IDENTIFICATION OF THE ADENOVIRUS TYPE 12 GENE PRODUCT(S) REQUIRED FOR INDUCTION OF CHROMOSOMAL ABERRATIONS IN HUMAN CELLS

IDENTIFICATION OF THE ADENOVIRUS TYPE 12 GENE PRODUCT(S) REQUIRED FOR INDUCTION OF CHROMOSOMAL ABERRATIONS IN HUMAN CELLS

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ABSTRACT

Unlike most RNA and DNA containing viruses, which induce cytogenetic damage at random sites throughout the human genome, the highly oncogenic adenovirus type 12 is also capable of inducing damage at specific chromosomal sites. Infection of human embryonic retinal or kidney cells with Ad12 results in the induction of heterochromatic gaps at specific (17q21-22, 1p36, 1q21, and 1q42-43) and random sites in the cellular chromosome. Previous work by Durnam et al. (1986) demonstrated that the viral early region 1 (E1) is sufficient for the induction of damage at band 17q21-22. The objective of the present study was to 1) identify the Ad12 E1 gene product(s) required for the induction of aberrations in human diploid cells, and 2) to determine whether the same or different functions are involved in the induction of damage at specific and random sites. To this end, adenovirus type 12/adenovirus type 5 recombinants with hybrid E1 sequences as well as viruses with mutations in the Ad12 E1B genes were used to map the Ad12 E1 function(s) required for the induction of chromosomal aberrations. The results of this study indicate that the expression of E1A proteins is not sufficient for this

effect. On the other hand, mutations within the E1B 55Kd protein but not the E1B 19Kd protein were found to affect the ability of the virus to induce both specific and random damage (Schramayr et al., 1990).

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This thesis is dedicated to my parents.

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LIST OF ABBREVIATIONS

Ad	adenovirus
bp	base pair
BCIP	bromochloroindolyl phosphate
cpm	counts per minute
Ci	curie(s)
°C	degrees celsius
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dl	deletion mutant
EDTA	ethylenediamine tetraacetic acid
FCS	fetal calf serum
ug, mg, g	microgram, milligram, gram
HEK	human embryonic kidney
HER	human embryonic retinal
HS	horse serum
hr(s)	hour(s)
IgG	immunoglobulin G
Kd	kilodalton
Kb	kilobase
ul, ml, l	microlitre, millilitre, litre
mu	map units

min(s)	minute(s)
uM, mM, M	micromolar, millimolar, molar
MW	molecular weight
MOI	multiplicity of infection
nm	nanometers
NBCS	new born calf serum
NBT	nitroblue tetrazolium
NP40	Non-Idet 40
nt(s)	nucleotide(s)
PBS	phosphate buffered saline
PFU	plaque forming units
pm	point mutant
PPO	2,5-diphenyloxazole
rpm	rotations per minute
sec(s)	second(s)
SSC	sodium chloride sodium citrate
SDS	sodium dodecyl sulfate
TCA	Trichloroacetic acid
TNE	TRIS/NaCl/EDTA
TRIS	Tris(hydroxymethyl)aminomethane
V	volts
v/v	volume/volume
w/v	weight /volume
wt	wild type

INTRODUCTION

1.1 The Role of Genetic Instability in Carcinogenesis

It is now well established that cancer arises from a stepwise accumulation of mutations in the genome (Klein and Klein, 1985; Bishop, 1987; Yunis, 1987; Klein, 1988). According to this theory, cancer originates from a single cell which in dividing develops a mutation. This original mutation may be spontaneous or it may be caused by any number of carcinogens, including chemicals, radiation or viruses. In successive divisions the mutated cells may accumulate additional mutations which may confer to them a selective growth advantage over normal cells causing them to grow faster and more aggressively. In fact, researchers have found that as many as ten distinct mutations may have to be accumulated in a cell before it becomes malignant (Marx, 1989). Although, for the most part, these mutations cannot be seen cytogenetically, microscopically visible damage to chromosomes have also been identified in many neoplasias.

The theory that karyotypic alterations might be responsible for the initiation and evolution of cancer is supported by a number of observations. First, most tumour

cells possess chromosome abnormalities (numerical and morphological alterations of the chromosomes) which are not present in other cells of the body. In addition, all the neoplastic cells in a given tumour frequently exhibit the same cytogenetic change, or related changes, attesting to their clonal origin (Nowell, 1976). One of the earliest accounts of a specific cytogenetic abnormality in cancer described the presence of a minute chromosome in the cells of individuals with chronic myelogenous leukemia (CML) (Nowell, 1960). This minute chromosome, which is now called the Philadelphia chromosome, is a reciprocal translocation between chromosome 22 and 9 resulting in a shortened 22 (Nowell and Croce, 1987). The Philadelphia chromosome occurs in 90-95% of individuals with CML and it has also been identified in a number of individuals with acute lymphocytic leukemia (ALL) (Nowell and Croce, 1987). The high frequency with which it occurs in CML suggests a strong causal relationship between this chromosomal abnormality and the disease. A second example of karyotypic abnormalities in cancer is the structural rearrangement of chromosome 1p present in approximately 70% of the tumours from individuals with neuroblastoma (Gilbert et al., 1984). In addition to the abnormalities of chromosome 1p, trisomies for the long arm of chromosome 1 and 17 occur in approximately 20% or more of these cases. Once again, the high frequency at

which abnormalities at chromosome 1p occur in neuroblastoma suggest a primary role in the development of this cancer. As the abnormalities of chromosome 1q and 17q occur to a lesser extent, and are not limited to neuroblastoma, it is unlikely that the gene changes produced by each will be necessary steps in neuroblastoma transformation. Each is however believed to represent a secondary event in tumour progression (Gilbert et al., 1984).

Second, the sites of genetic alterations often involve cellular proto-oncogenes or tumour suppressor genes. Activation of proto-oncogenes and/or inactivation of tumour supressor genes is thought to occur in both early and late stages of carcinogenesis, and can occur via a number of different types of damage. Proto-oncogenes are dominantly acting oncogenes that have been detected through their ability to induce cell proliferation and tumourigenicity. Many of the initial observations of proto-oncogene activation have come from the study of specific reciprocal translocations in various hematopoietic tumours such as Burkitt's lymphoma and CML. For example, in approximately 75% of individuals with Burkitt's lymphoma a reciprocal translocation between chromosome 8 and 14 is observed, with the remaining 25% of cases having a translocation between chromosome 8 and 22 or chromosome 8 and 2 (Nowell and Croce, 1987). It has been

demonstrated that in each of these translocations a transcriptionally active and rearranged immunoglobulin gene is brought into juxtaposition with the c-myc gene, resulting in transcriptional activation of the translocated c-myc (Taub et al., 1982; Adams et al., 1983). A similar situation is observed in the case of CML. A reciprocal translocation between chromosome 9 and 22 relocates a portion of the proto-oncogene c-abl and fuses it with a newly recognized genetic locus known as the breakpoint cluster region (bcr) (Shtivelman et al., 1985). The genetic fusion in turn creates a chimeric protein that includes the functional domain of the c-abl gene product, but whose enzymatic activity is more robust than that of the normal gene product (Ben-Neriah et al., 1986). In addition to translocation, chromosome changes in other tumours, reflecting gain or loss of genetic material have been identified and indicate a critical role for oncogene dosage in some instances of carcinogenesis (Nowell and Croce, 1987). For example, activation of Ki-ras by base substitution mutations has been observed as an early event in human colorectal tumourigenesis (Vogelstein et al., 1988), and amplification of N-myc has been associated with tumour progression of human neuroblastoma (Brodeur et al., 1987).

In contrast to proto-oncogenes, tumour suppressor genes are recessive oncogenes whose normal activity is to constrain or

limit cell proliferation. Deletion or mutational inactivation of such genes is thought to release cells from quiescence, enabling cell populations to expand unchecked. Extensive genetic and karyotypic evidence has suggested that one such suppressor gene determines susceptibility to retinoblastoma (Rb), and hence it is referred to as the Rb-1 gene. In 70% of Rb tumours, the long arm of chromosome 13 becomes homozygous for the chromosome containing the mutant Rb-1 allele (Cavenee et al., 1983). Patients with heritable Rb have also been shown to have a germ-line mutation in the candidate gene. The second RB-1 allele is inactivated most frequently by deletion or recombinational mechanisms during the initial stages of carcinogenesis (Hansen et al., 1985; Fung et al., 1987). In addition to being found in retinoblastoma, homozygosity for the mutated Rb-1 gene has been identified in a number of other human cancers, including osteosarcomas, small cell carcinomas of the lung and breast cancers (Hansen et al., 1985; Harbour et al., 1988; T'Ang et al., 1988; Minna et al., 1989). An additional tumour suppressor gene, referred to as p53, has been identified on the short arm of human chromosome 17 (McBride et al., 1986). Mutation of the p53 gene in transgenic mice has been shown to lead to development of lung tumours (Lavigueur et al., 1989). This gene also appears to be frequently affected in all types

of lung cancer in humans (Minna et al., 1989).

Third, most environmental carcinogens induce mutations in DNA and alterations in the number and structure of chromosomes. For example, cigarette smoking may account for up to one third of human cancers (Evans, 1984). A number of studies have evaluated the effects of cigarette smoke on chromosomal aberrations, and have found a highly significant increase in aberration frequencies in the blood cells taken from light smokers (Vijayalaxmi and Evans, 1982). An even larger increase in genetic damage was observed in heavier smokers (Obe et al., 1982). It is clear from these kinds of studies, and also from in vitro studies, that the majority of known chemical carcinogens are genotoxic and that in many cases the genetic damaging effects can be detected in the somatic cells of exposed individuals (Evans, 1984).

Fourth, inherited susceptibility to cancer is often associated with congenital cytogenetic defects or with impaired DNA repair. For example, autosomal recessive diseases such as Bloom's syndrome, Fanconi's anaemia, Xeroderma pigmentosum, and ataxia telangiectasia are associated with high frequencies of chromosome breakage and reunion, as well as with an increased incidence of leukemias and other cancers (Bloom et al., 1966; Veldhuisen and Pouwels, 1970). Cells from Fanconi's anaemia and Xeroderma

pigmentosum patients have also been shown to be more susceptible to transformation by Simian virus 40 (SV40) than normal cells (Todaro et al., 1966; Veldhuisen and Pouwels, 1970).

Finally, chromosome abnormalities are more extensive in advanced tumours. It is believed that the increased genetic instability of neoplastic cells is more likely to generate variants than in normal cells. Some of these variants may in turn possess additional selective growth advantages allowing them to survive and expand to become a predominant subpopulation within the tumour. For example, studies on the cells from individuals with CML have shown that in the early indolent stage of this disorder the cells typically show only the translocation between chromosome 9 and 22 involving the c-abl oncogene, but the terminal accelerated phase of the disease apparently represents overgrowth of this leukemia population by one or more subclones having additional karyotypic changes (Nowell and Croce, 1987). Similar associations have been demonstrated in other human leukemias as well as some solid malignancies in man and in animals.

Similar to the case of tumour development, immortalization and oncogenic transformation of human cells appears to be a multistep process, and chromosomal abnormalities are a consistent feature of cells transformed

in vitro by physical or chemical agents and by viruses (DiPaolo, 1983; Chang, 1986). Earliest studies investigating the relationship between karyotypic changes and the development of the transformed phenotype determined that chromosomal alterations are not necessary, nor are they prerequisite, for the morphologically transformed phenotype to be expressed but rather it appears that the transformation process per se causes chromosomal instability (Moorhead and Saskela, 1965). In experiments by Weinstein and Moorhead (1967) human cell cultures were infected with SV40 and karyotypic analyses were performed on the earliest serial culture in which cells were released from contact inhibition of division. In these cultures of diploid fibroblast-like cells these investigators found that the frequency of normal karyotypes was higher than statistically expected. These findings suggest that loss of contact inhibition of cell division appears to occur prior to the alteration of chromosome morphology. Similarly, Walen (1981) has shown that morphologically transformed progenitor cells of an experimental population of human epithelial cells have a normal diploid karyotype. The progenitor cells subsequently acquire the characteristics of chromosomal instability. This instability leads to chromosomal variation from which selection and evolution of new chromosomal stem lines can

occur over time in culture. This hypothesis is consistent with findings that alterations in both number and structure of the chromosome preceed the onset of crisis of the transformed population (Moorhead and Saskela, 1965; Graham et al., 1977; Walen, 1981; Chang et al., 1986). For example, analysis of human cells transformed by SV40 or by the SV40 early region has shown that chromosomal alterations occur as early events following expression of the viral oncogenes, and preceed the onset of crisis in the transformed population (Moorhead and Saksela, 1965; Walen, 1987; Chang et al., 1986). Similar observations have been reported for human 293 cells, which are transformed by adenovirus type 5 (Ad5) DNA and express only the viral early region 1 (E1). Substantial karyoptypic changes have been detected in these cells prior to crisis and the establishment of the immortal line (Graham et al., 1977; L. Wei and S. Bacchetti, unpublished). These observations suggest that the expression of viral oncogenes might correlate with chromosomal damage. This genetic damage could in turn give rise to populations of mutants amenable to selection for a variety of phenotypes including the ability to survive crisis and to induce tumors. It therefore seems possible that chromosomal alterations might play a role in the initiation of transformation <u>in vitro</u> and/or in the development of a fully transformed phenotype.

1.2 Adenoviruses as a Model System for Cancer Development

It has been known for a couple of decades that viruses can induce chromosomal aberrations both in vivo and in cultured cells. For the most part, both RNA and DNA viruses cause random chromosomal damage, which can vary in severity from single gaps and breaks to complete genomic pulverization (Nichols, 1983). The discovery of these viral effects on the mammalian genome came with early attempts to make a connection between viruses and human cancer (Trentin et al., 1962; Huebner et al., 1962). In particular, the human adenoviruses have been the subject of intense research as a model system for human cancer since the discovery that adenovirus type 12 (Ad12) induces tumours when injected into animals (Trentin, 1962). In addition, all strains of adenovirus have been shown to transform cells in vitro and the transformants can be tumourigenic. Although at present they do not appear to play a role in human cancer the adenoviruses continue to provide an excellent model for the study of oncogenic transformation, and to a much lesser extent, for elucidating mechanisms involved in the induction of chromosomal aberrations. The experimental approach taken in the present study consists of using highly oncogenic Ad12 as a model system for the induction of chromosomal aberrations in human

primary cells. Ad12 is of particular interest because, unlike most other RNA and DNA containing viruses, it induces heterochromatic gaps at specific sites on chromosome 1 and 17, as well as gaps and/or breaks at random sites throughout the human genome (Stich et al., 1964; Zur Hausen, 1967; McDougall, 1971; McDougall et al., 1974). Additionally, rearrangements involving the same regions on chromosome 1 and 17 as those that are modified by Ad12 have been detected in a number of human tumours (Rowley et al., 1977; Gilbert et al., 1984)

1.3 Adenovirus Classification

The adenoviridae family is composed of viruses with a linear, double stranded DNA genome enclosed in an icosahedral protein shell, and is divided into two genera; mastadenoviruses and aviadenoviruses. Classification into the two genera is based on the presence (Mast-) or absence (Avi-) of common antigenic determinants which are detected by either complement fixation assays or agar-precipitation tests. The mastadenoviruses have been isolated from a wide range of mammalian hosts, including higher primates (chimpanzees and humans). In contrast, the aviadenoviruses have only been isolated from a limited number of avian species.

The first human adenoviruses were isolated from tonsils and adenoidal tissue of infected individuals (Rowe, 1953; Hilleman and Werner, 1954). Over the ensueing 37 years, 42 antigenically distinct serotypes of human adenovirus have been isolated (Horwitz, 1990), and have been associated with a number of respiratory illnesses and inflammation of various tissues and organs of the body (Straus, 1984; Horwitz, 1990). The 42 human adenovirus serotypes have been classified into six main subgroups (A-F) based on a number of different biological and molecular criteria. These measures include haemmaglutination capacity (Rosen, 1962; Hierholzer, 1973), G and C content of the genome (Pina and Green, 1965), oncogenicity in rodents (Huebner, 1967), DNA homology (Green, 1979), molecular weight of virion polypeptides (Wadell, 1979) and restriction digestion pattern of the viral DNA (Wadell, 1980).

With few exceptions, all the methods of classification appear to agree and thus, can be used in conjunction to create an all encompassing scheme. By combining a number of the classification measures, six homology groups (A-F) were established which reflect various facets of relatively few biochemical properties shared by adenoviruses. Group A (Ad 12, 18, 31) which contains the highly oncogenic serotypes showed the greatest degree of heterogeneity with the genomic

sequence only having 48-69% homology to each other. Group B (Ad 3, 7, 11, 14, 16, 21, 34, 35) and C (Ad 1, 2, 5, 6) have intermediate or no oncogenic potential respectively, while group D (Ad 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42), E (Ad 4), and F (40, 41) contain less well studied and rarer isolates of adenoviruses. In all cases there is less than 25% homology between classes and greater than 85% within a class with the exception of class A, already mentioned above. For technical reasons, most of the research involving adenoviruses has dealt with serotypes 2 or 5. Unless otherwise stated, the information presented below will refer to these two serotypes however, it is considered valid for adenoviruses in general (Horwitz, 1990).

1.4 Virion Structure

Adenoviruses are non-enveloped, icosahedral viruses containing a single linear duplex molecule of DNA (Horne et al., 1959; Green and Pina, 1963; van der Eb and van Kesteren, 1966). The viral icosahedron is approximately 65-80 nm in diameter (Horwitz, 1990) consisting of an outer protein shell, or capsid, and an inner core structure of DNA and protein (Nermut, 1984). Twelve major virus encoded proteins

designated VPII, VPIII, VPIIIa, and VPIV-VPXII as well as several minor proteins make up the total structural components of the virus (Maizel et al., 1968a; Everitt et al., 1973; Nermut, 1984).

The viral capsid is composed of 252 distinct protein subunits which are referred to as capsomeres and are each approximately 7 nm in diameter (Horne et al., 1959). The capsomeres are divided into two main structural species called hexons and pentons (Ginsberg, 1966). The 240 hexons which fill the facets are made up of three VPII non-covalently bound subunits of 120 Kd (Maizel et al., 1968a; Everitt et al., 1973, 1975; Furcinitti et al., 1989). The 12 pentons are at the vertices of the icosahedron and are made up of two components: a penton base composed of five VPIII subunits of 70Kd, and a proteinaceous fibre which projects outward from the base, consisting of a trimer of VPIV subunits of 62Kd (Devaux et al., 1982; Nermut, 1984; Horwitz, 1990). The fibre has a length of 10-30 nm depending on the serotype, a diameter of 2 nm, and a knob, approximately 4 nm in diameter, at the end distal to the capsid (Valentine and Periera, 1965). The fibre polypeptide is oriented so that the amino terminus is attached to the penton base whereas the carboxy end contains the knob (Horwitz, 1990). A schematic representation showing the adenovirus polypeptides and their

positions in the virion is illustrated in figure 1.

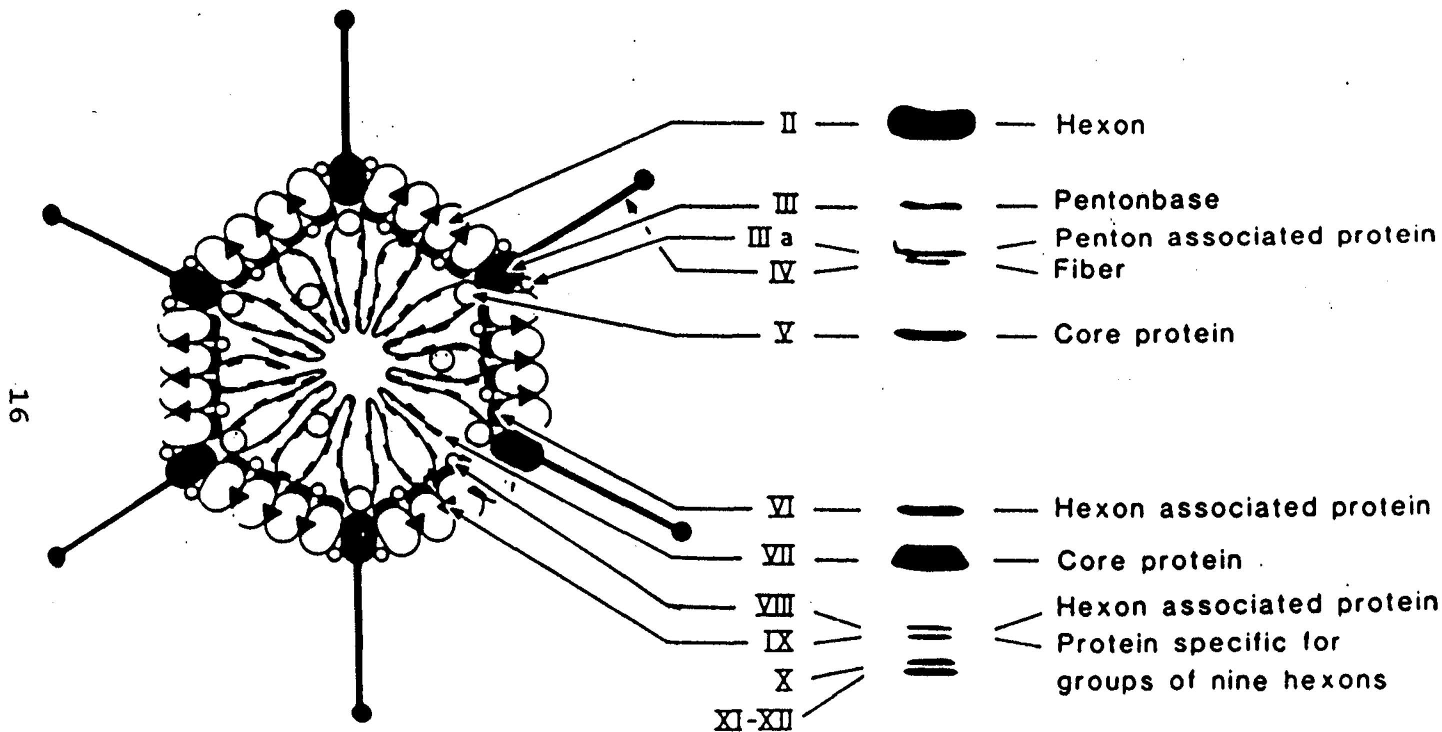
Analysis of virion proteins by SDS-polyacrylamide gel electrophoresis has resulted in the identification of a number of other, less abundant capsid components (Maizel, 1968a; Everitt et al., 1973, 1975). These viral components include VPIIIa (66Kd), which associates with the peripentonal hexons (Everitt et al., 1973, 1975), VPIX (12Kd) which acts as a cement between hexon capsomeres and binds them together in groups of nine (Everitt, 1973; Furcinitti et al. 1989), VPVI (24Kd) and VPVIII (13Kd), which are also hexon-associated and line the capsid interior, but are not part of the viral core (Everitt et al., 1973, 1975; Flint, 1981; Devaux et al. 1982; Horwitz, 1990). Polypeptide VPVI and VPVIII are synthesized as larger precursors and are cleaved during assembly (Bhatti et al., 1979).

The viral core consists of the viral genome and four additional viral proteins not appearing in the capsid (Maizel et al., 1968b; Robinson, 1973). The major core protein, VPVII (18.5Kd), comprises approximately 20% of the total virion protein with 1,070 copies of VPVII in each core (Maizel 1968a, 1968b; Everitt et al., 1973, 1975; Flint, 1981). VPVII, and another less abundant core protein, VPV (45.5K), are highly charged arginine rich proteins (Everitt et al., 1973; Nermut, 1984), and are believed to be non-covalently

FIGURE 1

Virion structure of human adenovirus. (Adapted from Horwitz, 1990).

Polypeptide SDS-gel Structural Unit



associated with the viral DNA causing it to fold into nucleosome-like subunits. Approximately 150 base pairs of DNA are associated with six VPVII subunits in each nucleosome-like structure (Mirza et al., 1982; Horwitz, 1990). In addition, a small highly basic peptide, protein u (4Kd), is believed to associate with the virion core as a minor component helping to condense and stabilize the core structure (Harpst, 1977; Vayda, 1983; Lundt, 1988; Anderson, 1989). Finally, at both 5' ends of the viral genome there is a 55 Kd terminal protein (TP) covalently attached via a phosphodiester bond between the hydroxyl group of a serine residue and the 5' phosphate of the terminal deoxycytidine residue (Sharp, 1976; Rekosh, 1977; Desiderio, 1981). The 55Kd TP and VPVII are synthesized as 80Kd (pTP) and pVPVII, respectively, then are cleaved during viral morphogenesis (Lischwe, 1977).

1.5 Organization of the Genome

As previously mentioned, the human adenovirus genome is a single linear molecule of double stranded DNA approximately 36,000 bp in length and with a molecular weight of $20-25x10^6$ daltons (Green and Pina, 1963; Green et al., 1967; van der Eb and van Kesteren, 1966; Wu, 1977). For reference, the genome is divided into 100 map units (mu) of 350 bp each with the

nucleotides being numbered from left to right (Sussenbach, 1984). The two DNA strands are separated into right (r) and left (l) strands based on the direction of transcription. The genome contains inverted terminal repeats ranging from 100-200 bp in length depending on the serotype; 103 bp for Ad2 and Ad5, 136 bp for Ad3 and Ad7, 164 bp for Ad12 (Sussenbach, 1984). A sequence at nucleotide 9-22 is believed to be the recognition signal for the binding of factors involved in viral DNA replication because of its stringent conservation among all serotypes (Sussenbach, 1984). The ITR's allow single stranded molecules to circularize by base pairing at the ends of the DNA forming a panhandle structure that is exactly like the ends of the original duplex molecule (Wolfson, 1972; Horwitz, 1990).

