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Cloning a cDNA for the pro-α2 chain of human type I collagen

(type I procollagen/human connective tissue disorders/recombinant DNA)

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ABSTRACT **Poly(A)-RNA enriched for type I procollagen sequences was isolated from normal human fibroblasts and used as template to synthesize double-stranded cDNA with avian myeloblastosis virus (AMV) reverse transcriptase. After the ends had been blunted with nuclease S1 and dGMP tails had been added with terminal deoxynucleotidyltransferase, the double-stranded cDNA was annealed with pBR322 DNA that had previously been cleaved with *Eco*RI, blunted with AMV reverse transcriptase, and dCMP-tailed with terminal deoxynucleotidyltransferase. The chimeric molecule was used to transform *Escherichia coli* strain HB101. Ninety-five recombinant clones were obtained and screened by dot hybridization analysis using ^{32}P-labeled cDNA synthesized from the original poly(A)-RNA collagen-enriched population. Three positive clones were isolated and further characterized by blot hybridization techniques and by *Eco*RII digestion. One clone with an insert of 2.2 kilobases was shown to contain sequences encoding for the pro-α2 chain of human type I procollagen. DNA sequence analysis of a 172-nucleotide fragment demonstrated that the cloned cDNA extends from amino acid position 450 of the α2 chain to the middle of the COOH-terminal propeptide.**

Collagen represents a major class of proteins in mammals and plays a fundamental role in the structure and function of most connective tissues (1, 2). Collagen is synthesized from procollagen, a precursor composed of three pro-α chains. At least five genetically and biochemically distinct types of collagen are found in different tissues, and they have a characteristic developmental distribution (1–3). Type I collagen, $[\alpha 1(I)]_2\alpha 2$, is present in bones, skin, tendons, and lungs, and it is believed to be directly or indirectly involved in inherited diseases of the connective tissue in man such as some forms of osteogenesis imperfecta (1). Although a great deal of information is available about posttranslational events leading to the maturation of the human procollagen chains, very little is known about the structure, the genetics, and the mode of expression of this family of genes in man.

In the past few years recombinant DNA techniques have proved to be a powerful tool for enhancing our understanding of the structure of eukaryotic genes and their altered phenotypic expression, as exemplified by the human globin genes (4). Recently cloned cDNAs for type I procollagen from chicken have been prepared (5–7), and the entire chicken pro-α2 gene (8–10) and part of the sheep pro-α2 gene have been isolated from phage libraries (11). The pro-α2 gene contains numerous introns interspersed with more than 50 coding units (exons), and its total length is about 40 kilobases (kb) or about 8 times longer than the pro-α2 mRNA. From these data and the observations of Avvedimento *et al.* (12) one can predict a complex pathway for maturation of the primary transcript of the pro-α2 gene; the possibility of processing mutations is increased in such a system.

MATERIALS AND METHODS

Materials. Reverse transcriptase (RNA-dependent DNA polymerase) from avian myeloblastosis virus (AMV) was purified (13) from plasma kindly supplied by Joseph Beard (Life Sciences, Gulfport, FL). Nuclease S1 was purchased from Miles. Calf thymus terminal deoxynucleotidyltransferase was a generous gift from Sidney Pestka (Roche Institute of Molecular Biology). Rabbit reticulocyte lysate was purchased from Amersham. Restriction enzymes were purchased from New England BioLabs and were used according to the supplier's recommendations. Nitrocellulose paper (0.45-μm pore diameter) was purchased from Schleicher & Schuell. Labeled nucleotides were from New England Nuclear.

RNA Purification, Cell-Free System Translation, and Product Analysis. In a typical preparation, 10–20 150-cm^2 flasks of confluent cultured human fibroblasts were harvested by mechanical scraping, and total poly(A)-RNA was purified according to the method of Burnett and Rosenbloom (14). RNA corresponding to a size class between 40 and 25 S was fractionated on a sucrose gradient after denaturation through a cushion of 10 mM CH_3HgOH according to a modification of the method of Payvar and Schimke (15). Yields of purified RNA ranged between 1 and 2 μg per original culture flask. The degree of enrichment for procollagen sequences was estimated by gel electrophoresis of the translational product from a rabbit reticulocyte lysate (16) with and without prior treatment with bacterial collagenase (17).

