Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

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Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **74** (1983), n.3, p. 173–181.

Accademia Nazionale dei Lincei

<http://www.bdim.eu/item?id=RLINA_1983_8_74_3_173_0>

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Accademia Nazionale dei Lincei, 1983.

Biologia. — *Chromatin transcriptional activity in the oviduct of the green frog*, Rana esculenta, *during the recovery phase.* Nota di ALBERTA POLZONETTI-MAGNI (*), GIANLUIGI GIANFRANCESCHI (*), ANNA GOBETTI (*) e VIRGILIO BOTTE (**), presentata (***) dal Corrisp. G. CHIEFFI.

RIASSUNTO. — È stata studiata l'attività trascrizionale della cromatina estratta dalle cellule dell'ovidutto dell'anfibio anuro, *Rana esculenta*, in due periodi, iniziale (Settembre) e finale (Gennaio), della fase di recupero. Nel corso di questi mesi le ghiandole dell'ovidutto sintetizzano ed accumulano una grande quantità di gelatina; questo fenomeno, controllato dagli ormoni della gonade, si accompagna anche ad un aumento significativo della sintesi di RNA. Progressivamente, a mano a mano che le mucine vengono impacchettate nel citoplasma, i nuclei delle cellule che le producono assumono un aspetto picnotico e questo fatto potrebbe essere l'espressione di una sorta di feed-back negativo del secreto sull'attività dei nuclei. Per verificare questa ipotesi è stata purificata la cromatina dai nuclei delle cellule dell'ovidutto prelevato da animali in fase di attiva sintesi (Settembre) della gelatina e quando questo processo è ormai completato (Gennaio). Su questi preparati si è valutata l'attività trascrizionale in *vitro*. Bisogna notare che è risultato pressocchè impossibile ottenere una preparzione pura di cromatina poichè in tutti i casi una sia pur limitata quantità di gelatina resta attaccata ai nuclei, anche dopo ripetute purificazioni. Ciononostante i risultati indicano che una più vivace attività trascrizionale è mostrata dalle preparazioni parzialmente purificate di cromatine ottenute in Settembre che non in quelle di Gennaio. Quando però si indaga su preparazioni più purificate di cromatina, tali differenze si annullano.

INTRODUCTION

In the green frog, *Rana esculenta*, after the breeding season (March-June) the spent oviduct resumes its annual development throughout a long " recovery phase " which lasts from August to January (Chieffi *et al.*, 1966; Chieffi *et al.*, 1966; D'Istria and Botte, 1970). During this period the oviduct glands synthesize and package in their cytoplasm a dramatic amount of jelly material (Andreozzi *et al.*, 1971). This phenomenon is correlated to an increase in total RNA content and is certainly under the control of ovarian sex hormones (Polzonetti-Magni *et al.*, 1976; Polzonetti-Magni *et al.*, 1976). Progressively, as mucins are stored in the cytoplasm, the nuclei of glandular cells assume a pyknotic aspect (Andreozzi *et al.*, 1971) and this phenomenon has been considered as a type of negative feed-back of the secretory products on the nuclei activity. It has been shown, however, that the nuclei pyknosis does not interfere *in vivo* with RNA and protein syntheis, since both at the beginning (September) and

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(***) Nella seduta del 12 marzo 1983.

at the end (January) of the recovery phase, the oviduct incorporates to the same extent labelled uridine into total RNA and labelled aminoacids into proteins (Polzonetti-Magni *et al.*, 1979). This observation, however, does not rule out the fact that gene expression or chromatin transcriptional activity can be modified during the course of the secretory phase. In fact, the study of RNA synthesis, shown by uridine uptake, is unable to demonstrate any negative regulatory feed-back mechanism of the secretory product, such as the jelly, on the nucleus. Therefore, it was considered of interest to purify chromatin from oviducts both when their glandular cells perform active jelly synthesis (September) and when this is completed (January) and then to evaluate the transcriptional activity of this chromatin *in vitro*.

Materials and methods

Animals. The frogs were collected near Colfiorito (Mc) at two different times in the recovery phase (September and January), and the oviducts were removed by dissection, weighed and treated as follows.

Isolation of nuclei and purification of chromatin.

Two different methods were employed.

