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Units of DNA Replication in Chromosomes of Eukaryotes

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I. Introduction

Considerable evidence has accumulated which indicates that replication in chromosomes is regulated in a way such that specific fractions or segments are replicated at specific times in the *S* phase. However, the size and the identity of the units that have been designated replicons (Taylor, 1963a) are still in doubt, and therefore the mechanisms by which the regulation is achieved are very incompletely understood. The idea that chromosome reproduction is somehow regulated with respect to specific sites probably goes far back to studies of differential pairing in meiosis and differential condensation in the mitotic cycle. However, the means for quantitative studies of the phenomenon were not available until thymidine-^{3}H was demonstrated to be a selective label which gives autoradiographic resolution sufficient for the study of segments of individual chromosomes (Taylor *et al.*, 1957; Taylor, 1958). The replication of specific segments of chromosomes at limited times in the *S* phase was clearly indicated by autoradiographic studies involving the X chromosome, the Y chromosome, and selected autosomes of the Chinese hamster genome (Taylor, 1960). Certain segments were replicated early in the *S* phase, while others were late. These studies were soon expanded to include the chromosomes of many species by laboratories all over the world. Studies of asynchronous replication have been reviewed rather extensively (Schmid, 1967; Lima-de-Faria, 1969; Taylor, 1969a; Rudkin, 1972; among others), and therefore little attention is given to this aspect of the problem of regulation. From these studies it has become clear that the heterochromatic segments, as first realized by Lima-de-Faria (1959), are among the late-replicating species of DNA. The correlation of late replication of one X chromosome in female mammals with genetic inactivation or prolonged repression through many cell divisions of certain loci, made the study of regulation of

more than passing interest (Lyon, 1961; Davidson *et. al.,* 1963; Taylor, 1969a). Instead of being merely a consequence of structural properties of the genome, its study appeared to be one approach to the problem of genetic regulation and cellular differentiation. The exploitation of this approach has been slow, however, because the identification of units involved in regulation, and evidence concerning mechanisms for their control, have been difficult to obtain.

Another approach to the study of regulation of DNA synthesis utilized the density label bromodeoxyuridine (BUDR) and asked the question: Do these segments of the DNA replicated early in one *S* phase also replicate early in subsequent *S* phase? The answer obtained in studies with synchronized cells indicated that there is a programming for early or late synthesis, and further suggested that there is a regulated sequence for all of the genome over the *S* phase (Braun *et al.,* 1965; Mueller and Kajiwara, 1966; Taylor *et al.,* 1971). The design of these experiments took advantage of the natural synchrony of mitosis in the slime mold *Physarum polycephalum* (Braun *et al.,* 1965) or induced synchrony in human (HeLa) cells with the folic acid analog aminopterin (Mueller and Kajiwara, 1966), and mitotic selection of Chinese hamster cells in culture (Taylor *et al.,* 1971), so that early replicating DNA could be labeled with thymidine containing either ^{14}C or ^{3}H. In some of the experiments the late replicating DNA was labeled with the alternate radioisotope. At the next cycle the cells were resynchronized, and BUDR incorporation forced during the early *S* phase. Since the BUDR density hybrid could be separated in cesium chloride isopycnic gradients, the proportion of labeled DNA, early- or late-replicating in the previous cycle, could be determined. In the experiments with *Physarum,* the DNA replicated in the first one-fifth of an *S* phase was nearly all replicated in the beginning one-fifth of the next *S* phase. Although the Chinese hamster cells were delayed about 3 hours when forced to begin *S* phase with BUDR, the sequence when replication finally occurred was comparable to that indicated by the pattern of labeling in the previous *S* phase.

Labeling of synchronized cells with thymidine-^{14}C or -^{3}H also showed that the DNA replicated early in *S* phase has a higher buoyant density, and therefore a higher G-C content, than that replicated in mid-*S* phase. The DNA replicated in mid-*S* phase has an average density similar to that of the whole genome while that replicated late has a lower buoyant density (Tobia *et al.,* 1970; Bostock and Prescott, 1971). Since there is no sequential replication of whole chromosomes as in bacteria (Bird and Lark, 1968), the basis for control must be programmed in smaller units.

In this article a critical look is taken at the evidence bearing on this problem. At the end a tentative model is proposed which takes into account what is known about the organization of the genomes of higher cells and their regulatory features.

