Manic Fringe and Lunatic Fringe Modify Different Sites of the Notch2 Extracellular Region, Resulting in Different Signaling Modulation*

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Three mammalian fringe proteins are implicated in controlling Notch activation by Delta/Serrate/Lag2 ligands during tissue boundary formation. It was proved recently that they are glycosyltransferases that initiate elongation of O-linked fucose residues attached to epidermal growth factor-like sequence repeats in the extracellular domain of Notch molecules. Here we demonstrate the existence of functional diversity among the mammalian fringe proteins. Although both manic fringe (mFng) and lunatic fringe (lFng) decreased the binding of Jagged1 to Notch2 and not that of Delta1, the decrease by mFng was greater in degree than that by lFng. We also found that both fringe proteins reduced Jagged1-triggered Notch2 signaling, whereas neither affected Delta1-triggered Notch2 signaling. However, the decrease in Jagged1-triggered Notch2 signaling by mFng was again greater than that by lFng. Furthermore, we observed that each fringe protein acted on a different site of the extracellular region of Notch2. Taking these findings together, we propose that the difference in modulatory function of multiple fringe proteins may result from the distinct amino acid sequence specificity targeted by these glycosyltransferases.

The Notch family of proteins consists of transmembrane receptors that play a critical role in cell fate choices and the formation of compartment border, preventing distinct cell populations from intermixing, during the development of both vertebrates and invertebrates (1–4). In higher vertebrates, four Notch genes, Notch1 through Notch4, have been identified (5–10). An important structural characteristic of these is the common presence of multiple repeats of epidermal growth factor (EGF)-like sequence in the extracellular domain. Ligands for the Notch receptors are designated Delta/Serrate/Lag2 (DSL) proteins (or DSL ligands), five of which have been identified thus far in mammals (Jagged1/Serrate1 (11–13), Jagged2/Serrate2 (14–16), Delta1 (17), Delta-like3 (18), and Delta4 (19)).

Drosophila and mammalian fringe proteins modulate the formation of compartment border in the developing embryo through affecting Notch activation by the ligands (20–25). Drosophila fringe inhibits a group of cells from responding to the ligand Serrate and potentiates them to respond to another ligand, Delta (20). In higher vertebrates, three fringe proteins, manic fringe (mFng), lunatic fringe (lFng), and radical fringe (rFng), have been identified (22, 26). The mammalian fringe proteins modulate Notch signaling when expressed in Drosophila (26), and lFng null mouse phenotypes are similar to those described for mice deficient in components of the Notch signaling pathway (27, 28). A weak sequence similarity shared by Drosophila fringe and a class of bacterial glycosyltransferases predicted that fringe proteins might be a glycosyltransferase (29). In a recent work, it was proven experimentally that Drosophila and mammalian fringe proteins have a fucose-specific β1,3-N-acetylglucosaminyltransferase activity that catalyzes the elongation of O-linked fucose on the EGF repeats of Notch (30, 31). However, it has not been determined whether these fringe proteins act in a distinct manner on the Notch molecules, and if they do, how the difference is generated.

In this study, we report functional diversity between mFng and lFng, namely a difference in their abilities to modulate Jagged1 binding to Notch2 and in the ligand-triggered Notch2 signaling. Of interest, we also found that the two fringe proteins modify different sites in Notch2, implying that each fringe species specifies the multiple putative consensus sequences for O-linked fucose glycosylation in the extracellular region of Notch2.

EXPERIMENTAL PROCEDURES

Preparation of Soluble Fusion Proteins—Soluble DSL proteins (sD1-Fc and sJ1-Fc) were prepared as described previously (32).

Cell-binding Assay—Binding of each soluble DSL protein to the surface of the pro-B cell line BaF3 was performed as described previously (13). Briefly, 3 × 10^5 BaF3 cells were incubated with 6.7 nM soluble DSL-Fc proteins in a cell-binding buffer (PBS containing 2% fetal bovine serum, 100 μg/ml CaCl2, and 0.05% NaN3) at 37 °C after blocking with 5 μl of rabbit serum. After a 10-min incubation, the cells were washed three times with the cell-binding buffer and further incubated with a phosphatidyethanolamine-conjugated anti-human IgG antibody. The cells then were analyzed using FACScaliber (Becton Dickinson Immunocyometry Systems).

