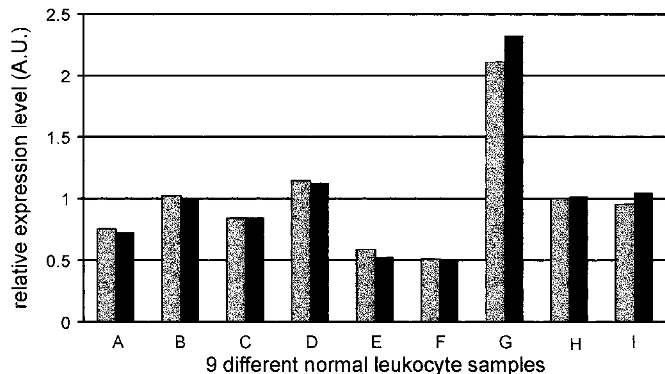

FIG. 3. Relative *HMBS* gene expression levels in nine normal leukocyte samples of which RNA was subjected to two parallel rounds (black and gray) of DNase treatment and cDNA synthesis.

longer restricted to those genes without any known pseudogenes. So far, we have analyzed 10 different housekeeping genes belonging to different functional and abundance classes in various tissues and cells. We clearly observed that no gene is really constantly expressed and multiple housekeeping genes are required for an accurate normalization (Vandesompele *et al.*, submitted for publication).

One-step RT-PCR reactions are often preferred over two-step reactions, because of a presumed minimization of experimental variation, as all enzymatic steps occur in the same tube under controlled thermocycler conditions. To address the issue of reproducibility, we determined the interassay variation of our established two-step RT-PCR protocol. We have therefore subjected nine different RNA samples to two parallel rounds of DNase treatment and cDNA synthesis and quantified the relative expression levels of each of four housekeeping genes (*HMBS, UBC, ACTB,* and *HPRT1*) in all samples using the comparative C_t method. The two-step protocol is highly reproducible with Pearson correlation coefficients ranging from 0.974 to 0.988 between the expression levels of the two parallel series of cDNA samples for the four tested genes, of which one is shown in Fig. 3. The interassay variation was calculated as the median coefficient of variation (standard deviation divided by the arithmetic mean) for the paired relative quantities obtained in the two parallel series for the four genes and amount to 7.7%. This figure is only slightly higher than the observed median intraassay variation of 5.8% (reflecting the variation between duplicated reactions in the same PCR run; based on 25 different genes tested on 30 samples, data not shown) and demonstrates the reproducibility of the two-step approach.

To summarize, SYBR green I is the detection format of choice for accurate and reproducible real-time transcript abundance measurements of a large series of genes. Obscuring PD observed in one-step RT-PCR reactions were eliminated by the use of a two-step and DNase treatment of RNA prior to cDNA synthesis was shown to be a prerequisite for accurate RT-PCR and facilitated primer design.

Acknowledgments. We thank Johanna Iso-Oja and Filip Pattyn for their excellent technical assistance.

REFERENCES

1. Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N. Y.)* **11,** 1026–1030.
2. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. (1996) Real time quantitative PCR. *Genome Res* **6,** 986–994.
3. Bustin, S. A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **25,** 169–193.
4. Lie, Y. S., and Petropoulos, C. J. (1998) Advances in quantitative PCR technology: 5′ nuclease assays. *Curr. Opin. Biotechnol.* **9,** 43–48.
5. Ririe, K. M., Rasmussen, R. P., and Wittwer, C. T. (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem.* **245,** 154–160.
6. Brownie, J., Shawcross, S., Theaker, J., Whitcombe, D., Ferrie, R., Newton, C., and Little, S. (1997) The elimination of primer–dimer accumulation in PCR. *Nucleic Acids Res.* **25,** 3235–3241.
7. Dunham, I., Shimizu, N., Roe, B. A., Chissoe, S., Hunt, A. R., Collins, J. E., Bruskiewich, R., Beare, D. M., Clamp, M., Smink, L. J., Ainscough, R., Almeida, J. P., Babbage, A., Bagguley, C., Bailey, J., Barlow, K., Bates, K. N., Beasley, O., Bird, C. P., Blakey, S., Bridgeman, A. M., Buck, D., Burgess, J., Burrill, W. D., O'Brien, K. P., *et al.* (1999) The DNA sequence of human chromosome 22. *Nature* **402,** 489–495.
8. Hattori, M., Fujiyama, A., Taylor, T. D., Watanabe, H., Yada, T., Park, H. S., Toyoda, A., Ishii, K., Totoki, Y., Choi, D. K., Soeda, E., Ohki, M., Takagi, T., Sakaki, Y., Taudien, S., Blechschmidt, K., Polley, A., Menzel, U., Delabar, J., Kumpf, K., Lehmann, R., Patterson, D., Reichwald, K., Rump, A., Schillhabel, M., and Schudy, A. (2000) The DNA sequence of human chromosome 21: The chromosome 21 mapping and sequencing consortium. *Nature* **405,** 311–319.

Effects of Sialic Acid Substitutions on Recognition by *Sambucus nigra* Agglutinin and *Maackia amurensis* Hemagglutinin[1]

Els C. M. Brinkman-Van der Linden,
Justin L. Sonnenburg, and Ajit Varki

Glycobiology Research and Training Center, Department of Medicine, and Department of Cellular and Molecular Medicine, University of California at San Diego, La Jolla, California 92093-0687

Received October 8, 2001; published online February 13, 2002

The lectin from elderberry bark, *Sambucus nigra* agglutinin (SNA),[2] and the two lectins from the legume

[1] This work was supported by USPHS Grants P01-HL57345 and R01-GM323373.

