LEC12 and LEC29 Gain-of-Function Chinese Hamster Ovary Mutants Reveal Mechanisms for Regulating VIM-2 Antigen Synthesis and E-selectin Binding*

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LEC12 and LEC29 are two gain-of-function Chinese hamster ovary glycosylation mutants that express the Fut9 gene encoding α(1,3)fucosyltransferase IX (α(1,3)Fuc-TIX). Both mutants express the Lewis X (Le-X) determinant Galβ(1,4)[Fucα(1,3)]GlcNAc, and LEC12, but not LEC29, cells also express the VIM-2 antigen SAα(2,3)-Galβ(1,4)GlcNAcβ(1,3)Galβ(1,4)Fucα(1,3)GlcNAc. Here we show that LEC29 cells transfected with a Fut9 cDNA express VIM-2, and thus LEC29 cells synthesize appropriate acceptors to generate the VIM-2 epitope. Semi-quantitative reverse transcription-PCR showed that LEC12 has 10- to 20-fold less Fut9 gene transcripts than LEC29. However, Western analysis revealed that LEC12 has ~20 times more Fut9 protein than LEC29. The latter finding was consistent with our previous observation that LEC12 has ~40 times more in vitro α(1,3)Fuc-T activity than LEC29. The basis for the difference in Fut9 protein levels was found to lie in sequence differences in the 5′-untranslated regions (5′-UTR) of LEC12 and LEC29 Fut9 gene transcripts. Whereas reporter assays with the respective 5′-UTR regions linked to luciferase did not indicate a reduced translation efficiency caused by the LEC29 5′-UTR, transfected full-length LEC29 Fut9 cDNA or in vitro-synthesized full-length LEC29 Fut9 RNA gave less Fut9 protein than similar constructs with a LEC12 5′-UTR. This difference appears to be largely responsible for the reduced α(1,3)Fuc-TIX activity and lack of VIM-2 expression of LEC29 cells. This could be of physiological relevance, because LEC29 and parent Chinese hamster ovary cells transiently expressing a Fut9 cDNA were able to bind mouse E-selectin, although they did not express sialyl-Le-X.

There are several established biological functions for mammalian α(1,3)fucosyltransferases (α(1,3)Fuc-Ts)1 (1), and it is apparent that regulation of α(1,3)Fuc-T activities at the transcriptional level may occur (2–6). One approach to identify factors that control α(1,3)Fuc-T expression at a transcriptional, translational, or activity level is to determine the molecular basis of gain-of-function glycosylation mutants that express an α(1,3)Fuc-T activity de novo (7). For example, three CHO mutants, termed LEC11, LEC11A, and LEC11B, express α(1,3)Fuc-TV from either the Fut6A or Fut6B gene (8). These mutants express sialylated Lewis X (sLe-X); SAα(2,3)Galβ(1,4)[Fucα(1,3)]GlcNAc, VIM-2 (CD65s), and all bind E-selectin (8, 9). Analyses of gene organization and expression of the CHO Fut6A and Fut6B genes in the LEC11 mutants provided insight into the mechanism of α(1,3)Fuc-T gene activation in each mutant, including evidence for a negative regulatory factor that represses the Fut6B gene in CHO cells (8). Expression cloning has recently identified three suppressors of α(1,3)Fuc-T activity in LEC11B cells.2

Four α(1,3)Fuc-T-encoding genes have been characterized in different CHO gain-of-function mutants. The Fut6A and Fut6B genes appear to be orthologous to the Fut3-Fut5-Fut6 cluster in humans (10) and are active in the LEC11 (Fut6B), LEC11A (Fut6A), and LEC11B (Fut6B) mutants (8). The Chinese hamster Fut4 ortholog is expressed in the LEC30 CHO mutant and the Fut9 ortholog is active in the LEC12, LEC29, and LEC30 CHO mutants (11). Neither of the latter mutants express the Fut4 gene (11). LEC12 and LEC29 do not bind anti-s-Le-X antibody, and their cell extracts do not fucosylate (2,3)sialylated N-acetyllactosamine (LacNAc) in vitro (9), indicating that they do not express the Fut6A, Fut6B, or Fut7 genes. Furthermore, Fut6 gene expression was not detected in LEC12 by RNAse protection (8) nor in LEC12 or LEC29 by Northern analysis.3 Thus, Fut9 appears to be the only α(1,3)Fuc-T-encoding gene expressed in these two mutants. It was therefore surprising to find that LEC12 and LEC29 have very different fucosylation patterns (9). Whereas LEC12 cells express the VIM-2 and Le-X epitopes at similar levels, LEC29 cells do not express VIM-2 above background in a binding assay with iodinated antibody (9). LEC29 cells are ~50-fold more sensitive to the toxicity of wheat germ agglutinin than LEC12 cells. They are also 3-fold less resistant than LEC12 to the leukoagglutinin from Phaseolus vulgaris termed L-PHA. In addition, LEC12 extracts have α(1,3)Fuc-T activity of 85.9 pmol/mg of protein/min compared with 2.4 pmol/mg of protein/min activity in LEC29 extracts (9).
CHO transfectants expressing a CHO Fut9 cDNA behave like LEC12 cells, and bind both anti-LeX and anti-VIM-2 antibodies suggesting that LEC29 cells express a compromised Fut9 (11). However, in vitro mixing experiments provided no evidence for a Fut9-degrading or -inhibiting activity in LEC29 cell extracts (11). In this report, we identify 5'-untranslated region (UTR) sequence differences in Fut9 cDNAs from LEC12 and LEC29 and provide evidence that they are largely responsible for the differences in Fut9 levels and fucosylated antigen expression in LEC12 and LEC29. We also show that regulation of Fut9 levels may have physiological significance in terms of E-selectin binding.

