (lane D), and highly purified RPV RNA polymerase (lane RP) were analyzed by electrophoresis on 13% SDS–polyacrylamide gels. The proteins were then visualized by staining with Coomassie blue as described in the text. The approximate molecular weights of the RNA polymerase subunits and of the standards are indicated in the left and right margins, respectively. Lane RP is from a separate gel.

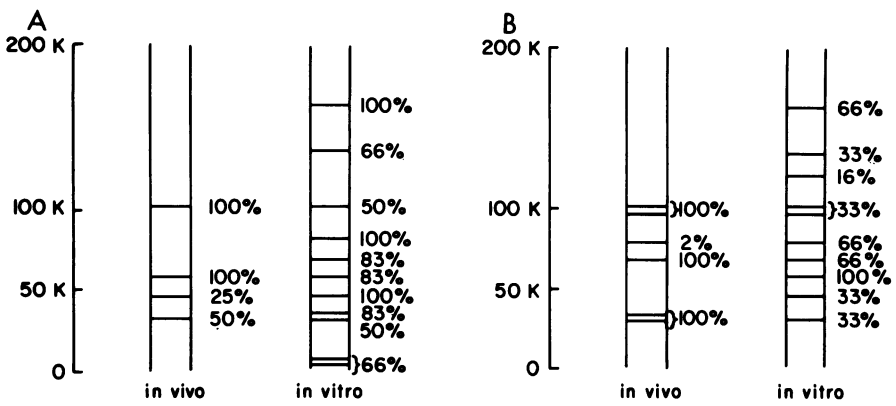

FIG. 3. Schematic depiction of the diversity of monoclonal antibodies from hybridomas produced by using different immunization protocols. By the immunization and fusion procedures described in the text, cells were dispersed into 24-well culture dishes in amounts that ultimately gave rise to approximately 10 different hybridomas per well. Culture supernatant fluids from polyclonal mixtures in a single well were screened by Western blot analysis as described in the text. The proteins recognized by monoclonal antibodies secreted by hybridomas produced by both the in vivo and in vitro immunization methods with mixtures of deoxycholate-soluble (A) or -insoluble (B) RPV proteins are indicated by a line drawing. The total numbers of wells screened were 24 and 48 for (A) and (B), respectively. The percentage listed at the right of each graph represents the percentage of the wells which contained in the culture fluid an antibody recognizing a protein of a given molecular weight. The scale of protein molecular weights is given at the left of each panel.

hybridomas obtained by the two immunization procedures. Our results, which are summarized as a schematic Western blot in Fig. 3., clearly show that with either immunogenic mixture, hybridoma cells secreting antibodies to the greatest number of different proteins are produced by the in vitro regimen of immunization. In addition, if one wishes to raise monoclonal antibodies against a protein with a rather high molecular weight, it appears that the in vitro method of immunization would generally be preferred. While it is tempting to conclude from these data that in vitro immunization might be the single method of choice, it is interesting to note that hybridoma cells secreting antibodies to some viral proteins are generated with high efficiencies only by the in vivo immunization procedure. The best examples are two detergent-insoluble proteins of approximately 100 and 35 kilodaltons (kDa) (Fig. 3). We have also noticed that with in vivo regimens, one generally tends to generate the highest number of antibodies against proteins present in the highest abundance. This can be readily seen by comparing the pattern of monoclonal antibodies (Fig. 3) with the pattern of Coomassie blue-stained proteins from the soluble and insoluble antigen mixtures (Fig. 2, lanes A and B). This is a feature of the in vivo immunization regimen which under some circumstances can be advantageous if one wants antibodies to a major protein constituent within a given mixture.

Several other points are of interest. The methods of antigen presentation to spleen cells under the two sets of immunization conditions may well be different, with the result that epitopes recognized by monoclonal antibodies derived by the two methods for a given protein might be quite different. Most of the monoclonal antibodies (>95%) derived by the in vivo procedure are of the IgG class, whereas only 60% of those generated by the in vitro procedure have been found to be of the IgG class, the remainder being of the IgM class. In view of all these considerations, we feel that the two procedures for immunization are complementary and that there are special advantages to each.

