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The *Dictyostelium* Developmental cDNA Project: Generation and Analysis of Expressed Sequence Tags from the First-Finger Stage of Development

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Abstract

In an effort to identify and characterize genes expressed during multicellular development in *Dictyostelium*, we have undertaken a cDNA sequencing project. Using size-fractionated subsets of cDNA from the first finger stage, two sets of gridded libraries were constructed for cDNA sequencing. One, library S, consisting of 9984 clones, carries relatively short inserts, and the other, library L, which consists of 8448 clones, has longer inserts. We sequenced all the selected clones in library S from their 3′-ends, and this generated 3093 non-redundant, expressed sequence tags (ESTs). Among them, 246 ESTs hit known *Dictyostelium* genes and 910 showed significant similarity to genes of *Dictyostelium* and other organisms. For library L, 1132 clones were randomly sequenced and 471 non-redundant ESTs were obtained. In combination, the ESTs from the two libraries represent approximately 40% of genes expressed in late development, assuming that the non-redundant ESTs correspond to independent genes. They will provide a useful resource for investigating the genetic networks that regulate multicellular development of this organism.

Key words: *Dictyostelium discoideum*; cDNA project; ESTs; multicellular development

1. Introduction

The protist *Dictyostelium discoideum* has been widely investigated as a useful model organism to study many basic problems in cell biology. One of the most remarkable aspects of this organism lies in its unique life cycle. *Dictyostelium* cells proliferate as unicellular amoeboid cells, but when their food source is exhausted, they aggregate and form a multicellular mass. It should be emphasized that the unicellular stage (growth phase) and multicellular stage (developmental stage) are clearly separated temporally and that the transition between these two stages is reversible until the onset of terminal differentiation. At the terminal differentiation stage, a fruiting body is formed with two distinct cell types, spores and stalk cells. This process includes most of the basic events of multicellular development such as intracellular signaling, cell differentiation, and pattern formation. Therefore, the elucidation of the molecular basis of morphogenesis of *Dictyostelium* may help us to reach a general understanding of the basic mechanisms involved in the establishment of multicellular organization in higher organisms.

In an effort to understand genetic networks involved in multicellular development, we initiated a cDNA sequencing project with the aim of identifying and characterizing genes expressed during development by analysis of expressed sequence tags (ESTs).[1] For this purpose, we constructed cDNA libraries from the first finger stage

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cells (14–16 hr of development). At this stage, cell differentiation and pattern formation have already been well established in the multicellular mass and the number of genes expressed is known to increase dramatically: about 7600 genes are estimated to be expressed as compared with about 4800 genes expressed during the growth phase and early development.[2] The cDNA clones were gridded out in 96-well multidishes.

In this paper, we report the construction and characterization of the cDNA libraries and summary of partial sequence analysis of cDNA clones. We have sequenced from the 3′-ends of selected clones from libraries named S and L, and deposited the obtained 3′-ESTs into the *Dictyostelium* cDNA database (*Dicty*_cDB, http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html) and GenBank/EMBL/DDBJ databases.

2. Materials and Methods

2.1. Strain, culture and development of D. discoideum

D. discoideum Ax4 cells were grown axenically in HL-5 medium[3] to 3×10^6 cells/ml and harvested by centrifugation. After repeated washing with LPS buffer (20 mM KCl, 0.24 mM $MgCl_2$ and 40 mM K_2HPO_4/KH_2PO_4, pH 6.4), cells were developed on nitrocellulose filters (TOYO Advantec) saturated with LPS buffer at a density of 5×10^6 cells/cm^2 at 22°C.

2.2. Isolation of total and polyadenylated RNA

Cells were harvested at the first-finger stage (14–16 hr development) on nitrocellulose filters, washed with 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.4) containing 20 mM EDTA and total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method using ISOGEN reagent (WAKO Chemicals Inc.) with the standard protocol of the manufacturer. Poly(A)$^+$ RNA was purified using oligotex-dT30 Super (Nihon-Roche), on which oligo-dT was covalently linked to latex particles.[4] The purification step was performed twice. From 4×10^8 cells, 18 μg of poly(A)$^+$ RNA was recovered.

