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Use of Transcriptional Repressors to Stabilize Plasmid Copy Number of Transcriptional Fusion Vectors

PAUL F. LAMBERT* AND WILLIAM S. REZNIKOFF

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706

Received 13 August 1984/Accepted 21 December 1984

Strong promoters cloned into transcriptional fusion vectors can adversely affect plasmid copy number. In this study, we investigated the use of transcriptional repressors, *lacI* and *tetR*, to stabilize the copy number of plasmids containing the *lacUV5* and *tetA* promoters, respectively. Repression of these promoters was found to prevent plasmid copy number variation. Transcriptional strength of these promoters, when cloned into transcriptional fusion vectors, was determined by measuring the rate of synthesis after derepression with inducer. By using this approach, promoter strength can be accurately measured in vivo, without the need to compensate for copy number variation.

An important advance in the study of transcriptional signals has been the use of transcriptional fusion vectors (9). These vectors contain a gene, the expression of which is under the control of a cloned promoter, and whose gene product is assayable. Unfortunately, plasmid replication is adversely affected by strong transcriptional signals, resulting in the variation of plasmid copy number (1, 13). This is thought to be due, in part, to the direct, transcriptional interference of primer RNA synthesis or processing at the origin of plasmid DNA replication (13). In addition, there are two possible mechanisms by which strong promoters could indirectly affect plasmid stability. One possibility is that an increase in the transcriptional and translational load placed on a cell by strong promoters could reduce the efficiency of plasmid DNA replication. A second possibility is that the accumulation of gene products from strong promoters is detrimental to cell growth, leading to the selection of those cells with reduced promoter-gene dosage (3).

Variation in copy number between plasmids containing different promoters leads to gross inaccuracies when comparing promoter strength. As a means of circumventing this problem with plasmid vectors, some investigators have chosen to readopt the use of lambda vectors in which single lysogens are assayed for promoter activity (2, 11, 18). Others have continued assaying promoter strength in plasmids by adjusting gene expression values to account for the differences in copy number (1, 4). A third approach is to stabilize plasmid copy number. Researchers in several laboratories have attempted this by inserting transcriptional terminators upstream of the plasmid replication origin so as to eliminate transcription readthrough (3, 13). This method succeeds in eliminating any direct effect of promoter strength on plasmid replication; however, it does not eliminate the indirect effects discussed above. In this study, plasmid copy number is stabilized by inhibiting the transcriptional activity of cloned promoters with transcriptional repressors. This results in plasmid copy numbers which are equal, irrespective of cloned promoter strength. With the use of inducers, promoter strength is determined by measuring the rate of gene induction over a short period of time (less than one cell doubling time).

* Corresponding author.

We studied the effect of transcriptional repressors on plasmid copy number for two promoters, the lactose operon (*lac*) promoter and the Tn*10* tetracycline gene (*tetA*) gene promoter. Plasmids used in these studies are illustrated in Fig. 1. The transcriptional fusion vector, pRZ5255 (L. Munson, Ph.D. thesis, University of Wisconsin, Madison, 1984), was used in studies of the *lac* promoters. Briefly, this vector is a derivative of plasmid pBR322 containing the W200 *trp-lac* fusion (12) positioned downstream from several unique restriction enzyme sites. Insertion of a DNA fragment containing a promoter upstream of the W200 *trp-lac* fusion leads to the expression of the *lacZ* gene product, β-galactosidase. The kanamycin resistance (Kan^r) gene (*nptII*) from Tn*5* has been inserted into the ampicillin resistance (Amp^r) gene (*blaA*) in pBR322, allowing for continuous selection by antibiotic in liquid culture. The 95-base-pair fragments containing the $lacP^+$ and *lacUV5* promoters were inserted between the *Eco*RI and *Sal*I restriction enzyme sites in pRZ5255, oriented correctly to direct the expression of *lacZ*. As cloned in pRZ5240, the *lac* promoter $lacP^+$ does not contain the catabolite activating protein-cyclic AMP binding site; consequently, this clone has very little transcriptional activity. The *lacUV5* promoter, cloned into pRZ5245, is a catabolite activating protein-independent mutant which has strong transcriptional activity. The strong *tetA* promoter was cloned into pBR322 by Wray and Reznikoff (16). In this construct, pRT301, the *tetA* promoter does not direct the expression of an assayable gene such as *lacZ*; however, the effect of the strong promoter on plasmid stability can still be observed and studied. The effect of promoter strength on plasmid copy number was measured by two methods: plasmid stability assays and plasmid copy number measurements. Transcriptional activity of *lac* promoter constructs was measured under various conditions, and these results are discussed with respect to plasmid stability.

