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Studies of Nondefective Adenovirus 2-Simian Virus 40 Hybrid Viruses

VIII. Association of Simian Virus 40 Transplantation Antigen with a Specific Region of the Early Viral Genome

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Two of the five nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrids induce SV40 transplantation resistance in immunized hamsters. These two hybrids, Ad2$^+$ND$_2$ and Ad2$^+$ND$_4$, contain 32 and 43% of the SV40 genome, respectively. The pattern of induction of SV40 transplantation antigen (TSTA) by the various hybrids differentiates TSTA from both SV40 U and T antigens. Since the SV40 RNA induced by both these hybrids is early SV40 RNA, these findings confirm that TSTA is an early SV40 function. By combining available data on SV40 antigen induction by these hybrids with electron microscopy heteroduplex mapping studies, the DNA segment responsible for the induction of SV40 TSTA can be inferred to lie in the region between 0.17 and 0.43 SV40 units from the site on the SV40 chromosome cleaved by *E. coli* R$_1$ restriction endonuclease.

The presence of a virus-specific transplantation antigen (TSTA) in simian virus 40 (SV40)-induced tumors and transformed cells has been well documented (5, 7, 8, 15, 17). This antigen is detected by the resistance in SV40-immune animals to tumor induction by SV40 or SV40-transformed cells. It has been distinguished from SV40 T antigen by the lack of serologically detectable T antibody in the sera of SV40-immune hamsters who resist the development of tumors (3) and by the sensitivity of the TSTA antigen to freezing and thawing (4). By cytotoxicity assays, SV40 TSTA has been found on the surface of SV40-transformed cells, and immunization of animals with membrane-rich subcellular fractions from such cells inhibits the development of tumors after challenge with viable cells, whereas immunization with nuclear or soluble cytoplasmic fractions affords no protection (34). In lytically infected cells, the development of SV40 TSTA was not affected by inhibitors of DNA synthesis or low levels of actinomycin D, but was inhibited by inhibitors of protein synthesis and by a concentration of actinomycin D that inhibits early and late viral functions (6). Thus, TSTA appears to be an early SV40 function which produces viral-specific alterations on the surface of transformed or lytically infected cells.

To begin to elucidate the mechanism by which SV40 induces specific changes on the cell surface, it would be useful to define further the TSTA function by associating it with a specific region of the viral genome. Rapp et al. (28, 30) initially demonstrated that a subunit of the SV40 genome contained in the defective adenovirus 7 (Ad7)-SV40 hybrid, E46$^+$, induced SV40 TSTA. The segment of SV40 DNA within the genome of the Ad7-SV40 hybrid particles seems to induce only early SV40-specific antigens and early SV40 RNA (12, 23, 26, 29, 32). However, this SV40 DNA segment appears to consist of 70% of the SV40 chromosome (14), which is about twice the suspected size of the early region of the SV40 genome.

Recently, a group of five nondefective Ad2-SV40 hybrid viruses have been isolated (20, 21). Designated Ad2$^+$ND$_1$ through Ad2$^+$ND$_5$, these agents induce only early SV40 antigens and/or early SV40-specific RNA (19, 21) and contain Ad2 DNA covalently linked to SV40 DNA segments which comprise up to 43% of the SV40 genome (see Fig. 1; references 10 and 13). During the characterization of the nondefective Ad2-SV40 hybrids, we were interested in determining whether any of these agents were capable of inducing SV40-specific TSTA.

The purpose of this report is to characterize further SV40 TSTA by associating it with the SV40 DNA segment contained in the Ad2$^+$ND$_2$

hybrid, and to present the experimental data which were used to order the known antigen-inducing regions in the early portion of the SV40 genome (13, 25).

MATERIALS AND METHODS

Viruses. The isolation of the nondefective Ad2-SV40 hybrid viruses from the nondefective B55 hybrid pool has been described (20, 21). Ad2$^+$ND$_1$, Ad2$^+$ND$_2$, Ad2$^+$ND$_3$, and Ad2$^+$ND$_5$ were propagated in human embryonic kidney (HEK) cells, while Ad2$^+$ND$_4$ was grown in African green monkey kidney cells (AGMK). All virus stocks used in these experiments represented the third tissue culture passage after plaque isolation (B55 and Ad2$^+$ND$_4$) or plaque purification (Ad2$^+$ND$_1$, Ad2$^+$ND$_3$, and Ad2$^+$ND$_5$). Infectivity titers ranged from 3×10^8 to 1×10^9 PFU/ml by plaque titrations in HEK cells.

