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Mapping of sequences with 2-fold symmetry on the simian virus 40 genome: A photochemical crosslinking approach

(hairpin turns on denatured simian virus 40 DNA/electron microscopy/restriction enzymes/hairpin map/genetic map of simian virus 40)

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ABSTRACT **Sequences with 2-fold axes of symmetry have been detected and mapped on the simian virus 40 (SV40) genome by their ability to form hairpin turns in single-stranded SV40 DNA. Supercoiled SV40 DNA (SV40 I) was digested with restriction enzymes *Eco*RI and *Hpa*II. The resulting linear DNA molecules with lengths of the complete SV40 genome were then denatured and photochemically reacted with 4,5′,8-trimethylpsoralen (trioxsalen) at 16.0 ± 0.5° and different ionic strengths. Secondary structures on the single-stranded SV40 DNA were crosslinked and their positions analyzed by electron microscopy. There were no observable hairpin turns on the denatured SV40 DNA when it was photoreacted in 1 mM Tris·HCl/0.1 mM EDTA at pH 7.0. In 20 mM NaCl, one specific hairpin turn was detected at 0.17 ± 0.02 map units on the map of *Eco*RI-digested SV40 DNA, where the 3′ ends of early 19S mRNA, late 19S mRNA, and 16S mRNA of SV40 have been mapped. In 30 mM NaCl there are five more major hairpin turns besides the most stable one. The centers of four of these specific hairpin turns were mapped at 0.26 ± 0.02, 0.68 ± 0.03, 0.84 ± 0.02, and 0.94 ± 0.01 units on the map of *Eco*RI-digested SV40. The fifth one is at or near the unique *Eco*RI cleavage site on SV40 DNA. The possible functions of these sequences are discussed in terms of the nature of the promoter sites, the replication origin, the processing of RNA precursors, and regulation at the translational level.**

Direct sequencing data have shown that many regulating segments of prokaryotic DNA contain sequences with 2-fold symmetry; these include the operators (1, 2), the promoters (3–8), and the origin of replication (9). This property may allow the formation of hairpin turns (hairpins) in these regions after strand separation and, possibly, the mapping of these secondary structures on the genome if the hairpins can be visualized directly by electron microscopy.

However, it has been difficult to detect hairpins on single-stranded or denatured viral DNA molecules by the protein monolayer-formamide spreading technique (10). Two possibilities could explain this failure. First, the hairpins, if there are any, are too short (<50 base pairs) to be discerned. Second, an examination of the regulatory sequences mentioned above indicates that few of them consists of perfect 2-fold symmetry. It may be that the hairpin forms with the lowest free energy are not stable under the formamide spreading conditions because of base mismatching in the foldback duplex. The latter problem can be solved by photochemically crosslinking the single-stranded or denatured virus DNA with 4,5′,8-trimethylpsoralen (trioxsalen). Trioxsalen molecules intercalate between base pairs of DNA and, upon irradiation with long wavelength UV, form covalent interstrand crosslinks (11–15). Using this technique we have shown previously that as many as eight hairpins can be stabilized by trioxsalen and visualized on fd DNA by electron microscopy. Furthermore, these hairpins appeared at specific sites on the fd genome and most of them are located near or in the *in vitro* fd promoter regions (16).

In this paper we describe the result of studying trioxsalen-crosslinked hairpins on the denatured form of the DNA of an animal virus, simian virus 40 (SV40). It will be shown that at least six regions of the SV40 genome are capable of forming discernible hairpins and, as in the case of fd, most of them coincide with positions of biological importance in the genetic map of SV40.

Abbreviations: trioxsalen, 4,5′,8-trimethylpsoralen; SV40, simian virus 40; SV40 I, supercoiled form of SV40 DNA; *Eco*RI-SV40 I and *Hpa*II-SV40 I, linear SV40 DNA generated by digestion of SV40 I with *Eco*RI and *Hpa*II enzymes, respectively.

