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Arrest of Motor Neuron Disease in *wobbler* Mice Cotreated with CNTF and BDNF

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Ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) each promote the survival and differentiation of developing motor neurons, but do so through distinct cellular signaling pathways. Administration of either factor alone has been shown to slow, but not to arrest, progression of motor neuron dysfunction in *wobbler* mice, an animal model of motor neuron disease. Because CNTF and BDNF are known to synergize in vitro and in ovo, the efficacy of CNTF and BDNF cotreatment was tested in the same animal model. Subcutaneous injection of the two factors on alternate days was found to arrest disease progression in *wobbler* mice for 1 month, as measured by several behavioral, physiological, and histological criteria.

The cytokine CNTF promotes the survival and differentiation of developing motor neurons in culture (*1*), rescues developing chicken motor neurons from death in ovo, and retards axotomy-induced death of facial nerve motor neurons in the neonatal rat (*2*). Administering CNTF to mice with inherited neuromuscular deficits slows but does not halt disease progression (*3*, *4*). BDNF, a member of the neurotrophin family of neurotrophic factors, promotes the survival or differentiation of rat and chicken motor neurons in vitro and in vivo (*5*, *6*), and synergistic interactions between BDNF and CNTF have been observed in studies with cultured rat neurons and in ovo (*6*, *7*).

To assess the potential synergistic effects of CNTF and BDNF in vivo, we administered these factors to *wobbler* mice, an extensively characterized animal model of motor neuron disease relevant to amyotrophic lateral sclerosis and spinal muscular atrophy (*8*, *9*). Mice with this inherited defect display forelimb muscle weakness beginning at 3 to 4 weeks of age, after which progressive paralysis, denervation atrophy, and contracture develop rapidly. Perikaryal vacuolar degeneration and neuron loss occur in the anterior horn of the spinal cord. Administration of CNTF alone (*4*) or (to a lesser extent) BDNF alone (*10*, *11*) has been shown to slow disease progression in these mice.

Upon diagnosis, 15 affected *wobbler* mice were randomly assigned to one of two treatment groups that received alternating doses of CNTF [1 mg per kilogram of body weight (mg/kg)] and BDNF (5 mg/kg) three times per week ($n = 8$) or vehicle solutions ($n = 7$) (*12*) for 4 weeks. All studies were blinded (*13*). Drug or vehicle was given by subcutaneous injection to the shaved lumbosacral area under halothane anesthesia. In a separate experiment, CNTF (1 mg/kg) or BDNF (5 mg/kg) alone was given five times per week to each of two groups of six *wobbler* mice for 4 weeks. All animals showed normal weight gain, and no adverse effects were seen in any of the treatment groups. The *wobbler* mice treated with vehicle solution showed a loss of grip strength (Fig. 1A), and paw position abnormalities progressed from mild to very severe (Fig. 1B). In contrast, the mice injected on alternate days with CNTF and BDNF showed no loss of mean grip strength, and paw position abnormalities showed no change. Several of

