Histone H5 and H1° cross-reacting material is restricted to erythroid cells in chicken

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Monoclonal H5 antibodies and a polyclonal antiserum, raised against the globular domain of chicken H5 (GH5) but which cross-reacts with histone H1° from mouse liver, were used to search for H5 or H1°-like proteins in chicken embryo and adult tissue sections by indirect immunofluorescence Chicken cell lines in culture were examined for H5 protein and H5 mRNA Histone H5 was detected only in erythroid cells in tissue sections of chicken embryos or adult livers H5 protein and H5 mRNA were found only in erythroid cells in culture No cross-reacting proteins were detected in any other tissue or cell line examined

Histone H5 *Histone H1°* *Indirect immunofluorescence* *Chicken embryo* *Chicken liver* *Anti-H5 antibody*

1 INTRODUCTION

Histone H5 accumulates as a major DNA-associated protein during erythrocyte maturation in birds, fish, reptiles and amphibians [1] It replaces the majority of H1 molecules on chromatin [2,3] and is correlated with chromatin condensation and a decrease in replication and transcription [2,3] A functional counterpart of chicken H5 is the H1° histone of mammalian species, which is found in many tissues and accumulates post-mitotically [4]. This class of histone may package chromatin in such a way that both DNA replication and transcription are significantly affected.

Histones H1° and H5 share a high degree of sequence homology [5], structural features [6] and immunological cross-reactivity [7,8]. The presence of common features in two proteins found in phyla as far apart in evolutionary terms as birds and mammals suggests a common function.

The search for H5 or H1°-like proteins in tissues other than erythrocytes in chickens has led to contradictory reports [9,10]. Here we have chosen the technique of indirect immunofluorescence on tissue sections to search for H5 and H1°-like proteins in embryonic and adult chicken tissues We have also probed RNA from chicken tissue-culture cells for H5 mRNA sequences. The results suggest that H5 is present only in erythroid cells and that H1° and H5-like proteins are absent from other chicken tissues.

2. MATERIALS AND METHODS

2.1 *Animals and cells*

Livers were obtained from white leghorn hens or Balb/C mice, 12–16 weeks old and used immediately for cryostat sectioning or histone extraction. Chicken embryos (4–6 days old) were frozen and sectioned immediately.

An AEV-transformed avian erythroid cell line (LSCC HD3 ts34 AEV [11]) was grown in DMEM with 2% chicken serum and 10% foetal calf serum An RSV-infected chicken fibroblast cell line grown in 10% tryptose phosphate broth, 5% foetal calf serum and 1% DMSO, and a Marek virus-transformed T cell line, grown in DMEM with 10% foetal calf serum, were also used

2 2. *Histone isolation*

Histones were extracted from purified nuclei by

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salt and acid extraction [12,13], dialysed against H_2O and freeze dried. Lysine-rich histones (H1, H5, H1°) were extracted from chicken or mouse nuclei with 5% perchloric acid and each purified by appropriate cation exchange chromatography [9,14]. The globular domain of H5 (GH5) was prepared by limited tryptic digestion of pure H5 [15], followed by purification of GH5 on a HPLC (Brownlee Guard Cartridge, C-18 300 Å pore, 10 μm particle size) reverse-phase column. Buffer A was 0.1% trifluoroacetic acid (TFA) in H_2O. Buffer B was 0.1% TFA in acetonitrile. A linear gradient of 15–50% buffer B was run over a 15 min period. The major peak corresponding to GH5 was collected, lyophilised and resuspended in PBS for injection into rabbits. N-terminal amino acid analysis was carried out to ensure that the purified material was authentic GH5 (amino acids 22–100 of H5).

2.3 *Antibodies*

Hybridomas secreting H5 monoclonal antibodies [16] were screened by radioimmunoassay (RIA) using ^{125}I-labelled rabbit anti-mouse IgG. Monoclonal antibodies were purified by protein-A Sepharose chromatography [17].

