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Mapping of Simian Virus 40 Early Functions on the Viral Chromosome

JOHN F. MORROW, PAUL BERG, THOMAS J. KELLY, JR.,[1] AND ANDREW M. LEWIS, JR.

Department of Biochemistry, Stanford University Medical Center, Stanford, California 94305, and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

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The simian virus 40 (SV40) DNA segment in the nondefective adenovirus 2-SV40 hybrid, Ad2$^+$ND$_4$, is colinear with the segment between 0.11 and 0.59 SV40 fractional length from the site at which the R_1 restriction endonuclease cleaves SV40 DNA. This specifies the region of the SV40 DNA molecule which induces the early SV40 antigens: U antigen, tumor specific transplantation antigen, and T antigen. A variant of Ad2$^+$ND$_4$, called Ad2$^+$ND$_{4del}$, was found which has a deletion of the DNA segment between 0.50 and 0.57 SV40 fractional length from the R_1 endonuclease cleavage point.

Two useful tools for the analysis of the molecular and genetic structure of simian virus 40 (SV40) DNA have recently been discovered: the cleavage of SV40 DNA at specific sites by bacterial restriction enzymes (2, 5, 13-15); and a set of nondefective hybrid viruses of adenovirus 2 (Ad2) and SV40 (6-12).

The *Eco* R_1 restriction endonuclease (R_1 endonuclease), from *Escherichia coli* carrying an *fi*$^+$ R-factor (R. N. Yoshimori, Ph.D. dissertation, University of California, San Francisco, 1971), breaks both strands of SV40 DNA at a specific site (5, 13-15). As illustrated earlier (14, 15), this site provides a convenient reference point or origin to which other structural and genetic features on the SV40 chromosome can be related.

The nondefective Ad2-SV40 hybrids are viable recombinants between Ad2 and SV40. These hybrids (designated Ad2$^+$ND$_1$ through Ad2$^+$ND$_5$) induce early SV40 specific antigens or SV40 specific RNA during lytic infection, or both (11). DNA-RNA hybridization experiments have demonstrated that these viruses contain different quantities of SV40 DNA and induce overlapping SV40-specific RNA sequences in proportion to the amount of SV40 DNA which they contain (6, 9). It has been demonstrated that each nondefective hybrid contains a single segment of SV40 DNA covalently inserted at the same unique site in the Ad2 DNA molecule; furthermore the SV40 DNA segments of these hybrid viruses form a completely overlapping set with a common end point. These data made it possible to relate specific regions of the SV40 genome to the induction of specific SV40 antigens (7).

The SV40 DNA segment contained in the Ad2$^+$ND$_1$ hybrid DNA is colinear with a sequence on SV40 DNA between 0.11 and 0.28 SV40 fractional length from the R_1 endonuclease cleavage site (14; Fig. 1). In this paper we show that the SV40 DNA segment in Ad2$^+$ND$_4$ lies between 0.11 and 0.59 SV40 fractional length from the R_1 endonuclease cleavage site on SV40 DNA. This confirms the earlier conclusion (7, 9) that the SV40 DNA segment in Ad2$^+$ND$_1$ is contained within that of Ad2$^+$ND$_4$. These findings allow us to locate, relative to the R_1 endonuclease cleavage site, the regions of the SV40 chromosome which induce the early SV40 antigens.

The Ad2$^+$ND$_4$ virions used for these experiments were found to contain two kinds of DNA molecules: one in which the SV40 segment is colinear with the corresponding region of SV40 and a subclass in which the SV40 segment in the Ad2$^+$ND$_4$ hybrid molecules contains a deletion. This deletion occupies the approximate region 0.50 to 0.57 fractional SV40 length from the R_1 endonuclease cleavage site.

MATERIALS AND METHODS

Viral DNAs. The plaque isolation and passage of Ad2$^+$ND$_4$ in African green monkey kidney cells has been described (10). A pool of virus was prepared by high multiplicity infection (10-50 PFU/cell) of human embryonic kidney cells with stock virus representing the third tissue culture passage after plaque isolation. Infected cultures were harvested, the virions were purified, and viral DNA was extracted by previously

[1] Present address: Department of Microbiology, The Johns Hopkins University, Baltimore, Md.

