

Characterization of the biochemical properties of recombinant ribonuclease III

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ABSTRACT

An *Escherichia coli* double strand specific endoribonuclease, RNase III, was cloned, expressed in large amounts, and purified to homogeneity. Enzyme activity was monitored by assaying fractions for the ability to correctly process exogenous RNA containing specific RNase III cleavage sites.

DEAE-Sepharose ion exchange chromatography in the presence of a linear KCl gradient (from 0.02 M to 0.75 M) demonstrated that RNase III exists as two distinct forms. One form elutes at a KCl concentration of 0.13 M and the other elutes at 0.33 M. The presence of stoichiometric amounts of the GTP-binding protein Era during purification results in the conversion of the low salt form into the high salt form. Size exclusion chromatography demonstrated that both forms exist as a dimer in solution. In order to investigate the nature of the dimer, protein cross-linking was performed and cross-linked products were detected by silver staining. The protein-protein dimer can be visualized at protein:cross-linker molar ratios as low as 1:15 within 1 minute of exposure to cross-linker in 0.1 M KCl. Upon addition of substrate RNA to the cross-linking reaction a second form of the protein-protein dimer (with a slightly smaller apparent molecular weight) becomes prominent. Induction of the new form is absolutely dependent upon the addition of substrate mRNA to the reaction mixture. We postulate that the RNase III dimer undergoes a dramatic conformational change upon recognition of RNA which we are able to trap by cross-linking.

INTRODUCTION

Processing of mRNA is an important means of regulating genetic expression in phages, viruses and cells. The *mnc* gene of *Escherichia coli* encodes a double strand specific endoribonuclease (RNase III) (1) which is involved in the maturation of RNA precursors. There are multiple RNase III cleavage sites in mRNA of several bacteriophages (2–5). Sites also exist in the *E. coli* rRNA precursors, and some *E. coli* mRNAs (6–8). Recently, an earlier prediction—that expression

of the *mnc* operon was auto-regulated due to the presence of an RNase III cleavage site within its own mRNA (9)—has been confirmed (10).

Although many of these processing sites have been mapped virtually nothing is known about the mode of action of this enzyme. There does not seem to be any sequence selectivity as the enzyme can process at specific sites as well as non-specific as demonstrated by its ability to digest double stranded reo virus genomic RNA (11) and yeast killer RNA (12).

In order to understand how this enzyme recognizes and cleaves its substrate we purified a large amount of RNase III from a recombinant clone. An earlier suggestion that RNase III is a dimer in solution was confirmed (13) and our data indicate that two forms of the RNase III dimer are present in cells harboring an RNase III expression vector. The conversion between the two species requires the presence of Era (an essential GTP-binding protein present in *E. coli*; refs. 14 and 15) in stoichiometric amounts. Protein-protein cross-linking experiments indicate that the recognition of substrate RNA is accompanied by a dramatic structural change in the dimer. The visualization of this event by cross-linking provides a simple, sensitive, and direct assay for investigating the protein and RNA determinants involved in RNase III action.

METHODS

Purification of RNase III

The bacterial strain SB221 harboring the plasmid pJHA002 was employed to express RNase III to a level of 5% of the total protein as described previously (9). Starting from a 4 l culture of that strain a 20 gm cell pellet was obtained by centrifugation. All subsequent procedures were performed at 0–4°C. Cells were lysed in a volume of 38 ml by treating with lysozyme /EDTA/detergent according to the protocol described previously (15). Efficient lysis was accompanied by a very large increase in the viscosity of the sample as DNA was released. DNA was digested by addition of DNase and physical shearing in a glass pipette. In order to prevent aggregation of RNase III the solution was adjusted to 1 M KCl by addition of KCl crystals. All cellular debris and membrane fragments were removed by ultracentrifugation for 1 hour at 100,000×g. RNase III was

