SYNCHRONIZATION OF MITOCHONDRIAL

DNA SYNTHESIS IN CHINESE HAMSTER

CELLS (LINE CHO) DEPRIVED OF ISOLEUCINE

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ABSTRACT

Mitochondrial DNA (mit-DNA) synthesis was compared in suspension cultures of Chinese hamster cells (line CHO) whose cell cycle events had been synchronized by isoleucine deprivation or mitotic selection. At hourly intervals during cell cycle progression, synchronized cells were exposed to tritiated thymidine ([^{3}H]TdR), homogenized, and nuclei and mitochondria isolated by differential centrifugation. Mit-DNA and nuclear DNA were isolated and incorporation of radioisotope measured as counts per minute ([^{3}H]TdR) per microgram DNA. Mit-DNA synthesis in cells synchronized by mitotic selection began after 4 h and continued for approximately 9 h. This time-course pattern resembled that of nuclear DNA synthesis. In contrast, mit-DNA synthesis in cells synchronized by isoleucine deprivation did not begin until 9–12 h after addition of isoleucine and virtually all [^{3}H]TdR was incorporated during a 3-h interval. We have concluded from these results that mit-DNA synthesis is inhibited in CHO cells which are arrested in G_1 because of isoleucine deprivation and that addition of isoleucine stimulates synchronous synthesis of mit-DNA. We believe this method of synchronizing mit-DNA synthesis may be of value in studies of factors which regulate synthesis of mit-DNA.

INTRODUCTION

Mitochondria exist in the cytoplasm of eukaryotic cells as semiautonomous organelles which contain their own genetic material and are capable of synthesizing RNA and certain proteins. Many questions remain to be answered concerning the biogenesis of mitochondria and to what degree mitochondrial replication is controlled or coordinated with other cellular events by the "host" cell. Answers to these questions might be provided by studying populations of mitochondria whose phases of development are synchronized, much as cell synchrony has been used to elucidate biochemical processes which occur during the cellular life cycle.

Attempts to correlate mitochondrial DNA (mit-DNA) synthesis with specific phases of the cell cycle have produced conflicting results. In the eukaryotic microorganisms *Tetrahymena pyriformis* and *Physarum polycephalum*, mit-DNA synthesis occurred throughout the cell cycle (Parsons, 1965; Parsons and Rustad, 1968; Charret and André, 1968; Guttes et al., 1967; Braun and Evans, 1969). In contrast to these results, synthesis of mit-DNA in yeast was detected after replication of nuclear DNA and before bud formation (Smith et al., 1968; Cottrell and Avers, 1970). Studies with cultured animal cells have also been inconclusive. Koch and Stokstad (1967) reported incorporation of tritiated thymidine ([^{3}H]TdR) into mit-DNA during the G_2 phase of Chang liver cells which had been synchronized by cold shock. Other investigators found synthesis of mit-DNA

during S and G_2 phases in mouse lyphoma cells (Bosmann, 1971) and HeLa cells (Pica-Mattoccia and Attardi, 1972). Incorporation of labeled DNA precursor into mitochondria was shown by Meyer and Ris (1966) to occur throughout the cell cycle in chick embryo fibroblast cells.

Chinese hamster cells, line CHO, can be synchronized in suspension culture by depriving them of isoleucine (Ley and Tobey, 1970; Tobey and Ley, 1971). These cells are arrested in the G_1 phase of their cell cycle and are unable to initiate nuclear DNA replication until supplied with isoleucine, at which time the cells reinitiate cell cycle traverse, replicate their DNA, and divide in synchrony. The objective of the present study was to investigate the effects of isoleucine deprivation on mit-DNA synthesis. Our results indicate that mit-DNA synthesis is inhibited in cells synchronized by isoleucine deprivation and that addition of isoleucine induces synchronous incorporation of [³H]TdR into mit-DNA.

MATERIALS AND METHODS

Cell Culture

Chinese hamster cells, line CHO (Tjio and Puck, 1958) were grown as suspension cultures in Ham's F-10 medium supplemented with 5% fetal calf and 10% calf sera, penicillin, and streptomycin. Cell concentrations were determined with a Coulter counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). Cells were routinely cultured for pleuropneumonia-like organisms (PPLO) with the medium of Chanock et al. (1962) which contained yeast extract prepared as described by House and Waddell (1967). No PPLO were detected during these studies.

