THE CELL-FREE ASSEMBLY OF A PANCREATIC DNASE-RESISTANT AND SALT-RESISTANT POLYOMA-LIKE PARTICLE FROM SEPARATELY PURIFIED POLYOMA EMPTY CAPSIDS AND POLYOMA DNA

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ABSTRACT

These investigations represent an effort to reassemble polyoma virions by incubating separately purified polyoma empty capsids and polyoma component I DNA in a cell-free system. A rapid dilution method (low ionic strength treatment) was developed which induces the formation of polyoma-like particles (PLPs). PLPs are stable in high salt concentrations (1.30 g/cc CsCl), and contain DNA which is resistant to pancreatic DNase (100 μg/ml). The buoyant density of PLPs (1.32 g/cc) is intermediate between full virions (1.34 g/cc) and empty capsids (1.29 g/cc). These properties indicate that the DNA of PLPs is located within the capsid.

The DNA of PLPs has been extracted and characterized. It consists of a homogeneous population of linear, double-stranded molecules, devoid of single strand nicks, with a molecular weight of 1×10^6 daltons (approximately one third the size of the polyoma genome). Up to 18% of the polyoma DNA added to the PLP forming reaction mixture is incorporated into PLPs. It is also reported that DNA from simian virus-40 and lambda, is taken up by polyoma empty capsids in similar experiments. The significance of these findings is discussed with reference to the possible use of polyoma capsids as vectors for gene delivery in mammalian tissue culture systems, and possibly in whole animals.

CHAPTER 1

INTRODUCTION

Viruses, in their simplest form, consist of an informational molecule of nucleic acid surrounded by a proteinaceous shell. Therefore, the essential step to be completed in the assembly of any virus is the enclosure of the viral nucleic acid within a polymeric capsid (encapsidation). With this in mind, the assembly of any virus can be assigned to one of three fundamental assembly models.

Fundamental Models for Virus Assembly

According to the first model, viral nucleic acid assumes a compact configuration prior to capsid assembly (Casjens and King, 1975, p. 562). Condensation of the nucleic acid results from an inherent high degree of secondary structure, and the addition of minor protein fractions forming a nucleoprotein core. Capsid subunits (capsomers) subsequently arrange themselves around this core to form the viral capsid. As capsomers are added the nucleic acid assumes its viral configuration. Viruses assembling in this manner include the single stranded RNA bacteriophages fr, f2, R17, MS2 and Q-beta.

The second model describes virus assembly as a "head filling" process, in which the nucleic acid enters a preformed empty capsid or pro-head (Casjens and King, 1975, p. 567). Most of the bacterial viruses, whose assembly mechanisms have been resolved, follow this

pattern. These include Salmonella typhinurium phage P22, and coliphages lambda, T2, T4, T7, and P4. A head filling assembly mechanism has also been described for the animal viruses adenovirus and poliovirus. The nucleic acid of these viruses is replicated as an oversize concatomer, which is cleaved enzymatically during the encapsidation process. Several variations exist in that both sequence-specific and non-specific cleavages occur (Sternberg and Weisberg, 1975). In T-even phages a headfull of DNA is packaged, containing slightly more than an entire genome. In this way the concatomer is cleaved into a population of circularly permuted molecules. Conversely, the complementary single-stranded ends of a lambda phage DNA are precisely defined by enzymes which cut the concatomer at specific nucleotide sequences. The encapsidation of P22 DNA initiates with a specific sequence, but terminates by cleaving the concatomer with a non-sequence specific endonuclease after a headfull of DNA has been packaged.

In the third assembly model the viral nucleic acid and protein shell condense simultaneously (Casjens and King, 1975, p. 560). Encapsidation initiates by the attachment of capsomers or aggregates of capsomers to an extended viral nucleic acid molecule. Additional capsomers or aggregates of capsomers are added, elongating the virus structure, and concomitantly internalizing the nucleic acid. The rod-shaped tobacco mosaic virus (TMV) is an example of viruses assembling in this manner.

Factors Contributing to Virus Assembly *In Vivo*

The models outlined above represent only the most fundamental aspect of viral morphogenesis: the enclosure of the nucleic acid. Each virus exhibits unique variations from these basic plans. Virus assembly *in vivo* may require the participation of additional components which may or may not be retained in the completed virion. Contributing factors may include non-capsid proteins, membranous components, virus or host-coded enzymes, polyamines, nucleotide triphosphates, or the presence of specific ions (Casjens and King, 1975).

The protein components of many viruses are significantly altered during assembly. Proteins may be added, lost, cleaved or fused in various combinations (Casjens and King, 1975, p. 568). For example, the empty capsid of P22 assembles around a preformed core of "scaffolding protein," which appears to exit as the viral DNA is encapsidated. The details of this exchange are unknown. Alternatively, major capsid proteins are added late in the assembly of lambda phage. In T4 assembly major coat proteins are cleaved prior to DNA encapsidation, and minor protein components cleaved during encapsidation. Some cleavage products are retained in the mature virion, others are not. Lambda phage assembly requires both protein cleavage and protein fusion. The fusion of proteins is thought to render the assembly process irreversible and may prove to be a general feature of virus assembly (Casjens and King, 1975, p. 571).

Cell-free Reassembly of Virions

The cell-free reassembly of virus particles, from separately isolated and purified viral components, has been accomplished with several viruses. Viral materials used in such reassembly experiments are derived either from disrupted virions, or from extracts of virus infected cells (Kaper, 1975). In other experiments, blocked intermediate virus structures, from temperature sensitive assembly mutants, are used as precursors for cell-free virus assembly (Edgar and Wood, 1966). The methodologies used to induce the cell-free reconstitution of virions have been extensively reviewed (Kaper, 1975) and are considered in detail in the experimental portion of this study.

Tobacco mosaic virus (TMV) was the first virus to be successfully reassembled in a cell-free system (Fraenkel-Conrat and Williams, 1955). To achieve this, virions were dissociated in either 67% acetic acid or 8 M urea, and their proteins isolated. The stepwise removal of the denaturing agents produced trimers of TMV protein subunits. After the addition of TMV-RNA, dialysis was continued to a pH of 7.5. The total dialysis period ranged from 25 to 37 hours, and produced rod-shaped structures. The length of these rods was determined by the length of TMV-RNA encapsidated. These experiments represent the first demonstration of the self-assembly concept for macromolecular systems.

Following the initial success with TMV, several other plant viruses were reassembled. The rod-shaped alfalfa mosaic virus, and icosahedral cucumber mosaic, brome mosaic, braod bean mottle, and cowpea chlaretic mottle viruses have all been reassembled in similar dialysis experiments (Bancroft, 1970). Virions were dissociated in

1 M NaCl and their protein and RNA fractions separated. A solution of phenol-extracted viral RNA was then added to produce a final concentration of 0.5 M NaCl. Dialyzing this solution to 0.01 M KCl induced the spontaneous reassembly of infectious virions. A rapid dilution technique has also been described for cucumber mosaic virus (Kaper and Geelen, 1971). In this case, an 0.5 M LiCl solution was diluted to 0.2 M with the concomitant reassembly of infectious virions. Tomato bushy and turnip crinkle viruses have been reconstituted by dialyzing solutions of viral components suspended in 0.5 M KCl - pH 9.0 to lower ionic strength and neutrality (Kaper, 1975, p. 378).

Several RNA containing bacteriophages, fr, f2, R17, and Q-beta, have also been reassembled in cell-free systems (Kaper, 1975, p. 364). In these experiments phage RNA, derived from phenol-extracted virions, was combined with capsid proteins, dissociated with 66% acetic acid. Dialyzing this solution to neutrality produced infectious virions. Alternatively, reassembly can be accomplished by a similar dialysis procedure in which 8 M urea, or 5 M guanidine hydrochloride is used to promote the dissociation of virus proteins. An additional protein component (A-protein) is required for infectivity in this group of viruses (Kaper, 1975, p. 370).

In 1975 Kaiser, Syvanen, and Masuda reported the reassembly of infectious lambda phage from purified lambda DNA, DNA-free "petite lambda particles," and a cell-free extract of induced lysogenic cells. An infectivity efficiency of one plaque formed per 1000 petite lambda and 1000 lambda DNA molecules was reported.

Attempts at reassembling infectious animal viruses have been generally unsuccessful. Although the reversible dissociation of polio virus, resulting in a reduction of infectivity to 0.05%, has been demonstrated (Drzenick and Bilello, 1972), to this author's knowledge no infectious animal virions have been reconstructed in physico-chemically identifiable quantities, from isolated viral components.

CHAPTER 2

POLYOMA VIRUS STRUCTURE

Polyoma is a relatively small animal virus, belonging to the Papovavirus group (polyoma virus, simian virus-40, and adenovirus). Polyoma causes a variety of tumors in new born mice (its natural host) and several other species. Polyoma virus also induces transformation in hamster cells grown in tissue culture. After the establishment of a suitable tissue culture method for mouse embryo cells (Stewart, Eddy, and Borgese, 1958) polyoma virus was studied as a physical particle. An abundance of structural information has resulted. Virions have a diameter of 45 nm and a particle weight of 28×10^6 daltons (Tooze, 1973, p. 271). They are composed entirely of DNA and protein, and contain no lipid or carbohydrate.

DNA

The DNA in polyoma virions exists in three distinct forms: component I, II, and III (Tooze, 1973). Polyoma component I DNA is a covalently closed, double-stranded molecule with an average of 21 super helical turns imposed upon it (Germond et al., 1975). Component I DNA is contained in a majority of infectious particles. In neutral sucrose gradients it has a sedimentation coefficient of 20S, while in alkaline gradients (pH 12.5 or above) it sediments at 53S due to the collapse of the super coiled configuration. The introduction of a single break in

one strand of component I DNA relaxes the super-coiled configuration, and generates component II DNA. Component II DNA sediments at 16S in neutral sucrose gradients, and separates into distinct 16S and 18S components in alkaline gradients, representing the separation of linear and circular single-stranded molecules under alkaline conditions. In some preparations linear double-stranded DNA molecules are encapsidated. These have been designated component III DNA and consist largely of cleaved host cell DNA, cut to 3.6×10^6 daltons and encapsidated. Particles containing host cell DNA have been termed pseudovirions (Michel, Hirt, and Weil, 1967).

Polyoma DNA exists as a nucleoprotein complex, both in extracts from polyoma infected cells and in mature virions (McMillen and Consigli, 1974). The protein complement of the nucleoprotein complexes is composed of low molecular weight basic proteins which have been identified as histone proteins coded for by the host cell genome (Frearson and Crawford, 1972). Nucleoprotein complexes closely resemble the structure of mammalian chromatin in that their DNA is organized into histone containing nucleosomes. This led Griffith (1975) to describe nucleoprotein complexes derived from simian virus-40 (SV-40) infected cells as "mini-chromosomes". The existence of similar structures in polyoma infected cells was reported by Cremisi et al. in 1976. One year later, G. Christiansen et al. (1977) reported that mini-chromosomes could be obtained from alkaline disrupted SV-40 virions. The structural similarity between mammalian chromatin and papovavirus mini-chromosomes provides a useful model system for studying the prohibitively complex mammalian material. However, in contrast to mammalian chromatin the nucleosomes

of mini-chromosomes are not evenly spaced along the DNA (Ponder, Crew, and Crawford, 1978). This may prove to be a consequence of the constraints placed upon the nucleoprotein complex as it exists within the viral capsid. Further, unlike mammalian chromatin the internucleosomal streches of DNA in polyoma virus nucleoprotein complexes lack histone proteins (Griffith, 1975). G. Christiansen et al. (1977) have reported that nucleoprotein complexes obtained from SV-40 infected cells contain loosely bound cellular histone H1, which is not found in mature virions. This H1 histone may temporarily occupy the internucleosomal streches of DNA prior to virus assembly.

Protein

Surrounding the nucleoprotein core is a protective capsid, constructed entirely of proteins. This hollow shell is composed of 420 protein molecules arranged into 72 capsomers (Tooze, 1973, p. 271). Three dimentional image reconstruction of electron micrographs show the capsid to be icosahedral in shape, with the capsomers organized on a laevo T = 7d surface lattice (Finch, 1974). Under extreme magnification the capsomers are observed to be hollow and slightly conical in shape, with a diameter of approximately 5 nm (Finch, 1974). Two distinct types of capsomers are found: (1) pentomers, which are coordinated with five neighboring capsomers, and (2) hexomers, which are coordinated with six neighbors (Wildy et al., 1960). Virions contain 12 pentomers, located at the apices of the icosahedron, and 60 hexomers filling in the faces of the structure (Finch and Crawford, 1975, p. 126). As will be detailed below, the composition of pentomers and hexomers has not been

conclusively determined, nor has any immunologic distinction been found, as is the case in adenovirus (Tooze, 1973, p. 426).

The proteins of polyoma virions can be resolved into at least seven distinct polypeptides by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Estes, Huang, and Pogano, 1971). These polypeptides have been designated VP1, VP2 . . . VP7, in order of decreasing molecular weight. The electrophoretic peaks corresponding to VP3, VP4 and VP6 are split into two distinct components designated a and b in each case (Gibson, 1974). VP1, VP2, and VP3 are the structural proteins which together comprise the viral capsid (molecular weights 48,000, 35,000, and 23,000 respectively), (Hewick, Fried, and Waterfield, 1975). VP1 is the major structural protein, and accounts for nearly 75% of the virion's particle weight (Finch and Crawford, 1975, p. 141). Tryptic peptide analysis has determined that VP3 is a proteolytic cleavage product of VP2 (Hewick et al., 1975). VP1 and VP2 are non-homologous in their peptide composition. The two genes that code for the three structural proteins in polyoma virions occupy approximately 43% of the coding capacity of the polyoma genome (Hewick et al., 1975).

