were examined so as not to introduce a bias for any known diseases. A 96-well plate was constructed, containing DNA from 76 of these individuals, 10 individuals from a three-generation Caucasian family, 7 individuals from a two-generation African-American family, and one chimpanzee, as well as positive and negative DNA controls. Immortalized cell lines derived from the two families were obtained commercially. The plate contained DNA from 82 unrelated individuals: 20 African-Americans, 20 Asians, 21 Caucasians, 18 Hispanic-Latinos, and 3 Native Americans.

11. Regions targeted for sequencing were amplified from genomic DNA isolated from the immortalized cell lines. Polymerase chain reaction (PCR) primer pairs were designed using the sequence and genomic organization in GenBank. The PCR products were purified using a Whatman Polyfiltronics 100 μl 384-well unifilter plate, essentially according to the manufacturer's protocol. The purified DNA was eluted in 50 μl of distilled water. Sequencing reactions were set up using Applied Biosystems Big Dye Terminator chemistry, essentially according to the manufacturer's protocol. The DNA primer used for the sequencing reaction was the M13 forward primer (5′-TGTAAAACGACGGCCAGT-3′) or the M13 reverse primer (5′-AGGAAACAGCTATGACCAT-3′). Reaction products were purified by isopropanol precipitation and analyzed on an ABI Prism 3700 DNA Analyzer. Sequences obtained were examined for the presence of polymorphisms by using the Polyphred program (*48*). The presence of a polymorphism was confirmed by sequencing both strands of DNA.
12. Each polymorphic site was compared with the public databases HGBASE (release 8 2000-11-1) and dbSNP (build 92, February 2001) to determine whether that site had been previously described.
13. A Web site containing the list of genes, base pair coverage, the number of SNPs, the number of haplotypes, and other characteristics described in this paper may be found at www.genaissance.com/genecharacteristics/genecharacteristics.pdf.
14. Splice sites were defined as the first 8 bases at the 5′ end of an intron and the last three bases at the 3′ end of an intron.
15. W. H. Li, C. I. Wu, C. C. Luo, *J. Mol. Evol.* **21**, 58 (1984).
16. To confirm the validity of SNPs that were observed only once as a single heterozygote, we resequenced the genomic regions from a subset of singleton SNPs. For 150 of 159 SNPs (94%), the presence of the polymorphism was confirmed by resequencing both strands of DNA.
17. J. C. Stephens *et al.*, unpublished data.
18. G. A. Watterson, H. A. Guess, *Theor. Popul. Biol.* **11**, 141 (1977).
19. The population-specific SNPs in our sample could occur in low frequency in one or more of the other populations but might not be detected because of the sample size. For instance, a SNP with a minor allele frequency of 0.0172 has only a 50% probability of being observed if the sample size is 20 people.
20. M. Slatkin, *Evolution* **39**, 53 (1985).
21. R. M. Cerda-Flores *et al.*, *Ann. Hum. Biol.* **19**, 347 (1992).
22. J. C. Stephens, D. Briscoe, S. J. O'Brien, *Am. J. Hum. Genet.* **55**, 809 (1994).
23. E. J. Parra *et al.*, *Am. J. Hum. Genet.* **63**, 1839 (1998).
24. M. D. Shriver *et al.*, *Am. J. Hum. Genet.* **60**, 957 (1997).
25. E. S. Lander, N. J. Schork, *Science* **265**, 2037 (1994).
26. N. Risch, K. Merikangas, *Science* **273**, 1516 (1996).
27. N. J. Risch, *Nature* **405**, 847 (2000).
28. W. H. Li, *Molecular Evolution* (Sinauer Associates, Sunderland, MA, 1997).
29. M. Nei, *Molecular Evolutionary Genetics* (Columbia Univ. Press, New York, 1987).
30. G. A. Watterson, *Theor. Popul. Biol.* **7**, 256 (1975).
31. F. Tajima, *Genetics* **123**, 585 (1989).
32. The standard neutral model (9) assumes that: a population is of a constant size; mating occurs randomly within the population; and mutations arise randomly at sites that are not already polymorphic and confer no selective advantage for any allele.
33. A. R. Templeton *et al.*, *Am. J. Hum. Genet.* **66**, 69 (2000).
34. A. G. Clark, *Nature Genet* **22**, 119 (1999).
35. J. G. Hacia *et al.*, *Nature Genet* **22**, 164 (1999).
36. J. A. Schneider *et al.*, unpublished data.
37. The haplotypes for each gene were constructed from the unphased genotypes using an extension of Clark's algorithm (*49*). We compared the haplotype assignments made for multiply heterozygous individuals in the two families. The agreement between our algorithm and those assignments made by Mendelian principles was at least 95%. In addition, we obtained similar results by comparing our algorithm with haplotype assignments made by molecular cloning.
38. T. Acharya *et al.*, unpublished data.
39. M. Nei, F. Tajima, *Genetics* **97**, 145 (1981).
40. The total number of haplotypes observed (50,471) was determined as follows: $(2 \times 82 \times 313) - (21 \times 41)$, in which the subtracted term is an adjustment for X-linked genes in males.
41. N. Saitou, M. Nei, *Mol. Biol. Evol.* **4**, 406 (1987).
42. Z. Mu *et al.*, unpublished data.
43. R. C. Lewontin, *Evol. Biol.* **6**, 381 (1972).
44. Not all populations had variability at the same position. Therefore, not all SNPs could be compared in the four populations. In addition, only those SNPs, with a minor allele frequency of at least five observations, were used. With these restrictions, 7607 SNP pairs were analyzed.
45. R. C. Lewontin, *Genetics* **140**, 377 (1995).
46. D. E. Reich *et al.*, *Nature* **411**, 199 (2001).
47. C. M. Drysdale *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 10483 (2000).
48. D. A. Nickerson, V. O. Tobe, S. L. Taylor, *Nucleic Acids Res.* **25**, 2745 (1997).
49. A. G. Clark, *Mol. Biol. Evol.* **7**, 111 (1990).
50. R. Grantham, *Science* **185**, 862 (1974).
51. We thank C. F. Aquadro, M. E. Kreitman, R. Myers, and N. Risch for insightful comments and M. Athanasiou, R. R. Denton, P. R. DiBello, A. E. Duda, G. Faith, M. G. Greene, C. Harris-Kerr, A. Kaul, S. Kliem, Z.-H. Lan, E. Lanz, S. A. Leonard, L. M. Malnick, Z. Mu, D. Murallo, C. L. Nicholson, C. Wan, C. Wilcox, A. J. Wood, and W. Zhao for their contributions.

