TRANSFORMING GENES AND GENE PRODUCTS OF POLYOMA AND SV40

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I. INTRODUCTION

The primary reason for studying SV40 and polyoma virus is that these small DNA-containing viruses cause tumors in vivo and transform cells in vitro. Polyoma virus was discovered as an agent causing parotid tumors.[1,2] It quickly became clear that the virus could cause a wide variety of tumors, and it was therefore called "polyoma virus".[3] SV40, which was discovered later,[4] was also found to induce tumors in hamsters.[5,6]

Polyoma virus and SV40 can also be studied to learn about immunological resistance to tumor formation. Although both viruses can cause tumors, the incidence of tumors in the natural population is quite low, even though the viruses themselves are widely distributed. Gross reported examining 10,000 mice and finding only one parotid tumor.[7] The ability of the immune system to protect animals from tumors caused by these viruses is great. Such immunological resistance could be fortunate for man, since human papova viruses such as BK or JC are widely disseminated in the human population.[8]

As polyoma virus and SV40 were studied in greater detail, their value as model systems for eukaryotic gene function became apparent. The viral chromatin contains cellular histones[9-13] and high mobility group (HMG) proteins.[14] Cellular enzymes are used for both viral DNA replication[15] and RNA transcription.[16-18] The processing of viral mRNA has been intensively studied, and much that is known about eukaryotic mRNA processing has been learned using these viruses.[19]

All these reasons for studying these viruses focus attention on their early regions. The early genes are responsible for neoplastic transformation. The early gene products are important in the production of the immunologic response needed for tumor rejection. Understanding the structure and function of the viral chromatin requires an understanding how the early gene products function. Analysis of the origin of early messenger RNAs demonstrates the way in which splicing can affect the use of coding sequences.

A variety of reviews on different aspects of SV40 and polyoma are listed in the references.[15,19-28] The most comprehensive review is *DNA Tumor Viruses* edited by John Tooze.[25] However, no single current review attempts to put the biology and biochemistry of SV40 and polyoma transforming genes into a single perspective. At a time when there is considerable uncertainty in the field about the degree of similarity or difference between the two viruses, such a perspective would seem important. This will be the goal of this article. It will soon become apparent that in some areas our understanding is wonderfully detailed; in others our knowledge is frustratingly incomplete.

II. INTRODUCTION TO THE VIRUSES AND VIRAL TRANSFORMATION

A brief introduction to the viruses and viral transformation will provide the reader with a context for the more detailed discussion of the early genes that follows.

Infection by polyoma virus or SV40 can have three results: productive infection resulting in progeny virus, "abortive transformation" in which the infected cells temporarily assume the transformed phenotype, and stable transformation which permanently alters the cellular phenotype. In permissive cells, which are mouse cells for polyoma and monkey cells for SV40, productive infection is the predominant response. In nonpermissive cells, such as rat or hamster cells for polyoma and rat, hamster, or mouse cells for SV40, abortive transformation is the common response. Both permissive and nonpermissive cells can become stably transformed. The frequency of stable transformation depends upon the cell line, but it is usually low.

Complementation studies define two early genes required for transformation by each virus: for polyoma virus these are the ts-a and hr-t genes, and for SV40, and A and F genes.[29-32,51] (I have used "F gene" in agreement with Tooze,[25] but the gene is often named by its mutants: dl [0.54 to 0.59] or dl 54/59.) Although some late mutations can affect viral transformation, such effects generally appear to be related to problems in penetration or uncoating.

It should be emphasized that the genes involved in transformation are needed for productive infection. For example, in UV inactivation experiments on polyoma virus, every survivor that can grow can also transform.[33-35,41a] This indicates that replication functions and transformation functions are not readily separated. Both the ts-a and hr-t mutants of polyoma virus, as well as the tsA mutants of SV40, were selected by their ability to grow productively under some conditions, but not others (*vide infra*). The importance of the transforming genes to productive infection is demonstrated by the fact that growth selections give rise to nontransforming viruses. Understanding the function of such genes, therefore, requires understanding their role in productive infection.

The gene products have been identified for each of the transforming genes. For SV40, the large T antigen is the A gene product,[46-49] and the small T antigen is the F gene product.[31,50-52] For polyoma virus, the large T antigen is the product of the ts-a gene.[53-59] Two primary products, the middle T and small T antigens, are altered by hr-t mutations.[56-59]

The characteristics of cells transformed by SV40 and polyoma virus are similar to those of cells transformed by other agents. Such cells, unlike their normal counterparts, show a relaxation of growth control. Many lines will grow efficiently in semisolid medium.[60,61] Transformants generally show a decreased serum requirement for growth[62,63] and reach a higher saturation density (see Benjamin[64] for a discussion). The morphology of transformed cells is altered in ways that are observable in single cells and in general populations. Transformed fibroblasts tend to be randomly oriented with respect to each other rather than showing a parallel alignment. Individual transformed cells appear less bipolar, becoming more rounded or stellate. Changes are observed in the actin architecture of transformed cells.[65-67] The structure of the plasma membrane is also changed as measured by increased agglutinability with plant lectins.[68,69] Changes can also be detected biochemically. Transformed lines can show changes in the amount of surface fibronectin.[70] Transport of various metabolites is increased.[71,72] Transformed cells can secrete large amounts of plasminogen activator into the medium.[73-75] Although such properties are generally associated with neoplastic transformation, it is possible to find exceptions in almost every instance. The nonselective isolation of transformants shows that all of these properties are not necessarily acquired in parallel.[76,77] The

observed variation in properties has led to the description of a variety of transformants ranging from "minimal" to "full".[45,76-79] "Minimal" transformants would grow in low serum, but not in soft agar. They would tend to have more ordered actin structures and secrete less plasminogen activator. "Full" transformants would grow in soft agar and exhibit the full range of properties associated with transformation. Martin[26] has reviewed the results of studies on 250 transformed clones; they show a continuous spectrum of properties rather than seeming to fall into discrete classes.

The cellular alterations described in the preceding paragraph have been observed in every instance for both polyoma virus and SV40 transformants. In the qualitative sense the two viruses affect cells in the same way. Whether the changes are quantitatively similar is more difficult to decide. Both older[80] and more recent[81-83] research suggests that these two viruses may not affect rodent cells in the same way. This point will be discussed in more detail later.

Quantitative assays for transformation are carried out in nonpermissive cells. This obviates the problem of subsequent reinfection and killing of the transformants by progeny virus. Rat or hamster cells are used to assay transformation by polyoma virus. In the case of SV40, hamster, mouse, or rat cells are used. A variety of assays have been employed to assess transformation: growth in agar or methyl cellulose,[60,61] dense focus formation,[84] colony morphology,[85,86] or growth in low serum.[87] The first two methods are those most generally used today. The ability to grow in a semisolid medium such as agar appears to be the property that most parallels tumorigenicity.[88,89] As discussed by Kahn and co-workers,[90] there are a number of exceptions — cell lines which grow in semisolid medium but fail to induce tumors in immunologically deficient nude mice. Sometimes this results from tumor immunity in the nude mouse.[90] In such cases growth in semisolid medium might even be a better assay for malignant potential than tumor induction in nude mice by avoiding problems of immunologic resistance.

III. THE EARLY GENES OF POLYOMA VIRUS AND SV40

The early genes of polyoma virus and SV40 are defined and their characteristics studied through the analysis of viral mutants. Two general sorts of approaches have been applied to the isolation of viral mutants. The first is to isolate conditional mutants that are restricted for growth at certain temperatures or on certain cell types. Selection for viability at low, but not at high, temperature allowed the isolation of the ts-a[36,37,487] (and tsA[39,40,92-98]) mutants. For polyoma, the hr-t mutants were selected by the ability to grow on polyoma-transformed 3T3, but not untransformed 3T3.[99,100] This protocol would be expected to detect mutations in functions required for transformation. It is worth noting that temperature selection has not yielded any hr-t mutants and that host-range selection did not identify any ts-a mutants. The probable reason for this will become apparent later. Second, the ability to manipulate DNA easily has permitted the isolation of a variety of nonconditional mutants. The ones most commonly studied are viable, but others are studied as defectives with a helper virus or as cloned DNAs. In SV40 the F mutants are one such class;[31,44,45,101,102] distal mutations in the A gene represent another.[49,101,103,104] For polyoma virus, mutations have been described that affect both the ts-a and hr-t gene products.[105-107,198] Over the next several years functions of these genes will be probed in great detail through the isolation of viruses with very specific mutations affecting early gene function.

A. The Ts-a Gene of Polyoma Virus

Ts-a mutants have been isolated by their ability to grow at low (31.5°) but not at high (38.5°) temperature. The prototype mutant ts-a was isolated by Fried in 1965.[487] Additional mutants were isolated by both Eckhart[36] and DiMayorca and co-workers.[37] Such

Table 1
SEQUENCE ALTERATIONS IN TS-a MUTANTS

Mutant	Nucleotide[a]	Base change	Change in large T	Ref.
ts-a	2193	G to A	ALA to THR	112
ts-25E	2883	G to T	GLY to CYS	112
ts-48	1228	C to T	THR to ILEU	113
	2341[b]	G to T	SER to ILEU	
ts-52	2883	G to A	GLY to SER	113

[a] Numbered according to Deininger et al.[189]
[b] Marker rescue experiments indicate this is the alteration responsible for the phenotype.

mutants form a single complementation group[29,30,32] and are usually temperature-sensitive for transformation as well as productive infection. There is one mutant (P155) that belongs to the ts-a complementation group based on analysis of growth, but retains the ability to transform at the nonpermissive temperature.[36,108] Marker rescue experiments map the ts-a gene to the distal portion of the early region.[109,110] Four such mutants have now been sequenced (Table 1). The mutations fall in two regions, both of which code only for the large T antigen.[111,112] One is near the extreme C-terminus. The other is the region 500 to 600 amino acids from the N-terminus. This region is highly conserved between SV40 and polyoma virus.[113] Interestingly, the tsA mutants of SV40 that have been sequenced show alterations in the same region of the molecule.[114]

Ts-a mutants are defective in viral DNA replication.[36-38,115] The block in viral DNA replication, like that for the tsA mutants of SV40, appears to be at the level of initiation.[115] Clertant and Cuzin have described an in vitro assay for initiation.[116] In their assay initiation can be blocked by Fab fragments of anti-T IgG, and the large T antigen of ts-a is temperature-sensitive for initiation. The ability of ts-a to induce cellular DNA synthesis does not seem to be impaired.[36,117] Since viral DNA replication does not occur, the late proteins are not synthesized.[37,38] Infections with a ts-a mutant lead to the overproduction of viral RNA.[118] This suggests that the polyoma ts-a gene product acts as a negative control element for early transcription. Intriguingly this overproduction does not depend on the temperature, suggesting the effects on RNA transcription and DNA replication are not coordinate.

Although ts-a mutants are defective in stable transformation, they are normal with respect to abortive transformation.[32,119,120] Ts-a mutants are also generally able to induce cell characteristics associated with the transformed phenotype when infections are carried out at the nonpermissive temperature. Thus, ts-a mutants cause rat fibroblasts to assume a transformed morphology even at the nonpermissive temperature.[117] They are unaffected in their wound serum requirement at 37°; cell movement in a wound is also normal.[121] Similarly the ts-a mutants cause a loss of stress fibers. The plasma membrane is altered to the agglutinable state after infection.[122] Similar changes in hexose transport are observed with wild type and ts-a mutant viruses.[123] In many cell lines transformed by ts-a mutants these kinds of characteristics can be temperature-sensitive,[124-127,532] suggesting that the ts-a function can have a continuing role in expression of the transformed phenotype. There has been considerable controversy on this point and it will be discussed in some detail in a later section after the experiments on tsA transformants of SV40 are described.

The ts-a function is involved in the process of integration. It can also be involved in excision of the viral genome. Integration of polyoma virus DNA into rat cells occurs primarily in partial or full head-to-tail arrangement.[128-133] Such tandem integration

appears to require an active ts-a function, since DNA transformation with ts-a DNA at the nonpermissive temperature results in a lower frequency of transformants that most often have single-copy insertions.[133] Two kinds of explanations have been proposed for this phenomenon. The most likely explanation is that head-to-tail dimers of viral DNA are generated during viral DNA replication. If, for example, polyoma virus DNA were replicating in a rolling circle fashion, then tandem viral insertions could occur. Such replication has been observed,[134] although the rolling circle is not the primary form of DNA replication, at least in permissive cells.[135] In this sense the requirement for ts-a in integration is just that of its requirement for viral DNA replication. However, the requirement of ts-a function to generate tandem inserts cannot be absolute, since ts-a DNA at the nonpermissive temperature will generate them with low (about 10%[133]) frequency, and since a restriction fragment lacking the C-terminal region of large T antigen resulted in a transformant with a head-to-tail insert.[136] A second possibility is that tandem integration could result from an *in situ* amplification. Colantuoni and co-workers[137] have shown that amplification of integrated sequences can occur in ts-a transformants at the permissive, but not the nonpermissive, temperature. Such a finding suggests that integration patterns can be altered even long after the initial event if an active ts-a gene is present.

The ts-a function affects the stability of viral DNA in transformed cells. Basilico and co-workers[129,130] have elegantly demonstrated that excision from integrated tandem repeats can lead to the appearance of phenotypic revertants. The frequency of this reversion is 50- to 300-fold higher in ts-a transformants at the permissive temperature than at the nonpermissive temperature. This "curing" appears to involve single copy loss following excision. The presence of free, unintegrated viral DNA molecules in transformed rat cells requires the presence of an active ts-a function.[138,139] In mouse cells, a similar induction of viral DNA replication was noted in down-shift experiments with a ts-a transformant.[140,141]

B. Host Range-Transformation (Hr-t) Gene of Polyoma Virus

Host range mutants of polyoma virus were first isolated by Benjamin in 1970 using polyoma-transformed 3T3 cells as a permissive host and normal 3T3 cells as a nonpermissive host.[99] The integrated polyoma genome of the transformed cells was expected to complement the growth of any mutant defective in functions expressed in transformed cells. For such a selection to succeed, functions expressed during transformation must be required for productive infection. Because the viral genome is only about 5000 base pairs, this is a reasonable assumption; the UV-inactivation experiments described earlier support this assumption directly.[33–35] A series of 19 hr-t mutants have been isolated using this selection protocol.[99,100] These mutants constitute a single complementation group and seem to have identical biological properties despite variation in their genomic alterations. These mutants are deficient in growth in the mouse, confirming the expectation implicit in the original selection protocol.[581,594] Mutations mapping in similar positions on the genome have also been generated in vitro in other laboratories.[142] As far as is known, such mutants are the same as other hr-t mutants.

Hr-t mutants are completely defective in stable transformation.[29,32,99,100,143] No stable transformant has ever been isolated, whether by focus formation or by growth in agar. Hr-t mutants are equally defective in inducing tumors in newborn hamsters.[144] Abortive transformation is not induced by hr-t mutants.[32,120] *De novo* infections with hr-t mutants do not result in specific cellular changes characteristic of the transformed state. Infection of rat fibroblast cells with wild type virus results in a change from end-to-end, side-to-side orientation to a disordered arrangement with underlapping of cells; cells change from an elongated shape to a more retracted multipolar shape.[117] These changes do not occur in infections with hr-t mutants. There is a loss of defined stress fibers after wild type virus

infection that can be seen directly or followed with antibodies against actin that is not observed in hr-t mutant infections.[117] Infection with, or transformation by, wild type polyoma virus leads to a change in plasma membrane structure that can be demonstrated by lectin agglutinability. Hr-t mutants fail to bring about this membrane change.[145]

Hr-t mutants do retain some ability to alter the cells they infect. Cellular DNA and histone synthesis are stimulated in both permissive and nonpermissive infections.[41c] The ability to induce cellular DNA replication and cell division has been most extensively studied in rat fibroblasts.[117] Hr-t mutants induce a single round of DNA replication and cell division, whereas wild type virus or ts-a mutants at 39° induce several rounds. Wild type virus, but not hr-t mutants, cause the appearance of a high frequency of polyploid cells.

The defect in productive infection by hr-t mutants is partial. This contrasts sharply with the absolute defect in transformation. A nonpermissive infection typically results in a burst approximately 2 to 5% that of wild type virus.[41-43,99,100] In nonpermissive infections hr-t mutants appear to be defective in assembly. Substantial amounts of viral DNA and capsid antigen are made in a nonpermissive infection, but the yield of infectious virus is low.[41c,100] The precise nature of the defect is not known. However, histones H3 and H4 from hr-t virions isolated after permissive infection are less highly acetylated than those of wild type virus.[146]

Although the rationale behind the isolation of the hr-t mutants was based on the expectation of direct complementation by the integrated viral genome, it soon became apparent that cells containing no integrated polyoma virus could also be permissive for the growth of the virus.[41-43,100] Baby mouse kidney cells, for example, are used for the routine preparation of high titer stocks. UCIB,[147] a spontaneous derivative of Balb/c 3T3 that is susceptible to transformation by murine leukemia virus, is used as the permissive cell in routine plaque assays. A wide variety of different cells have been found to support the growth of the virus.[41-43] Besides the baby mouse kidney epithelial cells, early passage mouse embryo fibroblasts are permissive for hr-t infections. However, from the first passage to the fourth passage permissivity drops 40-fold and late passage embryo fibroblasts are not permissive. Cells with a history of C-type virus infection are as permissive as the polyoma transformed cells. Sarcoma virus transformants or cells infected with murine leukemia virus are permissive. Attempts to show the activation of endogenous C-type viruses by wild type polyoma virus in parallel experiments to those demonstrating the effect of C-type viruses on hr-t permissivity have failed.[43] Interestingly, transformation by SV40 does not seem to render cells permissive for polyoma virus, although the permissivity values are very slightly above the untransformed controls.[42] A variety of phenotypic revertants isolated from polyoma transformed cells were found to be permissive for hr-t growth. Such results, and those with the SV40 transformants, indicate that there is no obligatory connection between anchorage independent growth and infectability by hr-t mutants. It must be emphasized that *cells permissive for the growth of hr-t mutants are not permissive for transformation by hr-t mutants.*

These growth and transformation characteristics of the hr-t mutants have led to a model for hr-t function.[100] The hr-t gene elicits from the cell factors required for productive infection as well as characteristic functions of the transformed state. Such permissivity and transformation factors must be at least partially distinct, since cells can be permissive without being transformed. (Cell lines expressing transformation functions, i.e., transformed cells, obviously cannot be "transformed" by hr-t mutants in any functional sense. As will be discussed in the next section, hr-t mutant DNA can be integrated into the genome of the cell; but such an integration has no obvious functional consequences.[142]) Cells can constituitively express the functions needed for productive infection at a particular embryonic state or in response to C-type viruses so that the need for the hr-t gene is bypassed.

Table 2
SEQUENCE ALTERATIONS IN HR-T MUTANTS

	Nucleotides changed[a]	Amino acid change middle & small T	Ref.
Deletion mutants			
SD15	435—575	—	150
6B5	462—640	—	148
A8	471—597	—	148
B2	504—744	—	148
NG18	527—713	—	148, 149
II5	649—749	—	150
Nondeletion mutants			
NG59	722: G to ATAA	ASP to ILEU-ASN	150
3A1	722: G to ATAA	ASP to ILEU-ASN	150
HA33	722: G to ATAA	ASP to ILEU-ASN	150

[a] Numbered according to Deininger et al.[189]

Marker rescue experiments localize the hr-t mutations to Hpa II fragment 4 in the proximal portion of the early region.[110] (See also Figure 9.) Of 19 hr-t mutants, 16 contain deletions.[100,110,148] Mapping experiments have positioned the deletions more precisely in this region.[148] Six of the deletion mutants have been sequenced, and the results are summarized in Table 2. Three hr-t mutants (NG59, 3A1, and HA33) do not contain deletions,[100,110,148] but rather show the same mutation.[150] The G at nucleotide 722 is altered to an A and three additional bases (TAA) are inserted. The appearance of an identical alteration in three different mutants is especially striking. It suggests that the possibilities for mutation in the hr-t gene are quite limited. Such limitations may partially explain why temperature-sensitive hr-t mutants have not been isolated so far.

