Transcripts within the replication origin, *oriC*, of *Escherichia coli*

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SUMMARY

Transcription start and termination sites were mapped in the *E. coli* replication origin, *oriC*. Outward transcription from within *oriC* (promoters *Pori-r* and *Pori-l*) was found to start *in vivo* at position 178 for *Pori-l* and at positions 294 and 304 for *Pori-r*, respectively. These transcripts were terminated after 100 - 150 bases, at terminators designated *Tori-l* and *Tori-r*. Transcription from the 16 kd promoter, which lies clockwise adjacent to *oriC* and promotes transcription toward *oriC*, started at position 757 and gave transcripts with 3' ends at several positions within and to the left of the minimal replication origin. However, the majority of transcripts traversed the whole *oriC* region, and were not terminated within the DNA segment tested. Transcription of the chromosomal 16 kd gene was negatively regulated by DnaA protein and positively affected by *dam* methylation. The possible function of these transcripts is discussed.

INTRODUCTION

In bacteria the regulation of DNA replication and cell division is controlled by the rate of initiation at the unique replication origin, *oriC* (1, 2). Therefore, one major interest in the study of DNA replication is to understand the molecular basis of the regulation of initiation.

Besides the action of *oriC* specific initiation proteins, initiation of DNA synthesis *in vivo* requires an RNA polymerase mediated transcription event (3-5). The role of the synthesized RNA(s) is not known. Two possible functions may be envisaged: priming of DNA synthesis or regulation by transcriptional activation of the origin.

Several approaches have been reported concerning the characterization of the transcription event(s) in the *oriC* region. Two divergently transcribed promoters are active *in vitro* (7; see Fig. 4), *Pori-l* and *Pori-r*. Attempts to identify these

promoters unequivocally in vivo failed until now (8-10). A further promoter activity promoting clockwise transcription in vivo has been located at the left border of oriC (10).

The minimal origin has been functionally defined as the minimal DNA fragment allowing autonomous replication of minichromosomes (6). However, the region clockwise adjacent, coding for a protein with a calculated M_r of 15,813 (henceforth called the 16 kd protein, see Fig. 4) seems to be involved in the control of initiation. The 16 kd gene was therefore named mioC (modulation of initiation at oriC; 20).

Here we analyze the influence of the DnaA protein on the transcription of the 16 kd gene in its chromosomal location by comparative S1 mapping. This analysis includes the precise mapping of the 5' end of the 16 kd transcript. In the -35 region of the 16 kd promoter a dam methylase recognition site (GATC) is present (21). Since adenine methylation of GATC sequences is known to influence oriC replication (22, 23) and promoter activities (24) we compared in vivo transcript levels by S1 mapping in the absence and presence of dam methylation. Termination of 16 kd transcription within and counterclockwise of the minimal origin was analyzed.

We demonstrate for the first time the activity of the origin promoters Pori-r and Pori-l in vivo and define the precise in vivo start of the origin transcripts by S1 mapping. The possible function of the transcripts in the origin region is discussed.

Table I. E. coli strains

Strain	Genotype	Ref.
CM987	<u>asnA</u> 31, <u>asnB</u> 32, <u>recA</u> 1, <u>relA</u> 1, <u>spoT</u> 1, <u>thi</u> -1	(25)
CM993	= CM987 containing pCM959	(25)
CM1671	Hfr, <u>asnA</u> ::Tn10, <u>asnB</u> 32, Δ (<u>asnA-oriC-gidA</u>)1071, <u>fuc</u> , <u>lysA</u> , <u>relA</u> 1, <u>spoT</u> 1, <u>thi</u> -1	(46)
EC559	<u>ara</u> -14, <u>argE</u> 3, Δ (<u>gpt-proA</u>)62, <u>galK</u> 2, <u>his</u> -4, <u>lacY</u> 1, <u>leuB</u> 6, <u>mtl</u> -1, <u>rpsL</u> 31, <u>supE</u> 44, <u>thi</u> -1, <u>tsx</u> -33, <u>xyl</u> -5	(66)
EC558	= EC559, <u>dnaA</u> 46	(66)
GM119	<u>dam</u> -3, <u>dcm</u> -6, <u>galK</u> 2, <u>galT</u> 22, <u>lacY</u> 1, <u>metB</u> 1, <u>mtl</u> -1(?), <u>supE</u> 44, <u>thi</u> -1(?), <u>tonA</u> 31(?), <u>tsx</u> -78	(67)
WM1332	= GM119 containing pdam118	(68)
WM1151	<u>lacI</u> ^Q L8, <u>recA</u> 56, <u>srlC</u> 300::Tn10	

