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Effects of Estradiol on Uterine Ribonucleic Acid Metabolism. Assessment of Transfer Ribonucleic Acid Methylation†

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ABSTRACT: Immature rats treated with estradiol for selected periods of time demonstrated both increased methylation of uterine transfer ribonucleic acid (tRNA) and methylase activities. Whereas the former parameter was assessed by incubating whole uteri with [*methyl*-^{14}C]methionine and measuring the incorporation of isotope into the tRNA, methylase activity was obtained by measuring the rate of incorporation of methyl groups from *S*-adenosyl[*methyl*-^{14}C]methionine into heterologous tRNA (*Escherichia coli* B) in the presence of uterine cytosol preparations (100,000*g* supernatants). Although increased methylation of tRNA during the estrogen response was demonstrated, additional studies indicated that these results were largely attributable to an increased rate of synthesis of tRNA rather than gross changes in either the type or amount of methylated constituents present. Evidence in this regard included the inability of estrogen treatment to alter significantly the (a) resulting patterns of *methyl*-^{14}C-methylated constituents of uterine tRNA, (b) the extent to which [2-^{14}C]guanine residues, incorporated into tRNA, become methylated, (c) the extent of methylation of precursor tRNA in the absence of tRNA synthesis, and (d) the types of methylase activities expressed in vitro.

That modification of tRNA may participate in or render some important regulatory function in cells or tissues is both intriguing and suggestive in view of the findings that the quantity and possibly quality of tRNA methyltransferases undergo significant alterations in a variety of biological systems experiencing dramatic changes in growth processes (Starr and Sells, 1969; Borek, 1971; Randerath and Randerath, 1973). Although the majority of these findings were derived from studies designed primarily to detect differences in methylase activities and, therefore, do not provide sufficient evidence for aberrant or hypermethylated tRNAs, additional support for their existence is suggested by numerous tRNA chromatographic profiles (Sharma and Borek, 1970; Kothari and Taylor, 1973) as well as altered methylation patterns of tRNAs derived from in vivo studies (Bergquist and Matthews, 1962; Craddock, 1969; Borek and Kerr, 1972).

The present investigation assesses the methylation of uterine tRNA which occurs following estrogen treatment of immature rats by examining both the in vitro activity of tRNA methyltransferases as well as by characterizing the types and amount of methylated constituents that appear on newly synthesized uterine tRNA at selected times following the administration of estradiol. The immature rat uterine system was selected for this study since earlier investigations have reported that estrogen treatment promotes increased and/or altered tRNA methylase activities (Lipshitz-Wiesner et al., 1970; Sharma et al., 1971; Baliga and Borek, 1974), altered tRNA chromatographic profiles (Sharma and Borek, 1970), and increased uterine tRNA synthesis and methylation (Billing et al., 1969; Munns and Katzman, 1971b).

Experimental Procedures

Treatment of Animals. Immature female rats, 21–22 days old and weighing 40–50 g, were purchased from National Laboratory Animal Co., Creve Coeur, Mo. After an acclimation period of 24 hr the rats were injected intraperitoneally with a single dose of 10 μg of 17β-estradiol (Sigma Chemical Co.) in 0.25 ml of aqueous 9.5% ethanol. At the prescribed times thereafter, the animals were sacrified by cervical dislocation and within 1 min the whole uterus was explanted free of adhering tissue and collected in ice-cold Eagle's minimum essential medium (MEM) for 5–10 min prior to incubation. The above MEM medium (no. 109, GIBCO) contained all of its defined ingredients except glu-

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tamine and methionine, the latter being added to the medium just prior to incubation as described below.

Incubation and Labeling Conditions. Groups of 6–12 uteri were placed in 50-ml beakers containing 5 ml of MEM medium which had been preequilibrated at 37° under an atmosphere of 95% O_2–5% CO_2 (Dubnoff incubator) and supplemented with glutamine (1.0 m*M*) and one of the following isotopes from Amersham Searle: [*methyl*-^{14}C]methionine (56 mCi/mmol), [*methyl*-^{3}H]methionine (2.6 Ci/mmol), and [U-^{14}C]guanosine (602 mCi/mmol). Under these conditions three labeling systems were utilized to assess the methylation of uterine tRNA. These included: (A) a 90-min pulse with [*methyl*-^{14}C]methionine (11.2 μCi/incubation) followed by a 60-min chase in the presence of unlabeled methionine; (B) a 120-min pulse with [U-^{14}C]guanosine (10.2 μCi/incubation) in the presence of unlabeled adenosine (5×10^{-6} *M*) and methionine (3.3×10^{-5} *M*) followed by a 60-min chase with actinomycin D (20 μg/uterus); and (C) a 90-min pulse with [*methyl*-^{3}H]methionine (500 μCi/incubation) in the presence of actinomycin D (20 μg/uterus) followed by a 60-min chase with unlabeled methionine. Whereas the methionine chases in labeling systems A and C were designed to minimize [*methyl*-^{14}C]methionyl-tRNA labeling, the actinomycin D chase associated with the [U-^{14}C]guanosine pulse was employed to chase [U-^{14}C]guanosine-labeled precursor tRNAs into mature forms. The latter was necessary since precursor tRNAs do not contain their full complement of methylated [U-^{14}C]guanine constituents (Munns et al., 1974a). The chase aspect of these experiments was performed simply by rinsing each group of uteri in ice-cold MEM medium prior to replacement in fresh medium preequilibrated as above and containing supplements of methionine (3.3×10^{-5} *M*), glutamine (1.0 m*M*), and in one instance actinomycin D (20 μg/uterus). At the conclusion of these incubations, uteri were again rinsed in ice-cold medium and processed immediately or stored at −80°.

