ADVANCES IN CANCER RESEARCH

Edited by

GEORGE KLEIN

SIDNEY WEINHOUSE

Vol. 25

ADVANCES IN CANCER RESEARCH

Edited by

GEORGE KLEIN

Department of Tumor Biology
Karolinska Institutet
Stockholm, Sweden

SIDNEY WEINHOUSE

Fels Research Institute
Temple University Medical School
Philadelphia, Pennsylvania

Volume 25—1977

ACADEMIC PRESS New York San Francisco London
A Subsidiary of Harcourt Brace Jovanovich, Publishers

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 52–13360

ISBN 0–12–006625–4

PRINTED IN THE UNITED STATES OF AMERICA

CONTRIBUTORS TO VOLUME 25

Numbers in parentheses indicate the pages on which the authors' contributions begin.

HERMAN FRIEDMAN, *Department of Microbiology and Immunology, Albert Einstein Medical Center, Philadelphia, Pennsylvania* (271)

F. L. GRAHAM, *Departments of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada* (1)

KRISHNA K. JHA, *Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts* (53)

ISAO KAMO, *Department of Microbiology and Immunology, Albert Einstein Medical Center, Philadelphia, Pennsylvania* (271)

DANIEL W. NEBERT, *Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland* (149)

HARVEY L. OZER, *Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts* (53)

ANTHONY E. PEGG, *Department of Physiology and Specialized Cancer Research Center, The Milton S. Hershey Medical Center, College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania* (195)

STEVEN A. ROSENBERG, *Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland* (323)

WILLIAM D. TERRY, *Surgery Branch and Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland* (323)

SNORRI S. THORGEIRSSON,* *Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland* (149)

ISAAC P. WITZ, *Department of Microbiology, The Dr. George S. Wise Center for Life Sciences, Tel Aviv University, Tel Aviv, Israel* (95)

* Present address: Laboratory of Chemical Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

CONTENTS

The *Ah* Locus and the Metabolism of Chemical Carcinogens and Other Foreign Compounds

SNORRI S. THORGEIRSSON and DANIEL W. NEBERT

Formation and Metabolism of Alkylated Nucleosides: Possible Role in Carcinogenesis by Nitroso Compounds and Alkylating Agents

ANTHONY E. PEGG

Immunosuppression and the Role of Suppressive Factors in Cancer

ISAO KAMO and HERMAN FRIEDMAN

Passive Immunotherapy of Cancer in Animals and Man

STEVEN A. ROSENBERG and WILLIAM D. TERRY

BIOLOGICAL ACTIVITY OF TUMOR VIRUS DNA

F. L. Graham[1]

Departments of Biology and Pathology, McMaster University, Hamilton, Ontario, Canada

I. Introduction

There are several advantages to using purified viral DNA to infect cells as opposed to using intact virions. First, and most important, it is possible to effect a practically infinite array of physical, chemical, and biochemical modifications of pure DNA that are not feasible with DNA packaged in (and protected by) the viral capsid. Provided methods exist for introducing pure DNA into cells, the biological effects of these modifications can be examined, and in certain cases new (and, it is hoped, useful) variant forms of viral DNA can be propagated. Clearly, it would be impossible to carry out more than a handful of the elegant experiments popularly referred to as genetic engineering if assays for biological activity of purified viral DNA were not available. Second, it may be possible to infect with DNA cells that are resistant to infection by intact virions as a result of blocks in adsorption, uptake,

[1] Research Scholar of the National Cancer Institute of Canada.

or uncoating. Third, ability to infect cells with purified DNA permits studies on the biological importance of different naturally occurring forms of viral DNA, such as altered structures that might be difficult to detect or to fractionate unless extracted from the virion, or forms of viral DNA that are never encapsulated in virions. And finally, ability to detect infectivity of purified viral DNA indicates a priori that the virions do not contain components, other than DNA, that are indispensable for the initiation of viral replication.

