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Peptide analysis of collagen produced from cDNA by transcription and translation *in vitro*

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When collagen CNBr-cleavage peptides are analysed by two-dimensional gel electrophoresis each peptide is resolved into a reproducible set of charged forms. To test whether this peptide heterogeneity resulted from polymorphic mRNA, collagen was produced by transcription and translation *in vitro* of a collagen cDNA clone, and the peptides were mapped by two-dimensional gel electrophoresis. A cDNA construct was produced by ligation of the 5′ end of the rat phenylalanine hydroxylase cDNA [Dahl & Mercer (1986) J. Biol. Chem. **261**, 4148–4153], containing the translation-initiation codon, to a human α1(I) cDNA [Chu, Myers, Bernard, Ding & Ramirez (1982) Nucleic Acids Res. **10**, 5925–5934] coding for a large portion of helical region including the complete CB7 and CB3 CNBr-cleavage peptides. This cDNA construct was ligated into the transcription vector pSP65, and cell-free translation of the mRNA transcribed from the pSP65 plasmid was performed with a rabbit reticulocyte lysate system. After CNBr cleavage of the hybrid protein translation products, the collagen CB7 and CB3 peptides were resolved by two-dimensional electrophoresis into the same multiple charged forms whether the mRNA was produced from the cDNA construct or was extracted from normal fibroblast cultures. This result demonstrated that the multiple peptide spots were not due to polymorphic mRNA species. The heterogeneity must result from some uncharacterized specific post-translational modification or chemical alterations during sample preparation. This method of expression and analysis of proteins from cDNA clones should be of considerable use in the identification and characterization of clones that code for mutant proteins.

INTRODUCTION

The interstitial or fibrillar collagen types I, II and III are the most abundant products of the collagen gene family (for review see Bornstein & Traub, 1979). Each collagen molecule consists of three polypeptide α-chains in a helical configuration. Fine structural analysis of the collagen α-chains has been facilitated by CNBr digestion, which produces a characteristic set of peptides for each collagen α-chain type (Scott & Veis, 1976). Each peptide is resolved by two-dimensional polyacrylamide-gel electrophoresis into a reproducible set of different charged forms (Cole & Chan, 1981; Benya, 1981). This type of charge heterogeneity has been observed with many proteins, and indicates the presence of several forms of each protein or peptide due to translational or post-translational differences. The blocking of the predominant post-translational modifications of the collagen α-chains, proline hydroxylation and lysine hydroxylation and glycosylation, does not alter this electrophoretic charge heterogeneity. Translational differences may therefore be responsible. Sequence heterogeneity of type I, II and III collagens has been suggested (Butler *et al.*, 1977; Seyer & Kang, 1981; Yamauchi *et al.*, 1982; Chu *et al.*, 1985) and may reflect the presence of minor gene products or allelic differences. Differences in RNA splicing during mRNA maturation can also lead to polymorphic protein sequences (Maeda *et al.*, 1985; Paul *et al.*, 1986).

In the present study we examined whether the α1(I) CNBr-cleavage peptide charge heterogeneity was due to multiple forms of the mRNA for this collagen α-chain. A collagen cDNA clone was expressed in a transcription system *in vitro*, the mRNA was translated into protein and the resultant collagen peptides were mapped. Since a cDNA clone derives from a single mRNA molecule, the examination of the peptides produced from this will determine whether the peptide spot heterogeneity exists in the product of a single mRNA species.

EXPERIMENTAL

Materials

Human dermal type I collagen was prepared by pepsin digestion and differential salt precipitation (Epstein, 1974). Further purification was achieved by DEAE-cellulose (Miller, 1971) and CM-cellulose (Gay & Miller, 1979) chromatography of the native protein. The collagen was dialysed against 0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.5, and concentrated to 0.5 mg/ml. The final purity was assessed by SDS/polyacrylamide-gel electrophoresis. This type I collagen preparation (1.0 ml) was mixed with Freund's complete adjuvant and injected subcutaneously into 6-month-old sheep. These were then injected with 0.1 mg of collagen at 2-week intervals. After 8 weeks, the animals were bled and immune serum was prepared. Antibody specificity was checked by enzyme-linked immunosorbent assay (Rennard *et al.*, 1982) and by immunoblotting to individual type I collagen CNBr-cleavage peptides (Szewczyk & Kozloff, 1985). The sheep anti-(human type I collagen) serum was specific for denatured type I collagen and reacted with all the major α1(I)- and

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α2(I)-chain CNBr-cleavage peptides. A monoclonal antibody to rat phenylalanine hydroxylase (PH8) was kindly given by Dr. R. Cotton (Jennings *et al.*, 1986).

