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Genetic Screen for Cloned Release Factor Genes

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Nonsense suppression reflects competition between a nonsense suppressor tRNA and a translational release factor. This provides a simple way to screen for release factor genes cloned into a multicopy plasmid. We have confirmed that the expected competition occurs with the gene for release factor 2, cloned by C. T. Caskey et al. (C. T. Caskey, W. C. Forrester, W. Tate, and C. D. Ward, J. Bacteriol. **158**:365–368, 1984), and used it to clone the gene for release factor 1.

Polypeptide chain termination requires the participation of release factors (RFs) which recognize specific terminator codons: RF1 catalyzes termination at UAA and UAG, whereas RF2 catalyzes termination at UAA and UGA (3, 4, 10). Termination suppressor tRNAs which read these codons thus compete with the corresponding RFs, as Beaudet and Caskey (1) have shown directly in the case of UAG suppression by *sup-3* tRNA.

This competitive relationship provides a simple way to screen for RF genes cloned into a multicopy plasmid. The increased RF concentration in the cells, due to the increased RF gene dosage, will reduce the efficiency of suppression. Suppression of terminator mutations in *lacZ* is readily visualized on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (8). Strains carrying a UAG mutation in *lacZ* and *supE444*, a glutamine-inserting UAG suppressor of low efficiency, produce pale blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Such a strain was used as a recipient for transformation by recombinant plasmids representing a pool of random chromosomal fragments.

We transferred a *lacZ* UAG allele ($U118^{UAG}$) into *Escherichia coli* CP79, which harbors *supE44* (see Table 1 for details). The pool was prepared by partially digesting DNA from this strain with restriction endonuclease *Sau*3A to yield an average fragment size of 5 to 20 kilobases. The fragments were then ligated into a cut and dephosphorylated *Bam*HI site in the *tet* gene of plasmid YRp7, a pBR322 derivative (11). The parental strain was then transformed with DNA from this pool, and primary ampicillin-resistant transformants were selected on minimal medium plates containing 40 μg of ampicillin per ml. Colonies inheriting recombinant plasmids (Amp^r and Tet^s) were screened for *lacZ* activity by replica plating onto minimal medium plates containing 2 mM isopropyl-β-D-thiogalactoside, 5 mM cyclic AMP, and ampicillin at 40 μg/ml. Since the insert DNA is isogenic with the chromosomal DNA of the recipient, any effect on galactosidase synthesis should derive from the increased gene dosage of plasmid-borne inserts.

The colony color of ca. 2,200 insert-containing primary transformants was surveyed for distinctly white colonies, as compared with the normal pale blue of the parent strain. Nine white colonies were found, and these were tested by a second criterion for reduced amber suppression. A bacteriophage T4 $r_{II}B$ amber mutant (X655am) was spotted on each of the nine white transformants; only one displayed reduced *supE44* suppression of X655am. When streaked on lactose-containing MacConkey agar plates, this clone was easily distinguishable (white colonies) from the parent (light red colonies) after 18 h at 30°C, with no differences in colony size. Streaks of this clone segregated lac^+ sectors on prolonged incubation. This sectoring was prevented when the plasmid DNA was introduced into a *recA* derivative of the parent strain.

Plasmid DNA (which we term pRF1) purified from this white transformant was submitted to preliminary analysis by restriction endonuclease digestion, revealing an insert size of 2.3 kilobases. The protein products encoded by this insert were examined by the maxicell procedure of Sancar et al. (9). We transformed CSR603 *recA uvrA* with pRF1 and also with the parental plasmid YRp7, and plasmid-encoded proteins were radiolabeled with [^{35}S]methionine 18 h after UV irradiation (Fig. 1). It is evident that pRF1 produced a protein of 48,000 kilodaltons which was not produced by the parental plasmid YRp7. This size is in good agreement with previous (2) and recent (5) estimates of the size of RF1. A gene coding for a protein of this size requires at least 1.3 kilobases of DNA and therefore comprises a major fraction of the inserted DNA. The identity of this protein as RF1 was confirmed by immunoprecipitation. Anti-RF1 serum specifically precipitated the 48,000-kilodalton protein from the [^{35}S]labeled extracts of maxicells (Fig. 1, lanes 4 and 5).