CHO cells were synchronized by depriving them of isoleucine as previously described (Ley and Tobey, 1970; Tobey and Ley, 1971). After 30–32 h in isoleucine-free F-10 medium which contained twice the usual concentration of glutamine and 15% dialyzed serum, the cells were released from G_1 arrest either by adding 2×10^{-5} M isoleucine or resuspending them in complete F-10 medium.

CHO cells were synchronized also by the mitotic selection technique as originally described by Terasima and Tolmach (1961, 1963) and modified by Tobey et al. (1967) and Petersen et al. (1968). Mitotically selected cells (initial mitotic index 0.90–0.96) were held in an ice bath for 2 h or less, and released into warm medium in spinner flasks. Each 2 h of collection provided a sufficient number of cells for two 1-h labeling periods during the cell cycle.

Labeling and Isolation of Mitochondrial and Nuclear DNA

At hourly intervals after resumption of cell cycle traverse, $0.5–1.0 \times 10^7$ cells were removed from the synchronized populations, pelleted, and suspended in 3 ml of thymidine-free F-10 medium to which had been added 15 μCi of [methyl-³H]thymidine (6–21.4 Ci/mmol; Schwarz-Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). After exposure to the isotope for 1 h in a shaker water bath at 37°C, cells were lysed with a Dounce homogenizer and mitochondria and nuclei were separated by differential centrifugation as described by Nass (1972). To guard against contamination of mitochondria with nuclear DNA, the isolated mitochondria were treated with 20 μg/ml DNase I (Worthington Biochemical Corp., Freehold, N. J.) for 30 min at room temperature. Mitochondria and nuclei were washed again, pelleted, and suspended in 4 ml of 0.25 M sucrose, 2 mM EDTA, 25 mM Tris buffer, pH 7.4.

Measurement of Incorporation of [³H]TdR into Mitochondrial and Nuclear DNA

Perchloric acid was added to suspensions of mitochondria and nuclei until the final concentration of acid was 0.25 N. Mitochondria and nuclei were chilled at 4°C for 30 min and then pelleted by centrifugation at 500 g for 15 min. After pellets of mitochondria and nuclei were treated with ethanol and ether, DNA was extracted twice for 1 h with 10% NaCl at 100°C (Nass, 1966). DNA was measured by the fluorometric method of Kissane and Robins (1958) as modified by Robertson and Tait (1971). Calf thymus DNA (Calbiochem, San Diego, Calif.) was used for standards and DNA was measured with a Perkin-Elmer 203 fluorescence spectrophotometer (Perkin-Elner Corp., Instrumental Div., Norwalk, Conn.). Incorporation of [³H]TdR into mitochondrial or nuclear DNA was determined on an aliquot of DNA extract equal to that used for quantitation of DNA. Aquasol (New England Nuclear, Boston,Mass.) was used as scintillation cocktail and the radioactivity was measured with a Packard TriCarb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downer's Grove, Ill.). Specific activity was calculated as counts per minute (³H]TdR) per microgram DNA.

RESULTS

Mit-DNA Synthesis in CHO Cells Synchronized by Mitotic Selection

Before the effect of isoleucine deprivation on mit-DNA synthesis could be evaluated, it was first

necessary to establish the pattern of mit-DNA synthesis in CHO cells which were synchronized by a different method. Mitotic selection was chosen as an alternate method for cell synchrony for the following reasons: (a) Time-course patterns of nuclear DNA synthesis and cell division in CHO cells synchronized by mitotic selection are similar to those in CHO cells synchronized by isoleucine deprivation (Tobey, 1973). (b) Mitotic cells are selected from a random population without the use of metabolic inhibitors and, therefore, are less biochemically perturbed (Tobey et al., 1972). (c) Published data exist concerning mit-DNA synthesis in HeLa cells which were synchronized by mitotic selection (Pica-Mattoccia and Attardi, 1972).

Because of the relatively small number of mitotic cells that detach each time the monolayer is shaken, these cells are routinely chilled in an ice bath to prevent cell cycle traverse until the desired number of cells have been accumulated (Tobey et al., 1972). Cell cycle progression is then induced by resuspending the chilled cells in warm medium. Ehmann and Lett (1972) have found, in studies with CHO cells, extended generation times which were proportional to the length of time the mitotically selected cells were held in the cold before release. In order to minimize possible cold-induced abnormalities in the synchronized cells, mitotic cells were held in the cold for 2 h or less before release.

