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Novel structure at 5′-ends of nascent DNA chains

(DNA replication/chain initiation/alkaline phosphatase/spleen phosphodiesterase/5′-blocking groups)

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ABSTRACT **Because of their association with protein, short nascent DNA chains in *Escherichia coli* can be separated from other cellular DNA by chromatography on hydroxylapatite. Protein-free DNA chains of less than 500 nucleotides in length are resistant to degradation from the 5′-end by alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1] and spleen phosphodiesterase (oligonucleate 3′-nucleotidohydrolase; EC 3.1.4.18). In contrast, DNA chains containing more than 500 nucleotides are degradable. From these results we conclude that short nascent DNA chains are structurally modified at their 5′-ends. The nature of this structure and its possible functions are discussed.**

Okazaki pieces, short DNA chains of about 1000 nucleotides in length, have long been considered the first intermediates in DNA replication (1). Recent evidence, however, shows that Okazaki pieces arise through the joining of many much shorter DNA chains (2, 3). Such short DNA chains accumulate in certain *dna* mutants (4, 5), suggesting that more than one reaction is involved in the synthesis of Okazaki pieces. The formation of a single Okazaki piece seems to involve many independent initiation events. Since all known DNA polymerases require a primer for the synthesis of a new DNA strand, we decided to study the 5′-ends of the short DNA chains, hoping that their structure might provide some clue to the mechanism of DNA chain initiation. In the course of these studies we found that the shortest replication intermediates, containing fewer than 500 nucleotides, carry an unusual group at their 5′-ends, which may function in the initiation or elongation of nascent DNA chains.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *Escherichia coli* strain 15 TAMT (*thy*$^-$, *arg*$^-$, *met*$^-$, *trp*$^-$) was grown at 14° in LSTL medium supplemented with 5 μg/ml of thymine and with 40 μg/ml each of arginine, methionine, and tryptophan, as described (2).

Labeling and Cell Lysis. In experiments where intracellular thymidine nucleotide pools were depleted, bacteria were incubated for 30 min in the absence of thymine and then pulse-labeled with 1 μg/ml of [^{3}H]thymine (50 Ci/mmol) for 10 sec as described (2). For steady-state labeling, an equal volume of LSTL medium, containing 5 μg/ml of [^{3}H]thymine, was added to the culture, and incubation was continued for 0.3–10 min (2). Incorporation of label was terminated with phenol-ethanol, and the cells were lysed as described (2), except that Pronase treatment was omitted.

Fractionation of Nascent DNA Chains. To reduce its viscosity, the lysate from 6×10^{10} cells was briefly treated with ultrasound. (^{14}C-Labeled DNA of 5000 nucleotides, added to the lysate prior to ultrasonic treatment, was not degraded.) The DNA was then separated from nonincorporated [^{3}H]thymine and nucleotide pools by gel filtration on Sephadex G-25 (fine, 0.9 × 100 cm column) in Tris-EDTA (20–1 mM, pH 8.0). Fractions eluting in the void volume and containing DNA chains of more than 6 nucleotides in length were pooled and adsorbed to hydroxylapatite (0.5 × 3 cm column) in 2 mM phosphate buffer (pH 6.8). DNA chains of increasing length were eluted from hydroxylapatite in a stepwise manner using 50, 100, 200, and 400 mM phosphate buffer. Individual peaks were rechromatographed on hydroxylapatite in the presence of 5 M urea to remove proteins.