MATERIALS AND METHODS

Materials. SV40 DNA was a gift from Drs. L. Hallick and J. Bartholomew and was isolated from SV40-infected TC-7 cells. The restriction enzymes *Eco*RI and *Hpa*II (Bethesda Research Laboratory, Inc.) were gifts from Dr. P. Modrich and H. Nagaishi, respectively. *Eco*RI-digested λ DNA fragments were provided by Dr. T. S. Hsieh. Tritium-labeled trioxsalen was prepared by S. Isaacs (17).

Restriction of Supercoiled SV40 DNA (SV40 I). *Eco*RI digestion was performed in 100 mM Tris·HCl (pH 7.6)/50 mM NaCl/5 mM $MgCl_2$/0.2 mM dithiothreitol/0.1 mM EDTA at 37° for 3 hr. For the *Hpa*II reaction, SV40 I was incubated with the enzyme at 37° in 60 mM Tris·HCl (pH 8.0)/7 mM $MgCl_2$ for 1 hr. After incubation, the reaction mixtures were first extracted with 100 mM Tris·HCl (pH 7.0)/saturated phenol and then with ether. The aqueous phase was dialyzed against 10 mM Tris·HCl/1 mM EDTA at pH 8.0.

Gel Electrophoresis. Electrophoresis of DNA samples in 1% agarose gels was performed as described (18, 19). *Eco*RI-cut λ DNA fragments were coelectrophoresed as molecular weight markers (20).

Denaturation of Restricted SV40 I DNA and Photochemical Crosslinking of Denatured SV40 DNA. The linear SV40 DNA resulting from the restriction enzyme reactions was denatured in alkali, neutralized, and dialyzed at 4° against 1 mM Tris·HCl/0.1 mM EDTA at pH 7.0 as described (16).

A typical DNA solution to be irradiated had a final volume of 100 μl and contained 5 μg of denatured SV40 DNA per ml and the appropriate NaCl concentration. This solution was mixed with 1 μl of 1 mg of trioxsalen per ml (in 100% ethanol) and sealed in a glass pipet. The pipet was placed into a chamber maintained at 16.0 ± 0.5° and irradiated with two 400-W General Electric mercury-vapor lamps, which provided an intensity in the spectral region from 300 to 400 nm of approximately 100 mW/cm^2 at the position of the pipet. Light with wavelengths between 340 and 380 nm was selected by circulating a 40% (wt/wt) cobaltous nitrate solution through the outer jacket of the chamber.

After irradiation for 10 min, the sample solution was mixed with another microliter of the trioxsalen stock solution and irradiation was continued for another 10 min.

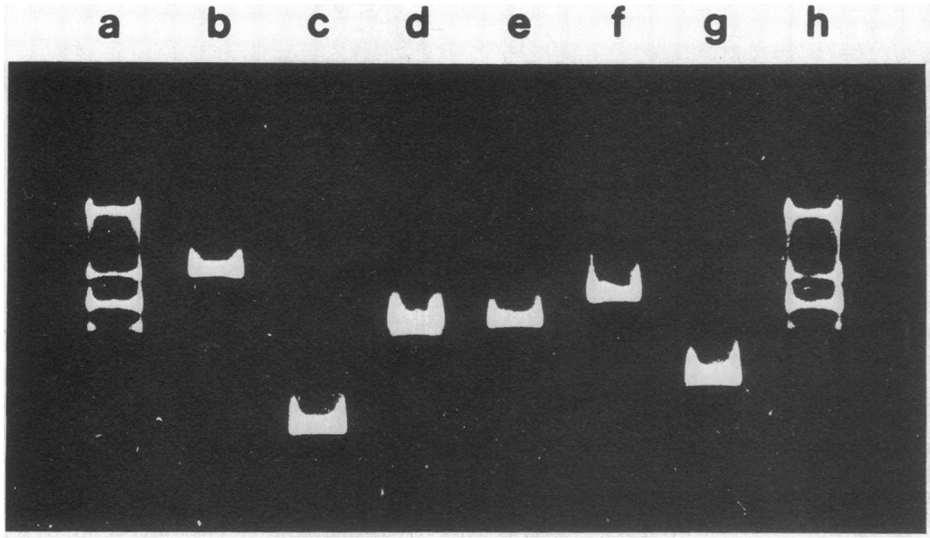

FIG. 1. Gel electrophoresis of SV40 DNAs in 1% agarose. (*a*) *Eco*RI-λ DNA fragments; (*b*) SV40 II; (*c*) SV40 I; (*d*) *Eco*RI-SV40 I; (*e*) *Hpa*II-SV40 I; (*f*) *Hin*dII-fd RF I; (*g*) fd RF I; (*h*) *Eco*RI-λ DNA fragments.