MATERIALS AND METHODS

Bacteria and plasmids

E. coli strains used are listed in Table 1.

pCM959 is a minichromosome containing exclusively chromosomal DNA from the region of the chromosomal replication origin, oriC, of E. coli (25, 26). pFD51 (27) is a promoter search plasmid derived from the galK vector pK01 (28). For our analysis, a small segment from the cloning region, between the HindIII and the SmaI sites, was deleted. pLSK1-32 is derived from pFD51 by cloning a 205 bp EcoRI fragment from pKB252 (29) containing the lacUV5 promoter into the EcoRI site.

pLSK5, used for overproducing DnaA protein, contains the 1.6 kb HincII-XhoI fragment of the dnaA gene (pos. 824-2437 in the sequence of ref. 30) with the dnaA reading frame and Shine-Dalgarno sequence but without the dnaA promoters, cloned into the SmaI and SalI site of the expression vector pJF118EH (31), behind the tac promoter (32). pLSK5 is able to complement dnaA temperature-sensitive mutations and upon induction of the tac promoter overproduces the DnaA protein substantially.

Cloning procedures

Plasmid preparations, restriction enzyme digests, agarose gel electrophoresis, ligations and transformations were done as described (33).

Assay for galactokinase activity

was done as described (18).

S1 mapping

Cells were grown to a cell density of $4-5 \times 10^8$ /ml, and the total cellular RNA was isolated (34). For S1 mapping of 5' ends restriction fragments (see figure legends) were labeled at their 5' ends using T4 polynucleotide kinase and (γ - 32 P)-ATP, followed by cleavage with a suitable restriction endonuclease and separation on polyacrylamide gels (33). In order to map the 3' ends of the 16 kd transcript, a 597 bp HincII - XhoI fragment and a 410 bp SacII - HindIII fragment were labeled at the 3' end of the XhoI site or the HindIII site, respectively, with (α - 32 P)-dNTP using DNA polymerase Klenow fragment (33), and used as hybridization probes.

S1 mapping was done as described (35) with the following

modifications: 150 µg RNA and 25,000 cpm of the probe were co-precipitated. The pellet was dissolved in 50 µl hybridization buffer. After denaturation at 85° C for 10 min, the samples were hybridized for 3 h at temperatures depending on the GC content of the probes (see figure legends; 33). 500 µl of cold S1 buffer were added, and the samples were digested with S1 nuclease (BRL, Gaithersburg, MA, USA) at 37° C for 30 min. The reaction was stopped with 0.5 M NH₄-acetate, 10 mM EDTA (final concentration). After precipitation with 2-propanol, the nucleic acids were resuspended in 80% formamide, 20%TBE, 0.025% bromophenol-blue and 0.025% xylene cyanol dyes and subjected to electrophoresis in 8% polyacrylamide, 8 M urea, gels in TBE buffer (33).

RESULTS

S1 mapping of the 5' end of the 16 kd transcript

To map the 5' end of the 16 kd transcript RNA was isolated from strain CM993 in the exponential growth phase. CM993 is CM987 carrying the minichromosome pCM959 at 10 copies per chromosome equivalent. The RNA was hybridized to the AvaII-MluI DNA fragment (pos. 619-777; all nucleotide positions refer to the sequence in ref. 21) labeled at the 5' end of the AvaII site (pos. 620) and digested with S1 nuclease. The probe was sequenced (36) and coelectrophoresed with the S1 protected DNA on sequencing gels (Fig. 1A). The predominant band (137 b) comigrated with the T fragment corresponding to pos. 757 when RNA of CM993 (pCM959) was analyzed. 16 kd transcription did not differ qualitatively on the chromosome (strain CM987) and minichromosome (strain CM993). The same protected DNA fragment was obtained with RNA from CM987 and CM993 (data not shown). However, CM993 contained 5 times more 16 kd transcript, which corresponds to the ratio of 16 kd genes on minichromosomes compared to the chromosome (10 copies of pCM959 per chromosome equivalent, and all chromosomes have two copies of the origin region).

Thus transcription of the 16 kd gene starts with an A at pos. 757, in agreement with the result obtained by measuring the length of in vitro transcripts (pos. 755±7; 37).

