学位申請論文

吉田秀郎

Molecular analysis of a new type of development-specific gene (dutA) of Dictyostelium discoideum.

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[ABSTRACT]

I found a new, developmentally-regulated gene of Dictyostelium discoideum, dutA, and analyzed its expression in development.

(1) Its expression is specifically induced at the early aggregation stage of development.

(2) Cellular interactions are essential for its induction.

(3) cAMP, a well-studied intercellular signaling substance in D. discoideum, is not involved in the induction.

Analysis of the structure of dutA RNA (1322nt) revealed its unique features as follows:

(4) it is encoded by a single nuclear gene

(5) it has no sustained ORFs (MAX=126nt)

(6) it is extremely AU-rich (83%)

(7) it contains peculiar sequence motifs (large palindromes, long AU-stretches and GC-clusters)

(8) it is localized in the cytoplasm but completely absent from ribosomes.

These features suggest that dutA RNA functions without being translated into protein.

Antisense mutagenesis and disruption of the dutA gene did not cause phenotypic changes, suggesting that the function of this gene is redundant. Overexpression of dutA RNA also caused no morphogenetic abnormalities. However, dutA appears to play an important role in the cell functions since its expression is strictly regulated in development and cognate sequences are widespread among organisms, from bacteria and yeasts to mammals and plants.

[INTRODUCTION]

Cells in the multicellular organism communicate with each other to integrate themselves into an individual. Such intercellular communication systems are quite diverse but indispensable, for example, growth factors (EGF, PDGF, ect.), hormones (PTH, thyroid hormone, etc.), neurotransmitters (acetylcholine, GABA, etc.), morphogens (activin, retinoids, etc.) and differentiation promoting substances (GM-CSF, erythropoietin, ect.).

Cellular slime mold Dictyostelium discoideum grows as a unicellular amoebae and performs its developmental process in a multicellular fashion (Fig. 1)(Loomis, 1982). It is naturally assumed that the ancestor of the cellular slime mold lived as a unicellular organism throughout its life cycle and individually formed a spore (S. Ishida, personal communication). In this view, in the course of evolution, it acquired the ability to form a multicellular aggregate and to differentiate another cell type (stalk) for their efficient survival. At the same time it also acquired the systems for intercellular signaling which regulate these processes. Since D. discoideum is one of the most primitive multicellular organisms giving rise to only two cell types (the spore and the stalk cell), the intercellular signaling systems in this organism may be prototypical and quite simple.

In D. discoideum, several substances acting in intercellular signaling systems have been identified. Among these, the effects of cAMP and DIF have been most extensively studied. cAMP works as a chemoattractant for developing cells (Janssens and Van Haastert, 1987) and induces the expression of many genes (Kimmel, 1988). DIF promotes cell type differentiation at later stages (Kay and Jermyn, 1983). Although the precise roles of these factors are still controversial, the developmental process of D. discoideum is regulated by these signaling substances.

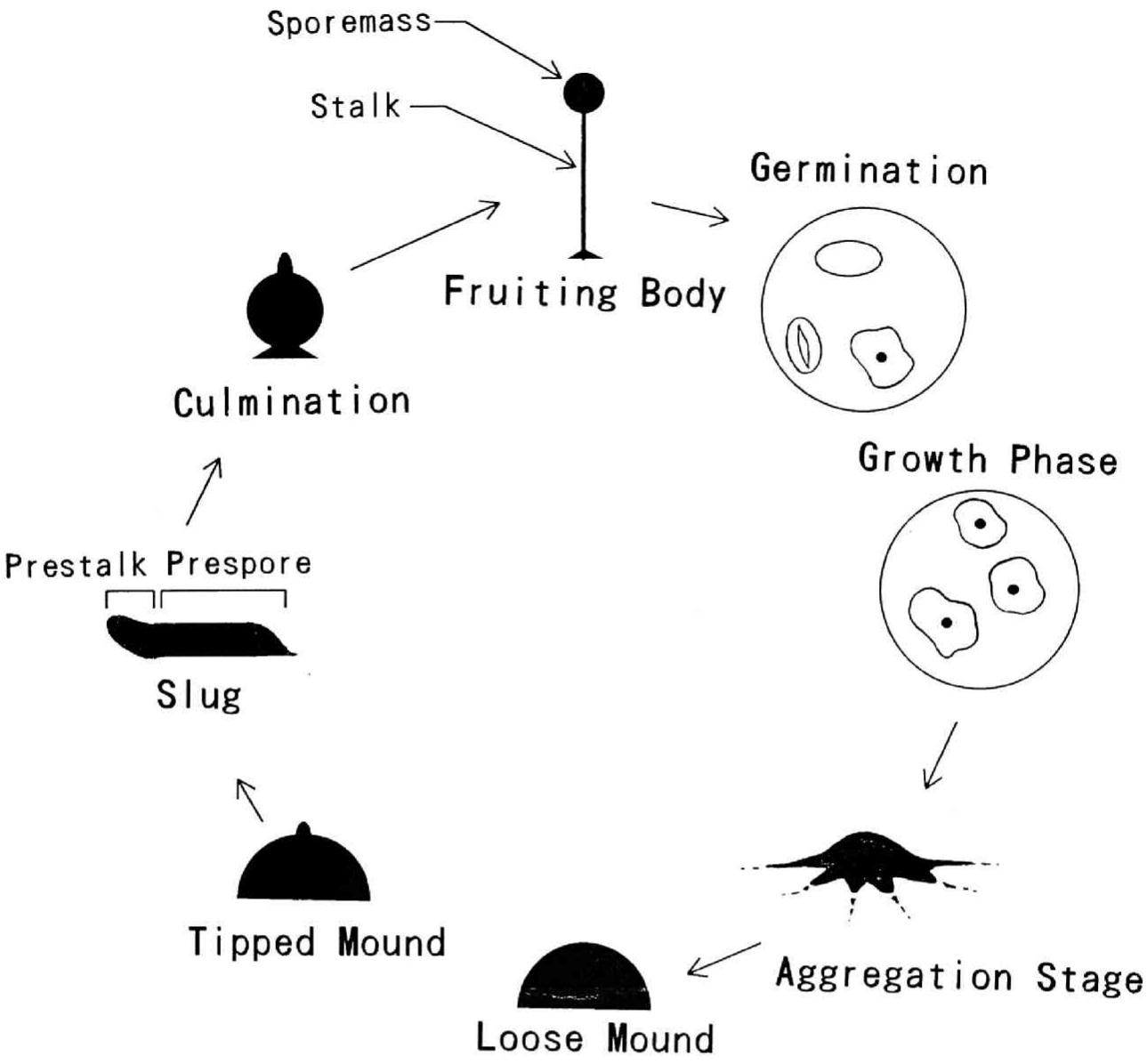


Fig.1. Schematic Representation of the Life Cycle of D. discoideum.
Spores germinate in the moist environment and grow as single amoebae. Upon starvation, they initiate multicellular development. In the early stages of development, cells gather to form a hemispherical aggregate emitting pulsatile cAMP as a chemoattractant. In the later stages of development, the aggregate transforms into an elongated shaped slug and by this stage cells have differentiated into two cell types (prespore and prestalk). A slug migrates on a substratum and finally forms a fruiting body consisting of spore mass and stalk.

At the start of my work, the molecular biological studies on intercellular signaling were just beginning in many organisms. We chose D. discoideum as a model organism for the analysis because of its simple life style (primitive multicellularity and a simple developmental system) and compactness of its genome ($5x10^7$bp in the haploid strain). We focused our study on the early stage of development (when two cell types are still undifferentiated) to identify the key factor for the differentiation. At this stage, amoebae emit pulsatile cAMP as a chemoattractant and assemble to form a hemispherical aggregate. We planned to isolate genes which are induced during this period and analyze how intercellular signaling regulates their expression.

Unexpectedly, the gene I cloned has peculiar sequences (high AU-content and long palindromes) with no sustained ORFs and shows complete absence from ribosomes, suggesting that it functions without being translated into protein. Thus I named this gene dutA (development-specific but untranslatable RNA).

There is a class of RNAs which exhibit their functions without being translated into proteins, such as rRNA, tRNA, RNAs in ribozymes (Guerrier-Takada, 1983; Cech, 1986) and spliceosomes (Brody and Abelson, 1985; Cech, 1990). In this thesis, I refer to these RNAs as structural RNAs according to Brown et al. (1992). Recently, a variety of structural RNAs having different functions have been reported, for example, guide RNA (Blum et al., 1990), snoRNA (Leverette et al., 1992; Sollner-Webb, 1993), mouse H19 RNA (Pachnis et al., 1988; Brannan et al., 1990) and human XIST RNA (Brown et al., 1992; Brockdorff et al., 1992). The nature of dutA RNA clarified in this study fits into the category of structural RNA. In addition to characterizing dutA RNA, I carried out overexpression experiments, antisense mutagenesis and gene disruption of dutA to investigate its function in development. Furthermore, I examined whether cognate sequences are present in the genomes of other organisms.

[RESULTS]

I. Expression of dutA

(A) Identification of dutA

First we tried to identify genes which were specifically induced at the early stage of development. For this purpose, a cDNA library of t(11) cells (cells developed for 11 hours) was prepared and differentially hybridized with ^{32}P-labeled cDNA probes prepared from poly(A) RNA of t(11) cells and growing cells. We obtained several clones that gave positive signals with a t(11)-cell probe and negative signals with a growing-cell probe. A clone, referred to as dutA (previously called DC6), whose corresponding mRNA accumulated in the highest amount, was used for further analysis.

(B) Expression of dutA RNA in Development

We examined the time course of dutA RNA expression in development by Northern blot (Fig.2). dutA RNA was about 1.3kb long and completely absent in growing cells. When cells were allowed to develop on filter (Fig.2, left), dutA RNA was induced at t(3) and reached a maximum at t(12) during the early stage of development, and remained throughout the late stage (t(12)-t(21)). In suspension cultures (Fig.2, right), similar induction occurred except that the onset of induction was slightly delayed and the level of the mRNA declined after 18h. We sometimes observed extra high-molecular-weight bands, for example, in suspension cultures (Fig.5A) or in slug cells migrating for a prolonged period (Fig.7). It is possible that some stressful conditions caused such an effect (see the section (F)).

Nuclear run-on experiments revealed that the expression of dutA RNA during the development is regulated at the level of transcription (Kumimoto,

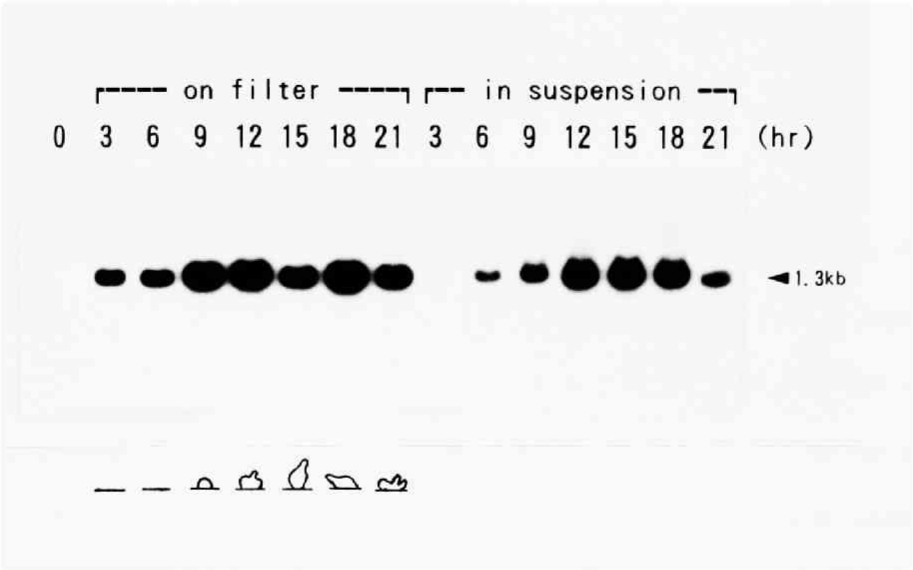


Fig.2. Time Course of dutA RNA Expression in Development.

NC4 cells were allowed to develop on filter pads or in suspension. RNA was extracted from cells at indicated times, separated, transferred and probed with dutA (C6A).

Yoshida and Okamoto, unpublished).

When cDNA library was re-screened with dutA cDNA, we usually obtained one positive clone per approx. 10000 library clones. Therefore, the abundance of dutA RNA is about 0.01% of poly (A)$^+$-RNA fraction.

(C) Intercellular Signals Induce dutA RNA Expression

Next we examined whether the expression of dutA RNA is regulated by intercellular signals (Fig.3). When aggregates of t(5.5) cells (which had accumulated a very small amount of dutA RNA) were dissociated and cultured in an isolated condition till t(11), only a small increase in the accumulation of the mRNA occurred (lane FM), in contrast to a rapid increase in undissociated cells (lane 11). This can be interpreted as meaning that cellular interaction is required for the accumulation of dutA RNA during the early stage.

On the other hand, if supplied with the medium conditioned with normally developed cells, dissociated and isolated cells were able to accumulate a considerable amount of dutA RNA (lane CM) as compared to control (without the conditioned medium; lane FM). Thus, factors secreted from developing cells mediate the inductive signal for dutA RNA expression, at least, in part. However, conditioned medium never recovered dutA expression to a normal level (lane 11). One of the explanations for this is that other signaling molecules that are not contained in the conditioned medium (for example, molecules associated to the cell surface) are also required for full induction. Another explanation is that if cells at a low density are unhealthy, then that could be the cause of low expression of dutA RNA.

(D) cAMP Signal Does Not Induce dutA RNA Expression

At the early stage of development, cAMP pulses secreted from cells work as a signal substance. cAMP binds to a specific receptor (belonging to a

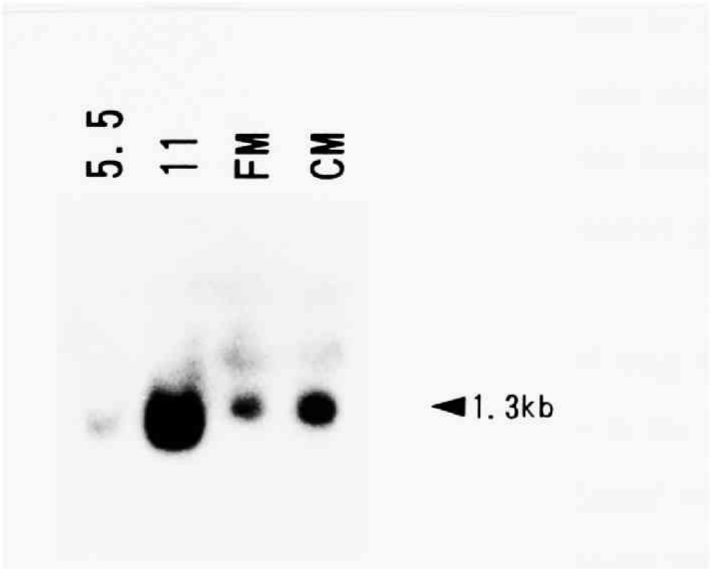


Fig.3. Effects of Cell Density and Conditioned Medium on dutA RNA Expression. NC4 cells were allowed to develop in suspension at high density ($1x10^7$cells/ml) for 5.5 hr (lane 5.5) or for 11 hr (lane 11). 5.5 hr developed cells were incubated for additional 5.5 hr at low density in the absence (lane FM) or presence (lane CM) of conditioned medium (see MATERIALS AND METHODS). RNA was extracted, separated, transferred and probed with dutA (C6A). The bands in lane FM and CM migrated slightly slower than those in lane 5.5 and 11 (as revealed by ethidium bromide staining), possibly due to the impurities (polysaccharides?) in the samples.

family of 7-transmembrane type receptors, such as β-adrenergic receptor and rhodopsin) located on the cell surface and the signal is transduced by a guanine nucleotide binding protein (G protein), causing diverse responses; for example, cAMP secretion (signal relay), chemotaxis and regulation of gene expression (Gerisch, 1987; Janssens and Van Haastert, 1987; Kimmel, 1988; Klein et al., 1988; Newell et al., 1988; Loomis, 1988; Firtel, 1989; Van Haastert et al., 1991).

To study whether cAMP pulses induce expression of dutA RNA, we examined its expression in mutant strains which are defective in the signal transduction pathways for cAMP pulses. The mutant strain Synag7 is defective in the protein (probably equivalent to the GTPase activating protein) that is required for G protein-mediated activation of adenylate cyclase, and consequently cannot secrete cAMP (Mann et al., 1988; Snaar-Jagalska and Van Haastert, 1988) In this strain, dutA RNA accumulates more abundantly than in its parental strain NC4 (Fig.4A). The mutant strain HC85 (Frigid A) has a deletion in the gene encoding the α 2 subunit of G protein and cannot activate adenylate cyclase or guanylate cyclase in response to cAMP pulses (Coukell et al., 1983; Kesbeke et al., 1988; Kumagai et al., 1989). In HC85, dutA RNA expression occurred somewhat precociously but to the same level as in its parental strain HC6 (Fig.4B). These results suggest that extracellular cAMP pulses and the related signaling pathway are not involved in the induction of dutA RNA expression.

This conclusion was confirmed by another set of experiments with reagents which block the production or the action of cAMP pulses. t(6) cells were treated with caffeine (known to inhibit cAMP-induced adenylate cyclase activation; Brenner and Thoms, 1984), adenosine (cAMP antagonist; Newell and Ross, 1982; Van Haastert, 1983; Theibert and Devreotes, 1984) and high concentration of cAMP (which downregulates cAMP receptors; Janssens and Van

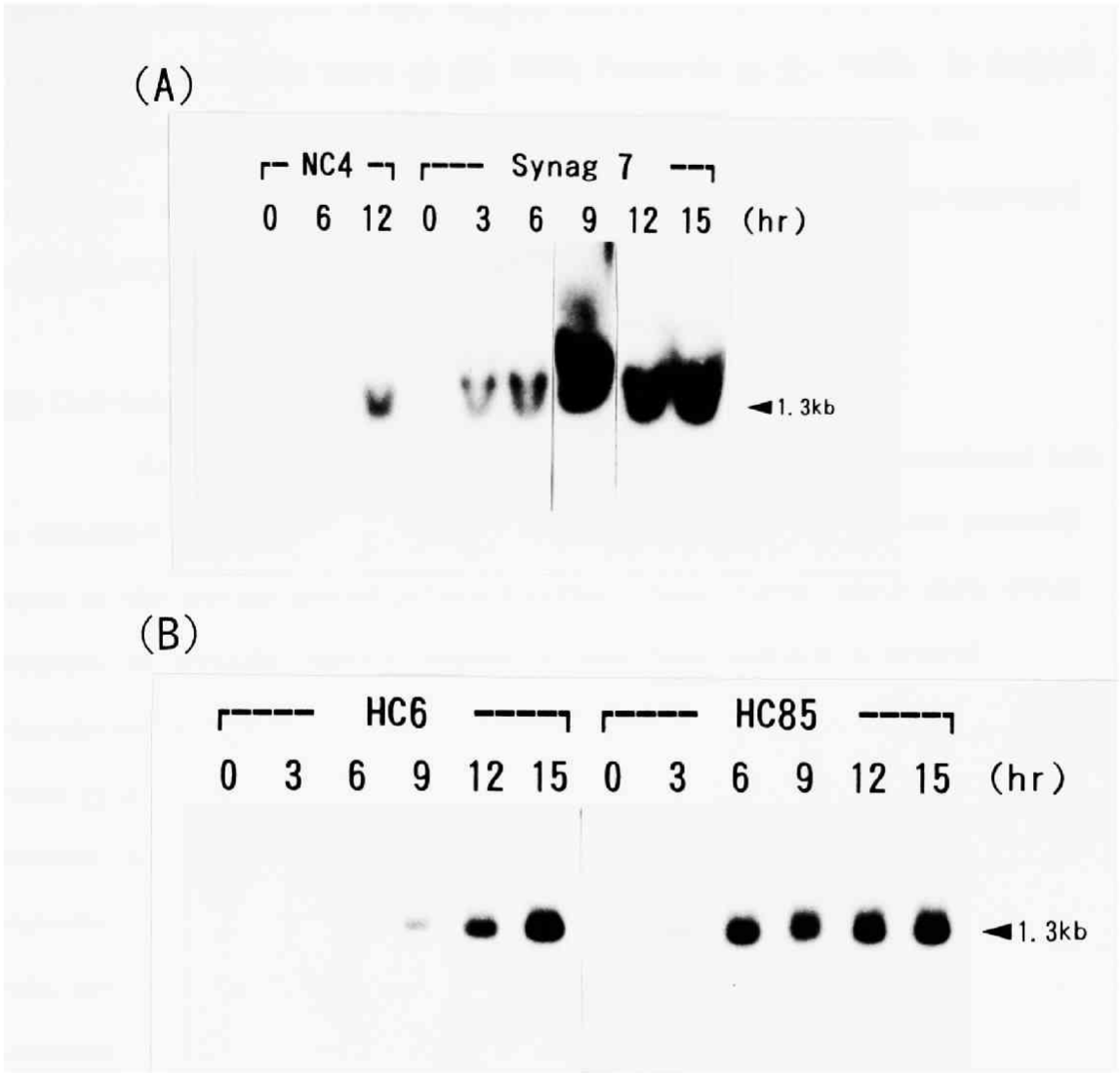


Fig.4. Regulation of dutA RNA Expression in the Mutants Defective in cAMP Relay Systems.

