Temperature dependence of strand separation of the DNA molecules containing integrated SV40 DNA in transformed cells

Allan H. Fried

Institut für Virusforschung, Deutsches Krebsforschungszentrum, 69 Heidelberg, West Germany

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ABSTRACT

It has previously been demonstrated that the DNA molecules containing a genetic marker from one region of a bacterial genome undergo complete strand separation at temperatures usually different from the molecules containing a genetic marker from another region of the genome. The experiments also showed that if a group of molecules undergo complete strand separation over a narrow temperature range, of the order of one to three degrees, it is highly likely that they all come from one region of the bacterial genome. The purpose of the work reported here was to establish appropriate procedures for doing a similar analysis of the DNA molecules containing the integrated SV40 DNA in transformed mouse cells. One result of interest is that in the 11A8 cell line about 40 per cent of the integrated SV40 DNA detectable by an RNA-DNA hybridization assay can be accounted for by a group of molecules which undergo complete strand separation within a 1.0 °C interval.

INTRODUCTION

It has been demonstrated that the DNA molecules containing a genetic marker from one region of a bacterial genome undergo complete strand separation at temperatures usually different from the molecules containing a genetic marker from another region of the genome (1-4). In addition, the same experiments provided a criterion for the homogeneity of a group of DNA molecules since they showed that if a group of molecules undergo complete strand separation over a narrow range of temperatures, of the order of one to three degrees, this means that it is highly likely that they all contain one region of the cellular genome in common. It was the purpose of the present work to establish procedures for doing a similar analysis of the DNA molecules which contain the integrated SV40 DNA in transformed mouse cells. It has previously been established that in SV40 transformed cells the SV40 DNA is integrated into the cellular DNA (5, 6).
In the case of the work with bacterial genetic markers a biological assay was used to determine that the DNA molecules had undergone complete strand separation. In the present work this assay was replaced with an RNA-DNA hybridization assay specific for single-stranded SV40 DNA (7). After the DNA molecules have been heated and cooled the single-stranded molecules will stick to a nitrocellulose filter and can be immobilized on the filter (7). The filters are then incubated with a solution of tritiated SV40 RNA that had been synthesized in vitro and that will hybridize with high specificity to the single-stranded SV40 DNA on the filters (7). The essential point here is that unless the strands of a DNA molecule are completely separated when they are heated they will completely regain their double-stranded structure upon cooling (8, 9). Therefore the appearance of single-stranded SV40 DNA sequences on the filter means that the entire molecule containing the SV40 DNA sequence has undergone strand separation.

The experiments were designed to establish the following basic aspects of the procedure: (1) that the cellular DNA molecules used were much larger than unit length SV40 DNA even after being heated to the temperatures used in the current experiments; (2) that the RNA-DNA hybridization assay gives the same $T_m$ for unit length linear SV40 DNA as other methods; (3) that the $T_m$ of the SV40-cellular DNA molecules is greater than the $T_m$ of unit length linear SV40 DNA. Since the $T_m$ of a DNA molecule is equal to the $T_m$ of its most heat resistant region (8, 10), this would mean that the $T_m$ is being controlled by cellular regions of the SV40-cellular DNA molecules; and (4) that the range of $T_m$'s possible for SV40-cellular DNA molecules is sufficiently large to give significance to a finding that a group of such molecules undergoes complete strand separation within a range of one or two degrees centigrade. The final object of the work was to see if in any of the three transformed cell lines studied a group of SV40-cellular DNA molecules could be found that undergoes complete strand separation within a temperature range of one or two degrees centigrade.

MATERIALS AND METHODS

Virus stocks. The plaque purified small plaque strain of SV40, SV-5 (11) was a gift of Dr. Malcolm Martin. It was subsequently passaged in my laboratory in CV-1 monkey cells. The strain of SV40, Rh911, isolated
by Girardi (12) had been subsequently plaque-purified three times by Dr. Gerhard Sauer.

