AD-A139 668 COMPARATIVE STUDIES ON THE STRUCTURE OF HUMAN ADENOVIRUS GENOMES 4 7 AND 21(U) MARYLAND UNIV BALTIMORE DEPT OF BIOLOGICAL CHEMISTRY R PADMANABHAN

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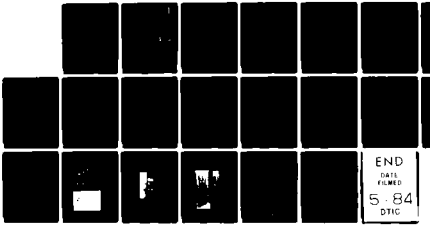

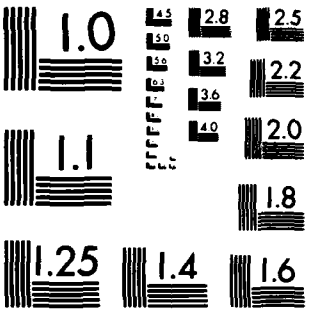

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"Comparative Studies on the Structure of Human Adenovirus Genomes 4, 7 and 21"

Annual Progress Report

by

Radhakrishnan Padmanabhan, Ph.D.

February 1, 1980
(for the period July 1, 1979 to January 20, 1980

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1. Abstract

Adenovirus Type 7 (Greider Strain) was grown in human KB cells. The virions were purified by CsCl equilibrium centrifugation, the DNA was extracted and purified. The viral genome was analyzed by restriction endonucleases EcoRI, BamHI, SmaI, HpaI, BclI, BstEII and KpnI. The restriction endonuclease patterns of the viral DNA was compared with those of three Ad 7 subgroups published by Wadell and Varsanyi (14). Patterns we obtained by cleaving Ad 7 with SalI, HpaI, BamHI and SmaI were identical to those obtained with strain 1058 and strain 55142 (Ad 7 vaccine strain) reported by Wadell and Varsanyi (14). However, EcoRI pattern of Ad 7 (Greider) was different from strain 1058 and 55142 in that one site mapped at 85.4 units from the left end of the DNA is missing.

DNA sequence at the inverted terminal repetition of Ad 7 DNA was analyzed in order to compare Ad 7 (Greider) with Ad 3 DNA, another member of "weakly oncogenic" group (B). Ad 2 (and Ad 5) member of non-oncogenic group (C) and with Ad 12, member of "highly oncogenic" group (A) of human adenoviruses. Our results indicate that the length of the inverted terminal repetition of Ad 7 (Greider) is identical to Ad 3 but different from Ad 2 (or Ad 5) and Ad 12 (or Ad 18). The nucleotide sequence homology in this unique region between Ad 7 and Ad 3 is 95%.

We purified the Ad 7 DNA-protein complex to establish conditions for DNA-transfection in Human Embryonic Kidney cells in vitro. We labeled the protein moiety with ^{125}I on tyrosine residues. We discovered that tyrosine-labeled peptide was still found to be covalently attached to the DNA even after pronase and protease K treatments. This finding enabled us to develop a highly sensitive method to label DNA from human adenoviruses at their termini and physically map the viral genome with several restriction endonucleases. The use of this method in physical mapping is demonstrated by constructing the physical map of Ad 7 (Greider) cleaved with BclI.

2. General Introduction

Human adenoviruses have been classified under DNA Tumor Virus group since they produce tumor in newborn hamsters. However, there is no evidence so far to link them as causative agents in human cancers. All human adenoviruses, except probably Ad 4 (since the data for Ad 4 is lacking), can transform rat embryo cells in vitro and the transformed cells are tumorigenic when injected to newborn hamsters or rats. All early experiments on in vitro transformation were conducted with cells of non-human origin such as rat embryo, and the difficulty of using human cells was due to the permissiveness of the cell for the replication of the virus. However, it was shown by Graham et al. (1) that sheared Ad 5 DNA could transform human embryonic kidney cells in vitro. The resulting transformed cells (293 cells) are now used for isolating mutants of Ad 5 defective in transformation (2). Integrated genes from the left-end of Ad 5 DNA present in 293 cells are able to complement the defective genes for transformation in the mutant. Such mutants could not replicate in HeLa or human KB cells.

Due to the involvement of human adenoviruses 4, 7 and 21 in the Acute Respiratory Diseases in military recruits and civilian populations, live vaccines against these viruses have been developed (3,4) which seemed to control this disease.

The central goal of the original research proposal is (1) to study the genome structure of these viruses which have been developed as vaccines (Ad 4, 7 and 21), using several restriction enzymes. The data will facilitate our future investigation of the physical organization of various genes, controlling the important functions such as DNA re-

plication, transformation and perhaps oncogenicity, the region coding for the synthesis of viral type-specific and group-specific antigens in the life cycle of the virus (2). We propose to generate mutants of adenovirus which are defective in transformation and oncogenicity. The need for such studies are re-emphasized due to recent reports in other laboratories that (1) sheared Ad 5 which are defective in its infectivity can transform human embryonic kidney cells in vitro (1) due to the preservation of the functional genes essential for transformation in the sheared DNA (since shearing is only a random process) (2) the region essential for transformation has been localized on the Ad genome to be at the left-end of the DNA between 4-16%. This part of the viral DNA gets integrated into the host genome during cell transformation and the cells are permanently transformed. (3) Using Ad 5-transformed human embryonic kidney cells as host, it has been possible to produce transformation-defective Ad 5 mutants by in vitro manupulation of Ad 5 DNA. These studies raised new questions such as whether Ad 5-transformed HEK cells could function as host for transformation defective, Ad 7 or Ad 21 mutants; whether these mutants are oncogenic in newborn hamsters.

In the past six months progress is made in the following aspects of structure and functional relationship of Ad 7 genome:

(1) Identification of a peptide linked covalently to the 5' termini of Ad 7 (weakly oncogenic) which seems to be common to Ad 2 (non-oncogenic) genome. This observation formed the basis of the development of a simple and sensitive method for physical mapping of adenovirus genome with restriction enzymes.

(2) Identification of an unique 14-nucleotide long homologous region near the termini of all adenovirus DNAs so far examined. Since all Ads replicate in the same host, this unique sequence may play a role in the infectivity of the virus. Experiments are in progress to check this possibility.

(3) Physical mapping of Ad 7 (Greider) strain using the sensitive method developed in our laboratory and comparison of the map with those of three Ad 7 subtypes (including vaccine strain 55142) mapped by Wadell and Varsanyi.

(4) Standardize conditions for measuring the infectivity titer of Ad 2 by plaque assay which will enable us to assay the infectivity of the live virus in the vaccine tablets.

(5) We chose to work with adenovirus type 7 (Greider) due to (1) its rapid growth in high yield in human KB cells in suspension cultures, (2) any methodology or information derived from such studies could readily be applied to other studies.

(6) We have recently obtained a virus stock from Ad 7 vaccine tablet through one passage in HEK cells (transformed by sheared Ad 5 DNA; also called 293 cells). This stock seems to have a low titer as it did not produce visible virus band in CsCl gradient when a suspension culture (500 ml) of human KB cells (1.5×10^8 cells) was infected with this virus stock. Under the same conditions, Ad 7 (Greider) stock we have prepared, yielded a band of virus from which about 200 μg of DNA was extracted. Normally, to obtain a good yield of virus, 10 - 25 PFU/cell or 6.9 - 17 $TCID_{50}$ per cell is used. In order to produce the same yield of virus, a vaccine virus stock of at least 1×10^9 $TCID_{50}$ (1.45×10^9 PFU) are to be added to infect 1.5×10^8 human KB cells. The virus released from one enteric-coated vaccine capsule (Ad 7) when suspended in 10 ml of Hank's balanced salt solution has a TCID of 1.6×10^6 (Dr. W. H. Bancroft and Dr. R. M. Scott, personal communication) which is approximately equal to 2×10^6 P.F.U. Hence, this has to be enriched 500 fold by serial passage through human KB, HeLa or HEK cells. We have recently established conditions for plaque assay of human Ad. This would enable us to measure the infectivity of the vaccine stock.

Original contract proposal which has been in effect since July 1, 1979 was awarded for the comparative studies of the genomes of Ads 4, 7 and 21. The personnel supported by the contract, their experience and their efforts expended during the duration

of the first year are given below.

Personnel	Experience	Period supported by contract
Morikazu Shinagawa, D.V.M., Ph.D. Visiting scientist currently Assistant Professor, Obihiro University School of Medicine, Obihiro, Japan.	Virology and Nucleic Acid Structure. Since 1977	July - August (End of his two year stay in this country).
Mrs. Raji Padmanabhan	Tissue culture, Virology and Nucleic Acids Structure. Since 1973.	July - September (joined NCI)
Mr. Igor Roninson, M.S.	Virology and Nucleic Acids For 5 years.	July - September (now a graduate student at M.I.T.)
Mr. Charles Burkaw, B.S.	Learning the techniques in Virology.	December - present
Ms. Monica Buescher, B.S.	First year medical student; three years of experience in Virology & Nucleic Acids at John Hopkins University.	January, 1980 Independent research elective study for credits.
Osamu Tokunaga, M.D., Ph.D.	Tissue culture and tumor biology.	January - present.