The multicopy plasmid pBR322, as well as its derivatives, are randomly segregated at cell division. Therefore, the likelihood of a daughter cell receiving no copies of the plasmid is a function of copy number. This correlation between plasmid copy number and plasmid stability has been established (6). For this reason, we used the plasmid stability assay, described previously (10), as one means of

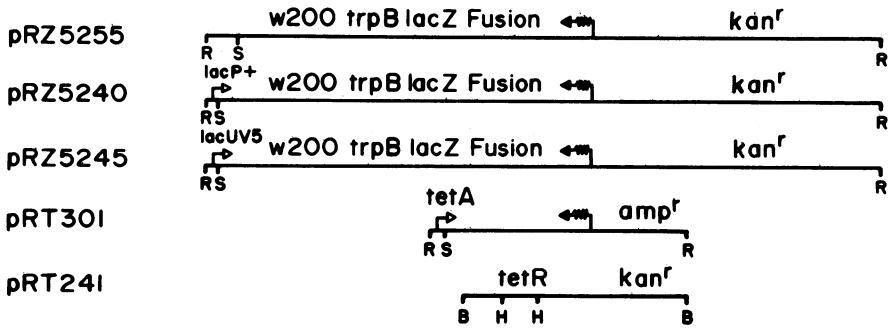

FIG. 1. Plasmids used in this study. The transcription fusion plasmid pRZ5255 and the derivatives pRZ5240 and pRZ5245 are drawn linearized at their unique *Eco*RI sites. The W200 *trp-lac* fusion (12) and Kan^r (*nptII*) gene are identified. Plasmids pRZ5240 and pRZ5245 are derivatives of pRZ5255 containing the 95-base-pair *Alu*I *lacP*$^+$ and *lacUV5* promoter fragments, respectively, cloned between the *Eco*RI and *Sal*I sites. PRT301 (14), illustrated in linear form cut at the unique *Eco*RI site, contains the *tetA* promoter as well as the Amp^r (*blaA*) gene. Plasmid pRT241, illustrated in linear form cut at the unique *Bam*HI site, is a derivative of the pBR322-compatible plasmid, pACYC177, which contains the *tetR* gene as well as the Kan^r gene (14). Symbols: ◄┐, position and orientation of transcription at the pBR322 origin of DNA replication; ┌►, direction of transcription from a cloned promoter. Restrictions enzyme sites are symbolized as follows: B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

measuring the effects of promoter strength on plasmid copy number. In these experiments, strains carrying the plasmid of interest are first grown in liquid culture under selective pressure (i.e., in the presence of drug), then transferred to medium lacking drug, and grown for 100 cell generations. At 20-generation intervals, cells are diluted into fresh medium, and a sample of cells is plated on nonselective, agar medium. One hundred colonies are subsequently scored for resistance to the original drug.

Figure 2A (open symbols) illustrates plasmid stability curves for plasmids pRZ5255, pRZ5240, and pRZ5245 grown in strain CSH26 (*ara* Δ*lac-pro thi*). Plasmid pRZ5245, containing the strong *lacUV5* promoter, is lost at a fast rate compared with the rates of plasmid loss measured for plasmids pRZ5255, containing no promoter, and pRZ5240, containing the weak *lacP*$^+$ promoter. The experimentally derived rate for loss of plasmid pRZ5245 increases faster with respect to time than that seen in computer-generated curves for plasmid loss based upon Gaussian distribution of randomly segregated plasmid populations (Fig. 2B). The reason for this difference is that the computer model does not take into account the growth advantage that cured cells have over cells harboring plasmids (7). It is still possible, however, to estimate plasmid copy number by comparing the initial slopes of experimental and computer-generated curves. By this approach a copy number of 4 is suggested for pRZ5245. In the cases of pRZ5255 and pRZ5240, there is so little plasmid curing that one can only indicate a minimum plasmid copy number between 6 and 8. These results indicate that the *lacUV5* promoter has reduced the plasmid copy number by 33% or more.

