Premature Chromosome Condensation of Synchronized Chinese Hamster Lung Cells

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PREMATURE CHROMOSOME CONDENSATION OF SYNCHRONIZED
CHINESE HAMSTER LUNG CELLS

A Thesis

Submitted to the Graduate Faculty of the New York State University
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by
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Premature Chromosome Condensation of Synchronized Chinese Hamster Lung Cells

Approved

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This thesis is respectfully dedicated to my wife, Mari E. Koerner Tucci, who sacrificed her own aspirations so that I might continue mine; and to Dr. Marlene Appley for her constant encouragement and support.
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ABSTRACT

The orderly, nonrandom pattern of nuclear chromatin is observable when it is condensed as visually distinct and structurally reproducible metaphase chromosomes. Interphase nuclei are susceptible to being induced to condense prematurely when by somatic cell fusion they become exposed to the influence of mitotic nuclei. The mitotic inducer has a differential effect on G1, S, and G2 chromatin and produces varied premature condensation products. In the following experiments metaphase-G1 fusions yielded single, elongated chromatids. The metaphase-S fusions resulted in pulverized condensation products. Metaphase-G2 fusions produced sister, elongated chromatids.

Sendai virus was used as the fusing agent in these experiments. The seed virus was injected into the allantoic cavity of 11-day chick embryos. Four days post injection the allantoic fluid was collected and titrated to give the desired virus concentration. The virus was subsequently inactivated by ultraviolet exposure. This inactive Sendai virus when suspended in medium with the Chinese hamster cells, produced cell fusion and the various premature condensation products.

The percentages of these various fusion products were able to be influenced by using synchronized cell populations. Thymidine, hydroxyurea and colcemid were the drugs of choice to bring about synchrony. Both thymidine and hydroxyurea have the capability to produce a transient inhibition of DNA synthesis. Colcemid interferes with the polymerization of microtubules and therefore arrests cells in metaphase. When synchronized populations were fused and compared to asynchronous population fusions,
INTRODUCTION

A low diploid number of chromosomes and the ease with which continuously propagating cell lines can be established, account for the wide use of Chinese hamster cells, *Cricetulus griseus*, in cytological investigations (Hsu and Zenzes, 1964). Hence the Chinese hamster lung (CHL) cell line was selected for the chromatin studies described in the text of this study. The first attempt to establish cell strains from this species was made by Ford and Yerganian (1958). However their results indicated a shift from the diploid chromosome number to a higher or lower than normal chromosome count. In 1961, Yerganian and Leonard were able to maintain the Chinese hamster cells in a diploid state for longer periods. Improved techniques and growth media were responsible for allowing these cultures to endure as diploid cells. In studies which require analysis of chromosomes, it is indeed desirable to have a stable cell line. Since a future goal of this work involves critical analysis of chromosomes and chromatin, a Chinese hamster line was a most appropriate choice. The diploid number of the Chinese hamster was demonstrated to be 22 by Hsu (1963). He furthermore, classified the 11 pairs into 4 natural groups with 2 pairs of large submetacentrics (group 1, pairs 1 and 2), 3 pairs of medium-sized submetacentrics (group 2, the six pair-3 and pairs 4 and 5), 3 pairs of medium-sized subteloctenrics (group 3, pairs 6, 7, and 8), and 3 pairs of small metacentrics (group 4, pairs 9, 10, and 11). The extensive chromosome studies previously reported, provided an excellent comparative basis for our Chinese hamster cytological investigations.
An early descriptive account of chromosomal events during division of both plant and animal cells was composed by Flemming in 1880. He recognized that the metaphase chromosomes arise from thread-like components in the interphase nucleus following a condensation process. In 1883, Van Beneden observed a linear pattern of chromomeres in Ascaris chromosomes. He also noted that this structure was duplicated in the daughter chromatids. For years the alterations that occur when interphase chromatin condenses to form the metaphase chromosomes was poorly understood. Recent research has evolved new theories concerning the phenomena of condensation, and of the decondensing process at telophase. Analysis of nuclear ultrastructure has established that interphase chromatin is composed of long bumpy fibers 200-300 Å thick (Ris, 1967). Such individual fibers are not able to be resolved with the light microscope. However, when they become closely packed, the aggregates as a whole become visible. Visible and invisible states of chromatin can be accounted for by alterations in the tightness of folding of such chromatin fibers (DuPraw, 1965). Transmission electron microscopy on whole mounted human chromosomes, first done by DuPraw (1965), enabled study of the chromatin fibers. He showed evidence for a folded fiber organization. In 1970, DuPraw demonstrated that chromatin from whole-mounted interphase and prophase nuclei revealed a fiber network with intermittent regions of denser fiber aggregations linked with many interconnecting fibers. Comings (1968) and Comings and Okada (1970, 1972) attributed structural order at these stages to specific attachment sites of the chromatin at the nuclear membrane. They suggested that these specific attachments might determine the pattern for the subsequent
folding of chromatin fibers. They further described a convergence of fibers at the annular pores of the nuclear membrane imposing an orderly pattern to chromatin condensation. The scanning electron microscope also contributed to chromatin structure theories. It was first used on human whole-mounted chromosomes in 1971 by Golomb and Bahr to demonstrate the 3-dimensional aspects of the surface-spread whole mounted critical point dried chromosomes. Existence of fiber bridges between chromatid pairs and among chromosomes was also demonstrated in that study. Further studies revealed the chromatin fiber to be arranged in two structural forms (Bahr et al., 1973). In one form the chromatin fibers appear to be arranged in parallel with the longitudinal axis of the chromatid. In the other structural forms demonstrated the chromatin fibers appear to be in a whorl of folded, looping fibers intimately associated with the longitudinal fibers. These whorls were shown to be comparable to the chromomeres of light microscopy in this same study. Differing stages of the condensation process show a change in ratio of longitudinal and chromomere fibers until at the end of condensation all of the fibers are in the whorl formations without intervening longitudinal fiber areas. Mass per unit length, diameter and unevenness or bumpiness are nearly the same for both longitudinal and chromomere fibers (Bahr et al., 1973). Alterations of the chromatin structure have been studied throughout the cell cycle using electron microscopy (Golomb and Bahr, 1974). They demonstrated that interphase chromatin begins to aggregate into prophase chromosomes that contain accumulations of chromatin whorls at complimentary positions along each chromatid. These chromatin whorls are again called
chromomeres. They further asserted that as prophase merged into metaphase, the chromomeres become less distinct and finally a condensed chromosome is observed. In contrast, interphase nuclei showed only a few parallel fibers and no chromomeres (Golomb and Bahr, 1974).

