A Convenient Micro Method for the Quantitation of Closed Circular Deoxyribonucleic Acid†

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ABSTRACT: A convenient and rapid micro method for the quantitation of covalently closed circular duplex DNA in the presence of nicked circular and linear DNA is described. The method relies upon the ability of closed circular DNA to renature spontaneously after brief exposure to either high temperature or high pH, both of which convert nicked and linear DNA to a denatured or single-stranded form. DNA that fails to renature can be digested to acid-soluble mononucleotides with single-strand specific S_1 nuclease leaving the renatured double-stranded DNA acid insoluble.

Closed circular bihelical DNA is found in a wide range of organisms including bacteria, bacteriophage, animal viruses, and eukaryotic cells (see review by Helinski and Clewell, 1971). A number of methods have been developed to separate closed circular duplex DNA from nicked circular and linear DNA. These include chromatography on columns of methylated albumin kieselguhr (Mandell and Hershey, 1960; Dulbecco *et al.*, 1965), differential sedimentation at alkaline or neutral pH (Vinograd *et al.*, 1965), dye–buoyant density centrifugation (Radloff *et al.*, 1967), and electrophoresis in polyacrylamide gels (Tegtmeyer, 1973). These methods have greatly facilitated the study of the synthesis and expression of circular DNAs, but are relatively slow and costly when applied to large numbers of samples.

This paper describes a convenient and rapid micro method for the quantitation of covalently closed circular duplex DNA in the presence of nicked circular and linear DNA. The method relies upon the ability of closed circular DNA to renature spontaneously after brief exposure to either high temperature or high pH, both of which convert nicked and linear DNA to a denatured or single-stranded form (Weil, 1963; Vinograd *et al.*, 1968). DNA that fails to renature can be digested to acid-soluble mononucleotides with single-strand specific S_1 nuclease (Ando, 1966) leaving the renatured double-stranded DNA acid insoluble.

Experimental Section

Materials. CV-1P, a line of African green monkey kidney cells, was propagated as previously described (Morrow and Berg, 1972). Simian virus 40 (SV40) strain Rh911, obtained from J. Vinograd, was grown and plaque purified on CV-1P cells. ^{3}H-Labeled SV40 DNA was prepared from infected cells as previously described (Beard *et al.*, 1973). Such preparations of SV40 DNA contain both covalently circular (SV40(I) DNA) and variable but small amounts of nicked circular (SV40(II) DNA) molecules, and were used directly without further separation of the covalently closed DNA. The infectivity of the DNA is generally of the order of 5×10^6 pfu/μg of DNA using a modification of the method of Pagano (1969). Full-length linear SV40 DNA labeled with [^{3}H]thymidine, formed by cleavage of circular SV40 DNA with a restriction endonuclease of *Hemophilus parainfluenzae* (HpaII) (Sharp *et al.*, 1973) was generously provided by J. Mertz and J. F. Morrow. S_1 nuclease from *Aspergillus oryzae* (Ando, 1966) was a gift of T. Ando.

Assay for Closed Circular DNA. The reaction mixture (0.1 ml) contained the radioactively labeled DNA to be analyzed and 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 200 mM NaCl, an excess (5 μg) of salmon sperm DNA as carrier, and 100 μg of sodium dodecyl sulfate. The sodium dodecyl sulfate prevents precipitation of protein during heating in cases where the sample contains protein and does not inactivate the enzyme activity (Vogt, 1973). Reaction mixtures were heated at 100° for 5 min and, after removal from the bath, 0.4 ml of an ice-cold solution of acetic acid containing 1.25 mM zinc chloride was added to adjust the pH to 4.6. The precise concentration of acetic acid required was predetermined for each set of stock solutions by titration of a mock reaction mixture. Samples were cooled in an ice bath, and after an aliquot of 0.2 ml was removed, 20–40 units (Beard *et al.*, 1973) of S_1 nuclease was added to the remainder of each reaction mixture. After incubation for 10 min at 37°, a second 0.2-ml aliquot was removed. Salmon sperm DNA (25 μg) was added to each aliquot, followed by ten volumes of cold 1 M HCl. The precipitates were collected on Whatman GF/C glass fiber filters, washed successively with 10 ml of 1 M HCl and 10 ml of ethanol, and the radioactivity was measured in a liquid scintillation spectrometer.

