Genetics and Developmental Biology: The Thomas Hunt Morgan Centennial Symposium

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GENETICS AND DEVELOPMENTAL BIOLOGY

The Thomas Hunt Morgan Centennial Symposium

Edited by Howard J. Teas
FOR
Richard S. Schweet
(1918-1967)
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The Thomas Hunt Morgan Centennial Symposium was planned not only as a tribute to Morgan's contributions to the beginnings of modern experimental biology but also as a discussion and presentation of the most modern aspects of genetics, developmental biology, physiology, and biochemistry that have evolved from beginnings in Morgan's work. It offered a unique occasion for bringing together many individuals who had known or worked with Morgan as well as those who are first, second, or third generation students of Morgan. And one feature of it was a series of personal reminiscences about Morgan. Excerpts from some of these—the banquet talk of George W. Beadle, a tribute by Albert Tyler, and comments by A. H. Sturtevant and Mrs. O. L. Mohr—are included in the introduction. These talks as well as personal notes and recollections of friends and fellow scientists that were collected in connection with the symposium are being assembled for future publication.

During the symposium University of Kentucky President John Oswald announced the formation of the Thomas Hunt Morgan School of Biological Sciences at the University. The concept of the Morgan School as a center for basic biological sciences in the College of Arts and Sciences as well as in the College of Medicine had been developed primarily by Richard S. Schweet, chairman of the newly formed Department of Cell Biology. Dr. Schweet, as chairman of the symposium organizing committee, was the prime influence in the symposium. His untimely death in 1967 terminated an already brilliant career, and his loss is sorely felt by his colleagues and students in this country and abroad.

The symposium organizing committee is grateful to the Office of Naval Research, the National Institutes of Health,
and the University of Kentucky for financial aid in holding
the conference. The committee would also like to thank
Mrs. Betty Cason of the Department of Cell Biology for her
help in connection with the symposium and the authors for
their cooperation in preparing their manuscripts for publi-
cation.

James Bonner
(Richard S. Schweet)
A. H. Sturtevant
Howard J. Teas
Albert Tyler
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INTRODUCTION

The development of modern genetics, A. H. Sturtevant has commented (1965), affords a striking example of the convergence of different disciplines. In the twentieth century it was first cytology that interacted with genetics, and rapid advances in both fields resulted. Subsequently, statistics, practical breeding, evolution theory, immunology, biochemistry, and biophysics all have contributed heavily. From syntheses afforded by this cross-fertilization have come new ideas, techniques, and dramatic developments in genetics as well as in related fields.

This symposium honoring Thomas Hunt Morgan on the centennial of his birth provides an example of the mutual enrichment that results from the interaction of diverse disciplines. Approximately 150 scientists, representing a variety of backgrounds and approaches, gathered in Lexington, Kentucky—Morgan's birthplace—to discuss recent developments in experimental biology that have derived from Morgan's work. Clearly the most important change has been the introduction of the molecular approach that has come about from the infusion of such areas or disciplines as biochemistry, biophysics, microbiology, and immunology.

The symposium papers reflect the transition and interrelationship of the classical and molecular. A review of this kind is particularly appropriate in view of the rapid changes taking place in the direction and in the experimental tools of experimental biology. This symposium volume thus combines a tribute to Thomas Hunt Morgan with an up-to-date presentation and synthesis of developmental biology and genetics that points the way to future research in these fields.

The symposium itself was arranged in six sessions, each of them directed by a person who had been either an associate
or a first or second generation student of Thomas Hunt Morgan and who was thus able to relate the subject of the session to Morgan's own work in that particular area of genetics. The first session, led by L. C. Dunn, concerned the gene. The second, chaired by A. H. Sturtevant, dealt with cellular mechanisms of inheritance. The third session treated molecular mechanisms of inheritance and was chaired by N. H. Horowitz. The fourth session, under the chairmanship of Richard Schweet, dealt largely with the mechanism of gene action illustrated in bacteriophage systems. Jack Schultz was in charge of the fifth session, which concerned the regulation of gene action. Finally, the sixth and concluding session, with Albert Tyler, had as its focus mechanisms of development.

In all six of these areas the work of Thomas Hunt Morgan played an important part either in making a direct contribution or in laying the groundwork for the later contributions of others. In the latter portion of his career, for instance, Morgan was much interested in exploring the basis for self-sterility in the hermaphroditic ascidians; and he saw in this work, Tyler suggests, a chance to bring closer together the fields of embryology, genetics, and immunology. The symposium has thus a particular appropriateness as a celebration of Morgan's centennial.

But what kind of person was this man whose work, so fundamental to modern genetics, won for him the Nobel prize for medicine in 1933, among many other awards and honors? As an integral part of the symposium a number of former associates presented their personal recollections of Morgan, recollections that give a glimpse of the man.

Morgan's career may be sketched briefly (see Sturtevant, 1959, for a more detailed account). He was born in Lexington, Kentucky, on September 25, 1866, descending on his father's side from an old Lexington family that included the Confederate general John Hunt Morgan and on his
mother's from a Baltimore family that included Francis Scott Key. In Lexington he attended the University of Kentucky and received the B.S. degree in 1886. From Kentucky he went to Johns Hopkins, where he received his Ph.D. in 1890. After a year of research in Jamaica and the Bahamas as a Bruce Fellow he was appointed an associate professor of biology at Bryn Mawr. In 1904 Morgan was appointed professor of experimental biology at Columbia. It was at Columbia that Morgan, who began as a descriptive embryologist, performed with Drosophila experiments that culminated in the demonstration of sex-linkage in chromosomes. This demonstration provided a crucial confirmation of the chromosome theory of inheritance. Four years later, in 1915, Morgan, together with A. H. Sturtevant, H. J. Muller, and C. B. Bridges, published The Mechanism of Mendelian Heredity, in which genetics was viewed in terms of chromosome theory. All subsequent work has been based on this theory. In 1928 Morgan went to the California Institute of Technology as professor of biology to set up there a biological division. He remained at that institution until his death in 1945.

All those in the symposium who spoke of Morgan personally testified to the enthusiasm and excitement which he generated in his students. Nowhere is this quality revealed more clearly than in accounts of his “fly room” at Columbia. Describing this room, Sturtevant recalls: “We [C. B. Bridges and Sturtevant] were fortunate enough, though both still undergraduates, to be given desks in Morgan’s laboratory. . . . This was about four months after the discovery of the white-eyed mutant and was at the very beginning of the major expansion of the work on Drosophila. We were at the right place at the right time! This laboratory, which came to be known as ‘the fly-room,’ was sixteen by twenty-three feet in area, with eight desks crowded in it, and in the early years usually a bunch of bananas hanging in one corner. . . . Here
the three of us raised *Drosophila* for the next eighteen years, with a steady stream of American and foreign graduates and postgraduate students sharing it with us. Rarely were there fewer than five occupants. The atmosphere was one of excitement. Everybody did his own experiments, with little or no supervision, but each new result was freely discussed by the group. There was no such thing as a coffee-break, or any special arrangement for discussion. Instead, we discussed, planned, and argued—all day, every day.”

Tone Mohr, the wife of the Norwegian scientist Otto Mohr, also remembered vividly the quality of Morgan’s laboratory. “It was a crowded lab, a little bewildering to us. We had, of course, expected something wonderful . . . but found it rather untidy, dusty, with milk bottles containing banana flies everywhere on tables and shelves. There was lively conversation going on, talking, joking, laughing—the professor in the midst of the group. . . . How different everything was from the European universities! We were used to a professor a little aloof, at a distance, who made a round in the lab now and then to guide and answer questions. But here in the new world, it was like a family having everything in common. We thought, ‘Well, this is the way in the United States.’ Only later we learned that the atmosphere of the Morgan group was indeed unique.”

Undoubtedly the enthusiasm which Morgan engendered in others sprang largely from the adventuresome, inquiring spirit which he himself evinced. A. H. Sturtevant mentions approvingly the description of Morgan as a “romantic” in science (Harrison, 1936), one to whom ideas came in quick succession, each to be tested for what usefulness it might prove to have. For the romantic a problem is only a stepping stone in his continuing investigation—sometimes in the right direction, sometimes not. Morgan fitted this description perfectly. Mrs. Mohr commented that the leading idea of the fly room was the experiment, and Sturtevant agrees that
Morgan was "devoted to the experimental approach. He was always ready to consider new or radical ideas—if they could be tested experimentally. One of his favorite expressions was 'Let's try it'—and trying meant carrying out an experiment."

Albert Tyler corroborated this characteristic. Morgan, he said, would be "best characterized as a brilliant explorer, rather than an analytical genius. He was always puzzling over natural phenomena of all kinds, biological ones in particular, and would constantly be outlining, usually on whatever scraps of paper would be at hand, experiments that he should do. He required very little excuse to do an experiment and was very rarely stumped by lack of equipment or materials." This resourcefulness and readiness was illustrated, for Tyler, in an incident which occurred at Corona del Mar, the marine biological station Morgan established at the California Institute of Technology. One day, discussing the problem of self-sterility in Ciona, Morgan said that acidified sea water might overcome the block to self-fertilization. It was suggested that weak acids might be better for this purpose than strong ones. Morgan immediately seized upon this approach, but no weak acids were available in the laboratory. He then took a lemon from his lunch basket, squeezed out the juice, and measured portions of it into dishes of sea water containing Ciona eggs. In a number of samples self-fertilization did occur. Later, of course, these results were carefully checked, and it was found that fertilization occurred in sea water at about a pH of 2.7 regardless of whether the acid was weak or strong.

Coupled with his inquiring mind, Morgan had a warm personality that engaged those who worked with him. Although his associates commonly referred to him as "The Boss" (not to his face), the term was one of affection, for, as George Beadle said, Morgan did not order anyone to do anything. He was an inspirer, not a commander. In 1945,
Morgan advised Beadle about coming to the California Institute of Technology as chairman of the biology division. Referring to his own invitation to come to that institution to head the division and be director of the laboratories, he had said that he would come as chairman but not as director, that he did not wish to be responsible for people who had to be directed.

A part of this warmth is the personal generosity which Morgan showed on many occasions. Both Beadle and Sturtevant referred to instances from their own careers where Morgan had provided crucial financial aid, though not wishing it to be known that he had done so. No doubt there were many others who at one time or another received the same kind of help. Acknowledged by the speakers generally was his helpfulness in advising on the presentation of lectures and the preparation of papers. On the other hand there are the many stories of his frugality with institutional funds; it was said, for example, that the half-pint milk bottles used for Drosophila culture at Columbia had often been retrieved from trash cans. At the California Institute of Technology he was reported to have turned down a request for a Harvard trip balance with the comment that “geneticists did not need elaborate equipment like that.” In a time when elaborate and expensive equipment is taken for granted, Morgan’s attitude may seem old fashioned, but though his economy may have no direct bearing upon his scientific accomplishments, it may also be taken as an aspect of a mind both quick to perceive a problem and quick to devise means for approaching and resolving it—a mind devoted to essentials. This was Morgan.

Morgan’s place in the history of genetics was suggested by George Beadle in a series of hypothetical questions:

“If Thomas Hunt Morgan had not discovered that first white-eyed fly, would Drosophila have been domesticated as a research organism?
"Would Sturtevant, Bridges, Muller, Weinstein, Payne, Metz, Gowen and a host of others, and their students, have become geneticists? . . .

"Would Neurospora research have become biochemical?

"Would Emery Ellis and Max Delbrück have recognized the virtues of bacterial viruses and made them objects of genetic study?

"Would Hershey and Chase have then demonstrated that DNA is the primary genetic information in some viruses and, in them, the sole species of molecule transmitted intact from generation to generation?

"Would Jim Watson then have teamed up with Crick to work out the molecular structure of DNA that has told us so much about how hereditary information is written, used in development and function, copied in replication, and through mutational change is responsible for a large part of organic evolution?

"No one knows the precise answers to these and a host of related questions.

"But we do know that without Morgan, our knowledge of the living world would be less complete by far. He and his three generations of intellectual descendants have come close to making meaningless the age-old question of the difference between the living and the non-living."

REFERENCES

Unless noted specifically the sources for this introduction are the reminiscences given by the various speakers at the symposium.

NONRANDOM SEREGATION
OF SISTER CHROMATIDS

Karl G. Lark

Prophase

DNA is perhaps the only molecular constituent of the cell that exists as a single entity which is replicated before division, following which each daughter molecule or chromosome must be carefully distributed into each daughter cell. This duplication and distribution poses a unique problem to the cellular physiologist. How can one entity—not to speak of more than forty—be carefully duplicated and then one copy of each separated into two cells? The problem becomes formidable when one considers that each of these objects is a strand or filament many millimeters long, all of which are coiled into a volume of less than 100 cubic microns.

Bacteria present a good system for the elucidation of such mechanisms in that they possess only a single chromosome, whose replication can be easily controlled by the investigator. Moreover, little is known about the cytogenetics of bacteria or the segregation of their genetic material. Thus, the bacterial physiologist is, to some extent, unfettered by prejudice.

In the course of experiments on chromosome segregation in bacteria, I formulated a model that postulated that segregation was a part of the replication process in that a polynucleo-tide strand became permanently attached to a segregation apparatus when it was first used as a template in replication. If such attachment could not occur, replication would not take place.

Such a model would account for the precise distribution of chromosomes into daughter cells of higher organisms, if one
assumed that all new templates were attached to a common segregation apparatus which was distinct from the one to which all old templates were already attached.

Experiments to test this were carried out by Drs. Consigli, Minocha, and myself. We concluded that this was indeed what happened in mammalian cells. To us it seemed gratifying that nature behaved so logically. We were, therefore, surprised to find that the majority of geneticists with whom we discussed these experiments found this conclusion surprising and somewhat disturbing. It was only then that I became aware of the extent to which the assumption of random segregation of genetic material pervaded genetic thinking.

Metaphase

I shall here discuss the segregation of sister chromatids in mammalian cells (Lark, Consigli, and Minocha, 1966; Lark, et al., 1966). The basic idea for these experiments comes from studies on bacteria that we undertook during the past two years. I am not going to discuss the data for these bacterial studies but shall summarize the results as supported by autoradiographic data from our laboratory (Lark and Lark, 1965; Lark and Bird, 1965; Lark, 1966) and by the P32 suicide data of Jacob, Ryter, and Cuzin (1966) and of Rownd (personal communication). The model that these data support is shown in Figure 1. This model requires that in order for a chromosome to replicate, it must have both of its polynucleotide strands—the two strands of the DNA double helix—attached to a segregation apparatus of some type. In bacteria, it has been presumed that this structure is the cell membrane (Jacob, Ryter, and Cuzin, 1966). Prior to replication, the completed chromosome is attached by only one of its polynucleotide strands (Figure 1A). The process of replication involves the separation of the other strand which
Figure 1. A model of chromosome segregation in bacteria: (A) The DNA double helix is attached by one polynucleotide strand to the bacterial cell surface. Replication is initiated by the scission of the other strand (B) (used as a template for the first time) and its attachment (C) to the cell surface. After replication (D) the attachment becomes permanent (E).

is going to serve as a template for the first time and its attachment to a new segregation apparatus (Figures 1C and D). Thus, the molecular unit of segregation is a single polynucleotide strand. We can explain all of the bacterial data by this model which, in its simplest terms, demands that strands used as a template for the first time are uniformly separated from those used as a template in a previous generation. Our bacterial data were obtained using *Escherichia coli* cells containing several identical genomes. In the experiments of Jacob, Ryter, and Cuzin (1966) and of Rownd (personal communication), the segregation of a chromosome from an episome—i.e., of two distinct replicons—was studied.
You will see that the model says that all of the "old" template strands of each replicon would be attached to the same segregation unit.

We may extend the model from two replicons per genome to the data of Taylor, Woods, and Hughes (1957), who showed that chromosomes of higher organisms are replicated semi-conservatively. Recent data of Cairns (1966) and of Taylor (see below, pp. 149-164) indicates that there are many sites of DNA replication (replicons) on each mammalian chromosome. One can see from our model how such a structure could replicate semi-conservatively. All of the old polynucleotide strands would be attached to one "backbone." (This says nothing about the nature of the "backbones" or the organization of the chromosome). Such a model yields a semi-conservative replication of a chromosome composed of several hundred replicating DNA units.

We have applied this model to the several chromosomes in the mammalian cell and asked the question, "Would all of them segregate according to the age of the template on which they were formed?" That is, is it possible that segregation is not random in a mitotically dividing cell?

The model of our experiment, shown in Figure 2, is essentially quite simple. We studied primary tissue cultures of mouse embryo cells, containing mouse fibroblasts. In Figure 2 the forty chromosomes of the mouse are represented schematically as four chromosomes. Each one contains two strands and will replicate semi-conservatively. After one generation of replication in radioactive medium (H\(^3\)-thymidine) each chromosome has given rise to two daughters, each of which contains a radioactive and nonradioactive strand. Thus, every chromosome is "half hot." Another round of replication in H\(^3\)-thymidine gives rise to one chromosome containing an original template strand which would be nonradioactive and one chromosome bearing a radioactive template. Since a radioactive strand is synthesized on this template, both strands will be radioactive. Such a chromo-
Figure 2. Alternative models of chromosome segregation during labeling with H\textsuperscript{3}-thymine. Cells are continuously labeled with radioactivity. At the end of G\textsubscript{1}, all sister chromatids possess one radioactive and one nonradioactive polynucleotide strand. After G\textsubscript{2}, two types of sister chromatids are present, those with two radioactive polynucleotide chains and those with only one. These may segregate at random (on right) or according to the age of the template used in replication (all 100 percent radioactive into one cell, all 50 percent radioactive into the other).

Some, then, would be “all hot,” the other “half hot.” You will see that there are two ways in which the two types of chromosomes could assort into daughter cells: Into cells that are half hot and cells that are all hot, which means that all forty chromosomes of one type go to one cell and
Figure 3. An experiment of the type described in Figure 2. A primary tissue culture of mouse embryo fibroblasts was incubated with H3-thymidine and cells were examined by autoradiography after (A) one, (B) two, or (C) three generations. The number of grains per nucleus were examined and the frequency of cells with a given radioactivity measured. The pairs of numbers in the lower right-hand corner represent second-generation dividing cells with two nuclei whose common parentage is therefore certain. The numbers of grains of the two members in each pair are tabulated side by side.

We found (Figure 3) that in the one generation of radioactive labeling most of the cells had about twenty-five grains per nucleus with a few cells with double this amount. After two generations of labeling, we had a bimodal distribution.
About half the cells had twenty-five grains and the other half about fifty. After three generations of labeling, we obtained almost three-quarters of the cells in the hot population and a quarter in the half-hot population. Our estimate of the number of generations that elapsed are based on the time normally observed for division (about twenty-four hours), not on cell counts.

Among the cells that are grown on coverslips, an occasional cell pair would be seen that was still connected by a cytoplasmic bridge. If we look at such pairs of nuclei arising from a single cell after the second generation of labeling, we find asymmetry in almost every pair.

We can also run the experiment in reverse and label cells for one generation and then grow them in nonradioactive medium (Figure 4). In this case, after one division in radioactive medium we again have a single population of nuclei (somewhat less homogeneous than in the previous experiment). After a second generation, now in nonradioactive medium, we find cells that have little or no radioactivity, that is, that contain less than five grains per cell. The proportion of such cells increases in the third generation and, again, where pairs of nuclei are observed in the same cell, they are asymmetrically labeled.

These results were observed with mouse embryo tissues. I should explain why the mouse system was so good. In the first place, only about 30 or 40 percent of the cell cycle is occupied by DNA synthesis, which occurs at the end of the cycle (Richards, Walker, and Deeley, 1956). This means that we will find most of the cells in the G1 period, where they contain one complement of DNA. This helps to keep our grain distribution fairly narrow. Few cells will be observed with twice the complement of chromosomes. A second thing that helped us was the inadvertent finding that when we prepared the mouse embryos for primary tissue culture,
Figure 4. The same type of experiment as in Figure 3 except that the cells were incubated with radioactive thymidine for one generation (A) and then in nonradioactive medium for a second (B) or third (C) generation. Primary tissue culture of mouse embryo fibroblasts was used.

the cells became synchronized. This also helped to homogenize the population with respect to DNA content.

On the other hand, if we subsequently pass these cells with trypsin, the result is not so clear as that in Figure 4. We do not find the two classes of cells so discretely separated. Many cells contain intermediate numbers of grains. However, we still observe a striking asymmetry. Thus, on continued passage in culture these cells, which are beginning to look
Figure 5. (A) An experiment similar to the type in Figure 4, but using Chinese hamster cells. Black area: cells grown to contain half-radioactive chromosomes. Stippled area: the same cells grown for another generation in nonradioactive medium. (B) A similar experiment to (A), but using HeLa cells.
less and less fibroblastic, also show less and less of a clear sister chromatid segregation pattern.

We also studied a strain of Chinese hamster cells (received from Dr. Herbert Taylor) using a cold passage experiment of the type in Figure 4. Figure 5A shows the result. The solid distribution represents the original labeled population. The stippled area represents the distribution of radioactivity after further passage in nonradioactive medium. The original population is more or less maintained and there is also a large population of lightly labeled cells. The pattern still bears out the idea that the chromosomes with the hot template, made in the previous generation, segregate from those with the cold template, made two generations before. However, this distribution is not as narrow as that of the mouse cells. In this case, we are hampered by the fact that S and G₂ occupies more than 60 percent of the cell cycle (Taylor, 1960), and we have therefore obtained cells in intermediate stages of DNA synthesis in which many cells have partially or completely duplicated their chromosomes. The radioactivity is accordingly disperse. This strain of cells is supposedly diploid.

When we studied a heteroploid strain—HeLa cells—where the chromosome content per cell may vary drastically, we found (Figure 5B) that segregation of radioactive and nonradioactive chromosomes no longer could be clearly observed. Even in these cells, however, there was a marked tendency of nonradioactive chromosome to segregate away from radioactive ones.

I have tried to present the positive and negative aspects of these experiments. Many more systems will have to be examined before we know whether the segregation models in Figures 1 and 2 are going to prove to be general. What our results say in the extreme case is that there is a common segregation during mitosis on the basis of old and new
centromeres and that the newly synthesized centromeres are all attached as a common unit. Clearly, this has to break down at meiosis and some method of re-aggregation must occur.

The usefulness of the model may be that it represents an indexing system by which one chromosome of every type would be distributed into daughter cells. However, this indexing system may become useless in meiosis where diploids must break down only later to be restored. Using current ideas derived from phage morphopoiesis or other structural assembly laws, we could construct ways in which such break-down and reassembly could occur. But such models would be even more speculative than our model as to whether or not sister chromatids segregate on the basis of the attachment of their template strand to centromeres of a common segregating unit.

OPEN DISCUSSION

DR. RICHARD SCHWEET: As I understand it, then, you must have something during interphase when the whole thing disappears that’s going to still hold this indexing system together?

