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Expression of a preproinsulin–β-galactosidase gene fusion in mammalian cells

(simian virus 40 vectors/transfection/COS-7 monkey kidney cells/*Escherichia coli*)

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ABSTRACT **As an approach to the study of mammalian gene expression, the promoters and translation initiation regions of the rat preproinsulin II and the simian virus 40 early genes were fused to the structural gene of *Escherichia coli* β-galactosidase, a sensitive probe for gene expression. These fusions were introduced into COS-7 cells, a simian virus 40 large tumor-antigen-producing monkey kidney cell line, where they directed the synthesis of enzymatically active hybrid β-galactosidase proteins. Conditions for transfection were varied to optimize the expression of β-galactosidase activity in the transfected cells. The pH optimum of this activity was found to be 7.0, the same as that of native *E. coli* β-galactosidase and distinct from the major lysosomal "acid" β-galactosidase. The fused preproinsulin–β-galactosidase was further characterized by gel electrophoresis of nondenatured cell extracts stained by a fluorogenic substrate and by immunoprecipitation and gel electrophoresis of ^{3}H-labeled cell proteins. These results all indicate that fully active tetrameric β-galactosidase hybrids can be produced in mammalian cells. The expression of preproinsulin–β-galactosidase activity was measured in the presence of high glucose, insulin, dexamethasone, or epidermal growth factor but no regulatory changes were observed.**

Gene fusions utilizing the *Escherichia coli lacZ* structural gene provide an especially suitable method for studying gene expression and regulation (1). Its product, β-galactosidase, can be assayed readily and does not require its NH_2-terminal amino acids for enzymatic activity (2, 3). Fusions lacking NH_2-terminal β-galactosidase gene codons can utilize not only the promoter and transcriptional regulatory elements but also the translational initiation and NH_2-terminal amino acid codons from other genes joined to the *lacZ* gene and result in the formation of enzymatically active hybrid proteins. Such β-galactosidase gene fusions have been used in bacteria and yeast for mapping and orienting genes and for locating their promoters, regulatory sites, coding regions, terminators, and other genetic elements (4–6). *LacZ* fusions also allow the use of *lac* operon genetic techniques as well as providing a convenient marker for subcellular localization (7) and purification (5, 8) which can be used for antibody induction (9) or amino acid sequence studies (10).

In the present study we investigated the feasibility of using *lacZ* translational gene fusions to study gene expression in mammalian cells. The rat preproinsulin II and the simian virus 40 (SV40) gene controlling elements were fused to *lacZ* and were found to direct the expression of enzymatically active β-galactosidase proteins in COS-7 monkey kidney cells.

MATERIALS AND METHODS

Plasmid DNA. DNA cloning methods have been described elsewhere (11). Plasmids were propagated in *E. coli* strains M182 [Δ(*lacIPOZY*) *X74*, *galK*, *galU*, *strA*] (12) and GM48 (*dam*$^-$) (13). The λrI2 clone was kindly provided by A. Efstratiadis (designated λrI22 in ref. 14). pSV2-β-globin (15) was generously provided by P. Berg.

Cell Culture and Transfection. COS-7 cells from Y. Gluzman were obtained via E. Kieff and maintained as described (16). Transfection procedures were based upon those of Gluzman (16) and Wigler *et al.* (17). In initial experiments, 100 ng of plasmid DNA and 20 μg of salmon sperm carrier DNA were precipitated by the calcium phosphate method of Graham and Van der Eb (18) and added to the medium on fresh monolayers of 6×10^5 cells per 100-mm plate. After 4 hr at 37°C, the medium was changed. Subsequent experiments incorporated the following changes: plasmid DNA concentration was increased to 50 μg per plate, salmon sperm DNA was omitted, incubation time with the calcium phosphate/DNA precipitate was increased to 18 hr, and 1 ml of 25% (vol/vol) glycerol in medium was added (to yield 2.5% glycerol) for 1 min at the end of this period (19).

Extraction and Assays. The cells were rinsed with 10 ml of phosphate-buffered saline (150 mM NaCl/10 mM sodium phosphate, pH 7.4). Then, 300 μl of buffer H (250 mM sucrose/100 mM sodium phosphate, pH 7.5) was added to the plate. Cells were transferred to a 1.5-ml tube and sonicated (Sonicator Cell Disrupter, Heat System/Ultrasonics, Plainview, NY) for 20 sec on ice and centrifuged for 10 min at 10,500 × *g*. In later experiments cells were disrupted by freeze-thawing three times in a dry ice/ethanol bath and a 37°C water bath and centrifuged as before.

