THE INDUCTION OF DNA SYNTHESIS IN THE CHICK RED CELL NUCLEUS IN HETEROKARYONS DURING THE FIRST CELL CYCLE AFTER FUSION WITH HeLa CELLS

R. T. JOHNSON AND A. M. MULLINGER
Zoology Department, University of Cambridge, Downing Street, Cambridge, England

SUMMARY

Induction of DNA synthesis in embryonic chick red cells has been examined during the first and second cell cycles after fusion with HeLa cells synchronized in different parts of G1 and S-phase. The data indicate that: (i) the younger the embryonic blood the more rapidly the red cells are induced into DNA synthesis; (ii) the greater the ratio of HeLa to chick nuclei in the heterokaryon, the more rapidly the induction occurs; (iii) DNA synthesis in the chick nucleus can continue after the HeLa nucleus has left S-phase and entered either G1 or mitosis; (iv) the induction potential of late S-phase HeLa is somewhat lower than that of early or mid S-phase cells; (v) less than 10% of the chick DNA is replicated during the first cycle after fusion and only a small proportion (15%) of the chick nuclei approach the 4C value of DNA during the second cycle after fusion; (vi) the newly synthesized DNA is associated either with the condensed regions of the nucleus or with the boundaries between condensed and non-condensed regions; (vii) the chick chromosomes at the first and second mitoses after fusion are in the form of PCC (prematurely condensed chromosomes); they are never fully replicated and are often fragmentary; (viii) DNA synthesis in the chick nucleus is accompanied by an influx of protein (both G1 and S-phase protein) from the HeLa component of the heterokaryon.

INTRODUCTION

It is possible to induce DNA synthesis in a G1 nucleus many hours before this would normally occur by fusing together cells in G1 and S-phase (Rao & Johnson, 1970). Similarly, cells which occupy a non-cycling pre-DNA synthetic stage, such as chick erythrocytes, peritoneal macrophages and spermatozoa, are induced into S-phase when fused with actively proliferating cells (Harris, 1965; Johnson & Harris, 1969a; Bolund, Ringertz & Harris, 1969; Gordon & Cohn, 1970, 1971a, b; Gledhill, Sawicki, Croce & Koprowski, 1972). The initiation of DNA synthesis in active or quiescent nuclei after fusion poses questions about the nature of the signals, specific or general, which are provided by the S-phase cell and which rapidly reprogramme the activity of the G1 nucleus.

Previous work on the induction of DNA synthesis in HeLa-chick erythrocyte heterokaryons has established that the activation of the chick nucleus, and the triggering of RNA and DNA synthesis, are associated with a rapid increase in nuclear volume and dry mass which is, at least in part, due to the uptake of HeLa proteins (Harris, 1967; Bolund et al. 1969; Appels, Bolund & Ringertz, 1974). DNA synthesis
in the chick nucleus occurs in synchrony with HeLa cell nuclei in heterokaryons, and
the presence of chick nuclei does not inhibit DNA synthesis in the HeLa nuclei within
the same cell (Johnson & Harris, 1969a). The processes accompanying activation of
the chick red cell nucleus in heterokaryons have been carefully documented by Bolund,
et al. (1969).

In this paper we extend these observations on the induction of DNA synthesis in
embryonic chick erythrocytes during the first cycle after fusion with HeLa cells
synchronized at various stages in the cell cycle. These experiments are designed to
examine both changes in the nature and concentration of inducing factors during the
HeLa cell cycle, and the effect that changes in the 'recipient' erythrocyte nucleus have
on the ability of inducing factors to stimulate DNA synthesis. In particular we examine:
(1) the relationship between the kinetics of initiation of DNA synthesis and the age of
the embryo from which the erythrocytes were derived; (2) the location and amount of
the newly synthesized DNA in the chick nucleus during the first and second cycles
after fusion; (3) the induction potential of HeLa cells from different regions of S-
phase; (4) the movement of HeLa cell protein into the chick nucleus following fusion
with synchronous HeLa cells.

MATERIALS AND METHODS

Cells and synchronization techniques

Stock HeLa cells with a modal chromosome number of 64 were grown in suspension culture
at 37 °C (Rao & Johnson, 1970). Mitotic cells were obtained by a nitrous oxide pressure arrest
(Rao, 1968). The degree of synchrony was assessed by microscopic examination of fixed and
stained cells and was routinely 95 % or greater. Synchronous cells in the G1-phase were obtained
by continued incubation of the mitotic cells in suspension culture. Cell division was monitored
by microscopic examination and cell counting. In suspension culture, between 80 and 90 % of
the mitotic cells completed division 2 h after the nitrous oxide block, and these cells
were used for the early G1 fusions. The remaining cells, at a concentration adjusted to 2 × 10^6/ml,
enter S-phase synchronously between 6 and 8 h later. The entry of the synchronized cells into
S-phase and their exit into G2 was monitored by pulse labelling with [3H]thymidine (0.5 μCi/ml;
22 Ci/mmol) and subsequent autoradiography (Fig. 3, inset). Populations of cells synchronized
in early, mid and late S-phase were selected at various times after the release of the mitotic
arrest and used for fusion with the erythrocytes. This method of S-phase synchronization
inevitably leads to some decay in synchrony, particularly in the late S-phase population, but it
was preferred to the double thymidine schedule which results in unbalanced growth and
possible abnormalities in the initiation of DNA synthesis (Studzinski & Lambert, 1969).

Erythroblasts and erythrocytes (which will hereafter be referred to as red cells) were obtained
from the blood of 4-19 day embryos either by teasing apart the small blood vessels of the
youngest embryos and removing the fluid or by cutting the allantoic vessels and bleeding into
the allantoic fluid. For the fusion studies, blood was collected from at least three embryos from
each age and pooled. The red cells were washed twice in phosphate-buffered saline (Dulbecco
& Vogt, 1954) and separated from contaminating debris and other cells by centrifugation in a
haemocrit. Cells in the lower half of the packed red cell column were shown by cytological
examination to be uncontaminated and were used for experiments. White Leghorn eggs,
incubated at 39 °C, were used.
Induction of DNA synthesis in red cell nuclei

Examination of the proliferative potential of red cells

Red cells from 3–19 day eggs were separately washed and purified as described above and placed in McCoy's medium supplemented with 10% foetal bovine serum in plastic dishes (Falcon Plastics, Inc.) in a humidified CO₂ incubator at 39 °C. These cells were pulsed with [³H]thymidine (5 μCi/ml; 22 μCi/mmol) for 6 h before cytocentrifuge (Shandon Southern Instruments Ltd) preparations were made. After extraction in 5% trichloroacetic acid (TCA) at 4 °C the preparations were coated with G5 emulsion (Ilford Ltd) diluted 1:2 with 1% glycerol and exposed for 2 weeks. Slides were stained in toluidine blue (0.1% in 0.01% sodium bicarbonate) and the labelling index was scored for each. Similar studies were made of the in vivo DNA synthetic potential of red cells by injecting embryos from each age (3–19 days) with [³H]thymidine (total of 25 μCi per egg) for 6 h before the removal of the blood cells, and preparation of autoradiographs. For each determination red cells were pooled from 5 eggs.

Cell fusion and examination of heterokaryons

Fusion between synchronized HeLa cells and red cells was achieved in suspension. 5 x 10⁶ HeLa cells and 5 times the number of red cells were mixed at 4 °C with 500 haemagglutinating units of ultraviolet-inactivated Sendai virus. The fusion mixture was left for 15 min at 4 °C and then transferred to 37 °C for 30 min. After fusion, the cells were plated out into plastic dishes in Eagle's MEM, supplemented with 5% foetal bovine serum and incubated at 37 °C in a humidified CO₂ incubator. Samples were pulsed with [³H]thymidine at doses ranging from 0.2 to 5 μCi per ml (22 Ci/mmol) at various times after fusion was completed (i.e. 45 min after the addition of virus) to monitor DNA synthesis; 10 mM hydroxyurea was added to the incubation medium in the presence of [³H]thymidine in certain experiments.

The examination of interphase heterokaryons was carried out on cytocentrifuge preparations. These slides were processed for autoradiography as described above. Heterokaryons in mitosis were collected by a high-pressure nitrous oxide block at times corresponding to both one and two complete HeLa cell cycles after the fusion. Chromosome preparations were made by the standard technique (Tjio & Puck, 1958) after incubating the arrested mitotic cells in the presence of colcemide at 0.25 μg/ml (Gibco-Biocult Ltd.), to disrupt the spindle, and thereby to facilitate spreading of the chromosomes. Prematurely condensed chromosomes (PCC) from 5-day embryonic red cells were visualized by fusing the chick cells with an equal number of mitotic HeLa cells in the presence of colcemide (0.25 μg/ml) as described by Waldren & Johnson (1974).