Transcription of the chromosomal 16 kd gene is negatively regulated by DnaA protein

Repression of the 16 kd promoter by DnaA protein was ob-

Fig. 1. S1 mapping of the 5' end of the 16 kd transcript.

A. RNA from strain CM993 (pCM959) was hybridized at 55° C to a 5'-(³²P)-AvaII-MluI fragment, pos. 619-777, digested with 100 units S1 nuclease, and subjected to electrophoresis on a 8% polyacrylamide-urea gel together with Maxam & Gilbert (36) sequencing reactions of the probe fragment. (a) probe, (b) S1 protected fragment.

B. RNA from strain WM1151 containing pLSK5 was hybridized at 54° C to a 5'-(³²P)-XhoI-MluI fragment, pos. 418-777, and digested with 100 units S1 nuclease. (a) without induction, (b) 3 h induction with 1 mM IPTG starting at $2 \cdot 10^8$ cells/ml, (c) 3 h induction with 1 mM IPTG starting at $4 \cdot 10^7$ cells/ml. Upper arrow: probe; arrow 340: protected fragment.

C. RNA from (c) the dam mutant strain GM119 and from (b) the same strain containing a dam⁺ plasmid (WM1332) were hybridized at 54° to a 5'-(³²P)-XhoII-MluI fragment, pos. 587-777, and digested with 150 units S1 nuclease. (a) marker, (d) probe.

served when this promoter was cloned in front of a marker gene in colEI derived plasmids (16, 18, 19). However, control may be different, at least quantitatively, in high copy number vectors. Therefore, we analyzed the effect of DnaA protein on transcription of the chromosomal 16 kd gene *in vivo*. We compared transcript levels in cells carrying the inducible DnaA overproducing plasmid pLSK5 in an uninduced and induced state. RNA was isolated from uninduced cells in early exponential growth phase and upon induction of dnaA transcription for 3 h with IPTG (1 mM) at two stages of the exponential growth phase (early and middle).

S1 analysis was performed using the XhoI-MluI DNA fragment (pos. 417-777) labeled at the 5' end of the XhoI site. The length of the S1 protected fragment (340 b, Fig. 1B) gives the same 5' end as mapped with the AvaII-MluI probe. Upon induction of the DnaA protein the transcription of the 16 kd gene was drastically repressed (Fig. 1B). Thus, DnaA protein acts as transcriptional repressor of at least two genes, the dnaA gene itself (38-40) and the 16 kd gene.

Absence of dam methylation reduces transcription from the 16 kd promoter

The occurrence of a GATC site in the -35 region of the 16 kd promoter prompted us to investigate the influence of dam methylation on the transcription efficiency from this promoter. RNA was isolated from both a dam mutant strain (GM119) and the same strain carrying a plasmid encoding the dam methylase (WM1332), and subjected to S1 analysis.

The 16 kd transcript level was reduced 2.5-fold when the GATC site was unmethylated (Fig. 1C). As internal standard we used transcript levels from the dnaA gene analyzed in the same RNA preparations. In keeping with previous results (38, 41) the level of the dnaA1 transcript was not changed in the absence of dam methylation (data not shown); like the 16 kd transcript, the dnaA2 transcript was reduced 2.5-fold in dam mutants.

Other promoters containing GATC sequences in the -10 and/or the -35 region analyzed so far, depend differently on methylation of GATC sites. Their activity is enhanced in the unmethylated state (24). The only known exception is the bacteriophage Mu mom promoter, but in this case the GATC sites are located upstream of the promoter (42).

Thus, transcription from the 16 kd promoter shares two properties with transcription of the dnaA gene: i) The negative regulation by DnaA protein; ii) The enhancement of transcription by dam methylation. It was suggested (41), that the dam methylation directly affects the interaction of RNA polymerase with the dnaA2 promoter, because of the reduced activity of the unmethylated versus methylated promoter in an in vitro transcription assay with purified RNA polymerase. A physical interaction of the DnaA protein and RNA polymerase has been proposed because