Rat phenylalanine hydroxylase cDNA was a gift from Dr. H. Dahl and Dr. J. Mercer (Dahl & Mercer, 1986), and the human α1(I) cDNA clone (Hf404) was a gift from Dr. F. Ramirez (Chu *et al.*, 1982). Restriction endonucleases and other nucleic acid-modifying enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) and used according to the manufacturer's specifications. Riboprobe pSP65 vectors, SP6 RNA polymerase and rabbit reticulocyte lysate were obtained from Promega Biotec, Madison, WI, U.S.A. L-[2,3,4,5-^{3}H]Proline (100 Ci/mmol) was purchased from Amersham Australia Pty., Sydney, N.S.W., Australia. Nitrocellulose and Elutip-d Minicolumns were obtained from Schleicher and Schuell, Keene, NH, U.S.A. Rabbit anti-(mouse immunoglobulin) and rabbit anti-(sheep immunoglobulin) antibodies were obtained from Dako Industries (Copenhagen, Denmark). ^{125}I-labelled Protein A was purchased from Amersham Australia Pty. All other chemicals were of analytical grade.

Construction of recombinant plasmids

The collagen α1(I) cDNA (Hf404) contained sequences coding for the central portion of the main triple-helical region from amino acid residue 247 to 861 of the α1(I)-chain. This included the complete α1(I) CB7 and CB3 peptides and portions of the CB8 peptide at the 5′ end and the CB6 peptide at the 3′ end (Chu *et al.*, 1982). However, this clone did not contain the 5′ translation-initiation codon. These sequences, required for efficient translation, were obtained from a rat phenylalanine hydroxylase (RPH1) cDNA clone (Dahl & Mercer, 1986). The RPH1 and Hf404 cDNAs were isolated from their respective plasmids by *Eco*RI digestion and electrophoresis in low-melting-point agarose. The bands corresponding to the cDNA inserts were excised and the cDNA was purified on Elutip-d Minicolumns (Schleicher and Schuell) according to the manufacturer's protocol.

Computer analysis of the cDNA sequences by using the Staden suite of programs (Staden, 1982) indicated that *Taq*I cleavage of the RPH1 cDNA would yield a 0.8 kb fragment containing the translation-initiation sequence (Fig. 1). *Taq*I cleavage of the Hf404 cDNA would yield a large fragment (1.7 kb) covering the triple-helical sequences corresponding to the α1(I) CB3 and CB7 peptides and small portions of the CB6 and CB8 peptides (Fig. 1). The *Taq*1-digestion products were fractionated by electrophoresis in low-melting-point agarose and purified on an Elutip-d Minicolumn. The 0.8 kb RPH fragment and the 1.7 kb Hf404 fragment were ligated (Fig. 1), and the 2.5 kb cDNA construct was isolated by electrophoresis in low-melting-point agarose and purification on an Elutip-d Minicolumn. pSP65 plasmids were cleaved at a single *Eco*RI site in the M13 polylinker region and dephosphorylated with alkaline phosphatase. The 2.5 kb RPH–Hf404 cDNA was mixed and ligated with this vector. $CaCl_2$-treated *Escherichia coli* (JM 101) cells were transformed with the recombinant vector.

Preparation of mRNA

Transcription *in vitro* of the RPH–Hf404 cDNA construct was performed as described by Melton *et al.* (1984). The linearized plasmid (2 μg) was added to 100 μl of 40 mM-Tris/HCl buffer, pH 7.5, containing 6 mM-$MgCl_2$, 2 mM-spermidine, 10 mM-NaCl containing 100 units of ribonuclease inhibitor (Promega Biotec), 10 mM-dithiothreitol, 0.5 mM each of ATP, CTP, GTP and UTP, and SP6 RNA polymerase (10–20 units). Transcription was performed for 1 h at 40 °C, following which template DNA was removed by incubation with 2 units of DNAase at 37 °C for 15 min. The mixture was extracted with phenol/chloroform and then with chloroform. Sodium acetate buffer, pH 5.0, was added to a final concentration of 0.3 M, and mRNA was precipitated with 70% (v/v) ethanol at −20 °C for 12 h. The precipitate, collected by centrifugation, was washed in 70% (v/v) ethanol. The pellet was dried and dissolved in diethyl pyrocarbonate-treated water.