A polyclonal anti-GH5 antiserum (GH5-1) was obtained from rabbits injected intradermally with 200 μg GH5 in 0 5 ml PBS 4 times at 3-weekly intervals. Rabbits were then bled, antiserum prepared and characterised by RIA.

2.4. *Western blots*

Histones were separated on 18% polyacrylamide SDS gels at pH 8.8 [18], transferred to nitrocellulose, treated with appropriate dilutions of the antibody preparation and detected with ^{125}I-goat anti-mouse IgG (1.5×10^6 cpm/ml) [19].

2.5. *Immunofluorescence on tissue sections*

Frozen tissue sections 4 μm thick were air-dried, fixed in cold acetone at -20°C for 10 min and dried. Sections were treated with either supernatant from hybridoma cells, purified anti-H5 antibody (25 μg/ml) or anti-GH5 antiserum (1:200 dilution in PBS) for 16 h at 4°C, washed with PBS for 0.5 h, followed by second antibody treatment Fluorescein or rhodamine-labelled rabbit anti-mouse (for monoclonal antibodies) or goat anti-rabbit (for polyclonal antisera) IgG were used. Staining was for 1 h at 37°C. The sections were washed as above, mounted in glycerol/PBS (9:1) and viewed with a Zeiss fluorescence microscope.

Tissue culture cells were either grown on coverslips or spotted on to slides in PBS, air-dried and fixed in acetone/methanol (1 · 1). Indirect immunofluorescence staining was performed as described for tissue sections.

2.6 *Northern blot analysis*

RNA prepared from chicken cell lines was glyoxylated, electrophoresed on a 1.5% agarose gel and transferred to nitrocellulose [20]. The filter was hybridized simultaneously with a nick-translated chicken H2b gene fragment [21] and a chicken H5 gene insert [22] labelled by the random priming method [23]

3 RESULTS

3.1. *Specificity of antibody preparations*

The specificity of monoclonal antibody preparations made against pure histone H5 was tested by both RIA and immunoblotting. No cross-reaction above background was observed with histones from monkey CV-1 cells used as a control. When these antibodies were tested against a perchloric acid extract of mouse liver nuclei (containing the H1 proteins and H1°) the amount of ^{125}I bound to the microtitre plate well was never above background (i.e. as for CV-1 histones) over a wide range of antibody and histone concentrations (not shown)

The specificity of the monoclonal antibodies was further tested by western blots. Total histones or perchloric acid extracts from chicken erythrocytes, mouse liver and monkey CV-1 cells were separated by electrophoresis on 18% polyacrylamide SDS gels and transferred to nitrocellulose (fig.1A). Fig.1B shows the reaction of one H5 monoclonal antibody (12E-H5) with these proteins. The amount of protein loaded on the tracks of the gel was adjusted to give approximately equal amounts of H5 and H1° proteins on the gel. 12E-H5 has a high affinity for H5. A weak cross-reaction is seen with H1° and other H1 proteins from both mouse liver and CV-1 cells but this is essentially a non-specific background as similar faint bands were visible over other core histone bands (fig.1C, lane 1) This monoclonal antibody (12E-H5) was

