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Bioassay for components regulating eukaryotic gene expression: A chromosomal factor involved in the generation of histone mRNA 3′ termini

(oocyte injection/regulator protein/gene control/transcription termination)

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ABSTRACT **We have adapted the oocyte injection procedure for the detection of regulatory components involved in the transcription of a eukaryotic mRNA gene. Injection of the histone gene repeat h22 DNA of *Psammechinus miliaris* into the *Xenopus* oocyte nucleus results in correct initiation of the histone mRNAs, but readthrough by RNA polymerase occurs at the 3′ end of the H3 histone gene (Hentschel, C. C., Probst, E. & Birnstiel, M. L. (1980) *Nature (London)* 288, 100–102). Coinjection into the oocyte of a chromosomal salt wash fraction derived from sea urchin embryos results in the generation of authentic 3′ termini of the histone H3 mRNA. We have partially purified the protein component by column chromatography and density gradient centrifugation. The regulatory factor binds to heparin columns and, hence, has the properties anticipated of an RNA- or DNA-binding protein. The sedimentation coefficient of the active component was determined to be about 12 S, suggesting a molecular weight of 200,000–250,000.**

Two general approaches can be used to study the mechanisms controlling gene expression. The first is to identify the regulatory signals by mutation, followed by an analysis of the expression of the mutated genes. Another approach is to characterize regulatory factors (proteins) interacting with these DNA signals. For an understanding of the processes of differentiation, the study of mRNA genes transcribed by polymerase II is of particular importance, because it is the protein-coding genes that primarily determine the cell phenotype.

There is now evidence that expression of the different sea urchin histone gene variants is under developmental control (reviewed in ref. 1). This regulation appears to occur both at transcription initiation and termination. Control at transcription initiation is suggested because the regulatory pattern can readily be reproduced by *in vitro* transcription of nuclei isolated from different embryonic stages of the sea urchin (2). That transcription termination also may be controlled developmentally can be inferred from a recent report (3) that transcription in the newt oocyte reads through the 3′ termini of histone genes, yielding the large transcripts typical for lampbrush chromosomes of this species.

Previous frog oocyte injection experiments have shown that the promoter (4, 5) and terminator signals (6) of all the genes of the sea urchin histone DNA clone h22 appear to be recognized by the frog oocyte transcriptional machinery, although with greatly different efficiencies (7). For the sea urchin H2A histone gene, the faithful generation of correct H2A histone mRNA 3′ ends is dependent on the presence of a highly conserved inverted DNA repeat that lies immediately upstream of the 3′ mRNA terminus 5′ A-C-C-A 3′ and on the presence of spacer sequences further downstream (6). The behavior of the H3 histone gene in the oocyte injection experiments is exceptional in that transcription initiation, although varying in different batches of the oocytes, usually occurs at a high rate, but transcription termination of this gene is always inefficient, giving rise to heterogeneous readthrough transcripts. A minority of these extend into the H2A terminal region further downstream, yielding a dicistronic mRNA (7). The simplest interpretation of this finding is that the frog oocyte lacks a cofactor required for transcription termination of the H3 gene.

We have adapted the oocyte injection technique (8, 9) and have developed a complementation assay for the search for such regulatory protein(s). In this paper we show that a chromosomal component can correct a lesion in the synthesis of H3 mRNA. Instead of a high level of readthrough transcripts, authentic H3 mRNAs with correct 3′ termini are generated in the presence of this protein fraction. Starting from chromatin prepared from sea urchin embryos at cleavage stage, this protein has been partially purified by selective salt extraction of the chromatin, followed by fractionation on heparin-Sepharose and DEAE-Sephacel columns. Furthermore it has been shown, by sucrose gradient centrifugation, to sediment at about 12 S, suggesting a molecular weight of $2–2.5 \times 10^5$.

METHODS AND MATERIALS

Enzymes and Reagents. Restriction enzymes were purchased from New England BioLabs or from Boehringer Mannheim. Phage T4 DNA ligase was obtained from Miles. Calf intestine alkaline phosphatase, S1 nuclease, and Klenow *Escherichia coli* DNA polymerase were purchased from Boehringer Mannheim, and T4 polynucleotide kinase was purchased from P-L Biochemicals.

