Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4134–4137, June 1986
Biochemistry

lac repressor blocks transcribing RNA polymerase and terminates transcription

(transcription regulation/repressor–operator function)

ULRICH DEUSCHLE*, REINER GENTZ, AND HERMANN BUJARD*†

Central Research Units, F. Hoffmann–La Roche and Co. AG, CH-4002 Basel, Switzerland

Communicated by Werner Arber, February 5, 1986

ABSTRACT **Operator sequences are essential elements in many negatively controlled operons. By binding repressors, they prevent the formation of active complexes between RNA polymerase and promoters. Here we show that the *Escherichia coli lac* operator–repressor complex also efficiently interrupts ongoing transcription. This observation suggests a mechanism of action for operators located distal to promoter sequences.**

It is generally believed that repressors of prokaryotic operons act exclusively by preventing the onset of transcription. This view is supported by a wealth of experimental data, of which the most convincing are the structural analyses of regulatory regions: operators are located within the DNA sequence covered by a promoter-bound RNA polymerase (i.e., between positions +20 and −50, where +1 is the first nucleotide transcribed) (1–3) or, as in the case of promoter P_1 of the *Escherichia coli gal* operon, within the cAMP–CAP (catabolite activator protein) binding sites (4). Thus, by occupying an operator, a repressor may either obscure a promoter sequence from being recognized by RNA polymerase or prevent the formation of an active complex between the enzyme and the promoter (5–7). However, within the *lac* operon, as well as within the *gal* operon, additional operator sequences were identified well downstream of the regulatory region (8, 9), and although an *in vivo* function for such an operator was demonstrated in the *gal* system (9), its mode of action has not been elucidated. The most straightforward mechanism, the direct interference of an operator–repressor complex with the transcribing enzyme, is generally ruled out (7, 10) despite suggestive genetic and biochemical data (11–13). Here we present evidence that the *lac* repressor–operator complex is indeed an efficient terminator of transcription *in vivo* and *in vitro*, suggesting an obvious mode of action for operator sequences found, for example, within structural genes of operons.

We had observed that, when transformed with plasmid pGBU207, *E. coli* cells showed differences in tetracycline resistance depending upon the internal level of *lac* repressor. In pGBU207 (14), a *lac* operator sequence is located between promoter P_{H207} and the coding sequence of the *tet* region; therefore, we analyzed the effect of an isolated *lac* operator sequence inserted into a transcriptional unit distal to the promoter.

MATERIALS AND METHODS

Plasmids and Bacteria. The pDS1 vector system and the promoters P_{G25} and $P_{D/E20}$ have been described (15, 16). The *lac* operator was obtained as a 54-base-pair (bp) *Hpa* II–*Alu* I fragment from pBU10 (14). Plasmid pDM1.1, which carries the *lacI*^{q} gene and the p15A replicon, was a gift of M. Lanzer (ZMBH, Univ. of Heidelberg). All plasmids and *in vivo* RNAs were prepared from transformed *E. coli* DZ 291 (14).

***In Vitro* Transcripts.** *In vitro* transcription was carried out under standard conditions (14, 16), whereby a 50-μl assay mixture contained 0.2 pmol of template (construct A, carrying promoter P_{G25} or $P_{D/E20}$ in plasmid pDS1; Fig. 1), 1 pmol of *E. coli* RNA polymerase, and [α-^{32}P]UTP whenever labeling of the transcription products was required. The reaction mixtures were incubated at 37°C in the absence or presence of *lac* repressor (gift of M. Lanzer). Repressor was inactivated by addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 200 μM. In general, incubation was for 3 min before samples were directly prepared for PAGE.

***In Vivo* Transcripts.** *E. coli* cells transformed with the proper plasmid were grown to an OD_{600} of 0.5 in M9 medium containing 10% Luria broth (17). Labeled RNA was obtained by adding 500 μCi (1 Ci = 37 GBq) of [^{3}H]uridine to 10 ml of the logarithmically growing culture. After 1 min at 37°C, cells were quickly chilled in liquid nitrogen and RNA was isolated according to Glîsin *et al.* (18). High intracellular levels of *lac* repressor were achieved by the simultaneous presence of the compatible plasmid pDM1.1. Repressor was inactivated by addition of IPTG (200 μg/ml) to the cultures 60 min before harvest.