Coprecipitation Using Soluble DSL Proteins—Coprecipitation experiments using the soluble DSL proteins were performed as described previously (32). Each soluble DSL-Fc (6.7 nm) was allowed to bind to 1 × 10^7 BaF3 cells in a buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, and 100 μg/ml CaCl2. Disuccinyl glutarate (Pierce), a cross-linking reagent, was then added to the soluble DSL-Fc-bound BaF3 at a final concentration of 20 μM followed by further incubation for 30 min.
Concentration of 6.7 nM. Cell-bound DSL proteins were analyzed by fluorescence-activated cell sorter. Human IgG (hiG) was used to determine the background. Reactions were incubated for 40 h with 5 × 10^4 (CHO(r)) cells with or without exogenous full-length Notch2 (N2) were precipitated with protein G beads. Luciferase activity in the mixture of CHO(r) cells then was measured using a luminometer.

**RESULTS**

*Endogenous and Exogenous mFng Is a Major Determinant for Binding of DSL Ligands to Notch2—* Recently, we showed that soluble Jagged1 (sJ1-Fc) binds less efficiently than soluble Delta1 (sD1-Fc) to Notch2 on a mouse pro-B cell line, BaF3, in a cell-binding assay and a coprecipitation assay (32). To determine the consistency of this relative relationship between Jagged1 and Delta1 in Notch2 binding, we conducted cell-binding assays for other Notch2-expressing cell lines and compared the binding amounts of Jagged1 and Delta1 in various cell lines. The results showed that the lower binding activity of sJ1-Fc observed in BaF3 was maintained in a group of cell lines, such as 32D (mouse myeloid progenitor) and mouse myeloid leukemia cells (designated group A; Fig. 1A). However, sJ1-Fc bound at a level equivalent to sD1-Fc in other cell lines such as CHO(r), NIH2-CHO (CHO(r)) with exogenously introduced full-length Notch2 (N2) were incubated for 3 × 10^5 in a 24-well plate and transfected with a Tp1-luciferase reporter plasmid, pGhG-1, by a liposome-base method (SuperFect, Qiagen). After transfection, the cells were co-cultured for 40 h with 5 × 10^5 of the parental or full-length DSL protein-expressing CHO(r) cell lines. Luciferase activity in the mixture of CHO(r) cells then was measured using a luminometer.

at room temperature. After the cross-linking reaction, the cells were solubilized in a TNE buffer containing 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.2 mg/ml aprotinin, and 1 mM EDTA for 30 min at 4 °C. The lysates were precipitated with protein G beads.

Transient Reporter Assay—The transient reporter assay was performed as described previously (32). Chinese hamster ovary ras clone-1 (CHO(r)) cells with or without exogenous full-length Notch2 (N2) were incubated at 3 × 10^5 in a 24-well plate and transfected with a TP1-luciferase reporter plasmid, pGashG-1, by a liposome-base method (SuperFect, Qiagen). After transfection, the cells were co-cultured for 40 h with 5 × 10^5 of the parental or full-length DSL protein-expressing CHO(r) cell lines. Luciferase activity in the mixture of CHO(r) cells then was measured using a luminometer.

**RESULTS**

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Results obtained with CHO(r) and C2C12 cells were similar to those with NIH2-CHO (data not shown). These results indicate that the binding of Delta1 and Jagged1 to Notch2 varies with the type of cell expressing Notch2 and further suggest that there must be a mechanism that modulates the binding between Notch2 and its ligands.

