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Reversal of Inhibition by the T7 Concatemer Junction Sequence on Expression From a Downstream T7 Promoter

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We have previously reported that placement of the phage T7 concatemer junction (CJ) just upstream of another gene on a plasmid in a T7 system proved to be inhibitory to expression of the downstream gene. We had hypothesized that the inhibition was a result of a readthrough transcript of the CJ element interacting with the translation start region of the downstream gene; also that in the absence of a T7 termination signal, transcription continued around the plasmid multiple times ("rolling circle" transcription), always juxtaposing the inhibitory CJ sequence proximal to the downstream gene mRNA. Two strong predictions were made from this model: 1) that introduction of a spacer sequence between the CJ element and the downstream gene should alleviate the inhibition, and 2) that reintroduction of a T7 transcription terminator should prevent rolling circle transcription, thereby reversing the inhibition by allowing some transcripts to be generated originating from the downstream promoter that did not contain the inhibitory CJ element upstream. We report here that both of these predictions have been fulfilled. However, the reversal of inhibition was only partial in the construct where the T7 terminator was reintroduced, indicating that there remains a residual inhibitory effect of the CJ element on expression of the downstream gene. A possible explanation is that the CJ element, acting as a pause site for transcription, blocks access to the downstream T7 promoter, thereby reducing transcription from that promoter. If this explanation is correct, steric hindrance of transcription starts resulting from an upstream RNA polymerase pause site may represent a previously unrecognized mechanism of transcriptional control.

T7 expression systems T7 transcription terminator RNA polymerase pause sites Steric hindrance of transcription starts

IN the course of attempting to develop a T7 vector from which the encoded mRNAs were less stable than messages from standard T7 constructs (whose mRNAs possess 3′ stem–loop structures), we previously tried utilizing the phage T7 concatemer junction sequence, which had been reported to contain an element that facilitates T7 transcription termination in vitro (6,8,9). In our previous study, to our surprise, we found that the 17 basepair T7 concatemer junction (CJ) sequence inhibited expression from a gene driven by a T7 promoter located immediately downstream (5). Because the downstream promoter was shown to be perfectly functional, we proposed that the basis for the inhibition was that transcription did not terminate efficiently in vivo at the CJ sequence, and that the readthrough mRNA assumed a conformation that obscured ribosome access to the translation start site of the downstream gene. We further hypothesized that in the absence of an efficient T7 terminator, putative giant readthrough transcripts (10,12) continuing around the plasmid ("rolling circle" transcription) prevented translation of the downstream gene even from mRNA that initiated at the downstream promoter, because in the readthrough

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transcripts the downstream translation start sequences were still mostly configured with the inhibitory CJ sequence juxtaposed just upstream (5).

Two strong predictions emerged from these hypotheses: 1) that insertion of a spacer DNA sequence between the CJ sequence and the downstream promoter should reactivate expression from the downstream gene even in the absence of efficient transcription termination, and 2) that introduction of a bona fide T7 terminator into the vector, to stop the rolling circle transcription, would also restore expression from the downstream gene because transcripts from the downstream promoter would no longer contain the inhibitory upstream sequences. In this report, we demonstrate that both of these predictions have been fulfilled. However, the extent of reversal of inhibition in the vector where a T7 terminator was introduced was not as high as expected. Thus, it remains possible that still another explanation previously considered—putative pausing by the T7 RNA polymerase at the CJ sequence—may also play a role after all, by transiently obscuring transcription starts at the downstream promoter. This could account for the less than robust reversal of inhibition in the vector with the T7 terminator reintroduced.

MATERIALS AND METHODS

Starting Vectors

The series of vectors constructed and tested was based upon vector pCT1 (11), previously used to study effects of rare codons on expression (3,4,11). With the exception of the CJ element, all the components of the vectors were assembled, as described in Rosenberg et al. (11), from pET vectors and plasmids carrying elements of T7 DNA, generously provided by Alan Rosenberg (Brookhaven National Laboratory). Vector pCT1.1 was generated in our previous study (5). Figure 1 illustrates the overall plasmid scheme (circle, top), with details of the various inserts and their differences between the *Bgl*II sites shown on the lines below.

