EFFECT OF REMOVAL OF f1-HISTONE ON THE CONFORMATION OF NUCLEAR CHROMATIN AND ON THE TRANSCRIPTION PROCESS

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1. Introduction

The investigation of the well-established stimulation effect of the endogeneous Mn^{2+}-dependent RNA synthesis in isolated nuclei by increasing ammonium sulfate concentrations [1–7] seems to offer a suitable way to get more insight into the mechanism of the transcription process of eukaryotic chromatin. In spite of the fact that the stimulation by ammonium sulfate creates very unphysiological conditions it may help to recognize what kind of changes at the template correlate with an activation of the transcription process under conditions at which association of new RNA polymerase molecules with the template are excluded. The influence of different ammonium sulfate concentrations on the removal of histones from the template with the chain length of the in vitro RNA synthesis product and on the conformational state of the chromatin as measured by hydrodynamic methods was checked as an attempt to elucidate the relationships between the template organization and the function of eukaryotic chromatin in the transcription process.

2. Materials and methods

Nuclei were isolated from 50-day-old Wistar rats by a modification of Chaveau's method [8]. For the sedimentation experiments as well as for viscosimetry measurements the nuclei were lysed in the presence of ammonium sulfate 0.1–0.4 M and 2 mM Mn^{2+}. The water clear nuclear lysates so obtained were centrifuged for 5000 *g* and 30 sec and the supernatant was used.

3. Results

In order to understand the molecular basis by which ammonium sulfate stimulates the process of the RNA synthesis in the isolated nucleus it seems to be of interest to notice that there are two steps of stimulation (fig. 1). The first step appears below 0.05 M ammonium sulfate. Approximately one half

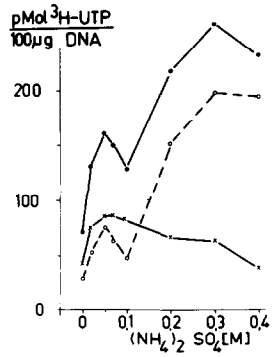

Fig. 1. Effect of ammonium sulfate concentration on the incorporation of [^{3}H]UTP in the isolated rat liver nuclei in the presence of 2 mM Mn^{2+}. Synthesis conditions: 10 min at 30°C, 20 µg nuclear DNA in 0.2 ml assay. (●——●) without α-amanitin; (×——×) with 2 µg α-amanitin; (○——○) calculated difference curve.

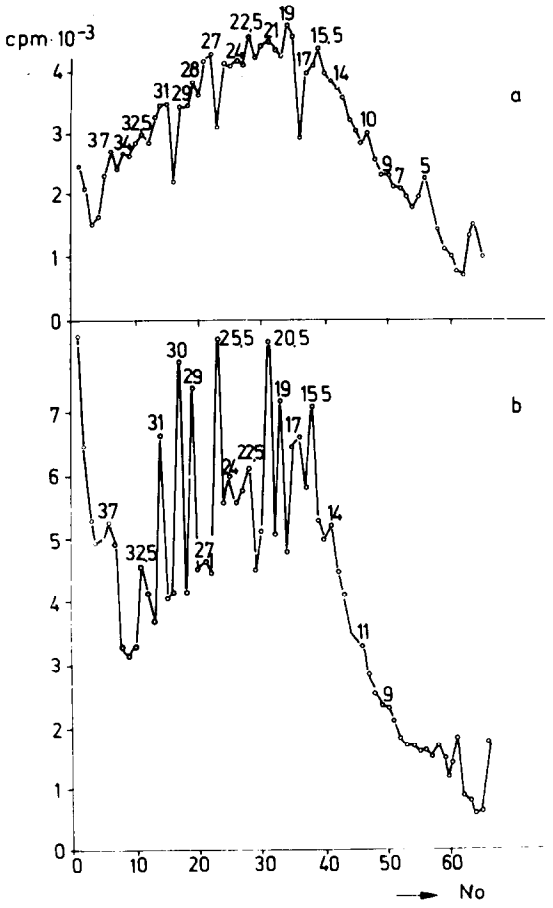

Fig. 2. Isokinetic gradient centrifugation analysis of RNA synthesized in vitro in rat liver nuclei in the presence of (a) 0.05 M ammonium sulfate; (b) 0.3 M ammonium sulfate. Synthesis conditions: 30 min, 30°C. Nuclei preparation and RNA synthesis were performed in the presence of the cytoplasmic RNase inhibitor isolated by affinity chromatography.

