

**INTEGRATION OF VIRAL DNA SEQUENCES IN INFECTED
AND
TRANSFORMED MAMMALIAN CELLS**

By

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ABSTRACT

Adenovirus infects human KB cells leading to cell lysis and production of progeny virus. It also transforms hamster cells which can induce tumors when injected into hamsters. An experimental system, the DNA-DNA reassociation technique was developed to study the integration of viral DNA sequences in infected and transformed cells. This technique detects less than one viral genome per cell.

The quantity of viral genome in tumor, transformed and lytically infected cells was measured. While multiple copies (up to 12) of viral DNA sequences per cell were found, the representation of viral genome was incomplete in transformed and tumor cells with a tendency for the right hand 45% of the Ad 12 genome being deleted.

The reiteration frequency of KB cellular DNA adjacent to an integrated viral genome was found to be representative of the cellular genome suggesting that in lytic infections, there are no specific sites. It was also found that the number of integrated viral sequences was enhanced by cellular proliferation as well as by blockage of cell DNA repair system with caffeine.

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ABBREVIATIONS USED

MW	Molecular weight
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
O.D.	Optical density
HAP	Hydroxyapatite
PB	Phosphate buffer
PBS	Phosphate buffered saline
TCA	Trichloroacetic acid
Ad	Adenovirus
SSC	Standard saline citrate
HS	Horse serum
FCS	Fetal calf serum
PI	Post infection
S	Svedberg
Krpm	Thousand revolutions per minute
H(h)	Hours
Ic	Incomplete

NOTE IN ADDENDUM

Since this thesis was written, the research group with Doerfler has shown that of the DNA in Ad 2 infected human KB cells, the 34-unit genome length DNA contains all parts of Ad 2. The 50-90S DNA contains the right molecular end in at least 10 to 15-fold greater abundance than the left hand end. The DNA sequence in the >100S fraction contains more of the left end of Ad 2 DNA. For more information readers are to see the article:

Fanning E. and W. Doerfler. (1977). Intracellular forms of adenovirus DNA. VI. Quantitation and characterization of the four size classes of adenovirus type 2 DNA in human KB cells, *Virology*, 81, 433.

INTRODUCTION

Viruses are a class of infectious agents. It has been estimated that many illnesses are the result of viral infections (Horsfall, 1965): Viruses are also unique in their sizes and in being obligatory intracellular parasites.

The smallest virus has a diameter of about 100 \AA ($1 \text{ \AA} = 10^{-10}$ meters) and the largest virus has a diameter of about 3000 \AA . A bacterium is at least ten times bigger than the largest virus.

Unlike cells which possess the potential to grow and divide, viruses depend on entering suitable host cells in order to multiply and produce progenies. The viruses are able to do so because they share the common characteristics of storing and transferring their genetic information in a format similar to that used by the cells.

The study of viruses has engendered tremendous interest. Understanding the biology of viruses would help to understand the diseases which they cause. Viruses can be a tool used to study cellular functions. Furthermore, some viruses can induce experimental cancer in animals. Knowledge acquired in studying this aspect of viral activity can be useful in understanding human neoplasms.

Classification of Viruses (Fenner *et al.*, 1974a)

Viruses are classified according to the type of nucleic acid in which the genetic information is stored. Thus, there are DNA and RNA viruses. They are also classified according to the host which they infect. Thus, there are animal DNA viruses, animal RNA viruses, bacterial and plant viruses. The term 'phage' or 'bacteriophage' refers to bacterial viruses.

Some DNA and RNA viruses can produce tumors in animals, they are collectively called tumor viruses. Whether a virus is a tumor virus or not also depends on the type of cell it infects. When a particular virus enters a cell of an animal normally its host, the end result is usually cell lysis and the release of more new virus particles. Such cells are said to be "permissive", in that they permit the virus to complete its life cycle, and the infection is "productive". When a tumor virus enters a cell which is not its natural host, viral particles often are not produced (the cell is "nonpermissive", the infection "nonproductive" of new virus), and the cell may or may not be transformed to possess tumorigenic potentials. Some viruses can productively infect one type of cell but it also can transform another type of cell. Human adenovirus type 12, for example, infects human cells productively, and transforms hamster cells. It is, therefore, a tumor virus.

RNA tumor viruses are classified into three categories, the A-type, B-type and C-type particles, by virtue of their appearance in the electron microscope. Mature C-type particles have a centrally located electron dense spherical nucleoid enclosed by a thin membrane. All known sarcoma and leukemia virus particles belong to this class (Tooze, 1973d). The B-type particles have a solid spherical nucleoid that is electron dense, and attaches to the inner surface of a thin membrane that encloses it. There are projecting spikes on the outer surface of the thin membrane. Mouse mammary tumor virus (MMTV) is a B-type particle. Immature B-type particles with their toroidal nucleus enclosed in a membrane are called A-type particles. The RNA extracted from RNA tumor viruses contains a fast sedimenting component and a slow sedimenting component, the 60-70S RNA and the 4-5S RNA. Although the amounts of RNA species vary from preparation to preparation, the ratio of 60-70S RNA to 4-5S RNA is about unity.

DNA tumor viruses are of four types. The first is the papilloma group, which infects man (with warts), rabbits, dogs, cattles and other animals. Because of the difficulty of growing it in culture, it has not been extensively studied. The second group, the polyomaviruses, includes the murine polyomavirus and the SV40 virus. These viruses each containing only 10 genes are extensively studied. Recently, SV40-related antigens were detected

in a metastatic human melanoma and in 3 of 7 meningiomas (Green, 1975). The other two groups of DNA tumor viruses are the adenoviruses and the herpesviruses. Of the 31 adenovirus types that infect man productively, 13 are either tumorigenic or can transform hamster cells. Since one member of the adenoviruses is the subject of this thesis, further discussion of this virus will take place later. Of all tumor viruses, herpesvirus is the candidate most likely implicated in human neoplasms. The Epstein-Barr virus, a member of the herpesvirus group which causes infectious mononucleosis, is causally related to Burkitt's lymphomas and to nasopharyngeal carcinoma. One must also consider the seroepidemiologic studies that have implicated human herpesvirus 2 in cervical carcinoma (Naib *et al.*, 1969).

Tumor Viruses

Virus as a causative agent for the induction of tumors was originally based on theoretical speculations by Borrell in 1903. The first demonstration of a virus being implicated in neoplasia - abnormal growth of cells, was in connection with fowl leukosis by Ellerman and Bang in 1908. Peyton Rous (1910, 1911) provided the first proof that a viral agent was capable of inducing solid tumors - sarcomas in chickens. This virus, now known as Rous Sarcoma Virus (RSV), has since proved to induce

tumors in ducks (Fujinami and Suzue, 1928), turkeys, guinea fowls, pigeons and a mammal, rats (Svet-Moldavsky, 1958). In the last seventy years, much evidence has been accumulated which strongly implies a viral etiology in the development of malignancy in subhuman primates, bovines, felines, canines, rodents, domestic fowls, fish and amphibians (Tooze, 1973; see Table 1). Simultaneous presence of viral particle or expression of viral antigens in tumors of these animals had been demonstrated by electron microscopy, immunology and molecular biological techniques. Virus inducing tumor is such an extensive biological phenomenon that if man were to be exempted, it would be a circumstance unparalleled in biology.

Adenovirus (Fenner *et al.*, 1974b,c)

Adenovirus is under study in the present work. This virus group was first discovered in 1953 (Rowe *et al.*, 1953; Hilleman and Werner, 1954). The name was suggested by Enders *et al.* in 1956 to designate this group of viruses isolated from respiratory tracts of man and other animals. While a review of the architecture, composition, cellular response to infection, and assembly and release of adenovirus is beyond the scope of this thesis, a brief mention of some of these aspects is warranted to provide background (in which the present work has been done).

The gross morphology of adenovirus resembles a

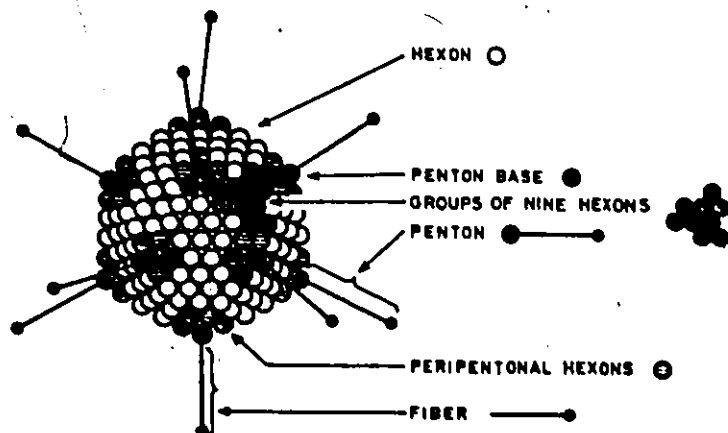
Table 1

A Brief Chronological List of Tumor Virology

<u>Researcher</u>	<u>Date</u>	<u>Events</u>
Ellerman and Bang	1908	Leukemia of chicken
Rous	1911	Chicken sarcomas
Creech	1929	Bovine papilloma
Shope	1932	Rabbit fibroma and papilloma
Lucké	1934	Frog renal carcinoma
Bittner	1936	Mouse adenocarcinoma
Gross	1951	Mouse lymphatic leukemia
Stewart and Eddy	1959	Polyoma virus causing sarcomas and adenomas in mouse, hamster, guinea pig, ferret and rabbit
Sweet and Hilleman	1960	SV40, hamster sarcomas
Trentin	1962	Human adenovirus causing hamster sarcomas
Stehelin <i>et al.</i>	1976	Isolation of cDNA _{sarc} *

* cDNA_{sarc} refers to the DNA sequences complementary to the sarcoma gene of Avian Sarcoma Viruses.

geometric structure called icosahedron. An icosahedron has 20 equilateral triangular faces, 12 vertices when the corners of five triangles meet, and 30 edges where sides of adjacent pairs of triangles meet (Fenner *et al.*, 1974b). There are 252 morphological units called capsomers to make up the icosahedral capsid. The capsomers at the vertices are called pentons. Each penton unit has a rod like projection with a knob attached at the distal end and is referred to as the fiber (Ginsberg *et al.*, 1966; Valentine and Pereira, 1965; Norrby, 1966). The proteins of the virion have been purified and studied by SDS-gel electrophoresis (Maizel *et al.*, 1968a,b; 1971; Everitt *et al.*, 1973; Anderson *et al.*, 1973) and they derived their names according to their positions in the SDS-gel. The following schematic drawing shows the arrangement of some components.



(Tooze, 1973e)

The double-stranded DNA molecule is protected by the capsid (Lawrence and Ginsberg, 1967). The molecular weight of DNA is 23×10^6 Daltons, large enough to have a coding capacity of about 40 proteins. Both strands are capable of forming intrastrand circles held together by hydrogen bonds between inverted terminal repetitions (Garson *et al.*, 1972; Wolfson and Dressler, 1972). Such terminal inverted repetitions have so far only been observed in adenovirus and adeno-associated virus (AAV) and their biological significance is unclear.

Thirty-one serotypes of human adenoviruses are known. Green (1970) has arranged them into groups A, B and C on the basis of homology of their DNA sequences. According to this scheme, group A includes types 12, 18, 31 and they are highly oncogenic in hamsters. Group B includes types 3, 7, 11, 14, 16, 21 and except Ad 11, they are weakly oncogenic. Group C includes types 1, 2, 5, 6 and they are not oncogenic. The virus studied in this thesis is Ad 12.

Rationale for Studying Tumor Viruses

In the long history of man's quest for knowledge about the world he lives in and about himself, he usually started by studying simple systems, extrapolated his knowledge from simple systems to more complex systems and formulated his approach to the more complex systems. This has also been the approach taken to understand

cellular mechanisms. The bacterial system was studied extensively. Much information about gene regulation in bacteria holds true for higher organisms as well. These include: storage of genetic information in codes in DNA; flow of information from DNA to protein via transcription into RNA and translation into protein; the energy-transfer pathways, to cite just a few. However, one does not expect that as rule, bacterial systems and mammalian systems are similar in their genetic organization and expression. Already, results show that Ad 2 mRNA contain nucleotide sequences which are coded remote from the DNA that codes the main sequences of mRNA (Chow *et al.*, 1977; Klessig, 1977; Dunn and Hassell, 1977). This suggests a new mechanism of transcription which is incompatible with the "independent promoter for each mRNA" scheme of the bacterial system (Losick and Chamberlin, 1976). Since mammalian viruses share many enzyme systems of the cell, it is likely that their modes of genetic expression would be similar to that of the cell. The study of the genetic expression of animal cells has so far been difficult because the genetic composition of animal cells is 10^3 times larger than that of bacteria. We may now circumvent this difficulty by studying viruses which are simpler than bacterial systems.

One can also study how tumor viruses disrupt normal cell functions. One mode of interaction between viral

cellular genomes is the insertion of the viral genome into the cellular genome. In the bacterial system, it has been shown that integration of the bacterial virus; phage Mu DNA might produce mutation if it is located in a position which destroys the continuity of a functional gene (Taylor, 1963; Boram and Abelson, 1971). Transcription of host DNA is altered after phage DNA integration with "reading through" from the phage DNA into the bacterial DNA (Yarmolinski, 1971). Messenger RNA containing viral and cellular information had also been observed in infected animal cells (Tonegawa, 1970). One would like to know more about how much of the viral genome is integrated in productive infection and also in transformation. The questions one is interested in asking would be: in what quantities they are integrated? are certain regions preferentially integrated? what conditions favor integration? what are the sites of integration?

To answer the above questions pertaining to Ad 12 infection, the amount of intracellular and integrated viral DNA was quantitated in the case of productively infected cells, in transformed cells and in cells isolated from Ad 12 induced tumors. The case of non-permissive cells was not studied because of technical limitations. Conditions which alter the level of integration were studied. The sites on the host chromosome adjacent to the integrated viral genome were characterized.

Adenovirus and Cell Interaction

Burger and Doerfler (1974) reported the integration of Ad 2 DNA into permissively infected KB cell DNA. This is consistent with earlier reports of integration of SV40 DNA into permissive monkey kidney cells (Hirai and Defendi, 1974a). The phenomenon of integration has also been investigated in the case of infection of non-permissive cells. By using radioactively labelled virus and substituting thymine in the cell DNA with 5-BudR so that its density was higher than virus DNA density, Doerfler (1968) found that infecting Ad 12 viral DNA was covalently linked to BHK21 cell DNA. Despite the challenge from zur Hausen and Sokol (1969) who reported that a significant amount of viral DNA was degraded and reutilized by hamster cells in a different cell line (Nil-2), Doerfler subsequently substantiated his claims (Doerfler, 1969; 1970; Doerfler *et al.*, 1973). The amount of adenovirus genome in tumor cells, however, has never been quantitated, although much work has been done on transformed cells. Part of the difficulty was in separating tumor cells from the tumors. Cells in a tumor may have several origins. By quantitating viral DNA in these cells, one is measuring the amount of viral DNA averaged over all the cells in the tumor. This approach does not yield information about one particular cell line. It gives information about all the cells. If a certain fragment of the viral genome is missing, then one

can safely conclude that not the entire viral genome is essential for the maintenance of the tumorigenic condition. The alternative approach would be to pick clones for the cells from the tumor and quantitate for viral genomes in each cell line derived from the clones. The objection to this approach is that *in vitro* growth is highly selective. The cells that pass this selectivity may not necessarily be the cells that represent the majority population of cells in the tumor. There are examples of cancer cells failing to grow *in vitro* as well (Shields, 1976).

A third approach is to transform cells with viruses. About 10 changes in cellular properties were noted following transformation but not all of these cell properties are altered in different cell types transformed by different viruses. Generally, a cell is changed morphologically, and antigenically; possesses integrated viral genes, and synthesizes viral mRNA and proteins. A surface change may be measured by agglutinating with plant lectins. Usually, *in vitro* transformed cells lose their density-dependent inhibition of growth. The limitation in studying transformed cells is that they do not necessarily proliferate into tumors when injected into animals. In some instances, manipulation of host immune system is required to allow these *in vitro* transformed cells to grow in animals. For example, primary rat embryo cells transformed by Ad 1 (McAllister *et al.*, 1969) and Ad 12 (Freeman *et al.*, 1967)

do not induce tumors in rats. McAllister (1969) suggested that Ad 1 and Ad 2 may produce strong transplantation antigens in these cells thus minimizing their chances of initiating a focus of tumor cells.

Integration of Adenovirus DNA

One general characteristic which transformed cells, tumor cells and probably permissively infected cells share is the presence of viral genome in these cells.

Several lines of evidence suggest that integration is a necessary step in viral transformation. Virus transformed cells contain viral genomes. This has been reported in the case of Ad 2, 7 and 12 transformed cells. The technique used was hybridization of cell DNA on filters with *in vitro*-synthesized viral complementary RNA (Green *et al.*, 1970). Experiments based on DNA reassociation kinetics were used to quantitate the number of copies and percentage viral genome in the transformed cells (Pettersen and Sambrook, 1973; Sharp *et al.*, 1974; Sambrook *et al.*, 1974; Green *et al.*, 1976). Strong evidence demonstrating the covalent integration of Adenovirus DNA into cellular DNA comes from Bellett's (1975) experiment on avian adenovirus transformed hamster skin cells and Tsuei *et al.* (1972) who reported that adenovirus transformed cells contained RNA molecules with linked viral and reiterated cellular base sequences. In 1975, Green and his coworkers

demonstrated that in an Ad 12 transformed hamster embryo cell line, HE C19, all the intracellular Ad 12 DNA was covalently integrated into the cell genome. A more recent report by workers in Doerfler's laboratory demonstrated that Ad 12 DNA is covalently linked to the high and intermediate repetitive sequences of transformed hamster cell DNA (Groneberg *et al.*, 1977). As far as the quantity of viral gene involved, the sequence of Ad 2 and Ad 5 viral DNA that can transform rat cells is as little as 7% of the left hand end of the viral DNA molecule (Graham, 1977).

Cellular DNA synthesis has been suggested as one of the many factors that would influence the integration frequency of viral DNA. Stimulation of hamster embryo cell DNA synthesis upon infection by Ad 12 and other serotypes of adenovirus has been reported (Shimojo and Yamashita, 1968; Takahashi *et al.*, 1969; Doerfler, 1969; Strohl, 1969). This is similar to the situation of polyoma infected cells (Dulbecco *et al.*, 1965; Weil *et al.*, 1966; Winocour *et al.*, 1965). In SV40-infected Chinese hamster cells, integrated viral DNA became detectable about 10 to 15 hours post infection, at the time when cellular DNA synthesis is stimulated (Hirai *et al.*, 1971). Furthermore, Hirai *et al.* (1974) showed that SV40 DNA was integrated even when the induction of cell DNA synthesis is decreased or abolished, however, they also found that integration level was dependent on the cell population that is in S phase.

Viral transformation has also been found to be dependent on cell DNA synthesis.

The event of integration has also been observed in the infection of cells with RNA tumor viruses. In this case, a virus specific DNA is replicated and integrated. The synthesis of viral DNA is dependent upon a virus-coded, RNA-directed DNA polymerase (Baltimore, 1970; Temin and Mizutani, 1970). The smaller genome of RNA tumor viruses allows one to account for all the genetic functions of the genome. Thus, it appears that integration is facilitated by cellular enzymes. When quail embryo fibroblasts were placed in stationary phase (G_0) by prolonged serum starvation and then released by serum replacement, viral DNA was integrated only into cellular DNA replicated during infection. Varmus *et al.* (1977), therefore, concluded that integration appeared to require cellular DNA synthesis. This event could be mediated by an enzyme present only during S phase, topographical changes of cell DNA in replication, replication itself, or factors associated with S phase.

This thesis reports work that has been done to study the interaction of Ad 12 DNA and mammalian cell DNA. An experimental system, the DNA-DNA reassociation technique, has been developed. This system can detect less than one viral genome per cell. It was used to determine the minimum amount of viral DNA required to

maintain the transformed and tumorigenic state of cells
The major aspects of this study are as follows:

- 1) Quantitation of viral genome in tumor, transformed, and productively infected cells.
- 2) Characterization of the site of integration of Ad 12 DNA into productively infected cells. The parameter characterized was the reiteration frequency in the cellular DNA sequences adjacent to an integrated viral DNA.
- 3) The exploration of the effect of factors which would potentially alter the frequency of viral DNA integration. These included factors having known effects on the synthesis of cellular DNA such as serum concentration, AraC, caffeine and ultra-violet irradiation.

MATERIALS AND METHODS

I. Tissue Culture Techniques

A. Media and solutions

i. Minimum Essential Medium (MEM)

MEM is a medium for monolayer cell cultures either on glass bottles or on petri dishes. It was purchased from Grand Island Biological Company, New York (catalogue number F-12).

ii. Joklik-Modified Minimum Essential Medium

This medium was used for suspension cell cultures. Its ingredients are basically similar to MEM mentioned above, without calcium chloride, potassium phosphate, magnesium sulfate, but with addition of magnesium chloride and increased concentration of sodium phosphate (catalogue number F-11).

iii. MEM alpha Medium (α MEM)

The composition of this medium is similar to MEM, with addition of sodium pyruvate, amino acids, and vitamins. This medium provided a more favorable nutritional basis for cells to grow. It was purchased also from GIBCO (catalogue number F-19).

iv. Antibiotics

Penicillin G and Streptomycin sulfate purchased from General Biochemicals, Chagrin Falls, Ohio, were routinely added to the growth media to a concentration of 100 μ g/ml of Streptomycin and 1.21 mg/ml of Penicillin G.

v. Fetal Calf Serum (FCS)

Fetal calf serum (FCS) was purchased from GIBCO, stored at -20°C and thawed just before being used. It was added to MEM or α MEM to a final concentration of 10%.

vi. Horse Serum (HS)

Horse serum (HS) was also purchased from GIBCO and handled exactly as for FCS. HS was added to Joklik's medium for suspension cell cultures to a final concentration of 5%.

vii. Fugizone

Amphotericin B was purchased from GIBCO and added to media to a concentration of 1%.

viii. Trypsin

Bacto trypsin as lyophilized powder was purchased from Difco Laboratories, Detroit, Michigan. It was reconstituted with citrate saline as a 1.25% stock

solution. Citrate saline contains 1% potassium chloride and 0.44% sodium citrate.

ix. Phosphate Buffered Saline (PBS)

The PBS without Mg^{2+} , Ca^{2+} was routinely used.

10 X PBS contains NaCl 80 gm; KCl 2.0 gm; Na_2HPO_4 11.5 gm; KH_2PO_4 2.0 gm in 1 litre solution.

B. Culture techniques

i. Monolayer Cultures

These were maintained at 37°C in a humid incubator with 5% CO_2 and 95% air. Confluent monolayer cell cultures were propagated by washing with sterile citrate saline at 37°C. Then they were trypsinized with 0.125% trypsin. Alternatively, some established cell lines can be propagated by scraping attached cells off glass with a sterile rubber policeman.

ii. Suspension Cultures

Cells were kept in suspension by constant agitation with a teflon magnetic bar held in motion by a magnetic stirrer. The concentration of cells were held between 2×10^5 to 5×10^5 /ml by regular dilution with suspension medium plus 5% HS.

iii. Secondary Cultures

Human KB cells. The cells, originally carcinoma

of the mouth, were obtained from Dr. M. Green, St. Louis University School of Medicine, St. Louis, Mo. These cultures were propagated either in monolayer or in suspension.

Hamster embryo cells. Thirteen day-old hamster embryos were removed from the mother under sterile conditions. They were rinsed with sterile PBS. Decapitated and enviserated, the carcasses were minced and treated with 0.25% trypsin for 15 minutes at 37°C under constant agitation. The viscous fluid was then filtered through several layers of sterile cheese cloth and the action of trypsin was terminated by adding 10 ml of FCS. Culture medium was added. Cells were pelleted by centrifugation for 5 minutes at 1 Krpm at room temperature in an International centrifuge. α MEM was added to resuspend the cell pellet. Subsequent cell cultures were grown in α MEM.

T₂ and T₆ cells. These are established cell lines or primary hamster embryo transformed by Ad 12 according to the procedure of Casto (1968). Details of transformation has been published in the Ph.D. thesis of J. Smiley (1977).

293-31 cells. This is a human cell line transformed by Ad 5 DNA (Graham, 1977).

C. Induction of tumors and isolation of tumor cell DNA

Newborn syrian hamsters were injected subcutaneously with 10^9 virions of Ad 12. After 6 to 8 weeks, tumors were excised, washed with phosphate buffered saline (PBS) several times, and minced finely. They were homogenized with a buffer containing 0.25 M sucrose, 3.3 mM CaCl and 0.01 M tris-hydrochloride (pH 7.4). The homogenization was carried out using a Sorvall Omni-Mixer (100 ml vessel) at medium speed (setting 4) for 3 minutes at ice temperature. The homogenate was then filtered through several layers of cheese cloth. Cells thus collected were washed extensively with PBS without Ca^{++} and Mg^{++} . This procedure yielded single cells, of which more than 90% were T-antigen positive, as shown by immunofluorescence (data not shown).

To extract tumor cell DNA, the procedure that has been described earlier was used with some modification (Lee and Mak, 1977). Briefly, the cells were lysed in a buffer containing 0.15 M NaCl, 0.015 M sodium citrate, 0.1 M Tris buffer (pH 8.0), 0.3 M sodium trichloroacetate, 5 mM EDTA, with 0.5% SDS. The lysate was homogenized in the Omni-Mixer vessel at setting 3 for 1 min. Pronase (Calbiochem) that had been pre-incubated for 2 h at $37^{\circ}C$ was added to 0.5 mg/ml. After incubating at $37^{\circ}C$ for 12 h, the sample was extracted three times with phenol and then precipitated with ethanol and dried. The nucleic acids were then resuspended with 0.3 M NaOH, boiled

for 20 min and neutralized. The DNA was precipitated with ethanol again and redissolved with 0.01 M Tris buffer (pH 7.0), 5×10^{-4} M EDTA, and 0.01 M NaCl, and dialyzed against the same buffer.

D. Fluorescent microscopy

Transformed cells were grown on cover slips. Tumor cells were dried on cover slips at -20°C . The cover slips were washed with PBS, air dried and fixed with carbon tetrachloride. The indirect immunofluorescence technique was used to detect the presence of Ad 12 T antigens. Anti-T antiserum was obtained from hamsters bearing Ad 12 induced tumors. Fluorescein-conjugated antisera against hamster was either purchased from Roboz Surgical Instrument Co., or as a gift from Dr. M. Buchmeier, Dept. of Pathology, McMaster University.

E. Complement fixation test

This was done according to the method of Kagan and Norman (1970). Ad 12 transformed cells were disrupted in PBS by sonication (Biosonik III, 30% maximum power, for one minute). Antiserum was collected from tumor-bearing hamsters and complement inactivated at 56°C for 30 minutes. Sheep red blood cells were obtained from Grand Island Biological Co. Complement was a gift from Dr. W. Rawls, Dept. of Pathology, McMaster

University. . HEP-2 cells were used as negative control and Ad 12 infected HEP-2 cells as positive control.

II. Virological Techniques

A. Viruses

Adenovirus type 2 (Ad 2) and type 12 (Ad 12) were originally obtained from Dr. M. Green, St. Louis, Mo. Ad 12 cyt mutants originally isolated by Takemori (1968) were obtained through Dr. H.F. Stich, University of British Columbia.

B. Cultivation of viruses

i. Ad 2 and Ad 12

KB cells grown in suspension cultures were pelleted by low speed centrifugation. Joklik medium with 1% FCS was added to resuspend the cells to 10^7 cells/ml. Virus was added to the cells at 300 particles per cell. The infected cells were maintained under constant agitation at 37°C for 90 minutes, then diluted to 2×10^5 cells/ml with Joklik medium with 5% HS. Virus was harvested at 48 to 72 h post infection.

ii. Cyt mutant

Cyt mutant (H12 cyt 12) of Ad 12 was propagated by infecting KB cells in suspension and incubated as monolayer cultures at 37°C. Virus was harvested at 48 h post infection.

C. Purification of virus

The method of Ginsberg (1966) was used. The infected cells were pelleted in the cold by low speed centrifugation and resuspended in 0.01 M phosphate buffer (pH 7.4) at 2.5×10^7 cells/ml. Sodium deoxycholate was added to a final concentration of 0.5%. After 30 min of lysis at room temperature, $MgCl_2$ was added to a concentration of 0.02 M, and deoxyribonuclease was added to 10 μ g/ml. The lysate was incubated at 37°C for 2 h and centrifuged at 2 Krpm in an International centrifuge for 20 min to remove unlysed cellular debris which would interfere with subsequent procedures. The supernatant was extracted with Freon 113 until clear. The viral suspension was then sedimented onto a cesium chloride cushion of density 1.44 gm/ml and banded isopycnicly twice in a cesium chloride of density 1.33 gm/ml at 33 Krpm for 24 h with Beckman rotor 60.

D. Infection of KB cells to study factors which alter integration frequencies

In the studies of effects of serum concentration, Ara C and caffeine, KB cells grown in suspension cultures at 3.5×10^5 cells/ml were infected with 2000 particles of Ad 12 per cell. After 1.5 h for adsorption under constant agitation, 3.5×10^7 cells were plated onto 150 mm diameter petri dishes with MEM containing

the appropriate concentrations of FCS, Ara C and caffeine. In the case in which the effect of UV-irradiation was studied, KB cells from spinner cultures were transferred to 150 mm diameter petri dishes 2 days prior to the experiment. A UV dose of 100 ergs/mm² (10 J/m²) was used. The UV light was produced by an 8 μwatt General Electric Germicidal tube, number G8T5. Incident dose was determined by a UV intensity meter (Blak-Ray short wave UV meter, model J-225, Ultra-violet Products, Inc., San Gabriel, Ca.).

III. Molecular Biology Techniques

A. Preparation of radioactive viral DNA

The 293-31 human cells were infected with Ad 12 (strain 1131) at about 500 virions/cell. At 15 h after infection, ³H-thymidine (20 Ci/mM) was added to a final concentration of 5 μCi/ml. The cells were harvested at 60 h after infection. Viral DNA was extracted from purified virus preparation. The specific activity of the viral DNA was about 2 x 10⁶ cpm/μg. On some occasions, KB cells were used instead of the 293-31 cells.

B. Restriction enzyme-generated viral DNA fragments

Eco RI restriction endonuclease was prepared from *E. coli* RY13 bacteria (Pettersen and Philipson, 1974),

a gift of Dr. L. Philipson, Uppsala, Sweden. This enzyme digested Ad 12 DNA into fragments which could be used as radioactive viral DNA probes.

³H-labelled viral DNA was digested with Eco RI. After digestion, the mixture was treated with chloroform-isoamylalcohol (24:1) and the DNA fragments were fractionated by 0.7% agarose gel electrophoresis (Mulder, 1974). DNA bands were located by staining with ethidium bromide and illuminating with UV light. DNA from the gels were recovered with a modified procedure reported earlier (Mulder, C., personal communication). Briefly, relevant gel segments containing DNA were cut, crushed finely, and dissolved in two gel volumes of 8 M sodium perchlorate. The solution was then diluted to 2.5 M sodium perchlorate. About 0.5 g of hydroxyapatite (HAP) was added and the slurry was loaded into a column. The HAP was then washed with 2.5 M sodium perchlorate, followed by 0.01 M sodium phosphate buffer (pH 6.8) and then 0.14 M phosphate buffer. The DNA was eluted with 4 bed volumes of 0.4 M phosphate buffer (pH 6.8). All operations were carried out at room temperature. The eluted DNA was then dialyzed against 0.01 M EDTA (pH 7.0) and concentrated by freeze-drying.

C. Determination of molecular weights of DNA

Molecular weights of DNA could be determined by alkaline sucrose gradients. Virus and cells were lysed in a solution of 0.5 M NaOH, 0.01 M EDTA at room temperature. The cell concentration was obviously important, but is a variable dependent on what centrifuge tubes were used. Extensive work on upper limits were done elsewhere (e.g. M. Pater and B. Palcic, Ph.D. theses). In all cases, the amount of material used in the present work was well within limits. The lysing time for virus was 5 h and for cells, 12 h. The sucrose gradients contain: 0.3 N NaOH, 0.001 M EDTA, 0.01% SDS, 5-20% sucrose, unless otherwise stated.

D. Separation of viral DNA from infected cell DNA

i. Alkaline Glycerol Gradient

Cell samples were lysed in 2 ml of the same lysing solution as stated in C for 12 h at room temperature before layering on top of 20-50% alkaline glycerol gradients in 0.1 N NaOH. SW27 buckets were used. The gradients were collected with the help of an ISCO fraction collector in fractions of 1 ml each. Optical density at 260 nm was monitored. The fractions were neutralized carefully with 1 M NaH_2PO_4 to pH 8, then dialyzed against 0.1 x SSC with four changes of dialysate during the first four h, and against 0.1 x SSC for

another 8 h. The sample was then extracted with chloroform-isoamyl alcohol (20:1) and then 5 times with equal volumes of ether to remove the alcohol and chloroform. The DNA was freeze-dried and stored.

ii. Network Technique

The method of Varmus *et al.* (1973) was followed. Unsheared DNA from infected cells was denatured by boiling for 10 min, and then chilled in ice bath. It was re-associated to Cot of 3 sec.mol/liter in 0.6 M NaCl, SDS was added to 1%, and centrifuged for 15 min at 140,000 x g in SW50.1 rotor. The network pellet was suspended in 0.3 N NaOH, boiled for 20 min to fragments as a preparation before reassociation. The nitrocellulose centrifuge tubes were siliconized, immersed in 50 µg/ml solution of denatured salmon sperm DNA, and rinsed with water.