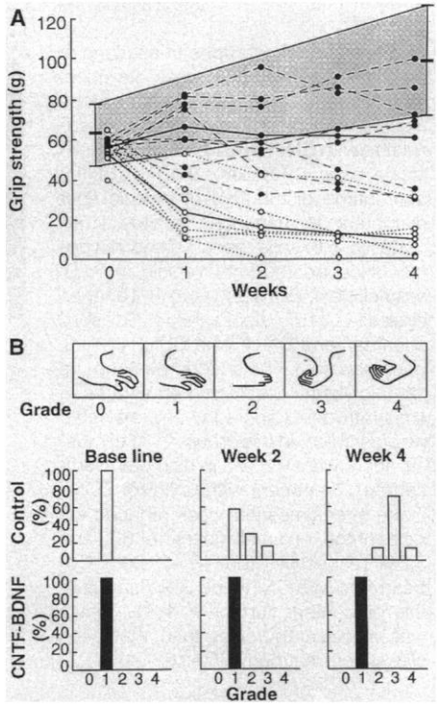

Fig. 1. Comparison of forelimb muscle function (**A**) and gradation of paw position abnormalities (**B**) in *wobbler* mice treated with vehicle solution ($n = 7$) or CNTF plus BDNF ($n = 8$). Shown in (A) are individual scores and means of grip strength measurements made at base line (3 to 4 weeks of age) and at weekly intervals for 4 weeks. Starting from the same base line, vehicle-treated mice (open circles) rapidly and progressively lost grip strength, whereas mice treated with CNTF and BDNF (solid circles) as a group maintained their initial grip strength. Grip strength differed significantly between the two groups at and after week 1 ($P < 0.0005$). The solid lines indicate the means for each group. Almost half the mice cotreated with CNTF and BDNF attained a grip strength that was comparable to that of 10 unaffected littermates, as shown in the shaded area (mean grip strength ± SD at 4 and 8 weeks of age). In (B), paw position abnormalities were graded as shown in the upper panel. All animals started as grade 1 (*12*). Over the course of 4 weeks, most of the vehicle-treated mice were judged to be grades 3 or 4, whereas mice cotreated with CNTF and BDNF did not go beyond grade 1.

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the affected mice cotreated with CNTF and BDNF showed gains in grip strength over 4 weeks that were similar to those of healthy littermates (shaded area, Fig. 1A).

The results of the CNTF and BDNF cotreatment experiment were compared to those obtained with CNTF or BDNF alone in our present and previous studies (*4*, *10*, *11*). Although treatment with CNTF or BDNF alone partially attenuated grip strength loss (Fig. 2A) and reduced running speed decline (Fig. 2B), only the combination of CNTF and BDNF arrested changes in these parameters and halted the progressive development of paw position abnormalities (Fig. 2C). In previous experiments, CNTF alone (three times per week; $n = 27$) slowed the latter (*4*), whereas with a small group of BDNF-treated animals ($n = 10$) no statistical differences from results in vehicle-treated animals were found (*10*, *11*). More frequent administration of BDNF alone (five versus three times per week) was not significantly more efficacious in retarding loss of grip strength in our study. Although treatment with CNTF alone five times per week slowed grip strength loss to a greater degree than treatment three times per week, this arrest was still significantly less effective than cotreatment with CNTF and BDNF.

CNTF and BDNF cotreatment preserved the forelimb musculature, as shown by the wet weight of the biceps muscle, which was 70% greater ($P < 0.005$) than that of vehicle-treated controls (*14*). Consistent with their arrest of the decline in grip strength and muscle weight, CNTF and BDNF cotreatment augmented muscle twitch tension by approximately twofold, as measured in vivo in the biceps muscle ($P < 0.001$) and in vitro in the biceps-triceps muscle pair ($P < 0.01$) (*15*).

Histological analyses (Fig. 3, A to C) revealed that the 4-week cotreatment with CNTF and BDNF attenuated muscle fiber atrophy (*16*). The vehicle-treated *wobbler* mice showed severe denervation-induced atrophy of the biceps muscle by 8 weeks of age (Fig. 3B). At this stage, the mean myofiber diameter of such mice was about 42% (~18 μm) that of normal littermates (Fig. 3D). Sections of biceps muscle from *wobbler* mice cotreated with CNTF and BDNF (Fig. 3C) displayed less myofiber atrophy than did vehicle-treated *wobbler* mice; the mean myofiber diameter (~29 μm) of these animals was 65% that of healthy littermates (Fig. 3D). Furthermore, the percentage of atrophied (presumably denervated) muscle fibers that were <8 μm in diameter was markedly reduced in the mice cotreated with CNTF and BDNF.

Finally, to assess the effects of CNTF-BDNF cotreatment on cervical motor neurons, we measured the mean number and diameter of myelinated fibers in the cervical ventral roots C5 and C6 and determined the mean number of choline acetyltransferase (ChAT)–positive neurons per section in cervical segments C1 through C7 (*17*). Most myelinated fibers in the ventral roots of vehicle-treated *wobbler* mice were <5 μm in diameter (Fig. 4A), whereas those in CNTF-BDNF–cotreated mice were typically >5 μm in diameter. Furthermore, CNTF-BDNF cotreatment reduced the loss of myelinated fibers evident in untreated *wobbler* mice (Fig. 4B). Vehicle-treated *wobbler* mice had fewer ChAT-immunopositive motor neurons than did normal littermates (Fig. 4C). This neuron loss was entirely prevented with cotreatment but was unaffected by treatment with CNTF or BDNF alone (*11*).

