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In Vivo Splicing of Protein: One Continuous Polypeptide from Two Independently Functioning Operons

(β-galactosidase/lactose operon)

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ABSTRACT **Active β-galactosidase (EC 3.2.1.23) is often formed in *rec*⁻ merozygotes containing a pair of mutations in the *z* gene of the *lac* operon. Contrary to expectation, with certain pairs of mutants this enzyme, upon dissociation, does not yield two independent polypeptides but only a single continuous protomer. Both genetic recombination and suppression have been ruled out as the source of this phenomenon. Therefore we believe we are observing the synthesis of one protein from two independently functioning genes.**

In this paper we demonstrate, using the lactose operon, that a single continuous polypeptide can be synthesized with information coming from two independently controlled, unconnected DNA sequences. Before describing our results it is necessary to present some background material on the enzyme β-galactosidase (EC 3.2.1.23) and alterations produced by mutations in its structural gene.

BACKGROUND

Native β-galactosidase of *Escherichia coli* consists of four identical subunits, each a polypeptide chain of molecular weight 135,000 (1, 2). These four protomers can be dissociated by various agents such as urea and sodium dodecyl sulfate. Urea dissociation is completely reversible; when β-galactosidase is inactivated and precipitated by boiling, the native structure can be reconstituted by dialyzing the boiled protein against urea (3, 4). The enzyme is stable within the bacterium (5).

The structural gene for β-galactosidase lies in the lactose operon, which has been extensively studied (6). Various mutations that inactivate the enzyme occur within the structural gene for β-galactosidase, i.e., the *z* gene. The mutations of primary interest to us are those that result in production of polypeptide fragments (7–9). Among these mutations are the "nonsense" mutations, which lead to premature termination of polypeptide synthesis. All nonsense mutations produce a polypeptide fragment extending from the normal N terminus to the point of the mutation. In addition, most nonsense mutants produce additional polypeptides starting at a reinitiation site within the gene and ending at the normal termination point (10–13). The quantity of reinitiation fragment produced differs widely from mutant to mutant.

For some nonsense mutants that are highly polar and produce small amounts of reinitiation fragment, it is possible to generate a new internal restart site that produces large amounts of reinitiation fragments by an additional mutation (10). An important feature of most termination and reinitiation fragments is that they are highly unstable and are rapidly degraded *in vivo* (5).

The points made above are illustrated by the data in Figs. 1 and 2. Fig. 1 is a map of the *z* gene of the *lac* operon. The operator and promoter region is to the left. Protein transcription goes from left to right, and the positions of the mutations of interest here are indicated on the map. Note that a "π" indicates the position of an internal restart; the name of that particular restart is in parentheses after the π. Numbers within parentheses after π refer to mutationally generated internal restarts. Greek letters within parentheses after π refer to naturally occurring restarts that have been identified.

Since both reinitiation and termination fragments are unstable, they can be detected only in pulse-label experiments. ^{14}C-labeled polypeptides from various mutants were separated according to molecular weight on Na dodecyl sulfate–polyacrylamide gels (Fig. 2). The figure illustrates the size and decay of various fragments.

By the use of episomes *E. coli* can be made diploid for a small region of its chromosome. This enables one to construct strains that have one *z*⁻ mutation on the chromosome and a different *z*⁻ mutation on the episome. These heterogenotes often produce active β-galactosidase, a phenomenon called intracistronic complementation (15–17). Complementation can also be demonstrated *in vitro*, with purified proteins (16, 17). It can even occur when one of the peptides is bound to a purified ribosome while the other is free in solution (18). Some complemented enzymes can be dissociated into the two mutant polypeptides and from this the belief has developed

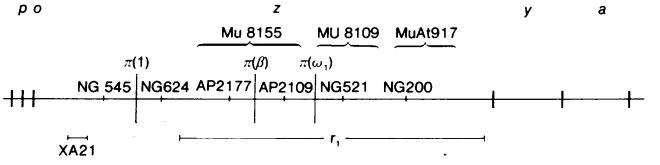

FIG. 1. Diagram of the *lac* operon. *o*, Operator; *z*, the structural gene for β-galactosidase; *y*, the structural gene for β-galactosidase permease; *a*, the structural gene for thiogalactoside transacetylase; $\pi(\beta)$ and $\pi(\omega_1)$, the naturally occurring sites of internal restart (12); $\pi(1)$, the mutationally generated restart site (10). Terminator mutations are shown above the line. The lines below indicate the extent of various deletions. Mutants used in these experiments have been described (29). The positions of Mu-induced *z*⁻ mutations have been mapped by deletions to within the indicated regions. However, the exact point of insertion has not been determined.