From stoichiometric evidence Finch (1974, p. 363) stated that "It is numerically possible that VP1 could account for the 60 hexomers and either VP2 or VP3 or both for the pentomers." This is in agreement with G. Christiansen et al. (1977) who found that 7S particles, identical in appearance to hexomers, can be released from SV-40 virions by exposing them to pH 9.8. Peptide analysis revealed only VP1 to be present in the 7S particles.

Alternatively, Ponder, Robbins, and Crawford (1977) have reported that VP1 exists in two electrophoretically distinct forms; phosphorylated and non-phosphorylated. The proportion of phosphorylated VP1 to non-phosphorylated VP1 is also approximately equal to the ratio of pentomers to hexomers. This has led Ponder to postulate that hexomers are composed of non-phosphorylated VP1 and that pentomers are composed of phosphorylated VP1.

Fingerprint analysis has identified VP4, VP5, VP6, and VP7 as cellular histones H2A, H2B, H3, and H4 (Lake, Barban, and Salzman, 1973). Mature virions lack histone H1, while nucleoprotein complexes extracted from SV-40 infected cells do not (G. Christiansen et al., 1977). At least a portion of the histones in polyoma virions are free to enter and leave the virus with relative ease (Frost and Bourgaux, 1978).

It should be pointed out that any meaningful review of polyoma virus structure and assembly should include data from studies of simian virus-40. These two closely related papovaviruses have virtually identical capsid structures and are generally considered to assemble via similar mechanisms. For this reason, evidence pertaining to both viruses will be presented in the remainder of this report.

CHAPTER 3

POLYOMA VIRUS DISSOCIATION

Several investigators have attempted to elucidate fine points of virus structure and assembly by careful observation of virions in controlled degradation experiments.

Huang, Estes, and Pogano (1972) found that exposure of SV-40 virions to a pH 10.5 disruption media containing beta-mercaptoethanol resulted in the dissociation of VP1 and VP2, leaving a "deoxynucleoprotein core I" (DNP-I) containing histones VP4, VP5, VP6 and all or part of structural protein VP3. Isopycnic centrifugation of DNP-I dissociates the histone proteins but not the VP3, producing DNP-II. Even though the polypeptides remaining attached to DNP-II were not identified, the absence of solubilized VP3 led the authors to conclude that "at least a portion of VP3 is tightly bound to the viral DNA." The omission of beta-mercaptoethanol from the pH 10.5 disruption buffer yielded reduced amounts of soluble VP1 and VP2 with the corresponding appearance of material with a molecular weight roughly equivalent to the sum of VP1 and VP2. This led the authors to conclude that "VP1 and VP2 might exist as compact structures cross-linked with disulfide bonds, perhaps forming the capsid."

In 1974 Walter and Deppert found that polyoma virions treated with 3% SDS, and analyzed by SDS-polyacrylamide gel electrophoresis in the absence of beta-mercaptoethanol, resulted in the solubilization of

all virus proteins except VP1. The SDS resistant VP1 was found to sediment at 140S in neutral sucrose gradients, a value equivalent to empty capsids. A 1.0 mM dithio-threithol (DTT) treatment was found to dissociate this 140S particle into monomeric VP1. In another experiment empty capsids, composed of VP1, VP2, and VP3 were exposed to increasing concentrations of SDS. An 0.1% SDS concentration was sufficient to remove VP2 and VP3 from the VP1 complex. From this, the authors concluded that "the capsid of polyoma virus represents a very stable, highly polymeric structure of VP1 protein subunits, which are covalently linked by disulfide bridges. VP2 and VP3 are bound to this structure by hydrophobic bonds"

Etchison and Walter (1977) demonstrated that polyoma virus proteins will progressively dissociate on treatment with increasing concentrations of SDS. Concentrations as low as 0.2% were found to transform virions either into empty capsids or into DNA containing particles, which sediment at 80S in neutral sucrose gradients. Presumably, the slow sedimenting 80S particles represent virions with partially extended DNA, as might result from the loss of minor protein components. Polyacrylamide gel electrophoresis of these resultant structures indicates the progressive dissociation of virus proteins. At a concentration of 0.02% SDS histone proteins VP4, VP5 and VP6 begin to dissociate. DNA release may occur prior to the complete dissociation of histones. However, DNA may be retained in SDS concentrations as high as 0.5%. The resulting empty capsids generally contain a nearly full complement of VP1, VP2, and VP3, with greatly reduced amounts of histones. SDS concentrations increasing from 0.03% to 0.5% progressively dissociate

the remaining histones, VP3 and VP2, with VP2 being the last to release completely. At 1% SDS empty capsid-like structures are produced which contain only disulfide cross-linked VP1. Electron microscopic observation of these particles revealed a structure similar to empty capsids, but with a markedly less regular pattern of capsomers, and a somewhat smaller diameter. Although no regular loss of pentomers is observed, it is possible that pentomers were lost causing a spontaneous reordering of capsomers.

In a recent report by G. Christiansen et al. (1977) SV-40 virions were treated with increasing pH in the presence of 1 mM DTT. At pH values up to 9.2 all capsid proteins sediment with the viral DNA in neutral sucrose gradients. Raising the pH to 9.8 yields 60S nucleoprotein complexes, and 7S material consisting of VP1 and minor amounts of VP2. Electron micrographs of the 60S complexes show them to be relaxed circular structures with an average of 21 beads (nucleosomes), joined by thin bridges. The 60S complexes appear to be similar to the 75S complexes which can be extracted from SV-40 infected cells, 25 hours after virus infection. The discrepancy in sedimentation rates is thought to be caused by the presence of cellular histone H1 in the extracted complex. Treatment of the 75S complex with 0.6 M NaCl selectively removes the H1 but not the other histones, and converts the 75S particles into 60S particles, identical to those obtained by pH 9.8 disruption of virions. Treatment of virions with pH 10.5 (as in Huang et al., 1972) results in a broad DNA containing peak centering at 30S in a neutral sucrose gradient. Here, most of the protein is found at the

meniscus of the gradient indicating the further degradation of 7S complexes.

CHAPTER 4

POLYOMA VIRUS ASSEMBLY *IN VIVO*

The *in vivo* steps leading to the formation of infectious polyoma virus remain obscure. In 1973 Tooze (p. 339) wrote that

> We know from immunological and electron microscopic studies that progeny virions are first found in the nuclei of cells productively infected with SV-40 and polyoma virus. But virtually nothing is known about the mechanism of maturation and assembly of progeny virus particles. The functions of the minor protein components of the virions, the role of cellular membrane sites, the contribution of self-assembly mechanisms and the time and way in which polyoma virus or SV-40 DNA is packaged are all open questions.

Since this was written, surprisingly little progress has been made toward understanding the assembly of SV-40 and polyoma virus.

Virions adsorb to receptor sites on the host cell surface (possibly containing neuraminic acid), and penetrate into the cytoplasm via pinocytotic vesicles. These vesicles accumulate around the nuclear envelope (Tooze, 1973). Polyoma virus uncoating is initiated by lysosomal action in the cytoplasm (Zolotor, 1970). However, Mackay and Consigli (1976) have demonstrated the simultaneous arrival of virus structural proteins with the nucleoprotein complex, in the nucleus of polyoma infected cells. Thus, at least part of the uncoating process occurs in the nucleus of infected cells.

The transcription of polyoma and SV-40 DNA occurs in two distinct phases, consisting of "early and late genes" (Tooze, 1973, p. 307). Before the onset of DNA synthesis 30-40% of the genome "the early genes"

are transcribed. The expression of these genes results in the induction of several enzymes related to nucleotide synthesis and DNA metabolism. These enzymes far surpass the coding capacity at the viral genome. Thus, at least some of the induced enzymes are of host origin. Viral DNA synthesis initiates around 18 hours post-infection (Yelton and Aposhian, 1972), with a concomitant stimulation of host cell DNA synthesis (Tooze, 1973, p. 323). Polyoma DNA is replicated as a nucleoprotein complex in close association with the host cell chromatin (Seebeck and Weil, 1974). As newly synthesized stretches of DNA are produced they are immediately bound by cellular histones.

Before the onset of viral DNA synthesis, a general stimulation of host cell protein synthesis occurs (Kiehn, 1973). Approximately 20 hours post-infection virus structural proteins are synthesized in the cytoplasm (Kiehn, 1973). This represents the expression of the remaining 60 - 70% of the viral genome, and constitutes "the late genes" (Tooze, 1973, p. 307). It has been demonstrated that the early genes are transcribed off of the opposite template strand from the late genes. This results in a non-symmetrical transcription in both polyoma and SV-40 (Salzman and Khoury, 1974). The very small cytoplasmic and nuclear pools of free SV-40 VP1 indicate that after its synthesis, VP1 is rapidly transported to the nucleus and there assembled into capsid material (Kiehn, 1973). Conversely, sizable pools of free VP3 are found in both the cytoplasm and nucleus (Kiehn, 1973), implying that VP3 is cleaved from VP2 in the cytoplasm. The actual sequence of events leading to the formation of viral capsids is not understood.

Productive polyoma infections generally result in the formation of two major types of viral particle: (1) full virions, sedimenting at 240S in neutral sucrose gradients, and (2) empty capsids, sedimenting at 140S (Crawford and Crawford, 1963; Murikami et al., 1968). Empty capsid preparations from our laboratory consistently sediment at 190S. This discrepancy may be caused by the incomplete removal of externally bound material in the reported 140S particles. A variety of aberrant particles may also result from productive polyoma infections (Tooze, 1973, p. 340). These may include: (1) defective particles which resemble full virions by most criteria but lack infectivity, (2) pseudovirions, containing linear double stranded host cell DNA within a polyoma capsid, and (3) several types of elongated structures, some of which contain DNA. These non-infectious particles contribute to the high particle to infectivity ratio (100 particles per plaque forming unit) observed for polyoma virus (Tooze, 1973, p. 273).

Pulse chase experiments (Ozer, 1972) have shown that radioactively labeled lysine is preferentially incorporated into SV-40 empty capsids, and subsequently can be chased into full virions. This evidence lends tentative support to the possibility that DNA-free empty capsids might function as intermediates in the formation of full virions. However, the possibility exists that empty capsids might be formed in loose association with the nucleoprotein complex. This association may be disrupted under the conditions used to extract empty capsids. If this is the case, nucleic acid would not appear to be associated with the viral capsid until it was stabilized internally later in virus maturation.

Yelton and Aposhian (1972) have published a time course study of polyoma infection, comparing the relative quantities of empty capsids, full virions and component I DNA, as derived from extracts of infected cells. Empty capsids are produced as early as 30 hours post-infection, while full virions are not detected in large numbers until 36-42 hours post-infection. The study also demonstrates the existence of large pools of excess empty capsids throughout the latter portion of the infectious cycle. It is possible that this excess of empty capsids may indicate the existence of two populations of empty particles: (1) defective particles which are not capable of maturing into full virions, and (2) non-defective particles which serve as precursors to full virions. The possibility also exists that the excess empty capsids might represent an unactivated state. Perhaps an empty capsid maturation step is required before they can function as intermediates in virus assembly. This would be in agreement with the differential rates of formation observed for SV-40 empty capsids and full virions (Ozer, 1972), and further, might help to explain the findings of Tan and Howe (1977) which show that only 16% of the radioactivity in SV-40 nucleoprotein complexes can be chased into full virions.

Tan and Sokol (1974) have demonstrated an arginine dependent step late in the SV-40 assembly process. If suitable host cells are grown on media lacking arginine and infected with polyoma virus, all viral proteins are produced, and are transported into the nucleus, but are not assembled into either empty capsids or full virions. With the subsequent addition of exogenous arginine, virus production occurs immediately, utilizing the viral components previously accumulated in the

nucleus. Some virus production is also observed to result from newly synthesized viral components. If cycloheximide is added with the exogenous arginine, no virus assembly is observed, indicating a requirement for protein synthesis in the final stages of virus assembly. However, if hydroxyurea is added with the arginine, empty capsids are formed but no mature virions, indicating a requirement for DNA synthesis in the production of full virions from empty capsids.

CHAPTER 5

POLYOMA REASSEMBLY EXPERIMENTS IN CELL-FREE SYSTEMS

The first notable attempt to elucidate polyoma virus assembly through cell-free reconstitution experiments was made by Friedmann (1971). Here, virions were exposed to an alkaline environment (0.2 M Na_2CO_3-$NaHCO_3$, pH 10.6) containing 0.01 M DTT. This treatment dissociated virions into their capsomeric subunits with a concomitant loss of all hemagglutination activity. Eighty percent of the protein was converted to 7S particles. Most of the remaining 20% sedimented at 10S with trace amounts being found at 1.5S and 2.6S. Dialyzing disrupted virions against 0.05 M Tris (hydroxymethyl) aminomethane (tris) (pH 8.0), 0.14 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM 2-mercaptoethanol, resulted in the formation of 100S particles which regained 17% of the lost hemagglutination activity. Electron microscopic observation of these particles revealed them to be "linear aggregates of capsomers" strung along circular DNA molecules. Amorphous clumps of capsomers were also observed. DNase treatment of 100S particles produced DNA containing 140S particles which were found to be non-infectious. Electron microscopy of these particles showed them to be roughly spherical in shape, with a diameter slightly smaller than full virions. However, the capsomers were observed to be "irregular and disrupted" in their arrangement. Although these reversible dissociation experiments may have no direct applicability to the <u>in vivo</u>

assembly process, they do unequivocably show that, under certain conditions, capsomers have the ability to bind to polyoma nucleoprotein complexes.

