NOTES

Relationship of Secretion Pattern and MPB70 Homology with Osteoblast-Specific Factor 2 to Osteitis following *Mycobacterium bovis* BCG Vaccination

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Significant homology was found between MPB70 and each of four repeat domains of osteoblast-specific factor 2 (OSF-2). Two internal homology regions within each repeat domain of OSF-2 presumed to be related to the active site(s) of this bone adhesion molecule showed the highest homology. A literature search concerning osteitis after *Mycobacterium bovis* BCG vaccination in neonates revealed that MPB70-high-producer sub-strains were associated with an increased incidence of osteitis following vaccination. These observations indicate that the function of MPB70 is related to the interaction between bacilli and the host following vaccination or infection with mycobacteria.

MPB70 was isolated by Nagai et al. (24). The protein occurs in different concentrations in the culture fluid after growth of various substrains of *Mycobacterium bovis* BCG, BCG Tokyo and BCG Copenhagen being prototype high-producer and low-producer strains, respectively (10, 22). Virulent strains of *M. bovis* produce and secrete large amounts of MPB70, while *Mycobacterium tuberculosis* culture fluids contain small amounts of the protein.

Sequencing of the entire gene was reported by Terasaka et al. (29). PCR studies on the *mpb70* gene in *M. bovis* BCG Tokyo showed the gene to be present in high-producer as well as low-producer substrains of BCG and in *M. tuberculosis* (4, 5, 14).

Although the major secreted proteins of mycobacteria are expected to be involved in the interaction between the mycobacteria and the infected host, the function of MPB70 is unknown.

The search for homology between MPB70 and other proteins was made with the ENTREZ molecular sequence retrieval system (release 10.0, 15 April 1994; ENTREZ is published by the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md., ISSN 1072-3072).

For alignment of the amino acid sequences to give maximal identity, the program PALIGN of the PC/GENE (release 6.8) sequence analysis software package was used (PC/GENE is distributed by IntelliGenetics Inc., Mountain View, Calif.). The comparison matrix used was the structure-genetic matrix described by Feng et al. (7). An open-gap cost of 7 and a unit-gap cost of 2 were found to be optimal. Accession numbers in the EMBL database are as follows: MPB70, M25386; mouse OSF-2, D13664; human OSF-2, D13666 and D13665. The homology has been submitted for entry into the database Prosite.

Takeshita et al. (28) reported the nucleotide and deduced amino acid sequences of mouse OSF-2. An open reading frame coded for a polypeptide chain consisting of a typical signal sequence followed by a cysteine-rich domain, a fourfold internal repeat domain, and a C-terminal domain but no transmembrane domains. The OSF-2 sequence was highly conserved between mice and humans. On the basis of amino acid sequence homology with fasciclin I, an insect homophilic adhesion protein (33), and the expression of OSF-2 in the bone and to a lesser extent in the lung, Takeshita et al. (28) suggested that OSF-2 functions as a homophilic adhesion molecule in bone formation.

Table 1 shows an alignment of the entire sequence of the mature MPB70 protein with each of the four repeat domains of mouse OSF-2. It is evident that extensive homology is present. The highest homology was observed between MPB70 and the second repeat domain and is illustrated in Fig. 1.

Takeshita et al. (28) pointed out that two regions within each of the four repeat domains showed high internal homology and also showed homology with the insect adhesion molecule fasciclin I. These regions are presented in Fig. 2 together with their consensus sequences for mouse OSF-2. The consensus sequence of the first internal homology region comprised 12 of the 13 residues; 10 of these 12 were identical and 1 was similar in MPB70, a concordance of 84.6%. The consensus sequence of the second internal homology region of mouse OSF-2 comprised 13 of the 14 residues. In this region, 6 of the 13 residues were identical in MPB70 and 3 others were similar, giving a concordance of 69.2%. It is also indicated in Fig. 2 that the distance between the two homology regions was similar in MPB70 and the four repeat domains of OSF-2.

The difference in production of MPB70 in different substrains of BCG and the homology between MPB70 and OSF-2 prompted a study of the relationship between the substrain used for vaccination and the frequency of osteitis following vaccination. The results are shown in Table 2.