The viral genes are grouped according to the time of expression during the lytic cycle with early, intermediate, and late phases being defined by the onset of viral DNA replication. In the early phase the early regions E1A, E1B, E2, E3, and E4 are transcribed. These genes encode primarily non-structural polypeptides, and some of the major late promoter driven genes. Expression of intermediate phase genes (VPIX ,VPIVa2 and VAs) is not dependent on viral DNA replication but these genes products are most easily detected during the late phase. The late phase includes viral DNA

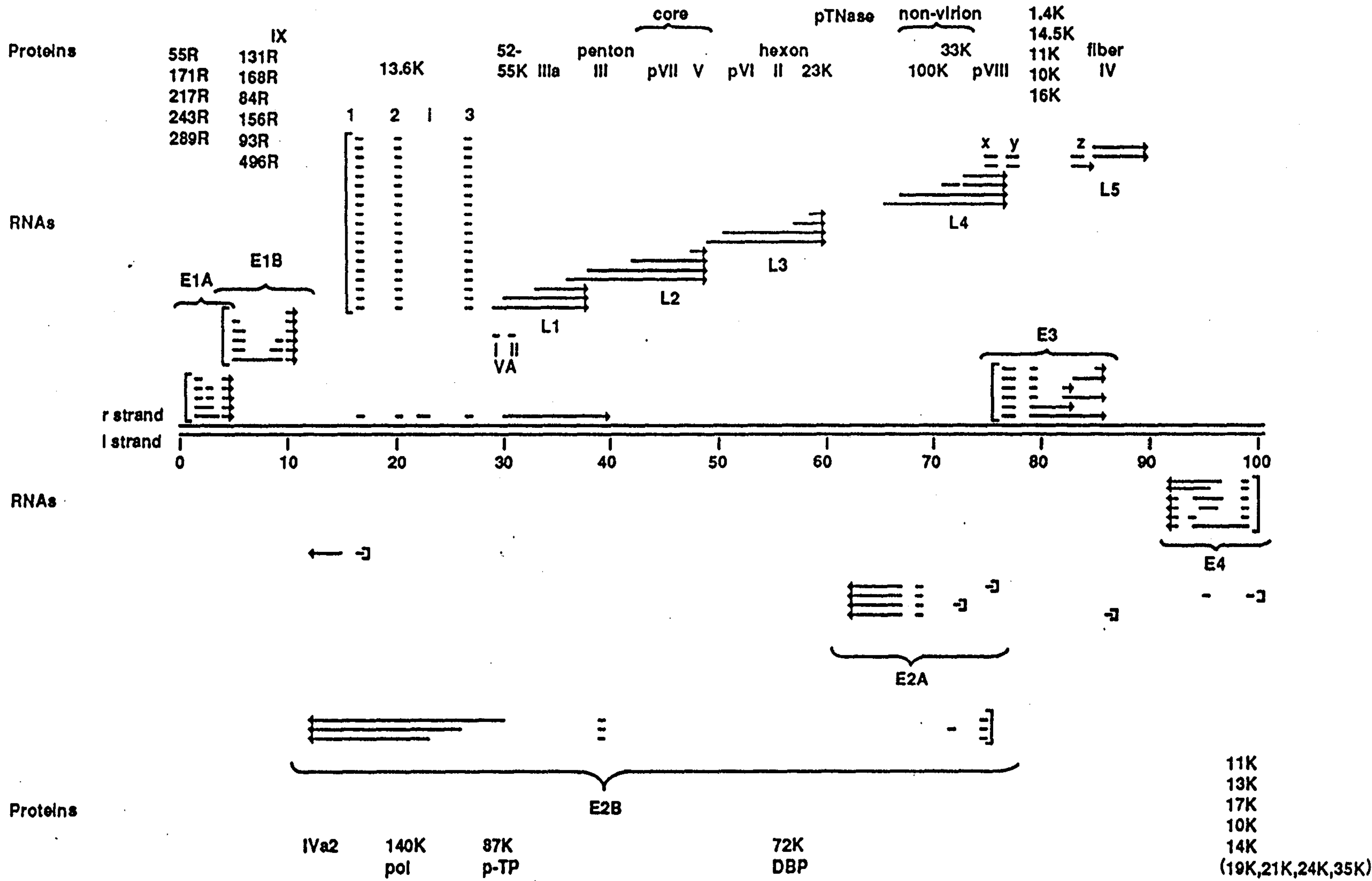
synthesis and transcription of the late genes (L1-L5), encoding mostly structural or assembly associated proteins (Horwitz, 1990). A schematic representation of the structure of the adenovirus type 2 genome is given in figure 2.

1.6 Productive Infection by Adenoviruses

A productive infection by human adenoviruses occurs in cells of their natural host, permissive for viral DNA replication and late gene expression. The replicative cycle is divided into various steps. Infection begins with viral adsorption to the target cell, penetration of the plasma membrane, uncoating of the virus and transport of the viral DNA to the nucleus of the cell. In the nucleus, early transcription occurs, followed by viral DNA replication and then late transcription. Finally, newly produced viral proteins and DNA are packaged into mature virions which are released to repeat the cycle in a neighbouring cell. In group C adenoviruses (eg. Ad5) the entire process lasts about 36 hrs and yields approximately 4,000-10,000 virions per infected cell (Green and Daesche, 1961; Horwitz, 1990). The cycle for group A viruses (eg. Ad12) is somewhat more prolonged (Horwitz, 1990).

FIGURE 2

Structure of the human adenovirus type 2 genome.
(Adapted from Horwitz, 1990).


Proteins
55R
171R
217R
243R
289R
IX
131R
168R
84R
156R
93R
496R
13.6K
52-
55K
IIIa
penton
III
core
pVII
V
pVI
hexon
II
23K
pTNase
non-virion
33K
100K
pVIII
1.4K
14.5K
11K
10K
16K
fiber
IV
1
2
i
3
x
y
z
RNAs
E1A
E1B
L1
L2
L3
L4
L5
I II
VA
E3
r strand
l strand
0
10
20
30
40
50
60
70
80
90
100
RNAs
E4
E2A
E2B
Proteins
IVa2
140K
pol
87K
p-TP
72K
DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

1.6.1 Adsorption, Penetration and Uncoating

Adsorption of adenoviruses to the target cell occurs through an interaction between the virion fibre and a specific receptor on the cell membrane (Londberg-Holm and Philipson, 1969; Svensson et al., 1981). Using KB cells, a number of researchers have purified a complex of three surface glycoproteins, referred to as gp78Kd, gp42Kd and gp34Kd, which likely constitute the adenovirus receptor (Meager et al., 1976; Hennache and Boulanger, 1977; Horwitz, 1990). Following attachment, a rearrangement of viral bound receptors is observed resulting in clustering of the adsorbed virus directly over the nucleus into structures called "caps" (Patterson and Russel, 1983). This is an energy dependent process which Patterson and Russel (1983) have suggested is mediated by the cytoskeleton, and which appears to play an important role in efficient viral penetration.

Within 5-10 minutes after attachment the virus is internalized into the cytoplasm of the cell. It is hypothesized that the virus bound to its receptor migrates within the plasma membrane to clathrin-coated pits to form endocytic vesicles or receptosomes (Pastan et al., 1987). The virus then enters the cell in the receptosome, where the pH drops to 5.5 causing an alteration in the viral

configuration. The altered virion surface causes the endocytic vesicle to rupture releasing the virion particle into the cytoplasm, with concomitant loss of the fibre and penton base components (Patterson and Russel, 1983; Fitzgerald et al., 1983; Svensson and Persson, 1984; Pastan et al., 1987). Alternatively, some researchers have suggested that adenoviruses enter by pinocytosis and that there is minimal loss of the penton (Chardonnet and Dales, 1970a,b; Horwitz, 1990). However, the virion DNA has been shown to become sensitive to DNase and to increase in density which may indicate a loss of some protein (Sussenbach, 1967; Svensson and Persson, 1984; Horwitz, 1990). Once internalized, the virus moves towards the nucleus via a process involving hexon\microtubule interaction (Dales and Chardonnet, 1973), and becomes closely associated with the nuclear pores. At the nuclear pores the viral core is released by a process likely involving a nuclear pore-associated ATPase (Chardonnet and Dales, 1972; Dales and Chardonnet, 1973). The naked DNA then enters the nucleus via the nuclear pores (Chardonnet and Dales, 1970a, 1972), and becomes tightly associated with the nuclear matrix (Younghusband and Mandrel, 1982). This process which is completed in 2 hrs at 37 C requires energy in the form of ATP and will not proceed at 0 C (Chardonnet and Dales, 1972; Horwitz, 1990). The viral genes are then

transcribed by DNA-dependent RNA polymerases II and III, and the DNA is replicated.

1.6.2 Early and Intermediate Gene Expression

As previously mentioned, early adenovirus transcription is the synthesis of viral RNAs from input templates before the onset of viral DNA replication (Horwitz, 1990). These mRNAs accumulate at approximately 0-8 hours post adsorption (Sharp, 1984). There are five early transcription units, termed E1A, E1B, E2A/E2B, E3 and E4. Each of these transcription units contains its own promoter and produces multiple overlapping mRNAs through alternate splicing events (Lewis et al., 1976; Berk and Sharp, 1977; Evans et al., 1977; Wilson et al., 1979; Chow et al., 1979). The E2 region is sometimes divided into two sets of non-overlapping transcripts, however both use a single promoter and are coordinately expressed (Stillman et al., 1981). The five early genes can be divided into two classes: the immediate-early gene E1A and the early delayed genes E1B, E2, E3 and E4 (Berk, 1986). The promoters of all the early genes require transactivation by the product of the E1A region (Jones and Shenk, 1979; Berk et al., 1979; Berk, 1986).

The E1A region is located at 1.3-4.5 mu on the r-strand of the adenovirus genome. Messenger RNAs from this region are the earliest transcripts identified, and can be detected by about 2 hrs after infection (Spector et al., 1978; Nevins, 1979; Rowe et al., 1984; Glenn and Ricciardi, 1988). The E1A gene produces two major mRNAs early in infection, a 12S and 13S mRNA (Berk and Sharp, 1978; Chow et al., 1979; Perricaudet et al., 1979; Kitchingman and Westphal, 1980), which share common 3' and 5' ends but differ in the size of the intron removed. A third mRNA, 9S, which accumulates later in infection (Spector et al., 1978; Chow et al., 1979), also shares the common 3' and 5' ends but has a much larger internal sequence spliced out. There are also two minor transcripts which appear primarily late in infection, the 10S and 11S mRNAs (Stephens and Harlow, 1987; Ulfendahl et al., 1987). Both contain exon one of the 9S mRNA as well as the common 3' and 5' ends but differ in that they have two internal splice sites rather than one. The estimated sizes of the products of the 13S, 12S, 11S, 10S, and 9S transcripts are 289R, 243R, 217R, 171R, and 55R, respectively, however a variety of proteins have been shown to arise from each transcript. Some, if not all of the heterogeneity found within the E1A proteins may be due to the fact that these products are differentially phosphorylated at various serine

residues (Yee et al., 1983; Tremblay et al., 1988; Tremblay et al., 1989; Dumont et al., 1989). Harlow et al., (1985) have suggested that E1A products can be resolved into as many as 60 polypeptide species using two-dimensional SDS-PAGE gels. A transcription and translation map of E1A is given in figure 3.

A number of functions have been ascribed to the 289R and 243R proteins in lytic infection. These functions include transcriptional activation (in cis or in trans) of the other viral early genes (Jones and Shenk, 1979, Berk et al., 1979); stimulation of transcription of cellular genes such as the heat shock protein 70 (Nevins, 1982; Kao and Nevins, 1983), the beta-tubulin gene (Stein and Ziff, 1984) and the cellular thymidylate synthetase gene (Zerler et al., 1987); repression of enhancer-driven transcription from all early promoters (Borrelli et al., 1984; Smith et al., 1986; Dery et al., 1987); stimulation of cellular DNA synthesis (Spindler et al, 1985; Zerler et al., 1987; Moran and Zerler, 1988; Smith and Ziff, 1988). In addition, E1A proteins have also been found to be involved in protection of infected cells against the anti-viral action of interferons (O'Malley et al., 1986; Reich et al., 1988). The function of the protein products of the 217R and 171R proteins, which have only recently been discovered, as well as the 55R protein are presently unclear.

Most of the functions ascribed to the 289R and 243R proteins have been mapped to regions highly conserved between the adenovirus serotypes termed conserved region (CR) 1, 2 and 3.

The transactivation function of E1A has been mapped to the 46 aa unique region (CR 3) of the 289R protein (13S product), which by itself has been shown to be sufficient to transactivate other viral genes (Montell et al., 1982; Lillie et al., 1986,1987; Jelsma et al., 1989). There may also be a possible role for CR 2 in transactivation (Jelsma et al., 1988; Schneider et al., 1989). Although the mechanism of transactivation is not clearly understood, the E1A proteins do not appear to bind DNA directly but rather appear to have an affinity for a variety of cellular transcription factors, such as ATF and E2F, which in turn are capable of binding to the promoter regions of the other early genes (Kovesdi et al., 1986; Wu et al., 1987; Jones et al., 1988; Fahnestock and Lewis, 1989; Pei and Berk, 1989). In one hypothesis, it is believed that E1A activates these cellular transcription factors by post-transcriptional modification, such as phosphorylation (Bagchi et al., 1989, Raychaudhuri et al., 1989), or by bringing appropriate factors together to form an active complex (Jalinot et al., 1987). These factors in turn enhance transcription. Alternatively, E1A may function in a fashion similar to a typical cellular activator (Lillie and

Green, 1989). Lillie and Green (1989) have proposed that E1A is directed to the promoter via an association between its own protein recognition domain and a promoter bound protein. In this model the activation domain of E1A, once it has been positioned at the promoter, is able to enhance transcription (Lillie and Green, 1989).

Other functions of E1A have been mapped to CR 1 which is present in both the 289R and 243R proteins. These functions include the ability to induce DNA synthesis in both human and rat quiescent cells (Spindler et al., 1985; Zerler et al., 1987, Moran and Zerler, 1988; Smith and Ziff, 1988), the stimulation of transcription of proliferating cell nuclear antigen (PCNA) (Zerler et al., 1987), and the repression of enhancer driven transcription from all early promoters (Borrelli et al., 1984; Smith et al., 1986; Dery et al., 1987). A fourth domain consisting of 5 amino acids at the carboxy terminus of 289R and 243R proteins has been shown to increase the rate of nuclear localization of the proteins (Krippl et al., 1985; Lyons et al., 1987).

The E1B region begins immediately downstream of the E1A polyadenylation site (4.6-11.2 mu), and contains its own promoter (Berk and Sharp, 1977). Transcription from this region is detectable by approximately 2 hrs after infection with maximum rates of synthesis being observed slightly later

than the other early regions (Spector et al., 1978; Nevins et al., 1979; Sharp, 1984; Glenn and Ricciardi, 1988). Five mRNA species are produced from a single primary transcript; 22S and 13S are the two major transcripts, while 14.5S, 14S and 9S are the three minor transcripts. Both major and minor transcripts initiate at the same start site (Bos et al., 1981), except for 9S which has its own promoter and encodes the structural protein IX. All the other transcripts use an identical 3' splice acceptor but differ in the size and number of the intron spliced out. The 22S message encodes both a 496R and 176R protein with overlapping sequences but which are translated in different reading frames (Bos et al., 1981). The 13S mRNA can also code for a 176R protein in addition to another 469R related protein in which the first 79 amino acids are spliced into the same reading frame as 176R. The 14.5S and 14S mRNAs produce additional 496R related products which when translated result in 155R, 92R and 82R proteins (Virtanen and Pettersson, 1985). Translation products have been identified for the 496R, 176R, 155R, and 92R open reading frames, however with the exception of the 496R and 176R products, their role in lytic infection is not yet clearly understood. A transcription and translation map of E1B is given in figure 3.

A number of investigators have determined that the E1B 496R and 176R open reading frames of Ad5 produce translation products of 58Kd and 19Kd, respectively (Anderson and Lewis, 1980; Green et al., 1983; Yee et al., 1983; Spindler et al, 1984). The size of the open reading frame and resulting translation product vary depending on the serotype: 55Kd and 19Kd for Ad 12, 53Kd and 21Kd for Ad 2. In addition, 58K sequence comparison of Ad12, Ad7 and Ad5 reveals that although the proteins are highly related they contain regions which are also highly divergent, especially at the amino terminus (van Ormondt and Hespler, 1983). This difference may account for the variation in oncogenicity among the various serotypes, an aspect which will be further discussed in the transformation section.

E1B 19Kd is an acylated protein (McGlade et al., 1987), and it has also been reported to be phosphorylated at a single serine residue (McGlade et al., 1989). Cell fractionation studies have shown that 19Kd is primarily associated with the nuclear membrane and perinuclear region (Rowe et al., 1983, McGlade et al, 1987). The 19Kd protein has been reported to be involved in viral replication since mutants affecting this protein are host range (Harrison et al., 1977), and are defective for viral DNA replication in both KB (Babiss and Ginsberg, 1984) and HeLa cells (Pilder et al., 1984). In

addition, mutants of this protein show a large plaque phenotype (Chinnadurai et al., 1983; Takemori et al., 1984) and a cytocidal cytopathic (cyt) effect (Pilder et al., 1984; Takemori et al., 1984; White et al., 1984; Barker and Berk, 1987). Many of these cyt mutants were also observed to have another phenotype, inducing extensive degradation of cellular DNA (deg phenotype) (Ezoe et al., 1981). The isolation of mutants which are cytocidal but do not degrade DNA suggests that these phenotypes involve separate functions of the protein (Subramanian et al., 1984; White et al., 1984). It has been demonstrated that the 19Kd protein is not the source of the nuclease activity since mutants lacking the entire coding region of this polypeptide are also cyt/deg mutants (Pilder et al., 1987). It has therefore been suggested that the nuclease is either induced or unmasked by adenovirus infection. In either case, the role of the 19Kd protein is, at least in part, to control and possibly regulate the nuclease (Pilder et al., 1987). It was further demonstrated by White and Stillman (1987), using E1A-E1B double mutants that expression of either the 13S or 12S E1A proteins produced the cyt as well as the deg phenotypes. The results of this study suggested that both the 13S and 12S E1A gene products could directly or indirectly cause the deg and cyt phenotype in cells infected with viruses defective for the E1B 19Kd gene

(White and Stillman, 1987). The ultimate role of 19Kd in the cyt/deg phenotype therefore appears to be to negatively regulate the effects by E1A proteins resulting in a preservation and maintainance of nuclear structure.

The 19Kd protein has also been reported to be involved in transactivation of the other early genes (Herrmann et al., 1987) and to relieve the repression activity of E1A on the early enhancer elements (Yoshida et al., 1987). However, this putative function of 19Kd remains controversial since other investigators have shown that 19Kd mutations have no effect on viral gene expression nor on E1A mediated enhancer repression in infected cells (Herbst et al., 1988). In contrast to the E1B 19Kd of Ad5, its Ad12 counterpart does not appear to be required for viral growth in human cells, but otherwise functions in infected human cells in the same manner as its Ad2 and Ad5 counterparts (Edbauer et al., 1988).

E1B 58Kd is a phosphoprotein with associated protein kinase activity (Branton et al., 1981; Yee and Branton, 1983). Immunofluorescence and cell fractionation studies have shown that 58Kd is mainly localized in the perinuclear region, but it has been observed in both the nucleus and cytoplasm (Sarnow et al., 1982; Rowe et al., 1983; Yee et al., 1983). In infected human cells 58Kd has been found to complex with the viral E4 34Kd protein (Sarnow et

al., 1984; Cutt et al., 1987), and the cellular anti-oncogene, p53 (Zantema et al., 1985). Although a similar association has not been demonstrated with Ad12 55Kd, p53 does appear to be stabilized in Ad12 infected cells (Mak et al., 1988). The 55Kd protein of Ad12 appears to be required for the efficient production of virus in infected human cells (Shiroki et al, 1986; Breiding et al., 1988). This requirement likely reflects the necessity of 55Kd for viral DNA replication (Shiroki et al., 1986; Breiding et al., 1988), a requirement which is apparently less stringent for the E1B 58Kd protein of Ad5 (Williams et al., 1986). 55Kd is also required for the efficient expression of the viral late proteins (Breiding et al., 1988) and mRNAs (Shiroki et al., 1986), as is the 58Kd of Ad5. In addition, 55 and 58Kd function to facilitate the transport of late viral message, as well as, to inhibit the accumulation of cellular mRNAs (Babiss and Ginsberg, 1984; Babiss et al., 1985; Cutt et al, 1987; Breiding et al., 1988; Leppard and Shenk, 1989). The latter function in Ad5 is believed to be related to the ability of 58Kd to complex with the E4 34Kd protein, thereby contributing to the shut-off of host protein synthesis during lytic infection (Babiss and Ginsberg, 1984; Babiss et al., 1985).

The E2 region is located at 69.7-61.5 mu and 27.8-14.2 mu for E2A and E2B, respectively, on the l-strand.

FIGURE 3

Transcription map of the E1 region of Ad5. (Adapted from Lewis and Anderson, 1987).

E1A

E1B

1 2 3 4 5 6 7 8 9 10 11 map units

1000 2000 3000 4000 base pairs

289R
13S

22S
176R
496R

243R
12S

13S
176R
84R

33

217R
11S

14S
176R
156R

171R
10S

14.5S
176R
93R

55R
9S

proposed
0.86Kb
131R
168R

9S
Protein IX

Transcription from the E2 promoter is detectable by approximately 2-4 hrs after infection with a sharp continuous increase throughout the early phase of infection (Glen and Ricciardi, 1988). During early infection the E2 region produces five mRNAs which encode several functions required for viral DNA replication.

E2A messages code for a 72Kd DNA binding protein (Ad-DBP), which has been observed in its full length form or as a 48Kd cleavage product lacking the amino terminal 120 aa (Sugawara et al., 1977; Axelrod, 1978). The 72Kd DNA binding protein is required for replication both in vivo and in vitro (Friefeld et al., 1983). It is a nuclear phosphoprotein with functional domains at both its carboxy and amino termini. The amino terminus which apparently is dispensible for DNA replication, possesses a number of functions including autoregulation and autophosphorylation (Morin et al., 1989), regulation of late gene expression (Klessig and Grodzicker, 1979; Brough et al., 1985), ATP binding capacity, and possibly protein kinase activity (Branton et al., 1985b). The carboxy terminus is involved in single stranded and double stranded DNA and mRNA binding (Klein et al., 1979; Cleghon and Klessig, 1986), down-regulation of viral early gene expression (Nevins and Winkler, 1980; Handa et al., 1983), and assembly of new virion particles (Nicolas et al., 1983). The 72Kd DNA

binding protein also appears to repress the initiation of E4 transcripts approximately 6 hrs after infection (Nevins and Winkler, 1980; Handa et al., 1983).

E2B messages encode the 80Kd terminal protein precursor (pTP) (Rekosh et al., 1977), as well as the 140Kd viral DNA polymerase (Ad-DNA pol)(Lichy et al., 1983). Both of these proteins are required for viral replication (Lichy et al., 1983), and will be discussed further below.

The E3 coding region is located at 76.6 to 86.3 mu on the towards the right end of the r-strand. Temporally, transcription of the E3 region occurs immediately after expression of E1A products (Nevins et al., 1979; Glenn and Ricciardi, 1988), and produces nine mRNAs. Four of the protein products, gp19Kd, 11.6Kd, 10.4Kd, and 14.7Kd, have been identified. Although the products of E3 are not required for viral growth in tissue culture, they play an important role in viral pathogenesis by modulating the host response to Ad infection (Ginsberg et al., 1989; Horwitz, 1990). The gp19Kd protein possesses a number of N-linked glycosylation sites allowing it to interact with the class I MHC antigens in the endoplasmic reticulum, thus preventing both processing and transport of MHC polypeptides to the cell surface (Burgert and Kvist, 1985). The 14.7Kd protein protects infected cells from lysis by the tumour necrosis

factor (Gooding et al., 1988), a function which once again helps to abrogate host mechanisms that recognize and kill infected cells in the early phase before viral progeny is produced (Horwitz, 1990). The 10.4Kd protein has been shown to bind to the epidermal growth factor receptor (EGF-R) (Carlin et al., 1989), the function of which is presently not clear.

The E4 region, which is located at 96.8-91.3 mu on the l-strand, may produce up to 24 messages. Similar to E3, the transcripts of E4 appear immediately after expression of E1A products. The products of the E4 region appear to be required for normal growth of the virus, since they play a role in the stability of late nuclear mRNAs (Weinberg and Ketner, 1986; Sandler and Ketner, 1989). In addition, E4 products are implicated in transactivation of E2 and activation of the E2F transcription factor (Reichel et al., 1989; Neill et al., 1990). These functions have been assigned based on the phenotypes of large deletion mutants and none of the proteins involved have been identified, except for the 34Kd product known to complex with the E1B 58Kd of Ad5 (Sarnow et al., 1984; Cutt et al., 1987). As previously mentioned, the complexing of 58Kd and E4 34Kd appears to play a functional role in the shut-off of host protein synthesis. The only other protein identified for this region is an 11Kd

nuclear matrix binding protein (Sarnow et al., 1982; Downey et al., 1983). This protein does not appear to be essential for lytic infection in tissue culture since mutants lacking a functional 11K polypeptide replicate at wild type levels (Horwitz, 1985). The E4 proteins are also believed to be required for transition of viral gene expression from early to late, and indirectly, for viral encapsidation (Weinberg and Ketner, 1986).

Additional transcripts described as intermediate, coding for the VPlVa2 and VPlX genes, as well as two species of low molecular weight virally associated RNAs (VA RNAs) are produced just before the onset of DNA replication (Lewis et al., 1976; Soderlund et al., 1976). The VPlX message is unusual in that it is the only coding mRNA of adenovirus that is not spliced (Alestrom et al., 1980). Unlike all the other adenovirus transcripts, which are transcribed by polymerase II, the virally associated RNAs (VA RNAs) are transcribed by polymerase III (Weinmann et al., 1976). These low molecular weight transcripts do not encode any protein and are required for efficient translation of viral messengers late in infection (Thimmappaya et al., 1982).

1.6.3 Viral DNA Replication

Viral DNA is replicated between early and late gene expression. It begins at approximately 6-8 hrs after infection with maximum rates of synthesis being achieved by 19 hrs (Kelly, 1984). The development of an *in vitro* replication system using Ad2 DNA has facilitated the identification and purification of viral and cellular factors required for initiation and elongation (Challberg and Kelly, 1979). The initiation of Ad replication in vitro was found to require three viral proteins and four cellular DNA binding factors. The viral polypeptides, previously mentioned, are encoded by the E2 region and include DBP 72Kd, pTP 80Kd, and Ad-DNA pol 140Kd. The cellular factors include nuclear factors I, II, and III (NF-I, NF-II, NF-III), and an origin recognition protein (ORP-A).