An extension of Friedmann's work was reported by Christensen and Rachmeier (1976). Using the same alkaline disruption medium (pH 10.6), Christensen found that a rapid neutralization, by adding 0.015 ml of 2 N HCl, produced a heterogeneous population of aggregates sedimenting closer to the full virion region of the gradient than did Friedmann's linear aggregates of capsomers. These aggregates contained varying amounts of DNA in a DNase sensitive form some of which were infectious. In another experiment, infectious particles were formed by rapidly neutralizing alkaline disrupted empty capsids in the presence of nucleoprotein complexes extracted from SV-40 infected cells. At pH 10.6, as in the formation of DNP-I (Huang et al., 1972), nucleoprotein complexes will exist as a beaded, somewhat extended circular complex. Unfortunately, no data are offered as to the relative infectivities of the novel particles as compared to full virions and nucleoprotein complexes. Further, the question of whether or not empty capsids reassemble prior to the formation of infectious particles is not addressed.

The data of Friedmann and Christensen provide indirect evidence that polyoma and SV-40 assembly might proceed via the addition of capsomers to an extended viral nucleoprotein complex, which could then collapse into a virus-like structure.

Another attempt to assemble polyoma virions in a cell-free system was reported by Aposhian, Thayer, and Qasba (1975). In these

experiments nucleoprotein binding complexes were formed by incubating purified polyoma empty capsids with histone-free polyoma component I DNA (separately purified from polyoma infected cells) in a media containing 0.015 M tris (pH 7.5), 0.01 M $MgCl_2$, and bovine plasma albumin. Sedimentation analysis and electron microscopy have identified the resultant binding complexes as consisting of single molecules of circular DNA bound by one, two or more empty capsids (monomer, dimer, etc.). The reciprocal situation of two or more DNA molecules binding to a single empty capsid is not observed. However, since the molar ratios of capsids to DNA were consistently held above unity such a reciprocal arrangement would not be favored. Complex formation is observed to be specific for empty capsids in that no complexes are formed when DNA is incubated with either full virions or capsomers prepared by the method of Friedmann (1971). The ionic nature of the binding complexes is indicated by their dissociation in CsCl gradients and their inability to form in concentrations of NaCl above 0.5 M. The complexes are also labile to a 50 ug/ml pancreatic DNase digestion.

The data clearly indicate the existence of a DNA binding site on the empty capsid. Unpublished data show this site to have a very high affinity for DNA, as demonstrated by a low rate of complex dissociation. The presence of a specific DNA binding/recognition site would be of great importance in a capsid filling mechanism for virus assembly. Alternatively, if viral capsids are formed by the addition of capsomer around a nucleoprotein core, such a binding site would serve no apparent use. Thus, the reported data provide additional tentative evidence supporting the intermediate role of empty capsids in polyoma

virus assembly. Although conclusive proof is lacking, the accumulated information implicates empty capsids as being precursors to full virions, and further that the observed binding complexes represent a necessary recognition step prior to the initiation of capsid filling.

From the evidence provided by virus dissociation studies, it seems likely that the DNA within virions is stabilized largely by ionic interactions (Kaper, 1975, p. 230). With this assumption in mind, experimental parameters were sought which might enhance such interactions and possibly induce the spontaneous encapsidation of the DNA attached to binding complexes.

CHAPTER 6

MATERIALS AND METHODS

Preparation of Polyoma Virions and Empty Capsids

Polyoma virions and empty capsids used in the binding experiments were produced by infecting primary mouse embryo cells grown in EC medium (Osterman, Waddell, and Aposhian, 1970). A small plaque variant of the Toronto strain of polyoma virus was used, at a multiplicity of infection of approximately one plaque forming unit per cell (PFU/cell). Virus production and harvesting was performed in accordance with the method of Crawford (1962). Virions and empty capsids radiolabeled with ^{14}C-arginine were produced by adding medium containing citruline and 1% dialyzed calf serum but lacking arginine to the cells after the virus adsorption period. Twenty hours post-infection ^{14}C-arginine was added, and the viruses harvested six days post-infection. Crude extracts were purified by the method of Winocour (1963). The peak fractions of virions and empty capsids were pooled after equilibrium centrifugation in CsCl, and subsequently subjected to two additional CsCl centrifugations. Finally, the peak fractions are sedimented through a neutral 10-40% sucrose gradient, and dialyzed against 0.01 M tris (pH 7.5), 0.05 M NaCl. The virions and empty capsids were located by radioactivities and/or hemagglutination assays.

Preparation of Polyoma DNA

Radioactively labeled polyoma DNA is produced by infecting baby mouse kidney cells grown in Dulbecco's modified Eagles medium containing 10% calf serum. The large plaque strain of polyoma virus was used at a multiplicity of infection of about 1 PFU/cell. Eighteen hours after infection ^{14}C-thymidine or ^{3}H-thymidine was added. Polyoma DNA was extracted 48 hours post-infection using the method of Hirt (1967). The DNA was extracted with phenol, precipitated with alcohol, and banded in two CsCl-ethidium bromide gradients. The ethidium bromide was removed by four extractions with isopropanol and dialysis against standard saline-citrate. Finally, the DNA solution was dialyzed against 0.02 M tris (pH 7.5), 0.02 M NaCl. Concentrations were determined directly on a Beckman DB-G Spectrophotometer, using an extinction coefficient of $E^{1\%}_{260}$= 200. Typically, a 280/260 absorbance ratio of 0.538 was obtained.

Sucrose Density Gradient Centrifugation

Analysis of binding complexes and virus particles was carried out in 10-22% neutral sucrose gradients. Sedimentation was performed in polyallomer centrifugation tubes, as will fit the Beckman SW-50.1 rotor. The sequential layering of nine 0.5 ml aliquots of sucrose stock solutions (10-22% sucrose, Schwarz and Mann ribonuclease free, in 0.01 M tris (pH 7.5) containing 0.15 M NaCl) will produce a non-linear neutral sucrose gradient after equilibration overnight at 4^{o}C. Immediately prior to centrifugation an 0.2 ml CsCl cushion (1.7 g/cc) is gently syringed into the bottom of each gradient. Pre-chilled samples are gently pipetted into the gradients, and loaded into pre-chilled buckets and

rotor. Centrifugation is performed for 90 minutes at 30,000 rpm, 4°C. Three drop fractions are collected with a tygon tubing drop spreader after puncturing the bottom of the tube.

The determination of sedimentation coefficients for either particles or DNA was performed in isokinetic neutral sucrose gradients. Here, 1.0 ml aliquots of the sucrose stock solutions described above are sequentially layered into polyallomer centrifugation tubes as will fit the Beckman SW-41 rotor. Allowing the tubes to equilibrate over night at 4°C. produces an isokinetic (linear) neutral sucrose gradient. Prior to centrifugation an 0.3 ml CsCl cushion is added. Centrifugation is performed for 2 hours at 25,000 rpm, 4°C. for particles, and for 6 hours at 40,000 rpm, 4°C. for DNA. Four drop fractions are collected after puncturing the bottom of the tube.

Five-20% Alkaline sucrose gradients were prepared with a Buchler Instruments gradient maker. In this process, two sucrose stock solutions were used: (1) 5% sucrose in 0.1 M NaOH, 0.5 M NaCl, 0.0025 M EDTA, and (2) 20% sucrose in 0.4 M NaOH, 0.5 M NaCl, 0.0025 M EDTA. Sedimentation of DNA preparations was performed at 49,000 rpm, for 90 minutes, 4°C., in an SW-50.1 rotor. Collection of fractions was as above.

Isopycnic CsCl Centrifugation

Analytical CsCl gradients were used in the characterization of complexes and DNA. Particles were banded in 1.30 g/cc CsCl (Harshaw Chemical Co., optical grade). Here, samples were made up to 4.0 ml with 0.01 M tris (pH 7.5), 0.001 M EDTA. Solid CsCl was weighed in to a final concentration of 1.30 g/cc and the density verified on a

refractometer (Bausch & Lomb). Samples were placed in cellulose nitrate centrifugation tubes as will fit the Beckman SW-50.1 rotor, balanced, overlayered with heavy mineral oil, and balanced again. Centrifugation was performed for 22 hours at 30,000 rpm, 20°C. Three drop fractions were collected (tygon drop spreader) after puncturing the bottom of the tubes. The density profile of the gradients was determined by taking 20 µl aliquots from every third fraction, and measuring their CsCl concentration with a refractometer. Radioactivities were determined as described below. A correction factor was used to compensate for the removal of the 20 µl aliquots.

In a similar procedure DNA was banded in pH 9.0 CsCl gradients. Here, 1.70 g/cc CsCl was used in a buffer containing 0.01 M tris (pH 9.0), 0.001 M EDTA. Centrifugation was performed for 70 hours at 30,000 rpm, 20°C., SW-50.1 rotor. Gradient preparation, fraction collection and the determination of CsCl densities are described above.

Determination of Radioactivities

Radioactivities were determined in a Beckman LS-200 liquid scintillation counter using a toluene based scintillation fluid containing 0.55% 2,5-diphenyloxazole (PPO), 0.01% 1,4-bis-(2-(4-methyl-5-phenyloxazolyl))-benzene (POPOP), 10% distilled water, and 33% Triton-x-100 (Rohm and Haas). A gain setting of 550 is typically used. In ^{14}C, ^{3}H double labeled samples channel cross-talk of 12% was typically obtained, and corrected for.

Other Methods

Hemagglutination assays were performed by the method of Crawford (1962), using guinea pig erythrocytes.

Protein concentrations were determined by the method of Lowry et al. (1951).

Formation of Polyoma-like Particles

Reaction mixtures were compiled in siliconized (Siliclad, Clay Adams Co.) glass test tubes. Typically, they contain 1.9 µg of polyoma component I ^{3}H-DNA (1.25×10^{5} cpm/ug), 25 µg of purified empty capsids, and distilled water to a final volume of 100 µl. The DNA is suspended in 0.02 M tris (pH 7.5), 0.02 M NaCl. The empty capsids are suspended in 0.01 M tris (pH 7.5), 0.15 M NaCl. Samples are incubated for 20 minutes at 37°C. Then 350 ul of distilled water are added and the incubation continued for 40 minutes. Next 50 µl of a pancreatic DNase (Worthington Biochemical Corp.; 2430 dornase units/mg) solution (100 µg/ml in 0.02 M tris (pH 7.5), 0.01 M $MgCl_2$) were added and the incubation continued for a final 20 minutes. The samples are then chilled in ice and analyzed by sedimentation through a 10-22% neutral sucrose gradient, as described above.

Extraction of Polyoma-like Particle DNA

Polyoma-like particle (PLP) containing fractions are collected and pooled from preparative sucrose gradients. The PLP containing sample is brought to 100 µg/ml in non-radioactive calf thymus "carrier" DNA, 5% (weight/volume) with crystaline sarkosyl (Geigy, NL 97), and then incubated in a 55°C. water bath for five minutes. After incubation the

sample is cooled to room temperature and then extracted three times with equal volumes of water saturated phenol (room temperature). Suspension of the phenol was accomplished by gentle mixing with a glass plunger. Phase separation was achieved by low speed centrifugation at 4°C. Each time the phenol layer is discarded (top layer). Following the last phenol extraction the aqueous phase is recovered, and dialyzed 18 hours against 0.01 M tris (pH 7.5), 0.01 M EDTA (three changes, one liter each, 4°C.). If necessary, the samples can be concentrated to a desired volume by packing the dialysis tubing in Aquacide II (Cal Biochemical Corp.).

Molecular Weight Determination for DNA

The molecular weight of DNA extracted from PLPs was determined from its sedimentation coefficient in a neutral, isokinetic sucrose gradient, in accordance with Studier's relation (Studier, 1965).

$$\log_{10} \text{M.W.} = \frac{\log_{10}(S_{w,20})}{0.346}$$

M.W. is the molecular weight of the DNA, and $S_{w,20}$ is the sedimentation coefficient of the DNA corrected from sucrose at 4°C., to water at 20°C.

CHAPTER 7

THE FORMATION OF A POLYOMA-LIKE PARTICLE

In an attempt to achieve the cell-free reconstitution of polyoma virus, an extension was made on the work of Aposhian et al. (1975).

Formation of Binding Complexes

Purified polyoma component I DNA will complex with one or more empty capsids on incubation in a cell-free system. The number of capsids binding to each DNA molecule is determined by the input mole ratio of empty capsids to DNA. Table 1 details the components of two reaction mixtures which produce binding complexes on incubation. These mixtures were asembled in pyrex test tubes treated with Siliclad (Clay Adams Co.; one minute in a 1% aqueous solution). After a 20 minute incubation at 37°C., the samples were chilled in ice, and layered onto 10-22% neutral sucrose gradients. Sedimentation was carried out for 90 minutes at 30,000 rpm, 4°C., in an SW-50.1 rotor. Fraction collection and the determination of radioactivities are described in Methods. Figures 1 and 2 represent the sedimentation profiles obtained from samples #1 and #2 respectively (Table 1).