MATERIALS AND METHODS

Antibodies, Chemicals, and Molecular Biology Reagents—Mouse IgM anti-SSAIA-1 (anti-LeX) monoclonal antibody (mAb) (12) was prepared previously in the laboratory (13). Purified, FITC-conjugated, mouse anti-LeX mAb (clone AHN1.1) was obtained from Calbiochem. Mouse IgM anti-VM-2 mAb (clone AHN1.1) was purchased from Biosearch GmbH. Mouse IgG1, rabbit, or goat anti-goat IgG, anti-mouse IgM, anti-mouse IgG + A + M, and horseradish peroxidase-conjugated goat anti-human IgG antibodies were purchased from Zymed Laboratories. Goat allophycocyanin-conjugated anti-mouse IgM (H + L) was from Caltag Laboratories. Purified recombinant mouse E-selectin fused to human IgG Fc fragment and produced in Escherichia coli and purified from mouse ascites (KM8621) or hybridoma supernatant (KM2681) were a kind gift of Dr. Hisashi Narimatsu, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan. Antibody C-17, an affinity-purified goat polyclonal antibody against a carboxy-terminal peptide of human Fut9, was purchased from Dr. E. Richard Stanley, Albert Einstein College of Medicine. Amino acid mix was purchased from Promega. Coomassie Brilliant Blue, Ponceau S, creatine phosphate, and creatine phosphokinase purified from rabbit muscle were obtained from Sigma. Hanks’ balanced salts mix was from Mediatech. Lysophosphocholine was supplied by Avanti Polar Lipids. Peptide N-glycanase F (PNGase F) purified from Flavobacterium meningosepticum was obtained from New England Biolabs. S-Adenosylmethionine was from ICN. Spermidine was purchased from Fluka. Zeomycin was from Invitrogen.

Cell Culture and Transfection—Parent Pro 5 CHO cells (17) and gain-of-function CHO glycosylation mutants LEC11 (18), LEC12 (19), LEC9 (11) and LEC29 (11) CHO cells were isolated previously. Cells were maintained in suspension culture at 37 °C in complete a-modified Eagle’s medium supplemented with 10% fetal bovine serum. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were sometimes added. Cell culture reagents were obtained from Invitrogen and Gemini. Adherent cells were transfected with DNA using Fugene6 (Roche Applied Science) or LipofectAMINE 2000 (Invitrogen) as per protocols suggested by the manufacturers.

Somatic Cell Hybridization—LEC12 and LEC29 cells were transfected with pcDNA1.carrying either a neomycin or zeocin resistance gene (Invitrogen). Stably transfected independent colonies, LEC12-neo, LEC12-zeo, LEC29-neo, and LEC29-zeo, were isolated after selection with neomycin (1.5 mg/ml active weight) or zeocin (75 μg/ml). LEC12-neo cells were fused with LEC29-zeo cells, and LEC12-zeo cells were fused with LEC29-neo cells as previously described (8). After 8–10 days, hybrid colonies were picked and expanded in culture.

Semi-quantification of Fut9 Transcripts by RT-PCR—RNA was prepared using 1 μl of TRIzol™ reagent (Invitrogen) per 100 μl cells. 10 μg of total RNA from LEC12 or LEC29 was reverse-transcribed at 48 °C using 1 μg of oligo(dt) and 100 ng of random hexamers with a mixture of Superscript IITM and avian myeloblastosis virus reverse transcriptase in 40 μl cDNA products were treated with RNase H (Invitrogen), and 1 μl of cDNA was subjected to PCR for 27, 30, 33, 36, 39, 42, or 45 cycles. Products were electrophoresed on a 1% agarose gel, and stained with ethidium bromide. NIH Image software was used to quantify by densitometry. Fut9 gene-specific primers were FS294 (5’-TGA CAA CAC AAT GAA CTG GGT CT) and FS299 (5’-CCA CAT GAA TAT CAT CAG CTG G). Glyceraldehyde-3-phosphate dehydrogenase gene-specific primers were FS270 (5’-TGA ATT CAT CAT TCA CCT CTA CAT) and FS271 (5’-AGA ATT CCT ACT CCT TGG AGG CCA).

Isolation of Microsome from Cells—Cells were grown to a density of 4–5 × 10^6/ml and pelleted by centrifugation at ~500 g for 10 min at 4 °C. After three washes with isotonic saline, cells were resuspended in 10 mm Tris, pH 7.4, at 6 × 10^6 cells/ml with 250 mm sucrose and placed on ice for 25 min before being homogenized by seven passages through a Balch ball-bearing homogenizer at 4 °C. Nuclei and unbroken cells were removed by centrifugation at 1,000 × g for 10 min. The post-nuclear supernatant was centrifuged at 10,000 × g for 10 min at 10,000 × g for 10 min at 4 °C to pellet the non-microsomal membrane fraction. Microsomes from the supernatant were isolated by centrifugation at 100,000 × g for 1 h at 4 °C. Both microsomal and non-microsomal membrane pellets were resuspended (3 × 10^7 cell equivalents/μl) in 1.5% Triton X-100 containing Complete EDTA-free protease inhibitor mixture (Roche Applied Science) and stored at ~80 °C in 20% glycerol.

Western Analysis—Protein concentrations were determined for membrane fractions and cell lysates by the Bio-Rad DC assay. Digestion of 100–200 μg of protein with 500 units of PNGase F was performed at 37 °C overnight. Digests were diluted in Laemmi SDS-PAGE sample loading buffer (20) containing β-mercaptoethanol (final concentration, 0.1%,), boiled 5 min, and electrophoresed under reducing conditions on denaturing Tris-glycine SDS-polyacrylamide gels. Gels (7.5% or 10%) were stained with Coomassie Brilliant Blue (Bio-Rad Laboratories, Scientific Instruments) apparatus or were purchased from Bio-Rad. Benchmark, MagicMark, or Mark12 mouse protein molecular weight markers (Invitrogen) were loaded in separate lanes. Staining of proteins was with Coomassie Brilliant Blue dye or Gelcode Blue stain ( Pierce Biotechnology). For immunoblotting, proteins were electrotransferred overnight to a Polyscreen polyvinylidene difluoride membrane (PerkinElmer Life Sciences) or SuperWest Pico™ (Pierce Biotechnology) reagents and exposure to Biomax™ MR x-ray films (Kodak).

