Acetylation of Nascent Polypeptide Chains on Rat Liver Polyribosomes in Vivo and in Vitro†

Angel Pestana‡ and Henry C. Pitot*

ABSTRACT: Rat liver polyribosomes may be acetylated in vivo utilizing [^{3}H]acetate as precursor and in vitro with [^{14}C]acetyl-CoA. The in vitro acetylation occurs almost completely in the amino terminal position while the in vivo acetylation (after correction for isotopic exchange and incorporation of tritium into nonacetyl positions of amino acids) was distributed equally between the amino terminal groups of a number of amino acids and the ϵ-amino groups of internal lysine residues. At least 50% of the labeled acetyl groups introduced in vivo as well as in vitro could be removed from polysomes as puromycin polypeptides or peptidyl-tRNA. The acetylated polypeptides have been resolved by gel filtration into two components, one with an average molecular weight of 20,000 and the other of 4000–7000. The results presented indicate that the N-terminal acetylation of nascent growing polypeptides is a post initiation event that occurs on small peptides (40–70 amino acid residues) and depends on the presence of a polysome-bound acetyltransferase which differs from other cytoplasmic acetyltransferases which catalyze predominantly the acetylation of internal amino groups of proteins.

The acetylation of proteins associated with rat liver polyribosomes has been observed in in vivo studies utilizing labeled sodium acetate as precursor (Liew and Gornall, 1973; Pestana and Pitot, 1974). The fact that acetylation of polysome-associated proteins was strongly inhibited by the administration of puromycin to the animal (Liew and Gornall, 1973) indicates that such acetyltransferase activity is closely related to protein synthesis. Further support for this hypothesis comes from the demonstration of labeled acetyl groups at the amino terminus of growing polypeptide chains (Pestana and Pitot, 1974; Liew et al., 1970). These experimental findings may be related to other concepts concerning the mechanism of amino terminal acetylation of proteins, a process that is probably post-initiational, the acetylation occurring during polypeptide chain growth (Strous et al., 1973, 1974; Shih and Kaesberg, 1973). Previous investigations concerned with the acetylation of polysomes in vivo have not been directed toward an understanding of the mechanism of attachment of the acetyl groups. In actual fact the acetyl group may be introduced as part of a complex process of initiation involving acetylated amino acids or during polypeptide chain elongation as suggested above or even subsequent to the process of translation in which the completed protein is modified. As a further experimental complication the use of labeled acetate as a precursor of acetyl groups in vivo will allow the isotope to enter the growing chain and finished protein as part of the structure (non-acetyl) of some amino acids such as glutamate, aspartate, alanine, or serine. The demonstration of the acetylation of proteins in polyribosomes and ribosomes occurring at the amino terminus after incubation in vitro with acetyl-CoA (Pestana and Pitot, 1973, 1974, 1975) offers an experimental system for the study of mechanisms involved in the acetylation of growing polypeptide chains as well as finished proteins. In this paper we have described a comparative study of the acetylation of rat liver polyribosomes in vivo with the process carried out in vitro.

Materials and Methods

Acetylation of Polyribosomes. A mixture of free and bound polyribosomes was prepared by treatment of the postmitrochondrial supernatant with sodium deoxycholate (Pestana and Pitot, 1975), followed by sedimentation through two layers of 1 and 2 *M* sucrose in TKM[1] (20 m*M* Tris-HCl–25 m*M* KCl–5 m*M* $MgCl_2$ (pH 7.5)). Sedimentation resulted from centrifugation of the discontinuous gradient for 14 hr at 40,000 rpm in a 50.1 Ti rotor (Beckman). In other preparations free and bound polysomes were prepared by a procedure similar to that described by Venkatesan and Steele (1972). Livers were homogenized in 0.3 *M* sucrose in TKM and centrifuged in an SW 25 rotor at 2000 rpm for 2 min followed by a second centrifugation of the supernatant for 15 min at 25,000 rpm in the same head. The supernatant (S-1) from this centrifugation was used as a source of free polysomes which were isolated by sedimentation through the two sucrose layers described above. The pellet from the 15-min centrifugation was resuspended by homogenization in 0.3 *M* sucrose–TKM containing 1% Triton and then centrifuged for 10 min at 12,000 rpm. This supernatant (S-2), containing the bound polysomes freed from the membranes, was adjusted to 1% sodium deoxycholate, mixed by gentle homogenization, and the detergent-released polysomes isolated in the same manner as the free polysomes. For the acetylation of polyribosomes in vivo, rats were given an intraperitoneal injection of 10 mCi sodium [^{3}H]acetate 5 or 15 min prior to sacrifice. Acetylation of polyribosomes in vitro utilizing polysomal pellets (20–40

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[1] Abbreviation used is: TKM, 20 m*M* Tris-HCl–25 m*M* KCl–5 m*M* $MgCl_2$ (pH 7.5).

A_{260} units/ml) was accomplished by resuspension in 20 m*M* Tris-HCl (pH 7.5) containing 1 m*M* dithiothreitol and incubation with [^{14}C]acetyl-CoA (0.2 μCi/ml). After 15 min at 37°, the incubation mixture was chilled and the acetylated polysomes were recovered by centrifugation for 1 hr at 40,000 rpm.

Isolation of Nascent Polypeptide Chains. Two procedures were utilized for the isolation of nascent polypeptide chains. In the first, polysomes were resuspended in 20 m*M* Tris (pH 7.5)–5 m*M* $MgCl_2$–0.5 *M* KCl, and incubated with 1 m*M* puromycin either alone (Blobel and Sabatini, 1971) or in the presence of 0.5 m*M* GTP (Chaterjee et al., 1972). After 20 min at 37° the polysomes were sedimented by centrifugation for 1 hr at 40,000 rpm. This procedure does not release all of the nascent chains of the polysomes (Blobel and Sabatini, 1971); in addition the high salt treatment utilized in the first step may of itself cause the release of some acetylated proteins bound to the ribosomes (Pestana and Pitot, 1975). As an alternate procedure, peptidyl-tRNA was isolated by ion exchange chromatography, taking advantage of the strong acidic charge on the nucleic acid component of the molecule. The technique followed was identical with that described by Cioli and Lennox (1973) but adapted to a larger amount of polysomes. The polysomal pellet (containing about 50–100 A_{260} units) was homogenized in 0.5% sodium dodecyl sulfate, 0.1 *M* NaCl, 0.1 *M* formate (pH 4.7), 0.1% Brij-35, and 6 *M* urea, freshly deionized. The homogenate (approximately 2–3 ml) was diluted with 10 volumes of starting buffer (0.1 *M* formate (pH 7.4), 0.1 *M* sodium chloride, 0.1% Brij-35, and 6 *M* urea) and passed through a column (2 × 5 cm) of ECTEOLA (Celex E, Bio-Rad) equilibrated with the starting buffer. The column was then washed with 75 ml of starting buffer to obtain fraction 1. The column was then eluted with a buffer of the same composition as the starting buffer but with a sodium chloride concentration of 1 *M*. The first 25 ml (fraction II) was found to contain peptidyl-tRNA (Figure 3). The peptide moiety of the complex was released by incubation of the peptidyl-tRNA at pH 10 at 37° for 15 min.

