POLARITY IN SEGMENTS OF THE *ESCHERICHIA COLI* *trp* OPERON WITH DELETED INTRAOPERONIC TRANSLATIONAL INITIATION SIGNALS

YASUNOBU KANO AND FUMIO IMAMOTO

*Department of Microbial Genetics, Research Institute for Microbial Diseases,
Osaka University, Yamada-kami, Suita, Osaka, Japan*

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ABSTRACT

The effect of deletion of the operator-distal genes of the *trp* operon, including the *trpE-trpD* intercistronic punctuation point, on the degree of transcriptional polarity (in this case the effect of a nonsense mutation on the level of mRNA from the distal part of the very gene where the mutation is located) was investigated. Double mutants which contain a nonsense mutation and a deletion in *trpE* were constructed, and the degree of transcriptional polarity was estimated by the decrease in messenger RNA for the operator-distal *trpE* beyond the nonsense mutation, as well as by the production of truncated messenger RNA for the region of *trpE* proximal to the nonsense mutation. The content of mRNA of operator-distal *trpE* and the size of the mRNA of operator-proximal *trpE* of the double mutants show that transcriptional polarity is not relaxed as a function of distance of the nonsense mutation from the operator-distal end of the *trpE* segment (at which the subsequent high efficiency translational initiation signal has been deleted). These findings are consistent with the conclusion that the degree of polarity depends on the distance of the nonsense mutation from the subsequent translation initiation signal, but not on its distance from the operator-distal end, including possible translational or transcriptional termination signals.

A nonsense mutation in one gene of an operon decreases the expression of all the operator-distal genes of the operon, in addition to resulting in inactivation of the product of the mutated gene. This phenomenon, called polarity (FRANKLIN and LURIA 1961; JACOB and MONOD 1961), has been observed for many bacterial operons that are transcribed into polycistronic messenger RNA molecules. The decrease in distal gene products correlates with the production of shortened mRNA molecules that are apparently terminated near the site of the nonsense mutation (CONTESSÉ, NAONO and GROS 1966; IMAMOTO and YANOF-SKY 1967a,b; IMAMOTO, KANO and TANI 1970). The decrease in mRNA levels is sufficient to explain the reduced expression of the distal genes (IMAMOTO and YANOF-SKY 1967a). The degree to which a nonsense mutation is polar has been observed to vary with its distance from an operator-distal cistron boundary. The closer the boundary, the less the polarity (NEWTON *et al.* 1965; NEWTON 1966; YANOF-SKY and ITO 1966; BAUERLE and MARGOLIN 1966; JORDAN and SAEDLER 1967; FINK and MARTIN 1967; BALBINDER *et al.* 1968; MARTIN and TALAL 1968;

BLUME, WEBER and BALBINDER 1968; YANOFSKY *et al.* 1971). Also, the polarity of a nonsense mutation in the *lac* operon decreases when a deletion mutation brings the nonsense mutation closer to the subsequent intercistronic boundary, but polarity is not affected when a deletion precedes the nonsense mutation (NEWTON 1966; ZIPSER and NEWTON 1967).

The critical feature of a cistron boundary in determining the degree of polarity is apparently the reinitiation site for protein synthesis. This can be inferred from the reduction of polarity when a new polypeptide reinitiation site is introduced by mutation close to but on the operator-distal side of a nonsense mutation (SARABHAI and BRENNER 1967; GRODZICKER and ZIPSER 1968). Also, the gradient of polarity in the *lacZ* gene is found to reveal peaks, corresponding to the position of sites that can serve as initiation sites when preceded in the map by a nonsense mutation (NEWTON 1969; ZIPSER *et al.* 1970).

However, it is unclear how the degree of polarity caused by a nonsense codon can be affected by a distal reinitiation site. Here we have asked whether the removal of the subsequent translational initiation signal affects polarity caused by a nonsense mutation. Is the degree to which a nonsense mutation is polar still determined by its position in the gene? In particular, we have studied the *trp* operon in *E. coli* with mutations in the first *trpE* gene, and asked how polarity is affected by deletion of the operator-distal *trpD*, *C*, *B* and *A* genes, including the *trpE-trpD* intercistronic punctuation point.

Polarity has been classically defined as the effect of a nonsense mutation in an operator-proximal gene on the expression of operator-distal genes. Here, we use the term "transcriptional polarity" to describe the effect of a nonsense mutation on the amount of mRNA from the operator-distal part of the very gene where the mutation is located. We report that transcriptional polarity is not significantly reduced by deletions, independent of the distance of the nonsense mutation from the operator-distal end of the remaining portion of the *E* gene. A preliminary account of some of these studies has appeared elsewhere (IMAMOTO, KANO and TANI 1970).