Identification of anti-RPV RNA polymerase monoclonal antibodies. To select for monoclonal antibodies directed against the RPV RNA polymerase, a screening assay was developed, based on the ability of the enzyme to be immunoadsorbed by individual monoclonal antibodies prebound to protein A-Sepharose beads. Briefly, samples of either crude extracts of virus (2, 32) or purified RNA polymerase (2, 27, 32) were exposed to protein A beads coated with a given monoclonal antibody, as described above, after which the potential enzyme-antibody-bead complexes were removed by centrifugation. The treated supernatant was then assayed for RNA synthesis, and the activity was compared with that of an identical sample which had not been treated with antibody-coated beads. An apparent inhibition of the soluble enzyme activity would occur when the (enzyme–monoclonal antibody–protein A-Sepharose bead) complex was removed after the beads were pelleted by centrifugation. One advantage of this assay is that antibodies need only bind but not necessarily inactivate the RNA polymerase to give a positive result. We arbitrarily considered a loss of >50% of the original activity after exposure to antibody-coated beads as indicative of recognition of the enzyme by a particular monoclonal antibody. Data for two monoclonal antibodies which fulfilled this criterion are shown in Table 1 together with a third antibody which did not. Each positive antibody gave >50% inhibition of soluble enzyme activity when either crude extracts or purified enzyme was tested. Further data will be provided to show

TABLE 1. Immunoadsorption of RPV RNA polymerase by protein A-bead-monoclonal antibody complexes

Antibody	% Immunoadsorption with the following enzyme source[a]:	
	Crude enzyme solubilized from virions	Purified enzyme
Mab-137	65	54
Mab-34	56	52
Mab-94[b]	24	37

[a] Percentage of the total soluble activity immunoadsorbed by the protein A-bead-monoclonal antibody complexes.

[b] Mab-94 is directed against a viral structural protein which is not a subunit of the viral RNA polymerase.

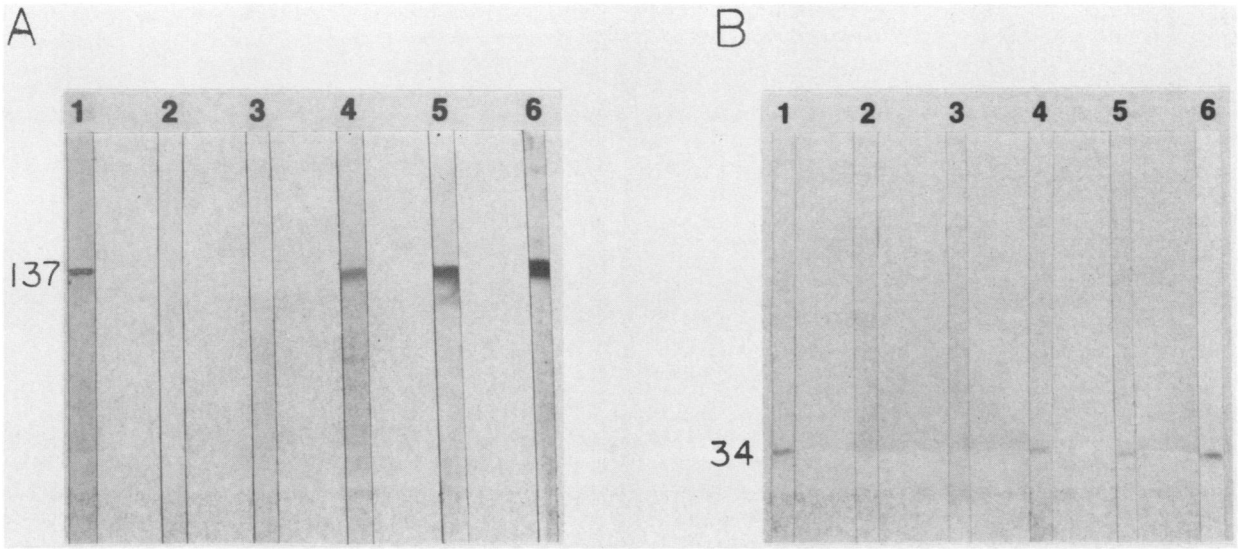

FIG. 4. Biosynthesis of RPV RNA polymerase 137-kDa (A) and 34-kDa (B) protein subunits in infected cells. Immunoblots are shown for the two subunits with: proteins of purified RPV (lanes 1), cytoplasmic extracts of uninfected RK cells (lanes 2), and cytoplasmic extracts of RK cells infected with RPV in the presence of cytosine arabinoside (lanes 3) or rifampin (lanes 4) or in the absence of inhibitors (lanes 5). All infected-cell extracts were prepared at 12 h postinfection. A sample of highly purified RPV RNA polymerase (see Fig. 2, lane RP) was also included (lanes 6). The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The blots were then probed with Mab-137 (A) or Mab-34 (B) as described in the text.

that one of the positive monoclonal antibodies recognized a viral protein of 137 kDa (Mab-137) and the second recognized a protein of 34 kDa (Mab-34); both proteins are constituents of the purified viral RNA polymerase. The third antibody (Mab-94) recognized a 94-kDa late protein which was cleaved to 62 kDa during viral maturation and is one of the major structural proteins of the mature virion (data not shown). As expected, Mab-94 showed much less inhibition of enzyme activity.