Nuclease S1 Mapping (19). A suitable DNA fragment for the characterization of the 3′ ends of *in vivo* and *in vitro* transcripts was obtained by cleaving construct A (Fig. 1) with *Acc* I and *Pvu* II. The *Acc* I cleavage site located 147 bp upstream of the operator sequence was filled in with [α-^{32}P]dATP, resulting in a 3′-labeled 318-bp fragment covering the entire operator sequence. About 0.01 pmol of the labeled DNA fragment was denatured and mixed with one-fourth of an *in vitro* transcription assay mixture or with 10 μg of total cellular RNA. The nucleic acids were allowed to hybridize (volume 30 μl, 80% formamide/0.4 M NaCl/40 mM Tris/HCl, pH 8) for 2 hr before 300 μl of S1 buffer (19) containing 20 units of nuclease S1 were added. After 2 hr at 14°C, the S1-resistant material was analyzed by electrophoresis in 8% polyacrylamide/8 M urea gels.

Quantitation of *in Vivo* RNA by Hybridization. RNA was labeled with [^{3}H]uridine and isolated as described above. Dihydrofolate reductase (DHFR)- and chloramphenicol acetyltransferase (CAT)-specific transcripts were quantified by hybridization with an excess of single-stranded M13 DNA carrying the proper DHFR and CAT gene sequences, respectively. The hybridized material was collected by filtration

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.

Abbreviations: bp, base pair(s); DHFR, dihydrofolate reductase; CAT, chloramphenicol acetyltransferase; IPTG, isopropyl β-D-thiogalactoside.

*Present address: Zentrum für Molekulare Biologie, Heidelberg, Im Neuenheimer Feld 282, D-69 Heidelberg, Federal Republic of Germany.
†To whom all correspondence should be addressed.

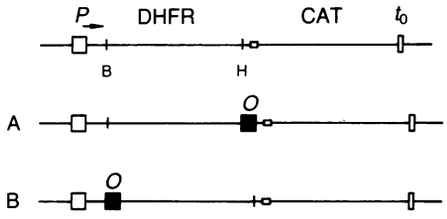

FIG. 1. Transcriptional unit used for operator insertion. The standard transcription unit of the pDS1 vector system (15) contains the coding sequence of the dihydrofolate reductase (DHFR) and the *E. coli* chloramphenicol acetyltransferase (CAT) genes, both of which can be brought under control of a single promoter (*P*). Transcripts of defined size are obtained by the function of terminator t_0 of phage λ, which also prevents extensive read-through into other parts of the plasmid (15). Expression of this unit *in vivo* under the control of promoter P_{G25} or $P_{D/E20}$ (16) yields exclusively CAT protein, since only this sequence carries a functional translational start signal (□). The *lac* operator (*O*) sequence (positions −17 to +34, ref. 7) was fused to either *Hin*dIII or *Bam*HI synthetic linkers and inserted into the *Hin*dIII (H) or *Bam*HI (B) site, resulting in constructs A and B, respectively. The distances between the promoter (*P*) and sites B, H, and t_0 are about 100, 670, and 1700 bp, respectively, depending somewhat upon the position of the promoter within the cloned fragment.

through nitrocellulose and its radioactivity was monitored. This method has been described previously (20, 21).

RESULTS

***lac* Repressor–Operator Complex Functions as a Regulatable Terminator.** The transcriptional unit used in these experiments has been described earlier as part of the pDS1 vector system (15). In these vectors, the coding sequence of DHFR and CAT genes are under the control of a single promoter, giving transcripts of ≈1700 nucleotides due to the terminator t_0 at the end of the *CAT* gene (Fig. 1). The transcription units analyzed here were controlled by either one of two promoters of coliphage T5 [P_{G25} or $P_{D/E20}$ (16)], and a *lac* operator sequence was inserted either between the *DHFR* and the *CAT* sequence (construct A) or into the *Bam*HI site near the promoter (construct B in Fig. 1). Since the *DHFR* sequence is not in-frame with any translational start site, the only protein expected from this expression unit is CAT.

