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**ISOLATION AND EVALUATION OF THE IMMUNIZING SUBUNITS OF BOVINE  
ADENOVIRUS TYPES 2, 3 AND 7**

*Iowa State University*

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**Isolation and evaluation  
of the immunizing subunits  
of bovine adenovirus types 2, 3 and 7**

by

**Jane Kwun-Lai Kan Battles**

**A Dissertation Submitted to the  
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## EXPLANATION OF DISSERTATION FORMAT

This dissertation consists of a general introduction, a literature review, two separate manuscripts, a general conclusion, references and acknowledgements. The manuscripts are presented in the format required for the dissertation.

The Ph.D. candidate, Jane Kwun-Lai Kan Battles, is the senior author and principal investigator for each of the manuscripts.

## GENERAL INTRODUCTION

Bovine adenoviruses (BAVs) have been associated with pneumonia, enteritis, pneumoenteritis, conjunctivitis, keratoconjunctivitis and weak calf syndrome (46). At least 10 serotypes of BAVs are recognized (46,64) and they have been divided into two subgroups based on antigenic and cultural characteristics (6,64). A serologic survey by D. E. Reed (personal communication, Molecular Genetics, Inc., Minnetonka, MN) has indicated that BAV-2, -3, -6, -7 and strain 9639 (a probable new serotype) are the most common in the Midwestern United States. It is thought that some degree of cross-protection exists within a subgroup and that the immunologic recall phenomenon acts within a subgroup (6,47,71,88). If a vaccine is to be constructed, it must, therefore, include members of both subgroups. In this study, BAV serotypes 2, 3 and 7 were chosen to represent both subgroups.

Purified viral subunits were prepared by preparative agarose immunoelectrophoresis, a method which has been used for production of monospecific sera to capsid subunits of human adenoviruses (14,15,16, 28,55). The viral neutralization potentials of the sera produced were analyzed using in vitro assays. Antigens involved in viral neutralization are primarily those associated with the hexon, but fiber antigens also have been implicated (67). Since hexon subunits are available in large quantities, efforts were concentrated on isolating hexon subunits that were capable of inducing viral neutralizing antibodies.

Before recombinant DNA technology can be utilized to manufacture these subunits, the relative locations of the genes coding for the major capsid proteins need to be identified on the genome. Since the genome of human adenovirus type 2 (HAV-2) has been well characterized, cross-hybridization studies were performed between HAV-2 and BAV-7 DNAs. Taken together with previous studies between HAV-2 and BAV-3 (42), the genomic homology data made it possible to align the genomes of the BAVs to HAV-2, and to study possible evolutionary relationships between these viruses.

The objectives of this study were:

- 1) To isolate and identify the immunogenic protein subunits of BAV types 2, 3 and 7. Particular attention was paid to the hexon subunits.
- 2) To prepare in rabbits monospecific sera against the isolated proteins.
- 3) To characterize these monospecific sera using viral neutralization, immunoprecipitation/sodium dodecylsulfate polyacrylamide gel electrophoresis, immunodiffusion and Western blot analysis.
- 4) To determine the apparent molecular weights of the major capsid polypeptides of BAV-2, -3 and -7.
- 5) To locate the relative positions of the genes coding for the major capsid proteins on the BAV-7 genome.
- 6) To study possible evolutionary relationships between the BAVs and HAV-2.

## LITERATURE REVIEW

Adenoviruses originally were discovered in human adenoidal cell cultures (79) and as agents causing respiratory infections of military recruits (40). The human adenoviruses (HAVs) are known to cause acute respiratory illness (40), epidemic keratoconjunctivitis (45) and infantile gastroenteritis (30). In addition, some HAVs possess oncogenic potential in nonpermissive cells (43,86).

Adenoviruses have been isolated from a variety of animal species, including monkey, cattle, pig, sheep, horse, dog, goat, mouse, opossum, tree shrew and birds (92). The first isolation of an adenovirus from cattle was reported by Klein et al. (49). Bovine infections frequently are inapparent, but in calves, bovine adenoviruses (BAVs) can cause pneumonia, enteritis, pneumoenteritis, conjunctivitis, keratoconjunctivitis and weak calf syndrome (46). Only one serotype, BAV-3, is known to be oncogenic for newborn hamsters (25).

### Bovine Adenoviruses

Presently, at least 10 serotypes of BAVs are recognized throughout the world (46,64). The BAVs are divided into two subgroups based on cultural and antigenic characteristics (6,64). The BAVs have been reviewed extensively (58,64), and their roles in bovine diseases have been summarized by Kahrs (46). The prototype strain of each serotype and the subgroups of BAVs are shown in Table 1.

Table 1. Bovine adenoviruses

Subgroup	Serotype <sup>a</sup>	Prototype strain	Reference
1	BAV-1	10	49
	BAV-2	19	50
	BAV-3	WBR1	27
2	BAV-4	THT/62	9
	BAV-5	B4/65	9
	BAV-6	671130	76
	BAV-7	Fukuroi	44
	BAV-8	Misk 67	11
	BAV-9	Sofia 4/67	36
	BAV-10	Nagano <sup>b</sup>	85

<sup>a</sup>Each serotype of adenovirus is now designated as a species, according to the latest report of the International Committee on the Taxonomy of Viruses (92).

<sup>b</sup>The only example in the literature of using strain Nagano as the prototype strain of BAV-10 was found in the cross-neutralization tests performed by Mohanty (64).

BAV-1 (strain 10) and BAV-2 (strain 19) were isolated from the feces of apparently normal cows upon culturing on primary calf kidney cells (49,50). Additional isolates of BAV-1 were reported from nasal samples of calves with pneumonia (25), calves with pneumoenteritis (81), normal calves (65), and calf kidney cell cultures (29). Additional isolation of BAV-2 was reported from embryonic kidney cell cultures as an adventitious contaminant (66). Both viruses are widespread among cattle populations, as demonstrated by the presence of neutralizing antibodies (32,48,49,50,64,77,97). In addition, BAV-2 strains have been found to cause natural outbreaks of respiratory diseases among sheep in Hungary (12).

BAV-3 (strain WBR1) was isolated from the eye of an apparently healthy cow upon culturing in bovine kidney cells (27). Additional isolates of BAV-3 were reported from a herd with pneumoenteritis (57), and cattle with acute respiratory disease (53). Serologic studies have indicated that BAV-3 is prevalent in the United States (13,54,57,64) and other countries (32,37). In addition, antibodies to BAV-3 have been detected in a herd of goats in Louisiana (31), and BAV-3 has been isolated from free-living African buffaloes (4).

BAV-4 (strain THT/62) and BAV-5 (strain B4/65) were isolated from calves with pneumoenteritis. Upon repeated passages in primary calf testicular cell cultures, these viruses were isolated from bronchial, nasal, conjunctival and fecal samples, as well as from a variety of tissues (9). Additional isolates of BAV-4 were reported from bulls with pneumoenteritis and respiratory disease (59), uninoculated bovine testicular cell (10,73), and free-living African Buffaloes (4). In addition, neutralizing antibodies to BAV-4 and -5 have been found in cattle populations (59,64,73,97).

BAV-6 (strain 671130) was isolated as a latent virus from primary calf testicular cell cultures (76). Additional isolates of BAV-6 include strain RG from calves with pneumonia (20,21), and strain KC-2 from cattle with keratoconjunctivitis (93). Serologic surveys by Mohanty (64) and Cole (22) indicated the presence of neutralizing antibodies by BAV-6 in cattle populations.

The prototype strain of BAV-7, strain Fukoroi, along with 8 other strains, was isolated from the blood, feces and viscera of cattle with

acute respiratory disease upon culturing in primary calf testicular cells (44,60). Additional isolations of BAV-7 were reported from tissues and feces of calves with pneumonia and enteritis (75), blood and synovial fluid of calves with weak calf syndrome (23,62,84), and bovine testicular cell cultures as an adventitious agent (73). Although large scale serologic surveys of BAV-7 have not been performed, the role of BAV-7 as an etiologic agent has been established (44,62,75). In addition, BAV-7 has been reported to cause abortion experimentally (83). Recently, antibodies to BAV-7 were found in apparently healthy goats in Louisiana (31).

BAV-8 (strain Misk 67) was isolated from calves with pneumoenteritis (11). Another strain of BAV-8 was isolated from a cow with leukosis (61). A serologic survey indicated that BAV-8 is prevalent in the cattle populations in Hungary (63). BAV-8 also has been isolated from free-living African buffaloes, along with BAV-3 and -4 (4).

BAV-9 (strain Sofia 4/67) was isolated from kidney and testicular cultures of healthy cattle (36,37). Additional isolations of BAV-9 include strain BIL from calves with pneumonia (20,21) and strain KC-6 from cattle with conjunctivitis (93).

BAV-10 (strain Nagano) was isolated from blood specimens of calves with mild respiratory disease upon culturing in primary calf testicular cells (85). Other probable new serotypes of BAV include an European strain isolated from cattle with pneumonia (51), a New Zealand strain isolated from a yearling heifer with systemic infection (41), and strain 9639 from South Dakota (D. E. Reed, Molecular Genetics, Inc.,

Minnetonka, MN, personal communication).

#### Adenovirus Nomenclature

The family Adenoviridae is divided into two genera, Mastadenovirus (mammalian adenoviruses) and Aviadenovirus (avian adenoviruses). This grouping originally was proposed because of the absence of immunologically cross-reactive proteins (92). Most adenoviruses isolated from mammalian hosts share a soluble complement fixation antigen (78). A notable exception to this is represented by the members of BAV subgroup 2 (6). Therefore, these serotypes are identified as adenoviruses based on their morphology, nature of nucleic acid content, and other physiochemical properties such as resistance to ether and chloroform (64).

The HAV species (formerly serotypes) have been classified into 5 subgenera (formerly subgroups) with respect to their biophysical, biochemical, biological, and immunological characteristics (92). Each adenovirus species is defined "on the basis of its immunological distinctiveness, as determined by quantitative neutralization with animal antisera. A species has either no cross-reaction with others or shows a homologous-to-heterologous titer ratio of >16 in both directions" (92).

#### Bovine adenovirus subgroups

The BAVs have been divided into two subgroups, based on antigenic and cultural properties (6,64). Members of subgroup 1 replicate in both bovine kidney and testicular cell cultures and possess the common soluble antigen present in Mastadenovirus. Members of subgroup 2 share only

traces, if any, of the common soluble antigen of the genus. These viruses are grouped on the basis of their failure to grow in bovine kidney cell cultures and their slow propagation rate in bovine testicular and lung cultures. Isolation of subgroup 2 BAVs often requires a series of blind passages. Characteristic differences in cytopathic effect also are observed between the two subgroups. Subgroup 1 viruses usually induce a single nuclear inclusion, irregular in shape, while subgroup 2 viruses form mainly multiple inclusions that are regular in shape. In addition, members of subgroup 2 are partially resistant to inactivation at 56°C for 30 minutes (6).

Recently, Adair and Curran (1) proposed a slightly different subgrouping of BAVs based on detailed studies on the cytopathology in cell cultures. Using immunofluorescence, electron microscopy, and Haematoxylin and Eosin cytology studies, they divided the BAVs into two subgroups with BAV serotypes 1, 2, 3, 7 and 9 in one subgroup and serotypes 4, 5, 6 and 8 in the other.

Although members of subgroup 2 do not share the soluble complement-fixation antigen with other mastadenoviruses, cross-reactions using other immunologic methods have been reported. Using the double immunodiffusion test, some workers reported cross-reactions (4,21) while others reported no cross-reactions (2,19,73,85). Using cross-fluorescent antibody tests, sharing of antigens was demonstrated between BAV subgroup 2 (BAV-5, -7, -8) and conventional mastadenoviruses (BAV-1, 2; HAV-2, -5) by Adair et al. (2). However, Mohanty (64) reported the lack of cross-reactions using the same tests. In addition, antibodies to BAV-7 have been

reported to neutralize HAV-11 (96).

#### Subgroups based upon polypeptide patterns

Most criteria used for grouping adenoviruses, such as hemagglutination and oncogenicity, are based on properties representing only minor regions of the genome. The structural polypeptides of the virion, however, represent a major portion of the adenovirus gene products (34). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis of different HAV subgroups have shown that members of a subgroup share an apparent molecular weight pattern for the internal structural polypeptides. However, each serotype displays a unique pattern for the capsid polypeptides II, III, IIIa and IV (hexon, penton base, peripentonal area and fiber, respectively). This makes SDS PAGE a useful tool in confirming the identification of a serotype and for the subgroup classification of adenoviruses (89).

#### Genomic homology studies

The ultimate subgroup classification will be based on nucleotide-sequence differences between the genomes of different serotypes (90) because the evolution of a virus is the direct result of gradual changes in the nucleotide sequence of the genome (3). Extensive sequence homology studies of HAVs have been performed (33,34,35). Genomic homology studies between HAVs and adenoviruses of animal origin have been performed by several groups of investigators. Larsen et al. (52) found two distinct regions of homology between HAV-2 and mouse FL at 11.2-14.9 map units

(m.u.) and 51.6-62 m.u. on the HAV-2 genome, corresponding to the IVa2- and hexon-coding genes, respectively. Studies between bovine subgroup 1 and HAV-2 showed even more homology. Niiyama et al. (68) reported 25% homology between BAV-3 and HAV-5, while Hu et al. (42) showed 80% homology between BAV-3 and HAV-2. Homology studies between avian and human adenoviruses also have been performed. Aleström et al. (3) found two distinct regions of homology between fowl adenovirus type 1 (CELO virus) and HAV-2 at 18.1-19.3 m.u. and 57-58 m.u. The first region does not correspond to any gene recognized so far, but the second region is within the hexon gene. Therefore, homology exists between the genomes of these two viruses even though avian and human adenoviruses do not cross-react serologically. Since structural constraints dictate certain similarities in the amino acid sequences of the hexon proteins from different adenoviruses, at least part of the hexon gene should be conserved in order to generate a capsid protein which can give rise to the characteristic adenovirus architecture (3).

#### Antigenic Determinants

The capsid components of adenoviruses display a wide range of antigenic specificities. Hexons carry group, intersubgroup, intrasubgroup and type specificities (70). Penton bases carry group, intersubgroup and intrasubgroup specificities (91). The fibers carry intersubgroup, intrasubgroup and type specificities (69).

The capsid subunits of adenoviruses are produced in excess by the infected cell as soluble antigens. This has facilitated the purification,

characterization and immunological identification of the virus capsid subunits (72). Crossed immunoelectrophoresis (CIE) has been used successfully to detect, identify and quantitate major HAV-2 soluble antigenic components (14,15,28,55,56). The CIE pattern of HAV-2 is comprised of 5 major peaks of immunoprecipitates. From the anodic to the cathodic side of the gel, they are: the hexons, free penton base, fiber determinants of pentons, penton base determinants of pentons, and the free fibers. The fiber and penton base determinants of pentons co-migrate, thus forming "stacked" peaks on the gel (56). CIE patterns are useful for the characterization of its mutants with structural alterations, and for analyzing monospecific sera from animals immunized with agarose immunoprecipitates of subunits (16,55,56).

### Vaccines

A subunit vaccine contains only the necessary viral structural components (subunits) required for eliciting a protective immune response (74,80). The development of adenovirus subunit vaccines free of any trace of nucleic acids is very important because of the oncogenic potential of some adenoviruses (26,80).

