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THE SIGNIFICANCE OF MEMBRANE ALTERATIONS SEEN IN DNA SYNTHESIS MUTANTS OF *ESCHERICHIA COLI*

ANDRÉE LAZDUNSKI* and BENNETT M. SHAPIRO

Department of Biochemistry, University of Washington, Seattle, Wash. 98195 (U.S.A.)

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SUMMARY

1. This study was undertaken in an attempt to evaluate the significance of several alterations in membrane composition which occur when mutants of *Escherichia coli* which are temperature-sensitive with respect to chromosomal replication are placed at the restrictive temperature. Certain mutants affected in initiation (*dna*A mutants) or continuation (*dna*B mutants) of chromosomal replication have been found to have a deficiency of membrane protein of molecular weight approx. 60000 and an excess of membrane protein of molecular weight approx. 30000. These membrane alterations are seen only when the strains are kept at the restrictive temperature, are not specifically localized to the cytoplasmic membrane, and persist when the DNA synthesis defect is suppressed. Thus, these alterations appear to be direct consequences of the mutation, but are not necessarily directly related to DNA replication *per se*.

2. The mutants are deficient in another membrane protein fraction of molecular weight approx. 40000. This component is deficient in all of the DNA synthesis mutants which have been examined, is localized to the cytoplasmic membrane fraction and disappears upon suppression of the DNA synthesis mutation. Thus, this membrane alteration seems to be directly related to DNA replication, although it may be a secondary response. This conclusion supports that of Inouye (Inouye, M. (1972) *J. Mol. Biol.* 63, 597–600).

INTRODUCTION

Recent studies from this laboratory[1–3] and that of Inouye *et al.*[4–6] have independently demonstrated that alterations in membrane composition accompany defects in DNA synthesis. On the one hand, temperature-sensitive mutants affected in either the initiation[1] or continuation[2,4] of chromosomal replication have been found to have membrane protein deficiencies and, likewise, inhibition of DNA synthesis by other means elicits similar membrane changes[3,5]. However, the results from this laboratory and that of Inouye have differed as to the nature of the membranous change. We have found that in both initiation and replication mutants *i.e.* *dna*A[1] and *dna*B[2] mutants, respectively, there is a relative deficiency of a mem-

* Present address: C.N.R.S. Laboratoire de Chimie Bacterienne, 31 Chemin Joseph-Aiguier, 13 Marseille, 9e, France.

brane protein component of molecular weight of approx. 60000 (MP60)* and a relative excess of component molecular weight of approx. 30000 (MP30)*, in addition to other minor changes. Inhibitors of DNA synthesis have, in our hands, confirmed the results seen with DNA synthesis mutants. Inouye and Gutrie[4] have found, in another mutant affected in continuation of chromosomal replication, a deficiency of a component of molecular weight approx. 34000–44000. One of the *dna*B mutants we studied was phenotypically suppressed[2] by the addition of 2% NaCl to the growth medium, under which conditions the DNA synthesis defect was reversed and the strain grew normally. The suppressed *dna*B strain preserved the changes at MP30 and MP60 which had been seen when the mutation was expressed[2]. We concluded from these results that the membrane alteration was a direct result of the mutation and not secondary to inhibition of DNA synthesis, and that the DNA synthesis defect might have been secondary to the membrane alteration. In the mutant studied by Inouye the deficiency at MP40, (which he called Band Y) was obliterated upon phenotypic suppression by NaCl. Thus it seems from Inouye's data that the membrane alteration which he has seen is somehow linked directly to DNA replication, perhaps occurring as a secondary consequence of inhibition of replication. The relationship between the changes seen in the *dna*A and *dna*B mutants and DNA synthesis remains obscure.

To further test the significance of the alterations at MP60 and MP30 we have performed the experiments reported in this paper. First, we attempted to study a second type of suppression. Lindahl *et al.*[7] have shown that *dna*A mutants can be suppressed by integration of the genome of phage P2 into the bacterial chromosome. From their studies, it was thought that initiation of chromosomal replication is placed under control of the replicon for the P2 genome in these *Escherichia coli* initiator mutants. Consequently, it is of some interest to see whether such suppressed strains of *dna*A mutants retain the alterations of MP60 and MP30, as seen by us previously with salt-suppressed *dna*B mutants, and, similarly, whether the smaller change at MP40 which we see in some of our strains (and is the one Inouye and Guthrie reported[4,6]) is preserved after such phenotypic suppression. We have found that the changes in MP30 and MP60 persist and the one at MP40 becomes effaced with such integrative suppression.

