

Loss of Simian Virus 40 DNA-RNA Hybrids from Nitrocellulose Membranes; Implications for the Study of Virus-Host DNA Interactions

(transformed cells/integration/multiplicity/SV40)

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ABSTRACT Complete hybrids of simian virus 40 (SV40)DNA and its complementary RNA (cRNA) are not retained on nitrocellulose membranes. At saturating cRNA concentrations, retention of the hybrids indicates incomplete homology between DNA and RNA, probably due to incorporation of host DNA in the viral DNA; this effect is most pronounced when DNA is produced in cells infected at high multiplicity. Hybrids between DNA of Chinese hamster cells transformed by SV40 and cRNA are retained if the DNA fragments are long, but they are lost if the DNA is sheared to less than the length of an SV40 DNA molecule. Hence, in cells examined with about six SV40 genomes per cell, each genome is individually integrated. The results may explain previous discrepancies in the estimation of the number of viral genomes in transformed cells.

The detection and quantitation of genomes of oncogenic viruses in transformed cells is based on DNA-RNA or DNA-DNA hybridization experiments (1, 2). Discrepancies in the numbers determined by different investigators suggest that the quantitation is unreliable. While studying the possible sources of variability in the hybridization assay based on retention of DNA-RNA hybrids on nitrocellulose filters, we found that the hybrids fail to be retained on the filters when the DNA molecules approach saturation with complementary RNA. The findings, which agree with those of other authors (3, 4), not only may account for the major discrepancies in the quantitation of viral genomes, but also offer a useful approach for study of the interaction of viral DNA with host DNA, both in lytic infection and transformation.

MATERIALS AND METHODS

Source of Viral DNA. All viral DNA preparations were derived from a plaque isolate of SV40 virus, strain 777. *Low-multiplicity* viral DNA was obtained by infection of BS-C-1 cells (a line of African green monkey kidney cells) with a 1/100 or 1/200 dilution of the first-passage supernate derived from the plaque isolate [input multiplicity of infection: 0.5-1 plaque-forming units (PFU) per cell]; DNA was harvested at 7-8 days. *High-multiplicity* viral DNA was made by infection with undiluted first-passage stock (multiplicity about 100 PFU per cell) or with later passages (at multiplicities between 10 and 1000 PFU per cell); cells were harvested at 3-4 days. ^{32}P -Labeled SV40 DNA was grown in the presence of 65 $\mu\text{Ci/ml}$ of $^{32}\text{PO}_4$ and 10 μM phosphate; the specific activity was 1 to 2 $\times 10^6$ cpm/ μg .

Abbreviation: SSC, 0.15 M NaCl-0.015 M $\text{Na}_3\text{citrate}$.

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Extraction and Purification of Superhelical (form I) SV40 DNA. Viral DNA was extracted according to Hirt (5). The supernatants were extracted twice with phenol and once with chloroform-isoamyl alcohol 24:1; they were then precipitated at -20° with two volumes of 95% ethanol. The precipitates were dissolved in 0.1 M Tris·HCl (pH 8.0)-0.01 M EDTA, and the viral DNA was purified by two ethidium bromide-CsCl density equilibrium centrifugations. The lower band, containing the superhelical form I DNA, was extracted with isopropanol-H₂O 9:1, dialyzed, and further purified by sedimentation through a 5-20% sucrose gradient in 0.01 M Tris·HCl (pH 8.0)-1 mM EDTA. The 20S band of form I DNA was dialyzed against 0.01 M Tris·HCl (pH 8.0)-1 mM EDTA, and was stored at 4°.

Preparation of Relaxed (form II) SV40 DNA. Form I-SV40 DNA was converted 50-70% to the relaxed form II DNA by DNase I action (incubation for 20 min at 37° with 25 ng of DNase per ml in 0.5 M NaCl-25 mM MgCl₂-0.01 M Tris·HCl-1 mM EDTA, pH 8.0). Form I and form II DNA were subsequently separated by ethidium bromide-CsCl density equilibrium centrifugation.

