
Studies of RNA release reaction catalyzed by *E. coli* transcription termination factor rho using isolated ternary transcription complexes[†]

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ABSTRACT

Protein factor rho catalyzes site-specific termination of transcription in a reaction requiring hydrolysis of nucleoside triphosphate with eventual release of RNA from RNA polymerase and DNA template. We have characterized the rho-catalyzed RNA release reaction using isolated transcription complexes. Transcription complexes containing T7 D111 DNA, RNA polymerase, and ³H-labeled nascent RNA were formed and isolated by gel filtration on an Agarose 5M column. When the ternary complexes were incubated with rho factor in the presence of ATP or dATP, significant amounts of nascent RNA were released from the complexes as determined in a membrane filtration assay. Gel electrophoretic analysis of RNA has revealed that rho releases selected species of discrete-sized RNA from among those originally present in the ternary complexes. These results show that rho essentially acts to release RNA from those ternary complexes which have come to pause, and that this reaction proceeds in a discrete step separately from the pausing of RNA synthesis. Under the conditions used, the extent of RNA release widely varied at individual pausing sites and thus the action of rho exhibited certain site-selectivity.

INTRODUCTION

The protein factor rho from *E. coli* causes termination of RNA synthesis at specific sites on the DNA template, with eventual release of RNA from DNA and RNA polymerase (1). It also possesses an RNA-dependent nucleoside triphosphate hydrolase activity (2), which is required for its termination function (3,4). Meanwhile, it has been shown that the rate of RNA chain elongation temporarily halts or slows down (pauses) even in the absence of any auxiliary factor at certain sites on the template including those assigned as rho-dependent termination sites (5,6). These findings have led to the current theory ('pausing' model) that rho essentially interacts with transcription complexes which have reached the 'pausing' sites, thereby effecting the release of RNA from them through an energy-requiring reaction (6,7). However, details of the assumed reaction processes are still poorly understood.

To further elucidate the precise molecular mechanism involved in rho-catalyzed termination, we have investigated the possibility that the putative

'paused' transcription complexes might be isolated intact so as to allow direct studies of the molecular interaction of rho factor with them. This paper describes the results of such studies using T7 and λ DNA as templates. Ternary transcription complexes containing DNA, RNA polymerase and nascent RNA, as isolated by gel filtration chromatography, were shown to retain reactivity with rho factor. Rho can release RNA from isolated ternary complexes in the presence of a single nucleoside triphosphate as a sole substrate for rho-dependent NTPase activity with a site-selectivity similar to that of rho-induced transcriptional termination. While this study was in progress, a brief report on a similar approach was presented by Galluppi and Conaway (8).

MATERIALS AND METHODS

1. Materials.

The deletion mutant of bacteriophage T7, Δ D111(9), and bacteriophage λ cI857S7 were grown and isolated as described previously (10, 11). DNA was isolated from purified phage by the phenol extraction method (11). RNA polymerase holoenzyme, purified from *E.coli* K12 according to Burgess and Jendrisak (12), was the generous gift of C.S. Park. Rho protein was purified from *E.coli* K12 by the method of Roberts (1) with some modification (13). Concentrations of RNA polymerase and rho protein were calculated from the absorbance at 280 nm using $E_{280\text{nm}}^{1\%}$ of 6.2 (14) and 6.0 (15), respectively. ^3H -GTP was purchased from Schwarz/Mann. Unlabeled nucleoside triphosphates, adenylyl imidodiphosphate (AMP-PNP) and cytidylyl adenosine (CpA) were purchased from PL Biochemicals, Inc., bovine serum albumin from Pentex, nitrocellulose membrane filter from Schleicher and Schull (Type BA85), and Biogel A-5M from Bio-Rad. Rifampicin was from Lepetit S.P.A. All other chemicals were of standard reagent grade.

