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Expression of functional beta-galactosidase containing the coxsackievirus 3C protease as an internal fusion

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Alpha complementation of beta-galactosidase (ßgal) is intracistronic and requires interaction between the alpha donor region (residues 3-41) and alpha acceptor fragment (produced by M15). We have constructed two plasmids which direct the synthesis of hybrid ßgal : coxsackievirus proteins in *Escherichia coli*. One plasmid, pBD1045, encodes an enzymatically active 3C protease of coxsackievirus B3 fused between the amino-terminal 79 amino acids of ßgal (containing the alpha donor region) and amino acids 80 to 1023 (alpha acceptor region). A second plasmid, pBD1043 encodes an inactive 3C protease and results in a fusion of 260 coxsackievirus amino acids between residues 79 and 80 of the ßgal monomer. Both hybrid proteins expressed by these constructs have beta-galactosidase activity regardless of whether the viral protease (183 amino acids) is autocatalytically cleaved out of the chimeric protein (pBD1045) or remains as part of a fusion protein (pBD1043). The implications of these results for structural flexibility of the complemented beta-galactosidase enzyme are discussed.

Wildtype ßgal is a tetramer of four, non-covalently linked, subunits (1, 2). Each monomeric subunit contains 1023 amino acids encoded by the structural gene, *lac* Z (3). The enzymatic activity of the protein beta-galactosidase (ßgal), which is encoded by the *lac* Z gene of *Escherichia coli*, is widely used to monitor the regulation of gene expression. Expression of the *lac* Z gene fused to procaryotic or eucaryotic transcriptional control elements provides a simple enzymatic assay to measure the functional activity of the linked control elements without the use of radioactiveisotopes (4, 5). ßgal activity can be regulated post-translationally by protein complementation between two, non-identical and sometimes overlapping, fragments of

Abbreviations: ßgal, beta-galactosidase; CVB3, coxsackievirus B3; HRPO, horseradish peroxidase; MUG, methylumbelliferyl-ß-D-galactoside; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; X-gal, 5-bromo-4-chloro-3-indolyl-ß-D-galactoside.

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the monomeric subunit (1, 2). Alpha complemetation, usually involves the interaction of the amino-terminus of ßgal (alpha peptide residues 3-92), with a separate ßgal molecule (M15) bearing an amino-terminal deletion of residues 11-41 (6). It is also possible to replace the sequences coding for either the amino-terminal 26 residues or the carboxy-terminal 2 residues of ßgal with a foreign sequence and not impair ßgal activity (7, 8). We show here that ßgal is able to tolerate an internal fusion of 260 amino acids, which includes an autocatalytic viral protease, and still retain enzymatic activity.

METHODS

Construction of chimeric *lac Z* : 3C protease plasmids. The *Hin*fI - *Sca*I fragment from pCIIIB35 (9), encoding the carboxyl-terminal 13 amino acids of the coxsackievirus 3A protein, 22 amino acids of 3B, all 183 amino acids of the 3C protease and the amino-terminal 42 amino acids of 3D from coxsackievirus type B3 (nucleotides 5255-6034) (10), was treated with the klenow fragment of *E. coli* DNA polymerase and subcloned into the *Sau*1 site of the *lacZ* gene in pCßgal7 (fig 1A). Expression of the chimeric *lacZ : CVB3* sequence is under the control of the lambda P_R promoter and the temperature sensitive cI857 repressor encoded by pCQV2 (11) (fig 1A). When the chimeric gene is expressed in *E. coli* the 3C protease and flanking sequences are thus positioned between amino acid residues 79 and 80 of ßgal (3). The DNA encoding a mutant 3C protease was similarly cloned as a *Hin*fI - *Sca*I fragment from pC11B9 into the *Sau*1 site of pCßgal7 (fig 1). The mutation in 3C does not disrupt the reading frame of the 3C RNA but results in the insertion of four amino acids (Pro-Asp-Pro-Asp) near the 3C catalytic site between residues Gly_{166} and His_{167}. This insertion completely abolishes 3C protease activity (12). The cloning of the coxsackievirus sequences containing the wildtype and mutant 3C at the *Sau*1 site of *lacZ* resulted in plasmids designated pBD1045 and pBD1043 respectively (fig 1B & C).