Electron Microscopy. The solution of photochemically crosslinked DNA was spread directly for electron microscopy by the 40% formamide spreading technique (10). Sometimes single-stranded circular fd DNA was included in the hyperphase as a length marker. Sample grids were examined in a Philips 201 Electron Microscope. Lengths of DNA segments were measured as described (16).

RESULTS

Formation of Hairpins on Denatured Nicked SV40 DNA (SV40 II). In order to see whether hairpins form on single-stranded SV40 DNA molecules, SV40 II was denatured and photochemically crosslinked with trioxsalen at 16° and 30 mM NaCl. Essentially all of the molecules have hairpins after the above treatment. Single-stranded SV40 DNA molecules, linear or circular, with as many as six hairpins were observed.

Generation of Linear SV40 DNA by Restriction Enzymes. SV40 I was subjected to digestion by the restriction enzymes *Eco*RI and *Hpa*II to generate full-length linear SV40 molecules (EcoRI-SV40 I and *Hpa*II-SV40 I, respectively). Each of the two enzymes cuts SV40 DNA at one specific site; the *Hpa*II cleavage site has been mapped at 0.74 units on the *Eco*RI map of SV40 (18, 21, 22).

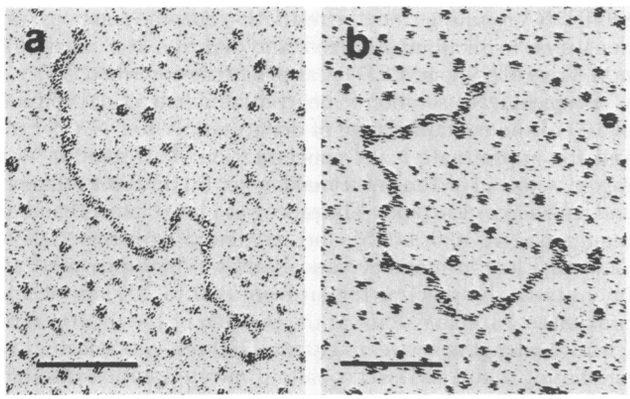

FIG. 2. Secondary structures on trioxsalen-crosslinked denatured *Eco*RI-SV40 I. Denatured *Eco*RI-SV40 I crosslinked at 16.0 ± 0.5° and (*a*) 20 mM NaCl or (*b*) 30 mM NaCl. The bars are 500 nucleotides long.

Fig. 1 shows the electrophoresis patterns of various forms of SV40 DNA on an agarose gel. The molecular weights of the linear SV40 DNAs, *Eco*RI-SV40 I and *Hpa*II-SV40 I, have been calculated to be 3.02×10^6 (4570 base pairs) from their positions in the gel relative to the *Eco*RI-λ fragments. Double-stranded fd was calculated to be 5750 base pairs long.

Hairpins on Crosslinked Denatured *Eco*RI-SV40 I Are Located at Specific Sites. *Eco*RI-SV40 I DNA was denatured and then crosslinked with trioxsalen at 16°. Over 50% of the molecules crosslinked at 20 mM NaCl showed at least one hairpin near one end (Fig. 2*a*). As the salt was increased to 30 mM NaCl, the average number of hairpins on the molecules also increased. Most of the molecules have one hairpin at one end and two at the other end, with another two to three hairpins located between the above three hairpins. Fig. 2*b* shows a typical molecule of denatured *Eco*RI-SV40 I crosslinked at 30 mM NaCl.