Initiation of viral DNA replication is primed by the 80Kd pTP bound to a dCMP molecule at the end of the template (Rekosh et al., 1977, Challberg et al., 1980). Formation of the pTP-dCMP complex is stimulated by the presence of the cellular factor, NF-I (Nagata et al., 1982), as well as the virally encoded DNA polymerase. The 140Kd Ad-DNA pol complexes in a 1:1 ratio with pTP and catalyzes the transfer of the dCMP to the pTP (Lichy et al., 1982). This is

accomplished through binding of the complex to the terminal end of the viral genome via a covalent attachment between a serine residue and the 5' phosphate of the dCMP nucleotide (Desiderio and Kelly, 1981; Lichy et al., 1982). The polymerase then uses the free 3' hydroxyl end of the nucleotide to initiate the polymerization reaction (Stillman et al., 1982). Synthesis of a daughter strand continues in a 5' to 3' direction to the end of the genome causing displacement of the parental strand of the same polarity (Lechner and Kelly, 1977). The 72Kd DNA binding protein appears to play a role in both initiation and elongation by increasing the affinity of NF-1 to the origin (Cleat and Hay, 1989), and by unwinding the genomic DNA to facilitate the initiation of the replication reaction (Lichy et al., 1983; Stuiver and van der Vliet, 1990).

The origin, which comprises nucleotides 1 to 51, contains three protein binding domains termed A, B and C, and occupation of these sites is required for efficient initiation of replication (Wides et al., 1987). Based on deletion studies, it appears that A is essential for replication and represents the minimum origin of replication, while B and C increase the efficiency of replication. NF-I binds to the B sequence (nucleotides 19-40)(Wides et al., 1987) to participate in initiation of DNA replication in the presence

of Ad-DBP (Nagata et al., 1982). ORP-A and NF-III (also referred to as ORP-C) bind to A sequences (nucleotides 1-18) and C sequences (nucleotides 41-51), respectively, to enhance the efficiency of replication (Rosenfeld et al., 1987). NF-II, which is a type I topoisomerase, is required later in the elongation process, presumably to reduce torsional stress (Nagata et al., 1983).

1.6.4 Late Gene Expression

The late messenger RNAs of adenovirus are transcribed during or after viral DNA replication by RNA polymerase II and III (VA RNAs only). Small amounts of some transcripts for protein IX, VPIVa2 and other VA RNAs I and II are detectable before DNA replication but are greatly enhanced after this process (Chow et al., 1980). In contrast, the major late promoter, which is located at 16.5 mu, requires newly replicated templates in order to direct high levels of transcription of late transcripts (Shaw and Ziff, 1980). Following DNA replication the primary transcript from the major late promoter extends all the way to 99 mu (Fraser et al., 1979). This long transcript is subsequently differentially cleaved and polyadenylated at one of five polyadenylation sites upstream from the termination site

giving rise to five families of late transcripts, L1-L5 (Shaw and Ziff, 1980). The viral genes transcribed late in infection code for all of the proteins associated with the viral capsid (discussed in "virion structure"), as well as some nonstructural proteins such as the 23Kd protease, 100Kd scaffolding protein and the 33Kd phosphoprotein. Following synthesis, the late proteins are transported to the nucleus where they are assembled into new virions (Flint and Broker, 1981).

1.6.5 Assembly

Virus assembly begins immediately after the synthesis of VPII, VPIII and VPIV proteins at which point these single polypeptides are assembled into the various capsomere components (Horwitz, 1990). Hexon capsomere assembly is mediated by the virus encoded 100Kd scaffolding protein within a few minutes after its release from the ribosome (Cepko and Sharp, 1983). The newly synthesized hexon capsomeres are then self assembled into an empty capsid shell over a period of 24 hrs (Horwitz et al., 1969). This is presumably accomplished through the formation of ninemers that would occupy each of the 20 facets of the virion (Horwitz, 1990). The penton base and fibre peptides are assembled into the

penton capsomere at a much slower rate, approximately 40% by 45 min, and the rest are assembled over a period of 10-11 hrs (Horwitz et al., 1969). The penton capsomeres do not associate with the empty shell until a later stage in virion development (Flint and Broker, 1981; Horwitz, 1990).

In the nucleus the empty shell, comprised of only hexon capsomeres, undergoes a series of developmental stages (or classes) before becoming a mature virion particle (Ishibashi and Maizel, 1974; D'Halluin et al., 1978; Flint and Broker, 1981; Horwitz, 1990). These developmental classes include "light intermediate capsid", "heavy intermediate capsid" and "young virion". Association of VPVI, VPVIII and VPVIIIa with the empty capsid constitutes the light intermediate capsid stage (D'Halluin et al., 1978). In addition, the light intermediate capsid is sometimes observed in association with a portion of the left end of the viral genome, which contains a recognition sequence for DNA packaging with the first 400 base pairs (Hammarskjold and Winberg, 1980). This DNA is probably an intermediate captured during the injection of the genome into the preformed capsid (Horwitz, 1990). It is postulated that the naked DNA enters the capsid through an opening at one of the vertices. Incomplete capsids containing full length DNA constitutes the heavy intermediate capsid (Flint and Broker, 1981). Finally, association of

penton capsomeres, protein IX (Gosh-Choudhury et al., 1987), precursors to the other minor capsid components and the virion core proteins constitutes the young virion (Ishibashi and Maizel, 1974; Flint and Broker, 1981; Horwitz, 1990). There does not appear to be a consensus over the hypothesis that the complete entry of viral DNA progresses in the absence of core proteins. Some investigators suggest that the core proteins are injected into the empty shell before the DNA enters, however it has also been proposed that the core proteins associate with the DNA and that they enter the capsid together (Horwitz et al., 1969; Sundquist et al., 1973; Philipson, 1984).

In the last stage of viral morphogenesis all the precursor molecules are cleaved by a 23Kd virus encoded protease from the L3 region (Weber, 1976; Bhatti and Weber, 1979). Cleavage of precursor capsid components yields mature products 55Kd TP, VPVI, VII, and VIII. Finally, a general compacting of the capsid occurs to give it a structure that is impermeable to nucleases (Horwitz, 1990). The mature virion remains associated with the cell for a long while after assembly, and is only released after the cell ruptures due to attrition (Horwitz, 1990).

1.7 Cell Transformation by Adenoviruses

The adenoviruses were the first human viruses shown to possess transforming properties (Trentin et al., 1962; Huebner et al., 1962). Although there is no evidence linking adenovirus to human cancer, the discovery that human Ad12 causes tumours when injected into newborn rodents, has spawned a great interest in these agents as a system to study molecular mechanisms involved in cell transformation.

As previously discussed, the variability in oncogenic potential of the different serotypes of adenovirus has been used as a source of classification. The highly oncogenic group A adenoviruses are capable of inducing undifferentiated sarcomas at the site of injection in rodents within 30-90 days (Trentin et al., 1962; Huebner et al., 1962). The group B adenoviruses are classified as weakly oncogenic since they require a much greater period of latency before the formation of tumours (200-400 days), and tumours are only observed in a small proportion of animals (Girardi et al., 1964; Larson et al., 1965). Finally, the non-oncogenic group C adenoviruses are not capable of inducing tumours in injected animals, even after a long period of latency (Trentin et al, 1962; Huebner et al., 1962; Lewis et al., 1974). However, all adenovirus serotypes are able to transform non-permissive

cells *in vitro* (Freeman et al., 1967) These cells have been shown to be tumourigenic when injected back into animals, however, the oncogenic potential of the transformed cells reflects that of the serotype. The type of host used also appears to play a role in whether or not tumours are induced. For example, cells transformed by group A and B viruses are capable of forming invasive tumours when injected into syngeneic rats (Freeman et al., 1967). Cells transformed by group C viruses can only induce tumours when injected into immunosuppressed newborn rats (Gallimore, 1972). This difference may be a reflection of the properties of the cells themselves. Ad12 transformed cells display characteristics similar to highly invasive tumour cells while Ad5 transformed cells do not (Bober et al., 1988). These features include, increased cell surface laminins and an increased affinity for type IV collagen. In addition, cells transformed by the highly oncogenic Ad12 appear to have an increased resistance to natural killer cells, as well as cytotoxic T-lymphocytes (Bernards et al., 1983; Raska and Gallimore, 1972) enabling them to evade the host immune system, an aspect which will be addressed below.

Several lines of evidence indicate that the region responsible for the transforming activity of adenoviruses map to the left end of the viral genome. Firstly, DNA-RNA

hybridization studies have shown that the viral genes transcribed in Ad transformed cells correspond to mRNAs during early phase of lytic infection (Green, 1970; Flint et al., 1975,1976; Graham et al., 1977; Aiello et al., 1979). Secondly, DNA-DNA hybridization studies determined that the left 12-14% of the viral genome is always present within transformed cells (Gallimore et al., 1974; Sambrook et al., 1974; Flint et al, 1976). Thirdly, using either sheared or restricted fragments of Ad DNA to transform cells the transforming region was mapped to between the left end 1 to 11% of the viral genome (Graham et al., 1974a; van der Eb et al., 1977), with a fragment corresponding to the left end 4.5% of Ad5 DNA being sufficient for immortalization of primary cells (van der Eb et al., 1977). Lastly, cells transformed by Ad5 DNA containing the left most 8% of the Ad5 genome were tumourigenic and did not appear to differ morphologically from cells which had been transformed by the intact virus, indicating that genes outside of the E1 region did not contribute to the transformed phenotype (Graham et al., 1974a,b ; van der Eb et al., 1977; Rowe and Graham, 1983; Rowe et al., 1984a). Similar DNA fragments from the left ends of both group A and B viruses were also shown to have transforming activity (Shiroki et al., 1977; Dijkema et al., 1979). It was hypothesized that since the E1 region is

transcriptionally active during early stages of productive infection (Chow et al., 1977; Berk and Sharp, 1977) at least part of the early proteins should play a role in transformation (Schrier et al., 1979), and a number of studies were carried out to identify E1 products involved in the transformation process.

The E1A proteins are sufficient for immortalization of primary cells (Dijkema et al., 1979; Shiroki et al., 1979; Houweling et al., 1980; Graham et al., 1984) but require the co-operation of E1B (van den Elsen et al., 1983), or of the human cellular oncogene Ha-ras for the expression of the morphological changes associated with complete transformation (Ruley, 1983; Branton et al., 1985; Lillie et al., 1986,1987). Immortalized cells are distinguished from transformed cells in that they can generally be passaged indefinitely but are more fibroblastic in morphology than fully transformed cells and do not possess the same characteristics, such as ability to grow in low serum and anchorage independent growth. Also, unlike fully transformed cells, immortalized cells do not have the ability to cause tumours when injected into animals.

Significant advances have recently been made in our understanding of the mechanism of how E1A induces cellular immortalization . It has been reported that E1A polypeptides associate with cellular proteins 300, 107 and 105Kd, in

infected and transformed cells (Yee and Branton, 1985), and that the binding of all three of these proteins correlates strongly with transforming activity (Egan et al., 1988; Whyte et al., 1989). The 105Kd protein, which has recently been identified as the product of the retinoblastoma gene, is an anti-oncogene whose deletion or inactivation triggers oncogenic transformation (Klein, 1988). An association between the E1A and Rb-1 proteins lead to the hypothesis that the complexing of these two proteins interferes with the normal function of the Rb-1 protein and thereby mimics what occurs when the Rb gene is lost such as in retinoblastoma (Whyte et al., 1988). While binding of Rb-1 is necessary for the transformation activity of E1A, it is not sufficient. Binding of 107 and 300Kd has also been found to be required (Egan et al., 1988; Whyte et al., 1989), although also not sufficient for complete transformation.

The products of both the 12S and 13S E1A messages are present in transformed cells (Rowe et al., 1984; Branton and Rowe, 1985), and have both been shown to contribute to the transformed phenotype (Graham et al., 1978; Montell et al., 1984; Weinberg and Shenk, 1984; Haley et al., 1984; Kuppuswamy and Chinnadurai, 1988). For example, mutants that do not produce a functional 12S product possess reduced transforming activity (Montell et al., 1984; Weinberg and Shenk, 1984),

while mutants defective for 13S products are capable of producing abnormal foci which lack some of the features of fully transformed cells (Graham et al., 1978; Montell et al., 1984; Weinberg and Shenk, 1984; Haley et al., 1984). In addition, some E1A mutants exhibit a cold sensitive transforming activity and studies with these mutants have demonstrated that a functional 13S product is required for the maintainence of transformation (Ho et al., 1982; Babiss et al., 1984).

E1A products are also required for the induction of tumours when Ad12 is injected into syngeneic animals (Byrd et al., 1988). This is a property of E1A that may account, at least in part, for the differences in oncogenic potential between Ad5 and Ad12. Using recombinant Ad5/Ad12 viruses to establish cell lines, it has been demonstrated that the presence of Ad12 E1A sequences correlates with the ability of of the cell lines to form tumours in syngeneic animals (Bernards et al., 1983, Sawada et al., 1988). Schrier et al. (1983) have suggested that this is due to the ability of Ad12 E1A to suppress expression of the class I major histocompatability antigen (MHC) resulting in a more successful evasion of the immune response directed against the transformed cells. A cellular protein that binds to the 5' end of the class I MHC gene has been shown to be present

in Ad12 transformants and absent in Ad5 transformed cell lines (Akrill and Blair, 1989). This factor is believed to be responsible for the reduction of MHC class I gene expression.

The E1B gene products alone are not capable of inducing transformation in either primary or established cells (van den Elsen et al., 1982; Ruley, 1983). Tumorigenicity however appears to depend on the E1A and E1B region but how much of the region is required depends both on the cell type, the method of transformation (virus or DNA mediated), and the adenovirus serotype. For example, in Ad5 mediated transformation the entire E1 region is required for fully transformed cells (Rowe and Graham, 1983). Alternatively, in Ad5 DNA mediated transformation assays E1A requires the contribution of the left half of the E1B gene, coding for the 19Kd protein and the amino terminus of the 58Kd protein, to fully transform cells (Graham et al., 1974a, b; Rowe and Graham, 1983; Rowe et al., 1984a). It has also been recently demonstrated that E1A proteins in combination with either the 19Kd protein or 58Kd protein are able to transform cells, but that transformation is more efficient in the presence of both proteins, suggesting that they may function in transformation via independent pathways (Edbauer et al., 1988; White and Cipriani, 1990). The induction of tumours upon injection of adenoviruses has only been demonstrated for the group A and

B serotypes (Trentin et al., 1962; Heubner et al., 1962). In Ad12, 55Kd is an important determinant of oncogenicity since mutations in this protein abolish tumour promoting activity when the virus is injected into nude mice or hamsters (Shiroki et al., 1986; Byrd et al., 1988).

19Kd is required for efficient transformation by both viruses and DNA since the transforming activity of various 19Kd mutants is reduced at least 10 fold relative to wild type (Mak and Mak, 1983; Chinnadurai, 1983; Pilder et al., 1984; Babiss et al., 1984; Bernards et al., 1986; Barker and Berk, 1987). It has also been shown that transformed cells which express the 19Kd protein possess gross disruptions in the intermediate filament (IF) cytoskeleton and that this phenomenon may be related to the mechanism by which this protein functions in transformation (White and Cipriani, 1990). White and Cipriani (1990) have suggested that the perturbations in the organization of IF's produced by the 19Kd protein may lead to anchorage-independent growth contributing to the transformed cell phenotype. In addition, 19Kd appears to function in a different manner in the process of transformation in the various serotypes. Ad12 19Kd is apparently dispensible for transformation of some cells such as baby rat kidney cell (Edbauer et al., 1988) but is required for the transformation of 3Y1 cells (Fukui et al., 1984;

Edbauer et al., 1988). Ad2 and Ad5 19Kd appear to be required for transformation of all cell lines tested.

No biochemical function of 58Kd has yet been linked with its role in transformation. However, the complexing of 58Kd and the cellular anti-oncogene p53 may prove to be of significance to this process. As previously mentioned, p53 is an anti-oncogene whose product is a suppressor of transformation (Hinds et al., 1989; Finlay et al., 1989). Mutations in this gene result in its ability to act as a promoter of transformation in rodent cells and to induce tumours in rodents. It can therefore be hypothesized that binding of 58Kd to p53 interferes with the normal function of this protein to constrain cellular growth mechanisms, resulting in a deregulation of cell proliferation. In support of this the transforming proteins of other DNA tumour viruses, such as SV40 large T antigen and HPV E6 also bind to p53 and this binding is required for transforming activity. Although efficient transformation by large T Ag in SV40 requires that it binds both Rb-1 and p53, the two corresponding binding domains exist on separate transforming molecules in adenovirus (Finlay et al., 1989).

1.8 Cytogenetic Damage by Adenoviruses

It has long been known that both DNA and RNA viruses induce chromosomal changes within infected host cells (Nichols et al., 1962, 1963; Stich et al., 1964; Zur Hausen, 1967). As previously mentioned, the occurrence of cytogenetic damage has been of prime interest to investigators with regards to cancer since chromosomal aberrations are often a feature of tumour cells and may contribute to the development of the neoplastic phenotype (LeBeau, 1986; Yunis, 1987). Similar alterations have also been observed in cells transformed by viruses <u>in</u> <u>vitro</u> (Graham et al., 1977; Chang, 1986; Walen et al., 1987). The adenoviruses in particular have been of interest in the study of cytogenetic damage since different serotypes of this virus vary in the type chromosomal aberrations that they induce (McDougall et al., 1971).

The mildly oncogenic and non-oncogenic adenoviruses have been shown to induce non-specific chromosomal aberrations in both permissive (Stich et al., 1964; McDougall et al., 1971; Caporossi and Bacchetti, 1990), and semi-permissive cells (Braithwaite et al., 1983; Murray et al., 1982a). These alterations consist primarily of chromatid gaps and breaks at seemingly random sites throughout the genome (Stich et al., 1964; McDougall et al., 1971; Braithwaite et al., 1983;

Caporossi and Bacchetti, 1990). However, work by Caporossi and Bacchetti (1990) has demonstrated that the Ad5-induced aberrations coincide with fragile sites. Studies with either semi-permissive rodent cells (Braithwaite et al., 1983), or permissive human embryonic kidney cells (Caporossi and Bacchetti, 1990) has indicated that expression of the gene products of the Ad5 E1A region is necessary for the induction of chromosomal aberrations. Viral DNA synthesis or other early viral gene products (E1B, E2A, E3) do not appear to play a role in the induction of chromosomal aberrations. Caporossi and Bacchetti (1990) have further shown that both the 289R and 243R proteins of E1A are involved in this process, with a critical requirement for the amino terminus, the unique region of 289R and the 3' 25% of exon 2.

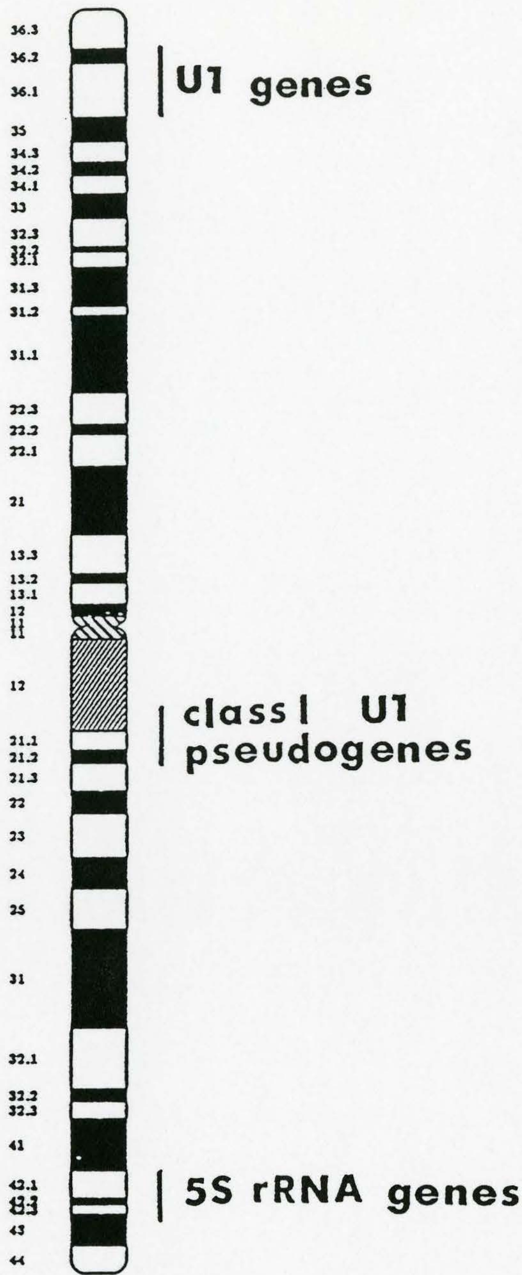
Unlike the mildly oncogenic or non-oncogenic adenoviruses the highly oncogenic group A adenoviruses (Ad12, 18 and 31) have been shown to induce chromosomal aberrations at very specific sites on chromosome 17 and 1, as well as gaps and/or breaks at random sites throughout the genome (McDougall et al., 1970, 1971; Durnam et al., 1986). Ad12 has been particularly well studied and hence, these sites are referred to as Ad12 modification sites (Durnam et al., 1986). At a low multiplicity of infection (MOI), nearly all detectable damage consists of gaps and breaks located at band 17q21-22 and most

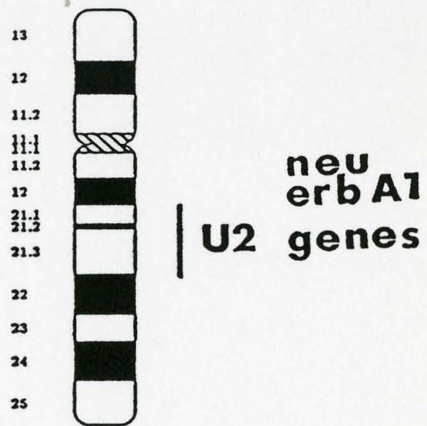
often involving both chromatids. With increasing multiplicities of infection, gaps and breaks involving one or both chromatids at three specific sites on chromosome 1 (p36, q21 and q42-43) as well as gaps or breaks at random sites throughout the genome are observed (Zur Hausen et al., 1967; McDougall et al., 1970, 1971; Durnam et al., 1986). Using scanning electron microscopy (SEM), Durnam et al. (1986) have shown that the specific damage induced by Ad12 at 17q21-22 consists of an uncoiling of the chromatin. It was hypothesized that this decondensed region is transcribed throughout mitosis, and that its breakage might be a secondary phenomenon. All of the Ad12 modification sites appear to map near U1 and U2 small nuclear RNA and 5s ribosomal RNA loci (Lindgren et al., 1985; Durnam et al., 1988). The biological significance of this is unclear at present (Lawler et al., 1989). In addition, the sites affected by Ad12 are common sites of chromosomal rearrangements in human cancers (Gilbert et al., 1984). A schematic representation of the chromosomes affected by Ad12 and their adenovirus modification sites are given in Figure 4.

A number of lines of evidence indicate that early viral genes are responsible for the induction of chromosomal aberrations by Ad12. Firstly, Raska and Strohl (1972) have shown that the time course of the appearance of chromosome

FIGURE 4

Location of the major and minor adenovirus type 12 modification sites. These sites have been mapped at 17q21-22 and 1p36; 1q21, with another minor site at 1q42-43. All of the Ad12 modification sites appear to map near U1 and U2 small nuclear RNA or 5s ribosomal RNA loci. ErbA1 and neu oncogenes also appear to map near the major modification site on chromosome 17.


U1 genes
class I U1
pseudogenes
5S rRNA genes
1


neu
erb A1
U2 genes
17

damage correlates with the appearance of early gene products. Secondly, chromosomal damage is observed in syrian hamster and mouse/human hybrid cells incapable of supporting late gene trancription or viral replication (MacKinnon et al., 1966; McDougall et al., 1973). Thirdly, Durnam et al. (1986) were able to induce Ad12 specific chromosome damage using an Ad5/Ad12 recombinant virus, containing the Ad12 E1 region in the place of Ad5 sequences. In addition, these investigators transfected a mouse/human hybrid cell line with an Ad12 E1 region clone and demonstrated that the clones incurred cytogenetic damage. In both cases, sequences from the E1 region appear to be involved in the induction of chromosomal aberrations, and the type of damage induced is primarily at site 17q21-22. None of the work thus far has been able to indicate whether specific and random aberrations are produced by a common or separate mechanism.

Adenoviruses induce a number of alterations to the cell growth cycle in infected cells which may be related to the viruses ability to induce karyotypic alterations both in number and structure of chromosomes. The effects of Ad5 infection on the cell cycle have been demonstrated in both quiescent and growing rodent cells (Younghusband et al., 1979; Murray et al., 1982). In quiescent rodent cells human adenovirus induces cells to progress into S phase and

synthesize cellular DNA under conditions in which normal cells remain arrested in G1 phase (Younghusband et al., 1979). Growing rodent cells are induced by Ad5 to prematurely reinitiate successive rounds of cellular DNA replication before segregation of the chromosomes and cytokinesis, resulting in aneuploidy and polyploidy of the cells. Those cells that do undergo mitosis frequently suffer structural chromosome aberrations and abnormal chromosome segregation (Murray et al., 1982). Cellular thymidine kinase is also stimulated in G1 arrested and growing rodent cells by Ad5 infection (Cheetham and Bellett, 1982). The induced progression of cells from G1 to S is believed to maximize the number of cells able to replicate viral DNA and the time that these cells spend in DNA replication. In addition to the induction of cellular DNA synthesis a number of events in the normal G1 to S phase do not occur. For example, G1 is shortened in some cells (Murray et al., 1982), and DNA replication is uncoupled from the synthesis of ribosomal RNA and polyamines (Pochran et al., 1980; Cheethem and Bellett, 1982).

The induction of chromosomal damage by adenoviruses appears to be related to virus-induced changes in cell cycle controls, since this damage shows cyclic variation in frequency. For example, studies using either Ad12 or Ad5 in

permissive or semi permissive cells (McDougall et al., 1974; McDougall, 1975; Caparossi and Bacchetti, 1990) have demonstrated that cells have to be infected in G1 or S phase to suffer the most damage, but that the damage is prevalently chromatidic (McDougall et al., 1974, 1975; Caporossi and Bacchetti, 1990). In addition, Bellett et al. (1982) suggested that adenovirus actually induces aberrations in the G2 phase of the cell cycle which preceeds the mitosis at which the damage is visible. This hypothesis arose from experiments in which spermine (a polyamine that binds to DNA and may play a role in the stability of the chromosomal structure) gave maximal protection against Ad5-induced aberrations when it was added at the beginning of G2. These findings suggest that the intracellular level of spermine is particularly important in G2, at least in virus-infected cells (Bellett et al., 1982). It may therefore be cells which are in G2 when a critical virus-induced event occurs which are most susceptible to damage (Murray et al., 1982). It is however still conceivable that the cell cycle stage at the time of infection is important to the susceptibility of the chromosomes. The reason for this is that polyamine synthesis occurs in G1 rather than G2 and hence, cells would have to be infected prior to the G1 stage in order to have reduced spermine levels and thus be more susceptible to damage in G2.

