Effect of Non-Histone Chromosomal Proteins on Transcription in vitro in Sea-Urchin

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Non-histone chromosomal proteins prepared from chromosomal material of the sea-urchin Paracentrotus lividus affect RNA synthesis in vitro. 1. The extent of transcription can be radically changed from inhibition to stimulation, depending on the DNA/non-histone chromosomal proteins ratio. 2. A correlation exists between stage of development and influence on transcription. 3. Non-histone chromosomal proteins exert their action by intervening directly on some initiation step of RNA synthesis, as shown by the numbers of initiation events that take place in their presence or absence. 4. Stimulatory activity is observed only in restrictive conditions of ionic strength and temperature. These observations are in agreement with models that predict for non-histone chromosomal proteins a regulatory role on the transcription process exerted through a modulation of promoter availability.

It is generally postulated that RNA polymerase activity is regulated by the selective control of promoter availability (Travers, 1976). Basically the process should require first the recognition of a sequence and then the interaction with a binding region; RNA synthesis starting from an RNA-initiation point (Prihnow, 1975) would then follow. The transition from the recognition complex to the stable pre-initiation complex involves local 'melting' within the promoter (Saucier & Wang, 1972); agents that affect the stability of the double helix, such as ionic strength (Mangel & Chamberlin, 1974b) or organic solvents (Nakanishi et al., 1974; Travers, 1974), effectively influence the transition necessary to initiate RNA synthesis. It has furthermore been observed that such transition occurs in vitro within a narrow range of temperature, as in the synthesis of Escherichia coli rRNA (Travers et al., 1973) and in the transcription of phage-λ gal DNA and of phage-λ sex DNA (Nakanishi et al., 1974). We have previously reported (Di Mauro et al., 1977) that the initiation of RNA synthesis is also temperature-dependent for sea-urchin DNA, where one initiation event takes place every $2 \times 10^4$ base-pairs at 37°C and every $10^5$ base-pairs at 12°C. Such temperature-dependent transitions were not observed for transcription of sea-urchin chromatin (Di Mauro et al., 1977; Tsai et al., 1976), where we found a 100-fold decrease in initiation events. This implies that some factors other than RNA polymerase and template sequence intervene to mediate the formation of initiation complexes in eukaryotes.

In the present paper we report observations on the action of sea-urchin non-histone chromosomal proteins that lend support to current theories that predict a regulatory role for such proteins. They have a marked influence on template activity, show correlation with developmental stage and seem to abolish the temperature-dependent transitions observed on purified templates, but absent on chromatin.

Experimental

Materials

Nucleoside triphosphates were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. [14C]UTP (14Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. E. coli B was grown in a complete medium (Burgess, 1969) to the late-exponential phase and kept as a frozen paste. Purified calf thymus histones (type II) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Spermatozoa from Paracentrotus lividus were obtained by two gastrointestinal injections of 0.55m-KCl. After thorough washing with sterile sea-water, spermatozoa were collected by centrifugation at 10000rev./min in a SS34 Beckman rotor and kept frozen.
homogenizer in hypo-osmotic medium [10mm-Tris/HCl (pH 8.0)/5mm-MgCl₂/5mm-dithiothreitol containing 200μg of phenylmethanesulphonyl fluoride/ml]; the resulting chromatin was washed twice and then resuspended in the same buffer made 1m with (NH₄)₂SO₄. The suspension was then sonicated six times for 20s with 1min cooling intervals [this was carried out with an MSE Sonifier (10mm probe at 20000rev./min) and centrifuged at 35000rev./min for 2h in a Beckman SW41 rotor. The supernatant containing the non-histone chromosomal proteins was made 65% saturated with (NH₄)₂SO₄ and centrifuged at 30000rev./min for 1h in a Beckman SW41 rotor. The resulting pellet was resuspended in 20ml of 10mm-Tris/HCl (pH 8.3)/5mm-MgCl₂/1mm-dithiothreitol containing 200μg of phenylmethanesulphonyl fluoride/ml and extensively dialysed for 36h against 6 litres (three changes) of the same buffer; contaminating lysine-rich histones are precipitated in this buffer and were removed by centrifugation (4h in a Beckman SW41 rotor at 37000rev./min, 4°C). The remaining clear supernatant, referred to as non-histone chromosomal proteins, was devoid of histones, as judged by electrophoresis (Panyim & Chalkley, 1969) (Fig. 1). The non-histone chromosomal protein solution was made 50% (v/v) in glycerol and kept at −20°C. In RNA-synthesis experiments non-histone chromosomal proteins were used as such. The non-histone chromosomal protein fraction was also devoid of contaminating nuclease activity; this was shown as described by Di Mauro et al. (1972).

