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Minireview

SV40: Cell transformation and tumorigenesis

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ABSTRACT

The story of SV40-induced tumorigenesis and cellular transformation is intimately entwined with the development of modern molecular biology. Because SV40 and other viruses have small genomes and are relatively easy to manipulate in the laboratory, they offered tractable systems for molecular analysis. Thus, many of the early efforts to understand how eukaryotes replicate their DNA, regulate expression of their genes, and translate mRNA were focused on viral systems. The discovery that SV40 induces tumors in certain laboratory animals and transforms many types of cultured cells offered the first opportunity to explore the molecular basis for cancer. The goal of this article is to highlight some of the experiments that have led to our current view of SV40-induced transformation and to provide some context as to how they contributed to basic research in molecular biology and to our understanding of cancer.

This is certainly not a history of SV40 and transformation. I am too young to either remember anything that can be called history, or to have the perspective necessary to place events in a historical context. Rather this is an attempt to understand how present day models of transformation and tumorigenesis emerged, and to highlight the role SV40 plays in the continuing development of these concepts. Hopefully this process will display gaps and weak points in current theory and thus help formulate the next generation of hypotheses and experiments.

Most major advances in our understanding of biology are the consequence of the development of new experimental methods. The driving force behind the development of new methodologies is the need to break through barriers that prevent scientists from satisfying their curiosities. This was certainly true in the 1960's when tumor viruses emerged as important experimental tools. Many of the most urgent questions of the day stemmed from a desire to understand cell biology. The fundamental questions were how do eukaryotes replicate their DNA, regulate gene expression, translate mRNA and synthesize proteins. How do cells know when to divide and when to remain quiescent? How is differentiation effected? What controls development? Still other questions of the day were disease focused. How does cancer arise? What causes birth defects? How can we prevent epidemics?

By 1960 the value of model systems in the discovery of fundamental molecular mechanisms was clearly established. The bacteriophages were extensively studied and proved to be particularly powerful models. Small, tractable genetic systems that are easy to grow and manipulate in the laboratory, bacteriophages allowed genetics and molecular biology to be combined, and this proved to be a fruitful marriage. This lesson was not lost on animal virologists. SV40 became a focus of study because it could be easily propagated in the laboratory, and because it formed well-defined plaques. The viral life cycle of approximately 4 days was short enough for practical experimental manipulation. Because it had such a small genome it was assumed that most viral functions (replication of viral DNA, expression of viral genes, assembly and release of progeny virions) would be carried out by cellular components. Furthermore, since SV40 induced tumors and transformed cells it offered one of the first molecular windows on cancer. Thus, understand the virus, and understand the cell.

Transformation and tumorigenicity

The ability to grow cells in culture drove major advances in cancer biology and virology. Viruses could be propagated in culture, free of the adaptive immune system, allowing genetic screens to identify viral genes, and provided fodder for the study of the molecular events occurring during infection such as nucleic acid replication, gene expression, and virion assembly. Furthermore, cell culture systems allowed the study of the effects of viral infection on cell biology, as well as the identification of the viral genes responsible for specific effects.

In cancer research, cultures of tumor-derived cells allowed the study of cancer cell biology. However, two important elements were missing, and without them the full promise of cell culture could not be fulfilled. First was the ability to compare the biology of cancer cell lines to that of their normal counterparts. Cancer cells were hard enough to establish in culture, and cells from noncancerous tissue remained viable for a limited number of passages at best. More importantly, the noncancerous cells that did culture were not of the same origin as the cultured tumor cell lines, making a comparison of limited value. This remains a vexing issue in cancer research today.

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The second missing element was the ability to mimic tumorigenesis in cell culture.

The link between animal cancer models and cell culture systems was provided by a landmark paper published by W. R. Earle and colleagues in 1943 (Earle et al., 1943b). In a series of carefully documented experiments, they showed that noncancerous cells cultured in the presence of methylcholanthrene, an agent known to induce tumors in animals, gradually "transformed" from a fibroblastic appearance to a more epithelial morphology. Furthermore, the "transformed" cells were tumorigenic when injected into mice (Earle et al., 1943a). Thus, the properties of transformed cells could be compared directly to the parental culture from which they were derived.

However, there was still an issue with these types of experiments. The primary cultures used in these experiments contained a mixture of different cell types. The gradual transformation observed by Earle most likely resulted from the overgrowth of the culture with the descendents of a small number (perhaps just one) of cells that acquired oncogenic mutations as a result of the treatment with methylcholanthrene. Thus, it was impossible to know the representation of that particular cell-type in the culture. If the transformed cells that overgrow the culture were derived from a minority cell-type in the population, then the utility of comparing the properties of the transformed cells with the parental culture would be limited. A solution to this issue was provided by the development of methods for the isolation and culture of single cells (Sanford et al., 1948).

