Enhanced polypeptide synthesis programmed by linear DNA fragments in cell-free extracts lacking exonuclease V

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Received 19 September 1983

Preparation of an in vitro coupled transcription-translation system from *E. coli* strains lacking exonuclease V has greatly improved the system for use with added linear DNA fragments. In fact, in extracts of these mutants linear fragments are stable for several hours. However the cell extracts show a high level of endogenous background. To avoid this complication extracts were prepared at 30°C from a mutant carrying a temperature-sensitive exonuclease V. Polypeptides coded by a specific DNA region, e.g., delineated by restriction endonuclease sites, can now be easily identified.

Exonuclease V *Zubay* E. coli rec*B* *DNA* *Polypeptide*

1. INTRODUCTION

We have described the use of a modified Zubay [1] in vitro system to direct the synthesis of polypeptides from various DNA templates. In those studies [2] we were able to demonstrate that supercoiling of the DNA template was not essential for efficient polypeptide synthesis. Consequently we were able to use successfully linear DNA restriction enzyme fragments in this system. Nevertheless we noted that substantially higher concentrations of linear templates were required to achieve results comparable to those obtained with the corresponding supercoiled or covalently closed DNA molecules. We conclude that this was probably due to exonuclease digestion of the DNA. We therefore investigated the role of the *rec*B,C exonuclease V, in the loss of template activity. In mutants completely lacking the enzyme high backgrounds were obtained due to large amounts of endogenous DNA fragments, active as templates. In this paper, we describe the capacity of cell-free extracts, derived from a mutant carrying a temperature-sensitive *rec*B allele, to support polypeptide synthesis programmed by DNA. We found that at 37°C linear templates were stable for at least 120 min in this extract, backgrounds are low and the efficiency of polypeptide synthesis approached that of covalently closed (relaxed) templates. Thus as little as 50 ng of a linear DNA fragment was sufficient to programme the synthesis of a specific polypeptide.

2. MATERIALS AND METHODS

Strain *E. coli* N138, *rec*B^{ts} was kindly provided by Dr G.G. Churchward, University of Geneva; MRE600 *rec*B^{+} was described in [2]. The plasmids used were pBR325 (*Ap,Cm,Tc*) 5.4 kb and its derivative pLG282 which carries a 2.2 kb *Eco*RI fragment from the *Col*I plasmid coding for *api*.

The preparation of all DNA templates including restriction endonuclease generated fragments and the standard procedure for the preparation of cell-free extracts (S30) for in vitro synthesis of polypeptides programmed by DNA have been described in [2]. For the extract prepared from strain N138 the cells were grown at 30°C rather than at 37°C and the extracts were preincubated at 30°C for 160 min to ensure complete digestion of endogenous DNA before storage in liquid nitrogen. All incubations were carried out at 37°C and contained 7.5 μl LMM (low molecular weight mix), 2 μl [^{35}S]methionine (or 2 μl unlabelled methionine),

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00145793/83/$3.00

Table 1

Strain	cpm	
	+ DNA	− DNA
MRE600	100396	1346
N138 (*recB*^{ts})	75698	2544

Incubations were performed with 3 μg of pBR325 as added template and either MRE600 or N138 (*recB*^{ts}) as source of S30. A 2 μl sample was removed after 30 min incubation and the [^{35}S]methionine incorporated into protein estimated by trichloroacetic acid precipitation. The figures presented are the average from several experiments

3.5 μl of 0.1 M magnesium acetate, 5 μl of S30 extract, DNA in 10 mM Tris, 1 mM EDTA (pH 7.0) and 10 mM Tris/acetate buffer (pH 7.0) to a final volume of 30 μl [2].