Figure 1 (13/1 mass ratio) contains three well defined peaks of ^{3}H-DNA. The identity of each peak was established stoichiometrically in double labeling experiments, and further by electron microscopy of

Table 1. Formation of binding complexes, mass ratios 13/1 and 7/1

Sample Number	#1	#2
Distilled Water	145 µl	145 µl
Tris 0.15 M (pH 7.5)	10 µl	10 µl
$MgCl_2$ 0.1 M	20 µl	20 µl
Bovine Plasma Albumin (5 mg/ml)	10 µl	10 µl
Empty Capsids (364 µg/ml)	10 µl	5 µl
^{3}H-DNA (58 µg/ml)	5 µl	-- --
^{3}H-DNA (27 µg/ml)	-- --	10 µl
Mass Ratio Capsid/DNA	12.5/1	6.7/1

peak ^{3}H-DNA containing fractions. The peak nearest the meniscus (fraction 33) contains unreacted component I DNA. Faster sedimenting material, centering on fractions 24 and 15 represent complexes consisting of a single DNA molecule complexed with one and two empty capsids respectively, (monomers and dimers). Increasing the mass ratio above 13/1 results in the formation of complexes of three or more empty capsids per DNA. Figure 2 illustrates the sedimentation profile obtained from a reduced capsid/DNA mass ratio (7/1). Under these conditions the formation of dimer complexes is suppressed.

In equivalent sucrose gradients, full virions are observed to co-sediment with dimers (240S). Monomers (144S) sediment significantly slower than the equal massed full virions due to the frictional drag produced by their extended DNA molecule. The addition of a second empty capsid, to form a dimer, nearly doubles the mass of the complex but only

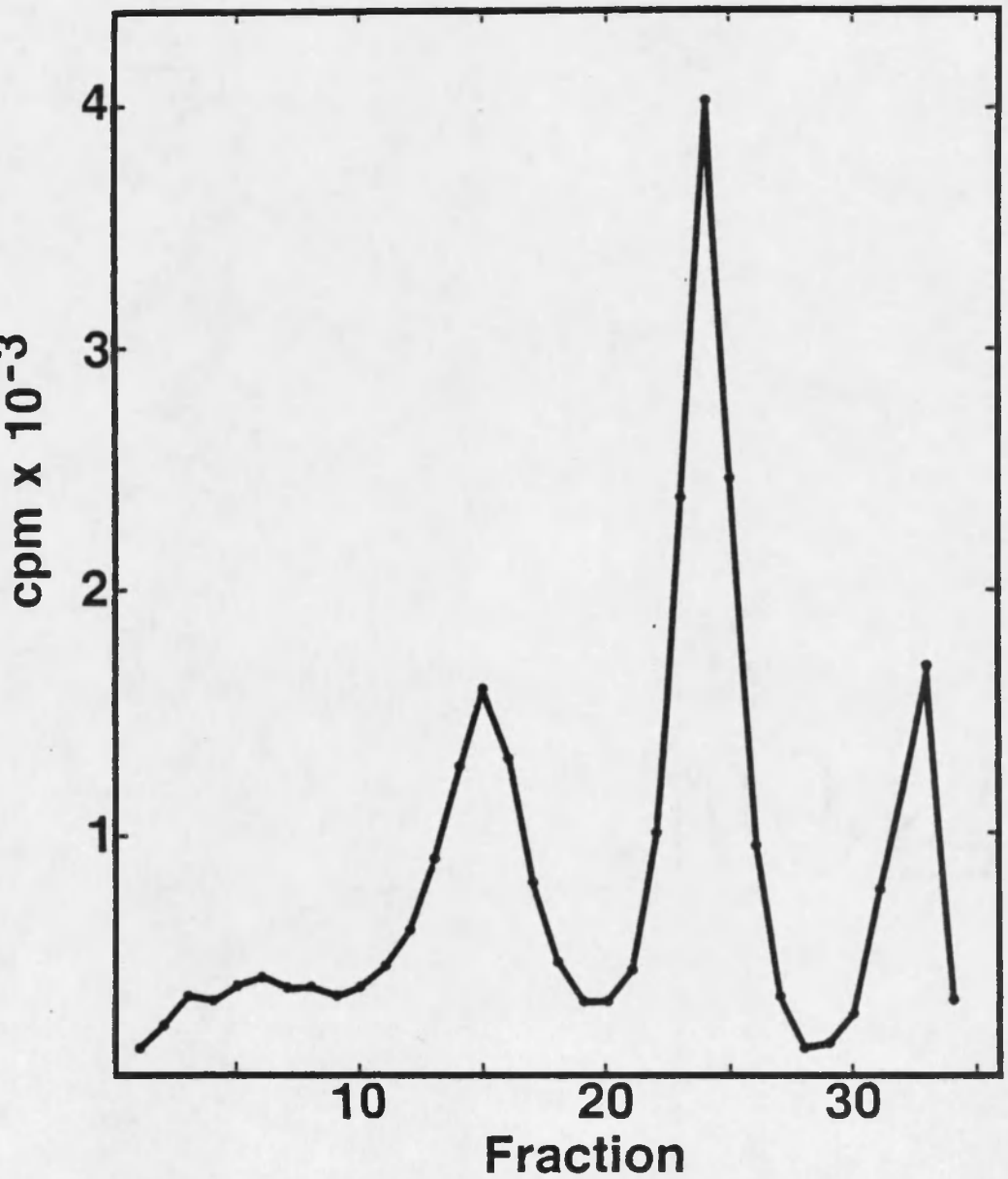

Figure 1. Formation of binding complexes (mass ratio 13/1). Neutral sucrose sedimentation.

Polyoma ^{3}H-DNA, 0.29 μg, and empty polyoma capsids, 3.64 μg, were incubated as described in sample #1, Table 1. The sample was layered on a 10-22% neutral sucrose gradient, and sedimented for 90 minutes at 30,000 rpm, 4°C., SW-50.1 rotor. Fraction collection and the determination of radioactivities were performed as described in Methods.

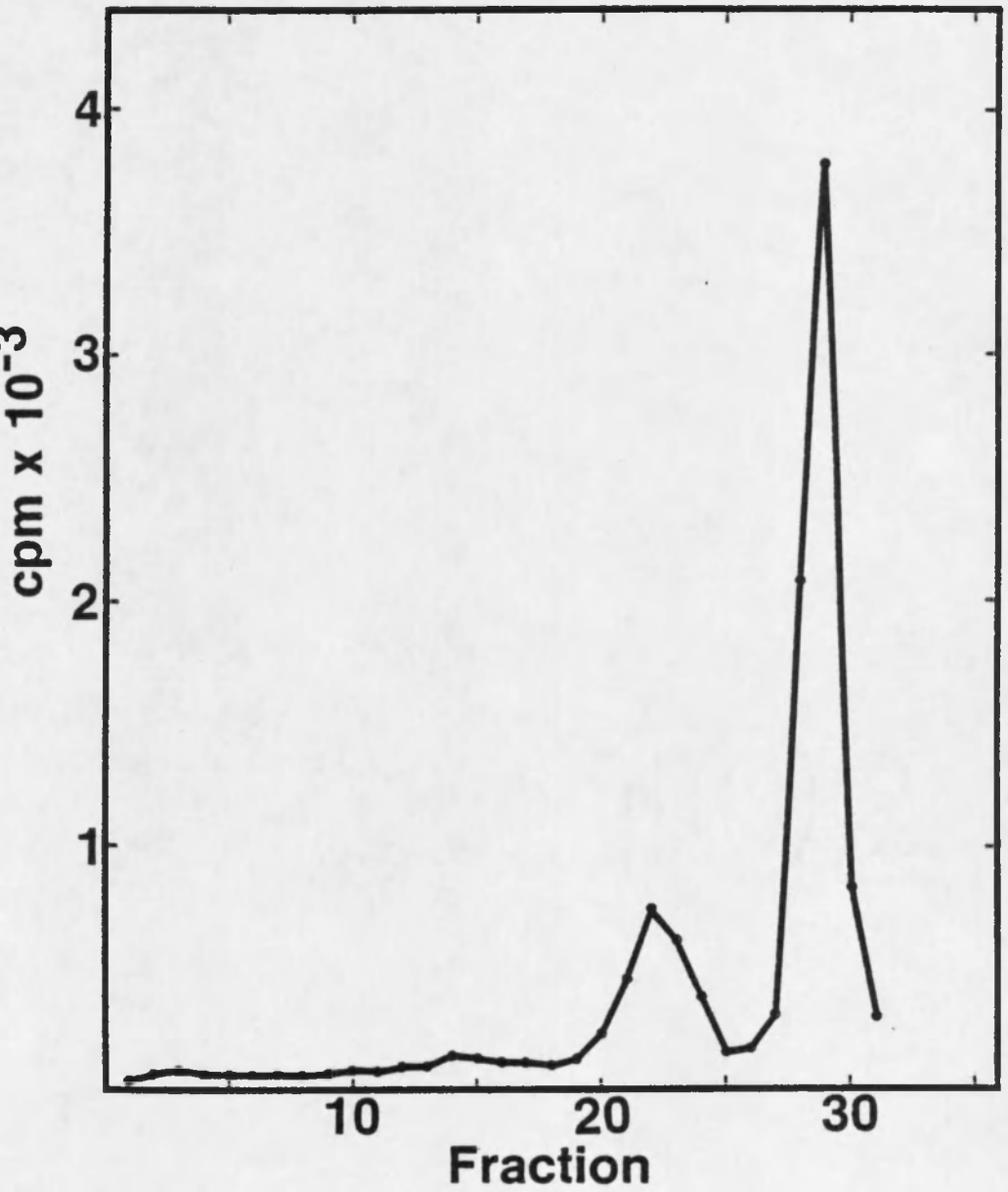

Figure 2. Formation of binding complexes (mass ratio 7/1). Neutral sucrose sedimentation.

Polyoma ^{3}H-DNA component I, 0.27 μg, empty polyoma capsids, 1.82 μg, were incubated as described in sample #2, Table 1. The sample was layered on a 10-22% neutral sucrose gradient, and sedimented for 90 minutes at 30,000 rpm, 4°C., SW-50.1 rotor. Fraction collection and the determination of radioactivities were performed as described in Methods.

slightly increases the frictional drag. This results in a faster sedimenting particle with a sedimentation coefficient coincidentally equal to that of full virions.

Sedimentation Assay

If these binding complexes can be induced to encapsidate their DNA, the frictional drag will be reduced, resulting in the formation of faster sedimenting particles. If only partial encapsidation occurs the length of the extended DNA would be shortened. This would remove a proportionate amount of the frictional drag and produce particles with a correspondingly higher sedimentation coefficient. Thus, any encapsidation which takes place should be recognized as a shift of binding complexes toward the bottom of the gradient.

Capsid/DNA mass ratios producing a minimum of dimers should be used in reassembly experiments, as this will avoid complication of the resulting sedimentation patterns by eliminating the presence of dimers in the region where full virions would be found. Figure 2 (sample #2) demonstrates a satisfactory suppression of dimers, thus providing a suitable capsid/DNA ratio for use in conjunction with a sedimentation assay. For this reason sample #2 will be used as the "standard binding complex reaction mixture." It should be noted, however, that Figures 1 and 2 represent experiments in which freshly purified viral components were used. With older preparations a higher mass ratio may be required to obtain a sedimentation pattern comparable to that shown in Figure 2. As the capsid preparations age a marked loss of binding efficiency is observed, often with a concomitant increase in the very fast sedimenting material held up by the CsCl cushion.

Experimental Parameters to be Varied

The following parameters were considered to be promising experimental variables for enhancing the ionic interactions assumed to be responsible for polyoma virus assembly.

I. pH. Varying the pH of the binding complexes might either create or eliminate charged groups. This could produce a charge configuration suitable for encapsidation to proceed. Alternatively, a change in pH might induce conformational changes in the structure of both DNA and protein, possibly achieving the correct spacial arrangement for encapsidation.

II. Temperature. Temperature could be a critical factor in overcoming an activation energy "barrier," or in establishing a near equilibrium state which may be necessary for the orderly and gentle loading of the capsid.

III. Ionic Strength. The addition of counter ions might neutralize repulsively charged groups and allow the formation of interactions necessary for encapsidation. Conversely, the removal of counter ions might expose attractively charged groups.

IV. Ethanol. The addition of 7-15% ethanol will reduce the degree of hydration of the viral components involved, without denaturing them. This might allow interaction between groups previously shielded by hydration layers.

V. Reducing Agents. The addition of SH-containing compounds (e.g., mercaptoethanol) might reverse oxidation induced conformations in the capsid protein.

VI. Detergents and Urea. Treatment of binding complexes with these agents might "loosen up" the capsid proteins either by weakening hydrophobic interactions or by removing easily accessible peptide chains.

VII. Polyamines. The addition of spermine or spermidine might effectively compete with the protein amino groups for the anionic DNA phosphate backbone. Under these conditions nucleic acid-capsid interactions could proceed in near equilibrium conditions. In such a reversible state only the most energetically optimal configurations will be selected, in this case the complete stabilization of the DNA within the capsid structure.

VIII. Histones. Polyoma DNA is always found in association with histones of host origin. These proteins may play an essential role in the stabilization of DNA within the capsid. Further, the attractive forces responsible for encapsidation might be protein-protein interactions between the capsid proteins and DNA associated histones. Thus the presence of histones might be a crucial requirement for encapsidation.

Preliminary Experiments

A series of experiments was designed to test the effectiveness of some of these variables in inducing DNA encapsidation. Sample #2

was used as a standard reaction mixture on which the following variations were imposed.

I. Reaction mixtures were incubated at raised temperatures; 20 minutes at 37°C., 45°C., and 55°C.

II. Prior to incubation, ethanol was added to final concentrations of 0, 5, and 10%.

III. Prior to incubation, spermine was added to final concentrations of 0, 5×10^{-5}, 5×10^{-4}, 5×10^{-3}, and 5×10^{-2} molar.

