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Analysis of Polyoma Virus DNA Replicative Intermediates by Agarose Gel Electrophoresis

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Agarose gel electrophoresis has been used to fractionate polyoma virus DNA replicative intermediates (RI) according to maturity. Approximate electrophoretic mobility versus maturity relationships were obtained for both intact (supercoiled) and nicked (relaxed) RI. There was considerable overlap between the supercoiled and relaxed RI populations after electrophoretic fractionation. Intact RI could be recovered from preparative agarose gels for further analysis by centrifugation, electron microscopy, re-electrophoresis, or nuclease digestion.

Replicative intermediates (RI) of simian virus 40 (SV40) DNA or polyoma virus (Py) DNA can be fractionated according to maturity by CsCl isopycnic centrifugation in the presence of ethidium bromide (EtBr) or propidium diiodide (2, 12). However, gel electrophoresis would be expected to provide much better resolution, and this paper reports the fractionation by Py RI by agarose gel electrophoresis, following an earlier study (19) with SV40 RI. Analysis of the effect of RI maturity on electrophoretic mobility has been facilitated by the use of an in vitro system (21), in which nuclei isolated from Py-infected mouse 3T6 cells are incubated with α-^{32}P-labeled deoxynucleoside triphosphates. A preliminary account of some of the results presented here was published earlier (11).

In electrophoretic analysis of viral DNA labeled both in vivo (^{3}H) and in vitro (^{32}P), the ^{3}H-labeled DNA was resolved into three major peaks (Fig. 1). The fastest migrating species was identified as Py DNA (I) on the basis of comparison with a sample of ^{14}C-labeled Py DNA (I) analysed on a parallel gel. Similarly, the middle ^{3}H peak in Fig. 1 was identified as Py DNA (II) after comparison with analysis of a sample of ^{14}C-labeled Py DNA (I) partially nicked with DNase I. The slowest moving ^{3}H-labeled peak in Fig. 1 was identified as a mixture of relatively high-molecular-weight cellular DNA, which escaped removal during the Hirt extraction procedure. This assignment was confirmed by analysis of DNA obtained from a mock-infected culture labeled with [^{3}H]thymidine. Previous investigators have reported that gel electrophoresis will resolve linear duplex DNA species only in a finite-molecular-weight range, and larger molecules all migrate as an unresolved band (3, 4, 14).

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The in vitro ^{32}P-labeled DNA (Fig. 1) migrated as a very broad band, but more slowly than Py DNA (I), and the result was tentatively interpreted as indicating separation of Py DNA RI according to maturity, on the basis of electrophoretic analysis of SV40 DNA (19). This assignment was supported by results of similar experiments in which the viral DNA was further purified by chromatography on benzoyl-naphthoyl-DEAE (BND)-cellulose, a technique in which the partial single-stranded nature of RI affects their separation from double-stranded DNA species, including Py DNA (I) and Py DNA (II). The additional purification step had no detectable effect on the profile of in vitro-labeled viral DNA, whereas the amount of all three peaks of in vivo-labeled DNA was markedly reduced. It should be noted, however, that although the BND-cellulose procedure was relatively efficient (>90%) in removing mature viral DNA from RI, removal was never complete, even after several cycles of chromatography.

As a first step to determining whether the range of electrophoretic mobilities of in vitro-labeled viral DNA represented a real fractionation, ^{32}P-labeled RI DNA was fractionated on a preparative agarose gel, and the DNA of some of the gel fractions was subjected to re-electrophoresis. DNA was recovered from preparative gel slices by electrophoresis into dialysis tubing (14); the mean recovery was 75%. Re-electrophoresis of the ^{32}P-labeled DNA yielded discrete peaks each with a relative electrophoretic mobility (R_m) almost coincident with those of the fractions from the preparative gel. R_m is defined as the electrophoretic mobility relative to that of Py DNA (I), which has an R_m of 1.