Localization of [³H]thymidine incorporation in red cell nuclei by electron microscopy

Ten- or twelve-day red cells were fused with HeLa synchronized in early G₁, as described above, and were plated out in plastic Petri dishes. At times ranging from 3.5 to 8 h after fusion (corresponding to times when the HeLa nuclei were in early or mid-S, respectively), 2 separate samples of the fusion mixture were pulsed with 50 μCi/ml [³H]thymidine for 10 or 20 min. After the pulse one sample was chased with medium containing 50 μM thymidine for 10 min (or in the case of the 10-day cells, for 2 min) and was then fixed immediately; the other sample was chased with thymidine (50 μM) for 30 min and was then incubated for a further 3.5 h in the presence of normal medium before fixation.

Cells were fixed for 1 h at room temperature in 3% glutaraldehyde in 0.1 M phosphate buffer containing 1% sucrose and 0.01% calcium chloride. They were then washed overnight at 4 °C in several changes of the same phosphate buffer, postfixed at room temperature in 1% osmium tetroxide in phosphate buffer, dehydrated in ethanol and embedded in Araldite. A parallel series of samples was extracted in 5% TCA at 4 °C before dehydration and embedding. Thin sections (silver-gold) mounted on celloidin-coated slides were stained with saturated uranyl acetate in 50% ethanol followed by lead citrate (Reynolds, 1963). A thin carbon film was deposited on the slides by vacuum evaporation and the sections were coated with Ilford L4 emulsion diluted 1:2 with 0.005% sodium lauryl sulphate. They were then exposed for 2 months in a dry atmosphere at 4 °C, and developed either in Microdol X (diluted 1:3) for 3 min at 20 °C after gold latensification for 2 min (Gupta, Moreton & Cooper, 1973) or in Phenidone.
Developer for 1 min without previous gold latentization. The sections were transferred to grids and examined in a Philips EM200 electron microscope operated at 60 kV.

The analysis of the autoradiographs followed the methods described by Gupta et al. (1973). The distribution of silver grains in the induced red cell nuclei was determined by measuring the minimum perpendicular distance from the grain centre both to the nearest boundary between condensed and non-condensed nuclear material and also to the nearest nuclear envelope. In the former case counts included all grains lying with their centre point within the nuclear envelope; in the latter all grains which either touched the nuclear envelope or were enclosed by it, were scored. Nuclei with less than three grains were not included. The sections used for detailed analysis had not been extracted with TCA.

The grain distributions were plotted as histograms which were compared with theoretical distributions of radioactivity from a perfect line source, using the methods of Salpeter, Bachmann & Salpeter (1969), and Gupta et al. (1973), by means of a Hewlett-Packard 910CB desk calculator and X-Y plotter.

**Determination of the amount of DNA synthesized by Feulgen densitometry**

Cytocentrifuge preparations of heterokaryons were fixed for 24 h in Carnoy’s fixative (3 parts ethanol to 1 part acetic acid) and air dried. Slides were hydrolysed in 5 N HCl for 40 min at 20 °C and stained with Schiff’s reagent according to standard procedures for Feulgen-DNA staining (Pearse, 1968). They were also counterstained very lightly with light green. The intensity of the Feulgen staining was measured using a Vickers M85 scanning Microdensitometer at 546 nm.

**Labelling the proteins of HeLa cells and HeLa-chick red cell heterokaryons and examination of protein migration in heterokaryons**

Seven-, eight-, and 15-day embryonic red cells were fused with HeLa cells synchronized in different parts of G1 or in S-phase after reversal of a nitrous oxide mitotic arrest. The precise schedules for the fusions and incubation of the cells in the presence of 3H-amino acids are presented in the Observations section. In each of these experiments, except where otherwise indicated, the 3H-amino acid mixture was composed of: [3H]arginine (5 Ci/mmole); [3H]lysine (250 mCi/mmole); [3H]histidine (30 Ci/mmole); [3H]leucine (46 Ci/mmole); [3H]methionine (2 Ci/mmole); [3H]tryptophan (1 Ci/mmole). Each amino acid was present at 2 μCi/ml. In some experiments, incubations were carried out in the presence of 25 μg/ml cycloheximide. After a period of incubation at 37 °C in a humidified CO2 incubator, the cell mixtures including the heterokaryons were removed from dishes by trypsinization, washed 3 times in warm complete medium, fixed in 3 % formaldehyde in phosphate buffer for 2 h, embedded in Araldite and sectioned (0-5- and 0-1-fim sections). The sections were coated with G5 emulsion diluted 1:2 with 1 % glycerol and exposed for 2 weeks. The autoradiographs were either stained in alkaline opa% methylene blue before examination or viewed unstained by means of phase contrast or Nomarski’s differential interference microscopy.

**Chemicals and radiochemicals**

All chemicals used in these studies, unless otherwise indicated, were purchased from Sigma Ltd. [Me-3H]thymidine, L-[3-3H]arginine, L-[4,5-3H]lysine, L-[2,5-3H]histidine, L-[4,5-3H]leucine, L-[2-3H]methionine and L-[3H]tryptophan were obtained from the Radiochemical Centre, Amersham, England.

**Observations**

**Inducibility of chick red cell nuclei as a function of the age of the embryo**

The occurrence of DNA synthesis and mitosis in circulating red cells from eggs at different stages of embryonic development was assessed by incubation in the presence
Induction of DNA synthesis in red cell nuclei of [H]thymidine, and by subsequent autoradiography. The results of such experiments are presented in Table 1, which shows the proportion of red cells synthesizing DNA, both in vivo and in vitro for embryos of different ages. After 7 days of embryonic development there is no further incorporation of [H]thymidine by the circulating red cells and mitotic figures are also absent from the population. This result confirms the work of Lemez (1964) and Weintraub, Campbell & Holtzer (1971) who showed that cell division and DNA synthesis cease in the peripheral red blood cells between the 6th and 7th days of development.

Table 1. DNA synthesis and mitosis in chick red cells from embryos at different stages of development

<table>
<thead>
<tr>
<th>Age of embryo, days</th>
<th>In vivo index of DNA synthesis, %</th>
<th>In vivo mitotic index</th>
<th>In vitro index of DNA synthesis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>55</td>
<td>0.65</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>0.47</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>0.17</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>0.8</td>
<td>0.05</td>
<td>0.9</td>
</tr>
<tr>
<td>8</td>
<td>0.02</td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>9-19</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

1000 cells were scored for each mitotic and DNA synthetic index.

Red cells which have reached the non-cycling stage can be induced to synthesize DNA by fusion with HeLa cells. In order to assess the relative inducibility into DNA synthesis of red cells that had been quiescent for different lengths of time, blood was collected from embryos of different ages, from 7 days onwards. The red cells were separated and fused with HeLa cells synchronized at the start of G1 or S-phase. The heterokaryons thus obtained were plated out into plastic dishes and incubated for various durations in the continuous presence of [H]thymidine (0.5 μCi/ml) before preparation for cytological examination. The induction of DNA synthesis in the red cell nuclei of different embryonic ages is shown in Fig. 1. The younger the red cell population the more rapid is the induction into DNA-synthetic activity, and the greater the proportion induced during an 8-h exposure to the S-phase environment of the HeLa cell. Thus the longer the red cell has occupied a non-cycling position, the greater is the lag before the signals for DNA synthesis in the heterokaryons take effect.

Additional experiments showed that the induction of DNA synthesis in the chick nucleus is a specific response to the S-phase in the heterokaryon. Fusion between red cells and HeLa cells at the start of the G1 period does not lead to initiation of DNA synthesis until the HeLa nucleus has entered S. Exposure of the red cell nucleus to the G1 heterokaryon does, however, result in a greater proportion of nuclei being induced into DNA-synthetic activity during the subsequent HeLa S-phase. This is particularly clear for older red cells. For example, exposure of 19-day red cell nuclei exclusively to 7 h of S-phase results in 24% induction, whilst exposure to 7 h of G1 plus 7 h of S results in induction in 71% of the population.
The nature of the induced DNA synthesis in the red cell nuclei was tested by incubation of the heterokaryons in the presence of hydroxyurea. Unscheduled DNA synthesis is known to differ from semiconservative replication in a number of important respects, including a marked insensitivity to inhibition by hydroxyurea (Cleaver, 1969). Heterokaryons prepared by fusion between 8-day red cells and HeLa cells in early $S$-phase were incubated in the presence of [3H]thymidine (1 $\mu$Ci/ml) and 10 mM hydroxyurea for up to 7 h. Subsequent autoradiography showed that no DNA synthesis had occurred in either the red cell or HeLa nuclei. The DNA synthesis that is normally induced would therefore not appear to be unscheduled in character.

