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DEPARTMENT OF BIOPHYSICS

GENERAL SUMMARY

Process of cell growth is composed of two types of biochemical events; one is a sequential progress of biochemical reactions and the other is the cyclic occurrence of specific biochemical events. The steady state growth of the cell is maintained through precise coordination of these two processes.

For nearly ten years we have studied regulation of DNA replication. Significance of our research is two fold. First, initiation of DNA replication is one of the main biochemical events during the cell cycle and may be directly involved in the regulation of another specific event, cell division. Second, DNA replication itself is composed of the two types of events mentioned above, i.e. sequential elongation and cyclic initiation.

I. Experiments with procaryotic cells.

We have shown that the chromosome of *B. subtilis* is composed of a single circular DNA molecule of about 2×10^9 daltons and replicates from a fixed origin bidirectionally and sequentially towards a fixed terminus. Rate of replication is controlled not by elongation rate but by a frequency at which replication is initiated from the origin. Therefore our interest has been focused on the molecular mechanism involved in the initiation events, and its regulation.

Analysis of mutants defective in the initiation showed participation of multiple proteins and RNA in the initiation. In order to analyse these proteins, a method was developed to isolate and identify proteins which have affinity for the cellular DNA (Abstract 28). Experiments with synchronously growing cells of these mutants revealed that a sequential action of these proteins and RNA resulted in the cell's ability to initiate new replication cycles (initiation potential). Although we know little about the nature of the initiation potential it is strongly indicated that a specific organization of the chromosome at the replication origin is related to this process.

From these lines of work our recent efforts are concentrated more on the elucidation of organization of the chromosome of the growing cell during the cell cycle. We found that genes near the replication origin formed a unique complex containing proteins and RNA (S-complex). The complex was purified and the chemical nature of its components was characterized. A unique structure of the DNA part was demonstrated using a recently developed technique of analysis of DNA fragments produced by restriction enzyme digestion (Abstract 29). In order to determine the location of the DNA relative to the origin, restriction fragments produced from DNA newly synthesized immediately after the initiation were

determined (Abstract 30). Thus DNA in the complex is located contiguous to and asymmetrically at one side of the origin (Abstract 29). Protein components of the complex were determined by reconstitution of the complex *in vitro* (Abstract 31).

In order to know the biological function of the S-complex we measured the change in structure of the origin during spore germination. We found that the replication origin formed DNA-protein complex prior to the onset of replication and then gradually bound to the cell membrane (Abstract 32).

Our hypothesis is that cyclic organization and disorganization near the replication origin of the chromosome has an essential role in activation and inactivation of initiation of the replication cycle.

II. Experiments with eukaryotic cells.

In parallel to the work with bacterial cells we have extended our interest to regulation of DNA replication and cell growth in eukaryotic cells. So far, our work is limited to the cultured cell or unicellular eukaryote because essentially the same methodology as we used for prokaryotic cells can be applied.

One of the unique features of the eukaryotic replication is that the chromosome is composed of many hundreds of replication units and replication occurs simultaneously at many sites. Our first experiment with eukaryotic cells was to ask whether or not this replication is regulated in an orderly fashion throughout the 10 hours of DNA synthetic phase in HeLa cells. We found that a definite temporal sequence is stable and not affected by mechanical and chemical treatment used to synchronize cell growth (Abstract 33).

Use of mutant cell lines is essential to analyze the complex process of replication as its usefulness has been proven in prokaryotic cells. In collaboration with Dr. Shiraishi we used a fibroblastic cell from Bloom's syndrome as a candidate for such a mutant. However no clear result was obtained as to the nature of the genetic defect in relation to DNA replication.

From these experiences we are currently working with a unicellular eukaryote, yeast *(Saccharomyces cerevisiae).* Our interest is two fold. One is that genetic analysis has been extensively performed with many genetic markers, and many mutants defective in cell cycle progress including initiation of the DNA replication cycle have been isolated. Second, microstructures such as spindle plaque and microtubules are thought to be involved in regulation of progress of the cell cycle and initiation of DNA replication. Throughout the past year we attempted to isolate mutants defective in DNA replication or altered in sensitivity to mitotic inhibitors. Chracterization of tubulin and its associated protein in yeast is also in progress (abstract 34 and 35).

ABSTRACT

(28) Two dimensional electrophoresis of DNA binding proteins.

S. Murakami and H. Yoshikawa

A new technique for two-dimensional electrophoretic separation of protein was developed by O'Farrell[1]. In this method proteins were separated by two different principles; first, by their isoelectric property and second, by molecular weight. Using this method some missense mutations can be detected due to the difference in the relative contents of acidic or basic amino acid residues.

We attempted to identify the *din* gene product of *B. subtilis* by this technique. In *din* mutants both initiation of the chromosomal replication and the prophage (SPO2) induction were inhibited reversibly at non-permissive temperatures[2]. We have analyzed proteins from whole cell lysates and those bound to DNA cellulose affinity column obtained from wild type as well as 10 different *din* mutants carrying different mutations in the *din* gene. Proteins were labeled by ^{35}S at permissive or non-permissive temperatures. Although essential procedures were the same as those by O'Farrell, the following modifications were necessary to analyze the whole cell lysate from *B. subtilis*. 1) ^{35}S-labeled cells were treated with lysozyme at 30°C for 20 min before nuclease digestion because the cell wall of *B. subtilis* was not disrupted easily by sonication alone. 2) Since proteolytic activity is high in the lysate, a potent inhibitor of proteases, diisopropylfluorophosphate was added to avoid ambiguity of the results due to proteolysis.

