Initiation of Synthesis of N-Terminal Acetylated Histones with Methionine†

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ABSTRACT: The studies presented were undertaken to clarify the unsettled question whether the synthesis of histones with a *N*-acetylserine residue at the amino terminal end is initiated with methionine. Histones were synthesized in vitro in a rabbit reticulocyte lysate, primed with a mRNA preparation from ascites cells. Initiation of polypeptide synthesis was investigated by using *N*-formyl[^{35}S]met-tRNA$_f^{Met}$ from yeast to label the *N*-termini. *N*-Formylmethionine was incorporated into histones H1 and H4 whose N-terminal amino acid is α-*N*-acetylserine. By comparison of tryptic peptides derived from these two histones labeled either with methionine or formylmethionine and from Edman degradation it is shown that N-terminal acetylated histones are initiated with methionine, as is the case for other eukaryotic and bacterial proteins.

It is generally accepted that the synthesis of eukaryotic proteins is initiated with methionine (Wilson and Dintzis, 1970; Housman et al., 1970; Smith and Marcker, 1970; Wigle and Dixon, 1970; Hunter and Jackson, 1971; Chatterjee et al., 1972) donated by methionyl-tRNA$_f^{Met}$, as is the case in bacteria. There is, however, still some controversy in the literature concerning the initiation of N-terminal acetylated proteins. It has been suggested that the synthesis of ovalbumin and histones is initiated with *N*-acetylglycine and *N*-acetylserine, respectively (Narita et al., 1969; Liew et al., 1970). On the other hand, the lens α-crystallin with a N-terminal acetylmethionine has been shown to become acetylated during its synthesis, but after initiation (Strous et al., 1973).

Three of the five major histones, H1, H2a, and H4, have a blocked N-terminal amino acid, namely *N*-acetylserine (Elgin and Weintraub, 1975). The N-termini of newly initiated polypeptides can be labeled in vitro with formyl[^{35}S]methionine donated by yeast methionyl-tRNA$_f^{Met}$ (Housman et al., 1970). This approach was recently used by Jacobs-Lorena and Baglioni (1973) to study the initiation of histone synthesis in an ascites cell-free protein synthesizing system. Although it was shown in their experiments by gel electrophoretic analysis that formylmethionine was incorporated into some of the major histone fractions, it was not demonstrated unequivocally that the N-terminal acetylated histones H1, H2a, and H4 were initiated with methionine.

We show in this paper by comparing tryptic peptides of in vitro synthesized histones labeled either with methionine or with *N*-formylmethionine that the synthesis of N-terminal acetylated histones H1 and H4 starts with methionine.

Material and Methods

Materials. Nucleoside triphosphates, creatine phosphate, creatine kinase, and tRNA from brewers yeast were obtained from Boehringer, Mannheim. [^{35}S]Methionine (sp act. 56 Ci/mmol) and L-[4,5-[^{3}H]lysine (sp act. 42 Ci/mmol) were purchased from New England Nuclear. Calcium-Leucovorin (calcium tetrahydroformylpteroylglutamate) was from Lederle; phenyl isothiocyanate, *N*-ethylmorpholine acetate, and trifluoroacetic acid were from Roth, Karlsruhe. Ribonuclease A was supplied by Serva, Heidelberg, and DCC-treated trypsin was from Sigma. All other chemicals were reagent grade from Merck, Darmstadt.

Preparation of Histone mRNA. Histone mRNA was prepared from logarithmically growing mouse ascites tumor cells kindly provided by Dr. P. Traub, Max-Planck-Institut für Zellbiologie, Wilhelmshaven. Cells were grown in suspension culture in Eagle's minimum essential spinner medium supplemented with 5% calf serum at a concentration of 5–7 × 10^5 cells/ml. Polyribosomes were prepared as described previously (Gallwitz and Mueller, 1969; Breindl and Gallwitz, 1973); they were suspended in 50 mM Tris-Cl, pH 7.5, 0.1 M NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate, and the RNA was extracted at room temperature with an equal volume of buffer-saturated phenol. A second extraction of the aqueous phase was performed with phenol–chloroform (1:1, v/v) and the RNA precipitated with 2 volumes of ethanol at −20 °C for 12 h. Fractionation of the polyribosomal RNA on sucrose gradients was performed as described (Gallwitz and Breindl, 1972), and the 5–10S RNA recovered from gradients was passed over poly(U)[1]–Sepharose as previously described (Gallwitz, 1975).