An essential equipment needed for the study of the infectivity of the virus and DNA was delivered only in the middle of October and a tissue culture microscope has still not been delivered and I am using the one in the laboratory of Dr. S.E. Polakis in our department.

The progress supported by this contract has resulted in the submission or preparation of the following manuscripts. Five complete sets with original figures are included and less satisfactory photocopies are included for the remainder of the required copies. If additional copies are required, they will be submitted as soon as possible.

MANUSCRIPTS SUBMITTED OR IN PREPARATION

(Since July 1, 1979).

1. Roninson, I. and Padmanabhan, R. (1980) Studies on the nature of the linkage between the terminal protein and the adenovirus DNA. Submitted to J. Biol. Chem.

2. Shinagawa, M. and Padmanabhan, R. (1980) Sequence analysis of the inverted terminal repetitions from A and B groups of human adenoviruses. Manuscript in preparation.

3. Buescher, M., Roninson, I. and Padmanabhan, R. (1980) Physical mapping of adenovirus genome by a novel terminal labeling method. Manuscript in preparation.

3. Isolation and Characterization of Adenovirus Type 7 Terminal Protein Complex

Adenovirus 2 and 5 DNAs have been reported to be covalently associated with a protein of molecular weight 55,000 daltons when extracted from the virions without the use of pronase, SDS and phenol (5-8). This DNA-protein complex has been shown to be about 15 fold more infective than the pronase and SDS treated Ad DNA (9,10). Therefore, we proposed to carry out DNA-transfection experiments using Ad DNA-protein complex. In the original research proposal, we outlined our plan to generate interserotypic recombinant strains in which the left end of oncogenic serotype to be replaced by the corresponding region from the non-oncogenic serotype. In these experiments, protein bound terminal fragments can orient themselves in only one possible way in the reaction catalyzed by DNA ligase (see also Section 5 of METHODS OF PROCEDURE in the renewal application). The prerequisite to carry out these experiments is to isolate Ad 7 DNA-protein complex and measure its infectivity in 293 cells.

Methodology

Ad 7 (Greider) was grown in spinner cultures of human KB cells and the virus was isolated and purified on CsCl gradients. The DNA-protein complex was isolated and purified on sucrose density gradients in the presence of 4 M guanidine hydrochloride (5,7). A typical separation of DNA-protein complex grom the virion proteins is shown in Fig. 1.

Results

The covalent nature of the protein attached to the DNA was tested by labeling the protein moiety with [^{125}I] using chloramine T as the oxidizing agent (11). This procedure labels the tyrosine residue of the protein (12). The [^{125}I] labeled DNA-protein complex was then electrophoresced on a 1.4% agarose gel in the presence of 0.1% SDS (13). The DNA was visualized by ethidium bromide fluorescence and the protein moiety was visualized by autoradiography. Fig. 2a & b shows that DNA as seen by ethidium bromide fluorescence (2a) is associated with [^{125}I] radioactivity (2b) and that this complex is not dissociated under the conditions of SDS-electrophoresis. These results are consistent with the covalent nature of the bond between the protein and Ad 7 DNA and are similar to the data obtained with Ad 2 (7,8,13).

Specific labeling with [^{125}I] at the termini of Ad 7 DNA.

The Sucrose gradient purified DNA-protein complex was labeled with [^{125}I] and subsequently treated with protease K. We calculated the percent of radioactivity associated with the DNA, measured as trichloroacetic acid (TCA) - precipitable cpm as well as TCA-soluble cpm. Greater than 90% of the radioactivity was released as TCA-soluble whereas about 10% of the radioactivity was still associated with the DNA. In order to locate [^{125}I] labeled moiety on the Ad 7 DNA and identify its chemical nature, [^{125}I] labeled Ad 7 DNA was cleaved with restriction enzymes BamHI and HpaI. There are 9 cleavage sites on Ad 7 DNA for BamHI and 2 sites for HpaI. These were fractionated on agarose gel (1.4%) electrophoresis. The ethidium bromide fluorescence of DNA fragments were photographed and the gel was subjected to autoradiography. Fig. 3a & c show the ethidium bromide fluorescence of DNA bands and b & d show the autoradiography of the gel. From these data, it is evident that the two terminal (F & J) fragments of BamHI digest and the two terminal A & C of HpaI digest are labeled with [^{125}I]. The physical

map of 8 different strains of Ad 7 using six restriction enzymes which fall into 3 groups was reported by Wadell and Varsanyi (14) (see Section 4). The association of [^{125}I] radioactivity with the terminal fragments of Ad 7 is consistent with the notion that a peptide containing a tyrosine moiety resulting from the protease K treatment of Ad 7 DNA-protein complex is still covalently attached to the termini of Ad 7 DNA.

Since all the human adenovirus serotypes replicate in human KB, HeLa or human embryonic kidney (HEK) cells, it was interesting to see whether DNA from Ad 2 virus (non-oncogenic, group C) has this unique iodinatable tyrosine residue. We did similar experiments of labeling Ad 2 DNA with [^{125}I] and analyzed the purified DNA with restriction enzymes. We found that the two terminal fragments, A & B of SalI (Fig. 3f) and A & B of BamHI (Fig. 3h) were labeled with [^{125}I]. From these results, it was evident that similar type of peptide with an iodinatable tyrosine residue seems to be linked to Ad 2.

In order to prove that [^{125}I] radioactivity is associated with tyrosine moiety of peptide and not the nucleotide moiety of Ad 7 DNA, labeled Ad 7 DNA was hydrolyzed with 2 M HCl and the acid hydrolysate was fractionated by two-dimensional thin-layer chromatography. Fig. 4 shows the autoradiography of the experiment. The dotted outline shows the mobility of authentic unlabeled monoiodotyrosine where the radioactive product from the acid hydrolysate comigrated. Spot X is presumably the partial decomposition product of labeled monoiodotyrosine under the conditions of 2 M acid hydrolysis (110^{o} in sealed glass ampules under nitrogen atmosphere for 24 hours). These data proved that tyrosine residue is indeed the [^{125}I] labeled moiety.

In order to probe into the nature of the linkage between the peptide and Ad 7 DNA, we digested Ad 7 DNA with a variety of nucleases, pancreatic DNAs I which generates 3'-OH and 5'-P ended oligonucleotides, micrococcal nuclease (3'-P and 5'-OH ended oligonucleotides) and single-strand specific S_1 nuclease (3'-OH and 5'-P-ended oligonucleotides). The products were analyzed by one-dimensional electrophoresis at 2400 volts using pH 1.9 buffer system and [^{125}I] was detected by autoradiography. The results are shown in Fig. 5. In this system, oligonucleotides attached to peptide which are generated by pancreatic DNAse I (Fig. 5a) are expected to move towards (+) anode due to the net negative charge due to phosphate groups of the oligonucleotide. However, if the oligonucleotide portion is digested away by an 3'$\rightarrow$5' exonuclease such as snake venom phosphodiesterase, then the peptide moiety due to the net positive charge is expected to move towards the cathode (Fig. 5c). [^{125}I] Ad 7 DNA was denatured by heating at 100^{o}C for 5 min, quick-cooled and subsequently treated with S_1 nuclease. The S_1 digest was analyzed by electrophoresis. Fig. 5b shows that S_1 nuclease cleaves the bond between the peptide and Ad 7 DNA.

The susceptibility of peptide-DNA bond to pancreatic DNAse I, snake venom phosphodiesterase and S_1 nuclease reveals that there should be a phosphodiester bond between the DNA-peptide (see Fig. 6). Treatment of [^{125}I]-Ad 7 DNA with micrococcal nuclease which cleaves to the left of 5' terminal phosphate moiety presumably resulted in the phosphate attached to the peptide (Fig. 5d). Dephosphorylation of the phosphopeptide by treatment with alkaline phosphatase resulted in the positively charged peptide. These data strongly support a phosphodiester type of linkage between the terminal protein and Ad 7 DNA (Fig. 6).

Significance of these studies: All adenovirus serotype seem to have a covalently attached terminal protein (15) which seems to play an important role in infectivity (9.10) and in DNA replication (16). When Ad virus DNA replicative intermediates have been shown to contain this covalently attached terminal protein. It is possible that an enzyme, coded by the viral DNA or a host genome, links the terminal protein to the 5' ends of daughter strands during viral DNA replication.

