Developmental Changes in the Methylation of Silkmoth Follicular Epithelial Messenger Ribonucleic Acid†

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ABSTRACT: The methylation of silkmoth follicular epithelial messenger ribonucleic acid (mRNA) was investigated at different stages of follicular development. Follicles from the late prechorionating and four chorionating stages were incubated in organ culture for 2 h with [*methyl*-^{3}H]methionine. The resulting polysomal-derived, poly(A+) mRNAs were characterized with respect to both size and composition of methyl-^{3}H-methylated constituents. As evaluated by sucrose density gradient centrifugation, prechorion mRNAs were heterogeneous in size (7-30S) in contrast to the relatively homogeneous chorion mRNAs which sedimented within the 7–14S region of the gradient. Analysis of the methyl-^{3}H-labeled products of mRNA digests (nuclease-P_1 or RNase T_2) via DEAE-cellulose and thin-layer chromatographic techniques revealed the following information. First, on the basis of nuclease-P_1 digestion, the predominating 5′-terminal cap structure in both prechorion (greater than 70%) and chorion (greater than 90%) mRNAs was m^{7}G(5′)ppp(5′)A^m, with minimal amounts of m^{7}GpppG^m and m^{7}GpppC^m (prechorion) and m^{7}Gpppm^{6}A^m (chorion) also detected. Second, on the basis of RNase-T_2 digestion, the ratio of cap type 1 structures (m^{7}GpppN^{m}pNp) to cap type 2 structures (m^{7}GpppN^{m}pN^{m}pNp) was 1:1 and 7:1 for prechorion and chorion mRNAs, respectively. Additionally, whereas prechorion mRNAs possessed an average of two internal m^{6}A residues per cap, chorion mRNAs were deficient in this or other internal methylated nucleosides, i.e., less than 0.1 m^{6}A/mRNA. As measured by the extent of incorporation of [^{3}H]methyl groups into 5′-terminal m^{7}GpppN^m structures, the synthesis of poly(A+) mRNA progressively increased during follicular development, ultimately exceeding the prechorion value by 20-fold. Collectively, these results indicate that significant alterations in the methylation patterns of silkmoth mRNA accompany transitions in the types and amounts of mRNA expressed during follicular development.

Posttranscriptional methylation of eukaryotic and viral mRNAs is a well-established phenomenon. As reviewed by Shatkin (1976) and Revel & Groner (1978), the bulk of methylated nucleotide products appear at the 5′ terminus of mRNAs as a unique m^{7}G-containing cap structure (m^{7}GpppN^m and m^{7}GpppN^{m}pN^m).[1] In addition to these methylated caps, many mRNAs contain a limited number of internal m^{6}A residues (Revel & Groner, 1978). While a variety of investigations (Both et al., 1975a,b; Muthukrishnan et al., 1975; Hickey et al., 1976; Chu & Rhoads, 1978; Munns et al., 1979a) have indicated the m^{7}G-containing caps are facilitory (if not obligatory) components for initiating mRNA translation, the functional significance of the internal m^{6}A nucleosides remains obscure (Revel & Groner, 1978). Because of their conservation during mRNA processing, it has been suggested that these latter methylated constituents provide recognition sites for various RNA processing and/or splicing enzymes (Revel & Groner, 1978; Canaani et al., 1979).

The kinetics of mRNA methylation (Frederici et al., 1976) and the sequence of reactions resulting in methylated cap structures (Ensinger et al., 1975; Moss et al., 1976) have been investigated. However, no information is available regarding the potential alterations that may occur in the methylation of mRNAs synthesized during the developmental career of a highly differentiated tissue. We have examined, therefore, the patterns of methylation of mRNAs derived from silkmoth epithelial cells at specific developmental stages (Paul et al., 1972; Paul & Kafatos, 1975; Gelinas & Kafatos, 1977). This particular system offers several advantages: (a) During any follicular developmental stage, only a limited number of mRNAs are expressed. (b) Clearly defined transitions in mRNA expression are observed during the course of differentiation. (c) Differentiating follicles are arranged such that representatives of each developmental stage are available from a single silkmoth pupa. (d) Follicles can be removed and maintained in organ culture for several hours without interruption to their development. The latter also permits mRNA to be labeled to high specific activities with a variety of isotopic precursors including [*methyl*-^{3}H]methionine.