Total RNA was prepared by the method of Liu *et al.* (1979) as modified by Wake & Mercer (1985). Briefly, post-confluent human skin fibroblast cultures were homogenized in 7.5 M-guanidinium chloride in 50 mM-sodium citrate buffer, pH 7.0, containing 0.1% (w/v) Sarkosyl and 100 mM-2-mercaptoethanol. The extract was layered over a 5.7 M-CsCl cushion and centrifugation was performed at 185000 *g* for 18 h at 4 °C. The resulting RNA pellet was redissolved in the guanidinium chloride solution prepared as described above but without 0.1% (w/v) Sarkosyl. The RNA was prepared from this solution by ethanol precipitation and redissolved in diethyl pyrocarbonate-treated water and re-precipitated with ethanol. The RNA yield was determined by measuring A_{260}.

Cell-free translation of mRNA

Cell-free translation of mRNA was performed in a rabbit reticulocyte lysate system. Typically such mixtures consisted of 35 μl of lysate, 1.3 μl of an amino acid mixture lacking methionine, 1.3 μl of an amino acid mixture lacking leucine, 5 μl of [2,3,4,5-^{3}H]proline and 2 μl (about 2 μg) of RPH–Hf404 mRNA or control fibroblast total RNA. Translation was undertaken for 2 h at 26 °C, and the translation products were then precipitated by the addition of $(NH_4)_2SO_4$ to 30% saturation and stirring for 12 h at 4 °C. The precipitate was collected by centrifugation and washed twice with 250 μl of 30%-saturated $(NH_4)_2SO_4$. The pellet was redissolved in 0.1 M-ammonium bicarbonate buffer, pH 7.8, and portions of the solution were freeze-dried for CNBr cleavage and electrophoresis.

CNBr cleavage

Freeze-dried samples of protein produced by cell-free translation of mRNA were suspended in 70% (v/v) formic acid containing 50 mg of CNBr/ml, and cleavage was achieved by the method of Scott & Veis (1976).

SDS/polyacrylamide-gel electrophoresis

The protein products of cell-free translation of mRNA were analysed on 5% (w/v) polyacrylamide separating gels with a 3.5% (w/v) polyacrylamide stacking gel. Collagen CNBr-cleavage peptides were analysed on 12.5% (w/v) polyacrylamide slab gels with a stacking gel of 4.5% (w/v) polyacrylamide. The sample preparation and conditions of electrophoresis are described elsewhere (Bateman *et al.*, 1984; Cole *et al.*, 1984).

Collagen CNBr-cleavage peptides were also analysed by two-dimensional polyacrylamide-gel electrophoresis.

Electrophoresis consisted of non-equilibrium pH-gradient electrophoresis in the first dimension and SDS/polyacrylamide-gel electrophoresis in the second dimension (Cole & Chan, 1981).

Radioactivity in ^{3}H-labelled protein products and peptides was determined by fluorography (Bonner & Laskey, 1974). Immunoblotting was performed as described by Szewczyk & Kozloff (1985), with a 25 mM-ethanolamine/glycine, pH 9.5, buffering system. This was found to be most suitable for basic proteins. Separated proteins and peptides were transferred to nitrocellulose by using an Electro-blot system (BioRad Laboratories, Richmond, CA, U.S.A.). M_r standards were also transferred on to nitrocellulose and were stained with a solution containing 0.1% Amido Black 10B, 45% (v/v) methanol and 10% (v/v) acetic acid for 3 min and destained in a solution of 10% (v/v) methanol and 7% (v/v) acetic acid. Non-specific antibody binding was blocked by incubating the nitrocellulose with 3% (w/v) bovine serum albumin (fraction V) in 20 mM-Tris/HCl buffer, pH 7.5, containing 0.9% NaCl. A polyclonal sheep anti-(human type I collagen) antibody preparation and a monoclonal mouse anti-[rat phenylalanine hydroxylase (PH8)] antibody were allowed to hybridize, followed by a second antibody, which was either rabbit anti-(sheep immunoglobulin) or rabbit anti-(mouse immunoglobulin) antibody. Hybridization was detected by binding of ^{125}I-labelled Protein A and autoradiography.