DNA-terminal protein complex is non-oncogenic in newborn hamsters and non-transforming, whereas removal of the protein by protease treatment attributes to increased oncogenicity of simian adenovirus 7 in newborn hamsters (17). From these data, it seems that the oncogenicity and transformation potential of Ad DNA are inversely proportional to its ability to replicate in either permissive or semi-permissive cells. This notion is supported by the fact that sheared Ad 5 DNA (which lost its ability to replicate into infectitious virion) was able to transform permissive human cells in vitro (1).

Nucleic acid-terminal protein complexes are not unique to adenoviruses, although it was discovered first in this system. Subsequently, Amros and Baltimore (18) and Rothberg et al. (19) independently studied the nature of the linkage and its involvement in the replicative intermediates of poliovirus RNA. Although poliovirus RNA from the virion as well as the newly synthesized nascent poliovirus (negative strand)→RNA molecules have been shown to contain the terminal protein of molecular weight 15,000 daltons, poliovirus mRNA lacks the terminal protein. An enzyme has been purified by Ambros and Baltimore which cleaves the poliovirus-terminal protein bond in the infected cells which seems to be a host enzyme (20). This might be a mechanism by which poliovirus RNA serves as a mRNA in the infected cells.

4. Physical Mapping of Ad 7 Genome (Greider) with Several Restriction Enzymes.

In Section 3, I described a novel method of terminal labelling which seems to be applicable to all human Ad serotypes due to a protein covalently attached to the genome in the virion (1,3,4). Subsequent to protease treatment, a short peptide (the length of this peptide is unknown) containing a tyrosine residue still attached covalently to the DNA. We labeled this tyrosine moiety with [^{125}I] and we obtained a labeled DNA with a specific activity of about 3×10^5 cpm/ μg. The advantages of this terminal labeling applicable to Ad serotypes are: (1) conventional labeling of 5' termini of a DNA involves the use of enzymes, bacterial alkaline phosphatase to dephosphorylate the 5' phosphate residues and then treat with polynucleotide kinase in the presence of [γ -^{32}P] ATP. This method is not applicable to human Ad DNA due to a block resulting from a covalent attachment of a peptide at the 5' termini (7,8,21). Alternate labeling methods depend on enzymes which catalyze the incorporation of [^{32}P] labeled nucleotides at the 3' termini such as deoxynucleotidyl terminal transferase, T_4 polymerase and DNA polymerase I. Labeling the tyrosine residue of the peptide with [^{125}I] by a chemical reaction is rapid and simple (and inexpensive) compared to the conventional methods although this method is applicable only to 5' termini of native Ad genome. [^{125}I] labeling of the termini of Ad genome is especially useful and highly sensitive to physically map the terminal restriction enzyme fragments of Ad DNA, as well as the ordering of the cleavage sites of restriction enzymes from the labeled terminus.

The original proposal was based on the observations of Wadell and Varsanyi (14) who showed that cleavage patterns of 8 different strains of Ad 7 fall into three groups (Table I). We analyzed the cleavage patterns of Ad 7 (Greider) and compared our patterns with those of Wadell and Varsanyi (14). Fig. 8 shows the cleavage patterns of [^{125}I]-Ad 7 (Greider) with EcoRI, BstEII, BclI, KpnI, SmaI and BamHI, SalI and HpaI. EcoRI and HindIII of Ad 7 (Greider) has been published (22) and it is identical to Ad 7-strain 1058, 55142 (Ad 7a subtype) and 879 (Ad 7b subtype) (see Table I). SmaI and BamHI cleavage patterns of the three groups (Table I) are characteristically different from each other. When Ad 7 (Greider) was digested with these two enzymes (Fig. 8a, lane 5 and 6), it gave rise to identical pattern characteristic of Ad 7a subtype (1058, 55142). SalI pattern is identical to all three subtypes of Ad 7 (see Fig. 7). Ad 7 (Greider) does not differ from these three types in SalI pattern. HpaI pattern of Ad 7a and Ad 7b types are identical but different from prototype Gomen strain. Cleavage pattern of Ad 7 (Greider) with HpaI resembles Ad 7a and Ad 7b subtypes.

Thus, Ad 7 (Greider) falls into Ad 7a subtype (strains 1058 and 55142) in five out of six restriction enzymes. It diverges from this group only in EcoRI pattern which is identical to Ad 7b subtype. From these data, it becomes clear that one has to analyze the DNAs from different strains using a larger number of restriction enzymes in order to be able to draw a line of demarkation between different subtypes. We are also analyzing Ad 7 (Greider) with restriction enzymes BstEII, BclI, KpnI. Their cleavage patterns are shown in Fig. 8a. The exact location of their cleavage site are being mapped using the newly developed method of terminal labeling (see below).

Fig. 9 shows the autoradiography of [^{125}I] labeled Ad 7 DNA digested completely with EcoRI, BstEII, BclI, KpnI, SmaI and BamHI. The sizes of these terminal fragments were determined using λ-EcoRI and λ-EcoRI + HindIII DNA fragments used as standard molecular weight markers (23). Thus, it was possible to identify the terminal fragments A and D in a particular cleavage pattern consisting of fragments A - H, as in Bcl I pattern (lane 3, Fig. 8a), A being the largest and H being the smallest.

The order of the fragments, A - H, with respect ot the left and right terminal fragments, A and D, respectively, were deduced using the procedure described by Smith and Birnsteil (24). Since BclI gives a simple spectrum of fragments easily separable from each other, we did not attempt to separate the two labeled termini before applying this procedure. Intact [^{125}I] Ad 7 DNA was digested with BclI under conditions which produced partial cleavages by reducing the amount of enzyme and the time of incubation required for a complete cleavage. A simple spectrum of partial digestion products were produced (Fig. 10) but the terminally labeled fragments form a simple overlapping series, all with a common labeled terminus. These were fractionated according to molecular weight by gel electrophoresis and detected by autoradiography and ethidium bromide fluorescence. The relative mobility of each labeled fragment allows determination of its molecular weight by comparison with λ-EcoRI and λ-EcoRI + Hind III digest as molecular weight standards and in turn locates the distance, in base pairs, of the respective restriction sites from the labeled terminus. The order of the fragments and their lengths thus corresponded directly to the order of restriction sites along the DNA molecule.

Table II gives the molecular weight estimates of 8 Bcl I fragments produced in a complete digest and Table III gives the values for the bands detected by ethidium bromide fluorescence and autoradiography in a partial digest.

Ordering of terminally labeled BclI fragments of Ad 7 DNA.

The first labeled fragment that appeared larger than D has a MW of 4×10^6 daltons. It comigrates with C (Table IV & Fig. 10). It can be deduced from the molecular weights of BclI fragments given in Table III that this fragment represents fused fragments D and F in the partial digest $(2.27 + 1.90) \times 10^6$) .

The second labeled fragment from D towards the origin has a MW of 4.8×10^6 daltons. It comigrates with B. This fragment represents fused fragments D + F + H (expected value of MW = 4.96×10^6 daltons).

First labeled fragment that appeared larger than labeled A has a MW of 9.9×10^6. This fragment represents fused fragments A+E (10.25×10^6 MW).

The second labeled band (11×10^6 MW) represents A+E+G, the third band (14×10^6 MW) represents A+E+G+C and the fourth band (17×10^6 MW) represents A+E+G+C+B.

The discrepancy between the values of molecular weight calculated as the sum of the individual fragments and those determined from their relative mobilities on the gel for bands representing A+E+G+C and A+E+G+C+B is due to the fact that values

TABLE 1. *Origin of the eight Ad7 strains and their separation into subtypes based on distinct DNA restriction site pattern*

Strain	Diagnosis	Source
Ad7 prototype		
Gomen	Pharyngitis	G. von Zeipel, Stockholm, Sweden
Ad7a subtype		
1058	Undifferentiated respiratory disease	G. von Zeipel
55142	Vaccine strain	M. Rosenbaum, Rockford, Ill.
1380	Healthy carrier	M. Lagercrantz, Stockholm
Ad7b subtype		
879	Protracted fever, diarrhoea	M. Lagercrantz
952	Protracted fever	M. Lagercrantz
860	Myocarditis	M. Lagercrantz
211	Febrile respiratory disease	M. Rosenbaum

(Copied from Wadell and Varsanyi)
see Ref.14

Table II

Restriction Enzyme Used	Number of Cleavage sites in Ad 7 (Greider) DNA	Sequence Cleaved	Terminal Fragments Left-terminus-Right-terminus
EcoRI	1	G↓AATTC	-
BamHI	9	G↓GATCC	J, F
SalI	2	G↓TCGAC	C, B
HpaI	2	GTT↓AAC	A, C
BclI	7	T↓GATCA	A, D
BstEII	7	CCC↓GGG	A, H
SmaI	7	CCC↓GGG	D, I
KpnI	3	GGTAC↓C	A, B

Table III

BclI Cleavage of Ad 7 DNA

(Complete digestion of [^{125}I] Ad 7 DNA followed by detection of bands using autoradiography and ethidium bromide fluorescence of DNA fragments).

