Readthrough transcription occurs at the rho dependent signal F1 T_{IV} in suppressor cells

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ABSTRACT

Suppressor cells infected with bacteriophage f1 yield phage encoded gene IV transcripts longer than those present in the *sup*^o host and identical to those found in a *rho*⁻ host. However, such longer transcripts do not appear in the suppressor-infected cell when, by changing the translation frame of gene IV, the ribosome is not allowed to proceed to the end of the gene IV message and thus to reach the rho dependent transcription terminator f1 T_{IV}. This suggests that ribosome movement beyond the natural gene IV stop codon disturbs the activity of that termination signal. In contrast to the *rho*⁻ behaviour, the suppressor does not accumulate high levels of gene IV messages indicating that the accumulation occurring in the *rho*⁻ mutant may not be a primary effect of the readthrough *per se*.

INTRODUCTION

Termination of transcription plays an important role in regulating gene expression in procaryotes (for reviews see: 1-3). Two different types of terminators have been identified in these organisms. Members of the first group, called rho independent, do not require the presence of any cellular factor. Their activity simply depends on transcript sites that contain a G-C rich region of dyad symmetry followed by a stretch of uridine residues. In contrast, the other class requires the presence of, at least, a specific factor called rho (4). The structural features of the rho-dependent terminators appear to be quite complex and are not fully understood. Evidence accumulated in many laboratories using different approaches indicates that at least two distinct, non overlapping, RNA segments are involved in executing the rho-dependent termination event (5-10). One directs the specific binding of the rho factor to the RNA and the other the actual termination of the transcript. This model has been further proved by the finding that in an *in vitro* transcription system addition of oligonucleotides complementary to the rho binding site leads to the appearance of transcripts that extend past the termination site (9).

Recent reports (11-13) suggest that some instances of polar

mutations might involve rho-dependent terminators activated by the premature falling off of the ribosome, indicating that the translating machinery might exert important effects on the event of rho dependent termination. This importance is further stressed by data suggesting that not only the movement of the ribosome *per se* but also the spacing between the transcription and translation complexes may activate these types of terminators (14). All this stimulates experiments which can shed more light on the interplay between ribosome movement and rho-dependent termination.

We thought that analysis of gene IV messages of the single-stranded DNA bacteriophage f1 (15) in suppressor cells could offer relevant data in this respect. This gene contains a rho-dependent terminator (16-19) located on a stem and loop structure 63 nucleotides from the stop codon (see figure 1). That codon will be read as a sense codon in an appropriate suppressor cell and the ribosome, instead of falling off, will proceed further. In this paper we show that infection of suppressor cells with phage f1 results in the appearance of longer gene IV transcripts which are identical in length to those found in a *rho*⁻ infected host (16). These longer transcripts do not appear when the distal part of gene IV is not engaged in translation. In contrast to the *rho*⁻ behaviour (16) however the suppressor cell does not yield high levels of gene IV messages. We discuss these data in the light of the complex nature of rho dependent termination.

MATERIALS AND METHODS

Phages and bacteria

Phage f1 and *E.coli* strains K37, K38, K819 and K143 were from Dr. N.D.Zinder (The Rockefeller University, N.Y.) and have been described (16,20). Plasmid pEG 25 containing a cloned wild type rho allele [described in (16)] was a kind gift of Dr. N.D.Zinder's laboratory.

RNA extraction

Twenty min after the infection with bacteriophage f1 *E.coli* cells were quickly poured onto frozen medium, centrifuged at 10,000 g for 5 min at 4°C and lysed by adding one volume (1 ml for 25 mls of cells) of lysis mixture (8M guanidine hydrochloride,

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Figure 1. The f1 TIV terminator. Based on (16). —○ indicates the main rho-dependent site; — the rho independent. The three nonsense codons (an amber, suppressible in *supD* and *supF* cells, on hairpin B and two close opal codons, not suppressible in *supD* and *supF*, on hairpin C) present downstream of the gene IV stop codon are underlined.

