DNA SYNTHETIC PATTERN IN THE NUCLEOLUS

I. Chicken Fibroblasts

SIBDAS GHOSH, RENATE LETTRÉ, and IRA GHOSH. From the Institute for Experimental Cancer Research, German Cancer Research Centre, 69 Heidelberg, Western Germany. Dr. Sibdas Ghosh's permanent address is Department of Botany, Calcutta University, Calcutta-19, India. Reprint requests and inquiries should be addressed to Dr. Lettré

INTRODUCTION

No definite information exists on the time of synthesis of desoxyribonucleic acid (DNA) in nucleoli during the S phase. The few reports in the literature are not in agreement. Harris (3) concluded from his results with thymidine-^{3}H that in rat fibroblasts DNA synthesis is initiated in the nucleolus. Ribas-Mundo (10) reported that DNA synthesis in human leukocytes occurs in the nucleolus-associated chromatin during the late S phase. In synchronized human tumor cells (strain CMP), Kasten and Strasser (5) observed two distinct periods of DNA synthesis in nucleoli: one period was at the beginning of the S phase, the other at about the middle of the second half of the S phase.

In one of our previous publications (2), we reported thymidine-^{3}H incorporation after a short pulse labeling in only one of the two nucleoli of chicken fibroblasts. Since there is no general agreement on the DNA synthesis pattern of nucleoli, we felt that further investigations should be undertaken. Our first aim was to determine the length of the S phase by observing the time between the first and the last appearance of a labeled cell in mitosis. The observation of the time between the end of incubation with thymidine-^{3}H and the appearance of prophase cells with labeled nucleoli allows us to decide whether the nucleolus synthesizes DNA at the beginning of the S phase or at its end. In addition, the observation of the labeling pattern in interphase nuclei was undertaken to provide information as to whether labeling of only one of two nucleoli might be observed with considerable frequency.

MATERIAL AND METHODS

As study material, we used chicken fibroblasts cultured in thin clots. Most of the cultures were incubated for 10 min in a medium containing thymidine-^{3}H (The Radiochemical Centre, Amersham, England, SA 5 Ci/M) at a final concentration of 1 μCi/ml. In some experiments, a 30 min pulse was used. After the pulse period, the cultures were promptly transferred to a medium containing non-radioactive thymidine at 40 times the concentration, and were kept there for another 10 min. The cultures were then washed in Hanks' balanced salt solution, and placed in fresh medium for different lengths of time. Some cultures were fixed every ½ hr, beginning at the 2nd to the 18th hr after incubation with the radioactive medium. In a second set of similar experiments, some cultures were fixed every 15 min during the periods of 3½–5 hr and 10–11½ hr after treatment with radioactive thymidine. For each experiment four cultures were used. The cultures were fixed in Serra's solution (Abs. alcohol:formalin:acetic acid 30:15:1). Ilford K.2 nuclear track emulsion was used for coating the cultures for radioautography. The preparations were developed after 6 wk and stained with hemalum.

OBSERVATIONS

The interphase nuclei which had incorporated thymidine-^{3}H showed the three following main

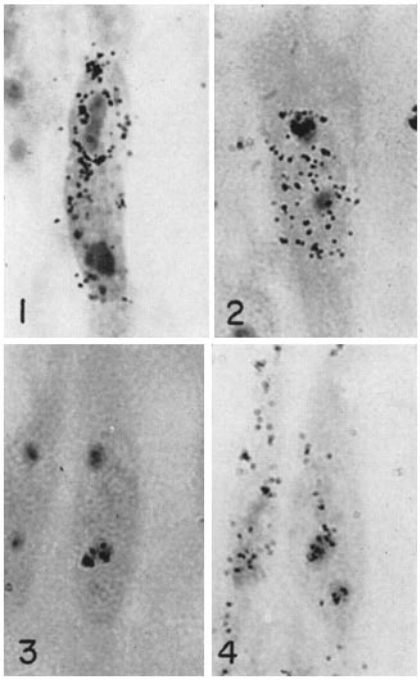

FIGURE 1 Labeling exclusively over the extranucleolar chromatin.

FIGURE 2 Labeling over both nucleoli and extranucleolar chromatin; only one of two nucleoli is labeled.

FIGURE 3 Only one of two nucleoli is labeled. No labeling in extranucleolar chromatin.

FIGURE 4 In rare cases both nucleoli are labeled, whereas extranucleolar chromatin is unlabeled.