Analysis of Enzymatic Digests of Acetylated Proteins and Peptides. The proteins were extracted from acetylated polysomes by the use of acetic acid in the presence of 0.1 *M* $MgCl_2$ (Hardy et al., 1969). Nascent polypeptides were isolated from acetylated polysomes as described above. Calf thymus histones (20 mg) were acetylated by incubation with a crude cytoplasmic acetyltransferase preparation (200 μg of protein from a 40% ammonium sulfate precipitate of a rat liver postmicrosomal supernatant) and [^{14}C]acetyl-CoA (0.2 μCi) in a medium containing 50 m*M* Tris-HCl (pH 7.5)–0.1 *M* KCl. After 30 min at 37°, the histones were extracted with 0.25 *M* H_2SO_4 and precipitated with 10 volumes of ethanol. Acetylation of endogenous components was accomplished by incubation of 10 mg of cytoplasmic acetyltransferase preparation with 0.2 μCi of [^{14}C]acetyl-CoA in the presence of 50 m*M* Tris-HCl (pH 7.5)–0.1 *M* KCl. After 15 min at 37°, the incubation mixture was chilled on ice and the proteins were precipitated with 50% ammonium sulfate. The protein samples to be analyzed were dialyzed extensively against water and subsequently freeze-dried. The dried material was resuspended in 0.1 *M* sodium carbonate (pH 8.1), and the mixture was treated with trypsin, Pronase, and carboxypeptidases A and B as previously described (Pestana and Pitot, 1975). The enzymatic digests were lyophilized, resuspended in 10 m*M* HCl, and loaded onto small (1 × 5 cm) Dowex 50 H^+ columns equilibrated with 10 m*M* HCl. The column was washed with 30 ml of the dilute HCl to obtain the "acidic" fraction containing amino acids with blocked amino groups (Maszluff and McCarty, 1970). All material retained on the column, which included N^{α}- as well as N^{ϵ}-acetyllysine, was eluted with 0.5 *M* NH_4OH and is referred to as the "basic" fraction. All fractions were evaporated to dryness and dissolved in small volumes of water. Paper chromatography and ion exchange chromatography of acetylated amino acids were carried out as described previously (Pestana and Pitot, 1974, 1975).

Gel Filtration of Nascent Peptides and Acetyltransferases. Acetylated nascent peptides released by puromycin were analyzed on a Bio-Gel P-10 column (1.8 × 80 cm) with 0.5% formic acid as the eluting buffer. Acetylated nascent peptides released by alkaline treatment of peptidyl-tRNA and of fraction 1 from ECTEOLA (Table II) were analyzed on a column (1.8 × 85 cm) of Bio-Gel P-60 by elution at room temperature with 0.1 *M* formate (pH 5.0) containing 0.1% sodium dodecyl sulfate. The presence of this detergent was found necessary to prevent protein aggregation. As molecular weight standards, myoglobin (17,800), cytochrome *c* (12,500), insulin (6000), glucagon (3500), and dinitrophenylated alanine were used to calibrate the columns and also included on each run where indicated. Fractions (2 ml) were collected using an LKB fraction collector. The radioactivity in each fraction was assayed directly.

Cytoplasmic and ribosomal acetyltransferases were separated according to their molecular size in a column (2 × 65 cm) of Bio-Gel A-15 equilibrated with and diluted with 20 m*M* Tris (pH 7.5)–0.5 *M* KCl–1 m*M* dithiothreitol. For each run, elution was performed at constant pressure with the help of a Mariotte flask.

Radioactivity Measurements. Radioactive components in soluble and cold Cl_3CCOOH were retained on glass fiber filters and washed five times with 5% Cl_3CCOOH (3 ml aliquots) and once with 15 ml of 50% ethanol in ether. The radioactivity on the dried filters was measured using a toluene-based scintillation mixture in a Nuclear-Chicago counter having an efficiency of 40% for ^{3}H and 60% for ^{14}C or ^{35}S when counted simultaneously. The radioactivity of samples in aqueous solution was measured after the addition of 5 or 10 ml of Scintisol (Isolab) in a comparable counter with the same efficiencies.

Chemicals. Acrylamide, *N,N'*-methylenebisacrylamide, Cellex E, Bio-Gel P-10, Bio-Gel P-60, Bio-Gel A-15, and mixed bed Amberlite (MB 3) were obtained from the Bio-Rad Co. Puromycin hydrochloride was a product of Nutritional Biochemicals. Calf thymus histones (Type II A) and Dnp-alanine were obtained from the Sigma Company while Pronase, trypsin, and carboxypeptidases A and B were products of Calbiochem. The molecular weight standards utilized as well as sucrose and urea were purchased from the Schwartz/Mann Corporation and N^{α}- and N^{ϵ}-acetyllysine were from Cyclo Chemicals. [1-^{14}C]Acetyl-CoA (52 μCi/mmol) and a tritiated amino acid mixture were products of the New England Nuclear Corporation. Sodium [^{3}H]acetate (2 Ci/mmol), [^{35}S]methionine, and [^{3}H]acetic anhydride were all obtained from the Amersham Corporation.

Results

Acetylation of Polyribosomes in Vivo and the Effect of Puromycin. Electrophoresis of whole polyribosomes in the

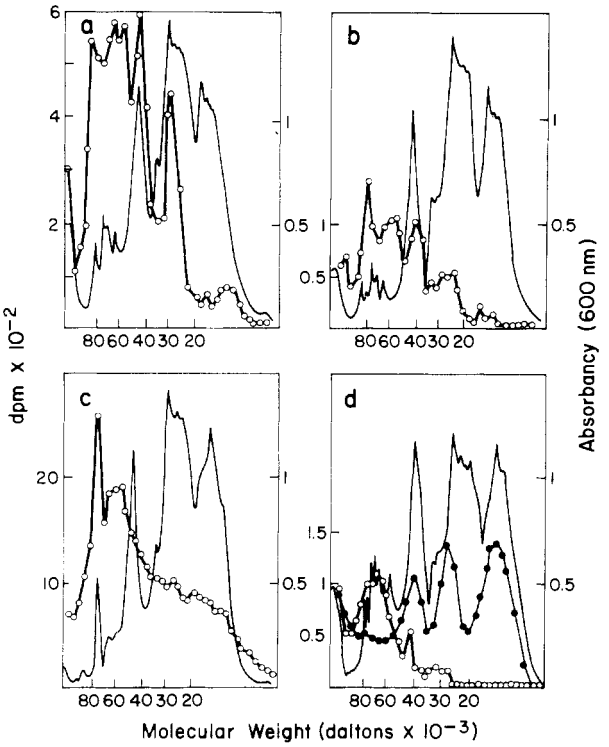

FIGURE 1: Electrophoretic analysis of acetylated polysomes. Polyribosomes were isolated from the liver of rats injected with sodium [^{3}H]acetate 15 min (a) and 5 min (c) before sacrifice. In b, puromycin (1 mg/100 g body weight) was injected just prior to the ^{3}H pulse given in a. Polysomes from two rats were pooled on each case. In d, polysomes labeled in vivo with ^{3}H amino acids and acetylated in vitro with [^{14}C]acetyl-CoA (see legend to Table II) were analyzed. Electrophoresis in 0.1% sodium dodecyl sulfate–10% acrylamide and radioactivity measurements were as described under Materials and Methods. Approximately 4 A_{260} units of polysomes were analyzed. Open circles represent tritium (acetate and amino acids) and closed circles [^{14}C]acetyl. The continuous line represents the absorbancy at 600 nm of stained gels, scanned with a Gilford spectrophotometer.