For assaying β-galactosidase, 20–100 μl of cell extract supernatant or *E. coli* β-galactosidase (3.73 g/liter) (Millipore, 67 units/mg) was added to pH 7.5 buffer Z (10 mM KCl/1 mM $MgSO_4$/50 mM 2-mercaptoethanol/100 mM sodium phosphate) (ref. 20, pp. 352–355) to 1 ml total volume and preincubated at 37°C for 5 min [pH 7.5 was used to maximize the discrimination against the endogenous "acid" β-galactosidase (see Fig. 4)]. Then 200 μl of *o*-nitrophenyl β-D-galactopyranoside (Sigma, 4 g/liter in 100 mM sodium phosphate, at pH 7.5) was added and incubation was continued at 37°C until a detectable yellow color was formed. The reaction was terminated by the addition of 500 μl of 1 M Na_2CO_3 and absorbance at 420 nm was measured. β-Galactosidase activity was expressed as units (nmol of *o*-nitrophenol formed per min) per mg of protein (21).

RESULTS

Construction of the Gene Fusions. The promoter and translation initiation region of the rat preproinsulin II and the SV40 early gene were fused to an abbreviated *E. coli lacZ* gene as

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Abbreviation: SV40, simian virus 40.

shown in Fig. 1. The abbreviated *lacZ* gene contains the coding region for the 1,023-amino acid β-galactosidase monomer (22) starting at the 10th amino acid codon.

The DNA sequence of the pDN26 preproinsulin–β-galactosidase fusion (Fig. 1*i*) encodes a fused protein that is nearly identical to *E. coli* β-galactosidase. Only nine NH_2-terminal amino acids of wild-type *E. coli* β-galactosidase [including the initiator formylmethionine residue which is cleaved off post-translationally (8, 22)] have been deleted (3) and these are replaced by seven amino acids, the first five derived from the NH_2 terminus of preproinsulin (14) and the two others from the fusion joint:

E. coli: M T M I T N S L A V V L . . .

Hybrid: M A L W I N P V V L . . .

The net charge of the hybrid protein thus remains the same, and its NH_2-terminal sequence is similar in composition to that of the wild-type enzyme.

