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The Biology of Viral Carcinogenesis

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Summary

Virus induction of tumors is the direct effect of a complex interaction of a single virus particle with the target cell. The factor which determines which virus has the potential for inducing tumors resides in the viral nucleic acid and of necessity must be related to the chemical structure of the viral genome. It has been suggested that the important chemical structure is a sequence of bases having a critical degree of commonness with the structure of certain segments of the cell's DNA. The susceptibility of cells to virus transformation might well be universal, but here also there is probably a spectrum of susceptibility dependent upon many structural, physiologic, and karyologic characteristics. However, even the effective entrance of the virus into the cell does not necessarily result in transformation. Replication of the cell DNA may be required for integration of the viral DNA based on homologous areas, and the low efficiency of transformation suggests that this probably requires precise timing of multifactored events. Early stimulation of cell DNA synthesis by induction of increased DNA synthetic enzyme activity may increase the opportunity for integration. Once integration has occurred, virus maturation is suppressed, but derepression of cell DNA synthesis continues. Early viral coded proteins demonstrated as new antigens may play a major role in this derepression. However, fortunately for us, if there is such a thing as tumor viruses of humans, not all virus-transformed cells grow into tumors, since new foreign antigens at the cell surface frequently cause their immunologic rejection.

Introduction

With the development of the field of molecular biology chiefly through work with bacterial viruses, it was not long before similar approaches were being made in animal virology once tissue culture and plaque assay technics were developed. It was only logical that a similar shift in approach should occur in tumor virus research when viruses such as Rous sarcoma virus (RSV) and polyoma virus were shown by quantitative methods to be capable of viral replication and cell transformation in *in vitro* tissue culture systems (32, 51).

In the last 8 to 10 years we have seen viral oncology shift from the technic where a poorly quantitated amount of crude virus material in a tumor emulsion was inoculated into an experimental animal, and then months or even a year or two later a tumor did or did not appear. Now cells of any species, including man, can be grown in tissue culture and directly inoculated with the virus being studied. Not only do certain tumor viruses readily multiply in these cultures, producing localized cell destruction called plaques, thus allowing accurate quantitation and efficient production of virus, but sometimes they cause a transformation of the normal cell to one having the properties of a tumor cell. And all this can be demonstrated not in months, but in as little as a few days!

Using these tissue culture technics in which virus induction of a tumor cell is taking place in a relatively simple, controlled system along with the older established *in vivo* methods such as transplantation, it has been possible to start applying many of the newer biochemical approaches so rapidly developing in studies of other cells and viruses. Because of this we can now ask and expect to get answers to many old and some new questions concerning the biology of tumor induction by viruses. Table 1 lists some of the more important of these questions.

In attempting to at least superficially answer the questions raised above, most emphasis will be placed on the DNA-containing papova viruses, especially polyoma virus, since more definitive biochemical studies have been made with them. Similarly, in the RNA tumor virus area most reference will be made to the RSV virus and the related avian leukosis group.

Criteria of *in Vitro* Transformation

Since *in vitro* transformation will be so importantly involved in this discussion, the criteria of this phenomenon should be mentioned. Table 2 lists the more readily demonstrable attributes of transformation. It is immediately obvious that all cells in culture transformed as the result of interaction with

Table 1

1. Does virus cause a tumor by direct or indirect action on a cell?
2. What makes a virus oncogenic?
3. What makes a cell susceptible to transformation?
4. What biochemical molecular events are responsible for viral oncogenesis?
5. Why do virus-transformed cells produce gross tumors?

Pertinent questions to be asked.

Table 2

1. Increased growth rate, shorter doubling time.
2. Unlimited lifetime in culture, establish as permanent lines.
3. Lack of contact inhibition, produce multi-layered clones.
4. Chromosomal abnormalities, increased incidence and types.
5. Production of tumors on transplantation to immunologically compatible host.

Criteria of *in vitro* transformation.

viruses—or for that matter due to any other type of induction—do not necessarily meet all these criteria. Exceptions to each have been demonstrated, but the last would be accepted most uniformly as the critical one. In general, most of the quantitative *in vitro* viral transformation studies have depended upon a combination of the first three attributes listed, but it must be realized that in many instances it is only an assumption that they are an equivalent to Number 5.

