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A B S T R A C T

Ph.D. Pierre Moreau Microbiology and Immunology

THE ARRANGEMENT OF INTEGRATED POLYOMA SEQUENCES IN TRANSFORMED CELLS

The frequency of transformation of rat cells obtained with recombinant plasmid DNAs which contained an intact early region of the polyoma genome (Bam clones) was not significantly different from those which carry an interrupted early region (Eco RI clones) or even part of the early region (Hind III-1 fragment transformant). The viral DNA within several Bam and Eco RI clones as well as fragment transformants was examined by Southern blotting. These cell lines contain integrated viral sequences which sometimes are tandemly arranged. We also found that the presence of large T antigen in transformed cells does not significantly alter the frequency at which tandem integration occurs. Furthermore, Rat-1 cells in culture were transformed with cellular DNA isolated from one Hind III-1 fragment transformant. These studies showed that the oncogenic potential of the viral DNA is maintained after integration. These findings demonstrate that only part of the polyoma early region is required to initiate and maintain transformation.

R E S U M E

Ph.D. Pierre Moreau Microbiologie et Immunologie

L'ARRANGEMENT DU DNA DU POLYOME INTEGRE DANS DES CELLULES TRANSFORMEES

Nous avons pu déterminer que la fréquence de transformation de cellules de rat obtenue avec le DNA de plasmides recombinants contenant une région précoce intacte (clone Bam) n'était pas, de manière significative, différente de celle obtenue avec les plasmides contenant une région précoce interrompue (clone R) ou même seulement une partie de la région précoce (fragment Hind III-1). Par ailleurs, nous avons examiné l'état physique des génomes intégrés dans les diverses lignes transformées. Ces lignées contiennent toutes des séquences virales intégrées qui sont quelquefois présentes sous forme de tandem tête-bêche. Il fut aussi démontré que la présence de "large T antigen" dans des cellules transformées n'affecte pas la fréquence de l'intégration en tandem. De surcroît, des cellules de rat furent aussi transformées par du DNA cellulaire isolé de cellules transformées par le fragment Hind III-1 indiquant bien que le génome viral conserve son potentiel oncogénique, même lorsqu'il est intégré. Ces études démontrent donc que seulement une partie de la région précoce du virus du polyome est nécessaire à l'établissement et au maintien de la transformation cellulaire.

A C K N O W L E D G E M E N T S

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INTRODUCTION

In 1951, Ludwig Gross working with murine leukemia virus noticed that newborn mice, previously injected with a cell free leukemic mouse extract, developed small bilateral tumors of the neck (1). By 1953, Gross had firmly established that these tumors were not caused by a leukemia virus but by a second, contaminating, oncogenic virus present in the extract (2).

Later, when large amounts of purified virions became available, it was quickly discovered that this virus could not only cause tumors of the parotid, as noticed by Gross, but could also cause tumors of the submaxillary, sublingual, epithelial and thymic glands; adrenal medullary and mammary tumors and lesions of the convoluted tubules of the kidney cortex. (For a review, see 3). Hence, because the virus discovered by Gross was capable of inducing such a wide variety of tumors, it was named poly oma - polyoma virus.

Since then, polyoma virus has stimulated the interest of scientists mainly because of its small genome and its capacity to transform cells in vitro thus providing an excellent model for studying the complex process of cellular transformation.

Although the transforming region of the polyoma viral genome had not been precisely defined when this study began, there was nevertheless a substantial amount of data

known on the virus. Since then, the advent of new powerful methods in molecular biology have greatly contributed to our present understanding of this small oncogenic virus.

It is well established that the polyoma virus particle is composed of proteins and DNA only and that there are no detectable lipids or carbohydrates present. Only three viral coded proteins make up the capsid of the virus (4); VPI which has a molecular weight of 47,000 daltons, VP2 - 35,000 daltons and VP3 - 27,000 daltons (5). These are assembled to form 72 capsomeres, organized in an icosahedral symmetry.

The polyoma virus capsid envelops a double stranded closed circular molecule of DNA which is associated with cellular histones (6). However, when the viral DNA is freed of histones, most of the molecules are found to have a superhelical configuration (form I). This "supercoiling" is thought to occur by the completion of the last phosphodiester bond while the DNA molecule is already distorted by its association with histones (7). Therefore, when the histones are removed from the DNA, the strain on the molecule is partially released by superhelical coiling. This same superhelical configuration is lost upon cleavage of a phosphodiester bond simply because free rotation about the phosphodiester bond opposite the nick is then possible (8). The molecule then becomes circular

(form II). The DNA molecule can also be linearized (Form III) by cleaving both stands of the DNA at the same site. These three DNA components have different electrophoretic mobilities and can readily be separated by gel electrophoresis. The three forms of viral DNA remain infectious although linearized molecules have only 1/10 of the infectivity of forms I and II DNA (9, 10).

A physical map of the circular viral genome was constructed some years ago by cleaving polyoma DNA with different restriction enzymes. The map shown in Figure I is subdivided into 100 units and is oriented according to the position of the single cut produced by Eco RI which is defined as map unit "0" (for a review, see 11). Furthermore, the nucleotide sequence of the entire viral genome has been recently determined (12).

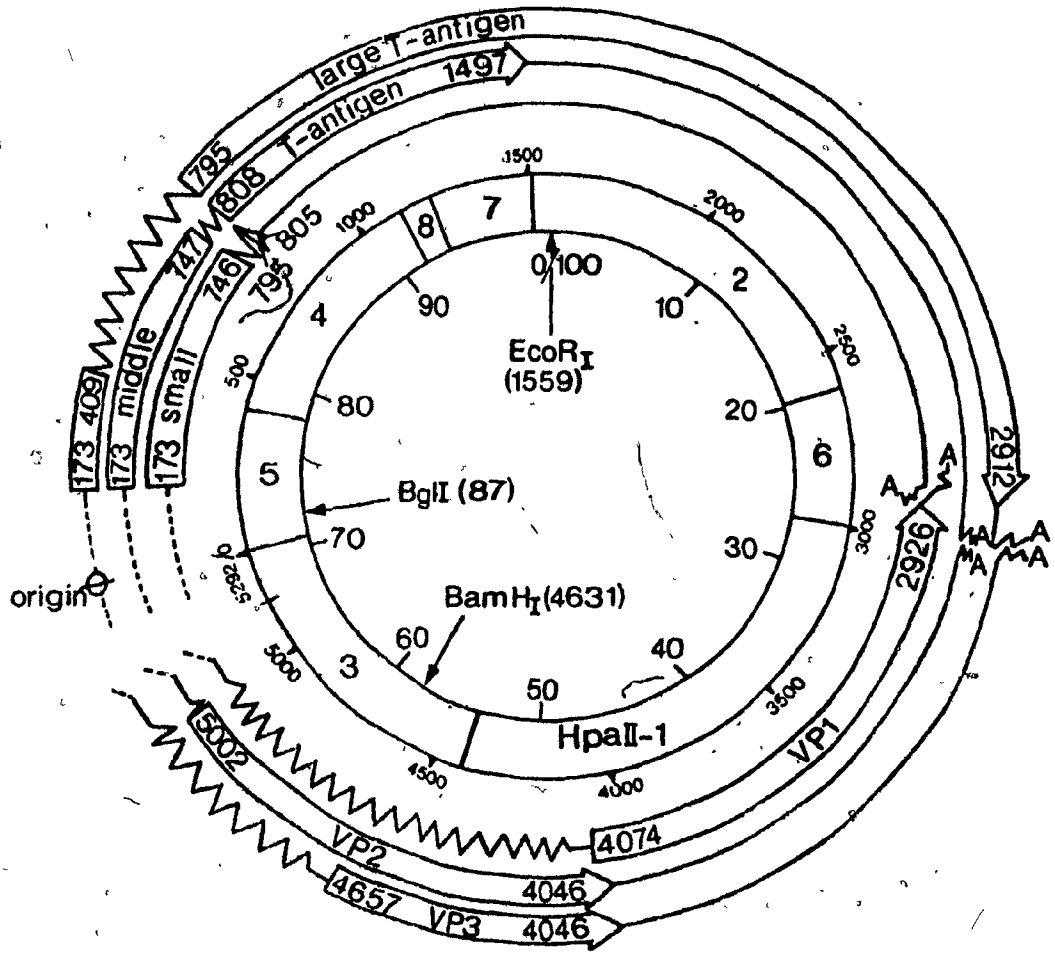
THE LYTIC CYCLE OF POLYOMA VIRUS

Infection of mouse cells (permissive cells) with polyoma virus results in cell death and the release of new virus particles. The infectious cycle is initiated by the reversible attachment of the viral particles to the virus receptor sites on the cellular surface. Prior to infection, attachment can be prevented by treating the cells with neuraminidase. Because of this, the cellular receptor sites are thought to be composed of neuraminic acid (13). After adsorption, the viral particles invade the cell by penetrating the cytoplasm, through which

Figure 1. Postulated landmarks on polyoma virus (A2 strain)

From Griffin, B.E., Soeda, E., Barrell, B.G. and Staden, R. (1980). Sequence and analysis of polyoma virus DNA. Molecular biology of tumor viruses, part 2. Cold Spring Harbor Laboratory. J. Tooze, ed.

POSTULATED LANDMARKS ON POLYOMA VIRUS (A2 STRAIN).



5292 bp

a. Bodies of middle and large T-antigens encoded within 2 different frames.

b VP2/VP3 and VP1 encoded within 2 different frames.

c. Alternative splice for middle T-antigen 765-795

they are transported by pinocytotic vesicles (14) to finally enter the nucleus, probably through nuclear pores (15). Once in the nucleus, the particles can be detected for two hours before they start disappearing, most likely because of uncoating. Subsequent events then occur in two distinct phases.

The Early Phase

The first noticeable change that occurs after the uncoating of the virus and before the onset of viral DNA replication is the appearance of specific viral mRNAs. These are transcribed from just a fraction of the viral genome (16, 17, 18), the "early region", which extends from 72 to 26 map units clockwise on the physical map (Figure 1). Because the viral early mRNAs represent only 0.01 to 0.001% of the total cellular RNA (19), their study has always been very difficult. However, Kamen and his co-workers have recently adapted the S1 nuclease gel electrophoresis technique of Berk & Sharp (20) to map the early mRNAs present in lytically infected cells (21). Briefly, they hybridized unlabelled RNA containing viral sequences, to denatured restriction fragments of polyoma viral DNA, under conditions where DNA-RNA hybrids were favored and DNA-DNA hybrids, excluded (22). The region of the restriction fragments which had not hybridized with the mRNAs were then left single stranded and were therefore susceptible to digestion by the single strand specific nuclease S1.

The S1-resistant DNA-RNA hybrids were separated by alkaline agarose gel electrophoresis (23), transferred to a nitrocellulose filter (24) and then annealed to ^{32}P-labelled viral DNA probes. By identifying the restriction fragments present, it became possible to establish a map of the viral sequences found in the mRNAs. Using results generated by a two-dimensional variation of the technique, where the S1-resistant hybrids were first separated by electrophoresis in a neutral buffer, then denatured *in vitro* and subjected to a second electrophoresis (perpendicular to the first one) in an alkaline buffer, Kamen *et al.* (25) also determined the different spliced structures of the early mRNAs. Their results have been recently reported (25).

The three major early viral mRNAs were found to have a common 5' end located at about 73.3 map units (25) very close to the putative initiation codon thought to be used for the translation of the early proteins (26). The three mRNAs were also found to be spliced. One mRNA is missing about 390 nucleotides between 78.3 and 85.6 map units, the second one about 50 nucleotides between 84.6 and 85.6 map units and the third one, about 65 nucleotides between 84.6 and 85.8 map units (25). Kamen *et al.* (25) have also shown that although the three mRNAs differ in the length of their coding regions, their 3' ends map at 25.8 map units just after the AATAAA sequence reported by Soeda *et al.* (26). This sequence is thought to be the signal for polyadenylation (27).

Besides the three major early viral mRNAs, Kamen and his group have also detected in lytically infected cells, the presence of additional mRNAs. The hybrids formed between these mRNAs and restriction DNA fragments have an additional S1 nuclease-sensitive site located at 93.2 map units (25). It is not yet known whether this new nuclease sensitive site indicates the presence of a second splice or an unknown RNA modification which would weaken the hybrid structure. Lytically infected cells also harbor a second minor population of mRNAs which are identical to the major mRNA species, except that they terminate in the middle of the early region, or at about 99 map units (25). The poly (A) tails of these mRNAs are thought to be added from a secondary polyadenylation site located near 98.7 map units on the viral DNA (26). The function of these minor species during lytic infection, is still unknown but Kamen et al. (25) have shown that they constitute the majority of viral mRNAs in certain cell lines transformed by polyoma virus.

Viral specific proteins are also synthesized during the early phase. Because these proteins can be precipitated by sera from animals carrying tumors induced by polyoma virus, and not by sera from normal syngeneic animals, they are called tumor antigens or T-antigens. It is now well established that there are three major tumor antigens encoded by polyoma virus, the most abundant one being large T antigen (M.W. of 100,000 daltons). The other two are respectively middle T antigen

(M.W. 55,000 daltons) found to be associated with plasma membranes and small T antigen (M.W. 22,000 daltons) found predominantly in the cytoplasm (28-33). The examination of partial peptide maps of these proteins has revealed that all three proteins share common peptides, that middle and small T antigens have peptides in common that are not present in large T antigen, and that each protein also contains unique peptides (31, 33). These observations were corroborated by recently published DNA sequencing data (26, 12). Only one reading frame is open for translation between map units 74 and 86. Between 86 and 26 map units (clockwise), there is a long uninterrupted reading frame that encounters its first termination codon at 25.6 map units. This same portion of the early region contains another open reading frame located between 86 and 98.5 map units.

If the inter-relationship among the early proteins is well established, their functions in the lytic cycle, on the other hand, are not yet completely understood. Nevertheless, large T antigen is known to autoregulate its own synthesis by blocking the transcription of early mRNAs (34). This observation stems from the study of temperature-sensitive polyoma virus mutants (tsA mutants) discovered by Fried in 1965 (35). This temperature-sensitive mutation was subsequently localized between 1 and 25 map units in the early region of the viral genome (36) and therefore does not affect the coding region of middle and small T antigens. When mouse cells are infected

with such a mutant at the non-permissive temperature, they synthesize a thermolabile form of large T antigen (37). These cells then accumulate 20 times more early viral mRNA than cells infected with wild type virus at the same temperature (34). However, when the same cells are co-infected with a temperature sensitive mutant and the wild type virus, there is no accumulation of the early mRNAs indicating that "normal" large T antigen (encoded by the wild type virus) inhibits their synthesis while the thermolabile form of large T antigen (encoded by the tsA mutants) was incapable of doing so (34).

The host-range, transformation-defective (hr-t) mutants (38), another group of polyoma early mutants which complement the tsA mutants, have also been very useful in determining the role played by the early proteins. The genomes of these mutants have deleted sequences between 78.4 and 85.2 map units on the physical map (39). Untransformed 3T3 mouse cells or mouse embryo fibroblasts at late passages cannot be lytically infected by the hr-t mutants (40,41). These mutants are also incapable of causing cellular transformation. Cells infected with hr-t mutants synthesize a large T antigen identical to the wild type induced large T, but synthesize middle and small T antigens in reduced amounts or fail to do so completely (28,31,42). The absence of middle and small T antigens results in a deficiency in acetylation of the encapsidated histones H3 and H4. This encouraged Benjamin and his co-workers to propose a model by

which the hr-t viral genes act as pleiotropic regulators of cellular gene expression (43, 44). The additional role played by the early proteins in cellular transformation will be discussed in the following section.

Apart from the appearance of the early viral mRNAs and the proteins they encode, the early phase of the lytic cycle is also characterized by an increase in the activity of some cellular enzymes including DNA polymerase, DNA ligase and thymidine kinase (45). Host cell DNA synthesis is subsequently initiated (46) (for a review, see 47) followed by viral DNA synthesis, thus marking the beginning of the second phase of the lytic cycle.

The Late Phase

Viral DNA replication can be detected about 12 to 15 hours after the infection of permissive cells by polyoma virus. How viral DNA synthesis is initiated is still not clear, but Eckhart et al. (48, 49) have shown that tsA mutants grown at the non-permissive temperature do not replicate their DNA. Large T antigen is therefore necessary for the initiation of viral DNA replication.

Polyoma viral DNA replication usually initiates at a fixed position on the viral genome and proceeds bidirectionally (50, 51). This single origin is located close to the junction between Hpa II fragments 3 and 5 or at about 71 units on the

physical map (50, 11). Because the replicating forks move at about the same rate along the circular viral genome, the termination point is reached 180° away from the point of initiation or at about 26 map units. Apparently, there is no special signal for the termination of replication (12). However, Bjursell (52) has shown the presence of rolling circle forms of polyoma DNA in lytically infected cells, but unlike Robberson et al. (53), Bjursell reported that this unidirectional replication originates from the same point as does bidirectional replication.

During the late phase, synthesis of early viral mRNAs and proteins continues and a new group of viral mRNAs and proteins start to appear. These late mRNAs are transcribed in a counterclockwise direction from a region of the viral genome which spans from 70 to 25 map units counter clockwise (16, 18) and encode the three proteins, VP1, VP2 and VP3 which constitute the viral capsid. In their original attempts to map the late mRNAs, Turler et al. (54) and Kamen and Shure (17) had assigned map positions on the assumption that the mRNAs were continuous with the DNA fragments from which they were transcribed. This assumption was subsequently invalidated by the discovery of mRNA splicing (55, 56, 57, 58). Kamen et al. (59) then reinvestigated the structure of the polyoma virus late mRNAs using the S1-mapping technique (20). They found that, although the three mRNAs have a common polyadenylated 3' end located at 25.3 map units, their 5' ends

have one of seven different capped oligonucleotides. These 5' ends are all transcribed from sequences located between 65.4 and 70.5 map units on the viral genome. Each of the mRNAs has a heterogeneous mixture of leader sequences (60) which contain at least two repeats of a basic sequence appearing on the viral DNA between 66 and 68 map units (61).

The nucleotide sequence of this region of the viral genome has been recently determined (62). Two long coding frames have been identified. One of them, which is thought to encode VP1, contains 383 consecutive sense codons and extends counterclockwise from 47.5 to 25.8 map units. VP2 and VP3 are thought to be encoded by the second reading frame, located within the proximal part of the late region, with its first initiation codon at 64.7 map units and its termination codon at about 46 map units (see Figure 1).

The first viral capsid proteins can be detected in the nuclei of infected cells 3 to 6 hours after the onset of viral DNA synthesis. About 24 hours after infection, the first viral progeny particles start to appear, therefore completing the lytic cycle of the virus. However, because the population of infected cells is not synchronized, the virus continues to be produced for another 24 hours. By then, most of the infected cells have detached from the surface of the dish and have died.

ABORTIVE INFECTION AND CELLULAR TRANSFORMATION CAUSED BY POLYOMA VIRUS

When non-permissive or semi-permissive quiescent cells are infected by polyoma virus, most of the cells divide several times before resuming the growth pattern of uninfected, untransformed cells. This "abortive transformation" was first observed by Stoker (63) after he infected BHK cells (Baby hamster kidney) with polyoma virus. During abortive infection however, a small fraction of the infected cells undergo stable transformation. In tissue culture, these cells grow continuously to form disorganized, multilayered foci, they require less serum growth factors, are easily agglutinable by plant lectins, have an increased sugar uptake and show no fibronectin (a major membrane protein with a M.W. of 250,000 daltons) on their cellular membrane. Transformed cells also lose their anchorage dependence. Finally, transformed cells exhibit very diffused actin cables, resulting in a depolymerized cytoskeleton. (For an excellent review of the morphological characters of transformed cells, see 47). By comparison, cultured, normal cells grow as monolayers, are readily agglutinated by lectins, have large amounts of fibronectin, are firmly attached to the substrate, and have very organized actin cables while they are resting. They do not release any plasminogen activators, nor can they grow in suspension cultures, and finally, they do not release any proteolytic enzymes in the medium.

