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DEPARTMENT OF BIOPHYSICS

GENERAL SUMMARY

Regulation of cellular growth and cell division is one of the basic problems to be solved in order to understand the nature of cancerous cells and to search for means to control them. Through studies on DNA replication and cell cycle in bacteria as well as in eukaryotic cells, it has been revealed that regulation of the DNA replication cycle plays a key role in constructing normal cell division cycles. At present our efforts mainly concern the regulation in bacterial cells because genetic methods as well as biochemical ones are available to solve the problem at the molecular level. We suspect that basic rules discovered in a simpler system may help to analyse similar phenomona in more complicated cells. In addition, experiments with an animal cell, HeLa cell, are being undertaken on a small scale, and we will briefly comment upon this.

Coupling between DNA replication and cell division occurs at two steps in the bacterial replication cycle. The first is at the initiation of the replication through a regulatory protein, an initiator, which accumulates at the same rate as that of the cellular growth. The second is at the termination which is known to produce a regulatory signal for the cell division. These two regulatory mechanisms together with the fact that the time necessary for completing a round of the replication cycle is constant (40 min.) in various media establish division cycles which are stable but adaptable to the changes in environmental conditions. However neither the molecular mechanism nor chemical entities involved in the initiation are known at the moment. Knowledge about the biochemical events following the termination of the replication cycle is also scarce.

Three lines of approach were chosen in our laboratory to investigate the mechanism and the regulation of the initiation in a bacterium, *Bacillus subtilis*.

One is to identify the mode of existence of the origin of the replication in the cell in terms of the association with the cell membrane and other cellular components such as protein and RNA. In *B. subtilis* we found that a circular chromosome is replicated from a fixed site, replication origin, bidirectionally and sequentially towards a fixed terminus. Various genes are identified in our laboratory and mapped near the replication origin. Among them *pur*A, *dna*I99 and *dna*56 are located nearest to the origin. Using these genes as genetic marks for the origin we found that the origin exists in two alternative states in the cell, one is tightly bound to the cell membrane and the other is a soluble complex with some proteins and RNA and readily solubilized when the cell is lysed with lysozyme. The latter soluble complex is characteristic because no part of the chromosome other than the molecule containing the origin and the neighbouring genes forms this type of complex. Taking advantage of this fact the

DNA molecule containing the replication origin was highly purified together with proteins and RNA bound specifically to it. Purification and properties, biochemical and as seen in an electron micrograph, of the complex will be shown below. From this line of work we expect to identify the chemical structure of the origin and to isolate protein and RNA components which interact with it specifically. The chemical aspect of the mechanism and regulation of the initiation will then be solved.

The second approach is a use of genetico-biochemical methods to analyse a sequence of events which lead the cell to initiate the replication cycle. We have isolated three kinds of temperature sensitive (ts) mutants which show defects in the initiation at high temperatures and one mutant in which the initiation is selectively prevented in the presence of phenethyl alcohol (PEA). Phenotypic defects of all the mutants are reversible, thus initiation of a new round of replication is resumed when the mutant cells are incubated at restricted conditions for a certain period of time (60 min) and then returned to the permissive conditions. Components required for the initiation are restored during incubation under the permissive condition. Analyses of the four mutants led us to postulate that a sequence of a multiple component process consisting of at least three proteins and RNA occurs before the initiation is resumed. Recent experiments on the initiation RNA and a gene product of one of the mutants will be summarized below.

The last is the most difficult problem in isolation and identification of a hypothetical regulatory protein, initiator, for the initiation. Since the proposal by Jacob et al. in 1961 many hypotheses have been offered speculating upon the nature of the initiator. The initiator accumulation theory and an auto-repressor model are most generally accepted. In the abstract following this, we describe a new experimental approach to clarify the nature of the hypothetical regulator. In this experiment a novel gene, dan-ind gene, was discovered. The product of this gene has control over the initiation of the replication of two independent replicons coexisting in the same cell, one is the host chromosome itself and the other is a prophage integrated in the chromosome. It may be therefore possible to isolate and identify the gene product because the prophage DNA is small and easy to handle *in vitro*.

As to the events which occur after the termination of the replication cycle and lead to the septum formation of the cell, we began to analyse an event which follows the termination immediately, i. e., segregation of the chromosome. Partition of the two daughter chromosomes should require a local membrane synthesis through which chromosomes attached to the membrane at the terminus can be separated physically. Such a membrane synthesis may also lead to the septa formation. From these considerations attempts were made to isolate mutants in which segregation of the chromosome is selectively blocked at elevated temperatures. Three genetically distinguishable mutants have been isolated and are described briefly in the abstract. As expected, the membrane of all mutants showed some abnormal properties in structure as well as biosynthesis at high temperatures. However most of the mutants continued to divide at the high tempera-

ture to produce anucleated cells. This apparent uncoupling between segregation and septation may be correlated with spore formation by the bacterium that requires a new specific type of septum formation. We will try to find a more specific condition under which precise coupling between termination-segregation and cell division can be examined.

