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Single-Stranded Structure of Newly Replicated DNA During Lens Regeneration (38837)

JAMES M. COLLINS

Department of Biochemistry and The Comprehensive Cancer Center, Medical College of Virginia, Health Sciences Division of Virginia Commonwealth University, Richmond, Virginia 23298

When the lens is removed from the eye of the newt, *Triturus* (Notopthalmus, Diemectylus) *viridescens viridescens*, a new lens will form from cells of the dorsal iris (for review, see Stone (1)). In the normal adult newt, the cells of the dorsal iris are in a G_0 state, or out of the cell cycle (for review, see Yamada (2)). Lentectomy induces these cells to enter the cell cycle (1, 2). By day 3 of lens regeneration, almost all of the cells are in G_1 (2). For the particular group of animals used for this experiment, most of the cells enter the S-phase before day 7 of lens regeneration.

Numerous reports have been concerned with the secondary structure of newly replicated DNA. Okazaki *et al.* (3), and Oishi (4) have presented evidence that in bacteria, newly formed DNA first appears as single-stranded molecules. Others have reported that part of the newly formed DNA is single stranded in animal systems as well (5–7).

We have extracted DNA from dorsal irises of newts that had been engaged in lens regeneration for 7 days. We have found that most of the newly formed DNA is single stranded, by virtue of its behavior when chromatographed on hydroxyapatite. Furthermore, this form appears subsequently to be converted to a double-stranded structure.

Materials and Methods. Adult newts were obtained from Glenn Gentry of Donelson, TN; [^{3}H]thymidine (1 Ci/mmole), generally labeled, was purchased from International Chemical and Nuclear Corporation. All procedures, surgical operation, conditions of incubation with [^{3}H]thymidine, isolation and purification of DNA, preparation of hydroxyapatite, and conditions of chromatography, have been described previously (8).

Results. Dorsal irises of newts that had been engaged in lens regeneration for 7 days (7-day regenerates) were incubated with 0.8 mCi of [^{3}H]thymidine for 15 min, or 60 min, as described previously (8). The 15-min incubation amounts to a "pulse." The 60-min incubation amounts to a "pulse-chase" as the kinetics of uptake of radioactive thymidine were linear for 45 min, then leveled off, indicating that the radioactive precursor pool had been depleted (8). DNA was extracted and purified essentially according to Marmur (9) as described previously (8). Specific activities obtained were 220 cpm/μg (15-min pulse) and 360 cpm/μg (60-min pulse-chase). DNA was chromatographed on freshly prepared hydroxyapatite columns (1 $\times$ 5 cm) with shallow linear gradients of sodium phosphate. Figure 1 shows the results obtained. For reference, the elution positions of native calf thymus DNA (Worthington Biochemical Corp.) and single-stranded calf thymus DNA (heat denatured in the presence of 1% formaldehyde to prevent reannealing) are indicated at the top of Fig. 1. These bands eluted with peaks at 0.1 *M* (single-stranded DNA) and 0.23 *M* (native DNA). This was determined after chromatography of the DNA labeled for 15 min and 60 min, with the same column.

After a 15-min pulse most of the radioactivity chromatographs as single-stranded DNA (Fig. 1a). This DNA possesses very high specific activity as evidenced by the fact that it has virtually no absorbance at 260 nm. The bulk of the DNA, after a 15-min pulse, contains very little radioactivity. The bulk of the DNA chromatographs as two absorbance bands that elute with peaks at 0.15 and 0.23 *M* sodium phosphate. Two absorbance bands are obtained, rather than a single band, due to the use of freshly prepared hydroxyapatite under conditions whereby partially denatured DNA separates from undenatured DNA (8). The band that elutes with a peak at 0.15 *M* is partially denatured DNA.

After a 60-min period, the radioactivity is found in the two bands of bulk DNA (Fig.

