Biochemical studies on the transcription antitermination function of the N gene product of coliphage λ.

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SUMMARY

An *in vitro* assay has been developed for the activity of the *N* gene product (pN) of bacteriophage λ, based on the N-dependent stimulation of *trp* mRNA synthesis from the DNA templates of λ*trp* transducing phages. Using this assay system, we show (a) that the stimulation of *trp* mRNA synthesis by pN requires the *nutL* sequence on the λ chromosome and (b) that pN can participate *in vitro* in the formation of RNA polymerase complexes that are competent for the transcription antitermination observed in our system.

INTRODUCTION

Efficient transcription of the delayed-early genes in phage λ is dependent upon the antitermination activity of the phage gene N product (pN). In the absence of pN function, transcription from the p_L and p_R phage promoters is terminated at sites, t_{L1} and t_{R1}, through the action of the host-specified rho factor (Roberts,1969).

The mechanism of the antitermination activity exerted by pN is not clearly understood. Some evidence indicates that pN at some stage interacts with RNA polymerase to effect antitermination (Georgopoulos,1971; Ghysen and Pironio, 1972; Epp and Pearson,1976; Baumann and Friedman,1976; Sternberg,1976). Salstrom and Szybalski (1978) have suggested that this association may precede the initiation of transcription, or possibly occur during transcription, at the nut site on the λ chromosome. From analyses of genetic hybrids formed in crosses between related bactriophages with diverse N-like gene functions (Salstrom and Szybalski,1978; Salstrom et al.,1979), and from the genetic and sequence analyses of $nutL^-$ phage mutants (Salstrom and Szybalski,1978; Rosenberg et al.,1977) defective in the recognition of pN for antitermination, the nutL site is inferred to lie just to the left of the p_L promoter.

Here we demonstrate in vitro that (a) pN stimulates trp mRNA synthesis on λtrp DNA by overcoming transcription termination barriers within the translocated trp operon segment, and (b) like in vivo, pN activity in this assay depends on the integrity of the nutL site of λ.

MATERIALS AND METHODS

a) Strains. All bacteria, bacteriophages and plasmids employed in this study are listed in Table 1. The rif^r mutant of E.coli A19trpAE1 was spontaneously isolated and mapped at the rpoB locus on the E.coli chromosome by P1kc transduction.

b) In vitro transcription system and RNA assay. The reaction mixture (0.42 ml) contained 40 mM Tris-HCl (pH 7.8), 6 mM β-mercaptoethanol, 50 mM KCl, 10 mM $MgCl_2$, 0.2 mM each of ATP, GTP,CTP and [^{3}H]-UTP (0.60 mCi/μmole), 1 mM K_2HPO_4, 5 μg of phage DNA and various amounts of nucleic acid-free S-100 protein. In the experiments employing purified E.coli RNA polymerase, the reaction mixture (0.2 ml) contained 40 mM Tris-HCl (pH 7.8), 6 mM β-mercaptoethanol, 50 mM KCl, 10 mM $MgCl_2$, 0.2 mM each of ATP,GTP,CTP and [^{3}H]-UTP (1.2 mCi/μmole), 5 μg of phage DNA and 3 μg of E.coli RNA polymerase. After incubation, reaction mixture was treated with 5 μg of pancreatic DNaseI for 5 min at 30° C and followed by the addition of 0.1 ml of 5 % SDS. The mixture was then quickly chilled to 0 °C. The RNA was extracted with phenol, precipitated with ethanol at -20° C and dissolved in 3 X SSC (1 X SSC: 0.15 M NaCl-0.015 M sodium citrate) containing 1 mM EDTA. The trp mRNA was assayed as described previously (Ishii and Imamoto 1976). The N mRNA was determined by DNA-RNA hybridization with l-strand DNA of λbio256 and λbio3h-1 phages.

c) Preparation of nucleic acid-free S-100 extracts. Cells of A19trpAE1, A19trpAE1rif^r and A19trpAE1(pYS5) were grown at 30° C in L-broth to 4 x 10^8 cells/ml. The cultures were quickly heated to 42° C for 12 min and then rapidly cooled to 37° C for 10 min further incubation. In the case

