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The sequence at the 3' terminus of mouse immunoglobulin secreted μ chain messenger RNA determined from cloned cDNA

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ABSTRACT

The 3' terminal nucleotide sequence of two clones containing DNA complementary to μ chain mRNA of IgM-secreting cells has been determined. The sequence shows a termination codon (UGA) adjacent to the terminal tyrosine codon for the secreted protein and a 3' non-coding region of at least 106 bases. The primary translation product of this μ chain mRNA seems to terminate at the tyrosine of the secreted protein.

INTRODUCTION

The primary structure of μ chains of human (1,2), dog (3) and mouse (4) shows the presence of a variable (V) region domain linked to four constant (C) region domains designated $C_\mu 1$, $C_\mu 2$, $C_\mu 3$ and $C_\mu 4$. In addition, a carboxy-terminal tailpiece (tp) of 19 amino acids occurs beyond $C_\mu 4$ (5). The first heavy (H) chain to be expressed during the development of the immune system is the μ chain (6,7). Initially, two μ chains are combined with two immunoglobulin light (L) chains to form a monomeric 7S IgM molecule acting as a cell surface receptor for antigen and forming an integral part of the membrane (8-10). When cells expressing surface IgM undergo differentiation into antibody-producing cells, the IgM is secreted in the form of 19S pentamers held together by J chains.

Little experimental evidence is available which explains the mechanism(s) involved in the change of membrane bound (m) to secreted (s) IgM. Several groups, however, have reported evidence that μ_m differs from μ_s in respect of an extra polypeptide segment at the C terminus of the μ protein (11,12). It has been proposed that this extra peptide takes the form of an additional hydrophobic piece located either at the C terminus of the μ_m (13,14) or between $C_\mu 4$ and the tp (15). Ultimately, in order to understand the mechnisms underlying the transition of μ_m to μ_s, the nucleotide sequences of the relevant regions will be needed. The putative primary sequence difference

between μ_m and μ_s can be explained in one of several ways. One possibility suggested by Williams et al. (14) is that the primary translation products of μ_m and μ_s mRNA are identical but that post-translational proteolysis of the μ_s protein produces the differences in the mature proteins. The sequence of the 3' terminal portion of μ mRNA from secreting cells is relevant to this hypothesis. The present paper describes the characterisation of two cDNA clones containing μ mRNA sequences and we have determined the 3' terminal sequence for this mRNA. Our data suggests that translational termination of μ chains for secretion occurs at a UGA codon adjacent to the triplet coding for the terminal tyrosine of the secreted protein.

MATERIALS AND METHODS

Cells

The cell line used for preparation of the μ chain mRNA was the Sp1/HL hybrid cell line derived from a fusion between X63-Ag.8 (8-azaguanine-resistant clone of MOPC 21 myeloma) and spleen cells derived from mice immunised against sheep red blood cells (16). This clone synthesises two chains - a specific μ heavy chain and $\varkappa$ light chain - and secretes IgM with anti-SRBC activity.

Molecular cloning of cDNA

A 19S H chain mRNA fraction was prepared from Sp1/HL cells as described (17). 30 μg mRNA were dissolved in 0.9 ml incubation mixture containing 50 mM Tris-HCl (pH 8.3) at 46° C, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 50 mM KCl, 10 μg/ml oligo$(dT)_{12-18}$ (P.-L. Biochemicals), 150 units/ml AMV reverse transcriptase, 1 mM of the unlabelled deoxynucleoside triphosphates dCTP, dGTP, dATP and dTTP in the presence of 150 μCi α-^{32}P-dATP ($\sim$ 300 Ci/mmole). The reaction mixture was incubated at 0° C for 10 min prior to the addition of enzyme. After 30 min incubation at 46° C, the reaction mixture was incubated in 0.3 NaOH at 50° C for 1 hr, neutralised, sodiumdodecylsulphate (SDS) added to 0.3% final and passed through a Sephadex SP50 column in 0.3 M Na-acetate, 1 mM EDTA pH 6.0. The DNA was EtOH-precipitated without addition of carrier. The single stranded cDNA was converted into double stranded cDNA by a second incubation at 46° C for 2 hr with AMV reverse transcriptase in 0.45 ml of the above solution. After chloroform/phenol extraction and passage over a Sephadex SP50 column the nucleic acid was ethanol-precipitated, dissolved in 250 μl S_1 buffer (50 mM Na-acetate pH 4.6, 100 mM NaCl, 1 mM $ZnSO_4$) and incubated at 37° C for 15 min. 18 units purified S_1 nuclease (18) were added and incubation was continued for 30 min at 37° C.