Our experience with HeLa cell is not very extensive. However we have found that too little is known about the mechanism and regulation of replication of chromatin to ask more elaborate questions on the role of DNA replication in the regulation of cellular growth and division. Our first aim is to find whether or not there is a defined sequence in replication of various replicons during the synthetic phase (S phase) of exponentially growing HeLa cells and then to ask how is the sequence if any controlled. An attempt to analyse the sequential replication in S-phase using a method to fractionate cells of various stages in S-phase without using any chemical agents to synchronize the cell division cycle will be presented.

This study was supported by a Grant-in-Aid for Scientific Research from the Misistry of Education, Science and Culture, Japan, by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan, by a Grant from Asahi Shinbun, a Grant from Kudo Zaidan and by a Grant from Matsunaga Kagaku Shinko Zaidan.

ABSTRACT

(23) **Isolation of a soluble DNA-protein complex containing a single chromosomal fragment derived from the replication origin of the *Bacillus subtilis* chromosome.**

K. Yamaguchi and H. Yoshikawa

In *Bacillus subtilis* the genetic structure near the origin has been well elucidated[1]. The origin is located between two genetic markers, purA and ts-dna8132. The distance between the origin and these two markers is estimated to be less than 2 to 5 x 10^7 daltons[1]. Using these genes as markers it is possible to isolate a chromosomal fragment containing the origin and cellular components which are specifically associated with it if a suitable isolation method is chosen to keep a complexity of the origin as intact as possible. For this purpose cell lysates were prepared by a mild treatment of lysozyme and a non-ionic detergent Brij-58 or treatment of the lysozyme alone and then gently shearing to produce fragments of some 2 to 4 x 10^7 daltons. Isolation and purification of the fragment containing the origin was performed by repeated sedimentation through a sucrose gradient with an optimum salt concentration to avoid dissociation of protein components from DNA.

Some 50% of chromosomal fragments containing *pur*A (*pur*A-DNA) in the lysate was found in a membrane fraction[2]. As reported previously the other 50% of *pur*A-DNA formed a complex which had a markedly higher sedimentation rate (70-100S) than

had the bulk of the supernatant DNA (40S). High salt concentrations, pronase and ionic detergents reduced the rate to 40S, indistinguishable from that of the bulk DNA, indicating that the *pur*A-DNA is a complex at least with some proteins[3]. Hence it is called "soluble complex" (S-complex). No genetic markers other than *pur*A and those closely linked to *pur*A was found in the complex. The origin label was concentrated in it but the pulse label in the replication point was not. Lysates without using detergent and mechanical shearing yielded the same amount of S-complex as did those with these treatments. These results suggest that S-complex is not a partial degradated product of the membrane bound *pur*A-DNA but is derived from another, membrane unbound, state of the origin DNA in the cell.

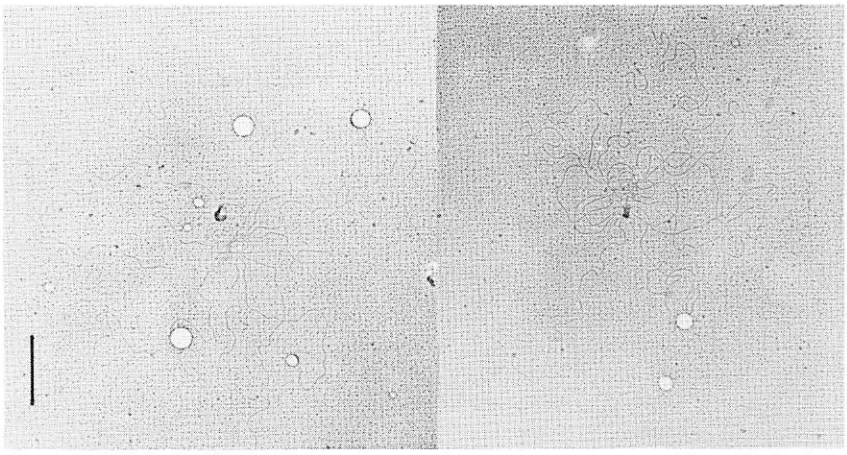
The S-complex in the lysozyme-Brij lysate was purified by repeating sedimentation 3 times in a sucrose gradient of pH 7.0 containing 0.1M KCl, as the complex is stable at these conditions. A purified complex (after the fourth sucrose gradient) contained essentially *pur*A-DNA as the sole DNA component indicating that only a single chromosomal fragment of some 3×10^7 daltons forms the S-complex. From the observed value of *pur*A/*his*A=190 the purity of the fragment was estimated as 60%. The amount of DNA recovered in the purified S-complex was some 2.5% of total cellular DNA.

