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Nucleotide sequences of the 3′-terminal untranslated region of messenger RNA for human beta globin chain

(RNA-dependent DNA polymerase/RNA polymerase/hemoglobins Cranston and Tak/frameshift mutation)

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ABSTRACT **In normal messenger RNA for the human β-globin chain, nucleotide sequences have been identified which can be matched to the amino-acid sequence of the abnormally long segment of the β-chain of hemoglobin Cranston. The finding of these sequences strengthens the hypothesis that the $\beta^{Cranston}$ chain arose by a frameshift mutation allowing the "readthrough" of the normal termination codon and translation of usually untranslated portions of the messenger RNA for the β-globin chain. The oligonucleotides which match the amino-acid sequence of hemoglobin Cranston provide a sequence of 36 nucleotides which follows the normal β-chain termination codon UAA.**

The origin of most abnormal human hemoglobins can be attributed to single nucleotide base substitutions, in the globin gene DNA and subsequently in the globin messenger RNA (mRNA), which lead to amino-acid substitutions in the affected globin chains. There are five different abnormal hemoglobins (1–5) which contain abnormally long α-globin chains of adult hemoglobin (Hb A: $\alpha_2\beta_2$). These α-chain variants have additional amino-acid residues at their carboxy-terminal end and their origin can be explained by chain termination or frameshift mutations of the α-chain mRNA (1–7). The additional amino-acid residues are thought to result from translation of usually untranslated 3′-terminal sequences of the mRNA. Normal globin mRNA, because of its molecular weight (8–11), is known to contain approximately 200 nucleotides more than are strictly necessary to code for the normal sized globin chains (9), and only 50 to 75 of these additional base residues can be accounted for by a 3′-terminal poly(A) sequence (12, 13). Direct nucleotide sequence analysis of normal human globin mRNA (14, 15) has in fact revealed predicted 3′-terminal nucleotide sequences of α-chain mRNA which match amino-acid sequences of the elongated portions of the variant α-chains.

Two abnormally long β-chain variants have been described, Hb Tak (16) and Hb Cranston (17). In the preceding manuscript (17), Bunn *et al.* have proposed that Hb Cranston may have arisen by frameshift mutation resulting from a two-base insertion: reduplication of the last two nucleotides of the codon for amino-acid 144. This mutation would allow readthrough of the out-of-phase termination codon and translation of usually untranslated 3′-terminal sequences of β-mRNA (17). The proximal portion of the new amino-acid sequence matches (with the frameshift) the previously proposed nucleotide sequence for this region of the normal β-chain mRNA (14, 15). Fitch (18) has proposed a similar mechanism for the origin of Hb Tak: frameshift by two-base insertion following the codon for residue 146, the usual carboxy-terminal amino acid of the β-chain.

We report here the detailed analysis of oligonucleotides present in normal β-chain mRNA, the sequences of which can be matched to the amino-acid sequence of the β-Cranston chain. The finding of these sequences in normal β-globin mRNA supports the hypothesis of the molecular basis of Hb Cranston by frameshift mutation allowing readthrough of usually untranslated sequences present in normal β-globin mRNA.

MATERIALS AND METHODS

Materials and methods are the same as previously described (14, 15). Human globin mRNA was isolated from reticulocytes and copied into complementary DNA (cDNA) by RNA-dependent DNA polymerase (reverse transcriptase) of avian myeloblastosis virus. ^{32}P-Labeled RNA (cRNA) was transcribed from the cDNA by *Escherichia coli* RNA polymerase in the presence of nucleoside [α-^{32}P]triphosphates. The ^{32}P-labeled cRNA was then digested with RNase T_1 and fractionated in two dimensions to yield a characteristic "fingerprint." Individual oligonucleotides were eluted and further digested with pancreatic RNase, U_2 RNase, or spleen phosphodiesterase (19).