presence of sodium dodecyl sulfate was used to study the acetylation of polysomal proteins in vivo. Previous studies had demonstrated that most of the incorporation of label from sodium [^{3}H]acetate was incorporated into the proteins of polysomes extracted with urea–LiCl (Liew and Gornall, 1973). In Figures 1a and c, the quantitative and qualitative changes in the acetylation of polyribosomes in vivo are demonstrated 15 and 5 min after the administration of the acetate pulse. The most significant difference seen in a comparison of these two plates is the decrease with time of the radioactivity bound to proteins of lower molecular weight. This displacement of radioactivity toward the higher molecular weight regions on the gel and the decrease in total radioactivity with time suggest the dilution of the label by endogenous precursors together with an elongation of radiolabeled acetylated molecules. That growing polypeptide chains are involved in these changes is further indicated by the effect of puromycin in vivo (Figure 1b). In fact, the administration of puromycin 15 min prior to the acetate pulse resulted in an 80% inhibition in the total acetylation of polyribosomes. Electrophoresis of the acetylated proteins of whole polyribosomes from puromycin-treated rats (Figure 1b) indicated that the acetylation of proteins of all sizes was inhibited by the antibiotic.

The effect of puromycin in vitro was investigated utilizing free and bound polysomes acetylated in vivo. In this experiment (Table I) polysomes were washed with 0.5 *M* KCl

Table I: The Effect of Washing Polysomes Labeled in Vivo in 0.5 *M* KCl on Subsequent Release of Label by Incubation with Puromycin in Vitro.[a]

	Radioactivity	
	dpm	%
A. Initial polysome pellet		
Free	44,600	100
Bound	73,000	100
B. Supernatant after puromycin		
Free	27,000	61
Bound	32,000	43

[a] Free and bound polysomes were prepared (see Materials and Methods) from rats injected with sodium [^{3}H]acetate 15 min before sacrifice. The polysomal pellets from each fraction were resuspended in 50 m*M* Tris-HCl (pH 7.5)–0.5 *M* KCl–5 m*M* $MgCl_2$ (buffer A) and pelleted again (60 min at 40,000 rpm) to obtain polysomes washed in 0.5 *M* KCl. The pellets of washed polysomes were resuspended in 1 ml of buffer A and centrifuged 10 min at 5000 rpm and the radioactivity insoluble in cold Cl_3CCOOH was measured in duplicate. The suspensions of free and bound polysomes were then diluted with 10 ml of buffer A containing 1 m*M* puromycin and 0.5 m*M* GTP, incubated at 37° for 20 min and centrifuged at 40,000 rpm for 60 min. Radioactivity insoluble in cold Cl_3CCOOH and protein was measured in duplicate aliquots of the puromycin supernatant.

before incubation with puromycin in order to dissociate the effect of the drug from the loss of acetylated peptides due to the high salt wash. Under these conditions incubation with puromycin of the high salt-washed polysomes still induces the release of a large amount of labeled, presumably acetylated, Cl_3CCOOH insoluble material from the polysomes (61 and 43% from the free and bound polysomes, respectively).

Acetylation of Polysomes in Vitro. Polyribosomes isolated from rat liver take up [^{14}C]acetyl from [^{14}C]acetyl-CoA into a Cl_3CCOOH insoluble form on incubation. The rate of incorporation of labeled acetyl was found to be linear over the range of free polysomes added (Figure 2). The low incorporation observed with bound polysomes appears to be the result of the use of detergents in the preparation since exposure of free polysomes to 1% sodium deoxycholate and Triton with subsequent centrifugation markedly depressed the incorporation of acetyl groups into the free polysome preparation. Studies in vivo (Table I) indicated that the extent of acetylation of free and bound polysomes is quite similar.

Acetylation of polyribosome components in vitro was analyzed utilizing sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In the same preparation the polysomes were labeled in vivo with 200 μCi/rat of a tritiated amino acid mixture (see Materials and Methods) administered intraperitoneally 5 min prior to sacrifice. As seen in Figure 1d, three peaks of [^{14}C]acetyl were found to be associated with protein fractions having apparent molecular weight ranges of 40,000, 30,000, and 10,000–20,000. This pattern of acetylated proteins is quite similar to that observed previously with ribosomes acetylated in vitro (Pestana and Pitot, 1975). Most of the tritium label derived from the amino acid mixture was found associated with proteins having molecular weights higher than 40,000. We did not attempt to compare the pattern of acetylated proteins derived from polysomes labeled in vivo and in vitro since the distribution of

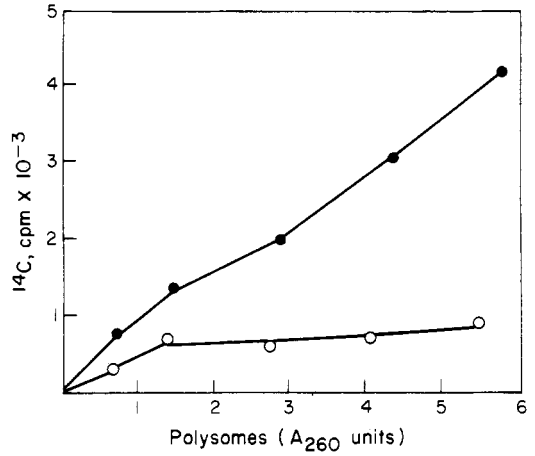

FIGURE 2: Endogenous acetylation of polyribosomes. Free and bound polysomes were prepared as described under Materials and Methods and were suspended in water (25 A_{260} units/ml). Aliquots were incubated in duplicate with 0.02 μCi of [^{14}C]acetyl-CoA in the presence of 50 m*M* Tris-HCl (pH 7.5)–0.1 *M* KCl–1 m*M* dithiothreitol in a final volume of 0.5 ml. After 15 min at 37°, 2.5 ml of 20% cold Cl_3CCOOH was added to each tube and the radioactivity in the Cl_3CCOOH precipitate measured as described under Materials and Methods. Closed and open circles stand for free and bound polysomes, respectively.

