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Isolation of Aspergillus niger mRNA and Synthesis and Cloning of its cDNA

Luo Jinxian Li Wenqing Zhang Tianyuan*

Abstract

Total RNA of Aspergillus niger was isolated by phenol-proteinase K extraction and LiCl precipitation. Poly(A)$^+$ mRNA was purified by two cycles of oligo dT)-cellulose affinity chromatography. The poly(A)$^+$ mRNA thus prepared was electrophoresed on denaturing agarose-6M urea gel to estimate the length and in vitro translated in a rabbit reticulocyte lysate supplemented with L-(^{35}S) methionine, which showed enough translation activity. The first and second strand cDNA were synthesized by AMV reverse transcriptase and RNase H-DNA polymerase I respectively using the total poly(A)$^+$ mRNA as template and oligo(dT)12-18 as primer. The yield rates of first and second strand cDNA synthesis are 25.8% and 96% respectively. The length of ds cDNA is about 200-5000bp which is similar to that of poly(A)$^+$ mRNA. The ds cDNA was first ligated with EcoRI linker, after digested with EcoRI, it was mixed with EcoRI-digested λgt10 and ligated with T4-DNA ligase. The resultant DNA molecules were packaged in vitro and then infected E. coli BNN93 and BNN102. 8x10^4 recombinant phages were obtained per ug cDNA. Rapid isolation and restriction analysis of two recombinant phages show the existence of 4.3Kb and 3.5Kb inserts respectively.

Keywords Aspergillus niger mRNA, cDNA synthesis, cDNA cloning

1 Introduction

The extreme metabolic versality makes the fungi very attractive for bioprocess and they have significant value in present and in future industrial developments[1]. Fungi have also proven to be valuable in studying eukaryotic genetics, metabolic regulation and development. With the development of recombinant DNA technology, attention turned rapidly to fungi but efforts have mostly focused on Saccharomyces cerevisiae, Neurospora crassa and Aspergillus nidulans[2,3,4,5]. A. niger is an industrial strain for glucoamylase and citric and gluconic acid product-

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* Department of Biology and Biotechnology Research Center

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ion, cloning and expression of its genes will be useful to fungal gene regulation and to strain improvement in Aspergillus. This paper describes the isolaton and purification of A.niger mRNA and the synthesis and cloning of its cDNA.

2 Materials and Methods

2.1 Strains and culture conditions

A. niger 3758 was obtained fron the Guangdong Food and Fermentation Institute.

The E. coli strains BNN93 and BNN102 for λgt10 cloning were from Shanghai Institute of Biochemistry.

E. coli SMR10 for preparing packaging extract was from Guangdong Institute of Microbiology.

For growth of A. niger, spores were inoculated in Zeiss medium containing 10% maize powder for 50 hours at 30℃ with shaking[6,7,8]. The cells were frozen in liquid nitrogen and stored at -70℃.

2.2 Enzymes and reagents

T4 DNA ligase, T4 polynucleotide kinase, EcoRI methylase, S-adenosyl methionine and EcoRI linker are purchased from Sino-American Biotechnology Company.

cDNA synthesis kit. (a-^{32}P)dCTP and (r-^{32}P)ATP were from Amersham Packaging extract was prepared according to the procedure of Rosenburg and one strain system was used for in vitro packaging[9]

2.3 Preparation of poly(A)$^{+}$ mRNA

10g of frozen cells was pulverized under liquid nitrogen in a mortar. Quartz was added to disrupt the cell wall.

The total RNA was extracted with phenol-proteinase K[10]and precipitated with LiCl. Poly(A)$^{+}$ mRNA was obtained by two cycles of chromatography on oligo(dT)-cellulose[11].

2.4 Cell free translation and electrophoresis

Poly(A)$^{+}$ mRNA from A.niger was electrophoresed on denaturing agarose-6M urea gel[12] and translated in a rabbit reticulocyte lysate supplemented with L-(^{35}S) methionine as described by the supplier to detect the integrity and translational activity of the mRNA,

2.5 cDNA synthesis

The total ds cDNA was synthesized using cDNA synthesis kit purchased from Amersham, according to procedures from the supplier[13]. The synthesized cDNA was identified by alkaline electrophoresis and

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autoradiography according to reference[11].

