Relaxed mutants of *Escherichia coli* RNA polymerase

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Received 25 January 1983

Unusual guanosine nucleotides synthesised during amino acid or energy source starvation are thought to be the effectors of the stringent response. In vitro experiments suggest that the magic spot compounds alter transcription specificity of RNA polymerase by binding to the enzyme. However, there is no good in vivo evidence for such an interaction. We define sites on the β-subunit of RNA polymerase which, when altered, yield *E.coli* mutants apparently insensitive to the presence of ppGpp.

RNA polymerase *Stringent control* *Nonsense suppression*

1. INTRODUCTION

When bacterial cells are starved of an amino acid or when aminoacyl-tRNA synthetases are inactivated, the presence of uncharged adaptor molecules in the ribosomal A site [1,2] activates the *relA*-mediated production of (p)ppGpp (or magic spot, MS), leading to a shut-down in the accumulation of stable RNA production [3] (a host of other cellular processes are also affected; reviewed in [4]). This stringent control is clearly an adaptive response which modulates cellular gene expression according to the available translation potential. It would appear that RNA synthesis (rather than degradation) is the target of stringent control [5]. Studies in vitro [6–8] suggest that MS acts upon RNA polymerase to limit transcription from particular promotors. (Such stringently-controlled regulatory sequences may have a G + C-rich region near to the start site of transcription [9]. However, there is no clear in vivo evidence for the involvement of RNA polymerase.

If (p)ppGpp acts on RNA polymerase (in the presence, perhaps, of other regulatory molecules), it should be possible to isolate mutants of the essential enzyme which are phenotypically relaxed despite the presence of a functional *relA* gene; that is, Rel$^-$ strains that have the potential to synthesise the effector molecule but lack a responsive target. We have investigated this possibility by testing a collection *E.coli* mutants which synthesise variants of RNA polymerase.

This collection was generated by the isolation of 95 independent, spontaneously-occurring mutants carrying amber lesions preventing the expression of the β structural gene, *rpoB* [10]. With a group of nonsense suppressors, 6 different but known amino acids can be inserted at each amber site (Gln, Leu, Lys, Ser, Trp, Tyr) [11]. Therefore, we can test the effect that such substitutions have on RNA polymerase function in vivo and in vitro, and determine the site of the substitutions by translational mapping [12]. We report that the exchange of certain amino acids in the third quarter of the β-subunit of *E.coli* RNA polymerase results in relaxed control over RNA synthesis.

2. METHODS

Isolation of the mutant strains and their phenotypic properties is described in [10]. The genetic background of these *E.coli* K12 strains is *rpoB*-(Am) *lacZ53*(Am) *rpsL argG metB recA1*/F110

Paper 5 in the series: Genetic studies on the β-subunit of *Escherichia coli* RNA polymerase

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Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

rpoB70(Rif-r) *metB*$^+$. RNA synthesis was followed by measuring incorporation of [^{14}C]uracil (0.5 μCi.ml^{-1}) into trichloroacetic acid-insoluble material before and after amino acid starvation induced by addition of valine (400 μg.ml^{-1}). Magic spot compounds were assayed by pre-labelling cells, grown in low phosphate (0.1 mM) minimal medium, with [^{32}P]orthophosphate (200 μCi.ml^{-1}) for one generation; induction of the stringent response was as above. Nucleotides (in 1 M formic acid) were separated on PEI-cellulose F coated TLC plates with 1.5 M KH_2PO_4 (pH 3.5) and visualised by autoradiography. Identification and sizing of amber fragments by immunoprecipitation is described in [12]. Briefly, we employed specific antiserum against RNA polymerase holoenzyme, in conjunction with formalin-fixed *Staphylococcus aureus* cells, to isolate [^{3}H]leucine-labelled proteins from crude cell extracts; short pulse-chase conditions at 30°C were employed to minimise proteolytic degradation [12].

3. RESULTS AND DISCUSSION

Chromosomal amber mutations in the β structural gene were isolated in a recombination-deficient (*recA*) background, and 6 different nonsense suppressors were introduced into each strain on F-prime plasmid derivatives. We have begun an analysis of the 331 suppressed *rpoB* strains for their stringent response (only about half of the 570 substitutions elicited permit viability [10]), by studying RNA synthesis upon valine-induced amino acid starvation. The ratio of the rate of incorporation of [^{14}C]uracil into RNA, following and before imposition of the block on amino acid synthesis, the relaxation index, is a measure of the degree of control over RNA accumulation (Breeden and Yarus, personal communication). An index of 1.0 reflects the relaxed phenotype whereas values of $\leqslant$0.1 are generally found for stringently-controlled *E.coli* strains.

The majority of our suppressed *rpoB*(Am) mutants studied to date had relaxation indices of about zero characteristic of the Rel$^+$ parent. Other amino acid changes gave considerably higher values: 0.88 ± 0.04, 0.42 ± 0.03 and 0.53 ± 0.03 for the Su5(Lys), Su2(Gln) and Su3(Tyr) derivatives of AJ5350, AJ5830 and AJ6040, respectively (table 1). Clearly, therefore, exchange of amino acids in the β-subunit of RNA polymerase is sufficient to prevent stringent control. Where are the sites involved?

Since fine mapping of mutations in essential genes is extremely difficult, we have chosen to locate the *rpoB* nonsense lesions by immunoprecipitation of amber fragments. The presence of a nonsense triplet within a structural gene results in premature translational termination, the size of the truncated polypeptide produced being directly dependent upon the position of the chain termination codon.