In addition we have attempted to see whether the protein differences are found in the cytoplasmic or outer membranes of *E. coli*. The cell surface of *E. coli* is composed of an inner cytoplasmic membrane, peptidoglycan, and an outer membrane containing lipopolysaccharide which is often considered a component of the cell wall[8]. One might anticipate that changes in membrane composition directly related to DNA synthesis would tend to be associated with the cytoplasmic rather than the outer membrane, especially if such membrane alterations reflect a disturbance in a DNA–membrane complex required for initiation and/or replication. Our results suggest that the changes at MP60 and MP30 are equally distributed between cytoplasmic and outer membrane, whereas a more modest change, similar to that seen

* MP60 is used to refer to a membrane protein fraction which has a molecular weight approx. 60000. In this operational definition, nothing is implied about the number of molecular components which comprise MP60, nor even that it represents a single complex species. The terminology is used merely to facilitate reference to areas of the electrophoretograms being discussed. Similarly, MP30 refers to a membrane protein fraction of molecular weight 30000.

by Inouye, is associated with the cytoplasmic membrane preferentially. All our data are compatible with the alterations at MP60 and MP30 being primary effects of the mutations, but not necessarily related to the DNA synthesis defects *per se*. The significance of these findings is discussed below.

METHODS

Most of the methods employed in this study have been used in our previous experiments and are published[1–3]; thus, only a short summary of specific methods will be given below.

Bacterial strains

The following strains of *E. coli* K-12 were employed: CRT 4614 *thi thy leu* str^r lac_Y *dna*A; CRT 4615, a temperature-resistant derivative otherwise isogenic with CRT 4614; MX74 T2 *thy* and MX74 T2-*ts*27 containing a temperature-sensitive DNA synthesis mutation were gifts of Dr M. Inouye[2,3]; PA 335 *thi thr leu his arg ilv* lac_Y gal_d *mtl xyl mal ara* str^r λ^r Tl^r; CRT 4624 is similar to PA 335 but contains *dna*A ts_{46} and is also *ilv*+; and CRT 4624 P2 *sig* 5, similar to CRT 4624 but containing prophage P2 *sig* 5 integrated into the bacterial chromosome[7]. This strain is temperature-resistant for growth and does not express the initiation defect of *dna*A ts_{46} as described by Lindahl *et al.*[7]. The last three strains were generously given by Dr Yukinori Hirota.

Media

The medium used in all of the experiments was a minimal salts medium as described previously[1] but supplemented with 4 mg/ml of glucose and 2 mg/ml Casamino acids (Difco). Isotopes were L-[U-^{14}C]leucine and L-[4,5-^{3}H]leucine obtained from New England Nuclear, as was the [^{14}C]thymine used for the measurement of DNA synthesis.

Growth of radioactively labeled bacteria and preparation of membrane fractions

Logarithmically growing bacteria cultures were used in all experiments as previously described[1–3]. For temperature shifts, radioisotopes were added 5 min after elevation of the temperature to 41 °C unless described otherwise. In each case 60 μCi of [^{14}C]leucine and 300 μCi [^{3}H]leucine were used per 100 ml of bacteria culture. Unless specifically mentioned, cultures were labeled for 3 h and growth was stopped by pouring over crushed ice, followed by centrifugation and washing with 0.05 M Tris–HCl (pH 8.1) as described[1,2]. All other preparation steps were exactly as our previous papers, as is the full description of the state of purity of the membrane vesicles obtained.