The importance of the functional aspects of chromomeres is evident when exploring models of genetic regulation of eukaryotic chromosomes as well as experimental data on the structural organization of chromosomal DNA and RNA units. The gene activation and regulation models of Britten and Davidson (1969), Georgiev (1969) and Davidson and Britten (1973) involve functions of genetic units as might be represented by a whorl or chromomere. These models all are based on the thought that the essential difference between prokaryotic and eukaryotic regulation are the more complex and probably much longer regulative segments in eukaryotic transcriptional units as compared to small bacterial operons as functional units. Sequence analyses of transcription products of eukaryotic cells have shown that the transcribed RNA is composed of two components: messenger RNA and the much longer heterogeneous nuclear RNA (Lewin, 1975). The mRNA is the copy of the producer gene, while the hn RNA is the copy of subsequent DNA at the end of producer unit. Based on the bacterial operon theory, the regulative segments are transcribed prior to the structural segments of producer genes. This means that the copy of regulatory segments must be at the 5' end of the total transcription product.

Georgiev (1969) proposed that the hn RNA represented the transcript of the regulatory segment of DNA. Crick (1971) suggested that the long regulative segments are located in chromomeric DNA.
In 1972, Paul further hypothesized that the activation of genetic units begins at one end of the chromomeric loop.

More recent chromatin studies have gone beyond the level of chromomeres to the composition of the fiber itself by proposing a model for the structure of DNA in chromatin sub-units. A substructure along the fibers was observed in electron micrographs of chromatin critical point-dried (Bram and Ris, 1971). Each sub-unit was proposed to have contained two turns of an inner coil, with a pitch of about 40 Å and an external diameter of 70 to 80 Å (Sahasarabuddhe et al., 1974). A larger amount of DNA was found to be wound around the inner coil at a diameter of about 150 Å (Bram, 1972). X-ray scattering and electron microscopy revealed the total length to be 600 to 700 Å or about 200 base pairs (Kornberg, 1974). Further studies indicated that the inner coil was protein rich and contained all of the histones except H 1. The H 1 histone was discovered to be associated with the outer coil (Baldwin et al., 1975).

This sub-unit has been termed a nucleosome (Oudet et al., 1975) or nu-body (Olins and Olins, 1974). Noll (1975) has suggested that the nucleosomes contain approximately 90 % of the total DNA in the eukaryotic genome. The nu-bodies have a DNA packing ratio of about 6.8:1, which is brought about by histone and DNA interactions.

Fusion of two cells in different phases of the cell cycle is a means by which the regulatory mechanisms for the initiation of DNA synthesis and mitosis can be examined and related to specific stages of the cell cycle. The orderly, non-random pattern of nuclear chromatin is observable when it is condensed as visually distinct and structurally reproducible metaphase chromosomes. The structural pattern of interphase chromatin, and the
orderly sequences by which chromatin condenses at prophase remain to be
determined and described. Through the experimental induction of pre-
maturely condensed chromatin, such systems might be studied. Interphase
nuclei are susceptible to being induced to condense prematurely when by
cell fusion they become exposed to the influence of mitotic nuclei (Rao
and Johnson, 1970). The mitotic cell has a differential effect on G1, S, and G2 chromatin, and produces varied premature condensation products
(Mazia, 1974). This premature condensation process occurs within 30-60
minutes, and apparently follows normal mitotic sequences (Johnson and Rao,
1970). Light microscopic observation of G1 chromatin condensation yielded
long and slender chromosomes with single chromatids. Such a configuration
was expected since DNA replication had not yet occurred in the G1 phase.
The condensation of S chromatin resulted in a fragmented appearance.
This phenomena has been previously referred to as chromosome pulveriza-
tion (Nichols et al., 1964; 1967; Sandberg et al., 1967; Norrby et al.,
1966; and Kato and Sandberg, 1967). Light microscopy studies showed the
pulverization effect to be distributed along the chromatin material. The
G2 prematurely condensed chromosomes were long and slender, but, consisted
of two chromatids. The appearance of sister chromatids in G2 chromosomes
is consistent with cell cycle information, since DNA replication has been
completed.

Sendai, a myxovirus, fuses cells together in the course of infection.
Okada (1962) described the massive fusion of Ehrlich ascites mouse tumor
cells to each other within minutes after the addition of Sendai virus.
Cells were first attached to one another by mutual adherence to the same viral particles. Fusion soon followed this event. Harris and Watkins (1965) showed that inactivating Sendai virus with ultraviolet irradiation prevented it from replicating, but did not prevent fusion. The cell fusion mediated by viruses is essentially a membrane phenomenon. Sendai viruses are absorbed on the cell membrane and cause an ill defined organizational alteration enabling membrane fusion to occur and multinucleated cells to be formed (Okada, 1962; Harris and Watkins, 1965, 1966; Harris, 1965, 1970). The infectivity of this virus was destroyed by doses of ultraviolet light (Coon and Weiss, 1969). The Sendai virus is a member of the para-influenza group of myxovirus. It is pleomorphic, roughly spherical, and an RNA virus which is surrounded by a lipoprotein envelope (Fenner and White, 1970). The fusing ability resides in the viral envelope, not in the nucleic acid component. Even fragments of this viral envelope are capable of producing some fusion. Removal of the lipid from the viral membrane completely abolishes the fusing ability (Cohen, 1969). A high enough dose of Sendai virus must be present to insure a high multiplicity of particles per cell, thus producing cellular clumping. The exact cell surface changes during fusion are not clear, but clumping takes place within a few seconds at 4°C (Takagiet al., 1969). This suggests that cell surface changes at this stage might not be caused by enzymatic activity. Fusion does not occur until the temperature is raised, preferably to 37°C (Scheebergen and Harris, 1966). Evidence has been raised to support the view that fusion is an energy-requiring reaction (Harris, 1965). The presence of calcium ions also appears to be essential (Harris 1965, 1970).
Cell fusion may be completed in less than 5 minutes at 37°F.