E. DNA-DNA Reassociation System

All DNA samples were subjected to alkaline fragmentation by boiling in 0.3 M NaOH for 20 min and then neutralized (Sharp *et al.*, 1974). The samples were then dialyzed extensively with 0.01 M Tris-hydrochloride (pH 7.0), 0.01 M NaCl, and 5×10^{-4} M EDTA. Tumor cell DNA or salmon sperm DNA (2.2 mg/ml) was mixed with appropriate amounts of ^3H -labeled viral DNA probe and denatured by boiling. The solution was adjusted to 1.0 M

NaCl, overlaid with mineral oil, and incubated at 68°C in stoppered glass tubes. Samples of 100 μ l were withdrawn at various times and immediately diluted 10-fold with 0.14 M phosphate buffer. Hydroxyapatite chromatography was used to separate the single- and double-stranded DNA (Gelb *et al.*, 1971). Radioactivity eluted from the hydroxyapatite column was determined by a liquid-phase counting system. Trichloroacetic acid was added to the 0.14 and 0.4 M eluates (4 ml) to final concentrations of 2.5 and 10%, respectively. A 15 ml portion of counting fluid made up of Triton X-114, xylene, and Omnifluor (New England Nuclear Corp.) (Anderson and McCure, 1973) was added. The counts per minute was then corrected for the differential quenching effects of 0.14 and 0.4 M phosphate.

F. Preparation of hydroxyapatite (HAP)

Hydroxyapatite crystals were prepared by the method of Muench (1971). Briefly, 1500 ml of 0.5 M CaCl₂ were dripped into 1800 ml of 0.5 M sodium phosphate buffer (pH 6.8) under constant agitation. The whitish crystals of CaHPO₄ were then washed with 3 litres of distilled water and then boiled for half hour in 3 litres of distilled water made alkaline by adding ammonium hydroxide using phenolphthalein as a pH indicator. The resultant crystals of hydroxyapatite were then washed seven times with 0.005 M sodium phosphate buffer (pH 6.8) and stored in 0.005 M sodium phosphate buffer at 4°C.

Under the light microscope with high power magnification, the crystals prepared by the Muench method were examined. They showed the typical conglomerations of blade-like crystals of hydroxyapatite (Bernardi, 1971).

G. S1-nuclease assay of DNA hybrids

S1-nuclease was used to assay for percentage of DNA in single- and double-stranded configuration. The conditions described by Leong (1972) were followed. DNA was digested by 1600-2000 units/ml of S1-nuclease in a solution of 0.03 M sodium acetate buffer pH 4.5, 1.8×10^{-3} M $ZnCl_2$, 0.3 M NaCl with 10 μ g/ml of denatured calf thymus DNA at 37°C for 2 h. After digestion, 30 μ g of salmon sperm DNA was added as carrier and TCA was added to 10% in ice temperature. The precipitate was collected on 0.22 μ millipore filters, dried and the radioactivity was determined by counting in 5 ml of toluene-Ommifluor mixture.

H. Fluorography to determine relative amount of 3H -thymidine in DNA fragments

Ad 12 DNA was digested with Eco RI endonuclease and electrophoresed on agarose gel. Fluorography was done according to Laskey and Mills (1975). The gel was immersed in 20 volumes of methanol for 30 min followed

by a second 30 min immersion in fresh methanol. The gel was then immersed in 10% w/w PPO in methanol for 3 h and dried in vacuum. Fluorograph was made by exposing the dried gel to X-ray film (Kodak RP/R-14) and developed. To quantitate the radioactivity in each DNA fragment, the film was scanned with a microdensitometer and the area under each peak was determined.

I. Incorporation of radioactive thymidine into KB cells

Spinner suspension cultures of KB cells were infected with Ad 12 virus and then transferred to 35 mm petri dishes containing appropriate concentrations of FCS. One-hour pulses of ^3H -thymidine at $0.5 \mu\text{Ci/ml}$ were performed. At the end of the pulse, cells were removed from the dishes, washed three times with PBS and pelleted by centrifugation. TCA (10%) was added and the precipitate was collected on fiber glass filters, dried and counted in 5 ml of toluene-Omnifluor fluid.

RESULTS

I. Establishment of the DNA-DNA Reassociation System

Denatured complementary copies of DNA renature to achieve a lower energy configuration (Marmur *et al.*, 1963). The rate of reassociation depends on DNA concentration, temperature, ionic strength and viscosity of the incubation medium (Wetmur and Davidson, 1968). Conversely, if all other variables are kept constant and rate of reassociation is known, the DNA concentration can be determined. In the experiments reported in this thesis, the rate of reassociation was determined by mixing an unknown sample with trace amounts of radioactively labelled "probe" DNA. The rate was monitored by assaying for the percentage of DNA in single stranded form using hydroxyapatite chromatography (Britten and Kohne, 1968).

The mathematical relationship used to describe DNA-DNA reassociation is:

$$\frac{C}{C_0} = \frac{1}{1 + KCot} \quad (1)$$

where

C_0 = Concentration of DNA in single-stranded form at initiation of reassociation;

C = Concentration of DNA in single-stranded form at time t during reassociation;

K = Reaction constant which depends on the genetic complexity of the genome being reassociated.

The plot of C/C_0 versus t is a first-order sigmoid curve. If there is more than one component in the reassociation mixture, a multiphasic curve will be found. Alternatively, the above equation can be transformed so that a first-order reaction gives linear plots.

Let f_{ss} = fraction of DNA in single-stranded form.

$$\therefore f_{ss} = \frac{C}{C_0} = \frac{1}{1 + KCot}$$

$$\frac{1}{f_{ss}} = 1 + KCot \quad (2)$$

The slope of $1/f_{ss}$ versus t is KC_0 . Hence, one could use the increase in the slope to quantitate unknown amounts of viral DNA provided that K could be found from the reassociation of a probe. Before the results of the application of these basic concepts are discussed, it is important to discuss the preparation of DNA samples and the establishment of assaying conditions.

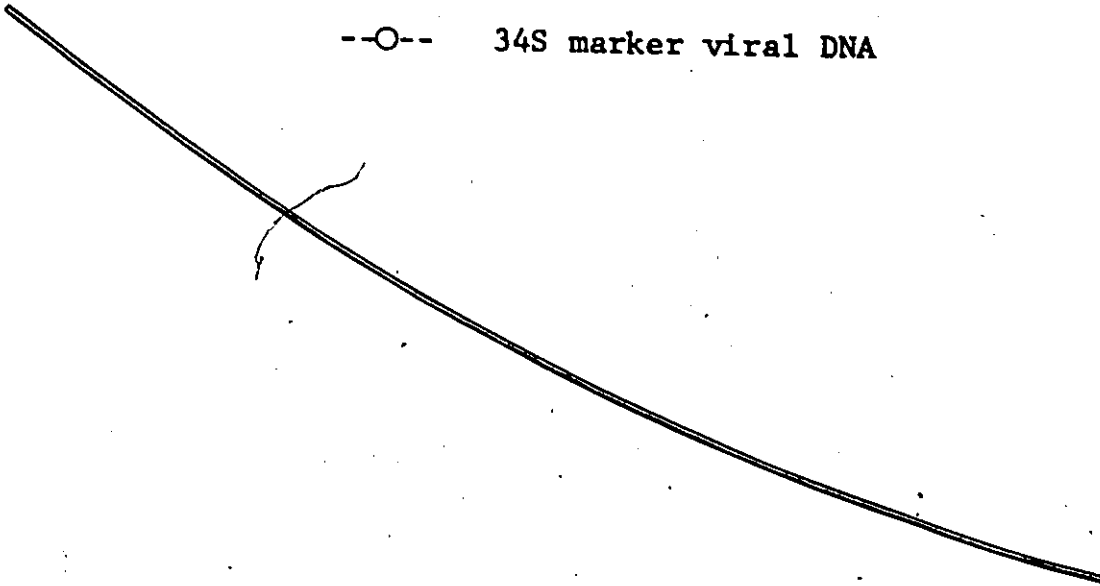
DNA extracted from various sources has different sizes depending on the extraction buffers, mechanical shearing, and length of time spent on different stages of extraction. Cellular DNA extracted according to the procedures described in Materials and Methods has a mean molecular weight of 3×10^7 Daltons, corresponding to a sedimentation coefficient of 54S in alkaline sucrose gradient using a 34S viral DNA as marker. This is shown in Figure 1.

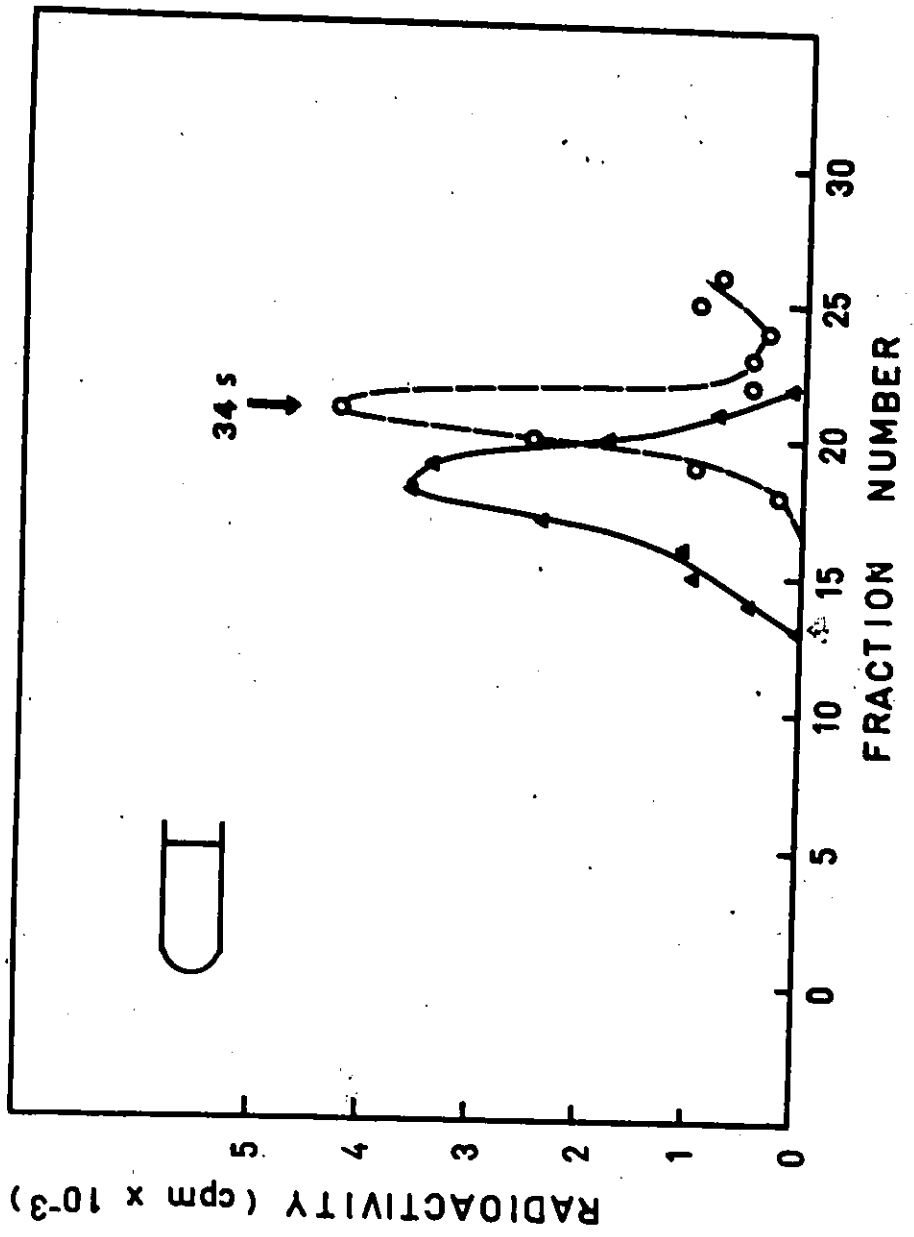
Figure 1

Profile and Size of Extracted Mammalian DNA

Cell DNA radioactively labelled with ^3H -thymidine was extracted according to the procedures outlined in Materials and Methods. It was mixed with a marker Ad 2 DNA labelled with ^{14}C -thymidine. Sedimentation conditions: 5-20% alkaline sucrose gradient in SW50.1 rotor at 40 Krpm for 1 h at 5°C .

—▲— extracted cell DNA
--○-- 34S marker viral DNA





In order to reduce variations of DNA sizes and also to minimize physical entanglement of DNA having large sizes, it is desirable to reduce the DNA into smaller, but more uniform size fragments.

The use of mechanical shearing to fragment DNA was explored. This was done by sonicating 2 ml of DNA samples in a glass Kimex tube held in an ice bath. Thirty percent of full power with a Biosonik sonicator adapted with a small probe was used. The S value of Ad 12 DNA was found to decrease with increasing sonication time. Figure 2 shows that its sedimentation coefficient drops from 34S for an intact molecule to 16S in the first min of sonication and levels off to between 8S to 10S after 2 min of sonication. Profiles of the fragmented Ad 2 DNA sedimented in alkaline sucrose gradients indicated that the size distribution became more homogeneous as the fragments were reduced to smaller sizes.

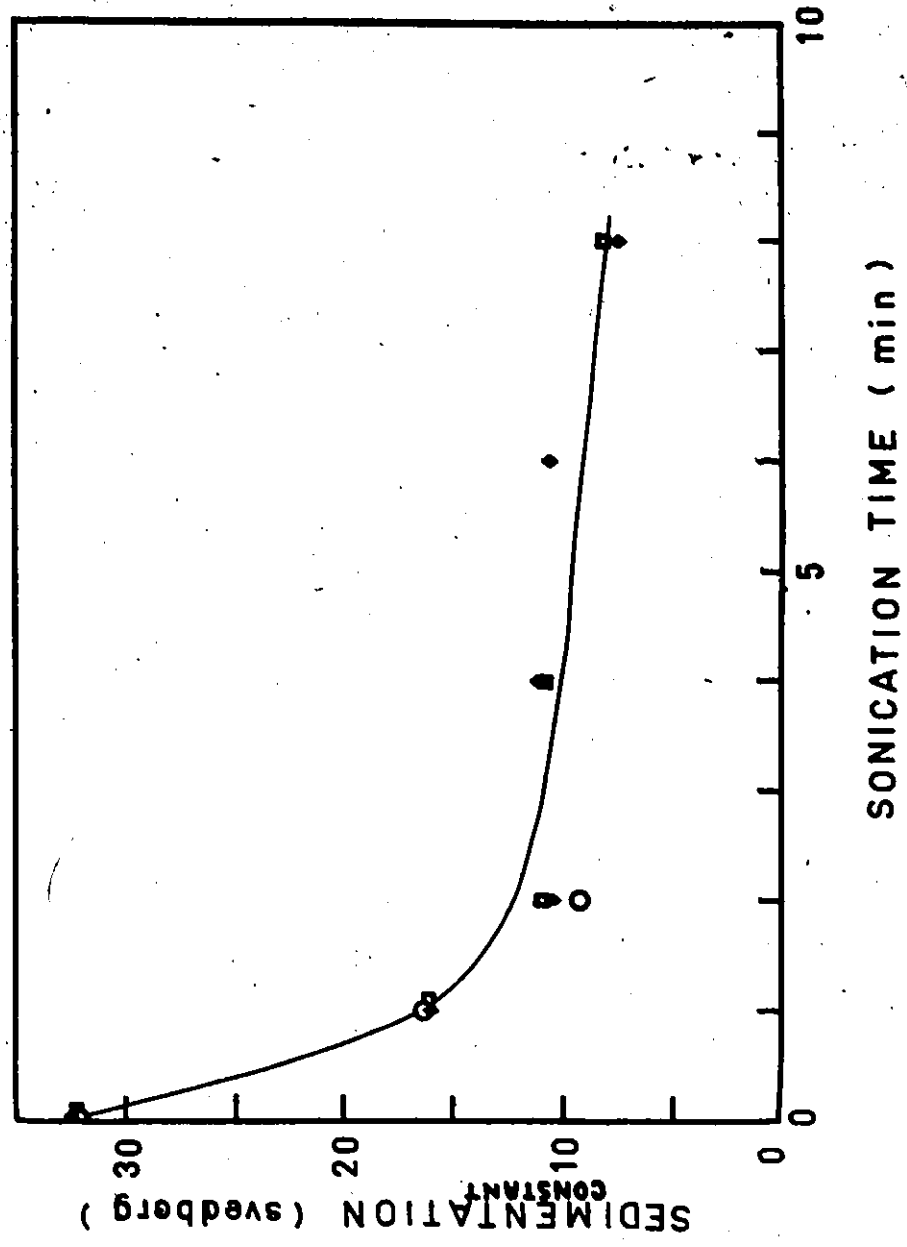
The method of fragmentation by alkaline degradation was used in many experiments reported in this thesis (Sharp *et al.*, 1974). Alkaline fragmentation would reduce Ad 2 virus DNA to about 6.5S in alkaline sucrose gradient after 15 min of boiling in 0.3 N NaOH. This corresponds to a molecular weight of 2.2×10^5 Daltons (about 600 nucleotides) (Studier, 1965). Again, the sizes of DNA fragments reach a more homogeneous distribution as the fragments are reduced to smaller sizes. Cellular DNA,

Figure 2

Size of Sonicated DNA
as Function of Sonication Time

Three different experiments with results
identified by three sets of symbols.

34a



which started out with a higher molecular weight and broader size distribution, retained a slightly broader size distribution although the mean sedimentation coefficient was the same as the viral DNA treated under similar conditions. This is shown in Figure 3.

The reassociated DNA was assayed by HAP chromatography. The temperature of the sample was maintained at 65°C by a water-jacket HAP column. Here, we have chosen to use hydroxyapatite crystals prepared by the method of Muench (1971) instead of the now highly popular brand BIO-GEL HTP of the Bio-Rad Company. The major difference is that BIO-GEL HTP was prepared at neutral pH before conversion to the final product in alkaline conditions (Tiselius, 1956), whereas the HAP using the Muench procedure was prepared at a lower pH before conversion.

Under the light microscope with high power magnification, the crystals prepared by the Muench method were examined. They showed the typical conglomerations of blade-like crystal morphology of hydroxyapatite. Compared with the crystals of BIO-GEL HTP, they were larger and more homogeneous in size. When stirred up, they settled more readily. Their flow rate was about 10 times higher. The DNA binding capacity was about 600 µg/gm of dry powder, and it was not affected by the decreased column contact time. The characteristics of the two types of crystals were further investigated by

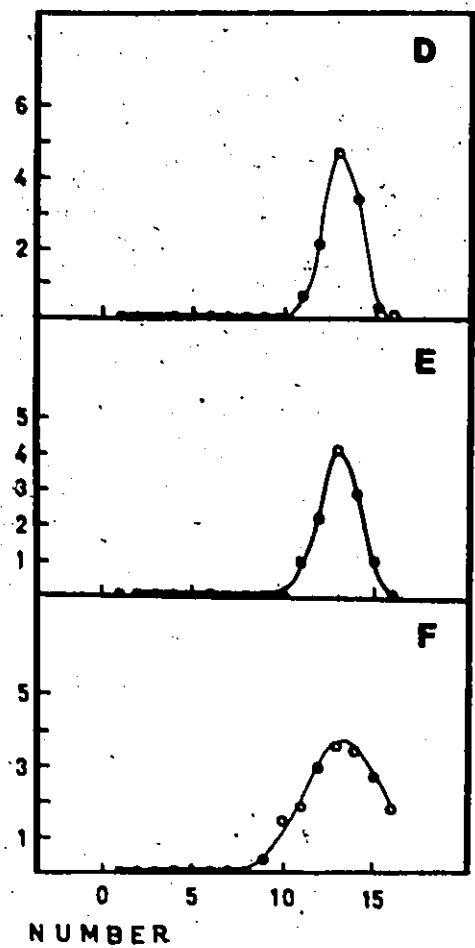
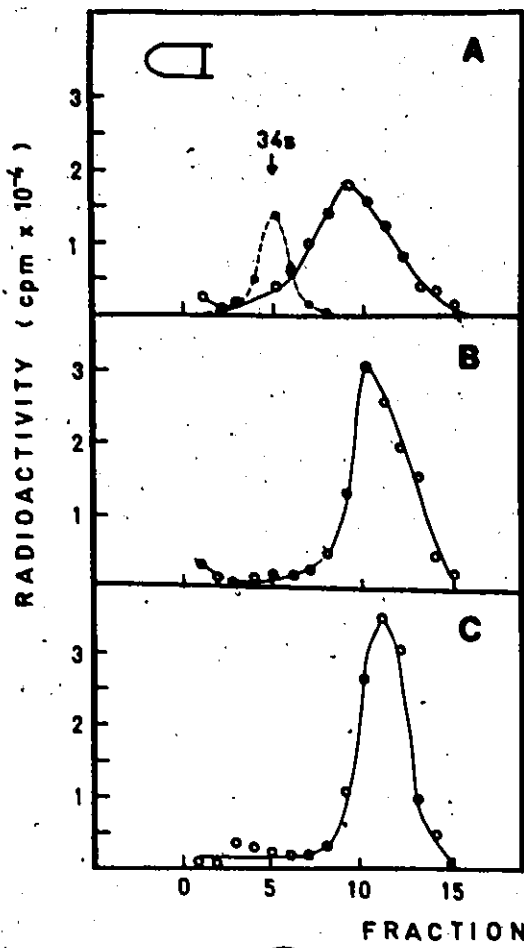
Figure 3

Size and Distribution of Fragmented DNA by
the Method of Alkaline Fragmentation

Alkaline sucrose sedimentation of fragmented Ad 2 and
KB cell DNA. Condition: SW50.1 rotor, in 5-20%
alkaline sucrose gradient centrifuged at 48 Krpm for
2.5 h.

Boiling time (min)

- A) 2
- B) 5
- C) 10
- D) 15
- E) 20
- F) 2 mg of KB cell DNA boiled for 20 min.
10 μ g layered on 5-20% alkaline sucrose
gradient centrifuged in SW50.1 rotor and
centrifuged at 48 Krpm for 2.5 h.



comparing the melting profiles of double-stranded DNA bound to the crystals. As shown in Figure 4, the melting profiles of DNA in 0% and 50% formamide in the two types of crystals were virtually identical. This shows that the DNA affinity of the crystals was the same when the DNA was intact, or partially unwound. In all subsequent experiments, the HAP prepared by the Muench method would be used.

Single-stranded DNA could be eluted from HAP crystals by 0.14 M sodium phosphate buffer and double-stranded DNA could be eluted with 0.4 M sodium phosphate buffer (Martinson, 1973a,b). To demonstrate the selective adsorption of single- and double-stranded DNA by the HAP crystals, elution profiles of double-stranded DNA were obtained. The double-stranded DNA was obtained by careful extraction of viral DNA from Ad 2 virus, and the completeness of DNA molecule was confirmed by sedimenting through alkaline sucrose gradients. Figure 5 shows that about 5% of radioactivity was eluted with 0.14 M PB, presumably representing single strands introduced in preparation of the sample.

The DNA used for reassociation was labelled with ^3H -thymidine. Counting efficiency of this radioactive isotope was greatly decreased by the amount of DNA in the samples, presence of PB and agents used to solubilize the sample. Both single-stranded and double-stranded

Figure 4

Melting Curves of Ad 12 DNA on Hydroxyapatite Columns

10^{-2} μ g of Ad 12 DNA radioactively labelled with ^3H -thymidine was bound to water jacketed column containing 1 ml of packed HAP. Melting was achieved by increasing the temperature of water circulating through the columns. Curves were obtained for melting in varying concentrations of formamide. Melting characteristics for HAP made according to the Muench method (1971) and as commercial products from Bio-Rad Co. (HAP-HTP) were compared.

Curves	HAP origin	Formamide concentration
A ○	Bio-Rad	0%
B ○	Muench	0%
C ○	Muench	10%
D ○	Muench	20%
E ○	Muench	30%
F ○	Muench	40%
G ●	Bio-Rad } Muench } overlap	50%

40 a

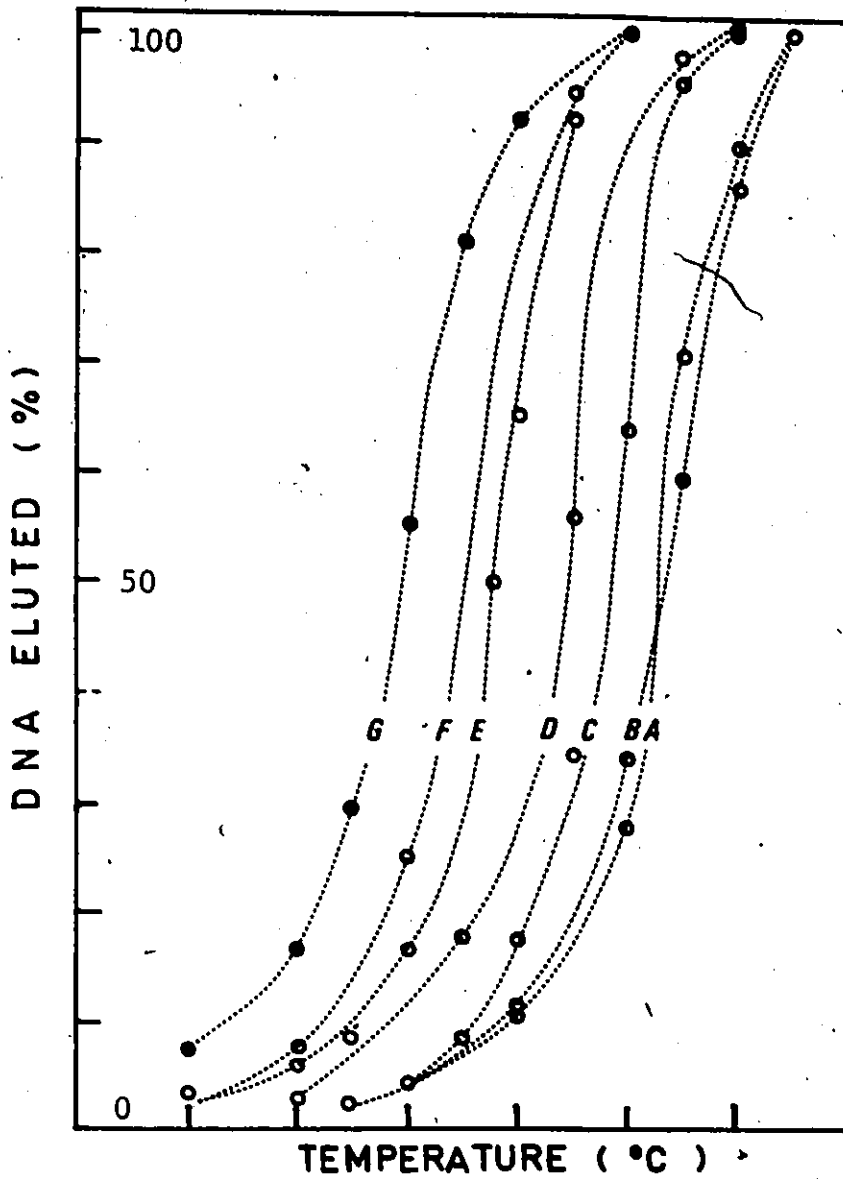


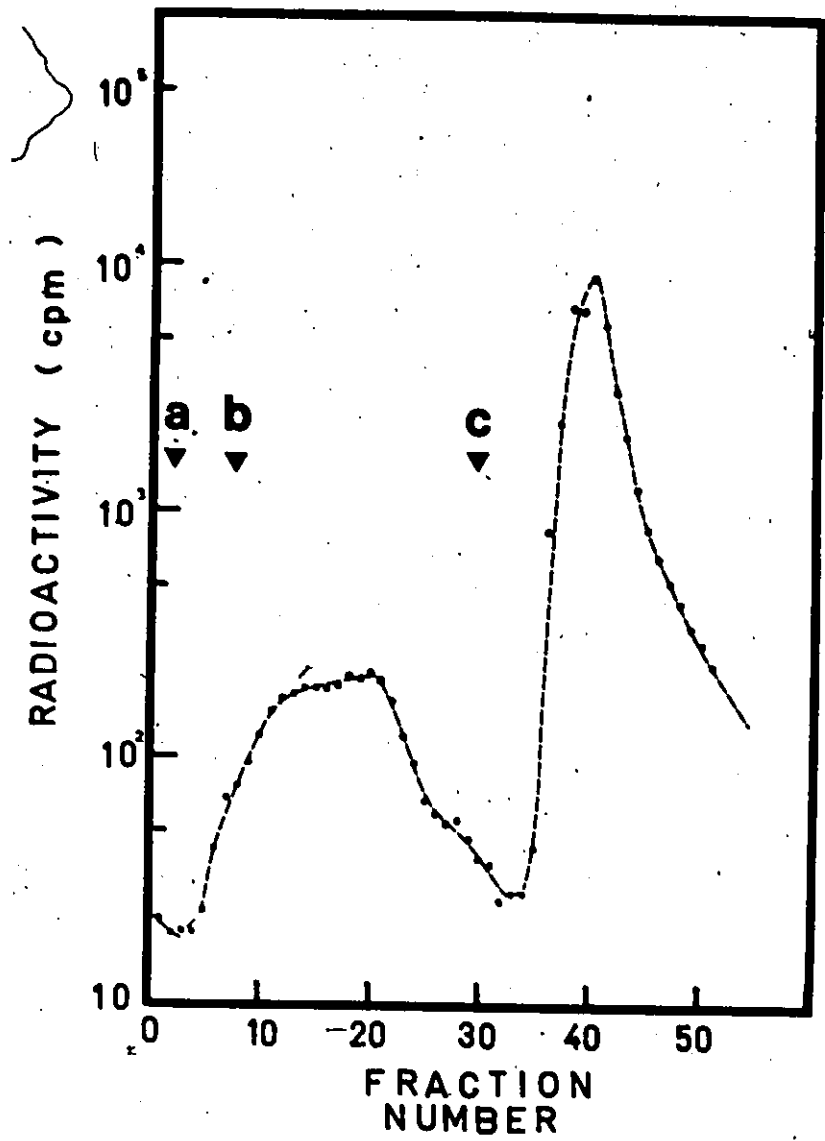
Figure 5

Elution Profiles of ^3H -labelled
Ad 2 DNA from Hydroxyapatite Column

2 μg of ^3H Ad 2 DNA extracted from virus particles was sonicated to a mean fragment length of 800 nucleotide pairs. The sample in 0.1 SSC was adsorbed for 20 min onto HAP. Phosphate buffer with concentrations as indicated in the graph was used to elute DNA from the columns. Void volume of the column was 4 ml.

- a) loading of sample in 0.1 SSC
- b) elution with 0.14 M phosphate buffer
- c) elution with 0.4 M phosphate buffer.

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fractions of eluates from HAP chromatography contain about 10 to 200 μ g of DNA. It was found that if these samples were precipitated by TCA and immobilized on nitrocellulose filters, dried and then counted, the scintillation counts would decrease as the DNA concentration on the filters increases (Figure 6). The obvious difference between radioactive counts on 0.22 and 0.45 μ filters had not been investigated. Theoretically, one could correct for this self-absorption, if the amount of DNA on the filter is known. However, the actual amount of DNA on the filter is usually unknown or only roughly estimated as this represents the reassociation of cellular DNA sequences. As an alternate approach, a method of counting DNA samples in solution was developed.

When phosphate eluates from HAP columns were mixed with a counting fluid containing Triton X-114, xylene and Omnifluor (Anderson, 1973), a white precipitate was formed. As this precipitate settled, the radioactivity counts dropped. The precipitate was probably a form of phosphate which decreased the radioactive counts. Since it was known that phosphates dissolve in acidic condition (Muench, 1971), TCA was added to the samples. The precipitates were observed to dissolve with a concomitant increase in radioactive counts as shown in Figure 7. It was also noticed that TCA itself quenched radioactivity as well (Figure 8). Different concentrations of TCA

Figure 6

Quenching of ^3H Radioactivity by DNA on Filters

Duplicate samples of radioactively labelled DNA on nitrocellulose filters of varying sizes.

- ▲ 0.45 μ nitrocellulose filter
- 0.22 μ nitrocellulose filter

43a

2

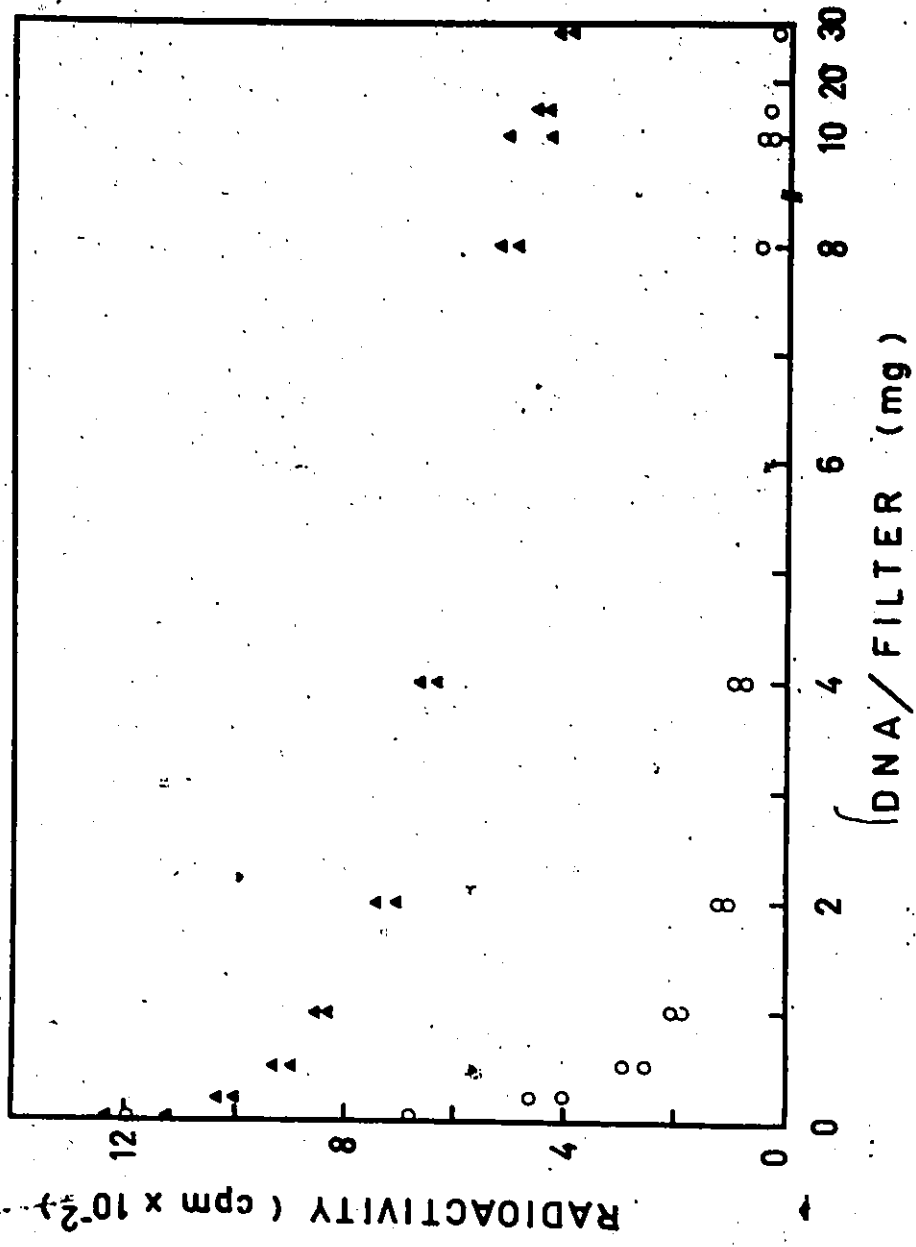


Figure 7

Scintillation Counting of Tritium in PB

Solubilizing phosphate buffer with acidic conditions for scintillation counting; time on x-axis starts with the addition of counting fluid; mixing was facilitated by shaking the vial container.

