1468–1474 Nucleic Acids Research, 1995, Vol. 23, No. 9

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

Jochen Heinrich, Hans-Dieter Riedel+, Beate Rückert, Rudi Lurz and Heinz Schuster*

Max-Planck-Institut für Molekulare Genetik, D-14195 Berlin, Germany

Received February 14, 1995; Accepted March 14, 1995

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·P53 and the *kil*A and the *rep*L genes, in that order. Transcription of the lytic replicon of P53 and synthesis of the product of *rep*L, but not *kil*A, are required for replicon function. We have identified an additional promoter, termed P53as (antisense), at the 5′-end of the *kil*A 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 *rep*L 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 (100 map coordinates) 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 *inc*A 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 *inc*A. 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 *kil*A, 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 P1 lytic replicon can only be maintained in a bacterial cell when the *kil*A 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 *rep*L 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 *lac*Z 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 *lac*Z 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 *kil*A 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 *lac*Z 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.

* To whom correspondence should be addressed

+Present address: Universitätsklinik Heidelberg, D-69115 Heidelberg, Germany

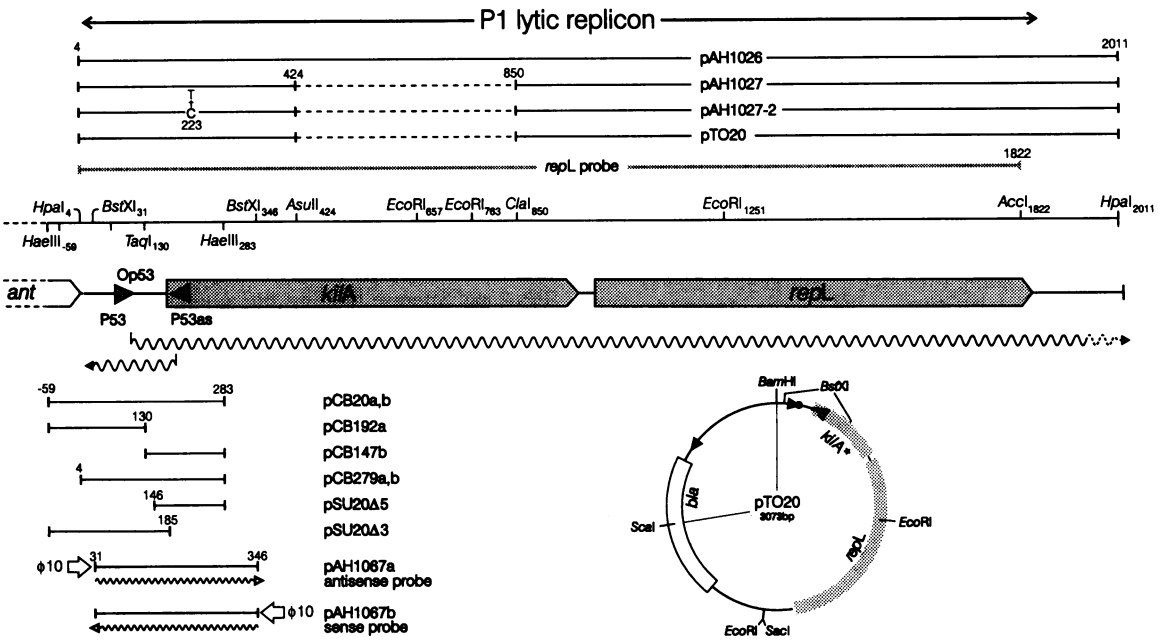

Figure 1. P1 lytic replicon and plasmids used. (Center) The P1 lytic replicon is located downstream of the anti-repressor gene *ant* of the P1 *imm*I operon and in a clockwise orientation on the P1 genetic map (1). It comprises the C1 repressor-controlled Op53 · P53 element (dot and triangle), the promoter P53as (this paper) and the genes *kil*A and *rep*L (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 *Bal*31 deletion end points at positions 146 and 185 (see Fig. 2). A 426 bp deletion in the *kil*A gene is indicated by the interrupted line. The reading frame is not altered in the resulting truncated *kil*A* 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 *rep*L 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.