All of the cell suspensions in this study were fixed in 3:1 (absolute ethanol: glacial acetic acid). The slide preparations were then air-dried and subsequently stained in either Giemsa for light microscopy or acridine orange for fluorescent microscopic observation. The Giemsa stain is widely used to demonstrate chromatin material. This stain is an extremely heterogeneous mixture which is prepared by mixing the acid dye eosin Y, a tetrobromo compound usually contaminated with lower homologues, with the basic dye methylene blue and certain of its demethylated and deaminated derivatives (e.g. azur A, thionin, and methylene violet bernthasen) as described by Lillie (1969). Chromatin material is stained a violet-blue color following exposure to Giemsa. Acridine orange was introduced into microscopy by Bukatsch and Haitinger as early as 1940, and since has been amply used in cytology. The spectrum of acridine orange bound to native DNA is essentially the same as that of the dye itself with a maximum emission of 535 mm (Rigler, 1966). This binding of the double-stranded DNA is attributed to the orthochromatic monomer form of the dye (Moutschien, 1976). Chromatin fluoresces a yellow-green color with acridine orange.

The aim of this project is to achieve synchronized cell populations. Synchronized cell populations can be a most useful tool in cytological investigations. Using autoradiographic techniques, Stanners and Till (1959) determined a time relationship between DNA synthesis and mitosis in L-strain mouse cells cultivated in vitro. They found that L-cells, following mitosis (M), were characterized by a long non-synthetic phase (G₁) involving half the generation time, followed by a DNA synthetic period (S).
with a duration about one-third the generation time, followed by a short premitotic, nonsynthetic period (G₂) which lasted for about one-fifth the generation time. Further studies by Stanner and Till revealed the probability of a cell being in any part of the cell cycle was proportional to the duration of that part of the cycle. Similar studies on the Chinese hamster cell lines yielded the generation time as well as the duration of the specific phases (Kato and Puck, 1967; Kato and Puck, 1968; Kato et al., 1969). From these studies and consistent results from our experiences, the generation time of the Chinese hamster lung line used in these experiments was approximately 12 hours. The durations of the different phases were as follows: G₁-3.0 hours, S- 6.0 Hours, G₂- 2.5 hours, and M- 0.5 hours.

It has been shown that thymidine at a high enough concentration inhibits DNA synthesis (Xeros, 1962). In these experiments 2mM thymidine was employed. Xeros explained the block in DNA synthesis by the capacity of excess thymidine to inhibit the formation of deoxycytidine triphosphate from cytidine-5'-phosphate (1962). After 24 hours, the thymidine is removed and the cells allowed to proceed through S phase to a somewhat synchronous mitotic burst. Using this method however, allowed cells to be trapped at whatever stage of the S period they were in when the excess thymidine was added. In 1964, T. Puck greatly improved the synchrony of cells by establishing the double thymidine block method. He postulated that the thymidine could act in either of two ways. It might prevent cells from entering the S phase or it might act to prevent DNA synthesis
at any point in the S period. His experiments showed the latter to be true, and he subsequently devised the double phasing method. After 24 hours in an excess of thymidine environment, some cells were distributed normally throughout the S period and the remainder were blocked at the end of G\textsubscript{1} just prior to DNA synthesis initiation (S phase). The thymidine is then washed out and the culture incubated for 8 hours at the end of which all the cells have passed through S. Thymidine is then readministered to the cells and the culture incubated for 14 additional hours. At this point, the culture consists of a highly monophasic population collected at the G\textsubscript{1}-S border. After the thymidine is removed, the cells synchronously pass through S.

An alternative method was proposed in which mitotic cells could be detached by shaking growing cultures, and these mitotic cells recovered and then plated out. Since cells in mitosis round up and become less firmly attached to a surface (Sinclair and Morton, 1963; Robbins and Marcus, 1964; Lindahl and Sorenby, 1966; Peterson et al., 1968) such a technique was feasible. An even simpler method for obtaining synchrony was described for separating mitotic or early G\textsubscript{1} cells by Shall and McClelland (1970). Cells that had just divided were smaller and thus fell less rapidly through a column of medium than do cells in other parts of the cell cycle. When the top 10 o/o of the column cells were recovered, they were found to be highly synchronous in early G\textsubscript{1} (Shall and McClelland, 1970).

Stubblefield (1964) discovered that Chinese hamster cells treated with colcemid (0.06 mg/ml) were not permanently blocked at metaphase, but overcame the inhibition about 12 hours later. Consequently in 1965,
Stubblefield and Klevecz successfully attempted to reverse colcemid inhibition simply by removing the drug after treatments of 2 hours. Metaphase cells were able to be removed from the monolayer culture by a technique described by Terasima and Tolmach (1963). Upon incubation of the metaphase cells in the absence of colcemid, mitosis resumed with anaphase occurring about 25-30 minutes later in most cells (Stubblefield and Klevecz, 1965).

Along with colcemid and thymidine, a third drug, hydroxyurea, was frequently used as a cell synchronizing agent (Sinclair, 1965 and 1967). Hydroxyurea had a differential effect on cultured Chinese hamster cells that were at different stages of their cell cycle. Cells that were synthesizing DNA at the time of exposure to the drug were lethally damaged (Sinclair, 1967). Cells which were in the other phases of the cycle progressed until they reached the $G_1-S$ border, at which point they were prevented from entering $S$ period (Sinclair, 1967). Upon removal of hydroxyurea, those cells which were at the $G_1-S$ border synchronously initiate and progress through the DNA synthetic stage of the cell cycle. Sinclair (1968) used $2\text{mm}$ hydroxyurea as a synchronizing concentration. In this same report Sinclair stated that cells in $G_1$, $G_2$ or $M$ phases of the generation cycle treated with the drug showed no chromosomal aberrations, while cells treated in the $S$ phase became moribund and eventually lysed. Hydroxyurea probably inhibited DNA synthesis in mammalian cells by interference with the enzyme ribonucleoside diphosphate reductase which reduces ribonucleotides to deoxyribonucleotides (Skoog and Nordenskjöld, 1971; Vogler et al., 1969).

