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FURTHER EXPERIMENTS ON THE ROLE OF THE NUCLEOLUS IN THE EXPRESSION OF STRUCTURAL GENES

I. DEÁK, E. SIDEBOTTOM AND H. HARRIS
Sir William Dunn School of Pathology, South Parks Road, Oxford, England

SUMMARY

When a chick erythrocyte nucleus is introduced into the cytoplasm of a mouse cell it undergoes reactivation and eventually determines the synthesis of chick-specific proteins in the cytoplasm of the heterokaryon. It has previously been shown that there is a close temporal correlation between the onset of synthesis of the chick-specific proteins and the appearance of nucleoli in the erythrocyte nuclei. We now show that the synthesis of chick-specific proteins in the heterokaryon decays if the nucleolus in the reactivated erythrocyte nucleus is irradiated with an ultraviolet microbeam. The synthesis of chick-specific proteins does not, however, decay if only one of two nucleoli in the erythrocyte nucleus or if extranucleolar areas of the nucleus are irradiated. These observations confirm that some function located at or near the nucleolus is essential for the full expression of structural genes.

INTRODUCTION

When a chick erythrocyte nucleus is introduced into the cytoplasm of a tissue culture cell of the same or a different animal species, it undergoes reactivation and resumes the synthesis of RNA and DNA (Harris, 1965, 1967). When the chick nucleus is introduced into the cytoplasm of a cell from a different species – for example, the mouse – it can be shown that chick-specific proteins are eventually synthesized in the hybrid cell (Harris, Sidebottom, Grace & Bramwell, 1969; Harris & Cook, 1969; Cook, 1970). It was observed, however, that the synthesis of the chick-specific proteins did not take place as soon as the erythrocyte nucleus began to synthesize RNA; only after a nucleolus developed in the erythrocyte nucleus did chick-specific proteins begin to be made in the cytoplasm of the heterokaryon. Clearly definable nucleoli may appear in the erythrocyte nuclei at any time between the second and the seventh day after cell fusion: nucleoli appear more rapidly in nuclei obtained from immature embryonic erythrocytes than in adult erythrocyte nuclei. The onset of synthesis of chick-specific proteins in the heterokaryon correlates well with the time of appearance of nucleoli in the erythrocyte nuclei, whether this occurs early, as in embryonic erythrocyte nuclei, or late, as in adult erythrocyte nuclei. Before they develop nucleoli, the erythrocyte nuclei synthesize large amounts of the high molecular weight RNA that shows 'polydisperse' sedimentation behaviour in sucrose gradients; but autoradiographic experiments involving the use of a microbeam of ultraviolet light demonstrate that during this period no detectable amount of the RNA made in the erythrocyte nuclei passes to the cytoplasm of the cell (Harris *et al.* 1969; Sidebottom &

Harris, 1969). When, however, nucleoli appear and synthesis of ribosomal RNA becomes detectable, passage of labelled RNA from the erythrocyte nuclei to the cytoplasm of the cell can be detected. A correlation thus exists between the time of appearance of nucleoli in the erythrocyte nuclei, the time at which passage of RNA from these nuclei to the cytoplasm of the cell can be detected, and the time at which chick-specific proteins begin to be synthesized. In the light of these observations the proposition was advanced that the nucleolus was involved in some way in the transfer from nucleus to cytoplasm of RNA carrying information for the synthesis of specific proteins (Harris *et al.* 1969).

Two alternative interpretations of this series of experiments have, however, been offered. The first postulates a species-specific restriction on the translation of RNA templates. Since the heterokaryons produced by the introduction of chick erythrocyte nuclei into mouse cells contain initially only mouse ribosomes, it has been proposed that the failure of the reactivated chick erythrocyte nuclei to determine the synthesis of chick-specific proteins prior to the development of nucleoli is due to the inability of mouse ribosomes to translate RNA made on chick genes. This interpretation resolves the difficulty posed by the temporal correlation between the passage of labelled RNA from the erythrocyte nuclei and the onset of synthesis of chick-specific proteins by calling into question the limits of resolution of the autoradiographic techniques used. It is argued that the amounts of RNA involved in the transfer of information from nucleus to cytoplasm are too small to be detected by autoradiographic techniques, so that even before the development of nucleoli in the erythrocyte nuclei RNA carrying specifications for the synthesis of specific proteins could have passed to the cytoplasm of the cell without being detected.

