Amino-Terminal Arginylation of Chromosomal Proteins by Arginyl-tRNA†

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ABSTRACT: Arginine was transferred from arginyl-tRNA to the amino-terminal end of chromatin proteins by L-arginyltransferase. The reaction was dependent on the presence of potassium ion and β-mercaptoethanol and was sensitive to RNase and trypsin. Treatment with DNase partially inhibited the transfer of arginine from arginyl-tRNA, suggesting that intact chromatin structure is necessary for modification of chromatin. The radioactivity incorporated into chromatin was sensitive to trypsin but not to DNase or RNase. Most of the incorporated radioactivity was recovered in the phenol fraction, supporting the notion that modification of chromatin takes place in proteins but not in nucleic acids of chromatin. Modification of the proteins by transfer of arginine from arginyl-tRNA takes place mainly in the nonhistone fraction of chromatin. Major portions of chromosomal proteins modified in this manner appear to be released from chromatin. Incubation of incorporated radioactive product with [^{12}C]arginyl-tRNA did not alter the product, showing that incorporated arginine is stable and does not exchange with added arginine or arginyl-tRNA. These observations suggest that aminoacyltransferase may function in the modification of chromosomal proteins and that modification of chromatin may alter the regulatory mechanisms of cellular functions.

Transfer of amino acids from aminoacyl-tRNA to the NH_2 terminus of preformed proteins has been shown in eukaryotic as well as prokaryotic cell-free systems (Kaji et al., 1963a,b). In an *Escherichia coli* cell-free system, leucine, phenylalanine, and tryptophan are transferred from the corresponding aminoacyl-tRNA to the NH_2-terminal end of the acceptor proteins which are present in the system (Kaji et al., 1963a, 1965a,b). In cell-free systems from rat liver, ascites tumor cells and thyroid transfer of arginine has been shown to take place from arginyl-tRNA to acceptor proteins in the presence of arginyltransferase (Kaji et al., 1963b; Kaji, 1968; Soffer and Mendelsohn, 1966; Tanaka and Kaji, 1974). These systems, which had been referred to as soluble systems in earlier publications (Kaji et al., 1963a,b; Kaji, 1968), consist of three components, aminoacyl-tRNA, the acceptor protein which is being modified, and aminoacyltransferase. Thyroglobulin and albumin can serve as acceptor proteins. Although the functional significance of this system is not yet clear, a recent observation has been made showing a mutant of *E. coli* lacking aminoacyltransferase having an altered rate of proline catabolism and hence exhibited abnormal growth characteristics (Deutch and Soffer, 1975). This observation suggests, therefore, that this system plays an important physiological role in cells as a means to modify preformed proteins so that the function of those proteins may be altered.

In a search for the functional significance of the protein-modifying system, the possibility that chromatin may be modified by NH_2-terminal addition was explored. There are two broad classes of proteins in chromatin, namely, histones and acidic proteins. It has been suggested that the acidic proteins in chromatin may play an important role in the transcriptional regulation of chromatin activity. We have found that arginine can be transferred in vitro from arginyl-tRNA to the protein fraction of chromatin, suggesting the possibility that biological activities of chromatin may be regulated by arginylation of chromosomal proteins.

Materials and Methods

Preparation of Crude and Purified Arginyltransferase. Mouse livers (5.1 g of BALB/c strain) were washed with phosphate-buffered saline, minced, homogenized in 30 ml of buffer A (0.3 M sucrose containing 4 mM magnesium acetate, 12.5 mM KCl, 0.01 M β-mercaptoethanol, 0.05 M Tris-HCl, pH 7.8), and centrifuged at 2000*g* for 5 min to remove nuclei. The supernatant was then centrifuged 20 min at 15 000*g*. Ribosomes were removed by a further centrifugation at 150 000*g* for 3 h. The soluble fraction was then brought to 70% saturation with $(NH_4)_2SO_4$ and centrifuged. he precipitate thus obtained was suspended in 10 ml of buffer A (without sucrose) and was dialyzed against this buffer A. Purified arginyltransferase was prepared as described (Soffer, 1970) except that 70% ammonium sulfate saturated solution of postmicrosomal supernatant of beef liver was used. All operations were carried out at 4 °C.