of phage infection, cultures of A19trpAE1 were grown to 4 X 10^8 cells/ml in L-broth (30° C) and infected at a multiplicity of 5. After a 15 min (stationary) adsorption period at 30°C, the cultures were incubated aerobically for 10 min at 30°C. The cultures were mixed with sufficient crushed ice to lower the temperature below 4°C within a few seconds. The cells were harvested by centrifugation, washed with buffer$I_{0.06}$[10 mM Tris-HCl(pH 7.8), 60 mM KCl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol] and then suspended in the same buffer. Cells were lysed by sonication and the lysate was centrifuged for 3 hrs at 37,000 rpm in the SW50.1 rotor of the Beckman model L ultracentrifuge. The clear supernatant was removed to yield the S-100. The extract was adjust to 0.4 M KCl and loaded onto a DEAE-cellulose column previously equilibrated with buffer$I_{0.4}$[10 mM Tris-HCl(pH 7.8), 0.4 M KCl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol]. The column was washed with the same buffer, and fractions containing the yellow color were pooled to yield the nucleic acid-free S-100. After addition of solid ammonium sulfate (56.1 g/100 ml of nucleic acid-free S-100), the resulting precipitate was resolved in buffer$I_{0.06}$ and dialyzed against buffer$I_{0.06}$. After dialysis, the extract can be stored for up to 6 months at -70°C before pN activity is assayed.

d) Preparation of phage DNA. Phage DNA's were prepared as described previously (Ishii and Imamoto,1978) and dissolved in a 10 mM Tris-HCl buffer (pH 7.8) after dialysis against the solution, and stored at 4°C. Isolated λcI90, λbio256 and λbio3h-1 phage DNA strands were prepared by the poly (U,G)/CsCl technique (Bøvre et al.,1971).

e) The other materials and methods used in the present experiments were as described elswhere (Ishii and Imamoto,1976).

RESULTS AND DISCUSSION

In vitro transcription system responding to pN function. Quantitative estimates of pN activity have been accomplished by using DNA-directed transcription-translation systems to synthesize particular proteins. Two such systems have been reported: in one, pN permits transcription originating at the p_R promoter to extend into gene R, resulting in the synthesis of endolysin (Greenblatt,1972) ; and in the other, pN allows anthranilate synthetase synthesis as a result of readthrough from p_L into trp on a λtrp DNA template (Dottin and Pearson,1973). We have developed a transcriptional assay for pN function in vitro based on the pN-dependent stimulation of trp mRNA synthesis from a λtrp DNA template, a reaction catalyzed by a nucleic acid-free extract of soluble protein (S-100) from E.coli. In the λtrp phages, the distal part of the trp operon (trp D,C,B,A) is fused to the N operon such that transcription of the trp operon segment depends on the p_L promoter (see Fig.1) and occurs in the absence of translational activities.

Transcription initiating at the p_L promoter of λtrp48 in the absence of N function is frequently terminated at at least two sites inside the trp operon segment (Nakamura et al.,1978a; Nakamura et al.,1978b). The first such site is located in the trpD gene and the second site is in the trpC gene (see Fig.1). However, termination of transcription at these sites in vivo appeares to be leaky, since some expression of the distal trp genes is observed. In an in vitro transcription system employing purified E.coli RNA polymerase, this background or "read-through" transcription of trp genes C,B and A can be almost entirely eliminated (a) by deletion of the low level p_{2trp} promoter and (b) by the addition of a relatively high concentration (35 μg/ml) of purified rho factor. In this system, the overall rate of trp D mRNA synthesis was reduced by about 30 % of the level observed in the absence

of rho factor (data not shown). Most of the trpD mRNA molecules produced in the presence of rho factor were short, sedimenting as a symmetrical peak of about 16 S (Fig.2a). Sedimentation of the RNA synthesized in the absence of rho factor, however, revealed the presence of an additional species of trpD mRNA of about 23 S (Fig.2b). This indicates the presence of at least one site where termination of transcription is facilitated by rho function at or near the middle of trp C-D region of λtrp48Δp_{2trp}.

When the nucleic acid-free S-100 extract of E.coli A19trpAE1 was used instead of purified RNA polymerase, the overall synthesis of trp mRNA directed by λtrp48Δp_{2trp} DNA was severely depressed (Table 1a). In these conditions the synthesis of N mRNA from the promoter-proximal portion of the N-trp operon proceeded at a virtually normal rate. The observed low-level of trp mRNA synthesis is therefore presumed to result from premature termination of p_L-initiated transcription at the specific site near to the trp D-E boundary, where rho factor (endogenously supplied) could function more efficiently in the S-100 extract than in the purified system. The data presented in Table 1a reveals a remarkable stimulation (about 40-fold) of trp mRNA synthesis when the S-100 extract was used derived from the thermo-induced E.coli A19trpAE1 carrying pYS5, a colE1-type plasmid constructed in vitro which carries the EcoRI-generated cIII-N-cI-O segment of phage λcI857 (ts) croam6 DNA. The two-fold higher level of trpCBA mRNA relative to trp D mRNA reflects coordinate synthesis of trp mRNA from the whole region of the translocated trp operon segment in λtrp48 (cf. Nakamura et al.,1978a). This is consistent with antitermination exerted at otherwise termination sites within the trp operon segment.

