Quantitative Analysis of Gene Expression Pattern in *Aspergillus nidulans* Mycelia by Sequencing of 3′-directed cDNA Clones

Yoon-Dong Park, Dong Whan Lee, Seog Jae Lee[1], Jong Hwa Kim[2] and Keon-Sang Chae*

Department of Molecular Biology,
[1]Department of Chemistry, Chonbuk National University, Chonju 561-756, Korea
[2]Department of Food Science and Engineering, Woosuk University, Chonbuk 565-800, Korea

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Since sequencing of randomly selected cDNA clones has been known to be a powerful approach to obtain information on gene expression pattern in specific cells or tissues, we have analyzed a 3′-directed cDNA library of vegetative mycelia of *A. nidulans* by single-pass sequencing of hundreds of randomly selected clones. Sequencing of 292 cDNA clones yielded 209 gene signatures (GSs) probably representing highly or lesser expressed genes in the vegetative mycelia. Among the 209 GSs, 25 (70 cDNA clones) appeared more than once and 184 only once. One GS appeared at a highest frequency of 6 times, 2 GSs 5 times, 4 GSs 4 times, 2 GSs 3 times and 16 GSs twice. About 6.6% GSs comprizing of 13 GSs showed alternative polyadenylation. Among 25 redundant GSs, three were common in both mycelia and sexual organs, and 22 were probably mycelia-specific. Out of 209 GSs, 36 were identified in GenBank showing of 70% or greater similarities. Only six GSs were for *A. nidulans* genes, and 13 GSs were of DNA or genes encoding cytoplasmic or organellar proteins. This pattern is similar to those in the human HepG2 cell line and in human colonic mucosa, although very few genes for nuclear proteins and for protein synthesis were in *A. nidulans*.

Key words: ***A. nidulans*, 3′-directed cDNA library, gene signature, single-pass sequencing, vegetative mycelia**

When *A. nidulans* having three life cycles grows asexually, vegetative mycelia grow at the apical tips with branches. During the growth, serial morphogenic developments proceed: formation of mycelia, foot cells, stalks, vesicles, buds of metula, phialides, and conidia. Various kinds of efforts to understand the above morphogenic developmental process have been addressed on a gene level. As results of the efforts, the mutants defective in each step were isolated by complementing defective mutations and the genes which participate in the asexual development, *trpC* (25), *brlA* (2, 3), *abaA* (9), *wetA* (13) and *yA* (15) were isolated. Using the mutants defective in conidiation, Clutterbuck calculated about 45~150 loci which may be involved in the asexual development (6), and Timberlake (24) demonstrated that the 1,200 cDNAs from conidiating mycelia may be involved in conidiation, but analyses have not been done yet.

Since only one 3′-directed cDNA molecule is known to be made from one mRNA molecule, single-pass sequencing of 3′-directed cDNA clones to generate gene signatures (GSs) is a rapid and efficient way to establish a detailed profile of gene expression pattern in a cell type. The GSs or expressed sequence tags (ESTs) can also be used for isolation of full-length cDNA clones. Okubo *et al.* (18) sequenced and analyzed about 1,000 randomly selected 3′-directed cDNA clones from more than 10 human cell lines and tissues including the HepG 2 cell line, the HL60 cell line, and the colon mucosa (7, 18, 19, personal communication). They found some abundantly appearing GSs in specific tissues. Recently, similar studies were carried out in many organisms including human (1, 11), *Plasmodium falciparum* (4), *Brassica napus* (20), mouse (14), and rice (23).

In this report, a 3′-directed cDNA library of vegetative mycelia of *A. nidulans* was constructed to analyze the gene expression pattern in the mycelia, and hundreds

* To whom correspondence should be addressed.

of randomly selected 3′-directed cDNA clones in the library were sequenced. Furthermore, similarities between cDNA clones, and between cDNA clones and GenBank entries were also analyzed.

Materials and Methods

Preparation of mRNA from *A. nidulans*

A wild type strain, *A. nidulans* FGSC4, was grown in a liquid complex medium for 18 hrs (8). Mycelia were harvested by filtration through a nylon filter. After disruption of the mycelia by a mortar in the presence of liquid nitrogen, total RNA was isolated by ultracentrifugation of the cell extract according to the method of Chirgwin *et al.* (5). Cellular poly(A)$^+$ RNA was prepared by oligo-(dT) cellulose column fractionation (21).