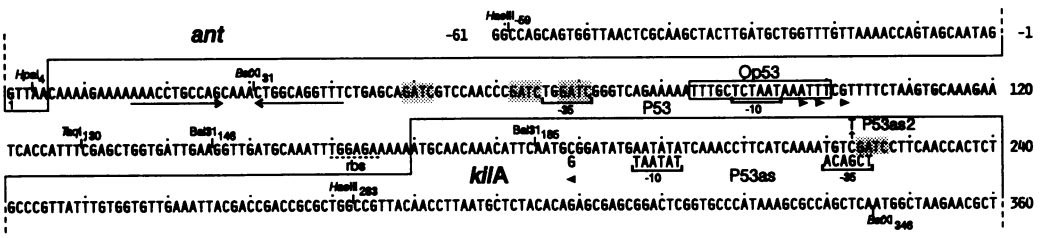

Figure 2. Nucleotide sequence of the regulatory region of the P1 lytic replicon. Nucleotides are numbered from 1 (*Hpa*I recognition site) to 360 in the *kil*A gene (5). The *Hpa*I site contains the termination codon, TAA, of the *ant* gene (9). Nucleotide positions upstream of the *Hpa*I site are indicated by negative numbers. Sequences of the *ant* and the *kil*A genes are framed. Upstream of *kil*A 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 *kil*A 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 *c*1.100 (14) (abbreviated to P1 and P1 *c*1ts respectively) and P1 *c*4.32 *ant*17 (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 *Pvu*II–*Bcl*I subfragment of P1 *Eco*RI fragment 7 with the *c*1 gene, its control region and the *coi* gene (18) inserted into a 2.5 kb *Dra*I fragment of the vector pKT101 Km^{r} (19). It was used to supply the C1 repressor. Plasmid pAM8 $c1^{+}coi^{-}lxc^{+}$ is a *coi*-defective derivate of pAM8 $c1^{+}coi^{+}lxc^{+}$(vector pKT101 Km^{r}) (19) that additionally contains the co-repressor *lxc* gene (20). The *coi* gene

was inactivated by insertion of two bases at the *Acc*I site (18). The plasmid was used to supply the C1 repressor and Lxc co-repressor. Plasmid pAM8 *c*1ts *coi*$^+$*lxc*$^+$ is a derivative of pAM8 *c*1$^+$*coi*$^+$*lxc*$^+$ in which the P1 *Bgl*II–*Pst*I fragment containing the C-terminal part of *c*1$^+$ is substituted by the analogous fragment of the temperature-sensitive mutant P1 *c*1.100 (14,21). It was used to supply the C1ts repressor and Lxc co-repressor.

Plasmids pAH1027 and pT020 contain a P1 lytic replicon and were constructed in the following way. A 16 kb *Sma*I–*Hin*dIII fragment of P1 *c*1ts, extending from map position 48 to 64 (1) was inserted into pT7-5 (*Sma*I/*Hin*dIII) to yield plasmid pAH16 (not shown). Next, a *Hpa*I$_4$–*Hpa*I$_{2011}$ fragment from pAH16 was inserted into pT7-6 (*Sma*I) 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 *c*1$^+$*coi*$^+$*lxc*$^+$, which supplies the C1 repressor and Lxc co-repressor. To inactivate the *kil*A gene we followed the procedure of Sternberg and Cohen (5) in deleting an *Asu*II$_{424}$–*Cla*I$_{850}$ fragment from pAH1026 to yield plasmid pAH1027 (Fig. 1). The latter contains the ColE1 replicon of the vector pT7-6, in addition to the *kil*A-truncated P1 lytic replicon. To eliminate the ColE1 replicon, a 2226 bp *Bam*HI–*Sca*I fragment of pAH1027 containing the lytic replicon was ligated to a 847 bp *Sca*I–*Bam*HI fragment of pJF118EH to yield plasmid pT020 (Fig. 1). Replication of pT020 solely depends on the *kil*A-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 *Sma*I (pCB302a)- or *Hin*cII (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 *Hae*III$_{-59}$–*Eco*RI$_{657}$ fragment (Fig. 1) in pT7-6 (*Sma*I/*Eco*RI). The plasmid was then mutagenized using the oligonucleotide 5′-GTTGAAGGATCAACATTTTG-3′. This oligonucleotide is complementary to the P1 DNA sequence (positions 234–215) with the exception of a G→A (underlined base) exchange at position 223 in the –35 region of P53as (Fig. 2). The plasmid carrying the P53as promoter-down mutation is termed pAH1020-2.

