A Colinear Map Relating the Simian Virus 40 (SV40) DNA Segments of Six Adenovirus–SV40 Hybrids to the DNA Fragments Produced by Restriction Endonuclease Cleavage of SV40 DNA

(SV40 DNA fragments/SV40-adenovirus hybrids/nucleic acid hybridization/genetic map)

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ABSTRACT The simian virus 40 (SV40) DNA segments present in a series of adenovirus-SV40 hybrids have been mapped with respect to the sites of cleavage of SV40 DNA by restriction endonucleases. Two approaches have been used. First, nucleic acid hybridizations were performed between equimolar quantities of the denatured DNAs. DNA molecules with adenovirus and adenovirus-SV40 hybrid transcripts of 11 DNA fragments obtained by cleavage of SV40 DNA by restriction endonuclease from Hemophilus influenzae. Secondly, selected fragments of SV40 DNA produced by the H. influenzae or H. parainfluenzae restriction endonucleases were used to form heteroduplex DNA molecules with adenovirus and adenovirus-SV40 hybrid DNA, which were then analyzed by electron microscopy. The two sets of data were consistent and have permitted alignment of the map of the SV40 segments of the hybrid viruses with the H. influenzae and H. parainfluenzae cleavage maps of SV40. Since cells infected with some of the hybrid viruses contain one or more SV40-specific antigens, the genetic determinants of these antigens could be localized on the cleavage map.

Two approaches to mapping the simian virus 40 (SV40) genome have recently been developed. One approach makes use of bacterial restriction enzymes that cleave SV40 DNA at specific sites. Of particular value for mapping studies are the R1 restriction endonuclease from Escherichia coli (1), which makes a unique double strand break in SV40 DNA (2, 3); restriction endonuclease from Hemophilus influenzae (4), which cleaves SV40 DNA into 11 fragments (5); and restriction endonuclease from Hemophilus parainfluenzae (6), which produces four fragments from SV40 DNA (7). The fragments produced by these enzymes have been ordered into a cleavage map of the SV40 chromosome (8). Using this map as a reference, it has been possible to locate the origin and terminus of DNA replication (9) as well as regions of the SV40 genome that serve as templates for “early” and “late” transcription of SV40 RNA in productively infected cells (10).

A second approach to mapping the SV40 genome involves the use of a series of nondefective adenovirus (Ad2)–SV40 hybrids (designated Ad2±ND; through Ad2±ND5) whose genomes contain specific, overlapping segments of SV40 DNA covalently inserted at a unique point into the Ad2 DNA molecule (11–15). The SV40 segments of the various hybrids have recently been mapped relative to one another (14) and relative to the unique R1 cleavage site (15). Since the nondefective Ad2–SV40 hybrids induce certain SV40 antigens during growth in susceptible cells (16), it has been possible to construct a map that relates regions of the SV40 genome to the induction of specific SV40 antigens (14, 15).

Utilizing nucleic acid hybridization methods of two types, the present study addresses itself to the relationship between the H. influenzae (Hin) and H. parainfluenzae (Hpa) restriction endonuclease cleavage maps of SV40 and the map of the SV40 segments of the hybrid viruses. In the first type of experiment, we employed E. coli RNA polymerase to prepare radiolabeled transcripts of the DNA fragments produced by H. influenzae endonuclease; these were then tested for homology with the SV40 segments of the various hybrids. In the second type of experiment, heteroduplex DNA molecules containing complementary strands derived from a restriction endonuclease fragment and from one of the hybrids (Ad2±ND) were visualized in the electron microscope, allowing direct determination of the homologous regions. Our results are consistent with those of Kelly and Lewis (14) and Morrow et al. (15) with respect to the size and relationship of the SV40 DNA segments in the hybrid viruses, and, in addition, place the common endpoint of these segments (14, 15) in the Hin-G fragment. Our results also permit construction of a colinear genetic map relating the specific fragments of SV40 DNA derived from H. influenzae and H. parainfluenzae restriction endonuclease cleavage of the SV40 genome to the segments of the SV40 genome contained within each of the adenovirus–SV40 hybrids. Thus, the genetic determinants of early SV40 antigens—T, TSTA, and T—could be localized on the SV40 cleavage map.