Construction of 3′-directed cDNA library

A 3′-directed cDNA library was constructed by the procedure of Okubo *et al.* (17) in pUC19 prepared from *dam*$^+$ *E. coli* JM109 (21). In this library, one cDNA molecule can be assumed to be made from one mRNA molecule.

Preparation of sequencing templates by asymmetric PCR

Sequencing templates were prepared by asymmetric PCR with a minor modification as described elsewhere (12). A small cell paste of a randomly selected transformant colony on the LB plate was used directly as a template for asymmetric PCR to minimize steps for preparation of templates. The primers used for asymmetric PCR were pUC/M13 universal primers (#1224 and #1233; New England Biolabs, MA, USA). The ratio of the one primer to the other was one-50th and dNTP concentration was reduced to 50 μM to minimize misincorporation errors. PCR was performed in a 50 μl reaction mixture containing 1.5 U of *Taq* DNA polymerase (Promega, WI, USA) on Temp-tronic PCR machine (Thermolyne, IA, USA) for 30 cycles at the following profile- 95℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 2 min followed by a final extension for 10 min at 72℃. After amplification, the single-stranded DNA products were identified on 1% TAE agarose gel (21).

DNA sequencing and GenBank search

DNA sequences were determined by the dideoxynucleotide chain termination method of Sanger *et al.* (22) using Sequenase (ver 2.0, USB, OH, USA) and [α-^{35}S]-dATP (Amersham, Bucks, UK). DNA sequence similarities between cDNA clones, and between cDNA clones and GenBank entries were analyzed using Blast program at the National Center for Biotechnology Information (NCBI, MD, USA). The individual cDNA sequences are available on request.

Table 1. Summary of cDNA sequence analysis

	No. of cDNA clones	GS species	Percentage (%)
Total	292		
<20 nt	38		
<20 nt	254	209	100
redundant group	70	25	27.6
solitary group	184	184	72.4
poly(A) identified alternative polyadenylation	150	111 13	59.1
poly(A) unidentified	104	98	40.9

Results and Discussion

Analysis of cDNA library by single-pass sequencing

A 3′-directed cDNA library was made using *A. nidulans* mRNA isolated from mycelia by vector-priming method (16). The cDNA moiety and the vector were selectively cleaved by *Mbo*I and *Bam*HI, respectively, and the 3′-directed regional cDNA library was constructed by religation of the products (17). By this method, one vector molecule has one cDNA insert. Hence, cDNA population in this library can faithfully represent mRNA population in a specific cell type.

Okubo *et al.* (18) reported that 51% of the 982 cDNA clones were abundant species consisting of 173 GSs and that the rest were less-abundant in the HepG2 cell line. Taken together, since the *A. nidulans* genome is smaller than the human one by an order of two, we assumed that about 300 cDNA clones were enough to represent abundant class cDNA species. Totally, 292 transformant colonies were selected randomly. The cDNA moieties were amplified by asymmetric PCR, and sequenced. Results of sequence analysis are summarized in Table 1. Among the 292 clones analyzed, 38 were excluded from further analysis due to having inserts shorter than 20 bp. Since 113 nucleotides per a clone were read in average, poly(A) sequence of only 111 species could be determined, and the exact insert sizes of some clones were difficult to determine from the sequences. Sequences of the 254 clones were compared with each other (Table 1). Among the 254 clones, 70 clones (27.6%) appeared more than once (redundant group) representing 25 different GSs, and 184 clones (72.4%) appeared only once (solitary group). The 27.6% of the redundant group was about half of 51% of the redundant group in the HepG2 cDNA library (18) and was similar to 27% of the redun-