that no peptide bonds are formed during complementation. The quantity of complemented enzyme in diploids of mutants with unstable fragments is considerably greater than would be expected from the stability of the fragments. This result shows that formation of active enzyme by complementation stabilizes the input fragments.

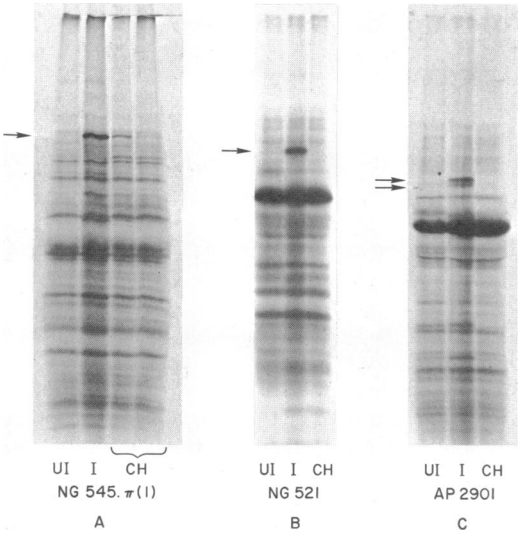

FIG. 2. Autoradiogram of Na dodecyl sulfate–polyacrylamide gel electrophoregram. Parallel cultures of F⁻NG545·π(1), F⁻NG521, and F⁻AP2901 were grown in the presence and in the absence of the inducer isopropyl-β-D-thiogalactoside at 37° in 50 ml of glycerol medium (per liter: 4 ml of 20% glycerol, 10 mg of tryptophan, 13.6 g of K_2HPO_4, 2 g of ammonium sulfate, 5.4 g of KOH, 1.5 mg of thiamine, 0.5 mM magnesium sulfate, adjusted to pH 7.6 with KOH). 0.25 ml of 0.1 M isopropylthiogalactoside was added to the induced culture. Growth was followed with a Klett spectrophotometer. At a Klett reading of about 100, the cultures were labeled for 4 min with 50 μCi of uniformly labeled L-[^{14}C]leucine (312 Ci/mol). A 10-ml sample was removed from the induced culture at the end of the labeling period and a 2000-fold excess of unlabeled leucine was added. 10-ml Samples were then removed at the end of 15 and 30 min from culture NG545·π(1). Only one sample was removed, at the end of 30 min, from cultures NG521 and AP2901. Uninduced cultures were sampled at the end of the 4-min labeling period. Over 70% of the label was incorporated into trichloroacetic acid-precipitable material. Cells from all samples were resuspended in 0.5 ml of TM buffer [10 mM Tris·HCl–1 mM Mg^{++} (pH 7.2)] and treated at 50% intensity for 30 sec in a Biosonik Sonicator. The extracts were centrifuged for 10 min at 10,000 × *g* and the pellet was discarded. 100 μg of purified β-galactosidase was added to all the samples, and the mixture remained at 4° for 10 min. 0.5 ml of antiserum against β-galactosidase was added to all the tubes. The tubes were mixed and allowed to stand at 4° for 2 hr. Antigen–antibody precipitates were collected by centrifugation, washed twice with TM buffer, and then dissolved in the sample buffer by heating for 10 min at 100°, to give a concentration of 10-30 μg/10 μl. 10-μl Samples were layered on 10% acrylamide-gel slabs, which were made and run according to Studier (19), with the discontinuous buffer system of Laemmli (20). The autoradiogram was exposed for about 4 days. *UI*, uninduced; *I*, induced; *CH*, chased. *Arrows* indicate the positions of the polypeptide fragments of interest. (*A*) π(1) restart fragment of 110,000 molecular weight. (*B*) 521 Nonsense fragment of 70,000 molecular weight. (*C*) AP2901 nonsense and $\pi(\omega_1)$ restart fragments (about 65,000 and 60,000 molecular weight, respectively). However, these have not been distinguished from one another. The samples taken at 15 and 30 min show the instability of these fragments. The prominent band in *B* and *C* is an unidentified crossreacting protein.

