Proc. Natl. Acad. Sci. USA
Vol. 78, No. 1, pp. 167–171, January 1981
Biochemistry

Organization of transcriptional signals in plasmids pBR322 and pACYC184

(gene organization in plasmids/promoter mapping/termination/expression of cloned DNA/overlapping promoters)

DIETRICH STÜBER AND HERMANN BUJARD

Molekulare Genetik der Universität Heidelberg, Im Neuenheimer Feld 230, 6900 Heidelberg, Federal Republic of Germany

Communicated by Stanley N. Cohen, September 29, 1980

ABSTRACT **Electron microscopic analysis of *in vitro* transcriptional complexes of pBR322 and pACYC184 revealed five and six major transcriptional units, respectively, in these two plasmid vectors. These units are transcribed with various efficiencies, depending upon the individual promoter strengths, which differ in pBR322 up to 10-fold. A most interesting signal arrangement was found at the beginning of the tetracycline resistance region, where two partially overlapping promoters (*P1* and *P2*) initiate transcription crosswise in opposite directions. Whereas *P2* is known to promote tetracycline resistance and to be inactivated by *Hin*dIII cleavage, *P1* is able to transcribe DNA integrated at that site and probably contributes to the expression of the β-lactamase gene in pBR322. In pACYC184, besides *P1*, *P2*, and the *cat* (chloramphenicol resistance) promoter (*P5*), two initiation sites (*P3* and *P4*) were mapped in a region that appears to be part of insertion sequence 1. The maps of transcription signals permit a more predictable utilization of these cloning vehicles and also allow the reinterpretation of earlier cloning results.**

Using various cloning vehicles to transfer genetic material into a more simple environment greatly facilitates the study of gene expression. Nevertheless, such analyses are often complicated by expression of the vector's own genes. Thus, diverse results are obtained when a particular piece of DNA is inserted into different cloning vehicles or different sites in the same vehicle. It has also proven difficult to clone DNA fragments carrying strong signals for gene expression such as efficient promoters, because such signals appear to upset the controlled expression of the vector's essential functions (unpublished data). We have determined the arrangement of transcriptional signals within the genetic text of the plasmids pBR322 (1) and pACYC184 (2) to allow their more rational use as cloning vehicles. The electron microscopic analysis of *in vitro* transcriptional complexes has yielded results that are in excellent agreement with *in vivo* data (3, 4). The transcriptional maps obtained with this technique and presented here show that these constructed plasmids are organized in well-defined transcriptional units; they explain results of cloning experiments hitherto not understood, despite the availability of nucleotide sequences, and confirm other data derived by various methods.

MATERIALS AND METHODS

All DNA probes of plasmids pBR322 (1), pACYC184 (2), pML21 (5), and pKK1 and pKK5 (6) were extracted with phenol. Restriction endonucleases were from Boehringer Mannheim (Federal Republic of Germany) or from New England BioLabs; streptolydigin was purchased from Upjohn (Heppenheim, Federal Republic of Germany). RNA polymerase from *Escherichia coli* and phage T4 gene *32* protein were isolated as described (3, 7).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Preparation of transcriptional complexes: In a typical assay (50 μl) containing 120 mM KCl, 20 mM Tris·HCl at pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM each ATP and GTP, and 0.05 mM CTP and UTP, 1–5 μg of DNA was preincubated for 3 min at 37°C. Transcription was started by the addition of RNA polymerase at ratios of 2–4 enzyme molecules per promoter and allowed to proceed between 0.5 and 4 min. The rate of chain elongation under these conditions was 15 nucleotides per sec. Transcription was stopped by diluting aliquots into a buffer suitable for restriction endonuclease digestion containing streptolydigin at 100 μg/ml and the amount of restriction enzyme sufficient to linearize the template DNA within 2 min at 37°C. The transcriptional complexes were then prepared for electron microscopy as described (3). For electrophoretic analysis of RNA, aliquots of *in vitro* assays containing [^{3}H]UTP were extracted with phenol, heated to 95°C in 70% (vol/vol) formamide for 2 min, and applied to 15% acrylamide/8 M urea gels. Heteroduplex analysis and quantitative evaluation of electron micrographs were carried out as described (3, 8).

