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# Purification of a Type 5 Recombinant Adenovirus Encoding Human p53 by Column Chromatography

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## ABSTRACT

**We have investigated the use of column chromatography for the purification of ACN53, a recombinant adenovirus type 5 encoding the human p53 tumor suppressor protein. Anion exchange, size exclusion, hydrophobic interaction, and metal chelating resins were tested; each was found to have distinct advantages and disadvantages. Based on these data, a rapid method was devised for the purification of ACN53. The resultant product was characterized and compared to cesium chloride density-gradient purified virus by SDS-PAGE, Western blot analysis, absorbance spectrum, total particle-to-infectious particle ratio, expression of p53 gene product in Saos-2 cells, growth inhibition of Saos-2 cells, and contamination by ATCC-293 host cell proteins. The results show that column chromatography offers an alternative to ultracentrifugation for the purification of recombinant adenoviruses for use in human gene therapy trials and other research applications.**

## OVERVIEW SUMMARY

**We have devised a chromatographic protocol for the purification of recombinant adenoviruses intended for use in human gene therapies that allows for production on an industrial scale. This method is intended to replace the current methodology of density-gradient ultracentrifugation. A comparison of the purity and potency of a recombinant adenovirus type 5 bearing the human p53 gene (ACN53) derived from chromatographic and ultracentrifugation methods is presented, and the advantages of virus purification by column chromatography are discussed.**

## INTRODUCTION

ADVANCES IN GENE THERAPY technology have brought new treatments for cancer and other serious disease states to the stage of clinical trials. In most cases, gene therapy involves viral vectors delivering genes to target cells. In striking contrast to the rapid advances that have taken place in genetic technology, purification of viruses has continued to rely upon density gradient centrifugation as a primary mode of purification for more than 30 years (Green and Pina, 1964). New methodologies for production and purification of viruses are needed if these promising experiments are to result in therapeutic products. The results reported here show that column chromatography offers significant potential as a method for large-scale virus production.

Our research has focused on new therapies based upon tumor suppressor genes and gene products, the two best characterized of which are p53 and retinoblastoma (Rb). Mutations in the p53 gene and subsequent loss of functional p53 protein have been implicated in the malignant behavior of a variety of human tumors (Bartek *et al.*, 1991; Hollstein *et al.*, 1991). Our strategy has been to suppress proliferating cells by introducing the normal p53 gene by infection with a recombinant adenovirus (rAd). The vector chosen for these studies, ACN53, was derived from an adenovirus type 5 (Ad 5) virus that has had the E1 coding sequences replaced with a 1.4-kb full-length p53 cDNA with expression driven by the human cytomegalovirus promoter (Wills *et al.*, 1994). Recombinant virions were produced in the human embryonal kidney cell line 293.

Previous published attempts to purify virus by chromatographic means has focused on size-exclusion chromatography (Hewish and Shukla, 1983; Albrechtsen and Heide, 1990). Size exclusion appears promising for bovine papilloma virus (Hjorth and Moreno-Lopez, 1982), and has been shown to be a superior method for the purification of tick-borne encephalitis virus (Crooks *et al.*, 1990). The use of size-exclusion chromatography has not yet become widespread, but is currently being employed for large-scale production of recombinant retrovirus

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(Mento, S. J., Viagene, Inc. as reported at the 1994 Williamsburg Bioprocessing Conference). Affinity chromatography, mostly using monoclonal antibodies (mAb), has been reported to be an effective method for the purification of antigens of viral origin (Njayou and Quash, 1991). Infective soybean mosaic virus (a virus that can survive pH 3) can be recovered using mAb affinity chromatography (Diaco *et al.*, 1986). Fowler *et al.* (1985) used affinity chromatography and density gradient centrifugation to purify Epstein-Barr virus.

Adenoviruses are large (diameter of approximately 80 nm) and somewhat fragile. A large literature base dealing with the relationship of structure to function has accumulated (for reviews, see Philipson, 1983; Horwitz, 1990). Little has been reported in the literature about chromatographic purification of infectious adenoviruses. Haruna *et al.* (1961) reported encouraging results using DEAE ion-exchange chromatography of Ad 1, 3, and 8. Klemperer and Pereira (1959) and Philipson (1960) reported disappointing results for the use of a similar method with Ad 2 and 5.

Chromatographic resins and equipment have been improved substantially since 1960. Given these recent advances and a wealth of available information about adenoviruses, we decided to revisit ion-exchange chromatography and to examine other modern chromatographic techniques for the publication of adenoviruses.

## MATERIALS AND METHODS

### *Production of infected ATCC 293 cells*

293 cells (American Type Culture Collection, CRL 1573) were grown in a 6,000-$cm^2$ Cell Factory (Nunc) in a humidified air/7% $CO_2$ incubator in 1.5 liters of DME high-glucose medium (Irvine Scientific) supplemented with 10% fetal bovine serum (FBS) (Hyclone).

Two to 2.5 days after seeding the Cell Factory, when cell monolayers reached about 50–60% confluency, the cells were infected at a multiplicity of infection (moi) of 5–10 infectious units (IU) per cell in 500 ml of fresh medium. The virus was added to the medium, mixed thoroughly, and introduced to the cells in the unit. Three to 4 days post-infection, the infected cells were ready for harvesting.

### *Harvest and lysis*

When cell monolayers showed signs of detachment from the surface of the Cell Factory, the cells were harvested by gentle tapping and centrifuged in a Beckman TJ-6 at 1,500 rpm for 5 min. Cells were resuspended and pooled in 25 ml of 100 m*M* Tris buffer pH 8.0 for use in the preparation of ultracentrifuge-derived standard virus. Samples destined for use in chromatography were resuspended in 25 ml of 50 m*M* HEPES buffer pH 7.5/150 m*M* NaCl, 2 m*M* $MgCl_2$, and 2% sucrose. The cells were lysed at this point by three cycles of freeze-thaw. Following the third cycle, cellular debris was removed by centrifugation in a Beckman TJ-6 at 1,500 rpm for 5 min. The supernatant from this step was adjusted to 2 m*M* $MgCl_2$, 2% (wt/vol) sucrose and 2.5% (wt/vol) $\beta$-cyclodextrin. Benzonase (American International Chemical, Inc. Naitick, MA) was added to a final concentration of 100 units/ml and allowed to incubate for 1 hr at room temperature. The treated material was clarified by centrifugation in a Beckman TJ-6 at 3,000 rpm for 10 min and filtration through a Gelman Sciences Acrodisc 0.8/0.2-$\mu$m filter.

### *Preparation of ACN53 standard material (CsCl-ACN53)*

Standard recombinant ACN53 virus was prepared by a three-step centrifugation procedure as described (Laver *et al.*, 1971) with the following modifications. Infected cells were lysed by three cycles of freeze-thaw and centrifuged at 15,000 rpm for 10 min, 4°C in a Sorvall SS34 rotor. The pellet was discarded, and the supernatant was treated with Benzonase at 133 U/ml for 30 min at room temperature. The treated material was layered onto a 1.25 g/ml and 1.40 g/ml CsCl discontinuous step gradient in 10 m*M* Tris pH 8.1, and centrifuged at 30,000 rpm for 60 min, 10°C in a Sorvall TST 41-14 rotor. The virus band from each tube was collected, pooled, mixed with 1.35 g/ml CsCl (in 10 m*M* Tris pH 8.1), and centrifuged overnight at 45,000 rpm, 10°C in a Beckman VTi 65 rotor. The virus band from each tube was collected and recentrifuged at 45,000 rpm as before for an additional 4 hr. The final virus pool from this step was dialyzed extensively against phosphate-buffered saline (PBS) supplemented with 2% sucrose and 2 m*M* $MgCl_2$.

### *Chromatographic parameters*

Column resins were tested for their separation characteristics in 6.6 × 50-mm (1.7 ml) borosilicate Omnifit columns fitted with 25-$\mu$m polyethylene frits. The columns were mounted on a PerSeptive Biosystems Biocad chromatography workstation. The chromatography was monitored on-line for pH, conductivity, and dual wavelength optical density detection at 280 nm ($A_{280}$) and 260 nm ($A_{260}$).

Anion-exchange resins were equilibrated in 50 m*M* HEPES pH 7.5, 300 m*M* NaCl, 2 m*M* $MgCl_2$, 2% sucrose at 1 ml/min. A 50 m*M* Tris buffer pH 8.0, (with 300 m*M* NaCl, 2 m*M* $MgCl_2$, 2% sucrose) was also used in certain trials for comparison. After samples were loaded and washed to baseline as monitored by absorbance, elution was performed with a 20-column volume 300–600 m*M* linear NaCl gradient and collected in 0.5-ml fractions. The column was then cleaned with two column volumes of 0.5 *M* NaOH followed by one column volume of 1.5 *M* NaCl.