During our study of *z*-gene mutant polypeptides we wished to purify and prepare them in large quantities. We thus decided to produce heterogenotes between pairs of mutations, one of which would supply a termination fragment and the other an overlapping reinitiation fragment. We planned to prepare the complemented enzyme from these diploids and then dissociate it into its constituent polypeptides, hoping in this way to greatly increase our yield of unstable material. This was the motivation for the following experiments.

RESULTS

We constructed, in a *rec*⁻ background, heterogenotes between the nonsense mutants NG624, NG521, and NG200 on the chromosome and NG545·π(1) on the episome. All of these produced β-galactosidase activity. In order to study the nature of the polypeptides constituting the enzyme molecule, we prepared extracts from these cells, precipitated the β-galactosidase activity with antiserum, washed the antibody–antigen precipitate, and dissociated it in hot Na dodecyl sulfate buffer. This material was then placed on 10% Na dodecyl sulfate–polyacrylamide gels (19, 20). After electrophoresis the protein bands were stained with the dye Coomassie blue (Fig. 3). What we had expected to find for each complementing heterogenote were protein bands corresponding to the input polypeptides. However, we found that in two of the cases there was a band that appeared, not at the position of the input polypeptides, but at the position of wild-type protomer. Since Na dodecyl sulfate–polyacrylamide gels separate polypeptides solely on the basis of molecular weight, this result indicated that in some of the heterogenotes a process occurred that had led to the production of a polypeptide the same size as the wild-type protomer. When NG200 was used as the terminator mutation and NG545·π(1) as the restart mutation, a band smaller than the wild-type protomer appeared. This band contained NG200 nonsense fragment and probably the π(1) restart fragment, which have very nearly the same molecular weights.

We repeated this experiment with various combinations of mutations (Table 1). A wide variety of combinations, but of course not all, produce protomer of normal size. We were thus faced with the possibility that two independently synthesized polypeptide chains can join *in vivo* to produce a wild-type protomer. The following experiments were designed to test this possibility.

We have provisionally called the protomer of wild-type size produced in heterogenotes "spliced protomer." Enzyme containing the spliced protomer was thermostable whereas enzymes containing complementing fragments were thermolabile. The question we now wished to answer was, did the spliced protomer have the same C- and N-termini as native β-galactosidase?

In order to determine this, we purified the wild-type and spliced enzyme protomers by precipitating β-galactosidase from extracts of the appropriate cells with antibody against β-galactosidase, running the peptides on polyacrylamide gels, and eluting the band at the position of the wild-type protomer. The first two N-terminal amino acids of the spliced and native protomer, determined by a modification of Edman's technique (21), were the same (threonine-methionine) (21, 22).

The C-terminal was analyzed with carboxypeptidase B (22, 23). A short incubation with this enzyme was followed by precipitation of the polymeric product. The free amino acids were coupled with dansyl chloride and analyzed by chromatography. Four amino acids were found in both the wild-type and the spliced protomer—lysine, glutamine, cystine, and tyrosine, the ones expected from published studies. (The technique we used is not quantitative, and so no ordering information was obtained.) This result suggests that the spliced protomer has the same C terminus as the native protomer. This evidence left us with no doubt that the spliced protomer was a continuous polypeptide chain, similar to or identical with the wild-type protomer.

Our next experiment was designed to test whether formation of spliced protomer required both mutant operons to be functioning. We constructed strains in which one of the operons in the heterogenote contained an O^c mutation making it partially constitutive, whereas the other operon was under O^+ control and therefore totally repressible (Table 2). Neither of the mutant operons functioning alone under O^c control produced more than a trace of active β-galactosidase. A heterozygous condition with both operons functioning, that is, with the cell induced with isopropylthiogalactoside, was required for formation of large amounts of enzyme. This observation rules out the possibility of suppression of the mutations. Thus we are led to conclude that both gene products must in some way interact to produce the spliced protomer.

