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Cloning and characterization of the complementary DNA for the B chain of normal human serum C1q

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Normal human C1q is a serum glycoprotein of 460 kDa containing 18 polypeptide chains (6A, 6B, 6C) each 226 amino acids long and each containing an N-terminal collagen-like domain and a C-terminal globular domain. Two unusual forms of C1q have been described: a genetically defective form, which has a molecular mass of approximately 160 kDa and is found in the sera of homozygotes for the defect who show a marked susceptibility to immune complex related disease; a fibroblast form, shown to be synthesized and secreted, *in vitro*, with a molecular mass of about 800 kDa and with chains approximately 16 kDa greater than those of normal C1q. A higher than normal molecular mass form of C1q has also been described in human colostrum and a form of C1q has been claimed to represent one of the types of Fc receptor on guinea-pig macrophages.

To initiate studies, at the genomic level, on these various forms of C1q, and to investigate the possible relation between the *C1q* genes and the procollagen genes, the complementary DNA corresponding to the B chain of normal C1q has been cloned and characterized.

Introduction

Normal human C1q (460 kDa), a subcomponent of the first component of complement, plays an important role in the activation of complement by immune complexes. During activation the C1q molecule binds to the Fc regions of immunoglobulin complexes and thus can initiate the activation of the proenzyme forms of the C1r and C1s subcomponents (Reid 1983). The normal C1q, found in the serum, is composed of 18 polypeptide chains (6A, 6B and 6C) each of which consists of a short N-terminal section of non-collagen-like sequence followed by a collagen-like region of approximately 81 residues and ending in a non-collagen-like, C-terminal region of 130 residues. A molecular model for C1q has been proposed in which an A, a B and a C chain are considered to combine to form a collagen-like triple helical region and a globular 'head' region, and the intact molecule therefore is composed of six triple helices and six globular 'head' regions (for review, see Reid 1983). The globular 'head' regions are considered to contain the site involved in the interaction between C1q and the Fc of aggregated IgG in immune complexes, while the collagen-like regions appear to be involved in the interaction with, and activation of, the other subcomponents of the C1 complex, that is, the C1r and C1s proenzymes. The collagen-like regions of C1q, after removal of the activated C1r and C1s by the control protein $C\bar{1}$-inhibitor, bind to fibronectin and to receptors on certain lymphoid cells and this property may be of importance in the clearance of immune complexes from serum since the C1q would still be bound to these complexes via its globular 'heads'. Thus C1q fulfils recognition and activation roles during the activation of the classical pathway of complement

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by immune complexes and perhaps a role in the clearance of these complexes by phagocytic mechanisms involving fibronectin or lymphoid cells or both.

The importance of C1q in the clearance of immune complexes is illustrated from studies of several families in which one, or more, of the members suffer from immune-complex-related renal disease probably as a result of possessing a defective form of C1q in their sera (Thompson

	molecular mass non-dissociating conditions/Da	molecular mass of chains in dissociating conditions after reduction/Da	
normal C1q	460000	6A 27500 6B 25200 6C 23800	(true molecular mass)
defective C1q	155000	2A, 2B } 70000 2C 28000	(apparent molecular masses in SDS-p.a.g.e.)
fibroblast C1q	800000	chains of ≈46000 (apparent molecular mass)	

C1q-like molecules also reported in: colostrum, epithelial cell lines, macrophages

Figure 1. Different forms of C1q.

et al. 1980; Chapuis *et al.* 1982; Hannema *et al.* 1982). The defect in these families appears to be the consequence of an abnormal *C1q* gene which produces an antigenically deficient, non-functional molecule. There was a complete lack of C1 and classical pathway activity in patients homozygous for the defect but complete activity could be restored to their sera by the addition of purified C1q. Structural studies, at the protein level, have shown that the defective C1q has a molecular mass approximately one third that of the normal molecule although it has a polypeptide chain and subunit structure similar in many respect to that of the normal molecule (Reid & Thompson 1983).

Other structural variants of C1q have been described (figure 1). Normal fibroblasts appear to synthesize a functionally active C1q molecule which has chains approximately 18 kDa greater than those of the normal serum C1q (Reid & Solomon 1977). It has been found (Skok *et al.* 1981) that skin fibroblast C1q appears to be under different genetic control from serum C1q, since the C1q synthesized *in vitro* by fibroblasts from one of the homozygote C1q deficient children was functionally normal and appeared structurally similar to the fibroblast C1q obtained from normal fibroblasts. A higher than normal molecular mass C1q-like molecule has been reported in human colostrum (Yonemasu *et al.* 1979) and a form of C1q has been claimed to represent one of the classes of Fc receptor on guinea-pig peritoneal macrophages (Loos 1983).

