Expression of Wild-Type and Mutant Murine α-Lactalbumin cDNAs in Baculovirus-Infected Insect Cells

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Production of wild-type and mutant mouse α-lactalbumins in baculovirus-infected insect cells is described. The efficiency of this expression system and its adequacy for studying the structure–function relationship in this subunit of lactose synthase are compared to those previously reported. Enzymatic characterization of the recombinant proteins is reported. The wild-type protein behaves as milk-purified α-lactalbumin, and one of the mutants was found to be unable to induce lactose synthesis *in vitro*.

Keywords: *α-Lactalbumin; baculovirus; expression; lactose synthase; mutagenesis*

INTRODUCTION

α-Lactalbumin (αlac) is one of the major whey proteins of milk. This calcium metalloprotein promotes lactose synthesis in the mammary epithelial cells by interacting with and modifying the substrate specificity of a UDPgalactosyltransferase (EC 2.4.1.22) (Ebner and Brodbeck, 1968). It has sequence similarity with *c*-type lysozymes, and the similar organization of both genes suggests a common ancestor (Quasba and Safaya, 1984). However, studies aimed at a better understanding of the evolution of αlac from lysozyme and of its structure–function relationship have just recently started with the development of efficient expression systems. These include the use of *Escherichia coli* (Wang et al., 1989; Kumagai et al., 1991, 1992; Grobler et al., 1994), *Saccharomyces cerevisiae* (Takeda et al., 1990; Viaene et al., 1991), and transgenic animals (Vilotte et al., 1989; Soulier et al., 1992; Hochi et al., 1992).

The present study investigates the baculovirus system as an alternative to produce αlac and reports the characterization of some recombinant αlacs.

MATERIALS AND METHODS

Reagents. AcMNPV-C6 (Kitts et al., 1990) and pVL941 DNA (Luckow and Summers, 1988), *Spodopteria frugiperda* cells (Sf9), and GRACE medium (Gibco, 074-90097P) were kindly provided by Dr. J. Cohen (INRA, Jouy-en-Josas). Cells were cultured at 28 °C in GRACE medium supplemented with 10% fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL). *Bsu*36I, *Taq* DNA polymerase, and uridine diphosphate galactose ([^{14}C]U-galactose) were purchased from New England Biolabs, Promega, and DuPont NEN, respectively, and all other chemicals were obtained from Sigma.

Procedure. *Site-Directed Mutagenesis.* cDNA λ9 (Vilotte et al., 1992), which encompassed the entire coding sequence of mouse αlac, was used as a template for site-directed mutagenesis using a polymerase chain reaction (PCR)-based method (Landt et al., 1990; Figure 1). Mutated cDNAs were cloned into pUC 19, sequenced using the chain termination method (Sanger et al., 1977), and subcloned into pVL941 at its *Bam*HI site. Orientation of the insert with regard to the polyhedrin promoter was determined by restriction mapping.

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Isolation and Amplification of Recombinant Baculovirus. Sf9 cells were transfected using a calcium phosphate coprecipitation method. One microgram of AcMNPV-C6 DNA linearized by digestion with *Bsu*36I was cotransfected along with 5 μg of recombinant pVL941 transfer vectors (Kitts et al., 1990). Separations of parental and recombinant viruses followed the procedure of Tosser et al. (1992).

Production and Characterization of Recombinant αlacs. Sf9 cells in six-well plates were infected at high multiplicity (10 pfu/cell) and culture medium (1.5 mL/10^6 cells) collected 72 h postinfection. Medium was clarified by centrifugation (1000*g* for 5 min). Quantification of recombinant αlacs present in the medium was performed by Western immunoblot analyses (Vilotte et al., 1989; Figure 1). αlac activity of the culture medium was measured according to the method of Hölpert and Cooper (1990) with the following modifications: Triton X-100, BSA, and glycerol were not added in the incubation solution, and the incubation time was reduced to 10 min. Separation of products from substrates was performed by Dowex 2 column chromatography.

RESULTS

Site-Directed Mutagenesis of Mouse αlac cDNA. Mutations were targeted against the invariant amino acids Phe-31, His-32, Gln-117, and Trp-118 of the aromatic cluster I of αlac (Acharya et al., 1989; Alexandrescu et al., 1992), which are components of, or adjacent to a galactosyltransferase-binding site (Richardson and Brew, 1980; Sinha and Brew, 1981; Shewale et al., 1984; Acharya et al., 1989; Alexandrescu et al., 1992), and against the invariant amino acids Asp-87 and Asp-88, which are parts of the calcium-binding loop (Stuart et al., 1986) involved in αlac stability. Oligonucleotides were designed to obtain three sets of nonconservative mutations: (Phe-31/His-32) → (Met-31/Asp-32), (Asp-87/88) → (Tyr87/88), and (Gln-117/Trp-118) → (Lys-117/Arg-118). However, due to PCR artifacts, five sets of mutations were actually obtained (see Materials and Methods and Figure 1).