Construction of pCT1.2

First, the Tϕ element (Fig. 2) was PCR amplified from vector pCT1. A 35-nt left primer (ggaaccttgacg tctgagttggctgctgccaccgc) introduced the *Aat*II (gac gtc) site at the 5′ end of the element, replacing the *Nhe*I site. A 24-nt right primer (gtgccacctgacgtctaa gaaacc) complemented the *Aat*II site at the 3′ end of the segment, utilizing the natural *Aat*II site in the vector (see Fig. 1, circle).

The polymerase chain reaction of pCT1 with these two primers yielded a 215-nt fragment that was cut with *Aat*II and ligated directly into the 5893-nt linear *Aat*II-cut pCT1.1. This inserted the Tϕ element of pCT1 into pCT1.1, at the *Aat*II site. PCR colony screening with the CJ element as left primer and a right primer (cccctcaagacccgtttagagg) complementary to a portion of the Tϕ element confirmed orientation of the insert. A schematic diagram of pCT1.2 from the *Bgl*II site upstream of the test gene through the *Aat*II site after the end of the Tϕ terminator is shown in Figure 1. This structure was confirmed by DNA sequencing performed by the NJ Medical School Molecular Resource Facility, under the direction of Robert Donnelly, Ph.D.

Construction of pCT1.3

We began with a limited *Bgl*II digestion of pCT1.1, in the presence of alkaline phosphatase to prevent religation. The resulting fragments were separated on low-melt agarose. The single-cut band was expected to include single cuts at the left, middle, or right *Bgl*II sites. A 2.4-kb *Bgl*II fragment from another vector, pJC27 (1), was then ligated into the *Bgl*II-cut pCT1.1 plasmid. The 2.4-kb *Bgl*II fragment from pJC27 contains the CAT gene for chloramphenicol resistance, and the p15a origin of replication, which is compatible with the ColE1 origin of pCT1 vectors; the fragment is not known to contain any sequences that could function as a terminator for T7 transcription. Transformants were selected on plates with both ampicillin and chloramphenicol. PCR colony screening identified clones with the 2.4-kb insertion at the middle *Bgl*II site of pCT1.1, using a left primer (cgtaaacagtgtcattga) from the portion of the test gene that is deleted in the control gene, and a right primer (gggaaaccgttgtggtctc) complementary to part of the ϕ10 element. These primers are indicated by the horizontal arrows above the schematic diagram of pCT1.3 in Figure 1. Although there are two ϕ10 elements in the vector, only the downstream element can amplify with the left primer. This screen worked because when the insertion is in the left or right *Bgl*II sites, PCR amplifies 438 nt, but when the insertion is at the middle *Bgl*II site, the amplification product is ~2.8 kb. The junction sequences and orientation of the 2.4-kb insert were determined by DNA sequencing carried out by the NJ Medical School Molecular Resource Facility. The CAT gene turned out to have been inserted in the same orientation as the test and control genes (from left to right