The study of these structural variants of C1q at the DNA level should help clarify the nature of the genetic defect involved in the immune-complex-related nephritic disease and also provide an interesting comparison between the genes coding for C1q and those coding for procollagen. To initiate these studies the cDNA corresponding to the B chain of normal human C1q has been cloned thus providing probes suitable for screening of genomic libraries or genomic blots.

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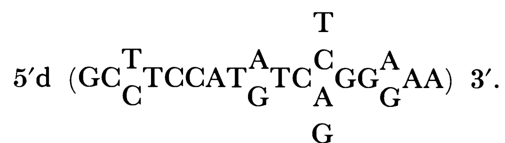
Methods used to isolate cDNA for human C1q

Synthesis and radiolabelling of the oligonucleotide probe

The probe was a mixture of 32 different 17-long oligonucleotides, synthesized by the solid-phase phosphotriester technique (Duckworth *et al.* 1981) based on the amino acid sequence Phe-Pro-Asp-Met-Glu-Ala which is located at positions 221–226 of the B chain of human C1q (Reid *et al.* 1982):



The 17-base oligonucleotide mix was 5′-labelled by using [γ-^{32}P] ATP and T4 polynucleotide kinase (Maxam & Gilbert 1980).

Preparation of the recombinant DNA *libraries*

Total RNA was extracted from approximately 10 g of human liver following the guanidine thiocyanate procedure of Chirgwin *et al.* (1979). The RNA was fractionated by centrifugation in 15–30% sucrose gradients to select molecules of more than 500 nucleotides in length, and then further purified by oligo-dT cellulose chromatography (Aviv & Leder 1972). Double-stranded DNA was synthesized from 80 μg poly A^+ RNA using standard procedures (Buell *et al.* 1978; Wickens *et al.* 1978).

Construction of the fragment library has been fully described (Bentley & Porter 1984). Briefly, half the double-stranded cDNA was desalted on a Sephacryl S-400 column (1.5 ml) then partially digested with Hae III before ligation into the Pvu II site of the plasmid vector pAT 153/Pvu II/8 (Choo *et al.* 1982). The products of the ligation were transformed into competent *E. coli* K12 strain MC1061 (Casadalan & Cohen 1980), grown in the presence of ampicillin and stored at −70 °C. The complexity of the fragment library was greater than 4×10^6 recombinants before amplification.

The full-length cDNA library was constructed as follows. The remaining double-stranded cDNA was treated with S1 nuclease, fractionated on a 15–40% sucrose gradient (5 ml) and all the fractions containing DNA of more than 500 base pairs were pooled. The DNA was repaired using DNA polymerase I (Klenow fragment) and cloned into pAT 153/Pvu II/8 as described above. The full-length library contained more than 1×10^5 transformants before amplification, of which approximately 50% contained inserts of greater than 1 kilobase (kb).

Isolation of cDNA *clones*

Approximately 200000 colonies of the human liver cDNA fragment library were plated onto ampicillin plates, then transferred to Whatman 541 filter paper (Suggs *et al.* 1981) and prepared for hybridization as described by Gergen *et al.* (1979). The filters were prehybridized by incubation at 60 °C, for 3 h in 0.9 M NaCl, 90 mM Tris HCl pH 7.5, 6 mM EDTA and 0.5% Nonidet P-40 containing boiled sonicated herring sperm DNA at 100 μg ml^{-1} and tRNA at 50 μg ml^{-1}. The filters were hybridized for 16 h at 46 °C in the same solution after addition of the [γ-^{32}P] end-labelled 17-base oligonucleotide probe mixture (1.5 ng ml^{-1}; 5×10^5 counts per minute per millilitre). The filters were washed with 0.9 M NaCl and 0.09 M sodium

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citrate, pH 7.4, once, at 4 °C for 30 min then three times at 46 °C for 30 min. After allowing the filters to air dry they were radioautographed at −70° overnight.