The mixture was again extracted with chloroform/phenol and nucleic acid was EtOH precipitated in the presence of 10 μg E. coli tRNA. The nucleic acids were dissolved in 20 mM EDTA (pH 8), heated for 20 min at 55°C and separated on a 6% Tris-borate polyacrylamide gel (19) using HaeIII restriction fragments of ØX174 phage as size markers. After the tracking dye (Xylene cyanol) had migrated 14 cm, an area containing molecules in the range of 1500-2000 base pairs was excised, DNA was eluted from the gel (20) and ethanol-precipitated. The yield with respect to mRNA input was 1.5%. The 3' termini of the double stranded cDNA were elongated with $(dA)_n$ homopolymeric tails using terminal transferase (Boehringer, Mannheim, West Germany) in a 180 μl reaction mixture containing 140 mM potassium cacodylate (pH 7.6), 30 mM Tris, 100 μM DTT, 1 mM $CoCl_2$, 100 μM ^{3}H-labelled dATP (100 μCi/mmole), 450 ng double stranded cDNA and 135 units terminal transferase (21). BamHI-digested pBR322 was similarly elongated with dT tails. After 15 min incubation at 37°C, approximately 100 dA residues had been added onto each 3' end of the double stranded cDNA and approximately 60 dT residues per 3' end of pBR322. For molecular cloning, tailed double stranded cDNA was annealed in a molar ratio of 1:1 to the pBR322 (22) and the recombinant DNA molecules were used to transform E. coli strain χ1776. Ampicillin-resistant, tetracycline-sensitive colonies harbouring immunoglobulin sequences were detected by colony hybridisation (23). Plasmid DNA was purified from bacteria treated for 6-12 hr with 12.5 μg/ml chloramphenicol (R. Curtiss, personal communication) by the cleared lysate procedure (24) and then banded in CsCl gradients containing ethidium bromide. Supercoiled DNA extracted from recombinant plasmids pμ/107 and pμ/118 was used to transform the E. coli strain HB101 (25).

Preparation of in vitro labelled DNA hybridisation probes for screening

H chain mRNA was further purified on a methyl mercury hydroxide agarose gel (26) and ^{32}P-labelled cDNA (specific activity 3 x 10^7 cpm/μg) was prepared using pT_{10} primer at 42°C as previously described (27) for use in the colony hybridisation experiment (23).

Hybrid arrest of translation

The translation of free and hybridised mRNA was carried out using a reticulocyte cell free lysate (28,29). The translation products labelled with ^{35}S-methionine were displayed on SDS polyacrylamide gels (30) which were subsequently treated with dimethyl sulphoxide (Me_2SO) followed by 22% 2,5-diphenyloxazole in Me_2SO_4, dried, and exposed to prefogged X-ray film (31).

Restriction endonuclease digestion

Digestion of recombinant DNA molecules with restriction endonucleases was carried out according to the instructions provided by the supplier (New England Biolabs).