MATERIALS AND METHODS

Bacterial strains

The origin of the strains employed is W3110 of *Escherichia coli* K12, except for *trpA28*, whose original strain is CR63 (IMAMOTO and YANOFSKY 1967a). Tyrosine auxotrophic mutants of W3110 that have amber alterations in a *tyr* gene were isolated by UV-irradiation in this laboratory. All the *trp* auxotroph markers studied were introduced into the mutant, W3110 *tyr-am*, by transduction with P1kc (Figure 1).

Phage stocks

The following phages were used as DNA sources: $\phi 80$, the non-transducing parental phage, and non-defective transducing phages $\phi 80trpE$ and $\phi 80trpE_{distal}$. The *trp* operon segments carried by the $\phi 80trp$'s are shown in Figure 1.

Growth media

Cells were cultured either in L-broth (LENNOX 1955) or in minimal medium (VOGEL and BONNER 1956). T1 dilution fluid (6×10^{-4} M $MgSO_4$, 5×10^{-4} M $CaCl_2$, 1×10^{-3} % gelatin and 6×10^{-3} M Tris buffer, pH 7.3) was used to suspend the phages P1kc or $\phi 80$.

Genetic procedure

P1kc lysate preparation: About 10^6 phage were spread on L-broth agar plates (1.2% agar) with L-broth soft agar (0.4% agar) containing about 10^8 bacteria. After incubation for 12 hr at 37° , 5 ml of T1 dilution fluid was poured on the plate and the plate left in a cold room (4°) for more than 6 hr. The phage solution was centrifuged to remove bacterial debris. Chloroform was added to sterilize completely. These procedures were repeated more than twice using the same bacteria to get P1kc lysates for transduction experiments. P1kc which can grow on *GalU* mutants were prepared by the method described by FRANKLIN (1969) and used when *galU*⁻ strains were studied.

P1kc transduction: Bacteria cultured in L-broth to late log phase were centrifuged and suspended in T1 dilution fluid. After infection with P1kc at m.o.i. 1 to 4 for 25 min at 37° in the presence of 2.5×10^{-3} M CaCl_2 , cells were washed with T1 dilution fluid and spread on selective plates (LENNOX 1955).

Preparation of *trp*-double mutants: *trpE9829-KAE44* and *trpE9829-KAD05* double mutants were isolated from *W3110 tyr-am trpE9829* as *tonB*⁻ (resistant to colicin B and V) (GRATIA 1966). The loci of these deletion termini in the *trp* operon were mapped by P1kc transduction of *trp* missense, ochre and frame shift mutations. The *supD* transductants of the double mutants were used as recipients. The other double mutants were prepared by recombination using *W3110 tyr-am cysB*⁻ *galU*⁻ as recipient strain. The *trpE* point mutation was introduced into the recipient by a co-transduction with *cysB*⁺. *Trp* deletion mutations were then introduced into the recipient by co-transduction with *galU*⁺. *Cys*⁺ recombinants were selected on minimal agar plates containing 1% D-glucose, and 50 µg/ml of L-tryptophan and L-tyrosine. *GalU*⁺ recombinants were selected on eosin-methylene blue minimal agar plates supplemented with 1% D-galactose, 50 µg/ml L-tryptophan and L-tyrosine. On this plate, cells having *tonB-trp_{del}* mutations can grow slowly and therefore very small dark-red-colored colonies are *GalU*⁺ *trp_{del}* recombinants. About 100 to 800 *galU*⁺ *trp_{del}* recombinants were purified and tested for their indole requirement and P1kc sensitivity. These *trp* deletion strains, as recipients, were recombined with various kinds of *trpE* and *trpD* point mutants and *trpB*⁺ were selected. The ratio of *Trp*⁺/*TrpB*⁺ recombinants was calculated to confirm the presence of double mutations. These double mutants were also shown to have the original *trpE* point mutation by recovery of the point mutation in recombination studies with the donor wild-type strain.

Suppression tests: An amber suppressor (*supD*) was introduced into the *trp* mutants by P1kc grown on *W3110 tyr-am supD* prepared by DR. OZEKI. *Tyr*⁺ cells purified twice were streaked on λ-agar plates (1.0% polypeptone, 0.25% NaCl and 1.2% agar). Then, phage λ*susO_g* which bears an amber mutation in the *O* gene (CAMPBELL 1961) was spotted on the cells. Cells sensitive to λ*susO_g* were stocked as *supD* transductants.

Assay of mRNA synthesis

Phage DNA preparation: Lysates of $\phi 80$ and $\phi 80trp$'s were prepared by lytic growth on sensitive bacteria (*W1485*). After treatment of the lysate with DNase (0.1 µg/ml), the phage particles were concentrated by centrifugation and washed by 3 cycles of high- and low-speed centrifugation. T1 dilution fluid was used to suspend the phage. The final precipitate of phage was suspended at a concentration of about 2×10^{12} phage particles/ml in 0.1 M sodium phosphate buffer (pH 7.1) containing 0.1 M NaCl. Phage DNA was extracted by phenol treatment (KAISER and HOGNESS 1960) and dissolved in a standard saline-citrate solution (0.15 M NaCl-0.015 M sodium citrate) after precipitation with ethanol. When needed, the DNA solution was dialysed against the same buffer to remove contaminating phenol.

