Initiation and Processing *in vitro* of the Primary Translation Products of Guinea-Pig Caseins

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1. Guinea-pig caseins synthesized in a mRNA-directed wheat-germ cell-free protein-synthesizing system represent the primary translation products, even though they appear to be of lower molecular weight when analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in parallel with caseins isolated from guinea-pig milk. 2. Identification of the *N*-terminal dipeptide of the primary translational product of caseins A, B and C and α -lactalbumin showed that all shared a common sequence, which was identified as either Met-Arg or Met-Lys. 3. Procedures utilizing methionyl-tRNA^{Met} or methionyl-tRNA^{Met} in the presence or absence of microsomal membranes during translation provide a rapid method of distinguishing between *N*-terminal processing of peptides synthesized *in vitro* and other post-translational modifications (glycosylation, phosphorylation), which also result in a change in mobility of peptides when analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 4. The results demonstrate that guinea-pig caseins, in common with most other secretory proteins, are synthesized with transient *N*-terminal 'signal'-peptide extensions, which are cleaved during synthesis in the presence of microsomal membranes.

Most, though not all, secretory proteins (see Palmiter *et al.*, 1978a) are synthesized with transient *N*-terminal signal sequences. The signal sequences are generally removed during protein synthesis, a membrane-requiring event (Blobel & Dobberstein, 1975a,b; Palmiter *et al.*, 1978b; Craig *et al.*, 1979). In addition to processing, cell-free synthesis in the presence of microsomal membranes is also coupled to core glycosylation (Rothman & Lodish, 1977; Lingappa *et al.*, 1978a,b).

Both proteolytic cleavage and glycosylation of peptides may give rise to a change in mobility of peptides, when analysed by SDS/polyacrylamide-gel electrophoresis (see Craig *et al.*, 1976). Although the glycosylated peptides can be identified by using lectin columns (Lingappa *et al.*, 1978a,b), the identification of transient *N*-terminal signal sequences is dependent on definitive identification of the primary translation products by using sophisticated micro-sequencing techniques (see Kemper *et al.*, 1976).

We have previously demonstrated (Craig *et al.*, 1976) that the guinea-pig milk protein α -lactalbumin is synthesized as a high-molecular-weight precursor polypeptide modified at the *N*-terminus in the wheat-germ cell-free system, and that this may be

processed to α -lactalbumin by the Krebs-II ascites-cell-free system, a membrane-requiring event. However, a similar analysis of the translation products of guinea-pig caseins synthesized in the wheat-germ cell-free protein-synthesizing system revealed peptide products of apparently lower molecular weight than those isolated from guinea-pig milk (Craig *et al.*, 1976). Guinea-pig caseins are heavily phosphorylated, and to a lesser extent glycosylated (Craig *et al.*, 1978). It seems likely that the presence or otherwise of these groups may well contribute to the anomalous mobility observed on polyacrylamide gels of the polypeptides synthesized *in vitro*.

In the present paper we describe rapid procedures utilizing formylated and unformylated [³⁵S]methionine donated by initiator tRNA which demonstrate that guinea-pig milk proteins, in spite of this anomalous mobility, are synthesized as the primary translational products in the wheat-germ cell-free system. Moreover, the caseins, like α -lactalbumin, are synthesized with transient *N*-terminal peptide extensions, which are processed in the presence of added membrane fractions.

The incorporation of formylmethionine from initiator tRNA has been used to study the initiation of protein synthesis in several eukaryotic systems (Housman *et al.*, 1970; Smith & Marcker, 1970; Smith, 1973). The procedure has proved particularly useful in experiments designed to determine whether

Abbreviation used: SDS, sodium dodecyl sulphate.

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certain viral proteins result from initiation of protein synthesis from single sites on individual mRNA species, or are derived from post-translational cleavage of larger polypeptides (Clegg & Kennedy, 1975; Glanville *et al.*, 1976; Siddell & Smith, 1978).

Experimental

Preparation of solutions and chemicals

All solutions were prepared with double-distilled water. Acrylamide and *NN'*-methylenebisacrylamide were recrystallized in chloroform and acetone respectively, as described by Loening (1967). *NNN'*-Tetramethylethylenediamine was redistilled *in vacuo* immediately before use.