Approximately 45000 colonies of the human liver cDNA full-length library were plated onto ampicillin plates then transferred to Whatman 541 filter papers and prepared for hybridization as described by Gergen *et al.* (1979). The filters were prehybridized and hybridized at 42° in buffer containing 50% formamide (Bernards & Flavell 1980). An 84 base-pair insert Clq cDNA probe was used for the screening after it had been labelled to a specific activity of approximately 10^8 counts per minute per microgram of DNA by nick translation (Rigby *et al.* 1977). The hybridization mixture contained 4×10^5 counts per minute per millilitre. After hybridization for 20 h the filters were washed with 0.15 M NaCl and 0.015 M sodium citrate, once, at 20 °C, for 15 min then twice, at 68 °C, for 30 min. After air-drying, the filters were radioautographed at −70° for 40 h.

Preparation and analysis of cloned DNA

Plasmid DNA was extracted from bacterial colonies by using the alkaline-sodium dodecyl-sulphate method (Birnboim & Doly 1979). DNA sequence analysis was carried out as described by Maxam & Gilbert (1980).

Cell-free translation of poly A^+ RNA

RNA was extracted from human liver, and from the epithelial cells of human small intestine, employing a previously described method (Carroll & Porter 1983). The total liver RNA was fractionated on a sucrose gradient and the fractions corresponding to the 18S peak (designated 18S-I) and the fractions corresponding to the trailing shoulder of the 18S peak (designated 18S-II) were saved for further studies. The poly A^+ RNA was prepared from intestine total mRNA, and from the liver 18S-I and 18S-II pools, by chromatography on oligo dT-cellulose. The fractions were translated in a rabbit reticulocyte cell-free system with [^{35}S]-methionine. Translation of C1q mRNA was determined by immunoprecipitation using affinity purified rabbit anti-human C1q (prepared as described by Reid & Thompson 1983). Antibody and 8 μg carrier C1q were added to the translation mixtures and the immune precipitates spun down after incubation, at 37 °C, for 60 min and, at 4 °C for 16 h. The immunoprecipitates were washed three times with ice cold immunoprecipitation buffer (0.5% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate, 150 mM NaCl, 1 mM EDTA and 62 mM Tris pH 6.8) before reduction and alkylation, in 4 M urea, 0.1 M Tris HCl pH 8.0 and 1% sodium dodecyl sulphate, and electrophoresis on a 50 mg ml^{-1} acrylamide gel, run in the presence of sodium dodecyl sulphate. Anti-human serum albumin and anti-ovalbumin were used to prepare positive and negative controls, respectively, from the cell-free translation mixtures. After electrophoresis the gel was dried down and radioautographed at −70 °C, for one to two weeks.

Results

Cell free translation of poly A^+ RNA

Initial experiments involved the isolation of poly A^+ RNA from human small intestine as well as from human liver. The intestine was used because the columnar cells of the small intestine are considered to be a major site of synthesis of the C1 complex (Colten *et al.* 1968). Sucrose gradient fractionation was only performed on the liver RNA preparation because of

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the low amounts of RNA available from the intestine preparation. The poly A+ RNA sedimenting in the trailing shoulder of the 18S peak (fraction 18S-II) in the sucrose gradient of the total liver RNA yielded C1q-like material in a cell-free translation system, as judged by immunoprecipitation with purified anti-C1q. The total poly A+ RNA from the intestine also gave immunoprecipitable C1q-like material in a cell-free translation system. In both cases a major band was seen at 48–49 kDa apparent molecular mass, along with, in the case of the intestine poly A+ RNA preparation, two minor bands at 22–25 kDa apparent molecular mass,