Expression of Wild-Type and Mutant Mouse αlac cDNAs in Baculovirus-Infected Insect Cells. Recombinant baculoviruses were obtained with the six cDNAs described in Figure 1 after they were subcloned into the transfer vector pVL941 and after cotransfection of Sf9 cells with these vectors along with wild-type AcNPV DNA. Secretion of recombinant αlacs in the culture medium of Sf9 cells 72 h postinfection was performed by Western analysis (Figure 1). Production

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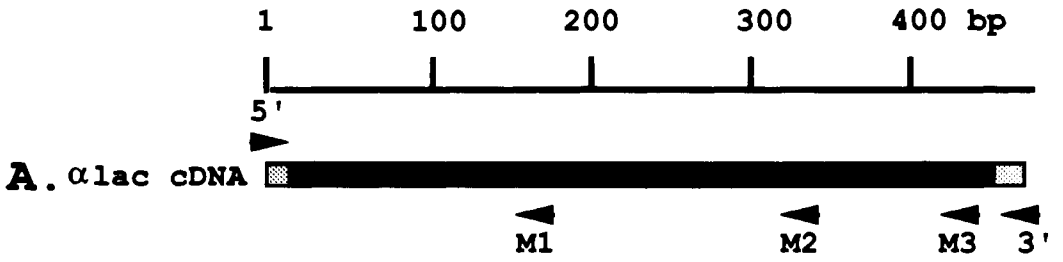

B.

Primer	Observed Mutations	Clone
-	-	PLVW
M1 : GCCACTGGTATCAATTAAAACACA	PHE- 31->MET HIS- 32->ASP	PLV17
M2 : CACGCTATGTAATAATCCAACTC	ASP- 87->TYR ASP- 88->TYR *ILE- 89->MET*	PLV2
M3 : CAACGCCTCTTTTCAAGCTT	GLN-117->LYS TRP-118->ARG	PLV10
	GLN-117->LYS TRP-118->ARG *CYS-120->TRP*	PLV6
	GLN-117->LYS TRP-118->ARG *CYS-120->STOP*	PLV23

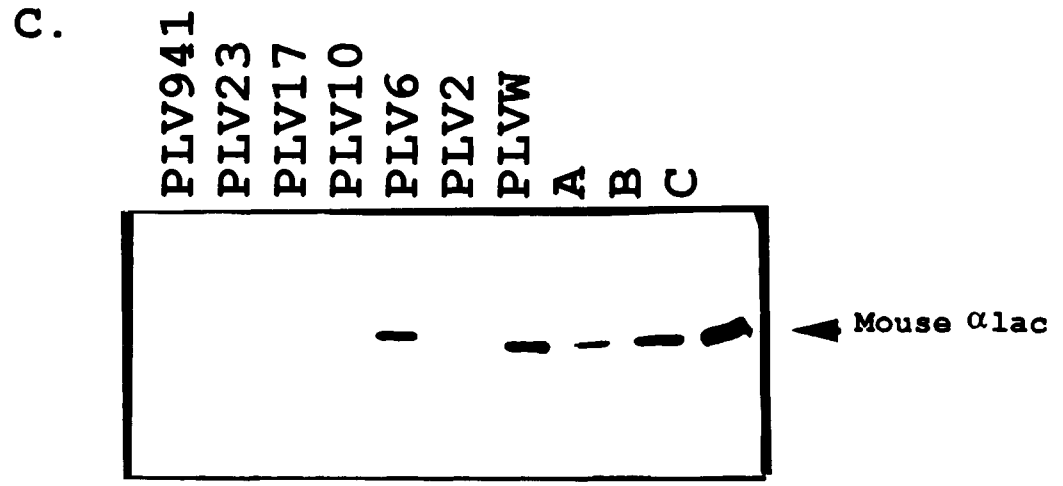

Figure 1. *In vitro* mutagenesis of mouse αlac cDNA and Western blotting analysis of recombinant αlacs. (A) Schematic representation of mouse αlac cDNA λ9. Gray and black sections represent untranslated and coding regions, respectively. Targets of the primers used for mutagenesis are indicated. (B) Sequences of the primers, observed mutations, and clone labeling. Underlined nucleotides are mutated nucleotides with regard to the cDNA sequence. Italicized mutations are unexpected mutations due to PCR artifacts. (C) Western blotting analysis of recombinant αlacs. Twenty microliters of culture medium from Sf9 cells infected with wild-type baculovirus (PLV941) or recombinant baculovirus (top margin) was used. (A–C) 100, 200, and 500 ng of milk-purified mouse αlac.