DR. KARL LARK: In terms of preconceived notions of the role of a structure, we have an advantage in working with a bacterial system. The whole structure is always invisible in the bacterial system. Nevertheless, it seems to be indexed continuously so that we have a very clear separation of old from new. All we know is that when the separation occurs, they appear to be neatly indexed. It would be most practical if this occurred when replication started. For this to happen there should be a new apparatus to which to attach the template which is going to be used for the first time. This is what we would like to believe, but we have no evidence for this.
DR. SCHWEET: But unless they are attached to a membrane somewhere all along, I mean, at all times you don't have.

DR. LARK: Yes, I would like to think this. As I say, there is no evidence for attachment to a membrane.

DR. SCHWEET: It is a requirement of the data though.

DR. LARK: No, all it says is that all the old strands are permanently attached to one kind of thing at all times. That is, they don't detach and later re-attach. However, we have no evidence for the membrane fulfilling this role.

QUERY: Are you suggesting that the centromere would be the same type of attachment that Jacob proposes for the bacterial system?

DR. LARK: No. I think that the attachment of individual polynucleotide strands of the DNA molecule must be very complex in the chromosomes of higher cells, because there are many replicons and these are somehow tied together. The simplest thing is to say that the many replicons which make up a chromosome are attached to one common structure. What seems to be happening in our experiments is that there is a tendency, at least in some of the systems, to tie these structures all into one master system as well.

QUERY: This would be difficult inasmuch as the nuclear membrane disappears, at least during the mitotic stages.

DR. LARK: Yes, if the nuclear membrane were to be the candidate for this, it would be difficult because it disappears in mitosis.

QUERY: I'm thinking very simply again of a membrane, as suggested by Jacob.

DR. LARK: One thing is certain, that the whole mammalian system is so much more complex that if the chromosome is attached to a membrane, then that membrane must serve as a forerunner of the much more complex system upon which further condensation would have to take place to form the mitotic apparatus.

QUERY: The only cytological evidence would be the Barr
body, then, as far as an attachment to a gross morphology—for example, the condensed X chromosome, which apparently is attached.

DR. LARK: You are referring to the material Dr. McClintock just talked about. That is, the attachment of a condensed chromosome to the membrane. Yes, except that we would go further and say that the attachment must always be with a certain orientation.

QUERY: One bit of evidence that perhaps mitotic chromosomes aren't so oriented is that when a mitotic chromosome has two centromeres, quite often two centromeres on the same chromosome will go to opposite poles.

DR. LARK: There is the postulate that dicentrics are unstable. However, it seems to me that the minute there is a sister chromatid exchange between the two centromeres then an old template becomes attached to a new one, which then goes in the opposite direction. If the number of sister chromatid exchanges is estimated, the probability of exchange is great enough so that the dicentric-within-two division in some systems would probably be lost.

QUERY: You would suggest then that a small dicentric would be more easily recovered than a large one?

DR. LARK: Yes, if the frequency of a sister chromatid exchange between the two templates in a dicentric is proportional to distance.

Anaphase

During the following afternoon I was encouraged by discussions with Dr. Taylor to study sister chromatid segregation in plants, which have no visible centriolar structure. Accordingly, I have been studying the distribution of radioactivity in anaphase figures in cells from plants of *Vicia faba* grown for twenty hours in radioactive thymidine and then for forty hours in nonradioactive medium. Figure 6 shows such a cell. It is clear that the radioactivity is not symmetric-
Figure 6. Autoradiogram of an anaphase in a cell from the root tip of *Vicia faba* labeled for 20 hours with H\(^3\)-thymidine and then grown in nonradioactive medium for 40 hours. Left: focus on the plane of the chromosome. Right: focus on the plane of the emulsion.

ally distributed between the two chromosome bundles. A survey of about three hundred such anaphase cells indicates that more than twice as much radioactivity is located in one set of chromatids as in the other. Thus, sister chromatid segregation in plants during mitosis also does not appear to be random. At present, it seems unlikely that these results are due to artifact, such as unequal absorption of \(\beta\) radiation over the two sister chromatid bundles.

We are faced with the conclusion that the distribution of chromosomes between daughter cells is not of necessity a random process. Similar conclusions are emerging from cytogenetic studies of meiosis in several systems (Walen, 1965; Feldman, 1966; Feldman, Mello-Sompayo, and Sears, 1966). These indicate that segregation of sister homologues is not always random.

The time may have arrived to re-examine genetic data in the light of what we know of cell physiology. If on the one
hand the distribution of chromosomes into daughter cells is precise, we must reconcile the eventual random segregation of genetic markers with this system of chromosome distribution both in mitosis and meiosis. The randomizing influence of sister chromatid and homologue exchange may be more important than most of us had realized.

It is clear that during meiosis the number of chromatids accommodated by a cellular indexing system is halved. What is no longer clear is whether the apportionment of homologues occurs at random during meiosis or is predetermined from the time of zygote formation onward.

Experiments of the type described above may yield an answer.

**Telophase (added in proof)**

Two years have elapsed since this article was written. During that time the experiments described above have been repeated in other systems. (In press are similar results with human and rat diploid cells (Jean Priest), *Aspergillus nidulans* (Kessel and Rosenberger), and wheat (Lark). However, contradictory results have been obtained in studies of the segregation of individual labeled chromosomes (Heddle, *et al.*, 1967 and Cuevas-Sosa, 1968).

Recent studies which I have made of wheat have demonstrated that asymmetric distribution of radioactive material depends on the genetic composition of the plant—tetra 5B nulli, 5D plants show nonrandom segregation of radioactive sister chromatids similar to Figure 6 whereas tetra-5D, nulli 5B plants show random segregation. Nevertheless, these tetra 5D, nulli 5B seeds germinate and produce normal seedlings, each cell of which contains a normal chromosome complement. It is clear, therefore, that nonrandom segregation of sister chromatids is *not* a necessary part of the segregation mechanism. Consequently, it also would appear that permanent attachment of chromatids to a common segrega-
SEgregation of sister CHROMATIDS

...tion unit is not a necessary feature of mitotic segregation, if indeed, it exists at all.

Thus, the cause of nonrandom segregation as well as the purpose which it serves remains obscure. It is possible that it functions as part of a mechanism for indexing chromosomes during segregation but it is equally feasible that it is a nonessential by-product of the segregation process.

In either case, it is clear that more experimentation is necessary before a mechanism can be established for the indexing and segregation of chromosomes.

ACKNOWLEDGMENTS

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MEIOSIS AND DNA REPLICATION IN CHLAMYDOMONAS

Noboru Sueoka, Kwen-Sheng Chiang,
& Joseph R. Kates

Meiosis is the cell division through which the chromosome number of the zygote is reduced to one-half and is the basis of segregation, linkage, and recombination of genes in higher organisms. The essential features of meiosis have been well characterized for more than half a century in terms of chromosome behavior: pairing of homologous chromosomes (synapsis), chiasma formation, and reduction in chromosome number (Rhoades, 1961). Elucidation of these processes at the molecular level in relation to recombination had to wait until recently because of the difficulty of overcoming technical problems in the control of biological systems to obtain meaningful interpretations of the results. One of the basic types of information for understanding the mechanism of meiosis is the relationship between chromosome behavior and DNA replication.

In 1958, Meselson and Stahl made an extremely important study on the mode of DNA replication in *Escherichia coli*. They proved the semi-conservative replication of DNA and also proved the technique of density gradient equilibrium centrifugation (Meselson, Stahl, and Vinograd, 1957) to be extremely powerful for the study of DNA replication. In eucaryotes, Taylor, Woods, and Hughes (1957) made a breakthrough in our understanding of chromosome replication with an autoradiographic analysis of plant mitosis. These works stimulated one of us (N. Sueoka) to analyze DNA
replication during meiosis. In 1958, the mechanism of crossing over in terms of DNA molecules was completely speculative. Copy-choice and break-and-reunion hypotheses were both prevalent. Other features of meiosis, synapsis and regular tetrad formation also were not accessible for biochemical analysis.

With this background, *Chlamydomonas reinhardi* was chosen as the experimental material and the study was initiated in 1958. *Chlamydomonas* DNA was first characterized by CsCl density gradient centrifugation and thermal hyperchromicity analysis, and the semi-conservative replication of DNA in mitosis has been reported (Sueoka, 1960). By 1960, several experiments on meiosis had been made which yielded information on some of the major features of DNA replication associated with meiosis: semi-conservative replication, existence of one round of DNA replication, and emergence of eight zoöspores from a zygote. M-band DNA (see below, pp. 32-34) was also observed, which was a complete mystery. However, these results were not definite because of the following difficulties: (a) mating efficiency on a large scale was extremely variable (0-90 percent mating) from experiment to experiment under the same experimental conditions; (b) contamination of vegetative cells was not completely eliminated; (c) preparation of DNA from mature zygotes was not refined in obtaining large molecular weight DNA. The variability of mating efficiency suggested the necessity of physiological studies of gametogenesis and mating. Since the participation of K. S. Chiang and J. R. Kates in 1962 in this project, major progress has been made. The problem of variable mating efficiencies was solved by recloning the mating types and making a preliminary test for mating before each experiment. Physiological conditions for the induction of gametes turned out to be important for high mating efficiency. Improvement of DNA isolation was essential for analysis of satellite DNA's (β and γ). The possibility of contamination of vegetative
Figure 1. The life cycle of C. reinhardi (octet strain).

Cells to account for M-band DNA (see below, pp. 34-36) was eliminated by repeated grinding of zygotes and extractions of DNA. A possible second round of DNA replication concealed by parental DNA precursor pool was also eliminated.

Thus, as summarized here, basic features of DNA replication associated with meiosis became clearer (for a detailed description, see Chiang, 1965; Sueoka, Chiang, and Kates, 1967). Replication of satellite DNA's (β and γ) will be reported elsewhere (Chiang and Sueoka, 1967).

Life Cycle of C. reinhardi and Its Synchronization

Figure 1 shows the life cycle of C. reinhardi (strain from R. P. Levine). Synchronization of mitotic cell divisions can be achieved by alternating light-dark periods (Bernstein, 1960;
Kates and Jones, 1964). Controlled induction of gametogenesis is crucial for effective mating. In general, those gametes induced from vegetative cells by nitrogen-withdrawal (Sager and Granick, 1954) toward the end of the growth phase gave the best mating efficiency. Caryogamy of the two nuclei followed approximately twelve hours after mating and the zygote did not divide but remained as a single cell and underwent maturation. The maturation period consisted of two days of light and seven days in the dark. When given light and salts, the mature zygote germinated, and the first two nuclear divisions constituted meiosis. The whole process of maturation and germination can easily be synchronized.

**DNA of C. reinhardi**

Figure 2 shows the banding pattern of *C. reinhardi* DNA in CsCl density gradient centrifugation. Three DNA components
TABLE ONE: *Buoyant densities in CsCl solution and abundance of DNA component in C. reinhardi (octet strain)*

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>DNA/cell x10^7 (μg)</th>
<th>Component</th>
<th>Density</th>
<th>%GC*</th>
<th>Relative amount of total DNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative</td>
<td>1.24 ± 0.081**</td>
<td>α</td>
<td>1.723</td>
<td>64.3</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β</td>
<td>1.695</td>
<td>35.7</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>γ</td>
<td>1.715</td>
<td>56.1</td>
<td>1</td>
</tr>
<tr>
<td>Gametes</td>
<td>1.23 ± 0.064†</td>
<td>α</td>
<td>1.723</td>
<td>64.3</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β</td>
<td>1.695</td>
<td>35.7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>γ</td>
<td>1.715</td>
<td>56.1</td>
<td>4</td>
</tr>
<tr>
<td>Freshly mated</td>
<td>2.46</td>
<td>α</td>
<td>1.723</td>
<td>64.3</td>
<td>89</td>
</tr>
<tr>
<td>zygotes</td>
<td></td>
<td>β</td>
<td>1.695</td>
<td>35.7</td>
<td>7</td>
</tr>
<tr>
<td>Zygospores</td>
<td></td>
<td>γ</td>
<td>1.715</td>
<td>56.1</td>
<td>4</td>
</tr>
<tr>
<td>(mature zygotes)</td>
<td></td>
<td>γ + M</td>
<td>1.715</td>
<td>56.1</td>
<td>41‡</td>
</tr>
<tr>
<td>Zoospores</td>
<td></td>
<td>α</td>
<td>1.723</td>
<td>64.3</td>
<td>50‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β</td>
<td>1.695</td>
<td>35.7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>γ + M</td>
<td>1.715</td>
<td>56.1</td>
<td>17</td>
</tr>
</tbody>
</table>

** Colorimetric measurements. Average value from 12 individual determinations and its standard deviation (Sueoka, Chiang, and Kates, 1967).
† Colorimetric measurements. Average value from 7 individual determinations and its standard deviation (Sueoka, Chiang, and Kates, 1967).
* Composition is obtained from the density, assuming no unusual bases: Density (ρ) = 1.660 + 0.098 (G + C) mole per cent (Schildkraut, et al., 1962).
‡ These values vary according to different experimental conditions. The value 41% represents mainly the M-band DNA (see text, pp. 32-34).

(a, β, and γ) are evident from Figure 2. The relative amounts of these components and their density are summarized in Table 1. The α-DNA is a major chromosomal DNA and the β-DNA is known as chloroplast DNA (Chun, Vaughan, and Rich, 1963; Sager and Ishida, 1963; Leff, et al., 1963). The cytological origin of γ-DNA has not been identified. The three kinds of DNA show typical denaturability. Different densities and elution patterns of the three DNA components suggest different base compositions (Table 1).
<table>
<thead>
<tr>
<th>DEVELOPMENTAL STAGE</th>
<th>MEDIUM</th>
<th>LIGHT CONDITION</th>
<th>DURATION OF DEV. STAGE</th>
<th>NITROGEN USED IN MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGETATIVE GROWTH</td>
<td>MINIMAL LIQUID MEDIUM</td>
<td>ALTERNATING LIGHT-DARK CYCLE</td>
<td>1-7 DAYS</td>
<td>N¹⁵</td>
</tr>
<tr>
<td>GAMETOGENESIS</td>
<td>LIQUID MEDIUM (NITROGEN-FREE)</td>
<td>CONTINUOUS LIGHT CYCLE</td>
<td>0-18 HOURS</td>
<td>N-FREE</td>
</tr>
<tr>
<td>MATURATION</td>
<td>4% AGAR MEDIUM</td>
<td>DARK PERIOD</td>
<td>1-7 DAYS</td>
<td>N¹⁴</td>
</tr>
<tr>
<td>GERMINATION</td>
<td>1% AGAR MEDIUM</td>
<td>LIGHT</td>
<td>2-10 HOURS</td>
<td>N¹⁴</td>
</tr>
</tbody>
</table>

Figure 3. General scheme of meiotic transfer experiment.

**Meiotic Transfer Experiment**

In order to analyze the timing and mode of DNA replication during the sexual cycle of *G. reinhardi*, the following transfer
Figure 4. Growth curves of *C. reinhardtii* (circle: minus mating type; star: plus mating type) in a meiotic transfer experiment. For experimental details, see Sueoka, Chiang, and Kates (1967).
experiment was carried out (Figures 3 and 4). The principle of the experiment is to label the two mating type cells with nitrogen-15 (N\(^{15}\)) and right after mating, to transfer the young zygotes to a nitrogen-14 (N\(^{14}\)) medium, then subject them to maturation (two days under light and seven days in the dark). The mature zygotes were germinated in a fresh N\(^{14}\)-medium under light. Germination took approximately ten hours and meiosis occurred between the eighth and ninth hours.

Cell samples were taken from various stages between mating and germination, and cell lysates were centrifuged in a CsCl density gradient. The results are shown in Figure 5.

The following features are clear for the replication of major DNA (a): (a) the density of major DNA (a) of zygotes remained unchanged throughout the maturation period (Figure 5a-c) and in the early part of the germination period (Figure 5d), indicating that replication of chromosomal DNA did not occur during these periods; (b) the fully N\(^{15}\)-labeled a-band (parental DNA) was completely replaced by a half-N\(^{15}\)-labeled (N\(^{15}\)-N\(^{14}\) hybrid) a-band approximately eight hours after the onset of the germination period (Figure 5e). At this time, germinating zygospores still possessed an undivided single nucleus as revealed by cytological evidence; (c) at the end of the germination process, when eight zoöspores were emerging from the zygospore, the hybrid a-band was still the sole a-DNA detected (Figure 5f).

These results show that the chromosomal DNA (a-band) completed its semi-conservative replication before the zygospore nucleus divided in the later germination period in which meiosis occurred. Moreover, although eight instead of four zoöspores were produced from each zygospore after meiosis, chromosomal DNA replicated only once throughout the maturation period of zygotes and the entire meiotic division cycle.

Figure 5 also shows that toward the termination of the
Figure 5. Microdensitometer tracings of UV-absorption photographs showing DNA bands resulting from density gradient centrifugation of purified DNA samples from a meiotic transfer experiment. See Figure 4 for growth curves and other experimental conditions. Conditions for density gradient centrifugation were the same as in Figure 2.

| Sample | Hours after transfer of N\textsuperscript{15} zygotes to N\textsuperscript{14} for matura\n| Description of sample |
|---------|--------------------------------------------------|
| a       | 0 Freshly mated N\textsuperscript{15} labeled gamete pairs (young zygotes) |
| b       | 52 At termination of 52-hr. light period in maturation on N\textsuperscript{14}-medium |
| c       | 220 0 At termination of maturation (52-hr. light plus 7-day dark period) |
| d       | 6 Germinating zygo-spores in N\textsuperscript{14} medium |
| e       | 8 Germinating zygo-spores, with single undivided nucleus (see Table 1) |
| f       | 12 8 zoöspores emerging from each germinating zygo-spore |
| g       | 12 Same as sample (f), except zygo-spores were germinated on N\textsuperscript{15}-medium |
initial 52-hour light period, a large newly synthesized DNA band appeared (Figure 5b). This DNA band will be referred to as “maturation band” or M-band DNA. The relative amount of the M-band DNA decreased rapidly when the α-band DNA was about to complete its duplication during the germination of zygotes (Figures 5d and e). Nevertheless, normally some of the M-band DNA remained throughout the germination process. From these results it became evident that although there was no chromosomal DNA replication during the zygote maturation period, an unexpected DNA component (M band) replicated extensively and appeared in large quantity. The appearance of the M-band DNA apparently did not interfere with normal chromosomal DNA replication, which took place in the later germination period. Therefore, the M-band appearance did not seem to affect our principal objective (meiotic chromosomal DNA replication) but seemed to be an interesting phenomenon in itself. As shown below, the possibility of M-band DNA’s being incorporated into the α-band DNA later has been eliminated.

**DNA Precursor Pool**

In order to interpret the results of the chromosomal DNA replication described above, it was important to establish that little N15-DNA precursor pool contributed to DNA replication throughout the entire course of the meiotic transfer experiment. For this reason, the gamete and zygote populations were tested for a DNA precursor pool under standard experimental conditions.

*Gametes.* If a sizeable DNA precursor pool exists in gametes, the transfer of N14-gametes to a high salt N15-medium without mating, which leads to dedifferentiation of gametes to vegetative cells, should not give discrete density shifts from light to hybrid and hybrid to heavy. The experimental results are exactly as expected for the absence of the precursor pool
Figure 6. DNA replication pattern during conversion of gametes to vegetative cells. N\textsuperscript{14} gametes were obtained by the nitrogen-withdrawal technique from N\textsuperscript{14}-grown vegetative cells at their competency state. After gametogenic differentiation was completed (tested by the gamete mating efficiency), minus mating type gametes were immediately transferred from the nitrogen-free medium into N\textsuperscript{15} \( \frac{1}{2} \) HSM for dedifferentiation. This culture was then subjected to synchronous culture conditions for vegetative growth. After taking sample DD-2, the dedifferentiation culture was diluted once, as indicated by the broken-line arrow. DNA samples were prepared from the cells sampled as indicated in the growth curve (Sueoka, Chiang, and Kates, 1967).

(Figure 6). The situation seems reasonable in view of the fact that the gametes were derived from vegetative cells by transferring the latter to a nitrogen-free medium, where cells divide, usually twice.

Zygospores. After the mating of two opposite mating type gametes, the zygospore undergoes maturation without cell division. The size of the zygospore increases considerably
TABLE TWO: Frequencies of uni-nucleate and multi-nucleate zygospores at the time when the a-band DNA has just completed its replication in the meiotic transfer experiment

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total zygospores counted</th>
<th>Uni-nucleate</th>
<th>Bi-nucleate</th>
<th>Tetra-nucleate</th>
<th>Octa-nucleate</th>
<th>% uni-nucleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500</td>
<td>469</td>
<td>22</td>
<td>7</td>
<td>2</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>917</td>
<td>63</td>
<td>15</td>
<td>5</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>815</td>
<td>762</td>
<td>39</td>
<td>13</td>
<td>1</td>
<td>94</td>
</tr>
<tr>
<td>4</td>
<td>735</td>
<td>682</td>
<td>41</td>
<td>9</td>
<td>3</td>
<td>93</td>
</tr>
</tbody>
</table>

during the maturation period and, therefore, an accumulation of DNA precursors may be expected. The following experiment, however, proves that there is no appreciable pool existent in the mature zygospore.

When the zygospore population, as in Figure 5c (i.e., N^{15}-labeled conjugated gamete pairs matured on an N^{14}-medium) is germinated on an N^{15}-medium, rather than on an N^{14}-medium as in the normal transfer experiment, the a-band detected after germination remains as fully N^{15}-labeled DNA (Figure 5g). This shows that little DNA precursor pool has built up during the maturation period, but DNA precursor was synthesized *de novo* after the onset of the germination process.

Results of the density transfer experiments in the present study (Figure 5) have demonstrated that during the sexual reproductive cycle of *C. reinhardi*, chromosomal DNA replicates only once semi-conservatively. DNA replication occurs during zygospore germination, when the germinating zygospore still has a single undivided nucleus (Figure 5e, Table 2). This means that DNA replication must have occurred in the prophase of meiosis. There is no further DNA replication until the end of the zygote germination, when eight zoöspores emerge from a single zygospore (Figure 5f). The production
of eight zoöspores indicates that a postmeiotic mitosis must have occurred. However, DNA replication did not take place after the meiotic divisions, but shortly before meiotic division of the zygote nucleus. Therefore, an obvious conclusion to account for the emergence of eight zoöspores from one zygote after one round of DNA replication is that the DNA content of the gamete should have been twice that of the zoöspore. The critical question, then, is the time of DNA replication with regard to the crossing-over event in the prophase of meiosis. A clue to the answer comes from genetic results. When the eight zoöspores which emerge from a zygote resulting from a two-factor cross are analyzed for marker segregation, the segregation pattern is typically tetrad, i.e., there are four pairs of zoöspores in which two cells of each pair have identical genotype (Levine, personal communication, 1965), as in the case of Neurospora crassa. This indicates that the crossing over occurs at the fourstrand stage and that DNA replication should occur after the crossing over. If the replication occurs before the crossing over, then the logical expectation of the segregation pattern is not tetrad but octad. The situation is illustrated in Figure 7.

