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Nucleotide sequence of 3' untranslated portion of human alpha globin mRNA

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ABSTRACT

We have determined the nucleotide sequence of 75 nucleotides of the 3'-untranslated portion of normal human α globin mRNA which corresponds to the elongated amino acid sequence of the chain termination mutant Hb Constant Spring. This was accomplished by sequence analysis of cDNA fragments obtained by restriction endonuclease or T_4 endonuclease IV cleavage of human globin cDNA synthesized from globin mRNA by use of viral reverse transcriptase. Analysis of cRNA synthesized from cDNA by use of RNA polymerase provided additional confirmatory sequence information. Possible polymorphism has been identified at one site of the sequence. Our sequence overlaps with, and extends the sequence of 43 nucleotides determined by Proudfoot and coworkers for the very 3'-terminal portion of human α globin mRNA. The complete 3'-untranslated sequence of human α globin mRNA (112 nucleotides including termination codon) shows little homology to that of the human or rabbit β globin mRNAs except for the presence of the hexanucleotide sequence AAUAAA which is found in most eukaryotic mRNAs near the 3'-terminal poly(A).

INTRODUCTION

Recent structural studies of eukaryotic mRNAs show that there are relatively long stretches of untranslated RNA between the termination codon and the 3'-terminal polyadenylic acid of the mRNA. Complete sequences are available for the 3'-untranslated regions of SV40 early and late mRNAs and for human and rabbit beta chain mRNAs; partial sequences are available for several other eukaryotic mRNAs. These various sequences show some homology in the region immediately preceding the polyadenylic acid. The remainder of the untranslated sequences are remarkable for their diversity, both in chain length and in nucleotide sequence. For example, the 3'-untranslated regions of rabbit and human beta globin mRNAs are substantially different (1,2,3), and more divergent than the translated sequences (1,2). Therefore it was desirable to extend these studies to determine if the 3'-untranslated sequence of human alpha globin mRNA demonstrates any homology with that of the human beta globin mRNA.

METHODS

(a) Enzymes and substrates.

The following restriction endonucleases were either obtained from New England Biolabs or prepared in this laboratory by published procedures: EcoRII (4); HpaII (5); HaeIII (4,6); Alu I. T_4 endonuclease IV was the kind gift of W. Sadowski; bacterial alkaline phosphatase was from Worthington; (γ - ^{32}P) ATP, 1000 to 4000 Ci/mmole, from ICN or New England Nuclear. T_4 polynucleotide kinase was purified from T_4 infected E. coli by the method of Richardson (7).

Sickle cell globin mRNA was isolated from reticulocyte RNA as previously described (8) and purified by oligo (dT) - cellulose column chromatography. Purified sickle cell mRNA from individual patients was then used as a template for the production of single or double stranded cDNA by the RNA-dependent DNA polymerase of avian myeloblastosis virus as described previously (1,9).

(b) End-labeling using polynucleotide kinase and (γ - ^{32}P) ATP.

For sequence analysis 6 to 10 µg of double stranded cDNA (synthesized in the absence of actinomycin D) were digested for 18 hr with restriction endonucleases as previously described (1). After digestion, the cDNA was treated with 0.4 to 0.6 µg of bacterial alkaline phosphatase for 60 min at 37^o in 60 mM Tris, pH 9.0. The reaction mixture was extracted twice with water-saturated phenol. The supernatant was collected after centrifugation, and traces of phenol were removed by a succession of ten ether extractions. The cDNA was precipitated by the addition of 1/10 volume of 3.0 M sodium acetate pH 7.0 and 2 volumes of ethanol at -20^o. The resulting precipitate was collected by centrifugation at 100,000 X G for 1 hr and redissolved in 100 µl of 70 mM Tris, pH 7.4, 20 mM $MgCl_2$, 5 mM dithiothreitol; 5 µl of 0.15 mM spermidine were added and the solution heated to 90^o for 3 min. After cooling,200 to 250 µCi of high specific activity (γ-^{32}P) ATP and 10 units of T_4 polynucleotide kinase were added and the solution incubated at 37^o for 60 min. The labeled digestion products were then separated by gel electrophoresis in a vertical 40 cm slab gel apparatus as described by Subramanian et al. (10), except that the upper 2/3 of the gel consisted of 10% acrylamide and lower 1/3, 15% acrylamide. After electrophoresis in the presence of 200 volts for18 hr the gel was covered with cellophane wrap and subjected to autoradiography. The gel segments corresponding to ^{32}P-labeled cDNA fragments were excised and ground in 0.1x SSC using a Tekmar tissue grinder. After centrifugation of the gel suspension, the cDNA

fragments extracted into the supernatant were precipitated by addition of ethanol and pelleted by centrifugation as described above.