Expression of chimeric ßgal : coxsackievirus proteins. DNA of plasmids pCQV2, pCßgal7, pBD1043 and pBD1045 was used separately to transform *E. coli* MC1061 (4, 8) by the calcium chloride method (13). Strain MC1061 is completely Lac^- because the entire *lacZ* gene has been removed. It is therefore a suitable host in which to examine ßgal activity without interference from endogenous ßgal alpha donor or acceptor fragments. Overnight cultures of plasmid-containg MC1061 cells were grown at 30°C in LB medium containing 100 micrograms/ml ampicillin. Fresh cultures were inoculated the following day at an $O.D._{600}$ of <0.1 and the cells grown at 30°C to an $O.D._{600}$ of 0.4. The cultures were transferred to 42°C for fifteen minutes to inactivate the cI857 repressor and then grown at 37°C for two hours. Aliquots were removed for Western blot analysis and ßgal activity assay.

Western blot analysis. Western blot analysis of total bacterial protein from induced cultures of E. coli MC1061 containing the plasmids described above was performed as previously described (14,

15). Briefly, equivalent amounts of bacterial protein, based on O.D.600, were suspended in Laemmli sample buffer and electrophoresed on a denaturing 7% PAGE, 0.5% SDS gel. Protein was transferred to nitrocellulose, blocked and probed as described (14, 15) with an anti-ßgal monoclonal antibody (Boerhinger Manheim) at 500 ng/ml. Bound monoclonal antibody was visualized with HRPO-conjugated rabbit anti-mouse IgG at 1:2500 dilution (Promega).

Enzymatic activity of chimeric ßgal : CVB3 proteins. To assess the level of ßgal activity in induced MC1061 cultures containing the various plasmid constructs, ßgal-mediated cleavage of methylumbelliferyl-B-D-galactoside (MUG) was monitored using a Dynatech MicroFLUOR reader (16). Aliquots of induced cultures of MC1061 cells containing plasmid pCQV2, pBD1043 or pBD1045 were placed in triplicate wells of a 96-well microtiter plate. To each well was added 1 drop of 0.1% SDS and 1 drop chloroform using a 200 microliter pipette tip to lyse the cells (17). The plate was agitated gently for five minutes. MUG assay mix (4:1 Z-buffer: MUG) was added in 100 microliter aliquots per well and incubated in the dark at 37°C. Fluorescence was monitored over time and in some experiments 100 microliters of 0.1 M glycine pH 10.4 was added to each well to amplify the signal prior to fluorometry. Addition of glycine terminates enzymatic activity and amplifies the MUG signal approximately four-fold.

