Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 349-357

DISTRIBUTION OF IP25 IN CHROMATIN AND ITS POSSIBLE INVOLVEMENT IN CHROMATIN CONDENSATION

Ruth Gjerset, Françoise Ibarrando, Sentob Saragosti and Harvey Eisen

Unité d'Immunoparasitologie and Département de Biologie Moléculaire. Institut Pasteur, 75015 - PARIS.

Received January 7, 1981

SUMMARY

We have examined the distribution in mouse liver chromatin of IP25, a lysine-rich chromosomal protein whose induction in tissue culture cells correlates with the appearance of differentiated characteristics of these cells. We find it concentrated in regions of the chromatin more resistant to nuclease action, where it comprises up to 40 % of the total lysine-rich histone. Purified IP25 condenses SV40 minichromosomes, consistent with a role in compacting or cross-linking distant chromosome regions.

INTRODUCTION

The chromosomal protein, IP25, appears in mouse Friend virus -transformed erythroleukemic cells when they are induced by various agents such as HMBA (hexamethylenebisacetamide) and DMSO (dimethylsulfoxide) to undergo terminal differentiation (1). Like histone H1, IP25 is extracted from chromatin by 5 % PCA or by 0.45 M NaCl, and is located internucleosomally (2). However, it displays a completely different array of tryptic peptides (2) and is unrelated to histone H1 antigenically (R. Gjerset, unpublished).

There are no other chromosomal changes in differentiating Friend cells as marked as the appearance of IP25, suggesting an important role for this protein in the structural transitions which must occur in chromatin during differentiation. Our interest has been to investigate the generality of IP25 occurrence in other tissue culture lines and in vivo, which

0006-291X/81/060349-09$01.00/0

will be the subject of forthcoming papers, and to elucidate its structural role in chromatin. In this paper we analyze the distribution of IP25 in mouse liver chromatin and demonstrate its involvement in chromatin condensation. We also show that like H1° IP25 is composed of two molecules of identical molecular weight which differ slightly in charge.

MATERIALS AND METHODS

1. Purification of IP25

Lysine rich proteins were extracted from moue liver as described previously (2) or by direct extraction with 5 % (V:V) perchloric acid. After incubation overnight at 4° C in 18 % (W:V) TCA , the precipitate was collected and washed with acetone containing 0.2 % HCl, followed by 2 washings with cold acetone. It was then dried and dissolved in 0.1 M phosphate buffer pH 6.8 containing 7 % guanidine HCl and aplied to a column of Bio-Rex 70 as described by Panyim and Chalkley (3). Fractions were dialized against 0.1 M acetic acid and lyophylized. Proteins were analysed by electrophoresis in 12.5% polyacrylamide gels in the presence of SDS (4) or in acid urea gels (5).

Treatment of proteins with cyanogen bromide (CnBr) was as in (6) and treatment of proteins with V8 protease was done as in (7). Degradation pruducts were analysed on 15 % SDS polyacrylamide gels as described in (8).

2. Preparation of liver nuclei and nuclease digestion

Livers from 7-8 adult mice mere minced and homogenized in 0.25 M sucrose containing 5 mM Pipes pH 7, 85 mM KCL, 10^{-3} M PMSF 50 ml total volume), using a Polytron tissue homogenizer. Nuclei were sedimented at 12000 x g for 15 minutes. They were then diluted with two volumes of 2.3 M sucrose in Pipes 5 mM, KCl 85 mM, PMSF 10^{3}M to make a final concentration of sucrose of about 1.6 M. 3 ml of each supernatant was distributed to 6 SW56 tubes and underlayed with 1 ml of 2.3 M sucrose in the same buffer. Nuclei were pelleted at 90 000 x g for 30 minutes. The upper layer was carefully aspirated and the tops of the tubes were cut. Nuclei were washed once in 5 % sucrose, 5 mM Pipes, 85mM KCl, 10^{-3}M PMSF, and resuspended in 1 ml of the same buffer. Yield was about 1-2 x 10^{8} niuclei. For nuclease digestions, the nuclear suspension was made 1 mM in $CaCl_2$ followed by the addition of either DNase I (Sigma) or micrococcal nuclease (Boehringer). The reactions continued 15 minutes on ice and were stopped by the addition of one volume of 10 mM EGTA 5 mM Pipes, 85 mM KCl, 10^{3} M PMSF. Nuclei were sedimented at 500 rpm for 5 minutes and the supernatant was assayed for released chromatin by measuring absorption at 260 mμ. For the analysis of digested chromatin on sucrose gradients, 250 μl of