Size-exclusion chromatography experiments were done in a 6.6 × 500-mm borosilicate Omnifit column packed with Toyopearl HW-75F resin and equilibrated at 1 ml/min in PBS/2% sucrose/2 m*M* $MgCl_2$. Injection volumes varied from 50 to 200 $\mu$l depending on the nature of the sample. Fractions of 0.5 ml were collected during elution.

For hydrophobic interaction chromatography, a 50 m*M* Tris pH 8/$(NH_4)_2SO_4$ buffer system and toyopearl butyl 650M resin were used. Samples were prepared for loading onto the column by mixing with an equal volume of 3 *M* $(NH_4)_2SO_4$ in 50 m*M* Tris buffer pH 8.0, incubated for 5–10 min, and centrifuged at 3,000 rpm for 5 min in an Eppendorf Microfuge (model 5415c) to remove any precipitate. The column was eluted with a gradient of 1.5 *M* to 0 *M* ammonium sulfate over 10 column volumes at 1 ml/min.

The immobilized zinc affinity chromatography (IZAC) system was prepared for metal charging by washing the column

sequentially with one volume of 100 m*M* EDTA and one volume of 0.2 *M* NaOH, flushing with water after each step. The matrix was subsequently charged with zinc by injecting one column volume of 100 m*M* $ZnCl_2$ in $H_2O$ acidified with 0.5 $\mu$l/ml glacial acetic acid and thoroughly washed in water prior to equilibration in 50 m*M* HEPES pH 7.5, 450 m*M* NaCl, 2% sucrose, and 2 m*M* $MgCl_2$. Sample loading did not require any prior manipulation; DEAE pool fractions or CsCl-derived material could be injected directly onto the column. After loading, the column was washed with a 10-column volume linear gradient from 50 m*M* HEPES pH 7.5, 450 mM NaCl, 2% sucrose, and 2 m*M* $MgCl_2$ to 50 m*M* HEPES pH 7.5, 150 m*M* NaCl, 2% sucrose, 2 m*M* $MgCl_2$. Elution was performed with a linear (0–500 m*M*) glycine gradient in 150 m*M* NaCl applied over 10 column volumes.

### *SDS-PAGE analysis*

For Coomassie blue staining, 100–200 $\mu$l of a sample (at approximately $1 \times 10^{11}$ particles/ml) were collected, desalted by trichloroacetic acid precipitation or by dialysis followed by concentration in a Speed-Vac. The sample was then resuspended in SDS-PAGE reducing buffer [125 m*M* Tris-HCl pH 6.8, 20% glycerol, 4% (wt/vol) SDS, 0.005% bromphenol blue, 0.5% $\beta$-mercaptoethanol] to approximately 30 $\mu$l, boiled for 5 min, and loaded onto a 1-mm × 10-well Novex 8–16% gradient Tris-Glycine minigel. Samples were electrophoresed for 1.5 hr at 140 V. The gel was then fixed in 40% methanol/10% acetic acid for 30 min, and Coomassie stained with the Pro-Blue staining system (Integrated Separation Systems, Natick MA.) according to the vendor's procedure.

Gels that were to be silver stained were loaded with 5–15 $\mu$l of sample. The sample was boiled with an equal volume of reducing buffer and electrophoresed as described for Coomasie detection. Gels were fixed in 10% trichloroacetic acid for 1 hr, washed three times in ultrapure water, and stained with the Daiichi silver staining kit according to the instructions provided (Integrated Separation Systems).

### *Western blot analysis*

An SDS-PAGE gel was run as described with approximately the same loading as that of a silver-stained gel. The bands were then transferred to a PVDF membrane pre-wetted in 100% methanol and equilibrated in Tris-buffered saline (TBS). The gel was also equilibrated in TBS. The proteins were transferred to the membrane using a Bio-Rad semidry transfer apparatus at 25 V for 30 min. The membrane was then blocked in 1% casein/0.01% sodium azide overnight at 4°C or at room temperature for 1 hr, and washed three times with TBS. The membrane was incubated with the primary polyclonal antibody (Cytimmune rabbit IgG $\alpha$-adenovirus type 5, Lee Biotechnology Research: San Diego, CA) at 5 $\mu$g/ml (in TBS) for 1 hr at room temperature. Following primary incubation, the membrane was washed three times with TBS and incubated with the secondary antibody (Amersham Life Sciences Horseradish peroxidase-conjugated anti-rabbit Ig) diluted to 1 $\mu$l stock antibody/1 ml TBS for 1 hr at room temperature. A final three-time wash was performed with TBS and the membrane incubated with Amersham ECL detection reagents for 1 min, exposed in the dark to Hyperfilm-ECL (Amersham) for various times (several seconds to minutes to give a selection of various contrasts), and developed in an X-ray film developer.

### *Particle number by absorbance at 260 nm in the presence of SDS*

The number of total virus particles (total = infection + noninfective particles) can be derived spectrophotometrically for pure virus. For this measurement, the virus sample ($\sim 1 \times 10^{12}$ particles/ml) was diluted 1:10 in 0.1% SDS in PBS. The sample was vortexed for 1 min and then centrifuged at 14,000 rpm in an Eppendorf Microfuge to remove any precipitate. A matched pair of curvettes were blanked with 0.1% SDS in PBS buffer by running a baseline scan on Shimadzu UV160U spectrophotometer. The SDS-treated virus sample was placed in the sample cuvette and scanned from 220 to 340 nm. If the absorbance between 310 and 320 nm was greater than 0.02, the sample was diluted further and remeasured. The $A_{260}/A_{280}$ ratio was also determined from this scan, and had to be between 1.2–1.3 to ensure that the product was pure enough to calculate particle number. If this condition was met, the absorbance value at 260 nm only was used to calculate the number of virions per ml. The conversion factor of $1.1 \times 10^{12}$ particles per absorbance unit at 260 nm (Maizel *et al.*, 1968) was used to calculate particle number with approximately 20% error.

### *Particle number by anion-exchange HPLC*

A 1-ml Resource Q (Pharmacia) anion-exchange column was also used to quantitate the number of total viral particles in various samples to ±5%. Unlike the measurement of absorbance at 260 nm, this assay may be applied to samples at any state of purification, from crude lysates to final product. Preparation of lysate samples was performed by first centrifuging the sample at 1,300 × *g* in a microfuge for 5 min. The supernatant was then adjusted to 2 m*M* $MgCl_2$ and 100 U/ml of Benzonase was added for 20–30 min at room temperature. The enzymatic treatment was necessary for assaying crude lysates only. Semipure or pure virus did not require such treatment and could be injected directly.

The column was equilibrated in 300 m*M* NaCl, 50 m*M* HEPES pH 7.5 at a flow rate of 1 ml/min on a Waters 625 chromatography system equipped with 717plus autosampler and a model 991 photodiode array detector (PDA). The chromatography was monitored on the PDA detector scanning from 210 to 300 nm. Elution was performed with a 2-ml wash in the equilibration buffer followed by a linear 10 ml 300–600 m*M* NaCl gradient. The resulting virus peak was quantitated at 260 nm by comparison to standard curves constructed by injecting $1 \times 10^8$ to $1 \times 10^{11}$ CsCl purified ACN53 virions that had been characterized for total particles by $A_{260}$ in 0.1% SDS ($\sim 1 \times 10^{12}$ particles/ml).

The assay was independent of injected sample up to a volume of 1 ml or more. Some standard autosamplers are limited to single-injection volumes of not more than 200 $\mu$l; this limitation could be overcome by repeated injections. Washing the column between 200 $\mu$l injections did not affect the chromatography. After the sample loading, the column was washed with two column volumes of equilibration buffer followed by a linear gradient from 300 to 600 m*M* NaCl in 50 m*M* HEPES pH 7.5 over 10 column volumes. The gradient was followed

with a two-column volume wash with 600 m*M* NaCl in 50 m*M* HEPES pH 7.5. After each chromatographic run, the column was cleaned with 2.5 ml of 1.5 *M* NaCl in 50 m*M* HEPES pH 7.5, and then reequilibrated for the next injection. The column was cleaned more vigorously after injection of crude samples by injecting 0.25 to one column volume of 0.5 *N* NaOH followed by a wash with 1.5 *M* salt. Injecting NaOH and then running the gradient was a convenient way to accomplish cleaning.

The validity of this assay was demonstrated by several tests. First, the peak area was found to increase linearly with virus particle number when CsCl-ACN53 was applied to the column. The area was also found to increase in the expected manner when uninfected lysates or infected lysates were spiked with increasing amounts of purified virus. Particle numbers measured by anion-exchange HPLC were in agreement with values obtained by spectrophotometry. Anion exchange was the preferred method for measurement of virus particles because of its speed, sensitivity, and accuracy. A full description of this assay will be presented elsewhere (Shabram *et al*, manuscript in preparation).