Preliminary studies with actinomycin D established that the amounts employed were sufficient to inhibit the synthesis of tRNA (see footnotes to Table I), while other studies have revealed that this level of antibiotic does not inhibit the activity of methyltransferase enzymes (Munns and Sims, 1975a).

Isolation and Characterization of Uterine tRNA. Total RNA from uterine homogenates was extracted with phenol–sodium dodecyl sulfate and the tRNA separated from the bulk of other RNAs by salt fractionation (1.2 *m* NaCl). Electrophoretic characterization of [*methyl*-^{14}C]methionine- and [U-^{14}C]guanosine-labeled tRNAs prepared in the above manner indicated that greater than 94% of the incorporated radioactivity migrated within the tRNA band of the gel. The above methods have been described in detail by Munns et al. (1974b).

To assess the effects of estradiol pretreatment in vivo on the extent of incorporation of the methyl group from [*methyl*-^{14}C]methionine and of [U-^{14}C]guanosine into the tRNA of intact uteri in vitro, aliquots of phenol-extracted, salt-fractionated tRNA were electrophoresed in 7.5% acrylamide gels and the radioactivity associated with the tRNA band of the gel determined (Munns and Katzman, 1971b). Measurements in this manner permit an accurate assessment of isotope incorporation into tRNA (uterus), since the percent recovery of tRNA from the rat uterus following phenol–dodecyl sulfate extraction and salt fractionation was found to be constant and therefore independent of estrogen stimulation (Munns and Katzman, 1971b; Munns et al., 1974b).

In Vitro Characterization of Uterine tRNA Methylases. tRNA methylase extracts from the cytosol fraction of uterine homogenates (i.e., 100,000*g* supernatant) were prepared according to the procedure of Pegg (1971) with minor modifications. These included (a) the addition of bentonite (100 μg/ml of homogenate) prior to homogenization, (b) extensive dialysis of the cytosol fraction against 0.5 m*M* 2-mercaptoethanol–10 m*M* Tris (pH 8.0), and (c) omission of an ammonium sulfate fractionation step. Dialysis was performed in an attempt to remove endogenous *S*-adenosylmethionine as well as low molecular weight dialyzable inhibitors of tRNA methylases reportedly present in such extracts (Halpern et al., 1971; Kerr, 1972; Leboy and Piester, 1973).

The standard assay medium for determining tRNA methylase activities contained: 20 μmol of Tris (pH 8.0), 0.02 μmol of *S*-adenosyl[*methyl*-^{14}C]methionine (61 mCi/mmol, Amersham–Searle), 4 μmol of spermidine phosphate, 0.6–0.7 mg of cytosol protein as determined by the method of Lowry et al. (1951), and 0.5 mg of heterologous tRNA (*Escherichia coli* B) in a final volume of 2.0 ml. The assay mixture was incubated at 35° for approximately 5 min prior to the addition of *S*-adenosyl[*methyl*-^{14}C]methionine and at frequent intervals thereafter 50-μl aliquots were withdrawn and processed for measurement of the rate of methylation of heterologous tRNA (Pegg, 1971). Methylase activity was expressed in terms of both the incorporation of radioactivity into heterologous tRNA per microgram of cytosol protein and per uterus (the latter activity being determined by the amount of protein contained in the cytosol fraction per uterus). Background radioactivity was accounted for by assaying in the absence of heterologous tRNA and represented less than 5% of the total radioactivity incorporated into heterologous tRNA during a 2-hr incubation. Reactions were terminated by the addition of 0.2 ml of 10% dodecyl sulfate, 200 μg of bentonite, and 1 ml of phenol. Following phenol–dodecyl sulfate extraction at 25°, the *methyl*-^{14}C-labeled tRNA in the aqueous phase was recovered by ethanol precipitation and processed for analysis of *methyl*-^{14}C-methylated constituents.

Determination of the Distribution of Radioactive Methylated Constituents of tRNA. Procedures employed for the determination of *methyl*-^{14}C-methylated constituents and of methylated [U-^{14}C]guanine constituents of uterine tRNA have been described in detail elsewhere (Munns et al. 1974a, b). These procedures permitted greater than 90% of the radioactivity incorporated into tRNA to be recovered and identified.