Materials

Unless specifically stated otherwise, all chemicals and solvents were obtained from sources previously described (Craig *et al.*, 1976, 1978, 1979; Glanville *et al.*, 1976). L-[³⁵S]Methionine (700–1350 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

mRNA isolation and cell-free protein synthesis

The isolation of poly(A)-containing RNA species from the lactating guinea-pig mammary gland has been described in detail elsewhere (Craig *et al.*, 1976, 1979). mRNA-directed protein synthesis in the wheat-germ cell-free system was carried out in assay volumes of 50 μ l–1.0 ml for 75 min at 21°C essentially as described previously (Craig *et al.*, 1976), except that either L-[³⁵S]methionine (80 μ Ci/ml, 800–1350 Ci/mmol) or *N*-formyl[³⁵S]methionyl-tRNA^{Met} (5×10^7 c.p.m./ml) or [³⁵S]methionyl-tRNA^{Met} was used as the radiolabelled precursor. In the presence of initiator tRNA, unlabelled methionine (4 μ M) was also included in the assay as a source of internal methionine residues. Dog pancreas microsomal membranes were prepared as described by Blobel & Dobberstein (1975a).

Preparation of *N*-formyl[³⁵S]methionyl-tRNA^{fMet}, [³⁵S]methionyl-tRNA^{fMet} and [³⁵S]methionyl-tRNA^{Met}

tRNA^{fMet} and tRNA^{Met} were prepared from total wheat-germ tRNA by chromatography on BD-cellulose (benzoylated DEAE-cellulose) as described by Smith & Marcker (1970). tRNA^{fMet} was charged with [³⁵S]methionine by using highly purified *Escherichia coli* methionyl-tRNA synthetase (Smith, 1973; Mellor & Smith, 1978), whereas tRNA^{Met} was charged by using crude wheat-germ synthetase (Siddell & Smith, 1978). Methionyl-tRNA^{fMet} was charged and chemically formylated as described by Glanville *et al.* (1976).

Immunoprecipitation and polyacrylamide-gel electrophoresis

Immunoprecipitation was carried out as described by Craig *et al.* (1976) by using a mixture of antisera raised in rabbits against caseins and α -lactalbumin (10 μ l/ml of initial assay) and the antibody-antigen complex recovered after precipitation with goat anti-(rabbit immunoglobulin G) antiserum (200 μ l/ml of initial assay).

For qualitative analysis these were dissolved in sample buffer and used directly for polyacrylamide-slab-gel electrophoresis as described elsewhere (Craig *et al.*, 1979) by the procedure of either Weber *et al.* (1972) or Laemmli (1970) and then fluorographed (Bonner & Laskey, 1974). When labelled proteins were to be eluted from polyacrylamide gels for subsequent peptide analysis, the total antibody precipitate was reduced, and then carboxamidomethylated in the presence of urea as described by Crestfield *et al.* (1963), except that iodoacetamide was used as the alkylating agent. The position of the peptides was determined by radioautography of the wet gel at 4°C.

'Fingerprinting' of labelled peptides

Proteins labelled with [³⁵S]methionine were eluted from polyacrylamide-gel slices by vigorous shaking at 37°C overnight in 50 mM-NH₄HCO₃ containing 0.1 % (w/v) SDS and 0.1 % (v/v) 2-mercaptoethanol, and the eluted protein was recovered and oxidized with performic acid in the presence of 100 μ g of the appropriate carboxamidomethylated milk protein as carrier (see Smith *et al.*, 1978). The recovered protein was then digested overnight with trypsin and a two-dimensional analysis of the peptide products performed on 20 cm $\times$ 10 cm cellulose-coated flexible poly(ethylene terephthalate) supports as described by Craig *et al.* (1978). [³⁵S]Methionine-containing peptides were detected after radioautography at room temperature for 1–6 days by using Fuji Rx X-ray film.

For further analysis of radiolabelled peptides, the plates were incubated for 2 h in an atmosphere of H₂O₂ and performic acid (1 : 20, v/v), the areas of cellulose corresponding to the radiolabelled peptides removed, and the peptides extracted with 1.0 ml of 0.1 M-HCl for 10 min. Cellulose particles were removed by centrifugation at 1000 *g*_{av} for 5 min at 20°C, and the supernatants freeze-dried. The extracted peptides were then either redissolved in 20–40 μ l of water and re-analysed directly, or subjected to Pronase digestion (140 μ g of Pronase/ml, for 2 h at 37°C) before further two-dimensional analysis. The dipeptide fMet-[¹⁴C]Arg was prepared as described by Smith (1973).