The development of mixed and clonal cultures of normal cells and the study of their properties led to the development of *in vitro* assays for transformation. For example, it was found that cells derived from skin or muscle exhibit contact inhibition of growth and movement in culture (Abercrombie and Heaysman, 1954). The observation that cells infected with Rous sarcoma virus do not growth-arrest or cease movement upon contact was the first evidence of *in vitro* transformation by the oncogenic retroviruses (Groupe and Manaker, 1956; Lo et al., 1955). The dense focus assay, which is widely used to assess oncogenic activity, was subsequently developed as a quantitative measure of *in vitro* transformation (Temin and Rubin, 1958). This assay depends on the ability of transformed cells to escape contact-mediated growth arrest and thus to continue to proliferate in the presence of neighboring growth-arrested normal cells. The result is the appearance of dense foci of multilayered cells growing on top of the monolayer of normal cells. Since each focus is the clonal outgrowth of a single transformed cell, the frequency of transformation can be determined under various conditions. Loss of contact inhibition and the ability to form dense foci correlate with tumorigenicity (Aaronson and Todaro, 1968).

Following the development of the dense focus assay, several other methods were developed to distinguish transformed and normal cells. These include anchorage independent growth, in which cells are suspended in dilute agarose or methocel so that they cannot interact with a surface (Macpherson and Montagnier, 1964). Many transformed cells growth-arrest under these conditions, although they may remain viable as single cells for weeks. In contrast, some transformed cells continue to proliferate and can be visualized as ball-like clusters under a microscope. Again, tumorigenicity is linked to anchorage independent growth (Shin et al., 1975). Another commonly used assay for transformation depends on the ability of transformed cells to survive and continue to proliferate in the absence of serum. Most cells are cultured in a rich basal medium supplemented by serum. Normal cells growth arrest and/or die in the absence of serum, while many types of transformed cells grow independent of serum. For example, SV40-transformed cells grow in the absence of serum (Smith et al., 1971). A more targeted form of this approach utilizes defined, serum-free medium. Some nontransformed cells proliferate in defined medium, supplemented with purified growth factors rather than serum. In the absence of an essential growth factor, the cells growth-arrest and undergo apoptosis. Transformed cells can be detected as those that bypass the requirement for an essential growth factor (Chiang et al., 1985).

Murine polyomavirus was shown to induce cell transformation in 1959 (Dawe and Law, 1959). Furthermore, murine polyomavirus-transformed cells are tumorigenic (Vogt and Dulbecco, 1960). Thus, by 1960 the refinement of cell culture techniques and studies of the biology of the oncogenic retroviruses and of murine polyomavirus set the stage for the *in vitro* studies of cancer. Three very different entities – chemical carcinogens, retroviruses and murine polyomavirus – were shown to share two common properties. Each induces tumors in animals, and each induces the transformation of cultured cells. At this point in time it was not clear whether transformation was induced by a single molecular action that then pleiotropically resulted in the simultaneous acquisition of the multiple properties of transformed cells, or if transformation resulted from multiple independent events. Viruses appeared to offer the most direct path to the answer, and the tumor viruses came under intense study.

SV40 induces tumors in laboratory animals and transforms cells in culture

The discovery of SV40 and the development of a robust plaque assay for the virus introduced a new model-transforming virus to the arena (Stinebaugh and Melnick, 1962; Sweet and Hilleman, 1960). SV40 was shown to induce tumors in newborn hamsters and was confirmed as a DNA tumor virus (Ashkenazi and Melnick, 1963; Black and Rowe, 1964; Eddy et al., 1961; Gerber and Kirschstein, 1962; Kirschstein and Gerber, 1962; Rabson and Kirschstein, 1962; Rabson et al., 1962). Furthermore, SV40 was shown to induce transformation of many different types of cultured cells, including both rodent and human cells (Black and Rowe, 1963; Black et al., 1963; Girardi et al., 1965; Jensen et al., 1963; Koprowski et al., 1962, 1963; Moyer et al., 1964; Ponten et al., 1963; Shein and Enders, 1962; Todaro and Green, 1964, 1966; Todaro et al., 1963, 1964, 1966). These early studies of SV40 transformation were greatly aided by the development of the first untransformed postcrisis cell line, 3T3 cells (Todaro and Green, 1963).

The development of untransformed established cell lines allowed the characterization of events following viral infection leading to transformation. Similarly, the infection of permissive cells with SV40 was shown to lead to a synchronous program that could be monitored with the emerging molecular techniques of the day. Typically productive infection was studied in one of several established African green monkey kidney cell lines, such as BSC or CV1. In these cells, the infectious cycle takes 3–4 days and results in cell death and the production of about 300 infectious progeny virions per infected cell.

In contrast, SV40 transformation was studied in rodent cell lines, such as the established mouse cell line, 3T3 (Todaro and Green, 1963). Rodent cells are nonpermissive for SV40 productive infection, and thus no progeny virions are produced when these cells are infected. However, viral attachment, penetration and uncoating proceed normally in these cells and the early viral proteins are expressed. Infection is blocked because viral DNA replication and late gene expression do not occur. In fact, within a few days following a high multiplicity infection of mouse or rat cells with either polyomavirus or SV40, the entire cell population acquires a transformed morphology (May et al., 1971; Smith et al., 1970, 1971; Stoker, 1968). This is termed abortive transformation, because the phenotype lasts for a few days before cells resume a normal untransformed appearance. Stable transformants emerge from this population with a low but measurable frequency, about one cell in every thousand converts to a stable transformed phenotype. However, these rare stable transformants are easily detected because after a few days of culture, their clonal descendants overgrow the monolayer to form dense foci.