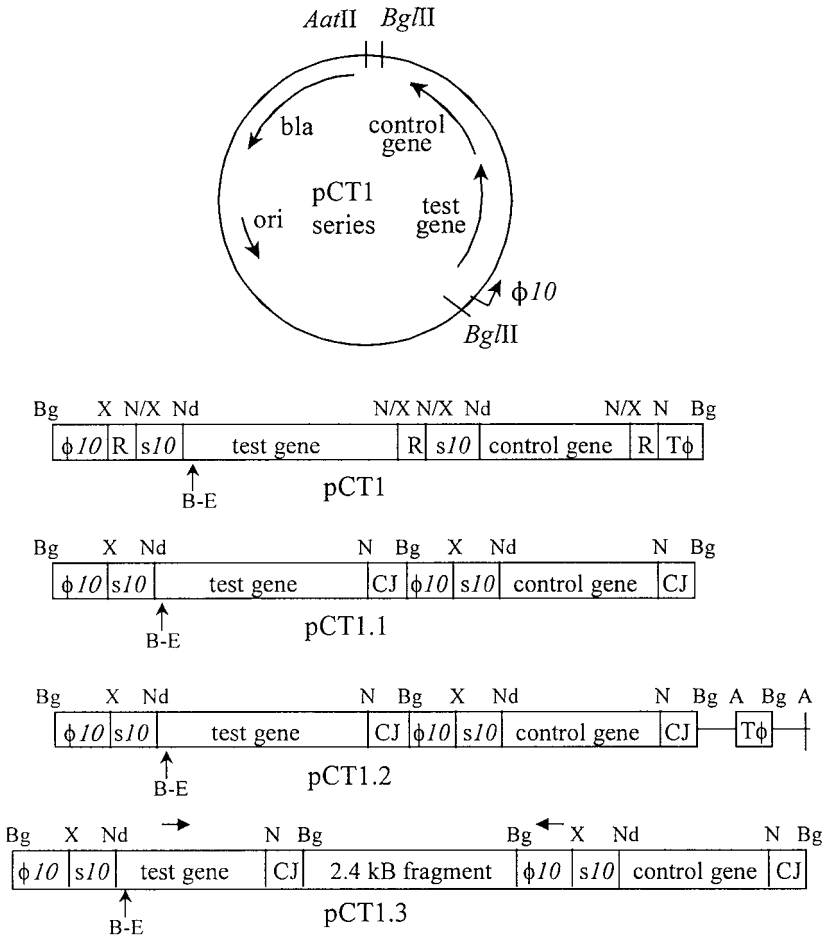

Figure 1. Arrangement of elements in the pCT1 series of vectors. ϕ10 represents a promoter for T7 RNA polymerase, s10 is a strong translational start site, and CJ is a 17-bp sequence from the T7 concatemer junction. The test and control genes are derived from T7 gene 9, with a 45 codon in-frame deletion from the test gene to derive the control gene. A *Bam*HI-*Eco*RI site in the test gene allows insertion of nucleotides after codon 13. R represents an RNase III cleavage site, and Tϕ is a transcriptional terminator for T7 RNA polymerase. *bla* represents the plasmid β-lactamase gene, and *ori* represents the plasmid's ColE1 origin of replication. The 2.4-kb fragment is from a *Bgl*II digestion of plasmid pJC27 (1). The arrows above pCT1.3 represent the primers used to identify the construct. Restriction sites: Bg, *Bgl*II; A, *Aat*II; X, *Xba*I; Nd, *Nde*I; N, *Nhe*I; N/X, *Nhe*I-*Xba*I fusion; B-E, *Bam*HI-*Eco*RI cloning site.