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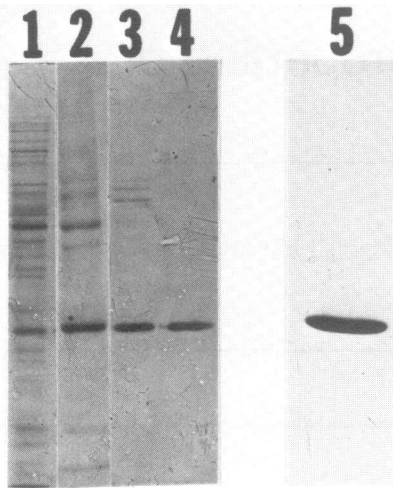


Figure 1. Purification of RNase III. Lanes 1–4 represent protein isolated at different stages of the procedure described in the Methods section. Lane 1; 0.1 μ l of the ammonium sulfate fraction enriched in RNase III protein. Lane 2; 4 μ l of the pooled RNase III peak fractions (total volume=53 ml) after Sephadex G-100 chromatography. Lane 3; 1 μ l of pooled peak fractions (total volume = 12 ml) collected at 0.13 M KCl following DEAE-Sephacel ion exchange chromatography. Lane 4; 1 μ l of the 2 M NH_4Cl effluence from the poly(rI · poly rC) agarose affinity column. Lane 5; same material as in lane 4 after concentration and dialysis (10 μ l loaded representing 4.8 μ gm of protein; total volume= 6.0 ml). Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 17.5% acrylamide gel (17). The gels were stained with Coomassie blue.

concentrated from the supernatant by ammonium sulfate precipitation as described by Dunn (13). The RNase III enriched precipitate was redissolved in 5 ml of buffer (50 mM Tris, pH 8.0, 0.7 M KCl, 0.02 M 2-mercaptoethanol, 2 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3) and loaded onto a Sephadex G-100 column with a bed volume of 480 ml. The column was developed with the same buffer and absorption of the effluence was monitored at 280 nm (all column chromatographic steps were accompanied by monitoring absorption). Fractions which contained absorbing material were subjected to SDS gel electrophoresis after dialysis, and were assayed for their ability to digest a RNase III specific substrate (see below). By this analysis most of the RNase III eluted with a molecular weight of 53,000 from the column (all absorbing fractions were subjected to these analyses throughout the entire purification procedure). Peak fractions were pooled, concentrated 10-fold on a dry bed of polyethylene glycol and brought back to the original volume with 50 mM Tris (pH=8.0). This fraction was loaded onto a DEAE-Sephacel column (bed volume= 180 ml) which was eluted with a linear KCl gradient from 0.02 to 0.75 M. The other components of the gradient buffers were the same as those employed during Sephadex G-100 chromatography. Greater than 90% of the applied RNase III was recovered at a KCl concentration of 0.13 M. This material was 95% pure as assayed by quantitative analysis of Coomassie blue stained gels. This mixture was subjected to affinity chromatography employing poly rI · poly rC agarose (Pharmacia) as described previously (13). In agreement with earlier results, we found that the RNase III elutes in the presence of 2 M NH_4Cl . The RNase III recovered from the affinity column was dialyzed overnight versus a 200 fold excess of 50 mM Tris (pH 8.0), 0.27 M KCl, 0.02 M 2-mercaptoethanol, 2 mM EDTA and concentrated to a