Experimental Procedures

Organ Cultures. Prechorionating follicles (numbers −5 to 0) and chorionating follicles (+1 to +8) were staged according to Paul et al. (1972), dissected, and incubated in organ cultures supplemented with [*methyl*-^{3}H]methionine (Munns et al., 1979a). For determinations of rates of RNA and protein synthesis in organ culture, [5,6-$^3H_2$]uridine or L-[4,5-$^3H_2$]-leucine was substituted for [*methyl*-^{3}H]methionine. In these latter experiments, the incorporation of [^{3}H]leucine and [^{3}H]uridine into trichloroacetic acid precipitable radioactivity was linear throughout a 4-h labeling period (data not shown). This information provides evidence as to the intactness of both transcriptional and translational systems of silkmoth follicles

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[1] Abbreviations used: RNase, ribonuclease; $NaDodSO_4$, sodium dodecyl sulfate; m^{7}G, 7-methylguanosine; m^{6}A, N^6-methyladenosine; pN and pN^m, 5′-phosphates of nucleoside and 2′-*O*-methyl nucleoside; pm^{7}G, pm^{6}A, pm^{6}A^m, and pm^{5}U, 5′-phosphates of 7-methylguanosine, N^6-methyladenosine, N^6,2′-*O*-dimethyladenosine, and 5-methyluridine; pG^m, pC^m, pU^m, and pA^m, 5′-phosphates of 2′-*O*-methylguanosine, -cytidine, -uridine, and -adenosine; m^{7}GpppN^m, m^{7}G(5′)ppp(5′)N^m.

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thoughout a 4-h incubation period.

Isolation of mRNA. The mRNA present in follicles previously labeled in organ culture with [*methyl*-^{3}H]methionine was isolated by initially preparing polysome pellets (Munns et al., 1979a). Pellets were solubilized in 10 mM Tris buffer (pH 7.5) containing 1% $NaDodSO_4$ and phenol extracted by the method of Palmiter (1974), and the RNA was isolated by ethanol precipitation. Polysomal RNA was dissolved in 10 mM Tris buffered (pH 7.5) 1 mM NaEDTA containing 0.2% $NaDodSO_4$, heated briefly to 90 °C (Desrosiers et al., 1975), quickly chilled on ice, and supplemented with 4 M NaCl to yield a final concentration of 0.25 M NaCl. In selecting for poly(A+) mRNA, the above heat-denatured preparation was warmed to 25 °C and applied to an oligo(dT)–cellulose (Collaborative Research, Inc., Type 2) column (4 × 10 mm) previously equilibrated with binding buffer (0.25 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM NaEDTA, and containing 0.2% $NaDodSO_4$). Following exhaustive washing with binding buffer, poly(A+) mRNA was eluted with 0.2% $NaDodSO_4$. Both heat denaturization and the oligo(dT)–cellulose chromatographic process were repeated. In this manner, the binding of poly(A+) prechorion and chorion mRNAs to oligo(dT)–cellulose was judged to be quantitative; i.e., greater than 90% was retained at 0.25 M NaCl. As evaluated by $NaDodSO_4$–sucrose gradient centrifugation and by analysis of methyl-^{3}H-methylated nucleotides, the above isolation procedure yielded mRNAs free of detectable rRNA and/or tRNA contaminants.

For assessment of the recovery of mRNA from prechorion and chorionating follicles, two approaches were employed. First, on the basis of three independent and identical labeling experiments, the amount of [*methyl*-^{3}H]methionine-labeled mRNA recovered from polysomes varied less than 14% of the mean value (data expressed as counts per minute of mRNA per 10 follicles). Second, techniques including phenol extraction and ethanol precipitation of mRNA as well as its adsorption to and elution from oligo(dT)–cellulose were greater than 90% efficient as monitored by exogenous [^{3}H]uridine-labeled chorion mRNA. These studies indicated that the recovery of mRNA from both prechorion and chorionating follicles is comparable and therefore permit an assessment of the extent of methylation of mRNA during follicular development.

Analysis of Methyl-^{3}H-Methylated Nucleotides and Caps. [*methyl*-^{3}H]Methionine-labeled poly(A+) mRNAs were digested with ribonuclease-T_2 or nuclease-P_1 (Munns et al., 1979a) and the resulting methyl-^{3}H-labeled mono- and oligonucleotides characterized by DEAE-cellulose (Munns et al., 1979a) and/or thin-layer chromatographic techniques. Details regarding salt-gradient, DEAE-cellulose chromatography appear in the legend of Figure 3.

Identification of methylated bases derived from formic acid digestion of RNA have been described elsewhere (Munns et al., 1974, 1979b). Briefly, these procedures consisted of hydrolyzing methyl-^{3}H-labeled mono- and oligonucleotides with 88% formic acid for 2 h at temperatures of 100 and 185 °C and separation of the resulting methylated bases via thin-layer chromatographic techniques.