RESULTS

Computer analysis of the known nucleotide sequences of RPH1 cDNA (Dahl & Mercer, 1986) indicated that after cleavage with *Taq*I the desired sequences including the initiation codon would be contained in a 0.8 kb fragment. The 1.7 kb *Taq*I fragment from the Hf404 cDNA included the entire α1(I) CB7 and CB3 peptides and portions of the CB6 and CB8 peptides. The ligation of these fragments maintained the correct codon reading frame. The 2.5 kb RPH–Hf404 cDNA fragment was inserted into the pSP65 vector (Fig. 1). The orientation of the insert was determined in five colonies by digestion of the plasmid with *Pvu*II and agarose-gel electrophoresis. Ethidium bromide staining showed the expected 0.7 kb, 0.9 kb, 1.1 kb and 2.9 kb fragments from the plasmids that contained the insert in the correct orientation. In contrast, the expected 0.3 kb, 0.7 kb, 0.9 kb and 3.7 kb fragments were obtained from the plasmids containing the insert in the reverse orientation (results not shown).

The mRNA obtained by transcription *in vitro* of the 2.5 kb RPH–Hf404 cDNA inserted into pSP65 vector was successfully used for cell-free translation with a rabbit reticulocyte system. Electrophoresis of the protein products revealed a single major protein band, which was shown by immunoblotting to contain both phenylalanine hydroxylase and collagen sequences (Fig. 2). The M_r of this band, as judged by electrophoretic migration, was also consistent with the expected M_r of the phenylalanine hydroxylase–collagen hybrid protein. There were also trace amounts of other labelled components, but their nature was not determined.

One-dimensional gel electrophoresis of the CNBr-cleavage peptides obtained from the translation products revealed three major and two minor bands (Fig. 3, lane 1). The upper major band was shown by

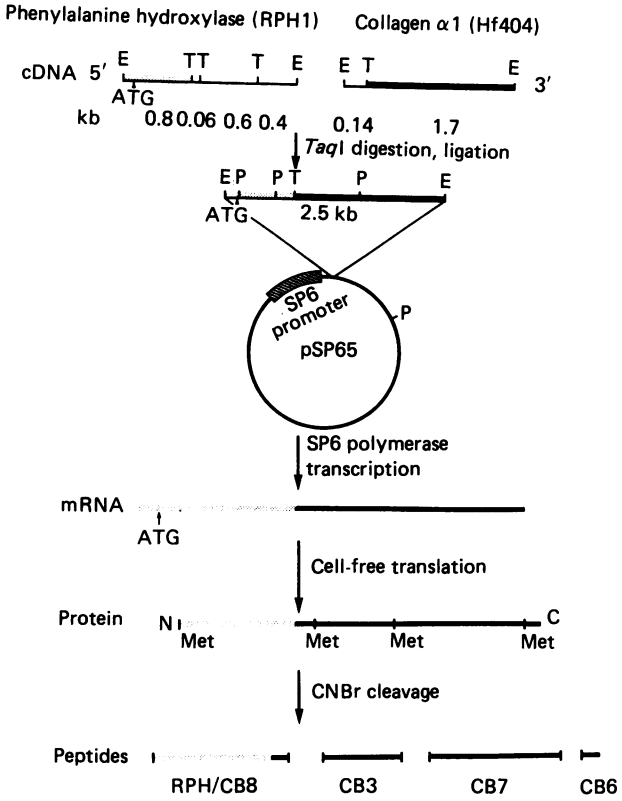

Fig. 1. Flow diagram of experimental plan

The diagram shows the construction of the hybrid RPH–Hf404 cDNA insert and transcription and translation of this into protein. Restriction-enzyme-cleavage sites in the cDNA are designated by T (*Taq*I), E (*Eco*RI) and P (*Pvu*II). The sizes of the fragments produced by these cleavages are given as kilo-base-pairs (kb). The initiation codon is indicated (ATG) as are the locations of the other methionine (Met) residues in the protein product. The collagen CNBr-cleavage peptides produced are shown, and the peptide containing some collagen α1 CB8 sequence as well as the phenylalanine hydroxylase sequence is designated RPH/CB8.