All non-permissive cells transformed by polyoma virus contain polyoma viral genetic material although no infectious virus can be found. As early as 1968, Westphal and Dulbecco (64) measured the amount of viral DNA in cells transformed by SV40 and polyoma virus. By hybridizing radioactive RNA, transcribed *in vitro* from purified viral DNA to transformed cell DNA attached to nitrocellulose filters, they found that while SV40 transformed cells carried about 5-30 viral genome equivalents per cell, polyoma transformed cells contained 4 to 10 copies of viral DNA per cell. However, their evaluation of the number of viral DNA copies per cell was wrong, first because the viral DNA they used as a template to synthesize the radioactive RNA contained covalently linked host cellular DNA and second, because the DNA-RNA hybrids were probably lost selectively from the nitrocellulose filters (65) during the reconstitution experiment resulting in an overestimation of the number of viral DNA copies per transformed cell. Subsequently, Botchan *et al.* (66) resolved these problems. It was then calculated that SV40 transformed cell lines contained between 1 and 10 SV40 DNA molecules per cell. Using a different approach which measured the rate of reannealing of small amounts of labelled viral DNA in the presence of high molecular weight cellular DNA isolated from transformed cells, Gelb *et al.* (67) found SV40 transformed cells to contain only 1-4 copies of viral DNA per cell. Using the same method,

Kamen et al. (16) found that mouse cells transformed by polyoma virus contained between 0.6 and 2.9 copies of viral DNA per cell and Zouzias et al. (68) reported that rat transformed cells contained up to 50 copies of polyoma viral DNA per cell. That the viral DNA sequences present in SV40 transformed cells were covalently linked to cellular DNA was first shown by Sambrook et al. (69).

Recently, Botchan et al. (70) have determined the arrangement of SV40 sequences in the genome of transformed cells. They fragmented cellular DNA with different restriction enzymes, fractionated the resulting DNA by gel electrophoresis and transferred the DNA onto a nitrocellulose filter (24). Subsequently, the transferred cellular DNA was hybridized with radioactive SV40 DNA. Their results showed that all the cell lines tested contained integrated viral DNA; in addition, seven of the eleven lines contained more than one separate insertion of viral DNA. The site of integration either on the viral or cellular DNA varied from one cell line to another. Since then, several groups have used this method to study integrated viral genes in polyoma transformed cell lines (71-74). Most of the cell lines studied appear to contain free viral DNA (68,72,73, 75,78) along with integrated tandem repeats of the viral genome ranging from less than 2 to more than 5 copies at one site (72). As in SV40 (70), the integration of polyoma viral DNA is not specific and can occur in more than one location on

the same cellular genome. The mechanism of integration of polyoma and SV40 viral DNA into host cell DNA is still unknown. Recently however, after studying in detail the arrangement of the integrated SV40 DNA in a transformed cell line, Botchan et al. (79) have suggested that the replicative form of viral or cellular DNA or both, are involved in the integration process. The exposure of a single stranded region of the DNA would then allow the initial base pairing between viral and cellular sequences to occur (79). Furthermore, the arrangement of the integrated viral genome, at least in SV40 transformed cell lines, appears to be very stable since no alteration of the integrated viral DNA and its flanking cellular sequences was noted even after hundreds of cell generations (70,80). In polyoma transformed cell lines, the integrated viral DNA also appears to be very stable but only in the absence of a functional, viral A gene product (81). However, when a functional A gene product is present, free viral DNA starts to appear as some of the integrated viral DNA is lost. This often leads to the reversion of some of the transformed cell lines, back to the normal phenotype (81). Zouzias et al. (68) have shown that tsA transformed cells lose their free viral DNA when shifted to the non-permissive temperature but that it reappears upon reincubation at the permissive temperature. This reappearing free DNA comes from the excision of the

integrated viral genome(s) (82). How this excision occurs is still unknown, but Botchan et al. (79) have recently proposed a model to explain the excision of integrated SV40 sequences when their transformed rat cells are fused with simian cells. By providing permissive factors, the simian cell allows viral DNA replication to be initiated. This replication would then raise the concentration of SV40 sequences at the integration point and therefore favor the recombination between homologous sequences. The model predicts that more than one complete genome must be integrated at one point to allow homologous recombination to occur. The same model could be used to explain excision in polyoma virus transformed rat cell lines. However, in these semi-permissive cells, only the presence of a functional A gene product would be required to initiate viral DNA synthesis. Alternatively, excision could take place by a mechanism where tandem integration would favor internal recombination which in turn, would be facilitated by the presence of the A gene product (81).

In 1966, T. Benjamin was the first to demonstrate that polyoma virus transformed cells contained polyoma virus-specific RNA (19). Later, in 1974, Kamen et al. (16) reported preliminary data suggesting that the RNA present in transformed cells was transcribed from the E strand of the DNA segment extending from within fragment Hpa II-5 to within fragment Hpa II-2. Recently however, Kamen et al. (25) have reported a

detailed study of the viral mRNAs present in either transformed mouse cells or transformed rat cells. In transformed mouse cells, no mRNA was found which extends over the entire early region (with the 3' end extending to 25.8 map units as in lytically infected cells). Instead, the 3' ends of most of the mRNAs extended to about 99 map units. However, the splicing pattern of the viral mRNAs appear to be similar to the patterns of early mRNA found during lytic infection. The 5' ends of the mRNAs in transformed cells map at 73.3 map units like the early mRNA produced in lytic infection. This observation suggested that the initiation of transcription occurs at a viral promoter. Kamen et al. (25) also reported that in one cell line containing integrated sequences from the late region, there was no late mRNA present in the cytoplasm or the nucleus suggesting the absence of functioning viral promoters in that region of the viral genome (25) and, at least in that particular cell line, that transcription from an efficient cellular promoter did not occur. Finally, Kamen et al. reported, for various transformed mouse cell lines, the existence of mRNAs containing both viral and cellular sequences, suggesting that mRNAs can be transcribed from DNA sequences beyond the junction of viral with host cell DNA (25).

Rat cell lines transformed by polyoma virus contain, in addition to the different species of viral mRNA found in mouse cells during a lytic infection (described earlier),

other shortened mRNAs transcribed from the early region of the viral genome (25). Included among these mRNAs are mRNA transcribed from the late region. The presence of these mRNAs correlates with the presence of free viral DNA in the transformed cell lines. Kamen et al. (25) have shown that in polyoma virus transformed rat cell lines containing a single insert of integrated viral DNA and no free DNA, there was no mRNA transcribed from the late region. The presence of viral mRNA transcripts extending into host sequences, like those found in polyoma virus transformed mouse cells, was also observed in transformed rat cells. As for the additional shortened mRNA found in some rat transformed cell lines, they were shown, by in vitro translation experiments, to encode truncated forms of large and middle T antigens (25).

Although it is well established that the early region of polyoma virus (and the three proteins it encodes) is responsible for cellular transformation, the role played by these proteins in the initiation and/or maintenance of transformation is still unclear. Previous studies have established that polyoma tsA mutants transform cells at the non-permissive temperature with efficiencies 2 to 3 orders of magnitude lower than at the permissive temperature (83,84,85). These studies have also shown that cells transformed at the permissive temperature with tsA mutants remain transformed when they are

shifted up to the non-permissive temperature (83,85). Because tsA mutants encode a defective large T antigen at the non-permi-sive temperature (37), these results suggest that large T antigen is necessary for the initiation of the transformed state but not for its maintenance. However, during this study we have shown that rat cells could be efficiently transformed by cloned fragments of the polyoma viral genome containing only part of the early region and therefore incapable of encoding large T antigen, (71). Moreover, Israel et al. (86) have shown that cloned fragments of polyoma DNA, also incapable of encoding large T antigen, induce tumors when injected in hamsters. These results therefore indicate that large T antigen is not absolutely necessary for the initiation of transformation. Furthermore, hr-t mutants (which encode a defective middle and small T antigens) cannot transform cells (38) even if they encode a normal large T antigen (32) suggesting that large T antigen alone is incapable of transforming cells. This conclusion has been recently confirmed by Lania et al. (73) who have isolated a cell line containing integrated and free viral hr-t mutant DNA. These cells contain large T antigen but, as expected, neither middle nor small T antigens is present (73). The morphology and the phenotype of the cells are identical to those of untransformed cells.

Seif and Cuzin (87) on the other hand, have shown that rat cells transformed by the tsA mutant of polyoma virus and subsequently isolated as foci on plastic Petri dishes (type N transformants) display a temperature sensitive phenotype while rat cells transformed by the same polyoma mutant, but isolated on agar, exhibit a transformed phenotype at both permissive and non-permissive temperatures. These experiments therefore indicate that, at least in some cells, large T antigen may play a role in the maintenance of transformation.

If large T antigen is not necessary for the initiation of transformation caused by DNA transfections and appears to play a role in the maintenance of transformation only in some cells, then small and/or middle T antigens must play a very important role in the transformation process. This suggestion has been confirmed by the recent characterization of several deletion mutants of polyoma virus.

In 1979, Griffin and Maddock (88) isolated mutants with deletions between map co-ordinates 88.5-91.5 (dl-8) and 91.5-94.5 (dl-23) a region thought to encode both large and middle T antigens. Cells transformed by these mutants have an altered phenotype; most of the cells transformed with dl-8 form larger colonies than wild type transformed cells while dl-23 form smaller colonies. After separation through gel electrophoresis, the small T antigen induced by these mutants is indistinguishable from the wild type protein but truncated forms of large and middle T antigens are detected. Other

mutants with deletions between map co-ordinates 92 and 99 have been isolated by Magnusson and Berg (89). Although these mutants grow as well as wild type virus in 3T3 cells, they transformed rat cells 0.2 to 0.05 as efficiently as the wild type virus (89). In view of these observations and because large T antigen does not appear to play a role in the maintenance of transformation (except in some cells), it is tempting to conclude that the absence of a "normal" middle T antigen is responsible for the inability of the mutants described by Magnusson and Berg to transform cells or for the changes in morphology seen in the cells transformed with dl-8 and dl-23. By contrast to the results described above, Bendig et al. (90) have isolated a mutant with a deletion of 66 base pairs located at the junction of the Hpa II 4/8 fragment (91.6 map units). This deletion is also within sequences encoding large and middle T antigens. This mutant produces a truncated form of large and middle T antigens, but causes no noticeable alteration in cellular transformation or viral replication. This may be because the truncated viral proteins, found in these cells, play an important role in the mechanism leading to the transformed phenotype.

Other deletion mutants containing deletions between map position 71 and 73 were isolated by Magnusson and Berg (89), Wells et al. (91) and Bendig and Folk (92). These mutations are not located in the sequences encoding the early proteins. Because these mutants grow as well as the wild

type virus in mouse cells and are not altered in their capacity to transform cells, the region between the origin of DNA replication and the initiation site of translation is defined as being non-essential for vegetative growth or transformation.

A protein kinase activity has also been found to be associated with polyoma virus middle T antigen *in vitro* (93, 94, 95). This observation is extremely interesting when we consider the possible mechanisms inducing cellular transformation since the product of the src gene of the Avian Sarcoma Virus which is thought to be entirely responsible for the establishment of transformation was also shown to be a protein kinase (96).

Taken together, these data suggest that middle or small T antigens or both are required to maintain the transformed state.

The purpose of this study was therefore to localize the smallest segment of the viral genome necessary to induce transformation. To accomplish this, we measured the transforming capacity of some cloned subgenomic fragments of the viral genome. The resulting, independently transformed cell lines, were subsequently isolated and their growth properties examined. The viral sequences within several of these transformed cell lines, were examined by Southern blotting (24)

according to the strategy previously described by Botchan et al. (70). The subsequent results suggest that only a restricted portion of the early region of polyoma virus is required to transform an established line of rat cells.

MATERIALS AND METHODS

A. Cell Culture

The established cell lines used in this study were either 3T6 cells, a line of mouse embryo fibroblasts (97) or Rat-1 cells, a subclone of Fisher rat F2408 cells (98) obtained from C. Basilico and recloned in our laboratory.

The cell cultures were maintained in DME (Dulbecco's Modified Eagle's Medium) (Flow) supplemented with 10% FBS (fetal bovine serum) (Flow) and kept at 37°C in an humidified, 5% CO_2 atmosphere. The cells were always subcultured before reaching confluence. To subculture, the cell layer was rinsed with warm (37°C) PBS (phosphate-buffered saline). One ml of trypsin solution (0.06% trypsin, 0.005 M EDTA in calcium and magnesium free (PBS) was added to each plate incubated at 37°C until all the cells had detached. Trypsinization was stopped by adding 5 ml of culture medium (DME with 10% FBS) per plate. The dislodged cells were diluted in culture medium and replated in 100 mm plastic Petri dishes (10 ml per plate).

B. Transfection of Rat-1 Cells with DNA

Rat-1 cells were transfected with viral and recombinant plasmid DNAs as described by Wigler et al. (99). In general, one ml aliquots of DNA solution was added to each Petri dish seeded the previous day with 5×10^5 cells. The cells were then incubated at 37°C for 2 to 3 weeks.

C. Establishment and Growth Properties of Transformed Rat Cell Lines

Individual foci on separate Petri dishes were isolated by placing over a focus of cells a stainless steel cylinder to which a few drops of trypsin solution were added. The detached cells were diluted in culture medium and plated in 60 mm Petri dishes. At confluence, the cells were recloned by replating at high dilutions and isolating individual colonies (as described above).

The saturation density of these newly transformed cell lines was defined as the density which the cells maintained for three consecutive days in culture. Growth curves of the various cell lines were obtained as follows. Twenty small dishes (60 mm) were seeded with 1×10^4 cells/cm^2. The number of cells in duplicate cultures was determined daily (using a hemocytometer), while the medium of the remaining dishes was changed every second day.

The capacity of newly transformed cells to grow in soft agar was tested by following the procedure described by Macpherson and Montagnier (100). Triplicate cultures, each containing 10^2, 10^3 and 5×10^3 cells were plated in 2 ml of 0.3% Noble Agar. Macroscopic colonies were counted after one to two weeks of incubation at $37^{\circ}C$.

D. Infection of Mouse 3T6 Cells with Polyoma Virus

Polyoma virus was routinely propagated in mouse 3T6 cells. To avoid the accumulation of defective virus particles, infections were performed at low multiplicity of infection (0.01 to 0.1 PFU (plaque forming units) per cell). After washing the cell layer with PBS, 1 ml of appropriately diluted virus stock solution was added to each 100 mm Petri dish. The plates were then incubated at 37°C and rocked every 15 minutes. After 2 hours, fresh culture medium was added and the cells were returned to the incubator. Three to four days later, or when CPE (cytopathic effect) was at its maximum, the cells were resuspended in 0.4 ml of Tris-saline at neutral pH and sonicated for one minute. The resulting cellular debris were incubated at 37°C overnight with 100 units of RDE (receptor destroying enzyme) (13). A drop of a 0.7% solution of sodium bicarbonate was then added and the cellular debris were centrifuged at 2,000 rpm for 10 minutes. The supernatant containing the virus particles was removed and kept frozen at -20°C. The concentration of virus particles in the stocks was determined by hemagglutination using guinea pig red blood cells (13).

E. Molecular Cloning of the Polyoma DNA

Polyoma viral DNA, used in the transformation of the Rat-1 cells, was cloned with the pBR322-*Escherichia coli* x1776 plasmid-vector system. *E. coli* x1776 was cultured either in x-broth (0.05 M Tris pH 7.6, 2.5% (w/v) bacto Tryptone, 0.75% (w/v) Bacto Yeast Extract and 0.4% (w/v) $MgCl_2$) and kept at 37°C in a shaking incubator, or on x-agar plates (1% Bacto agar in x-broth) also kept at 37°C. pBR322 is a ColE1 derivative plasmid (101). It has a molecular weight of 2.6×10^6 daltons and carries two genetic markers that confer to the harboring bacteria, resistance to ampicillin or tetracycline.

When *E. coli* cells are treated with calcium chloride solutions, they become "competent" and take up purified DNA (102). *E. coli* x1776 was therefore treated with 0.1 M calcium chloride for 24 hours at 0°C as described by Mandel and Higa (102) and Dagert and Ehrlich (103). Ten or 100 ng of recombinant plasmid DNA resuspended in 0.01 ml of TE (0.01 M Tris, 0.001 M EDTA) were added to 0.1 ml aliquots of competent bacteria. The DNA bacteria mixtures were incubated first on ice for 10 minutes then at 37°C for 5 minutes and diluted with 2 ml of x-broth. After incubating at 37°C for one hour, 100 ul or 10 ul of each bacterial culture were spread on agar plates containing either 100 ug/ml of ampicillin or 50 ug/ml of tetracycline. The plates were then kept at 37° until colonies appeared.

F. Colony Hybridization

Bacterial colonies harboring recombinant plasmids containing viral sequences were identified by colony hybridization (104). The bacterial colonies were transferred from the agar plates to nitrocellulose filters. The DNA within these bacteria was denatured by setting the nitrocellulose filters on layers of Whatmann paper soaked in an alkali solution (0.2 M NaOH, 0.6 M NaCl) for 10 minutes. To neutralize the DNA, the nitrocellulose filters were set on Whatman paper soaked in 0.6 M NaCl and 1.0 M Tris pH 7.4, again for 10 minutes. The nitrocellulose filters were subsequently baked at 80°C for 2 hours and hybridized with nick-translated polyoma viral DNA (as described in sections M and N). After autoradiography, the colonies containing polyoma viral DNA appeared black on the film. The parental colonies, left on the agar plates, were then picked and propagated.

G. Isolation of Recombinant DNA from Bacteria

Cloned polyoma DNA was isolated from bacteria harboring the recombinant plasmid as follows; The bacteria were washed in cold TE and resuspended in a chilled (4°C) solution of 25% sucrose in 0.05 M Tris pH 8.0. Fresh lysozyme was added to a final concentration of 2.5 mg/ml and the solution was gently swirled on ice for 5 minutes. 0.5 M EDTA was then added to a final concentration of 0.2 M and again the solution was swirled on ice for 5 minutes. Finally,

an equal volume of a 1% solution of Triton x 100 in 0.06 M EDTA and 0.05 M Tris was added and the mixture was left on ice for 10 minutes. The bacterial cell lysate was then centrifuged at 23,000 rpm (Beckman SW 27 rotor) for one hour at 4°C. The DNA within the supernatant fraction was purified by sedimentation through a CsCl density gradient containing 200 ug/ml of ethidium bromide. The autoforming gradients were centrifuged in an angle 65 rotor (Beckman) at 50,000 rpm for 20 hours at 25°C. The resulting purified DNA was disolved in TE and stored at 4°C.

H. Isolation of High Molecular Weight Cellular DNA

Total cellular DNA from transformed Rat-1 cells was isolated as described by Botchan et al. (70). In general, the cells were harvested by aspirating the medium from 10 Petri dishes (100 mm) and rinsing the cell layers with ice cold PBS. After rinsing, 5 ml of PBS were added to each Petri dish. The cells were resuspended in this buffer using a sterile rubber policeman and centrifuged at 1,000 rpm for 5 minutes at 4°C in a Sorval L-2B centrifuge. The cell pellet was then resuspended in 10 ml of TE to which were added 0.5 ml of 10% SDS (Sodium dodecyl sulfate) and 1 mg/ml of pronase (Calbiochem). After incubating for 5 to 8 hours at 37°C, the solution was extracted twice with 10 ml of phenol and once

with a 24:1 mixture of chlorophorm-isoamyl alcohol. The DNA solution was subsequently dialysed 3 times against 400 volumes of cold (4°C) TE and stored at 4°C.

I. Purification of Polyoma Viral DNA from Lytically Infected Cells

Polyoma viral DNA was extracted from lytically infected mouse 3T6 cells by following the procedure previously described by Hirt (105). The cells were washed with ice cold PBS and resuspended in lysis buffer (0.6% SDS, 0.01 M Tris pH 7.9, 0.01 M EDTA) for 5-10 minutes before 5 M NaCl was added to obtain a final concentration of 1.0 M. The resulting viscous solution was kept on ice overnight and subsequently centrifuged in a SW 27 rotor (Beckman) at 23,000 rpm for 60 minutes at 4°C. The supernatant fraction was removed, phenol extracted, dialyzed against TE and purified by sedimentation through an ethidium bromide (200 ug/ml) CsCl density gradient. Finally, the purified DNA was dialyzed, ethanol-precipitated and resuspended in TE.

J. Digestion of Cellular DNA with Restriction Enzymes

The cellular DNAs were digested with an excess of the restriction enzymes (2-3 units/ug of DNA) according to the conditions specified by the manufacturer (Bethesda Research Labs. and New England Biolabs.).

K. Gel Electrophoresis

The DNA fragments obtained by hydrolysis of cellular DNA with the various restriction endonucleases were fractionated either through neutral-agarose gels, according to Botchan et al. (70) or through alkaline-agarose gels as described by McDonell et al. (23). In general, 20 cm x 20 cm gels were run in an horizontal gel system under constant current conditions (not exceeding 5 v/cm). Depending upon the expected size of the DNA fragments, the concentration of agarose in the gel varied from 0.7% to 1.4%. Prior to electrophoresis, the DNA samples (5 ug of DNA for neutral-agarose gels, 50 ug of DNA for alkaline-agarose gels) were resuspended in 50 ul of electrophoresis buffer (0.04 M Tris base, 0.01 M NaH_2PO_4, 0.001 M EDTA for neutral gels and 0.03 M NaOH and 0.02 M EDTA for alkalin-agarose gels) containing 0.03% Bromophenol blue, 0.03% Xylene Cyanole FF and 8% (w/v) sucrose.