Additional thin-layer chromatographic techniques were developed to permit the complete resolution of 5′-,methylated nucleotides and m^7G-containing cap structures (m^7GpppN^m) generated during enzymatic digestion of mRNAs with nuclease-P_1. Initially, the [*methyl*-^{3}H]methionine-labeled mRNA was concentrated by ethanol precipitation in the presence of 5–10 μg of unlabeled RNA. It was redissolved in sodium acetate buffer (10 mM, pH 5.0) containing 10–20 μg of nuclease-P_1. Upon complete enzymatic digestion of the RNA (1 h at 37 °C), digests were evaporated under N_2 and redissolved in water (25–50 μL) containing 1–2 μg of authentic methylated nucleotide and cap standards (P-L Biochemicals), and 15–25-μL aliquots were applied to precoated cellulose thin-layer plates. Upon completion of two-dimensional development (see Figure 1 for additional details including solvent systems), the positions of the standard nucleotides and caps were located with the aid of a UV lamp. The cellulose adsorbent in each of the identified areas was recovered and transferred to scintillation vials containing 1.0 mL of 10% perchloric acid. Vials were incubated at 70 °C for 30 min prior to cooling and the addition of 9.0 mL of aqueous scintillant (PCS, Amersham Searle) for determination of radioactivity. Additional 5-μL aliquots of the RNA digest (not chromatographed) were processed similarly to determine the quantities of radioactivity applied to and recovered from thin-layer plates. In the present study, the recovery of radioactivity ranged from 83% to 92%.

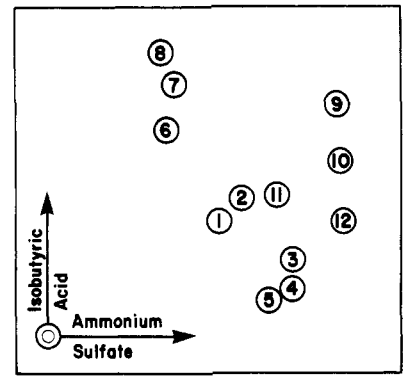

FIGURE 1: Separation of mononucleotides and m^7GpppN^m caps via thin-layer chromatography. This figure is a schematic representation of seven mononucleotides and five m^7GpppN^m cap standards resolved by two-dimensional development on precoated cellulose thin-layer plates (20 × 20 cm; Polygram Cell 400 UV_{254}; Macherey-Nagel and Co.). Nuclease-P_1 digests of [*methyl*-^{3}H]methionine-labeled mRNA containing 1–2 μg of each mononucleotide and cap standard were chromatographed in the direction indicated in isobutyric acid/acetone/glacial acetic acid/water/ammonium hydroxide (50:15:10:20:2 v/v). Plates were then air-dried (1–2 h) and developed in the second dimension in saturated ammonium sulfate/1 M sodium acetate/2-propanol (40:9:2 v/v). The positions of the standard mononucleotides and m^7GpppN^m caps were located with the aid of a UV lamp (UVS-11, Mineralight). The 12 standards and origin are represented numerically as follows: 0, origin; 1, m^7GpppA^m; 2, $m^7Gpppm^6A^m$; 3, m^7GpppC^m; 4, m^7GpppU^m; 5, m^7GpppG^m; 6, pm^6A; 7, pA^m; 8, pm^6A^m; 9, pC^m; 10, pU^m; 11, pG^m; 12, pm^5U.

The separation of seven methylated mononucleotides and five m^7GpppN^m cap standards is illustrated in Figure 1. The resolution of these constituents is reproducible with the exception that m^7GpppU^m (spot 4) and m^7GpppG^m (spot 5) are not resolved completely in all developments. Because of this problem and for superior resolution of all constituents, chromatographic development in the first dimension (i.e., the isobutyric acid system; see Figure 1) was conducted in a continuous manner as described by Munns et al. (1974).