Preparation of N-Formyl[^{35}S]methionyl-tRNA. Aminoacyl synthetases and transformylase were prepared from *Escherichia coli* according to the procedure described by Marcker (1965). After eluting the tRNA charging enzymes from DEAE-cellulose with 0.1 M potassium phosphate, pH 7.4, and the transformylase with 0.3 M potassium phosphate, pH 7.4, the aminoacyl synthetases were concentrated by ultrafiltration using a Diaflo UM-2 membrane, and the transformylase by 65% ammonium sulfate precipitation. The concentrated enzyme fractions were dialyzed for 10 h against 20 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and stored in aliquots at −80 °C. tRNA$_f^{Met}$ was charged and formylated in one step by incubating 1 mg of brewers yeast tRNA with *E. coli* charging enzymes (0.85 mg of protein/ml) and trans-

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[1] Abbreviations used are: poly(U), poly(uridylic acid); DTT, dithiothreitol; CM, carboxymethyl; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

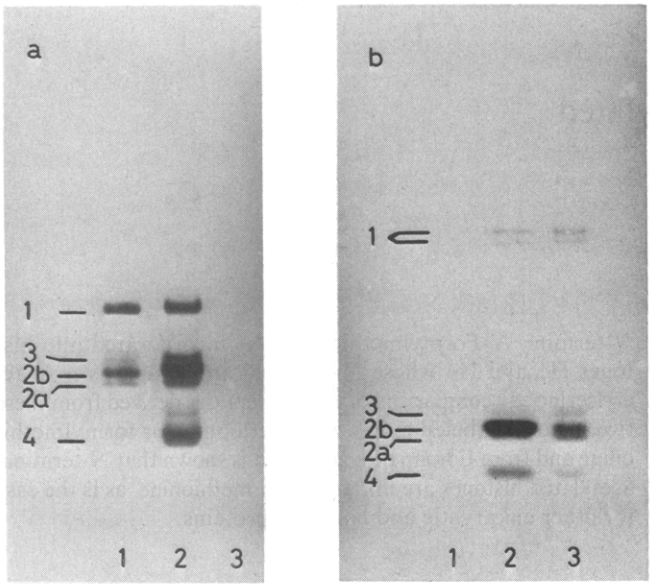

FIGURE 1: Histones synthesized in a rabbit reticulocyte lysate under the direction of ascites cell histone mRNA. In vitro synthesis was performed with [^{3}H]lysine (80 μCi/ml) as label, the acid-soluble proteins were extracted with 0.25 N HCl, and histones were separated from globin by CM-cellulose chromatography (Gallwitz and Breindl, 1972). In vitro synthesized histones and [^{14}C]lysine labeled marker histones were separated either on a 15% polyacrylamide slab gel containing 2.5 M urea (Panyim and Chalkley, 1969) (a) or on a 15% polyacrylamide-sodium dodecyl sulfate slab gel (Laemmli, 1970) (b). Radioactivity was detected by fluorography. (a) Slot 1: [^{14}C]lysine-labeled marker histone; slot 2: products of ascites mRNA; slot 3: products in the absence of exogeneous mRNA. (b) Slot 1: products in the absence of exogeneous mRNA; slot 2: products of ascites mRNA; slot 3: marker histones.

formylase (1.4 mg of protein/ml), 3 mM ATP, 5 mM $MgCl_2$, 25 mM Tris-Cl, pH 7.5, 0.5 mM DTT, 15 μg of calcium Leucovorin and 200 μCi of [^{35}S]methionine in a final volume of 0.45 ml. Incubation was performed at 36 °C for 20 min. The labeled tRNA was extracted from the incubation mixture with an equal volume of water-saturated phenol for 10 min at room temperature and the RNA was precipitated with 2 volumes of ethanol at −20 °C. To split the ester bond between unformylated methionine and tRNA the ethanol-precipitated RNA was treated with 10 mM $CuSO_4$, 0.2 M sodium acetate, pH 5.5, for 20 min at 37 °C (Schofield and Zamecnik, 1968), EDTA was added to a final concentration of 1 mM, and the RNA was passed over Sephadex G-25 fine equilibrated with 50 mM sodium acetate, 1 mM EDTA, pH 5.5. tRNA was precipitated three times from the same buffer solution with 2 volumes of ethanol. The extent of formylation was determined by hydrolyzing aminoacyl-tRNA for 60 min at 37 °C in 0.4 M NH_4OH, and high-voltage paper electrophoretic separation of [^{35}S]methionine and formyl[^{35}S]methionine on Whatman 3MM paper as described by Drews et al. (1972).