Nuclease Digestion of Nascent DNA Chains. Pulse-labeled DNA that was eluted from hydroxylapatite with 50, 100, or 200 mM phosphate buffer was dialyzed extensively against Tris-EDTA (20–1 mM, pH 8.0) and mixed with uniformly ^{14}C-labeled *E. coli* DNA of the same length. The ^{14}C-labeled DNA was produced by the action of DNase I on high-molecular-weight *E. coli* DNA and, therefore, contained 5′-phosphate groups; it was shown to be completely resistant to digestion by spleen phosphodiesterase (oligonucleate 3′-nucleotidohydrolase; EC 3.1.4.18) unless first treated with alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1]. (To ensure that the two types of DNA had exactly the same molecular weight they were cochromatographed on a Sepharose column.) To 1 ml of this mixture were added 50 μl of 1 M $MgSO_4$ and 1.5 units of alkaline phosphatase (Worthington). After incubation for 1 hr at 37°, the sample was made 0.1 M in sodium phosphate, pH 6.8, and incubated with 1 unit of spleen phosphodiesterase (Worthington) for 3 hr at 37°. (The amount of enzyme and the incubation time were increased for DNA of more than 1000 nucleotides in length.) The degree of breakdown to mononucleotides was determined by gel filtration on Sephadex G-100. The preparation of exonuclease I and the conditions for degradation of DNA with this enzyme have been described extensively (3). Molecular weights of DNA chains were determined by gel filtration on Sepharose CL-4B, which was calibrated using simian virus 40 fragments produced by *Hin* restriction nuclease (6).

RESULTS

Thymine-depleted bacteria were used for most pulse-labeling experiments because such cells incorporate [^{3}H]thymine at full specific activity and produce a greater proportion of short DNA chains as compared to steady-state cells (2). Gel filtration of the cell lysate on Sephadex G-25 effectively separated DNA from residual [^{3}H]thymine and nucleotides. The pulse-labeled material was then adsorbed to hydroxylapatite and eluted in a stepwise manner with phosphate buffer of increasing molarity (Fig. 1). Fraction I, eluting with 50 mM phosphate, contained single-stranded DNA chains of 50 to 100 nucleotides, as determined by gel filtration on Sepharose 4B. Fraction II, eluting with 100 mM phosphate, contained DNA chains of 100 to 500 nucleotides in length. The DNA in fraction III ranged in size from 500 to about 4000 nucleotides. This is the fraction in which purified, single-stranded DNA eluted. Fraction IV contained

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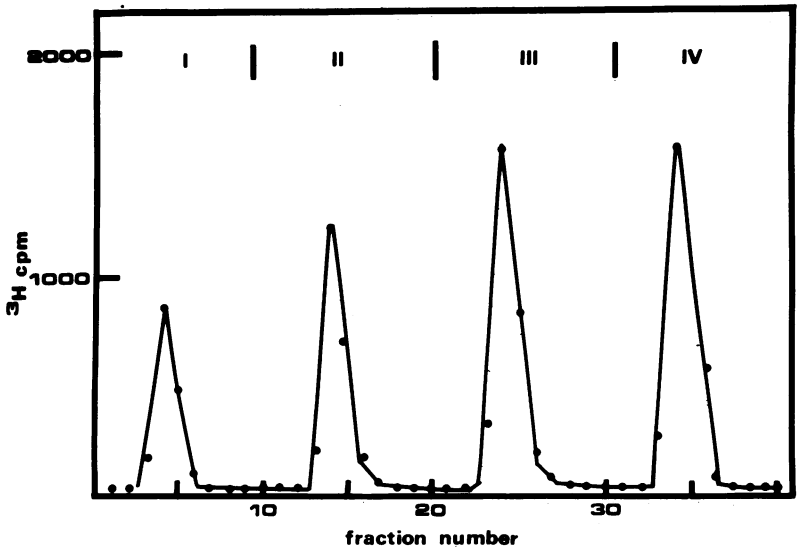

FIG. 1. Fractionation on hydroxylapatite of nascent DNA chains. Thymine-depleted bacteria were pulse-labeled for 10 sec at 14° with [³H]thymine, and the lysate was applied to Sephadex G-25. The DNA eluting in the void volume was adsorbed to hydroxylapatite and eluted with phosphate buffer of increasing molarity (fraction I, 50 mM; fraction II, 100 mM; fraction III, 200 mM; fraction IV, 400 mM).

double-stranded DNA. The pulse-labeled DNA in this fraction, when heat-denatured, had a similar size distribution as the DNA in fraction III with a larger proportion of longer chains. However, because of its association with high molecular weight unlabeled DNA it eluted as double-stranded DNA.