Disaggregation, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and fractionation of gels

Conditions were as previously described[1,2] with electrophoresis being performed in 5% gels and 0.1% sodium dodecyl sulfate after prior disaggregation in 1% sodium dodecyl sulfate, all in 0.1 M sodium phosphate buffer (pH 7.1). Electrophoresis was at 7 mA per tube and continued until the tracking dye (bromo-

phenol blue) had travelled 9 cm. Gels were fractionated with the aid of an Autogel divider (Savant), the gel being eluted with 0.1% sodium dodecyl sulfate directly into scintillation vials, after which 10 ml of Instagel (Packard) emulsifier-scintillant was added to each vial.

Scintillation counting and data processing

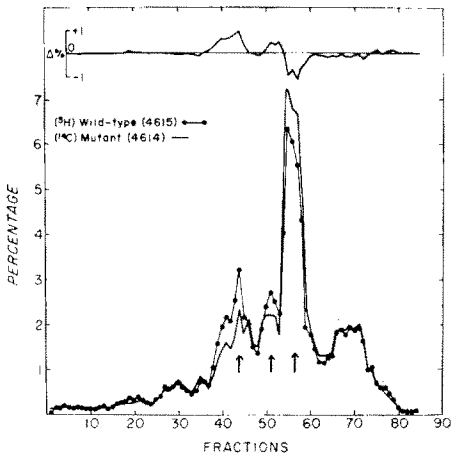
A Beckman Model LS 230 scintillation counter was used with window settings adjusted to keep the ^{3}H overlapping into the ^{14}C channel less than 1% and the ^{14}C spilled over into the ^{3}H channel around 6%. Under these conditions, efficiency for ^{3}H was approx. 30% and that for ^{14}C approx. 70%. The data were analyzed with an ALGOL modification of the program previously used.

Isolation and characterization of cytoplasmic membrane and outer membrane from E. coli

The *E. coli* membranes were resolved into three components designated L M and H as described by Osborn *et al.*[9]. The H (heavy) band is the outer membrane, L (light) band represents cytoplasmic membrane and M (middle) band represents unresolved material. Complete characterization of these three fractions has been made by Osborn[9]. We checked the purity of our preparations by measuring NADH oxidase activity (an index of inner cytoplasmic membrane) and lipopolysaccharide content (an index of outer membrane). These determinations were performed as described previously[9]. We are grateful to Dr Osborn for communicating her results to us prior to publication.

RESULTS

Fig. 1 illustrates the difference in membrane composition between two strains which are isogenic, with the exception that one of them carries the *dna*A mutation, making it unable to initiate DNA synthesis. The two major changes seen in the membrane composition are the differences at MP60 and MP30 which we previously reported[1]. In addition, there is a difference also in an area corresponding to MP40, which represents a deficiency in the membrane protein composition of the mutant strain. This material at MP40 is quantitatively less significant than the deficiency seen at MP60, but is the one corresponding to the deficiency seen by Inouye *et al.*[4,5]. Fig. 2 is an analysis of Inouye's mutant using our membrane preparation and our techniques. In this strain (as Inouye *et al.*[4,5] reported) there is no deficiency in the mutant at MP60 but only a deficiency in membrane protein at MP40. In addition the mutant has an excess of membranous material of molecular weight about 30000, when studied by our techniques. These differences are highly reproducible, as is shown in Table I. With the *dna*A strain we examined the deficiency at MP 60 accounts for some 3.5–4.5% of the total membrane protein. In contrast, the strain studied by Inouye has about 1.5–2% deficiency in MP40, no deficiency at MP60, and the excess of protein in the mutant at MP30 is from 1.5–3%, considerably more variable than that seen with the *dna*A strain. Although the data are not shown, we found (as Inouye has reported[6]) that the membrane protein compositional differences disappear in this strain upon phenotypic suppression