In this way, we identified fragments of M_r ($\times 10^{-3}$) 117, 83 and 100 in the 3 relaxed *rpoB*(Am) strains

Table 1

The effect of known amino acid substitutions in the β-subunit of *E.coli* polymerase on stringent control

Strain number	Mutant[a] class	Fragment[b] size	Stringent phenotype[c]: Amino acid inserted						
			None (Su$^-$)	Ser (Sul)	Gln (Su2)	Tyr (Su3)	Lys (Su5)	Leu (Su6)	Gln/Trp (Su7)
AJ5350	I	1041	S	S	–	–	R	S	S
AJ5830	G	736	S	S	R	–	–	–	S
AJ6040	A2	906	S	S	S	R	S	S	S

[a] The mutant collection has been separated into 16 groups according to which amino acids generate a functional RNA polymerase [11]: class A strains survive with all 6 substitutions whereas class G and I are viable with only Ser/Gln/Trp or Ser/Lys/Leu/Gln + Trp, respectively [the A mutants have been further distinguished since A2 (but not A1) Gln derivatives exhibit aberrant transcription termination]

[b] Refers to the size of amber fragment (in residues) synthesised by the *rpoB*(Am) strains

[c] S, stringent; R, relaxed; Sul, Su2, Su3, Su5, Su6 and Su7 are the phenotypic symbols for the nonsense suppressor alleles, *supD, SupE, supF, supG, supP* and *supU*, respectively

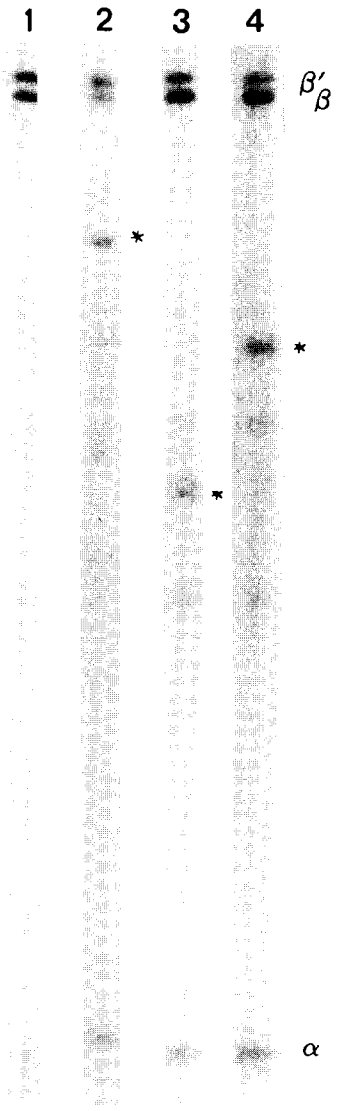

Fig. 1. Location of sites in the β structural gene involved in stringent control by immunoprecipitation of amber fragments from *rpoB*(Am) strains which exhibit a relaxed phenotype in the presence of certain nonsense suppressors. Radiolabelled, immunoprecipitated proteins were analysed on a 5–16% gradient SDS–polyacrylamide gel. Short chase samples (3 min) are shown: (1) AJ38 (*rpoB*$^+$/*rpoB70*); (2) AJ5350 (*rpoB1535*(Am)/*rpoB70*; (3) AJ5830 (*rpoB1583*(Am)/*rpoB70*); (4) AJ6040 (*rpoB1604*(Am)/*rpoB70*). The position of the amber fragments is marked by an asterix (the few other proteins precipitated stem from non-specific interaction with the carrier cells). The length of the truncated polypeptides (in residues) is calculated to be about: 1041 (2); 736 (3); 906 (4); we estimate a sizing error of 5%. These novel proteins exhibit differential stability (not shown). Note that the figure is a composite; hence, the bands in track (2) appear to migrate anomalously to those in the other tracks.

(fig. 1). That these are N-terminal polypeptides is strongly suggested by their high rate of synthesis in relation to β (reinitiation of translation, at its best, is reported to be only ~10% efficient [13,14]). Since the same precipitation procedure has identified a breakdown product of another fragment [12], and since the polypeptides found in the Rel$^-$ strains are all of different size and smaller than other β truncated proteins which we have observed (unpublished), it is likely that these proteins identified in SDS–polyacrylamide gels are not intermediates in a degradation pathway. The size of the 3 amber fragments implies, therefore, that the 3 sites responsible for the relaxed phenotype are relatively tightly clustered, between residues 736–1041 on the β-polypeptide (the complete subunit consists of 1342 amino acids [15]).

Substitution of certain amino acids in the third quarter of the β subunit of *E.coli* RNA polymerase is sufficient to perturb stringent control over bulk RNA synthesis. [While this paper was in preparation, we received a manuscript from Little, Ryals and Bremer reporting increased sensitivity of ribosomal gene activity to ppGpp in a Rif-r mutant of *E.coli* B/r.] One would predict, since the putative target site of MS (rather than MS synthesis, itself) is affected, that these partially relaxed *rpoB*(Am) strains retain the ability to produce the unusual nucleotide. Indeed, experiments with [^{32}P]orthophosphate (not shown) supports such a prediction.

ACKNOWLEDGEMENTS

We should like to thank Andrew Travers for introducing us to the MS assay and for helpful discussion, Jon Gallant for testing some of our relaxed strains, Linda Breeden and Hans Bremer for communicating results before their publication, and M.G. Hunter for assistance with the manuscript. The work reported was financed by a grant from the Nuffield Foundation to R.E.G.

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