2.3. Construction of cDNA library

DNA synthesis was performed using the SuperScript Plasmid System (GIBCO BRL, Life Technologies, Inc.) following the standard protocol of the manufacturer. Using 5 μg of poly(A)$^+$ RNA, the first strand cDNA was primed by an oligo $(dT)_{15}$ primer carrying a *Not* I adapter. After the second strand synthesis of cDNA, the termini of the cDNAs were made blunt by T4 DNA polymerase. *Sal* I adapters were ligated to both ends, and the *Not* I site at the 3′-end was generated by *Not* I digestion. The cDNAs were size-fractionated on a cDNA Size Fractionation Column (BRL) to recover the fraction over about 0.4 kb (fraction S). The size fraction over about 1.0 kb (fraction L) was obtained by 1% SeaPlaque GTG agarose (FMC) gel electrophoresis. The recovery of cDNAs from agarose was performed using GELase (Epicentre Technologies Corp.) according to the standard protocol of the manufacturer.

The cDNAs included in fraction S were ligated to the *Sal* I-*Not* I sites of pBluescript II KS- (Stratagene) and transformed into *Escherichia coli* DH5α by electroporation with *E. coli* Pulser (Bio-Rad) set at 18 kV/cm to generate library S which contained more than 6.8×10^5 recombinant clones. Complementary DNAs of fraction L were ligated into the corresponding sites of pSPORT1 (GIBCO BRL, Life Technologies, Inc.) and transformed into *E. coli* DL795 cells [K12 SH28 *Dhsd, mcrBC, mrr; e14 (mcrA)0; sbc201; recA*::Cm^r; *sup*E44], kindly provided by Dr. David Leach in University of Edinburgh and Dr. Masashi Fukuzawa in University of Dundee. In the same way, library L containing more than 1.8×10^5 recombinant clones was generated.

2.4. Template preparation and DNA sequencing

Template DNA was prepared as double-stranded plasmid DNA using a 96-Well Alkaline Lysis Miniprep Kit (Advanced Genetic Technologies Corp.) following the standard protocol of the manufacturer. Using the kit, 1–3 μg of plasmid DNA was obtained from 1 ml of culture. To measure the insert size of cDNA, the plasmid DNAs from library S were digested with *Sac* I and *Apa*I, and those from library L with *Pst* I and *Bam*HI, and subjected to 1% agarose electrophoresis.

Sequencing reactions were performed on plasmid templates using a Perkin-Elmer 9600 or 9700 Thermal Cycler and Applied Biosystems PRISM Ready Reaction Dye Terminator Cycle Sequencing Kit or Amersham DYEnamic Direct cycle sequencing kit. The reaction was primed by the M13 forward primer. Reaction products were precipitated with 99.5% ethanol and dried under a vacuum, and were stored at −20°C in the dark until electrophoresis. For sequencing, the products were suspended in sample loading buffer (83.3% formamide, 4.2 mM EDTA and 8.3 μg/ml blue-dextran) and run on a 373A, 377, or 377XL automated DNA sequencer (Perkin-Elmer, Division of Applied Biosystems).

2.5. Sequence data analysis

The nucleotide sequence data was deposited in the database (*Dicty*_cDB) after correction of ambiguous basecallings and removal of vector and primer/adapter sequences, and also in the GenBank/EMBL/DDBJ data banks with accession numbers AU033318–AU034147, AU036913–AU039189, AU039498–AU039526, AU040037–AU040094, C24606–C24688, C25499–C25576, C25578–C25687, C83826–C84252, C84677–C84905, C85032–C85043, C89607–C90875, C90877–C91581, C91900–C93423, C93682–C93774,