immunoblotting to contain phenylalanine hydroxylase sequences (Fig. 3, lane 2) and was therefore designated as RPH/α1 CB8 (see Fig. 1). The electrophoretic migration was also consistent with this designation. The two other major bands, which were solely collagen sequences (Fig. 3, lane 1), were identified as the α1(I) CB7 and CB3 peptides by comparison with the migration of unhydroxylated CNBr-cleavage peptides obtained from fibroblast cultures. The two minor bands that migrated more slowly than the RPH/α1 CB8 peptide were collagenous and probably represented incompletely cleaved collagen sequences (Scott & Veis, 1976).

Two-dimensional gel electrophoresis of the cell-free translation products of fibroblast total RNA demonstrated that most of the CNBr-cleavage peptides resolved into a number of changed forms (Fig. 4*a*). In particular, the α1(I) CB7 peptide separated into two major forms whereas the α1(I) CB3 peptide was only detected as a single major species. When the CNBr-cleavage peptides generated from the hybrid protein produced by cell-free translation were examined (Fig. 4*b*), both the α1 CB7 and α1 CB3 peptides showed peptide spot patterns identical

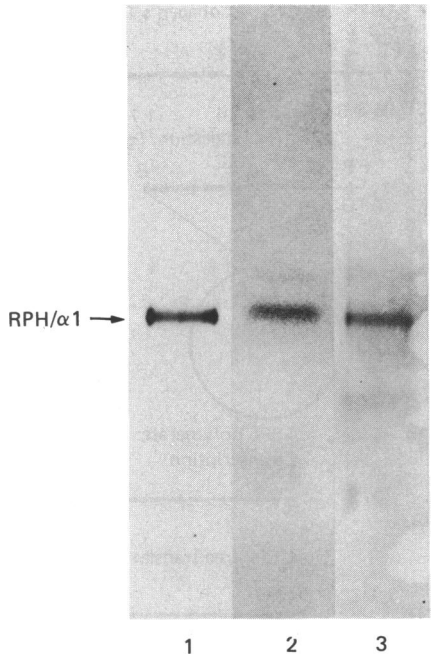

Fig. 2. SDS/polyacrylamide-gel electrophoresis of the products of translation *in vitro*

mRNA transcribed *in vitro* from the cDNA construct was translated in a rabbit reticulocyte lysate system (see the Experimental section for details) and analysed on a 5% (w/v) polyacrylamide separating gel. Lane 1, translation products labelled with L-[2,3,4,5-^{3}H]proline; lane 2, translation products identified by immunoblotting with monoclonal antibodies to phenylalanine hydroxylase (PH8); lane 3, products identified by immunoblotting with a polyclonal antiserum to type I collagen. All samples were reduced before electrophoresis. RPH/α1 identifies the migration position of the hybrid phenylalanine hydroxylase–collagen α1(I)-chain protein.

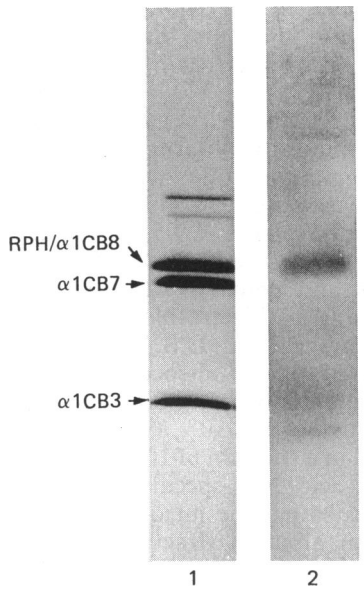

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the CNBr-cleavage peptides of the products of translation *in vitro*

The products of translation *in vitro* of the mRNA derived from the cDNA construct were digested with CNBr and analysed on 12.5% (w/v) polyacrylamide gels (see the Experimental section for details). Lane 1, L-[^{3}H]proline-labelled translation products; lane 2, immunoblot with monoclonal antibodies to phenylalanine hydroxylase. The migration positions of the type I collagen α1(I) CB3 and α1(I) CB7 peptides are shown. RPH/α1 CB8 identifies the peptide derived from the hybrid protein containing both phenylalanine hydroxylase and some α1(I) CB8 sequences.

with those of the corresponding peptides in the fibroblast total RNA translation (Fig. 4*a*). The hybrid RPH/α1 CB8 peptide also had a heterogeneous charge distribution.