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6132  ctaataacgc tagctgagtt ggctgctgcc accgctgagc aataactagc
6192  ataacccctt ggggcctcta aacgggtctt gaggggtttt ttgctgaaag
6242  gaggaactat atccggatcg agatctaatt cttgaagacg aaagggcctc
  25  gtgatacgcc tatttttata ggttaatgtc atgataataa tggtttctta
  75  gacgtcaggt ggcac
```

Figure 2. Sequence of Tϕ element in pCT1 (6258 nt) continuing downstream to *Aat*II site. *Nhe*I (g/ctagc) (nt 6141), *Bgl*II (a/gatct) (nt 6253), *Aat*II (gacgt/c) (nt 79) sites in underlined italics (β-Lactamase starts at nt 211.)

in Fig. 1); the ATG start codon is 1293 nucleotides downstream of the *Bgl*II site following the test gene and CJ element.

Measurements of mRNA and Protein Synthesis

The chromosome of the host cell for these experiments, BL27(DE3)pLysS (11), contains an integrated T7 RNA polymerase gene, controlled by the *lac*UV5 promoter. IPTG induces host RNA polymerase transcription of the T7 RNA polymerase gene; T7 RNA polymerase then transcribes the plasmid-borne test and control genes. Because the *lac*UV5 promoter is intrinsically leaky, there is basal activity of T7 RNA polymerase. To minimize this activity, another plasmid, pLysS, is added; pLysS carries the gene for T7 lysozyme, an inhibitor of T7 RNA polymerase. Only upon induction of the *lac*UV5 promoter is T7 RNA polymerase produced in large enough quantities to escape inhibition by T7 lysozyme, thereby amplifying expression of T7 target genes. Because pCT1.3 carries a 2.4-kb fragment containing both the CAT gene and a p15a origin of replication, both of which are present on the pLysS plasmid, it was not expected that pLysS would survive in cells transformed by pCT1.3. However, in at least in short-term expression experiments performed soon after transformation by pCT1.3, pLysS appeared to be maintained.

Labeling mRNA and protein in this system has been described by Gao and Goldman (2) and Harvey et al. (5). To briefly reiterate, cells were grown at 37°C in low-phosphate 3-[*N*-morpholino] propanesulfonic acid (MOPS) minimal medium with 50 μg/ml ampicillin and 25 μg/ml chloramphenicol to 2×10^8 cells/ml and induced with 1 mM IPTG. Ten minutes later, ~50 μCi/ml [^{32}P]phosphoric acid (Amersham) was added. Some cultures also had 100 μg/ml rifampicin added, shutting off host transcription while allowing T7 transcription. Thirty minutes after induction, a chase of 40 mM (nonradioactive) sodium phosphate, pH 7.4, was added. Samples (50 μl) were labeled for 2 min at various times with 20 μCi/ml, 50 Ci/mmol [^{35}S]methionine (Amersham) and stopped on ice with 25 μl 3× MOPS cracking buffer [60 mM MOPS, 24 mM sodium acetate, 3% SDS, 6 mM EDTA, 30% glycerol, and 0.15% each of bromophenol blue and xylene cyanole FF, stored at room temperature; 3% (v/v) β-mercaptoethanol is added before use], then boiled in capped tubes for 2 min; 10 μl of each sample was subjected to electrophoresis on sodium-dodecyl-sulfate polyacrylamide gels (SDS-PAGE) in which the 5% top gel was approximately equal in length to the 10% bottom gel. RNA species up to ~2 kb can be resolved in the top gel, and protein species in the bottom gel, as described (2). The dried gel was autoradiographed and also exposed in a phosphorimager.

The test gene, derived from T7 gene 9, is 316 amino acids in length (~38 kDa), the control gene, also derived from T7 gene 9, is 262 amino acids long (~31.5 kDa), while the CAT gene (introduced in pCT1.3 on the inserted 2.4-kb fragment) is 219 amino acids (~26 kDa), all easily distinguishable by SDS-PAGE. The test mRNA is ~1.1 kb, while the control mRNA is ~0.9 kb. Although the top part of the gel, in which these mRNAs migrate, contains SDS, it does not contain an RNA denaturant; thus, RNA migration is not necessarily proportional to size in these gels.

RESULTS AND DISCUSSION

Summary of Differences Between the Various Constructs

