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*A POLYCISTRONIC MESSENGER RNA ASSOCIATED WITH β-GALACTOSIDASE INDUCTION**

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A polycistronic messenger RNA molecule is one which contains information for more than one polypeptide chain. This implies that during the process of protein synthesis, more than one protein can be assembled on the same polyribosomal structure. It is frequently assumed that the induction of enzymes in bacterial systems is carried out through the formation of a polycistronic messenger RNA since the addition of an inducer substance is associated with the coordinated production of several protein molecules.[1] Several laboratories have demonstrated the formation of large-molecular-weight RNA molecules associated with bacterial enzyme induction and this is consistent with the interpretation of a polycistronic messenger molecule.[1-5] However, these experiments do not prove that a polycistronic messenger molecule is actually used during protein synthesis. For example, in mammalian cells, some large RNA molecules are broken down into smaller units before they are actually utilized.[6] Thus a more direct test is needed to be certain that a messenger RNA is polycistronic. We have chosen to demonstrate this using the polyribosomes on which the actual synthesis of proteins is occurring.[7] β-Galactosidase induction in *E. coli* is associated with the formation of three enzymes, β-galactosidase, galactoside permease, and thiogalactoside transacetylase.[8] It is possible to detect the presence of nascent β-galactosidase molecules attached to ribosomes and polysomes.[9-11] The size of the polysome is related to the size of the messenger RNA. Organisms which have a genetic deletion in the permease-acetylase region have smaller β-galactosidase polysomes than those seen in the wild type. From this we infer that the messenger RNA is polycistronic in this case.

Materials and Methods.—The general method used for preparing polysomes from *E. coli* has been described previously.[11] It involves pelleting penicillin spheroplasts, gently resuspending, and

lysing with a nonionic detergent. Here we have prepared polysomes from 20 different *E. coli* strains and have found that preliminary experiments were necessary with each strain in order to optimize the polysome yield. A sensitive factor is the rate at which the spheroplast preparation is cooled. In the wild-type strain Hfr 3000, spheroplasts are cooled by immersing the flask in an ice bucket at 0°. In other strains, such as A381, the spheroplasts are poured over ice to obtain a greater polysomal yield. Preliminary experiments were carried out with each individual strain to find conditions most likely to optimize the yield. In view of this variability, it is possible that the polysomal pattern observed in some cases does not represent the distribution found in the cell *in vivo*. In our standard method we sediment 40 ml of the spheroplast preparation (2×10^8 cells per ml) in a centrifuge tube in the cold room, and the tube is left in an ice bath after decanting.[11] After adding the detergent in 0.5 ml, the pellet is resuspended with a glass rod by stirring very slowly. This usually takes about 3 min per tube. Rapid stirring at this stage has been shown to disrupt the polysomal patterns.

The sucrose gradient technique was standardized in order to obtain reproducible data on the sedimentation behavior of the polysomes. A 25.2-ml sucrose gradient (15–30%) was made as described previously.[11] Onto this was layered 0.7 ml of the clarified spheroplast lysate and the material was centrifuged in the model L Spinco centrifuge SW25 rotor at 25,000 rpm for approximately 150 min. In constitutive strains where the level of free β-galactosidase is high, β-galactosidase polysomes were sometimes obscured by the movement of free β-galactosidase down from the top of the gradient. It was found that this could be eliminated by a simple procedure. The material was spun at 25,000 rpm for 15 min and then the rotor was allowed to come to a halt without braking. The centrifuge tube was removed from the rotor and 1.7 ml of the solution was removed from the top. This procedure removed most of the free β-galactosidase. The volume of fluid was then replaced by adding first 1 ml of the 15% sucrose solution with its normal buffer and then 0.7 ml of the lysate buffer.[11] The material was then centrifuged at 25,000 rpm for an additional 135 min. Control experiments showed that under the conditions described above, approximately 30% of the polysomes remained but less than 5% of the β-galactosidase activity. Mutants which were y^- in the lactose region were irradiated with ultraviolet light using a 15-watt GE germicidal lamp at 30 cm for 1 min and y^+ revertants were isolated.[12] At a survival level of 10^{-4}, the reversion rate was 0.2%.