2. Preparation of Ternary Transcription Complexes.

Unless otherwise specified, transcription reaction in most experiments was carried out in a reaction mixture containing 40 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 50 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), 50 $\mu\text{g/ml}$ bovine serum albumin, 6 $\mu\text{g/ml}$ RNA polymerase, 90 $\mu\text{g/ml}$ T7 D111 DNA, 10 μM each of ATP, CTP, UTP and ^3H -GTP (8 to 16 mCi/mmol) and 0.3 mM CpA. The complete reaction mixture except for omission of nucleoside triphosphates was preincubated at 37°C for 5 min and RNA synthesis was started by adding 4 nucleoside triphosphates. After 1 min of synthesis, initiation of RNA synthesis was halted by adding rifampicin (20 $\mu\text{g/ml}$). Subsequently, a 250 μl -

sample was taken out at various time intervals, immediately mixed with 10 μ l of 0.5 M EDTA to completely stop RNA synthesis, and quickly chilled on ice. Ternary transcription complexes were then isolated essentially as described by Rhodes and Chamberlin (16). All operations hereafter were done at 4°C. The above sample was loaded on a column (1 \times 6.5 cm) of Agarose-5M equilibrated with a buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT, 50 mM KCl and 5% (v/v) glycerol. Ternary complexes containing ³H-labeled nascent RNA was eluted in a void-volume fraction (about 1 ml), being completely separated from unincorporated nucleoside triphosphates. A column of minimum sufficient size was used here to minimize the time of elution (approximately 10 min) and thereby to prevent potential inactivation of ternary complexes during the isolation process. Ternary complexes thus isolated were kept at 4°C and used for reaction with rho factor within 2 to 3 hours.

3. Assay of RNA Release Reaction Catalyzed by Rho Factor.

A standard reaction mixture for measurement of RNA release contained, in a final volume of 50 μ l, 40 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.1 mM EDTA, 50 mM KCl, 50 μ g/ml bovine serum albumin, 0.5 mM dATP, 10 μ g/ml rho factor and 10 to 40 μ l of an isolated ternary complex solution (corresponding to 0.01-0.05 μ g of RNA polymerase originally used for RNA synthesis). After mixing all the constituents at 0°C, the reaction was started by shifting the temperature to 37°C, continued for 20 min, and stopped by the addition of heparin (20 μ g in 1 μ l water), a potent inhibitor of the rho termination activity (17). Subsequently, the amount of RNA released was determined by a membrane filtration method as follows. The whole reaction mixture was quickly chilled on ice, filtered gently through a nitrocellulose membrane (ϕ 23 mm) pre-soaked in washing buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.1 mM EDTA, 50 mM KCl and 5% (v/v) glycerol. The filter was further washed with three 0.5 ml-portions of cold washing buffer. The membrane retaining undissociated ternary complexes was dried, immersed in 10 ml of Econofluor (New England Nuclear) and counted for radioactivity in a scintillation counter. The filtrate and washings containing released RNA were pooled together, mixed with 14 ml of Aquasol (New England Nuclear) and counted.

4. Gel Electrophoresis of RNA.

For analysis of the size distribution of RNA, the reaction mixture for the RNA release reaction was appropriately scaled up so as to yield an RNA sample with a sufficiently high radioactivity (5×10^4 to 10^5 cpm). RNA

released from ternary complexes by rho was collected as filtrate passing through nitrocellulose membrane. RNA unreleased from ternary complexes and retained on the membrane was quantitatively eluted by washing the membrane with three 0.5 ml portions of washing buffer containing 0.2 % sodium dodecyl sulfate (SDS). RNA in each sample solution, obtained after fractionation by membrane filtration, was precipitated by adding 10 μ l of 5 mg/ml E.coli tRNA and 0.1 volume of 20 % (w/v) sodium acetate followed by 2 volumes of absolute ethanol. After standing at -20°C overnight, precipitated RNA was washed twice with 1-ml portions of cold 70 % (v/v) ethanol, dried in vacuo and then dissolved in 30 μ l of sample buffer containing 8 mM Tris-borate (pH 8.5), 0.25 mM EDTA, 8 M Urea, 5 % (w/v) glycerol and 0.025 % each of bromophenol blue and xylene cyanol FF. RNA samples were loaded onto 14 \times 18 \times 0.15 cm slab gels of 3 % acrylamide (30:1, acrylamide: bisacrylamide) in electrophoresis buffer containing 80 mM Tris-borate (pH 8.0), 2.5 mM EDTA and 7M urea, and run for 12 hours at 30 V. The following RNA was used as molecular weight markers: ^{32}P -labeled E.coli stable RNA (4S, 5S, 16S, 23S) and ^3H -labeled RNA produced by transcription of λ DNA in the presence of rho (4S, 6S, 8S, 12S) (1,18). After electrophoresis, the gel was fixed in 10 % (w/v) trichloroacetic acid solution, impregnated with 2,5-diphenyloxazole in dimethyl sulfoxide, and dried after soaking in water. Autoradiography was then performed by exposing dried gel to X-ray film (Kodak X-Omat R) at -70°C (19).

