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Molecular Cloning and Expression in *Escherichia coli* of a Rabbit Globin Gene

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유전공학적 방법에 의한 토끼 글로빈 유전자의 재조합과 대장균에서의 발현

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적 요

유전자 구조 및 유전정보 흐름의 차이로 인하여 고등생물의 유전자를 미생물에 직접 cloning하면 원하는 유전자 산물을 얻지 못하는 경우가 많다. 이것을 극복하기 위해서는 화학적인 방법으로 유전자를 합성하든지, 또는 역제효소를 사용하여 고등생물의 mRNA로부터 유전자를 합성하여 cloning하는 방법을 사용한다. 본 연구에서는 oligo(dT)-cellulose column 방법으로 순수분리한 토끼의 globin mRNA를 주형으로 이용하여 효소를 사용해서 DNA를 합성하고 plasmid pBR322의 Pst I site에 cloning하였다. 우선 AMV reverse transcriptase로 primary cDNA를 합성하고, 알칼리를 처리하여 주형 RNA를 제거했다. 이번에는 이 primary cDNA를 주형으로 Klenow enzyme과 reverse transcriptase를 차례로 처리하여 double stranded DNA를 합성하고, 이때 5′ end근처에 형성되는 hairpin loop을 Sl nuclease로 제거했다. Terminal deoxynucleotidyl transferase를 사용하여, 합성된 dsDNA에는 poly(dC) track을, Pst I endonuclease를 처리한 plasmid DNA에는 poly(dG) track을 각각 붙인다음 이들을 서로 annealing시키고 *E. coli*에 transformation시켜서 크기가 큰 plasmid를 갖는 clone을 cracking방법으로 일차 선별하였다. 이렇게 선별된 clone을 ***in situ*** hybridization방법으로 조사하여 globin DNA가 들어간 colony를 이차 선별하고 여러 restriction enzyme으로 잘라보아 globin DNA가 cloning된 것을 확인하였다. 토끼 hemoglobin으로 immunize한 rat (Wistar)에서 뽑은 제일차 혈청과 염소에서 뽑은 제이차 혈청의 antibody를 사용한 radioimmunoassay방법으로, cloning된 globin gene이 대장균내에서 발현되는 지의 여부를 살펴 보았는데, 박테리아의 β-lactamase와 토끼의 globin이 결합된 chimeric protein이 대장균내에서 다량 합성되며, 이 단백질은 토끼 hemoglobin의 antigenic determinant를 가지고 있음을 알 수 있었다.

INTRODUCTION

The availability of complementary DNA (cDNA) copies of mRNAs provides an extremely powerful tool for analyzing the structure, organization, and expression of eukaryotic genes (Breathnach *et al.*, 1979; Efstratiadis *et al.*, 1977; Sood *et al.*, 1981; Tilghman *et al.*, 1978). Besides the utility of cDNAs for defining the initiation, coding and the termination sequences of mRNAs, their use as hybridization probes makes it possible to search, isolate, identify, and characterize the corresponding genes from chromosomal DNA (Brack *et al.*, 1978).

In order to express eukaryotic gene sequences in bacteria, two basic approaches are available. In one case, the cloned eukaryotic coding sequences are spliced into the bacterial gene such that the bacterial initiation codon is followed directly by the codon for the first amino acid of eukaryotic protein. In the other, the coding sequence is inserted into an initial position in a bacterial gene so that the sequences are in phase with the bacterial coding sequences, which results in the synthesis of a hybrid bacterial-eukaryotic protein, from which eukaryotic protein has to be released (Goeddel *et al.*, 1979; Shine *et al.*, 1980). Although release can be a problem, there may be several advantages to the fusion protein approach. Firstly, it minimizes the chances of disturbance of the mRNA secondary structure in the region of the normal ribosome binding site and initiation codon, causing reduced rate of initiation of protein synthesis (Hall *et al.*, 1982). Secondly, degradation of the eukaryotic protein in the bacterium may be reduced when it is fused (Itakura *et al.*, 1977; Goeddel *et al.*, 1978; Davis *et al.*, 1981). Thirdly, the fusion protein may be secreted by the expressing bacteria if the eukaryotic DNA is attached to a bacterial sequence coding for a signal peptide that causes export of proteins to be translocated across membranes (Talmadge *et al.*, 1980). Finally, fusion protein itself may be used as antigens (Kleid *et al.*, 1981).