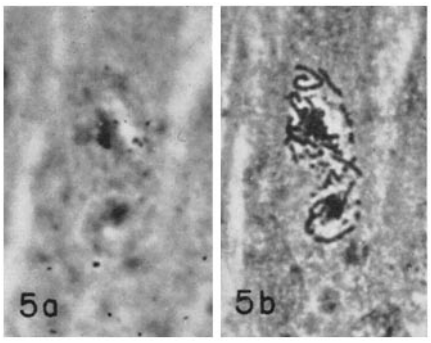

FIGURE 5 *a*, *b* Cell in prophase (first wave); labeling over only one of two nucleoli. *a*, Silver grains focused. *b*, Cell focused.

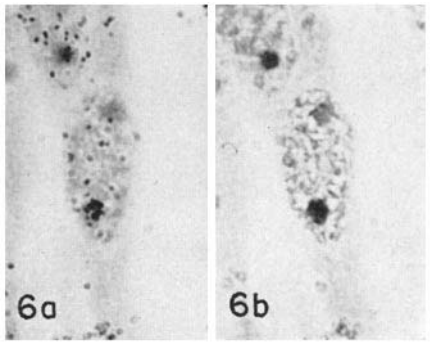

FIGURE 6 *a*, *b* Cell in prophase (second wave); only one of two nucleoli is labeled. *a*, Silver grains focused. *b*, Cell focused.

patterns of labeling (observations on 188 cultures). *Pattern 1:* most of these nuclei had their labeling exclusively over the extranucleolar chromatin (Fig. 1). *Pattern 2:* a low percentage of these nuclei exhibited labeling over both extra- and intranucleolar chromatin. In nuclei with two nucleoli, only one nucleolus was labeled (Fig. 2). *Pattern 3:* labeling here was confined mainly or exclusively to the nucleolus (in 1,000 cells in which counts were made pattern 3 was found in about 4% of labeled cells). Again, most of the nuclei revealed labeling in only one nucleolus if two were present (Fig. 3). In rare cases, however, labeling over both nucleoli was observed (Fig. 4).

The first labeled mitosis was detected 3½ hr after incubation with radioactive thymidine. The percentage of labeled mitotic cells in relation to the total number of cells in mitosis increased steadily up to 6 hr, when it reached 90%, and remained more or less constant for 4½ more hours. Thereafter, the percentage decreased. After 14½ hr, only 10% of the cells in mitosis were labeled. In the subsequent period, a slight increase occurred. For details, see Fig. 7 (about 100 mitotic cells were examined for each evaluation).

Some prophase cells with one labeled nucleolus became evident at 4:15 and 4:30 hr (Fig. 5 *a*, *b*). At 5:00 and 5:30 hr, only a few cells in prophase with labeled nucleoli appeared. In some of these prophase cells, extranucleolar chromatin was also labeled. From the 6th to the 10th hr, the prophase cells were devoid of nucleolar labeling. At 10:15 hr, however, a second wave of prophase cells with one labeled nucleolus could be seen. Such cells could be observed usually up to only 10:30 hr (Fig. 6 *a*, *b*). In one or two instances, however, prophase cells with labeled nucleoli were found at 11:30 hr, but they were completely absent in the subsequent hours.

DISCUSSION

In different cell types investigated so far, redundancy of the information for ribosomal RNA has been established (12, 16). In some organisms, e.g. *Xenopus* and *Drosophila*, containing 1,600 and 130 cistrons, respectively, per genome, mutation experiments gave evidence that all cistrons are accumulated on one chromosome of the genome (13, 16). In other organisms, e.g. rat (8), man, and Chinese hamster (see 4, 14), cistrons coding for rRNA are found to be located on several chromosomes. Our investigations presented here were performed on chicken cells, in which the information for the production of rRNA has also been reported to be localized on one chromosome of the genome (17). This statement is in agreement with our own observations which speak in favor of the assumption that one of the macrochromosomes carries the information for rRNA which, according to Ritossa et al. (12), consists of 100 cistrons per genome.