the nuclear suspension was treated with 50 µg of Dnase I or micrococcal nuclease, enough to release 40-50 % of the chromatin from the nucleus (which represented a plateau for liver chromatin) while rendering less than 2 % soluble in 10 % TCA.

3. Fractionation of digested chromatin and analysis of the fractions

Chromatin released from the nucleus following nuclease digestion was layered into a 10-30 % linear SW 41 sucrose gradient containing 5 mM Pipes pH 7, 85 mM KCl, 10 mM EGTA, 10^{-3} M PMSF and centrifuged at 200 000 x g for 3 hours. 1 ml fractions were collected using an ISCO fraction collector, and precipitated with four volumes of ethanol at -20° for 16 hours. The precipitate was recovered by centrifugation and analysed by SDS gel electrophoresis (4). Gels were stained and fixed in methanol:H_2O:acetic acid (5:5:1) containing 2.5 mg/ml Coomassie blue and destained in the same solution lacking Coomassie blue. For quantitation of the bands, gels were scanned and the peaks integrated using a Vernon scanning and integrating spectrophotometer. Standard curves were determined for known amounts of H1 and IP25 in order to verify that staining was proportional to the amount of protein in the gels.

4. Preparation of SV40 minichromosomes depleted of histone H1

SV40 minichromosomes were extracted from infected monkey MA cells 44 hours after infection as described (9). H1 was removed by a 5 minute treatment with 0.5 M NaCl as described (10) and H1 -free minichromosomes were purified by sedimenting through a 5-20 % sucrose gradient (0.1 M NaCl, 0.01 M triethanolamine HCl pH 7.5, 1 mM EDTA) for 90 minutes at 200 000 x g at 4°C using a SW41 rotor. The minichromosomes were adsorbed at 0.1 M NaCl to electron microscopy grids coated with a positively charged carbon film (11). Samples were stained with uranyl formate and rotary-shadowed with platnium palladium at an angle of 6°.

RESULTS

1. Purification of IP25

IP25 purified from mouse liver was analysed by SDS and acid -urea gel electrophoresis (fig. 1). Upon SDS gel electrophoresis a single band was found. Analysis on acid-urea gels revealed the presence of 2 bands, IP25 a and IP25 b. The two bands showed identical fingerprints after treatment with cyanogen bromide or protease V8 from Staphylococcus aureus. It thus appears that IP25 is identical to H1° isolated from calf or Ox liver (3, 12)

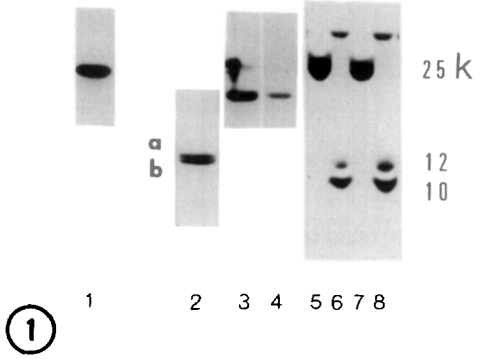
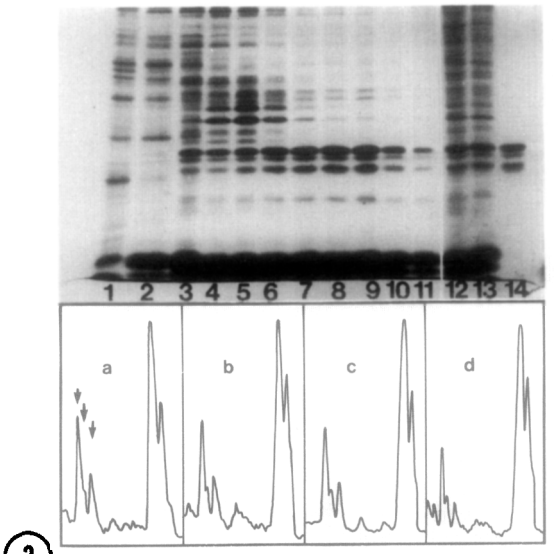


