Amino Acid-Sequence Variability at the *N*-Terminal Extra Piece of Mouse Immunoglobulin Light-Chain Precursors of the Same and Different Subgroups

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The proteins programmed in the wheat-germ cell-free system by the mRNA coding for the MOPC-63 mouse myeloma L (light) chain were labelled with six radioactive amino acids: [³⁵S]methionine, [4,5-³H]leucine, [3,4-³H]proline, [3-³H]serine, [4,5-³H]isoleucine or [2,3-³H]alanine. Amino acid-sequence analyses showed that over 90% of the total cell-free product was one homogeneous protein, which corresponds to the MOPC-63 L-chain precursor. In this precursor an extra piece, 20 amino acid residues in length, precedes the *N*-terminus of the mature L chain. The extra piece contains one methionine residue at the *N*-terminus, six leucine residues, which are clustered in two triplets at positions 6, 7, 8 and 11, 12, 13, one proline residue at position 16, and one serine residue at position 18. The closely gathered leucine residues, as well as their abundance (30%), suggest that the extra-piece moiety is hydrophobic. In the precursors, the extra piece is coupled to the variable region of the L chain. Partial sequences of precursors of L chains of the same and different subgroups that were labelled with the above six radioactive amino acids indicate that the extra piece is part of the variable region. Thus the precursors of MOPC-63 and MOPC-321 L chains, which are of the same subgroup, have extra pieces of identical size (20 residues), and so far their partial sequences are also identical (see above). On the other hand, in the precursor of MOPC-41 L chain, which is of a different subgroup, the extra piece is 22 residues in length. Further, the sequence of the MOPC-41 extra piece differs in at least ten positions from sequences of the extra pieces of the precursors of MOPC-63 and MOPC-321 L chains.

It has been repeatedly shown that the mRNA species coding for a variety of Ig (immunoglobulin) L (light) chains direct the synthesis of precursors that are larger than the mature L chain (Milstein *et al.*, 1972; Swan *et al.*, 1972; Mach *et al.*, 1973; Tonegawa & Baldi, 1973; Schmeckpeper *et al.*, 1974; Green *et al.*, 1975). We have shown that in the L-chain precursor an extra piece precedes the *N*-terminus of the mature protein (Schechter, 1973), i.e. the extra piece is coupled to the V (variable) region of the L chain. This finding immediately raised the question whether the extra piece is part of the V region or whether it represents a new constant polypeptide segment of the Ig chain. This issue can be resolved by sequencing the precursors of L chains of the same and different subgroups. The MOPC-63, MOPC-321 and MOPC-41 mouse myeloma L chains are of the κ -type and they share identical constant regions. The MOPC-63 and MOPC-321 L chains are of the same subgroup and differ at the V region only in eight out of 111 amino acid residues (McKean *et al.*, 1973). The MOPC-41 L chain (Gray *et al.*, 1967) is of a different subgroup and differs from MOPC-63 V region in 51 positions

(McKean *et al.*, 1973). We have previously shown that the precursors of MOPC-321 (Schechter, 1973; Schechter *et al.*, 1975; Schechter & Burstein, 1976a) and MOPC-41 (Schechter & Burstein, 1976b) L chains contain extra pieces which differ in size and sequence. We here report the size and partial sequence of the precursor of MOPC-63 L chain that was synthesized with the aid of mRNA in the wheat-germ cell-free system. Despite the fact that the sequence data are incomplete, it is evident that the extra pieces in the precursors of MOPC-63 and MOPC-321 L chains (which are of the same subgroup) are rather similar, and these differ extensively from the sequence of the extra piece in the MOPC-41 L-chain precursor.

Experimental

Materials

The MOPC-63 and MOPC-321 myeloma tumours, kindly given by Dr. M. Potter (National Institutes of Health, Bethesda, MD, U.S.A.), were maintained as solid tumours in female BALB/c mice (Potter, 1967).