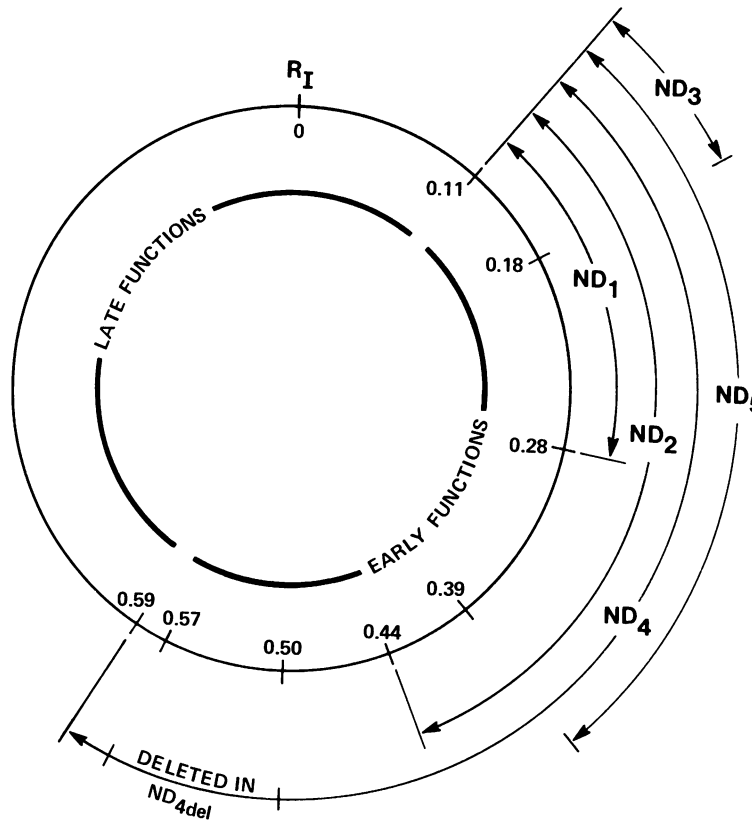

FIG. 1. *Map of the SV40 chromosome. The R_1 restriction endonuclease cleavage site is the origin. Positions are expressed in SV40 DNA fractional lengths from the origin. The innermost line marked "early functions" indicates the DNA segment which contains the early region. It does not imply that this DNA segment contains exclusively early functions (see Discussion).*

described techniques (7, 10). The same preparation of Ad2$^+$ND$_4$ DNA described by Kelly and Lewis (7) was used in these experiments. Ad2 was grown in KB-3 cells; a plaque isolated from SV40 strain Rh911 was grown in CV-1P cells at low multiplicity (14). The respective viruses were purified, and their DNAs were extracted as previously described (14). Covalently closed circular duplex SV40 DNA, SV40(I) DNA, was extracted from infected cells, purified, and converted to unit-length linear molecules, SV40 (L_{R1}), with R_1 restriction endonuclease (14). The R_1 endonuclease was the generous gift of H. W. Boyer and R. Yoshimori.

DNA denaturation and renaturation. Heteroduplexes involving Ad2 and Ad2$^+$ND$_4$ DNA strands as well as those including SV40(L_{R1}) were produced by alkaline denaturation and renaturation in 48% formamide (3). A mixture of 1.9 μg of Ad2$^+$ND$_4$ per ml, 1.0 μg of Ad2 per ml, and 0.25 μg of SV40(L_{R1}) DNAs per ml was annealed for 12 h at 25 C.

Electron microscopy. Samples were mounted for electron microscopy by the formamide technique (3) to permit measurement of single-stranded DNA. Shadowed grids were examined and photographed with a Philips EM300. Measurements were made with a Hewlett-Packard 9864A Digitizer and 9810A Calculator with a fully smoothed length calculation program giving an accuracy of ±0.5% and a higher degree of reproducibility on sample figures of known length.

