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CHANGES IN CHROMATIN STRUCTURE DURING SPERMATOGENESIS IN MATURING ROOSTER TESTIS AS DEMONSTRATED BY THE INITIATION PATTERN OF RIBONUCLEIC ACID SYNTHESIS *IN VITRO*

By CRISTOBAL MEZQUITA and CHING SUNG TENG

Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, *U.S.A.*

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To probe the structural change in the genome of the differentiating germ cell of the maturing rooster testis, the chromatin from nuclei at various stages of differentiation were transcribed with prokaryotic RNA polymerase from *Escherichia coli* or with eukaryotic RNA polymerase II from wheat germ. The transcription was performed under conditions of blockage of RNA chain reinitiation *in vitro* with rifampicin or rifamycin AF/013. With the *E. coli* enzyme, the changes in (1) the titration curve for the enzyme–chromatin interaction, (2) the number of initiation sites, (3) the rate of elongation of RNA chains, and (4) the kinetics of the formation of stable initiation complexes revealed the unmasking of DNA in elongated spermatids and the masking of DNA in spermatozoa. In both cases the stability of the DNA duplex in the initiation region for RNA synthesis greatly increased. In contrast with the *E. coli* enzyme, the wheat-germ RNA polymerase II was relatively inefficient at transcribing chromatin of elongated spermatids. Such behaviour can be predicted if unmasked double-stranded DNA is present in elongated spermatids.

Spermatids undergoing differentiation possess chromatin with unique properties compared with previous stages of spermatogenesis: (1) non-histone proteins and acetylated histones are removed and replaced by phosphorylated protamines (Platz *et al.*, 1975; Candido & Dixon, 1972; Grimes *et al.*, 1975; Mezquita & Teng, 1977); (2) the euchromatic and heterochromatic regions of the chromatin are replaced by fibres of uniform appearance (Walker, 1971; Tingari, 1973; Chevaillier & Gusse, 1975; Subirana, 1975); the beaded chromatin fibres are replaced by smooth fibres (Kierszenbaum & Tres, 1975); (3) the unmasking of DNA is revealed by the high capacity for binding of actinomycin D and cationic dyes (Lison, 1955; Loir & Hocherau de Reviers, 1972; Barcellona *et al.*, 1974; Mezquita & Teng, 1977) and by the high template capacity for RNA synthesis in the presence of *Escherichia coli* RNA polymerase *in vitro* (Mezquita & Teng, 1977); (4) the chromatin of differentiating spermatids is unable to support RNA synthesis *in vivo* (Monesi, 1964; Loir, 1972; Kierszenbaum & Tres, 1975; Mezquita & Teng, 1977).

At the end of spermatogenesis a massive condensation of the chromatin occurs. The DNA–protamine complex shows a degree of condensation similar to that found in the bacteriophage head and possesses low capacity for binding of actinomycin D and cationic dyes (Olins *et al.*, 1968; Darżynkiewicz *et al.*, 1969; Loir & Hochereau de Reviers, 1972; Mezquita & Teng, 1977). The nucleoprotamine is inactive in RNA synthesis *in vivo* and *in vitro* (Marushige & Dixon, 1969; Premkumar & Bhargava, 1972). All these observations indicate that a drastic rearrangement in chemical compositions and a change in structure are taking place in the genome. Since most of the information accumulated at present has been obtained with the electron microscope and by cytochemical methods, it is important to investigate the structural changes of the chromatin during spermatogenesis by using biochemical techniques. The deoxyribonucleases capable of specific interaction with the nucleoprotein complexes have been used as the tool for this purpose (Honda *et al.*, 1974). The prokaryotic and eukaryotic RNA polymerases, despite the possibility of 'unfaithful' initiations when added exogenously to chromatin, could also serve as a tool for probing the major changes in chromatin structure (Yamamoto & Alberts, 1976; Hirose *et al.*, 1976; Tsai *et al.*, 1976*b*). The technique of rifampicin (or rifamycin AF/013)-competition assay, developed by Sippel & Hartmann (1970) and Mangel & Chamberlin (1974) and subsequently adapted for the study of the initiation of RNA synthesis on chromatin (Tsai *et al.*, 1975, 1976*a*; Schwartz *et al.*, 1975), has been applied in the present study. This technique provided us with information on the structural changes of the chromatin during the transition from nucleohistone to nucleoprotamine in rooster spermatogenesis.

Materials and Methods

Animals and chemicals

Sexually maturing white Leghorn roosters (25–50 weeks old) were obtained from Hendricks Grain Farms, Houston, TX, U.S.A., and were used throughout this study.

