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# A coupled in vitro transcription–translation system for the exclusive synthesis of polypeptides expressed from the T7 promoter

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A coupled transcription–translation in vitro system has been developed in *Escherichia coli* specifically for the expression of genes under the exclusive control of the T7 promoter. This system consists of an *E. coli* crude extract (prepared from cells containing endogenous T7 RNA polymerase), rifampicin (an *E. coli* RNA polymerase inhibitor) and a labelled amino acid. When primed with a plasmid template encoding the target gene under exclusive control of the T7 promoter, this system has the capability to synthesize relatively large amounts of a unique, labelled polypeptide. This paper describes the characteristics and use of such a T7 RNA polymerase/T7-promoter specific in vitro system.

T7 RNA polymerase; Coupled transcription–translation system; Rifampicin; Specific expression; T7-S30

## 1. INTRODUCTION

The components of an ordinary *E. coli* in vitro coupled transcription–translation system include a cell-free, DNA-free extract of *E. coli* (S30), a labelled amino acid (usually [$^{35}$S]methionine) and a low mol. wt. mix which provides a supplement of factors including amino acids and nucleotide triphosphates (NTPs). The template DNA can be phage, plasmid, or linear DNA fragments. All genes encoded by the template are expressed and this includes the gene specifying the target polypeptide, as well as vector encoded proteins (the major products being the antibiotic resistance genes). We have taken advantage of the specificity of the T7 RNA polymerase and promoter by producing an *E. coli* in vitro transcription–translation system for the exclusive expression of target genes under the control of the T7 promoter. T7 TNA polymerase exhibits extreme specificity for its own promoter and will efficiently transcribe any DNA cloned downstream of the T7 promoter [1]. An S30 extract containing endogenous T7 RNA polymerase (T7-S30) was prepared and optimised for use in vitro. Rifampicin was added to the system in order to selectively inhibit *E. coli* RNA polymerase [2] and under such conditions, when primed with a plasmid template (encoding the target gene under exclusive control of the T7 promoter), the system can be used to synthesise unique, labelled target protein. In this paper we demonstrate the preparation, use and characterisation of this in vitro system.

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## 2. MATERIALS AND METHODS

Bacterial strain MC4100 (*ara*D139, *Δ* (*lac* IPOZYA) U169, *thi*A, *rel*A1, *rps*L) was used to host plasmid pAR1219. Plasmid pAR1219 confers ampicillin resistance (*β* lactamase) and also encodes the T7 RNA polymerase gene (gene *1*) under control of the inducible *lac* promote. [3]. T7-S30 extract was prepared from strain MC4100/pAR1219 as described [4] except that the cells were initially induced by the addition of 0.4 mM IPTG at an $A_{600nm}$ of 1, then incubated at 37°C for a **further three** hours. Extracts were prepared from MRE600 (RNase$^-$) **for comparison.** Plasmid templates used to direct polypeptide synthesis in ade pMJ216 [5] (+T7 promoter, Fig. 1), pLB8000 [6] (−T7 promoter), which encodes the outer membrane protein, LamB (47 kDa) and plasmid pYCP2 [7] which encodes a portion of the *his* operon (*hisJ*, *hisQ* and *hisM*) under control of the *lac* promoter. SDS-PAGE analysis was performed as described in [8] and fluorography as described in [9]. In vitro transcription reactions using commercially available T7 RNA polymerase (Promega Biotech) were carried out as described in [10]. Transcription–translation incubations were carried out as described in [4] except that 2 *μ*l rifampicin (10 mg/ml in 10% methanol) was added per 30 *μ*l incubation.