Preparation of pulse-labeled RNA: Bacteria were grown with aeration to 1.5×10^8 cells/ml in an enriched medium (L-broth) supplemented with L-tryptophan (50 µg/ml). In derepression studies, the cells were washed twice with cold minimal medium (without glucose), and suspended in the same medium to give a final density of about 2.5×10^{10} cells/ml. A portion (0.2 ml) of the cell suspension was transferred quickly to prewarmed (30°) minimal medium supplemented with 19 amino acids (each 0.5 mM) and glucose (1.0%), but without tryptophan, and the cell suspension was shaken vigorously in a 30-ml test tube in a water bath at 30° . L-tryptophan

(0.25 mM) was added in repression experiments. At a suitable time during incubation, the cell suspension (5 ml) was pulse-labeled with 100 to 500 μ Ci of tritiated uridine (15 to 24 Ci/m mole). After incubation with the isotopic compound, the suspension was rapidly poured onto 30 ml of crushed frozen medium, prepared just before use, containing 1×10^{-2} M Tris buffer at pH 7.3, 5×10^{-3} M MgCl_2 , 1×10^{-2} M NaN_3 , and 200 μ g/ml of chloramphenicol. RNA was prepared as described previously (IMAMOTO 1973).

Specific DNA-RNA hybridization technique: The hybridization assay was carried out essentially as described by NYGAARD and HALL (1963, 1964). DNA samples (100 μ g/ml) in a standard saline-citrate solution ($1 \times \text{SSC}$) were denatured by boiling for 15 min, then rapidly cooled to 0° . Excess heat-denatured DNA (5 μ g from a solution containing 100 μ g/ml) in $1 \times \text{SSC}$ and RNA sample in $1 \times \text{KCl-Tris}$ (0.5 M KCl-0.01 M Tris-HCl pH 7.3) were mixed in the ratio 1:4 in a final volume of 0.25 ml, incubated for 5 hr at 66° and then cooled slowly until they were below 40° . The mixtures were then transferred to 37° and incubated for 30 min with RNase (1 μ g/reaction mixture). Millipore filters were used for collecting DNA-RNA hybrids. Filters were washed with $1 \times \text{KCl-Tris}$ containing 5×10^{-4} % SDS at 50° . The filters were counted in a Beckman liquid scintillation spectrometer. Additional details of the assays for the mRNA specified by the *trp* operon have been presented elsewhere (IMAMOTO, MORIKAWA and SATO 1965). Results are reported as total radioactivity fixed on the filters and as the percentage of the total labeled RNA that is *trp*-mRNA. The average of duplicates is generally presented.

Sucrose density-gradient analysis: RNA preparations were sedimented in 5% to 30% linear sucrose gradients containing 0.01 M Tris buffer at pH 7.3 and 0.05 M KCl, for 5 or 6 hr at 36,000 rev/min in the cold in an SW39 rotor. After centrifugation the bottom of the tube was punctured and fractions were collected.

Reagents

[^3H]-uridine at 15 to 24 Ci/m mole was purchased from the Daiichi Chemical Company, Tokyo, and was used without the addition of carrier. Millipore filters, type HA (0.45- μ m pore size) were purchased from the Millipore Filter Company. DNase and RNase were obtained from the Worthington Biochemical Company. RNase was used after heating at 80° for 20 min in 0.15 M NaCl to inactivate any contaminating DNase.

RESULTS

In order to investigate how the degree of "transcriptional polarity" is affected by the length of the genetic region on the side operator-distal to the nonsense mutation site in strains in which intraoperonic translational initiation signals have been deleted, we employed sixteen double mutants, each of which retains a nonsense or a frameshift mutation in the operator-proximal portion of *trpE*, and a *tonB trp* deletion. Deletions *trpAD5* and *trpAD28*, which delete the operator-distal *trp* genes but retain *trpE* and the operator-proximal portion of *trpD* (including the translational initiation signal for *trpD*) intact, were used as control. Other deletion mutants, such as *trpAE11*, *trpAE10*, *trpAE9* and *trpKAE44*, retain only the operator-proximal portion of *trpE*. The exact map locations are indicated in Figure 1.