The following chemicals were obtained from the sources indicated: [^{3}H]UTP (specific radioactivity 17Ci/mmol), Tris and sucrose (ribonuclease-free) were from Schwarz/Mann (Orangeburg, NY, U.S.A.); EDTA, diphenylamine and ammonium sulphate were from Mallinckrodt (St. Louis, MO, U.S.A.); nucleoside 5′-triphosphates, heparin, tRNA (yeast), rifampicin and calf thymus DNA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); exponential-phase *Escherichia coli* (K12) paste was from Grain Processing Co. (Muscating, IO, U.S.A.); Proteinase K was from EM Laboratories (Elmsford, NY, U.S.A.); raw wheat germ was from General Mills (Kansas City, MO, U.S.A.); Polymin P was kindly given by BASF, Ludwigshafen, Germany; rifamycin AF/013 was a gift from Dr. G. Lancini (Gruppo Lepetit, Milano, Italy). All other chemicals were of analytical grade.

Preparation of nuclei and chromatin

The procedures for these preparations were as described by Mezquita & Teng (1977); all the subsequent processes were done at 0°C. Nuclei from the testis and from the spermatozoa of the vas deferens were isolated by the citric acid procedure and separated by centrifugation through a discontinuous sucrose density gradient and by sedimentation at unit gravity.

Five different fractions of nuclei were obtained: tetraploid primary spermatocytes (stage 1); small primary spermatocytes, secondary spermatocytes and spermatogonia cells (stage 2); round and elongating spermatids (stage 3); elongated spermatids and testicular spermatozoa (stage 4); spermatozoa from the vas deferens (stage 5). Chromatin from the different fractions of nuclei was prepared by stepwise decrease in ionic strength and suspended in 0.002M-Tris/HCl, pH7.5, before use.

Isolation of RNA polymerases

E. coli RNA polymerase containing the σ-subunit was isolated and assayed as described by Burgess & Jendrisak (1975). The final enzyme preparation was precipitated with 60%-satd. $(NH_4)_2SO_4$ and stored at −70°C in the storage buffer [0.01M-Tris/HCl, pH7.9, 0.01M-$MgCl_2$, 0.1mM-EDTA, 0.5mM-dithiothreitol and 50% (v/v) glycerol] at a concentration of 17mg/ml. The *E. coli* RNA polymerase was assayed with calf thymus DNA as a template, and the enzyme specific activity was 1000 units/mg of protein.

Wheat-germ RNA polymerase II was prepared and assayed as described by Jendrisak & Burgess (1975). The enzyme eluted from the phosphocellulose column was precipitated with $(NH_4)_2SO_4$ (50% saturation) and stored at −70°C in the same buffer used for the *E. coli* enzyme but also containing 2mg of bovine serum albumin/ml. The enzyme specific activity assayed with single-stranded calf thymus DNA was 500 units/mg of protein.

Conditions for RNA synthesis without reinitiation

Chromatin (5μg as DNA) was preincubated with *E. coli* RNA polymerase (diluted with 1mg of bovine serum albumin/ml) at 37°C for 15min in a preincubation buffer of 0.2ml final volume, containing 12.5μmol of Tris/HCl buffer, pH7.9, 0.25μmol of $MnCl_2$, 12.5μmol of $(NH_4)_2SO_4$ and 0.50μmol of 2-mercaptoethanol. At the end of the preincubation period, RNA synthesis was started by the addition of 37.5nmol of ATP, GTP, CTP and [^{3}H]UTP (75c.p.m./pmol) in 0.05ml and incubated at 37°C for 15min. Rifampicin (10μg) and heparin (200μg) were added together with nucleotides.

Wheat-germ RNA polymerase II was preincubated with 5μg of chromatin at 37°C for 15min in 200μl of preincubation buffer as described above. RNA synthesis was initiated by the addition of 50μl of a mixture containing 150nmol each of ATP, GTP and CTP, 15nmol of [^{3}H]UTP (500c.p.m./pmol) and 50μg of rifamycin AF/013. The reaction was carried out at 37°C for 15min. In either case RNA synthesis was stopped by the addition of 0.15ml of 0.2M-EDTA, pH7.9, and cooling at 0°C. Bovine serum albumin (150μg) was added, followed by 5ml of cold 5% (w/v) trichloroacetic acid containing 0.01M-sodium pyrophosphate. After standing for 30min at 0°C, the solution was centrifuged for 20min at 2380*g* in a Beckman model JS 7.5 rotor. The pellet was dissolved in 0.2ml of cold 0.2M-NaOH and immediately reprecipitated with 5ml of cold 5% (w/v) trichloroacetic acid. The precipitate was collected on glass-fibre filters (934-AH; Reeve Angel, Clifton, NJ, U.S.A.) and washed with 30ml of the cold 5% trichloroacetic acid. The filters were dried and transferred to counting vials containing 4ml of scintillation fluid [6g of 2,5-diphenyloxazole, 0.15g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene]. The radioactivity was determined in a Beckman model LS-250 liquid-scintillation spectrometer.