1. The isolation of nuclei was performed using a modification of the technique described by Yasmineh and Yunis, (1970). Frozen oviducts were homogenized (1 g/10 ml) in 0.32 M sucrose, 1.5 mM $CaCl_2$ in a Potter homogenizer (200 μ clearance; 7–10 mechanical strokes). From this suspension the thick jelly core was separated by centrifuging at 500×g for 20 min. The supernatant, which contained most of the nuclei and fluid jelly, was subjected to centrifugation at 800×g for 20 min to obtain a pellet with the nuclei and some cellular debris. This pellet was suspended in 1.62 M sucrose, 1.5 mM $CaCl_2$ and centrifuged for 10 min at 5000×g. The pellet of this centrifugation was resuspended twice in 2.2 M sucrose, 1.5 mM $CaCl_2$ and finally centrifuged at 12.000×g for 20 min. The final pellet was washed four times with 0.01 Tris–HCl (pH 7.1), 1.5 mM $CaCl_2$, followed each time by sedimentation of the nuclei through centrifugation at 650×g for 5 min. Then 10 volumes of distilled water were added to this preparation and the suspension was frozen and homogenized after partial thawing in a ground glass Potter Helvehyem homogenizer. This procedure was repeated three times. The unbroken nuclei were removed by centrifugation at 1000×g for 5 min. This method was preferred to sonication as indicated in the original paper of Yasmineh and Yunis, in order to obtain the chromatin as near as possible to the native state. The resulting suspension contained two fractions of chromatin with different states of condensation: heterochromatin which was collected by centrifugation at 3500×g for 20 min, and euchromatin which was sedimented by centrifugation at 78000×g for 1 h.

2. Frozen oviducts were homogenized in amphibian saline (1 g wet weight: /10 ml) α-amylase (100 μg/ml) and β-glucoronidase (100 μg/ml) were added to the homogenate and incubated at 37 °C for 6–10 h.

Then, the mixture was subjected to centrifugation at 1000×g for 10 min and the resulting pellet was homogenized twice in citric acid pH 3. Intact nuclei were collected by centrifugation at 800×g for 10 min. The nuclei were broken as indicated above and chromatin was collected by centrifugation at 2000×g for 1 h. The pellet was suspended in SSC×10^{-1} (SSC: 0.015 M trisodium citrate, 0.15 M NaCl) and this represented a crude chromatin preparation (fraction a). In some experiments 0.4 ml samples of the crude chromatin preparation were stratified on 8 ml. 1.5 M sucrose and centrifuged at 20000×g for 1 h. The floating chromatin complex was resuspended in SSC×10^{-1} and collected by centrifugation at 20000×g for 20 min. This was considered a partially purified chromatin preparation (fraction b).

The quantitative determinations of DNA, RNA and proteins were performed by diphenilamine, orcinol and Lowry's reactions respectively and evaluation of the jelly content of each preparation was done using the anthrone reaction for polysaccarides (Polzonetti–Magni *et al.*, 1979). All operations were done at 4 °C.

Incubation and assay for RNA synthesis.

The transcriptional capacity of oviduct chromatin was tested *in vitro* by DNA dependent RNA polymerase (from *E. coli* K–12 (Sigma)). System: 0.75 ml of the complete incubation mixture for RNA synthesis contained: 30 μmol Tris–HCl (pH 7.5), 3 μmol each of ATP, GTP, CTP and UTP, 2 μmol ^{3}H––CTP (22 Ci mmol^{-1}, Radiochemical Centre Amersham) 4 U RNA polymerase from *E. coli* and 10 μg chromatin DNA. The determinations of the radioactivity in the acid insoluble fraction were carried out on aliquots of 0.1 ml as previously described (Gianfranceschi *et al.*, 1975).

Thermal denaturation.

The melting profiles of chromatin were obtained utilizing a Zeiss spectrophotometer (mod. PMQ II) equipped with a Lauda Termostat Mod. K 2. The heating rate was 1 °C min^{-1}. Absorption changes at 260 nm were registered by means of Servagor RE 11 recorder.

RESULTS

Our preliminary interest was the preparation of chromatin free from jelly. This was quite difficult since a certain amount of anthrone positive material sticks to nuclei even after purification.

Therefore, transcriptional activity was initially evaluated on hetero—and eu-chromatin with a ratio between mucopolysaccarides and DNA, MU/DNA 4. This chromatin shows a ratio between total chromosomal protein and DNA of 1.3–1.5 which is considerably lower than that reported in other eukaryotic tissue

(rat or calf liver, mouse or rat or calf thymus). The presence of jelly residues could also explain the biphasic pattern of chromatin denaturation kinetics; moreover the melting curve demonstrates a low hyperchromatic index after heating at 95 °C.

$$\text{Hmax} = \frac{\text{A 260 nm 95 °C}}{\text{A 260 nm 30 °C}} = 0.18$$

(Fig. 1 a).

