

Gene expression and cell fusion analyzed by *lacZ* complementation in mammalian cells

(β -galactosidase complementation/fluorescence histochemistry/protein interactions)

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ABSTRACT Complementation reporter genes provide biological indicators of coincident expression of proteins in cells. We have adapted intracistronic complementation of the *Escherichia coli lacZ* gene for use in mammalian cells. Enzymatic activity detectable by quantitative biochemical assay, flow cytometry, or microscopy is produced upon convergent expression of two distinct mutant *lacZ* peptides within single cells, or upon fusion of cells expressing such mutants. A novel fluorescent substrate for β -galactosidase (Fluor-X-Gal) increases detection and permits simultaneous microscopic visualization of other fluorescent markers. The enzymatic complementation described here should facilitate studies of cell fusion, cell lineage, and signal transduction, by producing activity only when two proteins are expressed at the same time and place in intact cells.

Many biological studies would benefit from an examination of overlapping patterns of gene expression or determination of whether and where two proteins may interact within intact cells. Currently analyses in cultured cells or embryos are limited to a microscopic analysis of independent signals and subsequent correlation of protein locations. A means for generating a reporter enzyme that exhibits activity only when the products of two genes are expressed at the same time and place would enhance these studies. Furthermore, the development of a means for monitoring the physical interaction of proteins within living cells would be an attractive adjunct to coimmunoprecipitation from cell extracts or use of the yeast two-hybrid system. Our interests in the mechanism of myoblast fusion and in the interplay of networks of regulatory genes in muscle development led us to seek such a system.

lacZ is widely used in studies of gene expression and cell lineage in higher organisms (1–8). Several biochemical assays of β -galactosidase (β -gal) activity, including live-cell flow cytometry and histochemical staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal), make the product of the *lacZ* gene extremely versatile as a quantitative reporter enzyme, selectable marker, or histological indicator (9–11). An aspect of the *lacZ* gene that has not been exploited in eukaryotes is the classical bacterial genetic phenomenon of intracistronic complementation. In *Escherichia coli*, deletions of either the N or C terminus of *lacZ* produce enzyme that is inactive yet can be complemented by coexpression with a second inactive deletion mutant containing domains that are lacking in the first. Complemented β -gal activity arises by the concentration-dependent assembly of a stable hetero-octameric enzyme comprising all of the essential domains of the wild-type homotetramer. The N- and C-terminal domains involved in the two distinct classes of complementation are known as the α and ω regions, respectively (12–14). In this report, μ refers to the central peptide region between α and ω . We show here that complementation

by distinct *E. coli lacZ* mutants in mammalian cells permits analyses of cell fusion and detection of colocalized interacting proteins within single intact cells.

MATERIALS AND METHODS

Cell Culture. All cells were grown in DMEM plus 5% fetal bovine serum/15% iron-supplemented calf serum (HyClone). Differentiation of C2F3 cells was induced with DMEM plus 5% horse serum.

Plasmid Construction and Retrovirus Production. The N termini of *lacZ* genes from *E. coli* strains K802 (wild type) and DH5 α ($\Delta\alpha$) (15) were amplified by PCR with a pair of primers that altered three codons of N-terminal sequence (from MTM. . . to MG. . .). This alteration introduces a *NcoI* site at the 5' end of the gene that is necessary for the cloning and expression of the gene in the MFG retrovirus (16). Ligation of amplified fragments into the *FspI* site of *lacZ* from pBAG (1) created the full-length wild-type and $\Delta\alpha$ forms of the gene (the *FspI* site was destroyed). These full-length genes were cloned into the *NcoI* and *BamHI* sites of MFG (gift of R. C. Mulligan, Whitehead Institute, Cambridge, MA) to create MFG-KB5 and MFG- $\Delta\alpha$. The C-terminal 235 codons of KB5 were deleted by ligation of a blunted internal *ApaI* site to the blunted *BamHI* site of MFG. The resulting construct, MFG- $\Delta\omega$, is deleted after codon 788 and expresses an additional 3 amino acids encoded by vector sequence. MFG- $\Delta\mu$ was made by deleting 553 codons of KB5 between two in-frame *PvuI* sites. Proviral constructs were cloned into the *NheI* sites of the stable episomal plasmid vector LZRS-17 and expressed in BOSC 23 ecotropic packaging cells (17, 18) by calcium phosphate transfection and selection with 1 μ g/ml puromycin. The supernatant medium containing retrovirus from the packaging cells was harvested 24–48 h after packaging cells reached confluence and used to infect target cells in the presence of 8 μ g/ml Polybrene. The titer of the retrovirus in the supernatant is typically in the range of 10^6 – 10^7 transduction units/ml.