Figure 1 demonstrates the capacity of this gel electrophoretic system. Under our conditions the pH range in the first dimension was between 6.6 to 3.6 Using a uniform gel of 10% acrylamide in the second dimension, nearly 800 proteins were countable when a heavily exposed autoradiogram was carried out. By comparing autoradiograms of whole cell lysates

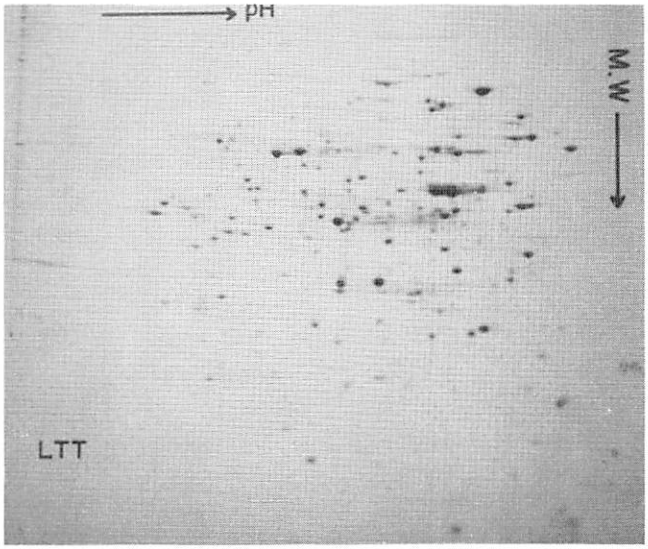

Figure 1. Separation of *Bacillus subtilis* proteins.

^{35}S-labeled whole cell lysate of *B. subtilis* 168 LTT contatining 90,000 cpm and approximately 5 μg of protein were loaded on the gel. The gel in the SDS dimension was a 10% acrylamide gel.

from 10 *din* mutants, we could not detect any spot whose position was shifted horizontally, i.e. changes in isoelectric point, from one mutant to another. There may be too many proteins in the whole lysate to detect a minor protein component. We therefore tried to concentrate the *din* gene product assuming that it binds to DNA.

^{35}S-labeled proteins in the lysate was precipitated by 60% ammonium sulfate. This fraction was applied onto a DNA cellulose column after dialysis. Proteins which were bound to the column were eluted by the elution buffer containing 2M NaCl. About 1.0 to 1.5% of the total cellular protein was bound to this column.

Figure 2 is a precise drawing of the autoradiogram of the two-dimensional gel for proteins bound to the DNA cellulose affinity column. The left side column illustrates major bands separated by a discontinuous SDS gel electrophoresis. We can detect reproducibly at least 100 proteins on the autoradiogram. This result suggests that more than 100 proteins interact directly or indirectly with DNA. These proteins distribute in wide range both in molecular weight and pH similar to those from the whole cell. Although the total number of proteins decreased and therefore better resolution was obtained by the electrophoresis, we did not find a spot which showed difference in mobility in either dimension resulting from the *din* mutation.

1) O'Farrell, P. H., J. Biol. Chem., 250, 4007 (1975).
2) Murakami, S., Murakami, Sh. & Yoshikawa, H., Nature, 259, 215 (1976).

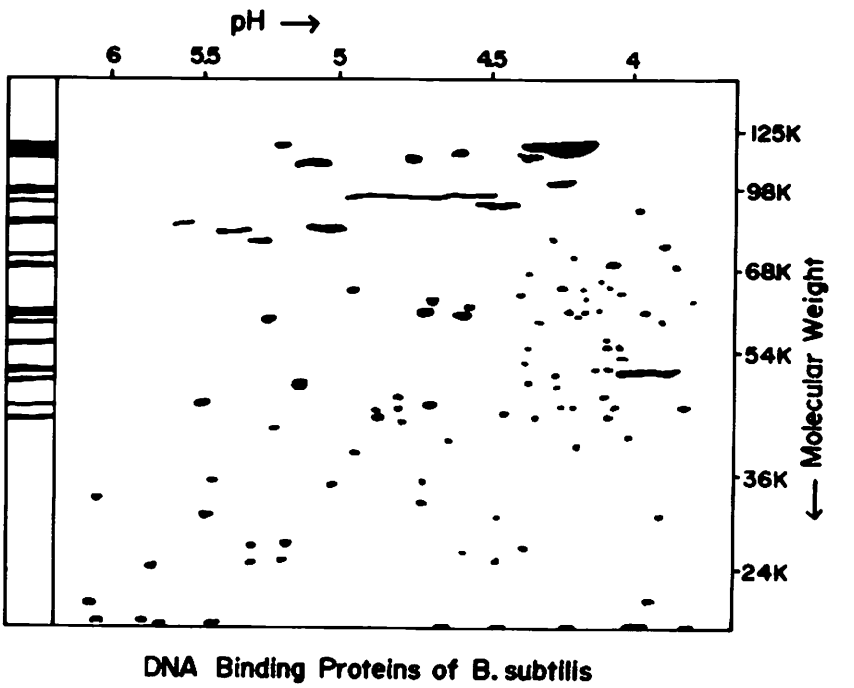

Figure 2. Two dimensional map of DNA binding proteins of *Bacillus subtilis.*

(29) Structure of DNA in a DNA-protein complex containing replication origin markers and its location on the *Bacillus subtilis* chromosome.