Fig. 2. S1 mapping of 3' ends of 16 kd transcripts. A. RNA from strain CM987 (1), CM993 (2) and CM1671 (3) were hybridized at 45° C to the 410 base probe (SacII-HindIII fragment, pos. -163 to +248, labeled at the 3' end of the HindIII site), and digested with 300 units S1 nuclease. B. RNA from strain CM993 (1) and t-RNA (2) were hybridized at 50° to the 597 base probe (HincII-XhoI fragment, pos. -177 to +421, labeled at the 3' end of the XhoI site), and digested with 200 units S1 nuclease. The minor bands at 410 bases (A, lane 3, band 1) and at 597 bases (B, lane 2, band 1) are due to probe hybridized to the complementary DNA strand. M: marker. The S1 protected DNA fragments are numbered from 1 to 15 (A) and 1 to 25 (B), respectively. The measured lengths of these fragments and the corresponding coordinates of their 3' ends in the oriC sequence are given in Table 2.

allele specific suppressor mutations of the dnaA (ts) phenotype have been mapped in the rpoB gene (β subunit of RNA polymerase; 43-45). The dnaA box of both promoters is located nearby and upstream of the -35 region. Therefore it seems possible that the effect of dam methylation on transcription is caused by an altered interaction of DnaA protein, RNA polymerase and DNA.

3' ends of transcripts from the 16 kd promoter

The reading frame for the 16 kd protein ends close to the rightward border of the minimal origin (see Fig. 4). In order to evaluate a possible effect of the 16 kd transcript on oriC we analyzed its termination. RNA was prepared from CM993 (pCM959) and from the isogenic plasmid free strain (CM987). Total RNA was hybridized to two probes: 1) A SacII-HindIII fragment (positions -163 to +248) labeled at the 3' end of the HindIII site, and 2) a HincII-XhoI fragment (positions -177 to +421) labeled at the 3' end of the XhoI site. Hybrids were digested with nuclease S1.

Table II. S1 mapping of the 3' ends of the 16 kd transcript

(A) Probe: <u>Sac</u> II- <u>Hind</u> III fragment				(B) Probe: <u>Hinc</u> II- <u>Xho</u> I fragment			
No.	Length (b)	Position	Intensity	No.	Length (b)	Position	Intensity
1	410	<-165	+++	1	597	< -177	+++
2	345	- 97	+	2	517	- 96	+
3	332	- 83	+	3	506	- 85	+
4	312	- 64		4	485	- 64	
5	298	- 50*	++	5+6	465	- 44*	++
6	290	- 42*	++				
7	263	- 15		8	420	1*	
8	249	- 1*	+				
9	225	23*	++	9+10	395	26*	++
10	221	27*	++	11	380	41*	+
11	210	38*	+	12	360	61*	
12	190	58*		13	348	73*	
13	180	68*		14	322	99*	
14	153	95*		15	312	109*	+
15	140	108*	+	16	300	121*	+
				17	291	130*	
				18	285	136	
				19	275	146*	
				20	250	171	+
				21	240	181	
				22	223	198	
				23	216	205	+
				24	206	215	+
				25	135	286	+

* 3' end coinciding with an RNA-DNA junction as mapped in (47).

50-60% of the full length probe was protected by both RNA preparations, demonstrating that more than 50% of the 16 kd transcription traversed the whole minimal origin and reached well into the adjacent gidA gene (Fig. 2). In addition, shorter protected fragments were observed: Four major bands with 3' ends at positions -50 (no. 5), -42 (no. 6), +23 (no. 9) and +27 (no. 10), and minor bands with 3' ends at several discrete positions (Fig. 2, Table 2). The same pattern was observed for the 16 kd transcript originating from the chromosome in the minichromosome free strain. A protected fragment was not found with RNA from strain CM1671 (46) in which the oriC region is deleted (Fig. 2). No substantial differences in the pattern were found when RNA isolated from dnaA mutants grown at 42° C was used in the S1 mapping (data not shown). Many of the termination sites observed

Fig. 3. S1 mapping of 5' ends of origin transcripts.

A. Transcripts from *Pori-r*. RNA was hybridized at 55° C to the probe shown, and digested with 100 units S1 nuclease. (1) marker, (2) probe, (3) RNA from strain EC558 (shifted for 2 h to 42° C) and (4) EC559 containing pLSK7-17 (with PlacUV5), (5) RNA from strain EC558 (42° C) and (6) EC559 containing pLSK2-27 (without PlacUV5).

B. Transcripts from *Pori-l*. RNA was hybridized at 55° C to the probe fragment shown in the lower part of the figure, and digested with 100 units S1 nuclease. (1) marker, (2) probe, (3) RNA from EC558 (grown at 30° C) containing pLSK56-17 (with PlacUV5) or (4) pLSK3-27 (without PlacUV5).

within the minimal origin coincide with termination sites found in a similar analysis (12) and are close to RNA-DNA transition sites mapped *in vivo* (47; Table 2).