[2] Abbreviations used: SNA, *Sambucus nigra* agglutinin; MAL, *Maackia amurensis* leukoagglutinin; MAH, *Maackia amurensis*

Analytical Biochemistry **303,** 98–104 (2002)
doi:10.1006/abio.2001.5539
0003-2697/02 $35.00
© 2002 Elsevier Science (USA)
All rights reserved.

tree *Maackia amurensis—M. amurensis* leukoagglutinin (MAL) and *M. amurensis* hemagglutinin (MAH) (1)—have been extensively used to detect α2–6- and α2–3-linked sialic acids, respectively (2–5). Studies on MAL have shown that the carboxyl group of sialic acid is primarily involved in interactions with this lectin (3, 4, 6). However, not all structural requirements for sialic acid recognition by either SNA or MAH are known (7). Sialic acids are 9-carbon acidic sugars that can have a variety of modifications. One of the major types is *N*-acetylneuraminic acid (Neu5Ac), which is the precursor of most others (8–11). One common variation is *N*-glycolylneuraminic acid (Neu5Gc), which is found in all mammals except humans (12–17). Various plant and animal lectins have been shown to prefer Neu5Ac and bind with lower affinity or not at all to Neu5Gc (11, 18–23). In contrast, other lectins prefer Neu5Gc (11, 20–22, 24–29). Furthermore, for some lectins, the glycerol-like side chain of sialic acid is essential for recognition and 9-O-acetylation of this side chain can block the interaction (11, 18–20, 22, 26, 30, 31). In other instances, 9-O-acetylation is required for recognition (11, 32–37). Here we have evaluated the importance of the *N*-acyl- and 9-*O*-acetyl groups of sialic acid for recognition by SNA and MAH.

Materials and Methods

Materials and cells. Unless otherwise stated the materials used were from Sigma Chemical Co. or Fisher Scientific. Products for cell culture were from Life Technologies. EBV-transformed lymphoblastoid cells derived from humans (provided by Dr. Peter Parham, Stanford University) were cultured in AIM-V with L-glutamine and 1% FCS (low serum was used to minimize incorporation of exogenous Neu5Gc into human cells) (15). EBV-transformed lymphoblastoid cells derived from orangutans (also from Dr. Parham) were cultured in RPMI 1640 with 10% FCS and L-glutamine. Recombinant soluble forms of human siglec-2 (CD22) fused with the Fc part of mouse IgG (hSiglec-2-Fc), of mouse siglec-1 (sialoadhesin) fused with the Fc part of human IgG (mSiglec-1-Fc), and of influenza C virus hemagglutinin–esterase fused with the Fc part of human IgG (CHE-Fc) were produced and purified as described previously (23, 38–41). For some experiments, CHE-Fc was used as a 9-*O*-acetyl sialic acid-specific esterase. For others, it was treated with DFP to inactivate the esterase activity—giving CHE-FcD, a probe that specifically binds to 9-O-acetylated sialic acids (33, 41).

SNA and MAH preference for Neu5Ac or Neu5Gc. Human EBV-transformed lymphoblastoid cells contain >95% Neu5Ac and <5% Neu5Gc, whereas orangutan EBV-transformed lymphoblastoid cells contain ~15% Neu5Ac and ~85% Neu5Gc (23). Humans and orangutans share ~97% genetic identity, and comparisons of their blood plasma show a high degree of conservation of protein expression and glycosylation (42). Thus, this pair of cells can be used to compare preferences of lectins to bind to either Neu5Ac or Neu5Gc (23).

Biotinylated MAH ("MAL-II") was obtained from Vector Laboratories (the name "MAL-II" was created by Vector Laboratories; Dr. Jim Whitehead, personal communication). Cultured cells ($0.3–1 \times 10^6$) were washed and then incubated for 1 h at 4°C with 100 μl of either biotinylated SNA (Vector) or biotinylated MAH. The lectins (5 or 10 μg/ml) were preincubated at 4°C for 30 min with PE-conjugated streptavidin (Jackson) (1:100). Binding was analyzed using flow cytometry (using a Becton–Dickinson FACScan machine). Cells were treated with mild $NaIO_4$ (2 mM in PBS for 30 min on ice) to specifically truncate the glycerol side chains of sialic acids (43–46). Sialidase treatment of cells was carried out at room temperature for 30 min, using 10 mU *Arthrobacter ureafaciens* sialidase/10^6 cells in 20 mM Hepes, pH 7.

Binding of SNA to 9-O-acetylated sialic acids. Bovine submaxillary mucin (BSM) was de-O-acetylated with 0.1 M NaOH for 30 min at room temperature (47). This mixture was subsequently neutralized with HCl. For a control sample, an already neutralized mixture of NaOH/HCl was added to the same amount of BSM. Both untreated and base-treated BSM were coated on microtiter wells (200 ng/well; plates from Nunc) in 50 mM carbonate/bicarbonate buffer, pH 9.5, for 15 h at 4°C. BSM on the plate was also treated with 2 mM $NaIO_4$ in PBS for 20 min at 4°C in the dark. Subsequently, the aldehydes formed by the $NaIO_4$ treatment were reduced with 10 mM $NaBH_4$ in PBS for 20 min at 4°C in the dark. Control wells received premixed aliquots of the same amounts of $NaIO_4$ and $NaBH_4$, which were added for 20 min at 4°C. All wells were then washed and blocked with ELISA buffer (TBS, 0.1% Tween, pH 7.45) for 1 h and incubated with biotinylated SNA (500 ng/well) for 2 h, followed by washing and incubation with alkaline phosphatase-conjugated streptavidin (Life Technologies) (1:1000) for 1 h. Development was done with 150 μl/well of the *p*-Nitrophenyl Phosphate Liquid Substrate System. Between incubations (all at room temperature) wells were washed 3× with ELISA buffer. Absorbance was determined at 405

