ABSENCE OF H1° FROM QUIESCENT CHICKEN CELLS

B. J. SMITH, Y. COOK, E. W. JOHNS and R. A. WEISS
Institute of Cancer Research, Fulham Road, London, SW3 6JB, England

Received 21 September 1981

1. Introduction

The histones are amongst the most evolutionally stable of proteins [1] and for H1 this is particularly true of the central, globular region of its molecule [2]. The same seems to be the case for H5 [3], a histone found only in mature nucleated erythrocytes of birds and other non-mammalian species. Data suggest that the role of H5 in these cells is one of repression of transcription (reviews [1,4]).

The conserved structure of H5's globular region occurs in a mammalian (bovine) nucleoprotein, H1° [5–7]. This similarity is especially striking in view of the phylogenetic distance between birds and mammals. Such strict conservation of structure may arise by conservation of a function which exerts harsh constraints on that structure. This would argue for the functions of H1° and H5 being the same, but the available data, while being circumstantial, indicate that they are different. The role suggested for H1° is one of repression of DNA synthesis [8]. However, why would one type of protein structure be linked with two different cellular processes? One or both of the suggestions for their functions could be incorrect, or both correct in that repression of syntheses of RNA from a DNA template and of DNA from a DNA template may be enough alike to require similarly structured proteins.

If H1° and H5 play different roles then they may both exist in the same cell or animal. If they have the same role, H5 should occur when mammalian cells require H1°. To reveal the functions of H1° and H5, these expectations were tested by a search for H1°/H5-like proteins in adult chicken livers and quiescent chicken cell cultures.

Abbreviations: GuCl, guanidinium chloride; SDS, sodium dodecylsulphate

2. Materials and methods

White Leghorn laying hens (~2 years old) were obtained from Orchard Farm, Bucks. Livers from freshly-killed hens were stored in liquid nitrogen until used.

SPAFAS White Leghorn chick embryo fibroblasts were grown in E4 medium supplemented with 10% tryptose phosphate broth, 4% foetal calf serum and 1% chick serum. Cells were incubated at 37°C in 10% CO_2 in air. The passage number of the cells was 6–12. For proliferating cells, subconfluent cultures containing rounded (mitotic) cells were used. To obtain cells which were not making DNA, cultures were either grown to confluence in normal medium or were maintained for 4 days in a medium containing 0.5% chick serum [9]. That such treatment inhibited DNA synthesis was confirmed by microscopic inspection of cultures (no rounded, mitotic cells seen), cell counting (no increase in cell numbers) and [^{3}H]thymidine incorporation (no labelled nuclei detected by autoradiography).

3T3 TK3 mouse fibroblasts were grown in E4 medium supplemented with 10% foetal calf serum. Incubation was at 37°C in 10% CO_2 in air. DNA synthesis was inhibited as in chick cells, except that 0.5% foetal calf serum was used.

Although chick and foetal calf sera did not contain any protein of electrophoretic mobility resembling those of the proteins of interest here, contamination of preparations by high levels of serum albumin interfered with electrophoresis. This was avoided by removal of albumin by incubation of cultures in serum-free medium for ~30 min.

Proteins were extracted with $HClO_4$ (5%, w/v) from whole, freshly-thawed chicken livers and the extract made 18% (w/v) CCl_3COOH. The precipitate was dissolved in water, made 5% (w/v) $HClO_4$ and re-precipitated by addition of HCl (0.3 M) and acetone (3.5 vol.) [5].

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

Proteins were prepared from cell cultures (~10^7 cells/preparation) in the same way as from livers, except that 6 vol. acetone were used to precipitate proteins in the final stage. This was necessary to obtain consistent preparations on a small scale, and resulted in co-isolation of HMG proteins. Proteolysis was reduced to a minimum by scraping up cells directly into $HClO_4$.

