A POSSIBLE TERMINATION FACTOR FOR TRANSCRIPTION IN ESCHERICHIA COLI

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Received November 26,1973

SUMMARY: DNA from both λdlac and λplac viruses direct mRNA-mediated synthesis of β-galactosidase (β-gal) in a coupled cell-free system. When λdlac DNA is used the system is almost completely dependent upon the presence of adenosine 3',5'-cyclic monophosphate (cAMP) which is required for normal initiation of transcription at the lac promoter. By contrast when λplac DNA is used about half the maximum amount of β-gal is synthesized in the absence of cAMP. It seems highly likely that this cAMP independent synthesis of β-gal is the result of read-through from a phage initiation site on the λplac DNA. Since there is believed to be at least one transcription termination signal before the lac operon it must be asked why this signal is not being recognized as such in vitro. We hypothesize that the normal bacterial termination factor is missing or inactive in the bacterial extract used to make the cell-free system. This view is supported by the finding that protein extract can be isolated from whole E. coli cells under mild conditions which prevents the cAMP-independent β-gal synthesis without inhibiting cAMP-dependent β-gal synthesis. The behavior of the active factor (called the ρ_Y factor) in this extract is different from either the Roberts ρ factor (called ρ_R here) or the Schafer-Zillig κ factor. Furthermore it has been found that the ρ_R factor cannot replace the ρ_Y factor.

Two events are essential to the termination of transcription. The elongation process must be halted and the newly synthesized RNA chain, which has been firmly held to the DNA-RNA polymerase complex during elongation, must be released. Evidence from in vitro studies suggests that there may be more than one mechanism for termination of transcription. Thus it has been demonstrated that so-called early transcription of λ bacteriophage DNA terminates at two loci on the DNA in the presence of a protein called ρ discovered by Roberts (1). In contrast in T_7 bacteriophage early transcription begins from one end of the chromosome and is terminated at a point about 20 per cent down the length of the chromosome without the involvement of any proteins other than the E. coli RNA polymerase (2). We have obtained evidence that bacterial genes (as opposed to phage genes) may require yet another protein for termination which we will tentatively call the ρ_Y factor (3, 4). For

purposes of distinction the Roberts ρ factor will be referred to as the ρ_R factor.

MATERIALS AND METHODS: Conditions for cell-free synthesis and assay. For cell-free synthesis of the enzyme β-galactosidase the S-30 extracts used were prepared from strain SB 7223 (5) containing a deletion of the entire lac region. Either λdlac DNA or λplac DNA were used for β-gal synthesis. Except for slight modifications, all procedures used for synthesis, enzyme assay, and preparation of bacterial S-30 extracts and DNA have been described in detail elsewhere (6). The incubation mixture contained per ml: 44 μmol Tris-acetate, pH 8.2; 1.37 μmol dithiothreitol (DTT); 55 μmol KAc; 27 μmol NH_4Ac; 14.7 μmol $Mg(Ac)_2$; 7.4 μmol $Ca(Ac)_2$; 0.22 μmole amino-acids; 2.2 μmol ATP; 0.55 μmol each GTP, CTP, UTP; 21 μmol phosphoenolpyruvic acid; 100 μg tRNA; 27 μg pyridoxine HCl; 27 μg triphosphopyridine nucleotide; 27 μg flavine adenine dinucleotide; 11 μg p-amino-benzoic acid; 0.5 μmol cAMP. These were preincubated for 3 min. at 37°C with 100 γ/ml of λdlac or λplac DNA with shaking before 9.0 mg S-30 protein extract was added. Incubation with shaking was continued for 60 min. at 37°C. After synthesis, a 0.2 ml aliquot was removed and assayed for β-gal with O-nitrophenyl β-D-galactoside. Enzyme assays were done at 29°C.

Isolation procedures used for ρ_Y factor. As a rule strain SB 7219 (5) containing a deletion of the entire lac region was usually used for preparation of the ρ_Y factor. Strain SB 7227 and several other strains containing lac deletions were also found to be effective sources of ρ_Y factor. Growth of bacteria and the preparation of S-100 fraction have been described (7). All preparations were carried out at 0-4°C. Samples were dialyzed against buffer I (0.01 M Tris-Ac pH 8.2, 0.014 M $Mg(Ac)_2$, 0.06 M KAc, 0.35 mM DTT, 5% glycerol) before assaying their effect on β-galactosidase synthesis.