IV. After 10 minutes of incubation, urea was added to final concentrations of 0, 1, and 3 molar. Incubation was continued for an additional 15 minutes.

With the exception of spermine, each of these variations on the standard incubation mixture produced sedimentation profiles indistinguishable from Figure 2. The addition of spermine to concentrations of 5×10^{-4} or greater progressively reduced the extent of monomer formation, with a concomitant increase of radioactivity in the unreacted DNA peak. No increase in the sedimentation rate of monomers was observed in any of these experiments. From this it can be concluded that under the experimental conditions tested, no detectable amount of DNA was encapsidated.

Low Ionic Strength Experiment

In another experiment, the response of binding complexes to a low ionic strength environment was tested. Reaction mixtures were prepared as described in Table 2. Tris buffer and $MgCl_2$ were omitted as they constitute the majority of the counter ions present in the standard

Table 2. Formation of binding complexes in high ionic strength, and low ionic strength plus ethanol

Sample Number	#3	#4
Distilled Water	90 µl	75 µl
Ethanol 95%	-- --	15 µl
Polyoma ^{3}H-DNA (11 µg/ml)	10 µl	10 µl
Empty Polyoma Capsids (1.08 mg/ml)	20 µl	20 µl

reaction mixture. The mixtures were incubated for 20 minutes at 37°C. Then 0.4 ml of distilled water was added to each sample and the incubation continued for an additional 20 minutes. Following incubation the samples were sedimented through 10-22% neutral sucrose gradients. Sedimentation, fraction collection, and the determination of radioactivities are described in Methods. Figure 3 illustrates the sedimentation profiles obtained. Here, sample #3 shows no significant amount of radioactivity below the monomer peak. Thus, the omission of tris buffer and $MgCl_2$ combined with a four fold dilution was not found to induce encapsidation. However, sample #4 (low ionic strength plus ethanol) shows a marked increase in the ^{3}H-DNA found below the monomer region. A loss of radioactivity from the free DNA peak is observed, but no change is apparent in the monomer peak.

DNase-Sedimentation Assay

The diffuse appearance of the fast sedimenting ^{3}H-DNA containing material indicates a heterogeneous population of complexes. These may

Figure 3. Formation of binding complexes in high ionic strength, and low ionic strength plus ethanol. Neutral sucrose sedimentation.

Polyoma ^{3}H-DNA, 0.11 μg, and empty polyoma capsids, 21.6 μg, were incubated as described in Table 2. The samples were layered onto 10-22% neutral sucrose gradients and sedimented for 90 minutes at 30,000 rpm, 4°C., SW-50.1 rotor. Fraction collection and the determination of radioactivities were performed as described in Methods. The gradient profiles from two separate sucrose gradients are depicted. -0-0-0- with low ionic strength treatment and ethanol; -0-0-0- low ionic strength treatment without ethanol (see Table 2).

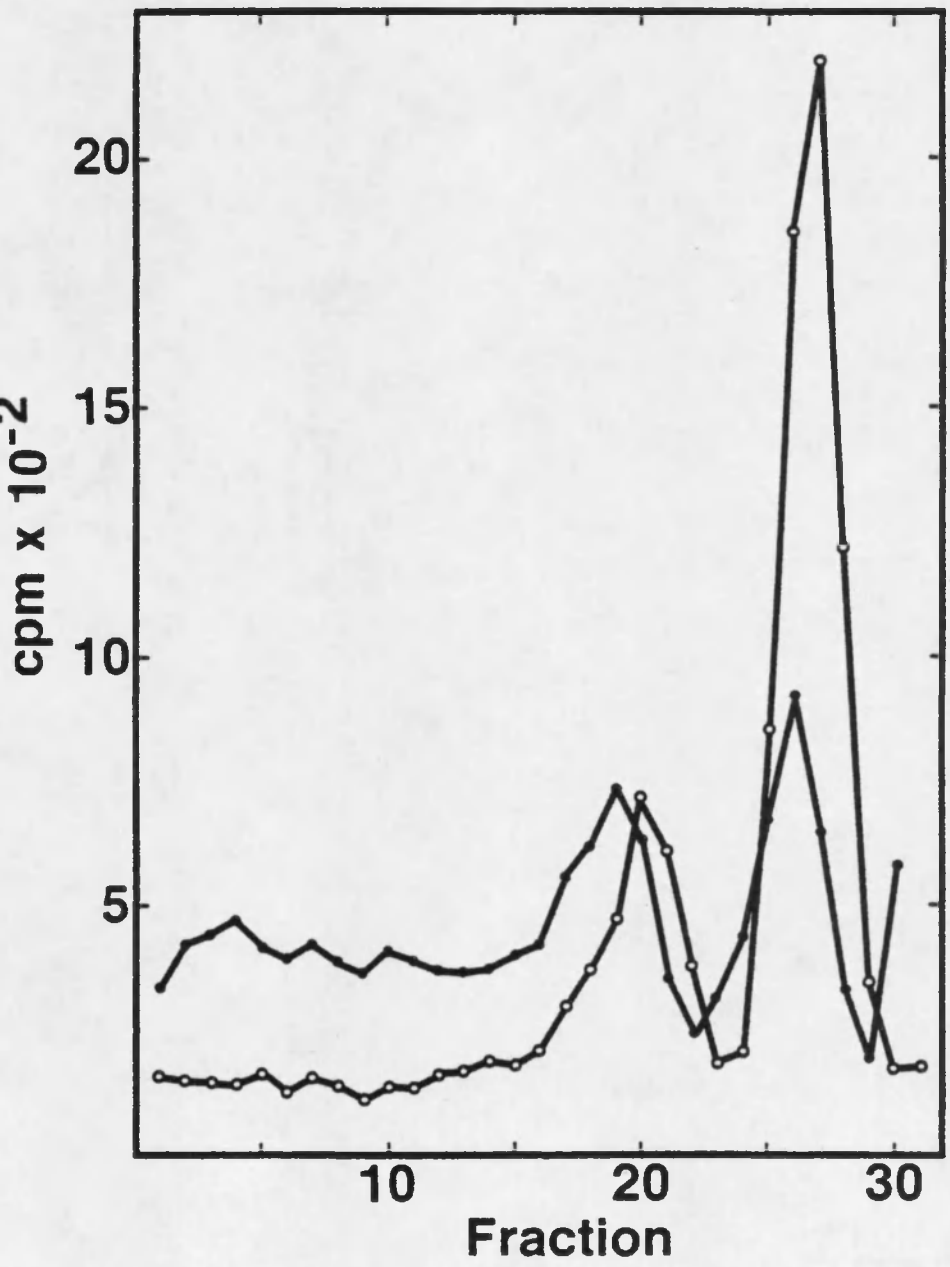

Figure 3. Formation of binding complexes in high ionic strength, and low ionic strength plus ethanol. Neutral sucrose sedimentation.

be the result of non-specific aggregation between DNA and capsid proteins. However, the possibility that some encapsidation might have occurred cannot be excluded. Any encapsidation which might have been induced, unless in very large proportion, would be masked by the superimposed background of aggregates. In addition, any dimers which might form as a result of the varied reaction conditions would be mistaken for encapsidated material. Clearly sedimentation alone is insufficient as an assay for DNA encapsidation. If sedimentation is to be utilized in a satisfactory assay for encapsidation, a method must be devised to eliminate the presence of any dimers and non-specific aggregates which might form. To this end, use was made of the fact that the DNA in polyoma virions is protected completely from the action of pancreatic DNase, while the DNA of binding complexes is not. Loose aggregates of DNA and capsid protein would also be expected to be labile to pancreatic DNase. The sedimentation of a DNase treated reaction mixture should permit the recognition of encapsidated DNA as rapidly sedimenting DNase resistant ^{3}H-DNA.

Formation of a Polyoma-like Particle

The low ionic strength experiment, described above, was repeated with minor variation, according to the protocol in Table 3. After the samples were incubated for 20 minutes at 37°C., 300 µl of distilled water was added to each sample and the incubation continued for an additional 20 minutes. Next, 55 µl of 95% ethanol were added to each sample, and the incubation continued for 20 additional minutes. Then, 50 µl of a pancreatic DNase solution (100 µg/ml; in 0.02 M tris (pH 7.5)- 0.01 M $MgCl_2$) was added to sample #6, and 50 µl of distilled water added to

Table 3. Formation of polyoma-like particles, with and without pancreatic DNase

Sample Number	#5	#6
Distilled Water	80 µl	80 µl
^{3}H-DNA	10 µl	10 µl
Polyoma Empty Capsids	10 µl	10 µl

sample #5. The samples were incubated a final 20 minutes at 37°C., and then sedimented through neutral 10-22% sucrose gradients. Sedimentation, fraction collection and the determination of radioactivities are described in Methods.

Figure 4 illustrates the sedimentation profiles of sample #5 and sample #6. The 10 µg/ml DNase treatment completely destroyed the monomer complexes (fraction 22). At the same time, a new peak (fraction 14), containing radioactive DNA that is resistant to pancreatic DNase, appeared below the monomer region of the gradient. Presumably, this DNase resistant material represents encapsidated DNA. In additional experiments, the DNase resistant DNA has been shown to withstand a ten times greater concentration of pancreatic DNase as is reported above (approximately 100 µg/ml). If empty capsids are omitted from the reaction mixture no fast sedimenting radioactive DNA is observed (Figure 5). This data eliminates the possibility that the DNase resistant peak observed in Figure 4 is a non-specific aggregation of DNA with non-capsid proteins. On the basis of these properties and others

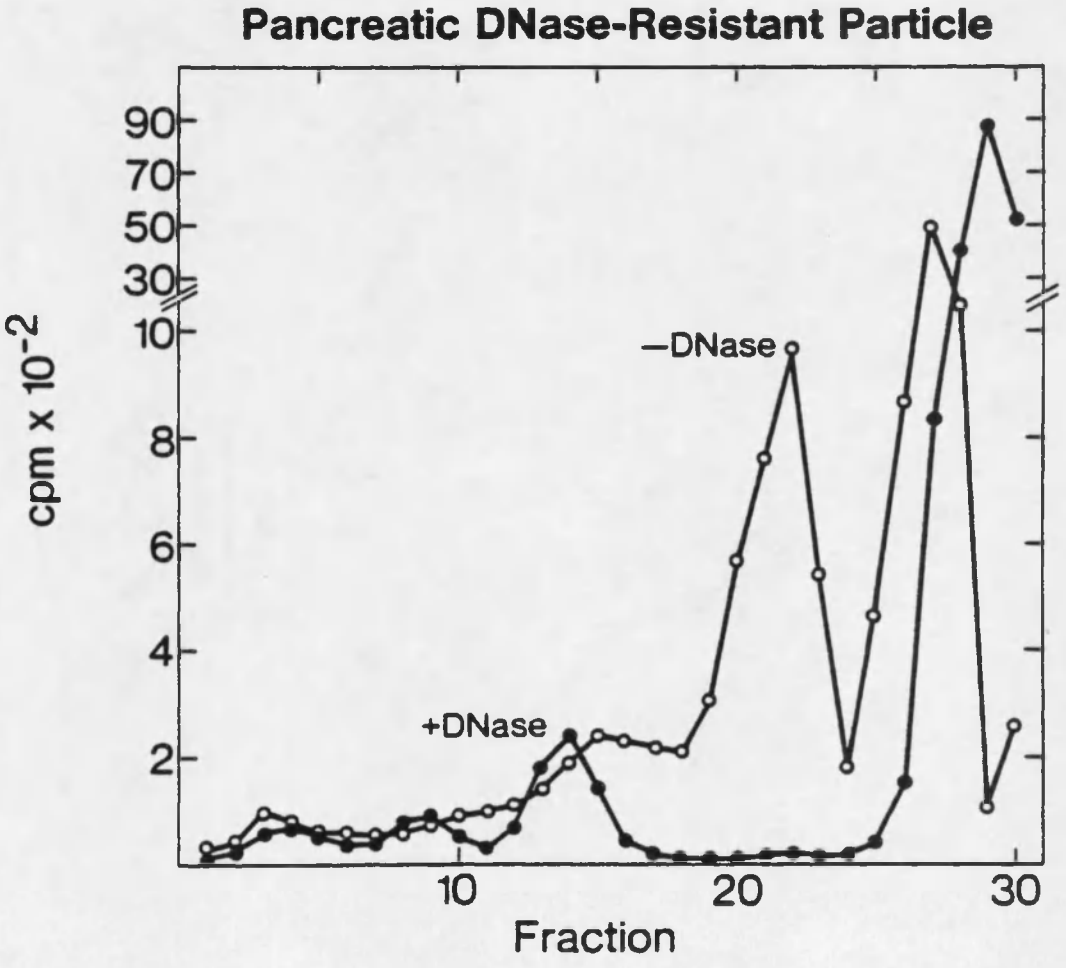

Figure 4. Formation of polyoma-like particles with and without pancreatic DNase. Neutral sucrose sedimentation.

Polyoma ^{3}H-DNA component I, 0.8 µg, and empty polyoma capsids, 25 µg, were incubated as described in Methods. With 10 µg/ml pancreatic DNase -●-●-●-; without pancreatic DNase -0-0-0- (see Table 3). Sedimentation in 10-22% neutral sucrose gradient, SW-50.1 rotor for 90 minutes at 30,000 rpm, 4°C.