Table 2. Summary of analysis of the redundant GSs

GS	Size	Freq.	Homo.sex.	>90%	80~89%	70~79%	GenBank No.	%	Start-term.	Target.total	Definition
Vg0222	111	6									
Vg0129	48	5				LACXPDAP	M58315	79%	310~344	3818	*L. lactis* cremoris dipeptidyl peptidase (pep
Vg0472	87	5	se0272								
Vg0568	30	4	se0605			HUMSWX14	L08881	77%	11~41	195	*H. chromosome*×STS sWXD145, single rea
Vg0544	121	4				PFTUBAI	X15979	70%	1180~1234		*Plasmodium flaciparum* α-tubulin I gene
Vg0226	95	4									
Vg0389	121	4									
Vg0174	207	3		ASNG034ARA			D32070	98%	750~861	935	*A. nidulans* heat shock protein
Vg0314	214	3									
Vg0795	216	2		ANADH3			X02784	96%	1514~1600	1676	*A. nidulans* alcohol dehydrogenase 3
Vg0433	39	2			BEUMTA		D38016	84%	1845~1869	2040	Mitochondrion sugar beet gene for NADH de
Vg0108	39	2				WESTO307	T02355	75%	167~199	257	*Caenorhabditis elegans* cDNA clone CEsAC62
Vg0123	106	2									
Vg0161	28	2									
Vg0248	93	2									
Vg0276	95	2									
Vg0342	126	2	se0428								
Vg0453	165	2									
Vg0479	212	2									
Vg0483	116	2									
Vg0677	130	2									
Vg0694	144	2									
Vg0720	119	2									
Vg0790	213	2									
Vg0791	127	2									

From left to right, each column represents GS number, size of the GS, frequency of the GS among 292 clones, clone number of identical sequence in a sexual organ-library, locus name, accession number of corresponding GenBank entry (target), similarity percent, and similarity region, size and definition of the target.

Table 3. Summary of analysis of GSs on similarity

GS	Size	Match. size	Freq.	Homo.sex	>90%	80~89%	70~79%	GenBank No.	%	Start-term.	Target.total	Definition
Vg0133	92	83	1		SMEPGK			M27549	100%	2078~2161	3720	*A. nidulans* 3-phosphoglycerate kinase
Vg0174	207	111	3		ASNG034ARA			D32070	98%	750~861	935	*A. nidulans* heat shock protein
Vg0795	216	86	2		ANADH3			X02784	96%	1514~1600	1676	*A. nidulans* alcohol dehydrogenase 3
Vg0326	49	44	1		EMEFLUG			L27817	95%		3960	*Emericella nidulans* fluG) gene, complete cds
Vg0379	69	55	1		ANU03520			U03520	94%	52/55	200	*A. nidulans* paba A9 biA1 internaltranscribed spacer 1&18
Vg0428	168	151	1	se0773	EMEACTINGA			M22869	93%	2170~2321	2686	*Emericella nidulans* gamma actin gene exon 1-6
Vg0122	31	22	1		APSYMSL			X61150	91%	2377-2399	2739	*A. pisum* symL gene for symbionin
Vg0154	29	21	1		BACHYP2			D31629	90%	13820~13841	18204	*Bacillus subtiis* gene for hypothetical protein
Vg0346	43	25	1			HUMMAGE12X		L18877	88%	4457~4482	4523	Human MAGE-12 protein gene
Vg0761	31	24	1			XELTGB		K02456	88%	192~216	232	*X. laevis* variant Met-tRNA-β gene
Vg0575	43	32	1			DDIDP87		D13973	87%	1485~1517	4458	Dictyostelium discodium Dp87 gene
Vg0113	42	22	1			HYDSTK		M25245	86%	1910~1932	2019	*H. attanuata* src-realated protein p57(STK)mRNA, comple
Vg0266	43	22	1			YSCL9476		U28372	86%	25264~25286	31184	*S. cerevisiae* chromosome IV cosmid
Vg0433	39	24	2			BEUMTA		D38016	84%	1845~1869	2040	Mitochondrion sugar beet gene for NADH dehydrogenase
Vg0251	27	25	1	se0143		ye16d02.r1		T90412	84%	180~205		*H. sapiens* cDNA clone117891 5'similar to contains Alu re
Vg0267	42	25	1			HUMHHIRE		D14436	84%	4452~4477		Human gene for hi-receptor, complete cds.
Vg0441	40	27	1			YSCATPSU8X		L20786	82%	2201~2228	2457	*S. cerevisiae*ATP synthase subunit 8 gene
Vg0139	36	24	1			HSHLTF1		Z46606	80%	2100~2124		*H. sapiens* HLTF-1 gene for helicase
Vg0143	28	25	1			GGASPAT		X15636	80%	1093~1118	1791	Chicken mRNA for aminotransferase
Vg0225	117	95	1			Yc01a02.S1		T57663	80%	174~170		*H. sapiens* cDNA clone
Vg0129	48	34	5				LACXPDAP	M58315	79%	310~344	3818	*L. lactis* cremoris dipeptidyl peptidase(pep XP)gene, compl
Vg0455	206	95	1				HTOHSP82	M55629	79%	2556~2651		*Histoplasma capsulatum* heat shock protein
Vg0464	65	60	1				WMMITDNA	X67618	79%	1145~1205	2184	*W. mrakii* mitochondrial DNA
Vg0634	169	97	1				NCRPSSU	X55637	79%	292~389	685	*N. crassa* mRNA for a ribosomal protein
Vg0148	37	29	1				UMPRPC4S	M62486	78%	78~107	428	Human c4b-binding protein gene
Vg0458	69	45	1				SHEFLAVCA	L04283	78%	8389-8434	3323	*Shewannella putrefaciens* flavocytochrom c gene
Vg0568	30	30	4	se0605			HUMSWX145	L08881	77%	11~41	195	*H. chromosome*×STS sWXD145, single read.
Vg0323	41	35	1				HIVED91DZ	M61346	77%	44~79	336	human immunodeficiency virus type 1(v91691), v3 region.
Vg0473	70	44	1				MUSFERRC	M59288	77%	943~987	2163	Musmusculus ferrochelatase mRNA, 3'region
Vg0108	39	32	2				WEST03076	T02355	75%	167~199	257	*Caenorhabditis elegans* cDNA clone CEsAC62
Vg0381	55	36	1	se0418			CEM04D8	Z32682	75%	17119~17155	35346	*Caenorhabditis elegans* cosmis M04D8
Vg0179	57	37	1				ASU03762	U03762	73%	7568~7605	20500	African swine fever virus Malawi Li 1-20/1 multigene fami
Vg0134	42	35	1				HRU07753	U07753	72%	1805~1840	2359	*Human hotavirus* 107E1B(VP4)gene, complete cds.
Vg0416	265	163	1				yc62c03.sl	R23340	72%	65~228		*H. sapiens* cDNA clone 131403
Vg0241	58	38	1				MHC4SLSP	L13459	71%	1983~2021	2170	*Mentha spicata* 4s-limonene synthase mRNA
Vg0544	121	54	4				PFTUBAI	X15979	70%	1180~1234		*Plasmodium flaciparum* α-tubulin I gene