Mab-137 and Mab-34 recognize the 137- and 34-kDa protein subunits of viral RNA polymerase which are synthesized late in infection. The synthesis of the viral proteins recognized by Mab-137 and Mab-34 in RPV-infected cells was monitored by immunoblot analysis (Fig. 4). The Mab-137 antibody recognized a 137-kDa protein present within purified virions (Fig. 4A, lane 1), whereas Mab-34 recognized a 34-kDa virion protein (Fig. 4B, lane 1). Each of these proteins is presumably of viral origin since no proteins were recognized in uninfected cells (Fig. 4A and B, lanes 2). Cells infected with RPV in the presence of cytosine arabinoside, an inhibitor which blocks viral DNA synthesis, were limited to the expression of early proteins. No proteins in cytosine arabinoside-infected cells were recognized by either Mab-137 or Mab-34, suggesting that, within the limits of the sensitivity of the immunoblot technique, both of these viral proteins accumulate after DNA replication and would by this criterion be classified as late proteins (Fig. 4A and B, lanes 3). When cells are infected in the presence of rifampin, viral DNA replication occurs, and late proteins are expressed. Morphogenesis, however, is blocked, and no mature virions are formed (12, 22). When both Mab-137 and Mab-34 were used to probe extracts of cells infected with RPV in the presence of rifampin, protein bands of 137 and 34 kDa, respectively, were readily visible (Fig. 4A and B, lanes 4). Similarly, in extracts of cells infected with RPV in the absence of inhibitors at 12 h postinfection when morphogenesis had begun, both the 137- and 34-kDa proteins were readily visible (Figs. 4A and B, lanes 5). Finally, both the 34- and the 137-kDa proteins are constituents of the highly purified viral RNA polymerase (Fig. 4A and B, lanes 6) whose protein composition is shown in Fig. 2, lane RP.

Localization of the 137- and 34-kDa subunits of the viral RNA polymerase within infected cells by indirect immunofluorescence. One stage in the development of poxviruses is characterized by the presence of cytoplasmic virosomes or factories, which serve as the site of viral transcription, DNA replication, and the onset of morphogenesis. Viral proteins utilized in these processes, such as the RNA polymerase, would be expected to localize to the virosomes during the infectious process. The intracellular localization of a protein can be directly visualized with the use of a specific antibody in conjunction with a second fluorescent antibody in indirect immunofluorescence experiments. These experiments afford the opportunity to reexamine the question of whether a portion of the viral RNA polymerase enters the nucleus, as has been reported (38). When uninfected cells were probed with either Mab-137 (Fig. 5A) or Mab-34 (data not shown), no immunofluorescence was detected, as expected. Cells were probed at 1 h postinfection with a control monoclonal antibody Mab-180 (Fig. 5B), which is directed against an early 180-kDa viral protein of unknown function (data not shown). The immunofluorescent pattern we observed is consistent with the localization of this protein at or near the cell surface rather than within virosomes. The pattern of staining shown with Mab-180 in Fig. 5B did not change as the infection proceeded and was not substantially affected by inhibitors of DNA synthesis (cytosine arabinoside) or of morphogenesis (rifampin) (data not shown).

To test the location of the RNA polymerase subunits, cells were infected with RPV in the presence of rifampin and processed for immunofluorescence at 4 and 12 h after infection. Rifampin was used because virosomes, rather than being dismantled for the production of virus, remain in place, albeit in a slightly altered morphological state within the cytoplasm (12, 26, 28). The staining with Mab-34 of cells 4 h postinfection showed a distinctive pattern, with the fluorescence localized to the discrete virosomes within the cytoplasm (Fig. 5C). By 12 h postinfection in the presence of