Results and Discussion

Effects of Estradiol Pretreatment in Vivo on the Incorporation of Isotopic Precursors into the tRNA of Intact Uteri in Vitro. As described under Experimental Procedures, three distinctly different labeling systems were utilized to assess the methylation of uterine tRNA. Whereas the [*methyl*-^{14}C]methionine and [U-^{14}C]guanosine pulse–chase systems (systems A and B, Table I) were employed to determine what types of *methyl*-^{14}C-methylated and methylated [U-^{14}C]guanine constituents appear in uterine tRNA, an additional [*methyl*-^{14}C]- or [^{3}H]methionine pulse in the presence of actinomycin D (system C) was constructed to assess the extent to which an existing population of precursor-tRNA continues to become methylated in the

Table I: Incorporation of [U-^{14}C]Guanosine and [*methyl*-^{14}C]Methionine into Uterine tRNA; Effects of Estradiol Stimulation.[a]

Labeling Systems	cpm Incorporated into tRNA/Uterus ± SD after Estradiol Pretreatment in Vivo (hr)				
	0 (Control)	1	6	12	24
A. [*methyl*-^{14}C]Methionine	2670 ± 210	3600 ± 260	11,800 ± 630	5800 ± 280	3330 ± 290
B. [U-^{14}C]Guanosine	2430 ± 170		8,500 ± 760		
C. [*methyl*-^{14}C]Methionine plus actinomycin D[b]	510 ± 30	840 ± 60	2,340 ± 190	1160 ± 160	600 ± 50
Ratio (A/B)	1.10		1.39		
Ratio (C/A)	0.19	0.18	0.20	0.20	0.18

[a] Triplicate groups of uteri were incubated with the appropriate isotopes and subsequently processed for isolation of tRNA and determination of isotope incorporation as described under Experimental Procedures. [b] Preliminary labeling experiments with [5-^{3}H]uridine in the presence and absence of actinomycin D indicated that the concentration of antibiotic employed (20 μg/uterus) was sufficient to inhibit the incorporation of [5-^{3}H]uridine into tRNA by greater than 96%.

Table II: Distribution of *methyl*-^{14}C-Methylated Constituents of Uterine tRNA; Effects of Estradiol Stimulation.[a]

Radioact. Accounted for as	% of [*methyl*-^{14}C]tRNA after Estradiol Pretreatment in Vivo (hr)				
	0 (Control)	1	6	12	24
1. 1-Methyladenine	11.4 ± 0.81	11.8 ± 0.62	11.0 ± 0.67	11.8 ± 1.02	12.0 ± 0.95
2. 2-Methyladenine	ND[b]	ND	ND	ND	ND
3. N^6-Methyladenine	ND	ND	ND	ND	ND
4. N^6,N^6-Dimethyladenine	ND	ND	ND	ND	ND
5. 1-Methylhypoxanthine	1.5 ± 0.20	1.6 ± 0.08	1.7 ± 0.1	1.6 ± 0.19	1.7 ± 0.09
6. 1-Methylguanine	7.4 ± 0.52	7.5 ± 0.60	7.4 ± 0.28	7.4 ± 0.57	7.2 ± 0.60
7. 7-Methylguanine	6.7 ± 0.45	6.7 ± 0.47	6.8 ± 0.52	6.5 ± 0.28	6.7 ± 0.36
8. N^2-Methylguanine	11.4 ± 0.72	11.5 ± 0.51	12.0 ± 0.59	11.6 ± 0.58	11.2 ± 0.63
9. N^2,N^2-Dimethylguanine	13.8 ± 1.10	14.0 ± 0.81	14.2 ± 1.07	14.0 ± 0.92	13.8 ± 0.85
10. 3-Methylcytosine	2.8 ± 0.23	3.0 ± 0.22	2.6 ± 0.18	3.0 ± 0.23	2.8 ± 0.20
11. 5-Methylcytosine	16.4 ± 1.55	15.9 ± 0.88	15.9 ± 1.33	17.1 ± 0.93	15.9 ± 1.06
12. 3-Methyluracil	ND	ND	ND	ND	ND
13. 5-Methyluracil	9.9 ± 1.02	9.4 ± 0.76	9.8 ± 0.74	8.9 ± 0.57	10.3 ± 0.83
14. 2′-*O*-Methylribose	13.2 ± 1.48	13.7 ± 1.61	12.4 ± 1.20	14.1 ± 1.53	13.5 ± 0.88
Methylated purines (1–9)	52.1	53.1	53.1	52.9	52.6
Methylated pyrimidines (10–13)	29.1	28.3	28.3	29.0	29.0
Methylated bases (1–13)	81.2	81.4	81.4	81.9	81.6
Methylated constituents (1–14)	94.4	95.1	93.8	96.0	95.1

[a] Aliquots of salt-fractionated uterine tRNA preparations previously labeled with [*methyl*-^{14}C]methionine (i.e., 90-min pulse, 60-min chase) were processed for determination of the distribution of *methyl*-^{14}C-methylated constituents as outlined under Experimental Procedures. [b] ND, not detected or less than 0.5% of [*methyl*-^{14}C]tRNA. Methylated base values represent the average of triplicate determinations with standard deviations as shown. The sum of the radioactivity recovered as methylated bases exceeded 10,500 cpm in all instances.