FIG. 1 - Gel analysis of mouse liver IP25 and its digestion products. Lane 1, IP25 a and b, SDS gel (12.5 %) ; lane 2, IP25 a and b, acid urea gel ; lane 3, IP25 a after CnBr treatment, SDS gel ; lane 4, IP25 b after CnBr treatment, SDS gel ; lanes 5 and 6, IP25 a before and after treatment with V8, SDS gel (8) ; lanes 7 and 8, IP25 b before and after treatment with V8, SDS gel (8).

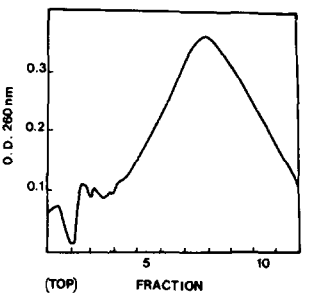
FIG. 2 - 10-30 % sucrose gradient of chromatin released from nuclei by micrococcal nuclease.

2. Association of IP25 with nuclease-resistant chromatin in mouse liver

We have examined the distribution of IP25 in chromatin released from nuclease digested liver nuclei. Figure 2 shows a profile of micrococcal nuclease digested chromatin following centrifugation through a 10-30 % linear sucrose gradient. The peaks corresponding to monosome up to 5 nucleosomes are indicated. DNA isolated from the heaviest region of the gradient migrated on agarose gels in a size range corresponding to 16-20 nucleosomes, while the bulk of the DNA remaining in the nucleus was larger than this (data not shown). Figure 3 shows an SDS gel displaying the proteins corresponding to the indicated fractions. It is evident that both IP25 and histone H1 are depleted in the more highly degraded material. However, as indicated in the scans of these gels (figure 3), IP25 is under-represented relative to histone $H1_1$ + $H1_2$ in the more highly digested material, and



3



4

FIG. 3 - SDS gel of proteins corresponding (from left to right) to fractions 1-11 from Fig. 2 (lanes 1-11) Nuclear proteins not released during digestion, (lane 12) ; total proteins from undigested nuclei (lane 13) ; and PCA extractable chromosomal proteins from liver (lane 14). Scans are (a) lane 13, (b) lane (12), (c) lane 10 and (d) lane 4. Arrows indicate (left to right) Hl_1, Hl_2, IP25.

FIG. 4 - 10-30 % sucrose gradient of chromatin released from nuclei by DNase I.

does not reach a level characteristic of total chromatin until the heavier fractions.

Figure 4 shows a profile of DNaseI digested chromatin following centrifugation through a 10-30 % linear sucrose gradient, and figure 5 shows the corresponding SDS gel displaying the proteins from the verious fractions. A depletion of IP25 as well as histone Hl in the more highly digested material is even more obvious here than with micrococcal nuclease. Again, upon examination of the gel scans, it is apparent that IP25 is underrepresented relative to histone Hl in the more highly digested material, and is enriched in the chromatin which remains in the