C. The Products of the Early Genes of Polyoma Virus

The products of the early genes of polyoma virus have been identified using serum from tumor-bearing animals. For that reason they have been called tumor (T) antigens. Figure 1 shows the proteins labeled with ^{35}S-methionine that can be precipitated from baby mouse kidney cells infected with wild type virus. The major product has an apparent molecular weight of 100,000. Four minor species of apparent molecular weights 63 K, 56 K, 36 K, and 22 K are also observed. Serum from tumor-bearing animals should recognize all virus-coded or virus-induced proteins that the host recognizes as foreign. With this serum, a band that appears to be actin is precipitated from both infected and uninfected cells. Reports on the number of species and their apparent molecular weights have varied from laboratory to laboratory. Table 3 summarizes this variation. In vitro translation of virus-specific RNA shows that the 100 K, 56 K, and 22 K are primary viral translation products.[59]

1. The ts-a Product: Large T Antigen

The major band with an apparent molecular weight of 100 K (large T antigen) is the product of the ts-a gene. It is thermolabile in pulse-chase experiments with ts-a mutants.[54,56,57] In a cell line transformed by a DNA restriction fragment lacking the distal portion of the sequences coding for the ts-a gene, the 100-K antigen is absent, but a truncated 34-K T antigen is observed.[136] The 100-K band is unaffected by hr-t mutations.[55-58] DNA sequencing suggests that the molecular weight of large T antigen should be 87,991.[25] The discrepancy between the predicted and observed values is probably a consequence of the postsynthetic modifications described below and anomalous

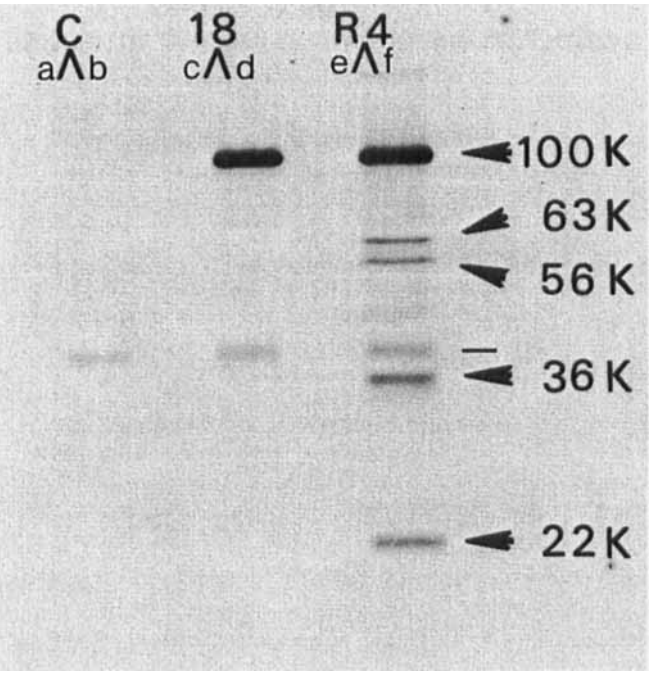

FIGURE 1. T antigens of polyoma virus. T antigens were extracted and immunoprecipitated from infected baby mouse kidney cells labeled with ^{35}S-methionine. The immunoreactive proteins were separated on discontinuous buffer SDS polyacrylamide gels of 12.5% acrylamide. (C) Proteins precipitated from mock-infected cells with [a] preimmune serum or [b] anti-T ascites fluid; (18) proteins precipitated from hr-t mutant NG18-infected cells with [c] preimmune serum or [d] anti-T ascites fluid; (R4) proteins precipitated from wild type (NG18R4)-infected cells with [e] preimmune serum or [f] anti-T ascites. The positions of large T (100 K), middle T (56 K) and small T (22 K), as well as the 63- and 36-K T antigens, are shown by the arrowheads. The line indicates actin. The data are taken from Schaffhausen et al.[55]

Table 3
T ANTIGENS OF POLYOMA VIRUS

Large T antigen	Middle T antigen	Small T antigen	Others	Ref.
100	57	23	61[a]	23
100—108	55	22	72, 67, 63, 55, 52	54
100	56	22	63[b], 36	55
90	60	22	55[c], 37[c], 33[a]	57
100	55	22	45[a], 39[a]	188
105	63	20	72, 56, 34	544

[a] Mapping experiments indicate this band is of viral origin.
[b] Mapping experiments indicate more than one component; the major is of cellular origin and the minor is a viral product.
[c] Mapping experiments indicate this band is of cellular origin.

behavior in SDS, since random coil chromatography in 6 *M* guanidine hydrochloride gives a lower estimate.[55] The amino acid sequence predicted from DNA and RNA sequencing results is shown in Figure 2.[25,153]

The large T antigen can be resolved into at least two species on one-dimensional SDS

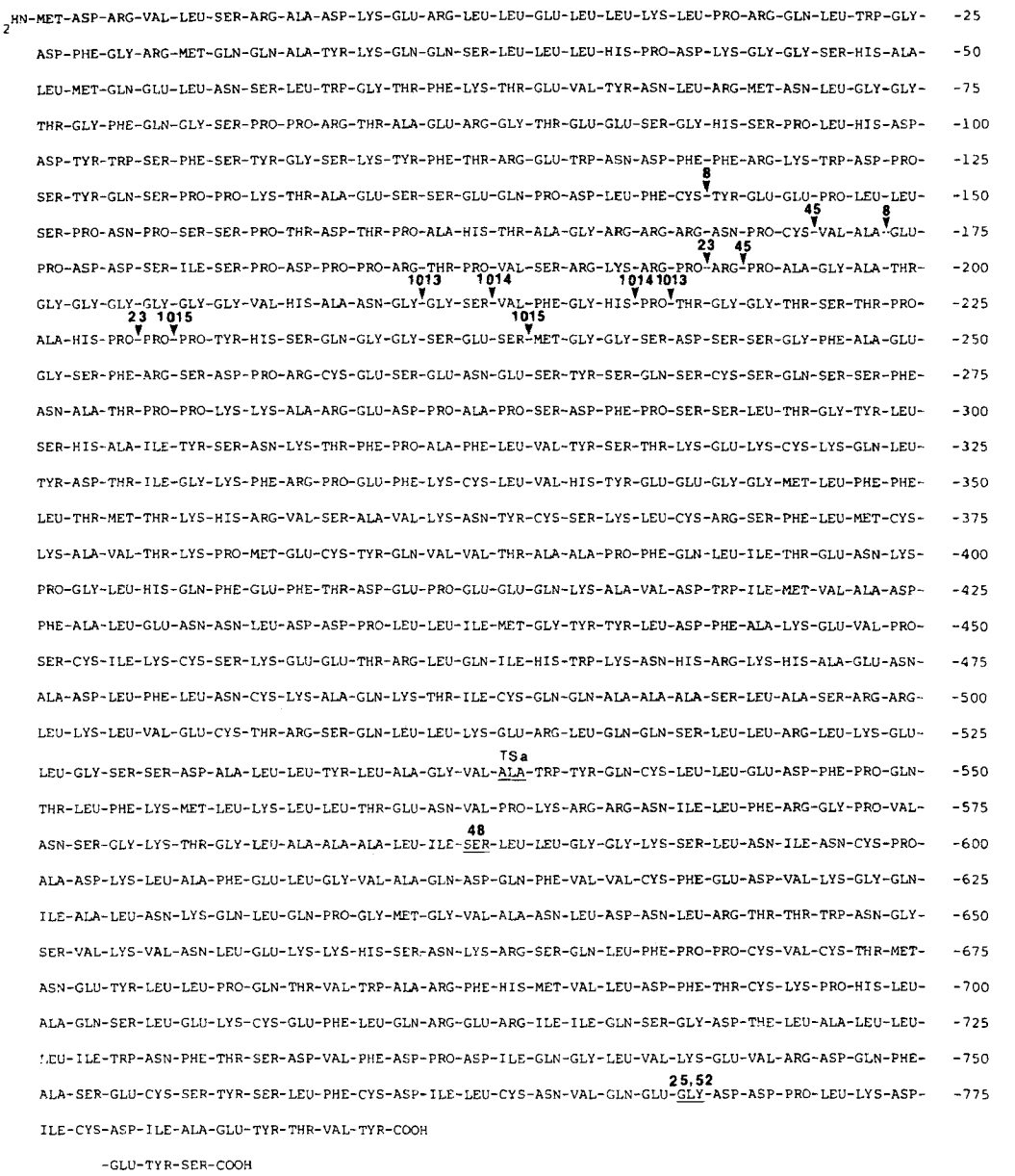

FIGURE 2. The predicted amino acid sequence of polyoma large T antigen. The amino acid sequence shown is predicted by Griffin and colleagues[25] for the large T antigen of the A2 strain. An alternative C-terminal sequence obtained for strain 3[189] is shown below the A2 sequence. The boundaries of deletions of various dl mutants are indicated by the arrowheads.[107,546,595] The positions of ts-a mutations are underlined.[112,113]

polyacrylamide gels.[23,55,143,151] It can be similarly resolved by two-dimensional gels using isoelectric focusing followed by SDS electrophoresis.[23] Türler[23,151] has shown that the slower migrating species predominates after viral DNA replication has begun, while the faster migrating species is observed early in infection. In pulse-chase experiments the apparent size of the large T antigen increases during the chase.[54,57] The product synthesized in vitro from polyoma RNA is apparently smaller than the species observed in vivo; this implies that there is posttranslational modification. Both forms are known

to incorporate phosphate to approximately the same specific activity.[55,154] However, the tryptic phosphopeptide maps are reported to be quite complicated,[23] and differences in a specific site would not have been detected in the previous experiments. Attempts to label large T with radioactive acetate[23] or carbohydrates[23,55] have so far proven unsuccessful.

The large T antigen is localized in the nucleus. The nuclear immunofluorescence obtained with anti-T serum is temperature-sensitive for some ts-a mutants.[155] Hr-t mutants give rise to normal nuclear immunofluorescence on infection.[100] Biochemical fractionation experiments also show that the major portion of the large T antigen is found in nuclear fractions.[56] A newly synthesized, highly phosphorylated fraction of large T antigen is found in the nuclear matrix.[156] Whether small amounts of large T antigen are also present in the plasma membrane as for the large T antigen of SV40 is not clear. The question has not been asked with the same zeal as for SV40. There is large T present in crude membrane preparations,[56] but this might well represent cross-contamination.

Purification of large T antigen has been only partially successful.[157,158] Large quantities of the protein will probably not be available until the gene is cloned. The results with partially purified material suggest that the protein can function in the initiation of DNA synthesis[116] and can act as an ATPase.[158] Partially purified preparations of polyoma large T antigen are resolved into two different fractions of DNA-independent ATPase by chromatography on DNA cellulose.[158] The activity of these preparations (6 to 12 μm/hr/mg) is comparable to that of the SV40 large T antigen. Like the SV40 activity, the polyoma virus ATPase activity is inhibited by anti-T sera. The large T antigen prepared from ts-a infected cells showed a 10 to 20-fold higher sensitivity to temperature than wild type. The ATP affinity labeling reagents, 8-azido-ATP and 2,3-dialdehyde ATP, can react with large T antigen in immunoprecipitates as would be expected for an ATPase.[582]

2. The hr-t Gene Products: Middle T and Small T Antigens

The hr-t gene has two primary products: small T antigen and middle T antigen. Translation of virus-specific RNA shows that both are virus-coded.[59] Both are absent from immunoprecipitates of hr-t deletion mutant T antigens.[35,55-58] Figure 1 shows this result for NG18 and Figure 3 shows the result for other hr-t deletion mutants. SD15, which has an in-phase deletion,[150] codes for a truncated product of 50 K, the size expected for a deletion of 47 amino acids. Figure 3 also shows that deletion mutants induce small amounts of 6- to 9-K species. The sizes of these products do not correlate directly to the size of the deletion. Presumably, they are stable breakdown products of altered polypeptides synthesized by these mutants.

Three nondeletion mutants (3A1, HA33, NG59) make normal amounts of mutant middle T and small amounts of mutant small T antigens. Based on DNA sequencing, the aspartic residue at 179 in wild type should be replaced by isoleucine-asparagine in the mutants.[150] The basis for the reduced amount of small T is not clear. The most likely explanation is that the mutant 22-K protein is less antigenically active. The altered product does not appear to be less stable in pulse-chase experiments.[583] Alternatively, the choice of RNA splices sites could be affected by the mutation so that less small T message is produced.

The middle T antigen is clearly important in transformation. Mutations that affect middle T, but not small T, can drastically alter the ability of the virus or viral DNA to transform cells. Such mutations will be discussed in the next sections. Treisman and colleagues have constructed a plasmid containing an early region encoding only middle T antigen.[568] The plasmid DNA transforms Fisher rat cells with an efficiency one half to one third that of a plasmid containing a normal wild type early region. The middle T

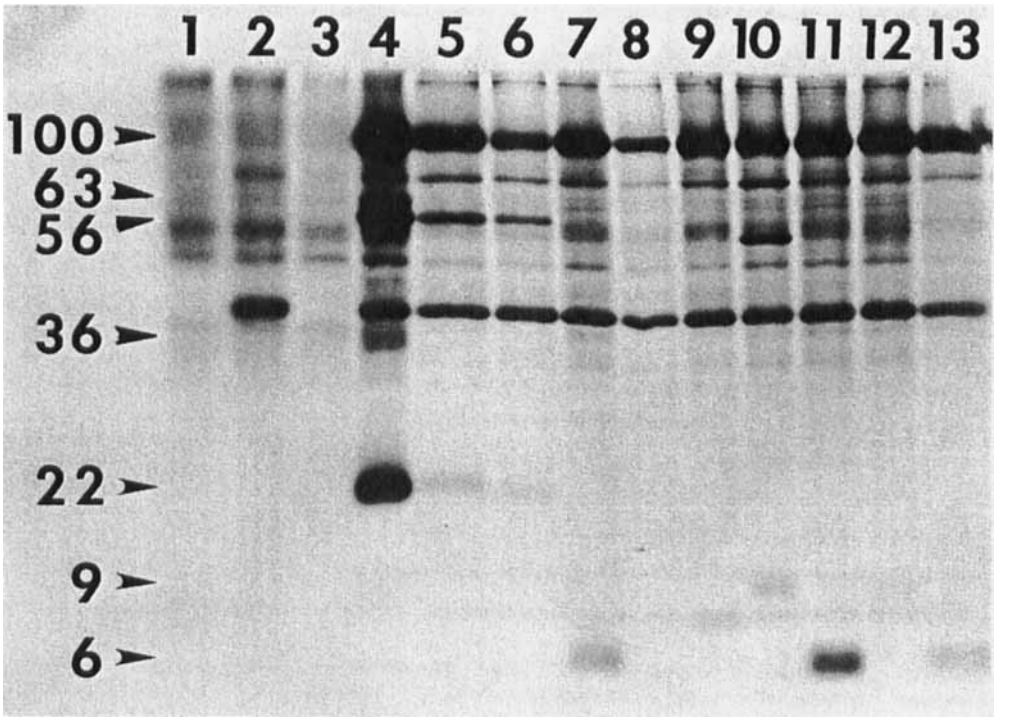

FIGURE 3. T antigens induced by hr-t mutants of polyoma virus. T antigens were extracted from 3T3 cells labeled with ^{35}S-methionine and precipitated with anti-T ascites fluid or control serum. 12.5% polyacrylamide gel. (1) Mock-infected cells, control serum; (2) mock-infected cells, anti-T ascites fluid; (3) wild type-infected cells, control serum; (4) wild type-infected cells, anti-T ascites, and extracts from hr-t infected cells precipitated with anti-T ascites fluid; (5) 3A1; (6) NG59; (7) 115; (8) A8; (9) NG23; (10) SD15; (11) 3A4; (12) 6B5; and (13) B2. The positions of the T antigens are indicated at the left. (Reproduced from Silver, J., Schaffhausen, B., and Benjamin, T., *Cell*, 15, 485, 1978. With permission.)

transformants obtained with the plasmid are similar in phenotype to those of wild type transformants, although many do grow poorly on plastic at very low cell density. So far, a specific role in hr-t function has not been experimentally demonstrated for the small T antigen.

Small T antigen has an apparent molecular weight of 22 K, in good agreement with the value of 22,785 predicted from DNA sequencing and RNA mapping.[25] Middle T antigen has an apparent molecular weight of 56 K, which is quite different from the value of 48,556 predicted from sequencing. Peptide mapping shows that middle and small T share a common set of N-terminal peptides with large T.[57,160,161,267] Middle T and small T share an additional set of peptides not found in large T. The small T antigen has four C-terminal amino acids, and the middle T a long C-terminal sequence, not shared with other species.

The predicted amino acid sequences of middle T (Figure 4) and small T (Figure 5) show some similarity to certain sequences found in peptide hormones. As first pointed out by Friedmann and colleagues,[162] there are two cysteine clusters (CYS-X-CYS-X-X-CYS) in the region common to small and middle T antigen that are also present in the α and β subunits of hormones such as TSH, LH, and FSH, as well as in SV40 small T. There is also a proline-rich cluster (residues 336 to 341) in middle T that is also found in the β subunit of human chorionic gonadotropin. Whether such sequences indicate a functional similarity as well is an intriguing question. (See Friedmann[162] and Benjamin.[163])

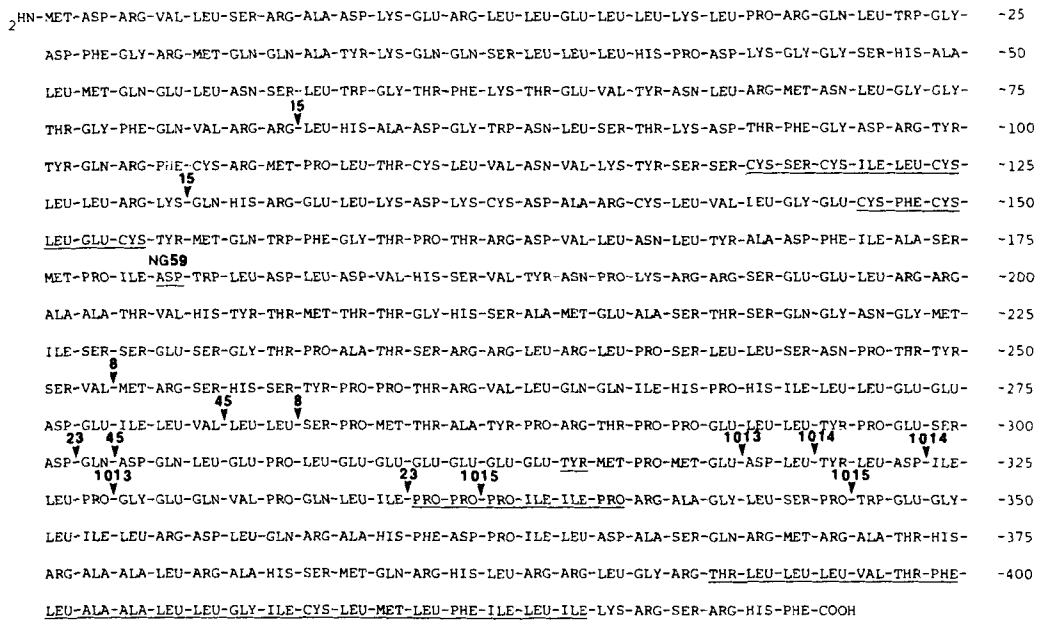

FIGURE 4. The predicted sequence of middle T antigen. The amino acid sequence is predicted by Griffin and colleagues[25] for the middle T antigen of the A2 strain of polyoma virus based on their DNA sequencing and the RNA mapping results of Kamen and co-workers.[195] The positions of the deletions in SD15,[150] dl 8,[546] dl 45,[107] dl 23,[546] and dl *1013* to *1015*[595] are indicated by the arrowheads under the mutant number. The deletion in dl 23 results in an additional proline residue, while the deletion in dl 1014 causes the insertion of an aspartic residue. Additional landmarks on the middle T molecule are underlined: the two cysteine clusters (residues 120 to 125 and 148 to 153) matching the sequences of TSH, LH, and FSH,[162] aspartic acid residue 179 that is altered to asparagine in hr-t mutants NG59, 3A1, and HA33 after the insertion of an additional isoleucine residue, tyrosine residue 315 that is a major phosphorylation site in the in vitro kinase reaction, the proline-rich sequence (residues 336 to 341) that is also found in the β-subunit of human chorionic gonadotropin,[163] and the stretch of 22 hydrophobic amino acid residues (394 to 415) likely to be involved in membrane binding.

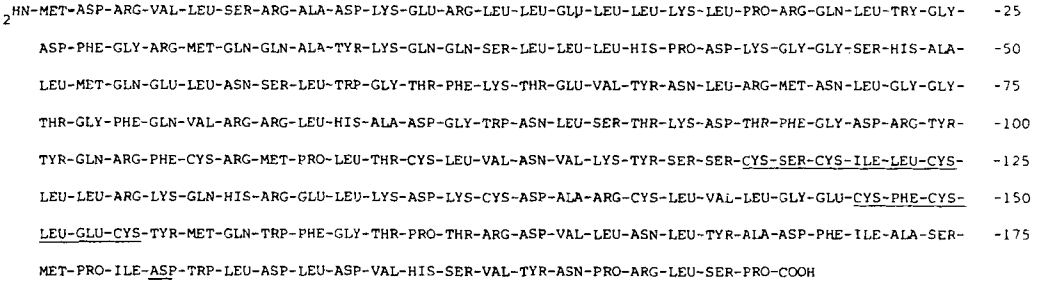

FIGURE 5. The predicted sequence of small T antigen. The amino acid sequence shown is predicted by Griffin and colleagues[25] for the small T antigen of the A2 strain of polyoma virus based on their DNA sequencing and the RNA mapping results of Kamen and co-workers.[195] The cysteine clusters (residues 120 to 125 and 148 to 153) also found in the sequences of FSH, TSH, and LH[162] are underlined. The aspartic acid residue 179 that is altered to an asparagine in hr-t mutants NG59, 3A1, and HA33 is also underlined; these hr-t mutations also cause an additional isoleucine to be inserted before the asparagine.[150]

Little is known about small T antigen. There is no evidence that small T is modified after synthesis. It appears to be localized in the cytoplasm.[56] No function has been ascribed to it so far. A fusion protein containing small T sequence has been cloned in

bacteria,[164] but the purification and biochemical study of small T will probably have to await the production of a cDNA clone.

Middle T antigen can be resolved into two species on SDS gels.[159,171] Most of the middle T has an apparent molecular weight of 56 K. This is the same size as the in vitro translation product. The minor form (referred to as 58 K) has an apparent molecular weight of 58 to 63 K depending on the gel conditions. The 58-K species is difficult to detect by ^{35}S-methionine labeling, but it is detectable by labeling in vivo with $^{32}PO_4$ or in vitro with ^{32}ATP. The difference between the two middle T species can be mapped to the C-terminal region by partial proteolysis. Mapping of deletion mutants places the difference between residue 200 and residue 300. Because the in vitro translation product is 56 K, the 58-K species is presumed to arise by postsynthetic modification.

Middle T antigen, but not small T, can be labeled in vivo with $^{32}PO_4$[55,107,154,159,171] (Figure 7). Both the 56- and 58-K forms are phosphorylated on serine or threonine residues, but the 58 K has a much higher specific activity ($^{32}P/^{35}S$).[171] The two species are phosphorylated at different sites in the C-terminal region upstream from residue 300. Because that is also the region to which the 56 K/58 K difference maps, phosphorylation may be involved in the resolution of the two forms.

The middle T antigen is associated with the plasma membrane.[56,152,165] Pulse-chase experiments[152] show that middle T continues to accumulate in the membrane during the chase period; this suggests that entry into the membrane may be a relatively slow process. Extraction with buffers containing Triton X-100 generates a cellular framework consisting of membrane lamina, nucleus, and cytoskeletal framework.[166,167] Middle T is also associated with this framework, although approximately 50% is also found in the soluble fraction.[159] The association of middle T with membranes is not surprising. The C-terminus contains a stretch of 22 hydrophobic amino acids bounded on either side by a cluster of basic amino acid residues (Figure 4). Such sequences are typical of other known membrane proteins.[168-170] Recently a termination mutant has been constructed for which the middle T lacks the last 37 amino acids.[584] This truncated middle T is found in the cytosol, not in membrane fractions. The orientation of middle T in the plasma membrane has not been firmly established. Based on protein kinase activity (see below), middle T appears to be oriented towards the inside of the cell, and not extracellularly.[159] Attempts to label middle T from the outside by iodination with lactoperoxidase or iodosulfanilic acid, to demonstrate its presence on the surface immunologically or to cleave it with protease treatment of the cell surface, have proven unsuccessful.[585] The hr-t mutant middle T antigens of SD15 or NG59 are also found in membrane fractions.[35] When the middle T is extracted from the membrane with Triton X-100, it is found in a high molecular weight complex.[159] This complex has not yet been characterized.