The major disadvantage in using purified DNA to infect cells is that biological activity is usually much lower for "naked" as opposed to encapsulated DNA, presumably as a result, at least in part, of inefficient uptake of pure DNA by animal cells. As will be discussed in the next section, several methods have been developed to enhance the biological activity of viral DNA. Although none of these methods, with perhaps one exception, approaches the efficiency of intact virions for introducing DNA into cells, assays for biological activity of viral DNA have become an extremely important tool in the study of tumor viruses.

For reviews dealing with techniques developed before 1970 and a discussion of biological activity (chiefly infectivity) of viral nucleic acids, the reader is referred to articles by Pagano (1969, 1970), Dubes (1971), and Butel (1973). The present article will deal with more recent studies on biological activity of viral DNA with emphasis on studies dealing with cell transformation rather than infectivity.

The term "transformation" as defined by Fedoroff (1967) refers to changes induced in cells by the introduction of new genetic material, a definition that clearly derives from bacterial genetics. Unfortunately, there is a tendency on the part of tumor virologists to adopt a more restrictive meaning for the expression transformation, i.e., a change to oncogenicity. There are at least two reasons for attempting to reverse this trend. First, one may hope that with the progress being made in the genetics of mammalian cells and with improved methods for infecting them with DNA it will ultimately be possible to demonstrate the acquisition and expression in cultured cells of genetic information from sources other than tumor viruses. Second, even the acquisition of genetic material from tumor viruses may result in stable alterations in cell cultures (other than cell death) which need not lead to oncogenicity. In what follows, the term transformation will be used in its broader sense; where oncogenicity of transformed cells has been demonstrated, this will be explicitly stated.

A list of the possible types of transformation of cultured cells would obviously be endless. A few of the most commonly observed transfor-

mations are changes in morphology, such as the conversion of fibroblast-like cells to epithelioid cells, changes in growth properties, such as growth rate, saturation density, or anchorage dependence, changes in metabolic requirements and in karyotype.

Oncogenic transformation is an event that alters cells in such a way that, after inoculation into a suitable host, they can grow to form a tumor. Oncogenicity is usually associated with several of the following properties of cells in culture: reduced sensitivity to contact inhibition resulting in growth to high saturation densities, reduced anchorage dependence resulting in the ability to grow in suspension, ability to grow in low concentrations of serum, ability to replicate indefinitely.

Most transformation assays are based on the detection of foci of cells having a characteristic morphology, or on the selection of cells having altered growth properties. Since alterations in cell cultures frequently arise spontaneously, it is important in any study on transformation to have a virus-specific marker to distinguish virus-transformed cells from spontaneously arising variants. The most commonly used markers are virus-specific RNA and DNA and viral antigens (usually T antigen), the latter being the most convenient markers for routine screening.

For reviews on transformation, see Black (1968), Macpherson (1970), Pontén (1971), Sambrook (1972), and Butel and Melnick (1972).

II. Properties of DNA Tumor Viruses

The six known groups of DNA viruses, with examples of members whose DNA has been demonstrated to have some form of biological activity, are listed in Table I. It is interesting that as many as four of the six groups contain viruses known to induce tumors either in their natural host or under certain laboratory conditions. Of these four groups, three, the papovaviruses, the adenoviruses, and the herpesviruses, contain members from which biologically active DNA has unquestionably been extracted. In this section a brief sketch is given of some of the properties of these three groups of viruses, concentrating on those aspects that are relevant to the biological activity of their extracted DNAs. For more detailed treatments a number of very useful books or review articles dealing with tumor viruses are available, and the reader is referred to books by Gross (1970), Tooze (1973), and Fenner *et al.* (1974) and to articles by Green (1970), Eckhart (1972), and Rapp and Jerkofsky (1975). In addition, several articles have discussed various aspects of SV40 and polyoma virus (Sambrook, 1972; Levine, 1974; Salzman and Khoury, 1974), adenoviruses (Schlesinger,

TABLE I
BIOLOGICAL ACTIVITY OF EXTRACTED VIRAL DNAS[a]