Northern and Southern blot analysis

DNA and RNA were extracted from bacteria as described (15,23) and transferred to Hybond-N membranes (Amersham). Transfer, immobilization and hybridization of nucleic acids were done as described by Pollard *et al.* for Northern blots (24) and according to the suppliers protocol (Amersham) for Southern blots. The *rep*L (Fig. 1) and *c*1 DNA hybridization probes were generated from isolated DNA fragments by random priming (25,26) using a Promega kit and [α-^{32}P]dATP (10 mCi/mmol; Amersham). The *c*1 probe was derived from the 1.6 kb *Acc*I DNA fragment of plasmid pAM8 encoding the *c*1 gene. The single-stranded sense and antisense probes (Fig. 1) were transcribed from *Eco*RI-linearized pAH1067b and pAH1067a *in vitro* using T7 RNA polymerase and [α-^{32}P]UTP (10 mCi/mmol; Amersham), according to Sambrook *et al.* (27). The plasmids were constructed by inserting the *Bst*XI$_{31}$–*Bst*XI$_{346}$ fragment in either orientation into pT7-6 (Fig. 1). Blots were analyzed with a PhosphorImager–Densitometer (Molecular Dynamics).

Electron microscopy

Binding of RNA polymerase (Promega) to supercoiled plasmid DNA was done with or without pre-incubation with C1 repressor as described (28). After fixation of the DNA–protein complexes with glutaraldehyde, the DNA was linearized with the restriction enzymes *Dra*II, *Sac*I or *Sca*I in order to pinpoint the P1-specific RNA polymerase binding site among several binding sites in the vector DNA. Adsorption of the complexes to mica followed by electron microscopy and evaluation of the data were as described by Spiess and Lurz (29).

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 *Hae*III fragment comprising the 3′-end of the *ant* gene, the Op53·P53 control region and the 5′-end of the *kil*A gene (Figs 1 and 2). The two sites can only be distinguished when the *Hae*III fragment is subdivided by *Taq*I into two *Hae*III–*Taq*I subfragments, 188 and 153 bp in size. The subfragments were inserted into *Sma*I-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 pSU20Δ3 DNA (Fig. 1) is comparable with that on linearized pSU20Δ3 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 pSU20Δ5 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