RESULTS

Analysis of Transcriptional Complexes Obtained with Covalently-Closed Circular DNA as Template. In contrast to linear phage DNA (3, 4) linearized plasmid DNA shows little template activity *in vitro* even at high ratios of RNA polymerase to promoter. Therefore, plasmid preparations with a high content of covalently closed circular DNA were used as templates; these, however, had to be linearized prior to the quantitative analysis of the originating transcriptional complex. Transcription was therefore halted by streptolydigin (9) at various times after chain initiation and the template was linearized by a single cut using an appropriate restriction enzyme. Under these conditions, high yields of complexes carrying, on average, 3–5 RNA chains and suitable for quantitative evaluation were obtained. Depending upon the individual preparation, 50–70% of the structures in a random microscope field appeared measurable, of which 70–85% could be arranged with respect to RNA initiation sites in an unequivocal way. For the various plasmids the following numbers of complexes with the respective number of RNA chains were evaluated: pBR322, 223/1059; pACYC184, 71/318; pKK1, 118/434; and pKK5, 100/376.

Promoters and Terminators Mapped in pBR322. Most of the transcription of pBR322 *in vitro* is initiated within two regions, one centering at around position 0 [*Eco*RI site of the plasmid (10)] and the other at around position 3000. Within the region containing the *Eco*RI site, transcription proceeds in both directions; the rightward transcripts are terminated at around position 650, while leftward transcription is terminated at around 3200 (Fig. 1). Because the *Eco*RI site (position 0) is lo-

Abbreviations: Tc, tetracycline; bp, base pair(s).