Indigogenic X-Gal Histochemistry for β -gal. Cells were fixed 5 min in PBS plus 4% paraformaldehyde and rinsed in PBS prior to staining. Indigogenic X-Gal staining was performed overnight at 37°C in PBS plus 1 mg/ml X-Gal, 1 mM MgCl₂, 5 mM K₃Fe(CN)₆, and 5 mM K₄Fe(CN)₆ (11).

Assay for β -gal Activity by Fluorescence Histochemistry. Cells were fixed and rinsed as for standard X-Gal detection. Following the method of Gossrau (19), X-Gal (GIBCO/BRL) and 5-bromo-6-chloro-3-indolyl β -D-galactopyranoside (Fluka; referred to as 5-6-X-Gal herein) were each tested for

Abbreviations: X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside; β -gal, β -galactosidase; 5-6-X-Gal, 5-bromo-6-chloro-3-indolyl β -D-galactopyranoside; DAPI, 4',6-diamidino-2-phenylindole.

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simultaneous coupling with 28[‡] different azo dyes (Sigma) on fixed cells expressing nuclear-localized *lacZ*. Of these dyes, fast red violet LB produced a fluorescent precipitate that was superior, in both brightness and fixation of the product, to all others tested. Spectrofluorimetry showed approximately twice the signal-to-background ratio with 5-6-X-Gal used in place of X-Gal in this azoindolyl coupling reaction. Proper fixation of the reaction product requires a minimum ratio of azo dye to enzyme substrate that increases as the absolute concentration of azo dye is reduced or the local enzyme activity is increased. Fluor-X-Gal histochemistry, as we have named the procedure using an azo dye in combination with either X-Gal or 5-6-X-Gal, was routinely performed in this study by a 30-min, 37°C incubation in PBS plus 100 µg/ml fast red violet LB salt and 25 µg/ml 5-6-X-Gal. Stock solutions of dyes and substrates were in dimethylformamide. Fluorescence micrographs were taken with a Zeiss Axiophot using a rhodamine/Texas red filter set (Ex 546/12; Em 590LP).

Immunofluorescence and Multiple Labeling. In multiple-fluorochrome experiments, immunofluorescent labeling was performed after fixation and prior to the Fluor-X-Gal reaction. Cells were blocked in PBS plus 10% horse serum at 4°C. Antibody incubations were performed at 4°C in blocking buffer with a rat monoclonal antibody to mouse NCAM (MAB310, Chemicon), biotinylated goat anti-rat secondary antibody (Vector Laboratories), and Cy5-labeled streptavidin (Amersham), at 1:1000, 1:250, and 1:100 dilutions of the commercial stock concentrations, respectively. 4',6-Diamidino-2-phenylindole (DAPI) was used at 100 ng/ml. Triple-labeled and deconvolved images were collected using software and a DeltaVision deconvolution microscope (Applied Precision, Mercer Island, WA), on loan to the Stanford Cell Sciences Imaging Facility. Filter sets for fluorescein isothiocyanate (Ex 490/20; Em 528/38), DAPI (Ex 360/40; Em 457/50), and Cy5 (Ex 640/20; Em 685/40) were used, and images represented in false color using Adobe PHOTOSHOP software.

Chemiluminescent Assay of β -Gal. Cells cultured in microtiter plates were lysed *in situ* in 50 µl of a 1:3 mixture of lysis and assay buffers containing Galacton Plus substrate from the Galactolight Plus assay kit (Tropix, Bedford, MA) (11). Reactions proceeded for 1 h at room temperature. After addition of Light Emission Accelerator solution, luminescence was measured on a MicroBeta 1450 scintillation counter (Wallac, Gaithersburg, MD).