Experimental Systems

Chart 1 schematically outlines a comparison of the typical interactions of a DNA tumor virus such as polyoma virus and an RNA tumor virus such as RSV with the corresponding susceptible host cells. In both cases cells may be transformed and new viral progeny may be produced. The striking difference is that both these events can occur in the same cell with the RNA viruses, whereas either one but not both can happen in the DNA virus-single cell interaction. Cells transformed by RNA viruses continue to undergo cell division and continue to produce and release infectious virus, while the DNA virus-transformed cell does not produce identifiable viral elements. In fact, when the DNA virus-transformed cell on a rare occasion does allow virus production, the cell dies in the process. These distinguishing characteristics of the two viral systems, although generally true, nevertheless have exceptions in special situations.

Viral Transformation–Direct or Indirect Effect?

Now the first of the questions which we asked can be answered in definite terms. The rapidity with which transformation of hamster cells in culture takes place on exposure to polyoma virus and its linear response to increasing doses of virus (41) firmly establishes that the change of a normal cell to a tumor cell is the *direct* effect of the tumor virus on the target cell and not the *indirect* result of virus infection somehow producing a secondary carcinogen. Even though in the case of polyoma virus the efficiency of transformation is quite low so that there is a chance of one transforming event occurring for every one million virus particles in the cell culture system, yet that one transformation appears to be due to the interaction of a *single* virus particle with a *single* cell. The same quantitative relationships also hold in the RSV-chick embryo cell culture system. Furthermore, in the DNA viruses the nucleic acid genetic core of the virus is the determinant of its oncogenic potential, for purified viral DNA is capable of producing cell transformation *in vitro* (9) and tumors in the experimental animal (10), but its efficiency is much lower than that of whole virus.

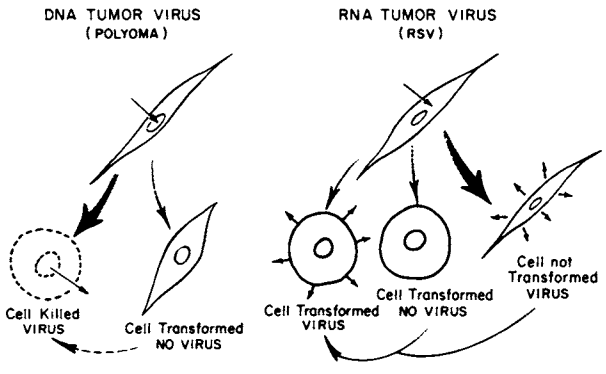

Chart 1. Possible interactions between tumor viruses and cells.

What Makes a Virus Oncogenic?

The first obvious requirement for oncogenicity is that the virus be capable of a dynamic interaction with the cell in which the cell not only survives but multiplies and, as will be further discussed later, a persistence of viral influence in that dividing cell. However, even this is not enough, for we know that carrier tissue cultures can be established *without* transformation by such nontumor viruses as mumps, parainfluenza, and rabies. A tumor virus must also be capable of modifying normal mechanisms responsible for *control* of cell replication.

Since the nucleic acid of all viruses is the source of the code that determines their characteristics, it is obviously important to examine the nucleic acids of tumor viruses for unique characteristics that might be correlated with oncogenic properties. For such a purpose, a comparison of the properties of tumor virus nucleic acids with those of similar but nononcogenic viruses is indicated. One of the difficulties here is to establish which virus is *not* oncogenic under any circumstances; hence the problem of selecting a valid control for such a comparison.

The determination of the properties of the nucleic acids of viruses requires that large amounts of purified and concentrated virus should be available as starting material. This becomes a principal limiting factor in the number of different viruses for which genetic material can be readily characterized. Although RSV and certain other avian leukosis viruses can be obtained in concentrated form, there is the difficulty of contamination with naturally occurring avian agents. Other RNA tumor viruses cannot readily be obtained in large amounts. For such reasons, attempts to characterize tumor virus nucleic acids have been limited mostly to the DNA viruses, including polyoma, SV40, papilloma, and adenoviruses.