The *trpAE* deletion mutants produce *trp* mRNA molecules of reduced size, directly reflecting the location of the deletion termini in *trpE*; *trp* mRNA size varies in relation to the distance from the beginning of the *trpE* to the site of the *trp* deletion termini (IMAMOTO, KANO and TANI 1970). The majority of the *trp* mRNA molecules produced by strong polar mutants are deficient in the mRNA regions corresponding to the operon region beyond the site of the nonsense alter-

FIGURE 1.—Genetic map of the *trp* operon and relative polarity values in mutants. The order of the mutationally altered sites and the *trpA* protein polarity values (in parentheses) were based on previous studies (YANOFSKY and ITO 1966; IMAMOTO and YANOFSKY 1967a). The *trpA* protein polarity value is the ratio, given as percent, of *trpA* protein production by the mutant to *trpA* protein production by the wild-type control. *Trp* operon segments carried by the deletion mutants or $\phi 80trp$ are indicated by solid lines. The relative sizes of the *trp* genes and distances between the mutation sites are only approximate. Deletion mutants KAE44 and KAD05 were isolated by Y. KANO and F. IMAMOTO. Phages were isolated by A. MATSUSHIRO ($\phi 80$ and $\phi 80trpE_{distal}$) and by J. P. GRATIA ($\phi 80trpE$). The locations of the termini of *trp* segments in $\phi 80trpE$ and $\phi 80trpE_{distal}$ were based on a genetic determination by Y. KANO. Abbreviations used are: *am*, amber mutation; *oc*, ochre mutation; *fs*, frameshift mutation.

ation. This is consistent with the appearance of truncated *trp* mRNA molecules corresponding to the portion of the operon operator proximal to the nonsense mutation (IMAMOTO and YANOFSKY 1967a). Comparison of the order of the size of the *trp* mRNA molecules with the order of the deletion termini and nonsense mutation sites on the genetic map indicates that the *trp* mRNA size is unambiguously correlated with the position of the deletion terminus or the nonsense alteration in *trpE*, and thereby suggests that synthesis of *trp* mRNA in the deletion and nonsense mutants apparently terminates at or near the site of the deletion termini or mutational alterations (IMAMOTO, KANO and TANI 1970). A transcriptional unit beginning from the *trp* gene segment in these deletion mutants is therefore assumed not to contain a foreign translational initiation signal as it would have had fusion between the *trp* gene segment and a gene adjacent to the end of the deletion occurred.

(a) *Effect of deletions that retain the translational initiation signal for trpD*

Operator-distal deletions that do not cover the *trpE-trpD* punctuation point do not affect the degree of polarity created by the nonsense mutations in *trpE*. The

double mutants employed contained a strong polar nonsense mutation (such as *trpE9829*, *trpE12242* or *trpE9758* and the deletion *trpAD28* or *trpKAD05*). As is shown in Figures 2c and d, in these double mutants, the production of *trpE* mRNA and *trpE_{distal}* mRNA decreased to the same level observed with the parental nonsense mutants (Figure 2a and b). Comparison of the degree of transcriptional polarity, expressed as the amount of *trpE_{distal}* mRNA produced in the double mutants relative to that in the parental deletion mutant (Figures 2c and d), with that observed with the parental nonsense mutants (Figures 2a and b), shows that transcriptional polarity in the double mutants remained as strong as in the parental strains.

Figure 3 presents the sedimentation profiles of *trp* mRNA from two double mutants possessing a strong polar mutation such as *trpE12242* or *trpE9758*, and the deletion *trpAD28*. In agreement with the above findings, the *trp* mRNA from double mutants peaked at a position of 12–13s, showing that most of the molecules were as small in size as in the parental nonsense mutants, rather than reaching the size seen in the deletion strain.

FIGURE 2.—*Trp* mRNA levels in various nonsense and double mutants. Tritiated RNA was prepared from derepressed cultures pulse-labeled for 2 minutes at the 10th or 20th minute after derepressions at 30°. The ordinate gives the relative values of *trpE* mRNA (difference between hybridization with $\phi 80trpE$ DNA and $\phi 80$ DNA) (a and c) and *trpE_{distal}* mRNA (difference between hybridization with $\phi 80trpE_{distal}$ DNA and $\phi 80$ DNA) (b and d) from the nonsense or double mutants compared to the value with *trp* mRNA from a *trp* deletion mutant *trpAD28* which was run in each assay of *trp* mRNA, except *trp* mRNA from *trpE9829* and *trpE9829-KAD05* which were compared to that of *trpAD5*. Under standard conditions, 40–50 μ g of [³H]RNA (specific activity, 13,000–17,000 cpm/ μ g) was annealed with 5 μ g of heat-denatured phage DNA. The typical data with the amount of *trpE* mRNA and *trpE_{distal}* mRNA were 0.237% and 0.009%, 0.252% and 0.021%, 0.478% and 0.092%, 0.131% and 0.002% or 0.498% and 0.105% for strain *E12242-AD28*, *E9758-AD28*, *AD28*, *E9829-KAD05* or *AD5*, respectively. The vertical lines joining points are added to avoid confusion with neighboring points. The other conditions are as described in MATERIALS AND METHODS.