**Cell lines.** SVT2 cells (13) and Balb/3T3 cells, clone A31 (14), were obtained from Dr. Charles Scher. SV3T3-47 cells (15) and 3T3 cells (16) were obtained from Dr. François Cuzin. 11A8 cells (17) were obtained from Dr. Malcolm Martin.

**Extraction of cellular DNA.** Cells were lysed in 0.1 M NaCl, 0.05 M Tris, 0.025 M EDTA, pH 7.2 (ENT Buffer) containing 0.6% sodium dodecyl sulfate. This was followed by the addition of 0.25 volumes of ENT containing 1 mg/ml pronase (Serva, Heidelberg) and incubation for one hour at 20°C. The pronase solution had been preincubated for one hour at 37°C. The DNA was extracted with phenol [80% (v/v) and containing 0.1% 8-hydroxyquinoline], precipitated with ethanol and dissolved in 0.015 M NaCl, 0.0015 M sodium citrate, pH 7.2 (0.1 x SSC). It was then incubated for 15 minutes at 37°C with pancreatic RNAse (25 µg/ml). The RNAse (1 mg/ml in H2O) had been preincubated at 80°C for 15 minutes. The DNA was extracted with phenol, precipitated with ethanol, dissolved in 0.1 x SSC and precipitated with isopropanol (18). The DNA was washed with ethanol and dissolved in 0.1 x SSC. The DNA concentration was measured by the diphenylamine reaction (19) using commercial calf thymus DNA preparations of known phosphorous content as a standard. This reaction gave values within 10 per cent of those obtained by measuring the A260λ of the DNA solution and assuming a value of 1.0 corresponds to 45 µg/ml.

For the preparation of tritium-labelled cellular DNA, cells were grown in the presence of [methyl-3H]thymidine.

**Preparation of SV40 DNA.** Unless noted otherwise, the following procedure was used. Confluent monolayers of CV-1 monkey cells were infected with SV40 at 2 plaque-forming units per cell. For the preparation of radioactive DNA either [methyl-3H]thymidine or [32p] orthophosphate was added to the medium 24 hours after infection. Cells were lysed 68 hours after infection in ENT buffer containing 0.6% sodium dodecyl sulfate. The cellular DNA was selectively removed in the presence of 1 M NaCl (20) and the SV40 DNA was extracted with phenol. The SV40 DNA was precipitated by adding ethanol and dissolved in 0.1 x SSC. It was then treated with ribonuclease as described for the extraction of cellular DNA, extracted with phenol, precipitated
with ethanol and dissolved in 0.1 x SSC. Superhelical SV40 DNA was isolated by CsCl equilibrium density centrifugation in the presence of 135 ug ethidium bromide/ml (21). The ethidium bromide was extracted with an isopropanol-CsCl solution (22) and the DNA was dialyzed against 0.1 x SSC.

In vitro synthesis of SV40 complementary RNA. Tritium-labelled SV40 complementary RNA was synthesized using Escherichia coli RNA polymerase containing sigma factor (purchased from Boehringer, Mannheim), SV40 DNA and tritium-labelled nucleotide triphosphates under the conditions described by Westphal (23). The RNA was extracted and banded on a CsCl-Cs2SO4 density gradient as previously described (24). The specific activity of the complementary RNA was calculated to be 27 x 10^6 cts/min/ug from the average specific activity of the nucleotide triphosphates and taking into account the counting efficiency of the liquid scintillation system. Normally SV-S strain DNA isolated from virions by a previously described technique (25) was used. The only exceptions were that the RNA preparation used in Figure 6 for experiments 6, 7 and 8 had been synthesized using Rh911 strain DNA as a template.

Standard Melting Curve Protocol. A solution of 8.37 M NaClO4, 0.175 M sodium citrate (buffer N) was first filtered through several glass fibre filters to remove undissolved impurities. A DNA-NaClO4 solution was made by taking 0.140 volumes of DNA that was dissolved in 0.1 x SSC and adding it to 0.860 volumes of buffer N (the final solvent is referred to as NSC buffer). Aliquots of the resulting solution were heated in closed screw top test tubes for 10 minutes at a temperature specified in a given experiment. The water bath temperature was held constant within ± 0.05 °C during the heating step. Tubes were placed in an ice-water bath and cooled before subsequent analysis.

