The lytic replicon of bacteriophage P1 is controlled by an antisense RNA

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ABSTRACT

The lytic replicon of phage P1 is used for DNA replication during the lytic cycle. It comprises about 2% of the P1 genome and contains the P1 C1 repressor-controlled operator–promoter element Op53, the kilA gene and the repL gene, in that order. Transcription of the lytic replicon of P53 and synthesis of the product of repL, but not kilA, are required for replicon function. We have identified an additional promoter, termed P53as (antisense), at the 5'-end of the kilA gene from which a 180 base transcript is constitutively synthesized and in the opposite direction to the P53 transcript. By using a promoter probe plasmid we show that transcription from P53 is strongly repressed by the C1 repressor, whereas that of P53as remains unaffected. Accordingly, the C1 repressor inhibits binding of *Escherichia coli* RNA polymerase to P53, but not to P53as, as shown by electron microscopy. Under non-repressed conditions transcription from P53 appears to be inhibited by P53as activity and *vice versa*. An inhibitory effect of P53as on the P1 lytic replicon was revealed by the construction and characterization of a P53as promoter-down mutant. Under non-repressed conditions transcription of repL and, as a consequence, replication of the plasmid is strongly enhanced when P53as is inactive. The results suggest a regulatory role for P53as on the P1 lytic replicon.

INTRODUCTION

The temperate phage P1 encodes two replicons for the replication of its genomic DNA. One, the lytic replicon, is active during the lytic cycle and requires one phage-encoded protein, RepL. The other, the prophage replicon, is responsible for maintenance of the P1 plasmid prophage and requires another phage-encoded protein, RepA. The two replicons are located about 12 map units apart on the 100 kb circular genome of prophage P1 (1) and can act independently of each other. The prophage replicon as a recombinant plasmid is controlled by the phage *incA* locus and depends on the DnaA protein of the host, but is unaffected by the phage repressor for lytic functions, C1. The lytic replicon, on the other hand, is independent of the RepA and DnaA proteins and is not affected by *incA*. It directs high copy number replication of an otherwise replication-defective λ vector (for all but selected references on P1 and P7 see 2,3).

The components of the P1 lytic replicon (Fig. 1) are: (i) a promoter, P53, which is regulated via the C1 repressor-controlled operator Op53 and whose activity is essential for replicon function; (ii) a promoter proximal gene kilA, whose product is not essential for replicon function, but which is lethal to the bacterial cell; (iii) a promoter distal gene region, which encodes the RepL protein and contains an as yet unknown origin of replication (4–6). Recombinant plasmids whose replication solely depends on the PI lytic replicon can only be maintained in a bacterial cell when the kilA gene is knocked out. Such plasmids, in turn, can no longer replicate when promoter P53 is shut off by the action of the C1 repressor (5). Transcription is repressed by binding of C1 to the operator Op53, whose asymmetric 17 bp sequence overlaps the promoter (7,8; Fig. 2). Thus binding of C1 prevents access of *Escherichia coli* RNA polymerase to the promoter. Transcription of repL is strictly required for the lytic replicon to be active. However, the promoter function of C1-regulated P53 can be replaced by other promoters (5,6). For example, when the inducible *lacZ* promoter of *E.coli* is used instead of P53, the extent of replication was shown to be proportional to the promoter activity. Surprisingly, however, the C1/P53-regulated replicon appears to be significantly more stable than the *lacZ* promoter-regulated replicon, although the copy number of the latter replicon is higher than is the copy number of the C1/P53-regulated replicon (5).

In the course of our studies on the C1 repressor-controlled operators of P1 we detected a second promoter in close proximity to the Op53-P53 element. We term this promoter P53as (for antisense) and its properties are described here. P53as is located at the 5'-end of the kilA gene and constitutively initiates transcription in the opposite (antisense) direction to that from P53 (Figs 1 and 2). We show that the transcriptional activity of P53as is essential for establishment of a plasmid whose replication is driven by the P1 lytic replicon. Since the *lacZ* promoter-regulated P1 replicon was originally constructed by deleting the region of promoters P53 and P53as (5), the instability of this replicon may be due to the absence of antisense transcription. These results suggest a crucial role for P53as in the stability of the natural P1 replicon.