3 ml of 0.12 M phosphate buffer with ^3H DNA in 10 ml of standard Triton X-114 counting fluid; at 40 min post mixing, the vial was shaken up and at 90 min post mixing, 0.15 ml of 50% TCA was added.

44 a

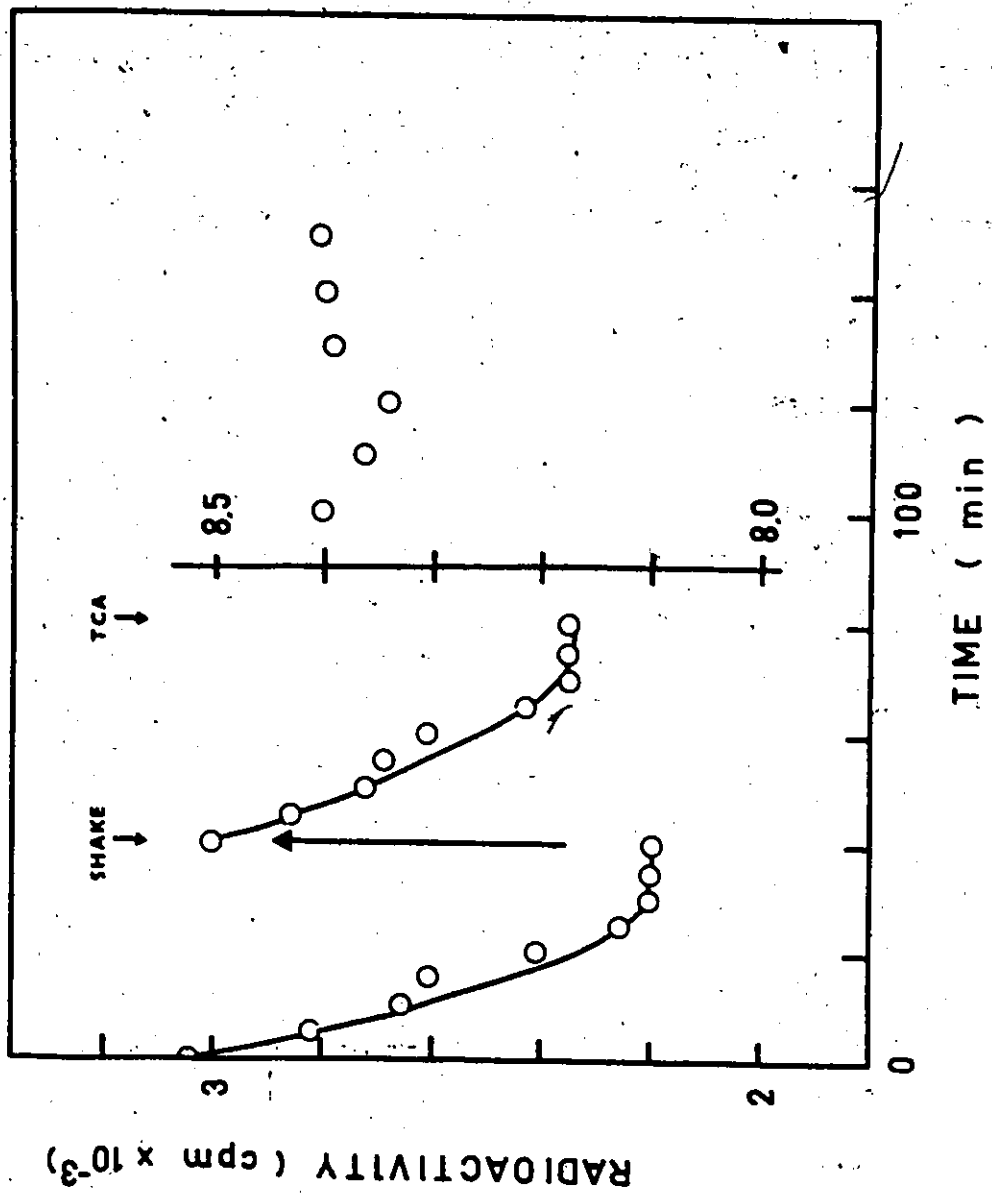
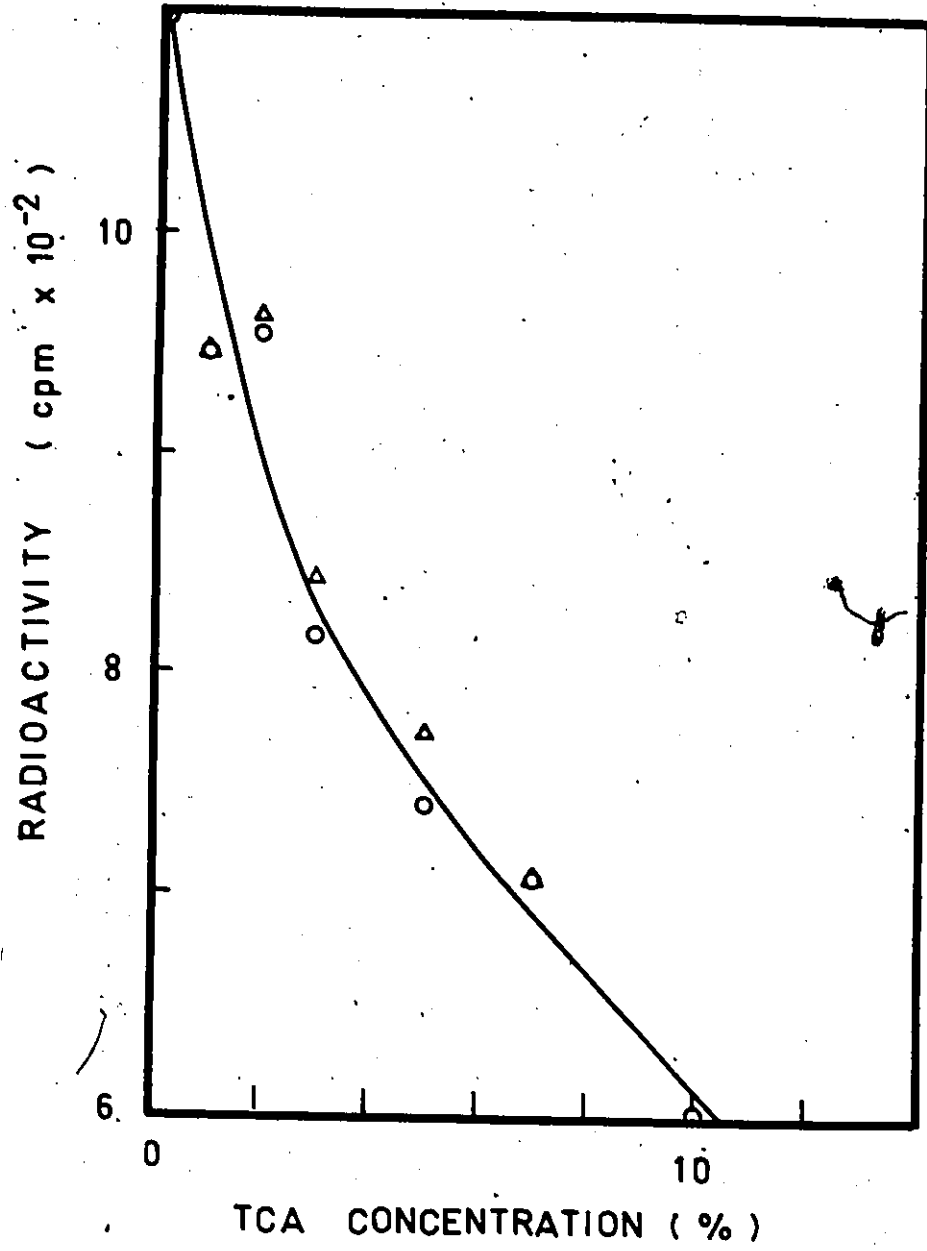


Figure 8

Quenching of radioactive counts by TCA
in Triton X-114 Counting Fluid

- △ ^3H in 0.5 ml 0.14 M PB, counted in 10 ml of Triton X-114 counting fluid and 1 ml TCA.
- ^3H in 0.5 ml 0.14 M PB, counted in 10 ml of Triton X-114 counting fluid and 1 ml TCA.

45a



were required to acidify the same volume of 0.14 M and 0.4 M PB. An optimum concentration of TCA to give the highest radioactive count was found to be one which just completely dissolved the phosphate crystals. Even under this condition the radioactive counts in the 0.14 M and 0.4 M PB fractions were not equal. Correction factors were required to adjust for this differential quenching. It was found that for 4 ml of 0.14 M PB solubilized with 0.2 ml of 50% TCA a correction factor of 1.14 times the radioactive counts was necessary to give the radioactive counts if the same amount of ^3H -labelled DNA was counted in 4 ml of water. Likewise, the correction factor for 4 ml of 0.4 M PB with 0.8 ml of 50% TCA was 1.86 (Table 2). In both cases the counting fluid was 15 ml of Triton X-114 solution as described in Materials and Methods.

DNA concentration played an important role in decreasing radioactivity when it was precipitated on filters. In order to find out whether DNA concentration still played such an influential part in liquid phase counting, the effect of DNA was investigated. Figure 9 shows that there was a drop of about 10% in radioactive counts as DNA concentration was increased from 100 μg to 1000 μg . Since in the experiments reported later on, the variation of DNA concentration in the samples would be less than 200 μg , one can safely assume that the effect of DNA concentration on quenching was small and

Table 2

Correction Factors for Quenching in Phosphate Buffer

³H-labelled sample of DNA in 0.14 M PB and 0.4 M PB acidified and counted in Triton x 114 fluid

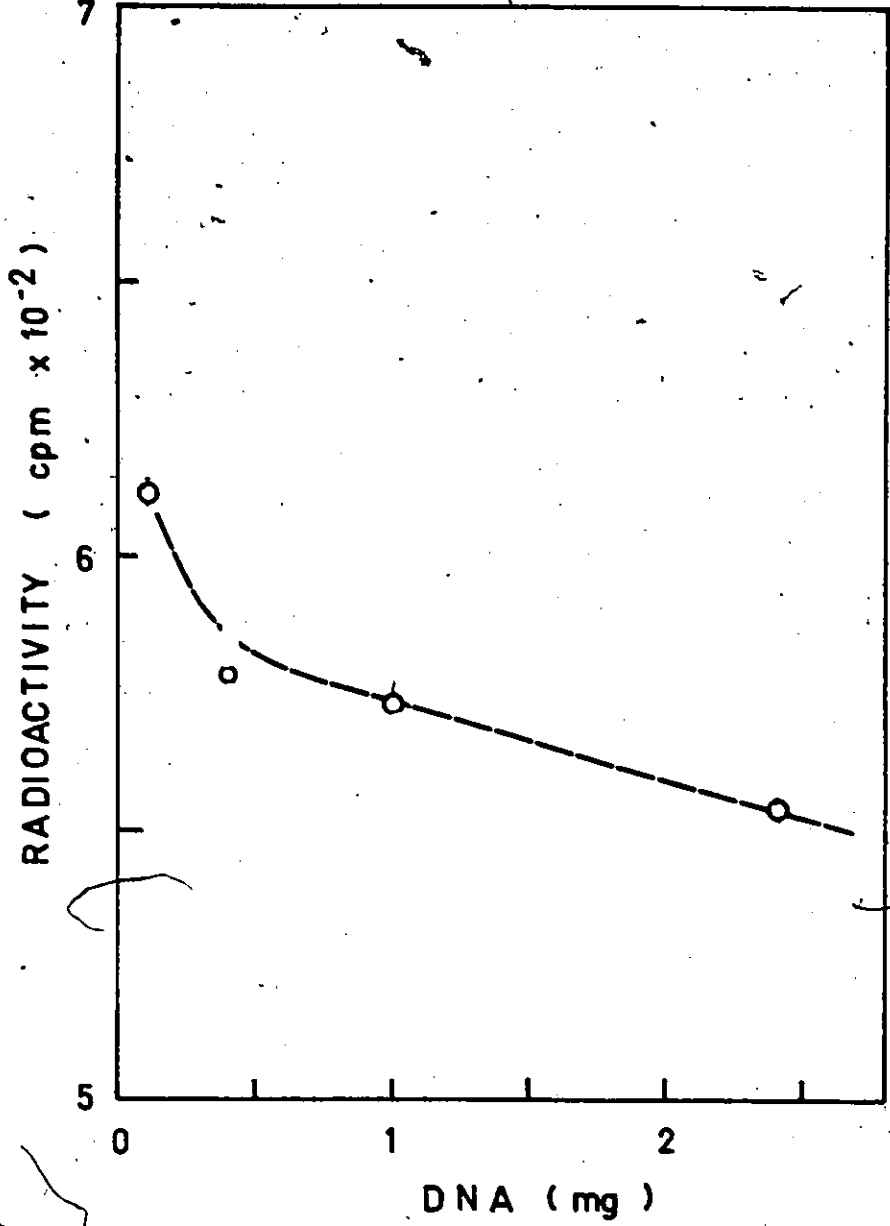
Sample	In 4 ml H ₂ O	In 4 ml 0.14 M PB plus 0.2 ml 50% TCA	In 4 ml 0.4 M PB plus 0.8 ml 50% TCA
1	6446	5740	3330
2	6362	5443	3654
3	-	5592	3346
Average counts	6404	5592	3443
Correction factor	1	1.14	1.86

Figure 9

Effect of DNA in Liquid Phase on the Quenching
of Tritium Radioactivity

^3H -labelled Ad 12 DNA in 4 μl of 0.4 M PE, counted
10 ml of Triton X-114 counting fluid with varying
amounts of salmon sperm DNA.

48



hence is not considered in calculations of single stranded percentages.

The quality of each newly made batch of hydroxy-apatite was monitored by its ability to bind single- and double-stranded DNA. The capacity to bind double-stranded DNA was found to be rather consistent from batch to batch. Some single-stranded DNA was not eluted from the HAP columns by 0.14 M PB. The amount of single-stranded which has this "zero-time binding" property varied from batch to batch ranging from 3% to 15%. Usually this can be handled by several procedures; like boiling the HAP crystals in SDS before use, introducing single-stranded calf-thymus DNA, or correcting it mathematically.

In the presentation of reassociation results, either the traditional plot of fraction of DNA reassociated against $\log C_0t$ (Kohne and Britten, 1971) or linear plot of $1/f_{ss}$ against time of reassociation (Lee and Mak, 1977) were used, as appropriate. C_0t is the product of DNA concentration (C_0) and the time (t) of reassociation. As rightly pointed out by Kohne and Britten (1971), C_0t is most easily calculated as the optical density at 260 nm of the dissolved DNA times the hours of incubation, divided by two. This approximates the usual units (second x mol/liter) since the extinction coefficient of DNA under these conditions is about 7200. The conventional unit of seconds-mol/liter has been used

throughout for the units of Cot so that results presented in this work can be interpreted and contrasted with those in the literature.

Ad2 DNA was fragmented by sonication and reassociated at 8.15 $\mu\text{g/ml}$ (Fig. 10). Ad12 DNA was fragmented by alkaline degradation and reassociated at 0.18 $\mu\text{g/ml}$. (Fig. 11) The slope of linear plot was determined by linear regression using the least square method or from a straight line drawn by eye. In both cases, the traditional and linear plots were displayed. The single sigmoidal component and the linearity of the traditional and linear plots, respectively, is consistent with the fact that adenovirus genome has only one genetic complexity. The terminal redundant sequences in these genomes represent less than 1% of the genome; hence they do not affect the reassociation kinetics.

The $Cot_{1/2}$ value can be read off Figure 10A to be 2.5×10^{-2} sec-mol/liter, or calculated from the slope* of Figure 10B:

$$\begin{aligned} \text{slope} &= KCo \\ &= \frac{(8-1) \times 60}{145} \\ &= 2.9 \\ \therefore K &= \text{slope}/Co \\ &= \frac{2.9}{(0.175 \times 0.5)} \\ &= 33.1 \end{aligned}$$

* Slopes determined by the methods described above were similar. In subsequent graphs on reassociation data, slopes were determined by lines drawn visually.

Figure 10

Reassociation of Ad 2 Viral DNA

8.15 $\mu\text{g/ml}$ of Ad 2 DNA sonicated to 8S denatured by boiling for 10 min, reassociated in 0.14 M phosphate buffer at 65°C and assayed with HAP columns.

- A) Plot of single-stranded DNA percentage versus Cot
- B) Plot of reciprocal of single-stranded DNA percentage versus time.

51 ✓

✓

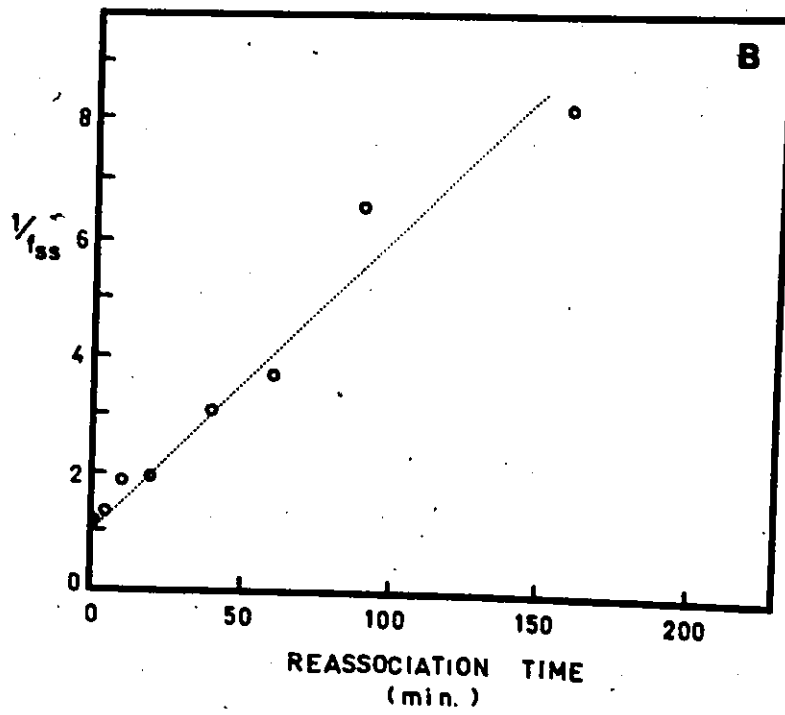
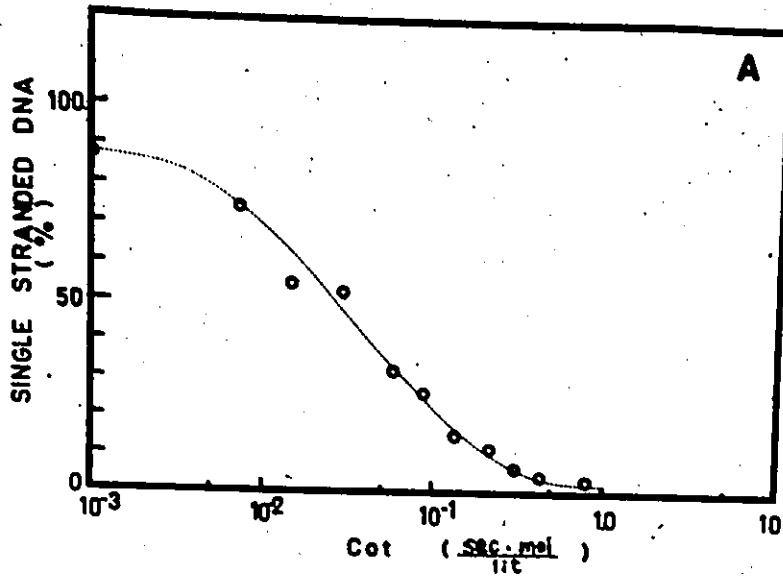


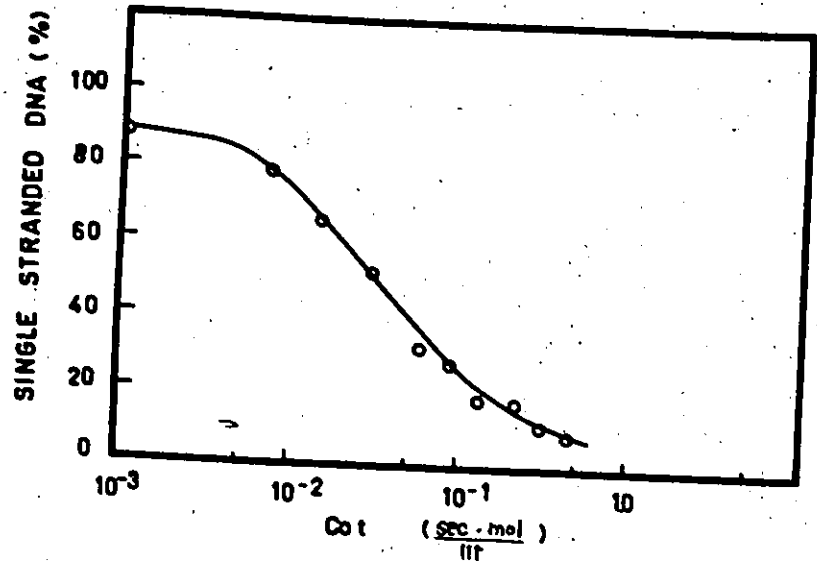
Figure 11

Reassociation of Ad 12 DNA

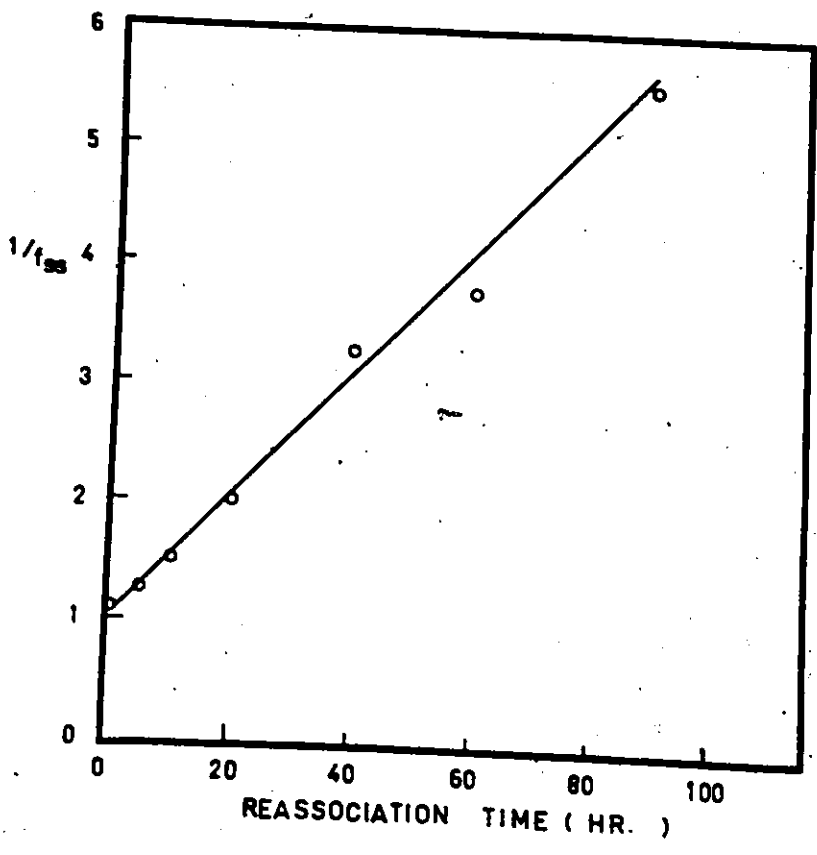
1.8×10^{-1} $\mu\text{g/ml}$ of fragmented Ad 12 DNA denatured and reassociated in 0.14 M PB at 65°C .

- A) Classical reassociation plot of percentage in single stranded form versus C_0t .
- B) Linear plot of $1/f_{ss}$ versus time.

52



R



from formula (2), at half reassociation,

$$\begin{aligned} 2 &= 1 + K \text{Cot}_{\frac{1}{2}} \\ \therefore K &= \frac{1}{\text{Cot}_{\frac{1}{2}}} \end{aligned} \quad (3)$$

hence

$$\begin{aligned} \text{Cot}_{\frac{1}{2}} &= \frac{1}{K} \\ &= \frac{1}{33.1} \\ &= 3 \times 10^{-2} \text{ sec-mol/liter} \end{aligned}$$

The $\text{Cot}_{\frac{1}{2}}$ value for Ad 12 DNA in 0.14 M PB was similarly determined to be 0.027 sec-mol/liter, consistent with the values for Ad 2 DNA. This value is also consistent with the $\text{Cot}_{\frac{1}{2}}$ value for adenovirus genome reported by others (Gallimore *et al.*, 1974; Green *et al.*, 1976).

To estimate the reproducibility of reassociation, a known amount of unlabelled Ad 12 DNA was mixed with Ad 12 radioactively labelled probe. The slopes of $1/f_{ss}$ versus time plot for reassociation of $1.5 \times 10^{-2} \mu\text{g/ml}$ of probe alone and driven by $1.5 \mu\text{g/ml}$ of unlabelled DNA was determined from Figure 12. Calculations showed that the reassociation rate was increased by 103 x in the presence of 100 times concentration of the probe, and the amount of unlabelled viral DNA was found to be $1.53 \mu\text{g/ml}$. Considering the input of $1.5 \mu\text{g/ml}$ represents about 2% deviation when the input of unlabelled virus is reduced this deviation is expected to increase. In subsequent experiments, reconstructions were frequently done to

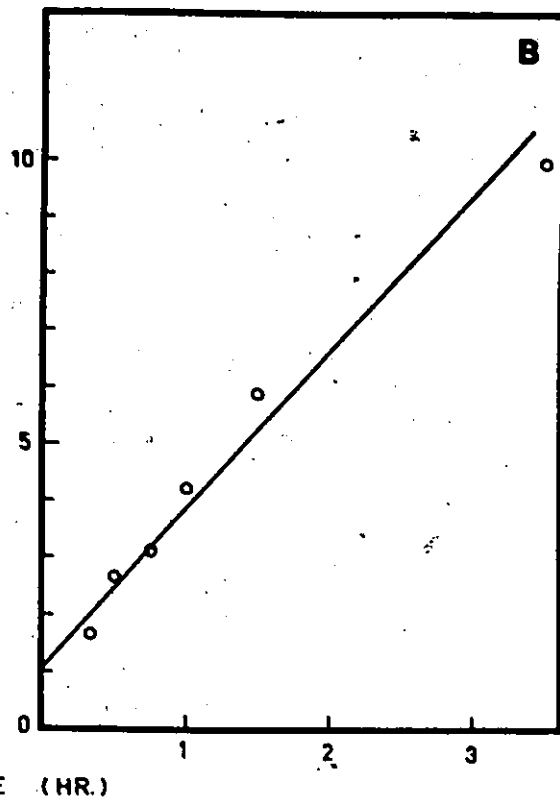
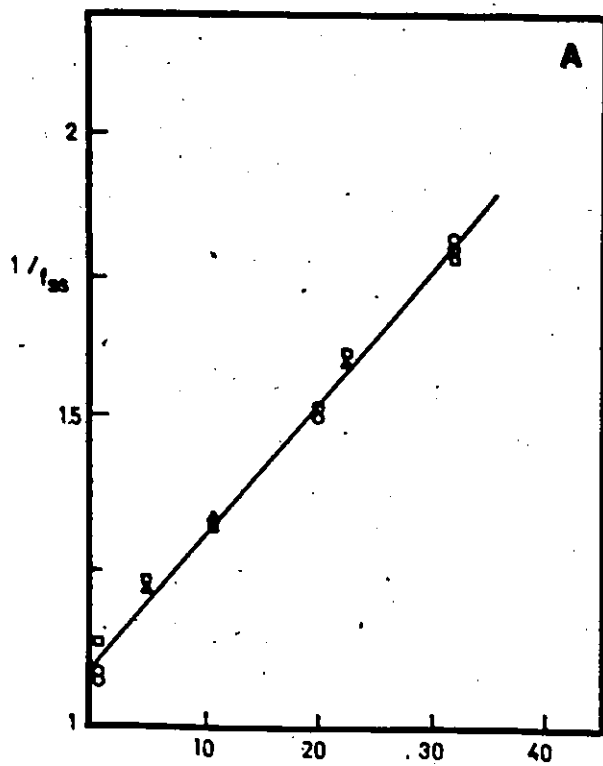
Figure 12

Reconstitution Experiment to Test
Reproducibility of Reassociation

○ Δ □ Plot of $1/f_{ss}$ versus time of the reassociation
of 1.5×10^{-2} $\mu\text{g/ml}$ of $^3\text{H-Ad 12 DNA}$ in 1 M
NaCl at 65°C assayed by HAP (triplicate expt.).

B
○ Plot of $1/f_{ss}$ versus time of the reassociation
of 1.5×10^{-2} $\mu\text{g/ml}$ of $^3\text{H-Ad 12 DNA}$ plus
 $1.5 \mu\text{g/ml}$ of unlabelled Ad 12 DNA in 1 M NaCl
at 65°C , assayed by HAP.

54a



estimate the accuracy of reassociation in the range of unknown DNA concentration. Table 3 shows the calculations involved in determining an unknown amount of reassociating DNA.

II. Viral Genome in Transformed Cells

Ad 12 can transform hamster embryo cells. These transformed cells contain viral DNA sequences (Green, 1970; Green, 1976) and synthesize virus-specific RNA (Fujinaga and Green, 1966; Smiley, 1977) and tumor (T) antigens (Huebner *et al.*, 1962). A transformed cell line, T₆ was examined for the presence of Ad 12 DNA. The method of DNA-DNA reassociation using viral DNA probe representing the entire viral genome was used (Gelb *et al.*, 1971; Petterson and Sambrook, 1973). Figure 13 shows the reassociation of 1.5×10^{-2} μ g of probe DNA and reassociation driven by the DNA extracted from T₆ cells. The reconstruction experiments were also included. An unlabelled viral DNA at 17.3 times the concentration of the probe DNA was used. From the increase in the rate of reassociation of the probe, the concentration of unlabelled viral DNA was calculated to be 19.5 times that of the probe. The DNA content of T₆ cells was determined by diphenylamine reactions to be 12 pg per cell. The viral genome equivalent was calculated to be 1.2 copies per diploid cell and 2.4 per transformed cell.