Results

Poly(A)-containing RNA isolated from the post-nuclear supernatant of the lactating guinea-pig

mammary gland directs the almost exclusive synthesis in the wheat-germ cell-free system of caseins A, B and C and α -lactalbumin polypeptides, as determined by antibody precipitation of [35 S]methionine-containing peptides synthesized *in vitro* (Fig. 1). Moreover, if *N*-formyl[35 S]methionyl-tRNA^{fMet} replaces [35 S]methionine as the radio-labelled precursor, in spite of a decreased incorporation of the label (2–3 %, compared with 35–40 % of the free amino acid), analysis of the products synthesized *in vitro*, by immunoprecipitation and gel electrophoresis, reveals a pattern identical with that observed with the free amino acid alone (Fig. 1).

These observations suggest that the labelled polypeptides represent the primary translation products of caseins A, B and C and α -lactalbumin, in spite of the anomalous mobility of the caseins when compared with the secreted proteins isolated from guinea-pig milk.

In order to justify this conclusion, we have characterized the *N*-terminal peptides of the primary translation products of [35 S]formylmethionine-

Fig. 1. Initiation of mRNA-directed milk-protein synthesis *in vitro* in a wheat-germ cell-free protein-synthesizing system. Cell-free protein synthesis with either [35 S]methionine or *N*-formyl[35 S]methionyl-tRNA^{fMet} as the radio-labelled precursor, antibody precipitation, SDS/gel electrophoresis in 10 % polyacrylamide gels (Weber *et al.*, 1972) and fluorography were carried out as described in the Experimental section. The relative electrophoretic mobilities of the authentic caseins and α -lactalbumin isolated from guinea-pig milk are indicated.

labelled caseins A, B and C and α -lactalbumin, synthesized on a large scale in the wheat-germ cell-free system. The radiolabelled milk proteins were recovered from the cell-free system by using antibody precipitation and the polypeptides then separated on preparative SDS/polyacrylamide slab gels essentially as described in the legend to Fig. 1 (results not shown). The individual peptides were then extracted from the gel and digested with trypsin (see the Experimental section).

Analysis of the [35 S]formylmethionine-containing peptide 'fingerprints' obtained from each eluted milk protein (Figs. 2a, 2b, 2c and 2d), revealed that each contained the same two predominant labelled peptides, a result confirmed by mixing all four digests before fractionation (Fig. 2f). In addition to these two peptides, a third peptide was also apparent in the casein-B preparation (Fig. 2b). After further treatment with performic acid to ensure that all peptides were oxidized to the sulphone derivative (Clegg & Kennedy, 1975), peptides 1, 2 and 3 were extracted, and either (i) re-analysed directly or (ii) digested with Pronase and then re-analysed. Performic acid treatment demonstrated that spots 1 and 2 represent different oxidation states of the same peptide, peptide 1 now co-migrating with peptide 2 (Figs. 3a and 3c). Pronase digestion had no effect on the mobility of this peptide, whereas performic acid treatment did not affect the mobility of peptide 3 (Fig. 3d). Peptide 3 was not further characterized.

As the predominant formylmethionine-containing peptide was positively charged at pH 3.5 (see Figs. 2 and 3), it seemed reasonable to predict that it might contain either lysine or arginine. This appeared to be justified (Fig. 3b) as the mobility after two-dimensional analysis was similar to that of performic acid-treated dipeptide fMet-[14 C]Arg. As no fMet-Lys standard was available, we were unable in this experiment to identify definitively the common formylmethionine-containing peptide of guinea-pig caseins A, B and C and α -lactalbumin as either fMet-Lys or fMet-Arg.

However, since these experiments were performed, we have found, using the wheat-germ cell-free system, that if [35 S]methionyl-tRNA^{fMet} (unformylated) is added as the radiolabelled precursor, the *N*-terminal initiating methionine residue of the milk-protein-mRNA directed peptides is not removed. Isolation and tryptic digestion of these peptides followed by analysis in parallel with methionyl-lysine obtained from [Met, Lys]bradykinin showed the mobility of all the peptides to be identical after two-dimensional analysis (A. Mellor & A. E. Smith, unpublished work).