absence of further tRNA synthesis. As shown in Table I, appreciable quantities of isotope were incorporated into tRNA under each of these labeling systems. Furthermore, the extent of isotope incorporation in each instance was increased significantly in those uteri pretreated in vivo with estradiol. For example, the incorporation of *methyl*-^{14}C groups (system A, Table I) into tRNA progressively increased during the initial 6 hr following hormone treatment, reaching a maximum at this time (approximately 400% of control values), and steadily declined thereafter to 225 and 130% at 12 and 24 hr, respectively. Evidence that these values reflect net increases in tRNA methylation rather than estrogen-induced fluctuations (increases) in the specific activities of the [*methyl*-^{14}C]methionine and *S*-adenosyl-[*methyl*-^{14}C]methionine pools has previously been provided by Munns and Katzman (1971a). Other data presented in Table I further indicate that these observed increases in tRNA methylation reflect to a large extent an increased rate of tRNA synthesis which accompanies the early stages of the estrogen response as shown by (a) the 350% increase in the incorporation of [U-^{14}C]guanosine into uterine tRNA 6 hr after initiation of the estrogen response and (b) by the 80% inhibition of tRNA methylation in the presence of an inhibitor of tRNA synthesis (Table I, ratio C/A).

If for the moment it is assumed that the incorporation of *methyl*-^{14}C groups and [U-^{14}C]guanosine reflects accurately the methylation and synthesis of uterine tRNA, respectively, then the ratio of the incorporation of these isotopes would provide a means of assessing the extent of methylation of tRNA accompanying the estrogen response. Analyses such as these are presented in Table I (ratio A/B, i.e. [*methyl*-^{14}C]tRNA/[U-^{14}C]guanosine tRNA) and seem to indicate that the tRNA synthesized 6 hr following estrogen treatment acquires approximately 25% more methyl groups than that synthesized in the unstimulated uterus (1.39/1.10 = 1.26). Although the above data suggest the occurrence of a hypermethylation phenomenon, an inherent difficulty associated with this type of measurement relates to the variability in the specific activity of labeled nucleoside precursors (Munns and Katzman, 1971a).

Distribution of the Radioactive Methylated Constituents of Uterine tRNA. To assess more fully the results presented in Table I, the patterns of radioactive methylated and nonmethylated constituents of the tRNA preparations were determined. Listed in Table II are the resulting distributions of *methyl*-^{14}C-methylated products derived from tRNA preparations previously labeled with [*methyl*-^{14}C]methionine. In each instance greater than 90% of the isotope incor-

Table III: Extent of Methylation of the [U-^{14}C]Guanine Residues of Uterine tRNA; Effects of Estradiol Stimulation.[a]

Recovered as	% of Total [U-^{14}C]Guanine Radioact. Recovered from tRNA Hydrolysates after Estradiol Pretreatment in Vivo (hr). 0 (Control)	6
Guanine	90.71 ± 0.89	90.69 ± 1.21
1-Methylguanine	2.01 ± 0.20	2.15 ± 0.28
7-Methylguanine	2.03 ± 0.26	1.83 ± 0.17
N^2-Methylguanine	3.10 ± 0.18	3.23 ± 0.24
N^2,N^2-Dimethylguanine	2.15 ± 0.31	2.10 ± 0.28
meG/Σ(G + meG)[b]	9.29 ± 0.49	9.31 ± 0.58

[a] Aliquots of salt-fractionated uterine tRNA previously labeled with [U-^{14}C]guanosine as described in Table I were processed for determination of the distribution of [U-^{14}C]guanine constituents as outlined under Experimental Procedures. The sum of the radioactivity recovered as methylated guanines (meG) and guanine (G), i.e., Σ(G + meG) always exceeded 8800 cpm. [b] meG/(G + meG) represents that percentage of [U-^{14}C]guanines in tRNA which became methylated. Each value represents the mean of triplicate determinations with standard deviations as shown. These data in conjunction with those presented in Table II indicate that the tRNA of control and 6-hr estrogen-stimulated uteri acquire 6.99 ± 0.38 and 6.77 ± 0.30 methyl groups, respectively (see discussion in text).

porated into tRNA was recovered and identified as 1-methyladenine, 1-methylhypoxanthine, 1-, 7-, N^2-, and N^2,N^2-methylguanine, 3- and 5-methylcytosine, 5-methyluracil, and 2′-*O*-methylribose constituents (the latter representative of a collective estimate of 2′-*O*-[*methyl*-^{14}C]nucleosides). Even though small differences were noted in the percentages of radioactivity associated with individual methylated constituents between control and estrogen pretreated tRNA preparations, the accuracy of the methods employed for these determinations was such that no statistically significant differences were noted (i.e., differences of mean values, Student *t* test). Preliminary data regarding the identity and distribution of the individual 2′-*O*-[*methyl*-^{14}C]nucleosides present in similarly labeled tRNA preparations further supported the above conclusion that estrogen treatment had little, if any, effect on the pattern of methylated constituents (unpublished observations). It is interesting to note that the distribution of methylated constituents presented in Table II resembles both qualitatively and quantitatively those reported for the tRNA of other mammalian cell systems (Munns et al., 1974a,b; Randerath and Randerath, 1973; Klagsbrun, 1972; Iwanami and Brown, 1968).