(c) Partial snake venom phosphodieterase digestion (SVPD). ^{32}P-labeled cDNA fragments were dissolved in 4 or 5 µl of 10 mM Tris HCl pH 7.4, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 6mM KCl and subjected to SVPD digestion as described by Maniatis et al. (11). Digested samples were applied to Cellogel strips and subjected to electrophoresis at 4000v for 1 hr in pH 3.5 buffer (12). The material was then transferred to thin layer DEAE-cellulose plates and fractionated by displacement chromatography (homochromatography) (13).

(d) T_4 endonuclease IV digestion. Single stranded cDNA synthesized in the presence of (α-^{32}P)dCTP (120 Ci/mmole, New England Nuclear) was digested with endonuclease IV and the products fractionated by electrophoresis at pH 3.5 in the first dimension and homochromatography in the second dimension (12,13). The oligonucleotides thus purified were eluted and subjected to SVPD digestion as described above.

(e) Analysis of cRNA.

The preparation and purification of cRNA transcripts from globin cDNA using E. coli RNA polymerase in the presence of (α-^{32}P) nucleoside triphosphates and the detailed procedures for sequence analysis of this cRNA have been described previously (9,12). In summary, pancreatic and T_1 RNase digests of the ^{32}P-labeled cRNA were fractionated in two dimensions as described above, and the oligonucleotides were subsequently eluted from the chromotography plates. The products from T1 RNase digests were further analyzed by digestion with spleen phosphodieterase, pancreatic RNase, RNase U_2 and alkaline hydrolysis. Primary pancreatic RNase digestion products were digested with T1 RNase and also analyzed further as above. Larger RNase T1 products were digested with carboxymethylated pancreatic RNase followed by pancreatic RNase digestion, or were analyzed by the combined use of carbodiimide reagent and pancreatic RNase (13).

RESULTS

A restriction endonuclease cleavage map for the human α globin cDNA is presented in Figure 1. Double stranded cDNA was synthesized from sickle cell mRNA using RNA-dependent DNA polymerase of avian myeloblastosis virus in the absence of actinomycin D. The resulting cDNA was digested with restriction endonucleases and the products separated by gel electrophoresis. These sickle cell cDNA fragments were assigned to either the α or β globin mRNA chain by comparison of similar gel separations of cDNA products

transcribed from mRNA of a patient with α thalassemia (Hb H disease)(1). Various cDNA fragments were correlated by molecular weight to cleavage sites thought to be present in human α globin gene DNA on the basis of sequence analysis of oligonucleotides of α cRNA, or on the basis of the genetic code from knowledge of the amino acid sequences of the normal and two abnormal (elongated) human α globin chains, (i.e. those of Hb Wayne (14) and Hb Constant Spring (15). Proposed cleavage sites were confirmed by matching the total nucleotide sequence of cDNA fragments extracted from the gel to specific amino acid sequences in α globin. Also, cleavage sites were reaffirmed by recutting isolated cDNA fragments a second time with a different restriction endonuclease.

(a) SVPD oligonucleotide maps.

Two-dimensional oligonucleotide maps of partial snake venom exonuclease digests of cDNA fragments are shown in Figs 2-8. From these figures, tentative nucleotide sequences can be deduced on the basis of relative mobility and mobility shifts between successively smaller exonuclease cleavage products (16). For example, from the oligonucleotide map K1-2, (Fig. 2), the sequence AACGGTAT was determined. Since the cDNA cleavage fragment was double stranded, this sequence either corresponds to that of the cDNA strand which is directly complementary to the alpha globin mRNA

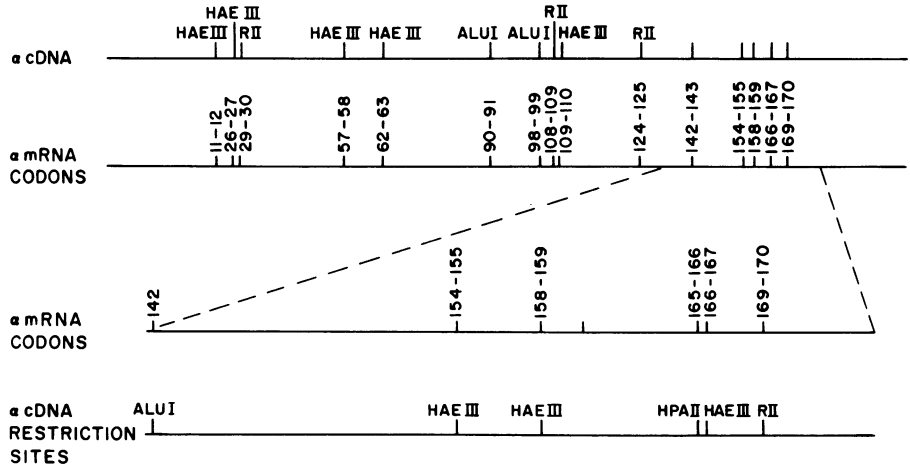

Figure 1. Restriction endonuclease cleavage map of human α cDNA.

