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Cloning and Expression of K11 Phage RNA Polymerase

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K11 RNA 중합효소의 Cloning 및 발현

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Using the PCR(polymerase chain reaction method), gene 1 of *Klebsiella* phage K11 coding for K11 phage RNA polymerase has been cloned and expressed under the control of *lac* promoter. K11 phage RNA polymerase was conventionally purified through the DEAE-sephacel and Affigel blue column chromatographies. The 0.2-0.3 M NH_4Cl fractions of DEAE-sephacel column chromatography showed K11 phage RNA polymerase activity and further purification with Affigel blue column chromatography showed nearly single protein band on SDS-polyacryl amide gel. K11 phage RNA polymerase, which is one of the T7 group phage RNA polymerases (*E. coli* phage T7, T3 and *Salmonella typhimurium* phage SP6 RNA polymerases), shares high degrees of homology with the other T7 group phage RNA polymerases. Previously we constructed T7 and SP6 promoter variants and revealed promoter specificity of T7 and SP6 RNA polymerases (Lee and Kang, 1993). To investigate the promoter specificity of K11 RNA polymerase *in vitro* K11 promoter activity was measured with SP6 promoter variants. The SP6 promoter variant which share highest degrees of sequence homology with K11 promoter sequence show strongest K11 promoter activity.

PCR 방법을 이용하여 K11 RNA 중합효소를 coding하는 *Klebsiella* phage gene 1을 cloning 하였고 *lac* 전사촉진제 조절 하에 발현시켰다. K11 RNA 중합효소는 DEAE-sephacel과 Affigel blue column chromatographies를 사용하는 상용 방법으로 분리하였다. DEAE-sephacel의 0.2-0.3 M NH_4Cl 분획에서 K11 RNA 중합효소의 활성을 보였고, 다음 단계의 Affigel blue column에서 SDS-polyacryl amide gel 상의 단일 band로 분리되었다. K11 RNA 중합효소는 T7 그룹 phage RNA 중합효소로 다른 T7 그룹 phage RNA 중합효소와 많은 상동성을 보인다 (대장균 phage T7, T3와 *Salmonella typhimurium* phage SP6 RNA 중합효소). 이미 우리는 T7과 SP6 전사촉진제 변이체를 제조한 바 있고 T7과 SP6 RNA 중합효소의 전사촉진제 특이성을 연구한 바 있다 (이상수와 강창원, 1993). K11 RNA 중합효소의 전사촉진제 특이성을 알아보기 위해 SP6 전사촉진제 변이체를 사용하여 *in vitro* K11 RNA 중합효소의 활성을 측정하였다. 이 변이체 중 K11 전사촉진제와 가장 유사한 것이 가장 높은 K11 RNA 중합효소 활성을 보였다.

Key words : K11 RNA polymerase, T7, T3 and SP6 phage, DEAE-sephacel, Affigel blue.

I. Introduction

Bacteriophage K11 RNA polymerase is expressed early in K11 bacteriophage infection of *Klebsiella* sp. 390 (03:K11) and plays an important role in regulating gene expression (Dietz *et al.*, 1990). The polymerase consists of a single polypeptide with a molecular weight of around 100,000 as the analogous enzymes which are expressed by other T7-like phages such as T7, T3 and SP6 phages (Chamberlin *et al.*, 1970, Beck *et al.*, 1989 and Butler and Chamberlin, 1982). These phage RNA polymerases show great specificity for its own cognate promoters which consist of 17-23 base pair one-stretch consensus sequence (Dunn and Studier, 1983, Rosa and Andrews, 1981, Beck *et al.*, 1989, Brown *et al.*, 1986 and Dietz, 1985). Thus the template specificities of these RNA polymerases is not only central to the strategy of T7-like phage infection but is also interesting and potentially useful to biochemists. Understanding the basis for this specificity is a challenge in its own right. Furthermore, the purified enzyme can be used to produce large amounts of specific RNAs simply by transcribing DNA that has been joined to a promoter for T7-like phage RNA polymerases.

