Flexibility in RNA priming of Okazaki pieces at the *E. coli* replication fork

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ABSTRACT

We present results which suggest considerable flexibility in the RNA priming of Okazaki pieces at the *E. coli* replication fork. Using film lysates on cellophane discs, we have identified RNA at the 5' ends of Okazaki pieces. All four ribonucleotides are found to be present at the RNA-DNA junction if all four ribonucleoside triphosphates are used. However, if only ATP, or ATP and GTP are used, then only 2'(3')AMP, or 2'(3')AMP and 2'(3')GMP are found at the RNA-DNA junction. A nearest neighbor analysis of RNA associated with Okazaki pieces using $\alpha^{32}\text{P}$ -CTP as a probe shows a similar dependence of nearest neighbor composition on the ribonucleoside triphosphate composition of the incubation mixture. Thus, the nucleotide composition of the RNA primers at the ends of Okazaki pieces varies as a function of the ribonucleoside triphosphates available.

INTRODUCTION

The experiments of Okazaki and co-workers demonstrated that the DNA of *E. coli* is replicated in a discontinuous fashion (1,2). Their analysis of *E. coli* replication forks revealed the transient presence of small DNA intermediates now called Okazaki pieces, which were then ligated to form the mature chromosome. In spite of extensive characterization of these Okazaki pieces, a number of questions remain, particularly with respect to the initiation of their synthesis. For example, although enzymatic studies clearly show that *E. coli* is capable of using RNA to prime synthesis of the Okazaki pieces, the primer-RNA formed at the *E. coli* replication fork has not been thoroughly characterized (3-8). In this communication, we present evidence for a class of RNA covalently attached to *E. coli* Okazaki pieces. Furthermore, we show that this RNA lacks a rigid nucleotide sequence, and that its nucleotide composition can be altered in vitro.

RESULTS

In an attempt to probe the mechanism of Okazaki piece initiation, we have made use of the cellophane disk in vitro DNA synthesizing system

Table 1. Nucleotide Distribution at the RNA-DNA Junction.

labeled [³² P] dNTP	total cpm sample	fraction of cpm transferred	rNMP labeled (% of total rNMPs released)			
			AMP	CMP	GMP	UMP
dATP	8.6 x 10 ⁵	1/2600	39	11	38	12
dCTP	3.9 x 10 ⁵	1/2700	29	36	16	19
dGTP	2.7 x 10 ⁵	1/1650	33	10	37	20
dTTP	3.6 x 10 ⁵	1/1400	33	27	20	20

E. coli, strain H560 (polA⁻ endI⁻) was grown, harvested and lysed as described previously (13). The four incubation mixtures (750 μ l each) contained: 20 mM morpholinopropane sulphonate acid (pH 7.4); 100 mM KCl; 5 mM MgCl₂; 0.1 mM EDTA; 20 μ M each of dATP, dCTP, dGTP, dTTP; 0.5 mM each ATP, CTP, GTP, UTP; 1 mg/ml NMN; and 0.25 mCi of one of the following, (a) [α -³²P] dATP, (b) [α -³²P] dCTP, (c) [α -³²P] dGTP, (d) [α -³²P] TTP. Each reaction mixture was divided into three, 250 μ l aliquots, used to support DNA synthesis of 6 x 10⁸ cells, lysed on a 2.5 cm diameter cellophane disk. Synthesis was at 30° for 15'. The reactions were stopped and the DNA purified by CsCl density gradient centrifugation as described previously (13). DNA-containing fractions were dialyzed against 1 M NaCl, 25 mM Tris (pH 8.0), 5 mM EDTA and finally against 10 mM Tris, 1 mM EDTA. Yeast t-RNA (5-10 A₂₆₀ units) and 5 M KOH (final conc., 0.33 M) were added, and each sample was incubated at 37° for 18 hours in polypropylene tubes. The solution was neutralized and desalted by the addition of 70% perchloric acid (24). rNMPs were analyzed by DEAE descending paper chromatography in 50 mM ammonium formate (pH 2.8) as described by Furlong (25). The paper was dried, cut into 1 cm strips, and ³²P radioactivity determined. The hydrolyzed tRNA served as U.V. markers to identify the 2'(3') rNMPs.

(Schaller et al. (9)). Previous results have shown that DNA synthesis in this system resembles synthesis *in vivo* by several criteria, including the rate of synthesis and the requirements for various protein factors (9-13). The Okazaki pieces synthesized on cellophane disks have been shown to originate from only one of the two DNA strands (lagging strand), presumably the result of *de novo* initiation events. In addition, use of this system allows inhibition of sealing of Okazaki pieces (by the addition of NMN) and the use of high specific activity ³²P-labeled nucleoside precursors.