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Loss of Huntingtin-Mediated BDNF Gene Transcription in Huntington's Disease

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Huntingtin is a 350-kilodalton protein of unknown function that is mutated in Huntington's disease (HD), a neurodegenerative disorder. The mutant protein is presumed to acquire a toxic gain of function that is detrimental to striatal neurons in the brain. However, loss of a beneficial activity of wild-type huntingtin may also cause the death of striatal neurons. Here we demonstrate that wild-type huntingtin up-regulates transcription of brain-derived neurotrophic factor (BDNF), a pro-survival factor produced by cortical neurons that is necessary for survival of striatal neurons in the brain. We show that this beneficial activity of huntingtin is lost when the protein becomes mutated, resulting in decreased production of cortical BDNF. This leads to insufficient neurotrophic support for striatal neurons, which then die. Restoring wild-type huntingtin activity and increasing BDNF production may be therapeutic approaches for treating HD.

Huntington's disease is a dominantly inherited neurodegenerative disorder characterized by chorea, cognitive abnormalities, and psychiatric disturbances beginning in mid-adulthood and progressing toward death (*1*, *2*). The disease is caused by a polyglutamine expansion in huntingtin that confers a toxic activity on this protein (*3*). Huntingtin is highly expressed in the brain, and particularly enriched in cerebral cortex and striatum (*4–6*). It is a cytoplasmic protein that is essential during development for gastrulation (*7–9*) and neurogenesis (*10*), and it is important for neuronal survival in the adult (*11–13*). Wild-type huntingtin is anti-apoptotic in neurons in the central nervous system (CNS) (*13*). Wild-type huntingtin also reduces the toxicity of mutant huntingtin in vivo (*14*). Huntingtin also is involved in vesicle trafficking in the secretory and endocytic pathways (*15*, *16*).