MATERIALS AND METHODS

Viruses, Host Cells, Virus Purification, DNA Extraction. Strain 777 SV40 and a small-plaque isolate of strain 776 SV40 were grown on BSC-1 cell monolayers (17). SV40 DNA I was purified by Hirt extraction (18) and CsCl-ethidium bromide centrifugation (19), as previously described (5, 20). Ad2 and Ad2–SV40 hybrid growth, purification, and DNA extraction have been described elsewhere (13, 20).
Derived from both strain 776 and 777 virus, were prepared as previously described (7). The previousy described mixtures containing 0.18 M KCl, 0.033 M Tris(HCl, pH 7.9, 6 mM mercaptoethanol, 3.3 mM MgCl2, 0.165 mM ATP, CTP, GTP, and UTP, 0.02-0.1 µg of a single fragment and 5 µg of E. coli 1113 RNA polymerase (EC 2.7.7.6) (21). [α-32P]ATP (sp. act. 20.6 Ci/mmol) or [3H]ATP, CTP, and UTP (sp. acts. 13.4, 19.8, and 24.5 Ci/mmol) were included in each polymerization. After 30 min at 37°, 1 µg of RNase-free pancreatic DNase (EC 3.1.4.5) was added and the reaction allowed to proceed for an additional 10 min. Reactions were terminated by sodium dodecyl sulfate-phenol treatment and the RNA was separated from substrate and precipitated as previously described (21).

Nucleic Acid Hybridizations. Nucleic acid hybridizations were performed by modification of the methods of Gillespie and Spiegelman (22) for 16 hr at 67° as previously described (20). For experiments comparing the extent of binding of fragment transcripts to the DNAs of SV40 and the Ad2-SV40 hybrids, equimolar amounts of DNA were fixed to filters—0.13 µg for SV40 (molecular weight, 3 × 106) and 1.0 µg for the hybrid viruses (molecular weight, 23 × 106). Constant molar quantities of the RNA transcripts of Hin-A-K were also introduced into the hybridization reaction in the comparative binding studies. Calculations were based on the percentage of the SV40 genome spanned by each DNA fragment (9).

Heteroduplex Formation and Electron Microscopy. Heteroduplexes containing strands from Ad2, Ad2+ND1, and Hin-B or Hpa-C DNA molecules were prepared according to the method of Davis et al. (23). A solution containing Ad2 DNA

**Preparation of RNA Complementary to DNA Fragments.** Hin fragment transcriptions were carried out in 31-p1 reaction mixtures containing 0.18 M KCl, 0.033 M Tris-HCl, pH 7.9, as the radiolabeled substrate; the specific activity of these transcripts was 10,600 cpm/pmol.

**Preparation of DNA Fragments.** The 11 DNA fragments derived from H. influenzae restriction endonuclease cleavage of intact SV40 DNA I, purified from cells infected with SV40 derived from both strain 776 and 777 virus, were prepared as previously described (5). These have been designated Hin-A through K. The H. parainfluenzae fragments were prepared as previously described (7); these have been designated Hpa-A through D.

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**Materials and Methods;** these transcripts had a specific activity of 12,800 cpm/pmol.

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**Table 1. Nucleic acid hybridization between RNA transcripts of simian virus 40 (SV40) DNA fragments and the DNA of adenovirus-SV40 hybrids**

<table>
<thead>
<tr>
<th>SV40 DNA fragment</th>
<th>Input* cpm</th>
<th>Total cpm hybridized Adenovirus 2 SV40</th>
<th>Total cpm hybridized† (percent of total cpm hybridized to SV40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>31,280</td>
<td>26</td>
<td>8,750</td>
</tr>
<tr>
<td>B</td>
<td>20,008</td>
<td>10</td>
<td>6,800</td>
</tr>
<tr>
<td>C</td>
<td>13,030</td>
<td>12</td>
<td>2,868</td>
</tr>
<tr>
<td>D</td>
<td>13,303</td>
<td>18</td>
<td>4,216</td>
</tr>
<tr>
<td>E</td>
<td>11,420</td>
<td>9</td>
<td>3,160</td>
</tr>
<tr>
<td>F</td>
<td>9,840</td>
<td>11</td>
<td>2,718</td>
</tr>
<tr>
<td>G</td>
<td>8,710</td>
<td>4</td>
<td>2,358</td>
</tr>
<tr>
<td>H</td>
<td>7,310</td>
<td>8</td>
<td>2,318</td>
</tr>
<tr>
<td>I</td>
<td>6,520</td>
<td>5</td>
<td>1,731</td>
</tr>
<tr>
<td>J</td>
<td>5,820</td>
<td>3</td>
<td>1,042</td>
</tr>
<tr>
<td>K</td>
<td>5,260</td>
<td>3</td>
<td>928</td>
</tr>
</tbody>
</table>