#### HAV subunit vaccines

Experimental subunit vaccines in human trials have been reviewed by Neurath and Rubin (67). The hexon and fiber subunits have been isolated in crystalline form from purified HAV-5 (39) and have been shown to induce protective immunity in humans (24). Analysis of purified hexons

and fibers of HAV-2 and -5 has shown that only type-specific antigenic determinants are active in stimulating neutralizing antibodies (94,95). Fiber antigens of several HAV types have been shown to induce viral neutralizing (VN) antibodies in human volunteers, but conflicting results have been reported concerning the formation of VN antibodies in experimental animals immunized with fiber antigens. In addition, the antibody response experienced in the human volunteers appeared to be subgroup-specific, but a type-specific VN antibody response was found in the experimental animals. One explanation for this could be ascribed to possible previous exposure of the adult volunteers to adenoviruses, so that the fiber antigen immunization induced a secondary response (67).

#### BAV vaccines

Inactivated and modified live BAV vaccines have been used in some European countries, but they have not gained acceptance in North America (46). Experimental BAV vaccines have been summarized by Bürki (17). Inactivated BAV vaccines have been produced against individual serotypes (5,17,18), in a combination of serotypes (7,8), and in multicomponent vaccines with parainfluenza -3 (82,87), and with both parainfluenza-3 and reovirus (82). Modified live BAV vaccines have been produced with attenuated BAV-4 (5), and with a ts mutant of BAV-3, in combination with parainfluenza-3 and infectious bovine rhinotracheitis in a trivalent vaccine (98). In addition, the use of hyperimmune serum as a prophylactic measure has been investigated (38). Although serologic responses were

• noted in the vaccinated animals, the protection conferred by these vaccines upon challenge with BAVs have not been studied.

**PART I. ISOLATION AND EVALUATION OF THE IMMUNIZING SUBUNITS OF BOVINE  
ADENOVIRUS TYPES 2, 3 and 7**

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Microbiology.**

**ISOLATION AND EVALUATION OF THE IMMUNIZING SUBUNITS OF BOVINE  
ADENOVIRUS TYPES 2, 3 and 7**

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## ABSTRACT

Monospecific sera against protein subunits of bovine adenovirus (BAV) types 2, 3 and 7 were produced in rabbits, using agarose immunoprecipitates prepared by preparative immunoelectrophoresis. Various forms of solubilized viral antigens were separated in line or crossed immunoelectrophoresis using type-specific hyperimmune sera. The specificities of these sera were determined by immunoprecipitation/sodium dodecylsulfate polyacrylamide gel electrophoresis (IP/SDS PAGE), Western blot and immunodiffusion. The viral neutralization potentials of these sera were analyzed using in vitro tests in homologous and heterologous systems. Since both viral neutralizing and non-neutralizing monospecific sera to hexons were obtained, a peptide mapping system using limited proteolysis in SDS PAGE was employed to detect any differences that may be present in the hexon polypeptides precipitated in IP/SDS PAGE. In addition, BAV-3 hexon subunits, produced by deoxycholate and heat treatment followed by DEAE BioGel A chromatography, were used to produce hyperimmune sera. These anti-hexon sera seemed to be superior to the monospecific sera produced from agarose immunoprecipitates in that they possessed subgroup-reactive neutralizing activities in addition to type-reactive ones. The presence of hexon subunits capable of inducing both subgroup- and type-specific viral neutralizing antibodies makes this subunit preparation an ideal candidate for use in a multivalent subunit vaccine.

## INTRODUCTION

Adenoviruses are non-enveloped, double-stranded DNA viruses. The viral particle consists of an icosahedron, 70-90 nm in diameter, with 252 capsomers. Of these, 240 are the non-vertex capsomers, or hexons, and 12 are the vertex capsomers, or penton bases. Either 1 or 2 fibers are attached to each penton base, depending on the genus of adenoviruses (37). In humans, adenoviruses are associated with respiratory (13), ocular (14) and gastrointestinal infections (11). In addition, some human adenoviruses (HAVs) are oncogenic in non-permissive cells (31).

Bovine adenoviruses (BAVs) are associated with pneumonia, enteritis, pneumoenteritis, conjunctivitis, keratoconjunctivitis and weak calf syndrome. Only one serotype, BAV-3, has been shown to be oncogenic for newborn hamsters (9). There are at least 10 serotypes of BAVs (15,24), which are divided into two subgroups based on cultural and antigenic characteristics (2,24). BAV types 1 through 3 are in subgroup 1 while BAV types 4 through 7 are members of subgroup 2.

A serologic survey by D. E. Reed, personal communication, Molecular Genetics, Inc., Minnetonka, MN) has indicated that BAV-2, -3, -6, -7 and strain 9639, a probable new serotype, are the most common in the Mid-western United States. Other serologic surveys also have documented the prevalence of BAVs in the cattle populations in this country (3,17,20,23, 24). It is thought that some degree of cross-protection exists within each subgroup and that the immunologic recall phenomenon acts within a subgroup (2,16,28,32). If vaccines are to be constructed, they must,

therefore, include members of both subgroups. BAV types 2, 3 and 7 were chosen for this study because they are common in the United States and represent both subgroups.

Adenoviruses elaborate a complex series of antigen specificities. Hexons carry group, intersubgroup, intrasubgroup and type specificities (27). Penton bases carry group, intersubgroup and intrasubgroup specificities (35). The fibers carry intersubgroup, intrasubgroup and type specificities (26). Analysis of purified HAV hexon and fiber subunits has shown that only type-specific antigenic determinants are active in stimulating neutralizing antibodies (38,39).

## MATERIALS AND METHODS

### Cell Culture

BAV-2 and -3 were propagated on Madin Darby bovine kidney (MDBK) cells. BAV-7 was propagated on low passage bovine lung (BLG) cells; HAV-2 was grown on HeLa cells. BAV-2, -3 and -7 reference strains were obtained from the American Type Culture Collection (ATCC), Rockville, MD. HAV-2 and HeLa cells were provided by Dr. S.-L. Hu (Molecular Genetics, Inc.). Cells were grown in Eagle's minimum essential medium with Earle's salts (MEM) supplemented with 10% betapropiolactone-treated fetal calf serum (FCS), 0.16% sodium bicarbonate, 8 mM N-2-hydroethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 0.2% lactalbumin hydrolysate, 2 mM L-glutamine and 62.7 ug per ml of ampicillin (sodium salt). Cell cultures were grown and maintained at 37°C in a humidified air atmosphere containing 2% carbon dioxide (CO<sub>2</sub>).

### Virus Stocks

All virus strains were passaged three times, as described below, prior to usage for antigen production. Cell cultures were infected at a multiplicity of infection (MOI) of 0.1 median tissue culture infective doses (TCID<sub>50</sub>) per cell. Infected cultures were maintained as described for all cultures except the MEM was supplemented with 5% FCS. When maximum cytopathic effect (CPE) was evident, infected cultures were harvested, frozen and thawed 3 times, and centrifuged at 600 X g for 10 minutes. The supernate was extracted with one tenth volume of chloroform, followed by low speed

centrifugation (600 X g, 10 minutes). The virus-containing aqueous phase was stored in aliquots at  $-70^{\circ}\text{C}$ .

Virus stocks were titrated in microtitration plates, using tenfold virus dilutions. Endpoints were determined on the basis of CPE development in unstained cultures (BAV-2, -3, HAV-2), or upon nuclear inclusion body formation in Giemsa-stained cultures (BAV-7). Titers were calculated by the Reed and Muensch method (30) and expressed as  $\text{TCID}_{50}$  per ml.

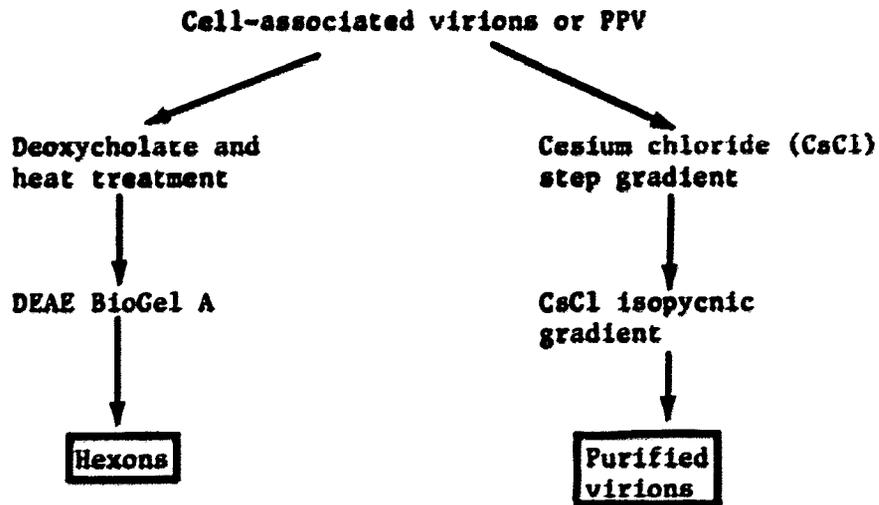
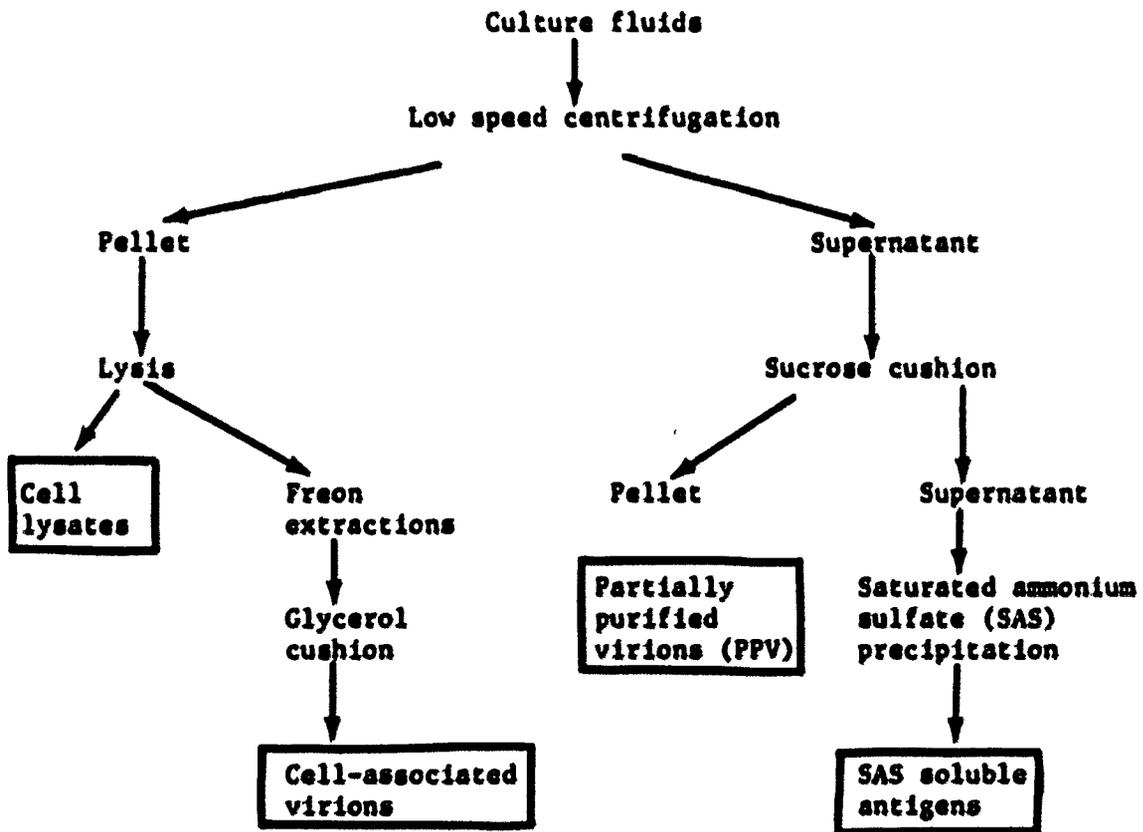
#### Antigen Preparation

Monolayers of cells were infected at a MOI of 3 to 5  $\text{TCID}_{50}$  per cell. Infected cultures were maintained in MEM supplemented with 5% FCS, 82 ug per ml of gentamycin sulfate, 62.7 ug per ml of ampicillin, 10 ug per ml of amphotericin B, and incubated as described for cell cultures. At maximum CPE, cultures were harvested and clarified by low speed centrifugation. The pellets were used for preparing cell lysates and cell-associated virions; the supernatant fluids were used for partially purified virions and soluble antigens. Both cell-associated virions and partially purified virions were used for preparing purified virions and hexons. The various antigen preparations used in this study are shown in Figure 1 and described below.

#### Cell lysates

Infected cell pellets were resuspended in lysis buffer (10mM Tris-HCl, pH 7.4, 1mM EDTA, 0.1 M NaCl, 0.5%, w/v, sodium deoxycholate, 1%, v/v, Nonidet P-40), placed on ice for 1 hour, and centrifuged at 13,000 X g for 15 minutes. The supernates were designated as "cell lysates".

**Figure 1. The various antigen preparations used in this study.  
Reagents and experimental conditions are described  
in the text**



### Cell-associated virions

Infected cell pellets were resuspended in a buffer containing 30 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% (v/v) Nonidet P-40 (NP-40) and 6 mM 2-mercaptoethanol, extracted with Freon at one-tenth the sample volume, and centrifuged at 350 X g for 5 minutes. The aqueous phase was centrifuged through a step gradient consisted of equal volumes of 5 and 45% glycerol (v/v in 30 mM Tris-HCl, pH 7.5) at 100,000 X g for 1 hour at 4°C. The pellets were resuspended and dialyzed in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and designated as "cell-associated virions".

### Partially purified virions (PPV)

PPV antigens were prepared by centrifuging culture supernatant fluids through 40% sucrose (w/v in 0.2 M phosphate buffer, pH 7.4) at 100,000 X g for 1 hour at 4°C. The pellets were resuspended in 10 mM Tris-HCl, pH 7.4, and designated as "PPV" antigens.

### Saturated ammonium sulfate precipitation of soluble antigens

Soluble antigens were purified by a method adapted from Boulanger and Puvion (5). After the culture supernates were centrifuged through 40% sucrose, the MEM and sucrose fractions were collected separately and dialyzed against TE buffer. The samples were adjusted to a final concentration of 55% with saturated ammonium sulfate (SAS), pH 6.8. The precipitates were stirred for 18 hours at 4°C and collected by centrifuging at 2,000 X g for 30 minutes. The pellets were resuspended and dialyzed in 0.025 M

Tris-Tricine buffer (4.3 g/l Tris-HCl, pH 8.6, 9.8 g/l Tricine, 0.11 g/l calcium lactate and 0.2 g/l sodium azide) containing 0.15 M NaCl (TTN buffer). These were designated as SAS/MEM and SAS/sucrose soluble antigens, respectively.

#### Purified virions

Virions were purified further from cell-associated virions or PPV preparations using two cycles of cesium chloride (CsCl) gradients. Samples were layered onto a preformed step gradient ( $\rho=1.2$  and  $1.4$  in 10 mM Tris-HCl, pH 7.4), and centrifuged at 100,000 X g for 1 hour at 4°C. The virus bands were collected and subjected to isopycnic gradient centrifugation ( $\rho=1.3$ ) at 100,000 Xg for 18-20 hours at 4°C. Virus bands were collected and dialyzed against TE buffer, pH 8.0.