Radioimmunoassay, an approach to detect small amounts of proteins, can be applied to identify the bacterial expression of eukaryotic gene. Besides, this system may detect not only a complete protein but also a fragment of protein or a fusion protein by virtue of its antigenic determinant. Using this approach, Chang *et al.* (1978) and Martial *et al.* (1979) reported the construction of plasmid expressing eukaryotic gene in *E. coli*.

In this study, therefore, the hybrid protein approach was chosen in an attempt to persuade bacteria to synthesize rabbit globin α- or β-chain, and the globin gene expression in *E. coli* was measured with competitive radioimmunoassay.

MATERIALS AND METHODS

Highly purified avian myeloblastosis viral reverse transcriptase was supplied from Life

Sciences Inc., St. Petersburg, Fla. Restriction enzymes, terminal deoxynucleotidyl transferase, and the Klenow fragment of *E. coli* DNA polymerase I were purchased from Bethesda Research Laboratories Inc. Goat anti-rat IgG serum was obtained from Cappel Laboratories, and rabbit hemoglobin was from Sigma. Sl nuclease was purified from *Aspergillus oryzae* crude α-amylase powder as described by Vogt (1973). New England Nuclear was the source of radiochemicals ([α-^{32}P]dGTP, [^{3}H]dTTP), and [^{131}I] was obtained from Institute of Atomic Energy, Seoul, Korea. Sephadex G-50, G-100 and Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, oligo (dT)-cellulose from PL-Biochemicals, and P-10 from Bio-Gel. Freund complete adjuvant was from Difco Lab., and all the other chemicals used were reagent grade.

Preparation of Rabbit Globin mRNA

Rabbit globin mRNAs were purified from rabbit blood by a modification of the method of Aviv *et al.* (1972) and Chirgwin *et al.* (1979). The process involves the isolation of polysomes using cushion method (Palacios *et al.*, 1972), phenol/chloroform extraction, oligo(dT)-cellulose chromatography, and sucrose gradient centrifugation.

Enzymatic Synthesis of cDNA

AMV reverse transcriptase was used to synthesize the first strand cDNA under the conditions described by Buell *et al.* (1978) with some modifications. The standard reaction mixture (100 μl) consisted of the following components; 100 mM Tris/HCl (pH 8.3), 10 mM $MgCl_2$, 140 mM KCl, 10 mM DTT, 1 mM dATP, 1 mM dGTP, 1 mM dCTP, 250 μM dTTP, 5 μM [^{3}H]dTTP, 50 μg/ml poly(A)-RNA, 50 μg/ml oligo(dT) $_{12-18}$, and 50 units of AMV reverse transcriptase. The reaction mixture was mixed well and incubated at 42°C for 3 hr. The reaction was stopped by adding 4 μl of 0.5 M EDTA (pH 8.0), followed by 50 μl of 150 mM NaOH. To free the transcript from its mRNA template, the reaction mixture was incubated for 8 hr at 37°C, and then neutralized the solution by adding 50 μl of 1.0 M Tris/HCl (pH 8.0) and 50 μl of 1.0 N HCl. The reaction was extracted with an equal volume of phenol/chloroform, and passed through a Sephadex G-50 spun-column. Small aliquot (10 μl) was saved for later analysis with formamide gel. The remainder of the reaction was ethanol-precipitated and used for the template of double stranded DNA (ds DNA) synthesis.

Enzymatic Synthesis of dsDNA Complementary to cDNA

The second strand cDNA synthesis was initiated by the Klenow fragment of *E. coli* DNA polymerase I as previously described by Efstratiadis *et al.* (1976), and then extended the strand by AMV reverse transcriptase under conditions modified from Monahan *et al.* (1976). The standard reaction mixture (100 μl) for the first step consisted of the following components: 0.1 M HEPES (pH 6.9), 10 mM $MgCl_2$, 2.5 mM DTT, 70 mM KCl, 0.5 mM four dNTPs, cDNA template, and 50 units of Klenow fragment of *E. coli* DNA polymerase I. The reaction mixture was assembled on ice and incubated at 15°C for 20 hr. The product was extracted with phenol/chloroform, passed through a Sephadex G-50

spun-column, and ethanol-precipitated.

The elongation of second strand was attempted by reverse transcriptase under following conditions; 100 mM Tris/HCl (pH 8.3), 140 mM KCl, 10 mM $MgCl_2$, 1 mM dNTPs, 10 mM DTT, cDNA template, and 40 units of reverse transcriptase. The reaction mixture was incubated at 42°C for 1 hr, and the reaction was stopped by adding 2 μl of 0.5 M EDTA. The mixture was extracted with phenol/chloroform, passed through a Sephadex G-50 spun-column, and precipitated with ethanol.