The whole corpus of experiments on interspecific hybrid cells argues against the notion that there are stringent species-specific restrictions on the translation of RNA templates. Man–mouse hybrid cells, for example, synthesize only mouse 28 s ribosomal RNA (Eliceiri & Green, 1969; Bramwell & Handmaker, 1971), but they synthesize a variety of human proteins. These cells continue to synthesize human proteins even after chromosome loss has eliminated all but one of the human chromosomes; and a number of different residual human chromosomes have been shown to be active under these conditions. In the case of hybrid cells produced by the introduction of chick erythrocyte nuclei into mouse A 9 cells a similar case against species-specific translational restrictions can be made.

As has previously been described (Schwartz, Cook & Harris, 1971), the two sorts of nuclei in A9-chick erythrocyte heterokaryons rarely enter mitosis together. The erythrocyte nuclei normally lag; and when the mouse nuclei undergo mitosis, the chromosomes of the erythrocyte nuclei undergo fragmentation by a process that has been termed 'chromosome pulverization'. Fragments of the 'pulverized' chick genetic material are apparently incorporated into the mouse nuclei during post-mitotic reconstitution. Cells bearing such fragments can be isolated by appropriate selective procedures and can be shown to synthesize chick proteins even though the amount of chick genetic material that they contain is too small to be detected in chromosome preparations (Schwartz *et al.* 1971; Paterson & Harris, 1972). Electro-

phoretic examination of the patterns of ribosomal RNA labelling shows that these cells, like man–mouse hybrids, produce only mouse 28 s ribosomal RNA: chick 28 s RNA cannot be detected (Bramwell, 1972). These findings together make it very improbable that there are stringent species-specific restrictions on the translation of RNA; and they indicate that in the A9-chick erythrocyte heterokaryons the failure of the reactivated chick erythrocyte nuclei to determine the synthesis of chick-specific proteins before they develop nucleoli is not due to the inability of mouse cytoplasmic components to translate chick RNA. This conclusion is, of course, strongly supported by recent experiments with cell-free and other systems which also demonstrate the absence of species-specificity in the translation of RNA (Lockard & Lingrel, 1969; Housman, Pemberton & Taber, 1971; Rhoads, McKnight & Schimke, 1971; Gurdon, Lane, Woodland & Marbaix, 1971).

The second alternative interpretation of the studies on reactivated erythrocyte nuclei proposes that the correlation between the appearance of nucleoli in the chick erythrocyte nuclei and the onset of synthesis of chick-specific proteins is essentially fortuitous: it is suggested that these two phenomena might well occur simultaneously during the process of nuclear reactivation without there being any functional connection between them. The experiments we now describe show that this second alternative can also be excluded. We present decisive evidence that in the heterokaryon the full expression of chick structural genes requires the operation of some mechanism located in, or close to, the chick nucleolus.

MATERIALS AND METHODS

All techniques used in this investigation have been previously described: cell lines and techniques of cell culture (Harris *et al.* 1969); cell fusion (Harris & Watkins, 1965); cytology and autoradiography (Harris, Watkins, Ford & Schoefl, 1966); autoradiographic assay of inosinic acid pyrophosphorylase activity (Harris & Cook, 1969); focal irradiation of cells with an ultraviolet microbeam (Sidebottom & Harris, 1969); mixed immune haemadsorption (Watkins & Grace, 1967).

RESULTS

Inosinic acid pyrophosphorylase (*E.C.* 2.4.2.8)

Fig. 1 plots the appearance of inosinic acid pyrophosphorylase activity in X-irradiated A9 cells into which nuclei from the erythrocytes of 9-day-old chick embryos have been introduced. A9 cells lack inosinic acid pyrophosphorylase and cannot therefore incorporate tritiated hypoxanthine into nucleic acids. As the chick erythrocyte nuclei undergo reactivation and develop nucleoli, chick inosinic acid pyrophosphorylase is synthesized in the heterokaryons, which thus acquire the ability to incorporate tritiated hypoxanthine into RNA. The amount of tritiated hypoxanthine incorporated per unit time rises sharply between the second and the fifth day and thereafter remains fairly steady. By the fourth day after cell fusion most erythrocyte nuclei have well developed nucleoli which label heavily with tritiated pyrimidine nucleosides. Erythrocyte nuclei usually develop one or two nucleoli.