Chromatin. Two different procedures were used for preparation of chromatin. (a) One procedure is essentially the method described by Seligy and Miyagi (1969). The nuclear pellet, obtained in the previous section, was suspended in 10 ml of buffer B (0.025 M KCl, 0.005 M $MgCl_2$, 0.05 M Tris,[1] pH 7.4) and centrifuged through 0.2 M sucrose containing buffer B at 2000*g* for 5 min. The pellets were purified with the above procedure using 0.5% NP40 in buffer B and this was repeated once more. The purified nuclei were homogenized in 10 ml of 0.01 M Tris, pH 7.8, and layered onto 20 ml of 1.7 M sucrose containing 0.01 M Tris, pH 7.8. The upper two-thirds of the tube was gently mixed and the sample was centrifuged for 3 h at 81 500*g* in a Spinco SW27 rotor at 4 °C. The resulting pellets were washed twice by resuspending in 5 ml of

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[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; ATP, adenosine triphosphate; NRK, normal rat kidney; RSV, Rous sarcoma virus.

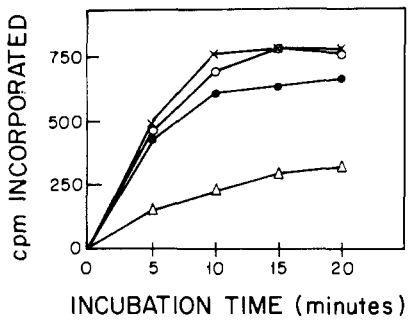

FIGURE 1: Transfer of arginine from arginyl-tRNA to chromatin—time course. Experimental conditions are as under Materials and Methods, except 20-μl aliquots were taken from 100 μl of reaction mixture at various time intervals for hot trichloroacetic acid insoluble radioactivity measurements. Reaction mixtures were as follows: transferase enzyme (△—△); transferase enzyme and 18 μg of chromatin (●—●); transferase enzyme and 36 μg of chromatin (○—○); transferase enzyme and 54 μg of chromatin (×—×).

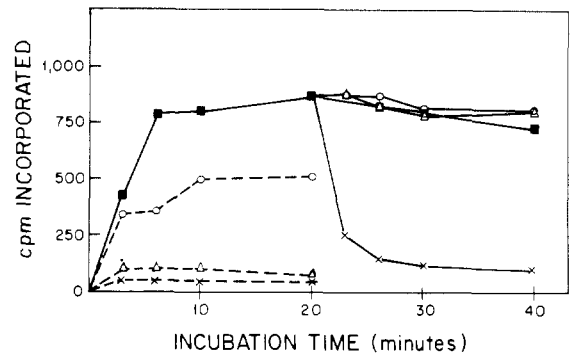

FIGURE 2: Effect of DNase, RNase, and trypsin on the transfer of arginine from arginyl-tRNA to chromatin. Aliquots (20 μl) were taken at various time intervals for hot trichloroacetic acid insoluble radioactivity measurements from 400 μl of the following incubation mixtures. (A) Transferase enzyme, 20 μg, chromatin, 72 μg, 80 000 cpm of [^{3}H]arginyl tRNA (■—■); (B) to 90 μl of A, after 20-min incubation at 37 °C, was added with 75 μg of RNase (△—△); (C) same as B, except that 25 μg of DNase was added (○—○); (D) same as B, except that the mixture received 5 μg of trypsin (×—×); (E) transferase enzyme, 5 μg, chromatin, 18 μg, 21 000 cpm [^{3}H]arginyl-tRNA in 100 μl of reaction mixture were incubated with 75 μg of RNase (△ - - - △); (F) same as E, but the reaction mixture was initiated with addition of 5 μg of trypsin (× - - - ×); (G) same as E, but the incubation was with 25 μg of DNase (○ - - - ○).