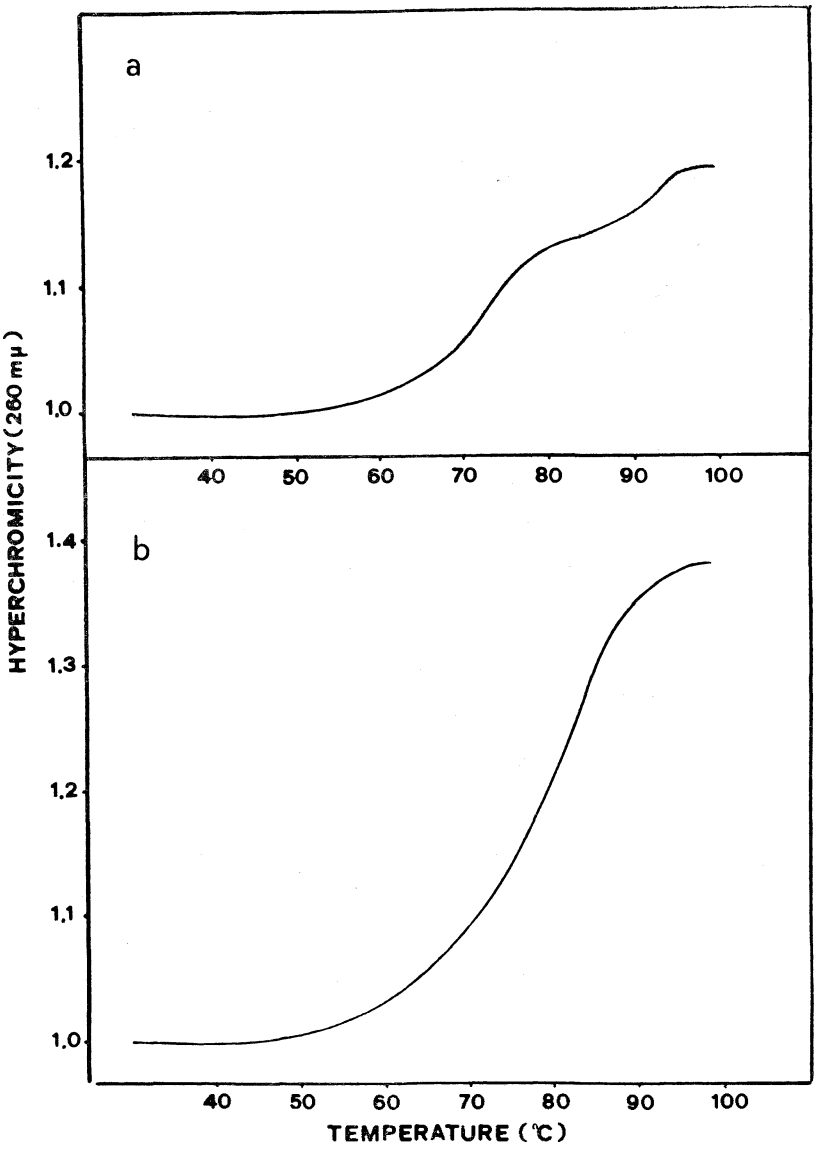

Fig. 1. - Continuous melting profiles of frog oviduct chromatin before (a) and after (b) digestion by α-amylase and β-glucoronidase. The enzymatic digestion of chromatin, the rate of heating and other conditions for chromatin DNA denaturation as given in Materials and Methods. Solvent is SSC × 0.01, pH 7.5.

In Tab. 1 the chromatin *in vitro* transcriptional activity is reported, compared to that of standard DNA (from calf thymus, Sigma). The results show that in spite of jelly presence, a good chromatin transcriptional activity, corresponding to about 10% of purified DNA is achieved. Euchromatin activity is more pronounced than that of heterochromatin, in agreement with different condensation levels.

TABLE 1

Time-course of chromatin transcription by RNA *polymerase from* E. coli K–12 *as compared to* DNA *transcription. Incorporation of* ^{3}H–CMP: *c.p.m.*

	2′	10′
DNA	11396	35468
Heterochromatin	568	2631
Euchromatin	1734	4563

In a subsequent experiment some attempts were made to eliminate jelly contamination from chromatin. The chromatin preparation obtained by centrifugation at 20000 × g for 30 min was suspended in 1.6 M CsCl and centrifuged for 5 h at 100 000 × g. This method failed to remove the jelly, even when the CsCl gradient dissociated the chromosomal protein components from DNA. This fact induces the belief that the jelly is combined with chromotin by hydrophobic bonds. The chromatin extractions with solvents such as chloroform caused a decrease in jelly content; but obviously the chromasomal protein assembly was completely impaired by the deproteinizing treatment. Experiments to remove jelly from chromatin by incubation at 37 °C for 1 hour with 2 units per ml of both β-glucoronidase and α-amilase were subsequently carried out. Following digestion, the chromatin was collected by centrifugation at 20000 × g for 1 h and resuspended in 10 mM NaCl; this procedure caused a significant decrease of jelly in the chromatin (ratio MU/DNA = 1). Correspondingly chromatin denaturation kinetics become monophasic (Fig. 1 b) and the maximum hyperchromicity increase by 0.4. On the contrary, the template capacity of the chromatin was not increased (Table 2).