Group	Member	Oncogenicity of intact virus	Biological activity of extracted DNA: Infectivity	Biological activity of extracted DNA: Transformation of cultured cells	Biological activity of extracted DNA: Tumor induction *in vivo*
Papovaviruses	Polyoma	+	+	+	+[b]
	SV40	+	+	+	NT
	BK	+[b,c]	+[c]	+[c]	NT
	RKV	−[d]	+[d]	NT	NT
	Shope papilloma	+	+[e]	NT	+[f]
	Bovine papilloma	+	−	+[g]	+[g]
Adenoviruses	Ad1	−[h]	+	NT	NT
	Ad2	−[h]	+	+	NT
	Ad5	−[h]	+	+	−[i]
	Ad12	+	NT	+	NT
	SA7	+	+[j]	+[k]	+
Herpesviruses	HSV1	?[l]	+[m,n]	+[n]	NT
	HSV2	?[l]	+[n]	+[n]	NT
	Pseudorabies	NT	+[n]	NT	NT
Parvoviruses	AAV-1	−[p]	+[q]	NT	NT
Poxviruses	Fowlpox	+	?[r]	NT	NT
Iridoviruses	African swine fever	−	+[s]	NT	NT

[a] Examples for which no references are given are well documented and are discussed in the text. In cases where at least one study has reported negative results and no positive results have been reported in the literature, the activity is listed as being absent (−). This is not meant to imply that more extensive studies will not detect the activity in question. Where no studies have been reported in the literature, the activity is listed as not tested (NT). A question mark has been used where conflicting reports have been given, or where the evidence is unclear.

[b] Shah *et al.* (1975).

[c] Van der Noordaa (1976), Takemoto and Martin (1976).

[d] Ito *et al.* (1966).

[e] Not infectious for cells in culture, but intact virions could be detected in DNA-induced tumors (Chambers and Ito, 1964).

[f] Ito (1961a,b), Ito and Evans (1961).

[g] Boiron *et al.* (1965).

[h] Nononcogenic, but induce oncogenic transformation of cells in culture.

[i] Burnett *et al.* (1975).

[j] Burnett and Harrington (1968b), Talas and Butel (1974).

[k] J. P. Burnett, personal communication.

[l] Oncogenicity of HSV1 and HSV2 is not yet firmly established, but oncogenic transformation of cells in culture has been demonstrated.

1969; Philipson and Lindberg, 1974; Philipson *et al.*, 1975), and herpesviruses (Rapp, 1974; Roizman and Furlong, 1974; zurHausen, 1975).

A. PAPOVAVIRUSES

Polyoma and SV40 have been the most intensively studied and are the best characterized of the DNA tumor viruses. The genomes of polyoma and SV40 are physically very similar, both consisting of double-stranded circles of molecular weight around $3 - 3.6 \times 10^6$ daltons (cf. Tooze, 1973). Extraction of DNA from purified virions yields three types of DNA: component I DNA consisting of covalently closed circles with a superhelical structure (usually the predominant form); component II DNA consisting of circles with one or more single-strand nicks resulting in a relaxed or open circle; and component III DNA consisting of double-stranded linear fragments of primarily host DNA (pseudovirion DNA). The relative amounts of component I and II DNA may depend on the extraction conditions (Vinograd *et al.*, 1965); component III DNA is seldom found in SV40 DNA preparations and is found in variable amounts in polyoma DNA (Crawford, 1969).

The three configurations of SV40 and polyoma DNA can be separated by velocity sedimentation through neutral sucrose gradients, components I, II, and III sedimenting at 21 S, 16 S, and approximately 14 S, respectively (cf. Winocour, 1969). Component I DNA can also be separated from components II and III by CsCl buoyant density centrifugation in the presence of ethidium bromide (Bauer and Vinograd, 1968). DNA extracted from SV40 or polyoma virus following repeated passages of the virus at high multiplicities of infection may also contain a large proportion of defective molecules containing deletions, substitutions, and repetitions.

For virus propagation and titration, primary or secondary mouse embryo cells or the mouse 3T3 line are commonly used for polyoma virus, and primary monkey kidney or established monkey cell lines such as

---

[m] Graham *et al.* (1973).

[n] Wilkie *et al.* (1974).

[o] Transfer of the HSV.2 thymidine kinase (TK) to TK$^-$ human cells (Bacchetti and Graham, 1976).

[p] Frequently causes persistent or latent infections, but oncogenicity has not been reported.