Patterns of incorporation of [³H]TdR into nuclear DNA and mit-DNA after release of mitotic cells are presented in Fig. 1. Synthesis of nuclear DNA began at about 3 h as the first cells traversed G_1 and entered S phase. Amounts of [³H]TdR incorporated into nuclear DNA decreased after 12 h as the cells moved out of S phase. Initiation of synchronous cell division began about 11 h after release. Incorporation of [³H]TdR into mit-DNA began 1–2 h after initiation of nuclear DNA synthesis and continued for approximately 9 h. The pattern of incorporation of isotope into mit-DNA was similar to that measured for nuclear DNA.

Effect of Isoleucine Deprivation on Mit-DNA Synthesis

To determine the effect of isoleucine deprivation on mit-DNA synthesis, exponentially growing CHO cells were suspended in isoleucine-free medium as described in Materials and Methods. During the ensuing 30 h, there was a 35–40%
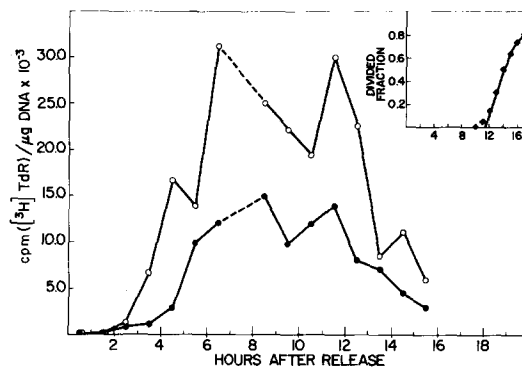

FIGURE 1 Time-course patterns of nuclear DNA and mit-DNA synthesis in CHO cells synchronized by mitotic selection. Cells were exposed to 15 μCi of [³H]TdR (21.4 Ci/mmol) for 1 hr and mit-DNA and nuclear DNA isolated as described in Materials and Methods. The early and late portions of the cell cycle were examined in separate experiments. The fraction of cells dividing was calculated as $N/N_o - 1$ (◆—◆). Incorporation of [³H]TdR is presented as cpm ([³H]TdR)/μg nuclear DNA (O—O) or mit-DNA (●—●).

increase in cell number because cells initially in S, G_2, or M, when deprived of isoleucine, continued to traverse the cell cycle until they reached G_1 phase (Ley and Tobey, 1970). Time-course patterns of incorporation of [³H]TdR into nuclear DNA and mit-DNA before and after release of cells from G_1 arrest by addition of isoleucine are presented in Fig. 2. The patterns of [³H]TdR incorporation into nuclear DNA and cell division were similar to those measured in cells synchronized by mitotic selection, i.e., nuclear DNA synthesis began about 3 h after addition of isoleucine and was followed by initiation of synchronous cell division at 13 h. In contrast to data gathered from mitotically selected cells, labeling of mit-DNA did not begin until about 12 h after addition of isoleucine, at which time the cells were in S or possibly early G_2 phase of the cell cycle. An example of the pattern obtained when the labeling period was shortened from 2 h to 1 h is shown in Fig. 2 B. In this instance, the peak of incorporation of [³H]TdR into mit-DNA was detected at 9–10 h and essentially all isotope incorporation occurred within a relatively narrow 3-h interval. In subsequent experiments, incorporation of [³H]TdR into mit-DNA occurred as early as 9–10 h or as late as 12–13 h after addition of isoleucine, whereas the time of onset of nuclear DNA synthesis and cell division remained constant between experiments.
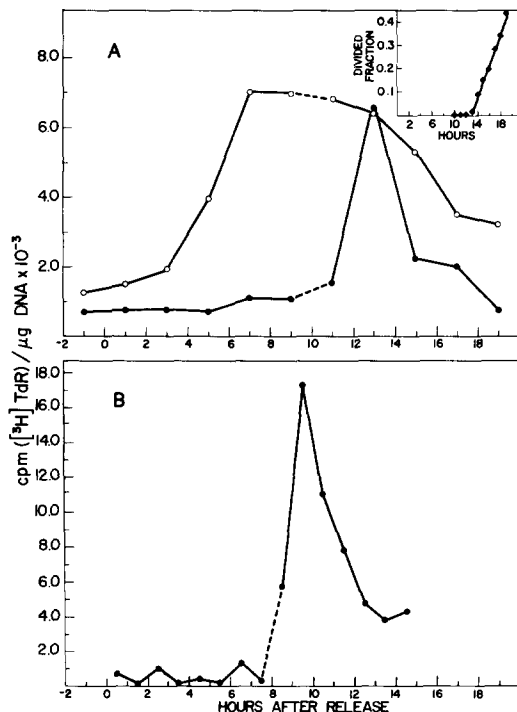
Figure 2 Time-course patterns of nuclear DNA and mit-DNA synthesis in CHO cells synchronized by isoleucine deprivation. (A) Nuclear DNA (O—O) and mit-DNA (●—●) were labeled by exposing cells to 15 μCi of [^{3}H]TdR (6 Ci/mmol) for 2 h. The fraction of cells having divided was calculated as $N/N_o - 1$ (◆—◆). (B) Mitochondrial DNA (●—●) labeled with 15 μCi of [^{3}H]TdR (21 4 Ci/mmol) for 1 h.