* RNA transcripts of the Hin-A, B, C, G, H, I, and J fragments were derived from polymerizations in which [3H]ATP, UTP, and CTP served as radio-labeled substrates as described in Materials and Methods; these transcripts had a specific activity of 12,800 cpm/pmol. RNA transcripts of the D, E, F, and K fragments were derived from polymerizations in which [α-32P]ATP (sp. act., 20.6 Ci/mmol) served as the radiolabeled substrate; the specific activity of these transcripts was 10,600 cpm/pmol.

† All values are averages of two or more determinations.
RESULTS

Transcripts of Hin Fragments. Transcription of all 11 fragments derived from H. influenzae restriction endonuclease cleavage of intact SV40 DNA were analysed by polyacrylamide gel electrophoresis in 100% formamide (results not presented) in an attempt to ascertain if they were transcribed in their entirety. For each fragment, the transcript consisted of one or more discrete bands of RNA. Each fragment transcript contained one band of approximately the size expected for the transcript of one entire strand of the template DNA fragment. For certain fragments, bands of RNA of larger size were noted. It thus appears very likely that all or nearly all of at least one strand of each DNA fragment is transcribed into complementary RNA.

Hybridization of Transcripts to SV40 and Hybrid Virus DNA. In an attempt to quantitate the representation of each DNA fragment in the genome of each hybrid virus, hybridization experiments were performed in which equimolar quantities of SV40 and hybrid virus DNA were introduced into the hybridization reaction. Under these conditions, equivalent binding of a fragment transcript to SV40 and hybrid virus DNA should indicate the presence of the entire fragment in the hybrid genome, whereas lesser degrees of binding should reflect the fraction of a given fragment represented in the hybrid genome. To test this supposition, the transcript of the SV40 Hin-B fragment was annealed to increasing quantities of SV40 and Ad2 + ND3 DNAs (Fig. 1). For SV40, binding increases linearly up to 0.13 µg per filter, after which a plateau is reached corresponding to binding of 35% of input counts. The shape of the binding curve for Ad2 + ND3 is essentially identical to that for SV40 with a plateau reached at 32.2% of input radioactivity, but with the linear portion of the curve reaching 1.0 µg of DNA per filter. The identical shape of these binding curves, the comparability of the plateau levels reached, and the identical extent of binding at equimolar quantities of DNA per filter indicate the presence of the complete Hin-B fragment in Ad2 + ND3 and support the validity of assessing quantitative representation of the DNA fragments in the hybrid viruses by means of binding to equimolar quantities of DNA.

The results of hybridizations between equimolar quantities of DNA from SV40 and the five nondefective Ad2-SV40 hybrids and radiolabeled RNA transcribed on each of the 11 Hin DNA fragments are presented in Table 1. Binding between the following specific Hin fragment transcripts and Ad2-SV40 hybrid DNAs is quantitatively equivalent to binding under identical conditions to an equimolar quantity of SV40 DNA: G transcript and Ad2 + ND3 DNA; G and B transcripts and Ad2 + ND3 DNA; G, B, and I transcripts and Ad2 + ND3 DNA; and G, B, I, and H transcripts and the DNA samples were mounted for electron microscopy by the formamide method (23) in order to visualize both single and double strands. Micrographs were taken on a JEM 100B electron microscope. Contour lengths of traced molecules were measured with a Keuffel and Esser 620300 map measurer.