Speculating that the fringe proteins might contribute to the different pattern in Jagged1 and Delta1 binding to Notch2, we next examined expression of the known mammalian fringe genes by Northern blot analysis. Strong expression of mFng was detected in BaF3, 32D, and mouse myeloid leukemia cells, but it was undetected in C2C12, CHO(r), and NIH2-CHO cells. In contrast, lFng and rFng were detected at a similar level in all of these cells (Fig. 2). In a reverse-transcriptase polymerase chain reaction, the expression of mFng was confirmed in BaF3 and 32D and not in C2C12 cells, whereas lFng and rFng were detected in BaF3, 32D, and C2C12 cells (data not shown). These results indicated that the expression of mFng correlated with the differential binding of sD1-Fc and J1-Fc to Notch2. To show the involvement of mFng in the differential binding, we generated N2-CHO clones expressing exogenous mFng (IN2/ mFng-CHO). At the same time, we also generated N2/CHO cells expressing exogenous lFng (IN2/lFng-CHO). When the data were transposed, it was revealed that mFng and lFng did not affect or marginally increased the binding of sD1-Fc to Notch2 receptors on the N2/CHO cell surface but reduced that of sJ1-Fc (Fig. 3B). The N2/mFng-CHO cells were similar to BaF3 and 32D rather than parental N2-CHO in that they had a preference for Delta1 (Figs. 1A and Fig. 3B). In contrast, although binding of sJ1-Fc to IN2/lFng-CHO was also less than that of sD1-Fc, the difference was less prominent than that in IN2/mFng-CHO. When the data were transposed, it was revealed that mFng and lFng did not affect or marginally increased the binding of sD1-Fc to Notch2 receptors on the IN2/CHO cell surface but reduced that of sJ1-Fc (Fig. 3C).

Coprecipitation assays then revealed that the amount of sJ1-Fc-coprecipitated N2TM was significantly less than that of sD1-Fc-coprecipitated N2TM in IN2/mFng-CHO cells, in contrast to the fact that the amount of N2TM coprecipitated with sD1-Fc and that with sJ1-Fc were closely similar in parental N1-CHO cells. In IN2/lFng-CHO cells, the amount of sJ1-Fc-coprecipitated N2TM was slightly less than that of sD1-Fc-coprecipitated N2TM (Fig. 3D). These observations indicate that the fringe proteins have the ability to modulate Notch receptors for interaction with their ligands and that each mFng and lFng executes this activity in a different manner.

Modulation of Ligand-activated Notch2 Signaling by Finge
Functional Diversity between Manic Fringe and Lunatic Fringe

Proteins—We then examined whether the two fringe proteins influenced ligand-triggered Notch2 signaling. In fN2-CHO, the three full-length DSL ligands, Delta1, Jagged1, and Jagged2, activated the transcription of a reporter gene driven by an RBP-Jκ-responsive promoter to a similar extent (see columns designated fN2-CHO in Fig. 4A), as was reported previously (32). Compared with this, Jagged1-activated transcription was decreased in both fN2/mFng-CHO and fN2/lFng-CHO cells (Fig. 4A). The degree of decrease was significantly greater in fN2/mFng-CHO than in fN2/lFng-CHO cells (Fig. 4B), demonstrating a difference in function between mFng and lFng. Delta1- and Jagged2-activated transcription was unaffected in fN2/mFng-CHO and slightly enhanced in fN2/lFng-CHO cells (Fig. 4, A and B). When the fringe-generated modulation of signaling through endogenous Notch receptors (Fig. 4D) was subtracted, results indicated that mFng profoundly reduced the Jagged1-triggered Notch2 signaling, and lFng reduced it to a lesser degree. After subtraction, neither fringe significantly modified the Notch2 signaling triggered by Delta1 or Jagged2.

It was reported that the expression of lFng in Delta1- or Jagged1-expressing cells did not affect the ligand-triggered Notch1 activity (33). To determine whether this is also true in the case of Notch2, we separately generated fringe protein-expressing D1-CHO and I1-CHO cell lines and performed a transient transcriptional activation experiment using these cells as stimuliators and fN2-CHO as a host of the reporter gene. The stimulator cells induced constant transactivation of the luciferase gene, irrespective of the exogenous introduction of mFng or lFng (Fig. 5). Furthermore, the addition of mFng or lFng purified from the culture supernatant of mFng-CHO or lFng-CHO cells did not affect the transcription induced by Delta1 or Jagged1 (data not shown). These observations indicate that the fringe proteins must be coexpressed with Notch2.