RESULTS AND DISCUSSION

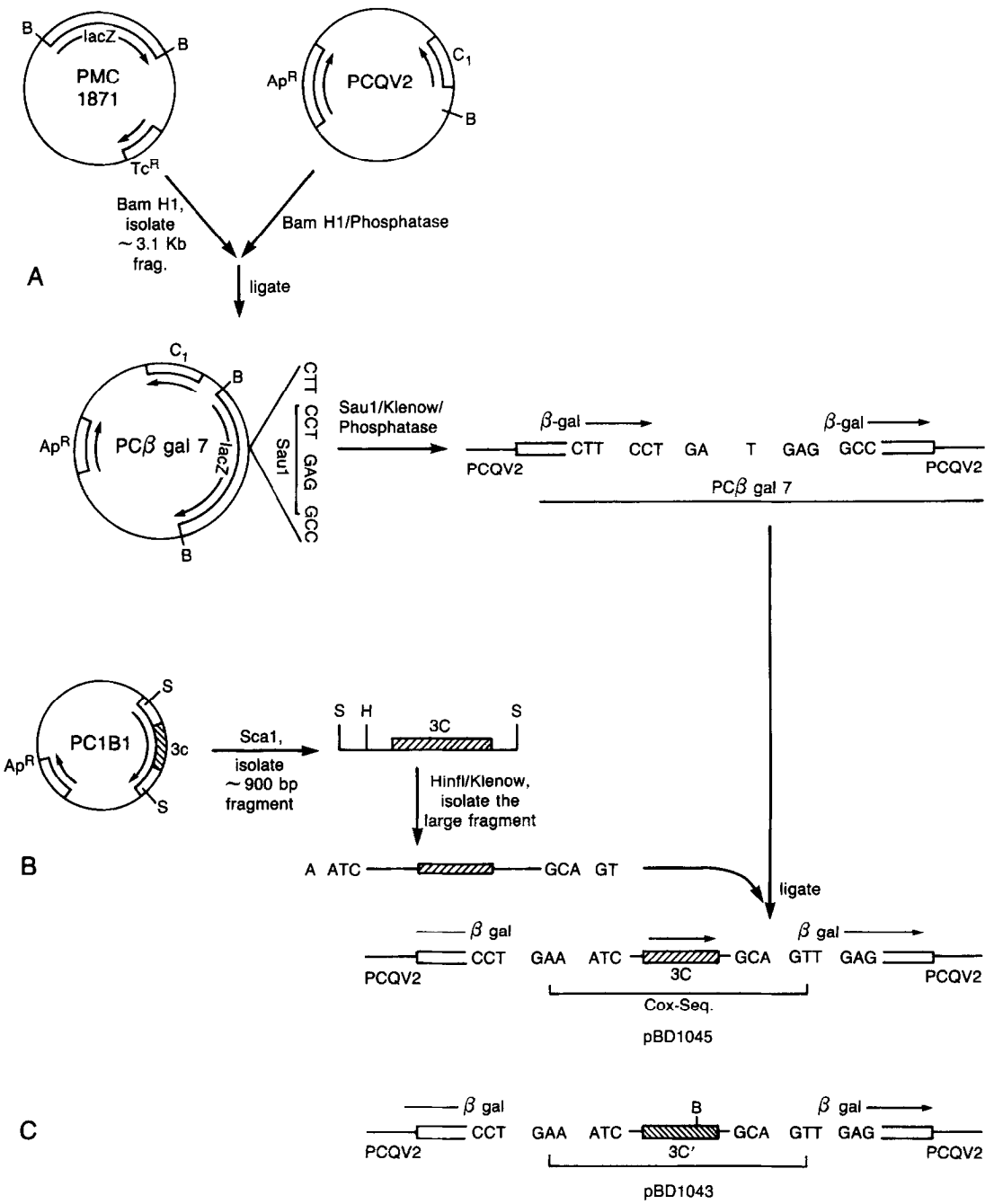
Two plasmids were constructed (Fig. 1) which direct the synthesis in *Escherichia coli* of either an active or inactive coxsackievirus 3C protease fused within beta-galactosidase. Western Blot analysis of total bacterial protein from induced bacterial cultures (Fig. 2) shows that nothing was detected in pCQV2-containing MC1061 cells (lane 1), whereas cells transformed with pCßgal7 produce ßgal which was detected as an intense band of 116 kilodaltons (lane 2). The ßgal produced by pCßgal7 also co-migrated with the ßgal protein electrophoresed as a molecular mass standard. Plasmid pBD1043 produced a chimeric ßgal : CVB3 fusion protein which migrated at approximately 145 Kilodaltons (lane 3) as expected and contains the amino-terminal 79 amino acids of ßgal (including the alpha peptide), the mutant 3C protease with flanking residues (260 amino acids) and the remainder of the ßgal monomer from amino acid 80 to 1023. The ßgal fragment detected in Fig. 2, lane 4, of approximately 112 kilodaltons results from the proteolytic processing of the chimeric ßgal : CVB3 protein by the active 3C protease contained within the fusion protein. The 112 kilodalton protein represents ßgal residues 80-1023 fused at its amino terminus to 42 residues of the coxsackievirus 3D protein left after cleavage by 3C at its own C-terminal glutamine-glycine processing site. The released

fragments, which include ßgal residues 1-79 and 13 amino acids of the CVB3 3A protein, are not detected by Western blot because the antibody used is specific for an epitope in the C-terminus of ßgal. These results indicate that the protease is autocatalytically active and processing at both 3C termini clevage sites appears to be complete.

The data in figure 3 indicate that fluoresence due to the MUG reagent alone or MUG reagent added to MC1061 cells containing plasmid pCQV2 is negligible and does not change significantly with amount of bacterial protein (fig 3) or over time (fig 4) up to 16 hour (data not shown). In contrast, significant ßgal activity is detected in induced MC1061 cultures which carry plasmid pBD1043 or pBD1045. The increase in ßgal activity in these cultures is approximately linear with respect to increasing amount of bacterial protein or time of incubation. The ßgal activity exhibited by the wildtype enzyme, expressed by plasmid pCßgal7, is several hundred-fold higher than that of either chimeric enzyme (data not shown). This is not unexpected since wildtype ßgal accumulates in MC1061 cells at higher levels than either of the two chimeric ßgal enzymes (Figure 2) and may also have a higher specific activity.