pMC1924 has the SV40 early gene promoter joined to the

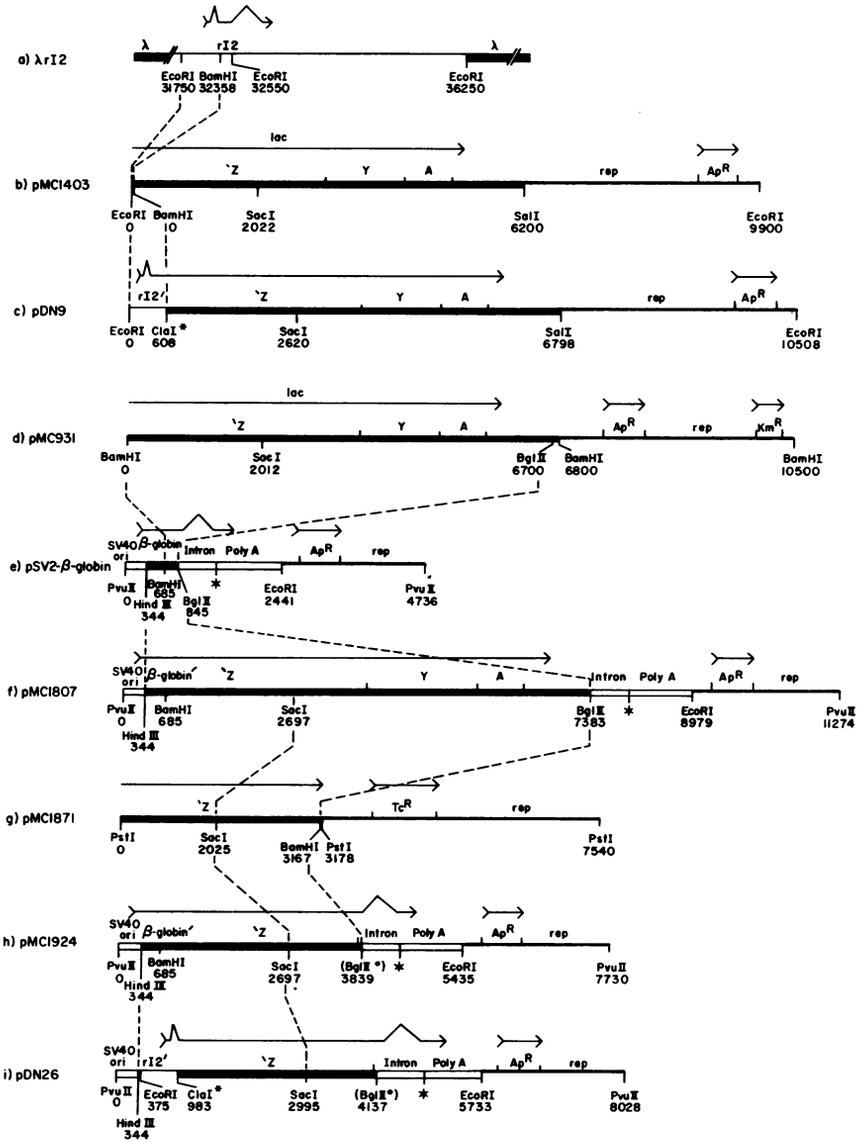

FIG. 1. Construction of rI2–*lacZ* (pDN26) and SV40–*lacZ* (pMC1924) gene fusions. λrI2 (*a*) is an *Eco*RI fragment containing the rat preproinsulin II gene (thin lines) ligated to the arms of phage λ Charon 4A (parallel lines) (14). pMC1403 (*b*) contains an abbreviated β-galactosidase ('Z) (thick lines) structural gene whose coding region starts in the 10th amino acid codon of β-galactosidase. The rat preproinsulin II controlling sequences on the 608-base-pair *Eco*RI–*Bam*HI fragment from λrI2 were joined to the *lacZ* segment of *Eco*RI/*Bam*HI cleaved pMC1403 (3) DNA to form pDN9 (*c*). To align the amino acid codons in frame, the *Bam*HI ends were filled in to form blunt ends prior to ligation. This duplicates the G-A-T-C sequence to create an A-T-C-G-A-T *Cla* I site (labeled as *Cla* I* in the figure). This site was verified by cleaving with *Cla* I after growth in a *dam*⁻ (13) *E. coli*. The SV40 early gene controlling region was joined to *lacZ* by inserting the abbreviated *lacZ* gene from pMC931 (*d*) into pSV2-β-globin (*e*) with *Bam*HI and *Bgl* II to form pMC1807 (*f*). pSV2-β-globin has the SV40 origin and early promoter preceding the cDNA coding for rabbit β-globin (15). * denotes the *Sau*3A junction between the SV40 intron and polyadenylylation sequences located after the β-globin segment. The DNA sequences beyond *lacZ* were removed by replacing the *Sac* I–*Bgl* II fragment of pMC1807 with the *Sac* I–*Bam*HI fragment of pMC1871 (*g*) to form pMC1924 (*h*). pMC1871 has a shortened *lac* sequence with a *Bam*HI site located after the β-galactosidase encoding region (4). *Bgl* II° denotes the ligation junction between the *Bgl* II and *Bam*HI ends. pMC1924 has the SV40 origin of replication and early promoter followed by the NH_2-terminal coding region of the β-globin cDNA fused in frame to *lacZ* codons to encode a hybrid β-globin–β-galactosidase protein. The rI2–*lacZ* gene fusion on pDN9 was joined to the SV40 origin of replication and intron plus polyadenylylation segments on pMC1924 by joining an *Eco*RI–*Sac* I fragment of pMC1924, with a 31-base-pair *Hin*dIII–*Eco*RI fragment from pBR322 as a linker to form pDN26 (*i*).

translation initiation site and first 298 nucleotides of the coding sequence of a rabbit β-globin cDNA clone (15) which are joined in phase to the 10th codon of β-galactosidase as in pDN26. These fusions were constructed with the pSV2–β-globin plasmid vector (15). The resulting plasmid contains a SV40 intron and polyadenylylation site just after the *lacZ* gene segment and the SV40 origin of replication.