Alternatively, the DNA was incubated for 10 min at room temperature in a solution (0.1 ml) containing 10 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl and 5 μg of salmon sperm DNA, pH 12.1–12.3 (adjusted with NaOH). The precise amount of NaOH required to achieve the indicated pH was determined for each set of stock solutions by titration of a mock reaction mixture. At pH values of 12.0–12.3 covalently closed DNA is incompletely denatured so that it renatures spontaneously on return to neutral pH (Vinograd *et al.*, 1968) whereas nicked and linear DNA remain single-stranded after neutralization. Reaction mixtures were cooled and adjusted to a final volume of 0.5 ml at pH 4.6, with a predetermined volume of cold acetic acid; the solution was made 1 mM zinc acetate and the quantity of radioactive DNA resistant to S_1 nuclease digestion determined.

Slight modifications of this assay procedure were used in some experiments, as indicated in the figure legends.

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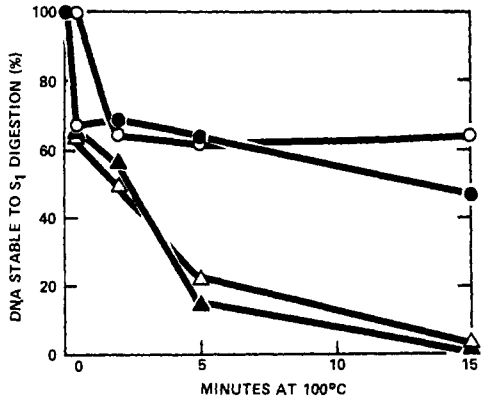

FIGURE 1: Denaturation of SV40 DNA at 100°. A mixture of ^{3}H-labeled SV40 (I) DNA (64%) and SV40 (II) DNA (36%) was heated at 100° at various concentrations of NaCl: 500 mM O—O. 100 mM ●—●, 10 mM △—△, no NaCl ▲—▲. At the indicated times aliquots of the mixture were removed, cooled in ice water, and brought to 60 mM sodium acetate (pH 4.6)–1 mM $ZnCl_2$, final volume 0.5 ml. The digestion with S_1 nuclease to determine the fraction of DNA resistant to digestion was carried out as described in the text.

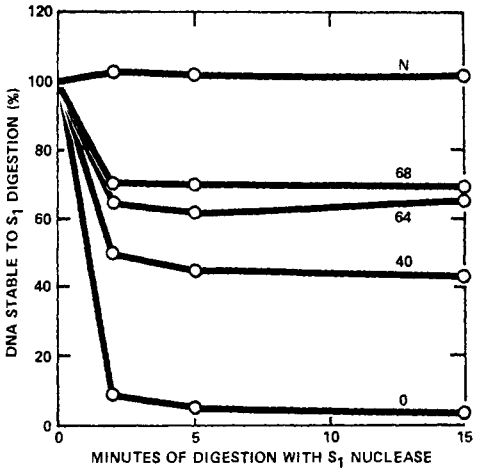

FIGURE 2: Time course of digestion of denatured SV40 DNA by S_1 nuclease. Mixtures containing various proportions of ^{3}H-labeled SV40 (I) and SV40 (II) DNA were denatured at 100° in 200 mM NaCl for 5 min, then cooled, and digested with S_1 nuclease as described in the legend to Figure 1. At the indicated times aliquots were removed and the amount of acid-precipitable radioactivity was determined. The numbers by each line indicate the per cent covalently closed DNA in that mixture measured by alkaline sucrose gradient velocity sedimentation; N denotes the mixture containing 40% SV40 (I) DNA digested with S_1 nuclease without prior heat denaturation.

Centrifugation. Sedimentation of SV40 DNA in neutral and alkaline sucrose gradients was performed as described previously (Beard *et al.*, 1973).

SV40 DNA Infections. CV-1P cells were infected with SV40 DNA in the presence of DEAE-dextran by a modification of the method described by Pagano (1969).

Results and Discussion

S_1 nuclease (Ando, 1966) degrades single- but not double-stranded DNA to acid-soluble products (Ando, 1966; Sutton, 1971; Vogt, 1973; Beard *et al.*, 1973). This fact plus the ability to denature nicked circular and linear DNAs under conditions where covalently circular DNA remains double stranded constitutes the basis for the present analytical procedure.