With construct A (in pDS1), no CAT synthesis is observed in *E. coli* cells containing high levels of *lac* repressor (Fig. 2). However, CAT production is rapidly induced to a high level by IPTG, as expected with these promoters (16). This experiment shows that the operator-bound *lac* repressor can efficiently interfere with ongoing transcription. It raises the question whether repressor merely blocks the transcribing enzyme or causes a true termination event. Analysis of *in vitro* and *in vivo* transcripts shows that the *lac* repressor–operator complex acts as a transcription terminator. In the absence of repressor, or in the presence of repressor and IPTG, transcripts of around 1700 nucleotides are the major products *in vitro* (Fig. 3*A*, lanes 1, 4, and 5). In contrast, when active repressor is included in the transcription assay, the vast majority of transcripts are terminated at three distinct positions (a, b, and c in Fig. 3*A*), yielding RNAs about 750 nucleotides long. Of these, only the smallest species can be converted into larger products (lanes 6 and 7). The others are not affected by prolonged incubation with unlabeled nucleoside triphosphates. When IPTG is added together with unlabeled nucleoside triphosphates, no increase in radioactivity is found in the 1700-nucleotide RNA species (data not shown). These results show that the repressor does not simply induce transcribing RNA polymerase to pause but rather triggers an active process of termination. The results

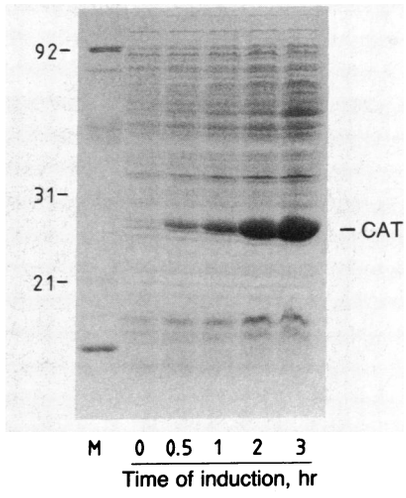

FIG. 2. Effect of operator insertion on CAT synthesis *in vivo*. A pDS1 plasmid carrying construct A with $P_{D/E20}$ as promoter was used to transform into *E. coli* cells carrying the compatible plasmid pDM1.1. The latter plasmid contains the *lacI*^{q} gene and provides high intracellular levels of *lac* repressor. Cultures of the transformed cells were grown to OD_{600} 0.7 before IPTG (200 μg/ml) was added. Aliquots of the culture were removed at times indicated and the pattern of the total cellular protein was monitored by $NaDodSO_4$/PAGE. The Coomassie blue-stained gel shows that the CAT protein, not visible at the time of IPTG addition, is the most prominent product after only 30 min. The size markers (lane M) are given in kDa at left.

of equivalent experiments carried out *in vivo* are shown in Fig. 3*B*. Again, in the absence of operator or in the presence of IPTG, the major plasmid-specified RNA is about 1700 nucleotides long (lanes 1 and 3). In cells containing high levels of repressor, however, two short transcripts of about 750 nucleotides are synthesized (lane 2). Lanes 3 and 4 of Fig. 3*B* show an additional RNA species of about 820 nucleotides (labeled x). This transcript is only observed in the presence of the operator-carrying fragment and when transcription is allowed to proceed past the operator either by addition of IPTG (lane 3) or by limiting amounts of intracellular repressor (lane 4), suggesting that an additional sequence acting as a terminator *in vivo* must be located downstream of the 60-bp operator fragment.

By quantifying DHFR- and CAT-specific RNA (refs. 20 and 21; unpublished work) we find as much as 90% termination *in vivo* (Table 1). This termination can be completely reversed by IPTG. Our data also indicate that the repressor-independent termination at position x (Fig. 3*B*) is ≈17% efficient (data not shown).

Topography of the *lac* Repressor–Operator Termination Signal. Where does an operator-bound repressor force the transcribing RNA polymerase to stop and to release the nascent transcript? To answer this question, we used construct A (Fig. 1), containing promoter P_{G25}, to produce transcripts in the presence or absence of *lac* repressor, and the 3′ ends of the RNAs terminated around the operator sequence were characterized by nuclease S1-mapping with 3′-labeled DNA fragments (Fig. 3*C*). When repressor is bound to the operator, transcription is terminated *in vivo* and *in vitro* at two sites upstream of the operator sequence (Fig. 4). The termination site observed *in vivo* when repressor is limiting or inactive has been mapped outside of but adjacent to the cloned operator fragment. In Fig. 4, the different sites are indicated by hatched columns. It appears most likely to us that the repressor terminates transcription at precise positions and that the regions of 3–5 nucleotides derived from S1-mapping experiments primarily reflect a heterogeneity of the S1 digest. The homogeneous transcript obtained *in vitro*