Alkaline conditions (>pH 8.0) and low salt concentrations (<0.02M) considerably reduced the sedimentation rate of the S-complex. Unlike the case of high salts and deproteinization treatments the reduced rate was significantly higher than that of the bulk or deproteinized DNA. The sedimentation rate of the complex was also influenced by its own concentration. These results suggest that the complex is an intermolecular aggregate of the *pur*A-DNA-protein complex. This was directly proved by the electron microscopic observation of the purified S-complex. Aggregates of several DNA molecules (average 3.5) forming a structure containing loops, bushes and amorphous materials stained black were seen when the complex was treated with glutaraldehyde before spreading with cytochrome c (Figure). Without glutaraldehyde treatment only normal linear duplexes of some 14μ were observed. When the S-complex was prepared in low salt concentrations and treated with glutaraldehyde, separate duplexes forming a local entangled structure with bushes and stained materials were seen in parallel with a decrease in aggregated molecules. These entangled duplexes may represent a monomeric form of the *pur*A-DNA-protein complex. Bushes seen in the complex disappeared by RNase treatment.

The S-complex purified from the lysozyme-Brij lysate contained many species of proteins which were qualitatively similar to those in the membrane fraction. The sedimentation profile of the proteins, however, did not coincide with that of the DNA or *pur*A of the purified complex. It is therefore hard to decide whether or not the S-complex was bound to small membraneous structures consisting of proteins and a minute amount of phospholipids. These structures may be an artificial aggregate of lipid depleted membrane proteins and cosedimented in the vi-

cinity of the S-complex. Accordingly protein components in the S-complex isolated without Brij were compared. In this case the sedimentation profile of proteins coincided with that of DNA and a significant quantity of phospholipids were found with the proteins. A preliminary electronmicrogram of the complex after glutaraldehyde treatment showed a different picture from those of the Brij treated S-complex. Aggregates with more than three molecules were scarcely seen and most of the molecules were separate molecules with a large structure containing bushes and stained material attached near the center of the molecule. Effects of pH and high and low salt concentrations were identical in both types of S-complex. From these results we tentatively conclude that a native S-complex is a complex between *pur*A-DNA-protein complex and a small membraneous particle. The Brij treatment might have caused detachment and aggregation of the *pur*A-DNA-protein complex on one hand and aggregation of lipid depleted proteins on the other. *In vitro* reconstitution of the complex is under investigation.

1) Hara, H., and H. Yoshikawa, Nature New Biol. 244, 200 (1973)
2) Yamaguchi, K., and H. Yoshikawa, Nature New Biol. 244, 204 (1973)
3) Yamaguchi, K., and H. Yoshikawa, Symposia Cell. Biol. 24, 99 (1973)



Electron-microgram of the purified S complex.
Magnification is x 8000 and the bar in the photo shows 1 μ.

(24) Molecular events involved in initiation of DNA replication cycle in *Bacillus subtilis*.

S. Murakami, N. Inuzuka and H. Yoshikawa.

Properties of one protein component and RNA required for the initiation and sequence in time of their function and interaction during formation of the initiation potential are studied using a ts-dna-initiation mutant dna27. Reinitiation of the DNA replication cycle which occurs when the mutant cell is treated at 47C for 60 min and then cooled to 30C was not affected by chloram-

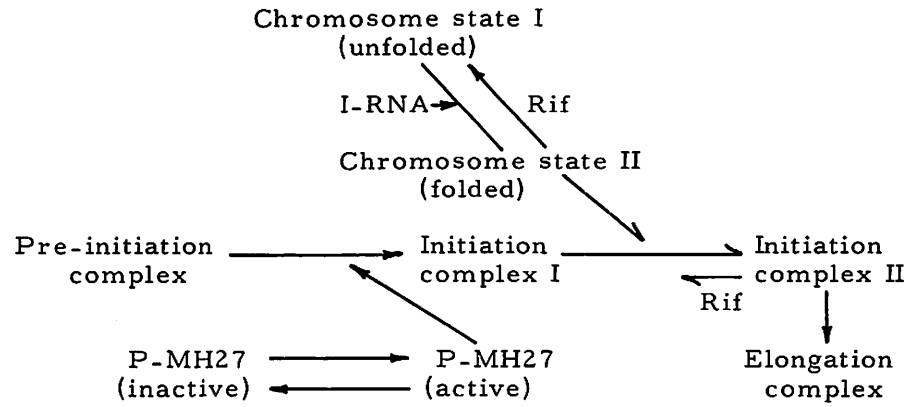
phenicol (CAP), but was severely prevented by either heating to 47C or by addition of rifampicin (Rif). However the reinitiation was no longer prevented by these treatments if cells were incubated at 30C in the presence or absence of thymine for 20 min prior to the treatments. Thus cells acquired initiation potential resistant to either heating to 47C or Rif. These results clearly show that RNA synthesis is involved in the initiation directly. The Rif-sensitive RNA synthesis which is required for the initiation is designated as initiation RNA (I-RNA).