Upon rechromatography on hydroxylapatite, all DNA eluted in the same fraction from which it had been isolated originally (Fig. 2a). When rechromatographed in the presence of 5 M urea, however, the pulse-labeled material from both fractions I and II eluted in fraction III as single-stranded DNA (Fig. 2b), suggesting that the first fractionation according to size was due to the presence of protein bound to the DNA chains. The same shift in the elution pattern was seen if the DNA was heated to 100° for 5 min prior to chromatography on hydroxylapatite. Treatment of the DNA with proteinase K (Merck) resulted in a partial (60–75%) shift, while incubation with phospholipases A (EC 3.1.1.4) and C (EC 3.1.4.3) or with ribonuclease A (EC 3.1.4.22) had no effect.

To eliminate the possibility that the short DNA chains eluting before single-stranded DNA (fractions I and II) were an artifact caused by thymine depletion, the same experiments were done with DNA isolated from cells that had been labeled under steady-state conditions. The pulse-length was increased to 20 sec to allow enough label to pass through the intracellular nucleotide pools. The analysis of the labeled DNA on hydroxylapatite showed the same basic elution profile (Fig. 3a). When the pulse length was increased to 2 min, the amount of label found in fractions I, II, and III increased, reflecting the higher specific activity of labeled nucleotides in the intracellular thymidine nucleotide pool (Fig. 3b). Because of the rapid and continuous conversion of nascent DNA chains into high-molecular-weight DNA, however, a much larger proportion of the incorporated label was found in fraction IV. The complete conversion of short DNA chains into high-molecular-weight DNA was observed if the [³H]thymine pulse was followed by 20 min of incorporation of unlabeled thymine (Fig. 3c). Under

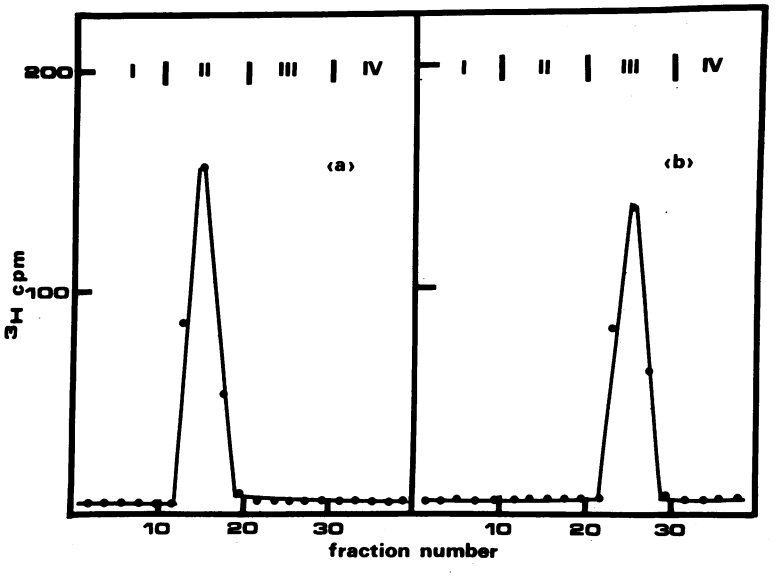

FIG. 2. Effect of urea on hydroxylapatite chromatography. DNA isolated from fraction II was rechromatographed on hydroxylapatite in the absence (a) or in the presence (b) of 5 M urea.

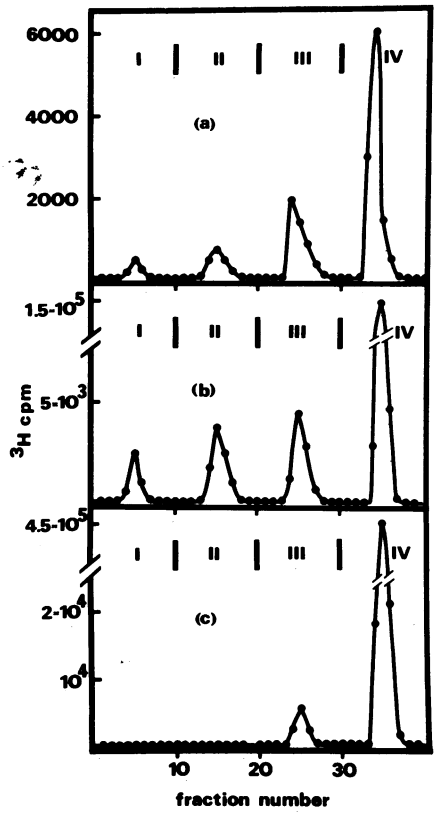

FIG. 3. Hydroxylapatite chromatography of DNA from cells labeled under steady-state conditions. (a) 20-sec pulse of [^{3}H]thymine, (b) 2-min pulse of [^{3}H]thymine, (c) 2-min pulse of [^{3}H]thymine followed by 20-min incorporation of unlabeled thymine.

steady-state conditions, it takes more than 10 min to completely replace the intracellular thymidine nucleotide pool (7).