S1 mapping of the 5' ends of the origin transcripts

First attempts to identify the origin promoters *Pori-r* and *Pori-l* *in vivo* have been done by fusing various DNA fragments from the origin region to the galactokinase gene. Only weak *galK* activity (about 1/30 of PlacUV5 activity, data not shown; 10), which was only slightly above background, has been detected by

this method. Therefore, we used the more sensitive S1 mapping technique in order to analyze whether the origin promoters defined so far only in vitro (7) are active in vivo.

Pori-r is contained in a HindIII-BclI fragment (pos. 244-356) from oriC, Pori-l in a HindIII-BamHI fragment (pos. 244-92). As templates for the S1 mapping we fused these fragments to the galK gene of pFD51, yielding pLSK2-27 (Pori-r) and pLSK3-27 (Pori-l), respectively. In addition, the lacUV5 promoter was inserted upstream of the origin promoters in these plasmids, resulting in pLSK7-17 (PlacUV5-Pori-r) and pLSK56-17 (PlacUV5-Pori-l), respectively (Fig. 3). Total cellular RNA was prepared from strain EC559 transformed with one of these galK fusion plasmids. The RNA was hybridized to a corresponding PvuII-EcoRV DNA fragment (shown in Fig. 3), derived from pLSK7-17 or pLSK56-17, respectively, ³²P-labeled at the 5' end of the EcoRV site in the galK gene, and digested with S1 endonuclease.

With plasmids containing both PlacUV5 and an origin promoter transcripts originating both from the lac promoter and from the origin promoter were found. With plasmids containing only oriC DNA in front of the galK gene the origin transcripts were seen at lower intensity. Protected fragments had lengths of 235 bases (Pori-l), and 199 + 209 bases (Pori-r), respectively (Fig. 3). The transcript starts were determined precisely by electrophoresis next to Maxam & Gilbert (36) sequencing lanes. The ori-r transcript started with about equal efficiency with U at position 294 or 295 and with U at position 304 or 305. The transcript originating from Pori-l was found to start with U at position 177 or 178 (data not shown).

The transcript starts obtained in vivo by S1 mapping deviate slightly from those determined by RNA sequence analysis of in vitro transcripts (7), probably due to the difficulty in reading the early part of the RNA sequence gel caused by secondary structure.

To analyze the influence of the DnaA protein on transcription originating at Pori-r and Pori-l S1 mapping was also performed with RNA prepared from the dnaA(Ts) mutant strain EC558 containing the galK fusion plasmids, after shift to the non-permissive temperature in the exponential growth phase. There was

Table III. Transcription termination within oriC

Plasmid	Inserted DNA fragment ^{a)}	GalK activity units ^{b)}	% of <u>Plac</u> activity
pLSK1-32	none	148	100
pLSK10-28	<u>HindIII</u> - <u>AccI</u> (244) (285)	325	220
pLSK7-17	<u>HindIII</u> - <u>BclI</u> (244) (352)	64	43
pLSK16-17	<u>HindIII</u> - <u>XhoI</u> (244) (417)	25	17
pLSK2-22	<u>HindIII</u> - <u>AluI</u> (244) (165)	85	57
pLSK56-17	<u>HindIII</u> - <u>BamHI</u> (244) (92)	56	38
pLSK78-17	<u>HindIII</u> - <u>BglII</u> (244) (38)	35	24
pLSK44-32	<u>HindIII</u> - <u>BamHI</u> (244) (1)	11	7

a) DNA fragments derived from pCM959 were inserted between PlacUV5 and galK in pLSK1-32

b) Measured in strain EC559.

no difference in the amount of S1 protected DNA fragment in the two strains (Fig. 3A). Therefore, a regulatory effect of the DnaA protein on the origin promoters is unlikely.

Termination of origin transcripts

Promoters within and close to oriC were first identified by mapping 5' ends of run off transcripts (7). The analysis of such transcripts suggested that transcription termination or pausing (48) might occur close to the origin promoters Pori-r and Pori-l, since some transcripts could neither be interpreted as run off transcripts nor as transcripts covering the complete restriction fragment.