A point which should be cleared here is that the classical terminology for description of meiosis is based on the behavior of chromosomes and not on DNA synthesis. To avoid common confusion, we propose a set of terms “pre-synaptic,” “synaptic,” and “post-synaptic” DNA syntheses. A critical reference period for describing the time of DNA synthesis is synopsis of the homologous chromosomes, in which the crossing over is thought to occur. The terminology seems, therefore, to represent a reasonable demarcation. According to this definition, in the present situation, the DNA synthesis associated with meiosis in an octet strain of C. reinhardi can be defined as post-synaptic and occurs in the prophase of the first meiotic division of the nucleus.

The corollary to this situation is the proposition of the “breakage-and-joining mechanism” of recombination as op-
Figure 7. Genetic segregation patterns expected from pre-synaptic (I) and post-synaptic (II) DNA replication. The present results support model II. In the diagram, the unit chromosome or chromatid is represented by a single line. The cytological status of the chromosome (separate chromosome or chromatid) in gametes and zygotes is not available. Shaded zoospores represent recombinants.

posed to the "copy-choice mechanism." Also excluded are models that assume concomitant occurrence of DNA synthesis and recombination. The proposition does not exclude, however, partial DNA synthesis, which may be required in the rejoining of the breaks (Whitehouse, 1963; Holliday, 1964).
In this connection, Hotta, Ito, and Stern (1966) have reported that in *Lilium* and *Trillium* there is a small amount of DNA synthesis (0.3 percent) during meiotic prophase and that the DNA synthesis is not in cytoplasm but in nucleus.

The breakage-and-joining mechanism of recombination was first demonstrated in a bacteriophage $\lambda$ by Meselson and Weigle (1961). In eucaryotes, the absence of a round of DNA replication at the time of synapsis has been indicated in spermatogenesis for a number of organisms (see Swift, 1953) and in plants (Taylor, 1953; Moses and Taylor, 1955). In these cases, DNA doubling occurs during leptotene or still earlier and thus before synapsis. In ascomycete *Neottiella*, chromosomal DNA replication takes place in the crozier before the fusion of two nuclei, thus completely separating DNA synthesis and synapsis (Rossen and Westergaard, 1966). All of these findings are consistent with the breakage-and-joining mechanism of recombination.

The fact that DNA replicates once, while a zygote originating from the fusion of two gametes gives rise to eight zoospores, is a peculiar characteristic of the octet strain. A different strain obtained from Dr. R. Sager, on the other hand, gives only four zoospores after germination (quartet strain). Results of DNA replication of the latter strain in meiosis will be presented elsewhere (Chiang and Sueoka, in preparation).

The nature of the M-band DNA is not clear at present. However, the appearance of the M-band DNA seems in no way to interfere with the principal objective of this study, namely, to define the mode of meiotic chromosomal DNA replication. A relevant point about this M-band DNA replication is that the buoyant density of the M-band DNA in the experiment presented in Figure 5 is 1.715, which is similar to that of the unlabeled $\gamma$-DNA satellite. During gametogenesis, there is a sudden increase of $\gamma$-DNA (Table 2). These observations suggest that the $\gamma$-DNA and M-band DNA might be of the same origin and that the M-band DNA is not unlabeled chromosomal DNA ($a$). Further studies on the replication of this
peculiar M-band DNA and its possible function with respect to the formation of mature zygotes are in progress.

The replication mode and regulation of satellite DNA's also became clearer through the study of the *Chlamydomonas* system which is discussed elsewhere (Chiang and Sueoka, 1967). Since the satellite DNA's also replicate in zygospores, a mere detection of DNA synthesis during the sexual cycle does not give a meaningful conclusion on the relation between DNA replication and genetic recombination.

**OPEN DISCUSSION**

**QUERY:** I'm interested in the "mystery" or M-band. Is this about coincident with a nonlabeled band? Is that about where its position is?

**DR. NOBORU SUEOKA:** The density of the M-band is not strictly reproducible. In most cases it is just the same as the N\textsuperscript{14} y-band DNA position, which is a little bit lighter in density than N\textsuperscript{14} a-band DNA. In some cases the density of M-band DNA is a little bit higher in density than N\textsuperscript{14} y-band DNA. A possible cause for this higher density is due to some incorporation of N\textsuperscript{15}. We do not think that M-band DNA is chromosomal (a-DNA).

**DR. RICHARD SCHWEET:** The M-band, since it only appears in the zygote and is unlabeled, must be from many, many rounds of replication of some small amount.

**DR. SUEOKA:** That's right. We have never been able to show the hybrid position of the M-band so far.

**QUERY:** They would have to disappear later, if that were the case? If there are many rounds of replication, death of some minor component, later it disappears?

**DR. SUEOKA:** That's right, but a y-band still remains, so that it looks as though the M-band DNA disintegrates during germination and the basic amount remains as y-band DNA. However, we have not demonstrated identity of M-band DNA as multiples of y-band DNA.
MEIOSIS AND DNA REPLICATION

DR. SCHWEET: This is like the extra DNA in many fertilized eggs in some sense, isn't it?

DR. SUEOKA: Yes, it looks like that.

QUERY: But the extra DNA in eggs is mitochondrial DNA, but aside from that I just wonder if the explanation for this might not be some kind of carbohydrate moiety which is attached to the DNA during this period which alters the density of a portion of the DNA?

DR. SUEOKA: We think that that is unlikely. Heat denaturation makes density of M-band DNA greater than that of undenatured by the right density increases (0.015 g/cm³). The M-band is also DNase sensitive.

QUERY: Halvorson has just reported a DNA in spores which is different from the exponentially growing DNA density and also has peculiar characteristics. It doesn't anneal with RNA and it doesn't self-anneal either. I wonder whether . . . .

DR. SUEOKA: But, remember, here the chromosomal DNA remains the same in density.

DR. PIOTR SLONIMSKI: If we do not do the transfer experiment, we should see an M-band.

DR. SUEOKA: Strangely enough, we haven't done that experiment. (Note: Since then, this experiment was performed and the new DNA with a density similar to that of γ-band DNA increases without density transfer).

QUERY: Dr. R. Grell did nice work on Drosophila and the increase of frequency of recombination of temperature treatment corresponds to the time of DNA replication in all genesis of Drosophila. Can a similar experiment be done?

DR. SUEOKA: I guess it can be done. As I remember, R. P. Levine reported the temperature effect on the recombination frequency in Chlamydomonas a long time ago.

QUERY: Do your experiments allow for the reutilization of the broken-down M-bands?

DR. SUEOKA: No, and the result shows that it cannot be reutilized (see text, pp. 35-36).

DR. SCHWEET: Would you say that the vegetative cells have
twice as much chromosomal DNA as the gamete—as the zoosperms, I mean?

DR. SUEOKA: Yes.

DR. SCHWEET: Is there some way to determine that directly?

DR. SUEOKA: Well, we have tried hard to determine the amount of DNA by the colorimetric method. Apparently, in algae it's a very difficult thing to do, because of all sorts of carbohydrates. For example, in germinating zygotes we get about ten times the DNA amount of vegetative cells by colorimetry, even though all the nucleotides are extracted, and we can't really tell. We have a rough estimate of the DNA amount of vegetative and gamete cells: 1.2 x 10^-7 μg per cell for both cases, which means seven times ten to the tenth molecular weight equivalent. However, the DNA amount measured colorimetrically in zygotes and zoosperms has been variable and irrational.

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Because of the unique and dominant role played by the genome, the cell is probably more affected by any disruption of covalent bonds in its genome than in any other cell constituent. Any irreparable damage to the DNA thread may result in a loss of function and lead to failure in cell reproduction and to cell death. It is hardly surprising, therefore, that cells should have evolved special devices for the preservation of the genome in adverse circumstances, such as when subject to damage by chemical mutagens, or high-energy radiations.

Several mechanisms for reconstructing damaged or fragmented genomes have been discovered in *Escherichia coli*, *Bacillus subtilis*, and *Micrococcus lysodeikticus*. Notable advances into this field followed investigations into the effects of ultraviolet (uv) light and into the manner in which cells respond to the formation of uv photoproducts in their DNA. Studies with uv light have been profitable, because this agent is known to form specific photoproducts in the DNA of the cell, which are stable and can be measured chemically. In this paper, after briefly summarizing the properties of the uv photoproducts and two well-known mechanisms for survival after uv irradiation, a third and possibly more versatile mechanism will be described.

**UV Photoproducts in DNA**

Exposure to uv light induces the formation of various photoproducts in DNA (Beukers, Ijlstra, and Berends, 1960;
Wacker, 1963; Smith, 1964; Setlow, J. K., 1966). Dimers are formed between adjacent pyrimidine bases in high yield and comprise 50 percent thymine-thymine dimers, 40 percent thymine-cytosine dimers, while cytosine-cytosine dimers represent only 10 percent of the yield (Setlow, R. B., Carrier, and Bollum, 1965; Setlow, R. B., 1966). These dimers are formed by linking the unsaturated double bonds to form a cyclobutane ring, and they must cause considerable local distortion of the phosphodiester backbone of the twin helix. The principal thymine-thymine dimer in uv-irradiated DNA is linked in the 5-5 and 6-6 positions in a cis-syn boat configuration, as would be expected if it is formed from adjacent bases in the same single strand of a twin helix. This isomer can be distinguished from others by chemical means (Blackburn and Davies, 1966). Pyrimidine dimers are probably the major products formed by uv light in DNA, but other products such as hydrates are formed in lesser amounts. As the yield of dimers is known, a defined number of these stable defects can be produced in DNA at will by exposing cells, or DNA, to the appropriate dose of uv light. The yield of pyrimidine dimers is about $1.3 \times 10^{-6}$ dimers per base pair per erg/mm$^2$ at 2537 A (Setlow, R. B., 1966; Wulff, 1963; Setlow, R. B., Swenson, and Carrier, 1963; Boyce and Howard-Flanders, 1964a). A uv dose of 1 erg/mm$^2$ will produce about six dimers in a genome of $10^7$ nucleotides (which is about the number of nucleotides in the genome of *E. coli* deduced by autoradiography (Cairns, 1963).

The biological effects of uv light are due, at least in part, to the formation of pyrimidine dimers and other photoproducts in DNA (Setlow and Setlow, 1962). Just how much of any biological effect is due to a particular type of radiation product is not readily determined because of the difficulty of producing only specific products in particular components of the cell. Moreover, many of the biological effects may be due to products formed in DNA, which are subject to the action of repair enzymes.
Fortunately for the development of the subject, pyrimidine dimers can be measured chemically and are stable. No less than three mechanisms are known for obviating the effects of such dimers in *E. coli*. These are: (a) photoreactivation, (b) excision, and (c) exchanges between sister duplexes following DNA replication. All three mechanisms can be effective in promoting colony formation in cells exposed to uv light.

**Photoreactivation**

When exposed to uv irradiation, many microorganisms exhibit a loss of viability and other biological functions. Partial recovery of biological activity may occur if the cells are subsequently exposed to light of about 3,500 Å wavelength. Photoreactivation, as this is called, is believed to reflect the action of a photoreactivating enzyme which has been extracted and purified (Rupert, 1962; Mohammed, 1966). If DNA containing pyrimidine dimers is treated with photoreactivating enzymes and exposed to 3,500 Å light, the dimers are removed and probably monomerized *in situ* (Setlow, R. B., Carrier, and Bollum, 1965; Setlow, J. K., Boling, and Bollum, 1965; Cook, 1967). Also of interest is the isolation of a mutant of *E. coli* defective in photoreactivation (Harm and Hillebrandt, 1962) and the finding that this strain lacks active photoreactivating enzyme (Rupert, 1965). It may be significant to understanding this phenomenon that photoreactivation can be mimicked by dye-sensitized photodissociation of thymine dimers in solution if a suitable dye is present (Lamola; 1966). This dissociation may reflect the transfer of energy from a triplet excited state in the dye to the dimer.

**DNA Repair by Excision of Defects**

After exposure to uv light, the repair of DNA is initiated by the excision of pyrimidine dimers. Both thymine-thymine
and thymine-cytosine dimers are released or excised from the DNA of certain microorganisms during incubation following irradiation. Pyrimidine dimers are released in wild type but not in certain uv-sensitive mutants, and they are recovered within acid-soluble oligonucleotides. Evidently, dimers are released from the DNA by excising short single-strand fragments (Boyce and Howard-Flanders, 1964a; Setlow, R. B. and Carrier, 1964). This release of dimers into an acid-soluble form has been reported in *E. coli*, *Micrococcus radiodurans*, *Bacillus megaterium*, T4-phage-infected *E. coli*, and *B. subtilis* (Setlow, R. B., 1966; Strauss, Searashi, and Robbins, 1966; Shuster, 1967). Evidence for an *in vitro* enzyme activity for the release of pyrimidine dimers from DNA has been obtained in extracts of *M. lysodeikticus* and there has been some success in the purification of a dimer-specific endonuclease (Carrier and Setlow, 1966; Miller, *et al.*, 1967). An enzyme activity which may be similar but was detected in extracts of the same organism by a biological assay has also been reported (Rorsch, Kamp, and Adema, 1964; Elder and Beers, 1965). Although these systems have been used to demonstrate the excision of dimers *in vitro*, further purification and investigation of the characteristics of the excision enzyme are still necessary.

The evidence that excision may constitute the first step in a process for the repair of DNA-containing dimers depends upon the properties of uv-sensitive mutants lacking the ability to release dimers, that have been isolated in several organisms. The greater sensitivity of these strains may reflect their inability to initiate DNA repair by the excision of the dimers. These excision-defective mutants are abnormally sensitive not only to uv light but also to bifunctional alkylating agents (Haynes, 1964), mitomycin C (Boyce and Howard-Flanders, 1964b), and nitrous acid (Finesilver, 1968). The range of mutagens to which these excision-defective strains are abnormally sensitive is much wider than the range for
which evidence of the photoreactivation of treated cells can be obtained. An interpretation of this difference in specificities is that whereas the photoreactivation may act by an enzyme-sensitized photodissociation and act only on a restricted class of structures, the excision enzyme acts by cutting covalent bonds in the phosphodiester backbone on either side of the damaged bases. To judge by its more catholic range of action, the excision enzyme may recognize a distortion of the DNA twin helix near the site of the damage rather than the specific product responsible. It may be significant that all the mutagens whose effects are more pronounced in excision-defective than in normal cells are cross-linking agents (Haynes, 1964; Boyce and Howard-Flanders, 1964b; Finesilver, 1968). Much interesting work remains to be done in exploring the stereo-chemical specificities of excision enzymes.

**DNA Degradation and Repair Replication**

When *E. coli* is uv irradiated and incubated, there is a degradation of the DNA in wild-type strains, but not in excision-defective mutants. This breakdown may be initiated by excision and reflect the action of an enzyme on the free single strands ending at these cuts. The total number of nucleotides released may be several hundred times larger than the number of dimers initially present in the DNA (Boyce and Howard-Flanders, 1964b; Howard-Flanders and Boyce, 1966). This suggests that the gaps left by excision may be substantially widened.

If the repair is to be completed, the gap must be filled with nucleotides complementary to those of the intact opposite strand, a process called repair replication. Evidence for this has been obtained, as radioactive, or density-labeled, nucleotides are incorporated into small patches in the DNA of uv-irradiated cells, rather than being restricted to the region of new DNA synthesis as in normal replication (Pettijohn and
Hanawalt, 1964; Hanawalt, 1967; Billen et al., 1967). Repair will be completed when the phosphodiester backbone is joined following the insertion of the last nucleotide into the gap.

The properties of certain enzymes of E. coli may be relevant to understanding the mechanism of DNA repair. Exonuclease III is effective on two-strand DNA and releases nucleotides from the 3' terminus (Richardson and Kornberg, 1964). E. coli DNA polymerase acts by the addition of 5' nucleotides to the 3' terminus (Richardson, et al., 1964). These enzymes have the specificities required for single-strand breakdown and repair synthesis and have been used to demonstrate the removal of nucleotides and the repair of λ-phage DNA (Richardson, Inman, Kornberg, 1964). However, there is no evidence to show whether these are the enzyme responsible for DNA degradation and repair synthesis in E. coli. If they are, it seems likely that the single-strand gaps formed by dimer excision are widened and then filled in by action on the 3' side of the gap. However, a direct test is still needed to show whether DNA breakdown and repair synthesis following excision proceeds in this fashion.

The formation of single-strand gaps and their subsequent repair in uv-radiated E. coli has been observed in experiments on the sedimentation of bacterial DNA in alkaline sucrose. Even when the DNA from a cell contains several thousand dimers, fewer than twenty, single-strand gaps, appear to be open at any one time (Setlow, R. B., 1967). Evidently, single-strand breakdown and joining follows rapidly once excision has taken place, as would be expected if excision enzyme is present in limited amount, while the breakdown and repair synthesis enzymes are abundant. Alternatively, the repair process may in some way be coordinated so that the required enzymes act sequentially in a single unit and complete the repair of one site before moving to the next site.
Single-Strand Joining

There is evidence from other systems to show that single-strand breaks produced in the DNA of *E. coli* in other ways are efficiently joined. For example, this is evident from the discovery that certain λ-bacteriophage recombinants contain joined fragments of DNA from both parents (Meselson and Weigle, 1961; Meselson, 1964), and from the low efficiency of killing by the decay of incorporated P$^{32}$ in the DNA of bacteria (Fuerst and Stent, 1956), and from the rapid formation of circular covalently bonded DNA by λ-phage, which can be recognized as fast-sedimenting material in alkaline sucrose gradients (Young and Sinsheimer, 1964; Bode and Kaiser, 1965). Gellert (1967) discovered that if phage λ-DNA is annealed *in vitro* to form hydrogen-bonded circular DNA, these circles can be converted by a crude extract of *E. coli* to the covalently bonded circular form, which can be detected as a fast-sedimenting component in alkali. Thus, hydrogen-bonded circles of phage λ-DNA are a natural substrate for the strand-joining enzyme. Olivera and Lehman (1967), Gefter, Becker, and Hurwitz (1967), and Zimmerman, Little, Oshinsky, and Gellert (1967) have purified this strand-joining enzyme, which is now generally referred to as polynucleotide ligase. Weiss and Richardson (1967) have purified the phage T4 enzyme from phage-infected cells.

Base Sequence Correction after Replication

*The Role of Genetic Recombination in the Survival of Colony-Forming Ability after UV Irradiation.* Although mutants defective in dimer excision are very uv sensitive in comparison with wild type, they nevertheless survive the production of many pyrimidine dimers in their DNA and retain the capacity for colony formation. On the average, about fifty pyrimidine
TABLE ONE: The numbers of pyrimidine dimers formed by the UV dose leaving 37 percent survival of colony-forming ability for various strains

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>uv dose for 37% survival in ergs/mm²</th>
<th>Corresponding number of dimers per 10⁷ nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>++</td>
<td>500</td>
<td>3,000</td>
</tr>
<tr>
<td>AB2463</td>
<td>recA13 ++</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>AB1886</td>
<td>+ uvrA6</td>
<td>8</td>
<td>50</td>
</tr>
<tr>
<td>AB2480</td>
<td>recA13 uvrA6</td>
<td>0.2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* The cells were harvested after overnight growth in yeast extract tryptone broth without aeration and plated on agar containing the same nutrients immediately before exposure to UV light. The plates were scored for visible colonies after incubation at 37°C for 18 to 24 hr. The relation between the fraction of cells forming colonies is approximately exponential only for the excision-defective strains AB1886 and AB2480.

dimers are formed in each genome per lethal event in this strain. Two interpretations may be considered. (a) Dimers may be tolerated in the genome and the consequent changes or deletions in the base sequence may be unimportant for colony formation. (b) Excision-defective cells may contain a mechanism for circumventing dimers so that the genomes of the daughter cells carry the correct base sequences, even though photoproducts remain in the irradiated chromosomes.

The first interpretation seems most improbable because of the remarkable precision exhibited by the gene-protein systems that have been investigated in detail. Moreover, it is hard to reconcile the first interpretation with the properties of strains that are forty times more UV sensitive and in which single pyrimidine dimers may sometimes suffice to prevent colony formation. As seen in Table 1, certain strains defective in both dimer excision and in genetic recombination are so sensitive that 0.2 ergs/mm² (which forms 1.3 pyrimidine dimers per genome of 10⁷ nucleotides) will reduce the number of colony-forming cells by one natural logarithm. If single pyrimidine dimers can be lethal in these double mutants, it
seems unlikely that great liberties can be taken in the base sequence of the daughter genomes.

The Construction of an Undamaged Genome in an Irradiated Haploid Organism Defective in Dimer Excision. As the greater survival of colony-forming ability in normal as compared to recombination-deficient mutants of *E. coli* does not depend upon a capacity for dimer excision, there may exist a third mechanism to promote cell survival following uv irradiation. If we assume as a working hypothesis that colony formation depends upon the construction of a daughter genome free from defects, and that the pyrimidine dimers remains in the parental DNA because the strain is excision-defective, then there must be a mechanism for deriving a daughter genome with correct base sequences from the damaged parental DNA. Evidently, this mechanism for promoting cell survival may depend in some way upon the machinery of genetic recombination. In this respect, it may parallel the mechanism of multiplicity reactivation. How do these mechanisms for survival work?

According to contemporary speculation, the initial act in genetic recombination may require the formation of a single strand with a free end, able to unwind from its own twin helix and pair with any homologous DNA that may be near. Indirect evidence for this idea comes from the efficiency with which fragments of transducing, or transforming, DNA are able to integrate into the homologous sites of the bacterial chromosome (Meselson, 1967; Lacks, 1966; Fox, 1966). It may therefore be asked whether free ends may also play a role in the mechanism by which recombination is increased following exposure to uv irradiation.

The Formation of Free Single-Strand Ends by Pyrimidine Dimers Without Excision. If single-strand cuts are required to initiate recombination, then the cuts formed by dimer excision may be responsible for the increased frequency of recombination following uv irradiation that is observed in
certain crosses between bacteriophage or bacteria (Jacob and Wollman, 1955). However, it has been noted that the frequency of genetic exchange between two markers can be increased very markedly by uv light in strains defective in dimer excision (Wilkins and Howard-Flanders, 1968). In view of this observation, it appears that single-strand ends may be formed at dimers in ways other than by excision. One possibility is that if dimers have not been excised at the time the DNA undergoes replication, then, because of the inability to form base pairs at this point, the daughter strand is formed with a gap opposite each dimer. The single-strand breaks so formed may be of unusual longevity because the presence of pyrimidine dimers in the opposite strand would inhibit joining by the cellular repair enzymes. This hypothesis led us to investigate the nature of the newly synthesized single strands formed when DNA containing pyrimidine dimers is replicated.