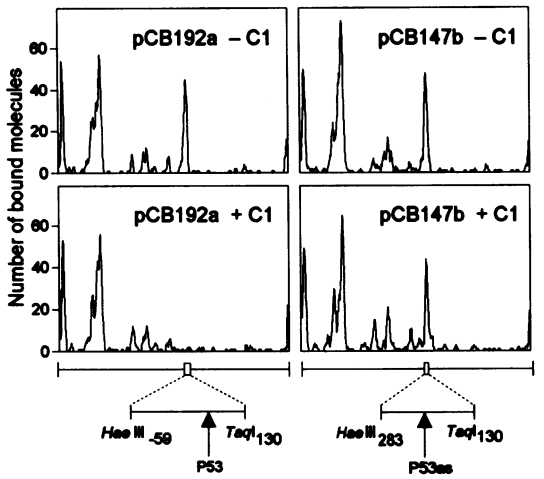

Figure 3. Interference of C1 repressor with the binding of RNA polymerase to promoter P53 but not P53as. The P1 *Hae*III$_{-59}$–*Hae*III$_{283}$ fragment (Fig. 2) was cut by *Taq*I into two subfragments, which contain the promoters P53 (left half) and P53as (right half) respectively. The subfragments were inserted into pCB302 DNA. After transformation and propagation in bacteria, the resulting supercoiled DNAs (425 ng pCB192a and 375 ng pCB147b; Fig. 1) were incubated *in vitro* with *E.coli* RNA polymerase (0.15 U, 5 min at 37°C) with or without prior incubation with the C1 repressor (80 ng, 15 min at 37°C) in a total volume of 30 µl. The DNA–protein complexes were prepared for electron microscopy as described in Materials and Methods. The abscissae represent the 7090 bp (left half) and 7055 bp (right half) plasmid DNAs linearized with *Dra*II (thin line, vector DNA; open box, P1 *Hae*III–*Taq*I insert; vertical arrow, position of the promoter). For each data set 110–150 DNA molecules were measured, which had a mean of 3.5–3.8 RNA polymerase molecules bound.

Table 1. Transcriptional activities of P53 and P53as

plasmid	Op53 P53 P53as	β-Galactosidase activity (units)				
		–	*E. coli* CB454 P1c4.32ant17 $c1^+cor^+lxc^+$	*E. coli* CB454 pAM2b $c1^+cor^+$	*E.coli* WM874 dam^+	*E.coli* WM874 dam^-
pCB20a	→	540	3	30	–	–
pCB20b	←	210	180	280	–	–
pCB192a	→	2,730	8	250	1,620	500
pCB147b	←	510	580	500	350	430
pCB279a	→	–	–	–	360	90
pCB279b	←	–	–	–	340	800

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 P1 DNA fragments inserted into the promoter probe vector. Bacteria were grown at 37°C. At $A_{600} = 0.5$, bacteria were prepared for the β-galactosidase assay as described (11). Activity is expressed as Miller units.

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).

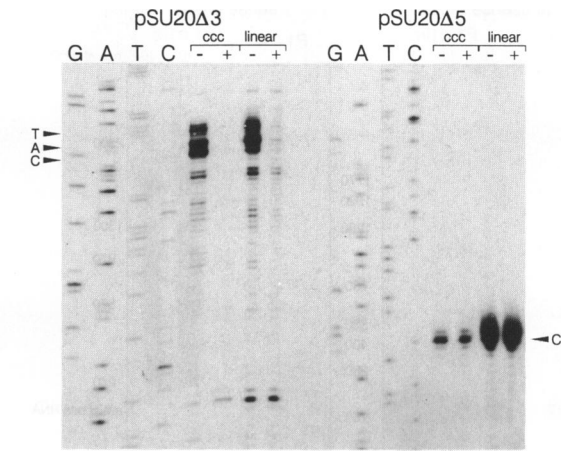

Figure 4. Identification of +1 transcript initiation sites of the promoters P53 and P53as. Plasmid DNAs (2.5 µg each of cccDNA or DNA linearized with *Asu*II) were incubated with *E.coli* RNA polymerase (4.4 U, 15 min at 37°C) with or without pre-incubation with the C1 repressor (50 ng/µl, 15 min at 37°C) in a total volume of 11.5 µl. Primer extension and DNA sequencing by the dideoxy chain termination reaction (31) were started with the 5′-^{32}P-labelled 17mer pUC primer 1221, (Biolabs; plasmid pSU20Δ3, left half) and the corresponding 17mer pUC reverse primer 1201, (Biolabs; plasmid pSU20Δ5, right half). Each half figure is subdivided into the sequencing reaction (left side) and the primer extension reaction with ccc- and linear DNA with or without C1 repressor pre-incubation (right side). Note that the nucleotides marked by an arrow are complementary to the 5′-terminal nucleotides of the transcripts (Fig. 2).