Isolation of RNA

After removal of the chromatin by centrifugation at 10000*g* for 20min, the synthesized RNA was treated with 20μg of Proteinase K/ml in an extraction buffer containing 0.5% (w/v) sodium dodecyl sulphate, 0.01M-NaCl, 0.01M-EDTA and 0.01M-sodium acetate, pH5.0, for 30min at 37°C. An equal volume of distilled phenol (saturated with extraction

buffer)/chloroform (1:1, v/v) was added, and the mixture was shaken at room temperature (21°C) for 30min. After centrifugation at 10000*g* for 10min, the aqueous phase was removed. Phenol and chloroform were added again and the operation was repeated until no protein interphase was observed. The aqueous fraction was removed and 50μg of purified tRNA was added as a carrier. The RNA was precipitated with 2.5vol. of 95% ethanol at −20°C for 18h. RNA was spun down at 4500*g* for 15min at 0°C in a Beckman model JA 20 rotor and subsequently freeze-dried.

Sucrose gradient centrifugation

RNA (approx. 25000c.p.m.) obtained by the previous procedure was suspended in 0.1ml of glass-distilled water, heated at 70°C for 1min, cooled quickly, layered on top of a linear 5–20% (w/v) sucrose gradient (4.9ml), and centrifuged at 189000*g* in a Beckman SW 50.1 rotor at 4°C for 2.5h. Twenty fractions (0.15ml/fraction) were collected, and the amount of 5%-trichloroacetic acid-precipitable radioactivity was determined. Purified cytoplasmic RNA (consisting of 28S, 18S, and 4S RNA from the hen oviduct) was used as a molecular-weight marker to standardize the gradients. The chain length of the synthesized RNA was determined as indicated by Spirin (1963) and Cedar & Felsenfeld (1973).

General procedures

These were as cited in Mezquita & Teng (1977).

Results

Titration of chromatin with E. coli and wheat-germ RNA polymerases

Chromatin prepared from nuclei at different stages of spermatogenesis was preincubated with increasing quantities of *E. coli* RNA polymerase for 15min at 37°C (Tsai *et al.*, 1975). RNA chain initiation was started by the addition of nucleotides, rifampicin and heparin, and RNA synthesis was allowed to occur for an additional period of 15min. Rifampicin at the concentration used allows initiation from the highly stable preinitiation complexes formed during the preincubation, but inhibits secondary initiation and reinitiations (Hinkle & Chamberlin, 1972; Mangel & Chamberlin, 1974). Heparin inhibits ribonuclease activity (Cox *et al.*, 1973). The rifampicin-resistant RNA synthesis exhibited two phases in the chromatin prepared from nuclear stages 1 and 2 (Fig. 1). A gradual enhancement of RNA synthesis on addition of increasing quantities of polymerase (0–16μg) was followed by a very small increase in nucleotide incorporation with further increases in polymerase concentration (16–30μg).

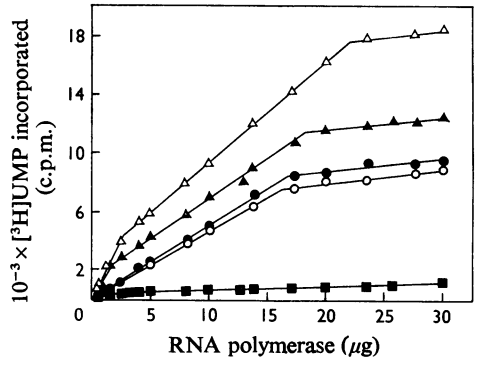

Fig. 1. *E. coli RNA polymerase saturation curves on chromatin from different stages of spermatogenesis*

E. coli RNA polymerase (0–30μg) was preincubated with 5μg of chromatin at 37°C for 15min in 0.2ml of preincubation mixture as described in the Materials and Methods section. After the preincubation, 50μl of nucleotides containing 10μg of rifampicin and 200μg of heparin was added and incubated at 37°C for an additional 15min. The RNA synthesized was precipitated with 5% (w/v) trichloroacetic acid and counted for radioactivity as described in the Materials and Methods section. ●, Chromatin from stage 1; ○, chromatin from stage 2; ▲, chromatin from stage 3; △, chromatin from stage 4; ■, chromatin from stage 5.

The titration curves of chromatin from nuclear stages 3 and 4 revealed three different phases of RNA synthesis (Fig. 1). Chromatin from stage-4 nuclei (elongated spermatids and testicular spermatozoa) showed an initial steep increase in RNA synthesis on addition of RNA polymerase (0–3μg), followed by a second phase with a lower rate of increment in RNA synthesis (3–22μg of RNA polymerase) in relation to the initial increase, and a third phase with very small increases in nucleotide incorporation with the additions of polymerase (22–30μg).

The titration curves of the chromatin from stage-3 nuclei (round and elongating spermatids) showed three different phases in an intermediate position between the titration curves of chromatin from stage-1 and stage-4 nuclei. The titration curve of the chromatin from stage-5 nuclei (spermatozoa from the vas deferens) showed very small increments of nucleotide incorporation on addition of RNA polymerase (0–30μg).