---

hemagglutinin; BSM, bovine submaxillary mucin; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; 6′SLL, Neu5Acα2–6Galβ1–4Glc polyacrylamide; RBC, red blood cells; siglec, sialic-acid binding immunoglobulin-like lectin; EBV, Epstein–Barr virus; FCS, fetal calf serum; IgG, immunoglobulin G; DFP, diisopropyl fluorophosphate; PE, phycoerythrin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline.

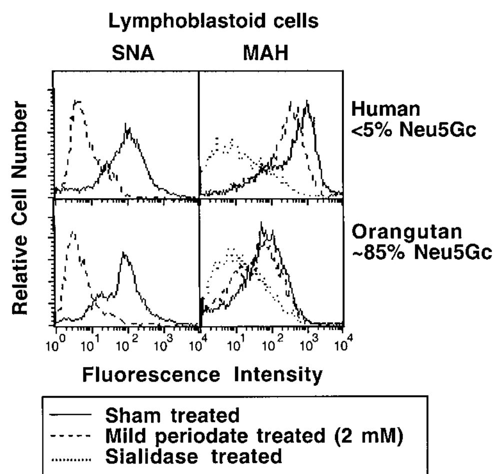

FIG. 1. Flow cytometry analysis of binding of SNA and MAH to EBV-transformed lymphoblastoid cells from humans and orangutans. Lymphoblastoid cells derived from humans or orangutans were incubated with biotinylated SNA or biotinylated MAH that had been preincubated with streptavidin–PE. After washing, binding was analyzed by flow cytometry. Solid lines show results for the sham-treated cells, dashed lines show profiles for mild periodate-treated cells (negative control for specificity of binding to SNA), and dotted lines show profiles for sialidase-treated cells (negative control for specificity of binding to MAH). Treatments were performed as described under Materials and Methods.

nm. As controls, binding was analyzed for CHE-FcD (specifically binds to 9-O-acetylated sialic acids) and for hSiglec-2-Fc (binding is blocked by 9-O-acetylation of sialic acids) (11, 19, 20, 22, 41). For the last two probes the incubation was followed by incubation with alkaline phosphatase-conjugated anti-human/mouse IgG (Bio-Rad) (1:500).

Binding of MAH to 9-O-acetylated sialic acid. Mouse red blood cells (RBCs), which are rich in 9-O-acetylated sialic acids (48), were treated with the CHE-Fc esterase (10 μg/10^6 RBCs) for 1 h at 37°C to remove 9-*O*-acetyl groups from sialic acid. Binding of MAH to these mouse RBCs (and sham-treated controls) was analyzed as described above for lymphoblastoid cells. As controls, binding was analyzed for CHE-FcD and for mSiglec-1-Fc (binding is blocked by 9-O-acetylation of sialic acids) (11, 20, 22, 31). The latter two probes were precomplexed with goat anti-human IgG–FITC (Caltag) (1:25).

Results and Discussion

EBV-transformed lymphoblastoid cells derived from humans and orangutans were used to analyze the preference of SNA and MAH for either Neu5Ac or Neu5Gc. As shown in Fig. 1, SNA binds equally well to the human cells (containing primarily Neu5Ac) and to the orangutan cells (containing primarily Neu5Gc). SNA could also bind to porcine submaxillary mucin (containing primarily Neu5Gc) and to ovine submaxillary mucin (containing primarily Neu5Ac) in an ELISA setup similar to the one described for BSM under Materials and Methods (data not shown). These data indicate that SNA can bind to both Neu5Ac and Neu5Gc. As expected for SNA (2), truncation of the side chain of sialic acid by mild periodate treatment of the cells abolished binding of this lectin (Fig. 1).

Similar to SNA, MAH binds both human and orangutan cells, indicating that it can bind ligands containing either Neu5Ac and Neu5Gc (Fig. 1). This confirms an earlier study reporting that porcine submaxillary mucin inhibits MAH hemagglutination of human erythrocytes (1). MAH binds the human cells better than the orangutan cells, suggesting that the lectin may prefer Neu5Ac-containing ligands. However, slight variations between the two cell lines in the ligand density, presentation, or structure may contribute to this observed difference. Periodate treatment of the cells did not reduce MAH binding appreciably, but binding was abrogated upon sialidase treatment. This demonstrates that MAH binding is reliant upon sialic acid, but does not depend upon its side chain.

SNA binds equally well to untreated and base-treated BSM (Fig. 2), showing that SNA can bind to sialic acids with or without 9-*O*-acetyl groups. In this assay CHE-FcD (binds specifically to 9-O-acetylated sialic acid) and hSiglec-2-Fc (binding is blocked by 9-O-acetylation of sialic acids) were used as controls. Most sialic acids in BSM are 9-O-acetylated, rendering them resistant to mild periodate treatment. As shown in Fig. 2, mild periodate treatment of BSM abrogates the binding of SNA only when 9-*O*-acetyl groups are first removed by base treatment. This finding was surprising for SNA, because it needs an intact glycerol side chain for recognition (2), which for many lectins correlates with the inability to bind to 9-O-acetylated sialic acid (11). Our data are in keeping with the findings of Roth and colleagues, showing that human colonic tissue sections were stained equally well with SNA, whether or not the tissue sections were pretreated with base (49).