BCG substrains can be divided into two main categories by their MPB70 secretion pattern, BCG Tokyo, Moreau, Russia,

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TABLE 1. Alignment of the MPB70 amino acid sequence with that of each of the four repeat domains of mouse OSF-2

OSF-2 repeat domain	Comparison with MPB70 residues		No. of gaps introduced	
	No. (%) identical	No. (%) similar	MPB70	OSF-2
R1	30 (23)	22 (17)	3	2
R2	36 (27)	33 (24)	4	3
R3	32 (25)	23 (18)	2	4
R4	35 (26)	20 (15)	2	2

and Sweden being high-producers and BCG Copenhagen, Pasteur, and Tice being low-producer strains (10, 22). BCG Glaxo has an intermediate position, producing somewhat higher amounts of MPB70 than the typical low-producer strains (1).

BCG osteitis has been reported mainly in countries where neonates are vaccinated with MPB70-high-producer BCG substrains. Concerning reporting in individual countries, changes in the incidence of osteitis have been associated with changes in the BCG substrain used for vaccination, at least in Finland, Sweden, and Czechoslovakia.

The Swedish MPB70-high-producer substrain, originally obtained from Calmette in 1926 (15), was used in Finland and Sweden until the mid-1970s, when Sweden terminated their compulsory vaccination program because of the high incidence of osteitis. In 1971, the production of BCG Sweden was transferred from Gothenburg to Statens Seruminstitut in Copenhagen. A concomitant increase in reported cases of osteitis was associated with the change in production localization; it rose from 7.3 to 36.9 cases per 100,000 vaccinated newborns in Finland and from 2.5 to 29 cases per 100,000 vaccinees in Sweden. This increased incidence has not been fully explained but may be related to a different number of viable units in the vaccines and to a higher level of general awareness of this complication. Finland had a higher incidence of tuberculosis and continued their vaccination program but changed to a vaccine based on BCG Glaxo. Following this change to BCG Glaxo, which secretes smaller amounts of MPB70, there was an immediate reduction in the number of reported cases of osteitis to 6.4 per 100,000 vaccinated neonates (13).

A strain termed BCG 725, obtained from Denmark in 1947, was initially used in Czechoslovakia (17, 20). BCG 725 is an MPB70-low-producer strain (1). In 1981, they changed to the Russian MPB70-high-producer strain, and concomitantly the first cases of osteitis were observed. When the dose was reduced by half in 1985, the incidence decreased from 3.7 to 2.3 per 100,000 vaccinated children (12).

In the Federal Republic of Germany, 14 cases of osteitis were reported during the period 1948–1974 (17). The Swedish strain was used during this period, and it is likely that these cases can be related to the Gothenburg vaccine.

Notification of adverse reactions to vaccines is inherently insufficient. Even in Sweden, where great attention was established, underreporting of osteitis was revealed (3, 9). It is obvious that the lack of reported cases of BCG osteitis does not imply that such cases do not exist. However, in a prospective study of adverse reactions to BCG vaccination, serious complications such as BCG osteitis were particularly looked for (16), and no cases were reported among more than 2.5 million children in other European countries vaccinated at less than 1 year old during the period from 1979 to 1982. In four of these countries, it is well established that MPB70-low-producer strains (BCG Copenhagen of BCG Pasteur) were used.

MPB70	31	GDLVGP	CAEY	AAANPT	GPASVQ	MSQDFV	AAASNN	FELTT	LAALSGQ
mOSF-2, R2	236	G-----	-----	-----	-----	-----	-----	-----	-----
MPB70	87	LNPQV	NLVD	TLNS	-GQY	TVFAP	TNAAF	SKLPA	STIDEL
mOSF-2, R2	261	-----	-----	-----	-----	-----	-----	-----	-----
MPB70	110	YHVV	-AGQ	TSPAN	VVGTR	-QTL	QASVT	VTGQ	GNLSL
mOSF-2, R2	285	-----	-----	-----	-----	-----	-----	-----	-----
MPB70	177	TANAT	VYMI	DSVL	MPPA				
mOSF-2, R2	355	-----	-----	-----	-----	-----	-----	-----	-----

FIG. 1. Alignment between MPB70 and the second repeat domain of mouse OSF-2 (mOSF-2). The MPB70 sequence is the deduced sequence from the gene cloned by Terasaka et al. (29); numbering is with methionine at position 1 in the signal sequence in both proteins. The sequence for repeat domain 2 (R2) of mOSF-2 is as given by Takeshita et al. (28). In the alignment, a colon shows identity and a period shows similarity. There were 36 identical (26.67%) and 33 similar (24.44%) residues. Four gaps were inserted in MPB70, and three gaps were inserted in mOSF-2 R2.