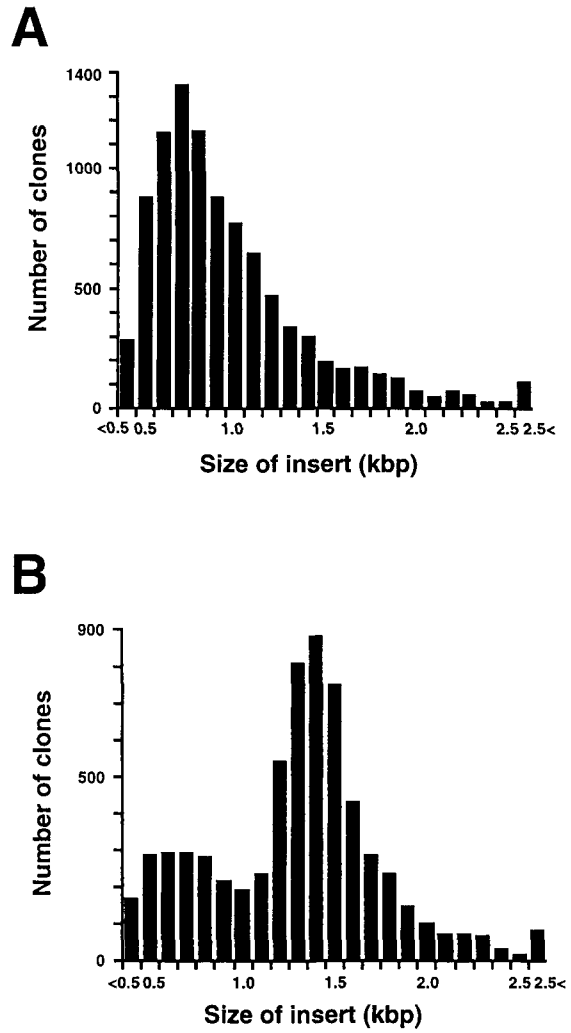

Figure 1. Distribution of inserted cDNA sizes in library S (panel A) and library L (panel B). The insert sizes were determined as described in Materials and Methods.

C93776–C93849, and C93851–C94532. The data were checked for overlap with the existing cDNA sequences and examined for similarities in the GenBank, EMBL, and DDBJ nucleic acid databases. They were also translated in all six reading frames, and each translation was compared with the protein sequence database Protein Information Resource (PIR) and SWISS-PROT. All similarity searches were performed using the BLAST algorithm.[5]

3. Results and Discussion

3.1. Construction and characterization of cDNA libraries

Poly(A)$^+$ RNA was isolated from the first finger stage of development of *D. discoideum* strain Ax4. Complementary DNA was synthesized using an oligo (dT) primer. The sizes of the obtained double-strand cDNA ranged from 0.2 kb to 10 kb (data not shown). Using the size fractionated cDNAs, two libraries, termed S and L, were constructed from the fraction greater than 0.4 kb and that greater than 1 kb, respectively. Both the cDNA fractions were unidirectionally ligated into vector plasmid, pBluescript II KS- or pSPORT1, and transformed into *E. coli*. For library S, 9984 clones were gridded out in 96-well microtiter plates, and 8448 clones were gridded for library L.

Table 1. Summary of insert data.

	Library S	Library L
Total Clones	9984	8448
With Insert	9378	6450
Uncut	64	712
No insert	330	1209
No plasmid	212	77
Mean Length Inserts (kb)	0.96	1.31

The insert sizes of the plasmid DNAs were determined as in Materials and Methods. With Insert: clones with cDNA insert longer than 400 bp, Uncut: clones produced no linear fragment by restriction enzyme digestion, No insert: clones with no cDNA insert, but only containing linearized vector plasmid, No Plasmid: clones with no DNA fragment found on electrophoresis

For preparation of sequencing templates, plasmid DNAs were isolated by the 96-well alkaline lysis method. After digestion with appropriate restriction enzymes to excise cDNA inserts, the distribution of insert sizes was determined by electrophoresis on 1% agarose (Fig. 1). For library S, 9378 clones gave discrete insert bands (represented as 'With Insert' in Table 1). The size distribution of the inserts showed a peak between 0.5 kb and 1 kb (Fig. 1A), and the mean length of the inserts was estimated to be 0.96 kb. For library L, the inserts could be determined for 6450 clones. The mean insert size was estimated to be 1.31 kb. As shown in Fig. 1B, the major peak of the inserts was present between 1.2 and 1.5 kb, although an additional small peak is also seen between 0.5 and 0.8. Insert size analysis of the two libraries apparently indicates that libraries S and L, respectively, represent the classes of genes coding for relatively short and long mRNAs.

Sixty-four clones in library S and 712 clones in library L, which are represented as 'Uncut' in Table 1, apparently showed the plasmid sizes containing inserts, but no inserts were generated by restriction enzyme cleavage. This might be due to partial deletion or rearrangement of cDNA inserts in *E. coli* cells. As some of these clones are expected to retain part of the cDNA inserts, they were also subjected to sequencing.