### *Measurement of infectious particles by $TCID_{50}$ assay*

The quantitation of infectious particles was accomplished by an end point titer assay (tissue culture infective dose of 50%, abbreviated $TCID_{50}$) similar to assays available in the literature (Philipson, 1961). Reagents, a materials list, and instructions for antibody staining are available from Chemicon International, Inc. (cat. # 3130, Adenovirus Direct Immunofluorescence Assay, Temecula, CA).

In brief, 293 cells were plated into a 96-well microtiter plate: 100 $\mu$l of $5 \times 10^5$ cells/ml for each well in complete MEM (10% bovine calf serum; 1% glutamine) media (GIBCO BRL). In a separate plate, a 250-$\mu$l aliquot of virus sample diluted $1{:}10^6$ was added to the first column and was serially diluted two-fold across the plate. Seven rows were used for samples, one was used for a negative control. A 100-$\mu$l aliquot of each well was transferred to its identical position in the ATCC-293 seeded plate and allowed to incubate a 37°C in a humidified air/7% $CO_2$ incubator for 2 days. The media was then decanted by inversion and the cells fixed with 50% acetone/50% methanol. After washing with PBS, the fixed cells were incubated for 45 min with a FITC-labeled anti-Ad5 antibody (Chemicon International #5016) prepared according to the kit instructions. After washing with PBS, the plate was examined under a fluorescent microscope (490 nm excitation, 520 nm emission) and scored for the presence of label. The titer was determined using the Titerprint Analysis program (Lynn, 1992). Typically, titers for purified material range from $1 \times 10^9$ to $2 \times 10^{10}$ (IU)/ml. The assay can measure infectious titers from $10^6$ to $10^{11}$ IU/ml. Error in the assay ranges from 20 to 35%.

### *Expression of p53 protein*

The activity of virus preparations was also tested by assaying for expression of the p53 gene product in Saos-2 cells (ATCC), a p53-negative osteosarcoma cell line. Saos-2 cells were seeded into a 6-well tissue culture plate at a concentration of $5 \times 10^5$ cells/well in 3 ml of media: Kaighn's nutrient mixture F12 (GIBCO BRL), DME high glucose (1:1 mixture), supplemented with 2 m*M* L-glutamine and 10% fetal bovine serum (HyClone). The cells were incubated in humidified air/7% $CO_2$ chamber at 37°C for 16–24 hr. Spent media was removed and replaced with 1 ml of fresh media, and the cells were infected at an moi of 20, 40, or 60 using purified virus. After incubation for 1 hr, an additional 2 ml of media was added and allowed to incubate for 8 hr. The cells were then washed once with Dulbecco's-PBS (GIBCO BRL) and lysed by adding 250 $\mu$l of: 50 m*M* Tris, 0.5% Nonidet P40, 250 m*M* NaCl, 5 m*M* EDTA, 5 m*M* NaF, 5 $\mu$g/ml Leupeptin, and 5 $\mu$g/ml Aprotinin/2 m*M* PMSF (Boehringer Mannheim). The plate was incubated on ice for 5 min, after which the lysates were transferred to individual 1.7-ml microcentrifuge tubes. These were centrifuged for 45 sec at 14,000 rpm in a microfuge. The supernatants were assayed for the presence of p53 protein by Western blot analysis with the primary anti-p53 monoclonal antibody 1801 (Vector Laboratories, Burlingame, CA) and a 1:1 mixture of sheep anti-mouse IgG-HRP and streptavidin-HRP (Amersham). The p53 protein band was detected using Amersham's ECL detection kit in accordance with the manufacturer's instructions.

### *Potency assay*

The antiproliferative activity of replication-incompetent adenovirus samples expressing p53 protein was assessed using a thymidine incorporation bioassay. Saos-2 cells were seeded in 96-well flat-bottomed microtiter dishes in 100 $\mu$l of Kaighn's nutrient mixture F12:DME high glucose (1/1) supplemented with 2 m*M* L-glutamine and 10% fetal bovine serum at a density of $1 \times 10^4$ per well. After an overnight incubation at 37°C in humidified air/7% $CO_2$ chamber, ACN53 and AC$\beta$GAL (a recombinant adenovirus that expresses $\beta$-galactosidase) were added in 100-$\mu$l aliquots; a serial three-fold dilution starting from an moi of 50 was used to infect the cells. Positive and negative control samples were incubated on each microtiter plate. All plates were returned to the incubator for an additional 48 hr. All dilutions were tested in duplicate. Cells were labeled with [$^3$H]thymidine (Amersham) at 0.5 $\mu$Ci/well for 6 hr at 37°C in a 7% $CO_2$ incubator. Next, cells were detached from the plastic substratum by adding 100 $\mu$l per well trypsin-EDTA (GIBCO BRL) at 37°C for 10 min and transferred to a 96-well glass fiber filter cassette (Packard Instrument Co., Meriden, CT) using a 96-well harvester (Packard Instruments). Sample filter cassettes were wetted with 50 $\mu$l/well MicroScint 20 scintillation cocktail, covered with Top-Seal sealant (Packard Instruments), and loaded into a Packard Top Count scintillation counter for analysis. Samples were counted for [$^3$H] for 1 min. Data (in cpm) were compared with media control values and plotted. Dose–response curves and values for $ED_{50}$ were determined using the general form of the four parameter logistic equation.

## RESULTS

### *Lysate treatment*

Nuclease-treated lysate was clarified to remove cellular debris and undissolved $\beta$-cyclodextrin through a combination of centrifugation and filtration techniques. Using analytical anion-

exchange analysis to measure recovery, it was found that centrifugation followed by filtration through a Gelman Sciences Acrodisc 0.8/0.2 $\mu$m two-stage syringe filter gave the best recovery (94%). Recovery of ACN53 depended on the pore size and type of membrane used for filtration; passage through 0.45-$\mu$m polysulfone, PVDF, and cellulose acetate-based membranes recovered between 34 and 75%.

Figure 1 shows that virus particles sediment during centrifugation of lysate samples. A 5-min centrifugation at 15,000 $\times$ $g$ in an Eppendorf Microfuge (model 5415c) at 4°C resulted in a 45% loss of virus particles as measured by anion-exchange HPLC, compared to a centrifugation of an identical sample at 1300 $\times$ $g$. The loss of particles in the sample correlates directly with the force of the spin.

Infected cell lysate contains contaminants both host cell and viral in origin. Some of these contaminants could be removed by treatment with nuclease prior to chromatography. Specifically, host cell, non-encapsulated, or incomplete ACN53 nucleic acids could be enzymatically degraded at this stage of the process with the addition of nuclease (Benzonase). Such treatment improved yields in ion-exchange chromatography. Benzonase was removed by subsequent purification steps as assayed by a commercially available ELISA kit (American International Chemical).

$\beta$-Cyclodextrin was added to lysate to reduce the contaminant load on a DEAE anion-exchange column (data not shown), providing better purification on the DEAE column and increasing the number of cycles the column could be run.

*Anion-exchange chromatography*

CsCl-ACN53 was injected onto a Fractogel DEAE-650M column equilibrated in 50 m*M* Tris pH 8 at 2 ml/min (350 cm/hr) and eluted with a 10-min (11.7 column volume) 0–1.5 *M* linear NaCl gradient. A single peak was detected with an on-line $A_{260}/A_{280}$ ratio of 1.23. The protein bands present in this fraction reacted with Ad 5 polyclonal antibody upon slot-blot analysis.

Several peaks were resolved when an infected cell lysate sample was applied to the DEAE column (Fig. 2). The composition of the peaks could be deduced from the on-line $A_{260}/A_{280}$ absorbance ratio (as opposed to a separate measurement in a stand-alone spectrophotometer with 0.1% SDS treatment). For example, the peak with a retention time of 9 min has an on-line $A_{260}/A_{280}$ ratio of 0.5, and was mainly protein. The 27-min peak had an on-line $A_{260}/A_{280}$ ratio of 2, suggesting that this material was nucleic acid. The ACN53 virus peak eluted at 19 min with a ratio of 1.23. The identities of these peaks were confirmed by spiking experiments and by running SDS gels of each peak. In our chromatography experiments, an on-line $A_{260}/A_{280}$ ratio of 1.23 $\pm$ 0.8 was found to be characteristic of virus peaks.