Transfection and Extraction. The rI2–*lacZ* fusion on pDN26 and the SV40–*lacZ* fusion on pMC1924 were used to transfect COS-7 cells. COS-7 cells produce SV40 large tumor antigen which directs the replication of these plasmids. The copy number of the pMC1924 plasmid in transfected cells was measured by gel electrophoresis and Southern blot analysis (23) of low molecular weight DNA (24). Approximately 10^4 copies per cell were detected (data not shown). However, the copy number per transfected cell is actually higher because only 15–90% of the cells have been reported (25–27) to take up DNA.

In the initial experiments, transfection with the SV40–*lacZ* plasmid DNA yielded 0.70 unit of β-galactosidase per mg of extracted protein, compared to 0.62 unit for mock transfection. The same background levels were obtained when transfection was carried out with pSV2–β-globin DNA (Fig. 1*e*), which lacks the *lacZ* sequence but contains the same SV40 sequences as does the SV40–*lacZ* fusion plasmid. Decreased β-galactosidase expression was seen when the cells were grown for longer or shorter periods of time after transfection (Fig. 2) and when the DEAE-dextran method (25) was used instead of the calcium phosphate method (17) (decrease by a factor of 0.5). Changes that increased β-galactosidase expression included: increasing the DNA concentration from 100 ng to 50 μg per plate, using the rI2–*lacZ* fusion instead of the SV40–*lacZ* fusion (50% increase), increasing the time of incubation with the calcium phosphate/DNA precipitate from 4 to 18 hr (2-fold), and shocking the cells with a 2.5% glycerol overlay (19). No change was detected when the cells were shocked with 10% dimethyl sulfoxide for 30 min (28) instead of with 2.5% glycerol. Higher levels of β-galactosidase expression were obtained with DNA preparations freed from RNA and low molecular weight molecules by passage through a Bio-Gel A-1.5m (Bio-Rad) column.

The extraction procedure was altered to minimize the contribution of the endogenous β-galactosidase activity. Initially, sonication was used to open the cells. Because a large proportion of the endogenous β-galactosidase was expected to be in the lysosomes (29, 30), an alternative procedure of freeze-thawing was used in an attempt to minimize the disruption of the lysosomes. Centrifugation of the freeze-thaw-disrupted cells removed almost half the endogenous β-galactosidase activity and approximately 40% of the total cellular protein without affecting the β-galactosidase retained in the supernatant. This resulted in a higher specific activity of the induced β-galactosidase in the extract. These expression and detection improvements yielded 275 units of β-galactosidase per mg of protein above the background. This represents 0.26% of the protein in the cell extract or approximately 3×10^6 molecules of β-galactosidase per transfected cell, assuming the specific activity to be the same as that of the wild-type *E. coli* β-galactosidase (20).

Characterization of the Hybrid β-Galactosidase. To demonstrate that there was not a coincidental increase in the production of endogenous β-galactosidase(s), the β-galactosidase activity was characterized in terms of its pH optimum, electrophoretic mobility, and immunoreactivity.

The β-galactosidase activities of extracts of transfected and mock-transfected cell as well as purified *E. coli* β-galactosidase were assayed at various pH values (Fig. 3). Mock-transfected cell extracts had a β-galactosidase optimum at pH 4.0, corresponding to that of the endogenous major acid β-galactosidase activity (30, 31). Purified *E. coli* β-galactosidase has a pH optimum of 7.0 (32). The pH curve of the transfected cell extract also had a large peak of activity with a pH optimum of 7.0, corresponding to the induced hybrid β-galactosidase, and a small shoulder at pH 4.0, corresponding to the lysosomal acid β-galactosidase.