**Methods**

**Isolation of E. coli RNA polymerase, preparation of DNA and growth of sea-urchin embryos.** These were done by procedures described by Di Mauro et al. (1977).

**RNA synthesis.** Conditions for RNA synthesis were as described by Di Mauro et al. (1977), both for reactions where reinitiation was inhibited and for normal synthesis. Incorporation without re-initiation was accomplished essentially by the procedure of Hymann & Davidson (1970), modified as described by Di Mauro et al. (1977).

**Preparation of non-histone chromosomal proteins.** Embryos were kept frozen at −12°C [freezing was done slowly in sea-water containing 10% (v/v) glycerol]. Thawed embryos were washed twice and resuspended in 3 vol. of buffer A [0.05m-Tris/HCl (pH7.6)/1mm-EDTA/10mm-β-mercaptoethanol], and 0.25m-sucrose, homogenized in a loose Dounce homogenizer and centrifuged at 8000rev./min in a Beckman SS34 rotor for 20min. The pellet containing the nuclei was kept at 0°C for 10−20min in buffer A containing 0.5% Triton X-100, washed twice in buffer A and resuspended in 60ml of buffer A containing 1.6m-sucrose. It was then layered in six centrifuge tubes each containing 20ml of buffer A containing 1.9m-sucrose and centrifuged at 22000rev./min for 60min in a Beckman SW 27.1 rotor. Purified nuclei were gently broken in a Dounce
Fig. 2. Template activity of sea-urchin DNA in the presence of non-histone chromosomal proteins from gastrulae
(a) Increasing amounts of DNA were incubated (50 μl total vol.) for 5 min at 37°C in the absence (○) or in the presence of 0.1 (●), 0.4 (■) or 1.2 (▲) μg of non-histone chromosomal proteins, then 0.7 μg of E. coli polymerase and the synthesis mixture (see the Experimental section) were added to 100 μl final volume. Reaction was stopped after 15 min at 37°C. In both preincubation and synthesis 0.1 M-(NH₄)₂SO₄ was present. (b) Preincubation of non-histone chromosomal proteins with DNA and synthesis were as in (a). Preincubation of the indicated amount of non-histone chromosomal proteins was done in the presence of 0.8 (▲), 2.0 (○) and 5.0 (●) μg of sea-urchin DNA. Incorporation in the absence of non-histone chromosomal proteins was respectively 1750, 2100 and 2050 c.p.m. and was taken as 100% value.

(20 μg/ml) and undersaturating (8 μg/ml). The results indicate that a DNA/non-histone chromosomal proteins ratio optimal for stimulation is observed only at oversaturating concentrations of template and that a decrease in DNA leads to a decrease in the relative template activity. Variations of the amount of RNA polymerase (in conditions of template excess) did not alter the observed pattern (results not shown), showing that the enzyme concentration is not involved in the inhibition/stimulation effect, which seems to be determined by DNA/non-histone chromosomal proteins ratio only.