M. Seiki, N. Ogasawara, K. Yamaguchi and H. Yoshikawa

A soluble complex of 70-100 S (S-complex) containing genetic markers near the replication origin, DNA and protein, was isolated from growing *B. subtilis* cells and purified to 80% homogeneity as to the DNA component. The molecular weight of the DNA was 3×10^7 daltons and contained two groups of genetic markers separated physically for about 10^7 daltons. DNA was purified from the complex by deproteinization, followed by RNase treatment and then digested with various restriction endonucleases, EcoRI, HindIII and BamHI. Fragments produced by these nucleases were separated by electrophoresis in agarose gel and relative amounts were determined either by staining with ethidiumbromide or by radioactivity in the DNA. Figure 1 shows several discrete bands of DNA fragments indicating that the DNA is derived from a specific chromosomal fragment. The total molecular weights of nearly equimolar fragments were estimated as approximately 2×10^7 daltons. This indicates that more than 1/2 portion of the randomly sheared DNA molecules contained an identical base sequence.

In order to determine whether or not this conserved sequence includes the origin sequence, fragments produced by restriction nucleases were compared with those produced from DNA newly synthesized immediately after the onset of the replication cycle (see abstract 30). Data in Table show that at a shorter period of replication, fragments from the replicated origin contained only a part of those from the DNA in the S-complex. After the longer period of replication newly replicated fragments included all fragments from the S-complex DNA. However, in all cases, approximately half of the fragments from newly replicated DNA did not find the corresponding homologues among the fragments from the DNA in the S-complex. Analysis with two kinds of nucleases, EcoRI and HindIII, gave identical results. A plausible interpretation of the results is that DNA in the S-complex is located contiguous to and asymmetrically at one side of the replication origin (Figure 2).

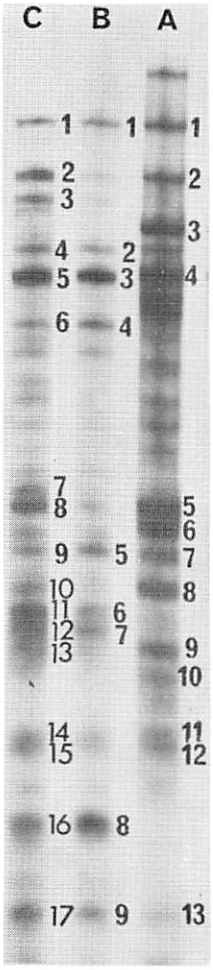

Figure 1. Fluorography of EcoRI fragments from s-DNA and pulse labeled origin.

DNA from uniformly labeled s-complex (A) and DNA pulse labeled near the origin for 1 min (B) or 10 min (C) were digested with EcoRI. Radioactive fragments were separated in 0.8% agarose gel and detected by fluorography.

	EcoRI fragments			
	Pulse labeled origin		sDNA	MW
	0–1 min	0–10 min		(X10^6 dalton)
Homologous to sDNA		(1)	1	7.2
		2	2	6.0
			3	4.9
	2	5	4	4.2
			5	3.9
			(6)	3.8
			(8)	2.9
		7	9	2.4
			(10)	2.3
	(4)	8	11	2.2
		9	12	1.9
		(12)	13	1.7
			14	1.4
		(13)	15	1.3
Total MW	4.2(6.4)	16.7(26.9)	37.1(46.1)	
Not homologous to sDNA		(3)		5.5
	(1)	4		4.6
	3	6	(7)	3.8
	(5)	10		1.8
	6	11		1.8
		(14)		1.3
	7	15		1.0
		(16)		1.0
	(8)	17		0.6
Total MW	6.6(13.6)	13.6(21.4)	(3.8)	

Table. Analysis of restriction enzyme fragments from S-complex and the pulse labeled origin. (Fragment numbers are the same as those in Fig. 1).

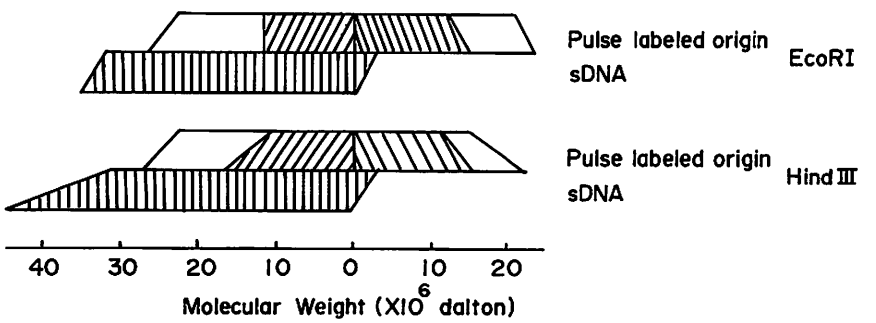

Figure 2. Schematic representation of assignment of the restriction enzyme fragments.
Shaded area of the pulse labeled origin represents the fragments labeled for 1 min and the open area represents those labeled from 1 to 10 min after the initiation began.