Mouse RBCs containing 9-O-acetylated sialic acids are reactive with CHE-FcD (Fig. 3). This binding is markedly reduced when the RBCs are pretreated with the CHE-Fc esterase, which removes the 9-*O*-acetyl groups from sialic acids (41). In contrast, Fig. 3 shows that binding of MAH to mouse RBCs is unaffected by the same esterase treatment. Thus, MAH can bind to sialic acids with or without 9-*O*-acetyl groups. mSiglec-1-Fc (binding is blocked by 9-O-acetylation of sialic acids) binding to mouse RBCs was observed only after removal of 9-*O*-acetyl groups by CHE-Fc. There are of course many other less common modifications of sialic acid in nature, which we have not studied (8–11), that may affect binding by SNA and MAH. Also, we have

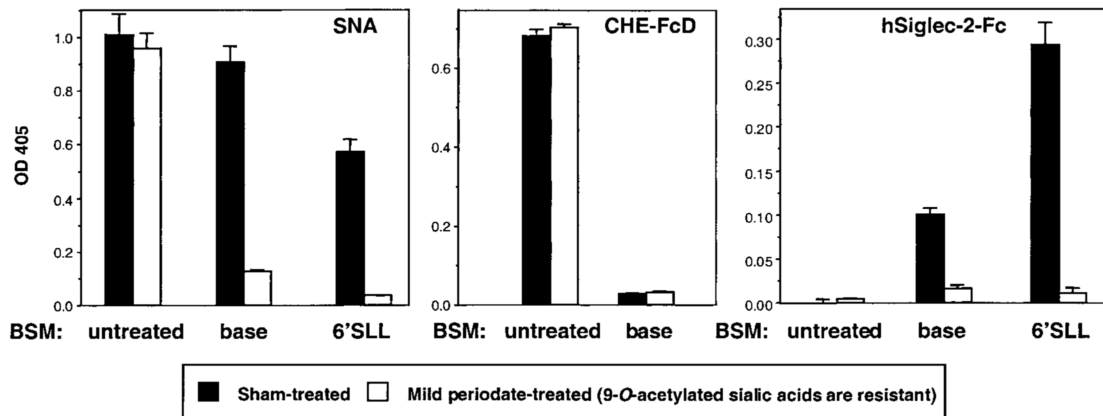

FIG. 2. Effect of 9-O-acetylation of sialic acid on binding of SNA to BSM. Binding of biotinylated SNA, CHE-FcD, or hSiglec-2-Fc to native or de-O-acetylated BSM was tested by ELISA as described under Materials and Methods. Binding of SNA and hSiglec-2-Fc to 6′SLL (Neu5Acα2–6Galβ1–4Glc polyacrylamide from Glycotech) shows that these lectins are active. Both untreated and base-treated BSM were also treated with mild periodate on the ELISA plate as described under Materials and Methods. 9-O-acetylation of sialic acids renders them resistant to mild periodate treatment. When no 9-O-acetylation is present this treatment truncates the glycerol side chain of sialic acids, showing the dependence of interactions on the intact side chain. Data shown represent means ± standard deviations of triplicates.

not addressed the issue of extended binding specificities beyond the sialic acid molecule itself (4, 6). For example, the amino-terminal octapeptide from human glycophorin A carrying three disialylated tetrasaccharide chains was reported to have a very strong affinity for MAH (5).

The seeds of the legume tree *M. amurensis* are known to contain two lectins: MAL and MAH (1). For this study we obtained "MAL-II" from Vector and assumed it to be MAH, based on the fact that their "MAL-I" has already been shown to be identical to MAL (6) and on the general properties for "MAL-II" reported by Vector Laboratories. Such confusion in nomenclature may arise from the differing molecular weights of these lectins from different sources. This could be explained by varying carboxyl-terminal processing (Dr. Richard D. Cummings, University of Oklahoma, personal communication) or possibly by different strains of the *M. amurensis* tree. It should also be noted that the name *M. amurensis* agglutinin (MAA) is used by some commercial sources. Depending on the isolation procedure used, such MAA can be either a mixture of both MAL and MAH or similar to MAL (1, 3, 5, 50).

It should also be kept in mind that the specificities of SNA, MAL, or MAH can vary somewhat with the experimental conditions used. For example, although clear specificity of SNA has been shown for α2–6-linked sialic acids and their side chains in various studies (2, 7, 11) including the present one, one report mentions that SNA can also recognize 6-O-sulfation of galactose linked β1–4 to GlcNAc when using affinity chromatography (51). Also, while strict specificity of MAL for Siaα2–3Galβ1–4GlcNAc has been shown using affinity chromatography (3, 4), this lectin shows significant binding to unsubstituted Galβ1–4GlcNAc, when used as a probe in flow cytometry, immunohistochemistry, or ELISA (Drs. Xiaomai Bai, Hiromu Takematsu, and Nissi M. Varki, UCSD; and Dr. Jim White-