Several physical properties of isolated virus DNA, such as its buoyant density and melting curve, can be determined directly. From these the molecular weight and strandedness can be estimated. Most animal virus DNA thus far examined, whether from oncogenic or from lytic viruses, have been double stranded, the molecular weights varying from 3 to 160 million. So far there has been no significant correlation of the size of the virus particle and molecular weight of its nucleic acid with oncogenicity. The form of the intact DNA molecule is of some interest since circular forms have been described. The DNA of polyoma (12) and SV40 (7) viruses, but not of oncogenic adenoviruses (20), has been shown to be circular, but that of most nononcogenic viruses has not been examined from this standpoint. Polyoma virus DNA is capable of both lytic infection and of *in vitro* transformation, whether it is circular or linear in form (9). It is not known at present what happens to the form of the DNA within the infected cell during viral replication or integration.

Most of the physical properties of the nucleic acids reflect

their chemical composition, and the content of bases can be calculated from them or determined by direct chemical analysis. There has been much interest in the base ratios of the nucleic acids, and it has been suggested that the percentage content of guanine plus cytosine (G + C) might be significant in determining the oncogenic potential of a given virus. This was based on the observation by Green (19) that adenovirus types 12 and 18, known to be oncogenic in hamsters, have a G + C content of 49%, compared to 56% in the apparently nononcogenic types 2 and 4. The hypothesis was that the G + C content of any oncogenic DNA virus might have to be of approximately the same order as that of mammalian cell DNA, which is 43%. The base ratios of polyoma, SV40, rabbit, bovine, canine, and human papilloma viruses have been found to be in the range of 41–48%. Herpes and vaccinia virus DNA's, on the other hand, have a G + C content at the opposite extremes of this range—68% for herpes (6) and 37% for vaccinia (54). Although these base composition findings are of interest, determinations on more strains of oncogenic and nononcogenic viruses are obviously needed before generalizations can be made. In fact, evidence that so simple a criterion is not likely to be valid is seen in recent work by Axelrod in my laboratory (unpublished results), in which the highly oncogenic SA 7 adenovirus of monkeys appears to have a G + C content of 58%.

However, the overall base composition of a nucleic acid is only a crude characterization, since it gives no evidence of the spatial arrangement of the bases in the functioning molecule. The recent development of methods of demonstrating hybridization of single strands of either RNA or DNA with another single strand of DNA having complementary base sequences has added specificity to the characterization of nucleic acids. By these methods it has been shown that it is possible for two DNA's having the same percentage base compositions to show no homology at all and to have no base sequences in common. These technics have already been applied in the tumor virus field. The DNA's of oncogenic adenovirus types 12 and 18 showed greater homology between themselves than with the nononcogenic types 2 or 4 (30). This suggested similarity in the base sequences of these two oncogenic adenoviruses. More concerning the importance of base sequences will be discussed later.

Relationship of Virus to Transformed Cell

Before discussing possible mechanisms of viral oncogenesis at the cellular level, it will be helpful if we look at the end result—the transformed cell. Is there any evidence that the interaction of virus with cell and the induction of the transformed state is anything more than a transient "hit and run" type of relationship? In the case of the RNA viruses, this evidence is direct since virus continues to be produced and released. Even with the DNA viruses, all the present evidence points to the persistence of the viral genome in the transformed cell despite the fact that infectious polyoma virus, for instance, cannot be demonstrated by any of a number of sensitive test procedures. The evidence for persistence of viral genetic material in the cells transformed by DNA viruses is summarized in Table 3.

Table 3

1. Infectious DNA extractable. Rabbit papilloma (Ito) (27).
2. Viral DNA hybridization with tumor cell DNA. Polyoma (Axelrod) (1).
3. Tumor cell RNA hybridization with viral DNA. Polyoma, SV40 (Benjamin) (4).
4. Infectious virus release. SV40 (Gerber) (14).
5. Viral marker rescue. Polyoma (Ting) (45).
6. Viral structural antigen in tumor cell. Adenovirus (Huebner) (25).
7. Virus-specific antigens in tumor cells. Polyoma, SV40, Adenovirus (Habel) (21), (Huebner) (26).