Hybridization Assay Protocol. A specified volume of DNA in NSC buffer was mixed with 1.5 volumes of 10 x SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 6.9) and the mixture sucked through a nitrocellulose membrane filter (Sartorius - Membranfilter, West Germany). The filters were baked at 80 °C for four hours (7) and incubated with one ml of a solution of SV40 complementary RNA (140,000 cts/min per ml) as described previously (7). The filters were washed and treated
with ribonuclease as described previously (7), dried and the amount of radioactivity determined in a liquid scintillation counter.

**Sedimentation analysis of DNA at alkaline pH.** DNA was dissolved in a specified volume of ALK buffer (3 volumes of 0.1 x SSC plus 1 volume of 3.6 M NaCl, 0.3 M NaOH) and layered on an 11 ml 5 to 20 % (w/v) sucrose gradient in a solution consisting of 0.9 M NaCl and enough NaOH to give a pH of 12.5. The gradient was then centrifuged at 20 °C in a Spinco SW41 rotor as specified in the text. Gradient tubes were collected from the bottom of the tube for analysis.

**Preparation of linear SV40 DNA using the R, restriction endonuclease.** Purified SV-S strain SV40 DNA labelled with $^{32}$P was further fractionated into superhelical DNA and nicked circular DNA by sedimenting the DNA on sucrose gradients as described previously (26). The superhelical DNA was incubated with the R, restriction enzyme (27) of *Escherichia coli* for one hour under conditions described elsewhere (27). The R, enzyme had been purified by Gary S. Hayward. The DNA was extracted with phenol, precipitated with ethanol and dissolved in 0.1 x SSC. The DNA was analyzed by sucrose gradient centrifugation at neutral pH as described previously (28) in the presence of $[^3H]$-labelled nicked circular DNA. It was found that 85 % of the $[^3P]$-labelled DNA sedimented as a homogeneous peak with a sedimentation constant 1.5 S units slower than the $[^3H]$-labelled DNA indicating that this peak of $[^3P]$-labelled DNA consisted of unit length linear SV40 DNA (28). Parallel gradients containing only the $[^3P]$-labelled DNA were also run and the fractions containing the unit length linear DNA were collected. The DNA was precipitated by adding ethanol and then dissolved in 0.1 x SSC.

**Treatment of DNA with S, endonuclease.** S, endonuclease was isolated using the procedure of Vogt (29) up to and including the DEAE chromatography step. Purification of the enzyme was done together with Inge Maichle. The reaction mixture for S, treatment contained 0.1 ng to 1.5 ng of $[^3P]$-labelled SV40 DNA, 25 ug of single-stranded monkey cell DNA and 200 ul of S, enzyme solution in one ml of the reaction buffer described by Vogt (29). After incubating the reaction mixture for 120 minutes at 35 °C, the amount of DNA that was precipitable in the presence of 100 ug of albumin and 5 % trichloroacetic acid was determined.
Production of nicked circular SV40 DNA with DNAse I. The reaction solution contained 3.5 μg of tritiated SV-S strain SV40 DNA with either zero (control DNA) or 5 ng of pancreatic DNAse (DNase I) (purchased from Schwarz/Mann) in 2 ml of 0.1 M NaCl, 0.02 M MgCl₂, 0.1 M Heps, pH 7.0. The tubes were incubated in an ice-water bath for 30 minutes and the DNA was purified by phenol extraction and precipitated with ethanol. The precipitate was washed with ethanol and dissolved in 0.1 x SSC.