II. Autoradiographic Studies of Initiation Sites

Autoradiographic studies first clearly indicated that chromosomes have many initiation sites. For example, some chromosomes may be labeled throughout their length during a short interval (10 minutes) of the *S* phase, while others are labeled in a few sites only (Taylor, 1960). In salivary gland chromosomes the labeling of single bands or clusters of bands was distinguished as independent sites of initiation (Plaut, 1963; Keyl and Pelling, 1963; Gabrusewycz-Garcia, 1964). Better estimates of the size of the segments that eventually grew from each initiation site were made when data on rates of chain growth became available. Several measurements based on different techniques yielded data indicating rates of the order of 1 μm per minute for mammalian cells growing at 37°C. By using thymidine-^{3}H of high specific activity and ingenious techniques for lysing cells and deproteinizing DNA with minimum shear, the extended labeled DNA strands were measured in autoradiographs (Cairns, 1966; Huberman and Riggs, 1968). The rate of polynucleotide growth was estimated to be 1 μm, or possibly 0.5 μm per minute. Measurements based on the size and density of BUDR segments attached to unsubstituted DNA after pulse labeling gave similar rates, and allowed the estimate that a Chinese hamster cell contains 5000 to 10,000 growing sites at any moment in the *S* phase (Painter *et al.*, 1966; Taylor, 1968). Since the amount of DNA in the nucleus of a diploid cell of the Chinese hamster is 7 pg at the G_1 stage (Huberman and Riggs, 1968), and there are 22 chromosomes, one can make an estimate of the number of units of a certain size in the chromosomes. Taylor (1968) estimated the maximum size to be between 200 and 400 μm based on the known growth rates for polynucleotide chains and the time required for the long arm of the X chromosome to replicate.

About the same time, Huberman and Riggs (1968) reported that cells blocked for 12 hours with fluorodeoxyuridine (FUDR) and released with thymidine-^{3}H of high specific activity had tandemly arranged labeled segments of DNA when spread by a procedure that allowed autoradiographs of extended DNA strands to be made. The center-to-center distance between labeled segments varied from 10 to 100 μm with most of the distances in the range 15–60 μm. When these investigators pulse labeled cells for 30 minutes and then removed the thymidine-^{3}H for another 30 minutes of growth, they noted many labeled segments with a tapering off of grains toward each end. This observation led them to propose that when initiation occurs at a site it typically moves in both directions from that point.

These experiments have recently been confirmed and extended by Callan (1972), utilizing cells of amphibians in culture. By studying two species, *Xenopus laevis* and *Triturus cristatus carnifex*, with quite different amounts of

DNA and differences in chain growth as well as length of *S* phase, he obtained some revealing evidence concerning units and patterns of replication. *Triturus*, with 29 pg of DNA per haploid set, has nearly 10 times the amount characteristic of *Xenopus* (3.1 pg per set of chromosomes). The rate of replication, that is chain growth in one direction, was found to be about 9 μm per hour at 25°C. This rate was based on the measurement of the length of labeled segments, many of which were tandemly oriented because of the stretching of DNA strands in preparing the autoradiographs (Fig. 1). The assumption was made that growth began at a site and proceeded in both directions, since a 2-hour labeling period followed by a 2-hour "chase" in which medium with thymidine-^{3}H was removed showed many labeled segments with a decreasing grain density at both ends (Figs. 2 and 3). As the thymidine-^{3}H was depleted, the specific activity of the DNA produced last would be expected to fall and thus produce the observed tailing off of the grain densities at the ends of labeled segments in the autoradiographs. From observations on cells blocked by FUDR for 20 hours before labeling, it was suggested that growth was initiated at some point and proceeded in both directions, as proposed by Huberman and Riggs (1968) for Chinese hamster and HeLa cells. The labeled segments were estimated by Callan to average about 36 μm after 2 hours' growth, and were about two times that length after 4 hours (Fig. 4). By considering these segments to be composed of two units, back to back, the growth rate was estimated. Center-to-center distance between labeled segments was about 60 μm, which was taken to be two replicating units initiated simultaneously.

