CONTROL OF DNA SYNTHESIS IN TISSUE CULTURE CELLS

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SUMMARY

Eukaryotic DNA is functionally divided into thousands of replicons, each of which may be duplicated at a characteristic time within the DNA synthetic (S) period. Our approach toward an understanding of the molecular mechanisms which control orderly eukaryotic DNA synthesis has been: (a) to devise a method of cell synchrony in a suitable tissue culture system wherein all cells in the population enter and traverse the S period with a high degree of synchrony; (b) to determine, utilizing this system, precisely when during the S period critical events and macromolecular syntheses occur; and (c) to examine, by polyacrylamide-gel electrophoresis, the spectrum of proteins which become associated with chromatin during the S period in such a way as to suggest their involvement with DNA synthesis. Possible mechanisms for control are discussed based on the results presented here.

Key words: DNA synthesis; replication controls; chromatin proteins; replicons; cell cycle synchrony.

INTRODUCTION

Most of what is known about the enzymology of DNA synthesis comes from studies on bacterial and bacteriophage chromosome replication [see Kornberg (1) for review]. The results of these studies have served as a model on which to pattern investigations in eukaryotic (usually tissue culture) cells. As various DNA polymerases, unwindases, ligases, etc. are discovered in animal cells, it is becoming apparent that the machinery utilized at a replicating locus on the eukaryotic chromosome is analogous in many respects to that utilized by microorganisms.

There are at least two major differences, however, which endow the eukaryotic cell with special problems during the course of chromosome duplication and indicate the need for sophisticated control mechanisms. The first relates to the intimate and extensive association of eukaryotic DNA with histone and nonhistone chromosomal proteins. These proteins undoubtedly serve a host of structural and regulatory functions, and clearly must be duplicated at some time during the cell cycle in order to re-establish the appropriate stoichiometric relationships with a doubled genome. The high binding constants of many histone and nonhistone chromosomal proteins (2) further indicate that macromolecules involved in DNA replication must be able to displace or at least transiently loosen these associations in order to copy all sequences with fidelity. Indeed, some aspects of the structure of the DNA-protein complex may even play a part in determining where DNA replication initiates on the chromosome (3).

The second important difference between pro- and eukaryotic DNA replication relates to the very large size of the DNA complement in higher organisms (about a 1000-times-larger number of base pairs than in E. coli). It has been known for many years that DNA replication in eukaryotes normally requires a matter of hours to complete and that replication occurs during a limited interval in the cell cycle (4). It also has been observed that the rate of DNA synthesis in synchronized DON C cells varies during the S period (5), and we have observed a tripartite rate curve in synchronized Chinese hamster ovary cells (6) (Fig. 3). These observations suggest that waves of DNA synthesis may occur during the S period in a single cell.

Duplication of the several chromosomes which comprise the eukaryotic genome occurs via thousands of replicons (7, 8), sometimes operating

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simultaneously (8) and apparently in a predetermined temporal order (9). Recent studies utilizing the technique of DNA fiber autoradiography define a replicating unit (replicon) with a centered origin from which DNA polymerization proceeds outward bidirectionally (8). The replication forks of a given unit coalesce with the growing forks extending from adjacent replicons, possibly at predetermined termini (8).

Thus the eukaryotic genome can be considered to be divided into thousands of replicons, each analogous in several respects (at least superficially) to the replicating chromosome of bacteria, bacteriophages and animal viruses. As with microorganisms, the origin of replication is expected to be the site for control of initiation (10) and hence for the control of replication of the entire genome. If waves of DNA synthesis during the S period are the result of different numbers of functioning replicons in operation at different times in S, then the implication is that DNA synthesis initiates at certain replicons at precise times during S.

The existence of the replicon and the apparent temporal order in which the many replicons function during the S period raise several questions: (a) Is the nucleotide sequence at the origin of replication unique? Or does initiation occur at random loci, perhaps determined by an inherent property of the structure of the nucleo-protein complex? (b) What molecules interact with the initiation sequence prior to polymerization, in order to effect controlled initiation? (c) How is the order of replication guaranteed? (d) How does the cell delay re-initiation at a recently duplicated locus until the next S period?

We are interested in all these questions, but will confine this discussion predominantly to the second and third aspects. We suspect that origins of replication will be unique nucleotide sequences, as they are proving to be in several animal viruses (11, 12). We assume at the outset that initiation of DNA synthesis in a replicon which was previously dormant (with respect to DNA synthesis) must involve the interaction of controlling elements with the origin of replication. We further assume that either the synthesis or activation of these controlling elements must be time-ordered, in order to explain the time-ordered replication of discrete loci during the S period.

Our approach has been: (a) to devise a method of cell synchrony in a suitable tissue culture system wherein all cells in the population enter and traverse the S period with a high degree of synchrony; (b) to determine, utilizing this system, precisely when during the S period critical events and macromolecular syntheses occur; (c) to examine, by polyacrylamide-gel electrophoresis, the spectrum of proteins which become associated with chromatin during the S period in such a way as to suggest their involvement with DNA synthesis; and (d) to assess the effect of these proteins on initiation in vitro.

**Materials and Methods**

**Cell culture, synchrony and inhibitor studies.** Chinese hamster ovary (CHO) cells were obtained from D. F. Petersen (Los Alamos Scientific Laboratories) and maintained on modified Ham's F-10 (13) medium supplemented with 10% bovine serum, 5% fetal bovine serum and antibiotics. Cells growing logarithmically on plastic Petri plates were synchronized as previously described (6); early G1 arrest was induced by isoleucine deprivation; complete medium containing 1 mM hydroxyurea (HU) then was added for 11 hr, at which time all cells had collected at the G1/S boundary; removal of HU promoted synchronous entry into S (6). For two cycles of S-phase synchrony, HU was administered 12 hr after removal of the first HU block, and the cultures again were maintained in the drug for 11 hr.

Cycloheximide and 5,6 dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) were administered during S in F-10 medium.

**Bromodeoxyuridine (BUdR) incorporation and UV irradiation.** In experiments involving BUdR incorporation, the analogue was made up at 5 µg per cc in thymidine-deficient F-10 medium supplemented with dialyzed serum and containing 2 µg per cc deoxyctydine and 0.2 µg per cc 5-fluorodeoxyuridine. Irradiation was performed through the plate bottom on cultures growing on glass cover slips in glass Petri dishes. The dish was exposed from below to 2 min of irradiation from a Hanovia sun lamp at a distance of 15 cm. The cells were in Earle's balanced salt solution during irradiation and cooled with a hair dryer directed at the plate bottom.

**[35S]Methionine ([35S]met) labeling, chromatin isolation and polyacrylamide-gel electrophoresis.** For examination of chromatin proteins synthesized at various times during the S period, cultures were labeled for 1 hr with [35S]met (12.5 µCi per cc) in F-10 containing nondialyzed serum (carrier methione was reduced to 20% of that in the standard F-10 formulation, and uptake into TCA-insoluble material was shown to be linear.