RESULTS AND DISCUSSION

Vectors Expressing Complementing Mutants of β -Gal. We constructed replication-defective retroviral vectors expressing several deleted versions of *lacZ* (Fig. 1A). First, a naturally occurring mutation, Δ M15 (21, 22), which we designate here as $\Delta\alpha$ for simplicity, was recreated by cloning from *E. coli*. Additional deletions were created based on the bacterial genetic map and biochemical data (12–14), the three-dimensional x-ray crystal structure (20), and the existence of convenient restriction sites within the gene (23). The proteins expressed by three such constructs are shown schematically in Fig. 1A. These deletion mutants express polypeptides representing an α -acceptor/ ω -donor ($\Delta\alpha$), an α -donor/ ω -acceptor ($\Delta\omega$), and an α -donor/ ω -donor ($\Delta\mu$).

[‡]The 28 dyes tested are as follows: fast red TR, fast bordeaux GP, fast red KL, fast red violet LB, fast red ITR, fast black K, fast garnet GBC, fast blue BN (*o*-dianisidine), fast blue RR, fast dark blue R, fast blue BB, fast yellow GC, fast orange GR, fast corinth V, fast brown RR, fast violet B, fast red GL, fast red 3GL, fast red AL, fast red GG, fast scarlet GG, fast scarlet R, fast red RL, fast red RC, variamine blue RT (4-aminodiphenylamine diazonium sulfate), fast red KB, fast red PDC, and fast red B.

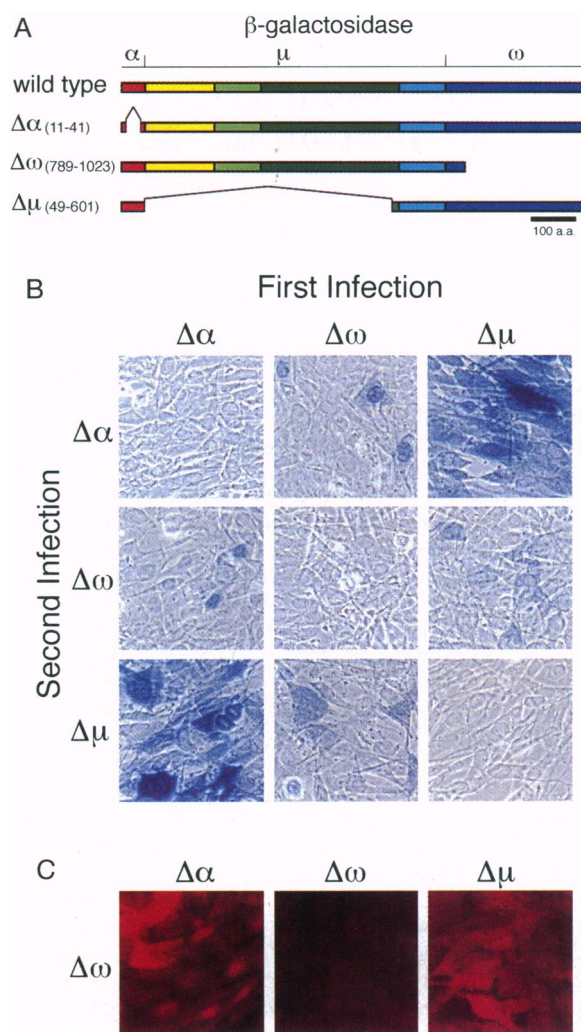


FIG. 1. Mutants of *E. coli lacZ* expressed from retroviral vectors complement to produce active β -gal enzyme in mammalian cells. (A) Schematic representation of expressed wild-type and deleted polypeptides $\Delta\alpha$, $\Delta\omega$, and $\Delta\mu$. Colored regions represent distinct domains of the β -gal monomer as portrayed by Jacobson *et al.* (20). The α and ω regions are thought to comprise the extreme N- and C-terminal domains, respectively. We designate μ as the central portion of the protein. Subscripts indicate the ranges of amino acids removed by the deletions. (B) Complementation of β -gal by sequential transduction with mutant *lacZ* genes. NIH 3T3 cells were subjected to two rounds of retroviral infection (24 h apart), and fixed cells were assayed with X-Gal at least 72 h after the last infection. Arrayed micrographs show blue X-Gal reaction product in sequentially transduced NIH 3T3 cells, with axis labels corresponding to the order of sequential infections with *lacZ* mutants. (Bar = 50 µm.) This experiment is representative of five sequential transduction experiments with either NIH 3T3 or C2F3 cells, all of which gave similar results. (C) Complementation in replicate cultures visualized by fluorescent histochemistry for β -gal using Fluor-X-Gal substrate. Sequentially transduced NIH 3T3 cells correspond to the middle row of B. (Magnification is the same as in B.)