Although the above results indicate that dramatic changes do not occur in the methylation patterns of tRNA syntheisized in the estrogen-stimulated uterus, they do not provide the necessary information to establish the extent of methylation of uterine tRNA. To provide an insight in this regard, tRNA preparations previously labeled with [U-^{14}C]guanosine were examined for their content of methylated [U-^{14}C]guanine constituents. These findings are presented in Table III and indicate that approximately 9.3% of the [U-^{14}C]guanine residues incorporated into the tRNA of both unstimulated and 6-hr estrogen-stimulated uteri became methylated. Further inspection of these data revealed that estrogen treatment did not significantly affect the relative proportions of methylated [U-^{14}C]guanine constituents (which were almost identical with those reported in Table II for the *methyl*-^{14}C-methylated guanines following the appropriate correction for N^2,N^2-dimethylguanine).

Table IV: Distribution of the *methyl*-^{3}H-Methylated Constituents Occurring on Uterine tRNA in the Absence of tRNA Synthesis; Effects of Estradiol Stimulation.[a]

Radioact. Accounted for as	% of [*methyl*-^{3}H] tRNA after Estradiol Pretreatment in Vivo (hr) 0 (Control)	6
1. 1-Methyladenine	8.2 ± 0.3	7.8 ± 0.5
2. 2-Methyladenine	ND[b]	ND
3. N^6-Methyladenine	ND	ND
4. N^6,N^6-Dimethyladenosine	ND	ND
5. 1-Methylhypoxanthine	0.8 ± 0.2	0.9 ± 0.3
6. 1-Methylguanine	3.2 ± 0.4	3.4 ± 0.5
7. 7-Methylguanine	3.7 ± 0.2	4.0 ± 0.4
8. N^2-Methylguanine	21.1 ± 1.4	20.9 ± 2.9
9. N^2,N^2-Dimethylguanine	7.3 ± 0.6	7.0 ± 0.5
10. 3-Methylcytosine	4.6 ± 0.4	4.7 ± 0.5
11. 5-Methylcytosine	14.0 ± 1.4	13.2 ± 1.1
12. 3-Methyluracil	ND	ND
13. 5-Methyluracil	7.0 ± 1.0	6.2 ± 0.7
14. 2′-*O*-Methylribose	25.6 ± 3.6	28.0 ± 3.4
Methylated purines (1–9)	44.3	44.0
Methylated pyrimidines (10–13)	25.6	24.1
Methylated bases (1–13)	69.9	68.1
Methylated constituents (1–14)	95.5	96.1

[a] Aliquots of salt-fractionated uterine tRNA previously labeled with [*methyl*-^{3}H] methionine in the presence of actinomycin D for 90 min prior to a 60-min chase period were processed for determination of the distribution of *methyl*-^{3}H-methylated constituents as described under Experimental Procedures. [b] ND, not detected or less than 0.5% of [*methyl*-^{3}H] tRNA. Values represent the mean of triplicate determinations with standard deviations as shown. The sum of the radioactivity recovered as methylated bases exceeded 10,200 cpm in all instances.

On the basis that mammalian tRNA contains approximately 24 guanine residues per molecule (Randerath and Randerath, 1973; Dayhoff and McLaughlin, 1972; Weinberg and Penman, 1968), the above results imply that an average of 2.23 guanine constituents become methylated per molecule of unfractionated tRNA (24 × 0.093 = 2.23). Correcting for the fact that N^2,N^2-dimethylguanine contains two methyl groups, the number of methyl groups incorporated into the [U-^{14}C]guanine residues of a tRNA molecule becomes 2.75 (i.e., 24 × [0.093 + 0.0215] = 2.75). This latter value, in conjunction with the percentage of *methyl*-^{14}C-methylated guanines in [*methyl*-^{14}C]tRNA (approximately 40%; see Table II), indicates that the tRNA of an unstimulated rat uterus acquires approximately 7 methyl groups per molecule of unfractionated tRNA synthesized ([100 − 39.3] × 2.75 = 6.99), while that of the 6-hr estrogen-primed uterus acquires 6.8 methyl groups ([100 − 40.4] × 2.74 = 6.77). It is important to note that the above estimation of the extent of methylation is based on the *distribution* of methylated and nonmethylated guanines from tRNA preparations previously labeled with [U-^{14}C]guanosine (Table III) and with [*methyl*-^{14}C]methionine (Table II) and hence is *independent* of the specific activities of the isotopic precursors employed (Munns and Sims, 1975b). On the other hand, the assessment of methylation based only on the *net incorporation* of these precursors into tRNA (data in Table I, i.e., the ratio [*methyl*-^{14}C]tRNA/[U-^{14}C]guanosine-tRNA) is *dependent* upon the specific activity of these isotopic precursors.