All fractions from the hydroxylapatite column were completely digestible by deoxyribonuclease I (EC 3.1.4.5) or exonuclease I (EC 3.1.4.25) from *E. coli*, showing that the label was incorporated into DNA and that the DNA chains had free 3′-hydroxyl groups.

To determine the nature of the 5′-ends of short nascent DNA chains, pulse-labeled DNA from fractions I and II was deproteinized by rechromatography on hydroxylapatite in the presence of 5 M urea, dialyzed, mixed with ^{14}C-labeled *E. coli* DNA of the same size containing 5′-phosphate groups, and subjected to enzymatic digestion by alkaline phosphatase followed by spleen phosphodiesterase. The reaction products were analyzed on Sephadex G-100, where undegraded DNA eluted near the void volume and nucleotides, released by the enzyme, eluted in the total volume. The results of this analysis show that short nascent DNA chains from thymine-depleted cells are completely resistant to the combined action of alkaline phosphatase and spleen phosphodiesterase. The extensive degradation of the ^{14}C-labeled control DNA shows that the enzymes were active (Fig. 4a). The identical result was obtained if the pulse-labeled material was not deproteinized in urea prior to enzymatic digestion. Similarly, the nascent DNA remained resistant to degradation by these enzymes after treatment with ribonuclease A or incubation in 0.3 M KOH at 65°C for 1 hr. However, if the nascent DNA was subjected to 1 M KOH for 24 hr at 37°, it became degradable by the combination of alkaline phosphatase and spleen phosphodiesterase. The phosphodiesterase alone did not digest the alkali-treated DNA, indicating that 5′-phosphate groups remain after exposure to 1 M KOH. In contrast to the pulse-labeled DNA from fractions I and II, DNA containing more than 500 nucleotides (fraction III) was degraded by alkaline phosphatase and spleen phosphodiesterase, without prior alkali treatment, to the same extent as the ^{14}C-labeled control DNA of the same size (Fig. 4b).

Again, it was possible that this lack of breakdown was an artifact created by thymine depletion. The same enzymatic digestions were, therefore, done with DNA isolated from cells that had been labeled with [^{3}H]thymine for 10 min under steady-state conditions. The results of this experiment were identical to those obtained with pool-depleted cells; DNA chains eluting in fraction I and II (less than 500 nucleotides in length) were completely resistant to enzymatic breakdown, while chains eluting in fraction III (longer than 500 nucleotides) were digestible. It appears, therefore, that some alkali-stable group or substituted nucleotide at the 5′-ends of short nascent DNA chains is blocking their enzymatic breakdown. This blocking