#### Hexons

Hexons were purified from cell-associated virions or PPV antigens using a method adapted from Pereira and Wrigley (29). The antigens were dialyzed against 5 mM Tris-HCl, pH 8.6, incubated at 56°C for 3 minutes in the presence of 0.5% (w/v) sodium deoxycholate (DOC), and purified by DEAE Bio Gel A chromatography (Bio Rad Lab., Richmond, CA). The column was equilibrated against 5 mM Tris-HCl, pH 8.6, and eluted using buffers of increasing ionic strength (0.05 M, 0.1 M, 0.2 M, 0.3 M and 2 M NaCl in 5 mM Tris-HCl, pH 8.6). Fractions eluted were monitored by conductivity and by absorbance at 280 nm. Protein-containing fractions were pooled, concentrated using YM 10 membranes (Amicon Corp., Danvers, MA), and

analyzed on SDS PAGE (see IP/SDS PAGE below). Protein concentrations were determined as described by Bradford (6).

### Hyperimmune Type-Specific Antisera

#### Calf reference antisera

Reference antiserum for BAV-2 (C87) was prepared in a colostrum-deprived calf. One ml of chloroform-treated stock ( $10^4$  TCID<sub>50</sub>) was emulsified in an equal volume of Freund's incomplete adjuvant (FIA), and injected intramuscularly (IM) on days 1, 21 and 36. Blood was collected 10 days after the last injection.

Reference antisera for BAV-7 (C72) and strain 9639 (GC10) were prepared in a colostrum-deprived calf and a gnotobiotic calf, respectively, by J. G. Wheeler (Iowa State University, Ames, IA). For C72, ATCC BAV-7 stock was injected intravenously (IV) on day 1, followed by IM injections of a FIA-emulsified BAV-7 field isolate on days 14 and 17. For GC10, chloroform-treated strain 9639 stock was injected IM on day 1, followed by an IM injection of a FIA emulsified stock on day 21.

#### Rabbit reference antisera

Reference antiserum for BAV-3 (RJ4) was produced in a rabbit using hexon subunits. Approximately 20 ug of BAV-3 hexons were emulsified in 1 ml of FIA and injected IM on days 1, 21 and 28. The rabbit was exsanguinated for serum one week later.

In addition, CsCl-purified virions were used to produce type-specific sera. The top (incomplete virions) and bottom (complete virions) bands of a BAV-3 CsCl isopycnic gradient were used to inoculate rabbits RJ39 and RJ40, respectively. Similarly, top and bottom bands of a BAV-7 CsCl isopycnic gradient were used for rabbits RJ44 and RJ45, respectively. All four rabbits were immunized using the same methods and schedule employed for RJ4.

Reference sera for HAV-2 (R106 and R107) were provided by Dr. S.-L. Hu (Molecular Genetics, Inc.). Briefly, HAV-2 stocks and Freund's complete adjuvant (FCA) were used for IM inoculations of both rabbits.

#### Globulin Precipitation

The globulins from antisera were prepared by saturated ammonium sulfate (SAS) precipitation according to Garvey et al. (12). Globulins were precipitated at 50% SAS (pH 7.8), stirred overnight at 4°C, and centrifuged at 2,000 Xg for 30 minutes. The pellets were resuspended in TTN and subjected to 2 more cycles of SAS precipitations. The final pellets were resuspended at one half the original sample volume, dialyzed extensively against TTN, and designated as "antibodies".

#### Monospecific Antisera Production

Monospecific antisera to BAV subunits were produced in rabbits using agarose immunoprecipitates prepared by preparative immunoelectrophoresis. All immunoprecipitates were prepared by line immunoelectrophoresis (LIE) except for the ones used in rabbits RJ 1-3, 11, 12, 14-16, which were

prepared from crossed immunoelectrophoresis (CIE) gels. The various antigens used in preparing the immunoprecipitates are shown in Table 1. In some instances, intermediate gels were used in LIE to facilitate the separation of antigen subunits. Inocula for rabbits RJ 37, 38, 42, 43, 46-48, 54-60 were prepared from immunoprecipitates using intermediate gels.

The rabbits were purchased from 2 local commercial sources. Rabbits RJ 1-3, 9-13 were obtained from one source while rabbits RJ 4-8, 14-60 were obtained from the other source. Each immunoprecipitate from an unstained gel was divided into 3 equal portions and stored in 0.5 ml of TT buffer. In the case of CIE gels, arcs from duplicate plates were used. The inoculum was emulsified in an equal volume of FIA and was injected IM on days 1, 21 and 28. The rabbit was exsanguinated for blood a week later. Antibodies were prepared from the serum using the globulin precipitation method described above.

The progress of the immunization was monitored by rocket immunoelectrophoresis. The final antibodies were characterized in CIE using the monospecific antisera as intermediate gels (1). In addition, viral neutralization, immunodiffusion, IP/SDS PAGE and Western blot techniques were used to characterize each antisera.

#### Crossed Immunoelectrophoresis (CIE)

Crossed immunoelectrophoresis was performed as described by Vestergaard (33) and modified by Lum and Reed (21). The agarose gels consisted of 1% agarose (Standard Low  $M_r$ , Bio Rad Lab.) in TT buffer containing 1%

**Table 1. The antigens used in preparing immunoprecipitates for monospecific sera production**

<b>Antigen preparations</b>	<b>Rabbit numbers (RJ)</b>
Sucrose pelleted culture supernatant (PPV) in 5% NP-40	1, 2, 3, 9, 10, 26, 27
Sucrose pelleted culture supernatant (PPV) in 0.5% DOC <sup>a</sup>	7, 8, 11, 12, 14, 15, 16
DEAE ion-exchange column purified hexons in 5% NP-40	5, 6, 22, 23
Saturated ammonium sulfate precipitated soluble antigens prepared from MEM fractions after pelleting through sucrose (SAS/MEM in 5% NP-40)	13, 17, 18, 19 20, 21, 24, 28, 33, 34, 35, 36, 37, 38, 42, 43, 46, 47, 48
Saturated ammonium sulfate precipitated soluble antigens prepared from sucrose fractions after pelleting through sucrose (SAS/sucrose in 5% NP-40)	25, 29, 30, 31, 32
Cell lysates in 5% NP-40	49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60

<sup>a</sup>PPV antigens were solubilized by heating at 56°C for 3 minutes in the presence of 0.5% deoxycholate.

each of Triton X-100 and PEG 6000 (TTT buffer). First dimension electrophoresis was performed on solubilized antigens at 10 V/cm for 90 minutes at 10°C. In the second dimension, the separated antigens were electrophoresed into agarose containing 5-20% antibodies, using 1.5 V/cm for 18 hours at 10°C. Gels were washed extensively in 0.1 M NaCl, pressed dried, and stained with Coomassie Blue R-250.

#### Line Immuno-electrophoresis (LIE)

Line immuno-electrophoresis was performed according to Kröll (18). The solubilized antigens were mixed with Sea Plaque agarose (1% final concentration, FMC Corp., Marine Colloids Div., Rockland, ME), placed into a trough, and electrophoresed into agarose (same as CIE agarose) containing 10-20% antibodies. Electrophoresis was carried out at 1-5 V/cm for 18 hours at 10°C.

#### Rocket Immuno-electrophoresis

Rocket immuno-electrophoresis was performed according to Weeke (36), using the same agarose and conditions as described for LIE. The gels were processed for staining as described for CIE.

#### Immunodiffusion (ID)

Double diffusion was performed using the same agarose as for CIE. Cell lysates were solubilized in 5% NP-40 and placed in the center well; antibodies were placed in the peripheral wells. Precipitin bands were allowed to develop for 48 hours at 25°C inside a humidity chamber.

The gels were processed for staining as described for CIE.

#### Viral Neutralization (VN)

The ability of each antiserum to neutralize homologous and heterologous serotypes was assayed by a constant virus-varying antibody test. VN tests were performed in microtitration plates, using 100 TCID<sub>50</sub> of virus and 0.1 ml of twofold dilutions of heat-inactivated (56°C, 30 minutes) serum per well. The tests were carried out to the 1:65,536 dilution for the homologous virus, and to the 1:256 dilution for heterologous viruses. VN titers were expressed as the reciprocal of the last dilution of antiserum showing CPE inhibition.

#### Immunoprecipitation/SDS Polyacrylamide Gel Electrophoresis

##### Radiolabeling of virus

Infected and mock-infected cultures in 75 cm<sup>2</sup> T-flasks were labeled with L-[<sup>35</sup>S]-methionine at 32 hour postinfection. The culture fluids were replaced with 100 uCi of L-[<sup>35</sup>S]-methionine (1200 Ci/mmol, Amersham Corp., Arlington Heights, IL) in 5 ml of methionine-deficient MEM and incubated for 2 hours at 37°C. The cultures were fed with 10 ml of methionine-deficient MEM and incubated until 72-96 hours postinfection. The cultures were harvested and processed for cell lysates and PPV antigens.

### Immunoprecipitation (IP)

<sup>35</sup>S-methionine labeled antigens were solubilized in 5% NP-40 and incubated with 10-25 ul of antibodies overnight at 4°C. The immune complexes formed were adsorbed onto 0.01 mg of Protein A Sepharose CL-4B beads (Sigma Chem. Co., St. Louis, MO) by incubating for 1 hour at 25°C. Sepharose beads were pelleted by centrifuging for 5 minutes at 13,000 X g and washed 3 times with TT buffer. The final pellet was dissolved in 50 ul of SDS PAGE treatment buffer (0.125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heated for 2 minutes at 100°C. 0.05% bromophenol blue was added as tracking dye.

### SDS PAGE

SDS PAGE was performed according to the discontinuous buffer system described by Laemmli (19). Samples were electrophoresed on 1.5 mm thick vertical slab gels (10% acrylamide, 2.7% N-N'-methylene-bis-acrylamide cross-linker) for 3 hours at 35 mA per gel. Molecular weight standards (Pharmacia Fine Chem., Piscataway, NJ) were co-electrophoresed on each gel. Gels were stained with Coomassie Blue R-250, destained, and dried. For <sup>35</sup>S-methionine labeled proteins, gels were subjected to fluorography (7) before drying. Autoradiography was performed at -70°C using Kodak XAR-5 film.

### Peptide Analysis

Peptide analysis by limited proteolysis in SDS PAGE was performed according to Cleveland et al. (8). Bands of interest were cut out from

a 10% SDS PAGE gel and placed into the wells of another SDS PAGE (15%) gel, along with 50 ug of S. aureus V8 protease (Miles Scientific, Naperville, IL). Electrophoresis was carried out as described for SDS PAGE except the current was turned off for 30 minutes, when the tracking dye was near the bottom of the stacking gel, to allow for proteolysis. Gels were processed for autoradiography as described for IP/SDS PAGE.

#### Western Blot Transfer

After SDS PAGE, gels were equilibrated in transfer buffer (0.025 M Sodium phosphate buffer, pH 6.8). Electrophoretic blot transfer was performed as described by Lum and Reed (21). After transfer, the nitrocellulose filters were cut into 1 cm strips and processed by 1 of the following methods.

##### Method 1

The nitrocellulose filters were processed and incubated with test antibodies as described by Lum and Reed (21), using the Tris-saline/BSA method. The bound antibodies were detected by the addition of 10 uCi of [<sup>125</sup>I]-Protein A (10 uCi/ug, New England Nuclear, Boston, MA).

##### Method 2

The nitrocellulose filters were processed as in Method 1 except 0.05% Tween 20 was used instead of BSA as a blocking agent. The bound antibodies were detected by adding 50 uCi of [<sup>125</sup>I]-goat F(ab')<sub>2</sub> anti-rabbit IgG (10 uCi/ug, New England Nuclear). When calf antibodies were

used, the strips were incubated first with rabbit anti-bovine IgG (Cappel Lab., West Chester, PA), followed by the [ $^{125}$ I]-goat anti-rabbit IgG incubation.

#### Autoradiography

Data were analyzed by direct autoradiography at  $-70^{\circ}\text{C}$ , using Kodak XAR-5 film and an intensifying screen.

## RESULTS

### Type-Specific Reference Sera

Using viral neutralization (VN) tests, none of the anti-BAV sera reacted to HAV-2 (Table 2). However, the HAV-2 antiserum, reacted to BAV-3. There were low level cross-reactions between the anti-BAV subgroup 1 sera and heterologous BAVs. The BAV-7 antiserum did not react with any of the subgroup 1 BAVs.

### Hexons

Figure 2a shows the DEAE BioGel A column profile of BAV-3 hexons. Fractions under the two peaks labeled "hexons", as determined by SDS PAGE (data not shown), were used to immunize rabbit RJ4. Figure 2b shows the crossed immunoelectrophoresis (CIE) patterns of BAV-7 PPV antigens before and after deoxycholate and heat treatment, and after DEAE ion-exchange chromatography.

### Monospecific Sera Against BAV-2 Subunits

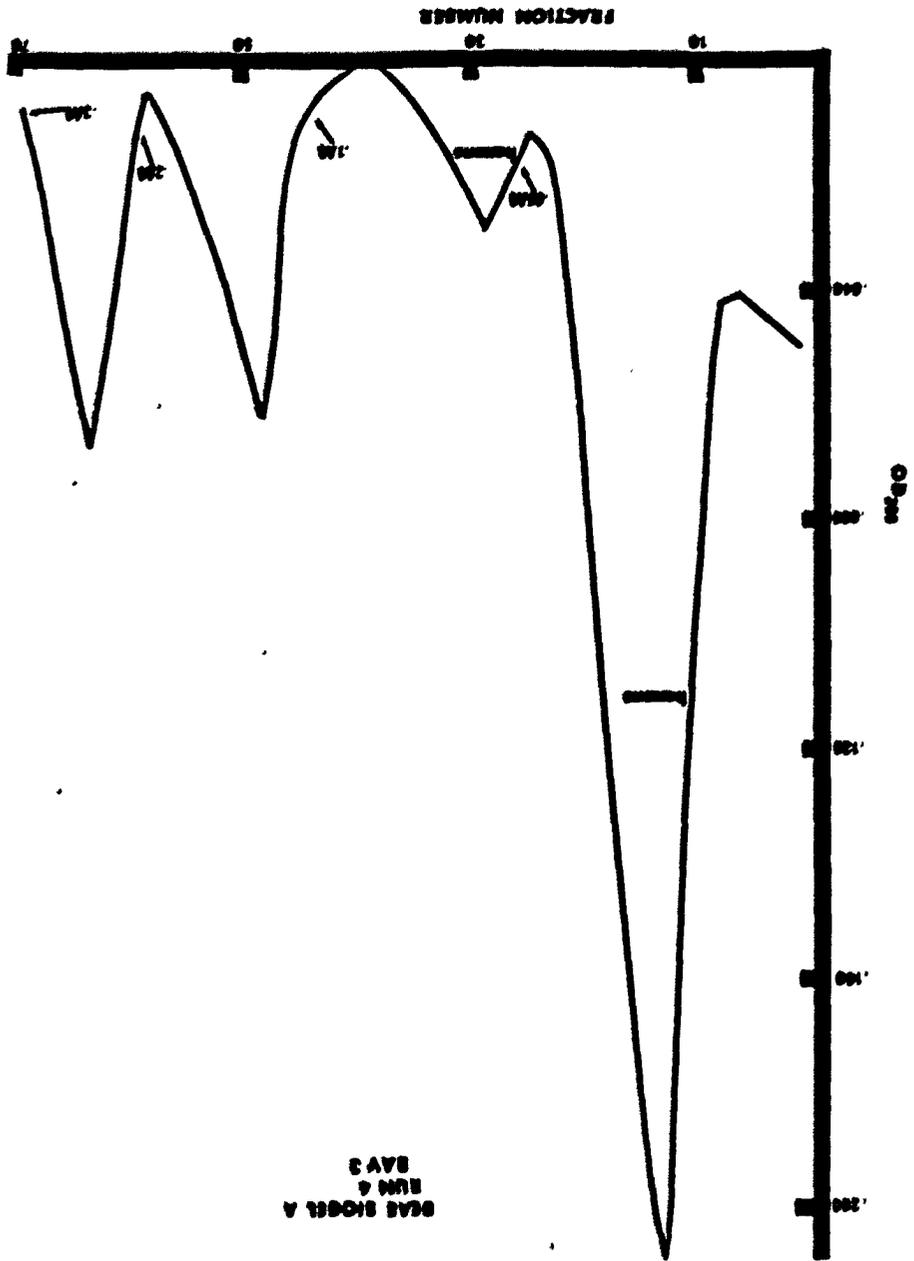
The various sera produced from immunizing rabbits using agarose immunoprecipitates of BAV-2 subunits are shown in Table 3. Both VN positive and negative antisera were obtained. Eight antisera were monospecific to hexons, with five showing VN titers of  $\geq 64$ . RJ19 and RJ48 were essentially monospecific with additional weak reactions to the "300K" polypeptides,

**Table 2. The cross-neutralization titers of type-specific reference sera**

Antiserum (Specificity)	Virus			
	BAV-2	BAV-3	BAV-7	HAV-2
C87 (BAV-2)	8,192	16	8	<2
RJ4 (BAV-3)	8	32,768	256	<2
C72 (BAV-7)	<2	<2	32,768	<2
R106 <sup>a</sup> (HAV-2)	2	256 <sup>a</sup>	<2	256 <sup>a</sup>

<sup>a</sup>Both homologous and heterologous VN assays for R106 were carried out only to 1:256. Data from plaque-reduction assays (S.-L. Hu, Molecular Genetics, Inc.) showed titers of >5,000 and >3,000 against HAV-2 and BAV-3, respectively. Preinoculation sera were negative. IP/SDS PAGE showed that R106 is type-specific to HAV-2 because it did not react with BAV-3 fiber. It is not known why R106 possesses VN or plaque-reduction titers to BAV-3.