Fig. 1. The induction of chick nuclei into DNA synthesis after fusion with cells in early $S$-phase, as a function of the age of the embryo and the time spent in the heterokaryon. Counts were restricted to heterokaryons with a total of 2, 3 or 4 nuclei per cell; 500 red cell nuclei were scored for each point. ○—○, 7-day nuclei; □—□, 8-day nuclei; ■—■, 9-day nuclei; ▲—▲, 10-day nuclei; △—△, 12-day nuclei; ▼—▼, 14-day nuclei.

Dose-response

The speed of induction of DNA synthesis in chick nuclei was assessed in relation to the ratio of chick to human nuclei in heterokaryons formed by fusion between red cells and HeLa cells synchronized at the start of $S$-phase. Among heterokaryons with only one HeLa nucleus it was found that, as the number of chick nuclei per cell increased from 1 to 3, there was a corresponding decrease in the proportion of chick nuclei which showed incorporation of [3H]thymidine during continuous incubation in the presence of 0.5 $\mu$Ci/ml (Fig. 2A). Correspondingly, among heterokaryons with only one red cell nucleus, an increase in the number of HeLa nuclei from 1 to 3 resulted in a progressive increase both in the number of red cell nuclei which were induced in a given time after fusion and in the speed with which DNA synthesis was initiated (Fig. 2B). These ratio effects were found for chick cells from different ages of embryo. It would thus appear that the induction of DNA synthesis in the chick
Induction of DNA synthesis in red cell nuclei

nuclei is controlled by the concentration of available inducing factors. The greater the supply of inducers the more rapidly the chick nucleus enters $S$-phase and the greater the proportion of nuclei which synthesize DNA during the period of time that the HeLa element itself spends in $S$.

Fig. 2. The induction of 8- and 14-day chick nuclei into DNA synthesis after fusion with HeLa cells in early $S$-phase, as a function of the ratio of HeLa to chick nuclei and the time spent in the heterokaryon. An average of 50 heterokaryons of each class was scored for each point. Where more than 1 red cell nucleus was present the value for induction into $S$-phase refers only to those heterokaryons in which there was a synchronous synthesis of the red cell nuclei. A, heterokaryons with 1 HeLa and 1–3 chick nuclei. Solid lines, 8-day nuclei; dotted lines, 14-day nuclei. Nuclear ratios in heterokaryons as follows: 1:1, 1 HeLa 1 chick; 1:2, 1 HeLa 2 chick; 1:3, 1 HeLa 3 chick. B, heterokaryons with 1 chick and 1–3 HeLa nuclei. Solid lines, 8-day nuclei; dotted lines, 14-day nuclei. Nuclear ratios in heterokaryons as follows: 1:1, 1 HeLa 1 chick; 2:1, 2 HeLa 1 chick; 3:1, 3 HeLa 1 chick.

A further point emerges from these studies of heterokaryons with high ratios of HeLa to chick nuclei (Fig. 2B). In the case of 8-day chick nuclei, the sharp rise in the proportion induced between zero and 1.5 h after fusion, followed by a plateau, may indicate the heterogeneity of the early embryonic red cell population in contrast to the steady rise in inducibility of the 14-day nuclei. This effect is not seen in heterokaryons where the HeLa:chick ratio is unity or less. It is known that mature erythrocytes in the embryo are derived from 2 proliferative sources, the primitive series formed in the yolk sac, and the definitive series produced in the embryo. Both regions of proliferation give rise to erythroblasts which can be distinguished by morphological criteria and which undergo maturation in the circulation, terminating as metabolically quiescent erythrocytes.

Some additional features of the dosage effect are seen from the results of another experiment, in which the heterokaryons were pulse-labelled with 0.5 $\mu$Ci/ml $[^3H]$thymidine for 30 min at different points after fusion (Table 2). One hour after the completion of fusion between 8-day red cells and HeLa in early $S$-phase the dose response is clear: the higher the ratio of HeLa to chick nuclei, the greater the proportion of chick nuclei induced. On the other hand, after 7 h there is little difference in the proportion of chick nuclei induced in the various classes of heterokaryons. It would appear that the supply of 'inducers' is limiting only for a relatively short period.
(though the length of this period varies somewhat between experiments depending on the precise conditions and state of the cells in each case). This would account for the fact that a dosage effect was not found by Johnson & Harris (1969a) with random HeLa–chick heterokaryons, since in the latter case the sampling of the heterokaryons was mostly carried out at a later time after fusion.

Table 2. Induction of DNA synthesis in 8-day chick nuclei as a function of nuclear ratios in the heterokaryons at two times after the completion of fusion

<table>
<thead>
<tr>
<th></th>
<th>Chick nuclei induced into $S$ in presence of S-phase HeLa, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 HeLa</td>
</tr>
<tr>
<td>A. 1 h after fusion completed</td>
<td></td>
</tr>
<tr>
<td>1 chick</td>
<td>14 (105)</td>
</tr>
<tr>
<td>2 chick</td>
<td>9 (66)</td>
</tr>
<tr>
<td>3 chick</td>
<td>2 (48)</td>
</tr>
<tr>
<td>B. 7 h after fusion completed</td>
<td></td>
</tr>
<tr>
<td>1 chick</td>
<td>72 (472)</td>
</tr>
<tr>
<td>2 chick</td>
<td>64 (538)</td>
</tr>
<tr>
<td>3 chick</td>
<td>67 (390)</td>
</tr>
</tbody>
</table>

The figures in parentheses refer to the total number of chick nuclei scored for each class of heterokaryon. Only in heterokaryons containing one chick nucleus will this figure represent the number of individual heterokaryons scored.

The data presented so far refer to heterokaryons in which all the HeLa nuclei were synthesizing DNA — that is they were in synchronous S-phase. In heterokaryons with asynchronous labelling of the HeLa nuclei (e.g. one in $S$ and one in $G_j$) the red cell nucleus was only very rarely found to incorporate $[^{3}H]$thymidine. This suggests some form of competition between HeLa and red cell nuclei for the inducing factors or the label.

Fusions between HeLa in different parts of $S$-phase and chick red cells

It is possible that the conditions which are responsible for initiating DNA synthesis in the chick nucleus vary throughout the HeLa S-phase. In order to examine this question red cells were separately fused with HeLa cells in 3 different regions of $S$-phase (early, mid and late $S$) and DNA synthesis in the red cell nuclei was monitored by pulse labelling with $[^{3}H]$thymidine at various times after fusion. The details of one such experiment were as follows.

The mitotic HeLa cells obtained after release from nitrous oxide arrest were incubated at 37 °C and allowed to proceed unperturbed into $S$-phase. Aliquots were removed for fusion at 6, 10 and 15 h after release of the mitotic block, when the
Induction of DNA synthesis in red cell nuclei

majority of the cells could be expected to be in early, mid or late $S$ respectively. At the same time, the passage of the unperturbed cell population into $S$-phase was monitored by pulse-labelling samples with $[^{3}H]$thymidine at hourly intervals and subsequent autoradiography. The small graph in Fig. 3 shows the position, with respect to DNA synthesis, of the synchronous population of HeLa cells used for each of the 3 fusions.

DNA synthesis in the heterokaryons of the fusion mixtures was also monitored at 2-h intervals by treatment of samples with 15-min pulses of thymidine at $0.25 \mu Ci/ml$,

Fig. 3. Induction of 8-day chick nuclei into DNA synthesis after fusion with HeLa cells at 3 different points in $S$-phase. The inset figure shows the entry of the parental HeLa population into $S$-phase, following the release of an $N_2O$ mitotic arrest. The data are based on pulse labelling with $[^{3}H]$thymidine and autoradiography. The arrows represent points in the progression through $S$-phase at which cells were removed and fused with chick red cells. The main figure shows: the induction of DNA synthesis in chick nuclei in heterokaryons consisting of 1 HeLa and 1 chick nucleus for each of the 3 fusions with HeLa in early (1) mid (2) and later (3) $S$-phase respectively, as a function of time spent in the heterokaryon. The continuous lines represent autoradiographic scores of labelled red cell nuclei, following 15-min pulses of $[^{3}H]$thymidine at $0.2 \mu Ci/ml$ (heavy lines) or $5 \mu Ci/ml$ (light lines); also, the proportion of mononucleate HeLa cells which incorporated $[^{3}H]$thymidine ($0.2 \mu Ci/ml$) during a pulse of 15 min, in each fusion mixture as a function of time (dotted lines). △—□, fusion 1, % of red cell nuclei induced, $0.2 \mu Ci/ml$ pulse; ●—△, fusion 1, % of red cell nuclei induced, $5 \mu Ci/ml$ pulse; ○—□, fusion 2, $0.2 \mu Ci/ml$ pulse; ○—○, fusion 2, $5 \mu Ci/ml$ pulse; ■—■, fusion 3, $0.2 \mu Ci/ml$ pulse; □—□, fusion 3, $5 \mu Ci/ml$ pulse; △—△, % of HeLa mononucleates in $S$-phase in fusion mixture 1; ○—○, % of HeLa mononucleates in $S$-phase in fusion mixture 2; □—□, % of HeLa mononucleates in $S$-phase in fusion mixture 3.
followed by autoradiography. The graph in Fig. 3 (continuous line) shows the time of initiation of DNA synthesis in red cell nuclei in binucleate heterokaryons in each of the 3 fusions. Both the rates and the extent of induction of DNA synthesis of chick nuclei are very similar after fusion with HeLa in the 3 different regions of S-phase. However, the final proportion of chick nuclei induced into S-phase is somewhat lower in the third fusion (i.e. with HeLa cells in the latter part of S-phase).