In order to test the terminator activity in the origin region in vivo, various restriction fragments were inserted into the vector pLSK1-32 between the lacUV5 promoter and the galK gene. All fusion plasmids had the same copy number as determined by a modification of the method described in (49). Therefore, a correction of galK activities for copy numbers was not necessary. Insertion of the HindIII-AccI fragment (pos. 244-285, containing part of Pori-r) between the lac promoter and galK resulted in a substantial increase in galK activity (Table 3). No

Fig. 4. Region of the *E. coli* replication origin.

Relevant restriction sites and coding regions are shown. Transcripts are represented by wavy lines, *dnaA* boxes and their orientation by filled bars, the gyrase binding site by a dashed bar.

such increase in activity was observed with fragments containing *Pori-l*.

In both directions successively lower *galK* activities were observed with increasing lengths of the fragments (Table 3). The major terminator for leftward transcription, *Tori-l*, was found to be located between the *BglII* site (pos. 38) and the *BamHI* site (pos. 1; compare pLSK78-17 and pLSK44-32 in Table 3). This termination coincides with a prominent termination site seen in the S1 mapping of 16 kd transcripts. Major termination for the *ori-r* transcript was found between the *BclI* site (pos. 352) and the *XhoI* site (pos. 417; compare pLSK7-17 and pLSK16-17 in Table 3). *In vitro* data (not shown) suggest that the major rightward terminator is located around position 406. We designate this terminator *Tori-r*.

DISCUSSION

A complicated array of overlapping transcription events was observed in the *E. coli* replication origin, *oriC* (Fig. 4). A transcript coding for a 16 kd gene was found to start *in vivo* at position 757 and transcribed toward and through the minimal origin. Two promoters within *oriC*, *Pori-l* and *Pori-r*, so far identified only *in vitro* (7), were found to promote transcription

starts in vivo at position 177/178 for Pori-l and at positions 294/295 and 304/305 for Pori-r.

Control of transcription in the origin region

The 16 kd transcript was synthesized in proportion to the number of gene copies in the cell. On high copy number vectors the promoter is negatively controlled by DnaA protein (16, 18, 19). S1 mapping employed here demonstrated that this is also true for the chromosomal 16 kd gene, and that repression by DnaA was nearly complete at elevated levels of DnaA protein (Fig. 1B). 16 kd transcription was enhanced in the presence of dam methylation (Fig. 1C). The same dependence was shown for the transcription of the dnaA gene from the dnaA2 promoter (38, 41).

The in vivo activity of the origin promoters Pori-l and Pori-r was at least 30 fold lower than the activity of the lacUV5 promoter (Fig. 3), in contrast to the in vitro situation where comparable transcription efficiencies for all promoters from the origin region were found (7, 37). This suggests that these promoters are very tightly controlled in vivo. Corroborating this interpretation we found substantially higher transcript levels originating from both origin promoters when these promoters were cloned downstream of a lacUV5 promoter (Fig. 3), possibly due to removal of a regulatory protein (repressor) by transcription from PlacUV5.

We found no indication for a regulatory effect of DnaA protein on the origin promoters Pori-l and Pori-r, although there are DnaA binding sites (dnaA boxes; 13) in both promoter regions. However, we cannot rule out that such a regulation is obscured by the high copy number of the plasmids carrying the origin promoters.

Termination of transcripts in the origin region

For both origin promoters we localized transcription termination sites. In an in vitro transcription system a strong termination signal was observed for the rightward transcript originating from Pori-r around position 406. Termination in vivo was more than 90 % effective for transcripts transcribed from the same DNA segment but originating from the lacUV5 promoter (and possibly Pori-r), using the galK assay (Table 3). We designate this terminator Tori-r.

Termination of leftward transcripts, originating from Pori-1, was in vivo progressively reduced at the BamHI (pos. 92), BglII (pos. 38) and BamHI (pos. 1) sites (Table 3). The most effective termination in this region was found between the BglII (pos. 38) and the BamHI (pos. 1) sites, respectively (Table 3). A major termination site for the 16 kD transcript was mapped in the same region, around the BglII site at position 22 (Table 2). We designate this region Tori-1. The net result for both, the rightward and the leftward origin transcription units are RNA molecules with lengths of 100 - 150 bases, and shorter ones. Leftward transcription termination in the same region was recently demonstrated by inserting oriC derived DNA fragments between the galK promoter and the galK gene (10, 16).