FIGURE 3.—Sedimentation profiles of *trp* mRNA from *trpAD28*, *trpE9758-trpAD28* and *trpE12242-trpAD28*. Pulse labeling was carried out with 300 μ Ci of [3 H]-uridine for 2 minutes at the 10th minute after derepression. Under standard conditions, 300–400 μ g of [3 H]RNA (specific activity, 40,000–50,000 cpm/ μ g) were sedimented in a linear sucrose density gradient. After sedimentation, each sample was fractionated into 30 fractions and a three-eighths portion of each fraction was assayed with DNA from ϕ 80*trpE* phage to determine *trp* mRNA (difference between hybridization with ϕ 80*trpE* DNA and ϕ 80 DNA). The *trp* mRNA values presented in the ordinate are on an arbitrary scale to adjust the peaks to the same height. One unit for strain *trpAD28*, *trpE9758-trpAD28* or *trpE12242-trpAD28* corresponds to a radioactivity of 1795 cpm, 717 cpm or 747 cpm, respectively. The abscissa presents fraction numbers for strain *trpAD28*. Sedimentation positions of 23 s rRNA, 16 s rRNA and 4 s rRNA from the strains are represented by arrows. The positions 23 s, 16 s and 4 s obtained from the other two strains, *trpE9758-trpAD28* and *trpE12242-trpAD28*, are aligned with the positions from strain AD28. The other conditions are as described in MATERIALS AND METHODS. $\circ$ — $\circ$, *trp* mRNA from strain *trpAD28*; $\bullet$ — $\bullet$, *trp* mRNA from strain *trpE9758-trpAD28*; $\circ$ — $\circ$, *trp* mRNA from strain *trpE12242-trpAD28*.

(b) *Effect of deletions of the distal genes including the translational initiation signal for trpD*

In this section, we show results obtained with 12 double mutants which retain various lengths of the *trpE* gene on the side operator-distal to the nonsense mutation site but not the intraoperonic translational initiation signal.

In a double mutant containing the *trpE9829* mutation in the *trpE* segment retained in deletion mutant *trpAE11*, transcriptional polarity created at the nonsense mutation remained apparently as strong as in the parental strain *trpE9829*. This is shown in Figure 4, in which the sedimentation profile of *trp* mRNA from the double mutant *trpE9829-trpAE11* as a whole is as small in size as the *trp* mRNA of the parental strain *trpE9829*. The *trp* mRNA from deletion mutant *trpAE11* showed a peak at approximately 17s, reflecting the location of the deletion terminus at the end of *trpE*.

FIGURE 4.—Sedimentation profiles of *trp* mRNA from *trpAE11*, *trpE9829-trpAE11*, and *trpE9829-trpAE11-supD*. Tritiated RNA was prepared from derepressed cultures pulse-labeled for 2 minutes at the 20th minute after derepression. After sedimentation, each sample was fractionated into 60 (*trpAE11*) or 30 (*trpE9829-trpAE11* and *trpE9829-trpAE11-supD*) fractions. The *trp* mRNA values presented are on an arbitrary scale to set the peaks to a suitable height for clear presentation. The abscissa represents fraction numbers for strain *trpAE11* and the positions of 23 s rRNA, 16 s rRNA and 4 s tRNA from this strain are given by arrows. One unit on the ordinate for strain *trpAE11*, *trpE9829-trpAE11* or *trpE9829-trpAE11-supD* corresponds to a radioactivity of 433 cpm, 816 cpm or 488 cpm, respectively. The other conditions and representations are as described in Figure 3. ○—○, *trp* mRNA from strain *trpAE11*; ●—●, *trp* mRNA from strain *trpE9829-trpAE11*; ◐—◐, *trp* mRNA from strain *trpE9829-trpAE11-supD*.

Introducing an amber suppressor (*supD*) into the double mutant led to an increase in the production of larger *trp* mRNA molecules the size of which corresponds to the *trpE* segment retained in *trpAE11* (Figure 4). This verifies the low production of *trp* mRNA molecules having the size of *AE11 trp* mRNA (i.e., conservation of strong transcriptional polarity) in the unsuppressed double mutant *trpE9829-trpAE11*, and also the existence of the deletion *trpAE11* in the double mutant.