After completion of electrophoresis, the DNA within the gel was stained by immersing the gels for 20 minutes in electrophoresis buffer containing 0.5 ug/ml of ethidium bromide (106). The gels were then photographed under U.V. illumination using a Polaroid HP-4 system and a Kodak 22A filter.

L. Transfer of DNA Fragments to Nitrocellulose Filters

After photography, the agarose gels were immersed in an alkaline solution (1.5 M NaCl and 0.5 M NaOH) for about 45 minutes at room temperature, rinsed in distilled water, and submerged in a second solution (3 M NaCl and 0.5 M Tris pH 7.0), again for 45 minutes. The cellular DNA fragments within the gels were then transferred to nitrocellulose filters essentially as described by Southern (24). By covering the top of the gel with first a sheet of nitrocellulose (BA83 or BA85 , Schleicher and Schuell) and then a stack of paper towels, both cut to the same dimension as the gel, a solution of 6 x SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate) was drawn up through the gel and the nitrocellulose filter to finally reach the dry paper towels. The DNA within the gel is eluted with the 6 x SSC and trapped on the nitrocellulose filters. These filters were then rinsed with 2 x SSC and dried for 2 hours in a pre-warmed vacuum oven at 80°C. The efficiency of transfer of the DNA from the gel to the nitrocellulose filters was tested by restaining the gel with ethidium bromide after the transfer process and comparing it with the photograph of the same gel taken before the transfer. In general, the cellular DNA fragments within 0.7% agarose gels were transferred with an efficiency of about 90%.

M. Hybridization of the Nitrocellulose Filters and Autoradiography

Before hybridization, the nitrocellulose filters were placed in a plastic bag and soaked for at least 4 hours in a solution of 5 x SSC, 0.1 M sodium phosphate buffer pH 7.0 and 4 x Denhardt's solution (Denhardt's solutions: 0.2% Ficoll, 0.2% polyvenypyrolidone, 0.2% BSA, dissolved in distilled water (107) heated to 68°C.

The solution in the plastic bag was then replaced by 5 ml of hybridization solution (5 x SSC, 0.1 M sodium phosphate buffer, 4 x Denhardt's solution and 0.1 ug of denatured ^{32}P-labelled polyoma DNA (specific activity 1×10^8 - 5×10^8 cpm/ug)) and hybridization was carried out for about 15 hours in a shaking water bath at 68°C.

Following hybridization, the nitrocellulose sheets were taken out of the plastic bags and washed at 68°C, first in a solution of 5 x SSC, 0.1 M sodium phosphate buffer pH 7.0, 1 x Denhart's solution and 0.5% SDS, then in a solution containing 1 x SSC, 0.1 M sodium phosphate pH 8.4 and 0.5% SDS and finally, in a solution of 0.75 x SSC and 0.3% SDS (2-3 hours in each solution). They were then briefly rinsed with distilled water, air-dried for 30 minutes, taped on a sheet of Whatman paper and subjected to auto-radiography at -80°C using Kodak RP-Royal film and

intensifying screens (Cronex Lightning Plus, Dupont) (108) for periods of 15 hours to 7 days.

By following this procedure, as little as 10^{-6} to 10^{-7} ug of polyoma DNA could be detected on the nitrocellulose filters.

N. In Vitro Labelling of DNA Molecules

Radioactive polyoma DNA was prepared in vitro by nick translation (109). In general, 0.1 ug of DNA was treated with 10 units of E. Coli DNA polymerase (Boehringer Mannheim) for 1 hour at 15°C. The reaction was performed in a volume of 0.05 ml containing 0.05 M Tris pH 7.8, 0.01 M Mercaptoethanol, 0.005 M $MgCl_2$, 50 ug/ml BSA and 25 uCi of each of the four ^{32}P labelled deoxynucleotide triphosphates (specific activity 2,000 - 3,000 Ci/mM, Amersham Corporation).

The labelled polyoma DNA was separated from the remaining free deoxynucleoside triphosphates by passage through a column (0.5 x 20 cm) of G-50 Sephadex equilibrated with TE. The specific activity of the labelled DNA usually varied from 1×10^8 to 5×10^8 cpm/ug.

R E S U L T S

When this study originated, the involvement of the early region of the polyoma viral genome during cellular transformation was well documented. However, whether the entire early region was necessary for the induction and/or maintenance of transformation was still unknown. To localize the smallest segment of the polyoma viral genome necessary to induce transformation, we directly measured the transforming capacity of cloned subgenomic fragments of the polyoma genome. The resulting, independently transformed cell lines were isolated and their growth properties examined. The viral sequences within several of these transformed cell lines were also examined.

A. Characterization of the Cloned Polyoma DNA

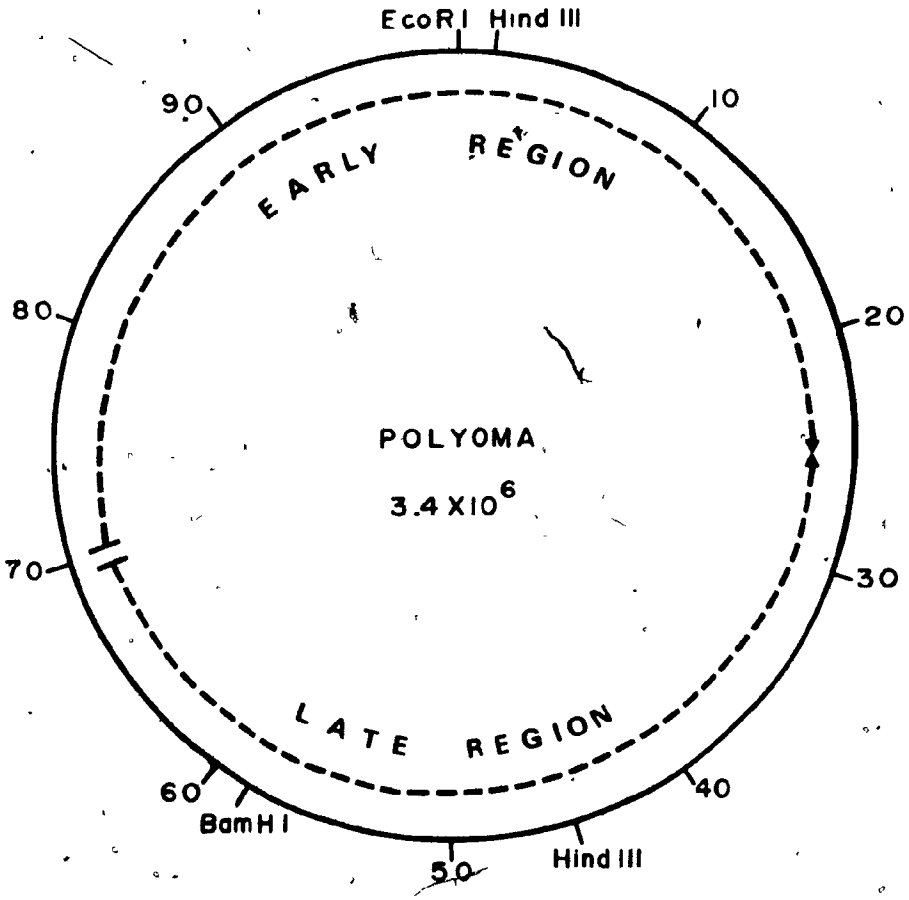
Polyoma viral DNA was cloned in the pBR322-*E. coli* plasmid vector system as described previously (section 2.E) and already published (71). Hydrolysis of the viral DNA with the restriction endonuclease *Hind* III yields 2 fragments, each containing about half of the early region. The *Hind* III fragments 1 (*Hind* III-1, 45 to 1.4 map units clockwise on the physical map) and 2 (*Hind* III 2, 1.4 to 45 map units) were

separately inserted into the unique Hind III site of pBR322 DNA (see Figure 2). The resulting recombinant plasmids were then used to transform E. coli x1776. Bacterial clones containing insertions of polyoma sequences were identified by colony hybridization (104). The insertion of the two Hind III fragments occurred in two possible orientations relative to the Eco RI site in pBR322 DNA. The recombinant plasmid DNAs containing the Hind III-1 fragment (pPH1-4 and pPH1-8) and the Hind III-2 (pPH2-41 and pPH2-2) were subsequently characterized by digestion with different restriction enzymes and hybridization with various DNA probes. A physical map of these recombinant DNA molecules is shown in Figure 3. The infectivity of the cloned viral DNA fragments was also determined. Plasmids containing either the Hind III-1 fragment or the Hind III-2 fragment were mixed and digested with Hind III. The resulting DNA fragments were ligated with Phage T4 ligase to reconstitute the entire polyoma genome. Both biochemical and biological assays demonstrated that the reconstituted viral genomes had not undergone any detectable alterations in E. coli.

The entire polyoma viral genome was also cloned in pBR322. These plasmids were constructed by cleaving both the viral and plasmid DNA with either Eco RI or Bam HI (both of these enzymes cleave polyoma viral or plasmid DNA

Figure 2. Physical maps of polyoma and pBR322.

The restriction endonuclease cleavage sites for polyoma DNA and the direction of transcription of the early and late regions are indicated. The sites of cleavage of Bam HI, Eco RI and Hind III, and the physical locations of the ampicillin resistance gene (Ap^r) and the tetracycline resistance gene (Tc^r) on pBR322 DNA are shown.


EcoR I
Hind III
10
20
30
40
50
60
70
80
90
EARLY REGION
LATE REGION
POLYOMA
3.4 X 10^6
Bam H I
Hind III

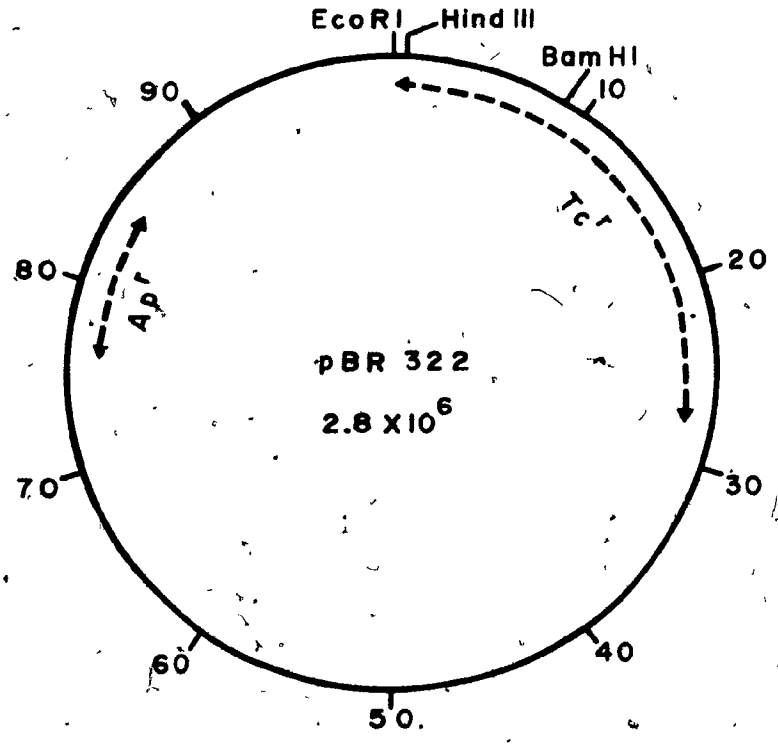
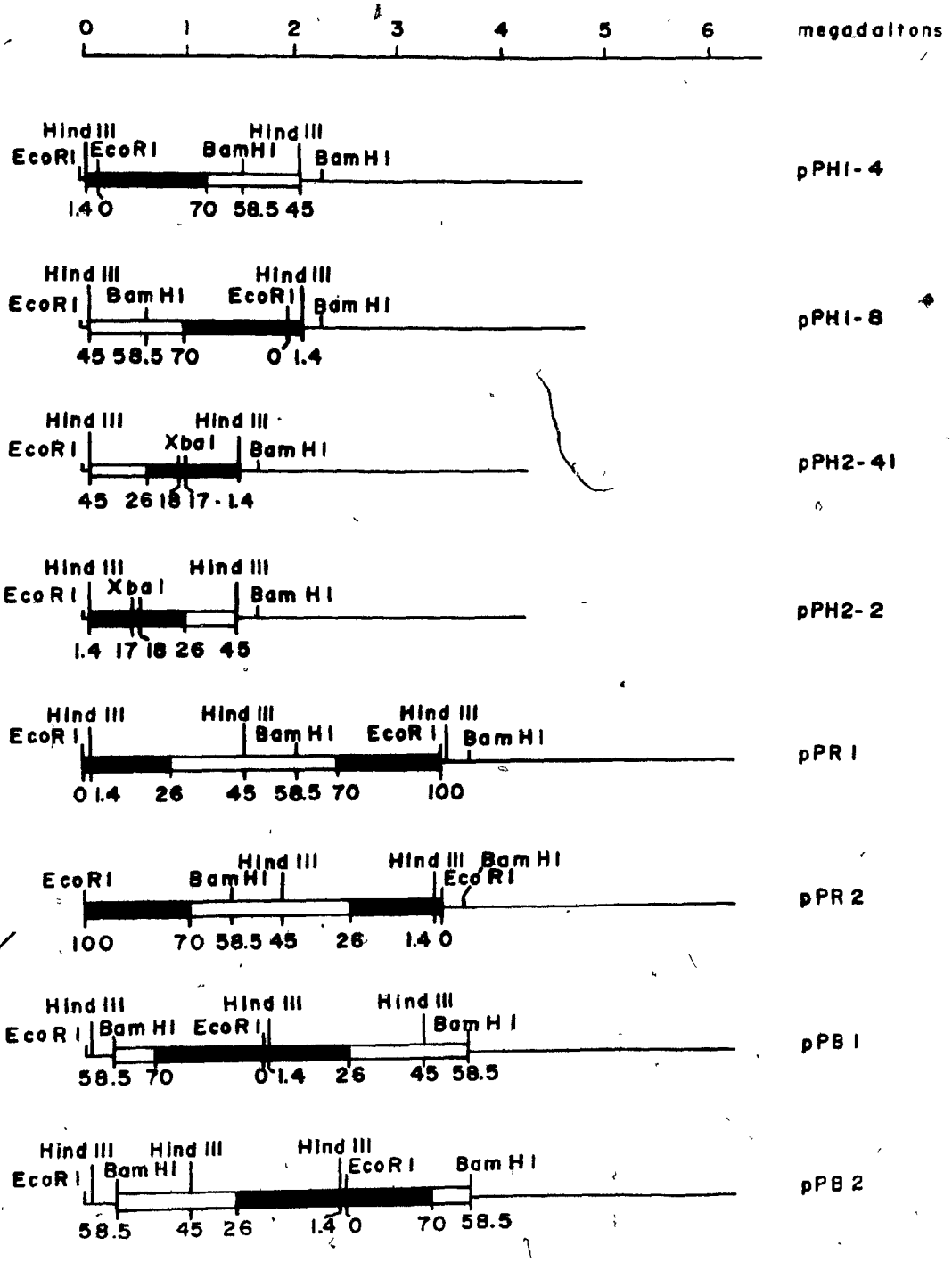

EcoR I
Hind III
Bam H I
10
20
30
40
50
60
70
80
90
Tc r
Ap r
pBR 322
2.8 X 10^6

Figure 3. Physical maps of polyoma-pBR322 recombinant plasmids.

Each circular plasmid has been opened at the Eco RI site within pBR322 DNA. The sites of cleavage of Bam HI, Eco RI and Hind III are shown. The boxed areas represent polyoma sequences and the darkened areas represent the early region of polyoma virus. The numbers below each map refer to the restriction endonuclease cleavage sites above and are shown here in polyoma map units. The recombinant plasmid genomes are drawn to scale.


0 1 2 3 4 5 6 megadaltons
Hind III EcoRI EcoRI BamHI Hind III BamHI
1.4 0 70 58.5 45
pPH1-4
Hind III EcoRI BamHI Hind III EcoRI BamHI
45 58.5 70 0 1.4
pPH1-8
Hind III EcoRI XbaI Hind III BamHI
45 26 18 17 1.4
pPH2-41
Hind III EcoRI XbaI Hind III BamHI
1.4 17 18 26 45
pPH2-2
Hind III EcoRI Hind III BamHI Hind III EcoRI BamHI
0 1.4 26 45 58.5 70 100
pPR1
EcoRI BamHI Hind III Hind III BamHI EcoRI
100 70 58.5 45 26 1.4 0
pPR2
Hind III EcoRI BamHI Hind III EcoRI Hind III BamHI
58.5 70 0 1.4 26 45 58.5
pPB1
Hind III EcoRI BamHI Hind III BamHI Hind III EcoRI BamHI
58.5 45 26 1.4 0 70 58.5
pPB2

once (see Figure 2)), and ligating the resulting DNAs with phage T4 ligase. The DNA of the recombinant plasmid was characterized as described above. Figure 3 shows the recombinant plasmids containing the entire polyoma genome either inserted into the Eco RI site (pPR1 and pPR2) or in the Bam H1 site of pBR322 DNA (pPB1 and pPB2).

B. Transformation with Cloned Polyoma DNA

To determine whether the entire polyoma early region is necessary to transform cells in vitro, the transforming capacity of the recombinant plasmid DNAs shown in Figure 3 was measured. Rat-1 cells in culture were transfected with DNA from each of the plasmids (99). Table 1 shows that with the exception of pPH2-41 and pPH2-2, all the recombinant plasmids tested are capable of transforming Rat-1 cells. Furthermore, these results revealed that recombinant DNA molecules which contained an interrupted early region (pPR1 and pPR2) or those which contained only part of the early region (pPH1-4 and pPH1-8) transform Rat-1 cells with frequencies comparable to those obtained by the recombinant plasmids containing an intact early region (pPB1 and pPB2). The results presented in Table 1 also suggest that the orientation of the polyoma sequences within the pBR322 DNA does not influence the transforming activity of each pair of recombinant plasmids.

Table 1. Transforming activity of various DNAs

DNA source	ug DNA/ dish	Foci/ dish	Average foci/ ug DNA
pPB1	0.138	112,120,163,195,78	968
pPB2	0.218	98,79,221,181,97	676
pPR1	0.161	103,91,128,153,105	720
pPR2	0.190	88,115,163,129,79	604
pPH1-4	0.194	91,95,117,158,123	602
pPH1-8	0.220	110,201,117,183,261	792
pPH2-2	0.230	0,0,0,0,0	0
pPH2-41	0.180	0,0,0,0,0	0
polyoma	0.120	210,251,163,221,181	1710
pBR322	1.84	0,0,0,0,0	0
salmon sperm	15.0	0,0,0,0,0	0

C. Properties of Rat Cells Transformed with Cloned Polyoma DNA Fragments

Cells transformed by polyoma virus and SV40 acquire a new set of properties that distinguish them from their untransformed counterparts (see Introduction). However, transformed cells do not acquire all these properties concomitantly (110), and it has been suggested that the different traits of transformed cells may be dependent on the separate activities of the early gene products of the virus (111,112). We were therefore curious to learn whether rat cells transformed with a segment of polyoma viral DNA capable of encoding small and middle T antigens only, displayed a partial or complete transformed phenotype. To determine this, we chose to study transformation-specific properties of cells transformed with the cloned Hind III-1 fragment (subsequently referred to as fragment transformants) and compare them to those of cells transformed with either polyoma virus or polyoma viral DNA.

Included among the acquired properties distinguishing transformed cells from untransformed cells is the capacity to grow to high saturation densities and the capacity to form colonies in soft agar medium (for a detailed description of the properties acquired by transformed cells, please refer to the Introduction). The results (Table 2) show that the fragment transformants and the polyoma DNA transformants grow to high saturation densities compared to

Table 2. Properties of transformed cell lines

Cell line	Transforming agent	Saturation density (cells/cm^2x10^{-4})	EOP* in agar (colonies/100 cells plated)
Rat -1	none	14	0.001
2-4a	pPH1-8 DNA	35	8.1
6-1a	pPH1-8 DNA	35	4.5
15-5a	pPH1-4 DNA	61	6.5
A4-13a	pPH1-4 DNA	50	12.5
A8-3a	pPH1-8 DNA	53	10.7
A8-8a	pPH1-8 DNA	40	4.3
A8-10a	pPH1-8 DNA	32	7.3
A4-19a	pPH1-4 DNA	43	2.7
16-2	polyoma DNA	18	8.1
18d2	polyoma DNA	50	7.0
19a1	polyoma DNA	27	30.0
18a1	polyoma DNA	26	9.6
33-3a	polyoma DNA	44	2.1
33-1a	polyoma DNA	36	3.6
38-1a	polyoma DNA	51	11.8
PyV1a	polyoma virus	61	10.0

*The efficiency of plating of cells in agar after 7-14 days of incubation at 37 degrees C.

the untransformed Rat-1 parent cell line. Table 2 also shows that all the various transformants are capable of growth in soft agar, unlike the parental Rat-1 cell line. The analysis of these two transformation criteria therefore shows that rat cells transformed with DNA containing only part of the viral early region (72 to 1.4) exhibit a fully transformed phenotype.