Table 3

Sample Calculations to Demonstrate the
Reproducibility of DNA reassociation

Slope of reassociation of 1.5×10^{-2} $\mu\text{g/ml}$ of $^3\text{H-Ad 12 DNA}$

$$S_c = \frac{4.2 - 1}{105} = 0.0305$$

Slope of reassociation of 1.5×10^{-2} $\mu\text{g/ml}$ of $^3\text{H-Ad 12 DNA}$
plus $1.5 \mu\text{g/ml}$ of Ad 12 DNA

$$S_s = \frac{6.0 - 1.0}{1.6} = 3.125$$

$$\begin{aligned} \text{Rate of increase in slope} &= \frac{S_s}{S_c} \\ &= \frac{3.125}{0.0305} \\ &= 102.5 \end{aligned}$$

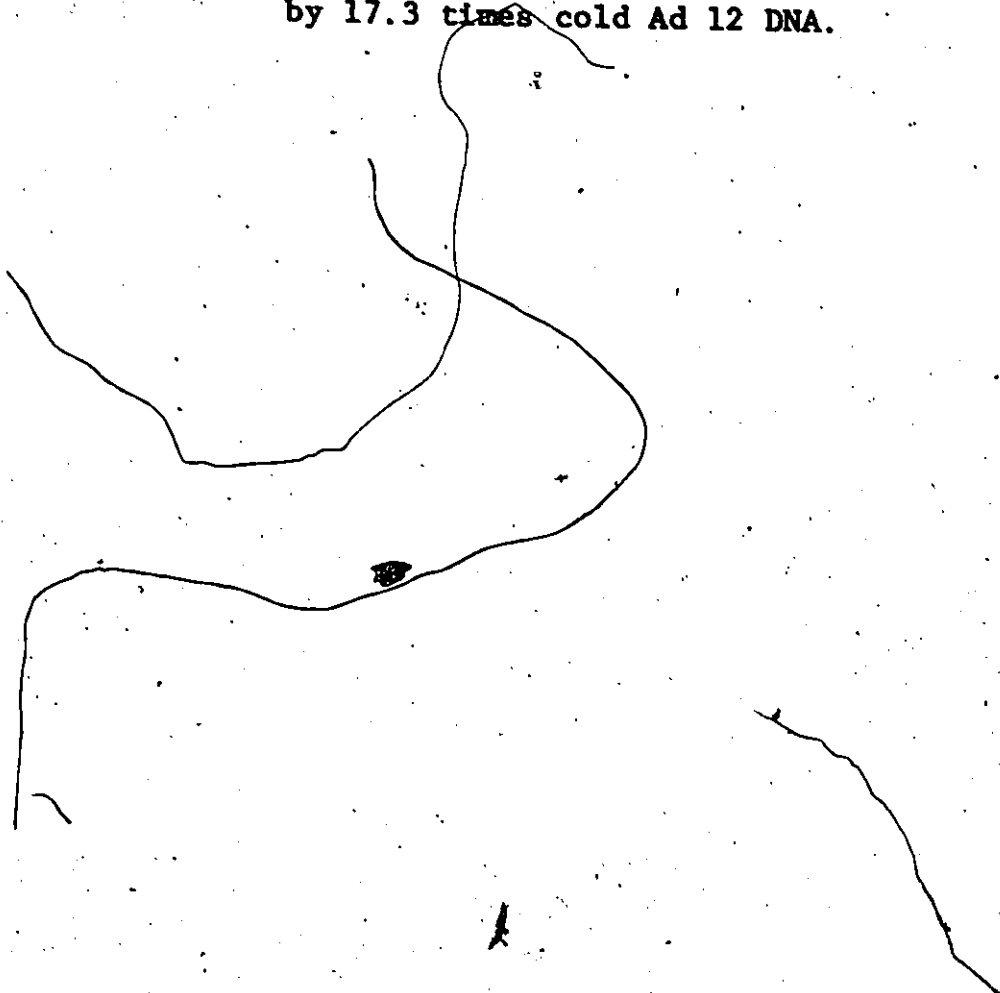
$$\begin{aligned} \text{Amount of viral DNA present} &= 102.5 \times 1.5 \times 10^{-2} \mu\text{g/ml} \\ &= 1.54 \mu\text{g/ml} \end{aligned}$$

$$\begin{aligned} \text{Amount of viral DNA added} &= 1.54 \mu\text{g/ml} - 1.5 \times 10^{-2} \mu\text{g/ml} \\ &= 1.53 \mu\text{g/ml} \end{aligned}$$

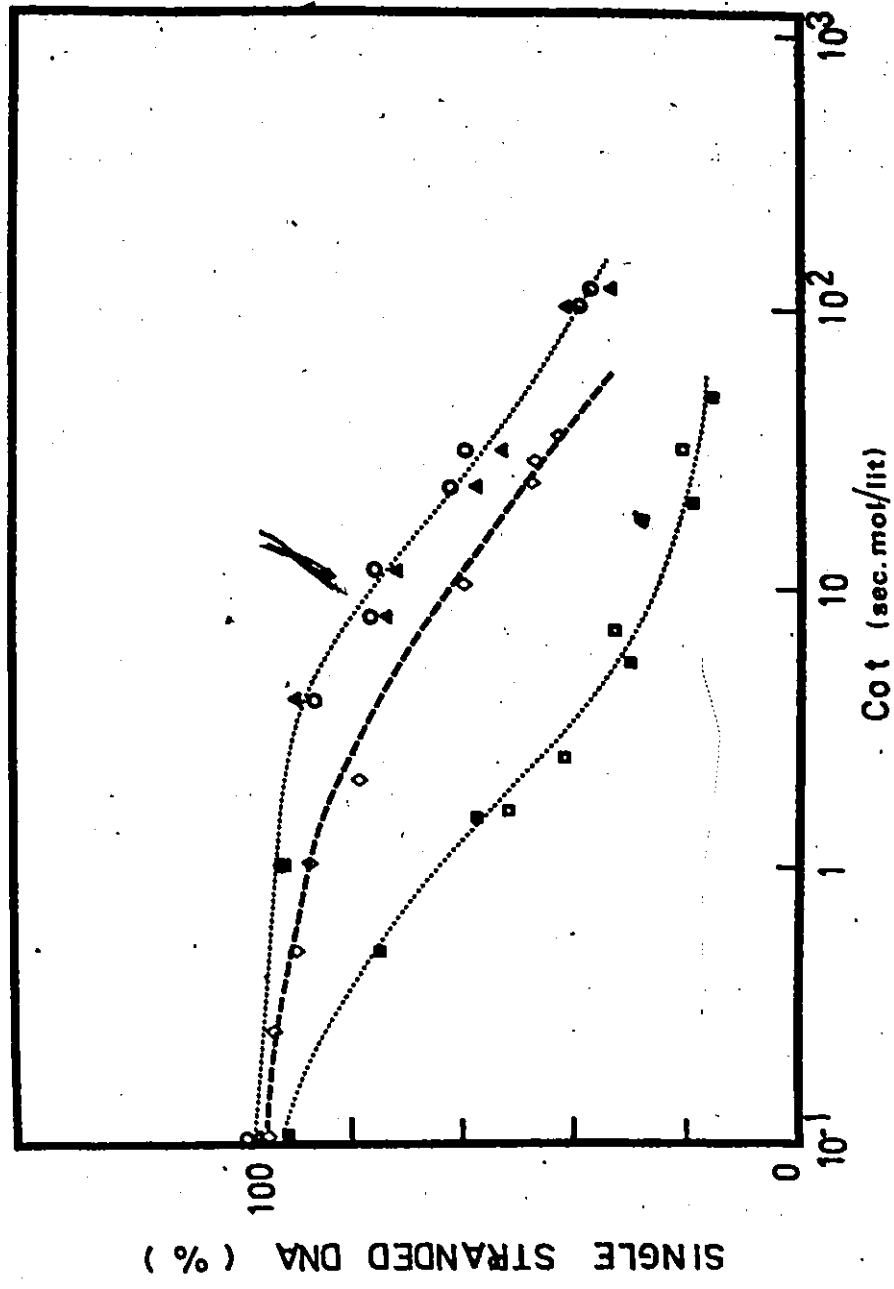
Figure 13

Reassociation of Probe DNA Driven by
DNA from T₆ Cells

- ³H Ad 12 + 2 mg/ml salmon sperm DNA.
- △ ³H Ad 12 + 2 mg/ml salmon sperm DNA.
- ◇ ³H Ad 12 + 2 mg/ml T₆ cell DNA.
- two independent reassociations driven
by 17.3 times cold Ad 12 DNA.



57a



DNA probes representing the whole viral genome were used in the above approach. This method is subject to some limitations. If the integrated genome is unequally represented in the transformed cells, for example, if a certain segment of the viral genome occurred several times more than others, the probe would be driven by this higher concentration of viral DNA to reassociate faster. Meanwhile, other segments of the probe would reassociate at a rate determined by the lower DNA concentration of these segments. The overall result would be misleading if several components co-exist in a reassociation.

One can use Eco RI restriction enzymes to digest Ad 12 DNA. This enzyme cleaves the double-stranded Ad 12 DNA at specific sites containing these base sequences:

5' - GAATTC - 3'

3' - CTTAAG - 5'

by introducing single-strand breaks between the guanine and adenine residue on each of the two DNA strands (Hedgpeth *et al.*, 1972). Digestion of Ad 12 DNA with Eco RI restriction enzyme results in the production of equimolar amounts of DNA fragments that can be isolated by electrophoresis. The digestion sites on the Ad 12 DNA molecule are as follows: (Mulder *et al.*, 1974)

16.3	11.5	27.4	7.2	2	35.6	% of length
C	D	B	E	F	A	Designation of fragment

Eco RI restriction enzyme was isolated from *Escherichia coli*, strain RY13 (Pettersson and Philipson, 1974). Figure 14A shows the radioactivity from the cleaved DNA fragments as they were separated by electrophoresis in agarose gels. The sequence and relative position of these peaks correspond to microdensitometer tracings of their autoradiographs as shown in Figure 14B. The area under each peak was determined and compared to the percentage of length of the viral DNA. It was important to determine whether thymidine incorporation is linearly proportional to the size of these fragments. In small quantities, radioactivity will be the only parameter available to estimate the amount of DNA used in reassociation.

The viral DNA fragments were retrieved from agarose gels through perchlorate treatment of the gels, bound to HAP and eluted. To ensure that these steps of extraction did not alter the reassociation kinetics of these fragments, their $Cot_{1/2}$ values were determined.

Table 4 shows comparisons of distribution of radioactivity and fractional length of fragments. It also displays theoretical $Cot_{1/2}$ and observed $Cot_{1/2}$ values for these fragments. Here, the theoretical $Cot_{1/2}$ values were calculated from the $Cot_{1/2}$ of the complete Ad 12 genome and the sizes of the fragments. No drastic differences were noticed, hence the five fragments recovered from the agarose gels were the fragments A, B, C,

Figure 14

Eco RI Fragments of Ad 12 DNA

- a. Radioactivity counts of ^3H thymidine labels
- b. Microdensitometer tracing of autoradiography of DNA in dried gels.

600

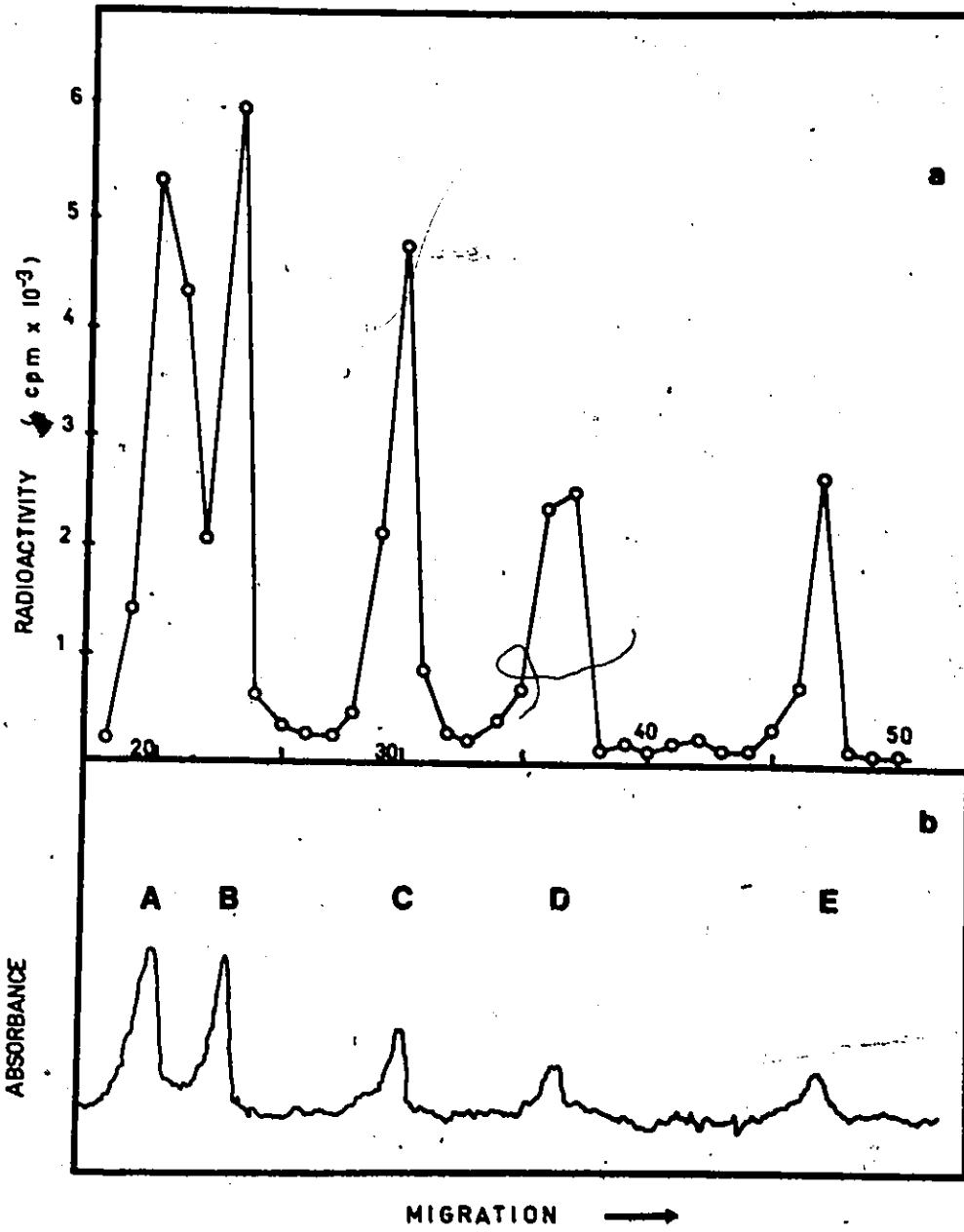


Table 4

Characteristics of Eco RI Generated Fragments of Ad 12 DNA

Fragment	Fractional length (%) (a)	Distribution of radioactivity	Theoretical Cot _{1/2} mol. sec/liter [Na ⁺] = 1.0 M	Observed Cot _{1/2} mol. sec/liter [Na ⁺] = 1.0 M
A	35.6	37.6	1.65 x 10 ⁻³	1.27 x 10 ⁻³
B	27.4	26.4	1.27 x 10 ⁻³	1.26 x 10 ⁻³
C	16.3	15.7	0.76 x 10 ⁻³	1.1 x 10 ⁻³
D	11.5	11.8	0.53 x 10 ⁻³	0.77 x 10 ⁻³
E	7.2	8.4	0.33 x 10 ⁻³	0.35 x 10 ⁻³

(a) Reference Mulder *et al.* (1974).

D, E as reported in the literature (Mulder *et al.*, 1974) and the extraction procedure did not alter their reassociation characteristics. Fragment F represents 2% of the viral genome. Its Cot_x is so small that it is not a suitable probe to quantitate less than 10 viral genomes per cell under the present experimental conditions.

Each fragment was used as a probe. The probe was mixed with DNA extracted from T6 cells and another Ad 12 transformed cell line T2 cells. The probe DNA concentration used was as follows: fragment A: $7 \times 10^{-3} \mu\text{g/ml}$; fragment B: $4.7 \times 10^{-3} \mu\text{g/ml}$; fragment C: $1.2 \times 10^{-2} \mu\text{g/ml}$; fragment D: $5.5 \times 10^{-3} \mu\text{g/ml}$; and fragment E: $1.2 \times 10^{-3} \mu\text{g/ml}$. It has been observed that viral sequences in transformed cells could vary upon passage of cells, as was in the case of the hamster cell line 333-2-29 transformed by HSV-2 (Frenkel *et al.*, 1976). To explore this possibility in the Ad 12 transformed cell lines, the viral genome in an early (21^{st}) passage and in a late passage (more than 60^{th}) of T6 was quantitated using Eco RI restriction enzyme generated probes.

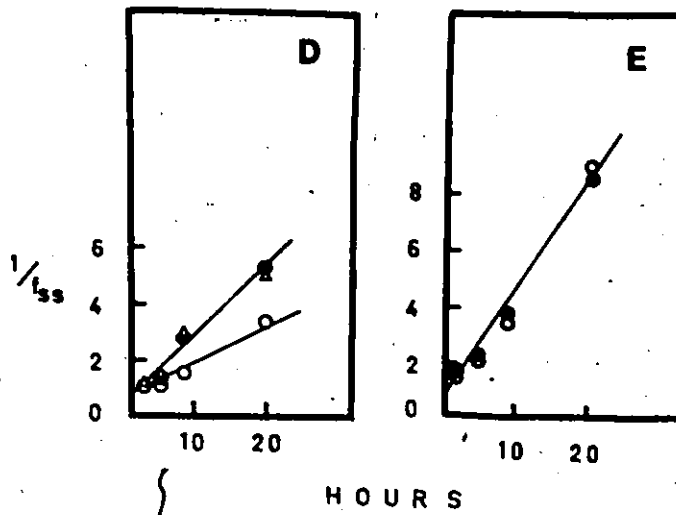
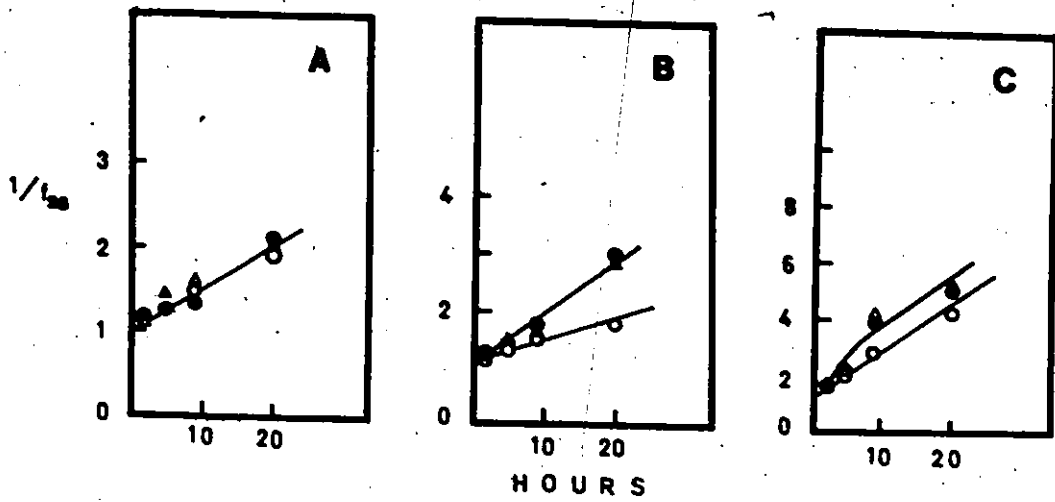
Figure 15 shows the reassociation kinetics of the probes alone and being with DNA extracted from the transformed cells (T6). The reassociation of fragment A was not accelerated either by the early or by the late passage T6 cell DNA, indicating that these cells contain no detectable amount of A fragments. The same situation was

Figure 15

Kinetics of Reassociation of Eco RI Cleavage Fragments of ^3H -labelled Adenovirus 12 DNA in the Presence of Salmon Sperm DNA and DNA from Early and Late Passages of T6 Cells

- Reassociation of viral probe in 2.2 mg/ml of salmon sperm.
- △ Reassociation of viral probe in 2.2 mg/ml early passage (21st) cultured T6 cells.
- Reassociation of viral probe in 2.2 mg/ml late passage (more than 60th) cultured T6 cells.

63 a



observed with reassociation of E fragment. The reassociation of C fragment is different from that of B and D fragments. Instead of being linear as in B and D, it is curved. This suggests that more than one component of viral genome representation is present. The acceleration in reassociation by early and late passages of T6 was identical, suggesting that there were no differences in the quantities of viral genomes. From the acceleration of the reassociation of these fragments, the viral equivalents were calculated as follows:

Fragment	T6 cells	T2 cells
A	non-detectable	0.4
B	1.2	2.2
C	2.1	2.0
D	3.2	2.7
E	non-detectable	1.7 Ic

The above result suggests that incomplete genome of Ad 12 was present in transformed cells. T2 cells contained less than one copy of Fragment A. This could be due to the fact that some transformed cells might have lost fragment A. Likewise, transformed cells are not always diploid, thus the viral copy numbers are underestimated. Despite that generally Ad-transformed cells contain T antigens (Huebner *et al.*, 1963; Pope and Rowe, 1964; Gilden *et al.*, 1968), both T2 and T6 cells were negative for immunofluorescence. The qualitative complement fixation test showed that T antigen was present in T2 cells but not in the T6 cells.

III. Viral Genome in Tumor Cells

When Ad 12 virus were injected into hamsters, tumors were induced. Cells from the tumors were isolated and the viral sequences in these cells were determined by reassociation techniques using probes derived from complete viral genomes. The probes were fragmented by sonication. Cells from four tumors were examined. Two components of reassociation were observed. From the point at which the reassociation started to deviate from a single component curve, the percentage of viral genome present was estimated. The frequency of occurrence of viral genome per diploid cell DNA was also calculated. These results are tabulated in Table 5.

In the above approach, one is limited to determine whether the entire genome was present in the cells. To improve the sensitivity of detection, DNA-DNA reassociation techniques using Eco RI cleaved Ad 12 DNA fragments was applied. Tumors induced by the highly oncogenic Ad 12 wild-type as well as by the weakly oncogenic cyt 70-mutant of Ad 12 were examined. A representative reassociation plot (from tumor #3) is shown in Figure 16. The amount of various viral DNA sequences present in the cells of six tumors, expressed as copies per diploid amount of DNA (6 pg) is shown in Table 6. Where more than one component in reassociation was observed, the number of copies was calculated from the initial slope of the

Table 5

Viral Genome in Tumor Cells
(complete viral DNA as probe)

Tumor	% of viral genome present	Frequency per diploid cell DNA
A	78	1.00
B	78	0.40
C	56	1.67
D	54	2.05

Figure 16

Kinetics of Reassociation of Eco RI Cleavage Fragments of ^3H -labelled Adenovirus 12 DNA in the Presence of Salmon Sperm DNA and DNA Extracted from Isolated Tumor Cells.

^3H -labelled adenovirus 12 DNA was prepared and cleaved by Eco RI endonuclease. The fragments of DNA were separated by electrophoresis as described in Materials and Methods. Each hybridization mixture contain appropriate amounts of radioactive probe and 2.2 mg/ml of either salmon sperm DNA or tumor cell DNA. The concentration of ^3H -labelled probe was Eco RI fragment A, 7×10^{-3} $\mu\text{g/ml}$; fragment B, 4.7×10^{-3} $\mu\text{g/ml}$; fragment C, 1.2×10^{-2} $\mu\text{g/ml}$; fragment D, 5.5×10^{-3} $\mu\text{g/ml}$; fragment E, 1.2×10^{-3} $\mu\text{g/ml}$.

- Renaturation of ^3H -labelled viral DNA probe in the presence of 2.2 mg/ml of salmon sperm DNA.
- Renaturation of ^3H -labelled viral DNA probe in the presence of 2.2 mg/ml of tumor cell DNA.
- Indicates points where the above (○) and (●) overlap. The percentage of DNA in single-strand of a reassociation reaction is expressed as $f_{ss} = 1/(1 + KCot)$

67a

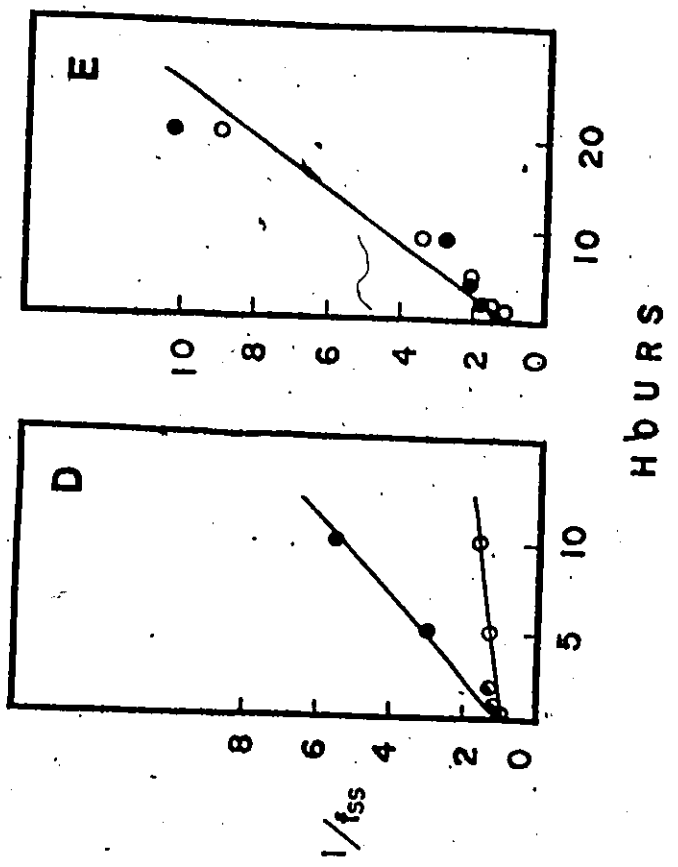
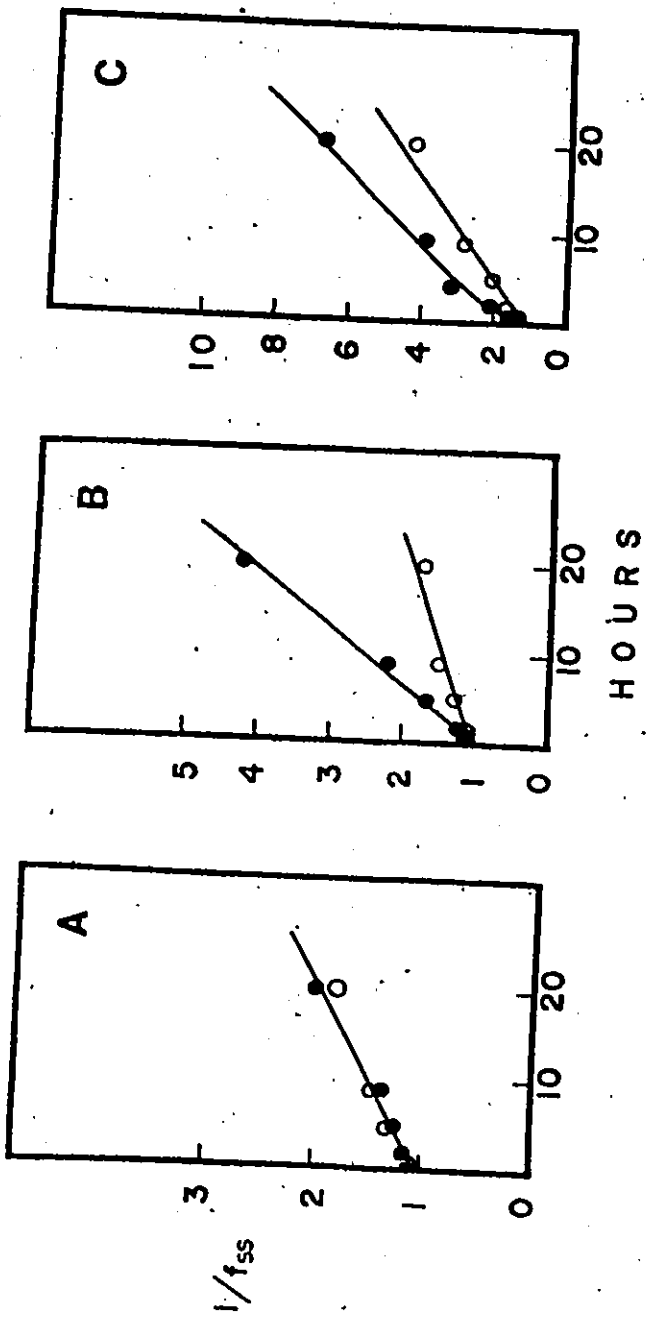


Table 6

Amounts of Adenovirus Type 12 DNA Fragments in Tumor Cells
(number of copies/diploid amount of DNA)

Tumor	Viral DNA fragments generated by Eco RI					
	C	D	B	E	F	A
1	5.6	2.7	3.5	N.D.	N.T.	N.D.
2	1.3	5.9	3.3 (I.C.)	N.D.	N.T.	N.D.
3	11.8 (I.C.)	10.6	3.6	N.D.	N.T.	N.D.
4	11.2	3.5	1.4	N.T.	N.T.	0.6
5	7.5	3.1	1.2	N.T.	N.T.	N.D.
cyt (1)	2.8	2.4	5.4	N.T.	N.T.	N.D.
(2)	3.6	N.T.	4.55	N.T.	N.T.	0.13

N.D. not detectable

N.T. not tested

I.C. incomplete

cyt cyt mutant induced tumor

reassociation curve. The viral DNA sequences in one tumor (cyt mutant induced) was measured twice to test reproducibility.

In all tumors tested, Eco RI fragment A was absent or present in very low amounts. Also fragment E was not detected in the tumors tested. It can also be seen that viral DNA sequences are not represented in equimolar quantities. The tumor induced by the low oncogenic cyt mutant shows a pattern of viral DNA sequences similar to those induced by the highly oncogenic Huie strain.

Since the number of copies of viral fragments varies within each tumor as well as between different tumors, it was essential to determine the sensitivity of our assay system. Since our ^3H -labelled probes were prepared using Ad 12 strain 1131, whereas several tumors tested were induced by the Ad 12 Huie strain, the rare occurrence of fragment A in the tumors could be due to the fact that the two strains of virus may not be completely homologous. To obviate this objection, reconstruction experiments were carried out. Figure 17 shows the reassociation kinetics of ^3H -labelled fragment A in the presence of different amounts of cold viral DNA (Huie strain). The amount of cold viral DNA added was equivalent to 1.5, 3.0 and 10 copies of fragment A per diploid amount of cell DNA. It can be seen that our assay system could detect the presence of about one

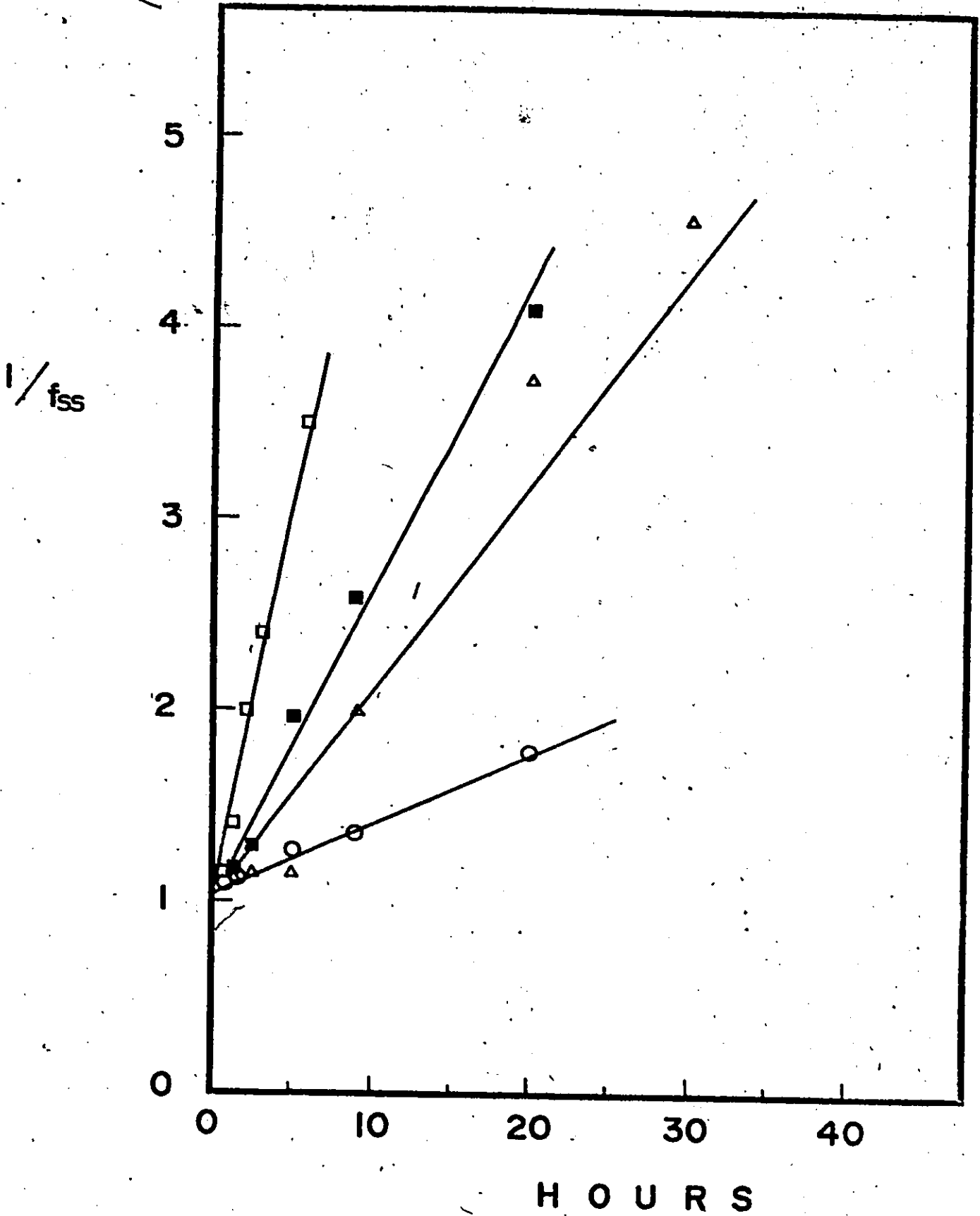
Figure 17

Reconstruction Experiment with Fragment A

7.5×10^{-3} $\mu\text{g/ml}$ of A fragment was co-incubated with various concentrations of unlabelled Ad 12 DNA (Hole strain).

- 7.5×10^{-3} $\mu\text{g/ml}$ of fragment "A" in 2.2 mg/ml of salmon sperm DNA.
- △ 7.5×10^{-3} $\mu\text{g/ml}$ of fragment "A" plus 2.2×10^{-2} $\mu\text{g/ml}$ of Ad 12 DNA in 2.2 mg/ml of salmon sperm DNA.
- 7.5×10^{-3} $\mu\text{g/ml}$ of fragment "A" plus 4.4×10^{-2} $\mu\text{g/ml}$ of Ad 12 DNA in 2.2 mg/ml of salmon sperm.
- 7.5×10^{-3} $\mu\text{g/ml}$ of fragment "A" plus 2.0×10^{-1} $\mu\text{g/ml}$ of Ad 12 DNA in 2.2 mg/ml of salmon sperm DNA.

702



copy of fragment A per diploid amount of tumor cell DNA.