**Figure 2a. DEAE BioGel A column chromatography after deoxycholate and heat treatment of BAV-3 cell-associated virions. Column elution was monitored by conductivity (as indicated by the molarities of NaCl), and by the presence of proteins (as indicated by absorbance at 280 nm)**



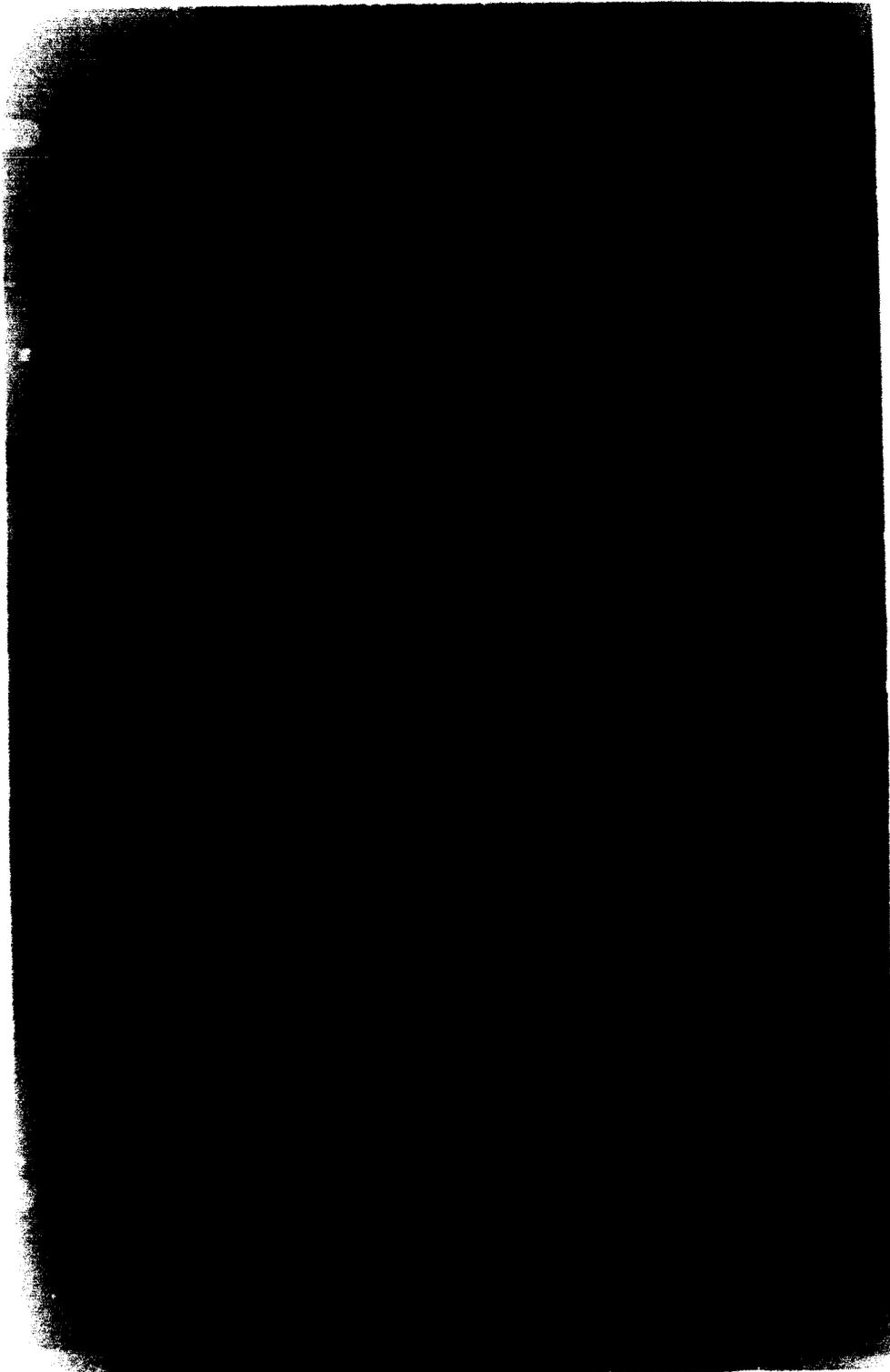
**Figure 2b. CIE patterns of BAV-7 PPV antigens used for isolation of hexon subunits**

**Antibodies: C72, reference antibody**

**Antigens: (A) PPV antigens before treatment**

**(B) PPV antigens after treatment**

**(C) Hexons fractions after DEAE BioGel A column purification**



**Table 3. VN titers and probable specificities of rabbit antisera produced from immunoprecipitates of BAV-2 subunits**

<b>Gel No.</b>	<b>Antigen preparation</b>	<b>Antibody</b>	<b>Intermediate gel antibody</b>	<b>Method of separation</b>
1	PPV	C87	-	LIE
2	SAS/MEM	C87	-	LIE
3	SAS/MEM	C87	-	LIE
4	SAS/Sucrose	RJ9	-	LIE
5	SAS/MEM	RJ10	-	LIE
6	SAS/MEM	RJ10	-	LIE
7	SAS/MEM	RJ9		LIE
8	SAS/MEM	C87	RJ31	LIE
9	Cell lysates	C87	RJ10	LIE
10	Cell lysates	C87	RJ31	LIE

<sup>a</sup>Precipitin lines in LIE were lettered from the anodic side of gel.

<sup>b</sup>Standard adenovirus SDS PAGE nomenclature was used: (II) Hexon; (III) Penton base; (IIIa) Peripentonal area; (IV) Fiber. "300K" refers to a high MW band seen when PPV antigens were used for IP/SDS PAGE. PPV antigens were used for sera showing VN titers of  $\leq 16$  and cell lysates were used for sera showing SN titers of  $\geq 32$ .

<sup>c</sup>Results of IP/SDS PAGE between cell lysates and RJ54-RJ59 (data not shown), from gels different than ones shown in Figures 3 and 4, were used in these designations.

Precipitin line <sup>a</sup>	Rabbit No.	Homologous VN titer	Polypeptides bound in IP/SDS PAGE <sup>b</sup>
A	RJ9	16	(300K), II, IIIa, IV
B	RJ10	256	II, III, IIIa, IV
A	RJ17	<2	II
B	RJ13	<2	(300K), II
A	RJ18	<2	(300K), II, IV (weak)
B	RJ19	<2	(300K), II
A	RJ29	128	II
B	RJ30	8	II
A	RJ31	4,096	II
B	RJ32	32	II, IV (weak)
A	RJ33	256	II
B	RJ34	4	(300K), II, IV
C	RJ35	16	II
Single	RJ36	4	(300K), II, III, IIIa, IV
A	RJ48	8	(300K), II
B	RJ43	256	II, III, IIIa, IV
C	RJ42	1,024	II
A	RJ54	<2	II, IV <sup>c</sup>
B	RJ55	16	II, IV <sup>c</sup>
C	RJ56	32	II, IV <sup>c</sup>
D	RJ57	64	II <sup>c</sup>
E	RJ58	2,048	II, III, IIIa, IV <sup>c</sup>
F	RJ59	64	II, III, IIIa, IV <sup>c</sup>
A	RJ60	2	(300K), II, IV (weak)

which are probably aggregates of hexons. All antisera were VN negative to heterologous viruses, except for RJ13 and RJ19. RJ13 and RJ19 showed VN titers of 64 and 16, respectively, against BAV-3. All pre-inoculation sera were negative ( $\leq 2$ ) to homologous and heterologous viruses. Monospecificity was determined by IP/SDS PAGE (Figures 3 and 4).

#### Monospecific Sera Against BAV-3 Subunits

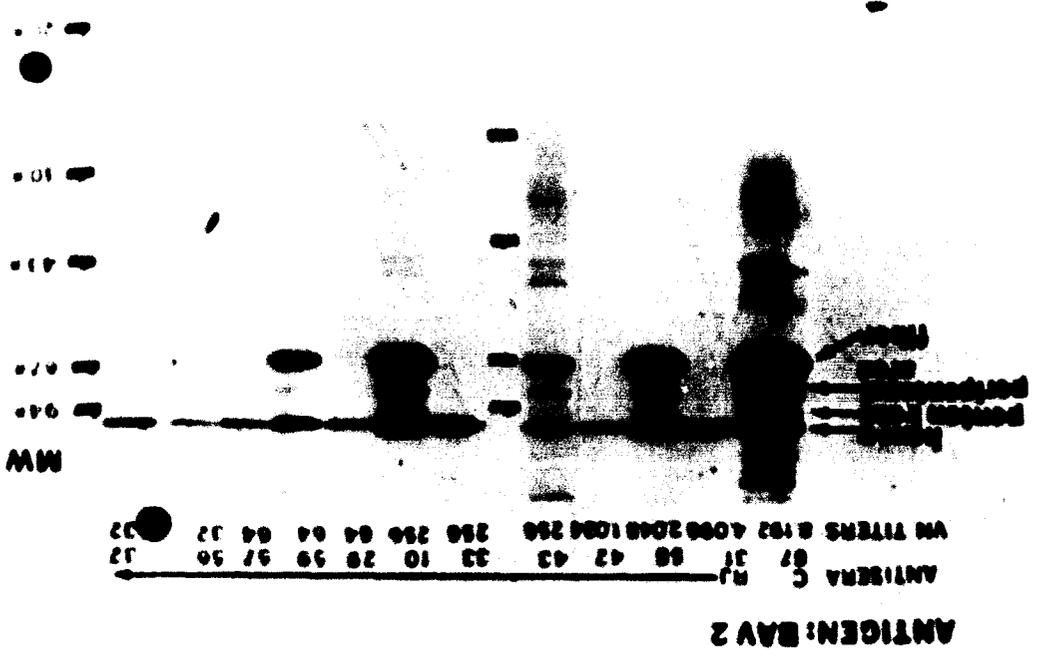
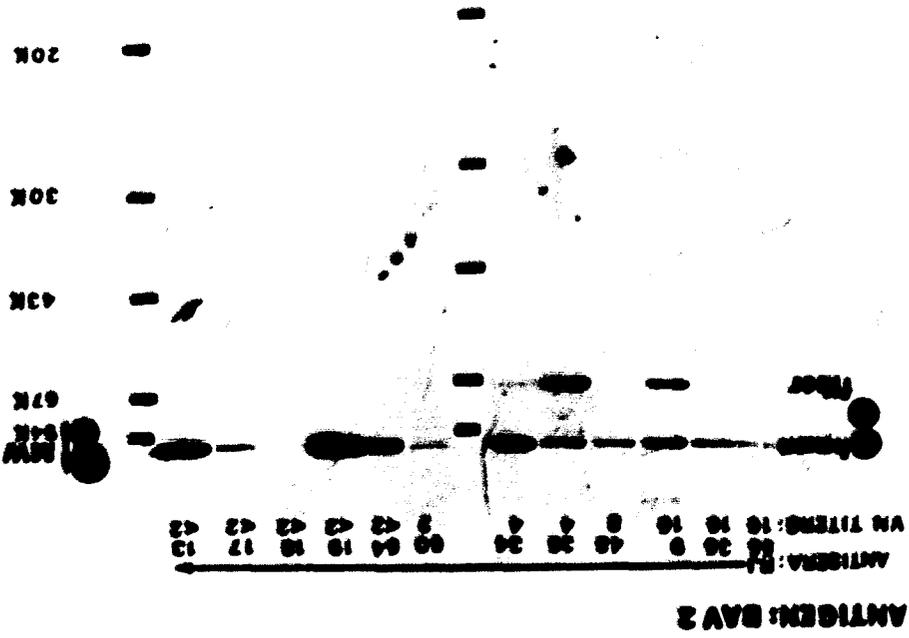
The various sera produced from immunizing rabbits with agarose immunoprecipitates of BAV-3 subunits are shown in Table 4. Eight antisera were monospecific to hexons, with 4 of these having VN titers  $\geq 256$ . CIE and ID results, in addition to IP/SDS PAGE results, were used to determine monospecificity because non-specific IP reactions were evident (data not shown). All of the antisera were negative in heterologous VN reactions and none of the preinoculation sera showed homologous VN activity.

#### Monospecific Sera Against BAV-7 Subunits

The various sera produced from immunizing rabbits using immunoprecipitates of BAV-7 subunits are shown in Table 5. Four monospecific sera were obtained. While all four showed VN titers of  $\geq 64$ , 2 were precipitating and 2 were non-precipitating in CIE and ID reactions. Monospecificities of RJ26 and RJ27 were determined by IP/SDS PAGE (data not shown). An antiserum having no anti-hexon specificity was obtained; similar sera were not obtained from BAV-2 and -3. This serum, RJ14, was reactive to the pentonal polypeptides such as penton base, peripentonal area and fiber. All antisera were negative in heterologous VN tests and

**Figure 3. Homologous IP/SDS PAGE results of reactions between VN positive (titer  $\geq 32$ ) antisera and [ $^{35}$ S]-methionine labeled BAV-2 cell lysates. Hexon, penton base, peripentonal area and fiber correspond to polypeptides II, III, IIIa, and IV, respectively, according to standard adenovirus SDS PAGE nomenclature**

**Figure 4. Homologous IP/SDS PAGE results of reactions between VN negative (titers  $\leq 16$ ) antisera and [ $^{35}$ S]-methionine labeled BAV-2 PPV antigens. Hexon and fiber correspond to polypeptides II and IV, respectively, according to standard adenovirus SDS PAGE nomenclature**



**Table 4. VN titers and probable specificities of rabbit antisera produced from immunoprecipitates of BAV-3 subunits**

<b>Gel no.</b>	<b>Antigen preparation</b>	<b>Antibody</b>	<b>Intermediate gel antibody</b>	<b>Method of separation</b>
1	Hexons	RJ4	-	LIE
2	SAS/MEM	RJ5	-	LIE
3	Hexons	RJ5	-	LIE
4	SAS/MEM	RJ6	-	LIE
5	SAS/Sucrose	RJ6	-	LIE
6	SAS/MEM	RJ5	RJ24	LIE
7	Cell lysates	RJ39	-	LIE
8	Cell lysates	RJ39	-	LIE

<sup>a</sup>Precipitin lines in LIE were lettered from the anodic side of gel.

