NUCLEAR PROTEINS IN GENETICALLY ACTIVE AND INACTIVE PARTS OF CHROMATIN

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1. Introduction

We have recently demonstrated a high rate of phosphate incorporation into nuclear proteins of genetically active tissues, including regenerating and premalignant rat livers and cells during virus multiplication [1–3]. In the meantime, it has been generally accepted that DNA and proteins are organized as nucleosomes within the chromatin [4–9]. After digestion of the chromatin by deoxyribonucleases, these units may be isolated as separate entities (mononucleosomes) or linked together in a polymeric form (polynucleosomes).

In the present paper the composition and the phosphorylation patterns of histones, derived from these two types of particles, are described. These investigations were prompted by the speculation that mononucleosomes and polynucleosomes might originate from sequences of the chromatin with differing genetic activity. The results obtained confirm this assumption and indicate that mononucleosomes are derived predominantly from the active parts of the chromatin, whereas polynucleosomes originate mainly from the genetically-inactive sequences.

In another approach, the chromatin of actively growing Zajdela ascites hepatoma cells was separated as in [10] into its active and inactive fractions. Most of the histone and non-histone proteins were found to be phosphorylated to a significantly higher degree in the active fraction than in the inactive one. This is in close agreement with the results obtained in the experiments with nucleosomes and emphasizes the significance of the phosphorylation of nuclear proteins during transcriptional and replicative activities. Furthermore these results tend to support current models of chromatin substructure.

2. Methods

Male Sprague-Dawley 3 month rats, were partially hepatectomized as in [11]. After 24 h they were injected intraperitoneally with 1.8 mCi [^{32}P]orthophosphate. After 1 h the animals were decapitated, the livers were excised and chromatin was prepared [12]. It was digested by incubation with DNase I for 20 min at room temperature [13] or with micrococcal nuclease for 3 min at 37°C [9,14]. The digest was then placed on the top of a 10–30% sucrose gradient and centrifuged for 24 h at 27 000 rev./min in an L3-50 Beckman centrifuge, rotor SW 27 [14]. After centrifugation, the tubes were emptied by use of a LKB Ultrorac fraction collector which was monitored by a Uvicord. Individual fractions (mononucleosomes, polynucleosomes, supernatant fraction and pellet) were dialysed for 24 h against distilled water and lyophylised. The position of the mononucleosomes within the gradient was checked by cosedimentation of catalase as in [14]. Histones were extracted with 0.2 N HCl and separated on 15% polyacrylamide gels [15,16]. The gels were scanned and the radioactivity of the individual histone fractions was determined after sectioning of the gels with a gel slicer as in [17].

Chromatin fractions, enriched with 'active' and 'inactive' regions, were obtained from day 3 *Zajdela hepatoma* ascites cells as in [10]. These fractions were identified through their ability to incorporate radioactivity after the intraperitoneal injection of 250 μCi [5-^{3}H]orotic acid 1 h prior to sacrifice.

3. Results

The chromatin from 25 h regenerating livers of

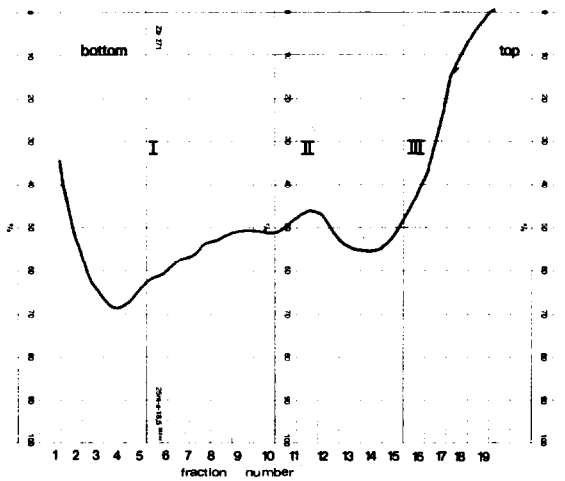

Fig.1. 10–30% Sucrose gradient of a DNase I digest of chromatin prepared from 25 h regenerating rat livers. Animals were injected intraperitoneally with 1.8 mCi [^{32}P]orthophosphate 1 h prior to sacrifice. Separation was performed as in [14] into polynucleosomes (I), mononucleosomes (II), and a top fraction (III).