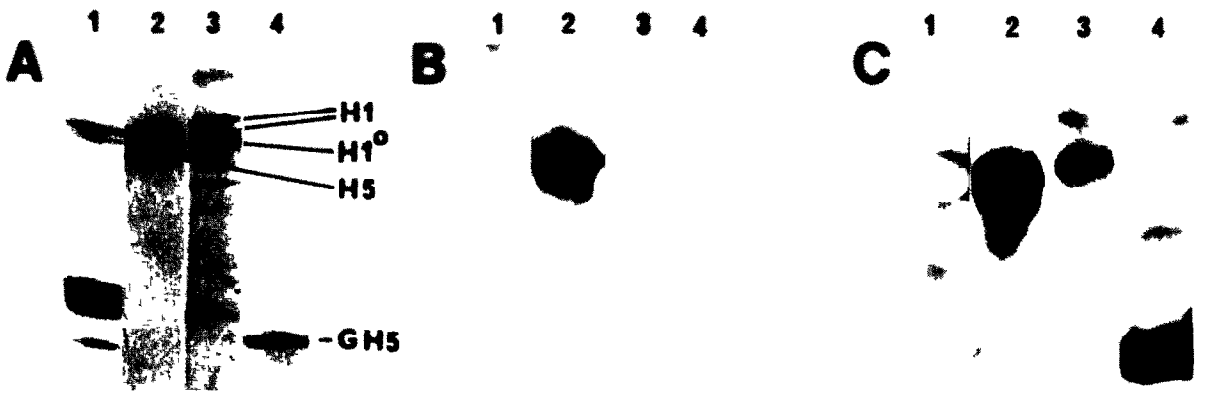

Fig 1 Immuno-blotting of histone proteins using anti-H5 antibodies (A) The proteins were separated on an 18% polyacrylamide SDS gel and stained with amido black Lane 1, histones from CV-1 cells, lane 2, perchloric acid extracted histones from chicken erythrocytes, lane 3, perchloric acid extracted histones from adult mouse liver, lane 4, the globular domain of H5, GH5 (B) The proteins were blotted onto nitrocellulose and treated with the monoclonal anti-H5 antibody 12E-H5 (C) An identical blot treated with anti-GH5 antiserum

purified from the hybridoma supernatant and used in subsequent experiments.

The most highly conserved region of the H1 proteins and H5 is the central globular region [10]. Monoclonal antibody against H5 (12E-H5) was tested for cross-reactivity with the globular domain of H5 (GH5). As shown in fig.1B, 12E-H5 does not cross-react with GH5 and is thus directed against the less conserved regions of the H5 protein and is an ideal preparation to search for H5 in chicken tissues.

A polyclonal antibody against GH5 (GH5-1) was raised in rabbits. Fig.1C shows that this antiserum cross-reacts with chicken H5, GH5 and with mouse H1° but not with other H1 proteins. GH5-1 was used to search for H1°-like proteins in chicken tissues.

3.2 *Immunofluorescence on tissue sections*

4–6-day-old chicken embryo sections were treated with the antibodies described above. Both the monoclonal 12E-H5 and the polyclonal GH5-1 cross-reacted strongly with H5 in erythroid cells in the vitelline membranes and developing blood vessels of the embryo (fig.2a). No fluorescence was visible in any other embryonic cells (fig.2a).

Because of the association of both H5 and H1° with fully differentiated tissues, livers from adult hens were examined for the possible presence of H5 with these antibodies; fig.2b shows a section of chicken liver treated with 12E-H5. Fluorescence was visible only in erythrocytes present in blood vessels or scattered throughout the tissue. The hepatocyte nuclei showed no fluorescence staining (fig.2b). The results obtained with GH5-1 were the same as those for the monoclonal antibody.

In order to test the specificity of the antibodies on tissue sections, adult mouse livers were sectioned and treated with 12E-H5 and GH5-1. 12E-H5 shows no fluorescence staining in any cells of the mouse liver (fig.2c) indicating the specificity of this antibody for histone H5 only On the other hand, GH5-1 stained the mouse hepatocyte strongly (fig.2d) in accordance with its previous cross-reaction (on Western blots) with H1° isolated from mouse liver.

In summary, H5-specific monoclonal antibodies only detect H5 in erythroid cells and not in other chicken tissues (chick embryos or adult liver). Polyclonal antibodies to the central domain of chicken H5 (GH5), although capable of specific binding to H1° of adult mouse liver, again detect cross-reacting material only in erythroid cell nuclei of chicken.