## 3. RESULTS AND DISCUSSION

To prepare an S30 extract capable of efficient coupled transcription–translation, which contained active T7 RNA polymerase and low endogenous mRNA and DNA, the existing method [4] had to be modified. Conditions necessary to induce the synthesis of sufficient levels of T7 RNA polymerase in the host strain were first determined. It was important to strike a balance between having sufficient T7 RNA polymerase present and avoid making the cells 'sick' through chronic overproduction which might affect the translational activity of the resulting S30 extract. Incubation of late logarithmic cells (MC4100/pAR1219) with 0.4 mM IPTG for varying lengths of time (between 0.5 and 3 h, unpublished results) indicated that a 3 h induction resulted in

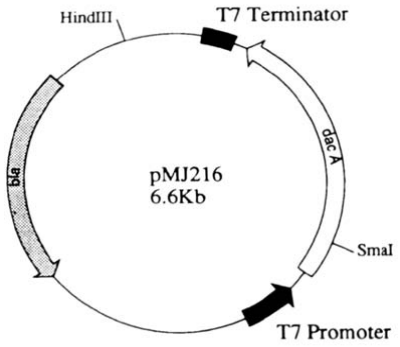


Fig 1 Map of pMJ216 Plasmid map of pMJ216 showing the relative positions of the T7 promoter and terminator sequences The direction of the arrows indicates the direction of transcription Relevant restriction enzyme sites are also shown

good expression of T7 RNA polymerase (unpublished results). Using these induction conditions, an S30 extract was prepared as described in [4]

Preliminary experiments showed that the T7-S30 extract had the ability to support *E coli* RNA polymerase-specific transcription–translation comparable with that in normal extracts prepared from MRE600 (unpublished results). On the whole, alteration of the procedure for S30 extract preparation had no significant effect on protein synthesis efficiency. However, the concentration of DNA template required to direct detectable levels of polypeptide synthesis did differ Identical incubations were set up containing varying concentrations of the DNA template pMJ216 which encodes the *E coli dac*A gene under control of the T7 promoter Both supercoiled and linear templates were compared. SDS-PAGE analysis showed that as little as 25 ng (Fig 2, track 3) of supercoiled pMJ216 was sufficient to direct polypeptide synthesis, compared with 250 ng in the normal extract (unpublished results) Similarly with a linear template, ordinary extract required in excess of 1 $\mu$g of linear DNA (unpublished results), whereas with the T7-S30 extract, 500 ng (Fig 2, track 13) of linear DNA was sufficient Moreover, T7-S30 extract exhibited little, if any, background incorporation, demonstrating that no endogenous mRNA and DNA was present in the extract Normally extracts are prepared from plasmid-free strains and so it was possible that plasmid pAR1219 could have survived the extract procedure to act as an endogenous template However, in T7 RNA polymerase-specific extracts, endogenous DNA templates would not be such a problem since host derived template expression can be selectively inhibited by rifampicin [2]

In order to determine the concentration of rifampicin needed to inhibit transcription by *E coli* RNA polymerase in the extract, plasmid pLB8000 (−T7 promoter) was used as a template The addition of methanol alone (0 7% v/v final concentration, Fig 3, track 2) had no effect upon expression, but increasing concentrations of rifampicin (in methanol, Fig 3, tracks 3–7) inhibited *E*