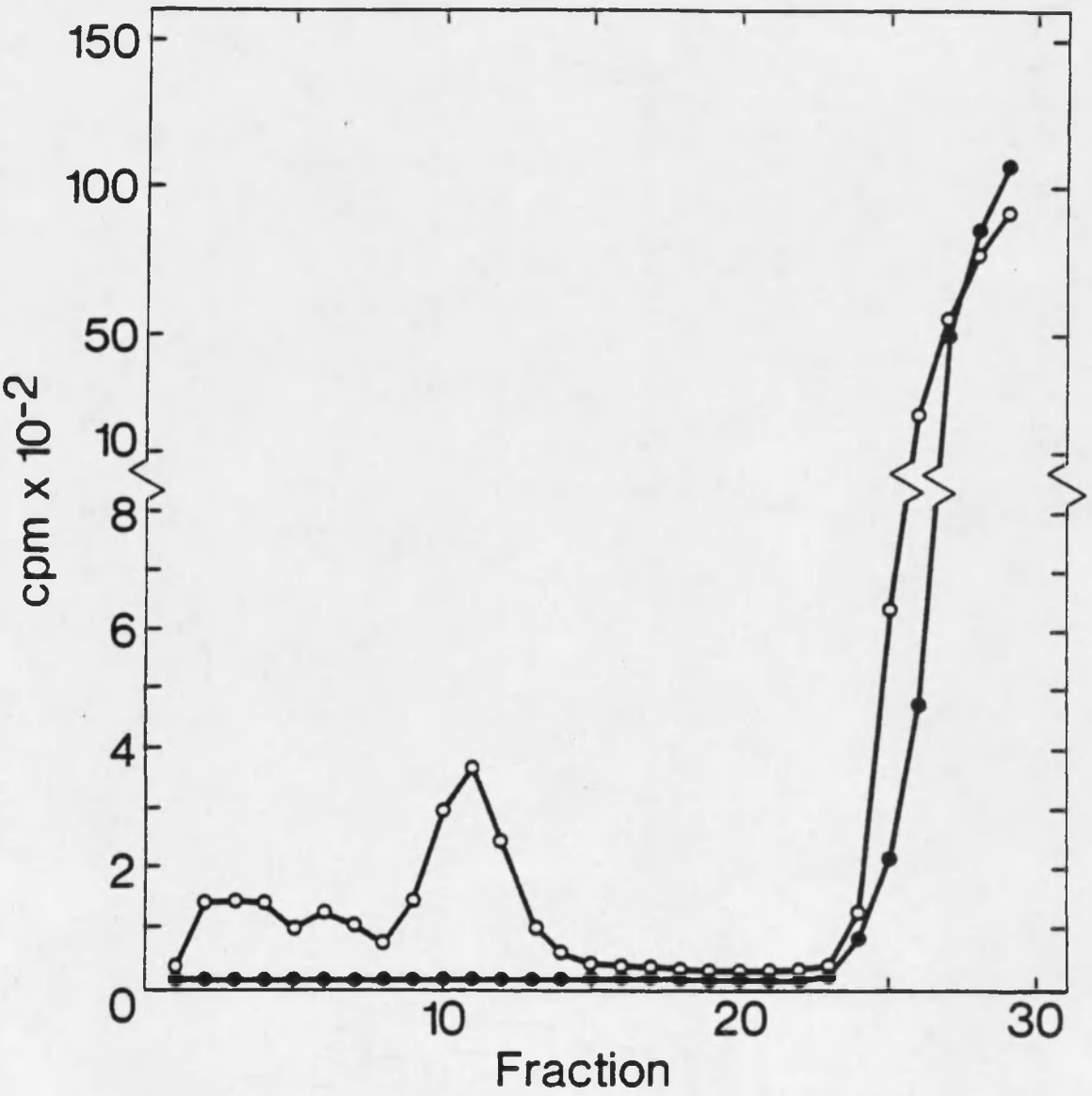

Figure 5. Formation of polyoma-like particles with and without empty capsids. Neutral sucrose sedimentation.

Polyoma ^{3}H-DNA component I, 1.9 μg, and empty polyoma capsids, 25 μg, were incubated and sedimented as described in Methods. Reaction with DNA and empty capsids -0-0-0-; DNA but no empty capsids -●-●-●-. Sedimentation in 10-22% neutral sucrose gradient, SW-50.1 rotor for 90 minutes at 30,000 rpm. 4°C.

discussed below, the novel, fast sedimenting, DNase resistant complexes are referred to as "polyoma-like particles" (PLPs).

The PLPs in Figure 4 comprise approximately 10% of the ^{3}H-DNA found in monomers before DNase treatment, and 4.5% of the total input ^{3}H-DNA. Recent experiments, in which bovine plasma albumin was added to the reaction mixture and the ionic strength reduced further have resulted in recoveries of up to 18% of the monomeric DNA as PLPs.

In these initial experiments PLPs were formed by the combined effects of low ionic strength and ethanol. Since then, PLPs have been formed by low ionic strength incubation alone. The necessity for the presence of ethanol has not been confirmed, although slightly higher yields (1-2%) are typically obtained. For this reason the PLP forming protocol described in Methods, and used in the remainder of this report, does not include the addition of ethanol.

In other experiments, exogenous calf thymus histones (Sigma Chemical Co. type II AS, without histone H1) were added to PLP forming incubation mixtures. The histones were added with the diluting distilled water to final concentrations of 0, 0.125, 0.375, and 2.5 μg/ml. Increasing histone concentrations were found to progressively inhibit the formation of PLPs, with a concomitant increase of the very rapidly sedimenting material accumulated on the CsCl cushion. A 0.125 μg/ml histone concentration was found to suppress PLP formation by approximately 75%, 0.375 μg/ml by 50% (as determined by a comparison of the areas under the peaks representing PLPs in sucrose sedimentation profiles). A 2.5 μg/ml histone concentration reduced PLP formation to background level. Equivalent concentrations of histones were also added to binding complex

forming incubation mixtures, both under the standard conditions (Table 1), and in reaction mixtures containing 0.15 M NaCl. Here again, no rapidly sedimenting DNase resistant material was produced.

CHAPTER 8

CHARACTERIZATION OF POLYOMA-LIKE PARTICLES

In a series of experiments, PLPs were analyzed with respect to several physical and chemical parameters. Information was obtained on the particle's buoyant density in CsCl, sedimentation properties in sucrose gradients, resistance to physical and chemical agents, and the amount and integrity of the DNA encapsidated.

Neutral Sucrose Sedimentation

PLPs were sedimented in isokinetic neutral sucrose gradients to determine their sedimentation coefficient. In this experiment, a single PLP forming reaction mixture was sedimented, the PLP containing fractions were pooled, and dialyzed, as described in the Methods. The resulting sample was sedimented through an isokinetic 10-22% neutral sucrose gradient (Methods). Prior to sedimentation ^{14}C polyoma virus was added as a sedimentation marker. The gradient profile, Figure 6 shows the PLPs (fraction 18) to sediment more slowly than the full virions (fraction 14). Using the ^{14}C virions and the meniscus as sedimentation markers a sedimentation coefficient of 190S is obtained.

Isopycnic CsCl Centrifugation

PLPs were characterized further with respect to their buoyant density in CsCl. Two PLP forming reaction mixtures were sedimented,

Figure 6. Sedimentation of polyoma-like particles in an isokinetic neutral sucrose gradient

Three PLP forming reaction mixtures each containing polyoma ^{3}H-DNA component I, 1.9 μg, and empty polyoma capsids 25 μg, were incubated and sedimented in three separate neutral sucrose gradients as described in Methods. Fractions containing PLPs were pooled and dialyzed against 0.01 M tris (pH 7.5), 0.001 M EDTA, and concentrated to 0.5 ml with Aquacide II. To this sample ^{14}C- polyoma full virions were added as a sedimentation marker, and layered onto a 10-22% isokinetic neutral sucrose gradient. Sedimentation was performed for 2 hours at 25,000 rpm, 4°C., SW-41 rotor. Sedimentation, fraction collection, and the determination of densities were carried out as in Methods. -0-0-0- ^{14}C- polyoma full virions (240S), and -0-0-0- ^{3}H- PLPs (190S).

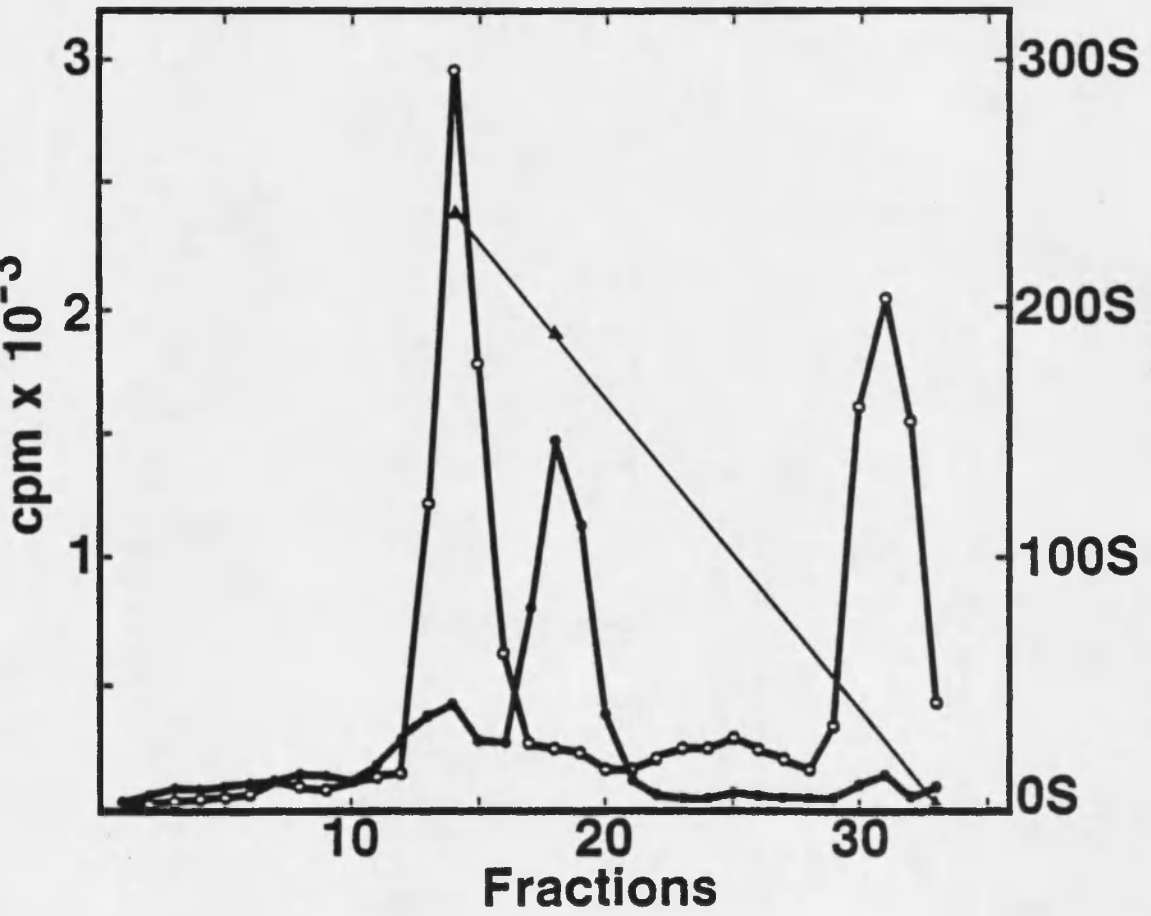

Figure 6. Sedimentation of polyoma-like particles in an isokinetic neutral sucrose gradient

pooled and dialyzed (Methods). Prior to centrifugation ^{14}C polyoma virus was added as a density marker (1.34 g/cc)(Tooze, 1973, p. 272). Isopycnic centrifugation was carried out as described in Methods (1.30 g/cc CsCl, 30,000 rpm, for 22 hours, 20°C. SW-50.1 rotor). The centrifugation profile, Figure 7, shows the PLPs to have equilibrated in a single narrow band (indicating a homogeneous population of densities) centering around a buoyant density of 1.32 g/cc. This density lies intermediate between that of full virions (1.34 g/cc) and empty capsids (1.29 g/cc), (Tooze, 1973, p. 272).

From this experiment it can be concluded that the encapsidation reaction is not reversible under conditions of high ionic strength. PLPs have been stored in 1.30 g/cc CsCl solutions for periods up to several months. Subsequent DNase treatment of these PLPs, followed by sedimentation through a sucrose gradient, has demonstrated their ability to withstand extended exposure to high salt concentrations. Such a resistance to high salt concentrations is not characteristic for the binding complexes described by Aposhian et al. (1975) which readily dissociate in 1.30 g/cc CsCl solutions. Thus further support is provided for the possibility that PLPs might represent DNA protected within an empty capsid. DNase resistant material resulting from a non-specific aggregation of DNA with capsid proteins would not be expected to withstand these conditions.