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 *c*1ts 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 in the P1 wild-type and a P1 *c*1ts lysogen at 28°C, the amount of which increases dramatically during P1 *c*1ts prophage induction (Fig. 5, left half). As expected, synthesis of this 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 *c*1ts prophage (Fig. 5, right half). Most probably they represent the mRNAs of the whole *rep*L operon, the

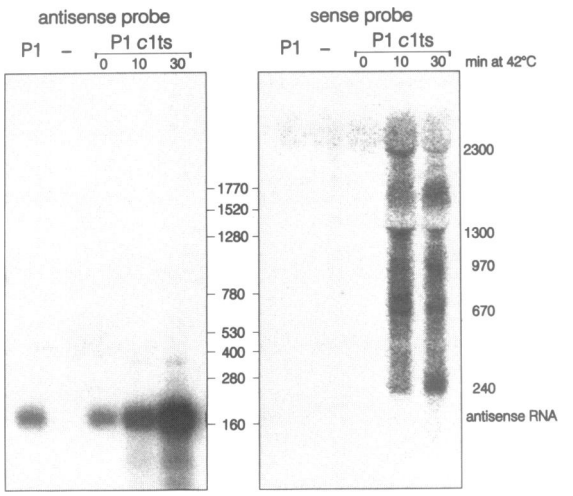

Figure 5. Sense and antisense transcription in the lytic replicon of prophage P1. *Escherichia coli* WM874 bacteria and their P1 wild-type and P1 *c*1ts lysogens were grown at 28°C. At A_{550} = 0.6 the temperature of the P1 *c*1ts lysogenic bacteria was shifted to 42°C by addition of an equal volume of 54°C pre-warmed medium. Incubation was then continued at 42°C for the indicated times before the bacteria were harvested. P1 wild-type and non-lysogenic WM874 bacteria were harvested at 28°C at A_{550} = 0.6. For Northern blots single-stranded RNA was transcribed with T7 RNA polymerase *in vitro* (27) using *Eco*RI-linearized plasmid pAH1067a (b) to yield a run-off sense (antisense) transcript (Fig. 1). The transcripts were used as antisense and sense probes respectively. Total cellular RNA (20 µg) was loaded in each lane. P1, P1 wild-type lysogen; (–), non-lysogen.

*kil*A and *rep*L genes individually and, possibly, some degradation products.

Promoter P53as down-regulates the P1 lytic replicon

To investigate the effect of transcription from P53as on the P1 lytic replicon we first constructed the plasmids pAH1027 and pT020 as described in Materials and Methods. Replication of pT020 solely depends on the P1 lytic replicon, with an (unknown) origin of replication which appears to reside in the *rep*L region, between the *Cla*I_{850} and *Hpa*I_{2011} cleavage sites (5; Fig. 1). Transcription from P53 is a prerequisite for the lytic replicon to function. Since the C1 repressor blocks the P53 promoter, plasmid pT020 cannot be maintained in cells containing the C1 repressor. However, plasmid pAH1027 carries a ColE1 replicon on its pT7-6 vector DNA, in addition to the P1 lytic replicon. Therefore, the function of the latter can be turned off by repressor C1 without impairing replication driven by the ColE1 replicon. In both plasmids the lethal effect of KilA on the bacterial cell is prevented by deleting 52% of the *kil*A gene, without impairing the reading frame.

Next we constructed a P53as promoter-down mutation as described in Materials and Methods. The 153 bp *Taq*I–*Hae*III and the 279 bp *Hpa*I–*Hae*III 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).

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

Plasmid	β-Galactosidase activity (U)	
	P53as	P53as2
pCB147b	730	110
pCB279a	800	1210

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.

We then tried to insert the P53as2 promoter-down mutation into plasmids pAH1027 and pT020 by replacing the *Bst*XI_{31}–*Bst*XI_{346} wild-type fragment with the corresponding mutant fragment of plasmid pAH1020-2. Since the two *Bst*XI 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 *c*1$^+$*coi*$^-$*lxc*$^+$ 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 WM874 bacteria (Table 3). These results indicate an essential function of the promoter P53as in controlling the P1 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). We conclude from these results that it is the function of promoter P53as to down-regulate *rep*L-specific transcription and replication. Obviously, overproduction of *rep*L transcripts and, as a consequence, stimulation of *rep*L-dependent replication is incompatible with plasmid stability and is, most probably, the reason why we failed to construct a pT020-2 mutant plasmid.