Results.—After induction, β-galactosidase activity can be seen associated with a discrete size of *E. coli* polysomes.[11] Previous work has shown that the distance which a polysome moves down a sucrose gradient is a function of the number of ribosomes associated with the polysome.[13] The first goal in this project was to study the reproducibility of the movement of the β-galactosidase polysome in a standard sucrose gradient. A typical analysis is shown in Figure 1*A*, in which sedimentation is to the left. The sharp peak in optical density (solid line) represents the 70*S* ribosomes which have moved a distance *a* down the gradient while the polysomes are further to the left. The dashed line representing β-galactosidase activity reaches a maximum near fraction 6 in the polysomes and then rises again near the top of the gradient due to the diffusion of free β-galactosidase. The dotted line shows the specific activity, i.e., the enzymatic activity per optical density unit. This curve has a maximum near fraction 3 which has traveled a distance *b* down the gradient. Two measures can be used for the migration of the β-galactoside polysome down the sucrose gradient, either the distance traveled by the peak of enzymatic activity (b') or the distance traveled by the peak of specific activity (b). Both of these distances have been measured relative to the distance (a) which the single 70*S* ribosomes have moved down the gradient. The figures list the value of the ratio b/a for the specific activity peak and tabular data are presented for both ratios b/a and b'/a.

Our experiments have shown that these ratios are constant over a wide variety of experimental conditions. For example, the effect of polysomal concentration is

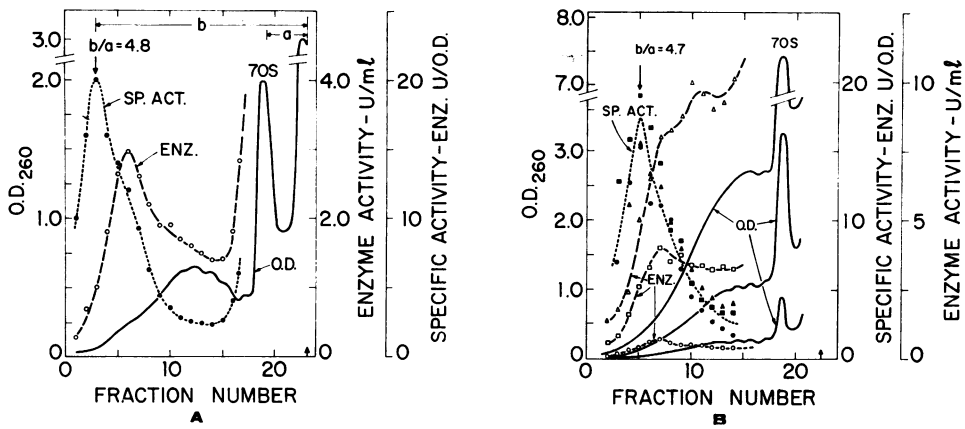

FIG. 1.—Sucrose density gradient of *E. coli* Hfr 3000 lysate after 10 min of induction with methyl-β-thiogalactopyranoside.[11] The lysate was prepared and centrifuged as described in *Methods*. Optical density at 260 mμ was continuously monitored in a Gilford spectrophotometer, and individual fractions were assayed for enzymatic activity.[11] Sedimentation is to the left, and the arrow at the bottom shows the position of the last fraction. (*A*) Definition of the ratio b/a. The related distance b' is less than b, and extends from the top of the gradient to the peak of enzymatic activity. (*B*) Cultures were induced for 30 min and then three different amounts of the lysate were centrifuged. Open symbols represent the enzymatic activity for the three different amounts of lysate. Closed symbols represent specific activities.

shown in Figure 1*B*. Here three different concentrations of lysate have been sedimented in the same rotor, and the enzymatic activity as well as optical density are indicated. The very high concentration of lysate does not show a well-characterized peak of enzymatic activity. Nonetheless, when the specific activity is plotted, as shown by the solid symbols, the peak values all cluster about the same dotted line with $b/a = 4.7$. Most analyses show a clear separation between the polysomal-bound enzymatic activity and the free galactosidase at the top of the gradient. This permits us to measure the distance traveled by the peak of polysomal-bound enzymatic activity (b') as well as the peak of specific activity (b). Lysates from the wild-type strain (Hfr 3000) were used as controls in each analysis of mutant strains. This allowed us to perform many measurements, and the results of 23 determinations are shown in Table 1. It can be seen that under these conditions the ratio b/a is 4.76 ± 0.12, so that within one standard deviation, the ratio is 4.6–4.9 for the wild-type organism.