1.9 Rationale for Study

This study was undertaken to: 1) identify the Ad12 E1 gene product(s) required for the induction of both specific and random aberrations in human embryonic kidney cells and 2) to determine whether the same or different functions are involved in the induction of damage at specific and random sites. The approach chosen was to use viruses with mutations in one of the Ad12 E1 genes in order to map the Ad12 E1 function(s) required for the induction of genetic damage. However, since mutants in the Ad12 E1A gene were not available at the time of this study, Ad12/Ad5 recombinants with all of Ad12 E1A or Ad12/Ad5 E1A hybrid sequences in an Ad5 background, were obtained from Tomas Jelinek (McMaster University), and were used to discern the role of these proteins in the induction of cytogenetic damage. To investigate the involvement of the major E1B proteins in this process, viruses with mutations or small deletions in the Ad12 E1B 19Kd and 55Kd genes were obtained from Shi-yun Zhang, Mike Schaller and Dr. Stanley Mak (McMaster University), and mutants with large deletions in the coding region for 55Kd were obtained from Dr. Kazuko Shiroki (Shiroki et al., 1986) and Dr. Philip Gallimore (Byrd et al., 1988). All the described viruses were used to infect HEK cells and metaphases

were analyzed for induction of chromosomal aberrations at both specific and random sites. Since some of the biological phenotypes of the mutants used in this study are known (viral DNA synthesis, transformation and tumourigenicity) it was thought possible to make a correlation between these viral functions and the ability of the virus to induce cytogenetic damage.

This study reports the findings of these experiments and the interpretation of the results.

MATERIALS AND METHODS

2.1 Cells

Human embryonic retinal (HER) and human embryonic kidney (HEK) cells were cultured in 100 mm dishes (Corning) in alpha-MEM (GIBCO Laboratories, Grand Island, NY) supplemented with antibiotics, l-glutamine and 10% fetal calf serum (FCS). The cells were obtained by trypsinization of embryonal retinas and kidneys, respectively, and were used as secondary cultures. Human 293 (HEK cells expressing Ad5 E1 proteins, Graham et al., 1977) and KB cells were grown in 150 mm dishes using F-11 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% new born calf serum (NBCS). Human MH12-C2 cells (2H; HEK cells expressing Ad12 E1 proteins; Mak and Mak, 1990) were grown in 150 mm dishes using alpha-MEM supplemented with 10% NBCS.

2.2 Viruses

Ad12 (Huie) was propagated in KB cells grown in suspension. Ad12 E1B mutant viruses were propagated in 2H cells and Ad12/Ad5 recombinant viruses were propagated in 293

cells. Virions purified by CsCl gradient centrifugation were used in most cases with the exception of dl207, del620, in602 and the Ad12/Ad5 recombinants. Virion concentrations were determined by optical density measurements, and titers of infectious virus were determined by plaque assays on 293 or 2H cells. Four Ad12/Ad5 recombinants were used: T12E1, which encodes the Ad12 E1 region in place of the Ad5 E1 region in an Ad5 genome; T1227, which encodes exon 1 of Ad12 E1A (up to Ad12 nucleotide [nt] 1142) joined to the remainder of the Ad5 genome from Ad5 nt 1227; T1461, which encodes the Ad12 E1A region in place of the Ad5 E1A region in an Ad5 genome; T2743, which encodes Ad12 E1A and a portion of Ad12 E1B (up to Ad12 nt 2525) joined to the remainder of the Ad5 genome from Ad5 nt 2743 (Tomas Jelinek and Frank L. Graham, unpublished data). In addition, the following Ad12 E1B mutants were used: pm1542 which contains a point mutation in the AUG of the 19Kd protein and dl1670 which contains an in-frame deletion of 6 amino acids between nts 1670 and 1688 of the 19Kd protein (Shi-Yun Zhang and Stanley Mak, unpublished data); pm1852 which has a point mutation introducing a stop codon at the third amino acid residue of the 55Kd protein; this mutant synthesizes an unstable form of this protein, probably as a result of reinitiation at amino acid 24 (Mak and Mak, 1990); and dl17, dl42, and dl1201 which contain in-frame deletions of 17, 42

and 33 amino acid residues, between nts 2083 and 2134, nts 2185 and 2311, and nts 2853 and 2955, respectively, within the coding region of the 55Kd protein; all three deletion mutants synthesize the 55Kd protein, but dl42 and dl1201 specify an unstable form of this product (Mak and Mak, 1990; Mike Schaller and Stanley Mak, unpublished data); in602 which contains an 8bp linker inserted at nt 2247 causing a frameshift and subsequent stop codon at nt 2270 (Byrd et al., 1988); del620 which contains an in-frame deletions of 232 amino acid residues between nts 2129 and 2825 within the coding region of the 55Kd protein (Byrd et al., 1988); dl207 which contains a deletion between nts 2323 and 3183 and a 9bp linker insertion at the deletion site resulting in a polypeptide consisting of 198 amino acid residues (Shiroki et al., 1986). in602, del620 and dl207 all synthesize the 55Kd protein. The map locations of the mutations and the phenotypes of the viral mutants are shown in figure 5 and Table 1, respectively.

2.3 Plaque Assay

In order to determine virus titres, tenfold serial dilutions of virus stock were plated on monolayers of 293 or 2H cells. The virus was allowed to adsorb to the cells for

FIGURE 5

E1 regions of mutant viruses. Viruses pm1542, and pm1852 carry point mutations at the corresponding nucleotides in the 19-and 55 Kd protein coding genes. The deletion (black boxes) in dl1670 involves nts 1670 to 1688 within the 19Kd protein coding region. The deletions (black boxes) in dl17, dl42, dl1201, dl207 and del620 involve nts 2083 to 2134, 2185 to 2311, 2853 to 2955, 2323 to 3183 and 2129 to 2825, respectively, within the 55Kd protein coding gene. in602 has a stop codon at nt 2270 resulting in a truncated protein (deleted portion also shown as black box). The E1 sequences in T12E1, T1461, T1227 and T2743 consist of the entire Ad12 E1 region, the entire Ad12 E1A region, exon 1 of Ad12 E1A (up to Ad12 nt 1142), and Ad12 E1A and a portion of Ad12 E1B (up to Ad12 nt 2525), respectively, placed in Ad5 background. KD=kilodalton

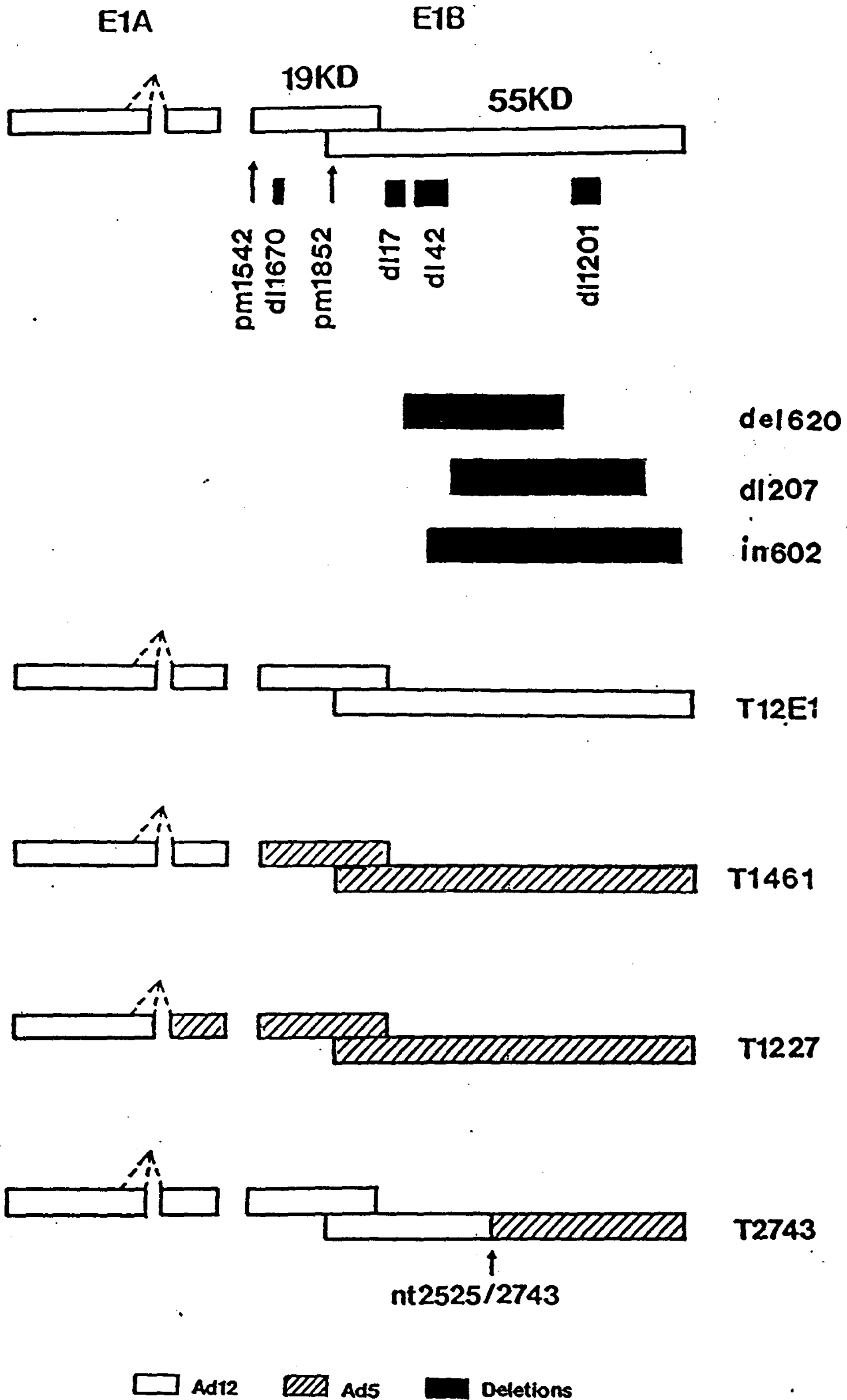

E1A
E1B
19KD
55KD
pm1542
dl1670
pm1852
dl17
dl42
dl1201
del620
dl207
in602
T12E1
T1461
T1227
T2743
nt2525/2743
Ad12
Ad5
Deletions

TABLE 1

Phenotypes of viral mutants

Virus	Mutated Ad12 protein (Kd)	Levels of protein expression[a]		
		E1A	E1B 19K	E1B 55K
pm1542	E1B(19)	wt	(-)	>>wt
dl1670	E1B(19)	wt	(-)	wt
pm1852	E1B(55)	wt	wt	US
dl17	E1B(55)	wt	wt	wt
dl42	E1B(55)	wt	<wt	US
dl1201	E1B(55)	wt	<wt	US
dl207	E1B(55)	<wt	ND	<wt
del620	E1B(55)	<wt	ND	<wt
in602	E1B(55)	<wt	ND	<wt
T12E1	None	<wt(Ad12)	<wt(Ad12)	<wt(Ad12)
T1461	None	<wt(Ad12)	<wt(Ad5)	<wt(Ad5)
T1227	E1A	wt(hybrid)	wt(Ad5)	wt(Ad5)
T2743	E1B(55)	(-)	(-)	(-)

a-Assayed in KB cells (see references in text) or HEK cells (this study) by immunoprecipitation of [^{35}S] methionine-labeled lysates or by immunoblotting and expressed relative to wt levels.
(-)=Undetectable
ND =not determined
US =unstable

1 hr at room temperature, after which the cells were overlayed with a mixture containing 0.5% agarose, 1x F-11 (supplemented with l-glutamine, penicillin/streptomycin and yeast extract), and 5% horse serum (HS). The cells were subsequently overlayed every 6 days until the appearance of plaques, at approximately 1 week for Ad5 and 2 weeks for Ad12.

2.4 Infections

Adenovirus infections were performed by removing the culture medium and plating an appropriate amount of virus diluted in phosphate buffered saline (PBS) on a monolayer of HEK or HER cells. Cells were partially synchronized by growing them in serum depleted medium (0.5% FCS) for 48 hrs, and adding 20% FCS 6 hrs prior to infection. This was done in all cases except when cells were infected for collection of proteins or DNA. The cells were incubated in the presence of virus for 1 hr at room temperature, after which the original conditioned culture medium was added and the infection allowed to proceed for 19 hrs before the cells were harvested. HEK and HER cells were generally infected with 2, 5 and 10 PFU per cell for Ad12 wt and mutants pm1852, pm1542 and dl1670. Multiplicities of 10, 50 and 100 PFU per cell were used for all other mutants.

2.5 Chromosome Preparation

Cells treated with colcemid for 4 hrs were harvested by trypsinization and incubated in 0.075 M KCl for 12 to 16 min at 37°C prior to fixation with methanol/acetic acid (3:1 v/v) for a minimum of 24 hrs. Chromosomes were dropped onto dry slides washed with 100% ethanol, or wet slides washed with liquid Knox soap, and stored at 4°C. If metaphases were not sufficiently spread out, cells were washed 1 or 2 times in 1:1 (v/v) methanol:acetic acid before being dropped onto slides. After fixation chromosomes were stained with 5% Giemsa stain for 5-10 min at room temperature and analyzed by light microscopy. The percentage of aberrant cells as well as the frequency and type of aberrations were determined by scoring 50 to 100 metaphases for each experimental treatment. The mitotic index for each experimental treatment was determined by counting the number of metaphases per 1000 cells.

2.6 Immunoprecipitation of Proteins

2.6.1 Radioactive Labeling

Labeling of infected HEK cells was generally at 17 hrs post infection. Each 60 mm dish of HEK cells was incubated for 45 min with 1.0 ml 199-methionine free medium (Gibco

Laboratories) supplemented with 1% dialyzed FCS, followed by the addition of 100 uCi of [^{35}S] methionine (Amersham Corp., Arlington Heights, Ill; specific activity, 1,300 Ci/mmol) diluted into 1.0 ml of the methionine free medium. The labeling period was generally for 2 hrs at 37°C.

2.6.2 Extraction of Proteins

After the labeling period, the radioactive medium was removed and the cells were rinsed two times with cold PBS and scraped into PBS using a plastic scraper or rubber policeman. The cells were pelleted by centrifugation (1000 rpm for 10 min) and the PBS was subsequently removed. The cell pellet was resuspended in RIPA (50 mM Tris [pH 7.2], 150 mM NaCl, 0.1% [wt/v] SDS, 0.1% [wt/v] sodium deoxycholate, and 1% Triton X-100) or NP40 (50 mM TRIS [pH 8.0], 150 mM NaCl, 1% NP40) buffer containing 0.004% [wt/v] aproteinin (Sigma). In most cases 100 ul of buffer was used per $2x10^6$ cells. After incubation on ice for 30 min the lysates were centrifuged at 10,000 rpm for 30 min at 4°C. The RIPA samples were sonicated 2 times 5 sec each before centrifugation. Following centrifugation, the supernatant was collected and the pellet discarded.

2.6.3 Immunoprecipitation of Proteins

The immunoprecipitations were set up as follows: 500 ul of cell lysate, 500 ul of RIPA or NP40 buffer plus aproteinin, 10-20 ul of antiserum (10 ul for M73, and 20 ul for Ab6aC3), and 200 ul protein A sepharose beads (Pharmacia) resuspended at a concentration of 10% in the appropriate buffer. Following thorough mixing, the samples were incubated for 2 hrs at 4°C with constant rotation on a wheel. The immunocomplexes were subsequently pelleted by centrifugation in a microfuge, and washed 3 times with NP40 buffer minus aproteinin followed by 2 washes with Tris-saline buffer (100 mM Tris [pH 7.2], 150 mM NaCl). In the case of lysates prepared in RIPA the beads were washed 5 times with RIPA minus aproteinin.

2.6.4 One Dimensional Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (1970). The stacking gel contained 5% polyacrylamide, while the separating gel used was 12%. The ratio of acrylamide to N-N'bismethylene acrylamide was 30 to 0.8.

Samples were prepared for SDS-PAGE by resuspending the pelleted immunocomplexes in 50 ul of 2x sample buffer (100 mM Tris [pH 6.8], 2% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue) and placing them in a boiling waterbath for 3 min. After centifugation to pellet the beads, 5 ul of the supernatant was removed and the radioactivity was quantitated in a Beckman liquid scintillation counter. The remaining 45 ul of the supernatant was loaded on a gel. Electrophoresis was at 80V for approximately 16 hrs or until the bromophenol blue dye front reached the bottom of the gel. The gel was washed with DMSO and fluorographed using PPO-DMSO (Bonner and Laskey, 1974) and dried for 2 hrs at 60°C before autoradiography using Kodak XAR-5 film at -70°C. In order to quantitate the amount of protein synthesized, films were scanned using an Ultrascan XL laser transmittance densitometer (LKB 2222-020) (Pharmacia LKB Biotech.).

2.7 Western Immunoblot

Protein samples to be analyzed by immunoblotting were prepared in the same manner as described above except that the cells were not radioactively labeled prior to harvesting. The proteins were also separated by SDS-PAGE as described

above. Following electrophoresis the gel was equilibrated for 1 hr in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol [v/v]). The nitrocellulose membrane (Bio-Rad) and sheets of Whatmann 3 mm paper were pre-soaked in transfer buffer and then assembled with the gel into a Bio-Rad Transblot Cell. Transfer was at 4°C at 70V for 4 hrs, after which the nitrocellulose sheet was, dried and stored at 4°C, or immediately blocked and probed. Dried blots were rehydrated and blocked using buffer A (0.05% instant skim milk [wt/v], 170 ul 30% Antifoam A, 5 ml 5% Tween 20 and 500 ul 0.1% Thimerosol made up to 500 ml with ddH2O) for 1 hr. The primary antibody, M73 (Oncogene Science, Inc.) or Ab6aC3 (Mak and Mak, 1990), was diluted 1:500 in buffer A, and incubated with the blot for 90 min. The blot was rinsed 4 times 5 min with buffer A. The Alkaline Phosphatase (AP) conjugated Goat anti-Rat IgG (for Ab6aC3) or AP conjugated Goat anti-mouse IgG (for M73) (Bio Can Scientific Inc.) was diluted 1:5000 in buffer A, and incubated with the blot for 1 hr, after which the blot was washed 2 times 5 min with buffer A and 2 times 2 min with borate buffer (0.93 g/l, pH 9.5). The alkaline colour reaction was performed by incubating the blot in the reaction mixture (5 ml 0.1% NBT in borate buffer, 0.5 ml of 5 mg/ml BCIP in dimethylformamide, 100 ul 2M MgCl2, 45 ml borate buffer [pH 9.5]). The colour development was allowed

to proceed for up to 2 hrs, after which the reaction was stopped by washing the blot with ddH2O.

2.8 Slot Blot

2.8.1 Harvesting of Viral and Cellular DNA

HEK cells from which total DNA was to be harvested were infected as described above. Total DNA was harvested by removing the medium from the plates and washing the cells 1 time with PBS. The plates were dried as well as possible, and 450 ul of lysing buffer (10 mM Tris [pH 7.4], 10 mM EDTA, 0.4% SDS) plus 50 ul of predigested pronase (5.0 mg/ml pronase in 10 mM Tris [pH 8.0], 20 mM EDTA) was added to each for approximately 6-8 hrs at 37°C. Total DNA was harvested by scraping the lysate into an Eppendorf tube using a rubber policeman. The samples were kept at 4°C until the DNA was extracted.

Total DNA was extracted from the samples as follows. The lysates were mixed with 1 vol 100 mM Tris (pH 8.0) saturated phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and 1/10 vol 30% sodium acetate, vortexed gently and mixed by hand for 15 min, after which the samples were spun in the microfuge for 5 min. The aqueous (top) phase was removed and a second extraction was performed by mixing the solution with an equal

volume of chloroform:isoamyl alcohol (24:1 v/v). The tubes were again vortexed gently, mixed by hand for 15 min and spun in the microfuge for 10 min. The aqueous phase was removed and mixed with 1/10 vol 1 M NaCl and 1/250 vol RNase (5 mg/ml stock in 10 mM Tris [pH 7.5], 15 mM NaCl and boiled for 5 min) followed by incubation of the samples at 37°C for 30 min. A third extraction was performed using 1 vol phenol:chloroform:isoamyl alcohol (without 1/10 vol Na Acetate), after which the DNA was precipitated using cold 100% ethanol at -20°C for 30 min. The DNA was spun down in the microfuge for 20 min and the pellet was washed with 70% ethanol, followed by 96% ethanol. The DNA pellet was allowed to air dry before being dissolved overnight in 100 ul ddH2O (sterile). The DNA was stored at -20°C until needed.

2.8.2 Quantitation of DNA by Fluorometry

To measure the concentration of DNA in each sample, 1 ul was mixed with 2 ml of 1x TNE buffer (10 mM Tris pH 7.4, 10 mM NaCl, 1 mM EDTA) plus 0.0001% Hoechst 33258 dye (1 mg/ml bis-benzimida-zole in ddH2O) in a cuvette and quantitated using the TKO 100 DNA fluorometer (Hoefer Scientific Instruments). 2 ml of the buffer alone was used to zero the

fluorometer, and a known concentration of calf thymus DNA was used as a standard to which all of the samples were compared. Once a concentration was determined in ng/ul, calculations were made to determine the volume of sample needed in order to obtain a given concentration of total DNA.

2.8.3 Slot Blot

The appropriate dilutions of DNA were made in 100 ul of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), to which 1/10 vol of 3 M NaOH was added, and incubated for 1 hr at 60°C. The samples were subsequently cooled to room temperature and an equal volume of 12x SSC (prepared from 20x SSC stock [pH 7.0] containing 3.0 M NaCl, 300 mM Na Citrate) was added. The nitrocellulose membrane and S&S GB003 paper were presoaked in deionized water, followed by 6x SSC, before being assembled into the filtration manifold (S&S Minifold I apparatus). Low vacuum was applied and the wells were rinsed 1 time with 6x SSC, after which the DNA samples (1.0 ng and 0.1 ng of DNA per slot per sample) were spotted, in duplicate, onto the nitrocellulose membrane. A positive control was also applied which consisted of 0.1 ng and 0.01 ng of purified Ad12 DNA. After application of the samples, the membrane was removed from the apparatus and baked for 1 hr at 80 °C. The membrane

was stored at 4°C until hybridization to a probe.

2.8.4 Hybridization of Probe to DNA Samples

A probe consisting of Ad12 DNA was radioactively labeled with alpha-[^{32}P] dCTP by nick translation using the BRL nick translation system (BRL Life Technologies, Inc.). The nitrocellulose filtre was presoaked in 200-300 ml of 2x SSC for 30 min and then placed in a plastic bag, which was sealed, for incubation in prehybridization buffer (50% formamide, 25% 20x SSC, 10% 50x Denhardt's solution [containing 104 ug sonicated and denatured salmon sperm DNA], 5% 1 M Tris [pH 7.5], and 10% ddH2O [sterile]) for 4 hrs at 42°C. Approximately 1-2x10^6 cpm/ml of probe in the hybridization buffer (50% formamide, 25% 20x SSC, 0.02% 50x Denhardt's solution with salmon sperm DNA, 2% of a 50% stock of Dextran Sulphate, 0.02% 1 M Tris [pH7.5]) was hybridized to the DNA on the nitrocellulose filter. Hybridization was allowed to proceed for approximately 16 hrs, after which time the radioactive hybridization buffer was carefully removed from the bag and saved in a Corning tube. The plastic around the filter was cut so that it could be removed and washed 3 times 5 min at room temperature in 2x SSC plus 0.2% SDS, followed by 3-4 times for 45 min at 65°C in 2x SSC plus 0.2% SDS. A

final wash in 0.1x SSC was done before drying the filtre and exposing it to Kodak XR-1 film for 3 days at -70°C.

2.9 Measurement of Cellular DNA Synthesis

2.9.1 Labeling of Cells with [^{3}H]-thymidine

Infections with 10 PFU per cell of virus were carried out as described above, at 6 hrs after the addition of serum. Cells were labeled for 30 min, at 37°C, with 50 uCi of tridiated thymidine (^{3}HTdR) (Amersham) at 3 hrs post serum addition as well as every hr between 6-18 hrs post serum addition. Following incubation, the radioactive medium was removed and the cells were rinsed 1 time with cold (4°C) PBS plus 10% NBCS, and 2 times with cold PBS alone. The cells were then scraped into PBS and frozen at -70°C.

2.9.2 Trichloroacetic acid (TCA) Precipitation

The frozen cell pellet was thawed and added to an equal volume of ice cold 20% TCA plus 10 mM sodium pyrophosphate (ppi). The mixture was vortexed and incubated on ice for 10-20 min. The millipore filtration apparatus was assembled and the glass fibre filtre was presoaked in 10% TCA plus 10 mM ppi prior to filtering of the samples. Low vacuum was applied

to the apparatus and samples were filtered one at a time onto individual glass fibre membranes, first being rinsed 2 times with 10% TCA plus 10 mM ppi and then 1 time with 95% ethanol. Filters were removed from the apparatus and allowed to air dry before being counted in toluene and PPO in a Beckman liquid scintillation counter.

RESULTS

3.1 Dose Response and Characteristics of Cytogenetic Damage by Adenovirus type 12

In order to assess cytogenetic damage induced by Ad12 in human diploid cells, initial experiments were performed to establish conditions yielding an adequate number of aberrant cells and aberrations for analysis while preserving good mitotic index and metaphase quality. Two types of human diploid cells were used for this study, subject to availability. Initially, human embryonic retinal (HER) cells were obtained from the Hospital for Sick Children in Toronto and were maintained in culture for approximately 10 passages. When HER cells became no longer available, human embryonic kidney (HEK) cells, prepared by Irene Mak (McMaster University) were used. A culture was seeded from frozen stocks whenever required and maintained in culture for a limited number of passages (approximately 3).