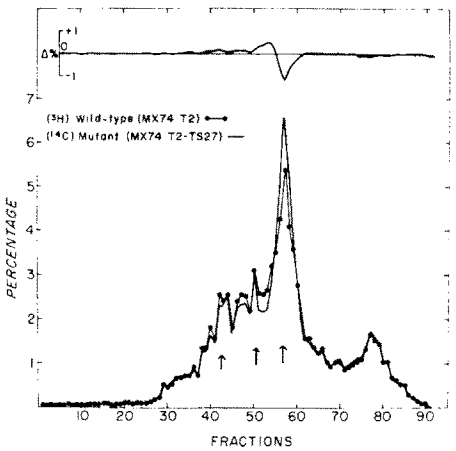


Fig. 1. Membrane protein composition of a *dna*A mutant at restrictive temperature. The mutant strain, CTR4614, was labeled with [^{14}C]leucine and the wild-type, CRT4615, with [^{3}H]leucine for 3 h growth at 41 °C. Membranes were purified and analyzed by gel electrophoresis as described in Methods. The graphs represent the percentage contribution of each fraction to the total radioactivity on the polyacrylamide gel; Δ% reflects the difference in radioactivity between the ^{3}H and ^{14}C contributions at each point, and thus is an index of the amount of altered material. Migration during electrophoresis was from left to right. In this and subsequent figures the positions MP60, MP40 and MP30 are indicated by arrows, positioned from left to right, respectively.

Fig. 2. Membrane protein composition of a temperature-sensitive DNA synthesis mutant. The strains were from Dr Inouye. MX74 T2, wild-type, was labeled with [^{3}H]leucine, and MX 74 T2-*ts*27, the mutant, was labeled with [^{14}C]leucine; the data are plotted as in Fig. 1.

TABLE I

QUANTITATION OF MEMBRANE PROTEIN ALTERATIONS

The difference in radioactivity seen at each of the peaks is obtained by integrating the area under difference peaks seen in experiments such as in Figs 1 and 2.

Strains	*Difference in radioactivity* (%) *seen at peak*		
	MP60	*MP40*	*MP30*
(A) Wild-type (4615) and a *dna*A mutant (4614)	4.6	1.4	3.8
	3.8	1.1	2.7
	3.6	1.3	2.6
	3.4	1.0	2.9
Mean value:	3.9	1.2	3.0
(B) Wild-type (MX74 T2)	0	1.5	1.5
and mutant (MX74 T2-*ts*27)	0	1.7	2.0
	0	1.8	3.2
Mean value:		1.7	2.3

by 2% NaCl; this is in contrast to what we saw with the *dna*B mutant that we had phenotypically suppressed in a similar fashion[2].

Suppression of the dnaA mutation

We have previously reported that the changes in MP30 and MP60 persisted in a phenotypically suppressed *dna*B mutant. We now have the opportunity to test

TABLE II

DNA SYNTHESIS IN A *dna*A STRAIN AFTER SUPPRESSION BY INTEGRATION OF PROPHAGE P2

Logarithmically growing cultures were incubated in $63B_1$ supplemented with 2 mg/ml Casamino acid (Difco). At the times shown, 1-ml samples were obtained and the incorporation of [^{14}C]thymidine (0.5 μCi; 0.223 mCi/mg) was measured during a 5-min pulse. The acid precipitable counts were measured by filtration onto Whatman GF/C filter paper followed by extensive washing with 5% trichloroacetic acid containing unlabeled thymidine (100 μg/ml).

Strains	*Rate of incorporation [cpm/absorbance of bacterial culture (× 10³)]*		
	30 °C	*Time after shift up at 41 °C*	
		1 h	*2.5 h*
Wild-type	23.4	57.8	48.4
Mutant	21.5	2.1	2.7
Suppressed mutant	18.3	55.5	50.4