Construction and Analysis of Recombinant Plasmids. Poly(A)-RNA, enriched for type I collagen sequences, was used as template for the synthesis of cDNA in the presence of AMV reverse transcriptase and an oligo(dT) primer (18). The product of this reaction was separated on an alkaline sucrose gradient, and fractions in the size class 2000–6000 nucleotides long were pooled and used as template for the synthesis of the second strand in the presence of AMV reverse transcriptase with the omission of the sodium pyrophosphate (19). The ends of the double-stranded molecules were blunted with nuclease S1, the molecules were fractionated on a sucrose gradient, and size fractions above 1500 base pairs were pooled and precipitated. An average of 15 dGMP residues was added to the 3′ end of the double-stranded (ds)-cDNA with terminal transferase (20). Plasmid pBR322 DNA was cleaved with *Eco*RI, ends were blunted with AMV reverse transcriptase, and oligo(dC) tails were added with terminal transferase (20). The ds-cDNA and the plasmid DNA were then annealed in equimolar amounts (20), and the result-

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Abbreviations: AMV, avian myeloblastosis virus; kb, kilobases; ds-, double-stranded; NaCl/Cit, buffer of 0.15 M NaCl in 0.015 M sodium citrate, pH 6.8.

ing chimeric molecules were used for transformation of *Escherichia coli* strain HB101 in the presence of $CaCl_2$ (21). Bacterial colonies containing plasmids were selected on ampicillin medium. All experiments were performed in accordance with the National Institutes of Health guidelines for recombinant DNA research.

Screening of the Bacterial Colonies. A rapid procedure (22) was used to isolate plasmid DNA from 6 ml of liquid culture grown without amplification. The DNA was resuspended in 60 μl of 6 mM Tris·HCl (pH 8.0)/6 mM NaCl/0.6 mM EDTA. Ten microliters was used for restriction analysis; the remainder was adjusted to 0.3 M NaOH, heated at 68°C for 15 min, and transferred in duplicate to a nitrocellulose filter. The filter was screened by dot hybridization analysis (23), using ^{32}P-labeled cDNA synthesized from the original total poly(A)-RNA population enriched for type I collagen sequences. Plasmids were isolated from the positive clones and purified by CsCl/ethidium bromide gradient centrifugation (24). The eukaryotic sequences were excised from the vector by endonuclease digestion, subjected to electrophoresis on agarose gel, electroeluted (25), and ^{32}P-labeled *in vitro* by nick translation (26).

Blot Hybridization Techniques and DNA Sequence Analysis. Hybridizations of DNA with filter-bound RNA were performed by using a variation of the method described by Thomas (27). Southern blottings were performed as described elsewhere (28). End-labeling, strand separation, and sequence analysis were performed according to Maxam and Gilbert (29).

RESULTS

Cloning and Analysis of Clones. Poly(A)-RNA was isolated from cultures of normal human skin fibroblasts with a procedure involving proteinase K digestion (14), chromatography on oligo(dT)-cellulose, and separation on sucrose gradient after denaturation in CH_3HgOH. Analysis of translation products by gel electrophoresis (not shown) suggested that about 10% of the RNA coded for pro-α1(I) and pro-α2 sequences. The poly(A)-RNA was used to synthesize ds-cDNA, which was then inserted into the *Eco*RI site of pBR322. About 25 ng of ds-cDNA tailed with oligo(dG) was annealed with 18 ng of pBR322 cut with *Eco*RI and tailed with oligo(dC). Ninety-five clones were obtained (Table 1). All the clones were true recombinants with inserts of various sizes between 0.7 and 5 kb. Of these clones, 84% had both *Eco*RI sites restored, 12% only one, and 4% none. When the chimeric molecules were subjected to dot hybridization analysis (23) with labeled cDNA prepared from the original enriched RNA population, three clones (Hf-18, Hf-32, Hf-90) gave a positive signal. The plasmids were isolated from these clones and analyzed. Clones Hf-18 (1.2 kb) and Hf-90 (4.5 kb) had both *Eco*RI sites restored, whereas Hf-32 (2.2 kb) lacked an *Eco*RI site at its 5′ end.

Identification of a Recombinant Plasmid Bearing Pro-α2 Collagen Sequences. Because of the possibility of preferential transcription of minor RNA species, we analyzed the three clones with respect to their size and their quantitative representation by blot hybridization with filter-bound RNA (27) (Fig. 1). Only one of the three clones (Hf-32) hybridized with a pattern similar in both size and intensity with the pattern previously reported for chicken collagen mRNA (30). Clone Hf-18 hybridized to a smaller RNA species (4200 nucleotides long).