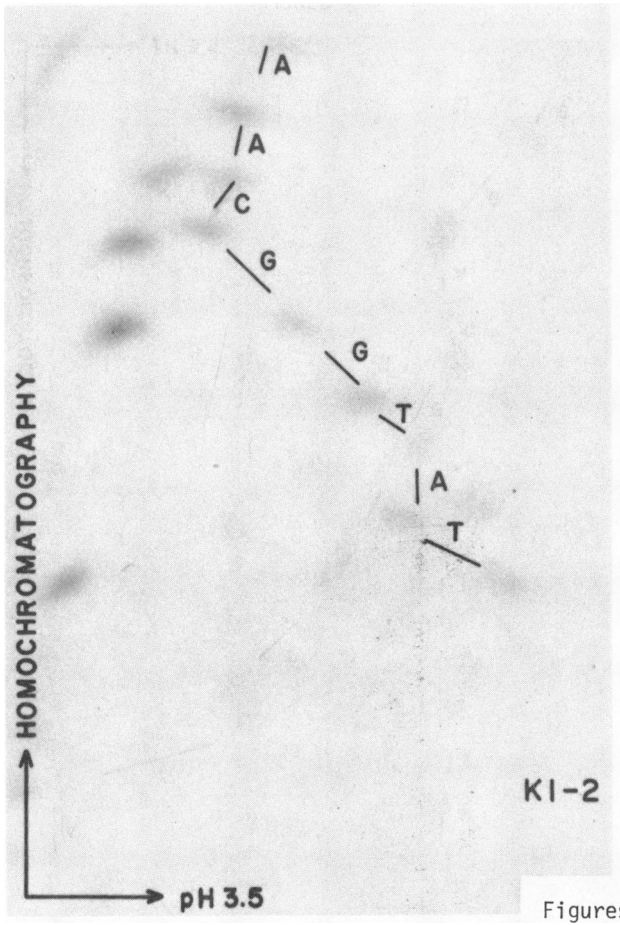

Figure 2.

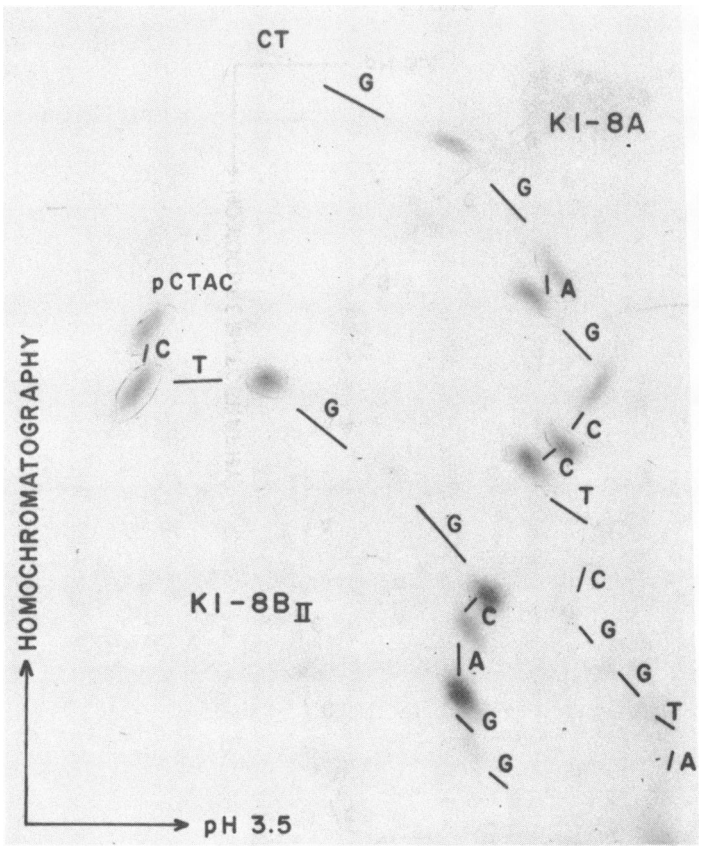

Figure 3.

Figures 2-8. Oligonucleotide maps of partial snake venom exonuclease digests of cDNA fragments.