Anti-VIM-5, Anti-LeX, and Anti-sLeX Binding to Cells by Flow Cytometry—Approximately 5 × 10^6 cells were washed twice with PBS, 1% bovine serum albumin, 2.7, calcium- and magnesium-free) or Hanks’ buffered salt solution (HBSS, 0.4% gelatin, 0.06% gelatin K_HPO4, 0.8% gelatin Na_HPO4, 1 mg% D-glucose) were preincubated in 200–400 μl of PBS or HBSS containing 2% BSA at 4 °C for 30 min. Cells were washed once with 3 ml of PBS or HBSS with BSA and resuspended in 200–400 μl of PBS containing 1–2 μg of purified anti-VIM-5, anti-leukocytes (anti-β2) vs. anti-leukocytes (anti-β2) vs. anti-VIM-2 monoclonal antibody. After incubation at 4 °C for 30 min, cells were washed once and incubated with 1–2 μg of rabbit anti-mouse IgM conjugated to FITC. After 30 min at 4 °C, cells were washed once and resuspended in 200 μl of PBS/BSA for analysis using a FACSscan flow cytometer (BD Biosciences). Propidium iodide was added at 1–2 μg/ml just prior to cytometry. All fluorescence data were collected using logarithmic amplification on 10,000–15,000 events (cell counts). Events considered for analysis were gated for either light scatter or low propidium iodide binding, or both. For double staining, an allophycocyanin-conjugated, goat anti-mouse secondary antibody (0.3–1 μg) was used instead of the FITC secondary antibody. Cells were washed with HBSS and incubated with 2 μg of FITC-conjugated anti-LeX antibody in a volume of 400 μl for 1 h at 4 °C. Fluorescence cytometry was performed on a FACScanCalibur™ cytometer (BD Biosciences) after a final wash with HBSS.

α(1,3)Fucosyltransferase Assay—α(1,3)Fuc-T activity was measured with LacNAc as acceptor as described previously (9). Each reaction contained 2.5 mmol of 2-morpholinophenylsulfonate buffer (pH 7.0), 5 mmol of NaCl, 0.25 mmol of MnCl2, 2–4 mmol of GDP-[14C]fucose (New England Nuclear), 0.1 μl of LacNAc (Dextra Labs), and 10 μl of cell extract (~50–100 μg of protein) prepared in buffer containing 1.5% Triton X-100 in 50 μl. After incubation at 37 °C for 60 min, the reaction was stopped by the addition of 1 ml of ice-cold water, and reaction products were separated on a small AG-IAX (Bio-Rad) column as described previously (21). Eluates were mixed with Ecolo (ICN Biochemicals) and counted in a scintillation counter (Beckman Coulter). Specific activities were calculated after subtracting the counts per mi

E-selectin Binding Induced by α(1,3)FucT-IX
nute incorporated into endogenous acceptors.

**Rapid Amplification of cDNA Ends—**The GeneRacer kit (Invitrogen) was used for 5'-RACE of LEC12 and LEC29 Fut9 transcripts. Reverse transcription of LEC12 and LEC29 mRNA was performed using a mixture of SuperScript II™ and avian myeloblastosis virus reverse transcriptases at 48 °C. RNase H-treated cDNA was amplified with Platinum™ Pfx polymerase using Fut9 gene-specific primer PS432 (5'-TAG TGA GAT GGC ACC CTT GG) and GeneRacer™ 5'-primer specific for the ligated oligonucleotide (5'-CGA CTA GAG CAC GAG GAC ACT GA). The PCR products were subjected to nested PCR using Fut9-specific primers PS537 (5'-TCT CGG GCT GCT GTA TCT AA) and GeneRacer 5'-nested primer (5'-GGA CAC TGA GAT CTA AAG TAA). PCR products were TA-cloned into pCR2.1 vector, and the inserts of at least three clones were sequenced using vector-specific primers PS616 (5'-CAG TGG TTT CCT TCG GCC CTT GCT CTT AGG TAA TGG A) and PS519 (5'-TTT TAA TGA AAG AAT AGA CAT TTC AAG AAC). Tag polymerase was used to add 5'-A overhangs to the purified product, which was cloned into pCDNA3.1/V5-His TOPO vector (Invitrogen). Sequencing confirmed that the insert was intact. The plasmid used with a Fut9 cDNA insert size of 2251 bp was designated pCMV-Full12F9. Similarly, a cDNA containing the 5'-UTR and coding regions of LEC29 Fut9 was obtained using primers PS617 (5'-TCT GCT TCT AGG AGC GGC CCA CCG CCG AAC CC) and PS343 (5'-TATA ATT CCA AAA CCA TTT CTT TCA ATT ACC CAC AG). This cDNA and pCMV-Full12F9 were digested with KpnI and BspEI restriction enzymes. KpnI cuts pCDNA3.1/V5-His TOPO vector just upstream of the BspEI site, and both LEC12 and LEC29 Fut9 cDNA once, at positions 793 and 792, respectively. The KpnI/BspEI-released fragment from the LEC29 clone was cloned into KpnI/BspEI-digested pCMV-Full29F9 to generate the LEC29 full-length construct pCMV-Full29F9 with an insert size of 2250 bp. The LEC12 and LEC29 constructs were identical to each other, except for the first exon in their respective 5'-UTRs.

**Generation of Tagged Fut9 and Fut11 Constructs—**The Fut9 gene ORF was amplified from pCMV-Full12F9 with Platinum Pfx polymerase using primers PS685 (5'-CCA CCA TGG AAC AAA AAC TCA TCT CAG AGG ATC TGA CAT CAA CAT ACG GCA TTC TTG GCT C) and PS343 (5'-TATA ATT CCA AAA CCA TTT CTT TCA ATT ACC CAC AG). The forward primer PS685 also encodes the c-Myc peptide sequence EQKLISEEDL. AmpliTag polymerase was used to add a 3'-A overhang to the purified product, which was then TA-cloned into pCR3.1 (Invitrogen). An N-terminal Myc-tagged mouse Fut11 expression construct was similarly generated by amplification from mouse placenta cDNA using primers PS686 (5'-CCA CCA TGG AAC AAA AAC TCA TCT CAG AGG ATC TGA CAT CAA CAT ACG GCA TTC TTG GCT C) and PS553 (5'-ATC CTC GAT GAT TTT TAT TTA TTG ATG TTT GTT TCT CAA GTC).