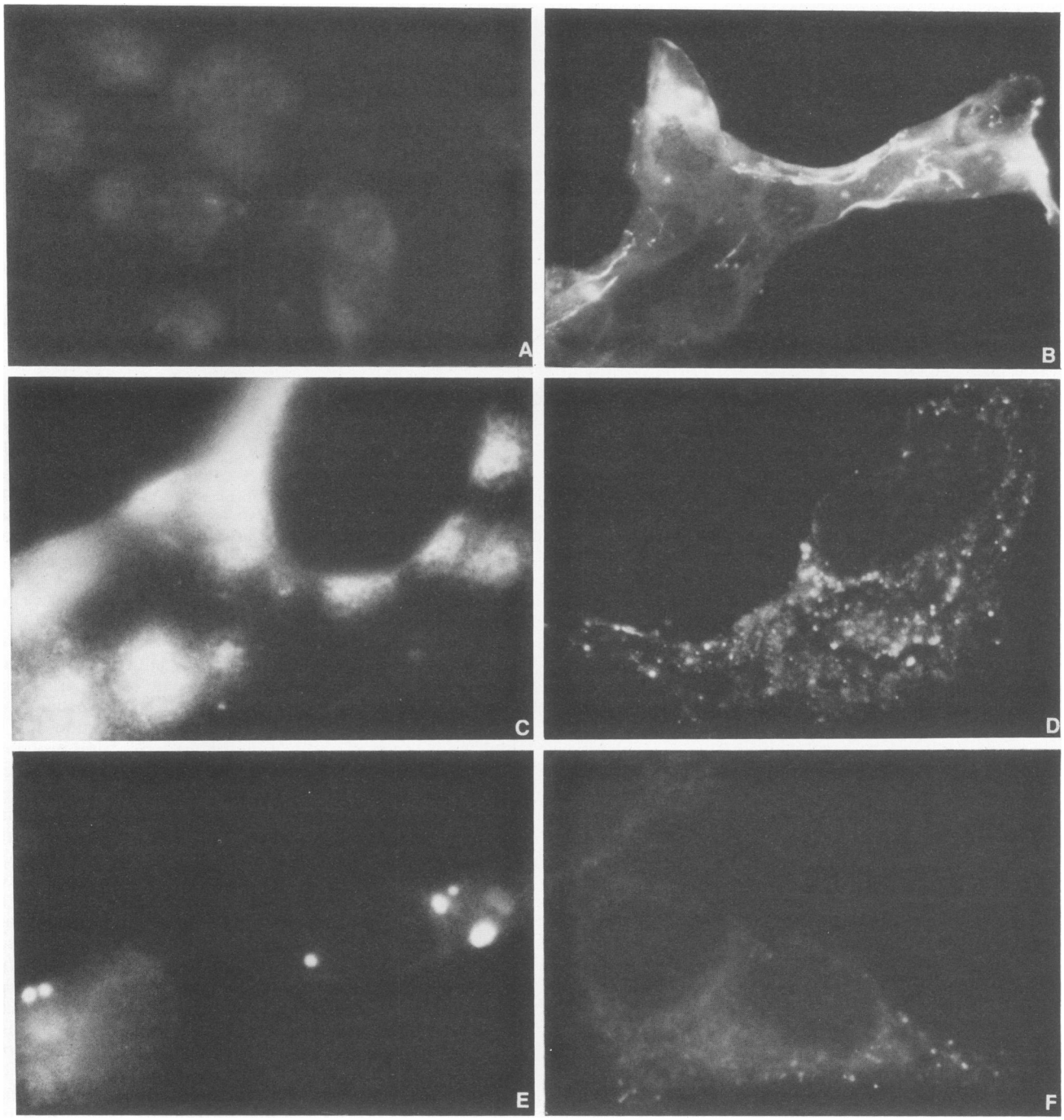

FIG. 5. Localization of the RPV RNA polymerase 137- and 34-kDa subunits within cells infected in the presence of rifampin by indirect immunofluorescence. Control and infected RK cells on cover slips were processed for indirect immunofluorescence staining as described above. Shown are uninfected cells probed with Mab-137 (A), infected cells probed with Mab-180 (B), infected cells probed with Mab-34 at 4 h (C) or 12 h (D) after infection, and infected cells probed with Mab-137 at 4 h (E) or 12 h (F) after infection. Infections (C through F) were done in the presence of rifampin. Relative magnifications: ×400 (A and B), ×1,000 (C, D, and F) and ×800 (E).

rifampin, virosomes persist but become considerably smaller (12, 26, 28); nevertheless, the 34-kDa RNA polymerase subunit remained within those smaller but discrete virosomes (Fig. 5D). Identical results were obtained with monoclonal antibody to the 137-kDa RNA polymerase subunit. At 4 h postinfection, a discrete localization of immunofluorescence was observed within the cytoplasm consistent with the protein being localized within virosomes (Fig. 5E). By 12 h after infection, the cytoplasmic localization was maintained and the staining was again mostly within discrete bodies which were smaller than those observed at 4 h after infection (Fig. 5F). The omission of rifampin, which resulted in a permissive infection, did not alter the virosomal staining patterns observed at 4 h postinfection with either Mab-34 or Mab-137 (Fig. 6). At 12 h after infection, in the absence of the drug, however, the staining remained exclu-