TABLE 2

Incorporation of ^{3}H–CMP: *c.p.m.*

	5′	10′
Chromatin without digestion	4526	6161
Chromatin after digestion	3644	5086

Chromatin transcription before and after digestion by β-glucoronidase and α-amilase (5 μg of chromatin DNA).

These results could suggest that the binding of jelly to chromatin induces a modification in stability and physical-chemical state but does not affect transcription by the exogen RNA polymerase system. In order to confirm these results, chromatin transcriptional activity was also studied *in vitro* by adding increasing amounts of jelly obtained from ovulated eggs. The results reported in Table 3 show that the transcriptional activity is inhibited only by adding large amounts of jelly which significantly modifies the medium viscosity.

TABLE 3.

Incorporation of ^{3}H-CMP: *c.p.m.*

	5′	10′
Chromatin (3 μg DNA)	2435	3648
Chromatin + 25 μg jelly	3216	3824
Chromatin + 50 μg jelly	861	1328

Chromatin transcriptional activity following addtion of increasing amounts of jelly from ovulated eggs.

This suggests that jelly does not have a specific control on trascriptional activity but only induces a modification of the chromatin components assembly.

The previous experiments were carried out on oviducts of *Rana esculenta* captured in September. However, the main aim of this work was to investigate the possible differences in transcriptional activity between the chromatin preparation from *Rana esculenta* oviduct nuclei at the beginning and the end of the recovery phase. For this purpose, the chromatin purification procedure was modified by taking into account the characteristics of *Rana esculenta* oviduct chromatin. Thus, an enzymatic digestion with β-glucoronidase and α-amilase was carried out directly on oviduct tissue homogenate taken both in September and January (near the end of recovery phase). The nuclear pellet, moreover, was homogenized in citric acid (pH 3), as suggested by Bush (1967), in order to reduce cytoplasmatic contamination without appreciably modifying the interactions between DNA and chromosomal proteins. Additionally, the chromatin obtained from broken nuclei by centrifugation at 20000 × g (crude preparation) was purified by 1.5 M sucrose gradient centrifugation (purified preparation), as described in Materials and Methods. With this second method, a better recovery of chromatin (500 μg chromatinic DNA from 10 g of oviduct tissue) was obtained. The components analysis (DNA, protein and sugars) and denaturation kinetics after heating show that this chromatin preparation has chemical and

physical characteristics very similar to that obtained by Yasmineh and Yunis (1970).

In Fig. 2, the chromatin absorption spectrum is reported.

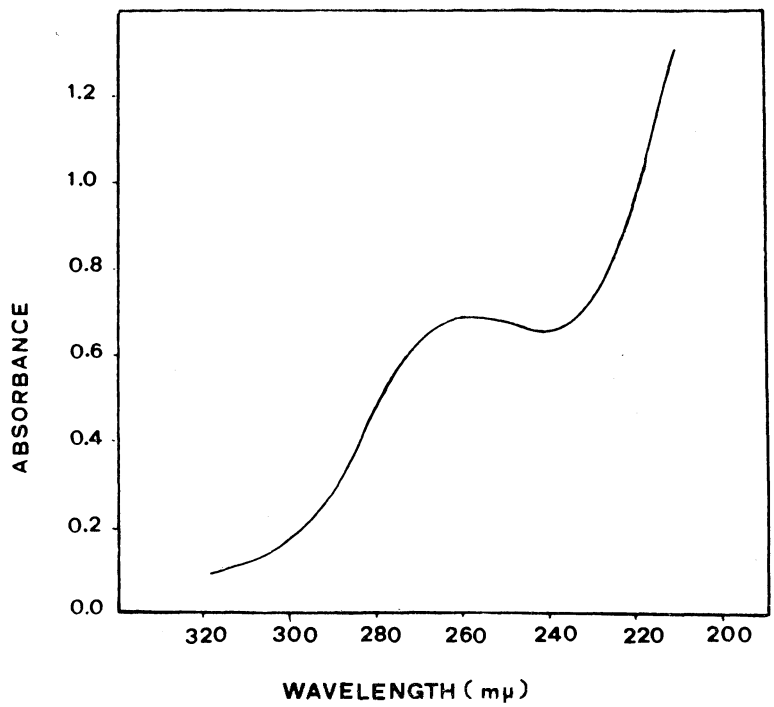

Fig. 2. – Absorption spectrum of oviduct chromatin. Continuous monitoring. Solvent is SSC × 0.01, pH 7.5.