If the development of the nucleolus in the erythrocyte nucleus were not functionally

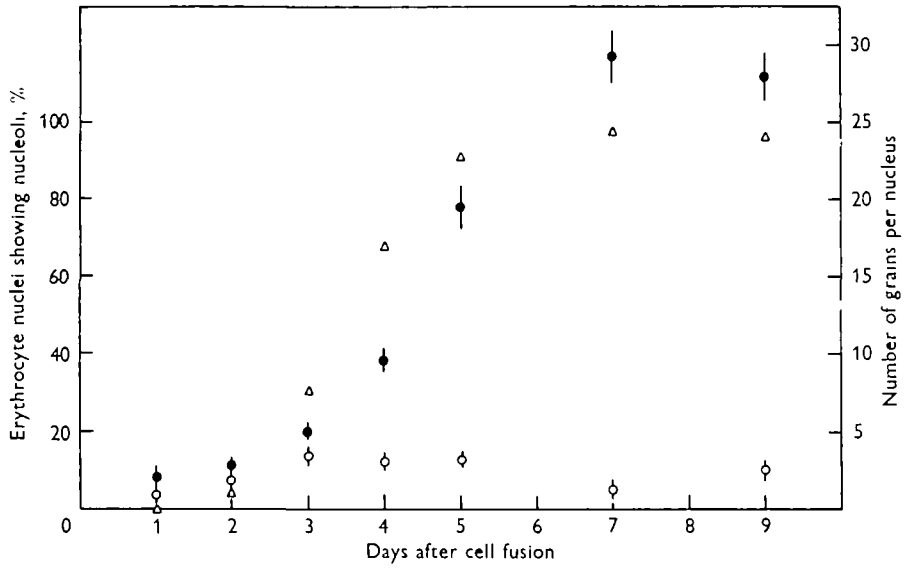

Fig. 1. Development of inosinic acid pyrophosphorylase activity in A9-chick erythrocyte heterokaryons, as measured by their ability to incorporate tritiated hypoxanthine into nucleic acid. The erythrocytes were taken from 9-day-old chick embryos, and incorporation of the labelled hypoxanthine was estimated by counting the number of grains over the A9 nuclei in autoradiographs. Hypoxanthine incorporation in the heterokaryons is initially only marginally greater than that in A9 cells, but between the second and the seventh day, as the erythrocyte nuclei develop nucleoli, incorporation increases markedly. ○, A9 cells; ●, heterokaryons; △, erythrocyte nuclei showing nucleoli. Vertical bars indicate standard errors.

related to the onset of synthesis of chick proteins in the heterokaryon then one would expect that synthesis of the chick proteins, once initiated, would be unaffected by subsequent inactivation of the erythrocyte nucleolus. Experiments were therefore done to test whether inactivation of the erythrocyte nucleolus after substantial amounts of chick inosinic acid pyrophosphorylase had already been synthesized had any effect on the subsequent ability of the cell to synthesize this enzyme. Five groups of heterokaryons, each containing one A9 nucleus and one chick erythrocyte nucleus, were compared. (1) Controls not subjected to any irradiation. (2) Heterokaryons in which a single nucleolus in the erythrocyte nucleus was irradiated with an ultraviolet microbeam on the fifth day after cell fusion. (The conditions of irradiation have previously been described: the organelle was exposed to the microbeam for 6 s. This dose of irradiation reduces RNA synthesis at the nucleolar site to less than 10% of the control level.) (3) Heterokaryons in which an extranucleolar region of the erythrocyte nucleus (nucleoplasm) was given the same dose of irradiation. (4) Heterokaryons in which one of two nucleoli of similar dimensions in a single erythrocyte nucleus was irradiated in the same way. (5) Cells in which the whole of the erythrocyte nucleus was irradiated with a larger microbeam. Cells in groups 2, 3, 4 and 5 were all irradiated at the same time so that individual coverslips bore cells of all five groups. At appropriate intervals after irradiation of the cells with the microbeam, the cultures