Conditions for Differential Denaturation of Nicked Circular and Linear DNA in the Presence of Covalently Circular DNA. The covalently circular form of SV40 DNA remains resistant to degradation by S_1 nuclease after heating at 100° at salt concentrations between 100 and 500 mM Na^+. The nicked circular form of SV40 DNA (and, though not shown in this experiment, the double-stranded linear SV40 DNA, as well) subjected to the same conditions rapidly becomes sensitive to the single-strand specific nuclease (Figure 1). If the heating is performed at 10 mM Na^+ or less, even the covalently circular molecules are progressively converted to a form which can be degraded by S_1 nuclease (Figure 1), presumably because single-strand breaks are introduced at the high temperature and low ionic strength (Vinograd *et al.*, 1968). Although the covalently closed DNA is most stable in 500 mM Na^+ at 100°, we have adopted as the standard condition for assay a 5-min heating period in 100–200 mM Na^+. This condition minimizes the renaturation of single-stranded DNA during the incubation with S_1 nuclease.

An alternative to heat denaturation is exposure of the DNA sample to pH 12–12.3. Although the covalently closed, nicked circular, and linear DNAs are each denatured under these conditions (Vinograd *et al.*, 1968; Pouwels *et al.*, 1968), *only* covalently circular DNA (except those molecules which contain small segments of RNA (*e.g.*, mt-DNA; J. Vinograd, personal communication) is renatured spontaneously after neutralization (Pouwels *et al.*, 1968). Following a brief incubation of the DNA sample at pH 12.1–12.3 (about 5 min at room temperature) and neutralization, more than 95% of nicked circular and linear DNA is degraded to acid-soluble products by S_1 nuclease; less that 5% of covalently closed DNA, however, becomes acid soluble following the same treatment.

The Amount of S_1 Resistant DNA Is a Measure of the Amount of Covalently Circular DNA. With DNA preparations containing different proportions of covalently circular and nicked plus linear SV40 DNA, the amount of DNA resistant to S_1 nuclease digestion following heat denaturation corresponds closely to the estimate arrived at by alkaline sucrose gradient analysis (Figure 2). Note that continued digestion with S_1 nuclease (or, in fact, the addition of more nuclease) does not appreciably alter the estimate of readily renatured DNA. Degradation of native SV40 DNA to acid-soluble fragments was not detected in this experiment. However in an experiment in which the amount of acid-soluble radioactivity released from native ^{3}H-labeled SV40 DNA by S_1 nuclease was measured directly (Beard *et al.*, 1973), the same preparation of S_1 nuclease degraded about 0.3% of native SV40 DNA to acid-soluble material in 30 min.

One complication in this procedure is the possibility that the single-stranded DNA may renature after cooling or neutralization. The critical parameters that determine the rate of reannealing are the concentration, complexity and size of the DNA, the ionic strength, and the temperature. To minimize this possibility our protocol calls for a rapid reduction of the ionic strength to 50 mM Na^+ or below and rapid cooling to below 10° following the denaturation step.

To test whether reannealing could occur during the standard assay, samples containing 0.05 or 0.5 μg per ml of full-length linear SV40 DNA (produced by cleavage of circular SV40 DNA with HpaII, a restriction endonuclease from *H. parainfluenzae* (Sharp *et al.*, 1973)) were heat denatured, cooled, and then digested with S_1 nuclease under the standard assay conditions. This DNA preparation contained no detectable circular DNA when analyzed by sucrose gradient sedimentation or electron microscopy. In each case more than 96%

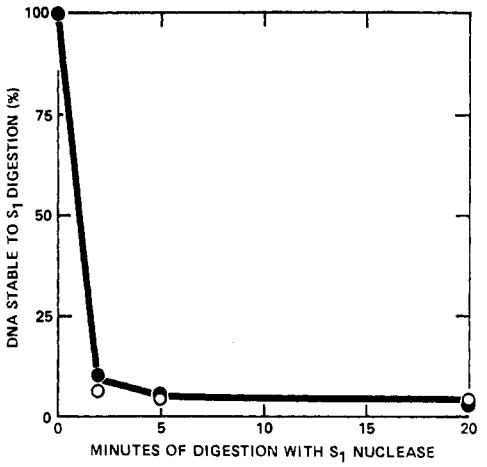

FIGURE 3: Lack of reannealing of denatured SV40 DNA during incubation with S_1 nuclease. Full-length linear SV40 DNA containing no circular DNA was denatured at 100° at DNA concentrations of either 0.5 μg/ml (○) or 0.05 μg/ml (●), cooled and incubated with S_1 nuclease as described in the legend to Figure 1. The amount of DNA which remained acid precipitable after various times of digestion by S_1 nuclease was determined as described in Methods.