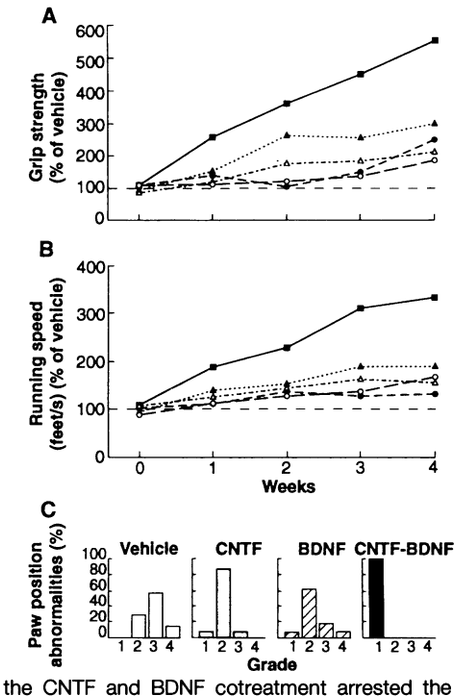

Fig. 2. Effects of treatment with CNTF alone, BDNF alone, and the CNTF-BDNF combination are compared from five separate experiments: CNTF and BDNF cotreatment (solid squares), CNTF five times per week (solid triangles), BDNF five times per week (open triangles), CNTF three times per week (*4*) (open circles), and BDNF three times per week (*10*, *11*) (solid circles). Each experiment had a vehicle-treated control group, and the results of treatment are expressed as a percentage of the mean of vehicle-treated control in each case (the broken line represents the control group at 100%). After 4 weeks, grip strength (**A**) in the CNTF-BDNF–cotreated group was 5.5-fold greater than that in vehicle-treated mice. ANOVA showed that the combination treatment was significantly better than any other treatment, with $P < 0.001$ for all groups tested. Similar effects were observed with running speed (**B**). Running speed in the cotreatment group was threefold greater than that of the vehicle-treated mice after 4 weeks. The cotreatment was better than treatment with CNTF or BDNF alone five times per week ($P < 0.01$) and also significantly better than CNTF or BDNF three times per week ($P < 0.001$). The symbols in (B) are the same as in (A). Whereas CNTF or BDNF three times per week slowed progression of paw position abnormalities (**C**), the CNTF and BDNF cotreatment arrested the progression of these abnormalities at grade 1.

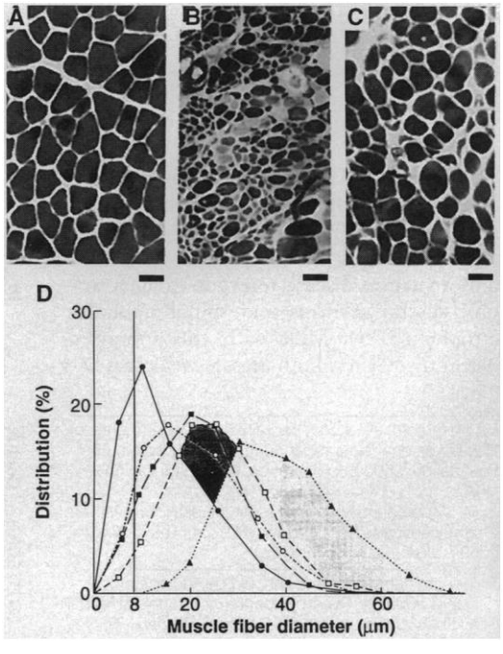

Fig. 3. Photomicrographs of sections of biceps muscles from 8-week-old mice. (**A**) Healthy littermate. (**B**) Vehicle-treated *wobbler* mouse. (**C**) *wobbler* mouse cotreated with CNTF and BDNF for 4 weeks (scale bars = 50 μm). (**D**) Plot of the percentage distribution of muscle fiber diameters in untreated healthy littermates ($n = 6$; solid triangles) and in *wobbler* mice treated with vehicle ($n = 9$; solid circles), BDNF alone ($n = 10$; open circles), CNTF alone ($n = 9$; solid squares), and CNTF plus BDNF ($n = 6$; open squares). The shaded peak on the left highlights the high percentage of small atrophied fibers in vehicle-treated *wobbler* mice. In this group, 14% of muscle fibers were <8 μm in diameter (vertical line). Treatment with CNTF or BDNF alone three times per week reduced the percentage of small fibers to 3.7 and 3.6%, respectively (both $P < 0.001$). Cotreatment with CNTF and BDNF reduced this percentage further to 1.3%, which was significantly lower than that seen with other treatments ($P < 0.001$).