For titration of the different types of chromatin with wheat-germ RNA polymerase II, the rifamycin derivative AF/013 was used instead of rifampicin (Meilhac *et al.*, 1972). To compare the titration curves of the chromatin with *E. coli* RNA polymerase and wheat-germ RNA polymerase II, both enzymes were incubated with chromatin in the presence of rifamycin

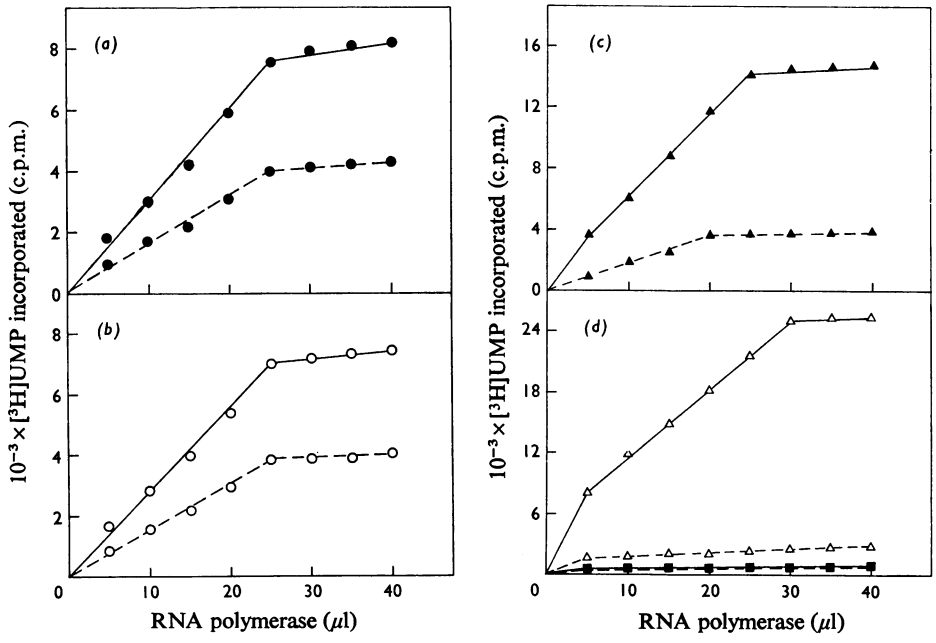

Fig. 2. *E. coli and wheat-germ RNA polymerase saturation curves on chromatin from different stages of spermatogenesis* *E. coli* RNA polymerase (——) and wheat-germ RNA polymerase (----) were preincubated with 5 μg of chromatin at 37°C for 15 min in 0.2 ml of preincubation mixture as described in the Materials and Methods section. Stock concentrations used were 0.4 μg/μl for *E. coli* RNA polymerase and 1 μg/μl for wheat-germ RNA polymerase. After the preincubation 50 μg of nucleotides containing 50 μg of rifamycin AF/013 was added and incubated at 37°C for an additional 15 min. The RNA synthesized was precipitated with 5% (w/v) trichloroacetic acid and counted for radioactivity. (*a*) ●, chromatin from stage 1; (*b*) ○, chromatin from stage 2; (*c*) ▲, chromatin from stage 3; (*d*) △, chromatin from stage 4; ■, chromatin from stage 5.

AF/013 as described in the Materials and Methods section.

Using chromatin from the nuclei of stages 1 and 2, we obtained similar enzyme-saturation curves with the prokaryotic and the eukaryotic enzymes (Figs. 2*a* and 2*b*). After a gradual increment in RNA synthesis on addition of RNA polymerase (0–25 μl), saturation was reached (25–40 μl). At the saturation point the ratio of RNA synthesized by *E. coli* RNA polymerase to that by wheat-germ RNA polymerase II was 1.8:1. The titration curves obtained for chromatin from stage-4 nuclei with the prokaryotic and eukaryotic enzymes were clearly different (Fig. 2*d*). The titration with the *E. coli* enzyme revealed three different phases of RNA synthesis: an initial steep increase (0–5 μl of added enzyme), followed by a second phase with a lesser slope (5–30 μl of enzyme), and then the saturation phase (30–40 μl of enzyme). With wheat-germ RNA polymerase II the saturation was reached after addition of a small quantity of the enzyme (5 μl), and the ratio of RNA synthesis by the prokaryotic to that by the eukaryotic enzyme was 15:1 at the saturation point.

The titration curves of chromatin from stage 3 (Fig. 2*c*) showed characteristics between those of stages 1 and 4. The titration with *E. coli* RNA polymerase revealed three different phases, as did the chromatin of stage-4 nuclei. The saturation point with wheat-germ polymerase was reached with 20 μl of enzyme, and the ratio of RNA synthesis by the prokaryotic to that by the eukaryotic enzyme was 3.7:1.

The titration curve of chromatin from spermatozoa showed very low increments in nucleotide incorporation with both RNA polymerases (Fig. 2*d*).