RESULTS

1. Isolation and Basic Properties of Ternary Transcription Complexes.

It has been shown that pausing of E.coli RNA polymerase during RNA synthesis is particularly enhanced when the transcription is allowed to proceed at very low concentrations of nucleotides (5,6). In view of the 'pausing' model of rho-mediated termination, it is interesting to examine whether ternary transcription complexes in such a pausing state might be isolated intact without losing their presumptive susceptibility to rho factor.

In the present study, DNA from a deletion mutant of T7 phage, Δ D111 (9), was mainly used as the template since it retains only one major promoter (A_1) in the early transcription region (20), thereby facilitating the analysis of product RNA. The transcription from A_1 promoter was selectively initiated and continued in the presence of a low concentration of ribonucleoside triphosphates (10 μM each) together with CpA (0.3 mM) as a primer (21). Shortly after the start of reaction, rifampicin was added to prevent reinitiation of RNA synthesis. A low molar ratio of RNA polymerase to DNA (about one mole-

cule of active enzyme per molecule of DNA) was used so as to minimize initiation from other minor promoters(2). At various time intervals, RNA synthesis was halted by addition of EDTA and the transcription complexes containing ^3H -labeled nascent RNA were isolated by fractionation on an Agarose-5M column as described in Materials and Methods. Ternary complexes thus isolated up to 40 min contained short RNA chains of discrete sizes (see Section 3 for details) indicating that RNA polymerase had been frequently pausing at many sites during the time period of RNA synthesis studied. Furthermore, the ternary complexes retained a full potential of elongation for at least several hours after isolation, if kept at 0°C . When they were incubated with unlabeled 4 nucleoside triphosphates (0.1 mM each), almost all the nascent RNA chains were chased into large RNA products corresponding to the entire early transcription region (Fig.3, lane 1b).

2. Release of RNA from Isolated Transcription Complexes by Rho Factor.

We next investigated whether rho might operate its RNA releasing activity against isolated ternary complexes using a membrane filtration method. The transcription complexes containing ^3H -labeled nascent RNA were incubated with rho factor in the presence of suitable nucleoside triphosphate as a substrate for rho factor. After arresting rho activity by addition of heparin, the reaction mixture was gently filtered through a nitrocellulose membrane. The addition of heparin was also found to be effective in preventing rebinding of dissociated RNA by RNA polymerase and rho. The amount of radioactivity retained on the membrane and that recovered in the filtrate were taken as representing intact ternary complexes and free RNA, respectively. Under similar conditions, rho alone caused little retention of free RNA molecules (less than 3 % of total), when this was tested using RNA isolated from ternary complexes (Table 1, (a)). In control experiments where either rho factor or nucleotide or both were omitted, the radioactivity of nascent ^3H -RNA was retained on the filter with an efficiency of 70 to 90 %, depending on the preparation and freshness of transcription complexes (Table 1). This efficiency tended to decrease gradually with the time of incubation at 37°C , presumably due to partial disruption of ternary complexes during incubation or contaminating RNase activity in the reaction mixture. That the intact ternary complexes were necessary for retention of nascent ^3H -RNA on the membrane was evident from the observation that the radioactivity retained on the membrane was almost abolished completely when the complexes were denatured by addition of SDS. When ternary complexes were incubated with both rho and ATP, the proportion of free RNA increased significantly over the

Table 1. Nucleoside Triphosphate Requirement for RNA Release by Rho Factor

Ternary complexes isolated after 10 min of RNA synthesis (containing 13000 cpm of ^3H -RNA and 0.05 μg of RNA polymerase) were incubated with or without rho factor (10 $\mu\text{g}/\text{ml}$) in the presence of various nucleoside triphosphates under otherwise the same conditions as described in the text: ATP, dATP, AMP·PNP, 1 mM each ; 4NTP, 10 μM each. The results of RNA release assay were expressed as the fraction of radioactivity retained on a membrane filter. Where indicated, ternary complexes were denatured by addition of SDS (final concentration 0.1 % (w/v)) before filtration, or RNA was isolated from the complexes by phenol treatment and then subjected to the filtration assay after incubation with or without rho. The values given in columns (A) and (B) represented means of duplicate measurements. The error of measurements was estimated to be $\pm 3\%$ or less.