Here we investigate whether wild-type huntingtin activity is important for the striatal neurons that selectively die in HD. These neurons require BDNF for their survival and differentiation (*17–19*). BDNF also protects striatal neurons from excitotoxin-induced neurodegeneration (*20*). Despite some reports of BDNF mRNA transcription in adult striatal neurons (*21*), most evidence demonstrates that striatal BDNF arrives by anterograde

transport from the cortex via cortico-striatal afferents (*22*, *23*).

We analyzed whether wild-type huntingtin influences BDNF production in normal and HD brains. We began by evaluating BDNF production and secretion in cultured CNS cells overexpressing full-length wild-type or mutant huntingtin (FLwt and FLmu cells, respectively) (*13*). Enzyme Linked Immunosorbent Assays (ELISAs) for BDNF, nerve growth factor (NGF), neurotrophin-3 (NT3), neurotrophin-4 (NT4), and ciliary neurotrophic factor (CNTF) were performed on the supernatant from parental (P) and clonally derived FLwt and FLmu cells (Fig. 1A) (*24*). Although there were no important changes in NGF and NT3 secretion among the various cell clones, BDNF release in FLwt cells was significantly higher than in parental cells (94.6% above parental). By contrast, FLmu cells secreted 52.5% less BDNF than parental cells. CNTF and NT4 were not detectable in the same supernatants. BDNF measurements in the cell lysates gave similar results (Fig. 1B, left). Analogous changes were also detected by Western blotting of the BDNF protein (Fig. 1B, right) (*25*). The BDNF level progressively increased in FLwt cells with time after plating and reached a plateau at 120 hours (Fig. 1C), whereas in FLmu cells the amount of BDNF produced always remained below that of parental cells. Thus, wild-type and mutant huntingtin modulate BDNF protein production differently. Furthermore, BDNF content is lower in cells expressing full-length mutant huntingtin.