(30) The arrangement of DNA sequence near the replication origin of the *Bacillus subtilis* chromosome.

N. Ogasawara, M. Seiki and H. Yoshikawa

Replication origin of the *B. subtilis* chromosome is located between two genetic loci, *pur*A and *ts*8132. We attempted to construct the restriction enzyme cleavage map of this region and to identify the exact location of the replication origin. For this purpose, restriction enzyme fragments from pulse labeled DNA at the initiation of replication were analysed.

The replication origin was labeled during synchronous initiation of replication by two methods, 1) germination of a thymine requiring mutant in the absence of thymine and 2) alignment of the chromosome using a temperature sensitive mutant defective in the initiation of DNA replication. Patterns of cleavage by various restriction enzymes of the labeled origin were analysed by fluorography after electrophoresis in agarose gel. When ^{3}H-bromouracil was used in place of ^{3}H-thymidine, labeled fragments were recovered in hybrid and heavy density regions in CsCl equilibrium density gradient centrifugation and gave the same patterns as those of ^{3}H-thymidine labeled DNA. These results show that the label was incorporated into newly replicated DNA at the origin and a random incorporation by repair processes was negligible if any.

In order to determine the replication order of these fragments we examined the digestion patterns of the DNA labeled for a different period of time. The comparison suggested that BamHI bands No. 1, 8.0×10^{6} daltons, or No. 5, 3.4×10^{6} daltons, were the first to be replicated (Figure).

The addition of novobiocin, an inhibitor of DNA replication, allowed a limited amount of replication after the initiation. Fragments synthesized in the presence of the drug consisted of only the right half of the fragments synthesized in normal initiation. These results suggest that, in spite of the bidirectional replication, the origin region has an asymmetrical structure.

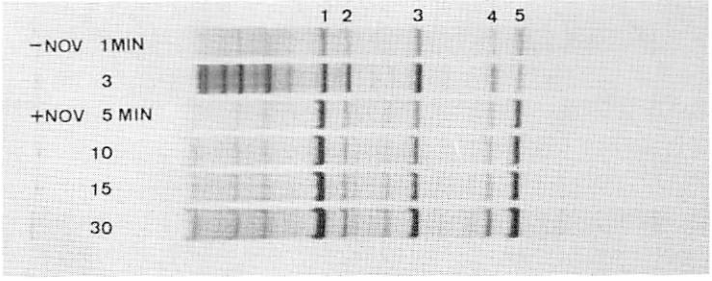

Figure. BamHI fragments from pulse labeled origin. Origin label was made by the method(1) for the period indicated in the figure with or without novobiocin.

(31) Proteins associated with the DNA located near the replication origin of the *Bacillus subtilis* chromosome.

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

The purified S-complex (see Abstract 29) contained proteins which were released from the complex either by high concentrations of salts or by various ionic detergents such as SDS and sarkosyl. When the complex was exposed to 0.5M KCl a drastic change in sedimentation rate was observed with each component of the complex. First, all DNA sedimented slowly with quite a homogeneous S-value of 30. On the other hand protein components were dissolved into two parts, one remained as large as 50-60 S (particulated proteins) and the other became completely solubilized into the supernatant fraction (soluble proteins).

We attempted to reconstruct the complex *in vitro* by mixing these three components in various combinations at high salt and then dialysing them against 0.1M KCl at 4°C. Figure shows that the addition of soluble protein fraction alone caused an increase in sedimentation rate of DNA. Acrylamide gel electrophoresis of the proteins reassociated with DNA *in vitro* revealed several discrete bands which were separated in the same gel. This indicates that these proteins are a minor component in the complex and have strong affinities to DNA. Native S-complex may be formed through interactions between proteins. Reassociation between proteins *in vitro* has not been reproduced yet. This partial reassociation of the complex was specific to the DNA from the S-complex, i.e. DNA near the replication origin (see Abstract 29).

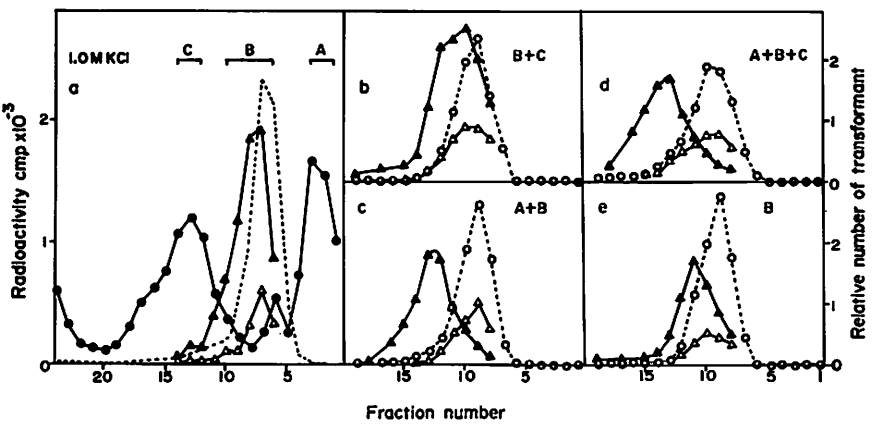

Figure. Reconstitution of DNA-protein complex containing *pur*A marker.