Determination of the number of RNA initiation sites for E. coli RNA polymerase on chromatin

The number of RNA initiation sites on the chromatin was determined by the following equation (Tsai *et al.*, 1975):

$$\frac{\text{Number of initiation sites}}{\text{pg of DNA}} = \frac{\text{c.p.m.} \times 10^{-12} \times 4 \times N}{\text{sp. radioact.} \times \text{DNA} \times \text{size}}$$

where c.p.m. is c.p.m. of UMP incorporated at transition point (RNA polymerase saturation point on chromatin); N is Avogadro's number; sp. radioact. is specific radioactivity of [^{3}H]UTP (75c.p.m./pmol); DNA is amount of chromatin DNA used (pg); size is number of nucleotides in the average chain length of RNA.

A sucrose-gradient analysis of RNA synthesized on chromatin from various stages of nuclei *in vitro* in the presence of rifampicin and heparin is presented in Fig. 3. According to the radioactivity profile and from Spirin's (1963) equation, the average lengths of the RNA chains were calculated to be 754, 764, 730, 823 and 508 nucleotides/chain for the respective chromatin stages 1, 2, 3, 4 and 5 (Table 1).

The initiation sites (in terms of $10^{-4}\times$sites/pg of DNA) were calculated as 7.16, 6.40, 10.04, 13.83 and 0.44 for the chromatin of stages 1, 2, 3, 4 and 5 respectively (Table 1).

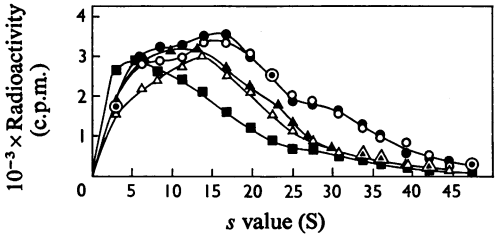

Fig. 3. *Sucrose-gradient analysis of RNA synthesized on chromatin in vitro*
Chromatin (25 μg of DNA, except 50 μg for spermatozoal chromatin) from various spermatogenic nuclei was incubated with 20 μg of *E. coli* RNA polymerase for 15min at 37°C. The conditions for RNA synthesis, extraction and sucrose-gradient centrifugation were described in the Materials and Methods section. The symbols representing the stage of spermatogenesis are as in Fig. 1.

Table 1. *RNA-chain-initiation sites and RNA chain length on chromatin at different stages of spermatogenesis*
The procedures for chromatin preparation, RNA synthesis, RNA product analysis on sucrose gradient, RNA chain initiation sites and chain-length determination were described in the Materials and Methods section.

Stage of differentiation	$10^{-4}\times$ No. of initiation sites/pg of chromatin DNA	Size of RNA synthesized (nucleotides)
1	7.16	754
2	6.40	764
3	10.04	730
4	13.83	823
5	0.44	508

Rates of RNA chain elongation

The propagation rates of the RNA chains were measured by preincubating *E. coli* RNA polymerase with different chromatins for 15min at 37°C. Chain initiation and elongation were started by the addition of the four nucleoside triphosphates, rifampicin and heparin. After 1, 2, 3 and 15min of incubation the rate of RNA chain elongation was estimated by dividing the initial rate of RNA synthesis by the total number of RNA chains synthesized, calculated as described above. The initial rates of elongation were 86, 89, 98 and 167 nucleotides/min for stages 1, 2, 3 and 4 respectively.

Kinetics of formation of stable binary complexes between E. coli RNA polymerase and chromatin

The processes of formation of the binary complex between RNA polymerase and its binding sites on DNA, as originally proposed by Chamberlin (1974), consisted of three steps: (1) the dissociation of polymerase from the non-specific sites, (2) the binding of polymerase to the initiation sites and the formation of a preinitiation complex (I complex), and (3) the formation of a stable complex (RS complex) on the initiation sites for initiating RNA synthesis.

Chromatin from nuclei at different stages of spermatogenesis was preincubated for various lengths of time to allow the formation of RS complexes capable of rapidly initiating RNA synthesis. The amount of RS complex formed was assayed by measuring RNA synthesis after the simultaneous addition of nucleotides and rifampicin. The formation of RS complexes on the chromatin of stages 1 and 2 reached a maximum after 40min of preincubation, whereas on the chromatin of stages 3, 4 and 5, the preincubation times required to reach a maximum were 30, 15 and 10min respectively (Figs. 4*a*–4*d*).

The semilogarithmic plots of the maximum rate of RNA synthesis minus the rate of RNA synthesis at each preincubation time versus the time of preincubation were linear for the chromatin of different stages (insets for Fig. 4). The half-time of formation of the RS complex ($t_{\frac{1}{2}}$) at 37°C was 9.5min for stage-1 and -2 chromatin, and decreased to 7.5, 5.5 and 2.0min for the chromatin of stages 3, 4 and 5 respectively (Table 2).