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Figure 1. P1 lytic replicon and plasmids used. (Center) The P1 lytic replicon is located downstream of the anti-repressor gene ant of the P1 immi operon and in a clockwise orientation on the P1 genetic map (1). It comprises the Cl repressor-controlled Op53 • P53 element (dot and triangle), the promoter P53as (this paper) and the genes kilA and repL (grey, arrowheaded bars). Arrowheaded wavy lines indicate the direction and maximal size of transcripts found. Only relevant restriction enzyme cleavage sites are shown. Their numbering is explained in the legend to Figure 2. (Upper and lower) P1 DNA inserts in vector DNAs are shown by horizontal lines. Numbers indicate the corresponding restriction enzyme cutting sites. The exceptions are the BglII deletion end points at positions 146 and 185 (see Fig. 2). A 426 bp deletion in the kilA gene is indicated by the interrupted line. The reading frame is not altered in the resulting truncated kilA* gene. Vectors used are pT7-6 (pAH series), pCB302a (pCB series) and pSU2719 (pSU series). a and b indicate opposite orientations of the P1 DNA insert in the vector (see Tables 1 and 2). The repL probe was used for Southern and Northern blotting. Single strand-specific RNA probes were prepared from the plasmids pAH1067a and pAH1067b as described in Materials and Methods. Plasmid pT020 is replicated via P53 • P53as-regulated synthesis of RepL protein.

Figure 2. Nucleotide sequence of the regulatory region of the P1 lytic replicon. Nucleotides are numbered from 1 (HpaI recognition site) to 360 in the kilA gene (5). The HpaI site contains the termination codon, TAA, of the ant gene (9). Nucleotide positions upstream of the HpaI site are indicated by negative numbers. Sequences of the ant and the kilA genes are framed. Upstream of kilA is a potential ribosome binding site (rbs) and the promoter P53, whose -10 and -35 regions are indicated by brackets (5). The -10 region of P53 overlaps the operator Op53 (boxed sequence). A 13 bp palindromic sequence further upstream is indicated by arrows below the sequence. The -10 and -35 regions of the promoter P53as are located at the 5'-end of the kilA gene. The base substitution in the P53as2 promoter-down mutation is marked by an arrow. The +1 positions of P53as and P53 are indicated by filled triangles and were determined by a primer extension assay (see Fig. 4). GATC dam methylation sites are shown by grey areas.

MATERIALS AND METHODS

Bacterial and phage strains

The E.coli K12 strains used were CB454 Δ lacZ galK recA56 (10) and WM874 ara Δ(lac-pro) thi (original name CSH 26; 11). WM874 dam' was prepared by transduction of WM874 with T4GT7 phage (12) which had been grown in E.coli GM2199 dam' (13). Phages used were P1 Cm, P1 Cm c1.100 (14) (abbreviated to P1 and P1 c1ts respectively) and P1 c4.32 ant17 (9,15).

Plasmid constructions

Vectors used for the construction of plasmids, presented in Figure 1 and Table 1, were pT7-6 (S. Tabor and C. C. Richardson, personal communication), pJF118EH (16), pCB302a (10) and pSU2719 (17). Plasmid pAM2b c1+coi+ contains the 2.27 kb PvuII-BclI subfragment of P1 EcoRI fragment 7 with the c1 gene, its control region and the coi gene (18) inserted into a 2.5 kb DraI fragment of the vector pKT101 Km' (19). It was used to supply the Cl repressor. Plasmid pAM8 c1+coi+lex+ is a coi-defective derivate of pAM8 c1+coi+lex+(vector pKT101 Km') (19) that additionally contains the co-repressor lex gene (20). The coi gene
was inactivated by insertion of two bases at the AccI site (18). The plasmid was used to supply the C1 repressor and Lox co-repressor. Plasmid pAM8 c1ts colP lac+ is a derivative of pAM8 c1ts colP lac+ in which the P1 BglII–PstI fragment containing the C-terminal part of c1+ is substituted by the analogous fragment of the temperature-sensitive mutant P1 c1.100(14,21). It was used to supply the C1s repressor and Lox co-repressor.

