Vol. 216, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 2, 1995 Pages 273-278

RNA POLYMERASE II TRANSCRIBES *Dictyostelium* UNTRANSLATABLE GENE, *dutA*, SPECIFICALLY IN THE DEVELOPMENTAL PHASE

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Received September 21, 1995

SUMMARY: In *Dictyostelium discoideum*, a novel type of RNA (*dutA* RNA), which is untranslatable, cytosolic and 1.3 kb in size, appears specifically after the aggregation stage [Yoshida, H. *et al.* (1994) Nucleic Acids Res. **22**, 41-46]. We here show that the *dutA* gene is transcribed by RNA polymerase II, based on its α-amanitin sensitive nature of in vitro transcriptional activity. We also show that the stage-specific accumulation of *dutA* RNA is primarily due to the stage-specific enhancement of the transcriptional activity of the gene.

Dictyostelium discoideum is one of the best suited organisms for studying the molecular mechanisms of cell differentiation because of its simple differentiation pattern. During the course of study of gene regulation in this microorganism, we found that an untranslatable RNA appears and accumulates during the aggregation stage and named its corresponding gene *dutA* (1, 2).

The *dutA* gene is present as a single copy in the nucleus without introns. *dutA* RNA (1322 nt) is extremely rich in A and U (83%), has no sustained ORF, and is not in association with ribosomes although it is localized in cytoplasm (2), strongly indicating that it functions without being translated into protein; namely it is untranslatable. Although the function of *dutA* RNA still remains unknown

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0006-291X/95 $12.00

since disruption (2) or overexpression (3) of the gene did not result in any detectable phenotypic changes, its stage-specific appearance during development implicates some important role.

Accumulating evidence indicates that there exist a variety of RNA in the cell besides conventional RNA, such as rRNA, tRNA and mRNA, and that some (4, 5, 6, 7, 8) of them play important roles in biological processes. Different species of RNA polymerase have been shown to be involved in the synthesis of these new types of RNA: H19 is transcribed by RNA polymerase II (9), while snRNAs are transcribed by RNA polymerase II or III (10, 11). In the present study, we first determined which species of RNA polymerase transcribes *dutA* gene. Furthermore, we also examined whether the amount of *dutA* RNA is regulated by stage-specific changes in the transcriptional activity, by run-on analysis using the nuclei prepared at different developmental stages.

MATERIALS AND METHODS

Growth and developmental conditions. The strain NC-4 was used throughout. Cells were grown in association with *Escherichia coli B/r*, harvested at the density of 3-5 X 10^6 cells/ml, washed free of bacteria and allowed to develop on nitrocellulose filters at a density of 4 X 10^6 cells/cm^2. The filters were placed on 2% (w/v) agar containing Lower Pad Solution (12). At various time points, cells were collected and nuclei and total RNA were isolated therefrom.

Northern analysis. Total RNA was extracted by phenol-chloroform containing 0.5% (w/v) SDS, size-fractionated by electrophoresis on a 1.2% (w/v) denaturing formaldehyde gel, transferred to nylon filters and hybridized with a random-primed ^{32}P-labeled *dutA* sequence as described in Maniatis *et al.* (13).

Isolation of nuclei. Nuclei were isolated as described previously (14) with some modification. About 1 X 10^8 cells were harvested at various time points of development from nitrocellulose filters, and resuspended in 950 µl of lysis buffer containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 10% (w/v) sucrose, 1 mM spermidine, 1 mM DTT, 20 mM KCl and 0.2 mM PMSF, mixed with 50 µl of 20% (v/v) NP-40, vortexed and kept on ice for a few minutes. After centrifugation at 3,000 X g for 5 minutes, pellet was suspended in 100 µl of storage buffer containing 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM spermidine, 1 mM EDTA, 1 mM DTT and 50% (v/v) glycerol. After being dispensed in small aliquots, nuclei were quickly frozen and stored into liquid nitrogen until use.