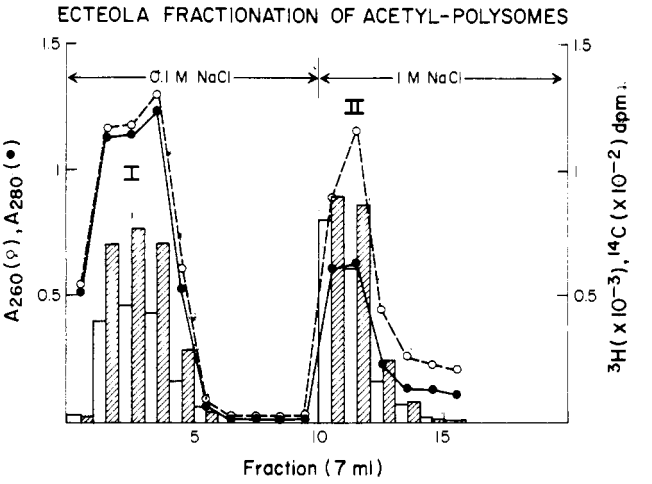

FIGURE 3: Fractionation of acetylated polysomes in ECTEOLA. Polyribosomes acetylated in vivo and in vitro (250 A_{260} units) were dissolved in urea–sodium dodecyl sulfate and eluted from ECTEOLA as described under Materials and Methods. Fractions of 7.5 ml were collected, and each fraction was assayed for absorbancy at 260 and 280 nm (open and closed circles, respectively) and also for the radioactivity insoluble in cold Cl_3CCOOH. Open bars represent tritium counts and the shaded bars the ^{14}C activity.

the in vivo label was a function of the length of the pulse (Figures 1a and c).

Isolation of Peptidyl-tRNA from Acetylated Polyribosomes. To substantiate further that the majority of the incorporation of acetyl groups occurred on nascent polypeptides of polyribosomes, a direct demonstration of the acetylation of peptidyl-tRNA isolated from polyribosome was carried out. Polyribosomes acetylated in vivo and in vitro as described under Materials and Methods were dissolved in 0.1% sodium dodecyl sulfate and 6 *M* urea, at a pH below 3, and then fractionated into RNA and protein on an ECTEOLA column as described under Materials and Methods. As seen in Figure 3, the 0.1 *M* NaCl eluate (fraction I) contained predominantly protein as judged from its spectral characteristics (A_{260}/A_{280} approximately 1). These proteins consist of ribosomal proteins, newly synthesized proteins, and contaminating soluble proteins including ferritin. Subsequent elution with 1 *M* NaCl (fraction II) is predominantly RNA as judged by its spectral characteristics (A_{260}/A_{280} ratio of approximately 2). This fraction includes peptidyl-tRNA and about 50% of the acetyl radioactivity insoluble in cold Cl_3CCOOH (Table IIA).

When polyribosomes having their nascent chains labeled in vivo with [^{35}S]methionine were utilized, 70% of the ^{35}S radioactivity was found in fraction II (Table IIB). To demonstrate that the labeled acetyl groups in fraction II were bound to the peptide component and not to RNA, aliquots of fraction II were subjected to mild alkaline hydrolysis, diluted with 10 volumes of the initial starting buffer, and rechromatographed on ECTEOLA columns. After this treatment all of the radioactivity was to be found in fraction I (Table II). The shift in the position of the radioactivity resulting from this treatment demonstrated that the acetyl groups are bound to the peptidyl moiety of peptidyl-tRNA by an alkali-labile bond, presumably that of an ester.

Species of Acetylated Molecules in Polyribosomes. In

Table II: Fractionation of Polyribosomes on ECTEOLA.[a]

	Fraction I				Fraction II			
	^{14}C or ^{35}S		^3H		^{14}C or ^{35}S		^3H	
	dpm	%	dpm	%	dpm	%	dpm	%
Experiment A								
$^{14}C/^3H$ acetylated polysomes	8,070	53	46,600	44	7,400	47	58,500	56
Fraction II after alkali treatment	5,600	100	43,000	100	0		0	
Experiment B								
[^{35}S]Met/^{3}H acetylated polysomes	11,000	31	33,000	46	24,000	69	39,000	54
Fraction II after alkali treatment	22,800	100	46,200	100	0		0	

[a] Polyribosomes were dissolved and fractionated in ECTEOLA as described under Materials and Methods. In experiment A, polysomes were acetylated in vivo ([^{3}H]acetate) and in vitro ([^{14}C]acetyl-CoA). In experiment B, polysomes were labeled in vivo with [^{3}H]acetate and [^{35}S]methionine given intraperitoneally 5 min before sacrifice. Cold Cl_3CCOOH insoluble radioactivity was assayed in 1-ml aliquots of the fractions (about 50 ml). The values given represent total radioactivity in each fraction.

Table III: Fractionation of Acetyl Amino Acids on Dowex 50 H^+.[a]

Acetylated Component	"Acidic" Fraction				"Basic" Fraction			
	^{14}C		^{3}H		^{14}C		^{3}H	
	dpm	%	dpm	%	dpm	%	dpm	%
Whole polyribosomal protein	2210	85	9,500	25	400	15	27,800	75
Puromycin peptides	505	99	440	12	30	1	3,200	88
Peptidyl tRNA from ECTEOLA	980	76	21,040	28	310	24	52,950	72
Endogenous cytoplasmic acceptor	750	30	8,100	27	1,720	70	21,960	73
Histones	3320	22			11,350	78		

[a] The source of proteins and peptides, the enzymatic digestion, and fractionation procedure have been described under Materials and Methods. In each case, ^{3}H represents isotope derived from [^{3}H]acetate given in vivo and the ^{14}C isotope from [1-^{14}C]acetyl-CoA on incubation in vitro. The percentages relate to the fraction of acidic or basic in one sample (^{14}C or ^{3}H) of acidic + basic fractions. Recovery of radioactivity from the column was always greater than 80%. See text for further details.

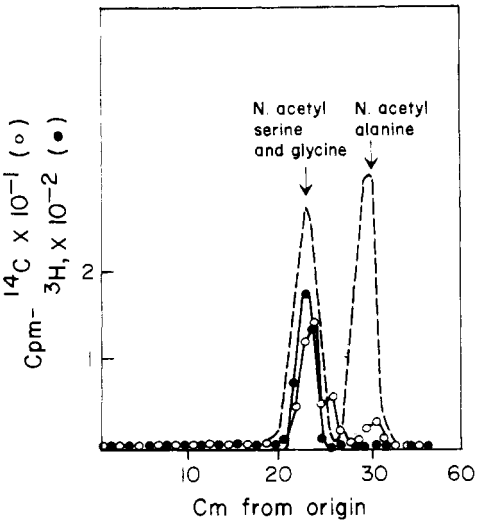

FIGURE 4: Paper chromatography of the "acidic" fraction obtained from polysomal proteins and polypeptides. The "acidic" fraction was obtained by Dowex fractionation of acetyl protein and polypeptides extracted from polysomes (see Materials and Methods, and Table III) and was analyzed by paper chromatography as described previously (Pestana and Pitot, 1975). Standard [^{3}H]-*N*-acetylserine, -glycine, and -alanine were prepared (Greenstein et al., 1953) and included in each run. Radioactivity in 1-cm strips was counted as described previously (vide supra). Solid circles and open circles correspond to ^{3}H and ^{14}C from the experimental samples. The continuous line represents the tritium label of the standards run in parallel on another strip.