The presence of RNA primers can be inferred by the alkali-induced transfer of ³²P, from labeled Okazaki pieces to a 2'(3') ribonucleotide (3,4). Such a transfer not only identifies the existence of a covalent linkage between DNA and RNA, but can provide information about the nucleotide sequence at the DNA/RNA junction. In the experiments shown in Table 1, DNA was synthesized in four reaction mixtures, each containing one [α -³²P] dNTP, three unlabeled dNTPs, and the four rNTPs. After purification by CsCl centrifugation, the DNA was treated with alkali, and exposed to a paper chromatography system designed to separate the individual (2'-3') rNMPs. As

Figure 1. Alkali treatment of ^{32}P -labeled DNA synthesized in the presence of only one or all four rNTPs.

A) ATP only. DNA was synthesized in vitro in a single reaction mixture as described in Table 1, with the following changes: the reaction mixture contained 0.125 mCi each of the four [α - ^{32}P] dNTPs, with 1 mM rATP as the only available ribonucleoside triphosphate; following alkaline hydrolysis, the sample was neutralized and analyzed by pH 3.5 paper electrophoresis. The sample was spotted on 3 MM paper and exposed to 4000 V for 60', using a buffer containing pyridine, acetic acid, water, v:v:v, 1:10:289. Marker 2'(3') rNMPs were visualized by UV light and the paper cut and assayed for ^{32}P radioactivity. Total DNA radioactivity was 1.5×10^5 cpm.

B) All rNTPs. DNA was synthesized and analyzed as in (A), except that the synthesis reaction mixture contained 0.5 mM ATP, 0.125 mM CTP, 0.2 mM GTP and 0.2 mM UTP instead of 1 mM ATP. Total DNA radioactivity: 5.4×10^5 cpm.

seen in Table 1, ^{32}P is transferred from all deoxynucleotides to all ribonucleotides. The frequency of transfer from each individual deoxynucleotide varies from 1/1400-1/2700, roughly consistent with the length of an Okazaki piece. Thus, not only can RNA-DNA junctions be identified, but they are quite random with respect to their sequence.

A peculiar feature of the in vitro DNA synthesizing system is that it does not require a full complement of rNTPs. Elimination of 3 rNTPs has no apparent effect on the frequency of initiation of Okazaki piece synthesis (14). Two explanations can be considered for these results. First, even if only one ribonucleoside triphosphate were added, turnover of RNA in the film lysate system may be sufficient to provide all four triphosphates. Alternatively, the RNA primers used may change in nucleotide composition in response to the ribonucleoside triphosphates provided. It is thus of some interest to know what effect ribonucleotide limitation has on the composition of the RNA found attached to the DNA.

Figure 1 illustrates the results of transfer experiments in which DNA was synthesized either in the presence of all four rNTPs or only in the presence

Figure 2. Sucrose gradient sedimentation of denatured, ^{32}P -labeled, DNA. DNA was synthesized as described in Fig. 1A except that the reaction contained 1 mM ATP and 1 mM GTP. The DNA was purified by CsCl centrifugation. Following extensive dialysis into 10 mM Tris, 1 mM EDTA, a 250 μl aliquot ($\sim 60\%$ of the sample) was denatured by dialysis into 95% formamide, 5 mM Tris, 1 mM EDTA for 45' at 20° (26), followed by 15' dialysis against 10 mM Tris, 1 mM EDTA, to remove the formamide. The sample was layered onto an 11.5 ml linear sucrose gradient (25%-5% sucrose in 25 mM Tris, 1 mM EDTA, 1 mM EGTA) and centrifuged for 5 hours at 40,000 rpm in an SW41 rotor in a Beckman L8 ultracentrifuge. Twenty-eight (0.4 ml) fractions were collected and a 20 μl aliquot of each assayed for radioactivity. Fractions were pooled, as indicated, and analyzed as described in Figure 3. Sedimentation is from right to left.

of 1 mM ATP. In the latter instance, transfer of ^{32}P is only to AMP. If both ATP and GTP are used in the incubation mixture, transfer to both AMP and GMP (but not CMP and UMP) occurs (results not shown). Apparently, composition of the ribonucleotides adjacent to the DNA is a function of the composition of the incubation mixture.

Though the previous results are consistent with the existence of RNA primers, it was desirable to show that the transferable phosphate originated from Okazaki pieces. To do this, a preparation of ^{32}P -labeled DNA synthesized in the presence of ATP and GTP was denatured and subjected to sucrose gradient sedimentation. This resulted in the distribution of nascent DNA into 2 size classes, shown in Figure 2, the larger pieces originating from the leading strand, the smaller from the lagging strand. Fractions from 3 regions of the gradient were pooled, as indicated, and individually treated with alkali. As seen in Figure 3, alkali-generated [^{32}P] rNMPs originated only from DNA in the Okazaki piece size class. Since this incubation mixture contained only ATP and GTP, transfer to only A and G was observed.