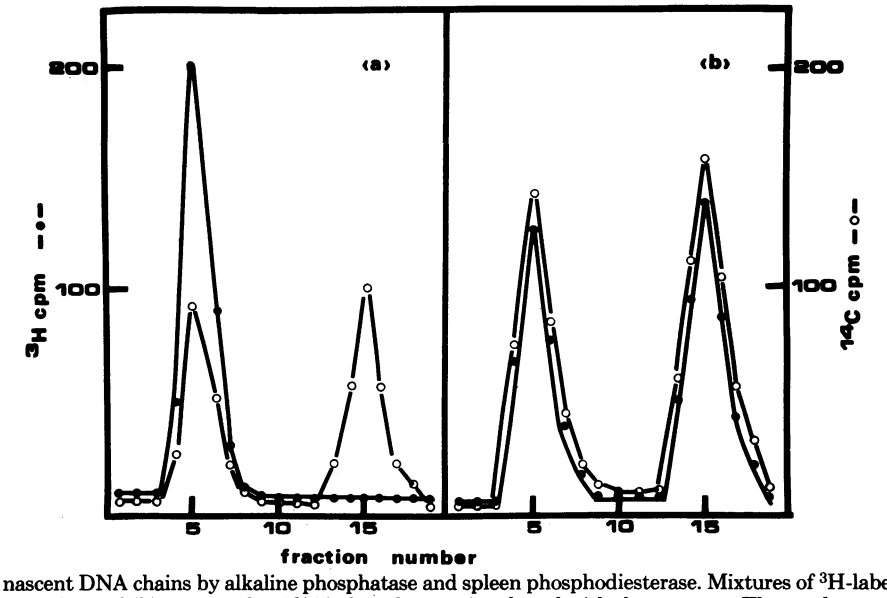

FIG. 4. Digestion of nascent DNA chains by alkaline phosphatase and spleen phosphodiesterase. Mixtures of ^{3}H-labeled DNA and ^{14}C-labeled control DNA, (a) 200 nucleotides and (b) 1000 nucleotides in length, were incubated with the enzymes. The products of this digestion were then analyzed on Sephadex G-100 (mononucleotides elute in the total volume, undegraded DNA elutes in the void volume).

group is apparently restricted to the smallest DNA chains and is not present in DNA of the size of Okazaki pieces.

There is a trivial explanation of our results. If, for example, the label were located predominantly near the 3′-ends of nascent DNA chains, little degradation by spleen phosphodiesterase would be observed since digestion of the control DNA was only 50% complete. The degradation from the 5′-end of the pulse-labeled DNA, although occurring, would not be seen because of the absence of label in this portion of the molecule. To rule out this possibility, pulse-labeled DNA and uniformly ^{14}C-labeled control DNA of exactly the same size were degraded with exonuclease I, and the time course of this degradation was followed by taking samples into trichloroacetic acid (Fig. 5). From the kinetics of the release of ^{14}C and ^{3}H label in acid-soluble form it is evident that the ^{3}H label is distributed as uniformly within the DNA molecule as the ^{14}C label is in the control DNA (8).

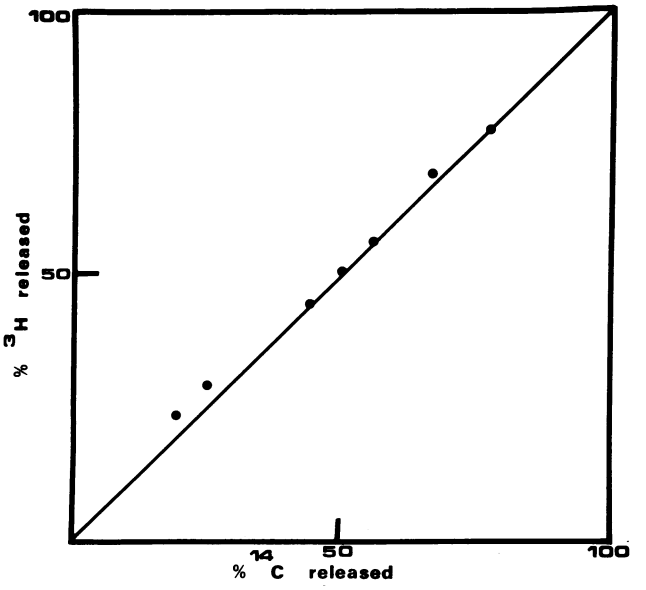

FIG. 5. Degradation of nascent DNA by exonuclease I. Pulse-labeled DNA chains, 200 nucleotides in length, and uniformly ^{14}C-labeled DNA of the same size were digested with exonuclease I. At different times samples were precipitated with trichloroacetic acid, and the extent of degradation was determined.