Cells of NC4, Synag7, HC6, and HC85 were allowed to develop in suspension. RNA was extracted at indicated times and analyzed by Northern hybridization with dutA (C6A).

Haastert, 1987), and dutA RNA expression was analyzed by Northern blot. To monitor the effect of the drugs, we also assayed the activity of cAMP phosphodiesterase (PDE; Barra et al., 1983; Podgorski et al., 1989). In drugged cells, the induction of PDE activity was considerably inhibited (Fig.5B), whereas the expression of dutA RNA occurred essentially as in drug-untreated cells (Fig.5A).

(E) Cell-type specificity of dutA RNA expression

At the late stage of development, the cell aggregate transforms into an elongated slug, in which at least two types of cells (prespore and prestalk) locate in the precise spatial pattern (Loomis, 1982). Genes which show either prespore- or prestalk- specific expression have been isolated in several laboratories (Barklis and Lodish, 1983; Mehdy et al., 1983; Jermyn et al., 1987; Ozaki et al., 1988). We separated prespore and prestalk cells by Percoll gradient centrifugation or by direct dissection with glass capillaries, and analyzed dutA RNA expression by Northern blot (Fig.6A and 6B). In prespore cells, only the 1.3kb transcript accumulated, whereas in prestalk cells, larger transcripts (1.6-2.5kb) were often (but not always) detected in addition to the 1.3kb transcript. We assume that suboptimal conditions of the slug (for example, the prolonged migration period) are associated with the accumulation of these transcripts (Fig.6C).

(F) Effect of Disaggregation of slugs on dutA RNA expression

It has been reported that the expression of many developmentally regulated genes is greatly affected by disaggregation of slugs (Barklis and Lodish, 1983; Mehdy and Firtel, 1983; Manrow and Jacobson, 1988; Mangiarotti et al., 1989; Takemoto et al., 1990). We therefore examined the effect of slug disaggregation on dutA expression. As shown in Fig.7, the 1.3kb transcript was

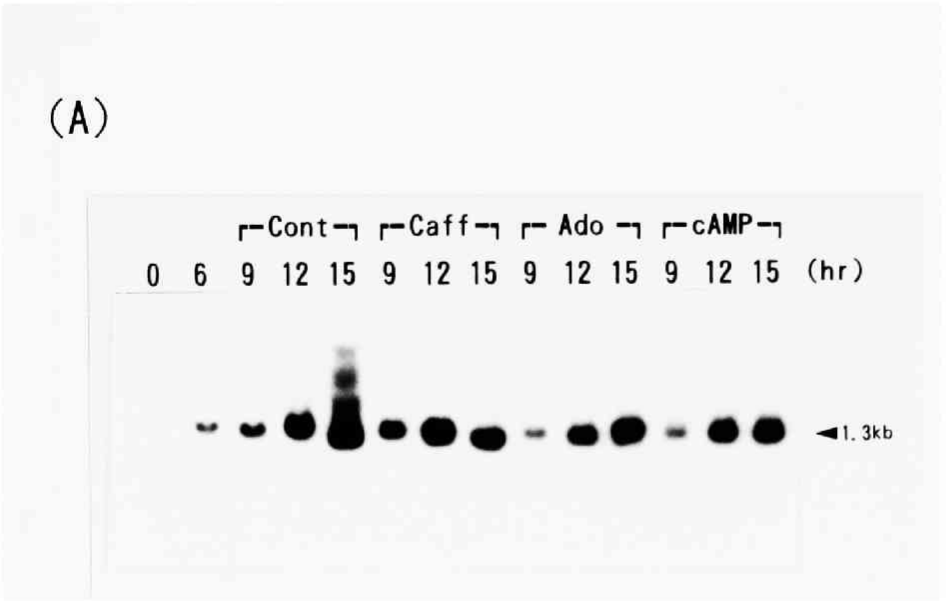


Fig.5. Independence of the dutA RNA Expression on cAMP.

(A) NC4 cells were allowed to develop for 6 hr in suspension, and were divided into four. They then received one of the treatments indicated below. Cells were harvested at 3 hr intervals and RNA was analyzed by Northern hybridization with dutA (C6A).

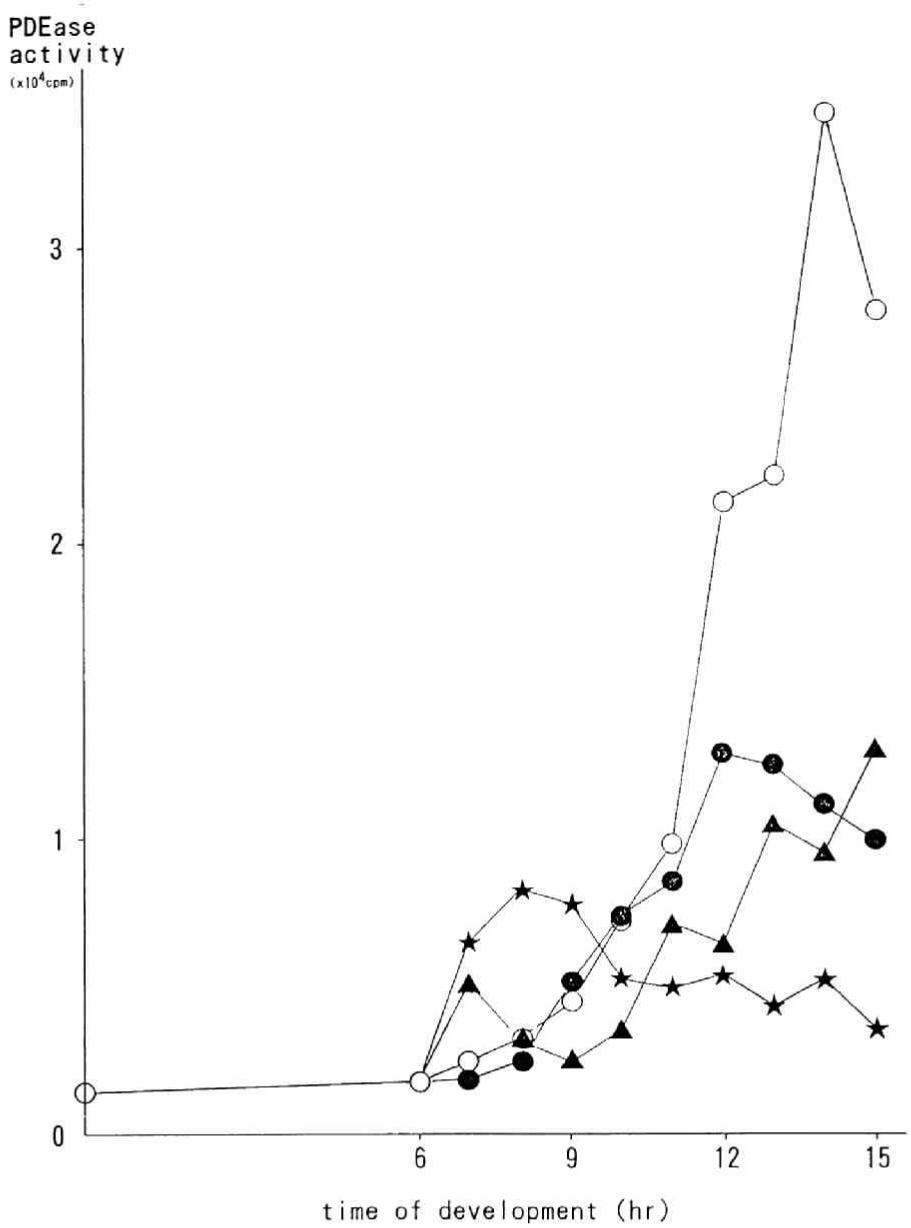
[Cont] no treatment

[Caff] caffeine (2mM initially, and then 1mM every 2 hr)

[Ado] adenosine (10mM)

[cAMP] a high concentration of cAMP (500μM initially, and then 100μM every hour)

(B)



(B) The cells in the same culture as in (A) were harvested at 1 hr intervals and assayed for phosphodiesterase (PDEase) activity.

○; no treatment

●; caffeine (2mM initially, and then 1mM every 2 hr)

▲; adenosine (10mM)

★; a high concentration of cAMP (500μM initially, and then 100μM every hour)

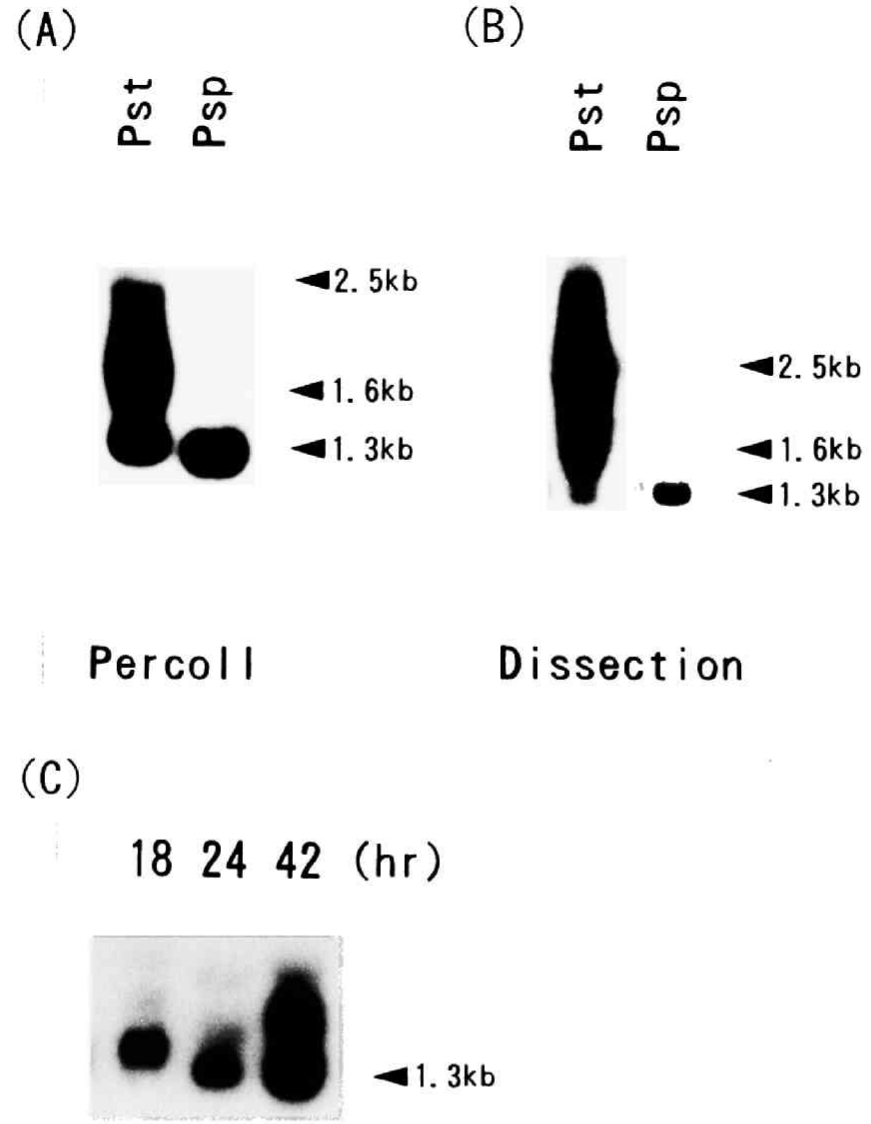


Fig.6. Cell Type Specificity of dutA RNA Expression.

Prespore (Psp) and prestalk (Pst) cells of strain NC4 were separated by density gradient centrifugation (A) or by dissection with a glass capillaries (B). RNA was extracted and analyzed by Northern hybridization with dutA (C6A).

(C) The effect of the length of the migration period on dutA transcripts. RNA was extracted from slugs which had migrated on filter for indicated times, and analyzed by Northern hybridization.

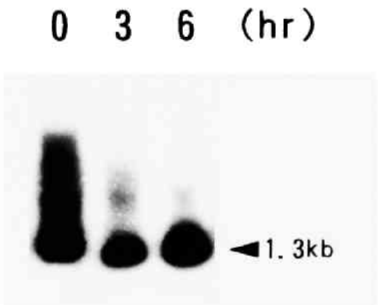


Fig.7. Effect of Disaggregation of Slugs on dutA RNA Expression.

Slugs (NC4) were disaggregated as described in MATERIALS AND METHODS. Disaggregated slugs (lane 0) were shaken for 3 hr (lane 3) or 6 hr (lane 6). RNA was extracted and analyzed by Northern hybridization with dutA (C6A).

not affected by disaggregation of slugs by pronase-BAL, but the larger transcripts disappeared after such treatment. We obtained essentially the same results when slugs were disaggregated by EDTA-NaCl (data not shown).

II. Structure of dutA

(A) Analysis of the dutA Gene

Despite of our great efforts to isolate full length cDNA for dutA RNA, standard methods for cDNA cloning did not work well. This is probably due to the peculiar sequences of dutA RNA as described later and we therefore first analyzed the dutA gene to facilitate cDNA cloning.

Genomic Southern analysis (Fig.8) shows that dutA is present as a single copy gene in the haploid genome. This conclusion was later confirmed by gene disruption experiments (see below). We also performed Southern Blot analysis at lower stringencies (blots were washed in 6xSSC, 4xSSC or 2xSSC at 42℃) but no related genes were detected (data not shown). Two genomic clones (EE2900 and ME900) were isolated and sequenced (Fig.9 and 17). Since restriction maps of these clones perfectly agreed with that derived from genomic Southern analysis, DNA rearrangement should not have occurred in the cloning process.

To determine the transcribed region in these DNAs, Northern blots of t(15) cells was probed with the fragments of genomic clones. Genomic subfragment ED211, DD386, AA379 and AX456 hybridized with dutA RNA (Fig.10A-D), whereas EE800 hybridized with transcripts of another gene, whose size and accumulation pattern are quite distinct from that of dutA RNA (Fig.10E). This indicates that 5'-end of the transcribed region does not extend into EE800. Since 3'-end of the transcribed region is delimited by C6A (see below), EE2900 and ME900 together must cover the entire region of the dutA gene.

To examine whether the dutA gene has introns, mung bean nuclease protection assay was performed. As shown in Fig.11, about 1.2-1.3kb protected fragment was observed, indicating that dutA RNA is encoded by one long exon. However, we cannot exclude the possibility of the presence of small exons at the

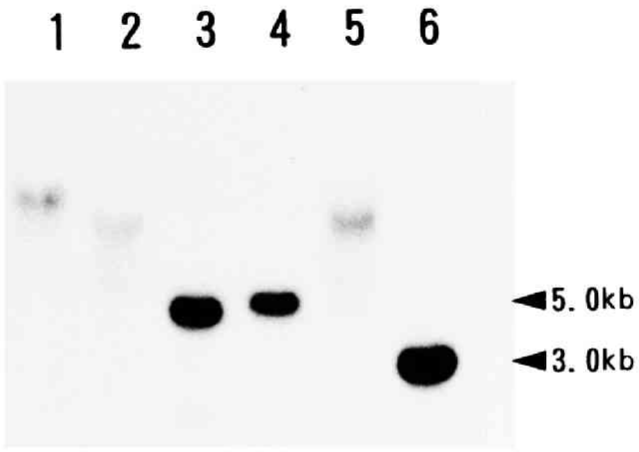


Fig.8. Southern Analysis of Nuclear DNA of the Strain Ax2.

Nuclear DNA was digested with restriction enzymes indicated below, separated on a 0.8% TBE-agarose gel, transferred to nylon membrane and hybridized with the ^{32}P-labeled C6A DNA. The numbers on the side of the blot indicate the length of DNA fragments in kb. The difference in the hybridization signal intensity could be due to the poor quality of our DNA preparation (enriched in low molecular weight fragments).

(lane 1) HindIII and KpnI (lane 2) BamHI and HindIII

(lane 3) XhoI and PstI (lane 4) BamHI and XhoI

(lane 5) BamHI and PstI (lane 6) EcoRI

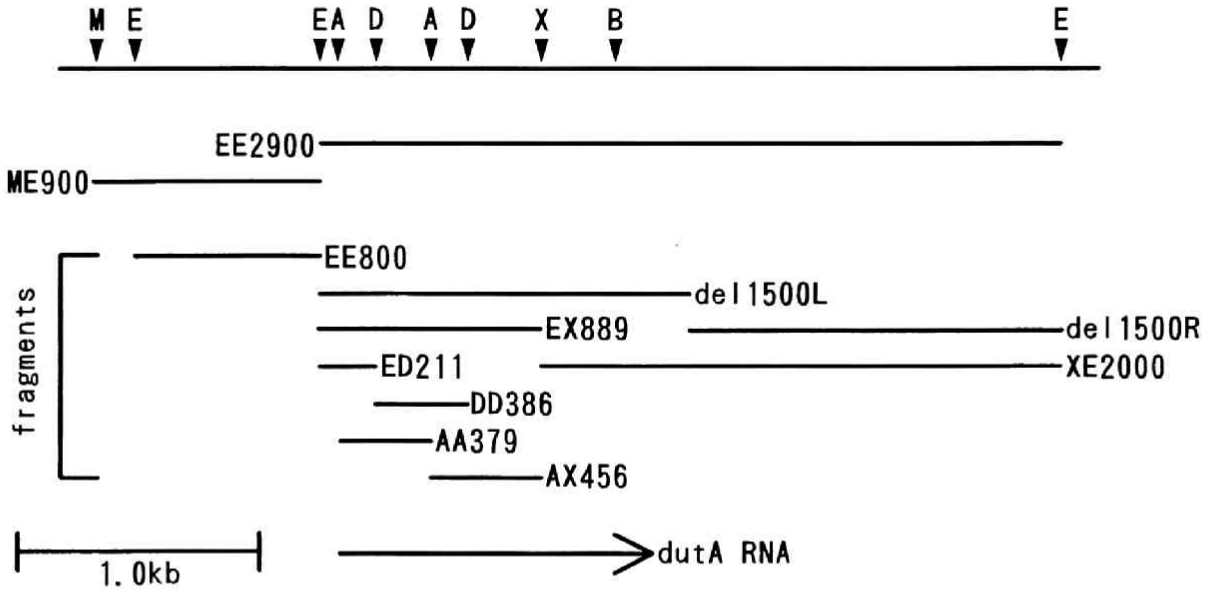


Fig.9. Restriction Map of the dutA Gene.

The locations of genomic clones and their fragments used in this study are also shown in lines. For preparation of EE2900 and ME900, see MATERIALS AND METHOD.

DNA fragments were subcloned after digestion of genomic clones with the restriction enzymes shown as prefix. Prefix del means a deletion clone digested by ExoIII nuclease from the downstream terminus (L) or the upstream terminus (R) of EE2900. The numbers in the designation indicate the length of each fragment. For instance, EX889 is an 889nt fragment which was subcloned from EE2900 digested with EcoRI (E) and XhoI (X).

