The EMBO Journal vol.9 no.11 pp.3761 - 3765, 1990

Periodic formation of the *oriC* complex of *Escherichia coli*

Shinyo Gayama[1,2], Takao Kataoka[1], Masaaki Wachi[3], Gakuzo Tamura[1] and Kazuo Nagai[1,3]

[1]Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113 and [3]Department of Bioengineering, Tokyo Institute of Technology, Ookayama, Meguro-ku, Tokyo 152, Japan

[2]Present address: Laboratory of Mitsubishi Gas Chemical Co., Niigata, Niigata 950 Japan

Communicated by F.Jacob

We examined formation of an *oriC*−membrane complex through the chromosome replication cycle by dot-blot hybridization using an *oriC* plasmid as a probe. In a wild-type culture synchronized for chromosome replication, *oriC* complex formation was observed periodically and transiently corresponding to the replication initiation event. Prior to initiation of replication the *oriC* complex was recovered in the outer membrane fraction as well as at the time of initiation of replication. Moreover, periodic formation of the *oriC* complex was observed even when further initiation of replication was suppressed by culturing an initiation ts mutant at the restrictive temperature. Similar periodic formation of the *oriC* complex was also observed when DNA elongation was inhibited by addition of nalidixic acid to the culture. However, the second periodic peak did not appear when rifampicin or chloramphenicol was added. Cells which formed the *oriC* complex at the restrictive temperature could immediately initiate chromosome replication when the cells were transferred to the permissive temperature. We conclude that the *oriC* region of *Escherichia coli* forms a specific complex periodically just before and at the time of initiation of chromosome replication and that *oriC* complex formation is a prerequisite for initiation of chromosome replication.

Key words: biological clock/chromosome replication/DNA−membrane complex/*E.coli*/*oriC*

Introduction

Replication of the *Escherichia coli* chromosome is strictly regulated at the stage of initiation (von Meyenburg and Hansen, 1987). Several models have been proposed but the true mechanism of regulation is yet to be understood. Since participation of the cell membrane in chromosome replication was suggested by Jacob *et al.* (1963) in the replicon model, many studies have been undertaken to study the interaction between the membrane and the replicon (see reviews of Tomizawa and Selzer, 1979 and Firshein, 1989). In *E.coli* a complex which contains the replication origin of the chromosome (*oriC*) was isolated by Nagai *et al.* (1980) and further purified by Hendrickson *et al.* (1982). The complex, designated as *oriC* complex, showed a density close to that of the outer membrane fraction. Since this complex was isolated from the cells at the time of initiation of chromosome replication (Nagai *et al.*, 1980) and early replicative intermediates were detected by electron microscopy (Yoshimoto *et al.*, 1986a,b), it was suggested that the *oriC* region forms a complex at the time of initiation of chromosome replication probably at the junction of the inner and outer membrane (Bayer, 1968). These studies suggest that the *oriC*−membrane complex has an important role in the regulatory mechanism for initiation of chromosome replication.

In the present communication, we show that formation of the *oriC* complex was observed periodically and transiently and that the complex was formed not only at the time of initiation of chromosome replication but also just prior to it. It was also suggsted that *oriC* complex formation is a prerequisite for initiation of chromosome replication.