DISCUSSION

Several lines of evidence suggest that Okazaki pieces are not the first intermediates in DNA replication but arise through the joining of many extremely short polynucleotide chains rather than by unidirectional chain elongation from a single initiation site (2–5). In this paper, we present evidence that these short nascent DNA chains are structurally modified at their 5′-ends and, as the result of this modification, become resistant to degradation by spleen phosphodiesterase. This resistance to enzymatic breakdown cannot be due to a 5′-phosphate group because treatment with alkaline phosphatase would remove it. Neither can lack of degradation be due to a di- or triphosphate group because it would be hydrolyzed during the incubation in 0.3 M NaOH at 75°. A covalently linked RNA primer (9, 10) can also be ruled out because the enzymes do not distinguish between RNA and DNA. In addition, RNA would be completely hydrolyzed after incubation in 1 M KOH for 24 hr leaving a hydroxyl group at the 5′-end of a DNA chain; such DNA chains would be degradable by spleen phosphodiesterase alone, which is contrary to our results. We can also rule out an artifactual modification of the 5′-ends of the short DNA chains because longer chains (Okazaki pieces), present in the same cell lysate and subjected to the same isolation procedure, carry 5′-phosphate groups and are degradable by phosphatase and spleen phosphodiesterase.

Other laboratories have reported that nascent DNA chains can be degraded by these enzymes (10–12). Since it is likely that *E. coli* lysates contain enzymes capable of removing the blocking groups, the reported presence of free 5′-phosphate and 5′-hydroxyl termini on nascent DNA chains could be the result of such enzyme action during the often time-consuming lysis procedures. Alternatively, it could be an artifact due to labeling with thymidine because we have shown previously that [^{3}H] thymidine is preferentially incorporated at the 3′-ends of longer DNA chains (Okazaki pieces) (13).

Concerning the nature of the blocking groups at the 5′-ends of nascent DNA chains, we must consider several possibilities. First, the 5′-terminal nucleotide may be modified and, as a result, becomes resistant to attack by spleen phosphodiesterase or alkaline phosphatase. Second, the chemical bond between the first and the second nucleotide may be different from a phosphodiester bond. [For example, it is possible that the 5′-terminal nucleotide is linked through a pyrophosphate bond to another nucleotide. Such a bond occurs at the 5′-ends of mammalian RNA viruses (14) and at the 5′-ends of DNA chains prior to ligation by polynucleotide kinase (15).] Third, a protein (or another type of macromolecule) may be linked to the 5′-terminus of a DNA chain through a phosphoamide bond or some other alkali-stable linkage. While the macromolecule itself would be destroyed by alkali, the last residue would remain linked to the DNA, thus preventing degradation. Fourth, the 5′-end of a nascent DNA chain may be attached to a metabolically stable, very short DNA molecule. The secondary structure of this "transfer DNA" (16) molecule or the nature of its linkage to the nascent DNA chain might make the latter resistant to enzyme digestion.

We can think of at least two functions for the blocking group at the 5′-end of a nascent DNA chain. First, the group may serve as a primer for the synthesis of a new DNA chain. Alternatively, it may function to activate the 5′-end prior to ligase action. We have previously shown that the molecular weight distribution curve of nascent DNA chains exhibits a change in slope at chain lengths of about 500 nucleotides (ref. 2; A. T. Diaz and R. Werner, submitted for publication). We concluded from this result that DNA chains shorter than this length are joined together by a mechanism different from that for longer chains, perhaps involving two distinct ligase enzymes. It may be significant that essentially all chains containing fewer than 500 nucleotides are blocked at their 5′-ends, while chains that are longer than 500 nucleotides carry 5′-phosphate groups.

In addition to being modified at their 5′-ends, nascent DNA chains are noncovalently bound to protein. This association with protein allows the elution of nascent chains from hydroxylapatite at phosphate concentrations that do not release single-stranded DNA, and, therefore, provides an excellent means for the separation of very short DNA chains from Okazaki pieces and larger DNA. [Other laboratories have used hydroxylapatite chromatography to demonstrate the single-strandedness of newly replicated DNA (1, 17) but have not observed the very short DNA chains that elute earlier than single-stranded DNA because they had removed proteins prior to chromatography.] Recent studies in our laboratory indicate that the association of nascent DNA chains with protein is probably rather specific. The DNA–protein complex is stable in Cs_2SO_4 gradients, exhibiting a buoyant density that is determined by the ratio of DNA to protein. Larger chains have a higher density than

shorter chains, suggesting that the protein is bound to only one end of the DNA chain (unpublished results). Since the DNA is freely digestible by exonuclease I, the protein is probably bound to the 5'-end.

No doubt, the special structure of 5'-ends of nascent DNA chains as well as its association with protein play a role in the replication process.

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