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Papovavirus Chromatin Associated Cellular Endonuclease Which Introduces One Double-Strand Cut in Superhelical Deoxyribonucleic Acid†

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ABSTRACT: Nuclear extracts from SV40-infected CV-1 monkey kidney cells and from polyoma-infected 3T3 mouse cells contain an endonucleolytic activity which cleaves circular viral DNA within the chromatin to full-length linear rods [Waldeck, W., Föhring, B., Chowdhury, K., Gruss, P., & Sauer, G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5964–5968; Scott, W. A., & Wigmore, D. J. (1978) *Cell (Cambridge, Mass.) 15*, 1511–1518]. Sedimentation of the nuclear extracts through sucrose density gradients revealed a preferential binding of the endonuclease to the viral chromatin. Deproteinized exogenous covalently closed superhelical DNA substrates such as SV40 and polyoma as well as Col E_1 and PM2 DNAs were linearized by the endonuclease by introduction of one double-strand break per molecule. The reaction products, FOIII unit length rods, were shown to be devoid of single-strand nicks by electrophoresis in denaturing agarose gels. The double-strand break was randomly located within the various substrates since redigestion of the FOIII with single-cut restriction endonucleases failed to generate discrete pairs of reaction products. Neither linear double-stranded nor nicked circular FOII DNA structures were accepted as substrates. The endonucleolytic activity does not require the presence of ATP but is sensitive to EDTA. The enzyme activity is of cellular origin since nuclear extracts from uninfected CV-1 cells converted exogenous superhelical DNA to FOIII structures with the same properties as those described above. The biological properties of the endonuclease are discussed in the light of its possible function in permitting genetic exchange between different circular genomes. Further, it may play an essential role late during the replication of papovavirus DNA when the catenated daughter molecules are liberated from each other by an as yet unidentified mechanism.

We have recently shown that the origin of DNA replication in Simian virus 40 (SV40) and polyoma chromatin is particularly accessible to endogenous chromatin-bound endonuclease which cleaves open the covalently closed circular viral DNA to form linear rods of unit length (Waldeck et al., 1978). Scott & Wigmore (1978) also reported that the origin of DNA replication in SV40 chromatin is exposed to the action of DNase I as well as endogenous endonuclease.

The reasons why this part of the viral chromatin is relatively vulnerable to nuclease action remain, at present, entirely speculative. The nature of the endogenous nucleases which are capable of cleaving the viral DNA at or close to the origin of replication is also unknown.

The endonucleolytic activity associated with SV40 chromatin in crude nuclear extracts from infected cells eventually degraded the endogenous SV40 DNA by randomly introducing single-strand and double-strand cuts. Protein-free exogenous SV40 FOI DNA,[1] when added as a substrate to unpurified SV40 chromatin preparations, was converted to FOIII and to FOII (Scott & Wigmore, 1978; Waldeck et al., 1978). Partial

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[1] Abbreviations used: FOI DNA, covalently closed double-stranded superhelical DNA; FOII DNA, circular double-stranded DNA bearing at least one nick in one of the strands; FOIII DNA, double-stranded linear rods of unit length; FOIV DNA, covalently closed relaxed double-stranded DNA; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; pfu, plaque-forming units.

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purification of the SV40 chromatin by sedimentation through sucrose gradients removed the bulk of the endonuclease(s) (Scott & Wigmore, 1978). However, the endonuclease which remained associated with the SV40 chromatin was capable of cleaving open the endogenous viral DNA at or close to the origin of replication. We have used such partially purified SV40 chromatin preparations to study the properties of the endonuclease in greater detail. This approach has led to the discovery that the papovavirus chromatin associated endonuclease acts exclusively on circular DNA molecules and does not attack linear double-stranded DNA. The circular DNA is cleaved open by introduction of one double-strand break per molecule at a completely random site.

A possible biological function of this linearizing enzyme may be sought in the late stage of replication of papovavirus DNA and perhaps also of mitochondrial DNA. The terminal step required to separate the two daughter molecules, which are linked together in a chain-like configuration, may conceivably be mediated by this endonuclease.

Experimental Procedures

Preparation of Nuclear Extracts and Purification of Viral Chromatin. A permanent line of *Cercopithecus aethiops* cells (CV-1) was infected with Simian virus 40 (SV40) strain 776 at multiplicities of about 50–70 plaque-forming units per cell. Nuclei were prepared from 10^8 cells at 40 h after infection as described (Winnacker et al., 1972). In the case of polyoma virus, 3T3 cells were infected at the same multiplicity, and the infection was terminated at 26 h after infection by isolation of the nuclei. The nuclear extracts were prepared as described by a modification of the method of Su & De Pamphilis (1976) by suspending the nuclei for 1 h in 0.5 mL of a hypotonic extraction buffer (10 mM Hepes–NaOH, pH 8.0, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride). During the extraction procedure, the nuclei were chilled in an ice bath (Waldeck et al., 1978).

For further purification, 600 μL of the combined nuclear extracts was centrifuged through sucrose gradients (5–30% sucrose in extraction buffer with a 1-mL 70% sucrose cushion) in a Spinco SW41 rotor at 40000 rpm at 0 °C. The peak fractions of the chromatin were pooled and used in the experiments.