A lot of study for T7, T3 and SP6 RNA polymerases were carried out and promoter specificities and amino acid sequence homologies of these RNA polymerases were elucidated (Dunn and Studier, 1983, McGraw *et al.*, 1985 and Kotani *et al.*, 1987). At recent, gene 1 of K11 phage for K11 RNA polymerase has been reported and its deduced amino acid sequence shows 71% homology to the T7 RNA polymerase (the product of T7 gene 1), 72% homology to the T3 RNA polymerase and 27% homology to the SP6 RNA polymerase (Dietz *et al.*, 1990). Also one K11 promoter sequence which is located down stream of gene for K11 RNA polymerase has been reported. Nevertheless, little is known about biochemical and genetical properties of K11 RNA polymerase. The yield of purified K11 RNA polymerase from infected cells is not good, because the enzyme is synthesized for only a few minutes during infection and does not accumulate to high levels. For the purpose to produce K11 RNA polymerase, gene 1 of K11 bacteriophage was cloned by using the PCR method and purified by conventional purification method of T7 and SP6 RNA polymerases (Butler and Chamberlin, 1982 and Davanloo *et al.*, 1984).

II. Materials and Methods

1. Bacterial strains, bacteriophage, and pla-smid vectors

E. coli. XL1-blue was used as the host for plasmid strains and *E. coli.* JM109 for expression of the cloned K11 RNA polymerase, and *Klebsiella* sp. 390 (03:K11) for the proliferation of phage K11. Phage K11 was the DNA donor for the cloning. *Klebsiella* sp. 390 (03:K11) and K11 phage were gratefully gifted from R. Hausmann (Freiburg Univ.). The plasmid pKU01 containing gene 1 of T7 phage for T7 RNA polymerase and plasmid pACYCSP6R containing gene 1 of SP6 phage for SP6 RNA polymerase were gifted from C. Kang (KAIST).

2. Cloning

Two deoxyoligonucleotides were synthesized by phosphoroamidate method on an automatic DNA synthesizer. These synthetic deoxyoligonucleotides were used as primers for the cloning of K11 RNA polymerase with PCR method. Sequences of primers are 5'ACTGGA TCCACAAAGAGGCACA3' and 5'CCTGGTACC TTACGCAAACGCGA3'. Purification of plasmids, agarose gel electrophoresis and transformation of cells, were mainly performed according to the procedures described by Maniatis *et al.* (1982).

3. Expression and purification of K11 RNA polymerase

When a culture of *E. coli* JM 109 containing the recombinant plasmid (pUC119K11) was grown at to an A_{600nm}=0.4 at 37℃ in LB broth IPTG(isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.2 mM and cultivation was continued for 3 hr. The cells were collected by centrifugation, washed with washing buffer (Tris-HCl 10 mM pH=7.0, 50 mM NaCl) and stocked at -70℃ until the enzyme assay was purified. Purification of K11 RNA polymerase was performed as described by Davanloo *et al.* (1984).

4. Assay for K11 RNA polymerase activity

Reaction mixtures contained 40 mM Tris · HCl at pH 7.9, 8 mM $MgCl_2$, 5 mM dithiothretol, 4 mM spermidine HCl, and 0.5 mM each of ATP, GTP, CTP and UTP. To monitor activity in crude extracts and during purification, incorporation of [α-^{32}p]CTP (30 μCi/μmol) into RNA in 30 min at 37℃ in a 20 μl reaction mixture 0.5 μg of K11 DNA was measured by insolubility in 5% trichloroacetic acid (Kang and Wu 1987).

III. Results and Discussion

1. Cloning the entire coding sequence for K11 RNA polymerase

Recently the DNA sequence for K11 RNA polymerase gene was reported (Dietz *et al.*, 1990). To clone gene 1 for K11 phage for K11 RNA polymerase by PCR method the complimentary DNA sequences which locate upstream and downstream region of gene 1 were synthesized (Materials and Methods). Also to insert PCR product into vector plasmid DNA more easily *Kpn* I and *Bam* HI restriction enzyme sites on each synthetic DNA were introduced. Using these synthetic DNAs as PCR primers and K11 phage DNA as a template, 2.8 Kb PCR product DNA was obtained (data not shown). After digestion of the PCR product DNA with *Kpn* I and *Bam* HI restriction enzymes the cleaved DNA fragment was inserted into *Kpn* I and *Bam* HI sites of pUC 119 (Fig. 1).

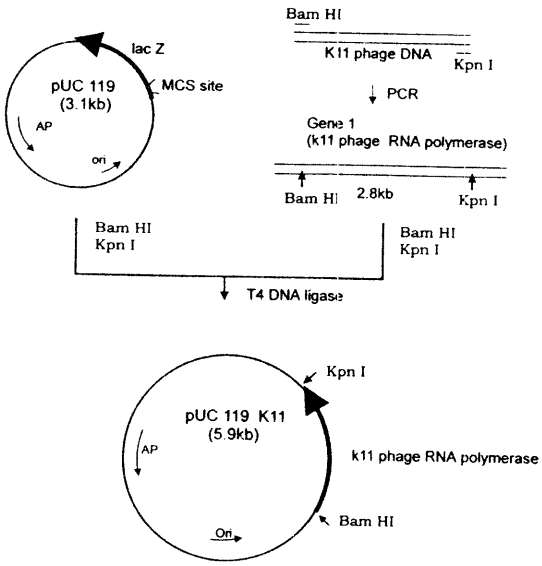

Fig. 1 Schematic illustration for the cloning of K11 RNA polymerase.