Source and Purification of Cellular DNA. DNA from *Escherichia coli* (strain K 12) cells was purchased from General Biochemicals. Animal cell DNA was extracted according to Marmur (6). Chinese hamster embryo DNA (from the fifth to tenth transfer) was further purified by density equilibrium centrifugation in ethidium bromide-CsCl. The upper band was treated with isopropanol and dialyzed against 0.01 M Tris·HCl (pH 8.0)-1 mM EDTA, and was stored at 4°. When required, the molecular weight of the DNA was reduced by sonication with a Branson sonifier. DNA of SV40-transformed cells was extracted from the seventh transfer of Cl 71 (Mut 295), a clone of secondary Chinese hamster embryo cells transformed by SV40 virus.

In vitro Synthesis of Virus-Specific Complementary RNA (cRNA). SV40 cRNA was synthesized according to Burgess (7). The reaction mixture (1.25 ml) contained 10 mM MgCl₂, 0.1 mM EDTA, 0.15 M KCl, 0.1 mM dithiothreitol, 0.3 mM (each) of ATP, CTP, and GTP, 68 μM UTP, 68 μM [^3H]-UTP (36.5 Ci/mmol, New England Nuclear Corp.), 15 μg of form I SV40 DNA, and 14 μg of RNA polymerase (from *E. coli* K-12, Miles, or from *Azotobacter*, kindly supplied by Dr. Joseph Krakow). After 90 min at 37°, 70-80% of the labeled UTP had become acid-precipitable; the reaction mixture was twice extracted with phenol and once with chloroform-

isoamyl alcohol; the cRNA (40–60 μg) was then precipitated with ethanol in the presence of 3 mg of yeast RNA. The specific radioactivity of the cRNA, calculated from the specific radioactivity of the incorporated UTP, varied from 1.3 to 1.75×10^7 cpm/ μg . Symmetric RNA was measured as the proportion that remained acid-precipitable after 45 min at 37° in $2 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M NaCl}-0.015 \text{ M Na}_3\text{ citrate}$) containing 20 $\mu\text{g}/\text{ml}$ of pancreatic RNase and 1 $\mu\text{g}/\text{ml}$ of T1 RNase. This proportion was not increased by self-annealing (3 hr at 65° in $2 \times \text{SSC}$, at a cRNA concentration of 1 $\mu\text{g}/\text{ml}$). The RNase-resistant fraction was 8–12% in different preparations; hence, 94–96% of the cRNA was transcribed from one strand (8). This strand will be referred to as the transcribed strand.

The size of the cRNA, determined by centrifugation in a dimethylsulfoxide gradient (9), was heterogeneous, with a mean sedimentation constant between 16 and 20 S. In some experiments, the cRNA was reduced to a mean size of 9–11 S by limited digestion with electrophoretically purified DNase (Worthington DPF), treated with iodoacetate (10) to remove most ribonuclease activity. The DNase (still containing some RNase activity) was used at a concentration of 50 $\mu\text{g}/\text{ml}$ in 5 mM MgCl_2 -0.1 M sodium acetate buffer (pH 5.3) to digest 5 μg of purified cRNA. After 1 hr of incubation at 37°, the reaction mixture was extracted twice with phenol and once with chloroform as described above.

Hybridization in Liquid. The hybridization mixtures contained, in a volume of 0.3–0.5 ml of $6 \times \text{SSC}$, viral DNA, cellular DNA, cRNA, and yeast RNA. Viral form II DNA was denatured at a concentration of 0.4 $\mu\text{g}/\text{ml}$ by boiling in $0.01 \times \text{SSC}$ (pH 7.1) for 10 min, followed by rapid chilling in ice and further dilution to 10^{-2} $\mu\text{g}/\text{ml}$ or less. Boiling at pH 7.1 did not break the viral strands, as determined by sedimentation of the denatured DNA in 5–20% sucrose gradients in the presence of 1 M NaCl (11). To obtain viral strands of smaller size, form II DNA was boiled for various times in $0.01 \times \text{SSC}$ of pH 3.5. Cellular DNA was denatured by boiling for 10 min in $0.01 \times \text{SSC}$ (pH 7.1). In early experiments, all hybridization mixtures contained 10 μg of cellular DNA and 1 mg/ml of phenol-extracted yeast RNA. In later experiments, the cellular DNA was omitted and the concentration of yeast RNA increased to 3 mg/ml. The cRNA was routinely filtered through a Millipore membrane filter (HAWP 02500) in a solution containing 1–3 mg/ml of yeast RNA before hybridization, a step found to effectively reduce the hybridization background.