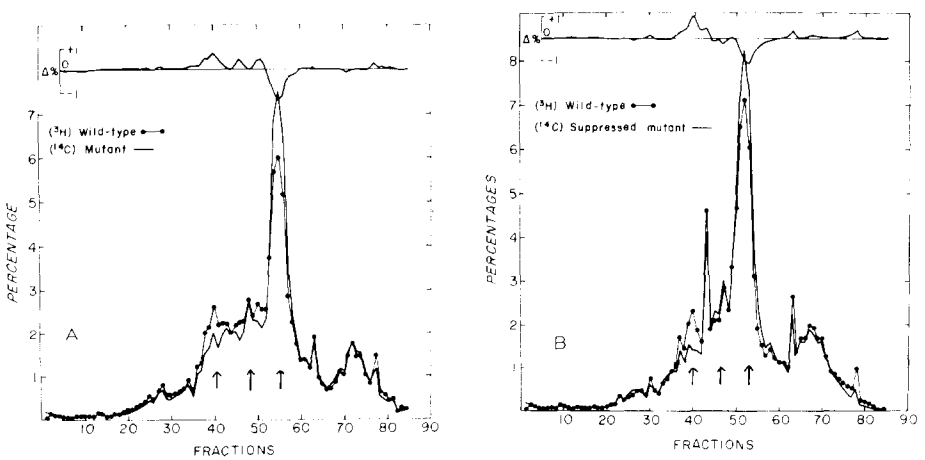


Fig. 3. Study of the suppression of the *dna*A mutation by insertion of P2 prophage into the chromosome. In both cases, growth was at 41 °C. Data were analyzed as in Fig. 1. (A) Membrane protein compositions of wild-type (PA 335) and the mutant CRT 4624 are compared. (B) Membrane protein composition of the wild-type (PA 335) and the mutant CRT 4626 (P2 *sig* 5) are compared.

whether this was also the case after suppression of a *dna*A strain. To do this we employed a strain containing the *dna*A mutation and a similar strain which has in addition the P2 prophage integrated into the *E. coli* genome. In the latter strain, the *dna*A mutation is not expressed, presumably because the lysogenic bacteria replicate under the control of prophage P2. Both of these strains were constructed by Drs Lindahl *et al.*[7] and were a gift from Dr Y. Hirota. In Table II is shown the rate of thymidine incorporation into DNA by the wild type, the *dna*A mutant, and the mutant which has been suppressed by insertion of the P2 prophage. As reported by Lindahl *et al.*[7], the rate of DNA synthesis in the suppressed mutant is identical to that in the wild-type strain, either 1 h or 2.5 h after temperature shift, and this rate is some 25 times that of the mutant. Fig. 3 shows the membrane protein composition of the wild type in comparison with the mutant, and with the mutant which has been phenotypically suppressed by insertion of the P2 prophage. Fig. 3A shows the characteristic difference seen between the wild type and mutant strains. These differences are the same as those seen in Fig. 1, when the same *dna*A mutation was present in a different genetic background. This is as previously described, the *dna*A mutation induced membrane alterations being independent of genetic background[1,2]. In Fig. 3B we see the comparison of membrane proteins from the wild type and the suppressed mutant. It is clear that the differences at MP60 and MP30 persist even upon such suppression. However, the peak at MP40 disappears in this suppressed mutant under conditions in which DNA synthesis is proceeding normally.

Localization of the membrane protein alterations

In order to see which of the differences in the *dna*A strain was localized to the cytoplasmic membrane and which to the outer membrane, we attempted to

TABLE III

ANALYSIS OF COMPONENTS OF CYTOPLASMIC AND OUTER MEMBRANE FRACTIONS

Membranes of the mixed, doubly labeled bacterial cultures used in Fig. 4 were fractionated and analysed for NADH oxidase and lipopolysaccharide as described by Osborn *et al.*[9]. The value for NADH oxidase in unfractionated membrane preparations was 1.9 μmoles/min per mg protein; that for lipopolysaccharide was 0.22 mg/mg protein. Thus, the cytoplasmic membrane is purified 2–3-fold over mixed membranes and is contaminated with some 10–30% of outer membrane, assuming that NADH oxidase and lipopolysaccharide are good markers for cytoplasmic and outer membranes, respectively. The validity of these assumptions has been previously discussed[9]. The purification of cytoplasmic and outer membranes, in our hands, has been achieved with variable success; the results in this table are of an average preparation.

Fraction	*NADH oxidase (μmoles/min per mg protein)*	*Lipopoly-saccharide (mg/mg protein)*	*Difference in radioactivity (%) of peak*		
			MP60	*MP40*	*MP30*
Cytoplasmic membrane (Band L)	4.30	0.08	3.2	2.7	2.1
Outer membrane (Band H)	0.48	0.31	4.1	1.7	3.8