In Tab. 4 the transcriptional activities of crude and purified chromatin from September and January oviducts are reported. Crude preparations show a good template activity in September oviducts; on the contrary, no appreciable transcription is evident in January oviducts. This difference is not confirmed when more purified chromatin preparations are used. This result could indicate that some inhibiting substances of transcriptional activity slightly aggregated to chromatin are removed by the sucrose purification gradient. However, we suggest the alternative hypothesis that the different transcriptional activity between September and January could be due to a different chromatin assembly. In fact, the high amount of jelly in January glandular cells could induce *in vivo* a close packing of chromatin, resulting in an inhibition of template capacity. Following extraction, this physicochemical structure of chromatin may be partially enlarged by flowing through the sucrose gradient.

TABLE 4.

Time-course of chromatin DNA (3 μg) *transcription: incorporation of* ^{3}H–CMP *c.p.m.*

		30″	5′	10′
September oviducts	Crude chromatin (fraction a)	238 ± 117	1318 ± 238	1911 ± 261
	Purified chromatin (fraction b)	464 ± 95	2035 ± 414	3324 ± 536
January oviducts	Crude chromatin (fraction a)	68	80	57
	Purified chromatin (fraction b)	298 ± 64	1789 ± 265	3141 ± 721
Crude chromatin September oviducts + 10 μg jelly		318 ± 116	1541 ± 531	2074 ± 418
Purified chromatin January oviducts + 10 μg jelly		253 ± 85	1630 ± 643	2985 ± 849

Transcriptional activity of crude and purified chromatin from September and January oviducts.

DISCUSSION

The studies *in vitro* carried out on the chromatin from oviducts at the beginning of the recovery phase (September) show a greater transcriptional actvity of euchromatin than of heterochromatin, obviously according to the different state of condensation. Therefore, for the same September oviducts, the attempts to remove the jelly give us a better preparation of chromatin in physicochemical state, but do not modify the *in vitro* transcriptional actvity.

The results obtained with chromatin at the beginning and at the end of the recovery phase show a greater transcriptional activity in the crude chromatin of September oviducts than in those of January; on the other hand, no significant difference was noticed in purified chromatin transcriptional activity for the two periods. Probably the sucrose gradient removes an inhibitor or induces a significant modification of chromatin packing so as to remove the inhibition of transcriptional activity. These results do not prove the role of the jelly in modulation of oviducal chromatin template activity; in fact, only by adding high amounts of purified jelly *in vitro* was transcriptional activity modified. However, *in vivo*, the great amounts of jelly stored in oviduct glands could induce an increase in chromatin packing which promotes inhibition in template activity.

REFERENCES

ANDREOZZI G., BOTTE V. and CHIEFFI G. (1971) – « Boll. Zool. », *38*, 261.

BUSH H. (1967) – In « Methods in Enzimology » (Ed. by Grossman L. Moldave K.) vol. XII, pp. 421.

CHIEFFI G., BELLINI-CARDELLINI L. and POLZONETTI-MAGNI A. (1966) – « Riv. Biol. », *59*, 41.

CHIEFFI G., BELLINI-CARDELLINI L. and POLZONETTI-MAGNI A. (1966) – « Ric. Sci. », *36*, 283.

DISCHE Z. (1955) – In « Methods of Biochemical Analysis » (Ed. by Glick D.), vol. II, pp. 313.

D'ISTRIA M. and BOTTE V. (1970) – « Atti soc. Pelor. Sci. fis. mat. nat. », *16*, 97.

GIANFRANCESCHI G. L., AMICI D. and GUGLIELMI L. (1975) – « Biochim. Biophys. Acta », *414*, 9.

POLZONETTI-MAGNI A., BELLINI-CARDELLINI L., BOTTE V. and CHIEFFI G. (1973) – « Riv. Biol. », *66*, 27.

POLZONETTI-MAGNI A., BELLINI-CARDELLINI L. and BOTTE V. (1976) – « Gen. comp. Endocrinol. », *28*, 96.

POLZONETTI-MAGNI A., GOBBETTI A. and BOTTE V. (1979) – « Rend. Acc. Naz. Lincei », *65*, 141.

YASMINEH W. G. and JUNIS J. J. (1970) – « Exp. cell. res. », *59*, 69.