Preparation of 'crude DEAE extract'. After overnight dialysis in buffer II (0.01 M KH_2PO_4-KOH pH 7.7, 0.35 mM DTT, 5% glycerol), the S-100 fraction prepared from 50 g of cells were passed through a 120 ml DEAE-cellulose, 0.91 meq/ml., pre-equilibrated in buffer II. After rinsing the column with 3 volumes of buffer II, the DEAE-cellulose bound protein was eluted with buffer II containing 0.3 M NaCl and precipitated by adding two volumes of cold saturated $(NH_4)_2 SO_4$ to the eluate. The resulting precipitate was collected by centrifugation at 10,000 rpm in a Serval GSA rotor and resuspended in 25 ml of the appropriate buffer for further purification. This DEAE-cellulose preparation is called the 'crude DEAE extract'. It has an O.D. 280/260 ratio greater than 1.4 which indicates that all except traces of nucleic acid have been eliminated.

DNA-cellulose column purification step. Double stranded calf thymus DNA-cellulose was prepared as described by Alberts (8). The DNA-cellulose prepared contained 1600 μg DNA/ml of column volume. The column was equilibrated in buffer IV (0.01 M KH_2PO_4-KOH pH 6.4, 0.35 mM DTT and 5% glycerol). 10 ml of DNA-cellulose was used for each 50 g of cells. After rinsing the column with buffer IV the ρ_Y activity was eluted with buffer IV containing 0.3 M NaCl.

RESULTS AND DISCUSSION: It has been reported that the λdlac DNA-directed cell-free synthesis of β-galactosidase (β-gal) is almost completely dependent upon the presence of cAMP (9); only 2 to 10 per cent of the optimal level of β-gal is obtained in the absence of cAMP. When λplac DNA is used instead of λdlac, 50 to 80 per cent of the optimal level of β-gal is obtained in the absence of cAMP. The same lac promoter-operator region is present in the

two viral DNAs but it is inserted into different regions of the virus chromosome. In a purified transcriptional system which contains only two proteins, E. coli RNA polymerase and the catabolite gene activator protein (CAP), a similar partial escape from normal controls has been noted with λplac DNA (10). In λplac the lac operon is inserted within the b_2 region (11). A possible explanation for the escape from total cAMP dependence is that transcription initiated in b_2 or some other nearby region on the phage DNA reads through the lac operon eliminating the necessity for initiation at the lac promoter to obtain β-gal synthesis.

The lac i gene is either very close to or immediately adjacent to the lac promoter-operator region (see Figure 1). Evidence has been presented

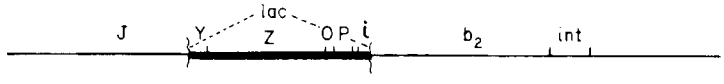

FIGURE 1. The genetic map of lac region in λplac 5 (modified from ref. 11). The heavy line shows the bacteria genome harboring lac i (partial) P, O, Z and Y (partial) inserted into the b_2 region of λ.

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that the i gene is transcribed from right-to-left (10, 12) implying the presence of a termination signal between the i gene and the promoter-operator region of the lac operon. If one is transcribing from a phage initiation site to the right of i, elongation into lac should not occur unless something is malfunctioning in the transcription termination process. A factor required for normal termination might be inactive or absent in the S-30 extract used for cell-free synthesis. Indeed some other proteins playing a role in the transcription-translation process are known to be inactive in the S-30. Such is the case for the ara C regulatory protein (5) and the SuA polarity factor (13). The idea that bacterial termination factor might be missing in the S-30 was tested by adding a crude protein extract (called a 'crude DEAE extract' in materials and methods) to the S-30 immediately prior

TABLE 1

Effect of ρ_y factor and cAMP on the synthesis of β-galactosidase directed by λplac DNA.[a]

Description of ρ_y containing extract added	β-gal (+ cAMP)	β-gal (- cAMP)	ρ_y activity[b]	mg of ρ_y containing protein added per ml of incubation mix
no addition	2.20	1.05	2.09	0
'crude DEAE extract'[c]	1.95	0.13	15.0	5.76
DEAE extract[d]	2.61	0.06	43.5	5.76
HA extract[e]	2.52	0.13	19.4	1.3
DNA-cellulose extract[f]	1.26	0.135	9.33	0.44

[a] Synthesis and assay are carried out as described in materials and methods. β-gal is reported as A_{420} corrected to an assay time of 200 min.
[b] The 'ρ_y activity' is defined as the ratio of β-gal synthesized with cAMP added to that synthesized without cAMP added. For a given extract, concentration dependence studies have shown that the amount of active ρ_y factor is approximately proportional to the 'ρ_y activity'.
[c] 'Crude DEAE extract' is described in materials and methods.
[d] DEAE extract is the peak fraction from a gradient elution on DEAE-cellulose as described in the caption to Figure 2.
[e] HA extract is a 0.18 - 0.35 M phosphate cut from a Hydroxyapatite column as described in the caption to Figure 3.
[f] DNA-cellulose extract is the 0 to 0.3 M NaCl fraction from a double strand DNA-cellulose column as described in materials and methods.

to cell-free synthesis of β-gal. Under these conditions, it was found that the cAMP-independent synthesis of β-gal is almost completely eliminated without appreciable effect on the cAMP-dependent synthesis (see Table 1).