Evidence for persisting DNA virus genome.

Thus, it is strongly suggested that persisting viral DNA is associated in some way with the cell DNA, coding for specific messenger RNA, which in turn produces virus-specific antigens. How much of the viral DNA is persisting and how much is required to induce transformation in the first place? It is apparent from inactivation studies with chemical and physical agents that, whereas the whole intact viral genome is necessary to establish a complete virus replication cycle, only from 20 to 50% of the polyoma genome is needed to transform a cell (3). But we must remember that the polyoma DNA has a molecular weight of only 2½ million and thus is probably capable of coding for only 6 to 12 proteins. Three have already been identified—the viral coat protein and two different types of specific induced antigens in the tumor cells, besides which a temperature-sensitive gene has been found in a mutant of polyoma virus, which is involved in viral DNA replication (13). In addition, another function probably requires a protein, and that is the initiation of cell DNA synthesis induced by the virus (11). If only 20 to 50% of the genome is necessary for transformation, then it is possible that as few as 1 to 6 proteins are involved, and yet there already are identified 4 possibly connected with functions important in the transforming process. With the future development of more polyoma virus mutants, it may be possible to pinpoint the gene and gene product that is critical for the transformation process.

Viral DNA synthesis at the time of infection is not required for cell transformation (46). In fact, there is some evidence that transformation is more efficient in cell types that are incapable of supporting a lytic cycle of virus replication (48). However, there is no reason to believe that the ability of the viral genome to be integrated or the incomplete virus cycle in the transformed cell are necessarily *dependent* upon a defect in the original invading virus' nucleic acid. In fact, in some systems such as the Shope papilloma of rabbits (40) and perhaps SV40 virus-transformed hamster cells (38), whole infectious virus can be recovered. It seems more logical that an important controlling factor of viral transformation is originating in the cell.

Cell Factors in Viral Transformation

Table 4 lists some of the ways in which it is known that the cell can influence the efficiency of viral transformation. Certainly the cell must have the proper receptor sites to allow attachment of the virus, permit penetration, and accomplish

Table 4

1. Attachment, penetration, and uncoating of virus particle.
2. Association of viral genome with cell genome, base sequence homology?
3. Physiologic state of cell, need for cell division, increased DNA target?
4. Karyologic state of cell, chromosome imperfections.

Cell factors in viral oncogenesis.

uncoating of the virus particle before the viral genetic core can interact.

The low efficiency of transformation and the dual choice in some systems of lytic or transforming infection suggested that there might be some mutant virus particle or a particular cell required. The evidence is against the possibility that a rare mutant virus particle is the one that causes transformation; all particles apparently are equally capable, although the degree of this capability may vary between different strains of the same virus. Likewise there is a variation in the relative susceptibility of different cell types from different species, but all cells of a given uniform culture are equally transformable. Transformability therefore appears to be a matter of physiologic rather than hereditable competence. In DNA tumor viruses, such as polyoma and SV40 and even in an RNA virus, Rous sarcoma virus (44), experimental evidence suggests a possible need for a specific interaction of the viral nucleic acid with the cell DNA. One of the intriguing findings with DNA tumor viruses is the fact that their DNA's seem to have base sequences in common with the DNA of the normal susceptible cell (1, 52). Perhaps such a commonness in chemical structure is a *prerequisite* for integration of the viral genome into the cell genome and thus for transformation. The degree of similarity of structure might vary so that a spectrum of potential oncogenicity would exist among viruses, from those with little base sequence homology rarely causing transformation, to others with longer common sequences having a greater oncogenic efficiency. On the other hand, the frequency of repetition of a given stretch of bases in the cell genome homologous to that in the virus might also influence the ease of viral integration. An interesting report that may be pertinent to this discussion concerning possible relationship in structure between tumor virus and cell DNA's is that of Winocour (53), who presents evidence that a small piece of mouse cell DNA is incorporated into the polyoma virus particle.

