FRACTIONATION OF CHICK OVIDUCT CHROMATIN: IV. ASSOCIATION OF PROTEIN KINASE WITH TRANSCRIPTIONALLY ACTIVE CHROMATIN

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SUMMARY

Chromatin isolated from the oviducts of estrogen treated chicks was fractionated into template active and template inactive components by sucrose gradient centrifugation and by ECTHAM cellulose chromatography. The fractionated chromatin was assayed for protein kinase activity. Chromatin protein kinase activity is enriched in the transcriptionally active fraction of chromatin.

INTRODUCTION

Considerable evidence has been accumulated in recent years implicating nonhistone phosphoproteins (NHPP) in gene regulation (1). Teng et al. (2) have demonstrated that NHPP are highly heterogeneous, tissue specific, and bind to the DNA or origin. Frenster reported an increased level of phosphoproteins in the "diffuse" or transcriptionally active region of chromatin (3). More recently, Shea and Kleinsmith (4) have reported that NHPP increase RNA synthesis in vitro. In light of these findings and in order to gain insight into the regulatory aspects of nonhistone phosphorylation, we have studied the nuclear protein kinases of the chick oviduct. In the present study, we were interested in determining if nuclear protein kinase activity is associated with the template active or template inactive region of oviduct chromatin. To carry out this study, we have used sucrose gradient centrifugation and ECTHAM

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cellulose chromatography to fractionate chromatin into template active and inactive regions. We find that chromatin protein kinase activity is considerably enriched in the fraction of chromatin which is more active transcriptionally.

MATERIALS AND METHODS

Preparation and Fractionation of Chromatin: Chromatin was prepared from purified nuclei isolated from oviducts of diethylstilbestrol (DES) treated chicks according to the method of Spelsberg and Hnilica (5). Chromatin was sheared in a French pressure cell and the active and inactive regions separated by centrifugation on linear sucrose gradients as described previously (6). Chromatin was also fractionated on ECTHAM-cellulose by a modification (7) of the method of Reeck et al. (8).

Chromatin Template Activity: E. coli RNA polymerase was prepared according to Burgess (9). Each template capacity assay of 0.25 ml contained: Tris-HCl (pH 7.9), 10 μmoles; $MgCl_2$, 2.5 μmoles; β-mercaptoethanol, 0.5 μmoles; KCl, 37.5 μmoles; 0.075 μmoles each of ATP, UTP, GTP, and CTP; 0.0015 μCi of [^{3}H]-GTP (Schwarz-Mann, 14.5 Ci/mmole) or 0.07 μCi of [^{14}C]-ATP (Schwarz-Mann, 47 mCi/mmole); 20 μg of polymerase and 5 μg of DNA as chromatin or purified chick DNA. Assays were incubated for 10 minutes at 37°C. Reactions were terminated with an excess of 5% CCl_3COOH. After 30 minutes, the acid precipitates were filtered onto Whatman GF/C glass fiber filters and washed with 2% CCl_3COOH. Filtered precipitates were digested with NCS tissue solubilizer (Amersham-Searle Corp.) at 50°C for 60 minutes and counted in a toluene-spectrofluor (Amersham-Searle Corp.). Samples were counted in a Beckman LS-250 liquid scintillation counter with fixed window isoset modules for ^{3}H and ^{14}C. Counts registered in the ^{3}H window were corrected for ^{14}C spillover. Template activity of chromatin or DNA was calculated from ^{3}H-GTP incorporation alone.

Protein Kinase Assay: Protein kinase was assayed in a total volume of 0.1 ml which contained 5.0 μmoles sodium phosphate buffer, 1 μmole $MgCl_2$, 1 nmole [γ-^{32}P]ATP (25 cpm per fmole) and 0.3 mg casein as exogenous substrate. Reactions were carried out for 6 minutes at 37°C, and stopped by the addition of 1.5 ml of 5% CCl_3COOH. The acid precipitates were filtered onto 0.45 nitrocellulose filters (Millipore Corp.) and washed with 5% CCl_3COOH. Filters were dried and counted in toluene-spectrofluor.