From left to right, each column represents GS number, size of the GS, matching size, frequency of the GS among 292 clones, clone number of identical sequence in a sexual organ-library, locus name, accession number of corresponding GenBank entry (target), similarity parcent, and similarity region, size and definition of the target.

dant group in sexual-specific cDNA library of *A. nidulans* (10). This result suggested that the gene expression pattern in the *A. nidulans* mycelia may be quite different from that of the HepG2 cell line, and that the abundance of specific mRNA populations both in mycelia and the sexual organs of *A. nidulans* may be similar. As shown in Table 2, of the redundant group, 1 GS appeared at the highest frequency of 6 times, 2 GSs 5 times, 4 GSs 4 times, 2 GSs 3 times, and 16 GSs twice. The percentage of 6 time appearance of the most abundant GS was 2.8%, similar to 2.2% of Okubo *et al.* (18). Furthermore, 7 GSs out of 25 were identified in GenBank.

Also, alternative polyadenylation was found (Table 1). Thirteen GSs out of the 25, which appeared more than twice with poly(A) sequence, showed different polyadenylation sites. The consensus sequence of polyadenylation signal in *A. nidulans* mRNAs is expected to be found from the 111 GSs.

Identification of mycelia-specific GSs

We also sequenced about three hundreds of 3′-directed cDNA clones of sexual organs of *A. nidulans,* and compared sequences of mycelial GSs with those of sexual organ GSs. Among 209 GSs, 20 GSs were also identified in the 3′-directed cDNA library of sexual organs (data not shown). Moreover, only three out of 25 redundant GSs were common in both mycelia and sexual organs, and the rest seemed mycelia-specific (Table 2). Seven GSs were identified in GenBank, and 18 were noble. Almost all redundant GSs in the sexual organ-cDNA library but not found in this mycelial 3′-directed cDNA library, hybridized with only sexual organ-RNA (10). This result indicated that the presence of redundant GSs only in a specific library can faithfully represent cell-type or tissue specific GSs. As a result, 16 GSs are probably mycelia-specific.