Correlation with the developmental stage

The effect of inhibition/stimulation of the template activity does not itself provide much information on the specificity attributes of the proteins under investigation. The sea-urchin system, however, offers the possibility of obtaining chromatin from embryos at different stages of development. If the proteins extracted had some physiological role in the control of transcription, one should observe a parallel in vitro with the RNA-synthetic activity in vivo. Fig. 3(a) shows the effect of non-histone chromosomal proteins purified from nuclei of freshly laid eggs, known to synthesize very little, if any, RNA [for a review of data on RNA synthesis in vivo during sea-urchin development see Giudice (1973)]. The effect of non-histone chromosomal proteins from eggs is only inhibitory, and a decrease in the amount of proteins used only leads to a decrease in the effect; we have never observed any appreciable stimulation by this material. Testing of the inhibitory capacity of the proteins extracted at different stages of development (Fig. 3b) shows that: (a) non-histone chromosomal proteins from eggs are able to block completely the transcription at a protein/DNA ratio of 15; (b) non-histone chromosomal proteins from embryos at higher stages of development have a decreased inhibitory capacity; (c) there is an increasing fraction of the template activity that cannot be blocked by very high concentrations of non-histone chromosomal proteins from developing embryos even at early stages. We have not observed, probably because of the limited sensitivity of the method, the same quantitative correlation in the stimulation effect.
Fig. 3. Effect of non-histone chromosomal proteins from various stages of development on transcription
(a) Template activity of sea-urchin DNA in the presence of non-histone chromosomal proteins from mature eggs. Increasing amounts of DNA were incubated for 5 min at 37°C in the absence (○) or in the presence of 0.05 (●) or 1.0 (■)μg of non-histone chromosomal proteins. Conditions were as in Fig. 2(a). (b) Differential inhibitory activity of non-histone chromosomal proteins purified from embryos at different stages of development. The effect of the amount of proteins shown in abscissa was tested, as above, on 0.8μg of sea-urchin DNA (subsaturating amount). Non-histone chromosomal proteins were from mature eggs (●), morulae (32 cells) (○), hatching blastulae (△) or mid-gastrulae (□). The 100% value (in the absence of non-histone chromosomal proteins) was 1850 c.p.m.

Mode of action

(a) Frequency of initiation sites. The natural target for regulatory proteins of the chromatin is one (or more) of the steps that lead to initiation of the synthesis of RNA. The fact (see Fig. 2a) that the amount of non-histone chromosomal proteins changes the saturation point of a DNA-saturation curve is a strong indication that their positive and negative activity is exerted on one of the initiation steps. At the optimum DNA/non-histone chromosomal proteins ratio, for instance, the DNA shows a 2-fold increase in transcriptional capacity. By directly calculating how many initiation sites are available, it can be shown that such an increase is due to an effective increase of availability of initiation sites. This can be conveniently achieved by the Hymann–Davidson technique (Hymann & Davidson, 1970) referred to in the Experimental section: the enzyme was preincubated with template and proteins (as indicated in the legend of Table 1) in a reaction mixture lacking one nucleoside triphosphate, in ionic conditions that allowed initiation and start of the synthesis, but not extensive elongation, to occur; after 15 min the missing triphosphate was added and ionic strength was increased to 0.4M-(NH₄)₂SO₄, thus allowing propagation, but no new initiation, to take place. The determination of incorporated radioactivity, the analysis of the length of RNA chains produced and the calculation of the initiation sites was performed as described by Di Mauro et al. (1977).

Table 1 reports the results of the calculation of available initiation sites in relation to the state of the template used: a decrease in the amount of RNA synthesis corresponds to a decrease in the frequency of initiation sites and vice versa. This evidence, taken together with the displacement of the DNA-saturation point, shows that non-histone chromosomal proteins act by altering the ability of the template to support synthesis.

(b) Conditions that alter template activity of DNA. It is known that factors that induce alteration of the conformation of the template [supercoiling (Hayashi & Hayashi, 1971), glycerol and other organic compounds (Nakanishi et al., 1974; Travers, 1974), ionic strength (Mangel & Chamberlin, 1974b) and temperature (Travers et al., 1973; Mangel & Chamberlin, 1974c)] have a strong influence on its template activity.