0.01 M Tris-HCl, pH 7.8. In some cases, chromatin was prepared according to the procedure described by Alfageme et al. (1974).

Preparation of tRNA and Aminoacyl-tRNA Synthetase. Established cell lines of normal rat kidney cells (NRK), transformed either by Rous sarcoma virus (Prague strain of subgroup A) or by a temperature-sensitive mutant of this Rous sarcoma virus (RSV) tsLA24 subgroup A (Wyke, 1973), were cultured in Dulbecco-modified Eagles medium supplemented with 10% calf serum. Cultures were washed twice with 5 ml of phosphate-buffered saline and cells were scraped from the petri dishes with a rubber policeman after the addition of 2 ml of a solution containing 0.01 M ethylenediaminetetraacetic acid, 0.15 M NaCl, and 0.4 mg/ml of bentonite. Cells were homogenized and the homogenate was extracted with an equal volume of phenol for tRNA preparation, and tRNA was purified as described below for arginyl-tRNA preparation. Aminoacyl-tRNA synthetase was prepared from the homogenate in 0.25 M sucrose, 0.15 M Tris-HCl, pH 7.8, 0.03 M KCl, and 0.006 M β-mercaptoethanol using previously described procedures (Yang et al., 1969).

Preparation of Arginyl-tRNA. The reaction mixture (0.5 ml) for preparing arginyl-tRNA contained the following in μmol/ml: cacodylate, 50, pH 6.9; KCl, 10; $MgCl_2$, 10; ATP, 2.0; and 1 μmol each of 19 of the [^{12}C]amino acids, except arginine. In addition, it contained 100 μg of tRNA, 150 μg of aminoacyl-tRNA synthetase, and 200 μCi of [^{3}H]arginine (sp act. 27.3 Ci/mmol). After 30-min incubation at 37 °C, the mixture was cooled in ice and applied to a DEAE column (1 × 3 cm), equilibrated with 0.01 M $MgCl_2$, 0.001 M EDTA, pH 4.5, containing 0.25 M NaCl at 4 °C. After washing with 30 ml of the same buffer, aminoacylated tRNA was eluted with 30 ml of this uffer containing 0.7 M NaCl. The aminoacyl-tRNA was precipitated with 2 volumes of ethanol and 0.1 volume of 20% potassium acetate, pH 4.5, overnight at −20 °C and recovered by slow filtration on a Millipore filter. The aminoacyl-tRNA was eluted from the filter in 0.5 ml of 0.01 M $MgCl_2$, 0.01 M sodium acetate, pH 4.5, and stored frozen at −60 °C.

Transfer of [^{3}H]Arginine from [^{3}H]Arginyl-tRNA to Chromatin. The reaction mixture (100 μl) contained the following in μmol/ml: Tris-HCl, pH 7.8, 100; KCl, 45; $MgCl_2$, 5; β-mercaptoethanol, 50; and 1 μmol each of the 19 amino acids, except arginine. In addition, the reaction mixture contained 48 μg of crude or 5 μg of purified arginyltransferase, various amounts of chromatin, and 20 000 cpm of arginyl-tRNA, unless otherwise noted. An aliquot of 20–25 μl was treated with hot trichloroacetic acid as described (Mans and Novelli, 1961).

Results

Transfer of Arginine from Arginyl-tRNA to Chromatin-Time Course. In the experiment illustrated in Figure 1, the time course of transfer of arginine from arginyl-tRNA to the acceptor proteins was studied. In the presence of added chromatin, the highest level of arginine transfer from arginyl-tRNA to protein was observed. The stimulation of arginine incorporation into protein by chromatin was observed both at the initial rate of arginine incorporation, as well as at the final level, since no further incorporation of arginine took place even when the mixture was incubated longer.

These observations suggest that arginine is transferred to chromatin by arginyltransferase in this system. Transfer of arginine from arginyl-tRNA to protein levels off at around 10 min, but the level of radioactivity remains stable, as can be seen in Figure 2, indicating that the major portion of arginylated chromatin as well as acceptor proteins are not degraded.

