Studies on Circular Deoxyribonucleic Acid

I. Isolation of Bovine Papilloma Virus and Characterization of Its Deoxyribonucleic Acid

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A simple method is described for the isolation of bovine papilloma virus and its deoxyribonucleic acid (DNA). As found with other representatives of this virus group, this DNA preparation contains two components, I and II, as shown by sedimentation and electron microscopic studies. Component I is a fast-sedimenting, twisted, circular DNA molecule and represents usually 70 to 90% of the DNA in the mixture. The direction of the twist in the superstructure is right-handed. Component II originates from I by one or more single-strand breaks and is the "re-laxed" circular form of the viral DNA.

Circular deoxyribonucleic acid (DNA) molecules are found in a variety of organisms, and are known to exist in different structural forms. Single-stranded, covalently closed double-stranded, and "hydrogen-bonded" double-stranded DNA rings have been found, and, in the case of covalently closed double-stranded rings, a twisted superstructure has been demonstrated (7). DNA of this twisted type has been isolated from tumor viruses of the papova group and from infected bacteria, where it is an intracellular form of the DNA of certain phages (*see* reference 6 for recent review). Covalently closed double-stranded rings have also been isolated from mitochondria of some mammalian tissue (3), and recently from HeLa cells (5).

Covalently closed, circular, double-stranded DNA is a potentially useful tool in a number of physical and biochemical studies; however, the isolation of such material from sources previously described usually involves laborious and expensive tissue culture techniques (e.g., for polyoma virus or simian virus 40), or rather complicated procedures in the case of intracellular forms of bacteriophage DNA, or access to large amounts of mammalian papilloma tissues (e.g., human or rabbit). The present paper describes a simple method for isolation of large amounts of bovine papilloma virus and its DNA. Bovine papilloma tissue can be obtained in amounts greatly exceeding that obtained from other sources.

MATERIALS AND METHODS

Isolation of bovine papilloma virus. A 5-g amount of frozen papilloma tissue was thawed in 40 ml of

isolation buffer (1.0 M NaCl, 0.02 M sodium phosphate, pH 8) and minced in a Sorvall Omnimixer at 2 C for 5 min at full speed. The homogenate was centrifuged at $9,000 \times g$ for 30 min. The pellet was resuspended in 40 ml of fresh isolation buffer, minced, and centrifuged as before. The two supernatant fluids were combined, and Sarkosyl 97 (Geigy) and trypsin (Difco) were added to a final concentration of 0.25 and 0.01%, respectively. After incubation for 1 hr at 37 C, the solution was stored in ice for 1 hr. The resulting precipitate, consisting mainly of collagen fibres, was collected by centrifugation at $9,000 \times g$ for 20 min and discarded. The supernatant fluid was layered into centrifuge tubes previously prepared in the following way. Cellulose-nitrate tubes for Spinco rotor SW 25.1 were filled first with 1.5 ml of a CsCl solution with a density of 1.5 g/ml. On top of this, 2 ml of a CsCl solution with density 1.3 g/ml was layered. Both CsCl solutions were prepared in 0.02 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.5). The CsCl solutions were overlaid with 8 ml of a linear sucrose gradient containing 0 to 30% sucrose in 1.0 M NaCl, 0.01 M sodium phosphate (pH 7.5), and 0.25% Sarkosyl 97. The samples were centrifuged at 25,000 rev/min for 150 min at 15 C. The papilloma virus was usually visible as a bluish opalescent band at the interface of CsCl solutions, and was isolated by piercing the tube and collecting fractions. The procedure yields very pure virus preparations, as can be seen in an electron microscope (Fig. 1). Up to 0.4 mg of virus per gram of starting material has been obtained. For large-scale preparations, instead of centrifugation through the sucrose gradient into the CsCl layers, the virus was pelleted at $100,000 \times g$ for 2 hr. The pellet was resuspended in 0.01 M sodium phosphate-0.001 M ethylenediaminetetraacetate (EDTA) (pH 7.5) and centrifuged in a CsCl solution with a density of 1.30 g/ml for 24 hr. Usually a single opalescent virus band was formed. After fractionation, the CsCl was removed

FIG. 1. Samples of bovine papilloma virus purified by combined sucrose gradient-CsCl centrifugation. Stained with phosphotungstic acid. $\times 7.0 \times 10^4$.

from the virus solution by dialysis against 0.2 M NaCl-0.01 M sodium phosphate-0.001 M EDTA (pH 7.5).

Extraction of DNA. DNA was extracted according to Weil (9). Solutions of 1 to 2 mg of virus per ml were used. Phenol was freshly vacuum-distilled under nitrogen. Phenol was removed from the aqueous phase by dialysis versus 0.01 M sodium phosphate-0.001 M EDTA (pH 7.5).

Sedimentation. A Spinco model E ultracentrifuge with monochromator and ultraviolet (UV) absorption optics was used for sedimentation and CsCl density gradient studies. The UV photographs were traced with a Joyce-Loebel Mark IIIc microdensitometer. Band centrifugation according to Vinograd et al. (8) was performed in 30-mm cells with the use of a 20- to 40- μ liter lamellar volume and a buffer containing 1.0 M NaCl, 0.01 M sodium phosphate (pH 7.5), and 0.001 M EDTA as the bulk solution. For the stepwise alkaline denaturation studies, the bulk solution contained 1.0 M NaCl and 0.1 M sodium phosphate. The desired pH was adjusted with NaOH and measured with a Sargent pH meter (model DR) equipped with a Sargent electrode (no. S-30070-10). No corrections for sodium ion concentrations were made. Denaturation of the DNA occurred in the centrifuge cell during gradient formation in alkali.