**Generation of Humanized Renilla Luciferase Reporter Constructs—**

The CMV promoter region of pSeCreTag2C-hygro vector (Invitrogen) was amplified with Platinum Pfx polymerase using primers PS650 (5'-ATG CAT CTC GAG CCA TGG ACA TAC TGG CCC) and an XhoI restriction site) and PS651 (5'-ATG CAT GAG GCC GGT AGC AGC GAG ATC CTT AGG TAA TAC GAC TCA GTA TGA TAG, with an Ncol restriction site). XhoI/Ncol-digested PCR products were cloned into pRL-null vector (Promega) between the XhoI and Ncol sites to obtain pRL-CMV. The pHRL-null vector has a T7 promoter upstream of humanized Renilla luciferase coding region such that the putative transcription start site lies 2 nt upstream of the first ATG start. For pRL-CMV, the CMV promoter-driven transcriptional start site is 26 nt upstream. Complete 5'-UTR and full-length LEC12 and LEC29 Fut9 were amplified from pCMV-Full12F9 and pCMV-Full29F9 cDNAs, respectively. The forward primers, bearing the T7 promoter region and a Ncol restriction site were for LEC12: PS647 (5'-ATG CAT GAG GCC GGT TAC TAC GAC TCA GTA TGA TAG GGA GCA CCA GAT GCT TCT GCC AGC ATG CC) and for LEC29, PS648 (5'-ATG CAT GAG GCC GGT TAC TAC GAC TCA GTA TGA TAG GGA GCA CCA GAT GCT TCT GCC AGC ATG CC). The reverse primer PS639 (5'-GGG CCA TGG TCT ATA TGG CAT TAT GAA TTT TGA TCT TCT AGG ACA GCG CCG CCA CCG CCG AAC CC) was used, except that the forward primers lacked the T7 promoter region: LEC12: PS647 (5'-ATG CAT GAG GCC GGT TAC TAC GAC TCA GTA TGA TAG GGA GCA CCA GAT GCT TCT GCC AGC ATG CC) and LEC29: PS648 (5'-ATG CAT GAG GCC GGT TAC TAC GAC TCA GTA TGA TAG GGA GCA CCA GAT GCT TCT GCC AGC ATG CC). In both these constructs, the transcription start site is 5 nt upstream of the first 5'-UTR whose end lies 11 nt upstream of the luciferase ATG start codon.

**Luciferase Assays—**Cells grown in 6-well plates were transfected with Renilla reniformis (sea pansy) luciferase reporter constructs using Fugene6 DNA transfection reagent. For normalization of transfection efficiency, a firefly (Photinus pyralis) reporter construct containing the phRL-CMVU12 and phRL-CMVU29, which have the luciferase coding region fused with the 5'-UTRs of Fut9, a similar strategy was used, except that the forward primers lacked the T7 promoter region: LEC12: PS646 (5'-ATG CAT GAG GCC GAT CAC TGG AGG TAT TTG CCA GCC TGC CC) and LEC29: PS646 (5'-ATG CAT GAG GCC GGT TAC TAC GAC TCA GTA TGA TAG GGA GCA CCA GAT GCT TCT GCC AGC ATG CC). In both these constructs, the transcription start site is 5 nt upstream of the first 5'-UTR whose end lies 11 nt upstream of the luciferase ATG start codon.

**Preparation of Translational Extracts from CHO, LEC12, and LEC29 Cells—**The method of Favre and Trepo (22) that incorporates sucrose and dimeric creatine kinase in lysis buffer was followed. Cells were grown to 90% confluence on a 15-cm plate. After washing once with 12 ml of cold wash buffer (150 mM sucrose, 33 mM NH₄Cl, 7 mM KCl, 45 mM Tris·HCl pH 7.4, 2.5 mM Mg[CH₃COO]₂, 1 mM dithiothreitol, 2.5 mM ATP, 1 mM GTP, 0.1 mM 5'-adenosylmethionine, 1 mM spermidine, 20 mM creatine phosphate, 40 units/ml creatine phosphokinase, 40 µM each amino acid mix) was spread over the cells. After 90 s at 4 °C, cells and buffer were scraped off and passed twice through a 26-gauge needle. After centrifugation at 100 x g for 2 min, the supernatant was aliquoted and stored at −80 °C. Protein concentration was measured using Bio-Rad DC assay with BSA as standard.

**In Vitro Translation—**The rabbit reticulocyte lysate-based T7-coupled transcription-translation kit (Promega) was used as per the manufacturer’s protocol with both RNA and DNA templates. Translation reactions using 15 µl of CHO cell extracts were performed in 20 µl containing in vitro transcribed RNA and 1 µl (50 µg) of creatine phosphokinase. When required, [35S]methionine or EasyTag Express™ 35S protein labeling mix (PerkinElmer Life Sciences) was added to translate probe products. Translation reactions were directly used in luciferase assays or electrophoresed on SDS-PAGE gels. Gels were either used for immunoblotting or dried and exposed to BioMax™ MR x-ray films (Kodak).
The Eμ promoter of the mouse immunoglobulin heavy chain gene (a kind gift of Dr. Barbara Birshtein, Albert Einstein College of Medicine) was cotransfected. Activities from both luciferases were measured sequentially from a single sample using the Dual-Luciferase reporter assay system from Promega. Cells were lysed after 2–3 days using 250 μl of Passive Lysis buffer (Promega). Lysates were assayed immediately or after storage at −20 °C. For in vitro translation reactions, samples were diluted 5- to 20-fold in Passive Lysis buffer. Measurements were performed on a Turner Designs TD-20e luminometer with a 2-s pre-read delay and a 10-s measurement period.