Reaction of Exogenous DNA with Purified Chromatin. The reaction mixtures contained 5 μL of the pooled sucrose gradient purified chromatin fractions and 0.5 μg of exogenous DNA in a total volume of 50 μL (or, multiple volumes thereof) of 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$. The exogenous DNA preparations were stored in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Since the endonucleolytic activity of the chromatin was inhibited by EDTA, the addition of $MgCl_2$ to the reaction mixture proved to be a necessary prerequisite to titrate the EDTA. The reaction was started by transfer of the reaction mixtures from 0 °C to a water bath at 37 °C. The reactions were terminated by addition of one-tenth of the volume of 8 M urea, 100 mM EDTA, 50% sucrose, and 0.05% bromophenol blue.

DNA Substrates and Restriction Endonucleases. SV40 DNA was prepared as described (Gruss & Sauer, 1977). The isolation of endogenous SV40 DNA from the viral chromatin has been detailed previously (Waldeck et al., 1978). Col E_1 DNA was a gift from W. Goebel, Universität Würzburg. The PM2 DNA was provided by P. Vosberg, Max-Planck-Institut, Heidelberg. The DNA as well as *Eco*RI, *Pst*I, and *Hpa*II was purchased from Boehringer, Mannheim. *Bam*HI was prepared in our laboratory. Prior to digestion with restriction endonucleases, the reaction mixtures containing exogenous DNA and chromatin were phenol extracted, precipitated with ethanol, and dissolved in the following buffer: 10 mM Hepes–NaOH, pH 7.5, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The reactions were terminated by addition of one-tenth the reaction volume of the buffer described above for termination of the endonuclease reaction.

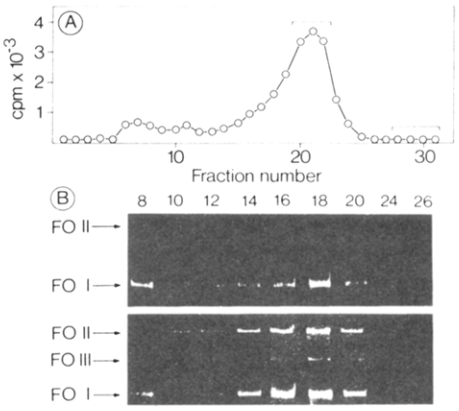

FIGURE 1: Purification of SV40 chromatin by sucrose density gradient centrifugation and analysis of the endogenous SV40 DNA. SV40-infected CV-1 cells were labeled at 40 h after infection for 30 min with 5 μCi/mL [^{3}H]thymidine, and, after washing, a nuclear extract was prepared and sedimented through a sucrose density gradient as described under Experimental Procedures. Fifteen drops per fraction were collected into chilled vials. (A) Sedimentation profile of [^{3}H]thymidine-labeled SV40 chromatin. Fractions pooled for further analysis are indicated by brackets. (B) Distribution of endogenous SV40 DNA. (Upper panel) Aliquots (100 μL) of each fraction of the gradient were incubated for 30 min at 0 °C, then phenolized, and subjected to agarose gel electrophoresis. Gradient fractions are indicated by numbers. (Lower panel) Aliquots (100 μL) of each fraction were incubated prior to phenolization for 30 min at 37 °C to allow the endogenous DNA to be converted to FOIII.

Agarose Gel Electrophoresis. The DNA was analyzed in 1.4% vertical agarose slab gels (19 × 20 cm, 3 mm thick) at 4 °C and 2.5 V/cm as described by Tegtmeyer & Macasaet (1972). For analysis under denaturing conditions, agarose blocks containing the DNA samples were excised under long-wavelength ultraviolet light after staining with EtBr. The blocks were sealed with melted agarose into a horizontal alkaline 0.7% agarose gel in 30 mM NaOH and 2 mM EDTA and electrophoresed at 4 °C and 2.5 V/cm. McDonell et al. (1977) have shown that DNA is denatured during electrophoresis under these conditions, and this was confirmed in our control experiments.

Electrophoretic patterns of ^{32}P-labeled SV40 reaction products derived from incubation with purified chromatin were determined by excision of EtBr-stained sections of the gel under illumination with ultraviolet light. The agarose blocks were incubated overnight in 3 mL of Instagel (Packard Instruments), and the radioactivity was then measured in a liquid scintillation counter.

Spectrophotometric Determination of Protein Concentration in Nuclear Extracts. The protein content of fractions of sucrose gradients was determined as described by Kalb & Bernlohr (1977) by measuring the absorbances at 260 and 230 mm against extraction buffer and calculating according to protein content (μg/mL) = (183) (A_{230}) − (75.8) (A_{260}).

