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STUDIES ON MAMMALIAN CHROMOSOMES

Final Report

David M. Prescott
Department of Molecular, Cellular and Developmental Biology
University of Colorado
Boulder, Colorado 80302

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ABSTRACT

1. The locations of satellite DNA sequences have been studied in Kangaroo rat cell lines by in situ hybridization. Satellite HS-β is located in the kinetochores of 32 of the 35 chromosomes. Satellite HD is located in the kinetochores of the other three chromosomes and in the short arms of most of the 35 chromosomes.

These satellite DNAs are replicated in a fixed order in the last half of S period.

Measurements of the DNA content of 7 species of kangaroo rats show that there is no precise relationship between amount of satellite DNA and genome size. In cultured cells, the amount of satellite DNA can be altered by treatment with bromodeoxyuridine.

2. The nuclear envelope of mammalian cells has been shown to have no role in the initiation and continuation of DNA synthesis.

3. a) Isolabeling of chromosomes has been acounted for by sister chromatid exchanges coupled with image spread of autoradiographs.

b) Sister chromatid exchanges occur preferentially at the kinetochore. Small differences in the frequency of exchanges occur at different positions in chromosome arms.

4. α and β polymerase have been shown by a new cell fractionation technique to be nuclear enzymes. Thymidine kinase is a cytoplasmic enzyme.

1. Satellite DNA in Kangaroo Rats

Satellite DNAs are present in most if not all mammals, although the amount of such DNA varies greatly from one species to another. In the genus Dipodomys, satellite DNA varies from a minor quantity in D. spectabulis and a few other species to a major quantity (60 percent of the total DNA) in D. ordii (Mazrimas and Hatch, 1972, Nature New Biol. 240:102).

Most of the research proposed in the original application required that we first establish cell lines in culture from several kangaroo rat species that are representative with respect to the various amounts of satellite DNAs carried within the genus. We attempted to obtain cell lines from kidney, spleen, and lung of D. ordii relying on spontaneous transformation to produce cell lines that would grow permanently in culture. These efforts failed, and we turned to transformation with simian virus-40. With SV-40 , cell lines have been established from lung tissue of D. ordii, D. merriami, D. panimintinus, and D. desertii. These lines grow vigorously in standard cell culture media. Each transformed line has undergone relatively slight change in karyotype, although the karyotype has been characterized in some detail only in D. ordii. In this case, the modal chromosome number is 70 , compared to normal diploid number of 72 . More importantly, the buoyant density profile of the DNA of the ordii cell line is exactly the same as in the original lung tissue.

Lung cells of a fifth species, D. spectabulis, have been put into culture, but these cells have not yet evolved to a rapidly growing line that can be used for experimentation.

In D. ordii, we have shown that the main band DNA (1.701 gm cm^{-3}) and the several satellite DNAs are replicated in a definite order within the S period of the cell cycle. These experiments were performed by pulse labeling with ^{3}H thymidine and next harvesting mitotic cells by the mitotic shake-off method at intervals after the labeling pulse. By this method of

"retroactive synchronization," we could obtain populations of cells that were in known positions within the S period at the time of the labeling with ^{3}H thymidine. The "retroactive synchronization" is most reliable for cells in the latter part of the cell cycle, since the longer the interval between labeling and the subsequent mitotic shake off, the greater the loss of synchrony in a cohort of cells. The method is also somewhat disadvantageous because the yield of synchronous cells (mitotic cells) limits the kinds of analyses that can be done. The procedure, nevertheless, permitted us to learn the following.

The first half of the S period is occupied by the replication of main band DNA. Little or no staellite DNA is synthesized during this portion of the S period, although the method is least accurate for this interval. The second half of the S period is taken up with the synthesis of the satellite DNAs. The second half of the S period is in turn divisible into two parts, the first concerned primarily with replication of the GC rich satellites (1.713 gm cm^{-3}) and the second occupied by the replication of moderately GC rich satellite DNA (1.707 gm cm^{-3}). Little or no main band DNA is replicated in the second half of the S period.