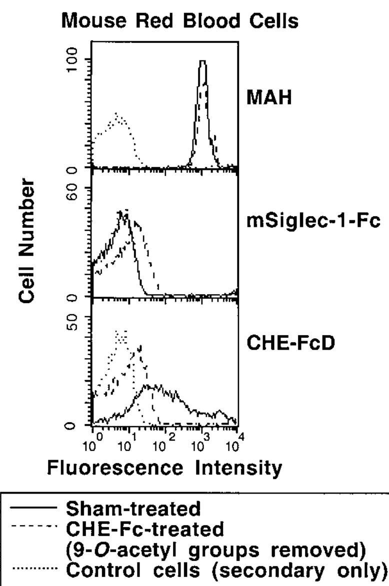

FIG. 3. Binding of MAH to mouse red blood cells: effect of 9-O-acetylation of sialic acid. Mouse red blood cells were incubated with biotinylated MAH preincubated with streptavidin–PE or with mSiglec-1-Fc or CHE-FcD preincubated with anti-human IgG–FITC. After washing, binding was analyzed by flow cytometry. Solid lines show results for sham-treated cells, dashed lines show results for CHE-Fc esterase-treated cells (performed as described under Materials and Methods). Dotted lines show profiles for control cells incubated only with either streptavidin–PE or anti-human IgG–FITC. CHE-Fc treatment removes 9-*O*-acetyl groups from sialic acids on the mouse RBCs.

TABLE 1

Comparison of Sialic Acid-Recognizing Plant and Animal Lectins

Lectin	Structural requirements for sialic acid recognition					References
	Linkage	Carboxylate	N-acyl group	Glycerol side chain	9-*O*-acetyl group	
SNA (*Sambucus nigra* agglutinin)	α2–6Gal(NAc)	Required	Ac or Gc	Required	No effect	This paper (2, 7, 11)
Siglec-2 (CD22)	α2–6Gal(NAc)	Required	Human: Ac or Gc Mouse: Gc (Ac not recognized)	Required	Blocks binding	(7, 11, 19, 20, 22, 23)
MAL (*Maackia amurensis* leukoagglutinin)	α2–3Gal	Required[a]	Ac or Gc	Not required	No effect	(3, 4, 7, 11)
MAH (*M. amurensis* hemagglutinin)	α2–3Gal[b]	Required	Ac or Gc	Not required	No effect	This paper (1, 5, 6)
Siglec-1 (sialoadhesin)	α2–3Gal (⪢α2–6Gal)	Required	Ac (Gc not recognized)	Required	Blocks binding	(7, 11, 20, 22, 23)

[a] MAL shows a sialic acid requirement in affinity chromatography (Ref. 3) but not in all other experimental settings (see text).
[b] MAH can also recognize 3-O-sulfation of galactose in Asn-linked glycans (Ref. 52).

head, Vector Laboratories; personal communications). In the present study, clear dependence on sialic acid was shown for SNA by mild periodate oxidation of the side chain and for MAH by sialidase treatment, indicating that these lectins are useful to distinguish between α2–6- and α2–3-linked cell surface sialic acids.

A comparison of some sialic acid-recognizing plant and animal lectins concerning their structural requirements for sialic acid recognition is shown in Table 1.

Acknowledgments. We thank Peter Parham (Stanford University) for the EBV-transformed cells, Ivan Stamenkovic (Massachusetts General Hospital) for the plasmid encoding hSiglec-2-Fc, and Paul R. Crocker (University of Dundee) for the plasmid encoding mSiglec-1-Fc. We gratefully acknowledge helpful discussions with Rick Cummings (University of Oklahoma), Irwin Goldstein (University of Michigan), and Jim Whitehead (Vector Laboratories).

REFERENCES

1. Kawaguchi, T., Matsumoto, I., and Osawa, T. (1974) Studies on competitive binding of lectins to human erythrocytes. *J. Biol. Chem.* **249,** 2786–2792.
2. Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B., and Peumans, W. J. (1987) The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(alpha 2–6)Gal/GalNAc sequence. *J. Biol. Chem.* **262,** 1596–1601.
3. Wang, W. C., and Cummings, R. D. (1988) The immobilized leukoagglutinin from the seeds of *Maackia amurensis* binds with high affinity to complex-type Asn-linked oligosaccharides containing terminal sialic acid-linked alpha-2,3 to penultimate galactose residues. *J. Biol. Chem.* **263,** 4576–4585.
4. Knibbs, R. N., Goldstein, I. J., Ratcliffe, R. M., and Shibuya, N. (1991) Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from *Maackia amurensis.* Comparison with other sialic acid-specific lectins. *J. Biol. Chem.* **266,** 83–88.
5. Konami, Y., Yamamoto, K., Osawa, T., and Irimura, T. (1994) Strong affinity of *Maackia amurensis* hemagglutinin (MAH) for sialic acid-containing Ser/Thr-linked carbohydrate chains of N-terminal octapeptides from human glycophorin A. *FEBS Lett.* **342,** 334–338.
6. Imberty, A., Gautier, C., Lescar, J., Pérez, S., and Wyns, L. (2000) An unusual carbohydrate binding site revealed by the structures of two *Maackia amurensis* lectins complexed with sialic acid-containing oligosaccharides. *J. Biol. Chem.* **275,** 17541–17548.
7. Varki, A. (1997) Sialic acids as ligands in recognition phenomena. *FASEB J.* **11,** 248–255.
8. Schauer, R. (1982) Sialic Acids: Chemistry, Metabolism and Function, Cell Biology Monographs, Vol. 10, Springer-Verlag, New York.
9. Varki, A. (1999) *in* Essentials of Glycobiology (Varki, A., Esko, J. D., Cummings, R., Freeze, H. H., Hart, G. W., and Marth, J., Eds.), pp. 195–210, Cold Spring Harbor Laboratory Press, Plainview, NY.
10. Zanetta, J. P., Pons, A., Iwersen, M., Mariller, C., Leroy, Y., Timmerman, P., and Schauer, R. (2001) Diversity of sialic acids revealed using gas chromatography/mass spectrometry of heptafluorobutyrate derivatives. *Glycobiology* **11,** 663–676.
11. Angata, A., and Varki, A. (2002) Chemical diversity in the sialic acids and related alpha-keto acids: An evolutionary perspective. In press.
12. Merrick, J. M., Zadarlik, K., and Milgrom, F. (1978) Characterization of the Hanganutziu–Deicher (serum-sickness) antigen as gangliosides containing *N*-glycolylneuraminic acid. *Int. Arch. Allergy Appl. Immunol.* **57,** 477–480.
13. Higashihara, T., Takeshima, T., Anzai, M., Tomioka, M., Matsumoto, K., Nishida, K., Kitamura, Y., Okinaga, K., and Naiki, M. (1991) Survey of Hanganutziu and Deicher antibodies in operated patients. *Int. Arch. Allergy Appl. Immunol.* **95,** 231–235.
14. Odaka, M., Yuki, N., Yoshino, H., Kasama, T., Handa, S., Irie, F., Hirabayashi, Y., Suzuki, A., and Hirata, K. (1998) *N*-glycolylneuraminic acid-containing GM1 is a new molecule for serum antibody in Guillain–Barre syndrome. *Ann. Neurol.* **43,** 829–834.
15. Muchmore, E. A., Diaz, S., and Varki, A. (1998) A structural difference between the cell surfaces of humans and the great apes. *Am. J. Phys. Anthropol.* **107,** 187–198.
16. Irie, A., Koyama, S., Kozutsumi, Y., Kawasaki, T., and Suzuki, A. (1998) The molecular basis for the absence of *N*-glycolylneuraminic acid in humans. *J. Biol. Chem.* **273,** 15866–15871.
17. Chou, H. H., Takematsu, H., Diaz, S., Iber, J., Nickerson, E., Wright, K. L., Muchmore, E. A., Nelson, D. L., Warren, S. T., and