Nuclear run-on assay. Nuclear run-on transcription was carried out as described previously (15) with some modification. The reaction was started by adding 20 ml of nuclei solution to the reaction mixture (80 µl) which contained 50 mM Tris-HCl (pH 7.9), 12.5 mM $MgCl_2$, 6.25% (v/v) glycerol, 313 mM NaCl, 125 mM DTT, 131 units RNase inhibitor, 0.25 mM ATP, CTP, GTP mix, 3.7 MBq of [a-^{32}P]UTP (>111 TBq/mmol). After incubation at 25 °C for 30 minutes, RNA was prepared by phenol-chloroform extraction, followed by Sephadex G-50 gel filtration.

Plasmids containing *dutA* (2), actin6 (15), tDNA (for $tRNA^{Val}$: gift from Dr. T. Dingermann) and rDNA (contains parts of the DNA for 17S and 26S and complete 5.8S RNA, gift from H. Tabuchi of this laboratory) sequences were digested with appropriate restriction enzymes, subjected to gel electrophoresis and blotted onto Hybond-N+ filter (Amersham). The filters were then used for hybridization with run-on transcripts. Hybridization was performed in 6 x SSC containing 50% (v/v) formamide at 42 °C overnight. The amounts of DNA in nuclei preparation were estimated by Bisbenzimide H 33258 Fluorochrome (CALBIOCHEM) (16), and used for normalization of transcriptional activity.

RESULTS AND DISCUSSION

The *dutA* gene is transcribed by RNA polymerase II

Nuclei prepared from 12-h developed cells were tested for sensitivity of transcriptional activity to α-amanitin. As shown in Fig. 1, the transcription of

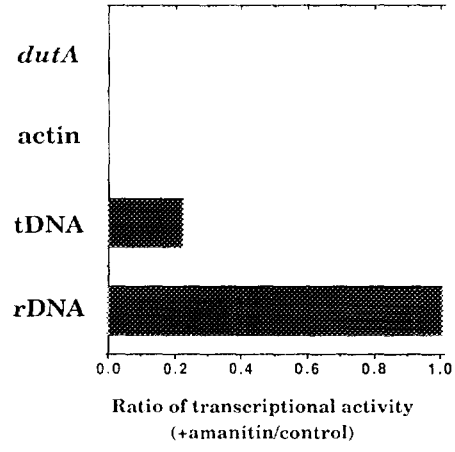

Fig.1. Sensitivity to α-amanitin of different RNA polymerases in *D. discoideum*. Nuclear run-on analysis was performed with and without 33 µg/ml α-amanitin using nuclei isolated from 12-h starved cells. ^{32}P-labeled RNA obtained by run-on transcription was hybridized with various DNA which had been fixed on nylon filters. Filters were washed with 0.1% SDS in 2 x SSC at 50 °C. Radioactivity was measured by a FUJIX imaging analyzer BA100. The ratio of the activity with α-amanitin to that without α-amanitin was displayed. The actual transcriptional activities for actin, *dutA*, tDNA and rDNA without α-amanitin were 1.8, 3.3, 2.2 and 29 arbitrary units, respectively.

the *dutA* gene, as well as that of the actin gene, used as a control of RNA polymerase II reaction, were completely inhibited by α-amanitin, while the transcription of the rRNA gene, executed by RNA polymerase I, was perfectly insensitive to the drug. The sensitivity to the drug (33 μg/ml) of these RNA polymerases in *D. discoideum* is consistent with a previous report (17). The synthesis of tRNA, known as polymerase III reaction, was partially-inhibited at this concentration of the drug. These results clearly indicate that the *dutA* gene is transcribed by RNA polymerase II.

As described earlier, it has been known that some of the untranslatable RNAs are transcribed by RNA polymerase II (such as H19 and a certain species of snRNA), while some are by RNA polymerase III. *dutA* are now proven to be grouped to the former. Among those, *dutA* might belong to the same category with H19, since they are both localized in cytoplasm but not in association with ribosomes. However, the existence of any functional similarity between these RNAs can not be discussed at present since the function of *dutA* RNA has not been clarified.