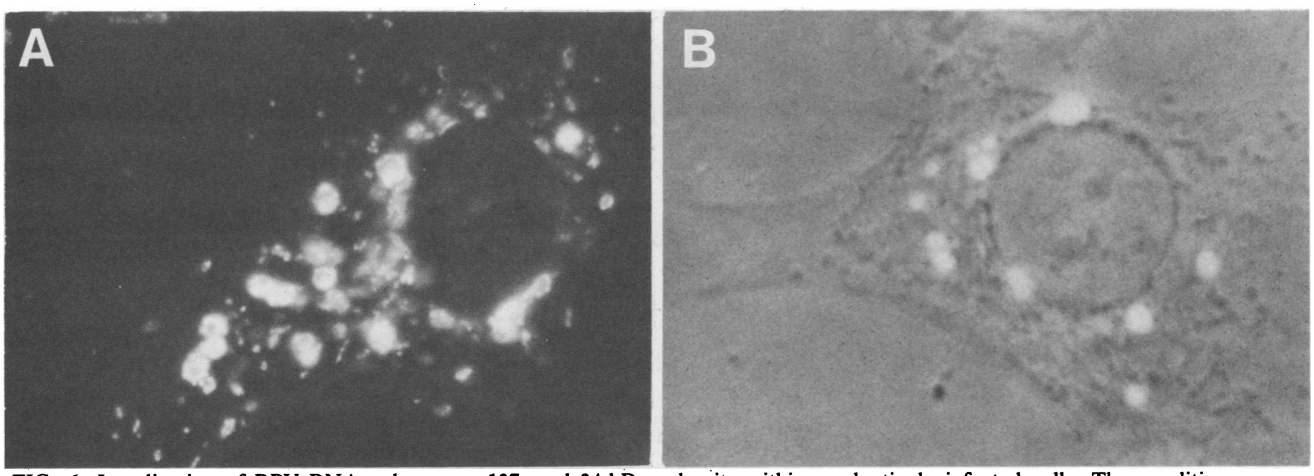

FIG. 6. Localization of RPV RNA polymerase 137- and 34-kDa subunits within productively infected cells. The conditions were as described in the legend to Fig. 5 except that cells were infected in the absence of rifampin. Cells were harvested at 4 h postinfection and probed with Mab-34 (A) and Mab-137 (B). Panel B represents a photographic superimposition of the phase and fluorescent images. Relative magnification, ×1,000.

sively cytoplasmic but was much more diffuse (data not shown), consistent with the production of individual mature virus particles. Interestingly, there was no detectable nuclear immunofluorescence within infected cells at any time or under any conditions with antibodies directed against either the 137- or 34-kDa RNA polymerase subunits (data not shown). It would appear, therefore, that both of these subunits are localized exclusively to the cytoplasm.

Mapping of the 137- and 34-kDa RNA polymerase subunits. The 137- and 34-kDa subunits were mapped by first isolating immunopositive phage plaques from a λgtll expression vector library constructed by the cloning of 3- to 5-kilobase fragments of sheared RPV DNA. Four immunopositive plaques were selected with Mab-137, and two were selected with Mab-34. The expression vector λgtll has been designed so that the insert site is in the distal portion of the gene

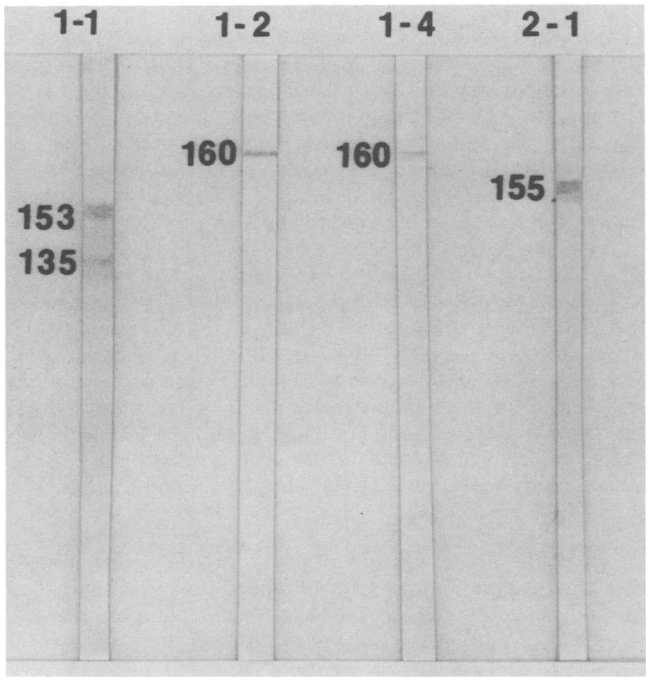

FIG. 7. Production of fusion proteins by four Mab-137-immunopositive λgtll clones. Fusion protein production was assayed by subjecting samples of induced bacterial cell cultures to immunoblot analysis and probing with Mab-137 as described in the text. Lanes 1-1, 1-2, 1-4, and 2-1 represent four independently isolated immunopositive clones.