The relationship between electrophoretic mobility and maturity of RI was investigated by fractionation of in vitro labeled DNA by prepar-

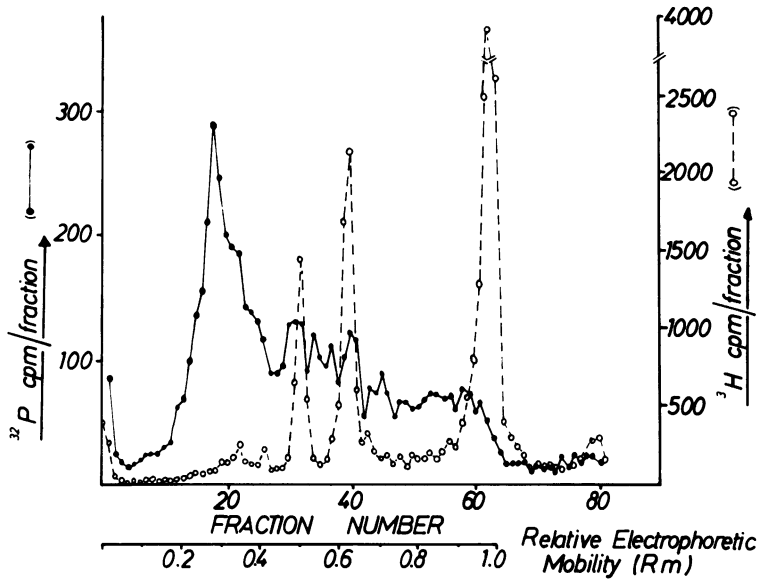

FIG. 1. *Agarose gel electrophoresis of Py DNA labeled both in vivo and in vitro. Cultures of 3T6 cells were infected with Py (21) and labeled with [^{3}H]thymidine (21) for 2.5 h. Nuclei were isolated (13) from the infected cells and incubated for 3 min at 25°C in the presence of α-^{32}P-labeled dGTP (15). Viral DNA was extracted by the Hirt procedure (6), purified by treatment with Pronase and chloroform-phenol (8), and finally dialyzed against E buffer (0.036 M Tris base-0.03 M NaH_2PO_4-0.001 M EDTA) containing 0.2% sodium dodecyl sulfate. A sample of the dialysate was analyzed by electrophoresis in 1.4% agarose gels. The gels were prepared essentially as described (19), except that glycerol was included to 10% to facilitate fractionation of the gel (20). Electrophoresis was for 7 h at 100 V. The 16-cm gel was frozen and cut into 1-mm slices, which were combined in pairs and digested in concentrated NH_3-30% H_2O_2 (1:50) prior to scintillation counting. The direction of migration is left to right.*

ative electrophoresis on agarose gels and subsequent analysis of pooled fractions by sedimentation through alkaline sucrose gradients (Fig. 2). In all cases, the alkaline sucrose gradient profile is bimodal. The peak S value of the lower-molecular-weight peak is 4 to 5 in all four gradients, but the S value of the second peak decreases progressively from pool A through D. These results indicate a general (inverse) relationship between electrophoretic mobility and maturity of RI. Also, it is clear that the small Okazaki fragments labeled in vitro are associated with RI at all stages of replication (16).

The general trend in the electrophoretic mobility versus maturity relationship was also confirmed by examining DNA extracted from preparative gels by electron microscopy; however, the number of molecules examined was not sufficient to enable quantitative analysis.

Further experiments revealed that the fractionation of Py RI DNA was complicated by the presence of both supercoiled and relaxed molecules. This was first demonstrated by extraction of ^{32}P-labeled RI DNA from adjacent fractions of a preparative agarose gel and analysis in propidium diiodide-CsCl gradients. Separation of relaxed and supercoiled RI DNA from a preparative agarose gel was also achieved by re-electrophoresis in a 1.4% agarose gel containing EtBr (7, 17), and, moreover, sedimentation analysis of DNA extracted from fractions of the EtBr-agarose gel showed that the relaxed and supercoiled RI populations had different maturities (see Fig. 4). However, the best demonstration of the presence of the two RI populations was from an experiment in which ^{32}P-labeled RI from fractions of a preparative agarose gel was digested with DNase I and then re-electrophoresed (Fig. 3). After DNase treatment, an additional population of RI molecules appeared with a much lower electrophoretic mobility. The ^{32}P-labeled RI from the original agarose gel fraction contained a mixture of relaxed (less mature) and supercoiled (more mature) molecules; the latter population, when relaxed by the DNase treatment, exhibited an expected decrease in electrophoretic mobility (R_m decreased from 0.50 to 0.24). Further nicking of the other population predictably had no effect on electrophoretic mobility. The small