<sup>b</sup>Only reactions to hexons and fibers were determined from IP/SDS PAGE due to the presence of non-specific reactions. Monospecificity was determined with the aid of CIE and ID patterns (data not shown). Specificities to penton base and peripentonal polypeptides may be present in any of the sera. In addition, more than one electrophoretic species may be present in CIE.

Precipitin line <sup>a</sup>	Rabbit no.	Homologous VN titer	Probable specificities <sup>b</sup>
A	RJ5	32	hexons
B	RJ6	256	hexons, fibers
A	RJ20	8	hexons
B	RJ21	<2	hexons
A	RJ22	256	hexons
B	RJ23	96	hexons, fibers
A	RJ24	256	hexons
B	RJ28	<2	hexons
Single	RJ25	32	hexons, fibers
A	RJ37	1,024	hexons
B	RJ38	1,024	hexons
A	RJ49	1,024	hexons, fibers
B	RJ50	1,024	hexons, fibers
C	RJ51	1,024	hexons, fibers
A	RJ52	1,024	hexons, fibers
B	RJ53	1,024	hexons, fibers

**Table 5. VN titers and probable specificities of rabbit antisera produced from immunoprecipitates of BAV-7 subunits**

<b>Gel no.</b>	<b>Antigen preparation</b>	<b>Antibody</b>	<b>Intermediate gel antibody</b>	<b>Methods of separation</b>
1	PPV	C72	-	CIE
2	PPV <sup>d</sup>	RJ1	-	LIE
3	PPV <sup>d</sup>	RJ1	-	CIE
4	PPV <sup>d</sup>	RJ1	-	CIE
5	PPV	RJ8	-	LIE
6	SAS/MEM	C72	RJ11	LIE

<sup>a</sup>Precipitin arcs in CIE were numbered from the cathodic side of the gel while precipitin lines in LIE were lettered from the anodic side.

<sup>b</sup>Due to difficulties in obtaining sufficient quantities of labeled or non-labeled BAV-7 antigens, a combination of methods were used, in addition to IP/SDS PAGE, to determine the specificities of the sera. Standard adenovirus SDS PAGE nomenclature was used: (II) Hexon; (III) Penton base; (IIIa) Peripentonal area; (IV) Fiber.

<sup>c</sup>These specificities were determined by Western blot analysis.

<sup>d</sup>PPV antigens were treated with 0.5% deoxycholate and heat (56°C, 3 minutes).

<sup>e</sup>Not determined.

<sup>f</sup>RJ14 did not react to hexons in IP/SDS PAGE, but was reactive to the other major polypeptides.

<sup>g</sup>RJ11 and RJ12 were obtained from a different commercial source from RJ15 and RJ16. Identical immunoprecipitates were used in the corresponding rabbits.

<sup>h</sup>RJ11 and RJ12 were monospecific, as determined by CIE and ID (data not shown). RJ26 and RJ27 reacted only to the hexon band in IP/SDS PAGE but they were nonprecipitating in CIE and ID.

Precipitin line or arc <sup>a</sup>	Rabbit no.	Homologous VN titer	Probable specificities <sup>b</sup>
1	RJ1	256	II, III <sup>c</sup>
2	RJ2	512	II and others
3	RJ3	1,024	II, III, IIIa, IV <sup>c</sup>
A	RJ7	32	N.D. <sup>e</sup>
B	RJ8	4,096	II and others
1	RJ14	<2	III, IIIa, IV <sup>f</sup>
2	RJ11	2,048 <sup>g</sup>	II <sup>h</sup>
3	RJ12	64 <sup>g</sup>	II <sup>h</sup>
1	RJ14	<2	III, IIIa, IV <sup>f</sup>
2	RJ15	16 <sup>g</sup>	N.D. <sup>e</sup>
3	RJ16	<2 <sup>g</sup>	N.D. <sup>e</sup>
A	RJ26	512	II <sup>h</sup>
B	RJ27	64	II <sup>h</sup>
A	RJ46	128	II and others
B	RJ47	384	II and others

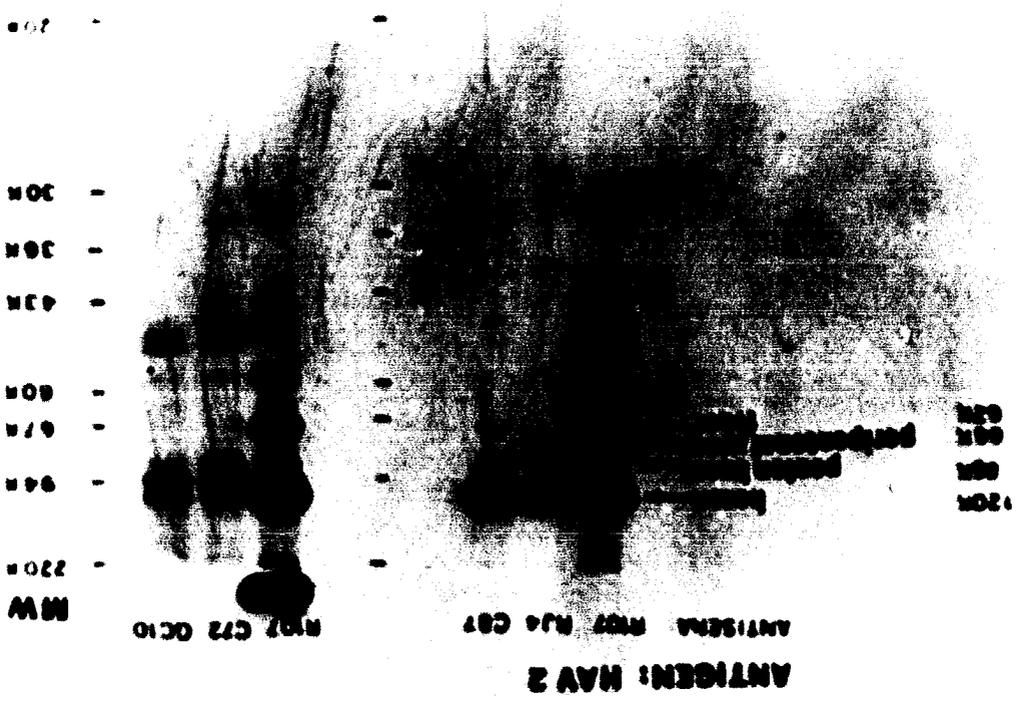
none of the preinoculation sera showed homologous VN activity.

#### IP/SDS PAGE Cross-reactions

The results of IP/SDS PAGE cross-reactions between the type-specific reference sera and [<sup>35</sup>S]-methionine labeled HAV-2, BAV-3 and BAV-2 cell lysates are shown in Figures 5, 6 and 7, respectively. GC10, an antiserum to strain 9639 (a probable subgroup 2 BAV type), was included for comparisons with anti-BAV-7 serum. [<sup>35</sup>S]-methionine labeled BAV-7 antigens were not available for this experiment. Mock-infected cell controls also were tested to insure the validity of these reactions. The controls for BAV-2 and BAV-3 IP/SDS PAGE are shown in Figure 8. All other controls (data not shown) also were negative. Varying degrees of inter-subgroups and intrasubgroup cross-reactions were evident for hexon, penton base, peripentonal area and fiber polypeptides. However, the homologous reactions of the fiber polypeptide remained the strongest, emphasizing the dominance of its type-specific determinants. No intersubgroup cross-reaction was obtained between BAV subgroup 2 antisera (C72 and GC10) and HAV-2 cell lysates for peripentonal area and fiber polypeptides. In fact, these two antisera showed a similar pattern (see Figure 5) that was distinct from BAV subgroup 1 antisera. The nonreactivities of RJ4 and C72 to BAV-2 lysates (Figure 8) may have been caused by errors in measuring test reagents and should not be used as conclusive results. There were cross-reactions for peripentonal polypeptides between BAV subgroup 2 antisera and BAV subgroup 1 viruses (BAV-2 and -3), as shown in Figures 6 and 7.

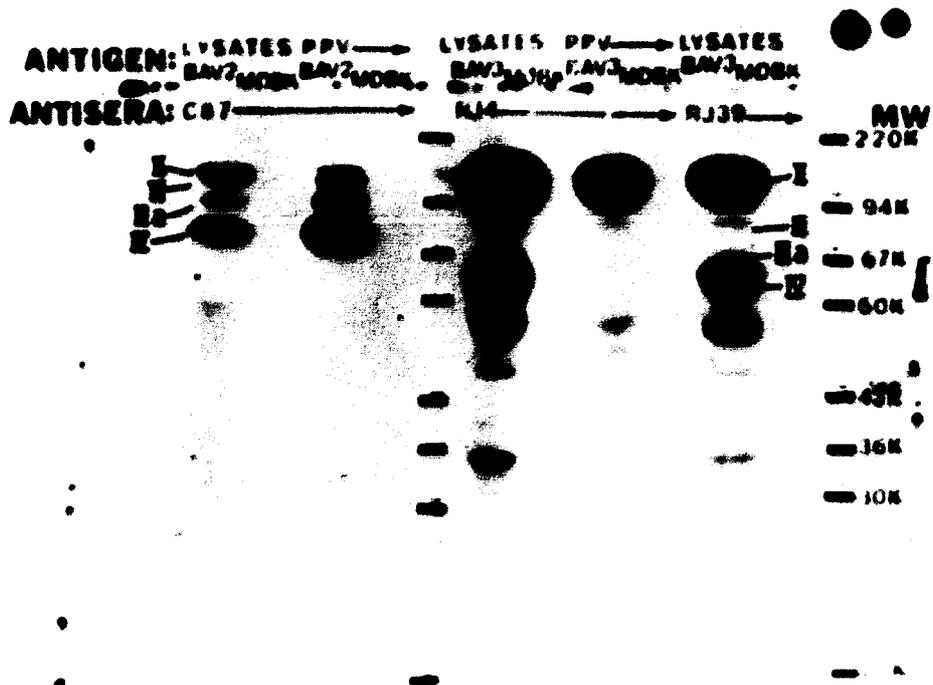
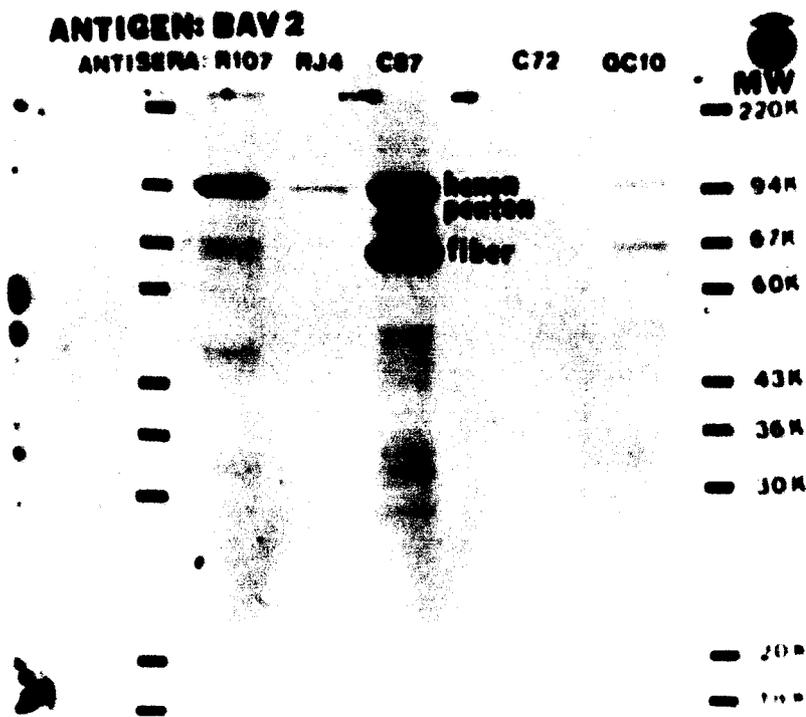
**Figure 5. IP/SDS PAGE of [<sup>35</sup>S]-methionine labeled HAV-2 cell lysates reacted to type-specific reference sera. The homologous serum is K107. Autoradiography was performed for 16 hours at -70°C. Molecular weights for HAV-2 capsid polypeptides were summarized in Tooze (31)**

**Figure 6. IP/SDS PAGE of [<sup>35</sup>S]-methionine labeled BAV-3 cell lysates reacted to type-specific reference sera. The homologous serum is RJ4. Autoradiography was performed for 16 hours at -70°C**



**Figure 7. IP/SDS PAGE of [<sup>35</sup>S]-methionine labeled BAV-2 cell lysates reacted to type-specific reference sera. The homologous serum was C87. Autoradiography was performed for 24 hours at -70°C**

**Figure 8. IP/SDS PAGE of [<sup>35</sup>S]-methionine labeled BAV- and mock-infected cell lysates and PFV. C87 is the reference serum for BAV-2. RJ4 and RJ39 are reference sera for BAV-3. Autoradiography was performed for 24 hours at -70°C. II: Hexon; III: Penton base; IIIa: Peripentonal area; IV: Fiber**



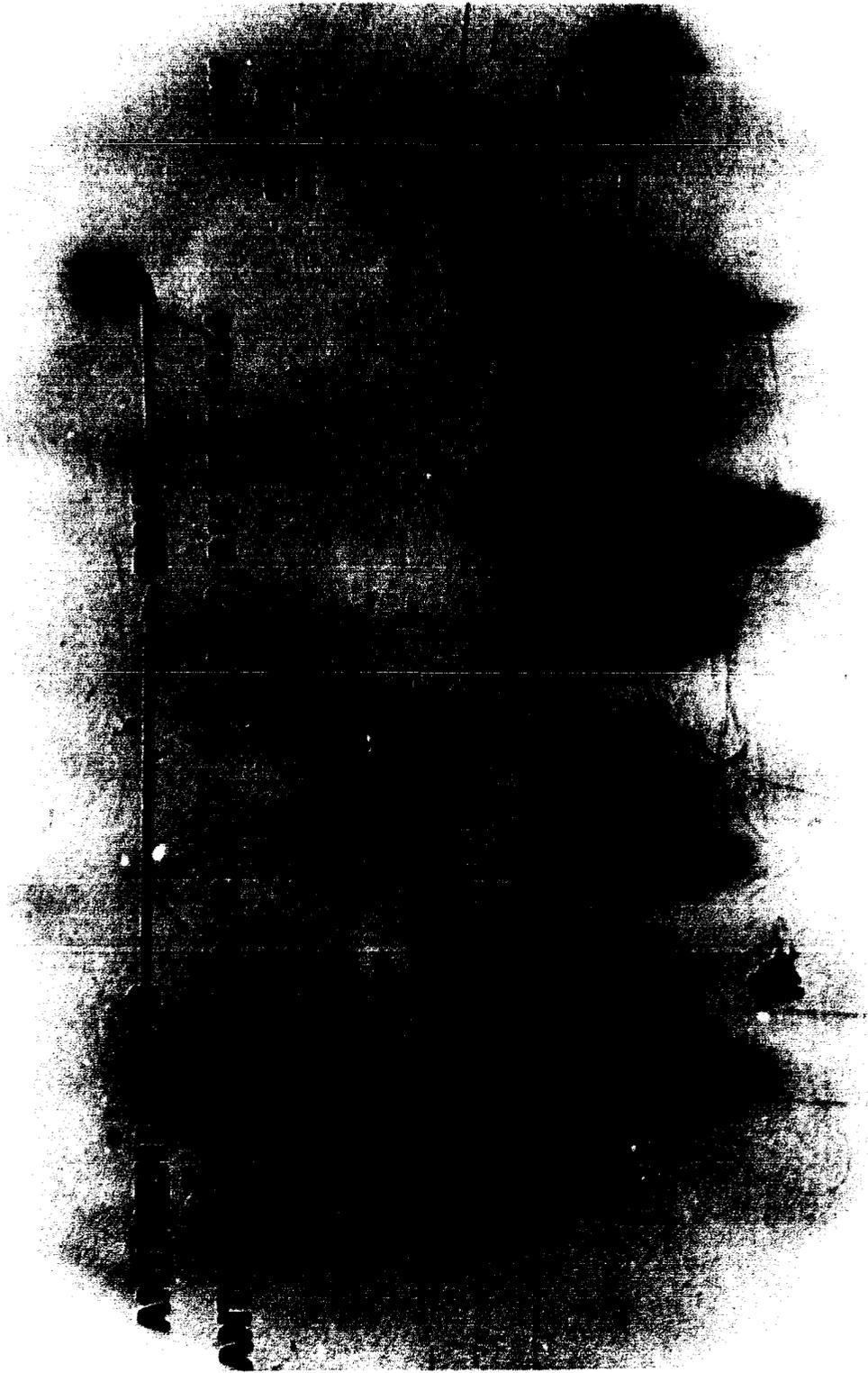
### Peptide Analysis

Since both viral neutralizing and non-neutralizing antisera were obtained, peptide analysis using S. aureus V8 protease was performed on hexon bands cut out from SDS PAGE gels. No difference was detected between the hexon bands immunoprecipitated by VN positive and negative sera (data not shown), using this particular enzyme and a one-dimensional gel. However, digests of slightly different molecular weights were obtained from hexon bands of HAV-2 and BAV-3 (see Figure 9). Varying degrees of reactions were also evident. For example, sera with high VN titers (RJ4 and RJ6) showed stronger reactions in comparison to RJ5, which only has a VN titer of 32. However, the difference could be attributed to concentrations of test reagents.