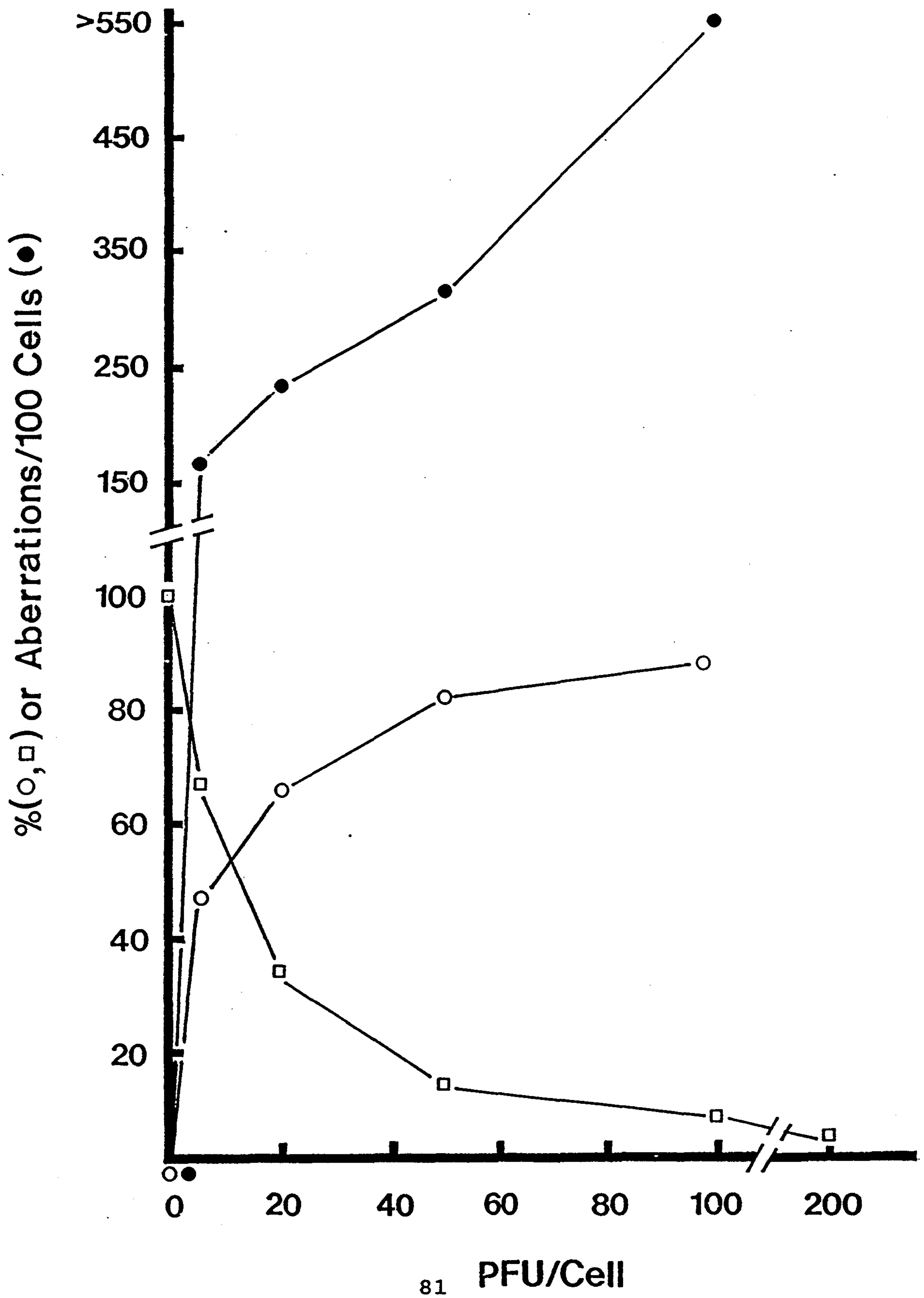
In the first set of experiments HER cells partially synchronized by serum starvation were infected with wild type Ad12 at multiplicities of 10, 20, 50, 100 and 200 PFU per cell for 19 hrs, and chromosome spreads were prepared as described in Methods and Materials. For all experimental points 1000

cells were analysed to determine the mitotic index. In addition, the percentage of aberrant cells and the frequency of aberrations were determined by scoring up to 100 metaphases per treatment.

The results of these experiments are given in figure 6 in which mitotic index, total number of aberrant cells and frequency of aberrations are plotted versus increasing dose of virus. Mitotic index decreased with increasing viral dose which made it difficult to score a statistically significant number of metaphases at doses of 100 PFU per cell and up. In fact, cells infected with 200 PFU per cell could not be scored because of an extremely low mitotic index (3% of mock infected cells). The decrease in mitotic index may, in part, have been due to a high rate of cell death, as suggested by the fact that the cell pellet from cultures harvested after 19 hrs of infection with 200 PFU per cell was considerably smaller than that of the other samples, even though an equal number of cells were infected at the outset of the experiment. Over the range of 10 to 100 PFU per cell, both the number of aberrant cells and the frequency of aberrations increased with viral dose up to a maximum of 88% aberrant cells and greater than 512 aberrations per 100 metaphases. Even at the lowest dose of virus (10 PFU per cell) levels as high as 154 aberrations per 100 metaphases, were observed. At higher

FIGURE 6

Dose response of cytogenetic damage induced by Ad12 in human embryonic retinal cells. Partially synchronized HER cells were infected for 19 hrs with the indicated doses of Ad12 wild type. The percentage of aberrant (○) and the frequency of aberrations (●) per 100 cells were determined by scoring up to 100 metaphases per treatment. Mitotic indices (□) were determined by analysing 1000 cells and are expressed as % of control cells (taken as 100 for control cells).


%(○,□) or Aberrations/100 Cells (●)
>550
450
350
250
150
100
80
60
40
20
0
20
40
60
80
100
200
PFU/Cell

multiplicities of infection the quality of the metaphases deteriorated as the chromosomes became progressively more fragmented thus making it difficult to identify the location of the damage and to accurately estimate the total number of gaps and breaks. The results of this experiment established a dose of 10 PFU per cell of Ad12 as being the upper limit for optimum analysis of chromosomal aberrations induced by this virus in human diploid cells.

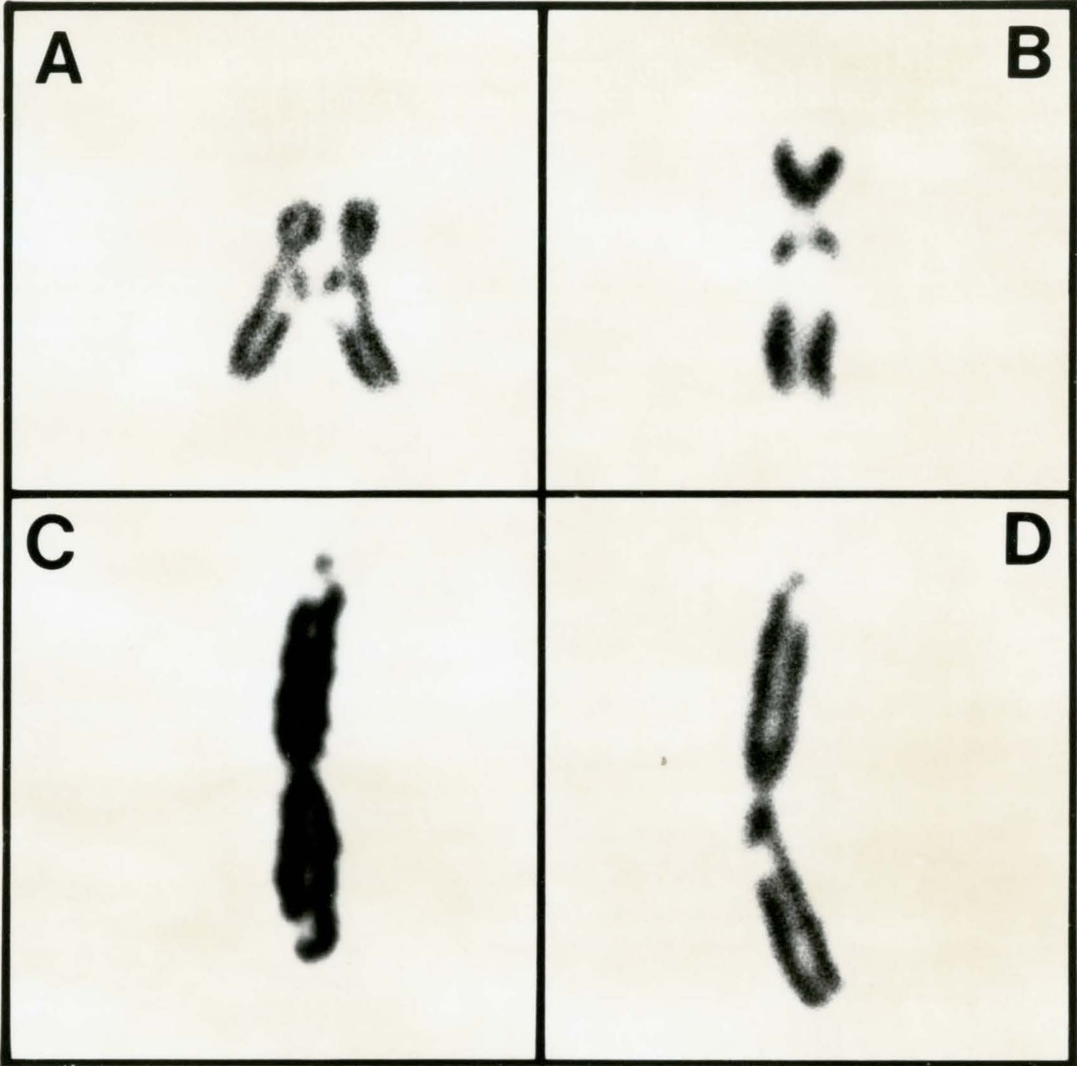
As outlined in the Introduction, previous work on the induction of cytogenetic damage by Ad12 established a gradation of sensitivity to the virus among different chromosomal regions in human cells (Stich et al., 1964; zur Hausen, 1967; McDougall, 1971; McDougall et al., 1974). Using G banding of the chromosomes, specific sites were located and the order of sensitivity was determined to be as follows: damage occurred primarily at site 17q21-22, followed by 1p36, 1q21 and 1q42-43, and finally, by the remainder of the genome. Having established with HER cells the upper bounds for dose of wild type Ad12, multiplicities of 10 PFU per cell and lower were assayed in order to quantitate the frequency of aberrations at various sites throughout the genome. Specific damage was identified in Giemsa stained, unbanded chromosomes, based on chromosome size, morphology and positioning of gaps and breaks relative

to structures such as the centromere and telomeres. For example, chromosome 17 is a small submetacentric chromosome with an Ad12 modification site located close to the centromere on the q arm at band q21-22. Chromosome 1 is the largest metacentric chromosome in the human genome and two of its three Ad12 modification sites (1q42 and 1p36) are found at opposite ends close to the telomeres: one at band q42 and the other at band p36. These two sites are difficult to differentiate in unbanded chromosomes because they occur at approximately the same distance from both telomeres. A third modification site is located close to the centromere on the q arm of the chromosome at band q21. Examples of alterations at all four modification sites are illustrated in figure 7. Aberrations observed at any other sites throughout the genome were considered random.

Partially synchronized HEK cells were infected with wild type Ad12 at multiplicities of 2, 5 and 10 PFU per cell for 19 hrs, harvested and analyzed cytogenetically as described above. The dose response for induction of damage was found to be reproducible over a number of experiments throughout this study and thus representative curves, based on one experiment, are given in figure 8A and B. The fraction of aberrant cells was found to increase with dose up to a maximum of 60% under the experimental conditions used (fig. 8A), and

FIGURE 7

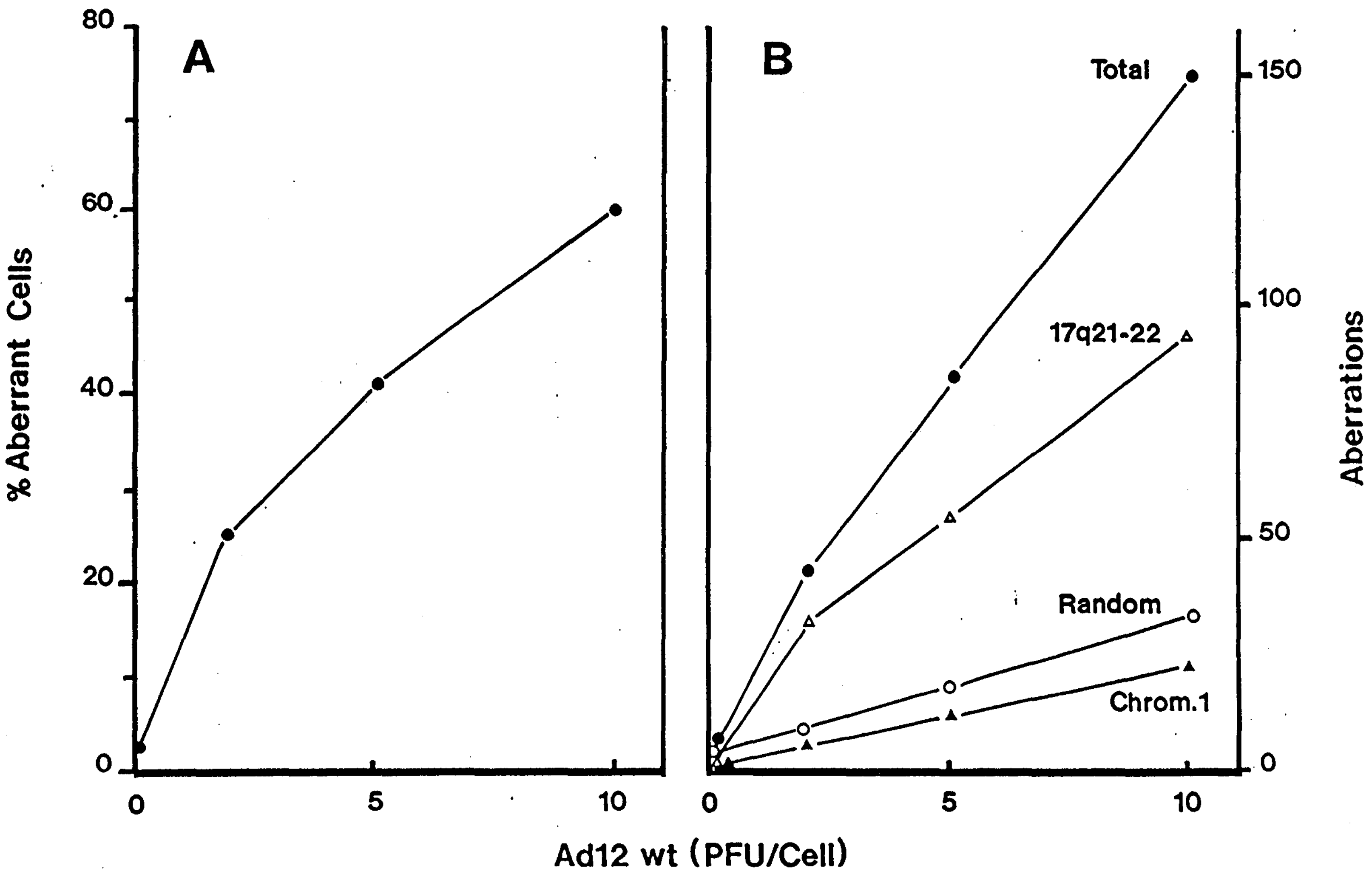
Site-specific, cytogenetic damage induced by Ad12: chromatidic (A) and chromosomal damage (B) at band 17q21-22 and chromatidic damage at bands 1p36 and 1q42-43 (C) and at bands 1p36 and 1q21 (D).


A
B
C
D

an essentially linear dose response was observed for the frequency of total aberrations (fig. 8B). With respect to the types of damage induced, at 2 PFU per cell the largest proportion of aberrations (76%) consisted of a heterochromatic gap at band 17q21-22, and the remaining 23% of aberrations were localized at one or more of the three Ad12 modification sites on chromosome 1 or at random sites throughout the genome. As the multiplicity of infection increased and more of the available 17q21-22 targets were affected, the dose response of this damage deviated from linearity. The frequency of aberrations on chromosome 1 or at random sites on other chromosomes, in contrast, continued to increase linearly with doses up to 10 PFU per cell. At 5 and 10 PFU per cell approximately 62% and 58% of aberrations occurred at 17q21-22, with the remaining 38% and 42% at sites on chromosome 1 or at random sites throughout the genome, respectively. These results are in agreement with those of others (Stich et al., 1964; zur Hausen, 1967; McDougall, 1971; McDougall et al., 1974), and confirm that sensitivity to the virus varies among different chromosomal sites. As expected, site 17q21-22 appears to be the most susceptible, followed by 1p36, 1q21, and 1q42-43, and finally, by the remainder of the genome.

FIGURE 8

Induction of cytogenetic damage by Ad12. Partially synchronized HEK cells were infected for 19 hrs with Ad12 wild type at the indicated multiplicities. Chromosome spreads were prepared and analyzed as described in Materials and Methods. (A) Percentage of cells carrying aberrations. (B) Frequencies of total, specific and random aberrations per 100 cells.


A
B
% Aberrant Cells
80
60
40
20
0
0
5
10
Total
17q21-22
Random
Chrom.1
150
100
50
0
Aberrations
Ad12 wt (PFU/Cell)

3.2 Cytogenetic Damage Induced by Ad12/Ad5 Hybrid Viruses

Previous work by Durnam et al. (1986) had demonstrated that the E1 region of Ad12 was sufficient for the induction of aberrations at band 17q21-22. In those experiments mouse/human hybrid cells were infected with an Ad12/Ad5 recombinant virus (RC15GT), which contains the Ad12 E1 region in place of Ad5 E1 (Bernards et al., 1984). As in the case of Ad12 infected cells, but unlike that of Ad5 infected cells, cytogenetic damage was observed predominantly in the human 17q21-22 portion of the translocated mouse/human chromosome. Comparable results were obtained when the hybrid cells were transfected with Ad12 E1 DNA sequences.

In order to address the question of which of the Ad12 E1 gene product(s) is involved in the induction of damage by this virus a similar approach, using Ad12/Ad5 hybrid viruses, was taken in this study. Four Ad12/Ad5 recombinant viruses were obtained from Tomas Jelinek (McMaster University): T12E1, which encodes the Ad12 E1 region in place of the Ad5 E1 region in an Ad5 genome; T1227, which encodes exon 1 of Ad12 E1A (up to Ad12 nucleotide [nt] 1142) joined to the remainder of the Ad5 genome from Ad5 nt 1227; T1461, which encodes the entire Ad12 E1A region in place of the Ad5 E1A region in an Ad5 genome; and T2743, which encodes Ad12 E1A and a portion of

Ad12 E1B (up to Ad12 nt 2525) joined to the remainder of the Ad5 genome from Ad5 nt 2743. HEK cells were partially synchronized and infected with the recombinants at multiplicities of 10 and 100 PFU per cell for 19 hours, harvested and analyzed cytogenetically as described above.

Results of these experiment are given in table 2. In agreement with results obtained by Durnam et al. (1986) using the recombinant RC15GT, T12E1 was found to have an Ad12 phenotype in that it caused primarily specific aberrations at Ad12 modification sites on chromosome 1 and 17, a property not shared by Ad5 (McDougall, 1971; Caporossi and Bacchetti, 1990). Quantitatively, however, T12E1 was much less efficient at inducing aberrations than wild type Ad12. In this respect, the virus was found to more closely resemble its Ad5 parent which induced a much lower frequency of total aberrations than did Ad12. At a multiplicity of 100 PFU per cell the level of damage observed was augmented to 36 aberrations per 100 metaphases, still considerably lower than levels observed for wild type Ad12 at 10 PFU per cell, or wild type Ad5 at 100 PFU per cell. With respect to specificity, approximately 80% of the aberrations occurred at site 17q21-22 and the remaining 20% of aberrations were localized on chromosome 1 or at random sites throughout the genome. Unlike T12E1, neither T1227, T1461 nor T2743 were able to

TABLE 2

Chromosomal damage induced by Ad12, Ad5 and recombinant viruses

Virus	PFU\cell	% Aberrant cells	No. of aberrations/100 cells	
			Total	Chromosomes 17 and 1
Ad12	10	56	168	132
T12E1	10	9	10	9
T12E1	100	20	36	29
T1461	10	0	0	0
T1461	100	0	0	0
T1227	10	0	0	0
T1227	100	0	0	0
T2743	10	0	0	0
T2743	100	0	0	0
Ad5	10	5	8	0
Ad5	100	34	86	4

induce aberrations, whether at specific or random chromosomal sites, even at a multiplicity of infection of 100 PFU per cell, a dose at which Ad5 too induces substantial random damage (table 2, and Caporossi and Bacchetti, 1990).

3.3 Expression of E1 Proteins by Ad12/Ad5 Hybrids

To rule out the possibility that the lack of cytogenetic damage by recombinants T1227, T1461 and T2743 was due to an inability of these viruses to express Ad12/Ad5 hybrid E1 polypeptides (in the case of T1227 and T2743) or Ad12 E1A polypeptides (in the case of T1461) it was necessary to demonstrate expression of these proteins. The recombinant T12E1 was also assayed to ascertain that it produced Ad12 E1 proteins. HEK cells were infected for 19 hrs with Ad12 wt at a multiplicity of 10 PFU per cell or with the recombinant viruses at multiplicities of 10 and/or 100 PFU per cell. Following electrophoresis of proteins on a 12% SDS-polyacrylamide gel and electroblotting of proteins to a nitrocellulose membrane, they were immunostained with the commercially produced monoclonal antibody M73 to stain the Ad5 E1A proteins or with a tumour serum, Ab6aC3, to immunostain Ad12 E1 proteins.

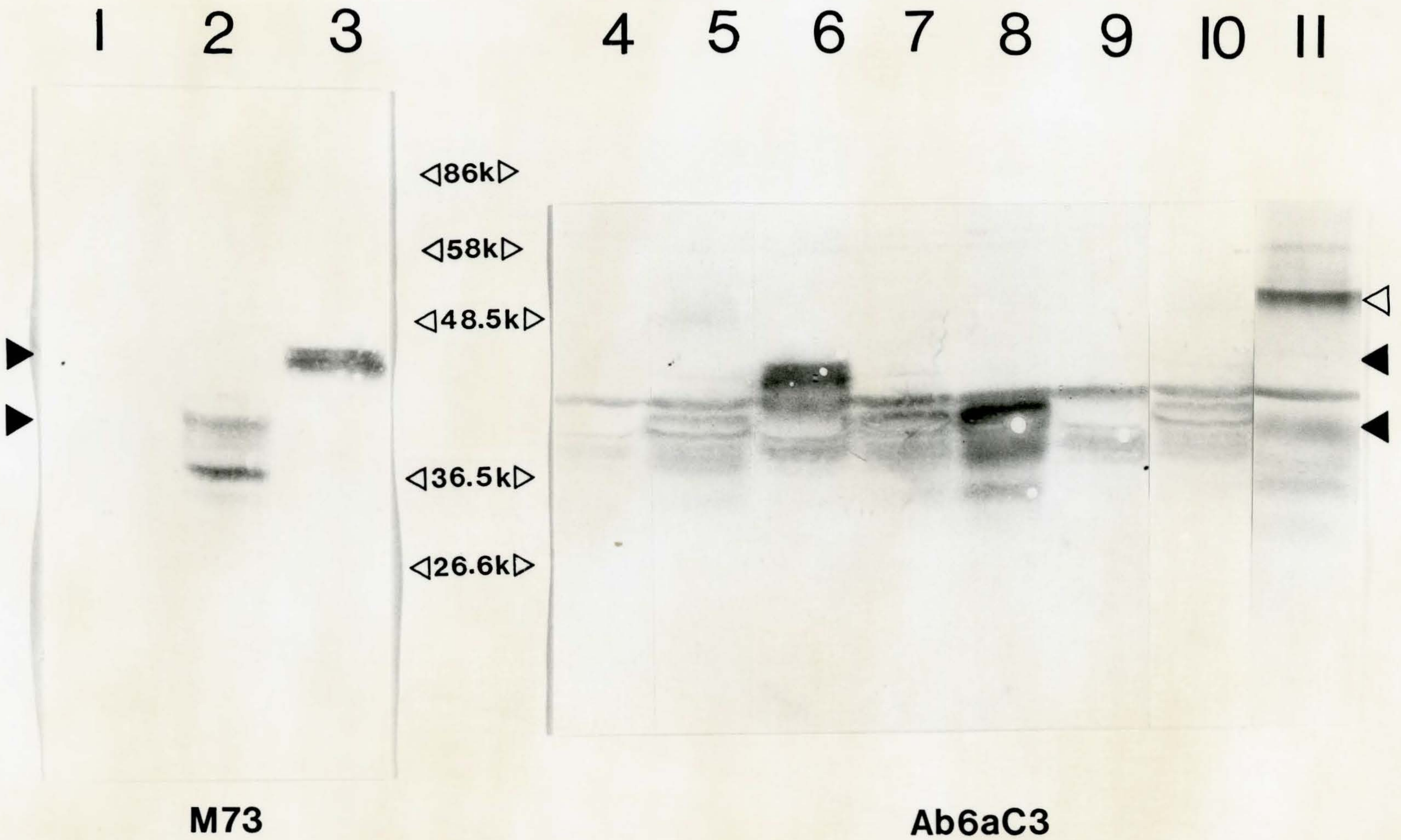
The results of one such experiment are shown in figure 9. Relative levels of protein expression were estimated by visual inspection of the stained gel. A number of observations could be made from this analysis. At a multiplicity of 100 PFU per cell T1227 produced amounts of E1A proteins higher than both Ad5 or Ad12 wt. In additional experiments (not shown) T1227 was found to produce amounts of E1A proteins comparable to either wild type virus when the same multiplicity of infection was used (10 PFU/cell). At 10 PFU per cell recombinants T12E1 and T1461 expressed E1A proteins at levels equal to, or only slightly lower, than wild type. On the other hand, at a multiplicity of 100 PFU per cell T1461 produced E1A proteins in amounts several times higher than those produced by Ad12 wt, while the amounts produced by T12E1 where only slightly higher as compared to wild type. Finally, expression of E1A proteins by recombinant T2743 was not detected even at a multiplicity of 100 PFU per cell, a result which was repeatedly obtained in several experiments.