Plasmids pAH1027 and pT020 contain a P1 lytic replicon and were constructed in the following way. A 16 kb Smal–HindIII fragment of P1 c1s, extending from map position 48 to 64 (1) was inserted into pT7-5 (SmalHindIII) to yield plasmid pAH16 (not shown). Next, a HpaII–HpaII fragment from pAH16 was inserted into pT7-6 (Smal) to yield pAH1026 (Fig. 1). Because of the lethal effect of KilA on the bacterial cell, pAH16 and pAH1026 can only be maintained in bacteria carrying the plasmid pAM8 c1ts colP lac+, which supplies the C1 repressor and Lox co-repressor. To inactivate the kilA gene we followed the procedure of Sternberg and Cohen (5) in deleting an AsnI-AsnI fragment from pAH1026 to yield plasmid pAH1027 (Fig. 1). The latter contains the ColEl replicon of the vector pT7-6, in addition to the kilA-truncated P1 lytic replicon. To eliminate the ColEl replicon, a 2226 bp BamHI–Scal fragment of P1H111 containing the lytic replicon was ligated to a 847 bp Scal–BamHI fragment of pRF118EH to yield plasmid pT020 (Fig. 1). Replication of pT020 solely depends on the kilA-truncated lytic replicon of P1. To test the promoter activity of P53 and P53as, the P1 DNA fragments (Fig. 1, lower part) were blunt-end ligated to Smal (pCB302a)- or HincII (pSU2719)-linearized vector DNA respectively. Fragments with sticky ends were treated with T4 DNA polymerase beforehand.

In vitro mutagenesis

The P53as promoter-down mutant was constructed by oligonucleotide-directed mutagenesis using double-stranded DNA (22), for which plasmid pAH1020 was used, which contains the P1 HaeIII–EcoRI fragment (Fig. 1) in pT7-6 (SmalEcoRI). The plasmid was then mutagenized using the oligonucleotide 5'-GTTGAAGGATCAACATTTTG-3'. This oligonucleotide is for which plasmid pAH1020 was used, which contains the PI c1s, extending from map position 48 to 64 (1) was inserted into pT7-5 (SmalHindIII) to yield plasmid pAH16 (not shown). Next, a HpaII–HpaII fragment from pAH16 was inserted into pT7-6 (Smal) to yield pAH1026 (Fig. 1). Because of the lethal effect of KilA on the bacterial cell, pAH16 and pAH1026 can only be maintained in bacteria carrying the plasmid pAM8 c1ts colP lac+, which supplies the C1 repressor and Lox co-repressor. To inactivate the kilA gene we followed the procedure of Sternberg and Cohen (5) in deleting an AsnI-AsnI fragment from pAH1026 to yield plasmid pAH1027 (Fig. 1). The latter contains the ColEl replicon of the vector pT7-6, in addition to the kilA-truncated P1 lytic replicon. To eliminate the ColEl replicon, a 2226 bp BamHI–Scal fragment of P1H111 containing the lytic replicon was ligated to a 847 bp Scal–BamHI fragment of pRF118EH to yield plasmid pT020 (Fig. 1). Replication of pT020 solely depends on the kilA-truncated lytic replicon of P1. To test the promoter activity of P53 and P53as, the P1 DNA fragments (Fig. 1, lower part) were blunt-end ligated to Smal (pCB302a)- or HincII (pSU2719)-linearized vector DNA respectively. Fragments with sticky ends were treated with T4 DNA polymerase beforehand.

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RESULTS

Two RNA polymerase binding sites exist in the control region of the P1 lytic replicon