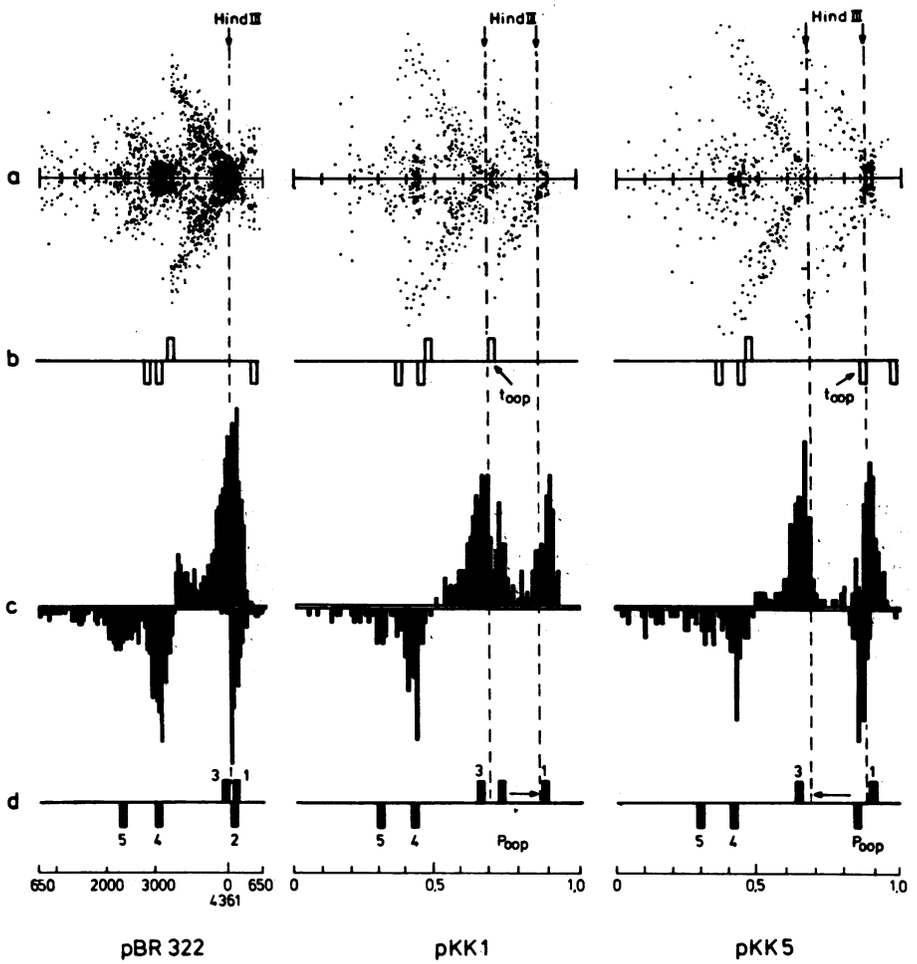

FIG. 1. Analysis of transcriptional complexes of pBR322, pKK1, and pKK5. The first line (*a*) shows graphs in which the *x* axis represents the plasmid DNAs linearized by *Sal* I cleavage. The points show the position (along the *x* axis) and the length (along the *y* axis) of RNA molecules (for computation the length of RNA molecules is doubled and each point is plotted above and below the *x* axis). In the plots the position of termination sites can already be identified because the length of the RNA chains is independent of incubation time (between 1.5 and 4 min at 15 nucleotides per sec). Similarly, the major promoters and their direction of transcription can be recognized. In *b* the position of the major termination signals is indicated. Peaks and symbols (□ for terminator, ■ for promoter) above and below the axis identify signals acting in leftward or rightward transcription, respectively. (*c*) Result of data processing as described in detail previously (3): the histograms are obtained after extrapolation of each RNA chain to its possible points of origin. The peaks represent, therefore, the major promoters which were numbered (*1* through *5* and P_{oop}) as shown in *d*. Integration of a 1080-bp λ DNA fragment into the *Hin*dIII site (pKK1, pKK5) separates *P1* and *P3*, at the same time destroying *P2* (*c*, *d*); it also shows the activity of P_{oop} and t_{oop}: in pKK1 transcripts from *P1* and from P_{oop} are terminated at t_{oop} at the left end of the integrated fragment, whereas in pKK5 *P1* transcripts cross this border; here the oop RNA is seen by the accumulation of points at the right end of the integrated DNA, where also some readthrough into the *tet* region is observed. The arrow in *d* identifies the orientation of the λ *O* gene, which in pKK5 is under the control of *P1*, allowing complementation of λ *O* mutants. The large number of points near the *x* axis at position 3000 of pBR322 represents the intense production of 104 RNA starting at *P4*, the strongest promoter of this system (4). The number of bp of pBR322 is according to Sutcliffe (10); the scales for pKK1 and pKK5 are fractional length.

cated close to the beginning of the coding region of the tetracycline (Tc) resistance genes (*tet*) (11), a promoter initiating transcription to the right (*P2*) was expected. Similarly, transcription to the left is a prerequisite for the expression of the β-lactamase (*bla*) gene (10). The broad range of positions of the *bla* promoter suggested, however, that more than one promoter may initiate transcription to the left, and more interestingly that one of them may be located to the right of the *tet* promoter (11). We have therefore analysed the pBR322 derivatives pKK1 and pKK5, which contain a 1080-base-pair (bp) fragment of phage λ DNA, integrated at the *Hin*dIII site (position 29) in both orientations (6). This λ DNA fragment carries the structural gene for the O protein as well as the promoter and terminator of the oop RNA (P_{oop}, t_{oop}). From the data obtained with pKK1 and pKK5, as shown in Fig. 1, we draw the following conclusions:

(*i*) pBR322 indeed carries two promoters initiating transcription towards the *bla* gene. One of these promoters (*P1*) is located to the right of the *Hin*dIII site at position 44 ± 80 bp. The other promoter (*P3*) is identified at 4200 ± 80 bp. The function of both promoters is independent of the orientation of the integrated λ DNA fragment.

(*ii*) The *tet* promoter (*P2*) located at 57 ± 80 is disrupted by integration of DNA into the *Hin*dIII site as described before (11).

(*iii*) The function of the transcriptional signals carried by the λ DNA fragment can be demonstrated: in pKK1, in which t_{oop} is in the proper orientation, it terminates the transcripts originating from *P1*, whereas with pKK5 transcription from *P1* proceeds across the λ fragment into the *bla* region. In both pKK1 and pKK5 extremely short transcripts (less than 100 nucleotides in length) are initiated at P_{oop} and terminated at t_{oop}. In pKK5, however, we have noticed some readthrough from P_{oop} into the *tet* region. This accounts for the observed Tc resistance of cells carrying pKK5 (5 μg/ml for pKK5 versus less than 2 μg/ml for