Infection with either SV40 or polyomavirus results in increased synthesis of cellular RNAs and proteins (Frearson et al., 1966; Kit et al.,

1966, 1967b; May et al., 1976; Oda and Dulbecco, 1968; Winocour and Robbins, 1970). Furthermore, infection of either permissive or nonpermissive cells with these viruses stimulates quiescent cells to undergo several rounds of DNA synthesis (Gershon et al., 1965, 1966; Hatanaka and Dulbecco, 1966; Henry et al., 1966; Kit et al., 1967a; Winocour et al., 1965).

By the early 1970s, these observations had coalesced to a model in which an SV40-encoded protein(s) induced quiescent cells to increase transcription and synthesize DNA in order to use the cell machinery to carry out viral infection. It was reasoned that the small genome size of SV40 and polyomavirus precluded them from encoding their own DNA polymerase or other proteins needed to replicate viral DNA. Furthermore, the large amount of viral DNA to be replicated (about 100,000 progeny DNA molecules/cell) required the increased synthesis of nucleotides, and thus increased amounts of the enzymes involved in their production.

The *A* gene of SV40 encodes large tumor antigen

SV40-induced tumors that were transplanted into fresh syngeneic hosts were rejected and cleared. The antigen that induced this rejection was termed tumor specific transplantation antigen or tsta (Defendi, 1963; Khera et al., 1963; Koch and Sabin, 1963). While it was known that tsta was only detectable in SV40-transformed or infected cells, it was not clear whether the antigen was virus-encoded, or if it was a host antigen that was unmasked by infection or transformation. Similarly, serum from tumor bearing hamsters or mice was used to detect an antigen (tumor antigen) induced by SV40 infection or transformation (Rapp et al., 1964). Such sera immunoprecipitated a protein of 90–100 kDa from ^{35}S-labelled cellular extracts. However, again it was not known whether this was a virus-encoded or host-encoded antigen. Tumor antigen was shown to be expressed within the first few hours following SV40 infection, prior to the replication of viral DNA and the synthesis of the viral capsid proteins: VP1, VP2, and VP3. Furthermore, unlike the capsid proteins, tumor antigen was expressed during the abortive infection of rodent cells.

Because SV40 grows well in culture and forms well-defined plaques it offered a tractable genetic system and a number of groups isolated temperature-sensitive viral mutants (Ishikawa and Aizawa, 1973; Kimura and Dulbecco, 1972; Robb and Martin, 1972; Robb et al., 1974; Rothschild and Lockwood, 1976; Tegtmeyer et al., 1970; Tegtmeyer and Ozer, 1971; Tevethia and Ripper, 1977; Yamaguchi and Kuchino, 1975). These mutants fell into four genetic complementation groups. Temperature-shift experiments established that groups B, C, and D replicated viral DNA but were defective for late-viral functions, while A mutants appeared to affect an early viral function and were defective for viral DNA replication at the nonpermissive temperature.

The discovery of restriction enzymes and their application to SV40 resulted in the first physical map of any DNA molecule (Danna and Nathans, 1971). This was especially valuable in these years prior to DNA sequencing. First, a physical map allowed all of the SV40 temperature-sensitive mutants to be physically located on the viral DNA (Lai and Nathans, 1974b; Lai and Nathans, 1975). DNA fragments could be isolated from specific locations on the viral DNA and used as probes in the newly developed Southern and Northern hybridization methods. Finally, deletion mutations could be generated *in vitro* by specifically excising DNA fragments from the viral genome (Lai and Nathans, 1974a).

A beautiful early application of *in vitro* mutagenesis demonstrated unambiguously that SV40 tumor antigen (T antigen) is virus encoded (Rundell et al., 1977). While wild-type SV40 DNA induced a T antigen that was detected as a 94 kDa protein following immunoprecipitation and SDS-gel electrophoresis, *dl1001*, a deletion mutant in which a DNA fragment was excised from the A gene, produced a 33 kDa T antigen. This was consistent with a series of other experiments in which restriction fragments containing the early promoter and A gene were microinjected into cells and shown to produce T antigen (Graessmann and Graessman, 1976; Graessmann et al., 1975), or in which purified A gene protein was microinjected into cells and shown to produce a protein that reacted with anti-tumor serum (Tjian et al., 1978). Similar approaches established that T antigen is tsta (Chang et al., 1979). Later experiments showed that cytotoxic lymphocytes (CTLs) select for T antigen variants in transformed cells (Lill et al., 1992).

SV40-transformed cells contain integrated viral DNA

SV40-transformed cell lines continue to express T antigen, and stably display the hallmarks of transformation through many generations. How is the T antigen gene passed from a dividing cell to its daughter? One explanation was offered by studies of the temperate bacteriophage, lambda. During lysogeny lambda integrates at a specific location, both with respect to the *E. coli* chromosome, and the lambda genome. Did SV40 DNA integrate into the cellular chromosome, thus explaining continued T antigen expression through multiple cell generations? If so, did the interligation occur at a specific site on the chromosome? Is there a specific viral mechanism to facilitate integration, as is found in bacteriophage lambda?