The prototype strain of Ad2 (strain Ad6) has been maintained by serial passage in HEK cells. The pool used in these experiments titered 6.9×10^8 PFU/ml in HEK cells.

SV40 strain 777 (2) has been maintained in our laboratory by serial, low-multiplicity ($<10^{-5}$ PFU/cell) passage in the BSC-1 line of AGMK cells (11). The three virus pools used in these experiments contained infectious virions in concentrations of 2×10^9, 3×10^8, and 4.4×10^8 PFU/ml in AGMK cells.

All virus pools were demonstrated to be free of mycoplasma by anaerobic culture on Hayflick medium (9), and all pools of Ad2 and hybrid viruses were shown to be free of adeno-associated viruses types 1 to 4 by complement fixation testing.

Cell lines. The SV40-transformed cell line (THK-1$_t$) used to challenge vaccinated hamsters was obtained from a subcutaneous tumor induced in weanling NIH hamsters by the THK-1 line (1) of SV40-transformed hamster kidney cells. After the fifth tissue culture passage, cells were concentrated by centrifugation ($500 \times g$ for 10 min); suspended in Eagle minimal essential medium containing 200 mmol of glutamine, 250 U of penicillin, and 250 μg of streptomycin per ml (EMEM), supplemented with 8% dimethylsulfoxide and 25% fetal bovine serum (FBS); and stored in sealed ampoules (50×10^6 cells/ml) in liquid nitrogen. Shortly before the hamsters were to be injected, the cells were rapidly thawed at 37 C and diluted in EMEM with 10% FBS. All THK-1$_t$ cells used for these experiments came from the same frozen lot.

Transplantation rejection tests. The techniques used to immunize and challenge hamsters were similar to those described by Rapp et al. (30). Groups (5–10 animals per group) of 3- to 4-week-old weanling hamsters were injected intraperitoneally at weekly intervals with 1 ml of virus stock for a total of three injections. Individual injections contained 10^7 to 10^8 PFU of SV40 per ml or 10^8 to 10^9 PFU of nondefective Ad2-SV40 hybrid viruses per ml. One week after the final injection the hamsters were challenged by subcutaneous injection of $10^{0.5}$, $10^{1.0}$, and $10^{1.5}$ tumor-producing doses (TPD$_{50}$) of THK-1$_t$ cells. Animals were examined weekly for the presence of tumors. The experiments were terminated 3 months after challenge.

RESULTS

Design of the animal experiments and titration of SV40-transformed THK-1$_t$ cells in hamsters. After initial tests to establish the optimum number of SV40-transformed cells to be used for the challenge, each experiment included nonimmune hamsters, hamsters immunized with a virus known to induce SV40 TSTA (either SV40 or a nondefective hybrid), and two to four groups of hamsters immunized with the agents to be tested for their ability to induce SV40 TSTA. With the exception of the B55 hybrid pool, all nondefective hybrids were tested in two separate experiments. The results of these experiments are presented in Tables 1 and 2.

The data in Table 1 indicate that approximately 3,000 THK-1$_t$ cells were required to produce tumors in 50% of the hamsters. Immunization with SV40 increased the TPD$_{50}$ (the number of THK-1$_t$ cells required to produce tumors in 50% of the hamsters) 30-fold.

These data were confirmed in another experiment by a 50-fold increase in resistance obtained by immunizing hamsters with the strain of SV40 isolated from the Ad2^{++} LEY hybrid (Table 2) (22).

Induction of SV40-specific TSTA by nondefective Ad2-SV40 hybrids. From the data in Table 2 it is apparent that immunization with the two nondefective Ad2-SV40 hybrids con-

TABLE 1. *Effects of SV40 immunization on the induction of tumors by THK-1$_t$ cells*

Immunizing virus	No. cells inoculated per hamster (log 10)	No. tumor-bearing hamsters/no. inoculated hamsters			TPD$_{50}$ (log 10)[a]	Resistance[b]
		Expt 1	Expt 2	Expt 3		
None	2.0	0/5				
	3.0	0/5				
	4.0	5/5	10/10	9/10	3.5	
	4.5		10/10	9/10		
	5.0	5/5	10/10	10/10		
SV40[c]	2.0	0/5				
	3.0	0/5				
	4.0	0/5	1/10	0/10	5.0	30
	4.5		6/10	1/10		
	5.0	3/5	9/10	2/10		

[a] To calculate the TPD$_{50}$, the data in experiments 1, 2, and 3 were pooled and evaluated by the method of Reed and Muench (31).