Because the fused preproinsulin–β-galactosidase was predicted to have the same charge as *E. coli* β-galactosidase and a nearly identical sequence, it would be expected to have the

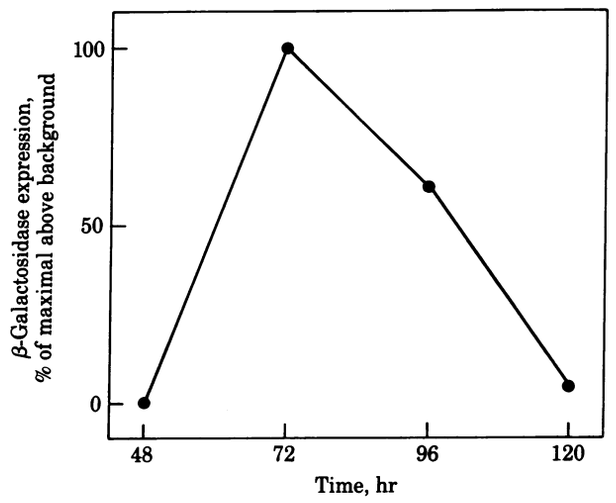

FIG. 2. β-Galactosidase expression as a function of time after transfection. Cells were transfected with pMC1924 and assayed, after the appropriate time, under the initial conditions described in *Materials and Methods*. Points represent the compilation of three experiments. Results are expressed as the β-galactosidase expression in the transfected cells minus the expression in the mock-transfected cells relative to the maximal difference observed.

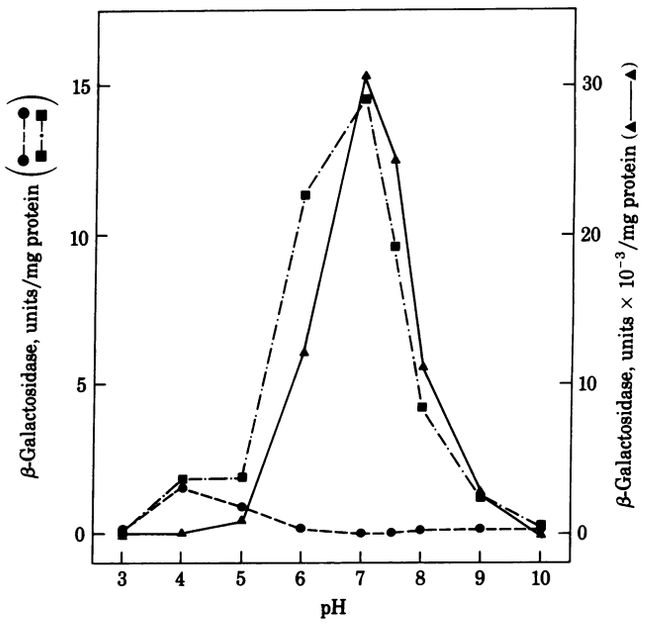

FIG. 3. pH dependence of β-galactosidase expression. Extracts of cells transfected (■) with 50 μg of pDN26, mock-transfected cells (●) and purified *E. coli* β-galactosidase (▲) in buffer H were assayed at the pH values indicated. The pH dependence of β-galactosidase was assayed in citrate buffer Z (10 mM KCl/1 mM $MgSO_4$/50 mM 2-mercaptoethanol/50 mM sodium phosphate/50 mM citric acid brought to the desired pH with NaOH.

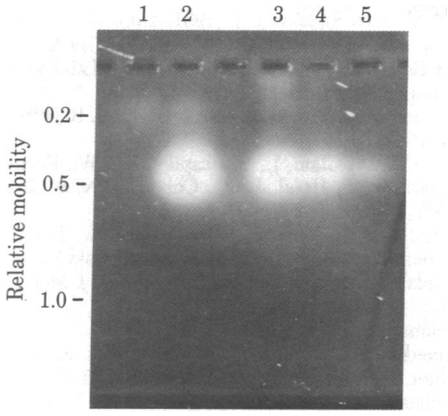

FIG. 4. Electrophoresis of cell extracts and *E. coli* β-galactosidase. Nine volumes of cell extract or *E. coli* β-galactosidase in buffer H was mixed with 1 vol of 25% glycerol/0.2% bromophenol blue, electrophoresed in a 0.5% agarose gel (type I agarose, Sigma) in 10 mM KCl/1 mM $MgSO_4$/100 mM sodium phosphate, pH 7.0, at 15 mA/cm for 3.5 hr. Gels were stained with the fluorogenic substrate 4-methylumbelliferyl β-D-galactopyranoside at 1 mM in buffer Z (pH 4.0) for 2.5 hr at 37°C and photographed in UV light with a yellow filter (no. 16) and a Polaroid camera with type 55 film. Lanes: 1, mock-transfected extract; 2, pDN26-transfected extract; 3, 4, and 5, *E. coli* β-galactosidase at 117, 23, and 2.3 milliunits, respectively.