The location of cistrons, coding for rRNA on one site of only one chromosome of the genome, facilitates the interpretation of asynchrony of the labeling of rDNA: when we observe two nucleoli in a diploid chicken cell, we can feel sure that in each nucleolus only one nucleolus-producing region is present; when we find only one nucleolus in a diploid cell, a fusion of both nucleoli must have taken place and there must be two producing regions, as shown by Weissenfels (17). Labeling over such a nucleolus does not permit us to determine whether DNA synthesis has taken place in only one nucleolus-organizing region or both. It should be mentioned that the photographs presented in the three papers cited (3, 5, 10) showed labeling mostly over two nucleoli of a cell. The cells used for these experiments (3, 5, 10), have a large number of chromosomes which have nucleolus-organizing regions. However, cells with a large number of nucleolus-organizing sites may not always show corresponding numbers of nucleoli. The mechanism by which the reduction of the number of nucleoli seems to be most frequently effected, is the fusion of nucleoli. This can easily be observed in living cells, as well as the "division" of a big nucleolus into smaller ones. Another interpretation of the possible mechanism of the reduction of the number of the nucleoli will be discussed later. In agreement with the observations of the previously mentioned authors (3, 5, 10), the labeling pattern of the interphase nuclei of chicken cells indicates that DNA synthesis in the nucleolus takes place at a different time from that in the extranucleolar chromatin. Since about 4% of all labeled cells show labeling exclusively or mainly over the nucleolus, it may be assumed that DNA synthesis in the nucleolus proceeds only for a period covering not more than 4% of the total length of the S phase.

From the results plotted in Fig. 7, it may be concluded that in cultures of chicken fibroblast cells about 90% of the cells have an S phase of approximately 11 hr and a G_2 phase of 3½ hr. The results of Firket and Verley (1), reporting, for the same material, a G_2 phase of about 4 hr, are in good agreement with our data. The second peak in the curve of Fig. 7 indicates that about 10% of the cells differ in the duration of their cell cycle from the majority of the cells. Further investigations will be needed to clarify whether the longer cell cycle might be related to a certain percentage of tetraploid cells.

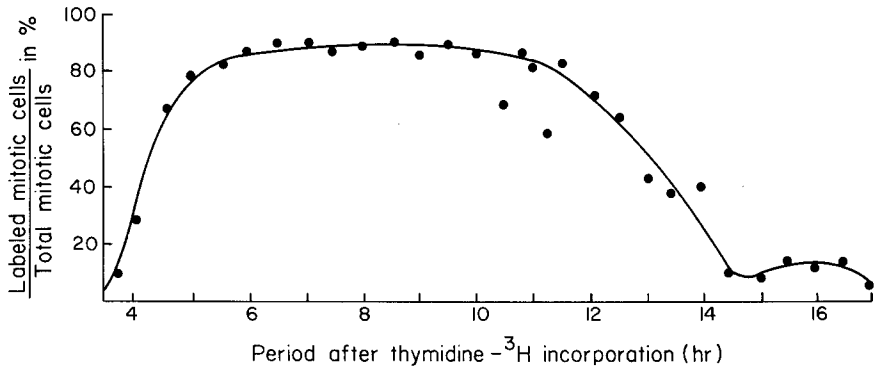

FIGURE 7 Diagram: percentage of labeled mitotic cells plotted against time after incubation.

Prophase cells with labeled nucleoli appeared twice (at 4:15 and 5:00 hr and 10:15 and 10:30 hr) during the 11 hr period after incorporation of radioactive thymidine, ranging from 3:30 to 14:30 hr. As this 11 hr period corresponds to the S phase in the cell cycle, it can be concluded that DNA synthesis in the nucleoli takes place twice in the S phase: once at about the 4th hr and later at about the 10th hr. Kasten and Strasser (5) also reported that DNA synthesis in the nucleolus occurs at two different periods in the S phase of synchronized human tumor cells, strain CMP. These authors believe that the early replicating "intranucleolar" chromatin corresponds to the nucleolar DNA, which acts as a template for rRNA, since during the first period of nucleolar DNA synthesis they observed a complete cessation of rRNA synthesis and a strong repression of all RNA synthesis. The authors are inclined to regard the late-replicating chromatin fraction (5, p. 139) "as genetically inactive or repressed (heterochromatin) and to be derived from the nucleolar organizer, since there are no significant RNA renewal changes in the nucleolus during its period of replication." (It should perhaps be mentioned that the term "nucleolar organizer" in the classical terminology means a heterochromatic knob, adjacent to the nucleolus (see reference 15, p. 133; for the modern use of the term, see National Cancer Institute Monograph, 1966, Vol. 23, pp. 145, 183, 446). Inactivity of part of the genes which carry the information for rRNA has often been discussed, both on the biochemical and morphological levels, by several authors (5, 7, 9, 11). It seems possible that a temporary repression (heterochromatization), i.e. shifts from the euchromatic state to the heterochromatic and vice versa, might serve as a regulatory system to accommodate the redundant rDNA to the needed production. The late replication of the second DNA fraction would correspond to the characteristic duplication pattern of heterochromatin, as was first described by Lima de Faria and Reitalu (6), who compared the euchromatic and heterochromatic segments of both X-chromosomes.