The ability to form a spliced protomer does not depend on the nonsense mutation itself, but on the particular pair of mutations. For example, NG200 forms spliced enzyme with $\pi(\omega)$ but not with $\pi(1)$ or $\pi(\beta)$. On the other hand, $\pi(1)$ and $\pi(\beta)$ produce reinitiation fragments which form spliced enzyme with many other nonsense mutants. This result suggests that details of protein structure enter into the decision as to whether to make spliced protomer.

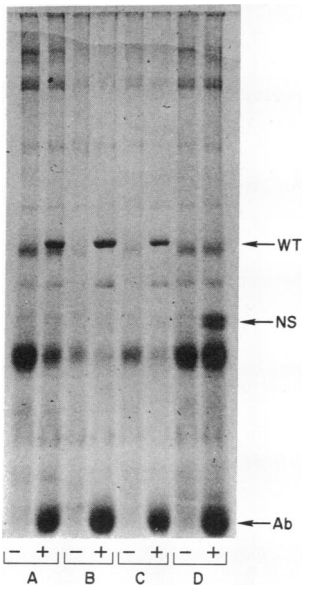

FIG. 3. Na dodecyl sulfate–polyacrylamide gel electrophoresis patterns of induced and uninduced cultures of "complementing" heterogenotes. Cultures grown overnight were diluted 1/20 in 5 ml of LB broth with and without isopropylthiogalactose. The cultures were incubated with shaking at 37° for 3 hr. They were centrifuged at 10,000 × *g* for 10 min, and the pellets were suspended in 0.5 ml of TM buffer [10 M Tris·HCl–1 mM Mg^{++} (pH 7.2)] and treated for 30 sec at 50% intensity in a Biosonik Sonicator. The extracts were centrifuged at 10,000 × *g* and the pellet was discarded. The supernatant fluids were mixed with 0.2 ml of antiserum against β-galactosidase and allowed to stand at 4° for 2 hr. Antigen–antibody precipitate was collected by centrifugation, washed twice with TM buffer, and finally dissolved in the Na dodecyl sulfate sample buffer by heating to 100° for 10 min, to give a concentration of 10–30 μg/10 μl. 10% Acrylamide gel slabs were made and run according to Studier (19), with the discontinuous buffer system of Laemmli (20). (−) and (+) indicate the absence or presence of isopropylthiogalactoside in the medium. (*A*) Wild-type F^-z^+; (*B*) F'NG545·π(1)/NG624; (*C*) F'NG545·π(1)/NG521; (*D*) F'NG545·π(1)/NH200. *WT*, β-galactosidase protomer of 135,000 molecular weight. *NS*, NG200 nonsense fragment of 110,000 molecular weight. *Ab*, heavy chain of the antibody molecule of 55,000 molecular weight. The prominent band below the band marked *NS* in all samples is an unidentified crossreacting protein.

TABLE 1. *Complementation and synthesis of spliced protomer in heterogenotes*

Strain		Enzyme activity	Protomer	Fragments	
Episome	Chromosome			N.S.	R.S.
NG545·π(1)	NG624	+	++	−	−
NG624	NG545·π(1)	+	++	−	−
NG545·π(1)	NG521	+	++	−	−
NG521	NG545·π(1)	+	++	−	−
NG545·π(1)	NG200	+	trace	+ (NG200)	N.D.
NG200	NG545·π(1)	+	trace	+ (NG200)	N.D.
NG545·π(1)	MuAT917	+	+	+	−
NG545·π(1)	Mu8109	+	++	N.D.	−
NG545·π(1)	Mu8155	+	+	N.D.	−
NG545·π(1)	AP2177·π(β)	+	++	−	−
AP2177·π(β)	NG545·π(1)	+	++	−	−
AP2177·π(β)	NG624	+	++	−	−
NG624	AP2177·π(β)	+	++	−	−
AP2177·π(β)	NG521	+	++	−	−
NG521	AP2177·π(β)	+	++	−	−
AP2177·π(β)	NG200	+	trace	+ (NG200)	N.D.
NG200	AP2177·π(β)	+	trace	+ (NG200)	N.D.
NG545·π(1)	AP2109·π(ω1)	+	++	−	−
AP2177·π(β)	AP109·π(ω1)	+	++	−	−
NG624	AP2109·π(ω1)	+	++	−	−
NG521	AP2109·π(ω1)	+	++	−	−
NG200	AP2109·π(ω1)	+	++	+ (NG200 & AR2109)	−
XA21	d545·r1	+	−	(XA21 deletion protein)	
NG624	XA21	+	−		

