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A NOVEL GENE TRANSFER SYSTEM FOR MAMMALIAN CELLS

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A NOVEL GENE TRANSFER SYSTEM

FOR MAMMALIAN CELLS

by

Steve N. Slilaty

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF CELLULAR
AND DEVELOPMENTAL BIOLOGY

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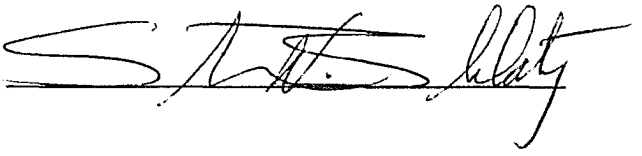
SIGNED: 

TO MOM & DAD

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ABSTRACT

Productive infection of mouse cells with polyoma virus yields mainly two types of particles: Complete virions and empty capsids. Empty polyoma capsids have been shown to be capable of interacting with DNA, *in vitro*, to form what has been referred to as polyoma-like particles (PLP). The particles are stable in high concentrations of salt and contain DNA protected by the capsid against the action of pancreatic DNase. The development of PLP into a gene transfer vehicle is the subject of the investigations described in the present dissertation. The approach has been to first, characterize the process of PLP formation and second, determine whether the genetic information contained in a specific DNA fragment and assembled into PLP *in vitro* can be transferred to cells and subsequently be expressed.

In terms of PLP characteristics, the experimental results described in this dissertation show that the DNA extracted from PLP is heterogeneous in size. It has a mean molecular weight of 1.2×10^6 with a standard deviation of $\pm 0.5 \times 10^6$. In addition, analysis of PLP DNA with restriction endonucleases revealed that a specific primary sequence or higher order structure is not required for PLP

formation. Either linear, circular or supercoiled polyoma DNA, as well as, single-stranded DNA, rRNA and the synthetic homopolymers poly(dA).poly(dT) and poly(dG).poly(dC) can be used for PLP formation.

Transfer of genetic information by PLP has been accomplished by using a restriction fragment containing the transforming sequences of polyoma DNA as a model gene. This fragment of polyoma DNA, which consists of 1,831 base pairs (approximately 1.2×10^6 daltons) and extends clockwise from the BclI site to the EcoRI site on the conventional polyoma map, causes the induction of the transformed phenotype in rat cells grown in culture. Infection of rat F111 cells by PLP, containing this DNA fragment, results in DNA-mediated oncogenic transformation of the cells as indicated by the formation of dense foci. This gene transfer activity of PLP is shown to be 50 to 150 times more efficient than the widely used calcium phosphate coprecipitation method of introducing DNA into mammalian cells.

CHAPTER 1

INTRODUCTION

Gene Transfer

Gene transfer studies provide some of the basic methodology necessary for investigating fundamental questions of molecular biology including the control of gene expression in mammalian cells and the functional consequences of DNA sequence organization. The same studies might also provide the experimental basis for the development of a gene therapy technique for treatment of genetic diseases at the level of the intact animal (e.g., Williamson, 1982; Motulsky, 1983). Currently, genes are introduced into cells by use of physical or biochemical methods. Physical methods involve either the mechanical microinjection of DNA into oocytes (Mertz and Gurdon, 1977; Kressman et al., 1978) or somatic cells (Diacumakos, 1973; Grassmann and Graessman, 1976; Capecchi, 1980) by use of elaborate micromanipulation equipment or the application of short (5-10 μs) electric pulses (5-10 kV/cm) to a suspension of cells and DNA (Wong and Neumann, 1982). The biochemical methods, on the other hand, are more extensive.

They include the fusion of recipient cells with donor microcells (Ege and Ringertz, 1974; Schor, Jhonson, and Mullinger, 1975; Fournier and Ruddle, 1977), RNA-loaded erythrocytes (Rechsteiner, 1976), or Escherichia coli (E. coli) protoplast (Schaffner, 1980; Sandri-Goldin et al., 1981); incubation of cells with DNA in the presence of facilitating agents such as polyornithine (Farber, Melnick, and Butel, 1975) or diethylaminoethyl-dextran (DEAE-dextran) (McCutchan and Pagano, 1968; Lai and Nathans, 1974; Sompayrac and Dana, 1981) or as a coprecipitate with calcium phosphate (Graham and Van der Eb, 1973; Wigler et al., 1979; Loyter, Scangos, and Ruddle, 1982). Other biochemical gene transfer methods have made use of calcium phosphate to coprecipitate and transfer metaphase chromosomes (McBride and Ozer, 1973; Spandidos and Siminovitch, 1977; Miller and Ruddle, 1978) or DNA encapsidated, in vitro, into λ heads (Ishiura et al., 1982); liposomes (Fraley et al., 1980; Schaefer-Ridder, Wang, and Hofschneider, 1982) or viral vectors assembled in vivo with the aid of coinfecting helper virions (Mulligan, Howard, and Berg, 1979; Goff and Berg, 1979; Sarver et al., 1981; DiMaio, Treisman, and Maniatis, 1982; Tabin et al., 1982).

Biochemical gene transfer methods generally rely on the use of a vector or an agent that has two important

characteristics: An ability to protect the DNA to be transferred from hydrolysis by nucleases present in the extracellular milieu and some specificity for interaction with the mammalian plasma membrane to result in the uptake of the DNA alone or of the DNA-vector complex. In microcell fusion for example, the genetic material to be transferred is protected by a plasma membrane which also serves as an efficient vector for fusion with recipient cells. Microcells are obtained by treatment of donor cells with colcemid, an agent that induces micronucleation. Enucleation of micronucleated cells is then accomplished by centrifugation of attached cells, cell-side-down, in the presence of cytochalasin B. This results in the release of structures containing limited amount of genetic material packaged in a micronucleus which is surrounded by a rim of cytoplasm and an intact plasma membrane (Ege and Ringertz, 1974; Schor et al., 1975). These structures which have been termed "microcells" are then used in fusion experiments with recipient cells whereby an undefined amount of genetic material is transferred.

The principle of DNA protection by a membranous structure followed by membrane-membrane fusion has been applied in several other approaches to biochemical gene transfer experimentation. Erythrocyte and liposome vectors rely on the entrapment of genetic material by what might be

considered a lipid vesicle. Similarly, the E. coli protoplast, generated by lysozyme digestion of the cell wall of E. coli cells harboring (in addition to the cellular chromosome) the desired DNA on a chimeric plasmid, might be considered a large lipid vesicle already containing the genetic material to be transferred.

Other methods of biochemical gene transfer have achieved DNA protection either by the use of polycationic polymers which tightly bind to the negatively charged phosphodiester backbone of the DNA molecule to prevent nuclease action or by entrapping the DNA into calcium phosphate precipitation crystals. In both cases, in addition to protection, the agents facilitate DNA entry into cells in culture perhaps by neutralization of the negative charges on the DNA in the case of the polycations DEAE-dextran and polyornithine and by phagocytosis enhancement when calcium phosphate precipitation crystals are present in the culture medium.

Another way of achieving protection and transfer capability has been accomplished by splicing genes into deleted simian virus 40 (SV40) (Mulligan et al., 1979; Goff and Berg, 1979), papilloma virus (Sarver et al., 1981; DiMaio et al., 1982), or Moloney leukemia virus (MLV) (Tabin et al., 1982) genomes, transfecting them into cells with calcium phosphate and allowing intracellular assembly

of these recombinant molecules into viral particles to proceed with the aid of coinfecting helper virions. The progeny, which contains recombinant particles and particles of the helper type, is then used to infect cells in which expression of the spliced gene is desired.

With the exception of the use of the calcium phosphate method for in vitro transfer of genetic information to bone marrow cells and their reinjection into the donor animal (Cline et al., 1980), and perhaps liposomes, the foregoing methods have limited potential for gene transfer to whole animals. On the other hand, in terms of gene transfer to cells in culture, the most extensively applied method is calcium phosphate coprecipitation. The other methods have not gained widespread use because they require highly specialized equipment and/or techniques. The efficiency of gene transfer by calcium phosphate coprecipitation, however, is in the range of spontaneous mutation rates making it difficult in some cases to distinguish between revertant cells and cells that have acquired the exogenous genetic material (Pellicer et al., 1980). In addition, the use of carrier DNA with calcium phosphate might interfere with a clear understanding of the mechanisms of gene integration and/or expression in the recipient cell. Thus, a gene transfer system consisting of the desired purified gene

protected by the coat of a polyoma virus that can be easily assembled in a cell-free environment should offer definite advantages including elimination of carrier DNA, improved efficiency and potential for use in studies of gene transfer to intact experimental animals.

Polyoma Virus

The papovavirus group (taxonomic family: Papovaviridae) consists of the papilloma and polyoma viruses (taxonomic genera: Papillomavirus and Polyomavirus). The first two letters from these two genera names, in addition to the first two letters from the early name of simian virus 40 (SV40), "vacuolating agent", were used to construct the group name prefix "papova" (Fenner, 1976). Papovaviruses are small (40-55nm diameter) DNA-containing viruses that replicate in the nucleus of mammalian cells. Depending on the host they infect, papovaviruses may be tumorigenic or may produce lytic, latent, or chronic infections (Salzman and Khoury, 1974; Tooze, 1980). Lytic infections are characterized by rapid viral replication followed by release of high levels of infectious particles. This is usually accompanied by severe clinical symptoms. Chronic infections on the other hand, are associated with low levels of viral replication

and release with little or no clinical consequences. Unlike lytic and chronic infections, latent infections involve a dormant presence of the virus, perhaps, in an integrated form into the host genome. While it is possible under some conditions to trigger the latent virus to undergo a lytic cycle, the host of a latent form of a viral infection is generally asymptomatic.

Polyoma virus was first observed by Ludwik Gross as an activity that specifically induced salivary gland (parotid) adenocarcinomas in Ak mice. Gross termed this activity the "parotid agent" (Gross, 1953a,b; Gross, 1955). Subsequent studies of the "parotid agent" showed that, in addition to parotid tumors, the agent occasionally induced a variety of other tumors including adrenal, thymic, mammary gland, renal and liver carcinomas (Stewart, Eddy, and Borgese, 1958). Because of the ability of this agent to transform so many different types of cells the name "polyoma virus" was proposed (Stewart et al., 1958) and is now universally accepted.

Polyoma virus, which infects (its natural host) mouse cells productively and causes oncogenic transformation in rat and hamster cells, is a member of the small-sized subgroup of the papovaviruses. Other members of this subgroup (genus: Polyomavirus) include SV40 and the ubiquitous human papovaviruses BK and JC. BK virus has

been associated with urinary tract infections and JC virus with progressive multifocal leukoencephalopathy (Tooze, 1980). The etiology of these agents, however, has only been observed in immunologically weakened individuals. Like BK and JC, SV40, which replicates in monkey cells, is a typical polyomavirus. In size, gross morphology, and basic biophysical properties SV40 virions are indistinguishable from the prototype polyoma virus. In addition, SV40 and polyoma have virtually identical capsid structures and are considered to assemble via similar mechanisms. For these reasons, evidence related to the architecture or assembly obtained for one virus is generally considered pertinent to the other. Therefore, where relevant, data from the SV40 literature will be discussed in the remainder of this dissertation.

Structure of Polyoma Virus

The construction of an isodimensional shell around a central point from a set of identical subunits can most economically be accomplished if icosahedral symmetry is employed (Casper and Klug, 1962). With this type of symmetry, the ratio of the number of required domains to the surface area covered is smallest (Casper and Klug, 1962). As tested by model building, the theory of

icosahedral shell geometry predicts important structural properties relevant to the architecture of spherical viruses (Casper and Klug, 1962; Klug and Finch, 1965). For example, an icosahedral shell will contain twenty equilateral triangular facets and twelve apexes. The shell is constructed from a number of morphological subunits (capsomeres) that are either six-coordinated (hexavalent) or five-coordinated (pentavalent). The notion of quasi-equivalent bonding, in relation to the construction of capsomeres from identical subunits, requires that the six-coordinated capsomeres be hexamers and the five-coordinated capsomeres be pentamers. The necessary number of morphological subunits is 10(T-1) hexavalent plus 12 pentavalent subunits. The symbol, T, is the triangulation number and is equal to pf^2 where p=1,3,7,13,19,21,31,37... and f=1,2,3,4... . If p is greater than or equal to 7, then the icosahedron is skewed and therefore must exist either in a right-handed (dextro or d) or a left-handed (levo or l) configuration. When at least one pentavalent capsomere is observed (two for unequivocal establishment) and the particle is isometric, then the subunit arrangement must be icosahedral. The ability of the subunits to undergo self-assembly into a shell of definite size is an additional property of icosahedral symmetry.

In attempting to solve the surface structure of polyoma virus, the foregoing principles have been applied to the analysis of electron micrographs of viral particles completely enveloped with stain and adsorbed to holey carbon films to allow for tilting and two-side observation (Klug, 1965; Finch, 1974). The presence of pentavalent capsomeres and the results from tilting experiments established that polyoma virus consists of 72 morphological subunits or capsomeres arranged on an icosahedral surface lattice with a triangulation number T=7d. In order for the quasi-equivalent bonding specificity to be conserved, a T=7 icosahedral shell requires the presence of 420 structural units in the form of 60 hexavalent hexamers and 12 pentavalent pentamers (Casper and Klug, 1962; Klug and Finch, 1965). On the basis of this, and the observation that polyoma virions contain 380 copies of the major capsid protein VP1 (43,000 daltons) and 60 copies of the other virus coded capsid proteins VP2 (35,000 daltons) and VP3 (23,000 daltons) (Tooze, 1980) it was proposed that VP1 is the constituent of the 60 hexamers (360 molecules) and VP2 or VP3 or both constitute the 12 pentamers (60 molecules) (Finch, 1974). Recent electron density maps from x-ray diffraction studies of polyoma capsid crystals at 22.5 angstrom resolution show, however, that both the hexavalent and the pentavalent morphological units are identical in

size (Rayment et al., 1982). This, in conjunction with phase refinement analyses and the fact that the capsid preparations studied contained little or no VP2 and VP3 molecules (Murakami et al., 1968), led to the conclusion that all of the polyoma morphological subunits consist of VP1 pentamers (Rayment et al., 1982). The earlier observation that the pentavalent morphological subunits are about the same size as the hexavalent subunits and that density differences were not evident between the two types of subunits (Finch, 1974) are consistent with this conclusion though a 60-hexamer-12-pentamer model was proposed (Finch, 1974). Construction of five- and six-coordinated pentameric capsomeres from the same protein subunit requires that the protein subunit alters its bonding specificity between pentavalent and hexavalent capsomeres. Changes in bonding specificity, however, are not consistent with the quasi-equivalence premise of the theory of icosahedral shell geometry (Casper and Klug, 1962). On the other hand, the finding that VP1 undergoes post-translational phosphorylation (Ponder, Robbins, and Crawford, 1977) and that it can be resolved into several different species by isoelectric focussing (Bolen et al., 1981) could provide the necessary variability in the bonding properties of the same polypeptide chain to account for its ability to form pentamers that can be either

hexavalent or pentavalent.