Terminal labelling of restriction fragments and DNA sequencing

5' Labelling of digested DNA or isolated restriction fragments was done as follows: DNA was suspended in 10 mM Tris pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 50 mM NaCl and incubated with 2.5 units alkaline phosphatase (Boehringer, Mannheim, West Germany) at 37° C for 30 min. After phenol extraction, residual phenol was removed by ether extraction. The DNA fragments were incubated for 1 hr at 37° C in 50 mM glycine-NaOH (pH 9.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 25% glycerol, 200-400 μCi γ-^{32}P-ATP (Radiochemical Centre, Ltd., Amersham, England; specific activity 3000-5000 Ci/mmole) and 5 units of T_4 polynucleotide kinase (Boehringer, Mannheim, West Germany). The DNA was phenol-extracted and precipitated by adding 2 vols ethanol. Labelled fragments, redigested with another restriction enzyme, were subjected to chemical modification (20) using the alternate G, alternate A, C and C + T degradation and fractionation of 8%, 12% or 20% acrylamide, 8 M urea gels (32).

Electron microscopy

Electron microscopy was carried out as described (33) using single stranded ØX174 as a size marker.

RESULTS

Isolation and characterisation of cloned μ cDNA

The cell line used for mRNA isolation was the hybrid cell line Sp1/HL which secretes an anti-sheep red blood cell antibody (anti-SRBC) of the IgM type (16). These cells have lost production of the myeloma-derived immunoglobulin heavy and light chains and only produce the μ and L chain products derived from the spleen cell. A semi-purified mRNA fraction was prepared from microsomes of Sp1/HL and double stranded complementary DNA (cDNA) (34) prepared with the modifications described in Materials and Methods. A poly (dA) tail was added to the double stranded cDNA with terminal transferase and annealed with pBR322 plasmid to which a poly(dT) tail had been added at the BamHI cleavage site. The recombinant plasmid molecules obtained were used to transfect E. coli χ1776. Since pBR322 loses resistance to tetracycline when foreign DNA is inserted at the BamHI site, selection for transformed cells was carried out by plating on ampicillin medium. Replica plating onto tetra-

cycline plates identified clones carrying inserted DNA (i.e. tetracycline-sensitive). These colonies were screened for cDNA inserts by colony hybridisation (23) using ^{32}P-labelled cDNA derived from Sp1/HL H mRNA which had been purified on a methyl mercury hydroxide agarose gel (26). A number of positive colonies were identified in this way and two clones were characterised further (designated pμ/107 and pμ/118). The presence of sequences in these clones complementary to μ H chain was confirmed by the ability of the plasmid DNA to inhibit the translation of the μ mRNA in a reticulocyte cell free system (29).

The length of the μ H chain cDNA inserts and the area of the mRNA included in the inserts of the two plasmids was determined by a combination of restriction enzyme mapping, filter hybridisation, nucleotide sequencing and, in the case of pμ/107, by electron microscopy. The plasmids were digested with HpaII, HaeII, HaeIII, EcoRI, BglII and TaqI and the mobilities of the resulting fragments compared to similar digests of pBR322. This analysis enabled us to distinguish fragments originating from the immunoglobulin cDNA insert and those originating from the pBR322. In order to deduce the relative arrangement of the restriction fragments, the nucleotide sequence of various fragments was determined and aligned to protein sequence of mouse μ chain (J.D. Capra, personal communication). These experiments allowed us to produce the partial restriction maps of pμ/107 and pμ/118 shown in Fig. 1.