B. subtilis cells were uniformly labeled with ^{3}H-thymidine and ^{35}S-sulfric acid. The crude S-complex was centrifuged in 5–20% sucrose gradient at 25,000 rpm for 4.5 hours in high salt buffer (a). Dissociated soluble protein (A), particulated protein (C) and DNA (B) fractions were mixed in various combinations as in the text and recentrifuged in low salt (0.1M KCl) buffer ((b) to (e)). Symbols are; broken lines and O···O; ^{3}H activity in DNA, ●—●; ^{35}S activity in protein, ▲—▲; *pur*A transformants, △···△; *his*A transformants.

(32) Formation of DNA-protein complex near the replication origin during spore germination of *Bacillus subtilis*.

H. Yoshikawa, N. Ogasawara and M. Seiki

During spore germination protein and RNA synthesis occurred before the initiation of DNA replication began. We have shown previously that a sequence of protein and RNA synthesis is prerequisite to the formation of the cell's ability to initiate DNA replication. In order to examine biological function of the S-complex (see Abstract 29), especially in relation to the initiation of replication, formation of the complex was measured during the early phase of germination.

A thymine requiring mutant spore was labeled with ^{14}C-thymine and germinated in the absence of thymine to inhibit the onset of DNA replication. Formation of the complex was assayed by measuring increases in sedimentation rate of the origin marker, *pur*A, in comparison with other chromosomal markers or bulk ^{14}C-labeled DNA.

Figure shows that the complex was formed *de novo* during spore germination depending on protein synthesis. Association of the origin with the membrane occurred in the later time suggesting that the S-complex is a precursor for the membrane bound state of the origin. DNA replication was initiated for a short period of time by the addition of a small amount of ^{3}H-thymidine of very high specific activity, and the distribution of the labeled DNA in the S-complex was determined. In contradiction to previous observations in vegetative cells, newly replicated DNA was not detected in the membrane fraction but was found in the S-complex region and in non-complexed DNA. This indicates that the complex was stable when the DNA in the complex was replicating. Organization of the replication origin itself immediately before the initiation is not clear at the moment.

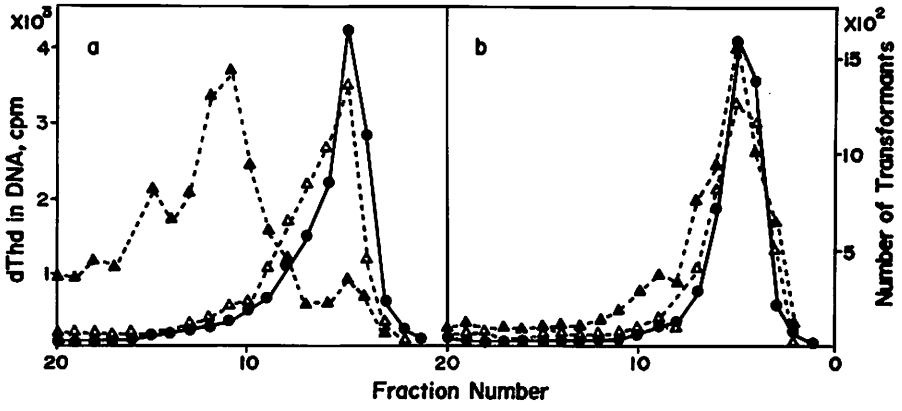

Figure. S-complex formation during spore germination.

Thymine requiring spores labeled with ^{14}C-thymine were prepared. They were germinated in the absence of thymine for 3 hours with (a) or without (b) required amino acids, leucine and tryptophan. Cell lysate was sheared and layered on top of the 5–20% sucrose gradient. Symbols are; ●—●: ^{14}C activity in DNA, ▲···▲: *pur*A transformants, △···△; *his*A transformants.

(33) Sequential replication of DNA during S-phase of HeLa cells.

S. Aoyama and H. Yoshikawa

Specific chromosomes such as X and Y chromosomes or specific chromosomal regions such as centromere are known to be replicated at a later period of the S-phase. Using synchronously growing cells, DNA labeled at the onset of a given S-phase was shown to be replicated at the early S-phase of the consecutive cell cycle. However no definite conclusion was drawn as to whether or not such a temporal sequence exists throughout the S-phase and in total DNA which is composed of several hundreds of replicons. In addition, unphysiological methods commonly used to synchronize DNA replication may affect the result. From these considerations we used HeLa cells and synchronized them by mechanical detachment of the mitotic cell populations. DNA was labeled uniformly for several generations with ^{3}H-thymidine and then pulse labeled with ^{14}C-thymidine for a short period of time. Immediately after the pulse labeling, cells entering into mitotic phase were collected sequentially in certain time intervals. Each cell population was then grown in the presence of 5-bromodeoxyuridine to measure the time of replication of the pulse labeled DNA. Figure 1 shows that the shorter the time between the pulse labeling and the mitotic shake off, the earlier the replication of the