D. Viral DNA in Transformed Cells

To show that the fragment transformed cell lines were transformed with DNA molecules containing only part of the polyoma early region and contained no other contaminating polyoma sequences, high molecular weight cellular DNA was prepared from several of these cell lines, digested with Hind III, fractionated by electrophoresis through an alkaline-agarose gel and screened for the presence of viral sequences using the Southern blot-hybridization technique (24). The three different DNA probes used in the hybridization experiment were labelled in vitro by nick translation (109). They were the polyoma Hind III-1 and Hind III-2 fragments, both separately isolated from an agarose gel after cleavage of pPH1-8 and pPH2-41 plasmid DNA with Hind III, and pBR322 DNA. The results of such an analysis with one representative fragment-transformed cell line (15-5a) is

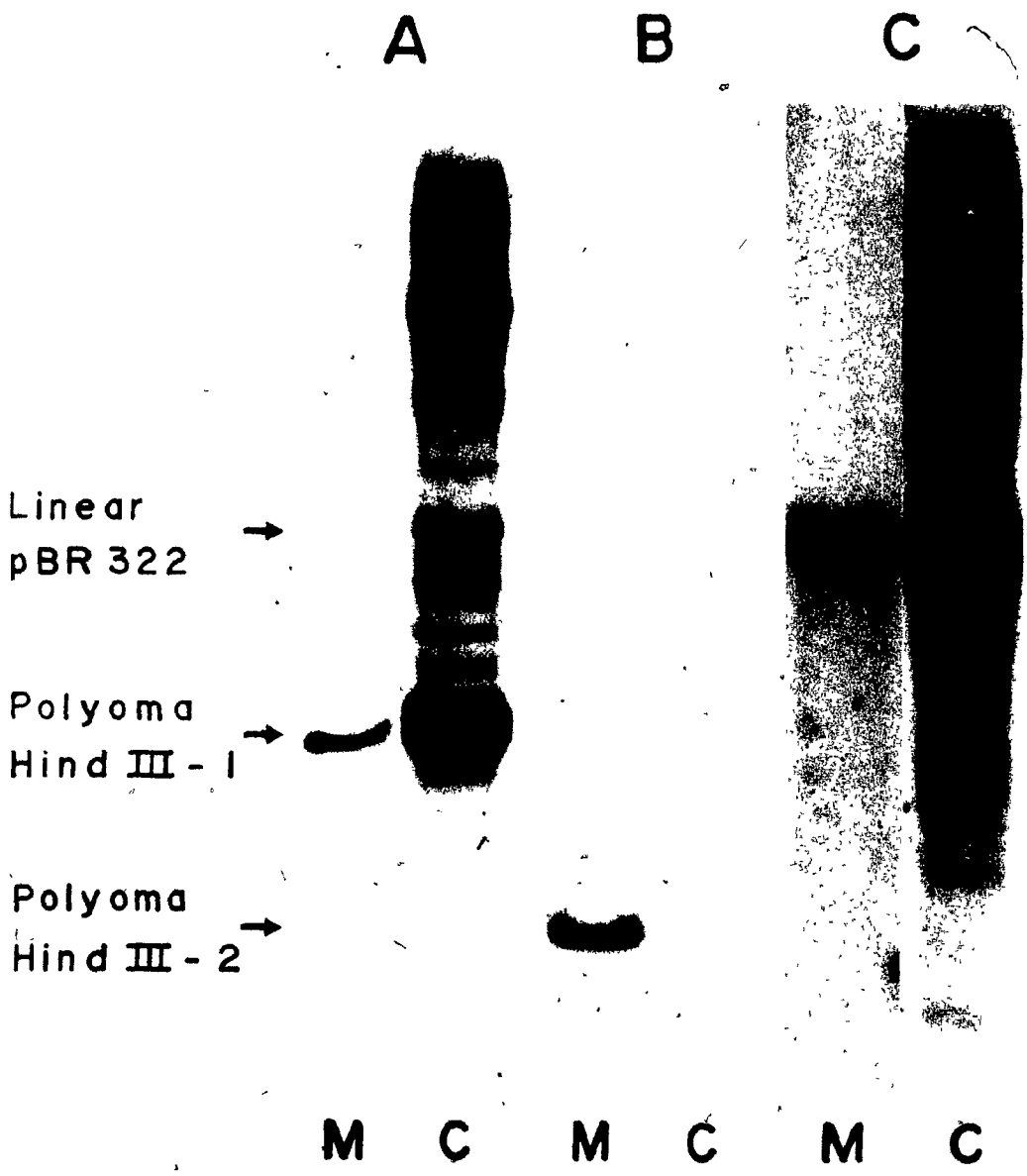
shown in Figure 4. Hybridization of 15-5a cellular DNA with the viral Hind III-1 fragment (Figure 4A) reveals after autoradiography the presence of several fragments complementary to the probe DNA, including an intense band which co-migrated with the marker Hind III-1 fragment. However, even after long exposure periods, no fragments are detected after hybridization of 15-5a cellular DNA with the Hind III-2 DNA probe (Figure 4B). Used as a probe, ^{32}P labelled pBR322 also reveals a multitude of fragments including one which co-migrated with linear pBR322 DNA. Because we can detect up to 10^{-7} ug of linear pPH1-8 DNA using this method, the analysis of 50 ug of cellular DNA can reveal as little as 0.002 copies of recombinant plasmid DNA per cell. Figure 4 thus shows that 15-5a cells contain less than 0.002 copy of the Hind III-2 fragment per cell, and therefore, probably does not contain this fragment at all.

E. The Arrangement of Viral DNA in Fragment-Transformed Cells

Until recently, the arrangement of viral DNA sequences in transformed cells was unknown. In 1976 however, Kettner and Kelly (113) and Botchan et al. (70) reported that in the case of SV40 transformed cells, multiple copies of intact and partial viral genomes integrate at different sites in the cellular genome and that the junction between the viral sequences and the cellular sequences differ from one insertion site to the other.

Figure 4. Detection of DNA fragments that contain polyoma or pBR322 DNA sequences after cleavage of 15-5a cellular DNA with Hind III.

A mixture of pPH1-8 and pPH2-41 DNA was cleaved with Hind III and 10^{-5} ug of the resulting DNA subjected to electrophoresis in adjacent slots (M) next to the 50 ug of 15-5a cellular DNA cleaved with Hind III (C). The fragments were transferred to nitrocellulose sheets, the sheets were cut into three strips and each of these was separately hybridized with:
(A) ^{32}P-labelled Hind III-1 fragment of polyoma DNA;
(B) ^{32}P-labelled Hind III-2 fragment of polyoma DNA;
(C) ^{32}P-labelled pBR322 DNA. The autoradiogram shown in A was exposed for 15 hours; in B for 33 hours; in C for 24 hours.

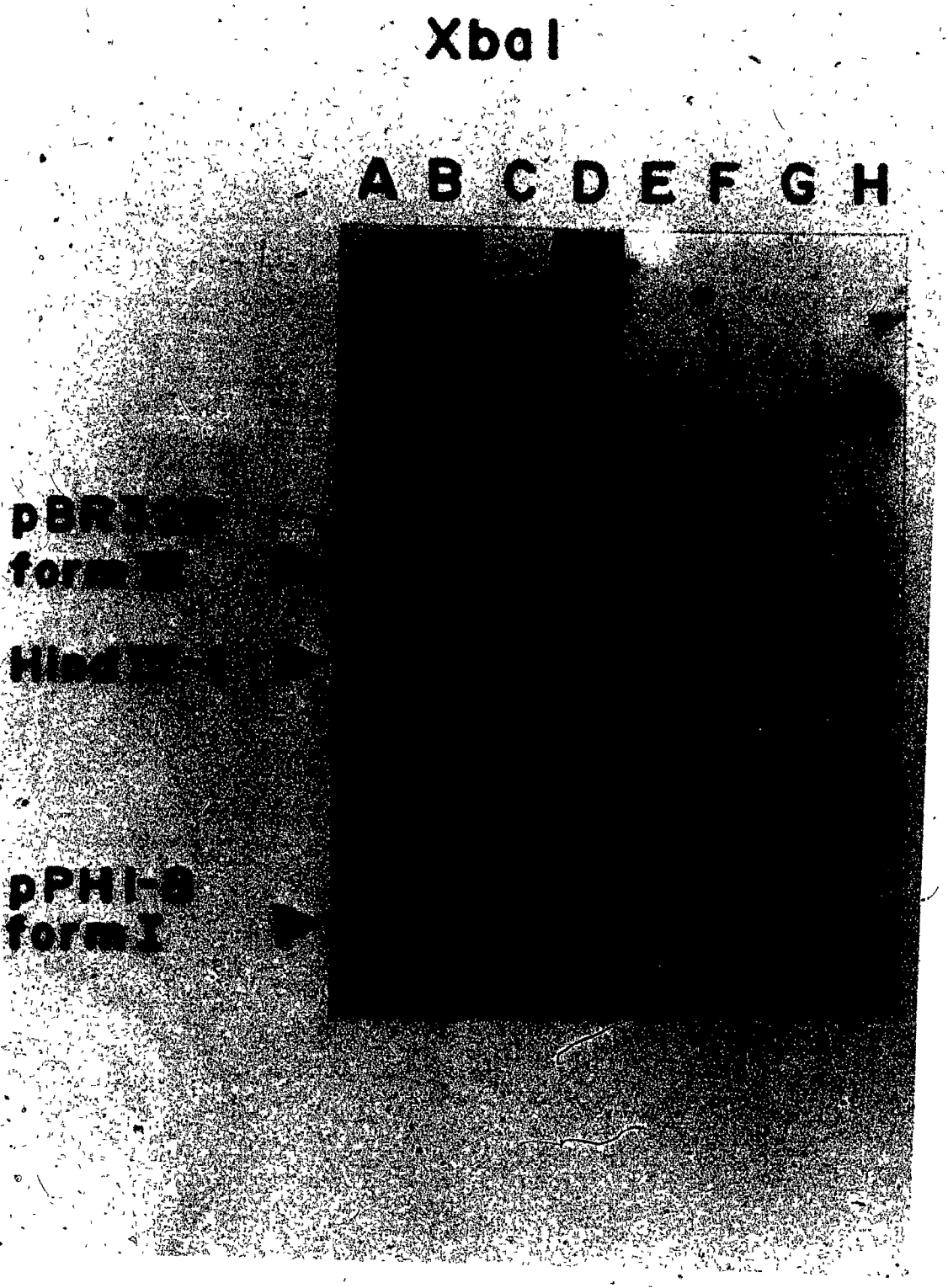

A
B
C
Linear
pBR 322
Polyoma
Hind III - 1
Polyoma
Hind III - 2
M C M C M C

To determine whether the polyoma viral sequences in transformed cells are arranged in a similar fashion, or whether there are specific sites, either on the viral sequences or on the cellular sequences, where integration takes place, we have examined, using Southern blot-hybridization (24), the arrangement of viral sequences within several fragment-transformed cell lines according to the strategy previously described by Botchan et al. (70).

To determine the number of separate insertions of integrated recombinant DNA present in the genome of fragment-transformed cells, their cellular DNA was digested with restriction enzymes which do not cleave pPH1-4 or pPH1-8 DNA (subsequently called no-cut enzymes). These enzymes cleave transformed cell DNA only at sites within cellular sequences. Thus, the total number of fragments containing polyoma viral sequences present after digestion of cellular DNA indicates the minimum number of separate insertions of viral DNA within the cellular genome. Figure 5 shows the results obtained when 5 ug quantities of cellular DNA from several transformed cell lines are cleaved with Xba I (a no-cut for pPH1-8 DNA) fractionated through an horizontal 0.7% agarose gel and hybridized to ^{32}P-labelled pPH1-8 DNA. Four of the seven fragment-transformed cell lines analyzed (24a, A8-3a, A8-8a and A8-10a) contain only one insertion of recombinant DNA. The remaining 3 cell lines contain either two separate insertions (A4-19a) or multiple

Figure 5. Detection of DNA fragments that contain polyoma sequences after cleavage of fragment-transformed cell DNAs with endonuclease Xba I.

Transformed cell DNAs (5 ug) were digested to completion with Xba I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPH1-8 DNA, the filter was subjected to autoradiography. The fragment-transformed cell lines analyzed are: 2-4a (B), 6-1a (C), 15-5a (D), A4-19a (E), A8-3a (F), A8-8a (G) and A8-10a (H). Lane A contains 10^{-5} ug of pPH1-8 form I DNA, 10^{-5} ug of Hind III fragment 1 and 10^{-5} ug of pBR322 form III DNA. The autoradiograms shown in A and D were exposed for 48 hours and in B, C, E, F, G and H for 5 days.


XbaI
A B C D E F G H

insertions (6-1a and 15-5a) of recombinant DNA. Similar results were obtained after digestion of fragment-transformed cellular DNA Hpa I (Figure 6), another no-cut enzyme for pPH1-8 DNA.

Very often after the electrophoresis of cellular DNA previously digested with various restriction endonucleases, the ethidium bromide stained gel shows co-migrating bands present in lanes containing cellular DNA. These bands are composed of tandemly repeated sequences present in the cellular genome (70). The ^{32}P-labelled probe DNA hybridizes "illegally" to the DNA within these bands which can therefore be seen on the autoradiograms. However, no polyoma sequences are present since these bands are also seen after digestion of untransformed cellular DNA. One good example of such co-migrating bands is seen in Figure 5.

In general, rat cells transformed with polyoma virus contain free viral DNA molecules (68, 72, 76, 77). Figures 5 and 6 show that none of the fragment-transformants contain free recombinant plasmid DNA molecules (sensitivity of the method: 0.02 copies of Hind III-1 fragment can be detected). Such free molecules, if present, would remain unaltered after digestion with Xba I or Hpa I and would therefore co-migrate with pPH1-8 form I DNA or the Hind III-1 fragment and pBR322 DNA shown in Figure 5A.

Figure 6. Detection of DNA fragments that contain polyoma sequences after cleavage of fragment transformed cell DNAs with endonuclease Hpa I.

Transformed cell DNAs (5 ug) were digested to completion with Hpa I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPH1-8 DNA, the filter was subjected to autoradiography. The fragment-transformed cell lines analyzed are: 2-4a (B), 6-1a (C), 15-5a (D), A4-19a (E), A8-3a (F) and A8-8a (G). Lane A contains (from top to bottom) 10^{-5} ug of pPH1-8 form III DNA, 10^{-5} ug of pPH1-8 form II DNA and 10^{-5} ug of pPH1-8 form I DNA. The autoradiograms shown in A, B and D were exposed for 48 hours in C, E, F and G, for 5 days.

Hpa I

To determine if the integrated recombinant plasmid sequences were arranged in a head to tail tandem repetition, the cellular DNA of the fragment-transformants was digested with Kpn I and Sal 1, each of which cleave pPH1-4 or pPH1-8 DNA at only one site. Digestion of the cellular DNA with such enzymes should generate full length linear recombinant molecules provided that the enzyme recognition site is duplicated at least once. These molecules will then be revealed as bands co-migrating with the linearized marker DNA. Digestion with Kpn I (Figure 7) reveals that of the 7 fragment transformants, only 15-5a contains a partial duplication of integrated pPH1-4 DNA. Similar results were obtained after digestion of 15-5a cellular DNA with Sal I (Figure 8). It is noteworthy that the black dots seen in a number of autoradiograms are caused by background hybridization of the ^{32}P-labelled DNA to the nitrocellulose filters (for example, see Figure 8).

The observation that the polyoma viral Hind III-1 fragment was sufficient to cause transformation of cells in culture was surprising but we were still curious to learn whether the entire Hind III-1 fragment was essential to cause cellular transformation. To determine this, we screened several fragment-transformed cell lines for the presence of an intact polyoma viral Hind III-1 fragment by cleaving the

Figure 7. Detection of DNA fragments that contain polyoma sequences after cleavage of fragment-transformed cell DNAs with endonuclease Kpn I.

Transformed cell DNAs (5 ug) were digested to completion with Kpn I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPH1-8 DNA, the filter was subjected to autoradiography. The fragment-transformed cell lines analyzed are: 2-4a (B), 6-1a (C), 15-5a (D), A8-3a (E), A8-8a (F) and A8-10a (G). Lane A contains 10^{-5} ug of pPH1-8 DNA cleaved with Kpn I. The autoradiograms shown in A and D were exposed for 48 hours and those in B, C, E, F and G, for 5 days.

Kpn I

A B C D E F G

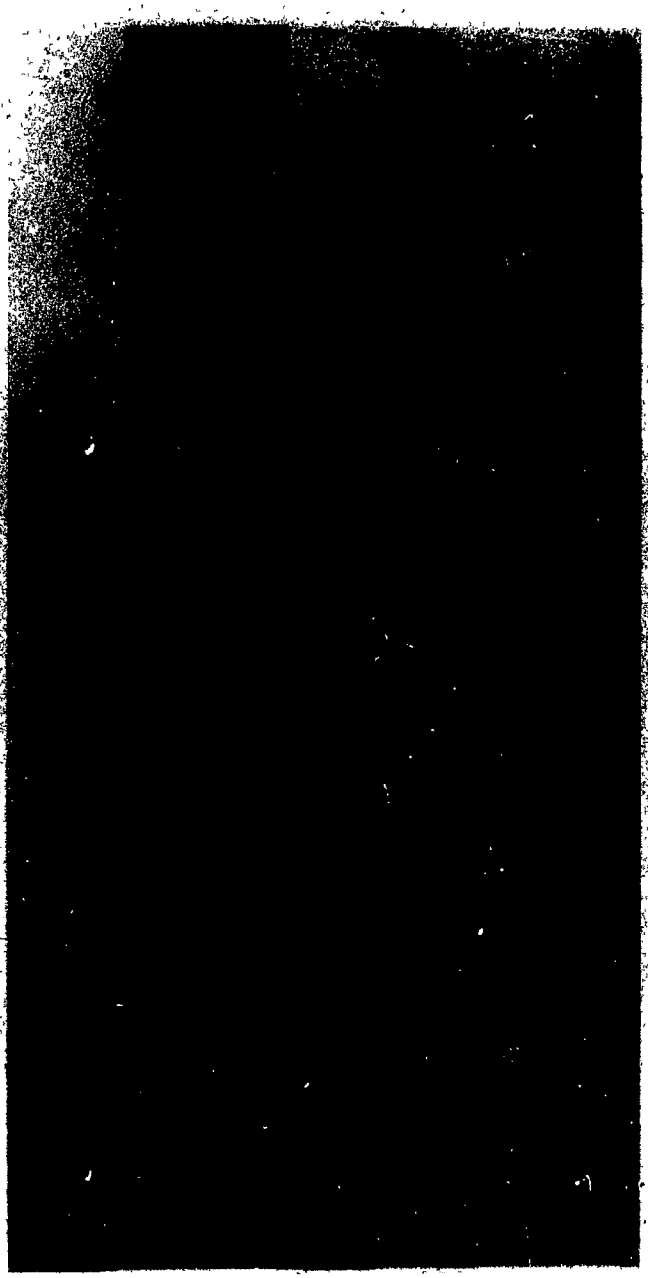
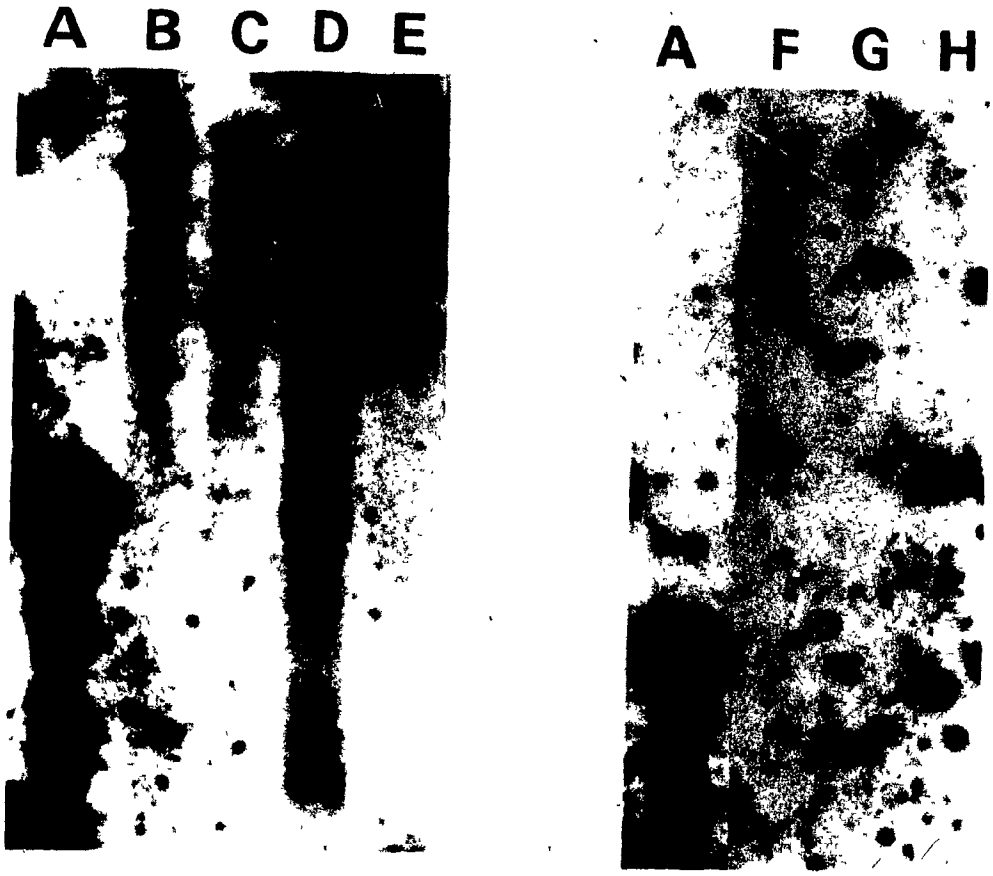


Figure 8. Detection of DNA fragments that contain polyoma sequences after cleavage of fragment-transformed cell DNAs with endonuclease Sal I.