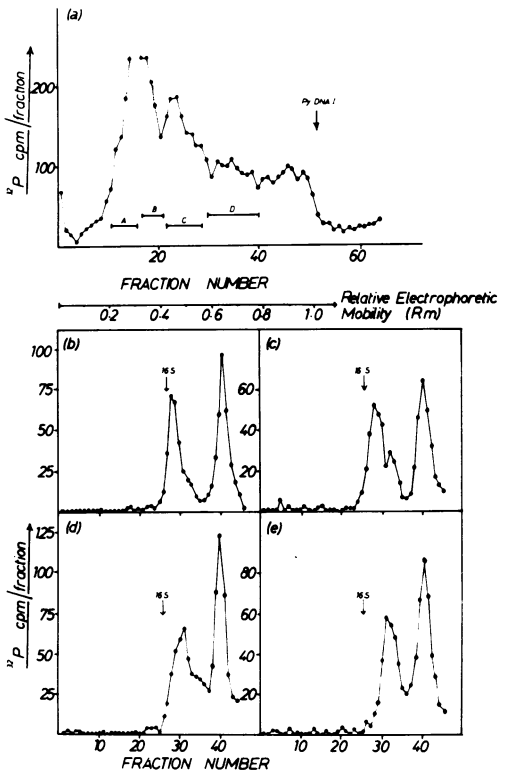

FIG. 2. *Sedimentation analysis of ^{32}P-labeled RI DNA fractions from a preparative agarose gel. (a) Nuclei from Py-infected 3T6 cells were incubated with α-^{32}P-labeled dCTP for 1 min at 25°C. Viral DNA was extracted and treated with Pronase and chloroform-phenol as for Fig. 1, and further purified by gel filtration through Bio-Gel P-30, followed by chromatography on BND-cellulose (8). The RI fraction from BND-cellulose chromatography was analyzed by electrophoresis in a 14-cm agarose gel for 7.5 h at 100 V. The gel was fractionated and the ^{32}P in each 2.2-mm slice was measured as Cerenkov irradiation. The marker ^{14}C-labeled Py DNA (I) was electrophoresed in a parallel gel. Gel fractions were combined into four pools A, B, C, and D as indicated. (b) through (e) DNA was eluted from pooled gel slices as described in the text and analyzed by sedimentation in alkaline sucrose gradients with an SW56 rotor (15, 21); the direction of sedimentation was right to left. The arrow indicates the peak fraction of the ^{14}C-labeled Py DNA (III) marker included in each gradient. The ^{14}C-labeled Py DNA (III) was prepared by EcoRI endonuclease digestion (15) of ^{14}C-labeled Py DNA (I). The samples analyzed were from pool A (b), pool B (c), and pool C (d).*

peak around fraction number 40, which appeared on re-electrophoresis both with and without DNase treatment, is inexplicable. In an analogous experiment to that described in Fig. 3, DNase treatment resulted in an R_m decrease of 0.73 to 0.41.

Figure 4 summarizes all the data relating RI maturity to electrophoretic mobility. Most of the data were obtained from the type of experiment illustrated in Fig. 2, although, in many cases, only one or two fractions from the preparative agarose gel were used, and an SW41 rotor (rather than an SW56) was used for alkaline sucrose gradient analysis. The experimental results with a preparative EB-agarose gel (described above) are also included in Fig. 4.

It was assumed that RI molecules with an electrophoretic mobility greater than that of Py DNA (II) are intact supercoiled molecules, so that the line of best-fit between data points with $R_m > 0.63$, and including the zero maturity point at $R_m = 1$ and the EtBr-agarose gel experiment data point, represents the maturity versus R_m relationship for supercoiled RI. Similarly, the data points at low R_m values, the EtBr-agarose gel experiment data point and the zero maturity point at $R_m = 0.63$ [i.e., for Py DNA (II)] have been used to indicate the R_m versus maturity relationship for relaxed RI.