Fragment	Distance (mm)	Relative Mobility+	M.W. x 10^{-6}	% Genome
A*	54	0.276	8.2	33.0
B	83.5	0.426	4.7	18.5
C	94.0	0.480	4.1	16.5
D*	147.0	0.750	2.27	9.1
E	155.0	0.791	2.05	8.2
F	162.0	0.827	1.90	7.6
G	235.0	0.199	0.86	3.5
H	242.0	1.235	0.79	3.2

+Relative mobility is calculated based on the mobility of λ-EcoRI + HindIII-M fragment (23) (M.W. 1.35 x 10^6) taken as 1.0 on the same gel.

*Denotes the labeled band detected by autoradiography due to terminal labelling.

Table IV

BclI Cleavage of Ad 7 DNA

(Partial digestion of [^{125}I] Ad 7 DNA followed by detection of bands using autoradiography and ethidium bromide fluorescence of DNA fragments).

Labeled Fragment detected by Autoradiography	Distance (mm)	Relative Mobility	M.W. x 10^{-6}	Order of Fragments Deduced
*	30.0	0.153	16.0 - 17.0	A+E+G+C+B
*	36.0	0.184	13.5 - 14.0	A+E+G+C
*	44.5	0.227	11.0	A+E+G
*	48.0	0.245	9.9	A+E
A*	56.0	0.286	8.0	A
	68.0	0.347	6.3	
	76.0	0.388	5.4	
B*	84.0	0.429	4.8	D+F+H
C*	96.0	0.490	4.0	D+F
	108.5	0.554	3.5	
	131.0	0.668	2.75	
D*	148.0	0.755	2.25	D
E	157.0	0.801	2.03	
G	236.0	1.204	0.86	

of MW greater than 4×10^6 daltons for λ-EcoRI + HindIII marker DNA fragments did not bear a linear relationship with their relative mobilities in this 0.7% agarose gel. These values have to be re-examined by carrying out the electrophoresis in a lower % gel (0.4 - 0.5%). However, the sequence of fragments from the left terminal A fragment (the orientation of the terminal A & D fragments were determined by digesting the labeled EcoRI A & B fragments with BclI), A+E+G and the sequence from the right-terminal D fragment, D+F+H are unambiguously deduced. The location of the remaining fragments B and C best fits the available data to give rise to the physical map of Ad 7 with BclI as A-E-G-C-B-H-F-D from left to right.

5. DNA Sequence Analysis at the Inverted Terminal Repetition (ITR) of Ad 7 (Greider).

Garon et al. and Wolfson and Dressler (1972) (25, 26) reported a unique form of terminal redundancy in adenovirus DNA molecules. When native adenovirus DNA molecules were denatured and reannealed, single-stranded circular molecules were observed by electron microscopy. This is possible only if the sequences from the 3' and 5' termini of single-stranded are complementary (1, 2....2' 1' where the numbers represent nucleotide sequences on the same strand of DNA). Almost all the DNA molecules from the human adenovirus serotypes so far tested possess this unique property as observed by EM.

Our laboratory has been interested in the comparative sequence analysis at the ITR region of Group A (highly oncogenic), Group B (weakly oncogenic) and Group C (non-oncogenic) Ads during the past several years. The rationale for this is the observations in different laboratories that this region is involved in (1) viral DNA replication and termination and (2) in the infectivity of the viral DNA. The exact mechanism by which ITR is involved in DNA replication is not clear although a model has been proposed (27, 28).

We sequenced the ITR region of Ad 7 (Greider) (Fig. 11, 12) and compared with our previously available data on Ad 2 (non-oncogenic) as well as Ad 12 and Ad 18 (highly oncogenic), and with the data of Pettersson on Ad 3 (another member of Group B Ads) and simian Ad 7 (29). Our data show that (1) the DNA sequence at the ITR of Ads in a particular group are either identical (Ad 2 and Ad 5) or highly homologous (2) the length of the ITR of Ads in a particular group is either identical (103 base pairs for Ad 2 or Ad 5 in Group C and 136 base pairs for Ad 3 or 7 in Group B) or nearly identical (162 base pairs for Ad 12 and 165 for Ad 18 in Group A) (3) the length of ITR of Ads increases from 103 to 165 as the oncogenicity of the Ads increases. The significance of this correlation is not clear at present because the role of ITR region in Ads is not known. However, it is interesting to note that the highly oncogenic simian Ad 7 has ITR longer than 160 base pairs (29) consistent with our observation with human Ads. Group B Ads (3, 7) which are weakly oncogenic in newborn hamsters have high degree of homology at ITR region (95%). The sequence of ITR of Ad 21 as well as Ad 4 is unknown at present.

6. Infectivity assays of Adenovirus Strains

It will be extremely useful to measure and quantitate the infectivity of adenovirus as Plaque Forming Units (PFU) for Ad vaccine strains rescued from the tablets. These data will augment the existing methodology of titering the virus by hemagglutination-inhibition assays, the neutralization test, the complement-fixation test, the fluorescent antibody test and the radioimmune assay.

Methodology and Results. We followed the procedure described by Williams (30) using HeLa cells as host in the presence of $MgCl_2$ which seemed to enhance plaquing efficiency. We titered our Ad 2 stock in order to standardize the conditions for the plaque assay. We are now currently titering the Ad 7 and Ad 4 vaccine tablets after releasing the virus particles from the enteric coated capsules following the procedure described (31). We are currently testing the plaquing efficiency of adenovirus in HeLa, 293 (HEK-transformed

by Ad 5) and vero cells. Infectivity of Ad 7 DNA isolated in the form of DNA-protein complex is also being studied in 293 cells.

7. Isolation of Vaccine Strains Adenovirus

The enteric coated vaccine tablets of Ad 4 and Ad 7 were obtained from Dr. William H. Bancroft and Dr. Robert M. Scott, Department of Viral Diseases, Walter Reed Army Institute of Research.

The objective of the original proposal is to study the restriction enzyme cleavage maps of the DNA from the vaccine strains and compare them with the established laboratory strains. These data would allow us to investigate whether several passages of a particular strain in the laboratory has resulted in any mutational changes detectable by differences in the restriction enzyme cleavage patterns. The data on two strains of Ad 7, prototype Gomen (32) and Ad 7 (Greider) (33) are available.

Methodology and Results. The virus particles were released from the Ad 7 vaccine tablets following the procedure described (31). It was then passed through HEK-cells (transformed by Ad 5) once and the virus stock showed cytopathic effect (CPE) in both 293 and HeLa cells. However, this stock seems to have a low titer (see Section 2). We are now currently trying to enrich the virus titer by serial passage through human KB cells in spinner culture.

8. Experiments to be Pursued or Completed During the Remainder of the First Contract Year.

A. Experiments to be completed:

1. Physical mapping of Ad 7 (Greider) with restriction enzymes. (1) We are currently mapping Ad 7 (Greider) with the restriction enzymes with SmaI, BamHI BstEII. We have completed the mapping of the genome with SalI, HpaI and BclI. EcoRI and HindIII map of Ad 7 (Greider) is already known (19). Ad 7 vaccine strain obtained after one passage in HEK-transformed by Ad 5 cells will be propagated in human KB cells. The DNA will be isolated and its cleavage patterns with a few representative restriction enzymes, such as SmaI and BamHI will be analyzed.

2. Identification of the amino acid attached covalently to the 5' termini of Ad 7 DNA. In poliovirus RNA, it has been clearly shown by two groups (Ambros and Baltimore, 1978; Rothberg et al. 1978; see Ref. 18 and 19) that tyrosine is linked covalently to the uridine residue via a phosphodiester bond.

In adenovirus system, we have shown that a phosphodiester type of linkage is involved between the protein and Ad 7 DNA. However, we do not yet have any evidence indicating which amino acid residue participates in the formation of the linkage. The tyrosine residue which was iodinatable is probably not linked to the terminal phosphate of Ad 7 DNA. It was reported that a free hydroxyl group is necessary for the incorporation of iodine atom to the phenyl ring of tyrosine (19, 34). Only three possible candidates, tyrosine, serine and threonine, are capable of forming a phosphodiester linkage with Ad 7 DNA. We will label the Ad 7 virus in vivo with ^{32}P and/or using ^{3}H labeled tyrosine, serine and threonine and identify the amino acid to which the terminal phosphate is transferred by micrococcal nuclease digestion. Since the terminal protein is involved in the replication of Ad DNA in human cells, it is possible that same type of linkage is involved in Ad 21 and Ad 4. We will investigate the nature of the linkage in Ad 4 and Ad 21.