### Western Blot Analysis

Western blots were performed to see if the antisera could react to sequential determinants, as in the case of bacterial fusion viral proteins produced using recombinant DNA technology. These preliminary results appear to indicate that the major polypeptides retained their immunological reactivities after the transfers. Western blots using BAV-7 PPV antigens and method 1 (BSA blocking), and BAV-3 cell lysates and method 2 (Tween 20 blocking), are shown in Figures 10 and 11, respectively. Method 2 was preferred over method 1 because some of the antisera reacted to BSA (data not shown), thus making interpretation of results impossible. RJ4 appeared to be able to react better with sequential determinants than the sera produced from agarose immunoprecipitates (Figure 11). As shown

**Figure 9. Peptide analysis of hexon bands from IP/SDS PAGE gels. Autoradiography was performed for 72 hours at -70°C**



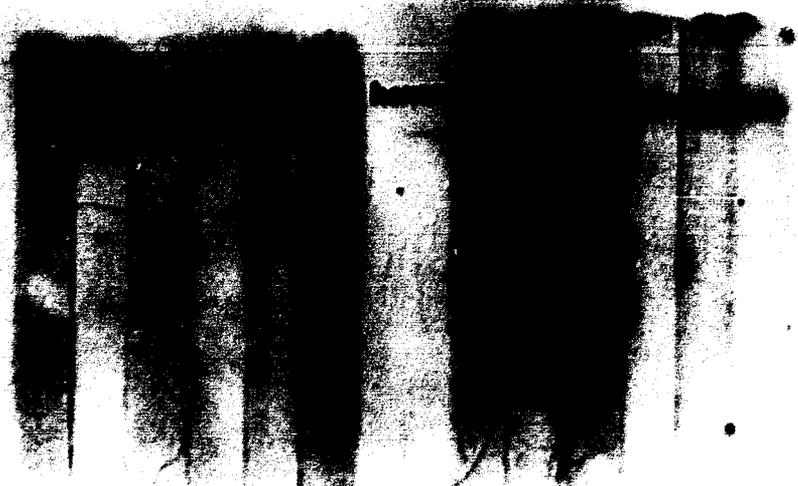
**Figure 10. Western blot analysis of BAV-7 PPV antigens and various antisera. C72 is the reference serum; RJ1 - 3 are anti-subunit sera. NRS: Normal rabbit serum. NCS: Normal calf serum. JW: Human control serum. C225: Control calf serum. Autoradiography was performed for 17 hours at -70°C (with screen)**

**Figure 11. Western blot analysis of BAV-3 cell lysates reacted to various BAV-3 antisera. Autoradiography was performed for 12 hours at -70°C (with screen)**



**ANTIGEN: BAV 3 CELL LYSATES**

ANTISERA: RJ  
63 4 62 61 60 49 48 39 38 37 24 22



in Figures 10 and 11, the capsid polypeptides remained reactive after transfer. However, the reactions to hexons were weaker than expected.

#### Molecular Weights of BAVs

The apparent molecular weights of BAV-2, -3 and -7 were determined by SDS PAGE. The MWs of the four major capsid polypeptides (II, III, IIIa, IV), along with published values, are shown in Table 6.

Table 6. The apparent molecular weights for BAV-2, -3 and -7 as determined by SDS PAGE

Polypeptide	Apparent molecular weights <sup>a</sup>				
	HAV-2 <sup>b</sup>	BAV-2	BAV-3	BAV-3 <sup>c</sup> (published)	BAV-7
II <sup>d</sup>	120K	105K	110K	113K	100K
III <sup>e</sup>	85K	95K	75K	68K	75K
IIIa <sup>f</sup>	66K <sup>g</sup>	82K	65K	64K	50K
IV <sup>h</sup>	62K <sup>i</sup>	68K	60K	52K	45K <sup>j</sup>

<sup>a</sup>Molecular weights are expressed as kilodaltons and represent averaged values from five gels.

<sup>b</sup>MW of HAV-2 polypeptides were taken from Tooze (31). MWs of polypeptides II, III and IV were published by Maizel et al. (22) and MW of polypeptide IIIa was published by Everitt et al. (10).

<sup>c</sup>MW of BAV-3 were published by Niiyama et al. (25).

<sup>d</sup>II: Hexon polypeptides.

<sup>e</sup>III: Penton base polypeptides.

<sup>f</sup>IIIa: Peripentonal area polypeptides (associated with peripentonal hexons).

<sup>g</sup>The apparent MW for IIIa of HAV-2 was about 75K in the present study.

<sup>h</sup>IV: Fiber polypeptides.

<sup>i</sup>The apparent MW for IV of HAV-2 was about 70K in the present study.

<sup>j</sup>The values for III, IIIa and IV of BAV-7 was calculated using only one gel.

## DISCUSSION

Agarose immunoelectrophoresis appeared to be a sufficient method for isolating subunits that are present in large quantities, such as hexons. The major problem encountered in line immunoelectrophoresis was the difficulty in obtaining homogeneous samples from a single precipitin line. Crossed immunoelectrophoresis appeared to provide better separation of subunits since it is a two-dimensional immunoelectrophoretic system. For example, subunits devoid of hexons were only obtained for BAV-7 using CIE. If these pentonal subunits have electrophoretic mobilities different from the hexon subunits, they will be separated well from each other. On the other hand, the relative position of a precipitin line in LIE is determined by the antigen/antibody ratio and on occasion different precipitin lines can superimpose on each other (18). This problem is compounded by the presence of hexon subunits having different electrophoretic mobilities (4). This may be one explanation of why so many agarose immunoprecipitates contained hexon subunits (see tables 3-5). A better approach would be to use LIE as a crude method only, to separate the subunits, and then further purify the antisera obtained from LIE using CIE.

The antiserum produced from deoxycholate and heat treated hexon subunits, RJ4, appeared to be superior to the sera produced from agarose immunoprecipitates in the following ways. It reacted better in Western blot analysis (see Figure 11), thus making it a good reagent in testing cloned viral polypeptides prepared as bacterial fusion products. RJ4

is viral neutralizing, making these subunits possible candidates for subunit vaccine antigens. In addition, these subunits were capable of inducing both subgroup- and type-specific viral neutralizing antibodies (see Table 2), making them ideal candidates for multivalent subunit vaccines.

The molecular weights obtained for BAV-3 appear to be in agreement with previously published values (25). The major difference was the higher molecular weight estimated for fiber (see Table 6). While the molecular weights of the hexons appear to be similar for the three BAV serotypes, the molecular weights of the other capsid polypeptides differ significantly. The peripentonal area and fiber polypeptides appeared to be the most variable. The apparent molecular weights for the peripentonal area polypeptides are 82K, 65K and 50K for BAV-2, -3 and -7, respectively. Fiber polypeptide molecular weights are 68K, 60K and 45K for BAV-2, -3 and -7, respectively. Only BAV-3 showed similar molecular weights to HAV-2 for these two polypeptides. The apparent molecular weights for penton bases are similar for BAV-3 and -7. The molecular weights of polypeptides III, IIIa and IV for BAV-2 are significantly higher than values obtained for HAV-2 and the other two BAVs.

It is believed that each adenovirus serotype displays an unique molecular weight pattern for polypeptides II, III, IIIa and IV (34). In addition, the size of the fibers (37), and to some extent, the sizes of hexons and penton bases (34) have been used in subgroup classification of HAVs. Additional studies are needed on the molecular weight patterns of BAVs. Together with genomic homology data, it may be possible to

devise a more detailed BAV subgroup classification scheme than the present two-subgroup system.

The capsid antigens involved in neutralization are primarily those associated with hexon, but fiber antigens also have been implicated (37). Since hexon subunits are abundant and can be easily isolated (5), they make good candidates for subunit antigens. The abilities of the hexon subunits isolated in this study to induce protective responses in calves need to be tested in vaccination/challenge studies.

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**PART II. GENOMIC HOMOLGY BETWEEN BOVINE ADENOVIRUS TYPE 7  
AND HUMAN ADENOVIRUS TYPE 2**

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**GENOMIC HOMOLOGY BETWEEN BOVINE ADENOVIRUS TYPE 7 AND HUMAN ADENOVIRUS  
TYPE 2**

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## ABSTRACT

Unlike most adenoviruses isolated from mammalian hosts, bovine adenovirus type 7 (BAV-7), a member of BAV subgroup 2, does not share the group-specific complement-fixation antigen with human adenovirus type 2 (HAV-2). In order to locate the genes coding for the major capsid proteins on the BAV-7 genome and to study possible evolutionary relationships between the viruses, cross-hybridization studies were performed on viral DNAs using cloned restriction fragments as probes. Consistent with the immunological observations, no hybridization was detected under normal conditions (annealing in 1M NaCl at 65°C and washing in 0.4M NaCl at 65°C). However, at reduced stringency (annealing and washing in 1M NaCl at 57°C), two distinct regions of homology were observed. One such region centered around 10.3 to 15.5 map units (m.u.) on the HAV-2 genome, corresponding to the IVa2 protein gene. The other region lay between 41 to 70 m.u. on HAV-2, with the most conserved region (50.1-58.5 m.u.) coding for the major capsid protein, hexon. These results allow the alignment of the two genomes. Furthermore, the present results are in contrast to the earlier observation that extensive homology exists between HAVs and BAV-3, which is a member of a different BAV subgroup. Taken together, these results strengthen the classification of these two bovine adenoviruses into separate subgroups, and lend support to the notion that subgroup differentiation of adenoviruses occurred before or shortly after the speciation of the hosts.

## INTRODUCTION

Bovine adenovirus type 7 (BAV-7) has been isolated from calves with pneumoenteritis (11,16,22), weak calf syndrome (3,17,26), and from bovine testicular cultures as an adventitious agent (21). In addition, BAV-7 has been reported to cause abortion experimentally (25).

Being a member of BAV subgroup 2, BAV-7 does not share the common soluble complement-fixation antigen with other mammalian adenoviruses such as human adenoviruses (HAVs) and BAV subgroup 1 viruses (2). BAV-7 was identified as an adenovirus based on its morphology, nature of nucleic acid content, and other physiochemical properties such as resistance to ether and chloroform (11).

The genome of BAV-7, including restriction mapping, has been studied (8). The size of its genome was estimated to be  $29.35 \pm 0.8$  kilobase pairs (8), which is considerably smaller than those of HAV-2 (27) and BAV-3 (12), and is closer to that of murine adenoviruses FL (13).

Genomic homology studies between different adenoviruses, besides providing valuable insights into their evolutionary relationships, can contribute to the development of a more rational approach to virus taxonomy (1). Genomic homology studies have been performed between HAV serotypes (5,6,7), and between HAVs and conventional mammalian adenoviruses of animal origin such as BAV-3 (9,18) and murine adenovirus FL (13). In addition, homology studies also have been performed between avian and human adenoviruses (1). Although these viruses do not cross-react serologically, regions of homology were observed between their

genomes (1).

The objectives of this study were to locate the relative positions of the genes coding for the major capsid proteins on the BAV-7 genome by alignment to the HAV-2 genome, and to study possible evolutionary relationships between human and bovine adenoviruses.

## MATERIALS AND METHODS

### Enzymes and Reagents

Restriction endonucleases Bgl II, Eco RI, Hind III, Hpa I, Kpn I, Sac I, Sma I, and Xho I were purchased from New England Biolabs (Beverly, MA). DNase I and DNA polymerase I (*E. coli*) were purchased from Bethesda Research Lab. (Gaithersburg, MD). [ $\alpha$ -<sup>32</sup>P]-labeled deoxynucleoside triphosphates were prepared at Molecular Genetics, Inc. (45 uCi/ul, used at 1 ul per 0.5 ug of DNA). All enzymatic reactions were performed under conditions specified by the manufacturer, unless otherwise indicated.

### Viral DNA

BAV-7 (strain Fukoroi) was propagated on low passage bovine embryonic testicular (BET) cells. BAV-3 (strain WBR-1) was propagated on HeLa cells. Cell cultures were grown in Dulbecco modified Eagle's minimum essential medium (D-MEM) supplemented with 10% fetal bovine serum, 100 I.U. per ml of penicillin, and 100 ug per ml of streptomycin sulfate. Cultures were infected at a multiplicity of 0.01 plaque forming units (PFU) per cell, maintained in D-MEM supplemented with 2% globulin-free calf serum, and harvested at 48 hours postinfection. Viral DNA was prepared from cesium chloride (CsCl) gradient-purified virions as described by Pettersson and Sambrook (20).

### Plasmid Clones

Fragments of viral DNAs generated by restriction enzymes were inserted into bacterial plasmids (pBR322) and cloned using standard cloning procedures (8,9,10,15). The plasmid clones chosen for this study were the Hind III clones of HAV-2 (10), the Hind III and Eco RI clones of BAV-7 (8), and the Hind III and Eco RI clones of BAV-3 (9).

### Agarose Gel Electrophoresis

Restriction endonuclease fragments of viral DNA were separated on 1% or 1.4% horizontal agarose (SeaKem ME, FMC Corp., Marine Colloids Div., Rockland, ME) slab gels in Loening's buffer.<sup>1</sup> Electrophoresis was performed at 2.5 V/cm for 16 hours. The DNA bands were stained with ethidium bromide and visualized with UV illumination.