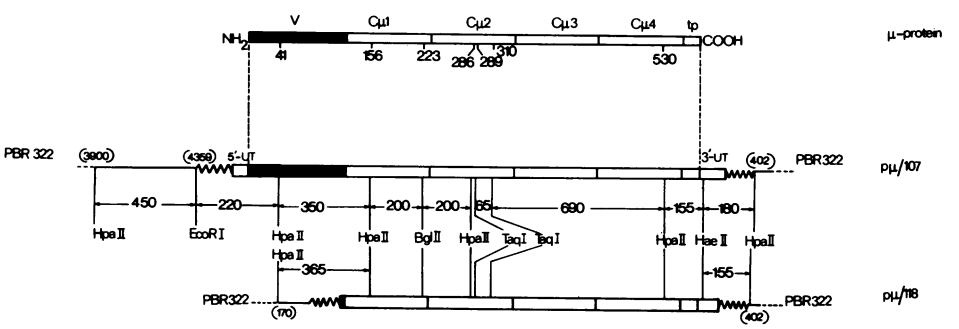

Figure 1. Restriction map of recombinant plasmids pμ/107 and pμ/118. The top line shows the schematic drawing of the secreted μ polypeptide chain (μ_S). Numbers relate to the amino acid position for relevant restriction sites of the recombinant plasmids pμ/107 and pμ/118. The homology region boundaries have been assigned by three-dimensional structure analysis (5). The two bottom lines show the schematic representation of the extent of μ mRNA sequence inserted in pμ/107 and pμ/118 recombinant plasmids. Restriction enzyme sites are indicated at the equivalent amino acid of the μ protein and the approximate distances (in base pairs) between restriction sites are shown. Numbers in parenthesis indicate pBR322 nucleotide positions (35). The poly(A/T) tails are indicated by ∧∧∧. UT: untranslated region.

One feature of pμ/107 which emerged from the restriction mapping data was the occurrence of a deletion of part of the pBR322 sequence, in pμ/107, between the EcoRI site [pBR322 nucleotide position 4359 (35)] and the BamHI site (pBR322 position 375). This deletion of pBR322 sequences was confirmed by heteroduplex mapping of pμ/107 with pBR322 (Fig. 2). pBR322 and pμ/107 were digested with SalI (an enzyme which cleaves pBR322 at position 650 but does not cleave within the H chain cDNA insert) and heteroduplexes were produced. Typical molecules showed the presence of four major regions: firstly a long duplex region "d" (about 3.6 kb) extending from the SalI site (pBR322 (position 650) to the beginning of the region deleted from pμ/107, whilst single stranded loop "c" represents the H chain cDNA inserted into the clone. Finally the duplex region "a" consists of the complementary portions of pBR322 and pμ/107 extending from the BamHI site (pBR322 position 375) to the SalI cleavage site (pBR322 position 650). The total length of the inserted DNA, including the homopolymeric tails, was estimated to be 2155 bases (± 130) relative to single stranded ØX174.

Nucleotide sequence of the region corresponding to $C_\mu 4$, tailpiece and part of the 3' untranslated region

The restriction mapping data revealed the presence of HpaII fragments, derived from the ends of the cDNA inserts of both pμ/107 and pμ/118, including the homopolymer tails used for the insertion of the double stranded DNA. The 335 base pair fragment of pμ/107 has been sequenced by the method of Maxam and Gilbert (20) and the nucleotide data is shown in Fig. 3. The strategy of the sequence analysis was to label the 5' end of the HpaII fragment with polynucleotide kinase followed by cleavage with HaeII to

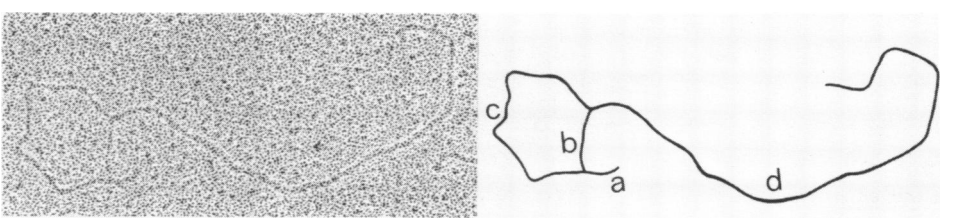

Figure 2. Electron micrograph of heteroduplex between pμ/107 and pBR322. Heteroduplex mapping was carried out between SalI cleaved pμ/107 and SalI cleaved vector pBR322 as previously described (33). The interpretative tracing (see text for full explanation) shows areas of duplex between pμ/107 and pBR322 (a,d) and single stranded region c (immunoglobulin cDNA insert) and b (pBR322 stretch deleted from pμ/107). Size measurements were made versus circular single stranded ØX174 assuming a size of 5375 bases (36).