To test the possibility of artificial modification of the chromatin during preincubation, chromatin was incubated without RNA polymerase for various lengths of time. The pretreated chromatin was preincubated for 15min with RNA polymerase, and the amount of the RS complex formed was measured as before. The amount of RNA synthesized was constant

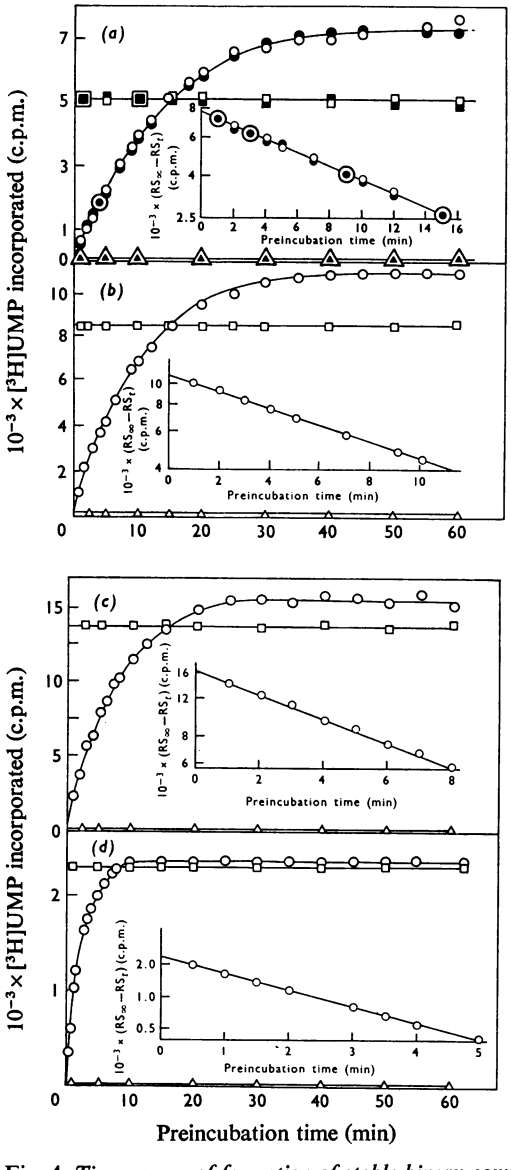

Fig. 4. *Time course of formation of stable binary complex*
Chromatin was prepared from the nuclei of (*a*) stages 1 and 2, (*b*) stage 3, (*c*) stage 4 and (*d*) stage 5. Chromatin (5μg of DNA, except 15μg of DNA for spermatozoal chromatin) from different stages of spermatogenesis were preincubated according to the following conditions: ○, chromatin was preincubated with 20μg of *E. coli* RNA polymerase; △, chromatin was preincubated without the addition of *E. coli* RNA polymerase for the indicated time intervals; □, chromatin was incubated at 37°C without the presence of *E. coli* RNA polymerase for the indicated time intervals, then followed by the addition of 20μg of enzyme and incubated for an additional 15min. After the preincubation, RNA synthesis was initiated by the addition of 0.05ml of ribonucleoside triphosphate mixture containing 0.2mg of rifampicin/ml. RNA was synthesized at 37°C for 15min. In (*a*) the solid symbol represents stage-1 chromatin, and the empty symbol represents stage-2 chromatin.

Table 2. *Effect of temperature on the formation of stable binary complex*
The value of $t_{\frac{1}{2}}$ and the ratio of $RS_{max.}$ was determined as shown in Fig. 3.

Stage of differentiation	$t_{\frac{1}{2}}$(min) 37°C	0°C	Ratio of $RS_{max.}$ (37°/0°C)
1	9.5	10.0	3.6
2	9.5	10.0	3.6
3	7.5	13.5	6.5
4	5.5	20.0	10.0
5	2.0	22.0	10.0

for chromatin pretreated for periods of up to 60min; the same amount was obtained with chromatin preincubated for 15min with RNA polymerase without previous pretreatment (Fig. 4).

To rule out the possibility that the rifampicin-resistant RNA synthesis might be produced by the endogenous RNA polymerase, we examined RNA synthesis in the absence of added exogenous RNA polymerase. There was no detectable RNA synthesis under this condition (Fig. 4). Evidently, then, endogenous RNA polymerase activity does not contribute to the total RNA synthesis.

Effect of temperature on RS-complex formation

During the processes of RS-complex formation in DNA, temperature is a critical factor; it accelerates the transformation of I complex into RS complex. This is a rate-limiting step of conversion. The $t_{\frac{1}{2}}$ for RS-complex formation measured at 0°C is therefore higher than that at 37°C (Mangel & Chamberlin, 1974).

In contrast, the formation of RS complex in chromatin is less dependent on temperature than in DNA, and is more dependent on the chromosomal proteins (Hirose *et al.*, 1976). An investigation of the effect of the preincubation temperature on RS-complex formation in the chromatin of various stages of spermatogenic nuclei could reveal more about the structure of chromatin in the initiating site region.