In order to include the data obtained by treatment of RI preparations with DNase in Fig. 4; the maturity values for the supercoiled RI molecules used (with R_m values of 0.50 and 0.73) were inferred from the R_m versus maturity relationship derived from the other data. The R_m shifts obtained with DNase treatment are in reasonable agreement with the R_m versus maturity relationship derived for relaxed RI.

The results in Fig. 4 clearly indicate that agarose gel electrophoresis provides a method for fractionation of Py DNA RI on the basis of maturity, and this confirms the results of an earlier study with SV40 DNA (19). However, fractionation is complicated by the presence of both nicked (relaxed) and intact (supercoiled) RI. The approximate R_m-maturity relationships depicted in Fig. 4 show that preparative agarose gel electrophoresis will only provide relatively pure populations of mature (>70%) relaxed RI molecules or immature (<60%) supercoiled ones. Intermediate regions of the gel ($0.3 < R_m < 0.6$) contain a mixture of relaxed and supercoiled molecules so that RI of a given R_m consist of a bimodal distribution of maturities. However, the limited resolution of the alkaline sucrose gradient analysis usually yielded only a single peak, representing an average of two populations; thus many of the experimental points in Fig. 4 lie between the two lines.

It is interesting to compare the electrophoretic mobilities of linear and circular duplex DNA molecules. The very large linear duplex molecules that are effectively "excluded" from