The Replication of DNA Containing Pyrimidine Dimers. It is a historical accident that investigations into the effects of uv light on DNA replication were first carried out in wild-type E. coli B and in the uv-sensitive mutant strain Bs-1, which is defective in dimer excision (Setlow, R. B. and Carrier, 1964). This strain incorporates very little thymidine after exposure to a low uv dose, 25 ergs/mm², and when synthesis estimated from the thymidine incorporation was compared with the amount expected, on the assumption that each dimer acts as a block to replication, there was an excess incorporation by a factor of about five. This led to the concept that pyrimidine dimers block DNA replication (Setlow, R. B., Swenson, and Carrier, 1963; Swenson and Setlow, 1966). However, this strain Bs-1 carries a mutation at the uvrB locus and a second mutation at the exr locus linked to metA, which also affects thymidine incorporation following uv irradiation. The results are therefore difficult to interpret. Strains of E. coli defective in dimer excision (but not double mutants such as Bs-1) show over half the normal rate of thymidine incorporation follow-
ing exposure to a uv dose of 25 ergs/mm². As these irradiated cells must contain nearly 150 pyrimidine dimers per genome, it seems likely that DNA replication may proceed past 100 or 200 dimers per hour, even if the polymerase is delayed a few seconds at each dimer (Rupp and Howard-Flanders, 1968).

Evidence That the Daughter Chromosome Is Made in Fragments and Joined in UV-Irradiated Cells

Methods have been developed for investigating the molecular weight of single-strand DNA from the E. coli chromosome by sedimentation in alkaline sucrose, taking precautions to avoid shear degradation. When E. coli is labeled with H³-thymidine and lysed directly in the top layer of an alkaline sucrose gradient, the DNA has been found to sediment at a rate that indicates the molecular weight to be between 1 and 2 x 10⁸ daltons (McGrath and Williams, 1966). Thus, the single-strand DNA fragments observed by this method correspond to about 1/10 to 1/20 of the whole bacterial chromosome. This method has been used to study radiation-induced breaks in DNA. If E. coli is exposed to uv light or X-rays, the rate of sedimentation of the DNA is reduced below the normal value, as if these radiations introduce single-strand breaks. However, in strains deficient in dimer excision because they carry mutation at either the uvrA or uvrB locus, there is no fall in the apparent molecular weight following UV irradiation (Rupp and Howard-Flanders, 1968). This is consistent with the idea that these cells lack the dimer-excision enzyme and so remain intact with the pyrimidine dimers still in their DNA.

This same method has recently been used to investigate the molecular weight of the DNA synthesized in cells containing pyrimidine dimers in their DNA. A strain defective for dimer excision was exposed to about 60 ergs/mm² of uv light and then incubated with H³-thymidine for ten minutes. The newly synthesized DNA was found to sediment more slowly than normal DNA (See Table 2), as if it contained gaps or
alkali-labile bonds. When the molecular weight distribution of this material, as determined by sedimentation in alkaline sucrose, was compared with the calculated distribution of the molecular weights of the single-strand lengths between pyrimidine dimers produced by this dose of uv light, the two distributions were remarkably similar (Rupp and Howard-Flanders, 1968). Thus, when DNA containing pyrimidine dimers is replicated, the newly synthesized DNA contains gaps, or alkali-labile bonds, in about the same numbers as the pyrimidine dimers, as if one is formed at each dimer, as illustrated in Figure 1.

Although these results do not show that the gaps are opposite the dimers, the following evidence from experiments on susceptibility to excision in conjugating systems suggests that this may be so.

Failure to Excise Dimers from DNA after Transmission Between Conjugating Bacteria

If an F+ strain, unable to excise pyrimidine dimers, is exposed to uv light and allowed to conjugate with an F− recipient, an episome containing uv-induced dimers is transmitted to the recipient. Such a strain may be permitted to conjugate with a normal or an excision-defective recipient, and the genetic activity of the transmitted episome can be
compared in the two strains. In three different experiments, it has been reported that the effectiveness of uv-irradiating the donor prior to conjugation is not influenced by the capacity for excision in the recipient. This was found to be the case in experiments on the indirect induction of λ-bacteriophage (Devoret, Monk, and George, 1965) in the production of lactose-fermenting colonies from a lac– strain (Rorsch, et al., 1962) and in the transmission of the ability to produce β-galactosidase in response to an inducer (Pardee and Prestidge, 1968). Thus, in three systems something appears to happen to the pyrimidine dimers during transmission which renders them no longer subject to the action of excision enzymes in the recipient.

A possible explanation is that the episome undergoes replication at the time of transmission (Jacob, Brenner, and Cuzin, 1963; Gross and Caro, 1966) and that the structure of the DNA in the region of the dimer differs after replication. In view of the finding that the average spacing between gaps in newly synthesized DNA is approximately equal to the spacing between dimers after normal replication, it seems possible that there are also gaps opposite the dimers in the transmitted episome. It has already been suggested that dimer-excision enzymes may recognize characteristic distortions of the DNA twin helix. Evidently, this enzyme might fail to act on a dimer if there is a gap in the opposite strand. It is still to be determined, however, whether the gaps are formed directly by the DNA polymerase, or whether special alkali-labile, or nuclease-sensitive, links are inserted into the daughter strand by the DNA polymerase in response to the presence of a dimer in the DNA undergoing replication.

Exchanges Between Sister Duplexes Following DNA Replication

As already stated, when excision-defection strains are exposed to uv light, incubated in the presence of H³-thymidine, and
TABLE TWO. Estimates of molecular weights of newly synthesized DNA from UV-irradiated E. coli sedimented in alkaline sucrose (Rupp and Howard-Flanders, 1968)*

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>D/D_T2 at peak of radioactivity in gradient</th>
<th>M x 10^-6 daltons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells labeled for 10 min.</td>
<td>1.5</td>
<td>160</td>
</tr>
<tr>
<td>Cells exposed to 60 ergs/mm² uv and then labeled for 10 min.</td>
<td>0.70</td>
<td>20</td>
</tr>
<tr>
<td>Cells exposed to 60 ergs/mm² uv, labeled for 10 min., and then incubated for 70 min.</td>
<td>1.3</td>
<td>120</td>
</tr>
</tbody>
</table>

* A thymine-requiring strain E. coli K-12 uvrA6 defective in dimer excision was grown in a glucose-casamino acids-salts medium with thymidine and subjected to the treatments listed above, using membrane filtration to effect transfer between the appropriate media. Labeling was with H³-thymidine at 100 μC/ml. After treatment, the cells were converted to spheroplasts in M9 buffer in 6% w/v sucrose and 0.1M tris at pH 8.1 by adding 1% lysozyme and 0.25% versene. After 5 min. at ice temperature, .01 ml was lysed directly on a 5 ml 2 to 20% w/v sucrose gradient containing .04 to .1 M NaOH at pH 12 (McGrath and Williams, 1966). The gradients were centrifuged at 30,000 rpm for 2 hr. at 20° C. in a SW 50 rotor, fractions were collected, and the H³ radioactivity was measured. The molecular weights listed above were determined from the positions of the peaks of maximum radioactivity in the gradients (Rupp and Howard-Flanders, 1968).

Then lysed so that their DNA can be sedimented in alkaline sucrose, the newly synthesized DNA appears to be of relatively low molecular weight. If these cells are incubated for a period of one hour between uv irradiation and labeling, this newly synthesized DNA also sediments slowly. The low molecular weight of the newly synthesized DNA presumably results from the fact that the pyrimidine dimers remain in the template DNA throughout the one-hour incubation. If, however, this experiment is repeated and the uv-irradiated cells are labeled for ten minutes and then incubated for one hour in non-radioactive medium before sedimenting in alkali, then the
sedimentation rate of the DNA synthesized after uv irradiation is more rapid and is close to the level observed in unirradiated control cells (see Table 2). Evidently, the daughter-strand fragments are in some way joined into high molecular weight material during the subsequent one-hour incubation (Rupp and Howard-Flanders, 1968).

These observations strongly suggest that there is some kind of reconstruction mechanism for assembling the newly synthesized fragments into a daughter genome. At any point where a duplex contains a dimer with a gap in the opposite strand, the sister duplex formed at replication will generally be intact. Exchanges between the sister duplexes could lead to the gap in the newly synthesized strand being filled in with the correct base sequence. Thus, the daughter-strand fragments may be assembled in a complex recombination-like process to form complete single strands of the genome. It may be these daughter strands that are replicated and give rise to viable daughter cells and are responsible for the survival of colony-forming ability.

This recombinational mechanism for survival following exposure to uv light may be more powerful and more universal than either photoreactivation or DNA repair by dimer excision. If we accept the greater survival of colony-forming ability of normal, as compared to recombination-deficient mutants, as evidence for the operation of such a mechanism, then it would appear to act after exposure to a wide variety of mutagens. The capacity of cells for excision has little effect on their survival after X-irradiation and almost none after treatment with monofunctional alkylating agents, such as methyl methane sulfonate. However, recombination-deficient mutants are abnormally sensitive to both these agents. Thus, it would appear that in addition to any enzymatic rejoining of X-ray-induced single-strand breaks that may occur, an important mechanism for survival following X-ray damage to the bases utilizes the machinery of genetic recombination
and thus may depend upon exchanges between the sister duplexes formed as the damaged DNA is replicated.

*Pyrimidine Dimers and Daughter-Strand Gaps as Intermediate Structures in Lysogenic Induction*

The discovery that the replication of DNA containing damaged bases can lead to the formation of a daughter strand with gaps opposite dimers is of interest in another connection. *E. coli* lysogenic for λ phage can be induced by exposure to UV light. We may therefore inquire whether the intermediate structure that causes induction might consist of DNA containing gaps opposite dimers. This hypothesis affords an explanation of the following observations. Indirect induction may take place when a λ lysogen is mated with a UV-irradiated F+ donor. This induction occurs with greater than normal efficiency if the donor is excision-defective but, as mentioned earlier, is independent of whether the recipient lysogen is excision-defective or not (Devoret, Monk, and George, 1965). As the episome is thought to be replicated during transmission (Jacob, Brenner, and Cuzin, 1963; Gross and Caro, 1966), there will be an opportunity for the postulated intermediate structure carrying dimers and gaps to be formed. Moreover, after transmission the dimers would no longer be subject to excision, which is in accord with experimental observations (Devoret, Monk, and George, 1965). It is also known that λ lysogens may be induced directly by exposure to UV irradiation, but this induction occurs only if the culture is actively growing (Jacob, 1952). Direct induction occurs with a greater yield-per-unit dose of UV light in excision-defective strains than in wild type (Howard-Flanders and Boyce, 1966; Harm, 1965; Mattern, Winden, and Rorsch, 1965). A coherent interpretation of these observations is afforded by the hypothesis that direct and indirect UV induction is due to an intermediate structure containing gaps opposite the UV dimers, formed by the replication
of a particular region of the uv-irradiated chromosome or episome.

Conclusions

In *E. coli* mutants defective in dimer excision, the effects of uv-induced pyrimidine dimers on the survival of colony-forming ability are largely circumvented by another mechanism. This mechanism appears to operate after DNA replication and to involve the daughter strand's being formed in fragments with gaps opposite each dimer. These daughter-strand fragments have been observed. There is indirect evidence to show that the gaps are probably opposite the photoproducts. Survival of colony-forming ability appears to depend upon the machinery of genetic recombination for the assembly of the daughter-strand fragments into a viable genome. The induction of a λ-lysogen by uv light may be due to a structure formed by the replication of some particular region of the bacterial chromosome (or an episome) containing pyrimidine dimers and the formation of gaps in the daughter strand opposite the defective bases.

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CHROMOSOME TRANSFER
IN BACTERIAL MATING

Thomas S. Matney
& Joan C. Suit

Genetic information essential to the growth of Escherichia coli K12 is contained in a continuous linkage group. Physically it appears to be a double-stranded molecule of deoxyribonucleic acid (DNA) some 1.3 mm in length. This "chromosome" behaves as a single replicating unit (Cairns, 1963). A growing cell of E. coli usually contains several such masses of DNA (Mason and Powelson, 1956).

Nonessential genetic information may exist as separately replicating units of DNA. The fertility factor (F) of E. coli K12 is an example. When it is present in the cells in an autonomous form the culture is referred to as F+. When F+ cells are mixed with cells devoid of F, designated F−, conjugal pairs are formed and F particles are transferred to the recipient cells from the F+ donors, converting the recipients to F+. Occasionally in F+ populations the F-factor may integrate into the large bacterial chromosome at one of a limited number of sites (Matney, Goldschmidt, and Erwin, 1963). Progeny of such a mutant cell inherit F at the fixed genomic site. When they are mixed with F− cells, conjugation occurs, the continuous donor chromosome breaks at the site of F attachment, and the resulting linear structure is transferred into the recipient cell such that the end with F attached to it is the last to enter. The high frequency of genetic recombinants produced by such a cross has led to the designation of donor derivatives containing F in the integrated state as Hfr.

The mating process may be divided into several phases:
formation of effective pairs, mobilization and transfer of chromosome, pairing of homologous regions of donor and recipient DNA in the zygote, recombination, and finally phenotypic expression.

Experimentally, there are two ways to effect pair formation. One is to mix the Hfr and F- bacteria together in liquid media where they come together by collision with the aid of whatever attractive devices they may have such as the F-specific pili or hair-like appendages of the male (Brinton, 1965); the other method to effect conjugal pairs is to force them into direct contact by either centrifuging them into a pellet or impinging them onto the surface of a membrane filter.

Jacob, Brenner, and Cuzin (1963) proposed a model that offered a possible mechanism for chromosome transfer. They suggest that when an F- cell conjugates with an Hfr it triggers the integrated F to initiate a new cycle of semi-conservative DNA replication. As the chromosome passes through the membrane-associated F replicator, one new daughter molecule is propelled into the F- recipient while the other remains in the Hfr. The model predicts that only DNA, newly synthesized in the donor, would be transmitted into the recipient.

Evidence for this model has been obtained in liquid matings. The autoradiography studies of Gross and Caro (1966) have shown the donor DNA in the zygote to consist of one old strand and one new strand, the latter synthesized in the Hfr during mating.

On the contrary, with forced matings on membrane filters a different picture has emerged. A family of Hfr's derived from a histidine requiring F+ strain was sharply limited in the ability to continue DNA synthesis when suddenly deprived of the required amino acid. A net increase of only 10 percent DNA was detected during the first thirty minutes of amino acid starvation. Prestarving the Hfr for histidine did not reduce its capacity to transfer chromosome and effect re-
Figure 1. Kinetics of $\text{Arg}^+_{\text{G6}}$ recombinant formation in a cross between Hfr G6 His$^-$ and F$-$ Arg$^-$.$^\dagger$. When the membrane filter matings were performed on unsupplemented soft minimal agar, 65 percent of the Hfr cells effected a recombinant; approximately twice as many recombinants resulted from matings on histidine-supplemented medium. Cells on one set of membrane filters were mated on minimal for 60 minutes and then transferred to histidine-supplemented medium; the starred curve denotes a second round of transfer. Mat- ing was interrupted by eluting the cells in saline, diluting, and vortexing for 30 seconds prior to plating on hard minimal agar to score for $\text{Arg}^+_{\text{G6}}$ recombinants.

combinants when mated on membrane filters resting on un-
supplemented soft minimal agar (Suit, et al., 1964).
TABLE ONE: Incorporation of C¹⁴-thymine by 
Hfr G6 His-323 Thy-158* impinging on membrane filters

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Cts/min/7 x 10⁷ Hfr cells†</th>
<th>Arg⁺ recombinants per 100 donor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hfr</td>
<td>Hfr x F—**</td>
</tr>
<tr>
<td></td>
<td>Hfr</td>
<td>+Hist</td>
</tr>
<tr>
<td></td>
<td>−Hist‡</td>
<td>+Hist‡</td>
</tr>
<tr>
<td>5</td>
<td>132</td>
<td>319</td>
</tr>
<tr>
<td>10</td>
<td>250</td>
<td>184</td>
</tr>
<tr>
<td>20</td>
<td>134</td>
<td>234</td>
</tr>
<tr>
<td>30</td>
<td>79</td>
<td>265</td>
</tr>
<tr>
<td>40</td>
<td>215</td>
<td>654</td>
</tr>
<tr>
<td>50</td>
<td>14</td>
<td>265</td>
</tr>
<tr>
<td>60</td>
<td>−27</td>
<td>239</td>
</tr>
<tr>
<td>70</td>
<td>79</td>
<td>434</td>
</tr>
</tbody>
</table>

* Prestarved 30 minutes for histidine.
† Counts adsorbed to blank filters, and to filters upon which only F— cells were impinged were subtracted from Hfr and Hfr x F— filters, respectively.
‡ Membrane filters were incubated on soft agar containing minimal salts, 0.4 percent glucose, 1.5 µg/ml C₁₂-thymine, 0.46 µg/ml, 0.1 µC/ml C₁₄-thymine and ± 100 µg/ml histidine. The filters were removed at the indicated times, washed with cold 5 percent trichloracetic acid, dried, and counted in the Tri-Carb-liquid scintillation counting system.
** F— Arg— in 9-fold excess.

The fertility of the G6 Hfr on membrane filter–minimal agar matings has consistently approached 75 percent (75 Arg⁺ recombinants per 100 donor cells). When histidine is added to the soft minimal agar mating medium the fertility doubles, as shown in Figure 1. In this experiment when the mating occurred on minimal agar the fertility was 65 percent and on minimal plus histidine 123 percent. The latter can only mean that many of the Hfr cells mated with two female cells simultaneously and effected two recombinants. Another feature of this experiment was to transfer several membranes after an hour of mating on minimal agar to minimal plus histidine to determine if a second round of transfer could be triggered by restoring the male's synthetic capacity. As may be seen from the starred curve, a second round of chromosome transfer did indeed occur and the final number of
recombinants obtained reached the minimal plus histidine control value.

To further substantiate that the G6 Hfr was transferring chromosome in the absence of detectable DNA synthesis, a thymine-less derivative was mated on C\textsuperscript{14}-thymine-containing medium with and without histidine. The incorporation of C\textsuperscript{14} into acid-insoluble cellular material during a seventy-minute time period is shown in Table 1. No increase in amount of label incorporated into acid-insoluble material could be detected during the minimal mating, while DNA synthesis proceeded rapidly during plus histidine mating. Twice as many recombinants were obtained in the plus histidine mating as with minimal. It appeared that during the minus histidine mating the recipient did not feed sufficient histidine to the Hfr to permit DNA synthesis (Krisch and Kvetkas, 1966).

We infer from these studies that in forced matings one chromosome may be transferred without detectable DNA synthesis while a second chromosome is transferred only if the synthetic capacity of the Hfr donor is restored. The second type of transfer may be the one in vogue in liquid matings.

It had been assumed from Fisher (1957) early data that the recipient cell did not play an active role in the transfer process. Curtiss and Charamella (1966) have shown that the recipient does have an energy requirement during transfer and, further, that the initial donor DNA entering the recipient must pair with its homologous region on the female chromosome—leaving us with the clearcut possibility that the female chromosome then revolves and reels in the donor DNA.

ACKNOWLEDGMENTS

This work was supported by Grant No. GM-12433 from the U. S. Public Health Service and Grant Nos. GB-1146 and GB-3744 from the National Science Foundation. Joan C. Suit
CHROMOSOME TRANSFER IN MATING

was a Faculty Research Associate of the American Cancer Society, Grant No. PRA-19.

REFERENCES


SUPPRESSION OF PHAGE MUTANTS
BY OCHRE SUPPRESSORS

Robert Haselkorn

In the spring of 1966 I realized that although I had been teaching for several years about the power of genetics in answering biochemical questions, I had taken little advantage of it in my own research. To remedy that situation, I spent that summer at Cold Spring Harbor, in C. R. Davern’s laboratory, studying the suppression of nonsense mutations in bacterial viruses. Nonsense mutations have been known for a long time, but it was only five years ago that their fundamental nature was described by Benzer and Champe, working on the rII cistrons of bacteriophage T4 (1962), and by Garen and Siddiqi, working on *Escherichia coli* alkaline phosphatase (1962). Nonsense mutations are ones in which a code triplet for polypeptide chain termination has been placed, by mutation from a triplet for an amino acid, in the interior of the corresponding RNA message—the result is premature polypeptide termination. The existence of nonsense mutations strongly suggests that there is something specific in the cell that causes termination of the polypeptide chain. And, obviously, nonsense mutations should be quite useful in studying polypeptide chain termination. For this purpose, the property of nonsense mutations to be exploited is their ability to be suppressed. One model for suppression is shown in Figure 1. In wild-type (su−) cells we assume that an sRNA bearing a substance x, which causes chain termination, responds to the nonsense codon UAG and displaces the growing polypeptide chain from the sRNA in the polypeptide position. In suppressor-carrying cells (su+) a suppressor substance is
Figure 1. Brenner's model for suppression. Suppressor sRNA competes with chain-terminating sRNA on the surface of the ribosome. In principle, any factor affecting this competition will influence the efficiency of suppression.

present. In the case of one suppressor strain (su 1), the suppressor substance has been shown to be a serine sRNA (Capecchi and Gussin, 1965; Engelhardt, et al., 1965). This serine sRNA presumably has an anti-codon which responds to UAG, competing with the chain-terminating substance in such a way that serine is put into the polypeptide chain, preventing chain termination. This is the model for the mechanism of suppression of nonsense mutations: competition between an
Two sets of nonsense mutations are known: ambers and ochres. There are two kinds of suppressors: the amber suppressors and ochre suppressors (Brenner and Beckwith, 1965; Gorini and Beckwith, 1966). Amber suppressors insert an amino acid in response to UAG only, whereas ochre suppressors insert an amino acid in response to either UAG or UAA. This fundamental asymmetry in suppression pattern provides the operational distinction between the two classes and is summarized in Table 1.

The experiments I shall describe had their origin in a paradox. The ochre suppressors were first identified by Beckwith as apparent revertants of nonsense mutations in β-galactosidase (Gorini and Beckwith, 1966; Beckwith, 1963). These suppressors were then used to define a new set of T4 rII mutants as ochres, according to the pattern in Table 1. From the pattern of mutagenesis relating amber and ochre mutants of T4, Brenner and coworkers deduced that the ochre codon was UAA (1965). At the same time, Garen and coworkers isolated a set of suppressors, different from the amber suppressors, as apparent revertants of nonsense mutations in alkaline phosphatase (Gallucci and Garen, 1966; Weigert, Lanka, and Garen, 1967). They also deduced that the nonsense codon responding to the new set of suppressors was UAA. The paradox was that the rII ochre mutants of T4 failed to plate on the Garen suppressor strains. I wanted

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**TABLE ONE: General plating pattern of T4 mutants on bacterial suppressor strains**

<table>
<thead>
<tr>
<th>Phage mutant</th>
<th>Bacterial suppressor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amber</td>
</tr>
<tr>
<td>Amber</td>
<td>+</td>
</tr>
<tr>
<td>Ochre</td>
<td>-</td>
</tr>
</tbody>
</table>

aminoacyl sRNA and the chain-terminating sRNA for the nonsense codon (Brenner, Stretton, and Kaplan, 1965).
**SUPPRESSION OF PHAGE MUTANTS**

**TABLE TWO: Plating pattern of T4 amber mutants on E. coli suppressor strains**

<table>
<thead>
<tr>
<th>Suppressor</th>
<th>Amber</th>
<th>Ochre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>E 4301</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N 58</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* + means clearing of the bacterial lawn when a drop containing $10^4$ phage is plated at 37° C. Bacteria: am 1 = S26 R1e; am 2 = S26 R1d; am 3 = R8a; Och A = U11 R1d; Och C = H12 R7a, all from Garen (1962); Och B = CA165 from Brenner. Phage mutants are from the Edgar collection via J. Speyer.

to see if this failure was fundamentally related to the mechanism of suppression, or trivial, in the sense that it was due to some peculiarity of the requirement of rII product in T4-infected cells.