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

Plasmid	Percent transformants[a]	
	WM874	WM874/pAM8 *c*1$^+$*coi*$^-$*lxc*$^+$
pAH1027	100	18
pAH1027-2	<0.02	11

[a]100% = 4200 transformants.

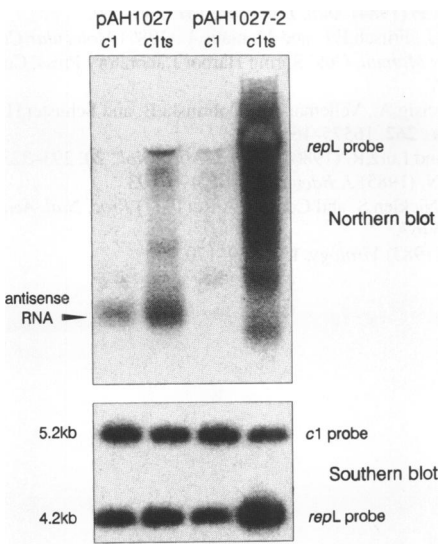

Figure 6. A P53as promoter-down mutation enhances repL-dependent transcription and replication. The bacterial strains used were WM874/pAH1027 and WM874/pAH1027-2. The strains carried the plasmid pAM8 *c*1$^+$*coi*$^-$*lxc*$^+$ or pAM8 *c*1ts *coi*$^+$*lxc*$^+$, from which the C1 wild-type (*c*1) or the thermolabile C1ts repressor (*c*1ts) was supplied. Bacteria were grown at 30°C. At A_{600} = 0.6 the temperature of the cultures was shifted to 42°C by the addition of an equal volume of 54°C pre-warmed medium. After 1 h at 42°C bacteria were harvested and 20 ml (2 ml) equivalents of each culture were used for the preparation of RNA (DNA). The *rep*L probe (Fig. 1) was used for measurements of lytic replicon-dependent transcription (upper part) and DNA replication (4.2 kb DNA, lower part). The pAM8 plasmids, 5.2 kb in size, were determined using the *c*1 probe (see Materials and Methods). Plasmid DNAs were linearized with *Pst*I before electrophoresis.

DISCUSSION

We have discovered an as yet unknown promoter of P1, which we term P53as and which directs the synthesis of a 180 base RNA. The characteristics of this transcript are typical of an antisense RNA. The RNAs initiating from P53as and P53 are transcribed from the same DNA in opposite and convergent directions. In contrast to the P53 sense transcript, the P53as transcript is a small molecule (with a half-life of about 14 min; data not shown) which affects P53 transcription negatively. The negative effect appears to be strong, because lytic replicon transcription is strongly enhanced in a P53as promoter-down mutant. Furthermore, a P53as-defective plasmid replicated solely via the P1 lytic replicon could not be established by bacterial transformation.

Various possibilities for interference by P53as RNA with P53 RNA may be envisioned. Antisense transcription from P53as may directly interfere with transcription initiated from P53 on the same DNA molecule. The P53as transcript may interfere with *Kil*A/*Rep*L protein synthesis by base pairing with the P53 transcript, by which means the ribosome binding site would be occluded and initiation of translation prevented. The P53 transcript may become more susceptible to degradation by the formation of double-stranded RNA. The outcome of our experiments do not allow us to distinguish between these possibilities. Also, we do not yet know whether the P53as transcript can act *in trans*. Nevertheless, we do not believe that P53as RNA encodes a protein. Although the 180 base RNA contains AUG initiation codons at positions +8 and +63, giving rise to open reading frames 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 *Eco*RI:14 fragment (positions –1226 to +657) containing expression vector pT7-6 or p*tac* in which the T7ϕ10 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 *rep*L 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 *rep*L 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.