The hybridization mixtures were incubated in stoppered-glass tubes for 16–18 hr in a water bath kept at 65°. After the incubation, the samples were diluted at room temperature with 5–7 ml of $6 \times \text{SSC}$, and poured over Millipore membrane filters that had been presoaked in $6 \times \text{SSC}$. The filters were allowed to drain by gravity on the filtration apparatus, washed with 5–7 ml of $6 \times \text{SSC}$, dried, punched to remove the rim, and incubated in scintillation vials with ribonuclease as described above. When 3 mg/ml of yeast RNA was used, the filters were sometimes washed twice with 20 ml of $2 \times \text{SSC}$ before RNase digestion. After the RNase treatment, each filter was washed five times by swirling it in the scintillation vial with 20 ml of $2 \times \text{SSC}$, then dried and counted. Each hybridization reaction was done in duplicate. In experiments with cellular DNA, the filters were counted and subsequently

assayed for DNA content by the diphenylamine reaction (13).

Hybridization with Filter-Immobilized DNA. DNA from Chinese hamster embryo and ^{32}P -labeled form I DNA from SV40 were denatured by boiling for 10–15 min in $0.1 \times \text{SSC}$, followed by rapid dilution into ice-cooled $0.1 \times \text{SSC}$. The DNA solutions were subsequently adjusted to $6 \times \text{SSC}$ and immobilized on Millipore membrane filters, according to the procedure of Gillespie and Spiegelman (12). For the hybridization, the filters carrying the immobilized DNA were incubated with light shaking for 18–22 hr at 65° in 1 ml of $6 \times \text{SSC}$, containing 0.5 mg of yeast RNA, 0.1% sodium dodecyl sulfate and various amounts of cRNA. After the hybridization, the filters were treated with RNase, washed, dried, counted, and assayed for DNA content as described above.

RESULTS

Hybridization in liquid of SV40 DNA with homologous cRNA. Bell-shaped hybridization curves

Fig. 1-I (curve A) shows the saturation curve obtained when low-multiplicity DNA was hybridized in liquid with different amounts of ^3H -labeled cRNA prepared on low-multiplicity SV40 DNA. The curve is bell-shaped; instead of the 2480 cpm of cRNA expected at saturation on the basis of the specific activity of the cRNA, only 500 cpm was bound to the filter at the highest point of the curve. The amount of viral DNA retained by the membrane rapidly dropped to about half of the original value. The concurrent loss of hybridized counts and of one-half of the viral DNA (presumably the transcribed strand) at high cRNA inputs suggests that extensively hybridized DNA molecules are not retained by the membranes. If so, it should be possible to correct for loss of hybridized counts from the membrane by normalization of the retained ^3H counts to the amount of the transcribed DNA strand retained by the filters. In fact, a plot of the inverse of the corrected values of the ascending part of the curve against the inverse of the cRNA concentration yields a straight line, which extrapolates to 2500 hybridized cpm, a value close to the expected saturation value. This result suggests that only a small nonhybridized segment of the DNA strand is sufficient for the retention of hybrid molecules. The correction is inaccurate for points in the descending part of the curve, due to the small number of cpm of ^{32}P retained. It seems, however, that the normalized ^3H counts decrease, suggesting that a small proportion of only partially hybridized molecules are held to the membrane at high cRNA concentrations. The possible nature of such molecules is discussed below.

The loss of DNA-RNA hybrid molecules was not prevented by addition of 10 μg of Chinese hamster or *E. coli* DNA to the incubation mixtures, either in long fragments or sheared to 100,000 daltons per single strand. All the bound cellular DNA was retained at the highest cRNA concentration, though 50% of the viral DNA was lost.