In several countries, MPB70-high-producer strains have been used for BCG vaccination, but there are almost no reports of BCG osteitis. The Russian strain was used in the former Soviet Union for vaccination of neonates. We have found one reference to osteitis in Moscow in 1971 (30). Given the Czechoslovakian experience with this strain, it is well documented that it is osteitis inducing, and it is highly likely that insufficient reporting explains the lack of reports from the USSR. It is more difficult to explain the lack of reports of osteitis with the BCG Tokyo MPB70-high-producer strain. This strain is widely distributed, with an annual production of more than 50 million doses (21). In Japan, more than 160 million persons were vaccinated in the period from 1948 to 1974, about 35 million of these being less than 5 years old. Children are usually vaccinated for the first time at 6 or 12 months of age. A 6-month delay from birth may partly explain a low incidence of osteitis in Japan. Apart from insufficient reporting, other possible explanations may be related to BCG Tokyo's being less potent than other BCG substrains (32).

The homology regions within the four repeat domains of

mOSF-2 R1	GSXNYEAFSNEAW	Distance	76	TNGVHVVIDRMLTQ
hOSF-2	--F-----			-----
mOSF-2 R2	GHFLLFAPTNEAF		74	KNGVIHLIDEVLIIF
hOSF-2	-----			N-----Q----
mOSF-2 R3	GEXLLAPVNNAF		67	RNGAIHIFREIIQF
hOSF-2	-----			-----K-
mOSF-2 R4	GDWLLFAPTNDAF		75	TNGVIHVVDKLLIYF
hOSF-2	-----V-----			-----
Consensus sequence	GnYTLFAPTNEAF	mean	73	TNGVIHVVIDEVLnF
	: : : : : : : :			: : : : : : : :
MPB70	GQYTVFAPTNAAF		71	ANATVYMIDSVLMP
	94	106		178
				191

FIG. 2. Comparison of amino acid sequence of MPB70 and OSF-2. Repeat domains R1, R2, R3, and R4 correspond to the fourfold repeat domains in mouse OSF-2 (mOSF-2) and human OSF-2 (hOSF-2). The boxed areas correspond to the two particularly conserved regions with greatest internal homology in the proteins. A dash indicates an identical residue in mOSF-2 and hOSF-2; an n indicates no consensus residue in mOSF-2. Residues that are identical in MPB70 and mOSF-2 are shaded. The distance is measured from the end of the first boxed internal homology region to the beginning of the second boxed region.

TABLE 2. Relationship between reported incidence of osteitis following BCG vaccination and MPB70 secretion by the BCG substrain used

Country (reference)	Period	No. of vaccinees <1 yr old	BCG substrain	Secretion of MPB70 ^a (reference)	No. of osteitis cases	No. of osteitis cases per 100,000 vaccinees
Finland (17)	1948–1974	1,646,700	Gothenburg	++	128	7.7
Finland (13)	1960–1970	726,000	Gothenburg ^b	++	53	7.3
Finland (13)	1971–1978	355,000	Gothenburg ^c	++	131	36.9
Finland (13)	1978–1988	594,000	Glaxo	+	38	6.4
Sweden (17)	1948–1974	2,830,100	Gothenburg	++	121	4.3
Sweden (3)	1960–1969	1,000,000 ^d	Gothenburg ^b	++	25	2.5
Sweden (3)	1972–1980	300,000 ^d	Gothenburg ^c	++	90	29
Czechoslovakia (17)	1948–1974	5,080,900	BCG 725 (CSR)	– (1)	0	0
Czechoslovakia (12)	1980–1985	754,700	BCG Russia	++	28	3.7
Czechoslovakia (12)	1985–1989	478,260	BCG Russia	++	11	2.3
Hungary (16)	1979–1981	435,088	Pasteur	–	0	0
Yugoslavia (16)	1979–1982	232,832	Pasteur	–	0	0

^a ++, high-producer strain; +, intermediate-producer strain; –, low-producer strain.