Electron Microscopy of Heteroduplex Molecules. As a separate approach to relating the SV40 cleavage and hybrid virus maps and in order to align the maps as precisely as possible, we prepared heteroduplexes between the DNA strands of Ad2, Ad2 + ND3, and an SV40 DNA fragment corresponding to one end of the Ad2 + ND3 SV40 segment. Since the Hpa-C fragment contains Hin-B and Hin-I (ref. 8 and Fig. 3) it should overlap the SV40 segment of Ad2 + ND3 (see Table 1). Under the conditions employed for mounting the DNA for electron microscopy both single and double strands are visible and distinguishable, so that the extent of homology between Hpa-C and the SV40 DNA segment of Ad2 + ND3 could be determined by length measurements. Fig. 2 shows a typical Ad2/Ad2 + ND3/Hpa-C heteroduplex. The heteroduplex between DNA strands of Ad2 and Ad2 + ND3 has been described previously (14). It contains a single substitution loop. One strand of this loop (labeled Ad2 in this diagram) corresponds to the Ad2 deletion in Ad2 + ND3 and measures 0.654 fractional Ad2 lengths. The other strand of the substitution loop is the SV40 segment of Ad2 + ND3 and measures 0.186 fractional SV40 lengths. In the Ad2/Ad2 + ND3/Hpa-C heteroduplex shown here, an Hpa-C DNA strand has hybridized with part of the SV40 segment of Ad2 + ND3. The portion of Hpa-C homologous with the SV40 DNA segment of Ad2 + ND3 is designated C1. The portion of Hpa-C that extends beyond the end of the SV40 DNA segment of Ad2 + ND3 is designated C2. The portion of the SV40 segment of Ad2 + ND3 that lies outside Hpa-C is designated S.

DNAs from both Ad2 + ND3 and Ad2 + ND4. Less than equimolar degrees of binding are seen between the following: B transcript and Ad2 + ND3 DNA; H transcript and Ad2 + ND3 DNA; and A transcript and the DNAs of Ad2 + ND4 and Ad2 + ND5. The significance of these lesser levels of RNA binding is supported by a linear increase in binding with increasing amounts of input RNA.
lengths and the portion of $Hpa$-C that lies outside the SV40 segment of Ad2'ND1 measures 0.075 fractional SV40 lengths. The sum of these two lengths agrees well with the observed length of $Hpa$-C [0.195 fractional SV40 lengths as determined by Sacc and Nathans(7)].

Since, as shown in Table 1, Ad2+ND1 DNA hybridizes with the RNA transcript of Hin-B but not with that of Hin-I, $Hpa$-C is oriented with respect to the SV40 segment of the hybrid such that the Hin-I portion is in the nonhybridized part of $Hpa$-C. This orientation has been confirmed by examining heteroduplexes between Hin-B, Ad2, and Ad2'ND1 DNA. These Ad2/Ad2-ND1/Hin-B heteroduplexes are similar to the Ad2/Ad2-ND1/Hpa-C heteroduplex shown in Fig. 2 except that the unpaired strand (corresponding to segment C2) is shorter by about 0.05 fractional SV40 lengths, the length of Hin-I. In summary, these data indicate that one end of the SV40 segment of the Ad2'-ND1 hybrid is 0.075 SV40 lengths from the Hin-I end of $Hpa$-C.

**Table 2.** Ad2/Ad2'-ND1/Hpa-C heteroduplexes

<table>
<thead>
<tr>
<th>DNA segment</th>
<th>Length (fractional SV40 lengths ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 (portion of $Hpa$-C homologous to SV40 segment of Ad2'-ND1)</td>
<td>0.122 ± 0.005</td>
</tr>
<tr>
<td>C2 (portion of $Hpa$-C not homologous to SV40 segment of Ad2'-ND1)</td>
<td>0.075 ± 0.003</td>
</tr>
<tr>
<td>Total</td>
<td>0.197</td>
</tr>
<tr>
<td>Length of $Hpa$-C [Sack and Nathans (7)]</td>
<td>0.195</td>
</tr>
<tr>
<td>S (portion of SV40 segment of Ad2'-ND1, not homologous to $Hpa$-C)</td>
<td>0.052 ± 0.003</td>
</tr>
<tr>
<td>C1 (portion of SV40 segment of Ad2'-ND1, homologous to $Hpa$-C)</td>
<td>0.122 ± 0.005</td>
</tr>
<tr>
<td>Total</td>
<td>0.174</td>
</tr>
<tr>
<td>Length of SV40 segment of Ad2'-ND1 [Kelly and Lewis (14)]</td>
<td>0.178</td>
</tr>
</tbody>
</table>