Although it is certainly difficult to evaluate the properties of the individual cell after it has been transformed by the virus, it appears that biologic and morphologic expression of the transformation requires at least one cell division after integration (47). In fact, there is some evidence that if cell division occurs shortly after virus infection, the efficiency of transformation is increased. This is consistent with the old observation that tissues with a high mitotic rate seem more susceptible to tumor formation. Also, very young, rapidly developing animals are most susceptible to virus-induced tumors, and at this age tumor viruses can cross species lines—for example, Rous sarcoma virus, a virus producing tumors in chickens under natural conditions, can also cause tumors when inoculated into newborn monkeys (34). However, enhancement of tumor induction by cell division may be only a reflection of the increase of the cell DNA target during the cell division cycle (2).

Even in the RNA virus transformation system there appears to be a requirement for cell DNA synthesis.

Sachs (39) has found in the case of *in vitro* polyoma virus transformation, as well as *in vitro* transformation by X-irradiation and carcinogens, that cell division must occur within 3 to 5 days after the transforming event for the transformed properties to be genetically fixed. This inhibition of expression of the transformed change by resting cells requires protein synthesis during the period in which the cell is kept from dividing.

The stability of the cells' chromosomal complement also appears to influence the efficiency of viral transformation. When chromatid breaks or other abnormalities have been increased by X-irradiation or aging, transformation occurs more efficiently or more rapidly, as shown in Chart 2 (18). In fact, Todaro *et al.* (49) quantitatively have tested the efficiency of *in vitro* transformation of human cells by SV40 virus and found that cultures from patients with Fanconi's anemia, an autosomal recessive disease associated with a high incidence of chromosome abnormalities and spontaneous neoplasms, were transformed 10 times as efficiently as normal human cells.

Biochemical Events during Polyoma Virus Replication

Before discussing possible mechanisms of transformation by viruses, we should look at the specific biochemical events that have been shown to occur during the replicative cycle of polyoma virus in mouse embryo tissue cultures. These are schematically presented in Chart 3 and represent a composite of the experimental findings of a number of investigators. The earliest specifically identifiable material synthesized is the T antigen (23), demonstrable by complement fixation and fluorescent antibody staining (43). It is assumed that specific early proteins perhaps different from the T antigen are also produced, since protein synthesis is necessary at early times for later production of infectious virus (17) but not for transformation (33). Next, there is an increased production of several enzymes required for DNA synthesis, such as thymidine kinase and DNA polymerase (11). Whether these DNA enzymes are specific and coded for by the viral genome is not completely clear, but there is evidence that some have characteristics different from those of normal cells (29); yet they appear not to be identical with the T antigen (28). Somewhat later viral DNA replication occurs (17), and at practically the same time there is a stimulation of cellular DNA synthesis (11). This cell DNA synthesis occurs even in systems such as rat cells, where polyoma virus transforms but does not replicate or synthesize new viral DNA (16). Finally, viral coat antigen appears followed by infectious virus.

In Chart 3 all the events prior to the point of the second arrow take place in the lytic interaction and probably during transformation, although the latter has not been directly demonstrated. It would therefore appear that in a given cell infected with polyoma virus, the decision between the two mutually exclusive pathways of virus maturation with cell death on the one hand and virus integration with transformation and lack