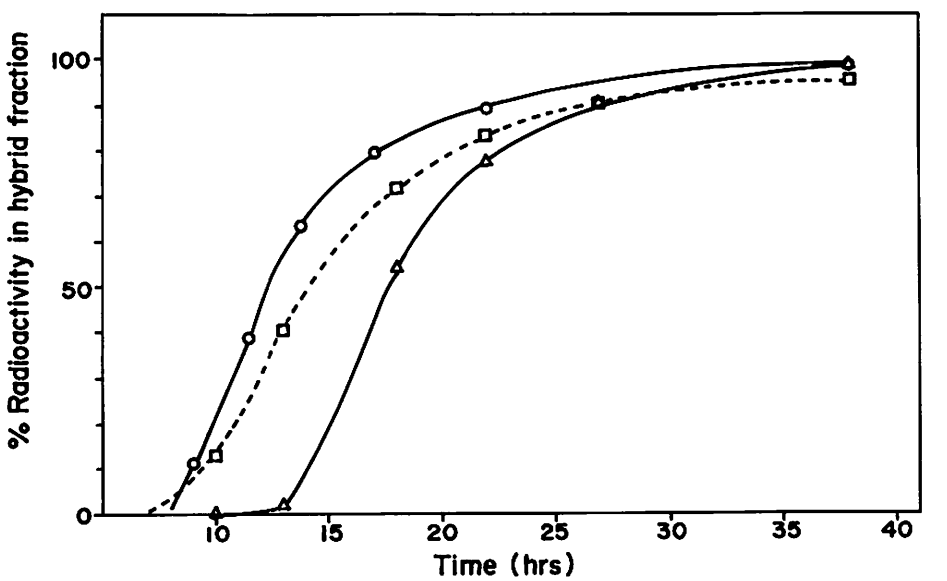

Figure 1. Replication of DNA, labeled in an exponential phase, during the following S-phase in the presence of 5-bromodeoxyuridine (5-BUdR).

HeLa S3 cells were grown and DNA was labeled as in the text. Mitotic cells were collected 1) 5–7 hours (△) and 2) 15–17 hours (○) after the pulse labeling. Each labeled cell was grown in the presence of 5-BUdR. At various times indicated samples were taken and DNA was analysed in CsCl equilibrium density gradient centrifugation. Radioactivities in hybrid DNA species (replicated DNA) were compared with those in original non-replicated DNA. Replication of ^{3}H labeled DNA (□···□) was used as an internal control.

pulse labeled DNA in the presence of 5-bromodeoxyuridine. This result indicates that DNA replicated at early, middle, or late in the S-phase during exponential growth was replicated again in the same sequence at the next cell cycle after the mitotic shake off.

This temporal sequence of DNA replication was also demonstrated by the second method in which cells collected by the mitotic shake off were synchronously grown for two consecutive generations. Cells uniformly labeled with ^{3}H-thymidine were synchronized and labeled for a short period of time with ^{14}C-thymidine at various times during the first S-phase. Each cell fraction labeled at different times was then grown into the second cell cycle in the presence of 5-bromodeoxyuridine. Figure 2 shows that the temporal sequence in the first cycle was completely reproduced in the second cell cycle. Together with the first experiment we conclude that DNA replication in HeLa cells maintained a definite temporal sequence for at least three generations.

Addition of hydroxyurea before the onset of the first replication cycle in the synchronous cells of the second experiment did not affect the temporal sequence indicating that the regulation of the replication sequence was not affected by the delay of the initiation due to the inhibition by hydroxyurea.

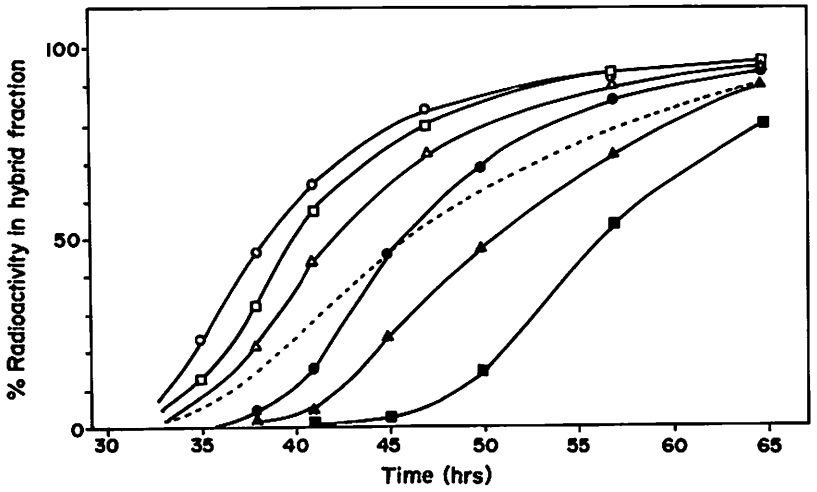

Figure 2. Replication of DNA during two consecutive S-phases in a synchronous cell culture.

Cells uniformly labeled with ^{3}H-thymidine were collected at mitotic phase as in the text (more than 95% homogeneous), and then labeled with ^{14}C-thymidine for 1 hour at 8–9 (○), 10.5–11.5 (□), 13–14 (△), 16–17 (●), 19–20 (▲) and 22–23 (■) hours after the onset of the first cell cycle. Each labeled population was added with 5-BUdR at the 30.5th hours and growth was continued. At times indicated after the addition of 5-BUdR, samples were taken and treated as Fig. 1. Replication of ^{3}H-DNA (○···○) was used as an internal marker for each sample and used to normalise them.

