EFFECT OF *ESCHERICHIA COLI* ρ FACTOR AND RNase III ON THE FORMATION OF φX174 RNA IN VITRO

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1. Introduction

Escherichia coli RNA polymerase initiates in vitro RNA synthesis on the three major promoters of φX174 replicative form I (RFI) DNA, giving rise to RNAs with pppApA. . . , pppApU and pppGpA. . . 5′-ends [1]. During transcription in a cell-free system the enzyme recognizes only one terminator [2], so that high mol. wt RNAs are formed and the synthesis pattern is very different from that seen in vivo [3]. However, when the termination factor ρ is added to the system, RNAs are synthesized that approximate in size to the φX-specific RNAs of infected cells [4,5]. These RNAs can serve in vitro as substrates for RNase III [5]. RNase III is responsible for the processing of *E. coli* ribosomal and phage T3 and T7 RNA precursors [6,7]. In this work, by initiating φX174 RNA on individual promoters using different limiting sets of ribonucleoside-5′-triphosphates (NTP), we have determined the sites of ρ-dependent termination of RNA synthesis. It has also been shown that RNase III acts upon virtually all φX174 RNA species. At least two sites of the enzyme action have been identified.

2. Methods

2.1. *Enzymes and DNA*

σ-saturated RNA polymerase holoenzyme was isolated by a modification [8] of the method in [9]. ρ factor, RNase III and phage φX174-infected *E. coli* were obtained as in [12]. φX174 RFI DNA was isolated from infected cells as in [10].

2.2. *Synthesis of ^{32}P-labelled φX174 RNA in vitro*

φX174 RNA was synthesized in a standard reaction mixture (1 ml) containing 40 mM Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 150 mM NaCl, 0.1 mM ethylenediamine tetraacetate (EDTA), 0.1 mM dithiothreitol, 200 μM each of four NTPs of which ATP and UTP were α-^{32}P-labelled at 50 mCi/mmol, 12 μg RFI RNA, 24 μg RNA polymerase and, where indicated 5 μg ρ factor and 20 μg RNase III. After incubating the samples for 15 min at 37°C, sodium dodecyl sulfate (SDS) was added to 0.5% and RNA was precipitated with isopropanol in the presence of 10 μg tRNA. γ-^{32}P-labelled RNA was synthesized under the same conditions but one γ-^{32}P-labelled NTP at 1 Ci/mmol was added instead of α-^{32}P-labelled one.

2.3. *Initiation of RNA synthesis on individual promoters by preincubation with a limited set of NTPs*

RFI DNA, 2.5 μg, and RNA polymerase, 5 μg, were preincubated in 0.1 ml with one or two NTPs (400 μM) for 5 min at 37°C, then 10 μg rifampicin, were added and the conditions of RNA synthesis were standardized. Incubation was continued for another 15 min to complete the synthesis of RNA molecules; SDS was then added and RNA precipitated with isopropanol.

2.4. *Isolation of individual RNAs synthesized with ρ factor and their treatment with RNase III*

The RNA labelled with the four [α-^{32}P]NTPs was synthesized in the standard reaction mixture (1 ml) in the presence of 10 μg ρ factor, isolated by the method in [11], and electrophoresed in a polyacrylamide–agarose slab gel. The slab was autoradio-

graphed, bands corresponding to individual RNAs were cut out, ground, and the RNAs were eluted with 0.45 ml of 0.3 M NaCl–0.5% SDS at room temperature overnight. Each eluate was purified on a Sephadex G-75 column washed with 0.01 M Tris·HCl (pH 7.9)–0.15 M NaCl. RNase III (20 μg/ml) and $MgCl_2$, EDTA and NaCl (to 10 mM, 0.1 mM and 0.2 M, respectively) were added, the samples were incubated for 15 min at 37°C, and RNA was isolated.

2.5. *Electrophoresis of [^{32}P]RNA*

The composition of polyacrylamide gel and electrode buffer was described in [12]. RNA samples were dissolved in 5 μl buffer with 1% SDS and 30% glycerol, heated for 5 min at 80°C and applied to 18 × 15 × 0.18 cm gel slabs. Electrophoresis was carried out at room temperature for 15 h at 40 V with bromphenol blue. The slab was dried and autoradiographed.