Strains were *rec*⁻ with the exception of MuAT917, Mu8109, and Mu8155. *Enzyme activity* column: "+" indicates the presence of β-galactosidase activity. *Fragments* column: "+" or "−" indicates the presence or absence of the nonsense (*N.S.*) or the restart (*R.S.*) fragment as seen on stained Na dodecyl sulfate–polyacrylamide gels; material in parentheses refers to the mutation donating the fragment; *N.D.* means not determined. d545·r1 is a double mutant. *Protomer* column: "++" indicates approximately the wild-type amount of the protomer; "+" indicates about 15–20%; "*trace*," less than 10% but a detectable amount of the wild-type protomer; "−" indicates absence of protomer.

TABLE 2. *Synthesis of β-galactosidase in homogenotes and heterogenotes*

Strain		Specific enzyme activity	
Episome	Chromosome	Uninduced	Induced
O^cNG521	O^cNG521	0.0024	0.06
O^cNG521	$NG545 \cdot \pi(1)$	0.0084	2.98
$O^cNG545 \cdot \pi(1)$	$O^cNG545 \cdot \pi(1)$	0.0068	0.12
$O^cNG545 \cdot \pi(1)$	NG521	0.015	4.09
	z^+	0.021	3.86

Cultures of the homogenotes and heterogenotes listed in the table were grown overnight and diluted $^1/_{20}$ in 5 ml of LB broth with and without isopropylthiogalactoside. The cultures were incubated with shaking at 37° for 2 hr. They were centrifuged at 10,000 × *g* for 10 min, and the pellets were suspended in 1 ml of TM [10 mM Tris·HCl-1 mM Mg^{++} (pH 7.2)] buffer. β-Galactosidase was assayed as described (3). A $^1/_{50}$ dilution of the suspension was used for measuring the absorbance at 550 nm. Specific enzyme activity is A_{420}/A_{550} × time.

Of particular importance is the formation of spliced enzyme in heterogenotes in which one mutation is a donor of a reinitiation fragment and the other is a phage Mu-induced mutation (24). It will be recalled that phage Mu inserts 25,000,000 daltons of its DNA into the *E. coli* chromosome, thus separating one end of a gene from the other. Table 1 also contains an example of a deletion mutation that complements well but does not form spliced protomer.

Mechanism

What is the mechanism by which this reaction takes place? DNA–DNA recombination has been ruled out as a mechanism, since splicing occurs in rec^- backgrounds, both z^- operons must function, and both are recoverable from the heterogenotes. Possibly splicing occurs by mRNA recombination. We can think of no plausible mechanism that would explain the mutant specificity for such recombination, and we consider an explanation of splicing based on mRNA to be a remote possibility.

Much more likely, we feel, is that splicing occurs at the level of polypeptide synthesis or between completed polypeptide chains. Here the tertiary structure of the native protomer would supply the information for specific joining. We have in mind two specific ways in which splicing could occur. The first is illustrated in Fig. 4. In this mechanism the polypeptides produced by the two z^- mutant operons are synthesized to completion. They then form a multimeric protein, which has nearly the tertiary structure of a normal protomer, but has excess polypeptides which hang out. Exopeptidases act on the multimeric protein producing a normal protomer with an accurate structure except for a single missing peptide bond. We envision that the exopeptidases would stop acting when the structure of a normal protomer is formed, since the ends would fit neatly into the structure and thus become unavailable for further proteolytic action. In Fig. 4*D* the single peptide bond has been formed by an enzyme which might be called "protein ligase." The mechanism described in Fig. 4 requires two kinds of enzymes for its successful completion, exopeptidases and protein ligase.

The mechanism shown in Fig. 5 also uses the tertiary structure of the native protomer as its source of specificity, but uses the normal polypeptide-synthesis system to form the peptide bond. In Fig. 5*A*, the restart fragment containing the normal C terminus has been completed. However, the nonsense terminator fragment containing the N terminus is still ribosome bound; it has not been synthesized up to the point where there is any overlap with the restart fragment.