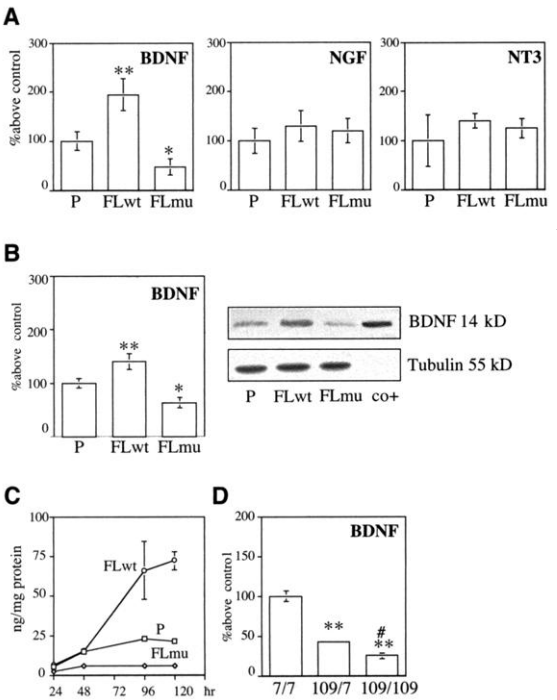

Fig. 1. Modulation of BDNF protein release and production by wild-type and mutant huntingtin. (**A**) ELISA for BDNF, NGF, and NT3 performed on the supernatant from parental cells (P) and subclones stably expressing FLwt or FLmu. BDNF level is significantly higher in FLwt cells (142.7 ± 45.3 ng/mg of protein lysate) whereas BDNF production is lower in FLmu cells (41.9 ± 12.3 ng/mg of protein lysate). Data are expressed as percentage of controls. Standard errors are from four independent experiments. Multiple cell clones (at 5th – or 6th passage) that expressed similar levels of FLwt and FLmu huntingtin were used and generated similar results. $*P < 0.05$, $**P < 0.01$ versus parental cells, analysis of variance (ANOVA) test. Web figures (*42*) show that FLmu and FLwt cells used in the study exhibit similar levels of expression of the transgene. (**B**) Left panel, ELISA assay on cell lysates. Data are expressed as percentage of controls (parental cells, P). Standard errors are referred to three independent experiments. $*P < 0.05$, $**P < 0.01$ versus parental cells, ANOVA test. Right panel, Western analysis performed on the same lysates. Equal loading is demonstrated by tubulin-immunoreactive band. Positive control (co+), human recombinant BDNF. (**C**) BDNF accumulation over time. Time zero is when cells are seeded. (**D**) Measurement of BDNF level in cultured CNS cells previously established from heterozygous (109/7) and homozygous (109/109) knock-in mice (*27*, *28*), and compared to wild-type littermates (7/7). BDNF levels (expressed as ng/mg of protein lysate) were as follows: 7/7 cells, 233.8 ± 16.3; 109/7 cells, 100.6 ± 0.4; and 109/109 cells, 50.1 ± 4.1. Shown is the mean of three independent experiments, $**P < 0.01$ versus 7/7 cells, $\#P < 0.01$ versus 109/7 cells, ANOVA test.

Modulation of BDNF levels by huntingtin is neuron-specific; no changes were observed in fibroblast 3T3 cells stably overexpressing the full-length wild-type or mutant huntingtin (*26*). Analyses of BDNF in CNS cells previously obtained from heterozygous and homozygous *Hdh* knock-in mice (*27*) [in which a 109 CAG triplet repeat has been inserted into the endogenous mouse huntingtin gene (*28*)] revealed a mutant huntingtin dosage-dependent decrease in BDNF levels (Fig. 1D). This implies either the loss of a potential protective effect of the wild-type protein or, alternatively, increased activity of a mechanism specific to the mutant protein.

Recent evidence indicates a role for transcriptional dysregulation in HD (*29*). In transgenic mice, the mutant amino-terminal huntingtin fragment affects the expression level of several genes, including neurotransmitter receptor genes and intracellular signal-

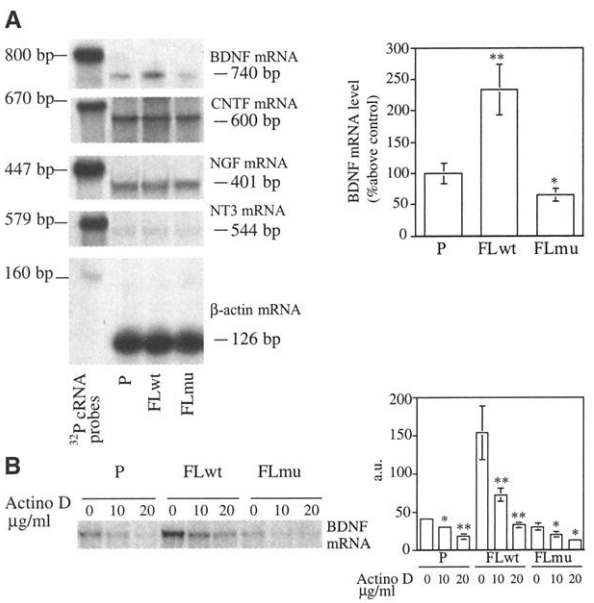

Fig. 2. Wild-type and mutant huntingtin differently modulate BDNF gene transcription. (**A**) Left, determination of BDNF, CNTF, NGF, and NT3 mRNA levels by RPA in P, FLwt, and FLmu cells. Higher BDNF mRNA levels were found in FLwt cells, whereas FLmu decreased BDNF levels. Actin probe is included as internal control. Right, quantitative analyses of the BDNF mRNA levels. The peak densitometric area was normalized over the peak densitometric area of the β-actin band. Data are expressed as percentage of controls. A mean of three different experiments is shown. $*P < 0.05$ and $**P < 0.01$ versus parental cells, ANOVA test. (**B**) Left, RPA. Cells were exposed for 6 hours to actinomycin-D. A similar decrease in BDNF mRNA level was found in all clones, including the FLwt cells. Right, densitometric analysis of the BDNF and β-actin mRNA levels, performed as described in (A). Data represent the average of three independent experiments. $*P < 0.05$ and $**P < 0.01$ versus untreated cells, ANOVA test.