Commercial wheat germ was supplied by the Bar-Rav Mill, Tel-Aviv, Israel. [^{35}S]Methionine (170 Ci/mmol), [4,5- ^3H]leucine (53 Ci/mmol), [3,4- ^3H]proline (43 Ci/mmol), [3- ^3H]serine (15 Ci/mmol), [4,5- ^3H]isoleucine (26 Ci/mmol) and [2,3- ^3H]alanine (31 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The ten [^{14}C]-labelled amino acids (alanine, arginine, glycine, isoleucine, leucine, lysine, proline, serine, threonine, valine, 90–320 Ci/mol) were obtained from New England Nuclear, Boston, MA, U.S.A.

Immunoglobulin L-chain proteins

The L chains were isolated from the urine of mice bearing the MOPC-63 or MOPC-321 tumours, as described by Potter (1967). Only one band, with electrophoretic mobility corresponding to mol.wt. about 24000, was observed when the reduced proteins were analysed on sodium dodecyl sulphate/polyacrylamide gels (Maizel, 1972).

Antibodies to MOPC-63 and MOPC-321 L chains

Hyperimmune sera were prepared in goats injected with either MOPC-63 or MOPC-321 L chain (3 mg per injection) in complete Freund's adjuvant, as described by Schechter *et al.* (1966). Quantitative precipitin curves of the reactions between the antisera and the L chains were prepared by a standard procedure (Schechter *et al.*, 1966).

Immunoglobulin L-chain mRNA molecules

The mRNA coding for the MOPC-63 L chain was prepared from MOPC-63 myeloma polyribosomes specifically precipitated by antibodies to MOPC-63 L chain, followed by chromatography on oligo(dT)-cellulose (Schechter, 1974). The mRNA molecules coding for the MOPC-321 and MOPC-41 L chains were prepared by the same immune precipitation procedure, as previously described (Schechter, 1973; Schechter & Burstein, 1976b).

Cell-free synthesis of L-chain precursors

Translation of the L-chain mRNA molecules was carried out in the wheat-germ cell-free system (Roberts & Paterson, 1973) with 30 mM-KCl, 87 mM-potassium acetate and 3.3 mM-magnesium acetate. Reaction mixtures contained 1.0 μM -[^{35}S]methionine, 2.9 μM -[^3H]leucine, 3.2 μM -[^3H]proline, 4.5 μM -[^3H]serine, 4.5 μM -[^3H]isoleucine or 4.0 μM -[^3H]alanine or a mixture of ten ^{14}C -labelled amino acids (each at 2.7 μM), complemented respectively with 19 or 10 unlabelled amino acids (each at 30 μM). A standard reaction mixture (40 μl) contained 0.007–0.009 E_{260} unit of mRNA. Incubation was at 25°C for 4 h. Radio-

active material precipitated by 10% (w/v) trichloroacetic acid was collected on Millipore filters. The dried filters were transferred to 10 ml of toluene containing 0.4% (w/v) 2,5-diphenyloxazole and 0.005% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene. The radioactivity was determined in a Packard model 3300 liquid-scintillation spectrometer with counting efficiency of 25% for ^3H and 60% for ^{35}S .

Radioactive amino acid-sequence analyses

The total cell-free products that were labelled by one radioactive amino acid at a time were analysed in the Beckman model 890C Automatic Sequencer as detailed elsewhere (Schechter & Burstein, 1976a). Each sample contained 5 mg of sperm-whale apomyoglobin carrier. Repetitive yields (degradation yield in the individual cycle; Edman & Begg, 1967) of the protein carrier and of the radioactively labelled materials ranged between 93 and 95%. All samples were sequenced twice. The pattern of radioactive peaks in the duplicate was identical, and the absolute yields varied at most by 8%.