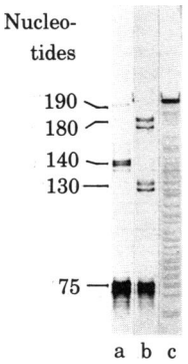

FIG. 2. Electrophoretic analysis of transcripts initiated by *P1* and *P2*. Lanes a and b show transcripts obtained using a 190-bp *Hae* III and a 178-bp *Hae* III/*Hpa* II fragment of pBR322 (10) as template, respectively; both fragments contain *P1* and *P2* (Fig. 4). Lane c depicts a single-stranded DNA marker. There are three major RNA species in lane a: the end-to-end transcript (190 nucleotides) and two transcripts initiating within the fragment (140 and 75 nucleotides). After *Hpa* II cleavage of the template (lane b) the 190- and 140-nucleotide transcripts are reduced in size by about 12 nucleotides, whereas 75-nucleotide RNA remains unchanged.

pKK1; measured as minimal inhibitory concentration on plates).

From the positions of *P1* and *P2* (Fig. 1) two transcripts of about 70 and 140 nucleotides can be predicted if the *Hae* III fragment, which spans position 4344 to 173, is transcribed (10). As seen in Fig. 2, such transcripts were indeed obtained. Shortening of the template at position 161 by *Hpa* II digestion (Fig. 4) reduced the size of the 140-nucleotide transcripts by about 10 nucleotides (Fig. 2); this RNA is therefore the product of *P2*. The sites of RNA chain initiation for *P1* and *P2* must be at 56 ± 10 and 32 ± 10, respectively.

The function of *P1 in vivo* was demonstrated by complementation experiments with pKK1- and pKK5-carrying cells and a λ *O* gene mutant: Only cells containing pKK5 in which the *O* gene is in the proper orientation (Fig. 1) allowed the development of λ*cIb2 O*am 125.

The second region in which extensive transcription is observed in pBR322 shows an unusual feature: as seen in Fig. 1, RNA chains are efficiently initiated as well as terminated around position 3000, resulting in transcripts less than 200 nucleotides long. It is difficult to determine the direction of transcription of such small transcripts. However, the high rate of chain initiation at site *P4* as well as the length of the transcripts suggest strongly that this promoter produces the 104 RNA described before (12). We conclude, therefore, that *P4* initiates transcription from left to right. The longer but less frequent transcripts seen in that region indicate the existence of further promoters, of which *P5* initiating transcription to the right could be identified; these transcripts are terminated around position 2700. A leftward promoter around 3100 giving rise to transcripts of about 500 nucleotides is suggested by our data; its precise position, however, is obscured by the high concentration of *P4* transcripts (Fig. 1*a*).

Analysis of pACYC184. This plasmid, which was constructed by scrambling various precursors, contains the p15 *A* replicon as well as genes for Tc and chloramphenicol resistance [*cat* gene (2)]. The position of the replicon within the plasmid was determined by heteroduplex analysis with pML21 DNA, which carries the closely related ColE1 replication system (5, 13). As seen in Fig. 3, this region extends to the left of the *Hin*dIII site spanning 810 ± 70 bp. Similarly, heteroduplexes between pACYC184 and pML21 (Fig. 3) as well as Tn9 (ref. 14, data not shown) revealed a 1300-bp homology between the region adjacent to the replication region and transposon Tn9 (fractional length 0.341–0.648). Restriction endonuclease analysis using *Hae* II, *Hha* I, *Hin*fI, and *Hph* I (data not shown) demonstrated sequence homology between pBR322 and pACYC184 extending from the right of the *Hin*dIII site to fractional length 0.211. Thus, the origin of only 10% of the plasmids DNA (fractional length 0.211–0.316) could not be identified.