A method for improving synchrony in the $G_2$ phase of the cell cycle
was devised by Pederson and Robbins (1971). Synchronization by selective detachment of mitotic cells from monolayers (Robbins and Marcus, 1964; Robbins and Scharff, 1966) or by exposure of random cells to 2 mM thymidine (Xeros, 1962; Bootswana et al., 1964) yielded populations which lost their synchrony by the time they reached the $G_2$ phase. Pederson and Robbins sought to better $G_2$ synchrony by selective detachment of cells from monolayers. These cells were allowed to pass through the subsequent $G_1$ phase in the presence of 2 mM thymidine. Following removal of thymidine, the cells moved through $S$ and $G_2$ with a high degree of synchrony (Pederson and Robbins, 1971).

The ultimate goal of this laboratory is to ultrastructurally compare fibers of both normally condensed and prematurely condensed chromatin. Before such a goal can be reached, preliminary experiments need be undertaken. Tissue culture techniques needed to be devised and standardized. Also, Sendai virus had to be cultivated since this virus was to be used as the fusing agent. The premature chromosome condensation experiments of Rao and Johnson needed to be repeated. Finally, to select for a specific fusion product, synchronized and timed cell populations had to be established. These preliminary experiments were the problems that this thesis project sought to solve.
MATERIALS AND METHODS

Cells. The diploid cell line, Chinese hamster lung (CHL) was obtained from the American Type Cell Culture Collection, Rockville, Maryland. The cells were cultivated at 37°C in RPMI no. 1640 medium (Grand Island Biol. Co.) supplemented with 10 o/o fetal calf serum (Grand Island Biol. Co.) and 1 o/o of 100x penicillin-streptomycin solution (100 ug/ml). The monolayer cultures were grown in sterile 75 cm², 250 ml plastic disposable tissue culture flasks.

Subculturing of the stock culture was performed to propagate the cell line. 3 mls of .25 o/o trypsin (Grand Island Biol. Co.) was warmed for each flask to be subcultured. The trypsin was warmed to optimal temperature. The medium was decanted from the flask containing the cells and the trypsin added for a period of one minute. After one minute the trypsin was poured off and the flask placed in an incubater at 37°C for 10 minutes. Then 20 mls of supplemented medium was added to the flask followed by gentle shaking to remove cells from the flask’s surface and place them in suspension. Using the hemocytometer, a cell count was done to determine the number of cells in suspension. The cells were diluted to 10⁷ cells/ml of medium and 20 ml aliquots placed in each T-flask.

Cell Counts. With a sterile Pasteur pipette, a drop of the cell suspension to be measured was placed onto the hemocytometer slide with the appropriate coverglass, being careful not to overflow into the trough. The slide was placed under the microscope at a power adequate for cell counting and the number of cells were counted in each of 4 sets of 16 squares which were
bordered on two adjacent sides by close parallel lines. The total of all 4 sets were added and divided by 4. This gave an average cell count. This average was multiplied by $10^4$ to obtain the number of cells per ml. The equation \[
\frac{\text{total cells}}{4} \times 10^4
\] adjusted the dilution of the suspension to the correct or desired concentration in cell/ml units.

cultivation, freezing and replating techniques needed to be devised. Using the hemocytometer, the cells were placed in a 10$^7$ cells/ml concentration in medium. To this was added glycerin to achieve a 10% solution (10 mls glycerin to 90 mls suspension.) 3 to 5 mls of this suspension was placed in sterile vials and the vials sealed. The vials were placed in a refrigerator for 3 hours, then in the freezer until frozen, and finally in the deep freeze at -70°C.

To neplate frozen cells, the ampule was placed directly into a water bath at 37°C with vigorous shaking. Thawing was to be rapid (within 40-60 seconds). As soon as the contents were melted the ampule was removed from the water bath and immersed in 70 o/o ethanol at room temperature. All operations from this point on were carried out under aseptic conditions in a sterile hood. The neck of the ampule was scored with a sharp file which was previously immersed in ethanol. The neck of the ampule was then broken between several folds of a sterile towel. Contents were then transferred to a sterile culture flask. A sterile pasteur pipette was used for this purpose. The cell suspension was then diluted with the supplemented RPMI 1640 medium previously described. One day after thawing, the culture
medium was changed in order to expedite removal of the glycerin.

**Virus Cultivation.** Air pockets were marked with a red crayon on 10 day old fertile eggs, and an area free of veins was likewise indicated. Eggs were placed in a high humidity incubator at 37°C with air pockets up. From this step on all procedures were carried out using sterile techniques to avoid secondary infections. On day 11, all eggs were swabbed with 70% ethanol. In the designated area of each egg a hole was bored through the egg shell about 1/4 inch in diameter. Precaution was taken not to pierce the shell membrane. The seed Sendai virus, acquired from Roswell Park Memorial Institute, was diluted 1/100 and 1/1000 in modified Hank's solution. Virus was injected using a 1 ml tuberculin syringe and a 23 gauge, 3/4 inch needle. A 0.1 ml quantity of virus was placed into the embryonic fluid of the allantoic cavity. 36 eggs were inoculated, 18 with 1/100 dilution and 18 with 1/1000 dilution of virus. Holes were sealed with parafin, and eggs were again incubated as described previously for 3 days. On the evening of the third day, all eggs were refrigerated overnight.