Additions or Treatment	^3H -RNA retained on membrane (% of total radioactivity)		Rho-dependent RNA release [(A-B)/A] $\times 100$
	-rho(A)	+rho(B)	
not incubated	79		
(a) denatured by SDS	0.5		
isolated RNA	1.0	2.5	
none	72	73	0
ATP	66	51	22
dATP	70	53	25
(b) dATP + 4NTP	61	12	80
AMP·PNP	67	72	0

control without these reagents. As a measure of the efficiency of rho-dependent RNA release, we use the percentage of rho-induced increase in free ^3H -RNA relative to the amount of membrane-retainable ^3H -RNA obtained from a control incubation without rho. For the RNA releasing activity of rho, ATP was completely replaceable by dATP but not at all by AMP·PNP. This suggests that the hydrolysis of nucleoside triphosphate by rho is required for its RNA releasing activity as it is for its termination activity (3,4). In the subsequent experiments, dATP was used throughout as a substrate for rho factor, since this deoxynucleotide might be sparingly used for RNA synthesis, if to any extent (22), and accordingly affect the functional states of ternary complexes less than ATP. Rho and nucleoside triphosphate-dependent release of RNA was also detectable by using other procedures for separation of ternary complexes and released RNA, such as gel filtration on an

Agarose-5M column and electrophoresis in Agarose gel (8,23). Compared with the membrane filtration procedure, however, these methods were more time consuming, and particularly, the electrophoretic procedure gave consistently higher background values (50 to 60 %) for free RNA in control experiments without rho, implying a greater disruption of ternary complexes during separation as reported previously (23).

The data presented in Fig.1 show the dependence of rho-induced RNA release on the time of incubation with rho, the concentration of rho and the concentration of dATP. With increase in each of these parameters, the apparent efficiency of release exhibited a plateau kinetics but never reached 100 % unless 4 nucleoside triphosphates were also added, see below. The maximal extent of rho-dependent RNA release critically depended on the duration of RNA synthesis before isolation of the ternary complexes, increasing steeply from a negligible value to 30 % or above as the time increased from 2 min to about 10 min, and leveling off thereafter (Fig.2). In a possibility deducible from the 'pausing' hypothesis, a limited RNA release by rho may signify that only a part of isolated transcription complexes has reached the 'pausing' sites or the potential sites for rho termination, at which the complexes become susceptible to the action of rho. According to this view, more RNA

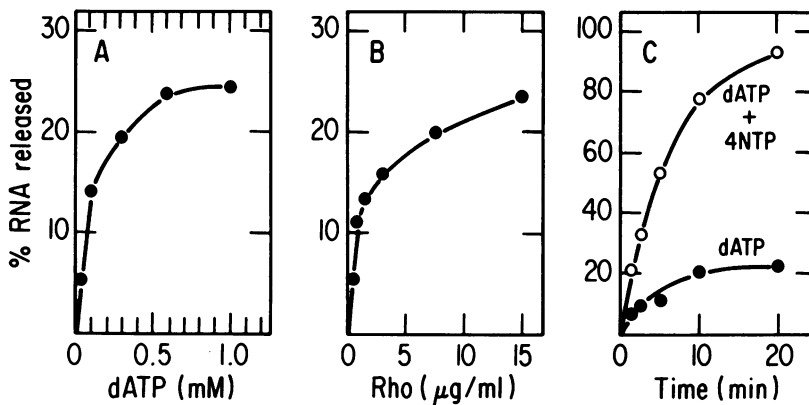


Fig. 1. Kinetics of Rho-dependent RNA Release. Ternary complexes isolated after 10 min of RNA synthesis (the same preparation as in Table 1) were used. The RNA release reaction was carried out under the standard conditions except that a single parameter was varied in each series of experiments: A, the dATP concentration; B, the rho concentration; C, the incubation time. In experiment C, the reaction mixture was supplemented with 4 ribonucleoside triphosphates (10 μM each) where indicated. The extent of rho-dependent RNA release was calculated in the same way as in Table 1.