Mechanism of Modulation of Notch Signaling by the Fringe Proteins—Next, we investigated whether the modification of the Notch2 molecule by the fringe proteins could be identified, and whether the functional diversity between them could be explained by the difference of such modification. We generated CHO(r) cells expressing either of the three deletion mutants of the extracellular domain of Notch2 with a FLAG tag (15N2-CHO, 22N2-CHO, and 29N2-CHO), for deletion mutants harboring the N terminus through the 15th EGF repeat, through the 22nd EGF repeat, and through the 29th EGF repeat, respectively. We further introduced fringe cDNA into these CHO cells. When the supernatant of each cell line was subjected to the gel and Notch2-FLAG was probed in a Western blot, we found that the migration of the 15N2 derived from lFng-expressing cells was slowed as compared with that derived from the parental 15N2-CHO cells (Fig. 6A). The introduction of mFng or the control vector did not affect the migration of the 15N2 protein. A similar result was observed in 22N2 (Fig. 6B). In contrast, the migration of 29N2 was up-shifted in the presence of either mFng or lFng (Fig. 6C), indicating an increase in the molecular weight of 29N2. These observations indicate that the fringe proteins directly modify Notch2, which is consistent with the recent finding that fringe is a glycosyltransferase that directly modifies Notch (30, 31). It was further indicated that lFng does this at a site from the N terminus through the 15th EGF repeat of Notch2, and mFng does so at a site from the 23rd through the 29th EGF repeat of Notch2.

DISCUSSION

In this study, we characterized the function of mFng and lFng and identified their functional diversities. Both fringe proteins differentially modulated the binding of DSL ligands to the Notch2 receptor as well as Notch2 signaling triggered by DSL ligands. We further obtained biochemical data suggesting that these fringe proteins modify the extracellular region of Notch2 at different sites.

Based on the results of in vivo studies using Drosophila, the notion that fringe inhibits Serrate/Jagged-dependent Notch activation and potentiates Delta-dependent Notch activation has been established (20, 21, 24). This notion was strengthened recently by in vitro analyses using mammalian versions of these molecules, namely lFng, Notch1, Jagged1, and Delta1 (33). Furthermore, the fringe protein was proved recently to be a glycosyltransferase that modifies the extracellular domain of Notch (30, 31). Given the recent finding that Drosophila fringe strengthened the binding of Drosophila Delta to Drosophila Notch (31), it was anticipated that the modification of Notch by fringe would influence binding between the Notch receptor and its ligand. However, it has also been reported that fringe does not affect binding between the Notch receptor and its ligand (30, 31, 34).

In the present study, the endogenous expression of mFng was clearly correlated to the finding that cell binding of Jagged1 to Notch2 was less than that of Delta1 (Figs. 1 and 2). It was also shown that the enforced expression of mFng reduced the binding of Jagged1 to Notch2 (Fig. 3D). We therefore suggest that the lower binding of Jagged1 to 32D and BaF3 cells than that of Delta1 resulted from the endogenous expression of mFng, which reduces the Jagged1 binding to Notch2. Furthermore, although mFng did not affect the binding of Delta1 to Notch2 (Fig. 3C), we observed that mFng strengthened the binding of Delta1 to Notch1 (data not shown). It is therefore true that a fringe protein could modify the binding of a DSL ligand to a Notch receptor in selected circumstances if not in all. Whether a certain fringe protein affects the binding of a DSL ligand to a Notch receptor largely depends on the combination of fringe protein, DSL ligand, and Notch receptor involved. The expression of exogenous lFng in CHO cells, which express endogenous lFng, also showed a binding modulatory activity, although in a lesser degree than that in mFng (Fig. 3). Expression level may therefore be a determinant of whether a fringe protein exerts modulatory activity on ligand-Notch binding.