Table 1. Transformation of *E. coli* with ds-cDNA-$(dG)_m$ and pBR322$(dC)_n$ hybrids

DNA	Colonies per plate	Colonies per μg of DNA	Transformation efficiency, %
pBR322	850	1,000,000	100
pBR322 (blunt-end)	2	100	0.01
ds-cDNA$(dG)_m$·pBR322$(dC)_n$*	30	3,800	0.38

* pBR322 DNA having an oligo(dC) tail that was annealed with human fibroblast ds-cDNA having an oligo(dG) tail.

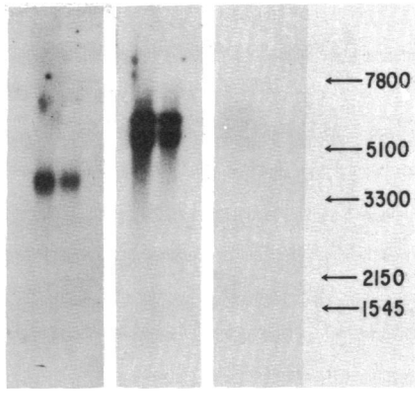

FIG. 1. Blotting analysis of total poly(A)-RNA isolated from human fibroblasts and hybridized to ^{32}P-labeled Hf-18 (*Left*), Hf-32 (*Center*), and Hf-90 (*Right*). The RNA was electrophoresed in a 0.7% agarose gel in 2 M formaldehyde, transferred to nitrocellulose paper, and hybridized to DNA of each clone at 5 ng/ml. The clones were ^{32}P-labeled by nick-translation to a specific activity of 5×10^8 cpm/μg. The two lanes in each panel represent 0.33 μg (left lane) and 0.16 μg (right lane) of total poly(A)-RNA. RNA markers that were run in a parallel slot and visualized by ethidium bromide staining were 35S poliovirus RNA (7800 nucleotides), 28S (5100 nucleotides) and 18S (2150 nucleotides) chicken embryo fibroblast RNA, and 23S (3300 nucleotides) and 16S (1545 nucleotides) *E. coli* ribosomal RNA. The size of the bands hybridizing with Hf-32 is 6200 and 5700 nucleotides. Preliminary experiments have demonstrated that the two RNA bands (*Center*) contain pro-α2-specific sequences and that they are present in both cytoplasmic and polysomal RNA.

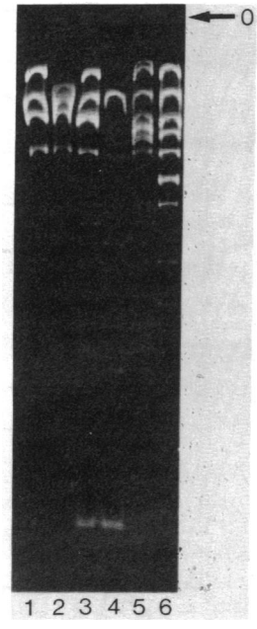

FIG. 2. Frequency of C-C-$\frac{A}{T}$-G-G sequences in Hf-32. One microgram of plasmid DNA was isolated, digested with *Eco*RII, and electrophoresed on a 12% polyacrylamide slab gel. Ethidium bromide staining is shown. Lane 1, pBR322 digested with *Eco*RII; lane 2, pBR322 digested with *Eco*RII and *Pst* I; lane 3, Hf-32 digested with *Eco*RII; lane 4, the 1.85-kb *Pst* I fragment of Hf-32 digested with *Eco*RII; lane 5, Hf-18 digested with *Eco*RII; lane 6, Hf-90 digested with *Eco* RII. O, origin of the gel.