Although *Triturus* DNA appeared to grow faster (ca. 20 μm/hr) the initiation sites were much further apart. The sites were so far apart that tandemly oriented labeled segments were not very common. The best estimate indicated that they were about four times as far apart as in the extended DNA of *Xenopus*. The *S* phase in *Triturus* is also about four times as long as that of *Xenopus*,

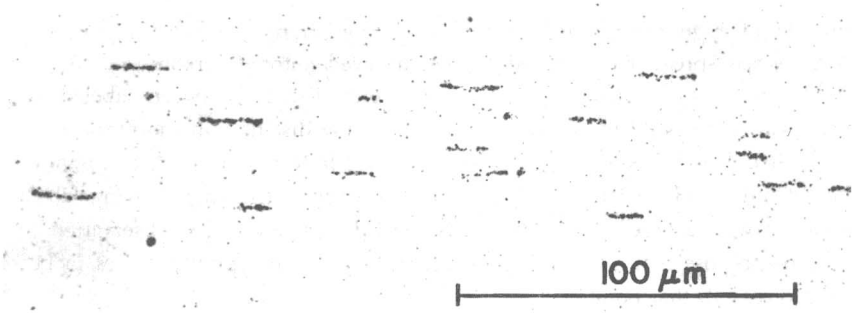
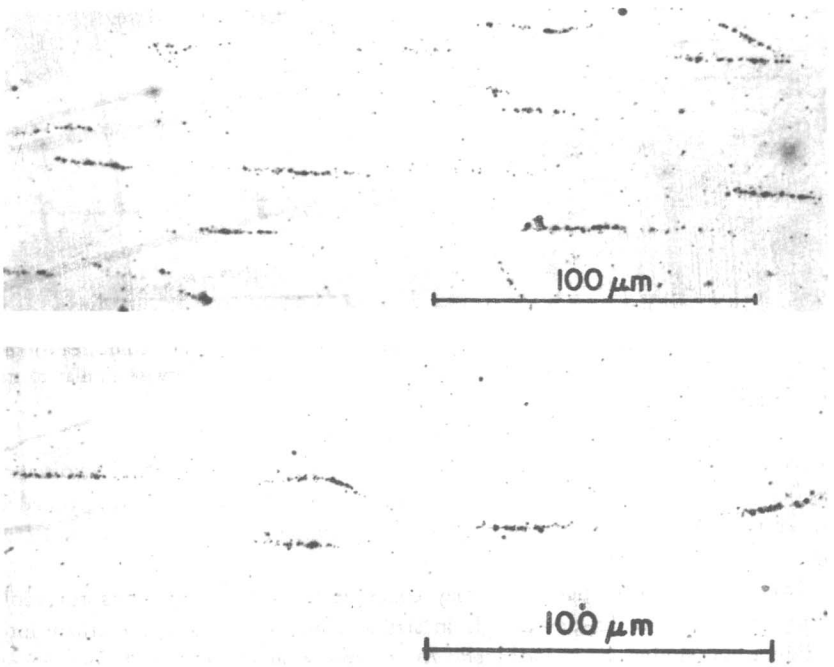


FIG. 1. Photograph of DNA fiber autoradiograph made from FUDR-treated cells of *X. laevis* which were pulse-labeled with thymidine-^{3}H for 2 hours at 25°C. The emulsion was exposed to the labeled DNA for 29 weeks. (From Callan, 1972.)



FIGS. 2 (*top*) and 3 (*bottom*). Photographs similar to that in Fig. 1 except that the cells had been grown for 2 hours in thymidine-^{3}H and then in medium free of labeled thymidine for 2 additional hours. Note the decrease in grain density at the ends of most segments. (From Callan, 1972.)

which is about 13 hours at 25°C. This means that nearly 10 times as much DNA can be replicated in the *S* phase of *Triturus* cells as in *Xenopus* cells with approximately the same number of units of replication (9 μm × 13 hours = 117 μm, and 20 μm × 52 hours = 1040 μm).