(i) Ionic strength. It is known that increased ionic strength affects enzyme conformation, the stability of binding to the DNA template and the rate and
Table 1. Influence of non-histone chromosomal proteins on the number of initiation sites

RNA polymerase (7.0 \mu g) was used in a transcripive system containing 10 \mu g (80\% of saturation) or 60 \mu g (3-fold excess) of sea-urchin DNA. The conditions of synthesis and of calculation of the initiation sites are reported in the text. The amounts of egg and gastrula non-histone chromosomal proteins used were 1.0 \mu g with limiting DNA and 10 \mu g of both non-histone chromosomal proteins with excess of DNA. The average chain length (+10\%) of the RNA chains produced was 390 nucleotides in the limiting DNA condition, and 480 nucleotides for the excess DNA condition.

<table>
<thead>
<tr>
<th>Relative DNA saturation</th>
<th>Transcription efficiency (%</th>
<th>10^{-4} \times \text{No. of base-pairs/initiation site}</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>80% 3-fold excess</td>
<td>80% 3-fold excess</td>
</tr>
<tr>
<td>Egg non-histone chromosomal proteins</td>
<td>100 100</td>
<td>2.8 12.0</td>
</tr>
<tr>
<td>Gastrula non-histone chromosomal proteins</td>
<td>32 65</td>
<td>7.8 19.0</td>
</tr>
<tr>
<td></td>
<td>125 80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0 14.0</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Influence of ionic strength on effect of non-histone chromosomal proteins

In (a) and (b) template activity of sea-urchin DNA was measured in the presence of 0.005 (\circ) or 0.1 (\bullet) \text{m-(NH}_4\text{)}_2\text{SO}_4. Assay conditions were as in Fig. 2(a). (a) Non-histone chromosomal proteins absent; (b) 0.2 \mu g of gastrula non-histone chromosomal proteins present. (c) Influence of \text{NH}_4\text{SO}_4 concentration on transcription in the absence (\circ) or presence (\bullet) of 0.2 \mu g of gastrula non-histone chromosomal proteins. Assay conditions were as in Fig. 2(a); 0.7 \mu g of RNA polymerase and 1.0 \mu g of sea-urchin DNA were present per assay.

duration of RNA-chain elongation (for a review see Chamberlin, 1974). It has in particular been shown that increasing salt concentrations progressively decrease the fraction of enzyme molecules that can initiate an RNA chain (Mangel & Chamberlin, 1974b), and evidence has been presented that low ionic strength favours the formation of a rapid-starting binary complex between DNA and RNA polymerase, whereas a non-starting or slow-starting complex is favoured at high ionic strength. In the E. coli enzyme–P. lividus template system, we can observe that a three-fold increase in the amount of DNA necessary to saturate a given amount of enzyme occurs when the (\text{NH}_4\text{)}_2\text{SO}_4 concentration increases from 0.005\text{m} to 0.1\text{m}, i.e. the availability of the template to the enzyme is decreased at higher ionic strength (Fig. 4a). The presence of gastrula non-histone chromosomal proteins has no effect at low ionic strength, where DNA displays more initiation sites to the enzyme, but markedly changes the template activity at 0.1\text{m-(NH}_4\text{)}_2\text{SO}_4: non-histone chromosomal proteins efficiently antagonize the decrease of template availability caused by increased ionic concentrations.

Taking into account the evidence obtained by Mangel & Chamberlin (1974b) that the lower amount
of initiation events at high salt concentration is due to a shift of equilibrium between non- or slow-starting and rapid-starting complexes in favour of the former, the experiment reported here is interpreted to mean that non-histone chromosomal proteins favour the formation of stable rapid-starting complexes; in low salt concentrations synthesis starts with an already high efficiency and the effect of non-histone chromosomal proteins cannot be seen.