Initially, complementation was tested in NIH 3T3 cells by sequential infection with retroviruses harboring different mutant constructs. Doubly infected cultures were assayed histochemically for β -gal activity (Fig. 1B). Cultures sequentially infected with the same mutant virus contained no blue X-Gal-positive cells. However, each culture sequentially infected with a pair of vectors encoding distinct mutant peptides showed histochemically detectable β -gal activity. Although there were differences among complementing pairs of mutants, for reasons discussed below, β -gal activity was similar for each pair of vectors, regardless of the order of infection.

The observed complementing properties of the various pairs of constructs are in accordance with predictions based on the sequence content of the three mutant gene products. $\Delta\alpha$ should be capable of either α - or ω -complementation with $\Delta\omega$, while $\Delta\mu$ should α -complement $\Delta\alpha$ and ω -complement $\Delta\omega$. Indeed, our data indicate that both α - and ω -complementation function in mammalian cells.

Fluorescent Histochemistry for β -gal. The activity of complemented β -gal in coexpressing NIH 3T3 cells is between 25-

and 200-fold weaker than that of wild-type *lacZ* expressed by the same vector (data not shown). This reduced activity poses problems for examining complemented β -gal by standard histochemical methods with X-Gal (9). Moreover, the chromogenic X-Gal reaction product hinders simultaneous analysis of other molecules, because it prevents microscopic detection of fluorescent markers. Existing fluorescein- and rhodamine-based galactoside substrates are useful in flow cytometry and have in a few cases been applied to microscopy (10, 24).