To assess the purification capabilities of DEAE chromatography, experiments were performed in which both noninfected 293 cell lysate and CsCl-ACN53 were applied to the column (Fig. 3). Most of the host cell material either passed through the column during the load or eluted at an earlier retention time

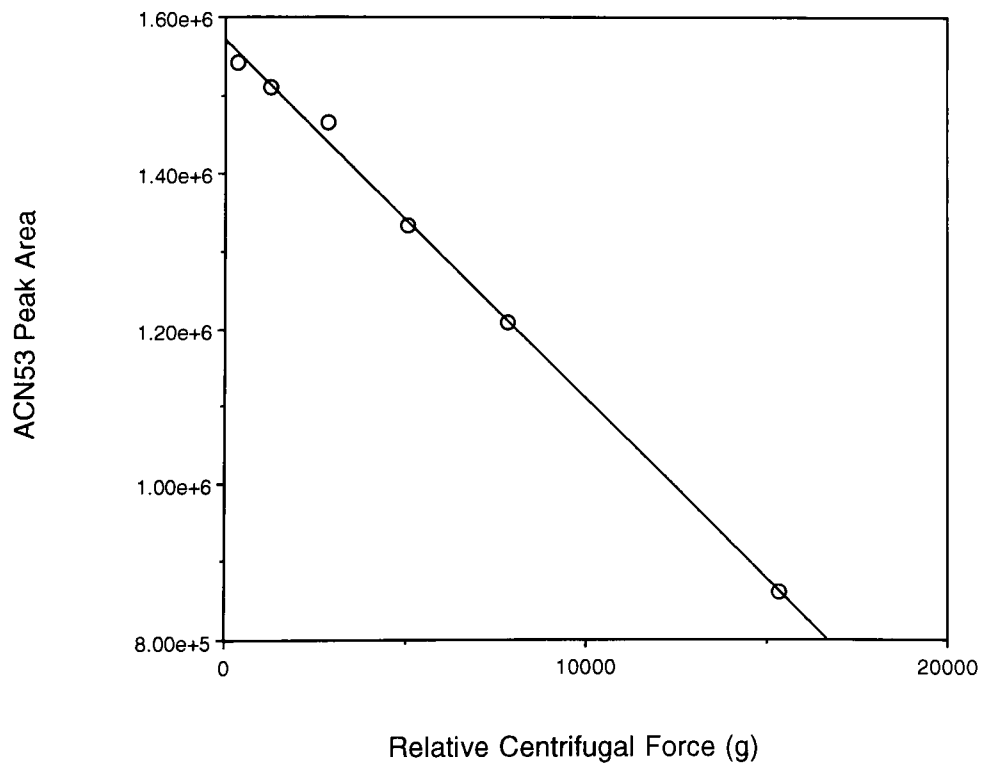


**FIG. 1.** Recovery of ACN53 from the supernatant of centrifuged lysate. Crude infected cell lysate was centrifuged for 5 min at different speeds in an Eppindorf Microfuge model 5415c at 4°C and analyzed for ACN53 by analytical anion exchange.

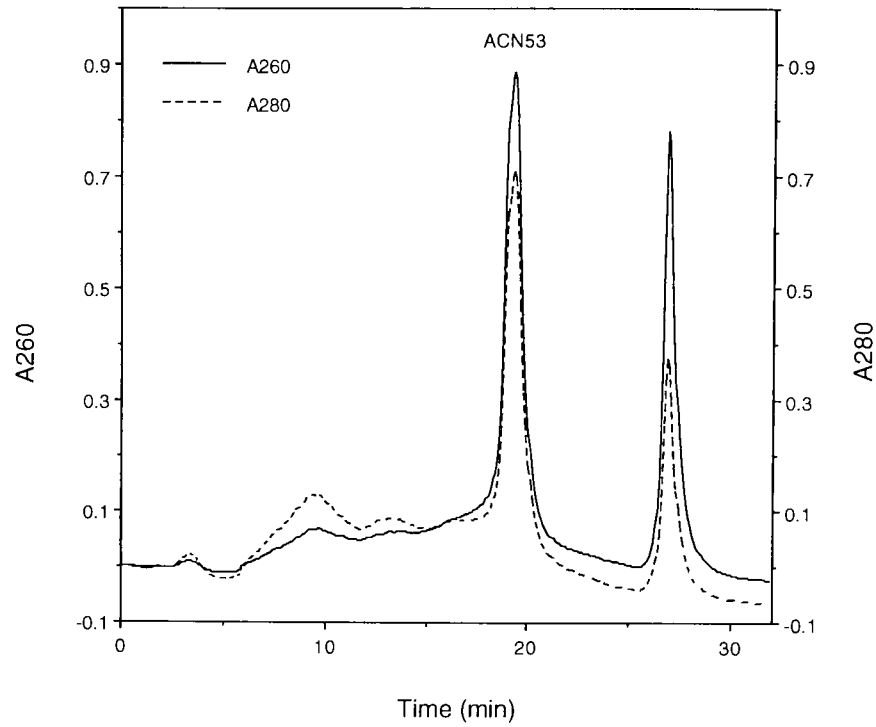


**FIG. 2.** Identification of ACN53 by dual-wavelength UV absorbance. Infected cell lysate components separated by DEAE chromatography can be identified according to their $A_{260}/A_{280}$-nm absorbance ratio.

than that of ACN53; however, a small peak eluted with the same retention time as ACN53. From these data it appeared that nonviral contamination of the ACN53 peak might be expected from host cell material. The peak eluting between 22–26 min in the cell lysate sample in Fig. 3 revealed an $A_{260}/A_{280}$ nm ratio of approximately 2, suggesting a high nucleic acid content. This peak was reduced or eliminated by treatment with Benzonase prior to chromatography.

A SDS-PAGE gel of DEAE pooled fractions shows a pattern similar to a CsCl-ACN53 standard (Fig. 4). The absorbance ratio $A_{260}/A_{280}$ of the DEAE pool measured in 0.1% SDS was 1.15–1.20, suggesting contamination by proteins. Product recovery from the column was assessed as 60–80% by infectious titer and analytical anion-exchange HPLC analysis. Most of the recovered infectivity was in the product peak, although a small amount of infectivity was found in the DNA peak and flowthrough pool (1–5%).

### *Size-exclusion chromatography*

The resin chosen for this study was Toyopearl HW-75F because of its large exclusion limit ($5 \times 10^6$ daltons) a 6.6 × 500-mm column was packed and equilibrated at 1ml/min in PBS, 2% sucrose, and 2 m*M* $MgCl_2$. A total of 50 $\mu$l of CsCl-ACN53 standard was injected to test the elution characteristics of ACN53 in this system. The resultant peak was very broad, eluting in a fraction size approximately 70% of the total column volume. This effect could not be overcome by increasing the salt concentation to 500 mM NaCl, ruling out ionic interaction of ACN53 with the resin matrix. The recovery off the column was also very low, with only 15–20% of the amount injected being eluted.

### *Hydrophobic interaction chromatography (HIC)*

Two hydrophobic resins, Toyopearl butyl 650M and Toyopearl phenyl 650M, were evaluated. Precipitation experiments performed with CsCl-ACN53 showed that the virus remained soluble in 1.5 *M* ammonium sulfate, and would therefore remain soluble under the loading conditions. CsCl-ACN53 was injected onto the column, and eluted with a gradient of 1.5 *M* to 0 *M* ammonium sulfate over 10 column volumes at 1 ml/min. Both phenyl and butyl resins bound and eluted ACN53, but the butyl column yielded a sharper peak.

After studying the chromatography of cell lysate on butyl 650M, it was determined that DEAE anion exchange was more effective as a first purification step. HIC was then investigated as a second purification step. A DEAE pool derived from an infected 293 cell lysate was diluted 1:1 with 3 *M* ammonium sulfate and purified over a butyl-HIC column. No precipitate was observed during this step. In the resultant chromatogram, the virus peak was well resolved from other components. Recovery of ACN53 ranged from 5 to 30% as measured by both infectious titer and analytical anion-exchange methods. After further analysis of HIC chromatography of the DEAE-derived virus pool indicated there was a problem with degradation of virus during HIC treatment, we decided to examine metal affinity chromatography.