Chromatin Preparation. Sea urchin embryos (≈64-cell stage) were grown at 10^4 eggs per ml in artificial sea water. The sea urchin chromatin was prepared essentially as described by Spelsberg and Hnilica (10): sea urchin embryos were harvested and washed once with 0.5 M sucrose in TKM buffer (50 mM Tris·HCl, pH 7.5)/25 mM KCl/5 mM $MgCl_2$), homogenized in the same buffer, and centrifuged for 10 min at 10,000 × g through a 1.5 M sucrose/TKM buffer cushion. The rough nuclei pellet was gently homogenized in 0.5 M sucrose/TKM buffer containing 0.2% Triton-X-100 and was centrifuged through a 1.5 M sucrose/TKM buffer cushion. The nuclei were lysed in 80 mM NaCl/20 mM EDTA/2 mM EGTA, pH 6.5, and centrifuged at 5,000 × g for 10 min. The chromatin was either directly used or stored at −20°C. All preparations were done at 4°C,

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Abbreviations: TKM buffer, 50 mM Tris·HCl, pH 7.5/25 mM KCl/5 mM $MgCl_2$; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

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and all buffers contained 1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$) in isopropanol, added directly before use.

Chromatin Salt Wash Fractions. The chromatin was subsequently extracted with 150 mM, 300 mM, 450 mM, 600 mM, and 2 M NaCl in 20 mM Tris·HCl, pH 7.0/1 mM EDTA/0.2 mM EGTA/0.5 mM dithiothreitol/0.5 mM $PhMeSO_2F$ and centrifuged at 4°C in a Beckman SW 27 rotor for 30 min at 22,000 rpm through a 10% (wt/vol) sucrose cushion in the same buffer. The chromatin salt extracts were concentrated by ammonium sulfate precipitation (0.35 g/ml) dissolved in and exhaustively dialyzed against protein injection buffer, which also served as storage buffer (5% glycerol/20 mM Tris·HCl, pH 7.0/100 mM NaCl/5 mM $MgCl_2$/1 mM EDTA/0.1 mM EGTA/0.1 mM $PhMeSO_2F$/0.5 mM dithiothreitol). The samples were stored at −80°C.

***Xenopus laevis* Oocyte Injection and RNA Analysis.** The protein fractions (25 nl) were injected into the cytoplasm of the oocyte prior to injection into the nucleus of 1-ng vector-free 6-kb circles of h22 (for preparation of DNA circles see ref. 11) together with 0.2 μCi (1 Ci = 3.7×10^{10} becquerels) of [α-^{32}P]GTP (Amersham) as described (9, 11). The extracted total RNA was used for electrophoresis (12) or purified on CsCl step gradients for nuclease S1 mapping.

Nuclease S1 Mapping. For the nuclease S1 mapping of mRNA 5′ and 3′ termini (13, 14), the multirestriction digests of h22 (7) were labeled at the 5′ or 3′ end. For nuclease S1 mapping of the 3′ terminus of H3 mRNA, the *Hpa* II fragment of h22 DNA containing the 3′ end of the H3 histone gene was purified and 3′ end-labeled with Klenow DNA polymerase. A 3′ end-labeled *Bst*EII–*Hpa* II fragment derived from a deletion mutant h22ΔE (4) that lacks a 335-base pair fragment of the H3-H2A spacer was used to demonstrate readthrough of the RNA polymerase II through the H3 gene terminus.

Columns and Density Gradient. DEAE-Sephacel and heparin-Sepharose CL-6B (Pharmacia) were prewashed and equilibrated as described (15). The density gradient of 5–20% sucrose was made up in 500 mM NaCl/50 mM Tris·HCl, pH 7.9/5 mM $MgCl_2$/1 mM EDTA/0.5 mM dithiothreitol/0.1 mM $PhMeSO_2F$ and run for 40 hr at 36,000 rpm in a Beckman SW 40.1 rotor at 4°C. The sucrose gradient was fractionated from the top, and the absorbance at 280 nm was monitored with an ISCO UA-4 monitor.