3. Results and discussion

Figure 1 shows the size distribution of the RNAs synthesized in vitro on RFI DNA of phage φX174 by RNA polymerase alone (a) and in the presence of ρ factor (b) and ρ and RNase III (c). The sizes of RNAs I, II and III synthesized without factors as well as the evidence from our experiments with selective initiation of these RNAs by limited sets of NTPs (fig.2) indicate that they correspond to the transcripts initiated on the three main promoters of phage φX174 RFI DNA and terminated on a ρ-independent site at the boundary of genes H and A [1,2]. This terminator is not 100% efficient since we invariably observed at least two more RNA groups of three bands each whose mol. wt were greater than those of RNAs I, II and III by a value which was a multiple of the genome size (fig.1(a)). In all cases of initiation in limited NTP sets (fig.2), we noted the appearance of shorter RNA molecules which were present only in trace amounts under conditions of normal initiation. These RNAs may appear as a result either of additional initiation or of additional termination. The latter possibility is less likely because the conditions of RNA termination did not differ from standard ones. Therefore, in a limited set of NTPs, initiation may occur also in several other sites of RFI, before the start of genes F, G and H (fig.7).

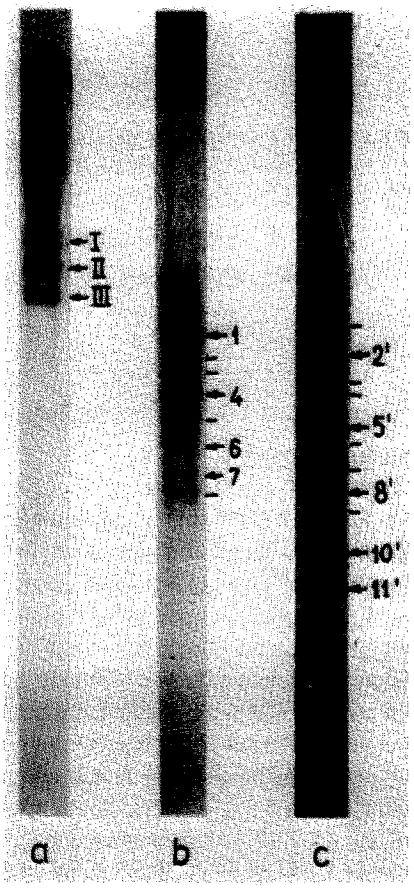


Fig.1. Size distribution of φX174 RNA synthesized in vitro. The conditions of RNA synthesis, isolation and gel electrophoresis were as in section 2. RNA synthesized in vitro (a) without factors, (b) with 5 μg ρ factor, (c) with 5 μg ρ factor and 20 μg RNase III. The mol. wt of RNAs were determined using 23 S and 16 S *E. coli* RNA as markers and expressed as the number of bases, assuming the weight of an average nucleotide unit to be 315 daltons. RNA I is 5400 bases long; II, 4500; III, 3600; 1, 2700; 4, 1770; 6, 1240; 7, 1000; 2', 2500; 4', 1760; 5', 1400; 8', 860; 10', 540; 11', 420; and 12', 280 bases long.

In the presence of ρ factor, there mainly form RNA species 1, 4, 6 and 7 (fig.1(b)). Experiments with selective initiation show that RNA 1 is initiated in the presence of ATP alone, RNA 4 in the presence of ATP and UTP, and RNAs 6 and 7 in the presence of CTP or GTP, i.e., on the three main promoters; consequently, RNAs 1, 4 and 7 are terminated on the first ρ-dependent terminator (T_1^{ρ}) and RNA 6 on the