(34) Effect of an antimitotic drug, demecolcin, on the growth of *Saccharomyces cerevisiae.*

S. Murakami

In eukaryotic cells, the G1 period is most variable in the cell cycle, e.g. increases in the cell's doubling time under poor growth conditions are caused by the elongation of the G1 period. This period is composed of the two types: non-programmed state and programmed state. Therefore the switching mechanism or transition events from non-programmed to programmed state may play a key role in regulation of the cell cycle.

Considerable important progress has been made to elucidate the mechanism of the cell cycle in eukaryotic cells using the *Saccharomyces cerevisiae* system by genetic-biochemical approaches. The most conspicuous observation in this line is that duplication of mitotic apparatus precedes, and is prerequisite for DNA replication (entry to S period)[1]. In *S. cerevisiae*, the mitotic apparatus is composed of three types of microtubules and spindle plaque which is a kind of microtubule-organizing structure located on the nuclear membrane.

(A) In order to elucidate the role of mitotic apparatus in regulation of the cell cycle I examined effects of antimitotic drugs on macromolecular syntheses and cell proliferation of the yeast. Among antimitotic drugs which are known to inhibit mitosis by interacting with tubulin, griseofluvin, colchicine, nocodazole and vinblastin sulfate have no inhibitory effect on *S. cerevisiae*. Only demecolcin(colcemid) inhibited cell proliferation and DNA synthesis under restricted conditions. In the presence of high concentrations of demecolcin (above 10mM), an increase in cell number was inhibited. This inhibitory effect was not observed when either a diploid strain was used instead of a haploid strain or when haploid cells were growing exponentially.

When increase in cell number was inhibited by the drug during synchronous growth after the stationary phase, DNA synthesis was also severely inhibited, while RNA and protein synthesis were scarcely inhibited. The remaining amount of DNA synthesis in the presence of demecolcin suggested that it did not inhibit the first round of DNA synthesis. To examine this possibility, haploid cells were grown exponentially in a synthetic medium containing ^{14}C-uracil (10μCi/20μg/ml) and then the culture was diluted with the same medium containing demecolcin and ^{14}C-uracil (same specific activity as before). As shown in the Figure the relative increase in DNA content reached almost 80% of that found at the time of the dilution. Cell proliferation was completely blocked under this condition. These results indicate that demecolcin dose not affect the cell's entry into the S period in the first cell cycle even when cell division is completely inhibited. This conclusion is also supported by the microscopic observation that nearly 90% of the cells developed buds when they were grown for 3.5 hours in the presence of the drug.

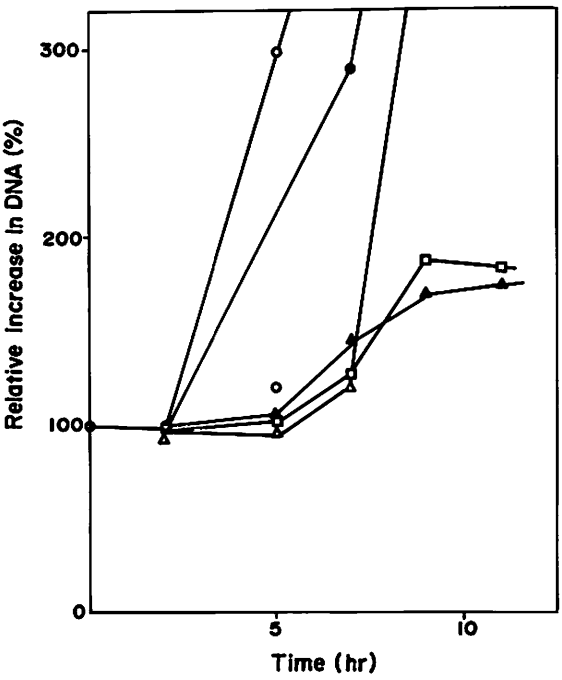

Figure. Effect of demecolcin on DNA synthesis of *S. cerevisiae*.

Methods were described in the text. Demecolcin was absent in the culture(O), or present at 1mM(●), 5mM(△), 10mM(▲) and 14mM(□).

(B) Two methods were used to isolate the conditional lethal mutant in which initial programming of the cell cyle was defective at the non-permissive temperature and sensitivity to demecolcin was altered.

1) Stationary cells of X2180-1A or A364A were mutagenized by methyl methane sulfonate and conditional lethal (no growth at 36°C or 20°C) mutants were selected by a replica method. One hundred and fifty four mutants were isolated from 5×10^4 surviving cells in 6 series of experiments. DNA synthesis and morphological changes of these mutants at non-permissive temperature were examined. Fourteen independent mutants were categorized as DNA-ts mutants in which DNA synthesis was arrested immediately after the temperature shift, or where increases in DNA content after the shift were within 40% of that at the time of the shift. In most of these mutants the percentage of unbudded large cells and/or large two cells increased after the shift. So far as it has been examined the sensitivity of these mutants to demecolcin were not altered.

2) Attempts to isolate hyper-sensitive mutants to demecolcin were not successful. Demecolcin-resistant mutants were isolated from the surviving cells which had been treated with 14mM demecolcin for three cycles during growth after the stationary state. More than 20 mutants which could initiate growth in the presence of the drug were isolated from the mutagenized cells. Due to a heavy mutagenization with nitrosoguanidine more than half of these mutants were ts or cs mutants. However sensitivity to the drug was not affected when revertants as to their sensitivity to the temperature were isolated. Properties of drug-resistant character and gentic analysis have not yet been examined.