The data in Table IV present a third argument against excessive methylation of uterine tRNA accompanying estrogen stimulation by demonstrating that in the absence of

Table V: Kinetics of Methylation of Heterologous tRNA (*E. coli* B); Uterine Methylase Activities and the Effects of Estradiol Stimulation.[a]

Estradiol Pretreatment in Vivo (hr)	In Vitro Incubation (min)	Methylase Act.[b] cpm/μg of Protein[c]	Methylase Act.[b] cpm × 10^{-3}/uterus[c]
0 (control)	15	74 (100)	32.1 (100)
	30	156 (100)	67.7 (100)
	60	308 (100)	134 (100)
	90	420 (100)	182 (100)
	120	528 (100)	229 (100)
1	15	70 (95)	30.4 (95)
	30	156 (100)	67.7 (100)
	60	300 (97)	130 (97)
	90	404 (96)	175 (96)
	120	536 (102)	233 (102)
6	15	91 (123)	57.3 (162)
	30	188 (121)	118 (174)
	60	344 (112)	217 (162)
	90	432 (103)	272 (150)
	120	524 (99)	330 (144)
12	15	84 (114)	60.0 (169)
	30	172 (110)	123 (182)
	60	396 (129)	283 (211)
	90	504 (120)	360 (198)
	120	644 (126)	474 (207)
24	15	118 (159)	118 (368)
	30	244 (156)	244 (360)
	60	496 (161)	495 (369)
	90	628 (150)	627 (345)
	120	704 (133)	703 (307)

[a] Standard conditions associated with the in vitro methylation of *E. coli* B tRNA with uterine methyltransferase enzymes are described under Experimental Procedures. [b] Methylase activity: cpm/μg of protein represents the amount of radioactivity incorporated into *E. coli* B tRNA per μg of cytosol protein, while cpm/uterus reflects the total methylase activity present in the cytosol fraction per uterus. [c] Number in parentheses is the percent of control activity at the prescribed incubation time. Each value represents the mean of duplicate determinations (variation from the mean never exceeded ±12%).

further tRNA synthesis (i.e., using actinomycin D), the methylation of an existing population of precursor tRNA is not affected by estradiol pretreatment. This latter approach assumes that if estrogen-induced hypermethylation of mature tRNA existed, its expression would be disproportionately elevated in the absence of further tRNA synthesis, since the methylation associated with normal tRNA synthesis and processing decreases rapidly under these conditions (Munns and Sims, 1975a; Bernhardt and Darnell, 1969). Although estradiol pretreatment had no demonstrable effect on the types of *methyl*-^{3}H-methylated constituents formed, the presence of actinomycin D not only inhibited the synthesis and methylation of uterine tRNA by 96 and 80%, respectively (Table I), but also resulted in a marked redistribution in the types of *methyl*-^{3}H-methylated constituents formed (compare Tables II and IV). Results similar to the above have been recently reported in KB cells and interpreted on the basis that specific types of methylation products occur at selected times during tRNA processing (Munns et al., 1974a; Munns and Sims, 1975a). According to this interpretation, the disproportionate increases in the percent of radioactivity associated with N^2-methylguanine, 2′-*O*-methylribose constituents, and 3-methylcytosine in tRNA preparations labeled in the absence of tRNA synthesis reflect predominantly those methylations that occur during the late stages of tRNA processing, while the relative decreases in 1-, 7-, and N^2,N^2-methylguanines reflect those

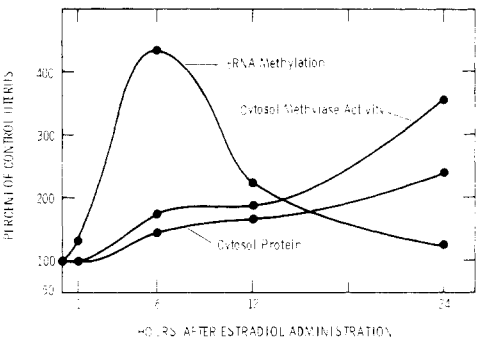

FIGURE 1: A summary of the effects of estradiol on the methylation of tRNA, cytosol tRNA methylase activity, and accumulation of cytosol protein in the immature rat uterus. The extent of methylation of tRNA was determined as described in Table I and cytosol protein (i.e., the protein in 100,000*g* supernatants of uterine homogenates) was determined by the method of Lowry et al. (1951). Cytosol methylase activities were determined as described in Table V and reflect the total methylase activity present in the cytosol fraction per uterus following a 30-min in vitro incubation.

methylation products that occur primarily during the early stages of processing. Although not presented in Table IV, findings similar to those reported above were obtained with uteri pretreated with hormone for 2, 12, and 24 hr.