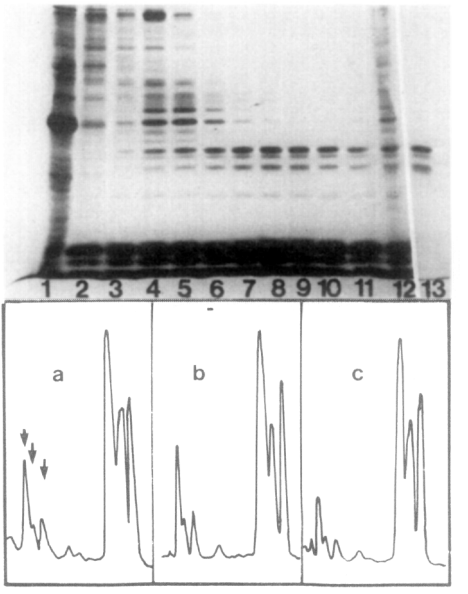


FIG. 5 - SDS gel of proteins corresponding (from left to right) to fractions 1-11 of FIG. 4 (lanes 1-11) to PCA extractable chromosomal proteins from liver (lane 13). Scans are (a) lane 12, (b) lane 10 and c lane 4. Arrows indicate (left to right) Hl_1, Hl_2, IP25.

nucleus following digestion. A summary of the distribution of IP25 in digested chromatin, analyzed by integrating the gel scans, is given in table I.

3. Effect of IP25 on the morphology of the SV40 minichromosome

In parallel experiments, we examined directly by electron microscopy the structural consequences of IP25 presence in chromatin. The SV40 minichromosome depleted of histone H1 as a chain of about 20 nucleosomes (Fig. 6 A). When these minichromosomes are incubated with purified IP25 for 5 minutes at room temperature at a weight ratio of IP25 to DNA of 1:1, the structure compacts, as shown in Fig. 6 B. The data support the participation of IP25 in the higher order condensation of chromatin, and are consistent with the localization of IP25 in regions of the chromatin less accessible to nucleases.

TABLE 1

Relationship of IP25 to Histone H1 in Fractions of Nuclease Digested Chromatin

	$\frac{IP25}{H1_1+H1_2}$
a) Total chromatin	0.41
b) Micrococcal Nuclease Digested Chromatin	
i) retained in nuclei	0.39
ii) released from nuclei	0.33
fractions 3-6	0.24
fractions 7-11	0.40
c) DNase I Digested Chromatin	
i) retained in nuclei	0.44
ii) released from nuclei	0.23
fractions 4-6	0.19
fractions 7-11	0.26

DISCUSSION

These results indicate that IP25, although located internucleosomally as is histone H1, is associated with more highly nuclease resistant regions of the chromatin. Whether IP25 is directly involved in conferring nuclease resistance either alone or in conjunction with other proteins is not known, but this would be consistent with observations that IP25 condenses SV40 minichromosomes. Since IP25 appears to be a dimer following 5 % PCA extraction of chromatin and ion exchange chromatography on Bio-Rex 70, it could be serving to organize and condense the superstructure of chromatin by cross-linking distant regions (H.Eisen, unpublished observations). The distribution among the lysine-rich histones of 40 % IP25 to 60 % $H1_1+H1_2$ suggests that there may be two IP25 molecules per six molecules of histone H1, or in view of the model of Thoma et al. (13), two molecules per turn of the solenoid supercoil.

It is well established that both micrococcal nuclease and DNase I preferentially digest transcriptionally active chromatin

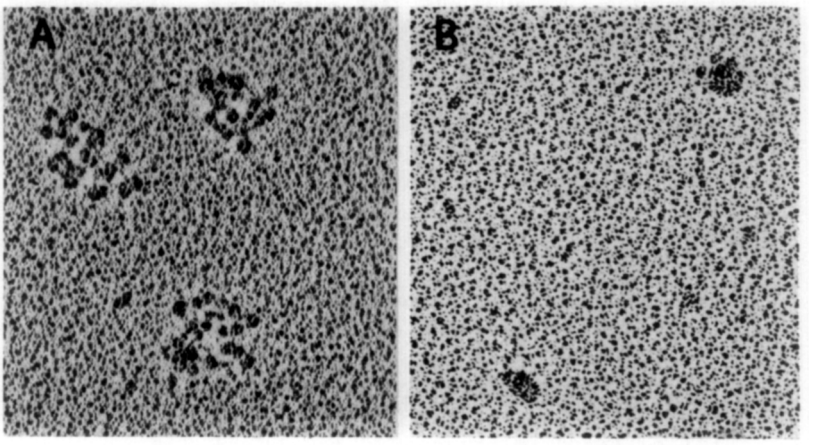


FIG. 6 - Electron micrographs of 0.5M NaCl treated SV40 minichromosomes (A), no protein added ; (B) IP25 added&