To determine whether or not the primary translational products of the caseins were synthesized with transient *N*-terminal peptide sequences, in an analogous manner to pre- α -lactalbumin (Craig *et al.*,

Fig. 2. Two-dimensional analysis of peptides labelled with formyl[^{35}S]methionyl-tRNA $^{\text{Met}}$ after mRNA-directed cell-free protein synthesis in the wheat-germ system

Radiolabelled milk proteins synthesized in the wheat-germ cell-free system (2 ml assay volume) in the presence of *N*-formyl[^{35}S]methionyl-tRNA $^{\text{Met}}$ were recovered by antibody precipitation, the antibody-antigen complex was reduced and carboxamidomethylated, and the peptides were separated on 10% SDS/polyacrylamide gels as described in the Experimental section. The radiolabelled peptides corresponding to the primary translation products of caseins A, B and C and α -lactalbumin were eluted, oxidized with performic acid, digested with trypsin (see the Experimental section), and the peptides

Fig. 3. Identification of the predominant formylmethionine-containing peptides

Peptides 1, 2 and 3 were extracted from the cellulose after performic acid treatment and re-analysed in parallel with an fMet-[^{14}C]Arg marker peptide. (a) Peptide 1, (b) fMet-[^{14}C]Arg, (c) peptide 2, (d) peptide 3. In practice (a) and (b) were spotted on the same cellulose thin-layer plate (see legend to Fig. 2), as were (c) and (d). The symbol $\times$ marks the point of application of each sample.

separated by electrophoresis in the first dimension and ascending chromatography in the second. (a) Casein A, (b) casein B, (c) casein C, (d) α -lactalbumin, (e) 1:1:1:1 mixture of all four peptide digests. Arrows mark the positions of the major radiolabelled peptides. In practice, caseins A and B (a and b) were spotted in parallel, one on the edge, and the other in the centre of a 20 cm $\times$ 20 cm cellulose thin-layer sheet. Electrophoresis was then performed simultaneously on both samples. The sheet was then divided into two, dried and separated in the second dimension by ascending chromatography, performed simultaneously in the same tank. The two strips were then re-aligned and radioautographed. This procedure was also followed for casein C and α -lactalbumin (c and d). Where a mixture of digests was analysed (e) to ensure maximum separation of the peptides in the second dimension, a single sample only was spotted on the edge of the cellulose sheet. The symbol $\times$ marks the point of application of each sample.

Fig. 4. *N*-Terminal cleavage of the primary translation products of guinea-pig caseins by a dog microsomal membrane fraction during mRNA-directed protein synthesis in the wheat-germ cell-free system

Guinea-pig milk proteins were synthesized in a mRNA-directed wheat-germ cell-free protein-synthesizing system, by using [35 S]methionine, [35 S]-methionyl-tRNA^{fMet} or [35 S]methionyl-tRNA^{Met} as the radiolabelled precursors. Synthesis was in the presence or absence of a dog microsomal membrane fraction. The [35 S]methionine-containing peptides were then separated on a SDS/polyacrylamide (15 %) gel (Laemmli, 1970). The relative electrophoretic mobilities of the authentic caseins and α -lactalbumin isolated from guinea-pig milk are indicated. Gels: (1) -mRNA, +[35 S]methionyl-tRNA^{fMet}; (2) -mRNA, +[35 S]methionyl-tRNA^{Met}; (3) -mRNA, +[35 S]methionine; (4) +mRNA, +[35 S]methionyl-tRNA^{fMet}; (5), as (4), +membranes; (6) and (7), as (4) and (5), but twice the radioactivity loaded on the gel; (8) +mRNA +[35 S]methionyl-tRNA^{Met}; (9), as (8), +membranes; (10) and (11), as (8) and (9), but with [35 S]methionine as the radiolabelled precursor.