Polyoma middle T antigen is associated with a protein kinase activity that phosphorylates either middle T itself[154,159,171-174] or middle T and IgG[173,174] in immunoprecipitates. There is a strong correlation between the ability of polyoma virus to transform cells or to induce tumors and the ability to carry out this in vitro kinase reaction. In many respects, but not all, this activity is similar to that observed for pp60^{src}, the product of the transforming gene of Rous sarcoma virus.[175-178]

When γ-^{32}P-ATP is added to T antigen immunoprecipitates, labeling of both the 56- and 58-K middle T antigen species is observed (Figure 6). The mobility of the 58-K form depends on the gel conditions and varies from 58 to 63 K.[159,171] Other laboratories observe only a single band.[172-174] However, the band can be broad and may represent two unresolved species. The ratio of the 56- and 58-K forms can differ in transformed cells; Py-3T3-6 cells, for example, show much more of the 56 K.[154,171] There is no question that the ^{32}P-labeled bands represent middle T antigen. Their mobilities are appropriately different in different viral strains and deletion mutants.[154,159,171,173] The partial proteolytic peptide maps obtained with chymotrypsin or *Staphylococcus aureus* V8 protease match

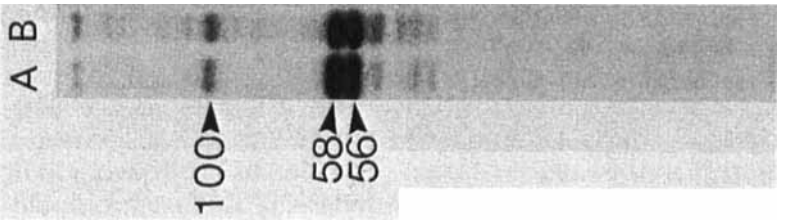

FIGURE 6. In vitro phosphorylation of polyoma T antigens. Immunoprecipitates were prepared from wild type- or ts25D-infected cells grown at 39.5° at 26 hr after infection. Ts25D is a mutant of the ts-a class.[37] The washed immunoprecipitates were incubated with γ-^{32}P-ATP, washed, and electrophoresed on discontinuous buffer SDS polyacrylamide gels of 9% acrylamide. The arrowheads indicate the position of the 100-K large T antigen and the 56- and 58-K middle T antigen species. (Lane A) ts25D; (lane B) wild type. (From Schaffhausen, B. and Benjamin, T., *Cell*, 18, 935, 1979. With permission.)

those obtained for ^{35}S-methionine labeled middle T.[154,159,171] Tyrosine is phosphorylated in the in vitro reaction,[172] as for the pp60^{src} reaction[178] or the Abelson virus p120 reaction.[179] The extent of the reaction is relatively low; at best 10% of the middle T antigen can be labeled in vitro. It would be interesting to know whether that 10% represents a specific subset of middle T antigen molecules.

The in vitro kinase activity is clearly a property associated with the hr-t gene. Figure 6 shows that immunoprecipitates from cells infected with ts-a mutants at the nonpermissive temperature are not defective in the in vitro kinase reaction. Kinase activity is also found in cells that contain no large T antigen.[154,172] Hr-t mutant immunoprecipitates are completely deficient in the in vitro kinase reaction (Figure 7).[154,159,171-173] Both hr-t deletion mutants and point-insertion mutants such as NG59, which have only a single "asp" to "ile-asn" change, are equally defective. Dl 23, which makes an altered middle T antigen but an apparently normal small T, is also defective in the in vitro reaction. This shows that small T alone is not sufficient for activity.

The correlation between transforming ability and the in vitro kinase reaction is generally supported by examination of non-hr-t deletion mutants and by examination of phenotypically normal revertant cells isolated from polyoma transformants. Dl 23 is defective in both the in vitro kinase reaction and in transformation.[159,173,174,198-200] Dl 8, which is especially effective in altering the cellular phenotype, exhibits a higher activity.[173,174,198-200] Dl 45, which appears not to be biologically affected by the deletion,[107] shows a normal in vitro kinase activity.[154] The only apparent exception is dl 1015,[105,570] which is defective in transformation, but shows wild type levels of kinase.[159] However, as will be discussed shortly, to dl mutants affect both the ts-a and hr-t gene products, and it is not known to what extent the dl 1015 defect is ts-a related. Phenotypically normal revertant cells isolated from polyoma transformants lack both the in vitro kinase activity and the ^{35}S-methionine labeled middle T antigen.[171] In no case so far, either in transformed cells or in mutant infections, is there an instance of a "transformation-plus" and "kinase-minus" phenotype.

The nature of the middle T substrate has been studied in some detail.[154,159,171] The 58-K form is an order of magnitude more active in the in vitro reaction than the 56-K middle T antigen. Both are phosphorylated at the same site(s) in the C-terminal region of the molecule. The major site is probably tyrosine 315. The region containing this tyrosine is deleted by the dl 23 mutation.[546] Other minor phosphorylations in the same region are possible. Because the 56- and 58-K forms differ in their in vivo phosphorylations, the second site phosphorylations could be the basis for the difference in specific activity

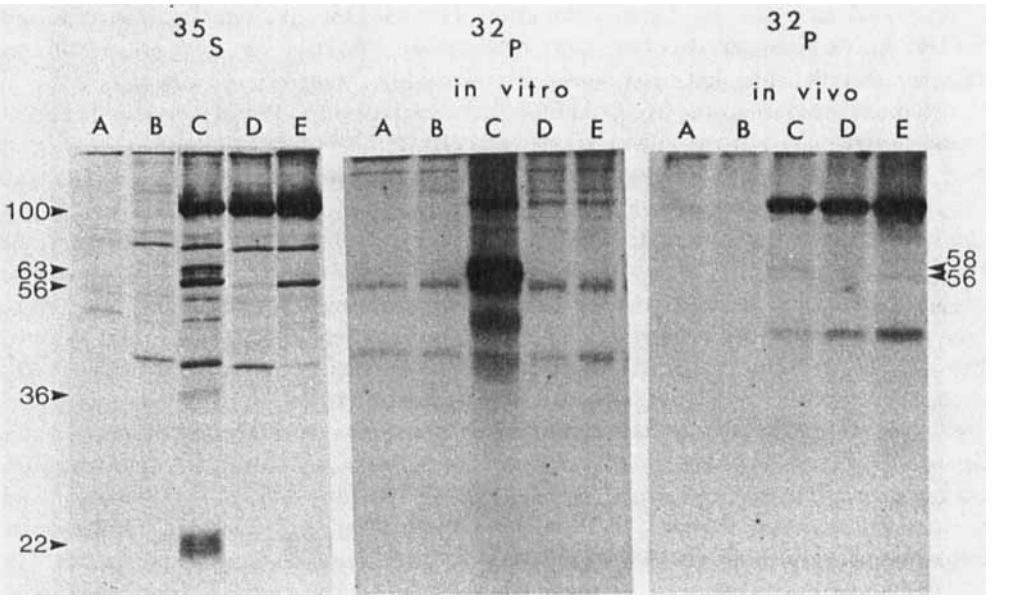

FIGURE 7. Phosphorylation of hr-t mutant T antigens in vitro and in vivo. Three sets of baby mouse kidney cells were infected with wild type virus or hr-t mutants. One set was pulse-labeled with ^{35}S-methionine. A second set labeled in the in vitro kinase reaction. The third set was pulse-labeled with $^{32}PO_4$ in vivo. The T antigens were electrophoresed on 12.5% acrylamide gels. (A) Wild type-infected cells, preimmune serum; (B) mock-infected cells, anti-T ascites; (C) wild type-infected cells, anti-T ascites; (D) hr-t deletion mutant NG18, anti-T ascites; (E) hr-t point-insertion mutant NG59. The arrows indicate the positions of the viral T antigens. The in vitro kinase reaction has resulted in so much labeling of the wild type 56- and 58-K middle T antigens that they are not resolved at this exposure. In the in vivo labeling experiment two species can be resolved in the middle T region of the gel. (From Schaffhausen, B. and Benjamin, T., *Cell*, 18, 935, 1979. With permission.)

between the two forms. Many cellular enzymes are known to be activated by phosphorylation.[181] The same in vivo labeling experiments have not shown labeling of tyrosine-315.[171] This raises the possibility that phosphorylation of middle T itself may be more important as a marker than as a reaction of direct biological importance. Mutants must be constructed to test this possibility directly.

Like middle T antigen itself, the kinase activity is associated with membrane fractions.[154,173] Fractionation experiments using Triton-X100 buffers show that the kinase activity is largely associated with the cellular framework.[159] Similar results have been obtained for the pp60^{src} kinase activity.[180] About half of the middle T antigen is solubilized by the Triton-X100 buffers in the preparation of cell frameworks; this middle T has a much lower activity in the kinase reaction. Comparisons of cell surface and cell framework labeling with γ-^{32}P-ATP suggests that the orientation of the kinase activity is to the inside of the cell and not extracellular.[159]

Whether the kinase activity is an intrinsic activity of middle T itself or whether middle T is associated with a cellular tyrosine kinase is not settled. The total failure of hr-t mutants in the in vitro reaction suggests that middle T is catalytically active. (See Schaffhausen and Benjamin[154,159] for a discussion.) However, middle T antigen can be fractionated into species of differing specific activity in cell framework preparations and also on Triton-X100-containing sucrose gradients.[159] Also, attempts to label middle T antigen with ATP affinity reagents, such as 8-azido ATP or 2,3 dialdehyde ATP, have not been successful.[582] If middle T antigen is associated with a cellular tyrosine kinase, such an interaction could modulate the activity of the cellular enzyme. The example of the ability of calmodulin to regulate cellular kinases is a model of such interactions.[181,182]

It is worth emphasizing that no comparable tyrosine kinase activity has been reported for SV40. The kinase activity that is associated with SV40 large T antigen preparations is clearly different from that associated with the middle T antigen of polyoma.[183-187]

Two other major bands, 63 K and 36 K, are observed on SDS polyacrylamide gels of immunoprecipitates from wild type infected cells.[35,55,56,58] These bands are absent from hr-t immunoprecipitates (Figure 1). Depending on the gel conditions, these bands may not represent single polypeptide species. As already indicated, the 58-K middle T antigen can have an apparent molecular weight of 63 K. Peptide maps of limit digests suggest that the 63-K region can also contain some fragments of large T antigen.[586] Partial proteolytic mapping experiments show that the major 63-K component has peptides distinct from any of the primary viral products.[159] This indicates that the major component is likely to be a cellular protein. The 36-K species migrates very closely to a cellular band present even when preimmune serum is used.[56] No information is available on the mapping of 36 K, but other laboratories have mapped proteins of similar molecular weight. Ito and co-workers have reported a 39-K species that appears to have N-terminal peptides common to all three T antigens as well as middle T-specific peptides.[188] Hutchinson and co-workers have reported a 33-K form that contains middle T peptides.[57] Pulse-chase experiments show that both the 63- and 36-K T antigens are relatively stable species.[56] In cell fractionation experiments, both are found in the cytoplasmic fractions. No activity has been discovered for the 63- and 36-K proteins.

3. The Anatomy of the Early Region of Polyoma Virus

Two laboratories have sequenced the complete genome of polyoma virus.[153,189] In the course of this work the sequences of the early region have been reported and discussed.[113,162,190-192] For the purposes of this discussion, the numbering system of Deininger and co-workers will be used.[189] Their numbering system starts at the beginning of a stretch of 8 Ts in the origin region that is homologous between polyoma virus and SV40. The sequences involved in coding the early proteins will be discussed here. Later the origin region will be considered; it is involved in controlling early expression and DNA replication. The large T antigen must bind directly in this region.

4. The Coding Region for the Early Genes of Polyoma Virus

Early mRNA sediments at about 20S.[193,194] In vitro translation of RNA fractionated by size shows that the messenger for large T antigen is smaller than the message for small T.[59] The major early mRNAs (Figure 8) share common 5′ ends near 73.3 map units and 3′ ends near 25.8 map units.[195] Fine-structure mapping places the 5′ ends near nucleotides 155 and 165 upstream from the common initiation codon found at nucleotides 188 to 190. At the end of the early region there is a sequence coding for AAUAAA (2937 to 2942), the polyadenylation signal common to eukaryotic mRNAs.[196] The introns for the early messengers have been determined by S1 mapping and comparison to consensus splicing sequences.[195] For the large T antigen mRNA, the intron extends from nucleotide 425 to nucleotide 809. Removal of the intron results in a change in the reading frame downstream of the splice so that the large T is encoded in an open reading frame until a termination codon is reached at nucleotides 2935 to 2937. The intron in the middle T mRNA extends from nucleotide 762 to 813. Removal of this intron changes the reading frame to a different one from that used in the large T message. The middle T termination codon is found at nucleotides 1513 to 1515. For small T antigen, removal of the intron from nucleotide 762 to 809 does not change to reading frame so that termination occurs at nucleotides 821 to 823. Some additional minor RNAs are observed.[195,197] There is an additional polyadenylation signal at nucleotides 1491 to 1496 which is used for RNA species in transformed cells. Such a messenger RNA could code for middle T or small T antigens, but not for large T antigen.

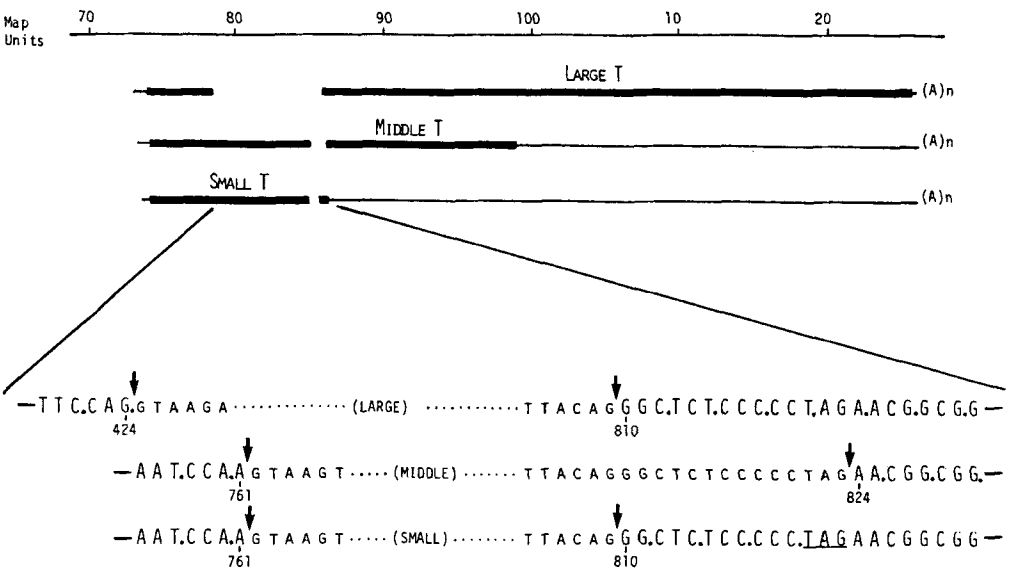

FIGURE 8. Messenger RNAs for the three primary early gene products. The arrangement of messenger RNAs that leads to three early gene products has been determined by Kamen and colleagues.[195] The 5′ ends of the early messages are found near nucleotides 155 and 165; the polyadenylation signal at the 3′ end is found at nucleotides 2937 to 2942. The large T splice joins nucleotides 424 and 810, causing a change in reading frame. Nucleotides 761 and 824 are joined by the middle T splice; this shifts the reading frame to the third frame, distinct from the original or that used to encode the C-terminus of large T. The small T mRNA has nucleotide 761 joined to 810, retaining the original reading frame. Nucleotides within the intron are shown in lower case. The triplets which would be read from the initiation codon are separated by the dots. The data are taken from Kamen et al.[195]

The DNA sequencing and RNA mapping results can be interpreted in terms of the protein sequences of the three T antigens (Figure 9). All three share a 79 amino acid N-terminal sequence. The large T mRNA is spliced so that the last 706 amino acids are unique to large T. The middle and small T mRNAs encode an additional 112 amino acids common to both in a region that is within the intron for large T antigen. All of the hr-t mutants have been mapped to this region. The intron for small T ends so that only four amino acids are coded downstream of the splice. The intron for middle T changes the reading frame so that an additional 230 amino acids are acquired in a frame distinct from those used for small and large T. These C-terminal amino acids for middle T are read from the same DNA sequence that code for an internal portion of the large T antigen in a different frame. Mutants in this region affect both the large T and middle T antigens, i.e, the ts-a and hr-t gene products. Their properties will be considered next. It is useful to note that the proteins already identified use all of the available open reading frames. As will be seen shortly, this is not true for SV40.

5. Mutants of Polyoma Virus Altering Both the ts-a and hr-t Gene Products

Four different laboratories have described deletion mutants in the coding region common to the large and middle T antigens.[105-107,198] The small T protein is not affected by these deletions. The positions of the deletions are indicated in Table 4. Different mutants are affected to different extents in either productive infection or transformation. Dl 8 grows less well than wild type, but transforms quite well.[198-200] Dl 23 grows well, but is defective in transformation.[83,198-200] Dl 45 is normal for growth and transformation,[107] while dl 1015 appears to be affected for both. Such a mixture of results is hardly surprising, since the ts-a, hr-t, or both ts-a and hr-t functions could be altered by such

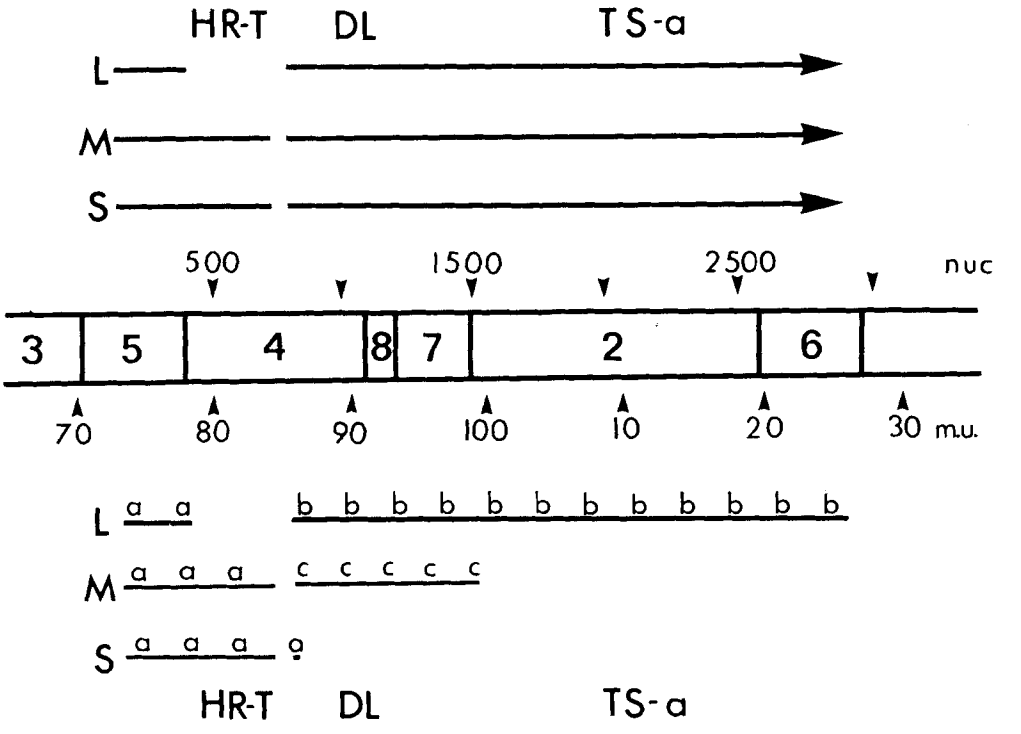

FIGURE 9. The coding sequences for the early gene products of polyoma virus. Peptide mapping,[57,160,544] RNA mapping,[195] and DNA sequencing[113,153,189] have been used to determine the coding sequences for the three primary gene products. The early region is shown in map units and nucleotide numbers, with the Hpa II fragments shown for reference. The RNAs coding for the three early gene products are shown above, with gaps left for the sequences removed by splicing. Below the line the positions of the coding sequences for large (L), middle (M), and small (S) T antigens are shown. The lower case letters indicate the reading frame used. Small, middle, and large T antigens share a common N-terminal sequence of 79 amino acids. Small and middle T antigen share an additional 112 amino acids encoded in the intron in the ts-a gene. The hr-t mutants map in this region, the proximal portion of Hpa II fragment 4.[110] Because of RNA splicing, middle T and large T are encoded in different frames after the splice so that each has its own C-terminal region. Deletion mutants (DL) have been isolated that alter the coding sequences for both proteins.[105,107,198] The ts-a mutants map in the distal portion of the early region (Hpa II, fragment 2).[109,110]

Table 4
SEQUENCE ALTERATIONS IN dl MUTANTS

Mutant	Deletion[a]	Bases deleted	Change in large T besides deletion	Change in middle T besides deletion	Ref.
dl 8	1005—1094	90	No	No	591
dl 45	1090—1155	66	VAL to GLY	No	107
dl 23	1154—1255	102	No	GLN to PRO	591
dl 1013	1206—1226	21	No	GLU to ASP	595
dl 1014	1213—1221	9	VAL to ASP	No	595
dl 1015	1260—1289	30	No	No	595

[a] The nucleotides are numbered according to Deininger et al.[189]

mutations. Preliminary complementation experiments indicate that such mutants do, in fact, have mixed phenotypes.[587] These mutants are nonetheless valuable, because they present the first possibility at estimating the relative role of middle and small T antigens

and simply because they provide useful markers in mapping and localization experiments.