Results

Endonuclease Cosediments with Viral Chromatin. Partially purified SV40 chromatin was prepared from extracts of SV40-infected CV-1 cells. The cells had been exposed at 2 days postinfection to [^{3}H]thymidine for 30 min to label the viral DNA in the nucleoprotein complexes. Figure 1A shows

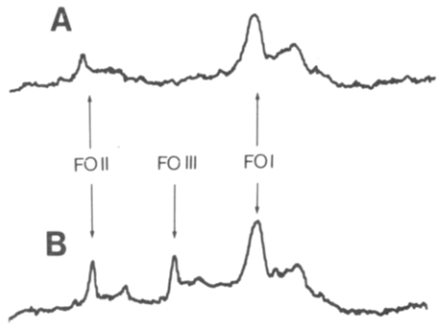

FIGURE 2: Absence of endogenous endonucleolytic activity from previrions. Sucrose density gradient purified SV40 previrions (obtained from a preparation described in Figure 1) and, for comparison, purified SV40 chromatin were incubated in extraction buffer for 30 min at 37 °C to assess the presence of endonucleolytic activity. Then the endogenous DNA was analyzed after phenol extraction by electrophoresis in an agarose gel. A densitometer tracing of the photograph from the EtBr-stained gel taken under ultraviolet light shows (A) previrion DNA and (B) DNA extracted from chromatin. The small peaks in front of the major components are due to the presence of some minicircular SV40 DNA.

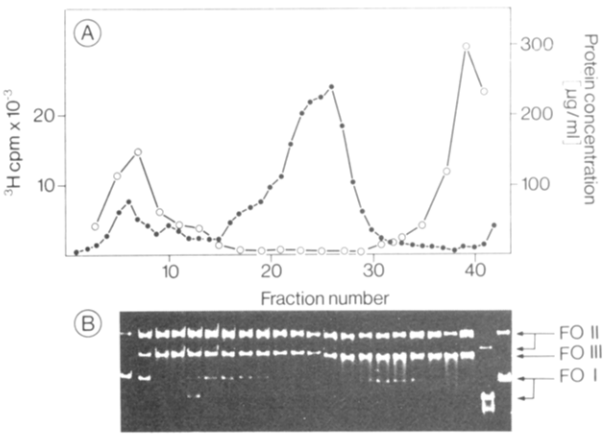

FIGURE 3: Distribution of endonuclease in a sucrose density gradient as revealed by reaction with exogenous DNA. A nuclear extract from SV40-infected CV-1 cells which had been labeled with [^{3}H]thymidine (1.5 µCi/mL) for 16 h was centrifuged in a sucrose density gradient. Ten drops/fraction were collected. (A) Sedimentation profile of [^{3}H]thymidine-labeled SV40 chromatin (filled circles) and of protein (open circles). The concentration of protein in alternate fractions was determined spectrophotometrically as described under Experimental Procedures. (B) Analysis of exogenous Col E_1 DNA after reaction with sucrose density gradient fractions. Aliquots (5 µL) from every second fraction were incubated for 30 min at 37 °C with 1 µg of Col E_1 DNA in 50 µL of 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$. The reaction products were analyzed by electrophoresis in a 1.4% agarose gel. For reference, the outermost two tracks of the gel at the right side contained 1 µg of SV40 DNA (containing some minicircular FOI) and 1 µg of the Col E_1 DNA substrate before exposure to the nuclear extract.

the sedimentation profile of the labeled chromatin in a nuclear extract in a neutral sucrose gradient. The viral chromatin sediments as a peak at 90–70 S (Su & DePamphilis, 1976; Varshavsky et al., 1979). Some fast-sedimenting material, probably virions, was collected on the 70% sucrose cushion. A small discrete peak of radioactivity at fraction 11 represents previrions sedimenting at 200–250 S (Garber et al., 1978; Baumgartner et al., 1979). Aliquots of individual fractions were incubated either at 0 °C, which prevents the action of the endogenous endonuclease(s) on the viral DNA (Waldeck et al., 1978), or at 37 °C to activate the endonucleolytic activity. Then the viral DNA was purified by phenol extraction, and the cleavage products were analyzed by neutral agarose gel electrophoresis. When incubated at 0 °C for 30 min, the viral chromatin contained mainly FOI DNA and very little FOII (Figure 1B). No FOIII of unit length was discernible under these conditions. The distribution of the SV40 DNA through the gradient, as detected under ultraviolet light after staining with ethidium bromide, followed the distribution pattern of [^{3}H]thymidine label, with a peak of viral DNA under the 90–70S peak of SV40 chromatin. The high multiplicities of infection used to increase the yield of chromatin (50–100 pfu/cell) lead occasionally to the appearance of some deleted minicircular SV40 FOI DNA (Chowdhury et al., 1979). The minicircular SV40 FOI DNA can be visualized as a weakly stained band which moves faster than the wild-type sized FOI.

When aliquots of the same fractions were incubated at 37 °C for 30 min, FOIII DNA structures were observed (Figure 1B, lower panel), as demonstrated earlier with unpurified nuclear extracts (Waldeck et al., 1978; Scott & Wigmore, 1978). Furthermore, the relative amount of FOII was greatly increased. This result shows that the endonuclease which cleaves the endogenous SV40 DNA to full-length linear rods and converts apparently FOI superhelical DNA to relaxed FOII cosediments with the viral chromatin.