Results

Size Characterization of Prechorion and Chorion mRNAs. The decrease in size and altered abundance of follicular proteins which accompany the developmental transitions between prechorinating and chorinating periods in silkmoth follicles (Paul & Kafatos, 1975) prompted us to determine if similar changes were apparent in polysomal-derived, poly(A)-containing mRNA preparations. Accordingly, follicles repre-

Table I: Identification and Distribution of Methyl-^{3}H-Labeled Mononucleotides and Methyl-^{3}H-Labeled Caps from Nuclease-P_1 Digests of Prechorion and Chorion mRNAs[a]

methylated constituents	% of total radioactivity applied to thin-layer plates					
	methyl-^{3}H-labeled mononucleotides		[*methyl*-^{3}H]m^{7}GpppN^m		unfractionated mRNA digests	
	prechorion	chorion	prechorion	chorion	prechorion	chorion
pA^m	6.4	8.2	ND	ND	3.0	2.0
pG^m	4.5	ND	ND	ND	2.0	ND
pC^m	ND[b]	ND	ND	ND	ND	ND
pU^m	16.3	58.4	ND	ND	7.9	8.2
pm^{6}A	65.2	21.3	ND	ND	39.8	3.5
pm^{6}A^m	ND	ND	ND	ND	ND	ND
pm^{5}U	ND	ND	ND	ND	ND	ND
m^{7}GpppA^m	ND	ND	64.4	86.1	27.9	71.8
m^{7}Gpppm^{6}A^m	ND	ND	ND	5.7	ND	4.6
m^{7}GpppG^m	ND	ND	17.2	ND	7.7	ND
m^{7}GpppC^m	ND	ND	7.0	ND	2.8	ND
m^{7}GpppU^m	ND	ND	ND	ND	ND	ND

[a] Nuclease-P_1 digests of prechorion and chorion mRNA were resolved into mononucleotides and m^{7}GpppN^m caps by DEAE-cellulose chromatography. Each fraction was recovered, desalted, and chromatographed in two dimensions on thin-layer chromatographic plates (Figure 1). Additional details regarding thin-layer chromatography and recovery of radioactive components are described under Experimental Procedures. Counts per minute data were obtained by 20-min counting cycles and the recovery of radioactivity from thin-layer chromatographs was approximately 90% of the total radioactivity (1500–4000 cpm) applied to each thin-layer plate. [b] ND, not detectable or less than 2% of the total radioactivity applied.

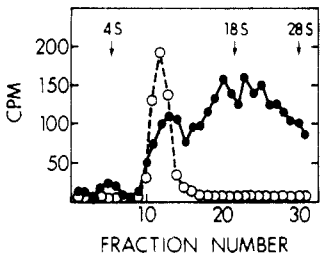

FIGURE 2: Analysis of prechorion and chorion mRNAs by sucrose density gradient centrifugation. Characterization of polysomal-derived, poly(A)-containing prechorion (closed circles) and chorion (opened circles) mRNA via NaDodSO$_4$–sucrose gradient centrifugation (linear 15–30% sucrose gradients). For details of this procedure as well as the isolation of methyl-^{3}H-labeled mRNAs, refer to Experimental Procedures and Munns et al. (1979a). Arrows depict the sedimentation of tRNA (4 S) and rRNA (18 S) standards in comparable gradients. The absorbance profiles (A_{254}), not shown, were similar to the above radioactivity profiles.

sentative of prechorionating (−5 to 0) and total chorinating (+1 to +8) stages were dissected and labeled in organ culture for 2 h with [*methyl*-^{3}H]methionine, and the methyl-^{3}H-labeled mRNAs were isolated and analyzed by density gradient centrifugation. As evaluated by the resulting profiles of radioactivity in Figure 2, prechorion mRNAs (solid circles) were heterogeneous in size (7–30 S), in contrast to a relatively discrete chorion mRNA population (opened circles) which sedimented within the 7–14S region of the gradient. These data are consistent with the observations of Paul & Kafatos (1975) and our unpublished observations (C. S. Morrow and J. R. Hunsley) regarding the size and distribution of proteins synthesized at these stages of follicular development. With respect to mRNA purity, further inspection of the sedimentation profile of chorion mRNA implied that these preparations were neither degraded nor contaminated with significant amounts of tRNA (4 S) and rRNA (18 and 28 S).

Characterization of the Methylated Constituents. Because the data in Figure 2 suggest significant alterations in the expression of mRNAs during follicular development, experiments were extended to determine the types and amounts of methyl-^{3}H-labeled constituents present in these mRNA preparations. This was accomplished by characterizing the products of mRNA digests derived from nuclease-P_1 digestion via DEAE-cellulose and thin-layer chromatographic techniques. DEAE-cellulose chromatography of both prechorion and chorion mRNA digests (not shown) indicated that all of the methyl-^{3}H-labeled constituents in both digests coeluted with either the mononucleotide (pA) or cap (m^{7}GpppA) standards. While this result demonstrated the completeness of the nuclease-P_1 digestion step, more importantly it revealed that the distribution of methyl-^{3}H mononucleotides and caps was significantly different when the prechorion and chorion mRNA digests were compared. Thus, approximately 60% and 40% of the radioactivity in the prechorion digest eluted as mononucleotides and caps, respectively, whereas the bulk of radioactive products in the chorion digest coeluted with cap structures (~80%).