B. Experiments to be pursued:

1. Physical mapping of Ad 21 and Ad 4 prototype and vaccine strains. The virions from vaccine strains 4 and 21 as well as prototype strains from American Type Culture Collection will be propagated in human KB cells. The source of Ad 4 strain obtained from ATCC (strain RI-67) was originally isolated from a case of primary atypical pneumonia during a Missouri winter in 1952-53 (Hillenan, M.R. et al., Proc. Soc. Exp. Biol. Med. 85: 183, 1954). The Ad 21 strain obtained from ATCC (Av-1645) was isolated from a conjunctival scrapings from a child with trachoma (Bell, S.D. et al., Amer. J. Trop. Med. Hyg. 9: 523, 1960).

The DNA will be isolated and their restriction enzyme cleavage sites will be mapped using our sensitive in vitro [^{125}I] labeling technique. In this regard, we will investigate the feasibility of labeling the terminal peptide of adenovirus DNA with ^{125}I directly isolated from the infected cells. Viral DNA will be extracted selectively from the cell DNA by a modified method of Hirt (35) and labeled with [^{125}I] for mapping studies. This procedure might cut down the time and the amount of viral DNA required for such studies.

2. DNA sequence analysis at the inverted terminal repetition of Ad 4 and Ad 21. There seems to be a correlation between the length of inverted terminal repetition (ITR) of an Ad serotype and its oncogenicity in newborn hamsters. The ITR is shortest in non-oncogenic (Ad 2 & Ad 5) serotypes (103) and longest in highly oncogenic (Ad 12 & Ad 18) Ads. The length of ITR for the weakly oncogenic group B (Ad 3, 7) Ads falls in the middle (136). ITR of Ad 21 (a group B Ad) is unknown. Ad 4 has been reported to be non-oncogenic, but it is classified into a unique group E from the DNA-DNA homology data (36). We will determine the length of ITR of Ad 4 and Ad 21 by actual DNA sequence analysis.

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FIGURE LEGENDS

FIGURE 1 Purification of Ad7 DNA-Protein complex.

Ad7 DNA-Protein Complex was purified by sedimentation on a gradient of sucrose containing 4M guanidine hydrochloride. The absorbance was measured using a U.V.monitor and a chart recorder.

FIGURE 2 Sodium dodecyl sulfate-agarose gel electrophoresis of Ad7 DNA-Protein Complex labeled with I-125.

The DNA band which migrates into the gel as seen by ethidium bromide flurescence (a) contains protein as seen by I-125 autoradiography (b). O denotes origin.

FIGURE 3 Cleavage of I-125 labeled Ad7 and Ad2 DNA with restriction enzymes.

a-d. Labeled Ad7 DNA was digested with BamHI (a&b) and HpaI (c&d).
e-h. Labeled Ad2 DNA was digested with SalI (e&f) and BamHl (g,h).
The DNA fragments were fractionated on a 1.4% agarose gel and the ethidium bromide flurescence was photographed (a,c,e andg). b,d,f andh represent the autoradiography of I-125 labeled DNA fragments, fractionated on gels a,c,e and g, respectively.

FIGURE 4 Two-dimentional thin layer chromatography.

Ad7 DNA labeled with I-125 was hydrolyzed in the presence of 6N HCl in sealed glass ampules under the atmosphere of nitrogen, at 110°C for 24h. The hydrolysate was chromatographed in two dimensions on cellulose-coated thin layer plate as described by Amrose and Baltimore(18). The position of unlabeled monoiodotyrosine marker was determined by ninhydrin staining and is indicated by dashed outline. The labeled spot X is probably the degradation product of labeled monoiodotyrosine generated during acid hydrolysis.

FIGURE 5 Electrophoretic Separation of I-125 labeled Ad7 DNA treated with various nucleases.
a) DNAseI (Worthington Biochemical Corp.) digestion of Ad7 DNA.
b) Denatured Ad7 DNA after treatment with single-strand specific S nuclease.
c) DNAseI, followed by snakevenom phosphodiestrase 1
d) micrococcal nuclease digestion
e) same as in d, followed by treatment with E.coli alkaline phosphatase.

Electrophoresis was carried out using formic/acetic acid buffer(pH 1.9) system at 2400 volts. At the end of electrophoresis, the paper was dried and autoradiographed.

FIGURE 6 Applications of our finding of a novel method of terminal labeling to 1) study the nature of the linkage between the DNA-protein 2) to physically map the genome with several restriction enzymes

FIGURE 7 Restriction site maps of the genomes of the Ad7 strains Gomen, 1058 and 879, obtained with SalI, EcoRI HpaI, SmaI HindIII and BamHI, by Wadell and Varsanyi(14).

FIGURE 8 Restriction enzyme patterns of Ad7(Greider) obtained with EcoRI, BstEII, BclI(lanes 1-3 from left to right), KpnI(lane5), SmaI(lane 6) and BamHI(lane 7). Lane 4 from the left contained lambda DNA digested with EcoRI used as molecular weight markers.

FIGURE 9 Autoradiography of the gel from FIGURE 8. Only terminal fragments are seen labeled. The terminal fragment H of BstEII digest (shown by arrow) is very faint. The reason why there is differential amount of I-125 label associated with one terminal fragment compared to the other is not well understood. One likely explanation is that there might be a small amount of protease contamination present in a particular restriction enzyme preparation . We are currently in the process of repeating this experiment in the presence of protease inhibitors.

FIGURE 10 Physical Mapping of Ad7 (Greider) with BclI. Ad7 DNA labeled with I-125 was digested with BclI partially and the partial digest was fractionated on a o.7% agarose gel. The molecular weight of each of the labeled bands were calculated using lambda DNA digested with EcoRI or EcoRI and HindIII as standard markers, run on the same gel. a) ethidium bromide flurescence and b) autoradiography of the gel. As shown in b, there are two labeled bands above the terminal fragment D (see FIGURE 9 for the two terminal fragments) and there are four bands above the terminal A fragment. The calculation of their molecular weights and the elucidation of their physical location on Ad7 DNA are given in TableIII.

FIGURE 11 Maxam-Gilbert's DNA sequencing gels. Ad7 DNA labeled at the 3' ends using dGTP(α-^{32}P) and T_4DNA polymerase was subjected to digestion with BamHI and the two terminal fragments F and J were separated on a 1.4% agarose gel. After elution of the two terminally labeled F and J fragments (see FIGURE 7 for the map of Ad7 (strains 1058 and 55142 cleaved with BamHI; our strain of Ad7(Greider) is identical to this pattern) from the gel, they were subjected to DNA sequence analysis by Maxam-Gilbert's procedure. Figure 11a,- 1 and 2 represent the two loadings of chemically degraded F and J fragments, applied on a 25% gel. 11b, BamHI-F fragment from another experiment was applied on a 25% gel. Both a&b give the sequence data starting from the mononucleotide upto nucleotide number 39. 11c, a 80x40x.075cm long gel(10%polyacrylamide) used to fractionate the degradation products. The sequence can be read from nucleotide number 20 (overlapping with the those in gel 11a) and the arrow in the middle lanes shows the end of terminal repetition.

FIGURE 12 Comparative sequence analysis of Inverted terminal repetitions from Ad serotypes. Ad7 DNA which is intermediate in oncogenicity has an inverted terminal repetition of intermediate length.