The bell-shaped hybridization curve has been obtained with larger viral DNA inputs (not shown). The peak was accordingly higher, and occurred at about the same cRNA to DNA input ratio as in Fig. 1.

Effect of the size of viral DNA on the hybridization curve

Fig. 1-II (curve A) shows a hybridization curve with the same cRNA used in Fig. 1-I and with viral DNA broken to about half the normal size, but heterogeneous. There is a

marked reduction in the height of the peak and a more rapid loss of DNA, suggesting that a minimum length of nonhybridized DNA is required for retention on nitrocellulose filters. In contrast, variation of the size of the cRNA in the range from 9 to 20 S had a small effect on the hybridization curve with DNA of normal length; only with the smaller cRNA was there a slight increase in the peak value.

Influence of the source of viral DNA on the hybridization curve

A marked variability in the final loss of hybrid counts was observed with different preparations of viral DNA. Thus, in Fig. 2 (curve A) the hybridization curve reaches a final level corresponding to 10% of the expected saturation value of 4350 cpm. Concurrently, the loss of the viral DNA is limited to only 80% of the transcribed strand. These results suggest again that in some of the RNA-DNA complexes, the DNA

could not be saturated, even at very high inputs of cRNA. The retained ^3H radioactivity, normalized to the retained fraction of the transcribed strand, fluctuates about 40% of the expected value at high cRNA concentration.

The shape of the hybridization curve correlated with the method of preparation of the SV40 DNA. Curves similar to that in Fig. 2 were obtained if the SV40 DNA used for hybridization or for synthesis of cRNA was derived from infection at high multiplicity. A possible explanation for the high residual level of hybrid counts under these conditions will be given below.

Loss of hybrid counts with filter-immobilized DNA

In Fig. 3, high-multiplicity SV40 DNA, immobilized on filters together with cellular DNA, was hybridized to cRNA made on low-multiplicity SV40 DNA. Although the curve of hybrid counts (curve A) has the aspect of a saturation curve, 40% of the viral DNA (curve B) or 80% of the transcribed DNA strand was lost, while the amount of cellular DNA retained on the filters remained constant (not shown). The apparent saturation level reached by the hybrid ^3H cpm is about 25% of the saturation value (23,600) expected from the specific activity of the cRNA. Normalizing the hybridized counts to the retained fraction of the transcribed strand and plotting the inverse of the normalized counts against the inverse of the cRNA concentration yields a saturation value (23,500 cpm) very close to that expected from the specific activity of the cRNA. Hence, the retention of hybrids to the membrane again required only a short nonhybridized segment of the DNA strand.

The loss of hybridized DNA from the nitrocellulose filter is probably equivalent to the failure of hybrid molecules to attach to the filter in the liquid hybridization experiments. As already suggested (3), the hybridized DNA is actually pulled off from the filter.