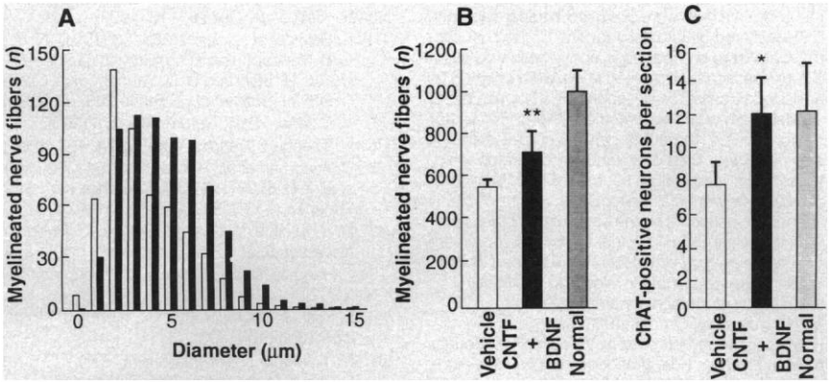

Fig. 4. Comparison of the diameter (**A**) and mean number (**B**) of myelinated ventral root nerve fibers (C5 and C6) and the number of ChAT-immunopositive motor neurons (**C**) in vehicle- and CNTF-BDNF–cotreated *wobbler* mice. CNTF and BDNF cotreatment (black) shifted to a larger fiber diameter than the vehicle treatment (white). The mean fiber diameter increased from 4.6 μm in vehicle-treated mice ($n = 5$) to 6.0 μm in cotreated mice ($n = 6$; $P < 0.002$). The mean number (±SD) of myelinated fibers in the cotreated *wobbler* mice was 30% greater than that in vehicle-treated animals but still less than that in healthy littermates ($n = 6$). The mean number (±SD) of ChAT-immunopositive neurons per section in the cervical spinal cord was greater in *wobbler* mice cotreated with CNTF and BDNF ($n = 6$) than in vehicle-treated animals ($n = 7$) and was similar to that found in healthy littermates ($n = 4$). A single asterisk indicates $P < 0.03$ and a double asterisk indicates $P < 0.01$ in comparison to vehicle-treated mice.

The synergism of CNTF and BDNF in the *wobbler* mouse is consistent with their synergistic effects on motor neurons in vitro and with the direct effect of CNTF on muscle in vivo (*6*, *18*). Unlike BDNF, CNTF may exert its beneficial effect in *wobbler* mice through direct action on muscle, as CNTF reduces atrophy of skeletal muscle induced by denervation (*18*). Although they have several overlapping activities, BDNF and CNTF are members of very different classes of molecules and signal through distinct cell surface receptors that activate distinct, as well as overlapping, signaling cascades. BDNF and the related factor neurotrophin-4/5 are highly basic polypeptides that, as homodimers, activate responsive cells by high-affinity binding to the tyrosine kinase receptor (TrkB), a member of the Trk family (reviewed in *19*) that is expressed in motor neurons (*6*, *20*). Receptor-mediated retrograde axonal transport of BDNF to motor neurons has been found after peripheral application of BDNF (*21*), providing a rationale for the systemic effectiveness of BDNF on neurons that have cell bodies within the central nervous system (CNS). Although mice homozygous for a null mutation in the gene encoding BDNF have a normal complement of motor neurons at birth (*22*), mice lacking functional TrkB receptors show a significant loss of motor neurons and die shortly after birth (*23*).

CNTF is related to the cytokines leukemia inhibitory factor (LIF), oncostatin M, and interleukin-6 and signals through the CNTF receptor complex, which consists of CNTFRα, LIFRβ, and gp130 (*24*). Although exogenous CNTF is very potent in promoting the survival of responsive neurons in vitro and after axotomy in vivo, the role of CNTF in neuronal development and maintenance is not clear, especially as CNTF is devoid of a signal sequence normally associated with secreted proteins. Furthermore, mice that are homozygous null for the gene encoding CNTF appear to develop normally and show motor neuron loss only as adults (*25*). It has thus been suggested that CNTF may function primarily as an injury factor, released from nonneuronal cells in response to nerve damage (*26*). CNTF is present normally in large amounts in myelin-associated Schwann cells, but these levels drop substantially after peripheral nerve damage (*27*). In contrast, CNTF levels are up-regulated in astrocytes surrounding sites of traumatic injury (*28*). After axotomy of basal forebrain cholinergic neurons in the adult rat, infusion of CNTF has been shown to prevent loss of the damaged neurons although ChAT levels are not restored (*29*). In the same paradigm, nerve growth factor and BDNF rescue both neurons and their appropriate ChAT phenotype (*30*).