At first glance I-RNA synthesis itself is temperature sensitive in this mutant and therefore the formation of the temperature resistant initiation potential was a direct result of the I-RNA synthesis. However, this was not the case and both activation of a product protein of the *dna*27 gene (P_{27}) and I-RNA synthesis occurred during the incubation at 30C. Evidence supporting this conclusion is summarized as follows. 1) Kinetics of the appearance of the temperature resistant potential and the Rif resistant potential are not unlike, but the latter precedes the former by 2 min. 2) About 30% of the cell fraction was arleady resistant to Rif at the time of the temperature shift from 47 to 30C, while no temperature resistant fraction was detected at that time. This residual Rif-resistant potential decreased rapidly when Rif was added at 47C prior to cooling. 3) Using a Rif-reversible mutant of *dna*27 in which RNA synthesis resumed soon after removal of Rif, formation of the temperature resistant potential was completely separated from that of the Rif resistant potential. Thus when cells were heated and cooled as usual with Rif present from the last 8 min at 47C through 30 min at 30C, cells acquired an initiation potential which is fully resistant at 47C but completely sensitive to Rif. In other words the temperature resistant potential can be formed rapidly (50% activation in 2.5 min) without I-RNA synthesis.

From the results described above together with other data which are not shown here in detail, the following picture can be drawn as to what is taking place in the mutant cell during heating at 47C and after cooling back to 30C. Immediately after heating P_{27} becomes inactive and in consequence the initiation of new replication cycles is arrested instantaneously. The other components required for the initiation, proteins and I-RNA, are synthesiszed continuously at this temperature. However no accumulation of I-RNA occurs at 47C and it turns over rapidly. Accordingly, if Rif is added at 47C, preexisting I-RNA decays quickly with a half life time of some 120 sec. When the heated cells are cooled to 30C, the initiation potential begins to be reformed depending on a rapid reactivation of P_{27}. The Activation of P_{27} occurs at 30C independent of I-RNA or its synthesis. Therefore unlike I-RNA, P_{27} can be accumulated at 30C even when I-RNA is absent and no initiation occurs. Reformation of the initiation potential requires a continuous I-RNA synthesis because even at 30C I-RNA turns over as rapidly as at 47C until the active P_{27} is recovered. Thus if Rif is added at various times during the reformation process, the amount of the initiation potential is determined by the combination of the rate of activation of P_{27}

and the rate of decay of I-RNA. Both activation of P_{27} and I-RNA synthesis occur independently of each other. Function of P_{27} is performed in the absence of I-RNA to form an initiation potential which is no longer sensitive to 47C but still requires I-RNA synthesis. The initiation potential at this stage can be defined experimentally by using the Rif-reversible mutant. Accordingly we call this intermediate stage as "initiation complex I"! In contrast to P_{27}, not only the function but also the stablility of I-RNA is completely dependent on the presence of the active "initiation complex I". Therefore we deduce that I-RNA participates at a stage after the formation of "initiation complex I" in forming a more advanced stage of the initiation potential, "intiation complex II". If all four deoxyribonucleotides are present the initiation occurs 10-15 min after the I-RNA has acted and "initiation complex II" is formed. At the moment we do not know the reason for the lag period of 10-15 min. If thymine is missing from the medium, no initiation occurs and "initiation complex II" can be maintained as long as I-RNA synthesis is continued.

Molecular events which lead to the formation of a completed initiation potential are summarized in the figure. In this figure a model for the function of I-RNA is included in addition to sequential steps involved in constructing the initiation potential. We adopted a folded chromosome structure as a candidate for the step in which I-RNA participates. The stability of the folded genome in *E. coli* resembles fairly well that of I-RNA. We assume that a specific folding at the replication origin is formed continuously throughout the cell cycle. This folding is not stable and turns over rapidly until "initiation complex I" is formed through an assembly of various protein components. Interaction of the folded structure at the origin with the "initiation complex I" at a certain period of the cell cycle triggers the initiation of chromosomal replication from the replication origin.



A model for a sequence of events involved in initiation of DNA replication.

(25) **A novel gene which controls the initiation of chromosomal replication and prophage induction in *Bacillus subtilis*.**

S. Murakami, Sh. Murakami and H. Yoshikawa.

We conducted a new experimental approach to clarify the nature of the hypothetical regulator, initiator, which is synthesized in harmony with the increase in cell mass and regulates the initiation of chromosomal replication. Based on the assumption that the induction of prophage and the initiation of host cell chromosome is controlled by a common regulator, it would be possible to isolate conditional mutants in which the two events are simultaneously prevented at non-permissive conditions. Induction of a temperate phage SPO2 in *B. subtilis* by 6-(para)-hydroxyphenylazouracil (HPUra) was used as a test system because it inhibited specifically chromosomal replication of *B. subtilis* without affecting either the repair synthesis of the cell or the replication of the SP02 DNA.