The *dutA* gene is regulated at the transcriptional level

dutA RNA is not detectable in the vegetative stage, but after starvation it begins to accumulate in the early aggregation stage, reaches a maximum level at the completion of aggregation and decreases a little thereafter (1). Fig. 2A shows that the changes in accumulation of *dutA* RNA in the same set of experiment carried out for run-on analysis described below.

To examine whether the regulation of *dutA* is due to the changes in transcripional activity or some post-transcriptional events, nuclei were prepared from cells at different developmental stages and subjected to run-on analysis. As Fig. 2B shows, in the vegetative stage, nuclei had essentially no transcriptional

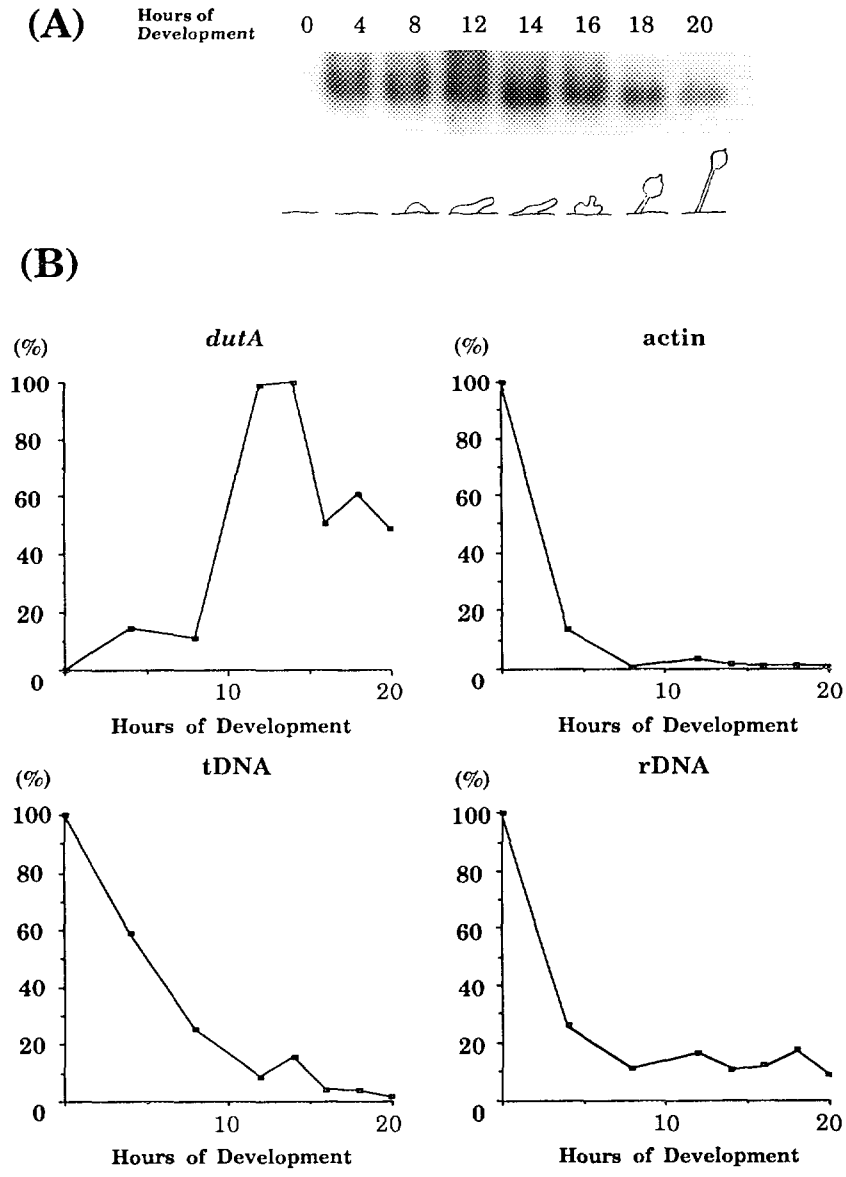

Fig.2. Changes in A) accumulation of *dutA* transcript (Northern analysis), and B) transcriptional activity (run-on analysis), during development. Nuclei isolated from cells at various time points of development were used for run-on analysis. After hybridization with various DNA which had been fixed on nylon filters, filters were washed with 0.1% SDS in 1 x SSC at 50 °C. Radioactivity was measured by a FUJIX imaging analyzer BAS 1500 and normalized by the DNA amount in nuclei used. Relative activity of transcription, taking the maximum value as 100, with respect to each DNA was displayed.

activity for the *dutA* gene, but the activity increased gradually after starvation and reached a peak level at 12 h. After 12 h of development, there was a decline in activity but even at 20 h there still remained considerably high

activity. These results indicate that the amount of *dutA* RNA is primarily regulated by its transcriptional activity rather than by post-transcriptional process. In contrast to *dutA* RNA, the transcriptional activity for actin, rRNA and tRNA genes, rapidly decreased after starvation. The decrease in transcriptional activity for the actin gene during development is in consistent with a previous work (18). The present work is probably the first to show that similar rapid decrease also occurs with the transcription of the tRNA and rRNA gene during development of *D. discoideum*.