**RESULTS**

**LEC29 Cells Do Not Possess Inhibitors of VIM-2 Synthesis**—LEC29 CHO cells have more Fut9 gene transcripts than LEC12 but ~40-times less α(1,3)Fuc-T activity (9, 11). LEC12 cells express the VIM-2 epitope on cell surface glycoproteins, but VIM-2 is barely detectable on LEC29. To determine whether activation of a negative factor or repression of a positive factor down-regulates expression of VIM-2 on the LEC29 mutant, somatic cell hybrids formed with LEC12 cells were examined for VIM-2 expression. This approach previously showed that CHO Fut6 genes may become active due to a cis mechanism correlated with gene rearrangement or a trans mechanism due to the loss of a negative regulatory factor that represses Fut6B expression in CHO cells (8). Neomycin or zeomycin resistance genes were introduced into LEC12 and LEC29 cells, allowing post-fusion selection for hybrid colonies in medium containing both antibiotics. Upon analysis by fluorescence cytometry, independent LEC12 × LEC29 hybrids were found to be as positive as LEC12 cells for cell surface VIM-2 expression (Fig. 1). Therefore, LEC29 cells do not express a dominant factor that represses VIM-2 synthesis.

**LEC29 Cells Have All the Factors Necessary for Expression of VIM-2**—The VIM-2 epitope includes fucose, sialic acid, and two lactosamine units. Thus, the virtual absence of VIM-2 on
LEC29 cells could be due to a defect in the generation of polylactosamines or α(2,3)sialylation or α(1,3)fucosylation. It is also possible that one or just a few glycoconjugates carry the VIM-2 epitope. Thus the anti-sLe\(^X\) mAb CSLEX-1 appears to detect one major sLe\(^X\)-carrying protein in CHO-K1 cells transfected with a Fut7 cDNA (23). To determine whether overexpression of Fut9 in LEC29 would generate VIM-2 as it does in CHO cells (11), the coding region of the Fut9 gene cloned into pCR3.1 (11) was transfected into LEC29 cells. Fut9 transcripts were positive for cell surface VIM-2 expression by fluorescence cytometry (Fig. 2A). Thus LEC29 cells have the acceptors necessary to generate the VIM-2 antigen. Coexpression of Le\(^X\) and VIM-2 structures was also examined on individual cells in transfected populations stably expressing a Fut9 cDNA by fluorescence cytometry. Depending on the site of integration in the genome and number of integrated copies, cells of these stable populations will vary in their expression of Fut9 protein. In Fig. 2B, it can be seen that there was a wide range of expression of fucosylated antigens. However, there was a direct correlation between Le\(^X\) and VIM-2 expression in both CHO and LEC29 transfected populations. This indicates that the level of Fut9 must be increased in LEC29 cells to allow synthesis of VIM-2.

**Fut9 Gene Transcripts in LEC12 and LEC29 Cells**—Although Fut9 gene transcripts can be detected by RT-PCR in LEC12 and LEC29, a signal was not observed for LEC12 by Northern blot analysis (11). Therefore, a semi-quantitative RT-PCR study was performed to compare the relative levels of Fut9 gene transcripts in the two cell lines. Total RNA of LEC12 or LEC29 was reverse-transcribed, and the cDNA was used as template for amplification of glyceraldehyde-3-phosphate dehydrogenase or Fut9 genes by PCR for different numbers of cycles. Forward and reverse primers were designed to recognize sequences on separate exons to ensure amplification of cDNA but not genomic DNA sequences. Glyceraldehyde-3-phosphate dehydrogenase products were detected in the 27-cycle reactions for both LEC12 and LEC29, and product increased almost linearly to 42 cycles (Fig. 3A). Fut9 cDNA products were easily detected in the 38-cycle reaction for LEC29 but not until the 45-cycle reaction for LEC12. After a longer exposure (Fig. 3A), LEC12 products were visible at 33 cycles. Thus, LEC12 expresses 10- to 20-fold fewer Fut9 gene transcripts than LEC29.

**LEC29 Cells Have Higher Levels of Fut9 Protein Than LEC29**—The CHO Fut9 protein has a deduced molecular mass of 42.2 kDa and three predicted N-glycosylation sites (11). Anti-Fut9 KM8621 antibody identified two major proteins in a reducing SDS-PAGE gel and transfer to polyvinylidene difluoride membrane.

**FIG. 3. LEC12 cells have fewer Fut9 transcripts but more Fut9 protein than LEC29 cells.** A, semi-quantitative RT-PCR of Fut9 RNA. LEC12 or LEC29 cDNA was amplified using glyceraldehyde-3-phosphate dehydrogenase or Fut9 gene-specific primers in reactions with different numbers of PCR cycles. Products were separated on an agarose gel and stained with ethidium bromide for imaging. The lowest panel is a longer exposure to better reveal LEC12 Fut9 transcripts. B, Western analysis was performed after electrophoresis in a 7.5% reducing SDS-PAGE gel and transfer to membrane, using anti-human Fut9 mAb KM8621. Each lane had 100 μg of protein from 1.5% Nonidet P-40 detergent extracts made from CHO, LEC12, or LEC29 cells. PNGase F treatment was used prior to electrophoresis to remove N-glycans. The star indicates a non-Fut9 cross-reacting band present in all CHO cells. C, microsomes and a non-microsomal fraction were prepared from CHO, LEC12, and LEC29 cells as described under "Materials and Methods," and 50 μg of protein from each were treated with PNGase F overnight. Immunoblotting to detect Fut9 was performed with mAb KM8621 after electrophoresis in a 7.5% reducing SDS-PAGE gel and transfer to membrane. The lower panel shows a longer exposure of the microsomal fraction. D, Fut9 protein is not selectively degraded in LEC29 cells. CHO, LEC12, and LEC29 cells were transiently cotransfected with expression vector for Fut9 and Myc tag at the N terminus. Cell lysates were treated with PNGase F overnight. Fut9 proteins with Myc tag at the N terminus were selectively cotransfected with expression vector for Fut9 and Myc tag at the N terminus. Cell lysates were treated with PNGase F overnight. Fut9 proteins with Myc tag at the N terminus were treated with PNGase F overnight. Fut9 proteins with Myc tag at the N terminus were treated with PNGase F overnight. Fut9 proteins with Myc tag at the N terminus were treated with PNGase F overnight.
ment while the Fut9 band moved to ~43 kDa (Fig. 3B). A probable Fut9 dimer at ~80 kDa was present only in LEC12. Further evidence for Fut9 assignment was obtained by cell fractionation. Microsomal membranes from post-nuclear cell extracts were enriched in Fut9 protein and contained much less of the ~55-kDa unknown protein compared with the non-microsomal fraction (Fig. 3C). This was the expected location of Fut9, which functions in the Golgi compartment. The amount of Fut9 protein was at least 20-fold higher in the LEC12 microsomal fraction compared with LEC29, and it was not detected in CHO. One explanation for this difference is that Fut9 protein in LEC29 gets degraded fast. Because available antibodies against Fut9 protein were ineffective for immunoprecipitation, pulse-chase studies to estimate the half-life of Fut9 protein in cells could not be performed. However, transfected myc-tagged Fut9 protein was found not to be selectively degraded in LEC29 cells (Fig. 3D). Differences at the level of translation were therefore investigated.