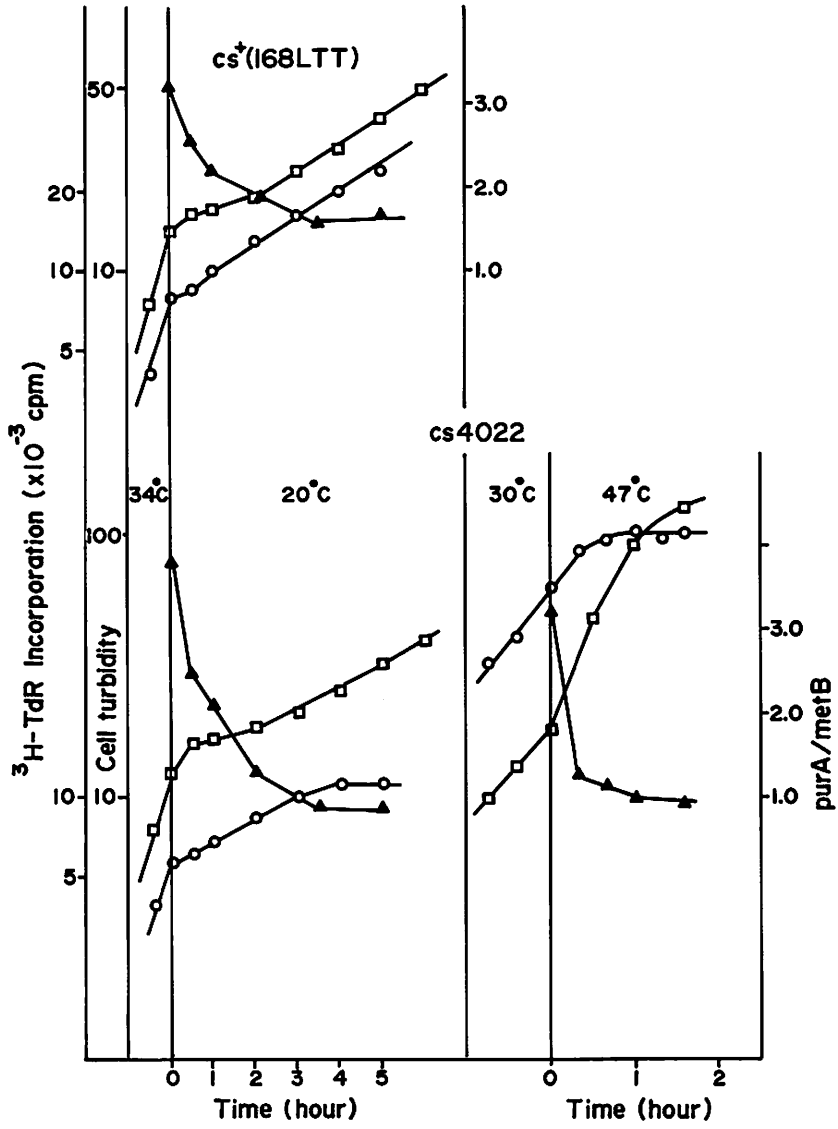
The mutagenized lysogens of *B. subtilis* 168 *trp*$^-$ were grown in a rich medium at 30C and then heated to 45C with the addition of HPUra at 5 min after heating. Ts mutants were isolated from the resultant surviving cells. Thirtyseven mutant lysogens which showed both ts growth and ts induction by HPUra were isolated either spontaneously or by the NTG treatment. The mutation occurring in the host chromosome was directly shown by introducing ts character into another recipient cell carrying *thy*$^-$ character. Surprisingly enough 10 out of 31 transformed mutant strains were found to be dna-ts-initiation mutants which was indicated by the decrease in marker frequency and the amount of the residual increase in ^{3}H-thymidine incorporation at 47C.

As expected when these mutants were lysogenized with a wild type SPO2 the prophage was induced by HPUra at 30C but not at 47C judging from the cell lysis and the incorporation of ^{3}H-TdR in the presence of HPUra a direct indication of the replication of the SPO2 DNA. The expression of early genes, the autonomous replication and maturation of SPO2 occurred normally at 47C in the presence of HPUra as indicated by a successful infection by SPO2 and induction by mitC at 47C even in the presence of HPUra. From this evidence we conclude that the mutation in this group of ts mutants affects at the non-permissive temperature both the initiation of chromosomal replication and prophage induction. Hence they are collectively named as "*ts-dna-ind*" mutants.[1)]

Recombination tests using three mutants carrying the dna-ind mutation as recipients revealed that all 12 ts-dna-ind mutations were not identical but closely linked with each other having recombination indices of 0 to 0.3, suggesting that they are located within one gene.