of the RNA synthesized in vitro at salt concentrations below 0.1 M ammonium sulfate represents the product of RNA polymerase B as can be concluded by the results obtained from α-amanitin inhibition. A further addition of ammonium sulfate up to 0.1 M yielded even a slight decrease of [^{3}H]UTP incorporation by the B-enzyme. The second increase of RNA synthesis is obtained between 0.1 M and 0.3 M ammonium sulfate. Further, the steepness of the stimulation between 0.1 and 0.2 M ammonium sulfate is remarkable. By chase experiments we could show that in the presence of ammonium sulfate up to 0.05 M no significant decrease of the incorporated counts was observed during a time of 30 min. Only without any addition of the salt some loss of RNA synthesized in vitro was found. The results of the chase experiments cannot exclude that the first stimulation peak at 0.05 M ammonium sulfate arises from an inhibition of remaining RNA-degrading enzymes of the nuclei which are active at lower ionic strength. They show, however, unequivocally that the increased amount of incorporated [^{3}H]UTP at ammonium sulfate concentrations higher than 0.1 M is based in fact on a stimulation of the transcription process.

Centrifugation profiles of the RNA synthesized in vitro at 0.05 M and 0.3 M ammonium sulfate, respectively, are shown in fig. 2. In both cases a heterogenous synthesis product of different chain lengths within a broad range was obtained. The analysis of 40 runs of different RNA preparations synthesized between 0.1 and 0.4 M ammonium sulfate showed that generally typical RNA fractions were found (table 1). The presence of these distinct RNA fractions in the in vitro RNA synthesis product was confirmed by re-centrifugation of a series of first run fractions. In the presence of α-amanitin these typical RNA fractions were not found in the RNA synthesis product. That means that the RNA syn-

Table 1
Frequency (n) of the appearance of RNA fractions with distinct sedimentation coefficients (s) related to 40 runs of different RNA preparations synthesized between 0.2 and 0.4 M ammonium sulfate

Approx. s	n	Approx. s	n	Approx. s	n
5	15	14	28	21.5	3
7	27	15.5	27	22.5	27
8	3	16	3	24	34
9	35	17	30	25.5	26
10	8	18	8	26.5	2
11	30	19	33	27	27
12.5	24	19.5	3	28	3
13	2	20	6	29	30
13.5	3	21	37		

Fractions with higher sedimentation coefficients are not listed. The underlined values represent RNA fractions with distinct chain lengths that are characteristic of the in vitro RNA synthesis product of RNA polymerase B.

thesized in vitro by the B-enzyme consists of a collection of distinct RNA fractions with different chain lengths (see table 1). Chase experiments performed at 0.3 M or 0.1 M ammonium sulfate, respectively, excluded the possibility that the distinct RNA fractions arose from a subsequent cleavage of original longer chains (see fig. 3). Chase experiments as well as a series of time experiments showed clearly that the RNA molecules were enlarged during the in vitro assay via the discrete chain lengths described above. It was further shown that the relative amounts of the distinct RNA fractions depend on the salt concentration in such a way that the more rapidly sedimenting RNA fractions increased with the ammonium sulfate concentration between 0.1 and 0.4 M as well as with time. If after a pretreatment of the nuclei at a higher salt concentration (see fig. 3) the RNA synthesis was performed at a lower salt concentration a similar distribution of the different RNA fractions was obtained as typical of the higher salt concentration. The work of Markov and Arion [9] may indicate that the slowly sedimenting RNA fractions could be present also under in vivo conditions. A series of in vivo rapidly labelled distinct minor RNA fractions with approximate sedimentation values of 5 S, 7 S, 9–10 S, 12–15 S and 16 S was demonstrated by these authors.