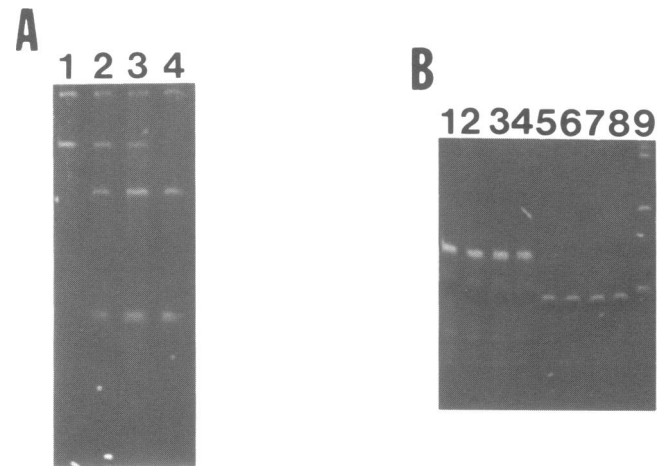


Figure 2. Characterization of the enzymatic properties of purified RNase III. Panel A; standard RNase III assay described in the Methods section using the 375 nucleotide runoff transcript. Incubation times were 0 min. (lane 1), 5 min. (lane 2), 15 min. (lane 3), 30 min. (lane 4). RNase III will cut the 375 nucleotide substrate at two positions (10). The identity of the bands (from largest, at top, to smallest) is; input template DNA, 375 nucleotide uncut transcript (lanes 1–3), 208 nucleotide 3' fragment (lanes 2–4), 84 nucleotide 5' fragment and 83 nucleotide internal fragment (not resolved, lanes 2–4). Panel B; standard RNase III assay using 100 ngm of *E. coli* 5s RNA (lanes 1–4), or 50 ngm of yeast phenylalanine tRNA (lanes 5–8) as substrate. Incubation times were as described for panel A. Lane 9 contains the products of a 20 min digestion of the transcript employed in panel A. Photographs of ethidium bromide stained 8% acrylamide gels are shown.

volume of 6.0 ml on a dry bed of polyethylene glycol. This fraction contained 3 mg of RNase III that is more than 99.8% pure. Protease inhibitors were present up to and including the ion exchange chromatography step, because significant losses were observed due to proteolysis in their absence.

RNase III activity assay

The standard RNase III assay contains 24 ngm of RNase III and 100 ngm of RNA in a total volume of 11 μ l. The assay buffer contained 0.02 M Tris (pH 8.0), 0.1 M KCl, 0.01 M MgCl_2 , 0.1 mM dithiothreitol, 0.1 mM EDTA. Incubations were carried out at 37°C for times varying between 5 to 30 min. Reactions were stopped by addition of sample loading buffer containing 95% formamide. Unless indicated otherwise, the RNA was prepared in vitro from pAR2656. The plasmid pAR2656 contains a subclone of the *mrc* operon inserted into a T7 expression vector (16) and was a gift from William Studier (Brookhaven National Laboratory). The *mrc* subclone contains a RNase III cleavage site as described previously (10). pAR2656 was linearized with the restriction enzyme *EcoRV* and 375 nucleotide run off transcripts were produced using T7 RNA polymerase from either BRL or Boehringer Mannheim. The in vitro transcription reactions were carried out precisely as detailed by the supplier. Approximately five μ gm of transcript was routinely obtained from 1 μ gm of template. This transcript is referred to throughout the text as RNase III specific substrate. The reaction products were separated on 8% polyacrylamide gels in the presence of 8 M urea, and visualized by staining with ethidium bromide.

Protein Cross-linking

The standard cross-linking reaction was carried out in the presence of 0.5 μ gm of RNase III, and 50 ngm of cross-linker