Results

Characterization of MOPC-63 L-chain protein and mRNA

The MOPC-63 and MOPC-321 L chains, which are of the same subgroup, have rather similar amino acid sequence (McKean *et al.*, 1973). We had therefore to ascertain that by mistake we were not studying the same L chain. It would have been rather difficult to distinguish between the MOPC-63 and MOPC-321 L chain precursors by sequence analyses, since this requires 47 degradative cycles. In the precursors 20 residues precede the N-terminus of the mature L chain (see below), and in the mature L chain the first amino acid difference occurs at position 27 (McKean *et al.*, 1973). However, the following experiments show that the myeloma tumours, from which the MOPC-63 and MOPC-321 mRNA species were prepared, were not identical. The L chains isolated from the urine of mice bearing the MOPC-63 and MOPC-321 tumours were different, since they elicited the formation of antibodies of different specificities (Fig. 1). Quantitative precipitin studies showed that antibodies induced by the MOPC-63 L chain reacted more readily with the homologous MOPC-63 L chain than with the MOPC-321 L chain. Cross-reaction with the heterologous MOPC-321 L chain was 85–90% (range from three assays). Conversely, anti-(MOPC-321 L chain) antibodies reacted more readily with MOPC-321 L chain than with the MOPC-63 L chain. In this system cross-reaction with the MOPC-63 L chain was 63–75% (range from seven assays). Also, the percentage of polyribosomes engaged in L-chain synthesis was different in these

EXPLANATION OF PLATE I

Radioautogram of sodium dodecyl sulphate/polyacrylamide gel of cell-free products programmed by MOPC-63 mRNA in the wheat-germ cell-free system

Total reaction mixtures labelled with ten ^{14}C -labelled amino acids were reduced and analysed on slab gels [13% (w/v) acrylamide, 16 V/cm, 1.5 h] as described by Maizel (1972). Results from reactions without (a) and with (b) added mRNA are given. Dots indicate the position of mature MOPC-63 L chain applied with the cell-free sample. Molecular-weight standards were ovalbumin (43 000), glyceraldehyde phosphate dehydrogenase (36 000), MOPC-63 L chain (23 920), myoglobin (17 200) and haemoglobin (15 500).

Fig. 1. Cross-precipitation reactions of anti-(MOPC-63 L chain) antibodies (a) and anti-(MOPC-321 L chain) antibodies (b)

E_{280} of solutions in 0.1 M-NaOH (1.0 ml) of immune precipitates obtained by the addition of MOPC-63 L chain (●) and MOPC-321 L chain (○) to 0.5 ml of antiserum from goats immunized with MOPC-63 L chain (a) or MOPC-321 L chain (b).

tumours. The fraction of polyribosomes specifically precipitated by anti-(L chain) antibodies (Schechter, 1974) was 6.6–7.7% and 16–17.5% (range from three assays in each case) of the total polyribosome population prepared from the MOPC-63 and MOPC-321 tumours respectively. These results show that the two tumours investigated were not identical, and we can assume with a high degree of confidence that they are the MOPC-63 and MOPC-321 mouse myelomas.

The MOPC-63 L chain mRNA effectively stimulated protein synthesis in the wheat-germ cell-free system when assayed with each of the radioactively labelled amino acids. The amount of trichloroacetic acid-precipitable radioactivity in the absence of mRNA was increased more than 12-fold when the mRNA was present in the cell-free extract. Gel electrophoresis of the total cell-free products (Plate 1) shows that the mRNA programmed the synthesis of several proteins, neither of which is of the same size as the mature MOPC-63 L chain (mol.wt. 23920, calculated from amino acid sequence; McKean *et al.*, 1973). The mol.wt. of the major component (which is

larger than the mature L chain) is about 25500. Other minor components ranged in size down to mol.wt. about 15500. The analyses quoted below show a distinct *N*-terminal sequence which accounts for over 90% of the total cell-free products. Therefore the vast majority of these proteins share the same *N*-terminus, and the differences in size probably originate from missing portions at the *C*-termini, owing to incomplete synthesis of the L chain. Detailed studies of the proteins programmed by L chain mRNA species in the Krebs II ascites (Schechter *et al.*, 1975) and wheat-germ (Schechter & Burstein, 1976b) cell-free systems have shown that this is indeed the situation. In these systems the total cell-free products, and several fractions isolated from them (the fractions ranged in size from 28700 to 15500 daltons), were analysed, and all of them were found to have an identical *N*-terminal sequence. These experiments strongly indicate that the size heterogeneity of the cell-free products is due to the initiation of mRNA translation at one point, with premature termination at several points (Schechter *et al.*, 1975; Schechter & Burstein, 1976b).