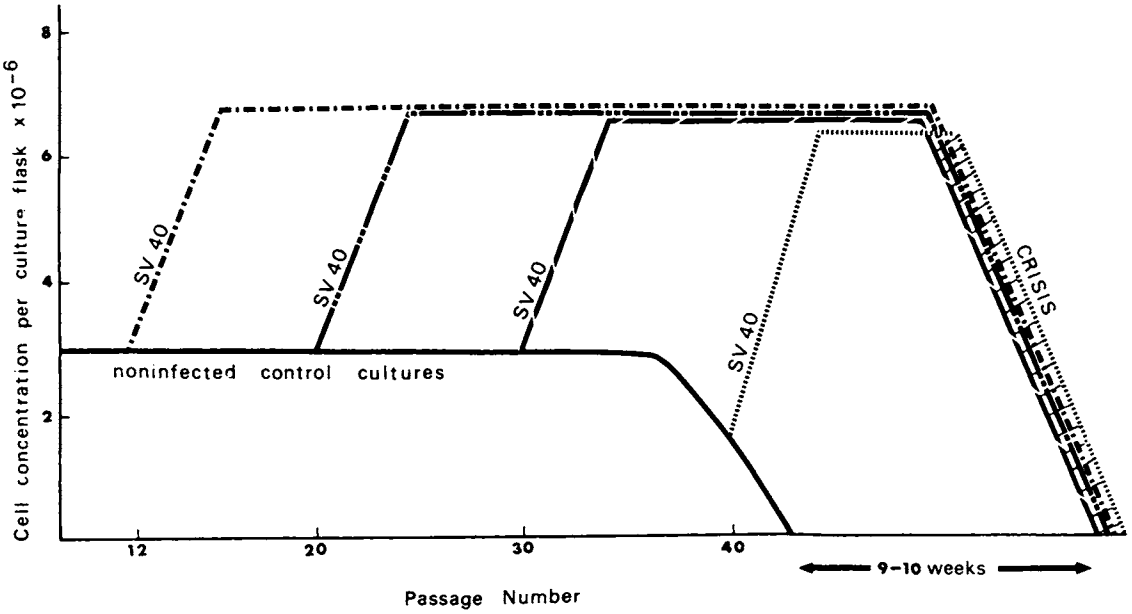

Chart 2. Human diploid cells exposed to SV40 virus at different stages of their life span. The "crisis" stage with marked cell lysis followed by establishment of transformed culture occurs at the same time in relation to age of culture (From Reference 18).

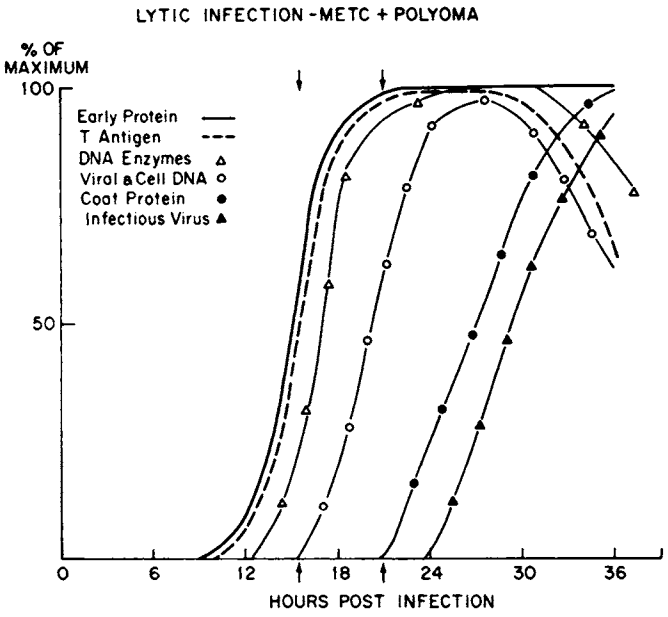

Chart 3. Schematic presentation of the kinetics of biochemical events occurring in the lytic cycle of infection of mouse embryo tissue cultures (METC) with polyoma virus.

of completion of virus replication on the other, must be made during the period between the two arrows.

Mechanisms of Virus-induced Transformation

At the time of polyoma infection one of the early events is a stimulation of cellular DNA synthesis, and correspondingly there is evidence suggesting that this may be necessary for integration of the viral genome into some permanent relationship with the genome of the cell. At the same time, there is inhibition of completion of the virus maturation cycle. It is logical to think that these events are interrelated, perhaps interdependent—that integration of virus DNA is responsible for the derepression of cell division and that uncontrolled cell division prevents virus maturation. That the latter may be true is suggested in the Shope papilloma DNA tumor virus system where virus maturation does occur in the tumor cell but not until that cell has come to the surface of the tumor where it becomes keratinized and dies (35). Yet it is interesting that in the polyoma system, even though there is suppression of virus maturation in the transformed cell, superinfection with the same virus is capable of initiating a complete lytic virus replication cycle, (24) even perhaps rescuing the original integrated and suppressed viral genome (45).