Results

Periodic formation of the oriC complex in synchronized cells

Since the *oriC*−membrane complex was recovered from cells when a temperature sensitive initiation mutant was synchronized for initiation of chromosome replication by temperature shift (Nagai *et al.*, 1980; Hendrickson *et al.*, 1982), but not from cells at an exponential phase of growth (Nagai *et al.*, 1980), it was expected that complex formation was transient and probably periodic. However, it was also possible that *oriC* complex formation was caused by temperature shift. Therefore we studied *oriC* complex formation in wild-type cells by a synchronization procedure other than temperature shift. In the present work, we adopted dot-blot hybridization using an *oriC* plasmid as a probe to detect the presence of the *oriC* region in the complex. By this procedure it was possible to assay formation of the *oriC* complex through the replication cycle. We studied *oriC* complex formation in the cells of W2252, which is wild-type in respect of DNA synthesis, synchronized by the stationary phase method of Cutler and Evans (1966). DNAs extracted from the washed membranes prepared from cells at various stages of the cell cycle were examined for the presence of the *oriC* DNA by dot-blot hybridization. The result is presented in Figure 1. Synchronized DNA synthesis was shown by incorporation of radioacive thymidine into acid insoluble products and the time points where half of the population initiated chromosome replication were at 20 and 60 min after transferring the cells to the fresh medium. The peaks of *oriC* complex formation were observed periodically at time points 10 and 50 min which were just prior to the initiation of chromosome replication. Since the cell number was doubled, the incorporation of [^{3}H]thymidine was expected to be twice the first round. The result, however, shows only a small increase. The reason for this discrepancy is not clear but it is partly because

© Oxford University Press

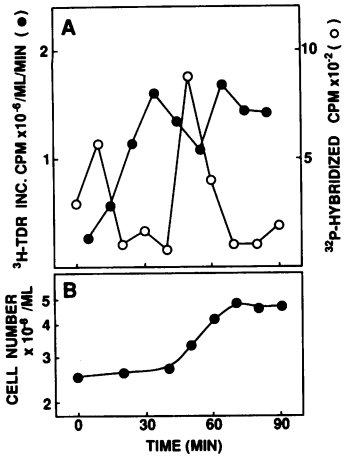

Fig. 1. Kinetics of *oriC* complex formation in W2252 cells synchronized by the stationary phase method. Time 0 is the time when the culture was shifted to the fresh medium. **A.** Cell DNA synthesis (●) and *oriC* complex formation in the washed membranes (○) were determined as described in Materials and methods. **B.** Cell number was counted microscopically. Doubling time during exponential growth was ~45 min.

synchronization was not complete in the second initiation of replication as suggested by broadening of the peak showing *oriC* complex formation. The stationary method was also applied to a temperature sensitive initiation mutant PC2 at the permissive temperature. However, we did not succeed in preparation of well synchronized cells of the strain.

Formation of the oriC complex prior to initiation of chromosome replication

In order to study more precise kinetics of *oriC* complex formation, that is, to examine whether the *oriC* complex was formed only just after initiation of replication, or if it was also formed just prior to initiation, we used a temperature sensitive initiation mutant to prepare cells at the stage just prior to initiation of chromosome replication as well as just after it. *E.coli* K12 strain PC2 (*dnaC2*) is a temperature sensitive mutant that will not initiate chromosome replication at the restrictive temperature (42°C). When the culture was kept at 42°C for about one generation and then shifted to 30°C, replication was initiated synchronously. In the previous report, using a synchronized culture of the strain, the *oriC* region specifically labeled with radioactive thymidine was shown to be enriched in the outer membrane fractions forming the complex (Nagai *et al.*, 1980).

Figure 2 shows the distribution patterns of *oriC* DNA in membranes prepared from cells at various stages of chromosome replication after fractionation by sucrose density gradient centrifugation. The outer membrane was distributed between fractions two to four and the inner membrane between fractions 11−13, as judged by the protein assay of Lowry *et al.* (1951) and SDS−PAGE. As expected from the results of Nagai *et al.* (1980), *oriC* DNA was detected primarily in fractions which corresponded to the outer membrane when the sample was prepared from cells at the time of initiation of chromosome replication (Figure 1D). Moreover, the same result was obtained using a sample prepared from cells at the stage just before initiation of replication (Figure 1C). These results indicate that the *oriC* complex is formed not only at the stage of replication initiation as reported previously but also just prior to