3.3 *Absence of H5 from chicken cells in culture*

3 3.1 Immunological studies

Cells from 3 chicken cell lines (erythroid, fibroblast and T cell described in section 2) were fixed and treated with 12E-H5 and GH5-1 an-

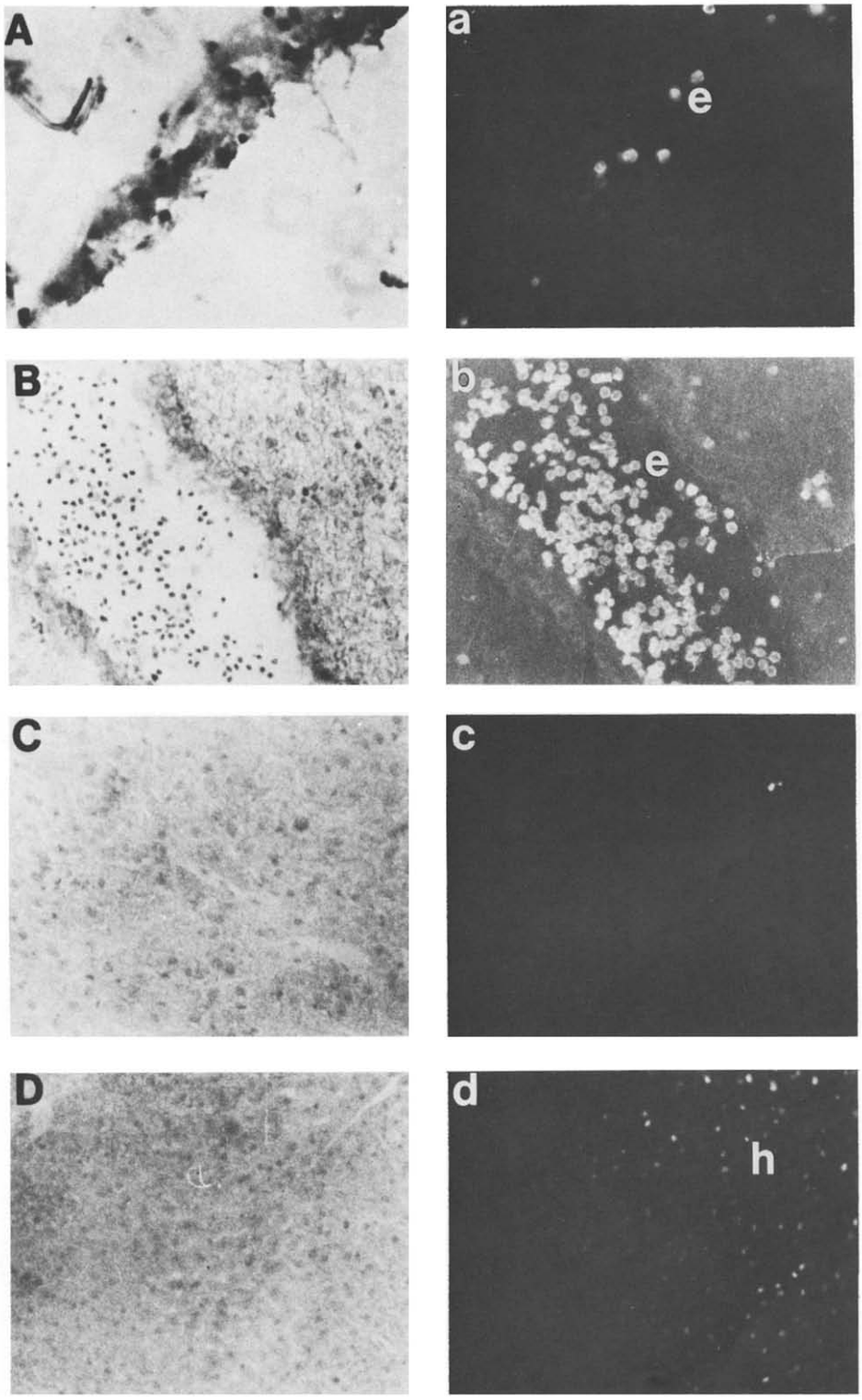

Fig.2 Immunofluorescence microscopy of chicken and mouse tissue sections. (A–D) Phase-contrast photographs, (a–d) fluorescence photographs (A,a) 4-day old embryo showing red blood cells stained with a monoclonal anti-H5 antibody (12E-H5); (B,b) adult chicken liver treated with the 12E-H5 antibody, (C,c) adult mouse liver treated with the same