### *Immobilized zinc affinity chromatography (IZAC)*

The interaction of virions with metals has been inferred from studies of viruses and bacteriophages (Lark and Adams, 1953, Brakke, 1956). We investigated the metal-binding properties of ACN53 by testing its ability to adsorb to a metal affinity col-

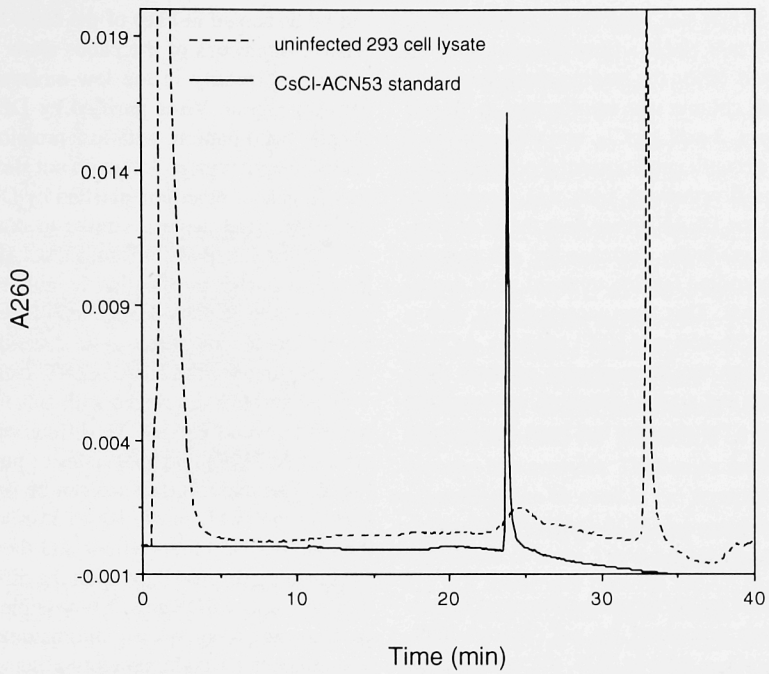


**FIG. 3.** Comparison of CsCl-ACN53 and host-cell contaminant retention times during DEAE purification. An uninfected 293 host-cell lysate blank was chromatographed over a DEAE column to assess how much contamination would coelute with the virus. The elution of CsCl-ACN53 is overlaid to compare retention times.

umn loaded with zinc ions. An ACN53-DEAE fraction pool purified over IZAC is shown in Fig. 5. Analysis of an IZAC fraction pool gives a yield of 49–65% and an $A_{260}/A_{280}$ ratios of 1.22–1.25. A gel and Western blot comparison of CsCl-ACN53, DEAE-purified, and DEAE-IZAC-purified material can be seen in Figs. 4 and 6. The CsCl-ACN53 and DEAE/IZAC materials were very similar, and the DEAE-only purified material was less pure by these criteria. The interaction of ACN53 with this column was shown to be zinc specific; injection of CsCl-ACN53 onto an uncharged column (a column not preloaded with zinc) resulted in a shift of the virus peak to the flowthrough.

Experiments were designed to study the effect of different IZAC buffer and elution systems. When a DEAE-ACN53 pool was split in half and purified over IZAC in HEPES pH 7.5 and Tris pH 8 buffer systems, the HEPES buffer yielded 2.5-fold more viral particles as measured by anion-exchange HPLC. IZAC could be run in the presence of 2% sucrose and 2 m*M* $MgCl_2$ without affecting the chromatography. Experience derived from CsCl-ACN53-based material indicated that sucrose and magnesium may help stabilize the virus, especially when freezing and thawing final product (data not shown).

The use of copper as the metal ion and imidazole as the elution agent were also tested (for a general review of metal affinity chromatography, see Kato *et al.*, 1986; Belew *et al.*, 1987). Of the four systems, zinc/glycine, zinc/imidazole, copper/glycine, and copper/imidazole, the zinc/glycine system performed best in terms of virus recovery. Decreasing pH gradients can also be used for elution but were avoided in the case of ACN53 because of its sensitivity to low pH.

ACN53 seems sensitive to abrupt changes in salt concentration. This phenomenon was first seen in HIC chromatography, and was also detected in the investigation of IZAC. When a DEAE fraction pool was diluted from its ionic strength of ap-

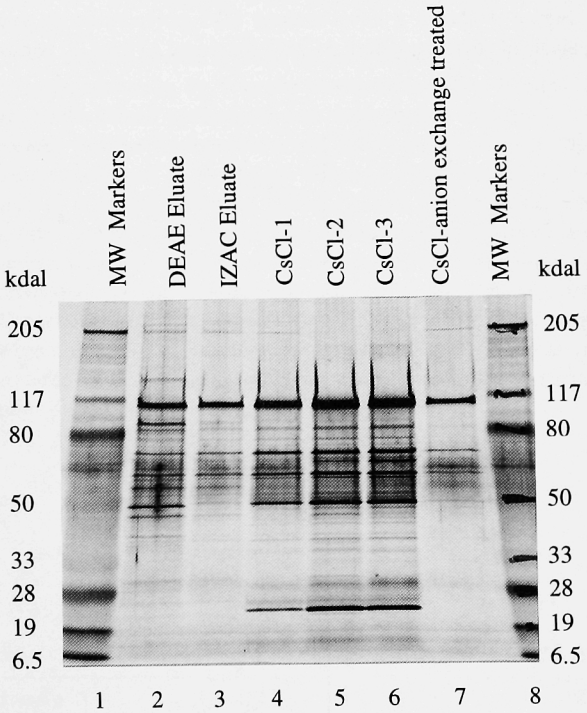


**FIG. 4.** SDS-PAGE comparison of ACH53 derived from column chromatography and CsCl ultracentrifugation. Samples were electrophoresed on an 8–16% gradient gel and silver stained. Lanes 2 and 3 are DEAE and IZAC eluate pools, respectively. Lanes 4–6 represent three different preparations (CsCl-1, CsCl-2, CsCl-3) of CsCl-ACN53 run side by side. Lane 7 represents the ACN53 peak recovered when a sample of CsCl-ACN53 is purified over a analytical anion-exchange HPLC column. Lanes 1 and 8 contain standard molecular weight markers.

proximately 450 m*M* to 150 m*M* NaCl and loaded onto an IZAC column equilibrated at 150 m*M* NaCl, a flowthrough peak with a high $A_{260/280}$ was observed. When the loading conditions were revised such that the IZAC column was equilibrated in 50 m*M* HEPES pH 7.5, 2% sucrose, 2 m*M* $MgCl_2$, and 450 m*M* NaCl (approximating the ionic strength conditions of the DEAE fraction pool), the size of the flowthrough peak was diminished. The objective was to load the DEAE eluate onto the IZAC column iso-osmotically, and gradually decrease the salt concentration to 150 m*M* in a controlled fashion to avoid virus degradation due to osmotic shock. This was accomplished with a 10-column volume linear 450–150 m*M* NaCl gradient. ACN53 was subsequently eluted with the same glycine gradient used before. Iso-osmotic loading and gradual desalting increased the product yield by 12% over a column that had been equilibrated and loaded at 150 m*M* NaCl.

### *Comparison of ACN53 virus purified by CsCl method and DEAE-IZAC chromatography*

Virus preparation purified by ultracentrifugation and by column chromatography were compared by six criteria: SDS-PAGE, Western blots, $A_{260}/A_{280}$ ratio in SDS, the ratio of total virus particles to infectious virus particles, expression of p53 gene product, growth suppression by the gene product, and an immunoassay for the presence of host-cell proteins in the final product.

Figure 4 shows a silver-stained SDS polyacrylamide gel analysis of several purified ACN53 fractions. At least 20 bands can be discerned in each of the three lots purified in CsCl; the relative intensities of the bands show some variation from lot to lot, particularly in the low-molecular-weight (10–35 kilodaltons) region. Virus purified by DEAE and IZAC shows a simpler band pattern, with less protein visible in the low-molecular-weight region. Although not shown in this figure, silver-stained gels of other lots purified by DEAE-IZAC also showed consistent band patterns similar to that seen in Fig. 4, lane 3. One of the lots purified using CsCl (Fig. 4, lane 6) was subjected to further purification by anion-exchange chromatography (see lane 7). Low-molecular-weight proteins were removed by this procedure, resulting in a band pattern very similar to virus purified by the DEAE-IZAC method.

Western blots developed with anti-Ad 5 polyclonal antibody revealed several bands. The differences between banding patterns of ACN53 in various states of purification can be seen in Fig. 6. The major difference can be seen in the relative intensities of the bands in the 10- to 35-kilodalton regions between the CsCl-ACN53 preparations and the chromatographic pools. This data sheds little light upon the differences in patterns seen in silver-stained SDS gels. It is possible that loosely bound virus proteins are removed by chromatography; alternatively, the low-molecular-weight proteins absent, or present in reduced levels, in DEAE-IZAC purified material may be cellular contaminants removed by chromatography but not by CsCl density-gradient centrifugation. In either case, the reduced levels of these proteins did not change the infectivity of the virus.