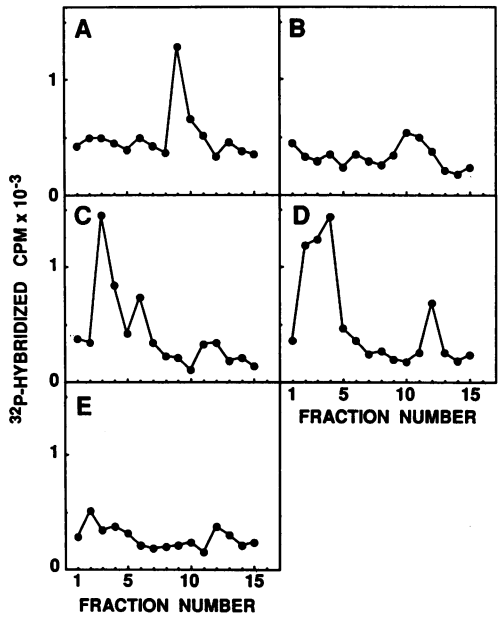

Fig. 2. *OriC* complex formation prior to initiation and at the time of initiation of chromosome replication in PC2 cells. The washed membranes were prepared from (**A**) cells at random growth at 30°C, (**B**) cells which were shifted to 42°C and cultured for 10 min, (**C**) cells which completed replication and were just prior to initiation after culturing for 60 min at 42°C, (**D**) cells which were shifted down to 30°C to allow initiation of chromosome replication synchronously and cultured for 3 min, and (**E**) cells which were cultured at 30°C for 30 min after temperature shift down, and were fractionated by sucrose density gradient centrifugation and the amount of *oriC* DNA was determined as described in Materials and methods. Doubling time at 30°C was ~55 min.

initiation. The *oriC* complex was not isolated from cells under other conditions examined here (see legend of Figure 2). The peak in Figure 2A, which does not coincide with either inner or outer membrane, was observed with poor reproducibility and was probably due to free DNA which was non-specifically sedimented with the membrane preparation.

OriC complex formation in PC2 at the restrictive temperature

Cells of PC2 which had been incubated at 42°C for 60 min were shifted to 30°C for 10 min and allowed to initiate chromosome replication synchronously. Then the culture was shifted back to 42°C to suppress further initiation of chromosome replication. Thus only one round of chromosome replication occurred. DNAs were extracted from the washed membranes at the times indicated and examined for the presence of the *oriC* region. The result is presented in Figure 3. *OriC* DNA was found to be significant in the sample prepared from cells just prior to initiation and reached a maximum when the cells initiated replication. As replication proceeded, the amount of *oriC* complex decreased and then increased again resulting in a peak 80 min after the temperature shift down. The latter *oriC* complex was presumably formed prior to the second round of initiation of chromosome replication. However, *oriC* complex formation was just transient and was not detected in the membrane fractions when the cells were harvested 110 min after the temperature shift down although there was no initiation of chromosome replication. Similar results were

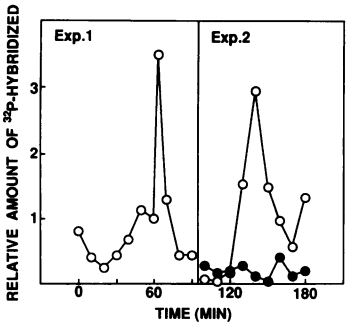

Fig. 3. Kinetics of *oriC* complex formation in PC2 cells. At time 0, cells were shifted up to 42°C and at 60 min the culture was shifted to 30°C to allow initiation of chromosome replication synchronously. At 70 min, the culture was shifted up to 42°C again to prevent further initiation. Washed membranes were prepared from cells at the times indicated and the amount of *oriC* DNA (○) and the *lac* region DNA (●) were determined as described in Materials and methods. The relative amount is shown in comparison with the amount of *oriC* DNA at 60 min (just before initiation).