Dl 23 behaves as a transformation defective virus that retains a slight residual activity. Because it grows as well as or better than wild type and gives comparable yields of viral DNA,[198,199] its ts-a function is likely to be normal. Transformants might not be detected in standard soft agar assays. However, when the assays are left for longer periods of time, small colonies are detected with a frequency similar to those arising from wild type infection.[83,198–200] When transformation is assayed by focus formation, foci appear two to three times more slowly than with wild type virus. Transformants picked as foci will regrow in agar slowly and inefficiently. Dl 23 transformants will induce tumors, although again more slowly than for wild type.[83,199]

Griffin and co-workers have shown that the actin structure in dl 23 transformants is not disordered and that the transformants do not release large amounts of plasminogen activator.[199] Seif has reported that trypsin-like activity is released from dl 23 transformants, but that this increase is 25-fold reduced from that of wild type virus.[83] Cellular adhesion is measured by the release of cells from the monolayer was intermediate between normal cells and wild type transformants. The T antigen patterns show truncated large and middle T antigens and a normal-size small T.[199,200] The electrophoretic mobility shift for middle T results in an apparent molecular weight that is now in accord with that predicted from DNA sequencing. This suggests that at least part of the anomalous gel behavior arises from sequences deleted by the mutant. The middle T antigen has little, if any, kinase activity associated with it.[173,174] This is not unexpected since dl 23 deletes tyrosine 315, the probable acceptor site.[171] The small amount of phosphate incorporated into dl 23 middle T in vitro maps differently from that of wild type.[171] Amounts of 63- and 36-K T antigens comparable to wild type are seen for dl 23 and the other dl mutants of this type.[583]

The behavior of dl 23 indicates an important role for middle T in transformation. This mutant shows that an intact small T alone is not sufficient to induce full transformation. The experiments with dl 23 do not differentiate between two different possibilities: that middle T alone is necessary and sufficient for transformation, or that middle and small T are both required for full transformation. The basis for the residual transforming activity of dl 23 is also not clear. Although it is possible that the middle T retains a partial activity, it is also possible that the residual transformation is a consequence of the large and small T antigens.

Dl 1015, isolated by Magnusson and Berg, is also substantially defective in transformation.[105,570] Like dl 23, the transformed colonies that do arise have been reported to be smaller. Dl 1015 is impaired in growth and viral DNA synthesis so that the ts-a function appears to be altered for this mutant. This is confirmed by the failure of dl 23 to complement ts-a for transformation; complementation studies with hr-t mutants have not been reported.[570] Magnusson and co-workers have argued that dl 1015 is also defective in hr-t function based on studies on induction of cellular DNA synthesis by the mutant.[570] However, dl 1015 is normal for the middle T antigen-associated kinase reaction.[159,171] If dl 1015 is defective in middle T function, but normal for kinase activity, this suggests the interesting possibility that the dl 1015 kinase has lost the ability to phosphorylate the appropriate cellular acceptors.

Dl 8 represents an opposite kind of result from dl 23 and dl 1015. Dl 8 grows five- to tenfold less well than wild type, suggesting the possibility of a ts-a defect.[198,199] However, the mutant transforms efficiently and the resulting transformants seem even more altered in their phenotype than wild type transformants.[198–200] Foci are more dense than those arising with wild type; dl 8 transformants grow better in soft agar. Tumors can be observed after 1 week in experiments where comparable doses of wild type transformed cells require 3 or 4 weeks to produce a tumor.[199] High levels of middle T kinase activity

are associated with dl 8 immunoprecipitates.[173] Such results may imply that the deletion generating dl 8 may increase the activity of the transforming protein(s).

To try to alter middle T function while minimizing possible effects on large T, two laboratories have created and isolated point mutants that have a premature termination codon in the middle T frame.[584,588] These mutants produce a large T with a single amino acid substitution and a truncated middle T antigen. The viruses grow well, but are highly defective in transformation. The truncated middle T antigens are defective in the protein kinase reaction. The middle T of 1387T[584] which lacks the last 37 amino acids at the C-terminal end does not associate with membranes, but rather is found in cytoplasmic fractions.

6. The Roles of Polyoma Early Genes in Transformation

Complementation studies have shown that both the ts-a and hr-t genes are required for transformation.[29,30,32] The evidence that has been discussed in the preceding sections indicates that these two genes have different roles in transformation. The hr-t gene elicits the transformed phenotype. The ts-a gene is primarily needed to stabilize the association (by integration?) of the virus with the cell. Once that association has occurred the ts-a function is no longer required. This section will review briefly the results already discussed that lead to this conclusion and then will expand on some additional data that bear on this view.

The functions of the genes can be inferred from the transformation assays themselves. After *de novo* infection of nonpermissive cells, a high percentage of cells act as though they are transformed for several generations.[61] This abortive transformation indicates the presence of the function required to induce the transformed phenotype. Ts-a mutants induce abortive transformation normally; hr-t mutants do not.[32,119,120] Only a small percentage of abortively transformed cells become stably transformed. The appearance of stable transformants involves the integration and continued expression of the viral genome. At the nonpermissive temperature ts-a mutants do not give rise to stable transformants.[29,32,36,37,91] Hr-t mutants, on the other hand, are likely to integrate with normal frequency.[142] The same conclusion can be reached from a consideration of individual parameters of transformation, such as actin organization and membrane structure. As discussed in the previous sections, hr-t mutants are defective and ts-a mutants competent to induce these phenotypic changes.

One set of experiments deals with the question of how much of the viral genome is necessary to induce stable transformation in vitro or tumors in vivo. Experiments utilizing viral DNA have shown that the proximal portion of the early region, i.e., the hr-t coding sequences, are sufficient for tumor induction in vivo or transformation in vitro. Israel and co-workers have shown that viral DNA cleaved in the ts-a region is not only capable of inducing tumors in hamsters, but is even better than intact form I DNA.[202,203] Blotting experiments verify that an intact early region was not reassembled during the integration process.[204,205] As would be expected, the ts-a gene product was not observed in cell lines derived from the tumors, but the hr-t gene products were.[203] A restriction fragment of DNA lacking the distal ts-a sequences entirely (ending at the Eco site at 0 map units) is also very efficient in tumor induction. The high tumorigenicity of DNA lacking intact ts-a coding sequences has not been explained. Israel and co-workers have pointed out that the ts-a product may be involved in transplantation immunity.[203] "Supertransformation" of a non-SV40 tumor cell by SV40 *decreases* its tumorigenicity.[209] However, animals immunized with polyoma reject tumor cells lacking large T with the same efficiency as cells that retain large T.[578]

Many laboratories have now shown that restriction fragments that include the hr-t coding sequences but lack the distal ts-a coding sequences can transform cells in

culture.[133,136,206-208] For example, a DNA fragment from 65 to 100 m.u. transforms in vitro and induces tumors in vivo.[136] Such a fragment terminates about 60 nucleotides after the termination codon for middle T antigen. The transformed cells contain the hr-t products, but contain only a 34,000 dalton fragment of the large T antigen. Hassell and co-workers have obtained similar results with a Hind III fragment (45:1 map units). Several cell lines derived, using this fragment, were compared to wild type transformants for different parameters of transformation. The efficiency of plating in agar, plasminogen activator production, and alterations in actin structure were all comparable. This suggests that none of these parameters is controlled by the ts-a gene. When the sequence coding for the C-terminus of middle T are deleted, plasmids containing hr-t coding sequences lose most of their ability to transform cells.[576]

The ts-a product alone cannot induce the transformed state. Lania and co-workers have described experiments attempting to transform Rat-1 cells with an hr-t mutant.[142] Of 50 clones isolated nonselectively, two were found to contain an integrated hr-t viral genome. This is approximately the frequency expected for transformation by wild type virus and suggests that hr-t mutants are not defective in integration. The two clones express the large T antigen. The cell lines are nonetheless phenotypically normal and are unable to induce tumors. Although complementation studies with ts-a mutants were not reported, the large T expressed in the clones is presumed to be functional because virus can be rescued by cell fusion. The possibility that the transformed phenotype is suppressed by a cellular mutation was ruled out by showing that the clones could be transformed by wild type virus. These results show that the ts-a gene is not sufficient to induce the transformed state.

If the ts-a gene were required only transiently for transformation, then the continuous presence of the large T antigen should not be required, while the hr-t gene products should be continuously present. A wide variety of cells have now been surveyed, and such results have been obtained.[57,58,131,188,203,210] Both primary hr-t products as well as the 63- and 36-K T antigens are always found in transformed cells. The large T antigen is often absent. In a survey of 13 different mouse lines, only two showed intact large T antigen.[58] The appearance of an intact large T antigen seems to be much more common in transformed rat cells, although clones of rat cells lacking large T have also been observed.[58,131,203,210] Not only can cells be shown to lack intact large T, but they can also be shown to lack the ts-a function. In contrast to the ability of Py-3T3 cells to support the growth of hr-t mutants, the cells cannot complement the ts-a defect.[29] This observation probably explains why ts-a mutants were not isolated using the host range selection procedure. Loss of large T has also been observed in vivo.[565] When large T-containing cells were used to induce tumors, cells reisolated from such tumors did not express functional large T antigen.

The loss of the ts-a product from transformed cells and the difference in the frequency of this event in rat and mouse cells is likely to be related to the role of ts-a in replication and excision and to the degree of permissivity of the cell. If an active ts-a gene were available in permissive cells, then viral DNA replication *in situ* might result in productive infection. 3T3 cells transformed by ts-a remain transformed at 39°, but initiate a productive infection when shifted to the permissive temperature.[140,141] Rat cells that do not in any case support efficient growth of the virus would exert much less selective pressure. However, even in rat cells *in situ* replication or excision controlled by ts-a affects integration patterns and can even lead to phenotypic reversion.[129,130]

Inactivation of the ts-a function can occur in a number of ways. RNA transcripts from the distal portion of the early region, or viral DNA sequences themselves may be absent from the transformed cells.[131,205,211] Alternatively, large T can be inactivated. SV40-transformed CV1 cells can contain a full-length large T antigen that is incapable of

complementing tsA mutants.[212] The PyB10·D_2 mouse transformant contains large T antigen,[154] but does not complement ts-a mutants.[590] A third method, applied only to SV40 so far, is the in vitro deletion of sequences around the replication origin; such deleted DNAs rise to transformants of permissive cells that contain an active tsA gene product.[213-215] The notion is that in the absence of an intact origin there will not be selective pressure against the presence of an active ts-a/tsA gene.

Although the kinds of experiments described here argue against an obligatory role for ts-a in transformation, such experiments obviously do not mean that ts-a is not involved in transformation. Della Valle and co-workers compared the transforming ability of ts-a DNA give to that of wild type at both the permissive and nonpermissive temperatures.[133] Although transformants arise with ts-a DNA at both temperatures, ts-a DNA is 10- to 25-fold less efficient at the nonpermissive temperature. Furthermore, the clones derived from wild type DNA or ts-a DNA at the permissive temperature show the common pattern of tandem integrations. Of 20 clones derived using ts-a DNA at the nonpermissive temperature, only two showed tandem inserts, while the rest showed single-copy integration.

Many transformed lines lacking intact large T do, however, contain fragments of that protein.[131,136,188,206,210] The presence of N-terminal fragments of large T raises the question of whether some functions coded by the ts-a gene might be retained by such fragments.[127] Fragments of the SV40 A gene do retain some activities. No definite conclusion for polyoma virus is yet possible. At the very least, there is no absolute requirement for ts-a function. The transformed line Rat-53 lacks large T fragments in vivo, and fragments are not detected in in vitro RNA translations.[131,210] Hamster transformants lacking large T fragments appear to express fully the transformed phenotype.[578]

The possibility that the ts-a gene may play a continuing role in transformation under certain circumstances has been suggested by experiments asking what happens when ts-a transformants are shifted from the permissive to the nonpermissive temperature. For both polyoma and SV40 this kind of experiment has generated considerable controversy. The result most often obtained for polyoma virus is that the ts-a transformants remain transformed.[32,36,37,91,129,201] Such a finding supports the idea that the ts-a gene is not required to maintain the transformed phenotype. On the other hand, Kimura[216] has reported the opposite result, and a series of experiments by Cuzin and colleagues indicates that either result may be obtained, depending on the conditions used to select the transformants.[124-127] Transformation of growth-arrested cells is reported to yield a high proportion of nontemperature-sensitive transformants. Cells that are temperature-sensitive in the expression of the transformed phenotype are more frequently isolated when the transformation is carried out under conditions allowing the cells to grow actively.[125-127] Similarly, low multiplicity of infection is reported to give rise to a large proportion of temperature-sensitive ts-a transformants. Because experimental tests of the variables affecting the outcome of these kinds of experiments have also been made for SV40, a discussion of the possible explanations for the observed variability will be deferred until later. Although these observations show that the ts-a gene may be important in the expression of the transformed phenotype in some cells, it should be emphasized that this does not appear to be true for most transformed cells.[32,36,37,91,129,201]

7. The Origin Region of Polyoma Virus

Both early and late transcription begin in the same area of the circular genome that contains the site of initiation of DNA replication. The large T antigen functions in this region. It is therefore of interest to review what is known about these sequences. Such a review will indicate that there is considerable homology between SV40 and polyoma and will also allow some educated guesses about which sequences are likely to be important.

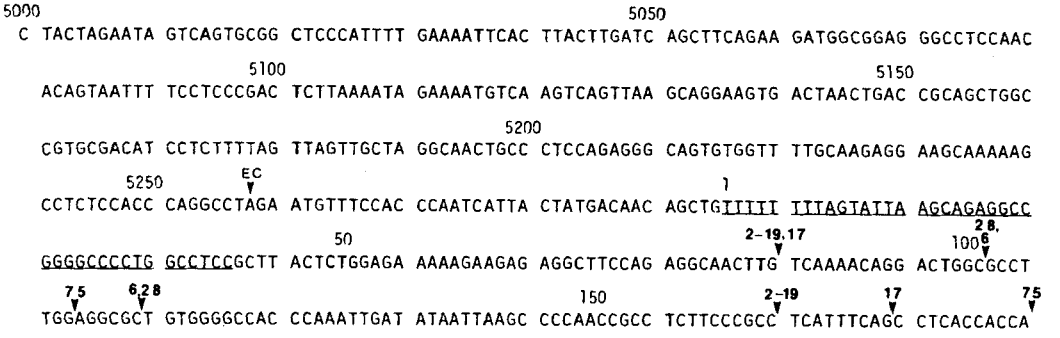

FIGURE 10. The origin region of polyoma virus. The sequence shown has been determined for strain 3.[113,189] The sequences deleted by mutants dl 6,[546] dl 28,[546] 17,[222] 2—19,[222] and 75[222] are indicated by the arrowheads. Nucleotide 5258, altered in many mutants capable of growing on embryonal carcinoma cells, is also indicated by an arrowhead. The region of similarity to the SV40 origin is underlined.[113,192]

FIGURE 11. Similarity of DNA sequence between SV40 and polyoma virus at the origin of DNA replication. The SV40 sequence is that reported by Buchman, Burnett, and Berg[25] based on the results of Reddy and colleagues[235] and Fiers and co-workers.[236] The polyoma sequences are those of Soeda and colleagues[192] (Py*) for the A2 strain or of Deininger and co-workers[189] (Py**) for wild type strain 3.

Figure 10 indicates the origin region of polyoma virus. The origin of DNA replication is around the HpaII 3/5 junction.[153,217] This region includes a stretch of 43 base pairs that show considerable homology between SV40 and polyoma; 34/43 base pairs are the same (Figure 11).[113,192] This set of sequences is retained in all viable deletion mutants. It is also found in the defective viruses that have been studied.[218–220] SV40 and polyoma differ significantly in the distance between the origin region and the initiation codon for the T antigens. Where SV40 has a stretch of only 28 nucleotides, polyoma virus has over 100. The distance can vary between strains; the A3 strain has a deletion of 11 base pairs compared to the A2 strain.[153] Upstream from the initiation codon (nucleotides 188 to 190) and the 5′ ends of the mRNA around 155 and 165, there is a putative "Hogness-Goldberg" box at nucleotides 135 to 143 that may be involved in initiation of RNA transcription. A ribosome binding site has been suggested at nucleotides 157 to 161 (C-T-T-C-C). On the late side of the origin there are 266 nucleotides before the intiation codon for VP2. As for SV40, this region contains several sequences of the type G-G-G-X-G-G.[153,189]

Several laboratories have mutagenized this area to study the effects of mutation on DNA replication and early gene expression.[105,106,198,221,222] Similar strategies have usually been employed. The Hae II and Bgl I sites are approximately equally distant between the T antigen initiation codon and the origin region. Viable deletion mutants have been isolated spanning these restriction sites. Mutants in this region are also discovered when DNase I is used to generate random deletions.[105] Of the mutants that have been

sequenced, overlapping deletions extend from nucleotide 86 to nucleotide 185 just before the initiation codon.

Most of the mutants are not affected in growth and transformation properties, but mutants such as dl 17[221] and dl 6[198] have altered plaque morphology. Dl 75, which has a deletion extending to within three nucleotides of the initiation codon, grows much less well than wild type virus and shows a fourfold reduction in viral DNA synthesis.[222] However, this defect can be overcome by coinfection with wild type virus, suggesting that the mutation affects the production of T antigen rather than the DNA sequences recognized by the T antigens. Direct measurement shows that dl 75 is markedly reduced in T antigen production, although no substantial difference is observed in the levels of RNA. The defect is presumably in the translation, but this has not been tested directly. Mutants dl 17 and 2—19 are somewhat altered in viral DNA replication (no more than twofold). Since this defect is not overcome by coinfection, the sequences altered in these mutants (nucleotides 86 to 109) may be involved in specifying the origin.[222] This region can be narrowed somewhat, because mutants dl 6 and 28 delete nucleotides 100 to 112[546,591] without affecting the growth of the virus. Both dl 17 and dl 75 delete the region coding for the 5′ ends of the early messenger RNA, while dl 17, dl 75, and 2—19 all delete the Hogness-Goldberg box. Since these mutants express early function, these sites cannot be absolutely required, and alternative sites may be used.

On the late side of the origin there are over 250 nucleotides preceding the initiation codon for the capsid protein VP2. Studies using teratocarcinoma cells indicate that these sequences are also involved in controlling early expression. Undifferentiated F9 or PCC4 cells do not support the growth of polyoma or allow the expression of the early proteins of SV40 or polyoma.[223-225] There is apparently a cellular component to the restriction since PCC4 are nonpermissive in a temperature-sensitive way even for wild type virus.[226] There has been some disagreement over the nature of the defect. F9 cells infected by SV40 are reported to express low levels of unspliced message.[225] However, Fujimura and colleagues have observed spliced early message in polyoma-infected F9 cells,[558] and Vasseur and co-workers have reported that the mRNA in PCC4 cells is spliced.[227] Differentiation makes the cells permissive for early expression.[228,229,558] Several laboratories have isolated polyoma mutants that will grow on either undifferentiated F9 or PCC4 cells.[227,230-234] All of these mutants show alterations between the late initiation codon and the origin. Even a single base change at nucleotide 5258 (A-T to G-C) is sufficient to allow permissivity.[232] This same base change has been independently observed in three different laboratories.[232-234] In some instances it is accompanied by duplication of other sequences in that region. Sequence duplication is a common theme in many of these mutants. In one pair of variants, for example, there is a deletion of sequences near the origin (20 nucleotides from the stretch of 8 Ts of PC204) and a duplication of sequences 100 to 200 nucleotides on the late side of the origin. Sekikawa and Levine have isolated variants containing the point mutation and 33 or 67 base pair tandem repeats in the same region.[233] Such changes have been discussed in terms of a "tRNA-like" structure that can be drawn for the viral DNA in that region.[234] The importance of host components is underlined by the observation that variants isolated to grow on PCC4 cells do not grow on F9,[231] and vice versa.[233]

IV. THE EARLY REGION OF SV40

A. The SV40 Coding Sequences

Two laboratories have completely sequenced the SV40 genome.[235,236] As for polyoma virus, individual papers have dealt with the sequences of the early region.[237-246] The numbering system used here is that described by Buchman, Burnett, and Berg,[25]

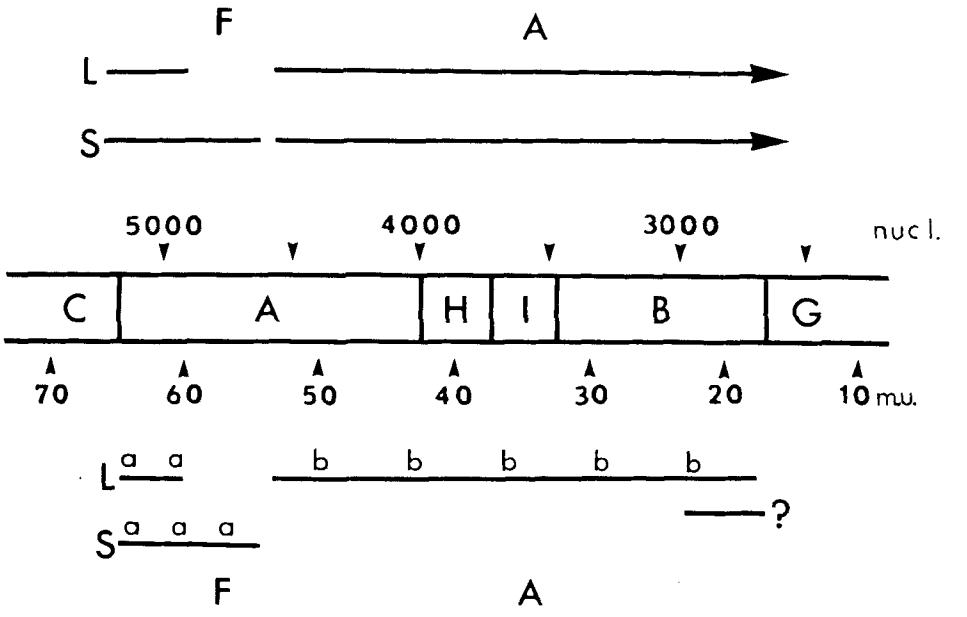

FIGURE 12. The arrangement of the SV40 early region. The SV40 early region is shown with the Hind II + III fragments indicated. The large T (L) and the small T (S) messenger RNAs are shown at the top, with gaps left to indicate the introns. The F gene mutations map within the intron for the large T mRNA.[31,44,45,102] The tsA mutants largely map to the Hind II + III H and I fragments.[258] The arrangement of coding sequences is shown below. Both large and small T antigens share a common N-terminal sequence of 82 amino acids. The last 92 amino acids of small T are encoded directly; the last 626 amino acids of large T are read downstream of the small T coding sequences because of messenger splicing. The heavy line indicated with the ? indicates a region containing an additional open reading frame which could be used to encode a third early protein.

corrected for an additional 17 nucleotides more recently discovered.[247] Figure 12 shows the general arrangement of the early region.