2.6 cDNA cloning and identification

cDNA was cloned in λgt10 by addition of linkers. The EcoRI linker was first kinased according to reference[14], then ligated with Eco RI-methylated cDNA[15], the ligated mixture was digested with EcoRI and passed through Sephadex G-100 column to remove free EcoRI linkers[14]. The EcoRI treated cDNA was ligated with EcoRI digested λgt10 at the ratio of 1:50(w:w)[16]. The ligated DNA was in vitro packaged with packaging extract prepared from E.coli strain SMR10. The packaged phage was amplified in E.coli BNN93 and BNN102.

Recombinant phage DNA was isolated from plague from E.coli BNN-102 and analysed by agarose gel electrophoresis.

3 Results and Discussion

3.1 mRNA purification and in vitro cell free translation

8 mg total RNA was obtained from 10 g of A.niger cells with an OD260:OD280=1.9. The total RNA was passed through oligo(dT)-cellulose for two cycles, poly(A)+ mRNA was collected from the salt-free buffer eluted fractions (third peak on Fig.1). 60ug poly(A)+ mRNA was obtained with an OD260:OD280=2.2, which means pure enough for cDNA synthesis.

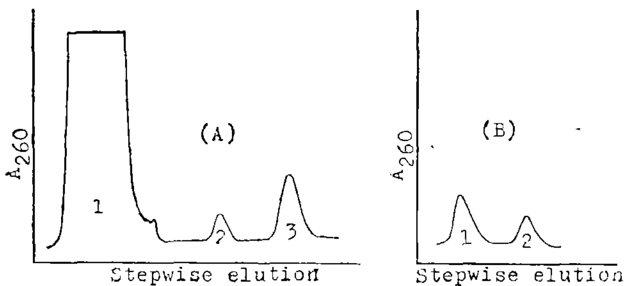

Fig.1 Chromatogrphy profile of A.niger RNA on oligo(dT)-cellulose column

(A) Peak 1 Flow through and rinsed fractions containing Poly(A)⁻RNA and small molecule tRNA

Peak 2 Medium salt buffer rinsed fraction containing residue rRNA and other unbound RNA

Peak 3 Eluted poly(A)+ mRNA

(B) Peak 1 Nonspecific bound RNA from flow through of second cycle oligo(dT)-cellulose

Peak 2 Poly(A)+ mRNA eluted from second cycle of oligo(dT)-cellulose

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Fig.2 shows the denaturing agarose-6M urea gel electrophoresis pattern, poly(A)$^+$ mRNA was dispersed between 25s and 17s rRNA, which shows the integrity of poly(A)$^+$ mRNA[12].

2μg of poly(A)$^+$ mRNA was in vitro translated in a rabbit reticulocyte lysate and (^{35}S)-methionine system, the results were shown in table 1 indicating that the total mRNA has a translation fold of 2.01 and a specific activity of 1.085x10^4.

Tab.1 Activity determination of A. niger poly(A)$^+$ mRNA

Samples	Counts of in vitro translation (cpm)	Translation folds (1)	Specific activity (2) (cpm/μg)
Control	1.08×10^4	-	-
Total mRNA (2μg)	3.25×10^4	2.01	1.08×10^4

(1) Fold of sample counts(net) to control counts

(2) Net counts of translation per μg mRNA(cpm/μg)

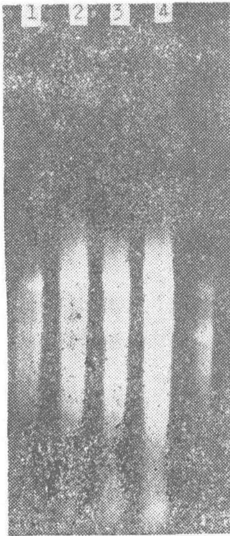

Fig.2 Agarose-6M urea electrophoresis of A. niger RNA
(2)—(4), Total RNA
(1), Poly(A)$^+$ mRNA after one cycle of oligo(dT)-cellulose
(5), Poly(A)$^+$ mRNA after two cycle of ligo(dT)-cellulose
The two sharp bands are 17s and 25s rRNA

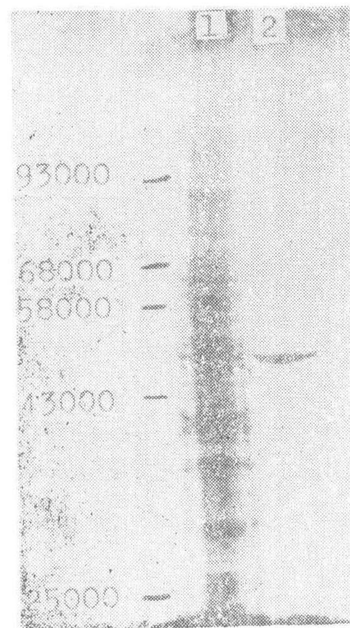

Fig.3 SDS-polyacrylamide gel electrophoresis, followed by autoradiography of in vitro translation products. Total poly(A)$^+$ mRNA extracted from A.niger was translated in a rabbit reticulocyte lysate with L-(^{35}S) methionine(lane 1), lane 2 is the control Left side shows the standard molecular weights

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The in vitro translation products were analysed by 12% SDS-polyacrylamide gel electrophoresis(Fig.3)in which protein bands ranging from 25000 to 93000 dalton were seen.