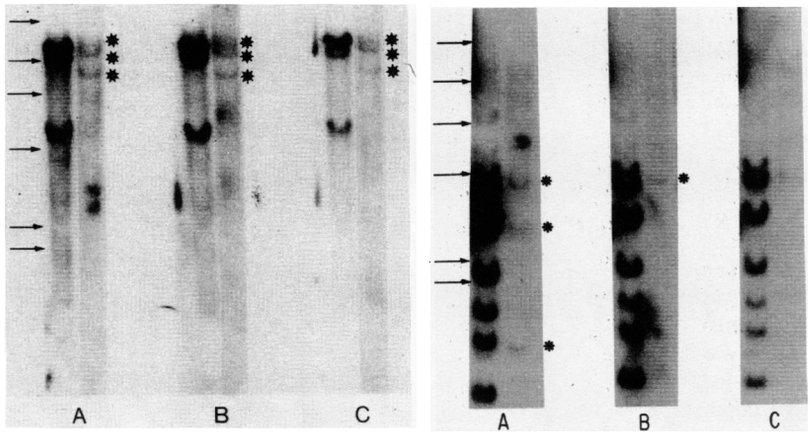

FIG. 3. (*Left*) Southern blotting analysis of chicken and human genomic DNA hybridized to ^{32}P-labeled probe specific for chicken pro-α1(I) sequences (pCg54). Both DNAs were digested with *Eco*RI. The hybridization was performed at 68°C (28) under decreasingly stringent conditions in 6× NaCl/Cit (A), 4× NaCl/Cit (B), and 2× NaCl/Cit (C). (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate, pH 6.8). In each pair, the left-hand slot contained the *Eco*RI-digested chicken DNA and the right-hand slot, the *Eco*RI-digested human DNA. The asterisks indicate the cross-hybridizing bands present in the human DNA. The arrows indicate the position of molecular weight markers, which consisted of λ DNA digested with *Hin*dIII. (*Right*) Experiment similar to that in *Left* except the hybridization probe used was specific for chicken pro-α2(I) sequences (pCg45).

Hf-90 did not show any distinctive banding pattern, implying that although it was a minor constituent of the original RNA population, it was preferentially transcribed by the reverse transcriptase. We excluded the possibility that it was a contaminant by showing a distinct restriction pattern with human genomic DNA in a Southern blotting analysis (not shown).

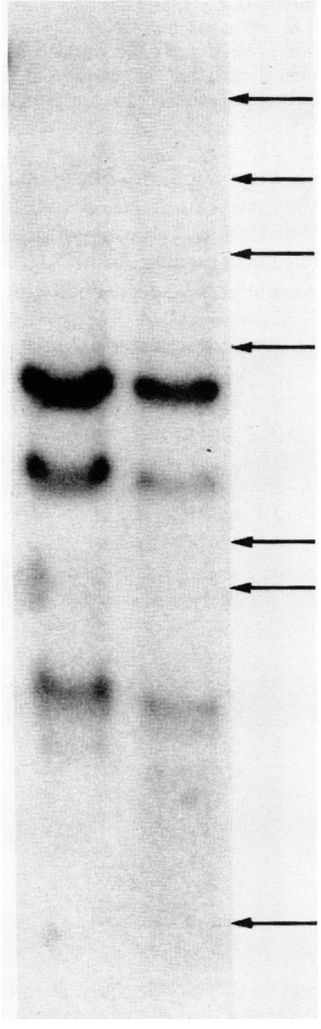

FIG. 4. Southern blotting analysis of human genomic DNA digested with *Eco*RI and hybridized to ^{32}P-labeled Hf-32. The hybridization was performed in 2× NaCl/Cit (28). Each slot contained human DNA from a different individual. The arrows indicate the position of the same λ *Hin*dIII markers used in Fig. 3.

To confirm these observations, another comparative experiment was performed on the three clones. Because the primary structure of the triple helical region of all collagen molecules has glycine as every third amino acid, and because glycine is frequently preceded by a hydroxyproline or followed by a proline, an enzyme recognizing a sequence such as C-C-N-G-G should extensively digest a DNA bearing collagen sequences. Lehrach *et al.* (5) exploited this unique feature to characterize a chicken pro-α2 cDNA clone by *Hae* III and *Hpa* II digestion. We have used a similar approach by digesting our three clones with *Eco*RII, which recognizes the sequence C-C-$\frac{A}{T}$-G-G and cleaves after the second C. When we compared the pattern of digestion obtained from our three clones, only Hf-32 clearly gave the expected pattern for a collagen cDNA (Fig. 2).

To characterize Hf-32 further, human and chicken genomic DNA was digested with *Eco*RI, and the DNA was analyzed by Southern blotting (27) under increasingly less stringent hybridization conditions with nick-translated pCg45 and pCg54, two well-characterized pro-α2 and pro-α1(I) chicken DNA clones (5, 6).

The *Eco*RI patterns for the pro-α1(I) and pro-α2 genes differ from each other in both species (Fig. 3). As shown in Fig. 4, the *Eco*RI pattern of human DNA blotted and hybridized to nick-translated Hf-32 resembles both in number and size of fragments the pattern observed when the same human DNA was cross-hybridized with the chicken pro-α2 probe. These data established that Hf-32 is a clone encoding a human pro-α2 chain.