Cell-Free Protein Synthesis and Separation of Histones. Histones were synthesized in a rabbit reticulocyte lysate under the direction of histone mRNA, as described previously (Gallwitz and Breindl, 1972; Breindl and Gallwitz, 1973). In a final volume of 0.5 ml, 1.6 A_{260} units of mRNA and either 40 μCi of [^{35}S]methionine or 34.6 × 10^{6}cpm of formyl[^{35}S]-methionyl-tRNA$_f^{Met}$ were used. When proteins were synthesized with formylmethionine, cold methionine (50 μM final concentration) was included in the incubation mixture to exclude any possibility of incorporation of unformylated [^{35}S]-methionine. After completion of in vitro synthesis, incubations were continued for 10 min in the presence of EDTA (10 mM) and 20 μg/ml of RNase A. The incubation mixtures were then cooled in an ice bath and an equal volume of 0.5 N HCl was added dropwise under stirring to extract acid-soluble proteins for 30 min in the cold. After centrifugation for 10 min at 25 000*g*, the soluble proteins were precipitated with 10 volumes of acetone at −20 °C for 24 h. In vitro synthesized proteins were then fractionated on CM-cellulose, as described previously (Breindl and Gallwitz, 1973), to separate globin and histones. In vitro synthesized histones were mixed with 20 mg of calf thymus histones and fractionated on a 2.5 × 160 cm Bio-Gel P-10 column equilibrated with 0.01 N HCl. Aliquots were taken from the column fractions and radioactivity was measured in Bray's scintillation fluid (Bray, 1960). Fractions corresponding to individual histone fractions were pooled and lyophilized.

Tryptic Digestion and Edman Degradation. Tryptic digestion was performed at 37 °C for 6 h in 0.1 M ammonium bicarbonate, pH 8.0, at a substrate–enzyme ratio of 25:1 (w/w). After 3 h of digestion, the same amount of trypsin was added. The tryptic digestion was terminated by adding a drop of glacial acetic acid to the incubation mixture and the peptides were then lyophilized twice. For Edman degradation proteins were deformylated in 0.5 N HCl at 90 °C for 20 min (Housman et al., 1970) and lyophilized. Edman degradation was performed as described by Gilmour and Dixon (1972). Protein (2–2.3 mg) dissolved in 500 μl of 5% *N*-ethylmorpholine acetate, pH 9.3, was reacted under nitrogen with 500 μl of 5% phenyl isothiocyanate in pyridine at 45 °C for 90 min. After evaporation the residue was taken up in 500 μl of trifluoroacetic acid, incubated under nitrogen at 45 °C for 30 min, and again evaporated to dryness. The dried material was taken up in 1 ml of 0.5 N acetic acid and extracted twice with butyl acetate. The combined upper phases and the residue were dried, dissolved in 200 μl of formic acid, and the radioactivity was then measured in 15 ml of Bray's scintillation fluid. Corrections for different quenching were made by external standardization.

High-Voltage Paper Electrophoresis. Tryptic peptides after lyophilization were dissolved in a small volume of water and any insoluble material was removed by centrifugation. Peptides were separated by high voltage paper electrophoresis on Whatman 3MM paper in acetic acid–formic acid, pH 1.9, for 150 min at 38 V/cm. The dried electropherograms were cut into 0.5-cm strips and counted in a toluene-based scintillation fluid.

Polyacrylamide Gel Electrophoresis. Acid-soluble in vitro products were either dissolved in 6 M urea, 0.1 M 2-mercaptoethanol and separated on 15% polyacrylamide slab gels, containing 2.5 M urea (Panyim and Chalkley, 1969) or they were dissolved in 50 mM Tris-Cl, pH 6.8, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 10% glycerol, heated for 1 min at 90 °C, and separated on 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (Laemmli, 1970). Radioactivity was detected by fluorography (Laskey and Mills, 1975).