RESULTS

Fingerprints obtained from T_1 RNase digests of human globin cRNA are shown in Fig. 1. Fig. 1A shows the pattern of normal total (α + β) human cRNA; Fig. 1B shows α-thalassemic cRNA which is greatly deficient in α-chain mRNA sequences (20, 21), and Fig. 1C, the cRNA of β-chain mRNA purified from total mRNA by formamide–polyacrylamide gel electrophoresis (21). The patterns in Fig. 1B and C are similar, and considerably simplified compared to that of Fig. 1A, as would be expected for isolated β-chain mRNA. Spots no. 6, 12, 17, and 41 are prominent in Fig. 1B and C, and, therefore, constitute nucleotide sequences of normal β-chain mRNA.

The results of pancreatic RNase digestion of these oligonucleotides and the partial nucleotide sequences deduced are listed in Table 1. Table 2 lists the results of U_2 RNase

Abbreviations: cDNA, DNA synthesized from mRNA by RNA-dependent DNA polymerase of avian myeloblastosis virus; cRNA, RNA synthesized from cDNA by *Escherichia coli* RNA polymerase; Hb, hemoglobin.

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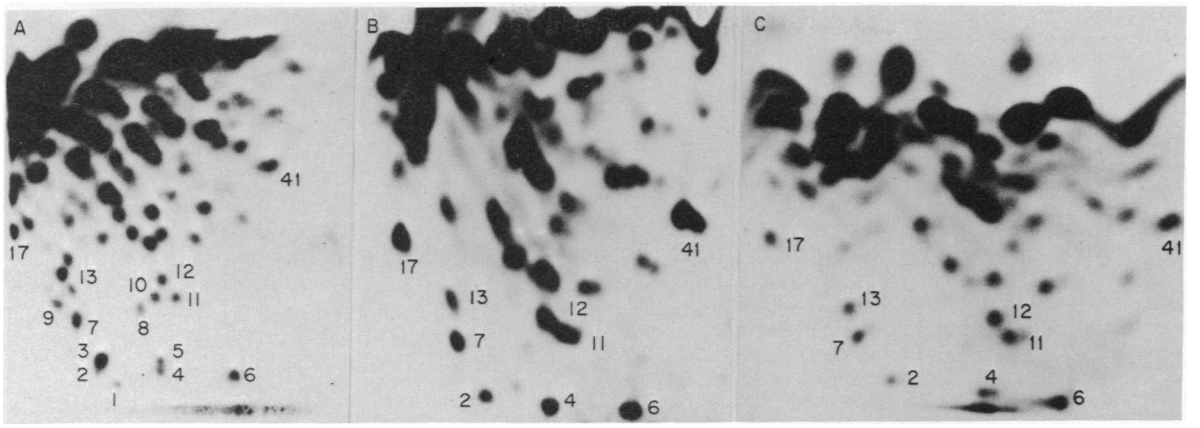

FIG. 1. Fingerprints of human globin cRNA. RNA was transcribed from globin cDNA using *E. coli* RNA polymerase, in the presence of [α-^{32}P]GTP. The RNA was then digested with RNase T_1 and the digest was fractionated in two dimensions: first, by high voltage electrophoresis at pH 3.5 on Cellogel strips (left to right), then by homochromatography on thin-layer plate of DEAE-cellulose (bottom to top) (19). Photos of radioautographs are shown. (A) cRNA of non-thalassemic human globin cDNA; (B) cRNA of α-thalassemic (Hb H disease) cDNA; (c) cRNA of cDNA synthesized from β-mRNA (slow band RNA), purified by formamide–polyacrylamide gel electrophoresis (21).

and spleen phosphodiesterase digestion of these same oligonucleotides.