Eco RI cleaves Ad 12 DNA into six fragments, and the order is C,D,B,E,F,A, C being the left-hand end (Graham *et al.*, 1974). Our data indicate that more than 35% of the right-hand end (fragment A) is missing in all tumors examined. The other fragments were represented in non-equimolar amounts. Qualitatively, these results are similar to those of Ad 2 and Ad 5 transformed cells but different from those of Ad 12 transformed cells and a tumor cell line (HT2) (Fanning and Doerfler, 1976; Green *et al.*, 1976).

The A fragment generated by Eco RI cleavage of Ad 12 DNA was consistently missing in tumors. This has necessitated more attention to the homology of A fragments in different strains of Ad 12. The homology in the DNA of Ad 12 strains 1131 and Huie were studied by saturation hybridization.

A trace amount of A fragment from strain 1131 was allowed to reassociate alone, driven by vast excess amount of DNA from strain Huie and strain 1131. The homologues after reassociation were assayed with S1 nuclease as well as hydroxyapatite chromatography. Hydroxyapatite binds DNA strands which contain a few base pair sequences (Bernardi, 1969). S1 nuclease is an endonuclease that cleaves single-stranded regions (Sutton, 1971).

Under exhaustive hybridization, S1 nuclease assay gives a more realistic idea of homologous regions than HAP. Figure 18 shows that under conditions when A fragment (from 1131) itself has minimal reassociation, and DNA from 1131 strain can saturate the hybridization to about 85%, DNA from Huie strain can only saturate to about 60% of the A fragment in hybrid form. From this experiment, it seems that 70% of A fragment of 1131 is homologous to DNA from Huie strain. Figure 17 shows that using Huie strain DNA, one can measure a minimum of one copy of fragment A per diploid amount of cell DNA and $1/f_{ss}$ plot was linear for most of about 70% of reassociation. This result agrees with those of Figure 18. The homology between these two strains was studied in finer details by Smiley (1977).

Cell DNA from one of the tumors (#3) was tested for the presence of subfragments of A. The enzyme Hind III cleaves Ad 12 DNA into the fragments F, D, C, A, G, J, K, L, B, H, I, E (Smiley, Ph.D. thesis), this, with the map for Eco RI to show corresponding positions are shown below:

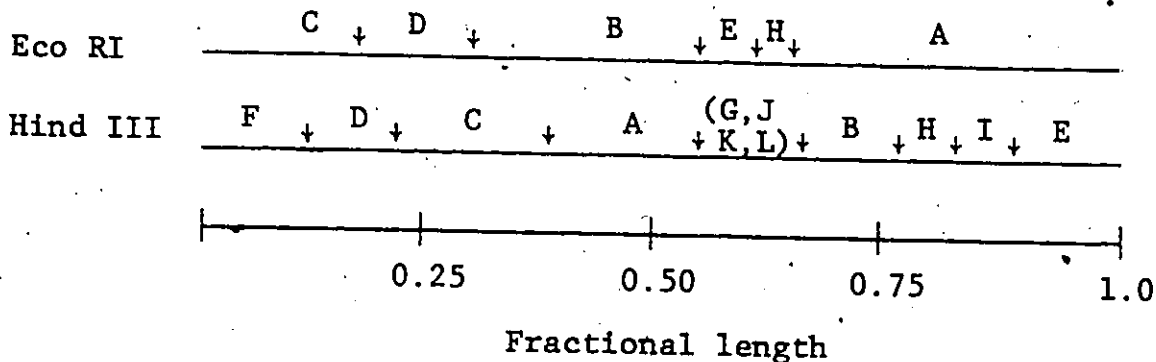
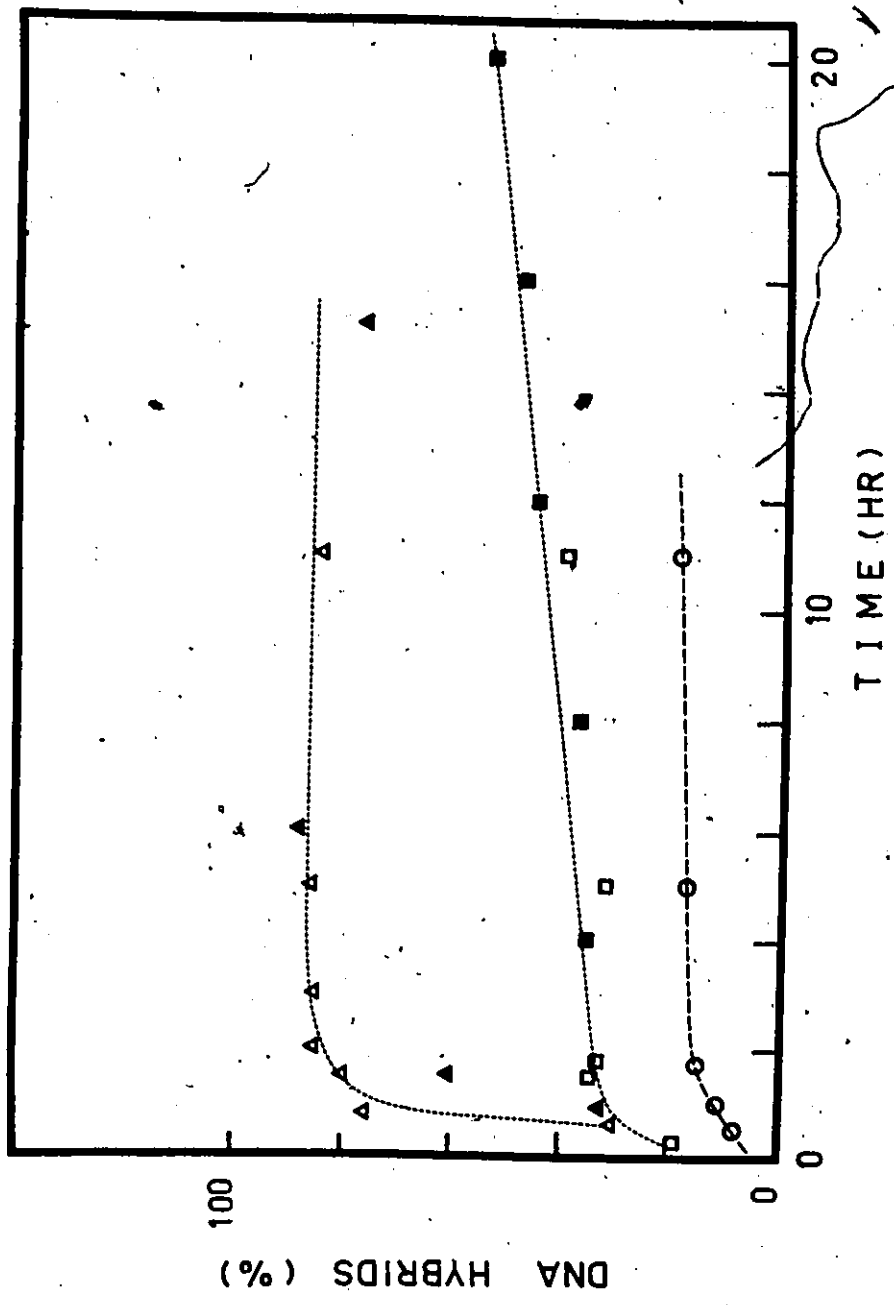


Figure 18

Saturation Hybridization of A Fragment from Strain 1131
Driven by DNA from Strains 1131 and Huie

- Reassociation of trace amount of radioactively labelled DNA from strain 1131.
 - □ Reassociation of the same amount of viral DNA driven by DNA extracted from Huie strain and present at 1.5 $\mu\text{g/ml}$ in the reassociation mixture (2 experiments), S1 nuclease assay.
 - ▲ △ Reassociation of the same amount of viral DNA driven by DNA extracted from strain 1131 and present at 1.5 $\mu\text{g/ml}$ in the reassociation mixture (2 experiments).
- △ S1 nuclease assay.
- ▲ HAP assay.

73 e



It can be seen that the A fragment of Eco RI corresponds to B,H,I,E fragment of Hind III. In order to test for minute amount of genome in this region, and with particular interest in terminal sequences which may control integration, the presence of Hind III H and E fragments in a tumor (#3) was tested. From Fig. 19 which shows reassociation of H and E in the presence of tumor cell DNA, no increase in reassociation rates were observed suggesting absence of these fragments.

It should be noted that the DNA reassociation experiments were carried out at high ionic strength and suboptimal temperature. Therefore, mismatching of bases occurred. This contributes to the error in the estimates of the number of copies of viral DNA sequences in tumor cells. Another source of error may be due to the variation in DNA fragment size. Under these experimental conditions the reconstruction experiment (Fig. 17) gave a difference of 20% between the theoretical copy calculated from the reassociate rate and the actual copy number determined from the known amount of viral DNA added to the reaction mixture. However, the major conclusion that different fragments of viral DNA were present in unequal molar amounts is still valid.

IV. Integration of Ad 12 Viral DNA Into Infected Cell DNA

Ad 12 infects human KB cells leading to the production of progeny viruses and subsequent cell death. The integration event of Ad 12 DNA into cellular DNA was next studied. To do so, viral DNA that is integrated into cellular DNA must

Figure 19

Quantitation for Hind III H and E Fragments
in DNA from Tumor # 3

A

E fragment

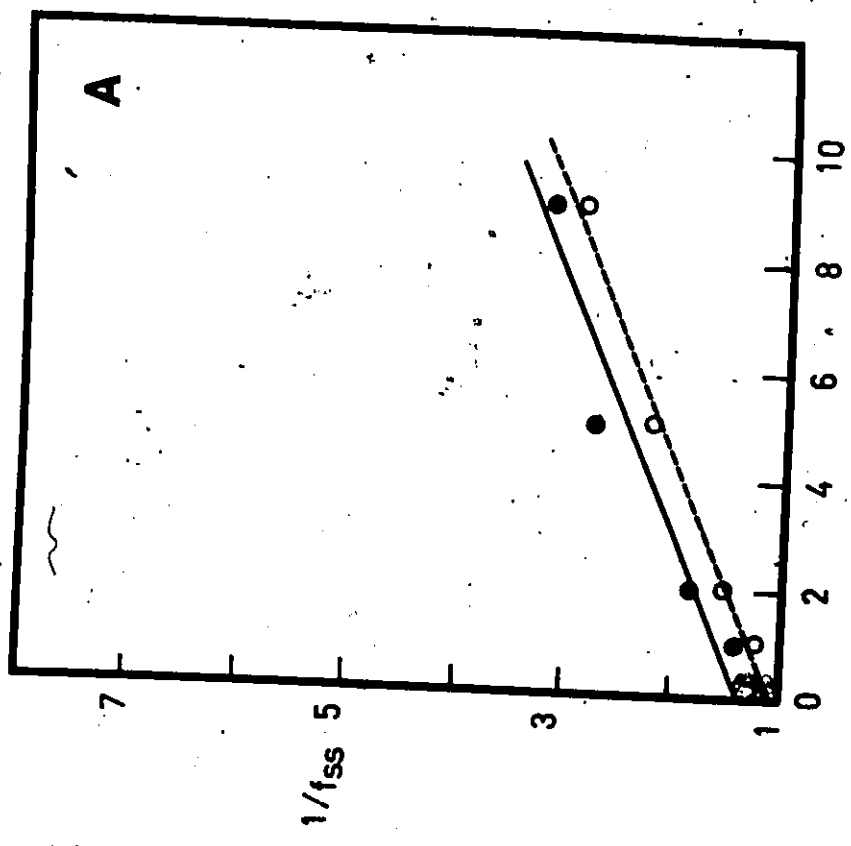
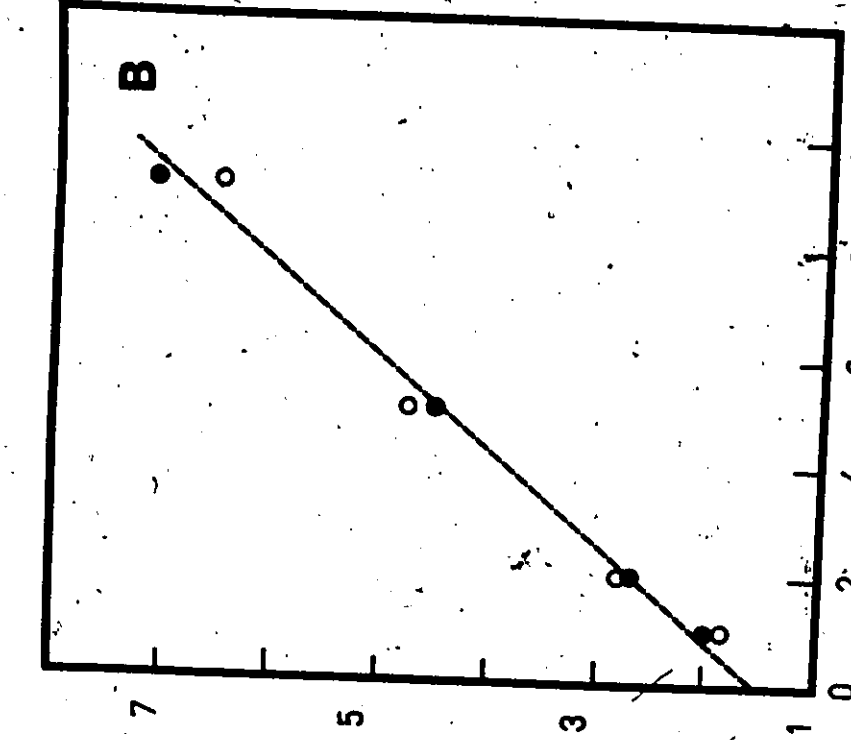
- reassociation of probe.
- reassociation of probe with
2 mg/ml of cell DNA.

B

H fragment

- reassociation of probe.
- reassociation of probe with
2 mg/ml of cell DNA.

75a



TIME (HR.)

$1/f_{ss}$

be separated from intracellular viral DNA that is free. Two methods were used: the network technique and the alkaline glycerol gradient sedimentation technique.

The network technique is based on the fact that mammalian DNA is interspersed with unique and reiterated sequences (Britten and Kohn, 1968; Sanders *et al.*, 1972). Hence, upon low Cot reassociation, the mammalian DNA forms networks due to reassociation of the highly reiterated sequences. This network sediments faster than viral DNA (Varmus *et al.*, 1973). In actual practice, some viral DNA is trapped in this network. A correct value of viral DNA that is covalently-linked to the cellular DNA can be obtained only when this trapped viral DNA is accounted for (Bellett, 1975).

Symbols: I = amount of viral DNA that is integrated
 F = amount of non-integrated viral DNA
 D_n = amount of cellular DNA that forms network
 D_s = amount of cellular DNA that does not form network
 C_n = amount of free viral DNA that is trapped in the network in mixing experiments
 C_s = amount of free viral DNA that remains free after network formation in mixing experiments
 V_n = viral DNA that is detected in the network
 V_s = viral DNA that is detected in the supernatant

Equation:

$$I = \frac{V_n \left[(V_s + V_n) \frac{C_n}{C_n + C_s} \right]}{1 - \left[\frac{D_s}{D_s + D_n} + \frac{C_n}{C_s + C_n} \right]} \quad (3)$$

$$I = V_s + V_n - F \quad (4)$$

In the above formulation $C_n/(C_n + C_s)$ is the fraction of free viral DNA that is trapped in a network.

It can be determined by:

1. measuring radioactivity of ^3H -labelled viral DNA trapped in the network of cell DNA;
2. mixing virions with cells and extracting DNA from the mixture, and then measuring the amount of viral DNA trapped by DNA-DNA reassociation techniques.

A trace amount of radioactively labelled viral DNA was mixed with 300 $\mu\text{g/ml}$ of KB cell DNA. The mixture was denatured and reassociated in 0.6 M NaCl to C_{ot} of 3 sec-mol/liter. Sedimentation using SW50.1 rotor or 50 Ti rotor gave consistent results. About 20% of viral DNA was recovered in the network which contained about 60% of the input cellular DNA. This result is presented in Table 7. Attempts to reduce the level of trapping by performing a second network technique did not reduce the percentage of trapped viral DNA. About 90% of cellular DNA was recovered in the network, however.

Other methods designed with the aim of reducing trapping in the network were tested. This included precipitating the cellular DNA as a complex with its histones in high concentration of NaCl (Hirt, 1967) and centrifuging the network onto a cushion of sucrose or formamide. None were satisfactory. Hence, in subsequent

Table 7

Trapping of Viral DNA in Network of Cellular DNA

Network was pelleted in SW50.1 rotor for 20 min at 40 Krpm, 5°C

Tube	Radioactive counts (cpm)		Recovery of cell DNA (μg)	
	supernatant	network	supernatant	network
		% in network		% in network
1	10578	2255	17.6	130
2	11245	2297	17.0	160
3	11325	2311	16.9	175
4	11531	2125	15.6	155
				165
				55
				56
				54
				55

experiments, networks were separated from free viral DNA and cellular DNA that does not participate in networks by reassociating to $C_{ot} = 6$ sec-mol/liter and centrifuging in SW50.1 at 40 Krpm for 15 min, 5°C. instead of 20 min. as in Tab

Reassociation was used to determine $C_n / (C_n + C_s)$ ratio. Since this ratio was crucial to calculate the level of integration, it was routinely done whenever networks were performed. From more than five determinations it was decided that this ratio was reproducible at $12\% \pm 3\%$.

The application of alkaline glycerol gradient method will be discussed later on.

KB cells infected with 2000 virions per cell were measured for the percentages of integrated viral DNA. Infected cells were harvested at different times post infection and stored in 4°C. DNA was extracted and the network technique and DNA-DNA reassociation methods applied. Figure 20 shows the reassociation of the radioactive probe DNA being driven by the two fractions of DNA.

The following aspects of the kinetics of Ad 12 DNA integration are revealed by the results:

- 1) integrated viral genome of infecting virus was first detected as early as 4 h post infection;
- 2) the amount of integrated viral DNA increased to about 150-200 genome equivalents per cell at about 17 h post infection and remained at this level;
- 3) the integrated viral genome was about 20-30% of the

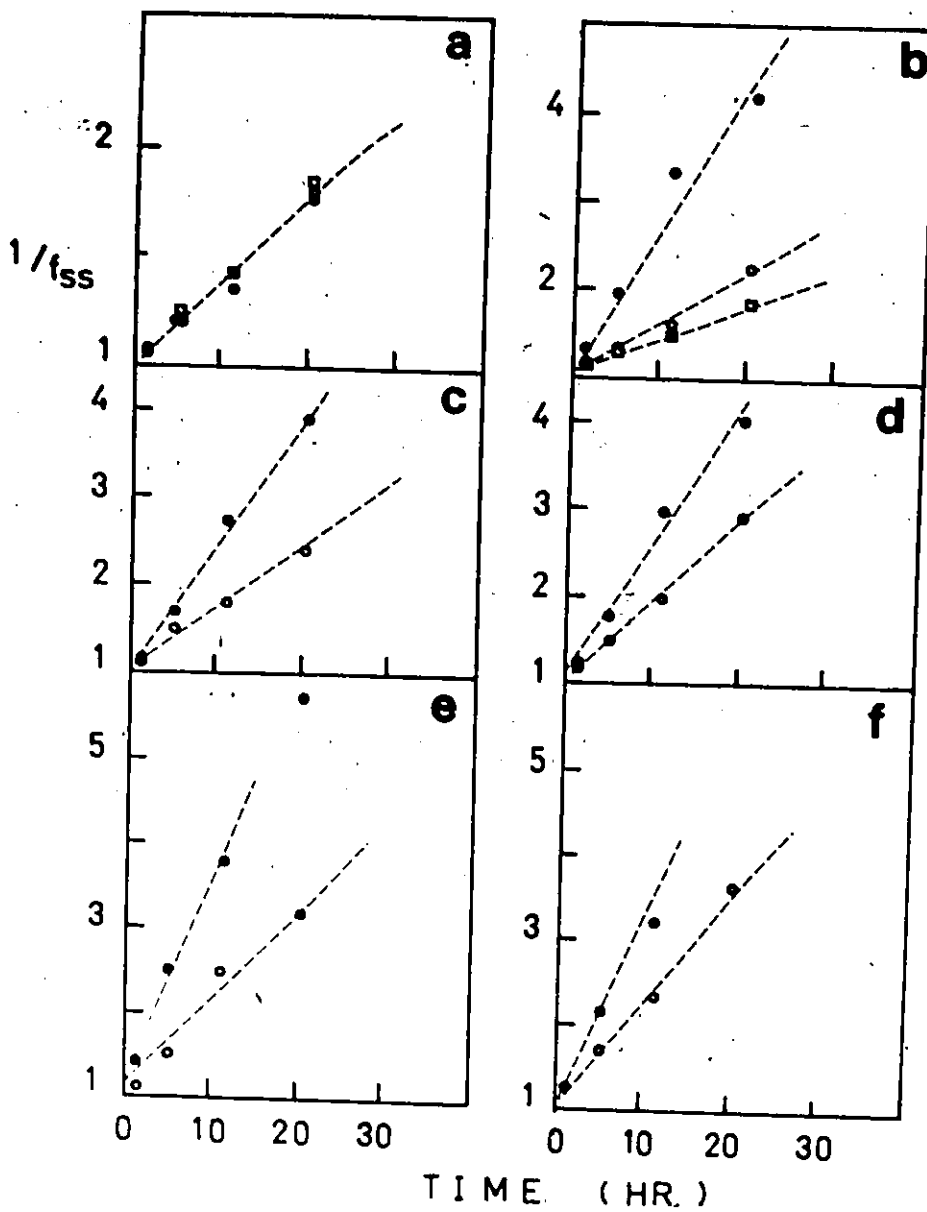
Figure 20

Reassociation of Ad 12 Viral DNA
with Infected KB cell DNA

$1/f_{ss}$ versus t plots of reassociation of radioactively
labelled Ad 12 probe (1.5×10^{-2} $\mu\text{g/ml}$) in the presence
of DNA extracted from network formation.

- reassociation of probe.
 - reassociation of probe with integrated viral DNA.
 - reassociation of probe with non-integrated viral DNA.
-
- A mock infection.
 - B 1.5 h post infection.
 - C 4 h post infection.
 - D 10 h post infection.
 - E 24 h post infection.
 - F 36 h post infection.

80a



total intracellular viral DNA.

These results are summarized in Figure 21.

The kinetics of integration of viral DNA into cellular DNA were also studied using the alkaline glycerol gradient method to separate free viral DNA from the integrated viral DNA. In order to sediment DNA from 10^8 cells, 20-50% alkaline glycerol gradients in 37 ml centrifuge tubes fitted for Beckman SW27 rotor were used. Figure 22 demonstrates that viral DNA can be separated from the bulk of cellular DNA. With SV40 there were suggestions that oligomeric forms of intracellular free viral DNA would co-sediment with high-molecular weight host DNA (Martin *et al.*, 1976). Mixing experiments were done. DNA-DNA reassociations indicate that little detectable trapping of viral DNA in the host DNA fraction occurred (Figure 23). The sensitivity of detection under the conditions of the mixing experiment was about 10 viral DNA copies per cell.

Using the alkaline glycerol gradient method to separate free viral DNA from cell DNA and viral DNA covalently linked to cell DNA, the kinetics of integration of viral DNA were studied. After infection the amount of integrated viral DNA increased until it levelled off after 16 h, at about 150 copies per cell. The percentage of intracellular viral DNA also peaked at 16 h post infection when about 25-30% was integrated.

Figure 21

Summary of Intracellular Viral DNA as a Function of
Post-infection Time

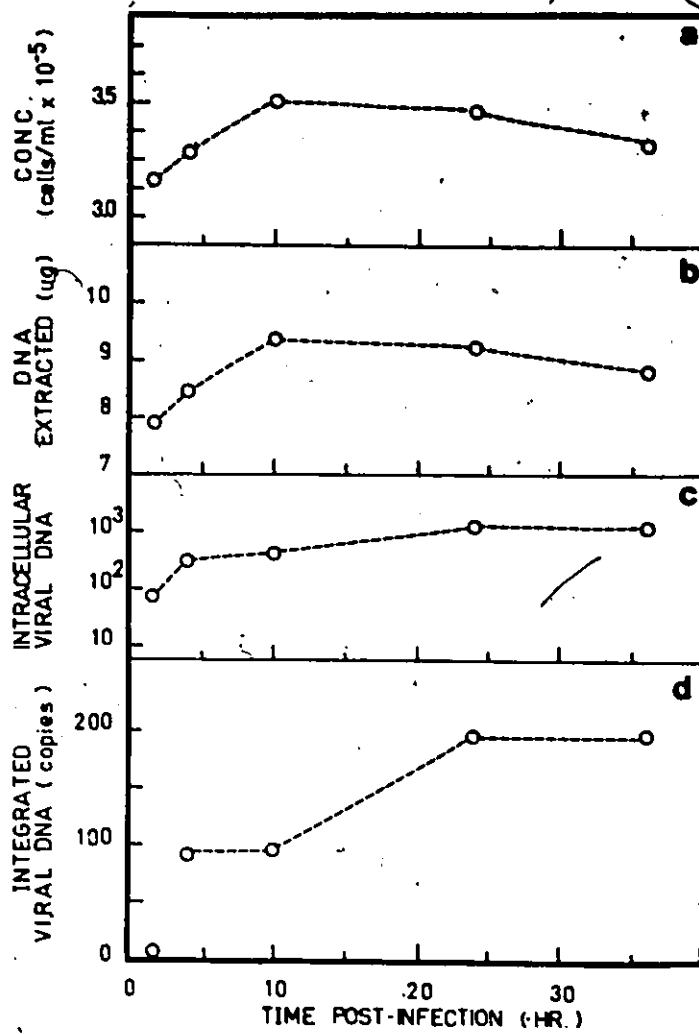


Figure 22

Separation of Viral DNA from Cellular DNA

Trace amount of ^3H -thymidine labelled Ad 12 virus was mixed with 4×10^7 cells and lysed. The lysate, together with a ^{14}C -thymidine labelled Ad 12 virus as a marker were centrifuged in 20-50% alkaline glycerol gradients in SW27 buckets (38 ml) for 5 h at 10°C .

- ^3H radioactivity of Ad 12 viral DNA.
- ^{14}C radioactivity of marker Ad 12 viral DNA.
- Optical density tracing of cellular components.

83a

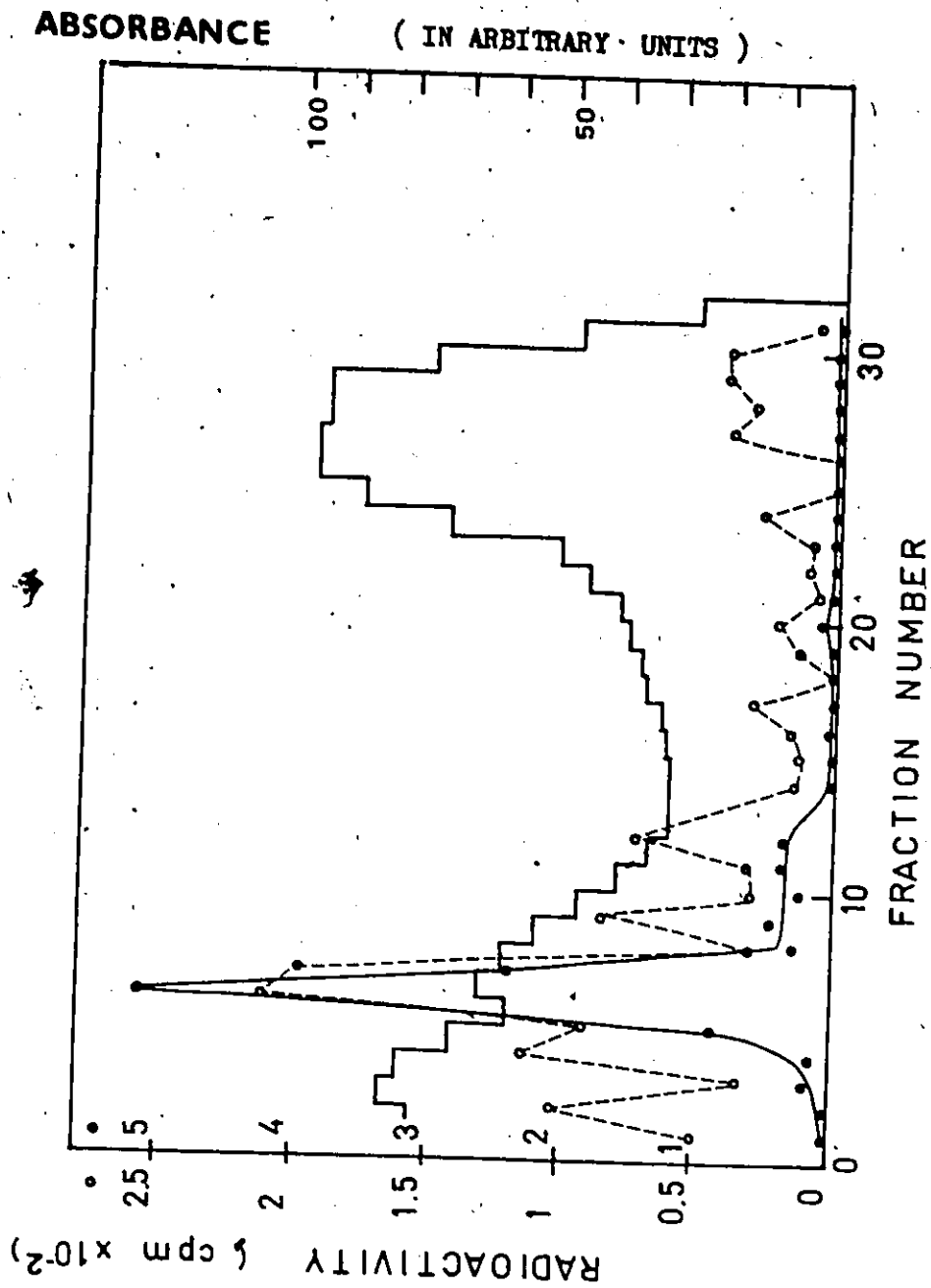


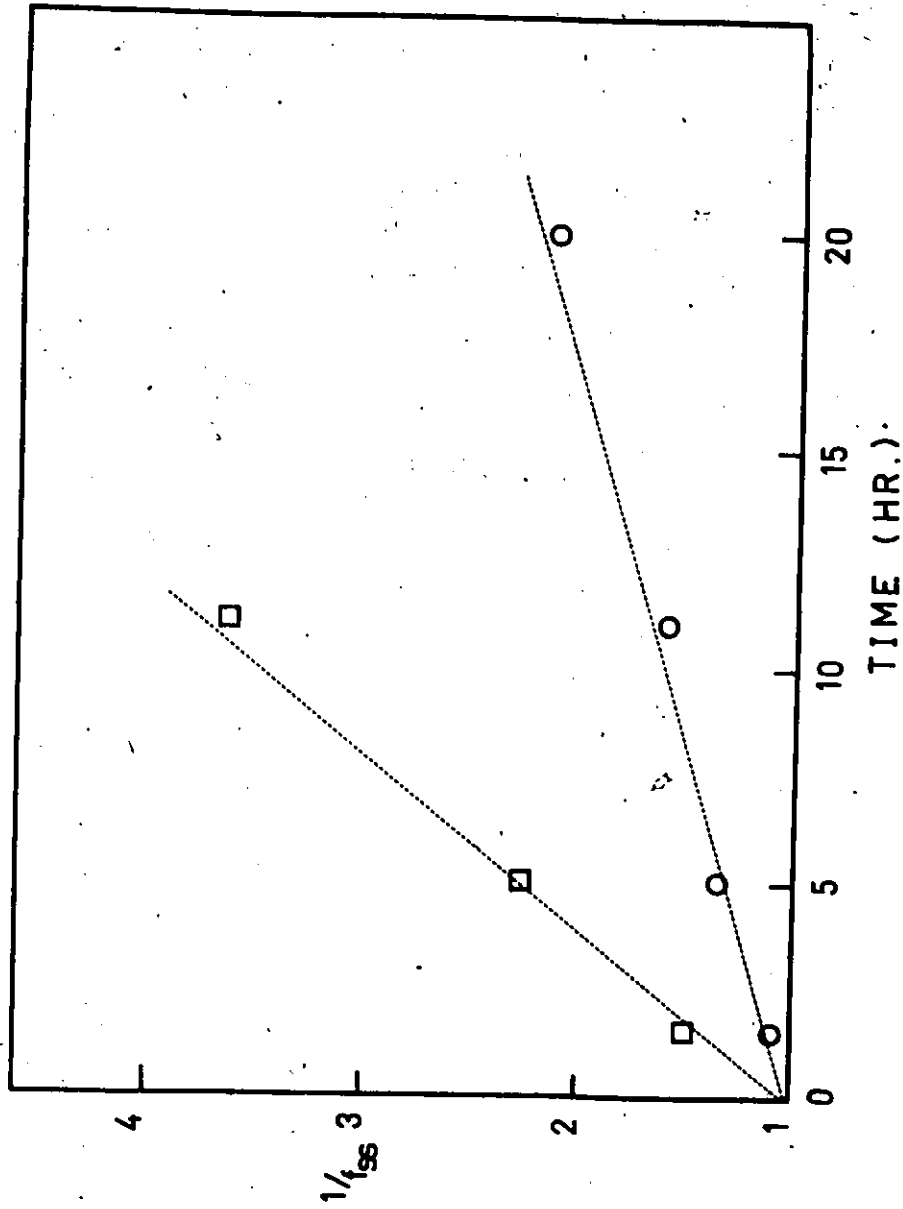
Figure 23

Mixing Experiment to Determine Trapping
of Free Viral DNA in Alkaline Glycerol Gradients

KB cells infected with 2000 virions per cell were processed with the alkaline glycerol gradient method. Viral DNA sequence in the supernatant and cell DNA fraction was measured by DNA-DNA reassociation technique.

- Reassociation of probe driven by 1/10 of DNA from the supernatant.
- Reassociation of probe driven by DNA from the cellular fraction.