For this purpose, I required a large set of mutants, preferably in the same gene, whose plating pattern could be examined on many suppressor strains. I chose amber mutants because they plate on both kinds of suppressor. The results of spot tests on six suppressor strains are shown in Table 2. In some cases the spot tests were verified by single-burst ex-
Robert Haselkorn

The E43 mutants are all in gene 43 of T4, which is the structural gene for the phage DNA polymerase (deWaard, Paul, and Lehman, 1965). The strains Sui, Su2, Su3, SuA, and SuC are derivatives of Hfr Cavalli isolated by Garen and coworkers. The SuB strain is a derivative of Hfr Hayes from Beckwith and Brenner. The amber mutants of T4 were isolated on CR63, which carries the Sui suppressor, so it is to be expected that they will all plate on Sui. Su2 has been shown to insert glutamine; the failure of some mutants to plate on Su2 could be due to one of two possibilities: either glutamine is inserted at the point of the mutation, and the protein with glutamine at that position is non-functional, or glutamine insertion at that particular position is inefficient. The same is true for Su3, which inserts tyrosine. Nevertheless, without being able to distinguish these alternatives, we can still use the spot-test pattern for rapid qualitative identification of suppressors without having to do protein chemistry or linkage analysis.

For our purpose the last three columns are of major interest. We see that T4 amber mutants can indeed be suppressed by Garen's ochre suppressors, so the failure of the rII mutants to plate on these strains requires some special explanation. It turns out that Hfr Cavalli requires an exceptionally high concentration of the rII product to permit phage growth; transduction of SuC from Garen's strain into Hfr Hayes gave a strain capable of suppressing rII ochres (Brenner, Kaplan, and Stretton, 1966). In this sense, the paradox we started with has a trivial explanation.

The last two rows in the table illustrate another feature of ochre suppression of T4 mutants. These mutations are in a structural gene for tail fibre and the structural gene for head protein, respectively, both of which are needed in large amounts. In general, none of the mutants in these genes plates on ochre suppressor strains. The correlation appears to be with the amount of suppressed protein needed, rather
than the time of gene expression, since an amber mutant in lysozyme, a late function like the phage structural components, plates on all the ochre suppressors.

Comparison of the last three columns suggests that the spot-test pattern of suppression of T4 mutants could be useful in identifying new ochre suppressors as they are isolated, without requiring extensive linkage analysis by transduction or mating. To see whether the spot-test pattern could really be useful, I applied it to the suppressor strains used by Yanofsky and Ito (1966).

These suppressor strains were isolated independently of the Garen-Brenner set and used by Ito and Yanofsky for studies on the suppression of nonsense mutants in tryptophan synthetase. The results are shown in Table 3 for two of their

### Table Three: Plating pattern of T4 amber mutants on E. coli ochre suppressor strains*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Su5 HfrC</th>
<th>SuA W3110</th>
<th>Su4 HfrC</th>
<th>SuC W3110</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4301</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>5</td>
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<td>8</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>11</td>
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<tr>
<td>12</td>
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<tr>
<td>16</td>
<td>+</td>
<td>+</td>
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<tr>
<td>17</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>18</td>
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</tr>
<tr>
<td>20</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Su4 = H12 R7a; Su5 = U11 R1d (see Table 2). The W3110 strains are from Yanofsky (1966).
ochre suppressors, together with the appropriate Garen strains. First, the overall similarity suggests that Garen's Su5 is the same as Yanofsky's SuA, and Garen's Su4 is the same as Yanofsky's SuC. Second, there are a few mutants, E4310 for example, that plate on the Yanofsky strain but fail to plate on the Garen strain. Su4 was transferred by transduction from Hfr Cavalli to another bacterial strain (FE 15), which then gave the W 3110 SuC spot-test pattern. The most obvious difference between the Hfr strain on the one hand, and W 3110 and FE 15 on the other, is sex. The latter two are females. Apparently, these two females contain something which makes ochre suppression more efficient, at least at some mutant sites. Recently, Daniel McMahon in my laboratory crossed Hfr C Su4 Sm• tryp+ with W 3110 SuC Sm• tryp−, selecting for tryp+ Sm•. Among 325 tryp+ recombinants, he found no su− cells, indicating that SuC and Su4 are closely linked, and possibly identical. Furthermore he found that about half of the recombinants which gave a spot-test pattern characteristic of W 3110 SuC were males. That is, segregation for a "modifier" of suppression occurs at a locus distinct from that of the suppressor, somewhere between tryp and the F factor in Hfr Cavalli.

Returning to the model of suppression, there are at least three ways a modifier could act to enhance suppression at the level of translation. It could increase the level of suppressor sRNA; it could decrease the level of chain-terminating sRNA; or it could alter the ribosome to favor competition for the chain-terminating codon by the suppressing sRNA. The first explanation is not likely in our case, since the effect has been observed with four different suppressors, which would require that the level of four different amino-acid activating enzymes, for example, be higher in W 3110 than in Hfr Cavalli. To decide between the other two alternatives, we must do some in vitro experiments.

In summary, we showed that Garen's ochre suppressors
work reasonably well on T4 amber mutants, provided the mutations are not in rII or T4 structural proteins; that T4 spot-test patterns can be useful in identifying suppressors; and that derivatives of two historic females of *E. coli* carry a gene whose product enhances suppression of several T4 amber mutants, relative to suppression of the same mutants in Hfr Cavalli.

ACKNOWLEDGMENTS

This work was supported by Grant No. GM-12344 from the U. S. Public Health Service. I should like to thank Drs. C. R. Davern, J. Cairns, and J. Speyer for their hospitality during the summer of 1966.

REFERENCES


GENE ACTION IN THE
CONTROL OF BACTERIOPHAGE
T4 MORPHOGENESIS

William B. Wood

The formation of individual protein molecules under the control of structural genes is now fairly well understood. Considerably less is known about what is for many proteins the next step: their assembly into more complex supra-molecular aggregates such as multi-enzyme complexes or cellular organelles. If we assume that this process also must be under gene control, then we are faced with the question of how genetic information is used to direct it. For a few simple systems such as the tobacco mosaic virus (Anderer, 1959) and the γ-globulin molecule (Haber, 1964; Whitney and Tanford, 1965), reconstitution experiments have shown that genetic specification of the subunit primary structure is sufficient to determine the size and shape of the aggregate. It is not yet clear, however, whether this self-assembly mechanism can explain the morphogenesis of more complex structures composed of many different gene products.

For the further exploration of this question, some of the larger bacteriophages offer several attractive features. They are built of many different kinds of protein subunits, and their morphologies are complex (Stent, 1963; Brenner, et al., 1959). They are amenable to genetic analysis, which has already provided considerable insight into the process of intracellular phage development (Epstein, et al., 1963; Edgar and Epstein, 1964). Most important, their morphogenesis has been opened recently to direct attack by biochemical
methods through findings that the assembly of bacteriophages T4 (Edgar and Wood, 1966), lambda (Weigle, 1966), and P22 (Israel, Anderson, and Levine, 1967) can be studied in vitro using viral components derived from infected cells.

Work in our laboratory has continued to focus on T4 morphogenesis. In this paper I wish to review the current status of this problem, with reference to recent experiments by Edgar and Wood (1966), Edgar and Lielausis (1968), Flatgaard (unpublished), King (1968), King and Wood (1969), and Wood and Henninger (1969). Experimental details of this work have been or will be published elsewhere.

**T4 Genes Affecting Phage Maturation**

The studies of Epstein, *et al.* (1963), show that many conditional lethal mutations in T4 lead to accumulation of recognizable phage components under restrictive conditions. By noting the components not present in electron micrographs of mutant-infected cell lysates, these authors have inferred the normal function of more than forty mutant genes essential for viral morphogenesis (see Figure 1). The results suggest a morphogenetic pathway consisting of three major independent branches. These lead to the formation of head, tail, and tail fibers, respectively, and are followed by steps in which the completed components are assembled into infectious virus particles.

From the DNA content of the phage, it can be estimated that only one-half to one-third of the total genes have so far been identified by mutation. It is therefore not unlikely that between fifty and one hundred gene products are required for virus assembly. If correct, these figures have two possible implications. The number of kinds of protein present in the phage particle may be far larger than the ten to twenty for which there is now evidence (Brenner, *et al.*, 1959; Epstein, *et al.*, 1963). Alternatively, we can speculate that some of
Figure 1. Defective phenotypes of T4 conditional lethal mutants. Mutationally identified genes are represented by shaded areas indicating relative locations and approximate map lengths where known. Enclosed symbols indicate defective phenotype as follows: DNA NEG., no DNA synthesis; DNA ARREST, DNA synthesis prematurely arrested; DNA DELAY, initiation of DNA synthesis delayed; MAT DEF., maturation defective, late functions not expressed; hexagon, free heads produced; inverted T, free tails produced; TAIL FIBER, tail fiberless particles produced. Gene 9 mutants produce particles whose sheaths are often contracted in electron micrographs; gene 11 and 12 mutants produce fragile particles which dissociate on standing to free heads and tails. From Edgar and Wood (1966).

these gene products play purely directive or catalytic roles in the assembly process, rather than contributing materially to the phage structure.
In Vitro Complementation Tests

The demonstration of T4 assembly \textit{in vitro} has opened a new approach to studying the nature of the morphogenetic pathway and the roles of some of the gene products involved. The method is based on a single type of experiment, which we have called the \textit{in vitro} complementation test (Edgar and Wood, 1966). If infectious phage are produced when two extracts made from mutant-infected cells defective in different gene products are incubated together, then the extracts complement each other; that is, one supplies in functional form a viral component which is nonfunctional in the other. We have now performed a large number of such pairwise tests (Edgar and Wood, 1966; Edgar and Lielausis, 1968; King and Wood, 1969). Almost all give unambiguous results: either no detectable increase or more than a 10-fold increase in the infective titer. On this basis we can arrange the defective extracts into \textit{in vitro} complementation groups, defined such that members of different groups complement whereas members of the same group do not. Thirteen such groups have so far been defined (Table 1). Since each represents a unit of assembly function, there are thirteen com-

\begin{center}
\begin{tabular}{ |c|c|c|c| }
\hline
Complementation group & Mutant genes & Defective phenotype: components recognizable by electron microscopy** & Inferred assembly function of missing component \\
\hline
I & 20,21,22,23, 24,31,40 & Tails, tail fibers & Head (formation) \\
I & 49,2,64,50, 65,4,16,17 & Heads, tails, tail fibers & Head (completion) \\
II & 53,5,6,7,8, 10,19,25 & Heads, tail fibers & Tail endplate \\
& 26,51,27,28, 29,48 & & \\
\hline
\end{tabular}
\end{center}
### TABLE ONE (continued)

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>Mutant genes</th>
<th>Defective phenotype: components recognizable by electron microscopy**</th>
<th>Inferred assembly function of missing component</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>54</td>
<td>Heads, tail endplates, tail fibers</td>
<td>Tail core</td>
</tr>
<tr>
<td>IV</td>
<td>13, 14</td>
<td>Heads, tails, tail fibers</td>
<td>Completion and/or attachment of heads, tails</td>
</tr>
<tr>
<td>V</td>
<td>15</td>
<td>Heads, tails, tail fibers</td>
<td>Completion and/or attachment of heads, tails</td>
</tr>
<tr>
<td>VI</td>
<td>18</td>
<td>Heads, tails, tail fibers</td>
<td>Completion and/or attachment of heads, tails</td>
</tr>
<tr>
<td>VII</td>
<td>9</td>
<td>Particles with contracted sheaths, free tail fibers</td>
<td>Completion of particles</td>
</tr>
<tr>
<td>VIII</td>
<td>11</td>
<td>Fragile particles with attached tail fibers</td>
<td>Completion of particles</td>
</tr>
<tr>
<td>IX</td>
<td>12</td>
<td>Fragile particles with attached tail fibers</td>
<td>Completion of particles</td>
</tr>
<tr>
<td>X</td>
<td>37, 38</td>
<td>Tail fiberless particles</td>
<td>Tail fiber component</td>
</tr>
<tr>
<td>XI</td>
<td>36</td>
<td>Tail fiberless particles, half-tail fibers</td>
<td>Tail fiber assembly</td>
</tr>
<tr>
<td>XII</td>
<td>35</td>
<td>Tail fiberless particles, half-tail fibers</td>
<td>Tail fiber assembly</td>
</tr>
<tr>
<td>XIII</td>
<td>34</td>
<td>Tail fiberless particles, half-tail fibers</td>
<td>Tail fiber assembly</td>
</tr>
</tbody>
</table>

* Heads attached to tails are designated "particles." All structural components observed are unattached unless otherwise indicated. Description of components as heads, tails, etc., does not imply that these structures are complete but merely that they are identifiable in electron micrographs.

Preparation of mutant-infected-cell extracts and testing for *in vitro* complementation were carried out as previously described (Edgar and Wood, 1966). Groups I and II are not rigorously defined in that not all of the possible pairwise tests have been performed. Each member of both groups was tested against representative members of I (23-extract) and II (27-extract). Assignment to I or II was made on the basis of these results and the previously observed defective phenotypes, which were mutually consistent. Extracts of group Ia do not complement those of Ib i.e., the observed Ib heads are nonfunctional for *in vitro* assembly. Extracts are referred to above and elsewhere by the number of the defective gene of the amber mutant used to infect the cells.

ponents whose interaction to produce complete infectious virus can be studied \textit{in vitro} under our present experimental conditions.

The above interpretation is supported by evidence from electron microscopy that mutants in the same \textit{in vitro} complementation group exhibit the same defective phenotype (with the exception of mutants in group I; see legend to Table 1). From the latter, we can infer something about the assembly function of the missing component corresponding to each group (Table 1). Components I and II, the head and the tail, are formed under the control of many genes, whereas the remaining components consist of only one or two gene products. This indicates that the head and the tail cannot be assembled \textit{in vitro} but that at least three other classes of morphogenetic reactions can take place: the union of heads and tails, the assembly of the tail fibers, and the attachment of tail fibers to fiberless particles.

The foregoing analysis provides only limited information concerning the nature and sequence of assembly functions. Further experiments must be carried out in order to determine more precisely the roles of components such as IV, V, and VI, and the order in which the thirteen components normally interact to form complete virus. These questions are taken up in order below.

\textbf{In Vitro Completion of Heads and Tails}

If the branches of the morphogentic pathway are independent as proposed above, then the heads present in a group II extract (e.g., 27-extract) should be complete, as should the tails in a group I extract (e.g., 23-extract). Supporting this idea is the finding that 27-heads and 23-tails, purified from the corresponding extracts by sucrose gradient sedimentation and mixed together, will unite spontaneously to form particles (Edgar and Lielausis, 1968). Since union does not appear to
require additional gene products, this result suggests that the defects in group IV, V, and VI extracts are in head or tail completion rather than attachment.

These alternatives have been distinguished by complementation tests using the isolated complete 27-heads and 23-tails. Extracts lacking the group IV component are complemented by heads but not tails (Edgar and Lielausis, 1968). Since a complete head can supply the missing function, component IV (genes 13 and 14) must be involved in completion of the head. Conversely, extracts defective in the group V or group VI functional components are complemented by tails but not heads, indicating that genes 15 and 18 are concerned with completion of the tail (Edgar and Lielausis, 1968).

*Sequencing the Morphogenetic Pathway*

To facilitate an understanding of the methods used to order the interactions of functional components, consider the schematic diagram shown in Figure 2. Assume that S is a large, easily isolatable structural component, for example, a precursor of the head, and that 1 and 2 are gene products which interact with the precursor $S_c$ to produce the complete component $S_a$ in extract complementation experiments. This process could occur by any one of the four paths shown. Possibilities A, B, and C represent possible sequential interactions, and D represents the possibility of no sequence—that is, where no two of the components will interact in the absence of the third. In principle, three of the four pathways can be distinguished as follows: The appropriate mutants are used to prepare a 1-extract (lacking component 1) and a 2-extract (lacking component 2). The precursor is then isolated from each of the extracts by sucrose gradient sedimentation and tested to ascertain whether it is in the form $S_c$ or $S_b$. It can be seen from the Figure that the results will allow a distinction between possibilities A, B, and C or D.
Figure 2. Possible interaction sequences of two gene products with a structural intermediate. See text for explanation of symbols. Extract complementation: 1-extract (X, 2) + 2-extract (X, 1) → Xa.

Experimentally, the problem lies in determining whether a gene product has acted or not; that is, whether the conversion $S_c \rightarrow S_b$ has occurred. The most general method is to test the isolated structure from one extract for ability to complement the other. For example, if isolated $S$ from 2-extract complements 1-extract, whereas the reciprocal test gives no complementation, it is clear that the precursor in 2-extract has been acted upon by 1 to produce $S_b$, and that the correct pathway is possibility A. If neither of the reciprocal tests gives complementation, then the correct pathway is probably C or D. Gene product action is more directly demonstrable if $S_b$ can be distinguished from $S_c$ by sedimentation behavior in sucrose gradients, antigenic properties, or appearance in electron micrographs. Examples of such cases will be encountered below.

The kinds of experiments described above have led to the formulation of T4 morphogenesis shown in Figure 3 (Edgar
Figure 3. The pathway of T4 morphogenesis. See text for explanation of symbols. Numbers in brackets denote gene products that are defined only by temperature-sensitive mutants and have not yet been extensively studied in vitro.

and Lielausis, 1968 and unpublished; Flatgaard, unpublished; King, 1968; King and Wood, 1969; Wood and Henninger, 1969). Solid arrowheads indicate steps that have been demonstrated in vitro, and numbers refer to the products of the corresponding genes. In the paragraphs below I shall describe some of the individual steps before discussing the general features of the pathway.

Steps in Head Morphogenesis

The early stages of head morphogenesis are under the control of seventeen mutationally identified genes, which can be divided into two classes on the basis of their defective phenotypes (see Table 1). The gene products under Y must function in order to produce a structure (He) recognizable as a head precursor in electron micrographs. Those under X are required for the conversion of He to Hb. Only the terminal
step in head completion has so far been demonstrated in vitro. The nature of this conversion remains unclear, since no structural differences have yet been detected between H_b and the complete head H_a.

The attachment of head structures to tails has never been observed in extracts prepared with mutants defective in genes 13, 14, or any of the preceding class of eight genes. It appears, therefore, that completion of the head is a prerequisite for head-tail union.

**Steps in Tail Morphogenesis**

The earliest structure so far isolated in tail morphogenesis is an 80S component identifiable as the endplate (E_a), which accumulates when the gene 54 product is lacking (Edgar and Lielausis, 1968). Preliminary experiments indicate a similar defective phenotype for gene 19 and 48 mutants, implicating these genes in core formation as well as 54. The fourteen gene products listed under Z are apparently required for endplate formation. All of the subsequent steps in tail morphogenesis can be demonstrated in vitro. The tail core, which is not found in the absence of endplate, is built onto the latter under the control of gene 54. The resulting 80S structure has the appearance in electron micrographs of a normal tail lacking the sheath (King, 1968). The completed tail (130S) is formed from this component by assembly of the sheath around the core under the control of genes 3, 15, and 18 (King, 1968; Edgar and Lielausis, 1968).

As in the preceding pathway, evidence from electron micrographs indicates that tail completion must precede attachment to the head. There is an exception to this rule, however. Mutations in genes 11 and 12, which normally act early in the tail pathway prior to 54, lead to the production of apparently normal tails which attach to the head, forming fragile defective particles (see Figure 1, Table 1). The latter
can be converted to active phage by exposure to an extract containing the gene 11 and 12 products. These are the only gene products so far discovered whose sequence of action relative to the rest of the pathway appears to be unimportant (Edgar and Lielausis, 1968).

**Steps Preceding Tail Fiber Attachment**

The presence or absence of phage tail fibers in a preparation can be determined by measuring its ability to block neutralizing anti-T4 serum (DeMars, 1955). Such serological tests, as well as electron micrographs, have shown that no tail fibers are attached to the complete tails or tail precursors isolated from appropriate mutant extracts by sucrose gradient sedimentation (King, 1968). This suggests that head-tail union is a prerequisite for the attachment of fibers to the tail end-plate. Moreover, Flatgaard (unpublished) has shown that the particles formed following infection by gene 9-defective mutants cannot serve as substrates for fiber attachment until acted upon by the product of gene 9.

**Steps in Tail Fiber Morphogenesis**

Six mutationally identified gene products are required for the formation and attachment of tail fibers (see Figure 1). Preliminary evidence regarding their sequence of action was provided by Edgar and Lielausis (1965), who studied the gene control of three distinguishable tail fiber antigens which they designated “A,” “B,” and “C.” The results of their experiments have been extended, using the techniques outlined above to give the tentative pathway of tail-fiber morphogenesis shown in Figure 4 (King and Wood, 1969). In the first step, not yet demonstrated in extracts, a structure corresponding in dimensions to one arm of the finished tail fiber is formed under the control of genes 38 and 37. This component
Figure 4. The pathway of T4 tail fiber morphogenesis (King and Wood, 1969). Heavy arrows indicate steps that have been demonstrated in vitro. Sedimentation constants of fiber structures were estimated by centrifugation of appropriate defective extracts through sucrose gradients, followed by localization of antigenic components in the collected fractions using adsorbed sera as previously described (Edgar and Lielausis, 1965). Structures of intermediates observed in the electron microscope by Eiserling, et al. (1967) were confirmed in our laboratory.

Interacts sequentially with the products of genes 36, 35, and 34 in that order, to produce the complete structure. The nature of these interactions remains unclear, as does the presumed role of gene 57 in the process (see Edgar and Lielausis, 1965). It is of interest that the sequence of gene product action so far elucidated parallels the map order of the corresponding genes. As in the preceding pathways, completion of the fiber appears to be a prerequisite for attachment to the phage. Particles produced by mutants blocked in any step of tail-fiber formation have never been observed to carry individual antigens or fiber precursors.

**Attachment of Tail Fibers**

The terminal step in T4 morphogenesis appears to be the attachment of completed tail fibers to the otherwise finished phage particle. We have studied this process (Wood and Henninger, 1969) in reaction mixtures containing purified fiberless particles (Edgar and Wood, 1966), high-speed supernatant fraction from a 23-extract as a source of tail
Figure 5. Fractionation of 23-extract by gel filtration. A 1 ml sample of 23-extract prepared as previously described (Edgar and Wood, 1966) was clarified by centrifugation for 1 hour at 49,000 rpm in the SW-50 rotor of a Spinco L-2 ultracentrifuge, run onto a 1 x 25 cm column of Bio-Gel P300 (Calbiochem), and eluted with .01 M Tris buffer pH 7.4 containing .005 M MgSO$_4$ at a flow rate of approximately 1 ml per hour. Fractions of 1 ml were collected and their optical density at 280 m$\mu$ determined in a Zeiss PMQII spectrophotometer (solid triangles).