The pattern of main band and satellite DNA replications has been correlated with the labeling pattern of chromosomes observed by autoradiography. When DNA was labeled with ^{3}H thymidine during the first half of the S period, most of the subsequent metacentric metaphase chromosomes were labeled only in their long arms. Two and possibly three pairs of chromosomes were labeled in both the long and short arms. These few chromosomes were subsequently shown to contain satellite DNA only at the kinetochore. When DNA was labeled with ^{3}H thymidine during the second half of the S period, most of the chromosomes were labeled in the kinetochore region plus the short arms. Three pairs of chromosomes were labled only in the kinetochore. These observations indicated that the satellite DNAs occupy the kinetochore of three pairs of chromosomes and the kinetochores plus the short arms of most or all of the remaining pairs.

This apparent location of the main band and satellite DNAs was also suggested by Giemsa staining of chromosomes treated with 0.007 N NaOH . Staining was intense in the kinetochores of all chromosomes and in the short arms of most of the chromosomes. Three pairs of chromosomes were intensely stained at the kinetochore only. The long arms of chromosomes were lightly stained. This staining pattern is consistent with the idea that satellite DNAs appear as constitutive heterochromatin in well-defined locations in the chromosomes.

The location of the satellite DNAs has been confirmed by in situ hybridization with ^{3}H RNA transcribed from satellite DNAs.

Finally, we have measured by cytospectrophotometry the amount of DNA per sperm (genome) for seven species. We have used mouse sperm as a standard for estimating the absolute amount of DNA per sperm in the kangaroo rats, using the value of 3.0 pg/mouse sperm established by others. The values for the seven species of kangaroo rats are:

D. ordii	4.4 pg
D. merriami	2.2 pg
D. desertii	4.0 pg
D. spectabulis	3.0 pg
D. panimintinus	2.8 pg
D. ingens	2.8 pg
D. microps	3.2 pg

It is clear that there is no precise relationship between the amount of satellite DNA in a species and the total amount of DNA in its genome. It is still notable that D. ordii has the most satellite DNA and the largest DNA value for its genome. The genus is also remarkable for the range of DNA values per genome. The genus covers a two-fold range and contains species with the least (2.2 pg) and the most (4.4 pg) DNA among mammals.

In collaboration with F. Hatch and J. Mazrimas of the Lawrence Livermore Laboratory, we have found that the HSβ satellite of kangaroo rat cells is unusually rich in the base 5' methyl cytosine. This satellite is localized in chromosome kinetochores in 32 of the 35 chromosomes in the cell line.

Newly synthesized ^{3}H RNA of kangaroo rat cells hybridizes with main band DNA but not with satellite DNAs. We interpret this to mean that satellite DNAs in cultured cells are not transcribed.

Finally, the synthesis of satellite DNAs in D. ordii occurs in the last half of the S period. By allowing incorporation of BUDR into DNA during the last half of the S period, we have changed the buoyant density profile of the satellite. All three satellites were decreased in amount and a new satellite (identified by buoyant density) appeared. These changes were permanent in the sense that they persisted after the BUDR had been diluted out by many generations of cell growth. We are now trying to establish new, similar lines with altered satellite contents and altered karyotypes.

Our thesis is that the entire short arms of ordii chromosomes should be expendable and unnecessary for cell proliferation because the short arms appear to be composed only of satellite DNA. This idea is in keeping with our failure to detect any RNA molecules in ordii cells that will hybridize with satellite DNAs.

2. The Nuclear Envelope and DNA Synthesis

An important facet of the problem of DNA replication in eukaryotes is whether initiation of replication occurs at points of attachment of the chromosome to the nuclear envelope. The question arises in part because of the evidence that the replicons of prokaryotic chromosomes initiate and then continue replication at points of attachment to the plasma membrane. Several kinds of experiments on prokaryotes suggest that the enzymes and other factors necessary for DNA replication may form a complex that remains associated with the plasma membrane during chromosome replication.