Digestion with Nuclease S1

The product of polymerases reaction was treated with Sl nuclease. The reaction mixture (30 μl) consisted of 0.2 M NaCl, 50 mM sodium acetate (pH 4.6), 1 mM $ZnSO_4$, the double stranded globin DNA, and 1 unit of Sl nuclease. The mixture was incubated at 30°C for 30 min, treated with phenol/chloroform, and precipitated with ethanol. The precipitation was redissolved in 18 μl of TE (pH 8.0), and added 2 μl of 3 M NaCl. The double stranded cDNA was fractionated into size classes by passing through a 1 ml column of Sepharose CL-4B equilibrated in 10 mM Tris/HCl (pH 8.0), 0.3 M NaCl, and 1 mM EDTA. Fifty μl fractions were collected, and then pooled the fractions that contain cDNA molecules greater than 500 base pairs in length.

Electrophoretic Analysis of cDNA

Electrophoretic sizing of DNA product was accomplished by using 5% polyacrylamide gels made in 98% formamide, as has been previously described (Retzel *et al.*, 1980). Ethanol-precipitated samples were resuspended in 25 μl of deionized formamide, heated at 60°C for 10 min and quenched on ice, and then added 5 μl of 20 mM (Na_2H-NaH_2) PO_4 buffer (pH 7.5) containing 0.03% (w/v) bromophenol blue. After the samples were layered onto (6×180)mm gels, electrophoresis was accmplished in 20 mM (Na_2H-NaH_2) PO_4 buffer (pH 7.5) under the constant voltage of 200V for 3 hr. Electrophoresed gels were frozen at −70°C, sliced into 2 mm sections, added LSC counting coctail, and counted in a Packard Tri-Carb liquid scintillation counter.

Homopolymer Tailing of Vector DNA and dsDNA

This reaction carried out by terminal deoxynucleotidyl transferase as described by Michelson *et al.* (1982), and Roychoudhurg *et al.* (1976). Thirty μg of pBR322 DNA Pst I-digested was added to 100 μl reaction mixture containing 140 mM sodium cacodylate, 30 mM Tris/HCl (pH 7.6), 2 mM DTT, 1 mM dGTP, 250 μg/ml BSA, and 1 mM $CoCl_2$. The reaction mixture was pre-incubated at 30°C for 10 min, and then 48 units of terminal transferase was added and incubated 15 min more at 30°C. The reaction was stopped by chilling to 0°C, and added 10 μl of 0.5 M EDTA (pH 8.0). The mixture was extracted with phenol/chloroform followed by chromatography on a column of Sephadex G-100 equilibrated in annealing buffer (0.1 M NaCl, 10 mM Tris/HCl (pH 7.8). The double stranded cDNA was tailed as above with dCTP and 24 units of terminal transferase by incubating at 37°C for 2 min.

Introduction of Plasmid into E. coli

Equimolar amounts of (dC)-tailed dsDNA and (dG)-tailed vector in annealing buffer were mixed at a final concentration of 1 ng/μl, heated to 65°C for 5 min, and allowed to anneal by incubating at 57°C for 2 hr. Using *E. coli* RRI cells, transformations were carried out in 50 mM $CaCl_2$ as described by Cohen *et al.* (1972). As the primary screening of cDNA clones, the transformation cells were smelt with cracking buffer (0.05 M Tris/HCl (pH 6.8), 1% SDS, 2 mM EDTA, 0.4 M sucrose, 0.01% BPB), and then the plasmids were analyzed by agarose gel electrophoresis. The colonies containing larger plasmids than vector DNA (pBR322) were selected for further characterization.

Colony Hybridization

Colony hybridization was accomplished as described by Grunstein and Hogness (1975) with a minor modification. Colonies were grown on nitrocellulose membrane over LB-agar media containing tetracycline. The bacterial DNAs including plasmids were immobilized on nitrocellulose membrane by the method of Vuorio *et al.* (1982). The membrane was then incubated for 2 hr at 42°C in 20 ml of pre-washing solution (50 mM Tris/HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, 0.1% SDS), and then the solution was exchanged for pre-hybridization solution (50% formamide, 5×Denhardt's solution, 5×SSPE, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, 2 μg/ml poly(A)). The SSPE (×20) solution consists of following components; 3.6 M NaCl, 200 mM NaH_2PO_4 (pH 7.4), and 20 mM EDTA (pH 7.4). The membrane was incubated at 42°C for 4 hr, and then added [^{32}P]-labeled cDNA probes (3.3×10^7 cpm) which were synthesized with reverse transcriptase using purified globin mRNA as template. The hybridization solution was incubated at 42°C for 24 hr. After washing and drying, the membrane placed under Agfa-Gevaert Curix/x-ray film for autoradiography.