Results

To study the possible involvement of methionine in the initiation of N-terminal acetylated histones we have used the approach originally described by Housman et al. (1970) to label the N terminus with *N*-formylmethionine, which cannot be cleaved off the growing polypeptide chains in eukaryotic cell-free systems. Histones were synthesized in vitro in a rabbit reticulocyte lysate (Gallwitz and Breindl, 1972) under the direction of ascites cell histone mRNA using either [^{35}S]-methionine or *N*-formyl[^{35}S]methionyl-tRNA$_f^{Met}$. First it had to be demonstrated that the mRNA preparation used con-

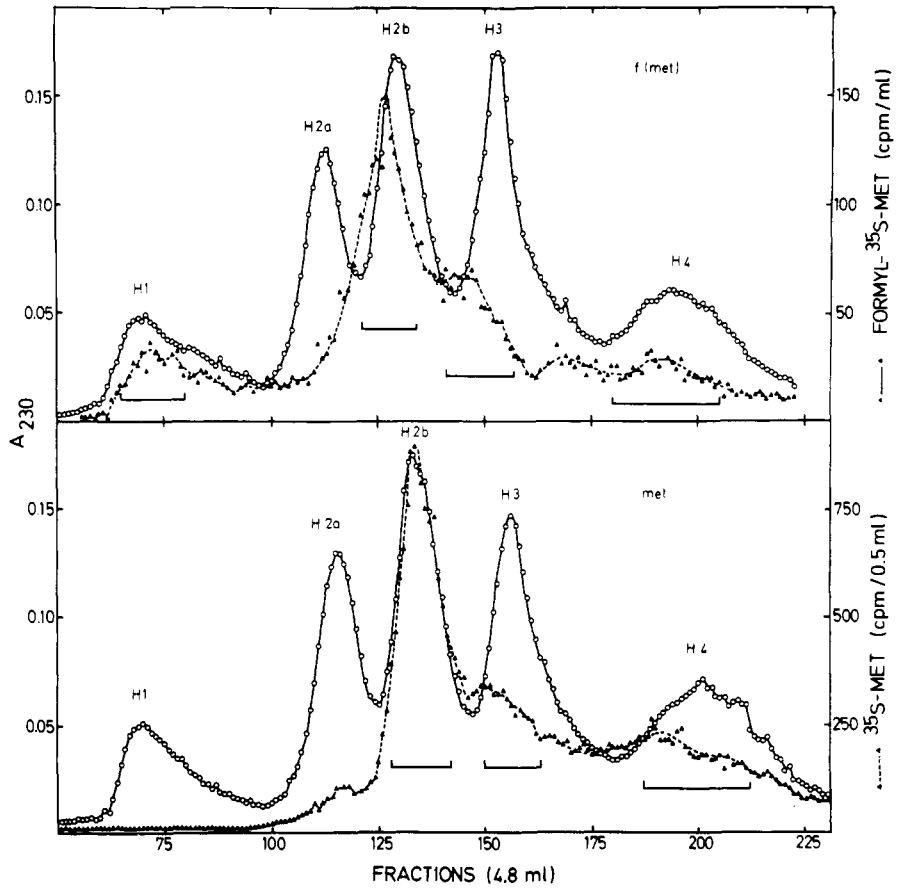

FIGURE 2: Bio-Gel P-10 column chromatography of in vitro synthesized histones. Histones synthesized in a rabbit reticulocyte lysate with either formyl[^{35}S]methionyl-tRNA$_f^{Met}$ or [^{35}S]methionine were mixed with 20 mg of calf thymus histones as marker and separated on a 2.5 × 160-cm Bio-Gel P-10 column equilibrated with 0.01 N HCl. Flow rate of the column was 12 ml/h. Fractions corresponding to individual histones were pooled, as indicated by the brackets for analysis of tryptic peptides.

tained the messengers coding for all individual histones. This was done by translating the ascites mRNA in the reticulocyte lysate using [^{3}H]lysine as label, since two of the histones, H1 and H2a, do not contain any methionine. The acid-soluble proteins synthesized in vitro were separated on acid–urea (Panyim and Chalkley, 1969) and sodium dodecyl sulfate polyacrylamide gels (Laemmli, 1970). The electrophoretic mobility of the products was compared to that of [^{14}C]lysine-labeled histones from HeLa cells. As shown in the fluorogram of Figure 1, all major histones are synthesized under the direction of the ascites cell mRNA, and histones H1 and H2b were the most intensively labeled fractions. In Figure 1a it can also be seen that the in vitro synthesized histone H4 moves to the same position in the acid–urea gel as the internally unacetylated form, which is the faster moving band of the H4 doublet. Figure 1b shows that the two forms of histone H1 which separate on sodium dodecyl sulfate gels are synthesized in vitro under the direction of the ascites cell mRNA used. After synthesizing histones in vitro under the same conditions but using methionine or *N*-formylmethionyl-tRNA, the reticulocyte lysate was extracted with 0.25 N HCl and newly made histones were purified from globin by CM-cellulose chromatography (Gallwitz and Breindl, 1972). The radioactively labeled histones were mixed with unlabeled calf thymus histones as markers and separated on a Bio-Gel P-10 column, as shown in Figure 2.