FIGURE 1

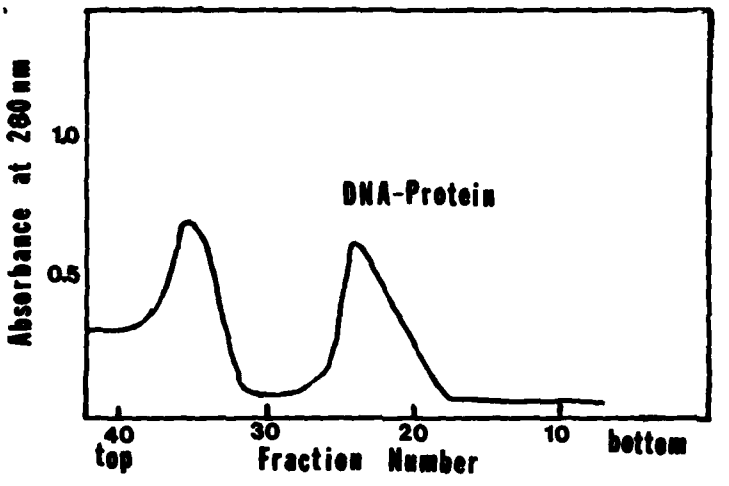


FIGURE 2

FIGURE 3

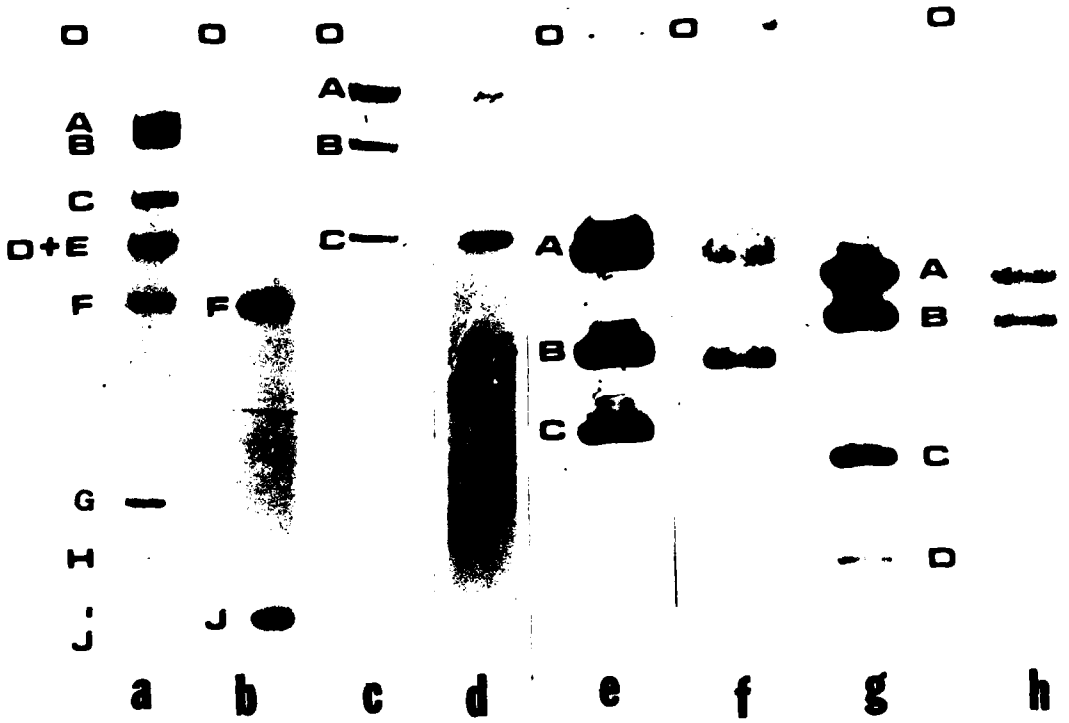


FIGURE 4

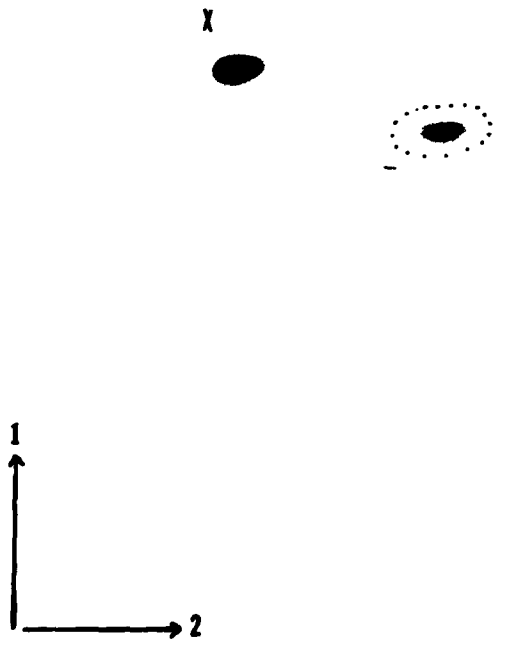


FIGURE 5

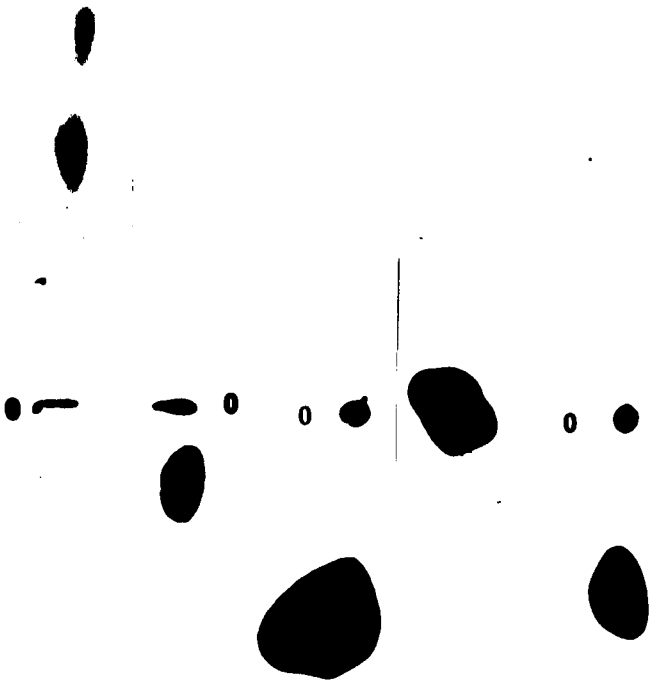


FIGURE 6

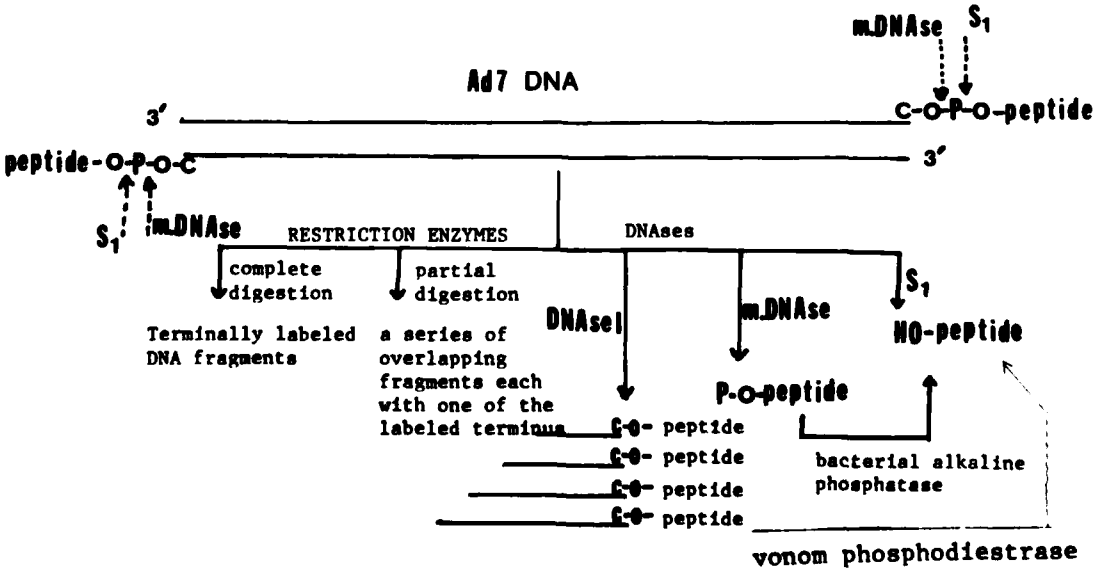
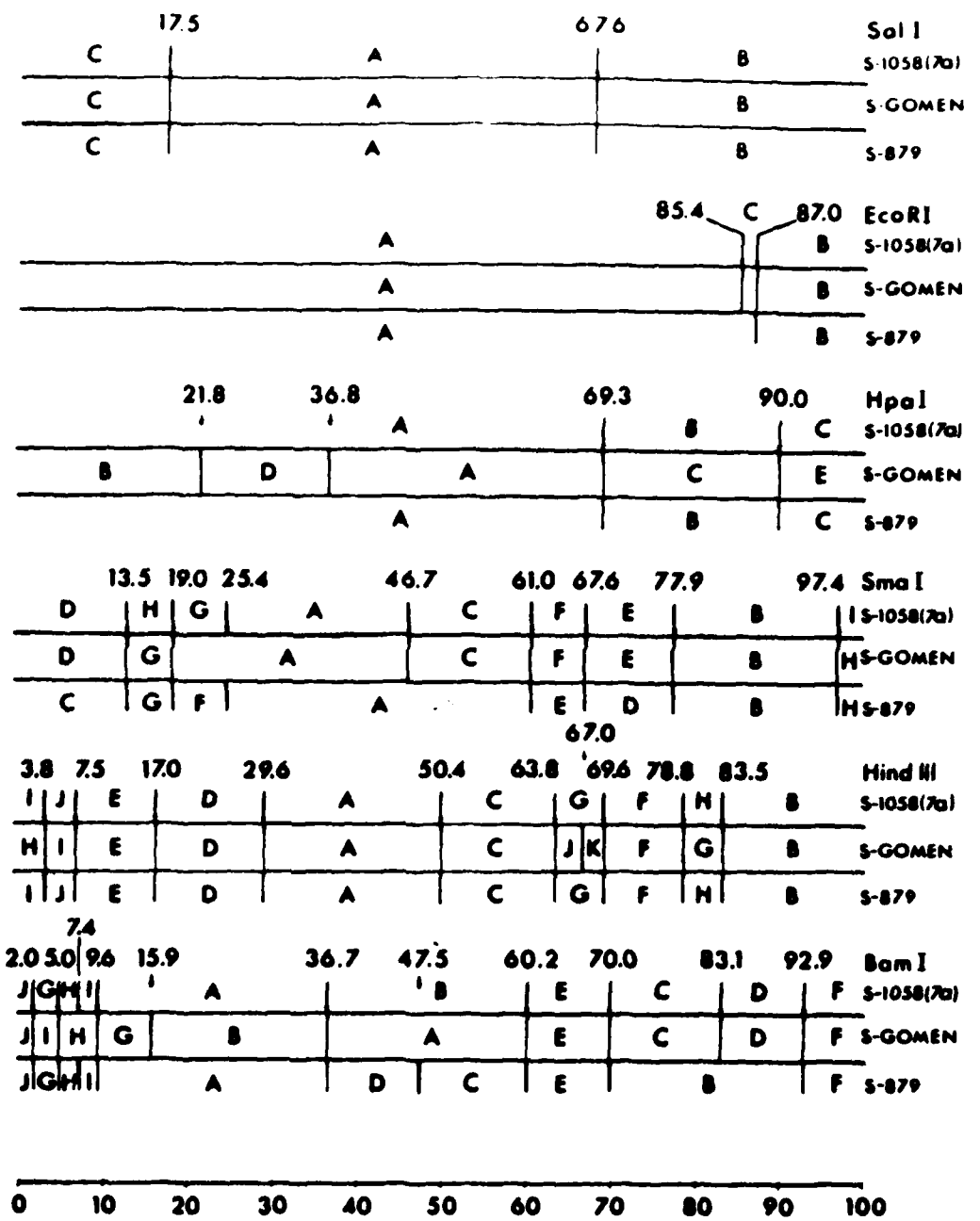