Mapping of Restriction Endonuclease Cleavage Sites

Colonies which gave positive hybridization were grown in liquid LB broth containing 10 μg of tetracycline per ml medium. The plasmid DNAs were isolated by means of alkaline lysis method previously described by Birnboim and Doly (1979). The plasmid DNAs were digested with EcoR I, Pst I, and BamH I restriction endonuclease in the common digestion buffer (33 mM Tris/HCl pH 7.0), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT), and then analyzed by 1.2% agarose gel electrophoresis.

Preparation and Storage of Antiserum

Using rabbit hemoglobin as immunogen, acceptable antisera to hemoglobin were raised in rat strain Wistar. Briefly, rabbit hemoglobin was suspended in saline solution (2 mg /ml), and emulsified with the same volume of complete Freund's adjuvant. The emulsion was injected into four intramuscular sites and four subcutaneous sites (1 mg Hb/rat). After four weeks, three booster injections were accomplished at intervals of ten days, and the rat was bled ten days later. Clear serum was prepared by centrifugation, added sodium azide to 0.05%, and then stored at −20°C.

Iodination of Hemoglobin

Rabbit hemoglobin was radiolabeled with [^{131}I] by the chloramine-T method (Greenwood *et al.*, 1963) with a minor modification. The reaction mixture (100 μl) contained 4 μg of hemoglobin, 0.5 mCi of [^{131}I], and 50 μg of chloramine-T in a buffer consisting of 50 mM Tris/HCl (pH 7.8) and 300 mM KCl. The reaction was allowed to proceed for 1 min and terminated by adding sodium metabisulfite. The free iodine was separated from [^{131}I]-labeled hemoglobin on Bio-Gel P-10 column (1×15 cm). Small aliquot (2 μl) of each fraction (0.4 ml) was taken to measure the radioactivity. The radioactivity of beta-emission of [^{131}I] measured by Packard Tri-Carb liquid scintillation counter was used throughout this study (Park and Rho, 1983).

Radioimmunoassay

Expression of the globin gene in *E. coli* was determined by radioimmunoassay (RIA) as described by Rho and Gallo (1979) with a slight modification. The antiserum was titrated by incubating about 10,000 cpm of the [^{131}I]-labeled hemoglobin with serial two-fold dilutions of rat antiserum. After incubation for 2 hr at 37°C and for 18 hr at 4°C, the antigen-antibody complexes were precipitated by adding proper amount of second antibody (goat anti-rat IgG serum). The iodinated hemoglobin was diluted in RIA buffer (50 mM Tris/HCl (pH 7.8), 300 mM KCl, 0.5% Triton X-100, 0.5% BSA, 0.05% sodium azide, and 10% glycerol), and all the following radioimmunoassays were performed in this buffer system. The radioactivity of the pellets was measured in beta scintillaton counter, and the results were expressed as the percentage of total TCA precipitable counts at each dilution.

Competitive radioimmunoassays were initiated at the condition where 30% of [^{131}I]-labeled hemoglobin proteins formed antigen-antibody complexes. The *E. coli* extracts used for RIA were prepared with 100 ml cultures of *E. coli* as described by Talmadge *et al.* (1980). Serial two fold dilutions of these solutions (80 μl each) were made in RIA buffer, and added 10 μl titrated anti-hemoglobin serum (titer 1:250). Reaction mixtures were incubated for 2 hr at 37°C and then added about 10,000 cpm of labeled hemoglobin diluted in 10 μl RIA buffer. After further incubation for 1 hr at 37°C, and for 18 hr at 4°C, antigen-antibody complexes were precipitated with second antibody, and the pellets were counted. The results were expressed as the percentage of total cpm in the pellet normalized to 100% binding in the absence of competing antigen.

The standard curve of competitive radioimmunoassay was obtained as above with rabbit hemoglobin instead of *E. coli* extract.