ACKNOWLEDGEMENTS

We appreciate the expert technical assistance of Jessica Priebe. We thank Dietmar Vogt for the preparation of plasmid DNA. We also thank Angelika Hofmann for critically reading the manuscript. This work was supported by the Fonds der Chemischen Industrie.

REFERENCES

1 Yarmolinsky,M.B. and Lobocka,M.B. (1993) In O'Brien,S.J. (ed.), *Genetic Maps*, 6th Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 1, pp. 1.50–1.61.
2 Sternberg,N. and Hoess,R. (1983) *Annu. Rev. Genet.*, **17**, 123–154.
3 Yarmolinsky, M. B., and Sternberg, N. (1988) In Calendar,R. (ed.), *The Bacteriophages*. Plenum Publishing, New York, NY, Vol. 1, pp. 291–438.
4 Cohen,G. and Sternberg,N. (1989) *J. Mol. Biol.*, **207**, 99–109.
5 Sternberg,N. and Cohen,G. (1989) *J. Mol. Biol.*, **207**, 111–133.
6 Hansen,E.B. (1989) *J. Mol. Biol.*, **207**, 135–149.
7 Eliason,J.L. and Sternberg,N. (1987) *J. Mol. Biol.*, **198**, 281–293.
8 Velleman,M., Dreiseikelmann,B. and Schuster,H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 5570–5574.
9 Heisig, A., Riedel, H. D., Dobrinski, B., Lurz, R., and Schuster, H. (1989) *J. Mol. Biol.*, **209**, 525–538.
10 Schneider,K. and Beck,C.F. (1987) *Methods Enzymol.*, **153**, 452–461.
11 Miller,J.H. (1977) *Experiments in Molecular Genetics*, 3rd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 431–435.
12 Wilson,G.G., Young,K.K.Y. and Edlin,G.J. (1979) *Nature*, **280**, 80–81.
13 Marinus,M.G., Carraway,M., Frey,A.Z., Brown,L. and Arraj,J.A. (1983) *Mol. Gen. Genet.*, **192**, 288–289.
14 Rosner,J.L. (1972) *Virology*, **49**, 679–689.
15 Sternberg,N. (1979) *Virology*, **96**, 129–142.
16 Fürste,J.P., Pansegrau,W., Frank,R., Blöcker,H., Scholz,P., Bagdasarian,M. and Lanka,E. (1986) *Gene*, **48**, 119–131.
17 Martinez,E., Bartolomé,B. and de la Cruz,F. (1988) *Gene*, **68**, 159–162.
18 Heinzel,T., Velleman,M. and Schuster,H. (1990) *J. Biol. Chem.*, **265**, 17928–17934.

19 Velleman,M. (1989) PhD thesis, Freie Universität Berlin, Berlin, Germany.
20 Velleman,M., Heinzel,T. and Schuster,H. (1992) *J. Biol. Chem.*, **267**, 12174–12181.
21 Heinrich,J., Riedel,H.-D., Baumstark,B.R., Kimura,M. and Schuster,H. (1989) *Nucleic Acids Res.*, **17**, 7681–7692.
22 Olsen,D.B. and Eckstein,F. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1451–1455.
23 Aiba,H., Adhya,S. and de Crombrugghe,B. (1981) *J. Biol. Chem.*, **256**, 11905–11910.
24 Pollard,J.W., Perry,C.R. and Thurston,C.F. (1988) *New Nucleic Acid Techniques*, **4**, 13–25.
25 Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13.
26 Feinberg,A.P. (1984) *Anal. Biochem.*, **137**, 266–269.
27 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28 Lurz,R., Heisig,A., Velleman,M., Dobrinski,B. and Schuster,H. (1987) *J. Biol. Chem.*, **262**, 16575–16579.
29 Spiess,E. and Lurz,R. (1988) *Methods Microbiol.*, **20**, 293–323.
30 Sternberg,N. (1985) *J. Bacteriol.*, **164**, 490–493.
31 Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
32 Cohen,G. (1983) *Virology*, **131**, 159–170.