The restriction of amber suppression by pRF1 was quantitated by direct measurement of β-galactosidase synthesis (Table 1). It can be seen that pRF1 reduced suppression of the *lacZ* UAG mutation by a factor of nine, whereas no such effect was produced by the parental YRp7 plasmid or by a control recombinant plasmid containing a different insert. The specificity of this effect for amber suppression was demonstrated by the fact that pRF1 had no effect on β-galactosidase synthesis in an isogenic $lacZ^+$ strain. Table 1 also demonstrates that pRF1 reduced the background leakiness of an unsuppressed UAG allele by a factor of five, probably due to competition between RF1 and the tRNAs which misread the UAG to produce background readthrough.

Reduced suppression of UGA can be used in an analagous manner to detect plasmid-borne inserts containing RF2. Our search for the RF2 gene was called off when it was isolated in

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TABLE 1. Plasmid pRF1 reduces UAG suppression and background unsuppressed UAG readthrough[a]

Relevant genotype[b]		Plasmid[c]	β-Galactosidase differential rate[d]	
lacZ-U118^{UAG}	*supE44*	YRp7	14.3 ± 1.8	(2)
lacZ-U118^{UAG}	*supE44*	pB1	14.0 ± 1.5	(2)
lacZ-U118^{UAG}	*supE44*	pRF1	1.55 ± 0.0	(2)
lacZ$^+$	*supE44*	none	76	(1)
lacZ$^+$	*supE44*	pRF1	88	(1)
lacZ1012^{UAG}	*supE*$^+$	none	0.7×10^{-3}	(1)
lacZ1012^{UAG}	*supE*$^+$	pRF1	$0.135 \pm 0.015 \times 10^{-3}$	(2)

[a] The indicated strains were cultivated at 30°C in glucose minimal medium supplemented with required amino acids, 0.05% casein hydrolysate, and 10% sucrose (which we find improves the stringency of selection for plasmid maintenance). Plasmid maintenance was kept under selection through the presence of 2 mg of carbenicillin per ml and confirmed by testing the frequency of carbenicillin-sensitive segregants in each culture (which was under 3% in all cases). The *lac* operon was induced by addition of 2 mM isopropyl-β-D-thiogalactoside and 5 mM cyclicAMP. Sonic extracts were prepared and assayed for β-galactosidase as described (7).

[b] The parental strain CP79 is *lacZ*$^+$ and carries *supE44*. A *supE*$^+$ derivative was constructed by cotransduction with a nearby Tn*10* element, by using suppression of phage UAG mutants as the screen for the *supE* genotype. The *lacZ* chain termination UAG alleles were introduced into appropriate *proC* CP79 derivatives by cotransduction with *proC*$^+$. All the host strains are thus nearly isogenic.

[c] Plasmid pRF1 is described in the text; YRp7 is the parental plasmid with no *E. coli* insert; pB1 is a control recombinant plasmid of YRp7 (obtained in the initial screening for pRF1) containing an insert in the *tet* gene which did not affect UAG suppression.

[d] Enzyme units per milligram of protein formed during the period of induction. The data record the mean ± standard error for those cases where duplicate cultures were processed. Measurements of enzyme activity and protein were performed in duplicate for each culture. The number of replicate cultures are in parentheses.

TABLE 2. Plasmids carrying RF2 reduce UGA suppression[a]

Plasmid[b]	β-Galactosidase synthesis[c]
None	1.0 ± 0.085 (4)
pLC42-13	0.17 ± 0.06 (5)
pRF2	0.32 ± 0.085 (6)

[a] The host strain is a derivative of RR1 constructed as follows. A *lacZ* UGA allele (*827z*) was transferred into strain RR1 by conjugation, and then UGA suppression was provided by transforming this strain with a plasmid (pPY1001) carrying a *supU* UGA suppressor. Plasmid pPY1001 was constructed by ligating the *Sau*3A fragment of plasmid pSWC101, which contains the *supU* UGA suppressor gene (2), into the single *Bam*HI site of pACY184, a plasmid compatible with pBR322 derivatives (6).