Besides the persisting viral nucleic acid, perhaps in some cases incomplete or defective, the only other regularly demonstrated viral-specific materials in the polyoma tumor cell are the T antigen in the nucleus and a new transplantation type of antigen on the cell surface. Normal cell division control mechanisms must certainly respond to stimuli at the cell surface, and the effect of such stimuli must be transmitted across the cytoplasm to the nucleus. The presence of the new tumor transplantation type antigen on the surface of the transformed cell may interfere with a regulatory signal coming from the cell's environment. Since the nuclear T antigen appears so early after the infection of the cell with polyoma virus, it is possible that it is in some way involved in derepressing the synthesis of cell DNA which takes place shortly thereafter. The production of T antigen does not require viral DNA syn-

thesis, the information for it being obtained from the original infecting virus particle (23). In the lytic infection cycle where new virus progeny is made and cell lysis and death are the end-result, there is not only early stimulation of cell DNA synthesis but later breakdown of cell DNA (5). It is interesting that this broken-down cell DNA has a molecular weight approximately equal to that of polyoma virus DNA (T. Ben-Porat and A. S. Kaplan, Correlation Between Replication and Degradation of Cellular DNA in Polyoma Virus Infected Cells. Virology, *32*: 457–464, 1967).

As mentioned earlier, there is now good evidence that the genome of the transforming polyoma virus persists in the tumor cell, but there is still the question of whether it is needed for continuation of the altered properties of the transformed cell. Certainly the viral genome is not easily eliminated, and in its integrated state it has characteristics different from those during complete viral replication. The ts-a gene, which is sensitive to heat in the lytic cycle, is resistant once transformation has occurred (13). Likewise, although virus replication and T antigen production are susceptible to inhibition by interferon before integration (37), there is no effect of interferon on T antigen production by the already transformed cell (36). Also the thymidine kinase in transformed cells is normal, whereas that stimulated by lytic infection is qualitatively different from normal enzyme (29). That the persisting viral genome is playing an important role is suggested by the fact that surface antigens and indeed cellular morphology is determined by the virus. Furthermore, the degree of oncogenicity of cells doubly transformed by polyoma virus and SV40 seems enhanced over singly transformed cells. Not only do viral genes persist, but in cell hybrids between polyoma-transformed and normal mouse cells, the oncogenicity and tumor antigen production are dominant (8). The fact that these hybrids are still tumorigenic suggests that this property was not due to lost genetic material in the original parental transformed cell and indicates a positive role of the integrated viral material. In other cell hybrids between virus-free SV40-transformed hamster or human cells and susceptible normal green monkey kidney cells, induction of maturation of infectious virus has been shown to occur at a low efficiency (15). It is not known in which nucleus of the hybrid cell the virus replicates, but at least some repressor of viral maturation in the tumor cell is rendered inoperative in the heterokaryon.

The point of action of the virus in upsetting cell control mechanisms need not be at only one site. In fact, this site may be different in the case of RNA and DNA tumor viruses or even between two different DNA viruses. The work of Todaro at NYU (50) and Takemoto (42) in my laboratory is of interest in this connection. They have shown that a single cell can be doubly transformed by two different DNA tumor viruses, and these cells appear to have a greater growth potential than tumor cells induced by only one virus. This not only suggests different sites of action on cell control mechanisms but also infers different sites for the possible integration of the viral genomes.

Now one of the very factors which at the *cellular* level may be involved in maintaining the malignant properties may actually help to protect the *intact animal* from development of a gross tumor. The new transplantation type of antigen on the surface of the virus-induced tumor cell is recognized as a foreign antigen by the immunologically competent host which reacts in a homograft type of rejection, thus suppressing or destroying its own transformed cells before they can divide sufficiently to produce a significant tumor (22). Naturally occurring polyoma virus infection is prevalent in many mouse colonies, yet a spontaneous polyoma tumor in these animals is practically never seen. However, as shown by Dr. Law of the National Cancer Institute (31), if such animals are made immunologically incompetent by thymectomy at birth, they do indeed develop polyoma tumors after such natural exposure to infection. These immunologic factors will be discussed more completely by Dr. Amos.

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