DISCUSSION

Collagen was produced by transcription *in vitro* of a collagen cDNA clone and cell-free translation of the resultant collagen mRNA. In this paper we describe this new approach to collagen structural analysis, and demonstrate its use in studying the derivation of the charge heterogeneity of collagen CNBr-cleavage peptides.

A requirement for the efficient production of proteins from cDNA by transcription and translation *in vitro* is that the cDNA contains the translation-initiation codon. Since the collagen cDNA clone available for this study was not full-length, and did not contain these essential sequences, they were obtained from a rat phenylalanine hydroxylase (RPH) cDNA clone. The ligation of this RPH cDNA restriction-enzyme fragment to a fragment from the collagen (Hf404) cDNA produced a hybrid clone with both phenylalanine hydroxylase and collagen coding sequences (Fig. 1). The correct codon reading frame was maintained. Translation *in vitro* resulted in a hybrid protein, and the collagen sequences of interest were studied after CNBr digestion, which removed the phenylalanine hydroxylase sequences and released the α1(I) CB3 and CB7 peptides.

Two-dimensional electrophoresis of the collagen CNBr-cleavage peptides produced from this cDNA showed that the α1 CB7 and CB3 peptides had the same peptide spot pattern as collagen produced from fibroblast total RNA or by human fibroblast cultures or extracted from human tissues. The generation of multiple charged forms of the α1 CB7 peptide from the expression of a single cDNA clone, derived from a single mRNA molecule, demonstrated that the charge heterogeneity was not due to multiple forms of the collagen mRNA. This finding is of importance in view of data showing that electrophoretically distinct forms of proteins can result from polymorphic mRNA species. Polymorphic mRNAs can arise from allelic differences (Mercer *et al.*, 1986), and in the case of collagens some sequence heterogeneity has been shown in type I, II and III collagens (Butler *et al.*, 1977; Seyer & Kang, 1981; Yamauchi *et al.*, 1982; Chu *et al.*, 1985). Polymorphic mRNA has also been shown to result from alternative RNA splicing of a single gene product leading to distinct protein species (Maeda *et al.*, 1985; Paul *et al.*, 1986). However, alternative pathways of RNA splicing have not been implicated in generating collagen protein diversity.

If these multiple two-dimensional-electrophoretic spots do not result from polymorphic mRNA, then what does cause them? Two possibilities exist. The first is