RESULTS

To determine the location of the SV40 DNA segment contained in Ad2$^+$ND$_4$ DNA with respect to the R_1 endonuclease cleavage site on the SV40 chromosome, we denatured and renatured a mixture of Ad2, Ad2$^+$ND$_4$, and SV40(L_{R1}) DNAs to permit reassortment of DNA strands. The various heteroduplex DNA molecules produced were examined by electron microscopy: Ad2-Ad2$^+$ND$_4$ (Fig. 2A); Ad2-Ad2$^+$ND$_4$-SV40(L_{R1}) (Fig. 2B); and Ad2$^+$ND$_4$-SV40(L_{R1}) (Fig. 2C).

The Ad2-Ad2$^+$ND$_4$ heteroduplex (Fig. 2A), formed from one strand of Ad2 DNA and the other from Ad2$^+$ND$_4$ DNA, has been described (7). The longer single-strand segment is the SV40 DNA segment contained in Ad2$^+$ND$_4$;

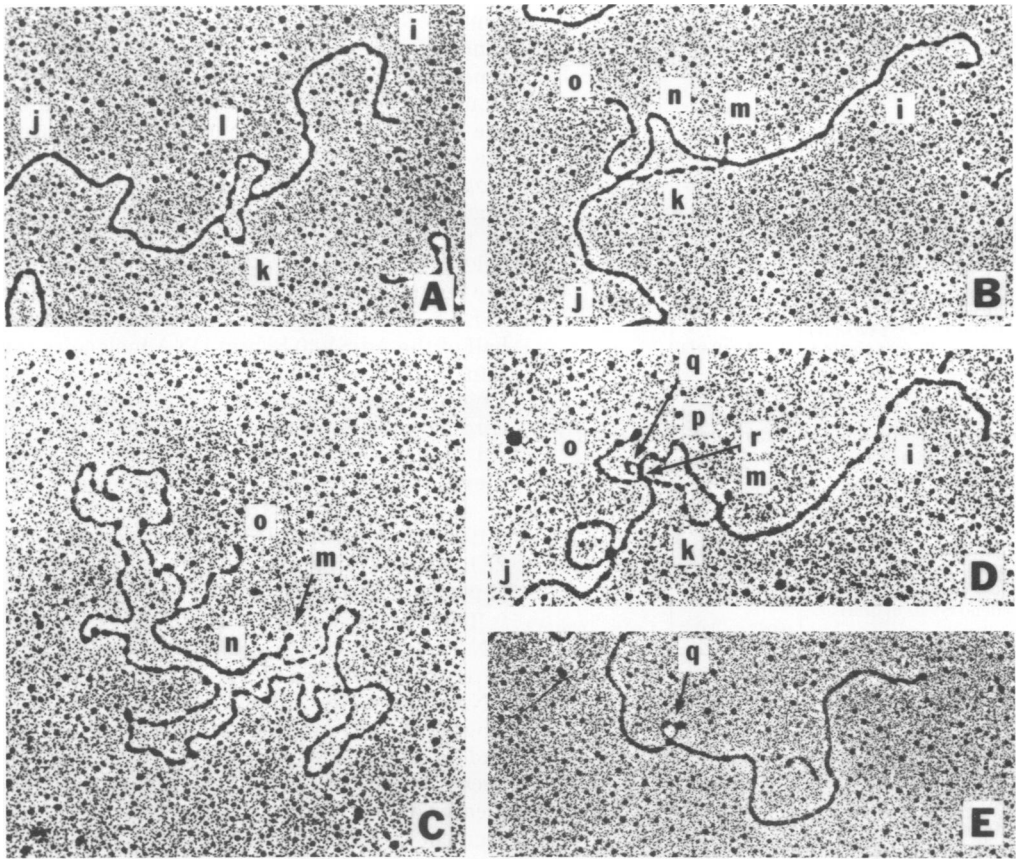

FIG. 2. *Electron micrographs of heteroduplex DNA molecules. A, A heteroduplex of Ad2 DNA and either Ad2⁺ND₄ or Ad2⁺ND₄del DNA. Segment 1: single strand from Ad2⁺ND₄ or Ad2⁺ND₄del DNA. Other segments as in B. B, An Ad2-Ad2⁺ND₄-SV40(L_{R1}) heteroduplex. Segments i and j: duplexes formed by Ad2⁺ND₄ and Ad2 DNAs. Segment n: duplex formed by Ad2⁺ND₄ and SV40(L_{R1}) DNAs. Segments m and o: single strands from SV40(L_{R1}) DNA. Segment k: single strand from Ad2 DNA. C, An Ad2⁺ND₄-SV40(L_{R1}) heteroduplex. Segments as in B. D, An Ad2-Ad2⁺ND₄del-SV40(L_{R1}) heteroduplex. Segment q: single strand of SV40(L_{R1}) DNA. Segments p and r: duplex formed by Ad2⁺ND₄del and SV40(L_{R1}) DNAs. Other segments as in B. E, An Ad2⁺ND₄-Ad2⁺ND₄del heteroduplex. Segment q: single strand present in Ad2⁺ND₄ DNA but absent in Ad2⁺ND₄del DNA. Magnification in each panel is ×30,000.*

the shorter single-strand represents the Ad2 DNA sequence deleted from Ad2⁺ND₄ (7). The molecule shown in Fig. 2B is a triple heteroduplex formed by annealing a strand of SV40 (L_{R1}) to the longer single-stranded segment of the Ad2-Ad2⁺ND₄ heteroduplex. These occurred at 10% the frequency