### Purification of Cloned DNA Fragments

The DNA of plasmid clones was purified from ethidium bromide-CsCl gradients using standard methods (15), cut with the respective restriction endonuclease, and separated by agarose gel electrophoresis. The DNA digests were separated either in a 1% SeaKem agarose gel as described above, or in a 1% low-melting-temperature (LMT) agarose (Sea Plaque, FMC Corp.) gel using E buffer.<sup>2</sup> The agarose bands containing the DNA

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<sup>1</sup>Loening's buffer is 0.36 M Tris, 30 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  and 1 mM  $\text{Na}_2$  EDTA.

<sup>2</sup>E buffer is 40 mM Tris-HCl, pH 8.0, 1 mM EDTA and 5 mM Sodium acetate.

inserts were cut out from the stained gels upon UV illumination. The DNA was recovered from the agarose using one of the following methods.

#### Method 1

DNA in SeaKem agarose was electroeluted at 10 V/cm for 3 hours in 0.5X TBE buffer,<sup>3</sup> followed by purification on DEAE-Sephacel (Pharmacia Fine Chem., Piscataway, NJ), as described by Maniatis et al. (15).

#### Method 2

DNA in LMT agarose was recovered by melting at 65°C for 10 minutes, followed by standard DNA purification procedures, as described by Maniatis et al. (15).

#### Subcuts of Purified Cloned DNA Fragments

Restriction endonuclease digests of cloned DNA fragments were separated on 1.2% or 1.5% SeaKem agarose gels as described for viral DNA. The HAV-2 fragments chosen for analysis were Hind III-A, -B, -C and -D. Hind III-A fragment was cut with Eco RI, Kpn I or Sma I. Hind III-B fragment was cut with Hpa I, Sma I or Xho I. Hind III-C fragment was cut with Bgl II, Sac I, Sma I, or Xho I. Hind III-D fragment was cut with Kpn I.

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<sup>3</sup>1 X TBE is 90 mM Tris, 8.9 mM Boric Acid, and 2 mM Na<sub>2</sub>EDTA.

### Transfer of DNA

DNA fragments were transferred from agarose gels to nitrocellulose filters essentially as described by Southern (24). After electrophoresis, the DNA in the gel was denatured by soaking in 0.2M NaOH and 0.6M NaCl for 45 minutes, followed by neutralization in 1M Tris-HCl, pH 7.4 and 0.6M NaCl for the same duration. The transfer was performed for 16 hours in 6XSSC.<sup>4</sup> Filters were air-dried, baked at 80°C under vacuum for 2 hours, and cut into strips before hybridization.

### Hybridization Conditions

Two sets of hybridization conditions were employed. For normal stringency, annealing was carried out in 6XSSC at 65°C, followed by washing in 2XSSC at the same temperature. For reduced stringency, both annealing and washing were done in 6XSSC at 57°C. Normal stringency was used for all homologous hybridizations, and for heterologous hybridizations between BAV-3 and HAV-2. Reduced stringency was used for all other heterologous hybridizations.

The filter strips were prehybridized for 2-4 hours in 6XSSC and 0.2% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone. Hybridization buffer was the same with the addition of 0.5% SDS, 1 mM EDTA and 0.125 ug of denatured probe. Probes of either viral DNA or cloned DNA fragments were radiolabeled in vitro with [ $\alpha$ -<sup>32</sup>P]-deoxynucleoside

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<sup>4</sup>1XSSC is 0.15M NaCl and 0.015M Sodium citrate. Both 6X and 2XSSC solutions were diluted from a 20X stock, which is 175.3 g/l of NaCl and 88.2 g/l of Sodium citrate.

triphosphates using nick-translation (23). Hybridizations were carried out for 16 hours. The hybridized filters were washed three times, air-dried and mounted for autoradiography.

#### Restriction Maps

Reference restriction maps for HAV-2, BAV-3 and BAV-7 were published by Tooze (27), Kurokawa et al. (12) and Hu et al. (8), respectively.

The base pair (bp) equivalents per map unit (m.u.) for HAV-2, BAV-3 and BAV-7 are 359 bp/m.u., 350 bp/m.u. and 293.5 bp/m.u., respectively.

## RESULTS

## Cross-hybridizations Between BAV-7 and HAV-2

Under normal conditions, no homology was detected between BAV-7 and HAV-2 (data not shown). However, at reduced stringency, homology was detected between the two viruses.

Experiment 1

Figure 1 shows the results when HAV-2 Sma I digests were transferred to filters and hybridized to nick-translated BAV-7 Hind III and Eco RI clones. Two regions of homology were detected between BAV-7 Hind III-B, -C, -F, Eco RI-G, -O<sub>1</sub>, -C clones and HAV-2 Sma I-F, -B, -D, -H, -A fragments.

Experiment 2

Figure 2 shows the results when BAV-7 Eco RI digests were transferred to filters and hybridized to nick-translated HAV-2 Hind III clones. (Results of using BAV-3 probes are described in a later section.) Basically, two regions of homology were observed between the two viruses, as in experiment 1. On the BAV-7 genome, these regions were located at 9.31-19.46 m.u. and 50.1-57.6 m.u., corresponding to 7.9-37.3 m.u. and 50.1-73.6 m.u. on the HAV-2 genome.

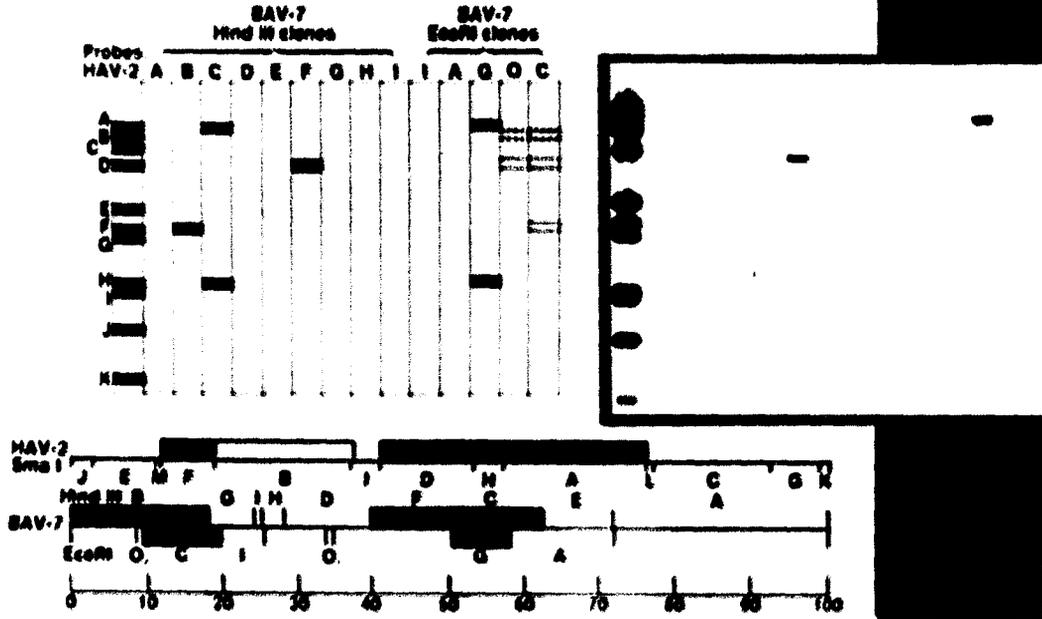
Experiment 3

To obtain finer boundaries within the 2 regions of homology, selected HAV-2 Hind III clones were cut further with various restriction

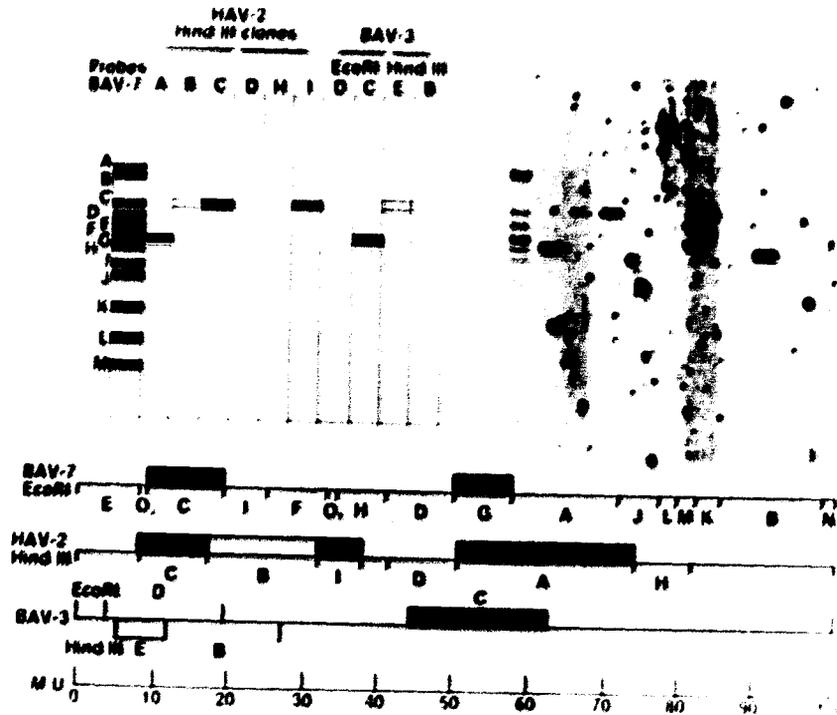
**Figure 1. HAV-2 Sma I digests hybridized to nick-translated BAV-7 clones. The first lane is the homologous hybridization using nick-translated HAV-2 probe. Autoradiograph is shown in the insert**

**Figure 2. BAV-7 Eco RI digests hybridized to nick-translated HAV-2 and BAV-3 clones. The first lane is the homologous hybridization using nick-translated BAV-7 probe. Autoradiograph is shown in the insert**

DNA on filters: HAV-2 Sma I digests  
 Nick-translated probes: BAV-7 clones



DNA on filters: BAV-7 EcoRI digests  
 Nick-translated probes: HAV-2 Hnd III clones and BAV-3 EcoRI and Hnd III clones



endonucleases, and hybridized to selected nick-translated BAV-7 Hind III clones. As shown in Figures 3 and 4, subcuts of HAV-2 Hind III-C, -B, -D and -A clones were hybridized to BAV-7 Hind III-B, -F, and -C probes.

#### Cross-hybridizations Between BAV-7 and BAV-3

No homology was detected between BAV-7 and BAV-3 under normal conditions (data not shown). However, under reduced stringency, homology was detected between the two viruses.

#### Experiment 1

Figure 2 shows the results of BAV-7 Eco RI filters hybridized to selected BAV-3 Eco RI and Hind III probes. Two regions of homology were evident. The first region was located at 9.31-19.46 m.u. on BAV-7 genome, corresponding to 5-11.9 m.u. on BAV-3. The other region centered around 50.1-57.6 m.u. on BAV-7 genome, corresponding to 44.3-62.7 m.u. on BAV-3.

#### Experiment 2

Figure 5 shows the results when BAV-3 Eco RI and Hind III digests were transferred to filters and hybridized to nick-translated BAV-7 Hind III clones. Again, two regions of homology were detected between the 2 viruses.

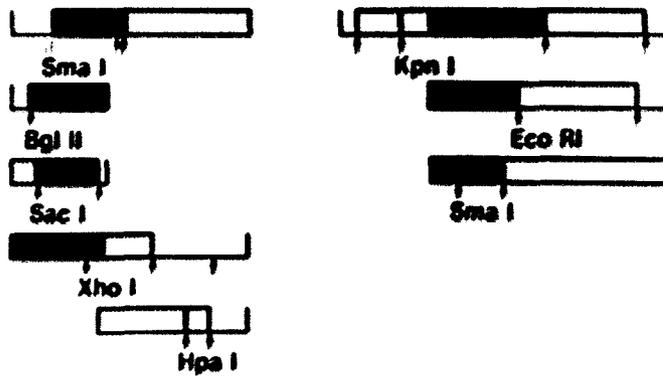
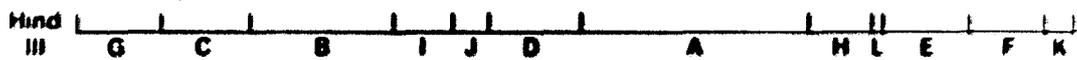
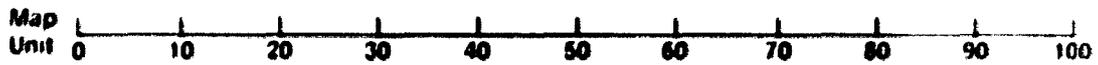
#### Composite Results

From the above experiments, the limits of genomic homology were defined, as shown in Figure 6. Between BAV-7 and HAV-2, one region of homology centered at 9.4 to 28.1 m.u. on HAV-2 genome, with the strongest

**Figure 3. Autoradiographs of HAV-2 Hind III clones digests hybridized to BAV-7 clones**

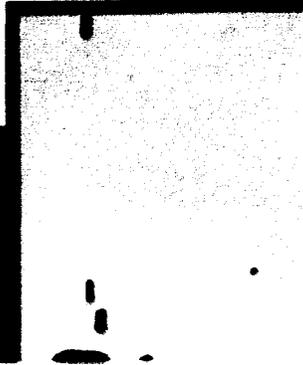
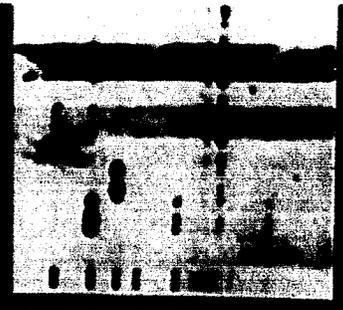
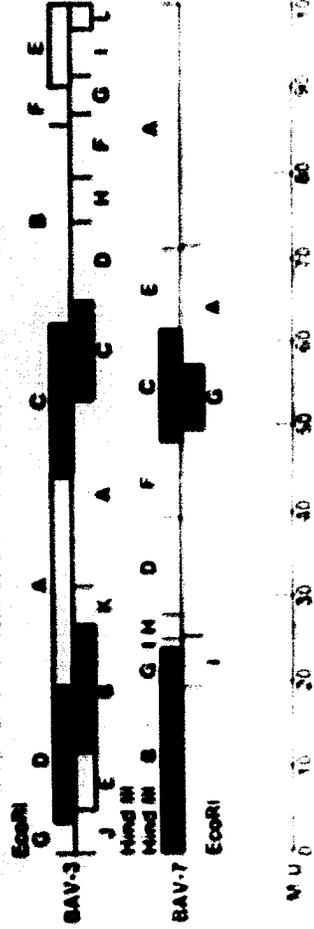
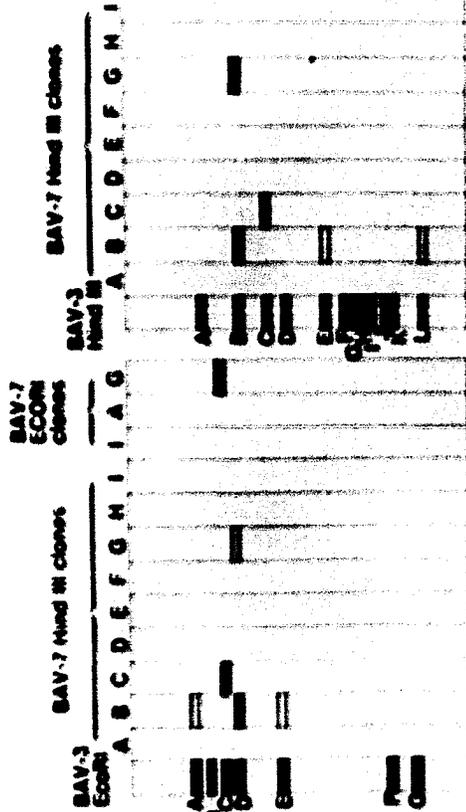
**Figure 4. Subcuts of HAV-2 Hind III clones hybridized to nick-translated BAV-7 clones**