Table 2. Summary of sequenced clones.

	Library S	Library L
Total ESTs	7663	1132
Non-redundant ESTs	3093 (40.4%)	471 (41.6%)
Known *Dictyostelium* genes	246 (8.0%)	78 (16.5%)
Homologs	910 (29.4%)	159 (33.8%)
Novel genes	1937 (62.6%)	234 (49.7%)

Non-redundant ESTs: EST groups considered to be derived from independent genes, Known *Dictyostelium* genes: genes showing significant similarity to *Dictyostelium* genes in the GenBank, EMBL and DDBJ nucleic acid databases with *P*-value <e-70, Homologs: genes whose longest open reading frame showed similarity to proteins of *Dictyostelium* or other organisms in the PIR and SWISS-PROT protein sequence databases with *P*-value < e-10.

3.2. Sequence analysis of library S

Clones expected to have cDNA inserts ('With Insert' and 'Uncut' in Table 1) were sequenced from their 3′-ends. Sequences less than 150 bp or those with more than 2% ambiguity were rejected. Among the 9444 clones sequenced, significant sequence data as ESTs that might permit identification of genes as ESTs were obtained from 7663 clones (81.2%, see Table 2). The obtained ESTs were compared with each other using the BLAST algorithm,[5] and those showing similarity with a *P*-value < e-60 were clustered into non-redundant EST groups. Out of 7663 ESTs, 3093 non-redundant groups were identified. Then the ESTs were examined for homology in the GenBank, EMBL and DDBJ nucleic acid databases. In addition, the sequences were translated into three possible reading frames, and the longest open reading frames were compared with the Protein Information Resource (PIR) and SWISS-PROT protein sequence databases. Out of 3093 independent groups, 246 groups were assigned to previously sequenced *Dictyostelium* genes and 910 groups to those with significant similarity (*P*-value < e-10) to genes of *Dictyostelium* or other organisms (Table 2). Assuming that the non-redundant EST groups correspond to independent genes, the frequency of gene appearance in library S was indicated in Fig. 2A. Among the independent genes, 2559 genes were represented by either one or two ESTs. As listed in Table 3, the cDNA sizes of the genes with the highest frequency of appearance were relatively short and ranged from 0.6 kb to 1.2 kb. This is consistent with the view that library S mainly represents genes encoding relatively short mRNAs, and thus the frequency of gene appearance in this library may reflect the expression profile of shorter genes.

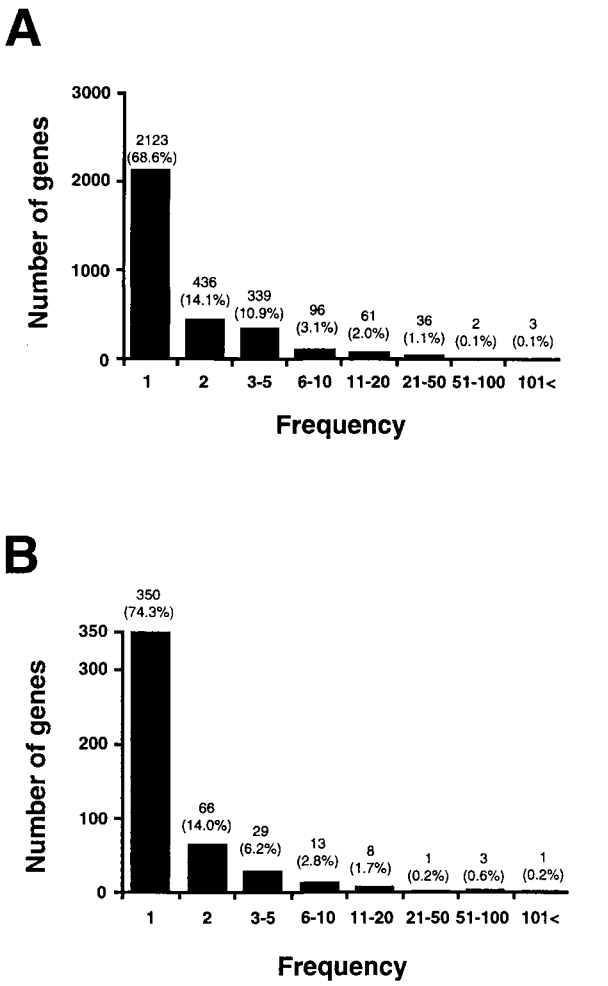

Figure 2. Frequency of gene appearance in the cDNA clones from library S (panel A) and library L (panel B). Among 7663 ESTs from library S and 1132 from ESTs library L, 3093 and 471 independent EST groups were defined respectively (see Table 2), and their frequency of appearance is shown.