of the Ad2-Ad2⁺ND₄ heteroduplexes. Because the duplex segment n (Fig. 2B) is continuous over its entire length, we may conclude that all of the non-Ad2 DNA in Ad2⁺ND₄ is colinear with SV40 DNA. This duplex segment has 47.3% of the length of SV40 DNA (Table 1). The two single-strand tails, m and o, are 0.114 and 0.395, respectively, SV40 fractional length (Table 1); consequently the SV40 DNA sequence in Ad2⁺ND₄ begins at 0.11 and continues to 0.59 SV40 fractional length from the R_1 endonuclease cleavage site. As expected the sum of the lengths of segments m, n and o equals, within experimental error, the length of an SV40 DNA molecule (Table 1). Similarly, the sum of lengths of segments i, k and j (Fig. 2B) is equal to the length of Ad2 DNA.

The molecule in Fig. 2C is a representative Ad2⁺ND₄-SV40(L_{R1}) heteroduplex. The Ad2⁺ND₄ strand is unpaired over most of its length but is circular because of an inverted terminal repetition (4, 19). The SV40(L_{R1}) strand pairs with the SV40 sequences in the Ad2⁺ND₄ to form a duplex segment n, while the rest of the SV40(L_{R1}) strand forms single-stranded segments m and o (Fig. 2C). Since these had the same lengths as the corresponding segments

TABLE 1. *Lengths of DNA segments of heteroduplexes shown in Fig. 2A, B*[a]

DNA segment	Fractional lengths	
	Ad2-Ad2$^+$ND$_4$-SV40(L_{R1})	Ad2-Ad2$^+$ND$_{4\ or\ 4del}$
SV40		
segment n or l (SV40 sequence in adeno-SV40 hybrid)	0.473 ± 0.013	0.432 ± 0.019
segment m [shorter single strand from SV40(L_{R1})]	0.114 ± 0.004	—
segment o [longer single strand from SV40 (L_{R1})]	0.395 ± 0.011	—
Total	0.982 ± 0.028	—
Ad2		
segment i (shorter duplex of Ad2 and adeno-SV40 hybrid)	0.139 ± 0.002	0.141 ± 0.002
segment j (longer duplex of Ad2 and adeno-SV40 hybrid)	0.794 ± 0.011	0.815 ± 0.013
segment k (Ad2 DNA deleted from adeno-SV40 hybrid)	0.050 ± 0.002	0.045 ± 0.002
Total	0.983 ± 0.015	1.001 ± 0.017