In order to interpret this experiment further in relation to the inducing potential of the different regions of the HeLa S-phase, it is necessary to make a more detailed comparison between the 3 fusions in terms of the nature of the HeLa environment to which the red cell nuclei were exposed throughout the 8-h period of observation following each fusion. This is made possible since in each of the fusion mixtures there remains an unfused mononucleate population of HeLa cells, and the proportion of these cells in S-phase at several points in the experiment was determined by autoradiography (dotted lines in Fig. 3). The manipulations involved in cell fusion and plating perturb the advancement of HeLa cells through the cell cycle in such a way that the passage into S-phase is somewhat delayed. Thus, the unfused HeLa cells of the second and third fusion mixtures are relatively more advanced into S-phase than the cells of the first fusion since they were allowed to pass through greater proportions of S before fusion took place. Since in each fusion mixture the HeLa cells pass through S in a relatively synchronous manner, the proportion of the HeLa mononucleate population in S at any time gives an indication of the position within S-phase of those HeLa nuclei that are still synthesizing DNA in heterokaryons consisting of one HeLa and one chick element. The earliest and latest pulsed samples from each fusion show that, although the proportion of mononucleate HeLa cells in S-phase varies during the 8-h period following each fusion, the extent of induction of the chick nucleus in heterokaryons is similar in each case, with the slight reduction in the late S-phase environment. This suggests that the potential for initiation of DNA synthesis does not vary greatly throughout S.

Parallel pulse experiments with 5 µCi/ml of [3H]thymidine for the 3 fusions revealed little difference in the proportion of chick nuclei induced, except for the early time in the last fusion (Fig. 3). This suggests that the incidence of DNA synthesis in the chick nuclei is probably not, in general, obscured either by transport effects, by variations of precursor pool size, by pool specific activity, or by rates of DNA synthesis. After a 15-min pulse of [3H]thymidine relatively few (less than 10%) of the heterokaryons exhibit a labelling pattern in which the chick nucleus shows incorporation while the HeLa does not, and such cells are only seen in the later samples of the second and particularly the third fusions. The red cell nucleus can continue to synthesize DNA when the HeLa nucleus has entered G2 phase (Fig. 8), and even when the heterokaryon has entered mitosis (Fig. 9). Such cells have not been included in Fig. 3, which therefore represents only those heterokaryons containing a HeLa nucleus synthesizing DNA. In a number of heterokaryons, especially from the earliest samples of the first fusion and the latest of the third, it is likely that during the pulse the HeLa nucleus moved from G1 into S, and from S into G2, respectively. This might influence the final proportion of chick nuclei induced, although the short pulse length (15 min)
Table 3. Induction of DNA synthesis in chick red cell nuclei after fusion with HeLa in different parts of S-phase as a function of time spent in heterokaryons and the ratio of HeLa to chick nuclei

Chick nuclei induced at 3 and 7 h after fusion completed, %

<table>
<thead>
<tr>
<th>Heterokaryon class</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Early S fusion</td>
<td>39 (36)</td>
<td>25 (50)</td>
<td>75 (83)</td>
<td>67 (134)</td>
<td>58 (50)</td>
<td>41 (70)</td>
</tr>
<tr>
<td>Mid S fusion</td>
<td>43 (51)</td>
<td>37 (51)</td>
<td>75 (54)</td>
<td>78 (100)</td>
<td>71 (52)</td>
<td>66 (50)</td>
</tr>
<tr>
<td>Late S fusion</td>
<td>54 (22)</td>
<td>—</td>
<td>86 (23)</td>
<td>—</td>
<td>78 (45)</td>
<td>54 (13)</td>
</tr>
<tr>
<td></td>
<td>40 (20)</td>
<td>—</td>
<td>62 (32)</td>
<td>—</td>
<td>49 (33)</td>
<td>—</td>
</tr>
</tbody>
</table>

Fusions were carried out with populations of HeLa cells which had been released from mitotic arrest for 8 h (early S), 13 h (mid S) and 16 h (late S) respectively. The cells were incubated either in the continuous presence of [H]thymidine (0.2 μCi/ml), or were pulsed with [H]thymidine (0.2 μCi/ml) for 45 min. The figures in parentheses indicate the number of heterokaryons scored for each point.
should minimize this potential distortion in the observations based on autoradiography.

The results from this type of experiment allow us to conclude that the conditions for initiating DNA synthesis in a heterologous nucleus do not fluctuate markedly during the HeLa S-phase, although there may be some decrease in potential towards the end of this period.

Table 4. Incorporation of \[^\text{3}H\]thymidine into chick nuclei induced to synthesize DNA after fusion with HeLa cells in different regions of the S-phase

<table>
<thead>
<tr>
<th>Position in the cycle, in h after release from mitotic arrest, of HeLa cells used in the fusions</th>
<th>% of chick nuclei induced in heterokaryons containing 1 HeLa and 1 chick nucleus</th>
<th>Average number of grains over chick nucleus ± s.e. mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>48</td>
<td>7.5 ± 1.7</td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>5 ± 3.8</td>
</tr>
<tr>
<td>10</td>
<td>52</td>
<td>9 ± 1.6</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>12</td>
<td>62</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>13</td>
<td>44</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>14</td>
<td>48</td>
<td>13 ± 3</td>
</tr>
</tbody>
</table>

Fusions between HeLa cells in different regions of S-phase and 8-day chick red cells were carried out at various times after release from mitotic arrest. For each fusion the heterokaryons were plated out and incubated for 1 h before adding \[^\text{3}H\]thymidine (2 \(\mu\text{Ci/ml}\)) for 1 h. The proportion of chick nuclei induced into S phase was determined by scoring 200 heterokaryons; grain counts were made on 30 such cells in each case.

Examination of the induction of chick nuclei as a function of the ratio of HeLa to chick elements in heterokaryons produced by fusion of HeLa cells in early, mid and late S-phase with red cells indicates that the inducibility of chick nuclei is similar in heterokaryons consisting of 2 HeLa nuclei and 1 chick nucleus, regardless of the position in S-phase which the HeLa occupied at the time of fusion. This contrasts with the inducibility at ratios of 1:1 and 1:2 where late S-phase HeLa are somewhat less efficient in promoting induction (Table 3).

As to the amount of DNA synthesis which occurs in the chick nucleus in response to exposure to different regions of S-phase, it is clear from autoradiography that there is less incorporation in both the early and late S fusions than for mid S fusions (Table 4). This is true whatever the concentration of \[^\text{3}H\]thymidine used, but it need not indicate that the rate of DNA synthesis is different. Further studies are in progress to elucidate this point.

Analysis of the extent of DNA synthesis in red cell nuclei by examination of interphase chromosome morphology and Feulgen densitometry

Fusion between HeLa cells in different regions of the cell cycle and chick red cells may result in the initiation of different patterns of chromosomal DNA synthesis. Experiments to investigate this possibility were carried out by fusing red cells
Induction of DNA synthesis in red cell nuclei

separately with HeLa cells early in $G_1$, at the $G_1/S$ boundary, or in late $S$, using a similar protocol to that described above. After fusion, heterokaryons in mitosis were accumulated by nitrous oxide pressure arrest at the end of both the first and second cell cycles so that the state of the chick chromosomes at the first and second divisions could be examined. Typical mitotic figures obtained in this manner from heterokaryons are shown in Figs. 10–13. The HeLa nucleus completes its $S$-phase and passes through the cycle, entering mitosis at an approximately normal rate, but in so doing it accelerates ahead of the chick nucleus in the same heterokaryon with the

Table 5. The nature of chick PCC in HeLa-chick red cell heterokaryons at the 1st and 2nd mitoses after fusion with synchronized interphase HeLa cells

<table>
<thead>
<tr>
<th>Type of fusion</th>
<th>1st mitosis</th>
<th></th>
<th>2nd mitosis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$G_1$ PCC (Class I),</td>
<td>Partial</td>
<td>$G_1$ PCC (Class I),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>completion</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Class II),</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Incomplete</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>fragments</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Class III),</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>HeLa early $G_1 \times 8$-day red cells</td>
<td>3 (2)</td>
<td>13 (10)</td>
<td>84 (66)</td>
<td>—</td>
</tr>
<tr>
<td>HeLa $G_1/S$ boundary $\times 8$-day red cells</td>
<td>3 (3)</td>
<td>7 (7)</td>
<td>90 (90)</td>
<td>—</td>
</tr>
<tr>
<td>HeLa late $S$ $\times 8$-day red cells</td>
<td>18 (9)</td>
<td>8 (4)</td>
<td>74 (37)</td>
<td>—</td>
</tr>
</tbody>
</table>

The figures in parentheses indicate the number of metaphase preparations with chick PCC of the various categories.

result that the chick nucleus enters mitosis prematurely. The morphology of these prematurely condensed chromosomes (PCC) of the chick nucleus provides specific information about the position that they occupied in the cell cycle at the time the HeLa element entered mitosis (Johnson & Rao, 1970; Johnson, Rao & Hughes, 1970).