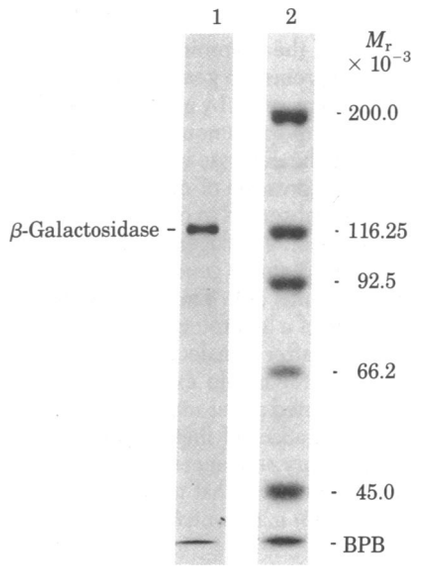

FIG. 5. Immunoprecipitation of hybrid β-galactosidase. Cells transfected as in later experiments with 50 μg of pDN26 were rinsed with phosphate-buffered saline after 48 hr and incubated for 24 hr with 4 ml of leucine-free RPMI 1640 (4.5 mM glucose; GIBCO) to which was added [^{3}H]leucine (120 μCi/ml; 1 Ci = 3.7 × 10^{10} Bq; Amersham). Extracts were passed through a 23 × 1 cm Sepharose 4B (Pharmacia) gel filtration column with 0.1 M NTM buffer (100 mM NaCl/10 mM sodium acetate/100 mM 2-mercaptoethanol/10 mM Tris, pH 7.6). Fractions with β-galactosidase activity were pooled and adsorbed on a DE52 (Whatman) ion exchange column and eluted with a gradient of 0.1 M NTM to 0.4 M NTM (0.1 M NTM plus 300 mM NaCl) (ref. 20, pp. 398–404). Pooled fractions with β-galactosidase activity (7.2 ml) were mixed with 800 μl of 10× detergent buffer (5% Triton X-100/5% sodium deoxycholate/1.5 M NaCl, 1 M sodium phosphate, pH 7.5) and 10 μl of anti-β-galactosidase antibody purified from rabbit anti-β-galactosidase antiserum (Cappel Laboratories, Cochranville, PA) by elution from a 1.5-ml affinity column of 35 mg of *E. coli* β-galactosidase coupled to Affi-Gel 15 (Bio-Rad) in phosphate-buffered saline at pH 2.5 containing 1 M glycine (33). After a 16-hr incubation at 4°C, 10 μl of a 10% suspension of heat-killed, formalin-fixed *Staphylococcus aureus* (IgGsorb, Enzyme Center, Boston) was added, and the mixture was incubated for 3 hr at 4°C. After six washes in 1× detergent buffer, the proteins were dissolved and electrophoresed through a 6% polyacrylamide gel (34). Gels were fixed, stained, impregnated with 2,5-diphenyloxazole (New England Nuclear), dried, and fluorographed on XAR-5 film (Eastman Kodak) (35). Lanes: 1, pDN26-transfected extract; 2, Coomassie blue-stained molecular weight standards.

same electrophoretic mobility. Accordingly, extracts from mock-transfected and rI2–*lacZ* transfected cells were electrophoresed in 0.5% agarose gels (Fig. 4) and these were stained with the fluorogenic substrate 4-methylumbelliferyl β-D-galactopyranoside to reveal the positions of active β-galactosidase. Extracts from mock-transfected cells gave a single band with a relative mobility of 0.2. Purified *E. coli* β-galactosidase produced a single band that migrated faster than the endogenous enzyme, with a relative mobility of 0.5. Extracts of transfected cells yielded two bands, the slower having the same mobility as the activity in the mock-transfected cell extract and the faster having the same mobility as the purified *E. coli* enzyme.

To validate further the production of a hybrid β-galactosidase, transfected cells were grown in the presence of [^{3}H]leucine to label the proteins synthesized 48–72 hr after transfection. Extracts were partially purified, immunoprecipitated with anti-β-galactosidase antiserum, electrophoresed, and fluorographed (Fig. 5). This procedure yielded a single band that migrated with *E. coli* β-galactosidase and was not present when the antiserum was preabsorbed with *E. coli* β-galactosidase.