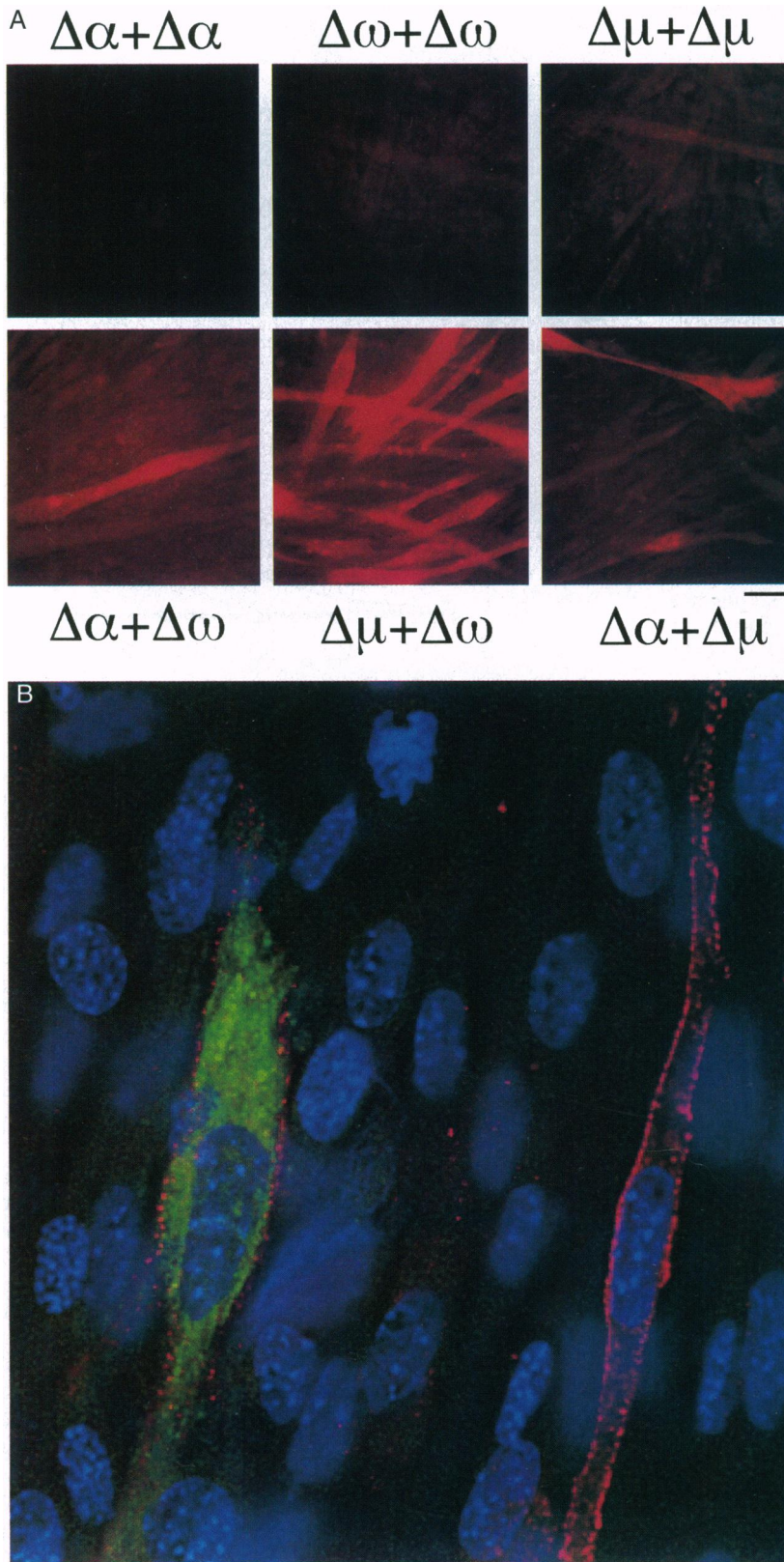


FIG. 2. Production of β -gal activity by fusion of myoblasts expressing complementing *lacZ* mutants. (A) Fluor-X-Gal substrate reveals β -gal in fused cells. Equally plated (25) one-to-one cocultures of pairs of C2F3 myoblasts were allowed to differentiate for 4 days before staining. Constructs expressed by cells in each culture are indicated. (Bar = 50 μ m.) (B) Triple-labeled image of a field of cocultured $\Delta\omega$ - and $\Delta\mu$ -expressing C2F3 cells reveals a binucleate myotube expressing β -gal formed by cell fusion (Left) and a mononucleate differentiated cell (Right) that does not express the enzyme. Fluor-X-Gal staining of β -gal activity (green) was viewed with an fluorescein isothiocyanate filter set. DAPI-stained nuclei (blue) and Cy5-immunolabeling for neural cell adhesion molecule (red) are also shown. (Scale: 12.5 mm = 10 μ m.)

However, these dyes are not able to produce a stable localized reaction product detectable by microscopy.

We therefore sought to develop a histochemical stain that, like the indigogenic X-Gal product, precipitates at the site of enzyme activity, yet unlike the product of the X-Gal reaction, produces a fluorescent signal. We found that the azo dye fast red violet LB, coupled to the enzymatic cleavage product of 5-6-X-Gal, results in a localized precipitate that fluoresces, when excited at 488 nm, with a broad emission spectrum peaking at 560 nm (not shown). The resulting product of this substrate formulation (which we designate Fluor-X-Gal, in view of its composition and fluorescent properties) may be viewed using either a standard rhodamine/Texas red (Figs. 1C and 2A) or fluorescein isothiocyanate (Fig. 2B) epifluorescence microscopy filter set. Fig. 1C demonstrates the increased histochemical signal generated with Fluor-X-Gal relative to conventional indigogenic X-Gal in replicates of the cultures shown in Fig. 1B, middle row. The enhanced sensitivity of this assay reveals that β -gal activity is detectable above background in the majority of cells infected with any pair of complementing vectors.

Single-Cell Assay of Myoblast Fusion by *lacZ* Complementation. Using Fluor-X-Gal, we tested whether complemented β -gal activity could serve as a visual indicator of myoblast fusion. C2F3 myoblast cultures (26, 27) were each infected with a vector harboring only one of the mutant *lacZ* constructs. Pairs of cultures were then plated together, the medium was changed to induce the myoblasts to differentiate, fuse and form myotubes, and the fused cultures were assayed histochemically for β -gal activity (Fig. 2A). β -gal-positive multinucleated myotubes were readily detected. As negative controls, cells containing the same mutant *lacZ* gene were cocultured and revealed no β -gal activity. Moreover, no Fluor-X-Gal positive signal was detectable in mononucleate cells in C2F3 cocultures (Fig. 2A and B) or in confluent cocultures of NIH 3T3 cells expressing complementing constructs (not shown). Thus, complementation of β -gal does not occur between adjacent transduced cells, indicating that cell fusion is a prerequisite for the formation of complemented enzyme. Fig. 2B depicts Fluor-X-Gal staining of complemented β -gal, with simultaneous fluorescent detection of nuclei and a cell-surface protein. The ability using Fluor-X-Gal to detect β -gal activity produced by cell fusion while employing multiple fluorochromes and advanced microscopy techniques is apparent.