Examination of the chick PCC in these heterokaryons reveals a great variety of chromosomal morphologies but in no case has a fully replicated set of chick chromosomes been observed; the majority are much more reminiscent of $G_1$ chromosomes. Three categories of morphology can be distinguished, although there is considerable overlap between classes. The first class (I) is essentially $G_1$ in type, consisting of single chromatids (Fig. 10). The second (II) is a class showing partial replication and is composed of chromosomes with mixtures of $G_1$ and $G_2$-like regions (Fig. 12). Finally, there is the class of incomplete fragments (III), consisting of those PCC which cannot be classified as either $G_2$ or $G_1$-like, and are probably in $S$-phase (Fig. 11). These
morphologies can be compared with control $G_1$, $S$ and $G_2$ PCC produced from a proliferative population of 5-day red cells by fusion with mitotic cells (Figs. 14–16).

Comparison of the different categories of mitotic figures obtained from the three fusions with HeLa cells synchronized in different parts of the cell cycle (Table 5) shows that the greatest proportion of partially completed $G_2$ PCC (Class II) is produced after fusion with early $G_1$ HeLa, although in no case was a fully replicated,
of the incompleteness of the chick material in these cells, they have not been included in the tabulated data.

It has so far proved impossible to determine whether different patterns of replication of the chick chromosomes are induced after fusion with the HeLa cells in different regions of S-phase, mainly because the amount of [3H]thymidine incorporated into chick chromosomes is very small in the course of a short pulse. In addition, the chick

![Fig. 5. Feulgen-DNA values of 12-day chick red cell nuclei during the first and second cell cycles in heterokaryons formed after fusion with HeLa in different parts of the G1 phase and also with random HeLa cells. A, G1 HeLa (1 h after release of mitotic block) × 12-day red cells. Mean Feulgen-DNA value 3.8 (±0.4) at 1 h after fusion (upper figure) and 4.7 (±1.3), at 51 h after fusion (lower figure) (n = 20). B, random HeLa × 12-day red cells. Mean Feulgen-DNA value 4.2 (±0.6) at 1 h after fusion (upper figure) and 4.5 (±1.0) at 51 h after fusion (lower figure) (n = 20).](image)

chromosomes at the first mitosis after fusion are always in the form of PCC and, further, the PCC are generally a mixture of G1, S and G2 elements, thus making specific localization difficult.

The increase in amount of DNA in chick nuclei during the first cycle after fusion with HeLa cells in G1 or S-phase was monitored by Feulgen densitometry. The results are shown in Fig. 4. They demonstrate that, during exposure of red cell nuclei to a complete 8-h S-phase HeLa environment, there is only a slight increase in the Feulgen-DNA value of the red cell nuclei. This result was found with red cells taken
from eggs ranging between 8 and 19 days of development. Moreover, exposure of the red cell nuclei to the complete G1 HeLa environment in the heterokaryon, prior to the passage of the HeLa nucleus into S-phase, did not substantially change the DNA-Feulgen value. On the other hand, exposure of red cells to 2 HeLa S-phases after fusion led to slightly greater increase in DNA-Feulgen values; and, in this case, a few red cell nuclei approached a 2-fold increase in DNA-Feulgen value (Fig. 5). A similar result was obtained after fusion between red cells and random HeLa cells. These studies complement the work on chick chromosomes at the first and second mitoses after fusion, confirming that although chick nuclei are induced to synthesize DNA soon after fusion with HeLa cells, they rarely undergo a complete round of synthesis, even when exposed to 2 HeLa cell cycles.

**Localization of newly induced DNA synthesis in the chick nucleus**

The distribution of newly initiated DNA synthesis within the chick nucleus has been examined by autoradiographic localization of incorporated [3H]thymidine. In these experiments HeLa cells synchronized in early G1 were fused with 10- or 12-day red cells. At various times ranging between 3-5 and 8 h after fusion, 10- or 20-min pulses of [3H]thymidine at a concentration of 50 μCi/ml were given to 2 samples; one sample was chased for 10 min (or for 2 min in the case of the 10-day red cells) in 50 μM thymidine and fixed immediately, and the other was similarly chased for 30 min before incubation in normal medium for 3-5-5 h, and subsequent fixation.

Examination of electron-microscope autoradiographs of HeLa–chick heterokaryons showed that the distribution of silver grains over the chick nuclei followed a similar pattern, regardless of: the time after fusion at which the pulse was given; the length of the pulse; the length of the period of chasing and incubation between the end of the pulse and the time of fixation; the age of the red cells; and TCA extraction during the specimen preparation. In each case the majority of silver grains appeared to lie close to the boundaries of the condensed regions and were not concentrated at the nuclear envelope (Fig. 17).

These points are illustrated in Figs. 6 and 7, which show the distribution of silver grains in red cell nuclei in heterokaryons under various conditions. To test the hypothesis that the silver grains are associated with the boundaries of the condensed regions (i.e. a line source) the minimum perpendicular distance from the grain centre...
Induction of DNA synthesis in red cell nuclei

Fig. 6. For legend see opposite.
to the nearest condensed boundary was measured. The grain distributions were plotted as histograms, with distances from the boundary as abscissa (Fig. 6A–C). Each histogram was also replotted to obtain a theoretical grain-density function (smooth sigmoid curves, Fig. 6A–C) according to the equation provided by Salpeter et al. (1969), and Gupta et al. (1973). The integrated curves were plotted with a half-distance of resolution (H.D.) of 120 nm, a value chosen both by comparison with previous work (Salpeter et al. 1969; Gupta et al. 1973), and by assessing the goodness of fit of the theoretical curves and the histograms.

There is good fit between each of the grain distributions plotted as histograms and the theoretical grain densities derived from them. This implies that the radioactivity probably originates as a 'line' source or a narrow band (i.e. the boundary of the condensed and non-condensed nuclear regions). The similarity between the grain distributions obtained after pulse labelling at different times after fusion (Fig. 6A and B) suggests that there is little difference in the localization of induced DNA synthesis as a function of the time the chick nucleus spends in an S-phase heterokaryon. In the pulse-chase experiment (Fig. 6C) the localization of grains is still predominantly at the boundary of the condensed region although proportionately more label now appears in the condensed regions.

The relationship of the grain localization to the nuclear envelope is shown in Fig. 7, which is typical of each experiment. There is essentially no pattern of association with the nuclear envelope.

In presenting these data, we recognize their limitations, namely: (1) that the silver grains in some cases obscured their exact position relative to the boundaries between condensed and non-condensed regions of the nucleus; (2) that we have measured the distance between each silver grain and the nearest condensed boundary in a particular section, without regard to condensed regions in other parts of the nucleus outside the plane of the section; (3) that Fig. 6A–C represents relatively small numbers of chick nuclei (only a small proportion of red cells in heterokaryons are labelled in these autoradiographs, particularly in the earlier samples); (4) that the degree of self-absorption of radioactivity may differ substantially between the non-condensed and condensed (heavy-metal-enriched) regions of the chick nucleus. This might lead to substantially

Fig. 7. Histogram as in Fig. 6B, except that the position of each grain is expressed in terms of the distance between the grain centre and the nearest nuclear membrane.
greater concentrations of silver grains in the non-condensed areas (or even at the boundary) than in the condensed areas. The fact that the proportion of grains in the condensed regions is, in our experience, always greater than in the non-condensed, implies that the specific activity of isotope in the dense regions may be even higher than we can see; (5) that comparison of our observations with the distribution of radioactivity from a perfect line source does not allow us to distinguish whether the source of emission is a line or a narrow band; (6) that the centre of the silver grain produced by Phenidone development does not necessarily overlie the exact source of $\beta$-emission.

There was variation in any particular sample with respect to the number of grains over different red cell nuclei, but there appeared to be no consistent difference in the pattern of grain distribution in heavily as opposed to lightly labelled nuclei. We were also unable to observe any differences in fine structure between heavily and lightly labelled nuclei.