Transformed cell DNAs (5 ug) were digested to completion with Sal I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPH1-8 DNA, the filter was subjected to autoradiography. Lane A shows the migration point of pPH1-8 linear molecules of DNA. The transformed cell lines analyzed are: 2-4a(B), 6-1a (C), 15-5a (D), A4-19a (E), A8-3a (F), A8-8a (G) and A8-10a (H). Lane A contains 10^{-5} ug of pPH1-8 DNA linearized with Sal I. The autoradiograms shown were exposed for 5 days.

Sal I



cellular DNAs with Hind III. The results (Figure 9) show that 5 of the 7 fragment-transformed cell lines (2-4a, 6-1a, 15-5a, A4-19a, A8-10a) contain a complete integrated copy of the Hind III-1 fragment of viral DNA. Two of the transformed cell lines (A8-3a and A8-8a) did not contain such a co-migrating DNA fragment (Figure 9). One possible explanation for the absence of an intact Hind III-1 fragment in these two cell lines could be that recombination between cellular DNA and recombinant plasmid DNA occurred within the polyoma Hind III-1 sequences.

To deduce the co-ordinates of the smallest continuous segment of viral sequences present, we investigated in greater detail the arrangement of polyoma viral DNA in A8-3a and A8-8a. To do this, we digested 10 ug of high molecular weight cellular DNA with a variety of restriction endonucleases whose sites of cleavage in pPH1-8 DNA are known. The resulting DNA was then fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with radioactive pPH1-8 DNA. By comparing the fragments obtained by digestion of pPH1-8 DNA with a particular restriction enzyme, with those derived from cellular DNA, it is possible to construct a map of the pPH1-8 sequences integrated in the cellular DNA. The results of such an analysis of A8-3a cellular DNA are shown in Figure 10 and summarized in Figure 11.

Figure 9. Detection of DNA fragments that contain polyoma sequences after cleavage of fragment-transformed cell DNAs with endonuclease Hind III.

Transformed cell DNA (5 ug) were digested to completion with Hind III and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPH1-8 DNA, the filter was subjected to autoradiography. The fragment-transformed cell lines analyzed are: 2-4a (B), 6-1a (C), 15-5a (D), A4-19a (E), A8-3a (F), A8-8a (G) and A8-10a (H). Lane A contains 10^{-5} ug of pPH1-8 DNA cleaved with Hind III. From top to bottom, the first band is linearized pBR322 DNA and the second one is polyoma Hind III-1 fragment. The autoradiograms shown in A, B and D were exposed for 48 hours and those shown in C, E, F, G and H, for 5 days.

Hind III

A B C D E F G H

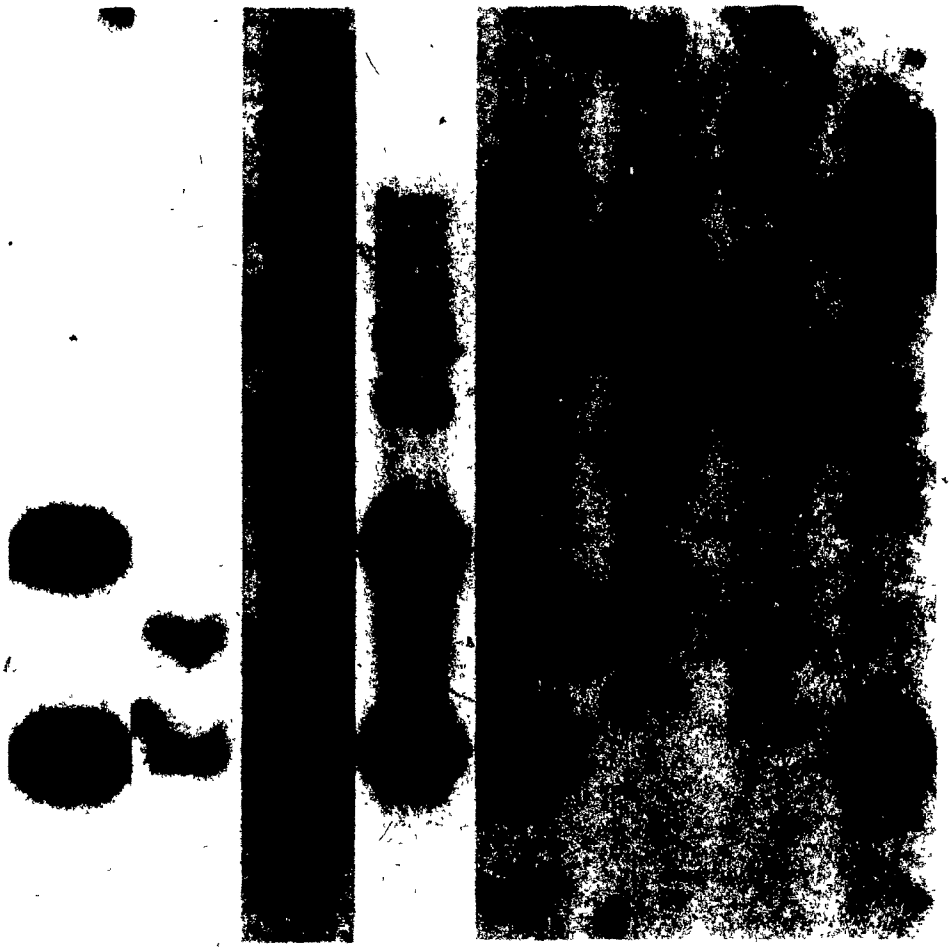


Figure 10. The arrangement of polyoma sequences in the DNA of A8-3a.

10 ug quantities of cellular DNA were digested with a variety of restriction endonucleases. The resulting fragments were fractionated through 0.7% agarose gels, transferred to a sheet of nitrocellulose and hybridized with ^{32}P-labelled pPH1-8 DNA. M refers to a marker of pPH1-8 DNA (10^{-5} ug) digested with the restriction endonucleases shown above. C refers to A8-3a cellular DNA.

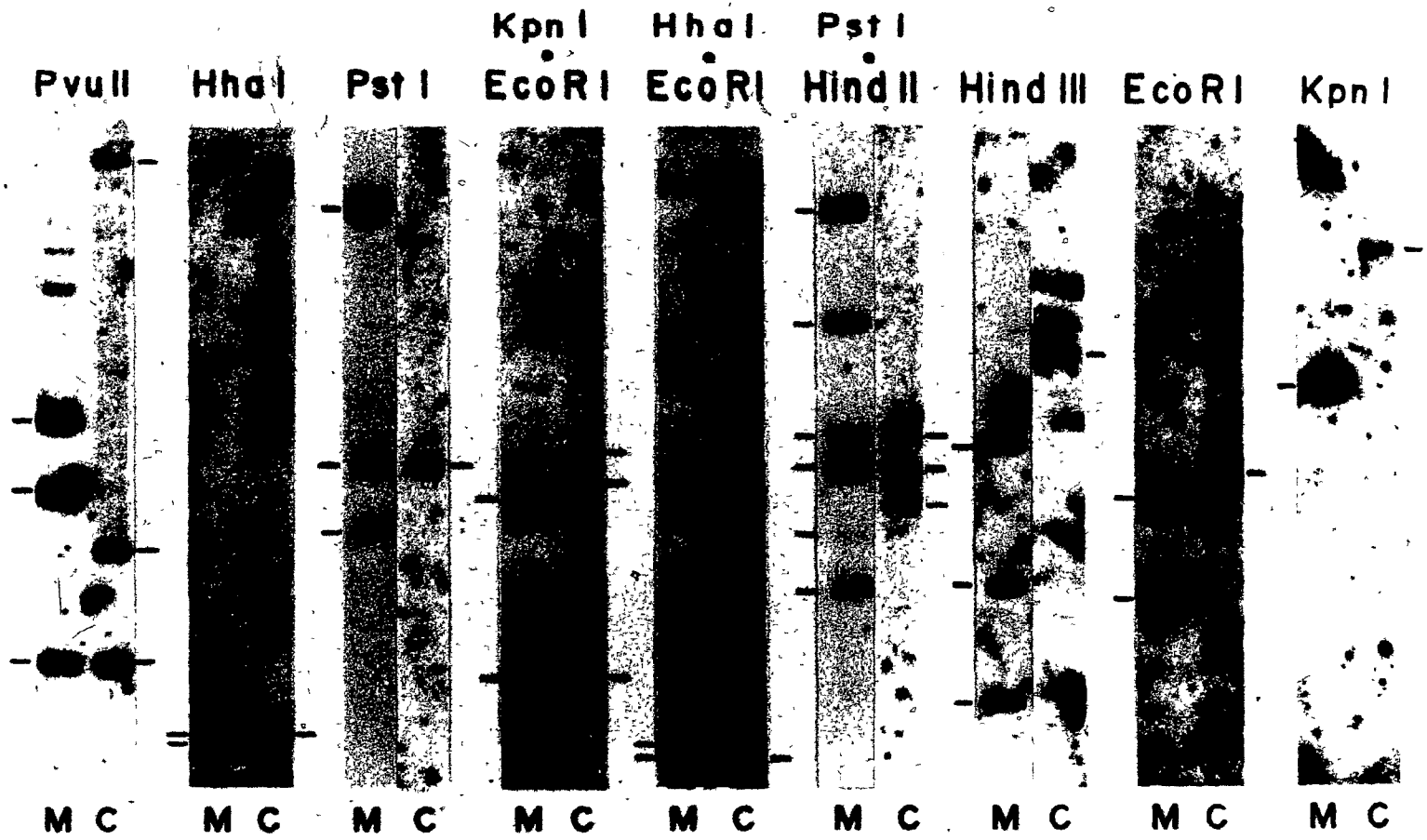
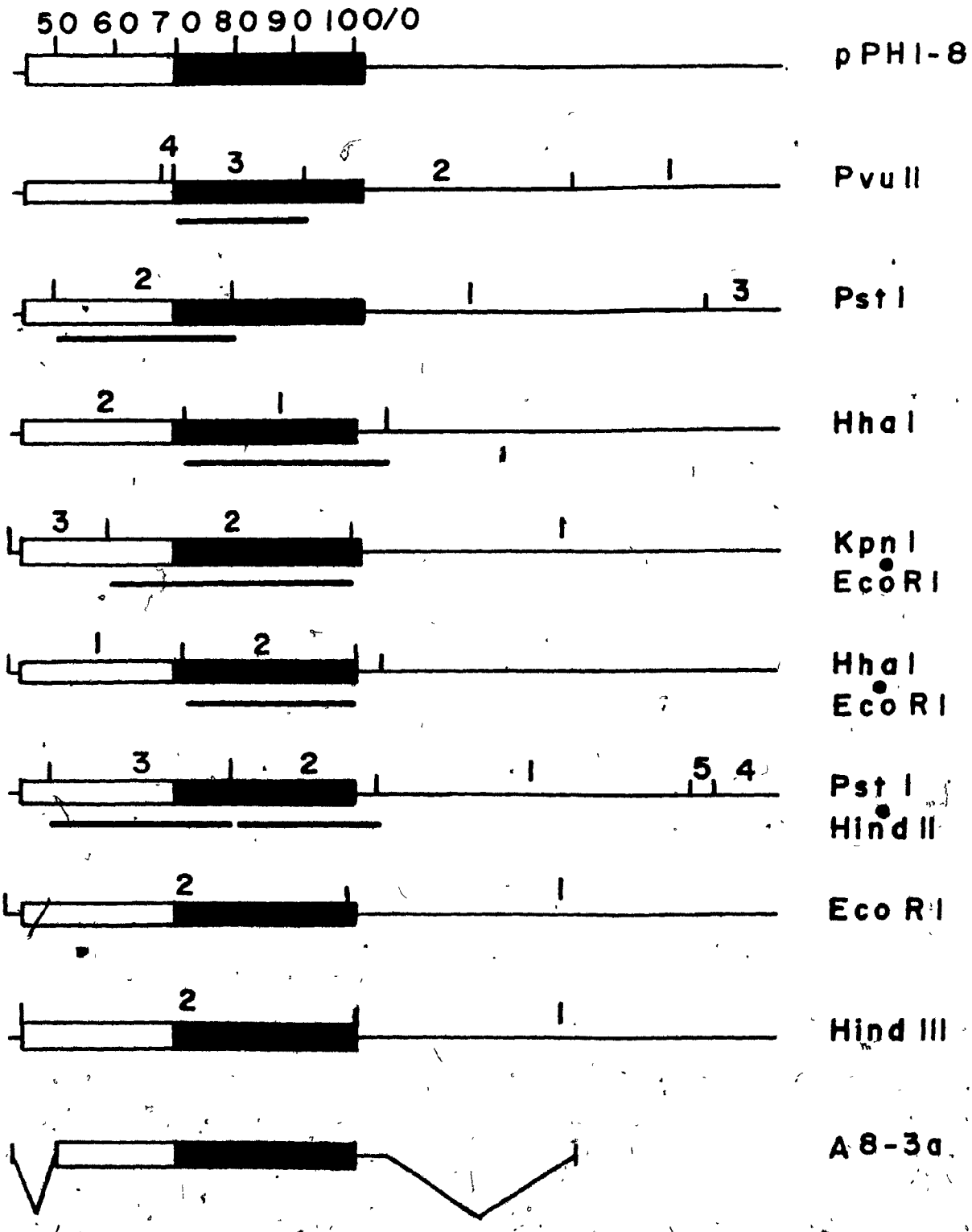

Pvu II
Hha I
Pst I
Kpn I
EcoR I
Hha I
EcoR I
Pst I
Hind II
Hind III
EcoR I
Kpn I
M C
M C
M C
M C
M C
M C
M C
M C
M C

Figure 11. Physical map of the pPH1-8 sequences integrated into the genome of A8-3a.

The data shown in Figure 10 is summarized here. Each circular plasmids have been opened at the Eco RI site within pBR322 DNA. The map at the top shows the structure of pPH1-8 DNA. The numbers refer to the map units on the polyoma physical map. The darkened areas indicate the early region sequences. The fragments containing polyoma sequences present in each restriction enzyme digest of pPH1-8 DNA are numbered according to their size. The underlined fragments represent those present in digests of A8-3a cellular DNA. Digestion of pPH1-8 DNA with Hha I yields a number of fragments, however only those which appear on the gel (Figure 10) are indicated here. The bottom map shows the structure of the integrated pPH1-8 sequences.


50 60 70 80 90 100/0
pPH1-8
4
3
2
1
PvuII
2
1
3
PstI
2
1
HhaI
3
2
1
KpnI
EcoRI
1
2
HhaI
EcoRI
3
2
1
5 4
PstI
HindII
2
1
EcoRI
2
1
HindIII
A8-3a

Those obtained after the analysis of A8-8a are showed in Figure 12 and summarized in Figure 13.

In both cell lines, one junction between cellular and pPH1-8 DNA occurs within polyoma sequences (between 45.0 and 50.3 map units) therefore destroying the polyoma Hind III site at 45 map units. The other junction between cellular and recombinant plasmid DNA occurs entirely within plasmid sequences. Therefore, in both A8-3a and A8-8a cell lines, the sequences of the early region contained in pPH1-8 DNA (from 70 to 1.4 map units) remain intact.

Although the junction between cellular and pPH1-8 DNA occurred within the same region of polyoma sequences (45 to 50.3 map units; however, it is not known whether integration occurred at the same point within this region) the integration points of pPH1-8 DNA into the genome of A8-3a and A8-8a are not identical. This conclusion is corroborated by the results obtained after cleavage of cellular DNA with various no-cut enzymes (Figures 5 and 6).

Figure 12. The arrangement of polyoma sequences in the DNA of A8-8a.

10 ug quantities of cellular DNA were digested with a variety of restriction endonucleases. The resulting fragments were fractionated through 0.7% agarose gels, transferred to a sheet of nitrocellulose and hybridized with ^{32}P-labelled pPH1-8 DNA. M refers to a marker of pPH1-8 DNA (10^{-5} ug) digested with the restriction endonucleases shown above. C refers to A8-8a cellular DNA.