These experiments suggest that RNA is covalently attached to Okazaki pieces, and that the sequence of the DNA-linked ribonucleotide is quite plastic. To determine if this plasticity extends beyond the junction

Figure 3. Alkali treatment of ^{32}P -labeled DNA after sucrose gradient sedimentation. To each of the pooled fractions from the sucrose gradient described in Figure 2, was added 20 μg of calf thymus DNA and 10 A_{260} units of yeast tRNA. The sucrose was removed by 15 hour dialysis into 20 mM Tris (pH 8.0), 2 mM EDTA, 2 mM EGTA, and each sample precipitated with ethanol. The precipitates were resuspended in 100 μl of 0.33 M KOH and incubated for 17 hours at 37° . Following neutralization, each sample was spotted on Whatman 3 MM paper followed by electrophoresis at pH 3.5, 4000 V, for 55 minutes.

The approximate number of ^{32}P cpm in each sample, following alkaline hydrolysis were: 1:100,000; 2:100,000; 3:10,000, and 1 represents fractions 1-4 (Pool 1); 2, 16-21 (Pool 2); and 3, 25-28 (Pool 3) from the sucrose gradient in Figure 2.

nucleotide, nearest neighbor analysis was performed on RNA found to co-purify with de novo synthesized DNA. Figure 4 illustrates an experiment in which DNA was synthesized in the presence of a low concentration [α - ^{32}P] rCTP (1 μM) and either: (A) 1 mM ATP or (B) 1 mM ATP and 1 mM GTP. The DNA was separated from the vast majority of the ^{32}P -labeled RNA by CsCl centrifugation, followed by nitrocellulose chromatography (11). A small amount of ^{32}P remained with the DNA fractions. If this material was exposed to alkali, all the radioactivity was found as either: (A) 2'(3') AMP or (B) 2'(3') AMP and 2'(3') GMP, depending on the reaction mixture in which it was synthesized. This sequence flexibility of the DNA-associated RNA contrasts markedly with that of the majority of the ^{32}P -labeled RNA. Figure 4C, for example, illustrates the transfer frequency for the non-DNA associated RNA labeled under conditions described in Fig. 4A ([α - ^{32}P] CTP and 1 mM ATP). The ^{32}P is

Figure 4. Nearest neighbor determination on RNA labeled with ^{32}P CTP.

A) **ATP only.** DNA was synthesized as described in Table I, with the following reaction mixture: (750 μl) 20 mM MOPs (pH 7.4); 100 mM KCl; 5 mM MgCl_2 ; .1 mM EDTA; 20 μM each of dATP, dCTP, dGTP, dTTP, 50 μCi ^3H -dTTP (ICN, 40-70 Ci/mmmole), 1 mM ATP and 0.2 mCi [α - ^{32}P] CTP (Amersham, 300 Ci/mmmole). Following purification by CsCl centrifugation, the sample (500 μl) was dialyzed in 10 mM Tris, 1 mM EDTA. The DNA was denatured by boiling for 3', made up to 0.5 M in KCl, and chromatogrammed on a 0.5 ml column of nitrocellulose prepared using the conditions of Boezi and Armstrong (27). Samples were eluted with 2 ml of 0.5 M KCl, 10 mM Tris, 1 mM EDTA, followed by 2 ml of 10 mM Tris, 1 mM EDTA. Sixteen fractions were collected and 25 μl aliquots from each assayed for both ^3H and ^{32}P radioactivity. Single stranded DNA was recovered on one fraction of the 10 mM salt elution and the sample was mixed with 5 A_{260} units of tRNA and incubated in 0.33 M KOH for 16 hours at 37° . Following hydrolysis, the sample was analyzed by pH 3.5 electrophoresis at 3,500 V for 45 min. One cm strips were cut and analyzed for radioactivity. ^3H -radioactivity in sample: 1320 cpm.

B) **ATP + GTP:** Identical to (A), except that the synthesis mixture also contained 1 mM GTP. ^3H -radioactivity in sample: 3100 cpm.

C) **DNA-free RNA:** Synthesis was performed as described in (A). However, a fraction was taken from the bottom of the CsCl gradient, and this consisted of ^{32}P -labeled RNA. This sample was passed over a nitrocellulose column (as in A), but the radioactivity eluted with the high salt wash. It too was hydrolyzed by KOH and analyzed as in (A).

obviously transferred to all four ribonucleotides even if only ATP is present. Thus the flexibility of nearest neighbors to incorporated C is a special property of DNA-linked RNA, and does not extend to the RNA presumably synthesized by RNA polymerase.