1) Hartwell, L. H., Bacteriol. Rev., 38, 164 (1974).

(35) Identification and partial purification of tubulin protein of *Saccharomyces cerevisiae.*

S. Murakami

The mitotic apparatus is suggested to play an important role in the regulation of the initial events of the cell cycle in *Saccharomyces cerevisiae*[1]. Although systematic efforts through genetical and morphological approaches have been focused on this subject, only a few preliminary studies were carried out to elucidate the biochemical properties of the molecular entities of the mitotic apparatus in the yeast[2&3]. In order to identify and purify tubulin protein of the yeast, the major component of the mitotic spindle, capability of the yeast tubulin to interact with mammalian tubulin or anti-tubulin antibodies was examined by a copolymerization system and an affinity column chromatography.

^{35}S-labeled protein fraction was prepared from *S. cerevisiae* X2180-1A and was coassembled with tubulin of pig brain prepared by the procedures according to Shelanski[4] in the presence of 1mM GTP and 4M glycerol at 30°C, and was disassembled in the absence of glycerol at 4°C. After two cycles of the assembly-disassembly processes, two protein bands having molecular weights slightly smaller than 55K were specifically concentrated. Interestingly, another protein band near the high molecular weight components of pig microtubules was also co-purified by this procedure.

The chromatography on three different kinds of affinity chromatography was carried out. The pig tubulin, anti-pig tubulin antiserum and non-immunized serum were conjugated separately with CNBr-sepharose. The ^{35}S-labeled whole lysate of the yeast cell was centrifuged at 100,000 xg to get a supernatant fraction which was then applied to each of the three affinity columns. Protein adsorbed by the columns (5 to 8% of the total) was eluted in two steps, first by applying buffer containing 2M NaCl and then by 50mM of glycine-HCl buffer (pH 2.7). Eluted proteins were analyzed by the

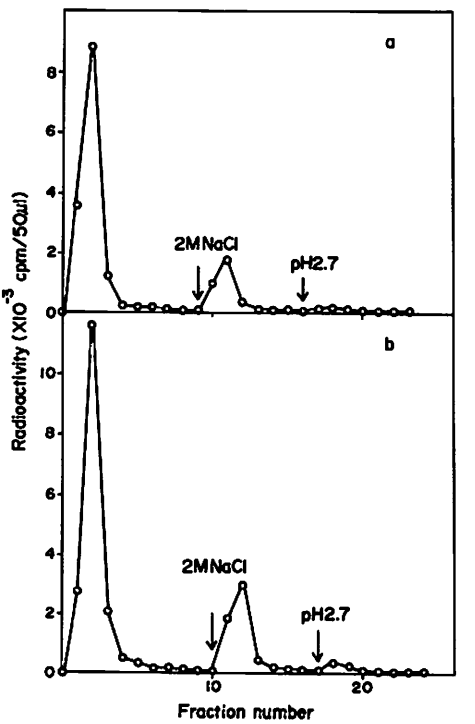

Figure 1. Elution profiles of affinity column chromatography.

Supernatant fraction of 100,000 × g centrifugation of ^{35}S-labeled yeast cell lysate was applied to pig tubulin sepharose column (a) or anti-pig tubulin antiserum sepharose column (b).

discontinuous SDS polyacrylamide gel and two dimensional gel electrophoresis after concentration by TCA and washing with acetone (see Abstract 28).

As shown in Figure 2, a protein band of 50 to 52K was not eluted in a flow through fraction and was eluted exclusively with 2M NaCl elution only from the tubulin-sepharose and anti-tubulin antiserum-sepharose column. This protein (tubulin) was also bound to the non-immunized serum-sepharose column with low efficiency by an unknown mechanism. Identification and partial purification of the yeast tubulin by these experiments strongly suggests that the structure and function of tubulin protein are highly conserved in these evolutinary diverged species.

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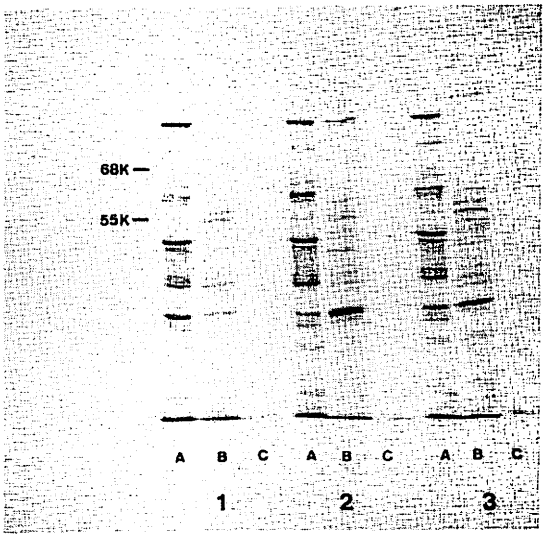

Figure 2. Separation of proteins by a discontinuous SDS gel electrophresis.

Flow through (a), 2M NaCl (b) and pH 2.7 fraction (c) of pig tubulin-sepharose (1), non-immunized serum-sepharose (2) and anti-pig tubulin antiserum-sepharose column (3) were applied to a discontinuous SDS gel.


TOTAL COUNT
SELECTED COUNT AREA
SELECTED COUNT
RECORD
CYTOFLUORIGRAF