Moreover these dna-ind mutations were found to be tightly linked to *dna-ts*27, a mutation carried by a ts-dna-initiation mutant isolated previously in our laboratory.[2)] Phenotype of the mutant as to the ts character of the initiation of chromosomal replication was indistinguishable from the dna-ind mutation. The results of three factor crosses by PBS1 transduction showed that the dna-ind gene is located near the middle of *arg*A and *leu*A. The reversion frequency of most of the dna-ind mutation was

more than 10^{-8} and the capacity to initiate chromosomal replication and to induce prophage in the presence of HPUra are simultaneously recovered after the incubation at non-permissive



Growth and DNA synthesis of cs-ts mutant of dna-ind gene. Exponentially growing cells of a wild type (the upper figure) and a cs-ts mutant (the lower figures) at 34C were cooled to 20C. Turbidity increase (□—□), DNA synthesis (○—○), and marker frequency (*purA*/*met*B: ▲—▲) were measured before and after the temperature shift down. In addition, the mutant cells were also grown at 30C and then heated to 47C in order to examine the effect of the temperature shift up on growth and DNA synthesis.

temperatures. This evidence indicates that both the initiation of chromosomal replication and prophage induction were affected by a single mutation occurring in the *dna-ind* gene. Since ts27 mutation prevented the initiation of the chromosomal replication without affecting the inducibility of the prophage by HPUra at high temperatures, these two functions are affected independently by the *dna-ind* gene product and a single mutation in this gene may cause the alteration in either one of the two functions or in both functions simultaneously.

Since the prophage SPO2 can be induced by HPUra even at 20C, attempts were made to isolate cold-sensitive (cs) mutants which could not induce prophage in the presence of the drug at 20C. 21 cs mutants were isolated spontaneously from 168LTT (SPO2) by a two successive induction by HPUra at 20C. None of these mutants grew on BHI plate at 20C and also at 47C. So far as examined, the initiation of chromosomal replication did not occur at either 20C or 47C in nonlysogen of these mutants (Figure). As expected these cs-ts mutations (4022) are mapped near the position of ts27, i. e. in the *dna-ind* gene.

In summary, a highly selective concentration of a specific type of dna-ts or dna-cs-ts mutants by the selection method used in this experiment clearly showed that a definite close relationship exists between the initiation of chromosomal replication and prophage induction. Furthermore the present finding of the dna-ind gene and pleiotropic effects of its mutation support the existence of the hypothetical substance.

1) Murakami, S., Murakami, Sh. and Yoshikawa, H. Nature, 259, 215 (1976)
2) Murakami, S., Inuzuka, N. Yamaguchi, K. and Yoshikawa, H. (1974) p58 in Molecular Biology Meeting of Japan.

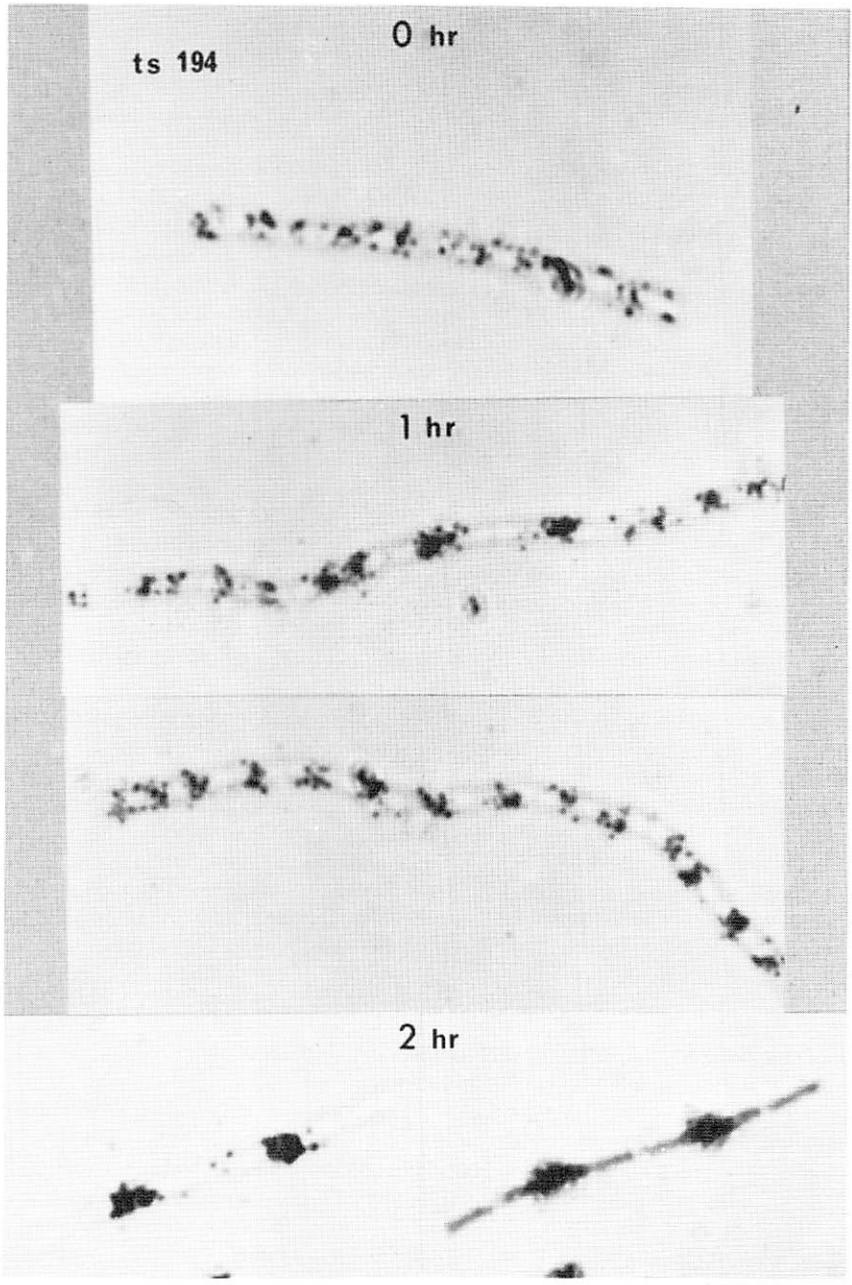
(26) Studies on the segregation of the bacterial nuclei: Isolation of mutants defective in nuclear segregation in *Bacillus subtilis*.