Thus, the emerging picture of the polyoma capsid appears to be a structure consisting of 72 pentomeric capsomeres composed of 360 molecules of VP1. Each capsomere is about 80 angstroms high and about 85 angstroms in diameter and contains a central hole that is approximately 40 angstroms in diameter at the surface and tapers shut 15 angstroms in. About 40 angstroms in from the surface, the basal part of the capsid is reached. At this level, the capsomeres are in direct contact with each other (Rayment et al., 1982) thereby forming an essentially sealed structure. The capsomeres are arranged on a T=7d icosahedral surface lattice. The icosahedron envelops the 5,292 base pair (Soeda et al., 1980; Tooze, 1980), covalently closed circular genome of polyoma virus in the form of a nucleoprotein complex. The term, nucleoprotein, rather than nucleohistone was used, because the absence of VP2 and VP3 from the viral shell necessitates that they occupy an internal position in the mature virions. Indeed, for the closely related papovavirus, SV40, it has been demonstrated that when virions are disrupted at pH 9.8 virtually all of the VP2 and VP3 molecules are found to be associated with the nucleohistone core (Christiansen et al., 1977). Furthermore, in addition to VP2 and VP3, the nucleohistone complex obtained by dissociation of polyoma

virus with ethyleneglycol-bis-N,N'-tetraacetic acid (EGTA) and dithiothreitol (DTT) has been reported to contain several copies of VP1 molecules (Brady, Winston, and Consigli, 1978). Thus, the polyoma DNA genome, which is complexed with the four core histones (H2a, H2b, H3 and H4) of host origin (Frearson and Crawford, 1972; Lake, Barban, and Salzman, 1973) to form approximately 24±2 nucleosomes (Cremisi et al., 1976; Ponder, Crew, and Crawford, 1978), is further complexed with VP2 and VP3, as well as, some VP1 molecules to form the core of mature virus particles. This core, without any associated VP1, VP2 and VP3 molecules is approximately 300 angstroms in diameter (Christiansen and Griffith, 1977; Muller et al., 1978). Since mature virions have a diameter of about 490 to 500 angstroms (Adolph et al., 1979; Rayment et al., 1982) and the pentamers forming the shell are about 80 angstroms high (Rayment et al., 1982) a 15 to 20 angstrom space surrounding the nucleohistone core, in addition to internucleosomal regions, remains available for the core-associated VP1, VP2 and VP3 molecules.

The foregoing picture of the structure of polyoma virus suggests a novel approach for the <u>in vitro</u> assembly of polyoma virions from individual viral components. Previous attempts at self-assembly of polyoma virus from dissociated virions in a cell-free system have either been

unsuccessful (Friedmann, 1971) or resulted in extremely low yields (0.05%) of infectious particles (Brady, Kendall, and Consigli, 1979). The suggested new approach involves the sequential addition of individually purified viral proteins to purified viral DNA to construct the viral core first, and the capsid around this core last. This might be accomplished as follows: First, supercoiled polyoma DNA is incubated with the four core histones (H2a, H2b, H3 and H4) to form the viral minichromosome (Griffith, 1975). Second, the VP2 and VP3 proteins, in addition to some VP1 molecules, are added in order to convert the minichromosome into the viral nucleoprotein core. Third, capsomeres, preassembled from purified VP1 molecules or isolated as capsomeres, are added to the cores to generate complete virions by coalescence around the preformed cores. The potential usefulness of this approach remains to be tested.

Polyoma Empty Capsids

When polyoma virus, produced in primary mouse embryo cells, is centrifuged to equilibrium in CsCl, two major bands appear (Winocour, 1963). One has a density of 1.339 g/cm^3 and represents complete virions. The other, at 1.297 g/cm^3, consists of empty capsids (Winocour, 1963; Crawford, Crawford, and Watson, 1962). Empty capsids are

devoid of nucleic acid (Crawford et al., 1962; Crawford and Crawford, 1963; Murakami et al., 1968) and histones (Frearson and Crawford, 1972; Aposhian, Thayer, and Qasba, 1975). They are composed of 360 molecules of the major viral coat protein, VP1 (43,000 daltons) (Rayment et al., 1982). These molecules are arranged into 72 pentamers with an overall icosahedral structure similar to that of complete virions (Adolph et al., 1979; Rayment et al., 1982). Polyoma empty capsids containing VP2 and VP3 in approximately the same proportion as complete polyoma virions can also be isolated (Aposhian et al., 1975; Finch and Crawford, 1975). It is important to point out that the polyoma empty capsids used in the experiments described in this dissertation have been prepared according to Aposhian et al., (1975) and do contain the VP2 and VP3 proteins. The presence of empty capsids has been reported in a number of other virus and bacteriophage preparations (Casjens and King, 1975).

The search for a biological role for empty capsids has led to their implication as assembly intermediates. For the bacteriophages T3, T4, T7, λ, and P22, and for poliovirus and adenovirus, empty capsids have been shown to be intermediates in morphogenesis (Casjens and King, 1975). With respect to the small DNA tumor viruses, however, the biological significance of empty capsids has not been

clarified. For example, when the viral coat proteins were the target of pulse-chase radiolabelling experiments, the results suggested that the capsid proteins first form an empty shell which in turn encapsidates the viral genome (Ozer, 1972; Ozer and Tegtmeyer, 1972). Precise analysis of these experiments, however, is difficult due to ambiguities regarding the structural stability of the various assembly intermediates under the experimental conditions used. Recently, experiments employing more gentle nucleoprotein extraction procedures, have shown that a pulse radiolabel in the viral DNA or proteins or both can be chased to mature virions via several steps of increasing protein to DNA ratio in the viral nucleoprotein complexes analysed (Garber, Seidman, and Levine, 1978; Coca-Prados and Hsu, 1979; Baumgartner, Khun, and Fanning, 1979; Fanning and Baumgartner, 1980; Garber, Seidman, and Levine, 1980; La Bella and Vesco, 1980). In addition, studies on the stability of SV40 nucleoprotein complexes, extracted from infected cells, suggest that empty capsids result from the disruption of immature virions in solutions containing high concentrations of salt (Garber et al., 1978; Fernandez-Munos, Coca-Prados, and Hsu, 1979; Seidman, Garber, and Levine, 1979; Coca-Prados and Hsu, 1979; Fanning and Baumgartner, 1980; Jakobovits and Aloni, 1980; Boyce et al., 1982). This, in conjunction with the

evidence from biochemical characterization of these complexes (Christiansen et al., 1977; Brady et al., 1978; Coca-Prados and Hsu, 1979; Fernandez-Munos et al., 1979; Garber et al., 1980; La Bella and Vesco, 1980) along with *in vitro* reassembly studies (Brady et al., 1979) provide substantial evidence that empty capsids are not involved in papovavirus morphogenesis and that virus assembly proceeds via the coalescence of capsid proteins around a nucleohistone core (Garber et al., 1978; Coca-Prados and Hsu, 1979; Fernandez-Munos et al., 1979; Baumgartner et al., 1979; Garber et al., 1980; Coca-Prados, Vidali, and Hsu, 1980; Fanning and Baumgartner, 1980; Jakobovits and Aloni, 1980).

Despite the uncertainty surrounding their function in the morphogenesis of polyoma virus, polyoma empty capsids provide an interesting system for studying: (i) the mechanisms of interaction between a protein superstructure and DNA, (ii) the physical and biochemical events associated with *in vitro* DNA packaging, and (iii) the feasibility of using them to develop a vehicle capable of efficiently transferring nucleic acids across the mammalian cell membrane.

Polyoma-Like Particle

Aposhian et al., (1975) reported that highly purified empty polyoma capsids bind polyoma DNA in a cell-free system to form a DNA-empty capsid binding complex. This binding complex however was not stable in high concentrations of salt (1.0 M NaCl) and its DNA was not protected against hydrolysis by pancreatic DNase. Similar observations using empty adenovirus capsids and double stranded DNA have been reported by Tibbetts and Giam (1979). Although the DNA of the adenovirus capsids-DNA complex was DNase sensitive, the complex was resistant to dissociation by treatment with high salt concentration. Barr, Keck, and Aposhian (1979) reported conditions for the cell-free formation of polyoma-like particles (PLP) from polyoma empty capsids and polyoma DNA. These particles were shown to be stable in 2.39 M CsCl and to contain a DNA fragment protected by the polyoma capsid from the action of pancreatic DNase. As compared to the 240S sedimentation coefficient of complete polyoma virions, PLP were shown to have a sedimentation coefficient (in sucrose at 4°C) of 190S (Barr et al., 1979). The buoyant density of PLP in CsCl was shown to be 1.32 g/cm^3 (Barr et al., 1979), which is intermediate between complete virions (1.34 g/cm^3) and empty capsids (1.30 g/cm^3). The DNA extracted from PLP was shown (Barr et al., 1979) to sediment at 11S,

a value corresponding to a double stranded molecular weight of approximately 1.1 x 10^6 as calculated by the method of Studier (1965). This method of DNA molecular weight determination, however, does not provide sufficient information concerning the possible presence of microheterogeniety in size. Such information can be readily obtained by use of agarose gel electrophoresis.

Present Study

In order to develop the PLP system into a gene transfer vehicle, the experimental approach involved the design of two major sets of experiments. The initial set was aimed at characterizing the PLP formation reaction in order to determine how the system might be applied to the transfer of genetic information to mammalian cells in culture. In these experiments, first, the molecular weight of PLP DNA was determined by agarose gel electrophoresis. Second, the specificity of possible binding sites on the polyoma empty capsids for a particular region of the polyoma genome was examined by the use of restriction enzymes. Third, the effect of nucleic acids structure on PLP formation was studied by carrying out PLP reactions using nucleic acids differing in their primary, secondary, or tertiary structure. Fourth, the effect of the size

(number of base pairs) of the DNA substrate on the efficiency of PLP formation was investigated. Finally, the effects of such factors, normally included in the PLP reaction mixtures, as bovine serum albumin and sodium chloride on the extent of PLP formation was examined.

In the second set of experiments, a DNA fragment that has measurable biological activity was used to construct PLP which in turn was used to infect appropriate cell cultures. Expression of that biological activity was then used to indicate whether gene transfer by PLP has occurred. The DNA fragment that was employed in these experiments is the transforming region of the polyoma genome (Novak, Dilworth, and Griffin, 1980; Chowdhury et al., 1980; Bastin, Bourgaux-Ramoisy, and Bourgaux, 1980; Hassell et al., 1980). The smallest restriction fragment containing this region, consists of 1,831 base pairs (1.2 x 10^6 daltons) and extends clockwise from the BclI to the EcoRI site on the conventional polyoma map (see Figure 10). Expression of this fragment has been shown to result in the induction and maintenance of the transformed state in rat cells growing in culture (Novak et al., 1980).

CHAPTER 2

MATERIALS AND METHODS

Virus and Empty Capsids

The small plaque variant of the Toronto strain of polyoma virus was produced by infecting primary mouse embryo cells in culture, at a multiplicity of infection of approximately one plaque forming unit per cell. Crude virus was harvested by the freeze-thaw and receptor-destroying enzyme (neuraminidase, Worthington Biochemical Corp.) method of Crawford (1962) and stored at -20°C. Freezing and thawing several times results in complete cell lysis and release of virus into the medium. Treatment of the lysate with receptor destroying enzyme eliminates neuraminidase sensitive adsorbtion of viral particles to cellular membrane fragments. Before use, empty capsids and virions were separated by the method of Winocour (1963) and further purified as described by Aposhian et al., (1975). Briefly, crude extracts were centrifuged in the Sorvall SS-34 rotor at 10,000 rpm and 20°C for 15 minutes to pellet cellular debris. Virus particles, present in the supernatent, were pelleted by

centrifugation in the Beckman Type 30 rotor at 25,000 rpm and 20°C for 3 hours then subjected to equilibrium centrifugation in CsCl density gradients. The peak fractions of virions and empty capsids were pooled and further purified by two rounds of equilibrium centrifugation in CsCl followed by sedimentation through a 10-22% sucrose gradient. Purified virions and empty capsids were dialyzed exhaustively against E buffer (10mM Tris-HCl, pH 7.5, 100mM NaCl, 1mM EDTA). It is pertinent to note that empty polyoma capsids derived from polyoma infected baby mouse kidney cells (Winocour, 1963) undergo PLP formation to a greatly diminished extent as compared to those obtained from primary mouse embryo cells. The reason for this is unclear at the present time.

DNA Preparation

Polyoma [methyl-^{3}H]dT-DNA (1.54×10^{5} cpm/μg) was produced by infecting Swiss mouse 3T3 cells with plaque purified virus (large plaque strain) at a multiplicity of infection of 2.5 plaque forming units per cell. Supercoiled polyoma DNA was extracted by the method of Hirt (1967) and purified by centrifugation to equilibrium using two rounds of CsCl-ethidium bromide density gradients (Radloff, Bauer, and Vinograd, 1967). DNA was extracted

with isopropanol to remove the ethidium bromide and dialyzed exhaustively against DNA buffer (10mM Tris-HCl, pH 7.5, 10mM NaCl, 1mM EDTA).

The restriction fragments of polyoma DNA used in the gene transfer experiments were generated by treating ^{3}H-labeled polyoma DNA with BclI followed by EcoRI (New England BioLabs). Other restriction fragments of polyoma DNA were generated by treatment with EcoRI, HindIII, PstI or HaeIII (New England BioLabs). The fragments were separated by electrophoresis on a 1.5% agarose gel (see below). The areas of the gel containing the restriction fragments were excised and the DNA purified from the agarose using two rounds of KI density gradients (Blin, Gabain, and Bujard, 1975) or by electroelution into a dialysis bag (McDonell, Simon, and Studier, 1977).

A modification of the proteinase K-sodium dodecyl sulfate (SDS) method (Gross-Bellard, Oudet, and Chambon, 1973) was used to isolate PLP DNA. Prior to the disruption of PLP (in E buffer) with SDS, the pH of the solution was increased to 8.3 by adding 2.0M Tris-HCl, pH 8.3 to a final concentration of 50mM. Proteinase K was added to a final concentration of 50μg/ml. The reaction mixture was incubated at 37°C for 20 minutes, then SDS was added to a final concentration of 1% and incubation continued for 60 additional minutes. The solution was then extracted twice

with phenol and the aqueous phase was dialyzed exhaustively against DNA buffer.

PLP Preparation

Although details of the PLP formation reaction have been described previously (Barr et al., 1979), minor modifications have been introduced. A typical reaction for PLP formation contained a 16:1 weight ratio of polyoma empty capsids (1-6μg of protein) to ^{3}H-DNA, 50μg of nuclease-free bovine serum albumin (BSA) (Miles-Pentex) and sterile distilled water to a final volume of 100μl. The empty capsids were in E buffer at a concentration of 200μg/ml. ^{3}H-DNA at a concentration of 10μg/ml (1.5×10^{5} cpm/μg), was in DNA buffer. After a 10 minute incubation at 37°C, 350μl of sterile distilled H_2O were added and incubation continued for an additional 20 minute period. At this time, 50μl of 200μg/ml pancreatic DNase I (Worthington Biochemical Corp., 2430 units/μg) in 20mM Tris-HCl, pH 7.5, 10mM $MgCl_2$ were added and incubation resumed for 10 more minutes. The reaction mixture was chilled and layered onto a 10-22% sucrose gradient (10.0 ml) that had been underlayered with 0.3 ml of a CsCl (1.7 g/cm^{3}) cushion. The sucrose gradients were prepared in 10mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, containing

0.5mg BSA/ml. Sedimentation was performed at 4°C in the Beckman SW41 rotor at 25,000 rpm for 120 minutes. Gradients were fractionated by puncturing the bottom of the tube and collecting the drops into fractions consisting of approximately 0.3 ml each. For analytical experiments, approximately 3.3 ml of scintillation fluid (30% (v/v) Triton x-100, 60% (v/v) toluene, 10% (v/v) water, 0.4% (w/v) 2,5-diphenyloxazole, 0.005% (w/v) 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene) were added to the entirety of each fraction and radioactivity determined using the Packard Model 3320 Liquid Scintillation Spectrometer. Otherwise, a sample from each fraction was assayed for radioactivity in an identical manner.