In order to determine whether these hairpins are located at specific positions, photographs of crosslinked denatured *Eco*RI-SV40 I were taken and the lengths of all the hairpins, as well as the center-to-center distances of adjacent hairpins, were measured and then converted to fractional lengths of SV40 DNA. The hairpin maps and histograms thus constructed are shown in Figs. 3 and 4. From Fig. 3 it can be calculated that the most stable hairpin has a length of 150 ± 60 base pairs and its center is located 0.17 ± 0.02 units from one end of *Eco*RI-SV40 I. At 30 mM NaCl (Fig. 4), the crosslinked denatured *Eco*RI-SV40 I showed five distinct peaks (I–V) in the hairpin histogram (Fig. 4*b*). The 34 molecules were aligned so that the ends with two hairpins were assigned to the right part of the molecule. The centers of each of these five hairpins have been determined to be 0.15 ± 0.02 (I), 0.26 ± 0.02 (II), 0.68 ± 0.03 (III), 0.84 ± 0.02 (IV), and 0.94 ± 0.01 (V) units away from the left end of *Eco*RI-SV40 I.*

* The position of the center of the most stable hairpin was calculated by averaging the center positions of that hairpin of all the molecules shown in Fig. 4*a* except molecule no. 21. The molecules in Fig. 5*a* used to calculate the center positions of hairpins I–V are listed as follows: hairpin I, all 34 molecules; hairpin II, molecules 8, 10, 11, 14, 18, 21, 24, 30, and 32–34; hairpin III, molecules 6, 7, 12, 13, 16, 18–23, and 25–34; hairpin IV, molecules 3, 7, 8, 11–13, 16, 17, 19–21, 23, 25–27, 29, 31, 32, and 34; hairpin V, 3, 6–14, 16, 17, 19–22, 25–27, and 29–34.

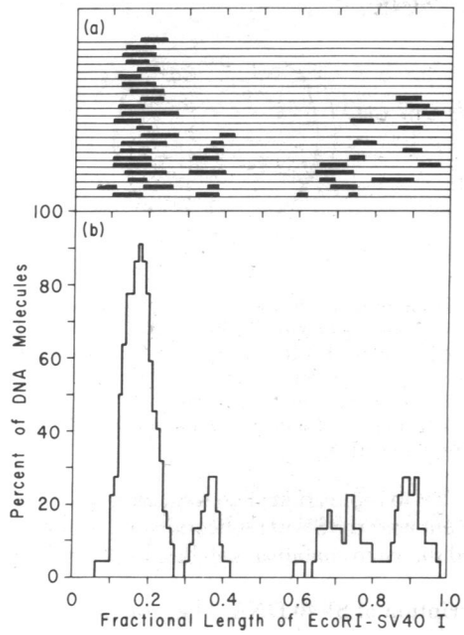

FIG. 3. Hairpin map (*a*) and histogram (*b*) of denatured *Eco*RI-SV40 I crosslinked at 16° and 20 mM NaCl. In the hairpin map, the hairpins that appeared between 0.1 and 0.2 units were assigned to the left part of the molecules. The histogram shows the percentage of the molecules measured that have a hairpin in a given length interval corresponding to one-hundredth of the SV40 genome size.

It is clear from the above experiments that the hairpins on trioxsalen-crosslinked denatured *Eco*RI-SV40 I are at specific positions. However, the polarity of neither of the hairpin maps in Figs. 3*a* and 4*a* has been determined. Also unknown is which one of the five hairpins in 30 mM NaCl corresponds to the most stable hairpin found in 20 mM NaCl. These questions can be answered by constructing a hairpin map of denatured *Hpa*II-SV40 I.

Sequences with 2-Fold Symmetry in *Hpa*II-SV40 I. After

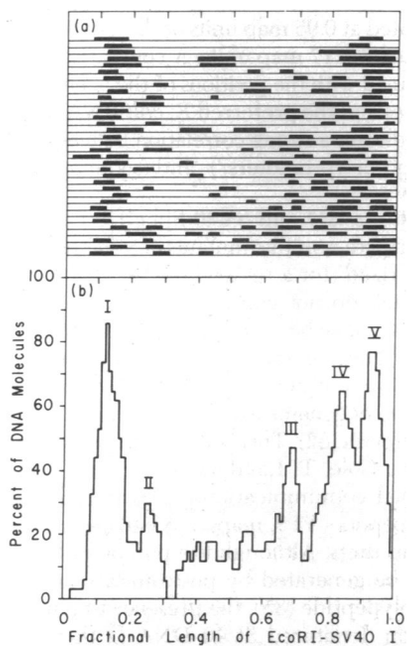

FIG. 4. Hairpin map (*a*) and histogram (*b*) of denatured *Eco*RI-SV40 I crosslinked at 16° and 30 mM NaCl.