[Abbreviations] A; AseI B; BglII D; DraI E; EcoRI M; MboI X; XhoI

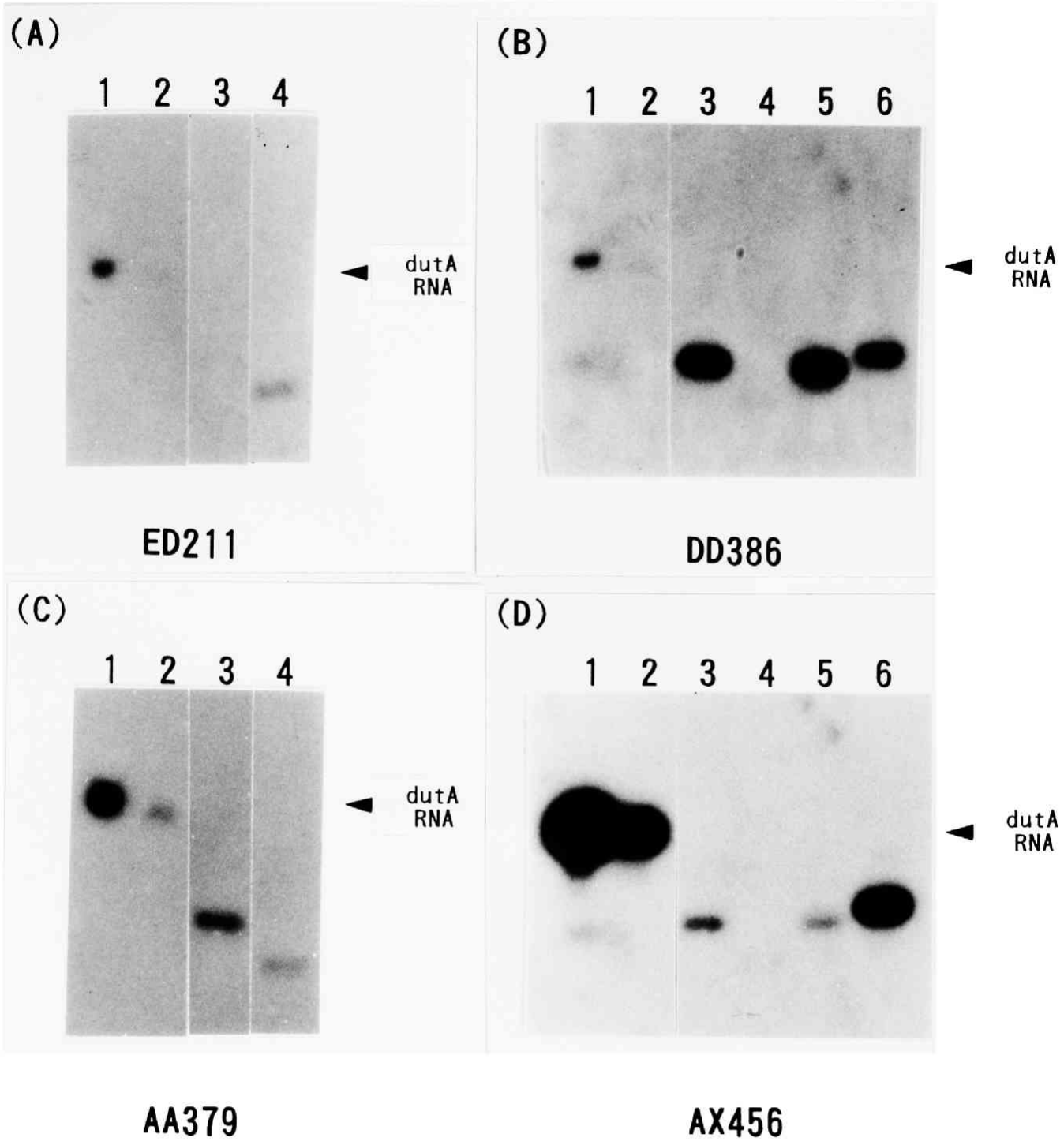


Fig.10. Northern Analysis with Genomic Subfragment

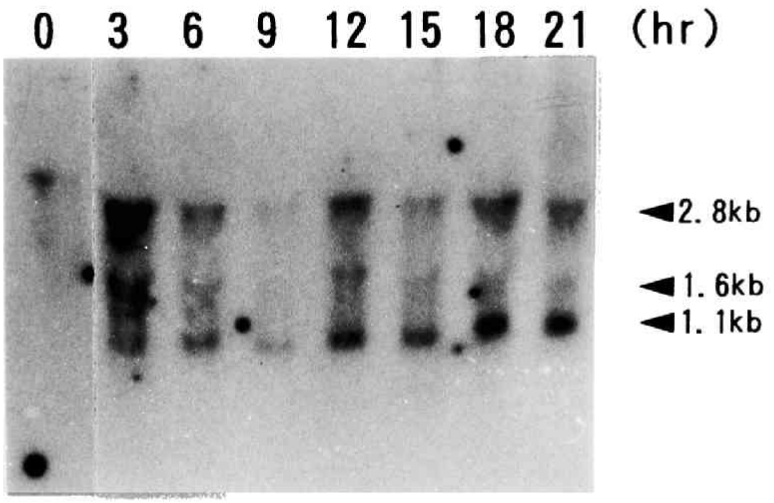
(A)-(D) RNA from t(15) cell was separated, transferred and probed with ED211, DD386, AA379 and AX456, respectively. Subfragment DNAs were also included in the blots to check the specificity of hybridization. Signal in lane 5 of (D) may reflect weak cross-hybridization.

(lane 1) 50μg of t(15) RNA (lane 2) 5μg of t(15) RNA

(lane 3) DD386 (lane 4) ED211

(lane 5) AA379 (lane 6) AX456

(E)



EE800

(E) The same blot as Fig.2 (RNA from cells developing in suspension) was probed with EE800.

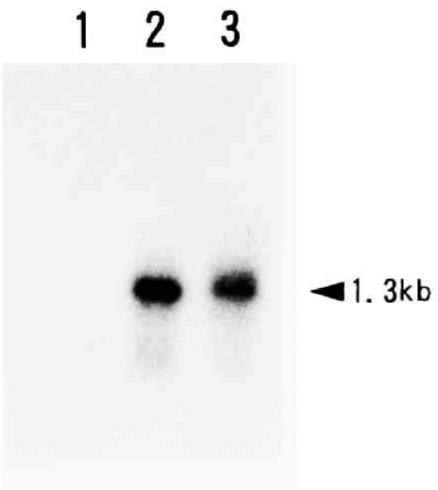


Fig.11. Mung Bean Nuclease Protection Assay.

Poly(A) RNA extracted from T(15) cells was hybridized with noncoding strand of EE2900, and then digested with mung bean nuclease. Digested fragments were separated on 0.8% NaOH-agarose gel, transferred to nylon membrane and probed with labeled EE2900.

(lane 1) mung bean nuclease 10unit/μl

(lane 2) 1unit/μl

(lane 3) 0.1unit/μl

ends of the gene (<50nt).

The restriction map of dutA gene is not consistent with that of mitochondrial DNA which was previously reported (Fukuhara, 1982), indicating that dutA is not a mitochondrial gene but a nuclear gene. This conclusion was confirmed by Southern analysis with purified mitochondrial DNA. As shown in Fig.12A and 12B, dutA was not detected in mitochondrial DNA, while mitochondrial rDNA, which was used as a control, was detected.

(B) The Sequence of dutA RNA

Since C6A, the cDNA clone that we first isolated (Yoshida et al., 1991), covers only a part of dutA RNA, we tried to isolate overlapping cDNA clones from oligo(dT)-primed cDNA library and internally-primed cDNA library, to determine the whole sequence of dutA RNA (Fig.13). However, by this standard method, only the clones that correspond to the 3'-half region of dutA RNA were obtained. To examine whether the extension of cDNA was inhibited, we performed primer extension experiment with primer C (Fig.14). At 42℃ of reaction temperature, four discrete bands of extended products were observed, indicating that the extension was terminated at several break points but not at random. The approximate location of break points was shown in Fig.18C. These termination may be caused by the secondary structure of dutA RNA. No improvement in cDNA extension was observed at 50℃. At 55℃, a band of 240nt was lost (probably due to the melting of local secondary structures) and the extension was rather inhibited (probably because of the inactivation of reverse transcriptase). Pretreatment of RNA with methylmercuric hydroxide, a denaturing reagent (Bailey and Davidson, 1976), was not effective at either 42℃ or 50℃, suggesting the tightness of its secondary structure.

To overcome this obstacle, we carried out reverse-transcription at very high temperature with Tth DNA polymerase (Tth pol). Since Tth pol is

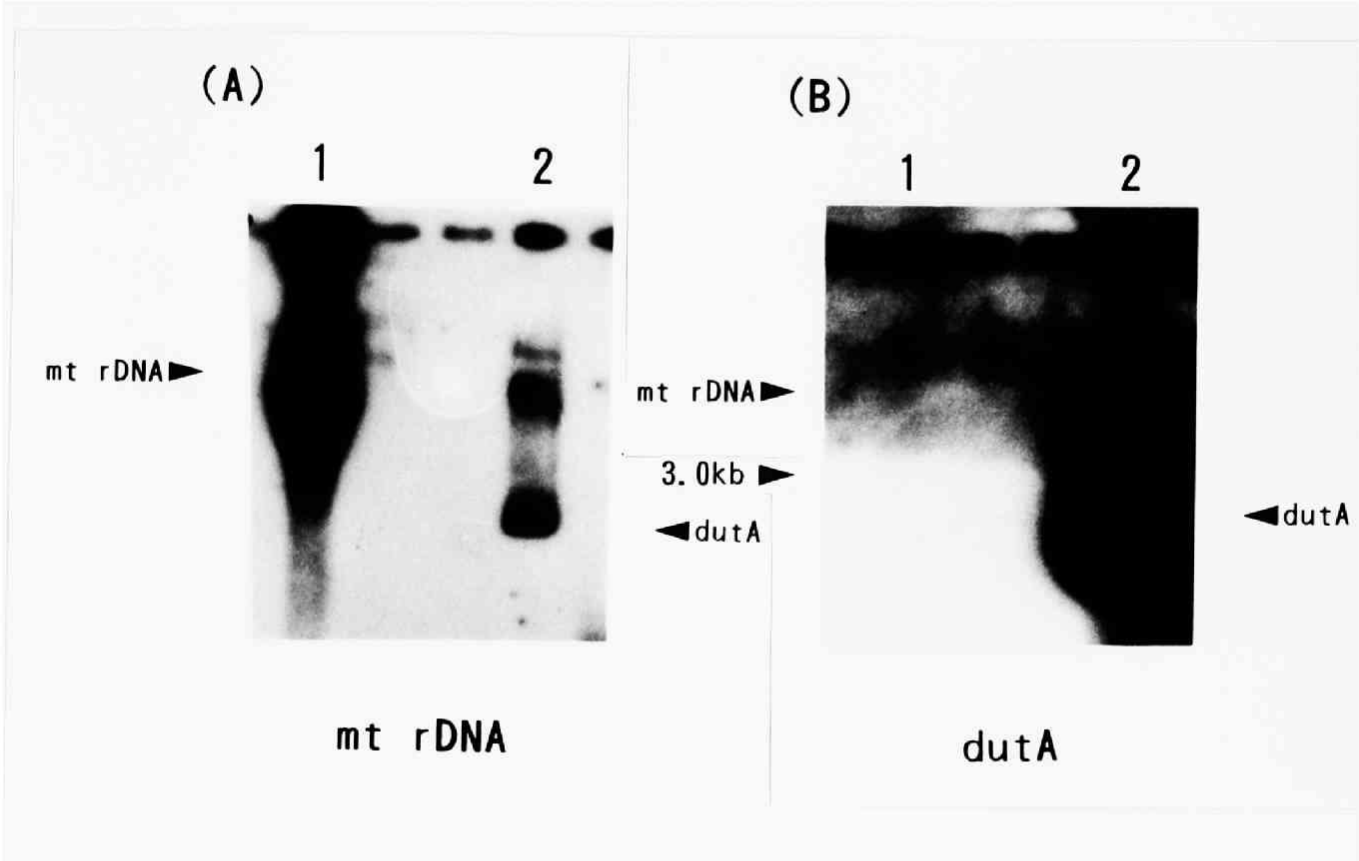


Fig.12. Southern Analysis of Mitochondrial DNA.

(A) DNA from purified mitochondria was digested with EcoRI, separated, transferred and probed with the fragment of mitochondrial large rDNA (lane 1). 10ng of pUC119-EX889 DNA was also included in the blot (lane 2). The weakly hybridized band in lane 2 is cross-hybridization to pUC119-EX889. This may be due to the contamination of pUC119 DNA in the rDNA preparation. No samples were loaded in the lanes between lane 1 and 2.

(B) The same blot as (A) was probed with EX889. The blot was exposed to X-ray film for 48hr to confirm no hybridization signal in lane 1 (the dutA gene should be seen at the position of 3kb, if it is located on the mitochondrial genome).

(lane 1) mitochondrial genome (10μg)

(lane 2) pUC119-EX889 DNA (10ng)

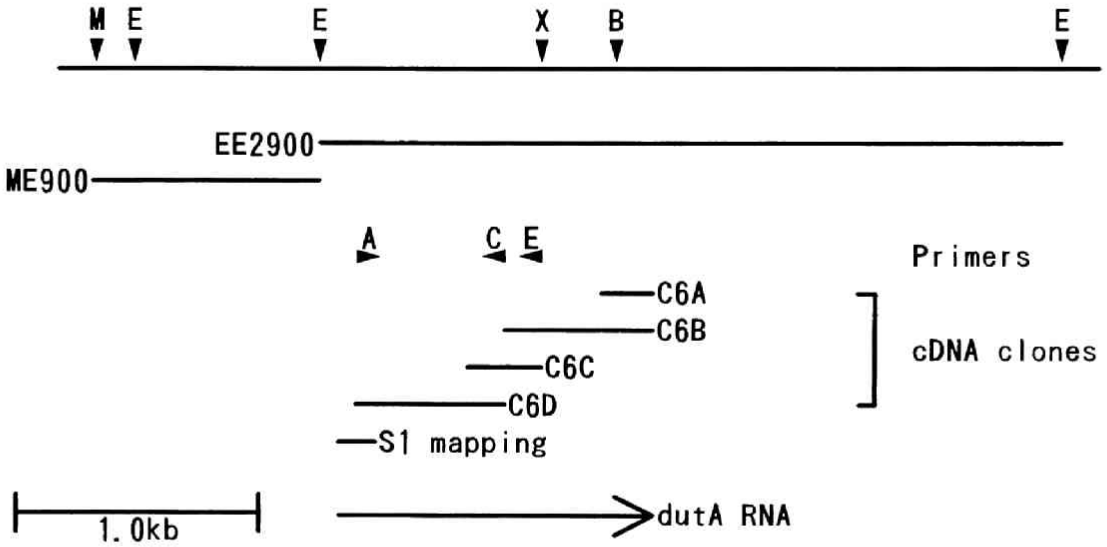


Fig.13. cDNA Clones of dutA.

C6A is a cDNA clone isolated by differential screening (see section I-(A)). C6B and C6C are the longest cDNA clones isolated from oligo(dT)-primed cDNA library and primer E-primed cDNA library, respectively. C6D was cloned by an RT-PCR method with primers A and C. The 5'-region of dutA RNA was determined by S1 nuclease mapping. For the restriction map and genomic clones, see Fig.9.

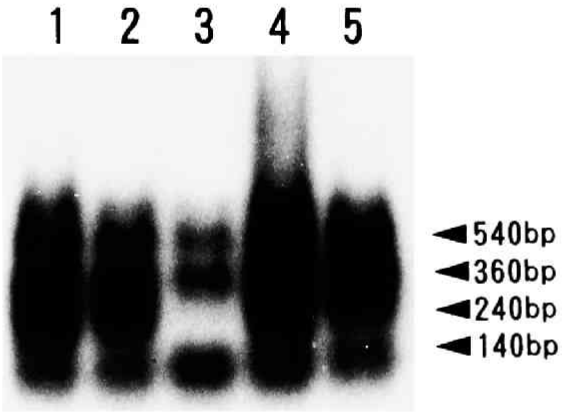


Fig.14. Primer Extension Experiment.

Poly(A) RNA extracted from T(15) cells was reverse-transcribed with primer C and reverse-transcriptase of rous associated virus (RAV-RTase) at various temperatures with or without the pretreatment of 10mM methylmercuric hydroxide.

(lane 1 and 4) 42℃

(lane 2 and 5) 50℃

(lane 3) 55℃

(lane 1-3) no pretreatment

(lane 4 and 5) pretreated with 10mM methylmercuric hydroxide

extremely thermostable, reverse-transcription can be done at 60-70℃ (Myers and Gelfand, 1991), at which temperature the secondary structure of RNA must be weakened. As reverse-transcriptase activity of Tth pol is relatively low, we amplified resultant cDNA by PCR. As shown in Fig.15, cDNA was amplified from t(15)-poly(A) RNA with primer A and C. This is probably not the product amplified from contaminating genomic DNA because amplification did not occur when template RNA was destroyed by NaOH (Fig.15). Amplified DNA was cloned and sequenced.

The initiation site of transcription was determined by S1 nuclease protection assay (Fig.16). All clones isolated from oligo(dT) library had the same 3'-terminus as C6A, indicating that the transcription of dutA RNA terminates at this point or its neighborhood. From these, we finally determined the entire sequence of dutA RNA (1322nt) (Fig.17).

The cDNA sequence is completely coincident with the corresponding region of the genomic sequence, implying that dutA RNA is encoded by one long exon without intron, consistent with the result of Mung bean nuclease mapping (Fig.12). As for a poly(A) tail, there is a poly(A) tract at the 3'-end of cDNA sequence but it is not certain whether this tract is posttranscriptionally added or transcribed from the genomic sequence, since a similar poly(A) tract is present at the corresponding position of the genome. The absence of the consensus sequence for the polyadenylation signal supports the latter possibility.

In the upstream non-coding region (Fig.17), there is a presumptive TATA box (-27bp) but neither G-rich element nor CA-rich element, which are the proposed cis-regulatory elements of transcription in D. discoideum (Kimmel, 1988). This agrees with its peculiar characteristics that its expression is not affected by cAMP (Yoshida et al, 1991) or DIF-1 (unpublished), which regulate the expression of many previously investigated genes of D. discoideum.

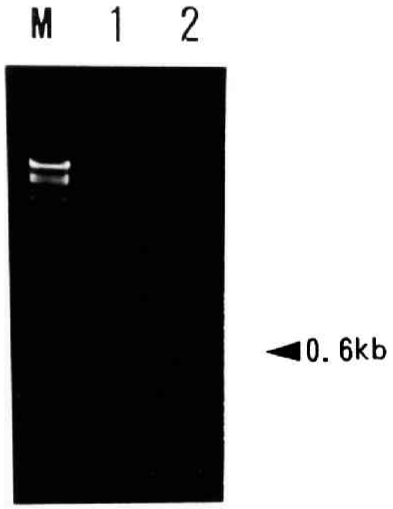


Fig.15. cDNA Amplification with Tth polymerase

RT-PCR was carried out with t(15)-poly(A) RNA (template RNA), primer A and C, and Tth polymerase. PCR product was separated and stained with ethidium bromide.

(lane 1) amplified with intact template RNA

(lane 2) amplified with RNA pretreated with 0.2N NaOH for 5min.