For molecular weight and restriction enzyme analysis of PLP DNA, preparative PLP reactions were carried out. They were performed by changing the volume of the initial incubation from 100µl to 1.0 ml and increasing the amounts of empty capsids and DNA 100 fold. The 16:1 ratio of capsids to DNA was, however, maintained. The volumes of sterile distilled water and DNase I solution were increased to 3.5 ml and 0.5 ml, respectively. After the final incubation, the reaction mixture (5.0 ml) was sedimented through a 10-22% sucrose gradient using the Beckman SW27 rotor at 24,000 rpm and 4°C for 120 minutes. Gradient

profiles similar to that shown in Figure 1 were obtained.

For the gene transfer experiments, PLP reactions were carried out as described above except that the mass ratio of empty capsids to DNA was 12:1 and the 10-22% sucrose gradients were formed in PLP buffer (10mM Tris-HCl, pH 7.5, 10mM NaCl, 1mM EDTA and 100µg BSA/ml). The mass ratio of empty capsids to DNA was decreased in order to minimize the formation of aggregates involving the association of more than one empty capsid per fragment of DNA. PLP buffer was used in order to maintain the ionic strength and BSA concentration at levels similar to those at which PLP formation takes place. This was done in order to prevent any possible dissociation of PLP into empty capsids and free DNA. A cushion of 0.5 ml of 66% (w/w) sucrose in PLP buffer was placed at the bottom of each gradient. The peak fractions of PLP from gradients similar in profile and yield to that shown in Figure 1 were pooled and concentrated by centrifugation onto a 66% (w/w) sucrose cushion using the Beckman SW41 rotor at 35,000 rpm and 4°C for 4 hours. Concentrated PLP preparations were dialyzed against PLP buffer without BSA using collodion bags (Schleicher & Schuell) with a molecular weight cut off of 25,000.

Gel Electrophoresis

A 1.5% agarose gel was used for the determination of the molecular weight of PLP DNA and for the separation of DNA fragments ranging in size from 350 to 23,000 base pairs. For the resolution of the fragments of polyoma DNA generated by digestion with HaeIII, a two-part vertical gel (5% acrylamide above 9% acrylamide) was employed. This gel was constructed by pouring into the gel chamber, to a height of approximately 18 cm, a solution of 9% (w/v) acrylamide, 0.46% (w/v) N,N'-methylene-bis-acrylamide, 0.15% (v/v) N,N,N'N'-tetramethylethylenediamine (TEMED), 0.05% (w/v) ammonium persulfate dissolved in electrode buffer (50mM Tris base, 20mM sodium acetate, 1mM EDTA adjusted with acetic acid to pH 8.1). After polymerization had occurred, the rest of the gel chamber (approximately 6 cm) was filled with a solution of 5% acrylamide, 0.26% N,N'-methylene-bis-acrylamide, 0.15% TEMED, 0.05% ammonium persulfate in electrode buffer.

Electrophoresis of all gels was carried out in a vertical slab apparatus, at room temperature, with buffer circulating at 6-7 ml/minute. During electrophoresis the gels remained in equilibrium with ambient temperature. Gels were 24 x 15 x 0.3 cm in size and were plugged at the bottom with a strip (1 x 15 cm) of 15-17 layers of Whatman #1 filter paper. Four volumes of DNA samples were mixed

with one volume of 5X Ficoll loading dye (250mM Tris-HCl, pH 7.9, 50mM EDTA, 15% Ficoll, 2.5% SDS, 0.2% bromophenol blue), heated at 65°C for 5 minutes, cooled rapidly in an ice water bath and loaded onto the gels. The electrophoresis schedule included a 60 minute pre-running period at 80 constant volts followed by sample loading and electrophoresis at 20 volts for 60 minutes. Voltage was then increased to 80 volts and kept constant for 9 and 16.5 hours for the agarose and the two-part acrylamide gel, respectively. Gels were stained for 2-3 hours in aqueous ethidium bromide (2μg/ml) and photographed over a long wave (366nm) ultraviolet lamp using Kodak Wratten filters #12 and #23A.

Virus, Empty Capsids and PLP Assays

Hemagglutination (HA) of guinea pig erythrocytes was used to quantitate polyoma virions, empty capsids and PLP (Crawford, 1962). One HA unit was assumed to correspond to 10^7 particles (Crawford, 1962). In addition, PLP were quantitated by the specific radioactivity of their DNA. Assays for plaque forming activity were performed on monolayers of mouse 3T3D cells in T25 flasks (Falcon) using an overlay of 0.9% agarose in Dulbecco's modified Eagle's medium (DME) supplemented with 2.5% horse serum and 100

units/ml each of penicillin and streptomycin (antibiotics).

Transformation Assay

Rat embryo F111 cells were used for the dense foci assay of transformation (Chowdhury et al., 1980). A stock culture was kindly provided by Dr. Mark Israel. The cells were grown at 37°C in a humidified incubator with a 10% CO_2 atmosphere using DME supplemented with 10% fetal calf serum and 100 units/ml antibiotics. Cell cultures in T25 flasks (Falcon) were infected at about 80% confluency (1×10^6 cells) with PLP, polyoma virus, or various controls, or transfected by a modification (Loyter et al., 1982) of the calcium phosphate coprecipitation method (Graham and Van der Eb, 1973). Briefly, coprecipitation of DNA with calcium phosphate was performed by mixing the DNA sample (10-30µl) into 0.25 ml of 25mM Hepes, pH 7.12, 250mM $CaCl_2$, 40µg/ml calf thymus DNA followed by the addition of 0.25 ml of 25mM Hepes, pH 7.12, 280mM NaCl, 1.5mM Na_2HPO_4. Coprecipitation crystals were allowed to form at room temperature without agitation for 30 minutes. The suspension was then added directly to the 5 ml of complete medium covering the cells to be transfected. Following a 4 hour incubation period at 37°C the medium containing the coprecipitate was replaced. From this point, these

cultures were treated in a manner identical to the other cultures comprising each complete experiment as shown in Table 1. Prior to infection of the cells with PLP, or with polyoma virus and the other controls, the monolayers were washed with Dulbecco's phosphate buffered saline (PBS) (Dulbecco and Vogt, 1954). Solutions for infection were prepared by mixing 0.5 ml of the infecting agent in PLP buffer with 0.5 ml of 2X DME containing 200 units/ml antibiotics. Infection was carried out at 37°C for two hours with gentle rocking of flasks every 15 minutes. Culture medium containing 10% fetal calf serum and 100 units/ml antibiotics was added without removal of the inoculum and changed every 3-4 days. Twenty-one days post infection, cells were fixed with 10% gluteraldehyde in PBS and stained with 0.03% Giemsa in water. The macroscopic, darkly stained, dense foci were then counted.

Other Materials and Methods

Kilham rat virus (KRV) ^{3}H-DNA was a generous gift from Dr. L. Salzman and ^{32}P-rRNA from BHK cells was kindly provided by Dr. M. Parker. Restriction enzymes were purchased from New England BioLabs and used as recommended. The homopolyomers [8-^{14}C]poly(dA).[2-^{14}C]poly(dT) and [8-^{14}C]poly(dG).poly(dC) were purchased from P-L

Biochemicals and their concentration was determined by absorbance at 260nm and 253nm using the molar (mole residue/l) absorption coefficients 6,000 and 7,400 respectively (Wells, Larson, and Grant, 1970). Similarly, absorbance at 260nm was employed for the determination of DNA and RNA concentrations using $A^{1\%}_{1cm}$= 200 for double stranded DNA and $A^{1\%}_{1cm}$= 250 for RNA and single stranded DNA. For the interconversion between molecular weight and number of base pairs, one deoxyribonucleotide pair was assumed to correspond to 650 daltons. Protein concentration was determined by the method of Lowry et al., (1951).

CHAPTER 3

RESULTS

PLP Formation

When the material resulting from the standard PLP formation reaction performed as described in "Materials and Methods" is analyzed on a sucrose gradient, the profile shown in Figure 1 is obtained. The radioactive peak at 190S (fractions 17-21) consists of PLP. Evidence for this has been presented by Barr et al., (1979). The sucrose gradient profile presented here demonstrates the reproducibility of the PLP formation reaction under the present conditions and serves as a reference for the ensuing experiments involving further characterization of the biochemical and biological properties of this 190S PLP peak.

Effect of Bovine Serum Albumin on PLP Formation

The initial report describing the *in vitro* conditions under which PLP formation occurs (Barr et al., 1979) mentions the inclusion of bovine serum albumin (BSA)

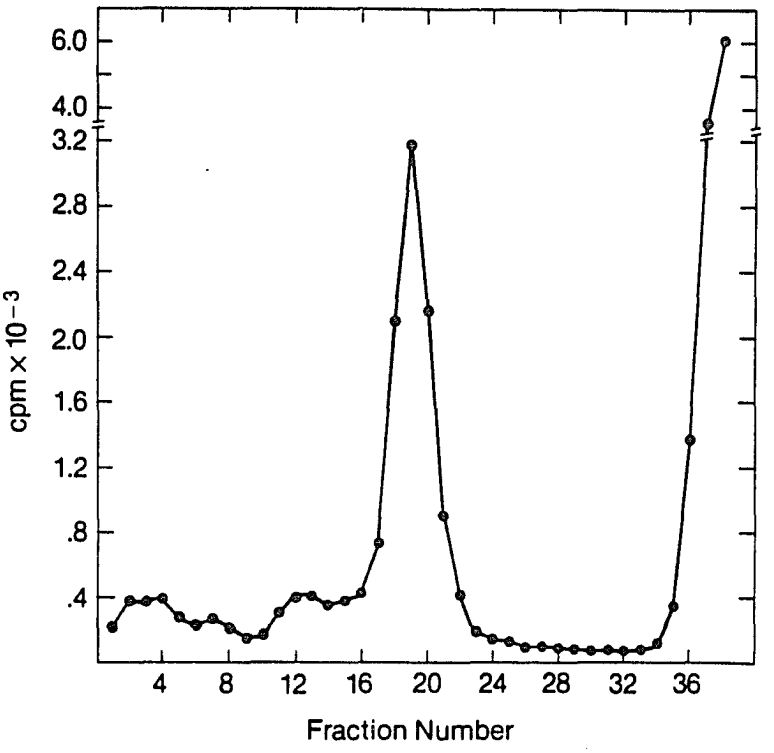

Figure 1. 10-22% sucrose gradient profile of a typical PLP reaction.

The reaction contained 3.8µg polyoma empty capsids and 0.24µg polyoma ^{3}H-DNA. It was carried out as detailed under "Materials and Methods". Typically, fractions 17-21 were pooled, dialyzed, and used for further studies. Sedimentation was from right to left and was performed in the Beckman SW41 rotor at 25,000 rpm and 4°C for 120 minutes. In a parallel gradient, complete polyoma virions, which have a sedimentation coefficient of 240S, peaked at fraction 14. Using this and the miniscus of the gradient (0S) as references, the PLP peak shown in this profile was calculated to be approximately 190S.

in the reaction mixture. However, whether PLP formation is dependent on the presence of BSA and if so at what optimum concentration was not discussed. In order to answer these questions, PLP reactions were carried out using various concentrations of BSA and PLP yield at each concentration was determined. The results are shown in Figure 2. It is evident that while PLP formation does occur in the absence of BSA, its inclusion at a concentration between 0.45 and 2.6µg/µl (at the 100µl level of the PLP reaction) enhances PLP formation by approximately 1.6 fold. Above the 2.6µg/µl level of BSA concentration, PLP yield shows a decline. The progress of the PLP reaction in the total absence of BSA argues against a direct role for BSA in PLP formation. On the other hand, enhancement of the reaction could be due to the action of BSA as a stabilizing agent in the process of PLP self-assembly.

Effect of NaCl Concentration on PLP Formation

The dilution step of the PLP reaction, performed by the addition of 350µl of water to the 100µl initial reaction mixture volume, results in 4.5 fold reduction in the ionic strength of the solution. This implies that PLP formation is related to the ionic strength of the environment in which the empty capsids and DNA are present.

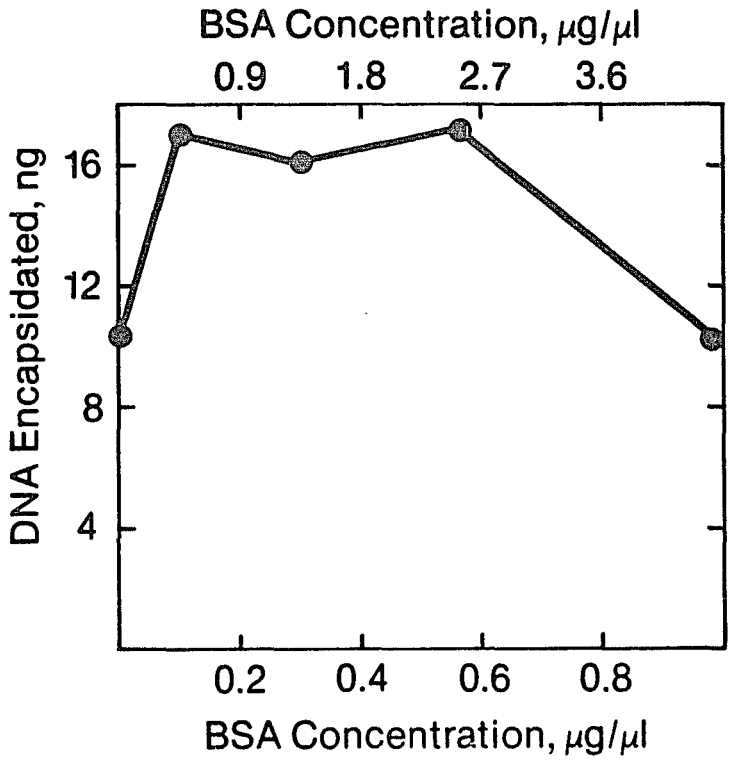

Figure 2. Inclusion of bovine serum albumin (BSA) in PLP reactions enhances the efficiency of PLP formation.

PLP reactions were carried out as described under "Materials and Methods" except that different amounts of BSA were added at the initial step. Each reaction mixture was analyzed by sedimentation through a 10-22% sucrose gradient and the resulting PLP peak was quantified (see Figure 1). The value of each PLP peak was then converted to ng DNA encapsidated per μg empty capsids and expressed on the ordinate. The abscissa reflects BSA concentration at the initial step of the PLP reaction (upper axis) or, following the dilution step (lower axis).

Substantial contribution to the ionic strength of the PLP reaction mixture results from the sodium chloride present in the empty capsids (100mM) and DNA (10mM) buffers.

To investigate the effect of ionic strength on the extent of PLP formation, a number of PLP reactions were performed in the presence of different concentrations of NaCl. The resultant reactions were analyzed by sedimentation through sucrose gradients and the peaks representing PLP (see Figure 1) were evaluated. Figure 3 shows the amount of PLP formed as a function of increasing NaCl concentration. It is evident that at final NaCl concentrations greater than approximately 80mM (post dilution) PLP yield begins to decline rapidly. In addition, there is a slight decrease in PLP yield at NaCl concentrations exceeding 13mM. Thus, for maximum PLP yield, it is important to maintain the salt concentration at or below 13mM. However, at a sacrifice of only a slight loss in yield (down to 90% of maximum) ionic strengths up to approximately 80mM may be tolerated. Above this level, however, PLP formation is seriously impeded.