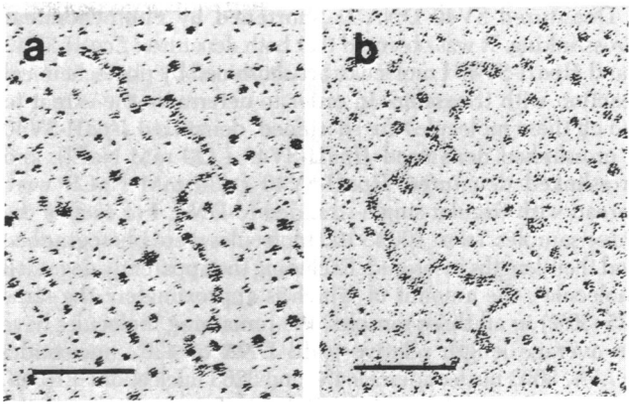

FIG. 5. Secondary structures on trioxsalen-crosslinked denatured *Hpa*II-SV40 I. Denatured *Hpa*II-SV40 I crosslinked at 16.0 ± 0.5° and (*a*) 20 mM NaCl or (*b*) 30 mM NaCl. The bars are 500 nucleotides long.

denaturation and crosslinking, *Hpa*II-SV40 I showed one specific hairpin at 20 mM NaCl near the middle of the molecule (Fig. 5*a*), whereas several hairpins were found in 30 mM NaCl (Fig. 5*b*). The hairpin histograms of crosslinked denatured *Hpa*II-SV40 I are shown in Fig. 6. The most stable hairpin is located at 0.39 ± 0.03 units from one end of the molecule. This leads us to conclude that the most stable hairpin is located at 0.17 instead of 0.83 units on the *Eco*RI map of SV40. Similarly, an examination of the hairpin histograms of both crosslinked denatured *Eco*RI-SV40 I and *Hpa*II-SV40 I at 30 mM NaCl indicates that the positions of hairpins I–V determined in the last section are their true positions on the *Eco*RI-SV40 map and that hairpin I corresponds to the most stable hairpin. Hairpin VI was not observed on crosslinked denatured *Eco*RI-SV40 I at 30 mM NaCl, suggesting that the sequence of this hairpin contains the *Eco*RI cleavage site. Alternatively, this hairpin might have escaped detection on the molecules in 30 mM NaCl because of its proximity to either end of the linear molecules.

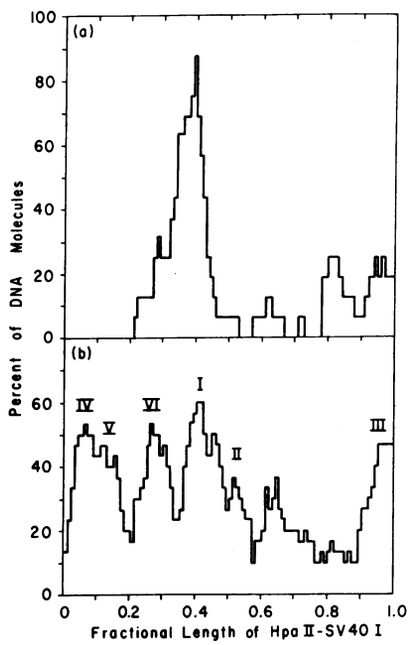

FIG. 6. Hairpin histograms of denatured *Hpa*II-SV40 I crosslinked with trioxsalen at 16° and two different ionic strengths: (*a*) 20 mM NaCl and (*b*) 30 mM NaCl.