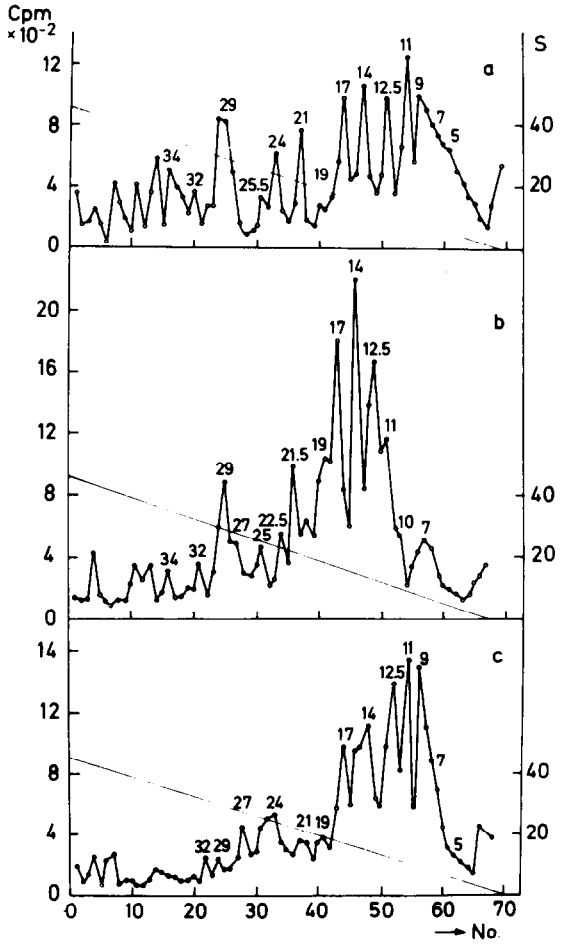

Fig. 3. Isokinetic sucrose gradient centrifugation analysis of RNA synthesized in vitro in rat liver nuclei without and with chase. (c) RNA labelled in vitro for 5 min with [^{3}H]UTP in the presence of 0.3 M ammonium sulfate; (b) RNA prelabelled in vitro for 5 min with [^{3}H]UTP. After that an excess of 50-fold cold UTP was added to the assay and the incubation was continued for another 15 min before the RNA was isolated; (a) conditions as in (b) but with the addition of cold UTP the ammonium sulfate concentration was lowered to a final concentration of 0.1 M ammonium sulfate by dilution.

Referring to the studies on in vitro RNA synthesis in the rat liver nucleus the removal of histones from DNA by increasing ammonium sulfate concentrations in the region between 0.1 and 0.4 M was investigated. Under the conditions of the in vitro RNA synthesis a

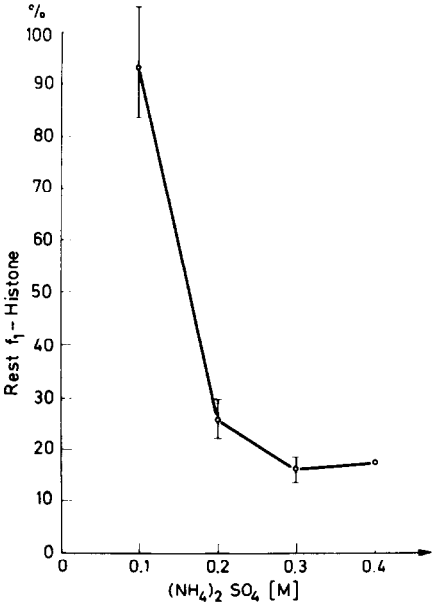

Fig. 4. Extractability of histone f1 from the salt-treated chromatin.

strong removal of histone f1 together with some f2b appeared between 0.1 and 0.2 M ammonium sulfate (fig. 4). This is the concentration region in which the steepest increase of in vitro RNA synthesis by ammonium sulfate was found (see fig. 1). Our results on the correlation of the removal of histone f1 and stimulation of the RNA synthesis by the endogenous RNA polymerase B complement the results of Georgiev et al. [10] who could show some time ago that in their salt-extracted chromatin removal of histone f1 leads to a strong increase of the template activity with *E. coli* RNA polymerase.

