Activation of two new $\alpha(1,3)$ fucosyltransferase activities in Chinese hamster ovary cells by 5-azacytidine

Barry Potvin*† and Pamela Stanley*

*Department of Cell Biology
Albert Einstein College of Medicine
New York, New York 10461

†Department of Biology
Yeshiva University
New York, New York 10033

Several mammalian $\alpha(1,3)$ fucosyltransferases ($\alpha[1,3]$ Fuc-T) that synthesize carbohydrates containing $\alpha(1,3)$ fucosylated lactosamine units have been identified. Although Chinese hamster ovary (CHO) cells do not express $\alpha(1,3)$ Fuc-T activity, the rare mutants LEC11 and LEC12, isolated after mutagenesis or DNA transfection, each express an $\alpha(1,3)$ Fuc-T that may be distinguished by several criteria. Two new CHO mutants possessing $\alpha(1,3)$ Fuc-T activity (LEC29 and LEC30) have now been isolated after treatment of a CHO cell population with 5-azacytidine (5-AzaC), ethylnitrosourea (ENU), or 5-AzaC followed by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Like LEC12, both mutants possess an *N*-ethylmaleimide-resistant $\alpha(1,3)$ Fuc-T activity that can utilize a variety of acceptors and both express the Lewis X (Le^x) determinant {Gal β [1,4](Fuc α [1,3])GlcNAc β 1} but not the sialyl $\alpha(2,3)\text{Le}^x$ determinant on cell-surface carbohydrates. However, LEC29 and LEC30 may be distinguished from LEC11 and LEC12, as well as from each other, on the basis of their unique patterns of lectin resistance and their abilities to bind the VIM-2 monoclonal antibody that recognizes carbohydrates terminating in NeuNAc $\alpha(2,3)$ Gal $\beta(1,4)$ GlcNAc $\beta(1,3)$ Gal $\beta(1,4)$ (Fuc α [1,3])GlcNAc β and also by the different *in vitro* substrate specificities and kinetic properties of their respective $\alpha(1,3)$ Fuc-T activities. The combined data provide good evidence that the LEC29 and LEC30 $\alpha(1,3)$ Fuc-Ts are novel transferases encoded by distinct gene products.

Introduction

The variable expression of particular carbohydrate structures has long been recognized as a

feature of glycoconjugates in developing mammalian tissues (Muramatsu, 1988). The relevant sugars are usually in terminal positions of carbohydrate chains and are highly antigenic (Feizi, 1985). The latter feature led to the identification of the stage-specific embryonic antigen 1 (SSEA-1)¹ (Solter and Knowles, 1978) that was later shown to comprise the sequence of sugars Gal $\beta(1,4)$ (Fuc α [1,3])GlcNAc β 1 (Gooi *et al.*, 1981). Early studies showed this antigen to be differentially expressed during embryonic development and present only in a restricted set of adult tissues (Fox *et al.*, 1981). However, the SSEA-1 antigen is often re-expressed in cells that become transformed and may be useful as a diagnostic marker of some human cancers (Kim *et al.*, 1988; Hakomori, 1989).

Interest in the SSEA-1 sequence of sugars (also termed Lewis X [Le^x] in their role as a blood group antigen) has recently increased due to the finding that Le^x appears to be a ligand of PADGEM, the platelet membrane glycoprotein that binds leukocytes (Larsen *et al.*, 1990). A related molecule, sialyl $\alpha(2,3)\text{Le}^x$ (SA α [2,3] Le^x), is recognized by the endothelial cell lectin epidermal growth factor-complement-binding cell adhesion molecule (LEC-CAM) termed endothelial-leukocyte adhesion molecule-1 (ELAM-1) (Lowe *et al.*, 1990; Phillips *et al.*, 1990; Walz *et al.*, 1990) and has recently been reported to

¹ Abbreviations used: 5-AzaC, 5-azacytidine; BSA, bovine serum albumin; CHO, Chinese hamster ovary; ELAM-1, endothelial-leukocyte adhesion molecule-1; EMS, ethylmethanesulfonate; ENU, ethylnitrosourea; FCS, fetal calf serum; $\alpha(1,3)$ Fuc-T, $\alpha(1,3)$ fucosyltransferase; LacNAc, N-acetyllactosamine (Gal β [1,4]GlcNAc); Lec^R, lectin resistance; LEC-CAM, lectin epidermal growth factor-complement-binding cell adhesion molecule; Le^x , Lewis X; L-PHA, lymphoagglutinin from *P. vulgaris*; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MOPS, 3-[*N*-morpholino]propanesulfonic acid; nLc₄, paragloboside; PADGEM, platelet activation-dependent granule-external membrane protein; RIC, ricin; sRBC, sheep red blood cells; SA, *N*-acetylneuraminic acid (sialic acid); SA α (2,3)LacNAc, α (2,3)sialyl-*N*-acetyllactosamine; SSEA-1, stage-specific embryonic antigen-1 Gal β (1,4)(Fuc α [1,3])GlcNAc β 1; WGA, wheat germ agglutinin.

be an even better ligand than Le^x for platelet activation-dependent granule-external membrane protein (PADGEM) or CD62 (Polley *et al.*, 1991). Studies with rare Chinese hamster ovary (CHO) cell mutants and human cancer cells have shown that the presence of Le^x and SA α -(2,3)Le^x determinants on cellular glycoconjugates is regulated by the expression of specific α (1,3)fucosyltransferases (α [1,3]Fuc-Ts) with different substrate specificities (Campbell and Stanley, 1983, 1984; Holmes *et al.*, 1985, 1986; Howard *et al.*, 1987). Therefore, determining the number and characteristics of the different α (1,3)Fuc-T enzymes encoded in the mammalian genome are important to understanding the biological roles played by α (1,3)fucosylated carbohydrates.