Since it is well known that the Bio-Gel matrix has ion-exchange properties (Candido and Dixon, 1972), some of the radioactive peaks were slightly displaced from the optical density profile. In the case of histone H2b the displacement of the two profiles is seen only with the formylmethionine-labeled but not with methionine-labeled protein, as it is expected from the incorporation of an additional amino acid at the N terminus carrying a formyl group. It is not understood, however, why the radioactive profiles of methionine-labeled histones H3 and H4 are also slightly displaced from the optical density profiles. A similar observation has already been made by Louie et al. (1973) in the case of arginine-labeled trout histone H3.

As expected from the known sequence data of histone H1 (Elgin and Weintraub, 1975), no radioactivity was associated with this histone when [^{35}S]methionine was used as label. In contrast, a significant amount of formyl[^{35}S]methionine, which cannot be cleaved off the N terminus, was found in histone H1. It can further be seen from Figure 2 that with the exception of histone H2a all other histones, H2b, H3, and H4, were labeled with methionine, as well as with formylmethionine.

The labeled histones were pooled from the Bio-Gel P-10 columns, as indicated in Figure 2, subjected to tryptic digestion, and the resulting peptides were separated by high-voltage paper electrophoresis at pH 1.9. As demonstrated in Figure 3, only one tryptic peptide labeled with formylmethionine was found in histone H1, as well as in histone H4. As already mentioned no methionine incorporation into histone H1 was observed because of the absence of methionine in the sequence. On the other hand, the tryptic digest of histone H4 contained one methionine-labeled peptide. This was expected from the known sequence data of histone H4 (DeLange et al., 1969) showing one internal methionine in the sequence. The tryptic

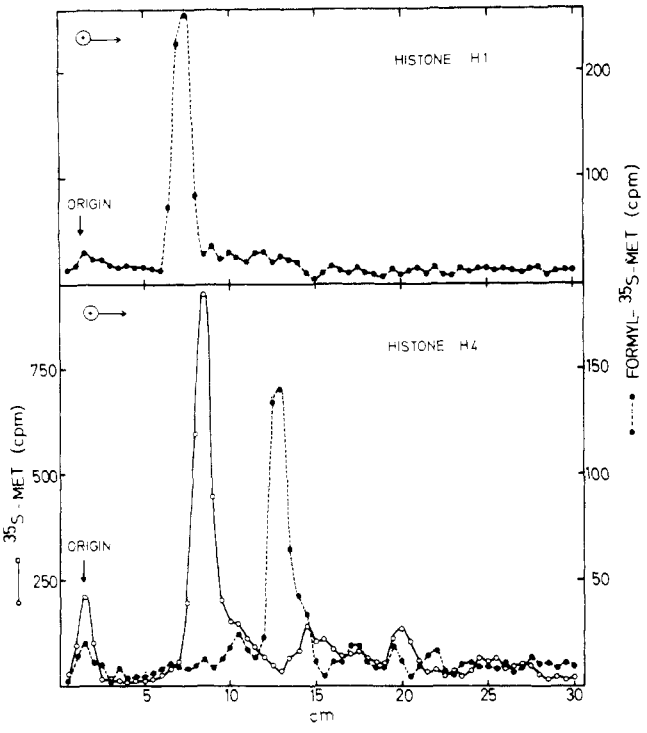

FIGURE 3: High-voltage paper electrophoresis of tryptic peptides. Histones H1 and H4 labeled in vitro with either methionine (O—O) or formylmethionine (● - - - - ●) were separated on a Bio-Gel P-10 column (Figure 2) and digested with trypsin. Tryptic peptides were separated on Whatman 3 MM paper in acetic acid–formic acid, pH 1.9. Radioactivity of 0.5-cm strips was measured in a toluene-based scintillation fluid.