84a



These reassociation experiments to quantitate viral genomes in infected cells are as shown in Figure 24. The non-integrated DNA fraction reassociates linearly up to 40 h post infection. At 72 h post infection the reassociation deviates from linear and it is not possible to say when the deviation starts since once there is more than one component reassociating, the curve is non-linear throughout (Sharp *et al.*, 1974). The important point to note is this: equimolar amounts of the entire genome are present in non-integrated form up to 40 h post infection. The 72 h post infection reassociation curve shows that parts of the viral genome exist in unequal proportions. The kinetics of integration are shown in Figure 25. The data presented in this graph are based on Figure 24. Viral integration starts as early as 4 h post infection. Maximum integration level of 160-170 viral DNA/cell is reached at about 36 h post infection. The maximum percentage of intracellular viral DNA integrated, however, is reached at about 20 h post infection, at that time, about 25% of viral DNA is integrated.

The effect of viral infection on cell growth has been investigated. The infected cells showed morphological changes. Under light microscopy, they appeared to be bigger, with increased reflection of light and the medium in which they grew tended to be more acidic. The number of cells increased until about 20 h post infection (Fig. 26).

Figure 24

Reassociation of Ad 12 Viral DNA
with Infected KB cell DNA

$1/f_{ss}$ versus t plots of reassociation of radioactively
labelled Ad 12 probe (1.5×10^{-2} $\mu\text{g/ml}$) in the presence
of DNA extracted from alkaline glycerol gradients.

- reassociation of probe.
- reassociation of probe with integrated
viral DNA.
- reassociation of probe with non-integrated
viral DNA.

- A mock infection.
- B 1.5 h post infection.
- C 4 h post infection.
- D 17 h post infection.
- E 40 h post infection.
- F 72 h post infection.

86

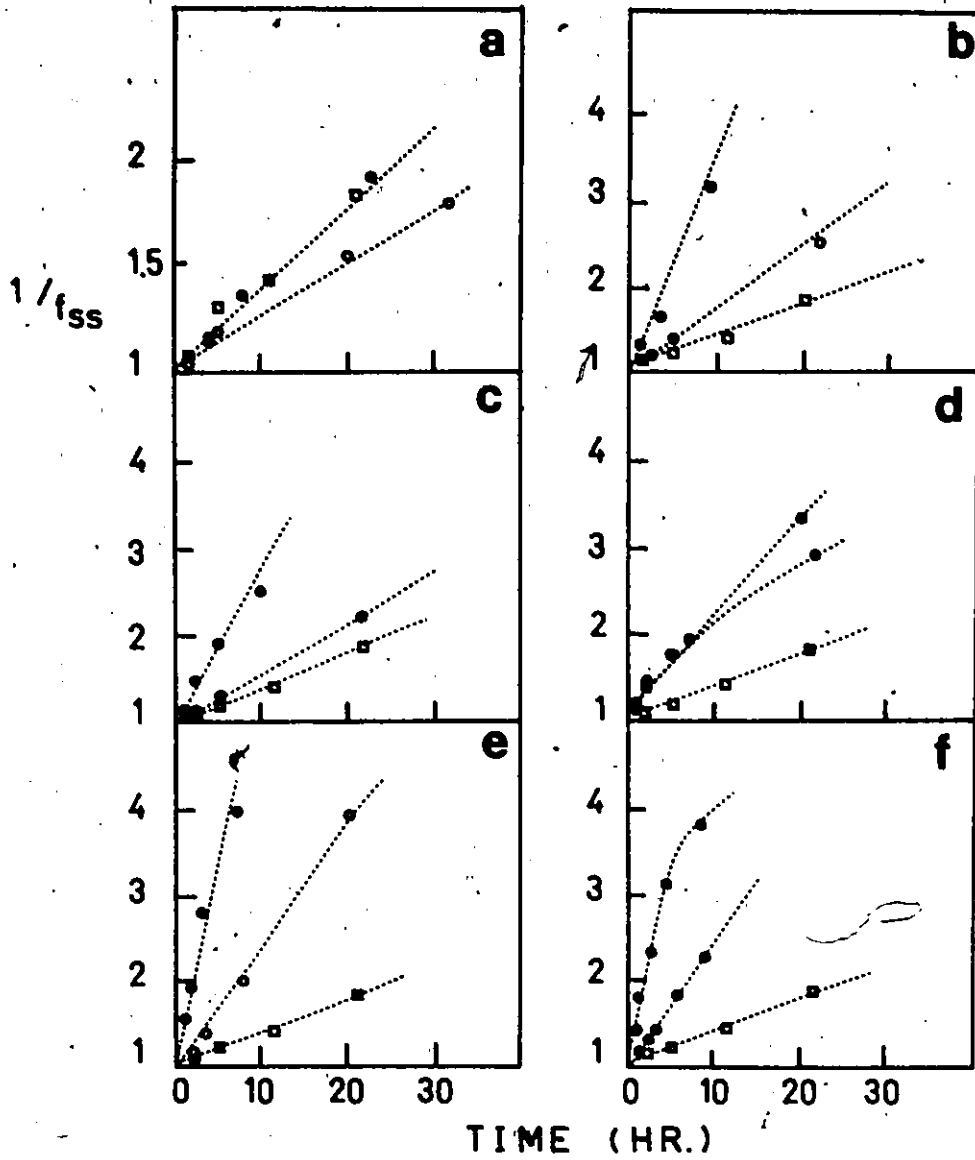


Figure 25

Percentage of Intracellular Viral DNA as a Function of
Time Post-infection (alkaline glycerol gradient method)

Duplicate experiments (Δ and \circ)

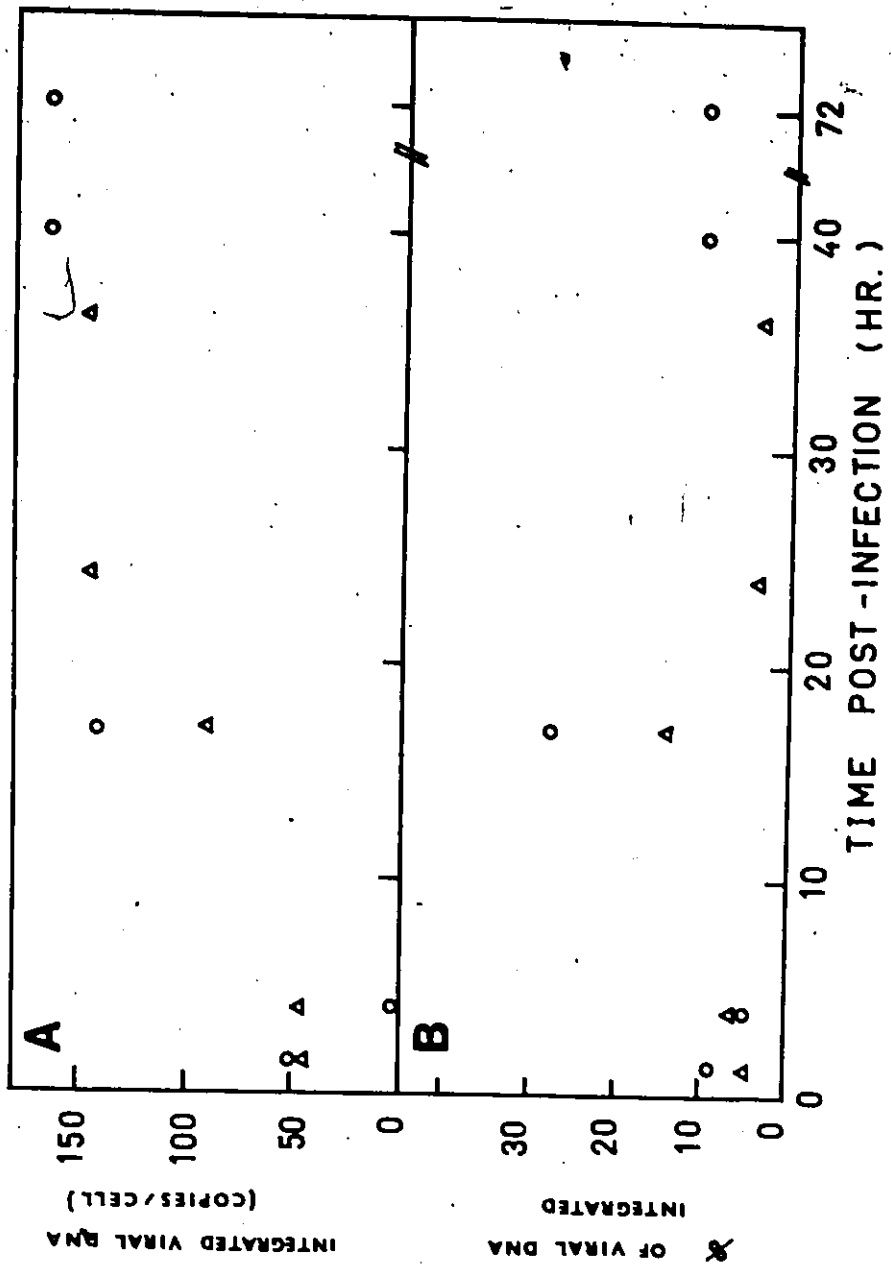
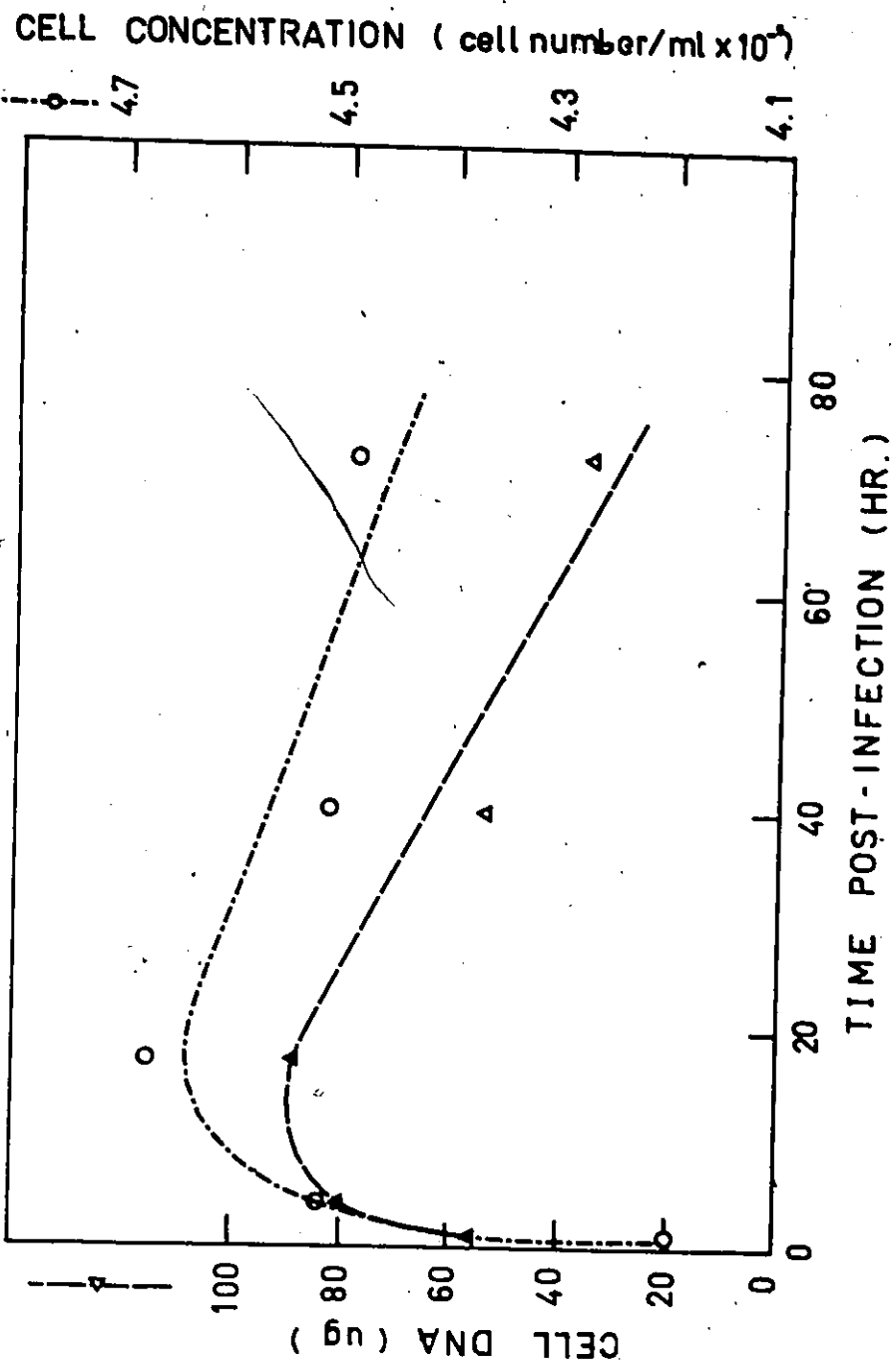


Figure 26

Cell Concentration and DNA Extracted from Ad 12 Infected
KB Cells as a Function of Time Post-infection

Cell DNA extracted refers to the DNA that was extractable from the alkaline glycerol gradients after infected cells were layered on top and centrifuged in these gradients.



After that the cell concentration fell with accumulation of some cell debris in the growth medium.

The DNA content of infected cells isolated on glycerol gradients was also quantitated with diphenylamine reaction (Colowick and Kaplan, 1957). The cell DNA extracted from a fixed volume of cell culture (200 ml) increased to a maximum at about 20 h post infection and then declined. This observation of cell DNA and the corresponding cell concentration at different times post infection is shown in Figure 26. The scattering of experimental points is due to the many steps involved in the extraction of DNA. The fact that this scattering is small shows that the procedures involved are highly reproducible.

V. Integration Sites

The human genome consists of 65% of unique sequences and 35% of sequences with a variety of repetitions (Saunders *et al.*, 1972). The rate of reassociation of denatured DNA is dependent on the frequency of repetitions (Britten and Kohne, 1968). One can isolate double-stranded DNA with HAP chromatography. Hence, DNA belonging to different classes of repetition can be isolated. Furthermore, the Ad 12 DNA sequences in each class can be quantitated. This approach was used to find out whether Ad 12 infected RB cell DNA with high repetition frequencies

and those with low repetition frequencies contained the same amount of viral DNA sequences. The scheme of fractionating KB cell DNA is presented in Figure 27 and the reassociation curve of KB cell DNA is shown in Figure 28.

Infected KB cell DNA was purified after recovery from alkaline glycerol gradients. The purified DNA was fragmented to 22S by boiling in 0.3 N NaOH for 2 min. The denatured cell DNA was reassociated to $Cot = 10$ sec-mol/liter in 0.14 M PB. Single and double strand fractions were recovered from HAP column as described in Materials and Methods. Under this condition, about 60-70% of DNA loaded on the HAP column could be recovered. Of this, 70% was in single-stranded form and 30% in double-stranded form.

Viral sequences in each fraction were determined by DNA-DNA reassociation (Figure 29). It was found that there were 132 viral DNA copies per 10 pg of cell DNA. Therefore, the density of integration sites was independent of frequency of repetition. However, it is possible that the long stretch of viral sequences integrated adjacent to the repetitive cellular DNA was sheared. The amount of viral DNA found associated with the repetitive sequences would be underestimated in this situation.

A similar approach to characterizing reiteration frequency of integration sites in SV40 transformed murine

Figure 27

Strategy for Fractionation of Cell DNA
According to Kinetic Complexity

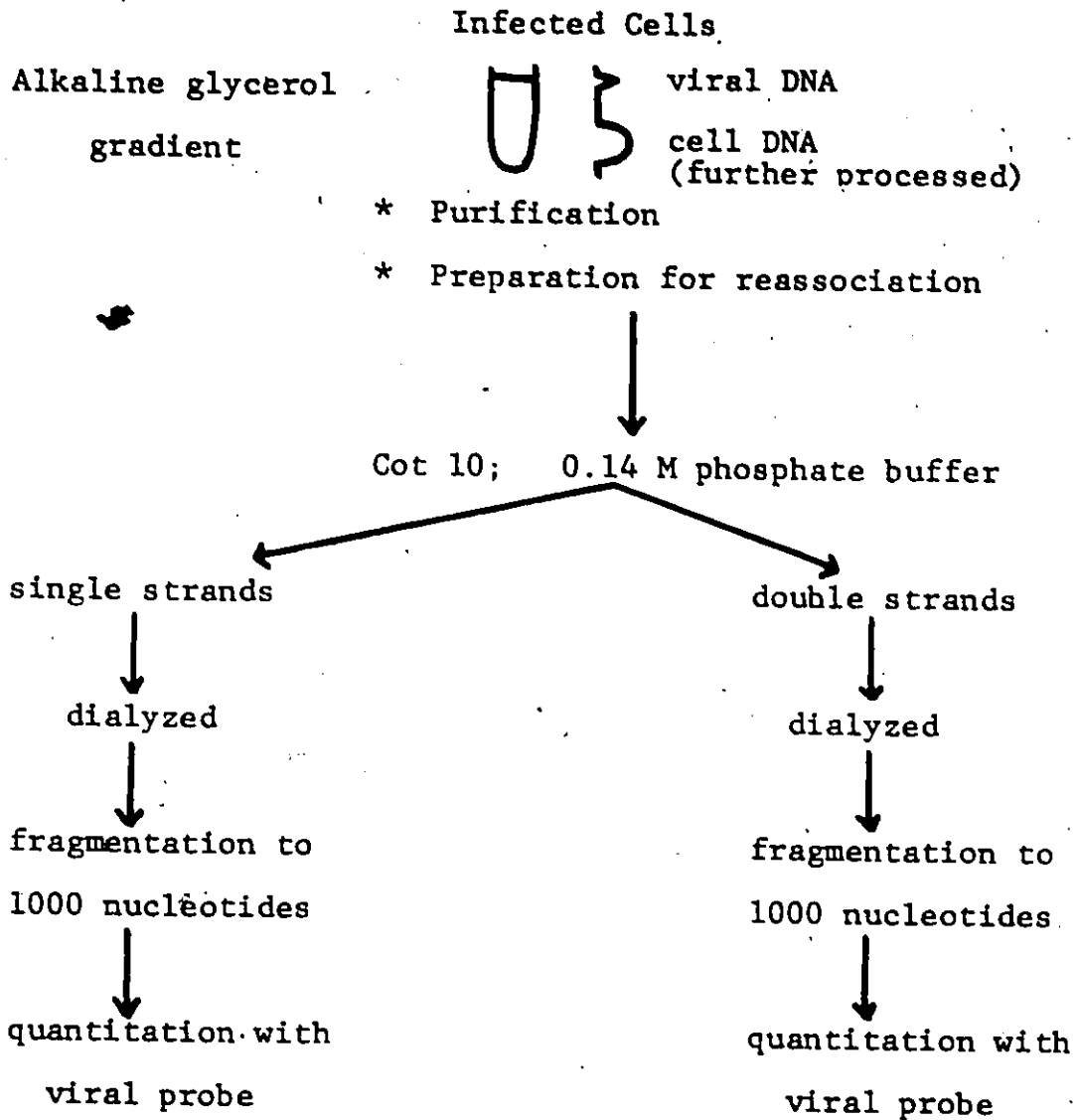


Figure 28

Reassociation of KB Cell DNA in 0.14 M PB

92a

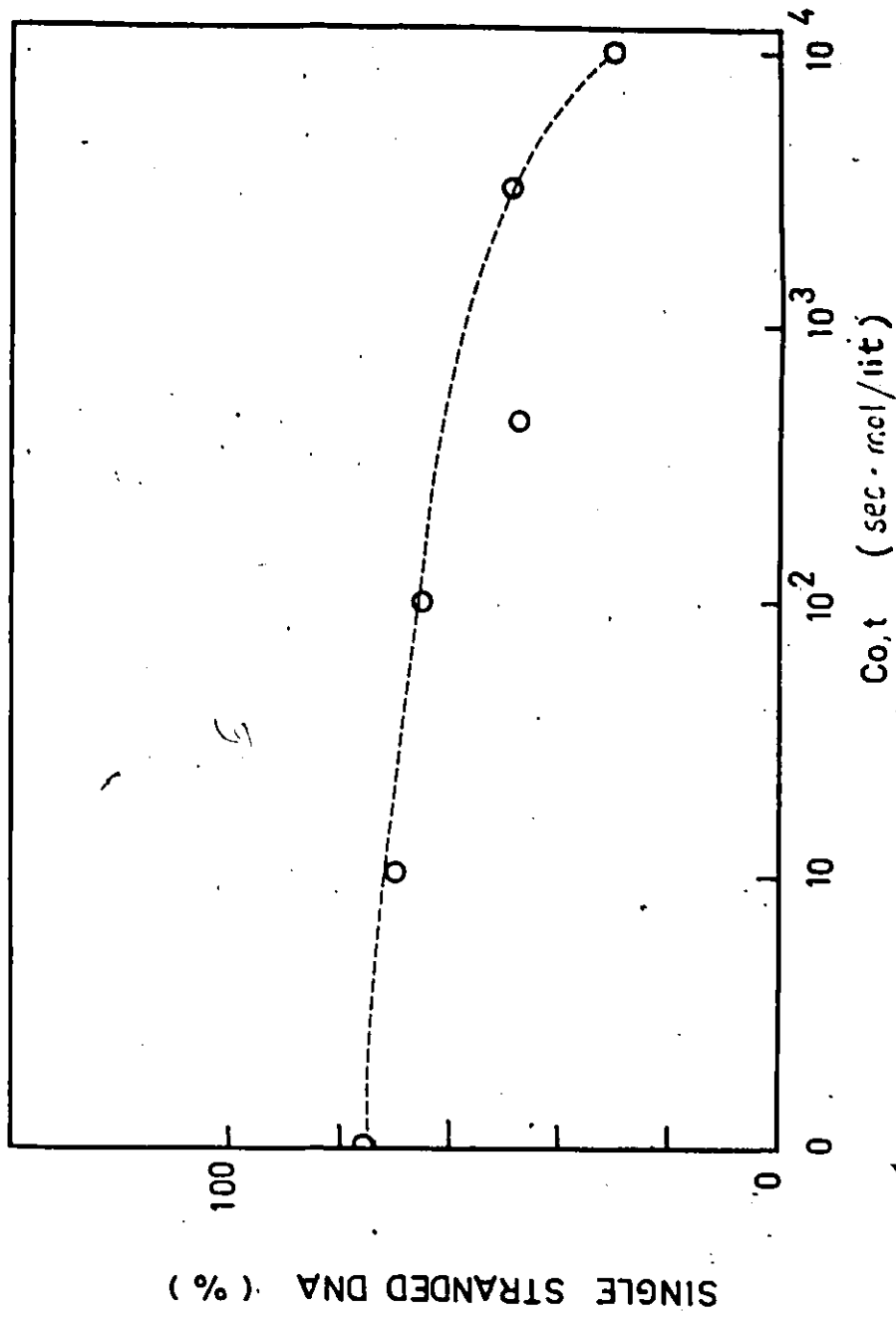


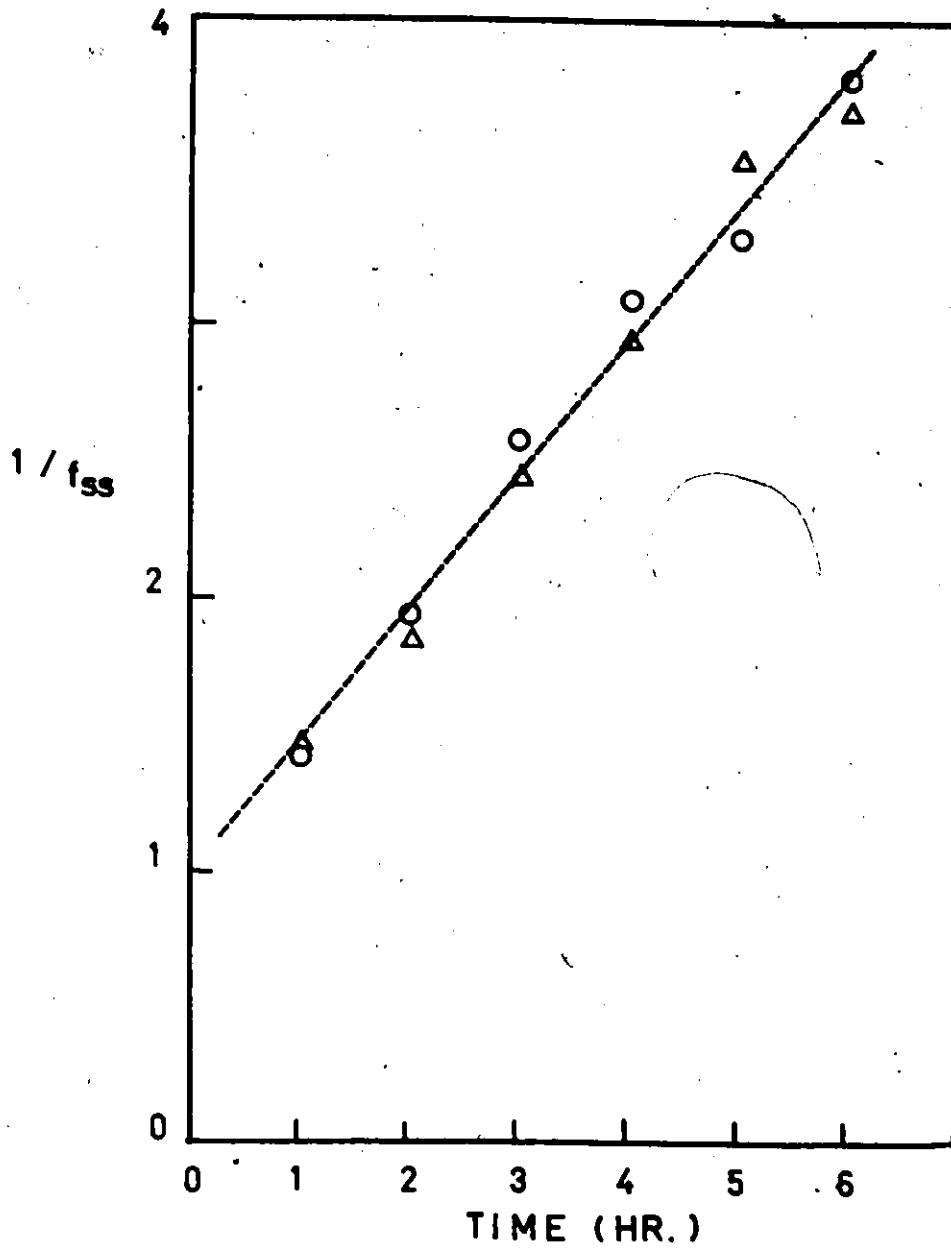
Figure 29

Reassociation of Viral DNA with Fractionated
Infected KB Cell DNA

After infection with Ad 12 at 2000 particles per cell for 36 h, 0.1 mg/ml of KB cell DNA isolated from alkaline glycerol gradients were reassociated at 0.14 M PB to Cot 10. Single-strand (slow reassociating fraction) and double-strand (fast reassociating fraction) were fractionated with HAP. Viral sequences in each fraction were quantitated with reassociation technique using complete viral probes.

- fast reassociating fraction.
- △ slow reassociating fraction.

93 a



cell lines was reported by Gelb and Martin (1973) who found that the integration site was exclusively in the unique fraction of cell DNA.

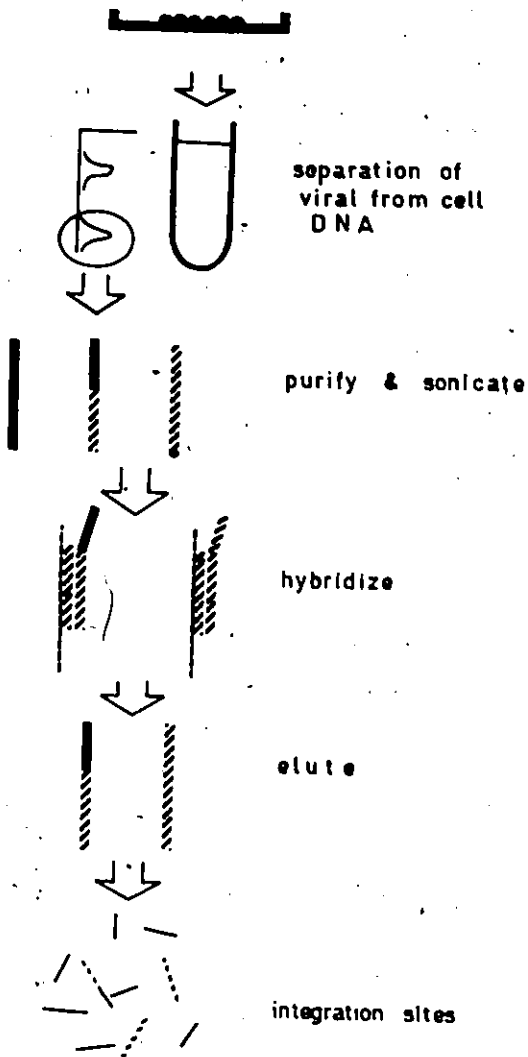
An alternative approach had been taken to characterize the integration sites by first selecting the integration sites and then characterizing them. The basic strategy is outlined in Figure 30. Monolayer KB cells grown in 50 cc α MEM were labelled with 0.5 mCi/32 oz bottle of ^3H -thymidine for 30 h. The radioactivity was then removed and the cells were washed with pre-warmed PBS. Fresh α MEM was added and the cells were allowed to grow for 12 h before being transferred to suspension culture by scraping. After 5 h of suspension culture the cells were infected with 2000 Ad 12 particles per cell. At 48 h post infection, the cells were lysed and centrifuged in alkaline glycerol gradients. The DNA purified from the gradients was sonicated for 1 min to reduce the molecular size to about 15S. This DNA was hybridized to 2 μg /filter of Ad 12 DNA according to the method of Grouse *et al.* (1972) in 5 x SSC and 50% formamide at 37°C for 48 h. Then the filters were washed carefully with the hybridization buffer. One ml of 0.3 N NaOH was added at 37°C for 2 h. The radioactivity recovered by this method using H^3 -DNA from infected cells was about 2.5 times that recovered when H^3 -DNA from non-infected cells was used. Thus substantial purification of cellular sequences adjacent to viral sequence was achieved.

Figure 30

Strategy for Isolation of Integration Sites
in Infected KB Cell DNA

- Cellular DNA sequences.
- Viral DNA sequences.
- — Sequences containing viral DNA
integrated into cellular DNA.

95a



If the selected integration site DNA was allowed to reassociate at this stage, the kinetics observed would be due to a) the kinetic complexity of the integration site DNA adjacent to viral sequences; b) the reassociation of viral sequences which were picked up by the filter hybridization. However, if one takes less than 0.1 μ g/ml of cell DNA it will virtually take 1000 hours of reassociation to attain a Cot of 1.0. If vast excess amount of unlabelled cell DNA were mixed with this integration site DNA, the reassociation kinetic would demonstrate the complexity characteristic of this class of DNA driven by the same class of unlabelled DNA. That is, if this integration site cell DNA belongs to a class that is fast reassociating, it would reassociate faster than the unlabelled DNA as a whole. But if this integration site DNA is representative of the whole cellular DNA, then its reassociation kinetics will not differ from the reassociation of KB cell DNA. To rule out that the reassociation is not due to the viral sequences one can monitor the viral DNA reassociation with 14 C-labelled Ad 12 DNA in the same reassociation mixture. The reassociation of infected KB cell DNA together with reassociation curve of KB cell DNA adjusted for ionic strength is displayed in Figure 31. The reassociation kinetics of the integration site DNA is not due to the viral fragment attached to it since the reassociation of

Figure 31

Reassociation of Integration Sites

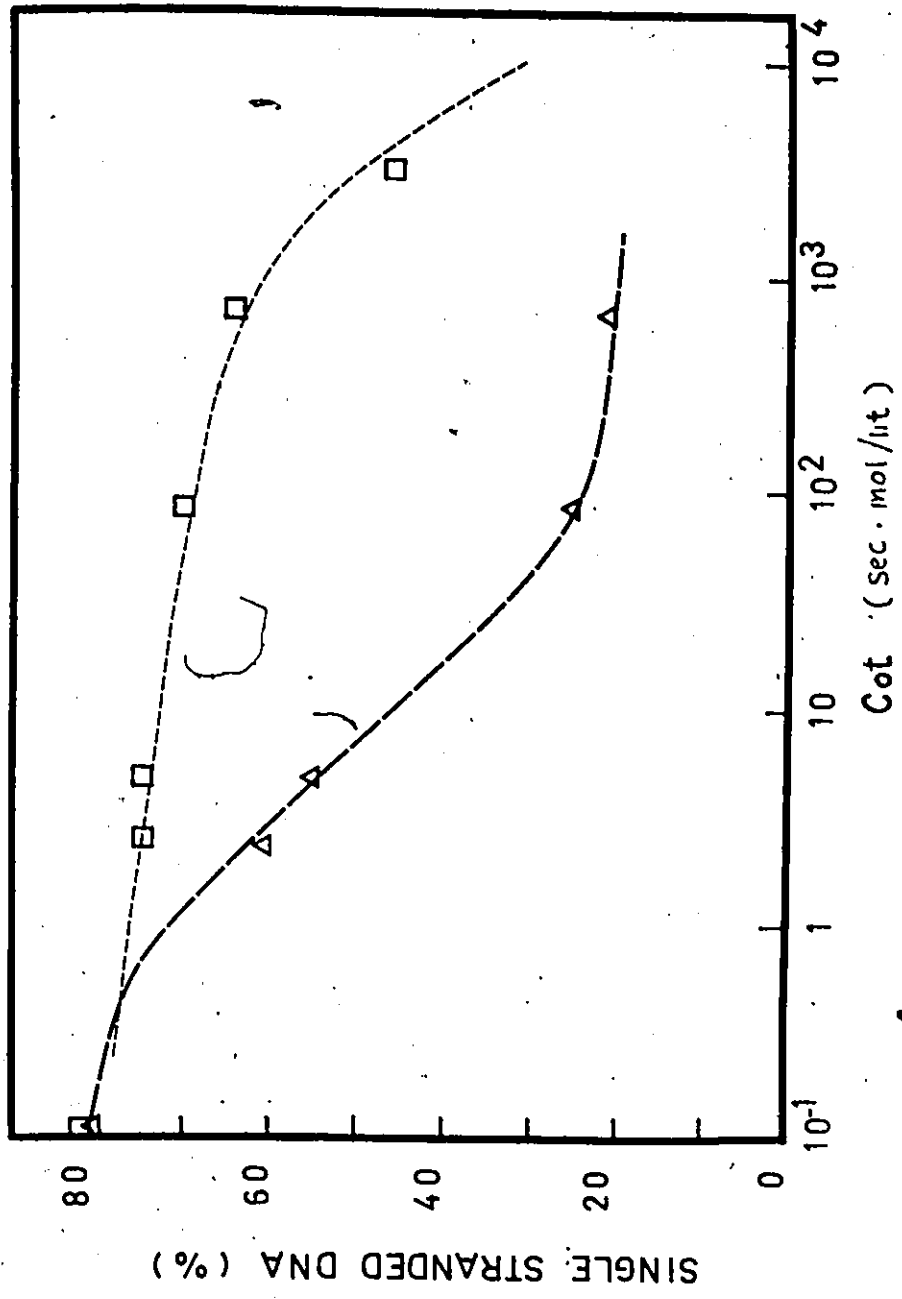
Reassociation medium contains 0.7 ml of integration site DNA at 4×10^4 ^3H cpm/ml; 0.1 ml of 1.1 mg/ml cold KB DNA; 0.2 ml of ^{14}C Ad 12 DNA; 0.2 ml of 3.3 M sodium phosphate buffer; total volume 1.2 ml.