In studies of human amnion cells, Comings and Kakefuda concluded from electron microscope radioautography that DNA replication is initiated at the nuclear envelope at the beginning of the period of DNA synthesis (S period). Contrary to this conclusion, Williams and Ockey have obtained

evidence by electron microscope radioautography that DNA replication at the beginning of the S period is not initiated at the nuclear envelope in Chinese hamster cells. According to them and to Huberman et al., DNA synthesis late in the S period is highly concentrated near the nuclear envelope; this result is to be expected because replication of DNA in heterochromatin dominates the later part of the S period, and heterochromatin tends to be tightly condensed against the nuclear envelope. The results of Williams and Ockey are to some degree supported by work of Erlandson and de Harven for HeLa cells and of Blondel for KB cells. In another eukaryote, Amoeba proteus, DNA replication is probably not associated with the nuclear envelope at any time during the S period.

To answer the question of association of initiation or continuation of replication with the nuclear envelope in mammalian cells requires a very high degree of synchrony of the cultured cells. Many of the agents used for synchronization are incompletely effective. Ockey, for example, showed that amethopterin does not block cells from entering the S period. A high thymidine concentration (thymidine block) also fails to block cells at the G_1-S border. We have done experiments that minimize the shortcomings of current methods of synchronization.

It would be a simple matter to determine whether initiation of DNA replication occurs at the nuclear envelope if all of the cells in a population could be reversibly arrested precisely at the G_1-S border. None of the synchronization techniques so far developed for cultured mammalian cells can provide such a population. An acceptable alternative for present purposes is a population in which only some of the cells are arrested at the G_1-S border, provided that none of the remaining cells is in any part of the S period. We have achieved this condition by using two synchronization techniques that, in combination, have a high probability of blocking about 20 percent of the cells in a population at the G_1-S border, with the remaining cells positioned at earlier parts of G_1. Cells were initially synchronized by the mitotic shake-off method, and the resulting G_1 cells were treated with two inhibitors of DNA synthesis, fluorodeoxyuridine and amethopterin,

in such a way the G_1-S border for times varying from zero min to a maximum of two hours. This short time of restraint with the inhibitors provided the maximum probability that the cells remained arrested at the G_1-S border.

Finally, we released cells from the inhibitors with 1- and 5-min pulses of ^3H thymidine. These short pulses minimize the distance that replication forks or newly replicated DNA could become displaced from the nuclear envelope. In 109 cells labeled after a 5-min pulse and six cells labeled after a 1-min pulse, not a single cell showed grains concentrated at the nuclear envelope.

We conclude from our experiments that DNA replication is not initiated at the nuclear envelope, but begins at interior regions of the nucleus. Secondly, in agreement with recent radioautographic studies of Fakan et al. and Huberman et al., our experiments show that the enzymatic machinery for DNA replication is not attached to membrane. Thus, the mechanism of DNA replication in higher eukaryotes may be significantly different from the mechanism in prokaryotes.

Our results and conclusions on sites of initiation of DNA replication agree with those of Williams and Ockey and disagree with those of Comings and Kakefuda. Concerning this disagreement, we can only reiterate the importance of synchronization procedures that require a minimum time of arrest of cells at the G_1-S border and completely eliminate the possibility that any cells in the population may be in the later parts of the S period.