As reported earlier (Kaji, 1968), a crude ribosome-free extract of eukaryotic cells contains acceptor proteins which accept arginine at the NH_2-terminal end from arginyl-tRNA. Thus, transfer of arginine from arginyl-tRNA was observed even in the absence of added chromatin when only arginyltransferase was used.

Effect of DNase, RNase, and Trypsin on Transfer of Arginine from Arginyl-tRNA to Chromatin. In the experiments shown in Figure 2, the effects of various degradative enzymes on the incorporation of [^{3}H]arginine into chromatin were studied. When DNase was added, prior to the addition of [^{3}H]arginyl-tRNA to the reaction mixture, a large portion of acceptor capacity of chromatin for arginine was lost, indicating that the transfer of arginine from arginyl-tRNA by arginyltransferase is dependent on the intact structure of chromatin. Although data are not shown here, the pretreatment of chromatin with DNase causes loss in the acceptor activity. Addition of RNase and trypsin completely inhibited the reaction because RNase hydrolyzed arginyl-tRNA and trypsin digested ac-

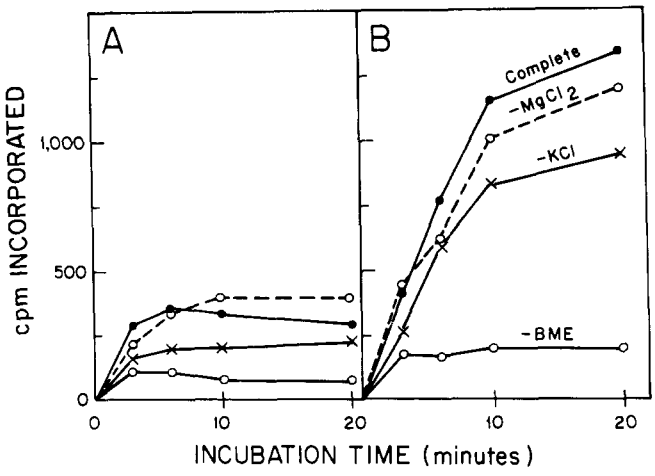

FIGURE 3: Dependence of arginylation of chromosomal proteins on the presence of potassium ion and β-mercaptoethanol. (1) Complete reaction mixture (●—●) contained various components as indicated under Materials and Methods; (2) no $MgCl_2$ was added (O - - - O); (3) no β-mercaptoethanol was added (O—O); (4) no KCl was added (×—×). Figure in A was carried out without chromatin addition and B was done with 18 μg of chromatin addition.

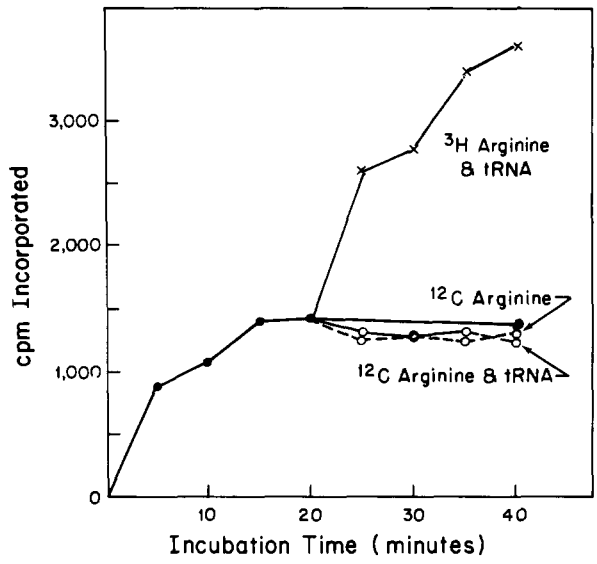

FIGURE 4: Lack of exchange of incorporated arginine with nonradioactive arginyl-tRNA or free arginine. Reaction mixture, as described under Materials and Methods, was incubated for 20 min and divided into 90-μl aliquots for further incubation with various components besides ATP, 0.8 μmol; phosphoenolpyruvate, 1.6 μg; aminoacylsynthetase 16 μg; or tRNA, 12 μg; derived from NRK cells transformed by a ts mutant of RSV (LA24PR-A). (A) Control experiment without further addition (●—●); (B) further addition of 0.2 μmol of unlabeled arginine (O—O); (C) same as above except no tRNA was used (O - - - O); (D) mixed with 0.2 μCi of [^{3}H]arginine (×—×).