Sequence Differences in the 5'-UTR of Fut9 Transcripts in LEC12 and LEC29—The coding region (1080 bp) of Fut9 cDNA from LEC12 and LEC29 have an identical sequence (11). Sequences of the 3'-UTR (969 bp) of Fut9 cDNA from LEC12 and LEC29 and the 92 bases immediately upstream of the coding region were also identical for the two sequences. This region of the hamster 5'-UTR is 70–80% identical to 5'-UTRs of mouse and rat Fut9 genes. However, the sequence further 5' was completely different in LEC12 and LEC29 cDNAs (Fig. 4A). The segment (1–109 nt) of the LEC29 5'-UTR sequence is composed of at least three exons, similar to human, mouse, and rat, which have three Fut9 exons separated from each other by large introns (~90–120 kb). The coding region and 3'-UTR lie in the third exon in each species. RT-PCR using a forward primer specific for either the LEC12 or LEC29 Fut9 exon 1 (PS616 or PS617, respectively) and a reverse primer specific for exon 1 of LEC29 Fut9. Genomic DNA (gDNA) gave no product as expected.

Fig. 4. LEC12 and LEC29 5'-UTR sequences differ in exon 1. A. alignment of 5'-UTR sequences of Fut9 cDNAs from LEC12 and LEC29. Residues shown in black belong to the putative first exon of the CHO Fut9 gene. Translation start and stop codons are underlined. B, the diagram shows the three exons of the CHO Fut9 gene as a cDNA with the positions of the primers used. Products obtained from reverse transcriptase reactions of LEC12 or LEC29 total RNA (+ and − RT enzyme) were subjected to PCR using reverse primer PS229 (specific to the Fut9 coding region) and forward primers PS616 specific for exon 1 of LEC12 Fut9 or PS617 specific for exon 1 of LEC29 Fut9. Genomic DNA (gDNA) gave no product as expected.
The LEC29 5'-UTR Efficiently Translates a Reporter Transcript—To investigate effects of the different LEC12 and LEC29 5'-UTRs on translation efficiency, reporter cDNA constructs containing the respective 5'-UTRs upstream of *Renilla* luciferase and downstream of a T7 or CMV promoter were generated. CHO, LEC12, or LEC29 cells were transiently transfected, and cell lysates were analyzed after 2–3 days. Surprisingly, all three cell types expressing the LEC29 5'-UTR construct had 3–5 times more activity than those transfected with the LEC12 5'-UTR reporter (Fig. 5A). There was no apparent difference in reporter gene transcripts between transfectants based on Northern analysis (data not shown). When the same cDNA constructs were assayed by coupled transcription-translation using the rabbit reticulocyte lysate-based TntT system, the presence of either the LEC12 or LEC29 5'-UTR inhibited translation 4–5 times compared with a construct that had no 5'-UTR. However, in this case there was no significant difference between luciferase activity generated from LEC12 and LEC29 5'-UTR constructs (data not shown).

Similar results were obtained from translation of *in vitro* generated RNA by translation extracts prepared from CHO, LEC12, or LEC29 cells, respectively. m7G-capped reporter RNA synthesized *in vitro* using T7 RNA polymerase was translated by each translation extract (Fig. 5B) and by a rabbit reticulocyte lysate (data not shown). As expected, the absence of a 5'-UTR improved translation considerably. However, the different LEC12 and LEC29 5'-UTRs did not differentially affect translation of the *in vitro* synthesized reporter luciferase RNA by any of the cell extracts (Fig. 5B).

The LEC29 5'-UTR Inhibits Translation from Full-length Fut9 cDNA Constructs—The absence of an inhibitory effect of the LEC29 Fut9 5'-UTR in luciferase reporter studies suggested that the 5'-UTR may interact with other regions of the transcript to regulate Fut9 mRNA translation. To address this, full-length Fut9 cDNA from LEC12 and LEC29 containing the 5'-UTR, coding region, and 3'-UTR, and differing only in the region corresponding to exon 1, were cloned into the pcDNA3.1/V5-His TOPO mammalian expression vector. As shown in Fig. 6A, lysates from LEC29 cells transfected with vector had a small amount of Fut9 protein detected by a polyclonal antibody. There was no nonspecific band with this commercial antibody. LEC29 cells transfected with the Fut9 ORF had the largest amount of Fut9 protein, including the dimer form. However, full-length LEC29 cDNA gave rise to much less Fut9 protein than observed in LEC29 cells transfected with full-length LEC12 cDNA. The levels of β-tubulin in each lysate were equivalent. In *vitro* α(1,3)Fuc-T enzyme activity in cell lysates were 90.2 (Fut9 coding region), 17.2 (full-length LEC12), and 5.3 (full-length LEC29 cDNA) pmol/mg of protein/min, respectively, correlating approximately with differences in Fut9 protein levels.