The initiation codon for the two known early proteins is found at nucleotides 5164 to 5162. Different 5′ ends of the early messages are found near nucleotides 5225, 5230, and 5234.[248-250,292] Upstream there is a form of the Hogness-Goldberg box from nucleotides 21 to 14. The polyadenylation signal AAUAAA is encoded from nucleotide 2609 to 2604, and the 3′ end of the early messages maps to nucleotide 2587.[248] Splicing of the large T RNA removes an intron from nucleotide 4919 to 4574 so that a termination codon is read from nucleotides 2693 to 2691. The termination codon for small T occurs at nucleotides 4641 to 4639, just before the intron in the small T gene (nucleotides 4639 to 4574). Alwine and Khoury have shown that the ratio of splices for small and large T antigen varies with temperature.[251]

There is an open reading frame in the distal portion of the early region distinct from that used for large T antigen.[235,236] There is sufficient information to code for a protein of about 11 K. Alternatively, this reading frame could be used with sequences coding for large T because of RNA splicing or a shift in reading frame occurring at the translational level. Such a hypothetical protein, differing in its C-terminal region from large T, has been termed "T★".[252] S1 mapping experiments have suggested the possibility of a third splice,[252] but Alwine and Khoury have suggested that a small RNA present late in infection gives rise to an additional S1 cleavage.[253] No protein so far is known to have sequences encoded by the open reading frame.

The results of RNA mapping and DNA sequencing can be combined with the results of

Table 5
A GENE MUTANTS

	Nucleotide(s) altered[a]	Bases deleted or changed	Amino acids changed	Ref.
Point mutants				
tsA 239	3639	G to C	TRYP to CYS	114
tsA 241	3639	G to C	TRYP to CYS	114
tsA 255	3552	G to C	TRYP to CYS	114
tsA 209	3538	C to T	PRO to LEU	114
tsA 1642	3461	C to T	PRO to SER	562
Deletion mutants				
tsA 1499	2932—2818	81	PRO added	98
dl 2194[b]	2903—2845	57	ASN added	104
dl 1263	2830—2798	33	PRO to THR	541
dl 1265	2720—2682	39	Nine deleted, 4 new C terminal amino acids	541

[a] Nucleotides numbered according to Buchman, Burnett, and Berg[25] with 17 additional nucleotides observed by van Heuverswyn and colleagues.[247]

[b] Dl 2194 is derived from dl 2122[31] which has a 234 bp deletion from nucleotides 4882 to 4649.

peptide mapping of the early proteins to describe the arrangement of coding sequences in the early region. The large T antigen (A gene product) and small T antigen (F gene product) share a common N-terminal sequence of 82 amino acids. This homology has been confirmed by peptide mapping.[254-256] The last 92 amino acids of small T are read directly, while those sequences are removed from the large T message by splicing. All of the viable deletion mutants (0.54 to 0.59) affecting small T map in this region. The remaining 626 amino acids of the large T antigen are coded by sequences distal to the small T termination codon.

B. Gene A of SV40

Both temperature-sensitive and nonconditional mutants of the A gene of SV40 have been studied. Temperature-sensitive mutants were isolated first,[39,40] and additional mutants have been isolated in several laboratories.[92-98] Such mutants are usually coordinately temperature-sensitive for productive infection and stable transformation, although the relative degree to which each process is affected can vary from mutant to mutant. TsA 1642 requires special comment because it differs from other tsA mutants in some respects and because its phenotype is similar to that of the hr-t mutants of polyoma.[562] The mutation is a single base change resulting in a proline to serine change in large T. Like the hr-t mutants, and unlike other tsA mutants, tsA 1642 synthesizes substantial amounts of viral DNA and capsid proteins at the nonpermissive temperature. The mutant is highly defective in transformation at 40.5°; its ability to transform at 33° depends on the cell type used in the assay. There are also mutants in which growth and transformation are not affected in a parallel manner. The mutant of Ishikawa and Aizawa[94] is temperature-sensitive for growth but not for transformation, like the P155 mutant of polyoma virus.[108] TsA 1499 is temperature-sensitive for growth, but cold-sensitive for transformation.[98,547] Many of the mutants have been mapped;[257,561,562] they largely map in a cluster in the middle of the early region between 43 and 32 map units. This region codes only for the large T antigen. Some of these have been sequenced (Table 5).[114,562] TsA 1499 contains a deletion at 21 map units, closer to the C-terminus of large T.[98]

In addition to the tsA mutants, a variety of other A gene mutants are available. There is a variety of deletion mutants.[101-104,259] Some of the mutants are viable, while others are defective and must be studied with helper virus or as cloned DNAs. Of particular interest is a series of deletion mutants that have been constructed by Pipas and colleagues; these span the entire A gene.[101] Second site revertants of replication origin mutants have been isolated which map between 43 and 50 map units.[260,261] This suggests that these sequences, which are upstream from the sites of tsA mutations, are important for the recognition of the origin by large T antigen.

The properties of the A gene have been deduced from four kinds of experiments. The first is comparison of the infections by tsA mutants at the permissive and nonpermissive temperatures. The second is the study of the phenotype of tsA transformants at the permissive and nonpermissive temperature. A third kind of experiment is the microinjection of DNA coding for the A gene or of the A gene product itself. The fourth sort of experiment involves study of different adenovirus-SV40 hybrids. These recombinant viruses contain sequences encoding differing portions of the A gene.[549] This makes it possible to study the biological effects of particular portions of the A gene. For example, the D2 protein from Ad2 + D2 contains the large T antigen sequences distal to 54 map units. It has been used to study both the DNA binding and enzymatic properties of large T antigen.[185,186,391,480] The last two kinds of experiments have shown that fragments of the A gene retain the ability to carry out some A functions. Such observations suggest the idea that different domains of the A gene may be important in different functions.

The A gene acts in a variety of ways. Its involvement in the initiation of viral DNA synthesis is perhaps the most important to the virus. The A gene can also regulate both early and late viral RNA transcription. It appears also to be involved in the induction of cellular DNA and RNA synthesis. The induction of cellular enzymes can be controlled by the A gene. The distal portion of the A gene provides a helper function for the efficient growth of human adenovirus in monkey cells. The appearance of the tumor-specific transplantation antigen (TSTA) parallels the appearance of the A gene in infected cells. There is considerable evidence that the A gene product itself is directly involved in the process of tumor immunity. Each of these subjects will now be discussed in more detail to illustrate the pleiotropic action of the A gene.

TsA mutants are defective in viral DNA replication.[40,262,275] Pulse-chase and temperature-shift experiments show that the defect is in initiation. Molecules that have initiated replication no longer require the A gene product to complete the process. Microinjection of SV80 large T antigen can complement the tsA defect.[263] When viral DNA is microinjected, only DNA coding intact A gene can complement the replication defect.[264] Since viral DNA replication does not occur at the high temperature, late viral products are not made.

The transcription of both early and late RNA is affected by the action of the A gene. Both the rate of synthesis and the amount of early RNA are greater in cells infected by tsA mutants at the nonpermissive temperature.[265,266,268,272] In tsA-infected cells shifted from the permissive to the nonpermissive temperature, for example, the synthesis of early RNA can be increased 15-fold.[266] Such overproduction of early RNA results in the overproduction of the large T antigen.[265] Experiments with inhibitors of DNA synthesis show that the RNA effect is not caused by the block in DNA replication per se.[268,278] Studies on viral transcription complexes extracted from cells confirm this.[272] These data suggest that the A protein controls its own transcription by negative feedback.[265,266] This has recently been demonstrated in an in vitro transcription system with large T or D2 protein, an analog of SV40 large T.[269,567] Some additional support for this model comes from experiments showing that inhibitors of protein synthesis cause overproduction of early RNA.[274] The interpretation of these inhibitor experiments is complicated by the

observation that they can cause an increase in RNA production, even in tsA infections at the nonpermissive temperature. The situation for late transcription is more complicated. The production of large amounts of late message appears to require an active A gene.[268,270-272] The appearance of late mRNA has been reported to depend on an active A gene.[271,273] Such results would imply that large T is a positive control element in late transcription. (See Parker and Stark[273] for a discussion of these results.) However, late message has been observed in tsA infections at the nonpermissive temperature and/or in the presence of inhibitors of protein synthesis so that the dependence is not absolute.[274,566] Temperature-shift experiments have given differing results. Usually a shift from permissive to nonpermissive temperature late in infection has little or no effect on late transcription even though DNA replication quickly stops.[268,270,272,320] In AGMK cells, on the other hand, a dramatic decrease in late RNA is observed.[320] Alwine and Khoury have suggested that such differences may arise from the nature of the interaction of the large T antigen with host cell factors.[320]

Although the data are somewhat conflicting, it appears that the A gene is involved in the induction of cellular DNA synthesis. Microinjection of the D2 protein stimulates cellular DNA synthesis.[283] Since the D2 protein lacks the N-terminal sequences of large T antigen, this suggests that the N-terminus is not required. Based on DNA microinjection experiments showing that DNA synthesis can be induced by a fragment ending at 32 map units, the C-terminal region appears not to be needed either.[264,282] Microinjection of cloned fragments suggest that the important sequences lie between 51 and 42 map units.[557] The number of apparent initiation points for cellular DNA synthesis is increased when transformed cells are grown in complete medium.[284,321] For tsA transformants, the frequency of initiation points is increased at the permissive temperature, but is like the untransformed controls at the nonpermissive temperature.[284] In labeling experiments to measure cell DNA synthesis, tsA mutants can be either somewhat[275] or largely[281] deficient at the nonpermissive temperature. Mutants that have a normal A gene, but lacking the small T, induce cellular DNA synthesis.[102,278,279,322] The basis for uncertainty is the repeated observation that tsA mutants induce cellular DNA synthesis at the nonpermissive temperature.[39,275,278,279,322] In the experiments of Hiscott and Defendi, tsA mutants could induce an initial round of DNA replication but not subsequent rounds.[279] This is in apparent disagreement with the observation that tsA mutants induce abortive transformation,[32] since abortive transformation requires multiple rounds of replication. Two sorts of explanations can be entertained to resolve some of the apparent discrepancies. The first is variability in the cells and protocols used. In the experiments of Hiscott and Defendi, for example, the cells are held in low serum prior to the start of the experiment; such a protocol differs markedly from that used for abortive transformation. The response may depend on cell type. AGMK and CV-1 cells respond differently to tsA mutants at the nonpermissive temperature.[278] Even wild type SV40 does not stimulate cell DNA synthesis in BSC-1 cells.[323] The second explanation says that the function required for viral and cell DNA replication are the same, but the functional requirements for viral replication (origin recognition?) are more stringent and more easily perturbed. Mutants are then detected that are defective in viral replication, but only slightly or not at all affected in the ability to induce cell DNA replication. (See Martin[26] for additional discussion of this point).

There is evidence connecting the A gene with changes in cellular RNA transcription. The best characterized system involves the expression of mouse rRNA in human-mouse hybrid cells.[285-287] In these hybrids the mouse ribosomal genes are not expressed; SV40 infection causes their expression. The activation of these genes is temperature-sensitive for tsA 58, but unaltered by mutations in the F gene. Adeno-SV40 hybrid viruses contain and express different portions of the A gene sequences.[297-300] Those viruses containing

the distal half of the A gene can cause this transcriptional activation.[287] Partially purified preparations of T antigen could stimulate rRNA synthesis in isolated nuclei.[288,289] It is worth noting that the sequences required for the induction of cellular DNA synthesis and the induction of cellular RNA synthesis appear to be different.[557] Postel and Levine have shown that the appearance of a particular form of thymidine kinase is temperature-sensitive in infections by a variety of tsA mutants.[291] Curiously, one of the tsA mutants out of seven was not temperature-sensitive in induction. Thymidine kinase is one of a collection of enzymes induced by infection with either polyoma virus or SV40.[324] Such induction is generally thought to occur at the level of transcription; in the case of dihydrofolate reductase induction by polyoma virus, this has been directly confirmed.[290]

One intriguing cellular effect that appears to be mediated by large T antigen is the production of factor(s) that can cause normal cells to grow in agar.[292] This work has been stimulated by the observation that murine sarcoma virus transformants produce a sarcoma growth factor (SGF) that can induce cells to grow in agar.[294] SGF binds to the epidermal growth factor (EGF) receptor. It is not clear that the production of the factor is the cause, rather than the result, of altered growth regulation. Cells that have lost the EGF receptor and the ability to respond to SGF can be transformed efficiently by Kirsten sarcoma virus.[325] For SV40, the production of the factor(s) depends on the A gene, and deletion mutants lacking small T can give rise to transformants producing the factor. The amount of factor secreted is correlated to the extent to which the transformant can grow without anchorage. The ability of the factor to act on transformed cells may be modulated by small T.

It has long been known that coinfection with SV40 helps human adenoviruses to grow efficiently in monkey cells.[295,296] Microinjection of large T antigen of the D2 hybrid protein provides helper activity.[283] Kimura has reported that tsA mutants can be impaired in helper ability at the nonpermissive temperature.[545] Deletion mutant 1265 that has a deletion at the C-terminal end of large T antigen is defective in helper function.[259] Dl 1263, which has a deletion near the C-terminus, may be somewhat deficient.[104,259] Adenovirus-SV40 hybrid viruses that contain only the information from the distal end of the early region (up to approximately 22 map units) are capable of efficient growth in monkey cells.[297-300] Some evidence suggests that the A gene product can replace the 72-K DNA binding protein of adenovirus either directly or indirectly.[302-304,326] Goldman and co-workers have argued in favor of a direct interaction.[303,304] The adenovirus helper function represents a clear example of a function of the A gene that can be genetically separated from the A gene function in SV40 replication or transformation. Dl 1265 is defective in helper function, but grows and transforms normally,[259] and tsA mutants can help adenovirus under conditions where SV40 DNA replication is blocked.[301]

Most studies on the characteristics associated with the transformed phenotype have been carried out on transformed cells and not after *de novo* infection. One exception to this is the study of actin architecture. Like the ts-a mutants of polyoma virus, the tsA mutants of SV40 are capable of disrupting the cellular "actin cables" even at the nonpermissive temperature.[279]

Many investigators have tried to assess the role of the A gene in transformation by asking what happens when tsA transformants are shifted from the permissive to the nonpermissive temperature. From a naive point of view, if the A gene is continuously required for expression of the transformed phenotype, cell transformation should be temperature-sensitive. If the A gene is not continuously required, then the cell transformation should not be temperature-sensitive. The Rous sarcoma virus system provided an encouraging example — mutants temperature-sensitive in $pp60^{src}$ are temperature-sensitive in parameters of the transformed phenotype.[327,328] For SV40, as

for polyoma, either result or both results can be obtained. Many laboratories have observed that SV40-tsA-transformants are temperature-sensitive for the expression of the transformed phenotype.[32,329-336] The different parameters of transformation are usually expressed in a temperature-dependent way. High levels of 2-deoxyglucose transport, plasminogen activator secretion, and loss of "actin cables" are observed at low, but not high, temperatures. The frequency with which temperature-sensitive clones are derived with tsA mutants of SV40 suggests that the A gene may often have a continuing role in transformation. However, other laboratories have observed temperature-independent phenotypes,[32,93,336-339] although this result is less common for SV40 than for polyoma virus. Many laboratories observe both results depending on the cell systems and experimental procedures, and a great deal of effort has been devoted to determining the basis for the outcome of this kind of experiment.[32,124-127,342-348] After a discussion of the observations, some of the possible explanations for the result will be considered. (See also Martin[26] and Fluck et al.[32] for additional discussion.)

The earliest results suggested that the method used to isolate the transformants was responsible for the result: transformants isolated as foci were sensitive to temperature shifts (N-type transformants) and those isolated in agar(A-type) were not.[124] It quickly became apparent that the growth state of the rat cell had a greater affect.[125,343] Cells that are actively growing early in transformation by tsA SV40 were more likely to be temperature-sensitive for transformation than quiescent cells.[126,342,343] The outcome also seemed to depend on the multiplicity of infection. High multiplicity infections more often resulted in temperature-independent transformants, while low multiplicity (around 1 to 10 PFU per cell) results in temperature-dependent transformants. In rat cells, the appearance of A-type transformants required that the virus have an active F gene; double-mutants in both A and F genes did not give rise to A types.[343] Different results are found in different kinds of cells. Hamster cells have predominantly given rise to N-type transformants.[277,341,345,435] However, A-type transformants are also observed, and their appearance does not require small T antigen.[346] Differences are observed even in different cell lines derived from the same species. Thus, mouse 3T3 cells gave temperature-independent lines, but embryo fibroblasts were temperature-dependent when transformed by tsA.[339] With all of these different variables it becomes very difficult to integrate the results from different laboratories into any one single model.

A variety of explanations can be considered for the appearance of a temperature-dependent or independent phenotype. The first involve control of the expression of early proteins. A-type transformants could arise from the overproduction of mutant T antigen at the high temperature; excess amounts of mutant protein might show enough activity to cause transformation at the nonpermissive temperature. In at least two instances, nontemperature sensitive transformants appear to overproduce T antigens.[333,334] An obverse argument would say that large T antigen plays no direct role in maintenance of transformation, but that a temperature-sensitive A gene product controls expression of F gene product or an undiscovered third protein. Temperature-sensitive expression of T antigens in tsA transformants has been observed in some instances.[347] Certainly neither of these explanations is universally applicable. A-type transformants do not always appear to overproduce T antigens nor does the expression of T antigen appear to be temperature-sensitive in all N-types.[32,334,343] The positive role for the A gene in the control of T antigen expression postulated in the latter case would be in conflict with its observed negative feedback role in early transcription discussed earlier.[265,266,268,272] A second class of explanations concerns the nature of the integration of the viral genome. Seif and Martin have proposed the possibility that N-type transformants cause transformation through the continuous action of viral proteins while the A-types could arise through effects on cellular growth controls by integration into control regions.[343] Some

information is now available on the integration sites in N- and A-type transformants.[312,348] Chepelinsky and co-workers examined the integration patterns in both rat and hamster transformants of both N- and A-types.[348] In hamster transformants grown at 41°, no free viral DNA is observed, so that growth at the nonpermissive temperature does not result from the presence of free genomes. Between one and three integration sites were observed for each type with little indication that the number of integrations was type specific. In rat cells, however, the N-type transformants tended to have only a single integration site, while the A-type transformants usually had multiple sites. Partial tandem repetitions were observed in some lines of each type. Three of their A-type rat transformants showed similar blotting patterns when "no-cut" enzymes were used. However, the blotting patterns showed no consistent differences in the integration sites for N- and A-type. Mougneau and co-workers studied the integration patterns in rat-transformed lines.[312] They too observed that N-type transformants usually showed only a single insertion, while the A-types usually showed multiple insertions. Southern blotting on three independent lines indicated that they had apparently identical integration sites! One of the three lines is an N-type showing temperature-dependent control of T antigen expression, and the other two are A-types having temperature-independent expression!! It is possible that the integration affects the control sequences for early expression; DNA sequencing will be needed to determine the integration sites more precisely. The third class of explanation suggests that cellular mutations can affect the expression of the transformed state. Wild type transformants can also be temperature-sensitive; in some cell lines, the frequency of temperature-sensitive wild type transformants is as high as those of tsA.[32] Chemical transformants are also often temperature-sensitive.[349] Renger and Basilico showed that a temperature-sensitive cellular mutation provided the basis for selection of a phenotypic revertant of an SV40 transformant.[350] It is possible that the A gene could be responsible for causing cellular mutations. Theile and co-workers have shown that wild type SV40, but not tsA 209, induces an increase in the frequency of mutation leading to resistance to 8-azaguanine.[351]

New antigens are observed in cells after infection or transformation by SV40. One of these, U antigen, is defined by nuclear membrane and perinuclear staining.[298,352] U antigen was originally discovered in adeno-SV40 hybrid virus-infected human and monkey cells. The U antigen can be differentiated from T antigen by its heat stability,[298,352] but it apparently represents some subset of viral large T.[352] Experiments with adeno-SV40 hybrid viruses indicate that the C-terminal portion of large T (28 to 11 map units) contains the U determinants. No function has been ascribed to the U antigen. A very important antigen is the tumor-specific transplantation antigen (TSTA or TrAg). This antigen is important in tumor rejection in vivo. It will be discussed in some detail below. Purified large T antigen is sufficient to induce tumor immunity.[353,3354]

1. The A Gene Product: Large T Antigen

The large T antigen is the A gene product. In most experiments large T is detected using antiserum from tumor-bearing animals.[49,309-311,313-315,579] Monoclonal antibodies have been obtained against large T antigen.[316-318,556] Antibodies have also been obtained using synthetic peptides of sequences from large T.[319] These two reagents permit the detection of different domains of the protein. The antibodies against the peptides recognize either the N-terminus or C-terminus of large T.[319] Interestingly, the large T, but not the small T, is recognized by the antibody against the N-terminus even though they have the same sequence. Different monoclonal antibodies recognize different portions of large T.[362,556] Some also react with cellular proteins.[551,556] One of these is a 68-K cellular protein found in the nucleus.[551]

The calculated molecular weight of large T is 81,632.[25] Electrophoresis in SDS gives