Several lines of evidence indicate that it is pN itself that is stimulating

overall synthesis of trp mRNA in the in vitro system. First, the stimulatory effect of trp mRNA synthesis on λtrp48Δp_{2trp} DNA was also observed with the nucleic acid-free S-100 extract from λ or λcI857tof12 -infected A19trpAE1 (Table 1). In these cases, however, production of mRNA from the promoter-distal trp genes was less than that seen with the extract from the strain carrying pYS5. Second, stimulation of trp mRNA synthesis was barely detectable in the S-100 extracts of A19trpAE1 cells infected with λNam7am53cI857tof12 or with λimm21 (Table 1). Third, the S-100 extract of λNts8-infected A19 trpAE1 showed little or no stimulation of trp mRNA synthesis at the non-permissive temperature (40° C), but at 30° C stimulated trpCBA mRNA as well as λ or λtof12 (see Table 1c). Therefore, we conclude that the observed stimulation of trp mRNA synthesis is due to the activity of pN rather than some other λ-coded protein whose synthsis is dependent on pN.

Comparing the relative factors of stimulation of trpD and trpCBA mRNA synthesis by the various pN-containing S-100 extracts on the λtrp48Δp_{2trp} DNA template, we further conclude that the pYS5 plasmid carrying strain, designed to over-produce pN after temperature induction of this strain, indeed yields about 25 times more pN activity than in the thermoinduced λcI857croam6 lysogen (unpublished data). Thus, using the pN-containing S-100 extract from pYS5-carrying cells, we compared the effectiveness of the λtrp48Δp_{2trp} DNA template relative to several other λtrp DNA templates (see Fig. 1). The results presented in Table 2 reveal that the factor of the stimulation caused by the pN-containing extract was greater on templates with fewer transcriptional termination signals. The decreasing order of stimulation was : λtrp48Δp_{2trp}, λtrp48, λtrp60-3(which retains a strong t_N-site (Nakamura et al.,1978b), and λtrp34(which bares two t_L sites and at least

one t_N- site) (see Fig.1). We note that in the experiment of Table2, the individual reactions were carried out in S-100 extracts of A19trpAE1 to which a smaller aliquote of the pN-containing S-100 extract was added. The amount of trp mRNA synthesized on λtrp48Δp_{2trp} DNA in the pN-free S-100 extract was nearly proportional to the amount of pN-containing extract added (see Fig. 4a). Further addition of the pN-containing extract (more than 1 mg/ml) caused a decrease in the level of trp mRNA synthesis. [Under these conditions, the initiation frequency at p_L in λtrp DNA was not markedly affected by the addition of increasing amount of the pN-containing extract to the pN-free extract, indicating an excess of RNA polymerase in the latter extract (cf. N mRNA levels in Table 1a and unpublished data).]

Interaction of pN with RNA polymerase. Using the foregoing transcription assay system, we (a) demonstrated the functional association of pN and RNA polymerase in vitro, an interaction which could be catalyzed by the nutL sequences of λ, and (b) ascertained in vitro the effect of a $nutL^-$ mutation, a defect in the leftward site of N utilization. In the experiments described below, increasing amounts of the pN-containing S-100 extract of thermoinduced A19trpAE1(rif^+) carrying pYS5 were added to a constant amount of the pN-free S-100 extract of A19trpAE1(rif^r), in the presence or absence of rifampicin, and the stimulation of trpmRNA synthesis from the λtrp48Δp_{2trp} DNA template was ascertained. First, in a control experiment, RNA polymerase from A19trp AE1 bearing the rif^r mutation was found to be quite resistant to rifampicin over the entire range of the drug concentrations tested (Fig. 3a); this is in contrast to the enzyme from the isogenic rif^+ strain and its pYS5-carrying derivative, which were found to be sensitive to very low levels of rifampicin (Fig.3, b and c). [In the experiments shown in Fig.3, a relatively low