CHO cells do not normally express α (1,3)Fuc-T activity and consequently have proved useful for cloning an α (1,3)Fuc-T gene by genomic DNA transfection (Potvin *et al.*, 1990; Kumar *et al.*, 1991) and for characterizing the transferase activities encoded in cloned α (1,3)Fuc-T genes (Goelz *et al.*, 1990; Lowe *et al.*, 1990). However the apparent de novo expression of α (1,3)Fuc-T activity in CHO cells has been observed in rare mutants obtained after mutagenesis (Stanley, 1983a) and after genomic DNA transfection (Potvin *et al.*, 1990)—possibly due to gene rearrangements. Such treatments gave rise to two different α (1,3)Fuc-T-positive CHO mutant types termed LEC11 and LEC12 whose α (1,3)Fuc-T enzymes represent distinct gene products (Campbell and Stanley, 1983, 1984; Howard *et al.*, 1987). LEC11 cells synthesize both Le^x and SA α (2,3)Le^x determinants and bind to ELAM-1 on activated endothelial cells as well as CD62 on activated platelets whereas LEC12 cells synthesize only Le^x and exhibit very little binding to either LEC-CAM (Howard *et al.*, 1987; Stanley and Atkinson, 1988; Phillips *et al.*, 1990; Polley *et al.*, 1991). The existence of the LEC11 and LEC12 CHO cell lines suggested the possibility of identifying other α (1,3)Fuc-Ts by activating quiescent α (1,3)Fuc-T genes in the CHO genome. Agents such as 5-azacytidine (5-AzaC) that demethylate DNA are known to activate silent genes in cultured cells, cancer cells, and during development (reviewed in Cedar, 1988; Cedar and Razin, 1990; Holliday and Ho, 1990; Jones and Buckley, 1990). We show in this paper that treatment of CHO cells with 5-AzaC, although it did not increase the frequency of LEC11 or LEC12 CHO mutants, resulted in the isolation of two new α (1,3)Fuc-T-positive CHO lines that express novel transferase activities.

Results

5-AzaC induces expression of the SSEA-1 antigen

Previous studies from this laboratory have shown that the CHO lines Pro⁻5 and Gat⁻2 have no detectable α (1,3)Fuc-T activity in direct transferase assays nor do they bind the monoclonal antibody α SSEA-1, which recognizes terminal α (1,3)fucosylated lactosamine units on glycoconjugates (Campbell and Stanley, 1983, 1984; Howard *et al.*, 1987). To determine whether 5-AzaC treatment might activate silent α (1,3)Fuc-T genes, Pro⁻5 cells were treated with varying doses of 5-AzaC, cultured for an expression period, and selected for resistance to wheat germ agglutinin (WGA), to which both LEC11 and LEC12 cells are highly resistant (Stanley, 1983a). The colonies that survived selection were screened for their ability to bind α SSEA-1 antibodies conjugated to sheep red blood cells (α SSEA-1/sRBC). To show that the 5-AzaC treatment had been successful, the frequency of reversion of Pro⁻ cells to auxotrophy (Pro⁺) was also monitored. The results in Table 1 show that, depending on the treatment regimen, from 0.1% to 4% of 5-AzaC-treated Pro⁻ cells were converted to Pro⁺, as reported previously by others (Harris, 1984; Patterson, 1985; Worton and Grant, 1985).

When a low concentration of WGA was applied to 5-AzaC-treated cells, the survival frequency was $\geq 10^{-3}$ as expected because all known WGA-resistant CHO mutants should survive this low concentration. Also, significant numbers of parental CHO cells would be expected to survive because 1–3 μ g/ml WGA kills only 90% of a CHO population (Stanley, 1983b). However, only 5-AzaC-treated populations gave rise to colonies that bound α SSEA-1/sRBC (Table 1). Interestingly, the number of α SSEA-1/sRBC-binding colonies increased in rough proportion to the number of Pro⁺ revertants, which generally also increased with the dose of 5-AzaC (Table 1). Within a single experiment and using a 4-d expression time, a direct correlation was observed after treatment with increasing concentrations of 5-AzaC. In the same experiment, cells allowed an expression period of 10 d also showed the correlation but had a much lower percentage of Pro⁺ revertants. In other experiments, even shorter expression periods gave high numbers of Pro⁺ revertants and α SSEA-1/sRBC-positive colonies. In all experiments, the only consistent correlation observed was that, given the same expression period, increasing concentrations of 5-AzaC gave rise to increas-

Table 1. 5AzaC treatment increases the frequency of α SSEA-1⁺ve CHO cells

5AzaC (μ g/ml)	Expression (d)	Percent Pro ⁺ revertants	No. WGA ^R colonies per 10 ⁶ viable cells	No. α SSEA-1/sRBC binding colonies per 10 ⁶ viable cells	Percent α SSEA-1 ⁺ ve WGA ^R
—	4	0.02	368	0	<0.1
—	10	0.01	350	0	<0.1
0.1	4	0.01	446	1	0.2
0.1	10	0.01	2585	0	<0.1
0.3	4	0.10	1245	25	2.0
0.3	10	0.05	878	1	0.1
1.0	4	3.80	945	154	16.3
1.0	10	0.53	835	6	0.7
1.5	4	4.44	1133	253	22.3
1.5	7	1.30	768	350	45.6
1.5	10	1.09	1270	10	0.8

Pro⁻5 CHO cells were treated with 5AzaC for 28 h, washed, and maintained at a density of $\sim 3 \times 10^5$ cells/ml for the expression times indicated. Colonies surviving selection in 3.5 μ g/ml WGA were screened with the α SSEA-1/sRBC conjugate to identify those that were producing the SSEA-1 cell-surface antigen. The data shown is from a single, internally controlled experiment. Similar results were obtained in two other experiments.

ing numbers of Pro⁺ revertants as well as α SSEA-1/sRBC-positive colonies. Treatment of Gat⁻2 CHO cells with 5-AzaC also gave α SSEA-1/sRBC-positive colonies after selection with 3.5 μ g/ml WGA (Table 2). When the concentration of WGA was increased, the number of survivors fell dramatically, and at WGA concentrations >5 μ g/ml, no α SSEA-1/sRBC-positive colonies were obtained. Thus, mutants with the LEC11 or LEC12 phenotype were not obtained from these selections.

Several colonies were isolated from the experiments described in Table 1 and characterized further. A high proportion ($\sim 90\%$) gradually lost the ability to bind α SSEA-1/sRBC on replating. However, one isolate, termed LEC29, retained stable expression of its phenotype in the absence of selection and a subclone was isolated by limiting dilution for further study.