Plasmid copy number also was measured directly (Table 1). Cultures were grown to an optical density at 550 nm of 0.5, and plasmid DNA was prepared by the procedure of Ish-Horowitz and Burke (5). Accurate cell counts were made of each culture. DNA samples, corrected for cell number, were electrophoresed on 1% agarose gels, and direct-to-gel hybridizations (14) were performed with ^{32}P-labeled pBR322 DNA. Autoradiographs were scanned, and plasmid bands were cut out and quantitated by liquid scintillation counting. Relative copy numbers were calculated based upon calculating the ratio of counts hybridized to the promoter clone (pRZ5240 or pRZ5245) to counts hybridized to the parent vector (pRZ5255). Plasmid copy number experiments were performed in triplicate; less than 10% error was found. The numerical value attained by this approach indicates the copy number of the promoter clone relative to the vector pRZ5255 (a value of 1.0 indicates that the copy number for the promoter clone is equal to that of pRZ5255). One cannot obtain an accurate absolute copy number by this approach. We chose this approach because it eliminated the need to estimate chromosomal DNA content which can lead to increased error. The results indicate that, although pRZ5240 has the same copy number as pRZ5255, pRZ5245 has 0.6 times that of the control plasmid, or a 40% reduction in plasmid copy number.

The stability experiments were repeated for the same three plasmids in the CSH26(F′ *lacI*^{q} *lacZU118 proA*$^+$ *proB*$^+$) background (Fig. 2A, closed symbols). The *lacI*^{q} mutation causes the overproduction of the *lacI* gene product in sufficient amounts to repress the *lac* promoter when present in multiple copies (see β-galactosidase assays, Table 1). In the *lacI*^{q} background pRZ5245 was no longer unstable, having a plasmid stability curve indistinguishable from that of pRZ5240 or pRZ5255. Plasmid copy number measurements also indicated that all three plasmids have equal copy numbers (Table 1) in the CSH26(F′ *lacI*^{q}) background. Thus, the adverse effects of the *lacUV5* promoter on plasmid copy number were inhibited by the *lac* repressor.

The tetracycline resistance gene (*tetA*) promoter is a strong promoter under tight regulation by the tetracycline repressor (15, 17). The presence of tetracycline in the medium causes the derepression of the *tetA* gene. When cloned into an ampicillin resistance, β-galactosidase transcriptional fusion vector, the *tetA* promoter expresses β-galactosidase at a high level and gives rise to *lacZ*$^-$ segregants at a high frequency in strains harboring this plasmid. These *lacZ*$^-$ cells have been characterized and found to contain plasmids deleted in the *tetA* promoter region (unpublished data). The apparent selection for loss of the *tetA* promoter may be indicative of a strong disadvantage to those plasmids containing this strong promoter. These preliminary observations led us to study the effect of the *tetA* promoter on plasmid stability. In plasmid stability experiments, plasmid pRT301 containing the *tetA* promoter was completely lost from strain CSH26 within 40 generations (Fig. 2C). However, when the compatible plasmid pRT241 (16) which contains the tetracycline repressor (*tetR*) gene is

introduced into the above strain, pRT301 is stably maintained (Fig. 2C). The presence of a sublethal dose of the inducer, tetracycline (0.1 μg/ml), sufficient to derepress the *tetA* promoter, causes the return of plasmid instability (Fig. 2C). Based upon a comparison with the computer-generated plasmid stability curves (Fig. 2B), pRT301 in *tetR*$^+$ background has a copy number of greater than 6 when grown in the absence of the inducer, tetracycline, and a copy number of 2 when grown in the presence of tetracycline.