Sequence analyses of the precursor of MOPC-63 L chain

The patterns of radioactivity obtained from sequencer runs of the total cell-free products programmed by the MOPC-63 L chain mRNA (Fig. 2) are different from those expected for the mature L chain. Peaks of radioactivity occurred at the indicated cycles from sequencing of products labelled with the following amino acids: [^{35}S]methionine, cycle 1; [^3H]leucine, cycles 6, 7, 8, 11, 12, 13, 24, 31, 35; [^3H]proline, cycles 16, 28; [^3H]serine, cycles 18, 27, 30, 34; [^3H]isoleucine, cycle 22; [^3H]alanine, cycles 29, 32. However, after 20 degradative cycles the positions of amino acids in the cell-free product show perfect homology with the positions of these residues in the mature L chain (Fig. 3), i.e., isoleucine-22, leucine-24, serine-27, proline-28, alanine-29, serine-30, leucine-31, alanine-32, serine-34 and leucine-35 in the cell-free product match with isoleucine-2, leucine-4, serine-7, proline-8, alanine-9, serine-10, leucine-11, alanine-12, serine-14 and leucine-15 in the mature MOPC-63 L chain (McKean *et al.*, 1973). The probability that this pattern of amino acids occurs by chance is practically nil. We have calculated the probability that the XIXLXXSPASLAXSLX sequence occurs by chance, where X denotes amino acid residues other than I (isoleucine), L (leucine), S (serine), P (proline) or A (alanine). If the probability for each amino acid is 0.05, as it would be if all amino acids occurred at random, the result is 1.8×10^{-22} . These results establish that the MOPC-63 mRNA programmes the synthesis of a precursor in which an extra piece, 20 residues in length, is coupled to the *N*-terminus of the

Determination of the absolute yield (i.e. the fraction of radioactive protein being sequenced from the total radioactivity in the sample) was detailed elsewhere (Schechter & Burstein, 1976a). Briefly, the expected radioactivity per residue at cycle 1 was calculated by dividing the radioactivity in the sample by the number of amino acid residues in the precursor, after correction (which was less than 8%) for endogenous activity of the cell-free extract. Recovered radioactivity per residue at cycle 1 from sequencer runs of the [^3H]leucine and [^3H]serine-labelled products was determined by plotting semilogarithmically the radioactivity in the peaks (Fig. 2) versus the number of sequencer cycles, and extrapolation to cycle 1 (Smithies *et al.*, 1971). The absolute yields (recovered/expected) were 102 and 99% for the [^3H]leucine- and [^3H]serine-labelled products respectively. Yields from other samples, which did not contain enough radioactive peaks for reliable lines to be drawn in the semilogarithmic plots, were determined as follows. The expected radioactivity at a particular cycle was calculated from the expected radioactivity at cycle 1 (see above) and from the repetitive yield (Edman & Begg, 1967) determined for the apomyoglobin carrier analysed in the same sequencer run. The recovered radioactivity at a particular cycle is given. The absolute yields (recovered/expected) were 91, 98, 90 and 88% from [^{35}S]methionine-, [^3H]proline-, [^3H]isoleucine- and [^3H]alanine-labelled products respectively. The yields obtained from analyses of product labelled with six different radioactive amino acids are comparable and are close to the expected theoretical value, as they should be when one protein is being sequenced. From these results we conclude that the MOPC 63 L-chain precursor comprises over 90% of the total cell-free products.