Extraction and Characterization of PLP-DNA

The intermediate density of PLPs can be explained in several ways. Each empty capsid might have taken up only a portion of the originally bound DNA, or alternatively, two empty capsids might have each

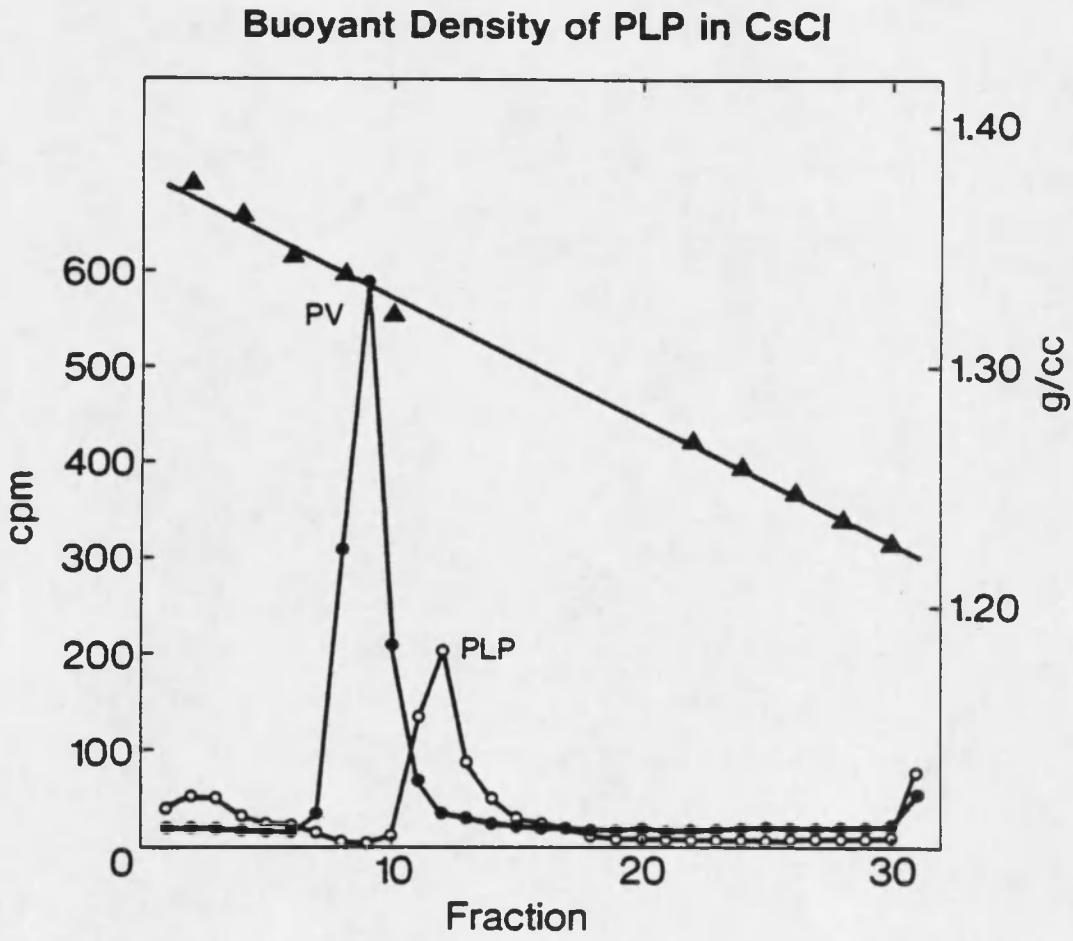

Figure 7. Isopycnic CsCl centrifugation of polyoma-like particles

Polyoma ^{3}H-DNA component I, 3.8 µg, and empty polyoma capsids, 50 µg, were incubated and sedimented in a neutral sucrose gradient as described in Methods. Sucrose gradient fractions containing PLPs were collected, pooled, and dialyzed three times against 0.01 M tris (pH 7.5)-0.001 M EDTA. ^{14}C-polyoma virus was added as a density marker (1.338 g/cc CsCl). CsCl was weighed into the dialyzed solution to bring the density to 1.30 g/cc as verified with a refractometer. The sample was centrifuged in the SW-50.1 rotor for 24 hours at 33,000 rpm, 20°C. Fractions were collected, CsCl density and radioactivities determined as described in Methods. Polyoma virus ^{14}C -●-●-●-; PLP-^{3}H-DNA -0-0-0-.

encapsidated opposite ends of the same DNA molecule. It is also possible that empty capsids have taken up single-stranded molecules of DNA.

In order to distinguish between these possibilities the DNA from PLPs was extracted and characterized. The extraction of PLP-DNA is described in Methods.

pH 9.0 CsCl Centrifugation

PLP-DNA was banded by pH 9.0 CsCl equilibrium centrifugation to determine if single or double-stranded DNA was taken up by empty capsids. Here, the DNA from three PLP forming reaction mixtures were extracted (Methods) and dialyzed for 18 hours against 0.01 M tris (pH 9.0)-0.001 M EDTA. After dialysis, ^{14}C-polyoma DNA component I was added as a density marker. A 4.0 ml portion of the sample was brought up to 1.70 g/cc with CsCl, and the densities checked with a refractometer. Isopycnic CsCl centrifugation (Methods) was carried out for 70 hours at 30,000 rpm, 20°C., in an SW-50.1 rotor. Figure 8 illustrates the resulting gradient profile.

The density of the native, double-stranded, polyoma component I DNA is 1.703 g/cc (Chemical Rubber Company, Handbook of Biochemistry, 1970). This value is in close agreement with the experimental values obtained for both the ^{14}C-marker DNA and the PLP-DNA. Single-stranded DNA generally assumes a more compact configuration than double-stranded DNA, and has a correspondingly greater buoyant density in CsCl. A difference of 0.015 g/cc has been reported for single- (denatured) and double-stranded lambda DNA (McCarthy, 1974). A shift of three fractions would be expected in Figure 8 if PLP-DNA were single-stranded. Both

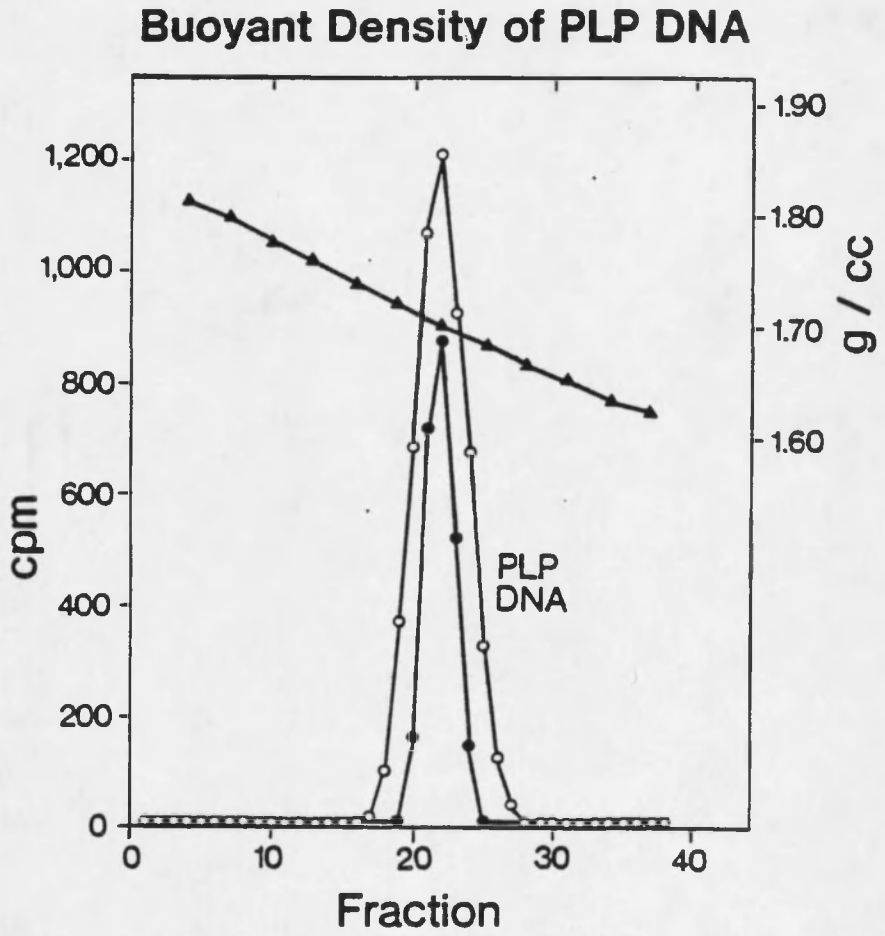

Figure 8. pH 9.0 isopycnic CsCl centrifugation of DNA extracted from polyoma-like particles.

Three PLP forming reaction mixtures, each containing polyoma ^{3}H-DNA component I, 1.9 μg, and empty polyoma capsids, 25 μg, were incubated and sedimented in three separate sucrose gradients as described in Methods. Fractions containing PLPs were collected, combined, and the DNA extracted as described in Methods. The final concentration of carrier DNA was 25 μg/ml. After phenol extraction the sample was dialyzed three times against 0.01 M tris (pH 9.0)-0.001 M EDTA. Polyoma ^{14}C-DNA was added as a density marker (1.703 g/cc CsCl). CsCl was weighed in to a concentration of 1.70 g/cc as verified by refractometry. The sample was centrifuged in the SW-50.1 rotor for 70 hours at 30,000 rpm, 20°C. Polyoma ^{14}C-DNA -0-0-0-; PLP-^{3}H-DNA -●-●-●-.

marker and PLP-DNA equilibrate in a single sharp band, centering around a buoyant density of 1.701 g/cc. From this data, it can be concluded that the DNA contained within PLPs is double-stranded.

Neutral Sucrose Sedimentation and Molecular Weight Determination

The molecular weight of PLP-DNA was obtained by sedimentation through an isokinetic neutral sucrose gradient. In this experiment, the DNA from three PLP forming reaction mixtures was extracted (Methods), dialyzed, and sedimented through an isokinetic 10-22% neutral sucrose gradient (Methods). Prior to sedimentation ^{14}C polyoma DNA containing both component I and II DNAs was added as sedimentation markers. The sedimentation profile, Figure 9, shows the PLP-DNA to sediment in a remarkably sharp peak centering around fraction 32. This suggests that a homogeneous population of DNA lengths are contained in PLPs. A sedimentation coefficient of 11S is obtained for PLP-DNA relative to the two ^{14}C-DNA sedimentation markers (component I DNA 20S, component II DNA 16S, Tooze, 1973, p. 279). This corresponds to a molecular weight of approximately 1.14×10^6 daltons, as calculated for double-stranded DNA by Studier's relation (see Methods). Thus, roughly one third of the native (3.6×10^6 daltons) polyoma DNA is contained within PLPs.

Alkaline Sucrose Sedimentation

The integrity of individual PLP-DNA strands was tested by sedimentation through a 5-20% alkaline sucrose gradient. Here, the DNA from one PLP forming reaction mixture was extracted (Methods), and concentrated to 0.5 ml. To this sample ^{14}C-polyoma DNA, containing component I

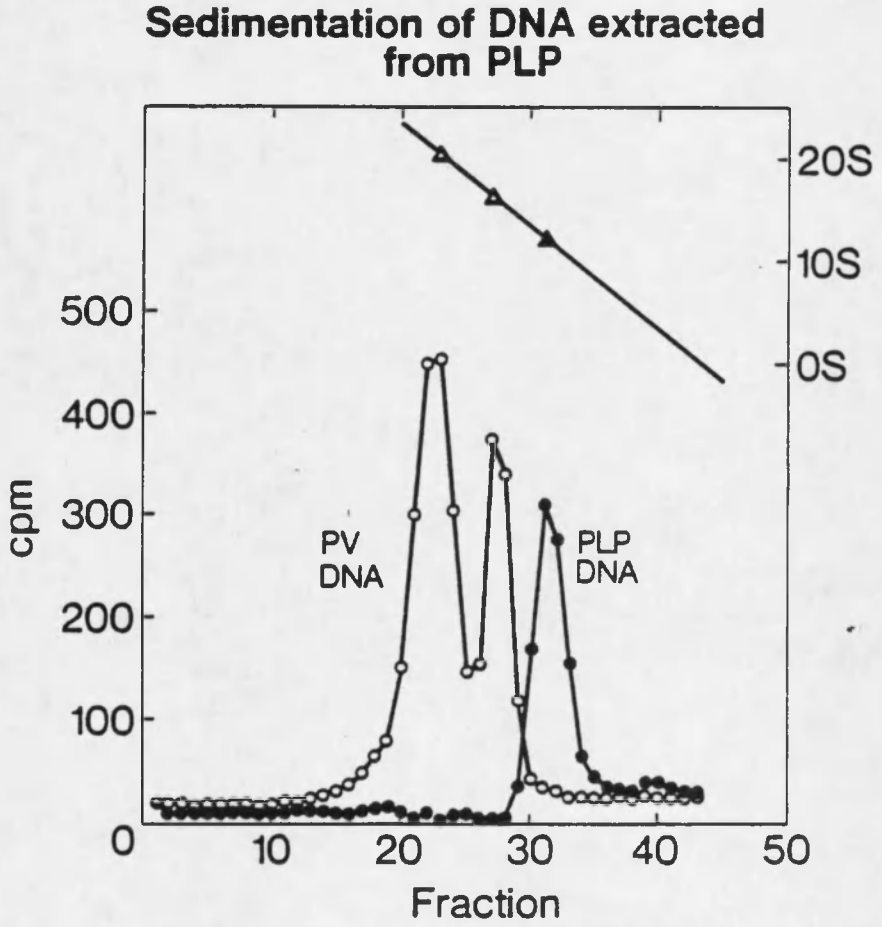

Figure 9. Sedimentation of DNA extracted from polyoma-like particles in an isokinetic sucrose gradient

Three PLP forming reaction mixtures, each containing polyoma ^{3}H-DNA component I, 1.9 μg, and empty polyoma capsids, 25 μg, were incubated and sedimented in three separate sucrose gradients as described in Methods. Fractions containing PLPs were collected, pooled, and the DNA extracted as in Methods. After phenol extraction the sample was dialyzed three times against 0.01 M tris (pH 7.25)-0.01 M EDTA. Polyoma ^{14}C-DNA containing components I and II were added as sedimentation markers and the mixture layered on an isokinetic sucrose gradient. Sedimentation was performed in the SW-41 Ti rotor for 6 hours at 40,000 rpm, 4°C. Fraction collection and the determination of radioactivities as in Methods. Polyoma ^{14}C-DNA -0-0-0-; PLP-^{3}H-DNA -●-●-●-.

and II DNAs was added as sedimentation markers. Centrifugation was carried out as described in Methods (49,000 rpm, for 90 minutes, 4°C., SW-50.1 rotor). In Figure 10 PLP-DNA is observed to sediment in a single narrow peak centering around fraction 22. No significant amount of radioactivity is observed above this peak. This indicates that the denatured PLP-DNA does not contain extensive single-strand nicks, as would be evidenced by low molecular weight fragments at the top of the gradient.