Varki, A. (1998) A mutation in human CMP-sialic acid hydroxylase occurred after the *Homo–Pan* divergence. *Proc. Natl. Acad. Sci. USA* **95,** 11751–11756.

18. Knibbs, R. N., Osborne, S. E., Glick, G. D., and Goldstein, I. J. (1993) Binding determinants of the sialic acid-specific lectin from the slug *Limax flavus. J. Biol. Chem.* **268,** 18524–18531.
19. Sjoberg, E. R., Powell, L. D., Klein, A., and Varki, A. (1994) Natural ligands of the B cell adhesion molecule CD22β can be masked by 9-O-acetylation of sialic acids. *J. Cell Biol.* **126,** 549–562.
20. Kelm, S., Schauer, R., Manuguerra, J.-C., Gross, H.-J., and Crocker, P. R. (1994) Modifications of cell surface sialic acids modulate cell adhesion mediated by sialoadhesin and CD22. *Glycoconjugate J.* **11,** 576–585.
21. Van der Merwe, P. A., Crocker, P. R., Vinson, M., Barclay, A. N., Schauer, R., and Kelm, S. (1996) Localization of the putative sialic acid-binding site on the immunoglobulin superfamily cell-surface molecule CD22. *J. Biol. Chem.* **271,** 9273–9280.
22. Kelm, S., Brossmer, R., Isecke, R., Gross, H. J., Strenge, K., and Schauer, R. (1998) Functional groups of sialic acids involved in binding to siglecs (sialoadhesins) deduced from interactions with synthetic analogues. *Eur. J. Biochem.* **255,** 663–672.
23. Brinkman-Van der Linden, E. C. M., Sjoberg, E. R., Juneja, L. R., Crocker, P. R., Varki, N., and Varki, A. (2000) Loss of *N*-glycolylneuraminic acid in human evolution—Implications for sialic acid recognition by siglecs. *J. Biol. Chem.* **275,** 8633–8640.
24. Smit, H., Gaastra, W., Kamerling, J. P., Vliegenthart, J. F. G., and de Graaf, F. K. (1984) Isolation and structural characterization of the equine erythrocyte receptor for enterotoxigenic Escherichia coli K99 fimbrial adhesin. *Infect. Immun.* **46,** 578–584.
25. Ono, E., Abe, K., Nakazawa, M., and Naiki, M. (1989) Ganglioside epitope recognized by K99 fimbriae from enterotoxigenic *Escherichia coli. Infect. Immun.* **57,** 907–911.
26. Mercy, S. P. D., and Ravindranath, M. H. (1993) Purification and characterization of *N*-glycolylneuraminic-acid-specific lectin from *Scylla serrata. Eur. J. Biochem.* **215,** 697–704.
27. Schultze, B., Krempl, C., Ballesteros, M. L., Shaw, L., Schauer, R., Enjuanes, L., and Herrler, G. (1996) Transmissible gastroenteritis coronavirus, but not the related porcine respiratory coronavirus, has a sialic acid (*N*-glycolylneuraminic acid) binding activity. *J. Virol.* **70,** 5634–5637.
28. Banerjee, M., and Chowdhury, M. (1994) Purification and characterization of a sperm-binding glycoprotein from human endometrium. *Hum. Reprod.* **9,** 1497–1504.
29. Delorme, C., Brüssow, H., Sidoti, J., Roche, N., Karlsson, K. A., Neeser, J. R., and Teneberg, S. (2001) Glycosphingolipid binding specificities of rotavirus: Identification of a sialic acid-binding epitope. *J. Virol.* **75,** 2276–2287.
30. Shi, W. X., Chammas, R., Varki, N. M., Powell, L., and Varki, A. (1996) Sialic acid 9-O-acetylation on murine erythroleukemia cells affects complement activation, binding to I-type lectins, and tissue homing. *J. Biol. Chem.* **271,** 31526–31532.
31. Strenge, K., Schauer, R., Bovin, N., Hasegawa, A., Ishida, H., Kiso, M., and Kelm, S. (1998) Glycan specificity of myelin-associated glycoprotein and sialoadhesin deduced from interactions with synthetic oligosaccharides. *Eur. J. Biochem.* **258,** 677–685.
32. Herrler, G., Rott, R., Klenk, H. D., Muller, H. P., Shukla, A. K., and Schauer, R. (1985) The receptor-destroying enzyme of influenza C virus is neuraminate-O-acetylesterase. *EMBO J.* **4,** 1503–1506.
33. Muchmore, E., and Varki, A. (1987) Inactivation of influenza C esterase decreases infectivity without loss of binding: A probe for 9-*O*-acetylated sialic acids. *Science* **236,** 1293–1295.