^b Produced in Sweden.

^c Produced in Denmark.

^d These figures have been calculated assuming an annual birth rate of 100,000. Compulsory vaccination was terminated in 1975.

OSF-2 and fasciclin demonstrated by Takeshita et al. (28) and illustrated in Fig. 2 are striking, indicating that they are involved in the function of the proteins. Zinn et al. (33) have proposed that fasciclin I is composed of two dimeric units, each of which contains a binding site for a ligand on the cell surface. The divalent molecule could function as a bridge between cells expressing the ligands. A similar function for OSF-2 is not unlikely, as indicated by Takeshita et al. (28). Their analyses indicated that the protein was expressed in mouse primary calvarial osteoblasts and in lung tissue, and the authors suggested that OSF-2 functions as an adhesion molecule in bone formation. The alternative splicings of human OSF-2 always occur C-terminally outside the repeats, also pointing to the importance of the repeats' being intact for the molecule to function.

The demonstration that the homology between OSF-2 and MPB70 is localized mainly to the two consensus regions points to a biological significance of the findings. They could be considered in relation to the function of MPB70 in the mycobacterial cell or, since it is a typical secreted protein, in relation to its significance for the interaction between the mycobacterial cells and the host. The homology may point to MPB70 being a pathogenicity factor in infections with virulent *M. bovis* and having an influence on the localization of multiplication following BCG vaccination.

Several other factors may contribute to the frequency of osteitis after BCG vaccination. Age at vaccination appears to be essential, since nearly all cases of osteitis (97%) have been observed in vaccinated neonates (17). Our analysis of the relationship between MPB70 production of the BCG substrain used for vaccination and incidence of osteitis has therefore been centered on countries that vaccinate mainly neonates. Osteitis lesions show a predilection for the proximal tibial end, distal femur, proximal humerus, rib, sternum, and the small bones of the hands and feet. The lesions are located mainly in the epiphyseal or metaphyseal regions of the respective bones (3, 20, 23, 25), where one would expect to find high osteoblast activities. This feature is an additional support for the role of MPB70 in the pathogenesis of these lesions.

The sequence homology with OSF-2 and association of BCG osteitis with MPB70-high-producer vaccine strains suggest that MPB70 is a mycobacterial bone adhesion protein. How firm the association of MPB70 is to the bacterial surface is crucial in this context. Our observations that MPB70 is efficiently secreted into Sauton medium during culture (31) suggest that this association is weak. However, efficiently secreted proteins

have also been observed in firm association with the bacterial surface (2, 26). In fact, MPB70 has been identified in the paracrystalline surface layer of BCG Russia, BCG Glaxo, and BCG isolated from three Finnish osteitis patients vaccinated with the Glaxo vaccine (18, 19).

Several proteins secreted by BCG are related to MPB70. MPB80 is the most closely related component and is usually coexpressed with MPB70 (11). There is also evidence for glycosylated variants of MPB70 (8). All these proteins could interact with a putative OSF-2 ligand and play a role in bone adhesion of disseminating bacilli. Although the *mpb70* gene has been demonstrated by the PCR technique, secretion of MPT70 is negligible during culture of *M. tuberculosis* H37Rv on Sauton medium (10), while large amounts of MPB70 are secreted by virulent *M. bovis*. However, current knowledge about MPT70 secretion by *M. tuberculosis* in vivo is insufficient. Both *M. tuberculosis* and *M. bovis* frequently cause bone lesions (27). The role of MPB70 and related proteins in mycobacterial bone tropism should therefore be further investigated.

The development of animal models to test directly whether a cause-effect relationship exists between MPB70 and osteitis is considered an extension of the present study. Furthermore, lesions of active bone tuberculosis caused by *M. tuberculosis* should be tested for MPT70 content to demonstrate whether secretion of MPT70 may occur in vivo and thus further point to the importance of MPB70/MPT70 in relation to bone involvement. Experiments are also in progress to study the interaction between MPB70 and the putative receptor for OSF-2 in relevant tissues.