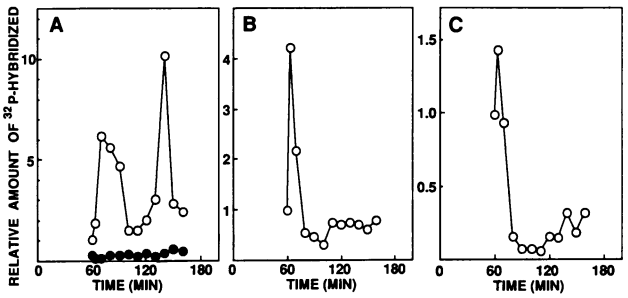

Fig. 4. Effects of inhibitors on *oriC* complex formation. Culture conditions were the same as in Figure 3 except nalidixic acid (100 μg/ml) (**A**), rifampicin (100 μg/ml) (**B**) or chloramphenicol (150 μg/ml) (**C**) was added to the culture at time 50 min. The amounts of *oriC* DNA (○) and *lac* region DNA (●) in the washed membranes were determined as described in Materials and methods. The relative amount is shown in comparison with the amount of the *oriC* DNA at 60 min (just before initiation). The arbitrary units are 415 c.p.m. in (A), 420 c.p.m. in (B) and 1615 c.p.m. in (C).

obtained with other temperature sensitive initiation mutants of *E.coli* N167 (*dnaA167*) and RS162 (*dnaB252*) (data not shown).

Specificity of DNA fragments in the oriC complex

When plasmid pMC1403 which carries the *lac* operon region of the chromosome was used as a probe, no significant amount of DNA from the membrane fraction was detected throughout the experiment (Figures 3 and 4A). Thus, DNA from the *lac* operon region does not form a complex such as the *oriC* complex. These results are in good agreement with previous observations (Nagai *et al.*, 1980) in which a significant amount of radioactivity incorporated into the *oriC* region but not into the whole chromosome was recovered in the complex. Therefore, chromosomal regions that form such a complex must be limited and the *oriC* region represents one of such specific loci.

Effect of inhibitors on oriC complex formation

We examined effects of inhibitors on *oriC* complex formation in cells cultured as described above. To block initiation as well as elongation, nalidixic acid (100 μg/ml) was added to

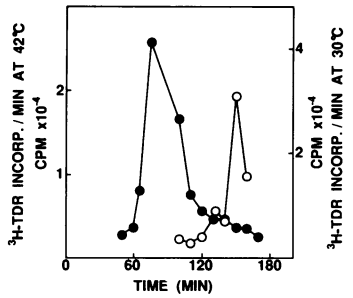

Fig. 5. Assay of initiation capacity of PC2 cells cultured at the restrictive temperature. Culture conditions were the same as in Figure 3. At the times indicated, a portion of the cells was assayed for DNA synthesis at 42°C (●) or at 30°C (○) as described in Materials and methods.

the culture 10 min before the shift from restrictive temperature to permissive temperature. However, there was slight incorporation of radioactive thymidine during the first several minutes (data not shown) suggesting that suppression of initiation was incomplete. Under this experimental condition, the first and second formation of the *oriC* complex were clearly observed although the width of the first peak was shown to increase as compared with that of the control experiment without nalidixic acid treatment (Figure 4A). It seems that the *oriC* region was retained in the complex for a longer period of time when initiation and/or elongation of DNA replication was affected by nalidixic acid. On the other hand, when rifampicin (100 μg/ml) or chloramphenicol (150 μg/ml) was added just before the temperature shift down, the cells were still able to initiate and complete one round of chromosome replication. Under this experimental condition, the first peak of *oriC* complex formation appeared and disappeared normally but the second peak was not detected significantly (Figures 4B and C). These results suggest that protein synthesis in the former replication cycle but not termination of replication was required for formation of the *oriC* complex for initiation of the latter replication. Apparent differences of the relative amounts of ^{32}P hybridized were mainly due to variability of the amounts of the *oriC* comlex formed just before initiation (60 min). The amounts of the *oriC* complex formed just after initiation (63 min) were almost constant.