To examine the effects of other mutagens on the induction of $\alpha(1,3)$ Fuc-T-expressing CHO lines, the parental CHO lines Pro⁻5 and Gat⁻2

Table 2. Isolation of SSEA-1⁺ve CHO mutants LEC29 and LEC30

Cell line	Mutagen	Expression (d)	WGA (μ g/ml)	No. WGA ^R colonies per 10 ⁶ cells plated	No. α SSEA-1/sRBC binding colonies per 10 ⁷ cells plated	Phenotypes identified
Pro ⁻ 5	—	NA	3.5	610	<1	
	MNNG	6	3.5	535	1	LEC11 (1)
	ENU	6	3.5	5460	5	LEC29 (5)
	5AzaC	4	3.5	1747	580	MIXED (4)
	5AzaC	8	3.5	2173	235	LEC29 (10)
	5AzaC } MNNG }	16	13.3	877	28	LEC30 (3)
Gat ⁻ 2	—	NA	7.5	36	<1	
	MNNG	6	7.5	65	8	MIXED (8)
	EMS	6	7.5	588	3	LEC11 (1)
	ENU	6	7.5	720	4	MIXED (4)
	5AzaC	4	3.5	1473	14	LEC29 (4)

Pro⁻5 and Gat⁻2 cells were treated with mutagens as described in Materials and methods. After the indicated expression periods, cells were plated in selective media at densities of 2×10^5 (for WGA concentrations of 3.5 and 7.5 μ g/ml) or at 1×10^6 (for WGA concentrations of 13.3 μ g/ml). Surviving WGA resistant colonies were screened with the α SSEA-1/sRBC conjugate, positives were picked, rescreened with the same conjugate, and finally characterized by their Lec^R phenotypes (see Figure 1). The number of isolates of each phenotype identified is given in parentheses.

were treated with different mutagens, selected for resistance to WGA, and screened for binding of α SSEA-1/sRBC (Table 2). No spontaneous α (1,3)Fuc-T-positive mutants were isolated. However, both point mutagens ethylmethanesulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) resulted in positives with a LEC11 phenotype as determined by the characteristics of their α (1,3)Fuc-T activities and by their resistance to the plant lectins WGA, ricin (RIC), and lymphoagglutinin from *Phaseolus vulgaris* (L-PHA).

LEC12 mutants, which have a characteristically different lectin-resistance (Lec^R) phenotype (Stanley, 1983a), were not obtained in these experiments. Although all the mutagenic treatments gave positives, many of the isolates had mixed phenotypes that were not easily categorized because they included phenotypes similar to previously characterized CHO mutants such as Lec1, Lec2, and Lec3 (Stanley, 1983b). However, several isolates with the characteristics of LEC29 obtained from 5-AzaC-treated Pro⁻⁵ cells (Table 1) arose from ethylnitrosourea (ENU)-treated Pro⁻⁵ cells and 5-AzaC-treated Pro⁻⁵ and Gat⁻² cells (Table 2). One novel phenotype that could be purified in stable form was termed LEC30. This was obtained after MNNG treatment of a 5-AzaC-treated population of Pro⁻⁵ cells.

LEC29 and LEC30 have novel Lec^R phenotypes

As expected for mutants expressing a new activity (i.e., the Le^x carbohydrate recognized by the α SSEA-1/sRBC conjugate), both LEC29 and LEC30 behaved dominantly in somatic cell hybrids formed with parental CHO cells. All hybrids bound α SSEA-1/sRBC and those tested individually were resistant to WGA and hypersensitive to RIC (data not shown).

Because it is not possible to distinguish between dominant mutant genes by complementation analysis, their novelty must be based on phenotypic comparisons before the precise nature of each dominant mutation is known. Both new mutants had a Lec^R phenotype distinct from LEC11 and LEC12 as well as from each other (Figure 1). The striking differences in their respective resistance to WGA and hypersensitivity to RIC strongly suggest that they add α (1,3)fucose to different arrays of cell-surface carbohydrates and therefore would be expected to express α (1,3)Fuc-T enzymes with a unique spectrum of substrate specificities.

Figure 1. Lectin resistance phenotypes of LEC11, LEC12, LEC29, and LEC30. Patterns of resistance or hypersensitivity to five cytotoxic lectins were determined for each cell line as described in Materials and methods. Results are shown compared with the parental (Pro⁻⁵) cell line. The concentration of each lectin necessary to kill 90% of parental cells is represented by the vertical line. The actual concentrations corresponding to the vertical line are as follows: L-PHA, 8 μ g/ml; WGA, 3 μ g/ml; CONA (concanavalin A), 25 μ g/ml; RIC, 0.010 μ g/ml; and LCA (agglutinins from *L. culinaris*), 25 μ g/ml. The numbers at the bottom of the figure represent x-fold resistance ■ or x-fold sensitivity ▨. The data represent the average of five separate experiments.

LEC29 and LEC30 express Le^x but not SA α (2,3)Le^x determinants

Monoclonal antibodies that bind α (1,3)fucosylated lactosamine units provide an approach to identifying the in vivo products of α (1,3)Fuc-T activities. Previous studies showed that both LEC11 and LEC12 CHO mutants bind the α SSEA-1 antibody that recognizes Le^x, whereas only LEC11 cells bind the CSLEX-1 antibody that recognizes SA α (2,3)Le^x (Campbell and Stanley, 1983; Howard *et al.*, 1987). The data in Figure 2 show that under these assay conditions, both LEC29 and LEC30 bound α SSEA-1 antibody equivalently to LEC11 and LEC12 cells. However, neither of the new mutants bound significant amounts of CSLEX-1 antibody. Controls using the parental Pro⁻⁵ CHO cells and the multiple mutant line Lec3.2.8.1 (which is unable to produce polylactosamine structures) failed

Figure 2. Binding of monoclonal antibodies to cell-surface antigens. Approximately 10^6 cells from each cell line were washed and assayed for their abilities to bind to the monoclonal antibody α SSEA-1 (which recognizes Le^x) $\square$ or CSLEX-1 (which recognizes $SA\alpha[2,3]Le^x$) $\blacksquare$. The results shown are from a representative experiment that was duplicated for each line at least once.

to bind significant amounts of either antibody. Interestingly, the abilities of LEC29 and LEC30 to bind α SSEA-1 were similar despite the marked difference in their Le^R phenotypes (Figure 1) and the fact that LEC29 cells bound the α SSEA-1/sRBC conjugate rather weakly compared with LEC30 (data not shown).