During the process of papovavirus maturation when the minichromosome is encapsidated, the endonuclease too might conceivably become an integral part of the virion. The absence of endogenous FOIII structures among the reaction products that arose after incubation of previrions (Figure 1B, fractions 12 and 13, lower panel) at 37 °C rules out, however, that activatable endonuclease was incorporated. This is further substantiated by the data in Figure 2 where in a parallel experiment comparable amounts of sucrose gradient purified SV40 previrions (Figure 2A) and of SV40 chromatin (Figure 2B) were incubated at 37 °C. The reaction products were electrophoresed into agarose, stained, and photographed. Densitometer tracings revealed the absence of FOIII structures as endogenous products in the previrions while they were present in chromatin.

The data in Figure 1 do not permit conclusions on the distribution of endonucleolytic activity in fractions other than those that contained endogenous FOI SV40 DNA as substrate for conversion to linear FOIII of unit length. Therefore, endonuclease activity was assayed with an exogenous substrate, purified deproteinized Col E_1 DNA, which can be distinguished from SV40 DNA by its larger size but shares superhelicity as a common feature. When incubated with aliquots from a sucrose gradient at 37 °C and analyzed in agarose gels for the conversion of FOI into relaxed FOII and linear FOIII structures, this substrate can be utilized to monitor the distribution of endonuclease within the gradient. A nuclear extract from infected CV-1 cells which had been labeled for 16 h rather than for 30 min (see Figure 1) was employed for this experiment to label the fast-sedimenting previrion and virion structures (Garber et al., 1978; Baumgartner et al., 1979). The distribution of labeled endogenous SV40 DNA and of protein after sedimentation of the nuclear extract through a sucrose density gradient is shown in Figure 3A. Some of the protein in the nuclear extract sedimented onto the 70% sucrose cushion at the bottom of the gradient while the bulk of the protein remained on top of the gradient. The pattern of the labeled SV40 DNA (filled circles) resembles the distribution of labeled viral DNA shown in Figure 1 except that the previrion peak (fraction 10) is somewhat accentuated. Aliquots from every second fraction of the gradient were used as a source of endonuclease and incubated with Col E_1 DNA at 37 °C for 30 min. Then the reaction mixture was analyzed in an agarose gel (Figure 3B). We consider here two sub-

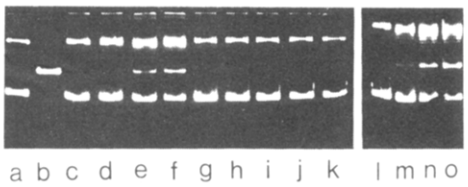

FIGURE 4: Exposure of exogenous Col E_1 DNA to purified and unpurified SV40 chromatin and analysis of the reaction products. Three different sources of endonucleolytic activity were compared with each other by reacting 5 μL of either nuclear extract or purified chromatin/1 μg of Col E_1 DNA as described in the legend to Figure 3. After 1, 5, 15, and 30 min (except k where the substrate was incubated for 60 min), aliquots were withdrawn from the reaction mixtures and analyzed by agarose gel electrophoresis. The Col E_1 substrate is shown in track a and *Eco*RI-generated Col E_1 FOIII in track b. (Tracks c–f) Purified pooled SV40 chromatin (see bar in Figure 1, fractions 20–22); (tracks g–k) pooled soluble proteins (Figure 1, fractions 28–31) were diluted 1:16-fold with extraction buffer to adjust the protein concentration to that of the purified chromatin batch; (tracks l–o) nuclear extract prior to sedimentation through the sucrose density gradient was diluted 1:32-fold to adjust the protein concentration as above.

strates, the endogenous SV40 DNA, which is only barely visible, and the exogenous Col E_1 DNA. The references for both DNA species are shown at the right side of Figure 3B. The conversion of FOI to FOIII serves as a measure for the activity of endonuclease. At first glance, it is evident that endonuclease is present throughout the entire gradient, although to a variable extent. Within the bottom fractions there was a preponderance of FOI while only relatively small amounts of FOIII were present. These fractions probably contained aggregates of virions which sedimented into the cushion. The fractions in tracks 3 and 4 contained aggregated chromatin which had been collected on top of the cushion (as revealed by electron microscopic examination; data not shown). A considerable amount of endonuclease must be associated with this material since Col E_1 FOI was completely converted to FOIII and FOII after incubation with these fractions. The fractions used in tracks 5 and 6, which correspond to the previrion peak (note also the lowermost band which represents endogenous SV40 FOI DNA), and tracks 7–9 probably contained smaller amounts of the endonuclease since FOI DNA persisted during the reaction period. In contrast, the peak of SV40 chromatin (tracks 10–14) contained the highest concentration of endonuclease, as evidenced by the complete conversion of the Col E_1 FOI DNA to FOIII and FOII. The persistence of endogenous SV40 FOI DNA suggests that the endonuclease may prefer the exogenous DNA as a substrate. The subsequent fractions, which are devoid of chromatin, again harbored smaller amounts of endonuclease, though the overall concentration of protein within these fractions of the gradient was markedly increased (see Figure 3A). Finally, the top fractions of the gradient containing high concentrations of soluble protein had sufficient endonuclease activity to cleave FOI DNA completely.