Sedimentation analysis (data not shown) of the DNA on sucrose gradients at neutral pH (26) revealed that 82% of control DNA was superhelical while 18% was nicked circular DNA. It also showed that for the DNA that had been treated with DNase, 40% of the DNA was superhelical and 60 per cent of the DNA was nicked circular DNA. Assuming that there are many equally probable attack sites for the DNAse, it was calculated that 61% of the nicked circular molecules in the DNase-treated preparation had only one nick by using the equation describing the Poisson Distribution.

RESULTS

Molecular weight of the cellular DNA molecules after exposure to different temperatures

The size of the DNA molecules isolated from SVT2 cells was measured after their exposure to different temperatures by employing sedimentation analysis at alkaline pH, conditions where the entire DNA population is converted to single-strand molecules (30). Unheated DNA and DNA heated to 50 °C or 65 °C for 10 minutes each displayed similar size distributions while DNA that had been heated to 80 °C showed evidence of being nicked by the heating procedure (Fig. 1). The average sedimentation constant for the DNA heated to 65 °C was estimated to be 48 S based on the positions of the SV40 DNA molecules of known sedimentation constant, which had been centrifuged on parallel gradients (Fig. 1). This corresponds to an average single-stranded molecular weight of 25 x 10⁶ (30). Hence the average double-stranded molecular weight of these molecules is about 50 x 10⁶. It will be seen that most of the SV40-cellular hybrid DNA molecules of interest undergo complete strand separation at temperatures which do not exceed 65 °C. Therefore at these temperatures the cellular DNA molecules were considerably larger than a complete SV40 DNA molecule.
Figure 1. Alkaline sedimentation analysis of cellular DNA after exposure to various temperatures. The Standard Melting Curve Protocol (see Materials and Methods) was followed for a solution containing 20 ug/ml of tritiated SVT2 cell DNA. DNA that was heated to a specified temperature was precipitated with ethanol and dissolved in ALK buffer. About 8 ug of each DNA sample in 500 ul of ALK buffer was then centrifuged on alkaline pH sucrose gradients at 33,000 revs/min for 285 minutes. The ordinate gives the cts/min in each gradient fraction that was precipitable in the presence of 100 ug albumin and 5% trichloroacetic acid. The arrows denoted by the sedimentation constants 53 S and 17 S give the positions of SV40 DNA I and II (26, 31), respectively, which had been centrifuged on parallel gradients in the presence of 20 ug of non-radioactive SV3T3-47 cell DNA. (a) Unheated DNA. (b) DNA heated to 50 °C. (c) DNA heated to 65 °C. (d) DNA heated to 80 °C.

which has a molecular weight of 3 x 10^6 (32).

The alkaline sucrose gradient sedimentation patterns obtained with unheated Balb/3T3 cell DNA were the same as those with unheated SVT2 cell DNA (data not shown).

Measurement of the Tm of linear SV40 DNA

SV40 DNA III molecules³ were generated using the R1 restriction endonuclease (27). Alkaline sedimentation analysis indicated that about 90 per cent of this DNA sedimented as a homogeneous population with a sedimentation constant of 16 S, the value expected for unit length single-stranded SV40 DNA (26) (Fig. 2). As a result it was concluded that about 80 per cent of the SV40 DNA III molecules had no nicks in either strand.
Figure 2. Sedimentation analysis at alkaline pH of the SV40 DNA III molecules. SV40 DNA III molecules labelled with $^{32}$P were prepared using the R1 restriction enzyme (Materials and Methods). SV40 DNA I labelled with tritium was also prepared and used when 29 per cent of the DNA had been converted to SV40 DNA II during storage. The DNA II molecules generate unit length single strand molecules (16 S) and single strand circular molecules (18 S) upon sedimentation at alkaline pH (26), and the arrows on the gradient are based on the positions of these tritiated molecules on the gradient. The $^{32}$P labelled DNA (0.04 ug), the tritium labelled DNA (0.16 ug) and non-radioactive 3T3 cell DNA (10 ug) were dissolved in 133 ul of ALK buffer and centrifuged on alkaline sucrose gradients of 31,000 revs/min for 15 hours. Fractions were collected on glass fiber filters, and the amount of radioactivity determined. (•••), 3H cts/min. (---•••), $^{32}$P cts/min. (a --- a), $^{32}$P cts/min.