The oriC complex and initiation capability

It is possible that formation of the *oriC* complex is required for initiation of chromosome replication to occur. Therefore, we examined the ability of PC2 cells cultured at 42°C to initiate chromosome replication within a short period when the cells were shifted back to 30°C. As shown in Figure 5, one cycle of chromosome replication was completed within 40 min as indicated by incorporation of radioactive thymidine in 1 min at 42°C. Thereafter no significant incorporation of radioactivity was observed suggesting that there was no further initiation of replication at this temperature. After completion of chromosome replication, a portion of the culture was shifted to 30°C so that the temperature sensitive DnaC protein could be reactivated. The cells were then allowed to incorporate [^{3}H]thymidine for 2 min. Only cells at 90 min after the temperature shift were significantly labeled during this period. This was about the time when formation of the *oriC* complex reached a maximum as shown in Figure 3 and incorporation was

probably due to initiation of a new round of chromosome replication. Therefore, only cells forming the *oriC* complex were ready to initiate chromosome replication immediately when the DnaC protein was activated. Thus it is suggested that formation of the *oriC* complex is a prerequisite for initiation of chromosome replication.

Discussion

In previous reports (Nagai *et al.*, 1980; Hendrickson *et al.*, 1982) it has been shown that a complex containing the *oriC* region and several proteins could be recovered from the cells when a *dnaC*^{ts} strain, PC2, was synchronized for initiation of chromosome replication by shifting back to the permissive temperature after being cultured at the restrictive temperature. The presence of early replicative intermediates of the chromosome and the *oriC* plasmid was revealed by direct electron microscopic examination of the complex (Yoshimoto *et al.*, 1986a,b). It was suggested that most of the chromosome would not be part of the complex because most of the radioactivity which had been evenly incorporated into whole chromosomal DNA was not recovered in the complex (Nagai *et al.*, 1980). Consistent with this earlier result, the *lac* region of the chromosome was not found significantly in the washed membrane fraction throughout the replication cycle. Thus, we conclude that the chromosomal regions in the complex are limited if any and the *oriC* region is one of such specific loci.

Applying dot-blot hybridization with an *oriC* plasmid as a probe, it was possible to study the time course of *oriC* complex formation throughout the replication cycle. Since the *oriC* complex was not detected in cells at an exponential phase of growth (Nagai *et al.*, 1980), it was expected that the complex formation was transient and it would be formed at the time of initiation of chromosome replication. The present results showed that a small but significant amount of *oriC* complex was detected in cells at the stage just prior to initiation and that the amount reached a maximum at the time of initiation. After this time the amount of complex decreased to the background levels. Complex formation again became obvious at the time corresponding to the second initiation round even when further initiation was suppressed. Moreover, a similar result was obtained when elongation of DNA synthesis was inhibited by nalidixic acid. These results suggest that the *oriC* region can form a complex without replication *in vivo*. As such they differ from observations made in an *in vitro* reconstitution experiment which showed that only hemimethylated *oriC* DNA could bind to the membrane (Ogden *et al.*, 1988).

Since cells which form the *oriC* complex at the restrictive temperature immediately initiated chromosome replication when shifted to the permissive temperature, cell components which are required for initiation of chromosome replication are likely to be assembled in the complex making an initiation complex 'initiosome' similar to the membrane complex of Laffan and Firshein (1987).