The absorbance ratio ($A_{260}/A_{280}$) is important because it is related to the ratio of protein and DNA in the product (discussed

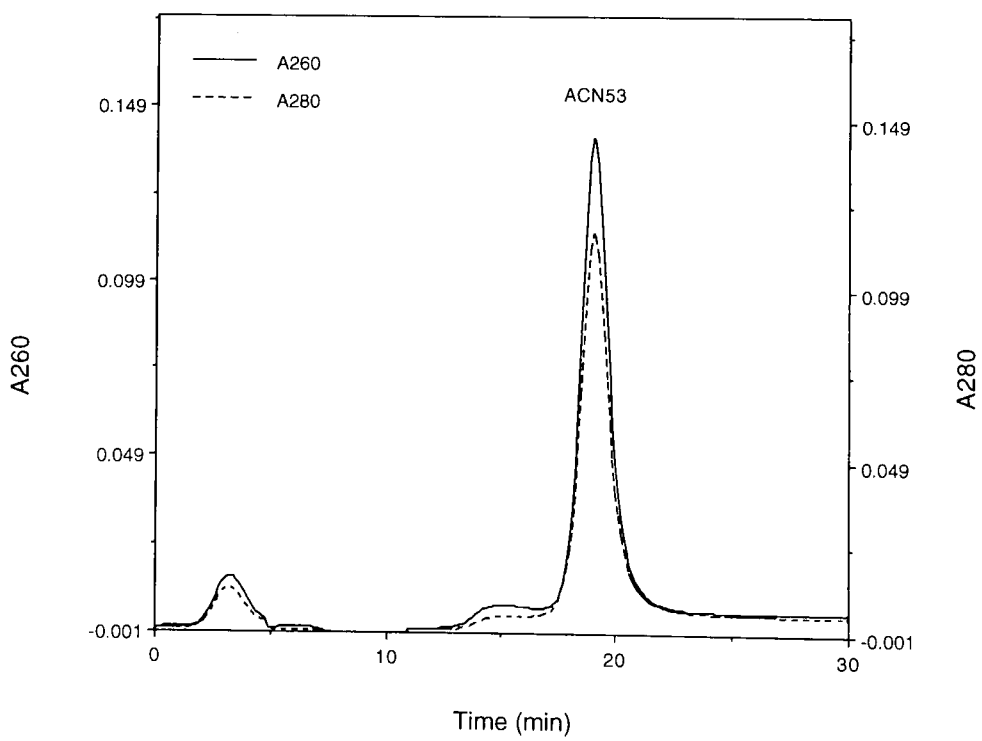


**FIG. 5.** Immobilized metal affinity chromatography of a DEAE-ACN53 fraction pool. A DEAE-purified ACN53 fraction pool was injected onto a 6.6 × 50-mm TosoHaas AF chelate 650M column charged with $ZnCl_2$ and eluted with a linear 0–500 m*M* glycine gradient.

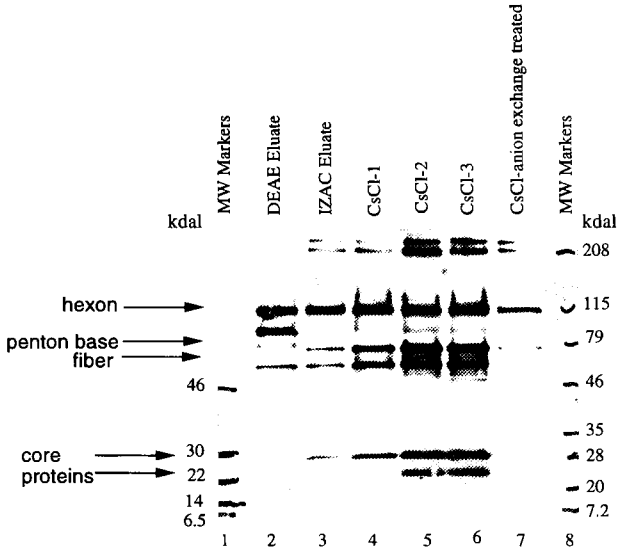


**FIG. 6.** Western blot comparison of ACN53 derived from column chromatography and CsCl ultracentrifugation. Samples identical to those described in Fig. 4 were electrophoresed on an 8–16% gradient gel and transferred to a PVDF membrane. The blot was incubated first with 5 μg/ml Cytimmune rabbit IgG anti-Ad 5 antibody, then with Amersham's horseradish peroxidase-conjugated anti-rabbit Ig (NA934) and developed using electrochemical detection.

below), and can be used as a measure of product purity. For various CsCl-ACN53 virus preparations measured in 0.1% SDS, the ratio was found to range from 1.2 to 1.3. The variation in the measured ratio reflects lot-to-lot variability as well as error in measurements. DEAE-IZAC products yielded ratios of 1.22–1.25, which are within the acceptable range. The $A_{260}/A_{280}$ ratio seemed less variable for DEAE-IZAC preparations than for CsCl-ACN53 virus preparations.

When samples purified by DEAE chromatography were subjected to this analysis, the range of ratio values for DEAE fraction pools was lower (1.14–1.20). These values could be related to how the fractions were pooled: wider pools gave lower numbers, consistent with the presence of contaminating proteins. The second chromatography step (IZAC) removed these proteins and improved the $A_{260}/A_{280}$ to values typical of CsCl-purified material (Table 1).

The ratio of total virus particles to infectious viral particles can vary widely from preparation to preparation. Values for CsCl-derived viruses ranged from 20(±7):1 to 130(±45):1. The calculation of this ratio in crude lysates or semipure fractions has been made possible by the anion-exchange particle assay. It cannot be performed by $A_{260}$ measurements as described in the Materials and Methods section. For crude samples, interfering absorbance from contaminants is too high. It is possible through the use of analytical anion exchange to observe if and how this ratio changes during purification, *i.e.*, whether the final product's total virus particle-to-infectious virus particle ratio is determined by the original ratio present in the raw material, or if it can be improved during purification. In following this value during a chromatographic purification, the total virus particle-to-infectious virus particle ratio of the crude lysate was 60(±21):1 (Table 2). DEAE-purified ACN53 had a particle ratio of 82(±29):1, while DEAE-IZAC, buffer-exchanged final product had a particle ratio of 88(±31):1.

The overall yield of total virus was approximately 32%; that of infectious virus was approximately 22%. These values are comparable to recovery values determined for ultracentrifugation procedures we currently use for the preparation of CsCl-ACN53. The yields from ultracentrifugation purifications was measured by analysis of crude lysate and final product by anion-exchange HPLC and infectious titer.

The recombinant adenovirus was designed to transfer a gene to a cell lacking a functional P53 gene. To assay for this activity, we incubated P53 null Saos-2 cells with DEAE and DEAE-IZAC virions, and after allowing for sufficient infection, assayed the cells for production of the p53 gene product by Western blot analysis. The results are shown in Fig. 7. p53 gene expression can be seen in the semipure DEAE-ACN53 as well as the DEAE-IZAC-ACN53-treated cells. The lower levels of expression in lanes 5 and 6 were due to the fact that they were run at a lower moi than the DEAE samples.

The ability of the gene product to suppress the growth of Saos-2 cells was assayed by measuring [$^3$H]thymidine incorporation (Fig. 8). Three different viral constructs were assayed for growth inhibition effects: CsCl-ACN53, DEAE-IZAC-ACN53, and CsCl-AC$\beta$GAL. Both of the p53 gene-bearing adenoviruses were able to inhibit the incorporation of [$^3$H]thymidine, while the CsCl-AC$\beta$GAL virus had no effect.

We assayed several types of samples for the presence of host cell contamination by Western blot analysis using polyclonal antibodies raised against 293 cell components. The results indicated that the final products of CsCl or DEAE-IZAC purification contained no detectable host cell contaminants. In the case of a partially purified DEAE-ACN53 fraction, there was a small amount of contamination seen: three major bands and several minor ones. The majority of host cell contamination was recovered in the flowthrough portion of the DEAE step. Contaminants that copurified with ACN53 in the DEAE step were removed by zinc affinity chromatography, and were recovered in IZAC flowthrough fractions prior to the introduction of the glycine gradient.

TABLE 1. YIELD AND PURITY OF CHROMATOGRAPHICALLY PRODUCED ACN53 BASED ON TOTAL PARTICLES

| *Step* | *Total viral particles*[a] | *Yield* | *$A_{260}$:$A_{280}$ ratio*[b] | *Purity*[c] |
|---|---|---|---|---|
| Lysate | $3 \times 10^{12}$ | — | — | 3% |
| DEAE load | $3 \times 10^{12}$ | | | |
| DEAE eluate | $2 \times 10^{12}$ | 67% | 1.17 ± 0.03 | 92% |
| IZAC load | $1.52 \times 10^{12}$ | | | |
| IZAC eluate[d] | $7.14 \times 10^{11}$ | 47% | 1.23 ± 0.02 | 98% |
| Overall | — | 32% | — | — |

[a]Determined by HPLC analytical anion-exchange assay.
[b]Measured in 0.1% SDS.
[c]Measured by integration of HPLC chromatogram at 260 nm.
[d]Assayed after dialysis into the storage buffer.