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ing proteins (*30*, *31*). Although wild-type huntingtin is predominantly a cytoplasmic protein, it is possible that small amounts of full-length protein or proteolytically cleaved fragments are localized in the nucleus (*32*) and are involved in transcriptional regulation of target genes. Alternatively, huntingtin may act within the cytoplasm by recruiting transcription factors. However, there are no reports of transcriptional activity of wild-type huntingtin. We analyzed whether full-length huntingtin influences BDNF protein production at the level of gene transcription. We performed RNase Protection Assay (RPA) (*33*) for BDNF, CNTF, NGF, and NT3 mRNAs in parental, FLwt, and FLmu cells (Fig. 2A, left). In FLwt cells, BDNF mRNA levels were increased 2.3-fold over those in the parental cells (Fig. 2A, right). In contrast, and consistent with the ELISA data, FLmu cells showed a 40% decrease in BDNF mRNA level as compared with parental cells (Fig. 2A, right). CNTF, NGF, and NT3 mRNA levels were not significantly changed in FLwt and FLmu cells from those in parental cells (Fig. 2A, left). Exposure of the cells to increasing doses of actinomycin-D, an inhibitor of gene transcription, generated a similar decrease in BDNF mRNA levels in all clones, including FLwt cells (Fig. 2B). Thus, huntingtin modulates BDNF production at the transcriptional level.

The structure of rat BDNF gene is complex: four 5′ exons are linked to separate promoters and one 3′ exon encodes the BDNF protein (*34*). These promoters are alternatively used, generating a tissue-specific (*35*) and stimulus-induced (*36*) pattern of BDNF expression. To evaluate whether the modulatory effect of huntingtin on BDNF gene transcription results from the preferential activation of one or more of these promoter regions, we performed reverse transcriptase–polymerase chain reaction (RT-PCR) using primers specifically recognizing each of the four BDNF exon-specific mRNAs (*37*). We found that parental cells (P, Fig. 3A) express exon II, III, and IV mRNAs (the two fragments in exon II mRNA lanes result from alternative usage of splice sites). FLwt cells showed an increase in exon II mRNAs (Fig. 3A). In contrast, a drastic depletion in exon II mRNAs as well as in exon III and IV mRNAs was seen in FLmu cells (Fig. 3A). RNAse protection experiments performed using cRNA probes specifically recognizing the various exons confirmed these data (Fig. 3B, left panel) and further indicated a 1.7-fold increase in exon II mRNAs in FLwt cells (Fig. 3B, right graphs) (*38*).

To assess quantitatively the influence of huntingtin on the transcriptional activity of each of the four BDNF promoters, we transfected parental, FLwt, and FLmu cells with different BDNF promoter-reporter gene constructs containing promoter regions II, III, and IV cloned upstream of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene (*39*). Promoter II activity increased ninefold in FLwt cells, whereas promoters III and IV were not affected (Fig. 3C). In contrast, promoter II, III, and IV activities were significantly lower in FLmu cells than in parental cells. Thus, wild-type and mutant huntingtin modulate BDNF gene transcription positively or negatively, respectively, by differentially influencing the activity of the BDNF promoters.

We next analyzed whether in vivo production and delivery of cortically derived BDNF to the striatum is similarly influenced by huntingtin. We used a transgenic mouse model of HD overexpressing wild-type or mutant full-length huntingtin (YAC18 and YAC72 mice, respectively) (*40*). In these mice, human huntingtin is expressed under its own promoter, which results in a developmental and tissue-specific expression pattern similar to that seen for endogenous huntingtin. No neurodegeneration is observed in YAC18 mice, but YAC72 mice develop a selective neurodegeneration of the striatal medium spiny neurons at 12 months of age (*40*). Cerebral cortex, hippocampus, and striatum were isolated from 9-month-old YAC18 and YAC72 mice (which are not symptomatic at this age) as well as wild-type littermates (L), and BDNF protein content was measured by ELISA (Fig. 4A). At this age, YAC18 mice exhibited a statistically significant increase in BDNF production in cerebral cortex. Similarly, the striatum of YAC18 mice contained more BDNF than wild-type littermates (*41*). BDNF was also increased in YAC18 hippocampus. Remarkably, when the same analysis was conducted in YAC72 mice, a statistically significant reduction in cortical and hippocampal BDNF was seen, as well as a 48% reduction in cortically derived BDNF in striatum (*41*).