$\alpha(1,3)$ Fuc-T activities of LEC29 and LEC30

The $\alpha(1,3)$ Fuc-T activity of the new isolates was assayed in detergent cell extracts using a variety of oligosaccharide, glycoprotein, and glycolipid substrates (Table 3). When the disaccharide $Gal\beta 1,3GlcNAc$ was used as substrate, no product was obtained showing that, like LEC11 and LEC12 (Campbell and Stanley, 1984), the new mutants could not transfer fucose to the Gal or GlcNAc residues in this disaccharide. By contrast, each of the isolates had activity with

N-acetylglucosamine ($Gal\beta[1,4]GlcNAc(LacNAc)$) that accepts fucose in $\alpha(1,3)$ -linkage. Previous studies have characterized the labeled product $Gal\beta 1,4[Fuc\alpha 1,3]GlcNAc$ obtained (Campbell and Stanley, 1983), and the monoclonal antibody data (Figure 2) clearly show that this terminal sugar sequence is expressed on intact LEC29 and LEC30 cells.

The assays in Table 3 using LacNAc as the acceptor were performed under saturating conditions and reveal an enormous disparity in the specific $\alpha(1,3)$ Fuc-T activities of the new mutants. The LEC29 transferase was markedly less active compared with the LEC11 or LEC12 transferases whereas the LEC30 transferase had a much higher specific activity than the LEC11 or LEC12 enzymes. The specific activity of the LEC30 $\alpha(1,3)$ Fuc-T ($\sim 73 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$) was higher than any glycosyltransferases previously studied in a crude CHO cell extract (Gottlieb *et al.*, 1975; Stanley *et al.*, 1975; Briles *et al.*, 1977; Narasimhan *et al.*, 1977).

The $\alpha(1,3)$ Fuc-T activities of LEC29 and LEC30 were like the LEC12 enzyme (and unlike the LEC11 $\alpha[1,3]$ Fuc-T) in that they were both resistant (86% and 97%, respectively) to 3 mM *N*-ethylmaleimide, they were active in the presence of 1 mM $ZnCl_2$ and they were able to use either Co^{++} or Ca^{++} instead of Mn^{++} (data not shown). An unusual feature of the LEC29 $\alpha(1,3)$ Fuc-T was that the optimal concentration of the detergent NP40 in the extraction buffer was found to be 0.5% rather than 1%. Higher concentrations of NP40 or the use of a variety of different detergents always yielded extracts with lower specific activities (data not shown).

As expected from the antibody binding results (Figure 2), the new mutants exhibited poor fu-

Table 3. $\alpha(1,3)$ Fuc-T activity of LEC29 and LEC30 cell extracts

Substrate	$\alpha(1,3)$ Fuc-T activity ($\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$)			
	LEC11	LEC12	LEC29	LEC30
$Gal\beta(1,4)GlcNAc$	50.1 (5)	85.9 (10)	2.4 (11)	1216 (11)
$Gal\beta(1,3)GlcNAc$	<0.1 (2)	<0.1 (2)	<0.1 (1)	<0.1 (1)
Fetuin	13.9 (2)	3.7 (4)	0.2 (2)	18.6 (2)
Asialo-fetuin	24.6 (5)	32.1 (5)	2.9 (3)	74 (1)
nLc ₄	21.2 (2)	30 (3)	<0.1 (1)	197.4 (2)
$SA\alpha(2,3)nLc_4$	28.4 (1)	<0.3 (1)	N.D.	93.8 (1)

$\alpha(1,3)$ Fuc-T activities were determined using the indicated oligosaccharide, glycoprotein, or glycolipid acceptor. The data shown are averages with the number of determinations given in parentheses. In every experiment, controls with LacNAc as the substrate were performed to demonstrate that each extract was active, even though it may have shown a complete lack of product formation with other substrates. N.D., not determined.

cose transfer to fetuin (which contains sialylated lactosamines) and considerably better transfer to asialofetuin. LEC29 had no detectable activity with the glycolipid substrate paragloboside ($n\text{Lc}_4$) whereas LEC30 was very active. As shown previously (Howard *et al.*, 1987), LEC11 but not LEC12 cell extracts transfer fucose to $\text{SA}\alpha(2,3)\text{nLc}_4$. Surprisingly, LEC30 extracts were also very active with this substrate, in spite of their inability to express $\text{SA}\alpha(2,3)\text{Le}^x$ on cellular carbohydrates (Figure 2). This apparent anomaly was explored by a more detailed examination of the products formed by LEC30 extracts when $\alpha(2,3)$ sialylated *N*-acetylglucosamine ($\text{SA}\alpha(2,3)\text{LacNAc}$) was compared with the same concentration of LacNAc for acceptor activity (Table 4). Products of the assays were differentially eluted from Dowex $1 \times 4 (\text{Cl}^-)$ to separate neutral products from those containing sialic acid. The former did not bind to the ion exchange column whereas the latter bound and were eluted with 3 ml of 0.15 M NaCl. GDP- ^{14}C -fucose also bound to the Dowex but control incubations lacking exogenous acceptor showed no release of ^{14}C -GDP-fucose with 3 ml of 0.15 M NaCl.