Molecular Weight of PLP DNA

The molecular weight of PLP DNA was determined by agarose gel electrophoresis and found to be heterogeneous

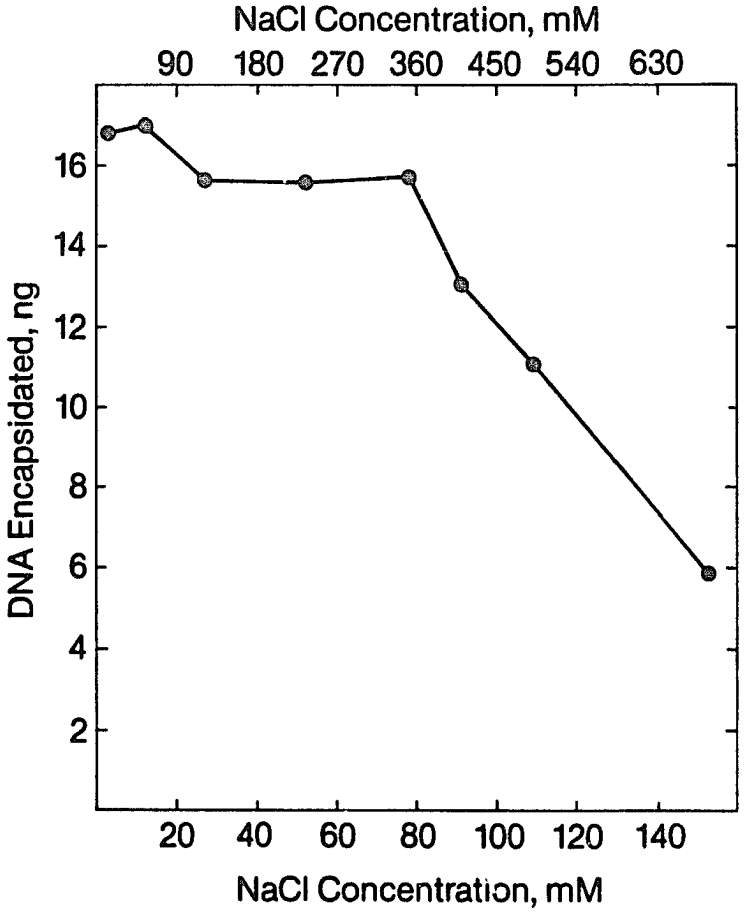

Figure 3. Dependence of PLP formation on the ionic strength of the reaction mixture.

To several PLP reactions, performed as detailed under "Materials and Methods", NaCl was added to different final concentrations at the initial step. After sedimentation of each reaction through a 10-22% sucrose gradient, the size of the PLP peak in each gradient was determined (see Figure 1). The values were converted to ng DNA encapsidated per μg empty capsids and expressed on the ordinate. NaCl concentrations at the initial step and following the dilution step are shown on the upper and lower horizontal axes, respectively.

(Figure 4, lane E). In order to be certain that the DNA extraction procedure did not shear or in some other way break down the PLP DNA, two controls were performed in parallel (Figure 4, lanes A and B). The first control consisted of λ DNA digested with HindIII and the second contained the same λ-HindIII digest to which polyoma empty capsids (protein:DNA mass ratio was 16:1) had been added. After a 10 minute incubation at 37°C, DNA extraction was performed. This allowed for the detection of possible DNA degradation, if any, due to experimental manipulations or the action of any nuclease activity contaminating the empty capsids preparation. No broadening or streaking of the λ-HindIII digests bands (Figure 4, A and B) occurred. The sharpness of the restriction bands indicates that neither the extraction procedure nor the presence of empty capsids result in the fragmentation of the DNA. This demonstrates that the molecular weight heterogeneity of PLP DNA observed in lane E of Figure 4 is not an experimental artifact of the isolation procedure.

Lanes C, D, F, and G of Figure 4 contain polyoma DNA digested with EcoRI, HindIII, PstI and HpaII, respectively. These, as well as the bands of lanes A and B, were used as molecular weight markers in the construction of the standard curve shown in Figure 5A. Figure 5B is a 600nm absorbance tracing of the photographic

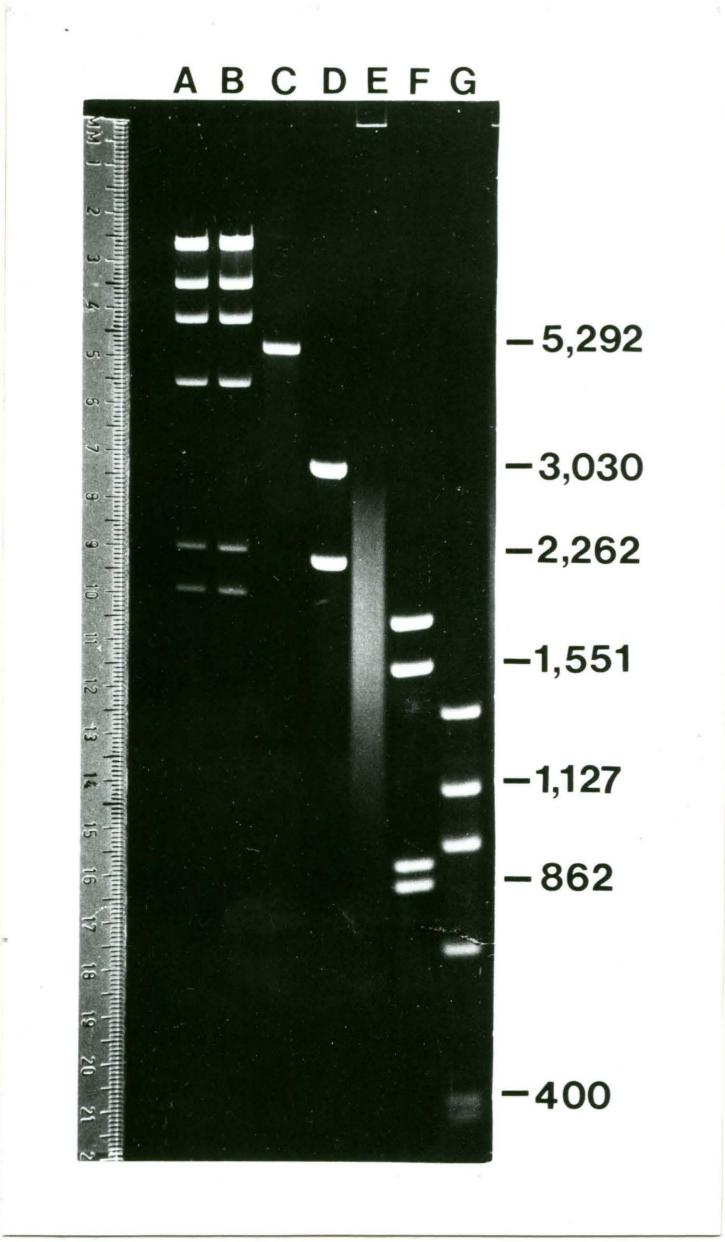

Figure 4. The DNA extracted from PLP is heterogeneous in size.

Electrophoretic analysis of, the DNA extracted from PLP, parallel extraction controls, and molecular weight markers, in a 1.5% agarose gel. (A), λ-HindIII digest; (B), λ-HindIII digest to which 40μg of polyoma empty capsids were added. These two samples were incubated at 37°C for 10 minutes, then extracted in parallel with PLP DNA as controls (see text). (E), DNA extracted from PLP. (C), (D), (F), and (G), molecular weight markers prepared by the digestion of polyoma DNA with EcoRI, HindIII, PstI, and HpaII, respectively. The number of base pairs of some of the polyoma restriction fragments is indicated.

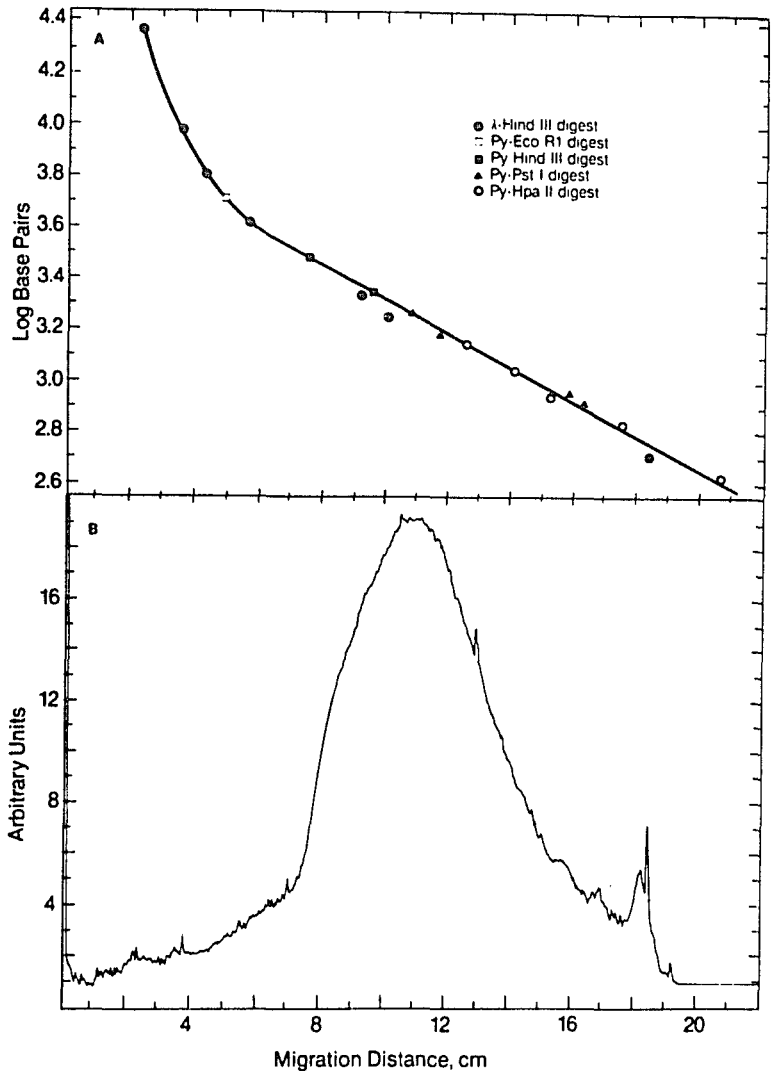

Figure 5. Analysis of the electrophoretic pattern of PLP DNA.

(A), Plot of the log of base pairs versus the migration distance for the molecular weight markers of Figure 4.

(B), Scan at 600nm of the photographic negative of Figure 4, lane E, (PLP DNA). Scanning was performed on a model 2400 Gilford recording spectrophotometer. The Gilford model 2410S linear transporter was used.

negative of the PLP DNA smear (Figure 4E). From this scan, it is evident that the migration of PLP DNA and therefore its molecular weight is represented by a wide distribution around a central maximum. Generally, functions of this type can be represented by the equation

$$\frac{C_i}{C_o} = e^{\frac{-(x_i - x_o)^2}{2\sigma^2}} \qquad \text{(I)}$$

where the Gaussian function has been expressed in terms of the concentration of each DNA species, C_i, relative to the concentration of DNA at the mean, C_o. The symbol x_o denotes the migration distance at the mean (C_o) and x_i refers to the migration distance corresponding to each C_i, whereas σ is the standard deviation. Taking the natural log of both sides and rearranging equation (I) yields:

$$-\ln \frac{C_i}{C_o} = \left(\frac{1}{2\sigma^2}\right)(x_i - x_o)^2 \qquad \text{(II)}$$

A graph of $-\ln C_i/C_o$ versus $(x_i - x_o)^2$ allows the computation of σ from the slope, provided a linear relationship is obtained (Vinograd and Hearst, 1962). Deviation from linearity, however, would indicate that the observed distribution departs from the Gaussian function and is, therefore, formed from a non-normal or skewed distribution of elements. Figure 6 shows such a graph for the right

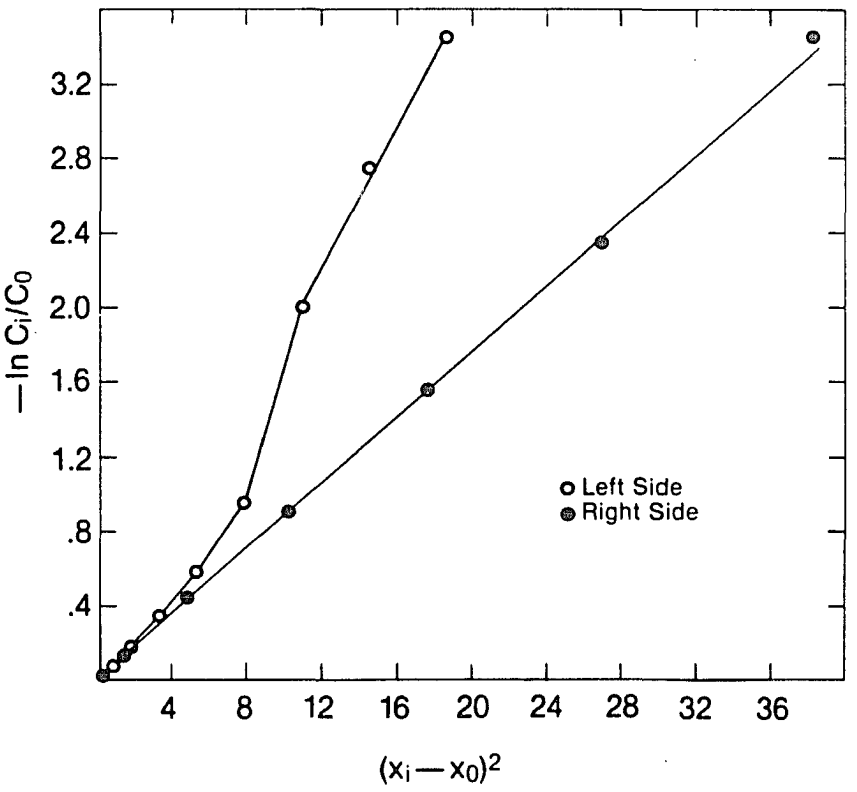

Figure 6. Graph of $-\ln C_i/C_o$ versus $(x_i-x_o)^2$ according to equation (II).

The data points were calculated from the scan of PLP DNA shown in Figure 5B. The line defining the center of the peak was arbitrarily drawn at 10.8 cm of migration. It was used to determine three necessary parameters: (i) the mean molecular weight from which dispersion was measured, (ii) C_o, and (iii) x_o. A baseline was drawn at 3.5 units of concentration (in Figure 5B) in order to eliminate the background.

side and for the left side of the PLP DNA molecular weight distribution depicted in Figure 5B. Clearly, the right side of this distribution is Gaussian in nature and yields a σ corresponding to approximately 0.5×10^6 daltons. This dispersion is from the 1.2×10^6 mean molecular weight value obtained from the arbitrarily chosen line defining the center of the peak at x = 10.8 cm of migration. A perfectly symmetrical distribution would be expected to yield superimposable curves irrespective of which side is analyzed. This, however, is not the case with respect to the left side of this distribution. Here, deviation from the normal distribution is evident by the non-linear arrangement of the data points. The presence of a region (at approximately 8 cm of migration distance, Figure 5B) which is characterized by a very steep slope indicates that the distribution is skewed and drops off rapidly. This rapid decline occurs at approximately 1.8×10^6 daltons. Given that the entire distribution falls in the linear region of the molecular weight standard curve (Figure 5) and assuming that size heterogeneity does not affect the electrophoretic behavior of DNA in agarose, it appears that the 1.8×10^6 daltons represent an upper limit on the size of DNA that can be encapsidated by polyoma empty capsids in the absence of histones.

