Detection of Human and Chick Nuclear Antigens in Nuclei of Chick Erythrocytes during Reactivation in Heterokaryons with HeLa Cells

(heterokaryons/immune fluorescence/autoantibodies)

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Communicated by Daniel Mazia, October 13, 1971

ABSTRACT Inactive nuclei of chick erythrocyte resume RNA synthesis and increase in volume and dry mass in heterokaryons made by virus-induced fusion of human tumor cells (HeLa) with chick erythrocytes. Nuclear growth is due primarily to migration of human macromolecules into the chick nucleus. Human nucleoplasmic antigens were detected in the nucleoplasm and human nucleolar antigens were detected in the nucleoli of reactivated chick erythrocyte nuclei. After some time, chick-specific nucleolar antigens appear in the nucleoli of both the reactivated chick nuclei and the HeLa cell-nuclei. The results suggest that human nuclear proteins play an important part in the reactivation of the chick genome.

The nuclei of mature chick erythrocytes are inactive with respect to RNA, DNA, and protein synthesis (1). Harris (2, 3) has demonstrated that in heterokaryons formed by fusing chick erythrocytes with HeLa cells with the aid of UV-inactivated Sendai virus, the chick-erythrocyte nuclei are reactivated and resume RNA synthesis. At an early stage of the reactivation process, the deoxyribonucleoprotein undergoes marked changes in dye-binding capacity and in the stability of the DNA component to thermal denaturation and to acid hydrolysis (4). Nuclear volume and dry mass increase and the condensed chromatin of the inactive erythrocyte nucleus is dispersed (3, 5). This process is paralleled by an accelerated RNA synthesis (2), but nuclear growth occurs also if chick RNA synthesis is blocked by UV-irradiation of the erythrocytes before cell fusion (3, 5). Chick-specific antigen synthesis has not been detected until after the formation of the nucleoli. Nucleoli, which are visible by the light microscope, appear 2-4 days after cell fusion (6). Since the erythrocyte nuclei undergo a 5- to 8-fold increase in dry weight during the first 48 hr, these results suggest that the erythrocyte nuclei grow by taking up proteins from the cytoplasm, the majority of which must be of human origin. In order to obtain direct evidence for this conclusion and with the purpose of elucidating factors involved in the reactivation process, we have examined the distribution of human nuclear antigens in chick erythrocyte x HeLa heterokaryons with the aid of antinuclear antibodies from patients with autoimmune diseases.

MATERIAL AND METHODS

Cells

We obtained chick erythrocytes from 15-day chick embryos by cutting the allantoic vessels and allowing blood to accumulate in the allantoic fluid. The erythrocytes were collected by centrifugation and washed twice in phosphate-buffered saline (pH 7.4).

HeLa cells were grown on glass slides in Eagles Minimum Essential Medium containing 10% calf serum. Heterokaryons were made by addition of 20-50 haemagglutinating units of UV-inactivated Sendai virus and 30 X 10⁶ chick erythrocytes in 10 ml of Earle's balanced salt solution to the HeLa cell monolayer. For further details of the procedure see ref. 7. The heterokaryons were maintained in Eagles MEM containing 10% calf serum. At various time intervals, slides were removed, rinsed in saline, and fixed in ethanol: acetone (1:1).

Immunological methods

Antibody binding was demonstrated with the indirect immune fluorescence technique (7). Slides were transferred from the fixative to distilled water (two changes, 10 min each) and then exposed to antiserum that were diluted 5-20 times with 10 mM sodium phosphate buffer (pH 7.0)–8% NaCl for 45 min, at 37°C. After rinsing (twice, 10 min each time) in buffer (pH 7.0) at 22°C, antibody binding was visualized by staining with fluorescein-conjugated rabbit anti-human immunoglobulin or sheep anti-rabbit immunoglobulin (Statens Bakteriologisk Laboratorium, Solna, Sweden), diluted 1:10 in buffer for 20 min at 37°C. After rinsing in buffer (twice, 10 min each time, at 22°C), the slides were mounted in a buffer–glycerol (1:9) mixture.