20 mM sodium acetate, pH 5.5 and 1mM EDTA). After complete clearing by incubation at 37°C, one volume of water-saturated phenol was added and mixed vigorously for 5 min; one volume of chloroform was then added, followed again by vigorous mixing. The mixture was then centrifuged at 4,000 g for 4 min at room temperature and the aqueous phase, made 0.25 M in NaCl, precipitated by two volumes of ethanol. After centrifugation at 12,000 g the sample was resuspended in extraction mixture (20 mM Na acetate pH 5.5, 0.5 % SDS and 1 mM EDTA) and extracted four times with one volume of water-saturated phenol and one volume of chloroform and once with one volume of chloroform alone. The aqueous phase, made 0.25 M in NaCl, was then precipitated twice with two volumes of ethanol. The RNA was then resuspended in water, made 40 mM in Na acetate pH5.5, 10 mM in MgCl₂ and treated with pancreatic DNase (final concentration: 20 microgr./ml, RNase free, Miles) for 30 min at 37°C. The RNA was then made 10mM in EDTA, extracted once with water saturated phenol and chloroform and once with chloroform alone, made 0.25 M in NaCl and precipitated by two volumes of ethanol. After centrifugation, the final pellet was resuspended in sterile water, divided into aliquots and kept at -30°C.

Northern hybridizations

Two microgram aliquots of RNA were fractionated on 1.8% agarose gels in the presence of 7.5 mM methylmercuric hydroxide (Alfa Ventron) according to (21) at 0.2 V/cm for 12 hrs at 20°C and then transferred onto nylon membranes (Zetaprobe, Biorad). The RNA was fixed by exposure to UV light. The filters were prehybridized in 4× SSPE, 0.5 % SDS, 5× Denhardt's solution and hybridized in the same medium to

the nick translated (22) probe (specific activity: 1×10^8 cpm/microgr. DNA) at 68°C. Following hybridization the filters were washed at 65°C in 2× SSC, 0.2% SDS for 10 min and in 0.1×SSC for 3 min.

Restriction and DNA modifying enzymes

Restriction enzymes *AccI*, *BanI*, *DraI*, *EcoRI*, *HindIII*, *NruI*, *PstI*, *SmaI*, *SspI*, *TaqI*, DNA Polymerase I, the large fragment (Klenow) of DNA Polymerase I, T_{IV} DNA Polynucleotide kinase were from Boehringer Mannheim.

Construction of plasmids pR 213 and 214

Phage f1 RF_I (replicative form I, double stranded covalently closed circular) DNA was partially digested with restriction enzyme *SspI* and a fragment spanning nucleotides 5024–5925 (numbered according to (23)) was purified. *ClaI* decamer (GCATCGATGC) linkers (synthesized in our laboratory) were ligated to it according to (24) and digested with the enzyme *TaqI*. In addition to cleaving the linkers, this digestion cuts the f1 DNA fragment at position 5683, just at the end of the B hairpin (see figure 1) present in the intergenic (I.G.) region. The largest fragment created by this digestion, corresponding to nucleotides 5024 to 5683 was then purified by agarose gel electrophoresis. This DNA segment contains the distal portion of gene IV down to the T_{IV} terminator. We have previously shown (17–18) that deletion mutant PII (25), which maintains only this part of gene IV, transcribed under the direction of the gene II promoter, displays a fully efficient rho dependent T_{IV} terminator. This implies that this gene IV segment contains any relevant sequences which might be involved in the rho dependent termination event at T_{IV}. Another f1 DNA fragment (nucleotides 5680–6407,

Figure 2. Construction of plasmids pR213 and 214. The various steps are detailed in the main text. pUC 9 and pBR322 plasmids are not drawn in scale. Transcription of f1 DNA, subcloned in the two plasmids, is initiated at the gene II promoter (Pr_{II}) and its direction is identical to that of the beta-lactamase gene in pBR322.

BanI-HindIII) containing the f1 gene II promoter was cloned into the *SmaI* site of plasmid pUC9 (26) giving rise to plasmid pR 210 (f1 *HindIII* site on the *HindIII* side of the polylinker). The DNA of the latter, after digestion with the enzymes *ClaI* and *AccI*, was ligated to the fragment containing the distal portion of gene IV, prepared as described above. One clone, containing f1 genes II and IV sequences oriented in the same direction was purified (pR 211). F1 gene IV sequences transcribed from this construct are translated according to the f1 natural reading frame. Two nucleotides, CG, were then inserted at the unique *ClaI* site of the DNA of plasmid pR 211, by opening it at that site, filling in its protruding ends with the large fragment (Klenow) of DNA Polymerase I and ligating them again. In this way the *ClaI* site disappears and a *NruI* site is created. F1 gene IV sequences transcribed from this second construct (pR 212) are translated in a different frame containing two ochre and two opal codons (non suppressible in *supD*). Phage DNA inserted into plasmids pR 211 and 212 was tested by sequence analysis, excised from the pUC vector with the enzymes *EcoRI* and *PstI* and transferred to plasmid pBR 322 (plasmids pR 213 and 214). Readthrough transcription at the main sites of the f1 T_{IV} terminator present on these plasmids results in the appearance of transcripts complementary to the distal portion of the beta lactamase gene beyond the *PstI* site of pBR 322 (see figure 2).