3.3. Sequence analysis of library L

A total of 1262 clones from library L were randomly sequenced from their 3′-ends, and 1132 ESTs (89.7% of the sequenced clones, Table 2) derived from 471 non-redundant EST groups were obtained. Searching against nucleotide and amino acid sequence databases revealed that the EST groups hit 78 known *Dictyostelium* genes, homologs of 159 known genes and 234 unknown genes (Table 2). The profile of frequencies of gene appearance was similar to that of library S: 88.3% (416) of the groups were represented by just one or two EST(s) (Fig. 2B). However, as shown in Table 4, the actin 15 gene, the elongation factor 1α gene (*efaA*), several prespore-specific genes (Dp87, *cotA*, *cotB*, *cotC*, *psvA* and *pspD*) and the cAMP-inducible, cysteine protease gene (*cprB*), which are known to be highly expressed during development,[6,7,8,9,10,11,12,13] were more abundant. Among them, *efaA*, Actin 15, Dp87 and *cotA* comprise

Table 3. Genes frequently appeared in library S.

Representative clone	Gene	Accession No.	mRNA size (kb)	Abundance (%)	References
SSA101	*dutA*	D16417	1.2	3.8	15
SSA360	BP74*	A34095	—	1.4	16
SSA238	*cbpA*	X82784	0.6	1.3	17
SSA357	—	—	—	0.9	—
SSA420	PEPT2*	Q63424	—	0.8	18
SSA419	*rasB*	M96622	0.7	0.7	19
SSA362	*ubqA*	M23752	1.1	0.6	20
SSA153	ORAB-1*	P22125	—	0.6	21
SSA225	—	—	—	0.6	—
SSA321	—	—	—	0.6	—
SSA592	*vatP*	X90516	0.7	0.6	22
SSA202	—	—	—	0.6	—
SSA213	*cprB*	X03344	1.3	0.6	13
SSA116	*pkiA*	U61986	0.7	0.6	—
SSB562	—	—	—	0.6	—
SSA132	—	—	—	0.6	—

Among 7666 ESTs of library S genes whose abundance is more than 0.5% were listed. Asterisks show putative homologs and their accession number in the PIR or SWISS-PROT protein sequence databases is shown.

Table 4. Genes frequently appeared in library L.

Representative clone	Gene	Accession No.	mRNA size (kb)	Abundance (%)	References
SLA107	*efaA*	X55972	1.8	10.8	6
SLA105	Actin 15	M14146	1.3	6.7	7
SLA106	Dp87	D13973	2.0	5.7	8
SLA112	*cotA*	X16491	2.2	4.5	9
SLA111	60S ribosomal protein L3	L08391	1.3	2.3	23
SLA246	60S ribosomal protein L1*	P49691	—	1.6	24
SLA121	*sahA*	M19937	1.6	1.6	25
SLA116	*psvA*	X57149	2.2	1.3	10
SLA195	*cotC*	M26239	1.8	1.3	11
SLA123	*cotB*	M26238	2.2	1.0	11
SLA203	*pspD*	U25144	2.1	1.0	12
SLA267	*cprB*	X03344	1.3	1.0	13
SLA193	*kspB*	X75263	2.4, 2.6	1.0	26

Among 1132 ESTs of library L genes whose abundance is more than 1.0% are listed. Asterisks show putative homolog and their accession number in the PIR or SWISS-PROT protein sequence databases is shown.

more than a quarter of all ESTs.

For further characterization of the genes, we are carrying out the following investigations: (1) determination of spatial and temporal expression profile of the identified genes by probing the high-density gridded filters on which the cDNA clones were blotted and *in situ* hybridization experiments,[14] and (2) analysis of the function of individual genes by antisense mutagenesis, gene disruption and ectopic expression.

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