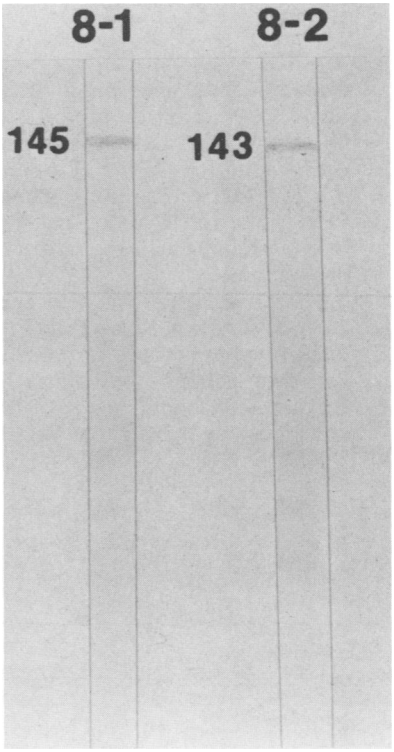

FIG. 8. Production of fusion proteins by two Mab-34-immunopositive λgtll clones. Experimental protocols are as described in the legend to Fig. 7 except that the immunoblots were probed with Mab-34.

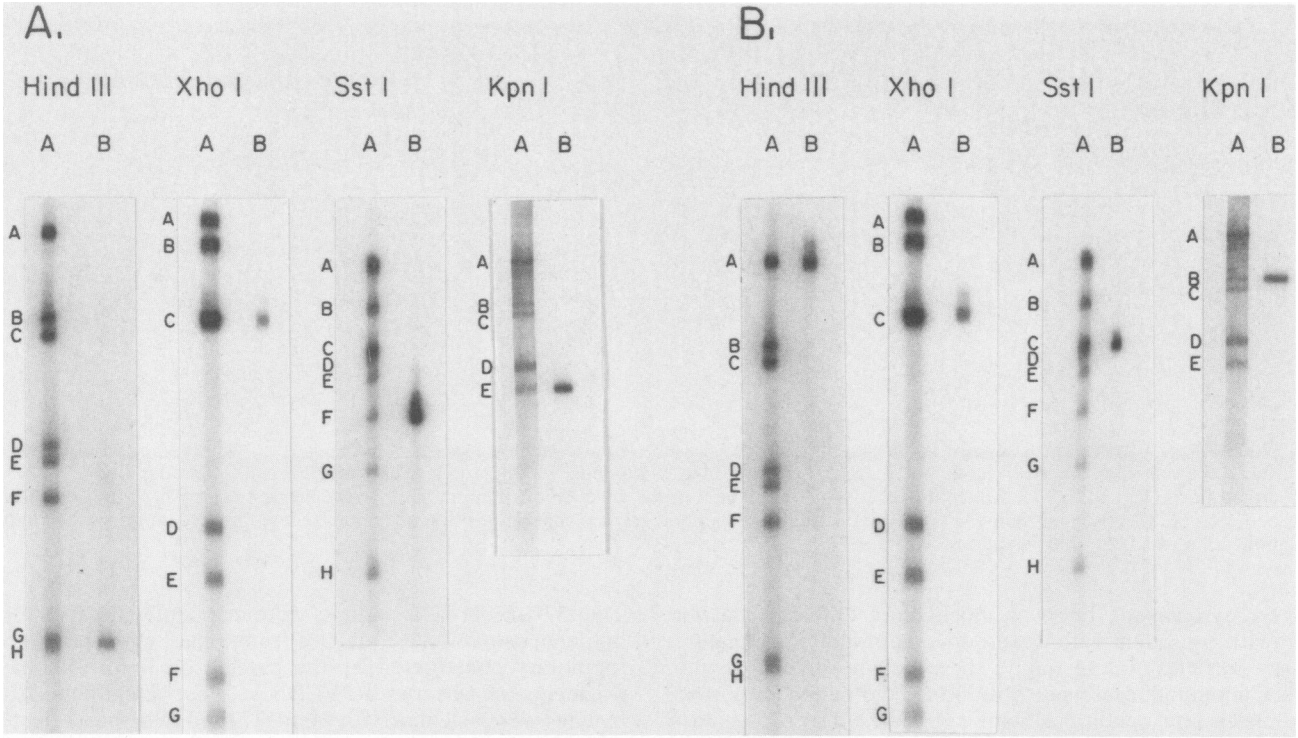

FIG. 9. Mapping of the 137- and 34-kDa subunits of RPV RNA polymerase by the hybridizations of immunopositive λgtll clones to digests of RPV DNA. The hybridization patterns of the Mab-137 immunopositive clones (2-1) and the Mab-34 immunopositive clone (8-2) are shown in panels A and B, respectively. Hybridizations were performed as described in the text and by Southern (31), with *Hin*dIII, *Xho*I, *Sst*I, and *Kpn*I digests of total RPV DNA. Each digest was hybridized both to nick-translated RPV DNA as a control (lanes A) and to the clone of interest (lanes B).