Proteins extracted from chicken livers were fractionated by chromatography on a column of Bio Rex 70 in sodium phosphate buffer (0.1 M, pH 6.8) with a gradient of GuCl (8–17%, w/v). 65 mg were loaded onto a column of 2.5 × 21 cm and eluted at 1 × 10 ml fraction/h. Elution was monitored by absorbance at 220 nm and the gradient was monitored by refractive index.

Proteins were analysed by electrophoresis in two systems:

(i) 15% polyacrylamide gel SDS (0.1%, w/v) and Tris–HCl (0.375 M, pH 8.8) [10];

(ii) 20% polyacrylamide in urea (2.5 M) and acetic acid (1 M, pH 3) with samples loaded in a 7.5% polyacrylamide gel containing urea (2.5 M) and acetic acid (0.3 M, pH 4).

In either case gel slabs of 0.1 × 14 × 14 cm were used. Protein bands on gels were stained with Coomassie brilliant blue R250 [11].

Treatment of proteins with cyanogen bromide was carried out as in [5]. N-Terminal amino acid identification was by the dansylation method [12].

3. Results

3.1. *Proteins of chicken liver*

The livers of adult mammals of various species contain H1° ([13,14], M. R. Harris, B. J. S., E. W. J., unpublished), so a detailed search for a chicken H1° was made in adult chicken liver by a method routinely used to prepare H1 and H1° from mammalian liver [5,11].

The chicken liver extract was electrophoresed in both acid–urea and SDS systems and H1 and H5 were identified. However, no protein of mobility similar to that of mammalian H1° was seen. The extract was then fractionated by chromatography as shown in fig.1. The steadily rising baseline in fig.1 is due to absorbance at 220 nm by the GuCl gradient. The first of the two distinct peaks in the profile (a) only contained material which had H1-like electrophoretic mobility in both acid–urea and SDS gel systems. Peak (b) only contained material with H5-like electrophoretic mobility. In the same system, bovine H1° elutes slightly later than H1 [11] but there was no significant peak in the equivalent position in fig.1, between peaks (a,b). No H1°-like material could be prepared from fractions in this region.

All H5s and H1°s studied so far contain a single methionine residue positioned towards the N-terminal end of the evolutionally conserved globular region ([3,5], M. R. Harris, B. J. S., E. W. J., unpublished). Treatment of these proteins with cyanogen bromide