Cell adhesion molecules are certainly essential for metazoans, while similar functions related to unicellular organisms are more difficult to envisage. Homology with a secreted protein, such as MPB70 of *M. bovis*, points to a function of secreted proteins related to the interaction between the multiplying microorganism and the infected host. Takeshita et al. (28) suggest that OSF-2 and fasciclin derive from a common ancestor. Current evidence from PCR studies (4) indicates that the *mpb/mpb70* gene is present only in the tuberculosis complex within the family *Mycobacteriaceae*, which is pathogenic for warm-blooded animals. How the tubercle bacillus, a gram-positive eubacterium, has acquired a protein related to these animal proteins is startling. The typical chronic course of mycobacterial infections, with a mainly intracellular localization of multiplying bacteria, makes horizontal gene transfer (6) a mechanism to be considered, suggesting that the gene product,

MPB70, has a function as a pathogenicity factor in the host-parasite relationship.

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REFERENCES

1. Abou-Zeid, C., I. Smith, J. Grange, J. Steele, and G. A. W. Rook. 1986. Subdivision of daughter strains of bacille Calmette-Guérin (BCG) according to secreted protein patterns. *J. Gen. Microbiol.* **132**:3047-3053.
2. Barnes, P. F., V. Mehra, G. R. Hirschfield, S.-J. Fong, C. Abou-Zeid, G. A. W. Rook, S. W. Hunter, P. J. Brennan, and R. L. Modlin. 1989. Characterization of T cell antigens associated with the cell wall protein peptidoglycan complex of *Mycobacterium tuberculosis*. *J. Immunol.* **143**:2656-2662.
3. Böttiger, M., V. Romanus, C. de Verdier, and G. Boman. 1982. Osteitis and other complications caused by generalized BCG-itis. *Acta Pædiatr. Scand.* **71**:471-478.
4. Cousins, D. V., S. D. Wilton, and B. R. Francis. 1991. Use of DNA amplification for the rapid identification of *Mycobacterium bovis*. *Vet. Microbiol.* **27**:187-195.
5. Cousins, D. V., S. D. Wilton, B. R. Francis, and B. G. Gow. 1992. Use of polymerase chain reaction for rapid diagnosis of tuberculosis. *J. Clin. Microbiol.* **30**:255-258.
6. Doolittle, R. F., D. F. Feng, K. L. Anderson, and M. R. Alberro. 1990. A naturally occurring horizontal gene transfer from a eukaryote to a prokaryote. *J. Mol. Evol.* **31**:383-388.
7. Feng, D. F., M. S. Johnson, and R. F. Doolittle. 1985. Aligning amino acid sequences: comparison of commonly used methods. *J. Mol. Evol.* **21**:112-125.
8. Fifis, T., C. Costopoulos, A. J. Radford, A. Bacic, and P. R. Wood. 1991. Purification and characterization of major antigens from a *Mycobacterium bovis* culture filtrate. *Infect. Immun.* **59**:800-807.
9. Foucard, T., and Å. Hjelmstedt. 1971. BCG osteomyelitis and osteoarthritis as a complication following BCG vaccination. *Acta Orthop. Scand.* **42**:142-151.
10. Harboe, M., and S. Nagai. 1984. MPB70, a unique antigen of *Mycobacterium bovis* BCG. *Am. Rev. Respir. Dis.* **129**:444-452.
11. Harboe, M., S. Nagai, M. E. Patarroyo, M. L. Torres, C. Ramirez, and N. Cruz. 1986. Properties of proteins MPB64, MPB70, and MPB80 of *Mycobacterium bovis* BCG. *Infect. Immun.* **52**:293-302.
12. Krepela, V., J. Galliova, V. Kubec, and J. Marik. 1992. The effect of reduced doses of BCG vaccine on the occurrence of osseous complications after vaccination. *Cesk. Pediatr.* **47**:134-136.
13. Kröger, L., E. Brander, M. Korppi, O. Wasz-Höckert, A. Backman, H. Kröger, K. Launiala, and M.-L. Katila. 1994. Osteitis after newborn vaccination with three different *Bacillus Calmette-Guérin* vaccines: twenty-nine years of experience. *Pediatr. Infect. Dis. J.* **13**:113-116.