Assays for activity were carried out as indicated below at 30° for 10 minutes in reaction mixtures of 100 $\mu$l containing 0.01 M Tris pH 7.4, 0.02 M MgSO$_4$, and $3 \times 10^{10}$ tail fiberless particles prepared as previously described (Edgar and Wood, 1966). Where indicated, the high-speed supernatant fraction of an extract prepared as above using a multiple mutant carrying am mutations in several tail fiber genes was used as a source of labile factor ("L") free of tail fibers. A sample of centrifuged 23-extract which had been heated 10 minutes at 50° C. was used as a source of tail fibers free of "L." Reaction mixtures contained either 70 $\mu$l of the indicated column fraction (open triangles—assay for fibers ["ABC"] plus "L"), or 70 $\mu$l of column fraction plus 20 $\mu$l of labile factor (solid circles—assay for fibers), or 30 $\mu$l of column fraction plus 60 $\mu$l of heated 23-extract (open circles—assay for "L"). Infectious phage titers were determined by plaque assay of an appropriate dilution of the reaction mixture on permissive _E. coli_ bacteria as described earlier (Edgar and Wood, 1966).
Figure 6. Effect of added labile factor on the rate and extent of fiberless particle activation. Tail fibers relatively free of labile factor were prepared from 23-extract using the column fractionation described under Figure 5. Three reaction mixtures were made up as in Figure 5 except that $2 \times 10^{11}$ fiberless particles and 40 µl of the tail fiber preparation were present in each. Labile factor was added in the amounts indicated, and the kinetics of infectious phage production were followed for 16 hours at 30°.

Approximately 2 between 20° and 30° C. No reaction is detected if the mixture is held at ice-bath temperature or if M++ is omitted. Under optimal conditions the initial kinetics of phage production are exponential and in good agreement with predictions based on the assumption that fibers are attached randomly and one at a time, and that a particle, to become infectious, must acquire three to four fibers.

Fractionation of 23-extract has led to the discovery of an additional factor required for the attachment of fibers to the particle (Figure 5). It is considerably more heat labile than the tail fiber itself (inactivated by heating 10 minutes
at 50° C.), and its behavior on gel filtration columns (Figure 5) is consistent with a high molecular weight. When added in increasing amounts to mixtures of purified fibers and fiberless particles, it increases the rate but not the final level of infectious virus production (Figure 6). This provides a method for assay of the factor and implies that it may act catalytically (Wood and Henninger, 1969). Labile factor activity is not detected in extracts of uninfected *E. coli* but appears in phage-infected cells approximately 4 minutes after infection at 30°. Recent evidence indicates that the factor is the product of phage gene 63. Purification and further characterization of the labile factor are in progress.

**General Features of the Morphogenetic Pathway**

The steps so far characterized *in vitro* represent only a fraction of the total number. It is already apparent, however, that there is a high degree of sequential order in the assembly process, and that this order is imposed at the level of gene product interaction rather than that of gene expression. We conclude this from the demonstration of several *in vitro* complementation groups corresponding to single genes (Table 1), which shows that only one gene product is lacking from the corresponding defective extracts.

The information so far obtained is primarily descriptive and limited to the specific process of T4 morphogenesis. Although a partial sequence of steps can be written, the manner in which the various gene products contribute to the assembly process remains unclear in all but a few instances. There is the suggestion that one gene product, the labile factor, acts catalytically in the attachment of tail fibers. Further study of this and other steps should eventually provide more insight into the general question of how genes direct the morphogenesis of supra-molecular structures.
ACKNOWLEDGMENTS

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REFERENCES

Haber, E. 1964. Recovery of antigenic specificity after de-


It is well known that organisms contain more than a single species of tRNA for many if not all amino acids. For example, in Neurospora crassa there are at least four leucine tRNA's (Barnett and Epler, 1966b) and in Escherichia coli (Weisblum, et al., 1965), at least five. Although the coding properties of multiple tRNA species have been examined in some detail (Barnett and Epler, 1966b; Weisblum, et al., 1965), their role in the whole of cellular protein synthesis is not completely understood. That is, it is not known whether all tRNA's participate in the syntheses of all proteins, or whether certain species are restricted in use to synthesis of a certain class of proteins. Such restrictions could result from codon recognition differences among the tRNA's, or by localization within subcellular organelles such as mitochondria or chloroplasts.

It has recently been observed (Barnett and Epler, 1966b; 1966a) that N. crassa contains two aminoacyl-RNA synthetases for both aspartic acid and phenylalanine. The minor synthetase component for each of these amino acids is unique in its tRNA acylation specificity. Aspartyl-RNA synthetase II (asp syn II) (Figure 1) acylates only one of the aspartic acid tRNA's (Figure 2) and similarly, phenylalanyl-RNA synthetase I (phe syn I) (Figure 3) acylates a single minor species of phenylalan-
Figure 1. Separation of two *Neurospora* aspartyl-rRNA synthetase activities by DEAE-cellulose chromatography (from Barnett and Epler, 1966a). Fractions (8.0 ml) were eluted from a diethylaminoethyl cellulose column (2 x 37 cm) with a linear gradient of KCl from 0.01 M (1,000 ml) to 0.4 M (1,000 ml); both contained 0.01 M potassium phosphate buffer (pH 8.0) and 0.01 M β-mercaptoethanol. Samples (0.1 ml) were assayed for synthetase activity using 155 μg of *Neurospora* tRNA.

Thus, the use of the individual synthetases, separated by column chromatography, permits the quantitation of individual subspecies of both aspartic acid and phenylalanine tRNA in any tRNA preparation. We have taken advantage of these observations and examined tRNA's prepared from *Neurospora* mitochondria for the possibility that one or more of the components of these multiple tRNA's (Figure 4).

**Table One:** Aspartic acid-acceptor activities of fractionated *Neurospora* tRNA preparations*

<table>
<thead>
<tr>
<th>tRNA</th>
<th>Specific activity (μmoles aspartyl-RNA formed per A_{260} unit)</th>
<th>asp syn I</th>
<th>asp syn II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole tRNA</td>
<td></td>
<td>2.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Cytoplasmonic tRNA</td>
<td></td>
<td>1.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Mitochondrial tRNA</td>
<td></td>
<td>1.1</td>
<td>5.5</td>
</tr>
</tbody>
</table>

* Preparation of the various cell fractions and the isolation of tRNA from them has been described (Barnett and Brown, 1967). The aminoacyl-RNA synthetase preparations and conditions for the assays have also been reported (Barnett and Brown, 1967).
Figure 2. Countercurrent distribution profiles of the *Neurospora* aspartic acid acceptor RNA's for asp syn I and II (from Barnett and Epler, 1966a). *Neurospora* tRNA (230 mg) was distributed in a 100-tube apparatus using the solvent system of Holley, *et al.* (1963). Samples (0.1 ml) were assayed using asp syn I (0.05 ml from enzyme fraction 65, Figure 1) and asp syn II (0.05 ml enzyme fraction 155, Figure 1).

tRNA systems is localized within this organelle (Barnett and Brown, 1966; 1967).

It may be seen in Table 1 that the species of aspartic acid tRNA which is acylated by asp syn II is found exclusively in the tRNA isolated from mitochondria. A similar result (Table 2) was observed for phenylalanine, in which the tRNA specific

<table>
<thead>
<tr>
<th>Specific activity (μmoles phenylalanyl-RNA formed per A260 unit)</th>
<th>phe syn I</th>
<th>phe syn II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole tRNA</td>
<td>0.09</td>
<td>3.3</td>
</tr>
<tr>
<td>Cytoplasmic tRNA</td>
<td>0.06</td>
<td>2.1</td>
</tr>
<tr>
<td>Mitochondrial tRNA</td>
<td>1.03</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Preparation of the various cell fractions and the isolation of tRNA from them has been described (Barnett and Brown, 1967). The aminoacyl-RNA synthetase preparations and conditions for the assays have also been reported (Barnett and Brown, 1967).
Figure 3. Separation of two *Neurospora* phenylalanyl-RNA synthetase activities by hydroxylapatite column chromatography (from Barnett and Epler, 1966a). Fractions (10 ml) were eluted from a hydroxylapatite column (2 x 17 cm) with a linear gradient of potassium phosphate (pH 7.0) from 0.01 M (1,000 ml) to 0.5 M (1,000 ml); both contained 0.01 M β-mercaptoethanol. Conditions for assays of enzyme activity were described previously (Barnett and Epler, 1966a).

for phe syn I is associated with the mitochondria. Preparations of mitochondrial tRNA have also been examined for acceptor RNA for sixteen other amino acids (by use of an unfractionated synthetase preparation from *Neurospora* hyphae) and tRNA's for all amino acids tested were present (Barnett and Brown, 1967). Treatment of the mitochondria with snake venom phosphodiesterase has no effect on the activity of the tRNA's subsequently isolated nor do the mitochondrial tRNA's exhibit a tendency to associate with or become attached to mitochondria during the sucrose gradient isolation techniques employed (Barnett and Brown, 1967). Thus, we conclude that the tRNA's are an integral part of the mitochondria.
The distinction between mitochondrial and cytoplasmic tRNA's raises the question of whether the mitochondria contain aminoacyl-RNA synthetases and whether they are demonstrably different from their cytoplasmic counterparts. Table 3 shows a comparison of aminoacyl-RNA synthetase preparations from mitochondria and cytoplasm, and it is apparent that they are indeed different. The mitochondrial synthetases for phenylalanine and aspartic acid acylate only mitochondrial tRNA's, whereas the cytoplasmic synthetases acylate both. These results are consistent with those described above using chromatographically separated phenylalanyl- and aspartyl-RNA synthetases (from extracts of whole cells) and indicate that phe syn I and asp syn II are of mitochondrial origin. The mitochondrial synthetase activities for leucine, lysine, serine, and
TABLE THREE: Transfer RNA specificities of mitochondrial and cytoplasmic aminoacyl-RNA synthetases*

<table>
<thead>
<tr>
<th></th>
<th>Mitochondrial synthetases</th>
<th>Cytoplasmic synthetases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mitochondrial tRNA</td>
<td>Cytoplasmic tRNA</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>0.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

*Transfer RNA and unfractionated synthetase preparations for mitochondrial and cytoplasmic fractions were prepared as described previously (Barnett and Epler, 1966a; Barnett and Brown, 1967).

Glutamine show a marked preference for mitochondrial tRNA (Table 4), suggesting that these enzymes may also be distinct from those in the cytoplasm.

These observations strongly suggest that the translation of genetic information into polypeptide sequences within mitochondria is completely independent of cytoplasmic protein syntheses, and that a unique set of tRNA's and aminoacyl-RNA synthetases are utilized by this organelle.

TABLE FOUR: Transfer RNA specificities of mitochondrial and cytoplasmic aminoacyl-RNA synthetases*

<table>
<thead>
<tr>
<th></th>
<th>Mitochondrial synthetases</th>
<th>Cytoplasmic synthetases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mitochondrial tRNA</td>
<td>Cytoplasmic tRNA</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.1</td>
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</tr>
<tr>
<td>Serine</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.6</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Transfer RNA and unfractionated synthetase preparations for mitochondrial and cytoplasmic fractions were prepared as described previously (Barnett and Epler, 1966a; Barnett and Brown, 1967).
ACKNOWLEDGMENTS

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REFERENCES


There is now evidence that the primary feature of processes of determination in embryonic development consists in the production of inactive messenger RNAs. Our purpose here is to direct attention to some of this evidence and to related experiments concerning the control of protein synthesis in embryonic development.

The Determination Process

The phenomenon of determination in embryonic development is a specification process whereby cells become progressively restricted with respect to their future fate. This process generally takes place without any microscopically visible change. It is detected by certain types of biological experimentation, particularly transplantation or explantation of the cells or tissues. When a presumptive tissue of a developing embryo is transplanted from one region to another of the same or another embryo, before the stage at which the relevant determination has occurred, the transplanted tissue will develop in accordance with its surroundings. After the process of determination has occurred, the transplanted, or explanted, tissue develops in accordance with the kind of differentiation it would have normally undergone if allowed
to develop in place. During the early stages of development the determination process generally specifies the fate of a part of the embryo with respect to an organ system. With the progress of development separate parts of the organ systems, and of the primary organ rudiments, become specified by additional steps in the determination process. For example, one may speak of the initial determination of the medullary plate, in response to induction by the underlying chordamesoderm, as a specification that restricts all descendant cells to differentiation in certain directions, mainly as one or another of the various kinds of nervous tissue. However, there is considerable interchangeability within this structure, the various parts becoming progressively more fully specified by further determination steps as development proceeds.

Extensive investigation by experimental embryologists during the past half-century has revealed that the determination process involves interactions between the various cells and tissues of the embryo. Certain of these interactions are largely unidirectional in that one member of the interacting tissues may exhibit a specific developmental response without, in turn, having any marked effect on the other tissue or tissues, at least initially. Such processes are designated "inductions." Other systems reveal reciprocal effects, the magnitudes of which generally relate in graded fashion to the position of the cells along the principal axes of the egg and embryo. The term "gradient-system interaction" is applied to such processes. These interactions are described in a number of treatises on experimental embryology (Morgan, 1927, 1938; Schleip, 1929; Hörstadius, 1935, 1950; Spemann, 1938; Needham, 1942; Willier, Weiss and Hamburger, 1955; Dalcq, 1957; Brachet, 1960; Yamada, 1961; Waddington, 1962; Saxén and Toivonen, 1962). Since the determination process occurs some time in advance of the actual differentiation of the tissue, one might assume, as a conceivable mechanism, that the cells acquire at the time of determination a specific substance or group
of substances that later cause the turning off and on of the relevant genes. Another possibility that has been proposed (Tyler, 1965, 1966, 1967; Tyler and Tyler, 1966a, 1966b) is that the relevant genes are in fact turned on at the time of determination but that the mRNA's that they produce are kept in an inactive (masked) form until a later time. The later differentiation of the cell would, then, occur after the accumulation of many molecules of the relevant masked mRNA's, which are unmasked by some additional process. The latter process need not be gene-specific. This proposition, namely, that mRNA's are initially produced in inactive form, and are later activated, in correspondence with determination and differentiation, is the one which is supported by the weight of the current evidence.

Evidence for a Masked Messenger RNA

Two kinds of experiments with sea urchin eggs have provided the primary evidence for a masked mRNA. In one of these, non-nucleate egg fragments were produced by means of centrifugation, and the fragments were activated parthenogenetically. Tests of their ability to incorporate radioactively labeled amino acid into protein were made on the intact fragments and on homogenates prepared therefrom by scintillation-counting methods (Tyler, 1963; Denny and Tyler, 1964) and in an independent study, on intact fragments by autoradiography (Brachet, Ficq, and Tencer, 1963). The results agree that the activated non-nucleate fragment incorporates amino acid into proteins as actively as does the nucleate fragments or even the fertilized whole egg. Use of the homogenates obviates the reservation that changes in permeability, or in free amino acid pool, may have been responsible for the results. Further studies (Tyler, 1965, 1966) have shown that the pattern of stimulation of incorporation, of each of the twenty "coded" amino acids, for the
activated non-nucleate fragments is similar to that for the activated whole eggs. This indicates that there are no major differences in the kinds of proteins that are synthesized. Frog eggs have also been examined for their capacity to incorporate amino acid into protein after enucleation (Smith and Ecker, 1965) and the results accord with those obtained with sea urchins.

The other type of experiment leading to the concept of a masked mRNA consists of treatment with actinomycin D (dactinomycin) (Gross and Cousineau, 1963; Gross, 1964), which is essentially a "chemical enucleation." This treatment also fails to prevent the increase in protein synthesis that occurs upon fertilization in sea urchin eggs, although RNA synthesis is inhibited. Puromycin, on the other hand, does prevent the increase in incorporation of amino acid into protein upon fertilization (Hultin, 1961a). Electrophoretic studies (Spiegel, Ozaki, and Tyler, 1965; Terman and Gross, 1965) show no significant differences in the radioactively labeled protein patterns, between actinomycin-treated and control sea urchin eggs in early development, nor any significant difference in pattern during the course of early development. Similar results have been obtained in experiments with frog eggs (Brachet, Denis, and deVitry, 1964).

Additionally, RNA with considerable template activity has been extracted from unfertilized sea urchin eggs, (Maggio, et al., 1964; cf. Monroy, 1965; Slater and Spiegelman, 1966a,b) and most of this resembles the mRNA of the developing eggs (Whiteley, McCarthy, and Whiteley, 1966; Glišin, Glišin, and Doty, 1966).

It is evident, then, that there is present in the cytoplasm of the egg before fertilization most, if not all, of the mRNA that is required for protein synthesis during early development. One reservation is that considerable cytoplasmic DNA is present in sea urchin eggs. This is now estimated to be about five to ten times the haploid value, depending on the
species (Pikó and Tyler, 1965; Pikó, Tyler, and Vinograd, 1967; Pikó, Blair, Tyler, and Vinograd, 1968) and, although it is orders of magnitude lower than many of the previously reported values, there is the possibility that this DNA might be turned on upon artificial activation in the non-nucleate fragment so as to produce mRNA's for active protein synthesis. However, the experiments with dactinomycin would argue against this possibility. In addition, this DNA is now known (Pikó, Tyler, and Vinograd, 1967) to be located principally in the mitochondria, and measurements (Nakano and Monroy, 1958; Giudice and Monroy, 1958) of their activity in protein synthesis show that it remains insignificant during the first three or four hours of development. Furthermore, the difference between fertilized and unfertilized eggs in protein-synthesizing capacity is shown by homogenate systems (Hultin, 1961b) from which the mitochondria had been removed.

Polyribosomes and Newly Synthesized mRNA

There has been some earlier (Hultin, 1961b), and newer (Hultin, 1964a; Monroy, Maggio, and Rinaldi, 1965) evidence that the relative inactivity of the unfertilized egg for protein synthesis was due to an inertness of the ribosomes. However, tests (Tyler, 1962, 1963; Nemer, 1962; Wilt and Hultin, 1962) with polyuridylic acid and other synthetic polynucleotides have shown that ribosomal preparations from unfertilized eggs can cooperate with these mRNA's so as to incorporate the corresponding amino acid into protein. The ribosomes from the unfertilized eggs are as effective in this regard as are those from the fertilized eggs or later embryos. Activation of the egg is also known to result in the formation of clusters of ribosomes, called polysomes, which are held together by a strand of mRNA and which are the effective sites of protein synthesis (Monroy and Tyler, 1963;
Hultin, 1964b; Stafford, Sofer, and Iverson, 1964). It appears, then, that most of the masked mRNA of the unfertilized egg is not attached to a group of ribosomes so as to form a polysome.

While the above-mentioned experiments show that the mRNA and other components of the protein-synthesizing system operating during early development are all present in the unfertilized egg there is, also, evidence (Nemer, 1963; Wilt, 1963, 1964; Gross, 1964; Brachet, Denis, and deVitry, 1964; Brown and Littna, 1964; Comb and Brown, 1964; Glišin and Glišin, 1964; Gross, Kraemer, and Malkin, 1965; Baltus, et al., 1965; Comb, et al., 1965; Spirin and Nemer, 1965; Davidson, et al., 1965; Bachvarova, et al., 1966; Siekewitz, Maggio, and Catalano, 1966; Whiteley, McCarthy, and Whiteley, 1966) that new RNA is formed during early development of various animals and that some, or most, of this is mRNA. From the previously cited evidence it would appear that this newly synthesized mRNA is also of the "masked" variety. Further indication of this appeared in pulse-labeling experiments (Spirin, Belitsina, and Aitkhozhin, 1964) with fish embryos at various stages to the beginning gastrula. These experiments have shown an incorporation of RNA precursors into an RNA-protein fraction that is smaller than the ribosomes. At the same time there is incorporation of labeled amino acids in the ribosomal complexes in which the RNA is not labeled. The apparently inactive fast-labeling RNA-protein has been termed an "informosome" and is presumably the equivalent of masked mRNA. However, extracts containing informosomes are reported to be active in an in vitro system without special treatment. This is not the case for the masked mRNA of unfertilized sea urchin eggs. These workers report that RNA's of the informosomes become associated with ribosomes after gastrulation, and new experiments (Spirin and Nemer, 1965) point to the existence of informosomes also in sea urchin eggs.
Overall, the present evidence indicates that, while the protein synthesis of early development utilizes principally the mRNA's that were unmasked upon fertilization, new mRNA's are being produced during this period and these in turn remain masked until a later stage, probably the time of gastrulation. As indicated below this situation apparently applies also to later stages of development.

*Nuclear Histones in Relation to Synthesis of mRNA*

Evidence has accumulated in recent years supporting the proposal of Stedman and Stedman (1950) that complexing with histones suppresses activity of the genes. This evidence is derived primarily from experiments showing inhibition of the stimulating action of DNA on protein synthesis by thymus nuclei (Allfrey, 1961; Allfrey and Mirsky, 1962), increased RNA synthesis upon removal of histones from nuclear preparations (Allfrey and Mirsky, 1962; Allfrey, Littau, and Mirsky, 1963), inability of DNA-histone complexes to "prime" in vitro for RNA synthesis in a preparation of pea-seedling nuclei (Huang and Bonner, 1962), and greatly increased RNA synthesis following deproteinization of such chromatin preparations (Bonner, Huang, and Gilden, 1963; cf. Bonner, 1965). On the basis of this inverse relationship between histone content and activity, and in accordance with the generally held view that the genes are mostly inactive until the time of gastrulation, one would expect the chromatin of the earlier stages to have a higher histone content than that from later stages. However, in cytochemical investigations of developmental stages of various animals, including mice (Alfert, 1958), snails (Bloch, 1963), and frogs (Horn, 1962; Moore, 1963), it has been found that the nuclei and chromosomes do not stain strongly for histones until the time of gastrulation. This apparent contradiction can now be resolved in favor of the histone-hypothesis on the basis of the
above-cited evidence that many genes are, in fact, active during early development, perhaps more per cell that at later stages, but the mRNA's that they produce remain in an inactive (masked) form until a later stage.

Thus, the evidence (cf. Hadorn, 1961; Brachet, 1960, for reviews) that there are no demonstrable "paternal" effects on the embryo until a late stage, generally corresponding to the time of gastrulation in most animals (in mammals the t12 mutant of mice is expressed in the late morula stage [Smith, 1956]) need not mean that the genes are turned off until that time. It is more likely that the absence of "paternal" effects at the early stages is due to inactivity of actively produced masked mRNA. While this re-evaluation of the evidence removes an inconsistency (low content of histone in cleavage nuclei) from the evidence concerning reversible gene-inhibition by histones, the lack of specificity exhibited by histones chemically in their interaction with DNA (cf. Johns and Butler, 1964), and biologically (cf. Brachet, 1964), constitutes a primary impediment to the formulation of a gene-control theory based on their manipulations.