Is The Sequence of PLP DNA Unique?

To determine whether a specific or random fragment of polyoma DNA is encapsidated during PLP formation, DNA from PLP was extracted and treated with restriction enzymes whose action on polyoma DNA is known. If PLP DNA is derived exclusively from a specific region of the polyoma genome, then only restriction bands characteristic of that specific region should appear following electrophoresis. On the other hand, if PLP formation represents encapsidation of random segments of polyoma DNA, then all fragments normally generated by that restriction endonuclease should appear. The results of the action of two different restriction enzymes, PstI and HaeIII, on intact polyoma DNA and PLP DNA are shown in Figure 7. In each case, it is evident that every fragment generated from polyoma DNA is also generated from PLP DNA. This indicates that all polyoma DNA regions are represented in PLP DNA and that formation of PLP does not involve a unique fragment or sequence of polyoma DNA. That this representation occurs with equal frequency is inferred from the fact that the relative intensity of the PLP DNA bands is essentially the same as that of intact polyoma DNA.

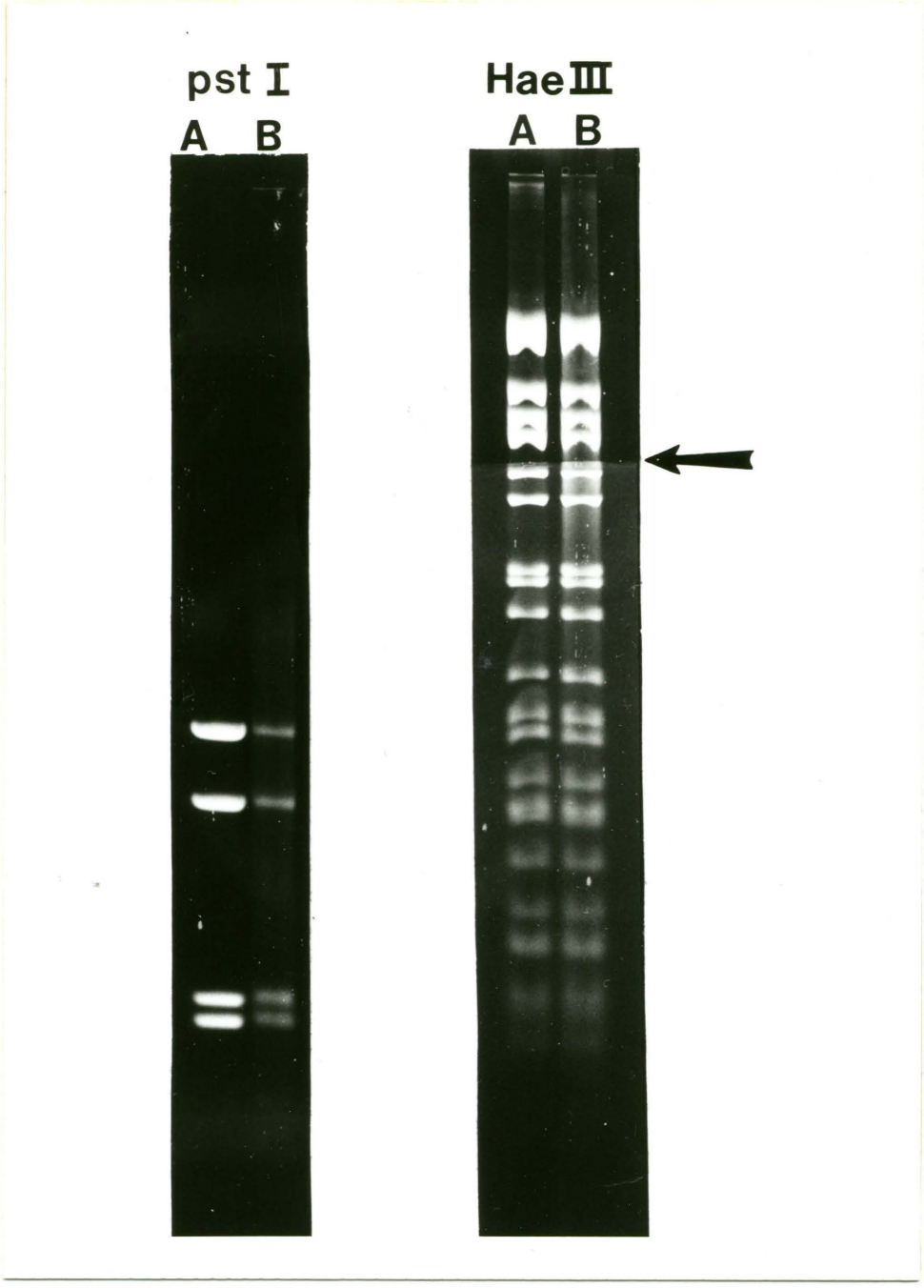

Figure 7. Restriction analysis of PLP DNA.

Polyoma DNA (A) and PLP DNA (B) were digested with the indicated restriction enzymes and subjected to electrophoresis in a 1.5% agarose gel for PstI and a two-part gel (arrow) of 5% acrylamide (5.5 cm) on top of 9% acrylamide (15.5 cm) for HaeIII. Supercoiled polyoma DNA was used to prepare PLP for this experiment.

Is There any Specificity for Nucleic Acid Structure in PLP Formation?

The data of Figure 7 indicate that a specific primary sequence of DNA does not appear to be required in the reaction of polyoma empty capsids with DNA to form PLP. The possibility remains, however, that a short stretch of AT or GC base pairs might be required as a signal for the initial capsid-DNA interaction. Visual inspection of the sequence of polyoma DNA (Soeda et al., 1980) revealed the presence of many triple and greater stretches of AT and GC base pairs. Furthermore, these AT and GC repeats are widely distributed along the polyoma genome. They are not clustered in one region. Therefore, a preference or a specificity for either the AT or the GC base pairs could have gone undetected by the foregoing restriction enzyme digestion experiments. In addition, a question arises as to whether secondary or tertiary conformation of DNA play a role in PLP formation.

In order to investigate these questions, the amount of PLP formed was measured as a function of increasing concentration of nucleic acids differing in their primary, secondary, or superhelical tertiary structure. The results of these studies are shown in Figure 8. Figure 8A depicts the effects of tertiary structural variation in double-stranded DNA (dsDNA) on the efficiency or extent of

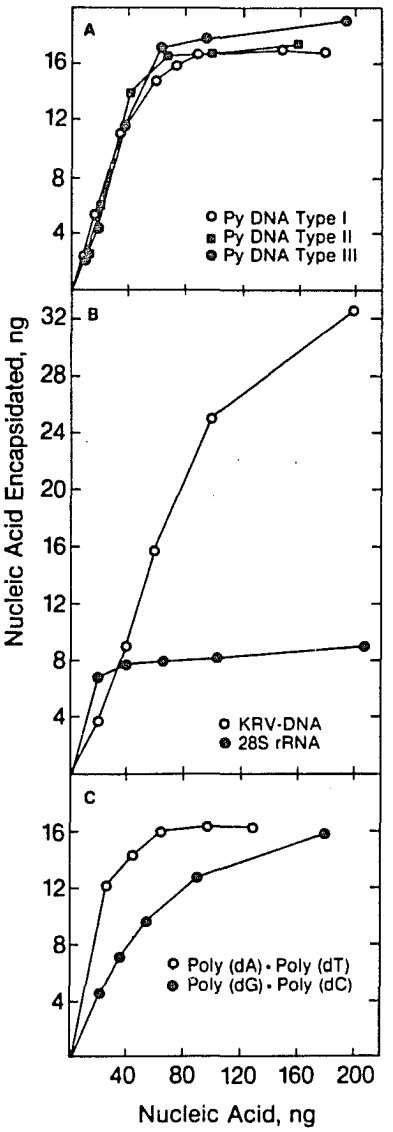

Figure 8. Nucleic acid structure and PLP formation.

For each data point, a PLP reaction was carried out as described under "Materials and Methods", except the amount of the particluar nucleic acid under investigation was varied. The amount of nucleic acid encapsidated per µg of empty capsids was determined from the sucrose gradient of each PLP reaction (see Figure 1) and expressed on the ordinate. The abscissa reflects ng nucleic acid present in each PLP reaction per µg of empty capsids.

its encapsidation. It is evident that the three forms of polyoma DNA, type I, supercoiled; type II, relaxed circular; and type III, linear; are encapsidated to approximately the same extent. In addition, the affinity of polyoma empty capsids for each of the three forms of polyoma DNA is also the same, as indicated by the equivalence of the three slopes in the linear region of the curves. With respect to the effect of the secondary structure of DNA, while the affinity of polyoma empty capsids for single-stranded DNA (ssDNA) (Figure 8B) is slightly lower than that for dsDNA (Figure 8A), nearly twice the amount of ssDNA was converted to PLP by the same amount of empty capsids. It is possible that the enhanced molecular flexibility of ssDNA is responsible for this increase in the extent of encapsidation. As for RNA, however, the extent of its conversion to PLP was greatly diminished when compared to DNA. Whether this is due to a different binding mechanism (perhaps a consequence of the 2' hydroxyl on the ribose backbone) or the adoption of extensive tertiary structures (tight-ball form) by the RNA molecule under the PLP reaction conditions is not known. Either poly(dA).poly(dT) or poly(dG).poly(dC) can be encapsidated (Figure 8C) indicating that polyoma empty capsids can interact with either the AT or the GC base pair to form PLP. The polyoma empty capsids, however, appear to

display significantly greater affinity for the AT than for the GC polymer. For the GC polymer, however, while reduced affinity was observed, under the same conditions, a plateau for the extent of its encapsidation was not reached. The reason for this is unclear, but may be related to the adoption of unfavorable double helical forms by the GC polymer (Wells et al., 1970).

In the course of the present investigations, it has also been observed that polyoma empty capsids are capable of forming PLP by interacting with DNA from a variety of sources including the bacteriophage λ, fd (supercoiled), ϕX174 (replicate form circular); the animal viruses SV40, and herpes simplex I, and E. coli, and mouse 3T3.

Effect of DNA Size on PLP Formation

In light of the experimental evidence presented thus far showing the non-specific interaction of polyoma empty capsids with DNA to form PLP, a question, concerning the effect of the polynucleotide size on this interaction, arises. To answer this question, restriction fragments of polyoma DNA ranging in molecular weight from 0.23×10^6 to 3.44×10^6 (full length of polyoma genome) were isolated and used to prepare PLP. The effect of DNA size on the efficiency of PLP formation was then determined by

measuring the amount of PLP produced (see Figure 1) by each of several different size classes. The values obtained, graphed as a function of the molecular weight of the DNA fragments used, are shown in Figure 9. It is important to note that since in any given empty capsids preparation the number of empty capsids that are competent in DNA uptake has been observed (in the course of the present investigations) to be constant, an increase in the amount of PLP formed reflects a greater extent of DNA encapsidation rather than a greater number of the empty capsids becoming competent in interacting with DNA as the molecular weight of DNA is increased.

The relationship obtained reveals an overall increase in the total amount of DNA encapsidated or converted to PLP as the size of the DNA substrate is increased (Figure 9). This increase, however, is biphasic. The curve displays two regions each characterized by a different slope. The very steep slope occurring below the level of approximately 500 base pairs indicates that little or no appreciable encapsidation of DNA fragments smaller than this size class takes place. This diminished interaction or binding ability indicates that for efficient encapsidation and/or activation of DNA binding domains, polyoma empty capsids require the DNA polynucleotide to be of a minimum length. Above this minimum polynucleotide

Figure 9. Extent of DNA encapsidation as a function of the size of the DNA fragments incubated with polyoma empty capsids to form PLP.

PLP reactions were performed as described in "Materials and Methods" using restriction fragments of different molecular weights (MW) obtained by treatment of polyoma DNA with EcoRI, HindIII, PstI or HaeIII. The 16:1 mass ratio of empty capsids to DNA was maintained regardless of fragment size. Individual fragments were purified following electrophoresis of the digests on a 1.5% agarose gel. The fragments used were: the 314 base pair (bp) and 403 bp HaeIII fragments for the 0.23×10^6 MW reaction; the 488 bp, 511 bp and 537 bp HaeIII fragments for the 0.33×10^6 MW reaction; the 673 bp and 718 bp HaeIII fragments for the 0.45×10^6 MW reaction; the 1,551 bp PstI fragment for the 1.01×10^6 MW reaction; the 2,262 bp HindIII fragment for the 1.47×10^6 MW reaction; the 3,030 bp HindIII fragment for the 1.97×10^6 MW reaction; and the 5,292 bp full length polyoma DNA generated by treatment with EcoRI for the 3.44×10^6 MW reaction. After sedimentation of each PLP reaction mixture through a 10-22% sucrose gradient (see Figure 1), the PLP peak in each gradient was evaluated and expressed as ng DNA encapsidated per µg empty capsids on the vertical axis. The horizontal axes depict the size of the DNA fragments in base pairs (upper axis) or in daltons (lower axis).

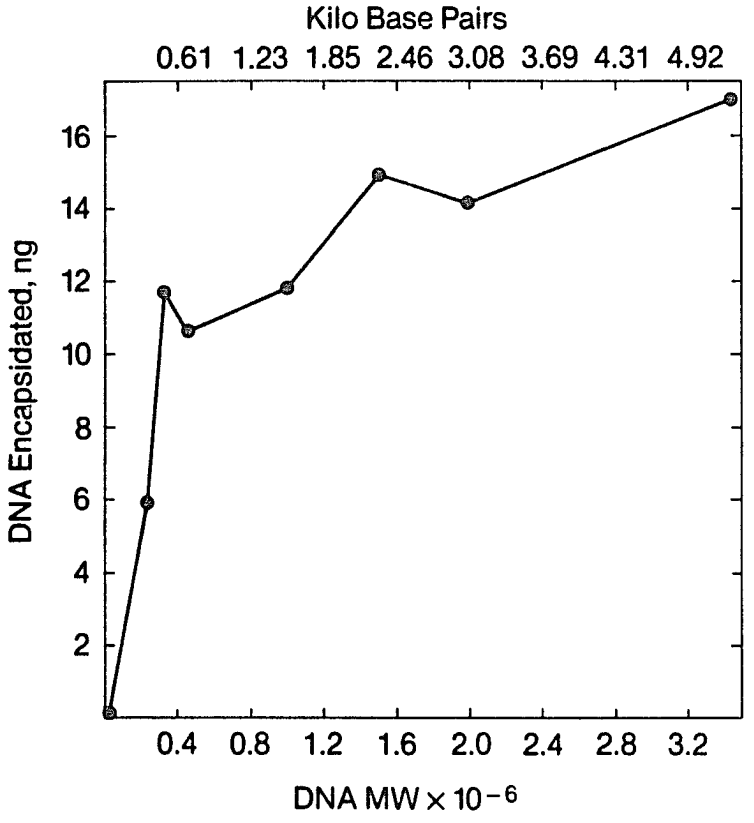

Figure 9. Extent of DNA encapsidation as a function of the size of the DNA fragments incubated with polyoma empty capsids to form PLP.

size, the second slope of the curve is encountered. Although this slope is reduced drastically as compared to the initial slope, it does not appear to approach zero. This indicates that the longer the DNA molecule the more efficient is its interaction with the polyoma capsid. It appears that this enhanced efficiency of interaction is a result of the presence of a lower energy state for the DNA molecule in association with the capsid rather than in the solution outside. Thus, the longer the DNA external to the capsid the more favored is the interaction. The enhanced efficiency of PLP formation with higher molecular weight DNA is reminiscent of the requirement of the <u>in vitro</u> λ packaging system, used in recombinant DNA cloning, for a large DNA substrate (Collins and Hohn, 1978).