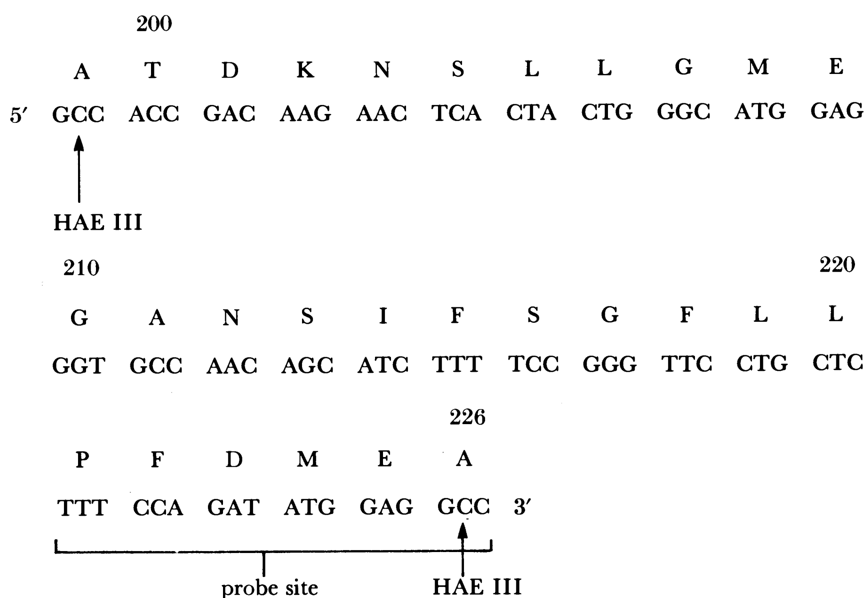

FIGURE 2. Isolation of cDNA coding for the C-terminal 28 amino acids of the B chain of human C1q. A mixture of 32 synthetic oligonucleotides each 17 bases long was used to probe the fragment library from human liver. Only the coding strand of the cDNA is shown. The arrows indicate the points of cleavage by the restriction enzyme HAE III which was used to prepare the cDNA fragments for the fragment cDNA library. The alanine residue at position 226 is the C-terminal amino acid of the B chain. The composition of the synthetic oligonucleotide probe mixture is given in the Methods.

when reduced immunoprecipitates were electrophoresed on SDS-polyacrylamide gels. The liver 18S-I poly A+ RNA fraction, on cell-free translation, followed by immunoprecipitation, with anti-albumin, and electrophoresis, yielded a well defined band of 65 kDa thus indicating the presence of albumin mRNA in the 18S-I fraction. The liver 18S-II fraction, on the other hand, contained relatively low amounts of the albumin mRNA as judged from the cell-free translation studies.

The poly A+ RNA fraction from intestine, despite not being enriched by fractionation on a sucrose gradient, was approximately three times more effective, on a mass basis, at producing radiolabelled immunoreactive C1q-like material in a cell free translation system, than the liver 18S-II fraction.

Identification and isolation of the C1q-B *chain* cDNA *clone*

By using the [γ-^{32}P] labelled, 17-long, oligonucleotide probe to screen the liver cDNA fragment library (approximately 200000 colonies) six positive clones were identified over background after a final wash at 52 °C. The cloned cDNA inserts were excised for Maxam–Gilbert sequence

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analysis by Cla 1/Bam H1 double digestion followed by selective filling-in of the Bam H1 end with [γ-^{32}P]dATP. All six clones contained the complementary oligonucleotide sequence (residues 221–226 of the B chain) along with the cDNA coding for residues 199–220 of the B chain (figure 2). This provided a cDNA probe, 84 base pairs long, which could be used, after labelling by nick translations, to screen the full-length cDNA liver library.

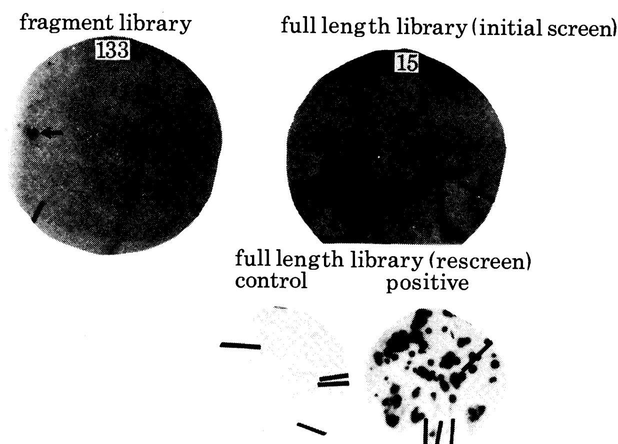

FIGURE 3. Use of the [γ-^{32}P] radiolabelled 84 base pairs probe in screening cDNA libraries. One positive colony is shown on Whatman 541 filters prepared for hybridization for an initial screen of the fragment library and an initial screen plus a rescreen of the full-length library. The negative control filter in the rescreen contained approx. the same density of colonies as the positive filter.