It can be concluded from these observations that the inability of the T1227 and T1461 recombinants to induce chromosomal damage is not due to a lack of E1A proteins, since both viruses were comparable to wild type in this respect. Conversely, despite producing reduced levels of E1A T12E1 is

FIGURE 9

Expression of E1A proteins in infected cells. HEK cells were infected with 10 PFU per cell of Ad12 wild type and 10 or 100 PFU per cell of Ad5/Ad12 hybrid viruses, and harvested at 19 hrs. Following electrophoresis of cell lysates and electroblotting of proteins to a nitrocellulose membrane the proteins were immunostained with the monoclonal antibody M73 specific for Ad5 E1a proteins (lanes 1-3) and tumour serum Ab6aC3 specific for Ad12 E1 (lanes 4-11). On the left and right side of the figure the immunostained bands corresponding to the Ad5 and Ad12 E1A polypeptides, respectively, are indicated with a closed arrow. The open arrow on the right of the firgure corresponds to the Ad12 E1B 55Kd protein. It should be noted that the mobility of the E1A polypeptides produced by T1227 was slower than that of wt proteins, presumably because of their hybrid nature.

	Virus	MOI
Lane 1	mock	-
2	Ad5 wt	10
3	T1227	100
4	mock	-
5	Ad12 wt	10
6	T1227	100
7	T1461	10
8	T1461	100
9	T2743	100
10	T12E1	10
11	T12E1	100


1
2
3
4
5
6
7
8
9
10
11
86k
58k
48.5k
36.5k
26.6k
M73
Ab6aC3

still capable of inducing low levels of specific and random aberrations. Taken together, these results suggest that T1227 and T1461 lack Ad12 sequences or functions which are necessary for the induction of aberrations, and point to a possible role of Ad12 E1B in this process. Lastly, since expression of E1 proteins by the recombinant T2743 was not detectable, the results obtained with this virus in the cytogenetic assays were uninterpretable.

3.4 Cytogenetic Damage Induced by Ad12 E1B Mutant Viruses

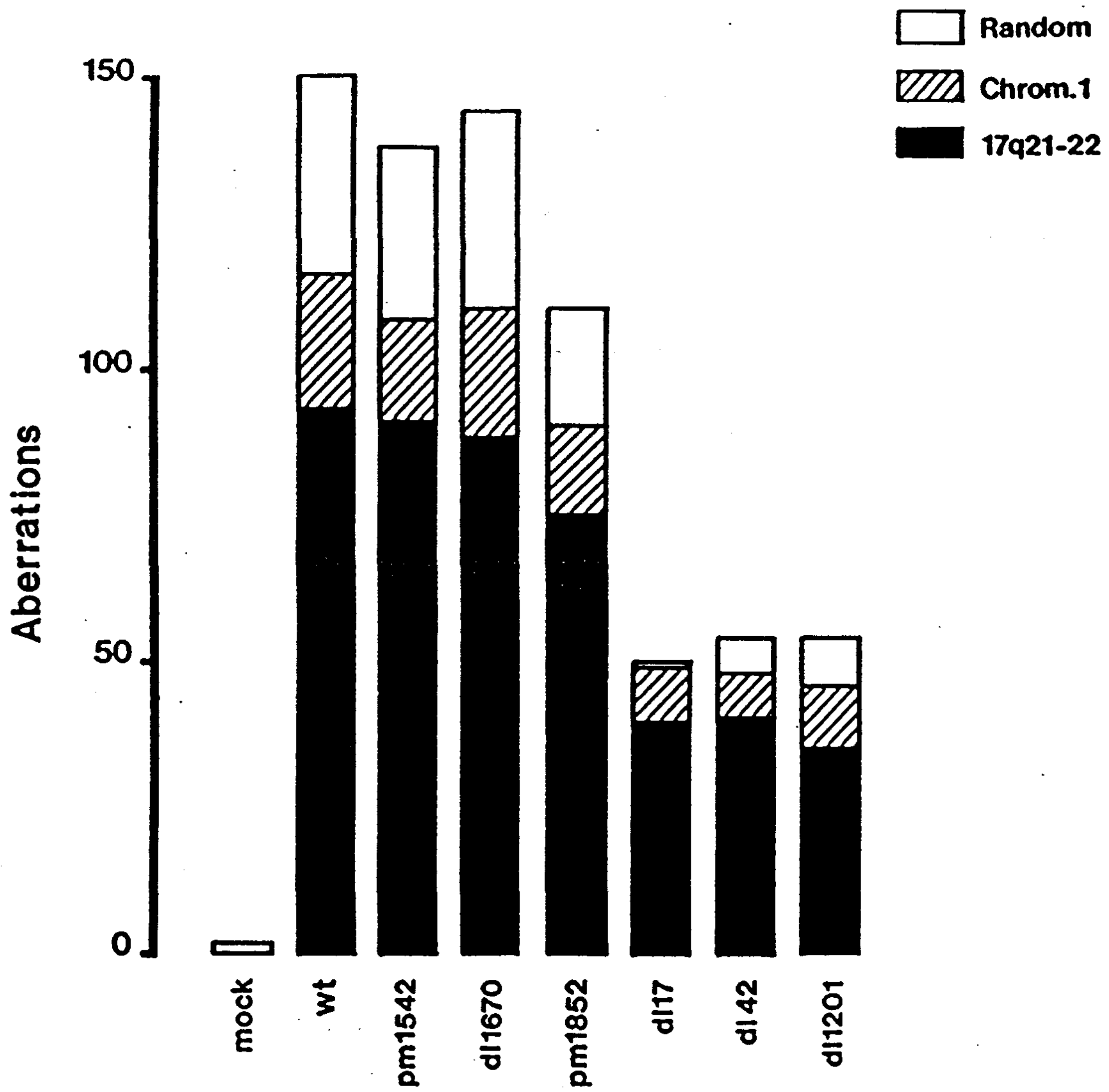
Having established that expression of E1A of Ad12 is not sufficient for the induction of either specific or random aberrations, the potential involvement of Ad12 E1B genes in the cytogenetic effect was investigated. These experiments involved the use of several Ad12 mutants with small deletions or point mutations in either of the E1B major gene products. Mutants pm1542, and dl1670 which have a point mutation (pm) or deletion (dl) in the coding region for the 19Kd protein were obtained from Shi-yun Zhang (McMaster University); pm1852, dl17 and dl42, which have either a point mutation (pm) or a deletion (dl) in the coding region for the 55Kd protein, were obtained from Irene Mak (McMaster University); and dl1201, which also has a deletion in the coding region of

55Kd, was obtained from Mike Schaller (McMaster University) (Refer to Methods and Materials, Fig. 6 and Table 2 for a more complete description). Initial experiments with the Ad12 E1B mutants were performed at a multiplicity of 10 PFU per cell to ensure the induction of all types of damage at levels sufficiently high for proper evaluation of the mutants.

The results of these experiments are given in figure 10 and are expressed in terms of frequencies of aberrations induced by the various mutants at specific and random sites. A number of observations could be made from these results. First, mutants that were impaired in the induction of cytogenetic damage were defective for aberrations at both specific and random sites. Second, viruses with mutations in the E1B 19Kd protein (pm1542 and dl1670) retained a wild type phenotype, whereas viruses with mutations in the E1B 55Kd protein were impaired to different degrees. Mutant pm1852, which synthesizes an unstable 55Kd protein initiating 24 amino acid residues from the normal start site (Mak and Mak, 1990), was still capable of inducing significant amount of chromosomal damage (73% of wild type). In contrast, mutants dl17, dl42 and dl1201, all of which synthesize a 55Kd protein with small internal deletions and, in the case of dl42 and dl1201, with reduced stability, were severely impaired in their ability to induce all types of aberrations (approximately 30% of wild type).

FIGURE 10

Cytogenetic damage induced by wild type and mutant viruses. Partially synchronized HEK cells were infected with 10 PFU of each virus per cell. The frequency of total aberrations per 100 cells is given by the height of the histograms.


Random
Chrom.1
17q21-22
150
100
50
0
Aberrations
mock
wt
pm1542
dl1670
pm1852
dl17
dl 42
dl1201

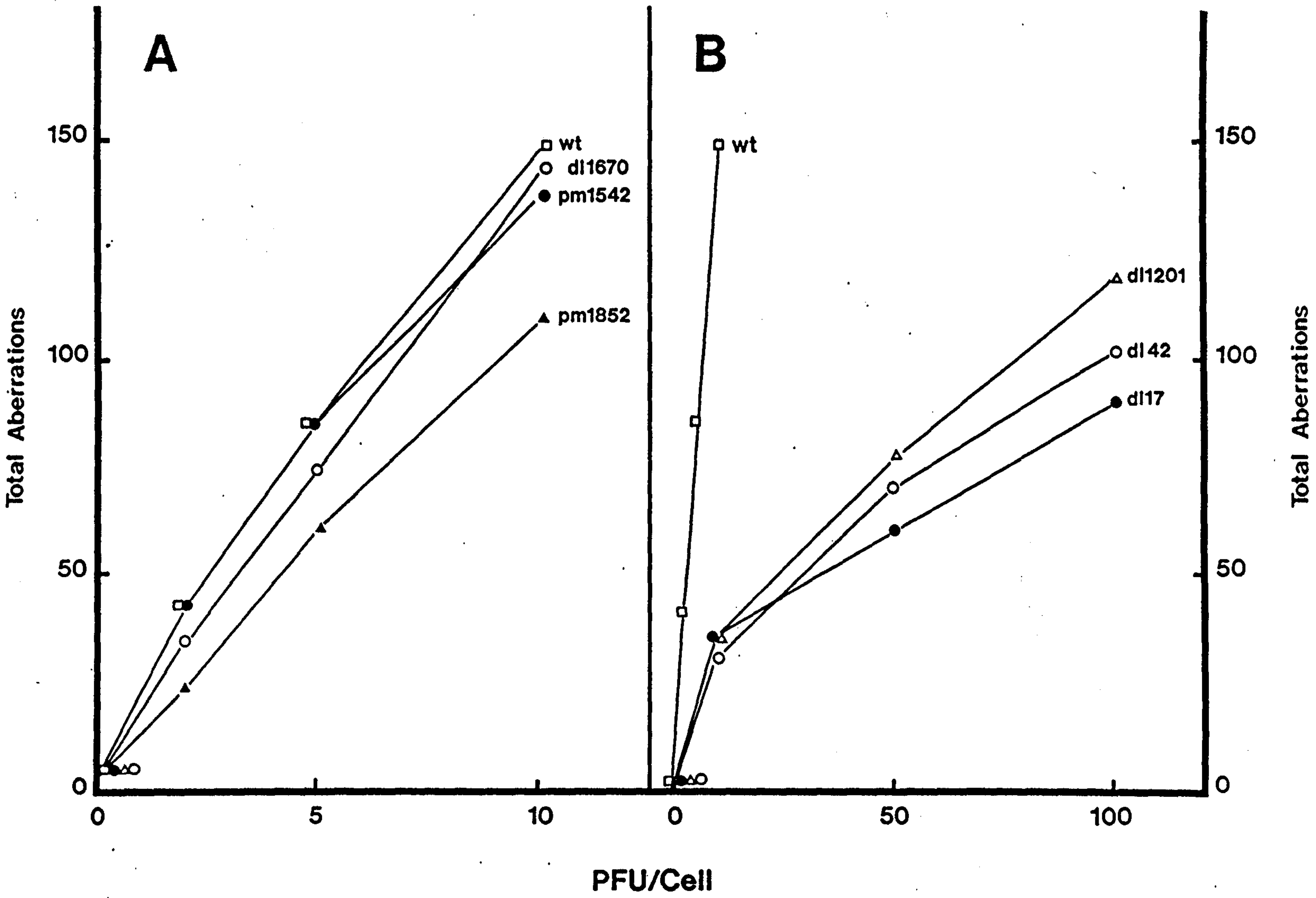
3.5 Dose Response of Damage Induced by Ad12 E1B Mutant Viruses

In order to obtain additional insight into the effect of the various mutations on the induction of chromosomal damage, E1B mutants with wild type or nearly wild type phenotypes (pm1542, dl1670 and pm1852) were assayed at lower multiplicities of infection (2, 5 and 10 PFU per cell). Analysis of the dose response over this range of MOI might reveal subtle differences not apparent at higher multiplicities of infection due to potential leakage, and would ascertain that comparison among viruses were done in the linear portion of the curve. On the other hand, mutants defective in the induction of damage (dl17, dl42 and dl1201) were assayed at higher multiplicities of infection (50 and 100 PFU per cell) in order to determine whether wild type levels of aberrations could be attained and again to ascertain the linearity of the dose response.

The results of these experiments are given in figure 11 in which frequency of total aberrations is plotted versus virus dose. The dose response obtained with pm1542 and dl1670 at 2 to 10 PFU per cell was indistinguishable from that obtained with wild type Ad12. Mutant pm1852, on the other hand, was attenuated at all doses (approximately 70% of wild type). With mutants dl17, dl42 and dl1201 the levels of

FIGURE 11

Dose response of damage induced by wild type and mutant viruses. Partially synchronized HEK cells were infected with 2,5 and 10 PFU of wild type virus and mutants pm1542, dl1670 and pm1852 per cell (A) or with 10, 50 and 100 PFU of mutants dl17, dl42 and dl1201 per cell (B). Only the frequency of total aberrations per 100 cells is shown.


A
B
Total Aberrations
150
100
50
0
0
5
10
0
50
100
wt
dl1670
pm1542
pm1852
wt
dl1201
dl 42
dl17
Total Aberrations
PFU/Cell

damage were augmented when the dose was increased to 50 or 100 PFU per cell but still remained considerably lower than those observed with wild type at 10 PFU per cell.

3.6 Expression of E1 Proteins by dl17, dl42 and dl1201

The reasons for investigating the expression of E1 proteins by the 55Kd deletion mutants were twofold. First, characterization of these mutants was originally done in KB cells (Mak and Mak, 1990), and it was necessary to confirm their phenotypes in HEK cells. Second, Byrd et al. (1988) have previously reported a role for the 55Kd protein in the expression of E1A in primary human cells. Thus it seemed necessary to ascertain whether the diminished ability of the deletion mutants to induce cytogenetic damage was due to a defect in the 55Kd protein or to a reduction in E1A levels. To this end, HEK cells were infected with 50 PFU per cell of Ad12 wild type and the 55Kd mutants (figure 12), or with 10 PFU per cell of Ad12 wild type and 100 PFU per cell of mutants (figure 13), the cells were labeled with [^{35}S]-methionine at 17 hrs post infection and harvested 2 hrs later. Cell lysates were prepared as described in Methods and Materials and immunoprecipitated using a tumour serum specific for Ad12 E1 proteins (Ab6aC3). Following electrophoresis of proteins,

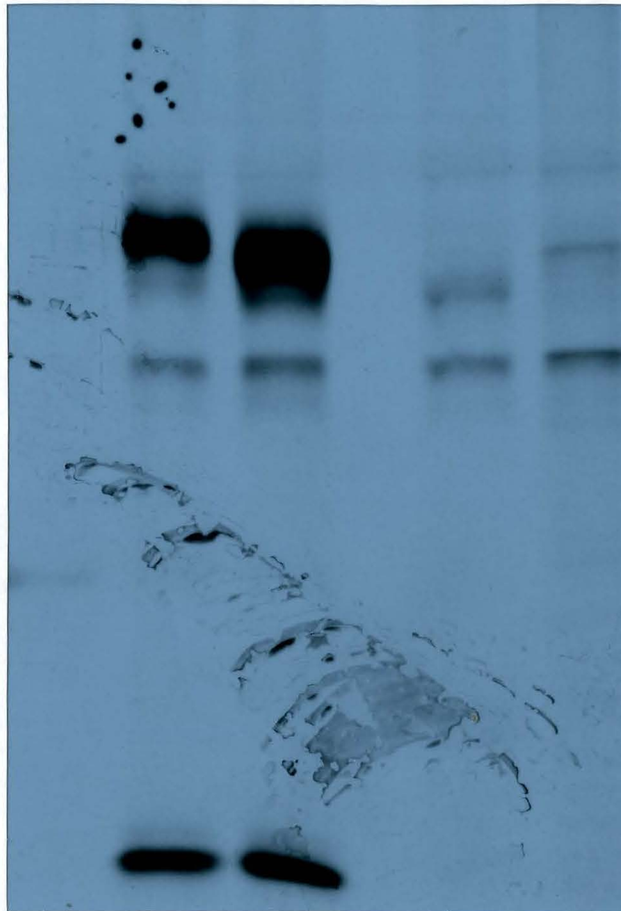
the gel was dried and exposed to Kodak XAR-5 film. In order to quantitate the amount of protein expressed, films were scanned using the Ultrascan XL laser densitometer described in Methods and Materials.

The autoradiographs and the amount of protein produced by mutant viruses relative to wild type are shown in figures 12 and 13, and in table 3, respectively. With respect to E1A, no significant differences in the amounts produced by 50 PFU per cell of wild type or 55Kd deletion mutants were observed in HEK cells (figure 12 and table 3). Similar results were reported for these mutants in KB cells (Mak and Mak, 1990). However, there was considerable differences in the ability of each of the 55Kd mutants to express the 19Kd or 55Kd protein. Dl17 produced amounts of either E1B protein only slightly higher than wild type, while dl42 and dl1201 were severely impaired in their ability to synthesize 19Kd or 55Kd. At multiplicities of 100 PFU per cell the mutants produced more E1A than did wild type at 10 PFU per cell (figure 13) and quantitation of the bands indicated an increase of 1.8-4.5 fold. Also at a multiplicity ten times higher than that of wild type, dl17 was found to express levels of 19Kd and 55Kd proteins comparable to or higher than wild type (10.2 and 1.6 fold, respectively), whereas dl42 and dl1201 still remained reduced for expression of 19Kd and 55Kd

FIGURE 12

Expression of proteins in infected cells. HEK cells were infected with 50 PFU per cell of Ad12 wt and E1B 55Kd mutants, and labeled with [^{35}S]-methionine 2 hrs prior to harvest. Cell lysates were prepared in NP40 buffer as described in Materials and Methods and immunoprecipitated using tumor serum Ab6aC3 for 4 hrs. Following electrophoresis of proteins the 12% polyacrylamide gel was dried and exposed to Kodak XAR-5 film for 3 days at -70°C. On the right of the figure, from the top, the open arrows indicate the differences in mobilities between the 55Kd proteins produced by the various Ad12 55Kd deletion mutants; the larger closed arrows correspond to the Ad12 E1A and E1B 19Kd polypeptides, respectively.

	Virus	MOI
Lane 1	mock	-
2	Ad12 wt	50
3	dl17	50
4	dl42	50
5	dl1201	50


1 2 3 4 5
69k
46k
30k

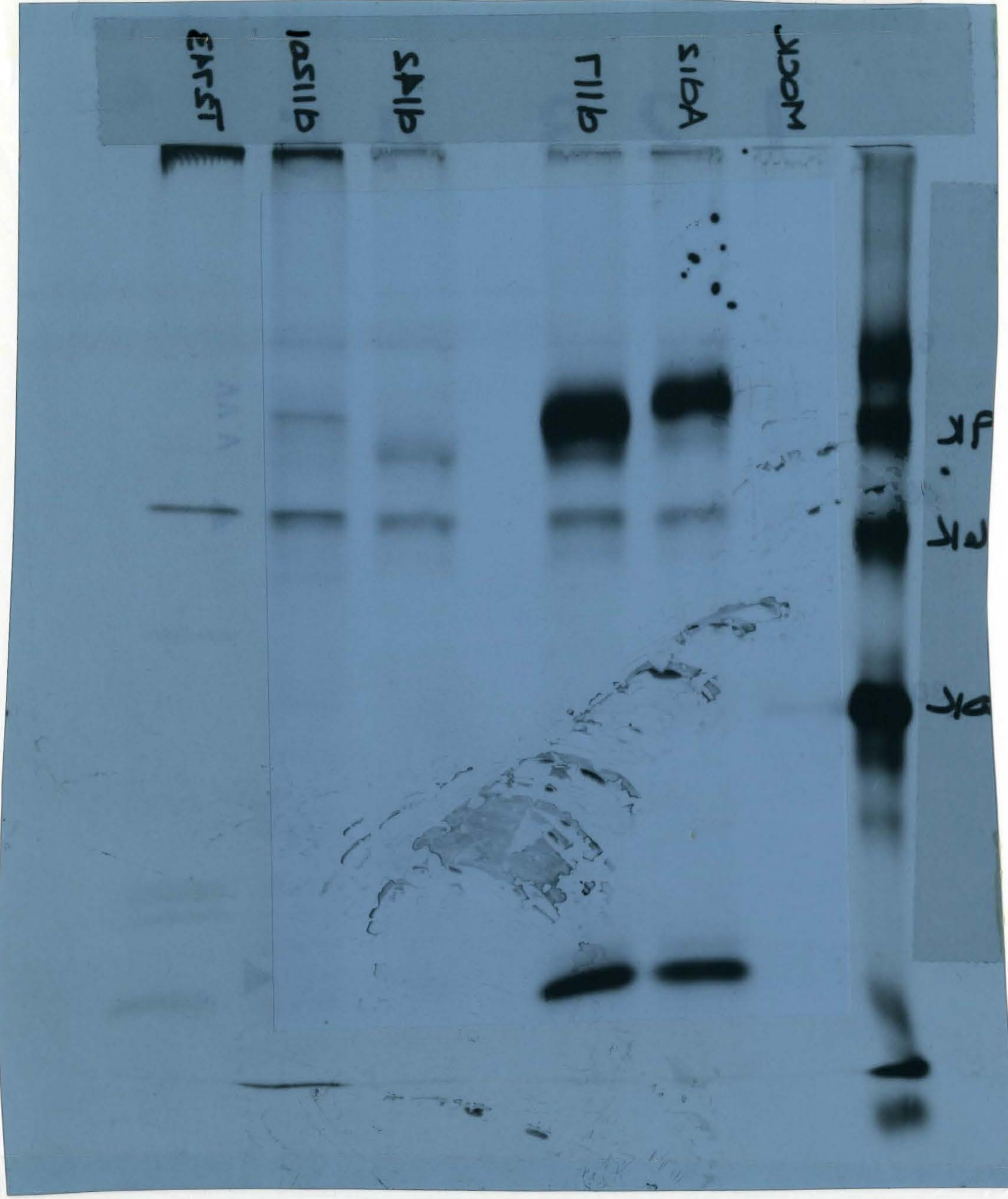
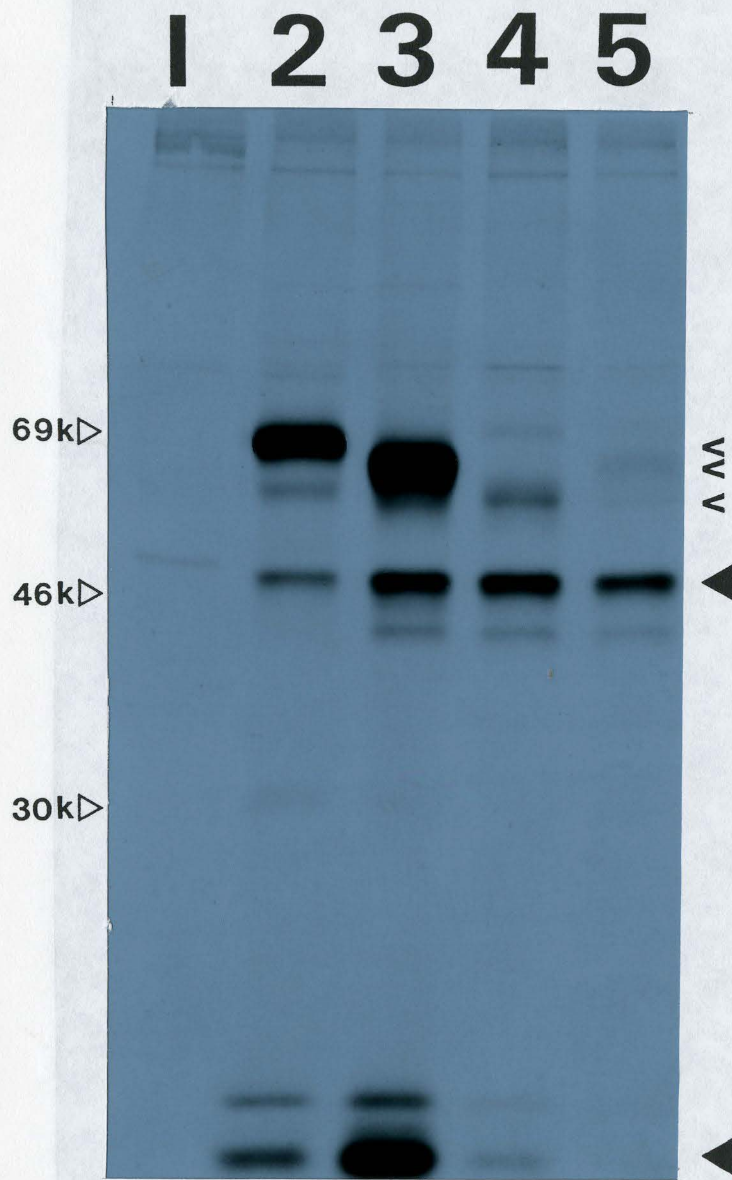


FIGURE 13

Expression of E1A proteins in infected cells. HEK cells were infected with 10 PFU per cell of Ad12 wt and 10 or 100 PFU per cell of mutants, and labeled with [^{35}S]-methionine 2 hrs prior to harvest. Cell lysates were prepared in NP40 buffer as described in Materials and Methods and immunoprecipitated using tumour serum Ab6aC3 for 4 hrs. Following electrophoresis of proteins the 12% polyacrylamide gel was dried and exposed to Kodak XAR-5 film for 3 days at 70°C. On the right of the figure, from the top, the open arrows indicate the differences in mobilities between the 55Kd proteins produced by the various Ad12 55Kd deletion mutants; the larger closed arrows correspond to the Ad12 E1A and E1B 19Kd polypeptides, respectively.