A population of SV40 DNA II molecules in which 61 per cent of these molecules had only one single strand break was generated using pancreatic DNAse (see Materials and Methods). There is evidence that a given region of a DNA molecule will undergo strand separation at the same temperature whether it is a form III molecule or a form II molecule with one nick (33). Hence the Tm of these molecules should be the same. The R1 endonuclease cuts the SV40 DNA at a single specific site (27, 28) while pancreatic DNAse is much less specific with respect to the base sequence it will attack (34). Therefore the form II molecules serve as a control to see if the R1 enzyme cuts the SV40 DNA at a site which leads to an unusually heat-sensitive form of SV40 DNA III.

The percentage of SV40 DNA III molecules that had undergone complete strand separation after being heated to various temperatures was measured by determining the percentage of the molecules that could bind to nitrocellulose filters (35) after heating the DNA both in the absence (Fig. 3(a)) and presence (Fig. 3(b)) of cellular DNA. A
similar experiment was done with the SV40 DNA II molecules (Fig. 3(c)). In the latter case, SV40 DNA I molecules were also present (40 per cent of the total DNA, see Materials and Methods), but only a few per cent of these molecules will bind to the filters after being heated to the temperatures employed in these experiments. This can be seen from the results with the control DNA in Fig. 3(c) in which 85% of the DNA is SV40 DNA I. In that case 82 per cent of the DNA did not bind to the filters after being heated to temperatures up to 80 °C. When samples containing 100 per cent SV40 DNA II were used, however, 100 per cent of the SV40 DNA II bound to the filters after being heated to 80 °C (data not shown). Hence in Figure 3(c) the melting curve is essentially the melting curve of the SV40 DNA II molecules in the population.
The melting curve of the SV40 DNA III population was also done by assaying for the amount of single-stranded DNA with the S1 endonuclease after heating and cooling the DNA (Fig. 4). This enzyme degrades single-stranded DNA at a much faster rate than it degrades double-stranded DNA (36).

![Figure 4. Determination of the melting curve of SV40 DNA III molecules using the S1 nuclease assay. The Standard Melting Curve Protocol was followed for a solution containing 2.1 ng/ml of [32P]-labelled SV40 DNA III. Each aliquot of DNA was then dialyzed against 0.1 x SSC buffer and treated with S1 nuclease as described in Materials and Methods.](image)

Finally, the Tm of the SV40 DNA III molecules, in the presence of untransformed cell DNA, was determined by using an RNA-DNA hybridization assay (7) specific for single-stranded SV40 DNA (Fig. 5). The SV40 cRNA used in the assay was transcribed from SV40 DNA using Escherichia coli RNA polymerase under conditions which have been shown to result in complete transcription of at least one of the SV40 DNA strands in its entirety (25, 37, 38). The cellular DNA concentration was the same as that subsequently employed in experiments where the melting curve of the SV40-cellular DNA molecules from transformed cells was measured. Also the amount of hybridizable SV40 DNA was comparable in magnitude since each heated 5 ml aliquot contained 5 x 10^-4 ug of SV40 DNA and 100 ug of untransformed cell DNA. It was determined in a separate experiment (data not shown) that 2.7 x 10^-4 ug of SV40 DNA plus 100 ug of untransformed cell DNA bound the same amount of SV40 cRNA in the hybridization assay as 100 ug of