Early studies looked for the presence of SV40 DNA in transformed cells. The experimental approach was based on DNA hybridization kinetics and was technically demanding. However, these experiments clearly indicated that SV40 DNA was present through many generations of transformed cells (Sambrook et al., 1968; Westphal and Dulbecco, 1968). Furthermore, viral early mRNA was present in SV40-transformed cells (Khoury et al., 1973; Lee and Nathans, 1975; Ozanne et al., 1973). The advent of Southern transfer and hybridization methods allowed this issue to be examined at a much higher resolution. SV40 transformed cells contain integrated SV40 DNA (Botchan et al., 1976; Ketner and Kelly, 1976). The SV40 DNA is integrated at random positions with respect to the cellular chromosomes. The exact integration site also appears to be random with respect to viral DNA. However, SV40 integration in all transformed cell lines is such that the SV40 early promoter and T antigen coding sequences are intact, thus insuring continuous T antigen expression.

Large T antigen is essential for transformation

With the introduction of multiple new experimental tools including restriction endonucleases, Southern and Northern hybridization, S1 nuclease and heteroduplex mapping, the genome organization of SV40 was gradually revealed. The map was centered by *ori*, the 64 bp minimal origin of viral DNA replication. The T antigen gene, or A gene, encompassed roughly half the DNA, and was expressed early after infection. The capsid proteins were encoded by the late region, a unit expressed after the onset of viral DNA replication.

Two lines of evidence were used to establish that T antigen function is necessary for transformation. First, restriction endonuclease fragments of SV40 DNA containing the early region are sufficient for transformation, while shorter fragments or fragments encoding the late region do not transform (Graham and van der Eb, 1973). Second, temperature-shift experiments with SV40 ts mutants showed that *A* gene function is required to maintain transformation (Brockman, 1978; Brugge and Butel, 1975; Kimura and Itagaki, 1975; Martin and Chou, 1975; Noonan et al., 1976; Osborn and Weber, 1975; Tegtmeyer, 1975).

Studies of revertants of SV40-transformed cell lines enforced the notion that T antigen was essential for transformation, and provided a clear demonstration that the multiple properties of SV40-transformed cells were not always coordinately displayed (Vogel and Pollack, 1973; Vogel et al., 1973). Experiments that examined the properties of SV40-transformed lines that were obtained without any phenotypic selection also enforced this point (Risser and Pollack, 1974). These

studies showed that transformants could be obtained that displayed a subset of the transformed phenotype.

The discovery of small t antigen had to await the development and refinement of several techniques: S1 protection and heteroduplex mapping techniques so that splicing could be discovered, DNA sequencing to obtain the early region open reading frames, and immunological reagents, particularly monoclonal antibodies, that recognized the T antigens. Mutants of large T antigen but not small t antigen were used to show that small t antigen does not transform when expressed alone, but does contribute to transformation under some conditions (Bouck et al., 1978; Seif and Martin, 1979; Shenk et al., 1976; Sleigh et al., 1978).

Large T antigen: genetic analysis of a multifunctional protein

Three developments greatly speeded the genetic analysis of SV40, and specifically of large T antigen: DNA sequencing, site-directed mutagenesis, and the ability to insert the SV40 genome into bacterial plasmid vectors. SV40 was the second genome to be completely sequenced (Fiers et al., 1978; Reddy et al., 1978). The availability of the sequence not only provided the first view of a self-replicating genetic unit in eukaryotes, but also proved a boon for the genetic analysis of the virus, and particularly T antigen. The effect of mutations on protein primary structure could now be determined. The contributions of individual nucleotides to the functions of *cis* acting elements, such as *ori*, the promoters, or the transcriptional enhancer could be assessed. The development of methods allowing site-directed mutagenesis, again using SV40 as the target, was the next key armament (Shortle and Nathans, 1978). Now mutations could be specifically designed to test specific hypotheses or to facilitate genetic screens. T antigen was a principal target, and the initial genetic assault focused on learning how a single protein controlled multiple aspects of the virus life cycle including both viral DNA replication and transformation.

The problem was that since T antigen is required for viral DNA replication, many T antigen mutants were defective for virus propagation. Thus, there was no easy way to generate stocks of these mutants. Nonetheless, heroic efforts were made. One approach was to introduce small deletion mutations into the T antigen gene, and then propagate them by coculture with an SV40 mutant carrying a large deletion in the late region. These mutations could complement in *trans* and thus a mixed viral stock could be obtained. Virions carrying the relatively small T antigen deletion could then be separated from virions carrying the larger late region mutation by CsCl buoyant density centrifugation (Scott et al., 1976).