ceptor proteins in chromatin as well as the arginyltransferase. On the other hand, when these inhibitors were added, after the incorporation of arginine into chromatin was completed, very little, if any, radioactivity was solubilized by RNase or DNase, whereas trypsin almost completely solubilized the radioactivity which had been incorporated into proteins. These observations indicate that radioactivity incorporated into chromatin is incorporated into protein but not into nucleic acids.

Dependence of Arginylation of Chromosomal Proteins on the Presence of Potassium Ion and β-Mercaptoethanol. In the experiments shown in Figure 3, arginylation of chromatin by arginyl-tRNA is performed in the presence or absence of various cofactors, such as magnesium chloride, potassium chloride, and β-mercaptoethanol. It is clear from this figure that β-mercaptoethanol is essential for arginylation of chromatin. In addition, potassium chloride appears to be required for complete arginylation of chromatin. In a comparative cytoplasmic system for the transfer of arginine from arginyl-tRNA to acceptor protein (Figure 3A) β-mercaptoethanol and potassium chloride were also required for the reaction.

Although data are not shown here, the pH dependence of transfer of arginine from arginyl-tRNA to chromatin showed a broad pH optimum range between pH 7.2 and 7.8. However, at all pH (6.9 ~ 8.5) studied, the stimulation of arginine incorporation by chromatin addition was observed.

Lack of Exchange of Incorporated Arginine with Nonradioactive Arginyl-tRNA or Free Arginine. In the experiments shown in Figure 4, transfer of arginine from radioactive arginyl-tRNA to chromatin, as well as to acceptor proteins present in the soluble system, was allowed to occur for 20 min. At this point, leveling off of transfer was observed due perhaps to exhaustion of arginyl-tRNA. This conclusion was reached on the basis that the addition of new [^{3}H]arginine and tRNA, together with an ATP-generating system, yielded more incorporation of arginine into hot trichloroacetic acid insoluble fraction. On the other hand, addition of nonlabeled arginine, tRNA, and ATP did not decrease radioactive arginine, which had been already incorporated into protein. This indicates that radioactive arginine once incorporated into chromatin is stable and does not exchange with nonlabeled arginyl-tRNA. Similarly, addition of free arginine in the absence of added tRNA or ATP did not release radioactive arginine from chromatin.

Evidence That Labeled Arginine Is Incorporated into Protein but Not into DNA. It is possible that incorporation of arginine into chromatin was due to incorporation into nucleic acids but not into proteins. To distinguish these two possibilities from each other, radioactive product was made after the reaction for an in vitro transfer of arginine from arginyl-tRNA to chromatin was completed. The labeled product was then mixed with phenol and both aqueous and nonaqueous layers were analyzed. As shown in Table II, the major portion of incorporated radioactivity moved into the organic phase, indicating that it was protein which was labeled in this reaction.

In addition, as indicated in Figure 2, DNase did not appreciably solubilize incorporated arginine when added to the reaction mixture after the transfer of arginine to chromatin was completed, indicating that radioactivity was incorporated into the protein portion of chromatin rather than onto DNA.

Amino-Terminal Analysis of Labeled Chromatin. The present system, which originally was called a soluble amino acid incorporation system and later referred to as an aminoacyltransferase or protein-modification system, involves transfer of an amino acid to the amino terminal of acceptor proteins. In order to establish that this is also true with arginine incorporated into chromatin, isolated chromatin, labeled with arginine by arginyltransferase, was subjected to amino terminal analysis.