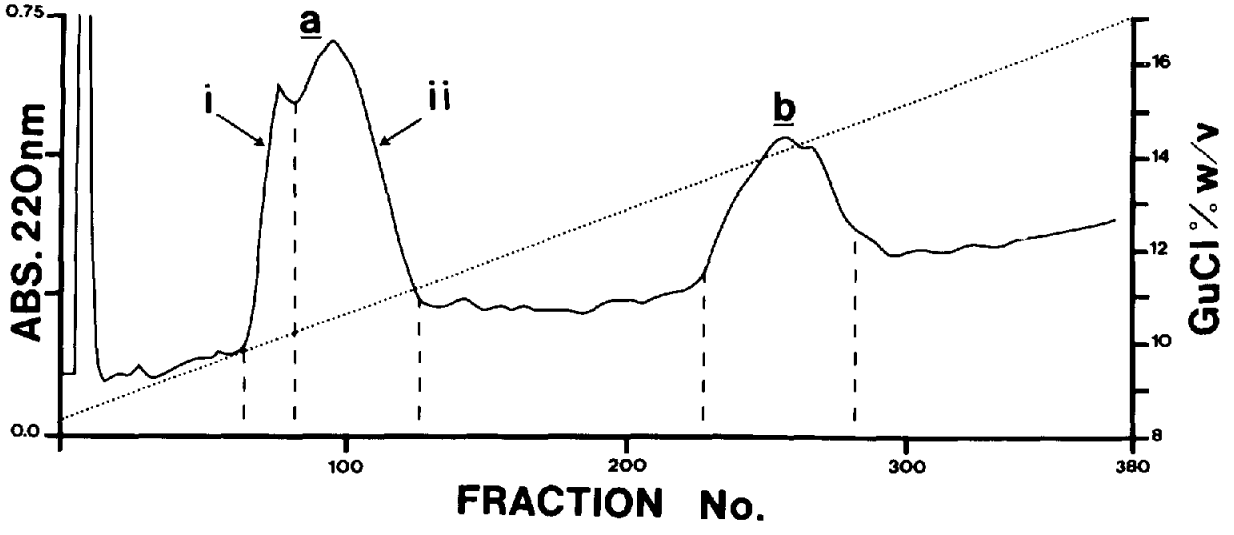

Fig.1. Profile of elution of chicken liver $HClO_4$-soluble proteins from a Bio-Rex 70 ion-exchange column, with a GuCl gradient (. . . .) in sodium phosphate (0.1 M, pH 6.8).

cleaves the molecules at the methionine residue and generates a large peptide which has characteristic mobility in SDS gels, slightly faster than the parent molecule [11]. This characteristic was used to investigate the possibility of an H1°-like protein co-chromatographing (and co-electrophoresing) with H1 in peak (a). Peak (a) was split into parts (i) and (ii) (fig.1) to concentrate any underlying small peak relative to the H1, and each was treated with cyanogen bromide. Although this treatment cleaved positive control proteins at their methionine residues, it did not alter the electrophoretic mobility of the peak (a) material. Neither did this treatment generate any free N-terminal amino acid residue in the peak (a) material, which has blocked N-terminal residues. Thus proteins in peak (a) contain no methionine and are unlike mammalian H1° in this respect. A similar search within peak (b) was impossible because the H5 in that peak would have given the same result as that expected of an H1°. However, since the overall amino acid content of H1° is very like H1 [11,14] it seems less likely that it would co-chromatograph (and co-electrophorese) with H5 in peak (b) than with H1 in peak (a).

To summarize, no H1°-like protein could be identified in a tissue which might be expected to contain it – adult chicken liver. Both H1 and H5 were identified (in fig.1 peaks (a) and (b), respectively) but the H5 possibly derived from erythrocytes trapped within the liver rather than from the liver cells themselves. Perfusion of chicken liver has been reported to remove all or most H5 (by virtue of removing erythrocytes) [15], but this was not done here because H1° has been found to be sensitive to attack by proteases, even in the presence of enzyme inhibitor [11].

3.2. *Proteins of cultured chicken cells*

Culture of chicken cells in the absence of erythrocytes provided an alternative system for investigation of the possibility of occurrence of H1° or of H5 in cells which were not synthesizing DNA. Inhibition of mouse cell division in vitro by deprivation of serum or growth to confluence results in an abundance of H1° in these cells [16]. Cell division and DNA synthesis were inhibited in chick embryo fibroblasts by these same 2 methods and their proteins prepared with minimal proteolysis.

Protein preparations were repeated at least once and gave the same results upon repetition. Some of these preparations are shown in fig.2, after acid–urea gel electrophoresis. SDS gels could not be used to analyse these preparations because the proteins HMG1 and 2 which they contained migrated with mobilities similar to those of H1° and H5 in this system.

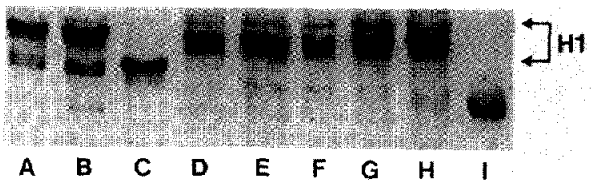

Fig.2. Polyacrylamide gel electrophoresis in acid–urea of $HClO_4$-soluble proteins from cultured cells: (A) serum-deprived mouse cells; (B) contact-inhibited mouse cells; (C) ox liver H1° (standard); (D,G) contact-inhibited chicken cells; (E,F) serum-deprived chicken cells; (H) proliferating chicken cells; (I) chicken H5 (from fig.1 peak (b)).