Plasmid copy number

Plasmid copy number was measured according to (27).

RESULTS

Suppressor cells yield f1 gene IV transcripts identical to those found in a ρ^- host

E. coli cells K37 (ρ^+ , *supD*), K38 (ρ^+ , *sup*^o) and K819 (ρ^- *sup*^o) were infected with bacteriophage f1, the RNA

Figure 3. Gene IV RNA synthesized in *sup*⁺, *sup*^o and ρ^- f1 infected cells. Lane a: RNA from K37 (*supD*, ρ^+); *supD* cells suppress amber mutations inserting serine residues at UAG codons. Lane b: RNA from K38 (*sup*^o, ρ^+). Lanes c and c': RNA from K819 (*sup*^o, ρ^-). Lanes a, b and c result from equivalent exposures (24 hrs, with intensifying screens) of the hybridized filter. Lane c' shows a shorter exposure (6 hrs, with intensifying screen) of lane c. The different cells were infected with bacteriophage f1, the RNA extracted, fractionated on a denaturing agarose gel, transferred onto nylon membranes and hybridized as described in Materials and Methods. Bands A, B and F represent transcripts terminated at the rho dependent T_{IV} sites, while C, D and G readthrough species. Band E corresponds to a molecule whose 3'OH end maps inside gene IV coding sequences and therefore it is irrelevant for the present analysis (La Farina: unpublished results).

extracted and the transcripts complementary to gene IV visualized by Northern hybridization. Figure 3 shows the pattern of bands found in each host. Transcription of f1 gene IV is initiated at

Figure 4. f1 gene IV transcripts. The genetic map is based on (20), the gene IV RNA map on (28). The rho dependent terminator T_{IV} is located just downstream of the gene IV stop codon (16–19). P indicates the position of the probe used in the hybridizations. I.G. is the intergenic region where the signals for DNA replication are located (15).

two different promoters (28): one located in front of gene III and one in front of gene IV (see fig.4). This activity results in the presence of two sets of transcripts which reach the T_{IV} terminator: one, quite rare, about 4 kb long (corresponding to band F or bands F, G in figure 3) and another, more abundant, whose slowest migrating species is about 1.46 kb long (bands A, B or A–D in figure 3). The pattern of 'terminated' transcripts observed in a ρ^+ cell (bands A, B and F: fig. 3, lane b;28) becomes more complex in a ρ^- host due to the presence of additional, readthrough transcripts (bands C, D and G: fig. 3, lanes c and c';16–19). The appearance of such readthrough bands in the RNA extracted from the ρ^- mutant confirm the data obtained by Moses and Model (16) using S_1 mapping. These Authors showed that in this host f1 gene IV transcription terminates not only on hairpin A as in the wild type, but also on hairpins B, C, D and E (see figure 1).

Interestingly, lane a of figure 3 shows that the readthrough gene IV messages (bands C, D and G) are also present in the ρ^+ $\sup^+$ host. In fact, in this host (lane a) transcripts initiated at the gene IV promoter show a pattern of bands (bands A–D) which exactly parallels that visualized in the ρ^- $\sup^0$ (lanes c, c'). Whether the end of the ρ^+ $\sup^+$ transcripts match exactly those of the ρ^- $\sup^0$ remains to be determined. In particular, it remains to be further studied whether the transcripts present in band A of the $\sup^+$ RNA terminate at the same site as in the ρ^+ or the ρ^- mutant (or elsewhere). The bands visualized in the suppressor however appear much less intense than those present in the ρ^- (see below). Gene IV transcripts initiated at the other (gene III) promoter also undergo readthrough in the suppressor. This is shown by appearance of a doublet, F and G, instead of the single band F in the region where the 4 kb long RNA migrates (resolution between bands F and G is quite poor in figure 3; however these two bands are much better resolved one from each other in figure 5). It is worth pointing out that small amounts of readthrough transcripts migrating in that region and visible only at very long exposure were detected in ρ^- cells by Moses and Model (16).