antibody, (D,d) adult mouse liver stained with the polyclonal anti-GH5 antiserum (e) Fluorescing erythroid cells, (h) fluorescing hepatocytes.

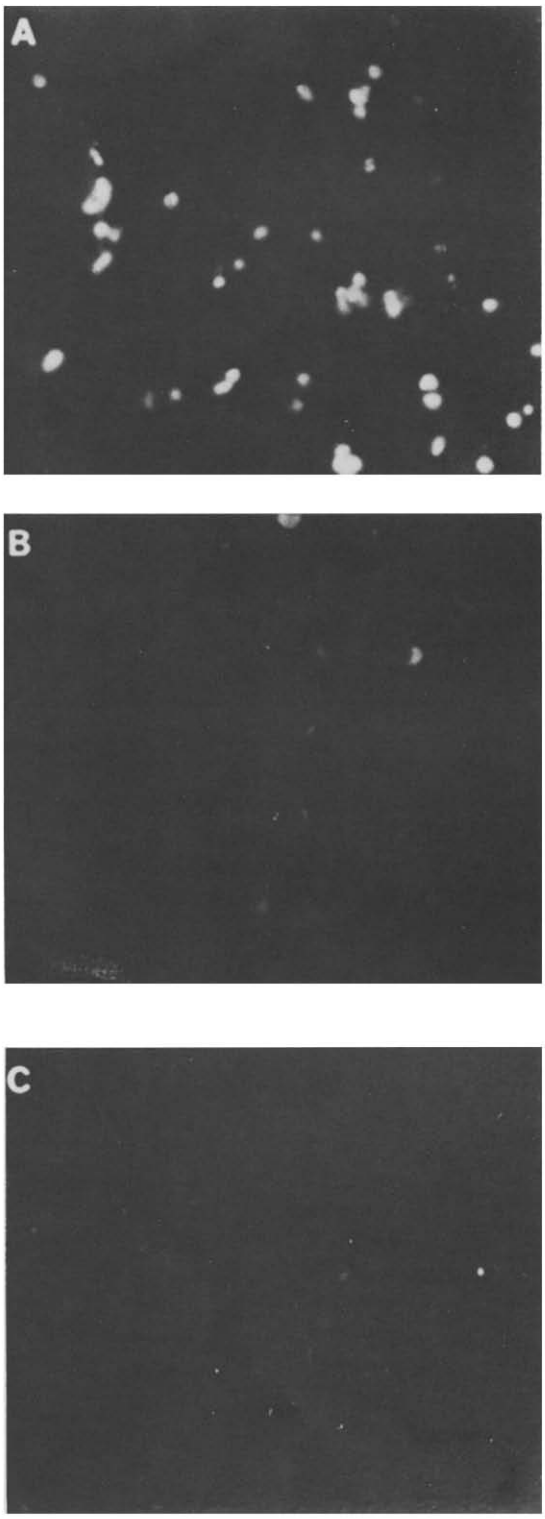

Fig 3 Immunofluorescence microscopy of chicken cell lines (A) AEV-transformed erythroid cells treated with the monoclonal anti-H5 antibody 12E-H5, (B) RSV-infected chicken fibroblasts treated with 12E-H5, (C) Marek virus-transformed T cells stained with 12E-H5

tibodies. The AEV-transformed erythroblast cell line was the only cell line in which fluorescent nuclei were seen with either of the above antibodies (fig.3).