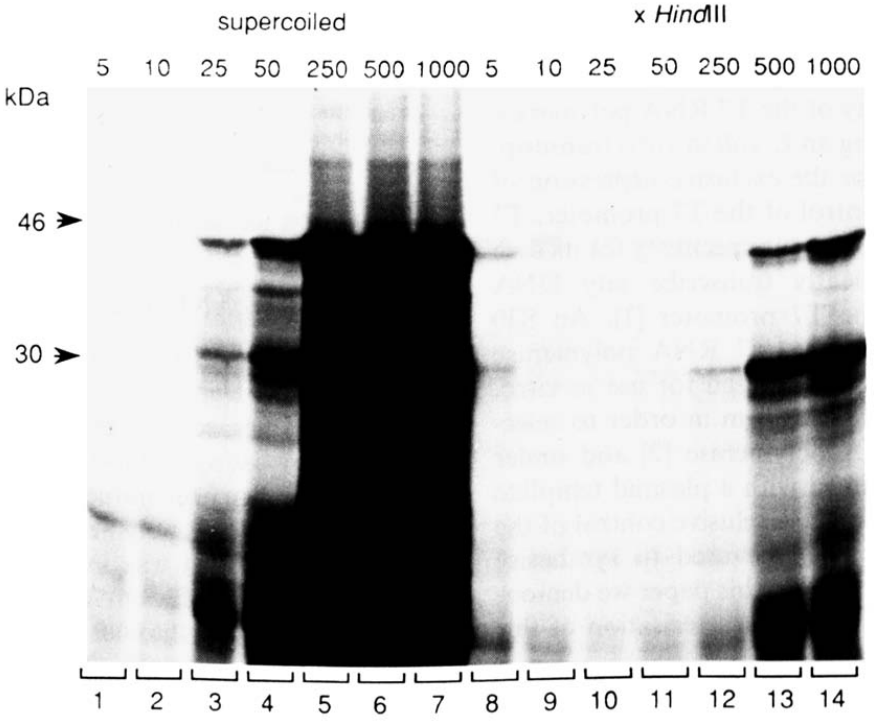


Fig 2 The effect of DNA concentration on polypeptide synthesis in T7-S30 extract In vitro transcription–translation incubations containing T7-S30 extract were primed with supercoiled pMJ216 (tracks 1 7), or pMJ216 cut at the unique *Hin*dIII site (tracks 8 14) and incubated at 37°C with [$^{35}$S]methionine for 45 min The amount of DNA added to each incubation is indicated in ng per 15 $\mu$l incubation Samples were analysed by SDS-PAGE and fluorography

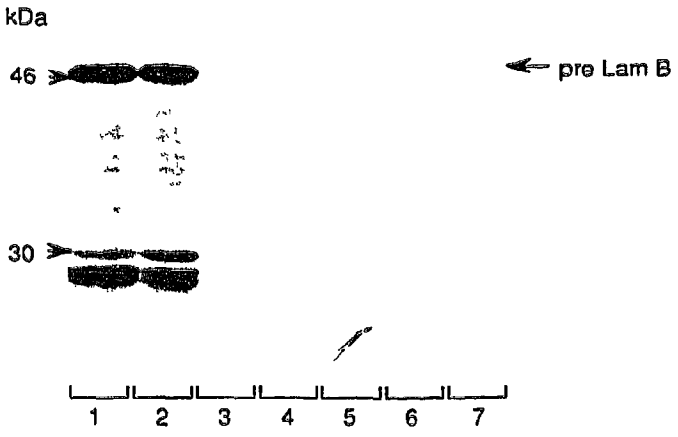


Fig 3 The effect of rifampicin on polypeptide synthesis in vitro In vitro transcription–translation incubations containing MRE600 extract were primed with 0 5 $\mu$l of pLB8000 in the presence of varying concentrations of rifampicin (tracks 3–7 contained 3, 13, 30, 350 and 700 $\mu$g/ml rifampicin in 0 7% v/v methanol final concentration, respectively) Incubations 1 and 2 contained no rifampicin but, in addition, 2 contained 0 7% v/v methanol final concentration After a 45 min incubation at 37°C the samples were analysed by SDS-PAGE and fluorography

*coli* RNA polymerase and therefore abolished protein synthesis However, on longer exposure of the autoradiograph faint bands were visible in tracks 3, 4, and 5 of Fig 3 (3, 13 and 30 $\mu$g/ml final concentration of rifampicin, respectively) and so for total inhibition, a final concentration of 350 $\mu$g/ml of rifampicin (2 $\mu$l of 10 mg/ml rifampicin in 10% methanol, Fig 3, track 7) was added to each subsequent incubation