This period of SV40 genetics defined a new paradigm in genetics. Mutate the DNA and screen for a phenotype, rather than the traditional screen for a phenotype and then map the mutation to the DNA. This, in fact, was the original meaning of "reverse genetics." Many new mutations were generated throughout the SV40 genome (Carbon et al., 1975; Cole et al., 1977; Lai and Nathans, 1974a; Mertz and Berg, 1974; Shenk et al., 1976). These new mutants were added to the already existing collection of temperature-sensitive mutants, which were now sequenced (Loeber et al., 1989). The development of techniques that allowed the microinjection of DNA and proteins into cells provided another avenue for assessing the biological activity of T antigen truncations (Galanti et al., 1981). Still another tool in the genetic analysis of SV40 came from the study of super-T antigens (Lovett et al., 1982; May et al., 1981, 1983).

Perhaps the most bizarre, yet most powerful genetic tool utilized during this period was the collection of human adenovirus-SV40 hybrids. Some types of human adenoviruses are defective for growth in monkey cells. In order to facilitate vaccine production, attempts were made to select for variants of these human adenovirus strains that could be propagated in monkey cells. Stocks of human adenoviruses were in fact obtained by passage through monkey cells. These stocks fell into two broad categories, both of which had unusual properties. Some stocks consisted of human adenovirus in mixed culture with SV40. It turns out that human adenoviruses can grow in monkey cells so long as these cells are coinfected with SV40. This activity of SV40 is carried by T antigen, and specifically maps to a carboxy-terminal domain called the HR domain (Cole et al., 1979; Pipas, 1985).

This first class of stocks was not of use for studying T antigen functions, but the second class proved incredibly useful. These stocks consisted only of human adenovirus virions. However, a fragment of SV40 DNA was integrated into a region of the adenovirus genome that is not essential for growth in cell culture. Different adenovirus isolates harbored different amounts of SV40 DNA, and these hybrid viruses were used to map the SV40 genome (Kelly and Lewis, 1973; Lebowitz et al., 1974; Lewis et al., 1974; Lewis and Rowe, 1973; Morrow et al., 1973). All of these adenovirus-SV40 hybrids carried a portion of the T antigen gene expressed as a fusion protein. All of the T antigen fragments contained an intact carboxy-terminal region of the protein, but were missing various amounts of the amino-terminal portion. Thus, as a collection they provided a set of T antigen deletion that expressed various sized carboxy-terminal fragments. The shortest piece of T antigen found in one of these isolates encoded just the last 38 amino acids of T antigen. Thus, the adenovirus-helping function of T antigen resides in this small carboxy-terminal fragment. The exact function of this T antigen domain is still unclear today.

The discovery and characterization of viable adenovirus-SV40 hybrids uncovered a paradigm directly relevant to emerging infectious diseases, which remains underappreciated today. The principle is that nonhomologous recombination across virus families can result in a virus with an extended host-range. Does this type of genetic exchange occur in nature, and if so, does it represent a mechanism by which new viruses can arise?

The final tool needed to accelerate the genetic analysis of SV40 was the demonstration that the SV40 genome could be cloned and propagated in bacteria as a recombinant plasmid, and rescued from that plasmid to regenerate infectious SV40 virions (Peden et al., 1980). This same paper included the first demonstration that oncogenes, in this case the SV40 T antigens, can induce transformation when expressed from a plasmid that had been propagated in bacteria. With the advent of cloning, T antigen mutants that were defective for virus growth could be amplified in bacteria prior to study in mammalian cells.

The availability of this collection of genetic tools drove a detailed dissection of T antigen's various activities. Different functions were mapped to distinct positions on T antigen's primary sequence. During this process a large number of truncation and amino acid substitution mutants were generated that screened for function (Clark et al., 1983; Kalderon et al., 1982; Kalderon and Smith, 1984; Peden and Pipas, 1992; Pipas et al., 1980, 1983; Soprano et al., 1983). The characterization of these mutants led to our current view of T antigen structure. Large T antigen consists of four structural domains: a J domain at the amino-terminus, a sequence specific DNA binding domain (OBD), a zinc binding domain, and finally an ATPase domain. The structure of each of these domains has been obtained (Kim et al., 2001; Li et al., 2003; Luo et al., 1996). The J domain is connected to the OBD by a relatively unstructured linker that contains docking sites for several cellular proteins. The structure of the HR/Ad helper domain at the carboxy-terminus has not been solved, nor is there yet a structure for the intact protein. Therefore, we know little about the orientation of one domain relative to another, nor of the dynamics that govern their movement. The structure of the complete small t antigen has been solved (Cho et al., 2007). Like large T antigen, small t antigen has a J domain at its amino-terminus, followed by a uniquely structured domain that governs its interaction with the cellular phosphatase, pp2A.