The sedimentation behaviour of the chromatin in the ammonium sulfate concentration range between 0.1 and 0.4 M has been examined. A strong dependence of the sedimentation coefficient was observed in this range (fig. 5). The difference in the sedimentation coefficient between 0.1 and 0.2 M ammonium sulfate was most prominent. It was more pronounced than found with chromatin prepared according to a modification of the method of Marushige and Bonner [11]. Moreover, after the salt concentration has once been raised up to 0.2 M a subsequent lowering to 0.1 M did not reproduce the original behaviour of the chromatin at this salt concentration. In contrast, the sedimentation behaviour of the DNA obtained by treatment with proteinase K of the respective chromatin preparations did not vary with the ammonium sulfate concentration. The values of the sedimentation analysis in combination with viscosity measurements (not shown) may be explained by changes in association as well as in conformation of the chromatin.

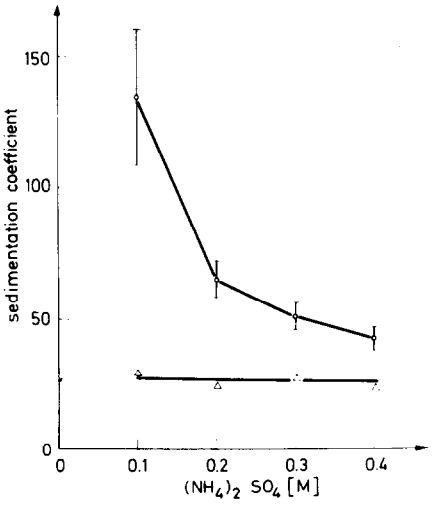

Fig. 5. Sedimentation coefficients of chromatin (○) (Extinction $E^{1\,cm}_{260\,nm}$ between 0.8 and 1.0) corrected for temperature and viscosity of the solution as a function of the ammonium sulfate concentration. Related DNA was prepared by proteinase K (△). Errors bars are standard deviations calculated for 10 experiments of 5 different preparations.

From our results we suggest that the chromatin material obtained directly from the nucleus with 0.1 M ammonium sulfate retains native characteristics in a kind of secondary level of organization. The native water clear solubilized chromatin complex at 0.1 M ammonium sulfate is characterized by a high sedimentation coefficient and viscosity as well as by the fact that histone f1 can be easily removed by salt extraction [12]. It further shows a low template activity. The native chromatin complex changes its properties gradually if chromatin is prepared according to commonly used methods. That means that histone f1 will be more tightly bound to the chromatin as it was shown by the change of the salt extractability with the isolation procedure [12]. Besides viscosity and sedimentation behaviour are already lowered at 0.1 M ammonium sulfate. The correlation of the removal of histone f1 from the chromatin with the changes of the hydrodynamic behaviour between 0.1 and 0.2 M ammonium sulfate suggests that histone f1 plays a prominent role in the maintaining of the secondary level of organization. A superstructure in native chromatin with cross-linkings between nucleohistone strands was deduced by other authors [13,14]. Chalkley and Jensen [14] who prepared calf thymus chromatin by gentle methods demonstrated its heterogeneous sedimentation behaviour. They could show that the rapidly sedimenting fraction of their chromatin preparation (about 130 S) behaved as would be expected for a cross-linked nucleohistone molecule. The most abundant 30 S material, however, lacked the intermolecular cross-linked structure. As in our experiments, the disruption of the complex structure of the 130 S material by higher salt concentrations was irreversible. The strong stimulation of RNA synthesis above 0.1 M ammonium sulfate supports the idea that decondensation of the chromatin after removal or changing of histone f1 leads to an activation of the chromatin template.