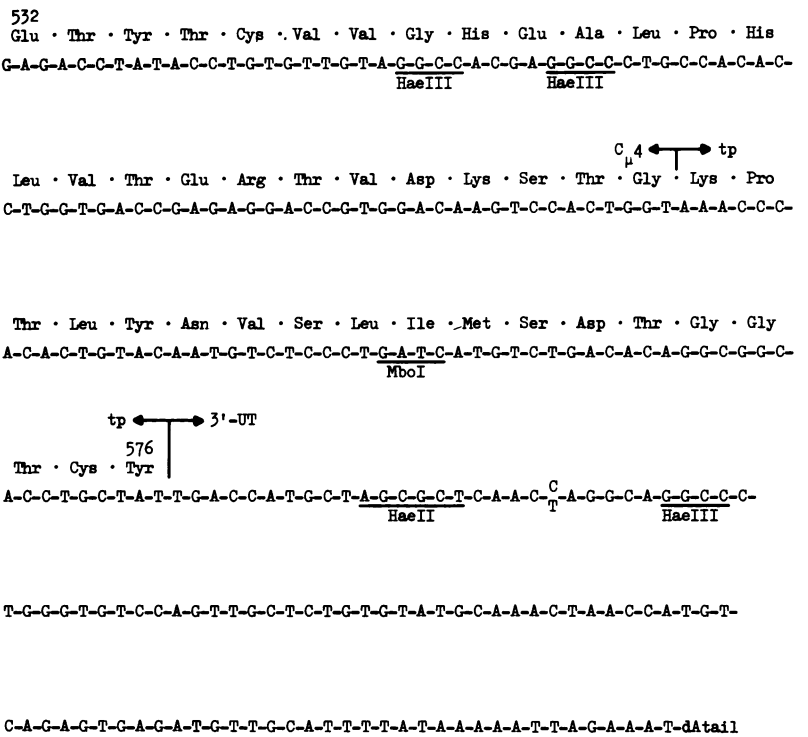

Figure 3. Partial nucleotide sequence of the coding strand of secreted μ chain extending from $C_{\mu}4$ into the 3' untranslated region. Boundaries between the last constant region domain $C_{\mu}4$ and the COOH terminal tailpiece (tp), and between tailpiece and 3' untranslated region (3'-UT) are indicated. The dA tail is the homopolymer tail added enzymatically to the ds cDNA and is not equivalent to the poly(A) of the mRNA. Cleavage sites for some restriction endonucleases are underlined. The residue at 21 bases from the Tyr codon may be C or T [unidentified since this is a methylated position (37)].

generate two fragments (155 and 180 base pairs) which were isolated from polyacrylamide gels and chemically degraded using the alternate G, alternate A, C and C + T degradation. Fractionation of the resulting mixtures was carried out on 8% and 20% polyacrylamide gels in the presence of 8 M urea (32). These fractionations enabled us to read the sequence from the amino acid codon 532 (glutamic acid) up to the start of the 3' untranslated region, and from the A residue 19 bases from the amino acid codon 576 (tyrosine) across the homopolymeric dA tails into the pBR322. The remaining sequence around the tailpiece and 3' untranslated region was obtained by digesting the 335 bp HpaII fragment with MboI, end-labelling and cleavage

with HaeIII followed by chemical degradation of the MboI-HaeIII fragment (59 base pairs). The comparison of our data with the published amino acid sequence of a secreted μ chain derived from the mouse myeloma tumour MOPC 104E (4) shows exact correspondence, except for a histidine-glutamic acid change at amino acid position 545. We also found a termination codon (UGA) adjacent to the tyrosine codon which is the final residue of the secreted mouse μ protein (amino acid 576). Sequence analysis of pμ/118 from the codon corresponding to amino acid 547 showed an identical sequence to pμ/107. The length of the 3' untranslated region which we found in clone pμ/107 consists of 105 bases (including the UGA). At the present time we do not know whether this encompasses the whole of the mRNA 3' untranslated region.