Upon completion of construction of pCT1.2 and pCT1.3, and confirmation of their sequences, these plasmids were placed in an expression host and assayed for production of test and control mRNAs and proteins, in parallel to cells containing parental plasmids pCT1 and pCT1.1. As diagramed in Figure 1, pCT1.1 contains the following differences from pCT1: a) the RNase III processing sites at the 3′ ends of the test and control genes have been replaced by CJ elements, and the RNase III site at the 5′ end of the test gene in pCT1 has been removed; b) the T7 transcription terminator (Tϕ) has been removed; and c) an additional T7 promoter (with *Bgl*II site at the 5′ end) was added just upstream of the control gene in pCT1.1. pCT1.2 is identical to pCT1.1 except for reintroduction of the T7 terminator just downstream of the pCT1.1 cassette. pCT1.3 likewise contains the same elements as pCT1.1, but with a 2.4-kb fragment as a spacer inserted between the test gene (ending with the CJ element) and the control gene (beginning with the downstream T7 promoter).

Inhibition of Synthesis of Control Protein in pCT1.1

The results reported in Harvey et al. (5) were confirmed in the present set of experiments. Labeled proteins and RNAs in the T7 system were subjected to SDS-PAGE in gels that allow both mRNA and protein to be detected (2). In Figure 3 (top) uninduced pCT1 (lane 1) and pCT1.1 (lane 4) showed low levels of test and control proteins and mRNAs. After induction, mRNA bands for test and control messages were abundant in pCT1 (lanes 2 and 3 of top panel). In these samples, ^{32}P was added 10 min after induction, and a chase of nonradioactive phosphate was added

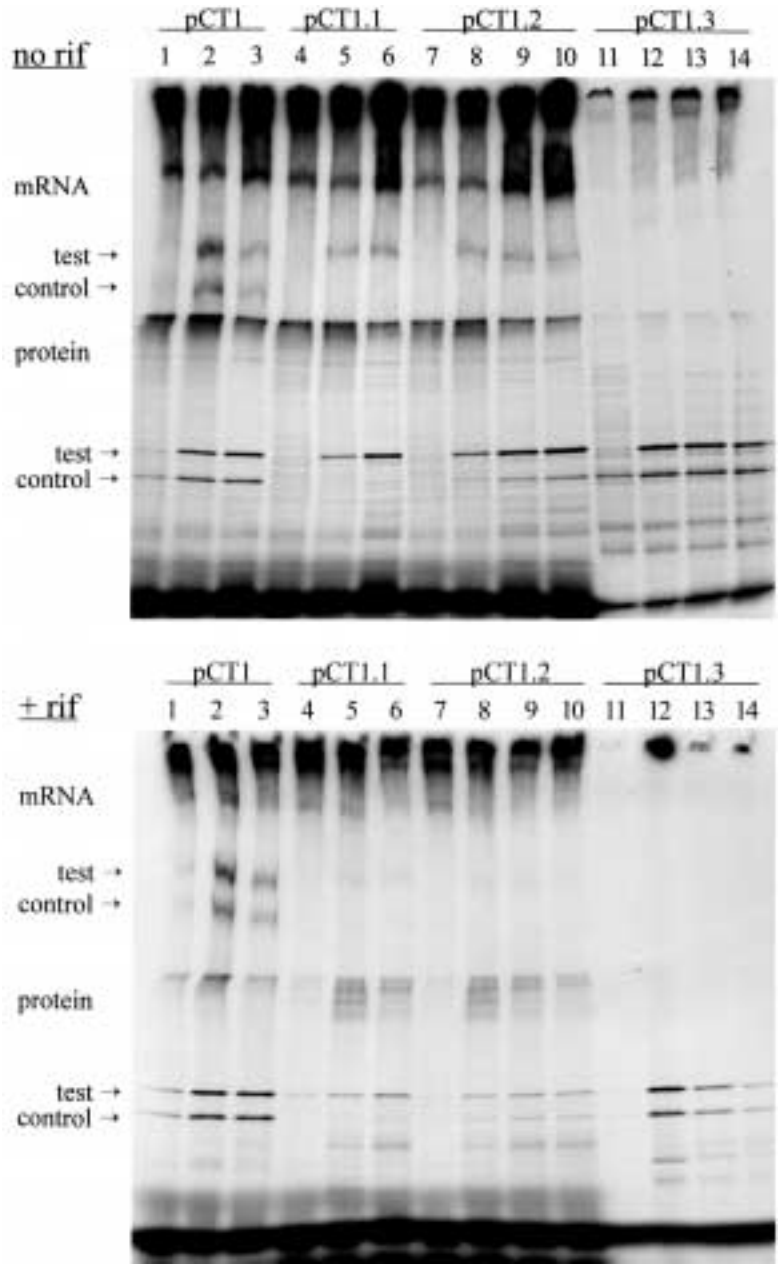

Figure 3. Test and control RNA and protein synthesis in cells containing the pCT1 series of vectors. Cultures were grown, labeled, and subjected to SDS-PAGE as described in Materials and Methods. pCT1: lanes 1–3; pCT1.1: lanes 4–6; pCT1.2: lanes 7–10; pCT1.3: lanes 11–14. Uninduced cultures were labeled for 20 min with [^{32}P]phosphate and 2 min with [^{35}S]methionine in the absence (lanes 1, 4, 7, 11, top panel) or presence (lanes 1, 4, 7, 11, bottom panel) of 100 μg/ml rifampicin (added at the same time as the ^{32}P label). All the other cultures were induced with IPTG at time 0, labeled with [^{32}P]phosphate at 10 min in the absence (lanes 2–3, 5–6, 8–10, 12–14, top panel) or presence (lanes 2–3, 5–6, 8–10, 12–14, bottom panel) of rifampicin (added at the same time as the ^{32}P label), and chased with sodium phosphate at 30 min. Samples (50 μl) were labeled for 2 min with [^{35}S]methionine at 30 min (lanes 2, 5, 8, 12), 45 min (lanes 3, 6, 9, 13), or 60 min (lanes 10, 14) postinduction. (Note that the 45- and 60-min samples were chased with nonradioactive phosphate starting at 30 min postinduction.) The dried gel was exposed to X-ray film for approximately 6 h.

at 30 min. Lanes 2 and 5 show incorporation after the initial labeling, while lanes 3 and 6 were chased for 15 min. Samples were also labeled for 2 min with [^{35}S]methionine at 30 min (lanes 2 and 5) and 45 min (lanes 3 and 6) after induction. There was good synthesis of both test and control proteins in pCT1 (top, lanes 2 and 3), but only the test protein was synthesized significantly in pCT1.1 (top, lanes 5 and 6), as previously reported (5).

Parts of the cultures were treated with rifampicin at the same time as ^{32}P addition (Fig. 3, bottom). After 20 min in rifampicin, pCT1 showed good syn-

thesis of test and control proteins even in the uninduced samples (bottom, lane 1) while pCT1.1 showed only a little detectable synthesis of test protein in the uninduced samples (bottom, lane 4). pCT1 samples treated with rifampicin showed similar RNA synthesis as samples without rifampicin, while the induced RNA band seen in pCT1.1 (top, lanes 5 and 6) is probably not T7 specific, because it isn't synthesized in rifampicin-treated samples (bottom, lanes 5 and 6). This band could result from host synthesis, because pCT1.1 does not shut off host transcription following induction compared with typical T7 vectors. Protein expression in the rifampicin samples shows pCT1 synthesizing both test and control proteins (bottom, lanes 2 and 3), while pCT1.1 synthesizes test but little if any control protein (bottom, lanes 5 and 6), as reported (5).