For identification and further quantitation of the individual methyl-^{3}H-labeled mononucleotides and methyl-^{3}H-labeled caps, the appropriate fractions were recovered and separated by two-dimensional, thin-layer chromatography. These results appear in Table I and indicate that the predominating methyl-^{3}H-labeled mononucleotides in prechorion and chorion mRNA are pm^{6}A and pU^m, respectively, while the major methyl-^{3}H-labled cap is m^{7}GpppA^m. Other methyl-^{3}H-labeled components identified in these digests include pA^m and m^{7}Gpppm^{6}A^m (chorion mRNA) and pA^m, pG^m, m7GpppG^m, and m^{7}GpppC^m (prechorion mRNA).

The absence of methyl-^{3}H-labeled caps in the mononucleotide fractions (and vice versa) provides sufficient evidence that these components are completely resolved from one another during thin-layer chromatography. In view of this finding and to obtain a more accurate distribution of all the methyl-^{3}H-labeled constituents present, unfractionated digests of prechorion and chorion mRNA were separated by identical thin-layer techniques. These results, also presented in Table I, confirmed and extended the findings originally obtained by DEAE-cellulose chromatography. Specifically, whereas greater than 50% of the radioactive methyl groups incorporated into prechorion mRNA were identified as pm^{6}A and pN^m mononucleotides, the bulk of the radioactivity incorporated into chorion mRNA was identified as m^{7}GpppN^m cap structures (~75%).

These data, however, provided no information as to whether the methyl-^{3}H-labeled mononucleotides (pN^m and pm^{6}A) are located adjacent to the m^{7}GpppN^m cap (i.e., m^{7}GpppN^{m}pN^m) or within internal regions of the mRNA molecule. For dif-

Table II: Composition of 2′-*O*-Methyl Nucleotides (pN^m) in m^7G-Containing Caps of Prechorion and Chorion mRNAs[a]

	% of total [*methyl*-^{3}H]pN^m				
prechorion mRNA	pA^m	pm^6A^m	pG^m	pC^m	pU^m
$m^7GpppN^{m'}$ where $pN^{m'}$ is:	72.7		19.4	7.9	
$m^7GpppN^{m'}pN^{m''}$ where $pN^{m''}$ is:	23.5		16.5		60.0
chorion mRNA					
$m^7GpppN^{m'}$ where $pN^{m'}$ is:	93.8	6.2			
$m^7GpppN^{m'}pN^{m''}$ where $pN^{m''}$ is:	12.3				87.7

[a] The information appearing in the above table is derived from data regarding the distribution of methyl-^{3}H-labeled mononucleotides and methyl-^{3}H-labeled caps appearing in Table I and Figure 3.

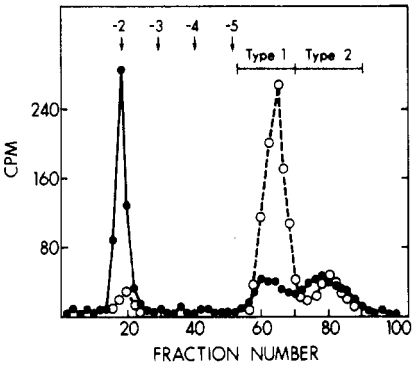

FIGURE 3: Elution profile of radioactivity obtained by linear salt gradient (0.05–0.20 M NaCl) DEAE-cellulose chromatography of RNase-T_2 digested prechorion (closed circles) and chorion (opened circles) mRNA preparations. The mono- (−2), di- (−3), tri- (−4), and tetraphosphates (−5) of adenosine were included as reference standards. Radioactivity eluting with 5′-AMP standard (−2) and those defined as type 1 and type 2 oligonucleotides were recovered, desalted, and hydrolyzed in formic acid. The resulting methyl-^{3}H-methylated bases and [*methyl*-^{3}H]N^m products were identified via thin-layer chromatographic techniques [see Experimental Procedures and Munns et al. (1979a,b)]. These data (not shown) revealed that all of the radioactivity coeluting with mononucleotides (fractions 18–20) was [*methyl*-^{3}H]pm^6A. Greater than 90% of the radioactivity present in type 1 and type 2 oligonucleotides was identified as [*methyl*-^{3}H]pm^7G (40–50%) and [*methyl*-^{3}H]pN^m (50–60%) nucleotides.