To further demonstrate that changes in striatal BDNF in YAC18 and YAC72 mice result from variations in cortical *BDNF* gene transcription, we analyzed *BDNF* mRNA levels in striatum as done for Fig. 3A. In agreement with others showing low BDNF mRNA levels in this brain region, no detectable bands were found after amplification of exon I, II, and IV mRNAs,

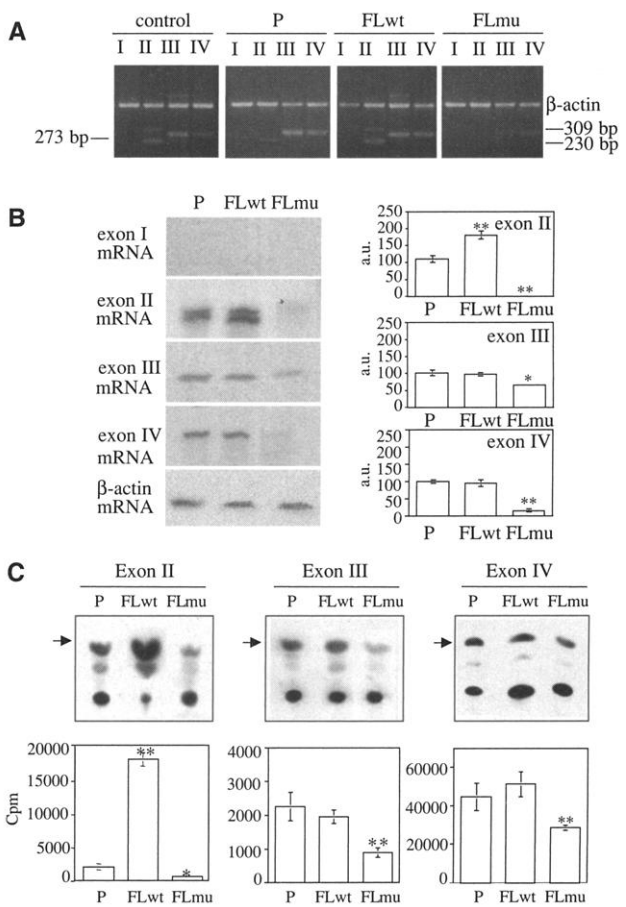

Fig. 3. Influence of huntingtin on the transcription of the four BDNF promoters. (**A**) Upper panel, RT-PCR analyses using exon-specific primers I to IV. Control, cDNA synthesized from 0.25 μg of RNA from adult hippocampus amplified in each lane. Exon I mRNA is not detectable in control hippocampus-derived RNA or in cells, consistent with the finding that this mRNA is transcribed only after kainic acid treatment (*34*). Instead, in FLwt cells, wild-type huntingtin affects positively the transcription of exon II mRNA. In contrast, mutant huntingtin abolished exon II, III, and IV mRNAs production. (**B**) Left panel, determinations of exon I, II, III, and IV mRNA levels by RPA. Probing with the BDNF exon II cRNA probe generates two protected bands corresponding to different spliced forms of exon II mRNA. Right panel, densitometric analysis of BDNF exons mRNAs and β-actin levels performed as described in Fig. 2A. Data represent the average of three different experiments. $*P < 0.05$, $**P < 0.01$ versus parental cells, ANOVA test. (**C**) Upper panel, CAT assay. BDNF promoter constructs encompassing promoter regions II, III, and IV cloned in front of the bacterial CAT reporter gene were transiently transfected in P, FLwt, and FLmu cells. FLwt activated BDNF promoter II, whereas FLmu repressed promoters II, III, and IV activity. Lower panel, quantification of the acetylated reaction products from three different experiments. $*P < 0.05$, $**P < 0.01$ versus parental cells, ANOVA test. Arrow, acetylated chloramphenicol.

