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Molecular Cloning of the Japanese Quail α A Globin cDNA

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ABSTRACT : Double stranded cDNA was synthesized using the poly(A)$^+$RNA of the Japanese quail reticulocytes and ligated into the EcoRI site of λgt 10 phage DNA. A recombinant phage, λQαG1 was selected by plaque hybridization using a 30mer synthetic oligonucleotide probe specific to the α globin gene. The size of the cDNA insert in the recombinant phage DNA was 0.53kb and the restriction map was similar to that of the chicken α A globin gene. Nucleotide sequencing of the cDNA insert indicated that it contained the entire coding information for the α A globin.

INTRODUCTION

There appear to be two major α globin components, α A and α D in adult avian erythrocytes. These adult α globins are already present in the definitive red blood cells at the late embryonic stage[1]. Yet, other embryonic α −type globins, π and π' are recognized in the primitive red blood cells of early avian embryo[1]. In chicken, the temporal and programmed switching in globin genes expression during the conversion from the primitive erythrocytes to the definitive erythrocytes takes place between days 5 and 9 after fertilization[2), 3)]. Molecular events involved in the change of globin gene expression have been studied extensively in erythrocytes of many species, however they remain mostly unresolved. For the elucidation of globin gene organization and of control mechanisms involved in globin gene expression, cloning of the globin gene cDNA is required. We have synthesized double stranded cDNA using poly (A)$^+$ RNA of the Japanese quail reticulocytes and isolated a α A globin cDNA clone by the use of recombinant DNA methodology. In this paper we describe the characterization and partial nucleotide sequencing of the cDNA insert which contains the entire coding information for the α A globin gene.

MATERIALS AND METHODS

1. Isolation of reticulocytes poly(A)$^+$ RNA.

Six adult quails were made anaemic by intramuscular injection of 0.1ml of 0.5% phenylhydrazine HCl for 5 consecutive days. 20ml of blood was collected from the anaemic birds. Red blood cells were isolated by centrifugation and washed five times in NKM solution (0.13M NaCl, 5mM KCl and 7.4mM $MgCl_2$). Red blood cells were hemolysed in the presence of 4M guanidine thiocyanate and 5mg of total RNA was recovered by the method described by CHOMCZYNSKI and SACCHI[4]. 160 μg of poly A tailed RNA was finally purified by oligo dT cellulose column chromatography and stored in 80% ethanol at −80℃[5].

2. In vitro protein synthesis.

5 μl (5 μg) of quail poly (A)$^+$ RNA was mixed with 40 μl of micrococcal nuclease treated rabbit reticulocyte in vitro protein synthesizing system (supplied by Wako Chemicals) and 5 μl (50 μ Ci) of [35S] methionine and was incubated at 30℃ for 60 min. 2.5 μl of the reaction mixture was combined with SDS polyacrylamide gel buffer and separated by SDS polyacrylamide gel electrophoresis. The gel was dried and autoradiographed on a X ray film.

3. Preparation and kinase labeling of the synthetic oligonucleotide probe.

The synthetic 30mer oligonucleotide having the sequence of GAAGTGGGGGAAGTAGGTC TTGGTGGTGGG was purchased from Nikkaki Co., Ltd., which is complementary to the sequence of the 37th codon to 46th codon of the α globin genes of most species. The 5' end of the oligonucleotide was radiolabeled with [γ−32P] ATP using T4 polynucleotide kinase (supplied by Takara Shuzo. Co., Ltd.)[5].

4. Northern blot analysis

5 μg of the RNA sample was first denatured with glyoxal and 50% dimethylsulfoxide and then electrophoresed on 0.7% agarose gel. The gel was soaked in 20 × SSC and was directly transferred to nitrocellulose membrane filters. The filters were prehybridized in a solution containing 5 × SSC, 9.75 × Denhardt's solution, 50% formamide, 0.1% SDS, and 100 μg/ml sonicated salmon sperm DNA at 42℃ for 5hr. The prehybridization solution was removed and the filters were hybridized with the labeled oligonucleotide (2.6×10^7cpm) in prehybridization solution at 42℃ overnight. After extensive washing in 2× SSC, 0.1% SDS and then in 0.1×SSC, 0.1% SDS, the filters were dried in the air and autoradiographed at−70℃ overnight[5].

5. Synthesis of double stranded cDNA.

5 μg of the quail reticulocyte poly (A)$^+$ RNA was used to prepare double stranded cDNA. A cDNA Synthesis Kit of Pharmacia was employed. EcoRI linker adaptor was ligated to the ds cDNA using T4 DNA ligase and T4 polynucleotide kinase supplied by Pharmacia and the EcoRI −ended cDNA was recovered by passing through a spun column.