Essentially similarly, a lack of effect of introduction of the deletions *trpAE9* and *trpKAE44* was observed on the degree of transcriptional polarity created by amber mutation *trpE9829*. Results presented in Figure 5 and Figure 6 show that the *trp* mRNA's produced from the double mutants *trpE9829-AE9* and *trpE9829-trpKAE44*, respectively, were almost the same size as that from the parental nonsense mutant *trpE9829*. In both double mutants, introducing an amber suppressor increased the proportion of larger *trp* mRNA molecules corresponding to the *trpE* segment retained by each deletion mutant. Thus the degree of transcriptional polarity created by *trpE9829* was not significantly affected by intro-

duction of deletion *trpAE9* or *trpKAE44*. On the basis of the physical map of transcription length (IMAMOTO, KANO and TANI 1970), these mutants delete the operator-distal 0.35 (for *trpAE9*) or 0.45 (for *trpKAE44*) portion of the *trpE* gene, assuming that the location of the *trpE-trpD* intercistronic punctuation point is in the middle of the region between the deletion end points of *trpAE11* and *trpAD28*. The nonsense mutant *trpE9829* is located at 0.83 with respect to the *trpE-trpD* punctuation point. Therefore, the double mutants retain *trpE* segments corresponding to 0.48 (for *trpE9829-trpAE9*) or 0.38 (for *trpE9829-trpKAE44*) portions of the whole *trpE* gene on the side operator-distal to the nonsense mutation *trpE9829*. The degree of polarity created by a nonsense mutation in *trpE* tends to be weakened when it is located distal to the nonsense mutation *trpE9758* (ITO and YANOFSKY 1966; IMAMOTO and YANOFSKY 1967a), which maps near the middle of the *trpE* gene.

In the experimental results presented in Figure 7, it is shown that the production of shortened *trp* mRNA by unsuppressed double mutants is accompanied by a deficiency in the mRNA segment corresponding to the region of *trpE* on the operator-distal side of the nonsense mutation site. Six double mutants containing the strong polar mutations, *trpE9829*, *trpE12242* or *trpE9758*, in the

FIGURE 5.—Sedimentation profiles of *trp* mRNA from *trpE9829*, *trpE9829-trpAE9* and *trpE9829-trpAE9-supD*. Tritiated RNA was prepared from derepressed cultures pulse-labeled for 2 minutes at the 10th minute after derepression. After sedimentation, each sample was fractionated into 30 fractions. The abscissa represents fraction numbers for strain *trpE9829* and the positions of 23 s rRNA, 16 s rRNA and 4 s tRNA from this strain are given by arrows. One unit on the ordinate for strain *trpE9829*, *trpE9829-trpAE9* or *trpE9829-trpAE9-supD* corresponds to a radioactivity of 668 cpm, 620 cpm or 225 cpm, respectively. The other conditions and representations are as described in Figure 4. ○—○, *trp* mRNA from strain *trpE9829*; ●—●, *trp* mRNA from strain *trpE9829-trpAE9*; ◐—◐, *trp* mRNA from strain *trpE9829-trpAE9-supD*.

FIGURE 6.—Sedimentation profiles of *trp* mRNA from *trpE9829-trpKAE44* and *trpE9829-trpKAE44-supD*. Tritiated RNA was prepared from derepressed cultures pulse-labeled with 300 μ Ci or 500 μ Ci of [3 H]-uridine for 2 minutes at the 10th minute after derepression. After sedimentation, each sample was fractionated into 30 fractions. The abscissa represents fraction numbers for strain *trpE9829-trpKAE44* and the positions of 23 s rRNA, 16 s rRNA and 4 s tRNA from this strain are given by arrows. A one-sixth portion of each fraction was assayed to determine *trp* mRNA from *trpE9829-trpKAE44-supD*. One unit on the ordinate for strain *trpE9829-trpKAE44* or *trpE9829-trpKAE44-supD* corresponds to a radioactivity of 677 cpm or 611 cpm, respectively. The other conditions and representations are as described in Figure 4. ●—●, *trp* mRNA from strain *trpE9829-trpKAE44*; ○—○, *trp* mRNA from strain *trpE9829-trpKAE44-supD*.

trpE segments retained in deletion mutants *trpAE10* or *trpAE11*, were examined for *trp* mRNA production during the steady state of transcription of the *trp* operon. *Trp* mRNA production by the mutants is expressed as the amount of *trp* mRNA detected relative to the amount found with an identically treated deletion mutant *trpAE10* or *trpAE11*. Although the reduction in *trpE_{distal}* mRNA appears to be slightly but distinctly less in the double mutants with *trpAE10* and *trpAE11* than in the double mutants containing the *trpE-trpD* punctuation point (cf. Figures 7b and d and Figure 2d), the observation of relatively little production of *trpE_{distal}* mRNA in the double mutants (Figures 7b and d) is significant and indicates that the diminished total *trpE* mRNA [40% to 60% of that of the control with the parental deletion mutants (Figures 7a and c)] detected resulted from a preferential reduction of the mRNA region corresponding to the region operator-distal to the nonsense alteration.