Sequence analyses of the precursor of MOPC-321 L chain

Analyses of MOPC 321 L-chain precursor, labelled with [^3H]leucine (Schechter, 1973; Schechter *et al.*, 1975), [^3H]serine, [^{35}S]methionine (Schechter & Burstein, 1976a), [^3H]proline and [^3H]isoleucine (Schechter & Burstein, 1976b), have been reported. These experiments showed that the *N*-terminal extra piece of MOPC 321 L-chain precursor is 20 residues in length, and that it contains one methionine residue at the *N*-terminus, six leucine residues at positions 6, 7, 8, 11, 12 and 13, one proline residue at position 16, one serine residue at position 18 and no isoleucine. For efficient comparison with the data for the MOPC-63 L-chain precursor, we synthesized and sequenced MOPC-321 L chain precursor that was labelled with [^3H]alanine. After 37 degradative cycles of this precursor, we found alanine residues only at positions 29 and 32, with an absolute yield of 101% (results not shown). These residues match with alanine-9 and

alanine-12 in the mature MOPC-321 L chain (McKean *et al.*, 1973). It is also evident that the MOPC-321 L-chain extra piece does not contain an alanine residue, similarly to the MOPC-63 L-chain extra piece.

Sequence analyses of the precursor of MOPC-41 L chain

Analyses of MOPC-41 L-chain precursor, labelled with [^{35}S]methionine, [^3H]leucine, [^3H]proline and [^3H]isoleucine, have been reported (Schechter & Burstein, 1976b). These experiments showed that the MOPC-41 mRNA programmes the synthesis of two precursors, in which 22 or 20 residues precede the *N*-terminus of the mature MOPC-41 L chain. About 75% of the molecules have a long extra piece (22 residues); the remaining molecules have a short extra piece (20 residues). In the short extra piece two residues are missing from the *N*-terminus; in the other 20 positions the two extra pieces show perfect sequence homology. The long extra piece contains two methionine residues at positions 1 and 3, two proline residues at positions 6 and 18, one isoleucine residue, at position 9, and a quadruplet of leucine residues, at positions 13–16. For efficient comparison with the data of the MOPC-63-chain precursor, we synthesized and sequenced MOPC-41 L chain precursors that were labelled with [^3H]serine and [^3H]alanine. In the following, positions of the residues are numbered according to the long extra piece (22 residues). After 33 degradative cycles of [^3H]serine-labelled precursor we found serine residues at positions 29, 31 and 32, with an absolute yield of 85% (results not shown). These residues match with serine-7, -9 and -10 in the mature MOPC-41 L chain (Gray *et al.*, 1967), and the extra piece does not contain any serine residue. After 30 degradative cycles of the [^3H]alanine-labelled precursor we found major radioactive peaks at cycles 5 and 7 and a minor peak at cycle 3, with an absolute yield of 72% (Fig. 4). Thus the long extra piece of the MOPC-41 L chain precursor contains two alanine residues at positions 5 and 7. As expected, the radioactive peaks from the short extra piece occur at cycle 3 and the other peak is included in cycle 5. Accordingly, in the short extra piece the alanine residues occupy positions 3 and 5.

Discussion

The MOPC-63, MOPC-321 and MOPC-41 L chains are of the κ -type. They share an identical constant region, but differ at the V region. The MOPC-63 and MOPC-321 L chains are of the same subgroup and differ at the V region in only eight out of 111 amino acid residues (7%). The MOPC-41 L chain belongs to a different subgroup and differs extensively in the V region from both MOPC-63 (46%) and

Fig. 4. Radioactivity recovered at each sequencer cycle from the total cell-free products programmed by MOPC-41 mRNA, and labelled with [^3H]alanine

This mRNA directs the synthesis of two precursors in which 22 (major component) or 20 (minor component) residues precede the *N*-terminus of the mature L chain. The two *N*-terminal residues in the long precursor (22 residues in extra piece) are missing from the short precursor (20 residues in extra piece) (see Schechter & Burstein, 1976b). The sample analysed contained 61000 c.p.m. The major radioactive peaks in cycles 5 and 7 originate from the long precursor. The short precursor yields a minor radioactive peak at cycle 3 and the second minor peak is included in cycle 5. Cycle zero: see legend to Fig. 2.