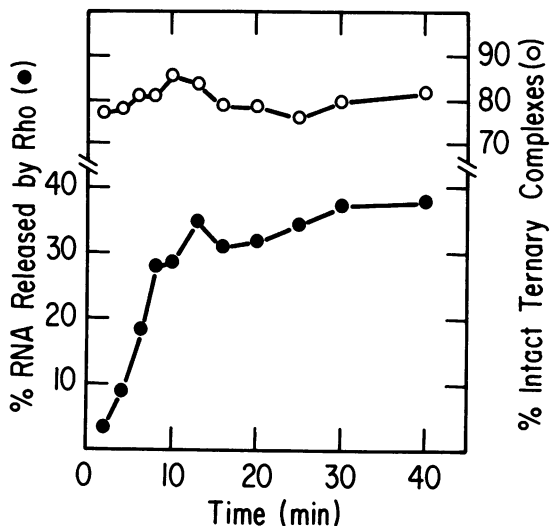


Fig. 2. Efficiency of RNA Release by Rho as a Function of the Duration of RNA Synthesis. Ternary complexes were isolated after the indicated durations of RNA synthesis. The extent of rho-dependent RNA release was determined in the same way as in Table 1. ○—○, Fraction of RNA retainable by the membrane filter without incubation with rho; ●—●, Rho-dependent RNA release.

might be released if the transcription complexes are challenged by rho factor in the presence of 4 ribonucleoside triphosphates so that each complex can resume transcription and eventually reach some termination sites. In fact, almost complete release of RNA by rho factor was observed when low concentrations of 4 nucleotides (10 μ M each) were supplied in addition to dATP (Fig. 1c, Table 1). Incidentally, it was found that incubation of the transcription complexes with a higher concentration of 4 NTP's (0.1 mM each) resulted in an extensive release of RNA even in the absence of rho, presumably because RNA chains were rapidly elongated to the end of the early transcription region (Fig. 3, lanes 1b and 3a), where transcription has been known to terminate spontaneously (20). These observations confirmed that the rebinding of free RNA by rho or RNA polymerase was negligible under the assay conditions. Another trivial possibility for the limited RNA release observed here is that a certain fraction of RNA chains might fail to pass through the membrane, albeit they had been actually terminated by rho. This was excluded from the following observation. Transcription complexes were treated with rho in the presence of dATP as above and subsequently incubated with a supple-

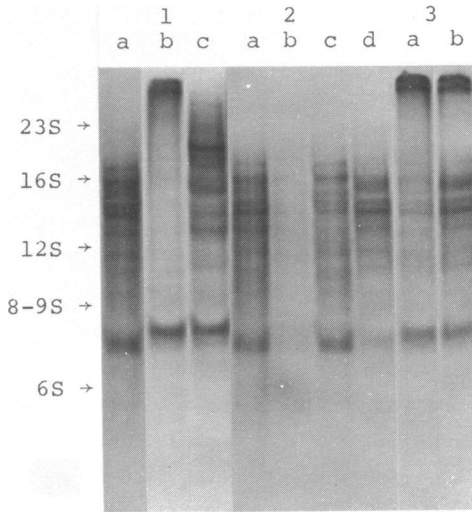


Fig. 3. Correlation between RNA Release and Termination by Rho Factor. Ternary complexes isolated after 10 min of RNA synthesis (approximately 1.7×10^5 cpm per sample) were treated as follows. RNA was analyzed by gel electrophoresis before or after fractionation by membrane filtration. 1, Total RNA from complexes without treatment (lane a) or from those incubated for 20 min with 4 ribonucleoside triphosphates (0.1 mM each) in the absence (lane b) or presence (lane c) of rho; 2, Unreleased RNA (lane a) and released RNA (lane b) from complexes incubated with dATP alone, or unreleased RNA (lane c) and released RNA (lane d) from complexes incubated with dATP and rho; 3, Total RNA from complexes which were incubated with dATP in the absence (lane a) or presence of rho (lane b) as in 2 and then allowed to elongate for 20 min by adding 4 ribonucleoside triphosphates (0.1 mM each) together with heparin (200 μ g/ml).