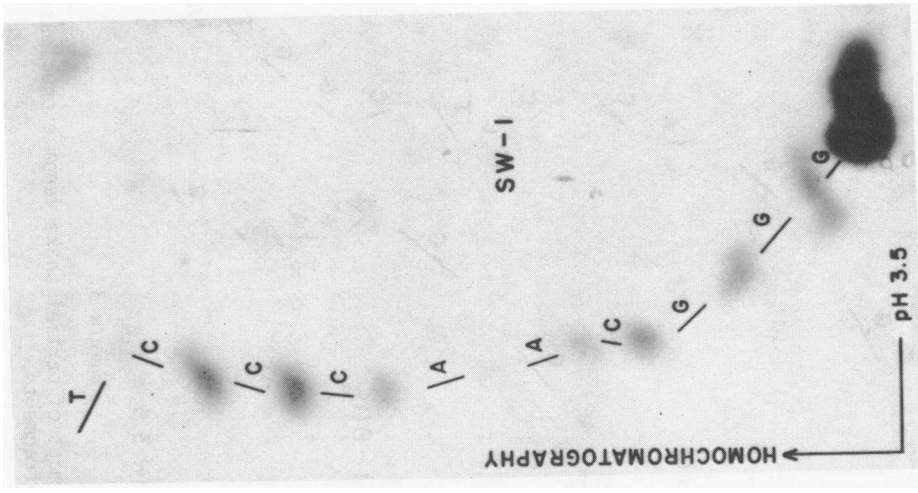

Figure 5.

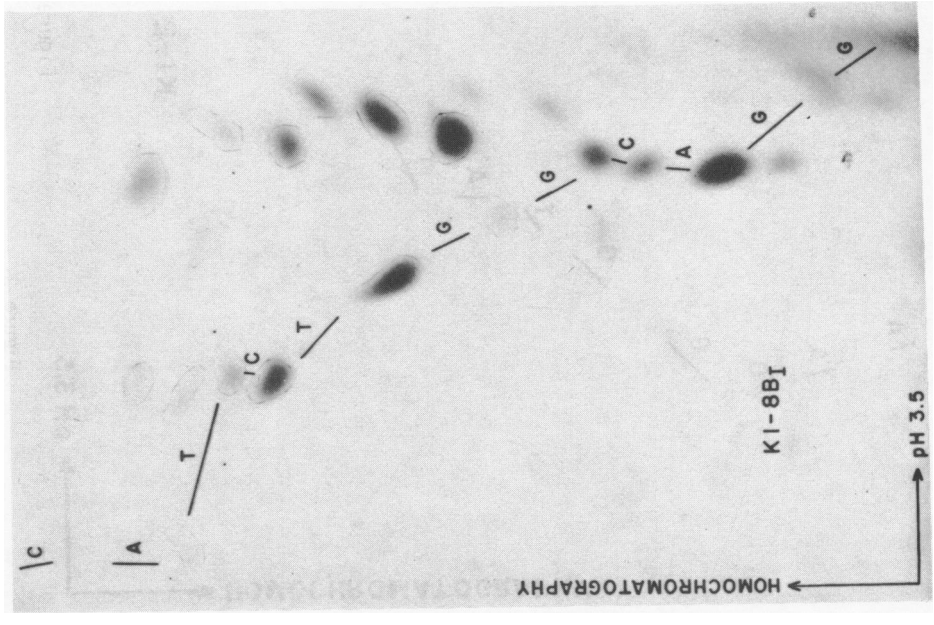

Figure 4.

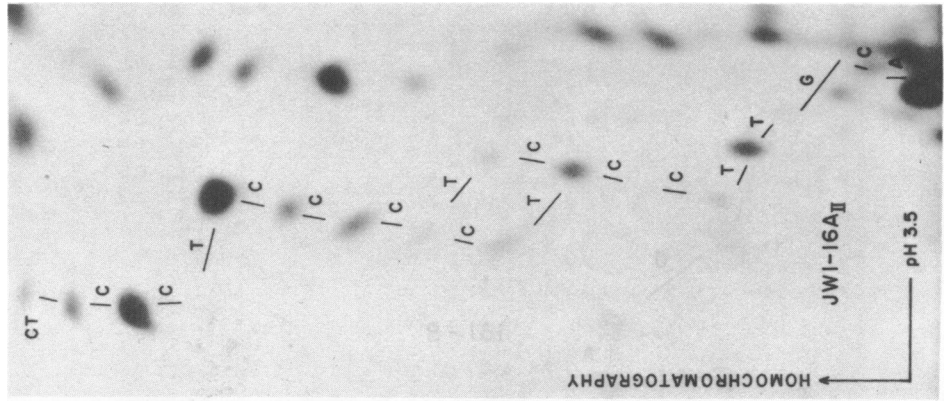

Figure 7.