Collectively, these observations are consistent with an encapsidation model involving DNA binding sites present on the internal rather than the external surface of the polyoma empty capsids. In this model, the process of DNA encapsidation would be governed by the interaction of two forces: Those favoring DNA encapsidation (E_{f1}, E_{f2}...) and those opposing it (E_{o1}, E_{o2}...). Until the forces favoring DNA encapsidation are equal to those favoring dissociation ($E_{f1}+E_{f2}...=E_{o1}+E_{o2}...$), DNA continues to be incorporated into the capsid. When equilibrium is reached, no further DNA encapsidation occurs. The energy resulting

from the unfavorable environment imposed on the DNA external to the capsid (E_{f1}) (perhaps due to the loss of counter ions following the dilution step) plus that of protein-DNA binding inside the capsid (E_{f2}), provide the majority of the force necessary for driving the DNA into the capsid to form PLP. Clearly, the longer the DNA extending outside the capsid the greater is the value of E_{f1} and therefore the greater is the overall magnitude of the PLP formation driving force. On the other hand, the energy stemming from the electrostatic repulsion between adjacent DNA strands inside the capsid (E_{o1}) plus that resulting from the resistance of double-stranded DNA to bending and coiling (E_{o2}) constitute the chief forces opposing DNA encapsidation.

Transfer of Genetic Information by PLP.

The data presented thus far (Figures 7 and 8) demonstrate that PLP formation does not require the presence of a specific primary, secondary or superhelical tertiary structure in the DNA substrate. Hence, a DNA fragment or a gene consisting of any given sequence may be reacted with polyoma empty capsids to construct PLP carrying specific genetic information. This provides the PLP system with a potential for use as a general gene

transfer vehicle for mammalian cells. To determine whether PLP can transfer genetic information to cells in culture, a restriction fragment of polyoma DNA that has measurable biological activity was used. This fragment is approximately 1.2×10^6 daltons in size and extends clockwise from the BclI site to the EcoRI site on the conventional polyoma map (Figure 10). It is the smallest of several restriction fragments encompassing the proximal part of the early region of the polyoma genome that has been shown to contain the genetic information necessary for the induction and maintenance of transformation* in rat cells (Novak et al. 1980; Chowdhury et al., 1980; Bastin, et al., 1980; Hassell et al., 1980). The remainder of the polyoma genome, which includes the distal part of the early region and extends clockwise from the EcoRI site to the BclI site, has no oncogenic activity (Novak et al., 1980). In this dissertation, these two fragments of DNA are referred to as the BclI-EcoRI transforming fragment and the EcoRI-BclI non-transforming fragment of polyoma DNA, respectively.

PLP preparations containing either the BclI-EcoRI transforming or, as a control, the EcoRI-BclI

*"Transformation" has been used in reference to both uptake of exogenous DNA by cells and cellular conversion to the oncogenic state. In this dissertation, "transformation" is used to indicate the latter meaning exclusively.

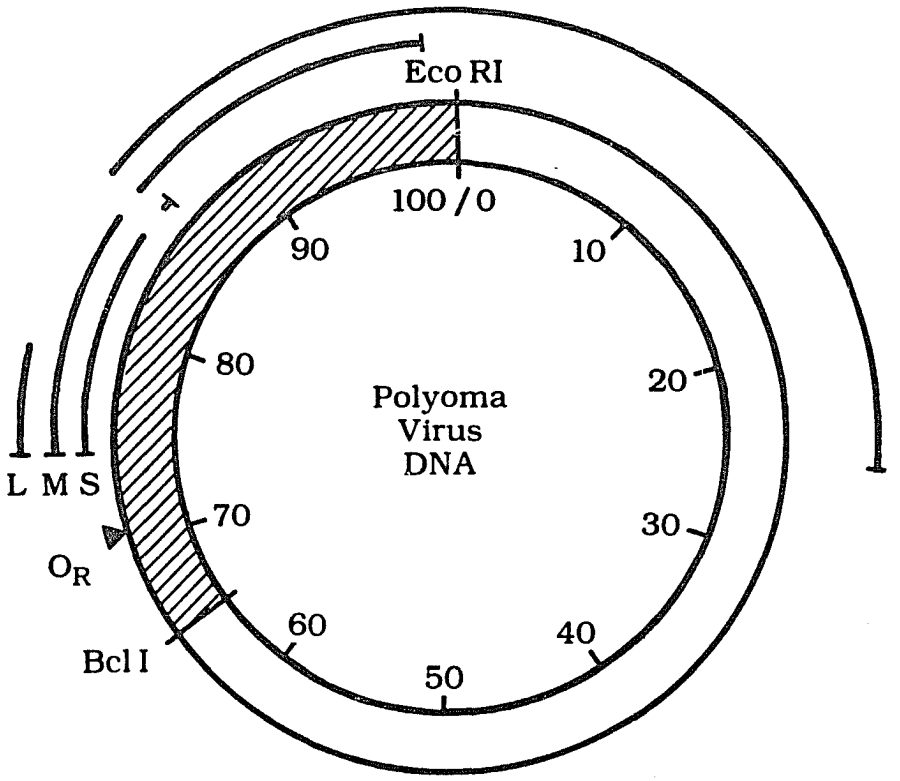

Figure 10. Physical map of the polyoma virus genome divided into 100 units.

The origin of DNA replication (O_R) and the coding locations of the early viral proteins small (S), middle (M), and large (L) tumor antigens are indicated. The fragment of DNA extending clockwise from the BclI site to the EcoRI site (shaded area) contains the coding sequences of the small and middle tumor antigens and is sufficient for the induction of the transformed phenotype (Novak et al., 1980). The other fragment which extends clockwise from the EcoRI site to the BclI site has no oncogenic activity (Novak et al., 1980).

non-transforming sequences of polyoma DNA were used to infect rat embryo F111 cells. The production of dense foci was used as an indication of oncogenic transformation and, therefore, of gene transfer and expression. Dense foci were found following infection with PLP containing the BclI-EcoRI transforming fragment (Figure 11A) or with polyoma virions (Figure 11B), or by treating the cells with a coprecipitate of the BclI-EcoRI transforming fragment and calcium phosphate (Figure 11C). Transformation was found to be associated only with the transfer of the BclI-EcoRI transforming fragment by PLP or calcium phosphate coprecipitation or as a part of the polyoma genome by polyoma virus. When the transforming fragment was absent, or a mechanism for its transfer not provided, transformation did not occur (Figure 11D,E,F,G,H). Transfer requires prior formation of PLP (by reaction of polyoma empty capsids with DNA), since an unreacted mixture of empty capsids and BclI-EcoRI transforming DNA failed to induce dense foci production (Figure 11H).

Induction of the transformed phenotype requires the delivery of a DNA fragment containing the required genetic information and is not the result of the penetration of the cells by empty capsids, PLP containing any DNA sequence, or virions contaminating the empty capsid preparations used to make PLP. This is indicated by the following:

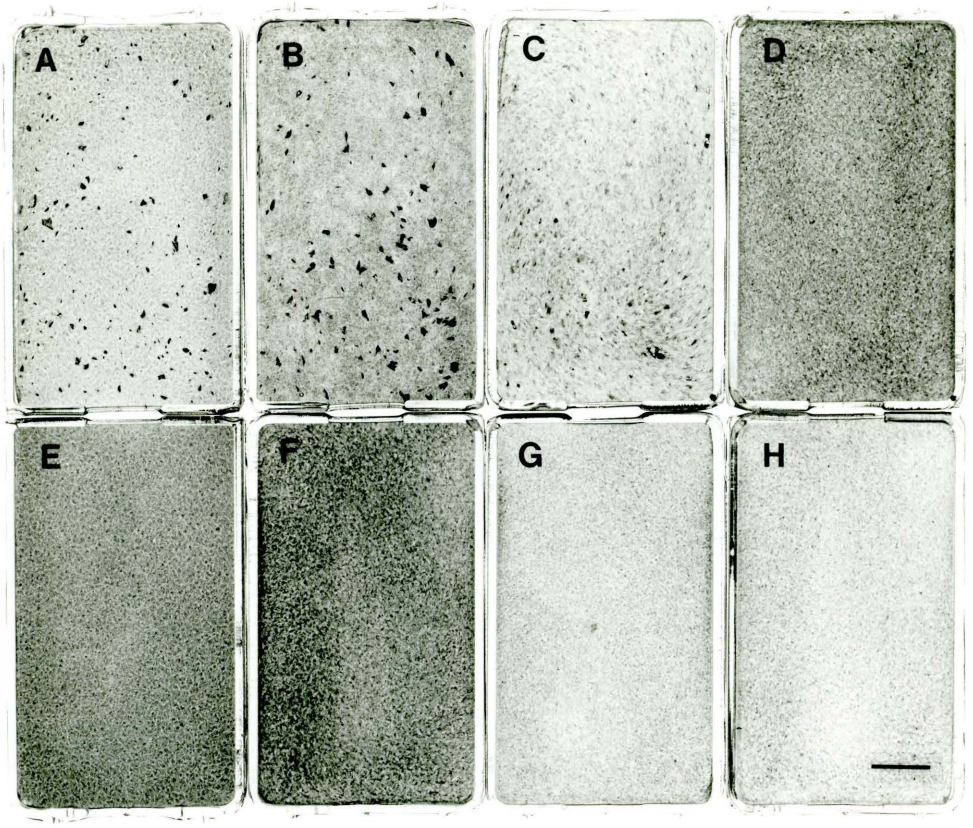

Figure 11. Macroscopic dense foci induced by the polyoma virus oncogenic sequences upon their transfer to rat cells by PLP, the calcium phosphate transfection method or as part of the complete polyoma genome by polyoma virions.

Slightly subconfluent rat F111 cells (1×10^6 cells) were inoculated, as described in "Materials and Methods" with (A), PLP containing the BclI-EcoRI transforming fragment (2.5×10^{10} particles containing 0.05μg DNA); (B), polyoma virus (0.5×10^{10} particles); (C), BclI-EcoRI transforming fragment coprecipitated with calcium phosphate (10×10^{10} molecules or 0.2μg DNA); (D), BclI-EcoRI transforming fragment as naked DNA (2.5×10^{10} molecules or 0.05μg DNA); (E), PLP containing EcoRI-BclI non-transforming sequences (2.5×10^{10} particles containing 0.05μg DNA); (F), polyoma empty capsids (2.5×10^{10} particles); (G), EcoRI-BclI non-transforming fragment coprecipitated with calcium phosphate (10×10^{10} molecules or 0.37μg DNA); and (H), an unreacted mixture of polyoma empty capsids (2.5×10^{10} particles) and BclI-EcoRI transforming fragment (3.5×10^{10} molecules or 0.07μg DNA). Bar represents 1.0 cm.

Transformation was not observed when cells were infected with empty capsids (Figure 11F) or with PLP made with EcoRI-BclI non-transforming polyoma sequences (Figure 11E) or with replicative form DNA from the bacteriophage ϕX174. Neither was oncogenic transformation observed when 10 times the amount of empty capsids was used as a control. In addition, when preparations of the empty capsids or of PLP containing the BclI-EcoRI transforming fragment were assayed for plaque forming activity at the same multiplicity of infection used in Table 1 no plaques were detected.

A quantitative summary of the gene transfer experiments comparing PLP with the calcium phosphate coprecipitation method is presented in Table 1. In the individual experiments performed, infection with the PLP gene transfer system produced dense foci 50 to 150 times more efficiently than did transfection with the calcium phosphate method. This corresponds to a frequency range of 5.6×10^3 to 6.5×10^3 transformants per 10^6 cells/μg DNA for PLP and 0.04×10^3 to 0.12×10^3 per 10^6 cells/μg DNA for the calcium phosphate coprecipitation method. Interestingly, the transforming efficiency of PLP appears to be only about 25% that of polyoma virions. This difference might result from a differential particle uptake and intracellular processing or from more efficient

TABLE 1. Transfer of the Oncogenic Sequences of Polyoma DNA by PLP or Calcium Phosphate Coprecipitation as Measured by Dense Foci Production on Rat F111 Cells.

Inoculum	Number of dense foci per flask (Average of 5 separate experiments)
PLP (BclI-EcoRI transforming fragment)	303 ± 23
PLP (EcoRI-BclI non-transforming fragment)	0
Ca/PO_4 (BclI-EcoRI transforming fragment)	4 ± 2
Ca/PO_4 (EcoRI-BclI non-transforming fragment)	0
BclI-EcoRI transforming fragment	0
Empty capsids	0
Empty capsids + BclI-EcoRI transforming fragment	0
Polyoma virus	1,195 ± 73

Slightly subconfluent cultures of rat F111 cells (1×10^6 cells) were inoculated with the above indicated agents as described in "Materials and Methods". Each of the five experiments was performed using approximately 2.5×10^4 particles or molecules of DNA per cell. Inoculum for the unreacted mixture of polyoma empty capsids plus BclI-EcoRI transforming fragment was prepared by mixing empty capsids (2.5×10^{10} particles) and the DNA fragment (3.5×10^{10} molecules or 0.07μg DNA) into 0.5 ml of 2X DME with antibiotics, then adjusting to a final volume of 1.0 ml with PLP buffer. The abbreviation, Ca/PO_4, refers to the use of the calcium phosphate coprecipitation method.

transformation by the complete viral genome.

Transfer of the polyoma oncogenic sequences by PLP, as with calcium phosphate coprecipitation crystals, results in stable expression of the transformed phenotype since cell cultures, established from individual dense foci induced by use of either method, displayed the usual growth properties and morphology characteristic of transformed cells. The mechanism of cellular transformation produced by these oncogenic sequences following their transfer to cells by calcium phosphate coprecipitation crystals or polyoma virions, has been shown to be a consequence of their continued expression following their stable integration into the host genome (Novak et al., 1980; Chowdhury et al., 1980; Bastin et al., 1980; Hassell et al., 1980). Presumably, similar mechanisms are responsible for the stable expression of the transformed phenotype by cells infected with PLP carrying these oncogenic sequences of polyoma DNA.

Stability of PLP During Infection

Whether during the infection process of gene transfer by PLP, structural changes possibly involving DNase-sensitive conformations occur, or whether PLP remain DNase-resistant 190S particles, has been investigated. PLP

reactions, using radiolabeled BclI-EcoRI transforming fragment of polyoma DNA, were performed and the resulting reaction mixtures without removal of DNase were used to infect cultures of rat F111 cells. As before, dense foci production indicating gene transfer activity was monitored. In addition, following the two hour infection period, the inoculum was removed and analyzed by sedimentation through a 10-22% sucrose gradient. With respect to dense foci production, no differences were noted in the frequency of foci induction under the conditions where DNase was present in the inoculum or under the standard conditions where PLP were purified prior to cell infection. This indicates that penetration of cells by PLP is not dependent on a DNase-sensitive intermediate stage and that PLP retain their DNase-resistance during infection. Furthermore, as can be seen from the sucrose gradient profile shown in Figure 12, the PLP that have not penetrated the cells also retain their 190S sedimentation coefficient. These results provide evidence that gene transfer by PLP involves particles having fundamentally the same properties as purified PLP.