concentration of the extracts (one fifth amount of the protein employed for standard assay) was used to alleviate premature termination of transcription.] Evidence that pN and RNA polymerase interact *in vitro* was obtained in the experiments represented in Fig.4, a and b. Synthesis of *trp* mRNA from λ*trp*48Δp_{2trp} DNA in an extract of A19*trpAE1* *rif*^{r} was progressively stimulated by increasing amounts of the pN-containing extract from the *rif*$^+$ strain carrying pYS5. The amounts of *trp* mRNA synthesized in the presence of rifampicin at all concentrations of pN tested (up to a maximum stimulation at a ratio of 4 of *rif*^{r} to *rif*$^+$ RNA polymerase) was about 70 % that produced in the absence of rifampicin (compare Fig.4, a and b). These results suggest that newly formed pN-*rif*^{r} RNA polymerase complexes participate efficiently in the transcription of the *N-trp* operon of λ*trp* DNA. We favor the possibility that pN forms its initial association with RNA polymerase (holoenzyme) in a post-initiational event, perhaps at the *nutL* site, to stimulate *trpCBA* mRNA synthesis.

When the DNA from λ*trp*48Δp_{2trp} phage carrying the *nutL44* mutation was used as template, the stimulation of *trp* mRNA synthesis by the addition of the pN-containing extract was barely detectable, both in the presence or absence of rifampicin (Fig.4, d and c). As a control to assess the overall capacity of the assay system to overcome transcription termination barriers on the template DNA, we examined the pN-dependence of transcription directed by the p_R promoter and normally controlled *in vivo* by the *nutR* site. The transcripts arising from the p_R promoter can be specifically assayed by using the *r*-strand of λ*cI90* DNA as a probe in DNA-RNA hybridization. Under the conditions employing λ*trp*48Δp_{2trp} *nutL44* DNA as a template, the addition of the pN-containing extract stimulated transcription originating at the p_R promoter. Stimulation was observed both in the presence and absence of

rifampicin, in contrast to the failure to stimulate transcription initiating at the p_L promoter (Fig.4, d and c). The stimulation of p_R-directed transcription was roughly linear, depending on the amount of pN-containing extract added, and was as efficient as that seen with $\lambda trp48\Delta p_{2trp}$ DNA (cf. Fig.4, a and b).

These results support a previous conclusion by Salstrom and Szybalski (1978) that the $nutL^-$ mutation specifically prevents the action of pN on transcription from the p_L promoter. Furthermore, since the cell-free system used lacks ribosomes and other essential components for translation, N-mediated antitemination does not require any contemporaneous translation of the mRNA. The *in vitro* transcription assay system is anticipated to be useful to determine the details of the interaction of pN and RNA polymerase.

ACKNOWLEDGMENTS

We are deeply indebted to Dr. W. Szybalski who was very generous in critical review of the manuscript prior to its submission and providing manuscripts relating to their most recent work on the role and mode of action of pN. We are also grateful to Dr. D. Schlessinger for critical reading of the manuscript.

Legends to Figures and Tables

Table 1. Bacteria, bacteriophages and plasmids employed in this study.

Figure 1. Simplified molecular maps of λ*trp* phages.

The genetic maps of transducing phages are based on the data reported previously (Nishimune, 1973; Fiandt *et al.*, 1974; Franklin, 1974; Jackson and Yanofsky, 1972). The map of phage λ*trp*34 is based on the data of heteroduplex mapping obtained by Fiandt and Szybalski (personal communication). t_N- indicates pN-sensitive transcription termination sites within the *trp* operon, observed in the absence of pN function in λ*trp*. Interpretation of (t_N-) is in the text. The dotted lines indicate deleted portions of the genome.

Table 2. *In vitro* synthesis of p_L-directed mRNA from λ*trp*48Δp_{2trp} DNA in the presence or absence of functional pN^{λ}.

The reaction mixture containing 5 μg of λ*trp*48Δp_{2trp} phage DNA and 500 μg of nucleic acid-free S-100 protein was incubated for 40 min at the temperature indicated. The other conditions are as described in Materials and Methods.

Table 3. pN-dependent stimulation of *trp* mRNA synthesis on various λ*trp* DNA templates.

150 μg of nucleic acid-free S-100 protein from A19*trpAE1*(pYS5) or A19*trpAE1* were added to the reaction mixtures containing 670 μg of nucleic acid-free S-100 protein from A19*trpAE1* and 5 μg of template DNA from various phages. The levels of *trpD* mRNA and *trpCBA* mRNA synthesized in the reaction mixture containing 670 μg of S-100 protein from A19*trpAE1* was 143 cpm and 89 cpm, respectively. The other conditions are as described in Materials and Methods.

Figure 2. Sedimentation profiles of *trpD* mRNA synthesized by purified RNA polymerse in the presence or absence of *rho* factor.