Denatured SV40 DNA Is Shortened by Photochemical Crosslinking. It was observed that both denatured *Eco*RI-SV40 I and *Hpa*II-SV40 I molecules are shortened by photochemical reaction with trioxsalen. In order to determine the extent to which these molecules are shortened, denatured *Hpa*II-SV40 I, crosslinked denatured *Hpa*II-SV40 I (20 mM NaCl), and crosslinked denatured *Hpa*II-SV40 I (30 mM NaCl) were spread with circular single-stranded fd DNA. For each of the three samples, from 100 to 160 molecules were photographed and their lengths measured. Excluding the apparently degraded molecules, the amount of which is approximately the same (25–30%) in all three samples, the remaining molecules have an average length relative to fd DNA of 4362 nucleotides ±7.5%, 3023 nucleotides ±9.4%, and 3263 nucleotides ±10.4%, respectively, for denatured *Hpa*II-SV40 I, crosslinked denatured *Hpa*II-SV40 I (20 mM NaCl), and crosslinked denatured *Hpa*II-SV40 I (30 mM NaCl). Thus, the denatured SV40 DNA has been shortened 30% by the photochemical crosslinking reaction. This is not caused by nonspecific breakage of the molecules because very few molecules (<5%) of the originally circular single-stranded fd DNA molecules were converted to linear forms after the same photochemical crosslinking treatment. Moreover, this shortening is not restricted to a specific region on SV40 DNA for, after correction of the distance between the *Hpa*II and *Eco*RI sites, the hairpin histograms of crosslinked denatured *Eco*RI-SV40 I (Figs. 3*b* and 4*b*) can be superimposed on those of crosslinked denatured *Hpa*II-SV40 I (Fig. 6).

Amount of Trioxsalen Covalently Bound to Denatured SV40 DNA. A sample of SV40 II was denatured and crosslinked with radioactive trioxsalen at different ionic strengths. The amount of covalently bound trioxsalen was determined (17). It was found that while the denatured SV40 DNA photoreacted in 1 mM Tris·HCl/0.1 mM EDTA at pH 7.0 has only one covalently bound trioxsalen molecule per 100 nucleotides, the molecules photoreacted in 20 mM and 30 mM NaCl have 5 and 10 trioxsalen molecules per 100 nucleotides, respectively. Considering the preferential binding of trioxsalen to double-stranded DNA (11) and pyrimidine bases (12), the above binding values are too high to assume that all of the trioxsalen bound exclusively to the observed hairpins. Instead, most of the trioxsalen molecules must be bound to those interhairpin regions, and the shortening we have observed is probably due to the presence of many short secondary structures stabilized by trioxsalen crosslinkage and yet not resolved in the electron microscope.

DISCUSSION

SV40 is one of the simplest and most well-studied animal viruses. A full understanding of its genome structure should help to reveal the mechanisms of the infection of animal cells by viruses as well as the details of genetic regulation of various activities in eukaryotic systems (for a recent review, see ref. 23). The evidence presented in this paper shows that long sequences with 2-fold axes of symmetry are located on the SV40 genome at specific positions. Four of the six regions that are capable of forming crosslinked hairpins have been mapped to positions of biological importance.

Replication of SV40 DNA. SV40 replication begins at a specific site (0.67 units on the *Eco*RI map of SV40) and proceeds bidirectionally, terminating about half-way around the circular DNA from the initiation point (24, 25). The fact that hairpins III (0.68 ± 0.03) and I (0.17 ± 0.02) are located in the initiation and termination regions of replication, respectively, suggests that these symmetric sequences may be important "start-stop" signals for SV40 replication. The sequence of a 28-base-pair-long segment near the SV40 replication origin has been determined and shown to contain a high degree of 2-fold symmetry (9).

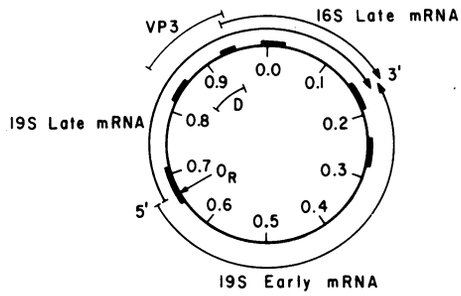

FIG. 7. Diagrammatic representation of the relationship between the positions of sequences with 2-fold symmetry of SV40 DNA (the blackened regions on the *Eco*RI-SV40 physical map) and the locations of the three SV40-specific mRNAs, the replication origin O_R, the tsD mutants (for a review, see ref. 23), and the SV40-specific polypeptide VP3 (S. Goff, C. Cole, T. Landers, S. Manteuil-Brutlag, and P. Berg, personal communication).