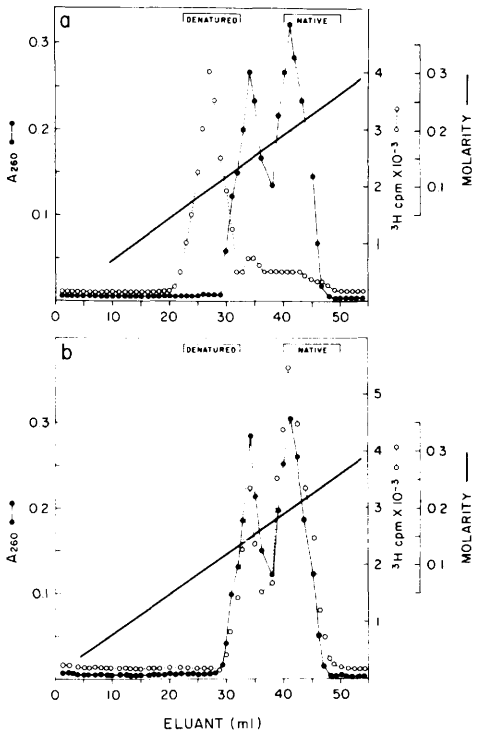

FIG. 1. Hydroxyapatite chromatography of DNA from irises of newts that had been engaged in lens regeneration for 7 days. Dorsal irises were incubated with 0.8 mCi of [^{3}H]thymidine for 15 min or 60 min. Elution was at 25° with a linear gradient of sodium phosphate, pH 7.0. The absorbance at 260 nm was determined with a Zeiss PMQII spectrophotometer. Aliquots of each fraction were applied to paper discs and counted in a liquid scintillation counter. (a) 145 μg of DNA (220 cpm/μg) from 20 irises labeled for 15 min, (b) 145 μg of DNA (360 cpm/μg) from 20 irises labeled for 60 min.

1b). This indicates that the single-stranded DNA (Fig. 1a) has been converted to double-stranded DNA (Fig. 1b). When the DNA-containing fractions of Fig. 1 were treated with pancreatic deoxyribonuclease (5 μg/ml, 37°, 3 hr) all radioactivity was converted from trichloroacetic acid-insoluble to soluble products. Pancreatic ribonuclease (50 μg/ml, 37°, 3 hr) had no effect.

Discussion. Habener *et al.* (7) have reported that the amount of single-stranded nascent DNA obtained from HeLa cells is dependent on the conditions of isolation, with higher temperatures affording greater yields. These authors reported that the use of organic solvents at 0° decreased the amount of single-stranded DNA obtained. In contrast to these reports, we have found that the method of Marmur (9) at 0–4°, gives excellent yields of single-stranded DNA from iris cells. Shearing the DNA before chromatography had little effect. However, our method includes a ribonuclease treatment followed by a pronase treatment (8), so our conditions are perhaps not comparable to those used by others (7).

It is difficult to account for the single-stranded nature of nascent DNA by a base-pairing mechanism of DNA replication. At present, two exclusive explanations have been offered (10). One is that a strand of DNA which is actively being replicated exists in a metastable state incompletely associated with the parental strand such that it can be dissociated by the drastic forces generated during isolation procedures (10). The other explanation is that the nascent DNA strand is temporarily incompletely hydrogen bonded to the parental strand so as to represent a true single-stranded DNA intermediate (10).

It seems clear that the single-stranded nature of the nascent DNA, reported herein, cannot be a result of general shear forces operating on any DNA, for we have detected single-stranded DNA only from cells actively replicating DNA. When irises from animals that had been engaged in lens regeneration for 0, 3, and 5 days were incubated with [^{3}H]thymidine for 15 min prior to extraction of DNA, the DNA's possessed only 1.1 % as much specific activity (cpm/μg) as did DNA from 7-day regenerates. Furthermore, when the DNAs from 0-, 3-, and 5-day regenerates were chromatographed, no single-stranded DNA bands could be detected. This indicates that the single-stranded nascent DNA appears as a result of the cells being in the S-phase, that is, actively replicating DNA. It seems plausible that the ability of these cells to convert the single-stranded nascent DNA into a double-stranded form (Fig. 1) is a consequence of *in vivo* mechanisms of DNA replication which at present are poorly understood.

Summary. Nascent (newly synthesized) DNA obtained from the regenerating lens-system of *Triturus* is first isolated as single-stranded molecules. These early intermedi-

ates are later converted into double-stranded molecules.

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