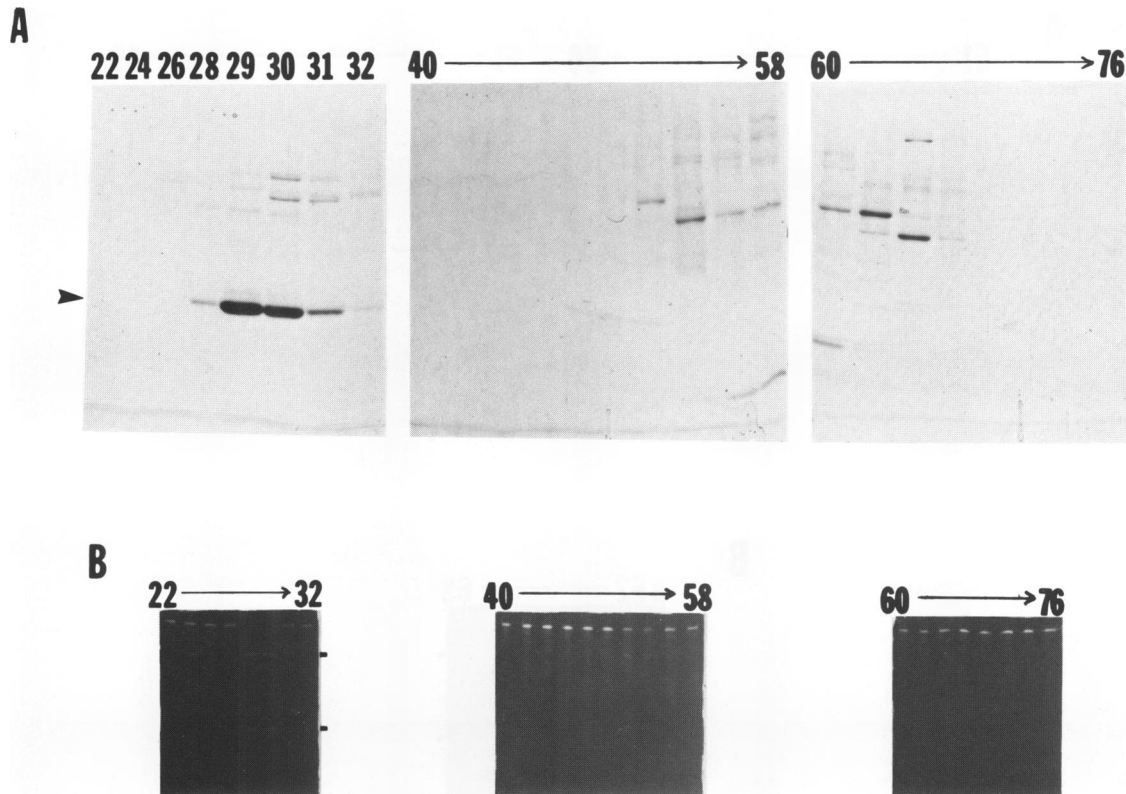


Figure 3. Analysis of fractions which absorb at 280 nm and were collected during DEAE-Sepharose ion exchange chromatography of cells harboring pJHA002. Panel A; Coomassie blue stained SDS-polyacrylamide gels. Fraction numbers analyzed are indicated at the top. A peak centered around fraction 29 (0.13 M KCl) was analyzed in the left gel. A second broad peak was analyzed in the center and right gels where every even number fraction from 40 to 76 is displayed. The arrowhead indicates the position of RNase III comigrating protein. Panel B; Standard RNase III assay of the fractions from (A) using 1 μ l of each fraction and 50 ngm of the 375 nucleotide run off transcript. The position of migration of the reactions products (208 nucleotide, 84 and 83 nucleotide fragments) as described in Figure 2 are indicated by the dashes. The concentration of KCl at fraction 53 was 0.33 M. Photographs of ethidium bromide stained gels are displayed.

(disuccinimidyl tartarate, DST; from Pierce Chemical Co.) in 11 μ l of the RNase III assay buffer described above (except that 0.02 M MOPS was substituted for Tris).

Cross-linking reactions were carried out for times of between 1 min. and 30 min. at either 4°C or 37°C. In reactions containing RNA, 0.5 to 1.0 μ gm of RNA was added to the cross-linking mixture. Reactions were terminated by addition of an equal volume of SDS sample loading buffer (containing 0.16 M Tris to scavenge unreacted DST). The reaction products were separated by SDS-polyacrylamide gel electrophoresis on 17% polyacrylamide gels (17) and visualized by silver staining (18).