6. Construction of the quail reticulocyte cDNA library.

0.2 μg of the ds cDNA attached with EcoRI linker adaptor was ligated to 2.5 μg of λ gt 10 arms supplied by Stratagene (Funakoshi) following a protocol provided by the company. The phage DNA was packaged in vitro using Gigapack purchased from Stratagene (Funakoshi). The phage particles were amplified on C 600 hfl host bacteria.

7. Plaque hybridization of the bacteriophage.

The λ gt 10 recombinant pharge library was distributed on agar plates and plaques were transferred to nitrocellulose filters. The DNA on membrane filters was denatured, prehybridized, and hybridized in the same manner as described in Northern blot analysis. The filters were dried and autoradiographed on X ray films at−70℃ overnight[6].

8. Restriction enzyme digestion.

λ phage recombinant DNA and the insert DNA fragment were digested to completion with restriction endonucleases, EcoRI, Hpa II, Hinc II and Taq I purchased from Takara Shuzo Co., Ltd. The DNA was electrophoresed, stained with ethidium bromide, and photographed.

9. Nucleotide sequencing

The cDNA insert of λ Q α G1 phage was subcloned into plasmids pUC 18 and 119 and the plasmid DNA was purified by the method of Birnboim and Doly[7]. Nucleotide sequence was determined by the dideoxy − ribonucleotide chain termination method[8] using M13 Sequencing Kit, 7−Deaza Sequencing Kit and M4 primer supplied by Takara Shuzo Co., Ltd.

RESULTS

Northern blot analysis of poly A tailed RNA purified from the Japanese quail reticulocytes clearly demonstrated that it contained 9S mRNA coding for the αA globin (Figure 1).

In vitro translation of the poly (A)+ RNA suggested that it was abundant for globin mRNA (data not shown). Double stranded (ds) cDNA was synthesized using 5 μg of the poly (A)$^+$ RNA and ligated into the EcoRI site of λ gt 10 DNA. After packaging, 1×10^4 of recombinant phage per μg of the mRNA was obtained. The recombinant phages were screened by plaque hybridization using a 30mer synthetic oligonucleotide probe specific to the α globin gene. A recombinant phage, λ Q α G1, which showed positive hybridization with the probe was isolated (Figure 2).

EcoRI digestion of λ Q α G1 phage DNA yielded the insert DNA in the size of 0.53kb along with 2 fragments of the vector DNA (Figure 3). The insert DNA was physically mapped with restriction enzymes, Hinc II, Hpa II and Taq I(Fig. 4A). Hinc II digestion of the

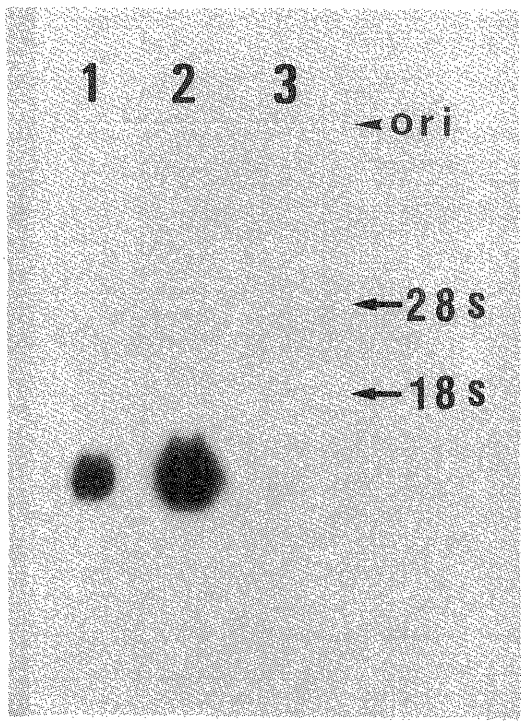

Fig. 1. Northern blot analysis of the quail reticulocyte poly(A)+RNA with a α-globin gene specific probe (see text).
Lane 1 : Quail reticulocyte total RNA.
Lane 2 : Quail reticulocyte poly (A)$^+$ RNA.
Lane 3 : Porcine liver poly (A) + RNA.