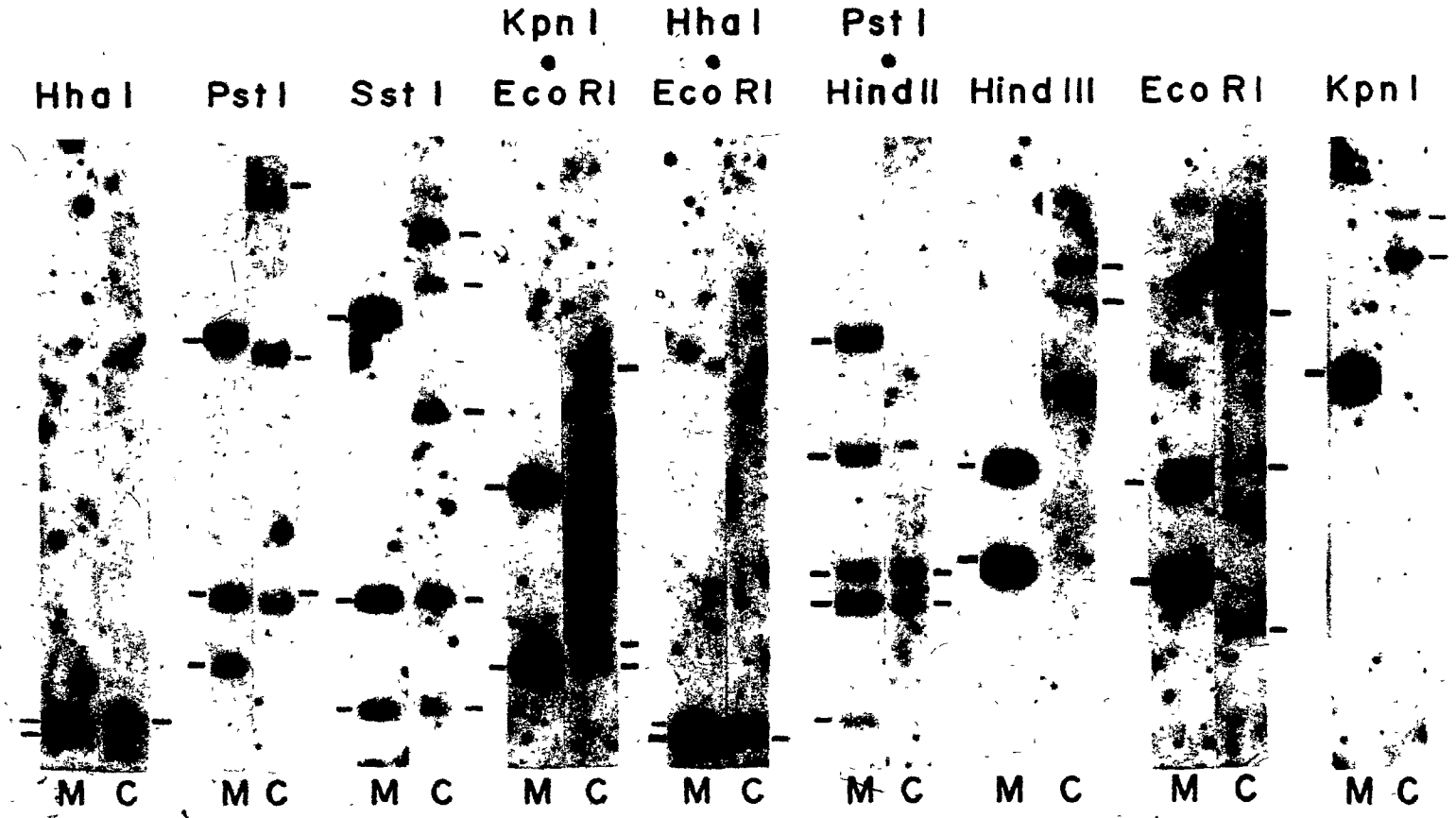
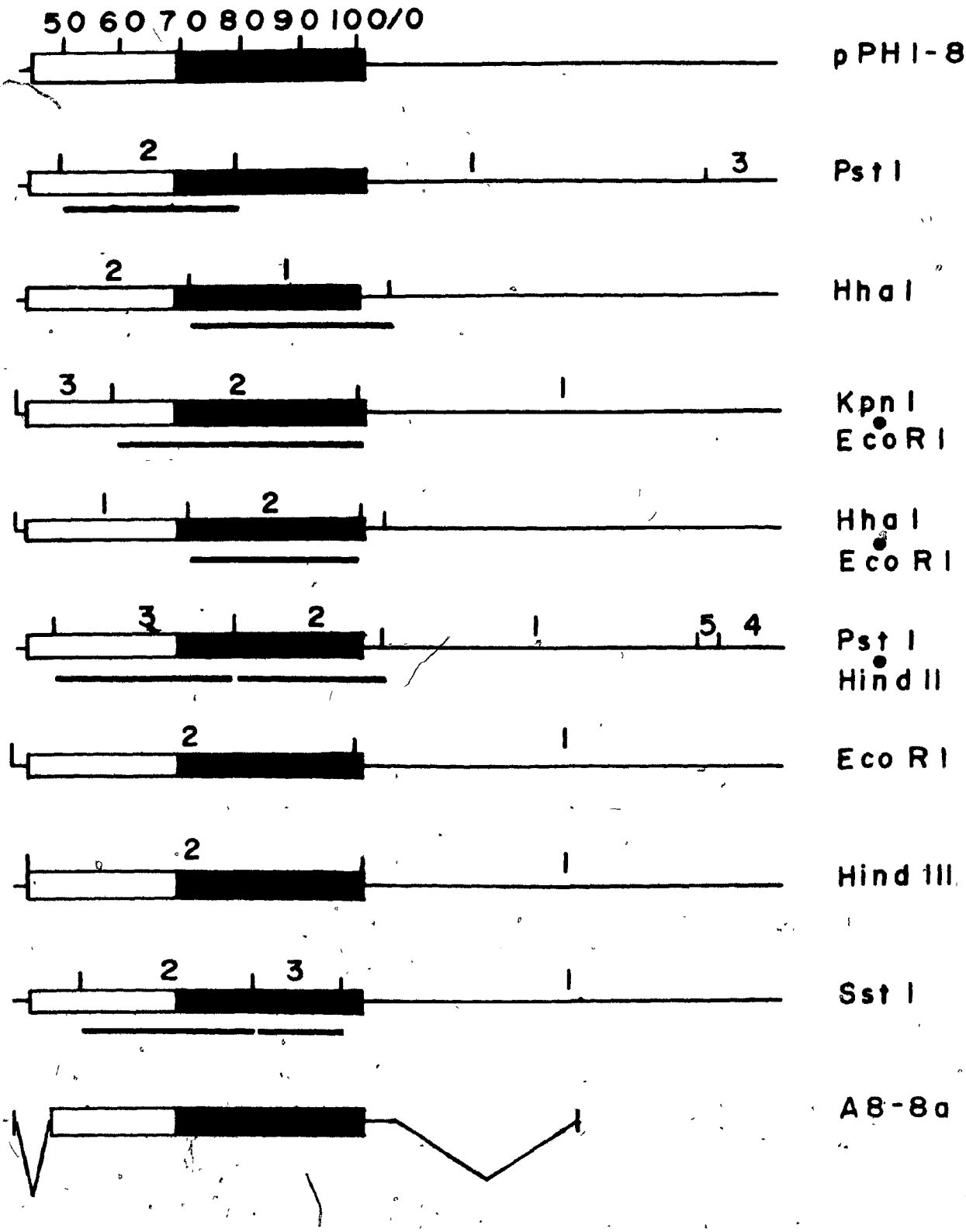

Hha I
Pst I
Sst I
Kpn I
Eco RI
Hha I
Eco RI
Pst I
Hind II
Hind III
Eco RI
Kpn I
M C
M C
M C
M C
M C
M C
M C
M C
M C

Figure 13. Physical map of the pPH1-8 sequences integrated into the genome of A8-8a.

The data shown in Figure 12 is summarized here. Each circular plasmids have been opened at the Eco RI site within pBR322 DNA. The map at the top shows the structure of pPH1-8 DNA. The numbers refer to the map units on the polyoma physical map. The darkened areas indicate the early region sequences. The fragments containing polyoma sequences present in each restriction enzyme digest of pPH1-8DNA are numbered according to their size. The underlined fragments represent those present in digests of A8-8a cellular DNA. Digestion of pPH1-8 DNA with Hha I yields a number of fragments. However, only those which appear on the gel (Figure 12) are indicated here. The bottom map shows the structure of the integrated pPH1-8 sequences.


50 60 70 80 90 100/0
pPH1-8
2
1
3
Pst I
2
1
Hha I
3
2
1
Kpn I
EcoR I
1
2
Hha I
EcoR I
3
2
1
5 4
Pst I
Hind II
2
1
EcoR I
2
1
Hind III
2
3
1
Sst I
A8-8a

F. The Arrangement of Viral DNA in Secondary Transformants

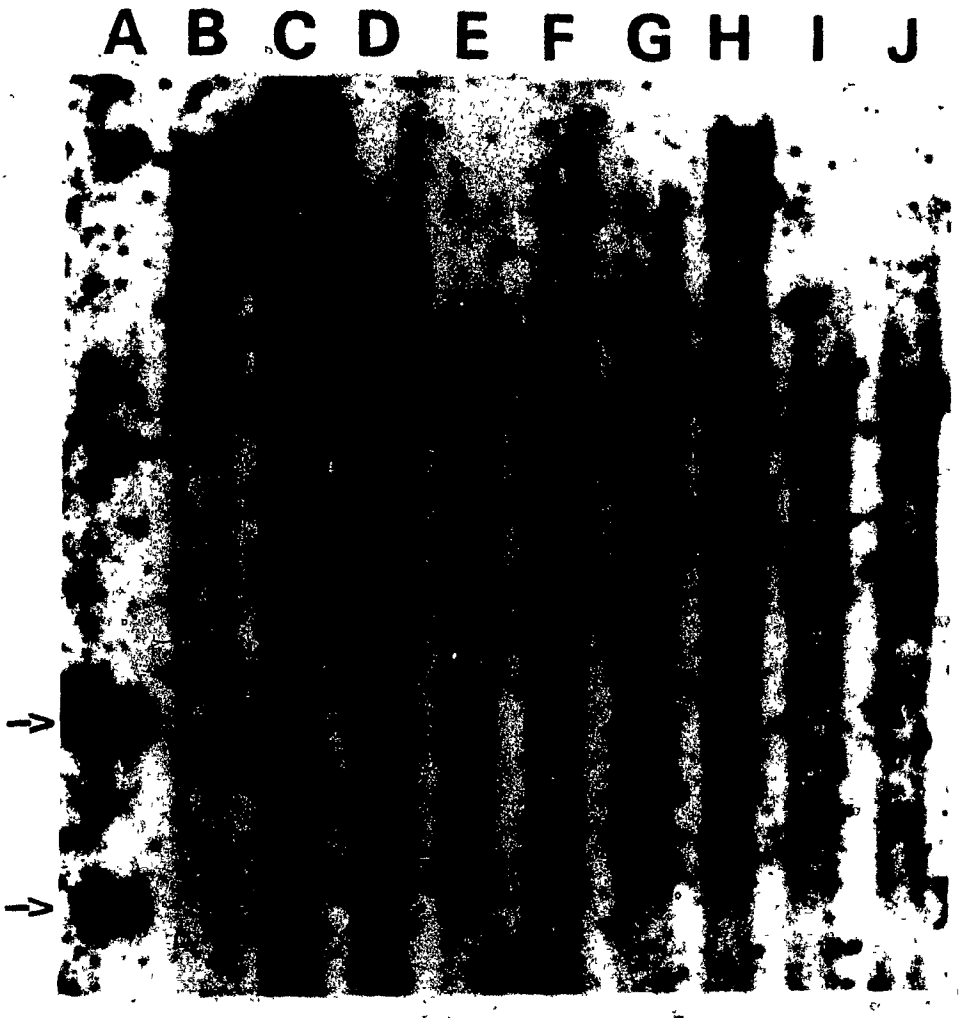
Different mechanisms have been proposed to explain integration of polyoma and SV40 sequences into cellular DNA. Included among these is one which suggests that integration occurs by legitimate recombination between viral and cellular DNA sequences. To test this hypothesis, we transfected Rat-1 cells in culture with cellular DNA isolated from one of the fragment transformed cell lines (15-5a). If integration occurs by homologous recombination, then the cellular DNA sequences flanking a particular insertion of pPH1-4 DNA should find homologous sequences on the cellular genome and integrate at that site. This would then be revealed by the presence of co-migrating fragments in the "secondary-transformants" and the parental 15-5a cellular DNA after digestion with a restriction enzyme which does not cleave pPH1-4 DNA.

To analyse the arrangement of pPH1-4 sequences within several secondary transformants, the cellular DNA of these cells was first digested with Xba I (a no-cut for pPH1-4). The resulting fragments were fractionated by gel electrophoresis, transferred to nitrocellulose filters and hybridized with ^{32}P-labelled pPH1-4 DNA. The results are shown in Figure 14. Four of the seven cell lines analyzed

Figure 14. Detection of DNA fragments that contain polyoma sequences after cleavage of secondary transformants cellular DNA with endonuclease Xba I.

5 ug of transformed cell DNAs were digested to completion with Xba I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPH1-4 DNA, the filter was subjected to autoradiography. The secondary transformed cell lines analyzed are: S15-1 (D), S15-2 (E), S15-4-1 (F), S15-5-1 (G), S15-5-2 (H), S15-5-4 (I) and S15-5-5 (J). The arrows in lane A show from top to bottom: pBR322 form III DNA and polyoma viral Hind III-1 fragment (10^{-5} ug of each). Lanes B and C show one and two ug of 15-5a DNA cleaved with Xba I. The autoradiograms shown were exposed for 60 hours.

Xba I



(S15-1, S15-2, S15-4-1, S15-5-1) contain only one insertion of recombinant plasmid sequences and one of them (S15-5-2) contains four. Only a smear could be detected in the remaining two cell lines (S15-5-4 and S15-5-5) and therefore, these were not further characterized. Similar results were obtained after digestion of cellular DNA from secondary transformants with Bgl II (Figure 15). The digestion of cellular DNA from cellular transformants with either Xba I or Bgl II did not generate fragments co-migrating with 15-5a cellular DNA fragments thus indicating that integration probably does not occur by homologous recombination mechanism.

15-5a cellular DNA contains a number of insertions of recombinant plasmid DNA (pPH1-4) arranged in tandem repeats (see previous section). To investigate if the genome of cells transformed by 15-5a cellular DNA (secondary transformants) contained a similar organization of recombinant sequences, the cellular DNAs of four secondary transformants were digested with Kpn I (which recognizes only one site of cleavage in pPH1-4 DNA). The results (Figure 16) show no fragments within the cellular DNA digests that co-migrate with linear pPH1-4 marker DNA, therefore indicating that none of the secondary transformants contains tandem repeats of recombinant plasmid DNA.

Figure 15. Detection of DNA fragments that contain polyoma sequences after cleavage of secondary transformants cellular DNA with endonuclease Bgl II.

5 ug of transformed cell DNAs were digested to completion with Bgl II and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPH1-4 DNA, the filter was subjected to autoradiography. The secondary transformed cells analyzed are: S15-1 (C), S15-2 (D), S15-5-1 (E) and S15-5-2 (F). The arrows in lane A indicate from top to bottom: 10^{-5} ug of pPH1-4 form II DNA and 10^{-5} ug of pPH1-4 form I DNA. Lane B shows 2 ug of 15-5a DNA cleaved with Bgl II. The autoradiograms shown were exposed for 5 days.

Bgl II

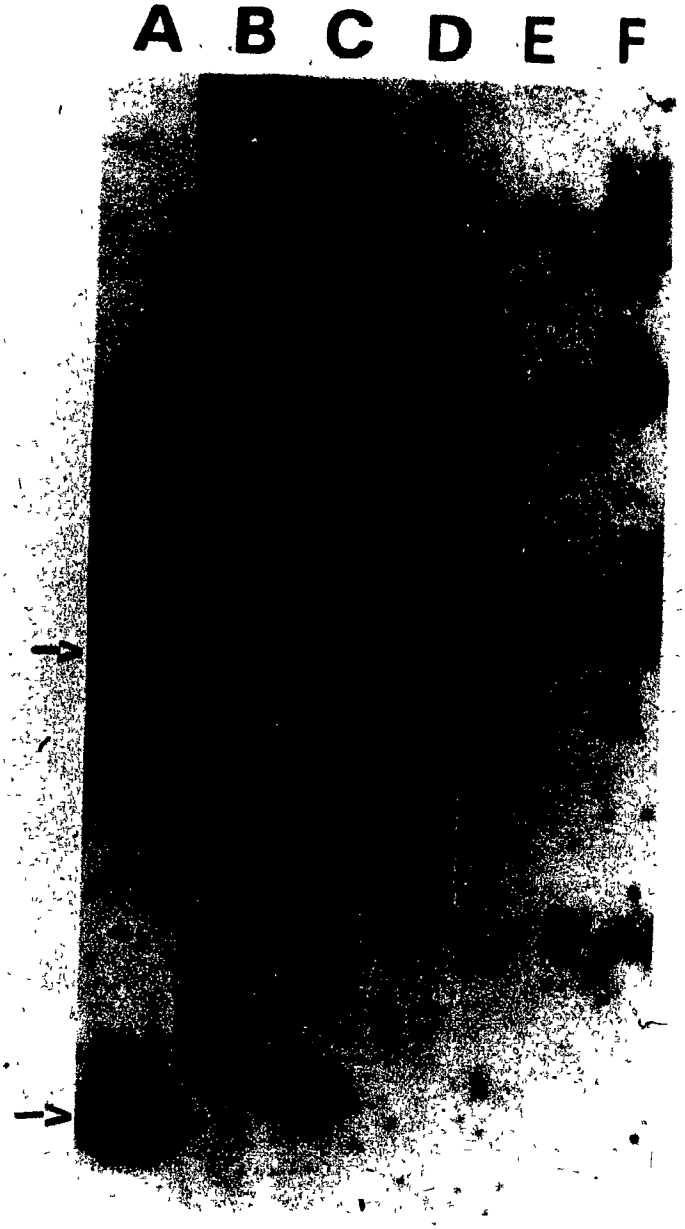
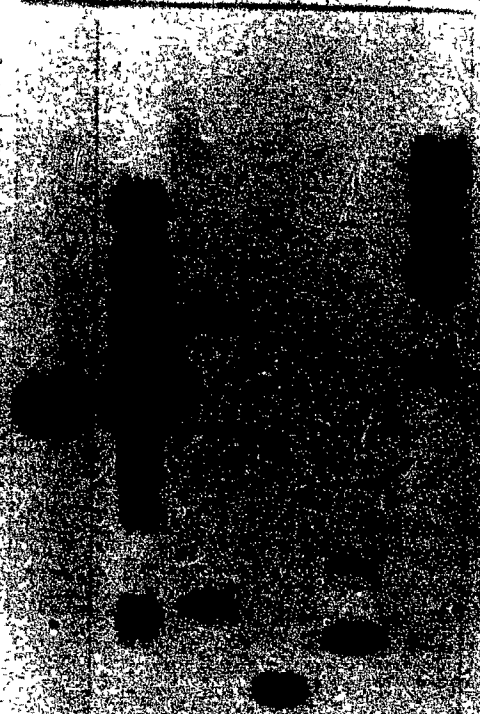


Figure 16. Detection of DNA fragments that contain polyoma sequences after cleavage of secondary transformants cellular DNA with endonuclease Kpn I.

5 ug of transformed cell DNAs were digested to completion with Kpn I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPH1-4 DNA, the filter was subjected to autoradiography. The secondary transformed cell lines analyzed are: S15-1 (C), S15-2 (D), S15-5-1 (E) and S15-5-2 (F). Lane A shows 10^{-5} ug of pPH1-4 form III DNA. Lane B shows 2 ug of 15-5a DNA cleaved with Kpn I. The autoradiograms shown were exposed for 15 hours.

Kpn I

A B C D E F



We were still curious to learn whether an entire Hind III-1 fragment (present in 15-5a cellular DNA) was necessary to cause cellular transformation. Secondary transformant cellular DNAs were therefore digested with Hind III and screened for the presence of an intact polyoma viral Hind III-1 fragment. The results are shown in Figure 17. Two of the four cell lines tested (S15-1, S15-5-1) contain an intact Hind III-1 fragment as revealed by the presence, in the cellular DNA digest, of fragments that co-migrate with the viral Hind III-1 fragment. Digestion of cellular DNA isolated from S15-2 and S15-5-2 cells however, fail to reveal the presence of such co-migrating fragments (Figure 17). The arrangement of polyoma viral DNA sequences in S15-2 was then investigated in greater detail as described in the previous section (for A8-3a and A8-8a). The results are shown in Figure 18 and summarized in Figure 19. Digestion of S15-2 cellular DNA with Pvu II, Sst I, Pst I & Hind II and Pst I reveals that the insertion of viral DNA contains sequences included between 52.7 and 1.4 map units. The sequences of the early region included in pPH1-4 therefore remain intact in S15-2 DNA.

Figure 17. Detection of DNA fragments that contain polyoma sequences after cleavage of secondary transformants' cellular DNA with endonuclease Hind III.

5 ug of transformed cell DNAs were digested to completion with Hind III and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPH1-4 DNA, the filter was subjected to autoradiography. The secondary transformed cell lines shown are: S15-1 (C), S15-2 (D), S15-5-1 (E) and S15-5-2 (F). Lane A shows from top to bottom: 10^{-5} ug of pBR322 linear DNA and 10^{-5} ug of polyoma viral Hind III-1 fragment. The autoradiograms were exposed for 15 hours.


Hind III
A B C D E F

Figure 18. The arrangement of polyoma sequences in the DNA of S15-2.

10 ug quantities of cellular DNA was digested with a variety of restriction enzymes. The resulting fragments were fractionated through 0.7% agarose gels, transferred to a sheet of nitrocellulose and hybridized with ^{32}P-labelled pPH1-4 DNA. The arrows indicate the position of the marker pPH1-4 DNA fragments obtained with the restriction endonucleases shown above.