The reaction mixture containing 5 μg of λ*trp*48Δp_{2trp} phage DNA and 3 μg of *E.coli* RNA polymerase was incubated for 30 min at 37° C in the presence (a) or absence (b) of 7 μg of *rho* factor. The other conditions are as described in Materials and Methods.

Figure 3. Effect of rifampicin on *trp* mRNA synthesis catalized by S-100 extracts of A19*trpAE1rif*^{r}, A19*trpAE1rif*$^+$ and A19*trpAE1rif*$^+$(pYS5).

The reaction mixture contained 130 μg of nucleic acid-free S-100 protein from A19*trpAE1rif*^{r} (a), A19*trpAE1rif*$^+$ (b) or A19*trpAE1rif*$^+$(pYS5) (c). Rifampicin was present at the concentrations indicated. O--O, *trpD* mRNA; ●—●, *trpCBA* mRNA.

Figure 4. pN-dependent synthesis of *trp* mRNA and λ mRNA from the λ*trp* DNA template carrying the *nutL*$^+$ or *nutL44* alleles.

The conditions employed were the same as described in Table 3 except that the reaction mixture contained 670 μg of nucleic acid-free S-100 protein from A19*trpAE1rif*^{r} instead of that from A19*trpAE1rif*$^+$. (a) λ*trp*48Δp_{2trp}*nutL*$^+$ DNA template, in the absence of rifampicin; (b) λ*trp*48Δp_{2trp}*nutL*$^+$ DNA template, in the presence of rifampicin (100 μg/ml); (c) λ*trp*48Δp_{2trp}*nutL44* DNA template, in the absence of rifampicin; (d) λ*trp*48Δp_{2trp}*nutL44* DNA template, in the presence of rifampicin (100 μg/ml). ●—●, *trpCBA* mRNA; O--O, p_R-promoted λ mRNA. The other conditions are as described in Materials and Methods.

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Table 1.

Strain	Relevant characteristics	Source or reference
Bacteria E.coli K12		
A19trpAE1	RNaseI$^-$ ΔtrpAE1 rif$^+$	Gesteland(1969)
A19trpAErif^r	RNaseI$^-$ ΔtrpAE1 rif^r	This study
Bacteriophages		
λtrp48Δp$_{2trp}$	trpΔDC181Nam7am53fed$^-$cIAt2 h$^\lambda$	Nakamura et al.(1978a)
λtrp48Δp$_{2trp}$nutL44	trpΔDC181Nam7am53fed$^-$cIAt2 h$^\lambda$nutL44	Salstrom and Szybalski(1978)
λtrp48	trp(D,C,B,A)$^+$	Franklin(1974)
λtrp60-3	trp(E,D,C,B,A)$^+$	Nishimune(1973)
λtrp34	trp(C,B,A)$^+$	This study
λcroam6cI857	N$^+$	Oppenheim and Oppenheim(1976)
λcI857tof12	N$^+$	Takeda et al.(1975)
λNam7am53cI857tof12	NN$^-$	Takeda et al.(1975)
λNts8	Nts	Tomizawa
λ	N$^\lambda$	
λimm21	N^{21}	
λbio256cI857	Δ(att-cIII:57.3-72.55%λ)	Szybalski and Szybalski(1974)
λbio3h-1nin5	Δ(att-N:57.3-73.5%λ)	Szybalski and Szybalski(1974)
λcI90		
ϕ80trpED		Sato and Matsushiro(1965)
ϕ80trpCBA		Deeb et al.(1967)
Plasmids		
pYS1	Ap^R* derivative of colE1	This study
pYS5	λcIII-N-cI-O segment of λcI857croam6 cloned in pYS1	This study