Experiments were carried out to test the effect of other variables on the ratio b/a. Cells were grown at 23°, 30°, and 37°C and then lysed and analyzed on the sucrose gradient. The ratios obtained at these three different temperatures were 4.7, 4.9, and 4.6, respectively, all within the limits of b/a as shown in Table 1. Thus the ratio b/a appears independent of generation time as it varied from 200 to 60 min in these experiments. It should be noted that the ratio of β-galactosidase to thiogalactoside transacetylase remains constant in the strain Hfr 3000 when grown at different temperatures even though this is not true for all strains of *E. coli*.[14] Changes in physiological state were also brought about by altering the concentration of amino acids. For example, in the strain A381 the casamino acid concentration was varied from 1 to 0.2 per cent, which brought about a change in generation time from 60 to 80 min. Nonetheless, polysomal material prepared under these conditions showed the same ratio of b/a. Other experiments showed

TABLE 1

THE SIZE OF β-GALACTOSIDASE POLYSOMES IN VARIOUS STRAINS OF *E. coli*

Strain	Genotype*	Source	b/a			Remarks
1. Hfr 3000	$i^+z^+y^+$	Luria (from Hayes)	4.7 4.7 4.6 4.8 5.0 4.9 4.7 4.8	4.7 4.8 4.8 4.7 4.7 4.7 4.9 4.8	4.9 4.6 4.7 4.7 4.8 4.7 4.7	Wild-type strain always used as a control tube in experiments to determine b/a ratio of the strains listed below. Average of b/a $4.7_6 \pm 0.1_2$. Measurement of enzyme peak (b') gives ratio $b'/a = 4.3 \pm 0.3$.
2. W4032 P1d13300	$i^-z^+y^+$	Luria (from Lederberg)		4.8		i^- Has no effect on b/a.
3. W6115	$i^+z^+y^-$	Lederberg		4.6		
4. W3153	$i^+z^+y^-$	Lederberg		4.6		
5. W4005	$i^+z^+y^-$	Lederberg		5.0†		
6. W4019	$i^+z^+y^-$	Lederberg		4.7†		
7. W3174	$i^+z^+y^-$	Lederberg	4.7 4.6	4.7	4.7	This strain has not been resolved from W6203 by the available point mutations.
8. 200P	$i^+z^+y^-$	Luria (from Monod)		4.7		
9. 300U	$i^+z^+y^-$	Cohn (from Jacob)		4.7		
10. 300R	$i^+z^+y^-$	Cohn (from Jacob)		4.7		
11. W2001	$i^+z^+y^-$	Cohn (from Lederberg)	4.8	4.6		
12. A381	$i^-z^+y^-_{del}$	Luria	4.0 4.0 3.9	4.0† 3.8† 4.0†	3.8† 3.7† 4.0†	This mutant has a large deletion covering most of the y region, as described in reference 2. Measurement of peak of enzymatic activity (b') gives ratio $b'/a = 3.5 \pm 0.2$.
13. 2E01c	$i^-z^+y^-$	Alpers (from Monod)	4.3	4.4		This mutant is labeled y_1^- in reference 8. It is believed to be a deletion mutant but this is not certain.
14. W6203	$i^+z^+y^-$	Lederberg	4.3 4.2	4.3 4.2	4.1†	Value of enzymatic peak ratio b'/a is 3.8.
15. W6203r	$i^+z^+y^+$		4.8 4.6	4.8	4.6	Obtained by UV treatment of W6203 and isolation of y^+ revertant. Value of enzymatic peak ratio b'/a is 4.3.
16. 766-*y* amber	$i^+z^+y^-$	Zipser	3.9	3.7†		
17. 766r	$i^+z^+y^+$		4.7	4.5†		Obtained by UV treatment of 766-*y* amber and isolation of y^+ revertant.
18. L142-*y* amber	$i^+z^+y^-$	Zipser	4.3	4.0†		
19. L142r	$i^+z^+y^+$		4.8	4.6	4.9	Obtained by UV treatment of L142.
20. 707-*y* amber	$i^+z^+y^-$	Zipser	4.7			