In mouse cell controls, the quiescent cells contained H1°, in similar abundance whichever method was used to inhibit DNA synthesis (fig.2A,B). However, no protein of H1°- or H5-like mobility could be seen in preparations from quiescent chicken cells (fig.2D–G) and no protein was more abundant (relative to H1) in these than in replicating cells (fig.2H). The absence of H1° or H5 from these chicken cells is probably not connected with them being short term, 'primary' cultures derived from embryos, for primary mouse embryo cell cultures brought to confluence contained H1° (not shown).

The H1 from replicating cells (fig.2H) appeared slightly more complex than that from cells which were not replicating but this was probably due to a higher level of H1 phosphorylation in the dividing cells [17]. A slight difference in H1 was observed between confluent and serum-deprived chicken cell cultures. In the former (fig.2D) the major H1 band of the 4 was the second from the top, whereas in the latter (fig.2F) it was the third.

Thus, neither an H1°- nor an H5-like protein could be identified in cultured chicken cells which were not synthesizing DNA.

4. Discussion

Various data have indicated an inverse correlation between the occurrence of H1° and cell division and/or synthesis of DNA [8,13,14] and this has led to the suggestion that H1° represses DNA replication [8]. The observed occurrence of H1° in mouse cells which are not synthesizing DNA fits in with this scheme. It

is therefore noteworthy that H1° was absent from adult chicken liver and both H1° and H5 were absent from quiescent cultured chicken fibroblasts. The methods used could detect H1° in the corresponding mammalian systems. The results suggest that H1° and H5 do not occur in the same animal and that H5 in birds does not regulate DNA synthesis. The distribution of H5 in birds remains limited to the nucleated erythrocyte. In [18] a protein from turtle was reported that was H1°-like in amino acid content. It was present in the liver (perfused) and to a lesser extent in nucleated erythrocytes but there was nothing in this reptile which more closely resembled avian H5. *Xenopus laevis* may also lack an erythrocyte-specific, H5-like protein [19]. Thus, perhaps reptiles and amphibians resemble mammals in their H1° types and distribution while birds are peculiar in having an H1°-like protein in erythrocytes only. This points to a fundamental difference between the cells of birds and mammals and perhaps other animals.

The absence of both H1° and H5 from a system which is not making DNA raises two possibilities:

(i) H1° does not repress DNA replication but is instead concerned with a process which is coincident with DNA synthesis in mammals. Birds may not require H1° function except in nucleated erythrocytes where that role may be fulfilled by H5;

(ii) H1° does regulate DNA synthesis in mammals, but birds achieve this end with a protein which is not of H1°- or H5-like character.

On this point it has been reported that chicken tissues of low cell division rate are characterised by a predominance of one type of H1 over other types [20]. This suggests involvement of an H1 subfraction in DNA synthesis regulation. The H1 pattern obtained here from serum-deprived chicken cells agreed with this report, the main H1 type being the third slowest on acid–urea gels. However, our result with confluent cultures did not agree, for the predominant H1 type was the second slowest. Possibly, the H1 pattern reflects only the general state of the cells' metabolism, with serum-deprived and contact inhibited cells being in slightly different states of arrest. It is already known that the pattern of H1 subfractions in cultured cells is affected by a change in metabolic state [21]. The conservation of an H5-like structure in mammalian H1°, perhaps dictated by function, also tends to argue against the second possibility. Whatever the case may be, mammalian cells seem to differ from avian cells (except nucleated erythrocytes) in their requirement for a protein of H5-like structure. Elucidation of this fundamental difference between 2 eukaryotic cell types would give further insight to the functioning of H1° and H5.

Acknowledgements

Our thanks go to Mrs C. Sigournay for technical assistance and Dr C. Marshall for the gift of the 3T3 TK3 cells. This work was supported by MRC and CRC grants.

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