Several important conclusions were reached regarding T antigen's transforming functions based on genetic studies. First, the large T

antigen/small t antigen common region (the J domain), as well as the conserved LXCXE motif located in the linker between the J domain and OBD, are both essential for transformation (Figge et al., 1988; Montano et al., 1990; Peden and Pipas, 1992; Pipas et al., 1983). Interestingly, a transformation-defective mutant mapping to the large T/small t common region can be complemented by the adenovirus E1A protein (Yaciuk et al., 1991). Second, sequences in the carboxy-terminal half of T antigen are required for transformation (Pipas et al., 1983; Zhu et al., 1992). Thus, T antigen possesses multiple activities that contribute to transformation (Dickmanns et al., 1994; Srinivasan et al., 1989). Furthermore, in some circumstances both the amino-terminal transforming sequences and the carboxy-terminal transforming function were required in concert to induce transformation, while under other conditions the amino-terminal functions were sufficient (Clayton et al., 1982; Colby and Shenk, 1982; Slinskey et al., 1999; Sompayrac and Danna, 1983; Srinivasan et al., 1989).

Another important result of this period was the identification of T antigen mutants that appear to separate different properties of the transformed phenotype. It was known that T antigen expression was sufficient to transform cells by several assays including immortalization, dense focus formation, anchorage independent growth and tumorigenicity in animals. Two models were considered to explain how this single viral protein altered so many cellular properties. In one model, T antigen functions as a pleiotropic effector. That is, all of the properties of a transformed cell are simultaneously acquired because T antigen acts on a key cellular target, and this key cellular regulator controls all of the phenotypes. Alternatively, T antigen could independently act on multiple targets, each regulating one cellular property. The observation that immortalization can be separated genetically from the induction of dense foci, and the finding that the induction of anchorage independent growth is genetically separable from tumorigenicity strongly supported the latter model (Tevethia et al., 1988; Thompson et al., 1990).

The intense focus on SV40 genetics and transformation, coupled with the availability of new molecular methods, led to many advances in cell biology tangentially related to transformation. These include the development of methods for mapping CTL recognition sites on T antigen (Anderson et al., 1988; Tanaka et al., 1988; Tevethia, 1983); the first description of non-homologous end-joining, or NHEJ (Wilson et al., 1982); and the discovery of the nuclear localization signal (Butel et al., 1969; Kalderon et al., 1984a, 1984b; Lanford and Butel, 1984). These genetic studies set the stage for uncovering the mechanisms by which SV40 induces cell transformation.

The relationship between viral replication and cellular transformation

The early observation that T antigen was required for both viral DNA replication and for the stimulation of cellular DNA synthesis led to a model for SV40 transformation. This model assumed that the mechanism that T antigen uses to initiate and drive viral DNA replication also applies to cellular DNA. Perhaps T antigen acted on cellular origins of replication, much as it does on the viral *ori*, and all the properties of a transformed cell are the downstream consequence of driving cells into S phase. This idea was supported by experiments showing that SV40 transformed cells have more replication origins than normal cells (Martin and Oppenheim, 1977).

A clear prediction of this model is that T antigen mutants that are defective for viral DNA replication should also be defective for stimulating cellular DNA synthesis and transformation. This fit with observations using temperature-sensitive mutants, since both viral DNA replication and transformation were lost at the nonpermissive temperature. However, the isolation of replication-defective T antigen mutants that retained the ability to transform clearly indicated that this model could not be correct (Gluzman and Ahrens, 1982; Kalderon and Smith, 1984; Manos and Gluzman, 1984, 1985; Peden and Pipas, 1985; Stringer, 1982). The description of the widely used COS cell line, a permissive monkey cell line expressing wild-type T antigen stemmed from these studies (Gluzman, 1981). Furthermore, mutants that retained the ability to replicate viral DNA, but were transformation-defective, were also obtained (Cosman and Tevethia, 1981; Peden et al., 1990).

SV40 induces transformation by binding key cellular proteins and altering their activities

The availability of high quality antibodies that specifically immunoprecipitated T antigen from cell extracts led to the development of combined genetic and proteomic approaches. These approaches were greatly enhanced by a collection of monoclonal antibodies specific for the SV40 T antigens (Harlow et al., 1981; Montano and Lane, 1984). These reagents set the stage for the discovery of cellular proteins that associate with the T antigens in infected or transformed cells. The combination of immunoprecipitation experiments with mutational analysis led to the identification of two critical targets that T antigen must act on to elicit transformation, the tumor suppressors p53 and pRb. Other chapters in this volume describe the details of these experiments. The salient points are summarized here.

T antigen–p53 complexes were identified both in SV40-transformed and infected cells (Lane and Crawford, 1979; Linzer and Levine, 1979). Furthermore, T antigen mutants defective for p53 binding are also transformation defective (Kierstead and Tevethia, 1993; Peden et al., 1989, 1998; Zhu et al., 1991). While the association of T antigen with p53 is necessary for transformation, it is not sufficient (Conzen and Cole, 1995). The interaction of T antigen with p53 blocks p53-dependent gene expression. This is partially due to the fact that T antigen binding blocks DNA binding by p53, thus it cannot associate with its target promoters (Bargonetti et al., 1992; Lilyestrom et al., 2006). Apparently T antigen also can block p53-dependent transcription and growth-arrest by mechanisms that are independent of direct association (Gjoerup et al., 2000; Quartin et al., 1994; Rushton et al., 1997). The interaction of T antigen and p53 also blocks T antigen's replicative functions (Lilyestrom et al., 2006; Tack et al., 1989; Wang et al., 1989). Under normal circumstances, p53 is unstable and does not accumulate to significant levels. However, as a consequence of its interaction with T antigen, p53 is stabilized and T antigen-transformed cells contain abundant amounts of the protein.