[b] Resistance = TPD$_{50}$ in immunized hamsters/TPD$_{50}$ in nonimmunized hamsters.

[c] Immunizing dose of SV40: Expt 1, 2×10^7 PFU/injection; Expt 2, 3×10^7 PFU/injection; Expt 3, 4.4×10^8 PFU/injection.

TABLE 2. *Effects of immunization with nondefective Ad2-SV40 hybrids on the induction of tumors by THK-1$_t$ cells*

Immunizing virus	Size of SV40 DNA segment within each hybrid[a]	No. cells inoculated per hamster (log 10)	No. tumor-bearing hamsters/no. inoculated hamsters		TPD_{50} (log 10)		Resistance[b]	
			Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Ad2$^+$ND$_1$	0.18	4.0	9/10	10/10	<3.8	<3.8	<2	<2
		4.5	10/10	9/10				
		5.0	10/10	8/8				
Ad2$^+$ND$_2$	0.32	4.0	0/10	1/10	4.6	4.8	13	20
		4.5	5/10	1/10				
		5.0	7/10	7/10				
Ad2$^+$ND$_3$	0.06	4.0	10/10	10/10	<3.7	<3.7	<1.6	<1.6
		4.5	9/10	10/10				
		5.0	10/10	10/10				
Ad2$^+$ND$_4$	0.43	4.0	1/10	0/10	4.8	4.8	20	20
		4.5	1/10	1/10				
		5.0	8/10	7/10				
Ad2$^+$ND$_5$	0.28	4.0	10/10	10/10	<3.7	<3.7	<1.6	<1.6
		4.5	10/10	10/10				
		5.0	10/10	10/10				
B 55	—[c]	4.0	2/10		≥5.1		≥40	
		4.5	1/10					
		5.0	1/10					
Ad2		4.0	8/10		<3.8		<2	
		4.5	10/10					
		5.0	10/10					
SV40/LEY[d]		4.0	0/10		5.2		50	
		4.5	0/10					
		5.0	1/10					

[a] Expressed as fraction of 1 SV40 genome equivalent (unit) as determined by electron microscopy (13).

[b] Resistance = TPD_{50} in immunized hamsters/TPD_{50} in nonimmunized hamsters = $10^{3.5}$.

[c] The size of the SV40 DNA segment within the genome of the hybrid virions in this pool has not been determined.

[d] Added as an additional control, SV40/LEY is the SV40 isolated from the Ad2$^+$ LEY hybrid (22). The pool used in this experiment was prepared in primary AGMK cells and contained 3×10^8 PFU of infectious SV40 virions per ml.

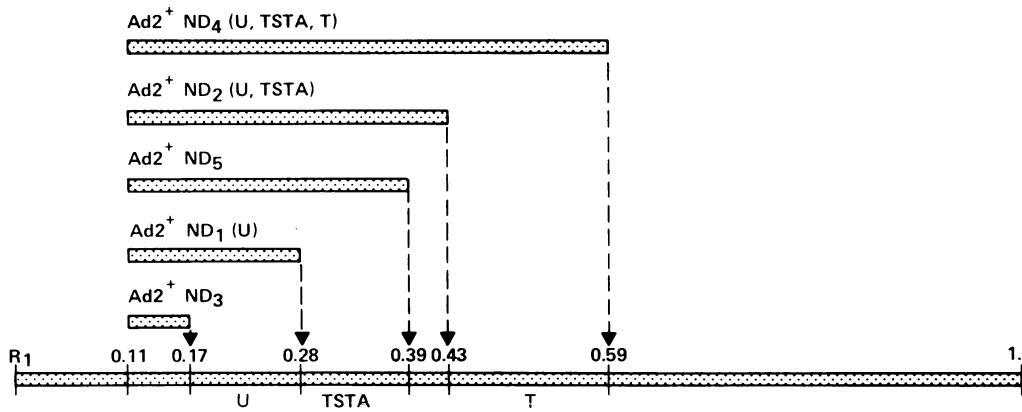

FIG. 1. *Location of SV40 DNA segments and functions in the nondefective hybrid genomes and the approximate location of these regions in the SV40 chromosome (adapted from Kelly and Lewis, and Morrow et al., 13, 24, 25). The placement of U, TSTA, and T along the R_1 linear should not be interpreted as the precise location of these antigen-inducing regions (see Discussion).*

taining the largest SV40 DNA segments ($Ad2^+ND_2$ and $Ad2^+ND_4$) increased the resistance of hamsters to tumor induction by THK-1_t cells by 13- to 20-fold.