				<b>HAV-2 HindIII-D</b> (Kpn I)		<b>HAV-2 Hind III-C</b> (Sac I, Xho I)	
<b>DIGESTS:</b>	<b>HAV-2 Hind III-C</b> (Sma I, Bgl II, Sac I, Xho I)			<b>HAV-2 Hind III-A</b> (Sma I, EcoRI, Kpn I)		<b>HAV-2 Hind III-B</b> (Hpa I, Xho I, Sma I)	
<b>PROBES:</b>	<b>BAV-7</b> Hind III-B (87°C)	<b>BAV-7</b> Hind III-B (89°C)	<b>HAV-2</b> Hind III-C	<b>BAV-7</b> Hind III-C	<b>BAV-7</b> Hind III-F	<b>BAV-7</b> Hind III-B	<b>BAV-7</b> Hind III-B



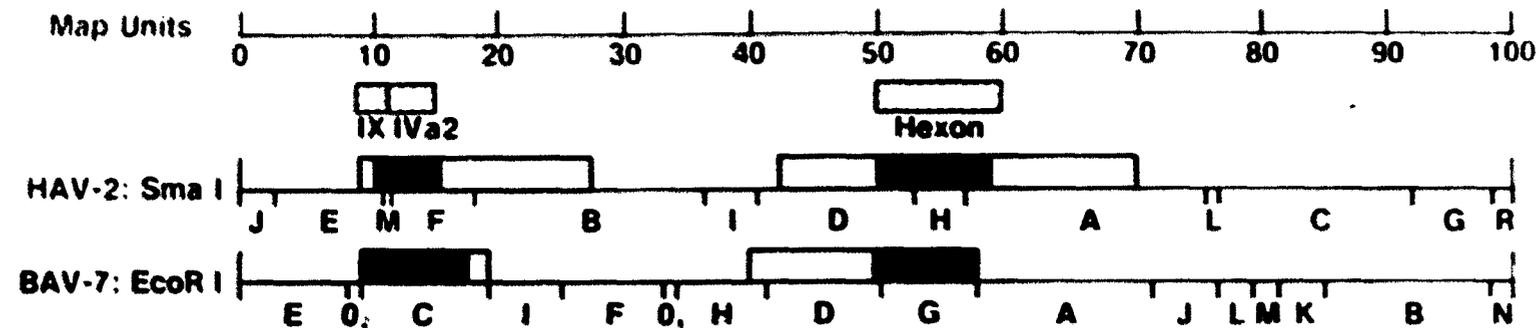
**Figure 5. BAV-3 Eco RI and Hind III digests hybridized to BAV-7 clones. The first lane of each gel is the homologous hybridization using nick-translated BAV-3 probe. Autoradiographs are shown in the inserts**

DNA on filters BAV-3 EcoRI or Hind III digests  
 Nick translated probes BAV-7 clones

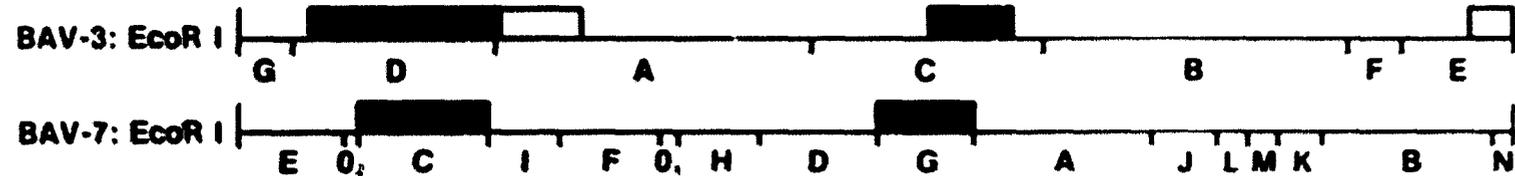


**Figure 6. Summary of results of cross-hybridizations between BAV-7 and classical mammalian adenoviruses (HAV-2 and BAV-3). The locations of the HAV-2 hexon, IVa2 and IX protein genes are shown**

**Genomic Homology Between HAV-2 and BAV-7**



**Genomic Homology Between BAV-3 and BAV-7**



hybridization at 10.3-15.5 m.u. (the IVa2 protein gene). The corresponding region on BAV-7 was located at 9.31 to 19.46 m.u. The other region of homology centered at 41 to 70.7 m.u. on HAV-2, with the strongest hybridization at 50.1-58.5 m.u. (the hexon protein gene). The corresponding region on the BAV-7 genome was 39.72-57.6 m.u. By aligning the BAV-7 genome to that of HAV-2, the locations for the hexon and IVa2 genes for BAV-7 were deduced as 50.1-57.6 m.u. and 9.31-17.82 m.u., respectively.

Between BAV-7 and BAV-3, one region of homology centered at 5-19.4 m.u. on BAV-3 and 9.31-19.46 m.u. on BAV-7. The other region lay between 53.8-59.9 m.u. on BAV-3, corresponding to 50.1-57.6 m.u. on BAV-7.

## DISCUSSION

In contrast to immunological data, genomic homology was detected between BAV-7 and HAV-2. As expected, homology was detected in the hexon-coding region. Since structural constraints dictate certain similarities in the amino acid sequences of the hexon proteins from different adenoviruses (9), at least part of the hexon gene would be expected to be conserved in order to generate a capsid protein that gives rise to the characteristic adenovirus architecture (1).

The other region of homology was detected in the IVa2 gene. This protein is believed to be a scaffolding protein involved in virion assembly (4,19). Since the IVa2 protein interacts with hexons, it is expected to be highly conserved among adenoviruses (8). The location of the hexon and IVa2 genes allowed the alignment of the BAV-7 genome to that of HAV-2.

Previous studies have indicated that extensive homology exists between BAV-3 and HAV-2 (8), and limited homology exists (in two distinct regions) between mouse adenovirus FL and HAV-2 (13), under standard conditions. Since the homology between BAV-7 and HAV-2 was detected only at reduced stringency, these results indicated that, of the three animal adenoviruses, BAV-7 was related most distantly to HAV-2, followed in that order by the murine adenovirus and BAV-3.

It is noteworthy that limited homology was observed between HAV-2 and CELO virus (1), an avian adenovirus which also does not possess the mammalian group-specific complement-fixation antigen. It seems that this virus would be more removed evolutionary from HAV-2 than BAV-7.

Homology studies among BAVs and between BAVs and HAVs are needed to obtain a better classification system for BAVs. Many of the subgroup 2 BAVs have not been studied adequately due to the absence of the complement-fixation group-specific antigen. These results indicate that BAVs can be studied using genomic homology studies instead of immunologic criteria.

Since previous studies (9,18) have indicated that extensive homology exists between HAVs and BAV-3, the present study strengthens the classification of BAV-3 and BAV-7 into two separate subgroups. In addition, it lends support to the notion that the subgroup differentiation of adenoviruses occurred before or shortly after the speciation of the hosts (28).

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### SUMMARY AND CONCLUSIONS

Monospecific sera against protein subunits of BAV types 2, 3 and 7 were produced in rabbits using agarose immunoprecipitates prepared by preparative line or crossed immunoelectrophoresis with hyperimmune type-specific sera. Monospecific anti-hexon sera that were viral neutralizing were obtained against all three serotypes. The sera produced were characterized by VN, IP/SDS PAGE, immunoelectrophoresis, immunodiffusion, and Western blot analysis. An anti-hexon subunit serum also was produced using deoxycholate and heat treatment followed by DEAE ion-exchange column purification. An attempt was made to analyze the differences between hexon subunits capable of inducing a viral neutralizing response and those that did not.

Genomic homology studies were performed between BAV-7 and conventional mammalian adenoviruses (HAV-2 and BAV-3). Under reduced stringency, two regions of homology were detected between BAV-7 and each of the two viruses. These results allowed the alignment of the BAV-7 genome to that of HAV-2 and the analysis of the evolutionary relationships among BAVs and HAV-2.

The following conclusions may be made:

1. Hexon subunits of BAV types 2, 3 and 7 are capable of inducing viral neutralizing responses in rabbits.
2. Sequential determinants of hexon polypeptides can be recognized by anti-hexon subunits sera.
3. Each of the 3 serotypes displays an unique molecular weight pattern for its major capsid polypeptides (II, III, IIIa, IV).

4. Two distinct regions of homology exist between the genomes of BAV-7 and HAV-2 even though the 2 viruses do not cross-react immunologically in complement-fixation tests.
5. The classification of BAV-7 and BAV-3 into two different subgroups was strengthened by their differences in the degrees of genomic homology with HAV-2.

Further research is needed in the following areas:

1. Monoclonal antibodies need to be developed, which would allow further characterization and purification of the hexon subunits.
2. The differences in hexon subunits which induce neutralizing and non-neutralizing responses should be analyzed by epitope mapping using different enzymes and two-dimensional gel systems.
3. The molecular weight patterns of all BAV serotypes, genomic homologies between these serotypes, and genomic homologies between the BAVs and HAV-2 should be studied in order to devise a more detailed subgroup classification of BAVs.

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**APPENDIX: REPRESENTATIVE IMMUNOELECTROPHORESIS AND IMMUNODIFFUSION  
RESULTS OF BAV-2, -3, AND -7**

**Figure A1. Representative immunoelectrophoresis results of BAV-2 subunits and antibodies**

**Antigens: BAV-2 SAS/MEM antigens**

**CIE: A to G**

**Rocket: H to I**

**Antibodies: (A) C87, reference antibody**

**(B) RJ9**

**(C) RJ10**

**(D) C87 with RJ31 as an intermediate gel**

**(E) C87 with RJ32 (bottom) and RJ31 (top) as intermediate gels showing RJ32 and RJ31 reacting to different subunits**

**(F) C87 with RJ42 as an intermediate gel**

**(G) RJ42 with RJ31 as an intermediate gel showing both antibodies having the same specificities and RJ31 blocking out the subunit reactive to RJ42**

**(H to J) Rocket patterns of RJ48, RJ43 and RJ42, respectively, showing antibodies from rabbits immunized with 3 different immunoprecipitates from the same LIE gel**

124

E

L

Z

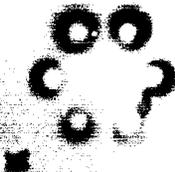
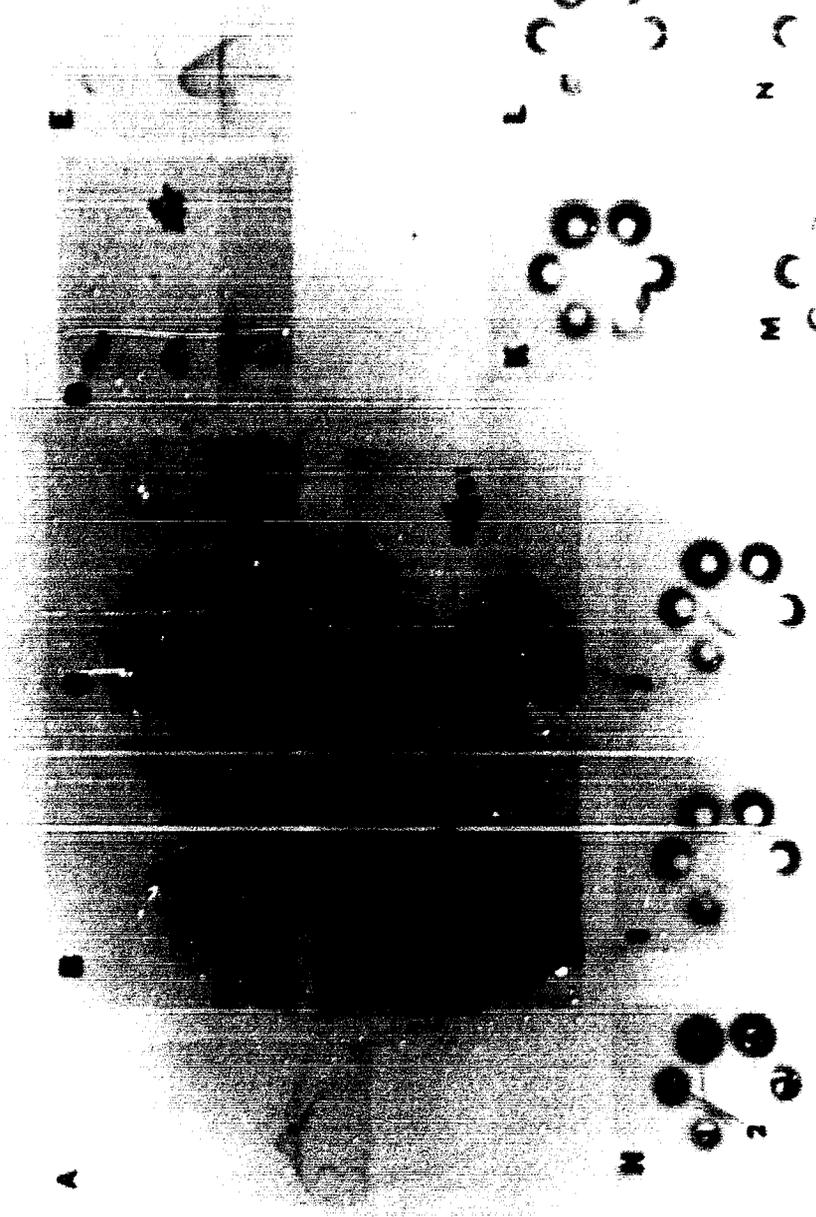
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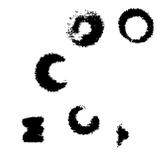
**Figure A2. CIE and ID patterns of BAV-3 antigens and monospecific antibodies**

**CIE (A to G): SAS/MEN antigens**

**ID (H to N) cell lysates antigens: (1) BAV-3; (2) BAV-2; (3) HeLa; (4) MDBK; (5) BAV-7;  
(6) HAV-2**

**Antibodies:**

- (A) RJ4, reference antibody
- (B) RJ5
- (C) RJ6
- (D) RJ4 with RJ5 as an intermediate gel
- (E) RJ4 with RJ6 as an intermediate gel
- (F) RJ4 with RJ24 as an intermediate gel
- (G) RJ4 with RJ21 as an intermediate gel
- (H) RJ4
- (I) RJ5
- (J) RJ6
- (K) RJ21
- (L) RJ24
- (M) RJ37
- (N) RJ38



**Figure A3. Representative immunoelectrophoresis results of BAV-7 subunits and antibodies**

**CIE: A to F, J and K**

**LIE: G**

**Rocket: H and I**

**Antigens: (A) PPV, (B to D) DOC and heat treated PPV, (E to K) SAS/MEM antigens**

**Antibodies: (A) C72, reference antibody  
Arcs 1, 2 and 3 were used to inoculate RJ1, RJ2 and RJ3, respectively**  
**(B) RJ1  
Arcs 1, 2 and 3 were used to inoculate RJ14, RJ11 and RJ12, respectively**  
**(C) RJ2**  
**(D) RJ3**  
**(E) RJ11**  
**(F) RJ12**  
**(G) C72 with RJ11 as an intermediate gel  
Lines 1 and 2 were used to inoculate RJ46 and RJ47, respectively**  
**(H) RJ46**  
**(I) RJ47**  
**(J) RJ11 with RJ46 as an intermediate gel**  
**(K) RJ11 with RJ47 as an intermediate gel**