Density determination. Harshaw optical grade CsCl in 0.02 M Tris buffer (pH 8) was used for de-

termination of the density of the papilloma DNA. DNA preparations from *Sarcina lutea* and *Pseudomonas rhodos* were used as density markers.

Electron microscopy. A Siemens Elmiskop IA electron microscope was used. Virus preparations were stained with phosphotungstic acid. DNA was prepared by the diffusion method described by Lang et al. (4).

RESULTS

As described above, this method for isolation of bovine papilloma virus provided good yields of high purity (Fig. 2). The DNA extracted from such preparations showed two components, I and II, upon sedimentation at neutral pH. Component I, representing usually 70 to 90% of the total DNA, had a sedimentation coefficient of 28.1S; component II sedimented with an $S_{20,w}$ of 20.8. At alkaline pH (bulk solution: 0.9 M NaCl, 0.1 M NaOH), I sedimented at 64S, whereas II gives rise to two bands with sedimentation coefficients of 23S and 20S, respectively. During stepwise alkaline denaturation between pH 11.1 and 11.4, the sedimentation velocity of I approached that of II before it increased to the value of the denatured compound above pH 12.5 (Fig. 3).

The density of native DNA in a CsCl gradient

FIG. 2. Twisted and "relaxed" double stranded circular papilloma DNA, prepared by the diffusion method (3). $\times 1.28 \times 10^5$.

FIG. 3. Sedimentation velocity-pH titration curve of papilloma DNA I (○) and II (△); ▲ = unresolved linear and circular single-strand molecules. Sedimentation coefficients are not corrected for viscosity of the solvent or buoyancy effects (40,000 rev/min).

at 40,000 rev/min was found to be 1.7065, corresponding to a guanine-cytosine content of 46.5%. Electron microscopy showed that the DNA preparations contain twisted (I) and relaxed (II) circular double-stranded DNA mole-

cules (Fig. 2). The contour length of nontwisted molecules prepared from ammonium acetate solution (ionic strength, 0.15 M NH_4^+ ; pH 6.4 to 7.0) was $2.54 \pm 0.07 \mu$. This corresponds to a molecular weight of 4.9×10^6 daltons (4).

DISCUSSION

Of the DNA isolated from a preparation of bovine papilloma virus, 70 to 90% can be characterized as covalently closed, double-stranded circles of 4.9×10^6 daltons molecular weight (component I). The percentage of component I varied among the different phenol extractions of a single virus preparation, which suggests that the yield of I depends on the isolation procedure rather than reflecting the existence of two forms of DNA (i.e., I and II) in the virus particles. The molecules of I have a twisted superstructure, as judged by their sedimentation properties in neutral and alkaline solution, as well as by electron microscopy. After alkaline denaturation, I exists in a tightly coiled, rapidly sedimenting form. This form results from the inability of the covalently closed circular DNA to release its helical and superstructural windings, which are a topological constant of the molecule. Superstructural windings can be introduced in such a circular molecule either by a deficiency or an excess of helical

twists before the ring closure. The formation of a normal Watson-Crick helix throughout the now circular molecule results then in right-handed (deficiency) or left-handed (excess) superstructural windings. Such windings can be released by a single break in one of the DNA strands, which introduces a swivel in the molecule and leads to a relaxed form (6, 7).

Component II is the relaxed circular molecule originating from I by one or more single-strand breaks (Fig. 2). Denaturation of this molecule yields two components: single-stranded circles ($S_{20,w}^{20} = 23$) and single-stranded linear molecules ($S_{20,w}^{20} = 20$). No band has been observed which sediments in correspondence to the double-stranded linear molecules of papilloma DNA.

The direction of the superstructural windings in I was determined by a technique similar to that used by Vinograd et al. for polyoma DNA (7). Thus, if twists in the molecule originated from a deficiency in helical turns before the ring closure, this superstructure should disappear if one untwists the Watson-Crick helix to an extent just corresponding to this deficiency. Such a molecule would no longer exhibit the sedimentation property of the compact form I; moreover, its sedimentation coefficient should approach that of a molecule relaxed by a single strand break (i.e., II). This partial unwinding of the superstructured molecule can be accomplished by partial denaturation of the DNA. The results of an experiment in which papilloma DNA is subjected to stepwise alkaline denaturation are shown in Fig. 3. It is clear that the sedimentation of neither I nor II changes significantly between pH 7.5 and 11.0. At slightly higher pH, however, the sedimentation coefficient of I drops to that of II, consistent with the loss of superstructural windings arising from partial denaturation. The sedimentation coefficient then increases, due to the formation of the tightly coiled denatured form of I. The uneven rise of the sedimentation coefficient above pH 11.4 (arrow) is not fully understood and is presently under study.

The sedimentation velocity pH curve of component II is that observed for double-stranded DNA without superstructural restrictions. First signs of denaturation are visible above pH 11.3; at pH 11.5, the sudden drop in sedimentation velocity indicates the separation into single strands.

The dip in the denaturation curve of I indicates an original deficiency of helical turns in the molecule, since partial denaturation converts I

into a configuration very similar to II. These results therefore suggest that the direction of the twists in the superstructure is right-handed.

The properties of papilloma virus DNA reported here are in agreement with those found earlier for DNA from the papova virus group (1, 2, 6).

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