To determine if the reduced translation of LEC29 Fut9 cDNA observed in transfectants was reflected by *in vitro* assays, full-length Fut9 cDNAs were subjected to *in vitro* transcription/translation in the coupled TntT reticulocyte lysate system. The coding region of the hamster Fut9 gene has six in-frame translation start codons that could generate multiple products (Fig. 6B). Three major species were obtained from the Fut9 coding region construct. Both LEC12 and LEC29 full-length Fut9 constructs gave fewer products, with a major band at 37.4 kDa in each case. The intensity of this band was less for full-length LEC29 Fut9 cDNA.

To investigate further, *in vitro* translation of full-length, capped Fut9 RNAs was examined using translation extracts prepared from CHO, LEC12, or LEC29 cells (Fig. 6C). Addition of *in vitro* synthesized RNAs to each translation extract resulted in several translation products of different sizes. The nonspecific band at ~55 kDa detected by this mAb (Fig. 3B) is marked with a star and provides evidence for relative loading. The ~42-kDa band present only in LEC12 extracts probably represents endogenous, unglycosylated Fut9 protein, because it was absent in the “-RNA” reactions of CHO and LEC29 extracts. Also, the intensity of this band was not increased when RNA was added, apparently because translation of this species did not occur efficiently in the extracts. Signals from the ~37-
after transfection were assayed for LEC12 and LEC29 Fut9 RNA. Cell lysates prepared 20 h translation reactions containing [35S]methionine. Reaction products were electrophoresed on a 7.5% reducing SDS-PAGE gel and treated for duplicate assays of lysates from duplicate transfections. Similar results were obtained in two experiments.

CHO—giving rise to less Fut9 protein and less ing or 3 5 LEC29 RNA.Taken together, these findings suggest that the vector alone, or vector containing the sialyl-LeX (9, 13, 25) and bind at only background levels to activated human endothelial cells (26), LEC12 cells bind to human E-selectin overexpressed in CHO cells (albeit less well than LEC11 (27), and a small subset of glycoproteins from LEC12 bind a mouse E-selectin-IgG chimera (28). Flow cytometry with the latter mouse E-selectin-IgG chimera showed that LEC12 CHO cells bound mouse E-selectin (Fig. 7A),4 and LEC12 cells bound ~2% of the amount of E-selectin bound by LEC11 cells (Fig. 7A). LEC29 cells that make good amounts of LeX but almost no VIM-2 (Fig. 2) did not bind mouse E-selectin (Fig. 7A). However, when CHO or LEC29 cells were transiently transfected with an Fut9 coding region cDNA, binding of mouse E-selectin was induced. Control human IgG1 did not bind to the transfectants. Furthermore, E-selectin binding was abolished in the presence of 1 mM CaCl2 and 5 mM EDTA, a condition that does not induce membrane shedding of glycoproteins (data not shown). Both mouse and human Fut9 have been shown not to generate sLeX following transfection, nor to fucosylate (2,3)sialylated LacNAc structures (29, 30). In agreement with this, LEC29 cells overexpressing Fut9 did not bind the CSLEX-1 mAb that recognizes sLeX, although LEC29 cells were capable of synthesizing sLeX if transfected with a Fut6 cDNA (Fig. 7B). This shows that levels of Fut9 expressed in LEC12 cells and induced by transfection of a Fut9 cDNA in CHO or LEC29 cells cause the synthesis of a fucosylated ligand for mouse E-selectin likely to be VIM-2.

DISCUSSION

When the LEC12 and LEC29 CHO mutants were initially characterized, their glycosylation phenotypes were different,

Fig. 6. The LEC29 Fut9 5'-UTR inhibits the translation of full-length Fut9 cDNA. LEC29 cells were transiently transfected with pCR3.1 vector alone, or vector containing the Fut9 gene coding region, or with a pcDNA3.1V5-His TOPO vector containing full-length (5'-UTR plus coding region) LEC12 or LEC29 Fut9 cDNA. A, lysates (~100 µg of protein) from LEC29 transfecants prepared in 1.5% Triton X-100 were analyzed for Fut9 protein level by immunoblotting with polyclonal C-17 anti-Fut9 antibody followed by anti-β-tubulin antibody. B, diagram of the CHO Fut9 cDNA coding transcript. There are six ATGs that are in-frame with the stop codon. Sizes of products arising from initiation of translation from each of the six ATGs are indicated as number of amino acid residues and predicted molecular weight. 0.5 or 1 µg of Fut9 coding region (Fut9 ORF), full-length LEC12 Fut9 (LEC12Fut9), or full-length LEC29 Fut9 (LEC29Fut9) cDNA was used as template in in vitro coupled transcription-translation reactions containing [35S]methionine. Reaction products were electrophoresed on a 7.5% reducing SDS-PAGE gel and treated for autoradiography. Exposure to film was for 36 h at -80 °C. The dash indicates no cDNA added. C, Western analysis of in vitro translation reactions. In vitro transcribed, capped, full-length LEC12 (12) or LEC29 (29) Fut9 RNA (1.5 µg) was translated in translation extracts prepared from CHO, LEC12, or LEC29 cells, and products were separated by SDS-PAGE, transferred to membrane, and immunoblotted with monoclonal KM2681 anti-Fut9 mAb. The star identifies the nonspecific band identified by this antibody. D, LEC29 cells were transfected with in vitro transcribed, full-length, LEC12, or LEC29 Fut9 RNA (2 µg) using the TransMessenger reagent. Cell lysates in 1.5% Triton X-100 were prepared after 20 h (50 µl of lysate per plate) and assayed for a(1,3)Fuc-T activity using LacNAc as substrate. Error bars indicate mean and range of data acquired in duplicate assays of lysates from duplicate transfections. Similar results were obtained in two experiments.