It appears from these data that endonuclease was bound preferentially to viral chromatin, rather than to virions and previrions. It should be pointed out here that essentially the same result was obtained when polyoma chromatin derived (1 day after infection) from productively infected mouse 3T3 cells was employed.

The notion of a preferential association of endonuclease with the viral chromatin was corroborated by the following experiment. The nuclear extracts were fractionated by sedimentation in sucrose gradients; the partially purified chromatin (fractions 20–22, Figure 1A) and the soluble proteins (fractions 28–31) were pooled and assayed for the presence of chromatin-associated endonuclease (Figure 4a–f) and soluble endonucleolytic activity (Figure 4g–k). Unfractionated nuclear extract was also examined (Figure 4l–o). The protein content of the top fractions of the sucrose gradient exceeded the protein content of the peak fractions of the chromatin by 16-fold. Therefore, in order to adjust the different reaction mixtures to comparable concentrations of protein, appropriate dilutions were employed. Exogenous Col E_1 FOI DNA (which contained some FOII; see Figure 4a) was added to the reaction mixtures. After a shift up to 37 °C, aliquots were removed at intervals, and the reaction products were examined in agarose gels. Purified SV40 chromatin contained cosedimenting endonuclease which generated visible amounts of FOIII after only 5 min of incubation (Figure 4c–f). For comparison, *Eco*RI-generated linear Col E_1 FOIII is shown in lane b. As the time of incubation was increased, more FOIII appeared. In contrast, the diluted chromatin-free fractions from the top of the gradient displayed no recognizable nucleolytic activity. On the other hand, unfractionated nuclear extract from SV40-infected cells behaved in the same fashion as purified viral chromatin (Figure 4l–o). From densitometer tracings of the photographs (not shown here), we can estimate that the rate of conversion of FOI to FOIII and FOII was almost as rapid as with purified chromatin when unfractionated nuclear extract was used. This result confirms that endonuclease sedimented preferentially with the viral chromatin.

Endonuclease Converts Superhelical DNA to Linear Rods of Unit Length by One Double-Strand Break. The persistence of exogenous FOIII DNA in the presence of exogenous FOI DNA is an intriguing observation which cannot be reconciled with the assumption that conversion of FOI to FOIII occurred via a FOII DNA structure bearing multiple random nicks in either strand to the extent that its circular configuration would eventually collapse and open linear rods would arise. Rather, the data indicate the presence of an endonuclease which cleaves open the circular DNA by one double-strand break.

For analysis of the kinetics of conversion of superhelical DNA to FOIII and FOII, radioactively labeled SV40 DNA was incubated at 37 °C as exogenous substrate with purified SV40 chromatin. After various periods of time, aliquots were withdrawn, and the distribution of radioactivity was examined after electrophoresis in agarose gels. As may be seen in Figure 5A, the rapid disappearance of FOI was paralleled by the appearance of FOII (and, as will be shown below, FOIV). Unlike FOII, which was generated very quickly, the FOIII structures appeared at a slower pace, and the reaction was not yet complete after 15 min. We have noticed, in fact, that the endonuclease remained active over at least 30 min.

The chromatin-associated nicking–closing activity (Champoux & Dulbecco, 1972; Keller et al., 1977) was also acting on the exogenous DNA substrate during the incubation period, thus generating partially unwound structures. As the time of incubation increased, completely relaxed covalently closed structures (FOIV), which are the final products of the nicking–closing reaction, accumulated in the position of FOII in the gel. Attempts to quench the activity of the nicking–closing enzyme while at the same time permitting the endonuclease to generate FOIII structures have failed. Removal of divalent cations from the reaction buffer by EDTA completely inhibited the endonucleolytic activity (data not shown) without reducing the activity of the nicking–closing enzyme (Keller et al., 1977). We were compelled, therefore, to devise an approach which would enable us to separate the relaxed covalently closed molecules from the circular FOII structures which bear at least one single-strand nick in one of either strand. The nicked