ment of 4 ribonucleoside triphosphates (0.1 mM each) after addition of heparin to abolish rho activity. The electrophoretogram of the reaction products (Fig. 3, lane 3b) showed that a majority of RNA species initially present were chased into large RNA products ($>23S$) except for those species whose electrophoretic pattern closely coincided with that of RNA released by rho at the first stage of incubation (Fig. 3, lane 2d). This result indicated that the transcription termination and the RNA release catalyzed by rho are in exact correspondence to each other in the T7 DNA transcription system studied here. One may still question whether rho-resistant ternary complexes might be artificially produced due to the special conditions used for RNA synthesis, such as the low concentration (10 μ M) of substrates in combination with a relatively high concentration of CpA (0.3 mM).

This possibility seems less likely because essentially similar results were obtained when the ternary complexes were prepared using a higher concentration of 4 nucleotides (0.1 mM each) but in the absence of primer (data not presented).

3. Site Selectivity of RNA Release by Rho Factor.

To determine whether the RNA releasing activity of rho is site-selective or not, we further investigated the size distribution of released RNA in comparison with that of the total RNA originally present in ternary complexes. Upon gel electrophoresis, total RNA samples from the ternary complexes obtained after successive intervals of RNA synthesis showed overlapping series of discrete bands which are indicative of pausing events at numerous sites (Fig. 4, lanes 2-5a). As many as 20 such sites were identifiable in the molecular weight range from below 4S to 16S at the present electrophoretic resolution. Released RNA also showed conspicuous banding patterns, which partly corres-

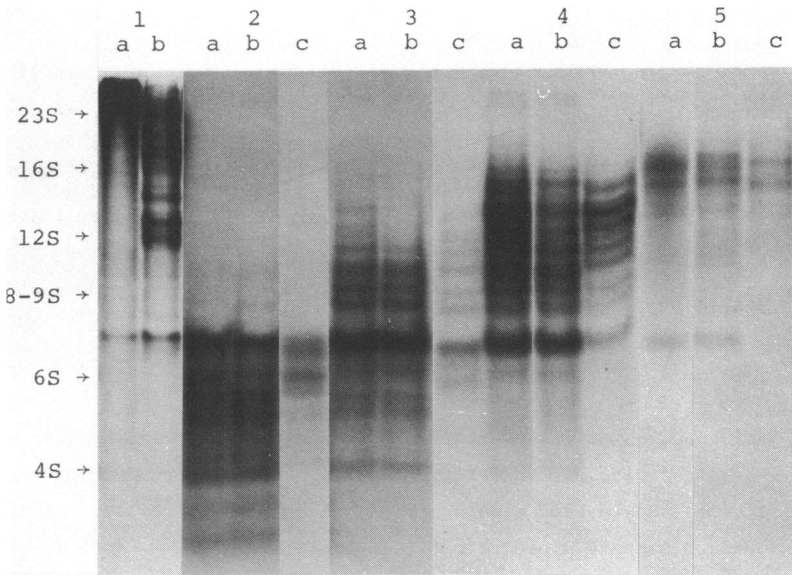


Fig. 4. Site-Selectivity of RNA Release by Rho Factor. Ternary complexes were isolated after varying durations of RNA synthesis: 2, 2min; 3, 4 min; 4, 8 min; 5, 16 min. Total RNA from untreated complexes (lane a), and unreleased RNA (lane b) and released RNA (lane c) from complexes incubated with rho were run in parallel on gel electrophoresis. In each series of analysis, the same amount of ternary complexes ($1.5-2.0 \times 10^5$ cpm) were used both for direct analysis of total RNA and RNA release reaction with rho. Also shown for comparison were the electrophoretic patterns of RNA formed by 20 min of transcription at 0.1 mM each of 4 ribonucleoside triphosphates in the absence (lane 1a) and presence (lane 1b) of rho factor.