RESULTS

Strategy for Identifying a Regulatory Protein. Because it was considered that regulatory proteins most likely were associated with the chromatin of maximally transcribed histone genes, sea urchin embryos at the 32- to 128-cell stage were chosen as the starting material. Chromatin of *Psammechinus miliaris*, or its close relative *Paracentrotus lividus*, was prepared (10) and extracted with salt at increasing concentrations. The extracted proteins were concentrated by ammonium sulfate precipitation and dissolved in and exhaustively dialyzed against storage buffer, which also served as a solvent for the subsequent injection experiments.

In a typical microinjection experiment, 1 ng (or $\approx 1.5 \times 10^8$ copies) of circularized plasmid-free h22 DNA was injected into each oocyte nucleus, a typical assay comprising about 10–20 centrifuged oocytes. Our working hypothesis was that the sea urchin chromatin contained regulatory proteins in roughly equimolar amounts to the histone genes. Based on this, enough protein was injected to approach a (calculated) 1:1 molar equivalent of injected histone genes and putative histone gene-specific regulatory protein(s). In practice, this amounted to coinjection of 1 ng of h22 DNA together with the chromosomal wash fraction of about 10,000 embryos into each oocyte. h22 DNA and [^{32}P]GTP were injected into the oocyte nucleus, whereas the proteins were introduced into the cytoplasmic compartment. This procedure was chosen to obviate the action of DNases invariably contaminating our chromosomal wash fractions. Our experiments appear to have borne out our prediction that cellular components would naturally partition into the nuclear and cytoplasmic compartments (16–18) because specific biological effects could be observed using this procedure.

Complementation of H3 Gene-Transcription Termination by a Chromosomal Factor. Oocytes injected with h22 DNA and different chromosomal wash fractions were incubated for 16–24 hr, and the RNA was extracted and analyzed by gel electrophoresis. As a control, buffer was injected into the cytoplasm of oocytes prior to inserting h22 DNA into the oocyte nucleus (Fig. 1*a*, lane 1). Two strong bands comigrating with genuine sea urchin histone mRNAs H2A and H2B (compare with Fig. 1*a*, lane H) could be observed. H1 and H4 mRNAs could not be detected after such a short autoradiographic exposure. Typically, only a faint, if any, band was observed at the position of the H3 mRNA (lane 1). As can be seen from the nuclease S1 mapping experiment (see Fig. 2*b*), in injection controls there was extensive readthrough through the terminus of the H3 gene, and transcripts were seen to extend at least to the breakpoint generated by the spacer deletion.

However, when the 300 mM chromatin salt wash was injected into the cytoplasm of the oocyte, the H3 mRNA band was enhanced and was seen, at high protein input, to approach the intensities of the H2A and H2B mRNA bands (Fig. 1*a*, lane 3). Injection of the 150 mM chromatin salt wash (Fig. 1*a*, lane 2) yielded a pattern of transcription similar to the control run, whereas the 450 mM chromatin salt wash was inhibitory to all h22 transcription (lane 4). This inhibition was even more pronounced when the 600 mM or the 2 M chromatin washes were injected (results not shown).

The question arose whether the augmented H3 mRNA synthesis observed with the 300 mM chromatin wash was ascribable to an increase in initiation or termination of transcription. To