Recent efforts have been directed at purification of the protein factor which inhibits the apparent read through using the inhibitory property to guide the purification. This factor is referred to as ρ_y. The 'crude DEAE extract' has been subjected to a variety of purification steps. 'Crude DEAE extract' has been fractionated by elution on a DEAE-cellulose column using a NaCl gradient. The active fraction containing ρ_y elutes as a sharp peak before the main body of the protein as shown in Figure 2. The 'crude DEAE extract' has also been subjected to fractionation on columns of hydroxyapatite

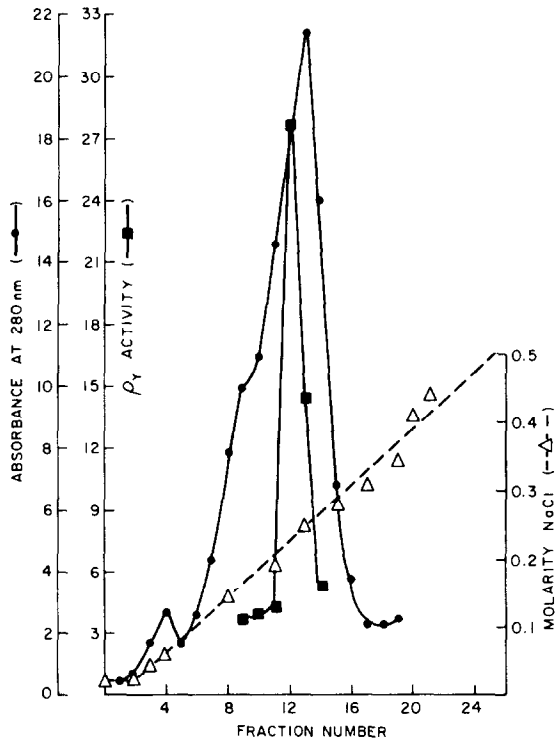

FIGURE 2. Gradient elution pattern on DEAE-cellulose. The 'crude DEAE extract' (described in materials and methods) dialyzed in buffer II overnight was applied to a 60 ml DEAE-cellulose (Whatman DE-52, 1.5 x 33 cm) column pre-equilibrated in buffer II with a linear gradient of 0 to 0.5 M NaCl. Fractions of 5 ml were collected, dialyzed against buffer I and assayed. The 'ρ_y activity' ('ρ_y activity' is defined in Table 1) elutes as a sharp peak around 0.22 M NaCl.

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and double-stranded DNA-cellulose. The 'ρ_y activity' elutes very late on hydroxyapatite (0.26 M phosphate) and it is estimated that about 50-fold enrichment is achieved in this step. Only about 1/10th of the protein of the 'crude DEAE extract' binds to a DNA-cellulose column in 0.01 M phosphate. About 1/6th of the bound protein containing the 'ρ_y activity' is released by 0.3 M NaCl. This suggests that a maximum of 60-fold enrichment is obtained by this procedure on DNA-cellulose. It is of great interest that 'ρ_y activity' is not retained by phosphocellulose even at low ionic strength. This suggests that the binding to DNA-cellulose involves something more than just the phosphate groups of the DNA. The enrichment of ρ_y by the above