* *y* Refers to the genes for thiogalactoside transacetylase as well as galactoside permease.
† Numbers marked with a dagger were judged to be less reliable since there was some breakdown in the polysomal distribution as seen in the optical density profile.

no change in this ratio as a function of time of induction.[11] In some preparations, the polysomal patterns appeared to be somewhat degraded as judged by the optical density curve. The changes in the pattern were probably associated with unknown variables in the preparative technique or in alterations associated with different strains. Despite this variability in optical density profile, the ratio b/a appeared to be constant. These experiments led us to believe that this ratio is a reliable index of the size of β-galactosidase polysomes.

A variety of mutant strains was obtained through the kind cooperation of several investigators, and the results of analyses are shown in Table 1. It was readily shown that the gene for inducibility, *i*, has no effect on the size of the β-galactosidase polysome. Most point mutants in the permease-acetylase (*y*) region do not alter the size of the β-galactosidase polysome, as shown by strains 3–11 in Table 1. However, the results are different if we look at the effect of large deletion mutations in the *y* region. Strain A381 has the genotype $i^-z^+y^-_{del}$ and has a large deletion which covers most of the *y* region.[2] The analysis of polysomal-bound β-galactosidase activity in this deletion mutant is shown in Figure 2 compared with the wild-type organism Hfr 3000. It is clear that a smaller β-galactosidase polysome exists in the deletion mutant. The ratio *b/a* has a value of 4.0 in contrast to the control value 4.9 for the wild-type Hfr 3000 (Fig. 2). The value of 4.0 for *b/a* is well outside the range of values seen in the experiments described above. Electron

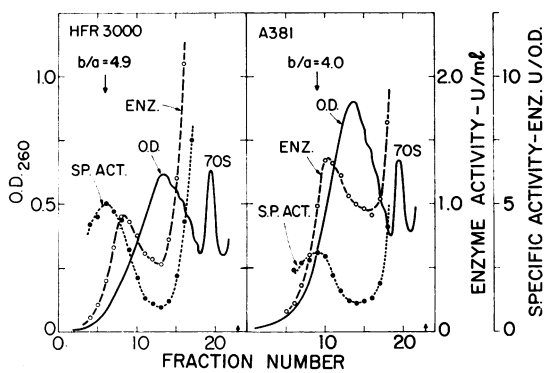

FIG. 2.—A comparison of parallel sucrose density gradient analyses of the wild-type organism Hfr 3000 and the *y* deletion mutant A381. Since A381 is a constitutive mutant with large amounts of β-galactosidase, the lysate at the top of the gradient was removed after 15 min of centrifugation from both strains, as described in *Methods*. This accounts for the fact that the 70*S* ribosome peak is smaller than normally seen. The deletion mutant has a β-galactosidase polysome with a smaller sedimentation constant.

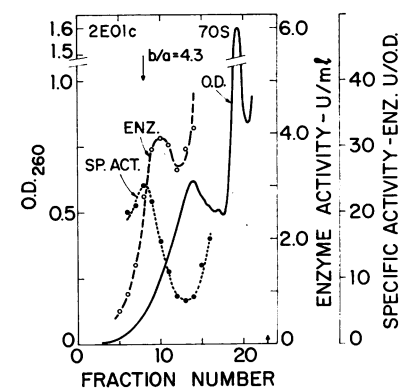

FIG. 3.—Sucrose density gradient analysis of strain 2E01c, a probable deletion mutant. The same procedure was used as described in Fig. 2.

microscopic studies of polysome fractions at the specific activity peak in the two cases show that the wild-type organism has polysomes containing approximately 50 ribosomes, while the peak in the deletion mutant A381 has polysomes containing about 30–35 ribosomes (unpublished observations). These figures should be taken as approximate, however, since the electron microscope examination of very large polysomes always shows many polysomal clusters which have been degraded. Another probable deletion mutant in the *y* region is the strain 2E01c, which has the polysome distribution shown in Figure 3, and the ratio *b/a* is 4.3 in this case.