Fig. 1. Construction of recombinant plasmids which express beta-galactosidase and chimeric CVB3 derivatives. (A) Construction of pCßgal7. A *Bam*HI fragment containing the coding sequence of ßgal was isolated from plasmid pMC1871 (4) and cloned into the *Bam*HI site of plasmid pCQV2 (15). A clone containing the ßgal sequence in the appropriate orientation with respect to the lambda P_R promoter was identified. In pCßgal7, expresssion of the *lacZ* gene is regulated by the lambda cI857 repressor. Also shown are the nucleotide sequences for the restriction endonuclease *Sau*1 and the sequences generated after digestion with *Sau*1 and treatment with klenow DNA polymerase. (B) Construction of plasmid pBD1045 which directs expression of a chimeric ßgal : CVB3 protein containing an active CVB3 3C protease. The plasmid PC1B1, containing CVB3 nucleotides 4947 to 6773 (10), was digested with *Sca*1. A 900 basepair *Sca*1 fragment containing the 3C protease coding sequence was digested with *Hin*fI and treated with klenow DNA polymerase. The large fragment containing the 3C coding sequence was isolated and ligated to pCßgal7 which had been linearized with *Sau*1 and treated with alkaline phosphatase and klenow DNA polymerase. The coding sequence of the 3C protease and its orientation are represented by the shaded box and arrow respectively. Nucleotides surrounding the CVB3 sequence are shown in triplets to indicate the reading frame of the *lacZ* gene is maintained. (C) Map of plasmid pBD1043. Construction of pBD1043 was identical to that of pBD1045 except that plasmid pC11B9 was used instead of plasmid pC1B1. Plasmid pC11B9 contains a 12 basepair linker (GGATCCGGATCC) inserted within the 3C coding sequence in plasmid pC1B1 (12). The letter "B" represents the *Bam*HI site in pC1B1 where the linker was inserted. The result of the linker mutation is the insertion of four amino acids, in-frame with 3C, during translation which abolishes proteolytic activity of the 3C protease (12).

The difference in βgal activity observed between the hybrid protein which contain the active 3C protease (pBD1045) and the inactive 3C protease (pBD1043) is relatively small but consistent. We cannot rule out that the slight differences in enzymatic activity may also be due to variation in the level of chimeric βgal protein synthesis although no significant differences were detected by Western blot



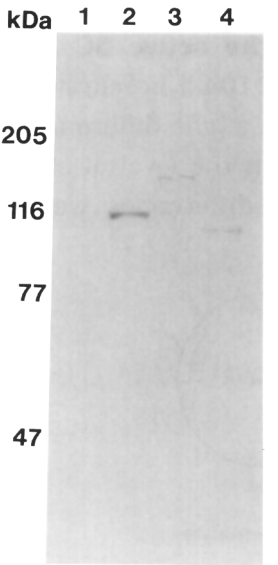
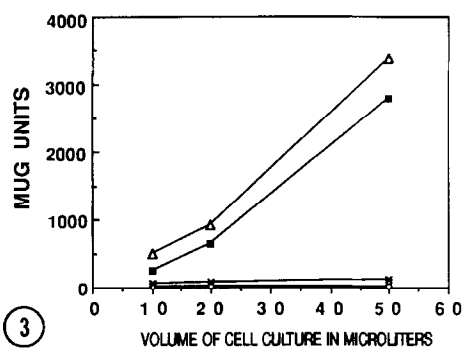


Fig. 2. Western Blot analysis of total bacterial protein from plasmid-containing cultures of MC1061 cells induced as described in the text. Equivalent volumes of induced *E. coli* cultures were suspended in Laemmli sample buffer and electrophoresed on a denaturing 7% PAGE-SDS gel. Total bacterial protein was transferred to nitrocellulose, blocked and probed as described (14) using an anti-ßgal monoclonal antibody (Boerhinger Manheim) at 500 ng/ml. Bound monoclonal antibody was visulized with peroxidase conjugated rabbit anti-mouse IgG at 1:2500 dilution (Promega). Lane 1: MC1061 cells containing plasmid pCQV2, Lane 2: MC1061 cells containing pCßgal7, Lane 3: MC1061 cells containing plasmid pBD1043, Lane 4: MC1061 cells containing plasmid pBD1045. Molecular mass standards are indicated at the left in kilodaltons (kDa).

analysis. By comparing the levels of protein synthesis (Fig. 2) with respective ßgal activity (Fig. 3 and 4), it is clear that the specific activities of the two chimeric enzymes are similar to each other but are reduced in comparison to wildtype ßgal. It is therefore unlikely that residual uncleaved ßgal (from pBD1045), if undetectable by Western Blot, could account for all of the enzymatic activity observed in cultures where clevage appears complete by Western blot analysis. Therefore, ßgal activity in induced pBD1045 cultures is likely a result of alpha complementation after protease cleavage of the ßgal : 3C fusion protein.