In Figure 8b, sedimentation profiles are presented for *trp* mRNA from a double mutant *trpE9758-trpAE9* in which a segment representing only 0.13 of *trpE* is retained on the side operator-distal to the nonsense alteration (cf. IMAMOTO,

FIGURE 7.—*Trp* mRNA levels in various double mutants. Tritiated RNA was prepared from derepressed cultures pulse-labeled for 2 minutes at the 10th or 20th minute after derepression. The ordinate is the relative values of *trpE* mRNA (a and c) and *trpE_{distal}* mRNA (b and d) from the double mutants compared to the value with *trp* mRNA from the parental deletion mutant, *trpAE11* (a and b) or *trpAE10* (c and d) which was run in each assay of *trp* mRNA. Under standard conditions, 40–45 μ g of [3 H]RNA (specific activity, 15,000–16,000 cpm/ μ g) (a and b) or 35–60 μ g of [3 H]RNA (specific activity, 12,000–19,000 cpm/ μ g) (c and d) was annealed with 5 μ g of heat-denatured phage DNA. Typical data for the amounts of *trpE* mRNA and *trpE_{distal}* mRNA were 0.192% and 0.025%, 0.283% and 0.020%, 0.244% and 0.024% or 0.465% and 0.073% for strain *E9829-AE11*, *E12242-AE11*, *E9758-AE11* or *AE11*, respectively (a and b), 0.150% and 0.018%, 0.279% and 0.035%, 0.256% and 0.028%, or 0.437% and 0.076%, for strain *E9829-AE10*, *E12242-AE10*, *E9758-AE10* or *AE10*, respectively (c and d). The other conditions and representations are as in Figure 2.

KANO and TANI 1970). It is striking that the majority of the *trp* mRNA molecules produced by the double mutant are obviously as short as those in the parental strain *trpE9758* (13 s) (Figure 8a). A relatively small number of mRNA molecules retaining full size for the *trpE* segment of *trpAE9* were produced in the double mutant, representing the relative extent of transcriptional polarity at the nonsense alteration. The *trp* mRNA species including the *trpE_{distal}* segment were obviously larger, sedimenting at a position of the *trp* mRNA of *trpAE9*, than the predominant *E9758 trp* mRNA species (Figure 8b). Thus most of the *E9758-AE9 trp* mRNA molecules sedimenting at a position of 13 s do not include RNA sequences for the operator-distal region beyond the *E9758* alteration. Consistently, relatively little *trpE_{distal}* mRNA was detected in the double mutant, demonstrating that the short *trp* mRNA molecules lack the mRNA region corresponding to the portion of *trpE* beyond the site of the nonsense mutation *trpE9758* (Figure 9). Thus, transcriptional polarity created by a *trpE9785* mutation remains quite strong even when a 0.35 portion of the region of *trpE* operator-distal to the mutation site is deleted.

FIGURE 8.—Sedimentation profiles of *trp* mRNA from *trpE9758*(a), *trpAE9*(a) and *trpE9758-trpAE9*(b). Tritiated RNA was prepared from derepressed cultures pulse-labeled for 2 minutes at the 10th minute after derepression. After sedimentation, each sample from strains *trpE9758* and *trpE9758-trpAE9* or *trpAE9* was fractionated into 30 or 60 fractions, respectively. The abscissa represents fraction numbers for strain *trpAE9*(a) and for strain *trpE9758-trpAE9*(b). The positions of 23 *s* rRNA, 16 *s* rRNA and 4 *s* tRNA from the respective strains are given by arrows. A three-eighths portion of each fraction was assayed with DNA from $\phi 80trpE$ phage (a and b) or with DNA from $\phi 80trpE_{distal}$ phage(b). One unit on the ordinate for strain *trpE9758* or *trpAE9* corresponds to a radioactivity of 429 cpm or 1167 cpm, respectively. One unit on the ordinate for strain *trpE9758-AE9* corresponds to a radioactivity of 1135 cpm or 114 cpm for *trp* mRNA or *trpE_{distal}* mRNA, respectively. The other conditions are as described in Figure 4. (a), ○—○, *trp* mRNA from strain *trpE9758*; ●—●, *trp* mRNA from strain *AE9*. (b), ○—○, *trp* mRNA from strain *trpE9758-AE9*; ●—●, *trpE_{distal}* mRNA from strain *trpE9758-AE9*.

DISCUSSION

The results demonstrate that the severity of transcriptional polarity is independent of the length of the region operator-distal to the nonsense alteration, as long as that region contains no punctuation point. Almost certainly the feature of punctuation that affects polarity is the translational initiation signal for the gene subsequent to the mutated gene (see beginning of paper). The distance from

FIGURE 9.—Comparison of *trp* mRNA levels in a double mutant *trpE9758-trpAE9* and deletion mutant *trpAE9*. Tritiated RNA was prepared from derepressed cultures pulse-labeled for 2 minutes at the 10th minute after derepression. The ordinate is the relative value of *trpE* mRNA(a) and *trpE_{distal}* mRNA(b) from the mutant *trpE9758-trpAE9* compared to the value with *trp* mRNA from the parental deletion mutant *trpAE9* which was run in each assay of *trp* mRNA. Under standard conditions, 45–55 μ g of [3 H]RNA (specific activity, 16,000–18,000 cpm/ μ g) was annealed with 5 μ g of heat-denatured phage DNA. Typical data for the amounts of *trpE* mRNA and *trpE_{distal}* mRNA were 0.204% and 0.004% or 0.332% and 0.038% for strains *E9758-AE9* or *AE9*, respectively. The other conditions and representations are as in Figure 2.