Similarity search of GSs in GenBank

All of 209 GSs were also compared with GenBank entries (Table 3). When a GS longer than 100 nt showed 70% or more similarity to the already known gene, over 40% of the GS length, or GS shorter than 100 nt showing similarity over a half of the GS length, it was regarded as the gene or the gene-like. Only 6 GSs (9 clones) were identified as *A. nidulans* genes in the GenBank: encoding a 3-phosphoglycerate kinase, a heat shock protein, an alcohol dehydrogenase, FluG protein, an internal transcribed spacer and γ-actin. The 30 GSs were identified as genes of other organisms in GenBank. In total, 36 GSs were identified in GenBank and they corresponded to 17.2% of 209 GSs. The percentage was much lower than 22.5% in the HepG2 library (18) and

Table 4. Identified genes and their frequencies

Gene	Locus	Frequency	subtotal
Protein synthesis			2
ribosomal protein	NCRPSSU	1	
Met-tRNA-B	XELTGB	1	
Secretory protein			1
c4b-binding protein	UMPRPC4S	1	
Cytoskelecton			2
gamma actin	EMEACTINGA	1	
a-tubulin I	PFTUBAI	4	
Cytoplasm & organella			13
heat shock protein	ASNGO34ARA	3	
alcohol dehydrogenase	ANADH3	2	
NADH dehydrogenase	BEUMTA	2	
ATP synthase	YSCATPSU8X	1	
amino transferase	GGASPAT	1	
heat shock protein82	HTOHSP82	1	
dipeptidyl peptidase	LACXPDAP	5	
3-phosphoglycerate kinase	SMEPGK	1	
flavocytochrom c	SHEFLAVCA	1	
mitichondrial DNA	VMMITDNA	1	
4s-limonene synthase	MHC4SLSP	1	
symS & symL	APSYMSL	1	
ferrochelatase	MUSFERRC	1	
Nuclear protein			1
helicase	HSHLTF1	1	
Membrain protein			1
Dp87	DDIDP87	1	
Regulatory protein			2
fluG	EMEFLUG	1	
src-realated protein	HYDSTK	1	
Virus protein			3
107EIB VP4	HRU07753	2	
Malawi Li 1-20/1	ASU03762	1	
V91691	HIVED91DZ	1	
Others			11
chromosome IV cosmid	YSCL9476	1	
STS sWXD145	HUMSWX145	4	
cosmid M04D8	CEM04D8	1	
hi-receptor	HUMHHIRE	1	
hypothetical protein	BACHYP2	1	
MAGE-12 protein	HUMMAGE12X	1	
internal transcribed spacer	ANU03520	1	
cDNA clones		4	
Total		51 clones	36 GSs

44.4% in human brain library (1). This may possibly arise from the fact that our cDNA clones are 3′-end sequences which are known to be unique and can faithfully be used to identify genes, and also that relatively small number of genes in *A. nidulans* have been sequenced.

Thirteen GSs out of 36 GSs were identified as DNA or genes encoding cytoplasmic or organellar proteins (Table 4). Similar gene expression patterns were observed in HepG2 cell line and in human colonic mucosa (18, 19). Distinctively and unexpectively, GSs for genes involved in protein synthesis were relatively low in their identification frequency. It may also arise possibly from

the fact that only a few *A. nidulans* genes have been cloned and sequenced.

We could expect to isolate the genes expressed highly in vegetative mycelia by screening of full-length cDNA library of mycelia using these GSs as probes. Considering the result that more than 70% of the clones showed the frequency only once, many of them may be expressed rarely in vegetative mycelia of *A. nidulans*. Furthermore, similar results were obtained when three hundreds of 3'-directed cDNA clones from sexual organ-specific library were sequenced (10). Therefore, GSs expressed abundantly in specific cell types could be identified by comparison of GSs obtained from specific cell types. At present, northern blot analysis is under way to confirm the vegetative-specific GSs and the full length cDNAs of the GSs are expected to be isolated from a full-length cDNA library.

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