The $t_{\frac{1}{2}}$ of RS-complex formation of different types of chromatin was determined at two different preincubation temperatures (37 and 0°C). Results presented in Table 2 indicate that RS-complex formation in chromatin of stages 1 and 2 has a low temperature-dependence. The $t_{\frac{1}{2}}$ for RS-complex

formation measured at 0°C compared with that at 37°C showed no significant increase (from 9.5 to 10min). However, a high temperature-dependence was observed in the chromatin of stages 4 and 5. The $t_{\frac{1}{2}}$ for RS-complex formation increased from 5.5 to 20min for stage 4, and from 2 to 22min for stage 5. Stage-3 chromatin showed a temperature-dependence intermediate between that of stage 2 and stage 4; the $t_{\frac{1}{2}}$ measured at 0°C is 13.5min and that at 37°C is 7.5min.

Correspondingly, the ratio of maximal RS-complex formation at 37°C to that at 0°C in stage-1 and -2 chromatin showed less temperature-dependence than that of stages 4 and 5.

Temperature-dependence of RNA synthesis by wheat-germ RNA polymerase

RNA synthesis on chromatin of meiotic nuclei showed low temperature-dependence. Meiotic chromatin (5μg) was preincubated with wheat-germ polymerase II (20μg) at 37°C and 0°C for 40min. RNA synthesis was initiated by the addition of nucleotides and rifamycin AF/013 under the conditions described in the Materials and Methods section. The ratio of RNA synthesized after 15min at 37°C to that at 0°C was 2.6:1. In contrast, chromatin from elongated spermatids showed a higher temperature-dependence, with a ratio of 5:1.

Discussion

The different patterns of initiation of RNA synthesis *in vitro* observed in chromatin from nuclei at various stages of spermatogenesis could be explained by the changes that chromosomal proteins undergo during the differentiation process.

Meiotic and premeiotic nuclei possess chromatin with somatic histones and a heterogeneous population of non-histone proteins (Platz *et al.*, 1975; Mezquita & Teng, 1977). The initiation pattern of RNA synthesis *in vitro* of chromatin from such nuclei by either the *E. coli* RNA polymerase or the wheat-germ RNA polymerase II is comparable with that reported by Tsai *et al.* (1976*a*,*b*) and Hirose *et al.* (1976) for chromatin of somatic cells.

In elongated spermatids the non-histone proteins decrease greatly (Vaughn, 1966; Zirkin, 1970; Mezquita, 1974; Platz *et al.*, 1975; Mezquita & Teng, 1977), and the histones are acetylated, removed, and replaced by phosphorylated protamines (Candido & Dixon, 1972; Louie *et al.*, 1974; Grimes *et al.*, 1975; Marushige & Marushige, 1975; Marushige *et al.*, 1976). These changes could alter the basic structure of the chromatin and be responsible for both the increase in initiation sites and the type of initiation pattern observed in elongated spermatids.

The initiation pattern of RNA synthesis *in vitro* obtained on native DNA by using *E. coli* RNA polymerase differs from the pattern observed on chromatin in the following ways: (1) increased number of binding sites (Cedar & Felsenfeld, 1973; Tsai *et al.*, 1975); (2) increased rate of propagation of growing RNA chains (Cedar & Felsenfeld, 1973); (3) presence of strong and weak polymerase-binding sites (Tsai *et al.*, 1975); (4) shorter half-time of formation of high-affinity enzyme–chromatin complexes (RS complexes) and a higher temperature-dependence of the RS-complex formation (Hirose *et al.*, 1976; Tsai *et al.*, 1976*b*). The eukaryotic RNA polymerase II enzyme is far less efficient for initiation of RNA synthesis on intact double-stranded DNA than is the prokaryotic enzyme (Meilhac & Chambon, 1973). The eukaryotic enzyme also shows higher temperature-dependence for RNA synthesis with native DNA than with chromatin (Tsai *et al.*, 1976*b*).

All the characteristics of the initiation pattern of RNA synthesis *in vitro* on chromatin from elongated spermatids reported in the present paper are similar to those of intact double-stranded DNA described above. This fact and our previous finding of a high capacity for binding of actinomycin D in elongated spermatids (Mezquita & Teng, 1977) indicate that unmasked stable double-stranded DNA is present in the chromatin of the elongated spermatids during the transition from nucleohistone to nucleoprotamine in rooster spermatogenesis.

Finally, in spermatozoa the massive condensation of the chromatin by a highly basic protein renders the DNA inaccessible to RNA polymerase (Marushige & Dixon, 1969; Shih & Bonner, 1970; Subirana, 1975; Mezquita & Teng, 1977), causing the great decrease in the number of initiation sites reported here.