the site of a nonsense mutation to the next translational initiation site has been shown to influence the degree of polarity (SARABHAI and BRENNER 1967; GRODZICKER and ZIPSER 1968; NEWTON 1969; ZIPSER *et al.* 1970). Our observations are complementary; i.e., in a single transcriptional unit lacking a distal highly efficient initiation signal, the length of the genetic region on the side operator-distal to the nonsense mutation site does not significantly affect the severity of transcriptional polarity.

Recent studies on transcription of the *trp* operon translocated into the early region of λ phage has demonstrated the existence of dual *trp*-transcription; one type is initiated at the authentic *trp* promoter and is controlled by the *trp* repressor, while another is initiated at the P_L promoter of gene *N* and is controlled by λ repressor (IMAMOTO and TANI 1972; SEGAWA and IMAMOTO 1974; FRANKLIN 1974). It has been recently shown by transcriptional studies using a λ *trp* phage which bears a strong polar nonsense mutation (*trpE9829* or *trpE9758*) in *trpE* of the translocated *trp* operon, that the nonsense mutation in the *trp* operon does not express polarity when the operon is transcribed by read-through from the P_L promoter (SAGAWA and IMAMOTO 1974). Similar results have also been observed in translational studies on polarity in the *trp* (FRANKLIN 1974) and *gal* (ADHYA,

GOTTESMAN and CROMBRUGGHE 1974) operons when transcription of the operon has been initiated at an outside P_L promoter of λ phage. In contrast to this observation, transcription originated at the *trp* promoter resulted in polarity effects, producing truncated *trp* mRNA from the part of the gene operator-proximal to the nonsense mutation. Thus, these findings indicate that the machinery by which polarity is elicited at the site of a nonsense mutation can selectively be picked up at either the promoter or some other unique region(s) located at the beginning of the *trp* operon, either during RNA polymerase activity or because of some structural feature of the transcript. One can therefore suggest that the translational reinitiation signal plays a role in such a way that it reduces the severity of polarity. In this sense, the reinitiation is formally analogous to a nonsense suppressor that permits the continuation of translation. The degree to which polarity is relaxed could depend on the distance of a nonsense mutation site from the subsequent reinitiation signal for protein synthesis (ZIPSER and NEWTON 1967). The molecular mechanism by which relaxation of polarity is exerted through reinitiation of translation is not clear.

In the presence of deletions, a slight but discernible increase in the level of *trp* mRNA production for the operator-distal segment of *trpE* beyond the nonsense alteration site was observed, however (Figure 7 and also IMAMOTO, KANO and TANI 1970). When the relative values of *trpE_{distal}* mRNA detected in the double mutant shown in Figure 7 are compared with those of the parental nonsense mutants indicated in Figure 2, the degree of transcriptional polarity was reduced 10% to 20% by the introduction of the deletion. This variation seems independent of the relative distance of the nonsense alteration from the deletion terminus. The conservation of transcriptional polarity was still significant when a deletion mutation was introduced operator-distal to the nonsense alteration, but the reason for this minor variability is not known.

The double mutant, *trpE9903*·*trpAE11* (Figure 1), also exhibited primarily small *trp* mRNA molecules like those of the parental strain *trpE9903* (unpublished data). This double mutant showed ratios of *trpE_{distal}* mRNA to *trpE* mRNA comparable to those of the parental strain *trpE9903* (IMAMOTO, KANO and TANI 1970). Although there is some difficulty in assay of the distal *trp* mRNA segment beyond the *E9903* mutation site (because $\phi 80ptE_{distal}$ DNA covers the *E9903* mutation), it seems that deletion of the *trpE*-*trpD* punctuation point did not significantly relax the degree of transcriptional polarity created by mutation *trpE9903*.

The possibility cannot be ruled out that the *trp* gene segment of the deletion mutants employed in the present experiments is fused to a very small genetic segment that contains a site functioning as a very low efficiency reinitiator for polypeptide synthesis. However, at least in the double mutants *trpE12242*·*trpAE10*, *trpE9758*·*trpAE10* and *trpE9758*·*trpAE9*, transcriptional polarity was significantly conserved when the region operator-distal to the nonsense alteration was shortened to 0.28, 0.21 or even 0.13, respectively, of the extent of *trpE*. It is known for the *trpE* gene that polarity created by nonsense mutations located in the region operator-distal to the *E9758* mutation site is relatively weak (Iro and YANOFSKY 1966; IMAMOTO and YANOFSKY 1967a).

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