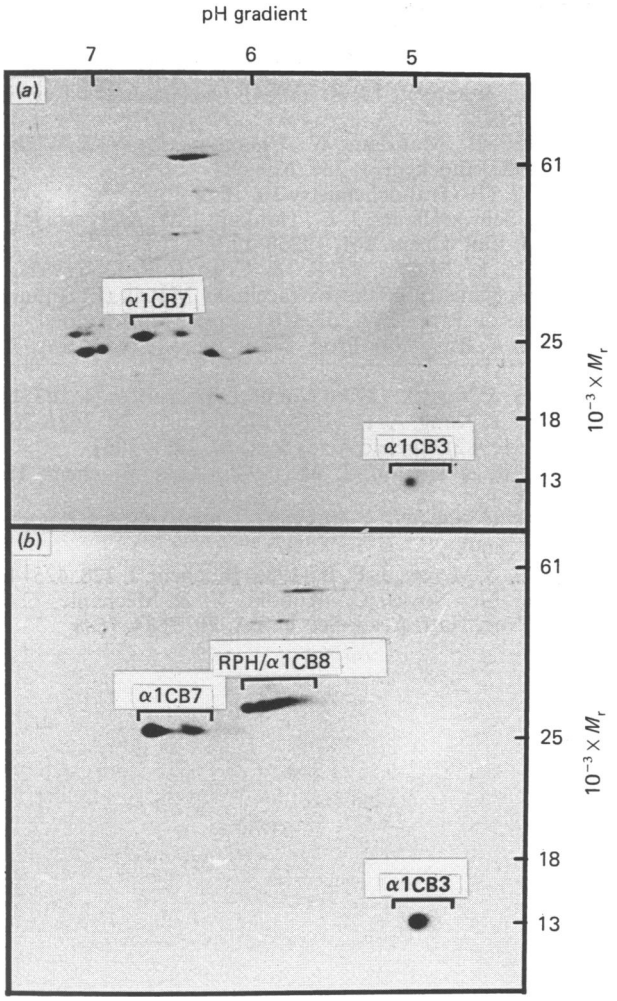

Fig. 4. Two-dimensional gel electrophoresis of the products of translation *in vitro*

The translation products labelled with [^{3}H]proline were CNBr-digested and analysed by two-dimensional gel electrophoresis as described in the Experimental section. (*a*) Translation products of total RNA extracted from control human skin-fibroblast cultures; (*b*) translation products of mRNA produced from the cDNA construct. The α1 CB7 and α1 CB3 peptides from the α1(I)-chain and the RPH/α1 CB8 peptide from the hybrid protein are indicated.

post-translational modification of the collagen either during synthesis or after secretion into the extracellular environment. The second possibility is chemical modification during sample preparation or analysis.

In the collagen α-chains the major post-translational modifications are proline hydroxylation and lysine hydroxylation and glycosylation (Bornstein & Traub, 1979). However, the charge heterogeneity has been shown to be unaltered by blocking these post-translational modifications in fibroblast cultures (Bateman *et al.*, 1984). Other modifications of collagens, such as the phosphorylation of hydroxylysine (Urushizaki & Seiffer, 1985) and sulphation of tyrosine in type V collagen precursors (Fessler *et al.*, 1986), have been reported. It is unlikely that such minor post-translational modifications would occur to the same extent or have the same specificity in systems *in vitro* such as cell-free mRNA translation and *in vivo*. Yet the heterogeneity of the type I collagen two-dimensional-electrophoretic peptide spot pattern is similar whether collagen produced in cell-free translation, cell culture or extracted from tissues was examined (Cole & Chan, 1981; Bateman *et al.*, 1984), and so we conclude that the heterogeneity is unlikely to derive from modifications *in vivo*.

The second possible source of charge heterogeneity is chemical modification of the peptide during sample preparation and electrophoresis. Although cleavage of methionine residues by CNBr can lead to two forms of homoserine, this was prevented by conversion of homoserine lactone into homoserine before electrophoretic analysis (Finlayson & Chrambach, 1971). In addition, intact collagen α-chains also display charge heterogeneity (results not shown), and thus it is unlikely that the CNBr cleavage contributed to the observed heterogeneity. The electrophoretic sample preparation and running conditions used here do not produce the heterogeneity, since the charged forms may be resolved by ion-exchange chromatography before electrophoresis (Cole & Chan, 1981). Chemical modification of asparagine and glutamine by deamidation can result in charge changes in peptides and proteins (Robinson, 1974), and this may be a factor in producing the charge heterogeneity. The extent of deamidation varies according to the surrounding peptide sequences, pH, ionic strength and time of reaction. The low pH during the CNBr-cleavage reaction would be conducive to deamidations so this is a possible source of charge heterogeneity.

The utility of the two-dimensional gel-electrophoresis technique remains undiminished by this charge heterogeneity, since these peptides spot patterns are highly reproducible both on multiple analyses of each sample and also between different control samples. A charge-change mutation resulting from a glycine-to-arginine substitution in the α1(I) CB8 peptide was readily distinguished over the consistent background of charge heterogeneity by using this method (results not shown).

This demonstration that detailed peptide analysis can be performed on collagen produced *in vitro* from cDNA clones has wider implications. In the study of many diseases cDNA cloning is the most expedient method for determining the nature of the protein defect. However, a limitation of this procedure is that in analysis of heterozygotes the cDNA clones obtained must be identified as coding either for the normal or for the mutant sequences. If a demonstrable protein defect is present, the expression of the cDNA into protein *in vitro*, which can then be peptide-mapped or otherwise analysed, should be of considerable help in selecting cDNA clones for DNA sequencing and further analysis.

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