Reversal of Inhibition of Control Gene Expression by pCT1.2 and pCT1.3

The two new constructs were predicted to alleviate the inhibition of control gene expression: in the case of pCT1.2, by preventing rolling circle transcription so that transcripts from the downstream T7 promoter would not contain the inhibitory upstream CJ sequences; in the case of pCT1.3, by inserting a large spacer to separate the inhibitory CJ sequences from the control gene mRNA. Both of these constructs accomplished this reversal. In the top panel of Figure 3, in the absence of induction, there is little (lane 7) or reduced (lane 11) synthesis of the test and control proteins. Upon induction for 30 min (lanes 8 and 12), 45 min (lanes 9 and 13), or 60 min (lanes 10 and 14), there is substantially greater synthesis of the control protein relative to the test protein than was observed in parental pCT1.1 at the first two time points [lanes 5 and 6; see also Harvey et al. (5)]. As was the case with parental pCT1.1, mRNAs were not detectable with pCT1.2 or 1.3 in this kind of assay, in contrast to the progenitor of this series, pCT1 (lanes 2 and 3). These results were also reproduced in the presence of rifampicin, in the bottom panel of Figure 3.

Quantitation of Incorporation Into Test and Control Proteins

The two gels shown in Figure 3, top and bottom, were exposed and quantitated in a phosphorimager (Table 1). The actual counts obtained in the protein bands corroborate the qualitative conclusions from looking at the gels. Because radiolabeling was performed as 2-min pulses of [^{35}S]methionine at the various times, these numbers reflect the rates of incorporation at the indicated times, and not the accumulation of label in the proteins.

The rate of incorporation into control protein in pCT1.1 is low, barely increasing above the noninduced (0 time) samples. In pCT1.2, the rate of control protein synthesis is elevated at least two- to threefold compared with pCT1.1, and perhaps more tellingly, continues to rise with longer induction times in the absence of rifampicin. In pCT1.3, control protein synthesis is elevated about an order of magnitude compared with pCT1.1, but for unknown reasons, the rates of both test and control protein synthesis decline at the later times both in the presence and absence of rifampicin. Possibly, the rolling circle transcription also hinders T7 expression in a nonspecific fashion; however, this decline is not observed for pCT1.1, which should also support rolling circle transcription.

Generally, the samples treated with rifampicin do not show substantial rate gains, if at all, at the later times for both test and control proteins; this is not surprising, however, because further production of T7 RNA polymerase is halted upon addition of rifampicin, and all T7 transcription from that point on is dependent on the fixed quantity of T7 RNA polymerase produced during the induction period prior to rifampicin addition.

Less Than Complete Reversal of Inhibition of Control Protein Synthesis in pCT1.2