Thus our results show that in strain K37, a ρ^+ $\sup^D$ cell, which is able to read UAG codons, a pattern of longer gene IV messages is generated, as with the ρ^- host. We have observed the same readthrough pattern infecting strain K143 (ρ^- $\sup^F$:

Figure 5. Gene IV RNA in f1 infected suppressor cells bearing a cloned ρ wild type allele. Lane a: RNA from K37 ($\sup^D$). Lane b: K143 ($\sup^F$); $\sup^F$ cells suppress amber mutations by inserting tyrosine residues at UAG codons. Lane c: K37/6 (K37 transformed with the cloned ρ gene). Lane d: 143/6 (K143 transformed with the ρ gene). Again, bands A, B and F are transcripts terminated at the rho dependent T_{IV} sites, while bands C, D and G are readthrough species.

fig. 5, lane b). To rule out the possibility that the ρ^- -like pattern observed in K37 and K143 was due in fact to an unknown defect in the rho function of these hosts, the experiments were repeated on K37 and K143 derivatives that harbored a functional rho gene cloned into a plasmid (16) and still the pattern of gene IV messages showed a readthrough (fig. 5, lanes c, d).

The ρ^- -like pattern of gene IV transcription observed in suppressor cells is abolished if the gene IV RNA is not engaged in translation

The experiments shown above suggest that the movement of the ribosome on gene IV RNA beyond its natural translation codon interferes with the f1 T_{IV} terminator and causes readthrough. To confirm such a model it is necessary to show that abolishment of translation of gene IV RNA results in disappearance of such events. To address this point more directly, we constructed and tested appropriate distal gene IV segments that contained identical nucleotide sequences decoded in two different reading frames (see Materials and Methods). The first corresponded to the unique f1 gene IV open reading frame while the second did not allow the ribosome to reach the end of the gene IV sequences both in $\sup^0$ and $\sup^+$ cells (29). Thus, if readthrough events at the f1 T_{IV} terminator in suppressor cells result from movement of the ribosome beyond the natural gene IV stop codon, they must be abolished when the RNA is derived from the second construct. Analysis of RNA extracted from $\sup^0$ and $\sup^+$ cells transformed with either of the two plasmid constructs confirmed this point. Figure 6 in fact shows that transcription of the first construct in the $\sup^+$ host (lane b) results in the appearance of longer RNA molecules (bands C and D), besides those (bands A and B) already observed in the $\sup^0$ host (lane a). In contrast, only bands A and B are present when the second construct is transcribed both in $\sup^0$ (lane c) and $\sup^+$ (lane d) cells. The

Figure 6. Gene IV RNA synthesized in *sup*^o or *sup*⁺ cells transformed with plasmids pR 213 and 214. Construction of the plasmids is described in Materials and Methods (see figure 2). Phage f1 distal gene IV RNA transcribed on plasmid pR213 is translated according to the phage natural reading frame; that transcribed on plasmid pR214 is translated out of this frame. K38 (lanes a, c) is *sup*^o, K37 (lanes b, d) is *sup*⁺. Lane a: K38/pR213; lane b: K37/pR213; lane c: K38/pR214; lane d: K37/pR214. A and B are transcripts terminated at f1 T_{IV}, C and D readthrough species terminated at the beta-lactamase tandem terminators.

Figure 7. Level of gene IV RNA in *sup*^o, *sup*⁺ and *rho*⁻ f1-infected cells. Two fold dilutions of RNA extracted from each host were spotted onto filters and hybridized to the probe.

relative mobility of the various transcripts makes it quite obvious to interpret bands A and B as molecules terminated at the f1 T_{IV} terminator, while bands C and D as readthrough molecules covering the distal portion of the beta lactamase gene and terminating at the two tandem terminators identified at its end (30). This suggests that movement of the ribosome over f1 T_{IV} interferes with its termination activity.

Suppressor cells do not yield high levels of f1 gene IV RNA

Infection of a *rho*⁻ host with phage f1 results not only in readthrough transcription beyond the f1 T_{IV} signal but also in accumulation of high levels of gene IV RNA (16). Our present results have confirmed this data. In fact, exposure of a RNA filter, after hybridization to the gene IV probe, for that length of time needed to visualize the bands present in the *rho*⁺ (lane b of figure 3) resulted in overexposure of those of the *rho*⁻ (lane c) which could be resolved one from each other only at a lower exposure (lane c'). In the *sup*⁺ host (lane a) however, the level of this RNA did not appear to exceed that found in the *sup*^o *rho*⁺. To estimate better the different gene IV RNA accumulation in each host, dilutions of the RNA were spotted

onto filters and hybridized to the gene IV probe. Figure 7 shows that while in the *rho*⁻ cell the level of gene IV RNA is at least 15 times that found in the *rho*⁺ *sup*^o (in full agreement with the data of 16), in the *rho*⁺ *sup*⁺ it does not show any significant increase. Analysis of the copy number of the phage RF_I [replicative form I, template for phage transcription, (31)] excluded the possibility that the higher level of gene IV messages found in the *rho*⁻ host could result from a higher accumulation of the templates in that cell.