The existence of smaller β-galactosidase polysomes is not confined to deletion mutants in the *y* region. In particular, there are certain classes of y^- organisms which are point mutants in the *y* region but nonetheless are characterized by smaller polysomes. The first case of this type which we studied was the strain W6203 (Fig. 4*A*). In this strain the ratio *b/a* is 4.3 which is smaller than that observed

with wild-type or other y^- mutants so that it acted as if it had a deletion in the y region. However, it is not a deletion mutant since ultraviolet radiation readily produced a large number of revertants to the genotype $i^+z^+y^+$, and the revertants had a normal polysome distribution with a ratio b/a of 4.8 (Fig. 4*B*). Thus, single mutational alterations in the permease-acetylase region are directly reflected in the size of the β-galactosidase polysome. In this case the modification in size was not related to the absence of a portion of the genome coding for y but was related to a modification of messenger RNA transcription or translation.

The work of Sarabhai *et al.*[15] on the amber mutants in the bacteriophage T4 showed that they were associated with incomplete polypeptide chain synthesis. This was most readily accounted for by a premature release of the ribosome from the messenger strand. Accordingly, it is of great interest to examine the polysomes of amber mutants in the y region. Dr. David Zipser kindly sent us three strains of *E. coli* which had amber mutations in the y region (Table 1, strains 16, 18, and 20). An

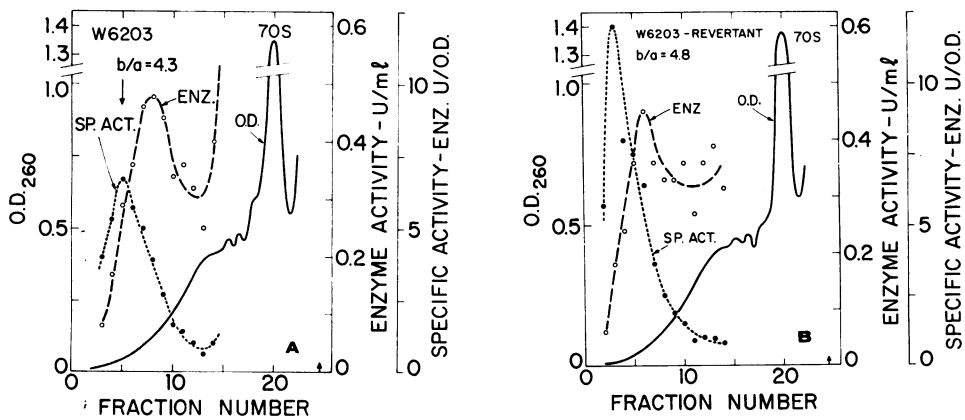

FIG. 4.—Density gradient analysis of strain W6203 with genotype $i^+z^+y^-$. Since the mutant was y^-, a 10^{-3} M solution of isopropylthiogalactopyranoside was used as an inducer for 30 min.[12] (*A*) Analysis of W6203. (*B*) Analysis of the y^+ revertant. The same inducer was used in (*A*) and (*B*).

analysis of the polysomal-bound β-galactosidase showed that two of these three mutants had ratios b/a that were substantially smaller than those observed in the wild type. Strain 766 had a ratio b/a of 3.9 while L142 had a ratio near 4.3. Strain 707 had a ratio 4.7 which is indistinguishable from that seen in the wild type and could occur if the mutation were near the end of the y region. y^+ Revertants from these amber mutants were obtained by irradiating with ultraviolet light. Revertants of strains 766 and L142 had wild-type polysomes with larger values of b/a.

Discussion.—The major finding reported here is that modifications of the genes for permease and acetylase in the lactose region are associated with changes in the size of the polysome which is manufacturing β-galactosidase. The size alterations are of two types, those associated with deletion mutants which irreversibly give rise to smaller β-galactosidase polysomes, and those found in point mutations, including the amber mutants which can be reverted to the wild type by ultraviolet irradiation. The reversion to the genetic wild type is associated with the reversion to the larger wild-type β-galactosidase polysome. The argument then for the poly-

cistronic nature of the messenger RNA during protein synthesis is that modifications in the permease-acetylase region are associated with changes in the size of the polysome on which β-galactosidase is being synthesized. This analysis is thus carried out directly on the structure in which protein synthesis is taking place.