pond to those of total RNA (Fig. 4, lanes 2-5c). However, some bands as well as diffuse stains extending between bands present in total RNA were missing or less dense in released RNA. Such tendency was particularly notable when the size of RNA was shorter than 8-9S. Beyond this size range, most of discrete RNA species detectable were released by rho to some significant extents, but not to completion. However, there also existed a few particular RNA species which were either almost completely released or conversely not released at all (cf. Fig. 3, lanes 2c and d; Fig. 4, lanes 4b and c). It should be emphasized that the electrophoretic pattern of RNA not released by rho factor (Fig. 4, lanes 2-5b) was complementary to that of released RNA in such a way that their patterns, if superimposed together, would give the original pattern of total RNA. This proves that the pattern of released RNA as observed is not an artifact due to degradation of RNA by contaminating RNase activity or fluctuation in electrophoretic separation. Thus, the above results can be taken as indicating that rho acts specifically, or at least preferentially, on certain populations of transcription complexes having reached some, but not all, of pausing sites. Also shown in Figs. 3 (lane 1c) and 4 (lane 1b) were the electrophoretic patterns of RNA terminated by rho during continuous RNA synthesis in the presence of 4 nucleotides. In these cases, major RNA bands distributed in the range of 12S to 16S closely coincided with those of RNA species which were effectively released from the ternary complexes by rho (Fig. 3, lane 2d; Fig. 4, lanes 4c and 5c). This suggests that the site selectivity of rho is essentially similar in its reaction with ternary complexes either during normal RNA synthesis or in the isolated state. We have also studied RNA releasing activity of rho using transcription complexes containing λ DNA template. The results were qualitatively very similar to those obtained with T7 DNA template (Fig. 5).

DISCUSSION

The present study has demonstrated that ternary transcription complexes containing DNA, RNA polymerase and nascent RNA can be isolated in a relatively stable state without losing the sensitivity to rho factor as well as the capacity for RNA chain elongation. In keeping with the previous report (8), rho was shown to cause release of RNA from ternary complexes in the presence of ATP as a sole substrate and under the conditions where RNA chain elongation was restricted. The RNA release activity of rho could be conveniently assayed by using the membrane filtration procedure in a semi-quantitative manner.

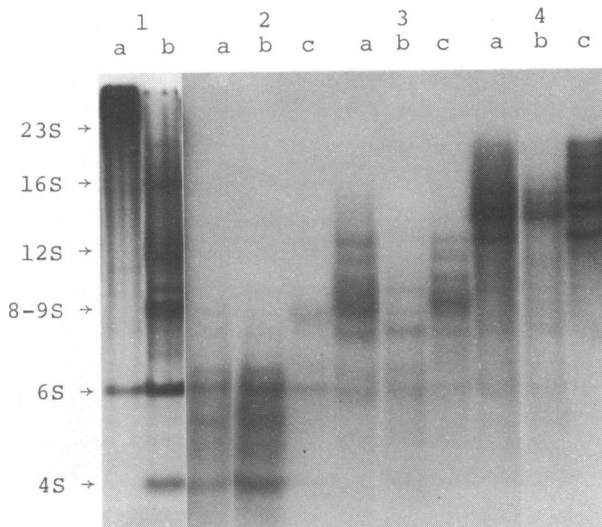


Fig. 5. The Pattern of Rho-Catalyzed RNA Release from λ DNA Transcription Complexes. Experiments analogous to those presented in Fig. 4 were carried out using λ DNA as template. 1, RNA synthesized at 0.1 mM each of 4 ribonucleoside triphosphates in the absence (lane a) and presence (lane b) of rho. For preparation of ternary complexes, RNA synthesis was carried out using 10 μ M each of 4 nucleotides and 0.3 mM CpA to facilitate selective initiations at P_L and P_R promoters. The duration of RNA synthesis used for isolation of ternary complexes were: 2, 2 min; 3, 5 min; 4, 10 min. In RNA samples numbered 2 to 4, lane a stands for total RNA, lane b for unreleased RNA and lane c for released RNA, respectively.