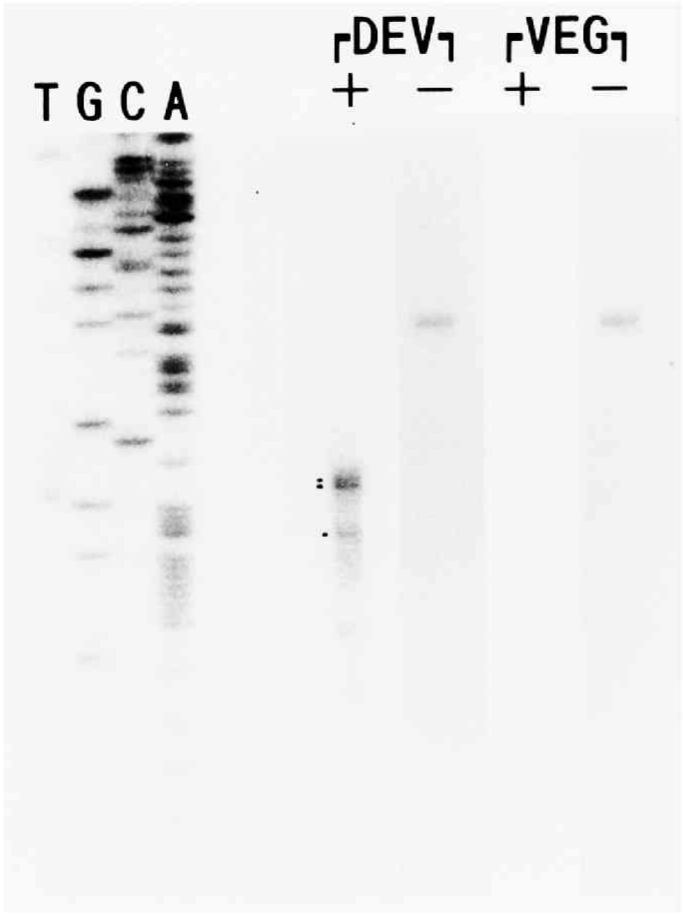


Fig.16. S1 Nuclease Protection Assay.

Poly(A) RNA from growing cells or t(15) cells was hybridized with labeled noncoding strand of ED211, digested with 0unit or 30units of S1 nuclease (reaction volume = 200μl), and separated on a 6% acrylamide-urea gel. Protected fragments were indicated by dots. A sequencing ladder of ED211 was run in parallel.

(VEG) RNA from growing cells

(DEV) RNA from t(15) cells

(-) S1 nuclease 0unit

(+) S1 nuclease 30units

Fig.17. Nucleotide Sequence of the dutA Gene.

The coding strand is shown in the 5'-to-3' direction. Numbers on the right indicate nucleotide sequence positions. Initiation sites of transcription (▼) and TATA box are marked. Nucleotide sequences that are transcribed into dutA RNA are in capital letters and those not transcribed are in lowercase letters. Location of the oligonucleotide primers used in this study are shown above or below the nucleotide sequence. >>> indicates the location of $(caa)_{20-25}$ repeat (I could not determine the precise number of its repeat, possibly due to its secondary structure). This sequence data has been submitted to DDBJ, EMBL and GenBank and assigned the accession number D16417.

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                                    gatcgtctgttaataattccatttttatttaatatttgta
catataaaatatgttaaaaaacatccattttgatatttttaatttaatttaatttttttt
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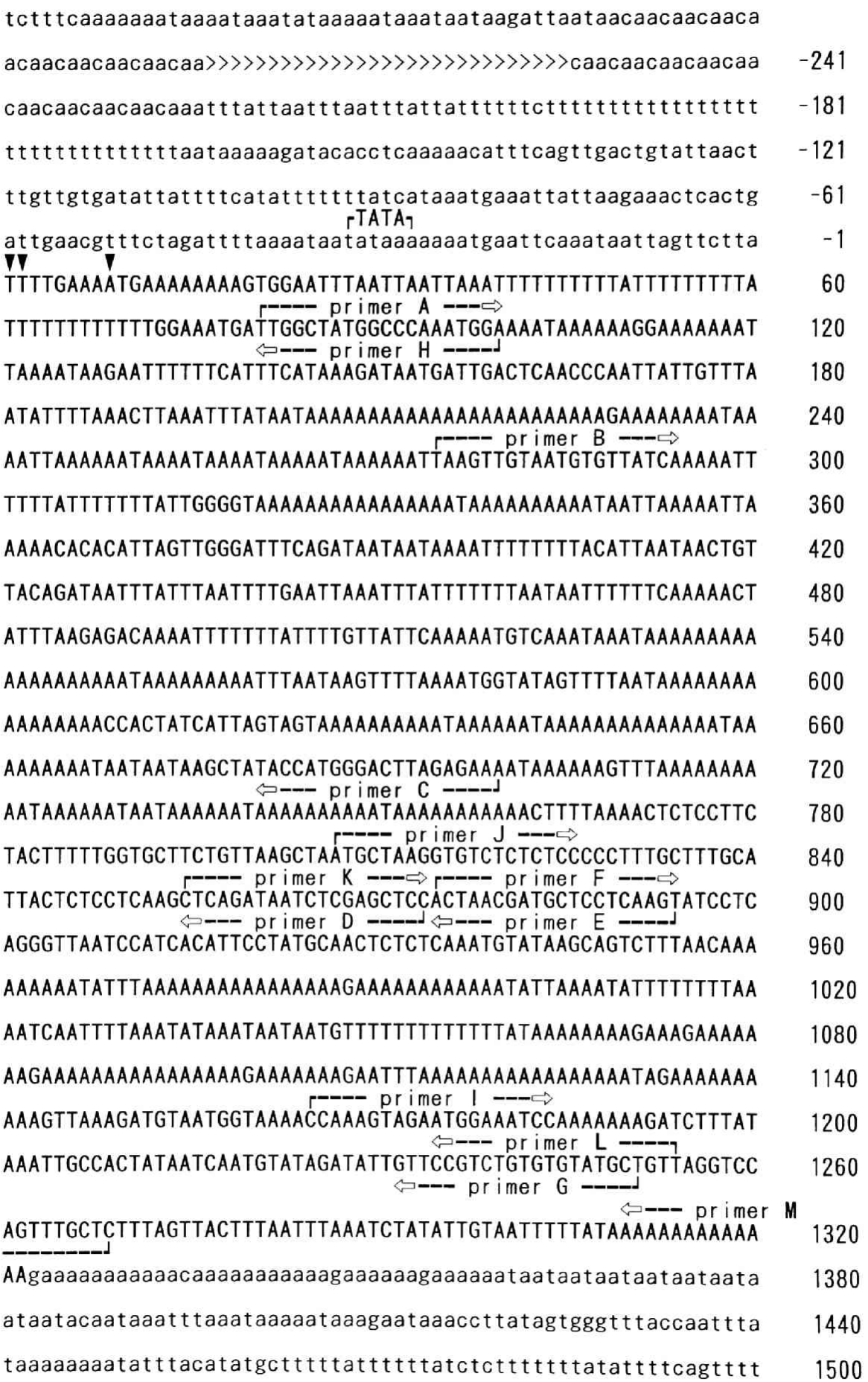
(continued)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primer H
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primer B
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TTTTATTTTTTTATTGGGGTAAAAAAAAAAAAAAAATAAAAAAAAAATAATTAAAAATTA 360
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AAAAAAATAATAATAAGCTATACCATGGGACTTAGAGAAAATAAAAAAGTTTAAAAAAAA 720
primer C
AATAAAAAATAATAAAAAATAAAAAAAAAATAAAAAAAAAAAACTTTTAAAACTCTCCTTC 780
primer J
TACTTTTTGGTGCTTCTGTTAAGCTAATGCTAAGGTGTCTCTCTCCCCCTTTGCTTTGCA 840
primer K
primer F
TTACTCTCCTCAAGCTCAGATAATCTCGAGCTCCACTAACGATGCTCCTCAAGTATCCTC 900
primer D
primer E
AGGGTTAATCCATCACATTCCTATGCAACTCTCTCAAATGTATAAGCAGTCTTTAACAAA 960
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AATCAATTTTAAATATAAATAATAATGTTTTTTTTTTTTTATAAAAAAAAGAAAGAAAAA 1080
AAGAAAAAAAAAAAAAAAAGAAAAAAAGAATTTAAAAAAAAAAAAAAAAAATAGAAAAAAA 1140
primer I
AAAGTTAAAGATGTAATGGTAAAACCAAAGTAGAATGGAAATCCAAAAAAAGATCTTTAT 1200
primer L
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primer G
primer M
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(continued)

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taaaaattaaaaatataaattttaaaaaaaacaatgatattactattttagataatgaaa 1620

tgtttaatttattaattttgtataaatatttatttattgagtattatcttcattattgtc 1680

atcatctgttggttgtttttttatcataaaatggttgaaattcatctctgaattttaaggt 1740

ttcggatgtttcaatttcttttgtttttacacaatctttccaatttaatttctctggtat 1800

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tattattattattattattattattattattattattattattattattattattattat 2580

tattattattattctattattgttattattataattattattgtattattaaaaaaag 2640

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tttttctttttatcattattaattggtgctaaagaaaggaatcttgttggttgtggatt 2940

tttatttttaaagttggtgtattgtaataaggtgctctttgaaagtaaaatgaattc 2998

(C) Protein Coding Potential of dutA RNA

To deduce the amino acid sequence of the putative dutA product, we searched the entire RNA sequence for any possible ORFs. However, multiple stop codons appear very frequently in all 6 reading frames (on the sense strand and the antisense strand) and thus no sustained ORFs could be found (Fig.18A). The longest possible ORF identified is only 126nt long, which is less than 10% of the length of dutA RNA (the longest ORF that initiates at a GUG codon is 156nt long but GUG has not been reported to be used as an initiation codon in D. discoideum). Moreover, the context of ATG in each ORF was quite different from the optimal consensus of the initiation context among D. discoideum genes (Table 1) (AAAATG: Yoshida and Okamoto, unpublished: some ORFs contain complete consensus sequence but are quite short ($\leq$ 27nt)). It is very unlikely that the sequence we determined was distorted by rearrangement during cloning process because the sequences of all genomic and cDNA clones, which were independently isolated, completely accord with each other.

Moreover, dutA RNA has some other peculiar characteristics: first, the nucleotide sequence is composed largely of A and U residues (A=51.1%, U=32.1%, G=8.6%, C=8.2%), which frequently appear as homopolymer tracts, especially in 5'-region. Second, G and C residues are not randomly scattered over dutA RNA but rather localized in two GC rich domains (Fig.18B). Third, the sequence of dutA RNA has six large palindromes and the largest one extends over 71nt (Fig.18C and 18D).

The above data are most consistent with the idea that dutA RNA does not encode protein but functions as a structural RNA. Computer searches with available data bases (by IDEAS program) could not find any other nucleotide sequences that had a significant similarity to dutA RNA.

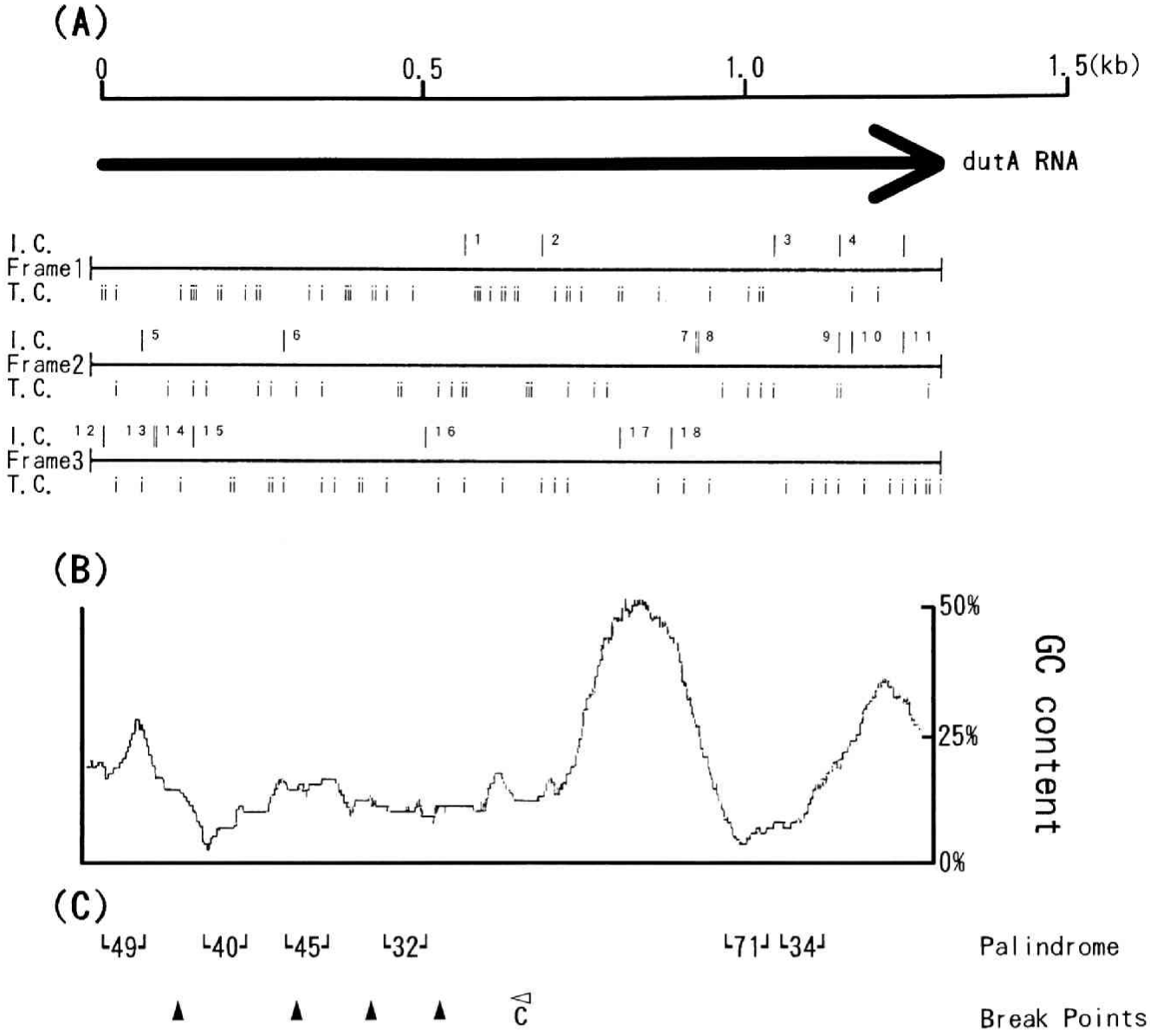


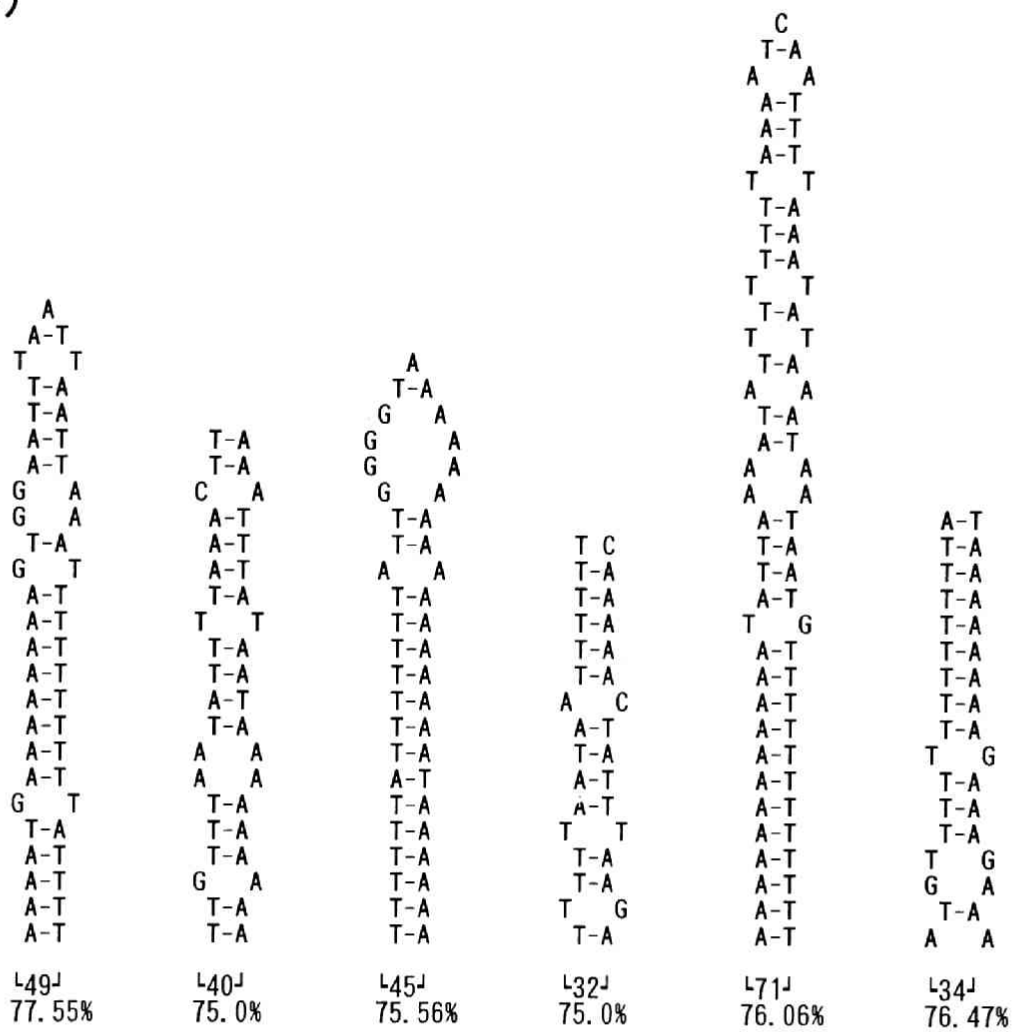
Fig.18. The Structure of dutA RNA.

(A) The location of initiation codon and termination codon. Three forward reading frames are shown. | and i indicate the location of initiation codon (I.C.) and termination codon (T.C.), respectively. Each ORF is numbered.

(B) Distribution of GC-content. Average GC-contents in a 100nt of window are calculated and plotted.

(C) The location of palindromes. Lower limits of length and matching are 30nt and 75%, respectively. Numbers indicate their length. Closed arrow heads indicate the location of break points in primer extension experiment (Fig.14). An open arrow head indicates the location of the primer C.

(D)



(D) The nucleotide sequences of palindromes.

TABLE I. Putative ORFs in dutA RNA.

The context of ATG in each ORF was compared with the consensus sequence of translational initiation site among D. discoideum genes (AAAATG). Identical nucleotides were in capital letters. Matching (shown in parentheses) was scored without ATG.

	Length (nt)	D. discoideum consensus
ORF1	6	AAAATG (3)
ORF2	27	AccATG (1)
ORF3	126	AtAATG (2)
ORF4	15	tcAATG (1)
ORF5	27	gAAATG (2)
ORF6	36	gtAATG (1)
ORF7	48	cctATG (0)
ORF8	33	cAAATG (2)
ORF9	3	AAgATG (2)
ORF10	111	AgAATG (2)
ORF11	39	tgtATG (0)
ORF12	27	AAAATG (3)
ORF13	39	gctATG (0)
ORF14	30	cAAATG (2)
ORF15	48	AtAATG (2)
ORF16	12	AAAATG (3)
ORF17	54	ctAATG (1)
ORF18	24	AcgATG (1)

III. Function of dutA

(A) Subcellular Localization of dutA RNA

The above results suggest that dutA RNA functions as a structural RNA but not as an mRNA. We analyzed the subcellular location of dutA RNA to assess whether it is transported into the cytoplasm, and whether it associates with the translational machinery of the cell. Cells in the slug stage were mildly disrupted and fractionated by differential centrifugation into first precipitate (cell debris), second precipitate (nuclear fraction), and second supernatant (cytosol-organelle fraction). The majority of dutA RNA was fractionated into cytosol-organelle fraction rather than nuclear fraction (Fig.19A), whereas that of Dd8 RNA (small nuclear RNA of D. discoideum: Kaneda et al., 1983) was in nuclear fraction as expected (Fig.19B).

The cytosol-organelle fraction was then further fractionated by sucrose density gradient centrifugation (Fig.19C-E). Actin RNA was fractionated into both polysomal fractions and lighter fractions, while dutA RNA was found only in the lighter fraction, indicating that dutA RNA does not associate with ribosomes. This is consistent with the absence of protein-coding potential of dutA RNA.