TABLE 2. YIELD AND PURITY OF CHROMATOGRAPHICALLY PRODUCED ACN53 BASED ON INFECTIVE PARTICLES

| *Step* | *IU/ml*[a] | *Total IU* | *Yield* | *Specific activity (P/IU)*[b] |
|---|---|---|---|---|
| Lysate | $1 \times 10^{10}$ | $5.0 \times 10^{10}$ | — | 60(±21):1 |
| DEAE load | $1 \times 10^{10}$ | $5.0 \times 10^{10}$ | | |
| DEAE eluate | $4.87 \times 10^{9}$ | $2.44 \times 10^{10}$ | 49% | 82(±29):1 |
| IZAC load | $4.87 \times 10^{9}$ | $1.85 \times 10^{10}$ | | |
| IZAC eluate[c] | $2.7 \times 10^{9}$ | $8.1 \times 10^{9}$ | 44% | 88(±31):1 |
| Overall | — | — | 22% | — |

[a]Determined by $TCID_{50}$.
[b]The ratio of noninfectious particles (P) to infectious particles (infectious units, IU) as determined by analytical anion exchange and $TCID_{50}$ assays.
[c]Assayed after dialysis into the storage buffer.

## DISCUSSION

### *Detection of ACN53 in process samples*

There are several methods available for the detection of viral product in process samples. These vary according to specificity, sensitivity, ease of use, throughput, and versatility in terms of identity and quantitation. $TCID_{50}$ is very sensitive ($10^5$–$10^{11}$ IU/ml) and has the advantage of being able to identify infective viral particles. It is not the assay of choice, however, for in-process sample analysis because of the time required (3 days). It also has the greatest error (35%). Absorbance measurements performed in a spectrophotometer can be used quickly for quantitation of total particles (±10%), but can only be used to measure the concentration of pure virus.

The quickest and most convenient assay is the analytical anion-exchange assay. This is capable of measuring total virus particles in any sample, from crude lysate to pure material in less than 30 min. It is by this method that we have been able to quantitate stepwise recoveries (±5%) during our purification process. With this assay, the identity, purity, and quantity of the virus can be established simultaneously be the UV absorbance characteristics.

The use of a dual-wavelength on-line spectrophotometric detector monitoring absorption at 260 (DNA $\lambda^{max}$) nm and 280 (protein $\lambda^{max}$) nm can provide real-time identification of ACN53 particles. This is possible because of the virus is composed of a complex of DNA and protein in a unique ratio as compared to free proteins (unassembled viral or free host-cell) or uncomplexed nucleic acids. A spectrum of pure DNA reveals an $A_{260}/A_{280}$ ratio of 1.8–2, whereas pure protein has a ratio of 0.4–0.6. A mixture of the DNA and protein has an $A_{260}/A_{280}$ ratio somewhere in-between depending on the relative amounts of each constituent. A spectrum of CsCl-purified, infective ACN53 has a distinct $A_{260}/A_{280}$ ratio of 1.2–1.3. A dual-wavelength chromatogram or on-line photo-diode array detector can easily distinguish between fractions containing mostly DNA, protein or virus (Fig. 2). Measurements of this sort can also be carried out on purified material in a benchtop spectrophotometer in the presence of 0.1% SDS for greater precision. Values <1.2 indicate contaminating protein, whereas values >1.3 indicate excess exogenous DNA present in the product.

SDS-PAGE and Western blot analysis are standard methods for the detection of proteins and are able to handle a large number of samples in a time frame of hours. The difficulties encountered here are concentration of dilute samples and interference by high salt. Furthermore, because ACN53 is a mixture of proteins, DNA, and carbohydrates, stained gels do not reveal a distinct single band but rather a complex pattern that must be compared to a CsCl standard. Coomassie blue or silver staining may be used. SDS-PAGE analysis is more easily interpreted as the sample becomes more pure, but is difficult to apply in crude lysates.

Western blot analysis is more specific than a stained gel and can unambiguously identify the presence of viral proteins in a complex mixture. This technique also reveals a pattern rather than a single band. Some column fractions will have unassembled viral proteins that are Western positive but are considered

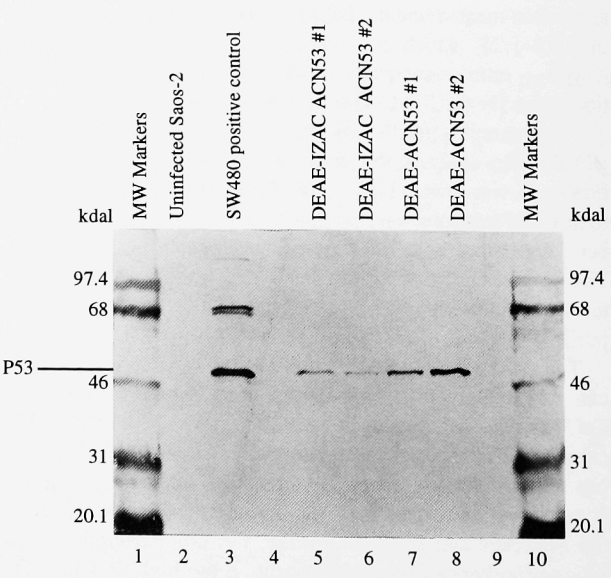


**FIG. 7.** Expression of p53 gene product in Saos-2 cells. Two different lots of chromatographically produced ACN53 were assayed by Western blot for their ability to affect gene transfer to p53-null Saos-2 cells. The semipure DEAE fractions are shown in lanes 7 and 8, the final product in 5 and 6. p53-expressing SW480 cells were used as a positive control; uninfected Saos-2 cells were used as a negative control.

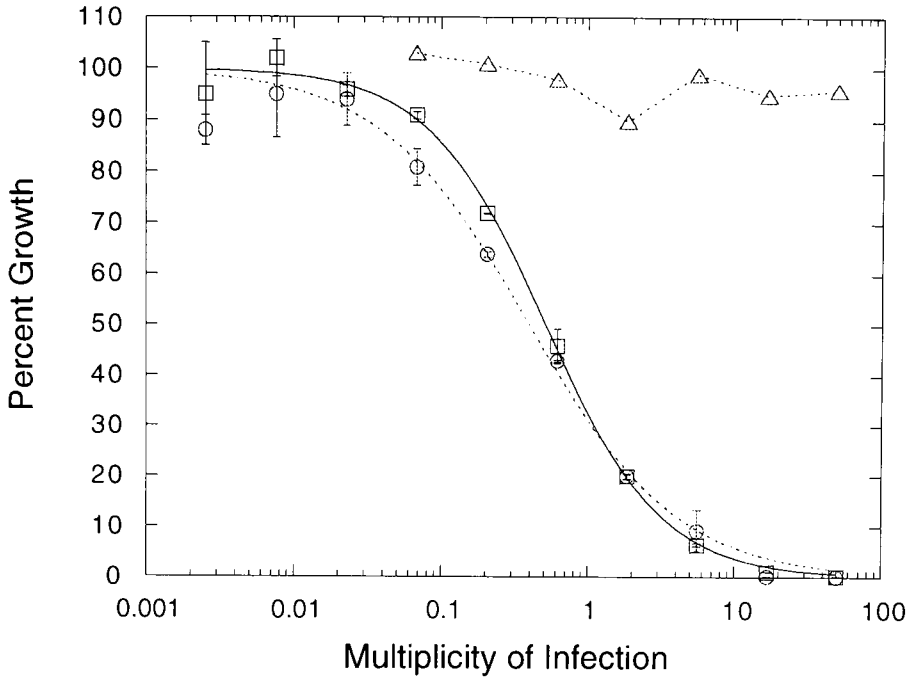


**FIG. 8.** Growth Inhibition of Saos-2 cells by ACN53. [$^3$H]Thymidine incorporation was measured in Saos-2 cells treated with DEAE-IZAC-ACN53 (squares) CsCl-ACN53 (circles), and AC$\beta$GAL (triangles). The percent inhibition of growth was calculated relative to a control sample in which cells were treated with media only. Calculated $ED_{50}$ values for the samples were: DEAE-IZAC-ACN53, 0.56 ± 0.04 moi; CsCl-ACN53, 0.45 ± 0.05 moi.

as impurities because they represent incomplete or degraded virus particles. For example, previrions form a lower-density band in the CsCl ultracentrifugation of ACN53. Electron micrographs show previrions to be comprised of incomplete viral particles (Moncany *et al.*, 1980). It is difficult to distinguish these inactive previrions from infective virions by Western blot or SDS-PAGE alone; additional information must be acquired (biological assays) and used in conjunction with gels and blots.

### *Chromatography of ACN53*

The treatment of lysate with nuclease is important for two reasons. First, it is necessary to remove unwanted DNA from the product, and second, to improve the performance of the chromatography in terms of yield and purity. Our goal was to obtain and characterize a nuclease that would be available in large quantity for a commercial process. We have chosen Benzonase for these reasons.

Clarification of the treated lysate was accomplished by a combination of filtration and centrifugation. Centrifugation was used to remove cellular debris, but needed to be brief to minimize loss of product (Fig. 1). The particles are large enough to be prone to sedimentation by centrifugation at low speed, as demonstrated in this study, as well as by sedimentation in ultracentrifuges. The yield as a function of centrifugal force can be quickly measured by analytical anion exchange. Loss of product during clarification by filtration was similarly evaluated and optimized for our vector. The types of filters examined in this study were 25-mm syringe tip units; as greater quantities of raw material become available, study on larger filters will be feasible.