The results in Table 4 show that, as expected, the LEC11 $\alpha(1,3)\text{Fuc-T}$ activity generated high levels of sialylated product when $\text{SA}\alpha(2,3)\text{-LacNAc}$ was the substrate. However, only neutral product was obtained from both LEC12 and LEC30 extracts. When $\text{SA}\alpha(2,6)\text{LacNAc}$ was the substrate, the LEC11 $\alpha(1,3)\text{Fuc-T}$ produced no product whereas the LEC12 and LEC30 enzymes gave small amounts of neutral products ($\sim 10\%$ compared with when $\text{SA}\alpha(2,3)\text{LacNAc}$ was the substrate; data not shown). The data suggested that LEC12 and LEC30 cells

possess a neuraminidase that removes the sialic acid from $\text{SA}\alpha(2,3)\text{LacNAc}$ (and to a lesser extent from $\text{SA}\alpha(2,6)\text{LacNAc}$), thereby generating LacNAc to be an acceptor in these extracts. This, in fact, proved to be the case as the presence of the neuraminidase inhibitor 2,3 dehydro-2-deoxy-*N*-acetylneuraminic acid (Usuki *et al.*, 1988) markedly inhibited neutral product formation by LEC12 and LEC30 extracts. The small amounts of sialylated products formed suggest that the LEC12 and LEC30 $\alpha(1,3)\text{Fuc-Ts}$ can inefficiently utilize the sialylated substrate. However, LEC12 extracts reproducibly gave a small amount of charged product even with the neutral substrate. This could reflect fucosylation of an endogenous protein substrate.

Direct assay of the neuraminidase activity of parental and mutant cell extracts using the fluoresceinated substrate methylumbelliferyl-*N*-acetylneuraminic acid under the same incubation conditions as those for the $\alpha(1,3)\text{Fuc-T}$ assay gave the following activities expressed in nmol sialic acid released $\cdot \text{h}^{-1} \cdot \text{mg protein}^{-1}$: CHO (Pro $^{-5}$), 66.4; LEC11, 27.6; LEC12, 54.0; LEC30, 106.5. Thus, parental CHO cells and all the $\alpha(1,3)\text{Fuc-T}$ positive cell lines, including LEC11, have substantial neuraminidase activity. Because within the cell, this CHO neuraminidase is presumably not localized to the Golgi apparatus, its presence should not be the primary reason that LEC12 and LEC30 $\alpha(1,3)\text{Fuc-Ts}$ fail to produce detectable amounts of $\text{SA}\alpha(2,3)\text{Le}^x$ at the cell surface (Figure 2). However, in detergent cell extracts, the CHO neuraminidase is clearly very able to remove SA from $\text{SA}\alpha(2,3)\text{LacNAc}$ at pH 6.5.

Table 4. $\alpha(1,3)\text{Fuc-T}$ activity of LEC29 and LEC30 for $\text{SA}\alpha(2,3)\text{LacNAc}$

Oligosaccharide substrate (1 mM)	Neuraminidase inhibitor (2 mM)	$\alpha(1,3)\text{Fuc-T}$ activity ($\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$)					
		LEC11		LEC12		LEC30	
		N	SA	N	SA	N	SA
Gal β 1,4GlcNAc	—	21.5	0.6	52.9	<0.1	311.1	<0.1
	+	19.0	<0.1	80.2	4.8	326.8	<0.1
$\text{SA}\alpha(2,3)\text{Gal}\beta$ 1,4GlcNAc	—	<0.1	28.8	22.8	<0.1	156.8	<0.1
	+	<0.1	28.4	2.7	2.1	13.3	6.2

The products of the $\alpha(1,3)\text{Fuc-T}$ activities present in detergent extracts of LEC11, LEC12, and LEC30 cells (using LacNAc and $\text{SA}\alpha(2,3)\text{LacNAc}$ as acceptors) were separated into neutral (N) and sialylated (SA) components by differential elution from Dowex 1×4 columns with distilled/deionized water followed by 0.15 M NaCl. The data shown is from a single, internally controlled experiment. Very similar results were obtained in three separate experiments except that LEC12 extracts usually gave lower levels of charged product (<0.1 – 2.2 instead of $4.8 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) with Gal β 1,4GlcNAc in the presence of the neuraminidase inhibitor.

Additional properties of LEC29 and LEC30 that distinguish them from LEC12 and each other

Although it is apparent from the combined evidence that both LEC29 and LEC30 do not synthesize $\text{SA}\alpha(2,3)\text{Le}^x$ moieties and therefore are distinct from LEC11 (Figure 2) (Campbell and Stanley, 1984; Howard *et al.*, 1987), their properties were qualitatively similar by several criteria to those of LEC12. Therefore, methods were sought to distinguish them from LEC12 and from each other.

As the LEC30 $\alpha(1,3)\text{Fuc-T}$ was so active, direct comparisons of its enzymatic properties were made with the LEC12 $\alpha(1,3)\text{Fuc-T}$ that has previously been characterized in some detail (Campbell and Stanley, 1983, 1984; Howard *et al.*, 1987). Apparent K_m and V_{max} values were obtained by varying the concentration of one of the substrates LacNAc or GDP-fucose while maintaining the other substrate at a saturating concentration (data not shown). Hydrolysis of GDP-fucose was $\sim 6\%$ during the incubation period and therefore did not significantly alter the concentration of this substrate. The apparent K_m for LacNAc of the LEC30 $\alpha(1,3)\text{Fuc-T}$ was ~ 0.65 mM, which is close to that previously determined for the LEC12 $\alpha(1,3)\text{Fuc-T}$ (0.8 mM in Campbell and Stanley, 1983). However, the apparent K_m s for GDP-fucose differed by at least threefold, being ~ 15 μM for LEC12 and ~ 53 μM for LEC30. Interestingly, the V_{max} s for each enzyme were also very different (~ 192 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for LEC12 and ~ 1472 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for LEC30), although this may reflect different amounts of enzyme in the respective cell extracts. These kinetic parameters are an average of the results of three experiments. In addition, the pH optimum for the $\alpha(1,3)\text{Fuc-T}$ of LEC30 was higher (pH 7.0–8.5) than that of LEC12 (pH 5.5–7.0). These results are consistent with the interpretation that the LEC12 and LEC30 $\alpha(1,3)\text{Fuc-T}$ s are different molecules.