rats which had been injected with [^{32}P]orthophosphate 1 h prior to sacrifice, was isolated and degraded by DNase digestion or, in some cases, nuclease digestion. The resulting nucleosomes were then separated on a sucrose gradient as demonstrated in fig.1. Essentially, 4 different fractions were obtained by this procedure:

(1) A pellet fraction P.
(2) A polynucleosomal fraction I.
(3) A mononucleosomal fraction II.
(4) A supernatant fraction III.

The distribution of proteins was significantly different in these 4 fractions. The polynucleosomal fraction contained the complete set of all histones, but was essentially free of non-histone proteins which were normally extracted together with histones under our conditions and migrate more slowly than histone H1. Mononucleosomes were highly depleted of histones of the H1 group, but besides this there were no significant differences as compared to the undigested chromatin serving as a control. In the supernatant fraction great amounts of H1 histones as well as non-histone proteins were found (fig.2).

Both nucleosomal fractions were characterized by a high phosphate content of histone H2A. However, H1 histones were highly phosphorylated only in the

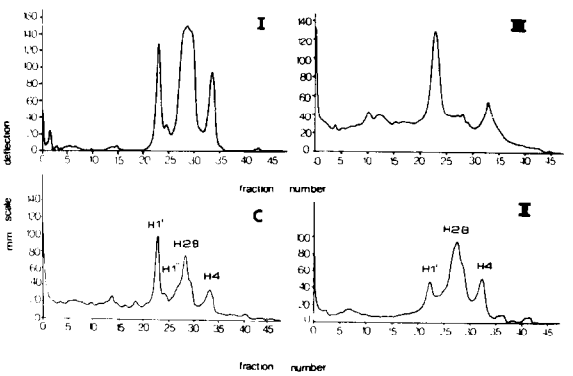

Fig.2. Densitometer tracings of histones, extracted from fractions I (polynucleosomes), II (mononucleosomes), and III (top fraction) after a gradient centrifugation as depicted in fig.1. C, control, demonstrating the histone pattern of undigested rat liver chromatin.

mononucleosomes, although only small amounts of these components could be detected in this fraction. Polynucleosomes were rich in H1 histones which showed, however, only low phosphate activity (fig.3).

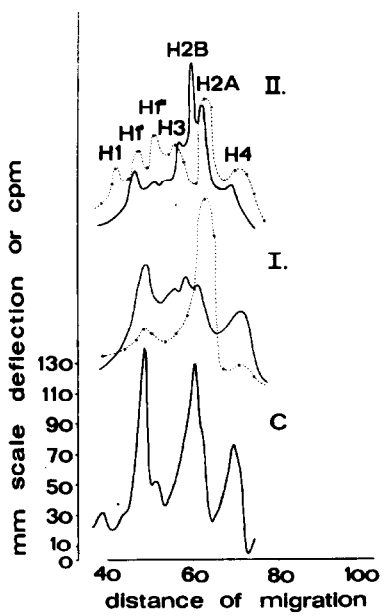

Fig.3. Densitometer tracings (——) and distribution of radioactivity (X– – –X) of ^{32}P-labelled histones, extracted from fractions I (polynucleosomes) and II (mononucleosomes) of a sucrose gradient, as demonstrated in fig.1. C, control; histone pattern of undigested rat liver chromatin.