RESULTS

Centrifugation of sheared oviduct chromatin on linear sucrose gradients as previously described (6) yielded the profile shown in Figure 1. When the gradient was divided into four fractions and assayed for template activity and ratio of incorporation of ATP to UTP into RNA calculated, the data shown in Table I were obtained. Template activity was consistently associated with the lighter sedimenting fractions, while the lowest activity was found in the pellet fraction (Table I). RNA polymerase assays carried out using [^{14}C]-ATP

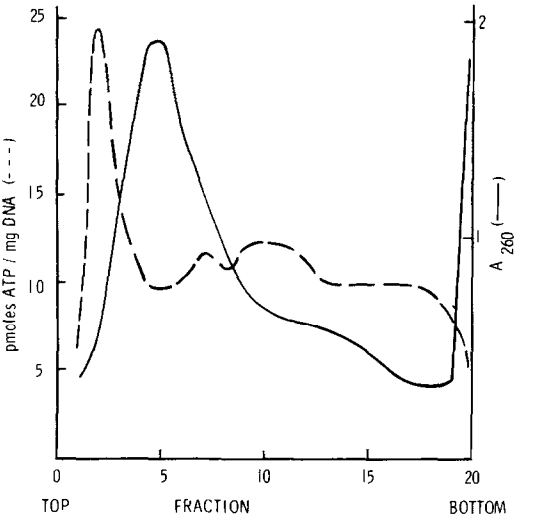

Figure 1. Distribution of protein kinase in sucrose gradient fractionated oviduct chromatin. Chromatin was fractionated and protein kinase activity was determined as described in Methods and Table 1. (——) absorbance at 260 nm, (---) protein kinase activity.

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and [^{3}H]-GTP revealed that, in addition to differences in template capacity, the base composition of the RNA products varied across the gradient. The RNA transcribed from the pellet was markedly enriched in G-C content over that transcribed from both the template active fraction and unfractionated chromatin.

In a separate experiment, chromatin was sheared and centrifuged as described above and the fractions were assayed for protein kinase activity. Figure 1 shows that the main peak of kinase activity occurred on the trailing edge of the major absorbance peak. To rule out the possibility that this kinase activity represented enzyme released from the chromatin, duplicate gradients were run containing salt-extracted nuclear kinase and sheared chromatin. The protein kinase activity in chromatin sedimented considerably faster than the free nuclear protein kinase, indicating that the enzyme remained bound to chromatin. Interestingly, none of the fractions tested were stimulated by the addition of 10^{-6} M cAMP in the protein kinase assay.

TABLE 1. Template activity of sucrose gradient fractionated chick oviduct chromatin.

CHROMATIN FRACTION	TEMPLATE ACTIVITY (UNFRACTIONATED CHROMATIN = 1.0)	$\frac{[^{14}C]\text{-ATP}}{[^{3}H]\text{-GTP}}$
Sheared Chromatin	1.00	1.68
Purified Chick DNA	4.00	1.72
Gradient Fractions 2-4	1.53	1.69
Gradient Fractions 5-7	1.33	1.69
Gradient Fractions 9-10	0.86	1.59
Gradient Fractions 11-15	0.57	1.35
Pellet Fraction	0.34	1.18

Chromatin (0.7 mg DNA/ml) was sheared in French pressure cell at 7000 psi. Insoluble material was removed by centrifugation at 10,000 x g for 20 minutes. The solubilized chromatin was layered onto a 13 ml gradient of 5 to 30% sucrose and spun at 154,000 x g for 6 hours. After centrifugation the gradients were fractionated and monitored at 260 nm. The fractions were then assayed for template capacity as described in Materials and Methods.