A comparison of DNA chain growth in spermatocytes with other cells of *Triturus* showed that the spacing of initiation sites is not necessarily constant within a species (Table I). The premeiotic *S* phase in *Triturus vulgaris at* 16°C is 9–10 days (Callan and Taylor, 1968), which is somewhat longer than the 2 days for somatic cells at 25°C. Callan's autoradiographic studies showed that at 18° the rate of chain growth, one-way replication, is about 12 μm per hour This is probably comparable to the 20 μm per hour measured at 25°C for somatic cells. Therefore the overall rate is not different in somatic and meiotic cells, but the initiation sites are farther apart in meiotic cells. However, embryonic cells during cleavage replicate their genomes in 1–2 hours at 18°C, that is, about 50 times as fast as somatic cells from adult tissues. Since the rate of chain growth

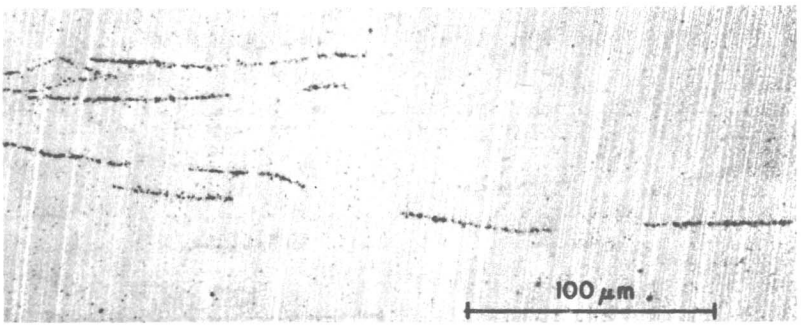


FIG. 4. Photograph of DNA strand autoradiograph produced from FUDR-treated cells of *X. laevis* grown for 4 hours in thymidine-^{3}H. Preparation was otherwise similar to that for the cells shown in Figs. 1–3. (From Callen, 1972.)

remains unchanged (about 12 μm per hour), the interval between initiation points is then as little as 24–48 μm, assuming that replication is bidirectional in the embryonic cells. The maximum size of the unit of replication is then 12–24 μm.

From these measurements one may conclude that regulatory units for replication in chromosomes are variable in size and may be even smaller than autoradiographic studies have indicated. An examination of structural components

TABLE I
PARAMETERS OF DNA REPLICATION IN TRITURUS[a]

Cell type	Length of *S* phase	Rate of chain growth (one-directional)	Sites of one-directional growth per haploid complement	Size of unit (μm)
Premeiotic spermatocyte	9–10 days (16° C)	12 μm/hour (18° C)	6,500	1,440
Somatic cell in culture	48 hours (25° C)	20 μm/hour (25° C)	19,500	480
Neurula	2–3 hours (18° C)	6–12 μm/hour (18° C)	312,000	15–30
Blastula	1 hour (18° C)	6 μm/hour (18° C)	1,560,000	6

[a] Approximations taken from Callan and Taylor (1968); Callan (1972) and personal communication. The data are from different species of *Triturus*, but the amount of DNA per genome is close enough to make pooled data useful for comparisons. The estimates of sites of growth and size of units are based on the minimum number of sites necessary to complete the complement at the given growth rates if growth at each site continues for one-half of the *S* phase, except in the blastula where growth for the whole *S* phase is assumed.

involved in replication by other means will no doubt be necessary to fully understand the situation.

III. Other Evidence for Bidirectional Initiation

Weintraub (1972) used a different technique to obtain evidence for bidirectional initiation and chain growth in chick embryo cells. By blocking with FUDR for 10 hours, 95% of the cells were accumulated in *S* phase. When these cells were pulse labeled for 5 minutes with BUDR and deoxyadenosine-^{3}H, the labeled single-chain segments had an average molecular weight of 2×10^6 daltons. When asynchronous cells were labeled in the same way, the labeled DNA averaged 15×10^6 daltons. The DNA from synchronized cells was of high density, indicating nearly complete substitution with BUDR, but that in asynchronous cells was very little changed in density. Using this as evidence that blocked cells accumulate labeled DNA during a subsequent pulse by semiconservative replication rather than by repair replication, Weintraub then proceeded to label such synchronized cells for 5 minutes with BUDR and then with thymidine-^{3}H for 15 minutes. Similar cells were labeled for the full 20 minutes with thymidine-^{14}C. DNA was extracted from both and centrifuged through an alkaline sucrose gradient after exposing it to ultraviolet light for 0–10 minutes. After 10 minutes' irradiation the DNA containing BUDR was reduced to about one-half the molecular weight of the marker DNA-^{14}C which was unaffected by the irradiation. Ultraviolet irradiation is known to sensitize DNA to alkaline breakage much faster if it contains BUDR rather than thymidine. From these experiments, Weintraub concluded that the BUDR segment was in the middle of the pulse-labeled DNA, and that initiation was bidirectional. However, he tempers the generality of this conclusion with the observation that initiation might be a statistical event dependent on the amounts of initiation proteins available. An increase in these proteins during FUDR treatment could be responsible for bidirectional growth, rather than a necessary polymerization mechanism.