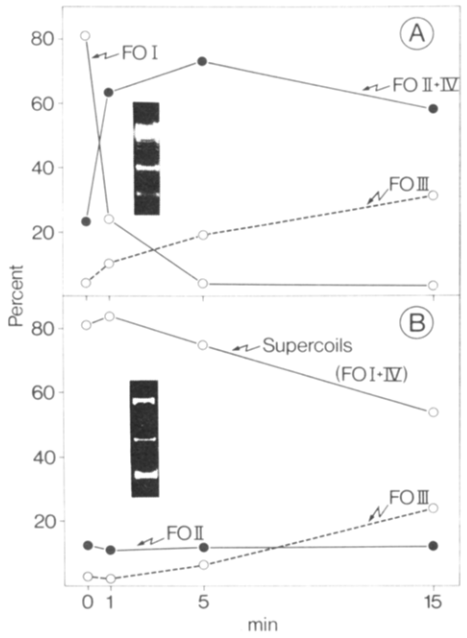

FIGURE 5: Kinetics of conversion of exogenous SV40 DNA to linear FOIII and relaxed DNA by exposure to SV40 chromatin-bound endonuclease. Purified SV40 chromatin (50 μL) was reacted (in 500 μL of 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$) with ^{32}P-labeled SV40 DNA (4 μg) as an exogenous substrate at 37 °C. After various periods of time, 50-μL aliquots (1200 cpm) were withdrawn, and the reaction was terminated as described under Experimental Procedures. A 50-μL aliquot from each sample was subjected to agarose gel electrophoresis in the absence (A) and in the presence of EtBr (1 μg/mL), both in the gel and in the electrophoresis buffer (B). The blocks containing the different reaction products were excised [in the case of (A), after staining with EtBr] under UV illumination, and the radioactivity was determined as described under Experimental Procedures. An example of a gel (SV40 DNA reacted for 2 min with chromatin) without (A) and with (B) EtBr is shown in the inserts.

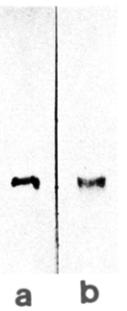

FIGURE 6: Analysis of ^{32}P-labeled SV40 FOIII DNA under denaturing conditions. The in vivo labeled FOIII DNA preparations were excised from neutral agarose gels, sealed into alkaline agarose gels as described under Experimental Procedures, electrophoresed, and detected by autoradiography. (a) FOIII DNA generated by endonuclease from purified SV40 chromatin. (b) *Eco*RI-generated FOIII DNA.

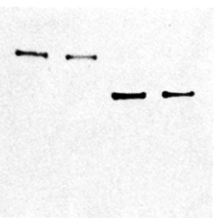

FIGURE 7: Exposure of nicked-circular and of linear SV40 DNA to purified SV40 chromatin. The in vivo ^{32}P-labeled SV40 DNA substrates (0.05–0.1 μg/reaction) were exposed for 15 min to 5 μL of purified SV40 chromatin and then autoradiographed after electrophoresis in an agarose gel. (a) SV40 FOII DNA generated by DNase treatment of FOI DNA (sham incubated in extraction buffer). (b) As above, but exposed to chromatin. (c) *Eco*RI-generated SV40 FOIII DNA. (d) *Eco*RI-generated SV40 FOIII DNA after exposure to chromatin.

circular FOII DNA can be elegantly revealed by treatment of the samples with ethidium bromide and subsequent electrophoresis in agarose gels in the presence of the dye. This procedure imposes superhelicity on all covalently closed circular structures which are devoid of single-strand nicks, thus also shifting partially relaxed molecules into the FOI position in the gels. Hence, the actual proportion of nicked-circular FOII DNA within the reaction mixtures can be precisely assessed by this procedure. As may be seen in Figure 5B, the proportion of nicked FOII molecules remained, rather unexpectedly, unchanged at 12% over the entire incubation period. Apart from the FOII DNA which was originally present in the exogenous substrates, no newly generated FOII molecules occurred even when the incubation period was extended to 30 min (data not shown here).

The simplest interpretation of this result is that an endonuclease, which copurified with SV40 chromatin, introduced one double-strand break per molecule into the exogenous covalently closed DNA. If any, then only very small amounts of endonuclease capable of introducing single-strand nicks in the exogenous DNA cosedimented with the chromatin. This was confirmed by a detailed analysis of the FOIII reaction product under denaturing conditions in an alkaline agarose gel. Freshly prepared SV40 DNA labeled in vivo with ^{32}P was incubated at 37 °C for 15 min with purified SV40 chromatin, the reaction mixture was subjected to agarose gel electrophoresis, and the FOIII DNA was excised from the gel. Then the agarose block containing the FOIII product was sealed on top of an alkaline agarose gel and electrophoresed together with *Eco*RI-generated SV40 FOIII DNA into the denaturing gel. The autoradiograph in Figure 6 shows that both the endonuclease and the *Eco*RI-generated linear rods of unit length migrated as sharp bands, and there was no indication of faster migrating material resulting from single-strand nicks. The data in Figure 5B also suggest that FOII did not serve as a substrate for the enzyme which generated FOIII since the reduction in the amount of supercoiled DNA was compensated by the accumulation of FOIII structures. Whether or not, however, FOII might represent, at least in part, a steady-state intermediate cannot be deduced from the data. The small discrepancy in the amounts of FOIII between parts A and B of Figure 5 can be explained by some partially unwound structures which were superimposed on FOIII in the gel shown in Figure 5A. Thus, the data in Figure 5B permit reliable comparisons between the different configurations of exogenous DNA.

That nicked FOII was not used as a substrate by the endonuclease was directly shown by incubation of FOII DNA, which had been generated by DNase treatment, with purified SV40 chromatin (Figure 7a,b). Furthermore, linear double-stranded DNA such as *Eco*RI-generated SV40 FOIII or Col E_1 FOIII (data not shown) was not attacked by the endonuclease and remained unaltered (Figure 7c,d).