3.3.2. Northern analysis

Total RNA from 3 chicken cell lines was probed with histone H5 and histone H2b-specific probes as shown in fig 4. It is clear that only the erythroid cell line contains H5 transcripts whereas (as expected) H2b transcripts are present in all 3 cell lines.

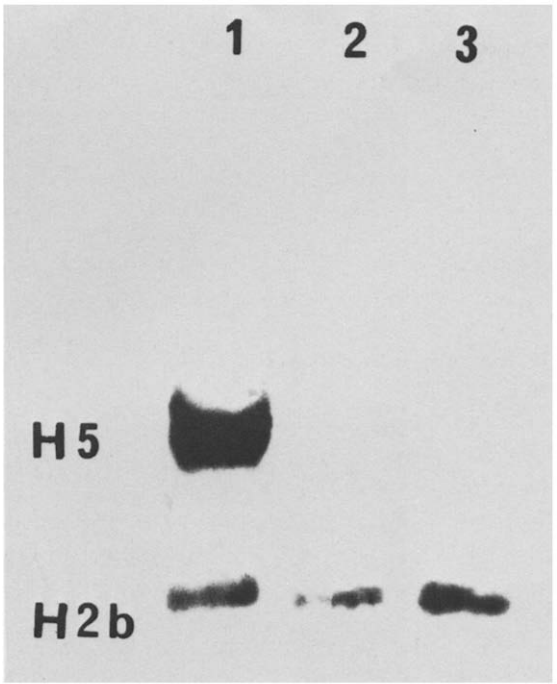

Fig 4 Northern blot analysis of RNA from chicken cell lines (1) AEV-transformed erythroid cells, (2) RSV-infected chicken fibroblasts, (3) Marek virus-transformed T cells

4. DISCUSSION

Two contradictory reports concerning the presence or absence of an H1°-like protein in chicken livers have recently appeared in the literature [9,10]. Immunological data presented in [10] suggest the presence of an H1°-like protein in chicken livers. However, the antisera used were not highly specific, cross-reacting strongly with other H1 sub-types from mouse liver. Our rationale for using GH5 as an antigen for H1°-cross reacting antibodies was to maximise the chance of detecting a protein equivalent to H1° in chicken tissues. A prerequisite was that the antibody preparation showed specificity for characterised H1° proteins, in this case from mouse liver. Our results for liver tissue (fig.2), for chick embryos (fig.2) and cells in culture (fig.3) support those of Smith et al. [9] who failed to find H1° in histone extracts of chicken liver and chicken cells in culture

It has been suggested that H1° plays a role in the regulation of cell proliferation [24] but there is some doubt since H1° accumulates predominantly after proliferation has terminated [4] The similarities between H1° and H5 would suggest a common function such as an ability to change the conformation of chromatin. This may restrict both transcription and replication. At low levels the effect may be minimal, for example, in the AEV line used here H5 does not prevent DNA replication or transcription. At intermediate levels, H1° or H5 may package chromatin such that a post-mitotic but transcriptionally active state is maintained. At higher levels, such as in the mature chicken erythrocyte, H5 may completely abolish transcription

The results presented here suggest that if there is a protein in non-erythroid cells of chickens which plays the same role as H1° or H5, then it is not immunologically related to these histones Other subtypes of H1 would be the most likely candidates for such a function and it is well known that the relative levels of H1 sub-types vary in different chicken tissues [25,26]. What is clear from our studies is that anti-chicken H5 antibodies capable of detecting H5 in chicken red cells or H1° in mouse liver nuclei, do not cross-react with chromatin-associated proteins, including H1 subtypes, in non-erythroid chicken tissues. Furthermore, at least in the cell lines examined, the restriction of H5 protein to erythroid cells in culture is reflected in the H5 mRNA levels in these cells.

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