[q] Hoggan *et al.* (1968), Boucher *et al.* (1971).

[r] Randall *et al.* (1966).

[s] Adldinger *et al.* (1966), L. Enjuanes and A. Carrascosa, personal communication.

BSC.1, CV.1, or Vero for SV40. Mouse and hamster cells are nonpermissive for SV40 replication, and human cells are semipermissive. In contrast, few, if any, cells seem to be totally nonpermissive for polyoma.

Transformation of cultured cells by polyoma virus, SV40, and most other viruses is usually assayed by one or two basic methods. The original procedure (Vogt and Dulbecco, 1960), still used most extensively, is to infect monolayer cultures with virus, then incubate for periods ranging from one to several weeks (in some cases detaching and reseeding cells at some time after infection). Under appropriate conditions, e.g., culture medium and cell density, foci or colonies of transformed cells are obtained; these are distinguishable from normal cells on the basis of morphology, saturation density, or other growth parameters. A second assay procedure involves reseeding infected cells in soft agar or methylcellulose suspension. Normal cells are unable to grow in suspension whereas many transformed cells divide to form colonies (Macpherson and Montagnier, 1964). Polyoma-induced transformation is usually assayed by the soft-agar suspension method using hamster cells (BHK21-C13 or Nil 2) whereas transformation by SV40 is more frequently assayed on monolayer cultures of mouse cells (3T3).

The papilloma viruses are only slightly larger than polyoma and SV40, containing genomes of around 5×10^6 daltons (Crawford, 1969). Like that of SV40 and polyoma virus, papilloma DNA is a double-stranded covalently closed circle. Although among the papilloma viruses are the first known DNA tumor viruses, including the human wart virus (the only virus known conclusively to induce tumors in man, albeit exceedingly benign ones), work on papilloma viruses has been extremely limited owing to the lack of cultured cells in which they can be propagated. Assays for biological activity of papilloma viruses are usually carried out in animals, although transformation of cultured cells by human papilloma virus (Noyes, 1965) and bovine papilloma virus (Black *et al.*, 1963; Thomas *et al.*, 1964) has been reported.

B. Adenoviruses

For detailed discussions of various aspects of adenoviruses, see reviews by Schlesinger (1969), Green (1970), Philipson and Lindberg (1974), and Philipson *et al.* (1975).

Adenoviruses are larger and more complex than viruses of the papova group, having a genome size in the range 20 to 30 $\times$ 10^6 daltons. Adenoviruses have been isolated from a great many species of animal,

but the best characterized are the human adenoviruses, especially serotypes 2 and 5 and to a lesser extent type 12. At least 31 different serotypes of human adenoviruses have been identified, and these can be divided into subgroups on the basis of hemagglutination tests (Rosen, 1960) or degree of oncogenicity (Huebner *et al.*, 1965). [For a recent discussion of classification of adenoviruses, see Philipson and Lindberg (1974).] Members of the same subgroup have a similar G+C content and a high degree of homology in their DNA; they also induce immunologically cross-reacting T antigens (reviewed by Green, 1970).

DNA extracted from purified adenovirions by conventional procedures (e.g., Pronase, SDS–phenol) is a linear, double-stranded, unnicked molecule, without sticky ends or terminal redundancies [see reviews by Philipson and Lindberg (1974) and Philipson *et al.* (1975)]. It has been pointed out (Watson, 1972; Bellett and Younghusband, 1972) that there are no known DNA polymerases that can completely duplicate such a molecule. That is to say, no mechanism is known for completion of the 5′ ends of the daughter strands of a linear DNA template lacking terminal redundancy. Recently, it has been shown that circular molecules of DNA could be extracted from the avian adenovirus CELO if proteolytic digestion is avoided (Robinson *et al.*, 1973). The DNA appeared to contain a "sticky" protein that could circularize the molecules by linking the two ends or could join together two or more molecules end to end to form concatemers of greater than genome length. Similar findings have been obtained with Ad5 (P. van Wielink, personal communication). Another unusual feature of adenovirus DNA is the presence of inverted repetitions at the ends of the molecule (Garon *et al.*, 1972; Wolfson and Dressler, 1972), which permit the circularization of single-stranded molecules to form circles with "panhandles" of double-stranded DNA. Both the "sticky" protein and the inverted terminal repetitions have been observed in DNA of more than one adenovirus type and are probably a general characteristic of adenovirus DNA. The inverted terminal repetition has also been observed in DNA from the defective parvovirus AAV (adeno-associated virus) (Koczot *et al.*, 1973; Berns and Kelly, 1974). The biological significance of these unusual structural features of adenovirus and AAV DNA is unknown, although a role in DNA replication has been postulated (Garon *et al.*, 1972; Wolfson and Dressler, 1972; Robinson *et al.*, 1973).