FIGURE 7



Restriction site maps of the genomes of the Ad7 strains Gomen, 1058, and 879, obtained with Sal I, EcoRI, Hpa I, Sma I, HindIII, and Bam I.

FIGURE 8

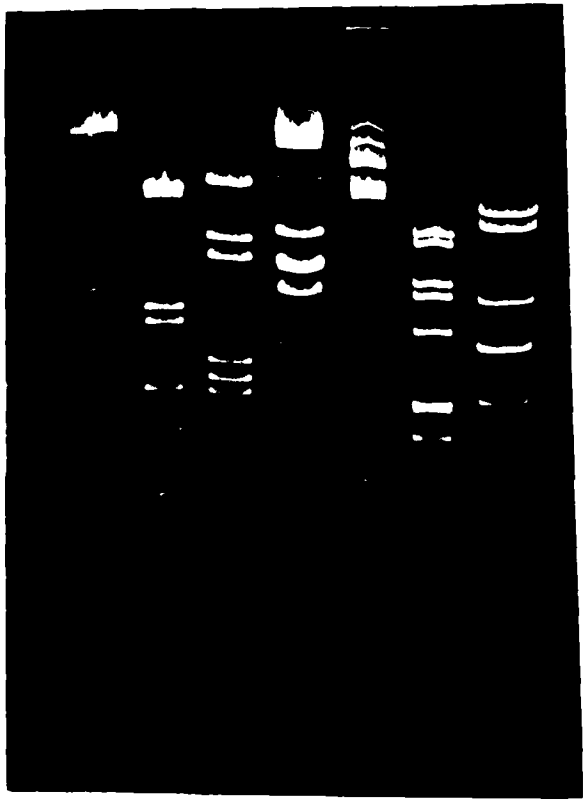


FIGURE 9

FIGURE 10

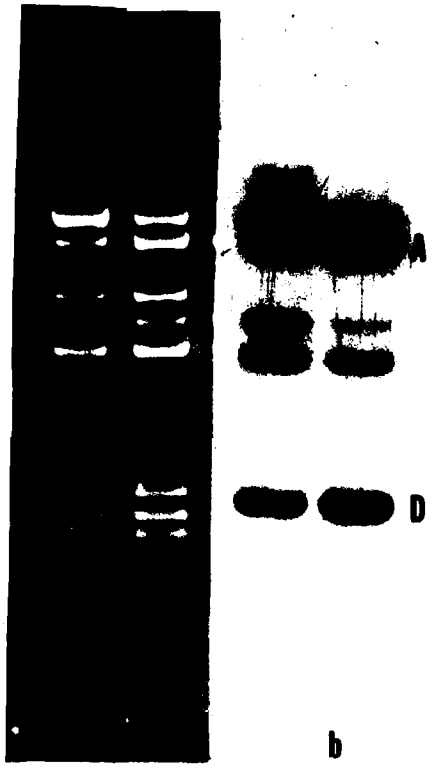


FIGURE 11

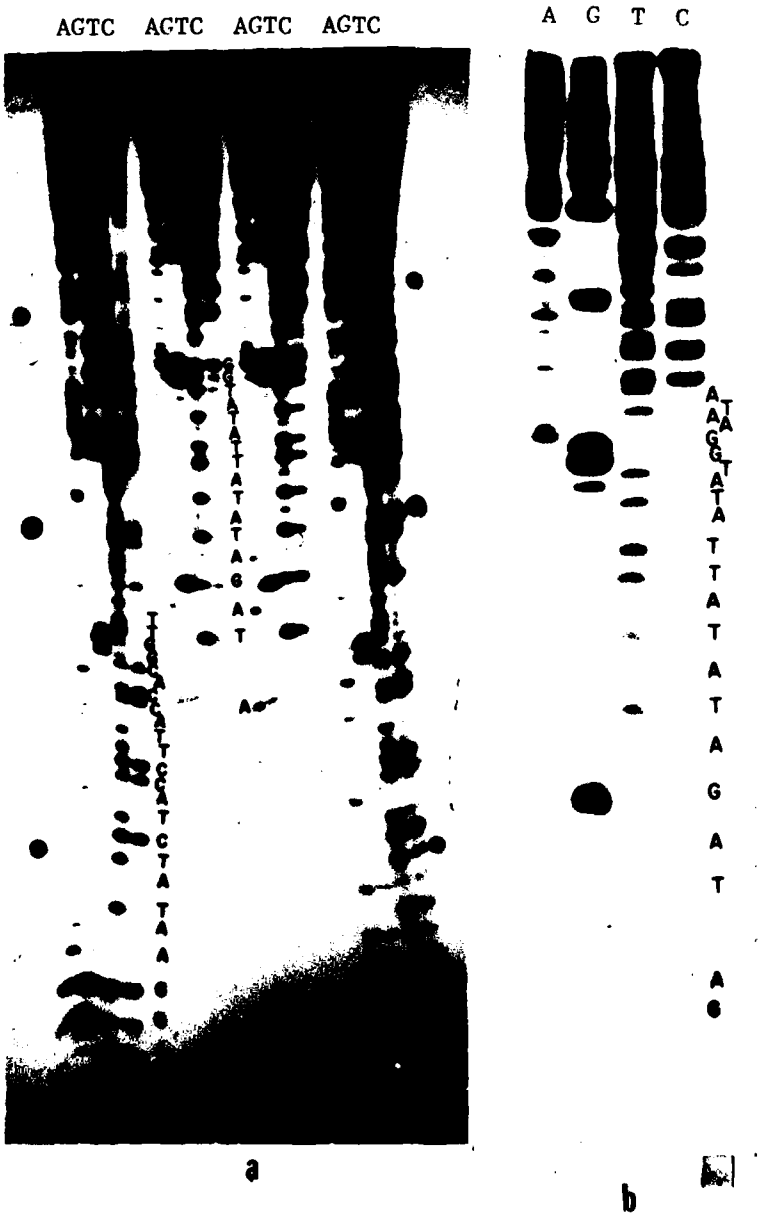


FIGURE 11c

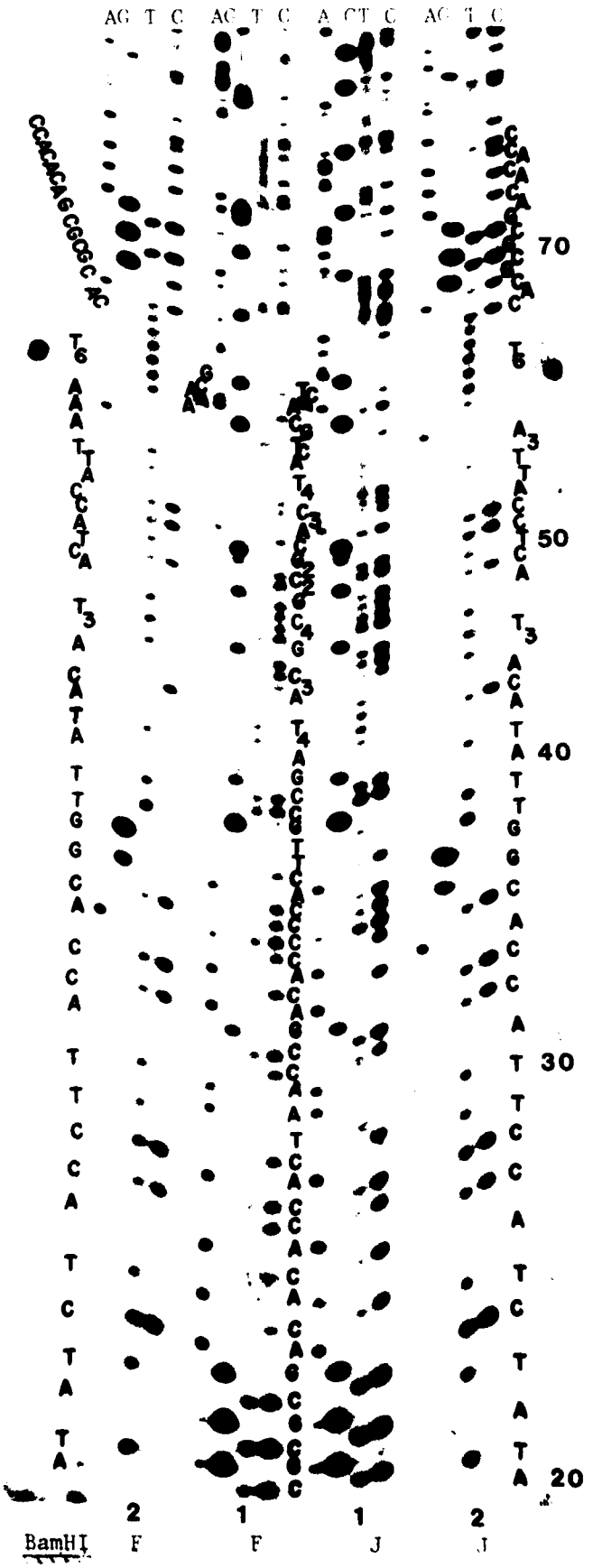
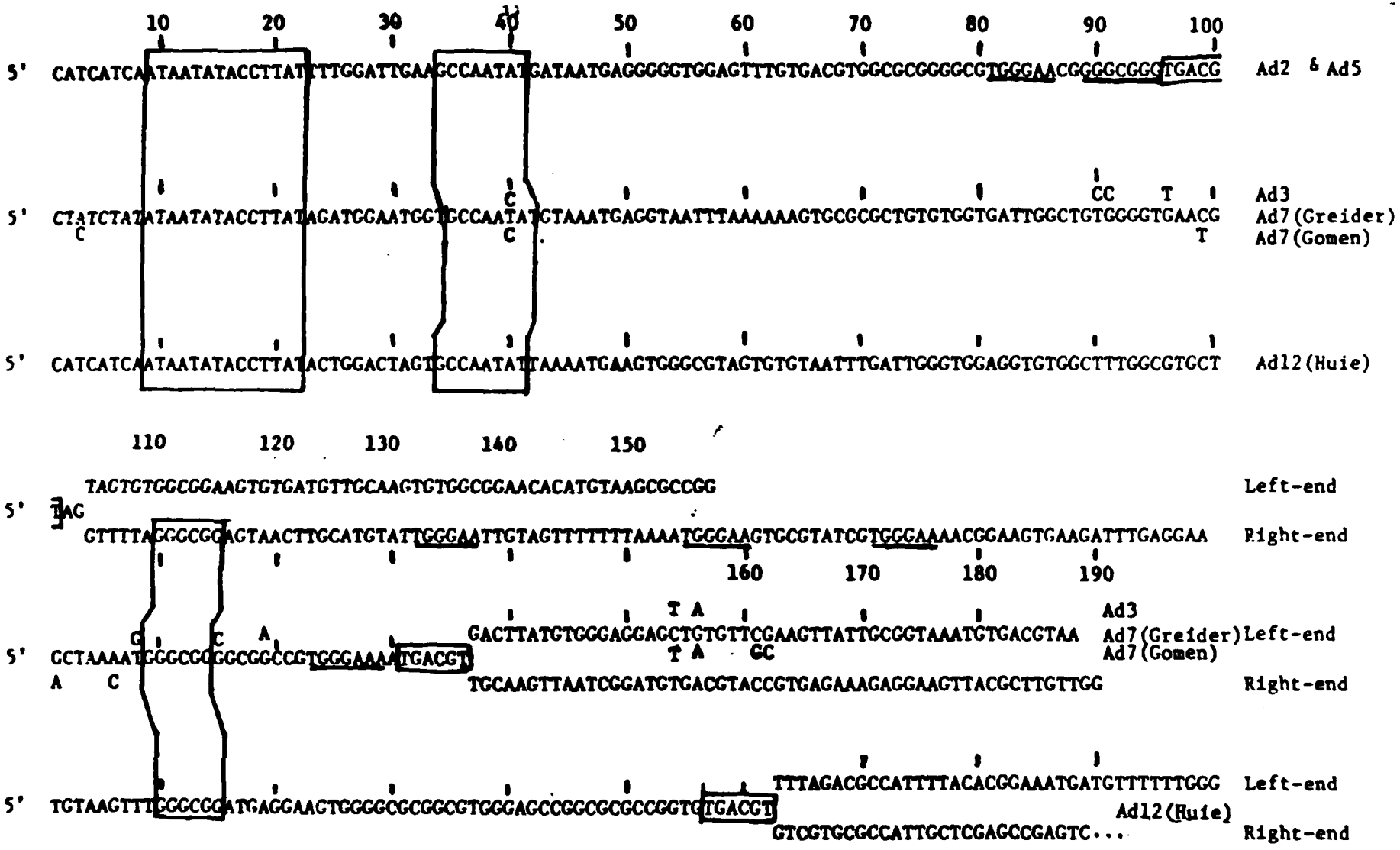


FIGURE 12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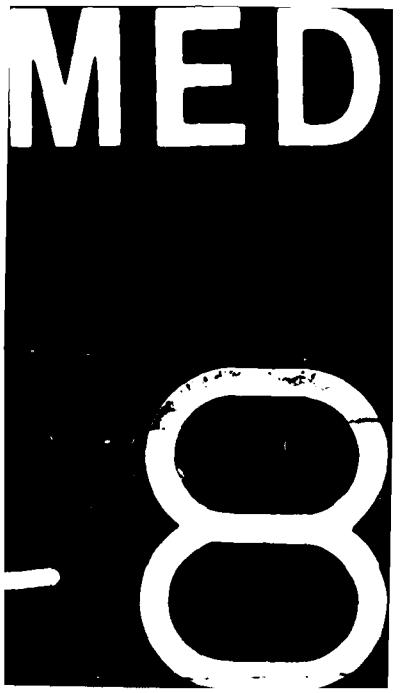
5' CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACG Ad2 & Ad5
5' CTATCTATATAATATACCTTATAGATGGAATGGTGCCAATATGTAAATGAGGTAATTTAAAAAAGTGCGCGCTGTGTGGTGATTGGCTGTGGGGTGAACG Ad3
C C CC T
Ad7(Greider)
T Ad7(Gomen)