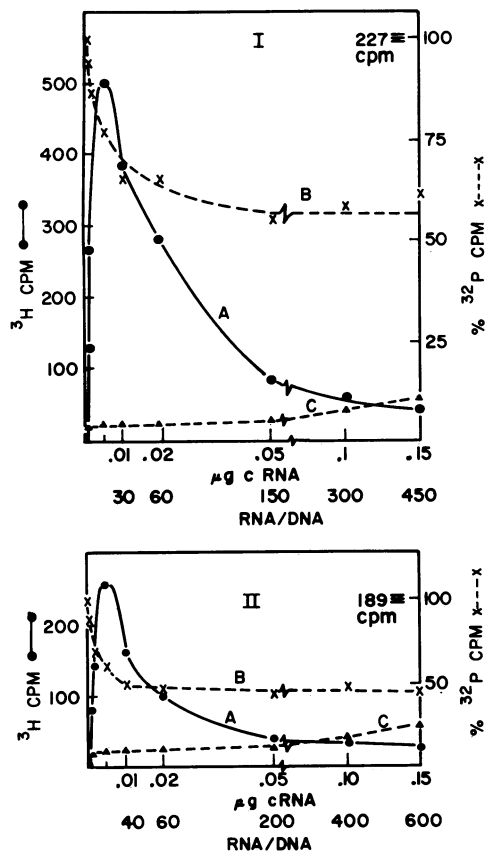


FIG. 1. Liquid hybridization of two sizes of ^{32}P -labeled SV40 DNA with different amounts of ^3H -labeled SV40 cRNA (specific activity = 1.5×10^7 cpm/ μg). The ^{32}P -labeled SV40 DNA and the viral DNA used as template for the synthesis of cRNA were low-multiplicity DNA. No cellular DNA could be detected in this viral DNA by DNA-DNA hybridization†. For each graph: curve A = hybridized (^3H) counts corrected by subtraction of nonspecifically bound counts (curve C); curve B = % SV40 DNA retained by filters: 227 cpm of ^{32}P (graph I) corresponds to 0.3 ng, 189 cpm of ^{32}P (graph II) to 0.25 ng of SV40 DNA; curve C = no DNA added to hybridization mixtures.

† We thank Drs. Lavi and Winocour for kindly having determined the amount of detectable host homology in the viral DNA preparations used for this work.

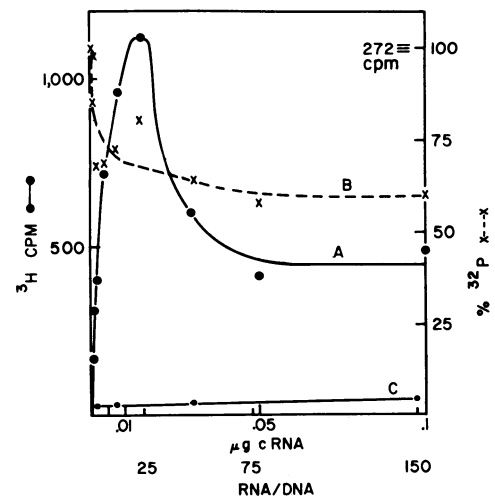


FIG. 2. Liquid hybridization of ^{32}P -labeled SV40 DNA with different amounts of ^3H -labeled SV40 cRNA. The SV40 DNA used in the experiment is the same used in the experiment shown in curve A (Fig. 1, graph I). The cRNA (specific activity as in Fig. 1) was synthesized on a template of high-multiplicity SV40 DNA. In this DNA, 3% of cellular DNA was detected by DNA-DNA hybridization†. Curves A, B, and C as in Fig. 1; 272 cpm of ^{32}P corresponds to 0.65 ng SV40 DNA.

Behavior of integrated SV40 DNA

DNA extracted from SV40-transformed Chinese hamster cells in the form of rather long fragments (Fig. 4) and hybridized in liquid to increasing amounts of cRNA (made on low multiplicity DNA) yielded a regular saturation curve. The saturation level corresponds to about six SV40 genomes per cell (calculated from the specific activity of the cRNA). However, after the cellular DNA was sheared to less than half of the length of SV40 DNA, a bell-shaped curve was obtained (Fig. 4). These results can be explained by the covalent linkage of the viral DNA to cellular DNA; in long DNA fragments, the cellular DNA portion, which cannot be hybridized, holds the hybrid segments to the membrane. When the molecules are short, some fragments lack cellular sequences and can form complete hybrids, which are lost. In order to determine whether the SV40 DNA molecules were integrated individually or together in a tandem reiteration, classes of DNA fragments of different length were hybridized to cRNA, and the amounts of hybrids retained on membranes were determined at a constant concentration of cRNA (0.15 $\mu\text{g}/\text{ml}$). Fig. 5 shows that the boundary between the partially and the completely retained fragments falls at about one SV40 DNA length (18×10^{-5} daltons), suggesting that viral DNA molecules are integrated individually. If the unhybridized DNA length required to anchor the hybrid is a small proportion of a viral strand, the experimental points should be near the dotted line in Fig. 5. The displacement to the left of many experimental points may be in part due to the high heterogeneity of the samples, since the longer fragments would contribute more molecules that are retained.