(B) Overexpression, Antisense Mutagenesis and Gene Disruption of dutA

To directly assess the function of dutA RNA in vivo, we first tried to overexpress dutA RNA in the cell. The overexpression construct (Fig.20A) was introduced into cells and transformants were selected in the presence of 20-500ug/ml of G418. Since dutA was linked to the actin 6 promotor, ectopic expression in vegetative cells was observed and the degree of overexpression in t(15) cells was 9.0 fold (quantitated by a Fuji Image Analyzer BA100) in all transformants (Fig.20B). However, they were normal in morphology. The copy number of the vector introduced into one of the transformants (TF5001) was more

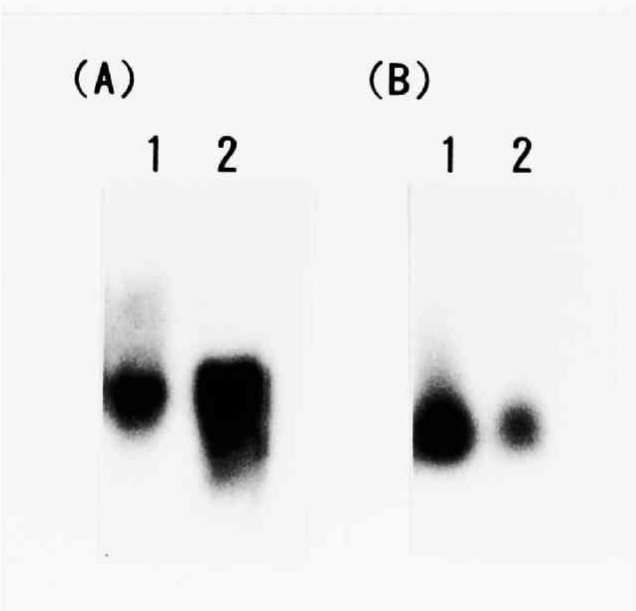
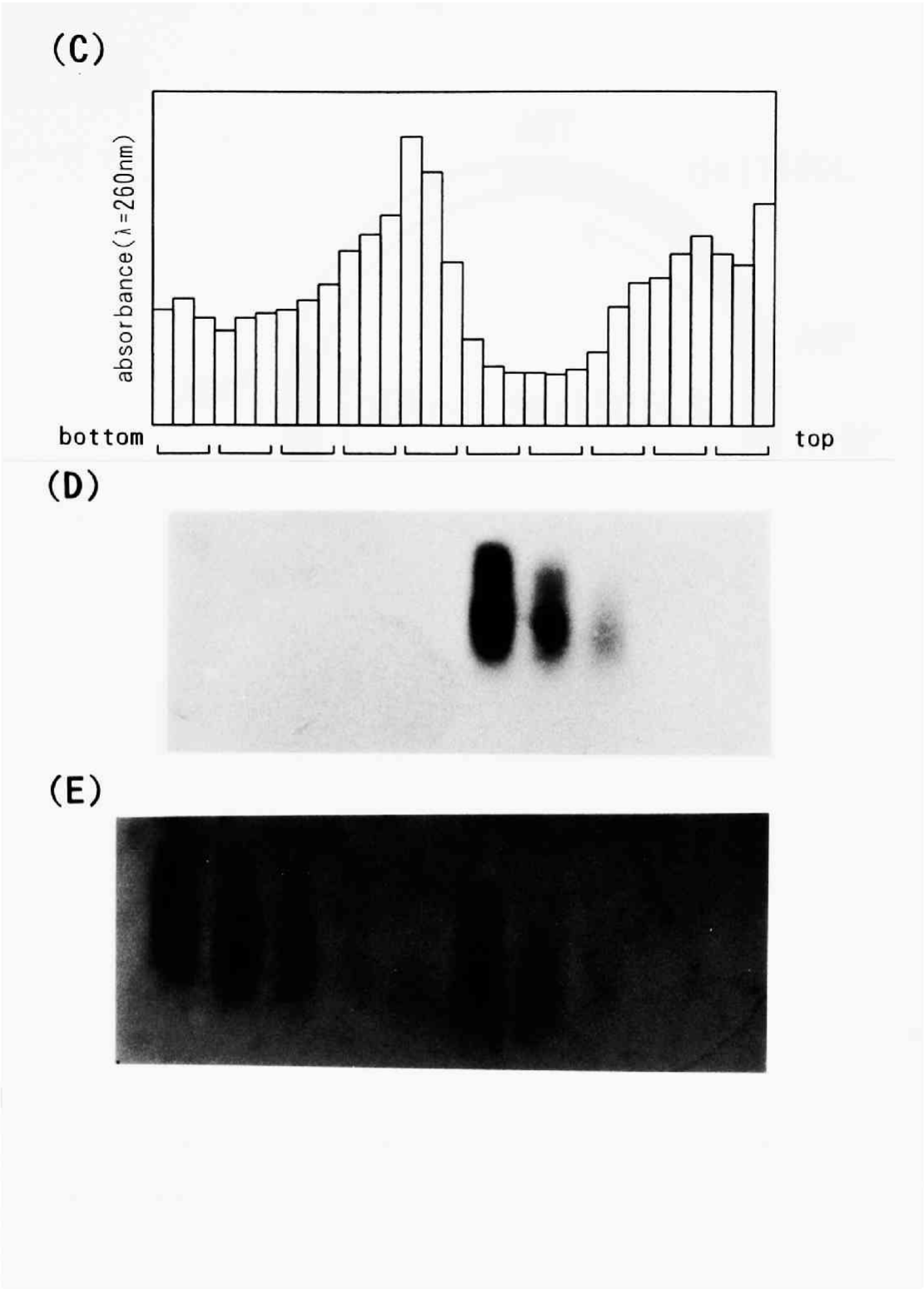


Fig.19. The Subcellular Localization of dutA RNA.

(A) Lysate of slug cells was fractionated into cell debris fraction, nuclear fraction (lane 1) and cytoplasm-organelle fraction (lane 2). RNA was extracted from each fraction, separated, transferred and probed with EX889.

(B) The same blot was probed with an oligonucleotide complementary to Dd8 RNA (5'-GGGTGTTCGAGTATTCATTG-3').



(C-E) Cytoplasm-organelle fraction obtained in (A) was further fractionated by sucrose density gradient centrifugation.

(C) The optical density profile (wave length = 260nm).

(D) Three consecutive fractions were combined and RNA was extracted therefrom, transferred and probed with dutA (EX889).

(E) The same blot was probed with actin 6 cDNA of D. discoideum.

(A)

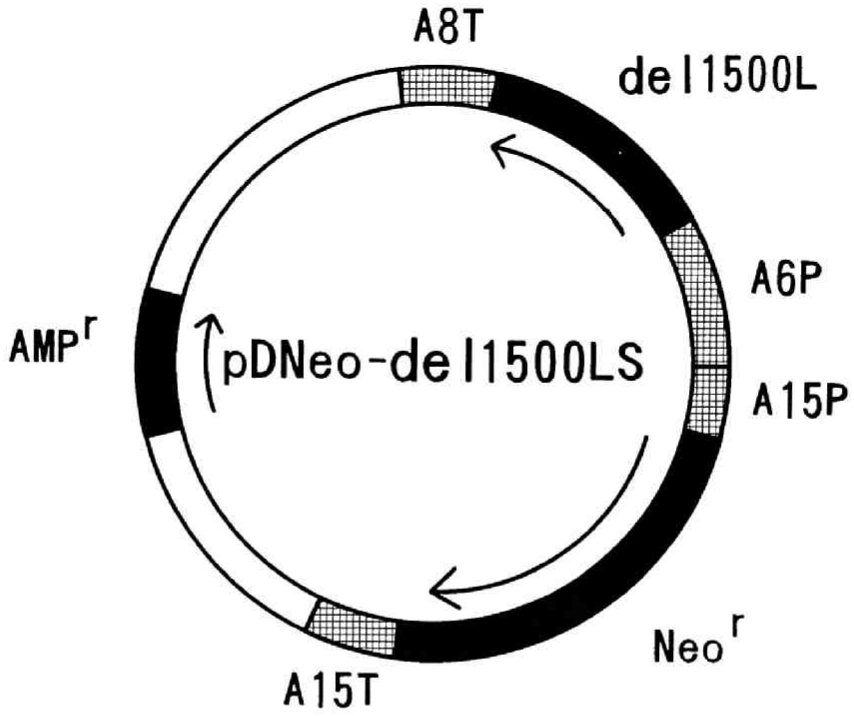
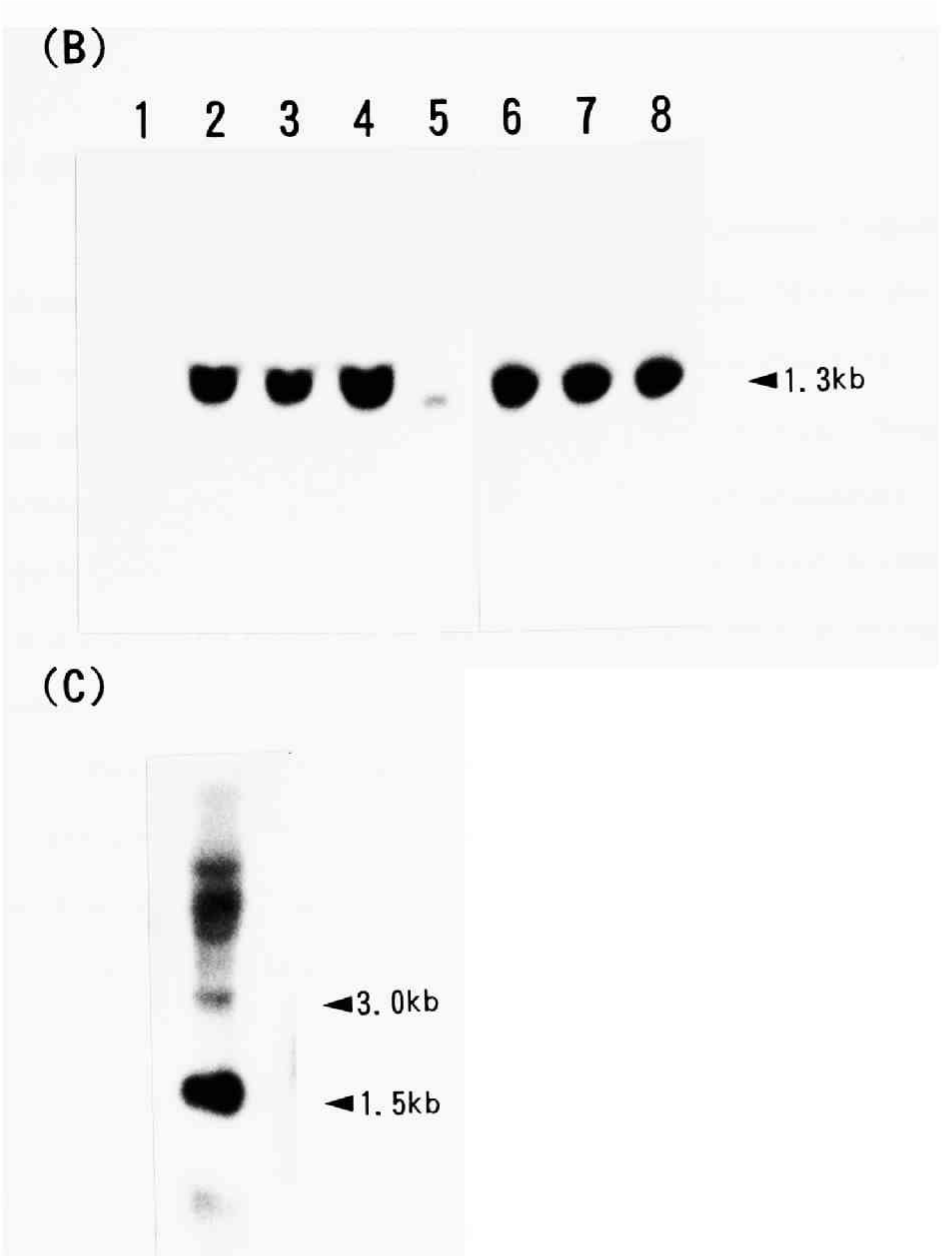


Fig.20. Overexpression Experiment.

(A) The structure of overexpression vector pDNeo-del1500LS.

This vector contains the Tn903 phosphotransferase gene (Neo') with D. discoideum actin 15 promotor and terminator, and del1500L fragment (which contains full length cDNA) inserted in the sense orientation with respect to the actin 6 promotor and actin 8 terminator. (also see Fig.25B)



(B) Northern blot analysis of overexpression transformants. Cells of transformants were developed in suspension for 15 hours and then RNA was extracted from them, separated, transferred and probed with del1500L.

(lane 1 and 5) parental strain Ax2

(lane 2-4 and 6-8) transformed strains (TF5001, TF5002 and TF5003).

(lane 1-4) growing cells

(lane 5-8) developing cells

(C) Southern blot analysis of an overexpression transformant. Genomic DNA of a transformant (TF5001) was cut by EcoRI+PstI, separated, transferred and probed with EX889. The 3.0kb band corresponds to the endogenous dutA gene and the 1.5kb band is derived from the introduced vectors. We quantitated the vector copy number by comparing the radioactivity of the 1.5kb band to that of the 3.0kb one.

than 30 (Fig.20C; quantitated by BA100).

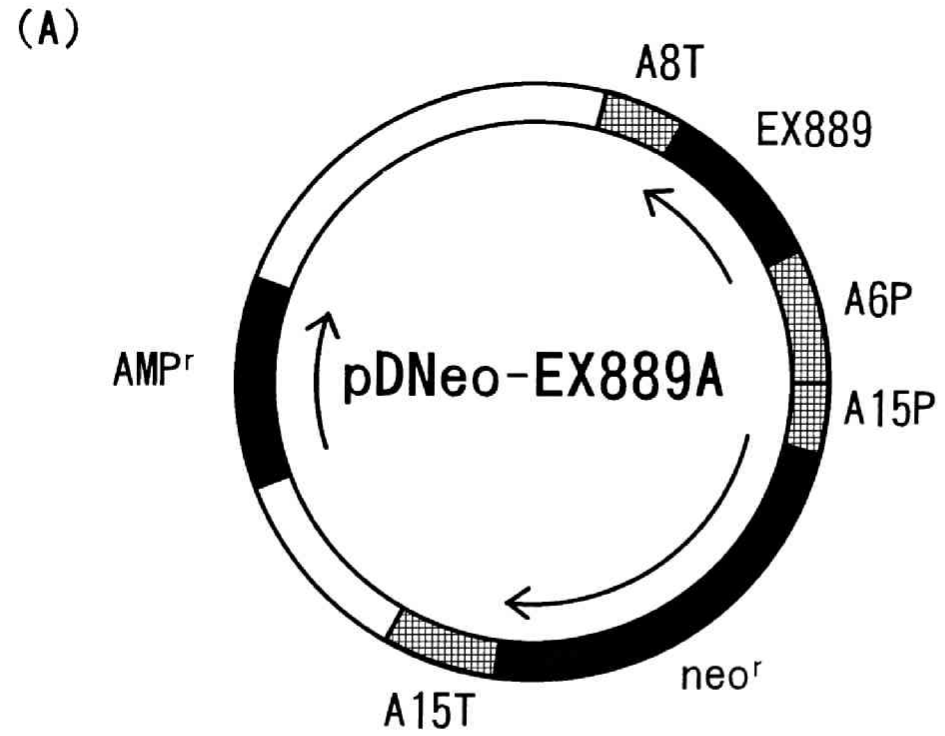
Next we performed antisense mutagenesis. Genomic fragment EX889 was inserted into the expression vector pDNeoII (Witke et al., 1987) in the antisense orientation to allow antisense RNA transcription (Fig.21A). The resulting plasmid pDNeo-EX889A was introduced into Ax2 cells. Though all of 60 transformants examined accumulated 0.9kb antisense RNA abundantly, the amount of 1.3kb dutA RNA was not reduced as compared with that found in cells of parental strain (Fig.21B). The phenotype of these transformants were all normal in development.

Then we disrupted the dutA gene by gene targeting in the haploid strain. The gene replacement construct EE800S-Neo^{r}A-XE2000S, which contains genomic fragment EE800 and XE2000 interrupted by the bacterial neomycin phosphotransferase gene (Neo^r; Witke et al., 1987) (Fig.22A), was introduced into Ax2 cells and transformants were selected in the presence of 7μg/ml G418. Disruptants were selected from transformants by genomic Southern hybridization (Fig.22B).

By these procedures, we obtained 4 disruptants out of 74 transformants. In these disruptants, dutA RNA was completely lost (Fig.22C). However, all of them showed normal morphology in development as far as examined. This suggests that dutA function is redundant in this organism.

(C) Cognate Sequences in Other Organisms

Next we examined whether sequences similar to dutA are present in the genomes of other organisms. As shown in Fig.23, cognate sequences in Saccharomyces cerevisiae and Schizosaccharomyces pombe were detected in the lower stringency genomic Southern blot. Cognate sequences in other organisms were also examined by using PCR and Southern hybridization. Genomic DNAs prepared from other cellular slime molds (D. mucoroides, D. rosarium and D.



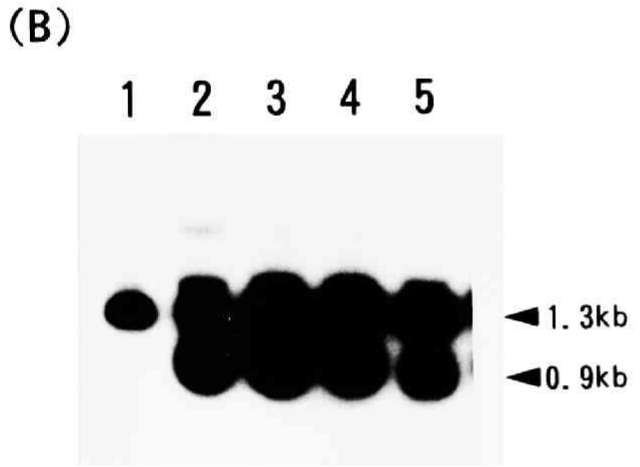


Fig.21. Antisense Mutagenesis of dutA.

(A) The structure of antisense RNA expression vector pDNeo-EX889A. This vector contains the Neo^r gene with actin 15 promotor and terminator, and EX889 fragment (which contains 5' part of cDNA) inserted in the antisense orientation with respect to the actin 6 promotor and actin 8 terminator (also see Fig.25B).

(B) Northern blot analysis of antisense transformants. Cells of antisense transformants were developed in suspension for 15 hours and then RNA was extracted from them, separated, transferred and probed with EX889.