In order to harvest virus, eggs were swabbed with 70% ethanol on the morning of the fourth day post inoculation. The parafin seals were removed and larger holes were bored in air pocket regions of shells. Precaution was again taken not to pierce the shell membrane. With a 10 ml syringe and 20 gauge 1.5 inch needle, fluid was withdrawn from the allantoic cavity by inserting the beveled edge needle through and just under the shell membrane. The egg was positioned almost horizontally and the needle was lifted up against the shell membrane. The needle was dipped in alcohol.
and flamed between each use. The fluid withdrawn, which should be white and cloudy, was kept iced in sterile 35 ml centrifuge tubes. Cell debris was removed by spinning down solutions in a clinical table top centrifuge. The supernatant was decanted into sterile graduates and the total volume measured. About 10 ml was set aside for future use as seed virus and for subsequent virus titration. The remaining virus was centrifuged in sterile 30 ml plastic tubes at 20,000 RPM for 30 minutes on a Rotor 30 of a Spinco Model L centrifuge. The supernatant was then discarded and the remaining pellets were iced until the titration procedure was completed. Following titration, virus was resuspended to obtain 20,000 hemagglutinating units per ml.

**Virus Titration.** 5 to 10 ml of fresh heparinized chicken blood was obtained. After settling in cold the plasma was removed and the remaining cells were suspended in Hank's without glucose. The suspension was centrifuged and the pellet was washed two additional times with the Hank's. Finally a 0.5 c/o red blood cell solution was made in Hank's without glucose. The stock virus was now diluted with Hank's without glucose. The stock virus was now diluted with Hank's without glucose to $\frac{1}{100}$, $\frac{1}{500}$, $\frac{1}{1000}$, $\frac{1}{1500}$, $\frac{1}{2000}$, $\frac{1}{3000}$, and $\frac{1}{4000}$ solutions. These dilutions were kept cold. A series of test tubes were set up, each containing 0.5 ml of the above virus dilutions along with a blank containing only 0.5 ml of Hank's without glucose. These test tubes were set up in duplicates. To each tube was added 0.5 ml of the red blood cell solution. All tubes were placed in a rack and gently shaken. After two hours of refrigeration the tubes were checked for agglutination. The virus particles attach to the membranes of red blood cells.
Since the virus are capable of joining to more than one cell, a sufficient quantity of virus could bring about attachment of many red blood cells and produce clotting. This process is termed hemagglutination. The highest dilution of virus which causes any detectable clotting is defined as the agglutination barrier. The dilution at this barrier determined the existing-hemagglutinating unit (HAU) of the stock solution. This was adjusted to the desired 20,000 HAU/ml. The virus solution was subsequently poured into the sterilized vials with 3mls in each. The vials were tightly capped and placed in the deep freezer at -70°C.

Virus Inactivation. The virus solution was spread into a thin layer in a shallow petri plate. While continuously swirling the plate, the virus was exposed to a 15 watt germicidal ultraviolet lamp at a distance of 30 centimeters. After four minutes of exposure, the virus suspension was returned to the proper vial. Some suspension was used to test for inactivation. This was done by reinjecting 10 day chick embryos and cultivating as previously described. After harvesting, a titration was performed to assay for any viral multiplication. If virus don't multiply inactivation can be assumed to be successful.

Synchronization. In order to place the CHL cells into the G1 phase of the cell cycle, reversal following colcemid inhibition method as described by Stubblefield and Klevecz (1965) was adapted. Chinese Hamster lung cultures were exposed to medium for 18 hours after subculturing. At the end of this time, the medium was removed by centrifugation and can be referred to as conditioned medium. Colcemid was added to 20 ml of conditioned medium for a final concentration of 0.06 mg/ml. Cells were exposed
to the colcemid supplemented medium for two hours after which metaphase cells could be removed by treating the monolayer for 30 seconds with 0.25 o/o trypsin at 4°C. Moderate agitation after 30 seconds detaches metaphase cells. The trypsin-cell suspension was immediately diluted with Hank's solution (Grand Island Biol. Co.) and centrifuged. The CHL cells were then resuspended in conditioned medium to avoid a lag phase, and incubated at 37°C. Since mitosis resumes in most cells with anaphase occurring in about 30 minutes (Stubblefield and Klevecz, 1965), the majority of cells would be in G₁ one-hour post-colcemid release. Earle et al. (1954) have studied quantitatively the minimum number of L-strain fibroblasts required in a given volume of medium in order to insure growth. Their investigations have emphasized the necessity for "conditioning" by the living cells of the nutrient media currently employed in tissue culture before multiplication can be supported (Puck and Marcus, 1955). The time required by the cells to condition the medium is the lag or latent period. Data obtained from Sanford et al. (1943) indicate that the culture medium supplemented with horse or fetal serum, is far from optimal for the growth of cells until the medium has been physically or chemically conditioned or altered by the cells.

To synchronize cells into the S phase, hydroxyurea was the drug of choice. Again CHL cells were grown for 18 hours after subculturing at which time hydroxyurea was added to the medium at a final concentration of 1mM. The medium was poured off after 6 hours of exposure and the cells collected using 0.25 o/o trypsin exposure at room temperature. Cells were resuspended in conditioned medium and incubated at 37°C to allow cells on G₁/S border to enter the S phase.
To select for cells in the G₂ phase, the colcemid inhibition reversal method was followed by a thymidine block. This technique was first illustrated by Pederson and Robbins in 1971. As previously described, subcultured cells were grown for 18 hours after which they were exposed for 2 hours to conditioned medium supplemented with 0.06 ug/ml of colcemid. CHL metaphase cells were detached with moderate agitation and resuspended in conditioned medium supplemented with 2 mM thymidine. After a 4 hour exposure the thymidine medium was poured off and conditioned medium was added. In approximately 6 hours, most of the cells were at the S/G₂ border.

Cell fusion. Ultraviolet-inactivated Sendai virus, containing 20,000 hemagglutinating units (HAU) per ml in glucose-free Hanks' balanced saline as a stock virus solution, was used for cell fusion experiments. Metaphase cells were harvested by 30 second 25 o/o trypsinization at 4°C followed by moderate agitation. G₁, S, G₂ cells were harvested by 2 minute 25 o/o trypsinization at room temperature. Four sets of experiments were performed. One set involved fusing metaphase cells with asynchronous cell population. A second set was done by fusing metaphase cells with cells synchronized in the G₁ phase. The third and fourth sets were achieved by fusing metaphase cells with S phase cells of G₂ synchronized cells respectively. The following protocol for cell fusion was the same in all four sets. Following trypsinization, 6 ml of Hanks' balanced saline was added to the flask with moderate agitation. The cell suspension was centrifuged and rewashed in Hanks's. Cells were finally resuspended at a concentration of 10⁷ cells/ml.