DISCUSSION

These experiments point out one important parameter in DNA-RNA hybridization experiments based on the reten-

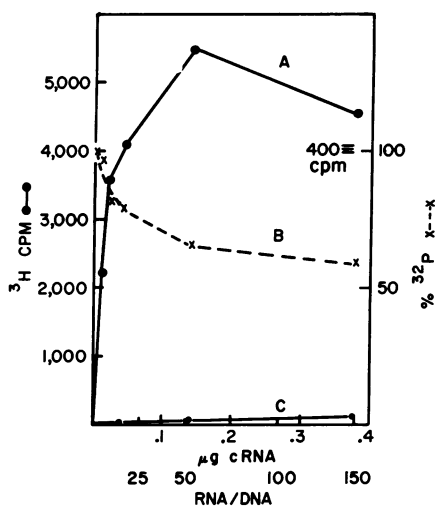


FIG. 3. Filter hybridization of high-multiplicity SV40 DNA with different amounts of low-multiplicity cRNA (specific activity as in Fig. 1). Each filter contained 8 μg of DNA from Chinese hamster-embryo cells sheared to about viral DNA size and 2.7 ng (=400 cpm) of ^{32}P -labeled SV40 DNA. Curve A = hybridized (^3H) counts corrected by subtraction of nonspecifically bound counts (curve C). Curve B = % SV40 DNA remaining on the filters. Curve C = ^3H counts bound to filters containing cellular DNA only.

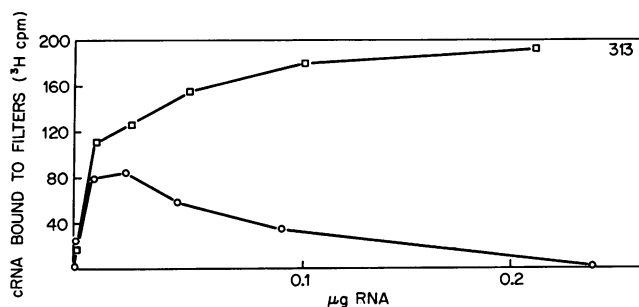


FIG. 4. Liquid hybridization of SV40 cRNA with 10 μg of sheared (○—○) and nonsheared (□—□) cellular DNA from SV40-transformed Chinese hamster-embryo cells (clone 71). The cellular DNA was denatured by boiling for 10 min in $0.1 \times \text{SSC}$. ^3H -Labeled cRNA (specific activity = 1.3×10^7 cpm/ μg) was synthesized on low-multiplicity SV40 DNA. The ^3H cpm are normalized to 10 μg of DNA per filter, and corrected for ^3H cpm retained from mixtures containing nontransformed cell DNA. (At the highest input of cRNA, the control filters retained 95 cpm of ^3H .)

tion of hybrids on nitrocellulose membranes, i.e., the requirement for a nonhybridized DNA segment. Its length is a small proportion of a SV40 DNA molecule, as indicated by the closeness between the extrapolated saturation value (at relatively low cRNA concentrations) and the expected saturation value. If the SV40 DNA molecules are sheared, the loss