..... (dotted line) imposition of curve for the reassociation of KB cell DNA, as from Figure 28.

□ reassociation of integration site DNA driven by KB cell DNA.

—△— reassociation of ^{14}C labelled Ad 12 DNA.

97a



^{14}C Ad 12 DNA follows different kinetics. The $\text{Cot}_{\frac{1}{2}}$ of the ^{14}C curve is 0.025 sec.-mol/liter which approximates the $\text{Cot}_{\frac{1}{2}}$ of Ad 12 DNA. The slight difference is due to unspecified amount of infecting Ad 12 DNA that might have been broken off from shearing the cell DNA. From Figure 31, it can be concluded that the reassociation of the integration sites is no different from the reassociation of uninfected KB cell DNA. Hence, one can conclude that the frequency of repetition and distribution of these sites are no different from the KB cell genome as a whole. This observation could be interpreted in either one of the following ways: a) there are no specific integration sites for Ad 12 in KB cells; b) there are specific integration sites in terms of short segments of nucleotide sequences, but these sites are randomly distributed throughout the cellular genome.

VI. Factors Which Alter Integration Frequencies

A. Serum concentration

The influence of serum concentration in the growth medium on the number of viral genome integrated into KB cell DNA was studied at 24 and 36 h post infection. Infected cells were grown in medium containing 1%, 5% and 10% FCS. At 24 and 36 h post infection, the cells were stored at 4°C. Network technique and DNA-DNA reassociations were used to quantitate integrated and non-integrated

viral DNA sequences. Duplicate experiments were done. Because of the high concentration of viral DNA in the non-integrated fraction, only 1/5 of the total amount of material in this fraction was used to drive the reassociation of the ³H-viral DNA probe. A representative graph of these experiments is shown in Figure 32. The results of the serum concentration on the level of integration at 24 and 36 h post infection were calculated based on the reassociation kinetics. These are presented in Table 8.

At 24 h post infection, decreasing the serum concentration from the routinely used 10% to 1%, resulted in decreasing the intracellular viral DNA copies from 550 per cell to half of that value. At the same time, integrated viral copies per cell was reduced from about 200 to 0. At 36 h post infection, the number of integrated viral genomes increased over that of 24 h, but no integration was observed with 1% FCS. It should be noted that the number of integrated copies in the present experiment at 24 h is comparable with that reported earlier (see section IV).

It has been shown that KB cells infected with Ad 12 continue to increase in DNA content until 20 h post infection (section IV). Experiments were done to study how this increase was affected by serum concentrations. Infected KB cells grown in MEM with different concentrations

Figure 32

Reassociation of Infected KB Cell DNA Grown on MEM with
Different Serum Concentration: Scored at 36 h, Post-
infection

Probe Ad 12 DNA concentration: 1.5×10^{-2} $\mu\text{g/ml}$ ○

Infected cell DNA ●

A 1% FCS network DNA

B supernatant DNA, 1/5 concentration used.

C 5% FCS network DNA

D supernatant DNA, 1/5 concentration used.

E 10% FCS network DNA

F supernatant DNA, 1/5 concentration used.

100a

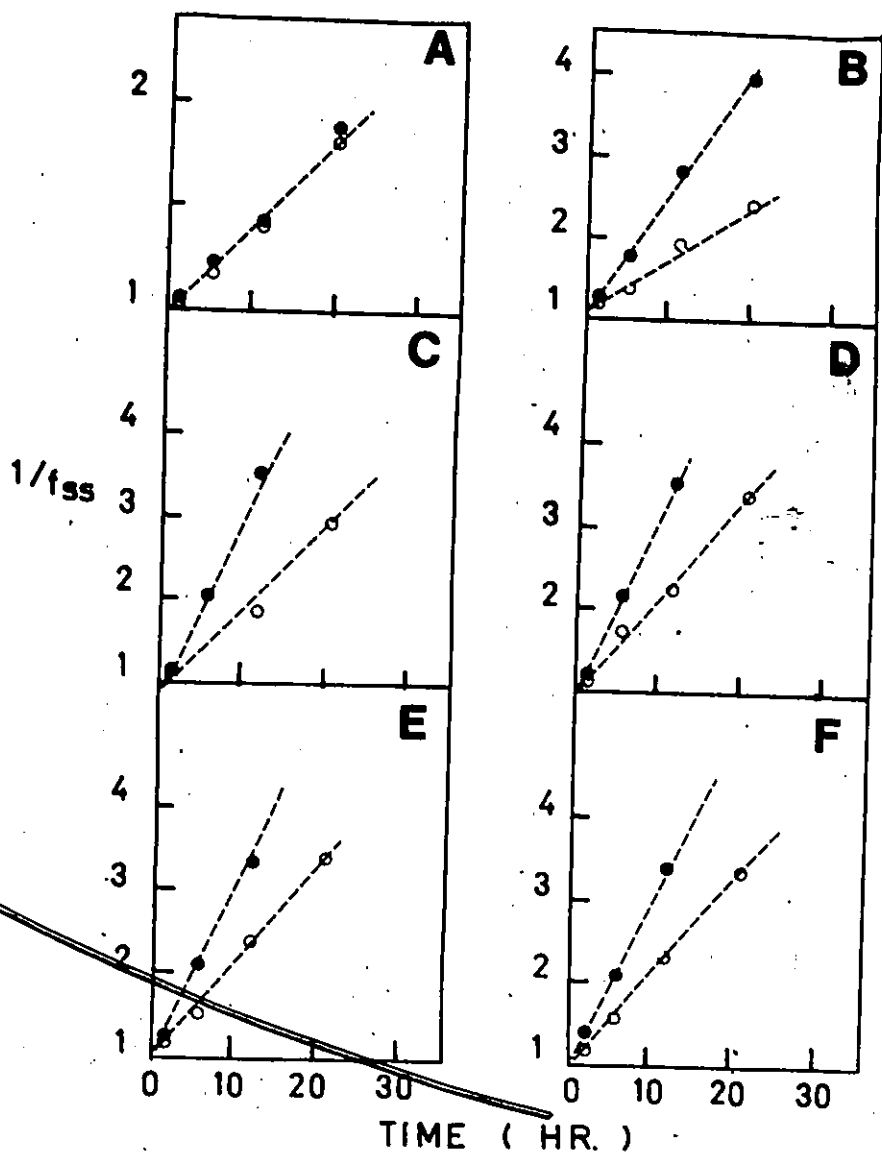


Table 8

Serum Effects on Intracellular Viral DNA in Infected KB Cells

	24 h post infection		36 h post infection	
	Non int./c.	Int./c. % int.	Non int./c.	Int./c. % int.
1%	253	0	243	0
5%	367	187	120	305
10%	550	183	543	390

of FCS were pulsed with radioactive thymidine. Incorporated radioactivity was scored as TCA precipitable counts (Figure 33). The rate of growth was affected by differences in serum concentration. In the case of infected cells, the rate of growth in 10% and 5% serum were similar after 10 h, whereas the rate of growth in infected KB cells in 1% was much lower. It was not intended to study in detail how much the growth rate was correlated with the level of integration. Hence, no further experiments were planned in this regard.

B. Cytosine arabinosides (ara C)

The effect of ara C, a DNA synthesis inhibitor, on the number of Ad 12 DNA sequences integrated into infected KB cell DNA has been studied. Experimental conditions were similar to those outlined previously. Following viral adsorption, 10% FCS and 20 μ g/ml of ara C were added to the culture medium. Again, the network technique and DNA-DNA reassociation were used to quantitate the number of integrated and non-integrated copies.

The inhibitive effects of ara C on cellular DNA synthesis is shown in Figure 34. The incorporation of ^3H -thymidine in infected KB cultured with 20 μ g/ml ara C decreased 15-fold during the first 5 h post infection.

Figure 33

Thymidine Incorporation of Cells at Different
FCS Concentrations, and Infected with 2000
Ad 12 Virions per Cell

Spinner suspension cultures of KB cells were infected with Ad 12 virus and then transferred to 35 mm petri dishes containing appropriate concentrations of FCS. One h pulse of H-thymidine at 0.5 μ Ci/ml were performed. At the end of the pulse cells removed from the petri dishes were washed 3 times with PBS and pelleted by centrifugation. 10% TCA was added and the precipitate was collected on fiber glass filters, dried and counted in 5 ml of Toluene Omnifluor fluid:

- A 1% Fetal Calf Serum (FCS)
- B 5% Fetal Calf Serum
- C 10% Fetal Calf Serum

△ Non-infected

▲ Infected

103 a

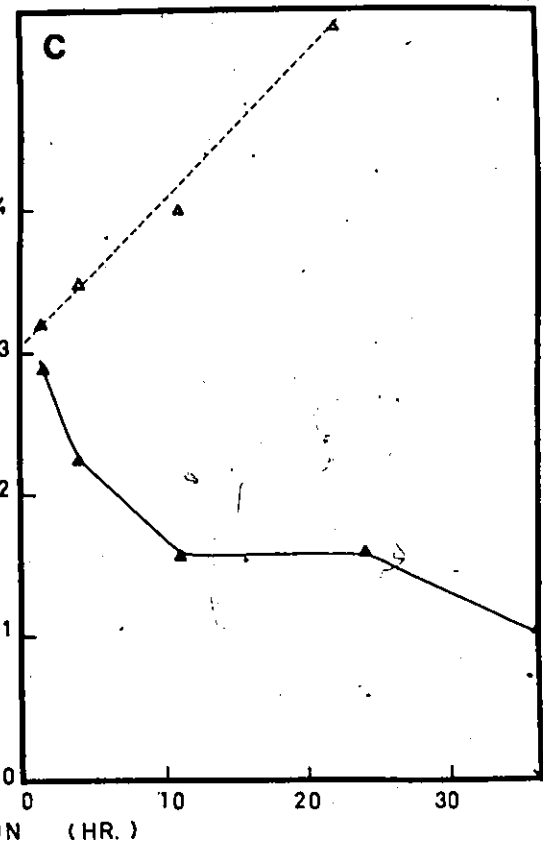
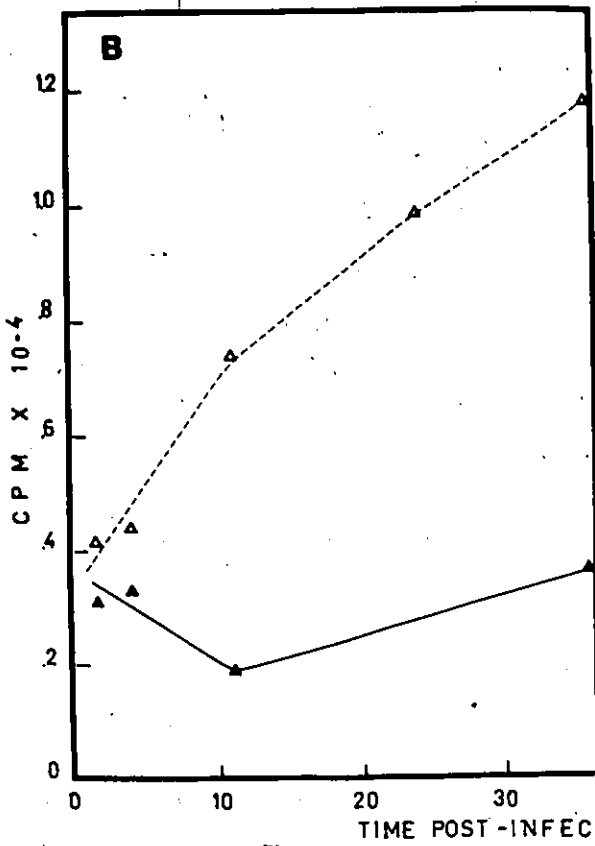
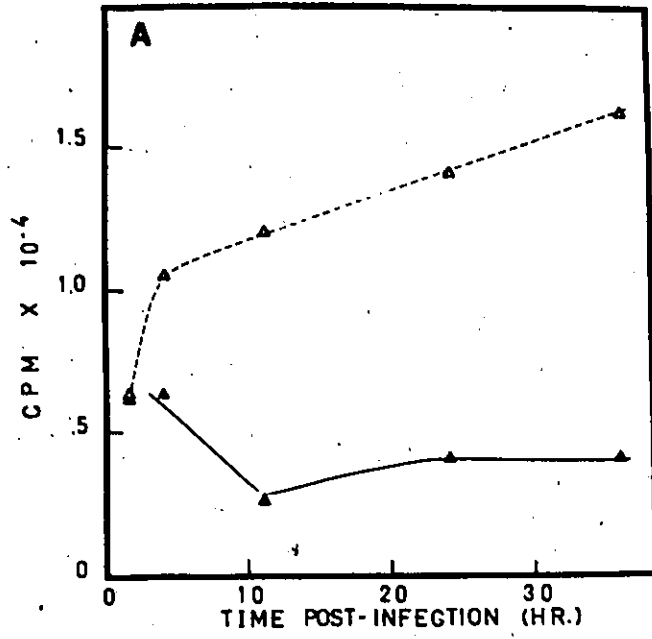
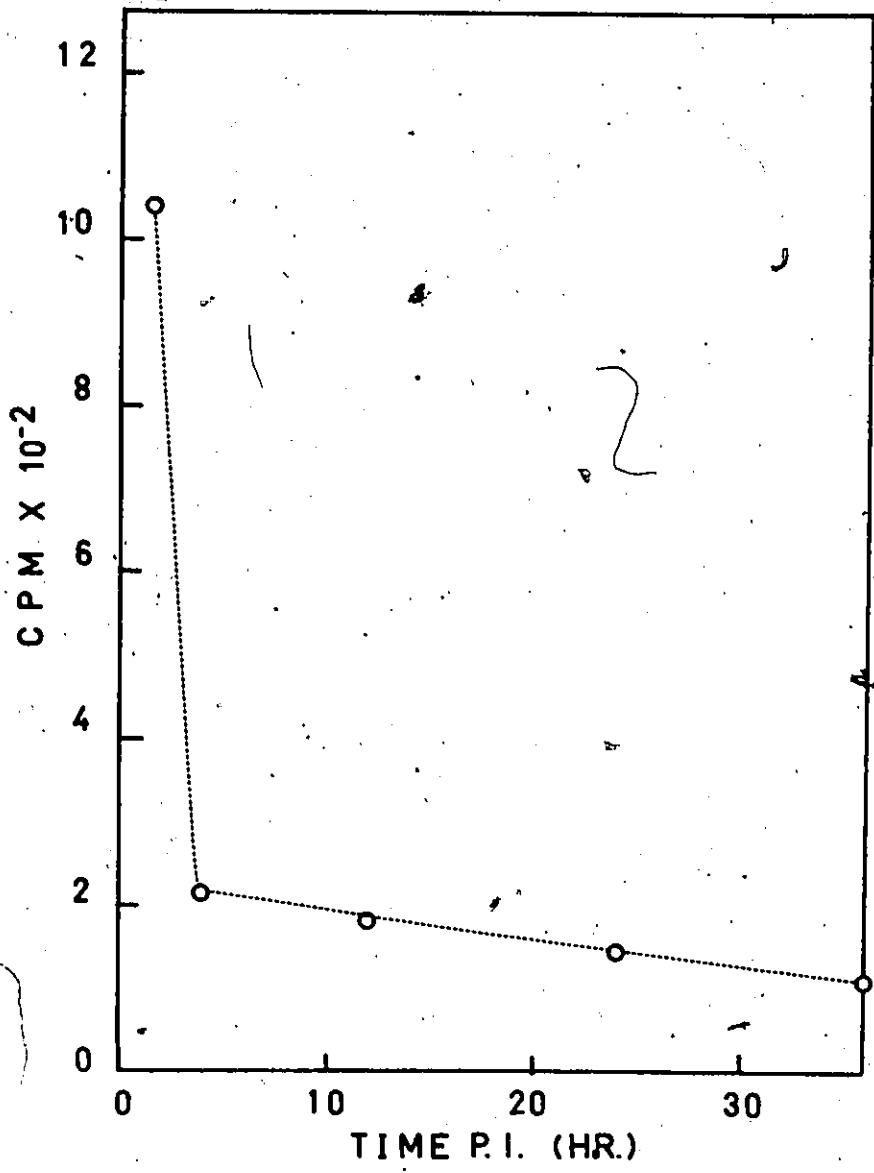


Figure 34

³H-thymidine Incorporation of Ad 12 Infected Cells at
20 µg/ml of Ara C. Detailed Experimental Conditions
Similar to Figure 33.



104a



Ad 12 DNA integration levels were scored at 24 and 36 h post infection. Duplicate experiments showed that at 24 h post infection, 20 copies of viral genome per cell were integrated, representing about 5% of intracellular viral DNA copies. The integrated viral DNA copies increased to 33 per cell representing about 16% of intracellular viral DNA. The data on DNA-DNA reassociation is shown in Figure 35.

C. Caffeine

The effect of caffeine, a DNA synthesis inhibitor, on the number of Ad 12 DNA sequences integrated into infected KB cell DNA was explored. Infecting conditions were similar to those described in Materials and Methods. After 1.5 h of virus adsorption, cells were plated on petri-dishes in medium containing either 1 mM or 5 mM caffeine. The amounts of intracellular and integrated viral DNA were scored by the network technique and DNA-DNA reassociation at 24 and 36 h post infection. The reassociation curves are shown in Figure 36. The amounts of intracellular viral DNA were calculated as shown in Table 9, which included data from experiments without caffeine. The total intracellular viral DNA concentration was suppressed in the presence of caffeine, but the integrated viral sequences were increased. This increase is more striking when the percentages of intracellular

Figure 35

Integration of Viral DNA into KB Cell DNA

Cells were infected with Ad 12 at 2000 virions per cell. After 1.5 h of adsorption, 20 $\mu\text{g/ml}$ of Ara C were added. Cells were harvested at 24 h and 36 h post-infection.

A 24 h post-infection. 7

B 36 h post-infection.

○● Reassociation of 1.78×10^{-2} $\mu\text{g/ml}$ of Ad 12 DNA probe.

△▲ Reassociation of Ad 12 probe in the presence of cell DNA from networks.

106a

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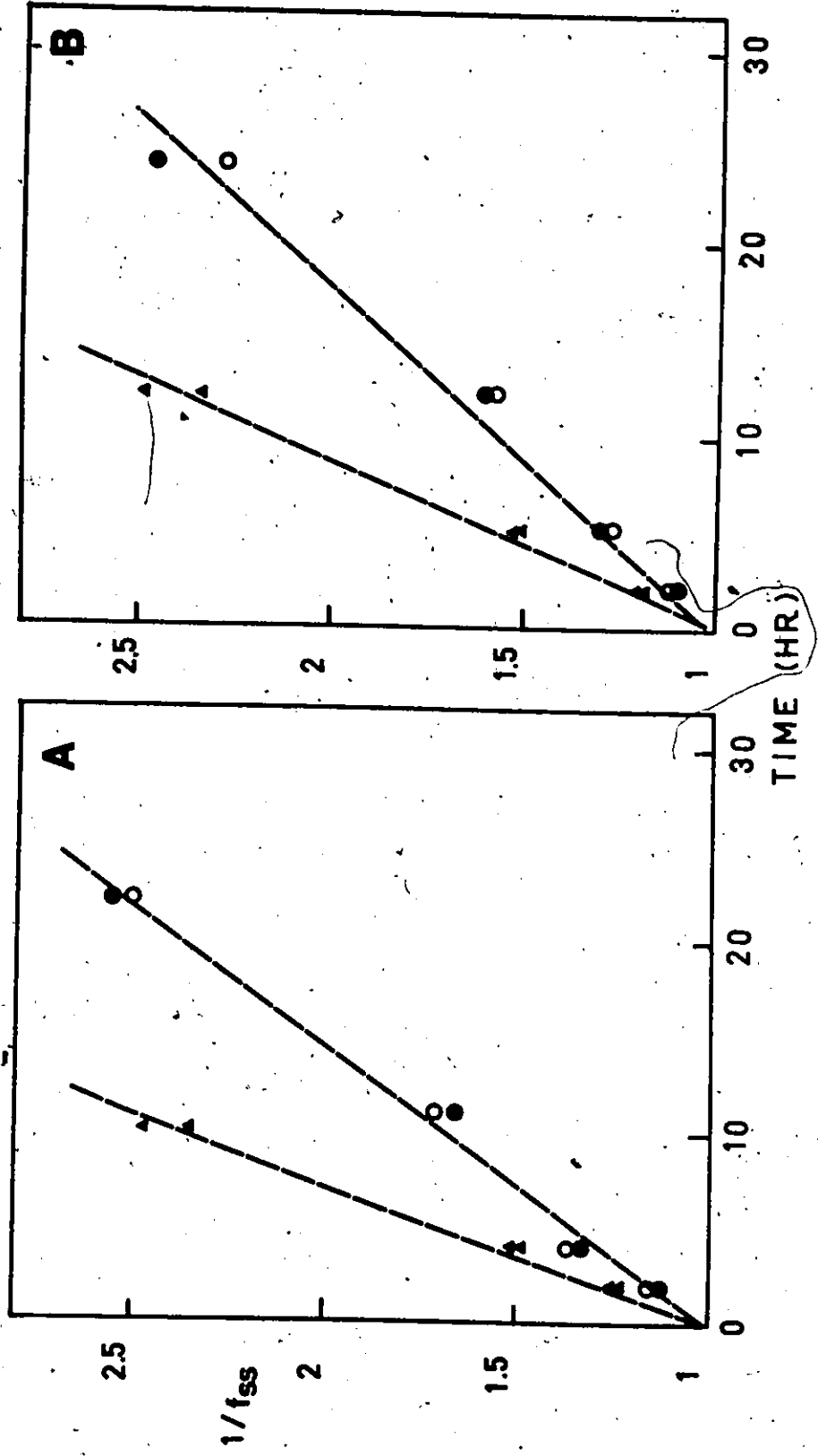


Figure 36

Integration of Ad 12 DNA into KB Cells
in the Presence of Caffeine

Cells were grown in 10% FCS and infected with 2000 virions of Ad 12 per cell. Caffeine was added right after infection.

- A 1 mM caffeine, 24 h post-infection.
- B 36 h post-infection.
- C 5 mM caffeine, 24 h post-infection.
- D 36 h post-infection.

- Integrated DNA.
- Non-integrated DNA.

107a

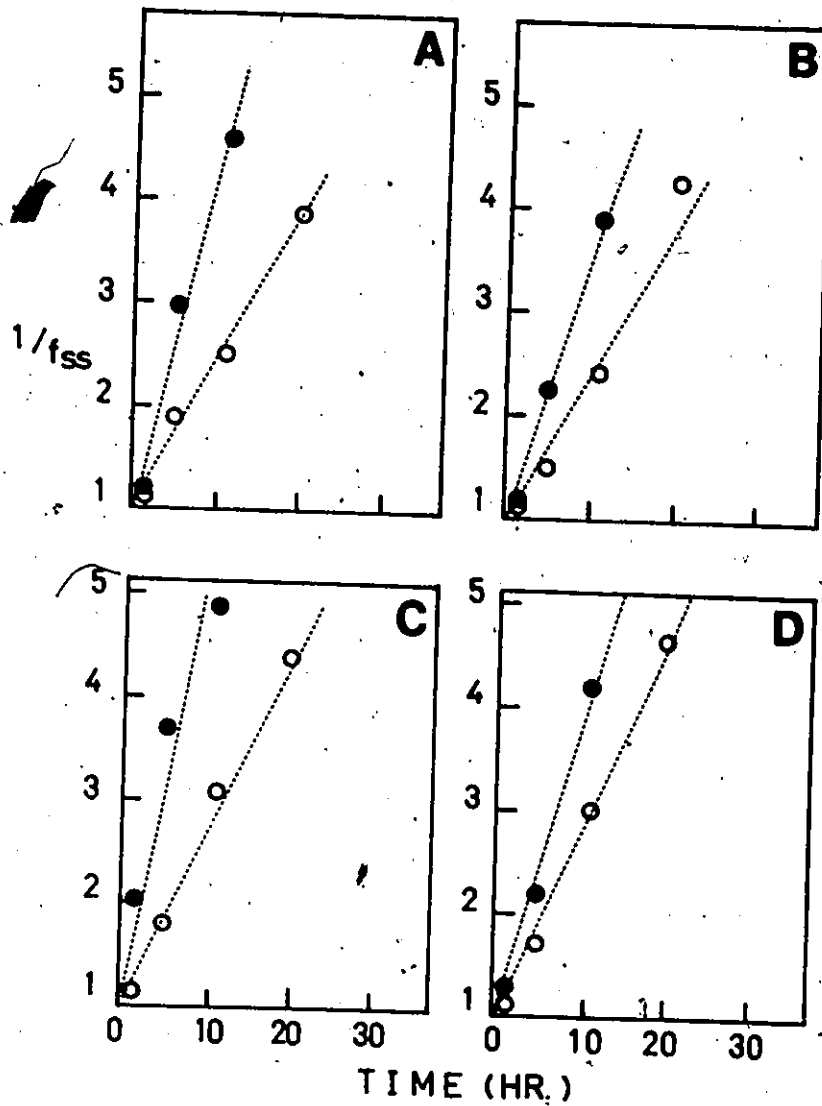


Table 9

Comparison of the Number of Integrated Viral DNA in the Presence of Caffeine

Post infection	No caffeine		1 mM caffeine		5 mM caffeine	
	integrated viral DNA per cell	% of intracellular DNA integrated	integrated viral DNA per cell	% of intracellular DNA integrated	integrated viral DNA per cell	% of intracellular DNA integrated
24 h	150	5	163	20	251	25
36 h	150	5	210	32	395	49

DNA in integrated form are compared. As a control to monitor the effect of caffeine on the DNA synthesis of Ad 12 infected KB cells, ^3H -thymidine incorporation was measured. TCA precipitable counts after a pulse of ^3H -thymidine were used to study the rate of DNA synthesis at different times post infection. Figure 37 shows that both 1 mM and 5 mM caffeine suppressed the rate of DNA synthesis to about 8% of the same cells grown in standard cultures without caffeine.

D. Ultra-violet light irradiation

The effect of UV irradiation was studied by irradiating KB cells with 10 J/m^2 of UV dose just before infection. The intracellular and integrated viral DNA per cell was studied by network technique and DNA-DNA reassociation at 1.5, 4, 10, 24 and 36 h post infection (Figure 38). The total intracellular viral copies per cell were less than that of infected cells without irradiation. No major differences between the number of integrated viral DNA sequences with or without UV irradiation were noticed (Table 10).

Figure 37

^3H -thymidine Incorporation of Ad 12 Infected Cells at
1 mM and 5 mM of Caffeine

Details of experimental condition similar to Figure 33.

- 1 mM caffeine.
- 5 mM caffeine.

110

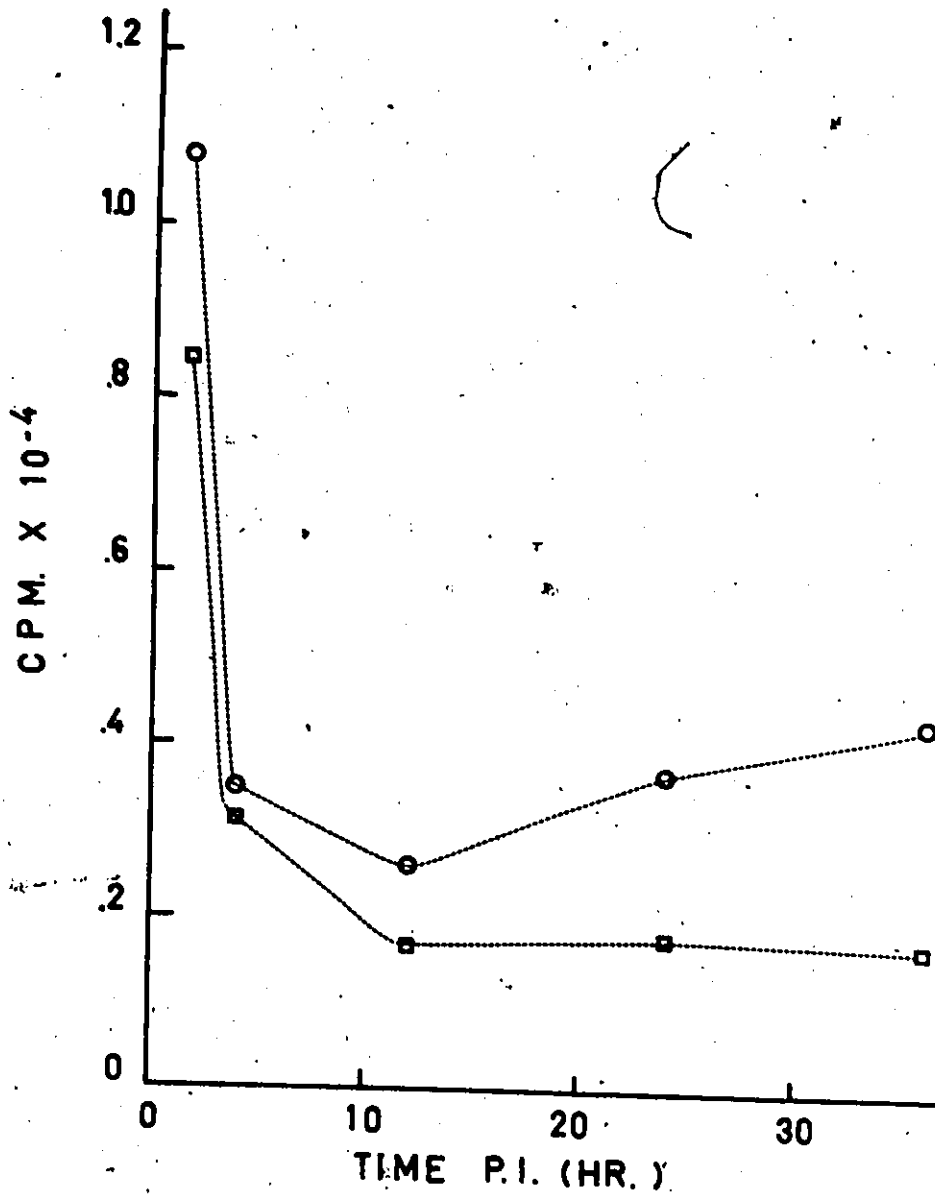


Figure 38

Integration of Viral DNA into KB Cell DNA

KB cells on petri dishes were irradiated with 10 J/m^2 of UV light just before infection. Cell DNA harvested were treated with the network technique to separate integrated and free viral DNA. 1/5 of recovered cell DNA used for reassociation. Probe DNA concentration: $1.5 \times 10^{-2} \mu\text{g/ml}$.

Reassociation of probe as shown in Fig. 32.

● ○ Duplicate experiment of reassociation of probe driven by cellular DNA network.

- A Mock infection.
- B 1.5 h post-infection.
- C 4 h post-infection.
- D 10 h post-infection.
- E 24 h post-infection.
- F 36 h post-infection.