RESULTS

Purification of RNase III

A recombinant clone which is capable of expressing RNase III to a level of 5% of the total protein in *E. coli* (pJHA002; ref. 9) was employed to purify large amounts of RNase III as described in the Methods section. In Figure 1 the protein recovered at various stages of the purification procedure is displayed. The major peak of RNase III protein and specific cleavage activity elutes with a molecular weight of 53,000 on a Sephadex G-100 column developed with 0.7 M KCl (Figure 1, lane 2). This is in excellent agreement with the data reported by Dunn (13) and strongly indicates that RNase III exists as a dimer in solution (the monomer molecular weight in denaturing

gels is 26,500; Figure 5, lane 1). After the final purification step approximately 3 mgm of RNase III was obtained with a purity of greater than 99.8%. Following electrophoretic separation of 5 μ gm of purified material, no additional bands can be detected by Coomassie blue or silver staining (Figure 1, lane 5). Twenty-four nanograms of protein will digest 100 ngm of specific substrate to completion within 30 minutes, but has no activity towards 5s RNA or tRNA (Figure 2). As expected the pure enzyme digests reo virus genomic RNA to the size of short oligoribonucleotides and correctly processes an *E. coli* rRNA precursor expressed in vitro from an *rnmB* operon fusion (19) (not shown). Based on these data it was concluded that the purified material shown in Figure 1, lane 5 is RNase III.

Analysis of Two Distinct Forms of RNase III

DEAE-Sepharose ion exchange chromatography was employed during the purification procedure presented above, and the results are presented in Figure 3. The column was eluted with a KCl gradient as described in the Methods section. The peak of RNase III protein (Figure 3A) and enzyme activity (Figure 3B) both eluted at a KCl concentration of 0.13 M (Fractions 29 and 30). A weak RNase III enzyme activity eluted much later (Figure 3B, fractions 52 to 54) at a KCl concentration of 0.33M. RNase III is encoded by a gene (*rnc*) whose transcription and translation is coupled to Era, a GTP-binding protein (10,14). Therefore, it was suspected that the two species of RNase III represent