**Discussion**

The results reported in this paper are summarized in Fig. 3, which shows a comparison of the cleavage map of SV40 with the map of the SV40 segments of the adenovirus-SV40 hybrids. The R1 restriction endonuclease cleavage site, which serves as the zero point of the map, has been located at 0.11 map units (i.e., fractional lengths of SV40 DNA from the R1 site) from the common endpoint of the SV40 segments of the nondefective hybrids (3, 15) and has been mapped within the Hin-F SV40 DNA fragment (8). On the basis of the DNA:RNA hybridization data presented in Table 1 the segments of SV40 DNA contained in the Ad2-SV40 hybrid viruses are colinear with the SV40 genome, originate near the end of the Hin-G fragment closest to the R1 cleavage site, and cover 0.064 (Ad2'-ND1), 0.200 (Ad2'-ND2), 0.273 (Ad2'-ND3), 0.365 (Ad2'-ND4), and 0.463 (Ad2'-ND5) fractional lengths of the SV40 genome. This quantitation is in good agreement with the data of Morrow et al. (15). The SV40 segment of the defective E46' hybrid is also colinear with SV40 DNA, begins at or near the same site as the SV40 segments present in the nondefective hybrid viruses, and includes 0.60 fractional lengths of the SV40 genome, terminating about one-half of the distance into the Hin-C fragment (24). It should be pointed out that the demonstration of colinearity of the hybrid SV40 segments with the SV40 genome by means of hybridization with transcripts of specific
Hin fragments independently establishes the order of these fragments in the SV40 DNA molecule.

The appearance of specific SV40 antigens in cells infected with the different hybrid viruses (16, 25, 26) coupled with the data of Kelly and Lewis (14) and Morrow et al. (15) has allowed the ordering of the genetic determinants of these antigens. The data also permit localization of these determinants on the SV40 map: U, TSTA, and T, proceeding distally from the R1 cleavage site, as shown in Fig. 3. The present nucleic acid hybridization data provide an independent line of evidence confirming the ordering of the genetic determinants of these antigens; the data also permit localization of these determinants on the SV40 cleavage map. Since these antigens appear prior to SV40 DNA replication, these genetic loci are part of the "early" region of the SV40 chromosome. Recently, Khoury et al. (10) have reported that stable "early" SV40 RNA isolated from infected cells hybridizes with Hin-H and I and part of A and B. On the assumption that the "early" region is a continuous segment of the genome, they have estimated that it extends from 0.26 to 0.57 map units (Fig. 3). Consistent with these limits are the findings that Ad2-ND1-infected cells contain all the species of "early" SV40 RNA found in SV40-infected cells (27), and that Ad2-ND1 DNA contains both "early" and "late" SV40 DNA sequences (28).

Khoury et al. (10) have also shown that "early" transcription of SV40 DNA in infected cells occurs from right to left as the map is drawn in Fig. 3. This suggests that transcription of the SV40 segment contained in some of the hybrids may begin within the Ad2 DNA to the right of the SV40 segment, yielding an Ad2-SV40 hybrid mRNA. It is possible that some hybrid RNA molecules of this type might fail to function as a messenger or be translated out of phase, thereby accounting for the lack of detectable SV40 antigens in cells infected with certain hybrid viruses.

Another interesting feature of the comparative maps is the common left end of the SV40 segments of all the nondefective Ad2-SV40 hybrids within fragment Hin-G (14, 15). Since these hybrids were all ultimately derived from a single virus pool, there is at present insufficient data to indicate a preferential integration site for the insertion of SV40 DNA into the adenovirus genome. Nonetheless, it is of great interest that the insertion of SV40 DNA into the Ad7 genome of E46¹ has also occurred in Hin-G at a site in close proximity to the site of origin of the SV40 segments in the nondefective hybrids. It is also curious that Hin-G also contains the site for termination of SV40 DNA replication (9), an event that is likely to require breakage and rejoicing of DNA strands, analogous to recombination during integration of SV40 DNA into adenovirus DNA. Whether these two reactions are related remains to be determined.

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