[b] Plasmids pLC42-13 and pRF2, both of which carry the RF2 gene, are described in the accompanying paper by Caskey et al. (5). Maintenance of these plasmids and the pPY1001 plasmid which provides UGA suppression was kept under selection through the presence of 50 μg of chloramphenicol per ml for pPY1001 and 1 mg of carbenicillin per ml for pRF2 and pLC42-13. Segregational loss of one or the other plasmid in strains harboring two compatible plasmids is not infrequent. Accordingly, we grew multiple cultures and assayed them for maintenance of both plasmids; several of these replicate cultures which exhibited a high frequency of segregation were eliminated from consideration.

[c] Methods of cultivation and assay are described in Table 1, footnote *a*. Enzyme synthesis was as defined in Table 1, footnote *d*, and is recorded as the mean ± standard error of the mean, with the number of replicate cultures in parentheses.

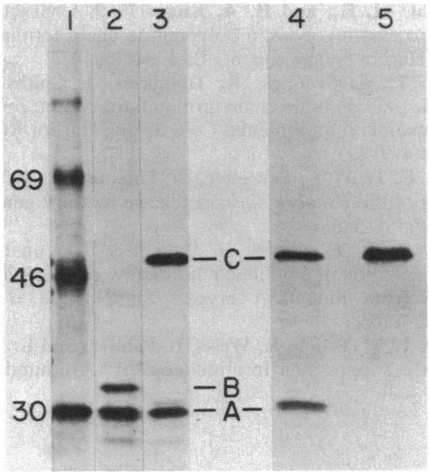

FIG. 1. Labeling of plasmid proteins in maxicells. Plasmid-carrying derivatives of *E. coli* CSR603 were grown (10 ml; 2×10^8 cells per ml) and labeled with [^{35}S]methionine (2.5 μCi/ml) by the maxicell cell procedure of Sancar et al. (9). The labeled cells were resuspended in a final volume of 400 μl. A ^{14}C-methylated protein mixture (Amersham Corp.) was used as a molecular-weight (MW) standard: bovine serum albumin (69,000 MW), ovalbumin (46,000 MW), and carbonic anhydrase (30,000 MW). Lane 1, MW markers; lane 2, CSR603 (YRp7); lane 3, CSR603 (pRF1). Immune precipitation of RF1 was done by pretreating 120 μl of maxicell extract with 30 μl of Staph A ghosts (Pan Sorbin; Calbiochem-Behring and Boehringer Mannheim Biochemicals) at 0°C for 30 min and then pelleting the ghosts by centrifugation (Microfuge; 3 min). Anti-RF1 serum (3 μl) was added to the supernatant, and the sample was incubated at 37°C for 20 min and then at 0°C for 18 h. Staph A ghosts (10 μl) were added, the sample was incubated at 0°C for 30 min, and the ghosts were pelleted by centrifugation (Microfuge; 30 s). The pellet was washed four times with buffer (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl), resuspended in 20 μl of sample buffer, and heated at 100°C for 4 min. Lane 4, CSR603 (pRF1), no immune precipitation; lane 5, CSR603 (pRF1), material recovered from immune precipitation. A, *blu* gene product (β-lactamase); B, *tet* gene product; C, RF1.

a different manner (as described in the accompanying paper by Caskey et al. [5]). Caskey et al. kindly provided us with the plasmids containing the RF2 gene, pLC42-13, and pRF2, and their ability to restrict UGA suppression is shown in Table 2.

The isolation of the RF genes may rekindle interest in the problem of polypeptide chain termination. RF reading of nonsense codons and misreading of sense codons can be explored by manipulating the level of RF1 and RF2 with these plasmids. The contribution of transcriptional and translational chromosomal DNA, errors to the basal leakiness of nonsense mutations in suppressor-free cells can be resolved. Structural questions concerning the relatedness of RF1 and RF2 can be posed. Schemes for selecting mutants in either the activity or specificity of the RFs can be devised, and the anti-suppression phenotype described here provides a convenient visual screen for the identification of such mutants.

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