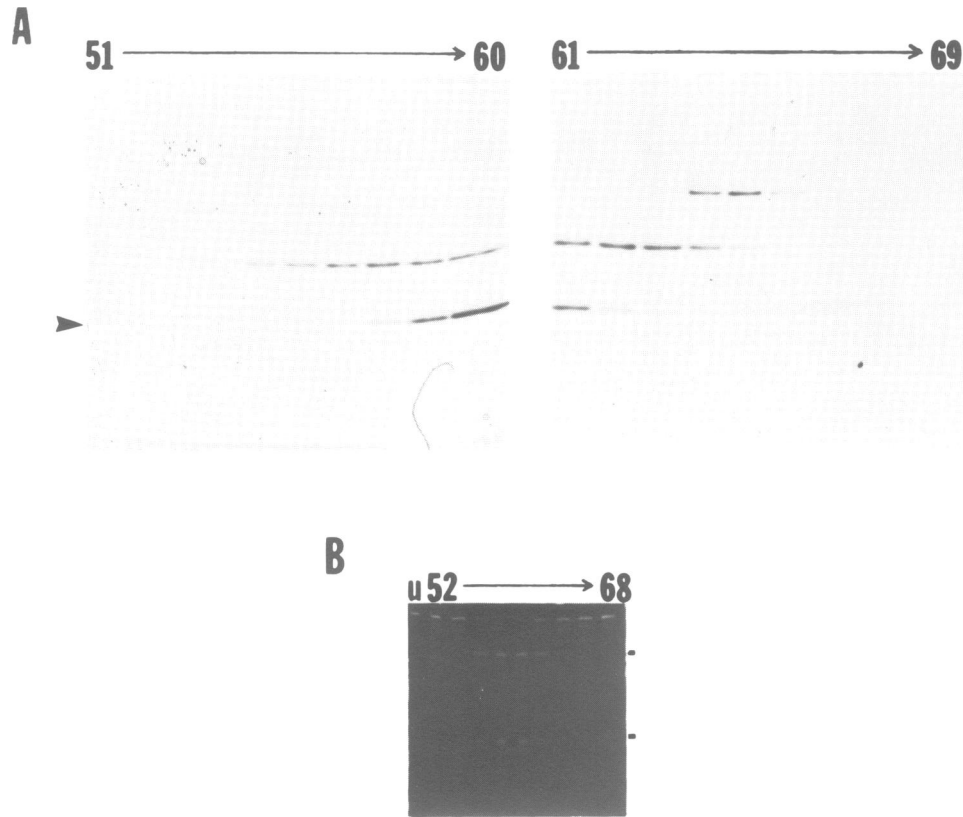


Figure 4. Analysis of fractions which absorb at 280 nm and were collected during DEAE-Sepharose ion exchange chromatography of cells harboring pJR002. Panel A; Coomassie blue stained SDS-polyacrylamide gels. The contents of every fraction between 51 and 69 is presented (the early peak shown is Figure 3, centered around fraction 29 was not observed). The arrowhead indicates the position of RNase III comigrating material. In this experiment the concentration of KCl at fraction 60 was 0.33 M. Panel B; standard RNase III assay of the even numbered fractions (52–68) shown in (A). Lane u contains uncut 375 nucleotide transcript. The dashes indicate the position of the reaction products (see Figure 3B).

different chemical forms and the conversion of one to the other may be modulated by Era. To test this possibility, the purification protocol was repeated on cells harboring a recombinant clone (pJR002) that expresses RNase III and Era in a 1:1 molar ratio (14). Fractions separated by DEAE-Sepharose chromatography were analyzed as shown in Figure 4. The peak of RNase III protein (Figure 4A, fraction 60) and enzyme activity (Figure 4B, fraction 60) occurred at a KCl concentration of 0.33 M. Neither protein nor enzyme activity was present at 0.13 M KCl. The high salt form of RNase III was purified to homogeneity as described above. Both forms shared the enzymatic properties demonstrated in Figure 2, except that the high salt form possesses a much higher activity towards the substrates used in this study (Figure 5). Both species also elute with an apparent molecular weight of 53,000 according to gel exclusion chromatography indicating they occur as dimers in solution, and they have identical one-dimensional peptide patterns upon partial trypsin digestion (not shown).

Characterization of the RNase III dimer in solution

In order to confirm the observation that RNase III is a dimer, protein-protein cross-linking was performed. In Figure 6 it is demonstrated that, in the presence of the cross-linking reagent DST, RNase III is efficiently modified resulting in the appearance of two major bands after separation by SDS-gel electrophoresis. A monomer band is present which migrates with an apparent



Figure 5. Activity of the high salt form of purified RNase III (lanes 2–5) or the low salt form (lanes 6–9) towards substrate RNA. The reactions were carried out as described in Figure 2 except that only 5 ngm of RNase III was employed in these reactions. Lane 1 contains uncut 375 nucleotide transcripts. Lanes 2 and 6 were incubated for 0 min, lanes 3 and 7 for 5 min, lanes 4 and 8 for 15 min, and lanes 5 and 9 for 30 min.

molecular weight of 26,500 and a dimer band is present with an apparent molecular weight of 50,000. The protein:DST molar ratio employed in this experiment was 1:15. Incubation in the presence of excess cross-linker (1:1,000 molar ratio) results in nearly complete conversion to the dimer species, but not the appearance of additional higher molecular weight species. If the