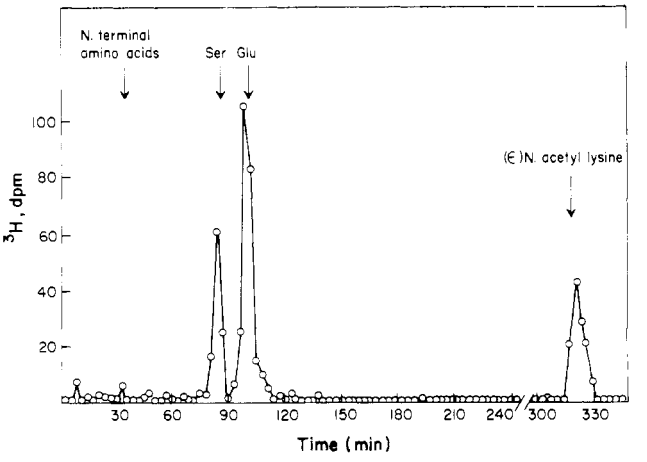

FIGURE 5: Ion exchange chromatography of the "basic" fraction of amino acids obtained from polysomal proteins and polypeptides. The "basic" fraction from an enzymatic digest of acetylated polysomal proteins (see Materials and Methods) was analyzed in an automatic amino acid analyzer as described (Pestana and Pitot, 1975). The effluent was split into two streams, one for the automated ninhydrin reaction and the other directed to a fraction collector for radioactivity measurements of 1-ml aliquots. The arrows indicate the time of elution of standard amino acids and acetylated amino acids. The circles correspond to tritium from [^{3}H]acetate administered in vivo.

order to characterize the acetylated components of polyribosomes, proteins were extracted from polysomes (Hardy et al., 1969) which had been acetylated both in vivo and in vitro. In addition polypeptides were obtained from polyribosomes after treatment with puromycin or by the alkaline treatment of peptidyl-tRNA as described under Materials and Methods. The samples of polysomal protein and polypeptides were extensively digested with proteolytic enzymes (see Materials and Methods) and the digests fractionated on Dowex 50 H^+ exchange resin into "acidic" and "basic" components (Marchis-Mouren and Lipmann, 1965). The acidic fraction was obtained by washing through the nonadsorbed material from the column with 0.01 *N* HCl. This fraction contains N-terminal blocked amino acids and peptides. The basic fraction which eluted with 0.5 *M* NH_4OH contains the adsorbed amino acids with free amino groups as well as α- or ϵ-aminoacetylated lysine. In Table III may be seen the distribution of ^{3}H and ^{14}C in these fractions from animals labeled in vivo and in vitro as before. In the case of polysomal protein and peptides most of the [^{14}C]acetyl radioactivity from the incubation in vitro with [^{14}C]acetyl-CoA was found in the acidic fraction (75–100%). When the label was administered in the form of sodium [^{3}H]acetate, only 12–28% of the label occurred in the acidic fraction. Analysis of the acidic fractions from both in vivo and in vitro labeling by paper chromatography showed one single radioactive peak having the R_f of *N*-acetylserine and *N*-acetylglycine (Figure 4). Much smaller amounts of label were associated with the peak corresponding to *N*-acetylalanine and a small unidentified peak between the two standards. These results are in accord with our previous report on the presence of acetyl groups at the amino terminus of nascent chains (Pestana and Pitot, 1974) and the preferential amino terminal character of the acetylation of ribosomes in vitro (Pestana and Pitot, 1975). In Table III may also be seen the fact that both histones and an endogenous cytoplasmic acceptor, as yet uncharacterized (Pestana et al., 1971), were both acetylated predominantly in the basic fraction and to a much lesser extent in the amino terminal position. This fact will be considered later in this section.

Some of the constituents of the basic fraction obtained

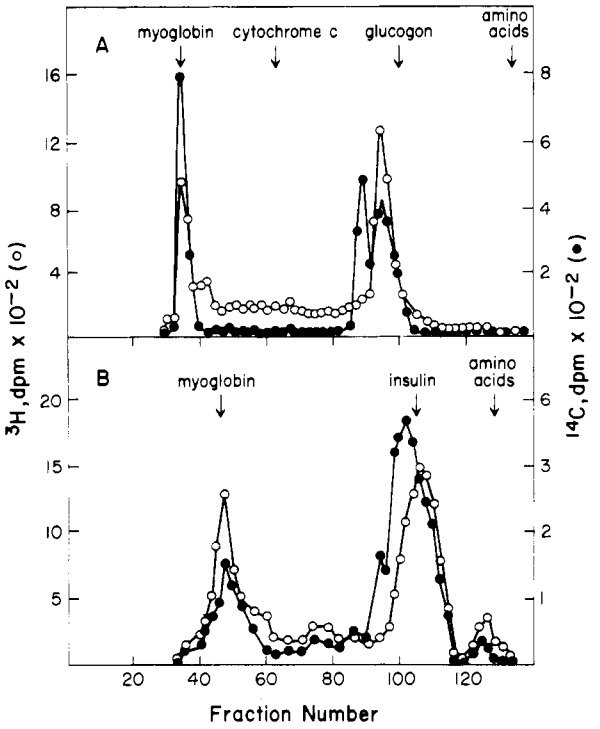

FIGURE 6: Gel filtration elution patterns of acetylated nascent peptides. Growing polypeptide chains were isolated from polysomes acetylated in vivo and in vitro. In A, the polypeptide chains were isolated by the puromycin method and chromatographed on Bio-Gel P-10 as described under Materials and Methods. In B, the polypeptides were obtained by the alkaline hydrolysis of peptidyl-tRNA and chromatographed on Bio-Gel P-60 described under Materials and Methods. The arrows indicate the elution of standards run simultaneously. Solid circles and open circles represent the radioactivity from [^{14}C]acetyl-CoA in vitro and sodium [^{3}H]acetate given in vivo, respectively.