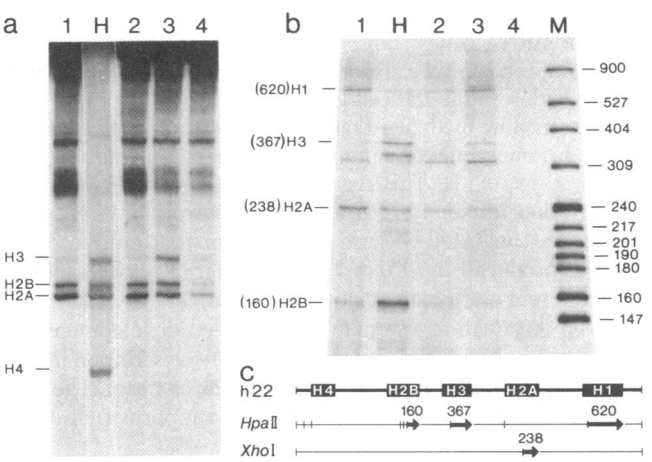

FIG. 1. (*a*) Autoradiogram of gel electrophoresis of total RNA extracted from injected oocytes. Aliquots (25 nl) of different chromatin salt washes were injected into the cytoplasm of the oocytes prior to injection of circularized h22 DNA (1 ng per oocyte) and [α-^{32}P]GTP into the nucleus of the *Xenopus* oocytes. The following solutions were injected: protein-injection buffer (lane 1), 150 mM chromatin salt wash (lane 2), 300 mM chromatin salt wash (lane 3), and 450 mM chromatin salt wash (lane 4). Lane H shows the ^{3}H-labeled histone mRNAs obtained from *P. miliaris* embryos. (*b*) Corresponding nuclease S1 mapping of the 3′ termini of the histone mRNAs with a multiprobe as described by Hentschel *et al.* (7). Lanes are as in *a*. Lane M shows molecular size in base pairs. (*c*) Schematic diagram of the h22 gene unit and protected DNA fragments. Sizes are indicated in base pairs.

resolve this, the number of 5′ ends of the histone mRNA transcripts, including the H3 mRNA, were quantified by the nuclease S1 protection method with the standard multiprobe (7). This revealed that the number of 5′ termini of the H3 mRNA had not increased relative to the H2A and H2B termini or to the H3 termini found in control oocytes (results not shown).

The 3′ termini of the histone mRNAs synthesized in the oocytes were also mapped by the nuclease S1 protection method with another multiprobe of the five histone genes. In the control experiment, authentic H1, H2A, and H2B 3′ termini were generated (Fig. 1*b*, lane 1). Authentic 3′ ends of the H3 mRNAs were recovered only in those oocytes that had been injected with 300 mM chromatin salt wash (Fig. 1*b*, lane 3).

These results demonstrate that a defined salt wash fraction of an actively transcribed sea urchin chromatin can elicit faithful generation of the H3 mRNA 3′ termini, whereas, in the absence of this fraction, transcription readthrough takes place (7).

Column Chromatography and Sucrose Gradient Sedimentation of the Chromosomal Termination Factor. To enrich the active component, we fractionated the protein extract by heparin-Sepharose chromatography (14, 15). The column was equilibrated with protein storage buffer (100 mM NaCl, final concentration), and the protein fraction was applied onto the column in the same buffer. The flow-through fraction (ca. 5–10% of the total amount of protein) was collected; the proteins retained by the heparin column were eluted with 500 mM KCl in the same buffer. Both fractions were concentrated by ammonium sulfate precipitation and subsequently dialyzed against injection buffer. The fractions from a constant number of embryos were injected into the oocyte in order to test their ability to generate authentic 3′ mRNA termini. $NaDodSO_4$/polyacrylamide gel electrophoresis of the extracted RNA and the corresponding nuclease S1 mapping of the 3′ ends are shown in Fig. 2 *a* and *b*, respectively. Injection of the heparin-bound fraction resulted in the generation of H3 mRNA 3′ ends (Fig. 2*a*, lane 2) as was the case for the 300 mM chromatin wash (lane 3). The flow-through fraction had no effect on the appearance of correct H3 termini (Fig. 2*a*, lane 1).

Next we fractionated the heparin-bound protein on a DEAE-Sephacel column. Proteins were applied onto the column in 100 mM KCl and were eluted stepwise from the column to yield a flow-through fraction and bound protein fractions that were eluted at 300, 600, and 1,500 mM KCl. The proteins were concentrated by ammonium sulfate precipitation as before, dialyzed, and tested for their ability to generate authentic 3′ ends to H3 mRNA. The active component was eluted from the column at 300 mM KCl (Fig. 3*a*). The 300 mM KCl eluate was then fractionated by sedimentation through a 5–20% sucrose density gradient containing 500 mM KCl (Fig. 3*b*). The proteins of the sucrose gradient pooled fractions 1–7 were precipitated with ammonium sulfate and tested for their biological activity. The appearance of the H3 mRNA 3′ termini, revealed by hybridization of the RNA to an H3-specific probe, was dependent on sucrose gradient pooled fractions 5 and 6 (Fig. 3*c*). When the pooled fractions 5 and 6 were coinjected with h22 DNA and the transcripts were mapped with the deletion mutant DNA probe described in Fig. 2, authentic 3′ termini could be detected together with readthrough products (lane 2). The final sucrose fractions 5 and 6 were only able to generate reduced amounts of correct 3′ termini as compared to the starting chromatin salt wash because of substantial losses of the active factor during purification.