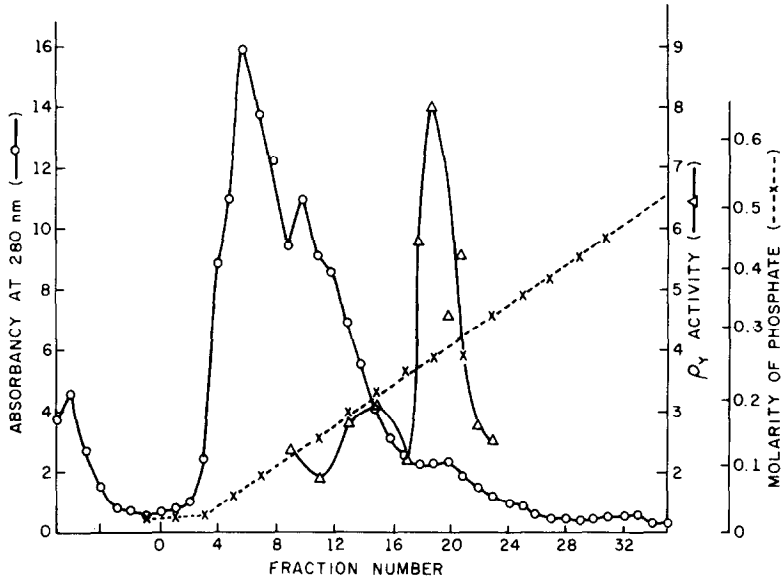

FIGURE 3. Gradient elution pattern on hydroxyapatite. Hydroxyapatite (Bio-gel HT) was pre-equilibrated in buffer III (0.01 M KH_2PO_4-K_2HPO_4 pH 6.8, 0.35 mM DDT and 5% glycerol). After overnight dialysis in buffer III, the 'crude DEAE extract' was loaded on a 32 ml column (1.5 x 18 cm.) and eluted with a linear gradient of 0 to 0.6 M phosphate. 3.5 ml fractions are collected and assayed for 'ρ_y activity' as described in Table 1. The peak of 'ρ_y activity' is found around 0.26 M phosphate.

described procedures is probably much greater than would be indicated from the specific activities of the various partially purified fractions (Table 1). This is because appreciable loss in activity occurs during purification. We are attempting to overcome this problem so that the various fractionation procedures may be effectively combined to achieve total purification of a highly active ρ_y factor.

In view of the hypothesized function of ρ_y factor in termination we were immediately drawn to the idea that we might be assaying for the same factor ρ_R that has been described by Roberts (1) which catalyses the termination and release of early transcription in λ. However, various tests indicate that ρ_y and ρ_R are different factors. Thus the Roberts ρ_R factor (either prepared by us or obtained from N. Minkley) was found to be ineffective

in preventing the cAMP-independent synthesis of β-gal. Furthermore the affinity properties of ρ_y and ρ_R on DEAE-cellulose and phosphocellulose are quite different. On DEAE-cellulose using a salt gradient ρ_y elutes before the main peak of protein whereas ρ_R is one of the last proteins to be eluted. On phosphocellulose ρ_y is not appreciably retained between pH 6.4-7.0 in 0.01 M phosphate whereas ρ_R is retained and elutes around 0.1 M phosphate at pH 7.0. It should also be mentioned that Schafer and Zillig have isolated a protein called κ which causes shorter pieces of RNA to be made from T5 DNA (14). The elution properties of κ on phosphocellulose distinguish it from both ρ_R and ρ_y.

The potential importance of the ρ_y factor described here is that it may be involved in termination of transcription for bacterial genes. The evidence presented is only indirect support for such a role and does not eliminate the possibility that other factors active in the S-30 extract may contribute to the termination process. We are purifying the ρ_y factor further so that its properties may be studied in greater depth.

ACKNOWLEDGEMENTS: We are grateful to N. Minkley for a sample of ρ factor isolated by the Roberts method. This work was supported by a grant from the National Institutes of Health (2-R01-GM-16648-04). We would like to thank Dr. M. Gefter for suggesting the use of hydroxyapatite in purification.

REFERENCES

1. Roberts, J.W. (1969) Nature 224, 1168; Roberts, J.W. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 121.
2. Millette, R.L., Trotter, C.D., Herrlich, P., and Schweiger, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 135.
3. Zubay, G., Yang, H-L., Reiness, G., and Cashel, M. (1973) in Molecular Cytogenetics, pp. 253-261, Plenum Pub. Corp.
4. Zubay, G. (1973) Ann. Rev. Genetics 7, in press.
5. Yang, H-L., and Zubay, G. (1973) Molec. Gen. Genet. 122, 131.
6. Zubay, G., Chambers, D.A., and Cheong, L.C. (1970) in The Lactose Operon, Beckwith, J.R. and Zipser, D., eds., Cold Spring Harbor Laboratory, p. 375.
7. Zubay, G., Morse, D.E., Schrenk, J., and Miller, J. (1972) Proc. Nat. Acad. Sci. 69, 1100.
8. Alberts, B. (1971) in Methods in Enzymology, L. Grossman and K. Moldave, eds., vol. 4, p. 198.
9. Chambers, D.A. and Zubay, G. (1969) Proc. Nat. Acad. Sci. 63, 118.
10. Eron, L. and Block, R. (1971) Proc. Nat. Acad. Sci. 68, 1828.
11. Malamy, M.H., Fiandt, M., and Szybalski, W. (1972) Molec. Gen. Genet. 119, 207.
12. Kumar, S. and Szybalski, W. (1972) J. Mol. Biol. 40, 145.
13. Wetekan, W. and Ehring, R. (1973) Molec. Gen. Genet. 124, 359.
14. Schafer, R. and Zillig, W. (1973) Eur. J. Biochem. 33, 201.