0.5 ml of the metaphase suspension and 0.5 ml of the second type of suspen-
sion and 0.5 ml of the second type of suspension was placed in a test tube in an ice bath at 4°C. To this 1 ml suspension was added 0.1 ml of the 20,000 HAU virus suspension (thus 2000 HAU/ml). This mixture was kept in the ice bath for 10 minutes. At this time the test tube was placed in a water bath at 37°C for 10 minutes with very gentle agitation to keep cells in solution. After ten minutes, the mixture was diluted with 6 ml of Hanks' balanced saline to bring volume up to 7 ml. The test tube was now kept in the 37°C water bath for 20 additional minutes with a gentle shaking every four minutes to keep cells in suspension. Cells were then collected by brief centrifugation at room temperature and prepared for chromosome spread technique.

Chromosome Spreads. The cell suspension was placed in a test tube and centrifuged as before to obtain a cell pellet. The fluid was decanted off and 0.6 ml of sodium citrate (15 mM) was added to hypotonically treat the cells. Sufficient swelling occurs after 7 minutes exposure to the sodium citrate solution. To this suspension was added 0.4 ml of ethanol: glacial acetic acid (3:1) as a fixative which was freshly prepared. This mixture was centrifuged and to the pellet, following decanting, was again added 3:1 fixative for a ten minute period. After centrifugation, the cells were resuspended in a couple of drops of 3:1. Slides were made by dripping a single drop of this suspension onto a cold precleaned slide. Slides were then allowed to air dry.

Staining. Air dried slides were stained either with acridine orange fluorescence or Giemsa. Both stains were obtained from Grand Island Biological Company. Slides were stained in Giemsa (ph 6.8) for 1.5 hours,
RESULTS

Virus. A total of 255 ml of virus solution was obtained from the inoculated chick eggs. From this 5.0 ml was removed for titration calculations. Dilutions of this solution were set up as follows: 1/100, 1/500, 1/1,000, 1/1,500, 1/2,000, 1/3,000 and 1/4,000. The 1/2000 dilution was demonstrated to be the agglutination barrier in both sets of dilutions. Thus, the remaining 250 ml of suspension had to be concentrated by a factor of 10 to obtain the desired 20,000 HAU. The suspension was centrifuged and virus pellet resuspended in Hanks solution to reach a final volume of 25 ml.

Following inactivation of the 20,000 HAU virus suspension, 0.1 ml of 1/100 and 1/1,000 dilutions were prepared. These were injected into the embryonic fluid of several eggs. After the appropriate incubation period, embryonic fluid was collected and tested by the viral titration. No detectable agglutination barrier was noticed. All suspensions were similar to the controls. It was determined that virus inactivation with ultraviolet light was efficiently performed.

Cell Fusion. The Chinese hamster lung cells were easily fused utilizing the Sendai virus as the fusing agent. Too large a virus count resulted in massive clumping of cells, while too few virus brought about small amounts of fused cells. All three metaphase-interphase types of fusion, which were described by Rao and Johnson (1970), were present in the slide preparations (i.e. M/G₁, M/G₂, and M/S). The percentages of each type of fusion varied according to the phase of the cell cycle that a cell was in when fused with a metaphase cell. For this reason, syn-
chronized cell populations were utilized. An asynchronous cell population was fused with one arrested in metaphase to serve as a control. Results were recorded in table 1. A M/M fusion was one in which two cells, both in metaphase of the cell cycle, were fused. The result of such a fusion was that both sets of chromosomes appeared normal with condensed sister chromatids in each chromosome. A M/G₁ fusion occurred when a metaphase cell and a cell in G₁ were fused. Metaphase chromosomes appeared normal, but, the G₁ chromatin condensed into less compact, chromosomes which consisted of only one chromatid. Such a phenomenon can be explained by the fact that in the G₁ phase, DNA replication did not yet occur. Thus sister chromatids could not have existed in the chromosomes (Fig. 1). The M/S fusion resulted when a metaphase cell unites with a cell in the S phase. Here again, the metaphase chromosomes remained condensed as normal, but, the prematurely condensed S chromatin formed a pulverized pattern (Fig. 2). Such a pattern was related to the uncondensed portions of DNA which at the time of fusion were undergoing replication alternating with segments of DNA which had already completed replication or had not yet begun. Finally a M/G₂ fusion was produced when a metaphase cell was fused with a cell in the G₂ period. As previously indicated, the metaphase chromosomes remained compact, but, the G₂ chromosomes exhibited a decondensed, elongated structure very similar to the G₁ prematurely condensed chromatin. However, in the case of the G₂ prematurely condensed chromatin sister chromatid exchanges were present. (Fig. 3). Such results were consistent with the literature since DNA replication was completed when these G₂ cells were fused.
Synchronization. One of the future aims of this work was to be able to study chromatin structure and function. Having successfully repeated the premature chromosome condensation experiments of Rao and Johnson (1970), a useful tool capable of studying chromatin was developed. However, in order to refine this system, synchronized populations were fused in an attempt to increase premature chromosome condensation of the chromatin from the desired phase of the cell cycle. Results were recorded in table 1. The counts of specific fusion types were tabulated in the following manner: (1) each type of fusion was done 4 times; (2) 25 fusions were counted from each of the 4 experiments (thus 100 total fusions were counted for each type; (3) fields of slides were randomly selected; (4) only bikaryons with at least one metaphase set of chromosomes were counted; (5) once a field was randomly selected, all bikaryons meeting the above qualifications were counted in that field. In instances when classification was doubtful because of poor staining, that fusion was not counted.