of the initial radioactivity became acid soluble after digestion with S_1 nuclease (Figure 3). The resistant fraction is probably not due to reannealing of separated full-length single strands since the rate of reannealing of separated DNA strands and consequently the fraction becoming double stranded would be expected to depend on the DNA concentration. Quite possibly, renaturation of single-stranded DNA could be troublesome at higher concentrations of DNA, with DNA of low complexity (highly reiterated DNA) or with DNA containing self-complementary sequences on the same strand.

Applications of the Analytical Procedure. The analytical procedure can be applied to the detection and analysis of any covalently circular DNA in the presence of nicked circular and linear DNAs. In our own investigations we have used the method to monitor the conversion of covalently circular DNA to the nicked and linear forms by the action of DNases and restriction endonucleases, and the conversion of partially

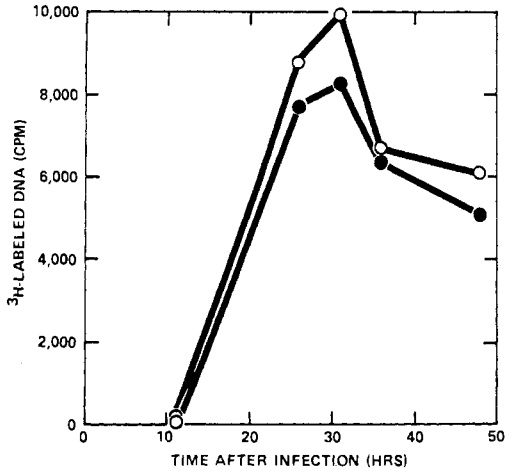

FIGURE 4: Synthesis of closed circular DNA after SV40 infection measured by alkaline sucrose gradient sedimentation (○) and by successive denaturation and S_1 nuclease digestion (●). See text for details. Sodium dodecyl sulfate (1 mg/ml) was added to the DNA solution prior to denaturation since the addition of NaCl to precipitate the cellular DNA also removes the detergent.

TABLE I: Detection of SV40 Viral DNA Replication in Cultures of 10^3 Cells Infected with SV40 Virus or SV40 DNA.[a]

	Expt	% of DNA Resistant to S_1 Digestion
SV40 virus infected	1	41.5
	2	28.3
Mock infected	1	4.2
	2	3.9
SV40 DNA infected	1	8.6
	2	6.6
Mock infected	1	3.8
	2	3.2

[a] Newly confluent monolayers of CV-1P cells in microtest plate wells (Falcon Plastics, approximately 1000 cells/well) were infected with either SV40 virus (5×10^4 pfu/well) or with SV40 DNA (2 ng of DNA (10^4 pfu) per well in the presence of 500 μg/ml of DEAE-dextran (Pagano, 1969)) or mock infected. From 24 to 48 hr after infection, DNA was labeled with [^{3}H]TdR (20 μCi/ml). Monolayers were then washed with Tris–saline solution (Smith *et al.*, 1960) and the cells were lysed with 20 μl/well of 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.1 M NaCl, and 5 mg/ml of sodium dodecyl sulfate. The lysate was transferred to a test tube, the volume brought to 0.1 ml by the addition of the same buffer without detergent, and the solution was homogenized by pipetting five times with a 100-μl micropipet. The pH was then brought to 12.1 by the addition of NaOH and after 5 min the samples were neutralized and the amounts of S_1 nuclease-resistant DNA were determined as described in the text.

replicated intermediates of SV40 DNA to mature, covalently circular molecules (DePamphilis and Berg, unpublished). Here we describe two experiments which demonstrate the applicability and sensitivity of the method for following the course of SV40 DNA synthesis in infected cells.

SV40 DNA SYNTHESIS IN MACRO CULTURES OF SV40 VIRUS-INFECTED CELLS. Confluent monolayers of CV-1P cells were infected with SV40 virus (input multiplicity 20) and [^{3}H]TdR (20 μCi) was added to the cultures at various times to label the DNA. One hour after the addition of the ^{3}H label, viral DNA was selectively extracted (Hirt, 1967), half of the sample was analyzed for SV40(I) DNA by conventional alkaline sucrose gradient sedimentation and the remainder by denaturation and S_1 nuclease digestion (Figure 4). The estimates of SV40 (I) DNA are virtually identical.