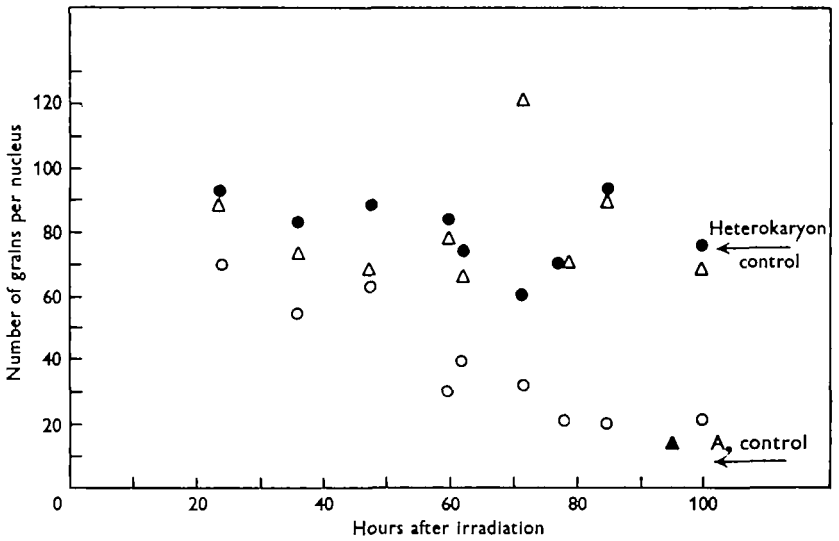

Fig. 2. Decay of inosinic acid pyrophosphorylase activity in A9-chick erythrocyte heterokaryons after selective irradiation of parts of the erythrocyte nucleus with an ultraviolet microbeam. ○, a single nucleolus in the erythrocyte nucleus irradiated; ●, one of two nucleoli in the erythrocyte nucleus irradiated; △, an extranucleolar area in the erythrocyte nucleus irradiated; ▲, the whole erythrocyte nucleus irradiated. Cells were irradiated at zero time. The controls are unirradiated heterokaryons and A9 cells.

were exposed for 6 h to tritiated hypoxanthine at a concentration of 5 μCi/ml, and autoradiographs of fixed preparations, treated so as to reveal incorporation of the isotope into RNA, were made in the usual way (Harris & Cook, 1969).

Figs. 2 and 3 show the effect of inactivating the erythrocyte nucleolus on the subsequent ability of the heterokaryon to incorporate tritiated hypoxanthine into RNA. Cells in which an extranucleolar region of the erythrocyte nucleus and those in which one of two nucleoli in the erythrocyte nucleus had been irradiated were indistinguishable from unirradiated cells in their subsequent ability to incorporate tritiated hypoxanthine into RNA. But cells in which a single nucleolus in the erythrocyte nucleus had been irradiated, progressively lost the ability to incorporate hypoxanthine into RNA; by the fourth or fifth day after irradiation they showed levels of inosinic acid pyrophosphorylase activity comparable to those found in A9 cells or in heterokaryons in which the whole of the erythrocyte nucleus had been irradiated. This experiment demonstrates that, even after the heterokaryon has developed the ability to synthesize chick inosinic acid pyrophosphorylase, inactivation of the erythrocyte nucleolus (where the erythrocyte nucleus contains only one such structure) results in decay of the synthetic system. That this system does not decay when an extranucleolar region of the erythrocyte nucleus is irradiated, or when only one of two nucleoli in the erythrocyte nucleus is irradiated, provides an adequate control for the specificity of the irradiation.

The loss of inosinic acid pyrophosphorylase activity in the heterokaryon after irradiation of the erythrocyte nucleolus indicates first that the enzyme itself undergoes