As a control, an asynchronous cell culture of the Chinese hamster lung line was fused with a metaphase population of the same. As can be seen in Table 1, 41 o/o of the counted fusions were of the metaphase-metaphase (M/M) sort. Such a high percentage was expected since approximately one-half of the cells were in the metaphase period at the time of fusion. The cells from the metaphase population comprised one-half of the fusion suspension while some cells from the asynchronous population also could have been in metaphase. Of the metaphase-G1 (M/G) types, 18% were observed. Such a percentage was also within explainable limits, since G1 was about
3.0 hours duration of a 12.0 hour cell cycle. The metaphase-S (M/S) kind of fusion occurred in 27 o/o of this type. S period was 6.0 hours long in the Chinese hamster lung line. Thus, this percentage was again consistent based on cell cycle phase lengths. Of the bikaryons counted, 140/o exhibited the metaphase - G2 type of fusion. Since G2 was 2.5 hours duration, this was once again explainable. Using these figures as controls, the degree of synchronization was determined.

After G1 synchronization and fusion of this population with metaphase cells, the following percentages were demonstrated: M/M - 42 o/o; M/G1 - 39 o/o; M/S - 14 o/o and M/G2 - 5 o/o. This was a significant rise in the M/G1 type from 18 o/o to 39 o/o with a coincidental drop in the M/S and M/G2 types. The M/M type remained about the same (41 o/o). Such a result was accounted for by the number of metaphase cells present in the fusion suspension. Results from the S phase synchronization and metaphase cells yielded these percentages: M/M - 44 o/o; M/G1 - 9 o/o; M/S - 47 o/o; and M/G2 - 0 o/o. A dramatic increase in the M/S types from 27 o/o to 47 o/o was observed. Also, a drop in M/G1 from 18 o/o to 9 o/o coupled with a decrease in M/G2 types from 14 o/o to 0 o/o confirmed the success of our synchronization techniques. Again the M/M type of fusion remained relatively constant at 44 o/o. Finally the effects of the G2 synchronized cells with the metaphase population was recorded. There were 36 o/o M/M, 3 o/o M/G1, 18 o/o M/S, and 43 o/o M/G2. Here the great increase of M/G2 from 14 o/o to 43 o/o was demonstrated. Furthermore, M/G1 decreased from 18 o/o to 3 o/o while M/S declined from 27 o/o to 18 o/o. Once again the change in M/M types was insignificant.
In each case shown in Table 1, an increase was demonstrated in the type of fusion desired. Additionally, since the numbers of metaphase cells remained relatively constant in all experiments, no significant M/M variation was noted. It was assumed that these increases in the selected type of were directly a result of the synchronization methods. The graphs in Figure 4 compare asynchronous fusion percentages of each type with the corresponding synchronous fusion percentages.
<table>
<thead>
<tr>
<th>Fusion Type</th>
<th>M/M</th>
<th>M/G₁</th>
<th>M/S</th>
<th>M/G₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metaphase + Asynchronous</td>
<td>41</td>
<td>18</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>Metaphase + G₁ Synchronized</td>
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<td>39</td>
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<td>5</td>
</tr>
<tr>
<td>Metaphase + S Synchronized</td>
<td>44</td>
<td>9</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>Metaphase + G₂ Synchronized</td>
<td>36</td>
<td>3</td>
<td>18</td>
<td>43</td>
</tr>
</tbody>
</table>
Figure 1a: normal Chinese hamster lung karyotype.

Figure 1: Fusion of a metaphase cell with a cell in the G1 phase of the cell cycle.

Figure 2: Fusion of a metaphase cell with a cell in the S phase of the cell cycle.

Figure 3: Fusion of a metaphase cell with a cell in the G2 phase of the cell cycle.
FIGURE 4

Comparison of Asynchronous and Synchronous Fusion
DISCUSSION

Considerable evidence has been gathered with autoradiography which indicates that replication of chromosomes is regulated in such a way that specific fractions or segments are replicated at specific times in the S Phase. The replication of specific segments of chromosomes at limited time intervals in the S phase was indicated by autoradiographic studies using the X chromosome, the Y chromosome, and selected autosomes of the Chinese hamster genome (Taylor, 1960). Certain segments were found to be replicated in early S phase, while others were replicated during late S. Follow up studies with synchronized cells indicated that the segments which were found to replicate late or early in S did so at the same time in subsequent S periods. These results indicated that there was a programming for early or late synthesis, and further suggested that there was a regulated sequence for all of the genome over the S phase (Braun et al., 1965; Mueller and Kajiwara, 1966; Taylor et al., 1971). Autoradiographic studies indicated that chromosomes had many initiation sites. Some chromosomes might have been labeled throughout their entire length during a short 10 minute interval of the S phase, while others were labeled in only a few sites during this time interval (Taylor, 1960). Measurements based on autoradiographic data allowed the estimate that a Chinese hamster cell contains 5,000 to 10,000 growing sites at any given moment in the S phase (Painter et al., 1966; Taylor, 1968). Furthermore, Huberman and Riggs (1968) reported that cells blocked for 12 hours with fluorodeoxyuridine and released with thymidine-3H of high specific activity showed a tandem arrangement of segments of DNA when spread by a procedure that allowed autoradiographs of extended DNA.
strands to be made. Further observations led them to propose that when initiation occurs at a site, it typically moves in both directions from that point. Thus DNA replication seems to proceed by the production of many short segments as has been demonstrated in both bacteria and cells of eukaryotes (Sakabe and Okazaki, 1966; Okazaki et al., 1971; Schandl and Taylor, 1969).

An analysis of the replication and integration of segments of DNA into chromosomes may prove to be one of the best ways to obtain evidence about the organization of the genome. With this concept in mind, this laboratory set out to study chromosomes and chromatin with a new method of cell cycle analysis, that of Premature Chromosome Condensation (PCC) using somatic cell fusion mediated by Sendai virus. Our future goal is to observe whole mounted chromatin in different phases of the cell cycle from both control cells and prematurely condensed cells. The significance of synchronized populations and standardization of the premature chromosome condensation technique of Rao and Johnson (1970) was of the utmost importance to obtain future experimental data. Therefore these techniques needed to be tested and established in this laboratory.

Establishing the virus preparation and titration procedures were most successful. Not only was an understanding of the techniques gained, but also an awareness of the caution which must be maintained in handling a potent viral agent such as the Sendai virus. The inactivation with ultraviolet exposure proceeded as scheduled and that too was successfully executed as demonstrated by the virus' inability to multiply in a suitable host.
organism. Enough virus was generated to supply this laboratory for several years of fusion experiments.