While the ratios of test to control protein synthesis in pCT1 and pCT1.3 don't quite make it to equivalence, running between one- and twofold, the ratios for pCT1.2 are decidedly elevated, running about five- to sixfold in the absence of rifampicin (although only twofold in the presence of rifampicin). This implies that the rescue of control protein synthesis by the introduction of the downstream T7 terminator is not complete (i.e., there is still some hindrance on expression of the downstream gene). It is possible that this simply reflects the sum of translation of two kinds of transcripts containing the control mRNA, both mRNA initiated from the downstream promoter and readthrough mRNA from the upstream promoter, which we already know is inhibitory on control gene expression. This explanation depends on the total amount of transcription being limiting. An alternative possibility is that pausing of the T7 RNA polymerase at the CJ site, which has been proposed by others (8,13) and supported in our previous study (5), acts to impede new transcription starts at the downstream promoter, which is only about 20 nt downstream from the CJ element in pCT1.1. Presumably, such a paused T7 RNA polymerase would be unable to initi-

TABLE 1
INCORPORATION OF [^{35}S]METHIONINE INTO TEST AND CONTROL PROTEINS

	Counts × 10^{-3}					
	No Rifampicin			+ Rifampicin		
Induction Time	Test Protein	Control Protein	Text/Control Ratio	Test Protein	Control Protein	Text/Control Ratio
pCT1						
0	14	27	0.52	29	32	0.91
30	129	96	1.34	204	140	1.46
45	169	109	1.55	198	146	1.36
pCT1,1						
0	14	6	2.33	15	3	5.00
30	69	10	6.90	37	4	9.25
45	221	14	15.79	62	10	6.20
pCT1.2						
0	10	4	2.50	8	4	2.00
30	88	14	6.29	28	13	2.15
45	192	39	4.92	39	20	1.95
60	301	53	5.68	35	17	2.06
pCT1.3						
0	11	56	0.20	4	5	0.80
30	219	99	2.21	258	135	1.91
45	149	86	1.73	70	48	1.46
60	76	59	1.29	21	20	1.05

Quantitation of test and control proteins synthesized in the pCT1 series of vectors. The [^{35}S]methionine bands in Figure 3 were quantitated following an 8-h exposure in a phosphorimager. The induction time is the time after addition of IPTG. Shown are the raw "volume" counts, uncorrected for background.

ate at the downstream promoter itself because it had not properly terminated [and T7 RNA polymerase is known to be highly processive, rarely if ever falling off template; Iost et al. (7)].

CONCLUSIONS

In this work, we have verified a number of aspects of the model proposed by Harvey et al. (5) to explain the inhibition by the T7 concatemer junction on expression of a gene immediately downstream. The introduction of a large spacer between the CJ element and the downstream gene has been shown to abolish the inhibition, consistent with our proposal that the CJ element inhibits the translation start of the downstream gene by means of a secondary or tertiary structure interaction in the mRNA. The reintroduction of a downstream T7 transcription terminator element also partially reversed the inhibition even with the CJ element in the same relative location to the downstream gene as before. This result supports our prior suggestion that "rolling circle" transcription in pCT1.1 allows little mRNA encoding only the downstream gene, while the readthrough transcripts juxtapose the inhibitory CJ element upstream of message encoding the downstream gene.

The observation that the reversal of inhibition by reintroduction of the T7 terminator was less than complete implies that there still is some inhibitory aspect in having a CJ element just upstream of a T7 promoter. While there seems to be an overall trend favoring expression from the mRNA most upstream on the plasmid, this is generally less than a factor of 2, whereas when the CJ element was immediately adjacent to the downstream promoter, the effect was five- to sixfold (in the absence of rifampicin). This could be explained by another suggestion considered in Harvey et al. (5), that the CJ element acting as a pause site for transcription inhibits, by virtue of its proximity, independent transcription initiation at the adjacent downstream promoter. This needs to be confirmed by additional experiments. If confirmed, this would be a novel method of control on transcription, by steric hindrance of transcription starts resulting from an upstream RNA polymerase pause site.

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