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Figure 5: Meltang curve of SV40 DNA III molecules using the RNA-DNA hybridization assay. The Standard Melting Curve Protocol was followed for a solution containing 10 x 10^-5 ug/ml of [32P]-labelled SV40 DNA III and 20 ug/ml of non-radioactive untransformed 3T3 cell DNA. A volume of 5 ml of each aliquot was then analyzed according to the Hybridization Assay Protocol. The ordinate gives the amount of tritiated SV40 cRNA that was bound to each filter in the assay. (o), experiment 1. (a), experiment 2. The results of doing the identical experiment at 80 °C with only 20 ug/ml 3T3 cell DNA and no SV40 DNA is given by (•), experiment 1 and (A) experiment 2. The shape of the melting curve of untransformed mouse cell DNA alone is determined in Figure 6(a) for 3T3 cell DNA and in Figure 6(c) for Balb/3T3 cell DNA and the shape of the dashed line in the present Figure is derived from those results and is drawn here to coincide with the points denoted by (e) and (A) at 80 °C. Based on 10 minute counts, the recovery of [32P]DNA on the filters was approximately 100 per cent for DNA that had been heated to at least 53 °C. However, since the amount of [32P] did not exceed 30 cts/min, these cts/min were not considered great enough to merit their display on the graph.

11A8 cell DNA when, in each case, the DNA had been fully denatured (heated to 80 °C).

The above experiments show that the Tm for SV40 DNA III molecules is between 52.0 °C and 53.0 °C and that the hybridization assay gives the same Tm as the nitrocellulose filter binding assay and the S1 nuclease assay.

Melting curves of the SV40-cellular DNA molecules

The melting curves of the SV40-cellular DNA molecules from three cloned transformed mouse cell lines were measured. The SVT2 cells and the 11A8 cells were derived by transformation of Balb/3T3 cells by the SV-S strain of SV40 (13, 17). The SV3T3-47 cells were derived by transformation of 3T3 cells by the 776 strain of SV40 (15).

The melting curves of the SV40-cellular DNA molecules from the transformed cell lines are shown in Figure 6. Either two or three independent experiments, each using an independently isolated DNA preparation, are shown for each cell line.

It has been shown previously that SV40 cRNA will also bind to untransformed cell DNA in the Hybridization Assay Protocol (7).
Figure 6. Melting curves of the SV40-cellular DNA molecules from transformed cells. The Standard Melting Curve Protocol was followed for a solution of cellular DNA (DNA concentration between 18 and 26 ug/ml depending on the experiment). A volume of each aliquot containing 100 ug of DNA was then analyzed according to the Hybridization Assay Protocol. The experiments are numbered from 1 to 9 in order to show whether the left or right hand ordinate should be used to read off the cts/min detected in the Hybridization Assay Protocol. (a) SV3T3-47 cell DNA, experiments 1 (o) and 6 (o); 3T3 cell DNA, experiment 2 (o). (b) 11A8 cell DNA, experiments 3 (o), 7 (o) and 8 (o). The dashed line is the curve determined for Balb/3T3 cell DNA in panel (c) of this figure. (c) SVT2 cell DNA, experiments 4 (o) and 5 (o); Balb/3T3 cell DNA, experiment 9 (o). The significance of the filled in symbols in this figure (•,•,•) is discussed in the text.
was taken into account by including, in each experiment with transformed cell DNA, an aliquot containing an equal amount of untransformed cell DNA that had been heated to 80 °C. In each panel in Figure 6 the value obtained with this untransformed cell DNA is given by a filled in symbol (e.g., ▲) for the experiment whose results are designated by the corresponding open point (e.g., △). The shape of the melting curve obtained with untransformed cell DNA was then determined in independent experiments (Fig. 6(a) and 6(c)) and drawn to scale so that it coincided with the filled in symbols at 80 °C. Therefore in any panel in Figure 6, the number of cts/min bound to integrated SV40 DNA sequences in a given aliquot of transformed cell DNA is given by the difference in height between the curves for transformed cell DNA and untransformed cell DNA at a given temperature.