The results shown in Figures 3 and 4 indicate ßgal activity for both hybrid proteins is similar irrespective of cleavage of the fusion protein by the internal 3C protease. These experiments clearly indicate that ßgal can tolerate the insertion of at least 260 amino acids at an internal site between the alpha peptide and alpha acceptor regions and still retain enzymatic activity. This result is also



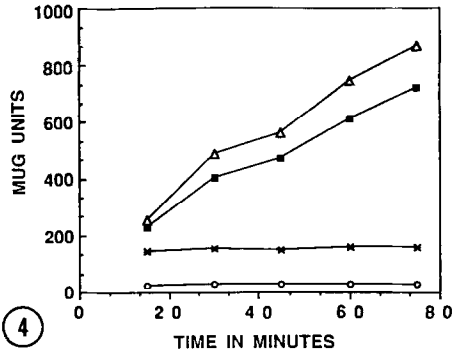


Fig. 3. ßgal activity is directly related to the volume of cell culture assayed. Increasing amounts of bacterial cultures, induced as described in the text, were assayed in triplicate in a 96-well microtiter plate. One drop each of 0.1% SDS and chloroform was added to each well using a 200 microliter pipeteman. The plate was gently agitated for five minutes followed by the addition of 100 microliters of MUG assay mix (4:1 Z-buffer:MUG) per well. After incubation in the dark at 37°C, 100 microliters of 0.1 M glycine, pH 10.4 was added to each well and fluoresence determined using a Dynatech MicroFLUOR reader. The data are expressed as the average fluoresence of triplicate wells in arbitrary MUG units. Z-buffer is 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$, 50 mM 2-mercaptoethanol, pH 7.0. MUG reagent was prepared as a 0.37 mM stock in water and stored in the dark at 4°C. MUG reagent only, ——o—— ; MC1061 cells containing pCQV2, ——x—— ; MC1061 cells containing pBD1043, ——■—— ; MC1061 cells containing pBD1045, ——△——.

Fig. 4. Beta-galactosidase activity of chimeric ßgal : CVB3 protein is similar over time. 50 microliter aliquots of MC1061 cultures, induced as described in the text, were assayed in triplicate in a 96-well microtiter plate. The assay was performed as described in the legend for Fig. 3 except that 50 microliter aliquots of cell cultures were used and fluoresence was monitored, without the addition of 0.1 M glycine pH 10.4, at the indicated times. The data are expressed as the average fluoresence of triplicate wells in arbitrary MUG units. MUG reagent only, ——o—— ; MC1061 cells containing pCQV2, ——x——; MC1061 cells containing pBD1043, ——■—— ; MC1061 cells containing pBD1045, ——△——.

supported by a recent report of the insertion of a ten residue cleavage site inserted after residue 79 of ßgal which does not abolish ßgal activity (16). These results indicate the flexibility of the region around amino acid 79 within ßgal. The apparant loss of ßgal activity due to cleavage of the chimeric ßgal protein by the HIV protease in *trans* (16), differs from the findings reported here possibly because of the greater sensitivity of a fluorometric assay for ßgal activity in comparison to the one employed by Baum et al. which uses X-gal. It is also possible that we detect ßgal activity after protease cleavage because the 35 coxsackievirus residues, which remain attached to the amino-terminal 79 residues of ßgal after 3C cleavage, serve to stabilize

the ßgal peptide and enhance complementation and the dependant ßgal activity.

The utility of ßgal is due, in part, to its flexibility in being able to retain enzymatic activity even when fused, at its amino or carboxy terminus, to large peptides. In this instance we have placed 260 amino acids from CVB3, which include a *cis*-acting viral protease, at a site within ßgal. In a coxsackievirus-infected cell, the positive-strand RNA genome of the virus directs the production of a polyprotein which is proteolytically processed by two, cis-acting, virally encoded proteases designated 2A and 3C (19, 20). The 3C protease autocatalytically cleaves itself out of the viral precursor polyprotein between glutamine and glycine pairs present 183 amino acids apart and thus creates its own amino and carboxy terminii (19, 20). We have shown that enzymatically active ßgal is produced, presumably by alpha complementation, after excision of the internally fused protease. Alpha complemented enzyme, produced by mixing ßgal peptide fragments derived from mutated bacteria or from cyanogen bromide cleavage of purified ßgal, usually contains some duplicated residues carried by both fragments. Our results show that the duplication of these residues is not absolutely required to produce active ßgal by alpha complementation since no duplicated residues are present in the complemented enzyme produced after 3C cleavage. Furthermore, an insertion of 260 amino acids within the ßgal monomer does not prevent the formation of active ßgal enzyme even when the 183 amino acids of the 3C protease are not removed. It is possible that the fused viral protein acts as a flexible hinge region, which allows the alpha peptide to occupy its normal position in the ßgal tetramer such that enzymatic activity is retained.

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