Assessment of Uterine tRNA Methylase Activities and the Effects of Estradiol Stimulation. In view of the findings presented in Table I regarding the estrogen-induced increases in uterine tRNA synthesis and methylation, it was of interest to determine if there was a concomitant increase in tRNA methyltransferase activity. Thus, following the appropriate periods of estradiol stimulation in vivo, the methylase activities of uterine cytosol fractions (100,000*g* supernatants) were assessed by their ability to transfer the *methyl*-^{14}C group of *S*-adenosyl[*methyl*-^{14}C]methionine to *E. coli* B tRNA. The results of this study are presented in Table V and indicate that uterine methylase activities progressively increased during the first 24 hr following estrogen treatment. Since an accompanying increase in the content of uterine cytosol protein occurred during the estrogen response (see Figure 1), methylase activities were equated both on the basis of radioactivity incorporated into *E. coli* B tRNA per microgram of cytosol protein (counts per minute/microgram of protein) as well as per uterus (counts per minute/uterus). Whereas the former reflects methylase activity relative to an increased accumulation of cytosol protein, the latter represents the total methylase activity contained in the cytosol fraction per uterus. Thus, as presented in Table V, not only was there a significant increase in total methylase activity following hormone treatment (approximately 250 and 350% of control activities at 12 and 24 hr, respectively), but also, this increase was disproportionately greater than the increased accumulation of cytosol protein.

Further analysis of the *methyl*-^{14}C-methylated products derived from these in vitro assays indicated that five methylase activities associated with the formation of 1-methyladenine, 1-, N^2-, and N^2,N^2-methylguanines, and 5-methylcytosine were expressed in significant quantities (Table VI). Although estrogen pretreatment increased total methylase activity (Table V), the distribution of the activities associated with the formation of the above five constituents remained proportional as reflected by their invariant distribution (Table VI).

Summary and Conclusions

Figure 1 illustrates the estrogen-induced increases in

Table VI: In Vitro Expression of Cytoplasmic tRNA Methyltransferase Activities from Estradiol-Stimulated Rat Uteri; Distribution of *methyl*-^{14}C-Methylated Constituents following Heterologous Methylations of *E. coli* B tRNA.[a]

Radioact. Accounted for as	% of [*methyl*-^{14}C] tRNA after Estradiol Pretreatment in Vivo (hr)				
	0 (Control)	1	6	12	24
1. 1-Methyladenine	22.5	24.3	24.7	26.5	23.0
2. 2-Methyladenine	ND[b]	ND	ND	ND	ND
3. N^6-Methyladenine	ND	ND	ND	ND	ND
4. N^6,N^6-Dimethyladenine	ND	ND	ND	ND	ND
5. 1-Methylhypoxanthine	ND	ND	ND	ND	ND
6. 1-Methylguanine	19.6	21.2	19.2	17.3	17.1
7. 7-Methylguanine	1.0	1.3	0.9	1.0	0.7
8. N^2-Methylguanine	9.9	9.7	10.8	10.1	11.0
9. N^2,N^2-Dimethylguanine	11.1	8.5	9.3	8.6	10.7
10. 3-Methylcytosine	ND	ND	ND	ND	ND
11. 5-Methylcytosine	25.4	28.5	28.8	29.0	27.9
12. 3-Methyluracil	ND	ND	ND	ND	ND
13. 5-Methyluracil	1.1	1.7	1.4	1.4	1.8
14. 2′-*O*-Methylribose	ND	ND	ND	ND	ND
Methylated purines (1–9)	64.1	65.0	64.9	63.5	62.5
Methylated pyrimidines (10–13)	26.5	30.2	30.2	30.4	29.7
Methylated constituents (1–14)	90.6	95.2	95.1	93.9	92.2

[a] In vitro assay of uterine tRNA methylase, isolation of heterologous tRNA, and determination of the distribution of radioactive methylated products as described under Experimental Procedures. [b] ND, not detected or less than 0.5% of [*methyl*-^{14}C] tRNA. Each value represents the mean of two experimental determinations with a variation from the mean of ±12% for values greater than 5.0. A minimum of 10,010 cpm was recovered as methylated bases from each experiment.

tRNA methylation, tRNA-methyltransferase activities, and uterine cytosol protein during the initial 24 hr following a single injection of estradiol into immature rats. In general, the data are in accord with those of a number of previous investigations which have demonstrated an enhanced synthesis of tRNA followed somewhat later by increased synthesis and accumulation of uterine protein (Mueller et al., 1958; Hamilton et al., 1968; Billing et al., 1969; Munns and Katzman, 1971a) and methyltransferase activity (Baliga and Borek, 1974). Of notable interest in Figure 1 was the finding that the increases in total uterine tRNA methylation and tRNA-methyltransferase activity were not coincident. For example, when tRNA methylation (which appears to be a direct reflection of tRNA synthesis; Munns and Katzman, 1971b) was maximal (430% of control, sixth hour), tRNA-methyltransferase activity was elevated only 175% of control activity. However, when these parameters were assessed at 12 and 24 hr, methylase activities were progressively increasing while tRNA methylation was decreasing. In view of these findings additional methylation parameters were assessed to determine if estrogen stimulation altered the type and/or amount of methylated constituents present in uterine tRNA. The latter included (1) the identification and distribution of methylated constituents present in uterine tRNA, (2) the extent to which these methylated constituents appear per molecule of unfractionated tRNA synthesized, and (3) characterization of uterine tRNA methylase activity. Results from these studies demonstrated, however, that estrogen treatment did not significantly alter either the type (Table II) or amount (Tables II and III) of methylated constituents appearing on newly synthesized tRNA. Additional evidence in this regard was provided by the inability of hormone stimulated uteri to demonstrate either altered patterns of methylation (relative to control patterns) in the absence of further tRNA synthesis (Table IV) or differences in the types of tRNA-methyltransferase activities expressed in vitro (Table VI).