whereas exon III mRNA was present at very low levels (Fig. 4B, lower panel). Striatal BDNF mRNA levels were not modulated by wild-type or mutant huntingtin; BDNF mRNA levels in YAC18 and YAC72 striata were indistinguishable from those in wild-type littermates. By contrast, analysis of mRNA levels from exons I through IV in cerebral cortex and hippocampus from YAC18 and YAC72 mice revealed an expression pattern similar to that observed in vitro (Fig. 4B, upper and middle panels). In particular, increased BDNF exon II and exon III mRNAs levels were observed in the cerebral cortex and hippocampus of YAC18 mice. In contrast, exon II mRNA was depleted and BDNF exon III and IV mRNAs were lower in the cerebral cortex and hippocampus of YAC72 mice. These results were also confirmed by RPA experiments on RNA extracted from the various brain regions of wild-type littermates YAC18 and YAC72 mice (*42*).

Thus, in vivo, wild-type huntingtin positively modulates the transcription of the *BDNF* gene. BDNF gene transcription and protein production are lost in cells and cerebral cortex of transgenic mice expressing mutant huntingtin, resulting in an in vivo downregulation of cortically derived BDNF in the striatum.

Analyses of BDNF levels in cortical tissue from HD patients support this finding. There was a 45% decrease in BDNF protein in the fronto-parietal cortex of HD brains (grades II and III) (Fig. 4C, lower graph). Semiquantitative RT-PCR determinations on a parallel sample from the same tissue also show a 65% loss in BDNF mRNA levels (Fig. 4C, upper panels) (*43*). The finding that huntingtin-mediated BDNF gene transcription and protein production is decreased in human HD brain regions that normally project to striatum suggests reduced trophic support for striatal neurons during the course of the disease. A similar semiquantitative RT-PCR analyses was performed to evaluate levels of NGF, CNTF, and NT3 in the same human cortical samples from control and HD subjects. Whereas NGF and CNTF mRNA levels were unchanged, those of NT3 decreased (*42*).

Transgenic mice expressing an NH_2-terminal fragment of mutant huntingtin and characterized by well-documented caspases activation are shown to express lower amounts of full-length wild-type huntingtin (*44*). We investigated whether BDNF levels depend on the presence of an intact full-length wild-type huntingtin protein. We exposed cultured CNS cells to apoptotic stimuli [previously shown to activate caspases in the same cells; see (*13*)], and measured full-length wild-type huntingtin protein and its mRNA levels as well as BDNF production (*45*). Endogenous full-length wild-type huntingtin protein (but not mRNA) was depleted after an apoptotic stimulus (Fig. 4D). This and other data (*44*) raise the possibility that activation of the apoptotic cascade leads to the loss of full-length wild-type huntingtin and other essential cellular proteins and may represent a general response of neurons to severe stress conditions imposed by environmental or genetic insults. Under the same experimental conditions, BDNF protein levels were lower than in untreated cells (Fig. 4D, lower graph).

HD is thought to be a gain-of-function disease affecting the brain's striatal neurons. But more recent results in neurons and in mice highlight the possibility that loss of wild-type huntingtin's beneficial functions may also contribute to the disease phenotype (*46*).