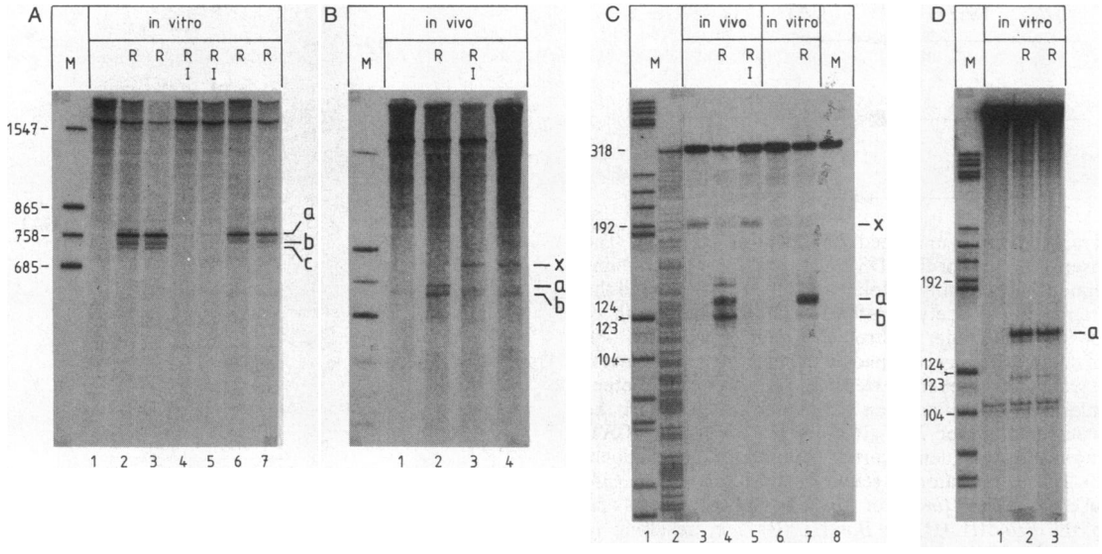

FIG. 3. Analysis of RNA synthesized in the presence or absence of *lac* repressor. (*A*) *In vitro* transcripts obtained from construct A in the absence (lane 1) or presence of 2 μg (lanes 2, 4, and 6) or 5 μg (lanes 3, 5, and 7) of purified *lac* repressor (R) per assay were analyzed by PAGE. Assays in which the repressor was inactivated by IPTG are indicated (I). The transcripts seen around position 1700 are terminated at t_0 (see Fig. 1). In the presence of functional repressor, three shorter species of RNA are identified (a, b, and c), of which c can apparently be "chased" upon addition of an excess of unlabeled UTP and 10 min further incubation (lanes 6 and 7). Addition of IPTG to the transcription assay completely abolishes termination (lanes 4 and 5). Markers (sizes in nucleotides at left) are a digest of pDS1* (15) with *Bam*HI, *Pst* I, and *Xba* I. (*B*) *In vivo* RNA specified by our transcription unit can be visualized directly due to the high efficiency of the promoters utilized. Lane 2 shows the RNA pattern from cells containing high levels of active repressor. The two RNA species visible resemble in size the *in vitro* transcripts terminated at sites a and b. Both species disappear if IPTG (I) is present in the culture (lane 3) or if *lac* repressor-producing plasmid pDM1.1 is absent (lane 4). The majority of transcripts synthesized under these latter conditions are 1700 nucleotides long and comigrate in these gel systems with rRNA. A new class of RNA (x) is visible in lanes 3 and 4. This transcript, which is 820 nucleotides long, is not present when the operator is deleted at the *Hin*dIII site (lane 1). Its termination is repressor-independent but requires the presence of the operator-carrying fragment. Markers are as in *A* but mixed with *Hae* III-cleaved pBR322. (*C*) Nuclease S1-mapping (19) of the 3′ end of *in vivo* and *in vitro* transcripts. Lanes 3 and 4 show the S1-resistant material obtained with *in vivo* RNA in the absence (3) or presence (4) of repressor (R). Lane 5 shows the effect of IPTG (I). Similarly, lanes 6 and 7 contain probes of *in vitro* RNA synthesized in the absence and presence of repressor, respectively. The positions of the 3′ termini of the various RNAs (a, b, and x) were determined by inference with size markers (M): a labeled *Hae* III digest of pBR322 (lane 1) and the G+A sequencing pattern of the 318-bp *Acc* I–*Pvu* II fragment labeled at the 3′ end. (*D*) Precision of *lac* repressor-induced termination. Construct B (Fig. 1) with promoter $P_{D/E20}$ was used to produce short transcripts (≈140 nucleotides) in the presence of repressor. Lanes 2 and 3 show these transcripts, whereas lane 1 contains the repressor-free control. Comparing the width of the bands in lanes 3 and 4 with those of the markers (M, *Hae* III digest of pBR322) suggests a precise termination (within 1–2 nucleotides). All gels contained 8 M urea and were 4% (*A* and *B*) or 8% (*C* and *D*) polyacrylamide. Size markers are denoted with M and given in nucleotides.

when construct B is used as template is in support of this (Fig. 3*D*).