M. Yamaguchi, K. Yamaguchi and H. Yoshikawa.

The bacterial cell division cycle can be briefly descibed as duplication of genetic material DNA (a cyclic process consisted in initiation, elongation and termination), segregation of replicated DNA, formation of a septum (or a cross wall) and physical separation of daughter cells in parallel with doubling of the cell mass. In recent years, many mutants defective in the cell division cycle have been isolated in order to study the mechanism and regulation of the bacterial cell division. Most of these studies have forcussed themselves on DNA replication, septum formation and relationship between these two phenomena. It was found that termination of the DNA replication cycle is required for the following cell division. However, little investigation has been done on the segregation of DNA which occurs soon after the termination of DNA replication and before the septum formation begins. Accordingly we tried to isolate bacterial mutants defective in nuclear segregation in order to facilitate genetic and biochemical analyses of the nuclear sergregation.

First, we isolated 630 temperature sensitive growth mutants

from cells mutagenized with nitrosoguanidine. Twelve mutant strains were then selected by examining nuclear segregation at



Replication and segregation of DNA in a seg⁻mutant. Mutant cells were labeled with ^{3}H-thymidine of a high specific activity at 30C. At time 0, temperature of the incubation was heated to 47C (restricted temperature). At 0, 1 and 2 hours after the heating, cells were fixed and radioautogram was taken. 0 hr: 4 to 8 clusters are seen in each cell. 1 hr: 2 to 4 clusters are seen in each cell. 2hr: only one large cluster is seen in each cell.

non-permissive temperatures under a phase-contrast microsope. Polyvinylpyrrolidone was used as a facile reagent for visualization of bacterial nuclei. In these mutant strains DNA was synthesized normally in parallel with the increase in cell mass even at nonpermissive temperatures. When mutant cells were grown at 30C and then heated to a nonpermissive temperature, the total number of nuclei in the culture stopped increasing, while the total cell number continued to increase resulting in a drastic decrease in the number of nuclei per cell and the appearance of anucleated cells. These results were further confirmed by autoradiography of cells labeled uniformly with ^{3}H-thymidine (Figure). To show more directly that nuclear segregation does not occur in mutant cells at nonpermissive temperature, DNA contents of each nucleus were determined by quantitative analyses of autoradiograms. DNA contents per nucleus in mutant cells increased 3-4 fold during a 2 hour incubation at high temperature, while in wild type cells DNA contents per nucleous remained constant under the same condition. These results indicate that in mutant cells nuclear segregation is specifically affected while DNA replication occurs normally at nonpermissive temperatures.

Through genetic analyses, these 12 mutations were divided at least into three groups inferring that at least three genes designated as *seg*A, *seg*B and *seg*C participate in nuclear segregation.

Genetic and biochemical characterization of these three groups of mutants are in progress.. No difference has so far been detected among mutants and their parent strains in the synthesis of macromolecules (DNA, RNA, proteins, phopholipids and cell wall) as a whole at the high temperature. However, in *seg*A mutants the composition of phospholipids was drastically changed. Thus relative amounts of caldiolipin increased from 5% of total phospholipid at the time of the temperature shift up to 30-50% of total phospholipid during incubation at the high temperature for 60 min. Some mutations in *seg*A simultaneously induced higher sensitivity to penicillin G or phenethyl-alcohol than the parent strain even at permissive temperatures, suggesting an interaction between a product of the *seg*A gene and the cell membrane. Further investigations are required to clarify the function of there *seg* genes.

(27) Regulation of DNA replication in HeLa cells.