encoding β-galactosidase (39, 40). Upon induction, each plaque generated readily detectable immunopositive fusion proteins (Fig. 7 and 8). All six fusion proteins were considerably larger (143 to 160 kDa) than β-galactosidase alone (116 kDa). Two immunopositive proteins were detected in cells infected with the Mab-137-positive phage clone 1-1 (Fig. 7). It is likely, however, that the smaller (135-kDa) immunopositive protein represents a degradation product of the large (153-kDa) protein.

The size of the RPV DNA insert was determined for each of the clones for which fusion proteins (Fig. 7 and 8) were obtained. The results are summarized in Table 2. The sizes are ~700 base pairs (bp) of RPV DNA for each of the two isolates expressing the Mab-34 epitope and from ~900 bp to 4 kilobases for the isolates expressing the Mab-137 epitope.

TABLE 2. Characterization of immunopositive λgtll recombinant clones selected by antibodies directed against poxviral RNA polymerase subunits

Clone no.	Selected by:	Insert size (kb)[a]	Size of fusion protein (kDa)
8-1	Mab-34	0.75	145
8-2	Mab-34	0.70	143
1-1	Mab-137	3.95	153, 135
1-2	Mab-137	3.20	160
1-4	Mab-137	3.45	160
2-1	Mab-137	0.88	155

[a] kb, Kilobases.

The clones containing the smallest inserts in each case (2-1 for Mab-137 and 8-2 for Mab-34) were selected for nick translation and hybridization to various restriction digests of genomic RPV DNA to map the genes for the subunits within the RPV genome. The results of the hybridizations are shown in Fig. 9A and B, respectively. The radiolabeled RPV DNA insert (~900 bp) of clone 2-1 encoding the 137-kDa epitope hybridizes to the unresolved G/H doublet, C, F, and E fragments of *Hin*dIII, *Xho*I, *Sst*I, and *Kpn*I digests of RPV DNA, respectively (Fig. 9A). The nick-translated clone 8-2 RPV DNA probe (~700 bp) which encodes the 34-kDa epitope shows hybridization to the A, C, C/D doublet, D, and B fragments of RPV DNA digested with *Hin*dIII, *Xho*I, *Sst*I, and *Kpn*I, respectively (Fig. 9B). For each subunit, similar results were obtained for each of the other clones described in Table 2. The combined results of these hybridization patterns allow assignment of approximate map locations for the two subunits within the RPV genome (Fig. 10).

DISCUSSION

One of the objectives of this work was to determine the best practical methodology for obtaining diverse monoclonal antibodies against the largest possible spectrum of poxvirus proteins. Toward this end, we compared the efficacies of obtaining hybridoma cells by both the in vivo and in vitro methods of immunization with two different mixtures of viral antigens. Generally, hybridoma cells secreting antibodies against the largest numbers of diverse molecular weight proteins are generated by the use of the in vitro immunization of spleen cells in culture. We also found that the in vivo method of immunization tends to generate hybridoma cells

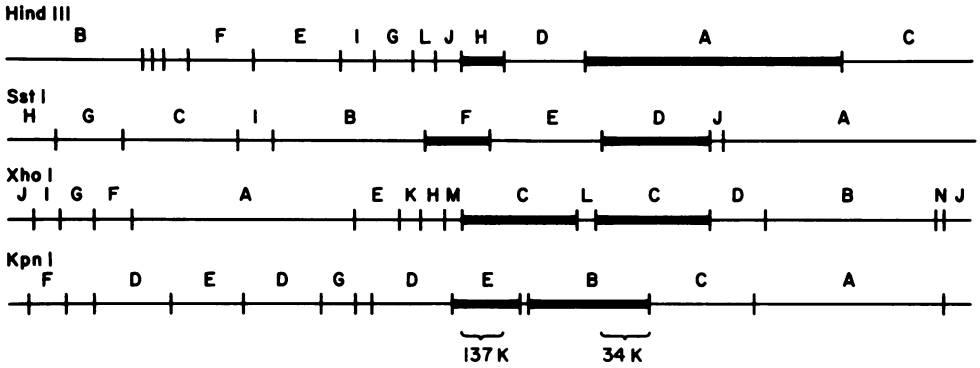

FIG. 10. Approximate map positions (heavy lines) of the 137- and 34-kDa subunits of the RPV RNA polymerase.

secreting monoclonal antibodies to the proteins present in major abundance within a mixture, whereas in vitro immunization results in higher numbers of monoclonal antibodies directed against minor components. Of the monoclonal antibodies from the in vitro procedure, roughly 40 and 60% are of the IgM and IgG classes, respectively. A much lower percentage of IgM antibodies (<5%) is found when in vivo procedures are followed. On balance, for the reasons discussed above, the two methods are complementary, and there are advantages to both procedures.