Human autoantibodies against nucleolar antigen(s) were obtained from a 52-year-old female patient suffering from polymorphic manifestations of lupus erythematosus.

Human autoantibodies against nucleoplasmic antigen(s) were obtained from a 17-year-old female patient suffering from a slight, but typical, erythematosus butterfly facial lesion.

Human autoantibodies against cytoplasmic antigen(s) were obtained from a 58-year-old female patient suffering from severe rheumatoid arthritis.

Rabbit anti-chick nucleolar antibodies were obtained inadvertently during the early phase of an immunization scheme, in which a crude preparation of skeletal-muscle actomyosin of chick was injected intravenously into rabbits (Carlsson, Savage, and Ringertz, unpublished data). This serum stains both cytoplasmic myofibrils and nucleoli in chick myotubes, by the indirect immune fluorescence method, whereas in chick epithelial cells and fibroblasts, only nucleoli give a strong positive reaction (see below).
Results

Specificity of antisera

The patient sera used in this investigation were selected from a large collection that was obtained from patients with autoimmune diseases. When applied to HeLa cells in the indirect immune fluorescence method, antibodies to human nucleoli gave a strong and well-defined nucleolar fluorescence (Fig. 1); antibodies to human nucleoplasm reacted mainly with antigens in the nucleoplasm (Fig. 2) but also with the nucleolus, whereas antibodies to human cytoplasm showed a predominantly cytoplasmic fluorescence (Fig. 3), the nuclei appearing as dark holes. Chick cells (fibroblasts, epithelial cells, and erythrocytes) reacted weakly or not at all with the human sera (Figs. 1–3). HeLa and chick cells stained with control sera from healthy humans showed a negligible reaction.

The rabbit serum directed against chick nucleolar antigen(s) reacted with chick nucleoli, and to some extent with cytoplasmic antigens (Fig. 4). Chick erythrocyte nuclei (which lack nucleoli) and HeLa cells did not react with antibodies to the nucleolar antigens. A summary of the properties of the different sera used is given in Table 1.

Immune fluorescence of chick erythrocyte nuclei in heterokaryons

Shortly after cell fusion, some chick erythrocyte nuclei, primarily those which seemed to enlarge most rapidly, gave

Fig. 5. Appearance of human and chick antigens in chick erythrocyte nuclei undergoing reactivation in chick erythrocyte x HeLa cell heterokaryons. Some difficulties were encountered in scoring the antichick fluorescence of very small chick nucleoli, since the transition from a background fluorescence level (weak cross reaction with human nucleolar antigens, Table 1) to a clearly positive reaction is a gradual one. For this reason we have chosen to consider the chick nucleoli as positive only if the HeLa nucleoli also showed a fluorescence clearly more than the background level. O—O, X⋯X, ▲, represent antisera to human nucleoli, nucleoplasm, and cytoplasm, respectively; •—•, represents antiserum to chick nucleoli.
a highly positive reaction with human antinuclear antibodies. The number of chick nuclei that reacted with antisera to human nucleoli and nucleoplasm increased gradually within a 42-hr period, whereas at all stages examined the chick nuclei remained negative with respect to the antiserum to human cytoplasmic antigens (Fig. 5).

As nucleoli formed in the chick nuclei, they gave a positive fluorescence reaction with both human- and chick-specific antinucleolar antibodies (Fig. 6, Fig. 10). The reaction with the human antibodies appeared sooner (Fig. 5) and was stronger than the reaction with the anti-chick antibodies. The pattern of fluorescence changed with increasing nuclear size from a few small and relatively weakly staining spots to a more generalized staining of the chromatin that became concentrated in 2-3 distinct nucleolar bodies in the largest nuclei, as illustrated by the sequence of pictures in Fig. 7. Human autoantibodies directed against antigens in the nucleoplasm also showed a very strong binding to enlarged erythrocyte nuclei (Fig. 8). At 28 hr after cell fusion, about 20% of the erythrocyte nuclei present in heterokaryons showed some reaction with the antiserum to human nucleoplasm, whereas close to 100% of these nuclei gave a positive reaction with the human specific antinucleolar antibodies.