Upon fusion of metaphase populations with either the G₁, S, or G₂ phase cells, the predictable premature condensation product was obtained in each instance. The experiments of Rao and Johnson (1970) were thus able to be consistently repeated. It has been amply documented by different approaches that premature chromosome condensation in fused cells represents an induction by metaphase cells of interphase cells to condense (Rao and Johnson, 1970; Matsui et al., 1972). Furthermore, the alterations in the interphase nuclei of such fused cells simulate both morphologically and biochemically the changes that occur during normal prophase (Chang and Gibley, 1968; Comings and Okada, 1970; Erlandson and de Harven 1971). The morphological changes were exhibited by nuclei of cells in the G₁, S, or G₂ phases before the appearance of premature chromosome condensation. Events in this prophase induction, analogous to those in normal prophase, are: (a) appearance in the light microscope of chromatin networks; (b) a condensation of chromatin along the inner nuclear membrane; (c) a disruption of nuclear membrane; (d) possible attachment of spindle tubules to chromatin; (e) the appearance of a structure resembling the kinetochore; (f) dissociation of polysomes; and (f) a release of nuclear RNA into the cytoplasm (Matsui et al., 1972).

The results obtained from our fusion experiments are in agreement with and further support the concept that a metaphase cell has the ability to trigger condensation events by fusing an interphase cell. The techniques described here have been specifically adapted for this laboratory to be
the basis for future research. A metaphase-\textsuperscript{G\textsubscript{1}} fusion, as described in the results section, directs condensation of the \textsuperscript{G\textsubscript{1}} chromatin to form a single chromatid chromosome. This is explainable since that cell had not yet gone through the DNA replication phase of the cell cycle. Thus, in the \textsuperscript{G\textsubscript{1}} phase the DNA and other chromatin proteins are in a state which allows them to condense if in the proper environment. A second type of fusion, the metaphase-\textsuperscript{S}, is more difficult to interpret. In this case our results again corresponded to the published reports of Rao and Johnson (1970). The pulverization effect was clearly observed in our experiments. This phenomenon is theorized to correspond with the replication of DNA in specific fragments or segments as demonstrated by Taylor (1960). It is possible that those segments which have either completed or not yet begun DNA replication are still associated with their corresponding chromatin proteins and thus similar to either \textsuperscript{G\textsubscript{1}} or \textsuperscript{G\textsubscript{2}} chromatin. Hence, these segments would be able to condense and stain with Giemsa. However, if a segment, at the time of fusion was involved in DNA replication, there might be some change of state in the chromatin. This could be a change within the DNA itself since the DNA helix must open for replication to begin, or there could be a change in the DNA- chromatin protein associations. Whatever the case, it is possible that these chromatin changes render the chromatin incapable of being induced to condense and stain with Giemsa. Thus, a pulverization effect of condensed and staining segments with alternating uncondensed segments could account for the observed results following fusion of a metaphase and \textsuperscript{S} phase cell. This recognizable discontinuity of replicating regions from non-replicating ones will serve as a model for quantifying replication sites at any fraction
of S-phase. The third type of fusion, metaphase- $G_2$, was observed in these experiments. Here, following fusion, the $G_2$ chromatin condensed into two chromatids, since the DNA synthetic phase had been completed. The usefulness of premature chromosome condensation as a tool for studying condensing phenomena of chromatin is most obvious. Future goals of this laboratory were to combine this technique with electron microscopic observation of the different fusion patterns. Such observations could help define the nature of normal as well as the experimental condensation events. The importance of these preliminary fusion experiments is essential to the future of such a project.

Using the electron microscope to study prematurely condensed chromatin patterns could be a tedious one, especially when searching for a particular type of metaphase-interphase cell fusion. Any method which would increase the frequency of a certain sort of fusion would indeed be a welcomed advantage. Hence, synchronized cell populations were utilized in the fusion experiments with the results recorded in Table 1. Figure 4 reveals the comparison of asynchronous versus synchronous fusions. Significant increases occurred in all metaphase-interphase fusion types when selected for by using synchronized cell populations. The metaphase + $G_1$ population fusion yielded an increase from 18 o/o to 39 o/o. The metaphase + S population fusion went from 27 o/o to 47 o/o. The metaphase + $G_2$ population fusion rose from 14 o/o to 43 o/o. Along with the rises in the sort of fusion selected for, was a corresponding decrease in the other metaphase + interphase fusion types. However, there was no significant change in the M/M
type of fusion. Such an observation can be accounted for by the fact that in each of the fusion experiments one-half of the cells were arrested in metaphase. Therefore, each metaphase cell had an equal chance of fusing with either another metaphase cell or an interphase cell. Since the number of metaphase cells involved in each of the fusion protocols were relatively constant, the M/M occurrences should have also been relatively constant. Similar homogeneous fusions of the interphase-interphase type must be considered when evaluating the results of Table 1. Such bikaryons or polykaryons are neglected in the table, but, do change the pools of interphase cells available to be fused with metaphase cells. Furthermore, the interphase-interphase bikaryon would not exhibit prematurely condensed chromatin in either nucleus since no mitotic inducer would be present within this cell. The Table 1 and Figure 4 data do show that the frequencies of the specific fusion types can be manipulated, and these increases can be of great assistance for electron microscopic studies at a future date.

In conclusion, these preliminary experiments paved a variety of pathways for reaching future goals of this laboratory. First, the necessary equipment was set up and proper materials acquired. Second, much of the literature relevant to performing such experiments was studied and selected protocols adapted for use. Third, the appropriate knowledge was learned for handling tissue cultures. Fourth, virus cultivation, titration, and inactivation were all successfully carried out yielding sufficient virus for experiments of future years. Fifth, cell fusion became a standardized routine with premature chromosome condensation patterns easily identifiable.
Sixth, synchronous cell populations were achieved using a combination of many techniques.


Lewin, B. 1975. Units of transcription and translation: Sequence components of hnRNA and mRNA. Cell 4: 77-93.