In our experiments with chicken fibroblasts, we found, in those cells in which labeling was exclusively or mainly confined to the nucleoli, that, in general, only one of the two nucleoli of a cell had incorporated thymidine-^{3}H. Such a phenomenon points to asynchrony of DNA synthesis in the two nucleoli. From the results which we obtained, it is tempting to assume that one of the nucleoli synthesizes DNA in the early S phase, whereas the other does so at the end of the S phase. However, the possibility must be kept in mind that both nucleoli synthesize DNA, one after the other, at both times during the S phase. If this were true, then, with a short pulse, labeling ought to be obtained in only one nucleolus of a cell. The fact that, in rare instances, we also observed cells with two labeled nucleoli speaks in favor of this replication pattern; the percentage of cells, however, was very low. In more than 60 preparations which were thoroughly examined, only seven such cells could be found.

With a longer pulse, both nucleoli could be expected to incorporate radioactive thymidine if DNA were replicated in one nucleolus after the other within the pulse period. Our attempt to demonstrate such a phenomenon with a 30-min pulse was unsuccessful: the nuclei were so heavily labeled that it became impossible to localize the label to the nucleoli accurately. Further investigations are necessary to solve the problem.

Received for publication 19 August 1969, and in revised form 17 November 1969.

REFERENCES

1. FIRKET, H., and W. G. VERLEY. 1958. Autoradiographic visualization of synthesis of deoxyribonucleic acid in tissue culture with tritium labelled thymidine. *Nature* (*London*). **181**:274.
2. GHOSH, S., and R. LETTRÉ. 1969. Demonstration of DNA synthesis in the filamentous structure of nucleolus. *Naturwissench.* **56**:140.
3. HARRIS, H. 1959. The initiation of deoxyribonucleic acid synthesis in the connective tissue cell, with some observations on the function of the nucleolus. *Biochem. J.* **72**:54.
4. HENEEN, W. K., and W. W. NICHOLS. 1966. Persistence of nucleoli in short term and long term cultures and in direct bone marrow preparations in mammalian materials. *J. Cell Biol.* **31**:543.
5. KASTEN, F. H., and F. F. STRASSER. 1966. Nucleic acid synthetic patterns in the synchronized mammalian cells. *Nature* (*London*). **211**:135.
6. LIMA DE FARIA, A., and J. REITALU. 1963. Heterochromatin in human male leukocytes. *J. Cell Biol.* **16**:315.
7. MCCONKEY, E. H., and J. W. HOPKINS. 1966. The relationship of the nucleolus to the synthesis of ribosomal RNA in HeLa cells. *Proc. Nat. Acad. Sci. U.S.A.* **51**:1197.

8. OHNO, S., and R. KINOSITA. 1956. On the nucleolus associated chromatin. *Exp. Cell Res.* **10**:66.
9. PHILLIPS, S. G., and D. M. PHILLIPS. 1969. Sites of nucleolus production in cultured Chinese hamster cells. *J. Cell Biol.* **40**:248.
10. RIBAS-MUNDÓ, M. 1966. Nucleolar DNA synthesis in leukocytes cultured in vitro. *Exp. Cell Res.* **41**:210.
11. RITOSSA, F. M. 1968. Non-operative DNA complementary to ribosomal RNA. *Proc. Nat. Acad. Sci. U.S.A.* **59**:1124.
12. RITOSSA, F. M., K. C. ATWOOD, D. L. LINDSLEY, and S. SPIEGELMAN. 1966. On the chromosomal distribution of DNA complementary to ribosomal and soluble RNA. *Nat. Cancer Inst. Monogr.* **23**:449.
13. RITOSSA, F. M., and S. SPIEGELMAN. 1965. Localization of DNA complementary to ribosomal RNA in the nucleolus-organizing region of *Drosophila melanogaster*. *Proc. Nat. Acad. Sci. U.S.A.* **53**:737.
14. SCHMID, W. 1963. DNA replication patterns of human chromosomes. *Cytogenetics.* **2**:175.
15. SWANSON, C. P. 1957. Cytology and Cytogenetics. Prentice Hall, Inc. New York. 133.
16. WALLACE, H., and M. L. BIRNSTIEL. 1966. Ribosomal cistrons and the nucleolar organizer. *Biochim. Biophys. Acta.* **114**:296.
17. WEISSENFELS, N. 1964. Struktur und Verhalten der Nukleolen von Hühnerherzmyoblasten in Gewebekultur während des Interphasewachstums und der Mitose. *Z. Zellforsch.* **62**:667.