Another method for distinguishing $\alpha(1,3)\text{Fuc-T}$ enzymes is on the basis of the $\alpha(1,3)$ -fucosylated structures expressed at the cell surface. Although the differences in Lec^R phenotype between LEC12, LEC29, and LEC30 (Figure 1) provided good evidence that they have different arrays of cell surface carbohydrates, there were no apparent differences in their abilities to bind the $\alpha\text{SSEA-1}$ antibody at two different dilutions (Figure 2 and data not shown), suggesting they express roughly the same amounts of the Le^x carbohydrate at the cell sur-

face. Another type of $\alpha(1,3)$ fucosylated lactosamine synthesized by LEC11 and LEC12 cells is $\text{NeuNAc}\alpha(2,3)\text{Gal}\beta(1,4)\text{GlcNAc}\beta(1,3)\text{Gal}\beta(1,4)\text{-(Fuc}\alpha[1,3])\text{GlcNAc}\beta 1$ (Macher, unpublished observations), and therefore it was of interest to examine LEC29 and LEC30 for binding of an antibody termed VIM-2 that recognizes this terminal sugar sequence (Macher *et al.*, 1988). The results in Table 5 show that significant differences in expression of this carbohydrate were evident between the previously isolated mutants LEC11 and LEC12 and the new mutants LEC29 and LEC30. Whereas both LEC11 and LEC12 cells bound VIM-2 at levels equivalent to $\alpha\text{SSEA-1}$, LEC29 exhibited essentially no binding of this antibody and binding of VIM-2 by LEC30 cells was reduced compared with $\alpha\text{SSEA-1}$ by $\sim 30\%$. These results suggest that the LEC29 and LEC30 $\alpha(1,3)\text{Fuc-T}$ s are novel because, if they merely represented an altered production of the LEC12 $\alpha(1,3)\text{Fuc-T}$, they should each have the same ability as that transferase to synthesize the VIM-2 relative to the Le^x carbohydrate determinant. The combined data from kinetic studies and VIM-2 binding assays provides good evidence for the uniqueness of the $\alpha(1,3)\text{Fuc-T}$ activities of LEC29 and LEC30.

Discussion

The ability of 5-AzaC to activate silent genes in CHO cells was first reported for a thymidine kinase gene by Harris (1982). Subsequently, high-frequency reversion of the proline auxotrophy of CHO cells was shown to be induced by 5-AzaC treatment (Harris, 1984; Patterson, 1985; Worton and Grant, 1985). Reversion of the Pro⁻

Table 5. Ability of LEC29 and LEC30 to bind the VIM-2 monoclonal antibody

Cell line	Percent ^{125}I cpm bound		Ratio
	$\alpha\text{SSEA-1}$	VIM-2	Percent cpm bound VIM-2/percent cpm bound $\alpha\text{SSEA-1}$
CHO	0.6	1.0	
LEC11	22.8	26.3	1.20 ± 0.21
LEC12	29.8	25.5	0.90 ± 0.10
LEC29	20.4	0.6	0.02 ± 0.01
LEC30	26.3	16.4	0.63 ± 0.15

The ability of cell-surface antigens to bind to the $\alpha\text{SSEA-1}$ and VIM-2 antibodies was determined as described in Materials and methods. The results shown are the averages of four separate determinations and the ratio in the last column is given $\pm\text{SD}$.

to a Pro⁺ phenotype is easy to assay and provides a good method for monitoring the effectiveness of 5-AzaC in activating other quiescent genes in CHO cells. Using this comparison, we have provided indirect evidence that at least one and possibly two $\alpha(1,3)$ Fuc-T genes can be activated in CHO cells by 5-AzaC treatment (Tables 1 and 2). The likely mechanism of this gene activation is by demethylation of relevant DNA sequences (reviewed in Cedar, 1988; Cedar and Razin, 1990). However, 5-AzaC treatment has also been shown to cause DNA rearrangements and other effects on cellular metabolism (Parrow *et al.*, 1989), so that determining the actual mechanism for the appearance of the LEC29 and LEC30 CHO phenotypes must await molecular genetic analysis. Similarly, the mutagens MNNG, ENU, and EMS have different effects on DNA. Although all cause point mutations by chemical modification of DNA (Newbold *et al.*, 1980), ENU and MNNG treatment have been correlated with gene activation and hypomethylation of DNA (Barr *et al.*, 1986; Ivarie and Morris, 1986; MacArthur *et al.*, 1986), whereas EMS can cause gene inactivation and hypermethylation of DNA (Farrance and Ivarie, 1985). Thus, the genetic origins of the different CHO mutants expressing an $\alpha(1,3)$ Fuc-T activity (Table 2) may be due to a mutational event, an effect on methylation status, or a DNA rearrangement.

The most interesting outcome of the mutagenesis experiments is the observation that different mutants resulted from different mutagenic treatments. Neither the LEC11 nor LEC12 mutant type was obtained after 5-AzaC treatment, and the LEC11 but not the LEC12 type was obtained after treatment with chemical mutagens. Other agents or treatment regimens that alter DNA in different ways may therefore give rise to additional CHO mutants that express other developmentally regulated $\alpha(1,3)$ Fuc-T activities. Evidence suggesting that methylation is a mechanism used by cultured cells to silence genes not required for cell survival (Antequera *et al.*, 1990) or to produce hemizyosity in CHO cells (Holliday and Ho, 1990) suggests that additional searches for the activation of developmentally regulated glycosyltransferase genes in CHO cells may be fruitful. Because hypomethylation of DNA occurs in some human cancers (Jones and Buckley, 1990), as does the activation of glycosyltransferase activities usually found only in the fetus (Hakomori, 1989), it is possible that determining the origins of the CHO mutants expressing a new glycosyltransferase will be helpful in focusing studies to identify

mechanisms of aberrant glycosyltransferase expression in cancerous tissues.