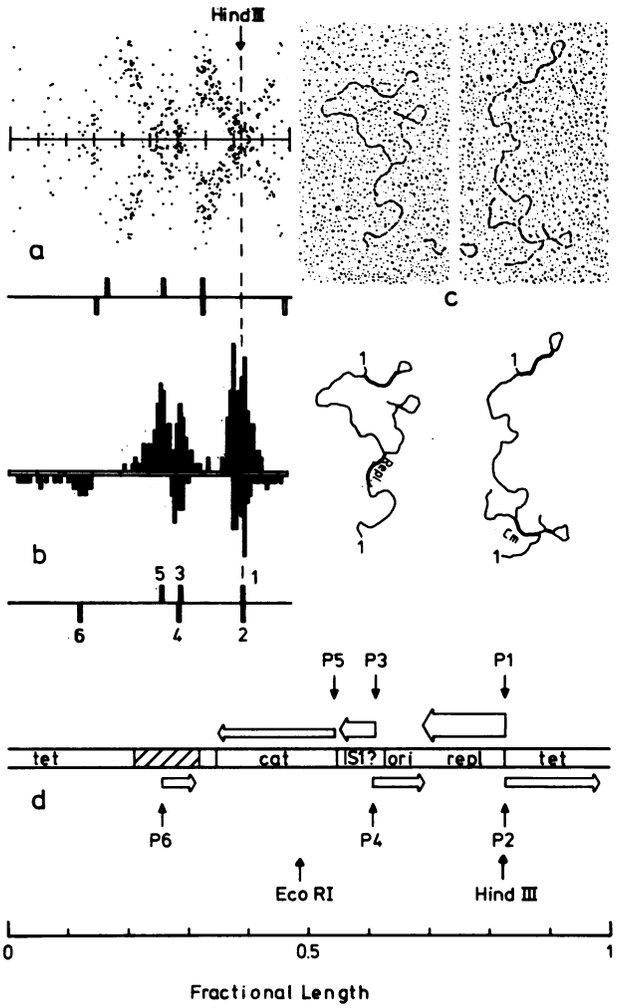

FIG. 3. Analysis of pACYC184. *a* and *b* show the mapping of transcriptional signals as described in Fig. 1. The *x* axis shows again the plasmid after linearization with *Sal* I and peaks and symbols (□ for terminator, ■ for promoter) above and below the *x* axis indicate signals for leftward and rightward transcription. Six major promoters (*b*) and corresponding terminators (*a*), which subdivide the plasmid DNA in distinct transcriptional units, were identified. (*c*) Examples of heteroduplex molecules between pACYC184 linearized with *Hin*dIII and pML21 linearized with *Sal* I. The latter molecule, 1, can be distinguished by the large stem-loop structure of the kanamycin region, whereas pACYC184 shows a characteristic small loop around the *Sal* I site. The evaluation of more than 20 such structures showed that the ColE1 replication region (Repl.) hybridizes to a region directly adjacent to the *Hin*dIII site of pACYC184; it is followed by sequences complementary to Tn*9* (data not shown) and to the part of pML21 that carries the COOH terminus of the *cat* gene (Cm). *d* combines the results of transcription and heteroduplex studies. The arrows delineate the six major transcriptional units of pACYC184 and their widths indicate the relative strengths of the various promoters.

Analysis of the transcriptional pattern of pACYC184 revealed promoters *P1* and *P2* at the beginning of the *tet* region just as in pBR322. Again the transcripts from *P2* are terminated at frac-

tional length 0.99 near the *Sal* I site. The transcription initiated at *P1*, however, proceeds into the replication region and is terminated just before the putative replication origin at fractional length 0.69 (Fig. 3). Within the sequence homologous to Tn9 three promoters were localized. Of these, two (*P3* and *P4*) are located at fractional length 0.61 and initiate transcription in opposite directions. They give rise to rather short transcripts (270 to 340 nucleotides). The third promoter (*P5*), however, at fractional length 0.549 initiates transcripts of about 800 nucleotides that cover the *Eco*RI site and are terminated at fractional length 0.35. It is known that integration of DNA into this *Eco*RI site destroys chloramphenicol resistance (15); furthermore, the transcript is long enough to code for a protein of the expected size [22,000 daltons, (16)]. We conclude, therefore, that *P5* is the *cat* promoter and that the orientation of the gene is as shown in Fig. 3. Because the *cat* gene is part of a 1300-bp fragment homologous to Tn9 and originally derived from R6/5, it appears likely that the promoters *P3* and *P4* are located within the insertion sequence 1 region adjacent to that gene in R6/5 (17). There is one more promoter (*P6*), at fractional length 0.259, which initiates short transcripts to the right (Fig. 3). It is located in that part of the plasmid whose origin could not be identified. The six promoters described in pACYC184 give rise to more than 80% of the *in vitro* transcripts; of these, the major classes are initiated at *P1*, *P2*, and *P5*.