DISCUSSION

Our results indicate that mit-DNA synthesis is synchronized in CHO cells which have been deprived of isoleucine. Although the time-course patterns of nuclear DNA synthesis and cell division were virtually the same in cells synchronized by isoleucine deprivation or mitotic selection, the patterns of [^{3}H]TdR incorporation into mit-DNA were different. After addition of isoleucine, the onset of synthesis of mit-DNA was abrupt and maximum incorporation lasted approximately 3 h (Fig. 2 B), whereas radioisotope incorporation into mit-DNA continued for about 9 h in cells synchronized by mitotic selection (Fig. 1). The broad pattern of mit-DNA synthesis in CHO cells synchronized by mitotic selection resembled those seen in other studies with synchronized populations of cells (Bosmann, 1971; Pica-Mattoccia and Attardi, 1972).

Caution should be exercised when attempting to correlate radioisotope incorporation with rate of net synthesis of DNA because of possible fluctuation in the size of precursor pools. In a study of mit-DNA synthesis in HeLa cells synchronized by mitotic selection, Pica-Mattoccia and Attardi (1972) measured the changes in cellular and intra-mitochondrial pools that occurred as a function of cell cycle progression. Correction of their isotope incorporation data because of fluctuations in thymidine pools allowed them to determine that the rate of mit-DNA synthesis was highest in G_2 rather than in S phase, as their uncorrected data had indicated. However, the overall pattern of mit-DNA synthesis was not significantly changed by these corrections because synthesis began in late G_1 and continued through G_2, a total of approximately 18 h.

The mechanism by which isoleucine deprivation prevents nuclear and mit-DNA synthesis in CHO cells is not known. Previous speculations concerning a role for isoleucine in nuclear DNA synthesis have centered around isoleucine-containing regulatory proteins or perhaps lipoproteins which are necessary for DNA replication (Tobey and Ley, 1971). Evidence that CHO cells deprived of isoleucine are relatively unperturbed, biochemically, has been provided by Enger and Tobey (1972), who showed that nuclear DNA synthesis is shut off very soon after removal of isoleucine, and that this early cessation of DNA synthesis precedes any appreciable effect on the RNA or protein synthetic capacity of the cell. Everhart and Prescott (1972) have recently shown that a degree of synchrony can be induced in CHO cells by growth in medium which contains suboptimal quantities of leucine; therefore, the effect of amino acid deprivation on DNA synthesis may not be specific for isoleucine. That amino acid deficiency can affect the structural form of mit-DNA has been reported by Nass (1969), who found that the frequency of circular dimers of mit-DNA increased from 9% in exponentially growing mouse L cells to 50% in cells starved for methionine or phenylalanine. This phenomenon was readily reversible in the case of methionine, but not phenylalanine. Whether or not mit-DNA synthesis was synchronized under these conditions is not known.

Evidence to support the notion that initiation of mitochondrial DNA synthesis is somewhat independent of other cell cycle related events is provided in the present study. Because the timing of

initiation of nuclear DNA synthesis and cell division were unchanged in replicate experiments, whereas initiation of mit-DNA synthesis varied by as much as 3 h, one might speculate that the mitochondria have a cycle of development which is only indirectly influenced by nuclear genome replication or cell division.

We have concluded from this study that depriving CHO cells of isoleucine not only provides large quantities of cells whose life cycle events are highly synchronized, but it also results in synchronization of mit-DNA synthésis. This method of cell synchrony may be of value for investigating factors which influence control of mit-DNA synthesis.

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