DISCUSSION

We have shown that in suppressor cells readthrough transcription occurs at the rho dependent termination signal T_{IV} of bacteriophage f1 resulting in f1 gene IV transcripts longer than those present in the *sup*^o and identical to those found in a *rho*⁻ host (figures 3 and 5). Such longer transcripts do not appear in the suppressor-infected cell when, by changing the translation frame of gene IV, the ribosome is not allowed to proceed to the end of the gene IV message and thus to reach the terminator site (figure 6).

A likely hypothesis to explain these data is that movement of the ribosome over the rho-dependent transcription termination signal results in a weakening of the terminator efficiency. F1 T_{IV} (16,18,19) is located at the end of the I region of transcription of bacteriophage f1 (19,28) with its main rho dependent site 63 nucleotides downstream of the gene IV stop codon. These signals lie on opposite sides of a stem and loop structure. Our results suggest that the sequences corresponding to this structure must remain untranslated in order for f1 T_{IV} to maintain its full termination efficiency. When this region is made available—through suppression—to the ribosomes, readthrough transcripts appear as if in a *rho*⁻ host. If, on the other hand, the reading frame of this sequence is altered and the ribosome falls off of gene IV message before reaching the terminator this effect is abolished: the readthrough transcripts no longer appear. This would imply that correct transcription termination of f1 gene IV messages depends on their correct termination of translation pointing out once more to the tight linkage that exists between the transcription and translation machineries in *E. coli*. Study of attenuation has shown that ribosome movement regulates the activity of certain rho independent terminators (for a review see 32). Our present results suggest that it can also affect the activity of rho dependent ones. What step(s) of the complex mechanism of rho-dependent termination is affected by the ribosome movement remains to be determined. At least two distinct, non overlapping RNA regions are known to play a role in the carrying out of this event: the segment to which the rho factor, in its hexameric form (3) binds and one at which termination actually occurs (1,2). Although no consensus sequence for the binding site has been found (6) it appears to correspond to a region of at least 80 nucleotides of seemingly little secondary structure (1,2). The features of the other region, corresponding to the 3'OH of the transcript, are not yet clear. While initially the occurrence of secondary structures at these sites had been questioned (33), later on, several authors, studying different rho-terminated transcripts, proposed that moderately strong secondary structures may be formed at the 3'OH end of these molecules (6,34–36). The 3'OH end of messages we have studied can, in fact, form a stem and loop structure (15). Consideration of the length (only 63 nucleotides) of the region separating the translation stop codon from the message end, as well as the capacity of this segment

to form a fairly stable secondary structure (about -26 KCal, calculated according to (37) makes it extremely unlikely that the rho factor binds to this segment. In contrast, inspection of the distal portion of the gene IV coding sequences reveal good similarity to several short sequences observed in presumptive rho binding sites of some other rho-terminated transcripts (6). Thus the organization of fl T_{IV}, with a rho binding-like region *upstream* of the stop codon and the entire sequence downstream of it forming a secondary structure, appears quite different from that of other well known terminators, such as lambda-tR1, lambda-tR2 and trp^t. In these, an unstructured region, corresponding to the rho binding site, is located downstream of the stop codon (6,9). Thus the readthrough effect observed on fl gene IV messages when the ribosome moves beyond the stop codon may not be the result of the ribosome interfering with the binding of the rho factor to the message. Other hypotheses may be proposed. For example, as is the case in other rho-terminated transcripts, the transcription bubble may pause at the site where the event of actual termination takes place (33,38). Disruption of the hairpin by the movement of the ribosome would eliminate that pausing, thus preventing the rho factor from catching the transcription complex. An alternative possibility, not based on the pausing hypothesis, would simply be that, independently of the formation of the hairpin structure downstream of the gene IV stop codon, the non translation of that RNA region is necessary for a physical interaction between the rho factor and the transcription complex, perhaps for the rho factor to elicit its helicase activity (10).

Suppressor cells, though yielding a pattern of fl gene IV messages identical to that found in a *rho*⁻ host, differ from that in not accumulating high levels of gene IV transcripts. This in turn opens the possibility that the accumulation observed in the *rho*⁻ cell may not be exclusively or even primarily an effect of the readthrough *per se* and that other types of interactions between the RNA and the factor may be involved. Further work is being carried out in our laboratory to shed light on this point.

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