RECOMBINANT αlac CLONE	MEDIUM αLAC CONCENTRATION	LACTASE ASSAY	
		MEDIUM VOL. (αlac ng)	αlac ACTIVITY detected (ng)
PLVW	15 mg/l	10.0 µl (150)	170 ± 15
		6.7 µl (100)	110 ± 10
PLV2	≤ 0.05 mg/l		
PLV6	7.5 mg/l	20 µl (150)	0
		13.3 µl (100)	0
PLV10			
PLV17	≤ 1 mg/l		
PLV23			

Figure 2. Levels of expression and enzymatic characterization of recombinant αlacs. αlac activity detected (ng) is expressed as equivalent amount of bovine αlac.

levels ranged from 15 mg/L for wild-type PLVW αlac to less than 0.05 mg/L for mutant PLV2 (Figure 1). These variations do not appear to be related to the relative RNA levels encoding these αlacs in infected Sf9 cells (data not shown). Furthermore, the polyclonal rabbit anti-bovine-αlac antibody used was previously shown to recognize all of the mutant αlacs efficiently. When expressed in *cos*-cells, following transfection with recombinant CMVO.AD expression vectors, similar amounts of the mutant proteins were detected in lysed *cos*-cells, while, as observed in infected insect cells, some αlacs were not detected in the culture medium (unpublished results). These observations suggest that differences in the levels of secreted αlacs are mainly related to post-translational mechanisms.

The apparent molecular weights of recombinant αlacs were found to be similar to mouse milk αlac for PLVW and 17, slightly higher for PLV6 and 10, and slightly lower for PLV2 and 23 (Figure 1 and data not shown). Again, similar observations were made when these cDNAs were expressed in *cos*-cells (unpublished observations).

Characterization of Recombinant αlac PLVW and PLV6. Recombinant αlacs PLVW and PLV6 were tested for their ability to induce lactose synthesis *in vitro* in the presence of UDPgalactosyltransferase (see Materials and Methods). In this regard, αlac PLVW has properties similar to those of milk-purified bovine αlac, whereas αlac PLV6 was devoid of any detectable enzymatic modulating activity (Figure 2). Mixing culture mediums from PLVW and PLV6 Sf9 infected cells did not affect the activity of αlac PLVW, suggesting the lack of enzymatic inhibitors in the medium from PLV6 cultures. Other αlac mutants were not tested because their concentration (less than 1 mg/L) was found to be too low for direct measurement of their activity in the culture medium without prior purification steps, due to the sensitivity of our *in vitro* assay.

DISCUSSION

Secretion of biologically active "wild-type" goat and bovine αlac by *S. cerevisiae* has been reported (Takeda et al., 1990; Viaene et al., 1991). However, the αlac concentrations observed in the culture media were relatively low (less than 2 mg/L). Expression of αlac cDNA in *E. coli* was more effective (up to 40 mg/L), but the protein was produced as an inclusion body of fused protein, needing proteolytic cleavage and refolding steps to obtain active proteins (Wang et al., 1989; Kumagai et al., 1991, 1992; Grobler et al., 1994). Expression levels were higher than those obtained in this experiment (up to 15 mg/L), but the baculovirus system allowed us to produce an active secreted αlac, which can potentially be post-transcriptionally modified; the efficiency of this system can probably be increased using new optimized vectors (Peakman et al., 1992). We also made several attempts to produce recombinant αlacs by transfecting *cos*-cells with CMVGO.AD recombinant plasmids. This system, convenient for its simplicity and speed, allowed us to obtain only low concentrations of αlac, less than 40 ng/mL (unpublished observations).

We have expressed a mutant αlac devoid of enzymatic activity (Figures 1 and 2). Since the mutations in PLV6 affect invariant residues of cluster I, this seems to confirm recent results suggesting that these residues have a functional role (Grobler et al., 1994). However, the artifactual extra mutation Cys-120 → Trp, which suppresses one of the four disulfide bridges of αlac, might be alone responsible for the inactivity of PLV6. Further analysis of a PLV10-enriched medium should clarify the functional importance of both Gln-117 and Trp-118.

Transgenic animals are the most efficient system so far in terms of levels of expression (Vilotte et al., 1989; Soulier et al., 1992; Hochi et al., 1992) but also probably the most costly and time-consuming. However, the recent obtention of αlac-deficient mice (Stinnakre et al., 1994) and the now available possibility to replace the endogenous mouse αlac gene by one carrying amino acid substitution(s) in ES cells (Stacey et al., 1994) open real opportunities to study *in vivo* the structure–function relationship in αlac when potentially important alteration will have been suggested using expression systems such as the baculovirus one.

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