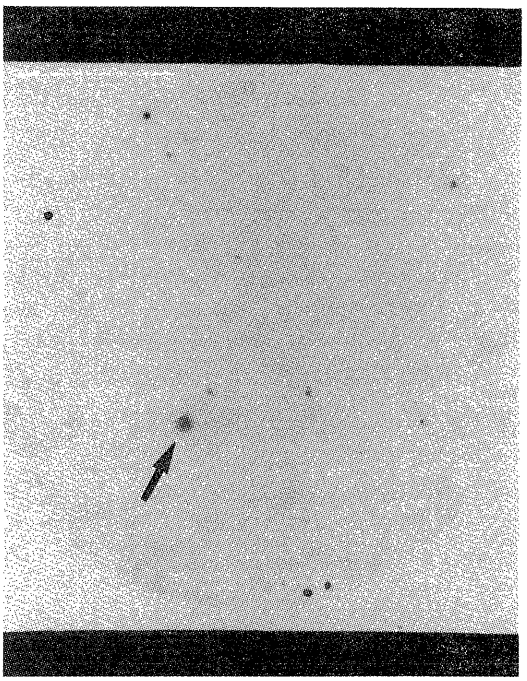

Fig. 2. Autoradiograph of plaque hybridization of the α -globin specific probe, with the recombinant λgt10 phage DNA.

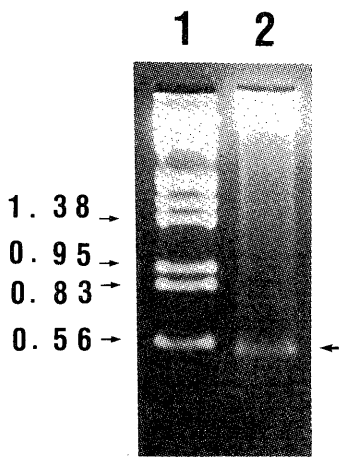

Fig. 3. Agarose gel electrophoresis of the cDNA insert in the recombinant phage λ Q α G1 DNA.
Lane 1 : DNA size marker (Hind III and EcoRI double digest of DNA).
Lane 2 : EcoRI digest of λ Q α G 1 DNA.
The arrow indicatez the position of the cDNA insert.

insert resulted in the DNA fragments of 0.32 kb and 0.21kb indicating a single restriction site. Whereas Hpa II digestion produced 0.3kb, 0.14kb and 0.08kb fragments. The insert DNA seemed to contain one Taq I sites, producing 0.3kb and 0.23kb fragments. These results were consistent with the predicted restriction sites based on the homology with the chicken α A globin gene(Figure 4B). The insert DNA was further subcloned into plasmid pUC 18. Two clones of recombinant plasmids pQ α G5 and pQ α G6 were isolated. Nucleotide sequence in the region around the termination codon of the quail α A globin gene was determined on pQ α G5 DNA. The sequence of 7 codons upstream of the stop codon was identical with the chicken α A globin. The extra 73 nucleotides sequence was found at 3' side of the termination codon (Figure 5). Sequencing of pQ α G6 DNA provided the information near the initiation codon of the quail α A globin.

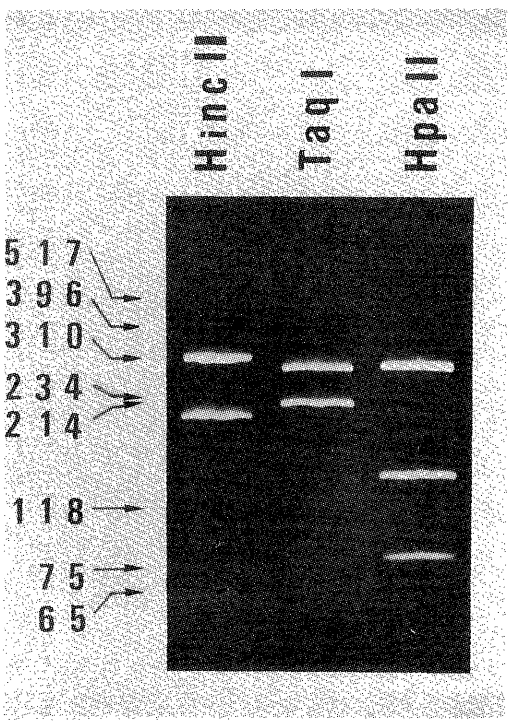

Fig. 4A. Polyacrylamide gel electrophoresis of the restriction enzyme digest of the λ Q α G1 cDNA insert. The standard DNA size markers (Hae III digest of φ X 174 DNA and Hinf I dinest of pUC 18 DNA) are indicated.