1112

$1/f_{ss}$

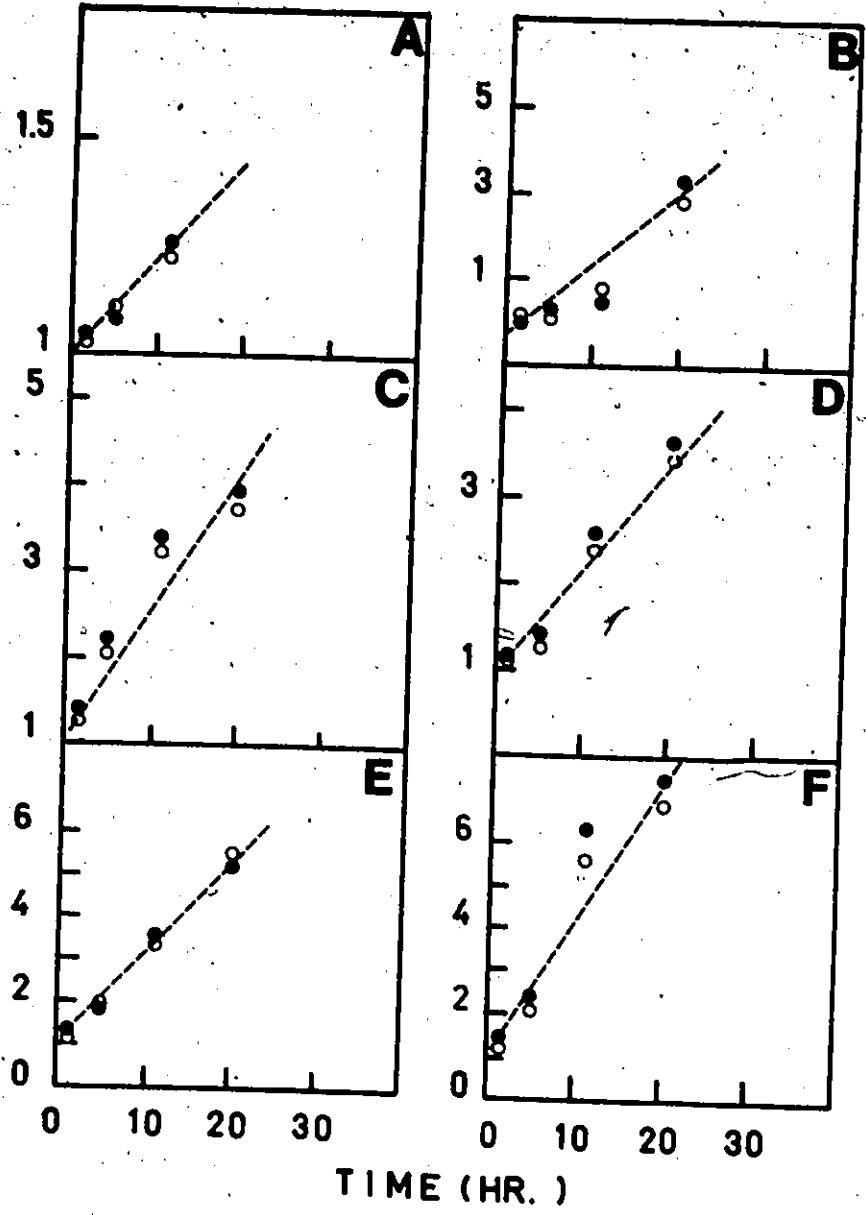


Table 10

Number of Integrated Viral DNA in UV Irradiated Cells

	Viral copies per cell at times (h) post infection			
	1.5	4.0	10	24
Total intracellular	333	504	553	618
Number integrated	70	111	177	167
% integrated	21	22	32	27

DISCUSSION

I. Establishment of the DNA-DNA Reassociation System

A system for DNA-DNA reassociation using hydroxyapatite chromatography for assaying the percentages of DNA in single-stranded configuration was established. The reliability of this system can be assessed by: 1) reproducibility of reassociation experiments; 2) comparison of $Cot_{1/2}$ values obtained in this system with published values; 3) results of reconstruction experiments.

Since the main objective of developing this system was to quantitate unknown concentrations of DNA, the last criterion was also the most important one.

The reproducibility of the system can be considered with reference to the $Cot_{1/2}$ values of Ad 2 and Ad 12 DNA in 0.14 M PB, the $Cot_{1/2}$ values of Ad 12 reassociating in 1 M NaCl, and the $Cot_{1/2}$ values of Eco RI fragments of Ad 12 DNA in 1 M NaCl. The $Cot_{1/2}$ values of Ad 2 in 0.14 M PB were 2.5×10^{-2} sec mol/liter, while that of Ad 12 was 2.7×10^{-2} sec mol/liter. The $Cot_{1/2}$ values for Ad 12 DNA reassociating in 1 M NaCl was 4.6×10^{-3} sec mol/liter. While there are many parameters that may affect $Cot_{1/2}$ values, e.g. fragment sizes, genome sizes, reassociating temperatures and viscosities, the major variable in

all these experiments is ionic strength. The effect of ionic strength and relative reassociation rate had been extensively investigated by Marmur and Lane (1960) and by Britten and Kohne (1968). The $Cot_{\frac{1}{2}}$ values obtained under different ionic strengths could be compared by converting these values to a corresponding ionic strength (Britten and Smith, 1970). The above $Cot_{\frac{1}{2}}$ values converted to 1 M $[Na^+]$ are listed in Table 11.

The $Cot_{\frac{1}{2}}$ values for reassociation of whole genome of adenovirus lie within 4.6×10^{-3} sec mol/liter and 5.0×10^{-3} sec mol/liter. There is a larger variation with the $Cot_{\frac{1}{2}}$ values derived from the reassociation of the Eco RI generated Ad 12 DNA fragments. The concentration of DNA in these experiments was determined entirely by radioactive counts. Hence it is affected by the distribution of thymidine in these fragments which was labelled with 3H -thymidine. The rate of reassociation is also affected by the distribution of G-C sequences (Wetmur and Davidson, 1968). Both thymidine distribution and G-C content in these sequences were uncertain. Therefore, the extrapolated $Cot_{\frac{1}{2}}$ values can only give an impression of the upper limit of variation, which varies from 3.56×10^{-3} to 6.75×10^{-3} sec mol/liter. It must be noted, however, that these values were obtained from only one preparation of Eco RI restriction fragments of Ad 12 DNA, although the reassociation

Table 11

$Cot_{1/2}$ Values from Various Experiments
in the Present Work

Experimental condition standardized at 68°C, fragment size
~700 nucleotides.

Adenovirus genome	$Cot_{1/2}$ sec.mol/lit.	$Cot_{1/2}$ in 1 M [Na ⁺] sec.mol/lit. complete genome complexity
Ad 2, 0.14 M PB	2.5×10^{-2}	4.64×10^{-3}
Ad 12, 0.14 M PB	2.7×10^{-2}	5.02×10^{-3}
Ad 12, 1 M PB	-	4.6×10^{-3}
Eco RI of Ad 12, A fragment		3.56×10^{-3}
Eco RI of Ad 12, B fragment, 1 M PB		4.60×10^{-3}
Eco RI of Ad 12, C fragment, 1 M PB		6.75×10^{-3}
Eco RI of Ad 12, D fragment, 1 M PB		6.70×10^{-3}
Eco RI of Ad 12, E fragment, 1 M PB		4.86×10^{-3}

values were measured numerous times for each of the fragments.

From the results of the reassociation of unfractionated Ad 12 genome the $Cot_{1/2}$ value of adenovirus types 2 and 12 in 1 M $[Na^+]$ and under the conditions described is about 4.6×10^{-3} sec mol/liter, with a variation of $\pm 20\%$ in the system established. This value is compared with published values below.

The $Cot_{1/2}$ values reported were obtained under different experimental conditions. The effect of ionic strength has already been discussed. Wetmur and Davidson (1968) and Britten and Kohne (1969) both pointed out that the rate of reassociation is directly proportional to the square root of length of fragments in the reaction. Hence, the $Cot_{1/2}$, which is proportional to the 1/rate of reassociation could be calculated if the sizes of fragments are known and the rest of the parameters affecting the rate were insignificant. The source of $Cot_{1/2}$ values, the experimental conditions and $Cot_{1/2}$ values converted to 1 M (Na^+) and size of 700 nucleotides are listed in Table 12.

Hydroxyapatite chromatography was used to estimate the percentages of single-stranded DNA in the present work as well as in the works cited in Table 12. Except for the $Cot_{1/2}$ reported by Pettersson and Sambrook (1973), the rest of the converted $Cot_{1/2}$ values were comparable with and fell within 25% of the $Cot_{1/2}$ values

Table 12

Published $Cot_{1/2}$ Values from Reassociation of Adenovirus DNA

Source	Experimental condition at 68°C	$Cot_{1/2}$ reported (sec.mol/lit.)	$Cot_{1/2}$ converted (sec.mol.lit.)
Pettersson and Sambrook (1973)	Ad 2, 1 M NaCl 500 nucleotides	2.25×10^{-3}	1.97×10^{-3}
Gallimore <i>et al.</i> (1974)	Ad 2, 1 M NaCl 300 nucleotides	1.00×10^{-2}	6.67×10^{-3}
Pettersson <i>et al.</i> (1973)	Ad 2 0.14 M Na PB 500 nucleotides	2.02×10^{-2}	3.75×10^{-3}
Green <i>et al.</i> (1976)	Ad 12, 0.72 M NaCl 400 nucleotides	5.00×10^{-3}	3.03×10^{-3}

in the present work, which was 4.6×10^{-3} sec mol/liter. Pettersson and Sambrook (1973) used their system to quantitate the number of copies of viral DNA in Ad 2 transformed rat cells and arrived at the conclusions of exactly 1.00 copy of Ad 2 DNA per diploid equivalent of cellular DNA. It is not possible to conceive any explanation for the large discrepancy between their $Cot_{1/2}$ values and those of others and ours.

The method of fragmentation of DNA to small numbers of nucleotides by alkaline degradation used in the present work is similar to that reported by Gallimore *et al.* (1974) and Sharp *et al.* (1974). The size of fragments they obtained was about 300 nucleotides. Despite that it is obvious that we are referring to the mean size of a distribution of sizes, their value is quite different from the value reported in the present work, which is 700 nucleotides. In both cases, however, the method of determining fragment sizes was alkaline sucrose gradients. In the present work, the DNA was fragmented by boiling for 15 min in 0.3 N NaOH. The above mentioned authors boiled DNA in 0.2 N NaOH for 30 min which they claimed yielded 500 nucleotide fragments (Pettersson *et al.*, 1973). In their subsequent publications, the conditions varied. In one instance, they boiled the DNA in 0.3 N NaOH for 25 min and found that DNA sizes were about 300 nucleotides. In another publication (Gallimore *et al.*, 1974) DNA

fragments were degraded to nucleotide segments of 300 bases by boiling for only 15 min in 0.3 N NaOH (see caption for Table 2 of Gallimore *et al.*, 1974). The rate of reassociation is inversely proportional to the square root of fragment length. A variation of fragment length by a factor of 2, for example, could change the reassociation rate by 1.4 times. The converted $Cot_{1/2}$ values from Gallimore *et al.* (1974) and Pettersson *et al.* (1973) are 6.67×10^{-3} sec mol/liter and 3.75×10^{-3} sec mol/liter, respectively. The explanation for this discrepancy is not obvious. The $Cot_{1/2}$ value of the present work, 4.5×10^{-3} sec mol/liter, incidentally, falls between these two values.

The reconstruction experiments fall into three categories: reassociations driven by 100 times DNA concentrations; reassociations driven by 17 times DNA concentrations, and reassociation of A fragment driven by 1.5 times, 3 times and 10 times DNA concentrations. Results of these experiments confirm that the DNA-DNA reassociation system is an accurate, reliable and reproducible system.

II. Viral Genome in Cells

Ad 12 virus transforms hamster cells which can potentially induce tumors when injected into hamsters. Alternatively, this virus can also induce tumors directly when virions are injected into the animals. The

transformed cells contain viral DNA sequences (Green *et al.*, 1970; Green *et al.*, 1976), synthesize virus-specific RNA (Fujinaga and Green, 1976) and tumor (T) antigens (Huebner, Rowe and Lane, 1962). It is impossible to find any evidence of infectious virus or infectious viral DNA in these cells. The type of rescue operations that can recover virus from transformed cells by SV40 and polyoma (Koprowski *et al.*, 1967; Fogel and Sachs, 1969) have never yielded adenovirus from adenovirus-transformed cells (Tooze, 1974).

Quantitation of viral genome in transformed and tumor cells would give information concerning the following aspects: a) is the failure to 'rescue' adenovirus from transformed cells attributable to incompleteness of viral genome in the cells?; b) what parts of the viral genome are required to maintain the transformed state or the tumorigenic state of a cell?; c) how are the viral DNA's incorporated into a host cell? how are they maintained?; d) are there any differences regarding the pattern of integrated viral segments between transformed cells, highly oncogenic Ad 12 wild type induced tumor cells and weakly oncogenic Ad 12 cyt mutant induced tumor cells?

The pattern of Ad 12 integrated into host cell revealed by the present work is that the right-hand end of the viral DNA is usually missing and the other

fragments are represented in unequimolar amounts. Apparently, the right-hand end is not necessary to maintain the transformed or tumorigenic state. This also accounts for the fact that Ad 12 viruses could not be 'rescued' by cell fusion or other physical-chemical manipulations.

The fact that viral DNA sequences integrated with different frequencies suggest that the viral DNA molecule is not integrated as one piece in total but that fragments of the molecule are able to find their own integration sites. It does not seem that any piece is particularly preferred. The higher frequency of 12 copies is found with the 'C' fragment in two tumors, but then some tumor cells contain as little as 2 copies of 'C' fragment.

In the latter case, the tumors may contain significant number of normal cells. Fragments 'A' and 'E' are usually missing. It is possible that animals selectively eliminate cells carrying these fragments. These fragments were present in low concentrations in *in vitro* transformed cell lines.

The pattern is consistent with results from Ad 2 transformed rat cells in which only 14% of the left-hand end of the viral genome were present (Gallimore *et al.*, 1974). It is at variance with Green *et al.* (1976) who found that in HE C19, a hamster embryo cell line transformed *in vitro* by Ad 12, 8 viral genome equivalents representing close to 100% of the viral genome were present. Fanning and Doerfler (1976) reported that in Ad 12 transformed

hamster cell lines HA 12/7, A2497-7 and T637, the lowest frequency of occurrence of viral segment was that of the Eco RI A fragment. They found that the rest of the Ad 12 genome was present in varying amounts. The trend they reported and the pattern found in the present work are similar.

It is possible that different strains and sub-strains of virus can affect the integration of viral genes in tumor cells. Also, *in vivo* growth of tumor cells may alter the viral gene content, as in the case for cells transformed by herpes simplex virus (Frenkel *et al.*, 1976). Cyt mutants of the highly oncogenic Ad 12 induce tumors in hamsters with low frequency (Takemori *et al.*, 1968). Despite this difference in oncogenicity between the cyt mutant and the Huie strain, we found that the pattern of viral information in the tumor cells induced by these virus strains is essentially the same. Thus, it appears that at least 45% of the right-hand end of the Ad 12 genome is not required for the maintenance of tumors *in vivo*.

In summary, both transformed cells and tumor cells were studied; the former represents a population of cells that were infected with tumor viruses and survived *in vitro* growth and the latter represents cells that were infected with virus and survived *in vivo* selection of the animal. In many transformed cells, only a limited number of viral DNA sequences were present.

Presence of an integrated viral DNA is not sufficient to maintain several properties of the transformed phenotype. This had been suggested with analysis of revertants of transformed cells (Shani *et al.*, 1972; Ozanne *et al.*, 1973). *In vitro* growth, besides having selected for numerous new cellular characteristics, presented to cells a challenge which is different from that of the animal. For example, in an animal system, despite its cell mediated immunity being eliminated, some transformed cells failed to produce tumors (Gallimore *et al.*, 1977). Likewise, epithelial cell lines derived from a number of human carcinomas could grow in suspension but failed to form tumors in nude mice (Shields, 1976). This shows that transformed cells, and tumor cells represent different systems.

The pattern of integrated Ad 12 DNA into transformed and tumors was indistinguishable from each other. Furthermore, the cytotoxic mutant of Ad 12 Huie strain shows a similar pattern of integration. This mutant has low oncogenicity in hamsters (Takemori, 1968). Thus, no correlation between oncogenicity and the viral DNA integration pattern was found. Since it was suggested that only anchorage-independent growth affected tumor production (Risser and Pollack, 1974), this particular characteristic does not depend on the number of viral DNA sequences present in the tumor cells.

III. Integration of Viral Genome into Cellular DNA

The previous experiments had demonstrated that viral genome persisted in transformed cells and tumor cells. The logical extension of these observations would be to find out how the infecting viral DNA was inserted into the host DNA. On a theoretical basis, this question could be attacked by setting up experimental designs such as infecting hamster cells with Ad 12, and searching for viral sequences in the infected cells. On practical grounds, one would have discovered that in order to reach the sensitivity to detect one viral genome per cell, the minimum number of cells required for each experiment is of the order 10^8 cells. It would be an enormous task to culture this many primary hamster cells in monolayers. In order to pursue the investigation of interactions between virus DNA and cell DNA, the system of permissive infection of KB cells by Ad 12 was studied.

The kinetics of Ad 12 DNA integrated into KB cell DNA show that some viral DNA was integrated prior to viral DNA synthesis which is about 17 h post infection (Mak, 1969). The majority of viral DNA sequences were integrated after viral DNA synthesis has started. Concomitant with the beginning of viral DNA synthesis and peak viral DNA integration, the number of cells as well as cell DNA scored as high molecular DNA declined. The decrease in cell number could be explained as cell lysis

due to some form of cytopathic effect. The decrease in cell DNA as high molecular weight DNA suggests that cellular DNA was converted into lower molecular weight. A similar event was observed by Pater (Ph.D. thesis, 1976) at high multiplicities of infection. In our case, the majority of cellular DNA remains in the high molecular weight fractions because the multiplicities of infection were low.

Two methods of separating free viral DNA from cellular DNA were used. The network technique did not show the unequal representations of viral genome in the free viral DNA fraction, whereas the alkaline glycerol method did. The finding using network technique was consistent upon repeating the experiment while that using alkaline glycerol gradients was not. In any event this represents an incidental finding, the validity of which has little bearing upon the goals of studying integration. It could be important, however, if one were concerned with studying generation of defective virions (Mak, 1971).

The integration of adenovirus genome into cell DNA prior to viral DNA synthesis was also observed by other workers. In the Ad 2 KB system, a fast sedimenting DNA (50-90S) was found to contain both viral and cellular DNA sequences (Burger and Doerfler, 1974). This fast

sedimenting DNA was first detected at 2-4 h post infection (Schick, 1975). It was not determined, however, what percentage of intracellular viral DNA was represented in, nor what portion of the viral genome participates in this fast-sedimenting segment.*

The results presented in this work are consistent with the finding that integration occurs prior to viral DNA synthesis. Furthermore, we did show that the whole viral genome was integrated in equimolar proportions.

The number of integrated viral genomes was about 200-300 copies per KB cell, after infection with 2000 particles per cell. KB cells infected with adenovirus type 2 at 100 pfu/cell resulted in 2000 to 7000 copies of viral DNA in the fast sedimenting form (Schick *et al.*, 1976). In the system of SV40 infecting permissive cells. Holzel and Sokol (1974) reported that 20,000 viral genome copies per cell were integrated. The genome of SV40 is 3 times smaller than adenovirus genome. As far as the integration of the amount of exogenous genetic material is concerned, the observation of Holzel and Sokol was consistent with that of Schick. The finding in the present work, appears to be different from these. It is tempting to speculate what would happen to the level of integration if the number of infecting Ad 12 virus particles were higher. Under high multiplicities of infection, virus yield would be suppressed (Ezoe, personal communication) and cellular

* See note in Addendum

DNA would be fragmented (Pater, 1976, Ph.D. thesis).

If one is content with the objective of studying viral DNA-cell DNA interactions under the conditions of attaining modest yields of viral progeny, rather than obtaining maximum number of fragments containing both viral and cellular information, the method and design used in this thesis are appropriate and realistic.

IV. Site of Viral Integration into Cell DNA

Hamster cells transformed by adenovirus show a set of new properties. (Tooze, 1973b). According to Green *et al.* (1976), all the intracellular Ad 12 DNA is covalently linked to cellular DNA. Hence integration is necessary to maintain the transformed properties of these cells. Mammalian genome contains 10^9 base pairs. One would like to know how many integrations could be introduced among these 10^9 base pairs, and whether there are specific sites?

A. Evidence of recombination between viral DNA and cell DNA

It has been demonstrated that viral DNA was among the cellular DNA recovered from "DNA networks" and from the high molecular weight DNA fraction of the alkaline glycerol gradients. Conversely, cellular DNA sequences could be recovered by hybridizing to viral DNA on filters. These experiments show conclusively that Ad 12 DNA is covalently integrated into the KB cell DNA.

Covalent linkage of viral and cellular DNA had been found in DNA sequences of defective supercoiled viral DNA molecules (Lair and Winocour, 1972; Martin *et al.*, 1973; Brockman *et al.*, 1973; Winocour *et al.*, 1974). Evidence for the integration of viral DNA into cell DNA in the lytic system was given by Babiuk and Hudson (1972), Ralph and Colter (1972) and Tuler (1977). Their results suggest that integration of polyoma viral DNA into mouse DNA occurs at about the same time as intranuclear T-antigen appears and coincides with the onset of cellular and viral DNA replication. About 20 to 300 genomes were integrated at the peak of viral DNA replication. Following viral DNA replication, approximately half of newly synthesized polyoma DNA were cell DNA-associated.

B. Characterization of integration sites by their frequency of repetition

In the lytic infection of KB cells result suggests that the integration sites reassociate identically to an uninfected mammalian genome. The frequency of repetition of these sites is similar to the rest of the cellular genome. In contrast, Ad 12 infects non-permissive cells and integrates into intermediate and highly repeated cellular sequences (Groneberg *et al.* 1977). Perhaps, integration is different in abortive and lytically infected cells.

Groneberg and co-workers (Groneberg *et al.*, 1977) have demonstrated that Ad.12 DNA is covalently linked to the high and intermediate repetitive sequences of the transformed hamster cell DNA. Their strategy included Sal I restriction enzyme to cleave cell DNA; filter hybridization to select cellular pieces adjacent to viral DNA and characterization of these cellular sequences following elution. Except for the techniques used for fragmentation of cellular DNA, the basic experimental approach used here was similar. The results we have obtained with KB cells are at variance with their results with transformed cells. Further discussion on the differences between integration sites of permissive and non-permissive cells will follow.

C. Specificity and number of integration sites

The number of integration sites found in the present work is about one order of magnitude less than that generally reported in other systems. The maximum number of integrated viral genomes reported is 2×10^4 (Holzel and Sokol, 1974). Each functional gene in mammals had been suggested to be composed of 2×10^3 nucleotides (Davidson *et al.*, 1975). It has also been suggested that the functional genes in a mammalian cell are about 10^4 to 10^5 .

If the integrated viral genome were distributed in a random fashion, this amounts to one integration site every few genes, which is a large scale recombinational event in a short time. Yet the cell number and intracellular DNA continue to increase up to about 20 h post infection; as we demonstrated in the present study. It seems that the lower number of integrated viral genome as found by this study is more reasonable. Another possible explanation is by proposing integration in tandem fashion. This would have required fewer sites. However, concatemers of adenovirus had never ~~been~~ reported. Recent results on Ad 5 infection of HEK cells showed that only 7 copies were integrated (Tyndall *et al.*, 1978). But in their system, the aim was not to demonstrate the number of integration sites. They did show, however, that viral DNA replication is not required for integration. This is consistent with our result that integration occurs before viral DNA synthesis.

As far as specificity of the sites is concerned, existing evidence results in quite conflicting interpretations. *In situ* hybridization and autoradiography has localized viral sequences on specific chromosomes of transformed cells in the cases of Ad 2, 7 and 12 (Loni and Green, 1973; McDougall *et al.*, 1974). Ad 12 also cause site specific breaks in chromosomes 1 and 17 (zur Hausen, 1967; McDougall, 1971).

Beyond the adenovirus system, Mary Weiss (1970) suggested that viral genetic material of SV40 may not be integrated at any specific site. In the system she studied, SV40 transformed human cell-normal mouse cell hybrids continuously shed human chromosomes. She was able to find virus-specific tumor antigen in several cells containing but a few remaining human chromosomes. This observation is consistent with biochemical evidence of Botchan *et al.* (1976). They cleaved the genome of SV40 transformed rat cells with restriction enzymes. The cleaved DNA was hybridized to SV40 DNA using the Southern technique (1975). The integrated viral genome was found to be integrated at different sites depending on transformed cell lines. Opposing evidence was given by the research group of Croce (Croce *et al.*, 1973; Khoury and Croce, 1975; Croce, 1977). They suggested that the site of integration of SV40 on human cells was on chromosomes 7 and 17. It should be noted also that specific attachment sites (att) on the viral DNA were also suggested. They were mapped at 31.2, 20.5 and 24.8 positions of the viral genome (Chow *et al.*, 1974).

So, here we are: whether there are specific sites or not remains a controversial topic. Our results are consistent with the idea of non-specific sites or specific sites randomly distributed. For the past 10 years, the research activity in DNA tumor virus had been concentrated on the expression of genetic information and

the induction of new growth characteristics of the cells. It is only recently that more people are paying attention to the equally important aspect of integration sites. Research into this aspect will ultimately solve the puzzle of how an exogenous DNA is introduced into a host, thereby resulting in alterations of growth.

Our results can be discussed in a more general context if one also includes data from RNA tumor viruses. Avian oncornavirus DNA sequences are vertically transmitted endogenous genes present in all apparently normal chicken cells (Shoyab and Baluda, 1975a,b). These endogenous proviral DNA sequences have been shown to be integrated in a region of cellular DNA with a repetition frequency of between 1000 to 2000 times per haploid chicken genome (Evans *et al.*, 1974). Exogenous avian myeloblastosis virus DNA was thought to be tandemly integrated into three sites (Shoyab *et al.*, 1976).

When the observations from different sources are arranged according to the number of integration sites (see Table 13) a pattern emerges.

The following trend can be deduced after a careful consideration of Table 13: 1) In all of the transforming systems, the integration is characterized by a low number of copies; with better defined sites; 2) In all of the productive infection systems, the integrated copies are numerous, the sites are random.

Table 13

Summary of Studies on Integration

System	Characteristics of sites	Source
<u>A. Low number of integrated genome copies (less than 50/cell)</u>		
Ad 12 - BHK 21		Doerfler (1970)
Ad 12 - hamster	intermediate and highly repeated sequences	Groneberg <i>et al.</i> (1977)
SV40 - transformed mouse	exclusively unique sequences	Gelb and Martin (1973)
Exogenous AMV DNA - chicken	tandemly integrated adjacent to proviral site	Shoyab <i>et al.</i> (1976)
Endogenous proviral - chicken	repeated 1000 to 2000 times	Evans <i>et al.</i> (1974)
<u>B. High number of integrated genome copies (more than 50/cell)</u>		
Ad 12 - KB	200-300 sites/cell, random distribution	Present work
Ad 2 - KB	sediment as 50-90S molecules 2000-7000 sites/cell	Schick <i>et al.</i> (1976)
CELO - check CEK	500-1000 sites/cell	Tyndall <i>et al.</i> (1978)
SV40 - CH-1	20,000 sites/cell	Holzel and Sokol (1974)
SV40 substituted DNA	mainly unique sequences	Winocour <i>et al.</i> (1974)

There is a report about the pattern of integration of infectious DNA of avian reticuloendotheliosis viruses in avian cells (Battula and Temin, 1978). There were multiple sites of integration in acutely infected cells and only a single site of integration in chronically infected cells with no cell death. At this stage it is still too early to make any final conclusion. But it is clear that more work is required. Discussion on this topic will continue in the following section concerning the factors that affect integration frequencies.

D. Factors affecting integration frequencies

As already discussed earlier, integration of viral genome is an important step in viral transformation. The frequency of integration is affected by factors such as specificity of integration sites, recombinational events and factors which would affect these events.

The earliest model of lysogeny was suggested to describe the integration of λ phage DNA into bacterial genome with a concomitant repression of most of the viral genes (Lwolf, 1973). Campbell (1962) suggested circularization and reciprocal recombination between specific sites on the viral genome and host chromosome as the mechanism for this integration. Perhaps biased by this view, some authors suggested that the inverted terminal repetitions of adenovirus would facilitate its integration by circularization (Garson *et al.*, 1972). This view is not necessarily

well founded because fragments of adenovirus could transform cells (Graham, 1976). The radiobiologist, Cleaver (1971), has suggested that unrepaired lesions in DNA would cause gaps to be left in the newly synthesized daughter strands; the gaps are filled by a recombinational process from the parental strand of the complementary DNA molecule (Rupp and Howard-Flanders, 1976). In *Escherichia coli* this recombinational process is error prone. Cleaver (1971) suggested that oncogenic viral DNA could be incorporated in a similar event.

i. Effect of Serum

The results presented in this work suggested that at low serum concentration, integration was suppressed. The effect of serum on integration and transformation had been studied in the SV40 Chinese hamster system (Hirai and Defendi, 1974a). Our results are consistent with theirs. Although they suggested that serum probably mobilized cells into the S phase of the cell cycle - hence, more cells are synthesizing DNA, it is difficult to conclude what step in DNA synthesis was linked with viral DNA integration. In the routine tissue culture, serum supplies about 10% of the media. Which substances in the serum were required remain a puzzle (Morton, 1970). Capacity to initiate cell division in nondividing cells, response of cells to added serum by growing to higher cell densities (Rubin, 1971;

Holley and Kierman, 1968), etc. are only a few of the many effects serum could have had on cells.

ii. Effect of Ara C on Integration

From the previous discussion, lowered serum concentration seemed to play a role in lowering the level of integration. Ara C inhibits cellular DNA synthesis; it would be interesting to know how this compound would affect integration. Ara C is metabolized to nucleotide derivatives which apparently successfully compete with normal metabolites at the catalytic center of enzymes converting cytidine nucleotide to deoxycytidine nucleotide and the enzyme incorporating deoxycytidine triphosphate into DNA, thus curtailing DNA synthesis. Our finding that ara C did inhibit integration is consistent with the results from experiments for serum effects. Some events in the S phase of cell cycle may be essential for the integration process. Whether this is the only process by which viral DNA can be integrated or not is not known.

iii. Effect of Caffeine on Integration Level

The results of serum dependence and ara C inhibition of integration level reported earlier show that cellular DNA synthesis may facilitate the integration of viral DNA. A natural question to ask then, is whether other means of inducing cell DNA synthesis would affect the integration

frequency. It has been reported that pretreating animal cells with x-rays, UV irradiation or radiometric drugs (e.g. 4-nitroquinoline-1-oxide) *in vitro* could increase the frequency of transformation by SV40 (Coggin, 1969; Pollack and Todaro, 1968; Diamond *et al.*, 1974); by polyoma virus (Stoker, 1963); and by human Ad 13 (Coggin, 1969). If transformation enhancement is mediated by cell DNA breakage and repair, one would like to examine the effect of caffeine on integration. The results reported in the present work suggest that caffeine does enhance integration. This observation is consistent with that reported for other systems. The explanation, however, is quite controversial. It has been reported that caffeine potentiates cell killing and produces chromosome aberrations and inhibits some form of repair system after UV or x-irradiation (Adler, 1970; Bishun *et al.*, 1973; Kihlman *et al.*, 1974; Rauth, 1970; Roberts, 1978). Casto *et al.* (1974) found that caffeine enhanced the frequency of adenovirus-induced transformation. In the SV40-cultured mouse cell C3H2K system, Ide *et al.* (1976) found that irradiation of cells with UV just before infection with subsequent cultivation in the presence or absence of caffeine, transformation would be enhanced. These results show that caffeine does two things: interferes with DNA repair systems and enhances viral transformation. The results obtained in the present work suggest that

integration is also enhanced. Caffeine may enhance transformation via enhancing integration. Since integration involves the insertion of viral DNA into host DNA, the opening of DNA strands by interfering with repair systems could have facilitated this process.

iv. Effect of UV Irradiation

UV irradiation causes breaks in DNA. Repair of these breaks necessitated DNA synthetic processes. Some attempts to identify the integration sites were reported. It was found that in productive systems, there are numerous integration sites. Reassociation experiments showed the frequency of repetition of these sites to be indistinguishable from that of the whole genome. However, none of these suggest that the integration sites are non-specific. On the contrary, UV irradiation, which causes specifically identifiable lesions of dimers did not enhance integration. This would have suggested that integration involves a certain degree of specificity, either on the level of sites, enzymes or combination of both. The enzymes involved in thymine dimer repair was believed to be endonuclease which cleaves the chain near a dimer to leave 3' phosphoryl and 5' hydroxyl termini, the latter adjacent to the dimer; a 3' phosphomonesterase to remove the terminal phosphate, an exonuclease to degrade the dimer-containing sequence, and two other enzymes, a polymerase and a ligase to complete the repair. Since the

patch size is about 200 nucleotides, it is difficult to conceive a mechanism by which large viral DNA pieces could be integrated into such sites (Cleaver, 1971). Post-replication repair (Rauth, 1970) is an alternative pathway by which cell DNA can repair dimer lesions. Unrepaired lesions in DNA cause gaps to be left in the newly synthesized daughter strands; the gaps are filled by a recombinational process from the parental strand of the other DNA molecule (Rupp and Howard-Flanders, 1967). The human XP cells, which are deficient in excision repair or its variant, deficient in post-replication repair, are more mutable by UV than normal cells (Maher and McCormick, 1976, Maher *et al.*, 1976). Lehmann and Bridges (1977) suggested that the higher mutability of XP variants is due to an error-prone repair pathway in human cells because an error-free process is absent in XP variants or because the number of mistakes were excess. The enhancement of integration level by caffeine could be due to its ability to interfere with post replication repair.

Caffeine has no effect on excision repair (Cleaver, 1969). It has been observed that caffeine inhibits the filling up of the post replicational gaps opposite the thymine dimers elicited in parental strands of DNA by UV irradiation (Cleaver and Thomas, 1969; Fujiwara, 1972; Fujiwara and Kendo, 1972). The persistent opening of gaps in the replicated host DNA would have given ample time for the viral DNA to be integrated.

E. Prospects of Further Experiments to Extend the Present Work

Some experiments can be planned to test some speculation outlined previously as well as to characterize further the integration sites. A straight-forward approach is to study the synergistic effect of caffeine on UV irradiation. If caffeine enhances integration by the mechanism suggested, then inducing more sites in the presence of caffeine would certainly further enhance integration.

Integration sites could be extracted following the strategy as outlined in Figure 30. Viral sequences could be removed by exhaustive reassociation to unlabelled viral DNA and HAP chromatography. The remaining integration sites can be characterized by restriction enzyme cleavage patterns. By characterizing integration sites from different cell lines which are permissive to Ad 12 infection, one can determine whether these sites are similar.

Early events in the integration of viral DNA sequences into non-permissive cells could be studied. Since only a very small amount of viral DNA sequences are integrated, radioactively labelled viral DNA reassociation probes with an activity ten times stronger is required. In this approach, information obtained belongs to a system which more approximates the tumorigenic state.

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