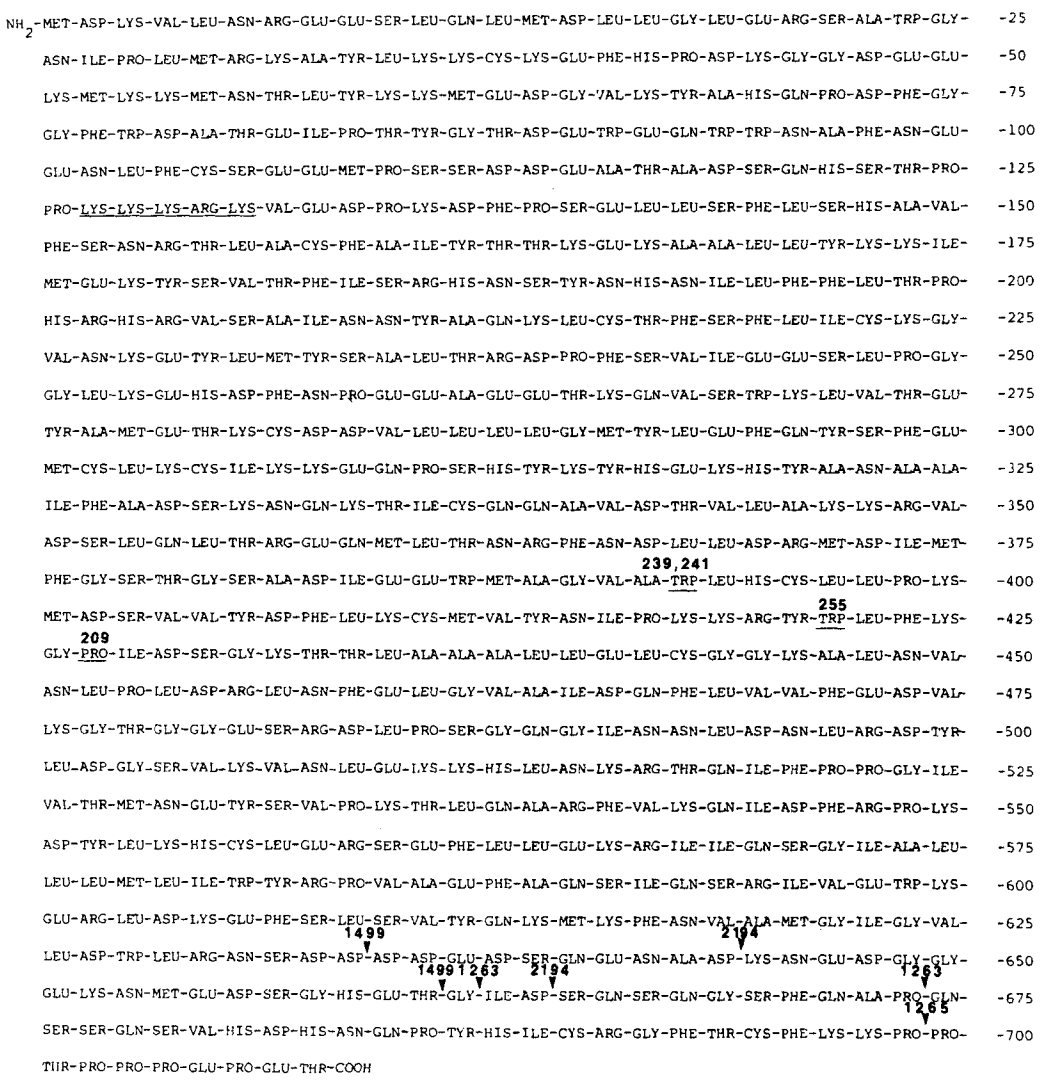

FIGURE 13. The predicted amino acid sequence of SV40 large T antigen. The amino acid sequence shown is predicted by Buchman, Burnett, and Berg,[25] based on the results of Reddy and colleagues[235] and Fiers and coworkers.[236] The amino acids shown to be altered by tsA mutations[114] are underlined. The boundaries of the deletions of tsA 1499,[99] dl 2194,[104] dl 1263,[541] and dl 1265[541] are indicated by the arrowheads. The deletion in tsA 1499 results in the addition of a proline residue, while the deletion of dl 2194 results in a new asparagine. The deletion in dl 1265 results in nine new C-terminal amino acids, since the usual termination codon is deleted.

higher values, but random coil chromatography on 6 *M* guanidine hydrochloride gives a value in agreement with the predicted molecular weight. Studies on dl 1263 and dl 1265 suggest that the C-terminal region contributes to the anomalous gel behavior.[355] Unlike the polyoma large T antigen, the SV40 product translated in vitro is similar or identical in size to the in vivo product.[357] Of the large T antigen sequence, the first 82 amino acids should be common to small T antigen as well.[235,236] Peptide mapping confirms this homology.[254-256]

The amino acid sequence of large T antigen is shown in Figure 13. It is instructive to compare the sequence to that of the polyoma large T as well as to the human papovavirus BK. The comparison to BK is useful because BK can complement the defect of tsA

mutants.[550] Structural changes between those two, therefore, are likely to be nonessential for function. Regions that are conserved are more likely to be essential. The large T antigens of polyoma and SV40 show considerable homology if the sequences are aligned to leave a gap in the SV40 sequence in the region that codes for the C-terminal portion of middle T in the polyoma sequence.[111,153] To put it another way, polyoma large T looks like SV40 large T with an additional insert. Deininger and co-workers have commented on the comparison to BK.[111] In general residues that differ between SV40 and BK also tend to be different in polyoma virus. Of 22 amino acid differences between BK and SV40, but conserved between SV40 and polyoma, i.e., positions of potential importance, 14 are conservative (e.g., LYS to ARG) changes in BK. The sequences coding for much of the C-terminus of middle and an internal portion of the large T antigen for polyoma virus are not obviously represented in SV40.[153] However, at the amino acid level, there is an arrangement of eight dicarboxylic acid amino acids including a stretch of six that is the same in polyoma middle T and SV40 large T.[113] Also the distal portion of large T of SV40 and the middle T of polyoma virus are relatively proline-rich.

Most of the large T antigen is located in the nucleus. This was shown quite early by indirect immunofluorescence[310,311] and immunoelectron microscopy.[358] D'Alisa and Gershey have shown that large T binds to muntjak chromosomes.[359,360] The banding pattern is the same as observed with Giemsa or quinacrine, Subcellular fractionation showed the presence of large T antigen in nuclear fractions.[359] Recently an SV40-adenovirus hybrid has been observed which does not concentrate its large T antigen in the nucleus.[361,362]

Since large T antigen is a DNA- and chromatin-binding protein, its presence in the nucleus is not surprising. More recently it has become apparent that a small amount of the large T antigen is associated with the plasma membrane. The impetus for discovering and characterizing this small amount was provided by experiments involving transplant immunity. A membrane T antigen was suggested with the observation of Deppert and Walter that HeLa cells infected by adenovirus-SV40 hybrid viruses had SV40 specific antigens in the plasma membranes.[363] The difficulty in analyzing large T in membrane fractions comes from two sources. First, there is the problem of contamination by a small amount of nuclear large T. Second, an immunologic demonstration of surface T antigen can be complicated by S antigens; these are surface antigens detected in transformed cells that are not virus-coded.[364-366]

Purified preparations of plasma membrane have large T antigen associated with them.[367-369] Deppert and Henning have been able to label large T antigen on the surface of SV80 cells using ^{125}I and lactoperoxidase.[370] If the cells are mildly trypsinized prior to labeling, then the yield of ^{125}I-large T is very much reduced. There is little detailed information about the large T antigen associated with the membrane fractions; the peptide maps of the nuclear and membrane forms of large T have not been compared. Schmidt-Ullrich and co-workers have reported that the large T associated with the membrane has a lower isoelectric point.[369] They have previously suggested that the large T antigen in the membrane can be labeled with glucosamine.[371] However, Soule and co-workers have been unable to confirm this in another cell system.[368] (If the distal open reading frame were used either through a small splice or some kind of suppressor activity, the resultant T antigen [T★] would be predicted to be less acidic than large T and not more acidic.)

Considerable immunochemical evidence supports the notion that a fraction of large T antigen is present on the cell surface.[372-376] Direct surface immunofluorescence on living cells that have not been treated with EDTA or mechanically removed from a monolayer has only been successful for mKSA cells grown in suspension.[375] Soule and co-workers have demonstrated surface fluorescence on both

transformed and infected cells treated with EDTA.[368] Deppert and colleagues have shown surface fluorescence on formaldehyde-fixed cells.[370,375,376] In temperature-shift experiments with a tsA transformant, there is coordinate loss or reappearance of both nuclear and surface T antigen staining.[374] Surface T antigen can also be demonstrated in similar assays using ^{125}I-protein A[368,375] or the binding of *Staphylococcus aureus* itself.[376] There is immunologic evidence indicating that the antigenic determinants recognized in large T on the cell surface and those in nuclear T can be different. There are antisera that will detect T on the cell surface, but will not detect large T in the nucleus of acetone-fixed cells.[368] The difference may result from acetone-sensitivity of nuclear T, since the same serum also precipitates nuclear large T from extracts of whole cells. Deppert and co-workers have made similar observations; the surface fluorescence can be demonstrated with antiserum against gel-purified SDS-denatured large T, but not with conventional antitumor serum.[376] When the fixed cells were lightly treated with trypsin, then surface fluorescence could be detected with both sera. This suggests that the sites usually detected by the antitumor sera are cryptic in the plasma membrane until exposed by manipulation of the cell surface.

The large T antigen can self-associate. In 1965 Gilden and colleagues observed that SV40 T antigen has an apparent sedimentation constant of 18S.[377] In 1969 Potter and co-workers resolved three forms of T antigen with apparent molecular weights of 56 to 75 K, 110 to 120 K, and 280 to 300 K on sucrose gradients.[378] Such experiments showed that the large T antigen could be found in higher-order forms. It is now clear that the large T can associate with itself and with cellular proteins as well. Experiments using the purified T antigen of SV80 have suggested the existence of three forms: a 5.5S monomeric form, a dimeric 7S form, and a 15.5S tetramer.[187] These correspond to the forms usually observed in cell extracts except that the 7S form may not be clearly resolved from the 5S monomer.[379-381] Interest in the self-association reaction was heightened by the observations of Kuchino and Yamaguchi[382] and Osborn and Weber,[383] that the large T antigen of tsA mutants does not undergo this association in infections at the non-permissive temperature. Pulse-chase experiments have been used to follow the conversion of large T antigen to the associated forms.[381] By the end of a 2-hr chase virtually all the large T antigen is converted to the larger form. In temperature-shift, pulse-chase experiments the associated forms of the tsA large T antigen assembled at the permissive temperature do not appear to be markedly unstable. Shift-down experiments show that antigen made at the nonpermissive temperature is still capable of carrying out the association after a shift to the permissive temperature. Although there is some disagreement over the differences, the associated and monomeric forms differ in a variety of characteristics. The monomeric form is less highly phosphorylated and may bind DNA less well.[187,379-381] The monomeric form has kinase activity associated with it, while the higher forms have both ATPase and an associated protein kinase.[187]

The large T antigen is subject to covalent modifications. The N-terminus is acetylated.[255,560] There is an antigenic determinant that is sensitive to periodate that has not been characterized.[384] A recent report demonstrates the ADP-ribosylation of large T.[559] Since only 1% of $^{32}PO_4$ incorporated into large T in vivo is released as iso-ADP ribose or ADP-ribose, the extent of this modification is likely to be low.

Tegtmeyer and co-workers were the first to demonstrate the phosphorylation of large T antigen.[49,385] The patterns of large T phosphorylation appear quite complex.[340,386-388] Both serine and threonine residues phosphorylated,[386] but there is no evidence that tyrosine can be phosphorylated. Mapping data suggest that the major phosphorylation site(s) occur in the sequences coded just after the splice site.[49,386,387] Schwyzer and colleagues have shown that the major phosphorylation occurs on a fragment extending from the N-terminus to approximately map unit 0.51.[387] Since small T antigen is not

phosphorylated, this argues, but does not prove, that the N-terminal common sequence is not modified. This places the phosphorylation site just after the splice from 0.54 to 0.51 map units. The general localization is supported by the observation that mutant dl 1001 retains SV40 sequences to the N-terminal side of 0.44 map units and the major phosphorylation site(s).[49] Walter and Flory have analyzed phosphorylation patterns in infections with the adenovirus-SV40 ND_4 hybrid virus.[386] Analysis of which hybrid proteins are highly phosphorylated places the major phosphorylation site(s) in the region from 0.62 to 0.59 or from 0.54 to 0.44. Proteolytic mapping data demonstrated that there must be another phosphorylation site between 0.32 and 0.24 map units.[387] Scheidtmann and colleagues have demonstrated that there is a threonine phosphorylated in the extreme C-terminal region and suggested that the threonine eight residues from the C-terminus is the one modified.[388] This phosphorylation appears to be a stable modification.[592] Pulse-chase experiments in hamster cells showed that the half-life of the large T phosphate is shorter than that of the protein.[389] In those experiments the turnover appeared to be biphasic; in the first six hours of the chase, the turnover was three or four times faster than later. Experiments with tsA mutants indicate there is relatively little difference in the turnover of wild type and mutant large T phosphate.[386,389] The tsA large T antigen may have its specific activity ($^{32}P/^{35}S$) reduced 20% relative to the wild type.[386] There are reports that the large T antigen associated in higher molecular weight structures is more highly phosphorylated than the free antigen.[381,411] It is difficult to be sure whether this represents a true difference or decreased lability of the phosphate in the associated forms to hydrolysis during isolation.

Attempts to study the enzymatic activities associated with SV40 large T antigen have used purified preparations of protein. Until now two different sources have been employed. The first is SV80 cells.[263,333] These transformed human fibroblasts contain from two to ten times as much large T antigen as other cell lines. The other source is the adenovirus-SV40 hybrid virus, Ad2 + D2.[391] The transcript of this hybrid virus codes for a 107-K protein that contains approximately 10 K of an adenovirus protein at the N-terminus followed by the C-terminal sequences of SV40. The SV40 sequences upstream from 54 map units are missing. The cells infected by this hybrid make ten- to 50-fold more of this D2 protein. Recently expression of intact large T has been put under the control of adenovirus promoters.[392,393] This should represent an even better source of protein for future studies, as might successful cloning in bacteria. Experimental results with the D2 protein and the SV80 large T show that large T antigen has an intrinsic ATPase activity associated with it. A protein kinase activity is also associated with preparations of large T antigen, although it is less clear whether this is an intrinsic activity of the protein or an associated cellular activity. One laboratory has reported that the SV80 large T antigen has DNA unwinding activity associated with it.[390]

Tjian and Robbins first reported an ATPase activity associated with the D2 hybrid protein.[185,186] Other laboratories have investigated the ATPase activity of the SV80 large T.[184,187,394] Several lines of evidence argue strongly that the ATPase is an intrinsic activity of the large T antigen. Both the protein and the activity copurify through a variety of purification procedures that result in apparently homogeneous preparations of D2 or SV80 T antigen.[184,186,187,394] The ATPase activity of the tsA30 large T is more thermolabile than that of wild type virus.[184] Anti-T sera and monoclonal antibodies against the large T antigen block the ATPase activity.[185,186,394] The ATPase activity, like the D2 protein itself, binds to DNA.[185,186] The ATPase activity depends on the self-association of large T in the same way that the DNA binding properties do.[187] The monomeric form lacks both the ATPase and DNA-binding activity, but the higher forms are active. The large T of SV40 can be labeled with ATP affinity reagents such as dialdehyde ATP.[593]

The observed ATPase activity ranges from about 1 to 13.5 μm/hr/mg. This activity is

comparable to that of the myosin ATPase.[186] Giacherio and Hager have studied the reaction in some detail.[394] There is a relatively broad nucleotide specificity. Nine triphosphates tested were hydrolyzed with difference in rate of about tenfold (ATP > TTP > UTP > GTP > CTP). The reaction requires Mg^{++} or Mn^{++}, although Ca^{++} is about half as effective. Poly (dT) stimulates the reaction more than fivefold. Of a variety of nucleotides tested, poly (dT) is the best, and long chain polymers work better than shorter polymers. The extent of poly (dT) stimulation depends upon the ATP concentration used in the reaction. This poly (dT) stimulation has attracted attention because SV40 and polyoma have a stretch of 8Ts in the origin region (Figure 11). It is possible that the ATPase reaction is important in the initiation of DNA synthesis. Some proteins involved in procaryotic DNA replication are known to be DNA-dependent ATPases.[553] However, the analogy to procaryotic systems is limited; the SV40 ATPase, for example, is not DNA-dependent.

The relationship of protein kinase activity to large T antigen is more controversial. Preparations of D2 protein[185,341] as well as of SV80 large T antigen[183,184,187] have protein kinase activity associated with them. This kinase activity phosphorylates both large T antigen and exogenous substrates such as casein or phosvitin. On casein, both serine and threonine residues are phosphorylated.[183] Some nuclear proteins,[341] but not histones,[185] can serve as substrates. The activity is not affected by cyclic nucleotides. As for the ATPase, some evidence supports the idea that the kinase activity is an intrinsic property of the large T. The activity associated with the tsA30 large T antigen is temperature-sensitive.[183] The kinase activity can be removed from solution using anti-T serum,[183] and the phosphorylation of the D2 protein can be blocked by anti-T gamma globulin.[185] Like the ATPase activity, the protein kinase activity will bind to DNA, but the bound material has a lower specific activity than the unbound.[185,186] Both monomeric and associated forms of the SV80 large T show associated kinase activity.[187] However, there is also evidence suggesting this activity might not be intrinsic to the protein. Giacherio and Hager have preparations of SV80 T antigen that lack kinase activity.[394] The D2 protein can be resolved into fractions that have no kinase activity and a smaller fraction that retains some activity.[185,186] Tjian and colleagues have argued that the specific activity of the protein is more than 1000-fold less than that of a cellular enzyme.[186] (Bradley and co-workers report values much closer to those of cellular enzymes.[187]) Finally, monoclonal antibodies that block the ATPase activity do not affect the kinase activity.[186] ATP affinity labeling reagents might help to settle the question one way or the other. Although the issue is not settled, it should be emphasized that an associated cellular enzyme would be most interesting. Such an activity would have the potential to modify the large T antigen, thereby altering its activity in DNA-binding or its association with cellular proteins.

Giacherio and Hager have reported that the SV80 T antigen can introduce negative superhelical turns into SV40 DNA.[390] ATP is not required for this unwinding. Interestingly, the SV80 large T does not introduce superhelical turns into pBR322 DNA. This activity has not been confirmed in other laboratories. The large T antigen isolated after cloning in adenovirus does not seem to have this activity associated with it.[392]

Large T antigen is both a DNA-binding and a chromatin-binding protein. The interactions of large T and DNA will be discussed in a later section along with studies on the DNA sequences involved in the initiation of DNA synthesis and the control of early transcription.

2. *The Association of Large T Antigen with Host Proteins*

Large T antigen has been shown to associate specifically with host proteins (MW 48 to 55 K). Such associations have the potential to modify large T function during either

productive infection or transformation. However, at the present time no clear conclusion can be reached about the importance of such associations.

Two kinds of evidence suggest that these nonviral T antigens could be important for transformation. First, there is a correlation between transformation by SV40 and the presence of large amounts of the host proteins. Untransformed Rat-1 cells do not have an appreciable amount of nonviral T antigen, but the SV40 transformants do.[395] Phenotypically normal revertant cells lack the nonviral T antigens, but retransformed cells isolated from the revertants again contain the proteins. *De novo* infections of mouse cells with tsA mutants at the nonpermissive temperature have only modest effects on these proteins, suggesting that their presence is controlled by the A gene.[396] The second sort of evidence is a general correlation between transformation and the presence of these cellular proteins. Screening of a wide variety of cells transformed by DNA viruses, RNA viruses, and chemicals, as well as spontaneous transformants, reveals a striking correlation between the presence of the host proteins and transformation.[397,398] Thirteen human tumor lines contain the 53,000 dalton protein; only HeLa cells do not.[399] However, evidence of a functional importance to these cell proteins in transformation is not available. Embryo cells have substantial amounts of these proteins at certain times, but would not be considered transformed.[400] HeLa cells are transformed, but do not have very much of the host protein.[398] Experiments with tsA mutants also argue against a functional role for the nonviral T antigens in the alteration of the cellular phenotype. As mentioned above, tsA mutant infections at the nonpermissive temperature do not much affect the amount of nonviral T, even though tsA mutants cause abortive transformation.[32] Shift-up experiments in tsA transformants alter the amount of nonviral T antigen only two- to three-fold in comparison to the 25- to 50-fold increase after transformation.[396]

A role for the nonviral T antigen in productive infection is equally uncertain. Temperature-shift experiments after tsA infections show very rapid effects on initiation of DNA synthesis.[40,262] By contrast, the entry of large T into a complex with the cell proteins appears to be a slow process.[381,404] The decay of complex formed at permissive temperatures also appears to be slow.[396]

Figure 14 shows the presence of nonviral T antigen in an immunoprecipitate from SV40-transformed Balb 3T3 cells and Fisher rat cells. This protein has an apparent molecular weight of 54 K; different laboratories report slightly different values (53 to 55 K). A second well-characterized antigen has an apparent molecular weight of 48 K.[401-404,412] These proteins are host-encoded. They are detected in embryonal carcinoma cells[405] or uninfected mouse cells.[396] Balb/c 3T3 cells transformed by SV40 and polyoma virus contain virtually identical proteins.[406] The nonviral T antigens are synthesized from RNA that does not hybridize to viral DNA[409] and have little peptide homology with either the large or small T antigens.[407-409]

Peptide mapping data indicate that the nonviral T antigens constitute a family of homologous proteins differing slightly from one species to another. Approximately 70% of the ^{35}S-methionine peptides of rat and mouse nonviral T comigrate.[409,410] Hamster transformants have nonviral T antigens with fewer homologuos peptides, and SV40-transformed human lines show approximately 40% homology with either rat or mouse proteins.[410] In some lines such as SV80, two distinct species are observed in the 55 K region.[356] Isoelectric focusing of the proteins from SV40 mouse transformants shows a series of spots between pI 6.0 and pI 7.5.[406] Since the nonviral T antigen is known to be phosphorylated,[405,569] some of this heterogeneity could represent postsynthetic modification.