These results indicated that the purified poly(A)$^+$ mRNA are mostly intact and has enough translation activity in vitro.

During the isolation process, proteinase K was added immediately after the cell lysis and followed by phenol-chloroform extraction so as to degrade and remove most nucleases and proteins. Specific precipitation of RNA by 2.5M LiCl favors to removal of most DNA and some polysaccharides. The oligo(dT)-cellulose column was heated to 70℃ during elution in order to get sharper peaks.

3.2 cDNA synthesis and determination of cDNA lengths

cDNA was synthesized according to the protocol provided by Amersham. During the first strand synthesis, both oligo(dT)12～18 and random primer were used, the results were summarized in table 2. The yield rates for oligo(dT)12～18 and random primer are 25.8% and 18.3% respectively, which coincides with the result(15～30%) of reference[13].

Tab. 2 Yield of total cDNA synthesis

cDNA	Template	Primer	Counts(cpm)		Calculation results		
			Total counts	Incorporated counts	Incorporation percentage(%)	Yield	Yield rate(%)
ss cDNA	mRNA(5μg)	oligo $(dT)_{12-18}$	158458	5829	3.68	1.29	25.8
ds cDNA	ss cDNA		106042	3833	3.01	1.26	97.1
ss cDNA	mRNA(1μg)	Random primer	179166	2340	1.31	0.183	18.3
ds cDNA	ss cDNA		156302	1977	1.26	0.177	96.7

As shown in Fig.4 and Fig.5, the length of first strand cDNA ranges between 200～5000 nucleotides similar to the length of mRNA. This indicates the synthesis of full length and nearly full length cDNA. These results show that both yield rate and length of first strand cDNA are very closed whether oligo(dT)12-18 or random primer are used,

Sodium pyrophosphate promotes the full length cDNA synthesis and prevents the formation of hair-pin structure[17], but it is a potential inhibitor of reverse transcriptase. Since sodium pyrophosphate and concentrated RNasin (20u/μg mRNA) are included in Amersham's cDNA synthesis kit, large amount of reverse transcriptase (20u/μg mRNA) was used. According to Watson[15], full length cDNA synthesis was obtained by adding RNasin but sodium pyrophosphate. In our experience,

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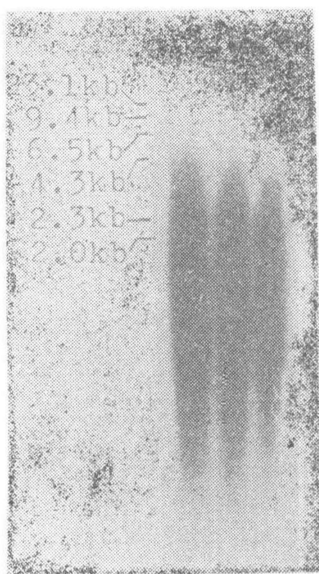

Fig.4 Agarose gel electrophoresis-autoradiography of the synthesised first and second strand cDNA. Left side is the standard molecular weight(λ/HindIII)
(1) ds cDNA
(2) first strand cDNA(oligo$(dT)_{12-18}$ primer)
(3) first stand cDNA(random primer)

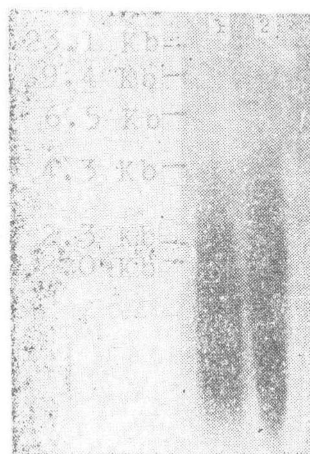

Fig.5 Agarose gel electrophoresis-autoradiography of the synthesised cDNA using random primer
(1) first strand cDNA
(2) ds cDNA
Left side is the molecular weigth markers(λ/HindIII)

a satisfactory result (19.8% yield rate) was obtained by decreasing RNasin(10u/μg mRNA), reverse transcriptase (10u/μg mRNA), omitting sodium pyrophosphate. Fig.6 shows the coincidence of the lengths of cDNA and mRNA.