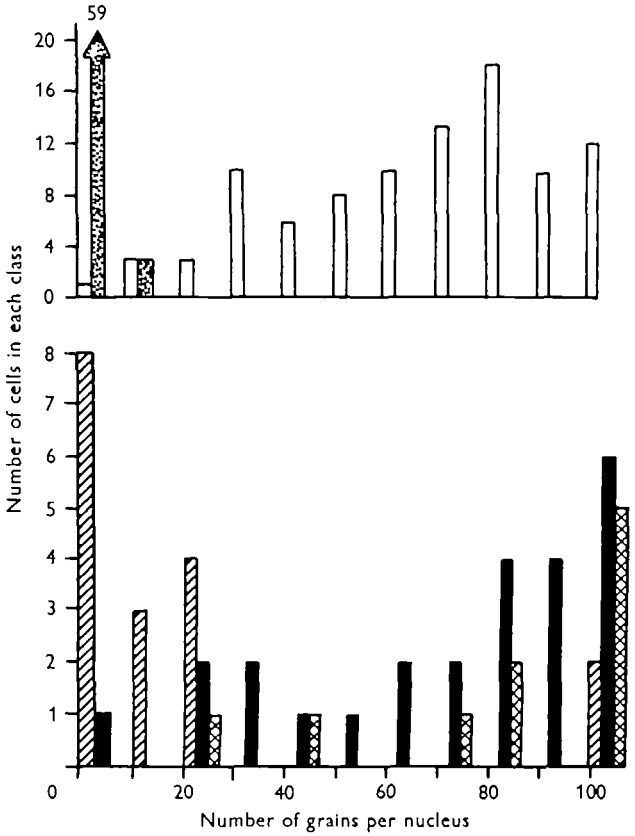

Fig. 3. Inosinic acid pyrophosphorylase activity in A9-chick erythrocyte heterokaryons 4 days after selective irradiation of parts of the erythrocyte nucleus with an ultraviolet microbeam. The histograms show the grain counts over the A9 nuclei in the various groups of cells. ▦, unirradiated A9 cells; □, unirradiated heterokaryons; ▨, a single nucleolus in the erythrocyte nucleus irradiated; ▩, one of two nucleoli in the erythrocyte nucleus irradiated; ■, an extranucleolar area in the erythrocyte nucleus irradiated.

degradation. This finding agrees with previous work (Harris & Cook, 1969), which indicates that the small amount of inosinic acid pyrophosphorylase introduced into the A9 cell during the process of cell fusion disappears by the third day. But loss of enzyme activity in the present experiments also implies that the RNA templates on which the enzyme is synthesized also decay, for the whole series of experiments on the reactivation of the erythrocyte nucleus indicates that if the templates for inosinic acid pyrophosphorylase are introduced into the cytoplasm of the heterokaryon they are eventually translated. It would thus appear that the level of inosinic acid pyrophosphorylase in the heterokaryon is maintained both by turnover of the enzyme itself and by a balance between supply and destruction of the relevant RNA templates. In any case, the results clearly show that the ability of the erythrocyte nucleus to determine the synthesis of chick inosinic acid pyrophosphorylase is dependent in some way on nucleolar activity.

Table 1. *Effect of irradiating the chick erythrocyte nucleolus on the synthesis of chick-specific surface antigens in the heterokaryon*

Days after irradiation	Proportion of cells showing chick surface antigen			
	Unirradiated heterokaryons	Single nucleolus irradiated	Nucleoplasm irradiated	One of two nucleoli irradiated
4	95/137 (69%)	12/30 (40%)	34/46 (74%)	10/17 (59%)
5	67/79 (85%)	10/30 (33%)	17/20 (85%)	13/18 (69%)
6	159/190 (84%)	8/26 (31%)	26/37 (70%)	20/23 (87%)

Chick surface antigen was scored as being present if 10 or more specifically sensitized sheep erythrocytes were adsorbed to the surface of the cell.

Surface antigens

It could, however, be objected that the gene coding for inosinic acid pyrophosphorylase might be located at, or close to, the nucleolar site, so that nucleolar irradiation inactivates the structural gene itself. This objection is improbable; for heterokaryons in which one of two nucleoli in the erythrocyte nucleus is irradiated retain the ability to synthesize inosinic acid pyrophosphorylase as efficiently as unirradiated cells – a finding that could only be accommodated in terms of direct inactivation of structural genes by supposing that one structural gene for the enzyme is present at each nucleolus and that the unirradiated gene compensates for the inactivation of its partner. Additional evidence against the view that the effects of nucleolar irradiation are due to direct inactivation of structural genes is, however, provided by observations on the synthesis of chick-specific surface antigens in a similar series of experiments.

As has previously been described (Harris *et al.* 1969), some chick-specific antigens are introduced into the surface of A9-chick erythrocyte heterokaryons by the process of cell fusion itself. These adventitious antigens are rapidly eliminated; but when nucleoli appear in the erythrocyte nuclei, chick-specific antigens reappear on the surface of the heterokaryon and progressively accumulate. The kinetics of appearance of the newly formed chick-specific surface antigens are essentially similar to those observed for the synthesis of chick inosinic acid pyrophosphorylase. Heterokaryons in which the synthesis of chick-specific surface antigens had already been established were therefore irradiated by the ultraviolet microbeam in the manner described for the experiments on inosinic acid pyrophosphorylase synthesis. The heterokaryons were irradiated by the microbeam 3–4 days after cell fusion and four groups of cells were studied: unirradiated controls; cells in which a single nucleolus in the erythrocyte nucleus was irradiated; cells in which an extranucleolar region was irradiated; and cells in which one of two nucleoli in the erythrocyte nucleus was irradiated. Four to six days after irradiation the four groups of cells were tested for the presence of chick-specific surface antigens by the immune haemadsorption reaction (Watkins & Grace, 1967). The results are shown in Table 1 and Fig. 4. It will be seen that where the