Mammals possess at least three $\alpha(1,3)$ Fuc-T activities that may be readily distinguished on the basis of their biochemical properties and their tissue specific expression (Mollicone *et al.*, 1990). Genes corresponding to the three enzymes have recently been cloned (Goelz *et al.*, 1990; Kukowska-Latallo *et al.*, 1990; Larsen *et al.*, 1990; Lowe *et al.*, 1990, 1991; Kumar *et al.*, 1991). One gene encodes an $\alpha(1,3/1,4)$ Fuc-T, another an $\alpha(1,3)$ Fuc-T that synthesizes Le^x but not SA $\alpha(2,3)$ Le^x, and the third, an enzyme that appears to synthesize both Le^x and SA $\alpha(2,3)$ Le^x (Lowe *et al.*, 1991). The results presented in this paper suggest that there may be at least two additional gene products that encode $\alpha(1,3)$ Fuc-Ts that synthesize only the Le^x determinant because LEC12, LEC29, and LEC30 CHO cells appear to express distinct $\alpha(1,3)$ Fuc-T enzymes. This conclusion is based on differences in their Lec^R phenotype that reflect the synthesis of different cell-surface carbohydrates (Figure 1), differences in their ability to bind the VIM-2 antibody compared with the α SSEA-1 antibody (Table 5), and differences in their substrate specificities (Table 3), apparent K_m parameters, and optimal assay conditions (see p. 993). Although definitive proof that we have identified four distinct CHO $\alpha(1,3)$ Fuc-T gene products will require cloning and direct sequence comparisons, the combined data are consistent with this hypothesis.

It is intriguing that mammals are capable of synthesizing several different molecules that can perform apparently very similar transferase reactions. This redundancy suggests that the addition of $\alpha(1,3)$ fucose to glycoconjugates is physiologically important. One approach to identifying specific biological roles for the different $\alpha(1,3)$ Fuc-Ts would be to manipulate their expression in transgenic mice. However, before this can be accomplished, specific probes that will hybridize to each $\alpha(1,3)$ Fuc-T gene product must be developed. CHO cells and the mutants that express $\alpha(1,3)$ Fuc-T activity (LEC11, LEC12, LEC29, and LEC30) provide an excellent model for characterizing such probes because CHO cells do not normally express any $\alpha(1,3)$ Fuc-T gene and each mutant apparently expresses only one $\alpha(1,3)$ Fuc-T gene product. In contrast, transformed human cells such as PC9 (Holmes *et al.*, 1986) and Colo 205 (Stroup *et al.*, 1990) have been reported to express more than one $\alpha(1,3)$ Fuc-T activity and normal leukocytes possess, in addition to the myeloid $\alpha(1,3)$ Fuc-T, low levels of the $\alpha(1,3)$ Fuc-T typical

of plasma (Mollicone *et al.*, 1990), or amniotic fluid (Hanisch *et al.*, 1988). Thus, individual tissues may well express several $\alpha(1,3)$ Fuc-T genes in either the same or different cell types making causal relationships difficult to define.

Another use for CHO mutants that express a single $\alpha(1,3)$ Fuc-T will be in characterizing the carbohydrate ligands recognized by LEC-CAM molecules such as ELAM-1 and PADGEM (reviewed by Brandley *et al.*, 1990; Springer and Lasky, 1991). The four $\alpha(1,3)$ Fuc-T-expressing CHO mutants differ in their abilities to produce the SA $\alpha(2,3)$ Le^x and VIM-2 or CD65 carbohydrates, although they appear to synthesize similar amounts of the Le^x carbohydrate (Figure 2 and Table 5). It seems likely therefore that they will also differ in their abilities to recognize LEC-CAM molecules. It is already established that LEC11 CHO cells bind as effectively as myeloid cells to ELAM-1 expressed on activated endothelial cells whereas LEC12 cells do not bind (Phillips *et al.*, 1990; Kumar *et al.*, 1991). Similar results have recently been reported for binding of LEC11 and LEC12 cells to activated platelets (Polley *et al.*, 1991). Characterization of the interaction of LEC29 and LEC30 cells with LEC-CAMs of different origins may reveal new carbohydrate recognition phenomena of physiological relevance.

Materials and methods

Materials

LacNAc, 2-acetamido-2-deoxy-3-O- β -D-galactopyranosyl-D-galactopyranose (Gal β [1,3]GlcNAc), fetuin, asialofetuin, WGA, RIC, sodium cacodylate, 3-[N-morpholino]propanesulfonic acid (MOPS), N-ethylmaleimide, Nonidet P-40, bovine serum albumin (BSA) (fraction V), ENU, 5-AzaC, 4-methylumbelliferone, 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid, and 2,3-dehydro-2-deoxy-N-acetylneuraminic acid were from Sigma (St. Louis, MO). The SA $\alpha(2,3)$ LacNAc and SA $\alpha(2,6)$ LacNAc were obtained from Oxford Glycosystems (Rosedale, NY). The glycolipid substrates nLc₄ and IV⁹NeuNAc_nLc₄ were gifts from M. Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA). GDP-[¹⁴C]-fucose was either purchased from Amersham Corp. (Arlington Heights, IL) (14 mCi/mmol) or from DuPont-New England Nuclear (Boston, MA) (225 mCi/mmol) and was diluted with unlabeled GDP-fucose from Biocarb (Lund, Sweden). The monoclonal antibody anti-SSEA-1 was prepared as previously described (Howard *et al.*, 1987) from ascites fluid generated by Caf./J mice injected with the hydridoma cell line 480 provided by Dr. Barbara Knowles (Wistar Institute, Philadelphia, PA). The purified CSLEX-1 monoclonal antibody (Fukushima *et al.*, 1984) was obtained from Dr. Paul Terasaki (University of California, Los Angeles, CA). The VIM-2 monoclonal antibody (Macher *et al.*, 1988) was the generous gift of Dr. Bruce Macher, San Francisco State University, San Francisco, CA. Rabbit anti-mouse IgM was a product of Zymed (San Francisco, CA) and ¹²⁵I-protein A (~10⁸ cpm/mg protein) labeled by the method of Bolton and Hunter (1973) was from Amersham. sRBC were ob-

tained from Scott Laboratories (Fiskeville, RI). The plant lectins L-PHA and agglutinins from *Lens culinaris* were products of Vector Laboratories (Burlingame, CA) or Wellcome Diagnostics (Temple Hill, Dartford, England) and concanavalin A was from Pharmacia (Uppsala, Sweden). Alpha (α) medium was obtained from GIBCO (Grand Island, NY), horse serum was from Hazleton Research Products (Lenexa, KS), and fetal calf serum (FCS) was from Flow Laboratories (McLean, VA). Dowex 1 $\times$ 4 (100–200 mesh) chloride was purchased from Bio-Rad (Richmond, CA). Ecolume was from ICN Biomedicals (Irvine, CA). EMS was obtained from Eastman Kodak (Rochester, NY), whereas MNNG was from ICN Biomedicals (Costa Mesa, CA). All other chemicals were reagent grade.