The protocol of Gubler and Hoffman[18] was used for second strand cDNA synthesis to avoid the unfavorable action of nuclease SI. As shown in table 2, the yield rate of second strand cDNA is 96% and its length is 200-5000 bp(Fig.4,Fig.5). Some of the second strand product is bigger than the first strand, this may due to the existence of hair-pin structure and hence elits the second strand synthesis[17].

3.3 Cloning and identification of ds cDNA

The synthesized ds cDNA was extracted with phenol and chloroform and precipitated with 2M NH_4Ac-ethanol to remove unincorporated free nucleotides. The ligation of ds cDNA and EcoRI-digested λgt10 was done as in Materials and Methods. The ligated mixture was packa-

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ged in vitro and then infected hosts E.coli BNN93 and E.coli BNN102. Either λgt10 or λgt10 hybrid phage can form plagues when BNN93 was used as host but only recombinant phage can form plagues with BNN 102 as host. $8x10^4$ recombinant phages were obtained per μg cDNA in our experiment. DNA of two recombinant phages were rapidly isolated and digested with EcoRI. The agarose gel electrophoresis pattern shows that the recombinant phages contain inserts of 4.3Kb and 3.5Kb respectively (Fig.7).

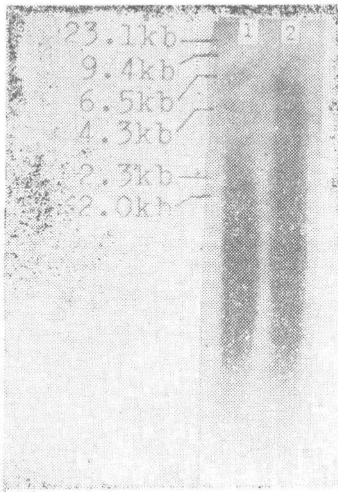

Fig.6 Agarose gel electrophoresis-autoradiography of the cDNA synthesised without soduim pyrophosphate
(1) first stranb cDNA
(2) ds cDNA
Left side is the molecular weight markers(λ/HindIII)

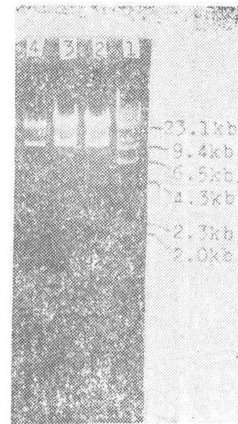

Fig.7 Restriction digestion analysis of recombinant phage DNA.
(1) λcI857S7 DNA + HindIII.
(2) (3) Recombinant phage DNA + EcoRI
(4) λgt10 DNA + EcoRI

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黑曲霉mRNA的分离及其cDNA的合成和克隆

罗进贤*　李文清　张添元

采用酚—蛋白酶K抽提和氯化锂沉淀法，从10g黑曲霉细胞中获得8mg总RNA，经二次寡聚(dT)-纤维素亲和层析纯化得60μg poly(A)$^+$mRNA。经紫外分光，6M尿素—琼脂糖凝胶电泳分析及体外翻译试验结果证明：纯化的poly(A)$^+$mRNA的纯度及完整性好，无降解并具翻译活性。以纯化的poly(A)$^+$mRWA为模板，以寡聚(dT)$_{12-18}$及随机的寡聚核苷酸为引物，用AMV反向转录酶合成了第一链cDNA。用RNaseH及DNA聚合酶Ⅰ水解模板mRNA并合成双链cDNA。第一链及第二链cDNA合成的产率分别为25.8%及96%。合成的cDNA的长度在200～5000bp之间与纯化的poly(A)$^+$mRNA的长度相等。合成的双链cDNA，经酚-氯仿抽提及2M醋酸钠-乙醇沉淀，除去游离的核苷酸后与EcoRI接头连接，再经EcoRI消化后与λgt10DNA连接，然后进行体外包装，包装物感染大肠杆菌BNN93及BNN102。λgt10及重组的λgt10都能在大肠杆菌BNN93上长出噬菌斑，而只有重组的噬菌体才能在BNN102上长出噬菌斑，按此法筛选，每μgcDNA可获得8×10^4个重组噬菌体。

关键词　黑曲霉mRNA，cDNA合成，cDNA克隆

* 生物学系及生物工程研究中心

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