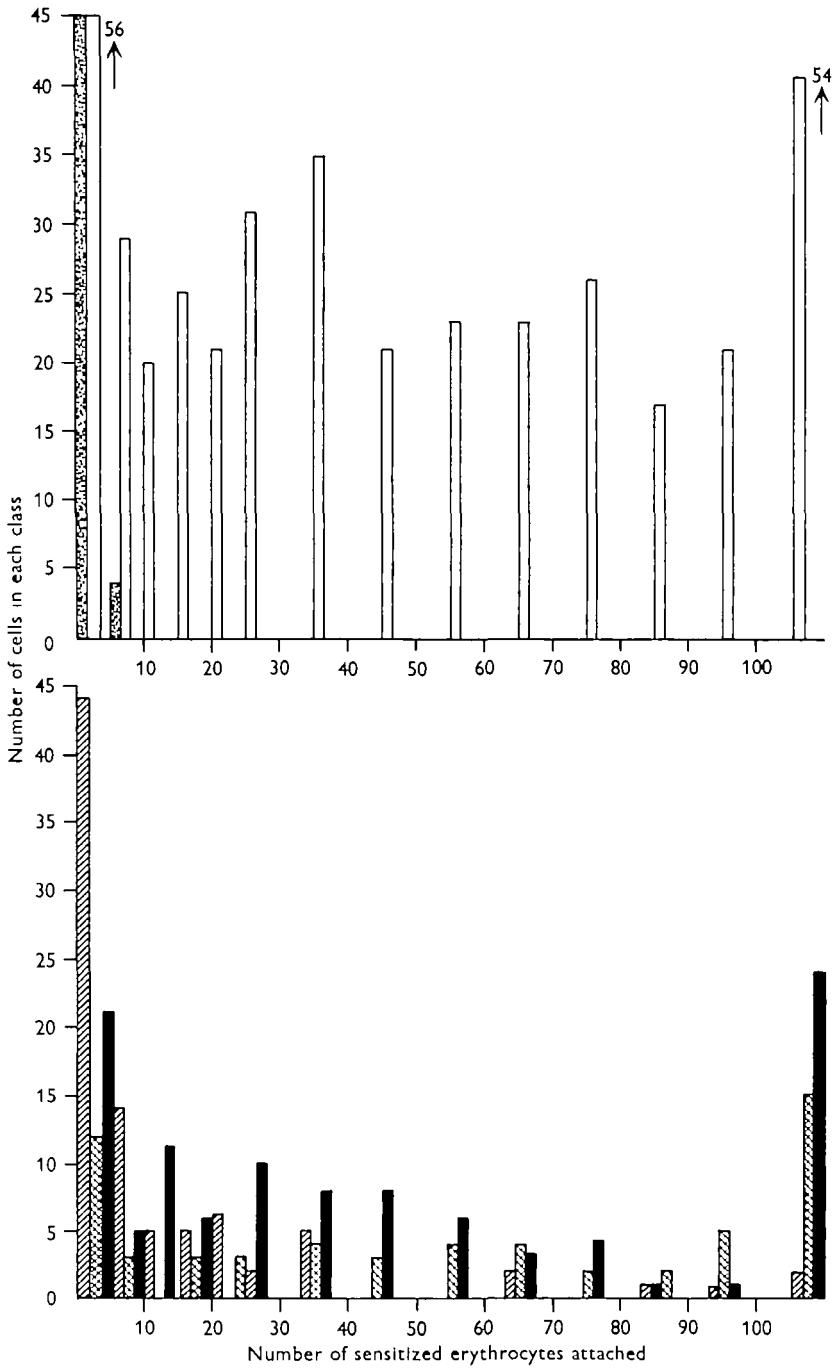

Fig. 4. Chick-specific surface antigens on A9-chick erythrocyte heterokaryons 4, 5 and 6 days after selective irradiation of parts of the erythrocyte nucleus with an ultraviolet microbeam. The results for all 3 days are pooled. The histograms show the numbers of specifically sensitized sheep erythrocytes adsorbed to the surface of the cells by mixed immune haemadsorption (Watkins & Grace, 1967). ▦, unirradiated A9 cells; □, unirradiated heterokaryons; ▨, a single nucleolus in the erythrocyte nucleus irradiated; ▩, one of two nucleoli in the erythrocyte nucleus irradiated; ■, an extranucleolar area in the erythrocyte nucleus irradiated.