Lane	Virus	MOI
1	mock	-
2	Ad12 wt	10
3	dl17	100
4	dl42	100
5	dl1201	100



1 2 3 4 5
69k
46k
30k

TABLE 3

Relative Amounts of Viral Proteins Produced by Mutant Viruses

Virus	MOI	E1 Protein		
		E1A	19Kd	55Kd
Ad12 wt	50	1.0	1.0	1.0
dl17	50	1.4	1.5	1.5
dl42	50	0.8	0.04	0.1
dl1201	50	1.2	0.03	0.1
Ad12 wt	10	1.0	1.0	1.0
dl17	100	4.5	10.2	1.6
dl42	100	4.2	0.2	0.07
dl1201	100	1.9	0.09	0.01

Note: Amounts of E1 proteins were determined by densitometric scanning of autoradiographs of immunoprecipitates run on 12% polyacrylamide gels. The amount of E1 protein present in the wt sample after 19 hrs infection was taken as 100%. These results were obtained from the autoradiographs shown in figures 12 and 13.

proteins. The mobility of the 55Kd protein was altered for all three mutant viruses indicative of a shortened protein as expected from their respective deletions. In agreement with findings from Section 3.3 these results indicate that apart from their role of E1A in transactivation of E1B, the E1A proteins are not involved in or at least are not sufficient for the induction of chromosomal damage. This is supported by the fact that despite increased levels at multiplicities of 100 PFU per cell the 55Kd mutants still retained their defective phenotype. Also in agreement with previous findings (Section 3.4 and 3.5), and by others (Durnam et al., 1986) the 19Kd protein appeared to be of no consequence to the cytogenetic effect since dl17, dl42 and dl1201 were equally impaired in the induction of aberrations and yet produced varying amounts of this protein. On the other hand, E1B 55Kd appeared to play a role in the induction of both specific and random chromosomal damage based on a correlation between mutations in this protein and the defect of the virus in the induction of chromosomal aberrations. The fact that all of the 55Kd mutants produced different levels of protein and yet were impaired to the same extent suggests that the conformation of the 55Kd protein, rather than its level of expression may play a role in the cytogenetic effect.

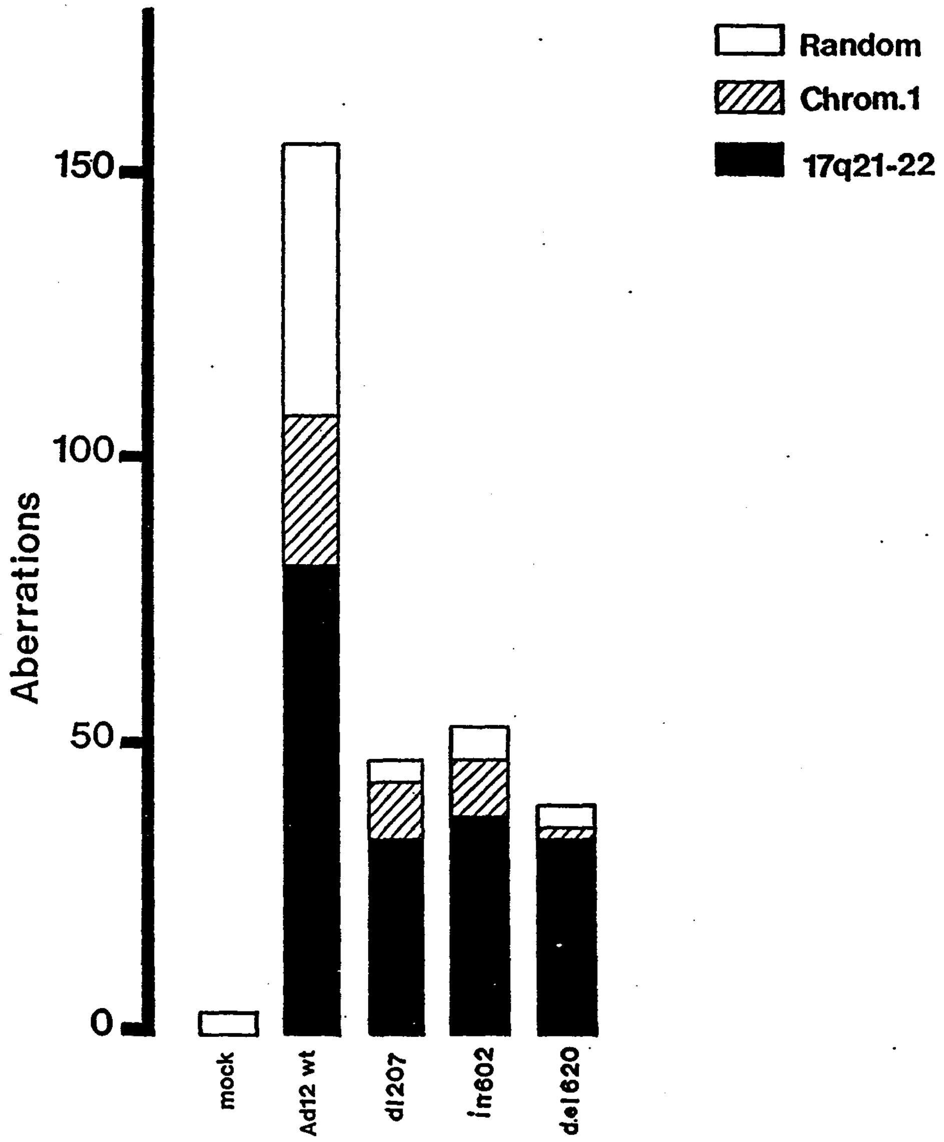
3.7 Cytogenetic Damage Induced by Additional E1B 55Kd Mutants

Ideally it would have been necessary to assay an E1B 55Kd null mutant to confirm this proteins involvement in the induction of chromosomal damage in human cells. At the time of this study no such mutants existed, however a number of mutants containing large deletions in the coding region of 55Kd and described as null for expression of the 55Kd protein were available. The E1B 55Kd mutant dl207 was obtained from Dr. Kazuko Shiroki (Shiroki et al., 1986), and mutants del620 and in602 were obtained from Dr. Gallimore (Byrd et al., 1988)(Refer to fig. 5 and table 1 of Methods and Materials for a complete description). Initially, partially synchronized HEK cells were infected with 10 PFU per cell of each of these mutants for 19 hrs, harvested and analyzed cytogenetically as described above.

The results of these experiments are given in figure 14 and are expressed in terms of frequencies of aberrations induced by each of these viruses at specific and random sites. Surprisingly, even at 10 PFU per cell each of the mutants was capable of inducing levels of specific and random aberrations essentially similar to those induced by mutants dl17, dl42 and dl1201. At a multiplicity of 10 PFU per cell the majority of aberrations were at site 17q21-22 (68-80%), with

FIGURE 14

Cytogenetic damage induced by wild type and mutant viruses. Partially synchronized HEK cells were infected with 10 PFU of each virus per cell. The frequency of total aberrations per 100 cells is given by the height of the histograms.



Random
Chrom.1
17q21-22
150
100
50
0
Aberrations
mock
Ad12 wt
dl207
in602
del620

the remaining 20-32% occuring at one or more of the Ad12 modification sites on chromosome 1 or at random sites throughout the genome.

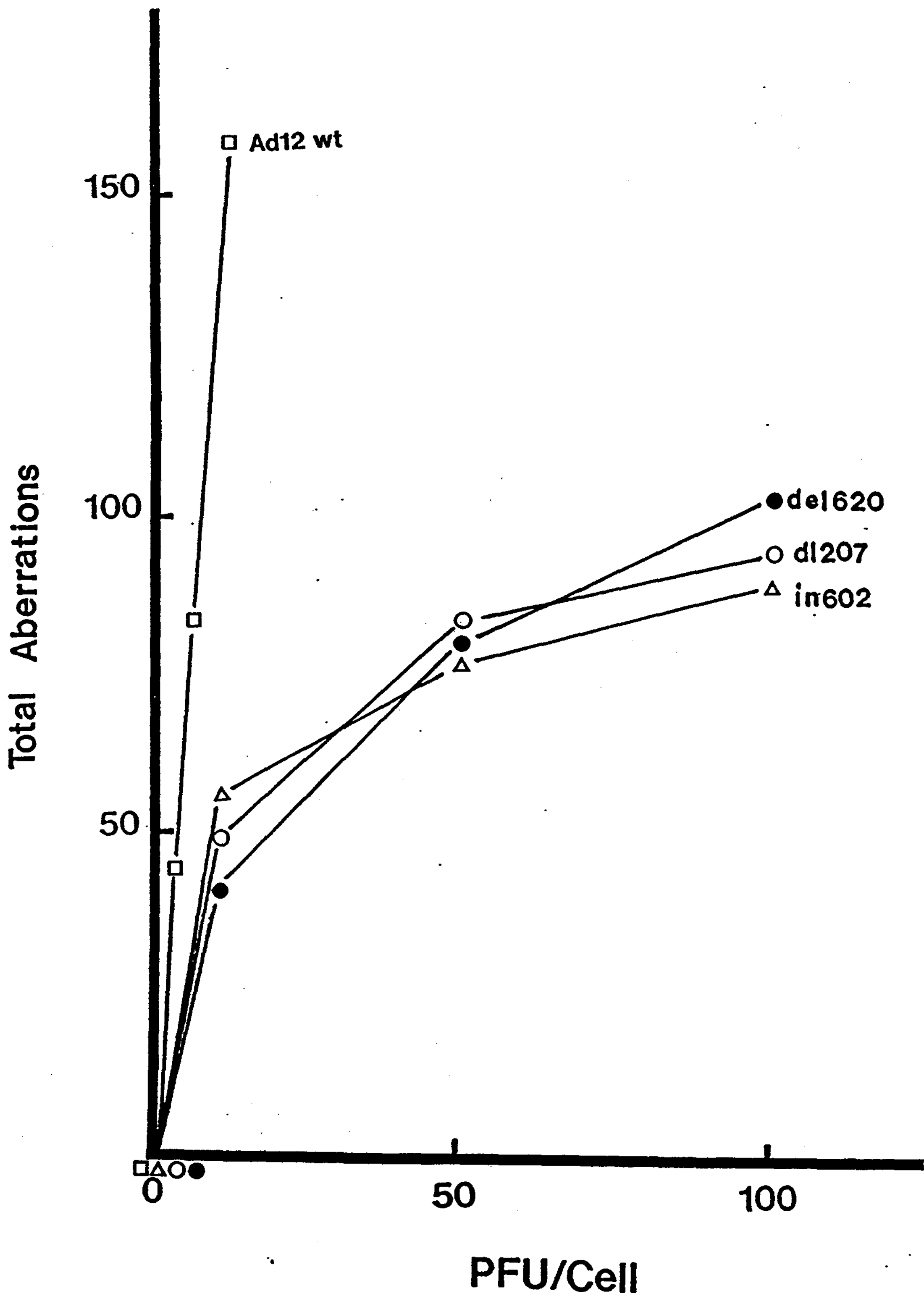
As previously done for the small 55Kd deletion mutants, multiplicities of 50 and 100 PFU per cell were assayed for the large deletion mutants in order to obtain additional insight into their effect on chromosomal damage. The results of these experiments are given in figure 15 in which frequencies of total aberrations are plotted versus virus dose. As the dose of virus was increased the level of damage augmented, however it remained considerably lower than the levels observed for wild type at 10 PFU per cell. As was the case with the small deletion mutants, dl207, del620 and in602 were all equally defective for the induction of cytogenetic damage, suggesting that there may not be one particular region of the 55Kd protein that is critical for this effect.

3.8 Expression of E1 Proteins by dl207, del620 and in602

Having established that mutant viruses dl207, del620 and in602 were capable of inducing low levels of aberrations, it was necessary to determine their phenotypes with respect to expression of E1 proteins. To this end, HEK cells were infected with 10 PFU per cell of Ad12 wild type and 10 or

FIGURE 15

Dose response of damage induced by wt and mutant viruses. Partially synchronized HEK cells were infected with 2,5 and 10 PFU per cell with wt or with 10, 50 and 100 PFU per cell of mutants dl207, del620 and in602. Only the frequency of aberrations per 100 cells is shown.



Ad12 wt
del620
dl207
in602
150
100
50
0
50
100
Total Aberrations
PFU/Cell

100 PFU per cell of the mutant viruses, and harvested at 19 hrs. Following electrophoresis of cell lysates and electroblotting of proteins to a nitrocellulose membrane the proteins were immunostained with tumour serum Ab6aC3 specific for Ad12 E1 proteins.

The E1A and E1B 55Kd proteins separated on a 12% gel are shown in figures 16 and 17, respectively. With respect to E1A, figure 16 demonstrates that levels of proteins produced by the 55Kd mutants are slightly lower than wild type levels at the same multiplicity of infection. Similar results were reported, by Byrd et al. (1988) for del620 and in602 in HEK cells. However, at multiplicities of 100 PFU per cell the mutants produced levels of E1A proteins clearly higher than those produced by wild type virus at 10 PFU per cell.

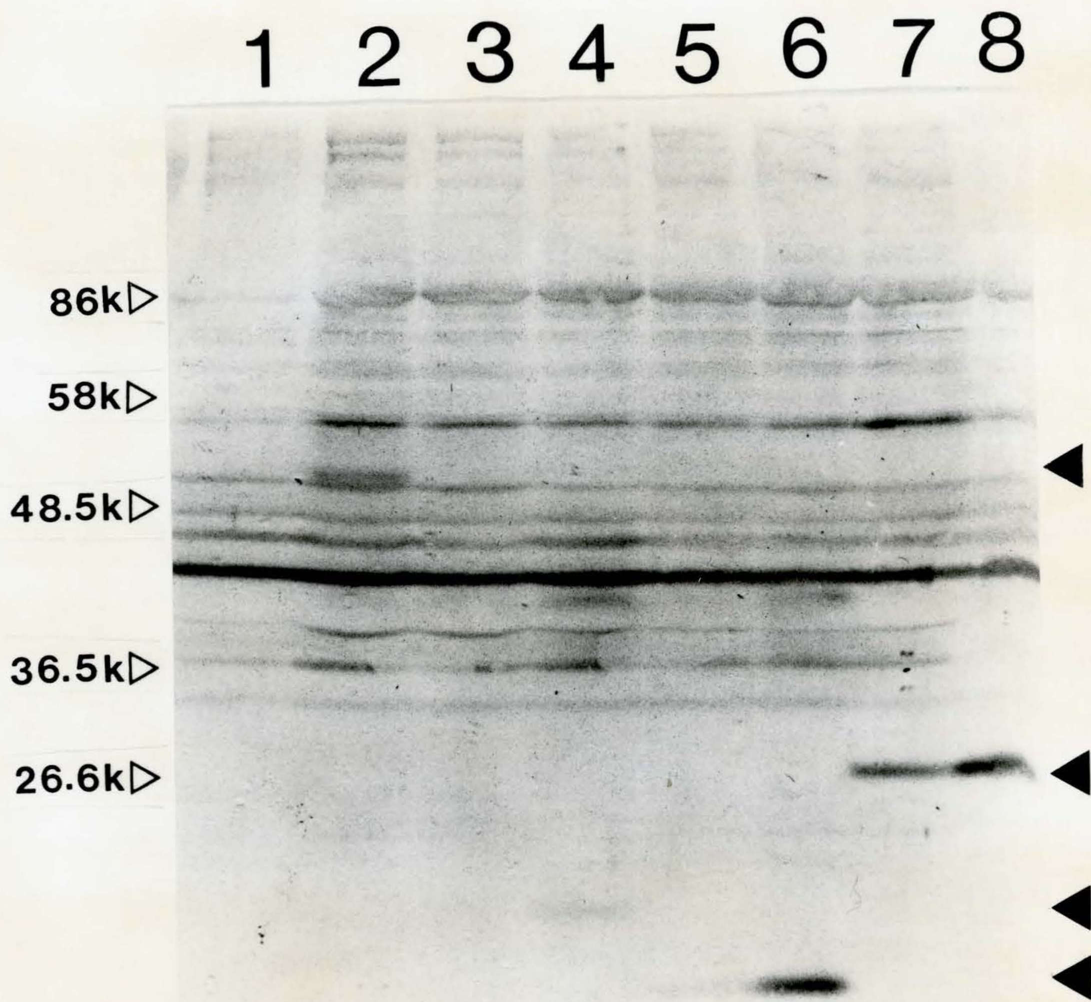
In figure 17 faster migrating, altered 55Kd gene products were detected for all three mutant viruses. The unique bands in each lane were found to correspond to the estimated size of the protein based on the size of the deletions. At a multiplicity of 10 PFU per cell the levels of 55Kd protein produced by dl207 and in602 were significantly lower than wild type, with the proteins produced by dl207 being barely detectable. On the other hand, del620 produced levels of 55Kd protein equal to or slightly higher than wild type Ad12.

FIGURE 16

Expression of E1A proteins in infected cells. HEK cells were infected with 10 PFU per cell of Ad12 wt and 10 or 100 PFU per cell of mutants, and harvested at 19 hrs. Following electrophoresis of cell lysates (prepared in NP40 buffer as described in Methods and Materials) and electroblotting of proteins to a nitrocellulose membrane the proteins were immunostained with tumour serum Ab6aC3 specific for Ad12 E1. On the right of the figure the immunostained bands corresponding to the E1A polypeptides are indicated by an arrow.

Lane	Virus	MOI
1	mock	-
2	Ad12 wt	10
3	dl207	10
4	dl207	100
5	del620	10
6	del620	100
7	in602	10
8	in602	100

1
2
3
4
5
6
7
8
86k
58k
48.5k
36.5k
26.6k


1 2 3 4 5 6 7 8
86k
58k
48.5k
36.5k
26.6k

At multiplicities of 100 PFU per cell the amount of 55Kd protein produced by each of the mutants was augmented but only del620 and in602 attained levels higher than wild type at 10 PFU per cell. The levels of expression of the 55Kd protein by dl207, in contrast, remained lower than those of wild type at 10 PFU per cell.

The results obtained in these experiments provided confirmation of the previous findings that E1A proteins are not sufficient for the induction of chromosomal aberrations in human cells. Once again, despite increases in E1A levels at multiplicities of 100 PFU per cell, the 55Kd mutants retained a defective phenotype. Findings in these experiments also confirmed the involvement of the 55Kd protein in the cytogenetic effect since for each mutant a correlation was observed between mutation in this protein and the defect of the virus in the induction of chromosomal damage. The fact that the mutants produced different levels of 55Kd protein and yet were equally defective again supports the hypothesis that the conformation of the protein rather than its level of expression plays an important role in the cytogenetic effect.

3.9 Analysis of All Mutants for Viral DNA Replication

The reasons for investigating viral DNA replication by all the mutants used in this study were twofold. First, previous findings by McKinnon et al.(1966) and McDougall et al. (1973) have suggested that the induction of chromosomal damage by Ad12 does not require viral DNA synthesis. These findings come from the observation that infection of mouse/human hybrid cells with Ad12, that have retained human 17q sequences, results in specific damage at the 17q band (McDougall et al., 1973; Durnam et al., 1986) in spite of the fact that viral replication does not occur in rodent cells (McKinnon et al., 1966). Second, the mutants obtained from Irene Mak, Shi-yun Zhang and Mike Schaller had been previously characterized in KB cells and in order to assess the role of viral DNA replication in the cytogenetic effect it was necessary to confirm their phenotypes in HEK cells. The mutants obtained from Dr. Shiroki and Dr. Gallimore were characterized in HEK cells and could serve as a confirmation for findings obtained in this study. HEK cells were infected with 10 PFU per cell of each virus, and harvested at 19 hrs. Following extraction and quantitation of total DNA from the cell pellets, 1.0 and 0.1 ug of DNA extract were spotted in duplicate onto nitrocellulose membranes using the S & S

Minifold I apparatus described in Methods and Materials. The nitrocellulose was hybridized to approximately 1-2x10^6 cpm/ml of a ^{32}P labeled probe consisting of purified Ad12 DNA. Following hybridization of the probe, the membrane was dried and exposed to Kodak XR-1 film for 3 days at -70°C.

The results of these experiments and their interpretation are given in figure 18. It should be noted that these results are preliminary since the experiment, although attempted a number of times, was hampered by technical problems. For example, it can be seen from both films that the apparatus leaked, probably because of inappropriate suction or a poor seal. In addition, difficulties were encountered in the loading of correct amounts of total DNA onto the membranes. It can be seen that in some lanes (10, 11 and 12) the viruses appear to over produce viral DNA when 1.0 ug of total DNA was applied, yet this phenotype was not observed with 0.1 ug. Lastly, there was an unexplained problem with the wild type control (lane 2) since DNA could be detected in the 1.0 ug lane, but not in the 0.1 ug one, for one film (I), and vice versa for the duplicate film (II).

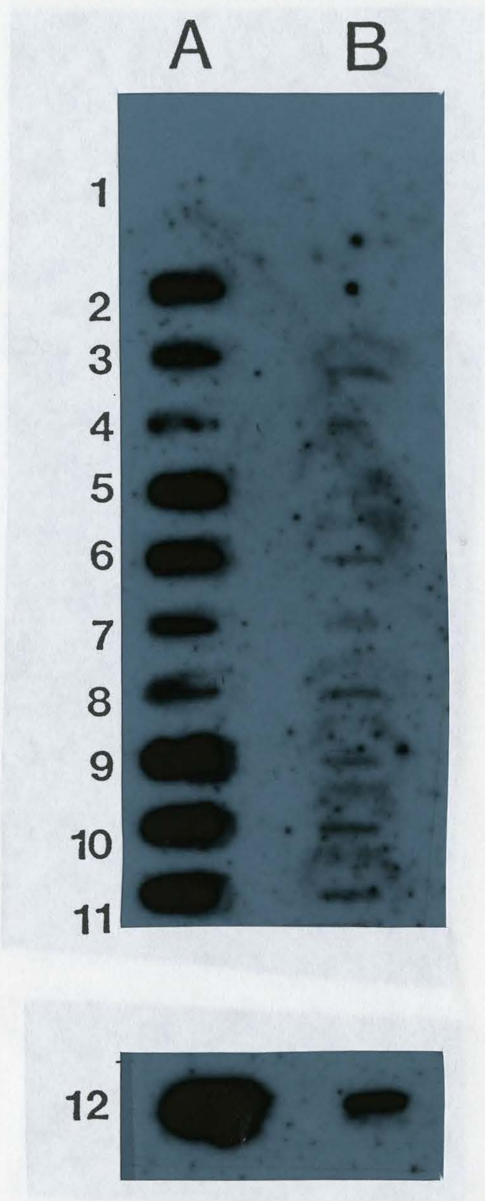
Thus, as far as they can be interpreted the results of these experiments are as follows: based on film A, pm1542, dl1670 and dl17 produced wild type amounts of DNA and were therefore considered DNA positive. Mutants pm1852, dl1201

FIGURE 18

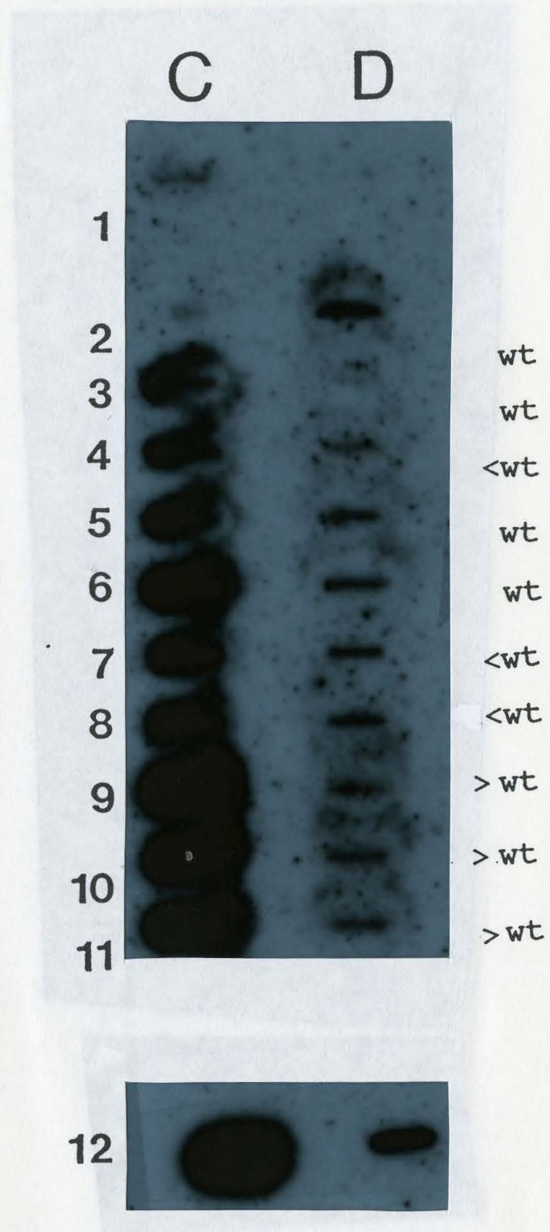
Viral DNA produced by wt and mutant viruses. HEK cells were infected with 10 PFU per cell with Ad12 wt and mutant viruses, and harvested at 19 hrs. Following extraction and quantitation of total DNA from cell pellets, 1.0 ug (Lane A and C) and 0.1 ug (Lane B and D) of DNA extract were spotted in duplicate (I and II) onto nitrocellulose membranes using the S & S minifold I apparatus. The nitrocellulose was hybridized to approximately $1\text{-}2\times10^6$ cpm/ml of a $[^{32}P]$ labeled probe (consisting of purified Ad12 DNA), dried and exposed to Kodak XR-1 film for 3 days at -70°C. 0.1 ug (Lane A and C) and 0.01 ug (Lane B and D) of purified Ad12 DNA was used as a positive control.

Row 1 mock
2 Ad12 wt
3 pm1542
4 pm1852
5 dl1670
6 dl17
7 dl1201
8 dl42
9 dl207
10 in602
11 del620
12 purified Ad12 DNA

I



II



and dl42 synthesized lower amounts of DNA than wild type. Although the results for these mutants are in agreement with those obtained in KB cells they are still considered tentative. The results obtained for dl207, del620 and in602 on the other hand are not in agreement with previous findings by Shiroki et al. (1986) and Byrd et al. (1988) since these viruses appeared to over produce DNA in the present experiments, yet they were previously found to be negative for DNA synthesis. In any case, since variation in the production of viral DNA did not appear to correlate with the ability to induce aberrations, the results obtained in the present experiments are comparable with previous findings that viral DNA replication does not play a role in the induction of chromosomal damage by Ad12.

DISCUSSION

It is generally accepted that carcinogenesis is a multifactorial process involving the stepwise accumulation of mutations throughout the genome. Thus, genetic instability which is an inherent feature of tumours could contribute to the initiation and evolution of cancer (Klein and Klein, 1985; Yunis, 1987; Klein, 1988). Similar to the case of tumor development, immortalization and oncogenic transformation of human cells appears to be a multistep process, and chromosomal abnormalities are a consistent feature of cells transformed *in vitro* by physical or chemical agents and by viruses (DiPaolo, 1983; Chang, 1986). In the case of virally transformed cells it has been shown that chromosomal aberrations occur as early events following the expression of the viral oncogenes, and that these alterations occur prior to crisis and establishment of the immortal line (Graham et al., 1977; Chang et al., 1986). These findings would therefore suggest that, as is the case *in vivo*, mutations of cellular genes might contribute to the initiation of transformation *in vitro*, and to the development of the transformed phenotype.