**mRNA Synthesis and Embryonic Determination**

The demonstration (Reich, *et al.*, 1962, and others; cf. Reich, 1964) that actinomycin D (dactinomycin) inhibits DNA-dependent RNA synthesis without impairing DNA replication has provided a useful tool, along with various direct-acting inhibitors of protein synthesis, for exploring problems of embryonic determination. The primary question in the present context is whether dactinomycin blocks differentiation when applied before, but not after, the time at which a specific determination process has taken place, whereas the antimetabolites to protein precursors show immediate effects. The pertinent evidence, which is largely affirmative, has been
accumulating in many investigations with various kinds of developing systems. The following account gives a brief listing of some of the relevant work.

In sea urchins, in addition to work discussed above, there have been a number of investigations of the action of dactinomycin, and other antimetabolites, with reference to embryonic determination (cf. Ficq, 1964; Hörstadius, 1963; Giudice and Hörstadius, 1965; Lallier, 1964; Markman, 1963; Markman and Runnström, 1963; Wolsky and Wolsky, 1961) and the results (including tests of isolated parts) are in general accord with the stated proposition. One investigation (Scarano, De Petrocellis, and Augusti-Tocco, 1964) concerns deoxycytidylate monophosphate aminohydrolase, the activity of which decreases during normal development but is maintained in embryos developing in dactinomycin. Here the dactinomycin-inhibited mRNA would appear to function as an accessory controlling agent, but this need not be at the gene level, as the authors propose.

In amphibia the inability of dactinomycin to halt developmental progress during early stages has been demonstrated in several experiments (Brachet and Denis, 1963; Brachet, Denis, and deVitry, 1964; Wallace and Elsdale, 1963; Flickinger, 1963). It should also be noted that in this group of animals, too, there is evidence of RNA synthesis even at very early stages (Brown and Littna, 1964; Decroly, Cape, and Brachet, 1964). There have also been tests with dactinomycin on the primary embryonic induction system (Toivonen, Vainio, and Saxén, 1964) and on the lens-regenerating system (Yamada and Roesel, 1964), the results of which indicate the synthesis of an inactive mRNA at the time of determination.

Studies with cells of the erythroid line have provided early evidence for an mRNA that is not only stable but also temporarily inactive, inasmuch as the hemoglobin synthesis occurs some time after RNA synthesis has ceased (cf. Borsook, et al.,
1962; Borsook, 1964). In experiments with chick embryos effects of dactinomycin and other metabolic inhibitors, including X-rays, on this and other differentiations have been examined in explants of the young embryos and in de-embryonated blastoderms (Heilpom-Pohl, 1964; Hell, 1964). The evidence is not easily evaluated. For hemoglobin it is complicated by the report that the globin is detectable immunologically in the unincubated blastoderm (Wilt, 1962; cf. Lemez, 1964). However, recent experiments with chick embryos (Wilt, 1965), demonstrating a time lag between the acquisition of insensitivity to amounts of dactinomycin that suppress RNA-synthesis and the time of hemoglobin-synthesis, are interpretable in terms of the synthesis of an inactive mRNA at the time of determination. The delayed teratogenic effects of dactinomycin (Tuchmann-Duplessis and Mercier-Parot, 1960; Pierro, 1962) are in the same direction, as are also the results of explantation experiments with ocular lens, feather, and other tissues (cf. Scott and Bell, 1964). The studies on the down feathers (Bell, et al., 1965) have indicated that inactive mRNA may be associated with polysomes comprised of four ribosomes in the form of a square. In this form the polysomes are insensitive to RNase. When "keratinization" of the feather begins, at about the thirteenth day of incubation, the squares open up, the polysomes become sensitive to RNase and are capable of functioning in protein synthesis.

Further evidence that production of masked mRNA's represents the primary event in determination is supplied by dactinomycin experiments with isolated induction-systems for pancreas (Rutter, Wessels, and Grobstein, 1964; Wessels, 1964; Wessels and Wilt, 1965), kidney tubules (Jainchill, Saxén, and Vainio, 1964), heart and thigh muscle cells (Yaffe and Feldman, 1964), and chick axial system (Klein and Pierro, 1963). These systems, which are being so well explored in many directions (cf. Grobstein, 1964), are very favorable subjects
for such analysis. Again, the experiments reveal an acquisition of dactinomycin-insensitivity that correlates in time with the determination process. Dactinomycin treatment has also been done with cleavage-stage mammalian eggs (Silagi, 1963; Mintz, 1964) and inhibition of RNA-synthesis demonstrated along with a relatively early inhibition of development, which would be consistent with the evidence mentioned above for relatively early (late morula stage) gene-expression in this group of animals.

**Summation**

The demonstrations that mRNA's can occur in an inactive form and become activated upon a specific developmental event, such as fertilization, and that the masked condition may represent the customary form in which the mRNA's are initially released from the nucleus during development, have provided the basis for interpretation of the process termed "determination." The evidence is provided largely by coincidence in the time at which determination, as revealed by the results of transplantation or explantation experiments, occurs in a particular prospective tissue or organ and the time at which the system becomes insensitive to inhibition by dactinomycin and, in some experiments, by X-rays or other agents that inhibit DNA-dependent RNA synthesis. The evaluation of the evidence is in many instances obscured by the fact that the inductive systems represent a sequential series and that there is often reciprocal action. In sum, however, it now appears that there are sufficiently cogent arguments to warrant the proposition that each determination process is represented chemically by the production, in masked form, of the specific mRNA's required for the later differentiation of the tissue. This also provides a new outlook toward the problem of specification of the particular developmental events and the time at which specific genes are turned on.
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I remember a time, long ago, when I was a graduate student. As happens to all graduate students, or to most graduate students, I ultimately came to the end of my graduate student career, climaxed by an oral examination. As the chairman of the examining committee, Professor Alfred Henry Sturtevant, sat down, he lit his pipe and said, “Tell me, please, what is your idea of a gene?” Luckily I’ve forgotten what I answered. As a matter of fact, I’ve forgotten all the other questions too and can remember only that first one. But if Professor Sturtevant were to ask me today, I would say, “Well, I think a gene is a stretch of DNA which contains the information for some cellular function or structure, together with all of the ancillary material which determines whether that gene shall be derepressed and put out this information in a messenger RNA, or shall be repressed—inert.” By my definition of the gene, I think I take our discussion from the realm of pure DNA husbandry into the realm of DNA as it occurs in nature and thus in chromosomes.

I think that most of us agree that differentiation and development, the development process, is due to the properly programmed sequential turning off and turning on of the genes, so what we really want to learn about, if we are to apply our knowledge of molecular biology to differentiation, is to find out how the act of transition of a gene from a repressed or derepressed state occurs, or vice versa. So, to study this, we have to find out something about the material basis of repression itself.
I'm not going to talk today at all about the ways in which genes are changed from the repressed to the derepressed state, because I don't know anything about it, and I'm not going to talk about developmental processes, either. But what I do want to talk about is the information that has been accumulated about materials with which DNA, as it occurs in the structure of the chromosome, is complexed. If we want to study this matter, the straightforward thing to do is go and isolate some chromosomes and study them. I will say right away that DNA, as it occurs in chromosomes, has protein complexed with it. One would think at first that maybe the way to approach the study of chromosomes would be to lyse some Escharichia coli and from it to pellet or otherwise obtain DNA, together with the materials to which it is complexed in the cell. But this turns out to be a difficult matter. The proteins that are complexed with the DNA isolated from E. coli are generally like all of the other proteins in E. coli. We have no way to tell whether we have isolated something that nature made or whether we have merely isolated some artifacts, suitable for publication in Acta Artifacta. In the chromosomes of higher creatures, on the contrary, the DNA is complexed with a characteristic group of proteins, the histones, which only occur in chromosomes. When we isolate DNA from higher creatures we can tell, since the chromosomal proteins have their own signature, whether what we have isolated is a natural entity rather than an artifact.

My colleague, Professor Ru-chih Huang, and I began seven years ago to try to isolate chromatin, interphase chromosomes, from cells of higher creatures (Huang, Maheshwari, and Bonner, 1960). Following the lead of Mendel we started, naturally, with peas. We have a motto: "Anything you can do, you can do better with peas." But let us not talk about pea chromatin first. Now we have progressed to rats. Let us consider the chromatin of rat liver.

Chromatin of rat liver, isolated by published methods
(Marushige and Bonner, 1966), contains DNA and a small proportion of RNA, about 5 percent as much as DNA. It also contains histone—in the case of rat-liver chromatin in a mass ratio of histone to DNA of about 1:1. Chromatin contains also some nonhistone protein, of which more later. Such isolated chromatin possesses a nontrivial property, the power to conduct DNA-dependent RNA synthesis provided it is supplied with the four riboside triphosphates. It has the power to conduct this synthesis because isolated chromatin carries with it its own chromosomal RNA polymerase, and because a portion of the DNA acts as template for this polymerase. However, chromosomes as we isolate them contain a relatively small amount of RNA polymerase, and indeed the addition of more RNA polymerase increases the rate of generation of RNA; chromatin can act as template for exogenous RNA polymerase. In addition, chromosomes of higher creatures is not particular about where the RNA polymerase for which it is to serve as template comes. For example, isolated liver chromatin serves admirably as template for RNA synthesis catalyzed by *E. coli* RNA polymerase and all of the work on chromatin as template for RNA synthesis which I shall discuss is done with such polymerase.

Figure 1 concerns a template saturation curve in which a constant amount of RNA polymerase is incubated with different amounts of DNA given as a purified rat liver chromatin or as pure DNA. It is clear that rat-liver chromatin is about one-fifth as effective as template as is an equivalent amount of DNA supplied as pure DNA. Figure 1 also shows that if we supply twice as much enzyme to the reaction mixture, then twice as much DNA, either as chromatin or as purified DNA, is required to saturate the enzyme. We take this to mean that the number and availability of binding sites for RNA polymerase are the same in rat-liver chromatin and purified rat-liver DNA.

Let us now ask, what is the component of the chromatin
Figure 1. Comparison of the template activities of rat-liver chromatin and of the deproteinized DNA of rat-liver chromatin. The reaction mixtures, 0.25 ml, were of the composition specified by Marushige and Bonner and contained E. coli RNA polymerase F4, 30 µg (A), or 60 µg (B). After Marushige and Bonner (1966).
that makes its template activity be less than that of pure deproteinized rat-liver DNA? Keiji Marushige has shown that it is the histone fraction of chromatin. If rat-liver chromatin is extracted with 0.2 N HCl (in which histones are soluble and DNA is not), the histones are removed from the DNA, although the nonhistone proteins remain complexed to it. Chromatin thus dehistonized has the template activity of completely deproteinized rat-liver DNA. Histones may also be removed from chromatin by dissociation in salt solutions. The data of Figure 2 show that when rat-liver chromatin is pelleted from increasing concentrations of salt, in this case sodium perchlorate which is particularly effective in histone removal, histone is removed and template activity of the chromatin increased. At a salt concentration of 0.5 M sodium perchlorate, for example, 90 percent of the histone is removed, two-thirds or more of the nonhistone protein remains associated with DNA, and the template activity for RNA synthesis of the dehistonized chromatin is nearly that of completely deproteinized DNA.

Why is it that the template activity of chromatin for RNA synthesis is less than that of DNA? Is it because all of the DNA is less efficient in the support of RNA synthesis by RNA polymerase? Or is it because a portion of the DNA is totally inactive in RNA synthesis, and a further portion active as template for support of RNA synthesis by RNA polymerase? In the case of pea chromatin, it is easy to show that the latter alternative is true (Bonner and Huang, 1963). If we shear pea chromatin so that the DNA is reduced to a molecular weight of approximately ten million and then make the solution 0.15 M in salt, the solution separates into two fractions. A major portion of the DNA precipitates. This portion contains DNA stoichiometrically complexed with histone—it is a well known property of nucleohistone that it is insoluble in 0.15 M salt, although it is soluble in both lower and higher salt concentrations. A further portion remains in solution. These
Figure 2. Effects of various salt treatments upon chemical composition and template activity of several chromatin. Chromatins isolated from rat liver, rat spleen, and calf thymus were treated with NaClO$_4$ at concentrations ranging from 0.25 to 2 M. The histone:DNA ratios for rat-liver, rat-spleen, and calf-thymus chromatin subjected to the same treatment but without salt were 1.10, 0.87, and 1.14, and the nonhistone protein:DNA ratios 0.72, 0.24, and 0.33, respectively. For determination of template activity, 10 µg of DNA in the form of chromatin were incubated with 100 µg of RNA polymerase F$_3$ under standard conditions given in Marushige and Bonner; RNA synthesis by F$_3$ alone subtracted. Open circles, fraction of histone removed; closed circles, template activity of remaining nucleoprotein; triangles, fraction of nonhistone protein removed. After Marushige and Bonner (1966).

two fractions differ in that the DNA which is stoichiometrically complexed with histone is template inactive for RNA synthesis, whereas that portion which remains in solution in 0.15 M NaCl, and which is impoverished in histone and contains the bulk of the nonhistone protein, has a high template activity. The behavior of rat-liver chromatin is somewhat different. If we shear it to DNA molecular weight ten million, the resulting solubilized material has the same template activity and the same chemical composition as the initial chroma-
Keiji Marushige has shown (personal communication, 1967), however, that all we have to do to separate rat-liver chromatin into fractions of greater and lesser template activity is to shear it so that the DNA is of still smaller size. Suppose we shear DNA of rat-liver chromatin to average molecular weight 500,000 (by use of endonuclease II) and then make it 0.15 M in sodium chloride. The majority of the DNA precipitates. A further portion remains in solution. The portion that precipitates possesses a DNA: histone ratio of near stoichiometric (histone positively charged groups equal number of negatively charged phosphate groups of DNA). The great bulk of the nonhistone protein remains in solution complexed to DNA but contains little histone. The DNA of the nucleohistone fraction is stabilized against melting as compared with DNA. The fraction impoverished in histone and enriched in nonhistone protein is less stabilized against melting. And too, the template activity of the portion of the chromatin that is impoverished in histone is greater than the template activity of the portion of the chromatin that is enriched in histone.

We are led, then, to the conclusion that chromatin of higher creatures, peas, rats and the like, is composed of two portions: a portion of the DNA, in general the great majority of it, is complexed with histone in the form which we know and refer to as native nucleohistone. This portion has low template activity for RNA synthesis. A further portion of the DNA has bound to it the great bulk of the nonhistone protein of chromatin. This portion has high template activity.

Let us digress for a moment to comment on the properties of native nucleohistone. These properties have been studied by many people over the last three or four years, and they include the following: DNA stoichiometrically complexed with histone has an electrophoretic mobility different from DNA. It has a negative charge and its mobility depends as a first approximation on the number of free carboxyl groups in the
histone. These are determined by the dicarboxylic acid content of the histone. In nucleohistone, also, the DNA is deformed. When nature puts histone on DNA it strains the base pairs and causes the DNA to become hyperchromic as compared to pure DNA. The reason that the DNA is strained and that base-pair interaction is less in nucleohistone than in DNA is, we believe, due to the fact that in native nucleohistone the DNA double helix is wound into a supercoil. This has been shown both by X-ray diffraction and by electron microscopy. In the supercoiled form, three coils of the DNA double helix are included in one gyre of a supercoil, which has a repeat length along the nucleohistone axis of 110 Å.

So much for the physical chemistry of nucleohistone. Let us now consider the properties of the histones themselves. Histones are interesting. By interaction with DNA they confer upon the DNA properties that it does not otherwise have: stabilization against melting, low template activity, supercoiling, etc. The chemistry of histones has been studied a great deal since Miescher discovered them one hundred years ago. There are now methods for the separation of the several histones from one another and we are beginning to gain knowledge of how many different species there are, as well as ways in which histones of different creatures are similar to or different from one another. These methods have been developed in the laboratories of Professor Butler of the Chester Beatty Institute, of Harris Busch of Baylor University Medical School, and of J. Murray Luck of Stanford University and his colleague Kenneth Murray. We have found the methods of Kenneth Murray (Rasmussen, Murray, and Luck, 1962) most satisfactory.

Histone is first separated from purified chromatin, for example, of peas, by acid extraction and then precipitated with ethanol. The histone precipitate is dissolved, dialyzed against acetic acid, lyophilized, and then dissolved in guani-
COLUMN CHROMATOGRAPHY OF PEA BUD HISTONES
COLUMN SIZE 0.6x55 cm
RESIN: AMBERLITE CG50

Figure 3. Fractionation of pea-bud histones by column chromatography on Amberlite CG-50. Protein concentration in the effluent fractions was determined by absorbance at 400 m\(\mu\) of the turbid solutions resulting when the 0.26 ml fractions were mixed with 1.1 M trichloroacetic acid in a total volume of 1.56 ml. Circles, protein concentration (absorbance); squares, concentration of guanidinium chloride (GuCl). After Fambrough and Bonner (1966).

Guanidinium chloride. It is next applied to a column of a weak cationic exchanger, CG-50, from which it is eluted with a gradient of guanidinium chloride. By the column procedure, histones are separated from one another on the basis of their sizes and charges. They emerge as three major groups, shown in Figure 3, known respectively as lysine-rich histones I, a and b, slightly-lysine-rich histones II, a and b, and arginine-rich histones III and IV. Each of the fractions separated by this procedure is still heterogeneous, as can be shown by disc electrophoresis in 10 M urea. By disc electrophoresis one can not only determine the number of components present in a histone mixture but also ascertain the homogeneity of each fraction. As is clear in Figure 4, each of the column-separated
Figure 4. Fractionation of pea-bud histones by disc electrophoresis in polyacrylamide gel. Electrophoresis in gel 15 percent in polyacrylamide, and containing 6 M urea. The figures are microdensitometer tracings on the Canalco Model E microdensitometer. The gels were stained with amidoschwarz and destained electrophoretically. The gels were stained with amidoschwarz and destained electrophoretically. The gels were then scanned. Tracing A shows the electrophoretic fractionation of whole pea-bud histone. Tracings B, C, and D show, respectively, the electrophoretic fractionation of histones I, IIa, and III-IV, all prepared by column chromatography on Amberlite CG-50. Peaks at far left indicate origin of gel, not stained material. The forward shoulder on the histone IIa peak in tracing A is histone IIb. After Fambrough and Bonner (1966).
COLUMN CHROMATOGRAPHY OF CALF THYMUS HISTONES

COLUMN SIZE 0.6 x 55 cm
RESIN AMBERLITE CG50

Figure 5. Fractionation of calf-thymus histones by column chromatography on Amberlite CG-50. Protein concentration in the effluent fractions was determined by absorbance at 400 m\textmu; of the turbid solutions resulting when the 0.26 ml fractions were mixed with 1.1 M trichloroacetic acid in a total volume of 1.56 ml. After Fambrough and Bonner (1966).

histones is contaminated by others. Luckily, however, when the original column-separated fractions are passed for a second time through other columns, fractions are obtained that are homogeneous as judged by disc electrophoresis.

We conclude from this exercise that the histone species of peas include histones Ia and b, histones IIa,2 and b, and histones III and IV. III yields several peaks by disc electrophoresis which, however, yield identical tryptic peptides, which we judge to be merely oligomers of one another. Thus, in pea-bud chromatin there are apparently seven major histones only. The histones of pea buds are remarkably similar to the histones of other creatures. For example, the histones of calf-thymus chromatin, which are the most studied histones,
separate on the CG-50 column in much the same way as do pea-bud histones (Figure 5). Again histones Ia and b appear (there is more of Ib in thymus than in peas), as well as IIa and b (there is more of IIb in calf thymus than in peas), and histones III and IV. These, again, are separated by a second column chromatography into fractions that are homogeneous by disc electrophoresis. Again, there would appear to be seven major histones in calf-thymus chromatin. The corresponding histone fractions of pea bud and calf thymus are identical to one another with respect to electrophoretic mobility in disc electrophoresis and in amino acid composition (within the limits of the Spinco amino acid analyzer). They yield similar, but not identical, tryptic peptides. There are similarities too in their N-terminal amino acids. In histones Ia and b of both pea and thymus the N-terminal groups are blocked; they are acetylated. The N-terminal amino acid of histone II is proline and of histones III and IV is alanine in both cases. The same conditions are true also for the histones of rats.

We may conclude then that the functions that the histones perform are functions for which it is important to have particular properties. The nature and composition of histones, determined long ago in evolution, are such that they perform these functions. The requirement that these functions be retained has apparently greatly retarded evolutionary change in the histones. The common ancestor of peas and cows, which undoubtedly occurred long, long ago, must have had genes for making histones and these genes have been retained essentially unaltered ever since.

There are then only a few kinds of histones. They sit on DNA and by doing so prevent the DNA from being transcribed by RNA polymerase. A few years ago people used to talk about the possibility that there might be a different kind of histone for each gene that is to be repressed. We see that this is not possible because there are many fewer kinds
Figure 6. CsCl density gradient centrifugation of P³²-labeled pea-bud nucleohistone. The nucleohistone had been previously pelleted from 0.4 M NaClO₄ to remove histone I. The pellet was redissolved in 2.09 M CsCl and centrifuged at 39,000 rpm to equilibrium. The DNA of the nucleohistone is pelleted at the bottom of the tube and is not recovered. Histone and associated RNA bands with the peak density indicated. After Huang and Bonner (1965).

of histones than there are genes to be repressed. The same kinds of histones must repress many genes. Histones, however, have little ability to read base sequence, have little ability to know what kind of DNA they are sitting on. If, for example, histone is mixed with a mixture of poly dAT and ordinary DNA at low ionic strength, the histone binds impartially with both kinds of DNA. In order to find out more about how histones recognize which portion of the genome to sit on, and to find out more about the organization of histones in chromatin, Professor Huang and I have chosen to develop new methods for the separation of histones from DNA and have undertaken a general study of histone-DNA
interaction. One approach is shown in Figure 6. Purified nucleohistone, in this case of pea-plant chromatin, is dissolved in cesium chloride, 2.09 M. At this ionic strength the histone dissociates from the DNA. The solution is then centrifuged to density gradient equilibrium. At the concentration of cesium chloride used, DNA pellets. The histone bands at its point of neutral buoyancy, a density of 1.286. The nucleohistone used in the experiment of Figure 6 was obtained from pea plants that grew from babyhood in P\textsuperscript{32}, so that their nucleic acids are P\textsuperscript{32}-labeled. It is clear that there are P\textsuperscript{32} counts in the banded histone. The occurrence of P\textsuperscript{32} in histone dissociated from chromatin with salt is due to RNA which is complexed to the histones.

Figure 7 shows histone similarly banded in cesium chloride, but after extraction with acid in the way classically used for
purification of histones. Histone that has been so treated loses its RNA. In addition, it bands at a lower density, namely, 1.233. It also bands much more diffusely than does the histone obtained directly by salt dissociation. The increment in density of the salt-dissociated nucleohistone is that which would be expected on the basis of its content of RNA. It contains about 8 percent as much RNA as histone and this amount of RNA would be expected to increase the density of the histone from 1.233 to 1.286. The fact that the acid-extracted histone is more highly dispersed than the salt-dissociated material is due to the fact that the salt-dissociated histone has a higher molecular weight and hence diffuses less rapidly. On the basis of the band width of salt-dissociated histone, we calculate that the molecular weight of the histone-RNA complex units is of the order of a few hundred thousand, between 200 and 500 thousand. The complexes in the bands are therefore composed each of many histone molecules of the average molecular weight of acid-extracted histone, which is about 20 thousand. Notice, too, that with salt-dissociated histone there is no histone which bands at the density characteristic of acid-extracted, RNA-free, histone.