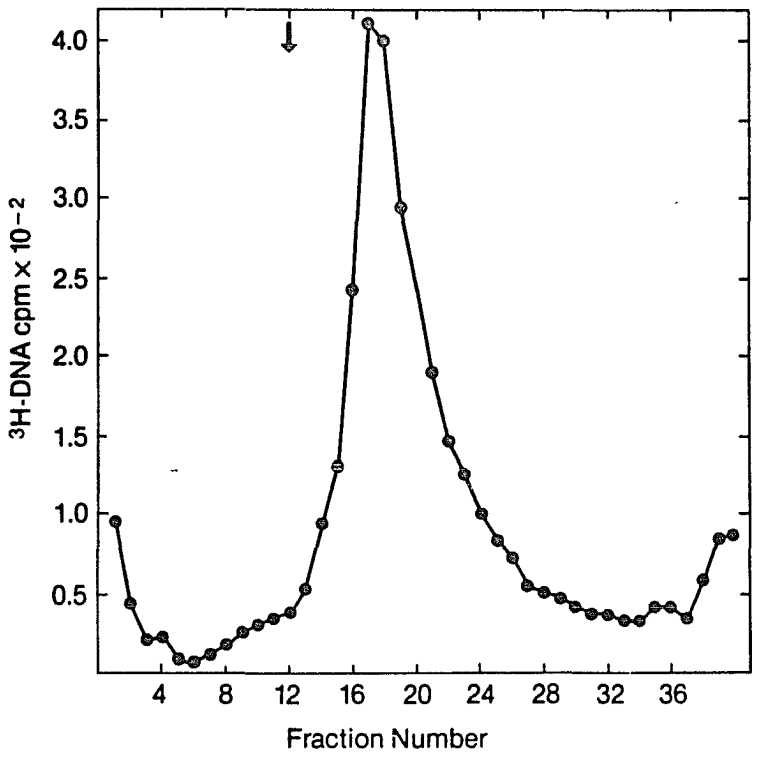

Figure 12. Sedimentation of a PLP inoculum removed from a cell culture at the end of the two-hour infection period.

A PLP reaction, performed as described in "Materials and Methods" using ^{3}HdT BclI-EcoRI transforming fragment of polyoma DNA and without removal of DNase, was mixed with an equal volume (0.5 ml) of 2X DME with antibiotics and used to infect rat F111 cells. At the end of the two-hour infection period, the inoculum (1.0 ml) was removed and layered onto a 10-22% sucrose gradient (10.0 ml) in PLP buffer. Centrifugation, fractionation and radioactivity determination were performed as described in "Materials and Methods". Sedimentation was from right to left. The 240S sedimentation position of complete polyoma virions in a parallel gradient is indicated by the arrow. Using this and the miniscus of the gradient (0S) as markers, the sedimentation coefficient of the peak shown is calculated to be approximately 190S. Broadness of the peak is a consequence of the large sample volume used.

Dose-Response Characteristics of the PLP Gene Transfer Vehicle.

The number of dense foci produced as a function of the number of PLP containing the polyoma BclI-EcoRI transforming fragment used to infect 1 x 10^6 rat F111 cells is depicted in Figure 13. At PLP inputs up to 4 x 10^{11} particles per 1 x 10^6 cells, the relationship closely approximates a curve of the general form $y = x^n$ where y and x represent the variables indicated on the ordinate and the abscissa, respectively, and the exponent, n, is about 0.4. Above this multiplicity of infection, a plateau is observed. One of the factors contributing to this plateau effect is the decrease in resolution inherent in the dense foci assay system as higher numbers of foci per unit area are approached. For example, at high multiplicities of infection, transformation events occurring in adjacent cells could result in overlapping and thus may be scored as a single dense focus.

It is interesting to note that when the calcium phosphate coprecipitation method was used to assay the infectivity of adenovirus DNA, Graham and Van der Eb (1973) obtained a similar logarithmic relationship but with a different exponent. For their system, the exponent, n, was found to be 3±1, a value yielding a concave instead of a convex curve as observed for the transfer of the

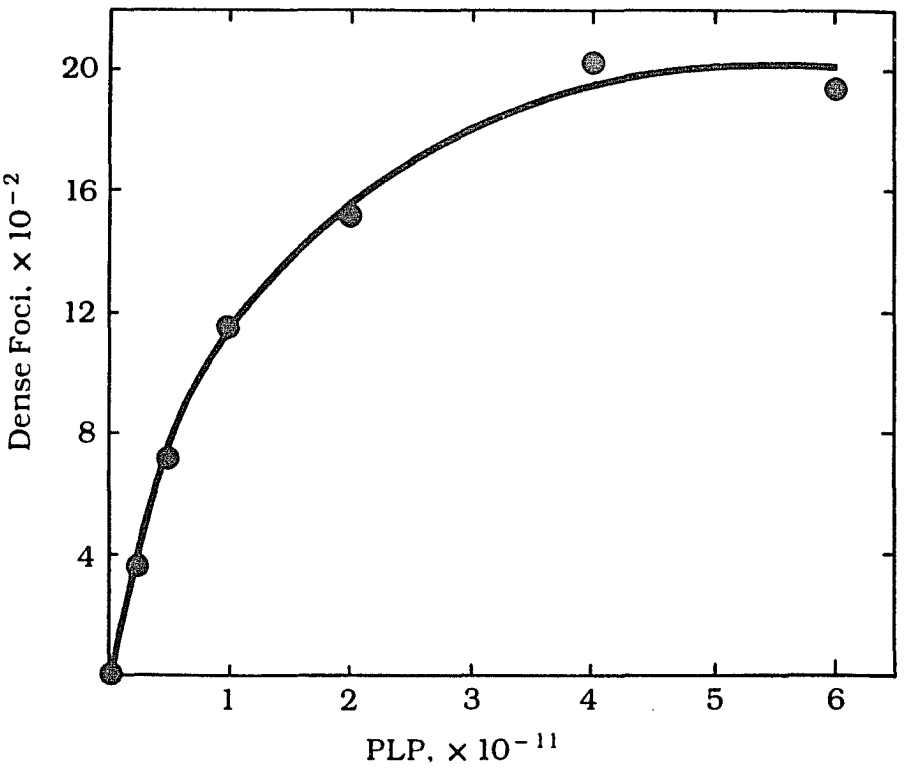

Figure 13. Dose-response curve for PLP-mediated transfer of the oncogenic sequences of polyoma DNA.

Rat F111 cells (1 x 10^6 cells) were infected, as described in "Materials and Methods", with different amounts of PLP containing the BclI-EcoRI transforming fragment of polyoma DNA. Dense foci overgrowing the normal monolayer were stained and counted twenty-one days post-infection.

BclI-EcoRI oncogenic fragment of polyoma DNA by PLP. Provided that the events leading to the expression of these two different DNA molecules are comparable, it is possible to postulate that the convexity of the PLP curve is a reflection of the greater efficiency of PLP in gene transfer ability (see Table 1). Furthermore, it suggests a different mechanism of DNA-vector uptake into the cells.

CHAPTER 4

DISCUSSION

PLP: Formation and Characteristics

The interaction of polyoma empty capsids with DNA to form PLP has been investigated. The evidence presented demonstrates that the highly ordered multimeric structure of polyoma empty capsids (Adolph et al., 1979; Rayment et al., 1982) is capable of interacting with a variety of nucleic acids. Previous observations (Barr et al., 1979) in conjunction with the present results are consistent with a model in which a DNA fragment is encapsidated and has become protected within a previously empty capsid. The previous observations concerning the physical and biochemical characteristics of PLP included the resistance of the DNA component of PLP to nuclease activity, and the stability of the particle in solutions of high salt concentration (Barr et al., 1979). Classically, these are the two main types of evidence that a nucleic acid moiety occupies an internal position in a virus particle. The application of other methods, such as x-ray crystallography, low angle x-ray scattering, and neutron

diffraction, however, offers more rigorous proof for the localization of the nucleic acid component in a nucleoprotein complex.

The main observations made here in support of this encapsidation model are (i) the presence of a relatively sharp upper limit on the size of PLP DNA and (ii) the requirement of a large DNA substrate for efficient PLP formation. One interpretation of these two observations is that they reflect the saturation of a finite number of DNA binding sites and/or represent the physical filling of the capsid's vacant core. Since in normal virions the nucleohistone core including the internucleosomal regions of presumably naked DNA is first surrounded by the core proteins then by the capsid proteins, any DNA binding sites would more likely be directed towards the inside rather than the outside of the capsid. In fact, dissociation experiments have revealed that VP2 and VP3 are associated with the viral minichromosome following its release at pH 9.8 (Christiansen et al., 1977). Consequently, these two viral proteins were implicated for an internucleosomal binding function replacing the role of H1 in mature virions (Pett, Estes, and Pagano, 1975; Polisky and McCarthy, 1975). Thus, saturation of DNA binding sites and physical filling of the empty core may not be readily distinguishable if the DNA is envisioned to thread its way

from one binding site to the next at random until all binding sites are occupied or the physical volume of the capsid core is filled. This, however, is restricted by interstrand repulsion and by the physical characteristics of the DNA molecule and its ability to undergo bending or coiling without the aid of the organizing function of the core histones. Actually, when the histone cores are present (i.e. as in normal virions) approximately twice the amount of DNA (5,292 base pairs) can be compacted in the same amount of space.

In addition to the mechanical and physical difficulties associated with packaging naked DNA, thermodynamic considerations (Riemer and Bloomfield, 1978) indicate the existence of a positive free energy barrier stemming from electrostatic repulsion residing in the acidic nature of the DNA backbone. Given charge neutralization and orderly folding of the DNA upon interaction with histones, it would be expected that a greater amount of DNA can be packaged in their presence. Thus, for the _in vitro_ assembly of polyoma virions that are indistinguishable from those formed _in vivo_, the assembly reactants must include the core histones as present in native virions.

In the case of _in vitro_ self-assembly of tobacco mosaic virus, tobacco mosaic virus proteins are able to

discriminate nicely between their own and other types of RNA (Casjens and King, 1975; Richards and Williams, 1976). It has been proposed that specific nucleotide binding domains recognize and attach to a nucleation region approximately 100 nucleotides in length. Whether the tobacco mosaic virus proteins (or disk-like structures) recognize a sequence periodicity of the form AXXAXX... within this 100 nucleotide region, however, has not been clarified (Richards and Williams, 1976). In contrast to this specific recognition process, polyoma empty capsids are shown not to require or prefer a specific DNA sequence for PLP formation. In terms of polyoma assembly, these data suggest that a specific recognition mechanism for encapsidation of the polyoma genome in vitro, and probably in vivo, is lacking. This might apply whether polyoma morphogenesis proceeds via "the engulfment of the minichromosome" model or "the coalescence of capsid proteins around the nucleohistone core" mechanism. The lack of a specific recognition mechanism may be the underlying reason for pseudovirus production in which mouse (host) DNA instead of polyoma DNA is encapsidated (Aposhian, 1975).

The lack of sequence specificity in polyoma empty capsids binding to polyoma DNA led to the investigation of the binding efficiency when structural differences other

than primary sequence are present (Figure 8). It is not surprising that double stranded DNA (dsDNA) even in the supercoiled form is more efficient than single stranded DNA (ssDNA) or 28S ribosomal RNA in PLP formation. The reduced extent of PLP formation with RNA as a substrate is probably due to excessive secondary structure. The lower affinity for single strandedness is consistent with the fact that polyoma has dsDNA and thus its internucleosomal regions may be better stabilized by enhanced affinity for dsDNA. The extent of PLP formation with poly(dA).poly(dT) is about the same as that for dsDNA. The shape of the curve suggests a similar mode of binding. On the other hand, the smaller slope of the poly(dG).poly(dC) curve suggests a different type of binding mechanism and a lower affinity for this homopolymer. The reduced affinity may be due to a different type of helical structure for the poly(dG).poly(dC). The observations on non-specific binding of dsDNA are consistent with a recently proposed model by Kim, Sussman, and Church (1975) for non-specific interaction of protein with DNA. According to this model, contact occurs at the narrow groove of DNA. Each of the two DNA strands run anti-parallel to the backbone of the peptide with which hydrogen bonds are formed. The peptide is in the β structure, the backbone of which fits nicely into the minor groove.

Hydrogen bonding type of interaction is consistent with high salt stability. Environments of high salt are disruptive to forces involving ionic but not hydrogen bonding. Thus, the polyoma empty capsids-DNA complex and its conversion to PLP provides a novel model system for studying protein-DNA interactions. Clearly, protein recognition of nucleic acids is one of the most fundamental of molecular processes. While numerous examples of DNA-protein complexes exist, the polyoma empty capsid-DNA system is unique in that, eventhough the protein moiety involved is a complex superstructure, it is probably not as intricate as the ribosome, for example. In addition, the fact that empty capsids interact with a variety of nucleic acids suggests that the interactions are simple non-specific ones, the study of which might provide a stepping stone for dissecting more complex systems.

PLP as a Gene Transfer Vehicle

The second group of experimental results demonstrate that PLP containing the BclI-EcoRI transforming fragment of polyoma DNA can transfer this DNA fragment to rat cells. The genetic information of the fragment is then expressed and cellular transformation results. That this oncogenic transformation is the result of the transfer and

subsequent expression of the viral oncogenic sequences is indicated by the failure of PLP formed with EcoRI-BclI non-transforming sequences or with ϕX174 replicative form DNA to induce dense foci formation. In addition, the various controls performed show that the observed activity cannot be accounted for by polyoma virions contaminating the empty capsid preparations used to make PLP.

The fact that transformed cell cultures can be established from individual dense foci produced by infection with PLP containing the BclI-EcoRI transforming fragment of polyoma DNA and maintained indefinitely, suggests that the DNA transferred continues to be expressed and that transformation of the cells is stable rather than transient. Stable expression of a DNA fragment following its introduction into a mammalian cell is generally accompanied by stable integration into the recipient cell's genome. Indeed, this has been found to be the case for the BclI-EcoRI transforming fragment of polyoma DNA following its delivery to rat cells by the calcium phosphate coprecipitation method (Novak et al., 1980). As for the transfer of this DNA fragment by PLP, it appears reasonable to assume that with this system the fragment also becomes integrated. Direct evidence for the physical integration of exogenous DNA into the genome of recipient cells can be obtained by the application of the Southern blotting

technique (Southern, 1975). In this technique, total DNA from recipient cells is isolated, treated with one or more restriction endonucleases and fractionated by gel electrophoresis. After denaturation, fractionated DNA is transferred to a nitrocllulose membrane and immobilized. The presence of integrated donor DNA sequences is then determined by hybridization to a radiolabeled donor DNA probe followed by autoradiography.