As shown in Table I, a major portion of arginine in the chromatin reacted with dinitrofluorobenzene, indicating that it had free amino groups. Very little arginine was transferred to chromatin when the reaction was carried out in the absence of enzyme. In conformation of previous results, the endogenous acceptor proteins present in the arginyltransferase system, which are used for arginylation of chromatin, accept arginine from arginyl-tRNA at the NH_2-terminal end (Kaji, 1968). It should be pointed out that the chromosome-associated proteins

TABLE I: Amino Terminal Analysis of Labeled Chromatin.[a]

	(1) Chromatin With Transferase	(2) Chromatin Without Transferase	(3) Isolated Chromatin	(4) Isolated Supernatant
Arginine (Internal)	143	103	127	125
N_2P-arginine (NH_2 terminal)	2485	225	665	1543

[a] (1) Arginyltransferase, 25 μg, chromatin, 120 μg, and 100 000 cpm of [^{3}H]arginyl-tRNA were incubated at 37 °C for 20 min in 0.5 ml of reaction mixture, as described under Materials and Methods. The mixture was treated with 10 μg each of RNase and DNase for 15 min at room temperature, then mixed with trichloroacetic acid to a final concentration of 20% and the mixture was centrifuged. Pellets thus obtained were further washed once in 20% Cl_3CCOOH and twice in acetone containing 0.2% HCl. Cl_3CCOOH-treated pellets were suspended in 0.2 ml of solution containing 0.6 M $(NH_4)_2CO_3$ and 0.5 ml of DNFB in 95% ethanol. (2) Same as (1) except transferase was omitted. (3) Same as (1) except the total reaction mixture was centrifuged for 15 min at 10 000*g* to separate chromatin from supernatant (4). Pellets were suspended in 0.5 ml of 0.01 M Tris-HCl, pH 7.8. Amino terminal analysis of each of the dinitrophenylated (N_2P) product was carried out as described previously (Kaji, 1968). Approximately 40% of the hydrolyzed materials were processed for chromatography and values here represent cpm recovered on paper.

TABLE II: Phenol Extraction of Incorporated Product.[a]

Phase	cpm	% Recovery
H_2O	1 729	10.4
Phenol	14 949	89.6

[a] The reaction mixture contained 96 μg of crude arginyltransferase and 36 μg of chromatin, besides components listed under Materials and Methods. Water (200 μl) was added after 40-min incubation at 37 °C, and the whole mixture was shaken with 0.4 ml of water-saturated phenol. Both aqueous and phenol fractions were treated with hot 5% trichloroacetic acid and counted as described (Mans and Novelli, 1961).

are released from DNA after arginylation of chromosomal proteins by arginyltransferase. This observation was further supported by the following experiment in which we have attempted to identify labeled proteins by fractionating them into histone and nonhistone proteins as described (Johns, 1971).

Electrophoresis of Arginine-Labeled Proteins. After treatment of chromatin with the complete arginylating system, chromatin was centrifuged down and the acid-soluble proteins of the pellet and supernatant fractions were prepared as described (Panyim and Chalkley, 1969). The patterns of these proteins in acid–urea gels are shown in Figure 5. Staining with Coomassie blue reveals that all of the histones were still bound to the chromatin after the incubation (gels 1A and 2A), but that several nonhistone proteins were released. The supernatant contains contaminating proteins (gels 3A and 4A) which are similar to those observed in the crude mouse liver arginyltransferase preparation by itself (5A). Gels were further processed by fluorography in order to detect the [^{3}H]arginylated proteins (B series of Figure 5). No detectable radioactivity can be seen in histones (1B and 2B). Essentially all of the arginylated proteins are found in the chromatin-free supernatant (3B and 4B). One interpretation of these results is that the chromosomal proteins are released from DNA and subsequently