This method has the advantages in speed, minimum inactivation of the ternary complexes during manipulations, and ease of the subsequent analysis of fractionated RNA, over other available methods such as gel filtration chromatography, electrophoresis in agarose gel (8, 23), and differential centrifugation (1). Characterization of rho function in the present system has provided evidence which lends support to and helps further define the pausing model of transcription termination. The RNA releasing activity of rho has been known since its discovery (1). However, it has remained to be established whether the RNA release is an indispensable step of rho-mediated termination or a gratuitous consequence of the latter. In the present study it was shown that the RNA release activity required the presence of hydrolyzable nucleoside triphosphate as does the termination activity. RNA terminated by rho was shown to closely correspond to released RNA both in their

quantity and size distribution (Fig. 3, lanes 2d and 3b). Moreover, RNA chains could be almost quantitatively released from ternary complexes under conditions where rho effected a complete termination of RNA synthesis (Fig. 1c). These results are consistent with the view that the primary function of rho is to release RNA (7, 24).

Another important finding is that the rho-catalyzed RNA release from isolated ternary complexes operates in a site-selective manner as does the termination in a normal transcription system. This contention is supported by the following observation: (1) The extent of RNA release showed a plateau in the presence of saturating amounts of rho and ATP (or dATP) after a sufficient duration of incubation, the level of the plateau depending on the conditions of RNA synthesis prior to isolation of the ternary complexes (Table 1, Figs. 1 and 2); (2) Rho preferentially released RNA species of discrete sizes, which were presumed to have reached the 'pausing' sites (Figs. 3 and 4); (3) RNA chains terminated by rho in a normal transcription system were similar in size to those released from isolated ternary complexes (Figs. 3 and 4).

The implication of these results is two-fold. First, the pausing of transcription and the release of termination of RNA by rho factor can proceed in discrete steps completely separable in time. Second, the action of rho factor is not triggered by a mere reduction in the rate of RNA chain elongation since not all RNA species were released even when RNA polymerization was grossly restricted in the absence of nucleoside triphosphates other than ATP or dATP. It thus appears that ternary complexes at the pausing sites might have some special properties which allow them to serve as the target of rho. The present demonstration that such presumptive properties can be stably maintained after isolation of the ternary complexes points to the possibility of direct molecular characterization of pausing transcription complexes and their interaction with rho. But a question arises why the release of RNA even at saturating concentrations of rho was only partial at a majority of the pausing sites. The most likely explanation may be that the ternary complexes located at a given pause site could exist in more than one state with differing sensitivity to rho factor. It is possible that the ternary complexes may be actually pausing at multiple neighboring positions, which are indistinguishable by the gel electrophoresis used here. Interesting to note in this connection, it has been demonstrated that within a number of rho-dependent and independent termination sites sequenced so far, the product RNA generally ends not at a single base position but rather at several adja-

cent residues with various frequencies (18, 28). On the other hand, the complexes may assume heterogeneous conformations even if they are pausing at the same base position. The heterogeneity could lie either in the secondary structure of nascent RNA or in the overall molecular integrity of the ternary complexes. In either case, the proper interaction between rho and the ternary complexes seems to depend critically on some stringent conditions in addition to the pausing event. An alternative explanation for the partial release of RNA by rho is that the reactivity of the ternary complexes with rho may be partly damaged during their preparation or somehow inhibited under the assay conditions used. The possibility of improving the yield of RNA release is currently being pursued.

It should also be noted that the amount of rho required for maximal RNA release (ca. 10 $\mu\text{g/ml}$) is much higher than that needed for efficient rho-dependent termination (less than 1 $\mu\text{g/ml}$) (1). As discussed before, the isolated ternary complexes probably contain a considerable fraction of subpopulations which have intrinsically low sensitivities to rho factor. Existence of termination sites requiring similarly high levels of rho has been demonstrated in a few bacterial operons (29). Previous studies of rho-dependent termination have been centered at major termination sites which are readily detectable in standard transcription systems with relatively high concentration of substrates (0.1 mM or more). In contrast, the present system allowed us to detect many more sites for termination on T7 D111 DNA: as many as 10 sites could be counted in the range of molecular size from 6S to 16S at the present resolution of gel electrophoretic analysis. Although the biological significance for such a number of termination sites is not known at the present, their further characterization in comparison with the known major termination sites (17, 25-27) may be instructive in broadening our scope into the molecular basis for the site selectivity and the regulation of transcription termination by rho factor.

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