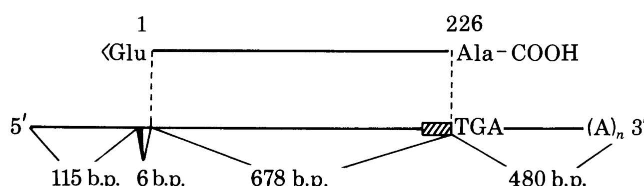

FIGURE 4. Isolation of cDNA coding for amino acid residues 1–226 of the B chain of human C1q. < Glu denotes a 'blocked' glutamyl residue, that is, having no free α-amino group. ▨ The location of the 84 base pair probe isolated from the fragment library. The 115 base pair at the 5′ of the strand shown is probably present as the result of a cloning artefact, thus the minimum length of mRNA coding for the B chain is $6+678+480 = 1164$ base pairs.

By using the [^{32}P] labelled 84 base pair cDNA probe to screen the liver cDNA full-length library (approximately 45000 colonies) three positive clones were identified over background after three washes of 30 min at 68 °C, in 0.15 M NaCl and 30 mM Tris HCl, pH 7.4 (use of the 84 base pair probe is shown in figure 3). All three positive clones appeared to have the same insert of 1269 base pairs as judged by restriction enzyme mapping. The sequence of one of the cloned inserts was determined by the Maxam and Gilbert procedure which established that the cDNA coding for residues 1–26 of the B chain was present (that is, the entire chain is found in serum (C1q) (figure 4). A termination codon was found immediately after the codon for alanine, at B-226, followed by 468 nucleotides of presumably 3′ non-coding sequence and ending in a poly A tail. At the 5′ end of the insert (as represented in figure 4) the first 115 nucleotides appear to be present as the result of a cloning artefact since they are complementary,

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and in a different orientation, to those found at the 3′ end of the insert (except in the length of the tail, 15 Ts being found at the 5′ end and 9 As being found at the 3′ end). A sequence of six nucleotides (CAGGCC) was found between the first 115 nucleotides at the 5′ end and immediately before the codon for the N-terminus of the B chain (as it is found in serum C1q). This indicates that the C-terminal sequence of any pre- or pro-section of the B chain is -Glu-Ala-.

Discussion

Early biosynthetic studies implicated the intestine as one of the major sites of synthesis of C1q in guinea-pigs, humans and pigs (Colten *et al.* 1966; Colten *et al.* 1968; Day *et al.* 1970). Columnar epithelial cells were considered to be the principal cell type involved in the small intestine and this view is supported by the report by Morris *et al.* (1978) that human fetal intestine columnar epithelial cells can synthesize, and secrete, up to 3700 times more haemolytically active C1 than several other cell types. However, it is well established that a variety of other cell types such as human fibroblasts (Al-Adnani & McGee 1976; Reid & Solomon 1977) human macrophages (Bensa *et al.* 1983) and mouse or guinea-pig macrophages (see Loos (1983) for a review) synthesize material that is antigentically and functionally similar in some respects to serum C1q. In view of the wide variety of cell types which possess the potential to synthesize C1 functional activity, or C1q immunoreactive material, it was decided to isolate the RNA from both human intestine and human liver for this study.

The cell-free translation experiments showed that both the intestine poly A^+ total RNA and the liver poly A^+ 18S-II RNA fraction produced a main polypeptide chain of 46–48 kDa, and in addition the intestine poly A^+ total RNA produced two chains of 22–25 kDa. The 46–48 kDa chain is of a similar size to the chains of a C1q-like molecule synthesized and secreted by human fibroblasts (Reid & Solomon 1977; Skok *et al.* 1981) and could possibly represent a pro-form of the serum C1q chains. The presence of minor chains of 22–25 kDa in the immunoprecipitate from the cell free translation of the intestine poly A^+ total RNA indicates the possibility that the chains of normal serum C1q (of expected molecular mass 24–28 kDa, Reid 1983) are synthesized with only very short N-terminal extensions but another explanation may be that there has been limited proteolysis of the 46–48 kDa chain during the immunoprecipitation period at 37 °C. Also, the finding that immunoprecipitable C1q-like material was located in the 18S-II fraction of human liver is consistent with the chains of C1q having a larger mRNA than would be expected for polypeptides in the 24–28 kDa range.