~32-, and ~26-kDa species with full-length LEC12 Fut9 RNA were more intense than those with full-length LEC29 Fut9 RNA in reactions with all three translation extracts, even though the CHO extract was not as active as LEC12 or LEC29 extracts. It is clear from these in vitro translation assays that full-length LEC29 Fut9 RNA with 5'- and 3'-UTRs is translated less efficiently than full-length LEC12 Fut9 RNA.

To assay full-length Fut9 RNAs in vivo, LEC29 cells were transfected with capped, in vitro synthesized, full-length LEC12 and LEC29 Fut9 RNA. Cell lysates prepared 20 h after transfection were assayed for a(1,3)Fuc-T activity (Fig. 6D). LEC12 RNA gave more a(1,3)Fuc-T activity than the LEC29 RNA. Taken together, these findings suggest that the 5'-UTR of LEC29 Fut9 mRNA acting together with its coding or 3'-UTR region reduces translation of Fut9 gene transcripts giving rise to less Fut9 protein and less a(1,3)Fuc-T activity.

CHO Cells Expressing Fut9 Bind a Mouse E-selectin-IgG Chimera—LEC11 CHO cells express sialyl-LeX (9, 13, 25) and bind E-selectin (8, 26). Although LEC12 cells do not express sialyl-LeX (9, 13, 25) and bind at only background levels to activated human endothelial cells (26), LEC12 cells bind to human E-selectin overexpressed in CHO cells (albeit less well than LEC11 (27), and a small subset of glycoproteins from LEC12 bind a mouse E-selectin-IgG chimera (28). Flow cytometry with the latter mouse E-selectin-IgG chimera showed that

4 M. Asada and P. Stanley, unpublished observations.
and it was considered unlikely that they expressed the same Fut gene (9). They have different lectin resistance phenotypes, express a different spectrum of \( (1,3) \) fucosylated cell surface antigens, and have very different levels of \( (1,3) \) Fuc-T activity in cell extracts. Thus it was surprising to find that both mutants express the Fut9 gene (8, 11). However, the mutant with the most Fut9 gene transcripts, LEC29, had the least \( (1,3) \) Fuc-T activity. Here we identify a molecular basis for the discrepant phenotypes of LEC12 and LEC29, provide insight into translational control of Fut9 gene transcripts, and reveal potential biological consequences of regulated expression of \( (1,3) \) Fuc-TIX. A comparison of LEC12 and LEC29 properties is summarized in Table I.

Fig. 7. A, mouse E-selectin binds to CHO cells overexpressing Fut9. LEC11 and LEC12 CHO mutants and LEC29 or CHO cells transiently transfected with empty vector (+ vector), or with vector containing the coding region of Fut9 (+ Fut9) were analyzed by fluorescence cytometry for binding of 6 \( \mu \)g of mouse E-selectin-IgG fusion protein or human IgG1 detected with anti-human IgG1 secondary antibody. LEC29 + Fut9 cells were also tested in the presence of EDTA as described under "Materials and Methods." The inset enlarges the area of difference in the LEC29 + Fut9 panel. B, sialyl-Le\( ^{\text{X}} \) is not generated on LEC29 cells overexpressing a Fut9 cDNA. LEC29 cells that were transiently transfected with empty vector (+ vector), or with vector containing the coding region of the Fut6B (8) or Fut9 Chinese hamster genes (LEC29 + Fut9, left panel; LEC29 + Fut6, right panel) were analyzed by fluorescence cytometry for binding of 3 \( \mu \)g of CSLEX-1 mAb.

FIG. 7. A, mouse E-selectin binds to CHO cells overexpressing Fut9. LEC11 and LEC12 CHO mutants and LEC29 or CHO cells transiently transfected with empty vector (+ vector), or with vector containing the coding region of Fut9 (+ Fut9) were analyzed by fluorescence cytometry for binding of 6 \( \mu \)g of mouse E-selectin-IgG fusion protein or human IgG1 detected with anti-human IgG1 secondary antibody. LEC29 + Fut9 cells were also tested in the presence of EDTA as described under "Materials and Methods." The inset enlarges the area of difference in the LEC29 + Fut9 panel. B, sialyl-Le\( ^{\text{X}} \) is not generated on LEC29 cells overexpressing a Fut9 cDNA. LEC29 cells that were transiently transfected with empty vector (+ vector), or with vector containing the coding region of the Fut6B (8) or Fut9 Chinese hamster genes (LEC29 + Fut9, left panel; LEC29 + Fut6, right panel) were analyzed by fluorescence cytometry for binding of 3 \( \mu \)g of CSLEX-1 mAb.
**E-selectin Binding Induced by α(1,3)Fuc-T-IX**

49725

**Table I**

| Characteristics of CHO, LEC12, and LEC29 cells |
|------------------------------------------------|
| Properties of LEC12, LEC29, and CHO cells are summarized from this study (Figs. 2, 3A, 3C, and 7A) and Refs. 8 and 10. | |
| negative; +, positive; the number of | |
| indicates relative quantitation. | |

| CHO | LEC12 | LEC29 |
|-----|-------|-------|
| Fut9 transcripts | – | ++ | ++ |
| Fut9 protein | – | +++ | + |
| α1,3Fuc-T activity | – | +++ | + |
| Leα expression | – | +++ | + |
| VIM-2 expression | – | – | – |
| E-selectin binding | – | – | – |

**Table II**

| Downstream coding region | Assay | 5’-UTR |
|---------------------------|-------|--------|
|                            | None  | LEC12 Fut9 | LEC29 Fut9 |
| Renilla luciferase         | DNA transfection | ND | + | ++ |
| CHO Fut9 (coding plus 3’-UTR) | In vitro RNA translation | ++ | + | ++ |
|                            | DNA transfection | + | + | + |
|                            | In vitro RNA translation | ND | + | + |
|                            | RNA transfection | ND | + | + |
|                            | In vitro transcription and translation | +++ | + | + |

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**Table II**

Effect of Fut9 5’-UTRs on the translation of downstream coding regions

The data in Figs. 5 and 6 are summarized. –, negative; +, positive; the number of + signs indicates relative quantitation. ND, not done.
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