It was reported previously that mFng and lFng inhibited Notch1-mediated signaling triggered by Jagged1 and enhanced that triggered by Delta1, and either Jagged1- or Delta1-triggered Notch2 signaling was enhanced by lFng (33). In contrast, we describe here that mFng and lFng inhibited Notch2-mediated signaling triggered by Jagged1, in which mFng showed stronger activity, and that neither fringe affected Notch2-mediated signaling triggered by Delta1 (Fig. 4). This apparent discrepancy may be because of the difference in the cells that were used to overexpress mFng and lFng. From our results, modification of Notch2 binding to Jagged1 (reduction) and...
Fig. 3. Modulation of Notch2 binding by fringe proteins. A, the generation of fN2-CHO cell lines expressing FLAG-tagged mFng and IFng proteins, designated fN2/mFng-CHO and fN2/IFng-CHO, respectively. The expression of each fringe protein was verified by Western blot analysis with an anti-FLAG antibody. B and C, the binding of two DSL proteins to fN2-CHO, fN2/mFng-CHO, and fN2/IFng-CHO cells was analyzed by fluorescence-activated cell sorter. The concentration of incubated DSL proteins was 6.7 nM. C, the binding data were re-aligned to show the effect of mFng and IFng on ligand binding to the Notch receptors more clearly. D, coprecipitation analysis. After incubation of 6.7 nM of the respective DSL-Fc protein or control human IgG (hIgG) with fN2-CHO, fN2/mFng-CHO, and fN2/IFng-CHO cells, protein G beads were added directly to the lysates to precipitate an Fc-containing complex. Notch2™ in each fN2-CHO is shown in the right panel. Two bands represent Notch2™ generated by introduced Notch2 cDNA (exo N2™) and endogenous Notch2™ expressed in CHO(r) (endo N2™).

Fig. 4. Modulation of ligand-activated Notch2 signaling by fringe proteins. A, reporter gene activation by ligands in fN2-CHO cells with or without exogenous fringe proteins. fN2-CHO cells with or without introduced fringe cDNAs were transiently transfected with a TP1-luciferase reporter plasmid, pGa986–1, and then co-cultured with the parental or full-length DSL protein-expressing CHO(r) lines, fD1, fD1-CHO; fJ1, fJ1-CHO; fJ2, fJ2-CHO. B, to make the comparison among the ligands clear, the ratio of each ligand-induced transcriptional activity to fD1-induced transcriptional activity was plotted. C, generation of CHO(r) cell lines expressing mFng and IFng (mFng-CHO and IFng-CHO). The expression of each fringe protein was verified by Western blot analysis. D, reporter gene activation by ligands in CHO(r) with or without exogenous fringe proteins.
The supernatants of the three kinds of sN2-expressing CHO proteins. In the present study, we reveal the existence of functional diversity among the fringe proteins. Given that it is known that the strength of Notch signaling is critical for the exact cell fate decision based on the fact that abnormal phenotypes are exhibited by haploinsufficiency of DSL ligand (37, 38), we propose that multiple fringe proteins with diverse signal-modulation activities exist in higher vertebrates to rigidly control the strength of Notch signaling. We also show that Jagged2-triggered signaling is much less affected by either fringe protein than Jagged1-triggered signaling, despite significant structural similarities between the two ligands. We believe that these findings will facilitate our understanding of the complexities of Notch signaling in higher vertebrates, in which multiple Notch receptors, DSL ligands, and fringe proteins exist.