These findings, coupled with the widespread distribution of CNTFRα in the CNS (*31*), support the hypothesis that CNTF is a broadly acting neuroprotectant that can act in concert with more specific neurotrophic agents to restore neuronal function after trauma. Not only is CNTFRα expressed on adult motor neurons, but like BDNF, CNTF undergoes receptor-mediated retrograde axonal transport to the cell bodies of injured motor neurons (*32*). CNTF and BDNF cotreatment arrested the development of severe motor dysfunction that occurs rapidly in *wobbler* mice after the onset of an inherited motor neuron disease at around 4 weeks of age. The additive and possibly synergistic effects of CNTF and BDNF cotreatment were predicted from previous in vitro studies and probably reflect the distinct receptors and signaling pathways of these two factors.

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12. Recombinant human BDNF (2.5 mg/ml; Amgen-Regeneron Partners) and recombinant human CNTF (0.5 mg/ml; Regeneron) were produced by expression and purification from *Escherichia coli* and inactive vehicle solution as described (*21*) [P. Masiakowski *et al.*, *J. Neurochem.* **57**, 1003 (1991)]. Vehicle for BDNF was 0.9% saline and bovine serum albumin (0.5 mg/ml) and that for CNTF was 12 mM phosphate and 10 mM lactate with 5% mannitol (pH 4.5).
13. All animal experiments were conducted under protocols approved by the Cleveland Clinic Institutional Animal Research Committee in accordance with NIH guidelines. Body weight, semiquantitative paw and forelimb grading scales, and forelimb grip strength were determined weekly in *wobbler* mice treated with CNTF and BDNF ($n = 8$), with CNTF alone for 5 days ($n = 6$), with BDNF alone for 5 days ($n = 6$), and with vehicle ($n = 7$) and in normal littermates ($n = 10$). The semiquantitative grading scales were classified as grade 0, normal; grade 1, atrophy of paw fingers; grade 2, curled fingers; grade 3, curled wrists; and grade 4, forelimbs flexed to chest. When *wobbler* mice were diagnosed at 3 to 4 weeks of age, paw abnormalities were graded as grade 1. Grip strength of the fore-

paws—the maximum muscle strength in the forelimbs (measured in grams)—was measured by holding the mice in the air and allowing them to grasp with both forepaws a horizontal wire strain gauge connected to a dynamometer [W. E. Kozachuk, H. Mitsumoto, V. D. Salanga, G. J. Beck, F. Wilber, *J. Neurol. Sci.* **78**, 253 (1987)] (*4*). The differences between the two groups (treated and normal) in quantitative measurements were analyzed with the unpaired *t* test. For multiple group comparisons, analysis of variance (ANOVA) was used. All tests were two-sided; the significance level (α) was set at 0.05.

14. The mean (±SD) biceps muscle weight was 7.9 ± 2.4 mg in cotreated mice ($n = 8$) and 4.4 ± 0.9 mg in vehicle-treated mice ($n = 7$) ($P < 0.005$).
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16. The right biceps muscle was dissected, weighed, rapidly frozen, serially sectioned (10 μm), and stained with acid, intermediate, and routine alkaline phosphatase. The mean diameter of muscle fibers was calculated with a morphometric program (Jandel Sigmascan, San Rafael, CA) and was derived from counts (performed in a blinded fashion) of >1000 muscle fibers in randomly selected areas.
17. For analysis of ventral root fibers, a separate group of six *wobbler* mice was cotreated with CNTF and BDNF as described in the text. At the end of treatment, the animals were perfused intracardially with 3.6% buffered glutaraldehyde. The C5 and C6 ventral roots were embedded in epoxy resin and cut in 0.5-μm sections. Myelinated fibers were counted by a blinded observer as described (*8, 9*) by use of the Jandel Sigmascan histometric system. For motor neuron counts, the entire cervical cord (C1 to C7) was dissected, fixed overnight in 4% paraformaldehyde, and stored in 30% sucrose. Serial 30-μm frozen sections were stained for ChAT with a polyclonal antibody (Chemicon) on the basis of techniques previously described (*6*). Spinal motor neurons were counted in every twelfth section by a blinded observer. Six untreated *wobbler* mice and four healthy littermates were studied separately (*11*).
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TECHNICAL COMMENTS