Transcription of SV40 DNA. The sequencing data of promoters (3–8), including the preferred initiation site for *E. coli* RNA polymerase on SV40 DNA at position 0.18 of the *Eco*RI-SV40 map (3, 4), shows that most of them contain sequences of approximate 2-fold symmetry. The results of the photochemical crosslinking experiment done on fd DNA (16) are consistent with this idea.

Three RNAs have been detected in and isolated from SV40-infected cells (refs. 26 and 27; Fig. 7). The early 19S mRNA has been mapped on the physical map of SV40; it is transcribed from the early strand, with its 5′ terminus located near the replication origin and the 3′ terminus located near the termination site of replication (28–31). Late 19S mRNA and 16S mRNA have been detected at the late stage of SV40 infection (27) and mapped on the SV40 genome (26, 28, 30). The late 19S mRNA is transcribed from the late strand of SV40 from a position near the replication origin at 0.67 map units (5′ mRNA end) to the termination site of replication at approximately 0.17 map units (3′ mRNA end). The 5′ end of the 16S mRNA is located at 0.95 map units of the *Eco*RI-SV40 map and also terminates at 0.17 map units. A comparison of the hairpin map we obtained with the positions of the 5′ ends of these three mRNA species and the preferred *E. coli* RNA polymerase initiation site indicates a strong correlation between initiation sites for transcription (promoters?) and sequences with 2-fold symmetry axes.

SV40 Mutants tsD and SV40 Specific Polypeptide VP3. Many temperature-sensitive mutants of SV40 have been isolated and characterized (for a review, see ref. 32). One group, tsD mutants, which do not complement with mutants in other groups (33, 34), have been thought to be defective in virus uncoating at high temperature. All the D mutants map between 0.85 and 0.94 map units on *Eco*RI-SV40. These facts suggest that the portion of genome associated with mutants D may code for a virion protein (32). This point has been confirmed recently by S. Goff, C. Cole, T. Landers, S. Manteuil-Brutlag, and P. Berg (personal communication), who found that the virus-specific polypeptide, VP3, maps on SV40 genome between 0.84 and 0.95 map units. Although the polypeptide VP3 has been proposed to be generated by posttranslational cleavage of a precursor polypeptide (35), the presence of hairpins (hairpins IV and V) on denatured SV40 DNA at these two positions suggests that the symmetric sequences at 0.84 and 0.94 may play important roles in the expression of VP3 at transcriptional

and translational levels. They may function in the production of VP3-coding mRNA either by initiating transcription at position 0.84 and terminating transcription at 0.95, or by providing posttranscriptional cleavage sites on a precursor RNA. On the other hand, the two hairpins may appear on the late 19S mRNA and punctuate and control the translation of the mRNA segment between them to produce VP3. Baltimore *et al.* (36) have suggested, however, that initiation of more than one discrete polypeptide on a single eukaryotic mRNA is unlikely, an argument against translational control on a polycistronic message.

Posttranscriptional Cleavage of RNA. It has been shown *in vitro* (37) that RNase III of *E. coli* cleaves transcripts of the early region of T7 phage to small molecules identical to the phage-specific mRNAs in the infected cells. Similar enzymes have been found in animal cells (38). Some evidence has been presented to show that the SV40-specific late 19S mRNA may be a precursor to the 16S mRNA (26). The presence of symmetric sequences on SV40 near the 5′ ends of all three SV40-specific mRNAs suggests that these sequences may appear on the primary transcripts of SV40 (27, 39) or the late 19S mRNA as hairpins and be recognized by processing enzymes.

Summary. Our results are summarized in Fig. 7, in which the positions of the six hairpin-forming sequences, as well as the known maps of SV40-specific mRNAs, the SV40-specific polypeptide VP3, and regions of tsD mutants, are indicated on the *Eco*RI-SV40 physical map. Complete sequencing data of SV40 DNA and refined mapping of the hairpins should reveal the exact locations of these sequences with 2-fold symmetry and facilitate the study of their biological functions.

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