3. RESULTS AND DISCUSSION

Initial experiments with a *recB* mutant showed that S30 extracts completely lacking exonuclease V gave extremely high background incorporation in the absence of DNA templates, rendering such a system quite inoperable. This was presumably due to substantial amounts of endogenous DNA which is normally degraded by exonuclease V during preparation of the extracts. To overcome this difficulty we decided to use an alternative strain car-

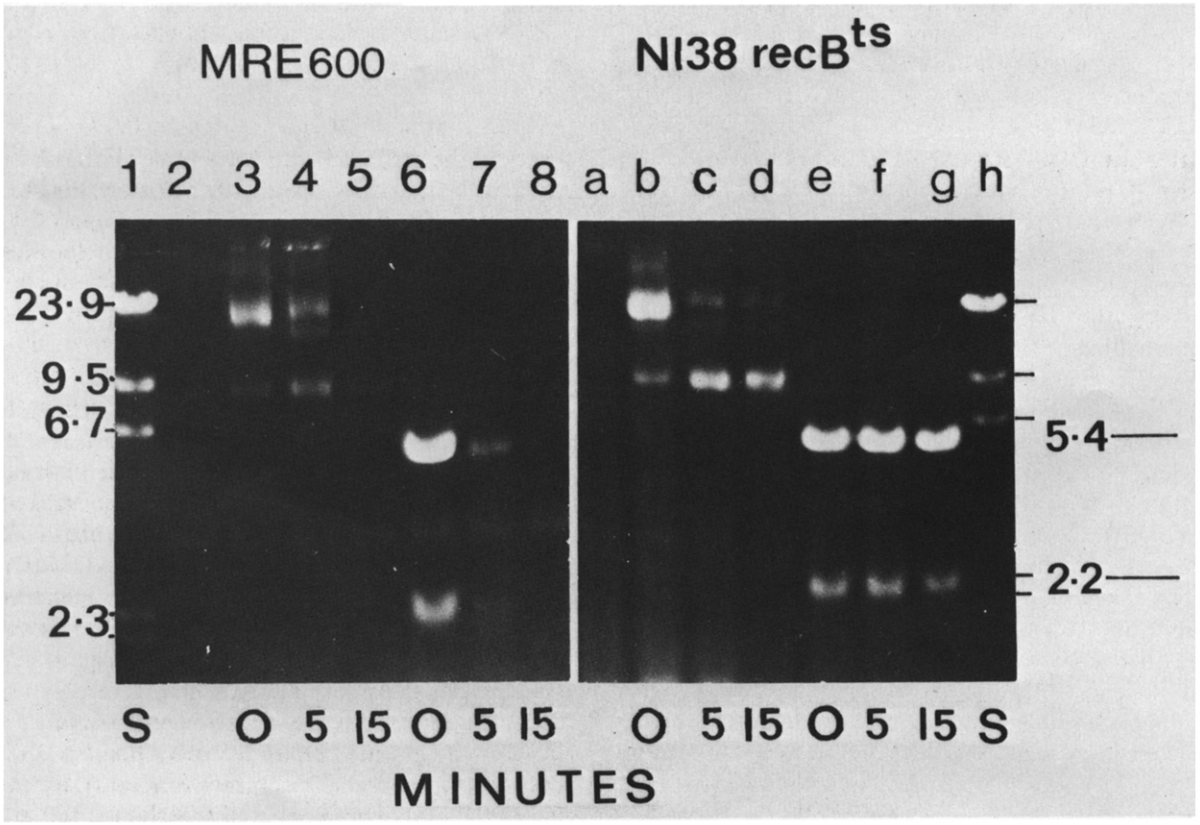

Fig.1. Stability of linear DNA templates in a *recB*^{ts} extract. S30 extracts were prepared from MRE600 (*recB*$^{+}$) and N138 (*recB*^{ts}) and incubated with 2.5 μg of either pLG282 or pLG282 cut with *Eco*RI. 5 μl aliquots were removed at intervals and analysed on a 0.6% agarose gel stained with ethidium bromide. (Tracks 1 and h) molecular mass standards (kb); (2 and a) no DNA; (3,4,5,b,c and d) 2.5 μg of pLG282; (6,7,8,e,f and g) 2.5 μg *Eco*RI fragments. Samples (0.5–1 μg DNA) were removed at the times indicated. Further samples removed at 60 and 120 min were identical to the 15 min sample in each case.

rying a temperature-sensitive mutation in *rec*B [3]. Extracts were prepared at 30°C as described in section 2 and preincubated at 30°C to reduce background incorporation. As shown in table 1 a low level of incorporation in the absence of added DNA comparable to that of the MRE extract, could be achieved by this strategy.