(lane 1) parental strain Ax2

(lane 2-5) antisense RNA expressing strains

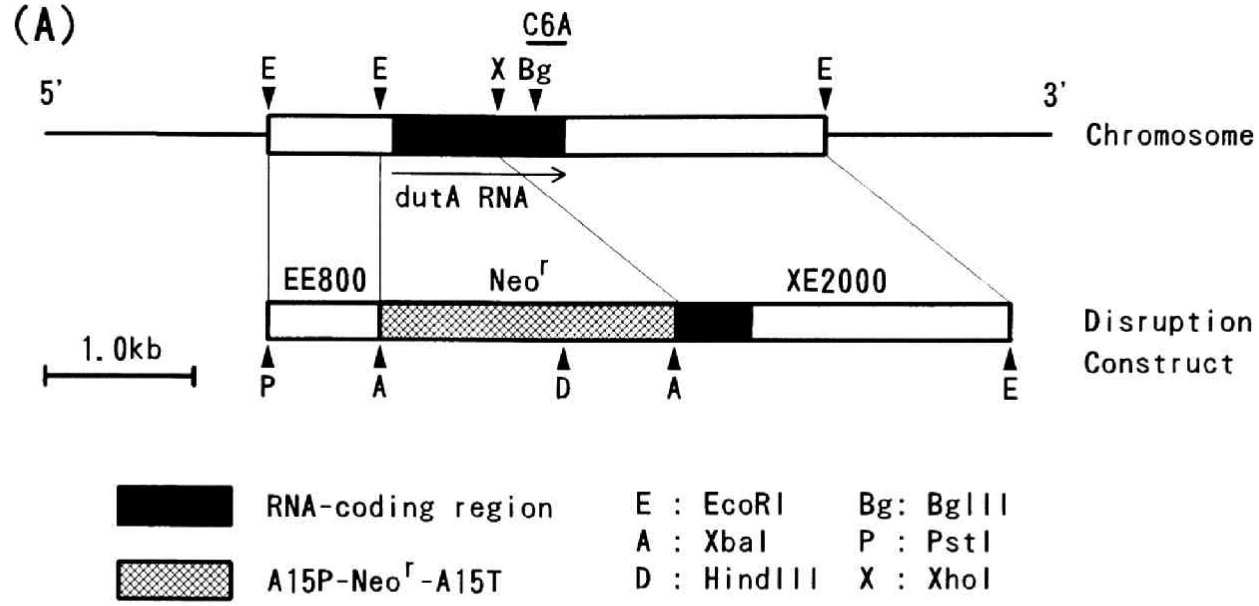
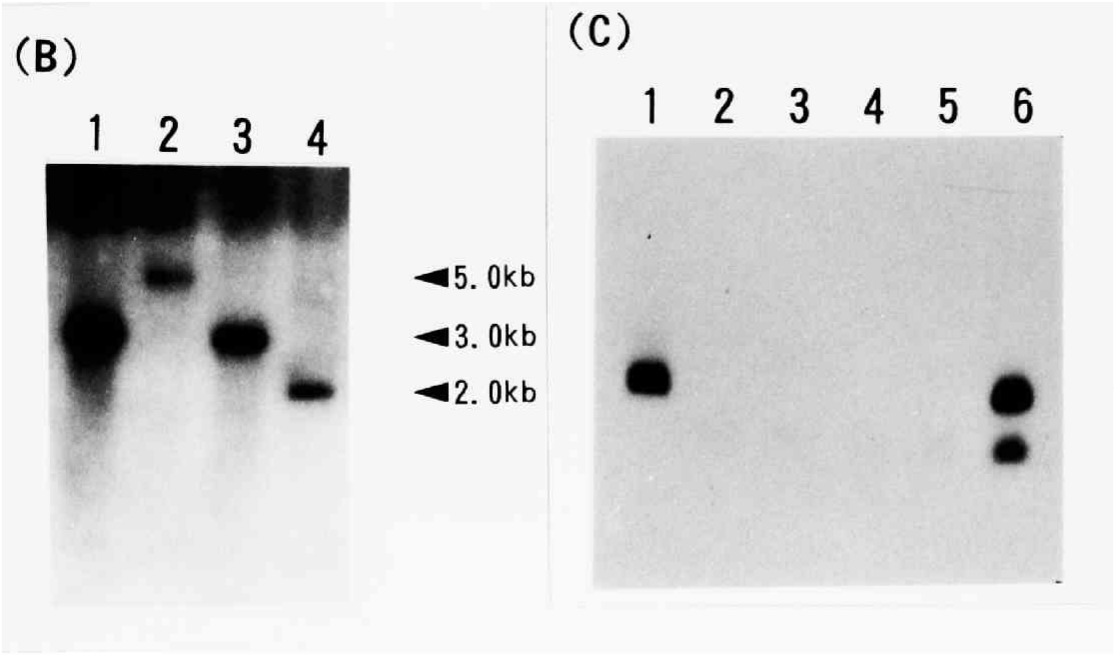


Fig.22. Disruption of dutA Gene

(A) The strategy for the disruption of dutA gene. The disruption construct has EE800 and XE2000 fragment interrupted by the Neo^r gene with the actin 15 promotor and terminator (also see Fig. 25A).



(B) Southern blot analysis of transformants. Genomic DNA of the parental strain and transformants was digested with restriction enzymes indicated below, separated, transferred and probed with C6A.

(lane 1 and 3) parental strain Ax2

(lane 2 and 4) transformant TF503

(lane 1 and 2) EcoRI

(lane 3 and 4) EcoRI+XbaI

(C) Northern blot analysis of disruptants. Cells of disrupted strains were developed on filter for 15 hours and then cellular RNA was extracted from them, transferred and probed with C6A. The upper band in lane 6 corresponds to the endogenous dutA RNA (the gel slightly smiled). The lower band in lane 6 could be a transcript derived from a vector randomly inserted near the promotor of the other gene.

(lane 1) parental strain (Ax2)

(lane 2-5) disruptants (TF503, TF505, TF511 and TF555)

(lane 6) undisrupted transformant (TF541)

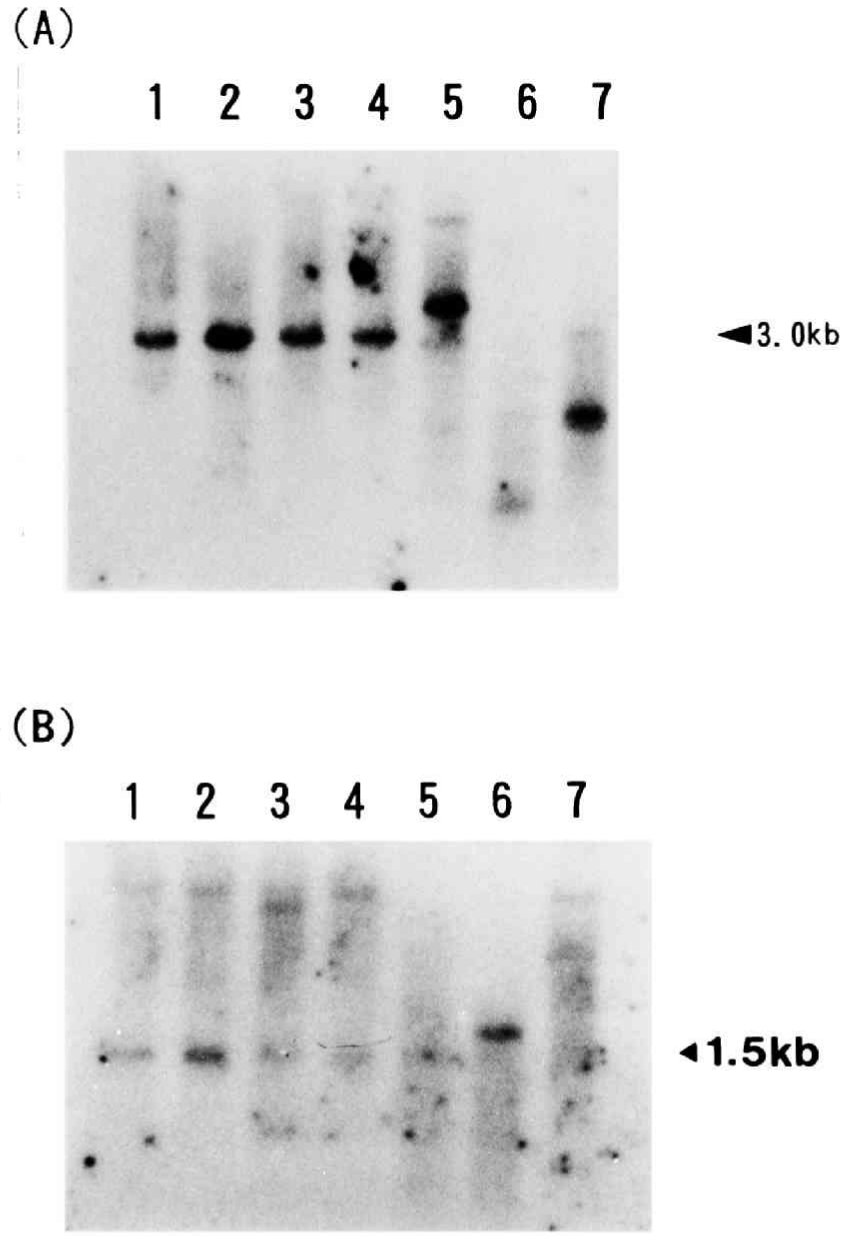


Fig.23. Southern Analysis of Yeast Genome at Lower Stringencies

Genomic DNA of yeasts was digested with restriction enzymes indicated below, separated, transferred and probed with dutA (DEL1500L). The blots were washed 2xSSC at 42℃.

(A) S. cerevisiae (B) S. pombe

(lane 1) EcoRI (lane 2) EcoRI+PstI (lane 3) EcoRI+BamHI

(lane 4) EcoRI+XhoI (lane 5) DraI (lane 6) MboI

(lane 7) HaeIII

purpureum), S. cerevisiae, S. pombe, Drosophila melanogaster, Oryzias latipes (killifish), Mus musculus (mouse), Bos taurus (bovine), Arabidopsis thaliana, Lemna paucicostata (duck weed) and Escherichia coli (bacteria) were used as templates and PCR was performed with 7 pairs of primers (Fig.24A). Amplified DNA fragments were Southern-blotted and probed with dutA (del1500L). As shown in Fig.24C-G (data of several organisms are not shown), hybridizable bands were detected in all of the organisms examined and in some of the pairs of primers, 2-3 bands were detected in a lane. However, the bands detected by ethidium bromide staining did not always coincide with these hybridizable bands. The size of the bands of E. coli was quite different from those of eukaryotes. It is unlikely that these were amplified from contaminated DNA of D. discoideum because their sizes were not completely identical to those expected from D. discoideum (Fig.24B), some of the pairs of primers did not yield hybridizable bands, and no bands were detected if a template DNA was omitted (data not shown). These results suggest that dutA like sequences are widespread from bacteria and lower eukaryotes to mammals and plants. However, we must await the results of actual sequence determination to ascertain that those sequences are indeed the cognates of the dutA gene.

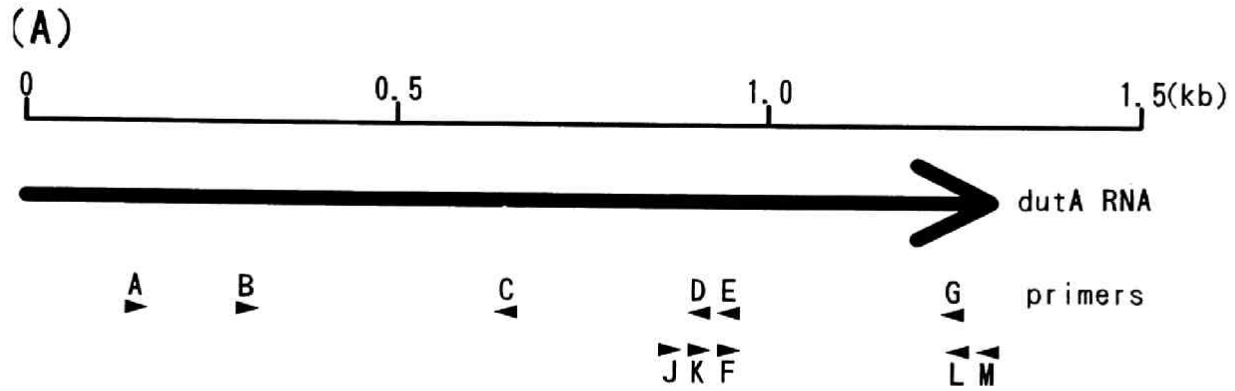
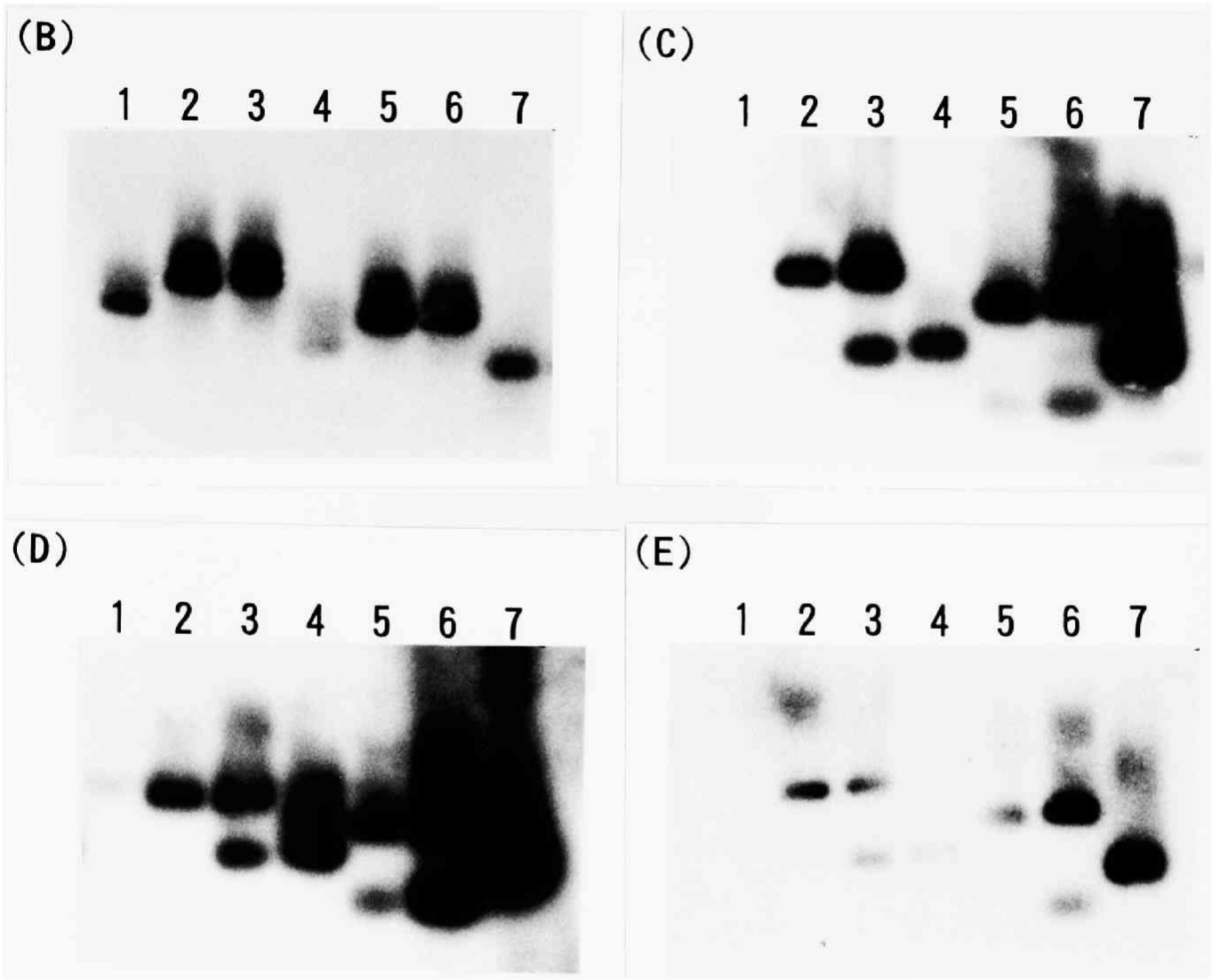


Fig.24. Cognate Sequences in Other Organisms.

(A) The location of primers for PCR study. The length of primers is 20nt. Precise location is shown in Fig.1G.



(B)-(F) PCR was done with genomic DNA of various organisms. Amplified DNA was separated, transferred and probed with DEL1500L.

(B) D. discoideum

(C) Saccharomyces cerevisiae

(D) Drosophila melanogaster

(E) mouse (balb/c)

(lane 1) primer A and C

(lane 2) primer A and D

(lane 3) primer A and E

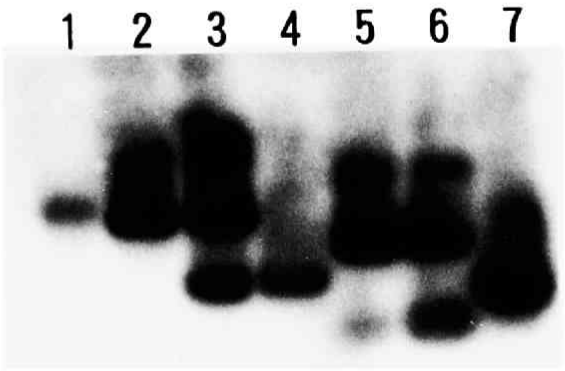
(lane 4) primer B and C

(lane 5) primer B and D

(lane 6) primer B and E

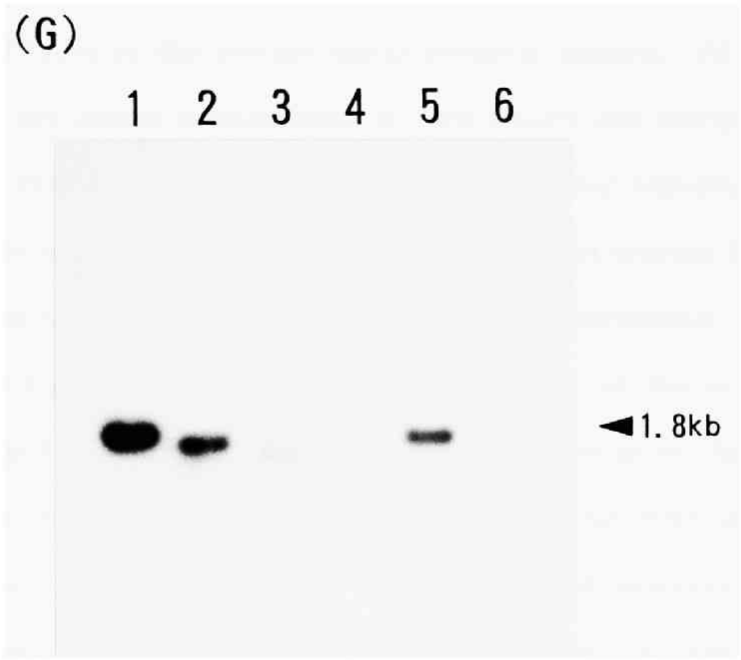
(lane 7) primer F and G

(F)



(F) PCR was done with the genomic DNA of Arabidopsis thaliana.

(lane 1) primer A and C
(lane 2) primer A and D
(lane 3) primer A and E
(lane 4) primer B and C
(lane 5) primer B and D
(lane 6) primer B and E
(lane 7) primer F and G



(G) PCR was done with the genomic DNA of Escherichia coli.

(lane 1) primer J and M (lane 2) primer J and L

(lane 3) primer J and G (lane 4) primer F and M

(lane 5) primer F and L (lane 6) primer F and G

[DISCUSSION]

I. dutA RNA Expression Is Regulated By Intercellular Signals

In the development of D. discoideum, starved cells differentiate into two distinct cell types at the precise spatio-temporal pattern. At the time of starvation, cells are almost homogeneous in their nature and pluripotent for the differentiation. Previous studies indicate that intercellular signaling systems play a major role in the regulation of this differentiation process (Loomis, 1982). As a first step toward understanding how the intercellular signaling system regulates the gene expression in the development of this organism, I cloned a gene dutA, which is expressed at a certain time after the onset of development, and examined it in detail. I found that dutA RNA is induced by unidentified signals, but not by cAMP. Recently, several intercellular signaling molecules have been identified in D. discoideum. In near future, experiments with a [dutA promotor::β-galactosidase] fusion construct will clarify whether such molecules induce dutA RNA expression.

II. The Structure and Function of dutA RNA

For more precise examination, I carried out molecular biological analysis of the dutA gene and RNA. To my surprise, dutA RNA had unusual nucleotide sequences, having no reasonably long ORF, and was present in the cytoplasm but not associated with ribosomes. In addition, sequences similar to dutA widely exist in other organisms.

(A) Does dutA encode a protein?

D. discoideum uses universal codons in nuclear and mitochondrial genes (Nellen et al., 1987). When the sequence of dutA RNA is converted into the

amino acid sequence according to the universal codons, stop codons appear so frequently in all 3 reading frames that any potential ORFs are too short (≦126base) to encode a protein. Moreover, dutA RNA shows extremely low GC-content (17% G/C), compared to the minimal GC-content necessary to encode an average protein (36% G/C). In D. discoideum, protein coding regions are relatively GC-poor (~38% G/C) but not to that extent (Nellen et al., 1987). However, we cannot strictly rule out the possibility of the existence of a dutA protein product. It is formally possible that one of these short ORFs encode a very small polypeptide (not more than 126nt = 42 amino acid residues ≒ 4.6kDa). The Drosophila heat shock gene hsrω has been proposed to encode a protein of only 27 amino acids (Fini et al., 1989). However, ORFs in dutA do not contain the consensus sequence of the initiation context (some ORFs have consensus sequence but they are quite short; Table 1), suggesting that they do not encode peptides.