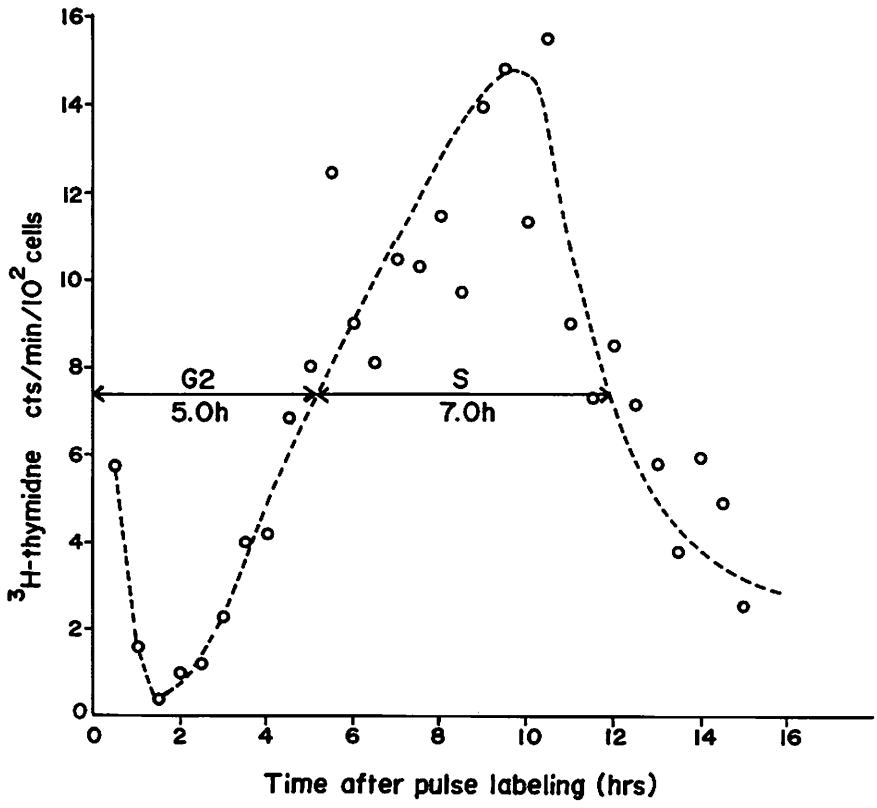
S. Aoyama and H. Yoshikawa.

Autoradiographic and kinetic studies of eukaryotic cells have revealed that DNA synthesis occurring within nuclei appears to involve any discrete DNA units which are made at different times and are distributed non-randomly among chromosomes and along the segment of individual ones. Given DNA sequences replicate during the same portion of the S phase from one cell generation the next. However it is not known whether temporally ordered gene replication is controlled by genetic factors or chromosomal structure itself.

In order to approach this problem it is necessary to establish a sensitive assay system to determine the ordered sequence of

DNA replication by eukaryotic cells growing under usual conditions as well as unusal conditions which may cause structural changes in the chromosome.

HeLa S3 cells were grown in monolayers on a flat glass surface of Loux's bottles at 37C using a low-calcium Eagles's medium supplemented with 10% calf serum. Tenuously attached mitotic cells were selectively detached and collected at intervals through the cell cycle from exponentially growing monolayer cultures by manually shaking the bottles. Since cells composing the monolayer continued to enter the mitotic phase, it was possible to continuously collect mitotic cells for about one generation. The mitotic cell fraction of each preparation detached every 20 or 30 min exceeded 90%. After an exponentially growing culture was pulse-labeled with ^{3}H-thymidine, mitotic cells were collected every 20 min and the radioactivities in DNA were measured as a function of the time at which cells were detached from the bottle. This gives the rate of DNA synthesis in mitotic cells as a function of the time in the cell cycle when they were labeled with ^{3}H-thy-



Analysis of cell cycle in exponentially growin HeLa cells. HeLa cells exponentially growing in a monolayer were labeled with ^{3}H-thymidine for 10 min. Every 20 min after the labeling, loosely attached mitotic cells were removed from the monolayer by mannually shaking bottles. ^{3}H-thymidine incorporated in DNA was measured with each detached cell sample.

midine (Figure). The sooner a mitotic cell was detached from the monolayer, the older the cell was in the cell cycle when it was labeled. The length of the S phase obtained from this pattern was about 7 to 8 hours agreeing well with previous findings by usual cell cycle analysis.

Exponentially growing cells were also pulse-labeled with 5-bromodeoxyuridine in place of ^{3}H-thymidine and DNA was isolated from each cell fraction collected by the technique described above. Through banding twice in neutral CsCl gradients, newly replicated hybrid DNA molecules were isolated. In this way one can isolate newly replicated chromosomal segments at various times during a given S phase. Using DNA-DNA hybridization between 5-Bu-DNA and ^{3}H-thymidine labeled DNA fractionated as described above, we are at present trying to examine the ordered sequence of DNA synthesis under normal conditions.

In further study we will try to test whether or not the sequence of DNA synthesis can be changed when the cell is grown for more than one generation in the presence of 5-bromodeoxyuridine which is known to cause localized changes in chromatin condensation probably through modification of chromosomal protein-DNA interaction.