[a] Lengths are presented as mean fractional length ± twice the standard error of the mean. Measurements were made on 173 SV40(L_{R1}) molecules and 21 Ad2 homoduplexes in the same photographs with the 36 Ad2-Ad2$^+$ND$_4$-SV40(L_{R1}), 14 Ad2$^+$ND$_4$-SV40(L_{R1}), and 21 Ad2-Ad2$^+$ND$_{4\ or\ 4del}$ heteroduplexes. The length of SV40(L_{R1}) DNA (5,400 base pairs) was found to be 0.141 ± 0.002 of Ad2 DNA length. The designation of the various DNA segments is shown in Fig. 1. Contour lengths of single-stranded DNA segments k, l, m, and o were converted to double-stranded DNA equivalent lengths by multiplying the single-stranded lengths by 1.229 ± 0.026, the ratio of duplex SV40 DNA length to single-stranded SV40 DNA length on this grid (173 and 98 molecules, respectively).

in Ad2-Ad2$^+$ND$_4$-SV40(L_{R1}) heteroduplexes they are presented together in Table 1.

Occurring at 75% the frequency of the Ad2-Ad2$^+$ND$_4$-SV40(L_{R1}) heteroduplexes (Fig. 2B) were molecules identical to them except for a deletion loop in the duplex segment corresponding to the SV40 DNA sequence (Fig. 2D). For reasons presented below, we believe that this type of heteroduplex results from the presence of a variant of Ad2$^+$ND$_4$ present in this virus pool. This variant contains a short deletion within the SV40 DNA sequence and will be referred to as Ad2$^+$ND$_{4del}$.

In the Ad2-Ad2$^+$ND$_{4del}$-SV40(L_{R1}) heteroduplexes, the SV40(L_{R1}) strand pairs with the Ad2$^+$ND$_{4del}$ strand to form two duplex segments p and r, in Fig. 2D. These segments are 0.39 and 0.02 SV40 fractional length, respectively (Table 2). Separating them is a single-stranded DNA loop, q in Fig. 2D, with a length of about 0.07 SV40 fractional length. Since less than one in fifty of the SV40(L_{R1}) DNA strands contained any deletion, as judged by the absence of deletion loops after denaturation and renaturation, it seems likely that loop q results from a deletion in the SV40 DNA segment of the Ad-SV40 hybrid. Supporting this conclusion are measurements of the various segment lengths (Table 2); the sum of the lengths of segments m, p, q, r, and o from heteroduplexes of the type illustrated in Fig. 2D, as well as Ad2$^+$ND$_{4del}$-SV40(L_{R1}) heteroduplexes, equals the length of SV40 DNA (98.9%; standard error [SE], 1.7%). It appears, therefore, that nearly half of the Ad-SV40 hybrid DNA molecules contains a deletion of about 0.07 SV40 fractional length, within the SV40 DNA sequence. Accordingly, denaturation and renaturation of the Ad-SV40 hybrid DNA should yield homoduplexes and heteroduplexes with a deletion loop of about 0.07 SV40 fractional length located 0.194 Ad2 fractional length from one end (Fig. 2E); such heteroduplexes were found with the expected frequency.

The heterogeneity in the Ad-SV40 hybrid DNA population was not readily detectable in heteroduplexes formed from Ad2 and Ad2$^+$ND$_4$ DNAs (of the type in Fig. 2A). The single-strand lengths of the SV40 DNA segments in Ad2$^+$ND$_4$ and Ad2$^+$ND$_{4del}$ should not be distinguishable because of the variance of DNA lengths (3). Our measurements of the length of the SV40 DNA segment in such heteroduplexes shows a broad

TABLE 2. *Lengths of DNA segments of Ad2$^+$ND$_{4del}$-SV40(L_{R1}) heteroduplexes shown in Fig. 2D*[a]