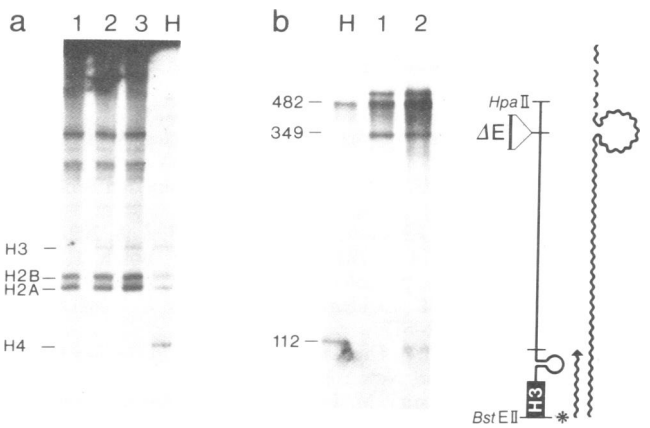

FIG. 2. (*a*) Autoradiogram of gel electrophoresis of total RNA extracted from injected oocytes. Aliquots (25 nl) of different protein fractions were injected into the cytoplasm prior to injection of circularized h22 DNA (1 ng per oocyte) and [α-^{32}P]GTP into the nucleus of the *Xenopus* oocytes. The following protein fractions were injected: heparin-Sepharose flow-through (lane 1), heparin-Sepharose-bound fraction eluted with 500 mM KCl (lane 2), and 300 mM chromatin salt wash (lane 3). Lane H shows the histone mRNAs from *P. miliaris*. (*b*) Nuclease S1 mapping of the 3′ termini of the H3 mRNA of sea urchin embryos (lane H) and of injected oocytes in the absence (lane 1) or presence (lane 2) of the regulatory factor. A 3′ ^{32}P-labeled *Bst*EII–*Hpa* II probe of the H3–H2A spacer deletion mutant (h22ΔE, ref. 4) was used to map the transcripts. Sizes are shown in base pairs. (*Right*) Details of the anticipated RNA·DNA hybrid molecules.

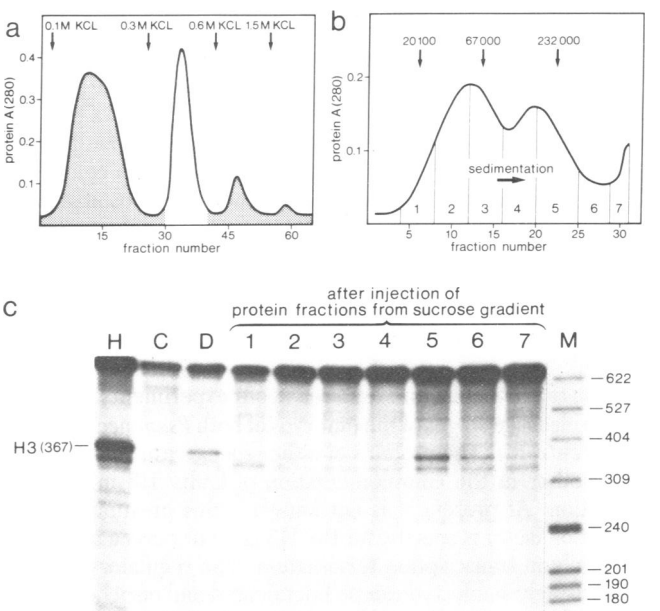

FIG. 3. (*a*) DEAE-Sephacel chromatography. The 500 mM eluate from the heparin-Sepharose column was loaded onto a 3-ml DEAE-Sephacel column as described. The bound proteins were eluted stepwise as indicated. The absorbance at 280 nm was monitored, and fractions of 0.5 ml were collected. The fraction from the clear area contained the termination activity, whereas the fraction from the shaded areas had no effect on the transcription termination. (*b*) Sucrose density gradient. The fractions 30–40 from the DEAE-Sephacel column were loaded onto a 5–20% sucrose gradient as described. After centrifugation, the gradient was fractionated and the absorbance at 280 nm was monitored. Fractions were pooled as indicated. Arrows and numbers at the top of the figure show the positions of marker proteins (trypsin inhibitor, bovine serum albumin, and catalase) run in a parallel gradient. (*c*) Nuclease S1 mapping of histone H3 3′ termini against an H3 gene single probe obtained from sea urchin histone mRNAs (lane H), RNA from control oocytes coinjected with h22 DNA alone (lane C), oocytes coinjected with proteins of the 0.3 M KCl eluate and h22 (lane D), and oocytes coinjected with h22 DNA and the proteins of sucrose gradient fractions 1–7 of Fig. 3*b* (lanes 1–7, respectively). Size markers are shown in base pairs (lane M).