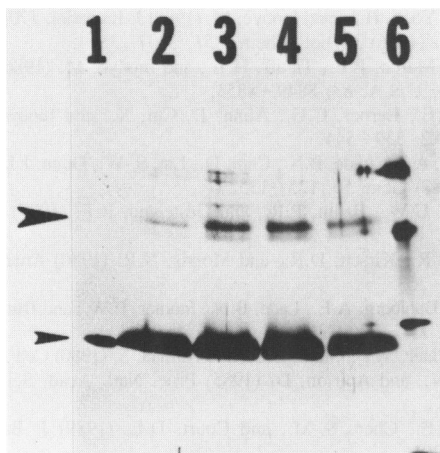


Figure 6. Cross-linking of purified RNase III with DST. Lanes 1–5 contain 0.5 μgm of purified RNase III and (except lane 1) 50 ngm of DST. Incubation times were 1 min (lane 2), 5 min (lane 3), 15 min (lane 4), 30 min (lane 5) at 4°C. Lane 6 contains molecular weight markers; bovine serum albumin (molecular weight = 66,000), ovalbumin (molecular weight = 46,000), carbonic anhydrase (molecular weight = 29,000) and lysozyme (molecular weight = 14,000). The small arrowhead indicates the position of RNase III monomer. The large arrowhead indicates the position of the 50,000 molecular weight cross-linked dimer species. Bands in lanes 2–5 with molecular weight > 50,000 are artifacts of silver staining. They are present in the absence of cross-linker (Figure 7, lane 1) and also in the absence of protein (not shown). The photograph of a silver stained SDS-polyacrylamide gel is shown.

dimer band were the result of non-specific contact between monomers one would expect to be able to detect trimers and tetramers, etc., after exhaustive cross-linking. There is precedent for the presence of endogenous RNA as an active component of an RNase (20). In order to examine whether the higher molecular weight form of RNase III contains RNA we have stained cross-linked and uncross-linked material (up to 2.4 μgm) with ethidium bromide after electrophoresis and have not been able to demonstrate staining material. In addition, treatment of cross-linked material with RNase A does not result in a change in the molecular weight of the dimer band (not shown). These data indicate that RNase III is a stable protein dimer in solution. These experiments do not address the possibility that low molecular weight RNA may be present in our preparations.

Cross-linking of RNase III in the presence of RNA

In order to understand the nature of the interaction between RNase III and its substrate, cross-linking experiments were performed in the presence of RNA (Figure 7). Interestingly, in addition to the monomer and the dimer band a third band (apparent molecular weight = 48,000) was present when specific substrate RNA was added to the reaction mixture (Figure 7, lane 3). The new band is a protein-protein dimer as determined by the fact that its mobility is not affected by treatment with ribonuclease A. In addition, using ^{32}P -labeled specific substrate no radioactivity was incorporated into any of the bands. The formation of the 48,000 molecular weight species is absolutely dependent upon the presence of the specific substrate for RNase III. As shown in Figure 7 the band does not occur when ribonucleotides (lane 7), 5s RNA (lane 5), or tRNA (lane 4) are added to the reaction mixture. All cross-linking experiments shown were performed using the low salt species of RNase III, but the results are identical in experiments in which high salt RNase III was employed.

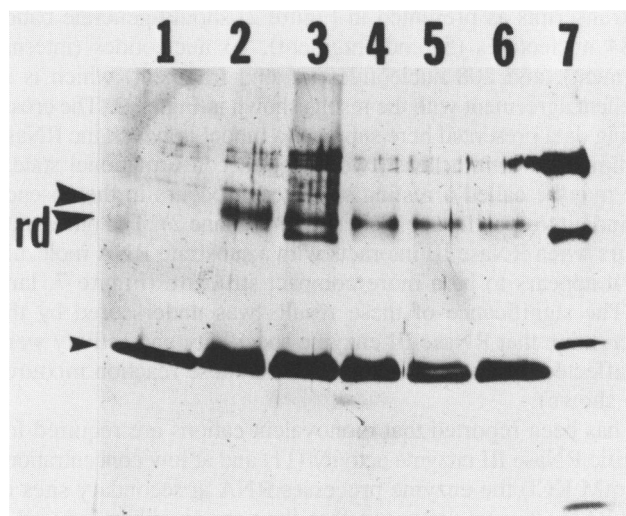


Figure 7. RNase III protein-protein cross-linking in the presence of RNA. All samples contain 0.5 μgm of RNase III and (with the exception of the material shown in lane 1) 50 ngm of DST. Samples in lanes 2 to 6 contain; no additional RNA (lane 2), 1.0 μgm of the 375 nucleotide transcript (lane 3), 0.5 μgm yeast phenylalanine tRNA (lane 4), 0.5 μgm *E. coli* 5s RNA (lane 5), or 0.1 mM rNTP (lane 6). Lane 7 contains molecular weight markers as described in the legend in Figure 6. Incubations were carried out for 15 min at 4°C. The small arrowhead indicates the position of the monomer, the large arrowhead indicates the position of the 50,000 molecular weight dimer and rd indicates the position of the RNA induced dimer. *E. coli* 5s RNA and yeast tRNA were purchased from Boehringer Mannheim. The photograph of a silver stained gel is shown.