DISCUSSION

A class of RNA is synthesized in the cellophane disc system which has properties consistent with the primer for Okazaki piece synthesis. This RNA bands with DNA in a density gradient, and is detected by phosphate transfer from ^{32}P deoxynucleotides in DNA to a 2'(3') ribonucleotide after alkali treatment. Treatment with alkali does not detectably change the sedimentation profile of denatured DNA intermediates in a neutral sucrose gradient. This evidence is consistent with RNA covalently linked at the 5' terminus of DNA.

The demonstration of covalent linkage between RNA and DNA does not necessarily mean that the RNA is associated with Okazaki piece synthesis. This material could conceivably have no relationship to DNA synthesis at all, and instead be the artifactual product in the in vitro system of an enzyme such as polynucleotide phosphorylase. However, there are two additional lines of evidence that make it likely that the RNA being studied is primer RNA. First, after analysis in a neutral sucrose gradient, the RNA is found to be associated with Okazaki pieces. Perhaps equally important, it is absent from both the bottom of the sucrose gradient, the position to which the bulk of the cellular DNA sediments, and from the top of the gradient, where small RNAs might be expected to band. Furthermore, the frequency of junctions found approximately correlates with the number of Okazaki pieces synthesized. These latter data make it likely that RNA detected by these phosphate transfer experiments is covalently attached to the Okazaki pieces synthesized in the in vitro system.

The most interesting feature of this RNA is the flexibility of its nucleotide composition as detected by phosphate transfer experiments; it changes as a function of the relative concentration of ribonucleoside triphosphates in the incubation mixture. Perturbations in the pool of rNTPs appear to have no major effect on the rate of priming, but markedly affect the nucleotide composition of the RNA.

The RNA primers at the ends of Okazaki pieces are presumably synthesized by the dnaG primase (4). Since this enzyme is a DNA directed RNA polymerase, how can the flexibility observed in the nucleotide composition of the primer be rationalized? One possible explanation involves the switching event from RNA to DNA. Thus, if only ATP were provided in the in vitro system, the dnaG primase would presumably pause after incorporating an A residue, if the next nucleotide were one of the absent rNTPs. Switchover from RNA to DNA might then occur. However, if all four triphosphates were present, the primase could continue to incorporate ribonucleotides and the switch-over point would

then reflect a more random distribution of RNA nucleotides.

The RNA primers have been studied mainly by phosphate transfer experiments which probe for selected positions in the primer chain. Although these studies in the in vitro system indicate flexibility in RNA priming, the data do not eliminate a certain degree of specificity. For example, there is a purine bias for the RNA nucleotide at the RNA/DNA junction (see Table 1). Additionally, transfer experiments with only GTP and UTP at 1 mM concentration in the in vitro reaction (results not shown) yield approximately twice as much 2'(3') G as 2'(3') U from the RNA junction after alkali treatment (and no A or C). Another facet of the data which may suggest specificity in priming are the experimental results shown in Figures 1a and 4a, in which Ap is essentially the only product of the phosphate transfer from DNA, or the $\alpha^{32}\text{P}$ -CTP probe. The absence of pAp, ppAp or pppAp suggest that both the DNA junction and any C residues in the primer must have as their "nearest neighbor" an internal A residue in the RNA primer chain and that neither the DNA junction nor a C residue in the primer are next to a 5' terminal ribonucleotide. Substantial transfer to a 5' terminal A residue processed in the in vitro system to a 5' OH terminus is the alternative possibility.

The plasticity of the sequence of RNA primers in the E. coli system can be contrasted to other well characterized in vitro DNA synthesizing systems. In the case of coli phage T7, for example, the primer sequence is quite fixed with respect to sequence and length, and cannot be synthesized in the absence of the appropriate precursors (15-18). Mammalian systems (19-21) and phage systems using purified E. coli DNA-replication enzymes (22,23), respond to nucleotide starvation by substituting deoxynucleotides within the RNA-primer. We have found no evidence of such a dNTP substitution mechanism under these conditions, as only ribonucleotides are found within the primer RNA. If deoxynucleotides were incorporated within the primer, ^{32}P -dinucleoside diphosphates, as well as more complex products should have been detected in the $\alpha^{32}\text{P}$ -CTP transfer experiment described above. Significant levels of such products were not found.

One intention in characterizing this primer RNA was that peculiarities in its structure would answer the following questions about the initiation of Okazaki piece synthesis: where along the template does priming begin and where does the synthesis of the RNA cease and the DNA begin? The experiments presented above suggest considerable flexibility in E. coli Okazaki piece priming by RNA. The flexibility in priming may arise because a large excess of primer sequences can potentially be used, and only a small fraction of the

total are actually chosen to prime Okazaki pieces at a particular fork; alternatively the priming mechanism may be intrinsically flexible.

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