Although limited to some extent by the size of DNA that can be encapsidated and therefore transferred, PLP, as a gene transfer system, offers significantly enhanced efficiency over the widely used calcium phosphate coprecipitation method. In addition, the PLP system does not require the use of carrier DNA, which might interfere with a clear understanding of the mechanisms of gene integration and/or expression in the recipient cell. Other properties of the PLP system offer additional advantages. The papovavirus coat provides PLP with structural specificity for penetrating the cell membrane of various mammalian hosts including human cells (Takemoto et al., 1978). Furthermore, the ability of the polyoma empty capsids to encapsidate, <u>in vitro</u>, DNA of any given sequence allows any gene of the appropriate molecular weight to be encapsidated for transfer.

Collectively, these characteristics of the PLP

system provide a novel capability for application to in vitro and, potentially, in vivo gene transfer studies. In terms of in vivo gene transfer investigations, the rat preproinsulin gene (1,620 base pairs) including its intervening sequences and the regulatory signals at the 5' and 3' termini (Sarver et al., 1981), among a number of other genes (e.g., Hanggi and Zachau, 1980; Lowy et al., 1980; Benyajati et al., 1981; Wagner, Sharp, and Summers, 1981; Canaani and Berg, 1982), is of a suitable size for encapsidation. PLP containing this gene, for example, might be tested in the Wistar rat (Nakhooda et al., 1977) which has insulin-dependent diabetes mellitus.

Conclusions

In summary, the data from the experiments conducted permit the formulation of the following conclusions:

1. Optimum PLP yield occurs when PLP formation reaction is carried out at a NaCl concentration below 15mM and BSA is included at a concentration between 0.45 and 2.6µg/µl at the initial step of the reaction.

2. Efficient PLP formation requires the DNA substrate to be greater than or equal to 500 base pairs. Larger DNA molecules result in even greater PLP formation efficiency.

3. PLP DNA is heterogeneous in size with an average molecular weight of 1.2×10^6 and a standard deviation of $\pm 0.5 \times 10^6$.

4. A specific primary, secondary or superhelical tertiary structure in DNA is not required for PLP formation.

5. PLP can transfer genetic information to mammalian cells in culture as indicated by the stable transformation of rat cells following infection by PLP containing the transforming sequences of polyoma DNA.

6. Transfer of genetic information by PLP is 50 to 150 times more efficient than by the widely used calcium phosphate coprecipitation method of introducing DNA into mammalian cells.

REFERENCES

Adolph, K.W., Caspar, D.L.D., Hollingshead, C.J., Lattman, E.E. Phillips, W.C. and Murakami, W.T. (1979) Science 203, 1117-1120.

Aposhian, H.V. (1975) in "Comprehensive Virology" (Fraenkel-Conrat, H. and Wagner, R.R. eds) Vol. 5, pp. 155-218, Plenum Publishing Corp., New York.

Aposhian, H.V., Thayer, R.E. and Qasba, P.K. (1975) J. Virol. 15, 645-653.

Barr, S.M., Keck, K. and Aposhian, H.V. (1979) Virology 96, 656-659.

Bastin, M., Bourgaux-Ramoisy, D. and Bourgaux, P. (1980) J. Gen. Virol. 50, 179-184.

Baumgartner, I., Kuhn, C. and Fanning, E. (1979) Virology 96, 54-63.

Benyajati, C., Place, A.R., Powers, D.A. and Sofer, W. (1981) Proc. Natl. Acad. Sci. USA 78, 2717-2721.

Blin, N., Gabain, A.V. and Bujard, H. (1975) FEBS Lett. 53, 84-86.

Bolen, J.B., Anders, D.G., Trempy, J. and Consigli, R.A. (1981) J. Virol. 37, 80-91.

Boyce, F.M., Sundin, O., Barsoum, J. and Varshavsky, A. (1982) J. Virol. 42, 292-296.

Brady, J.N., Winston, V.D. and Consigli, R.A. (1978) J. Virol. 27, 193-204.

Brady, J.N., Kendall, J.D. and Consigli, R.A. (1979) J. Virol. 32, 640-647.

Canaani, D. and Berg, P. (1982) Proc. Natl. Acad. Sci. USA 79, 5166-5170.

Capecchi, M.R. (1980) Cell 22, 479-488.

Casjens, S. and King, J. (1975) Ann. Rev. Biochem. 44, 555-611.

Casper, D.L.D. and Klug, A. (1962) Cold Spring Harb. Symp. Quant. Biol. 27, 1-24.

Chowdhury, K., Light, S.E., Garon, C.F., Ito, Y. and Israel, M.A. (1980) J. Virol. 36, 566-574.

Christiansen, G., Landers, T., Griffith, J. and Berg, P. (1977) J. Virol. 21, 1079-1084.

Christiansen, G. and Griffith, J.D. (1977) Nucleic Acids Res. 4, 1837-1851.

Cline, M.J., Stang, H., Mercola, K., Morse, L., Ruprecht, R., Browne, J., and Salser, W. (1980) Nature (London) 284, 422-425.

Coca-Prados, M. and Hsu, M.-T. (1979) J. Virol. 31, 199-208.

Coca-Prados, M., Vidali, G. and Hsu, M.-T. (1980) J. Virol. 36, 353-360.

Collins, J., and Hohn, B. (1978) Proc. Natl. Acad. Sci. USA 75, 4242-4246.

Crawford, L.V. (1962) Virology 18, 177-181.

Crawford, L.V., Crawford, E.M. and Watson, D.H. (1962) Virology 18, 170-176.

Crawford, L.V. and Crawford, E.M. (1963) Virology 21, 258-263.

Cremisi, C., Pignatti, P.F., Croissant, O. and Yaniv, M. (1976) J. Virol. 17, 204-211.

Diacumakos, E.G. (1973) in "Methods in Cell Biology", ed. Prescott, D.M. (Academic Press, New York, NY), Vol 7, pp. 287-311.

DiMaio, D., Treisman, R. and Maniatis, T. (1982) Proc. Natl. Acad. Sci. USA 79, 4030-4034.

Dulbecco, R. and Vogt, M. (1954) J. Exp. Med. 99, 167-182.

Ege, T. and Ringertz, N.R. (1974) Exp. Cell Res. 87, 378-382.

Fanning, E. and Baumgartner, I. (1980) Virology 102, 1-12.

Farber, F.E., Melnick, J.L. and Butel, J.S. (1975) Biochim. Biophys. Acta. 390, 298-311.

Fenner, F. (1976) Intervirology 7, 20-54.

Fernandez-Munoz, R., Coca-Prados, M. and Hsu, M.T. (1979) J. Virol. 29, 612-623.

Finch, J.T. (1974) J. Gen. Virol. 24, 359-364.

Finch, J.T. and Crawford, L.V. (1975) in "Comprehensive Virology" (Fraenkel-Conrat, H. and Wagner, R.R., eds), Vol. 5, pp. 119-154, Plenum Publishing Corp., New York.

Fournier, R.E.K. and Ruddle, F.H. (1977) Proc. Natl. Acad. Sci. USA 74, 319-323.

Fraley, R., Subramani, S., Berg, P. and Papahadjopoulos, D. (1980) J. Biol. Chem. 255, 10431-10435.

Frearson, R.M. and Crawford, L.V. (1972) J. Gen. Virol. 14, 141-155.

Friedmann, T. (1971) Proc. Natl. Acad. Sci. USA 68, 2574-2578.

Garber, E.A., Seidman, M.M. and Levine, A.J. (1978) Virology 90, 305-316.

Garber, E.A., Seidman, M.M., and Levine, A.J. (1980) Virology 107, 389-401.

Goff, S.P. and Berg, P. (1979) J. Mol. Biol. 133, 359-383.

Graessmann, M. and Graessmann, A. (1976) Proc. Natl. Acad. Sci. USA 73, 366-370.

Graham, F.L. and Van der Eb, A.J. (1973) Virology 52, 456-467.

Griffith, J.D. (1975) Science 187, 1202-1203.

Gross-Bellard, M., Oudet, P., and Chambon, P. (1973) Eur. J. Biochem. 36, 32-38.

Gross, L. (1953a) Proc. Soc. Exp. Biol. Med. 83, 414-421.

Gross, L. (1953b) Cancer 6, 948-958.

Gross, L. (1955) Proc. Soc. Exp. Biol. Med. 88, 362-368.

Hassell, J.A., Topp, W.C., Rifkin, D.B. and Moreau, P.E. (1980) Proc. Natl. Acad. Sci. USA 77, 3978-3982.

Hanggi, U.J. and Zachau, H.G. (1980) Gene 9, 271-285.

Hirt, B. (1967) J. Mol. Biol. 26, 365-369.

Ishiura, M., Hirose, S., Uchida, T., Hamada, Y., Suzuki, Y. and Okada, Y. (1982) Mol. Cell. Biol. 2, 607-616.

Jakobovits, E.B. and Aloni, Y. (1980) Virology 102, 107-118.

Kim, S.H., Sussman, J.L., and Church, G.M. (1975) in "Structure and Conformation of Nucleic Acids and Protein-Nucleic Acid Interactions" (Sundaralingam, M. and Rao, S.T. eds) pp. 571-575, University Park Press, Baltimore.

Klug, A. (1965) J. Mol. Biol. 11, 424-431.

Klug, A. and Finch, J.T. (1965) J. Mol. Biol. 11, 403-431.

Kressmann, A., Clarkson, S.G., Pirrotta, V., and Birnstiel, M.L. (1978) Proc. Natl. Acad. Sci USA 75, 1176-1180.

LaBella, F. and Vesco, C. (1980) J. Virol. 33, 1138-1150.

Lai, C-J and Nathans, D. (1974) Virology 60, 466-475.

Lake, R., Barban, S. and Salzman, N. (1973) Biochem. Biophys. Res. Com. 54, 640-647.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

Lowy, I., Pellicer, A., Jackson, J.F., Sim, G-K., Silverstein, S. and Axel, R. (1980) Cell 22, 817-823.

Loyter, A., Scangos, G.A. and Ruddle, F.H. (1982) Proc. Natl. Acad. Sci USA 79, 422-426.

McBride, O.W. and Ozer, H.C. (1973) Proc. Natl. Acad. Sci USA 70, 1258-1262.

McCutchan, J.H. and Pagano, J.S. (1968) J. Natl. Cancer Inst. 41, 351-357.

McDonell, M.W., Simon, M.N., and Studier, F.W. (1977) J. Mol. Biol. 110, 119-146.

Mertz, J.E., and Gurdon, J.B. (1977) Proc. Natl. Acad. Sci. USA 74, 1502-1506.

Miller, C.L. and Ruddle, F.H. (1978) Proc. Natl. Acad. Sci USA 75, 3346-3350.

Motulsky, A.G. (1983) Science 219, 135-140.

Muller, U., Zentgraf, H., Eicken, I. and Keller, W. (1978) Science 201, 406-415.

Mulligan, R.C., Howard, B.H. and Berg, P. (1979) Nature (London) 277, 108-114.

Murakami, W.T., Fine, R., Harrington, M.R. and Sassan, Z.B. (1968) J. Mol. Biol. 36, 153-166.

Nakhooda, A.F., Like, A.A., Chappel, C.I., Murray, F.T., and Marliss, E.B. (1977) Diabetes 26, 100-112.

Novak, U., Dilworth, S.M. and Griffin, B.E. (1980) Proc. Natl. Acad. Sci. USA 77, 3278-3282.

Ozer, H.L. (1972) J. Virol. 9, 41-51.

Ozer, H.L. and Tegtmeyer, P. (1972) J. Virol. 9, 52-60.

Pellicer, A., Robins, D., Wold, B., Sweet, R., Jackson, J., Lowy, I., Roberts, J.M., Sim, G.K., Silverstein, S. and Axel, R. (1980) Science 209, 1414-1422.

Pett, D.M., Estes, M.K. and Pagano, J.S. (1975) J. Virol. 15, 379-385.

Polisky, B. and McCarthy, B. (1975) Proc. Natl. Acad. Sci. USA 72, 2895-2899.

Ponder, B.A.J., Robbins, A.K. and Crawford, L.V. (1977) J. Gen. Virol. 37, 75-83.

Ponder, B.A.J., Crew, F. and Crawford, L.V. (1978) J. Virol. 25, 175-186.

Radloff, R., Bauer, W. and Vinograd, J. (1967) Proc. Natl. Acad. Sci USA 57, 1514-1521.

Rayment, I., Baker, T.S., Casper, D.L.D., and Murakami, W.T. (1982) Nature (London) 295, 110-115.

Rechsteiner, M. (1978) Natl. Cancer Inst. Monogr. 48, 57-64.

Richards, K.E., and Williams, R.C. (1976) in "Comprehensive Virology" (Fraenkel-Conrat, H. and Wagner, R.R. eds) vol. 6, pp. 1-37, Plenum Publishing Corp., New York.

Riemer, S., and Bloomfield, V.A. (1978) Biopolymers 17, 785-794.

Salzman, N.P. and Khoury, G. (1974) in "Comprehensive Virology" (Fraenkel-Conrat, H. and Wagner, R.R., eds), Vol. 3, pp 63-141, Plenum Publishing Corp., New York.

Sandri-Goldin, R.M., Goldin, A.L., Levine, M., and Glorioso, J.C. (1981) Mol. Cell. Biol. 1, 743-752.

Sarver, N., Gruss, P., Ming, F-I., Khoury, G. and Howley, P. (1981) Mol. Cell. Biol. 1, 486-496.

Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) Science 215, 166-168.

Schaffner, W. (1980) Proc. Natl. Acad. Sci. USA 77, 2163-2167.

Schor, S.L., Johnson, R.T. and Mullinger, A.M. (1975) J. Cell Sci 19, 281-303.

Seidman, M., Garber, E., and Levine, A.J. (1979) Virology 95, 256-259.

Soeda, E., Arrand, J.R., Smolar, N., Walsh, J.E. and Griffin, B.E. (1980) Nature (London) 283, 445-453.

Sompayrac, L.M. and Danna, K.J. (1981) Proc. Natl. Acad. Sci. USA 78, 7575-7578.

Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.

Spandidos, D.A. and Siminovitch, L. (1977) Proc. Natl. Acad. Sci. USA 74, 2943-2947.

Stewart, S.E., Eddy, B.E. and Borgese, N. (1958) J. Natl. Cancer Inst. 20, 1223-1243.

Studier, F.W. (1965) J. Mol. Biol. 11, 373-390.

Tabin, C.J., Hoffman, J.W., Goff, S.P. and Weinberg, R.A. (1982) Mol. Cell. Biol. 2, 424-436.

Takemoto, K.K., Bond, S.B., Haase, A.T. and Ting, R.C. (1978) J. Virol. 25, 326-330.

Tibbetts, C. and Giam, C-Z. (1979) J. Virol. 32, 995-1005.

Tooze, J., ed. (1980) "DNA Tumor Viruses" Part 2, Cold Spring Harbor Laboratory, New York.

Vinograd, J. and Hearst, J.E. (1962) Fortschritte Der Chemie Organischer Naturstoffe 20, 372-422.

Wagner, M.J., Sharp, J.A. and Summers, W.C. (1981) Proc. Natl. Acad. Sci. USA 78, 1441-1445.

Wells, R.D., Larson, J.E. and Grant, R.C. (1970) J. Mol. Biol. 54, 305-316.

Wigler, M., Sweet, R., Sim, G.K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. and Axel, R. (1979) Cell 16, 777-785.

Williamson, B. (1982) Nature (London) 298, 416-418.

Winocour, E. (1963) Virology 19, 158-168.

Wong, T-K., and Neumann, E. (1982) Biochem. Biophys. Res. Com. 107, 584-587.