14. Li, H., J. C. Ulstrup, T. Ø. Jonassen, K. Melby, S. Nagai, and M. Harboe. 1993. Evidence for absence of the MPB64 gene in some substrains of *Mycobacterium bovis* BCG. *Infect. Immun.* **61**:1730-1734.
15. Lind, A. 1983. Letter to the editor: the Swedish strain of BCG. *Tubercle* **64**:233-234.
16. Lotte, A., O. Wasz-Höckert, N. Poisson, et al. 1988. Second IUATLD study on complications induced by intradermal BCG-vaccination. *Bull. Int. Union Tuberc. Lung Dis.* **63**:47-64.
17. Lotte, A., O. Wasz-Höckert, N. Poisson, N. Dumitrescu, M. Verron, and E. Couvet. 1984. BCG complications. *Adv. Tuberc. Res.* **21**:107-193.
18. Lounatmaa, K., and E. Brander. 1989. Crystalline cell surface layer of *Mycobacterium bovis* BCG. *J. Bacteriol.* **171**:5756-5758.
19. Lounatmaa, K., and E. Brander. 1990. Immunoelectron microscopic localization of 22kDa protein antigen in the surface layer of *Mycobacterium bovis* BCG strains, p. 894. *Proc. XII Int. Congr. Electron Microscopy*. San Francisco Press, San Francisco.
20. Marik, I., R. Kubát, J. Filipský, and J. Galliova. 1988. Osteitis caused by BCG vaccination. *J. Pediatr. Orthop.* **8**:333-337.
21. Milstien, J. B., and J. J. Gibson. 1990. Quality control of BCG vaccine by WHO: a review of factors that may influence vaccine effectiveness and safety. *Bull. W.H.O.* **68**:93-108.
22. Miura, K., S. Nagai, M. Kinomoto, S. Haga, and T. Tokunaga. 1983. Comparative studies with various substrains of *Mycobacterium bovis* BCG on the production of an antigenic protein, MPB70. *Infect. Immun.* **39**:540-545.
23. Mortenson, W., O. Eklöf, and H. Jorulf. 1976. Radiologic aspects of BCG-osteomyelitis in infants and children. *Acta Radiol. Diagn.* **17**:845-855.
24. Nagai, S., J. Matsumoto, and T. Nagasaka. 1981. Specific skin-reactive protein from culture filtrate of *Mycobacterium bovis* BCG. *Infect. Immun.* **31**:1152-1160.
25. Peltola, H., I. Salmi, V. Vahvanen, and J. Ahlqvist. 1984. BCG vaccination as a cause of osteomyelitis and subcutaneous abscess. *Arch. Dis. Child.* **59**:1157-1161.
26. Rambukkana, A., P. K. Das, J. D. Burggraaf, S. Young, W. R. Faber, J. E. R. Thole, and M. Harboe. 1992. Heterogeneity of monoclonal antibody-reactive epitopes on mycobacterial 30-kilodalton-region proteins and the secreted antigen 85 complex and demonstration of antigen 85B on the *Mycobacterium leprae* cell wall surface. *Infect. Immun.* **60**:5172-5181.
27. Savage, W. G. 1929. The prevention of human tuberculosis of bovine origin. Macmillan, London.
28. Takeshita, S., R. Kikuno, K. Tezuka, and E. Amann. 1993. Osteoblast-specific factor 2: cloning of a putative bone adhesion protein with homology with the insect protein fasciclin I. *Biochem. J.* **294**:271-278.
29. Terasaka, K., R. Yamaguchi, K. Matsuo, A. Yamazaki, S. Nagai, and T. Yamada. 1989. Complete nucleotide sequence of immunogenic protein MPB70 from *Mycobacterium bovis* BCG. *FEMS Microbiol. Lett.* **58**:273-276.
30. Wasz-Höckert, O., A. Backman, E. Brander, M. Pasila, and J. Rapola. 1976. Further studies on chronic osteitis in infancy caused by BCG-vaccination in newborns. *Bull. Int. Union Tuberc.* **51**:251-253.
31. Wiker, H. G., M. Harboe, and S. Nagai. 1991. A localization index for distinction between extracellular and intracellular antigens of *Mycobacterium tuberculosis*. *J. Gen. Microbiol.* **137**:875-884.
32. World Health Organization. 1965. Report from the Expert Committee on Biological Standardization, WHO/BS802.65. World Health Organization, Geneva.
33. Zinn, K., L. McAllister, and C. S. Goodman. 1988. Sequence analysis and neuronal expression of fasciclin I in grasshopper and drosophila. *Cell* **53**:577-587.