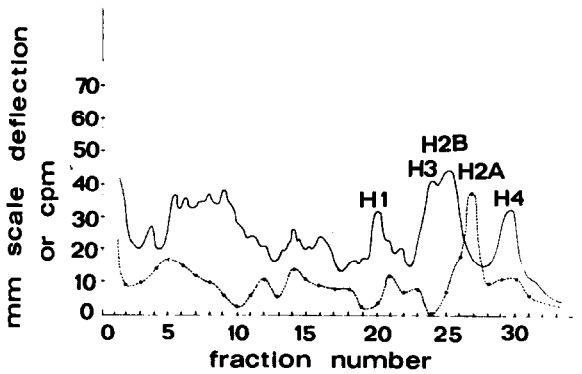

Fig.4. As in fig.3, but from the pellet fraction.

An even lesser degree of H1 phosphorylation was demonstrated in the pelleted material (fig.4). On the contrary, both H1 histones and non-histones were highly phosphorylated in the supernatant fraction (fig.5). Phosphates were bound covalently to the histone molecules, which was indicated by the fact that the radioactivity of the individual bands remained unchanged after reprecipitation. Moreover, phosphate attachment withstood *N*-bromosuccinimide cleavage under acidic conditions as demonstrated for lysine-rich histones of the regenerating rat liver [1]. On the other hand, the action of alkaline phosphatase resulted in a complete loss of the phosphate residues. This is strong evidence for covalent binding between phosphates and histones [28,29].

These results indicate that mononucleosomes might originate through nuclease attack of active sequences

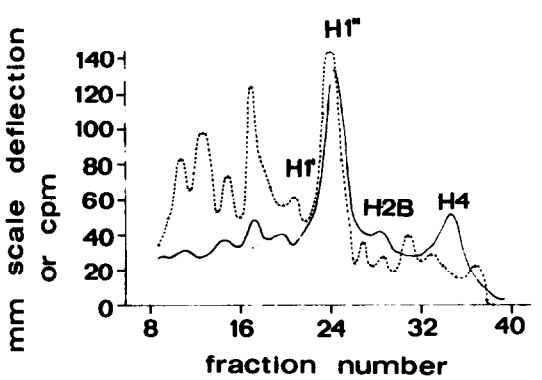

Fig.5. As in fig.3, but from the top fraction of the gradient.

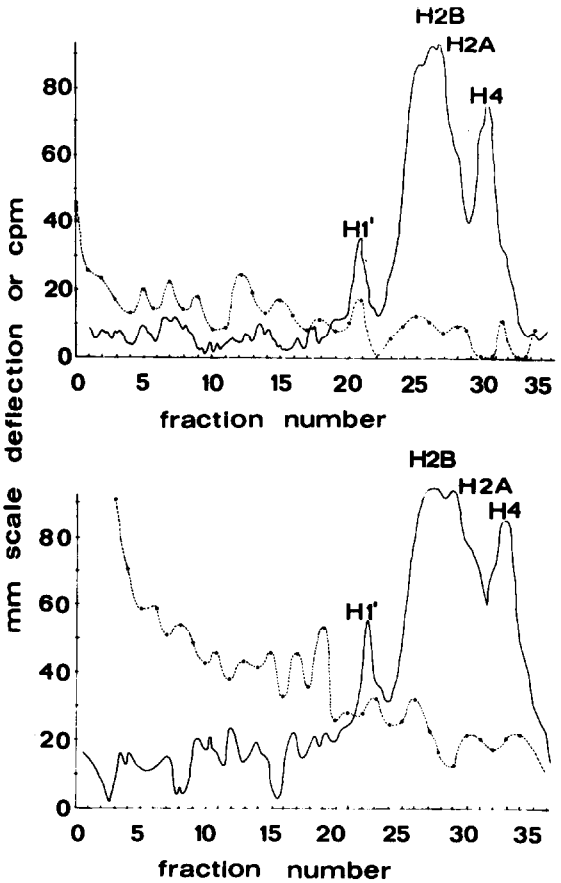

Fig.6. Densitometer tracings (——) and distribution of radioactivity (X– – –X) of 'active' (lower graph) and 'inactive' (upper graph) fractions of chromatin from growing *Zajdela ascites* hepatoma cells, prepared and separated as in [10]. Animals were injected intraperitoneally with 1.8 mCi ^{32}P 1 h prior to sacrifice.