A serum reacting strongly with human cytoplasmic antigens, but not with human nuclei, failed to stain erythrocyte nuclei of the chick at all stages examined (Figs. 5 and 9).

**Immune fluorescence of HeLa nuclei in heterokaryons**

The staining pattern of HeLa nuclei in heterokaryons with antisera to human nucleoli, nucleoplasm, and cytoplasm was the same as that of nuclei in mononucleated HeLa cells that were not subjected to cell fusion. With the chick-specific antinucleolar antibodies, the following pattern was noted: immediately after cell fusion, HeLa-cell nuclei showed only a weak reaction, but as the chick-erythrocyte nuclei in the heterokaryons grew in size and developed nucleoli, the
Antigens in Chick Erythrocyte Nuclei 3231

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Human antiserum to:</th>
<th>Rabbit antiserum to chick nucleoli</th>
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<tr>
<td></td>
<td>Nucleoli</td>
<td>Nucleoplasm</td>
</tr>
<tr>
<td>HeLa nucleoplasm</td>
<td>(+)†</td>
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<tr>
<td>HeLa nucleoli</td>
<td>++</td>
<td>+++†</td>
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<tr>
<td>HeLa cytoplasm</td>
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<td>(+)</td>
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<tr>
<td>Chick fibroblasts</td>
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<tr>
<td>nucleoplasm</td>
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<td>(+)</td>
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<tr>
<td>nucleoli</td>
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<td>−</td>
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<tr>
<td>cytoplasm</td>
<td></td>
<td>−</td>
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<tr>
<td>Chick erythrocytes</td>
<td>−</td>
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<tr>
<td>nucleus</td>
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<tr>
<td>cytoplasm</td>
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<tr>
<td>Heterokaryons (HeLa cells x chick erythrocyte)</td>
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<tr>
<td>chick nucleoplasm</td>
<td>(+)</td>
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<tr>
<td>chick nucleoli</td>
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<td>HeLa nucleoplasm</td>
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<td>cytoplasm</td>
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</table>

* Comparisons can be made between cell types but not between different sera. The ratings are based on subjective estimations of fluorescence intensities after staining with the indirect immune fluorescence method. Antibodies to human nucleoli, human nucleoplasm, and chick nucleoli were also checked by microfluorimetric measurements.

† (+) represents slight positive reaction in some experiments; variation may have been due to different batches of fluorescein-conjugated antibodies.

‡ The evaluation of nucleolar fluorescence is difficult due to the strong fluorescence of the surrounding nucleoplasm.

HeLa and chick nucleoli became strongly positive. Microfluorimetric measurements revealed (after taking the larger size of the HeLa nucleoli into consideration) that the staining of the HeLa nucleoli was as intense as that of the nucleoli in the chick nuclei (Fig. 10). 76 hr after cell-fusion, some mononucleated, large cells with a morphology resembling that of HeLa cells also reacted with the chick-specific antinuclear antibodies, suggesting that synkaryons containing both human and chick genomes had formed.

The intensity with which HeLa nucleoli reacted with the antiserum to chick nucleoli in heterokaryons was clearly dependent upon the ratio of HeLa- to chick-cell nuclei. With increasing ratios of HeLa/chick nuclei, the fluorescence intensity of individual HeLa-cell nucleoli decreased. Within the individual heterokaryons, the HeLa-cell nuclei closest to the chick-erythrocyte nuclei gave the strongest reaction (see HeLa nucleoli in Fig. 10), suggesting the presence of a gradient of chick nuclear antigens throughout the cytoplasm of the heterokaryon.