Human adenoviruses replicate in cells of human origin, with human embryonic kidney cells, or the established lines HeLa, KB, or Hep-2 the most commonly used for virus propagation and titration. In addition, many other cell types exhibit some capacity to support the replication of human adenoviruses, and probably permissive or semiper-

missive cells are more common than totally nonpermissive cells, at least from mammalian species. The degree of "permissivity" of any particular cell type may vary greatly from one adenovirus serotype to another (cf. Philipson *et al.*, 1975). For example, Syrian hamster cells are nonpermissive for Ad12 but semipermissive for Ad2 and Ad5. Rat cells are nonpermissive for Ad5 and Ad12 but apparently semipermissive for Ad2 (Gallimore, 1974).

Infection of permissive cells with adenoviruses results in the production of often quite large amounts of viral DNA, RNA, and proteins, and large numbers of progeny virus, inevitably causing the death of the host cell. Consequently, transformation by adenoviruses is usually limited to nonpermissive cells (a possible exception is transformation of rat cells by Ad2) in which viral replication is blocked but certain early functions are expressed, among them those which induce T antigen(s). Cells transformed in culture by adenoviruses, like the cells of adenovirus-induced tumors, have a characteristic and rather easily recognized morphology. They are usually epithelioid, are smaller than most cultured cells, and have a relatively small amount of cytoplasm (cf. Philipson and Lindberg, 1974). Cells transformed in culture by the oncogenic adenovirus Ad12 or by weakly oncogenic Ad3 will induce tumors after injection into syngeneic hosts (Freeman *et al.*, 1967a,b) whereas rat cells transformed by the nononcogenic serotypes Ad 1, 2, and 5 will not induce tumors (McAllister *et al.*, 1969; Graham *et al.*, 1974b) unless the recipient animals are immunosuppressed (Gallimore, 1972).

Of considerable value for studies on transformation by adenoviruses was the observation (Freeman *et al.*, 1967a,c) that adenovirus transformed cells had a selective advantage over normal cells in medium containing reduced concentrations of calcium ions. Transformation assays could be improved in efficiency, and the time required for growth of colonies reduced, by incubating cultures in low calcium ion medium after exposure of cells to adenoviruses. Their distinctive morphology and the ability to replicate in reduced calcium ion concentrations are two of the main criteria for distinguishing foci of adenovirus-transformed cells from normal cells or spontaneously arising variants. Once colonies are isolated and subcultured, the cells can be tested for the presence of the appropriate adenovirus T antigen and ultimately for the presence of viral RNA and DNA.

C. Herpesviruses

The largest viruses that will be discussed in this article are the herpesviruses. These contain a genome approximately 10^8 daltons in

size (Becker *et al.*, 1968; Kieff *et al.*, 1971), thus large enough to code for approximately 100–200 average-sized proteins. Certain of the herpesviruses are the only DNA tumor viruses known to cause malignant neoplasms in their natural hosts, but, presumably because of their complexity, the herpesviruses have until recently been studied much less intensively than some of the papovaviruses or adenoviruses. However, herpesviruses have commanded increasingly close attention in the last few years because of evidence that Epstein–Barr virus may be the causative agent in certain human malignancies (reviewed by Klein, 1973) and because of seroepidemiological studies linking Herpes simplex virus (HSV) type 2 to human cervical carcinoma (reviewed by Rapp, 1974). The brief discussion following below, as well as the later discussion on biological activity of herpesvirus DNA, are concerned mainly with HSV.