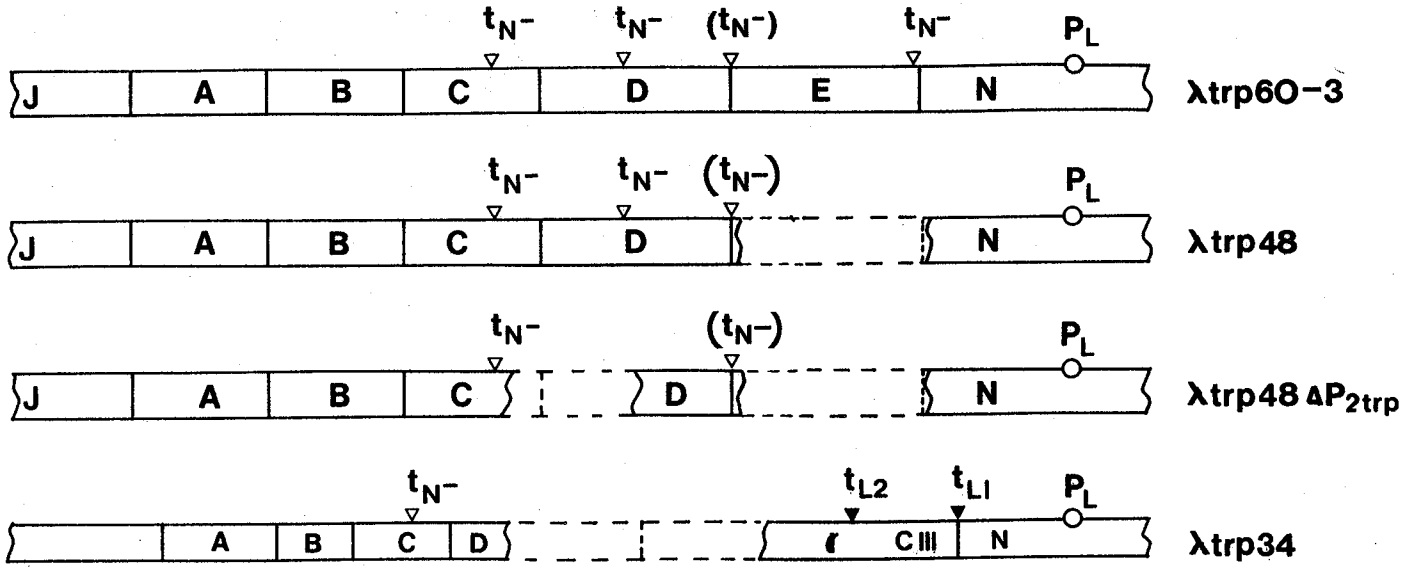
* Ap^R: ampicilin resistant

Table 2.

	Source of pN	Temperature of assay	Presence of functional pN^{λ}	mRNA synthesis (cpm)		
				trpCBA	*trpD*	*N*
(a)	pYS5	30°C	+	2,342	1,260	1,040
	none	30°C	-	61	150	840
(b)	λ*cI857tof12*	30°C	+	686	482	
	λ*Nam7am53cI857tof12*	30°C	-	41	76	
(c)	λ*Nts8*	30°C	+	467	318	
	λ*Nts8*	40°C	-	30	100	
	λ	30°C	+	494	305	
	λ	40°C	+	450	288	
(d)	λ	30°C	+	452	297	
	λ*imm21*	30°C	-	101	323	

Table 3.