Table 3 lists the complete nucleotide sequences for spots no. 6, 12, 17, and 41, derived from the nucleotide sequence data, and the amino-acid sequences of the β^{A} and $\beta^{Cranston}$ globin chains to which they can be matched. The sequence of spot no. 17 matches the amino-acid sequence of β-chain residues no. 142–144. The sequence of spot no. 12 matches the sequence of the two carboxy-terminal amino acids of the normal β-chain (residues no. 145 and 146); the RNA sequence then contains the termination codon UAA followed by two additional bases: GC. The nucleotide sequences proposed by Bunn *et al.* (17) and Fitch (18) for the mRNA of the $\beta^{Cranston}$ and β^{Tak} chains, respectively, are also listed below the normal sequence of spot no. 12, and the proposed two-nucleotide sequence reduplications are underlined. The resulting frameshifts in the reading of spot no. 12 would lead to the synthesis of the first five and three new amino acids observed in the $\beta^{Cranston}$ and β^{Tak} chains, respectively, as illustrated in Table 3. Spot no. 41 is a mixture of two different sequences, 41a and 41b (Table 3). Both sequences occur in β-mRNA and both sequences can be matched to the amino-acid sequence of residues no. 150–152 of the $\beta^{Cranston}$ chain. Further studies are necessary to establish which of these two sequences corresponds to this specific "untranslated" region of β-mRNA. Finally, the nucleotide sequence of spot no. 6 matches the sequence of the last four amino acids (nos. 154–157) of the $\beta^{Cranston}$ chain and has an "in phase" termination codon, UAA, following the codon for the C-terminal amino-acid tyrosine ($\beta^{Cranston}$ no. 157). The oligonucleotide A-A-A-G-G-U-U has been isolated from pancreatic RNase digests of β-chain cRNA and it probably overlaps with the U-A-A-A-G-G sequence of spot no. 6. Our sequences thus assign five additional base residues beyond the new termination codon.

From all of these results, a long linear sequence can be proposed for normal β-chain mRNA, starting at the codon for amino-acid no. 142, and it is shown in Fig. 2. In this sequence of 54 nucleotides, one base residue indicated by the arrow at codon position no. 149 remains totally ambiguous, and because of the ambiguity in the assignment of the sequences of spots no. 41a and 41b, two alternative base residues (C or U) must be proposed at three other sites of the sequence, as indicated in Fig. 2.

DISCUSSION

The finding, in normal β-chain mRNA, of nucleotide sequences which match amino-acid sequences in the abnor-

Table 1. Results of pancreatic RNase digestion of T_1 oligonucleotides

Spot no.	Isotope used and digestion products obtained: [γ-^{32}P]-ATP	[α-^{32}P]ATP	[α-^{32}P]CTP	[α-^{32}P]GTP	[α-^{32}P]UTP	Sequence deduced
6	U	U, C, AAU, AAAG	U, C	AAAG(G)	AAU, AU, C, U	U(AAUU,AUU,CC,[U,C]U) AAAG(G)
12	U	AAG, C, U	AAG, AC, AU	AAG	AU, AC	U(AUC,ACU)AAG(C)
17	C	AAG, AC, C	AC, C	AAG	AAG	C(CC,AC)AAG(U)
41	C	—	C, U, G	U	G, C, U	C(UU,UCU)UG(C or U) and CCUUUUUG(C or U)

Oligonucleotides of T_1 RNase digests of labeled RNA were eluted from DEAE-cellulose plates after two-dimensional fractionation of the digests as in Fig. 1. The oligonucleotides were then completely digested with pancreatic RNase and the digestion products were identified by their position after electrophoresis on DEAE-cellulose paper at pH 3.5 (19). The digestion products that are underlined contain substantially more radioactivity than the other products of the same digest. The spot numbers refer to Fig. 1. The RNA analyzed was cRNA synthesized from globin cDNA in the presence of the indicated [α-^{32}P]NTP. The 5′-terminal (left) nucleotide of each oligonucleotide was also determined (second column) by labeling a T_1 RNase digest of natural globin mRNA by use of [γ-^{32}P]ATP and polynucleotide kinase. The position of single nucleotides in parentheses at the 3′-terminus (right) of oligonucleotide sequences was determined by nearest neighbor analysis.