DISCUSSION

The analysis of transcriptional complexes described here permits the identification and localization of promoters and terminators, the determination of the direction of transcription, and an estimate of the relative strengths of the signals. Promoters mapped in this way can be positioned along the template with an accuracy of 0.5–2%, depending on the size of the DNA studied. Thus, in pBR322 and pACYC184 the position of promoters has been determined with a standard deviation of ±80 bp. By taking into account the number of RNA chains initiated in various regions of the template in a given time, the relative strengths of various promoters were estimated and a 10-fold range in signal strength was observed. The accuracy in mapping terminators is lower (±150 bp) because only the longest transcripts can be used for their identification.

In pBR322 five major promoters were identified. They initiate more than 85% of the *in vitro* transcripts. The most interesting arrangement was found at the beginning of the region coding for Tc resistance: there are two promoters (*P1* and *P2*) within a stretch of 110 nucleotides that share around 20 bp for their RNA polymerase binding sites and initiate transcription in opposite directions as shown in Fig. 4. The function of *P1* is demonstrated not only by *in vitro* transcription of pBR322, pKK1, pKK5, and pACYC184 but also by its *in vivo* activity. By using pBR322 derivatives pKK1 and pKK5 (Fig. 1), which carry the *O* gene of phage λ, *in vivo* complementation of a λ *O* mutant is observed if the *O* gene is under control of *P1* (Fig. 1). The function of *P1* also explains data described previously (19, 20).

P1 initiates transcription 1.5 times more efficiently than does the *tet* promoter *P2*, and, although we cannot exclude some termination in front of *P3*, our data suggest strongly that transcription from *P1* proceeds into the *bla* region and contributes to its expression. The two promoters at the beginning of the *tet* region (*P1* and *P2*) reflect a rather intricate regulatory organization: not only do they share parts of their RNA polymerase binding sites, which probably excludes simultaneous binding of the enzyme to both promoters, but also there are extensive sequence symmetries (11), which may be targets for the binding of additional regulatory factors. Because this region of pBR322 is derived from pSC101 (21), a plasmid carrying a constitutive and an inducible Tc resistance (22), this promoter arrangement may be an important part of a more complex *tet* operon.

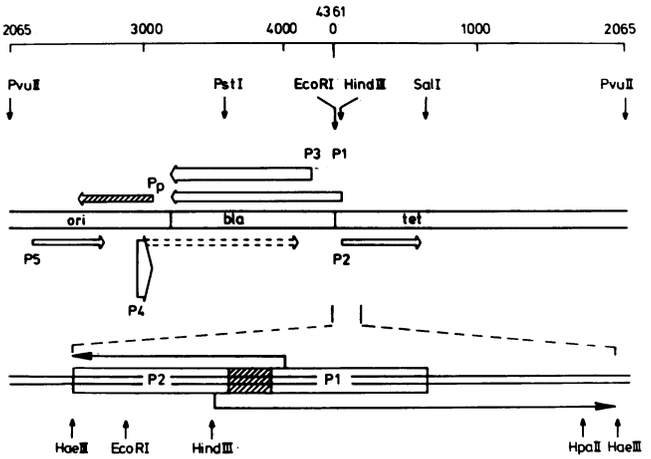

FIG. 4. Transcription map of pBR322. The upper part of the figure gives a schematic view of pBR322 linearized by *Pvu* II. The transcriptional units, defined by the various promoters (*P1* through *P5*) and corresponding termination signals, are delineated by arrows. The widths of the arrows reflect the relative promoter strengths. It can be seen that the *bla* gene is heavily transcribed because two promoters (*P1* and *P3*) contribute to its expression. Under the conditions used transcription from *P2* is terminated near the *Sal* I site, leaving most of the *tet* region untranscribed. The most efficient promoter of the system is *P4*, which produces the 104 RNA. Some of these transcripts appear to overrun the adjacent termination signal and extend along the noncoding strand of the *bla* gene. P_p denotes a leftward promoter initiating transcripts that prime DNA synthesis (hatched arrow) (18). The lower part of the figure shows an expansion of the *Hae* III fragment spanning positions 4344 to 173 (10) that was used for the analysis of *P1* and *P2*. The arrows indicate the 70- and 140-nucleotide transcripts originating from these promoters, of which only the 140-nucleotide transcript is affected by *Hpa* II cleavage of the template. It can be seen that the RNA polymerase binding sites (boxed areas) of *P1* and *P2* overlap for about 20 bp (hatched area). The scale in the upper part is given in bp according to Sutcliffe (10).