34. Ravindranath, M. H., and Paulson, J. C. (1987) *O*-Acetylsialic acid-specific lectin from the crab *Cancer anntenarius. Methods Enzymol.* **138,** 520–527.
35. Vlasak, R., Luytjes, W., Spaan, W., and Palese, P. (1988) Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. *Proc. Natl. Acad. Sci. USA* **85,** 4526–4529.
36. Schultze, B., Gross, H.-J., Brossmer, R., Klenk, H.-D., and Herrler, G. (1990) Hemagglutinating encephalomyelitis virus attaches to *N*-acetyl-9-*O*-acetylneuraminic acid-containing receptors on erythrocytes: Comparison with bovine coronavirus and influenza C virus. *Virus Res.* **16,** 185–194.
37. Kunkel, F., and Herrler, G. (1993) Structural and functional analysis of the surface protein of human coronavirus OC43. *Virology* **195,** 195–202.
38. Stamenkovic, I., Sgroi, D., Aruffo, A., Sy, M. S., and Anderson, T. (1991) The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and α2–6 sialyltransferase, CD75, on B cells. *Cell* **66,** 1133–1144.
39. Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., and Seed, B. (1990) CD44 is the principal cell surface receptor for hyaluronate. *Cell* **61,** 1303–1313.
40. Powell, L. D., Jain, R. K., Matta, K. L., Sabesan, S., and Varki, A. (1995) Characterization of sialyloligosaccharide binding by recombinant soluble and native cell-associated CD22. Evidence for a minimal structural recognition motif and the potential importance of multisite binding. *J. Biol. Chem.* **270,** 7523–7532.
41. Klein, A., Krishna, M., Varki, N. M., and Varki, A. (1994) 9-*O*-acetylated sialic acids have widespread but selective expression: Analysis using a chimeric dual-function probe derived from influenza C hemagglutinin-esterase. *Proc. Natl. Acad. Sci. USA* **91,** 7782–7786.
42. Gagneux, P., Amess, B., Diaz, S., Moore, S., Patel, T., Dillmann, W., Parekh, R., and Varki, A. (2001) Proteomic comparison of human and great ape blood plasma reveals conserved glycosylation and differences in thyroid hormone metabolism. *Am. J. Phys. Anthropol.* **115,** 99–109.
43. Van Lenten, L., and Ashwell, G. (1971) Studies on the chemical and enzymatic modification of glycoproteins A general method for the tritiation of sialic acid-containing glycoproteins. *J. Biol. Chem.* **246,** 1889–1894.
44. McLean, R. L., Suttajit, M., Beidler, J., and Winzler, R. J. (1971) *N*-acetylneuraminic acid analogues. *J. Biol. Chem.* **246,** 803–809.
45. Reuter, G., Schauer, R., Szeiki, C., Kamerling, J. P., and Vliegenthart, J. F. G. (1989) A detailed study of the periodate oxidation of sialic acids in glycoproteins. *Glycoconjugate J.* **6,** 35–44.
46. Murray, M. C., Bhavanandan, V. P., Davidson, E. A., and Reinhold, V. (1989) Modification of sialyl residues of glycoconjugates by reductive amination. Characterization of the modified sialic acids. *Carbohydr. Res.* **186,** 255–265.
47. Diaz, S., Higa, H. H., Hayes, B. K., and Varki, A. (1989) O-acetylation and de-O-acetylation of sialic acids: 7- and 9-O-acetylation of α2,6-linked sialic acids on endogenous *N*-linked glycans in rat liver Golgi vesicles. *J. Biol. Chem.* **264,** 19416–19426.
48. Varki, A., and Kornfeld, S. (1980) An autosomal dominant gene regulates the extent of 9-O-acetylation of murine erythrocyte sialic acids. A probable explanation for the variation in capacity to activate the human alternate complement pathway. *J. Exp. Med.* **152,** 532–544.
49. Murayama, T., Zuber, C., Seelentag, W. K. F., Li, W. P., Kemmner, W., Heitz, P. U., and Roth, J. (1997) Colon carcinoma glycoproteins carrying α2,6-linked sialic acid reactive with *Sambucus nigra* agglutinin are not constitutively expressed in nor-