3. Sister Chromatid Exchanges in Rat Kangaroo (Potorous tridactylis) Chromosomes

a) Isolabeling

The autoradiographic demonstration of semi-conservative segregation of DNA in the chromosomes of Vicia faba by Taylor also revealed the phenomenon of sister chromatid exchange. In subsequent studies of sister chromatid exchanges in Vicia faba chromosomes, Peacock described an autoradiographic

pattern which he termed "isolabeling." Isolabeling, observed by autoradiography in sister chromatids at the second or later metaphases after incorporation of ^{3}H thymidine, has sometimes been ascribed to an exchange between the multiple DNA duplexes in polynemic sister chromatids. An analysis carried out by us on the frequency and size of isolabeled regions in chromosomes of the rat kangaroo showed that all isolabeling can be accounted for by sister chromatid exchanges coupled with the image spread that can occur in tritium autoradiographs. Hence, in this case, it becomes unnecessary to postulate binemy or polynemy to explain isolabeling.

b) Frequency of Exchanges

The frequency of sister chromatid exchanges in the chromosomes of a cell line from the Tasmanian rat kangaroo was determined to be 0.79 exchanges per chromosome for two cell cycles. Twenty-five percent of these exchanges occur at the kinetochore. The mean frequency of exchanges per chromosomal arm is roughly proportional to the length of the chromosome, with the exception of a mean frequency of 0.20 exchanges per chromosome found at the kinetochore of all chromosomes regardless of length. Thus, the kinetochore is a highly preferential site for sister chromatid exchanges. Compared to the main portion of the chromosomal arms, the exchange frequency was somewhat lower adjacent to the kinetochore and at chromosome ends. The number of exchanges per unit length also tends to be lower for the short arm of chromosome 1. No correlation was found between the frequency of exchanges and late-replicating DNA.

4. Intracellular Locations of DNA Polymerases and Thymidine Kinase

The major eukaryotic DNA polymerase (polymerase α) has repeatedly been found in the cytoplasmic fraction of disrupted mammalian cells. We have reexamined the cellular location of polymerase α by measuring activity in L 929 cells which have been enucleated in vivo in a centrifugal field during treatment with cytochalosin B. This treatment produces cytoplasts

(enucleated cells), which contain most of the cytoplasm surrounded by a plasma membrane (two percent to eight percent retain their nuclei), and karyoplasts, which consist of nuclei surrounded by a small amount of cytoplasm and a plasma membrane. Crude extracts of cytoplasts and karyoplasts were assayed for DNA polymerase activity. Up to 95 percent of the DNA polymerase α activity was found in the karyoplast fraction, and the total DNA polymerase α activity in the cytoplasts and karyoplasts equaled the activity in whole cells (900 pmole TMP incorporated/hour/10^6 cells). In addition, DNA polymerase β, found by others to be in the nucleus, was almost entirely in the karyoplasts. Thus, we find that both DNA polymerases are predominantly in the karyoplasts, and we conclude that they are associated with the nucleus in vivo. We cannot distinguish between an intranuclear or a perinuclear location. We assume that the cytoplasmic location of polymerase α reported by others is the result of separation or extraction of the enzyme from the nuclei during cell disruption or nuclear isolation.

In contrast to DNA polymerases, almost all thymidine kinase activity in the cell is located in the enucleated portions of mouse L cells. The small amount of kinase associated with the nucleus-containing portion of the cell is probably due to the shell of cytoplasm present in this component.

Bibliography of Publications Associated with This Contract

Mouse cell DNA polymerases are associated with the nucleus. In preparation.

The cell cycle and the control of cellular reproduction. D. M. Prescott. 1975. In Advances in Genetics (Caspari, E. W., ed.), Vol. 18, Academic Press, Inc., New York. In press.

Frequency and sites of sister chromatid exchanges in rat kangaroo chromosomes. D. A. Gibson and D. M. Prescott. 1974. Exptl. Cell Res. 86:209-214.

Location of satellite DNAs in the chromosomes of the kangaroo rat (Dipodomys ordii). D. M. Prescott, C. J. Bostock, F. T. Hatch, and J. A. Mazrimas. 1973. Chromosoma 42:205-213.

Sister chromatid exchanges in isolabeling. D. A. Gibson and D. M. Prescott. 1973. Exptl. Cell Res. 83:445-447.

Initiation and continuation of DNA replication are not associated with the nuclear envelope in mammalian cells. G. E. Wise and D. M. Prescott. 1973. Proc. Nat. Acad. Sci. USA 70:714-717.