Table 2. Results of partial digestion of T_1 oligonucleotides

Spot no.	Isotope	Nuclease	Digestion products
6	[α-^{32}P]CTP	U_2 RNase	UU(U,C)UA, UCCA
	[α-^{32}P]UTP	U_2 RNase	UU(U,C)UA, UUA
12	[α-^{32}P]UTP	U_2 RNase	CUA, UA(U)
17	[α-^{32}P]CTP	U_2 RNase	CCCA
41	[α-^{32}P]GTP	SPD	UG, UUG, CUUG, UCUUG

Oligonucleotides from T_1 RNase digests of globin cRNA were partially digested with either U_2 RNase or spleen phosphodiesterase [SPD] (19). The digestion products were identified by their position after electrophoresis on DEAE-cellulose paper at pH 1.7 (7% formic acid) and/or pH 3.5; certain digestion products were subsequently eluted and their sequence was further analyzed by alkaline hydrolysis or pancreatic RNase digestion.

mally long β-chain of Hb Cranston supports the hypothesis that this Hb variant arose by a mutation allowing translation of usually untranslated sequences of the β-chain mRNA. This mutation could not be a simple chain termination mutation, because the amino-acid sequence of the β^{Cranston} chain changes from that of normal β-chain at a position corresponding to two full codons prior to the termination codon. Bunn *et al.* (17), however, pointed out that the new amino-acid sequence can be explained by reduplication of the last two bases of the codon (AAG) for amino-acid residue no. 144 (Lys). The resulting frameshift in the translation of the nucleotide sequence previously proposed for this region of the mRNA (spot no. 12) would give the amino-acid sequence described in Hb Cranston (17) (Table 3). This type of two-nucleotide insertion could result from nonhomologous crossing-over of chromosomes, or from other defects in DNA replication or repair.

The other abnormally long β-chain variant, Hb Tak, has a slightly different amino-acid sequence from that of Hb Cranston. Its sequence is normal through amino-acid residue no. 146 (the usual carboxy-terminal histidine); the additional amino-acid residues then follow. The precise sequence of these residues has not yet been published, but the amino-acid composition of the elongated segment of Hb Tak is very similar to that of Hb Cranston. The β-Tak chain is difficult to explain by a chain termination mutation, because a single base substitution in the termination codon could not give threonine (ACN), the amino acid in position no. 147. However, the sequence of the β-Tak chain can be explained by a frameshift mutation due to reduplication of the last two bases of the codon (CAC) for $\beta^{146\ \text{His}}$ (Table 3) (18).

The total extent of the untranslated nucleotide sequences of β-chain mRNA is not known with certainty. The difference in molecular weight between α- and β-chain mRNA's of mouse (22), rabbit (11, 22–26), and man (21, 27) is considerably greater than expected if the β-mRNA differs from α-mRNA only by the additional 15 base residues that it must contain to code for five additional amino-acid residues (β:α::146:141 amino-acid residues). It is estimated that the rabbit β-mRNA contains approximately 65 more nucleotides than the rabbit α-mRNA (26). There is no evidence that the difference in size between α- and β-mRNAs is due to differences in the length of the 3′-terminal poly(A) sequence of these mRNAs (12, 13, 28). The bulk of the untranslated sequences in human α-chain mRNA appear to be situated at the 3′-terminal extremity of the mRNA: 96 residues must be