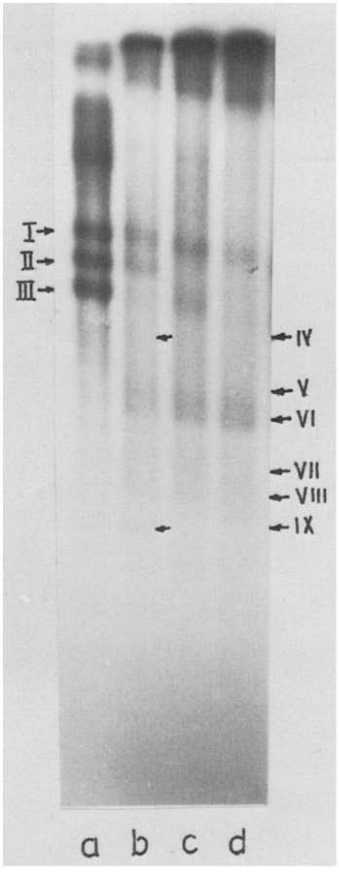


Fig.2. Selective initiation of ϕX174 RNA on individual promoters by preincubation of RFI DNA and RNA polymerase with ATP (d), ATP + UTP (b), and ATP + GTP (c); without preincubation (a). The lengths of RNAs: IV, 2900; Y, 2000; VI, 1800; VII, 1250, VIII, 1100; and IX, 900 bases.

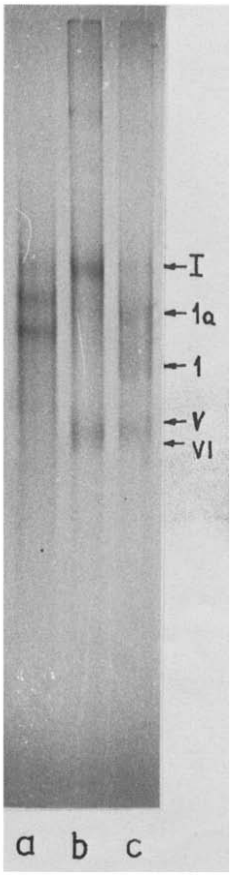


Fig.3. Termination of the synthesis of ATP-initiated ϕX174 RNA in the presence of an insufficient amount of ρ factor. (a) Initiation and synthesis in standard conditions; (b) initiation with ATP alone; (c) initiation with ATP alone followed by synthesis with 2 μg/ml ρ. RNA Ia is 3750 bases long.

second ρ-dependent terminator (T_2^ρ) which are spaced ~200 base pairs apart (fig.7). Moreover, there seems to exist one more ρ-dependent terminator (T_3^ρ) since we observed longer RNA molecules (1a in fig.3) in experiments where ρ was not present in a saturating concentration.

We have shown that RNase III cuts ϕX174 RNAs in vitro [5]. It has been possible to establish some sites of RNase III action by combining the results of three types of experiment. At first we analyzed the action of RNase III on isolated individual 1, 4, 6 and 7 RNAs synthesized in the presence of ρ factor (fig.4, table 1). Then limitation by selective initiation

Table 1
Effect of RNase III on individual ϕX174 RNAs

Individual RNA[a]	RNA species formed by the action of RNase III[b]								
1		2′	4′				10′	11′	12′
4				5′	7′	8′	10′	11′	12′
6							10′	11′	12′
7							10′	11′	12′