Host–Origin of the Endonuclease. The question arises whether the chromatin-associated endonuclease is of cellular origin or whether it may be virus coded. One could speculate, for example, that T-antigen, being bound to the chromatin (Mann & Hunter, 1979; Reiser et al., 1980), might be a possible candidate. Therefore, the chromatin from the *tsA30* mutant of SV40 (Tegtmeyer, 1972) was examined under nonpermissive conditions for the presence of active endonuclease. Although the T-antigen of the *tsA30* mutant is

FIGURE 8: Determination of the site of cleavage. SV40 DNA was incubated for 15 min at 37 °C with either purified SV40 chromatin (a–d) or a nuclear extract from uninfected CV-1 cells (e–h). Five microliters of purified chromatin or nuclear extract was used per 1 μg of SV40 DNA. The SV40 DNA reaction products were electrophoresed into an agarose gel, and the FOIII products were excised from the gel. The FOIII DNA was recovered by subsequent electrophoresis into a dialysis bag. After concentration by precipitation, the DNA was redissolved and digested with restriction endonucleases. The products were electrophoresed in an agarose gel and revealed by hybridization with nick-translated ^{32}P-labeled SV40 DNA according to the method of Southern (1975). Purified FOIII (a) generated by exposure to SV40 chromatin was digested with (b) *Hpa*II, (c) *Bam*HI, and (d) *Pst*I. Purified FOIII generated by exposure to a nuclear extract from uninfected CV-1 cells (e) was digested with (f) *Hpa*II and (g) *Pst*I. For reference, SV40 DNA FOI was digested with *Pst*I (h).

temperature sensitive, there was, nevertheless, endonuclease activity demonstrable (unpublished experiments).

For further confirmation of the cellular origin of the endonuclease, nuclear extracts from uninfected CV-1 cells were reacted with exogenous Col E_1 FOI DNA. The result, formation of linear rods of unit length in the presence of FOI and FOII structures, clearly revealed the cellular origin of the endonuclease (see also Figure 8e, where SV40 DNA was employed as an exogenous substrate). The FOIII reaction products, when analyzed under denaturing alkaline conditions, were shown to be devoid of single-strand nicks (data not shown).

Thus, like the cellular nicking–closing enzyme, which acts on covalently closed circular DNA, the endonuclease displays a strong affinity for viral chromatin, a feature which fortuitously permitted its detection.

Cleavage Occurs at Random. The endonuclease thus cleaves superhelical DNA molecules by introduction of one double-strand break. The question arises then whether the cleavage occurs specifically or at randomly distributed sites. A specific site could be revealed, for example, by redigestion of the FOIII with single-cut restriction endonucleases and by analysis of the two resulting products. Lack of such discrete fragments and, instead, a FOIII band with smaller diffuse products would signal random distribution of the sites of cleavage. The latter possibility is borne out by the data in Figure 8. SV40 DNA was exposed to purified SV40 chromatin or to a nuclear extract from uninfected CV-1 cells to generate FOIII DNA. After purification by electrophoresis, the FOIII produced by exposure to SV40 chromatin (Figure 8a) was cleaved with the single-cut endonucleases *Hpa*II (Figure 8b) and *Bam*HI (Figure 8c) and with *Pst*I (Figure 8d) which cleaves SV40 FOI DNA twice. In neither case was the FOIII converted to pairs of fragments, the sum of whose sizes would correspond to unit length, and digestion with *Pst*I, too, revealed the authentic cleavage pattern (for reference, see Figure 8h). Similarly, digestion of the FOIII obtained by incubation with a nuclear extract from uninfected cells (Figure 8e) with *Hpa*II (Figure 8f) and *Pst*I (Figure 8g) gave the same

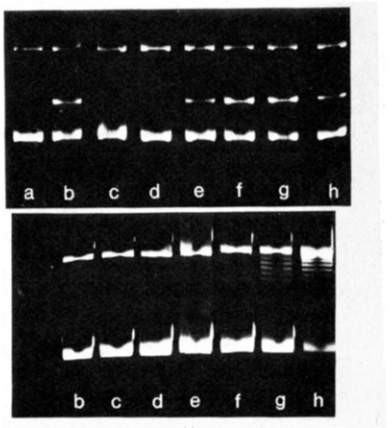

FIGURE 9: Partial purification of the endonuclease. Sucrose density gradient purified SV40 chromatin (4 mL) was adsorbed to hydroxylapatite, and batches (4 mL each) were eluted with increasing concentrations of sodium phosphate as described in the text. Exogenous Col E_1 DNA (0.5 μg) was reacted with aliquots (50 μL) from each batch to reveal the presence of either endonuclease (FOIII structures in the upper panel) or nicking–closing activity (partially unwound structures in the lower panel). The reaction products were revealed after electrophoresis in 1.4% agarose gels. (Lane a) Col E_1 DNA substrate; (lanes b–h) the enzyme activities obtained after elution with 0, 100, 200, 300, 400, 500, and 600 mM sodium phosphate.

results, namely, authentic cleavage patterns with diffusely distributed smaller products.