A remarkable concordance between cancer specific chromosomal abnormalities and the location of both heritable and common fragile sites has been observed (LeBeau, 1986; Yunis, 1987; Glover and Stein, 1988). These sites are, by definition, specific points on chromosomes that exhibit fragility under appropriate conditions of induction. Cytogenetically, this fragility is seen as a nonstaining chromosome or chromatid gap, or break (Glover and Stein, 1988). Viruses such as Ad12 and other group A adenovirus serotypes induce the expression of specific fragile sites. These sites are referred to as Ad12 modification sites, and are located at band 17q21-22 and at three locations on chromosome 1 (1q21, 1p36 and 1q42-43)(Stich et al., 1964; Zur Hausen, 1967; McDougall et al., 1974). Durnam et al. (1986) have determined that the damage induced by Ad12 in the 17q21-22 region results in a decondensation of chromatin. All of the Ad12 modification sites have been found to map near the U1 and U2 small nuclear RNA and 5s ribosomal RNA loci (Lindgren et al., 1985; Durnam et al., 1986), although the biological significance of this observation is not understood at the present time (Lawler et al., 1989). In addition to specific damage, Ad12 is also capable of inducing chromosomal aberrations at random sites throughout the human genome, but at a considerably lower frequency.

Previous work by Durnam et al. (1986) described chromosome damage induced in mouse/human hybrid cells by an Ad12/Ad5 recombinant virus, encoding Ad12 E1 sequences in an Ad5 genome, as well as by plasmids containing the Ad12 E1 region. In either case cytogenetic damage was observed predominantly in the human 17q21-22 portion of the translocated mouse/human chromosome suggesting that the E1 region of Ad12 is sufficient for the induction of aberrations at band 17q21-22. The present study ensued from these observations and was undertaken to specifically identify the Ad12 E1 region(s) required for the induction of chromosomal aberrations in human diploid cells and to determine whether the same or different viral functions are involved in the induction of damage at specific and random sites.

The results obtained in this study indicate that the expression of the E1B 55Kd protein is required for the induction of both specific and random chromosomal damage, based on the defective phenotypes of T1227 and T1461, and of the 55Kd protein defective mutants pm1852, dl17, dl42, dl1201, dl207, del620 and in602. Although a role for the 55Kd protein in the expression of E1A in HEK cells had been previously reported (Byrd et al., 1988), data presented in this study (Sections 3.3 and 3.6; Figures 11, 14 and 15) indicate that the mutant phenotype does not result from

defects in E1A synthesis since there was no significant difference in the levels of E1A proteins produced by wild type or E1B deletion mutants. In fact, at multiplicities 10 times greater than those used for wild type Ad12, the mutants produced higher levels of E1A and still retained their defective phenotype. These results suggest that apart from their role in transactivation of E1B, E1A proteins are not involved in the induction of chromosomal aberrations by Ad12. In agreement with findings by Durnam et al. (1986) using a 19Kd protein defective mutant, 19Kd appeared to be of no consequence to the cytogenetic effect, since mutants pm1542 and dl1670 had a wild type phenotype over a range of multiplicities from 2 to 10 PFU per cell (Section 3.4 and 3.5).

The mechanisms by which the 55Kd protein induces aberrations, whether direct or indirect, are presently unknown. There was no correlation between levels of expression of defective 55Kd proteins and cytogenetic alterations suggesting that the conformation of the 55Kd protein may be of importance to the mechanism of induction of damage. The Ad12 E1B proteins are multifunctional, and mutational analysis has identified discrete regions of the molecule which are essential for different processes (Mak and Mak, ; Shiroki et al., 1986). As indicated in Table 5, and

as was determined in this study (Section 3.9), the mutants used in this study have different phenotypes with respect to viral DNA replication, cell transformation, and tumourigenicity of the transformants, and there appears to be no obvious correlation between any of these phenotypes and the ability to induce cytogenetic damage. It therefore appears that the cytogenetic effect may involve other properties of the 55Kd protein.

These findings give rise to a number of questions concerning how the 55Kd protein is able to elicit the induction of chromosomal aberrations. For example, does the 55Kd protein act on its own to induce chromosomal aberrations or does it operate in concert with other viral and cellular factors? Insight into this question may come from results obtained with the recombinant virus, T12E1. One possibility is that the 55Kd protein does act on its own. Thus, in the case of T12E1 it can be concluded that wild type levels of aberrations are not attained because Ad12 E1B gene products are not fully functional in an Ad5 background. This interpretation is in agreement with the observation that T12E1 and similar recombinants although expressing wild type levels of E1 protein have a host range phenotype (Sawada et al., 1988; Jelinek and Graham, unpublished data). On the other hand, the very low levels of damage induced by T12E1 may be

TABLE 4

Biological Properties of Viral Mutants[a]

Virus	Transformation[b]	Tumorigenicity[c]	Cytogenetic damage
pm1542	wt	ND	wt
dl1670	<wt	ND	wt
pm1852	<wt	wt	<wt
dl17	<<wt	-[d]	<<wt
dl42	<<wt	<wt	<<wt
dl1201	<wt	<wt	<<wt
T12E1	wt	ND	<<wt
T1461	wt	ND	-
T1227	wt	ND	-
dl207	<<wt	-	<<wt
in602	-	-	<<wt
del620	-	<<wt	<<wt

a.wt= wild type levels
<wt= reduced (10%) relative to wt
<<wt= reduced (2% or less) relative to wt
ND= not done.
b.Assayed in baby rat kidney cells with virus and/or plasmid (see references in text).
c.Tumorigenicity of transformed cells and/or virus was assayed in weanling rats (see references in text).
d.-, Undetectable.

indicative of the fact that 55Kd on its own is not sufficient to induce wild type levels of damage. It is therefore another possibility that 55Kd interacts with other proteins to attain levels of aberrations comparable to wild type. The fact that mutations in different regions of the 55Kd protein reduce to the same extent the ability of the virus to induce damage suggests that the entire protein may be required for this process. This suggestion would be consistent with the hypothesis that the 55Kd protein interacts with other viral and cellular proteins.

If other proteins do in fact interact with 55Kd to induce chromosomal aberrations what is their source? One protein of viral origin that is known to form a functional complex with the E1B 58Kd protein of Ad5 is the 34Kd protein encoded by the E4 region (Sarnow et al., 1984; Cutt et al., 1987). The Ad12 55Kd protein may have a similar functional association with the Ad12 E4 protein. The idea that 55Kd may interact with other viral proteins is also supported by findings in this study using the mutant pm1542. Despite the fact that pm1542 over-expresses 55Kd by as much as 10 times (Shi-yun Zhang, personal communication), it only shows wild type levels of aberrations, suggesting that an additional viral protein, with which 55Kd interact, may be the rate limiting factor affecting this virus' ability to induce higher

levels of aberrations. A contribution to wild type yields of damage by Ad12 functions other than those encoded by E1 would not contradict results obtained with transfected cells (Durnam et al., 1986), since the efficiency of induction of aberrations cannot be accurately measured in this system. The prospect of a direct interaction between the 55Kd protein and a cellular protein seems less likely. The reason for this is that a cellular protein is present in all samples including that of the recombinant virus, T12E1, as well as cells transfected with Ad12 E1 sequences (Durnam et al., 1986). It therefore does not seem plausible that this type of interaction is involved in the induction of chromosomal aberrations. A third possibility is somewhat more complicated whereby an interaction between the 55Kd protein, another viral protein(s) and cellular protein(s) are required to achieve wild type levels of damage. In such a mechanism, 55Kd might interact with one or a number of other viral proteins to stabilize or activate a cellular protein whose function is to induce chromosomal damage. If the other viral protein is missing or is of the wrong serotype, as in the case of T12E1, then the stabilizing effect is reduced. Additionally, if 55Kd is mutated (cf. dl17), then the ability of this complex to stabilize or activate the cellular protein is also reduced. One example of a concerted effort between

viral proteins to ameliorate a process is that of trans-activation of E2 transcription by adenovirus E1A and E4 proteins. Neill et al. (1990) have identified a direct interaction between the 19Kd protein of E4 and The E2F transcription factor that stabilizes the binding of this factor to the E2 promoter region. In the absence of E4 the E2F factor still binds to DNA with specificity but it forms an unstable complex. E1A also activates E2F binding to the E2 promoter with subsequent E2 transcription. However, together with E4 the two transactivators lead to a much more enhanced activation of E2F than on their own.

Insight into the nature of the protein(s) involved in the cytogenetic effect may be gained from work by Durnam et al. (1986). As described above, using SEM these investigators have shown that the specific damage induced by Ad12 at 17q21-22 consists of an uncoiling of the chromatin. It is therefore conceivable that the protein causing this effect is one whose function is to decondense DNA. The 55Kd protein has not been shown to posses this capability and thus it may interact, either directly or indirectly, with a protein that is able to uncoil DNA. A number of types of proteins have been identified that have the capacity to decondense chromatin, including viral and cellular helicases, helix-destabilizing proteins and topoisomerases.

The results presented in this study are in surprising contrast to those obtained regarding the induction of random cytogenetic damage by Ad5 in HEK cells(Caporossi and Bacchetti, 1990). In the latter case, as already reported for rodent cells (Braithwaite et al., 1983), the expression of exon 1 and of the transactivating domain of E1A appeared sufficient for the induction of damage, and as already mentioned E1B was found to be dispensable. The behaviour of T1227 and T1461 exemplify the differences between the two serotypes of adenovirus. These viruses lack both the Ad5 and Ad12 sequences required for the induction of aberrations by the wild type parents, and as would be expected they are both incapable of inducing cytogenetic damage. These observations suggest that different mechanisms might be involved in the induction of aberrations by the two serotypes of virus, or conversely, that different polypeptides in Ad12 and Ad5 are able to induce cytogenetic damage. Although the Ad12 and Ad5 major E1B proteins seem to fulfill equivalent roles in the lytic cycle, precedents have also been set for differences in their functions. For example, the requirement for Ad12 E1B 55Kd in viral DNA synthesis and in virus or DNA mediated transformation is much more stringent than in the case of Ad5 E1B 58Kd (Byrd et al., 1988; Breiding et al., 1988). In fact, Ad12 E1B 55Kd even appears to be dispensible for

transformation by Ad5 DNA (Graham et al., 1974a,b). Finally, although infection with both serotypes of adenovirus results in stabilization of the p53 cellular protein, the Ad5 58Kd protein binds this protein whereas an association between Ad12 55Kd and p53 has not been observed (Sarnow et al., 1982; Mak et al., 1988).

The idea that the two serotypes of adenovirus each possess a different polypeptide capable of inducing cytogenetic damage is further complicated by the fact that Ad12 is able to induce specific as well as random aberrations while Ad5 is only able to induce random damage. Thus if the function required for the induction of cytogenetic damage has been transposed from E1A to E1B in Ad12 then it has also somehow been altered allowing it to induce damage with specificity. Since the existence of two mechanisms of damage induction, both governed by the expression of the 55Kd protein, would seem unlikely the question of how a single mechanism is able to direct two types of damage remains. One possibility is that the 55Kd protein interacts with a DNA binding protein, such as a transcription factor, which has different affinities for a number of promoters with strongest binding capacity for sequences in Ad12 modification sites. Conversely, the DNA binding protein interacts with promoters common to many genes but has increased affinity for certain

target sequences when it is bound by the 55Kd protein. As the specific sites become saturated the factor binds to other sites. Once again, the association between the 19Kd protein of E4 and the E2F transcription factor demonstrates the way in which complex formation between a viral protein and a cellular DNA binding protein can result in increased affinity for certain DNA sequences (Neill et al., 1990).

The fact that to date an extensive functional analysis of Ad12 E1B 55Kd has not yet been carried out complicates the investigation of possible mechanisms by which 55Kd might induce chromosomal damage. Recent studies (Byrd et al., 1988; Breiding et al., 1988; Mak and Mak, 1990), and those which are up and coming will likely begin to discern additional functional domains of these polypeptides. A 55Kd null mutant is presently being developed in our laboratory which will serve to confirm this protein's involvement in the induction of chromosomal aberrations, as well as to address the possibility of other proteins being involved in this process. Another approach to this question would be to generate mutants which are defective for other early gene(s), such as E4. Finally, an additional way to establish the sufficiency of 55Kd for damage induction is to link its coding sequence to an inducible (metallothionine) or constituitive (beta-actin) promoter and to express it in a human cell on its

own. A coordinated effort among all the studies mentioned above might ultimately identify the mechanism by which Ad12 is able to induce aberrations at both specific and random sites.

A final issue which remains to be addressed is the significance of the specificity of the Ad12 modification sites. The location of U1 and U2 small nuclear RNA and 5s ribosomal RNA genes near chromosome bands specifically damaged by Ad12 is not likely to be a mere coincidence. It has been demonstrated by Lawler et al. (1989) that Ad12 infection of KB cells does not result in stimulation or repression of snRNA gene expression and thus the biological significance of the involvement of these sites in Ad12 infection is unclear. Alternatively, it may be that the specificity of the Ad12 modification sites resides in the target sequences. A prominent feature of U1, U2 snRNA and rRNA gene is their reiterated structure and thus it is a possibility that such sites might be more susceptible to relaxation than other less reiterated sequences. Finally, it may be the environment in which these sequences reside that is of importance. These issues can be addressed by introducing the target genes into other chromosomal sites of human cells as well as into cells of other species, such as rodent cells.

It was apparent in the mock infected cells that there were two waves of cells entering the S phase with approximately 4 hrs between the two peaks of DNA synthesis. The first and second wave began at 8 hrs and 13-14 hrs post serum addition, respectively. Each cycle of DNA replication was approximately 5 hrs in length. The bi-phasic pattern of DNA synthesis was not as apparent for HEK cells infected with either Ad12 wt or mutant dl1201. As expected, infection of HEK cells with Ad12 wt had the effect of reducing cellular DNA replication. However, both waves of synthesis appeared to be equally affected by the virus. The amount of total DNA synthesized, in both the first and second waves of incorporation, was reduced by approximately 40% as compared to mock infected cells. It was difficult to determine the exact reduction in cellular DNA synthesis because a certain amount of the synthesis represented by the curves likely corresponds to viral DNA replication, which is known to occur at approximately 9-12 hrs post infection (Green and Daesche, 1961; Horwitz, 1990). The results obtained for dl1201 were not interpretable since the counts obtained for each sample were, for the most part, below background levels.

To measure the length and frequency of mitosis, HEK cells were synchronized as described in Methods and Materials. 20 hrs after the addition of serum (and every hr thereafter for

5 hrs) colcemid (0.2 ug/ml) was added and the cells were harvested 1 hr later. To determine the effect of Ad12 and dl1201 infection on mitosis the cells were infected with 10 PFU per cell of each virus at 6 hrs post serum addition and treated as described for the mock infected cells. For each time point the mitotic index was calculated and plotted versus hrs post serum addition.

The results of these experiments are given in Figure 20. For all of the treatments the same pattern of entry into mitosis was observed but the mitotic index varied. As was also observed for the S phase in synchronized HEK cells, the cells did not appear to be fully synchronized but rather reached mitosis in two waves with approximately 3-4 hrs between the two peaks of maximum mitotic index. The length of each mitotic wave was approximately 3 hrs, with the first and second wave beginning at 20 and 23 hrs post serum addition, respectively. Infection with either virus did not alter the length of the waves. Ad12 wt infection of HEK cells had the most profound effect on mitotic index. In the first wave the mitotic index was not decreased significantly (89% of mock infected cells) but by the second wave a drastic drop in mitotic index was observed (13% of mock infected cells) suggesting that the cells may have been blocked or retarded from reaching mitosis. These findings would also

suggest that in the second wave cells are more susceptible to damage. Increased sensitivity to damage may be the case since the second population of cells have been exposed to the virus for an additional 3-4 hrs thereby allowing an accumulation of viral gene product(s). To test this hypothesis the frequency of aberrations and percent aberrant cells were determined for the peak of each wave. The results of this analysis showed the two populations of cells to be equally damaged with similar frequencies of aberrations (78-90 aberrations/ 100 metaphases) and aberrant cells (38%). Dl1201 infection also had the effect of reducing mitotic index but to a lesser extent than wild type. Once again, in the first wave the mitotic index was not significantly affected (80% of mock infected cells) but it was decreased to 66% of mock by the second wave.

The results of these experiments indicate that synchronization of HEK cells by serum starvation is incomplete resulting in cells that are passing through the cell cycle in two waves separated by approximately 3-4 hrs. These findings may be indicative of the fact that the cells become blocked at various points in the G1 phase depending on the cell cycle stage that they are in at the time of serum depletion. For example, cells that are in G1 at the time of serum depletion proceed through this stage and are subsequently blocked before

entering DNA replication, that is at the G1/S boundaries. Alternatively, cells that are not in G1 at the time of synchronization will pass through the other cell cycle stages before being arrested at the beginning of G1. Cells are released from the block by addition of 20% FCS and those that were halted at the end of G1 will proceed immediately into S while cells at the beginning of G1 will lag behind. Although unlikely, it is also possible that the two wave pattern may have been caused by the synchronization protocol. It may have been that in changing the medium to 0.5% FCS a residual amount of serum from the growth medium was left behind. A solution which could remedy this situation is to rinse the plates several times with PBS before making a medium change. One way of determining whether residual serum interferes with complete synchronization is to use a different method, such as addition of hydroxyurea to the cells. This chemical blocks cells in the S phase by inhibiting DNA synthesis. The findings in these experiments of essentially two populations of cells (separated by 3-4hrs in the cell cycle) do not affect the data for chromosomal aberrations since samples scored in those experiments consisted of pooled populations of cells collected over the 4 hr interval.

FIGURE 19

Effect of Ad12 wt and dl1201 infection on cellular DNA synthesis. HEK cells were labeled for 30 min with [^{3}H] thymidine at 3 hrs post serum addition as well as every hr between 6-18 hrs post serum addition. Infections with 10 PFU per cell of virus were carried out at 6 hrs after the addition of serum. After the labeling period the cells were harvested and the DNA was precipitated using trichloroacetic acid (TCA). The radioactivity in each sample was quantitated in a Beckman liquid scintillation counter and expressed as counts per minute (cpm). For each time point cpm is plotted versus hrs post serum addition.

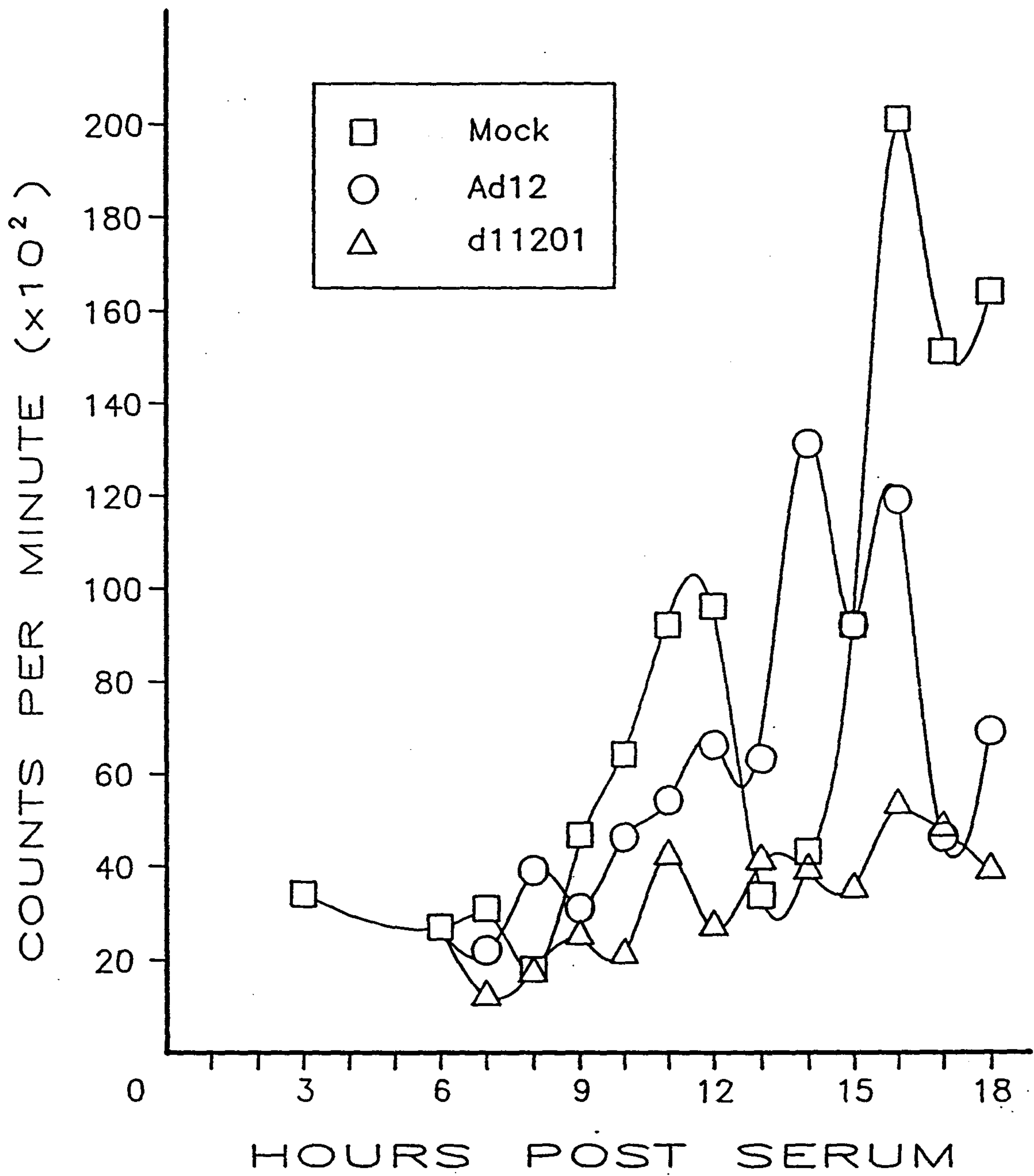
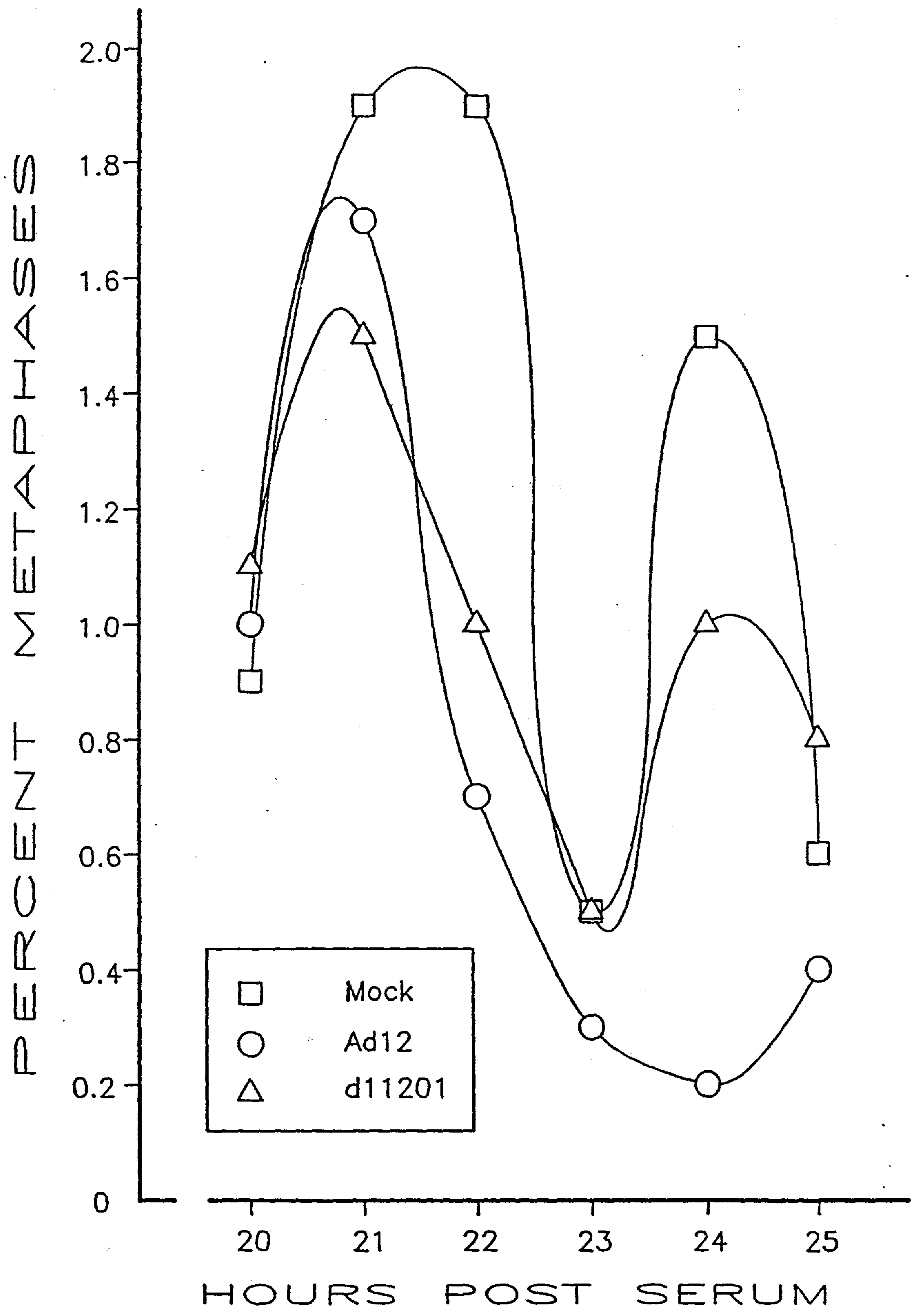

Mock
Ad12
d11201
COUNTS PER MINUTE (x10²)
200
180
160
140
120
100
80
60
40
20
0
3
6
9
12
15
18
HOURS POST SERUM

FIGURE 20

Effect of Ad12 wt and dl1201 infection on length and frequency of mitosis. HEK cells were infected with 10 PFU per cell of Ad12 wt or dl1201 6 hrs post serum addition. 20 hrs after the addition of serum (and every hr thereafter for 5 hrs) colcemid (0.2 ug/ml) was added and the HEK cells were harvested 1 hr later. For each time point the mitotic index was calculated and plotted versus hrs post serum addition.


PERCENT METAPHASES
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
20
21
22
23
24
25
HOURS POST SERUM
Mock
Ad12
d11201

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