In a single test, immunization with the B55 hybrid pool, which is biologically indistinguishable from $Ad2^+ND_4$ and from which all the nondefective Ad2-SV40 hybrids were derived, produced a 40-fold increase in the TPD_{50}.

Neither nonhybrid Ad2, $Ad2^+ND_3$ (containing 0.06 SV40 units [for a definition of SV40 unit see footnote *b*, Table 2]), $Ad2^+ND_1$ (0.18 SV40 units), nor $Ad2^+ND_5$ (0.28 SV40 units) induced detectable transplantation resistance in these experiments.

DISCUSSION

The experiments described in this paper correlate the size of the SV40 DNA segments contained within the nondefective hybrid genomes with the ability of these viruses to induce SV40 TSTA. Since SV40 U antigen is induced by the smaller SV40 DNA segment in the $Ad2^+ND_1$ genome and SV40 T antigen induction requires an additional segment of SV40 DNA present in the $Ad2^+ND_4$ genome, the induction of SV40 TSTA by the SV40 DNA segment within $Ad2^+ND_2$ differentiates this antigen from SV40 U and T antigens.

Girardi and Defendi (6) demonstrated that the development of SV40 TSTA in lytically infected cells was unaffected by inhibitors of DNA synthesis and concluded that this antigen represented an early SV40 function. In support of this conclusion, we point out that more than 90% of the SV40 RNA sequences present at 24 h in $Ad2^+ND_2$- and $Ad2^+ND_4$-infected Vero cells are identical (by nucleic acid hybridization competition) to SV40 RNA made early during the lytic cycle (19). Furthermore, Khoury et al. (submitted for publication) have shown that, like early RNA from SV40-infected cells, the SV40 RNA present at 24 h in $Ad2^+ND_2$- and $Ad2^+ND_4$-infected Vero cells reacts only with the minus or E strand of SV40 DNA (16, 33). Since it is likely that, in the inoculated hamsters, transcription of the SV40 DNA segments in the nondefective Ad2-SV40 hybrids is the same as transcription of these segments in lytically infected Vero cells, these data indicate that SV40 TSTA is induced by the SV40 DNA sequences on the minus SV40 strand and is an early SV40 function.

The findings presented in this paper have been used to order the known SV40 early antigen-inducing regions within the SV40 genome and to relate these segments to the R_1 site on the SV40 genome (Fig. 1; references 13, 24, and 25). Combining the data on SV40 antigen induction by the nondefective hybrid viruses with the electron microscopy heteroduplex mapping studies, it would appear that SV40 DNA lying between the termination site of the $Ad2^+ND_1$-SV40 DNA segment at 0.29 SV40 units from the R_1 site and the termination site for the $Ad2^+ND_2$-SV40 DNA segment at 0.43 SV40 units is required for TSTA induction. However, these data do not fix the precise location of the TSTA-inducing region of the SV40 genome. With reference to the orientation of the diagram in Fig. 1, the right-hand end of the TSTA-inducing DNA segment must lie between 0.29 and 0.43, while the left-hand end could extend to the left of 0.29 into the $Ad2^+ND_1$-SV40 DNA segment. Moreover, Patch et al. (27; and manuscript submitted for publication) have shown that all of the nondefective hybrid SV40 DNA segments contain late as well as early SV40 DNA. These data suggest that one or more late templates may exist within the early SV40 region as well as on either side. The exact manner in which these late SV40 DNA sequences interdigitate with the early SV40 DNA sequences in this region of the SV40 genome is not yet known. Thus, more detailed knowledge of the topography of the early region of the SV40 genome will be required to identify precisely the regions which induce the early SV40 antigens.

It should be noted that the $Ad2^+ND_5$ hybrid, which contains almost as long an SV40 DNA segment as $Ad2^+ND_2$, but fails to induce serologically detectable SV40 antigens, also failed to induce detectable SV40 TSTA. This could indicate that the right-hand end of the TSTA-inducing region lies to the right of 0.39 SV40 units; more likely, $Ad2^+ND_5$ fails to induce TSTA for the same unknown reason (perhaps a frameshift) that it fails to induce SV40 U antigen.

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