DISCUSSION

The *mc* operon of *E. coli* encodes *mc*, *era* and *recO* (9,14,21–23). In previous reports we have shown that *era* encodes a GTP-binding protein with intrinsic GTPase activity (14,15). A functional *era* allele is required for cell viability (15,22,24) and conditional mutants are blocked in a very early step of cell division (25). The *mc* gene encodes RNase III, and although transcription and translation of *mc* and *era* are linked (10,14) there have been no previous reports describing a coupling of the function of these genes or their products. The data presented here demonstrate that the presence of Era during the purification of RNase III induces a change in the chemical properties of RNase III. Since RNase III is not essential for viability in *E. coli* (22), our results suggest that RNase III occurs in a coupled, but non-obligatory pathway with Era. The mechanism by which the properties of RNase III have been modified are currently under investigation. It is tempting to speculate that the modification is a phosphorylation event catalyzed either directly or indirectly (via an unidentified kinase) by Era. Phosphorylation of RNase III has been demonstrated upon infection with bacteriophage T7 where it has been shown that RNase III is phosphorylated by the T7 protein kinase at serine resulting in a 4-fold stimulation of RNase III enzyme activity (26). It is important to note that phosphorylation via protein kinase is a common mechanism by which GTP-binding proteins elicit a cellular response in many signaling pathways.

Little is known about the mechanism by which RNase III cleaves double stranded RNA. It is known that the cleavage of the *mc* mRNA by RNase III occurs at bp –38 and –120 (10). These sites are on opposite strands of the stem of a previously proposed stem-loop structure occurring in the 5' leader region of the mRNA (9). Cleavage of this region (when present on run-

off transcripts as presented in Figure 2) should generate bands of 84 nucleotides (5' end fragment), 83 nucleotides (internal fragment), and 208 nucleotides (3' end fragment) which is in excellent agreement with the results shown in Figure 2. The cross-linking data presented here supports a model in which the RNase III dimer exists in at least two different conformational states. One may be called a resting state and it occurs in the absence of binding to specific substrate (Figure 7, lane 2). The other state occurs when RNase III interacts with a substrate RNA molecule and it appears to be a more compact structure (Figure 7, lane 3). The significance of these results was underscored by the observation that RNase III enzyme specificity and activity were not affected by the presence of DST in these reaction mixtures (not shown).

It has been reported that monovalent cations are required for specific RNase III enzyme activity (11) and at low concentrations (20 mM KCl) the enzyme processes RNA at secondary sites in vitro (13). It was suggested that the cations either induced a conformational change in the dimer or shifted the monomer-dimer equilibrium (13). Cross-linking experiments were carried out in the presence of 0.01 M, 0.1 M and 0.3 M KCl. At low KCl concentrations the cross-linked dimer is nearly undetectable, and the amount of dimer observed increased with increasing salt concentration (data not shown), indicating that monovalent cations shift the monomer-dimer equilibrium in favor of dimer formation.

A convenient and sensitive assay for investigating the interaction of a purified ribonuclease with its substrate in vitro has not previously been reported. Gel shift assays are of limited use because the RNA is processed during incubation with the enzyme. Here we show changes in the mobility of protein species which specifically require the presence of substrate RNA. These properties can be utilized to understand, in detail, the mechanism by which RNase III recognizes and cleaves RNA. Current studies are aimed at understanding what residues on the RNA and the protein are required for these events.

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