2. Amino acid sequence of K11 RNA polymerase

The amino acid sequence of K11 RNA polymerase shown in Fig. 2.

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  1 MNALNIGRND FSEIELAAIP YNILSEHYGD GAAREQLALE HEAYELGRQR
 51 FLKMLERQVK AGEFADNAAA KPLVLTLHPQ LTKRIDDWKE EQANARGKKP
101 RAYYPIKHGV ASELAVSMGA EVLKEKRGVS SEAIALLTIK VVLGNAHRPL
151 KGHNPAVSSQ LGKALEDEAR FGRIREQEAA YFKKNVADQL DKRVGHVYKK
201 AFMQVVEADM ISKGMLGGDN WASWKTDEQM HVGTKLLELL IEGTGLVEMT
251 KNKMADGSDD VTSMQMVQLA PAFVELLSKR AGALAGISPM HQPCVVPPKP
301 WVETVGGGYW SVGRRPLALV RTHSKKALRR YADVHMPEVY KAVNLAQNTP
351 WKVNKKVLAV VNEIVNWKHC PVGDVPAIER EELPPRPDDI DTNEVARKAW
401 RKEAAAVYRK DKARQSRRCR CEFMVAQANK FANHKAIWFP YNMDWRGRVY
451 AVSMFNPQGN DMTKGSLTLA KGKPIGLDGF YWLKIHGANC AGVDKVPFPE
501 RIKFIEENEG NILASAADPL NNTWWTQQDS FFCFLAFCFE YAGVKHHGLN
551 YNCSLPLAFD GSCSGIQHFS AMLRDSIGGR AVNLLPSDTV QDIYKIVADK
601 VNEVLHQHAV NGSQTVVEQI ADKETGEFHE KVTLGESVLA AQWLQYGVTR
651 KVTKRSVMTL AYGSKESLVR QQVLEDTIQP AIDNGEGLMF THPNQAAGYM
701 AKLIWDAVTV TVVAAVEAMN WLKSAAKLLA AEVKDKKTKE VLRKRCAIHW
751 VTPDGFPVWQ EYRKQNQARL KLVFLGQANV KMTYNTGKDS EIDAHKQESG
801 IAPNFVHSQD GSHLRMTVVH ANEVYGIDSF ALIHDSSGTI PADAGNLFKA
851 VRETMVKTYE DNDVIADFYD QFADQLHESQ LDKMPAVPAK GDLNLRDILE
901 SDFAFA
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Fig. 2 The amino acid sequence of bactericphage K11 RNA polymerase.