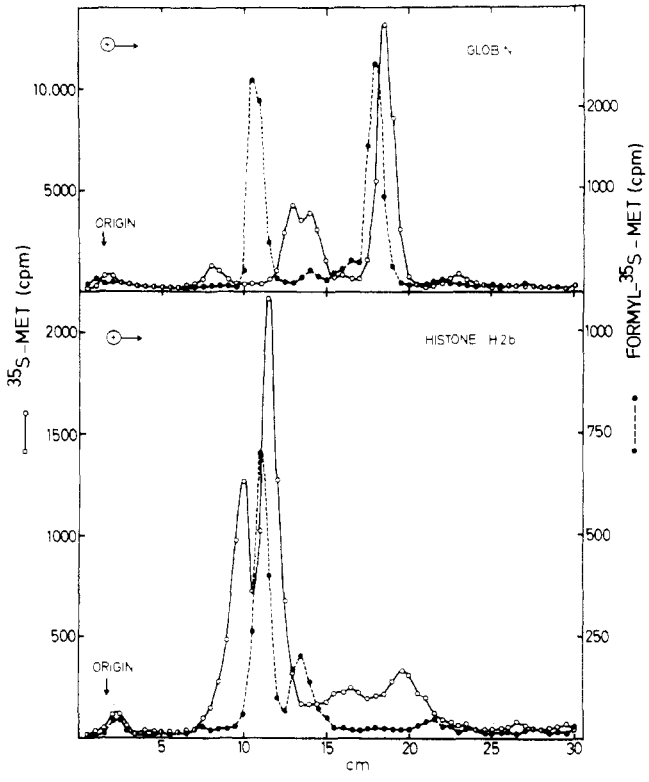

FIGURE 4: High-voltage paper electrophoresis of tryptic peptides. Histone H2b and globin, labeled in vitro with either methionine (O—O) or formylmethionine (● - - - - ●), were digested with trypsin and the resulting peptides separated by high-voltage paper electrophoresis at pH 1.9 and 38 V/cm.

peptide containing the internal methionine has 12 amino acids and is different in size and charge from the N-terminal tryptic peptide which, when initiated with formylmethionine, would have the structure *N*-fMet-Ser-Gly-Arg (the sequence data mentioned have been taken from calf thymus histone H4, but there is overwhelming evidence now for a high evolutionary stability of the primary sequence of this histone. All mammalian histones H4 are therefore very likely to have identical or nearly identical primary structures and this has been observed in a number of studies (DeLange et al., 1969; Wilson et al., 1970; Sautiere et al., 1971)). The different electrophoretic behavior of the two differently labeled tryptic peptides derived from histone H4, as shown in Figure 3, is therefore fully compatible with their expected structures.

As an example for the initiation of a histone with an unblocked N terminus, tryptic peptides derived from histone H2b, labeled either with methionine or with formylmethionine, were analyzed as shown in Figure 4. Again, one major formylmethionine-labeled peptide was found, but to our surprise we observed two major methionine-labeled peptides with slightly different electrophoretic mobilities at pH 1.9. This finding was unexpected, since according to published sequence data (Iwai et al., 1970) only one methionine-labeled tryptic peptide should be present in histone H2b. We therefore labeled total histones with [^{35}S]methionine in cultured ascites cells, purified histone H2b, and compared the radioactive tryptic peptides from the in vivo and in vitro synthesized protein. The same two major methionine-containing peptides were found from either of the two histone preparations (data not shown).

For comparison, rabbit globin labeled in the same lysate with methionine or formylmethionine was subjected to tryptic digestion. As shown in Figure 4, two formylmethionine-containing peptides derived from the α and β chains were separated electrophoretically. In the same figure, three methionine-labeled peptides can be seen. This was also found by Housman et al. (1970) and interpreted as result from chymotryptic cleavage of one of the two expected methionine-containing peptides.

TABLE I: Edman Degradation of Acid-Soluble in Vitro Synthesized Proteins.[a]

Label	Radioactivity (dpm) in Org. Phase	Residue	% of Radioactivity N-Terminal	Internal
[^{35}S]Met	3 497 000	169 000	4.6	95.4
f[^{35}S]Met	145 000	530 000	78.5	21.5

[a] Total acid-soluble proteins (2–2.3 mg) synthesized in a rabbit reticulocyte lysate with either [^{35}S]methionine or formyl[^{35}S]-methionyl-tRNA were subjected to Edman degradation as described in Materials and Methods.

To show that the formylmethionine was at the N terminus of the proteins newly synthesized in the reticulocyte lysate, Edman degradation was performed with total acid-soluble in vitro products, including globin. As shown in Table I, after one round of degradation only 4.6% of the radioactivity derived from the methionine-labeled proteins, but 78.5% of the radioactivity from formylmethionine-labeled proteins, was found to be N terminal.