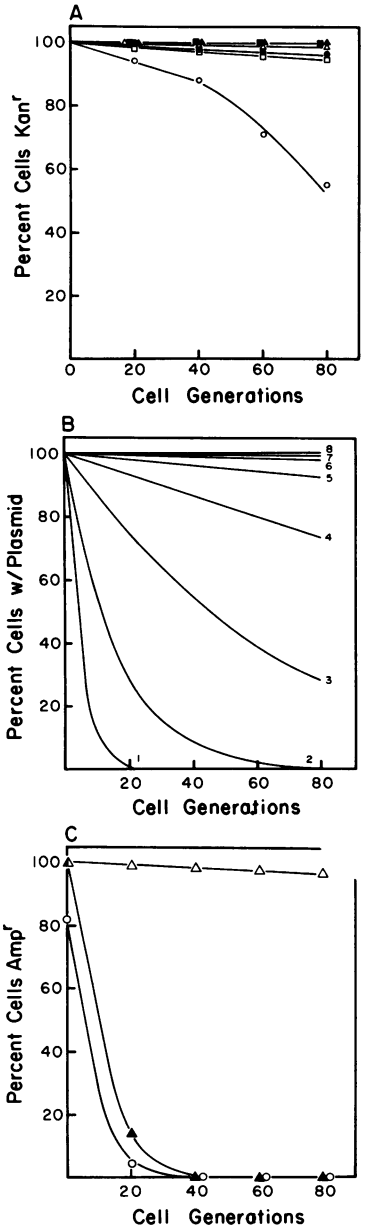

FIG. 2. (A) Plasmid stability curves for plasmids pRZ5255 (□,■), pRZ5240 (△,▲), and pRZ5245 (○,●) grown in strain CSH26 (open symbols) or strain CSH26(F′ *lacI*^{q}) (closed symbols). Each point represents the average of three stability experiments. (B) Theoretical plasmid stability curves were generated by computer based upon the iterative equation: $A(T) = (1 - 0.5^{2C})^T$, where T = generation number, C = copy number, and $A(T)$ = percentage of the cell population that contains plasmid. Plasmid copy numbers are indicated for each curve. (C) Plasmid stability curves for strains harboring plasmid pRT301 which contains the *tetA* promoter. Curves represent experiments performed in strains CSH26 (○) and CSH26 harboring plasmid pRT241 grown in the presence (△) or absence (▲) of subinhibitory levels of tetracycline (0.1 μg/ml).

TABLE 1. β-Galactosidase measurements[a] and relative copy numbers[b] for transcriptional fusion constructs

Plasmid	Promoter	Steady-state values for:		Rate of induction values for CSH26(F′ *lacI*^{q})
		CSH26	CSH26(F′ *lacI*^{q})	
pRZ5255	None	7.6 (1.0)	6.0 (1.0)	1.0 (1.0)
pRZ5240	*lacP*$^+$	5.2 (1.1)	4.3 (0.9)	8.0 (0.9)
pRZ5245	*lacUV5*	370 (0.6)	7.4 (1.0)	830 (1.0)

[a] Steady-state values are reported in units of β-galactosidase; rate of induction values are reported in (units of β-galactosidase synthesized per minute) × 100.

[b] Relative copy number values are presented within parentheses. Relative copy number is described in the text. Briefly, this value represents the ratio of the plasmid copy number for the promoter-containing plasmid relative to the vector-only plasmid.

To illustrate the extent of plasmid loss due to transcriptional activity by the *tetA* promoter, plasmid DNA was isolated from strain CSH26 harboring both pRT301 and pRT241 and electrophoresed on agarose gels (Fig. 3). When DNA is isolated from a culture grown in the presence of ampicillin but in the absence of the inducer, tetracycline, both plasmids are present (lane 1). The same result is obtained (lane 2) when the strain is grown for 40 generations in the absence of both drug selection (ampicillin) and inducer (tetracycline). This indicates that, even in the absence of selective pressure, pRT301 is stable when the *tetA* promoter is transcriptionally inactive. DNAs from duplicate cultures grown for 40 generations in the absence of drug selection (ampicillin) but in the presence of the inducer, tetracycline, were run in lanes 3 and 4. Here, derepression of the *tetA* promoter results in the complete loss of pRT301 DNA. Lanes 5 and 6 contain DNA samples taken from strains harboring pRT241 or pRT301, respectively. Together with results of the plasmid stability assays (Fig. 2C), these experiments point to the ability of the tetracycline repressor to stabilize plasmid pRT301 containing the *tetA* promoter.