within the chromatin, while polynucleosomes are degradation products of inactive parts. We therefore tried to prepare two fractions enriched with active and inactive parts of the chromatin, respectively. These experiments were performed with *Zajdela hepatoma* cells which were in the active growth phase. The separation of the chromatin in a discontinuous sucrose gradient, as in [10] for rat ventral prostate chromatin, resulted in essentially two fractions. In the prostate experiments, the upper fraction, remaining near the top of the gradient, was characterized as active chromatin by:

(i) A 5-fold higher endogenous RNA-polymerase activity.

(ii) A 10–20-fold higher template activity.

(iii) A 10-fold higher trichloroacetic acid-precipitable radioactivity after the incorporation of radioactive uridine as compared to the lower fraction containing the genetically less active components.

We checked this for our conditions by comparing the incorporation of [^{3}H]orotic acid into these two fractions and found, in excellent agreement with [10], a 9.5-times higher radioactivity in the upper fraction than in the lower one (1380 : 145 cpm/unit A_{260}). In fig.6 it is clearly demonstrated that qualitative and particularly quantitative differences are to be found between the composition of non-histones in these fractions. In addition, a significantly higher degree of phosphorylation is observed in histones and non-histones of the active chromatin than in the inactive one.

4. Discussion

During recent years several authors have developed a model for chromatin substructure, according to which DNA and histone fractions H2A, H2B, H4 and H3 are arranged as bead-like structures termed nucleosomes. These nucleosomes are connected by short stretches of DNA which in turn are complexed with histone H1 [4–9]. Other authors have demonstrated that there exists a dependence of the processes of transcription, replication and cell division on the phosphorylation of specific histone fractions [1–3, 18–21]. There is only limited evidence, however, concerning the significance of changes in the structure of chromatin with respect to these reactions.

As we have shown, genetic activity is paralleled first of all by the phosphorylation of H1 histones [1–3]. In addition, an incorporation of phosphate into other, specific histones is observed. We argued that this is reflected in the degree of phosphorylation of different chromatin fractions which had been enriched with active and inactive sequences, respectively. In fact, we could demonstrate that nuclear proteins are phosphorylated to a higher degree in active than in inactive chromatin (fig.6). This was observed not only with H1 histones, but also with other histone fractions and non-histones proteins.

Extensive H1 phosphorylation is demonstrated also in the mononucleosomal fraction of 25 h regenerating livers. In these liver remnants, high rates of RNA and particularly DNA synthesis are observed [1,22]. It is therefore suggested that these particles originate from the active portions of the chromatin. According to the 'asymmetric hairpin' model [23–25], H1 histones are located on the short DNA stretches connecting the nucleosomes and may form crosslinks with neighbouring portions of DNA, thus resulting in the formation of loops. The phosphorylation of these H1 molecules seems to be a prerequisite for an extension of the chromatin fibre and for nuclease attack. Nucleosomes originating from these sites are either free of adhering DNA–H1-complexes or still bear those highly phosphorylated connecting segments. We therefore find in our nucleosomal fraction only little but highly phosphorylated H1 histone. On the other hand, the H1 of the inactive chromatin is phosphorylated only to a limited degree and degradation reactions by nucleases cannot take place. As a consequence, only larger structures, i.e., polynucleosomes, can originate from those regions. From this it is evident that polynucleosomes are characterized by a histone pattern typical for unfractionated chromatin with only limited amounts of phosphate incorporated into H1 histones. Interestingly enough, polynucleosomes are completely devoid of nonhistone proteins. This interpretation is supported by the histone pattern obtained from the pellet fraction, since this nuclease-resistant fraction exhibits the lowest H1 phosphorylation of all.