Other possibility is that a functional ORF is generated either by frame shift translation (recoding) (Gesteland et al., 1992) or by posttranscriptional nucleotide insertion (RNA editing) (Simpson and Shaw, 1989; Mahendran et al., 1991; Walbot, 1991). Since there are many stop codons throughout dutA RNA in any reading frames, recoding or editing must occur over the entire region of dutA RNA to create a functional ORF. However, such frequent recoding is quite unlikely and has not been reported in any organisms. As for RNA editing, there is no report of editing occurring in the cytoplasm where the majority of dutA RNA is localized. Moreover, we have never observed edited dutA RNAs in any cDNAs we cloned. Therefore, it is unlikely that recoding or editing makes dutA RNA translatable.

Finally, the most compelling evidence for dutA RNA's not being mRNA is the absence of dutA RNA from ribosomes in the cytoplasm. From these, we concluded that dutA RNA does not encode a protein.

(B) How does dutA exhibit its function?

Our observation indicates that dutA RNA functions without being translated into protein. Thus the action mechanism of this extraordinary RNA is an interesting issue in our next study.

One possibility is that the transcription of dutA makes the local chromatin structure loose and then promotes the transcription of other genes. Such transcription-induced changes in chromatin structure have been proposed to play an important role in immunoglobulin class switching, where nascent RNA transcripts may stabilize transcription-induced triplex DNA conformers by base pairing with their DNA templates (Reaban and Griffin, 1990).

An alternative possibility that could account for our observation is that dutA RNA works as a structural RNA. Its peculiar nucleotide sequence (large palindromes, AU tracts and GC islands) might be important for the conformation of the RNA, association with proteins and its function. In fact, our observation that cDNA extension in vitro was inhibited at several break points strongly supports the possibility of the formation of very tight secondary structures. An attempt to calculate the most stable secondary structure of dutA RNA (by MFOLD program) was unsuccessful, probably due to too many possibilities of pairing among AU-rich regions.

Recently several new candidates for a structural RNA have been reported. For example, vertebrate snoRNA (small nucleolar RNA) is a large family of intron-encoded RNA and implicated in the ribosome biogenesis or function (Leverette et al., 1992; Sollner-Webb, 1993). Kinetoplastid gRNA (guide RNA) works as a "guide" in RNA editing (Blum et al., 1990). E. coli 10SaRNA has a tRNA like domain and is involved in cell growth (Y. Komine and H. Inokuchi, personal communication). Silkworm TFIIIR (transcriptional factor IIIR) is a macromolecule containing RNA (Young et al., 1991). In prokaryotes,

endogenous antisense RNAs regulate the replication of plasmids (ColEI, R1, IncF, etc.), the lytic processes of phages (lamda, P1, etc.), the transposition of a transposon (IS10) and several bacterial genes (ompF and crp) by various mechanisms, for example, primer sequestration, mRNA destabilization, translational suppression and transcriptional termination (Green et al., 1986; Eguchi et al., 1991). As for eukaryotes, human and Xenopus bFGF antisense RNA mediates, via hybrid formation, covalent modification of both strands and regulates translation of bFGF (Kimelman and Kirschner, 1989). The C. elegance heterochronic gene lin-4 encodes small RNAs (encoded by an intron of another gene) which negatively regulate lin-14 translation via an antisense mechanism (R. C. Lee et al., 1993). D. discoideum EB4 antisense RNA is proposed to regulate the accumulation of EB4 mRNA (Hildebrandt et al., 1992). These RNAs are mostly small RNA species (~200nt).

H19 RNA of mouse and human is induced during embryogenesis, associates with a cytoplasmic particle and has been shown to have a tumor-suppressor activity (Pachnis et al., 1988; Brannan et al., 1990; Bartolomei et al., 1991; Hao et al., 1993). The XIST gene of human (and Xist of mouse) is expressed from only one of the two X chromosomes, localized in the heterochromatic Barrbody and possibly involved in the inactivation of X chromosome (Davis, 1991; Brown et al., 1991A, 1991B; Brown et al., 1992; Brockdorff et al., 1992; Kay et al., 1993). Budding yeast sme2 RNA is essential for meiotic process and associates with mei2 protein (M. Yamamoto, personal communication). sme2 RNA, H19 RNA and XIST RNA are assumed to be structural RNAs but the molecular mechanisms by which they operate are still unknown. Since the length of these RNAs are relatively large (1-20kb), their action mechanisms may be quite complex. Although the length and the subcellular localization of dutA RNA is similar to those of H19 RNA, they are not similar in their nucleotide sequences.

(C) Does dutA have a role in the development of D. discoideum?

We found that the expression of dutA is strictly regulated by cellular interaction during the development (Yoshida et al., 1991) and dutA RNA accumulates relatively abundantly (~0.01% of poly(A) RNA). Moreover, the sequences similar to dutA are widespread among organisms, from prokaryotes and lower eukaryotes to mammals and plants. These facts support the notion that dutA has some important role in D. discoideum and other organisms.

Although none of gene disruption, antisense mutagenesis and overexpression cause phenotypic changes, this does not necessarily imply that dutA RNA is a useless RNA. There are many reports that disruption and antisense mutagenesis of D. discoideum genes, as well as mammalian genes, caused no phenotypic changes (Witke et al., 1987; Elkins et al., 1990; Saga et al., 1992). This may result from the redundancy of the gene function and it is even possible that more important genes have more redundancy. Another possibility is that under optimal conditions for growth and development provided in the laboratory, phenotypic defects may not be detectable. We are now trying to isolate and disrupt the cognate gene in S. cerevisiae. Comparison between the D. discoideum and S. cerevisiae RNA could enable us to identify conserved regions or features which might be important for the dutA function.

These structural RNAs are reminiscent of the "RNA world". These RNAs must have a conserved and fundamental role in the cell function. Since dutA cognates exist in lower eukaryotes, such as yeasts, it will be suitable for the molecular analysis of structural RNAs. Furthermore, the outcome of such studies may be applied to the structural RNAs in higher eukaryotes because the cognates also reside in them.

[MATERIALS AND METHODS]

(A) General Methods

The strains NC4, V12M2 and Ax2 of D. discoideum were used as wild type strains and all manipulations of cells were performed according to Sussman (1987) and Yoshida et al., (1991). The mutant strains Synag7 and HC85 were kindly given by Dr. M. B. Coukell and Dr. A. Kumagai, respectively. We usually used cells (T(15) cells), which developed for 15hr in suspension (20mM Na_2HPO_4/KH_2PO_4 pH7.0, 2mM $MgSO_4$ at the density of $1\sim2x10^7$cells/ml). cAMP phosphodiesterase assay was carried out as described by Okamoto (1979). Transformation of Ax2 cells was carried out by the calcium phosphate method (Nellen et al., 1984).

DNA and RNA manipulations were performed as described by Maniatis et al. (1989). DNA labeling and cDNA library construction were done with a multiprime DNA labeling system (Amersham) and a You-Prime cDNA synthesis kit (Pharmacia LKB), respectively. In Southern and Northern analysis, 10μg of DNA or RNA was loaded in each lane unless otherwise mentioned. Filter hybridization with DNA probes was carried out for 12hr at 42℃ in the presence of 50% formamide and 5xSSPE, and washed at 37℃ or 50℃ in 0.1xSSC and 0.1% SDS.

PCR was performed with thermal cycler B-641 (KURABO) according to the manufacture's instruction. For RT-PCR, poly(A) RNA was reverse-transcribed and resultant cDNA was amplified with Tth polymerase (TOYOBO) according to Myers and Gelfand (1991).

(B) Culture in an Isolated Condition (low-cell-density culture)

NC4 cells developed in suspension for 5.5h were dispersed by pipetting in 10mM phosphate buffer pH6.4 containing 2mM EDTA-0.9% NaCl, suspended at a

density of $1.5x10^4$ cells /ml in either (1) 20mM phosphate buffer pH7.1 or (2) conditioned medium, and spread onto agarose-coated Nunc dishes at a density of $1x10^3$ cells /cm^2. After 5.5h incubation, 2mM EDTA was added to the medium and the dishes were incubated for 5min. Cells were then detached from substrata by vibrating dishes vigorously and collected by centrifugation at 3000 rpm for 5min. RNA was extracted therefrom.

(C) Preparation of Conditioned Medium

Cells were allowed to develop for 6h in suspension at $1x10^7$cells/ml and then removed by centrifugation. The supernatant was filtered through glass filters (Whatman Paper Ltd., Kent, England; GF/C) and stored at -20℃ after lyophilization.

(D) Cell Type Fractionation

NC4 cells were allowed to develop on filter pads until the migrating slug stage. Prespore and prestalk cells were separated by three cycles of Percoll density gradients (Tsang and Bradbury, 1981; Ratner and Borth, 1983) or by dissection with glass capillaries. When slugs were separated by Percoll gradient, the purity of prespore fraction was more than 99% and that of prestalk fraction was more than 90%, while when separated by dissection, the purity was 90% and 95%, respectively, as judged by immunocytochemistry (Takeuchi, 1963).

(E) Disaggregation of Migrating Slugs

Slugs developed on filters were collected, disaggregated by either pronase-BAL or EDTA-NaCl (Takeuchi and Yabuno, 1970) and suspended in 20mM phosphate buffer (pH6.4) containing 1mM $MgSO_4$ and 1mM EDTA at a density of $1x10^7$ cells /ml. After shaking in suspension for 3h or 6h, cells were harvested for

RNA analysis.

(F) Isolation of Genomic Clones.

The genomic clone EE2900 was isolated from a genomic mini-library. Nuclear DNA of Ax2 cells was digested with EcoRI and separated on a 0.8% TAE-agarose gel. 2.3-4.3kb fragments of digested DNA were collected and inserted into plasmid pUC118. This library was screened with the labeled C6A probe (C6A is a cDNA clone isolated by the differential screening as described in RESULTS). ME900 was isolated by inverse PCR (Triglia et al., 1988; Fig.25). Nuclear DNA which was digested with MboI and ligated to be circularized was used as a template, and amplification was carried out with primers H and I (Fig.17).

(G) Fractionation of Cellular Components

Developed Ax2 cells (in the slug stage) were mildly lysed in the lysis buffer (50mM HEPES (pH7.5), 1% Triton X-100, 10% sucrose and 5mM $MgSO_4$). Lysate was fractionated by differential centrifugation, i.e., at 400g for 5min and then at 2000g for 5min. The first precipitate (P1: cell debris) and the second precipitate (P2: nuclear fraction) were resuspended in lysis buffer. The second supernatant (S2: cytosol-organelle fraction) was further fractionated by sucrose density gradient (15-40%) centrifugation at 100000g for 3hr.

(H) Construction of the Disruption Vector, the Antisense Vector and the Overexpression Vector

The disruption vector was constructed as follows (Fig.26A): EE2900 was inserted into pUC119 at the EcoRI site and a plasmid in which the upstream region of EE2900 was located near the BamHI site was selected (pUC-EE2900S). EE800 fragment was blunted with Klenow fragment and inserted into pUC119 at the HincII site and a plasmid in which the upstream region of EE800 was located near

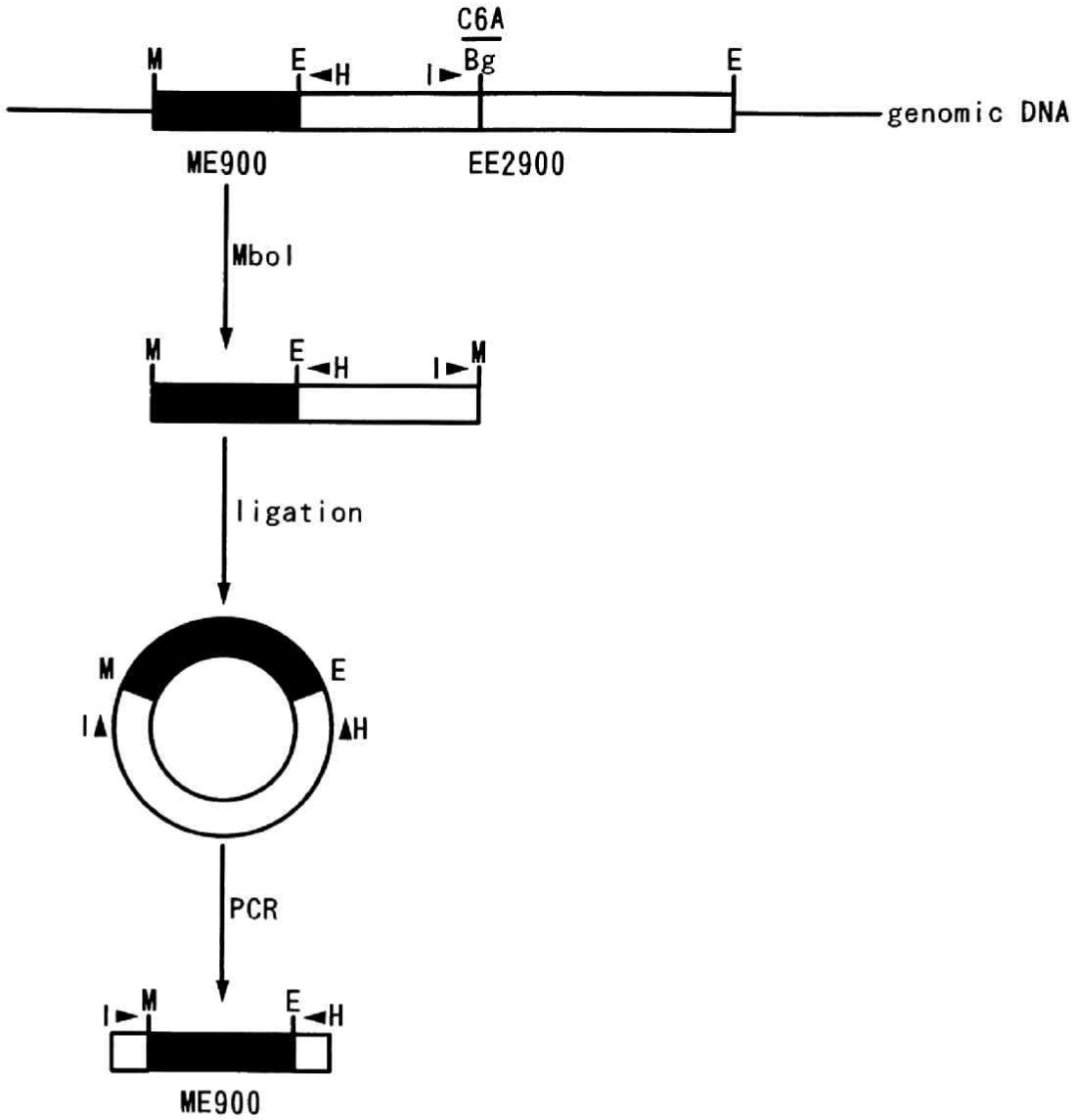


Fig.25. Cloning of ME900 by Inverse PCR.

[Abbreviations]

M ; MboI E ; EcoRI Bg; BglII

(A)

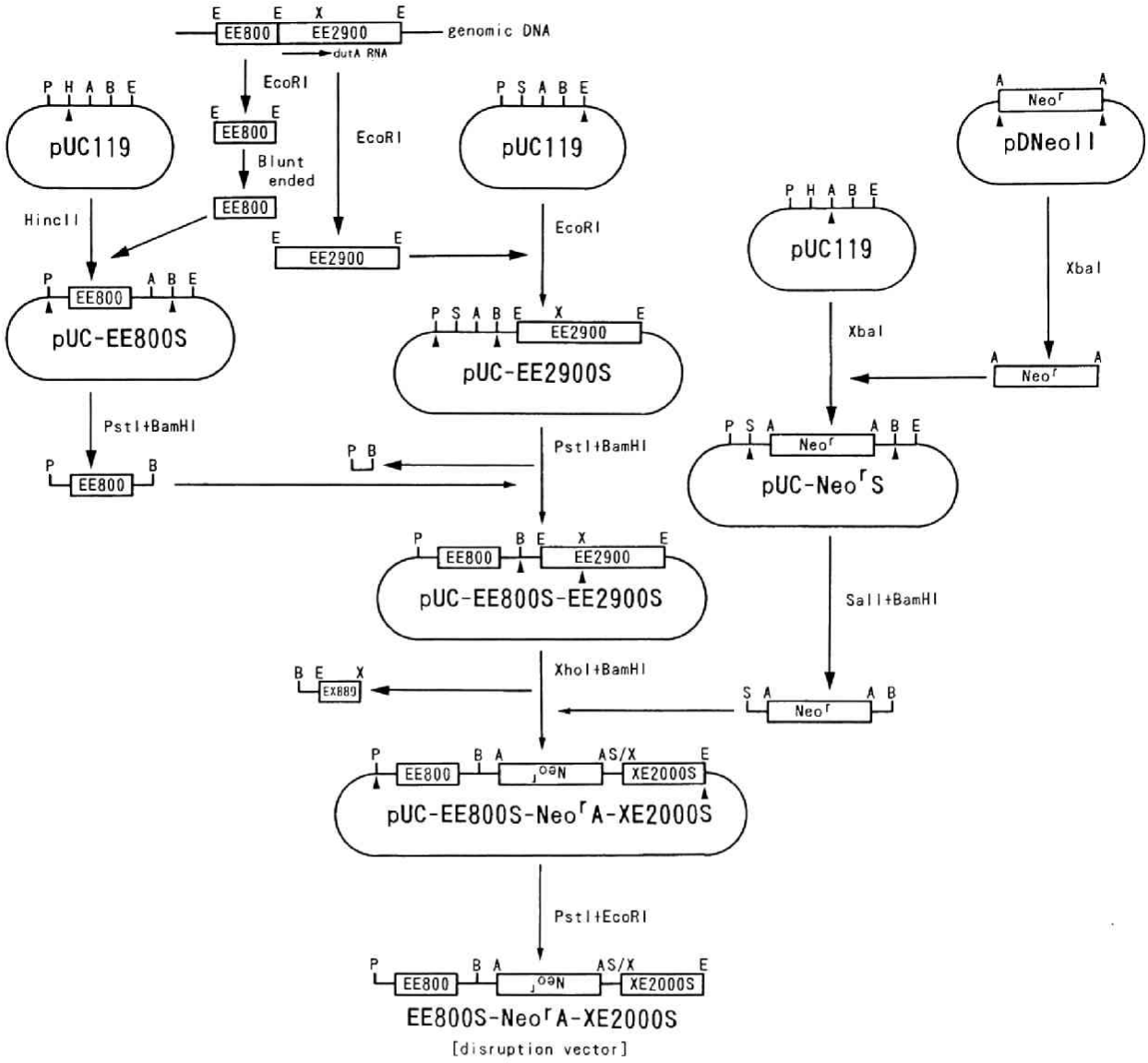


Fig.26. Construction of the Vectors to D. discoideum.