The relationship between the appearance of distinct RNA groups with distinct chain lengths in the in vitro RNA synthesis experiments and the postulated superstructure of the chromatin remains unsolved as yet. The RNA synthesized in vitro in the nuclei is, of course, the result of the transcription of a big number of different transcription units. From the fact that discrete groups of RNA with distinct chain lengths can be demonstrated which grow up via distinct RNA fractions with time as well as with increasing ammonium sulfate concentrations we suggest that the discrete lengths of the RNA molecules reflect a general organization principle of chromatin. The simplest way to explain the fact is the assumption that at least on parts of the transcription units of the isolated nuclei 'transcription blockers' are localized. They appear to be arranged at distinct places and give rise to a temporary or final stop to the travelling RNA polymerase molecules. These hypothetical transcription blockers may be partly removed or inactivated by increasing salt concentrations as well as with time at a distinct salt concentration above 0.1 M ammonium sulfate. Between two blockers the RNA polymerase may travel more or less unchecked. The question why in vitro only a part of the endogenous RNA polymerase travels along the transcription units if the ammonium sulfate concentration is not raised above 0.1 M is an open one up to now. A latent RNA polymerase activity which can be unmasked by high salt concentrations was found in fixed mouse kidney epithelial cells (Auer et al. [14]). From the in vitro experiments with isolated rat liver nuclei it seems probable that in the nucleus of the liver cells also only a part of the existing RNA polymerase is in fact simultaneously engaged in the RNA synthesis process in the living cell. The other part may be associated with the template in any 'pre- or post-initiation' state which can be unmasked in the vitro system by the appropriate salt concentration. This activation of additional transcription units in vitro seems to be correlated with the removal of histone f1 from the template. In order to get more insight into the processes of the in vitro RNA synthesis a simulation model was constructed.

Appendix: Computer simulation model

The aim of this model is to test the phenomenological effects of the assumed transcription-inhibiting blockers. By means of the comparison between the experimental results and the simulation results of the computer model it is possible to verify hypotheses about the internal system parameters. The real system to be mapped is the transcription apparatus with its structure and function elements (1) DNA-sequence; (2) blocker sites inhibiting RNA-synthesis in such a manner that RNA chains with distinct lengths arise, each blocker with a time-dependent probability of being removed; (3) polymerase molecules synthesizing RNA with a certain velocity and with a probability depending on time to be unable to synthesize further RNA, i.e. to have the velocity zero and with a certain distribution over the template at the beginning of the experiment (start distribution).

Because the experimental results do not concern a single template's behaviour but they are integrative results concerning a set of transcribable DNA-sequences as a whole, a probabilistic approach is suitable. According to the nature of the real process, the model is a discrete one.

The basic idea of the transcription model is as follows. We define a transcription system τ consisting of transcription units $T_k \epsilon \tau$. On each unit T_k ($k = 1, 2, \ldots K$) open for transcription during the experiment blockers B_j are localized at template sites A (B_j) on each template T_k. This is suggested immediately from experimental results (necessary conclusion). Let M_{ik} be an RNA molecule of length x_i synthesized on the k-th template. We get in τ the sets $\mathbf{M}_i$ with elements $M_{ik} \cdot p(x_i)$ is the probability that a randomly chosen RNA molecule M is an element of $\mathbf{M}_i$ (see the following scheme)

	M_1	$M_2, \ldots$	M_i	M_N
T_1	M_{11}			
T_2	M_{12}			
.				
.				
.				
T_k	M_{ik}	.....	M_{ik}	
..				
.				
.				
.				
T_K	M_{iK}	.....		M_{NK}

Let n_{ik} be the number of M_{ik} within M_i.

$n_i = \sum_{k=1}^{K} n_{ik}$ is the number of RNA molecules of length x_i in the whole system τ and $= \sum_{i=1}^{K} n_i$ the total number of RNA molecules.

We define $\frac{n_i}{n} = p(x_i)$, i.e. the probability $p(x_i)$ that a randomly chosen RNA molecule from τ is of the length x_i is equal to the relative amount of all RNA molecules of the length x_i. The model is based on these probabilities $p(x_i)$ defined as relative amounts. This approach is needed because in the real experiment n and the n_i are never estimable but the $p(x_i)$ is.

The process is described by the following 5 internal parameters (basic parameters B).

B 1: number of blockers: b

B 2: blocker position vector $B = (B_1, \ldots, B_j, \ldots, B_b)$

B 3: probability dependent blocker removal on time: $P_t(B_j)$

B 4: polymerase start distribution of the template: $C^{to} = (C_0^{to}, C_1^{to}, \ldots, C_i^{to}, \ldots, C_N^{to})$

B 5: polymerase velocity: V

B 6: probability dependent polymerase lethality on time: $P_t(\ell)$.