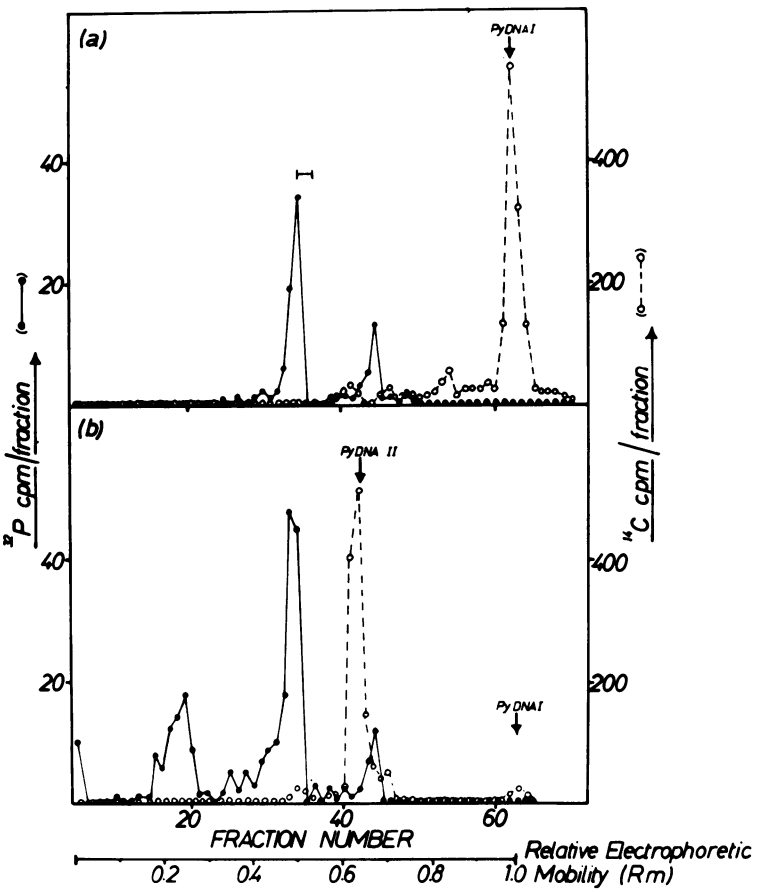

FIG. 3. *Effect of DNase treatment on electrophoretic mobility of RI. ^{32}P-labeled RI DNA was prepared from a 30-min (25°C) nuclear incubation and purified and fractionated on a preparative agarose gel as for Fig. 2. DNA from one of the gel fractions (R_m = 0.52) was eluted by electrophoresis and purified by heating in 0.1 M Tris-hydrochloride (pH 7.5) containing 1% sodium dodecyl sulfate for 2 h at 55°C, followed by two extractions with chloroform-phenol (1:1), and was finally filtrated through a Bio-Gel P-30 column. The purified ^{32}P-labeled RI DNA was then mixed with ^{14}C-labeled Py DNA (I). Part of the mixture was subjected to re-electrophoresis (a), and the remainder was treated with DNase (2 × 10^{-5} μg per μg of DNA) and then analyzed by agarose gel electrophoresis (b). Both gels were 14 cm in length, and electrophoresis was for 8 h at 100 V. The bar in (a) indicates the R_m of the ^{32}P-labeled RI in the original preparative agarose gel.*

the gel move with a much higher R_m (~0.5) than some of the much smaller RI molecules. Moreover, the double-stranded branched structure that results from *Eco*RI cleavage of mature RI also has a lower R_m (Fig. 5) than the much larger linear molecules. It has been suggested that the anomalous behavior of high-molecular-weight duplex DNA can be explained in terms of long molecules migrating through the pores of the gel in a "threading" fashion (4).

Py DNA has only one cleavage site for the *Eco*RI restriction endonuclease, and this site has been mapped at 0.29 units from the origin of replication (12). For a relatively mature (>58%) RI, in which one of the two forks had progressed beyond the *Eco*RI cleavage site, treatment with the endonuclease will produce cuts in each of the two daughter strands. Cleavage of mature RI molecules with *Eco*RI resulted in a reproducible increase in electrophoretic mobility (Fig. 5). Note the mobility of Py DNA (III), the *Eco*RI cleavage product of Py DNA (I); the R_m value obtained from several experiments was found to be 0.78. In a similar experiment, DNA was eluted from gel fractions containing the *Eco*RI-cleaved ^{32}P-labeled RI, and analysis by electron microscopy revealed molecules of the expected branched configuration.

In an experiment illustrating an application

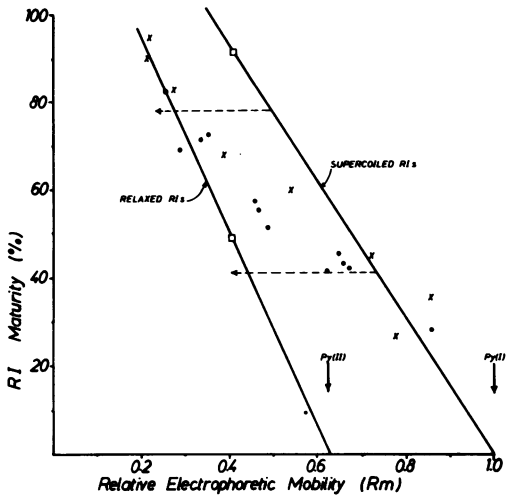

FIG. 4. *Relationship between RI maturity and electrophoretic mobility. In experiments of the type shown in Fig. 2, the average maturity of* ^{32}*P-labeled RI was determined by calculating (1, 18) the size of the growing daughter strand relative to the internal* ^{14}*C-labeled Py2 DNA (III) marker (·). In similar experiments,* ^{32}*P-labeled RI DNA was eluted from just one or two fractions of preparative agarose gels and analyzed by sedimentation in alkaline gradients with a SW41 rotor (×). Also included is data from experiments involving electrophoresis in a preparative EtBr-agarose gel (see text) (□), and DNase digestion as in Fig. 3 (←- -).*

of the electrophoretic technique for fractionation of Py RI, ^{32}P-labeled RI was prepared from a 30-min nuclear incubation (25°C) and fractionated on a preparative agarose gel as before. The long in vitro incubation time was used to