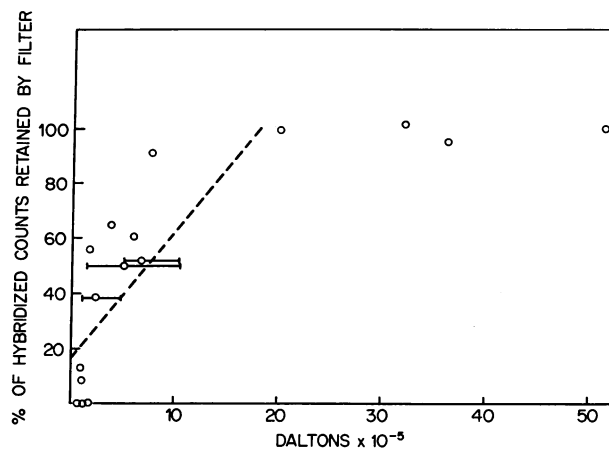


FIG. 5. Influence of the size of cellular DNA fragments on the amount of hybridized counts retained by nitrocellulose membranes. DNA from SV40-transformed Chinese hamster cells was sheared by sonication, and classes of fragments corresponding to various sizes were isolated by sedimentation velocity in neutral sucrose gradients. The size distribution of each class was determined on a sample denatured by boiling (10 min in $0.1 \times \text{SSC}$) and sedimented in a 5–20% sucrose gradient in the presence of 1 M NaCl. An aliquot of 10 μg of each DNA fraction was hybridized with 0.15 μg of low-multiplicity cRNA (specific activity = 1.7×10^7 cpm/ μg). The ^3H counts were normalized and corrected as for Fig. 4. The circles correspond to the peaks of the DNA bands. However, the bands were wide, indicating considerable heterogeneity, the degree of which is indicated for some points by the attached lines. The dashed curve gives the expectation for singly integrated SV40 molecules, with negligible anchoring length, corrected for 17% residual retention of complete hybrids at 0.15 μg of cRNA per ml (see Fig. 4).

of hybrid counts is proportionally larger and more rapid, suggesting that a minimum length of nonhybridized DNA is required for retention on nitrocellulose filters. If the cRNA and DNA are completely homologous, the saturation curve in liquid is bell-shaped (Fig. 1). Partial lack of homology causes a residual level of retained hybrids at high cRNA concentrations; such an effect is observed if DNA of high multiplicity is used for the hybridization or for the synthesis of cRNA.

The residual retention of hybrids at high-cRNA concentrations affords a sensitive method to detect the presence of small nonhomologous sequences in the DNA and cRNA used for hybridization. The results obtained with DNA from SV40-transformed cells show that one possible source of nonhomology is the presence in the same molecule of cellular and viral sequences. Insertion of host-cell DNA into viral DNA prepared at high multiplicity of infection may also contribute nonhomologous sequences to free form I DNA (14). With free-viral DNA, another possible source of nonhomology is structural aberrations, such as inversions. Due to the asymmetry of cRNA, segments of the nontranscribed strand inserted in the transcribed strand would remain nonhybridized and could anchor the hybrid to the filter. The possible presence of viral molecules with structural aberrations in high-multiplicity DNA is being investigated. The low degree of saturation of the DNA molecules retained at high-cRNA concentrations could be explained by the presence of molecules with relatively long cellular insertions or inversions, which would leave a large segment of the DNA strand nonhybridized.

The finding that cRNA synthesized on high-multiplicity DNA and hybridized to low-multiplicity DNA equally helps the retention of hybrids (Fig. 2) could be attributed to deletions of viral sequences in DNA molecules with cellular insertions (15) with corresponding deletions in their RNA-transcripts. Hybridization with such deficient cRNA molecules would leave nonhybridized segments in the DNA. Possibly due to steric hindrance, these unpaired stretches may not be covered by other cRNA molecules. A similar situation could hold for hybrids with RNA-transcripts of viral DNA molecules with inversions.

Nonhomologous recombination between viral and cellular DNA, and possibly among viral DNA molecules, occurring especially at high multiplicity of infection, may be caused by a viral gene product and may represent an aberrant form of integration.

The application of these findings to study of the state of viral DNA in transformed cells leads to two main results: (i) Previous estimates from this laboratory of the number of viral genomes per cell (1), based on comparison of the amounts of cRNA hybridized to transformed cell DNA with those hybridized to free viral DNA, are in considerable excess; probably most hybrids formed by the free viral DNA in

reconstruction experiments were lost, whereas those formed by the integrated viral DNA were not. When this phenomenon is taken into account, the revised estimates fall in the lower range obtained with other methods (2). (ii) The regular shape of the saturation curve with transformed cell DNA, and its change to a bell-shaped curve after fragmentation of the DNA (Fig. 4), is in agreement with integration of viral DNA in the cellular DNA. Furthermore, the results of shearing the DNA show that the lengths of the integrated viral segments correspond to no more than one genome. Since the hybridization results indicate six molecules of viral DNA per cell in the transformed Chinese hamster line examined, they would imply that integration occurred at more than one chromosomal site in this near-diploid cell line.

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