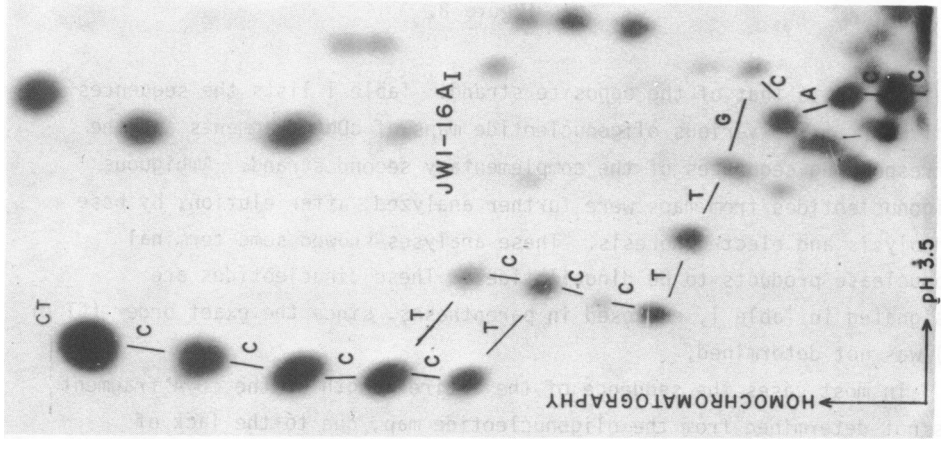

Figure 6.

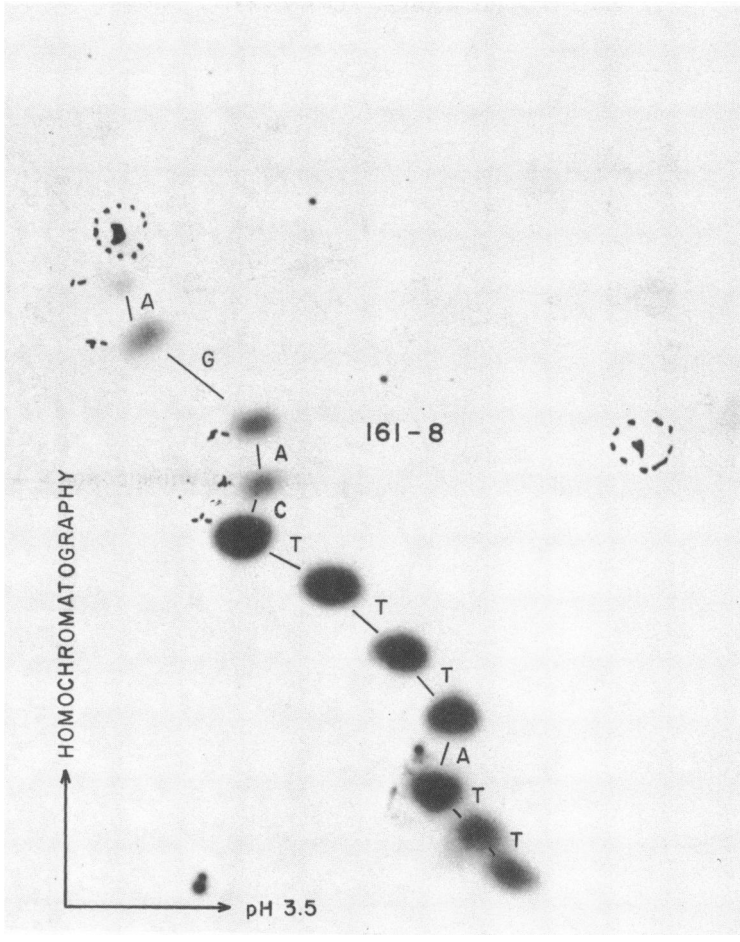

Figure 8.

sequence or to that of the opposite strand. Table 1 lists the sequences derived from the various oligonucleotide maps of cDNA fragments and the corresponding sequences of the complementary second strand. Ambiguous oligonucleotides from maps were further analyzed, after elution, by base hydrolysis and electrophoresis. These analyses showed some terminal exonuclease products to be dinucleotides. These dinucleotides are designated in Table 1, enclosed in parentheses, since the exact order (CT or TC) was not determined.

In most cases the sequence of the entire length of the cDNA fragment was not determined from the oligonucleotide map, due to the lack of separation of the very large oligonucleotide products by the 2 dimensional

Table 1

Sequences Derived From SVPD Oligonucleotide Maps of cDNA Fragments.

Fragment Number	Fig. No.	Sequence	Complementary Sequence	α mRNA Codon Position
K1-2	2	AACGGTAT	ATACCGTT	139 - 142
K1-8A		(CT)GGAGCCTCGGTA	TACCGAGGCTCC(AG)	143 - 147
K1-8B$_I$	4	CATCTGGCAGG	CCTGCCAGATG	151 - 154
K1-8B$_{II}$	3	(C_2TA)CTGGCAGG	CCTGCCAG(ATG$_2$)	151 - 154
SW1	5	TCCCAACGGG	CCCGTTGGGA	156 - 159
JW1-16A$_I$	6	(CT)CCC$^{TC}_{CT}$CCTTGCAC	GTGCAAGG$^{GA}_{AG}$GGG(AG)	161 - 165
JW1-16A$_{II}$	7	(CT)CCTCCC$^{TC}_{CT}$CCTTGCA	TGCAAGG$^{GA}_{AG}$GGGAGG(AG)	160 - 165
161-8	8	AGACTTTATT	AATAAAGTCT	172 - 175