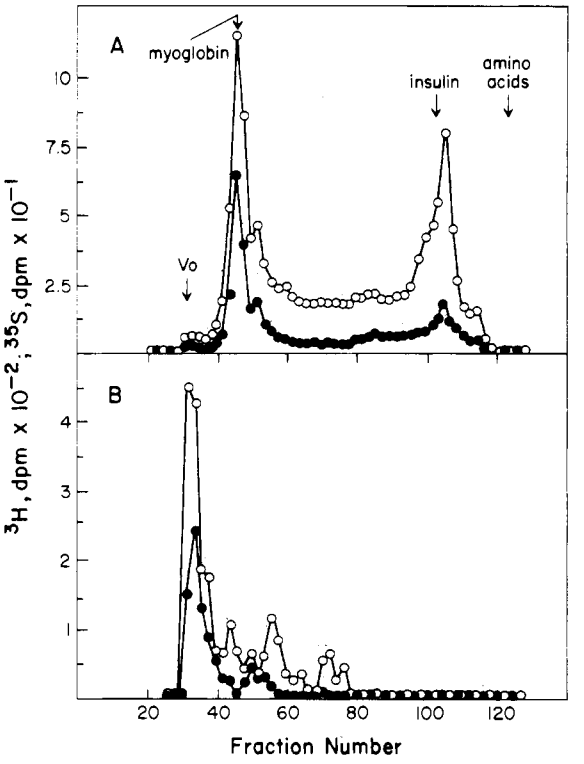

FIGURE 7: Gel filtration analysis of ECTEOLA fractions. Polysomes were labeled in vivo with sodium [^{3}H]acetate and [^{35}S]methionine given intraperitoneally 5 min before sacrifice. Fractionation of nascent chains on ECTEOLA was performed as described under Materials and Methods. The fractions were concentrated by ultrafiltration in a pressurized cell (Amicon) before analysis on Bio-Gel P-60 as described under Materials and Methods. (A) Elution pattern of labeled peptides obtained from fraction II after alkaline hydrolysis; (B) elution pattern of peptides obtained from fraction 1; (○) ^{3}H; (●) ^{35}S. See text for further details.

from Dowex 50 were characterized separately by ion-exchange chromatography (Figure 5). In this figure only the tritium radioactivity is demonstrated since the amount of ^{14}C obtained in the various fractions of the elution pattern was not sufficiently high to be analyzed (see Table III). Approximately 30% of the radioactivity in the basic fraction emerges at the same time as the standard, N^{ϵ}-acetyllysine, while the remainder of the tritium emerged with the glutamic acid (50%) and serine (20%) standards. The presence of the tritium from acetate administered in vivo in serine and glutamic acid residues of proteins has been previously reported (Liew and Gornall, 1973; Maszluff and McCarty, 1970). These results indicate that the amount of tritium incorporated from sodium [^{3}H]acetate in vivo into proteins is not a proper measurement of their degree of acetylation. However, after amino acid analysis as carried out in Figure 5, it is possible to correct for the contribution of label not present in acetyl groups in the protein. When this is done in the case of acetylated ribosomal protein and polypeptides it appears that the acetyl groups introduced in vivo are equally distributed between the ϵ-amino group of lysine and the amino terminal group of other amino acids.

Size Distribution of Acetylated Nascent Polypeptides. In order to gain further insight into the acetylated species, the size distribution of nascent peptides enzymatically labeled by acetyl-CoA in vitro or sodium acetate in vivo were carried out by gel filtration.

In Figure 6A, acetylated nascent polypeptide chains released from polysomes upon incubation with puromycin were chromatographed on Bio-Gel P-10. The elution pattern of radioactivity showed two major peak areas. One of these was coincident with the standard, myoglobin, and was not well resolved from the void volume. The other emerged slightly ahead of the standard, glucagon. A third peak seen only in the in vitro acetylated nascent chains was also consistently observed eluting prior to that near the glucagon standard. Nascent polypeptides released from peptidyl-tRNA by mild alkaline treatment (see Materials and Methods) were also analyzed for their molecular size distribution. For this analysis Bio-Gel P-60 was used in order to resolve proteins having a molecular weight above 20,000. As can be seen in Figure 6B, the labeled acetyl groups introduced either in vivo or in vitro were also found in two peaks coincident with the standards, myoglobin and insulin. These results suggest that there is a preferential acetylation of nascent polypeptide chains occurring in two discrete size ranges. One size class occurs in the region of the void volume of 20,000 molecular weight or greater while the other is between 4000 and 7000. It is also possible that acetylation of the growing chains occurred at random on all sizes of the nascent polypeptide chains and that subsequently a selective release of certain chains took place during the isolation of peptidyl-tRNA. In order to study this possibility, [^{35}S]methionine labeled polysomes were used as an internal control during the fractionation of peptidyl-tRNA on ECTEOLA columns. In Figure 7 may be seen the size distribution of the radioactivity of fractions I and II eluted from the column. Analysis of fraction II (peptidyl-tRNA) shows two

Table IV: The Effect of $MgCl_2$ on Free and Polysome-Bound Acetyltransferase Activities.[a]

Enzyme Source	[^{14}C]Acetyl cpm Incorporated	% Inhibition by Mg^{2+} Ions
Polysome bound acetyltransferase	503	
0.1 m*M* $MgCl_2$	294	42
Free cytoplasmic acetyltransferase (I)	985	
0.1 m*M* $MgCl_2$	918	7
Free cytoplasmic acetyltransferase (II)	6,823	
0.1 m*M* $MgCl_2$	6,511	6

[a] The source of polyribosome-bound acetyltransferase was the supernatant from polysomes resuspended in 20 m*M* Tris (pH 7.5)–0.5 *M* KCl and centrifuged for 1 hr at 40,000 rpm. The free cytoplasmic acetyltransferases I and II were obtained by gel filtration of postmicrosomal supernatant (40% ammonium sulfate precipitate) on Bio-Gel A-15 (Figure 8). The histone acetyltransferase activity was assayed in the presence or absence of 0.1 m*M* $MgCl_2$ by the method of Pestana et al. (1971).