MOPC-321 (48%) L chains (McKean *et al.*, 1973). To gain information on the relation between the *N*-terminal extra piece and the V region, which are linked in the precursor molecule, we compared the sequences of extra pieces of L chains of the same and different subgroups, which were labelled with the same six radioactive amino acids. Although the sequence data are incomplete (Fig. 5), it is evident that the extra piece of L chains of different subgroups (MOPC-41, compared with MOPC-63 and MOPC-321) differ in size and sequence (Table 1). On the other hand, the extra pieces in the MOPC-63 and MOPC-321 L chain precursors are of the same size, and they share identical sequences of methionine, leucine, proline and serine residues. The probability that the MOPC-63 and MOPC-321 L chain extra piece sequence indicated in Fig. 5 occurs at random is 6×10^{-16} . It seems therefore that the large sequence homology found at the V regions of L chains of the same subgroup is retained at the extra pieces of the precursors of these L chains. These findings strongly

Fig. 5. Comparison of the size and partial sequence of the *N*-terminal extra piece in the precursors of MOPC-63, MOPC-321 and MOPC-41 L chains. Data are based on analyses of precursors that were labelled with [^{35}S]methionine, [^3H]leucine, [^3H]proline, [^3H]serine, [^3H]isoleucine and [^3H]alanine. Sequences are lined up to give maximal homology with the *N*-terminal extra piece of the MOPC-63 precursor. The extra pieces of the two MOPC-41 L chain precursors are given (Schechter & Burstein, 1976b). Homologous regions are enclosed.

Table 1. *Sequence heterogeneity at the N-terminal extra piece of κ -type L-chain precursors*

Data referring to the extra piece represent minimal values, since they are based on partial sequences given in Fig. 5. Data for the V region are based on sequences of the mature L chains (McKean *et al.*, 1973). Amino acid substitutions are compared with MOPC-63 L-chain precursor. The extra pieces of the two MOPC-41 L-chain precursors are given (Schechter & Burstein, 1976b).

Precursor	Residues in extra piece	Amino acid substitutions in			
		Extra piece		V-region	
		No.	%	No.	%
MOPC-63	20				
MOPC-321	20	0	0	8/111	7
MOPC-41 (a)	22	10/22	46	51/111	46
MOPC-41 (b)	20	8/20	40	51/111	46

indicate that the *N*-terminal extra piece is part of the V region. At the genome level, these results indicate that the V-region gene is larger than hitherto realized.

Despite the fact that the extra pieces exhibit sequence variability, all of them contain large (and rare) clusters of leucines: two triplets in MOPC-63 and MOPC-321, a quadruplet in MOPC-41, and closely gathered five leucine residues in the extra piece of MOPC-104E L-chain precursor (Burstein & Schechter, 1976). This suggests that the extra-piece moiety would be quite hydrophobic. Indeed, although not all hydrophobic residues were analysed, in the MOPC-41 L-chain extra piece, 50% of the residues are already known to be hydrophobic (Fig. 5). Further, preliminary data on the sequence of MOPC-321 L-chain precursor labelled with several ^3H -labelled amino acids show that 70% or more of the extra piece is composed of hydrophobic residues. The marked hydrophobicity suggests that the role of the extra piece is to favour interaction of the precursor with the endoplasmic membranes or with the cell surface (or both).

The finding of *N*-terminal methionine in all precursors investigated (three precursors reported here; also the MOPC-104E L-chain precursor, Burstein & Schechter, 1976) suggest that these molecules represent the direct product of mRNA translations within the cell. This is based on the fact that there is no methionine in the position preceding the *N*-terminal residue of the mature L chain (Fig. 5), and that methionine is the initiator residue for protein synthesis in eukaryotes (reviewed in Schechter & Burstein, 1976a). Therefore translation of the L-chain mRNA is contingent on the integrity of the nucleotide sequence coding for the *N*-terminal extra piece. This segment is located at the 5' end of the mRNA, where mRNA translation is initiated. In agreement,

we have shown that L-chain mRNA molecules which are deficient at the 5' end are untranslatable in a cell-free system (Schechter, 1975).

The vast majority of precursor molecules synthesized within the cell should be processed to the mature protein (cleavage of the extra piece) rather quickly, because only mature L chains were isolated from myeloma tumours or body fluids (Svasti & Milstein, 1972; Schechter, 1973). Therefore the cleavage step(s) converting the precursor into the mature protein may provide a point of metabolic control to regulate L-chain secretion.

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