Fragment no. 161-8 was obtained from endonuclease IV digestion of ^{32}P-labeled single stranded cDNA; all other fragments were obtained by restriction endonuclease cleavage of double stranded cDNA. The underlined sequences are those represented in Fig. 9.

fractionation procedure. In several cases, residual nucleotides that were undetermined by the two-dimensional procedures, were deduced from knowledge of the sequence specificity of the endonuclease used to produce the given cDNA fragment. For example, SW-1, and JW1-16A represent fragments produced by cleavage of the cDNA with restriction endonuclease Hae III. This endonuclease recognizes the sequence GGCC, cleaving between the GC residues. Therefore, fragments produced by Hae III cleavage contain CC at the 5' terminus and GG at the 3' terminus.

One region of the 3' untranslated portion of the α globin mRNA which was particularly difficult to sequence was the region between the termination codon (which is involved in a recognition site for endonuclease AluI) and codon no. 154 (which is involved in a Hae III recognition site). The fragment produced by cleaving the cDNA with both of these endonucleases was shown by gel electrophoresis to have a length fo approximately 36 nucleotides. By use of SVPD oligonucleotide maps, we were able to deduce 15 nucleotides of the 5' terminus (K1-8A) and 12 nucleotides of the 3' terminus (K1-8B, I-II) of this fragment. Of the remaining 9 bases spanning the midpoint of this fragment, 6 were assigned on the basis of sequence analysis of cRNA oligonucleotide no. T26 (Table 2). This oligonucleotide (U_4C_4G) was found in a single molar yield in α cRNA and uniquely matched the

sequence of this position of the α globin mRNA. From the preceding analysis, only the sequence of codon no. 148 was left ambiguous. The first two bases of this codon were deduced to be GC by analysis of the amino acid sequences of Hb Wayne and Hb Constant Spring (14). The third base was deduced to be A on the basis of the following reasoning: If the third position of codon no. 148 was G or C, one would expect to find, respectively, GGUU in pancreatic RNase digests, and CCG in RNase T1 digests of α cRNA. In fact, analysis of cRNA yields no GGUU oligonucleotides (eliminating G) and very little CCG (eliminating C). Furthermore, if the third position was T (or U in the mRNA)codon nos. 147 and 148 would contain a recognition site for AluI. Since this fragment was not recut by AluI, the third base cannot be a T. Therefore, the third base in codon no. 148 is most probably an A residue.

Analysis of oligonucleotide fingerprints JW1-16A_I and JW1-16 A_{II} (Figures 6 and 7) raised the possibility of two alternative sequences, in the same region of the mRNA, varying from each other by a possible inversion of two nucleotides (CT). We believe that this may represent the occurrence at this site of a genetic polymorphism (see discussion).

Table 2

RNase T1 Oligonucleotides of Human Globin cRNA
Corresponding To 3'-Untranslated Sequences of α mRNA

Spot no.	Sequence derived from analysis of cRNA alone	α mRNA sequence assignment (Fig. 9)	α mRNA codon position
T3	(G)ACC(U,C)CAAAUACCG(U)	GACCUCCAAAUACCGU	136 - 141
T5	(G)C(C_n, UC_n, U)UG(C)	GCCCUCCUCCCCUCCUUGC	159 - 165
T14	(G)C(C,UCC,C)AACG(G)	GCCUCCCAACGG	155 - 158
T20	(G)AAUAAAG(U)	GAAUAAAGU	172 - 174
T26	(G)(U,U,C)C_3U_2G(C)	GUUCCUCCUGC	149 - 152
T27	(G)(C,C,C,U)C_2U_2G(G)	GCCCUUCCUGG	166 - 170
T51*	(G)(C,CU)CG(G)	GCCUCGG	145 - 147
T56*	(G)UUAAG(C)	GUUAAGC	141 - 143
T60	(G)(UC,U,U)UG(A)	GUCUUUGA	170 - 172

The spot numbers refer to those illustrated in Refs. 8 and 26.
*This spot contains a mixture of different oligonucleotides with the same overall base composition but different sequences.

(b) Derivation of the sequence of the 3' untranslated region of human α mRNA.

The abnormal hemoglobin, Hb Constant Spring, is believed to have arisen by a single base substitution in the α chain termination codon (UAA) to give CAA, a codon which specifies glutamine (15). This mutation would then allow readthrough of the termination codon and translation of usually untranslated sequences of the α mRNA until a new "in phase" termination codon is encountered. In the case of Hb Constant Spring this occurs 31 codons later since the α Constant Spring globin chain contains 31 additional amino acid residues at its C-terminus. From the amino acid sequence of Hb Constant Spring beyond the normal termination codon, (Figure 9 Line A), one can predict a partial nucleotide sequence for this region of the α globin mRNA (Figure 9, Line B). The sequences derived from partial snake venom exonuclease digests of cDNA fragments (Table 1) and those derived from analysis of cRNA (Table 2) can be aligned with the predicted sequence as illustrated in Figure 9, Lines C and D.