As reported previously (1), *dutA* gene appears to be regulated in a way distinct from other developmentally regulated genes in *D. discoideum*. Elucidation of the mechanism for its regulation is now in progress in our laboratory.

REFERENCES

1. Yoshida H., Yamada, Y., and Okamoto, K. (1991) Differentiation 46, 161-166.
2. Yoshida, H., Kumimoto, H., and Okamoto, K. (1994) Nucleic Acids Res. 22, 41-46.
3. Yoshida H. (1994) Thesis (Kyoto University).
4. Berget, S. M., and Robberson, B. L. (1986) Cell 29, 691-696.
5. Borsani, G., Tonlorenzi, R., Simmler, M. C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., and Lawrence, C. (1991) Nature 351, 325-329.
6. Hao, Y., Crenshaw, T., Moulton, T., Newcomb, E., and Tycko, B. (1993) Nature 365, 764-767.
7. Watanabe, Y., and Yamamoto, M. (1994) Cell 78, 487-498.
8. Baer, M. F., Reilly, R. M., McCorkle, G. M., Hai, T. Y., Altman, S., and RajBhandary, U. L. (1988) J. Biol. Chem. 263, 2344-2351.
9. Brannan, C. I., Dees, E. C., Ingram, R. S., and Tilghman, S. M. (1990) Mol. Cell. Biol. 10, 28-36.
10. Carbon, P., Murgo, S., Ebel, J. P., Krol, A., Tebb, G., and Mattaj, I. W. (1987) Cell 51, 71-79.
11. Mattaj, I. W., Dathan, N. A., Parry, H. D., Carbon. P., and Krol, A. (1988) Cell 55, 435-442.
12. Newell, P. C., Longlands, M., and Sussman, M. (1971) J. Mol. Biol. 58, 541-554.
13. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Jacobson, A., Firtel, R. A., and Lodish, H. F. (1974) J. Mol. Biol. 82, 213-230.
15. McKeown, M., and Firtel, R. A. (1981) Cell 24, 799-807.
16. Cesarone, C. F., Bolognesi, C., and Santi, L. (1979) Anal. Biochem. 100, 188-197.
17. Yagura, T., Yanagisawa, M., and Iwabuchi, M. (1976) Biochem. Biophys. Res. Comm. 68, 183-189.
18. DeSilver, D. A., Benedict, M. A., and Ratner, D. I. (1991) Biochim. Biophys. Acta. 1089, 309-319.