The induction of DNA synthesis in chick nuclei may be influenced to some extent by their position in the heterokaryon. There is a tendency for those chick nuclei closest to the HeLa nucleus to be induced at a higher frequency than those further away. Moreover, chick nuclei are sometimes seen in both light- and electron-microscope autoradiographs with grains confined to regions nearest the HeLa nucleus (Fig. 18); such chick nuclei always lie close to the HeLa nuclear membrane. In some sections the outer nuclear membranes of HeLa and chick nuclei are in continuity with each other (Figs. 17, 19). These observations suggest that not only might there be a gradient of cytoplasmic inducers in the heterokaryons but also, at least in some instances, a direct nucleus-to-nucleus interaction.

Movement of protein into the chick nucleus following fusion with synchronous HeLa cells

Entry of eukaryotic cells into $S$-phase is accompanied by a marked redistribution of protein between nucleus and cytoplasm such that cytoplasmic protein rapidly accumulates in the nucleus, which therefore grows disproportionately faster than the whole cell (Zetterberg, 1970). We have therefore examined whether HeLa cell protein moves rapidly into chick nuclei during the first few hours after fusion and whether this is restricted to fusions between red cells and $S$-phase HeLa.

The experiments consisted of fusions between red cells and synchronous HeLa cells in $G_1$ or early $S$-phase, followed by incubation of the heterokaryons in medium containing radioactive amino acids. After a period of incubation the cells were fixed and embedded in Araldite before 0.5-μm sections were cut for subsequent autoradiographic localization of label. Other experiments involved fusion of red cells with synchronous HeLa cells that had been prelabelled for various durations with radioactive amino acids. These heterokaryons were also incubated before fixation, embedding and sectioning.

The details of these experiments were as follows (see Table 6). (1) Heterokaryons formed by fusions between HeLa cells at the start of $G_1$ and either 7- or 14-day red cells were incubated in medium containing radioactive amino acids for a duration less than the average length of $G_1$-phase (5 h). This experiment shows that protein
synthesized in $G_1$ moves into the red cell nucleus during $G_1$. A control experiment carried out in the simultaneous presence of cycloheximide shows that the final labelling pattern represents protein, rather than adventitiously bound amino acids.

(2) HeLa cells that had been incubated for 5 h in $G_1$-phase in the presence of radioactive amino acids were fused at the start of $S$-phase with red cells. Some of the heterokaryons were allowed to move through the cycle unperturbed, while others were incubated in the presence of cycloheximide for an equivalent period before fixation. These experiments show that $G_1$ protein continues to move into chick nuclei when the HeLa element has itself moved into $S$-phase. Furthermore, the cycloheximide experiment shows that the perturbations introduced into the system by cell fusion did not stimulate radically different patterns of nuclear protein synthesis or exchange between nucleus and cytoplasm. (3) Heterokaryons formed by fusion between unlabelled HeLa cells at the start of $S$-phase and red cells were incubated in medium

Table 6. Migration of HeLa cell protein into chick red cell nuclei during the first cell cycle after fusion

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Age of embryonic red cell population, days</th>
<th>Type of fusion</th>
<th>$^3$H-amino acid incubation schedule for HeLa</th>
<th>Heterokaryons</th>
<th>Red cell nuclei labelled in heterokaryons, % and (no. counted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (a)</td>
<td>7</td>
<td>Red cells x early $G_1$ HeLa</td>
<td>Labelled for 5 h after fusion i.e. during 'HeLa' $G_1$-phase</td>
<td></td>
<td>91 (193)</td>
</tr>
<tr>
<td>(b)</td>
<td>14</td>
<td>Same expt. as 1 (a)</td>
<td>Heterokaryons fixed before HeLa element moved into $S$</td>
<td></td>
<td>84 (100)</td>
</tr>
<tr>
<td>2 (a)</td>
<td>7</td>
<td>Red cells x early $S$ HeLa</td>
<td>Labelled for 5 h in $G_1$</td>
<td>Incubated for 5 h after fusion in the absence of label and then fixed</td>
<td>76 (101)</td>
</tr>
<tr>
<td>(b)</td>
<td>14</td>
<td>Same expt. as 2 (a)</td>
<td></td>
<td></td>
<td>54 (98)</td>
</tr>
<tr>
<td>(c)</td>
<td>7</td>
<td>Red cells x early $S$ HeLa</td>
<td>Labelled for 5 h in $G_1$</td>
<td>Incubated for 5 h in cycloheximide after fusion in the absence of label and then fixed</td>
<td>84 (58)</td>
</tr>
<tr>
<td>(d)</td>
<td>14</td>
<td>Same expt. as 2 (c)</td>
<td></td>
<td></td>
<td>61 (36)</td>
</tr>
<tr>
<td>3 (a)</td>
<td>7</td>
<td>Red cells x early $S$ HeLa</td>
<td>Labelled for 5 h after fusion, i.e. during 'HeLa' $S$-phase only and then fixed</td>
<td></td>
<td>82 (82)</td>
</tr>
<tr>
<td>(b)</td>
<td>14</td>
<td>Red cells x early $S$ HeLa</td>
<td></td>
<td></td>
<td>79 (48)</td>
</tr>
</tbody>
</table>

Details of the mixture of $^3$H-amino acids used in these experiments is given in Materials and methods. Parallel experiments to 1 (a), (b), and 3 (a), (b) were carried out in the simultaneous presence of cycloheximide (25 µg/ml) and $^3$H-amino acids. No radioactivity was found in the fused or unfused cells.
Induction of DNA synthesis in red cell nuclei 475

containing radioactive amino acids. These experiments show that protein synthesized exclusively during the S-phase also migrates into chick red cell nuclei.

We have therefore demonstrated that protein synthesized both during G1 and S-phase migrates into the majority of red cell nuclei after fusion, and that this is true for both 7- and 14-day red cells. The experiments do not attempt to identify the nature of the migratory proteins.

The ability of proteins synthesized during different regions of the G1-phase to migrate into chick nuclei was examined in a similar manner. Table 7 presents the data for migratory proteins synthesized during the first 3 h of G1 versus the next 3-h period, and demonstrates that proteins synthesized during the 3rd to 6th hour of G1 subsequently migrate into chick nuclei more efficiently, or are retained there more effectively than those synthesized earlier in the G1 phase.

Table 7. Protein migration into 8-day chick red cell nuclei following fusion with synchronized HeLa cells, prelabelled in different parts of G1

<table>
<thead>
<tr>
<th>Chick nuclei labelled, %</th>
<th>3 h after fusion</th>
<th>6 h after fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa prelabelled for 3 h in early G1 before fusion</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>HeLa prelabelled during the 3rd to 6th hour after mitotic reversal before fusion</td>
<td>50</td>
<td>61</td>
</tr>
</tbody>
</table>

The mixture of ³H-amino acids consisted of [³H]leucine, [³H]tryptophan and [³H]arginine, each at 2 μCi/ml; 100 heterokaryons were scored for each point. Both experiments were also carried out in the presence of cycloheximide (25 μg/ml), added during the fusion and continuously present for up to 6 h. No significant differences in the numbers of red cell nuclei labelled were found.

DISCUSSION

A number of studies have been made of the heterokaryons which result from fusion between actively proliferating cells and chick red cells (Harris, 1965, 1967; Schneeberger & Harris, 1966; Johnson & Harris, 1969; Bolund et al. 1969; Bolund, Darzynkiewcz & Ringertz, 1969; Gordon & Cohn, 1971; Ringertz, Carlsson, Ege & Bolund, 1971; Ege, Carlsson & Ringertz, 1971; Darzynkiewicz, Chelmicka-Szorc & Arnason, 1974 a, b). In this paper we have examined some of the short-term interactions that are associated with the initiation of DNA synthesis during the first cycle after fusion between synchronous HeLa cells and chick red cells of different ages. These studies have a bearing on the production of proliferating hybrids between an active cell and a chick red cell, since the genetic constitution of such hybrids is probably largely determined by the events which occur during the first cell cycle after fusion (Schwartz, Cook & Harris, 1971; Boyd & Harris, 1973; Kao, 1973; Klinger & Shin, 1974).

Fusions between synchronous populations of cells allow us to examine the inter-
actions between specific phases of the cell cycle (Rao & Johnson, 1970). In the case
of the HeLa-chick fusions, such information is important if we are to understand the
mechanisms which reprogramme the chick nucleus in the heterokaryon. Moreover,
they provide information both about how the quiescent chick nucleus responds to and
is activated by different phases of a proliferative cell cycle and conversely about the
nature of the different phases of the HeLa cell cycle, in terms of the response of the
chick nucleus.