Heavy Polyoma-like Particles

Figure 4 and Figure 6 illustrate the presence of a small peak of DNase resistant material sedimenting immediately below the PLPs. These "heavy PLPs" sediment very near the region where full virions would be found. They may comprise as much as 4% of the total input radioactivity. To determine if this material represents particles which have encapsidated more DNA than PLPs, heavy PLP-DNA was extracted and compared with PLP-DNA in an isokinetic neutral sucrose gradient. A sedimentation coefficient of 11.5S was obtained for heavy PLP-DNA and 11.2S for PLP-DNA. The molecular weights of these two DNAs are nearly equivalent. As such, the heavy PLP material does not represent the encapsidation of more than 1×10^6 daltons of DNA. Instead, heavy PLPs may result from the aggregation of PLPs under the low ionic strength conditions used.

Integrity of Polyoma-like Particle Capsid Structure

The data presented are consistent with a model in which double-stranded molecules of DNA are taken up by empty capsids. The integrity

Figure 10. Sedimentation of DNA extracted from polyoma-like particles in an alkaline sucrose gradient

Three PLP forming reaction mixtures, each containing polyoma ^{3}H-DNA component I, 1.9 µg, and empty polyoma capsida, 25 µg, were incubated and sedimented in three separate sucrose gradients as described in Methods. Fractions containing PLPs were collected, pooled and the DNA extracted as in Methods. After phenol extraction the sample was dialyzed three times against 0.01 M tris (pH 7.25), 0.01 M EDTA. Prior to sedimentation the sample was brought up to pH 9.0 with NaOH, and ^{14}C-polyoma DNA containing components I, and II was added as a sedimentation marker. Samples were layered onto an alkaline sucrose gradient (0.5 M NaCl, 0.0025 M EDTA, 5-20% sucrose, with 0.1 M NaOH in the 5% sucrose, and 0.4 M NaOH in the 20% sucrose). Gradients were layered with a Buchler Instruments gradient maker and used immediately. Sedimentation was performed for 90 minutes at 49,000 rpm, 4°C., SW-50.1 rotor. Fraction collection and the determination of radioactivities as in Methods. Polyoma ^{14}C-DNA -0-0-0-; PLP-^{3}H-DNA -0-0-0-.

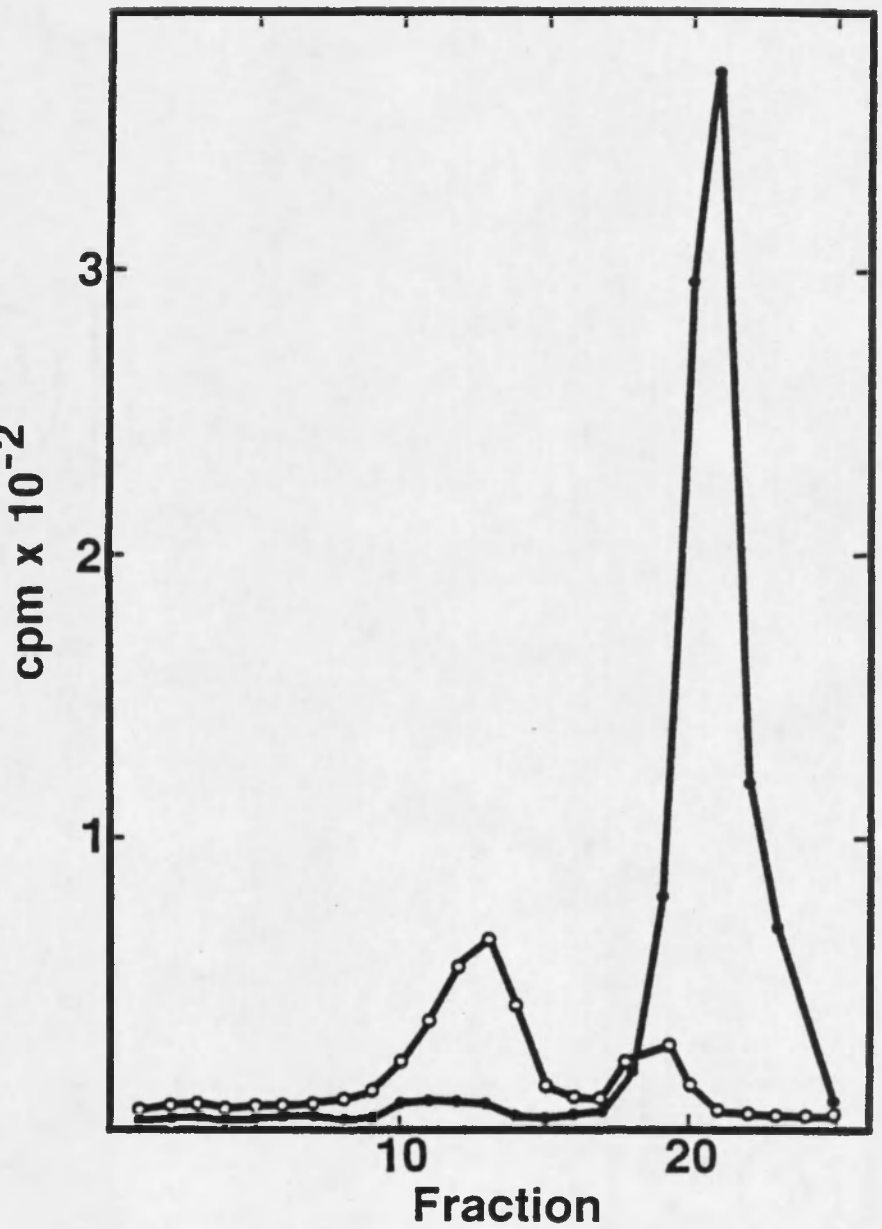

Figure 10. Sedimentation of DNA extracted from polyoma-like particles in an alkaline sucrose gradient.

of the latter structure was suggested by the correspondence of hemagglutination activity with the ^{3}H-PLP containing fractions in a sucrose gradient. However, the unreacted empty capsids were found to sediment too near the PLPs to permit their adequate separation. In addition, Walter and Deppert (1974) have demonstrated that 0.1% SDS treated empty capsids, containing only VP1, were as active as untreated empty capsids in their hemagglutinating ability. Thus an intact capsid structure is not required for a positive hemagglutination test.

Recent electron microscopic observation of PLP containing CsCl gradient fractions has revealed their capsid structure to be intact. Negatively stained samples have shown the arrangement of capsomers to be regular and undisturbed.

Polyoma-like Particle Formation with Non-homologous DNA

Recent experiments have shown that PLPs can be formed with non-homologous DNAs. To date, DNase resistant PLPs have been formed using SV-40 -DNA and lambda phage DNA. In both cases the DNA was found to have a molecular weight close to 1×10^{6} daltons, equal to that of PLP-DNA. This indicates that the termination of the encapsidation process is specific for the conditions used in the cell-free system and not for sequences on the DNA.

Preliminary experiments using ^{32}P-poliovirus RNA demonstrate the ability of polio RNA to form binding complexes with polyoma capsids. However, as of yet the encapsidation of poliovirus RNA by polyoma empty capsids has not been achieved.

CHAPTER 9

DISCUSSION

A cell-free system has been defined for the formation of a polyoma-like particle from purified polyoma DNA and empty polyoma capsids. The resultant particles contain a double-stranded molecule of DNA (approximately 1×10^6 daltons) that is resistant to the action of pancreatic DNase. The particles are also resistant to dissociation in high salt concentrations. These data are consistent with a model in which the DNA attached to a monomer binding complex is drawn inside an empty capsid. The exact physical location of the PLP-DNA is not readily obtainable without detailed structural analysis such as X-ray diffraction or neutron scatter studies.

The low ionic strength incubation used to induce the formation of PLPs may act in one of the following ways. Dilution of the reaction mixtures may serve to reduce the number of counter ions bound to the viral components. These counter ions may neutralize charged groups required to initiate or maintain the encapsidation process. If this is the case, the dilution of these counter ions may uncover attractive charges on the capsid and DNA, allowing the spontaneous encapsidation of DNA. Alternatively, the low ionic strength environment may serve to swell the empty capsid permitting the DNA to enter through the enlarged spaces between capsomers. These spaces may be too small for

molecules of pancreatic DNase to enter, thus conferring a portion of the DNA molecule with DNase resistance. The addition of magnesium ions with the DNase may rapidly reverse this swelling and trap some of the DNA inside. The extent of capsid swelling in low ionic strength media is presently being investigated by analytical ultracentrifugation.

Regardless of the mechanism involved, the pieces of incorporated DNA are remarkably uniform in size. This suggests that the encapsidation process is not terminated randomly. Rather, it seems more likely that encapsidation is blocked at a specific step. Additional conditions may be required for encapsidation to proceed further. Blockage may also result from irreversible errors occurring early in the encapsidation reaction. For example, charge misalignments may cause an inefficient utilization of capsid space resulting in incomplete packaging of DNA. Alternatively, the absence of histones in the PLP forming reaction mixture may cause the incomplete condensation of the DNA. Here again, an inefficient use of capsid space would result.

Whether or not this cell-free system in any way reflects the in vivo assembly process is not clear. In several respects the conditions used in PLP formation differ from the in vivo conditions under which viruses are normally formed. For example, the low ionic strength environment used to induce PLP formation is not likely to occur in the nucleus or any other compartment of an infected cell. In addition, the empty capsids used in PLP formation are purified through a minimum of two CsCl centrifugations. This harsh ionic treatment would be likely to dissociate any ionically bound proteins which might normally be found within the capsid. Such proteins may not be found in the mature virion, but may be required for the orderly packing of empty capsids.

Assembly experiments using polyoma capsids derived from crude lysates of infected cells (not exposed to CsCl) may alleviate this deficiency.

Similarly, the DNA used in the formation of PLPs is purified free from cellular histones. It is well established that polyoma DNA is always found in a nucleoprotein complex, even during DNA replication. The omission of histones from attempted reassembly experiments would eliminate capsid-histone interactions which may be essential for encapsidation. Thus, if polyoma virus assembles via a capsid filling model, empty capsids should be able to complex with nucleoprotein complexes extracted from infected cells. Preliminary experiments have shown that 53S nucleoprotein complexes will form binding complexes in both high and low ionic strength reaction mixtures. However, exposure of these complexes to pancreatic DNase did not produce any DNase resistant material. In other experiments (Chapter 7) the addition of exogenous histones were not found to enhance further encapsidation. This may be due to the inability of histones, added under these conditions, to organize the DNA into nucleosomes.

Even though the conditions of this cell-free reassembly system do not closely match the environment found with in an infected cell, the formation of PLPs clearly demonstrates the ability of empty polyoma capsids to incorporate exogenous DNA. This provides additional support for the possibility that polyoma virus assembly might proceed via a head filling model.

The question of whether or not a specific portion of the polyoma genome is incorporated into PLPs is unresolved. This issue can be explored by restriction endonuclease mapping of the PLP-DNA. It is likely,

however, that the PLP-DNA is a random fragment of the polyoma genome, as polyoma capsids are known to take up random fragments of host DNA, producing pseudovirions. In addition, recent experiments have shown that PLPs can be formed using SV-40 and lambda DNAs. In both cases the protected DNA was found to have a molecular weight equal to that of PLP-DNA. This indicates that the termination of the encapsidation reaction is specific for the conditions used in the cell-free system and not for sequences on the DNA.

Polyoma-like particles containing non-homologous DNA are analogous to the naturally occurring pseudovirions. Polyoma pseudovirions have been used to infect mouse fibroblast cells (Osterman et al., 1970) and human embryo cells (Qasba and Aposhian, 1971) grown in tissue culture. In these experiments pseudovirus uncoating was demonstrated. Further, it was established that at least a portion of the pseudoviral DNA arrives in the nucleus of the infected cells. At the present time the successful expression of a transferred gene using polyoma pseudovirions as a vector has not been achieved. Nonetheless, it is reasonable to assume that a genetic transfer analogous to specialized transduction is possible using polyoma capsids filled with specific DNA fragments. A procedure for encapsidating specific fragments of DNA would effectively increase the chances of a successful gene transfer over a random selection of DNA fragments, as is the case in pseudovirions. The formation of PLPs, presented in this report, may provide such a mechanism for the packaging of pre-selected DNA fragments in polyoma capsids. As such, the polyoma pseudovirus system may provide a potential vector for the delivery of genetic material to

mammalian cells grown in tissue culture, and perhaps to the whole animal. The implications and experimental problems involved in such an undertaking are considerable, and are extensively reviewed by Aposhian, Barr, and Keck (1978).

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