From the position of the protein on the sucrose gradient and by comparison with the molecular weight standards, a sedimentation value of about 12 S and a molecular weight of 2–2.5 $\times 10^6$ could be estimated by assuming the protein to be globular in shape. The biological activity was sensitive to treatment with trypsin. Starting with 1.2×10^8 (125 ml packed) embryos containing an estimated 5 g of proteins (19), purification yielded 300 μg of proteins in the final preparation after sucrose gradient fractionation. The preparation remained active for months when stored at −80°C.

DISCUSSION

The developmentally controlled highly repeated histone genes of the sea urchin provide a unique opportunity to analyze the enzymology of transcriptional control because, for these genes, the titer of regulatory proteins can be anticipated to be correspondingly high and to be detectable by means of a sensitive bioassay system. Here we report the detection, isolation, and preliminary characterization of a factor apparently necessary for the generation of correct 3′ termini of sea urchin H3 histone mRNAs.

It was observed that authentic 3′ ends to H3 mRNA were found in high yield only when the proteins extracted from at least 10,000 sea urchin embryos at the 32- to 128-cell stage [each cell comprising 600–1,000 histone gene repeats (20, 21)] were injected into each oocyte. Losses of the active component during isolation of the chromatin, incomplete partitioning of the proteins in the oocyte, and the presence of antagonists in the final preparation notwithstanding, the chromosomal proteins of about 5×10^8 actively transcribed histone genes of the embryos were apparently just sufficient to correct the faulty transcription of 1.5×10^8 sea urchin histone H3 genes coinjected into the frog oocyte. The concentration of chromosomal wash proteins in the 25 nl used for each oocyte injection is equivalent to that of the proteins of 10^{10} cells dissolved in 1 ml. A simple calculation shows that to carry out a comparable oocyte experiment challenging unique genes, regulatory proteins would need to be concentrated to about 10^{12} to 10^{13} cell equivalents per ml.

The regulatory factor identified in our experiments is present at the cleavage stages of the embryos of both *Psammechinus* and *Paracentrotus*. The proteins of either species can substitute for each other in the complementation of faulty H3 mRNA termination. At present it is not known if this presumptive termination factor is specific for the H3 gene or plays a more general role in transcription termination. The regulatory factor is surprisingly stable and can be fractionated and enriched by column chromatography, followed by sucrose density gradient centrifugation. The latter suggests a sedimentation constant of about 12 S, equivalent to a molecular weight of approximately $2–2.5 \times 10^5$. However, it is not clear whether this protein sediments in a multimeric form or is associated with other proteins. The regulatory factor binds to heparin columns and, hence, has the properties anticipated of an RNA- or DNA-binding protein. The active component is unlikely to represent RNA polymerase II because of its molecular weight. Furthermore the polymerase II elutes from DEAE columns at higher salt concentrations (400–500 mM KCl; ref. 22), whereas the active factor was recovered in the 300 mM KCl wash.

Prokaryotic models show that regulator proteins often interact with DNA sequences showing dyad symmetries (23). The termini of all histone genes sequenced to date, with the sole exception of those of yeast, show a highly conserved hyphenated DNA repeat (reviewed in ref. 1) not unlike that seen in ρ-dependent bacterial genes (24, 25). When subjected to a mutational analysis, histone gene terminator signals show striking similarities to those of their prokaryotic counterparts (4). It is conceivable that the regulatory factor detected in our experiments interacts with such palindromic and less conserved sequences, either at the level of DNA or of the RNA transcript. Clarification of its exact mode of action awaits its complete purification and utilization of *in vitro* reconstituted transcriptional systems.

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