It can be noted in Figure 6 that if one considers the ratio of the cts/min bound to 100 ug of transformed cell DNA that was fully denatured (heated to 80 °C) to the cts/min bound to 100 ug of untransformed cell DNA that was fully denatured, one reaches the conclusion that the three different types of transformed cell DNA have different capacities to bind SV40 cRNA in the hybridization assay. This was confirmed (data not shown) by comparing equal amounts of fully denatured DNA from each cell line in the same Hybridization Assay Protocol experiment. After subtracting out the cts/min that bound to an equal amount of untransformed cell DNA (3T3 and Balb/3T3 cell DNA bound the same amount of SV40 cRNA) – the following results were obtained: 11A8 cell DNA bound 1.5 times as much SV40 cRNA as SVT2 cell DNA and 0.5 times as much SV40 cRNA as SV3T3-47 cell DNA.

It can be seen from the results in Figure 6 that the shape of the melting curve is specific for the SV40-cellular DNA molecules from each transformed cell line. It can also be seen that the great majority of these molecules undergo complete strand separation at temperatures greater than 53 °C, the temperature required to completely separate the strands of SV40 DNA III molecules.

SV40-cellular DNA molecules can be found to undergo complete strand separation at temperatures as high as approximately 68 °C and as low as approximately 55 °C (Fig. 6(a)). In addition there are examples of these molecules undergoing complete strand separation at various intermediate temperatures (Figs. 6(a), (b) and (c)). This range of temperatures is within the range over which cellular DNA molecules undergo complete strand separation as evidenced by optical density
Figure 7. Melting curves of transformed cell DNA based on the measurement of the A260 of the DNA. The Standard Melting Curve Protocol was followed for a solution of cellular DNA, the DNA concentration being in the range 17 to 20 μg/ml depending on the experiment. The value of A260 was then measured at 22 °C. The per cent hyperchromicity is equal to \( \frac{[A_{260}(T) - A_{260}(22)]}{[A_{260}(80) - A_{260}(22)]} \) where A260(T) is the value of A260 for DNA that had been heated to a temperature T. The values A260(22) and A260(80) were 0.45 and 0.59, respectively, for SVT2 DNA, 0.50 and 0.66 for 11A8 cell DNA and 0.52 and 0.68 for SV3T3-47 cell DNA. (△), results with SVT2 cell DNA; (○), results with 11A8 cell DNA; (•), results with SV3T3-47 cell DNA.

For the purposes of the present communication only the transition between 56.0 °C and 57.0 °C with 11A8 cell DNA will be considered as evidence that a group of SV40-cellular DNA molecules can be found which display a sharp melting curve. The shape of the curve is based on three independent experiments and is probably the most reliable evidence for a sharp transition in the present experiments. The percentage of the total integrated SV40 DNA that is accounted for by this sharp transition can be calculated in the following manner:

The increase in the height of the melting curve between 56.0 °C and 57.0 °C for 11A8 cell DNA (Fig. 6(b)) can be defined as H, in arbitrary units. The increase in the height of the melting curve for untransformed cell DNA in the same temperature interval (Fig. 6(b)) is then 0.17 H. Therefore the increase in the height of the curve with 11A8 cell DNA due to integrated SV40 DNA is 0.83 H. (If one does the same calculation for each of the two neighbouring intervals, 55.0 °C to
56.0 °C and 57.0 °C to 58.0 °C, the increase in the height of the 11A8 curve due to integrated SV40 DNA is less than 0.1 H, confirming that the transition between 56.0 °C and 57.0 °C is a sharp transition.) The total amount of integrated SV40 DNA is given by the difference in the heights of the curves for 11A8 cell DNA and untransformed cell DNA when both are fully denatured, i.e., heated to 80 °C. At this point, however, some uncertainty is introduced into the calculation since the difference in the height between these two curves is actually greater at 62 °C than 80 °C. Therefore taking the value for the difference in the height of the curves at 62 °C leads to a lower, more conservative, value for the percentage of the total SV40 DNA accounted for by the sharp transition. The difference in the height of the curves at 62 °C is 2.1 H. Therefore the percentage of the total integrated SV40 DNA accounted for by the sharp transition between 56.0 °C and 57.0 °C is [(0.83 H/2.1 H) x 100] or 40 per cent.