1976), we have synthesized milk proteins in the wheat-germ cell-free system by using [35 S]methionine, [35 S]methionyl-tRNA^{fMet} or [35 S]methionyl-tRNA^{Met} as the radiolabelled precursors, each in the presence and absence of a dog pancreas stripped-microsomal-membrane preparation. The results (Fig. 4) demonstrate firstly a very marked change in distribution of those peptides with methionine residues donated by the free amino acid or methionyl-tRNA^{Met} as a result of added membranes (tracks 9 and 11). Processing of the primary translation product of casein C to a smaller peptide was particularly marked. However, the primary translation

products of caseins A and B, although not well separated by the Laemmli (1970) polyacrylamide-gel system, clearly underwent processing in the presence of microsomal membranes, as judged by the appearance of two additional peptides, one of slightly increased and the other of slightly decreased electrophoretic mobility. Examination of the fate of peptides labelled only at the *N*-terminal methionine by using [35 S]methionyl-tRNA^{fMet} but synthesized in the presence of microsomal membranes (tracks 5 and 7) demonstrated that the effect of membranes was to eliminate [35 S]methionine-labelled peptides corresponding to the processed casein polypeptides, results consistent with an *N*-terminal cleavage event as a result of adding microsomal membranes.

Throughout these experiments evidence for the processing of pre- α -lactalbumin was less evident, as, although these particular separation conditions (15 % polyacrylamide) revealed processing of the caseins, α -lactalbumin and pre- α -lactalbumin were not resolved. Moreover, the disparate mobility of the caseins isolated from guinea-pig milk when compared with those synthesized *in vitro* under different electrophoretic conditions (see Figs. 1 and 4), emphasizes the inconsistency that can result from molecular-weight estimations by using SDS/polyacrylamide-gel electrophoresis alone.

Discussion

Our observations using *N*-formyl[35 S]methionyl-tRNA^{fMet} as the radiolabelled precursor for cell-free protein synthesis, followed by extensive characterization of the resulting formylmethionine-containing peptides, demonstrate that, in spite of their anomalous mobility on SDS/polyacrylamide gels when compared with the purified milk proteins, each represents the primary translation product of the appropriate mRNA species.

Analysis of the formylmethionine-containing peptides identified either fMet-Arg or fMet-Lys as the *N*-terminal sequence of the primary translation products of caseins A, B and C and α -lactalbumin. Casein B exhibited some heterogeneity, in keeping with our previous observations (Craig *et al.*, 1978). Comparison of the known *N*-terminal amino acid of the proteins isolated from guinea-pig milk (Table 1) with those of the primary translation products demonstrates that the primary translation product of casein B, in addition to α -lactalbumin (known), is also synthesized with *N*-terminal amino acids (fMet-Arg or fMet-Lys) not present in the purified secreted protein. Similar comparison of the remaining peptides was less definitive, as all possess lysine as the *N*-terminal residue in the secreted form (see Brew, 1972; Craig *et al.*, 1978).

Identification of the formylmethionine-containing peptides is essential in all experiments involving

Table 1. Comparison of the amino acids found at the *N*-terminus of the primary translation products and the authentic guinea-pig milk proteins

	Casein A*	Casein B*	Casein C*	α -Lactalbumin†
<i>N</i> -Terminal amino acid of caseins and α -lactalbumin isolated from milk	Lys	Met	Lys	Lys
<i>N</i> -Terminal amino acid of the primary translation products	Lys/Arg	Lys/Arg	Lys/Arg	Lys/Arg

* From Craig *et al.* (1978).

† From Brew (1972).

formylmethionyl-tRNA^{Met} in eukaryote systems, as, although the presence of the formyl group prevents the removal of the *N*-terminal methionine residue after initiation of peptide synthesis (Houseman *et al.*, 1970), it is not a physiological initiator in eukaryote systems, and so competes poorly with endogenous initiator tRNA (Smith, 1973). Consequently, as we used wheat-germ tRNA as the starting material, it was important to ensure that incorporation was not due to small amounts of contaminating methionyl-tRNA^{Met} in the formylmethionyl-tRNA^{Met} preparation, resulting in the incorporation at internal methionine residues. An assessment of methionyl-tRNA^{Met} preparations showed them to be over 95% pure, as judged by analysis of the primary translation product of the SV40 capsid protein VP1, which has an *N*-terminal sequence of Met-Lys-Met. Peptide analysis of protein VP1 labelled *in vitro* by using [³⁵S]methionyl-tRNA^{Met} demonstrated that over 95% of the incorporated label was in the *N*-terminal methionine (A. Mellor, unpublished work).

Our observation that the unformylated initiating methionine was not removed from the nascent polypeptide chain was not unexpected, as sequencing studies of many different proteins have shown that *in vitro* the initiating *N*-terminal methionine is retained (Burstein *et al.*, 1977; Palmiter *et al.*, 1978b; Mellor & Smith, 1978).