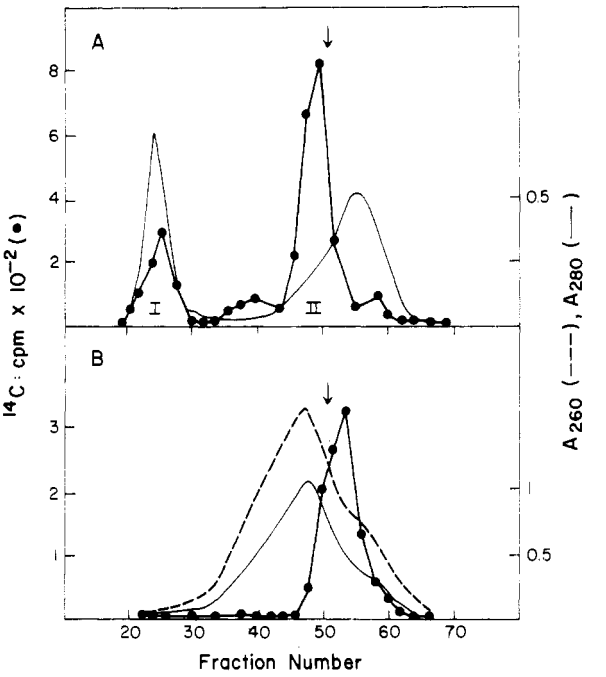

FIGURE 8: Free and polysome-bound acetyltransferases. The preparion of free cytoplasmic acetyltransferase was a 40% ammonium sulfate precipitate prepared from a rat liver post-microsomal supernatant. Before analysis, the precipitate was resuspended in the eluting medium described under Materials and Methods (20 m*M* Tris-HCl (pH 7.5)–0.5 *M* KCl–1 m*M* dithiothreitol) and dialyzed for 4 hr against the same medium. For the analysis of polysome-bound acetyltransferase, rat liver polyribosomes were resuspended in the eluting medium, supplemented with 1 m*M* $MgCl_2$ and stirred for 4 hr at 0–4°. Gel filtration on Bio-Gel A-15 was carried out as described under Materials and Methods. Aliquots of 0.5 ml were used for the assay of histone acetyltransferase activity (Pestana et al., 1971). Closed circles represent the isotope incorporation into histone, after correction for blank values without histones. A and B correspond to the elution profiles of the free cytoplasmic and polysome-bound activities, respectively. The arrows indicate the elution of the marker rat liver ferritin, purified according to Drisdale and Munro (1965).

peaks of [^{3}H]acetate and [^{35}S]methionine. Unlike the ^{3}H label, most of the ^{35}S was found in the higher molecular weight peak. Analysis of fraction I (Figure 7B) demonstrated that most of the ^{3}H and ^{35}S emerged close to the void volume and ahead of the myoglobin standard. The absence of ^{35}S and ^{3}H from the lower molecular weight regions of the elution profile indicates that little or no hydrolysis of peptidyl-tRNA took place during the isolation procedure at least within the range corresponding to nascent polypeptides between 3000 and 20,000 molecular weight. If such hydrolysis had occurred during the procedure there would have been a shift of radioactivity from fraction II to fraction I (Table II). Whether the acetylated material having molecular weights higher than myoglobin (Figure 7B) represents ribosomal proteins, newly synthesized protein, or nascent polypeptides released during the experimental manipulation of the polysomes cannot be ascertained from the experiments thus far performed.

Partial Characterization of Free and Polysome-Bound Acetyltransferase Activities. Upon washing polyribosomes with 0.5 *M* KCl a 75% decrease in the acetylation of the particles was observed when they were incubated with [^{14}C]acetyl-CoA. Simultaneously there was the appearance of an histone acetyltransferase activity in the KCl supernatant (Table IV). These findings were in accord with previous studies (Pestana and Pitot, 1975) that have demonstrated the endogenous enzymatic acetylation of rat liver ribosomes with a pH optimum of the thermolabile reaction of approximately 7.5. This earlier study also demonstrated the sensitivity of the ribosomal-bound enzyme to $MgCl_2$ as well as its inhibition by nucleotide triphosphates.

In preliminary investigations (Saxholm, Pestana, and Pitot, unpublished data) we have observed that the histone acetyltransferase activity of the cytosol may be resolved into two components by gel filtration on agarose (Bio-Gel A-15). The smaller peak of activity was found in the void volume together with some low molecular weight RNA, but most of the activity was found in a second peak eluting well after the void volume but ahead of ferritin added as a marker. We reasoned that the rather large apparent molecular weight of the acetyltransferase found in the void volume could have resulted in cosedimentation of this activity with the polysomes thus giving rise to the endogenous polysomal acetyltransferase activity described above. In order to determine whether this was actually the case, polyribosomal-bound and free cytoplasmic acetyltransferase preparations were compared with regard to their behavior on Bio-Gel A-15. Both preparations of acetyltransferase were treated with 0.5 *M* KCl in a similar manner prior to gel filtration and were eluted with a buffer containing 0.5 *M* KCl as described under Materials and Methods. In Figure 8A one can see the elution profile of the free cytoplasmic acetyltransferase activities. This pattern does not differ from the elution pattern of this preparation in the absence of 0.5 *M* KCl as described above. The elution of polyribosomes in 0.5 *M* KCl from Bio-Gel A-15 (Figure 8B) demonstrates the acetyltransferase activity eluting slightly after the ferritin marker, unlike the second peak of acetyltransferase activity found free in the cytosol. On this basis one may suggest that the molecular size of the polyribosome bound acetyltransferase is significantly different from the two species of molecular size of the free cytoplasmic acetyltransferase. Further evidence in support of a difference between the polysome-bound form of the acetyltransferase and those found free in the cytoplasm may be seen from their sensitivity to inhibition by Mg^{2+}. In Table IV one can see that 0.1 m*M*

$MgCl_2$ resulted in a greater than 40% inhibition in the polysome bound activity compared with essentially no inhibition by this ion of either of the cytoplasmic acetyltransferase activities. Furthermore, the data seen in Table III reveal a marked difference in the extent of N-terminal acetylation resulting from the activity in vitro of the ribosome-bound acetyltransferase and N-terminal acetylation which predominates from the action of the cytoplasmic enzymes. The endogenous acceptor is found to elute from the Bio-Gel A-15 column in the same region as the cytoplasmic acetyltransferase II (Figure 8A).

Discussion

In this paper using two different experimental methodologies we have demonstrated that 50% or more of the acetyl groups introduced into polysomes in vivo and in vitro are attached to growing polypeptide chains. In Table I it was demonstrated that puromycin treatment of both free and bound polysomes acetylated in vivo resulted in the release of 61 and 43%, respectively, of the labeled proteins. On the other hand, acetylated nascent polypeptides isolated as peptidyl-tRNA were found to contain 47 and 56% of the acetyl groups introduced in vitro and in vivo, respectively (Table II). It is quite likely that these are minimal values since hydrolysis of some peptidyl-tRNA during the isolation procedure may well have occurred.

Analysis of the enzymatic digests of acetylated polysomal proteins and nascent peptides demonstrated that 20% of the acetylation in vivo is amino terminal. In contrast, almost 80% of the acetylation occurring in vitro was found to be amino terminal (Table III) which is in agreement with previously reported data (Pestana and Pitot, 1975). However, when the basic fraction obtained from an enzymatic digest of polysomal proteins and nascent peptides eluted from a Dowex 50 H^+ was subjected to amino acid analysis, half of the isotope from [^{3}H]acetate given in vivo was found in the amino acid structures of serine and glutamate while less than 30% of the label occurred in the ϵ-amino group of lysine (Figure 5). This result indicates that about half of the tritium label found in polysomes after a short pulse labeling with sodium [^{3}H]acetate is due to its incorporation into the structure of two or more amino acids. The remainder of the tritium is present in the acetyl radicals, approximately equally distributed between amino terminal and ϵ-aminolysine residues. Since the endogenous acetylation of polysomes and ribosomes studied in vitro has a predominantly amino terminal structure (Table III; Pestana and Pitot, 1975) whereas the acetylation catalyzed by free cytoplasmic acetyltransferase activities is predominantly on internal lysine ϵ-amino groups (Table III), one may propose that the acetylation of polyribosomes in vivo (after correction for the non-acetyl isotope) results from the combination of the activities of free cytoplasmic and ribosomal-bound acetyltransferases. The ribosomal or polysomal acetyltransferase activity appears to be directed predominantly toward the amino terminal acetylation of growing polypeptide chains. However, further studies will be required to characterize the free and bound cytoplasmic activities further (Figure 8) and to determine both the substrate specificity (protein vs. nascent polypeptide) and the site of acetylation (amino terminal vs. internal free amino groups).