Table 3. Nucleotide sequences of human β globin mRNA

Spot no.	Sequence	Chain
6	UCCAAUU(U,C)UAUUAAAG	
	154 155 156 157 158	β^{Cranston}
	Ser- Asn- Phe- Tyr- Term	
	UCC-AAU-UUC-UAU-UAA-AG(G)	
12	UAUCACUAAG	
	144 145 146	β^{A}
	Lys- Tyr- His- Term	
	[AA](G)-UAU-CAC-UAA-G(C)N	
	144 145 146 147 148 149	β^{Cranston}
	Lys- Ser- Ile- Thr- Lys- Leu	
	AAG-AGU-AUC-ACU-AAG-C[U]N	
	144 145 146 147 148 149	β^{Tak}
	Lys- Tyr- His- Thr- Lys- Leu	
	AAG-UAU-CAC-ACU-AAG-C[U]N	
17	CCCACAAG	
	142 143 144 145	β^{A}
	Ala- His- Lys- Tyr	
	(G)CC-CAC-AAG-(U)[A]N	
41a	CUUUCUUG	
	150 151 152 153	β^{Cranston}
	Ala- Phe- Leu- Leu	
	(G)CU-UUC-UUG-$\binom{(C)}{(U)}$[U]N	
41b	CCUUUUUG	
	150 151 152 153	β^{Cranston}
	Ala- Phe- Leu- Leu	
	(G)CC-UUU-UUG-$\binom{(C)}{(U)}$[U]N	

The sequences listed are based on the data shown in Tables 1 and 2. The position assignments of the C and U shown in parentheses in spot no. 6 are ambiguous and can be interchanged on the basis of the nucleotide sequence information alone. Listed under the linear sequences are the unique amino acid sequences of the β-globin chain which match the nucleotide sequences in the listed codon groupings. The nucleotides in parentheses at the 5′-terminus (left) and 3′-terminus (right) of the hyphenated nucleotide sequences are not contained in the numbered oligonucleotides but are known to be present in the indicated position by the nature of the nuclease digest (T_1 RNase) and "nearest-neighbor" analysis, respectively; additional nucleotide assignments on the basis of the genetic code alone are shown in brackets. N indicates an unknown nucleotide in a codon. The proposed frameshifts due to double base repetitions in the β^{Cranston} and β^{Tak} mRNA sequences are underlined. The numbers over the sequences refer to the position of the indicated amino acid in the designated β-globin chain.

present to account for the elongated α-chain of the chain-termination mutant Hb Constant Spring (1, 6), and analysis of α-chain cRNA sequences reveals additional large untranslated 3′-terminal oligonucleotides which account for 15 to 20 more nucleotides (Forget, Marotta, and Weissman, unpublished observations). In the case of human β-cRNA, in addition to the 3′-untranslated sequence of 36 nucleotides which matches the elongated amino-acid sequence of Hb Cranston (Fig. 2), there are only four additional large oligonucleotides (totalling approximately 45 nucleotides) which cannot be matched to normal amino-acid sequences of the β-chain and which presumably represent 3′-terminal untranslated sequences of the β-chain mRNA situated to the 3′-side of the β-Cranston termination codon (Forget, Marotta, and Weissman, unpublished observations). Thus only about 81 nucleotides of the approximately 200 to 260 un-