The induction of semiconservative DNA synthesis in the chick nucleus is a specific
response to the S-phase environment provided by the HeLa cell, whilst RNA synthesis
is induced after fusion with cells in each phase (Harris, 1967). Once DNA synthesis
has been initiated by an S-phase environment, however, it can continue in the
heterokaryon even though the cellular environment is G₂ or mitotic in type. As
judged by grain counts, these chick nuclei incorporate as much [³H]thymidine as they
do during a pulse of comparable duration in the presence of a HeLa S-phase nucleus.
These observations suggest that the conditions for the support of DNA synthesis
continue to exist beyond S-phase, thus confirming the previous work of Johnson &
Harris (1969a).

DNA synthesis occurs very rarely in red cell nuclei in heterokaryons which also
contain HeLa nuclei in both G₁ and S-phase. This suggests that the G₁ HeLa nucleus
in some manner prevents the red cell nucleus from entering S-phase, and is remi-
niscent of the competition observed between HeLa and Ehrlich ascites nuclei in hetero-
karyons (Johnson & Harris, 1969b). The finding that red cell nuclei can synthesize
DNA while the HeLa nucleus is in G₂ suggests that in this situation there is less
competition among the nuclei of the heterokaryon.

The speed of initiation of DNA synthesis in S-phase red cell fusions is related to the
ratio of HeLa to chick nuclei present, and the proportion of chick nuclei induced
during the 7-h period of exposure to the HeLa S-phase is highest in heterokaryons
with high ratios of human to chick elements. These observations provide a means of
measuring the factors in the HeLa cell which are responsible for the initiation of DNA
synthesis, as in the case of HeLa-Ehrlich ascites heterokaryons and heterophasic
HeLa homokaryons, in which a similar dosage effect has been described (Johnson &
Harris, 1969b; Rao & Johnson, 1970). A similar measurement of factors involved in
stimulating RNA synthesis in chick nuclei in heterokaryons has been observed by
Ringertz, Carlsson & Savage (1972) and Darzynkiewicz et al. (1974b), who report that
the degree of enlargement of chick nuclei after fusion is related to the ratio of nuclei
present: the lower the proportion of chick nuclei in the heterokaryon the greater is
their nuclear enlargement.

The position of the chick nucleus with respect to the HeLa nucleus in the hetero-
karyon may influence the activation and induction of DNA and RNA synthesis
(Holmes & Porter, 1971). In this paper we describe the continuity between the outer
nuclear membranes of the HeLa and chick nucleus, and the observation that those
nuclei lying closest to the HeLa nucleus are most frequently induced. These observa-
tions support the hypothesis that there may be a direct nucleus-to-nucleus transfer of
material (Ringertz et al. 1972). It should be noted, however, that the cytoplasm of
Induction of DNA synthesis in red cell nuclei

active cells can alone stimulate limited RNA and DNA synthesis, as in the case of chick red cell and rabbit macrophage nuclei respectively after fusion with cytochalasin B-enucleated cells (Ladda & Estensen, 1970; Poste & Reeve, 1972).

Little is known about the mechanisms which control the initiation of DNA replication during the cell cycle. However, it seems likely that events which occur during $G_1$ are involved with this control (Temin, 1971; Smith & Martin, 1973; Pardee, 1974). The nature of the underlying molecular changes that take place during the $G_1$ phase is poorly understood; neither do we understand the modulations of structure and function which are undertaken by the cell in preparation for DNA synthesis. Such preparations might, however, include the modification of the $G_1$ chromosome so that the DNA template becomes accessible for semiconservative DNA synthesis (Gurley, Walters & Tobey, 1974; Schor, Johnson & Waldren, 1975). ‘Priming’ the red cell nuclei in $G_1$ before exposure to the factors involved in initiating DNA replication results in a greater proportion being induced into DNA synthesis, although no corresponding increase in the amount of DNA replicated has been measured. The question remains as to whether the $G_1$ exposure results in specific events which lead the red cell nucleus more naturally into DNA synthesis, or whether it represents a general activation of the quiescent nucleus, ensuring that the template is made more accessible for the processes of DNA replication. We have shown that $G_1$ protein migrates into the red cell nucleus during the $G_1$ phase in heterokaryons, and also find considerable changes in morphology of the chick nucleus after fusion with both $G_1$ and $S$ cells (in preparation). It is possible that the mechanism of priming which occurs during exposure to $G_1$ resembles the proteolytic activation described by Darzynkiewicz et al. (1974a). The lag before DNA synthesis is induced in red cells after fusion with $S$-phase HeLa may represent a period during which the quiescent template is made available; a similar lag has also been described previously by Gordon & Cohn (1970, 1971a, b) for macrophage activation.

The speed of induction is related not only to the ratio of HeLa to chick elements in the cell but also to the age of embryo from which the red cells are taken. Thus, in a given time after fusion with HeLa cells in early $S$-phase, the proportion of red cell nuclei induced is highest in those cells which most recently arrived in the quiescent state. This is presumably related to the physicochemical state of the chick chromatin in these cells. Little is known of the mechanism whereby the proliferating erythroblast enters the quiescent state and undergoes maturation into the erythrocyte, but the changes that occur in the nucleus are striking and well documented. The nucleus undergoes a series of biochemical and morphological changes, the best illustrated being the loss of nuclear protein and protein phosphokinase (Dingman & Sporn, 1964; Gershey & Kleinsmith, 1969); an accumulation of a specific histone fraction (Edwards & Hnilica, 1968); a decrease in the frequency of nuclear pores (quoted in Franke, 1974; our unpublished data); the condensation of chromatin into dense nuclear bodies and a reduction in the rate of RNA synthesis (Davies, 1968; Cameron & Prescott, 1963; Madgwick, Maclean & Baynes, 1972); a reduction in the ability of the chromatin to intercalate dyes such as acridine and ethidium and a decreased sensitivity to thermal denaturation of the chromatin (Kernell, Bolund & Ringertz, 1971). These changes can
be reversed, the most carefully studied example being in HeLa–chick red cell hetero-
karyons (Bolund et al. 1969a, b), although some of these phenomena are reversible by
incubation of the cells in buffered solutions without serum (Ringertz & Bolund, 1969).
Our data show that the further the erythroblast has progressed towards the mature
erthrocyte state, the greater is the difficulty in reactivating it into DNA synthesis.
Similarly, in heterokaryons with low ratios of HeLa to chick nuclei those factors
which produce a reversal in the red cell maturation process are considerably diluted,
and thus their effects are not observed until considerably later.

HeLa cells in different regions of S-phase, with the exception of those in late S,
show similar potential as inducers of DNA synthesis in red cell nuclei. In late S-phase
there is a reduction in the induction potential in heterokaryons consisting of one HeLa
and one red cell element. This is in agreement with the findings of Gordon & Cohn
(1971a) who examined the induction potential of melanocytes in late S-phase in
heterokaryons of melanocyte-peritoneal macrophage parentage. At ratios of HeLa to
chick nuclei greater than 1:1, however, the proportion of chick nuclei induced after
fusion with HeLa cells in late S was as high as in 1:1 heterokaryons derived from
fusions that occurred earlier in the S-phase (Table 3).

In contrast to Bolund et al. (1969), we do not find in heterokaryons a large propor-
tion of red cell nuclei with a 4C DNA content at the end of the second cycle, despite
the fact that we fused cells in synchronous phase and that this might be expected to
promote the development of internal nuclear synchrony. There is little difference in
the amounts of DNA synthesized by red cell nuclei of different ages (8-day versus
15-day) over this period, although the speed of induction into S-phase is clearly
related to the age of embryo from which the red cells were taken. During the first cell
cycle after fusion less than 10% of the chick genome is replicated, regardless of
whether the fusion partner was in early \( G_1 \) or early S. Small differences in DNA
values would, however, not be measurable because of the Feulgen artifact.

The induced DNA synthesis in chick red cell nuclei is apparently concentrated at
the boundary between condensed and non-condensed areas, though there appears to
be a preferential localization of \(^{3}H\)thymidine within the condensed regions. This
distribution closely parallels the pattern of DNA synthesis revealed by pulse-labelling
experiments with a number of other cell types such as mouse P815 cells (Fakan &
Hancock, 1974), lymphocytes stimulated by phytohaemagglutinin (Milner, 1969) and
regenerating limb bud cells in amphibia (Hay & Revel, 1963). In all these cases, a
relatively large proportion of the nuclear volume is occupied by condensed chromatin.
In chick red cells, the position of newly replicated DNA does not change substantially
during a subsequent incubation period of up to 4 h. This confirms the positional
stability of DNA which has previously been described in other systems such as
Chinese hamster fibroblasts and KB cells, where DNA synthesis occurs in the non-
condensed regions of the nucleus (Blondel, 1968; Williams & Ockey, 1970).