Hemoglobin Wayne is believed to have arisen by deletion of one nucleotide in codon no. 139 of the normal α globin mRNA (14). This deletion results in a frameshift mutation which allows readthrough of the

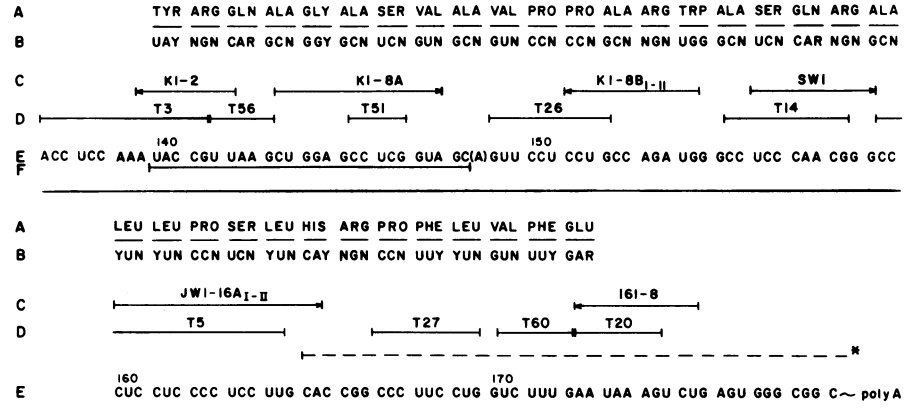

Figure 9. Nucleotide sequence of 3'-untranslated region of α mRNA. A, amino acid sequence of Hb Constant Spring; B, mRNA sequence predicted from Hb Constant Spring amino acid sequence; C, SVPD cDNA sequence assignments (Table 1); D, cRNA sequence assignments (Table 2); E, experimentally derived sequence of α mRNA; the bracket under the sequence represents the mRNA sequence predicted by Hb Wayne; the dashed line over the sequence represents the sequence determined by Proudfoot and coworkers (17,18).

termination codon and synthesis of five additional amino acids by translation of usually untranslated sequences of the α mRNA. By comparing the amino acid sequences of both Hb Constant Spring and Hb Wayne, a unique nucleotide sequence of 26 base residues can be predicted for this region of the α mRNA, including 17 base residues beyong the termination codon (14). This predicted sequence is indicated by the bracket under the sequence listed in Fig. 9, Line E, and does not disagree with the sequence which we have derived for this portion of the α mRNA.

The sequence of human α mRNA from residue no. 66 beyond the normal termination codon to the 3'-terminal poly (A) has been determined by Proudfoot, and co workers (17,18), and is indicated by the dashed line in Fig. 9; some of our α cRNA sequences and T_4 endonuclease IV oligonucleotide sequences can be matched to portions of this sequence. Assuming no internal duplication of sequences, our sequence overlaps the 3'-terminal sequence proposed by Proudfoot and coworkers. We therefore did not conduct any further detailed analysis of the very 3'-terminal portion of human α mRNA.

αGLOBIN mRNA

UAA GCU GGA GCC UCG GUA GCA GUU CCU CCU GCC AGA UGG GCC UCC CAA CGG GCC CUC
CUC CCC UCC UUG CAC CGG CCC UUC CUG GUC UUU GAA UAA AGU CUG AGU GGG CGG C polyA

βGLOBIN mRNA

UAA GCU CGC UUU CUU GCU GUC CAA UUU CUA UUA AAG GUU CCU UUG UUC CCU AAG UCC
AAC UAC UAA ACU GGG GGA UAU UAU GAA GGG CCU UGA GCA UCU GGA UUC UGC CUA AUA
AAA AAC AUU UAU UUU CAU UGC polyA

Figure 10. Comparison of 3'-untranslated regions of human α and β globin mRNAs.

DISCUSSION

The nucleotide sequence determined for the 3'-untranslated portion of human alpha globin mRNA is entirely consistent with the sequence of the C-terminus of Hb Constant Spring and provides further confirmation of the hypothesis that this hemoglobin arose by a mutation resulting in a base substitution in the termination codon of the α globin mRNA allowing readthrough of the termination codon and translation of usually untranslated 3'-terminal sequences of the mRNA (15). The sequence also fully agrees with the sequence of 26 nucleotides predicted for a portion of this region of the α mRNA, on the basis of comparison of the amino acid sequence of the

frameshift mutant Hb Wayne with that of Hb Constant Spring (Ref. 14 and Fig. 9).