Transcriptional activity was measured for the *lac* promoter constructs and correlated with plasmid stability meas-

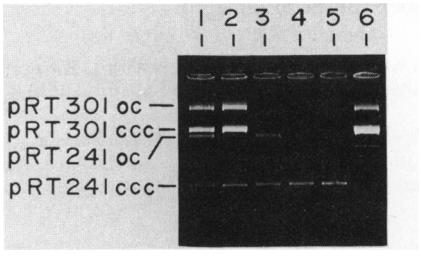

FIG. 3. Agarose gel electrophoresis of plasmid DNAs isolated from strain CSH26 harboring both pRT301 and pRT241 (lanes 1 to 4). Lane 1 is DNA taken from cells grown in the presence of ampicillin. Lane 2 is DNA taken from cells grown for 40 generations in the absence of ampicillin. Lanes 3 and 4 are samples taken from duplicate cultures grown as described for lane 2 but in the presence of the inducer, tetracycline. Lanes 5 and 6 are DNA samples taken from strain CSH26 harboring pRT241 (lane 5) or pRT301 (lane 6). Supercoiled (ccc) and relaxed (oc) forms of the two plasmids are labeled.

urements. Steady-state β-galactosidase assays were performed as previously described (8) on the *lacUV5* (pRZ5245) and *lacP*$^+$ (pRZ5240) clones in both strains CSH26 (*lacI*$^-$) and CSH26(F′ *lacI*^{q}) (Table 1). In the *lacI*$^-$ background the strong *lacUV5* promoter gave 380 U of β-galactosidase activity, or 70 times the level of β-galactosidase measured in the *lacP*$^+$ strain, whereas, in the *lacI*^{q} background the *lacUV5* promoter gave only 6 U, less than 2 times the background. This result suggests that the *lacI*^{q} strain produces sufficient repressor to completely inhibit the *lacUV5* promoter present in multiple copies and correlates well with the stabilization of plasmid copy number observed under these same conditions.

Rates of β-galactosidase expression on plasmids pRZ5240 and pRZ5245 were measured in strain CSH26(F′ *lacI*^{q}) after induction with 2 mM isopropyl-β-D-thiogalactopyranoside. The rate measurements were obtained over a 45-min period after induction with isopropyl-β-D-thiogalactopyranoside; during this time we saw no change in the copy number of any plasmids (data not shown). In the *lacI*^{q} background the *lacUV5* promoter is 120 times as active as the *lacP*$^+$ promoter (Table 1). This value is approximately twice as great as that measured in the *lacI*$^-$ strain under steady-state conditions. We believe this reflects the stabilization of plasmid copy number by transcriptional repression. Indeed, if the steady-state values for β-galactosidase expression in the *lacI*$^-$ background are corrected for differences in relative copy number (Table 1), the strength of *lacUV5* with respect to *lacP*$^+$ more closely equals the value determined in the *lacI*^{q} induction experiment.

It is interesting to note that in the induction experiments the rate of β-galactosidase expression off the *lacP*$^+$ construct was reproducibly higher than the rate of expression off the control plasmid, pRZ5255. In the steady-state assays, however, no difference in expression could be detected above experimental error. This increased sensitivity, by using the rate of induction assay, may be of value in studying weak promoters.

We found that transcriptional repressors stabilize the copy number of plasmids carrying strong promoters, thus eliminating the need to compensate for differences in plasmid copy number when assaying promoter strength or multicopy vectors. This approach is applicable to the study of promoters which are tightly regulated, including those promoters under the control of attenuators or positive regulators as well as repressors. By repressing transcriptional activity, both direct and indirect effects of strong promoter on plasmid copy number can be eliminated.

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