RESULTS

Purification of Globin mRNA

The poly(A)-containing mRNA was purified through the treatment of phenol/chloro-

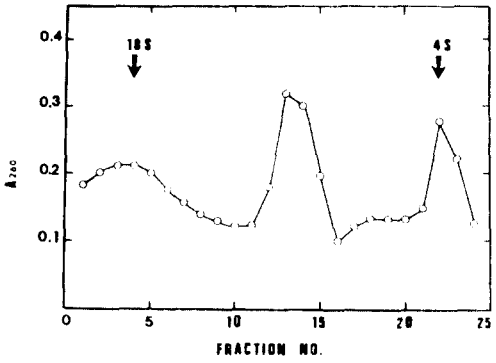

Fig. 1. Absorbance of RNA purified by sucrose density gradient centrifugation after oligo(dT)-cellulose chromatography. The absorbance of each fraction was measured at 260 nm, and normalized to 1 ml sample.

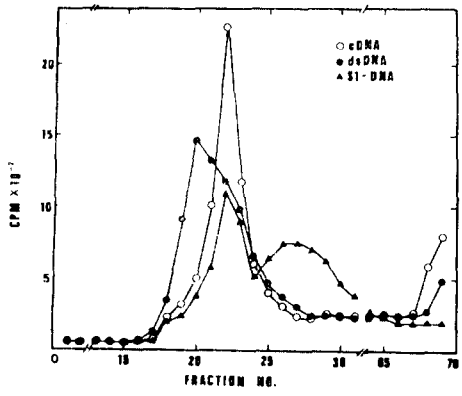

Fig. 2. Electrophoretic profile of cDNA and dsDNA synthesis and Sl nuclease cleavage. Gel electrophoresis and the radioactivities of the slices were counted as described in Materials and Methods. The small fragments of plasmid pBR322 digested by EcoR I/BamH I (375 bases) EcoR I/Pst I (752 bases), and BamH I/Pst I (1, 127 bases) were used as size marker.

form, oligo(dT)-cellulose chromatography, and then fractionated by sucrose gradient sedimentation. Three major peaks were appeared on sucrose gradient as shown in Fig. 1. mRNA banded at 9 S region was pooled for further studies.

Enzymatic Synthesis of Double Stranded DNA in vitro

To determine if cDNA synthesis was accomplished successfully, small amount of [^{3}H] dTTP was added in the reaction mixture of primary cDNA synthesis. The degree of DNA synthesis and S1 nuclease cleavage was analyzed on denaturant formamide gel (Fig. 2). The electrophoretic profile of primary cDNA synthesis gave prominant peak at 650 bases, in which the full length globin cDNA expected to be located. After double stranded DNA synthesis, a new peak was obtained at 1, 300 bases approximately double the size of cDNA. When the 1, 300 bases long DNA was treated with S1 and analyzed by the formamide gel, the major 650 bases long peak and broad peak appeared. These results suggested that the complementary DNA of Hb mRNA was synthesized, and the dsDNA had S1 nuclease sensitive site as expected.

Homopolymer Tailing and Transformation of E. coli

The most common approach currently employed for the cloning of double stranded DNA copies of mRNA is to anneal the cDNA, which has short homopolymeric tracks of (dC), with Pst I-linearized pBR322 that has been similarly modified by (dG) tails. This procedure provides for the regeneration of the original vector Pst I site on both sides of the insert sequence (Otsuka, 1981). The reaction temperature and the incubation time

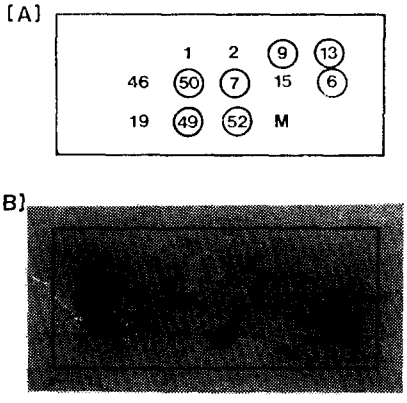

Fig. 3. Hybridization of globin cDNA to transformants. The procedures for colony hybridization and autoradiography were described in Materials and Methods. [A]. Colonies grown on nitrocellulose membrane are illustrated with number. The members enclosed with circles show that the colonies have responded positively. M indicates the standard colony containing plasmid pBR322. [B]. The autoradiograph of hybridized colonies corresponding to above colonies.

were determined to yield six to twelve (dG) or (dC) tracks. After the annealing and transfromation process, approximately 1,300 transformants resistant to tetracycline were obtained per microgram pBR322 annealed with cDNA.

Colony Hybridization

To estimate the yield and identify the clones containing α-and β- globin cDNA, colony hybridizations were performed on bacterial transformants, which were selected by primary screening (Fig. 3). Fig. 3B shows that about 60% of the transformants contained recombinant plasmids with DNA sequences homologous to the globin cDNA. This results reflected the abundance of globin mRNA among red blood cell RNA containing poly (A) and high efficiency of cDNA synthesis.