**DISCUSSION**

The experiments can be seen to have established the following four basic aspects of the melting curve procedure pertinent to its use in the study of integrated SV40 DNA molecules:

1. The size of the cellular DNA molecules is about 17 times that of a unit length SV40 DNA molecule even when the DNA is heated to 65 °C for ten minutes. This is an advantageous situation since the greater the molecular weight of these molecules the greater probability that a given molecule will contain a cellular base sequence with a $T_m$ greater than that of unit length SV40 DNA.

2. The use of the RNA-DNA hybridization assay gives the same $T_m$ for a unit length SV40 DNA molecule as the use of the $S_1$ nuclease assay or the nitrocellulose filter binding assay. Also the width of the transition in the melting curve is of comparable sharpness, not more than two degrees, using all three assays. This means that the RNA-DNA hybridization assay is a reliable method to decide whether a molecule containing SV40 base sequences has undergone complete strand separation or not.

3. The $T_m$ of almost all of the SV40-cellular DNA molecules is greater than that of unit length linear SV40 DNA. This implies that the $T_m$ is being controlled by a cellular region of the SV40-cellular
DNA molecule in each case. Therefore if a group of such molecules shows a sharp transition in its melting curve, the homogeneity reflected by the sharpness of that transition results from homogeneity with respect to those cellular base sequences that determine the $T_m$ of the molecules and not from SV40 base sequences.

(4) If one combines the results from all three transformed cell lines, it is possible to find SV40-cellular DNA molecules that undergo complete strand separation at temperatures as low as about 55 °C and as high as about 68 °C as well as at many intermediate temperatures. Therefore the finding that a particular group of SV40-cellular DNA molecules all have undergone complete strand separation within a range of one or two degrees would mean that, within the limits of resolution of the experiments, it is a homogeneous group of molecules with respect to those cellular base sequences which determine the $T_m$ of the molecules. Given the complexity of organization of the base sequences in mammalian DNA, this result by itself would not prove that all the molecules in the group come from one site in the mouse genome. It would however be supporting evidence for such a hypothesis with respect to that group of molecules.

Given the above reasoning it is of interest that in 11A8 cells there is a group of SV40-cellular DNA molecules that accounts for about 40 per cent of the integrated SV40 DNA and undergoes complete strand separation between 56.0 °C and 57.0 °C. This observation is highly specific for 11A8 cell DNA since no comparable percentage of the SV40 DNA can be accounted for by a transition at this temperature in DNA from the other two transformed cell lines. It indicates that it is indeed possible to detect sharp transitions in the melting curves of the SV40-cellular DNA molecules from a transformed cell line. Hence the melting curve technique may turn out to be a useful method for obtaining evidence that a group of SV40-cellular DNA molecules originate from one site in the cellular genome.

The SVT2 and 11A8 cell lines are among those transformed cell lines with the lowest SV40 DNA content per diploid cell genome and indeed were thought at one point to have between 1 and 2 SV40 genome equivalents per diploid cell genome (17,39,40). Recent, more detailed analysis has shown, however, that in SVT2 cells this number is closer to 4 (41). It can be seen from the present communication and previous work (17,39) that 11A8 cells have even more SV40 DNA per ug of cell
DNA than SVT2 cells. As pointed out by Botchan et al. (41), quantitation and fractionation techniques involving SV40 DNA in transformed cells will have their greatest value for correlating the physical state of the SV40 DNA with its biological effects when transformed cell lines become available that are diploid in nature, have all been transformed from the same homogeneous stock of SV40 and have a very small number of integrated SV40 DNA pieces, preferably one.

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REFERENCES

Abbreviations used: SV40, Simian virus 40; Tm, the temperature that results in complete strand separation for 50 per cent of the DNA molecules; SV40-cellular DNA molecules, the cellular DNA molecules containing integrated SV40 DNA; SV40 DNA I, II and III are the superhelical, nicked circular and linear forms of double-stranded SV40 DNA, respectively; SV40 cRNA, SV40 complementary RNA.