Since the complex ultimately disappeared in the absence of replication initiation, its capacity to initiate chromosome replication may be quickly lost (such as by inactivating components in the initiosome e.g. the *dnaC* gene product). In other words, the timing of initiation of chromosome replication could be regulated by a biological clock which triggers the formation and degradation of the *oriC* complex, the mechanism of which is yet to be elucidated. In this context, possible contributions of cardiolipin, a membrane phospholipid, to activation and inactivation of the DnaA protein which is dependent on complex formation with ATP or ADP (Sekimizu *et al.*, 1988a,b) may be of particular interest. The DnaA protein is known to bind selectively to the *dnaA* boxes in the *oriC* region (Fuller *et al.*, 1984; Matsui *et al.*, 1985) and activated DnaA protein is indispensable for initiation of replication at *oriC in vitro* (Sekimizu *et al.*, 1988a) and *in vivo* (Kohiyama *et al.*, 1966). Therefore it is possible that such modifications of the DnaA protein can take place on the membrane as reported by Yung and Kornberg (1988) and that activated DnaA protein triggers the construction of the *oriC* complex at a specific domain of the membrane. Novel protein synthesis but not DNA synthesis might then be necessary for this process of membrane activation.

The specificity of components which constitute the *oriC* complex will be determined by preparing the complex in pure form. Isolation and identification of the protein components in the complex are in progress in our laboratory. Such information should contribute to understanding the mechanism which determines the timing of initiation of chromosome replication in the cell cycle.

Materials and methods

Bacterial strains and growth media

E.coli K12 strain W2252 (*thy met*) was provided by T.Beppu. Strain PC2 (*dnaC2 leu thy str*) (Carl, 1970) was provided by Y.Hirota. Cells were grown in L-broth containing 1% Bacto-tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl and 0.1% glucose supplemented with 5 mg of thymine and 1 mg of thiamine per liter. For synchronization by the stationary phase method, cells of W2252 were grown in M9 medium supplemented with 0.4% glucose and 0.2% casamino acids.

Synchronization of cells

Strain W2252 was synchronized by the stationary phase method as described by Cutler and Evans (1966). For synchronization of PC2, cells were grown at 30°C in L-broth to OD_{550} of 0.2 and the culture was shifted to 42°C and incubated for 60 min. The culture was then shifted down to 30°C to allow initiation of chromosome replication synchronously. In order to prevent further initiation of replication, the culture was shifted up again to 42°C after culturing at 30°C for 10 min.

Preparation of the oriC DNA in the complex

Cells suspended in TKE1 buffer (20 mM Tris–HCl pH 7.8, 100 mM KCl, 1 mM EDTA) were broken by passing through a French pressure cell (Ohtake, Tokyo, Japan) at 700 kg/cm^2. After removing unbroken cells, membrane fractions were collected by ultracentrifugation (189 000 *g*, 45 min, 4°C) and the membrane fractions were washed with the same buffer. For fractionation of the outer and inner membranes, the crude membranes were layered on 10 ml of a 30–50% (w/w) linear sucrose gradient in TKE1 buffer and centrifuged at 160 000 *g* for 16 h at 4°C in a Hitachi RPS40T rotor. The washed membranes or fractions after sucrose density gradient centrifugation were treated with RNase A (100 μg/ml) for 30 min at 37°C and then with 1% SDS for 10 min at 65°C. DNAs were extracted by repeated treatment with phenol–chloroform and chloroform, respectively, and recovered by ethanol precipitation.

Dot-blot hybridization

DNAs were blotted onto a nitrocellulose filter after denaturation by boiling and alkali treatment as described by Maniatis *et al.* (1982). Hybridization was carried out at 66°C as described by Southern (1975). An *oriC* plasmid pTSO125 (Sugimoto *et al.*, 1979) was provided by M.Takanami. Plasmid pMC1403 (Casadaban *et al.*, 1980) was provided by K.Yoda. Plasmid DNA was labeled with [α-^{32}P]dCTP using a nick translation kit from Amersham. Hybridized radioactivity was measured in a liquid scintillation counter.

Assay of DNA synthesis

A portion of 0.5 ml of cultures were pulse labeled with 1 μCi of [^{3}H]thymidine for 1 min at 42°C or for 2 min at 30°C. The reaction was terminated by adding 0.5 ml of 10% trichloroacetic acid. Resulting

precipitates were collected on a glass fiber filter (0.45 μm) and washed first with 5% trichloroacetic acid and then with 1% acetic acid. Radioactivity was measured in a liquid scintillation counter.