Restriction Mapping

A group of colonies responding positively to colony hybridization were analyzed further by restriction enzyme digestions to determine the size of the cDNA inserts and to identify either α- or β-globin cDNA. According to previous reports, α-globin DNA contains one EcoR I site, but BamH I site. However, β-globin DNA contains both one EcoR I and BamH I site (Efstratiadis *et al.*, 1977; Heindell *et al.*, 1978). EcoR I endonuclease digestion of the α-globin cDNA cloned plasmid, therefore, gave two fragments (1,105 and 3,795 bases long), and BamH I gave the full sized linear plasmid DNA (Fig. 4). In the case of β-globin cDNA clones, both EcoR I and BamH I endonucleases produced two fragments: EcoR I cleaved the plasmid into 1,105 and 3,815 bases, and BamH I into 1,415 and 3,505 bases (Fig. 5). Each of these plasmid DNAs, containing α- and β-globin cDNAs, was digested with Pst I endonuclease at the reconstructed Pst I restriction site adjacent to the oligo (dG:dC) join. Most of the clones, judged to contain nearly full sized cDNA by the EcoR I endonuclease digestion, contained two Pst I restriction sites and yielded the fragments of original pBR322 DNA (4,362 bases) and cDNA (about 560 bases).

Expression of Globin Antigen

To explore if the cloned globin was expressed in *E. coli* we investigated the clone with competitive radioimmunoassay. After immunization of rat with rabbit Hb, bleeding was

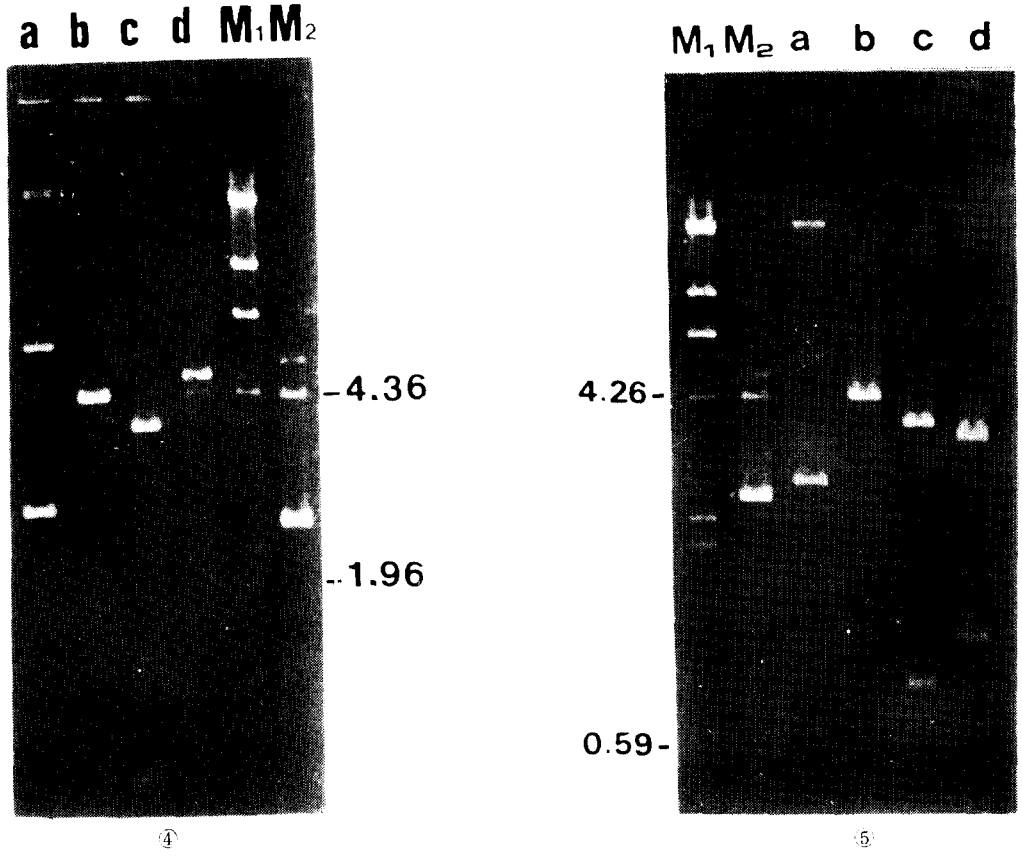

Fig. 4. Restriction endonuclease analysis of plasmid containing α-globin gene. Plasmid DNA prepared from number 50 colony (lane a) was digested with Pst I (lane b), EcoR I (lane c), and BamH I (lane d), and then electrophoresed on 1.2% agarose gel. The DNA size marker M_1 was prepared by digesting λ DNA with Hind III endonuclease, and M_2 illustrated the open circle, linear, and supercoil from of plasmid pBR322 from top to bottom.