Although these results imply that gross changes in the methylation of tRNA are absent in the estrogen-stimulated uterus, it is equally important to note that the procedures employed in the present investigation would not detect dramatic changes in the methylation of a single or small number of discrete tRNA species. This latter uncertainty results from both the inability of existing methodologies to provide suitable criteria for determining what tRNAs (if any) are aberrantly modified as well as the appropriate fractionation procedures required for their isolation and subsequent characterization.

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Transcription of Fractionated Calf Thymus Chromatin by RNA Polymerase of Calf Thymus and *Escherichia coli*†

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ABSTRACT: Calf thymus chromatin has been sheared and fractionated on sucrose gradients. Approximately 5–10% of the chromatin is resolved from the bulk of the input chromatin as a slowly sedimenting fraction. The protein/DNA ratio of the slowly sedimenting fraction is not greatly different from the protein/DNA ratio of the more rapidly sedimenting chromatin fraction. Analysis of DNA of the chromatin fractions by CsCl equilibrium density gradient centrifugation indicates that DNA of the slowly sedimenting fraction is depleted in the satellite DNA banding at 1.716 g/cm^3. The template properties of the chromatin fractions have been examined with *Escherichia coli* RNA polymerase and with form II and form III RNA polymerases of calf thymus. At rate-limiting concentrations, the slowly sedimenting fraction is twofold more active than the rapidly sedimenting fraction as a template for *E. coli* RNA polymerase. Homologous form II and form III RNA polymerases are respectively 30-fold and 16-fold more active with the slowly sedimenting fraction than with the rapidly sedimenting fraction. The activity of form II RNA polymerase in transcribing the slowly sedimenting fraction exceeds its activity in transcribing an equal concentration of native DNA. Kinetic studies, in which RNA polymerase activity is assayed at various concentrations of chromatin, indicate that the greater activity of *E. coli* RNA polymerase with the slowly sedimenting fraction is due to an increased rate of transcription at saturating concentrations of template (V_{max}), and is not due to a lower concentration required for half-maximal rate of transcription (K_m). In contrast, the increased rates of transcription of the slowly sedimenting chromatin fraction by the homologous polymerases are due to a decrease in concentration required for half-maximal rate of transcription rather than an increased rate of transcription at saturating concentrations of template. The relative decrease of satellite DNA in the slowly sedimenting fraction of chromatin and the enhanced template activity of the slowly sedimenting fraction suggest that this fraction is equivalent to nuclear euchromatin while the more rapidly sedimenting chromatin is equivalent to nuclear heterochromatin.

Chromatin is a complex of DNA, histones, non-histone proteins, and possibly RNA, the interactions of which are believed to restrict transcription to only a selected set of genes in differentiated cells. On the microscopic level, genes selected for active transcription are visualized as euchromatin while genes which are inactive, either facultatively or constitutively, are visualized as highly condensed heterochromatin (see review by Frenster, 1974). These visualized structural differences between active and inactive chromatin have led to a number of experiments in which chromatin has been separated into portions active and inactive for transcription in vitro.

In vitro studies of transcription of chromatin have been performed on chromatin fractionated by differential centrifugation (Frenster et al., 1963; Chalkley and Jensen, 1968; Murphy et al., 1973; McCarthy et al., 1974), gel filtration (Janowski et al., 1972), differential solubility (Marushige and Bonner, 1971; Arnold and Young, 1974), and ion-exchange chromatography (Simpson, 1974). Except for the studies of Murphy et al. (1973) and Howk et al. (1975), *Escherichia coli* RNA polymerase (ribonucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) rather than homologous RNA polymerases was used.

Although isolated mammalian chromatin has been shown to yield tissue specific RNA when transcribed in vitro by *E. coli* RNA polymerase (Gilmour and Paul, 1973; Axel et al., 1973), it is reasonable to assume that at least some of the in vivo controls of transcription will not operate when chromatin is transcribed by bacterial RNA polymerase. In particular, controls affecting only one of the multiple forms of RNA polymerase found in mammalian cells (Roeder and Rutter, 1969) can be studied only by using homologous polymerases.

We have fractionated sheared calf thymus chromatin by sedimentation through sucrose gradients and have evaluated the fractions as templates for form II and form III RNA polymerases from calf thymus, as well as for *E. coli* RNA polymerase. Fractions which differ in template activity have been obtained and kinetic studies indicate that there are important qualitative as well as quantitative differences between *E. coli* and mammalian RNA polymerases in the

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