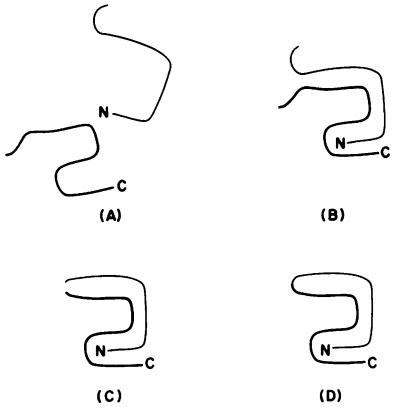

FIG. 4. Peptidase–ligase splicing mechanism: The reinitiation fragment, containing the normal C terminus, is *thickened* to distinguish it from the fragment that contains the normal N terminus. In this mechanism the polypeptides specified by the two z^- mutants are synthesized to completion (*A*). After completion of their synthesis, these two fragments form a multimeric protein (*B*). This protein has nearly the tertiary structure of a normal protomer but has excess polypeptides which hang out. (*C*) Result of the action of exopeptides on the structure of *B*. The structure of the normal protomer is now accurate except that a single peptide bond is missing. (*D*) The missing bond has been formed by an enzyme, "protein ligase."

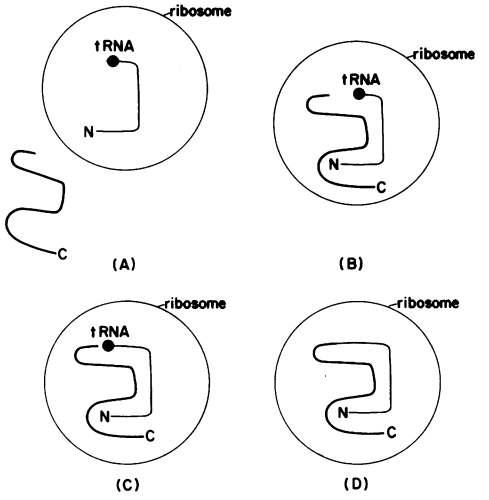

FIG. 5. Ribosome-associated splicing mechanism: (*A*) Reinitiation fragment containing the normal C terminus has been completed. The fragment containing the N terminus is still ribosome-bound and incomplete. In this situation there is no region of overlap between the two fragments. (*B*) Result of interaction between the reinitiation fragment and the nascent fragment on the ribosome. The structure formed has a gap which represents that part of the N-terminal fragment that has not yet been synthesized. (*C*) Synthesis has continued until all amino acids necessary for a native protomer are present. At this point only one peptide bond needs to be made in order to complete the native protomer. This is formed (*D*) by the normal polypeptide-synthesis system.

The restart fragment and the nascent growing termination fragment interact on a ribosome, forming the native protomer structure containing a gap, which represents that part of the N-terminal fragment that has not yet been synthesized. Synthesis continues until all the amino acids necessary for a native protomer are present. At this point only one peptide bond needs to be made in order to construct a native protomer. We conceive of this bond being formed by the normal polypeptide-synthesis system. The only difference from the usual situation is that the amino acid to which the bond is transferred is not held by a second tRNA but is the N terminus of the restart fragment fixed in position by the tertiary structure of the protomer. This mechanism using ribosome-bound interaction between the two polypeptide fragments has the feature that it requires no new enzymes. That interaction between fragments can occur on ribosomes has been demonstrated by the occurrence of complementation on ribosomes.

The data we have presented here show clearly that information from two unconnected, independently controlled copies of the *z* gene can end up in the same continuous polypeptide chain, without genetic recombination. We do not know what significance this splicing is to bacteria or other organisms. It is, however, reasonable to surmise that it will have some significance in various biological systems. One possibility is the synthesis of antibodies. A variable and a constant region, which may be specified by unlinked genes, end up in the same continuous polypeptide chain of immunoglobin (25–28). The system we have described in bacteria could possibly serve as a model for synthesis of immunoglobin. We are of course aware that some evidence interpreted against our hypothesis exists (30, 31).

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