Now let us go to some of the characteristics of the RNA that is associated with histone of chromatin. First of all, its occurrence seems to be general. Michael Dahmus has shown that it is present in rat liver and in rat ascites tumor chromatin. Tom Shih has shown that it is present in calf-thymus nucleo-histone, and Professor Huang has shown that it is present in the chromatin of chicken embryos (Huang, 1967). All contain histone-associated RNA. This class of RNA is characterized by special properties. In the first place, it possesses a short chain length. End-group analysis shows that pea-chromatin RNA has a chain length of forty nucleotides. It is characterized too by a relatively high content of a strange base, dihydrouridylic acid. The dihydrouridylic acid plays a role in the organization of the histone-RNA com-
Figure 8. CsCl density gradient centrifugation of P32-labeled histone-RNA complex in the presence of 1 M GuCl. The complex obtained by CsCl banding was redissolved in 1.85 M CsCl containing 1 M GuCl and centrifuged at 39,000 rpm to equilibrium. After Bonner and Huang (1966).

Professor Huang (1967) has shown that it is through the dihydrouridylic acid that RNA is covalently bound to protein.

So much for the RNA. Let us now consider to which of the histones the RNA is fastened. We have studied this by two methods. One consists in dissolving the histone-RNA complex in cesium chloride containing guanidinium chloride, the latter a hydrogen-bond-breaking agent. We then density gradient centrifuge the solution. Under these circumstances the RNA, together with a portion of the protein, pellets and
Figure 9. Amberlite CG-50 column fractionation of P32-labeled histone-RNA complex obtained by CsCl banding. The complex was dissolved in 8 percent GuCl and fractionated with a gradient of GuCl as described by Fambrough and Bonner (1966). After Bonner and Huang (1966).

when rebanded exhibits a density of 1.46. This indicates that a complex of RNA and protein containing about 40 percent RNA and 60 percent protein has been broken off from the RNA-histone complex. The remaining histone bands with a density of 1.233, as is shown in Figure 8. Alternatively, the histone-RNA complex can be dissolved in guanidinium chloride and then passed through a CG-50 column in the way previously described for histone separation. Figure 9 shows what happens when a preparation of P32-labeled histone-RNA complex (obtained by cesium chloride banding) is dissolved in guanidinium chloride and then separated in a CG-50 column. There is very little P32, and hence very little RNA associated with histone I or II. The majority of RNA is associated with the minor protein which is first eluted. A further portion is associated with histone III-IV, but we have subsequently found that rechromatography of this material causes it to dissociate into histone III-IV and protein similar to the initial peak material. Apparently, the bonds between these proteins
are broken by the high concentration of guanidinium chloride required to elute histone III-IV. Similar binding of a protein containing bound nucleic acid to arginine-rich histone has been described by Hnilica and Bess (1965).

It appears that the RNA of histone-RNA is bound to just one component, not to all. Since all of the histone bands with the density characteristic of histone-RNA complex, it is clear that the other kinds of histone molecules must in turn be bound to the RNA-binding protein. These bonds are broken by acid or by guanidinium chloride.

Let us now consider the role of the RNA portion of the chromosomal histone-RNA. In native chromatin, or native nucleohistone, the RNA is bound in such a way that it is resistant to attack by RNase. It can be released from this mode of binding either by treatment of the nucleohistone with heat, which melts the base pairs, or by treatment of the nucleohistone with DNase, which destroys the DNA. These facts suggest, though they do not prove, and we do not know any way to prove it, that in native chromatin the RNA is bound to DNA by base pairing.

In order to decide what to do next about finding out the role of the RNA of histone-RNA, we must speculate; speculation is the guide to action. We speculate that chromosomal RNA may be involved in helping the histone complexes to read base sequence—that this kind of RNA may be an adaptor by means of which the proteins that are associated with DNA in chromatin are enabled to read base sequence and hence bind to the particular portion of the genome with which they are supposed to associate. If this speculation were true, it is required that the RNA of histone-RNA be vastly heterogeneous. There are many different sites, many different genes in the genome to be detected and to be complexed with histone. That the RNA of histone-RNA is physically heterogeneous has been shown by Frank Fujimura. The question of sequence heterogeneity of chromosomal RNA may be approached by DNA-RNA hybridization. The chromosomal RNA
Figure 10. Hybridization of the RNA of histone-RNA complex to whole genomal pea DNA. Hybridization was by the method of Gillespie and Spiegelman (1965). Protein was removed from this complex by treatment with pronase. The ethanol-precipitated RNA was subsequently purified on a DEAE Sephadex column by elution with a gradient of NaCl in 7 M urea. After Bonner and Widholm (1967).

is purified to rid it of all protein. We then see with how much of the nuclear DNA it will hybridize. In order to be sure that we know how to hybridize RNA and DNA by the fine method of Gillespie and Spiegelman (1965), we first determined how much of the nuclear DNA hybridizes with ribosomal RNA. We have found 0.25 percent pea nuclear DNA hybridizes with pea ribosomal RNA, which is an agreeable accord with the previous determination of 0.29 percent made by Chipchase and Birnstiel (1963). We have also determined the fraction of nuclear DNA with which the transfer RNA of peas will
hybridize. The value obtained, 0.052 percent, is higher than the 0.025 percent obtained by Goodman and Rich (1962) and by Giacomoni and Spiegelman (1962) for transfer RNA of *E. coli*, but is still a relatively low value.

The data of Figure 10 concern the hybridizing characteristics of chromosomal RNA. A rather substantial proportion of the genome hybridizes with chromosomal RNA, 5.05 percent. In fact, chromosomal RNA hybridizes with whole nuclear DNA to an extent that approximates the proportion of chromosomal RNA and DNA in native chromatin.

To summarize, then, the emerging picture of the organization of chromatin of higher creatures is one in which a small fraction of the DNA is associated with RNA of a special class. In addition, RNA is bound to protein of a particular class. This protein-RNA complex is, in turn, associated with histone molecules. The histone molecules in turn are associated into long strings—strings capable of complexing with DNA of 300 to 600 or more base pairs. The histones of these units are of but a few kinds, I, II, and III-IV. The histone complexes are therefore generally similar to one another. The RNA molecules that are associated with the histone complexes are very dissimilar. And so, we speculate that in chromatin of higher creatures, it is the RNA that does the base-sequence detecting, and it is the histone, which by sitting upon the DNA, causes repression, the inability of the DNA to support RNA synthesis.

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REPLICATION OF CHROMOSOMAL DNA AND MECHANISMS OF RECOMBINATION

J. Herbert Taylor

Just a few days more than ten years ago, we reported to a group of biologists in an overcrowded room at the AIBS meetings in Storrs, Connecticut, the results of some experiments that Philip S. Woods, Walter L. Hughes, and I (1957) had just completed, which showed the semi-conservative distribution of DNA in chromosomes labeled with tritiated thymidine. This was not only the first experimental evidence indicating that DNA might be replicating as predicted by Watson and Crick (1953), but also the beginning of a series of experiments with tritiated thymidine and other nucleosides in autoradiographic studies. These studies have thrown light on two problems in which Professor Morgan maintained a lively interest, namely, gene or chromosome reproduction and the mechanisms of crossing over. I would like to give briefly the highlights of these studies by ourselves and by others, some who used the same tools and some who used other techniques which yielded information bearing directly on these problems. I cannot mention all of the related studies, and therefore this report should not be taken as a review, but rather as a personal account of experiences and changing concepts over a ten-year period.

Units of Replication

It seemed to many of us then (1956) that a chromosome must consist of many strands of DNA and protein interwoven into
a complex structural fabric. Soon after the initial studies with the H\(^3\)-thymidine, we began using this same tool for determining the pattern of sister chromatid exchange. The experiments led to the concept that the two chromosomal subunits which were segregating during reproduction were not identical in their ability to recombine, i.e., the subunits had properties similar to the two polynucleotide chains of a DNA duplex. The difference could be an opposite sense or polarity which restricted reunion to like members of the four subunits in two sister-chromatids (Taylor, 1959). This finding, along with the demonstration by Meselson and Stahl (1958) that the two subunits of a DNA duplex were being distributed in a semi-conservative fashion during replication, led to a reconsideration of the possible structure of a chromosome. It seemed certain that it was composed of a single duplex of DNA. To obviate the necessity of unwinding such a long piece of DNA, nearly a half-meter in the largest chromosomes, it was proposed that the DNA existed as rather short pieces held together by some sort of linking material (Freese, 1958; Taylor, 1959). Since then, various schemes for folding or coiling the DNA to form a compact, microscopically visible structure have been suggested, but most of these retain the original concept that the basic structure is a tandem arrangement of units of replication, and perhaps function, which we now call replicons (Taylor, 1963). Our best working model of a chromosome is the one presented to us in the oocytes of amphibians and some other vertebrates during diplotene. These structures have been called "lampbrush" chromosomes. They consist of a central axis from which functionally active loops extend (Rückert, 1892; Gall, 1952; Callan and Lloyd, 1960). Callan and McGregor (1958) showed that the structural element of the loops as well as the main axis is DNA. Later Gall (1963) performed kinetic experiments with deoxyribonuclease that indicated that a single duplex forms the structural core of the loops, which are to be regarded as
Figure 1. Diagrammatic model of a segment of a chromosome. The model shows only the DNA, a single duplex for each chromatid, and the hypothetical "replication guides" which are assumed to be coupled into a flexible column or axial element. Other components are not shown, but it may be noted that a prominent feature is a series of loops which represents the units of replication. Even though a whole arm might consist of a single DNA duplex, one chain is assumed to be broken during replication on each side of a replication guide and then rejoined following replication (after Taylor, 1966).
the linear extensions of single chromatids. The axis, on the other hand, which consists of two chromatids closely associated, was found to act as if composed of two DNA duplexes, four DNA chains. Since no evidence has been obtained that any non-nucleotide linkers exist, a model (Figure 1) based on the assumption that a chromosome arm consists of a single DNA duplex without linkers has been proposed (Taylor, 1966). It is by no means certain that linkers do not exist, but our present knowledge of the existence of nucleases which could open DNA chains and enzymes which can effect reunion of single chain breaks make the concept of a non-nucleotide linker obsolete. Furthermore, we cannot be sure that all chromatids consist of a single DNA duplex, but this idea fits most of the experimental evidence and the model will serve until data requiring a revision are in hand.

Note should be taken that it still seems necessary to assume that something holds the long chromosomal DNA strand in shape. These structures may be proteins attached to the DNA (Figure 1) but do not necessarily interrupt its continuity. In the model and in a hypothetical scheme of reproduction which has been proposed (Taylor, 1966), these units are called "replication guides." They are assumed to be stacked into some type of flexible column which is probably attached to the centromere. If the loops were supercoiled and disposed about the column in the metaphase chromatid, it would consist of a cylindrical rod similar to that typically seen with the light microscope. Some larger chromosomes may have a helical coil formed with this axis as the stabilizing element, especially in meiotic chromosomes. Such a model can even account for the visible doubleness long known to be a property of anaphase chromosomes. The two ranks of loops or rings would stack to give a bipartite chromatid in which the two half-chromatids would be relationally coiled by twisting of the axis formed of the replication guides. Such a bipartite structure would have no direct
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relation to the DNA subunits demonstrable with tritiated thymidine.

Another contribution of the studies with H³-thymidine has been the concept that the sequence of replication of chromosomal sectors, perhaps controlled at the level of small units of replication, is reproducible and therefore predictable once determined for a particular cell type. Furthermore, the pattern of replication of the DNA changes as cells differentiate. A correlation between function, genetic transcription (Klevecz and Hsu, 1964), or expression of mutant genes (Davidson, Nitowsky, and Childs, 1964) has been established in a few cases and is assumed to be an important mechanism of genetic regulation in higher cells.

Regulation of Replication

The first evidence that the sequences of replication of DNA in chromosomes is regulated was obtained in autoradiographic studies of root cells of Crepis (Taylor, 1958), grasshopper spermatocytes (Lima-de-Faria, 1959), and somatic cells of Chinese hamster (Taylor, 1960). The latter illustrates the point best and provides the most complete picture to date. Original studies were made on two established cell lines, one from a male embryo and one from a female embryo (Taylor, 1960). After a pulse label with H³-thymidine, samples of the unsynchronized cell population were fixed at hourly intervals and incorporation of H³-thymidine into various chromosomes was examined in autoradiograms. The most striking variations in labeling of large blocks of DNA occurred in the sex chromosomes but was by no means restricted to these. To summarize, the pattern for these chromosomes were as follows: (a) In cells derived from the male soma, the Y chromosome was replicated in the last half of S (DNA synthetic phase) and the X chromosome had its short arm replicated in the first half of S while the long arm of the X was replicated in
the last half of S. \((b)\) In cells derived from the female, one X chromosome was replicated almost completely in the last half of S, while the other X followed a pattern very similar to the X of the male cells.

Since these first studies, the ordered replication of the whole or sectors of X and Y chromosomes, as well as autosomes, has been established in many species of both animals and plants. That the pattern is consistent for a given type of cell from generation to generation was indicated by autoradiography but was perhaps more strikingly demonstrated for all the DNA by Mueller and Kajwara (1966). They labeled a partially synchronized culture of HeLa cells in early S and noted that the same DNA molecules labeled in early S were the first to be labeled in a second S phase.

Evidence that the pattern of chromosomal DNA replication may vary in different tissues of an animal is strikingly illustrated by a comparison of the somatic cells and spermatogonial cells. Utakoji and Hsu (1965) have shown that those regions of the X and Y chromosome which replicate late in the somatic cells are replicated early in spermatogonial cells, where these regions might be reasonably expected to be active. The basis for this correlation between late replication and genetic activity is not yet understood, but the widespread occurrence of the phenomenon and its changes during differentiation suggest a major role in the regulation of gene action in higher cells.

**Size of the Replicon**

Before much progress can be made in understanding the control of DNA replication, we need to know much more about the molecular units, the size, and structure of the replicons. One way to estimate the maximum size is to find the rate of growth of DNA strands during replication. With this rate, the amount of DNA in a chromosome and total time required
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Figure 2. Distribution of DNA in a CsCl gradient. The DNA was isolated from Chinese hamster cells grown for 10 minutes in a medium with BUdR-H3 under conditions known to force essentially complete substitution of BUdR for thymidine. The molecular weight of the DNA is probably greater than 50 x 10^6.

for its replication, the maximum size of the replicon can be given. Recently we have determined the rate of growth for DNA in Chinese hamster cells in culture (Taylor, 1967). The method is based on the use of BUdR-H3 (bromouracil deoxyriboside-H3) of high specific activity as a density label. Cells were grown in a medium containing BUdR-H3 (10^-5 M) and FUdR (fluorouracil deoxyriboside), which is a potent inhibitor of thymidylate synthetase. Under these conditions the thymidylate in the cell is rapidly depleted and BU is substituted for thymine in the newly formed DNA. Cells were depleted of thymidylate by ten minutes' pretreatment with
Fig. 3. Distribution in a CsCl gradient of another sample of the DNA shown in Fig. 3 sheared to a mol. wt. of about $9 \times 10^6$.

FUdR ($2 \times 10^{-6}$ M), grown for ten minutes in BUdR-$H^3$ plus FUdR, and then for twenty minutes in unlabeled thymidine. The DNA was prepared by a method that preserves a high molecular weight (probably more than $50 \times 10^6$ daltons). When a sample of this DNA was banded in a solution of CsCl in the SW39 rotor (Spinco Model L2), dripped from the bottom and the radioactivity and optical density determined for each three-drop fraction, the profile shown in Figure 2 was obtained. The bulk of the DNA bands at a mean density of about 1.70, but the BUdR-$H^3$ did not band in a position typical of BU hybrid strands. Evidently, the BU-DNA was in relatively short pieces which were part of larger molecules of unsubstituted DNA. When a sample of the same DNA was sheared by shaking over isoamyl-alcohol—chloroform and
passage through a column of Bio-Gel P-60 and then was centrifuged as above, the profile shown in Figure 3 was obtained. A large proportion of the $\text{H}^3$-labeled DNA then banded in the position typical of BU-hybrid (density about 1.75). When a sample of DNA from cells grown for only five minutes with BUdR in an experiment similar to the one described above was centrifuged, a profile similar to that shown in Figure 2 was obtained. When the DNA was sheared by similar treatment and banded, the profile shown in Figure 4 was obtained. It may be noted that very little of the $\text{H}^3$-
labeled DNA now sediments in the position typical of fully substituted hybrid. Presumably, the strands of BU-DNA are too short to be completely freed of unsubstituted DNA by this amount of breakage. From these two experiments one may deduce the approximate rate of growth of the new BU strands by the following reasoning: If the stretches of BU hybrid are relatively short compared to the total DNA and the breakage of DNA by shear is random, no pieces of pure DNA hybrid will be produced unless the pieces produced by breakage are smaller than the stretches of BU-DNA. When the stretches of BU-DNA are just two times the mean size of the pieces produced by breakage, one-half of the BU-H\(^3\) will appear in pieces of pure BU hybrid and one-half will appear in fragments attached to unsubstituted DNA. This condition occurs at about ten minutes (Figure 3 and 4).

The molecular weight of the particles in the CsCl gradient is not accurately known, but an estimate was obtained by sedimenting a sample of the same H\(^3\)-labeled DNA through a preformed sucrose gradient, 5 to 20 percent (w/v) sucrose dissolved in 0.15 M NaCl and 0.015 M Na citrate, pH 7.0 at 4° C. When the distance moved was corrected for differences in viscosity and compared with sedimentation rates given by Burgi and Hershey (1963) for DNA's of similar size, an average molecular weight of about 9 \times 10^6 was obtained for these particles. Two times this value gives an estimate of the size of runs of BU-DNA in the chromosomes after a ten-minute growth in BUdR. These pieces would be 18 \times 10^6 daltons, or about nine microns in length.

If all of the DNA of intermediate density is produced by transition regions between BU-substituted DNA and thymine-containing DNA, it may be estimated that the counts in hybrid BU-substituted DNA equals the total counts in partially substituted DNA in about nine or ten minutes (Figure 3). Therefore, the average rate of growth of DNA chains in these cells is nine microns in nine or ten minutes (about one micron per minute).
The interval required for the replication of the long arm of the X chromosomes can be estimated to be three and one-half hours (200 minutes). This arm is about three microns long in a total length of 124 microns in the metaphase diploid complement (Hsu and Zenzes, 1964) and therefore contains about 2.7 percent of the total DNA per diploid cell, which has been estimated by Huberman and Riggs (1966) to be $7 \times 10^{-6}$ µg. Therefore, the DNA in the long arm of the X chromosome would be 58,000 microns long. Since it all replicates in about 200 minutes, it must grow at the rate of $58,000 / 200$ (290 microns) per minute. However, our measurements indicate a growth of only one micron per minute at each growing point. There must be at least 290 growing points in the long arm of the X and each unit of replication would be about 200 microns in length (mol. wt. $400 \times 10^6$). As mentioned at the beginning, this is a minimum number and a maximum length based on the assumption that each replicon grows for the full 200 minutes.

**Genetic Recombination**

We turn now to an account of the experiments on genetic recombination which demonstrate that a physical exchange of chromosomal segments occurs during or preceding meiotic prophase. Since the experiments have been published elsewhere (Taylor, 1965), the account will be brief. Male grasshoppers (*Romalea*) were injected with H$_3$-thymidine at stages during the third or fourth instar. Testes were fixed twenty to thirty days later when some of the cells labeled at pre-meiotic stages were reaching the meiotic divisions after the long meiotic prophase. Stages at diplotene, metaphase I, and metaphase II, among others, were found to be labeled. The sixteen spermatocytes in a cyst proceed through the various stages in synchrony and when any of these were labeled, all showed similar patterns. However, only a few cysts were labeled at stages early enough to show segregation of labeled
Figure 5. Autoradiograms of metaphase II chromosomes from secondary spermatocytes of a grasshopper (Romalea) which had been injected with H3-thymidine when these chromosomes were in spermatogonial cells at the DNA synthetic phase one division before premeiotic interphase. By the premeiotic interphase the H3-thymidine had been depleted and the chromosomes replicated once more without incorporating additional H3. They arrived in meiotic prophase with one labeled and one unlabeled chromatid and were fixed 24 days after injection of H3-thymidine when the cells with labeled chromosomes had arrived at the second meiotic division. Since centromeres are terminal or near terminal, the metaphase II chromosomes consist of two chromatid arms joined at the centromere (arrows). (a) Dyad with no visible exchanges. 3,600X. (b) Dyad with one clear reciprocal exchange of labeled segments and a distal nonreciprocal exchange. 3,600X. (c) Dyad with two nonreciprocal switch points with respect to labeled and nonlabeled segments in one arm. These nonreciprocal exchanges are presumably the result of exchanges between homologous chromatids and the corresponding segments are frequently in chromatids of another dyad. 3,600X. Autoradiograms are from a paper by Taylor (1965).
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chromatids at meiosis. Nevertheless, from the few observed, it was quite clear that distribution of DNA followed the pattern typical of chromosomes in mitotic cycles. At the $X_1$ division (the first after being labeled) all chromatids were regularly labeled. Unlabeled segments were seen due to asynchrony of DNA replication, but when a segment of a chromatid was labeled the sister chromatid was also labeled at the same position. If the meiotic divisions were the $X_2$ divisions (the second division after being labeled), the chromosomes showed the semi-conservative pattern of segregation (Figure 5). Since there is no DNA replication between the two meiotic divisions, segregation of label will not be different at the two divisions, except that analysis at the chromatid level is seldom if ever possible before anaphase I, when the chromatids separate. Dyads (chromatid pairs) at anaphase I and metaphase II proved to be the best stages for determining the distribution of labeled segments. If no segmental exchanges occurred except sister chromatid exchanges, each dyad would be expected to contain the equivalent of one complete labeled chromatid as in the $X_2$ divisions of the mitotic cycle. Exchanges between homologous chromatids could, of course, alter such a regular distribution. It was clear from the analysis of dyads from a few cysts, where chromosomes were labeled throughout their length, that exchanges between homologous chromatids were the most frequent events. Since only one of the two chromatids in a dyad was labeled at, or near, the centromere, it was also clear that the centromeres of homologous chromatids regularly segregated at anaphase I. The observed frequency of exchanges, based on switch points in labeled pairs of chromatids (dyads), was exactly that expected if each chiasma counted at diplotene represented an exchange between homologous chromatids. Sister chromatid exchanges appeared to be either absent, or at least infrequent, during the period of meiotic crossing over.

These observations on exchange of chromatid segments
during meiosis support the concept that reciprocal exchange of genetic loci will be found to be correlated with breaks and exchanges between homologous chromatids. Other types of phenomena which might be associated with reciprocal exchanges, such as repair or replacement of small segments of DNA (Taylor, 1968), cannot be studied until more sensitive techniques are available. However, nothing in these studies of exchange rule out other mechanisms of recombination which may be a part of the meiotic process.

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