We became aware of the existence of a second promoter in the Op53-P53 control region of P1 (Fig. 1) when we studied interference by the C1 repressor with binding of RNA polymerase to the known promoter P53 by electron microscopy. Two RNA polymerase binding sites were found on a 341 bp HaeIII fragment comprising the 3'-end of the ant gene, the Op53-P53 control region and the 5'-end of the kilA gene (Figs 1 and 2). The two sites can only be distinguished when the HaeIII fragment is subdivided by TaqI into two HaeIII–TaqI subfragments, 188 and 153 bp in size. The subfragments were inserted into Smal-linearized pCB302a, yielding plasmids pCB192a and pCB147b respectively (Fig. 1). As expected, binding of RNA polymerase to the 188 bp insert in pCB192a can be inhibited by the C1 repressor (Fig. 3, left half), because the overlapping Op53-P53 control element is located within the region (Figs 1 and 2). However, binding of RNA polymerase to the 153 bp insert in pCB147b was not affected by the C1 repressor (Fig. 3, right half). This RNA polymerase binding site represents a constitutive promoter which we term P53as (Figs 1 and 2). The additional three to four RNA polymerase binding sites (Fig. 3) are all located within the vector part of the recombinant plasmids.

Two promoters, P53 and P53as, initiate transcription in opposite and convergent directions

We verified the existence of the two promoters P53 and P53as, arranged in opposite directions to each other, by two methods: (i) the +1 transcript initiation sites were determined by primer extension of in vitro transcripts from appropriate plasmids (Fig. 4); (ii) transcriptional activities were determined by measuring β-galactosidase activity using the promoter probe plasmid pCB302a (Table 1). Transcripts from promoter P53 were found to start at three positions located in close proximity to each other. The transcriptional activity of P53 on supercoiled pSU20A3 DNA (Fig. 1) is comparable with that on linearized pSU20A3 DNA. Both are strongly inhibited in the presence of the C1 repressor (Fig. 4, left half). Transcripts from P53as, on the other hand, start at a unique position and transcription is not inhibited by the C1 repressor, but is strongly stimulated when linearized instead of supercoiled pSU20A5 DNA (Fig. 1) is used (Fig. 4, right half). These findings are discussed in detail below. The data are supported by the results of the β-galactosidase assay (Table 1). In this assay the enzyme activity was measured with recombinant promoter probe plasmids.
carrying the promoters P53 or P53as respectively (pCB192a and pCB147b; Fig. 1) or both promoters together (pCB20a and pCB20b; Fig. 1). In E. coli CB454, P53 promoter transcription from pCB192a is strongly inhibited in the presence of both the C1 repressor and the Lxc co-repressor (supplied from the P1 prophage), but less strongly in the presence of C1 alone (supplied from plasmid pAM2b). In contrast, P53as transcription from pCB147b remains unaffected by C1, with or without Lxc. The repressibility of sense transcription from P53 and constitutive antisense transcription from P53as are also observed when the promoter probe plasmids pCB20a and pCB20b are used (Table 1).

Table 1. Transcriptional activities of P53 and P53as

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>E. coli CB454</th>
<th>E. coli WM874</th>
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<tbody>
<tr>
<td>pcB192a</td>
<td>+30</td>
<td>+30</td>
</tr>
<tr>
<td>pcB192b</td>
<td>+20</td>
<td>+20</td>
</tr>
<tr>
<td>pcB193a</td>
<td>-20</td>
<td>-20</td>
</tr>
<tr>
<td>pcB193b</td>
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</tr>
<tr>
<td>pcB20a</td>
<td>+10</td>
<td>+10</td>
</tr>
<tr>
<td>pcB20b</td>
<td>+20</td>
<td>+20</td>
</tr>
</tbody>
</table>

Escherichia coli strains of the CB454 and the WM874 series were transformed with one of the plasmids shown in the first column. Relevant P1 immunity genes, which are present on the prophage or plasmid of the recipient strain, are indicated. The letters a and b of the plasmid (first column) mark the relative position of the lacZ gene (arrowhead, second column) for C1 DNA fragments inserted into the promoter probe vector. Bacteria were grown at 37°C. At A600 = 0.5, bacteria were prepared for the β-galactosidase assay as described (11). Activity is expressed as Miller units.

It is striking that the sequence 5'-GATC-3', which is recognized specifically by the methylase coded for by the bacterial dam gene (30), occurs three times close to and within the −35 region of P53 and once overlapping the −35 region of P53as (Fig. 2). 6-Methyladenosine (6-meAde) is the product of the reaction catalyzed by the bacterial methylase and a lack of 6-meAde has been correlated with decreased replication of replicons (30). In E. coli WM874 dam −, P53 promoter transcription from pCB192a and pCB279a is 3- to 4-fold stronger than in the corresponding dam + strain. In contrast, P53as transcription is not affected by methylation when P53as is the only promoter on the plasmid (pCB147b), however, an ~2-fold reduction is found when P53 is present on the same plasmid (pCB279b) (Table 1). We assume that this is due to the increase in activity of P53, which negatively affects P53as transcription.