DNA segment	Fraction of SV40 DNA length
segment r [shorter duplex of AD2$^+$ND$_{4del}$ and SV40(L_{R1})]	0.023 ± 0.004
segment q (single-stranded loop)	0.066 ± 0.006[b]
segment p [longer duplex of Ad2$^+$ND$_{4del}$ and SV40(L_{R1})]	0.391 ± 0.009
Total	0.480 ± 0.019

[a] Measurements are presented as mean fractional length ± twice the standard error of the mean, relative to 173 SV40(L_{R1}) molecules in the same photographs with the 27 Ad2-Ad2$^+$ND$_{4del}$-SV40(L_{R1}) and 12 Ad2$^+$ND$_{4del}$-SV40(L_{R1}) heteroduplexes measured. The DNA segments are as in Fig. 2D. Contour length of single-stranded DNA segment q was converted to duplex DNA equivalent length as described in the legend to Table 1.

[b] One of us (T.K.) made measurements of segment q in 16 Ad2$^+$ND$_4$-Ad2$^+$ND$_{4del}$ heteroduplex molecules and found it to be 0.098 ± 0.006 SV40 fractional length, located 0.193 ± 0.003 Ad2 fractional length from one end of the molecule.

distribution about a mean of 0.432 SV40 fractional length (SE, 0.9%), which agrees closely with an earlier determination by Kelly and Lewis (7). That length is very likely the average of the SV40 DNA segment lengths in the $Ad2^+ND_4$ and $Ad2^+ND_{4del}$ DNAs. Taking account of the estimated frequencies of the two types of DNA molecules, we would expect a mean length of 0.447 SV40 fractional length (SE, 0.8%).

DISCUSSION

The site at which the R_1 restriction endonuclease cleaves the SV40 DNA molecule can be used as a point of reference to locate or map discrete features of the SV40 genome (14, 15). In effect, by defining the R_1 endonuclease cleavage point as the origin of the map, other points on the DNA molecules can be located with respect to the origin in SV40 fractional length units. By using that convention, the SV40 DNA segment contained in $Ad2^+ND_4$ has been shown to be colinear with the region between 0.11 and 0.59 SV40 DNA map units (Fig. 1). Since the SV40 DNA segments of each of the other Ad2-SV40 nondefective hybrids are contained within the SV40 DNA segment of $Ad2^+ND_4$, and all share a common end point (6, 7) we can specify the location of each of these segments on the SV40 DNA map as well (Fig. 1).

An unexpected, interesting development from this study was the discovery of a subclass of $Ad2^+ND_4$ DNA molecules which contain a deletion within the SV40 DNA segment. The deletion occurs between 0.50 and 0.57 SV40 map units (Fig. 1). The deletion, therefore, lies outside the SV40 segments of the other nondefective hybrids. Studies to learn whether the deletion occurs within the DNA segment required for induction of T antigen are in progress, since $Ad2^+ND_4$ but not $Ad2^+ND_2$ induces the production of SV40 T antigen after infection.

The SV40-specific RNA synthesized after infection by $Ad2^+ND_4$ contains all of the RNA species made during the early phase of SV40 infection (9). Consequently, it appears that the SV40 DNA segment between 0.11 and 0.59 contains the region responsible for the expression of early functions (Fig. 1). It is likely, however, that this segment also has regions which are only transcribed late in SV40 infection, i.e., after SV40 DNA replication begins. Patch, Lewis, and Levine (17) have estimated that only about 36% of the SV40 DNA segment contained in $Ad2^+ND_1$ (the region between 0.11 and 0.28) is complementary to the RNA made early in SV40 infection; the remaining 64% of that region hybridizes only to RNA made late in infection. Assuming that the DNA segment specifying the early RNA species is continuous and that there is no other late SV40 RNA template region in $Ad2^+ND_4$, it is then reasonable to infer that all the DNA specifying early RNAs (and possibly functions) is contained in the SV40 DNA segment that lies between 0.22 and 0.59 on the SV40 DNA map. The size of this segment (0.37 SV40 DNA length) agrees well with earlier estimates of 0.3 to 0.4 (1, 16, 18) for the amount of SV40 DNA transcribed into the early RNAs in lytic infection.

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