(ii) Temperature. RNA synthesis in vitro is strongly affected by temperature. Essentially every step of the synthesis is influenced, from modification of the structure of the DNA itself (Von Hippel & McGhee, 1972) to the rate of RNA-chain elongation (Manor et al., 1969). The most dramatic effect has, however, been observed (Mangel & Chamberlin, 1974c) on the formation of the binary complex between RNA polymerase and DNA, which is able to start synthesis; it was therefore decided to test in this respect the influence of the sea-urchin non-histone chromosomal proteins in the transcription of the homologous template.

To study the initiation step separately from subsequent synthetic stages we made use of the technique described by Di Mauro et al. (1977) that allows separation of initiation from elongation. Fig. 5 shows how template efficiency of sea-urchin DNA is affected by the temperature. The initiation step was allowed to occur at 0°C, the elongation at 37°C. In the absence of non-histone chromosomal proteins the amount of DNA necessary to saturate 0.7μg of the enzyme at 0°C is 7μg, a value 5 times that necessary to saturate the same amount of enzyme at 37°C (see Fig. 1a). This effect of temperature on the initiation step is generally well known, and we have described it for sea-urchin DNA (Di Mauro et al., 1977). Fig. 5 also reports the influence of non-histone chromosomal proteins on the template capacity at 0°C. The effect is clear-cut, and shows that in the presence of non-histone chromosomal proteins (a) DNA is more available, and (b) the saturation obtained at 0°C is identical with that obtained at 37°C in the presence of proteins (see Fig. 2a) (in the presence of non-histone chromosomal proteins the saturation occurs at 1μg at both 0 and 37°C; in their absence it occurs at 2μg at 37°C, or at 7μg at 0°C). An increase in temperature thus increases the initiation sites available; at 0°C non-histone chromosomal proteins mimic the temperature effect, giving the DNA at 0°C a transcriptional capacity even higher than that shown at 37°C by the DNA alone.

This result is in agreement with that of the ionic-strength experiment: the action of the non-histone chromosomal proteins is mostly evident in conditions (high salt or low temperature) that disfavour rapid starting-complex formation.

**Discussion**

Current theories on the regulatory function of the chromatin imply a fundamental role of non-histone chromosomal proteins (Paul, 1972). Evidence is rapidly accumulating that a specific regulatory role can also be maintained by these proteins in vitro (Gilmour & Paul, 1973; Haxel et al., 1973; Barret et al., 1974), in different transcriptive systems: chromatin is not after all a particularly delicate material. RNA polymerase activity is thought to be regulated by the selective control of promoter availability, and much direct evidence for this has accumulated in prokaryotes (Travers, 1976); in eukaryotes such a selective control is supposed to be basically of the same nature, although much more complex because of the higher integrative necessities of the eukaryotes (Britten & Davidson, 1969).

The present paper describes some general properties of the sea-urchin non-histone chromosomal proteins and tests whether their function can be correlated with promoter availability.

The developing sea-urchin embryo is an appropriate system for this kind of study, because it offers a well-synchronized material for the observation of stage-related differences of the chromatin components and because of a programmed RNA synthesis [i.e. rRNA is not synthesized before the gastrula stage and histone mRNA is essentially the only product during the first few cleavage divisions; for a review of this subject see Guidice (1973)].

The experiments reported in which a specificity of
the chromatin function was shown made use of
E. coli RNA polymerase and not of the corresponding
homologous multiple forms, showing that basic
specificity resides on the template and not on the
enzymic system (thought to provide only a gross
control). Moreover, detailed knowledge of the
various steps of the E. coli RNA polymerase-
template interaction is available. In this respect
particularly useful is the demonstration (Mangel &
Chamberlin, 1974a) that the discriminatory factor in
initiation is the existence of an equilibrium between
two forms of binary complex, the slowly or non-
initiating and the rapidly starting, and that ionic
strength and/or temperature affect the synthesis
of RNA by influencing this equilibrium (Mangel &
Chamberlin, 1974b, c).