Fig. 5. Restriction endonuclease analysis of plasmid containing β-globin gene. All the procedures of analysis were the same as described in Fig. 4, but number 52 colony containing rabbit β-globin gene in plasmid was used instead of number 50 colony.

performed very cautiously to avoid hemolysis. Since the three dimensional structure of all mammalian hemoglobins is conserved, rat hemoglobin itself may crossreact with the antiserum.

Rabbit hemoglobin was radiolabeled with [^{131}I] and was separated from free iodine by Bio-Gel P-10. The specific activity of [^{131}I]-labeled hemoglobin, eluted in void volume, was 22.5 μCi/mg. Using this [^{131}I]-labeled antigen, antiserum titration was performed as shown in Fig. 6.

To establish the standard curve of competitive radioimmunoassay, titrated antiserum was incubated with known amounts of hemoglobin and precipitated the antigen-antibody

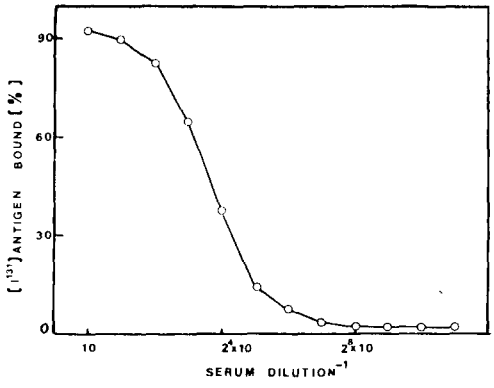

Fig. 6. Titration curve of rat anti-rabbit Hb serum. About 10,000 cpm of [^{131}I]-labeled rabbit hemoglobin was incubated with serial two fold dilutions of antihemoglobin serum.

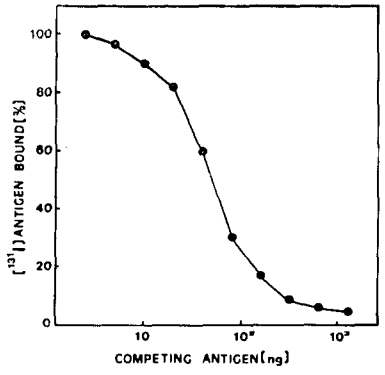

Fig. 7. Standard curve of competitive radioimmunoassay established with hemoglobin. About 10,000 cpm of [^{131}I]-labeled hemoglobin was competed with known amounts of highly purified rabbit hemoglobin.

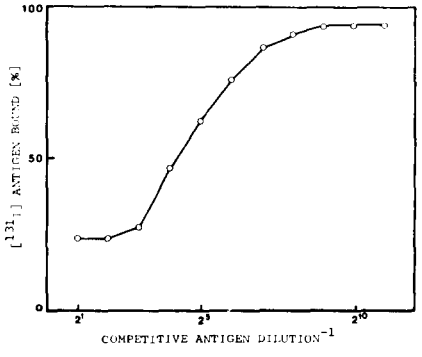

Fig. 8. Competitive radioimmunoassay for bacterial globin gene product. The radioimmunoassay was accomplished as described in Materials and Methods, and the *E. coli* extracts used here were from number 50 clone containing α-globin gene in plasmid.

complex with double antibody method as shown in Fig. 7. The amount of serum chosen for this assay was 1:250 serum dilution, which could precipitate 30% of labeled antigen. The expression of globin gene in *E. coli* was measured with this assay system using bacterial extracts instead of standard rabbit Hb we concluded that about 9,400 molecules of chimeric protein, composed of β-lactamase and globin, existed in full grown *E. coli*. In this calculation, we used the amounts of cell extract required for 50% inhibition of the binding of radiolabeled antigen. As presented in Fig. 8, the inhibition curve by bacterial protein, however, was not identical but similar to standard curve. That is, the slope of competition curve was slaker than the standard curve, and tailed off earlier. The phenomena suggested that the globin gene products in *E. coli* were similar to rabbit globin and having antigenic determinant.

DISCUSSION

The cDNA cloning method described here would be a general and applicable to purified, mixed mRNAs, viral RNAs, or cellular RNAs, all of which have poly(A) tails.