Chromatin was also fractionated by ECTHAM-cellulose chromatography. Simpson and co-workers (8) have shown that this technique separated chromatin into early (I) and late (II) eluting fractions which exhibited properties expected of active and inactive chromatin, respectively. Using a modification of this method we have fractionated chick oviduct chromatin on ECTHAM-cellulose and assayed Fractions I and II for protein

kinase activity. Table II shows that Fraction I, representing the transcriptionally active portion of the genome, possessed approximately 10-fold more protein kinase activity per μg of DNA than Fraction II. To eliminate the possibility that protein kinase originally bound to Fraction II might have been extracted between Fractions I and II due to the salt gradient used, the material eluting between I and II was collected and assayed (Fraction IIa, Table 2). This fraction was found to contain relatively little kinase activity, indicating that protein kinase was not extracted from the chromatin during chromatography. Hence, the results obtained from both sucrose gradient centrifugation and ECTHAM chromatography support the conclusion that protein kinase activity is associated with the transcriptionally active region of chromatin.

Table 2. Protein kinase activity in unfractionated chromatin and Fractions I and II from ECTHAM-cellulose.

	Protein Kinase Activity pmole ^{32}P [1]	
	+ Casein	- Casein
Unfractionated Chromatin	6.15 ± 0.01	0.95 ± 0.01
Fraction I	8.21 ± 0.01	1.25 ± 0.01
Fraction IIa [2]	0.67 ± 0.01 [3]	---
Fraction II	0.85 ± 0.01	0.11 ± 0.01

[1] incorporated per μg chromatin - DNA

[2] pooled column fractions eluted from 100 mN NaCl to the beginning of chromatin Fraction II

[3] calculated using the chromatin DNA content in fraction II and assuming that all kinase activity in Fraction IIa is eluted from Fraction II

DISCUSSION

In the present work both sucrose gradient centrifugation and ECTHAM-cellulose chromatography were employed to separate the template active and inactive regions of oviduct chromatin. With respect to the former procedure, we have previously shown that sheared chromatin which sediments in the lighter, more transcriptionally active peak has additional properties expected of "active" chromatin (6). Although the significance of the variation in base composition of RNA's synthesized from these fractions (Table 1) is uncertain, the differences do indicate that the DNA sequences accessible to E. coli polymerase were not identical in the active and inactive chromatin fractions. It is important to realize, however, that DNA sites accessible to E. coli polymerase in vitro are not necessarily identical to those transcribed in vivo. The origin of the relatively G-C rich sequences transcribed in the pellet fraction could be a heavy satellite DNA in which is a minor component of the chick genome. Satellites of mouse, guinea pig and crabs have previously been shown to be contained in heterochromatin. Studies of ECTHAM fractionation to be reported elsewhere (7,11) demonstrated that Fractions I and II possess both physical and biological properties exepcted of active and inactive chromatin, respectively.

In the protein kinase assay performed on the various chromatin fractions, casein was employed as a substrate. However, significant phosphorylation also occurred if casein was omitted from the assay (Table 2), indicating that endogenous proteins were phosphorylated. It was of interest to ascertain the nature of these endogenous protein substrates. Several lines of evidence suggest that they were predominantly nonhistones. First, if oviduct nuclei were phosphorylated in the presence of $AT^{32}P$ and the nuclear proteins were fractionated (12), 90-95% of the protein bound radioactivity was associated with nonhistones. Second, after extraction and partial purification of the oviduct nuclear protein kinase, substrate specificity studies revealed that acidic proteins, such as casein and phosvitin, were considerably better sub-

strates than basic proteins, such as histone and protamine (13). Third, protein kinases which phosphorylate histones were generally present in the cytosol; this has been shown to be the case with the oviduct histone kinase (14). We therefore feel that the protein kinase activity we observed was primarily responsible for the phosphorylation of nonhistones. The preferential association of this activity with the template active fraction is of interest in light of the proposed role of nonhistone protein phosphorylation in gene regulation of eukaryotes.

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