For comparisons between experimental and computer results 3 external parameters (phenomenological parameters P) are introduced:

P 1: time-dependent distribution $\rho_t(x_i)$ of chain lengths.

P 2: time-dependent number $m(t)$ of polymerase molecules active in the synthesis process.

P 3: time-dependent amount Y of total synthesized RNA during the experiment.

Because the P's are functions of the B both in the real system and the model falsification of hypotheses about the parameters B comparing P of the real system with P^M of the model is possible. If P^k and P^M do not agree (using a generalized distance parameter) the assumed parameter vector B^M cannot be accepted. For instance, one of the computer experiments is shown in fig. 6.

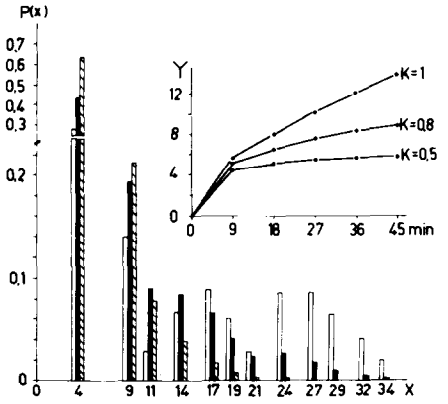

Fig. 6. Distribution $p(x)$ of chain lengths x depending on the probabilities of blocker removal $P_t(\bar{B}_j)$ after 45 min of RNA synthesis and time dependent amount Y(t) of total synthesized RNA. In this case it was predicted that all RNA polymerase molecules (pm) are in front of the first blocker at the beginning of the experiment and assumed that no polymerase dies during the experiment. [K indicates the reduction of $P_t(\bar{B}_j)$].
$K1$ = normal $P_t(\bar{B}_j)$ [empty (1st) columns]; $K = 0.8\ P_t(\bar{B}_j)$: 20% reduced [filled (2nd) column]; $K = 0.5\ P_t(\bar{B}_j)$: 50% reduced [striped (3rd) column].

References

[1] Widnell, C. G. and Tata, J. R. (1966) Biochim. Biophys. Acta 123, 478–492.
[2] Blackburn, K. J. and Klempere, H. G. (1967) Biochem. J. 102, 168–176.
[3] Pogo, A. O., Littau, V. C., Allfrey, V. G. and Mirsky, A. E. (1967) Proc. Natl. Acad. Sci. U.S. 57, 743–750.
[4] Ro, T. S. and Busch, H. (1967) Biochim. Biophys. Acta 134, 184–187.
[5] Chambon, P., Ramuz, M., Mandel, P. and Doly, J. (1968) Biochim. Biophys. Acta 157, 504–519.
[6] Pogo, A. O. (1969) Biochim. Biophys. Acta 182, 57–65.
[7] Mandel, Jr., J. L. and Chambon, P. (1969) Biochem. Biophys. Res. Commun. 35, 868–874.
[8] Chauveau, J., Moulé, Y. and Rouiller, Ch. (1956) Exptl. Cell Res. 11, 317–321.
[9] Markow, G. G. and Arion, V. J. (1973) Eur. J. Biochem. 35, 186–200.
[10] Georgiev, G. P., Anaieva, L. N. and Kozlow, J. V. (1966) J. Mol. Biol. 22, 365–371.
[11] Marushige, K. and Bonner, J. (1966) J. Mol. Biol. 15, 160–174.
[12] Fenske, H., Eichhorn, I. and Lindigkeit, R. (submitted for publication).

[13] Littau, V. C., Allfrey, V. G., Frenster, J. A. and Mirsky, A. E. (1964) Proc. Natl. Acad. Sci. U.S. 50, 1026–1032.

[14] Chalkley, R. and Jensen, R. H. (1968) Biochemistry 7, 4380–4387.

[15] Auer, G., Moore, G. P. M., Ringertz, N. R. and Zetterberg, A. (1973) Exptl. Cell Res. 76, 229–233.