(14,19). In addition, replicating chromatin is preferentially sensitive to micrococcal nuclease (20). The absence of IP25 in regions of the chromatin known to be enriched in transcribing or replicating sequences is consistent with a role in impeding these functions. It is also consistent with physiological observations with tissue culture cells, where its appearrance precedes the cessation of DNA synthesis and cell division and the indiction of specialized functions by HMBA and DMSO (1) and by butyric acid (manuscript in preparation). By closing down certain regions of the chromatin, IP25 may serve to limit the cell's differentiative and proliferative capacity. Observations on the in vivo distribution of IP25 also suggest a role in fixing or limiting the cell's developmental capacity (22).

Proteins with properties similar to IP25 have been identified in the rat (21), the calf and the ox (12), and have been termed Hl_3 (21) or Hl° (12). In the case of the rat, it has recently been demonstrated (2) that the protein corresponding to IP25 is associated with micrococcal nuclease resistant regions

of the chromatin, in agreement with our finding. It is likely that thi protein, or one similar to it, occurs in all mammals and plays a key role in the control of cell proliferation and differentiation.

ACKNOWLEDGEMENTS

We greately acknowledge the excellent technical assistance of Madame Elizabeth Khoury. We thank Professor François Jacob for providing space and facilities. This work was supported by grants from the C.N.R.S. and I.N.S.E.R.M.

REFERENCES

1. Keppel, F., Allet, B. and Eisen, H. (1977) Proc. Natl. Acad. Sci. USA 74, 653-656.
2. Keppel, F., Allet, B. and Eisen , H. (1979) Eur. J. Biochem. 96, 477-482.
3. Panyim, S. and Chalkley, R. (1969) Biochem. Biophys. Res. Comm. 37, 1042-1049.
4. Laemmli, U.K. (1970) Nature 227, 680-685.
5. Traub, P. and Boeckmann, G. (1978) Hoppe-Seyler's Z. Physiol Chem. 359, 571-579.
6. Walker, J.M., Godwin, G.H. and Johns, E.W. (1976) Eur. J. Biochem. 62, 461-469.
7. Houmard, J. and Drapeau, G.R. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3506-3510
8. Swank, R.T. and Munkers, K.D. (1971) Anal. Biochem. 39, 162-177
9. Varshavsky, A.J., Sundin, O.H. and Bohn, M.J. (1978) Nucleic Acids Res. 5, 3469-3478.
10. Müller, U., Zentgraph, H., Eiken, I., and Keller, W. (1978) Science 201, 406-415
11. Dubochet, J., Ducommun, M., Zollinger, M. and Kellenberger, E., (1971) J. Ultrastructure Res. 35, 147-167.
12. Smith, B.J. and Johns, E.W. (1980) FEBS Lett. 110, 25-29
13. Thoma, F., Koller, Th. and Klug A. (1979) J. Cell Biol. 83 403-427
14. Weintraub, H. and Groudine, M. (1976) Science 193, 848-856.
15. Gazel, A. and Axel, R. (1976) Proc. Natl. Acad. Sci. USA 73, 3966-3970
16. Bellard, M., Gannon, F., and Chambon, P. (1978) Cold Harbor Symp. Quant. Biol. 42, 779-791
17. Bloom, K.S. and Anderson, J.N. (1978) Cell 15, 141-150
18. Reeves, R., (1978) Biochemistry 17, 4908-4916
19. Levy-Wilson, B. and Dixon, G.H. (1979) Proc. Natl. Acad. Sci USA 76, 1682-1686
20. Worcel, A., Han, S., and Wong, M.L. (1978) Cell 15, 969-977
21. Gorka, C. and Lawrence, J.J. (1979) Nucleic Acids Res. 7 , 347-359
22. Gjerset, R., Gorka, C., Hasthorpe, S., Ibarrondo, F., Lawrence, J.J. and Eisen, H. (1981) Proc. Natl. Acad. Sci. U.S.A. in press