Extracts prepared from both *E. coli* MRE600 and *E. coli* N138 (*rec*B^{ts}) were incubated at 37°C together with either supercoiled pLG282, or the *Eco*RI restriction fragments (5.4 kb and 2.2 kb) of pLG282 as DNA templates. Samples were removed at intervals and analysed by agarose gel electrophoresis to determine the stability of the added DNA. As shown in fig.1 using the MRE600 extract the restriction fragments were almost completely degraded within 5 min, whilst in the N138 extract no DNA degradation could be detected even after 120 min of incubation. These results indicate that under these conditions exonuclease V is the major degradative activity in wild-type extracts but is largely absent in N138 extracts at 37°C. When this extract was incubated at 37°C in the presence of an added linear DNA template (fig.2) stimulation of

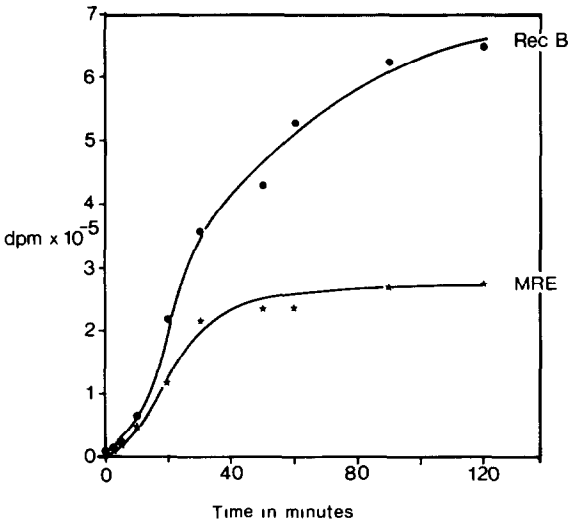

Fig.2. Protein synthesis programmed by a linear template. 10 μg of pBR325 cut with *Hin*dIII was used as a template to programme protein synthesis with either MRE600 or N138 as the source of S30; [^{35}S]methionine was included to label the synthesised peptides and 2 μl samples were removed at intervals and the radioactivity incorporated into protein determined by trichloroacetic acid precipitation.

DNA synthesis was obtained in a reaction which could be sustained for up to 120 min and significantly longer than that obtained with extracts from *E. coli* MRE600 containing exonuclease V.

As a further test of the ability of N138 extracts to support the synthesis of polypeptides programmed by linear DNA templates, standard 30 min reactions were carried out over a range of DNA concentrations. The template used was the 5.4 kb plasmid pBR325, linearised with *Pst*I which cuts within the β-lactamase gene. This plasmid codes for a 24.8 kDa protein, chloramphenicol acetyl transferase (CAT). As shown in fig.3 as little as 375 ng of template was sufficient to promote readily detectable amounts of [^{35}S]methionine-labelled CAT with the *rec*B^{ts} extract compared to more than 5 μg with extracts prepared from the MRE600 strain. In fact, longer exposures of the gel in fig.3 detected CAT programmed by 50 ng of DNA.

In our initial studies [2] with linear DNA templates we reported that extracts of MRE600 did support the synthesis of a number of polypeptides, although with a reduced efficiency compared to covalently closed circular molecules. In the present study we found, as indicated in fig.2, that in contrast, linear molecules were rapidly degraded and were quite inefficient as templates. Similar results, using the basic system described in [2], have also been obtained with another *E. coli* K12 strain (J. Merrick, personal communication). The basis for this apparent discrepancy has now been satisfactorily resolved with the finding that freshly prepared extracts of strain MRE600 and K12 strains contain high levels of an exonuclease activity, presumably exonuclease V which decays upon storage of extracts. Thus, extracts kept, for example, for 1 year in liquid nitrogen have quite low levels of exonuclease activity.

In conclusion, we now find a substantial improvement in the efficiency of linear DNA templates in this cell-free extract system, reflecting the increased stability of the template when extracts were prepared from a *rec*B^{ts} mutant. Furthermore, provided the preparative procedures and the preincubation period indicated in section 2 are followed, the high backgrounds, which normally accompany the complete absence of exonuclease V (see also [4]) can be successfully avoided.