DISCUSSION

It seems likely that at least one facet of the change from membrane to secreted IgM is the presence of an extra polypeptide segment in the former (absent from the latter) probably at the C terminus (11,13,14,38). The occurrence of an extra peptide segment in two apparently otherwise identical proteins is intriguing and may occur by one of several mechanisms. However, the information present in the mRNA for μ_m and μ_s could be precisely the same but yielding the different peptides by translational or post-translational mechanisms. One possibility is that the initial transcription products in secreting and nonsecreting cells are identical, but the μ_s is generated by proteolytic degradation of the C terminal extra peptide (14). Alternatively the μ_m component could originate by tRNA suppression of a termination codon adjacent to the C terminus of μ_s and utilisation of a different terminator in the so-called untranslated region (39-41).

We have determined the nucleotide sequence at the 3' end (spanning $C_\mu 4$, tailpiece, plus about 100 residues of the 3' untranslated region) of two randomly selected cDNA clones. Both sequences are identical, and consist of continuous nucleotides in agreement with the published amino acid sequence (4) except at one internal position (Fig. 3). The most important feature of this sequence is the presence of a UGA termination codon adjacent to the final (tyrosine) codon of secreted μ. The μ_s polypeptide would, therefore, arise from chain termination at the UGA and the initial translation product would not possess an extra polypeptide at the C terminus to be subsequently removed. Furthermore, no extra sequence was found between the $C_\mu 4$ and the tailpiece sequence which might code for a peptide inclusion in this region. The possibility remains that a proportion of the μ mRNA in Sp1/HL cells is of the

μ_m type, and this mRNA might therefore not have been represented in the recombinant clones we have analysed even though two independent clones possess the same nucleotide sequence.

It is still conceivable that the UGA codon, of the sequences we have obtained, does not function as a chain terminator since evidence is accumulating that suppressor tRNAs exist in eukaryotes which allow read-through of terminators (39-41). In this respect it has been reported that an extra peptide of about 20 residues can be detected at the C terminus of the L chain from MOPC 321 when this mRNA is translated in a cell free system (42). This extra peptide possibly results from suppression of the initial chain termination of L chains and read-through of the 3' untranslated region to a UAA terminator at 21 codons from the terminator (43). The nucleotide sequence shown in Fig. 3 argues against a similar situation having a physiological role in formation of μ_m. We find that a UAG termination codon occurs two codons from the UGA and the only other termination codons in the correct phase are repeats of UGA (25 codons from the initial UGA) and of UAG (33 codons from the initial UGA). Suppression of UGA and UAG termination codons therefore seems very unlikely to occur in this mRNA. Thus it seems most probable that an extra peptide present in μ_m will have its origin in an extra nucleotide stretch within the μ_m mRNA. One possible site for creation of the difference between μ_m and μ_s mRNA might be at the level of nuclear RNA processing. It is thought that RNA splicing events occur in the formation of both L chain mRNA (44,45) and H chain mRNA (46) so that differential splicing routes could give rise to an additional nucleotide stretch in an otherwise identical sequence originating from a unique DNA sequence.

CONTAINMENT

All work involving recombinant DNA was carried out according to the GMAG guidelines for recombinant DNA, i.e. disabled host-vector biological containment (χ1776, pBR322) and Category III physical containment (Category III facility of the Biochemistry Department, Imperial College of Science and Technology, London). Non-disabled biological containment (HB101) and Category II physical containment were used in work involving plasmids pμ/107 and pμ/118 after characterisation of these clones.

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