[a,b] Correspond to fig.1(b) and fig.1(c), respectively

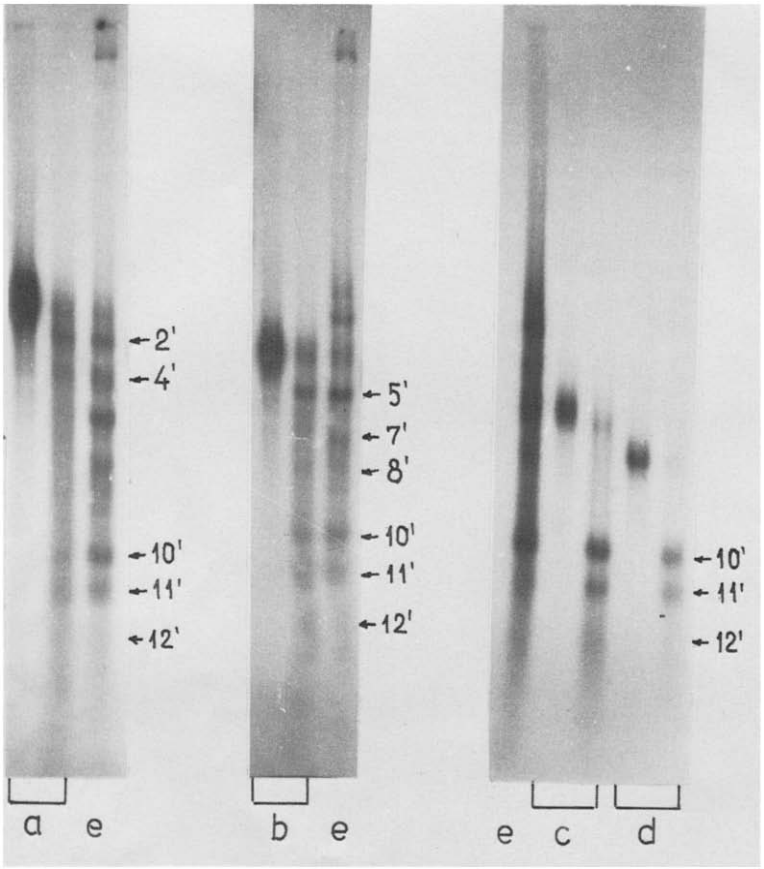


Fig.4. Effects of RNase III on individual ϕX174 RNAs synthesized in the presence of ρ factor. (a), (b), (c) and (d) correspond to the action of RNase III on RNA species 1, 4, 6 and 7 in fig.1b. (e) RNAs synthesized in the presence of ρ + RNase III.

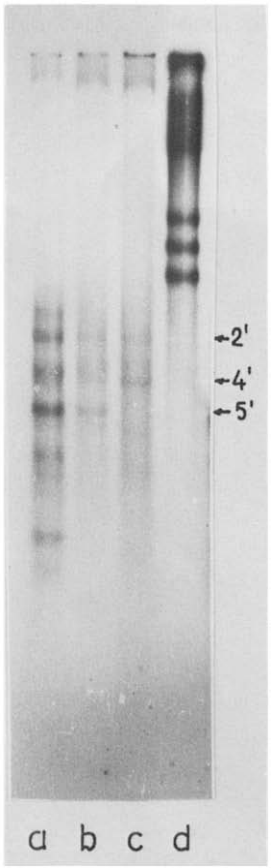
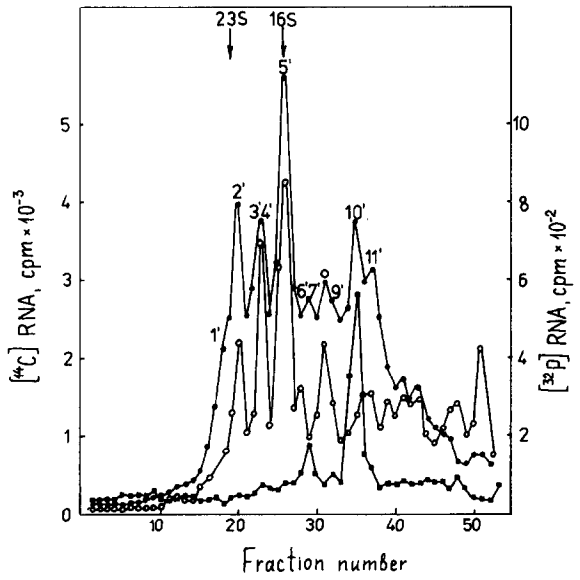


Fig.5. Analysis of the RNA synthesized in the presence of ρ factor and RNase III and initiated (a) with all four NTPs, (b) with ATP + UTP, (c) with ATP and (d) without preincubation and without factors.



of the set of RNAs synthesized in the presence of ρ factor and RNase III (fig.5) has shown also that RNAs 2′ and 4′ form upon preincubation with ATP alone, but that ATP and UTP must be present during preincubation for the formation of RNA 5′. The incorporation of [γ-^{32}P]NTP into the RNA synthesized in the presence of ρ factor and RNase III has shown that RNAs 2′, 4′, 5′ and 8′ all retain their [γ-^{32}P]ATP whereas RNA 10′ contains nearly all incorporated [γ-^{32}P]CTP (fig.6).