In the presence of rifampicin, peptide synthesis would indicate that active T7 RNA polymerase was present in the S30 extract. In vitro transcription–translation incubations containing T7-S30 extract and rifampicin were therefore primed with plasmid pMJ216 (+T7 promoter) In parallel, pMJ216 cut at the unique *Hin*dIII site and pMJ216 cut at the unique *Sma*I site (Fig 1) was also used to prime identical incubations SDS-PAGE analysis (Fig 4) revealed the presence of two labelled bands, pre-DacA and pre-$\beta$ lactamase in the incubation primed with supercoiled pMJ216 (Fig 4, track 1), whereas in the incubation primed with pMJ216 cut at the unique *Hin*dIII site (Fig. 4, track 2), pre-DacA was the major product In the incubation primed with pMJ216 cut at the unique *Sma*I site, no polypeptides were synthesised. Firstly, we conclude that active T7 RNA polymerase was present in the extract, since polypeptide synthesis was achieved in the presence of rifampicin. Secondly, it was expected that only DacA would be expressed from supercoiled pMJ216 because of the presence of a T7 terminator site immediately after the *dac*A gene However, since $\beta$ lactamase was also synthesised, we conclude that the T7 terminator was functioning inefficiently. It has been shown in previous work that the T7 terminator is not entirely efficient in vitro [1] and we (unpublished results) and others [11] have demonstrated that this terminator is also inefficient in vivo Thirdly, use of pMJ216 cut at the *Sma*I site as a template for polypeptide synthesis showed that the T7 RNA polymerase did not recognise any other site on the plasmid as a start site for transcription This further indicates that the synthesis of $\beta$ lactamase arose due to a failure of T7 RNA polymerase to terminate transcription The transcripts from these various pMJ216 templates were generated using in vitro transcription reactions containing commercially available T7 RNA polymerase Agarose gel electrophoresis (unpublished results) showed that in the presence of supercoiled plasmid a smear of RNA was present, probably representing a large number of different sized RNA transcripts produced as a result of transcription (by T7 RNA polymerase) proceeding many times round the plasmid Reactions primed by the linear DNA template pMJ216 resulted in discrete bands of RNA suggesting that run-off transcripts were produced However, use of the template pMJ216 cut at the *Sma*I site (where the T7 promoter was effectively removed from any downstream genes) also resulted in transcript production It is possible that the polymerase molecule is able to swing round and transcribe the opposite strand of the DNA molecule, as has been demonstrated with other templates [12], resulting in longer transcripts Subsequent translation of the transcripts (Fig 5) showed that the *Sma*I transcripts (track 3), contrary to expectation, encoded

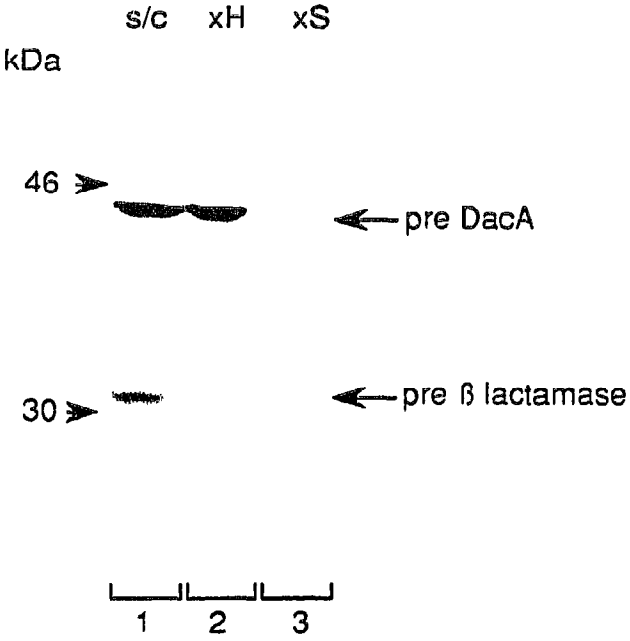


Fig 4 Polypeptide synthesis in T7-S30 extract primed with pMJ216 in the presence of rifampicin In vitro transcription translation incubations containing T7-S30 extract and rifampicin (350 $\mu$g/ml) were primed with 0 5 $\mu$g of pMJ216 Supercoiled pMJ216 (track 1), pMJ216 cut at the unique *Hin*dIII site (track 2) and pMJ216 cut at the unique *Sma*I site (track 3) After a 45-min incubation at 37°C, the samples were analysed by SDS-PAGE and fluorography