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REFERENCES

1. Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. (1995) Science 268, 225–232.
2. Doherty, D., Feger, G., Younger-Shepherd, S., Jan, L. Y., and Jan, Y. N. (1996) Genes Dev. 10, 421–434.
3. Weinmaster, G. (1997) Mol. Cell. Neurosci. 9, 91–102.
4. Greenwald, I. (1998) Genes Dev. 12, 1751–1762.
5. Weinmaster, G., Roberts, V. J., and Lemke, G. (1991) Development 113, 199–205.
6. Weinmaster, G., Roberts, V. J., and Lemke, G. (1992) Development 116, 931–941.
7. Kopan, R., and Weintraub, H. (1993) J. Cell Biol. 121, 631–641.
8. Réau, A. G., Conlon, R. A., Zirngibl, R., Yamaguchi, T. P., and Rossant, J. (1992) Dev. Biol. 154, 377–387.
9. Lendahl, U., Dahlstrand, J., and Lendahl, U. (1994) Mech. Dev. 46, 123–136.
10. Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D., and Kitajewski, J. (1996) Development 122, 2251–2259.
11. Lindsell, C. E., Shabbeer, C. J., Boulter, J., and Weinmaster, G. (1995) Cell 80, 969–977.
12. Li, L., Milner, L. A., Deng, Y., Iwata, M., Banta, A., Graf, L., Marcovina, S., Friedman, C., Trask, B. J., Hood, L., and Torok, S. B. (1998) Immunity 8, 43–55.
13. Shimizu, K., Chiba, S., Kuman, K., Hosoya, N., Takahashi, Y., Kanda, Y., Hamada, Y., Yazaki, Y., and Hirai, H. (1999) J. Biol. Chem. 274, 32961–32968.
14. Shabbeer, C. J., Boulter, J., Lindsell, C. E., and Weinmaster, G. (1996) Dev. Biol. 180, 370–376.
15. Luo, B., Aster, J. C., Hasseljian, R. P., Kuo, F., and Sklar, J. (1997) Mol. Cell. Biol. 17, 6057–6067.
16. Valsecchi, C., Ghezzi, C., Ballabio, A., and Rugari, E. I. (1997) Mech. Dev. 69, 203–207.
17. Bettenhausen, B., Hrobe, A. M., Simon, D., Guenet, J. L., and Gossler, A.
18. Dunwoodie, S. L., Henrique, D., Harrison, S. M., and Beddington, R. S. (1997) *Development* **124**, 3065–3076
19. Shutter, J. R., Scully, S., Yeo, C. Y., Pisenti, J., Henrique, D., Abbott, U. K., Fallon, J. F., and Tabin, C. (1997) *Nature* **386**, 366–373
20. Panin, V. M., Papayannopoulos, V., Wilson, R., and Irvine, K. D. (1997) *Nature* **387**, 908–912
21. Cohen, B., Bashirullah, A., Dagnino, L., Campbell, C., Fisher, W. W., Leow, C. C., Whiting, E., Ryan, D., Zinyk, D., Boulia, G., Hui, C., Gallie, B., Phillips, R. A., Lipshitz, H. D., and Egan, S. E. (1997) *Nat. Genet.* **16**, 283–288
22. Cho, K. O., and Choi, K. W. (1998) *Nature* **396**, 272–276
23. Klein, T., and Arias, A. M. (1998) *Development* **125**, 2951–2962
24. Johnston, S. H., Rauskolb, C., Wilson, R., Prabhakaran, B., Irvine, K. D., and Vogt, T. F. (1997) *Development* **124**, 2245–2254
25. Evrard, Y. A., Lun, Y., Aulehla, A., Gan, L., and Johnson, R. L. (1998) *Nature* **394**, 377–381
26. Zhang, N., and Gridley, T. (1998) *Nature* **394**, 374–377
27. Yuan, Y. P., Schultz, J., Mlodzik, M., and Bork, P. (1997) *Cell* **88**, 9–11
28. Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S., and Vogt, T. F. (2000) *Nature* **406**, 369–375
29. Bruckner, K., Perutz, L., Clausen, H., and Cohen, S. (2000) *Nature* **406**, 411–415
30. Shimizu, K., Chiba, S., Hosoya, N., Kuman, K., Saito, T., Kurokawa, M., Kanda, Y., Harada, Y., and Hirai, H. (2000) *Mol. Cell. Biol.* **20**, 6913–6922
31. Hics, C., Johnston, S. H., diS Belgium, G., Vogt, T. F., and Weinmaster, J. (2000) *Nat. Cell Biol.* **2**, 515–520
32. Kleg, K. M., and Muskavitch, M. A. (1999) *J. Cell Sci.* **112**, 3289–3297
33. de Celis, J. F., and Bray, S. J. (2000) *Development* **127**, 1291–1302
34. Ju, B. G., Jeong, S., Bae, E., Hyun, S., Carroll, S. B., Yim, J., and Kim, L. (2000) *Nature* **405**, 191–195
35. Oda, T., Elkahloul, A. G., Pike, B. L., Okajima, K., Krantz, I. D., Genin, A., Piccoli, D. A., Meltzer, P. S., Spinner, N. B., Collins, F. S., and Chandrasekharappa, S. C. (1997) *Nat. Genet.* **16**, 235–242
36. Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J., Costa, T., Pierpoint, M. E., Rand, E. B., Piccoli, D. A., Hood, L., and Spinner, N. B. (1997) *Nat. Genet.* **16**, 243–251