ferentiation between these possibilities, prechorion and chorion mRNA were digested with RNase T_2 and the resulting mononucleotides and m^7G-containing caps characterized by DEAE-cellulose chromatography. These results are presented in Figure 3 and revealed that approximately 45% of the radioactivity of prechorion mRNA digest eluted with the mononucleotide fraction (subsequently identified as [*methyl*-^{3}H]pm^6A; see legend to Figure 3), while greater than 95% of the methyl-^{3}H-labeled products of chorion mRNA digests eluted as m^7G-containing caps. Identification of oligonucleotides designated types 1 and 2 in Figure 3 as m^7GpppN^mpNp and m^7GpppN^mpNp is based upon (i) the release of [*methyl*-^{3}H]pm^7G after treatment with tobacco acid pyrophosphatase, (ii) formic acid hydrolysis data which indicated the presence of approximately equal quantities of [*methyl*-^{3}H]pm^7G and [*methyl*-^{3}H]pN^m constituents, and (iii) the immunospecific retention of these oligonucleotides with anti-m^7G antibodies (Munns et al., 1979a,b). The assignment of these structures as m^7G-containing caps is also supported by the results presented in Table I.

On the basis of the accumulated data in Figure 3 and Table I, the identity and distribution of the pN^m constituents of type 1 and type 2 caps are summarized in Table II. It is evident from this summary that the transition between prechorionating and chorionating periods is accompanied by marked changes in the composition of pN^m nucleotides adjacent to the 5′-terminal caps, notably, a loss of pG^m and pC^m nucleotides with the appearance of small quantities of pm^6A^m. Further, the ratio of type 1 to type 2 cap structures (calculated from data in Figure 3) increases from 1:1 in prechorion mRNA to 7:1 in chorion mRNA. Lastly, while prechorion mRNA contains an average of approximately two internal pm^6A residues, the amount of this internal methylated nucleotide in chorion mRNA appears insignificant, i.e., less than 0.1 pm^6A/mRNA during either a 2-h (Table I) or 4-h pulse (not shown) with [*methyl*-^{3}H]methionine.

Extent of Methyl-^{3}H Incorporation into Cap Structures as a Function of Developmental Stage. The possibility that additional transitions might occur during discrete stages of choriogenesis was also examined. In these experiments, groups of chorionating follicles representative of four discrete stages of development were labeled separately with [*methyl*-^{3}H]-methionine, and the chorion mRNAs were isolated as before. Following nuclease-P_1 digestion, the resulting methyl-^{3}H-labeled mononucleotides and [*methyl*-^{3}H]m^7GpppN^m caps were separated and identified by thin-layer chromatography. These results (not shown) revealed no significant alteration in the pattern of methylated constituents throughout the chorionating periods examined (follicles +1 to +8); i.e., the type and distribution of methyl-^{3}H-labeled constituents were identical with those reported in Tables I and II for total chorion mRNA. Consequently, changes in the distribution of methylated nucleotides during the chorionating period are absent or too minimal to be detected by the procedures employed in these investigations.

However, one change that was evident from the above investigation was the dramatic increase in the *extent* of incorporation of [^{3}H]methyl groups into the polysomal-derived, poly(A+) mRNA m^7GpppN^m caps during the course of follicular development. These changes appear in Figure 4 and revealed that the largest increase in the rate of cap methylation occurs during the transition between the prechorionating follicles (−5 to 0) and early chorionating (+1 to +2) stages. During later stages of choriogenesis, cap methylation progressively increases and ultimately exceeds the prechorion values by 20-fold. With evidence lacking to the contrary, it is assumed that the extent of incorporation of [^{3}H]methyl groups into these cap structures reflects the net rate of synthesis of mRNA molecules; i.e., approximately a 20-fold increase in capped mRNA production was achieved during these developmental stages.