Discussion

To our knowledge there is only one example where it has been shown that acetylation of the α-N-amino group of a

protein occurs during the synthesis of that protein, but after initiation. This is the eye lens α-crystallin whose N-terminal amino acid is α-*N*-acetylmethionine (Strous et al., 1973). This protein represents a special case, however, since the N-terminal methionine is not cleaved off the growing polypeptide chain.

The aim of the studies reported here was to decide whether histones having a N-blocked amino terminal end are initiated with methionine, as has been shown for bacterial and other eukaryotic proteins. It was reported by Liew et al. (1970) that in rat liver the synthesis of N-terminal acetylated histones might be initiated with *N*-acetylserine donated by *N*-acetylseryl-tRNA. In earlier studies, however, other investigators were unable to detect tRNAs carrying α-N-acetylated amino acids or to find enzymatic activities acetylating amino acids bound to tRNA (Marchis-Mouren and Lipmann, 1965; Haenni and Chapeville, 1966).

The availability of histone mRNA (Gallwitz and Breindl, 1972; Jacobs-Lorena et al., 1972) made it possible to investigate the possible involvement of methionine in the initiation of the synthesis of α-N-acetylated histones in vitro. To translate histone mRNA the rabbit reticulocyte lysate was chosen as the in vitro protein synthesizing system because of its good initiation properties (Breindl and Gallwitz, 1973). All histones synthesized in vitro under the direction of ascites cell mRNA were labeled with *N*-formylmethionine donated by formylmethionyl-$tRNA_f^{Met}$ from yeast, except histone H2a, which was hardly labeled. Most interestingly, *N*-formylmethionine was incorporated into the N-terminal acetylated histones H1 and H4, and in each case one labeled peptide was obtained after tryptic digestion of the two histones, respectively. Histones synthesized under identical conditions but using methionine as labeled amino acid did not show any incorporation of radioactivity into histone H1, which was expected because of the absence of methionine in the sequence. This result in itself clearly establishes that the formylmethionine incorporation into histone H1 was at the amino terminal end. According to the known sequence data, the α-N-acetylated histone H4 has one internal methionine in position 84 (DeLange et al., 1969). Therefore, tryptic digestion was expected to give one methionine-labeled peptide with different electrophoretic mobility as the peptide derived from the amino-terminal end carrying *N*-formylmethionine. This indeed was found experimentally, as reported above.

That unblocked histones are as well initiated with methionine was demonstrated in the case of histone H2b, which was highly labeled with formylmethionine as well as with methionine. Here again, one major formylmethionine-containing peptide, besides a minor labeled one, was found with a different electrophoretic mobility as the two predominant methionine-containing tryptic peptides derived from this histone. The two minor methionine-labeled peaks seen in the same electropherogram might result from protein contaminating histone H2b. Such nonhistone proteins can be seen in the autoradiogram of the polyacrylamide gels to electrophorese between histones H2a and H4. The observation of two major tryptic peptides radioactively labeled with methionine was an unexpected finding, since histone H2b from calf thymus has two methionine residues in positions 59 and 62 separated by glycine (position 60) and isoleucine (position 61). The ascites cell histone H2b must have a different structure, since also after in vivo labeling two methionine-containing peptides were found. To account for this finding, there could either be a third methionine within the sequence or one of the two amino acids in between the two methionine residues could have been changed in a conservative manner to lysine or arginine.

In summary, we have conclusively shown that at least two of the three α-N-acetylated histones, H1 and H4, are initiated with methionine in a rabbit reticulocyte lysate. It is therefore very likely that the initiation mechanism in vivo is the same. In light of the data presented in the literature and from our own finding, we believe that the initiation of protein synthesis with methionine is the general initiation mechanism of all eukaryotic proteins including those whose N-terminal amino acid residues have an α-N-blocked amino group.

Acknowledgment

We thank Mrs. R. Seidel for competent technical assistance and Dr. D. Doenecke for critical reading of the manuscript.

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Regulation of Membrane Phospholipid Synthesis by the *relA* Gene: Dependence on ppGpp Levels†

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ABSTRACT: A series of experiments using a pair of isogenic *rel*$^+$ strains of *Escherichia coli* differing only in the *spoT* locus has demonstrated a quantitative correlation between the inhibition of phospholipid synthesis and the intracellular level of ppGpp. The conditions examined were (1) amino acid starvation; (2) release from amino acid starvation; and (3) balanced growth. We also have been shown the presence of a third gene (in addition to *relA* and *spoT*) concerned with ppGpp metabolism and have found that the level of ppGpp during amino acid starvation is unaffected by an increase in the dosage of the *relA* gene.