Table 2. *Effect of irradiating the erythrocyte nucleolus on the sensitivity of the heterokaryons to diphtheria toxin*

Class of heterokaryon	No. of heterokaryons before addition of toxin	No. of heterokaryons surviving after addition of toxin	Percentage surviving
Unirradiated	183	11	6
Single nucleolus irradiated	85	73	86
Nucleoplasm irradiated	72	21	29
One of two nucleoli irradiated	44	14	32

erythrocyte nucleus contained a single nucleolus, irradiation of this structure resulted in the disappearance of chick-specific antigens from the surface of the heterokaryon; but these antigens did not disappear when an extranucleolar region of the erythrocyte nucleus or when one of two nucleoli in the erythrocyte nucleus was irradiated. It thus appears that even after the synthesis of chick-specific surface antigens has been established by the reactivated chick erythrocyte nucleus, this synthesis, like the synthesis of chick inosinic acid pyrophosphorylase, remains dependent on the activity of the nucleolus in the chick nucleus: if this nucleolus is inactivated, synthesis of chick-specific surface antigens decays. It has, however, been shown that the genes determining species-specific surface antigens are widely distributed throughout the chromosome set (Weiss & Green, 1967), so that in this case, the effect of nucleolar irradiation cannot be explained by *chance* proximity between the nucleolus and the structural gene. It could, of course, be argued, although there are grounds for considering the idea improbable, that *all* active structural genes are located in close proximity to the nucleolus; but a model of this kind implies some function for the nucleolar region in the expression of structural genes and is thus a version of our general proposition and not an argument against it. It is, in any case, clear that the observed association between the appearance of the nucleolus in the chick nucleus and the onset of synthesis of chick proteins is not coincidental.

Sensitivity to diphtheria toxin

Mouse cells in culture are at least 100000 times more resistant to the destructive action of diphtheria toxin than chick cells (Dendy, 1972). This difference is apparently due to the presence on the surface of chick cells of a receptor or receptors for the toxin absent from the surface of mouse cells. In chick–mouse hybrid cells sensitivity to the toxin may thus be used as a chick-specific marker over a wide range of toxin concentrations. When A9-chick erythrocyte heterokaryons are exposed to the toxin at a concentration of 0·1 Lf* units per ml, they remain resistant to its action until nucleoli appear in the erythrocyte nuclei; when this occurs, sensitivity to the toxin develops with kinetics similar to those observed for other chick-specific markers (Dendy, 1972). Experiments were therefore done to see whether sensitivity to diphtheria toxin, once established in the heterokaryon, decayed after inactivation of the chick nucleolus in

* Lf = 'Limes flocculation': an immunological measure of toxin concentration.

the same way as inosinic acid pyrophosphorylase and chick-specific surface antigens. The experimental design was the same as that used for the study of these other markers: the nucleoli and extranucleolar areas of the reactivated chick erythrocyte nuclei were irradiated by the microbeam and the sensitivity of the various groups of heterokaryons to diphtheria toxin was examined. The toxin, at a final concentration of 0·1 Lf/ml, was added to the cultures 4 days after the cells were irradiated and the number of surviving heterokaryons scored 60 h later. A9 cells, like other mouse cells, multiply at a normal rate in this concentration of toxin. The results are shown in Table 2. It will be seen that, like the synthesis of chick inosinic acid pyrophosphorylase and chick surface antigens, sensitivity to diphtheria toxin decays after inactivation of a single nucleolus in the chick erythrocyte nucleus, but much less after inactivation of extranucleolar regions. It thus appears that a range of different chick-specific markers all fail to be synthesized before the development of the nucleolus in the chick erythrocyte nucleus and all cease to be synthesized when this nucleolus is inactivated.

DISCUSSION

In the original interpretation given to the experiments on nuclear reactivation, it was proposed that the nucleolus was involved in regulating the passage to the cytoplasm not only of the RNA made at the nucleolar site but also of the RNA made elsewhere in the nucleus (Harris *et al.* 1969); and, in the light of the autoradiographic findings, the suggestion was made that the flow of both these families of RNA was in some way coordinated. This still seems to be the simplest interpretation of the observations we have made. It is worth considering whether there is any experimental evidence from other sources that is incompatible with it.