The immunoaffinity screening assay that was developed to select for anti-RVA RNA polymerase antibodies should be applicable to any enzyme, and particularly to multisubunit enzymes. The method should also be useful in screening for antibodies directed against regulatory proteins which are themselves devoid of enzymatic activity but which may bind to a given enzyme. The advantage of constructing insoluble immunoaffinity reagents for rapid screening is that antibody recognition and binding, but not concomitant inactivation of the enzyme, are necessary to lower the levels of enzyme activity that remain in solution.

We have described two monoclonal antibodies, Mab-137 and Mab-34, which were determined by inhibition of polymerase activity and direct binding to purified RPV RNA polymerase to be directed against the 137- and 34-kDa subunits, respectively, of the virion-associated RNA polymerase (Table 1; Fig. 4A, lane 6; Fig. 4B, lane 6). The two antibodies were then used to study the biogenesis of the RNA polymerase, to localize the enzyme in situ within infected cells, and to locate the genes encoding the proteins within the viral genome.

The biosynthesis of these two proteins was examined through immunoblot analysis. Although both proteins are readily detectable within purified virus, purified RNA polymerase, productively infected cells, and cells infected in the presence of rifampin, we were unable to detect the presence of either subunit within infected cells when viral DNA synthesis was blocked (Fig. 4A and B, lanes 3). We conclude from these results that within the limits of sensitivity of the immunological techniques used in this analysis, both RNA polymerase subunits are late proteins.

The availability of monoclonal antibodies directed against given poxvirus proteins, when used in conjunction with a random library of RPV DNA cloned into expression vector, allows the mapping of genes by a relatively easy procedure. The alternative procedure for this purpose, which involves the hybrid selection of viral mRNAs by specific DNA restriction fragments followed by the subsequent translation of the selected mRNAs in vitro, is much more laborious. The use of expression vector libraries together with monoclonal antibodies as a means for gene mapping also circumvents one of the major problems associated with the in vitro translation of mRNAs, namely, the poor translation of proteins of very high molecular weights such as the 137-kDa protein described here. We have created such an RPV DNA library and have mapped the genes for the two RNA polymerase subunits. Based on the hybridization patterns of cloned RPV DNA insert probes, the 137-kDa subunit maps within the left two-thirds of the *Hin*dIII H fragment (Fig. 9A), whereas the 34-kDa subunit maps in the left third of the *Hin*dIII A fragment. Although the map locations are not precise, the genes do not appear to be closely linked.

We have also used Mab-137 and Mab-34 to determine the intracellular localizations of these two RNA polymerase subunits within infected cells by indirect immunofluorescence, a study which is relevant to the question of the role of the cell nucleus in poxvirus development. Despite reports of both viral RNA synthesis within the nucleus of infected cells (3, 9) and the presence within the nucleus of the virus-associated RNA polymerase activity (38), results of immunofluorescence studies with Mab-137 and Mab-34 (Fig. 5) indicate that these subunits are found only within the developing virosomes in the cytoplasm and that neither subunit enters the nucleus. It would appear, therefore, that at least these two subunits of the enzyme are not involved in any intranuclear phase of viral gene transcription.

Results of other studies in our laboratory involving in situ hybridization of RPV DNA to intact infected cells have likewise suggested that there is no evidence for the entry of viral DNA into the nucleus and that the DNA remains within the cytoplasm (H. Minnigan and R. W. Moyer, submitted for publication). Therefore, all of our results suggest that the requirement of the nucleus for a productive poxvirus infection is not likely to depend upon the intranuclear entry of viral components. Additional studies in our laboratory indicate that RPV infection induces the movement of at least one subunit of RNA polymerase II of the host cell from the nucleus, first to the cytoplasmic virosomes and thereafter into mature virus (manuscript in preparation). A direct involvement in some way of host cell RNA polymerase II in poxvirus development would explain the sensitivity of the infection to α-amanitin (14, 30). More specifically, the direct utilization of some form of RNA polymerase II to transcribe RPV late genes is consistent with the observation (Fig. 4) that late genes of the virus are actively transcribed in virosomes before significant amounts of the late 137- and 34-kDa subunits of the virion-associated RNA polymerase are synthesized.

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