cDNA libraries were made from both intestine poly A^+ total RNA (by insertion of double stranded cDNA into the plasmid pBR322 using the dGdC tailing method and then using the treated plasmid to transform *Escherichia coli*) and liver poly A^+ total RNA (as described in Methods). The use of the intestine cDNA library is not described in detail since it yielded only 3 'false positive' clones on screening with the 17 long oligonucleotide probe, that is, the inserts (800–1000 base pairs) from the plasmids in these clones contained a complementary region of DNA which was a 15/17 mismatch with the probe and did not appear to be related to the serum C1q sequence in any other area. The finding of a relatively abundant number of positive clones in the liver fragment and full-length cDNA libraries is consistent with the previous reports that a wide variety of cells, besides intestinal epithelial cells, can synthesize C1q-like material. At present, only a minimum size, of 1164 base pairs, can be estimated for the C1q B chain mRNA and a measurement of the true size will have to be obtained by isolation of a clone

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with a longer 5′ section or by the use of the cDNA probes in the analysis of mRNA by Northern blotting. However, it should be noted that the finding of messenger RNA for C1q-like chains (as judged by immunoprecipitation of cell free translation products) in the 18*S* sucrose-gradient fraction indicates the presence of an mRNA molecule of approximately 1800 nucleotides.

The amino acid sequence coded for at the N-terminus of the B chain is -Gln-Ala-$Gln^{B\text{-}1}$-$Leu^{B\text{-}2}$- (where B-1 = the N-terminal amino acid of the B chain as it is found in serum C1q). The -Ala-$Gln^{B\text{-}1}$-bond may be split by a peptidase in a similar manner to the procollagen N-terminal peptidase which splits the bond -Ala-Gln- in procollagen α2(1) (figure 5). In both cases, the N-terminal glutamine residue becomes 'blocked' after the cleavage since no free α-amino group can be found on N-terminal sequence analysis.

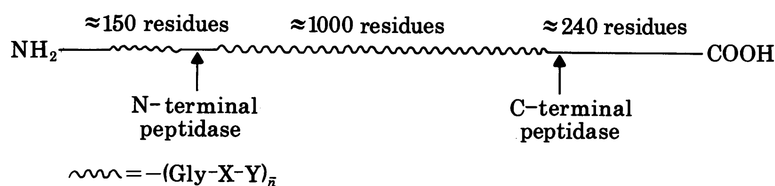
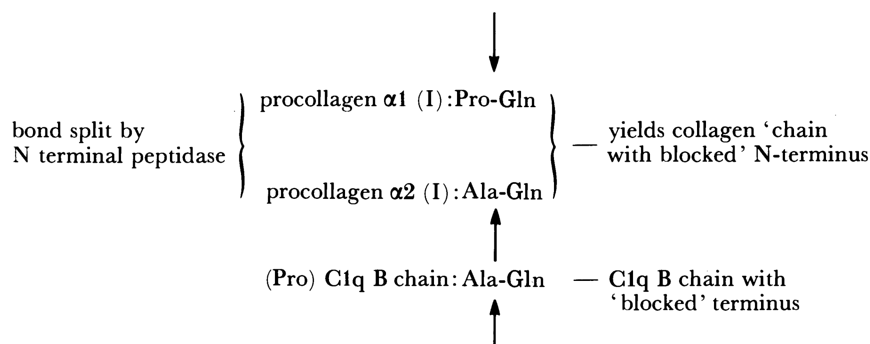


FIGURE 5. One chain of a three chain procollagen molecule is represented in the diagram. The approximate sizes of the -(Gly-X-Y)$_n$-repeating triplet portions (∿), involved in triple helix formation, and non-helical portions of the N- and C-extension (—) are shown. The N- and C-extension are split from the procollagen by N- and C-terminal peptidases (↑) to yield the collagen molecule. The α1 (I) calf procollagen and α2 (I) chick procollagen sequences are taken from Glanville *et al.* (1984). The C1q cDNA sequence predicts that a similar type of N-terminal peptidase may be involved in the conversion of a pro-C1q B chain to yield the chain as it is normally found in serum.



The nucleotide sequence coding for residues B-1 to B-226 is entirely consistent with the published amino acid sequence (Reid *et al.* 1981) except at position B-73 where the nucleotide sequence predicts a glycine residue (that is, a sequence Gly-$Gly^{B\text{-}73}$-Pro) while the published amino acid sequence gives a proline that is, -Gly-$Pro^{B\text{-}73}$-Pro-. However this difference is due to a misinterpretation of the protein sequence data (K. Reid, unpublished results) rather than to the presence of two types of C1q B chain in human serum.

The cDNA for the B chain of normal C1q will allow the isolation of cosmids containing genomic DNA coding for the B chain and, in view of the high sequence homology over certain stretches of the A, B and C chains of C1q (Reid 1983) also should allow the isolation of cDNA coding for the A and C chains. Having obtained appropriate probes for all three chains of normal C1q a complete study of the structural variants (figure 1) of C1q can be made.