Partial Purification of the Endonuclease. For purification of the enzyme, 10^8 CV-1 cells were harvested 40 h after infection with SV40, and a nuclear extract was prepared. Then the nuclear extract was sedimented through a sucrose density gradient, and the fractions containing the viral chromatin were isolated and dialyzed against 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 0.5 mM dithiothreitol in 40% glycerol overnight at 4 °C. Then the partially purified SV40 chromatin preparation was adjusted to 0.8 M NaCl and adsorbed to 0.5 g of hydroxylapatite (in a centrifuge tube), and batches were eluted with the buffer described above, except that the glycerol concentration was reduced to 10%. The elution took place stepwise by increasing the phosphate concentration of the buffer from 0 to 600 mM as indicated in Figure 9. The phosphate was removed by dialysis, and the presence of the endonuclease was revealed as described in the previous sections by reacting aliquots of the eluates with exogenous FOI Col E_1 DNA. Electrophoresis of the reaction products in 1.4% agarose gels (Figure 9, upper panel) revealed free endonuclease (lane b) which did not bind to the hydroxylapatite. Lane a shows the exogenous substrate from which the FOIII structures were generated by the endonuclease. There remained, however, endonuclease bound to the hydroxylapatite, the bulk of which was eluted with phosphate concentrations mainly between 300 and 500 mM (lanes e–g). Since the endogenous SV40 DNA was concomitantly eluted (data not shown), we conclude that the endonuclease within these batches was still chromatin bound.

Interestingly, when aliquots from the eluates were assayed under reaction conditions which quench the endonucleolytic activity but favor the nicking–closing enzyme (Champoux & Dulbecco, 1972), it was found that the latter enzyme copurified with the SV40 chromatin, as shown by the partially unwound reaction products in Figure 9 (lower panel, lanes e–g). There was no nicking–closing activity eluted at phosphate concentrations below 200 mM. We have purified, therefore, endonuclease to the extent that at least endogenous DNA and nicking–closing activity were removed from the enzyme preparation (upper panel, lane b). This preparation was used

to define one unit as the amount of endonuclease required to convert 50% of superhelical DNA (0.5 μg input) to FOIII within 30 min at 37 °C.

Discussion

In the experiments reported here, we describe a papovavirus chromatin associated enzyme, an endonuclease, which accepts superhelical DNA as a substrate and which linearizes the circular DNA by introduction of one randomly located double-strand break per molecule. In addition to SV40 and Col E_1 DNA, which are dealt with in this report, polyoma and PM2 phage FOI DNAs were also accepted as substrates (data not shown). Such endonucleolytic activity is not unprecedented. There are the bacterial type I restriction endonucleases (*Eco*B, *Eco*P1) which, once attached to circular DNA, travel along the substrate and conduct only one, but randomly located, double-strand break (Linn & Arber, 1968; Smith et al., 1972; Adler & Nathans, 1973). Similarly, Sundin & Varshavsky (1979) have reported that staphylococcal nuclease cleaved SV40 DNA within the chromatin only once, although more than one cleavage site were shown to be accessible to multiple-cut restriction endonucleases. Furthermore, S_1 nuclease makes one double-strand break per molecule of circular double-stranded SV40 DNA at a random site, similar to the endonuclease investigated here (Waldeck et al., 1976). The S_1 nuclease does not require superhelicity as an essential feature of the substrate, but also cleaves covalently closed (Chowdhury & Sauer, 1976) and nicked circular DNA molecules (Chowdhury et al., 1975), while not attacking linear double-stranded DNA.

The endonuclease described in this paper and the nicking–closing enzyme, on the other hand, do not appear to be identical entities. The endonuclease activity is not dependent on ATP (up to 0.5 mM displayed no influence; data not shown) but requires the presence of divalent cations. The presence of EDTA completely inhibits the formation of FOIII DNA structures while the nicking–closing enzyme remains active under the same conditions. These results show that very probably the chromatin-associated endonuclease which cleaves exogenous FOI to FOIII is identical with the activity which is capable of converting endogenous FOI DNA to linear FOIII rods of unit length (Waldeck et al., 1978; Scott & Wigmore, 1978). The presence of FOIII structures that had been opened close to or at the origin of DNA replication in chromatin from productively infected cells and in virion particles (Waldeck et al., 1978) indicates that the endonucleolytic cleavage of SV40 DNA may be a naturally occurring process whose significance, however, is unclear.

Conceivably, one might associate the biological function of the endonuclease with a terminal step during the replication of circular DNA. Mitochondrial DNA, for example, replicates (Kasamatsu et al., 1971) like papovavirus DNA (Jaenisch et al., 1971; Sebring et al., 1971) along covalently closed circular maternal templates, a process which finally results in the synthesis of two catenated daughter molecules which must be liberated from each other by introduction of at least one double-strand break into one of the rings. Recombinational events between covalently closed circular DNA molecules, such as different mutated coinfecting SV40 genomes (Goff & Berg, 1977), could be readily initiated by an endonuclease with the characteristics described here. In such a case, the enzyme, together with a ligase activity, would represent one of the essential components of a cellular recombination system.

Acknowledgments

We thank Ellen Fanning for critical reading of the manuscript and Mathilde Theobald for expert technical assistance.

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