Whole-Culture Assay of Myoblast Fusion by *lacZ* Complementation. Based on the extensive β -gal complementation observed visually in Fig. 2, we selected $\Delta\mu$ and $\Delta\omega$ for development of techniques to study the extent and kinetics of myoblast fusion by quantitative biochemical assay. A sensitive dioxetane-substrate-based chemiluminescent assay of β -gal activity in cell lysates was employed, which yields a signal more than two orders of magnitude above background in extracts of complementing fused myotubes (Fig. 3A) (11).

To determine whether the assayable activity was formed exclusively within fused cells, lysates were prepared from individual cultures transduced with different mutant constructs, and the lysates were then mixed and assayed. No significant complementation was detected in the mixed lysates, consistent with the concentration dependence of the complementation reaction and the large dilution of the peptides that occurs during cell lysis. Thus, the β -gal activity detected in lysates from complementing cocultures was derived from the fusion of intact cells. Fig. 3B shows a time course of fusion. These data show that the fusion-dependent increase in complemented enzyme activity provides a rapid and quantitative method for analysis of the kinetics of cell fusion.

Mutant-Specific Differences in Complementation. Reproducible differences in the efficiency of complementation with different pairs of *lacZ* mutants were observed, by histochemistry and by biochemical assay. Our choice of retroviral ex-