Examination of chick chromosomes at the first mitosis after fusion confirmed the
observation, based on Feulgen densitometry, that only a small proportion of the chick
DNA is replicated during the first cycle. No complete set of mitotic chick chromo-
somes was found at the first mitosis, although there was some evidence that more
normal chromosomes were obtained either when the red cells were taken from the
youngest embryos (e.g. 7 days) or when they were fused with HeLa in early $G_1$, rather
than in early or late $S$. In all these heterokaryons, the HeLa element advances through
the cycle at a normal rate, and ahead of the red cell nucleus which is consequently not
permitted to complete its own round of DNA replication. The incomplete and
prematurely condensed chick chromosomes which appear at the first mitosis demon-
strate this point. The majority of these partially completed sets of chick chromosomes
are probably lost from the fused cells within the first few cycles. No complete $G_2$
chick PCC have been found in the fused population at the second mitosis. These
observations help to explain why complete, identifiable chick chromosomes have not
been discovered in hybrids formed between actively proliferating mouse 3T3 cells or
3T3, or Chinese hamster Don cells and red cells (Schwartz et al. 1971; Boyd & Harris,
1973; Klinger & Shin, 1974). (In these studies chick genetic material is clearly
integrated into the genome of the cell and is functional as determined by enzymic
criteria. Klinger & Shin also found a few fragments of chick chromosomal material.)
The generation of gross internal asynchrony within heterokaryons of this type when
the red cell is not allowed to activate rapidly enough to accompany the other nucleus
leads to premature chromosome condensation and subsequent elimination of these
elements (Rao & Johnson, 1972). Only when the active partner is arrested in some
manner before the first mitosis can the chick nucleus pass into division with the host
nucleus in a normal manner. In such cases (Kao, 1973), complete sets of chick
chromosomes are found in the first hybrid mitoses. Very occasionally the HeLa
element may pass through mitosis without inducing the lagging chick nucleus into
PCC. In these cells the chick nucleus segregates into one of the daughter cells (Johnson
& Rao, 1972). It might be supposed that in such instances, where there is no destruc-
tive induction of PCC at the first division, the chick nucleus might pass into the second
cycle competent to duplicate its entire genome.

A number of studies have shown that activation of the chick red cell nucleus in
heterokaryons is accompanied by an influx of HeLa cell protein (Ringertz et al. 1971;
Appels et al. 1974). Moreover, much of the protein that accumulates in the chick
nucleus becomes associated with morphologically defined regions in the nucleus, as
demonstrated by immune microfluorimetry (Ringertz et al. 1971; Ege et al. 1971).
There is little information about the functions of the numerous proteins which enter
the chick nucleus after fusion, although it is known that the F2C chick histone is
replaced by human F1 histone (Appels et al. 1974). Some of the human proteins may
be enzymes which specifically act at the level of the DNA template, such as those
involved in excision repair of DNA (Darzynkiewicz, 1971). The extent to which the
proteins constitute the signals for general activation of the red cell genome is still
obscure. However, the red cell nucleus, in comparison with actively dividing chick
cells, is poor in non-histone nuclear proteins (Dingman & Sporn, 1964) and the rapid
replacement of such material, albeit from a heterologous cell, may underlie a general
mechanism of activation which is followed by specific regulation of transcription and
replication. The question still remains as to whether the reactivation of the chick
nucleus into DNA synthesis is wholly operated by human polymerases or is
dependent on endogenous chick enzymes which are activated after fusion. Endogenous chick RNA polymerase is presumably present since it has been demonstrated in adult hen erythrocytes (Scheintaub & Fiel, 1973).

In our experiments the incorporation of radioactive amino acids into red cell nuclei, as detected by autoradiography, may be considered to represent uptake of radioactive HeLa cell protein since it is completely suppressed by cycloheximide. Moreover, prelabelling of the HeLa cell before fusion followed by exposure to cycloheximide immediately after fusion still results in the uptake of radioactivity into the red cell nuclei even though virtually all protein synthesis has been stopped. This finding implies that the red cell nuclei are labelled by the transfer of radioactive protein from the HeLa cell partner of the heterokaryon. In terms of the cell cycle, therefore, we now know that proteins synthesized during the $G_1$ and $S$-phase migrate into the chick nucleus very rapidly after fusion is completed. There appear to be differences in the migratory ability of proteins synthesized during the first 3 h of $G_1$, as compared with the subsequent 3-h period. The uptake of protein in this system closely resembles that described for somatic nuclei injected into the cytoplasm of unfertilized eggs of *Xenopus laevis*. In this case the nuclei are induced to synthesize DNA, and simultaneously take up a considerable amount of protein from the cytoplasm (Arms, 1968; Merriam, 1969). These experimental systems may provide information about the nature of $G_1$ versus $S$ cytonucleoproteins and may also allow us to determine whether there is a causal relationship between migration of proteins and the induction of DNA synthesis.

We gratefully acknowledge the technical assistance of Mrs A. Welberry-Smith and Miss M. Duller. We also wish to express our thanks to Dr S. L. Schor for discussion, to Dr B. L. Gupta for discussion and help with the electron-microscope autoradiography and to the Huntingdon Research Centre for the use of their scanning integrating densitometer. This work was supported by the Medical Research Council and the Cancer Research Campaign. R.T.J. is a Research Fellow of the Cancer Research Campaign and gratefully thanks Peterhouse, Cambridge for a Research Fellowship.

REFERENCES


Induction of DNA synthesis in red cell nuclei


Induction of DNA synthesis in red cell nuclei


(Received 30 January 1975)
Figs. 8, 9. Localization of silver grains over one chick red cell nucleus in the absence of incorporation into the HeLa nucleus of the same heterokaryon. The heterokaryon was one of a population produced by fusion between 8-day red cells and HeLa cells in late $S$ (i.e. 14 h after the release of a mitotic arrest). A 15-min pulse of $[^{3}H]$thymidine at 5 $\mu$Ci/ml was given 8 h after fusion was complete. The scales represent 10 $\mu$m.

Fig. 8. HeLa nucleus in $G_1$.

Fig. 9. HeLa in mitosis.

Figs. 10–13. Chromosome preparations of HeLa/chick red cell heterokaryons at the first and second mitoses after fusion. The scales represent 10 $\mu$m.

Fig. 10. Autoradiograph of heterokaryon formed between 9-day red cells and late $S$-phase HeLa at the first mitosis after fusion, showing the $G_1$-like chick PCC (Class I). No incorporation of $[^{3}H]$thymidine (15-min pulse) can be detected in the chick PCC.

Fig. 11. Heterokaryon formed between 9-day red cells and early $S$-phase HeLa at the first mitosis after fusion, showing the heterogeneous and possibly $S$-like nature of the chick PCC (Class III).

Fig. 12. Heterokaryon formed between 9-day red cells and early $G_1$-phase HeLa, showing the partial replication of the chick chromosomes (Class II). Some $G_2$ elements are visible in the chick PCC (arrows). First mitosis.

Fig. 13. Heterokaryon formed between early $S$-phase HeLa and 9-day red cells, showing the fragmentary and incomplete nature of the chick PCC at the second mitosis after fusion.
Induction of DNA synthesis in red cell nuclei
Figs. 14–16. Chick PCC in chromosome preparations of heterokaryons formed between mitotic HeLa and 5-day chick red cells. The scales represent 10 μm.

Fig. 14. $G_1$ PCC – monovalent chromosomes.
Fig. 15. $S$ PCC – heterogeneous elements.
Fig. 16. $G_4$ PCC – bivalent chromosomes.
Figs. 17, 18. Autoradiographs of heterokaryons formed by fusion between 12-day red cells and early G1 HeLa. At 8 h after fusion, the heterokaryons were pulsed for 10 min with [3H]thymidine at 50 μCi/ml and chased with excess thymidine for 10 min before fixation. Autoradiographs were developed in Microdol X after gold latensification.

Fig. 17. The silver grains are concentrated at the boundaries of the condensed areas in the red cell nucleus (r). The outer nuclear membrane of the chick nucleus is in continuity (arrow) with the outer membrane of a HeLa nucleus. n, HeLa nucleolus.

Fig. 18. The silver grains are localized at the boundaries of the condensed areas in the red cell nucleus (r); they are also situated only in the regions of the chick nucleus lying closest to the HeLa nucleus.
Induction of DNA synthesis in red cell nuclei
Fig. 19. Autoradiograph as in Figs. 17 and 18, except that Phenidone developer was used. The outer nuclear membranes of the chick and HeLa nuclei are in continuity (arrow). r, red cell nucleus.

Fig. 20. Protein migration in HeLa-chick red cell heterokaryons. Autoradiograph of 0.5-μm section showing silver grains over a red cell nucleus (arrow). HeLa cells were synchronized in early G₁-phase and incubated for 5 h in the presence of a mixture of ³H-amino acids (details in Materials and methods) before fusion with 14-day chick red cells. The heterokaryons were incubated for a further 5 h in the absence of ³H-amino acids but in the presence of cycloheximide. Viewed by Normarski differential interference microscopy. rc, unfused red cells.