(A) Disruption Vector EE800S-Neo'A-XE2000S

[Abbreviations]

E; EcoRI B; BamHI A; XbaI S; SalI

H; HincII P; PstI X; XhoI D; HindIII

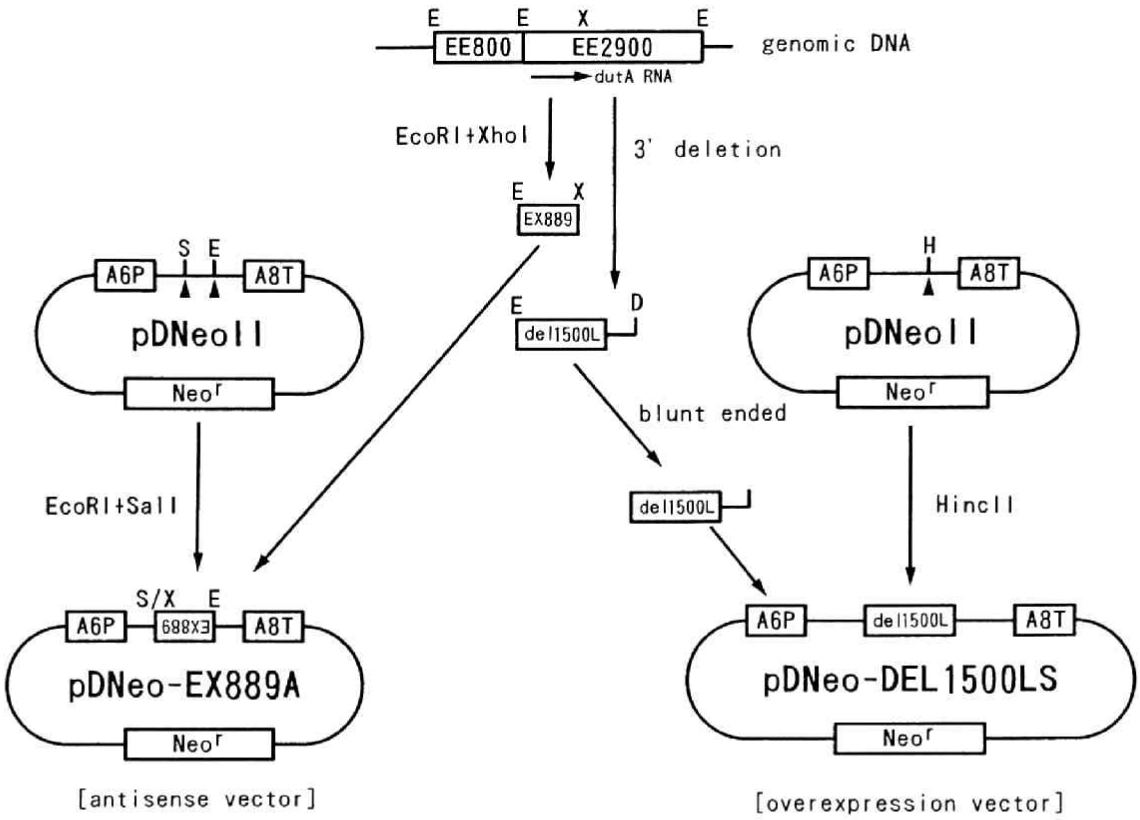
A6P; actin 6 promotor A8T; actin 8 terminator

Neo'; Tn903 neomycin resistant gene with actin 15 promotor and terminator

the PstI site (pUC-EE800S) was selected. BamHI-PstI fragment containing EE800 was excised from pUC-EE800S and inserted into pUC-2900S at the BamHI-PstI site (pUC-EE800S-EE2900S). XbaI-XbaI fragment containing actin 15 promotor-aminoglycoside 3'phosphotransferase gene-actin 15 terminator (Neo^r) was excised from pDNeoII and inserted into pUC119 at the XbaI site and a plasmid in which the upstream region of Neo^r was located near the SalI site was selected (pUC-Neo^{r}S). BamHI-SalI fragment containing Neo^r was excised from pUC-Neo^{r}S and inserted into pUC-EE800S-EE2900S at the XhoI (located in EE2900)-BamHI site (pUC-EE800S-Neo^{r}A-XE2000S). This plasmid was cut with EcoRI and PstI to liberate the insert (EE800S-Neo^{r}A-XE2000S) and Ax2 cells were transformed with it.

To create the antisense vector (Fig.26B, left), EX889 fragment was inserted into plasmid pDNeoII (Witke et al., 1987) at the EcoRI-SalI site to allow the antisense RNA expression (pDNeo-EX889A). As for the overexpression vector (Fig.26B, right), DEL1500L fragment was inserted into the same plasmid in the sense orientation to the actin promotor.

(B)



(B) Antisense Vector pDNeo-EX889A (left)

Overexpression vector pDNeo-del1500LS (right)

[Abbreviations]

E; EcoRI	B; BamHI	A; XbaI	S; SalI
H; HincII	P; PstI	X; XhoI	D; HindIII

A6P; actin 6 promotor A8T; actin 8 terminator

Neo'; Tn903 neomycin resistant gene with actin 15 promotor and terminator

[REFERENCES]

Bailey, J. M. and Davidson, N. (1976). Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. 70, 75-85.

Barklis, E. and Lodish, H. F. (1983) Regulation of Dictyostelium discoideum mRNAs specific for prespore or prestalk cells. Cell 32, 1139-1148.

Barra, J. (1980) pdsA, a gene involved in the production of active phosphodiesterase during starvation of Dictyostelium discoideum amoebae. Mol. Gen. Genet. 177, 607-613.

Bartolomei, M. S., Zemel, S. and Tilghman, S. M. (1991). Parental imprinting of the mouse H19 gene. Nature 351, 153-155.

Berks, M. and Kay, R.R. (1988) Cyclic AMP is an inhibitor of stalk cell differentiation in Dictyostelium discoideum. Dev. Biol. 126, 108-114.

Blum, B., Bakalara, N. and Simpsom, L. (1990) A model for RNA editing in kinetoplastid mitochondria: "Guide" RNA molecules transcribed from maxicircle DNA provide the edited information. Cell 60, 189-198.

Brannan, C. I., Dees, E. C., Ingram, R. S. and Tilghman, S. (1990). The product of the H19 gene may function as an RNA. Mol. Cell. Biol. 10, 28-36.

Brenner, M. and Thoms, S. D. (1984) Caffeine blocks activation of cyclic AMP synthesis in Dictyostelium discoideum. Dev. Biol. 101, 136-146.

Brockdorff, N., Ashworth, A., Kay, G. F., McCabe, V. M., Norris, D. P., Cooper, P. J., Swift, S. and Rastan, S. (1992). The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell 71, 515-526.

Brody, E. and Abelson, J. (1985). The "spliceosome": yeast pre-messenger RNA associates with a 40S complex in a splicing dependent reaction. Science 228, 963-966.

Brown, C. J., Ballabio, A., Rupert, J. L., Lafreniere, R. G., Grompe, M., Tonlorenzi, R. and Willard, H. F. (1991A) A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature 349, 38-44.

Brown, C. J., Lafreniere, R. G., Powers, V. E., Sebastio, G., Ballabio, A., Pettigrew, A. L., Ledbetter, D. H., Levy, E., Craig, I. W. and Willard, H. F. (1991B) Localization of the X inactivation centre on the human X chromosome in Xq13. Nature 349, 82-84.

Brown, C. J., Hendrich, B. D., Rupert, J. L., Lafreniere, R. G., Xing, Y., Lawrence, J. and Willard, H. F. (1992). The human XIST gene: analysis of a 17kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. Cell 71, 527-542.

Brunkow, M. E. and Tilghman, S. M. (1991) Ectopic expression of the H19 gene in mice causes prenatal lethality. Genes Dev. 5, 1092-1101.

Cech, T. R. (1986) Biological catalysis by RNA. Annu. Rev. Biochem. 55, 599-629.

Cech, T. R. (1990) Self-splicing of group I introns. Annu. Rev. Biochem. 59, 543-568.

Clarke, M., Kayman, S. C. and Riley, K. (1987) Density-dependent induction of discoidin-I synthesis in exponentially growing cells of Dictyostelium discoideum. Differentiation 34, 79-87.

Coukell, M. B., Lappano, S. and Cameron, A. M. (1983) Isolation and characterization of cAMP unresponsive (frigid) aggregation-deficient mutants of Dictyostelium discoideum. Dev. Genet. 3, 283-297.

Davies, K. (1991) The essence of inactivity. Nature 349, 15-16.

Eguchi, Y., Itoh, T. and Tomizawa, J. (1991) Antisense RNA. Annu. Rev. Biochem. 60, 631-652.

Elkins, T., Zinn, K., McAllister, L., Hoffmann, F. M. and Goodman, C. S. (1990).

Genetic analysis of a Drosophila neural cell adhesion molecule: interaction of fasciclin I and abelson tyrosine kinase mutations. Cell 60, 565-575.

Fini, M. G., Bendena, W. G. and Pardue, M. C. (1989). Unusual behavior of the cytoplasmic transcript of hsrω: an abundant, stress-inducible RNA that is translated but yields no detectable protein product. J. Cell Biol. 6, 2045-2057.

Firtel, R. A., Van Haastert, P. J. M., Kimmel, A. R. and Devreotes, P. N. (1989) G protein linked signal transduction pathways in development: Dictyostelium as an experimental system. Cell 58, 235-239.

Fukuhara, H. (1982). Restriction map and gene organization of the mitochondrial DNA from Dictyostelium discoideum. Biol. Cell 46, 321-324.

Gerisch, G. (1987) Cyclic AMP and other signals controlling cell development and differentiation in Dictyostelium. Annu. Rev. Biochem. 56, 853-879.

Gesteland, R. F., Weiss, R. B. and Atkins, J. F. (1992). Recoding: reprogrammed genetic decoding. Science 257, 1640-1641.

Green, P. J., Pines, O. and Inouye, M. (1986) The role of antisense RNA in gene regulation. Annu. Rev. Biochem. 55, 569-579.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. and Altman, S. (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell 35, 849-857.

Hao, Y., Crenshaw, T., Moulton, T., Newcomb E. and Tycko, B. (1993) Tumour-suppressor activity of H19 RNA. Nature 365, 764-767.

Hildebrandt, M. and Nellen, W. (1992) Differential antisense transcription from the Dictyostelium EB4 gene locus: implications on antisense-mediated regulation of mRNA stability. Cell 69, 197-204.

Jain, R., Yuen, I. S., Taphouse, C. R. and Gomer, R. H. (1992) A density-sensing factor controls development in Dictyostelium. Genes Dev. 6, 390-400.

Janssens, P. M. W. and Van Haastert, P. J. M. (1987) Molecular basis of

transmembrane signal transduction in Dictyostelium discoideum. Microbiol. Rev. 51, 396-418.

Jermyn, K. A., Berks, M., Kay, R. R. and Williams, J. G. (1987) Two distinct classes of prestalk-enriched mRNA sequences in Dictyostelium discoideum. Development 100, 745-755.

Kaneda, S., Gotoh, O., Seno, T and Takeishi, K. (1983). Nucleotide sequence of Dictyostelium small nuclear RNA Dd8 not homologous to any other sequenced small nuclear RNA. J. Biol. Chem. 258, 10606-10613.

Kay, R. R., Garrod, D. and Tilly, R. (1978) Requirements for cell differentiation in Dictyostelium discoideum. Nature 271, 658-660.

Kay, G. F., Penny, G. D., Patel, D., Ashworth, A., Brockdorff, N and Rastan, S. (1993) Expression of Xist during mouse development suggests a role in the initiation of X chromosome inactivation. Cell 72, 171-182.

Kesbeke, F., Snaar-Jagalska, B. E. and Van Haastert, P. J. M. (1988) Signal transduction in Dictyostelium fgd A mutants with a defective interaction between surface cAMP receptors and a GTP-binding regulatory protein. J. Cell. Biol. 107, 521-528.

Kimelman, D. and Kirschner, M. W. (1989) An antisense mRNA directs the covalent modification of the transcript encoding fibroblast growth factor in Xenopus oocytes. Cell 59, 687-696.

Kimmel, A. R. (ed.) (1988). Molecular Biology of Dictyostelium discoideum development. Alan R. liss, Inc.

Klein, P. S., Tzeli, J. S., Saxe III, C. L., Kimmel, A. R., Johnson, R. L. and Devreotes, P. N. (1988) A chemoattractant receptor controls development in Dictyostelium discoideum. Science 241, 1467-1472.

Kumagai, A. and Okamoto, K. (1986) Prespore-inducing factors in Dictyostelium discoideum. Developmental regulation and partial purification. Differentiation 31, 79-84.

Kumagai, A., Pupillo, M., Gundersen, R., Miake-Lye, R., Devreotes, P. N. and Firtel, R. A. (1989) Regulation and function of G-alpha protein subunits in Dictyostelium. Cell 57, 265-275.

Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843-854.

Leverette, R. D., Andrews, M. T. and Maxwell, E. S. (1992) Mouse U14 snRNA is a processed intron of the cognate hsc70 heat shock pre-messenger RNA. Cell 71, 1215-1221.

Loomis, W. F. (ed.) (1982) The Development of Dictyostelium discoideum. Academic Press, New York.

Loomis, W. F. (1988) Signals that regulate differentiation in Dictyostelium. ISI ATLAS OF SCIENCE: IMMUNOLOGY pp.25-30.

Mahendran, R., Spottswood, M. R. and Miller, D. L. (1991) RNA editing by cytidine insertion in mitochondria of Physarum polycephalum. Nature 349, 434-438.

Mangiarotti, G., Bulfone, S., Giorda, R., Morandini, P., Ceccarelli, A. and Hames, B. D. (1989) Analysis of specific mRNA destabilization during Dictyostelium development. Development 106, 473-481.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Mann, S. K. O., Pinko, C., Firtel, R. A. (1988) cAMP regulation of early gene expression in signal transduction mutants of Dictyostelium. Dev. Biol. 130, 294-303.

Manrow, R. E. and Jacobson, A. (1988) mRNA decay rates in late developing Dictyostelium discoideum cells are heterogenous, and cyclic AMP does not act directly to stabilize cell-type-specific mRNAs. Mol. Cell. Biol. 8, 4088-4097.

Mehdy, M. C., Ratner, D. and Firtel, R. A. (1983) Induction and modulation of cell-type-specific gene expression in Dictyostelium. Cell 32, 763-771.

Myers, T. W. and Gelfand, D. H. (1991). Reverse transcription and DNA amplification by a Thermus thermophilus DNA polymerase. Biochemistry 30, 7661-7666.

Nellen, W., Silan, C. and Firtel, R. A. (1984). DNA-mediated transformation in Dictyostelium discoideum: regulated expression of an actin gene fusion. Mol. Cell. Biol. 4, 2890-2898.

Nellen, W., Datta, S., Reymond, C., Sivertsen, A., Mann, S., Crowley, T. and Firtel, R. A. (1987) In Spudich, J. A. (ed.), Dictyostelium discoideum: molecular approaches to cell biology. Methods in Cell Biology 28, Academic Press, Florida, Vol. 28, pp67-100.

Newell, P. C. and Ross, F. M. (1982) Inhibition by adenosine of aggregation centre initiation and cyclic AMP binding in Dictyostelium. J. Gen. Microbiol. 128, 2715-2724.

Newell, P. C., Europe-Finner, G. N., Small, N. V. and Liu, G. (1988) Inositol phosphates, G-proteins and ras genes involved in chemotactic signal transduction of Dictyostelium. J. Cell Sci. 89, 123-127.

Okamoto, K. (1979) Induction of cyclic AMP phosphodiesterase by disaggregation of the multicellular complexes of Dictyostelium discoideum. Eur. J. Biochem. 93, 221-227.

Ozaki, T., Hasegawa, M., Hamada, Y., Tasaka, M., Iwabuchi, M. and Takeuchi, I. (1988) Molecular cloning of cell-type-specific cDNAs exhibiting new types of developmental regulation in Dictyostelium discoideum. Cell differ. 23, 119-124.

Pachnis, V., Brannan, C. I. and Tilghman, S. M. (1988). The structure and expression of a novel gene activated in early mouse embryogenesis. EMBO J. 7, 673-681.

Podgorski, G. J., Franke, J., Faure, M. and Kessin, R. H. (1989) The cyclic nucleotide phosphodiesterase gene of Dictyostelium discoideum utilizes alternate promoters and splicing for the synthesis of multiple mRNAs. Mol. Cell. Biol. 9, 3938-3950.

Ratner, D. and Borth, W. (1983) Comparison of differentiating Dictyostelium discoideum cell types separated by an improved method of density gradient centrifugation. Exp. Cell Res. 143, 1-13.

Reaban, M. E. and Griffin, J. A. (1990). Induction of RNA-stabilized DNA conformers by transcription of an immunoglobulin switch region. Nature 348, 342-344.

Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T. and Aizawa, S. (1992) Mice develop normally without tenascin. Genes Dev. 6, 1821-1831.

Simons, R. W. (1988) Naturally occurring antisense RNA control: a brief review. Gene 72, 35-44.

Simpson, L. and Shaw, J. (1989). RNA editing and the mitochondrial cryptogenes of kinetoplastid protozoa. Cell 57, 355-366.

Snaar-Jagalska, B. E. and Van Haastert, P. J. M. (1988) Dictyostelium discoideum mutant synag 7 with altered G-protein-adenylate cyclase interaction. J. Cell Sci. 91, 287-294.

Sobolowski, A. and Weeks, G. (1988) The requirement for DIF for prestalk and stalk cell formation in Dictyostelium discoideum: a comparison of in vivo and in vitro differentiation conditions. Dev. Biol. 127, 296-303.

Sollner-Webb, B. (1993) Novel intron-encoded small nucleolar RNAs. Cell 75, 403-405.

Sussman, M. (1987) In Spudich, J. A. (ed.), Dictyostelium discoideum: molecular approaches to cell biology. Methods in Cell Biology 28, Academic Press, Florida, Vol. 28.

Symous, R. H. (1992) Small catalytic RNAs. Annu. Rev. Biochem. 61, 641-671.

Takemoto, K., Takeuchi, I. and Tasaka, M. (1990) cAMP regulation of the expression of prespore-specific genes, SP96 and Dp87, in disaggregated slug cells of Dictyostelium discoideum. Cell Diff. Dev. 31, 89-96.

Takeuchi, I. (1963) Immunochemical and immunohistochemical studies on the development of the cellular slime mold Dictyostelium mucoroides. Dev. Biol. 8, 1-26.

Takeuchi, I. and Yabuno, K. (1970) Disaggregation of slime mold pseudoplasmodia using EDTA and various proteolytic enzymes. Exp. Cell Res. 61, 183-190.

Theibert, A. and Devreotes, P. N. (1984) Adenosine and its derivatives inhibit the cAMP signaling response in Dictyostelium discoideum. Dev. Biol. 106, 166-173.

Triglia, T., Peterson, M. G. and Kemp, D. J. (1988). A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res. 16, 8186.

Tsang, A. and Bradbury, J. M. (1981) Separation and properties of prestalk and prespore cells of Dictyostelium discoideum. Exp. Cell Res. 132, 433-441.

Van Haastert, P. J. M. (1983) Binding of cAMP and adenosine derivatives to Dictyostelium discoideum cells. Relationships of binding, chemotactic and antagonistic activities. J. Biol. Chem. 258, 9643-9648.

Van Haastert, P. J. M., Janssens, P. M. W. and Erneux, C. (1991) Sensory transduction in eukaryotes; A comparison between Dictyostelium and vertebrate cells. Eur. J. Biochem. 195, 289-303.

Walbot, V. (1991) RNA editing fixes problems in plant mitochondrial transcripts. Trends in Genet. 7, 37-39.

Weeks, G. (1984) The spore cell induction activity of conditioned media and subcellular fractions of Dictyostelium discoideum. Exp. Cell Res. 153, 81-90.

Williams, J. G., Ceccarelli, A., McRobbie, S., Mahbubani, H., Kay, R. R., Early,

A., Berks, M. and Jermyn, K. A. (1987) Direct induction of Dictyostelium prestalk gene expression by DIF provides evidence that DIF is a morphogen. Cell 49, 185-192.

Witke, W., Nellen, W. and Noegel, A. (1987). Homologous recombination in the Dictyostelium α-actinin gene leads to an altered mRNA and lack of the protein. EMBO J. 6, 4143-4148.

Wurster, B. (1990) New roles for DIF-effects in early development in Dictyostelium. Dev. Biol. 140, 189-195.

Yamada, Y. and Okamoto, K. (1990) Three steps in prespore differentiation in Dictyostelium discoideum with different requirements of cellular interaction. Differentiation 44, 81-87.

Yoshida, H., Yamada, Y. and Okamoto, K. (1991). DC6, a novel type of Dictyostelium discoideum gene regulated by secreted factors but not by cAMP. Differentiation 46, 161-166.

Young, L. S., Dunstan, H. M., Witte, P. R., Smith, T. P., Ottonello, S. and Sprague, K. U. (1991) A class III transcription factor composed of RNA. Science 252, 542-546.

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