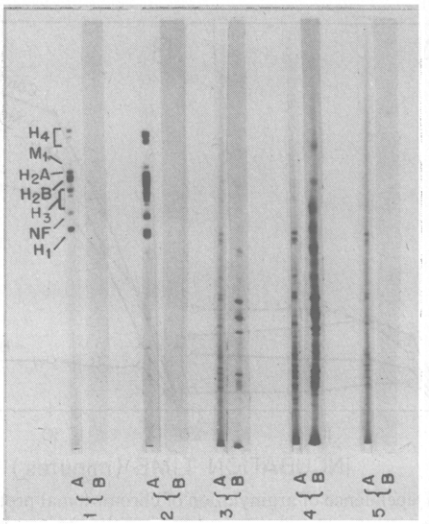

FIGURE 5: Localization of modified proteins in chromatin by fractionation and gel electrophoresis. The incubation was identical to that of Figure 1, except that the amounts of the constituents were 270 μg of chromatin, 480 μg of crude arginyltransferase, and 200 000 cpm of [^{3}H]arginyl-tRNA in 1 ml. After 20-min incubation at 37 °C, the mixture was centrifuged for 10 min at 10 000*g* to sediment the chromatin (a). H_2SO_4 was added to the chromatin and supernatant (b) at a final concentration of 0.25 N, to separate acid-soluble and -insoluble proteins. (c) Chromatin was not added to the reaction mixture. The proteins were precipitated with 90% ethanol, suspended in 50 μl of water, and analyzed by gel electrophoresis as described (Panymn and Chalkeley, 1969). (A) Stained with coomassie blue; (1,2) (of a) 1 and 3 μl of acid-soluble proteins of the chromatin pellet; (3,4) (of b) 1 and 3 μl of acid-soluble proteins of the supernatant; (5) (of c). (B) Gels were processed for fluorography (Bonner and Laskey, 1974).

arginylated. Alternatively, these proteins may be released from the chromatin after NH_2-terminal arginylation. Although a number of proteins can be detected in the crude arginyltransferase enzyme preparation in te absence of added chromatin by Coomassie blue staining (5A), no visible radioactive proteins can be detected (gel 5B). Similar results were obtained from the experiment where the crude mouse liver soluble enzyme was replaced with a purified beef liver arginyltransferase (data not shown): histones were not labeled, but proteins were found dissociated from chromatin after the reaction mixture was arginylated. No visible protein bands nor radioactivities were seen with the purified transferase alone.

Discussion

It has been suggested recently that histones may function largely in the maintenance of chromatin structure, while nonhistone proteins may regulate transcription from the DNA template (Paul, 1970; Baserga and Stein, 1971; Spelsberg et al., 1972; Hnilica, 1972; Stein et al., 1974). Modification of chromosomal proteins has been reported to alter the regulatory capacity of these proteins on the function of chromatin. Thus, phosphorylation of histone as well as nonhistone proteins appears to take place in a distinct manner during the cell cycle (Langan, 1968; Ajiro et al., 1975). Phosphorylation of proteins perhaps reduces the positive charge of chromosomal proteins, resulting in a loose association of these proteins with DNA. Another mode of modification of chromosomal proteins known at present is an acetylation of proteins at a free amino group (Phillips, 1963a,b; 1968) which would reduce the positive charge of chromosomal proteins, and thus weaken the association of basic proteins with DNA. The exact role of these modification systems in the regulation of chromatin function remains to be explained.

The data reported in this communication suggests the possibility that a new mode of protein modification may exist for chromosomal proteins and this modification may be related to their biological activity. The transfer of arginine from arginyl-tRNA to chromosomal proteins, as indicated in this communication, may play an important role in the function of chromatin. It is most unlikely that arginylation of protein fraction of chromatin involves a histone fraction, since most of labeled proteins are fractionated into nonhistone proteins. Furthermore, most of the sequenced histones are known to be blocked at the NH_2-terminal end and the incorporation of arginine into chromatin was studied by following the incorporation of radioactive arginine into a fraction insoluble in hot trichloroacetic acid. This treatment is known to solubilize histone F_1.

It is, therefore, probable that arginine is incorporated into the acidic proteins of chromatin. Because of the basic nature of arginine, the incorporation of this amino acid into the acidic proteins may lower the negative charge of the acidic proteins, resulting in altered association of these proteins with DNA. This might lead to a modification of transcriptional capacity.

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