It should be pointed out that the sensitivity in measuring polysome size by sedimentation on sucrose gradients is not very great, and small deletion mutants in the *y* region would be indistinguishable from point mutants. We would expect only larger deletion mutants to show a substantial change in the polysome size. This is in part related to the fact that the β-galactosidase is a large molecule with a molecular weight near 500,000 which is made of four subunits each of which is apparently composed of separate subcistrons.[16] In contrast to this, in the *y* region the enzyme thiogalactoside acetylase has a molecular weight of 64,000 and is made of two subunits.[14] The size of the β-galactosidase polysome is determined by many factors, one of which is the length of the messenger RNA which holds the ribosomes together. In hemoglobin synthesis, about five ribosomes are found on a messenger which is coding for a polypeptide of molecular weight near 17,000.[7] The ribosomes on the messenger have a gap between them on 50–150 Å, and similar interribosomal spacing has been seen in other mammalian cells.[13] Bacterial polysomes show roughly comparable spacings in the electron microscope but much more work will be necessary to characterize their ribosomal distributions. If we make an estimate that the β-galactoside messenger molecule has ribosomes on the average as close together as in the hemoglobin polysome, then we conclude that the galactoside permease molecule cannot have a large asymmetric unit, and it may be even smaller than the acetylase.

With a messenger RNA molecule of given length, other factors influence polysome size such as the rate of ribosomal attachment, detachment, and movement on the polysome. For polycistronic messenger molecules, these rates may differ in different cistrons. Zabin[14] has shown that in fully induced cells 8–10 times as many β-galactoside subunits are made as acetylase subunits. This suggests that ribosomes do not travel down the entire polycistronic messenger but come off, presumably at intercistronic markers. Ribosomes may also attach at such places, as is suggested from studies with viral RNA's.[13,17] Thus, different synthetic rates in the various cistrons of the messenger may be associated with differences in ribosomal packing and these effects might modify the over-all size of the polysome.

Another method of confirming the association of the β-galactosidase enzyme with nascent acetylase or permease on the same polysome is to look for direct evidence of these molecules. Only a small number of nascent protein molecules show enzymatic activity while they are still attached to polyribosomes; nonetheless, it was considered worth while to look for this activity. Accordingly, in collaboration with Dr. David Alpers, the assay was carried out using a very sensitive method.[18] No enzymatic activity could be detected on the polysomes of induced *E. coli*. However, it may be possible to use a different approach involving specific antibodies for acetylase in attempting this identification on polysomal material.

We have shown that certain classes of point mutations give rise to smaller polysomes. The amber mutations are believed to occur through the formation of a nucleotide triplet which brings about a premature detachment of the ribosome and a subsequent abridgment of polypeptide chain synthesis.[15] It is clear that experi-

ments could be carried out with a large number of amber mutants in the y region which might show a correlation between the size of the β-galactosidase polysome and the position of the amber mutations on the genetic map. It is interesting to note, however, that the point mutant W6203, which had a smaller β-galactosidase polysome than that seen in the wild-type organism, is insensitive to an amber suppressor. Dr. J. Beckwith has informed us that this strain already contains the suppressor C-600. It is likely that the polysome is shorter because of ribosomal detachment at the mutation site. However, further work must be carried out to determine the nature of the mutational change in this strain.

Summary.—The relative size of polyribosomes showing β-galactosidase activity has been measured for over 20 strains of *E. coli*. Organisms which have deletions in the acetylase-permease part of the lactose region have smaller polysomes than the wild type. Some point mutations, including amber mutants, also have smaller polysomes. Wild-type revertants of these mutants have the larger wild-type polysomes. It is concluded that the messenger RNA for β-galactoside induction is polycistronic during the translation process as well as in transcription, and contains information for making β-galactosidase as well as the other proteins of the lactose region.

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