Studies on the molecular size of acetylated nascent polypeptides using gel filtration demonstrated the occurrence of two relatively discrete peaks of peptides acetylated both in vivo and in vitro (Figures 6 and 7). That peak eluting close to the insulin standard may represent the earliest peptide chains exhibiting amino terminal acetylation. This peak also contains most of the acetyl groups introduced in vitro. Its elution volume corresponds to polypeptides having 40 to 70 amino acid residues, a size that compares with the value reported for the complete acetylation of nascent chains of the protein, crystallin (Strous et al., 1974). In addition, experiments in vivo labeling both with sodium [^{3}H]acetate and [^{35}S]methionine (Figure 7) revealed that the lower molecular weight peak of acetylation contains the tritium label at a specific radioactivity ($^3H/^{35}S$) five times greater than the peak of larger molecular weight acetylated peptides and small proteins eluting with the void volume. Thus unless the frequency of methionine in the first 50 residues of most liver proteins is much less or that of serine and glutamate much greater than in the remaining portion of the proteins then, even after correcting for the tritium incorporated into glutamate and serine, these data indicate that these smaller peptides have been acetylated by a mechanism other than that of protein synthesis itself. The relatively low acetyl label consistently found in the intermediate molecular weight zone up to those peptides eluting with or just after the void volume (Figures 6 and 7) is also suggestive of a distinctive role for the high level of acetylation to amino acid incorporation seen in the 4000–7000 region. The mechanism which we feel best fits the data at present is that most if not all peptide chains are acetylated relatively nonspecifically during the early stages of protein synthesis while the chain is less than 70 amino acid residues. As the chain length increases selective deacetylation proceeds. The fact that specific peptidyl-tRNA deacylases occurring in rat liver ribosomes have already been described (Menninger et al., 1974) is in accord with this hypothesis.

Alternative explanations for these data would also include the specific acetylation of discrete sizes of polypeptides (50 and 200 amino acid residues) followed by termination of the protein. Examples of proteins which might fit into these two size classes are histone f_{2a1}, an acetylated protein of approximately 10,000 molecular weight (50–70 residues) and histone f_1 or the ferritin subunit having molecular weight ranges of 18,000–20,000 (150–200 amino acid residues). It is also possible that acetylation of peptides begins and ends in the 40–70 residue size. While one might expect a continuous level of acetyl radioactivity if this were so, the gap observed in Figures 6 and 7 may be explained by a slowing down of the rate of elongation possibly due to experimental manipulation of the system. The determination of which of these alternatives most closely represents the actual mechanism of the acetylation of nascent chains on polyribosomes is presently under study.

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Comparative Ability of RNA and DNA to Prime DNA Synthesis in Vitro: Role of Sequence, Sugar, and Structure of Template · Primer†

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ABSTRACT: The priming efficiency of oligo(RNA) vs. oligo(DNA) in a homopolymer template · homooligomer primer system was compared using four DNA polymerases. The templates included $(dT)_n$, $(dA)_n$, $(dC)_n$, and $(dI)_n$. Primers were the oligomers of the complementary DNA or RNA with chain lengths of 6 to 23. The DNA polymerases used were from *Micrococcus luteus,* avian myeloblastosis virus (AMV), and *Escherichia coli* (polymerase I and polymerase III). The polymerases demonstrated a preference for the DNA primers with $(dC)_n$, $(dA)_n$, and $(dI)_n$ templates. However, when $(dT)_n$ was the template, all but the AMV polymerase preferred $(rA)_{11}$ more than 200-fold better than $(dA)_{12}$. This preference was due to the physical structure of the initiation complex. The structures of the oligo · polymer complexes were characterized by mixing curves, melting curves, and analytical buoyant density analyses. $(rA)_{11} + (dT)_n$ formed predominantly a duplex structure, whereas $(dA)_{12} + (dT)_n$ formed the three-stranded structure, $(dA)_{12} \cdot 2(dT)_n$. The K_m of the duplex with *E. coli* Pol III was 2.9 μM $(rA)_{11}$. The K_i of the triplex was 2.2 μM $(dA)_{12}$, indicating that Pol III could bind to the triplex but would not elongate the $(dA)_{12}$ primer. The influence of structure on priming also was demonstrated with longer oligomers, $(dA)_{23}$ and $(rA)_{23}$, where the $(dA)_{23}$ formed more duplex-like structures and primed more than the $(dA)_{12}$. $(dT)_{10} + (dA)_n$ complexes also were shown to form triplex structures that inhibited priming. These results show that template · primer structure has more influence on priming than the sugar moiety or the sequence of the nucleic acid.

Prior studies from this and other laboratories (Green and Gerard, 1974) were aimed at establishing the specificities for template · primers of DNA polymerases isolated from several different sources. Studies were performed with polymeric DNAs, RNAs, and DNA · RNA hybrids as well as with natural nucleic acids. These studies documented that DNA polymerases are qualitatively similar in their requirements but that quantitative differences existed in their capacities to utilize certain template · primers. Bacterial DNA polymerases as well as the "reverse transcriptase" from AMV[1] had a pronounced preference for DNA rather than RNA templates (Green and Gerard, 1974) except in certain cases when deoxyribooligonucleotide primers were utilized (Baltimore and Smoler, 1971; Goodman and Spiegelman, 1971; Wells et al., 1972).

Prior studies from this and other laboratories also have demonstrated that RNA, as well as DNA, can serve as a primer for DNA synthesis in vitro and in vivo (for a review see Wells and Inman, 1973). Early reports showed a stimulatory role for RNA (suggesting its role as primer); more convincingly, recent work provided direct chemical analyses of the RNA–DNA covalent bonds formed at the initiation site (Wells et al., 1972; Leis and Hurwitz, 1972; Flügel and Wells, 1972; Keller, 1972; Verma et al., 1972, and later papers). In those cases where the homologous high molecular weight RNAs and DNAs were compared as primers, the RNAs generally primed as well (Wells et al., 1972) or a little less efficiently (Chang and Bollum, 1972) than the DNAs.

We wished to compare further the ability of RNA and DNA to serve as primer for DNA synthesis in vitro in well-characterized systems. Since a goal was to quantitate initial

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[1] Abbreviations used are: AMV, avian myeloblastosis virus; Pol I, *Escherichia coli* DNA polymerase I (polA); Pol III, *E. coli* DNA polymerase III (polC or dnaE).