Promoter P53as is active in non-induced and induced P1 prophage

To determine the transcripts in the Op53-P53 control region of an intact P1 genome, the P1 wild-type and a P1 cts lysogen were used. Northern blot analysis using single-stranded sense and antisense RNA probes revealed two sets of transcripts. (i) A 180 base long antisense RNA is found only upon induction of the P1 cts prophage (Fig. 5). (ii) A 140 base long sense RNA in the P1 wild-type and a P1 cts lysogen at 28°C, the amount of which increases dramatically during P1 cts prophage induction (Fig. 5, left half). As expected, synthesis of this antisense RNA is initiated at the promoter P53as and terminates in the palindromic terminator structure, as judged from its size (Fig. 2). No such RNA is observed in a non-lysogen. (ii) Sense transcripts ranging in size from 240 to 2300 bases are found only upon induction of the P1 cts prophage (Fig. 5, right half). Most probably they represent the mRNAs of the whole repL operon, the...
and Taql-HaeUl described in Materials and Methods. The 153 bp activity by a factor of -1.5 (Table 2).

Mutation in pCB279a, on the other hand, leads to an increase in P53 activity to -15%. The same mutation in pCB147b reduces P53as activity to -15%. As judged by (J-galactosidase activity, the P53as promoter-down mutation in pCB302a driven by the ColEl replicon. In both plasmids the lethal effect of pAH1027 carries a ColEl replicon on its pT7-6 vector DNA, in addition to the PI lytic replicon. Therefore, the function of the latter depends on the PI lytic replicon, with an (unknown) origin of replication which appears to reside in the repL region, between the CiaI and HpaI cleavage sites (5; Fig. 1). Transcription from P53 is a prerequisite for the PI lytic replicon to function. Since the Cl repressor blocks the P53 promoter, plasmid pT020 cannot be maintained in cells containing the C1 repressor. However, plasmid pAH1027 carries a ColEl replicon on its pT7-6 vector DNA, in addition to the PI lytic replicon. Therefore, the function of the latter can be turned off by repressor C1 without impairing replication driven by the ColEl replicon. In both plasmids the lethal effect of KilA on the bacterial cell is prevented by deleting 52% of the kilA gene, without impairing the reading frame.

Next we constructed a P53as promoter-down mutation as described in Materials and Methods. The 153 bp Taql–HaeIII and the 279 bp Hpal–HaeII DNA fragments of P1 carrying this mutation were inserted into the promoter probe plasmid pCB302a to yield the plasmids pCB147b and pCB279a respectively (Fig. 1). As judged by β-galactosidase activity, the P53as2 promoter-down mutation in pCB147b reduces P53as activity to -15%. The same mutation in pCB279a, on the other hand, leads to an increase in P53 activity by a factor of -1.5 (Table 2).