DETECTION OF SV40(I) DNA SYNTHESIS IN MICRO CULTURES INFECTED WITH SV40 DNA. Infection of monolayers of CV-1P with even saturating quantities of SV40 DNA (Pagano, 1969) is relatively inefficient as only about 1–5% of the cells become T-antigen positive (DePamphilis, Wilson, and Berg, unpublished). Where the quantity of DNA available for infection is limited (*e.g.*, 0.01–10 ng), the efficiency of infection is even less; under these circumstances it is very difficult to detect viral DNA synthesis by conventional extraction and sedimentation. Moreover, there are several experimental protocols which require monitoring the ability of a particular DNA to replicate even though it cannot be encapsidated in a virion; for example, SV40 DNA containing deletions, insertions or other structural anomalies (Jackson *et al.*, 1972; Mertz and Berg, unpublished results) cannot propagate as a virion,

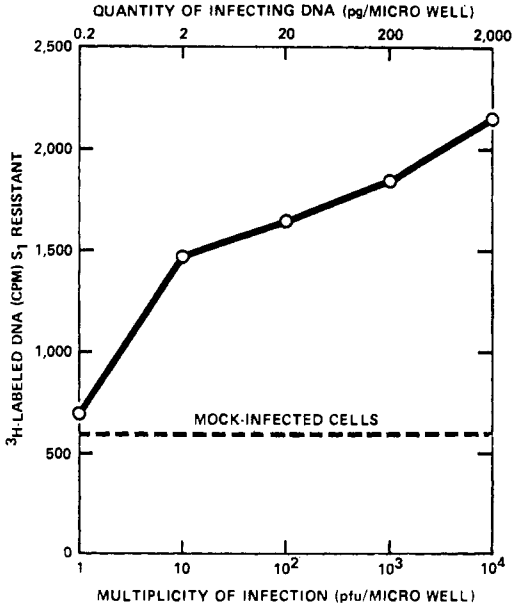

FIGURE 5: Synthesis of SV40 DNA in CV-1P cells infected with various multiplicities of SV40 DNA. Monolayers of 10^5 cells/micro well were either infected with the indicated amounts of SV40 DNA in 5 μl or mock infected. Labeling of DNA, cell lysis, and assay of covalently circular DNA were as in Table I. The level of S_1 nuclease-resistant DNA in lysates of mock-infected cells is indicated by the horizontal line.

and therefore cannot produce a plaque or a disseminating infection.

Newly confluent monolayers of CV-1P cells in microtest plate wells (Falcon Plastics; see Robb, 1973) were either infected with SV40 virions, SV40 DNA, or mock infected. Twenty-four hours after infection the DNA was labeled by replacement of the growth medium with medium containing [^{3}H]thymidine (20 μCi/ml). Twenty-four hours later cells were lysed with sodium dodecyl sulfate and the lysate (containing both the viral and cellular DNA) was analyzed for SV40(I) DNA by the denaturation-S_1 digestion assay. In this case DNA was denatured by raising the pH to 12.1, since even in the presence of sodium dodecyl sulfate a precipitate was produced at 100°.

In mock-infected cultures about 3–4% of the labeled cellular DNA is not degraded by S_1 nuclease after denaturation and neutralization (Table I). This background may be due to circular cellular DNA, rapidly reannealing DNA (perhaps of low genetic complexity or self-complementary sequence) or to incomplete digestion at low DNA concentration by S_1 nuclease (Sutton, 1971). In SV40 virus infected cells, about one-third of the labeled DNA is covalently circular as judged by its resistance to S_1 nuclease. In cells infected with SV40 DNA the S_1 nuclease-resistant DNA is between two and four times the level in uninfected cells, a value consistent with the likelihood that only 2–5% of the exposed cells was infected by the SV40 DNA. Figure 5 shows the results of an experiment in which the amount of SV40 DNA used to infect each micro culture was varied. Even when the infection was performed at an input multiplicity of 10 pfu/micro well (2 pg of DNA/micro well), there was a significant and reproducible increase in the amount of labeled DNA resistant to alkali denaturation as judged by its S_1 nuclease insensitivity, but this is near the limit of detection of the method. Development of ways to infect a higher proportion of cells exposed to SV40 DNA could overcome this limitation.

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