Fig.6. Incorporation of [γ-^{32}P]ATP (—○—○—) and [γ-^{32}P]-CTP (—■—■—) into ϕX174 RNA synthesized in vitro in the presence of ρ factor and RNase III. The RNA was isolated and electrophoresed as in [5]. (—●—●—) uniformly labelled ϕX174 [^{14}C]RNA.

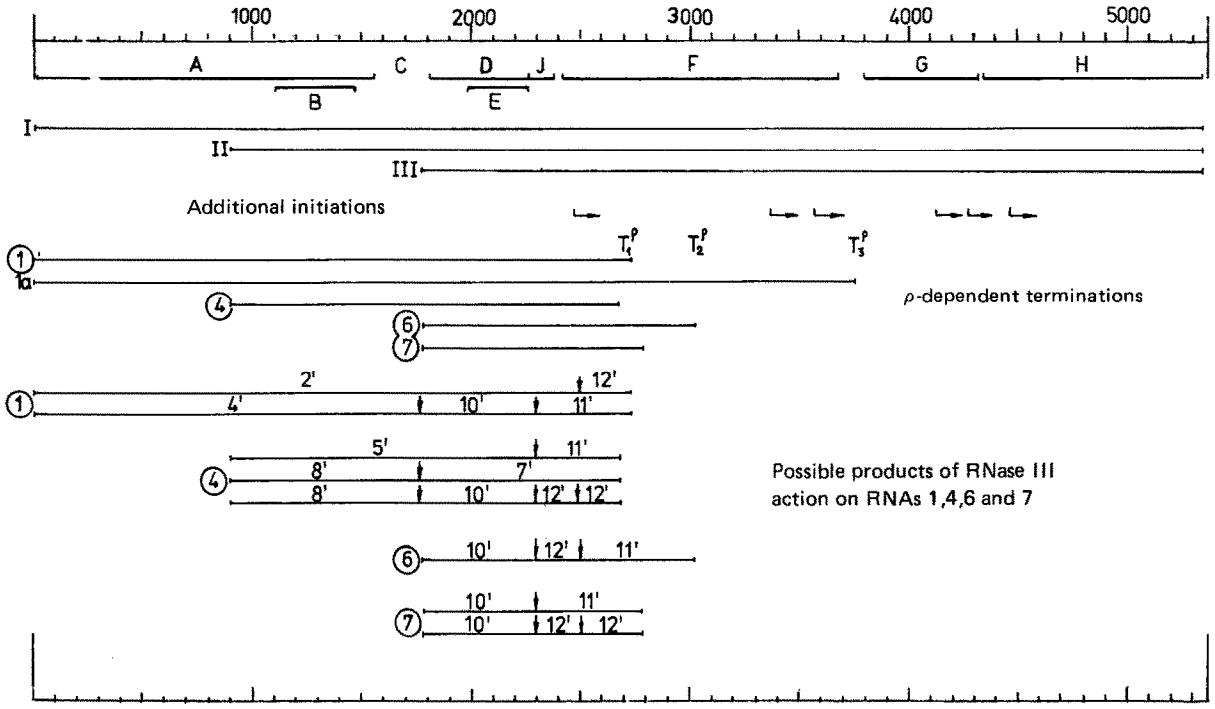


Fig.7. Locations of control sites on the Sanger's genetic map of ϕX174 [13].

It is thus possible to localize three sites of RNase III action on RFI DNA (fig.7). Evidently that 2′ and 4′ RNAs and 5′ and 8′ RNAs are the 5′-terminal fragments of RNAs, initiated on the main promoters I and II respectively, whereas RNA 10′ can result both upon excision from the RNAs initiated on promoters I and II and upon cutting the 5′-terminal fragment from the RNA initiated on promoter III. 10′ RNA appears to represent a block of genes D, E and J. One may assume that the processing of ϕX174 RNA is necessary for regulating the synthesis of phage-specific proteins.

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