Discussion and Conclusions

By restricting our analyses to polysomal-derived, poly(A)-containing mRNA, we enhanced the probability of obtaining mRNA actively engaged in protein synthesis. Thus a necessary prerequisite in this investigation was to establish the purity of previously uncharacterized prechorion mRNA in view of the finding reported by Efstratiadis & Kafatos (1976) that substantial rRNA contamination was present in follicular mRNA selected by oligo(dT)–cellulose chromatog-

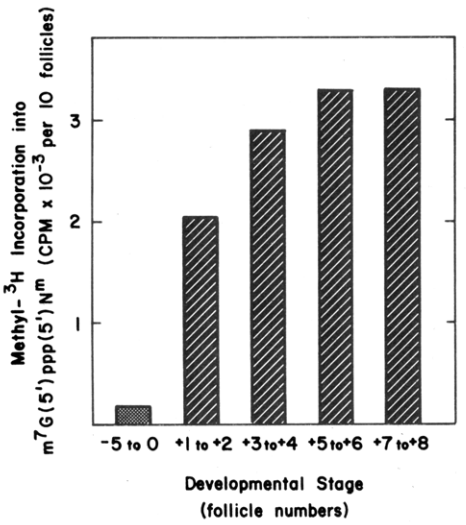

FIGURE 4: Extent of methylation of m^7GpppN^m caps of mRNA as a function of follicular development. Follicles from the above indicated developmental stages were dissected from the same silkmoth. They were labeled with methyl-^{3}H in organ culture (2 h) and the polysomal-derived, poly(A)-containing mRNA was isolated as described under Experimental Procedures. The amount of radioactivity incorporated into cap structures of the type m^7GpppN^m was determined by separating the products of nuclease-P_1 digestion of mRNA via thin-layer chromatography (see Experimental Procedures and Table I).

raphy. This difficulty, however, was overcome by utilizing a heat denaturization step just prior to oligo(dT)–cellulose chromatography (Desrosiers et al., 1975) and yielded mRNA free of other RNA contaminants, as evaluated by a number of criteria. First, gradient centrifugation of chorion mRNA (Figure 2) failed to reveal significant radioactivity sedimenting at either 4 S (tRNA) or 18 S (rRNA). Second, characterization of a RNAse-T_2 digest of mRNA via DEAE-cellulose chromatography (Figure 3) revealed a lack of methyl-^{3}H-labeled di- (−3), tri- (−4), or tetranucleotides (−5), which are representative of similar digests of tRNA and rRNA (Maden & Salim, 1974; Khan et al., 1978). Last, the absence of tRNA contamination was confirmed by our failure to detect [*methyl*-^{3}H]pm^5U in nuclease-P_1 digests (Table I), a methylated nucleotide prevalent in both pro- and eukaryotic tRNA preparations (Munns & Sims, 1975).

A second consideration critical for the interpretation of our results is the kinetics of mRNA methylation. As a result of their examination of the time course of mRNA methylation, Friderici et al. (1976) concluded that the various nucleotide constituents are methylated at different stages of mRNA maturation. Consequently, determinations of the number of particular methylated nucleotides per mRNA (unfractionated) should be based upon uniform methyl-^{3}H labeling as well as a knowledge of the rate of degradation of mRNA. Since these parameters cannot be rigorously established in the in vitro organ culture system employed, the types and amounts of methylated constituents appearing in prechorion and chorion mRNAs are presented here as tentative estimates. However, the use of identical labeling conditions throughout these experiments (2-h pulse) permits a valid assessment of the qualitative *differences* observed in mRNA methylation, particularly with respect to the presence and absence of internal m^6A nucleosides in prechorion and chorion mRNAs, respectively.

It remains to be determined whether the observed changes in follicular epithelial mRNA methylation are the consequences of transitions in the methylase activities, or in the types and stabilities of mRNAs expressed during the course of development or if such changes modify mRNA metabolism and/or translational utilization. It is of interest, for example, to examine what *functional* or *structural* features chorion mRNAs may have in common with the other small histone and globin mRNAs (Perry & Scherrer, 1975; Moss et al., 1977), which are also deficient in internal m^6A residues. Additionally, the finding that 2′-O-methylation can protect RNA from exoribonucleolytic hydrolysis (Stuart & Rottman, 1973) warrants consideration of the possibility that the divergent compositions of cap type 1 and type 2 structures between prechorion and chorion mRNA may be related to their differential stabilities.

In summary, our results indicate that as development proceeds from the prechorion to the chorionating period the (i) average size of mRNA decreases, (ii) a marked decline in the ratio of type 2 to type 1 caps as well as in the proportion of internal m^6As within mRNA molecules occurs, and (iii) a dramatic 20-fold increase in the extent of methyl-^{3}H incorporation into m^7GpppN^m cap mRNA appears. Lastly, a thin-layer chromatographic procedure is presented that rapidly and reproducibly separates the methylated nucleotides and m^7G-containing caps present in eukaryotic mRNAs.