Cell lines and cell culture

Two CHO lines Pro⁻⁵ and Gat⁻² (Stanley *et al.*, 1975) were used as the parental cell populations in these studies. The origins and properties of the two dominant mutant cell lines LEC11 (Pro⁻LEC11.E7) and LEC12 (Pro⁻LEC12.1B) have been previously described (Stanley, 1983a,b; Campbell and Stanley, 1983, 1984; Howard *et al.*, 1987). The isolation and properties of the Lec3.2.8.1 multiple mutant (which produces only severely truncated N-linked carbohydrates that cannot be used as acceptors by $\alpha(1,3)$ Fuc-Ts) has also been previously described (Stanley, 1989). All cells were routinely maintained in suspension culture at 37°C in complete α + medium supplemented with either 10% FCS or 10% horse serum and 1% FCS.

Mutagen treatments

Suspension cultures of Pro⁻⁵ or Gat⁻² CHO cells at densities of ~2 $\times$ 10⁵ cells/ml were treated with EMS, MNNG, or ENU as previously described (Sallustio and Stanley, 1989). Exposure of parental cell types to 5-AzaC at concentrations of 0.1, 0.3, 1.0, or 1.5 μ g/ml were performed exactly as described by Sallustio and Stanley (1989). Expression times varied as described in text.

Selection and screening

After appropriate expression intervals, surviving cells were plated at densities of 2 $\times$ 10⁵–1 $\times$ 10⁶ in 100-mm tissue culture dishes containing WGA at concentrations of either 3.5, 7.5, or 13.3 μ g/ml in α + medium supplemented with 10% FCS. Colonies arising after 8–12 d were screened for the expression of the Le^x determinant on cell surface carbohydrates using the α SSEA-1/sRBC conjugate as previously described (Potvin *et al.*, 1990).

Lectin resistance and cloning

Presumptive mutant colonies were picked into 17 $\times$ 100 mm sterile plastic tubes and expanded for further characterization and storage. Lec^R phenotypes were determined using techniques described by Stanley (1983b). Cloning was by limiting dilution and always preceded further testing.

Somatic cell hybridization

Cloned isolates were fused to parental cells by the polyethylene glycol/dimethylsulfoxide method previously described by Stanley (1983b). Hybrid cell colonies were characterized for their ability to bind the α SSEA-1/sRBC conjugate. Some colonies were picked, expanded in culture, and tested for Lec^R to WGA and RIC and for chromosome number as described previously (Stanley, 1983b).

Monoclonal antibody binding

The ability of the newly isolated cell lines to bind to the anti-SSEA-1, CSLEX-1, or VIM-2 antibodies was quantified using the indirect method described by Howard *et al.* (1987), except that incubation times were reduced to 1 h and antibodies were diluted 1:50 before use. Briefly, 10^6 cells were washed and resuspended in phosphate-buffered saline containing 2% BSA in a volume of 200 μ l. The percentage of cpm bound was determined using 125 I-protein A in the presence and absence of the first antibody (α SSEA-1, VIM-2, or CSLEX-1).

$\alpha(1,3)$ Fuc-T assays

Cell extracts were prepared in extraction buffers containing 1% Nonidet P-40 (or 0.5% Nonidet P-40 in the case of LEC29) as previously described (Howard *et al.*, 1987). The assay for $\alpha(1,3)$ Fuc-T activity was also as previously described (Howard *et al.*, 1987; Potvin *et al.*, 1990) except that 2% Triton CF-54 was not generally added to the reaction mixtures. Unless otherwise specified, all reaction tubes contained 2.5 μ mol MOPS buffer (pH 6.5), 5 μ mol NaCl, 0.25 μ mol MnCl_2 , and 5–10 μ l of cell extract containing ~ 100 μ g of cell protein. Unless specified in the Tables or text, oligosaccharide acceptors were used at 1 μ mol/reaction, fetuin and asialofetuin at 1 mg/reaction, the glycolipid acceptors nLc₄ and IV⁹NeuNAc₄Lc₄ were at a concentration of 40 μ g/reaction, and GDP-[14 C]-fucose (~ 25 000 cpm/nmol) was present at 1 or 2 nmol/reaction.

When SA $\alpha(2,3)$ LacNAc or SA $\alpha(2,6)$ LacNAc was used as a substrate, the Dowex columns were first eluted with 2 ml of deionized, distilled water and subsequently with 3 ml of 0.15 M NaCl. Neutral products were obtained from the water wash whereas the sialylated products were eluted by the NaCl wash. Fractions were collected into scintillation vials and counted in Ecolume. Under these conditions, 14 C-GDP-fucose remained bound to the Dowex resin as determined in control reaction mixtures included in each assay.

Neuraminidase assays

Neuraminidase assays generally followed the procedure described by Montreuil *et al.* (1986) and used the sodium salt of 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid at a concentration of 100 μ mol/reaction as the substrate. To measure the neuraminidase activity under conditions similar to those employed for $\alpha(1,3)$ Fuc-T assays, 2.5 μ mol MOPS buffer (pH 6.5) was substituted for the McIlvane buffer. Protein concentrations were as described for the $\alpha(1,3)$ Fuc-T assay and the final reaction volume was 250 μ l. All tubes were incubated for 1 h at 37°C and the reactions were stopped by the addition of 0.6 ml of 0.2 M glycine buffer (pH 10.7). The amount of free methylumbelliferone was determined by spectrofluorimetry using a Perkin Elmer fluorescence spectrophotometer (model LS-5, Perkin Elmer, Norwalk, CT) with an excitation wave length of 390 nm and an emission wave length of 450 nm. Standard curves were constructed using dilutions of an aqueous solution of the sodium salt of 4-methylumbelliferone.

Protein determinations

Protein concentrations were determined by the use of the Bio-Rad protein assay reagent under conditions described by the manufacturer.

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