The 54-K nonviral T is located in the nucleus.[411,414] (It is worth noting that Soule and Butel have reported a similar protein in both the nucleus and the plasma membrane of

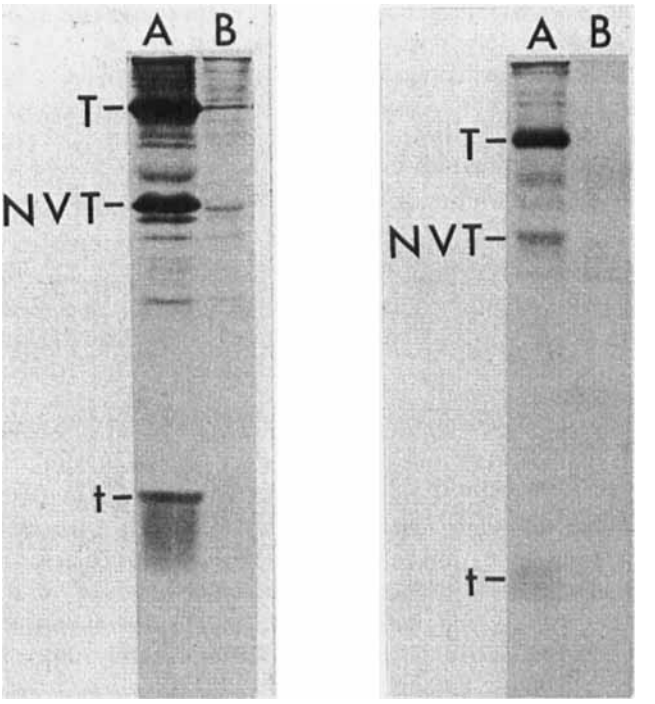

FIGURE 14. SV40 T antigens from SV40-transformed cells, SV40-transformed Balb/c 3T3 mouse cells (left) or SV 40-transformed Fischer rat (F111) cells (right) were pulse-labeled with ^{35}S-methionine. The T antigens were extracted, immunoprecipitated with hamster serum, and separated on discontinuous buffer SDS polyacrylamide gels. (A) Anti-T serum; (B) preimmune serum. The 94-K large T antigen (T), the 55-K cellular nonviral T antigen (NVT), and the 17-K small T antigen (t) are indicated by the lines.

transformed cells.[367]) Based on its sedimentation properties, McCormick and co-workers have suggested the 54 K exists in solution as a tetramer.[563] The nonviral T is phosphorylated[405,411,414,569] and appears to have multiple sites for phosphorylation.[414] Immunoprecipitates of 54 K, whether or not from virus-transformed cells, have an associated protein kinase activity that phosphorylates the protein on serine and threonine residues.[569]

De novo infection of mouse cells by SV40 induces a 25- to 50-fold increase in the quantity of the 54-K nonviral T.[396] While infection with tsA mutants at the nonpermissive temperature does not markedly increase the amount of 54 K, infection by mutants lacking small T or transformation by those mutants still results in increased levels of the protein.[396,409] This suggests that small T antigen is not involved in the induction. The appearance of 54 K after infection of monkey cells has been harder to document. While some workers did not detect it at all,[405,411] others have observed the protein.[412,413] Harlow and co-workers observed a fivefold increase in CV-1 cells, although the appearance of 54 K was delayed relative to the mouse system.[413] The increase in the levels of 54 K appears to come from greater stability of the protein in SV40-infected or transformed cells.[564] There are similar levels of 54 K mRNA in 3T3 and SV40-transformed 3T3, and the protein has a much longer half-life in the transformants.[564]

Lane and Crawford first suggested the nonviral T antigen was in a complex with large T.[415] They observed that antibody directed against gel-purified large T antigen could

precipitate gel-purified large T, but not gel-purified 54 K. In nondenatured cell extracts both proteins were precipitated. Both large T and nonviral T eluted together from a Sephacryl S200 column. Monocloneal antibodies against SV40 large T precipitate the nonviral T antigen from cells containing large T, but not from embryonal carcinoma cells.[405] Monoclonal antibodies active against 54 K can precipitate large T only when it is associated with the cell protein.

Sucrose gradient analysis of extracts from transformed mouse cells[411,414] or monkey cells[381,413] reveals a complex of approximately 23S. The molecular weight of this complex has been estimated between 600 K and 1 M. Pulse-chase experiments show the conversion of the 5-6S monomeric large T to the complex form.[381] The large T in the complex is preferentially labeled in vivo by $^{32}PO_4$.[411] As for the self-associated species, tsA mutant large T does not enter the complex at the nonpermissive temperature, but complex formed at the permissive temperature is relatively stable on shift up. Because the D2 protein associates with the 54 K, N-terminal large T sequences are apparently not involved in binding.[563] It is likely to be the association of 54 K with large T that leads to its increase stability in SV40-transformed or infected cells.

The amounts of free large and nonviral T can be estimated immunologically. Crawford and colleagues have reported that most, if not all, nonviral T in transformed cells is complexed to the viral large T.[406] Using monoclonal antibodies, Gurney and co-workers suggested about 80% of the nonviral T was complexed.[317] It has been suggested that the lability of the complex may account for differing results.[413] In SV80 cells only about 20% of the large T is associated with the cell protein.[317] In a productive infection the ratio of free large T to complexed large T can be as great as 1000/1.[413]

Studies on a 48-K cellular protein parallel those on the 54-K nonviral T antigen. This protein is antigenically unrelated to the 54-K protein and has a different partial proteolytic map.[402,403] As for the 54 K, it appears in a rapidly-sedimenting complex with large T.[404] The large T antigen in the complex appears to be highly phosphorylated. The increase in the quantity of the 48 K depends on an active A gene, but does not require small T antigen. Immunofluorescence shows that the 48 K is nuclear.[403,404] One potentially interesting difference between the 48- and 54-K nonviral T antigens is that the 48 K does not seem to be ubiquitous in transformed cells. Although at least one methylcholanthrene-induced tumor line shows 48 K by immunofluorescence, other non-SV40 transformants do not.[402] At least one SV40 transformant (SV80) seems to lack 48 K.[402]

3. Abnormal Forms of Large T Antigen

Different forms of the large T antigen can be classified according to their molecular weight: (1) "super T" antigens having molecular weights larger than that of large T antigen, typically between 110 and 130 K; (2) species of T antigens with molecular weights very close to that of large T, referred to as doublets and triplets; and (3) truncated forms with molecular weight substantially smaller than larger T and usually larger than small T.

Super T has been observed in both rat and mouse cells.[407-409,416-420] Peptide mapping typically shows that these proteins contain principally the peptides of large T antigen and that some of those peptides are present in greater than molar amounts, indicating some kind of duplication.[408,409,418,419] There are probably a variety of such proteins. Smith and colleagues have demonstrated that the super T from REF 2006 cells contains the N-terminal peptide of large T.[409] However, Chang and co-workers have reported a super T from mouse transformants that lacks the N-terminal sequences common to large and small T antigens.[408] Whether the super T antigens retain function is not clear. Cell lines that contain a super T almost always contain a normal-size large T as well. There is one rat cell line (subclone 7) that appears to make no large T.[418,419] Is has so far been

impossible to rescue virus from such cells, suggesting that the super T may be inactive for replication of integrated viral DNA replication. However, full-sized viral DNA can occasionally be detected in southern blots of extracts from heterokaryons of subclone 7 and monkey cells. A protein that probably corresponds to a super T of polyoma virus has also been observed.[188]

Often the large T antigen appears as a doublet or triplet with a 94-K large T antigen and one or more smaller species.[49,52,254,420,421] Several lines of evidence indicate that these are simply proteolytic cleavage products. Peptide analysis indicates that the smaller fragments (about 88 K, about 85 K) share most peptides with full-size large T.[49,254,420] Mapping of adenovirus-SV40 hybrid protein indicates that the cleavages occur at the C-terminal end.[422] The appearance of these fragments depends upon the conditions used for the extraction. The addition of inhibitors of proteolysis, for example, decreases the yield of the smaller forms.[421]

Truncated products of sizes between large T and small T antigen are also observed in many cell lines. Smith and colleagues have observed a 33-K protein in SWSV/3T3 cells.[254] This protein has the N-terminal peptides, including those that are shared with small T antigen, but is missing many large T peptides. SV80 cells produce an 8-K polypeptide.[423] This polypeptide contains sequence common to small and large T antigens. It is synthesized in a cell-free translation system. This and other data suggest that it is not an artifact of extraction. Its function, if any, is unknown.

C. Gene F of SV40

The second early gene which has been described is the F gene. The gene, and mutants defective in the gene, are often called "dl (0.54 to 0.59)", which indicates the portion of the genome to which they map. Shenk and colleagues were the first to describe mutants of this class.[44] Many additional mutants have now been isolated in different laboratories.[31,45,102] Table 6 lists the mutants which have been sequenced. Many of the mutations map completely within the coding region for small T antigen, but others (dl 884, for example) delete the sequence for the small T splice. It is generally agreed that the mutants grow well in culture,[31,44,45] although Topp has reported that some may grow with a slightly reduced efficiency relative to wild type SV40.[427] It should be noted that these mutants have not been selected on the basis of their growth properties, but rather have been isolated based on the presence of deletions in their DNA. The transformation properties of the dl (0.54 to 0.59) mutants have been somewhat difficult to sort out, because they depend on the kinds of cells and transformation assays used. However, dl (0.54 to 0.59) mutant-transformed cells can induce tumors in hamsters.[428,429]

De novo infections of permissive cells by the F mutants have not been extensively studied, since the viruses show no strong growth defect. There is no indication that the dl mutants exhibit any host range properties similar to the hr-t mutants of polyoma virus. Neither is there any significant defect in the acetylation of H3 and H4 histones in the virion.[452,583]

Mutants lacking small T antigen induce cellular DNA synthesis.[102,279,322,344] Hiscott and Defendi have shown that these mutants induce a first round of DNA replication after infection, but fail to induce subsequent rounds.[279] This finding is similar to that reported for the hr-t mutants of polyoma virus.[117] (Unlike ts-a mutants of polyoma, tsA mutants of SV40 were also found to be unable to induce subsequent rounds of DNA replication in the same series of experiments.[279]) The induction of cellular DNA synthesis by the dl mutants can be blocked by concentrations of theophylline that do not affect induction by wild type virus.[322] Mutants in the F gene are normal in the ability to induce thymidine kinase after infection.[322]

The F gene may also be involved in altering the cellular phenotype. Dl (0.54 to 0.59)

Table 6
F GENE MUTANTS

Mutants	Deletion	Bases deleted	Type of mutation	Ref.
dl 2112	4904—4636[a]	269[b]	Splice junction[c]	531
dl 1441	4899—4865	35	Frame-shift	424
dl 1442	4891—4751	141	In-frame	424
dl 891	4891—4867	25	Frame-shift	424, 531
dl 2006	4891—4642	250	Frame-shift	424
dl 2121, 2122	4882—4649	234[d]	Frame-shift	531
dl 1440	4853—4586	268	Splice junction	424
dl 884	4830—4584	247	Splice junction	424, 531
dl 890	4828—4802	27	In-frame	424, 531
dl 2102	4755—4741	15	In-frame	531
dl 886	4754—4745	10	Frame-shift	436
dl 885	4749—4733	17	Frame-shift	436
dl 2007	4745—4670	76	Frame-shift	436
dl 888	4693—4626	68	Splice junction	436
dl 883	4671—4614	58	Splice junction	424, 531

[a] Nucleotides numbered according to Buchman, Burnett, and Berg[25] with the addition of the 17 additional nucleotides observed by van Heuverswyn and coworkers.[247]
[b] Two additional nucleotides not found in wild type virus are present.
[c] Splice junction mutations remove the proximal splice for small T antigen.
[d] Four additional nucleotides not found in wild type virus are present.

mutants do not efficiently disrupt actin cables during a *de novo* infection, although some of the infected cells do show altered structure.[279] Microinjection of wild type SV40 DNA or a restriction fragment sufficient to encode the small T antigen leads to a loss of actin cables, but microinjection of dl mutant DNA or purified D2 protein does not.[430] Topp and Rifkin report that dl transformants are less likely to have disordered actin structures than those of wild type virus, but they obtain different results with different mutants.[79] In the same series of experiments, dl (0.54 to 0.59) transformants almost always produced small amounts of plasminogen activator. Wild type viruses induce host antigens in the centriolar region; F mutants do not.[431,432] This is potentially of interest because the centriolar region is of importance in mictrotubule assembly[433] and because deciliation of the centriole is an early cellular response to growth factors.[434] (However, SV40 does not appear to mimic growth factors in causing deciliation.[434])

The first tests of transformation with the dl (0.54 to 0.59) mutants showed that they induced transformants capable of growth in low serum.[44] However, the mutants were quickly found to be defective in soft agar transformation assays[31,32,51] or in dense focus assays.[45] For example, Feunteun and colleagues reported that dl 2112 has no more than 1/1000th the transformation ability of wild type virus.[31] Fluck and Benjamin also noticed a greatly reduced efficiency of transformation by the dl mutants, although they did detect some clones that would grow in soft agar.[32] It should be emphasized that no system shows the F mutants to be completely deficient for transformation. This is a striking difference between the F mutants and the hr-t mutants of polyoma virus. Complementation studies demonstrate that the F gene and the A gene represent two different genes required for transformation.[31,32,51] The dl (0.54 to 0.59) mutants are also defective in assays for abortive transformation.[32]

The observation that F mutants are normal for transformation in one sort of assay and highly defective in another led to a series of investigations on the nature of the F defect.

Since the ability to grow in low serum is a characteristic of "minimal" transformants, one possibility is that dl (0.54 to 0.59) mutants are capable of inducing "minimal" but not "full" transformation. Sleigh and co-workers examined a series of rat transformants and found that wild type transformants isolated on the basis of colony formation could grow in semisolid medium, but that dl (0.54 to 0.59) transformants could not.[45] A subsequent series of reports have indicated that such cells may not fully express the transformed phenotype.[78,79,429] Secretion of plasminogen activator, for example, was usually not elevated in dl transformants.[79] An extensive series of investigations by Martin and colleagues reached the opposite conclusion.[277,344,346,435] Both Chinese hamster lung cells and Fischer rat 3T3 cells could be "fully" transformed by the F mutants. The transformed cells are able to grow in agar and grow to high saturation densities. Frisque and co-workers were able to confirm these results in hamster cells.[429] Dl (0.54 to 0.59) transformants of hamster embryo fibroblasts showed altered actin organization and high levels of plasminogen activator secretion. Comparisons of early passage and late passage mouse embryo fibroblasts showed that the early passage cells transformed by the F mutants were more like wild type transformants, but that dl transformants of late passage mouse embryo cells were less fully transformed than those of wild type.[429] It is important to emphasize that dl (0.54 to 0.59) transformed hamster cells give rise to tumors in the animal.[428,429]

The second question concerned the frequency with which F mutants could transform cells. In standard soft agar assays, the frequency of transformation by the dl (0.54 to 0.59) mutants is much reduced.[31,32,45,51] Martin and co-workers have reported that the result depends on the state of the cells at the time of the transformation assay.[277,344,346,435] Cells that are actively growing at the time of transformation can be transformed by the dl (0.54 to 0.59) mutants with efficiencies similar to wild type virus even in agar assays. Resting cells are not transformed efficiently. However, resting cells can be transformed with slightly greater efficiency if the cells are also treated with phorbol esters which are tumor promoters.[435] In cells that are growth arrested by starvation for platelet-derived growth factor(PDGF), the block in transformation by the F mutants can be partly overcome by the addition of the PDGF.[277]

1. The F Gene Product: Small T Antigen

The product of the F gene is the small T antigen (Figure 14). The amino acid sequence predicted from the DNA sequence is shown in Figure 15.[25] SV40 small T is quite homologous to the small T antigen of polyoma virus. Both proteins have the two cysteine clusters (CYS-X-CYS-X-X-CYS) separated by 22 amino acids like the cluster in the pituitary hormones LH or FSH.[162] The immediate sequence around amino acid 179 in polyoma, the site of the nondeletion hr-t mutations, is not conserved in SV40.

The usual molecular weight observed on SDS polyacrylamide gels is between 17 and 20 K. Crawford and O'Farrell have observed that the value can be affected by the reduction of disulfides.[437] The dl (0.54 to 0.59) mutants induce altered small T antigens. Some have truncated products and others have none at all.[31,45,50,102,436] Khoury and colleagues have pointed out that when the deletion affects the splicing sequence, a truncated product is not usually observed.[426,436] Dl 884 is an exception to this: Khoury and co-workers have suggested that either an alternative splice is used or the truncated product is synthesized from an unspliced message.[436]

Two approaches have been used to try to obtain sufficient quantities of small T antigen for biochemical analysis. The first is purification from infected CV-1 cells.[438] The second is the cloning of small T in bacteria. Attempts to purify small T from procaryotic or eucaryotic sources have encountered problems with aggregation. So far, these efforts have not yet yielded purified protein for biochemical analysis.

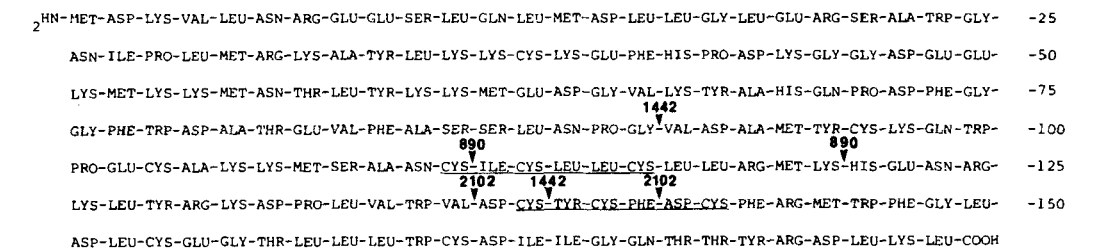

FIGURE 15. The predicted amino acid sequence of SV40 small T antigen. The amino acid sequence shown is predicted by Buchman, Burnett, and Berg,[25] based on the data of Reddy and colleagues[235] and Fiers and co-workers.[236] The two cysteine clusters (residues 111 to 116 and 138 to 143) similar to those of polyoma virus small T and the hormones TSH, LH, and FSH[162] are underlined. The boundaries of the in-frame deletions of dl 1442,[424] dl 890,[424,531] adn dl 2102[531] are indicated by the arrowheads.

Some properties of the small T antigen have been deduced from experiments with labeled cell extracts. Tegtmeyer and colleagues have estimated that there are approximately 10^6 small T antigen molecules in an infected cell based on continuous labeling experiments.[438] The small T antigen is found in the cytoplasm as is the small T antigen of polyoma. Unlike the large T antigen the small T does not appear to be phosphorylated.[389] The small T antigen does not bind to DNA.[380,481] Small T antigen associates with two cellular proteins.[441-443] This conclusion is based on the observation that a protein of 56 K and another of 32 K are found in immunoprecipitates of wild type, but not dl (0.54 to 0.59) mutants. These two proteins can be coprecipitated from labeled extracts of polyoma-transformed or uninfected cells, if unlabeled extracts of wild type infected cells are added. There is no function associated with these proteins as yet.

D. The Roles of the A Gene and the F Gene in SV40-Transformation

The characteristics of the two early genes discussed in the earlier sections suggest that their roles in transformation are not completely analogous to the two early genes of polyoma. For SV40 the A gene is required both for the establishment and maintenance of transformation of cells in culture. Besides the evidence already discussed several additional lines of data support this view.

Unlike the DNA fragment experiments for polyoma virus, DNA fragment transformation results with SV40 indicate the entire early region is required for transformation.[101-444] In particular, fragments including the small T coding sequences are not sufficient to transform. Deletions in the sequences distal to the small T coding sequences strongly impair the ability of the DNA to transform. TsA 1499 is temperature-sensitive for growth, but generates cold-sensitive transformants.[98] TsA 1499 contains a deletion around 21 map units, outside the coding sequences for the F gene. One dispensible portion of the A gene appears to be near the C-terminus. Deletion mutants 1263, 1265, and 2194 are not altered in transformation ability because of their deletions in that region.[103,104]

Many laboratories have surveyed the T antigens of transformed cells. Unlike the results obtained for polyoma virus, the SV40-transformants always retain the large T antigen. Recent experience suggests that phenotypically normal revertants no longer express large T antigen.[445-447] This differs from earlier experience that suggested that T antigen-positive revertants were usually obtained.[448-451] The basis for this difference needs to be established. However, the results of Gluzman and co-workers show that a large T antigen-containing cell need not be positive for A function measured by virus growth.[212] Some transformed CV-1 cells contain full-length T antigen, but fail to complement the tsA defect.

These two kinds of results are supported by other results already discussed, to arrive at the conclusion that the A gene can function to control cellular phenotype: (1) cells can be fully transformed by dl (0.54 to 0.59) mutants; (2) the majority of cells transformed by tsA mutants are temperature-sensitive for the transformed phenotype; and (3) purified T antigen (D2 protein) or fragments of DNA containing most of the A gene coding sequences can induce cellular DNA synthesis.

Some arguments can be made that the A gene might not be involved in altering cellular expression. The tsA mutants induce abortive transformation at the nonpermissive temperature.[32] Some tsA transformants are not temperature-sensitive for the transformed phenotype. Transformed cells, as indicated above, do not necessarily retain A function as measured by complementation of tsA mutants.