The transcripts from the DnaA regulated 16 kd promoter were found to enter the minimal origin. More than 50 % of these transcripts traversed the whole minimal origin (Figs. 2 and 4, Table 2) and are thus - unlike transcripts originating from the lacUV5 promoter - not terminated efficiently at Tori-1. Of the 25 termination sites found 11 are at the same positions as those found in a recent report (12). In our analysis we found in addition 7 termination sites to the left outside the minimal origin. Many of the 3' ends found for the 16 kd transcript (13 out of 25; see Table 2) within and outside the minimal origin coincide with RNA-DNA transition sites mapped in vivo (47, 50; Table 2).

Possible functions of the origin transcripts

Both temperature sensitive mutations in the RNA polymerase (51, 52) and the dnaA gene (53-57) lead at intermediate temperatures to an altered DNA concentration (origin/mass ratio) in the cell, indicating that both proteins are involved in controlling the rate of initiation at oriC. Primer synthesis in in vitro replication can be mediated by either RNA polymerase alone (58) or by DNA primase alone (59). However, in vivo RNA polymerase is absolutely required for initiation (3, 4). DnaA regulated transcription of the 16 kd gene into and through the minimal origin could be part of the regulatory circuit in timing of initiation.

Transcription, but not the expression of the 16 kd gene is important. The 16 kd gene is translated (9, 60), but no function could yet be assigned to the protein. It seems not to be an

essential protein, neither for DNA replication nor for other cellular functions (16, 46).

The importance of 16 kd transcription and its regulation by DnaA protein is supported by the following observations: i) Initiation of in vitro replication of minichromosomes is preceded by DnaA dependent repression of transcription only if the 16 kd gene is present on the template (18). ii) Sequences in the 16 kd region are required for bidirectional replication in vivo (11). iii) Transposon insertions in the chromosomal 16 kd gene change the initiation frequency in slowly growing cells (17). iv) The presence of the 16 kd promoter including the dnaA box on oriC-pBR322 joint replicons confers incompatibility towards minichromosomes (14-16) as well as towards the chromosome in dnaA(Ts) mutants (16, 17). v) Deletion of the 16 kd promoter or alterations in the promoter region or the dnaA box result in a reduced copy number of minichromosomes (our unpublished results; 16, 61), as does the insertion of a transcriptional terminator between the 16 kd promoter and the minimal origin in the terminating but not in the reverse orientation (15, 16). vi) The similarity in control of the 16 kd promoter and the dnaA promoters by DnaA protein and by dam methylation is intriguing. This is especially true in view of the observation that initiation at oriC in vivo requires the methylation of GATC sites (22, 23).

Termination of leftward (counterclockwise) transcription from the 16 kd promoter and from heterologous promoters, PlacUV5 or PgalK, was found to occur at the same positions (19). Therefore, the specificity but not necessarily the efficiency (compare Fig. 2 and Table 3) of termination resides in the DNA segment from oriC. Counterclockwise leading strand DNA synthesis was found to start in vitro in the same segment (62), and termination sites coincide at the nucleotide level with RNA-DNA transition sites found in vivo (see above). Therefore, the ori-1 transcript and/or the 16 kd transcript are candidates for priming leading strand counterclockwise synthesis in vivo.

If the 16 kd transcript is used as a primer, backup systems for priming must exist since initiation is possible, albeit at a reduced rate, in the absence of 16 kd transcription.

Such a backup system could be the ori-l transcript and/or DNA primase. The rightward transcript from Pori-r is a countertranscript to parts of the 16 kd transcript. This situation is very reminiscent to the colE1 origin region (63) and other plasmid systems except that the Pori-r transcript is present in vivo in small amounts only.

In view of these restrictions, we think it is more likely that Pori-l and Pori-r are responsible for the synthesis of primers for both leading strands of bidirectional DNA synthesis. The 16 kd transcript may be used for transcriptional activation of the origin. In addition to the negative regulatory role of DnaA protein at the 16 kd promoter and the dnaA promoter DnaA protein is required positively at oriC (64), mediating the assembly of a replication complex (65). Transcription from P16kd through oriC may increase the binding efficiency of DnaA protein to the four dnaA boxes in the origin. Thus a stimulation of binding of DnaA protein to oriC at low DnaA protein concentrations due to increased transcription from the 16 kd promoter may be successively reduced with increasing DnaA protein concentration. Such a homeostatic control of DnaA protein binding to oriC is a testable hypothesis for the mechanism of transcriptional activation of oriC.

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