The herpesvirus genome is a double-stranded linear DNA molecule like that of the adenoviruses but containing single-strand nicks or alkali-labile bonds (Kieff *et al.*, 1971; Wilkie, 1973). Molecules of HSV DNA can circularize following partial digestion with exonuclease III (Sheldrick and Berthelot, 1974) or λ exonuclease (Grafstrom *et al.*, 1974), suggesting the presence of redundant termini. In this respect, HSV DNA seems to be quite different from adenovirus DNA and the replication of HSV DNA would not be expected to have any unusual features, at least as far as completion of the 5′ ends of newly synthesized daughter strands is concerned.

Few if any mammalian cells seem to be totally nonpermissive for HSV replication, and since HSV is highly cytolytic any demonstration of transforming activity requires some method of blocking infectivity. Since the target size of viral DNA sequences required for transformation is less than that of sequences required for infectivity, UV or photodynamic inactivation of HSV will reduce infectivity at a greater rate than transforming activity. This rationale has been used to demonstrate transformation of mouse, hamster, rat, and human cells by HSV.1 and HSV.2 as well as human cytomegalovirus, another member of the herpesvirus group (Duff and Rapp, 1971a,b, 1973, 1975; Munyon *et al.*, 1971; Albrecht and Rapp, 1973; Rapp *et al.*, 1973; Kutinová *et al.*, 1973; Boyd and Orme, 1975).

Another approach used successfully to obtain transformation of cells in culture is to infect cells with temperature-sensitive mutants defective in lytic replication but not in transformation, and this has led to the isolation of HSV-transformed mouse, hamster, and rat cell lines (Macnab, 1974; Takahashi and Yamanishi, 1974; Kimura *et al.*, 1975; Hughes and Munyon, 1975). The characterization of HSV-transformed

cell lines is still at a rather preliminary stage. The morphology of HSV-transformed cells is variable with foci of both epithelioid and fibroblast cells observed. Oncogenicity of transformed hamster cells has been demonstrated (Duff and Rapp, 1971a,b 1973), but only limited success has been reported in attempts to induce tumors in animals inoculated directly with HSV (Nahmias *et al.*, 1970; Munoz, 1973). Consequently, tumors induced by transformed cells cannot be compared to tumors induced directly by HSV, nor can a comparison be made between cells transformed by inactivated virus and cells cultured from virus-induced tumors.

III. Assays for Infectivity of Viral DNA

Unaided, the uptake and expression of exogenous nucleic acids in animal cells seems to be a very inefficient process. Consequently, attempts to assay biological activity (usually infectivity) of viral nucleic acids have led to the development of a number of methods for introducing nucleic acids into cells and detecting their expression. A brief description of infectivity assays is given in this section, with emphasis on recent studies relating to infectivity of viral DNA.

A. Hypertonic Saline Method

This technique was originally developed as a method for detecting infectivity of poliovirus RNA (Koch *et al.*, 1958; Alexander *et al.*, 1958). It was shown to be suitable for the detection of polyoma virus DNA infectivity by DiMayorca *et al.* (1959) and later by Weil (1961) who improved and standardized the procedures for extracting and assaying polyoma virus DNA. The hypertonic saline technique, whose mechanism of action is largely unknown, basically consists of exposing cells to viral DNA or RNA in hypertonic saline solutions (usually in the range 0.5–1.0 *M* NaCl). For more detailed discussions of this technique, see Colter and Ellem (1961) and Pagano (1970). This type of assay has now been largely superseded by newer more efficient methods, at least for the detection of infectivity of viral DNA. The hypertonic saline technique has been used by a number of workers to study infectivity of both polyoma (Weil, 1961; Crawford *et al.*, 1964; Bourgaux *et al.*, 1965; Winocour, 1967; van der Eb and Cohen, 1967) and SV40 DNA (Gerber, 1962; Black and Rowe, 1965a). Specific infectivities in the range 1000–3000 PFU/μg have been reported for polyoma and SV40 DNA (Bourgaux *et al.*, 1965; Black and Rowe, 1965a; Winocour, 1967).