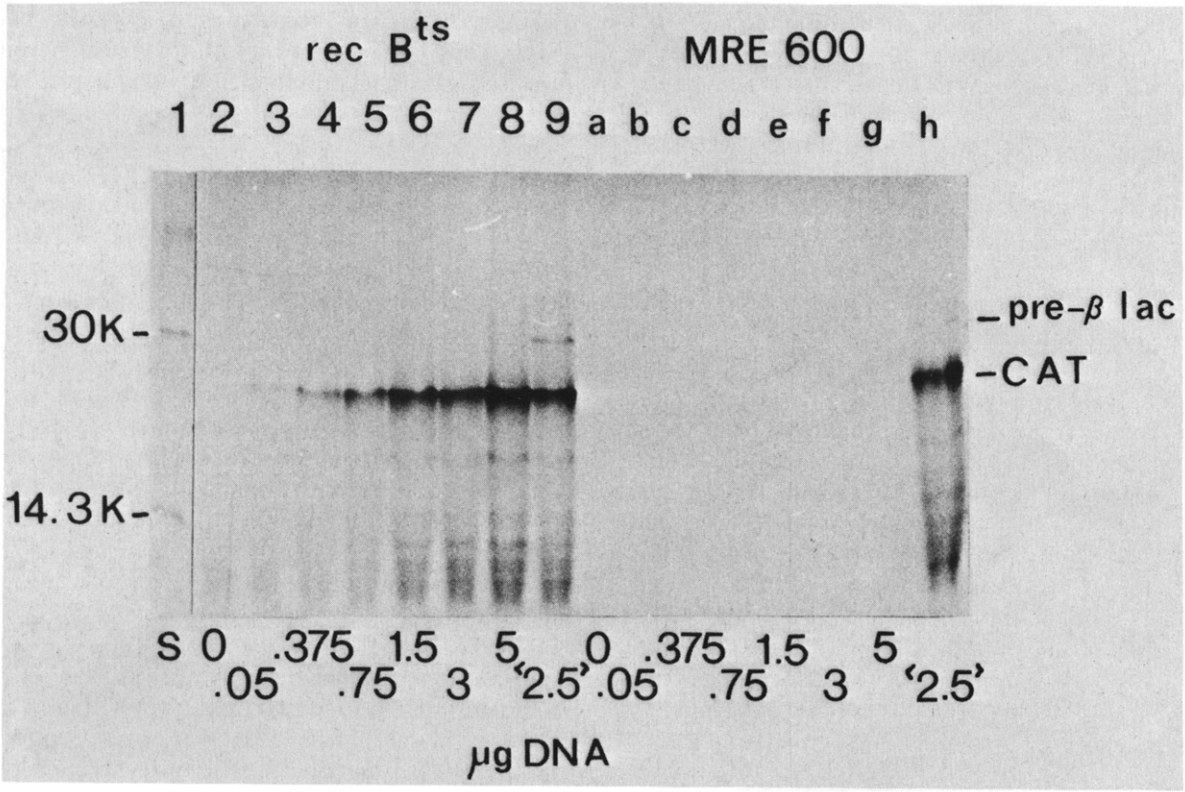

Fig.3. Effect of DNA concentration on polypeptide synthesis. pBR325 coding for major products, CAT and pre-β-lactamase was cut at the single *PstI* site and the linear fragments incubated in the presence of [^{35}S]methionine with N138 (*recB*^{ts}) S30 extract (tracks 1–9), or MRE600 (*recB*$^{+}$) S30 (tracks a–h). After 30 min incubation samples were removed and analysed by SDS–PAGE (15% acrylamide) and autoradiography. The amount of template DNA used in each incubation is indicated; tracks 9 and h programmed by 2.5 μg supercoiled pBR325. Molecular mass standards, carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa). Note that longer exposures of samples programmed by 3–5 μg linear DNA in the MRE600 extract revealed a band at the CAT position. K, kDa.

ACKNOWLEDGEMENTS

M.J. acknowledges the receipt of a Medical Research Council Graduate Studentship. We also wish to acknowledge the support of the Medical Research Council through a Project Grant Award (G978/839/C).

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