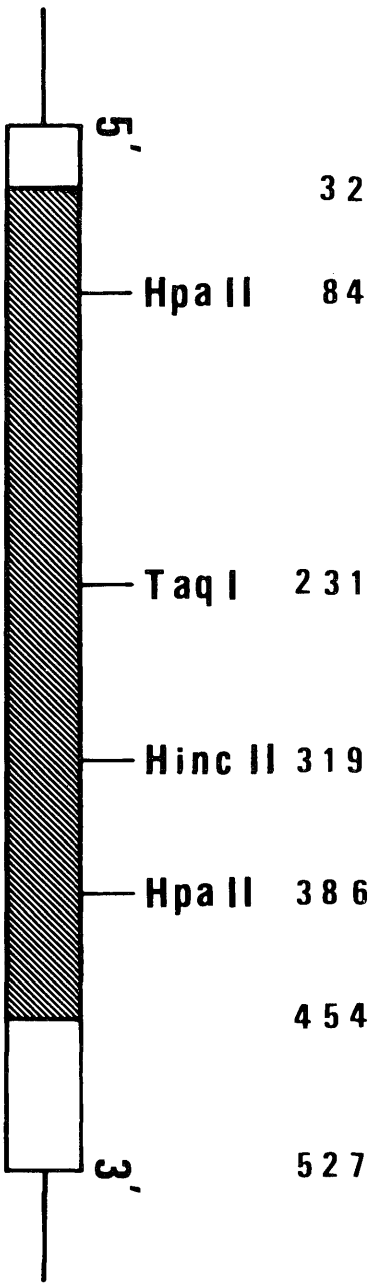

Fig. 4B. A proposed restriction map of the quail α A−globin cDNA. The quail α A globin coding region is indicated by a shaded box. Coordinate numbers for the endnuclease cleavage sites and the globin coding region are indicated.

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                                          1
                                    ***  val  leu  ser  ala  ala  asp  lys  thr
AATTCGGCACGAGCGCAGAGGTGCAACC ATG GTG CTG TCC GCT GCT GAC AAG ACC

                                          18                 84
asn  val  lys  gly  ile  phe  ser  lys  ile  ala                ser  asp  leu  his
AAC GTC AAG GGC ATC TTC TCC AAA ATC GCC - - - - AGC GAC CTG CAT

                                          96   97
ala  gln  lys  leu  arg  val  asp  pro  val  asn  phe  lys  leu  leu  gly  gln
GCC CAG AAG CTG CGC GTG GAT CCT GTC AAC TTC AAA CTC CTG GGC CAA
                                         HincII
                                                    114                135
cys  phe  leu  val  val  val  ala  ile  his  his  pro                val  leu  thr
TGC TTC CTG GTG GTG GTG GCC ATC CAC CAC CCT - - - - GTG CTG ACC

                  141
ala  lys  tyr  arg  ***
GCC AAG TAC CGT TAA GATGGCACGGCGGGTAGAGCTGGGGGGCAACCCACCACCAGCC


CTCCGACAGCGAGCAGCCCTCGTGCCG
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Fig. 5. Nucleotide sequence of the λ Q α G1 cDNA insert containing the quail α A−globin gene. The sequence corresponding to the EcoRI linker adaptor is underlined.

The coding sequence for the first 18 amino acid residues of the globin was determined and found to be nearly identical with the chicken α A globin. The insert DNA contained extra 31 bp prior to the initiation codon (Figure 5). Two DNA fragments obtained by the Hinc II digestion of the EcoRI insert of the phage λ Q α G1 were subcloned into plasmid pUC 18. Two types of recombinant plasmid clones were isolated; namely pQ α GH14 which contained the 320 bp insert DNA and another pQ α GH15 having the 210 bp insert DNA. Nucleotide sequencing of the two clones of recombinant plasmid DNA revealed the sequence spanning from 84th to 114th codons of the quail α A globin including the Hinc II restriction site (Figure 5).

DISCUSSION

Avian species are among the most convenient animal models for the study of embryonic development. Haematopoietic cell differentiation in chicken and quail has been studied extensively and the globin gene expression is a useful marker for the erythrocyte differentiation. In an attempt to elucidate the α globin gene organization, we have cloned the cDNA for the α A globin of the Japanese quail.

Evidence in support of the cDNA clone specifically encoding the quail α A globin was obtained as follows. First, the poly $(A)^+$ RNA of the anaemic quail reticulocytes contained abundant α globin mRNA, as confirmed by Northern blot analysis and in vitro translation experiments. Second, the recombinant phage was selected by plaque hybridization using a oligonucleotide probe specific to the α globin sequence. Thirdly, physical mapping of the cDNA insert revealed that its restriction sites were quite similar to those found in the chicken α A globin gene. Finally and most convincingly, the nucleotide sequence of the cDNA insert indicated that it contained the entire coding information of α A globin and the sequence was similar to that of the chicken α A globin gene. Partial amino acid sequence of the α A globin (QII α) of the Japanese quail is known[9]. Our preliminary

nucleotide sequence data does not contradict with the protein information. This is quite contrary to the situation reported in the α A globin of chicken[10, 11, 12]. The complete nucleotide sequence of the α A globin gene, when available, should provide the full information on the primary structure of the quail α A globin. The cDNA clone we isolated should also be useful for the analysis of the α globin gene organization.

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