One basic characteristic of the non-histone
cromosomal proteins that we have purified from
developing sea-urchin embryos is that their stimula-
tory–inhibitory action is a function of the amount
of the template present (that is of the relative propor-
tions enzyme/template/non-histone chromosomal
proteins).

Many examples of more or less purified protein
factors that stimulate RNA synthesis in vitro have
been reported (Stein & Hausen, 1970; Jacquet et al.,
1971; Di Mauro et al., 1972; Lee & Dahmus, 1973;
Seifart et al., 1973; Sudgen & Keller, 1973; Teissere
et al., 1975). Some exhibit pronounced enzyme
specificity and have much greater stimulatory effect
on eukaryotic RNA polymerase form B (Stein &
Hausen, 1970; Seifart et al., 1973; Sudgen & Keller,
1973). Other factors, such as 7-factor purified from
Saccharomyces cerevisiae (Di Mauro et al., 1972),
seem to stimulate some step of the transcription
process that is common to a large number of enzyme-
template combinations; we have in addition ob-
served that 7-factor has a stimulatory–inhibitory
action that depends, as for sea-urchin non-histone
cromosomal proteins, on the amount of template
present (Di Mauro et al., 1974). A correlation of the
stimulatory activity with the excess of template has
been reported also for the E. coli factor H (Jacquet
et al., 1971). The widespread occurrence (E. coli,
yeast, sea urchin) of this effect leads one to think that
some general aspect of the initiation of transcription
in vitro is affected by this type of protein [defined as
type-II factors (Lukacs & Stein, 1976)]. The observa-
tion also that spermine, spermidine, polylysine or a
mixture of histones (Hall et al., 1973) in conditions
of enzyme shortage, or histone I alone (Lukacs &
Stein, 1976; Konishi & Koide, 1971), in the appro-
priate range of concentration, have a dose-dependent
effect on the amount of RNA synthesis, further
generalizes the interest of the system and indicates
that it may be possible to construct simple model
systems.

Besides these general aspects correlated with the
mode of action, it is noteworthy that sea-urchin non-
histone chromosomal proteins are also endowed in
vitro with some specificity, as shown by the lack of
stimulation by non-histone chromosomal proteins
prepared from eggs and by an inhibitory power
differentially correlated with the synthetic capacity
in vivo. It will be of interest to test the populations of
RNA synthesized in vitro for similarities with those
made in vivo. Such observations of relative specificity,
although only quantitative and therefore not com-
pletely satisfactory, are, however, sufficient to rule
out the most trivial explanations and indicate a
physiological role. We suggest that such a role is of
the kind described for non-histone chromosomal
proteins in the model proposed by Paul (1972).

The evidence that supports such a hypothesis is:
(a) the action of the proteins, being a function of the
DNA/non-histone chromosomal proteins ratio, is
exerted directly on the DNA; (b) their influence is
exerted directly through initiation; (c) the condi-
tions that shift the equilibrium from rapid-starting
complex to non- or slowly-starting complex, dis-
favouring the productive synthetic events, are
counter-balanced by non-histone chromosomal
proteins; noteworthy is the removal of the tempera-
ture-dependent transitions normally exhibited by the
purified template.

The profound effect exerted by sea-urchin non-
histone chromosomal proteins on the template
activity of the DNA seems to render it more similar
to the chromatin state. This consideration may help
to understand the observation that at high protein/
DNA ratio the promoter enhancement is lost and
substituted by inhibition; considering that the main
function of chromatin proteins should be the im-
position of specific limitations on the sequences
transcribed, at high protein/DNA ratio this limita-
tive function may become the quantitatively more
important one. Another explanation of the observed
inhibition at high protein/DNA ratio might be that
enhancement and inhibition are each due to different,
unequally active, protein subpopulations. The results
presented here, however, are not sufficient to allow
us to speculate more on this point: the possibility of
obtaining purified ribosomal (E. di Mauro & M.
Pomponi, unpublished work) and histone (Birnstiel
et al., 1974) genes in this material will provide the
means of studying more precisely the function of
non-histone proteins.

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