The conclusion can be further criticized in that the reduction in molecular weight by irradiation would proceed during irradiation as observed, if the BUDR segments were localized mainly at one end of the ^{3}H-labeled segments but were also connected to unlabeled segments which had grown before pulse labeling with BUDR. Although Weintraub states that the cells were blocked at the interface between G_1 and *S* phase, he presented no critical evidence on this point. An examination of Chinese hamster cells in culture has shown that synchronized cells make 5–10% of their DNA before being arrested by FUDR treatment (Taylor *et al.*, 1973a). It is at least possible that this could happen in the chick cells used by Weintraub

Although it is clear from autoradiographs that some labeled segments grow

from both ends within 2 hours in the case of *Xenopus* (Callan, 1972), and within 30 minutes in the case of Chinese hamster cells (Huberman and Riggs, 1968), this pattern may not be a necessary one and may give little fundamental insight into the regulatory systems of DNA replication.

IV. Molecular Subunits of DNA

Although large functional subunits or replicons may be present in chromosomes, information to date does not allow one to identify these unequivocally. Evidence is accumulating which indicates that essentially all the DNA in a chromosome exists as a single duplex, as the semiconservative distribution of DNA originally suggested (Taylor *et al.*, 1957). However, at that time it seemed inconceivable that such long polymers could exist or be replicated without numerous interruptions. Within a short time evidence was obtained that the two semiconservatively distributed longitudinal subunits are of opposite polarity (Taylor, 1958, 1959), and the conclusion was reached that even large chromosomes consist of a single DNA duplex (Taylor, 1963b). However, the idea of some type of linker, or at least a change in polarity at intervals along the helix, was retained in these early models. With the failure to find any evidence of protein linkers (Macgregor and Callan, 1962), and the discovery of ligases which could repair nicks in DNA, the idea of linkers was abandoned in unineme models of chromosomes (Taylor, 1966, 1969a). Two additional types of evidence now make the unineme models almost a certainty. One is the demonstration by Laird (1971) that the rate of renaturation of DNA of *Drosophila melanogaster* is consistent with a high percentage of the genome being present in only one copy, that is, one unique DNA helix per chromatid. The other is a more recent and critical addition to the accumulating evidence. Kavenoff and Zimm (1973) used an improved technique for measuring viscoelastic recoil of long DNA strands and correlated these measurements with molecular weights. The method cannot be described in detail in the space available here, but the reader is referred to articles describing calibrations made with the DNA of *Escherichia coli* phages T7 and T2 and with whole genomes of *E. coli* (Chapman *et al.*, 1969; Klotz and Zimm, 1972a,b). Extrapolation of the measurements to *Drosophila* chromosomes, which are more than 20 times as large, leaves some uncertainty about the absolute size of the DNA molecules but makes it clear that probably only one exists per G_1 chromosome (Kavenoff and Zimm, 1973), or at the most two, considering the largest error in the measurement. The DNA was exhaustively digested with pronase without affecting the length, which appears to rule out any protein linkers in these long polymers. Other unusual linking elements and single-chain nicks would not be revealed, however. Even though some uncertainty remains in extrapolating to the absolute size of the molecules, the fit

with other measurements on the amount of DNA per chromatid by cytophotometric methods is remarkably close. For example, the viscoelastic technique indicates 41×10^9 daltons for the two largest chromosomes of *D. melanogaster*, while Rudkin's (1964) cytophotometric measurements of the amounts of DNA indicate that these same chromosomes have an amount that would make a duplex of 40×10^9 daltons for chromosome 2, and 43×10^9 daltons for chromosome 3. Comparison of lengths in a translocation strain of *D. melanogaster* with a longer chromosome indicates a corresponding increase in molecular weight to 58×10^9 daltons, while *D. americana* with chromosomes nearly two times as long has the largest DNA, with a molecular weight correspondingly higher, 79×10^9 daltons. The accuracy of comparative measurements allows the conclusion that the polymers are not interrupted at the centromeres. *Drosophila virilis* has telocentric chromosomes a little more than one-half the length of the two long metacentric chromosomes of *D. americana*. The corresponding molecular weights are 47×10^9 and 79×10^9 daltons, respectively. However, comparisons of molecular weights in wild-type *D. melanogaster* with an inversion strain making a nearly telocentric chromosome of chromosome 3 revealed no significant difference in the molecular weights of the largest polymers from the two strains.