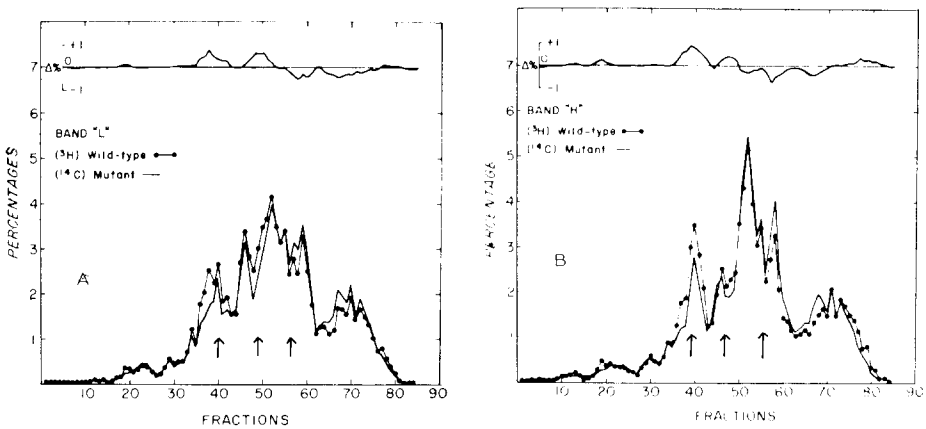


Fig. 4. Comparison of the protein composition of "inner" and "outer" membranes at restrictive temperature. The *dna*A mutant strain, CRT 4614 was labeled with [^{14}C]leucine and the wild-type, CRT 4615 with [^{3}H]leucine for 2 h growth at 41 °C. The culture was harvested, washed and mixed; the mixed, doubly labeled preparation was then treated as described by Osborn[9]. (A) Cytoplasmic membrane fraction (Band "L"); (B) Outer membrane fraction (Band "H").

separate inner and outer membranes by the isopycnic density centrifugation technique described by Osborn *et al.*[9]. Table III illustrates the purity of the two separated fractions. There is 10 times more NADH oxidase activity in the light band (corresponding to the cytoplasmic membrane) than in the heavy (outer) membrane band. Lipopolysaccharide is enriched some 3.3-fold in the outer membrane fraction. Thus, although our purification of these preparations was not absolute, the inner membrane is some 3–10 times purified away from outer membrane material. Fig. 4 shows the membrane protein composition of cytoplasmic and outer membrane preparations purified from a mixed *dna*A–wild type membrane preparation. The overall composition of membrane protein in the inner and outer bands differs, as was previously reported[9]. All three differences between mutant and wild type, discussed above, are seen in the cytoplasmic membrane fraction. Although there is no enrichment of MP60 and MP30 in this fraction (compare the data in Table III with the average value and the distribution of values seen for several experiments in Table I), there is a significant enrichment of MP40, the value being twice that seen for the mixed membrane preparations examined in Table I. Naturally, there is a degree of variation in these measurements, which are semiquantitative at best. Nonetheless, the difference at peak MP40 accounts for 2.7% of the radioactivity in the cytoplasmic membrane, whereas it accounts for between 1.0–1.4% of that in the mixed membrane preparation. This enrichment in MP40 is in agreement with that seen for NADH oxidase, which is used as a marker for the cytoplasmic membrane. Although the alterations in the outer membrane are more complex than usually seen, the membrane protein differences of interest are represented in the outer membrane to the same extent as in the mixed membrane preparation. Thus, a comparison of the data in Fig. 4 and Table III with that of Table I suggests that the only component clearly enriched for in this membrane fractionation is that at MP40, which seems to be associated with the cytoplasmic membrane.

Kinetics of appearance of the membrane alterations

Because of the results above, all of which suggested that the alteration of MP40 might be more directly linked to DNA replication than those at MP30 or MP60, we analyzed the kinetics of appearance of the three different peaks after a temperature shift from 30 to 41 °C. To do this the cultures were labeled with 0.5-h pulses either immediately upon temperature shift, after 1 h growth at the elevated temperature, or after 2 h growth at 41 °C. The results are shown in Fig. 5. When the pulse is given during the first 0.5 h after temperature shift (Fig. 5A) the difference at MP40 is more prominent than the differences at MP60 and MP30 and accounts for about 4% of the membrane protein. However, after 1 h growth at the restrictive temperature, a 0.5-h pulse shows membrane alterations (Fig. 5B) at the 3 loci identical to that seen for the 3-h labeling period (as in Fig. 1) The pulse after 2 h gave an identical protein pattern as that seen in Fig. 5B.