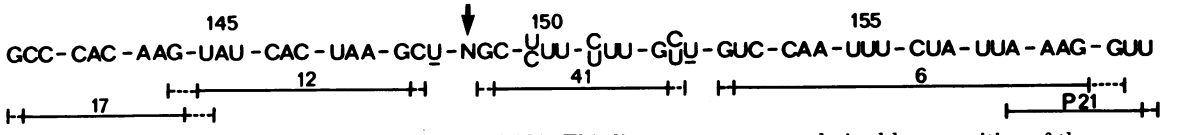

FIG. 2. Partial nucleotide sequence of human β-globin mRNA. This linear sequence was derived by apposition of the sequences of the individual oligonucleotides listed in Tables 1–3, on the basis of the information provided by the amino-acid sequence of β^{A} and $\beta^{Cranston}$ globin chains. The nucleotide sequences of the individual numbered T_1 RNase oligonucleotides of Fig. 1 and Tables 1–3 are represented under the linear sequence by brackets; nucleotides assigned on the basis of "nearest neighbor" sequence information are encompassed by the dashed extensions of the brackets. Oligonucleotide P21 is present in fingerprints of pancreatic RNase digests of human β-chain cRNA. The nature of the underlined base residues (in codons 148 and 152) was determined on the basis of the genetic code and the amino-acid sequence of the $\beta^{Cranston}$ chain (Tables 1 and 3). The numbers over the sequence represent the codon positions of the normal β-chain mRNA, without the $\beta^{Cranston}$ frameshift. Only one base residue, indicated by the arrow in codon no. 149, is totally undetermined. In three other positions (in codons 150, 151, and 152) the sequence may contain either a C or a U, because of the two sequences of spot no. 41, both of which can be matched to the same $\beta^{Cranston}$ amino-acid sequence.

translated nucleotides thought to be present in β-mRNA can be definitely assigned to the 3′-end of this mRNA. It is possible that in β-mRNA the 5′-terminal untranslated sequence is considerably longer than in α-mRNA. This portion of the mRNA sequence would not be detected in the fingerprints of the cRNA because the cDNA used as a substrate for cRNA synthesis does not contain sequences corresponding to the 5′-extremity of the natural mRNA (14, 15). Alternatively, the 3′-untranslated sequence of human β-mRNA may be as long as or longer than that of the α-mRNA, but this sequence may be rich in guanylic acid residues and may, therefore, be hydrolyzed by T_1 RNase into many small oligonucleotides which cannot be definitely designated as untranslated versus translated sequences.

Proudfoot and Brownlee (29) have published a sequence of 52 nucleotides adjacent to the 3′-terminal poly(A) sequence of rabbit β-globin mRNA. This long RNA sequence contains a sequence of 10 nucleotides [situated 17 base residues from the poly(A)] which, apart from one base difference, is homologous to the last 10 nucleotides of our spot no. 6: C-U-A-U-U-A-A-A-G-G (human) versus C-U-A-A-U-A-A-A-G-G (rabbit). This sequence in rabbit β-mRNA appears to participate in a theoretical hairpin loop which may be common to the 3′-terminus of different eukaryotic mRNA's (29). In the case of human β-mRNA, if this sequence constitutes part of a similar loop, it is unexpectedly close to the normal termination codon, and probably unexpectedly distant from the poly(A), because as stated previously, at least 45 more base residues appear to be present to the 3′-side of spot no. 6. It is, however, feasible that spot no. 6 in *normal* β-mRNA may in fact be situated close to the 3′-terminal poly(A): the β-Cranston chain may have arisen by a crossover phenomenon which deleted DNA sequences corresponding to a portion of the terminal translated and much of the adjacent untranslated portions of the normal β-mRNA. If this were the case, however, one would have to designate as fortuitous the match between spot no. 12 (with the proposed two-nucleotide reduplication) and the new amino-acid sequence of the β-Cranston chain ($\beta^{Cranston\ 145-148}$) (Table 3). It is also possible that a sequence similar to C-U-A-A-U-A-A-A-G-G may occur more than once in the 3′-terminal sequence of mRNA's and represent a repeated signal, the function of which could be related to termination of transcription, poly(A) addition, mRNA secondary structure, or sites of RNA–protein interaction.

Note Added in Proof. After this work was completed, we learned that the complete amino-acid sequence of the elongated segment of Hb Tak had been determined (Lehmann, H., Casey, R., Lang, A., Stathopoulou, R., Imai, K., Tuchinda, S., Vinai, P. & Flatz, G. (1975) *Br. J. Haematol.* **31**(Suppl.) 119–131. Its sequence is identical to that of Hb Cranston from residue 147 to 157. This finding strongly supports the hypothesis that both Hb variants arose by frameshift mutations which allowed the translation of usually untranslated regions of normal globin mRNA.

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