Radioactive materials and reagents

[α-^{32}P]dCTP (410 Ci/mmol) was purchased from Amersham International plc, England and [^{3}H]thymidine (102.2 Ci/mmol) from New England Nuclear, Boston, MA, USA. Nick translation kit N5000 was purchased from Amersham. Other reagents were commercial products.

Acknowledgements

We thank Drs M.Schaechter and M.Yamasaki for their valuable discussions and interest in the present work. We are grateful to Dr W.Firshein for his critical reading and help in preparation of this manuscript and to Dr M.Takanami for the generous gift of an *oriC* plasmid pTSO125. This work was supported by research grants 01615003 and 01560117 from the Ministry of Education, Science and Culture, Japan.

References

Bayer,M.E. (1968) *J. Gen. Microbiol.*, **53**, 395–404.
Carl,P.L. (1970) *Mol. Gen. Genet.*, **109**, 107–122.
Casadaban,M.J., Chou,J. and Cohen,S.N. (1980) *J. Bacteriol.*, **143**, 971–980.
Cutler,R.G. and Evans,J.E. (1966) *J. Bacteriol.*, **91**, 469–476.
Firshein,W. (1989) *Annu. Rev. Microbiol.*, **43**, 89–120.
Fuller,R.S., Funnell,B.E. and Kornberg,A. (1984) *Cell*, **38**, 889–900.
Hendrickson,W.G., Kusano,T., Yamaki,H., Balakrishnan,R., King,M., Murchie,J. and Schaechter,M. (1982) *Cell*, **30**, 915–923.
Jacob,F., Brenner,S. and Cuzin,F. (1963) *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 329–348.
Kohiyama,M.D., Cuzin,A., Ryter,A. and Jacob,F. (1966) *Ann. Inst. Pasteur* (Paris), **110**, 465–486.
Laffan,J. and Firshein,W. (1987) *J. Bacteriol.*, **169**, 2819–2827.
Lowry,O.H., Rosenbrough,N.J., Farr,A.L. and Randall,R.J. (1951) *J. Biol. Chem.*, **193**, 265–275.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Matsui,M., Oka,A., Takanami,M., Yasuda,S. and Hirota,Y. (1985) *J. Mol. Biol.*, **184**, 529–533.
Nagai,K., Hendrickson,W., Balakrishnan,R., Yamaki,H., Boyd,D. and Schaechter,M. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 262–266.
Ogden,G.B., Pratt,M.J. and Schaechter,M. (1988) *Cell*, **54**, 127–135.
Sekimizu,K., Brumhill,D. and Kornberg,A. (1988a) *J. Biol. Chem.*, **263**, 7124–7130.
Sekimizu,K., Yung,S.Y. and Kornberg,A. (1988b) *J. Biol. Chem.*, **263**, 7136–7140.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Sugimoto,K., Oka,A., Sugisaki,H., Takanami,M., Nishimura,A., Yasuda,S. and Hirota,Y. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 575–579.
Tomizawa,J. and Selzer,G. (1979) *Annu. Rev. Biochem.*, **48**, 999–1034.
von Meyenburg,K. and Hansen,F.G. (1987) In Neidhardt,F.C. *et al.* (eds), *Escherichia coli and Salmonella typhimurium.* American Society for Microbiology, pp. 1555–1577.
Yasuda,S. and Hirota,Y. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5458–5462.
Yoshimoto,M., Kambe-Honjoh,H., Nagai,K. and Tamura,G. (1986a) *EMBO J.*, **5**, 787–791.
Yoshimoto,M., Nagai,K. and Tamura,G. (1986b) *Mol. Gen. Genet.*, **204**, 214–220.
Yung,B.Y. and Kornberg,A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7202–7205.

Received on June 5, 1990; revised on July 23, 1990