5' CATCATCAATAATATACCTTATACTGGACTAGTGCCAATATTAAAATGAAGTGGGCGTAGTGTGTAATTTGATTGGGTGGAGGTGTGGCTTTGGCGTGCT Ad12(Huie)
110 120 130 140 150
TACTGTGGCGGAAGTGTGATGTTGCAAGTGTGGCGGAACACATGTAAGCGCCGG Left-end
5' TAG
GTTTTACGGCGGAGTAACTTGCATGTATTGGGAATTGTAGTTTTTTTAAAATGGGAAGTGCGTATCGTGGGAAAACGGAAGTGAAGATTTGAGGAA Right-end
160 170 180 190
T A Ad3
G C A GACTTATGTGGGAGGAGCTGTGTTCGAAGTTATTGCGGTAAATGTGACGTAA Ad7(Greider) Left-end
5' GCTAAAATGGGCGGGCGGCCGTGGGAAAATGACGT T A GC Ad7(Gomen)
A C TGCAAGTTAATCGGATGTGACGTACCGTGAGAAAGAGGAAGTTACGCTTGTTGG Right-end
TTTAGACGCCATTTTACACGGAAATGATGTTTTTTGGG Left-end
5' TGTAAGTTTGGGCGGATGAGGAAGTGGGGCGCGGCGTGGGAGCCGGCGCGCCGGTGTGACGT Ad12(Huie)
GTCGTGCGCCATTGCTCGAGCCGAGTC... Right-end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8