The initial cDNA copy of mRNA was synthesized with AMV reverse transcriptase using primer oligo(dT) annealed to the polyadenylated tail of the mRNA (Efstratiadis *et al.*, 1976; Heindell *et al.*, 1978). Since the efficiency of the primary cDNA synthesis is affected by the integrity and secondary structure of the mRNA, the quality of the reverse transcriptase, and the reaction conditions, this process was accomplished with highly purified reverse transcriptase under the optimal conditions described by Buell *et al.* (1978) and Retzel *et al.* (1980). Much efforts were exercised to obtain a better yield of full length and double stranded cDNA by carrying out a reaction with reverse transcriptase after the reaction with Klenow polymerase, because the stopping points of dsDNA synthesis were different for Klenow enzyme and reverse transcriptase. To retain full structural genome of cDNA until after the removal of the hairpin loop of dsDNA, we lowered incubating temperature and raised the salt concentration from the normal conditions performing Sl nuclease digestion by way of stabilizing the hydrogen bonds and lessening the activity of Sl nuclease. The dsDNA was, subsequently, introduced into the Pst I site of pBR322 vector DNA by homopolymer tailing method to facilitate the later analysis and the subcloning of insert DNA.

Modifications of this cloning process, such as use of a plasmid vector which itself serves as the primer for first strand cDNA synthesis (Okayama *et al.*, 1982), or tailing the oligo (dC) at the 3′ end of primary transcript to eliminate the need for the nuclease digestion of 5′-proximal sequences (Land *et al.*, 1981), can be used to improve the yield of full length cDNA or to produce cDNA with intact 5′-proximal nucleotide sequences.

To express rabbit globin antigenic determinant in *E. coli*, we made use of the fusion protein approach. In this experiment, globin DNA sequences were expressed as a part of a fusion protein whose amino terminus was encoded by bacterial β-lactamase sequences and whose carboxyl terminus was encoded by eukaryotic globin sequences. This fusion protein approach has been used as a general method to express eukaryotic (viral or cellular) proteins which otherwise may be difficult to produce in large quantity because of degradation or instability of the eukaryotic proteins in bacteria (Davis *et al.*, 1981; Goeddel *et al.*, 1979; Itakura *et al.*, 1977; Shine *et al.*, 1980). Furthermore, the β-lactamase region composing the amino terminus of fusion protein serves as a signal peptide which carries the fusion protein to the periplasmic space results in facilitating the purification of the protein (Talmadge *et al.*, 1980).

Considering that the three-dimensional structures of α- and β-chain of hemoglobin are strikingly similar even though there are some differences in the amino acid sequences of

these two polypeptide chains (Perutz, 1974), and that the antigenic determinant resides in a very small portion (several amino acids) of the antigen (Evans *et al.*, 1983), we can anticipate the antigenic determinant of α- and β-chain to be conserved. And if it is true, both α- and β- chain can interact with the same antiserum produced by hemoglobin. In practice, bacterial extract, expected to contain chimeric protein (α-globin and β-lactamase), competed considerably with standard hemoglobin as presented in Fig. 8. This suggests either the antigenic determinants of hemoglobin are the same (or similar) in α- and β-chain, or the rat, from which antiserum was obtained, was immunized mainly by the α-chain.

Further investigation of α- and β-globin gene expression in *E. coli* is needed to evaluate these possibilities.

ABSTRACT

The structural gene of rabbit hemoglobin was cloned into Pst I site of pBR322 in *E. coli*. The complementary DNA (cDNA) was synthesized from rabbit globin mRNA with avian myeloblastosis viral reverse transcriptase, and then RNA was destroyed at pH 11. The double stranded cDNA was synthesized with both Klenow fragment of *E. coli* DNA polymerase I and reverse transcriptase and then the hairpin loop was opened with Sl nuclease. Double stranded cDNA was subsequently tailed with dCTP and annealed to dGMP-tailed vector DNA. After transformation and initial screening of appropriate clones by plasmid size, the cloned colonies were identified by *in situ* colony hybridization using [^{32}P]-labeled cDNA probes and characterized the inserts with restriction endonucleases. The expression of cloned globin gene was investigated by standard radioimmunoassay using rat anti-rabbit Hb serum as primary antibody and goat antirat IgG serum as secondary antibody. The result suggested that the chimeric proteins (the part of β-lactamase from the vector pBR322 and globin from rabbit) were supposedly produced in *E. coli* and the product had the antigenic determinant of rabbit hemoglobin.

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