Table 1. Efficiency of transcriptional termination by the *lac* operator–repressor complex

Promoter	Repressor	IPTG	Labeled RNA, cpm DHFR	Labeled RNA, cpm CAT	E,* %
P_{G25}	+	−	14,764	1,652	89
			14,361	1,628	89
	+	+	21,593	16,931	22
			20,748	16,622	20
$P_{D/E20}$	+	−	20,618	2,094	90
			20,097	2,055	90
	+	+	27,356	23,199	16
			26,750	25,140	10

E. coli cells harboring pDS1 (carrying construct A) and pDM1.1 (for repressor production) were grown to an OD_{600} of ≈0.4 before the cultures were divided and IPTG given to one of them. After further incubation at 37°C for 30 min, RNA of both cultures was labeled with [^{3}H]uridine, extracted, and quantified as described in *Materials and Methods*. For both promoters, P_{G25} and $P_{D/E20}$, <10% of transcripts are CAT-specific when IPTG is absent. Upon induction, the CAT-coding region is expressed, though not with the same efficiency as the DHFR sequence. This difference of ≈17% is due to the termination signal identified at position x (Fig. 4).
*Termination efficiency.

DISCUSSION

The data presented above show that the complex between *lac* repressor and operator can efficiently halt transcribing RNA polymerase and cause the release of nascent RNA. The two sites where RNA synthesis is interrupted both lie upstream of the operator sequence. Of these, the major site utilized *in vivo* and *in vitro* (site a in Fig. 4) immediately borders the operator sequence, indicating that the active center of the transcriptional elongation complex can move very close to the hindering repressor–operator complex. This suggests that, in contrast to the promoter-bound enzyme, the transcribing RNA polymerase barely extends in front of its catalytic site. The second site (b in Fig. 4), where release of RNA occurs, is 10 bp upstream of site a. *In vivo*, both sites are utilized with about the same frequency, whereas site a is the preferred one *in vitro*. The intracellular concentration of repressor may have an effect on this phenomenon. The weak termination signal identified at site x is most likely created by integrating the operator sequence into this particular environment, since it occurs about 15 bp outside of the inserted fragment and at a distance 45 bp from the center of the operator sequence.

Although several lines of evidence have indicated that an operator-bound *lac* repressor may interfere with ongoing transcription (9, 12), the view that a transcribing RNA polymerase would "peel off" such DNA-bound proteins was generally accepted. This was also suggested by *in vitro* data (12) that showed that, in the presence of *lac* repressor and RNA polymerase, the *lac* UV5 promoter/operator sequence

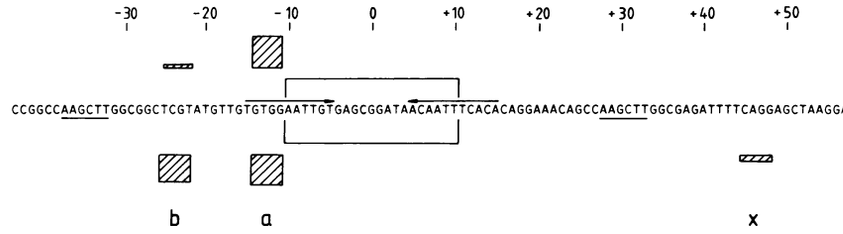

FIG. 4. Sequences involved in repressor-induced transcriptional termination. The central region of the operator sufficient to bind repressor (1, 22) is boxed, and the inverted repeat of the sequence is delineated by arrows. The G in the center of the operator sequence has been used to define position 0. The sites where transcription is terminated are indicated by the hatched columns. The width of the columns reflects the heterogeneity of the S1-resistant material and the height of the columns represents the relative frequency of termination at the respective site. The columns above and below the sequence describe the *in vitro* and *in vivo* results, respectively. Sites a, b, and x correspond to the designations used in Fig. 3. In the presence of repressor, transcripts are terminated upstream of the operator sequence. Termination at site x occurs outside of the original operator fragment. The *Hin*dIII cleavage sites used to insert the 54-bp fragment between the DHFR and the CAT sequence yielding construct A (Fig. 1) are underlined.

can only transiently block a transcriptional elongation complex. By contrast, our data demonstrate that a *lac* repressor–operator complex located distal to a promoter sequence can directly interfere with gene expression by efficiently terminating transcription. This sheds new light on the possible role of operators found outside of the primary regulatory region. Thus, operator/repressor systems could have functions in addition to the one commonly considered—namely, (*i*) to prevent readthrough from upstream regions into the repressed operon and (*ii*) to establish a polarity pattern within an operon that is dependent on the level of inducer and the affinity between a particular operator sequence and a repressor.