Restriction Endonuclease Mapping. The 2.2-kb insert of Hf-32 contained cleavage sites for the enzymes *Ava* I, *Bgl* I,

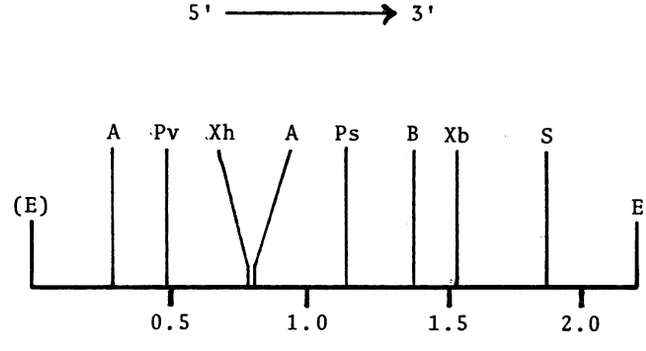

FIG. 5. Restriction endonuclease map of the insert of Hf-32. A, *Ava* I; Pv, *Pvu* II; Xh, *Xho* I; Ps, *Pst* I; B, *Bgl* I; Xb, *Xba* I; S, *Sac* I; E, *Eco*RI. The direction of transcription is indicated by the arrow at the top of the diagram. The numbers indicate the size of the clone in kb. (E) indicates the unrestored *Eco*RI site.

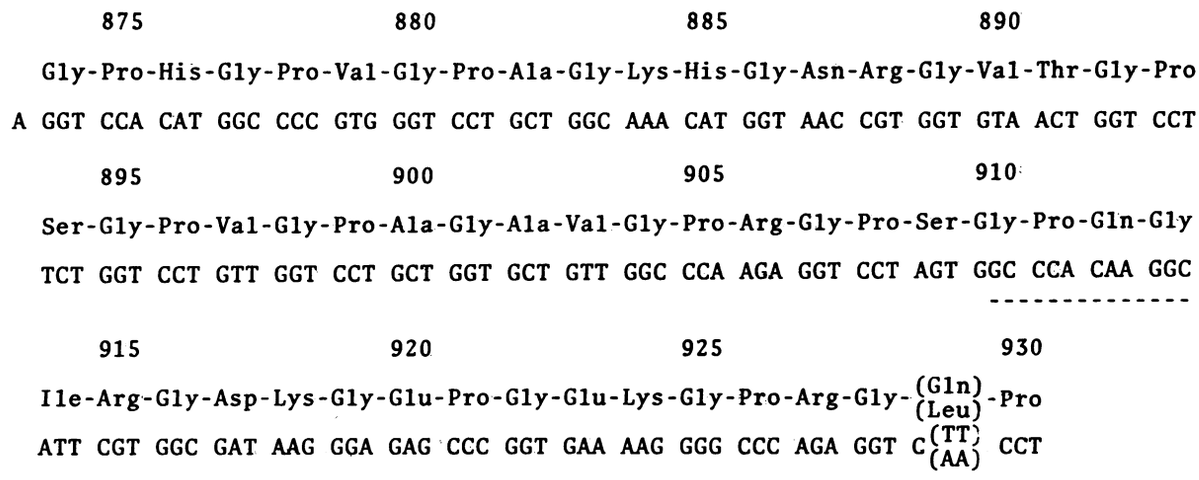

FIG. 6. Sequence of a 172-nucleotide long *Eco*RII fragment of Hf-32. The protein sequence derived from the DNA is also shown. Numbers indicate the amino acid residues of the $\alpha 2$ chain (Fig. 7). The broken line indicates the *Bgl* I site. The sequence coding for residue 929 was not definitively resolved.

*Eco*RII, *Hae* III, *Hin*cI, *Mbo* II, *Pst* I, *Pvu* II, *Sac* I, *Xba* I, and *Xho* I. Some of them were mapped as shown in Fig. 5. Other enzymes did not cleave; these included *Bam*HI, *Bgl* II, *Eco*RI, *Hin*dIII, *Hpa* I, *Kpn* I, *Pvu* I, *Sal* I, and *Sma* I. In order to orient Hf-32 with respect to the direction of transcription, we digested it with *Pst* I, generating two fragments of 4.65 and 1.85 kb. The 1.85-kb fragment, which contained 750 nucleotides of pBR322, was isolated and further digested with *Eco*RII. It was clear from the *Eco*RII pattern shown in Fig. 2 that this portion of the clone contained most of sequences coding for the α chain domain, suggesting that the genetic maps of the vector and the insert have the same orientation. This conclusion was confirmed by nucleotide sequence analysis of the clone.