mal human colon mucosa and are distinct from sialyl-TN antigen. *Int. J. Cancer* **70,** 575–581.
50. Johansson, L., Johansson, P., and Miller-Podraza, H. (1999) Detection by the lectins from *Maackia amurensis* and *Sambucus nigra* of 3- and 6-linked sialic acid in gangliosides with neolacto chains separated on thin-layer chromatograms and blotted to PVDF membranes. *Anal. Biochem.* **267,** 239–241.
51. Yamashita, K., Umetsu, K., Suzuki, T., and Ohkura, T. (1992) Purification and characterization of a Neu5Acα2 $\rightarrow$ 6Galβ1 $\rightarrow$ 4GlcNAc and HSO_3^- $\rightarrow$ 6Galβ1 $\rightarrow$ 4GlcNAc specific lectin in tuberous roots of *Trichosanthes japonica. Biochemistry* **31,** 11647–11650.
52. Bai, X. M., Brown, J. R., Varki, A., and Esko, J. D. (2001) Enhanced 3-*O*-sulfation of galactose in Asn-linked glycans and *Maackia amurenesis* lectin binding in a new Chinese hamster ovary cell line. *Glycobiology* **11,** 621–632.

One-Step Unidirectional Cloning of Tandem Repeats of DNA Fragments: An Application for Fusion Protein Production

Zsolt Jobbagy, Jeffrey L. Ward, Shuy-Vang Toan, George P. H. Leung, and Chung-Ming Tse[1]

Gastroenterology Division, Department of Medicine, Johns Hopkins University, 720 Rutland Avenue, Ross Research Building, Room 930, Baltimore, Maryland 21205

Received October 22, 2001; published online February, 11, 2002

Biochemical characterization of mammalian proteins is often hampered by the availability of the purified proteins. Bacterially expressed fusion proteins are thus utilized in a diverse array of biochemical and cell biology studies such as ligands for affinity chromatography, tools for protein–protein interaction, and purified antigens for antibody production (for example see 1, 2). Most expression systems provide an easily purifiable protein (e.g., maltose-binding protein or glutathione *S*-transferase) (3–5) or a short affinity tag (e.g., 6×His) (6) as fusion partner. Unfortunately, in the case of relatively short protein fragments of interest (or epitopes) that are under 10 to 15 kDa, the resolution limit of the standard SDS/PAGE decreases the convenience of expression systems that offer short affinity tags (7). Furthermore, molecules of less than 5 kDa are generally not good immunogens and have to be coupled onto a carrier for antibody production (8). To circumvent these problems in many applications of fusion proteins, it is desirable to express fusion proteins containing multiple copies of an epitope. Furthermore, epitope tagging is widely used for biochemical and functional characterization of mammalian proteins (9). In many cases, insertion of multiple copies of an epitope into mammalian proteins facilitates the purification, and increases the sensitivity for quantitation, of the epitope-tagged proteins expressed in mammalian cells (10).

Tagging a protein with multiple copies of an epitope can be achieved by sequentially inserting each copy of the epitope into the protein while manipulating the respective cDNA. This method is tedious and time consuming. Alternatively, a long oligonucleotide encompassing multiple copies of an epitope can be inserted into the protein via PCR. However, the purity of oligonucleotides >50 bp in length is not reliable and this approach is feasible for small protein epitopes.

We describe here a shortcut to cloning tandem repeats of cDNA coding for a relatively short protein fragment. This method takes advantage of restriction enzyme pairs with compatible but not identical recognition sites (e.g., *Bam*HI/*Bgl*II, *Xba*I/*Nhe*I, and *Cla*I/*Acc*I), allowing for selection of clones containing DNA fragments in the desired orientation (Fig. 1). It is fast, simple, and reliable, and it allows researchers to generate a set of constructs containing multiple copies of an epitope in a single step.

As an example to test the feasibility of this cloning and expression approach, we expressed and affinity purified the third cytoplasmic loop of the human equilibrative nucleoside transporter 1 (hENT1)[2] as antigen for antibody production. Because of the high homology of hENT1 with other members of the ENT family, we restricted the length of our protein fragment to 53 amino acids that are unique to hENT1. We used the Xpress System (Invitrogen) for the expression of the recombinant protein. Figure 1 shows the general scheme of the experimental procedures. The pTrcHis C vector was cut with the compatible restriction endonuclease pair *Bam*HI–*Bgl*II and then treated with calf intestinal alkaline phosphatase (CIP) (New England Biolabs) for 1 h to prevent self-ligation of the vector. The DNA fragment coding for the predicted third cytoplasmic loop of hENT1 was obtained by PCR with the primer pair gcca**agatct**ACCTGGCCAATAAATCATCCC (5′primer with *Bgl*II site) and ccca**ggatcC**GGAAGACAGTGAAGACTGAAGG (3′ primer with *Bam*HI site). The PCR was performed with the following parameters: 95°C, 2 min, 1 cycle; 95°C 1 min, 55°C 45 s, 72°C 1 min, 25 cycles; 72°C 7 min, 1 cycle. The PCR fragment was analyzed by agarose gel electrophoresis and the predicted 170-bp PCR fragment was isolated (Fig. 2A, lane 2). The purified DNA fragments were digested with *Bgl*II and *Bam*HI for 2 h, repurified, and

[1] To whom correspondence should be addressed. Fax: (410) 955-9677. E-mail: mtse@mail.jhmi.edu.

[2] Abbreviations used: hENT1, human equilibrative nucleoside transporter 1; CIP, calf intestinal alkaline phosphatase; IPTG, isopropyl-β-D-thiogalactopyranoside; VSVG, vesicular stomatitis virus glycoprotein.

Analytical Biochemistry **303,** 104–107 (2002)
doi:10.1006/abio.2001.5548
0003-2697/02 $35.00
© 2002 Elsevier Science (USA)
All rights reserved.