The synthesis of membrane phospholipids as well as the synthesis of stable RNA species is regulated by the *relA* locus of *Escherichia coli* (Sokawa et al., 1968; Merlie and Pizer, 1973; Polakis et al., 1973; Nunn and Cronan, 1974, 1976; Nunn et al., 1975). Amino acid starvation of stringent (*rel*$^+$) strains results in a two- to four-fold decrease in the rate of phospholipid synthesis, whereas relaxed (*relA*$^-$) strains synthesize phospholipids normally during amino acid starvation.

In vivo, control has been shown to be exerted at the levels of both fatty acid synthesis and phospholipid synthesis (Nunn and Cronan, 1974, 1976). The molecular mechanism causing the decreased rate of lipid synthesis is thought to involve the inhibition of fatty acid and phospholipid biosynthetic enzymes by guanosine 3′,5′-bis(diphosphate) (ppGpp).

Cashel and Gallant (1969) showed that this unusual nucleotide accumulates during amino acid starvation of *rel*$^+$ (but not *relA*) strains (see review by Cashel, 1975). The case for the involvement of these nucleotides in *relA* gene control of lipid synthesis is based on both in vivo and in vitro experiments. Merlie and Pizer (1973) showed an inverse relationship between the rate of phospholipid synthesis and the presence of ppGpp during the onset and release of stringency. However, these data were not sufficiently detailed to allow a quantitative comparison between the ppGpp concentration and the rate of phospholipid synthesis. The in vitro experiments showed that ppGpp inhibits certain lipid biosynthetic enzymes (Merlie and Pizer, 1973; Polakis et al., 1973; Lueking and Goldfine, 1975; Ray and Cronan, 1975).

Although these experiments strongly suggest a role for ppGpp in the inhibition of lipid synthesis, the interpretation of these data is not completely straightforward. First, the enzymatic experiments are comprised by the knowledge that such enzyme inhibition data can be misleading. Enzymes are known in both the phospholipid (Merlie and Pizer, 1973) and nucleotide (Erlich et al., 1975) synthetic pathways which can be inhibited by ppGpp but which do not appear to play a role in stringent control.

The in vivo experiments of Merlie and Pizer (1973) do not greatly strengthen the enzymatic studies. These authors fell short of demonstrating a quantitative relationship between the level of ppGpp and the rate of phospholipid synthesis and thus the present case for involvement of ppGpp in the stringent control of lipid synthesis is not compelling.

Our previous experiments (Nunn and Cronan, 1974, 1976) were designed to clarify interpretation of the enzyme inhibition data. The present paper demonstrates a quantitative relationship between the intracellular levels of ppGpp and the rate of membrane phospholipid synthesis. In this work we used *spoT*$^-$ strains of *E. coli,* which are deficient in the turnover of ppGpp (Laffler and Gallant, 1974, 1975; Stamminger and Lazzarini, 1974). We also have examined the effects of dosage of the *relA* gene on ppGpp content, RNA synthesis, and phospholipid synthesis during the stringent response.

Experimental Procedures

Bacterial Strains. The relevant phenotypes of the *E. coli* K12 strains are given in Table I. Several of these strains are constructed in the course of this work. The genetic procedures we used for strain construction have been described previously (Nunn and Cronan, 1974; Cronan, Silbert and Wulff, 1972).

Strains WY3 and WY4 are *relA*$^+$ strains derived from strain NF161 which are isogenic excepting the *spoT* locus. Strain NF161 was converted to *str*^{R} and then converted to a *met*$^+$, *pyrE*$^-$ strain via conjugation with strain WN18. This recombinant strain was then transduced to *pyrE*$^+$ with P1 phage grown on strain WN30 (*spoT*$^+$). Strain WY4 is a strain which acquired the *spoT*$^+$ allele whereas strain WY3 is a *pyrE*$^+$ recombinant which remained *spoT*$^-$.

Strains WN30 and WN31 are a similarly constructed set of isogenic *spoT* strains derived from strain AT2538 by transduction to *pyrE*$^+$ with phage grown on NF161. Strain WN33 is an *arg*$^+$ (by transduction) *thyA*$^-$ (by trimethoprim

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* NIH Career Development Awardee (1-K4-GM 70,411).