Our experiments with a membrane-supplemented cell-free system, using [³⁵S]methionyl-tRNA^{Met} or the free amino acid, demonstrate that the addition of microsomal membranes results in the processing of all the milk-protein primary translation products. In these experiments we cannot exclude changes in electrophoretic mobility of the caseins, caused in part by other post-translational modifications, a supposition supported by reports of coupled cell-free synthesis, segregation and core glycosylation of integral membrane (Rothman & Lodish, 1977) and secretory proteins (Lingappa *et al.*, 1978a,b). However, our experiments, using methionyl-tRNA^{Met} in the presence and absence of microsomal mem-

branes, suggest that the addition of membranes results in a proteolytic cleavage at the *N*-terminus of all the caseins synthesized *in vitro*.

Interpretation of this result depends very much on the assumption that the initiating methionine, whether formylated or otherwise, is not removed by eukaryote aminopeptidases (Houseman *et al.*, 1970; Mellor & Smith, 1978), particularly in the present experiment by a membrane-associated enzyme. Evidence concerning the timing of this event is limited, but is consistent with a rapid removal of the initiator methionine. Thus it is removed from globin when the polypeptide is 15–20 residues long (Jackson & Hunter, 1970), and from ovalbumin when the nascent chain is 19–22 residues long (Palmiter *et al.*, 1978b). Ovalbumin is a secreted protein, which, although devoid of a transient *N*-terminal peptide (Palmiter *et al.*, 1978a), is sequestered in an identical manner to those proteins containing such a sequence (Lingappa *et al.*, 1978a). Thus it seems reasonable to speculate that *N*-terminal specific methionyl aminopeptidases capable of removing the initiating methionine are not present in the membrane preparation, as calculations suggest that attachment of the nascent polypeptide chain of secretory proteins to the endoplasmic reticulum occurs after the addition of at least 40–50 residues (Rothman & Lodish, 1977; Palmiter *et al.*, 1978b; Craig *et al.*, 1979). These observations tend to preclude any possibility that the evidence that we have presented represents only the removal of the initiating methionine residue from guinea-pig caseins as a result of added microsomal membranes.

We conclude that, in spite of the anomalous electrophoretic mobility on a variety of SDS/polyacrylamide-gel systems of the caseins synthesized *in vitro* when compared with their counterparts isolated from guinea-pig milk (see Craig *et al.*, 1976, 1978, 1979; Zehavi-Willner & Lane, 1977), it is apparent that the primary translation products of guinea-pig caseins, like α -lactalbumin, are synthesized with transient *N*-terminal peptide extensions of undetermined length, and that these are removed when

synthesis occurs in the presence of added microsomal membranes, an observation consistent with current concepts concerning the segregation of secretory proteins (Blobel & Dobberstein, 1975a,b). The rationale behind our approach has been substantiated by reports from several laboratories which demonstrate, by sequence analysis of milk proteins synthesized *in vitro* in the absence of membrane preparations, that rat α -lactalbumin (Lingappa *et al.*, 1978b), sheep α -lactalbumin (Mercier *et al.*, 1978a), β -lactoglobulin (Mercier *et al.*, 1978b), α_{s1} -, α_{s2} -, β - and κ -caseins (Gaye *et al.*, 1977) are all synthesized with *N*-terminal peptide extensions. It is also noteworthy that the *N*-terminal peptides were found to be Met-Lys for sheep β -lactoglobulin, α_{s1} -, α_{s2} - and β -caseins, Met-Arg for sheep κ -caseins, and Met-Met for sheep α -lactalbumin and rat α -lactalbumin.

Our approach therefore provides a simple rapid method that may be used to distinguish rapidly between proteolytic cleavage of transient *N*-terminal 'signal' sequences, and co-translational or post-translational modifications of secretory proteins, both of which may affect the mobility of the resulting peptides when analysed by SDS/polyacrylamide-gel electrophoresis. This conclusion is supported by similar experiments which have recently demonstrated in an identical manner that the glycoprotein (G) of vesicular stomatitis virus is synthesized with a nascent *N*-terminal peptide extension, which is removed during cell-free protein synthesis in the presence of membranes (Irving *et al.*, 1979).

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