Phylogenetic Analysis of a Reported Complementary DNA Sequence

In the sequence reported as the complementary DNA (cDNA) of 12-kD human B cell growth factor [(*1*), original GenBank entry accession number M15530], 54 out of 124 codons were predicted to be derived from an Alu fragment. In addition to the originally described Alu element that was predicted to contribute the 32 COOH-terminal amino acids, we identified a second Alu fragment that coded for the 22 NH_2-terminal residues (Fig. 1). The unusually high Alu content suggested that these repeats might be instrumental in the emergence of new proteins. To reconstruct the underlying events, we undertook the phylogenetic analysis of the corresponding sequence. The contributing repeats (Fig. 1) belong to the old Alu subfamilies, Sx and J, which have been present in primates for over 35 million years (*2*). We studied this region in several primates including New World monkeys and prosimians. Six amplification primers based on the reported sequence (*1*) were used in different combinations in order to compensate for possible mismatches between species. DNAs from Old World monkeys (OWM), gibbon, great apes, and humans amplified well with most primer combinations. The apparent length of the PCR products were as predicted from the M15530 clone, indicating that the latter was colinear with the genomic locus. We cloned and sequenced PCR products that were amplified with the use of U-L primer pairs from three humans and from chimpanzee, gorilla, gibbon, baboon, and macaque (*3*).

All human sequences that we sequenced differed from the reported M15530 cDNA (*1*) by two substitutions and two deletions (Fig. 1). Three of these changes interrupted the M15530 open reading frame: the transversion at position 119 created a stop codon TGA, while both deletions resulted in the frameshifts and premature termination. These differences with M15530 also existed in all nonhuman primate sequences analyzed. The authenticity of M15530 cDNA could be thus questioned, unless (i) our PCR products were amplified from an intronless pseudogene, (ii) M15530 was recently derived by duplication from the genomic locus analyzed here, or (iii) the differences represented an edited transcript.

The first possibility is contradicted by the following analysis. The locus in question is at least as old as the phylogenetic divergence of OWM. Therefore, a similar number of changes should separate the intronless pseudogene in OWM, apes, and humans from the original active sequence evolving independently (dotted line in Fig. 2). However, this cannot be the case for M15530, which appears as the closest relative of the genomic human sequence (Fig. 2). It differs at only four positions from the human genomic sequence and at 11, 14, 21, 50, and 51 positions from chimpanzee, gorilla, gibbon, baboon, and macaque genomic sequences, respectively (*4*).

The second scenario is improbable as it would require a series of fortuitous events during a short evolutionary time period (Fig. 2). Duplication of the genomic locus analyzed here would have to be followed by nucleotide substitution and two deletions extending the reading frame, gain of a tissue-specific promoter, and an intron capture, which would render the resulting locus refractory to PCR.

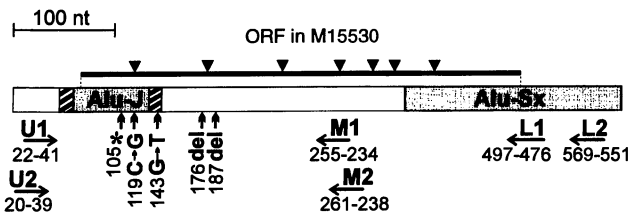

Fig. 1. Structure of the 12 kD BCGF cDNA reported by Sharma *et al.* (*1*). Alu segments are shown as shaded boxes, hatched blocks represent direct repeats, and the horizontal line above the locus corresponds to the open reading frame in M15530 cDNA. Horizontal arrows show localization of PCR primers; vertical arrowheads, STOP codons; vertical arrows, substitutions and deletions with respect to the orginally reported M15530 sequence. The asterisk indicates the location of a six-nucleotide sequence CGCGGC present in OWM and in the corresponding Alu-J consensus sequence.