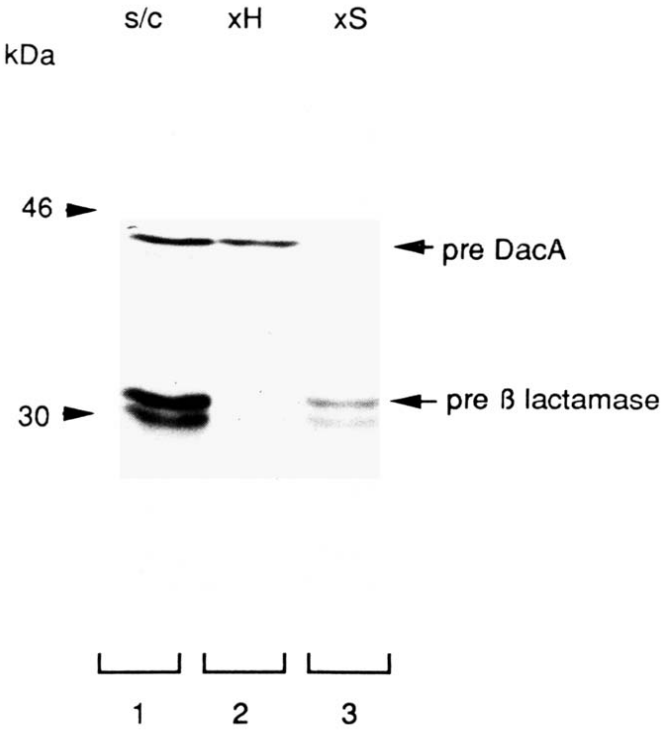


Fig 5 In vitro translation of T7-specific transcripts of pMJ216 In vitro transcription–translation incubations containing MRE600 extract in the presence of rifampicin (350 $\mu$g/ml) were primed with half of the transcripts synthesised in in vitro transcription reactions which had been primed with supercoiled pMJ216 (track 1), pMJ216 cut at the unique *Hin*dIII site (track 2) and pMJ216 cut at the unique *Sma*I site (track 3) After a 45 min incubation at 37°C the samples were analysed by SDS-PAGE and fluorography