One of the more striking results of the analysis of the coding portions of the rabbit and human beta globin mRNAs has been the marked bias in the use of synonym codons for certain amino acids (1,2). Similar results have been found in the case of the coding portions of human α mRNA (unpublished observations). For example in the case of leucine, serine, and alanine the following codons have not yet been found in the nearly completed coding sequence of the human β globin mRNA: UUA and UUG (Leu); UCG and UCA (Ser), and GCG (Ala) (1). CGN codons for arginine are used in the α mRNA but not in the β mRNA. The bias in codon usage is similar in the case of rabbit β globin mRNA (2) and correlates well with the levels of different tRNA isoacceptor species present in rabbit reticulocyte tRNA (Hatfield, Mathews and Caicuts, personal communication). It is possible that this phenomenon may play a role in the regulation of globin synthesis. Within the additional portion of the α mRNA that is translated in Hb Constant Spring, the codons UUG (Leu), UCG (Ser) CGG (Arg) and GCG (Ala) are found. Each of these codons is infrequent or absent in the coding sequence of the normal human globin chain mRNAs. If the tRNA species for these rare codons are deficient in the cell, then this could possibly explain the low amounts of Hb Constant Spring present in red cells: in individuals with Hb Constant Spring, the variant Hb is found to comprise only a low percentage (1-3%) of the total hemoglobin (15). Its synthesis may be limited to early erythroid precursor cells (19), although some evidence to the contrary exists (20). Perhaps, as the red blood cells mature the various tRNAs segregate and the variant chains cannot be translated effectively due to the use of the codons which are scarce in normal globin mRNAs and which require tRNAs which are present in low amounts in reticulocytes. The hypothesis that the variant Hb Constant Spring may be synthesized in low amounts because of the presence of codons in its mRNA which are not usually utilized in normal globin chain synthesis does not however appear to apply to the case of Hb Wayne which is also synthesized in low amounts (4 to 6% of the total Hb, Ref. 14) but the mRNA for which does not contain rarely used globin mRNA codons other than one CGG for its C-terminal Arg.

Most, if not all, human populations appear to have two alpha chain loci per haploid set of chromosomes (21). One might therefore expect to see genetic polymorphism in the translated or untranslated regions of α

chain mRNA. To date, no definite evidence of such polymorphism has emerged either from analyses of α chain variants or from amino acid sequence studies of normal α globin chains (22). However, on the basis of cDNA sequence analysis (SVPD digestion of cDNA fragments), we have noted in at least two different individuals, the apparent interchange of the nucleotides TC and CT at position nos. 61 and 62 (codon no. 163) beyond the termination codon in the 3'-untranslated region of the alpha globin mRNA (Ref. 23, Table I, Figs. 6 and 7).

The finding of a possible polymorphism of the 3'-untranslated sequence of human α globin mRNA is of particular interest because it occurs at a site, codon no. 163, which should be represented in Hb Constant Spring. One of the sequences determined for this region of the mRNA (-CCC-UCC-UUG) (codon nos. 162-164) is in agreement with the amino acid sequence found in Hb Constant Spring: Pro - Ser - Leu; the alternative sequence (-CCC-CUC-UUG) codes for the amino acid sequence Pro-Leu-Leu which was not detected in the original Hb Constant Spring sample (24) or in a number of additional samples obtained from various oriental subjects (J.B. Clegg, personal communication). One explanation for this finding is the possibility that the Hb Constant Spring mutation specifically affects only one of the two linked α globin genes. An alternative explanation is that the observed polymorphism (in mRNA isolated from black patients with sickle cell anemia) is specific for that particular racial group and not present in oriental subjects. Polymorphism has also been described in the 3'-untranslated sequence of rabbit α mRNA (25) but this polymorphism in the rabbit occurs at a more distal site than the human polymorphism: 30 nucleotides from the poly (A) in the rabbit, versus 48 and 49 nucleotides from the poly (A) in the human.

A comparison of the 3'-untranslated sequence of the β globin mRNA from rabbit and human shows striking sequence homologies in the distal region of the mRNAs (adjacent to the poly (A)) but little homology in the more proximal region adjacent to the normal termination codon (1-3). A comparison of the distal region of the rabbit and human α globin mRNAs also shows striking sequence homology (17,18). The sequence of the entire 3'-untranslated sequence of rabbit α globin mRNA has recently been determined by Proudfoot et al (27); with the exception of the first 10 nucleotides following the normal termination codon which are identical in both α mRNA's, the proximal portion of the 3'-untranslated rabbit α mRNA shows less homology with the human α mRNA than does the sequence

adjacent to the poly (A). In our present study, we have found no striking homologies in comparing the complete sequence of the 3'-untranslated region of the human α and β mRNAs (figure 10) other than the hexanucleotide sequence AAUAAA which is present near the poly (A) in many eukaryotic mRNAs (17). Therefore there does not seem to be any extensive species specific sequence homology between the 3'-untranslated sequences of closely related mRNA.

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