Another unexpected result was the site at which transcripts from *P2* are terminated. Whereas transcription from *P1* and *P3* proceeds to a termination site at the distal end of the *bla* gene, the transcripts of *P2* are terminated within the *tet* region around position 650. A transcript of this size can code in one reading frame for a 19,000-dalton protein, which is not sufficient, however, for conferring Tc resistance (4). The function of this termination site is therefore unclear, but it is of interest that the sequence around it includes an attenuator-like structure (i.e., an overlapping hairpin loop structure can be constructed between positions 618 and 666).

It is obvious from Fig. 1 that the *bla* gene in pBR322 is heavily transcribed and that this transcription is efficiently terminated at position 3200. The intense transcription within the adjacent region is due to *P4* at position 2900, the most efficient promoter of the system. This promoter gives rise to transcripts less than 200 nucleotides in length. Such transcripts are expected in that region, which codes for the so-called 104 RNA (12). These short RNA molecules, which appear to be involved in the plasmid's replication (23), are the most abundant transcription products (4). Both the position and the signal strength of *P4* suggest that it initiates transcription from left to right (Figs. 1 and 4). Furthermore, our data indicate that, although these short transcripts are terminated effectively around position 3000, some readthrough into the *bla* gene occurs. This transcriptional activity probably explains why promoter-free DNA

[e.g., the dehydrofolate reductase gene of the mouse, (24)] inserted in the *Pst* I site of the *bla* gene can be expressed independently of its orientation. The high occurrence of *P4* transcripts interferes with the evaluation of the longer transcripts observed between positions 2200 and 3100 bp, the region carrying the replication origin [position 2532–2534 (10, 25)]. Thus, whereas *P5* could clearly be identified, our data only suggest another promoter around position 3100 initiating transcription to the left. Such a promoter (P_p in Fig. 4) was in fact described by Itoh and Tomizawa (18), who showed that transcripts from this promoter are involved in the priming of DNA replication.

Plasmids pACYC184 and pBR322 are homologous for about 1500 bp, which include the Tc resistance region. Consequently, the transcriptional pattern of that part of the two plasmids is identical: *P1* and *P2* initiate transcription in opposite direction and with the expected relative frequencies. Similarly, in pACYC184 transcripts from *P2* are terminated at fractional length 0.99 near the *Sal* I site. To correlate the residual transcriptional pattern with the genetic text of the plasmid, the various regions had first to be identified by heteroduplex analysis (Fig. 3). It became obvious that transcripts from *P1* proceed into the replication region to the left of the *Hin*dIII site, terminating just in front of the replication origin. Further to the left, three promoters (*P3*, *P4*, and *P5*) are located within the fragment originally derived from R6/5 (2). Two of these promoters (*P3* and *P4*) initiate short transcripts in opposite directions, and it appears from our data that these promoters share some sequences within their RNA polymerase binding sites. It is of special interest that both promoters map in a region that is most likely part of insertion sequence 1. The third promoter, *P5*, causes intense transcription of the region containing the *cat* gene. The transcript, around 800 nucleotides long, is terminated within the sequence homologous to Tn9. The position of this promoter within pACYC184 has led to its isolation on various restriction fragments and to its further characterization (26). It should be noted that the 104 RNA identified in acrylamide gels is synthesized close to the *Hin*dIII site; its promoter is not revealed, however, by electron microscopy because of the intense transcription initiated at *P1* and *P2*.