Search for Regulation. β-Galactosidase expression was used as an indicator of regulation of the preproinsulin–β-galactosidase gene fusion (pDN26). Cells were transfected with rI2–*lacZ* and grown for 2 days in medium containing 4.7 mM glucose. The cells were subsequently grown in the presence of either 4.7 mM glucose or 16.5 mM glucose for 26 hr and assayed. No significant differences in the levels of β-galactosidase expression were seen. Addition of insulin (1 μg/ml), dexamethasone (1 μM), or epidermal growth factor (500 ng/ml) at 20 hr prior to harvest also had no significant effect on β-galactosidase expression.

DISCUSSION

These results demonstrate that bacterial β-galactosidase can be expressed as a fusion product in animal cells at sufficiently high levels for convenient assays. Peak levels of enzyme activity were obtained 72 hr after transfection and were approximately 300-fold above background in some experiments. The hybrid enzyme could be distinguished clearly from the endogenous β-galactosidase on the basis of its pH optimum (Fig. 3), apparent molecular size on $NaDodSO_4$ gel electrophoresis (Fig. 5), and charge and tetrameric structure as assessed by electrophoresis (Fig. 4), as well as sucrose density gradient centrifugation (data not shown). In all these respects it resembled the parental wild-type β-galactosidase of *E. coli*, from which it differed only minimally at its NH_2 terminus. The hybrid enzyme also was precipitated by antibodies against *E. coli* β-galactosidase and displaced by the *E. coli* enzyme, a further indication of its close similarity to the native enzyme. It thus seems reasonable to conclude that the level of enzymatic activity achieved is a valid measure of hybrid protein production. While this work was in progress, two other groups reported the production of β-galactosidase activity in animal cells but did not characterize this activity further (36, 37). These results thus confirm and extend their findings, indicating that the observed changes in β-galactosidase activity result from production of the bacterial form of the enzyme and that peak levels of induction are obtained 72 hr after transfection in several systems.

The expression of the fusion protein is apparently not due to

titration of a negative control factor by the high copy number in the COS-7 cells of the preproinsulin control region because the endogenous preproinsulin gene is not detectably expressed upon transfection as measured by insulin radioimmunoassay (data not shown). The complete genomic rat preproinsulin II gene has been reported to be expressed in transfected COS cells (38). Similar anomalous expression of other cloned genes such as β-globin (39) has been observed in cell lines that do not normally express the corresponding endogenous genes; in erythroid cells, correct regulation of β-globin genes has been obtained for some but not all transformants (40). This expression is also not likely to be due to the use of a heterologous species because β-globin genes are expressed after transfection of both homologous and heterologous species. Possible explanations for inappropriate expression of transfected genes may include alterations in chromatin structure (41), a lack of methylation (42), or the absence of additional regulatory sequences. An additional possibility in the system we have used is that nearby SV40 72-base-pair enhancer sequences (43) may contribute to the activation of this gene.

Insulin expression is normally regulated in pancreatic beta cells. High glucose concentration increases preproinsulin mRNA levels (44) and stimulates insulin biosynthesis (45) and secretion (46) in normal or cultured pancreatic islets. Injected insulin decreases the circulating levels of endogenous insulin and C-peptide *in vivo* (47) whereas dexamethasone inhibits insulin release from cultured pancreatic monolayers (48). Neither these compounds nor epidermal growth factor was found to influence preproinsulin–β-galactosidase expression in this system. The lack of regulation of preproinsulin–β-galactosidase expression is not surprising because the COS-7 cells are not differentiated to produce insulin and thus may lack insulin-controlling signals. Alternatively, the preproinsulin–β-galactosidase hybrid may be transcribed from the adjacent SV40 early promoter instead of the insulin gene promoter. However, Lomedico (38) has shown that insulin expression from a vector with an adjacent SV40 early promoter is initiated from the insulin promoter. In addition, Hall *et al.* (37) have recently found that β-galactosidase fusions to the long terminal repeat of mouse mammary tumor virus on a similar SV40 vector apparently use the virus promoter because their expression is inducible by glucocorticoids in Ltk^- cells. Thus, to detect insulin gene regulation and to identify specific regulatory factors it may be necessary to use cells differentiated to express insulin.

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