Furthermore, the viscoelastic measurements of Kavenoff and Zimm (1973) indicate that the shear involved in cell lysis, even without agents to increase viscosity, are not sufficient to break polymers as long as 1–2 cm. Therefore it seems unlikely that the 100- to 150 μm segments produced when cells are lysed at pH 10.0–10.5 are the products of breakage by shear (Taylor, 1969b; Taylor *et al.*, 1970), although no measurements by the viscoelastic method were made at such elevated hydroxyl ion concentrations. Measurements of the sedimentation coefficients of DNA segments from chromosomes of Chinese hamster cells indicate that chain separation is difficult to attain, and sedimentation coefficients may be a poor indication of the length of single chains (Taylor, 1969b; Taylor *et al.*, 1970). However, sedimentation in denaturing solutions of sodium trichloroacetate and sodium perchlorate plus urea (Taylor *et al.*, 1970, and unpublished results) indicates that DNA has nicks about every 100 μm in both poynucleotide chains if the pH never falls below 10.5. If the proteins are digested at 37°C with excess pronase at pH 9.0 the nicks are produced about every 20 μm (Taylor *et al.*, 1970). It appears likely now that nuclease activity could be responsible for these nicks after lysis. Those nicks spaced at the 100-μm or longer intervals probably exist in the functioning cells, because these are present in DNA lysed at higher pH where enzymes are not likely to be active.

The existence of smaller functional subunits of DNA is indicated by several lines of investigation. These subunits are revealed by reannealing studies, the appearance of subunits which can be released during replication, and electron

microscopic studies after partial melting. They may never exist as separable components of the genome and are, therefore, joined into long polynucleotide chains at most stages of the cell cycle. Nonnucleotide linkages are not necessarily ruled out, but have not been demonstrated, and since most properties of the genome can be accounted for without assuming such linkages, we will assume the chromosome is two continuous complementary polynucleotide chains. Nicks are induced during replication, repair and possibly during transcription.

Britten, Davidson, and co-workers have presented evidence for three classes of DNA based on studies of rates of reannealing (Britten and Davidson, 1971; Davidson *et al.*, 1972). The first is a very fast reannealing fraction which may contain satellite DNAs clustered in restricted parts of the genome as well as a fraction widely distributed through the genome which they call the "zero time reannealing fraction." The second is intermediate repetitive DNA which is also widely scattered in the genome. The third is a fraction which anneals very slowly and is presumably composed of unique segments. A recent study of the genome of *X. laevis* utilized segments produced by shearing to the size classes averaging 250, 700, 870, 1500, and 3700 nucleotides in length. From the rates of reannealing of each of these fractions, which indicated the proportion of segments with intermediate DNA, they concluded that 50–55% of the genome consists of unique segments 900–1100 nucleotides in length separated by intermediate repeated sequences of 300 ± 100 nuceotides (Fig. 5). Another 25% consists of unique segments of 4000 to 8000 nucleotides in length separated by similar segments of repetitious DNA. The remainder of the genome appeared to be longer pieces of unique DNA and some very repetitious material. Using a quite different technique, Wu *et al.* (1972) annealed segments of intermediately repetitious DNA to long single chains of DNA from *Drosophila* cells and spread these by a modification of Kleinschmidt's (1968) technique. They then measured the contour lengths between unpaired tails from the intermediately repetitive DNA along the single chains. They also measured the paired double chain segments formed by the repetitive DNA with longer chains. From these measurements they estimated that unique segments about 750 nucleotides long were separated by repetitious segments of 150 to 200 nucleotides.

An interesting correlation with the above reports may be observed from measurements made by Evenson *et al.* (1972) of the distance between regions with low melting points in DNA of Chinese hamster cells and chick fibroblasts (Fig. 6). DNA was melted to a limited extent in 0.01 *M* sodium phosphate (pH 7.0) by holding it at 50°C for 10 minutes in the presence of 11% formaldehyde. The DNA was then spread by the method of Kleinschmidt (1968), and the length of the melted regions measured. The mean contour of the melted segments was a little more than 0.1 μm (300 to 400 nucleotide pairs) while the