**DISCUSSION**

The present results offer direct evidence that human macromolecules move into the chick-erythrocyte nucleus during its reactivation in HeLa cytoplasm. Human antigens characteristic of the nucleus and the nucleoplasm appear in analogous parts of the chick nucleus as it grows in size. Some antigens characteristic of human cytoplasm, which are not present in any major quantities in HeLa-cell nuclei do not accumulate in the chick nucleus. This shows that nuclear growth is not merely a passive swelling process with a random uptake of human proteins from the cytoplasm. It appears, on the contrary, to be a highly specific process, since certain types of human antigens, those found in human nuclei, are concentrated in the chick nuclei. This specificity may result from a selective penetration through the nuclear membrane and/or a selective binding of certain types of proteins to the deoxyribonucleoprotein in the chromatin.

Virtually all proteins found in the nucleus as part of the deoxyribonucleoprotein, or in the nucleolus, appear to be synthesized in the cytoplasm (9-12). Some of these proteins shuttle back and forth between the cytoplasm and the nuclear compartment, whereas others, after entering the nucleus, tend to remain there (nonshuttling proteins) (9, 10). Altered properties of the deoxyribonucleoprotein, such as has been observed in this system (4), may be the direct cause of the trapping of certain proteins in the nuclear compartment causing an increase in nuclear weight (5). The fact that human nuclear antigens accumulate in the chick nucleolus and not randomly in the chromatin is most likely due to a specific affinity of some nuclear antigens for the nucleus-organizing chromatin regions. That the state of the chromatin seems to determine the binding of the antigens studied here is further stressed by the observation that during mitotic chromosome condensation in HeLa cells, the antigens detected by the antisera to human nucleolus and nucleoplasm seem to become evenly distributed throughout the cytoplasm; the condensed chromosomes showed little fluorescence (to be published).

Small intranuclear bodies that were stained with the
antinucleolar antibodies appeared well before 30 hr after cell fusion, which is considerably earlier than previously observed (3, 6). This discrepancy is probably mainly due to the greater resolving power of the immune fluorescence method, as compared to normal phase contrast microscopy (unstained cells) or light microscopy (stained cells). The observation that chick-erythrocyte nucleoli stain positive with human-specific antinucleolar antibodies before they turn positive with the anticock antibodies (Fig. 5) may have several explanations. On the basis of observations made by Harris et al. (3, 6) concerning the role of the nucleolus in RNA transport, it may be that the chick nucleus has to form its initial nucleolus from human macromolecules before a messenger molecule coding for the chick nucleolar components can be transported into the cytoplasm and translated there into chick nucleolar proteins.

The appearance of chick nucleolar antigens in the HeLa-cell nucleoli after the reactivation of the chick nucleus is analogous with the observation of human-nucleolar antigens in the chick nuclei. Chick and human nucleolar components appear to be similar in their tendency to accumulate in nuclear compartments and in binding to the nucleolar-organizing chromatin regions. The fact that the nuclei come from cells representing different species seems to be of little importance. Yet human and chick proteins are different enough to permit a high degree of immunological species specificity.

The nature of the antigens detected by our sera is not yet known. Nucleolospecific staining patterns have been observed previously in connection with autoimmune diseases (14, 15). The antigen studied by Swanson Beck is destroyed by RNase but cannot be absorbed by RNA alone (14). The staining pattern observed with nucleoplasmic antigens corresponds to the "speckled" pattern observed in some cases of systemic lupus erythematosus. This antigen is believed to be a soluble glycoprotein that is present in nucleoplasm (16).

It appears on the basis of the results reported here that human autoantibodies against nuclear components can provide a useful tool in elucidating the manner by which the chick genome is reactivated in chick erythrocyte x HeLa-cell heterokaryons.

We thank Drs. A. Fagraeus, J. Jonsson, B. Edgren, and T. Fischer for generous help in collecting the patient sera, and Mrs. K. Järnebrand, U. Kronnahl, G. Blomgren, and G. Jacobson for excellent technical assistance. This investigation was supported by grants from the Swedish Cancer Society and the Swedish Natural Science Research Council.