Similarly, the interaction of T antigen with the Rb family of proteins, pRb, p107 and p130, is essential for transformation (Chen and Paucha, 1990; DeCaprio et al., 1988; Ewen et al., 1989; Ludlow et al., 1989, 1990). In fact, it appears that all polyomavirus-encoded large T antigens interact with Rb proteins through their conserved LXCXE motif (Dyson et al., 1990). The current model is that T antigen must block the growth-suppressive functions of all three Rb proteins in order to induce transformation. This is consistent with the observation that the T antigen LXCXE motif is not required to transform cells in which an upstream negative regulator of the Rb proteins has been ablated, or that are lacking Rb proteins (Chao et al., 2000; Zalvide and DeCaprio, 1995). Interestingly, when the LXCXE motif is moved to the carboxy-terminus of T antigen, transformation is not affected (Tevethia et al., 1997b).

As discussed above, the third region of T antigen that is essential for transformation is the amino-terminal domain it shares with small t antigen. This region is a J domain, and T antigen functions as a DnaJ molecular chaperone (Srinivasan et al., 1997). The T antigen J domain is required to inactivate Rb proteins and, thus for transformation (Srinivasan et al., 1997; Stubdal et al., 1997; Zalvide et al., 1998). The function of the J domain is to recruit the cellular hsc70 chaperone protein so that energy derived from hsc70-mediated ATP hydrolysis can be used to disrupt the association of Rb proteins with E2F transcription factors (Sullivan et al., 2000, 2001). This model is

consistent with the structure of a T antigen fragment with pRb (Kim et al., 2001).

These efforts established that interaction of T antigen with three cellular targets contribute to transformation. The interaction of T antigen with hsc70 and the Rb proteins is required to free E2Fs from Rb-mediated repression, thus driving cells into S phase, while its interaction with p53 blocks this tumor suppressor's growth-repressive and proapoptotic functions. This model leaves a major discrepancy unresolved: how does this reconcile with earlier observations that both the amino-terminal half and the carboxy-terminal half of T antigen can independently induce transformation under some circumstances? This question will be discussed further below.

While the inactivation of Rb proteins and of p53 is essential for transformation, a number of studies indicate that these actions are not sufficient (Manfredi and Prives, 1990; Sachsenmeier and Pipas, 2001). Does SV40 act on additional cellular targets to elicit transformation? Several additional potential targets of T antigen have been identified (for a review, see Ahuja et al., 2005). Clearly small t antigen's action on the cellular phosphatase pp2A is required for transformation in some cases (Hahn et al., 2002; Pallas et al., 1990). In addition, large T antigen interactions with CBP/p300, TEF-1, Cul7, and Bub1 have been implicated in transformation (Ali et al., 2004; Berger et al., 1996; Borger and DeCaprio, 2006; Eckner et al., 1996; Hein et al., 2008; Kasper et al., 2005; Lill et al., 1997; Poulin et al., 2004). Finally, the IGF pathway has been reported to play a role in SV40 transformation (DeAngelis et al., 2006; Fei et al., 1995; Porcu et al., 1992; Sell et al., 1993). How these interactions contribute specifically to the transformed phenotype is the subject of current investigations.

Combining mouse and viral genetics: how does SV40 action on cellular targets contribute to tumorigenesis?

SV40 T antigen was the first viral oncoprotein to be expressed in transgenic mice and the fact that these mice developed neoplasia of the choroid plexus opened the door to specific phenotypes displayed in cell culture to tumorigenesis in animals (Brinster et al., 1984). Among the first mutants to be tested was dl1137, a deletion mutation that expresses the first 121 amino acids of T antigen fused to 11 missense amino acids followed by a premature stop codon. The resulting T antigen fragment includes the J domain and the LXCXE motif, but terminates just short of the NLS. This mutant had been shown to stimulate cellular DNA synthesis and transform some established cell lines (Soprano et al., 1983; Srinivasan et al., 1989). Two other mutants were also studied: one carrying an amino acid substitution in the LXCXE motif that renders it unable to associate with Rb proteins, while the other carries an in-frame deletion that inactivates the J domain, but is capable of binding Rb proteins and p53. These studies established that both the J domain and LXCXE motif were required to induce choroid plexus neoplasia (Chen et al., 1992; Saenz Robles et al., 1994; Symonds et al., 1993).