It has frequently been demonstrated, in a wide range of eukaryotic cells, that cytoplasmic fractions may contain complexes in which a 'rapidly labelled polydisperse' RNA is associated with ribosomes containing little or no radioactivity (see, for example, Perry & Kelley, 1968); and it has also been suggested that some 'rapidly labelled polydisperse' RNA passes to the cytoplasm of the cell under conditions in which ribosomal RNA synthesis is inhibited (Roberts & Newman, 1966). Observations of this kind might be thought to contradict the interpretation we have proposed for our results; but this view overlooks the complexities of RNA labelling in higher cells. The nuclei of almost all eukaryotic cells contain a substantial store of RNA of ribosomal type; indeed the great bulk of the RNA in eukaryotic nuclei has the general characteristics of ribosomal RNA. On the other hand, the nuclear store of 'polydisperse' RNA is so small that it cannot be detected by ultraviolet absorption; the presence of this RNA is revealed only by the rapidity with which it becomes labelled when the cell is exposed to a radioactive RNA precursor. Even if this 'polydisperse' RNA did pass to the cytoplasm of the cell, and its passage were rigidly coupled to the flow of ribosomal RNA, RNA labelling would none the less show the 'rapidly labelled polydisperse' RNA associated with ribosomes of much lower specific activity. And this asymmetrical labelling pattern would persist until the preformed nuclear stores of ribosomal RNA were fully labelled. This would also be true under conditions in

which ribosomal RNA synthesis was inhibited, provided that the procedure used to inhibit this synthesis did not impede the flow of preformed ribosomal RNA to the cytoplasm. Unless accurate corrections can be made for precursor pools and preformed stores of RNA (and present knowledge does not permit this), kinetic observations of this kind do not provide any information either for or against the idea of a coordinated flow of nuclear RNA components to the cytoplasm of the cell.

The relevance of the RNA labelling experiments to our own observations is, in any case, based on two assumptions, both questionable. The first is that the 'rapidly labelled polydisperse' RNA found in cytoplasmic fractions of cell homogenates represents RNA that has reached the cytoplasm as a result of physiological transfer from the cell nucleus; but there is strong evidence that most of this material is derived from cell nuclei broken during the process of cell fractionation (Plagemann, 1969; Ivanyi, 1971). The second is that this cytoplasmic 'rapidly labelled polydisperse' RNA is the RNA that carries the nuclear instructions for protein synthesis. It need hardly be said that this proposition is at least open to doubt. It could be argued that the only acceptable test at present available for the identification of an RNA template is that it supports the synthesis of a specific protein. If this argument is accepted, then observations based on RNA labelling alone provide no information at all about the flow from nucleus to cytoplasm of the RNA that bears instructions for the synthesis of specific proteins.

Some mention should perhaps be made of experiments on developing embryos. It is not immediately obvious that results obtained on embryos, which are systems of continuously multiplying cells, can be compared with those obtained in the present study, which deals only with somatic cell interphase, for at mitosis the nuclear membrane undergoes dissolution. Even so, it needs to be recognized that present evidence does not permit one to decide when, during early embryonic development, new information for the synthesis of specific proteins passes to the cell cytoplasm. In sea-urchin, amphibian and ascidian embryos, it is clear that embryonic development can proceed to a remarkably advanced stage on the basis of pre-formed maternal information present in the egg before fertilization (Barrett & Angelo, 1969; Wright & Subtelny, 1969; Lambert, 1971); and no experiments comparable to the present series on somatic cell heterokaryons have yet been reported for any embryonic material. 'Rapidly labelled' and/or 'polydisperse' RNA has indeed been found in cytoplasmic fractions prepared from sea-urchin embryos at stages of development preceding those at which the synthesis of ribosomal RNA was thought to begin (Kedes & Gross, 1969); and similar cytoplasmic RNA fractions have been found in embryos of the 'anucleolate' mutant of *Xenopus laevis*, in which little or no synthesis of ribosomal RNA occurs (Gurdon & Ford, 1967). These observations do not call for special comment; they are subject to all the kinetic and methodological uncertainties that have already been discussed. In the case of the experiments on sea-urchin embryos the observations themselves have been largely undermined by recent work which indicates that ribosomal RNA synthesis begins much earlier than had previously been supposed (Emerson & Humphreys, 1971); in the case of the 'anucleolate' *Xenopus* mutant an additional uncertainty arises from the presence of very large stores of preformed

ribosomal RNA and from the fact that in this mutant the cell nuclei contain numerous structures which have the electron microscopic appearance of small nucleoli and which shows clusters of RNA-containing particles indistinguishable from those seen in normal nucleoli (Hay & Gurdon, 1967).

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