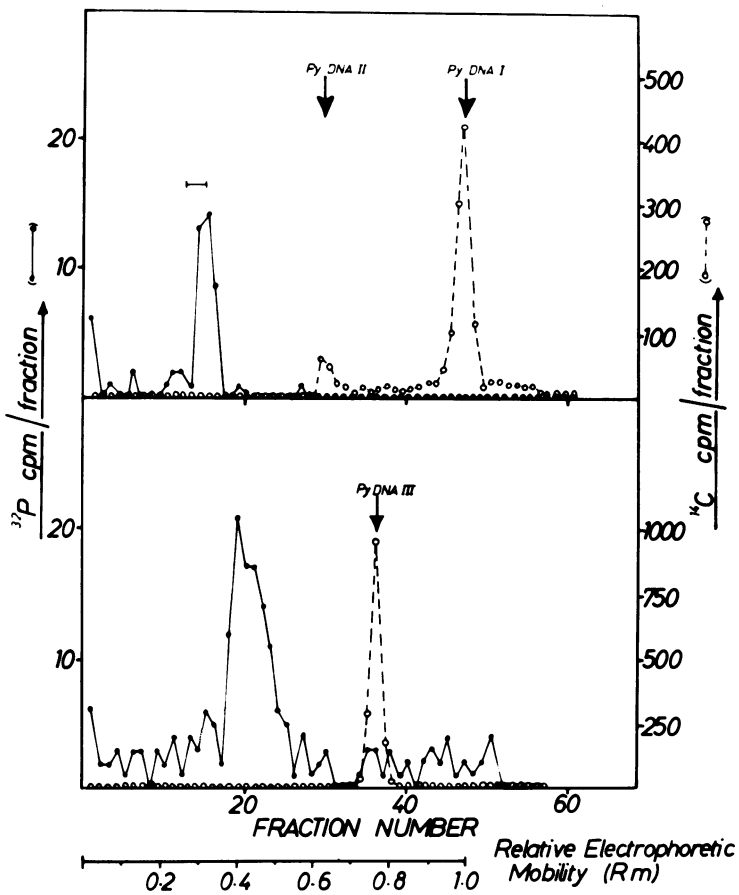

FIG. 5. *Cleavage of RI with EcoRI endonuclease: effect on electrophoretic mobility. Purified* ^{32}*P-labeled RI from two gel fractions (mean* $R_m = 0.30$*) was prepared as described for Fig. 3 and mixed with* ^{14}*C-labeled Py DNA (I). Part of the mixture was subjected to re-electrophoresis on an agarose gel (a). The bar indicates the* R_m *value for the* ^{32}*P-labeled RI in the original preparative gel. The remainder of the* ^{32}*P-labeled RI-*^{14}*C-labeled Py DNA (I) mixture was incubated with EcoRI endonuclease (15) and then analysed by agarose gel electrophoresis (b). Electrophoresis of the 14-cm gels was for 6.3 h at 110 V.*

minimize the proportion of labeled DNA appearing as 4 to 5S Okazaki fragments (10). The DNA from two 2.2-mm gel fractions was extracted and purified as described for Fig. 3. Analysis of part of the ^{32}P-labeled RI sample in an alkaline sucrose gradient (SW41) gave an S value of 14.8, which corresponds to an RI maturity of 0.82. The remainder of the ^{32}P-labeled RI was digested with *Eco*RI endonuclease. Analysis of the digest in an alkaline sucrose gradient (SW41) gave peaks at 7.2S and 13.6S. The size of each of the cleavage products of the ^{32}P-labeled daughter strands of the RI were used to calculate the maturity of the RI, and the results were 0.86 and 0.76 for the smaller and larger fragments, respectively. The ratio of ^{32}P activity between the *Eco*RI cleavage products of the daughter strand was approximately 2, in favor of the larger fragment. Since this ratio is more than 1, it can be deduced that elongation in vitro progressed through the *Eco*RI cleavage site; in fact, the observed value of approximately 2 indicates that the extension of the daughter strands in vitro was approximately 1,000 nucleotides per replication fork.

The agarose gel electrophoresis technique described here has been used to fractionate immature RI that accumulated during hydroxyurea treatment in vivo (9). Application of the technique in analysis of Py and SV40 DNA replication should extend to other duplex DNA molecules, including the double-stranded replicative forms of genomes consisting of single-stranded circular DNA molecules.

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