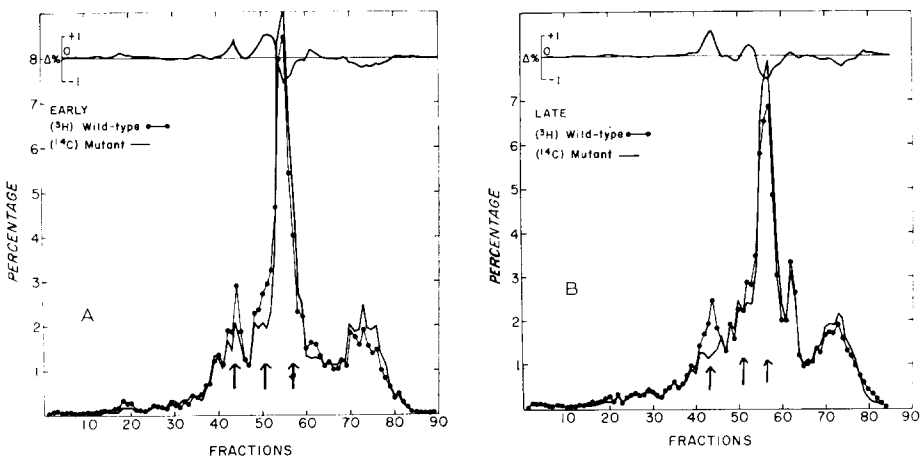


Fig. 5. Membrane protein synthesis at different times of growth at restrictive temperature. (A) Mutant and wild-type have been labeled during the first 0.5 h of growth at 41 °C. (B) Mutant and wild-type have been labeled by a 0.5-h pulse after 1 h growth at 41 °C.

DISCUSSION

The assignment of a specific role in DNA replication to any of the fractions which are altered in our experiments[1–3] is extremely difficult. We have extensively discussed in our previous publications many of the problems associated with the interpretation of such membrane alterations. This discussion will not be repeated here. However, the experiments in this paper do suggest that the membrane alteration at MP40, which corresponds to that reported by Inouye *et al.*[4,5] is more likely to be directly associated with DNA replication than those at MP60 and MP30. In the first place, the alteration is suppressed upon integrative suppression of the *dna*A strain examined in this paper, and was also seen to be suppressed when the strain of Inouye and Pardee[5] was phenotypically suppressed by a completely different mechanism. In the second place, the alteration at MP40 seems to be enriched in the cytoplasmic membrane fraction, which is what one might expect for a membrane

protein directly involved in DNA replication. Finally, this change is the first change seen upon elevation of the temperature in a temperature-sensitive *dna*A mutant.

Nonetheless, the changes at MP60 and MP30 seem to be a direct result of the temperature-sensitive mutation. First, the differences in MP30 and MP60 are not seen when either *dna*A or *dna*B mutants are compared with wild-type strains at 30 °C (see refs 1 and 2). These results were found with essentially isogenic strains, prepared by transduction, which decreases the possibility that other temperature-sensitive mutations were present in the *dna*A and *dna*B strains examined. Secondly, as we reported before for the *dna*B strain we previously examined[2], the membrane alterations persisted at MP30 and MP60 after phenotypic suppression by 2% NaCl. This phenomenon was seen when the mutation was present in two completely different genetic backgrounds. Similarly, after suppression of the *dna*A mutation by integration of prophage P2 the alterations at MP30 and MP60 persist as reported above. Thus, these changes are induced in the membranes of certain temperature-sensitive mutants in comparison with wild-type cells upon growth at the restrictive temperature. However, they do not seem to be directly related to DNA replication. Our data suggest that the *dna*A and *dna*B mutations may have pleiotropic effects, one of which is to alter membrane protein composition. The relationship of these alterations to the gene products of the *dna*A or *dna*B loci remains unclear. It is possible that mutations might be reflected in altered membrane assembly, in addition to altered DNA synthesis, and that only the latter be phenotypically suppressed. We are purifying the MP60 fraction in order to examine, by immunological methods, its role, if any, in the cell division cycle.

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