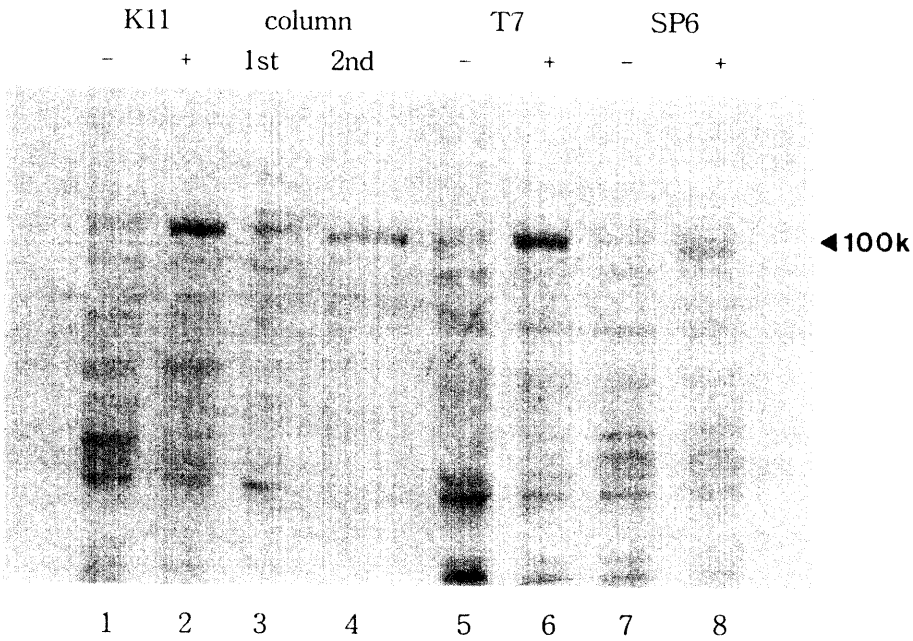

Fig. 3 SDS-polyacrylamide gel electrophoresis of crude extracts and purified fractions from cells haboring pUC119K11. Lane 1; unind-uced cloned K11 RNA polymerase (-), 2; induced cloned K11 RNA polymerase (+), 3; 1st DEAE-Sephadex column chromatography purified 0.2-0.3 M NH4Cl fractions of cloned K11 RNA polymerase, 4; 2nd Affigel-blue column chromatography purified 1.5 M NH4Cl fractions of cloned K11 RNA polymerase, 5; uninduced cloned T7 RNA polymerase (-), 6; induced cloned T7 RNA polymerase (+), 7; uninduced cloned SP6 RNA polymerase (-), 8; induced cloned SP6 RNA polymerase (+)

	-15 -10 -5 +1	
k11	A **A** T T A G G **G C** A C A C T A T A G **G G**	K11 promoter
SP6	A T T T A G G T G A C A C T A T A G A A	activity
		(cpm)
	A T T T A G G * * * C A C T A T A G A A	
	G C A	42092
	G G A	13473
	T **C A**	39315
	A **C A**	40178
	G C T	8998

Fig. 4 *In vitro* K11 promoter activities of SP6 promoter variants with K11 RNA polymerase. The *in vitro* transcription activities of the variants were measured in separate, parallel, reactions containing each variant plasmid.

The sequence consisted of 904 amino acid residues including f-methione. This gives a molecular weight of 100383 daltons which is well in agreement with the value of the polymerase from purification as estimated by the SDS-PAGE analysis (Fig. 3).

3. Expression and purification of K11 RNA polymerase in *E. coli*

To examine whether the cloned DNA segment actually encodes the functional K11 RNA polymerase, expression experiments were tried. The cloned K11 RNA polymerase gene does not contain the promoter-like sequence, so that I placed the segment under the control of the *lac* promoter. The *Kpn* I-*Bam* HI PCR fragment was inserted downstream of the *lac* promoter of pUC119 (Fig. 1). *E. coli* JM109 cells containing pUC119K11 was cultured in the presence or absence of the inducer IPTG, and the cell lysates were analyzed by SDS-polyacryl amide gel electrophoresis. The SDS-PAGE analysis of cell lysates with or without IPTG induction and the fractions of purification steps is shown in Fig. 3. When induced by IPTG, one major band appeared at the positions of 100,000 daltons. The position of this band is nearly same with that of T7 and SP6 RNA polymerases bands. Based on these observations, I concluded that the cloned DNA segment actually encodes the functional K11 RNA polymerase and is efficiently expressed in *E. coli* cells. The nucleotide sequence is now available, and it is possible to produce a large amount of the enzyme in the *E. coli* system.

Through two column chromatography after cell lysis and 55% ammonium sulfate precipitation K11 RNA polymerase was purified and analyzed by SDS-PAGE. The 0.2-0.3 M NH_4Cl fractions of first DEAE-sephacel column chromatography showed many protein bands and further purification with Affigel blue second column chromatography showed nearly single protein band on SDS-polyacryl amide gel. Therefore it seems that K11 RNA polymerase has similar protein tertiary structure and properties to T7 and SP6 RNA polymerases

4. Promoter specificity of K11 RNA polymerase

Previously we made T7 and SP6 promoter variants and revealed promoter specificity of T7 and SP6 RNA polymerases (Lee and Kang, 1993). The base pairs at -8, -9 and -10 of the SP6 consensus sequence promoter were singly and multifully substituted by T7 specific base pairs; -8A by T, -9G by C, and -10T by A (T7) or G (SP6). To investigate the promoter specificity of K11 RNA polymerase, *in vitro* K11 promoter activity was measured with these SP6 promoter variants (Fig. 4). The SP6 promoter mutant which share highest degrees of sequence homology with K11 promoter sequence (-8A, -9C and -10G) shows strongest K11 promoter activity. The single substitutions at -8 (A → T) and -9 (C → G) considerably reduced *in vitro* K11 promoter activity to 21% and 32% of wild type K11 basepairs at these positions. On the other hand, The single substitutions at -10 (G → T or A) does not reduce *in vitro* K 11 promoter activity (93% and 95%).

IV. Acknowledgements

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