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5′ Domain and Nucleotide Sequence of an Adult Chicken Chromosomal β-Globin Gene†

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ABSTRACT: A 6.1-kilobase chromosomal chicken DNA fragment containing an adult β-globin gene has been cloned from total *Eco*RI-digested chicken DNA. The complete β-globin gene is approximately 1600 nucleotides in length and is flanked by 0.9 and 3.6 kilobases of DNA in this cloned fragment at its 5′ and 3′ termini, respectively. The chicken β-globin contains two intervening sequences at the same locations as the mammalian globin genes. The CAP site of the globin gene was determined by electron microscopic and S1 nuclease mapping, and the nucleotide sequence at the 5′ end of the gene was established. The gene contains 80 nucleotides at its 5′-untranslated region. At 29 nucleotides preceding the CAP site is the Hogness box sequence GATAAAA. The nucleotide sequences surrounding this AT-rich region are extremely GC rich (70%). The occurrence of CG dinucleotides is unusually frequent in this region, resulting in the appearance, immediately preceding the Hogness box sequence, of six *Hpa*II sites (recognition sequence CCGG) which could be targets for DNA methylation in the genome. A stretch of 16 dG residues which is not present in other globin genes is located 194 nucleotides preceding the CAP site. However, limited nucleotide conservation at the region between the Hogness box and the CAP site was observed between the avian and mammalian β-globin genes, but not between the chicken β-globin gene and the chicken ovalbumin, ovomucoid, and conalbumin genes. Although there are additional sequence homologies between the avian and mammalian β-globin genes further 5′ from the Hogness boxes, none is as striking as the pentanucleotide sequence CCAAT located exactly at 45 nucleotides preceding the Hogness box sequence in the avian and mammalian β-globin genes but not the other chicken genes.

Recombinant DNA technology has permitted the dissection of the eucaryotic genome. Structural and sequencing analyses after gene isolation by molecular cloning have established that mammalian globin genes are comprised of three mRNA coding segments separated by two intervening sequences (Tilghman et al., 1978; Tiemeier et al., 1978; Leder et al., 1978; Konkel et al., 1979; Nishioka & Leder, 1979; Lawn et al., 1978; Lacy et al., 1979; Hardison et al., 1979; van den Berg et al., 1978; Bernards et al., 1979). More recently, such a structure has also been observed in an adult chicken β-globin gene (Dodgson et al., 1979; Ginder et al., 1979). In the past, we and others have cloned the chromosomal ovalbumin and ovomucoid genes from total chicken DNA and shown that both of these estrogen-inducible genes in the chick oviduct contain seven intervening sequences (Woo et al., 1978; Dugaiczyk et al., 1978, 1979; Mandel et al., 1978; Catterall et al., 1979; Lai et al., 1979). Nucleotide sequencing analysis at the 5′ ends of these two genes has revealed some similarities (Lai et al., 1979). In order to compare the nucleotide sequences flanking the 5′ ends of the hormone-inducible genes with that of a steroid irresponsive chicken gene and those flanking the 5′ ends of mammalian and avian globin genes, we have cloned an adult chicken β-globin gene and determined its 5′-nucleotide sequence.

Materials and Methods

Materials. White Leghorn laying hens were purchased from Rich-Glo Farm, La Grange, TX, and the animals were made anemic by daily injection of phenylhydrazine. On the seventh day, the animals were sacrificed, and reticulocytes were obtained from plasma by low-speed centrifugation. Their livers were rinsed with cold 0.9% NaCl solution and frozen in liquid nitrogen immediately after dissection. Liquified phenol and reagent grade chemicals were purchased from Fisher Scientific Co. Formamide was obtained from Fluka. Restriction enzymes, T4 DNA ligase, and sequence grade polyacrylamide were purchased from Bethesda Research Laboratories. S1 nuclease was from Miles Laboratories. DNase and bacterial alkaline phosphatase were from Worthington. *Escherichia coli* DNA polymerase I and T4 polynucleotide kinase were from Boehringer Mannheim. Radioactive deoxyribonucleoside triphosphates were purchased from Amersham.

Methods. Reverse-Phase Column Chromatography. The method has been successfully used to enrich the mouse β-globin gene from total mouse DNA for its subsequent cloning (Tilghman et al., 1977). One hundred milligrams of *Eco*RI-digested chicken liver DNA in 10 mM Tris[1]–acetate, pH 7.4, 1.5 M NaOAc, and 0.1 mM EDTA was chromatographed on a RPC-5 column (1 × 50 cm) by the procedure of Hardies & Wells (1976). The DNA was eluted from the column in

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; kb, kilobase; bp, base pairs; cDNA, complementary deoxyribonucleic acid; rRNA, ribosomal ribonucleic acid.

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