These studies confirmed that in the mouse, as in cell culture, the J domain and Rb-binding functions of T antigen were required for transformation. Results obtained with the mutant dl1137 were more intriguing and established an important paradigm of tumorigenesis (Saenz Robles et al., 1994; Symonds et al., 1993). This mutant induced choroid plexus neoplasia, but the tissue expanded much slower than seen with full-length T antigen. The number of proliferating cells was identical as that seen in transgenic mice expressing full-length T antigen. Furthermore, while little or no apoptosis was apparent in the choroid plexus expressing wild-type T antigen, abundant apoptosis is detected in dl1137 transgenic mice. Thus, the rate of tissue expansion is controlled by the balance of two factors, cell proliferation and cell death. Expression of dl1137 in p53 null mice results in rapidly growing tumors showing little apoptosis. Thus, the increased cell death seen in the dl1137 transgenic mice was due to this protein's inability to block p53. The dl1137 mutant, renamed T121, induces cell proliferation in a number of different mouse tissues showing that this effect is not limited to the choroid plexus (Simin et al., 2004).

When T antigen is expressed in the enterocytes of the mouse small intestine, these terminally differentiated cells are driven back into the cell cycle resulting in intestinal hyperplasia (Kim et al., 1994). The mutant N136 expresses the first 136 amino acids of T antigen, which includes the J domain, LXCXE motif, and NLS and no missense amino acids, and also induces intestinal hyperplasia. Furthermore, both the LXCXE motif and the J domain are required to induce enterocyte proliferation (Kim et al., 1994; Rathi et al., 2007). Thus, unlike the choroid plexus, the inactivation of Rb proteins does not induce a p53 response, and T antigen–p53 interactions play no role in the transformation of this cell type (Markovics et al., 2005). Similar results were obtained in the pancreas, where T antigen's ability to induce tumors is independent of its ability to bind p53 (Tevethia et al., 1997a).

This simple system allows a direct test of an important part of the SV40 transformation model. The model predicts that, as a consequence of Rb protein inactivation, E2Fs are freed to drive gene expression and cell proliferation. Thus, T antigen-mediated transformation should require E2F-dependent transcription. This prediction has been difficult to test in cell culture systems because of the functional redundancy of multiple E2Fs. However, in many tissue settings only a few or even a single E2F are expressed. Two major activating E2Fs, E2F2 and E2F3a, are induced upon expression of T antigen in enterocytes and their ablation greatly reduces T antigen-induced enterocyte proliferation (Saenz-Robles et al., 2007).

Studies from both transgenic and cell culture systems established that T antigen possesses at least two functions, Rb-inactivation and p53 inactivation, that contribute to transformation. In many instances, these functions cooperate to induce transformation. Similarly, different cellular oncogenes cooperate to transform cells (Land et al., 1983; Ruley, 1983). Both wild-type T antigen and the amino-terminal truncation mutants, such as dl1137, cooperate with the cellular oncogene ras to induce transformation (Michalovitz et al., 1987; White et al., 1992). In fact, T antigen possesses two independent activities, each of which can cooperate with ras (Beachy et al., 2002; Cavender et al., 1995).

Where are we now?

As of this date, our understanding of how SV40 induces transformation in cell culture and tumors in animals is incomplete. However, a number of the pieces of the puzzle are in place and the immediate questions are fairly clear. T antigen drives cells into the cell cycle by its action on the Rb proteins: pRb, p107, and p130. Quiescence is maintained, in part, by the presence of a repressive complex on the promoters of E2F-responsive genes. This complex, termed DREAM, is anchored on these promoters by E2F4, which directly binds to DNA, and p130, that is bound to E2F4. T antigen binds to E2F4 via its LXCXE motif and, in a reaction that requires hsc70-mediated ATP hydrolysis, destroys the repressive complex by dislodging E2F from p130, and sending p130 to the proteasome for degradation. This results in the derepression of E2F1, E2F2, and E2F3a, the activating E2Fs, that then bind to these same promoters and stimulate gene expression and S phase entry. This explains early observations that amino-terminal fragments of T antigen, as short as 121 amino acids, are capable of stimulating cellular DNA synthesis. This model also explains why truncated T antigens induce neoplasia in transgenic mice. However, we cannot yet conclude that T antigen action on Rb proteins is sufficient for these effects. The amino-terminal fragments encoded by dl1137 and N136 also contain binding sites for Cul7 and Bub1, both of which have been implicated in SV40 transformation. Thus, it is possible that T antigen action on these, or other yet to be discovered, targets is also required for transformation.

In many cell-types, the unscheduled stimulation of S phase resulting from Rb protein inactivation results in the activation of the

p53 pathway. The stabilization of p53 and subsequent transcription of p53-dependent genes results in cell cycle arrest and/or apoptosis. T antigen blocks this response by binding to p53 and preventing p53-dependent transcription. However, this may only be part of the story. As a consequence of its interaction with T antigen, p53 is stabilized and SV40-transformed cells contain large amounts of T antigen–p53 complexes. It is possible that these complexes contribute to some aspect of transformation, a so-called gain of function model. For example, the transcriptional adapter proteins CBP and p300 are recruited to T antigen–p53 complexes via their interaction with p53. One possibility is that the indirect interaction of T antigen with CBP/p300 plays some role in transformation, as it does with the adenovirus E1A protein.

Finally, a number of lines of evidence indicate that T antigen action on the Rb proteins and on p53 is not sufficient for transformation. A number of potential additional targets have been discussed above. One of the next challenges is to understand how these different T antigen actions are coordinated to induce the transformed phenotype.

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