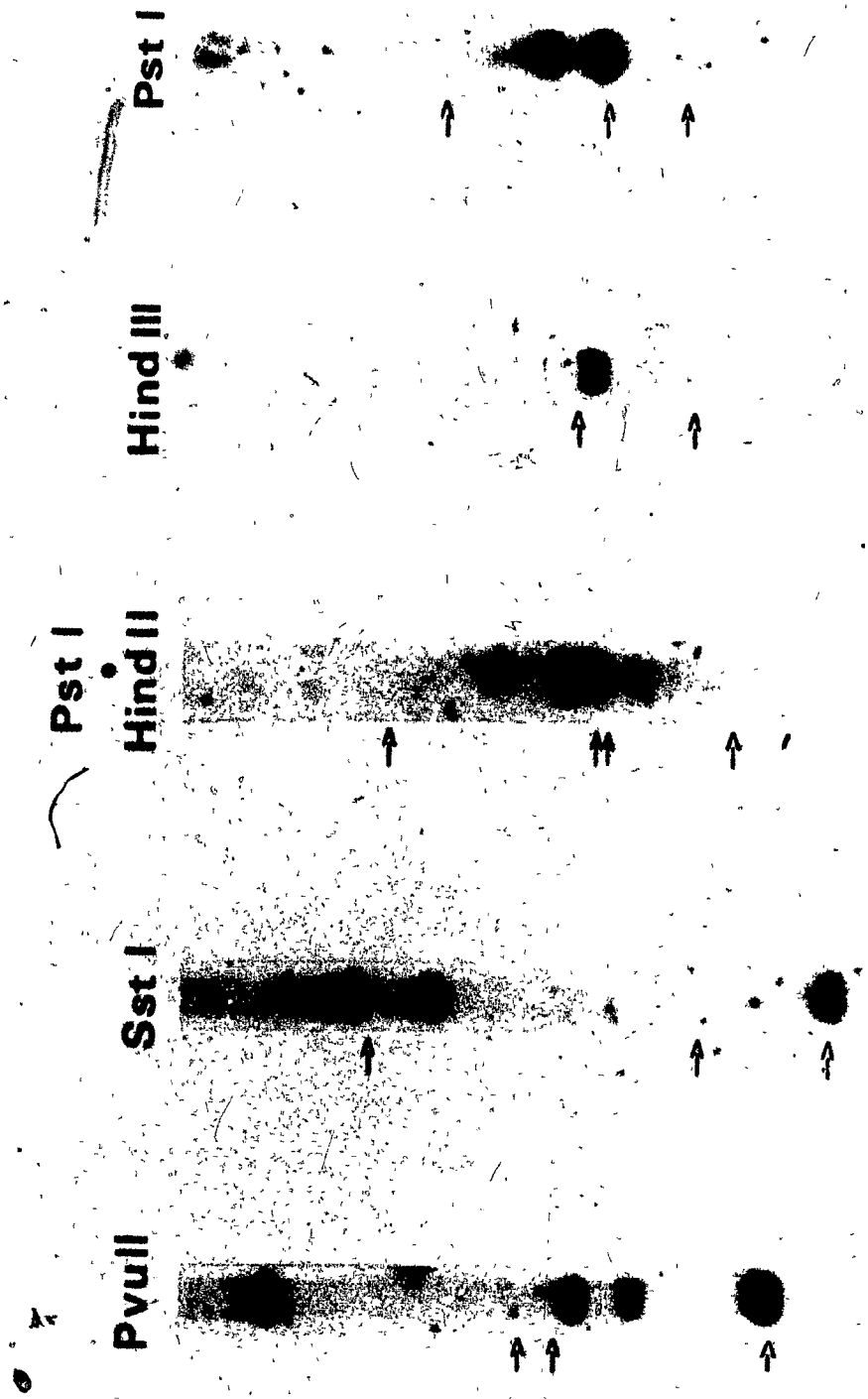
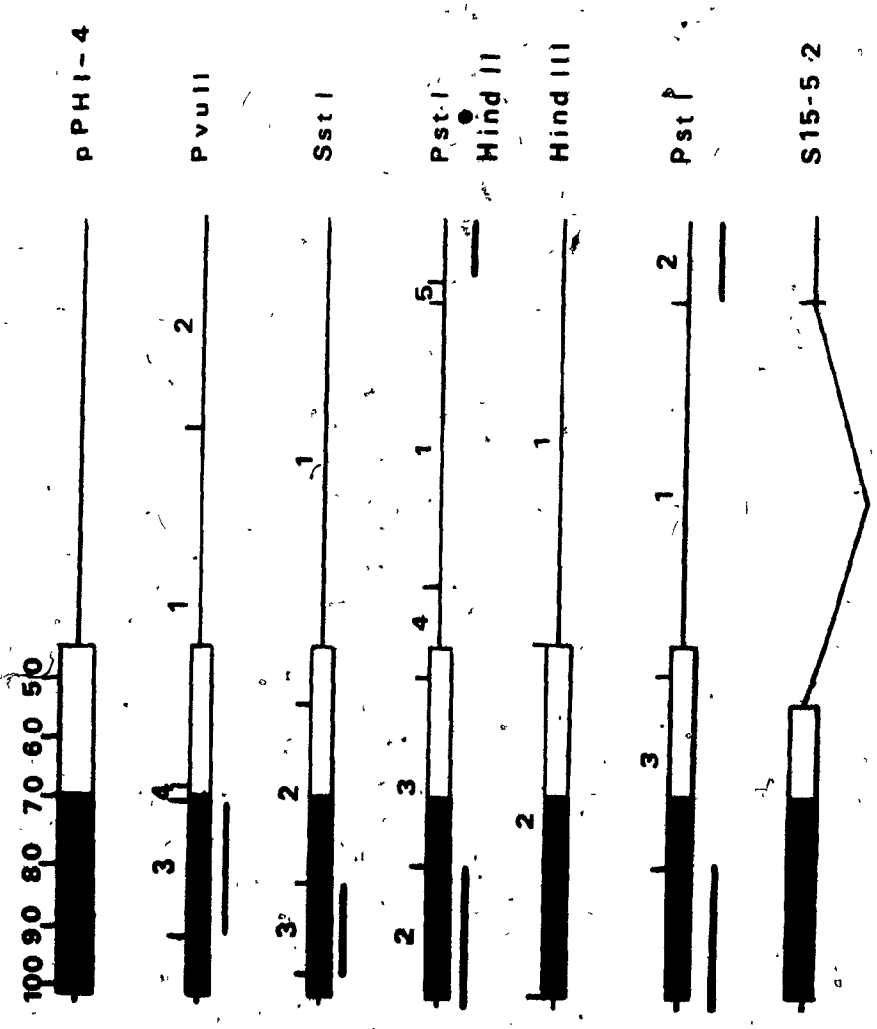

Pst I
Hind III
Pst I
Hind II
Sst I
Pvu II

Figure 19. Physical map of the pPH1 4 sequences integrated into the genome of S15-2

The data shown in Figure 18 is summarized here. Each circular plasmid have been opened at the Eco RI site within pBR322 DNA. The map at the top shows the structure of pPH1-4 DNA. The numbers refer to the map units on the polyoma physical map. The darkened areas indicate the early region sequences. The fragments containing polyoma sequences present in each restriction enzyme digest of pPH1-4 DNA are numbered according to their size. The underline fragments represent those present in digests of S15-2 cellular DNA. The bottom map shows the structure of the maximum pPH1-4 sequences integrated.


pPHI-4
PvuII
SstI
PstI
HindII
HindIII
PstP
S15-5'2
100 90 80 70 60 50

G. The Arrangement of Viral Sequences in R and B Transformants

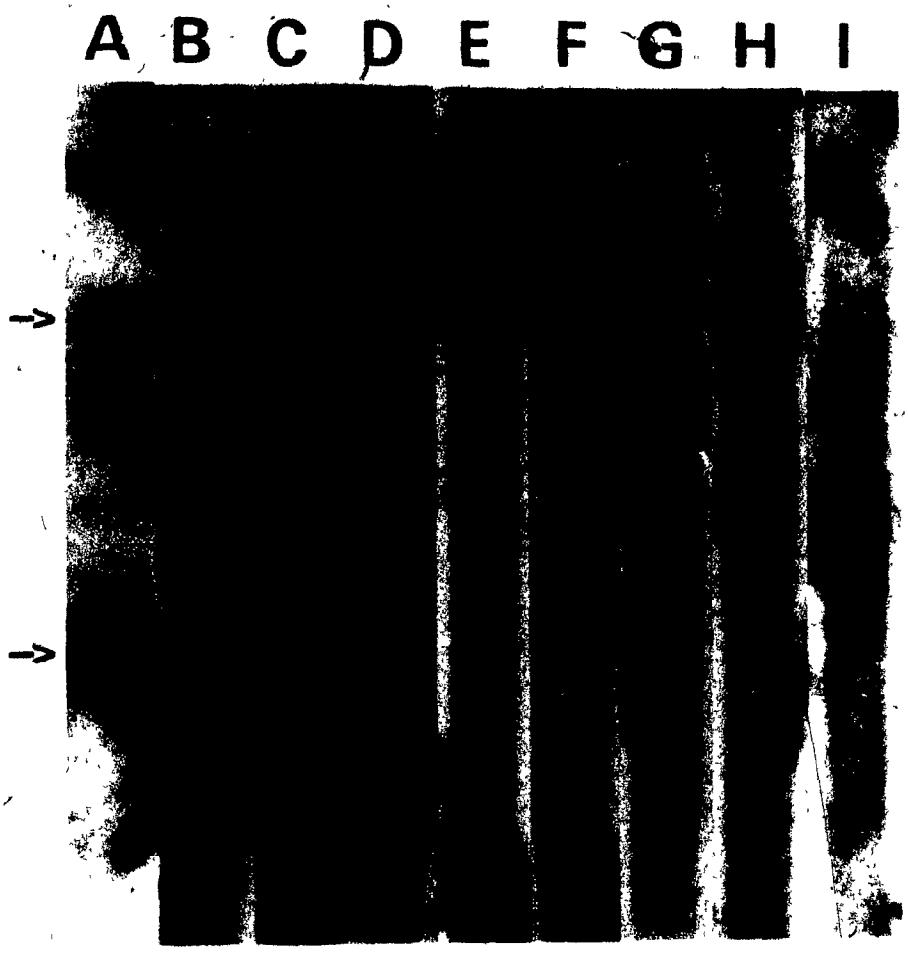
Rat cells transformed by polyoma virus have been shown to contain integrated tandem repeats of viral DNA sequences (72). How these tandem repetitions of viral sequences arise in the cellular genome of transformed rat cells is still unknown but different mechanisms have been proposed (79). Most of these suggest that single stranded regions exposed in rolling circle or replicative intermediates of the Cairns type during viral DNA replication are involved in the initial base pairing between the viral and cellular DNA sequences. Because large T antigen is essential for the initiation of viral DNA replication (48, 49), the models predict that large T antigen should be present in cells harboring tandemly integrated viral sequences.

To elucidate the role played by large T antigen during the integration process, we compared the arrangement of integrated viral DNA sequences in Rat-1 cells transformed by recombinant plasmid DNA molecules incapable of encoding large T antigen (pPR1 and pPR2) with those of cells transformed by recombinant plasmids capable of doing so. Initially, we were interested in learning whether the number of separate insertions of viral sequences within the cellular genome of host cells depended on the activity of large T antigen. Figure 20 shows the results obtained after

Figure 20: Detection of DNA fragments that contain polyoma sequences after cleavage of R-transformed cell DNAs with endonuclease Bgl II.

Transformed cell DNAs (5 ug) were digested to completion with Bgl II and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPR1 DNA, the filter was subjected to autoradiography. The transformed cell lines analyzed are: R1-1 (B), R2-1 (C), R3-1 (D), R4-4 (E), R5-2 (F), R6-1 (G), R9-1 (H) and R10-2 (I). Lane A contains from top to bottom: 10^{-5} ug of pPRI form II DNA and 10^{-5} ug of pPRI form I DNA. The autoradiograms were exposed for 6 days.

BglII



cleavage of high molecular weight DNA isolated from cells transformed by pPR1 or pPR2 recombinant DNA (subsequently referred to as R transformants) with Bgl II which does not recognize any cleavage site in pPR1 and pPR2 DNA. Five of the eight cells lines tested (R2-1, R5-2, R6-1, R9-1, R10-2) contain a single insertion of recombinant plasmid DNA. R1-1 cells show two separate insertions of pPR1 DNA while R3-1 and R4-4 show 13 and 10 different insertions of recombinant plasmid DNA respectively (Figure 20). These results are confirmed by the digests of the R transformed cellular DNA with Hpa I, another no-cut enzyme for pPR1 DNA (Figure 21). For comparison, the cellular DNA isolated from 8 cell lines transformed by pPB1 or pPB2 DNA (B transformants) was also cleaved with Bgl II and Hpa I. The results are shown in Figures 22 and 23 respectively. Four of the eight clones analyzed (B1-1, B7-1, B13-1 and B15-2) contained only one insertion of recombinant plasmid DNA while the remaining cell lines either contain two separate insertions (B3-1) or multiple insertions (B2-1, B4-1 and B14-1) of recombinant DNA. In summary, five of the eight R transformed cell lines (incapable of encoding large T antigen) and four of the eight B transformants (capable of encoding large T antigen) contained single insertion of viral sequences within their cellular genome.

Figure 21. Detection of DNA fragments that contain polyoma sequences after cleavage of R-transformed cell DNAs with endonuclease Hpa I.

Transformed cell DNAs (5 ug) were digested to completion with Hpa I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPR1 DNA, the filter was subjected to autoradiography. The transformed cell lines analyzed are: R1-1 (B), R2-1 (C), R3-1 (D), R4-4 (E), R5-2 (F), R6-1 (G), R9-1 (H) and R10-2 (I). Lane A contains from top to bottom: 10^{-5} ug of pPRI form II DNA and 10^{-5} ug of pPRI form I DNA. The autoradiograms were exposed for 6 days.

Hpa I

A B C D E F G H I

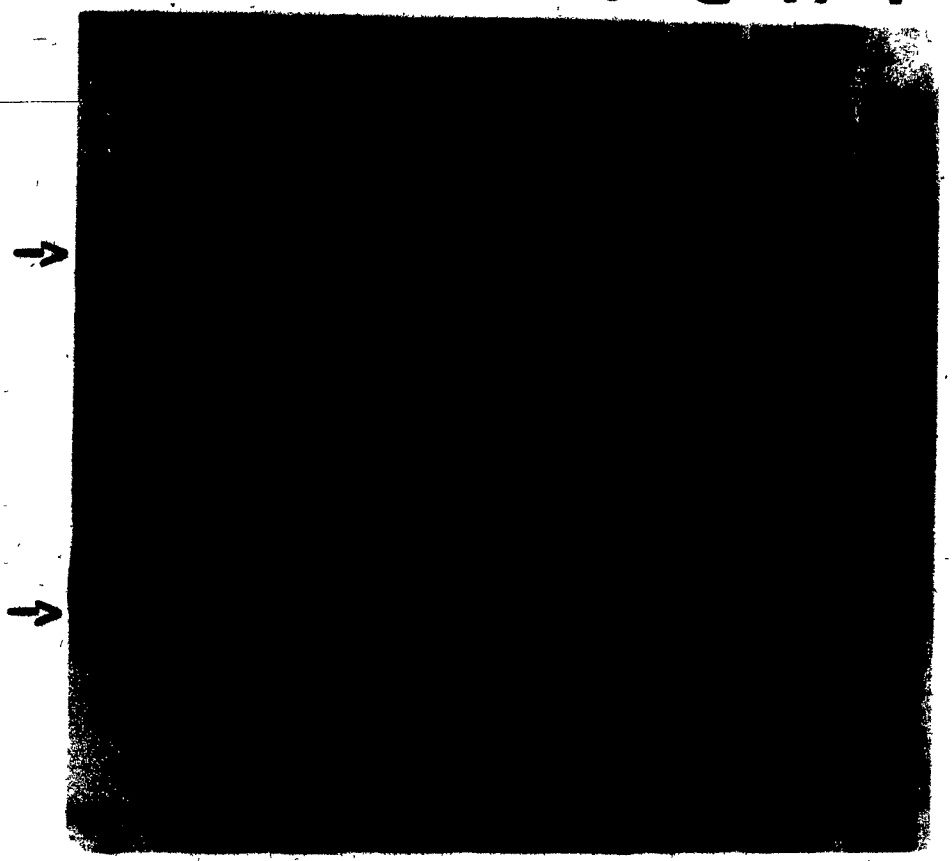


Figure 22: Detection of DNA fragments that contain polyoma sequences after cleavage of B transformed cell DNAs with endonuclease Bgl II.

Transformed cell DNAs (5 ug) were digested to completion with Bgl II and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPB1 DNA, the filter was subjected to autoradiography. The transformed cells analyzed are: B1-1 (B), B2-1 (C), B3-1 (D), B4-1 (E), B7-1 (F), B13-1 (G), B14-1 (H) and B15-2 (I). Lane A shows from top to bottom: 10^{-5} ug of pPB2 form II DNA and 10^{-5} ug of pPB2 form I DNA. The autoradiograms were exposed for 6 days.

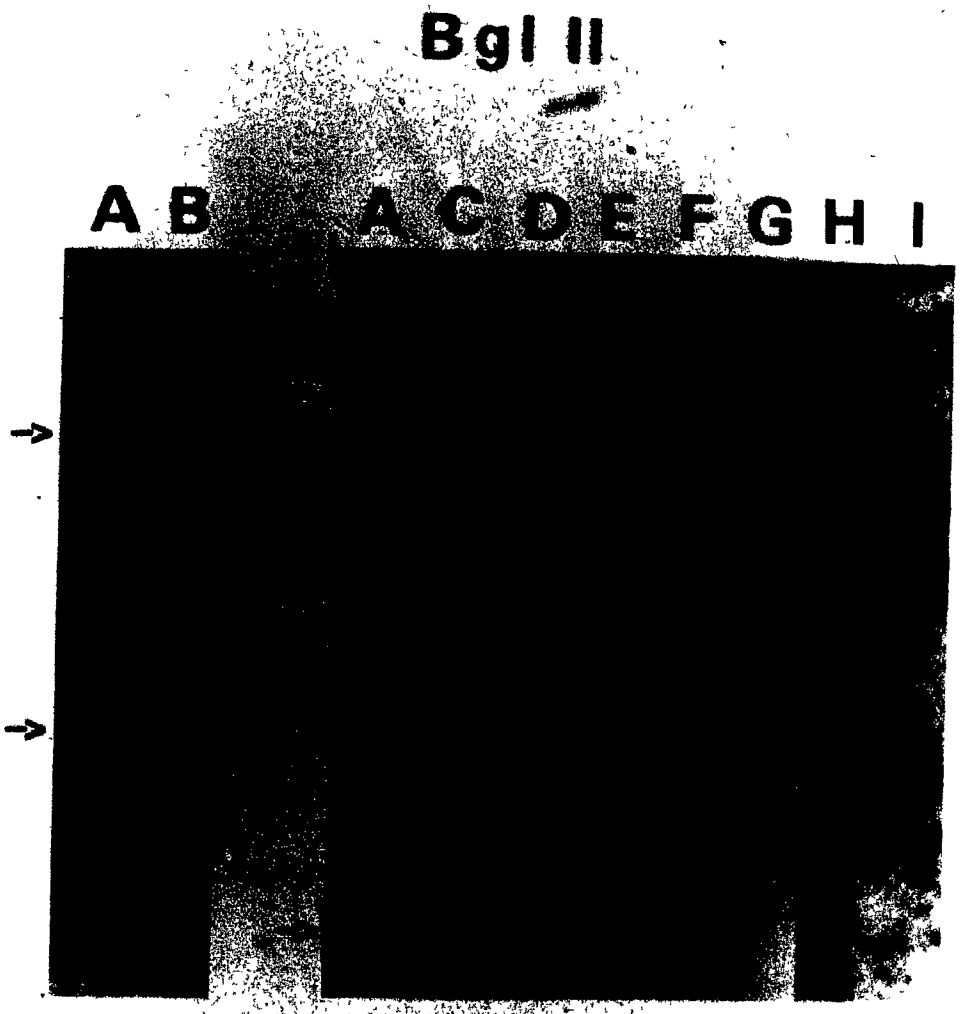

Bgl II
A B A C D E F G H I

Figure 23. Detection of DNA fragments that contain polyoma sequences after cleavage of B transformed cell DNAs with endonuclease Hpa I

Transformed cell DNAs (5 ug) were digested to completion with Hpa I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPB1 DNA, the filter was subjected to autoradiography. The transformed cells analyzed are B1-1 (B), B2-1 (C), B3-1 (D), B4-1 (E), B7-1 (F), B13-1 (G), B14-1 (H) and B15-2 (I). Lane A shows from top to bottom: 10^{-5} ug of pPB2 form II DNA and 10^{-5} ug of pPB2 form I DNA. Lane J shows 10^{-5} ug of pPB2 form III DNA. Lane K shows from top to bottom: 10^{-5} ug of polyoma (A2) DNA form II and I.

Hpa I

Consequently, the number of separate viral DNA integration sites does not depend on the presence of large T antigen. Figures 20 to 23 also show that none of the R or B clones contained free viral DNA.