There are two different ways to resolve these apparent discrepancies. The more likely explanation is that the A protein is multifunctional and that it is possible to mutate the function needed for DNA replication without affecting the function involved in altering the cellular phenotype. The ability of tsA mutants to provide the adenovirus helper effect at the nonpermissive temperature is one clear example of this.[259,301] TsA 1642 is a virus in which the functions required for transformation and production of infectious virus are severely impaired, while the functions required for DNA replication are only slightly affected.[562] Transformed lines containing large T that is unable to complement the tsA defect could be another example of mutation affecting domains of the A gene differently.[212] The alternative possibility is that there is a third early gene and early protein. As discussed earlier, there is a second open reading frame in the distal portion of the early region which could be used to code for a small protein or to produce a large T variant (T*). Differential affects on A function in growth and transformation would arise in the same way mutations can alter both middle T and large T antigens in polyoma virus. So far, no third protein has been discovered, and mutations in the region containing the second open reading frame do not seem to alter the ability of the virus to transform.[103,104]

The role of the A gene in integration and excision has not been tested in the same ways as for polyoma. SV40-transformants can contain tandem inserts as do the polyoma transformants.[308,312,348] Transformants of CV-1 cells obtained with UV-irradiated virus contain only a single insert.[456] This is likely to be analogous to the result that ts-a mutants of polyoma largely yield transformants containing a single insert at the nonpermissive temperature.[129] Endogenous virus is not recovered from the transformed CV-1 cells, suggesting that replication function has been lost; tsA mutants are not complemented by the large T in such cells, again suggesting that A gene replication function has been lost.[212,456] Botchan and colleagues have directly demonstrated that a functional A gene is required for the excision of the integrated viral genome.[455] Some transformed cell lines have free viral DNA.[454,457] The maintenance of the ability to harbor free viral DNA appears to require an active A gene product, since it is lost in tsA mutants grown at the nonpermissive temperature.[457]

The F gene is not absolutely required for transformation. Every report describes at least a low level of transformation with the dl (0.54 to 0.59) mutants. The frequency with which transformants are obtained and the degree of alteration of the cellular phenotype appears to depend on the nature of the cells used. The F gene may function in the transformation of resting cells by inducing them to grow.[277,429,435] In a sense this idea may be related to the earlier results of Todaro and Green.[459,460] They observed that cellular replication was needed to "fix" the transformed state. When cells were not permitted to grow, the frequency of transformation was considerably reduced. Their results would be explained by saying that the cells could not respond to small T. According to this model, small T acts as a growth factor. Indeed, when Balb/c 3T3 cells are grown in medium depleted in platelet-derived growth factor, the defect in transformation observed for the dl (0.54 to 0.59) mutants can be partially "complemented" by PDGF.[277]

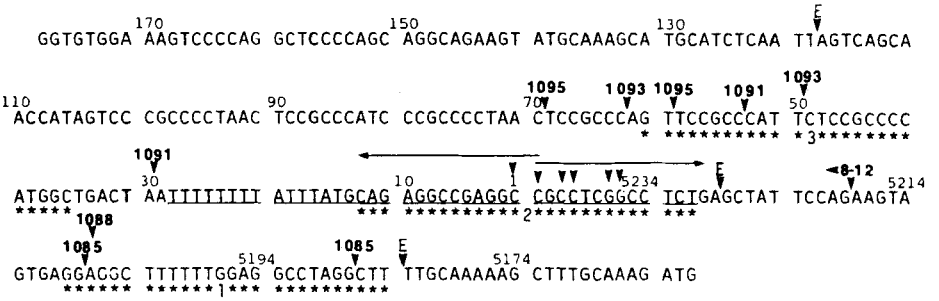

FIGURE 16. The origin region of SV40. The DNA sequence shown is reported by Buchman, Burnett, and Berg,[25] based on the data of Reddy and colleagues[235] and Fiers and co-workers.[236] The region of sequence similarity to the polyoma origin is underlined. The 27 base pair palindrome is indicated by the opposing arrows; the arrowheads underneath indicate the bases at which point mutations are known to affect origin expression.[464] The sequences to the late side of the origin precede the palindrome, while the early side of the origin comes after it. The sequence from nucleotide 107 to 178 represents the first 72 base pair repeat. The boundaries of the deletions in some viable deletion mutants are indicated by the arrowheads on the late side of the origin.[461,462] The deletion $8\text{—}12^{214}$ removes all the nucleotides preceding 5218. CS 1088 has a mutation creating a new Hind III site indicated by the arrow; the sequences deleted by CS 1085 are indicated by the arrowheads.[464] The boundaries of large T antigen binding deduced from exonuclease III digestion experiments are indicated with an arrowhead under an E.[483] The three T antigen binding sites deduced from studies with the D2 protein are underlined (***1***,***2***,***3***).[480]

In some cell lines the presence of small T antigen appears to be required for a full expression of the transformed phenotype. In such cells, small T could function to potentiate the cellular response to the A gene. Cells that constituitively express whatever factors are required for the potentiation would not require the F gene for the continued expression of the full transformed phenotype. In this sense, F mutations define a "host-range" for full transformation in a fashion similar to the way hr-t mutations of polyoma virus define a host range for productive infection. A crude analogy can be drawn between this kind of function for small T and the function of "competence factors" such as PDGF in the mitogenic response of 313 — each is necessary, but not sufficient, for a biological response. Alternatively, small T could act directly in some cell types to induce aspects of the transformed phenotype. The loss of actin cables, for example, may be a direct response to small T.[430]

E. The Origin Region of SV40

Between the initiation codon for the small and large T antigens and the start of the late genes, there are over 600 base pairs containing the origin of viral DNA replication and sequences important in the control of transcription. Viral mutants define sequences that are either dispensable or required for these functions. Large T antigen binds to this region. After a brief discussion of the general topography, the interactions of large T with these sequences will be considered in more detail.

Figure 16 shows the SV40 sequences surrounding the origin of DNA replication. The boundaries of the origin region are defined by studies on deletion mutants.[461-463] It comprises approximately 63 base pairs, within which there is a striking 27 base pair palindrome. Point mutations within this palindrome can affect viral DNA replication positively, negatively, or conditionally.[464] This is the region which is homologous to the polyoma origin of DNA replication (Figure 11). This area contains the 5′ ends for both the early and late RNAs.[248-250,293] It should be noted that SV40 and polyoma virus differ somewhat in genomic arrangement in this region. For polyoma, the ends of the early RNA are downstream from the origin sequences. There is also a Hogness-Goldberg box.

Although the Hogness-Goldberg box is not required for viral gene expression,[458,465] it appears to be important in positioning the initiation of transcription.[465,542,543] On the late side of the origin there is a sixfold repeat of the sequence $G_{3-4}CG_2Pu_2$, which is common to polyoma virus and SV40 as well as adenovirus[466] and BK virus.[467] Viable deletion mutants may lack these sequences, suggesting that they may be nonessential.[247,461] Beginning at base 107 there are two 72 base pair repeats. One of these can be removed by mutation, but mutations altering both repeats drastically reduce early transcription.[468,542] Curiously, this region appears to be devoid of nucleosomes in a fraction of viral minichromosomes.[469-472]

Sequences homologous with those of the SV40 origin have been detected in human or monkey cell DNA.[473,474] McCutchan and Singer have described a rare sequence of 300 base pairs in monkey DNA that is homologous with the palindrome sequence and both areas of repeats on the late side of the origin. Dhruva and colleagues have reported an evolutionary variant of SV40 that contains an insert of monkey DNA.[474] This insert is quite similar to a portion of the origin to the early side of the sequences common to SV40 and polyoma and slightly beyond. The insert and origin share a 14 base pair sequence similar to that present in the Alu family of human sequences. The Alu family includes many of the repeated sequences of DNA that are interspersed at regular intervals in single-copy DNA in the human genome.[475-477] The presence of these sequences in heterogeneous nuclear RNA, and near gene coding sequences, has suggested that they could be important in control of RNA transcription or processing; their similarity to the viral origins of DNA replications suggests that these sequences could be involved in the initiation of cellular DNA synthesis.[477]

F. Binding of Large T Antigen to SV40 DNA

Large T antigen preferentially binds to SV40 DNA at the origin.[391,478-480] The large T antigen of tsA mutants is temperature-sensitive for DNA binding.[48,479] An elegant biological confirmation that the interaction of T antigen with the origin region is important for virus growth comes from experiments on mutant viruses that have alterations in the origin region. Second-site revertants have been isolated that have regained a wild type phenotype.[261] The mutations giving rise to the second site revertants map in the coding region for large T antigen.

Studies on large T antigen DNA binding have used a number of different techniques: (1) nitrocellulose filter binding assays;[479] (2) DNA cellulose binding assays;[481,482] (3) DNase I protection experiments;[391,480] (4) exonuclease III protection experiments;[483] (5) immunoaffinity binding assays using anti-T sera;[484-486] and (6) dimethyl sulfate protection experiments.[480,567] Filter binding assays shows that the dissociation constant for the binding of large T antigen to DNA is approximately 10^{-12} M.[479] Immunoassays give a similar value (3.2×10^{-12} M) and indicate that the origin DNA-large T complex has a half-life of approximately 100 min.[486] Both of these assays detect additional binding sites. In the filter binding assays Hind II + III fragment A (65 to 43 m.u.) and B (32 to 17 m.u.) will also bind large T, while immunoassay detects a binding site in the Eco RI fragment D (83 to 73 m.u.) of the late region. The Eco RI D binding has a more rapid decay rate than the origin binding.

Nuclease digestion experiments have defined the binding of large T antigen to the origin more precisely. The D2 hybrid protein can protect sequences of approximately 30, 75, and 120 base pairs of SV40 DNA from digestion with DNase I.[391,480] Higher D2 concentrations protect larger fragments, suggesting that these represent sequential binding sites. Mapping the protected fragments locates the first binding site between nucleotides 5184 to 5209, the second between 5231 to 13, and the third from 36 to 61 (Figure 16). Exonuclease III digestion experiments using 5'-^{32}P-labeled DNA and SV80 large T also define the sequences involved in binding.[483] An exo III stop is observed at

nucleotide 5228, approximately at the edge of the second D2 binding site. At somewhat higher concentrations of large T an exo III stop is also discovered at nucleotide 5183, the edge of the first D2 binding site. When digestion is carried out from the other direction, a stop is detected at nucleotide 118, which is within the first of the two 72 base pair repeats.

Studies using origin mutants have been used to further evaluate the nuclease digestion results. DNA from CS 1085 which has 21 base pairs deleted from the first T antigen binding site binds D2 protein much less efficiently than wild type DNA.[485] Deletions in the second or second and third binding sites have little effect on DNA binding.[486] A mutant Hind III fragment from CS 1088 that contains only the second and third binding sites is bound only poorly, suggesting that binding at these second and third sites may not be independent of that at the first site.[485] Protection of dl 23 DNA, which contains only sites 2 and 3, occurs only at very high (>1000:1) protein-to-DNA ratios, whereas sites 2 and 3 are protected from DNase digestion at much lower ratios when site 1 is present.[567] These results have been interpreted in terms of cooperative binding of large T to DNA. Myers and Tjian have shown that mutants defective in D2 protein binding in vitro are also deficient in replication in monkey cells.[484] The first binding site may also be involved in control of early transcription. Rio and colleagues have shown that the D2 protein can block transcription from DNA that contains site 1, but not from DNA that has lost site 1 and part of site 2.[269] However, site 1 is apparently not sufficient, because replacement of site 2 with bacterial sequences renders large T ineffective in blocking transcription.[567] McKay and DiMaio have reported that both CS 1085 and CS 1088 overproduce early message and proteins in vivo.[485]

It is worth noting that the degree of analogy between T antigen-DNA binding in vitro and in vivo is not completely clear. Despite the fact that CS 1085 and CS 1088 show altered DNA binding in vitro, both mutants grow normally at 37°. The basis for this discrepancy is not clear. One suggestion is that the redundant sequence AAAAGCCT present both in site 1 and the mutants may be sufficient for viability.[461] An alternative is that site 1 binding is more important for transcription than for the initiation of DNA replication.

The complex forms of SV40 large T can be active in DNA binding. Bradley and colleagues have shown that the dimer and tetramer forms of SV80 large T are active in DNA binding, but the monomer form is not.[187] Electron microscopic results indicate that the major DNA binding species of the D2 protein is the tetramer; the tetramer can undergo conversion to a dodecameric structure.[576] Somewhat different results have been obtained in crude extracts. Here a 5S form which may represent a monomer/dimer mixture is the major DNA binding form, while the 16-18S species does not exhibit tight binding to origin region DNA.[379] McKay has also observed reduced binding of higher molecular weight forms.[486] Comparisons of "new" and "old" T antigen showed that the newly synthesized material was better able to bind to DNA.[481] It is likely that an understanding of the behavior of the different forms will require a more complete understanding of their biochemistry; the degree of phosphorylation, for example, is an uncontrolled variable in the T antigen preparations.

Studies on the domain of the large T protein required for binding to the DNA have only recently begun in earnest. The D2 protein is lacking N-terminal sequences of large T, but of course binds DNA. Prives and co-workers have shown that an 82-K fragment synthesized in vitro from cRNA will bind to DNA. This fragment likely includes all the sequences distal to the splice. Smaller fragments including one at 72 K are not DNA-binding. They have concluded that these smaller fragments share a common C-terminus with the 82 K.[380] This suggests that the 10-K region just downstream from the splice is crucial to DNA binding. It has been suggested that a sequence of LYS-LYS-LYS-ARG-LYS in this region may be important in DNA binding.[482] Two other observations support the idea that this portion of the molecule is involved in origin binding. Mutant dl 1001

produces a truncated 33-K large T that contains sequences upstream from 42.5 map units; this truncated T antigen binds to DNA cellulose.[49] Also, the alterations in second-site revertants of origin mutants map to the region between 53 and 50 map units.[257,258] Interestingly, this region involved in DNA binding contains the major phosphorylation sites.[49,386,387]

The binding of large T antigen to viral chromatin has been less extensively studied than binding to DNA. The first experiments involved the association of partially purified T antigen to extracted viral chromatin.[489] The addition of T antigen caused the chromatin complex to adsorb to nitrocellulose filters. Two to three times as much T antigen was required to cause the binding of viral DNA. Mann and Hunter showed that the large T antigen can be detected in viral chromatin.[490] Calculation suggested that as many as four molecules of large T antigen could be bound per viral DNA molecule. The large T is associated with both the replicative forms and the minichromosomes containing the mature form I DNA.[491,492] From 50 to 80% of the replicative forms have large T antigen associated with them, while less than 20% of the "mature" minichromosomes contained T antigen. The association of large T with the viral chromatin is temperature-sensitive in shift-up experiments with tsA mutants.[490,492] The T antigen associated with the chromatin can be classified as weakly binding or tightly binding, based on dissociation by 0.5 *M* NaCl-0.4% Sarkosyl; the tight-binding large T can be mapped to the origin region of the DNA.[492] Examination of the large T phosphopeptides indicates some minor differences between the chromatin-associated and free large T antigen.[493] Paradoxically, tsA mutant large T in viral chromatin shows the pattern of phosphorylation typical of free T antigen even at the permissive temperature.

1. The Tumor-Specific Transplantation Antigen (TSTA)

Immunization of animals with polyoma or SV40 results in virus-specific resistance to tumor induction by subsequent challenge by virus or transformed cells.[494-500] This transplantation immunity is presumably responsible for the failure of polyoma and SV40 to act as efficient tumor-causing agents in their natural populations. The ability of SV40 to cause tumors in hamsters, for example, has been ascribed to disruption of cellular immune defenses.[501,502]

Thymus-derived lymphocytes are of primary importance in the immunologic response.[503,572,573] This can be studied in vivo by tumor induction or in vitro in a lymphocyte-mediated cytotoxicity assay. The H2 gene complex controls the response; syngeneic, but not allogeneic, transformed cells are killed in the cytotoxicity assays.[504-514] The killing can be blocked by anti-H2 antibodies.[507-508] There are some indications that other immune mechanisms may also operate. Non-T cell effectors can be found in the peritoneum of immune animals.[505] SV80 fails to induce tumors in Balb/c nude mice, but will cause tumors in CBA/N nude mice that have an additional defect in thymus-independent B cell function.[90] The details of the cellular immunity are beyond the scope of this discussion and the reader is referred to some of the recent literature.[507-514] The role of the viral gene products will be discussed here. For SV40 a considerable body of evidence implicates the large T antigen directly in the process of transplantation immunity. Very little information is available for polyoma.

The ability of the large T antigen to induce transplantation immunity has been clearly demonstrated. Kinetic studies show that T antigen and the ability to induce transplant immunity appear concomitantly.[518,519] The appearance of TSTA can be temperature-sensitive in tsA infections, suggesting that an active A gene product is required.[519] Dl (0.54 to 0.59) mutants induce TSTA so that small T antigen does not appear to be involved. The large T antigen and TSTA have been shown to copurify in several laboratories.[353,515,516] Both the purified D2 protein and purified large T from SV80 cells immunize animals against tumors.[353,354] As little as 0.6 μg of SV80 large T antigen can

protect against tumor challenge.[353] Studies on adenovirus-SV40 hybrid viruses indicate that the coding sequences between 0.28 and 0.17 map units are sufficient for TSTA.[517]

Cell-mediated immune reactions leading to cytolysis require interactions with target cell membranes. The evidence supporting a membrane localization for a fraction of the large T antigen has been detailed earlier. Small T antigen is apparently not involved in specifying the target, since dl transformants are killed in a normal fashion.[520] Experiments to block cytolysis with anti-T sera have not been successful. This prevents a formal demonstration that the viral proteins themselves are directly involved in the target. However, antibodies directed against the antigens required for killing have been successful in blocking cytolysis only in special cases.[521,522] It has recently been reported that a 100-K protein can be precipitated with antiserum directed against H2.[512] Whether this will represent the beginning of a biochemical definition of the target remains to be seen.

Much less information is available for polyoma virus. Where cell fractionation experiments with SV40 indicate that most of the TSTA-inducing activity is localized in the nucleus, fractionation of a polyoma-transformed mouse cell shows much more activity in the plasma membrane fractions.[523] Since the distribution of T antigens in the cells that were used is not known, interpretation of this experiment is difficult. It suggests that the middle T antigen can act as an immunogen. On the other hand, NG18 is able to induce transplantation immunity, suggesting that the large T antigen is also capable of induction of TSTA.[594] Although there have been earlier reports of killing of polyoma transformants in vitro, only recently has a system been developed that can be used to evaluate the importance of the different T antigens in the cytotoxic lymphocyte assay.[552] Such studies should help to understand the immune response to polyoma tumors.

V. CONCLUDING REMARKS

Two kinds of explanations for transformation can be entertained *a priori*. The first is that the continuous action of viral gene products is required to maintain the transformed state. The second is that the virus permanently alters cellular growth controls as a result of a single nonrecurring event. Examples of such an event might be integration of viral DNA into a cell control sequence, or a virally-induced somatic mutation of some element important in cellular regulation, such that normal growth properties are permanently lost. Whether any single explanation is sufficient for all transformation by SV40 and polyoma virus is not clear.

Most of the evidence supports the view that transformation results from the continuous action of the viral gene products. The viral DNA sequences required for transformation match the coding regions for the gene products. Transformed cell lines continuously express the viral gene products. The reversion to normal phenotype is very often accompanied by the loss of the expression of those viral gene products. The microinjection of purified SV40 T antigens mimics the effects of transformation on some aspects of the cellular phenotype. Considerable effort has been devoted to studying the sites of integration of viral DNA in transformed cells.[305-308,312,348,524-526,575] Such studies have not generally observed any indication of specific sites of integration within the host genome. The rate of somatic mutation is increased by SV40, but that increase is much less dramatic than the increase in the number of transformed clones.[533-536]

Although the viral gene products appear to be generally required to induce the transformed state, both the specificity of integration and the possible role of somatic mutation need to be examined further. Earlier results indicating little specificity in integration may be misleading because the integration pattern can change after the integration event for both polyoma[137] and SV40.[571] Also, the host sequence adjoining the integrated genome can be rearranged;[526] such rearrangements could obscure similarities

in integration patterns. Two recent reports indicate that independent transformed lines seem to have very similar, if not identical, patterns of integration.[312,348] Examination of SV40 DNAs that have acquired cellular sequences has shown that particular sequences are commonly found.[527-530] This may be another indication of specificity in the interaction between viral and cellular DNA. Theile and colleagues have argued that the rate of somatic mutation induced by SV40 can be very similar to the frequency of focus formation.[351]

The detailed arrangement of the early regions of SV40 and polyoma virus are clearly different. To begin to evaluate this difference, it is first necessary to know whether SV40 and polyoma virus have the same effects on cellular phenotype. Certainly the two viruses alter the same cellular properties, but whether there is a quantitative difference is less certain. In animal experiments it has generally been easier to obtain tumors with polyoma virus than with SV40. It has long been known that differences in transformation can be observed in culture as well. Todaro and colleagues reported in 1965 that the morphology of SV40 and polyoma transformants is different.[80] The fact that SV40-transformed rat cells grow more slowly in agar than polyoma transformants has been used to select cells transformed by both viruses.[81] Perbal and Rassoulzadegan have shown that SV40 transformants secrete reduced amounts of plasminogen activator and show smaller increases in hexose transport.[82] Seif has studied all of these kinds of properties in wild type SV40 transformants and in cells transformed by dl 23 of polyoma virus.[83] He concluded that the two kinds of transformants are similar and suggested that SV40 "differs" from polyoma virus by the addition of a functional middle T antigen. Examination of the regions of homology between the early regions of the two viruses, as discussed earlier, suggests the possibility that the stretch of information coding for the unique portion of middle T has been "added" to the SV40 early region to generate polyoma virus. The difficulty in comparing transformed cells is that the outcome may be dominated by the degree of homology of the cellular targets in the host being tested and the natural host of the virus. It would be instructive to carry out such comparisons in monkey or human cells. If similar results were obtained, then the hypothesis that polyoma has an additional activity might not be unreasonable. It is interesting to note that Takemoto and colleagues have described polyoma-transformed human cells that are morphologically altered, but fail to grow in agar.[548]

If the transformation capacities of the two viruses are the same, then there are at least two possible explanations for the differences between SV40 and polyoma virus. The first is that there is another early gene and protein for SV40 which remain to be discovered. The existence of an open reading frame in the distal portion of the early region is strongly suggestive of another early protein. No such protein has yet been detected, and mutations in that region of the genome do not seem to alter transforming ability.[103,104] However, if the gene product is essential for growth and encoded colinearly with the A gene product, then mutants might not have been detected and might have to be constructed very carefully. This would mean that there are three SV40 early genes. For polyoma virus one can anticipate the construction of mutants providing only small or middle T, which might also lead to the definition of three early genes.

An alternative is that the SV40 A gene has functional domains analogous to the hr-t gene of polyoma. Specifically the A gene product could function as both a large T and a middle T antigen. If there is no third SV40 early product, then the large T antigen must be able to induce the transformed phenotype. However, the functions that can be specifically associated with the hr-t gene, such as effects on actin architecture, plasma membrane structure, or associated tyrosine kinase activity have not yet been associated with the large T of SV40.

The details of how these viruses alter the cellular phenotype are not known. For SV40 it has been proposed that transformation results from the ability of the A gene to initiate

cellular DNA synthesis. (See Martin[26] for a discussion of this possibility.) If polyoma virus and SV40 induce transformation in the same way, this does not seem probable. The initiation of DNA synthesis is a function associated with the ts-a gene of polyoma. For polyoma virus the tyrosine kinase activity associated with middle T antigen is likely to be involved. This kind of activity is associated with a variety of retrovirus transforming gene products as well.[175-178,554-555] (See Langan,[537] Hunter,[538] and Marx[539] brief reviews.) In such a case phosphorylation of specific cellular targets would be expected. For Rous sarcoma virus, for example, the phosphorylation of vinculin has been suggested to be important in virus-induced alterations of cellular morphology.[540]

For the moment one must have a high tolerance for ambiguity in considering transformation by SV40 and polyoma virus. Because of technical progress in manipulating viral genomes and in developing immunological reagents to detect the proteins or their domains, understanding of the viral genes and their products will soon be possible. Translating that knowledge into an understanding of the cellular response will be difficult. The reported variation among different cells is quite striking, and our understanding of the molecular basis for the cellular changes caused by these viruses is quite limited. Nonetheless, one can hope that we will progress from papers describing the differences between cells to papers explaining the molecular basis for those differences.

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