DNA Sequence Analysis. Because there is essentially no structural information available about human collagen chains (2, 31), it was not possible to make a direct comparison between the amino acid sequences derived from Hf-32 and the sequences obtained by protein analysis. There is, however, a high degree of homology in the primary structure of the type I α chains found in different species. This phenomenon made it possible to further characterize Hf-32 by comparing it to α chains whose primary structures are known. We determined the sequence of an *Eco*RII fragment from Hf-32 that was 172 nucleotides long and contained the *Bgl* I site of the clone (Fig. 6). The fragment was found to correspond to a segment extending from residue 905 to residue 925 of the calf pro-$\alpha 2$ and from residue 905 to residue 930 from chicken pro-$\alpha 2$ (Fig. 7). These data and the presence of the unique *Bgl* I site within this portion of the molecule (Figs. 6 and 7) establish that the 5′ end of Hf-32 begins at about residue 450 of the $\alpha 2$ chain and that the clone extends to the middle portion of the COOH-terminal propeptide.

DISCUSSION

We report in this paper the synthesis, cloning, and identification of a 2.2-kb cDNA bearing specific sequences for the pro-$\alpha 2$ chain of human type I collagen. By comparing the amino acid sequences derived from the clone (Hf-32) with those previously obtained by amino acid sequence analysis of α chains in calf and by nucleotide sequence analysis of cDNAs for chicken type I procollagen, we established that Hf-32 contains about 40% of the coding sequences. Sequence analysis of this cDNA will make it possible to determine a large portion of the primary structure of the human pro$\alpha 2$ collagen. Furthermore, we will be able to address several biological and genetic questions about the localization, linkage, and expression of this gene. Hf-32 should make it possible to isolate the entire human pro-$\alpha 2$ gene from phage libraries, a necessary step in the elucidation of genetic defects of connective tissues in which collagen has been implicated (1). In addition, restriction enzyme polymorphism associated with the pro-$\alpha 2$ gene in families at risk for inherited collagen defects may prove to be as useful in the ante-natal diagnosis of these disorders (33, 34) as it has been for sickle cell anemia and thalassemia (4).

Note Added in Proof. Further experiments similar to those shown in Fig. 1 recently demonstrated that a third, minor species of pro-$\alpha 2$ mRNA (about 5500 nucleotides) is present in both cytoplasmic and polysomal fractions of human fibroblasts, suggesting at least three polymorphic mRNAs for pro-$\alpha 2$.

We are grateful to Dr. Helga Boedtker for supplying us with the two clones for chicken type I procollagen (pCg45 and pCg54) and the nucleotide sequences of these clones. We also thank Drs. Arthur Bank, Michael Bernard, Peter Fietzek, Maria Morabito, Bjørn Olsen, and

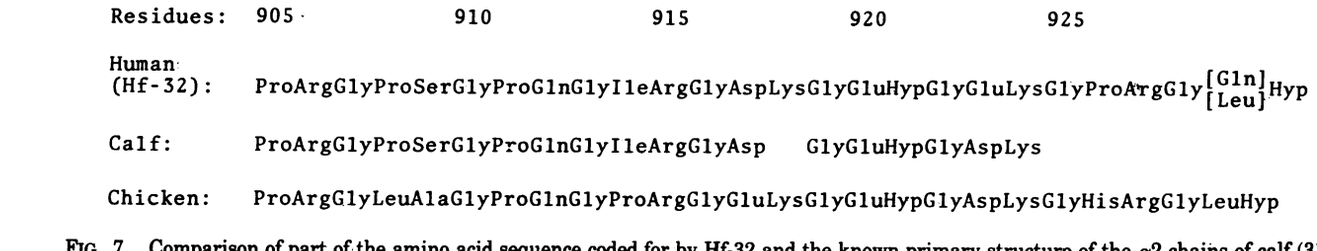

FIG. 7. Comparison of part of the amino acid sequence coded for by Hf-32 and the known primary structure of the $\alpha 2$ chains of calf (31) and chicken (32). The chicken sequence was derived from the DNA sequence of the clone pCg45 (32). Hyp, hydroxyproline.

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