Our data show that *in vitro* various parts of plasmids pBR322 and pACYC184 are transcribed with very different efficiencies. Both DNA strands of both plasmids contain coding regions with proper termination signals that prevent extensive symmetrical transcription. How meaningful are these *in vitro* results? Previous experiments with phage templates have shown that strand selection and relative promoter strength derived by this method agree well with *in vivo* data (3, 4). The localization in this study of the *bla*, the 104, and the *tet* promoter (*P3*, *P4*, and *P2* in pBR322) as well as the identification of the *cat* promoter (*P5* in pACYC184) and of *P1* (in both plasmids) show the value of such an analysis. Furthermore, the identification of several termination sites revealed a highly structured transcription map and indicates the importance of these signals. In fact, we have strong indications that for plasmid stability the signal strength of promoters and terminators has to be compatible (unpublished data). We wish to emphasize that this functional analysis has not only revealed signals of remarkable strength such as *P1*, which had not been identified previously even though the nucleotide sequences were available, but also uncovered an interesting signal arrangement that may play a major role in the control of Tc resistance.

We thank Dr. A. Klein for supplying pKK1, pKK5, and mutants of phage λ. This work was supported by the Deutsche Forschungsgemeinschaft (Bu 338/8-10) and by Dr. S. Schmus.

1. Bolivar, F., Rodriguez, R. L., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977) *Gene* **2**, 95–113.
2. Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **134**, 1141–1156.
3. Stüber, D., Delius, H. & Bujard, H. (1978) *Mol. Gen. Genet.* **166**, 141–149.
4. Stüber, D. (1980) Dissertation (Universität Heidelberg, Heidelberg, Federal Republic of Germany).
5. Hershfield, V., Boyer, H. W., Chow, L. & Helinski, D. R. (1976) *J. Bacteriol.* **126**, 447–453.
6. Kuypers, B., Reiser, W. & Klein, A. (1980) *Gene* **10**, 192–203.
7. von Gabain, A. & Bujard, H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 189–193.
8. Koller, B., Delius, H., Bünemann, H. & Müller, W. (1978) *Gene* **4**, 227–239.
9. Siddhikol, C., Erbstoeszer, J. W. & Weisblum, B. (1969) *J. Bacteriol.* **99**, 151–155.
10. Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77–90.
11. Boyer, H. W., Betlach, M., Bolivar, F., Rodriguez, R. C., Heyneker, H. L., Shine, J. & Goodman, H. M. (1977) in *Recombinant DNA-Molecules*, Miles Symposium Series, eds. Beers, R. F. & Basset, E. G. (Raven, New York), Vol. 10, pp. 9–19.
12. Chan, P. T., Lebowitz, J. & Bastia, D. (1979) *Nucleic Acids Res.* **7**, 1247–1262.
13. Goebel, W. & Schrempf, H. (1972) *J. Bacteriol.* **111**, 696–704.
14. Chow, L. T. & Bukhari, A. I. (1978) *Gene* **3**, 333–346.
15. Chang, S. & Cohen, S. N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4811–4815.
16. Marcoli, R., Iida, S. & Bickle, T. A. (1980) *FEBS Lett.* **110**, 11–14.
17. Slocombe, P. M., Ely, S. & Timmis, K. N. (1979) *Nucleic Acids Res.* **8**, 1469–1484.
18. Itoh, T. & Tomizawa, J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2450–2454.
19. Barry, G., Squires, C. L. & Squires, C. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4922–4926.
20. Emtage, J. S., Tacon, W. C. A., Catlin, G. H., Jenkins, B., Porter, A. C. & Carey, N. H. (1980) *Nature (London)* **283**, 171–174.
21. Cohen, S. N., Chang, A. C. Y., Boyer, H. W. & Helling, R. B. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3240–3244.
22. Tait, R. C., Rodriguez, R. L. & Boyer, H. W. (1977) *Mol. Gen. Genet.* **151**, 327–331.
23. Conrad, S. E. & Campbell, J. L. (1979) *Cell* **18**, 61–71.
24. Chang, A. C. Y., Nunberg, J. H., Kaufmann, R. J., Ehrlich, H. A., Schimke, R. T. & Cohen, S. N. (1978) *Nature (London)* **275**, 617–624.
25. Tomizawa, J., Ohmori, H. & Bird, R. E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1865–1869.
26. Le Grice, S. F. J. & Matzura, H. (1980) *FEBS Lett.* **113**, 42–46.