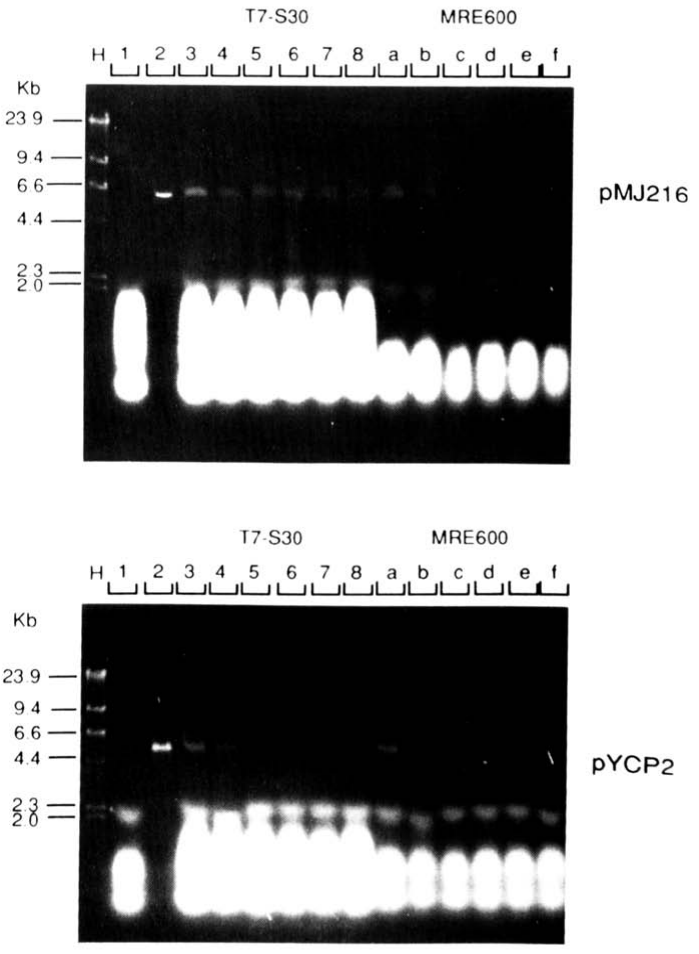


Fig 6 Stability of linear DNA template in T7-S30 extract 150 $\mu$l in vitro transcription–translation incubations containing MRE600 extract (track a–f) and T7-S30 extract (tracks 3–8) were primed with 5 $\mu$g of plasmid pYCP2 cut at the unique *Eco*RI site (bottom photograph) and pMJ216 cut at the unique *Hin*dIII site (top photograph) Incubation was carried out at 37°C in the absence of rifampicin Samples were removed at various time intervals (0 15, 30, 45, 60, 100 min, tracks 3–8 and tracks a–f, respectively) and added to an equal volume of agarose gel sample buffer (containing 4% w/v SDS) The samples were analysed by agarose gel electrophoresis on a 1% (w/v) gel then stained with ethidium bromide (0 5 mg/ml) for 30 min For reference track 1 shows extract without added DNA and track 2 shows 0 5 $\mu$g of the relevant plasmid template Track H shows $\lambda$*Hin*dIII standards

polypeptides of the same size as $\beta$ lactamase, whereas the *Hin*dIII transcripts encoded DacA and the supercoiled transcripts encoded both DacA and $\beta$ lactamase Comparison of the amount of protein synthesised from the transcripts with that synthesised from the T7-specific in vitro coupled transcription–translation incubations indicated that more efficient protein synthesis occurred in the coupled system

We have shown here that the inclusion of a transcription terminator sequence does not preclude the necessity to linearise plasmid templates for unique expression Restriction endonuclease digestion is not only another step to perform but also linear DNA is rapidly degraded by endogenous endonuclease V activity in the extract preparations [13]. Therefore, to investigate the half-life of linear DNA in the two extracts, two plasmids pMJ216 (+T7 promoter) and pYCP2 (−T7 promoter) were chosen because of their similarity in size. Both plasmids were used to prime incubations containing either T7-S30 extract, or MRE600 extract Agarose gel electrophoresis showed that linear pMJ216 is still present after 100 min incubation in the T7-S30 extract (Fig 6, top photograph, track 8), but is rapidly degraded in the ordinary S30 (tracks a–f). In contrast, pYCP2 is rapidly degraded in both extracts (Fig. 6, bottom photograph) Therefore, linear DNA only survives well when a T7 promoter is present on the plasmid and T7 RNA polymerase is present in the extract T7 RNA polymerase elongates RNA chains five times faster than *E coli* RNA polymerase [2] and so it is possible that the increased frequency of transcription prevents degradation of the linear template Therefore, in the presence of T7 RNA polymerase, a DNA template encoding the T7 promoter (and therefore recognised by T7 RNA polymerase) is more stable than DNA not recognised by T7 RNA polymerase. This explains why less linear DNA

was required to productively prime the T7-S30 extract (Fig. 2 and unpublished results) when compared with a normal S30 extract.

In summary, we have developed a coupled transcription–translation system for the generation of polypeptides cloned downstream of the T7 promoter This coupled system is not only simple to prepare and use, but also yields more polypeptide product from a given amount of DNA template than when the processes of transcription and translation are uncoupled.

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