We then tried to insert the P53as2 promoter-down mutation into plasmids pAH1027 and pT020 by replacing the BstXI131–BstXI346 wild-type fragment with the corresponding mutant fragment of plasmid pAH1020-2. Since the two BstXI recognition sequences differ from each other (Fig. 2), reinsertion of the fragment is only possible in the natural orientation. The ligation mixtures were used to transform WM874 and WM874/pAM8 c1*cor-lac+ bacteria. Only recombinants from mixtures of pAH1027 and pAH1020-2, but not of pT020 and pAH1020-2, yielded transformants of C1 repressor-containing bacteria. Transformants of WM874 bacteria were not obtained. The C1 repressor requirement of the recombinant plasmid was proven by repeating the transformation. Compared with plasmid pAH1027 (wild-type), mutant plasmid pAH1027-2 again yielded transformants only of C1 repressor-containing bacteria. Transformants of WM874 bacteria were not obtained. The requirement of C1 repressor and Lxc co-repressor for the maintenance of plasmid pAH1027-2 was studied at 42°C under C1-repressed and C1ts-derepressed conditions (Fig. 6). As expected, antisense RNA from P53as is only found in plasmid pAH1027 and not in pAH1027-2. In the presence of C1 wild-type repressor, lytic replicon transcription is slightly derepressed in pAH1027-2 when compared with pAH1027, but the mutation has no significant effect on replication of pAH1027-2. In contrast, under derepressed conditions lytic replicon transcription and replication of pAH1027-2 increases ~10- and 3-fold respectively, when compared with plasmid pAH1027. At the same time, replication of plasmid pAM8 is reduced about 2-fold (Fig. 6). We conclude from these results that it is the function of promoter P53as in controlling the PI lytic replicon. To find out what this function might be, C1-controlled transcription and replication of pAH1027 and pAH1027-2 were studied at 42°C under C1-repressed and C1ts-derepressed conditions (Fig. 6). As expected, antisense RNA from P53as is only found in plasmid pAH1027 and not in pAH1027-2. In the presence of C1 wild-type repressor, lytic replicon transcription is slightly derepressed in pAH1027-2 when compared with pAH1027, but the mutation has no significant effect on replication of pAH1027-2. In contrast, under derepressed conditions lytic replicon transcription and replication of pAH1027-2 increases ~10- and 3-fold respectively, when compared with plasmid pAH1027. At the same time, replication of plasmid pAM8 is reduced about 2-fold (Fig. 6).

Table 2. Effect of a P53as promoter-down mutation on the transcriptional activities of P53 and P53as

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>β-Galactosidase activity (U)</th>
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<tbody>
<tr>
<td>P53as</td>
<td>730</td>
</tr>
<tr>
<td>P53as2</td>
<td>110</td>
</tr>
<tr>
<td>pCB147b</td>
<td>800</td>
</tr>
<tr>
<td>pCB279a</td>
<td>1210</td>
</tr>
</tbody>
</table>

Escherichia coli CB454 was transformed with the plasmids pCB147b or pCB279a, which carry the P53as wild-type or P53as2 promoter-down mutant. Growth of bacteria and β-galactosidase assay were done as described in the legend to Table 1.

Table 3. Requirement of C1 repressor and Lxc co-repressor for the maintenance of plasmid pAH1027-2

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>WM874</th>
<th>WM874/pAM8 c1*cor-lac+</th>
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<tbody>
<tr>
<td>pAH1027</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>pAH1027-2</td>
<td>&lt;0.02</td>
<td>11</td>
</tr>
</tbody>
</table>

*100% = 4200 transformants
of 31 and only seven codons respectively, neither initiation codon is preceded by a potential ribosome binding site. Moreover, no protein was detected upon expression of a P1 DNA EcoRII:14 fragment (positions -1226 to +657) containing expression vector pT7-6 or ptac in which the T7<sub>φ10</sub> or the tac promoter is superimposed on P53as.

The identification and characterization of promoter P53as reveals a second control element of the P1 lytic replicon aside from the C1-regulated operator–promoter element Op53-P53. Both elements have a negative effect on transcription of the P1 lytic replicon, apparently without affecting each other. Stimulation of P53 activity by methylation (Table 1), on the other hand, indicates that synthesis of repL protein is related to replication of the P1 DNA. What might be the function of the P53as transcript? Keeping in mind that P53as activity is much stronger on linear than on supercoiled DNA (Fig 4), we suggest that transcription from P53as serves to down-regulate P1 DNA replication at two stages of the P1 life cycle: (i) at the very beginning, upon infection, before the linear DNA is circularized by the phage-specific lox–cre recombination system (3); (ii) during the late stage of infection, when replication of supercoiled P1 DNA is superceded by rolling circle replication and linear P1 DNA concatemers accumulate (32). In accordance with this hypothesis is the finding that the amount of P53as transcript increases strongly in the late stage of P1 development (Fig. 5, left part). In contrast, the amount of repL transcripts, which is roughly the same at 10 and 30 min after induction (Fig. 5, right part), is expected to decrease per P1 DNA molecule.

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