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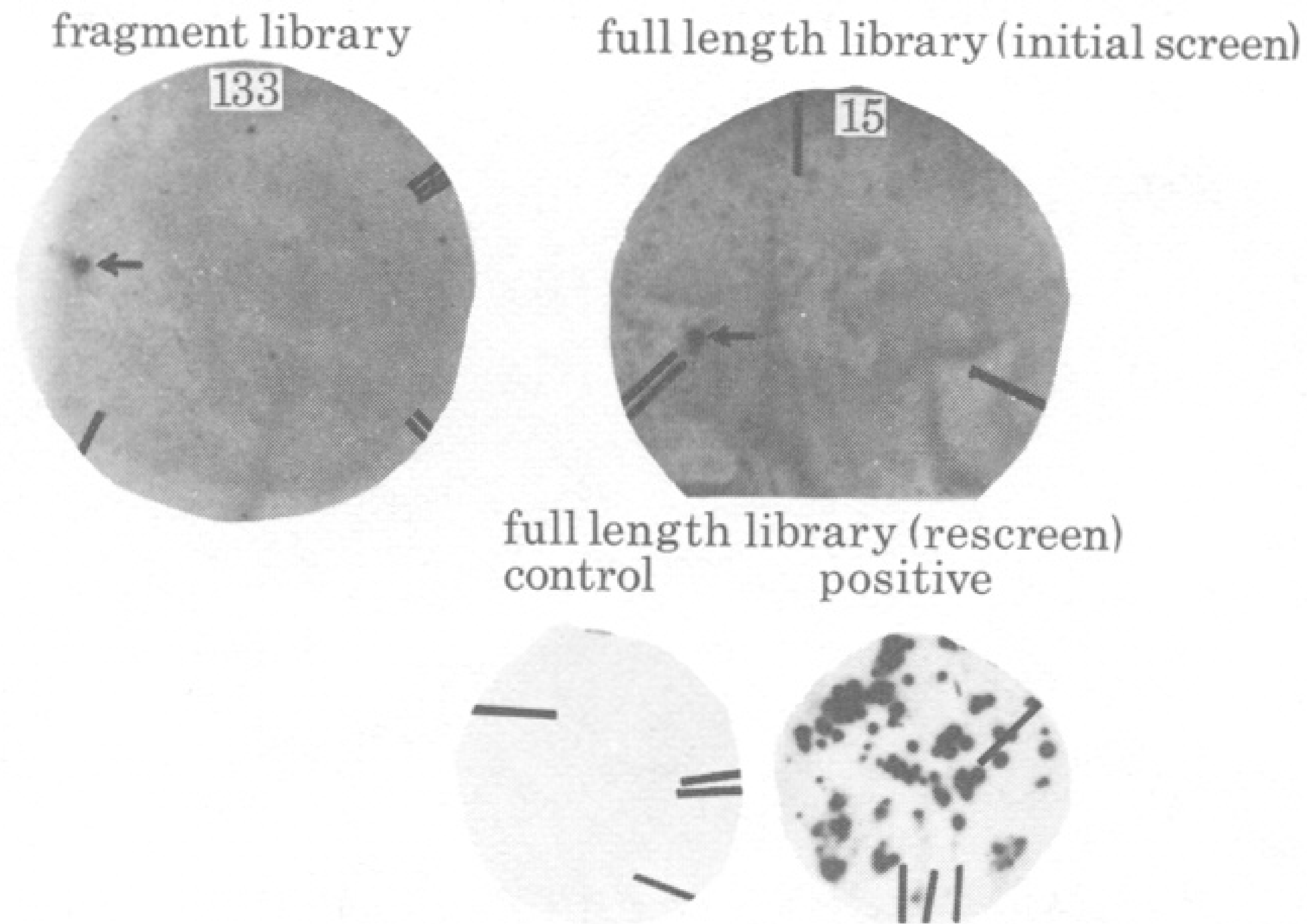

[FI]GURE 3. Use of the [γ-^{32}P] radiolabelled 84 base pairs probe in screening cDNA libraries. One positive colony is shown on Whatman 541 filters prepared for hybridization for an initial screen of the fragment library and an initial screen plus a rescreen of the full-length library. The negative control filter in the rescreen contained approx. the same density of colonies as the positive filter.