	DNA template	Presence of pN	mRNA synthesis (cpm)	
			trpCBA	*trpD*
(a)	λ*trp*60-3	+	782	984
		-	78	85
(b)	λ*trp*48	+	991	1,217
		-	107	117
(c)	λ*trp*48Δp_{2trp}	+	1,104	1,461
		-	48	76
(d)	λ*trp*34	+	289	318
		-	38	71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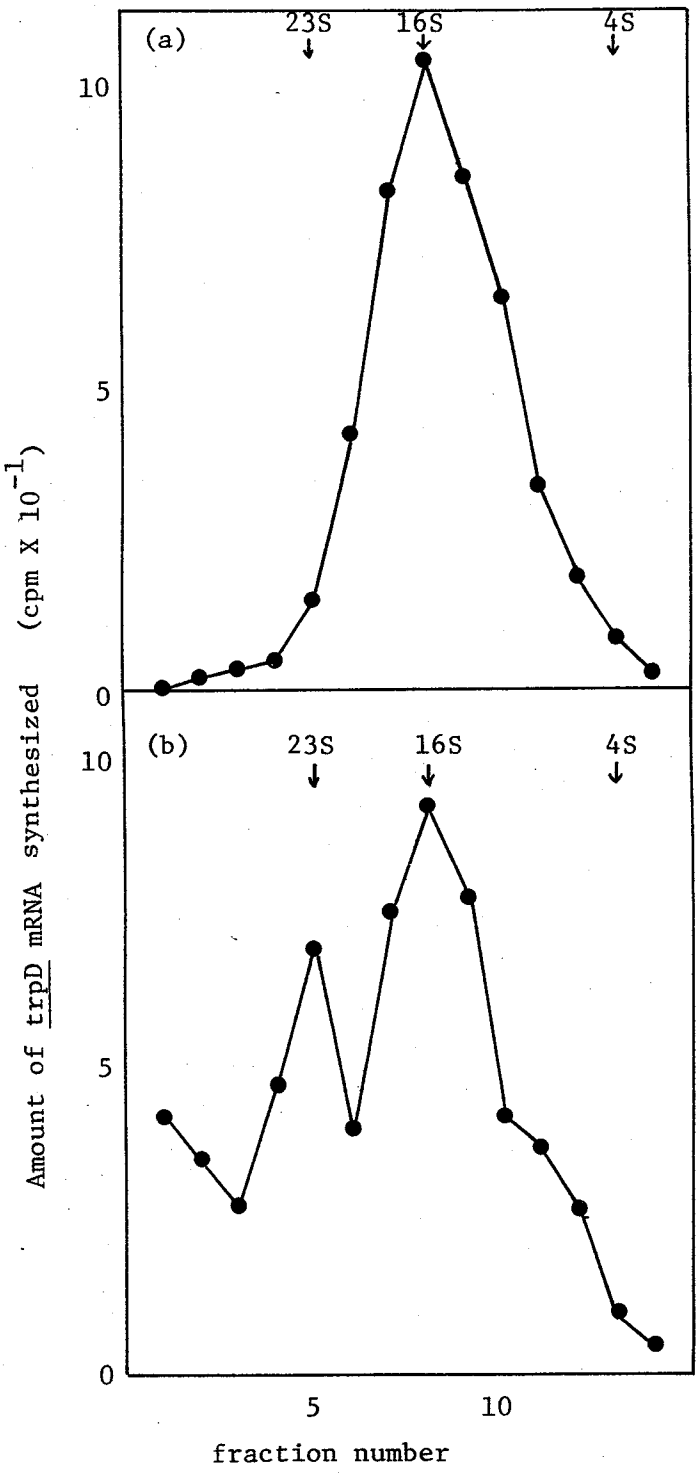
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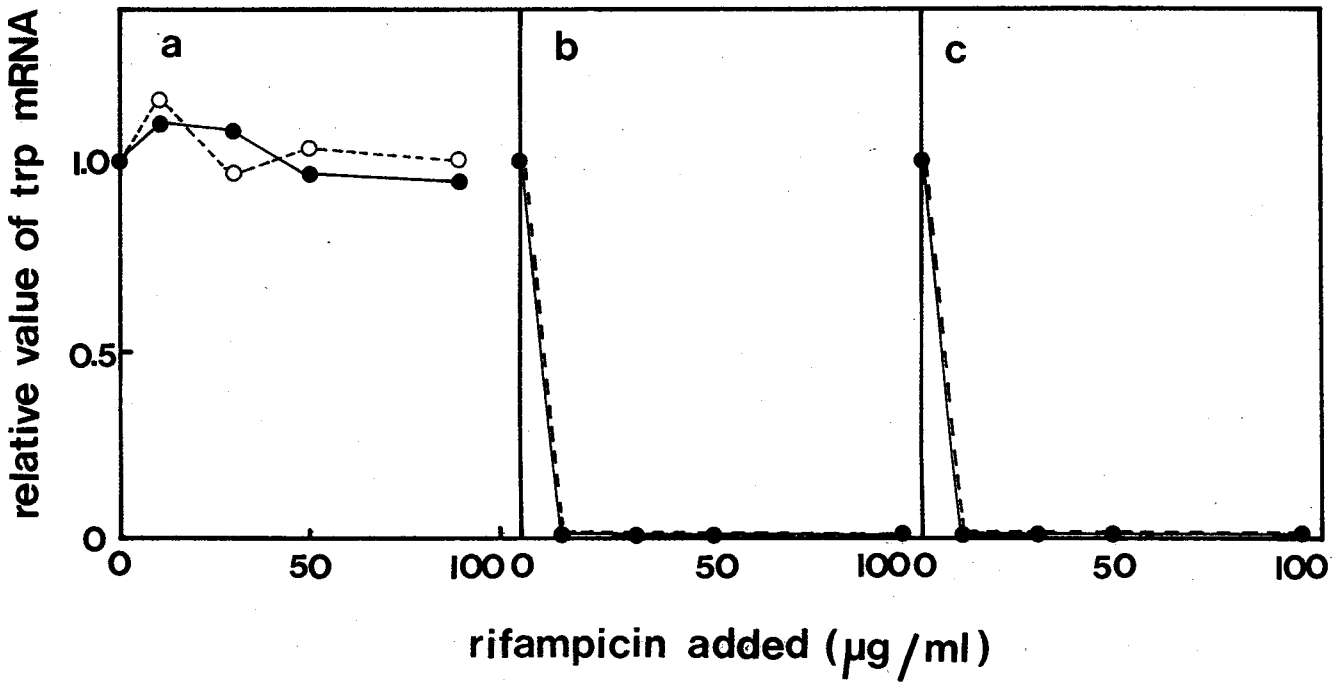
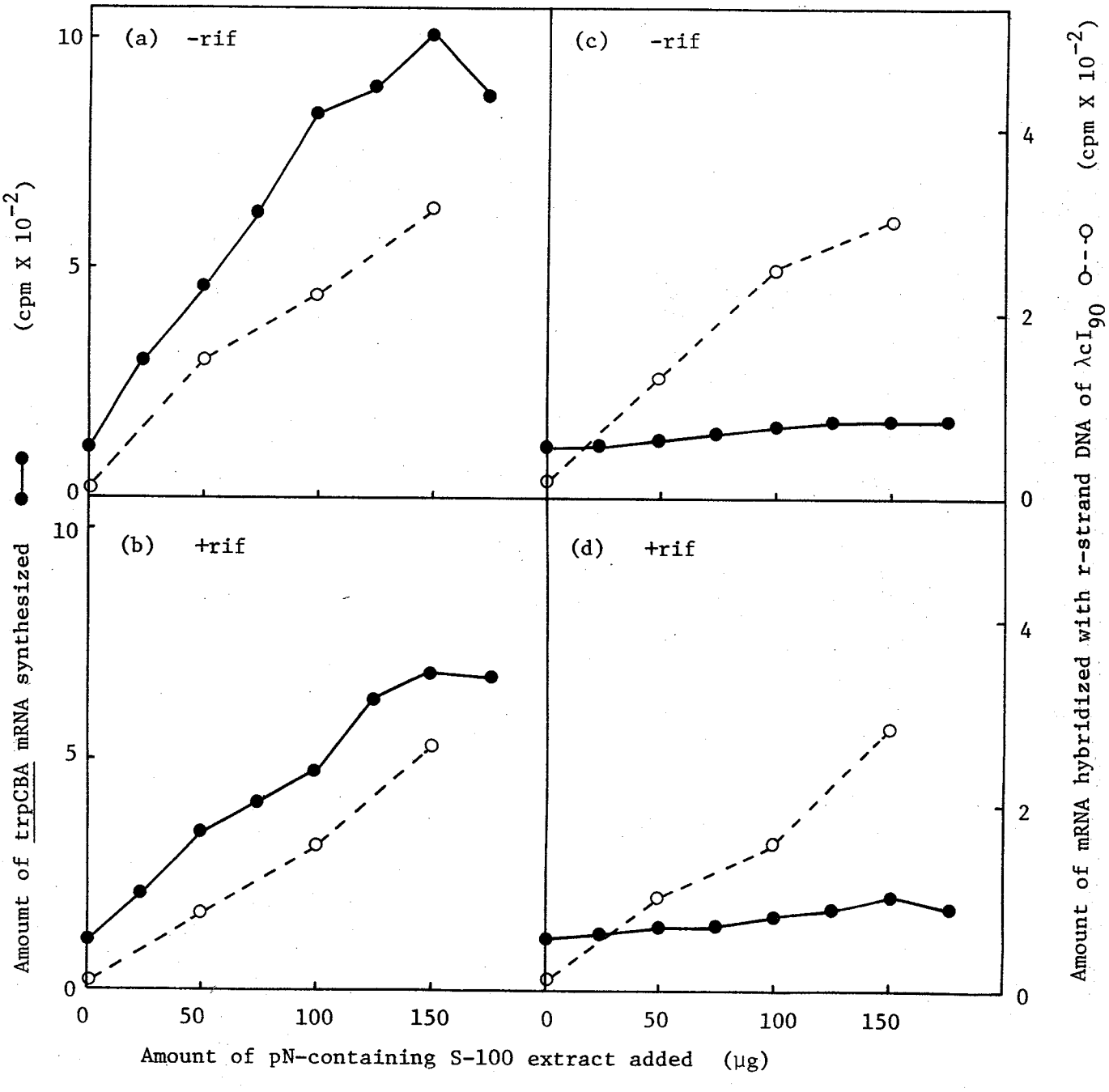
(b)
23S
16S
4S
10
5
0
5
10
fraction number
Amount of trpD mRNA synthesized (cpm X 10^{-1})


a
b
c
relative value of trp mRNA
1.0
0.5
0
0
50
100
0
50
100
0
50
100
rifampicin added (μg/ml)


(a) -rif
(c) -rif
(b) +rif
(d) +rif
10
5
0
4
2
0
50
100
150
Amount of trpCBA mRNA synthesized (cpm X 10^{-2})
Amount of mRNA hybridized with r-strand DNA of λcI90 (cpm X 10^{-2})
Amount of pN-containing S-100 extract added (μg)