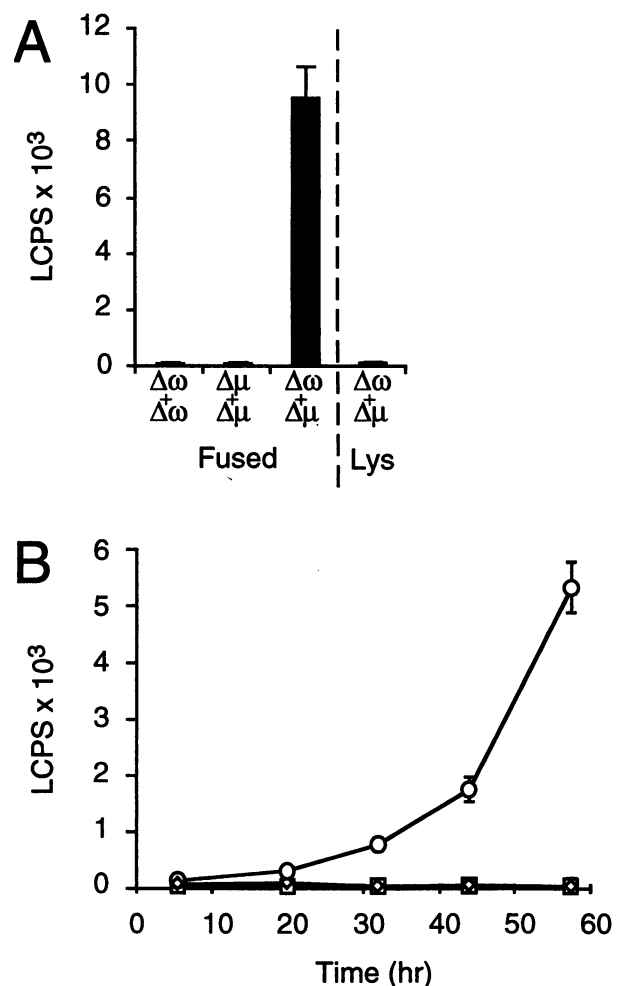


FIG. 3. Quantitative biochemical assay of complementation of β -gal determines extent and kinetics of cell fusion. (A) Chemiluminescent assay of β -gal in lysates from fusing cocultures of $\Delta\omega$ - and $\Delta\mu$ -expressing C2F3 cells. Cultures were allowed to differentiate for 4 days before cells were lysed and assayed. Assays were performed on lysates of cocultured cells that formed myotubes (Fused), as well as on a mixture of lysates from cultures expressing either $\Delta\omega$ or $\Delta\mu$ alone (Lys). Data are in luminescence counts per second (LCPS). Each value is the mean of eight replicates. Error bars show standard deviation. A representative of five experiments is shown, all of which gave similar results. (B) Time course of cell fusion by assay of complemented β -gal activity. Replicate C2F3 cocultures, as described in A, were lysed and assayed at the indicated time after exposure to low serum differentiation medium (27). Values are shown for cocultured $\Delta\omega$ - and $\Delta\mu$ -expressing C2F3 cells (\circ), and for unmixed cultures of cells expressing either $\Delta\omega$ or $\Delta\mu$ alone (\square and \diamond , respectively). Each data point is the mean of six replicates. Error bars show standard deviation.

pression system (18) and the results of Fluor-X-Gal detection (Fig. 1C) suggest that these differences are not due to wide variation in viral titer or infection efficiency, since the majority of cotransduced cells exhibited enzyme activity. Instead, as reported for *E. coli*, these disparities are likely due to mutant-specific differences in protein stability, efficiency of complex formation, or catalysis rate of the complemented enzyme (12–14, 28–35). Interestingly, the ability of a single gene product, $\Delta\mu$, to function as both α - and ω -donor also has precedent in *E. coli* (12–14) and suggests the as yet untested possibility of three-part complementation by mutants containing only intact α , ω , and μ regions, respectively. Among other applications, this might allow detection or selection of cells expressing three distinct proteins based upon a single reporter enzyme activity.

The spatial distribution of β -gal within complemented cells (either cotransduced NIH 3T3 or C2F3 cells or fused C2F3 myotubes) differed depending upon the identities of the mutant partners. Complemented β -gal formed with $\Delta\alpha$ and either $\Delta\omega$ or $\Delta\mu$ was prevalent in the nucleus, perhaps because the deletion in $\Delta\alpha$ creates or reveals a cryptic nuclear localization signal. By contrast, wild-type and $\Delta\mu/\Delta\omega$ -complemented enzymes exhibited predominantly cytoplasmic distributions in fibroblasts, myoblasts, and myotubes. Furthermore, $\Delta\mu/\Delta\omega$ complementation appeared generally more robust than $\Delta\alpha/\Delta\mu$ in syncytia formed by fusion (Fig. 2A), whereas the reverse was true for cotransduced C2F3 myoblasts (not shown) or NIH 3T3 cells (Fig. 1B). Possibly, the different efficiency of complementation in the context of a syncytium results from a combination of restricted localization of the $\Delta\alpha/\Delta\mu$ complex and domain-specific localization of gene products characteristic of individual nuclei within myotubes (36). The observed differences among mutants in complementation efficiencies and cellular distribution may make a given pair of lacZ mutants more appropriate for use in certain studies, for example $\Delta\mu/\Delta\omega$ for cell fusion, than another pair that is optimal for coexpression of genes within mononucleate cells or for potential use in studies of protein-protein interactions.

Conclusions. Our results show that the utility of *lacZ* for the study of higher organisms can be further extended in multiple new directions. The development of a fluorogenic histochemical substrate for β -gal will permit novel microscopic applications of *lacZ* in general, and of complementation in particular. As shown here, *lacZ* complementation is of use as a selectable marker for cotransduction of cells with two and possibly three vectors. The complementing properties of mutant *lacZ* genes can be exploited for a wide range of studies directed at determining whether proteins derived from two active genes are coincident or colocalized within cells. The potential application of the system to a biological problem, the microscopic detection, quantitation and kinetics of myoblast fusion is shown herein. Other applications include transgenic animals expressing complementing *lacZ* mutants from two promoters of interest, which should clearly reveal cell lineages in which the products of both genes coincide spatially and temporally. It may be possible to study protein-protein interactions *in vivo* by observing the influence (enhancement or inhibition) of domains of interest upon the complementation of peptides within chimeric proteins. Use of *lacZ* mutants engineered to contain specific sequences that direct sorting to different intracellular compartments may permit studies of translocation of proteins to be monitored in a biological manner, including not only microscopy but also quantitative and kinetic analysis.

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