The power of the analytical anion-exchange assay is evident in its speed, versatility, and throughput. We now have a fast method for the detection of total viral particles in cell culture samples, as well as process step samples. Having an assay of this sort is critical for quantitating step yields during purification and for protocol optimization, and can be applied many ways, from the optimization of growth conditions in culture to the stability of viral particles under a variety of parameters. The assay was derived from data collected in the study of DEAE chromatography of CsCl-purified virus. The adaptations required to change from a preparative mode to an analytical one were to select a higher-resolution column that could withstand multiple injections of crude lysate, and its installation on a high-performance chromatographic system. Qualitatively, the chromatograms obtained from a preparative DEAE column and an analytical quarternary amine are similar. The two anion-exchange methods differ in that one has been optimized for production and the other for quantitation.

Overall, the characteristics of DEAE chromatography were found to be very consistent, and loading studies with high titer lysate ($3 \times 10^{12}$ virus particles/ml) showed a linear response between volume injected and ACN53 peak area recovered (data not shown). Elution of a DEAE column by introduction of a linear salt gradient gave three major peaks. The first of these was a protein peak with a $A_{260}/A_{280}$ ratio of $\sim 0.5$. Next was the ACN53 peak ($A_{260}/A_{280} = 1.23$) followed by a DNA ($A_{260}/A_{280} = 2$) peak at the end of the gradient. This holds true whether run in a HEPES or a Tris buffer system. If run at pH 7.5, less contaminating material bound to the column; pretreatment of lysate with $\beta$-cyclodextrin removed additional contaminants. Further studies on DEAE chromatography buffer sys-

tems showed that 2% sucrose and 2 m*M* $MgCl_2$ (which have been implicated in other studies in our laboratory for optimal activity; data not shown) did not interfere with the chromatography. Chromatography in phosphate buffers gave poor yields and less well-resolved peaks (consistent with Philipson, 1960). HEPES pH 7.5, NaCl, sucrose, and $MgCl_2$ was selected as the buffer system. DEAE chromatography yielded a high degree of initial purification. Immunochemical analysis of a DEAE fraction pools using polyclonal antibodies directed against ATCC-293 cell components revealed contaminating host cell proteins present. IZAC purification of the DEAE pool removed these contaminants.

The behavior of ACN53 on a size-exclusion column was explored with the goal of taking advantage of the large size of virus particles. The molecular weight of a virus particle can be estimated to be approximately $2 \times 10^8$ daltons with a diameter of 70–90 nm (Green *et al.*, 1967; Van der Eb *et al.*, 1969). The expectation was that this technique could separate mature virions from proteins and DNA fragments. Unassembled or immature virions should elute differently because of their less compact architecture. Most of the literature available concerning the use of chromatography for the purification of virus focuses on size-exclusion techniques (Hjorth and Moreno-Lopez, 1982; Hewish and Shukla, 1983; Olivon *et al.*, 1986; Albrechtson and Heide, 1990; Crooks *et al.*, 1990). We found several disadvantages involved with the use of size-exclusion chromatography for the purification of virions. Most commercially available resins are intended for separation of proteins of less that $10^6$ daltons or for desalting/buffer exchange applications. It may be possible to utilize size exclusion by collecting ACN53 in the excluded frontal peak and the lower-molecular-weight contaminants in later fractions. Another concern is the injection volume constraint of a sizing column and the need to concentrate virus prior to injection onto the column. The use of a size-exclusion column increased the process time by introducing the need for sample concentration steps prior to injection and after product collection.

The positive attributes of butyl-HIC chromatography were that it could purify a DEAE fraction pool to an $A_{260}/A_{280}$ nm ratio of 1.2–1.25 without any sample conditioning other than the addition of ammonium sulfate. Butyl-HIC chromatography exhibited good resolving power and could be run in Tris pH 8 or HEPES pH 7.5. Product yield, however, was low. Chromatograms obtained by butyl-HIC revealed several peaks, more than should have been present given the relative purity of the DEAE-derived starting material. Either DEAE copurified substantial amounts of DNA and protein fragments along with viral particles, or butyl-HIC chromatography was disrupting viral particles. SDS-PAGE analysis of HIC fractions compared with those of DEAE purified virus did not show an excess of proteins. Explanations for this phenomenom could be that the short time in which column-bound ACN53 is subjected to a change in osmotic pressure from 1.5 to 0 *M* ammonium sulfate causes a disruption of the virion, or that the virions were denatured upon elution due to selective desorption of pieces of the virion as opposed to desorption of the entire particle. HIC did show promise, but needed further development work to resolve the yield and degradation issues.

Immobilized zinc affinity chromatography had several advantages over both size-exclusion chromatography and HIC for purification of ACN53: IZAC gave higher product recovery and did not require sample manipulation of the DEAE fraction pool prior to loading. Impurities removed by this method eluted in the flowthrough peak and were well resolved from product, leading to simpler pooling criteria. Another advantage of IZAC was that it could be used to reduce the salt concentration of the DEAE pool in a slow, controlled fashion to minimize product loss through osmotic shock damage. IZAC was found to be reproducible, and when used in conjunction with DEAE provided a two-column purification protocol capable of delivering pure ACN53 as specified by SDS-PAGE gels and Westerns, $A_{260}/A_{280}$ ratios, and total virus particle-to-infectious virus particle ratios. Stepwise and overall recovery in terms of total virus particles and infectious virus particles are summarized in Tables 1 and 2.

The stepwise recoveries from the DEAE and IZAC ranged from 44 to 67%. The loss of virus after each step could be attributed to two main factors. First, there was selective pooling of the column fractions. A greater source of loss was the physical trapping of virus in the column resin itself. Virus particles are large enough to be trapped within the resin pores. When the column was cleaned with NaOH, the particles were dissolved and eluted in a degraded form. In optimizing a chromatographic protocol for the purification of virus, column resins must be chosen and tested for the effectiveness of separation due to their chemical functionalities as well as recovery due to their physical architecture.

Analysis of the biological activities of DEAE-IZAC-ACN53 demonstrated that chromatographically produced ACN53 is equal to or better than CsCl-ACN53 in terms of purity and activity. The chromatographic procedure takes less than a day to perform. The ultracentrifugation protocol requires 3 days. A major advantage to the chromatographic procedure is that it can be scaled up using standard, automation-capable procedures, and process-scale chromatography equipment. The CsCl purification method, in contrast, is limited by the capacity of laboratory centrifuges.

The preferred process for the purification of ACN53 is outlined in Fig. 9. Infected cell lysate is treated with nuclease prior to the chromatographic steps. Clarification is then accomplished by step filtration through 0.8-$\mu$m followed by 0.2-$\mu$m membranes. If necessary, a larger-pore (*e.g.*, 5 $\mu$m) prefiltration step can be added for more viscous suspensions. Adjustment to pH 7.5/300 m*M* NaCl is then performed in preparation for loading onto a DEAE column. The product peak, as detected by the $A_{260}/A_{280}$-nm ratio or the characteristic photo-diode array spectrum, is pooled and directly injected onto a zinc-charged, isoosmotically equilibrated metal affinity column. The ionic strength of the buffer is then gradually lowered to approximate phospate-buffered saline (~150 m*M* NaCl) prior to elution of product with a glycine gradient. This material is then dialyzed into the final formulation.

The use of chromatography for the purification of recombinant adenoviruses for use in gene therapies provides an effective alternative to cesium chloride density gradient ultracentrifugations. There are several advantages related to this methodology, including quality, consistency, decreased process time, system automation, and the ability to process large

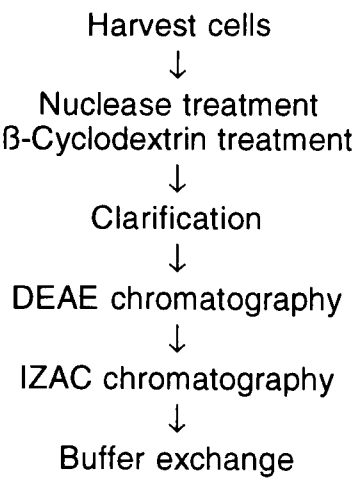


**FIG. 9.** Process flow diagram for the purification of ACN53. A scalable process for the purification of adenoviral vectors is presented. Details of the process are presented in the Materials and Methods section.

amounts of crude lysate. This method can also be used to purify other recombinant Ad 5. The purification scheme developed specifically for our ACN53 vector selects for product based on the surface characteristics of the virion. These characteristics should not change with different internal DNA constructs, leading to similar chromatographic behaviors independent of the target gene inserted inside the vector. We have begun preliminary investigations into the purification of recombinant Ad 5 vectors containing genes other than human p53 and have found them to behave the same as ACN53.

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