H1 histones with high phosphate activity, which had been liberated during the degradation of active chromatin to mononucleosomes, are found in the top fraction of the gradient. In this fraction also most of the non-histones can be detected. These proteins are characterised by an extremely high degree of phosphorylation. We have not yet any reliable information concerning the localization of non-histones within the chromatin. However, our results possibly indicate a relatively close arrangement at least of some non-histones and H1 histones on DNA. This would be in agreement with the idea that part of the non-histones possibly play a role in maintaining chromatin structure

[26] and with [27], where only limited occurrence of those proteins in nucleosomes was found.

References

[1] Letnansky, K. (1975) Cell Tiss. Kinet. 8, 423–439.
[2] Letnansky, K. and Wenzel, J. (1977) Cancer Res. 37, 800–804.
[3] Letnansky, K., Dostal, V. and Reiss-Gutfreund, R. J. (1978) in preparation.
[4] Olins, A. L. and Olins, D. E. (1974) Science 183, 330–332.
[5] Sahasrabuddhe, C. G. and Van Holde, K. E. (1974) J. Biol. Chem. 249, 152–156.
[6] Clark, R. J. and Felsenfeld, G. (1974) Biochem. 13, 3622–3628.
[7] Hewish, D. R. and Burgoyne, L. A. (1973) Biochem. Biophys. Res. Commun. 52, 504–510.
[8] Kornberg, R. D. (1974) Science 184, 868–871.
[9] Noll, M. (1974) Nature 251, 249–251.
[10] Anderson, K. M., Chance, H. and Kadohama, N. (1975) Exp. Cell Res. 94, 176–190.
[11] Higgins, G. M. and Anderson, R. M. (1931) Arch. Path. 12, 186–202.
[12] Butterworth, P. H. W., Cox, R. F. and Chesterton, C. J. (1971) Eur. J. Biochem. 23, 229–241.
[13] Chae, C.-B. (1974) Biochemistry 13, 1110–1115.
[14] Honda, B. M., Baillie, D. L. and Candido, E. P. M. (1974) FEBS Lett. 48, 156–159.
[15] Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337–346.
[16] Panyim, S. and Chalkley, R. (1969) Biochem. 8, 3972–3979.
[17] Letnansky, K. and Reisinger, L. (1972) Biochem. Biophys. Res. Commun. 29, 312–320.
[18] Wilhelm, J. A., Spelsberg, T. C. and Hnilica, L. S. (1971) Biochemistry 1, 39–65.
[19] Balhorn, R., Balhorn, M., Morris, H. P. and Chalkley, R. (1972) Cancer Res. 32, 1775–1784.
[20] Gurley, L. R., Walters, R. A. and Tobey, R. A. (1975) J. Biol. Chem. 250, 3936–3944.
[21] Garrard, W. T., Kidd, G. H. and Bonner, J. (1976) Biochem. Biophys. Res. Commun. 70, 1219–1224.
[22] Rabes, H. M. and Brändle, H. (1969) Cancer Res. 29, 817–822.
[23] Varshavsky, A. J., Ilyin, Y. V. and Georgiev, G. P. (1974) Nature 250, 602–606.
[24] Varshavsky, A. J. (1976) Physiol. Chem. 357, 340.
[25] Varshavsky, A. J., Bakayev, V. V. and Georgiev, G. P. (1976) Nucleic Acids Res. 3, 477–492.
[26] Albrecht, J. and Hemminki, K. (1977) Biochim. Biophys. Acta 475, 152–159.
[27] Augenlicht, L. H. and Lipkin, M. (1976) Biochem. Biophys. Res. Commun. 70, 540–544.
[28] Sherod, D., Johnson, G. and Chalkley, R. (1970) Biochemistry 9, 4611–4615.
[29] Balhorn, R., Rieke, W. O. and Chelkley, R. (1971) Biochemistry 10, 3952–3959.