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References

Barcellona, W. J., Brackeen, R. B. & Brinkley, B. R. (1974) *J. Reprod. Fertil.* **39**, 41–48

Burgess, R. R. & Jendrisak, J. J. (1975) *Biochemistry* **14**, 4634–4638

Candido, E. P. M. & Dixon, G. H. (1972) *J. Biol. Chem.* **247**, 5506–5510

Cedar, H. & Felsenfeld, G. (1973) *J. Mol. Biol.* **77**, 237–254

Chamberlin, M. J. (1974) *Annu. Rev. Biochem.* **43**, 721–775

Chevaillier, P. & Gusse, M. (1975) *J. Microsc. Biol. Cell.* **23**, 153–164

Cox, R., Hines, M. & Carey, N. (1973) *Eur. J. Biochem.* **32**, 513–524

Darżynkiewicz, Z., Bolund, L. & Ringertz, N. R. (1969) *Exp. Cell Res.* **55**, 120–123

Grimes, S. R., Chae, C. B. & Irvin, J. L. (1975) *Arch. Biochem. Biophys.* **168**, 425–435
Hinkle, D. C. & Chamberlin, M. J. (1972) *J. Mol. Biol.* **70**, 157–185
Hirose, M., Tsai, M. J. & O'Malley, B. W. (1976) *J. Biol. Chem.* **251**, 1137–1146
Honda, B. M., Baillie, D. L. & Candido, E. P. M. (1974) *FEBS Lett.* **48**, 156–159
Jendrisak, J. J. & Burgess, R. R. (1975) *Biochemistry* **14**, 4639–4645
Kierszenbaum, A. L. & Tres, L. L. (1975) *J. Cell Biol.* **65**, 258–270
Lison, L. (1955) *Acta Histochem.* **2**, 47–67
Loir, M. (1972) *Ann. Biol. Anim. Biochem. Biophys.* **12**, 411–429
Loir, M. & Hochereau de Reviers, M. T. (1972) *J. Reprod. Fertil.* **31**, 127–130
Louie, A. J., Candido, E. P. M. & Dixon, G. H. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 803–819
Mangel, W. F., & Chamberlin, M. J. (1974) *J. Biol. Chem.* **249**, 2995–3001
Marushige, K. & Dixon, G. H. (1969) *Dev. Biol.* **19**, 397–414
Marushige, K., Marushige, Y. & Wong, T. K. (1976) *Biochemistry* **15**, 2047–2053
Marushige, Y. M. & Marushige, K. (1975) *J. Biol. Chem.* **250**, 39–45
Meilhac, M. & Chambon, P. (1973) *Eur. J. Biochem.* **35**, 454–463
Meilhac, M., Tysper, Z. & Chambon, P. (1972) *Eur. J. Biochem.* **28**, 291–300
Mezquita, C. (1974) M.D. Thesis, University of Barcelona
Mezquita, C. & Teng, C. S. (1977) *Biochem. J.* **164**, 99–111
Monesi, V. (1964) *J. Cell Biol.* **22**, 521–532
Olins, D. E., Olins, A. L. & Von Hippel, P. H. (1968) *J. Mol. Biol.* **33**, 265–281
Platz, R. D., Grimes, S. R., Meistrich, M. L. & Hnilica, L. S. (1975) *J. Biol. Chem.* **250**, 5791–5850
Premkumar, E. & Bhargava, P. M. (1972) *Nature (London) New Biol.* **240**, 139–143
Schwartz, R. J., Tsai, M. J., Tsai, S. Y. & O'Malley, B. W. (1975) *J. Biol. Chem.* **250**, 5175–5182
Shih, T. Y. & Bonner, J. (1970) *J. Mol. Biol.* **50**, 333–344
Sippel, A. & Hartmann, G. R. (1970) *Eur. J. Biochem.* **16**, 152–157
Spirin, A. (1963) *Prog. Nucleic Acid Res.* **1**, 301–346
Subirana, J. A. (1975) *Biol. J. Linn. Soc.* **7**, *Suppl.* **1**, 239–244
Tingari, M. D. (1973) *J. Reprod. Fertil.* **34**, 255–265
Tsai, M. J., Schwartz, R. J., Tsai, S. Y. & O'Malley, B. W. (1975) *J. Biol. Chem.* **250**, 5165–5174
Tsai, M. J., Towle, H. C., Harris, S. E. & O'Malley, B. W. (1976*a*) *J. Biol. Chem.* **251**, 1960–1969
Tsai, M. J., Tsai, S. Y., Towle, H. C. & O'Malley, B. W. (1976*b*) *J. Biol. Chem.* **251**, 5565–5574
Vaughn, J. C. (1966) *J. Cell Biol.* **31**, 257–278
Walker, M. H. (1971) *Chromosoma* **34**, 340–354
Yamamoto, K. R. & Alberts, B. M. (1976) *Annu. Rev. Biochem.* **45**, 721–746
Zirkin, B. R. (1970) *Chromosoma* **31**, 231–240