These properties could play a role in the fine tuning of gene expression at the transcriptional level and may be considered as a type of attenuation. Systems to examine this hypothesis could be the *gal* as well as the *lac* operon (8, 23, 24). Both operons contain a second operator sequence about 50 and 400 bp downstream of the RNA initiation site, respectively. In the *gal* operon, operator 2, which is located within the structural gene *galE*, may affect transcription from both promoters P_1 and P_2, but primarily from P_2 by attenuation, whereas operator 1 is the main control element of P_1. Finally, Sellitti and Steege (25) reported that transcription from the *lacI* promoter is "punctuated" within the *lac* control region and that this punctuation is strongly influenced by the *lac* repressor. These data suggest that the mechanism of action for repressor/operator systems proposed here is utilized in the *E. coli lac* system.

We thank M. Lanzer for supplying purified *lac* repressor and pDM1.1; W. Kammerer, M. Lanzer, and D. Stueber for helpful discussions; and J. Scaife and S. Le Grice for critical comments and reading of the manuscript. The help of Y. Kohlbrenner in preparing the manuscript is gratefully acknowledged.

1. Gilbert, W. & Maxam, A. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 3581–3584.
2. Gunsalus, R. P. & Yanofsky, C. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7117–7121.
3. Adhya, S. & Miller, W. (1979) *Nature (London)* **279,** 492–608.
4. Shanblatt, S. H. & Revzin, A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1594–1598.
5. Reznikoff, W. S. (1972) in *RNA Polymerase*, eds. Losick, R. & Chamberlin, M. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 441–454.
6. Majors, J. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4394–4399.
7. Reznikoff, W. S. & Abelson, J. M. (1980) in *The Operon*, eds. Miller, J. H. & Reznikoff, W. S. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 221–243.
8. Gilbert, W., Gralla, J., Majors, J. & Maxam, A. (1975) in *Protein-Ligand Interactions*, eds. Sund, H. & Blauer, G. (de Gruyter, Berlin), pp. 193–210.
9. Irani, M. H., Orosz, L. & Adhya, S. (1983) *Cell* **32,** 783–788.
10. Mitchell, D. H., Reznikoff, W. S. & Beckwith, J. (1975) *J. Mol. Biol.* **93,** 331–350.
11. Reznikoff, W. S., Miller, J. H., Scaife, J. G. & Beckwith, J. R. (1969) *J. Mol. Biol.* **43,** 201–213.
12. Horowitz, H. & Platt, T. (1982) *Nucleic Acids Res.* **10,** 5447–5465.
13. Herrin, G. L. & Bennet, G. N. (1984) *Gene* **32,** 349–356.
14. Gentz, R., Langner, A., Chang, A. C. Y., Cohen, S. N. & Bujard, H. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4936–4940.
15. Stueber, D. & Bujard, H. (1982) *EMBO J.* **1,** 1399–1404.
16. Gentz, R. & Bujard, H. (1985) *J. Bacteriol.* **164,** 70–77.
17. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 431–435.
18. Glîsin, V., Crkvenjakov, R. & Byus, G. (1974) *Biochemistry* **13,** 2633–2637.
19. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
20. Deuschle, U. (1986) Dissertation (Univ. of Heidelberg, Heidelberg).
21. Bujard, H., Deuschle, U., Kammerer, W., Gentz, R., Bannwarth, W. & Stueber, D. (1985) in *Sequence Specificity in Transcription and Translation*: UCLA Symposia, eds. Calendar, R. & Gold, L. (Liss, New York), pp. 21–29.
22. von Hippel, P. H. (1979) in *Biological Regulation and Development*, ed. Goldberger, R. E. (Plenum, New York), Vol. 1, pp. 279–347.
23. De Crombrugghe, B., Busby, S. & Buc, H. (1984) *Science* **223,** 831–838.
24. Majumdar, A. & Adhya, S. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6100–6104.
25. Sellitti, M. A. & Steege, D. A. (1985) *J. Cell. Biochem.* **85,** 205.