To determine if the recombinant plasmid sequences were tandemly integrated in these cells, the cellular DNA of the R transformants was digested with Xba I and Sal I. Both of these enzymes cleave pPR1 and pPR2 DNA only once. (Xba cleaves at two sites separated by only 45 base pairs in the distal part of the early region of the polyoma genome). The results obtained after digestion of the cellular DNA with Xba I are shown in Figure 24. Of the eight clones analyzed, two (R3-1 and R4-4) contain at least a partial tandem duplication of the integrated pPR1 DNA. On the other hand, digestion with Sal I reveals the presence of tandem repetitions of recombinant sequences in two additional cell lines; R5-2, R9-1 (Figure 25). For comparison, cellular DNAs isolated from B transformed cell lines were also cleaved with Xba I to determine whether the recombinant plasmid sequences were integrated in tandem repetitions. The results (Figure 26) show that three of the seven cell lines analyzed (B3-1, B4-1 and B14-1) contain tandem repeats of recombinant

Figure 24. Detection of DNA fragments that contain polyoma sequences after cleavage of R-transformed cell DNAs with endonuclease Xba I.

Transformed cell DNAs (5 ug) were digested to completion with Xba I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPR1 DNA, the filter was subjected to autoradiography. The transformed cell lines analyzed are: R1-1 (B), R2-1 (C), R3-1 (D), R4-4 (E), R5-2 (F), R6-1 (G), R9-1 (H) and R10-2 (I). Lane A shows 10^{-5} ug of pPR1 form III DNA.

Xba I

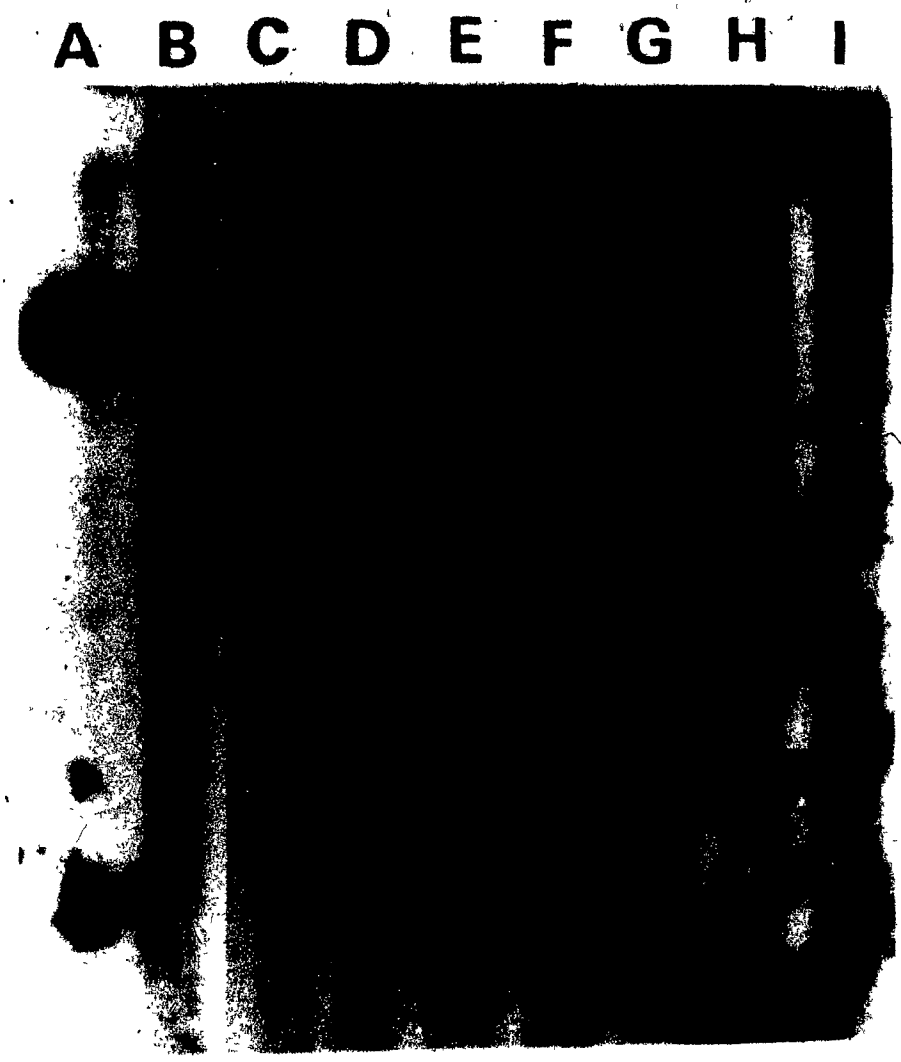


Figure 25. Detection of DNA fragments that contain polyoma sequences after cleavage of R-transformed cell DNAs with endonuclease Sal I

Transformed cell DNAs (5 ug) were digested to completion with Sal I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPR1 DNA, the filter was subjected to autoradiography. The transformed cell lines analyzed are: R1-1 (B), R2-1 (C), R3-1 (D), R4-4 (E), R5-2 (F), R6-1 (G), R9-1 (H) and R10-2 (I). Lane A shows 10^{-5} ug of pPR1 form III DNA.

Sal I

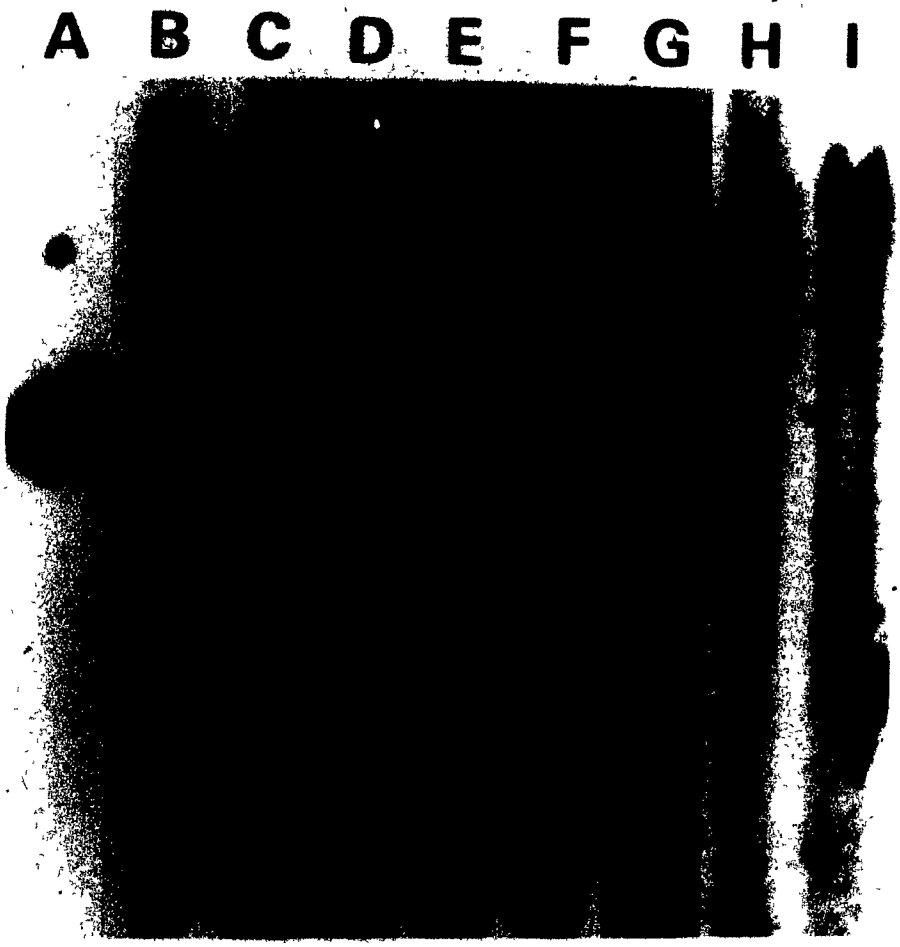
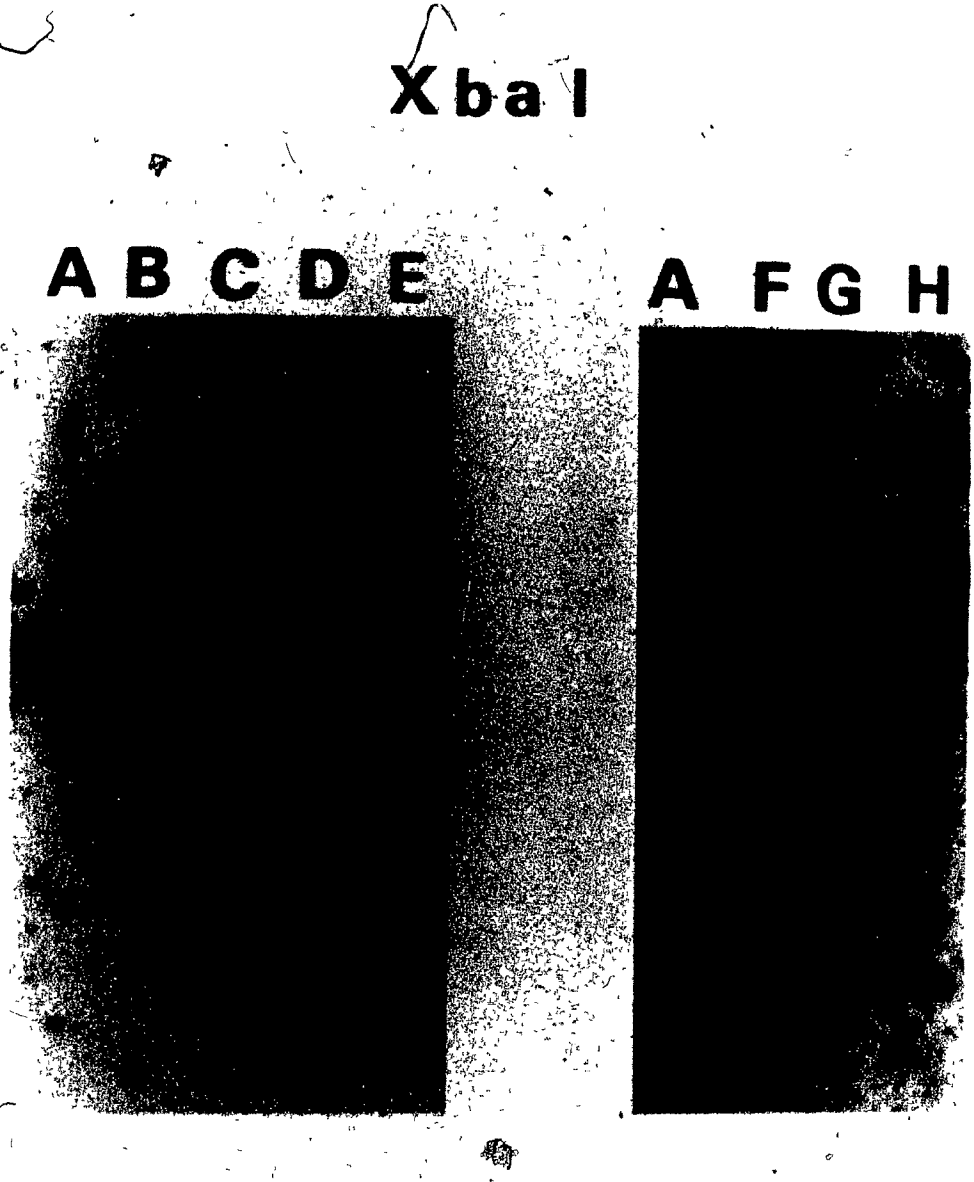


Figure 26. Detection of DNA fragments that contain polyoma sequences after cleavage of B transformed cell DNAs with endonuclease Xba I.

Transformed cell DNAs (5 ug) were digested to completion with Xba I and the resulting fragments were fractionated by electrophoresis through 0.7% agarose gels. After transfer of the DNA to a sheet of nitrocellulose and hybridization with ^{32}P-labelled pPB1 DNA, the filter was subjected to autoradiography. The transformed cells analyzed are: B1-1 (B), B2-1 (C), B3-1 (D), B4-1 (E), B7-1 (F), B13-1 (G), B14-1 (H) and B15-2 (I). Lane A contains 10^{-5} ug of pPB2 form III DNA. The autoradiograms were exposed for 6 days.


Xba I
A B C D E
A F G H

plasmid sequences. Therefore, in summary, the presence of large T antigen in transformed cells does not significantly alter the frequency at which tandem integration occurs since four of the eight R transformants and three of the seven B transformants analyzed contained integrated tandem repetitions of recombinant plasmid DNA.

D I S C U S S I O N

We have measured the transforming capacity of eight recombinant plasmid DNAs (Figure 3) containing either the entire or only part of the polyoma viral genome. The results we have presented show that only part of the early region, the 5'-proximal half (70-100 map units) is required to establish transformation of Rat-1 cells. This conclusion stems from the observation that recombinant plasmids containing either the entire polyoma genome interrupted at the viral Eco RI site (0/100 map units) or the Hind III-1 fragment of polyoma DNA (45-1.4 map units) are capable of transforming rat cells. The cloned Hind III-2 fragment of polyoma viral DNA (1.4-45 map units) which does not include sequences from the proximal portion of the early region, is incapable of inducing the transformed phenotype. Moreover, the frequency of transformation obtained by infection with recombinant plasmid DNAs capable of encoding large T antigen (pPB1 and pPB2) and those incapable of doing so (pPR1 and pPR2, pPH1-8 and pPH1-4) are approximately the same. Because the coding sequences for small and middle T antigens are located exclusively in the proximal part of the early region (70-100 map units), whereas the sequences encoding

large T antigen are located in the proximal as well as in the distal part of the early region, these results suggest that large T antigen neither enhances nor inhibits the establishment of DNA mediated transformation. This conclusion contradicts the observation that large T antigen is necessary for the initiation of cellular transformation (83, 84, 85). One explanation for this apparent discrepancy could be that we have studied transformation after DNA transfection. It is possible that during DNA infections, a small number of cells take up large amounts of viral DNA. This increased quantity of polyoma DNA within the cell could then overcome the need for large T antigen to initiate transformation.

Because we reasoned by analogy to SV40 that the entire early region of polyoma virus would be required to induce transformation, the observation that large T antigen is not essential to transform cells was surprising. Using the blot-hybridization technique, we therefore screened several fragment transformants for other (contaminating) DNA sequences than those included in the Hind III-1 fragment and found none. Without any doubt then, these rat cells are transformed by sequences incapable of encoding the entire large T antigen.

These results are confirmed by several recent studies. First, large T antigen is not found in the fragment-transformants (Pomerantz, B., Ito, Y. and Hassell, J.A., unpublished data) nor is it found in hamster cells transformed by polyoma virus (31) or in hamster tumor cell lines established by infection with polyoma viral DNA (115). Furthermore, Israel et al. (114) have reported that recombinant plasmids containing pBR322 DNA inserted into the Eco RI site of the polyoma viral genome (0/100 map units) and therefore interrupting the sequences of the early region, was tumorgenic when injected into hamsters. Kamen et al. (25) analyzed the polyoma viral mRNAs found in mouse and rat transformed cells and reported that some cell lines did not contain RNA sequences derived from the distal portion of the early region. Lania et al. (73) have analyzed 13 Rat-1 cell lines transformed by polyoma virus. Included among these were two cell lines which contained only a single insertion of viral DNA with an incomplete early region. Neither of these cell lines synthesized large T antigen (73). Taken together, these data strongly suggest that large T antigen is not required to maintain the transformed state. Furthermore, rat cells containing an integrated hr-t viral genome and synthesizing a functional large T antigen but no middle and

small T antigens display a normal phenotype (73), thus indicating that large T antigen alone is not sufficient for the maintenance of transformation.

Whether middle and small T antigens are essential to maintain the transformed state is still not clear. However, these proteins have been detected in fragment transformed cell lines (Pomerantz, Ito and Hassell, unpublished data) and are always detected in polyoma-transformed cells (28, 31, 115), therefore establishing a strong correlation between their presence and the maintenance of the transformed phenotype. In addition, cells transformed by polyoma virus containing deletions within the sequences encoding middle and large T antigens (88, 89) transform cells less efficiently than the wild type virus. Therefore, if small T antigen alone is not sufficient to fully transform cells and large T antigen alone is not capable of transforming cells (discussed earlier), then middle T antigen appears to be required to maintain the transformed state. Nevertheless, it can still be argued that the combined action of large and small T antigen is responsible for maintaining the transformed phenotype. No mutant with lesions in sequences encoding middle T antigen alone has yet been isolated. Such polyoma

mutants, without a middle T antigen would then resemble a SV40 genome which encodes only large and small T antigens. Unlike polyoma virus however, SV40 large T antigen alone is capable of transforming certain cells *in vitro*.

Our results also show that there are no specific sites in the cellular genome of rat cells at which integration of polyoma preferentially occurs and additionally, there are no specific sequences on polyoma DNA at which attachment to the host chromosome takes place. These results were obtained by comparing the arrangement of the viral DNA sequences within the cellular genome of seven *Hind* III-1 fragment transformed cells, eight R transformants and eight B transformants. Similar results have been obtained from the analysis of several polyoma transformed rat and hamster cells (72-74). In addition, integration of SV40 sequences within rat cellular DNA has also been shown to be non-specific (70, 113). The integration of polyoma virus and SV40 therefore contrasts sharply with that of bacteriophage λ into the *E. coli* genome. Here, unit length λ viral DNA molecules always integrate into a well defined chromosomal site. Botchan *et al*. (70) recently proposed that unlike λ viral DNA, integration of polyoma

and SV40 viral sequences into cellular genomes could be a generalized phenomenon that occurs by legitimate or illegitimate recombination between viral and cellular sequences. However, the arrangement of viral sequences within the genome of our secondary transformants suggests that integration does not occur by homologous recombination. Nevertheless, a small degree of homology between viral and cellular sequences at the viral and cellular DNA junctions could exist and could not be detected by the methods used. These could only be detected by determining the sequence of nucleotides at the site of linkage between viral and cellular DNAs. Botchan et al. (79) have already done such an analysis on a SV40 transformed rat cell line and found no evidence of partial homology at the junction of cellular and viral sequences. They also postulate that single stranded regions exposed in rolling circle or replicative intermediate of the Cairns type during viral DNA replication are involved in the initial base pairing between the viral and cellular DNA sequences. Further evidence to support this hypothesis comes from the arrangement of integrated viral sequences in various rat transformed cells. Indeed, polyoma and SV40 sequences are always (Polyoma) and often (SV40) found to be integrated in head to tail tandem

repetitions within the cellular genome of rat cells (72, 73, 70, 113, 116). These tandem arrays of viral sequences were shown to contain up to 5 unit-length copies of the viral genome (72), suggesting that viral DNA replication took place before integration. Large T antigen should therefore be present in these cells. We have investigated the role played by large T antigen during the integration process by comparing the arrangement of integrated viral sequences in Rat-1 cells transformed by recombinant plasmid DNA molecules incapable of encoding large T antigen (R cells) with those of cells transformed by recombinant plasmid capable of doing so (B cells). We found no significant difference in the organization of the viral sequences within the two types of transformed cells. These experiments therefore seem to suggest that large T antigen is not responsible for the tandem integration of the viral DNA. It is of interest to note, however, that among the seven Hind III-1 fragment transformed cells (also incapable of encoding large T antigen), only one (15-5a) contains tandemly repeated recombinant plasmid sequences. This finding is considerably different from that obtained from the R transformants. To resolve this discrepancy, we propose that recombinant plasmids containing the polyoma

genome inserted into the RI site could encode a truncated form of large T antigen which could still promote, to a certain degree, viral DNA replication and therefore tandem integration. Cells transformed by the viral Hind III-1 fragment inserted into the Hind III site of pBR322 DNA could not encode such functioning truncated proteins and therefore would not contain integrated tandem repetitions of viral sequences. The viral specific proteins present in fragment-transformed cell lines have been analyzed (Pomerantz, Ito and Hassell, unpublished data). Among all the fragment transformants studied, only 15-5a contained what seemed to be a truncated form of large T antigen. Accordingly, this cell line was also shown to be the only fragment transformant containing viral sequences tandemly integrated.

To explain the absence of free viral or recombinant plasmid sequences within the transformed cell lines analyzed, we can postulate that although the hypothesized truncated large T antigen present in these cells is sufficient to initiate viral DNA replication, it is incapable of mediating excision of the viral sequences from the integrated state as it has already been suggested as a function for large T antigen (81).

The observation that the cellular DNA isolated from 15-5a cells transforms Rat-1 cell in culture confirms that the oncogenic potential of the viral DNA is maintained after integration. In addition, this further confirms the observation that the proximal part (70-1.4 map units) of the early region of polyoma virus encodes all the information needed to stably transform rat cells in culture.

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