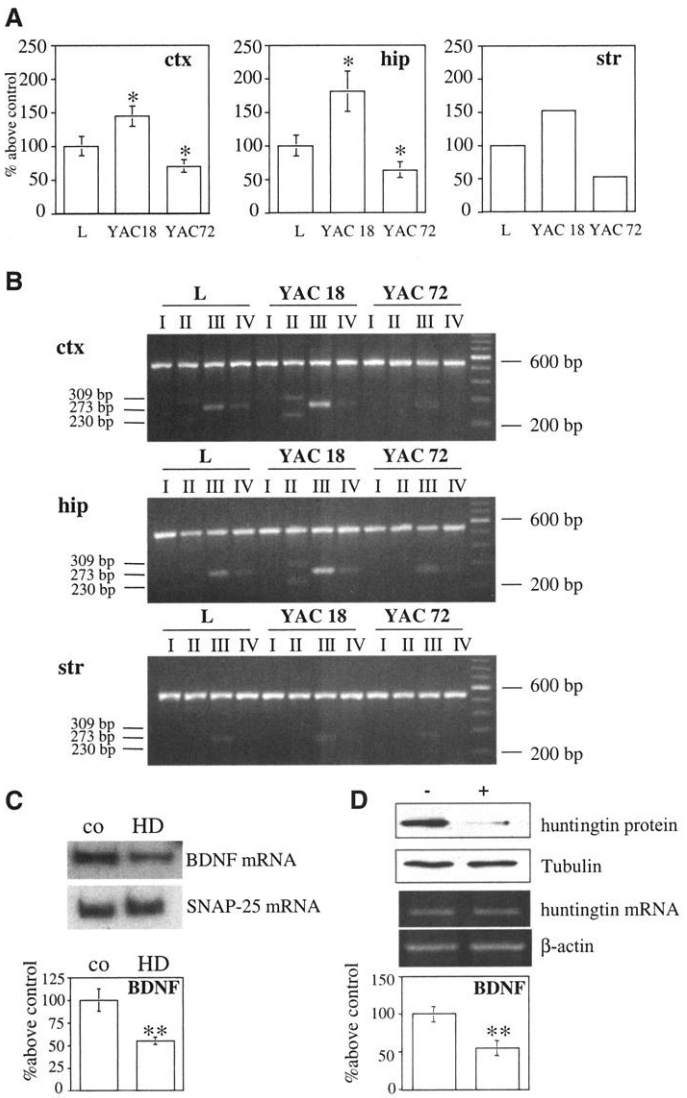

Fig. 4. Modulation of wild-type and mutant huntingtin on cortical BDNF production. (**A** and **B**) Changes in BDNF protein and mRNA levels in YAC18 and YAC72 mice overexpressing the wild-type or mutant protein, respectively. (A) BDNF ELISA assay of lysates from ctx, hip, and str from 9-month-old YAC transgenic mice. Increased BDNF levels were found in ctx, hip, and str of YAC18 mice. In contrast, YAC72 mice, which are presymptomatic at this age showed significantly lower BDNF levels. Data are expressed as percentage above control (L). Standard errors refer to four independent experiments. Duplicate samples for striatum were analyzed due to the more limited amount of tissue available. One of the two measurements is reported; the second generated similar results. $*P < 0.05$, versus wild-type littermates, ANOVA test. (B) Huntingtin influences BDNF gene transcription in vivo. Levels of BDNF exon I to IV mRNAs were measured as described in Fig. 3A. The levels of exon I to IV mRNAs in the striatum of YAC18 and YAC72 (lower panel) transgenic mice were no different from those of L. In contrast, in YAC18 mice, exon II and exon III mRNA levels in cerebral cortex and hippocampus were higher than in wild-type littermates. In the same tissues from YAC72 mice, exon II mRNA was not detected, whereas exon III and exon IV mRNAs were decreased. (**C**) BDNF levels in samples from frozen frontoparietal cortex obtained from an autopsy of age-matched controls (co) and HD patients (two patients were analyzed for each group, grade II and III). One of the two measurements conducted on separate samples is shown. Upper panels, analysis of BDNF mRNA levels evaluated by semi-quantitative radioactive RT-PCR. SNAP-25 mRNA levels are also shown. BDNF mRNA levels were lower in HD cerebral cortex than in controls. Lower graph, ELISA of BDNF protein levels. BDNF protein was lower in HD cerebral cortex (45.2% less than control). Similar BDNF levels were observed in another HD patient. $**P < 0.01$ versus control, ANOVA test. (**D**) Measurement of huntingtin protein (upper autoradiograms) and its mRNA levels (middle panels), before (–) and after (+) exposure of CNS cells to apoptotic stimuli, such as 1 mM 3-nitropropionic acid for 30 hours. The same result was obtained after 18 hours (not shown). Cultures of conditionally immortalized CNS cells grown at the permissive temperature (33°C) were used (*13*, *27*). No changes in cell number were found in these conditions at the time point analyzed. Depletion of intact full-length huntingtin protein occurs in apoptotic cells as compared to untreated cells. Huntingtin mRNA level remained unchanged. Lower graph, BDNF production is reduced in the conditions described above. Shown is a mean of three independent experiments. $**P < 0.01$ versus untreated cells, ANOVA test.

Huntingtin is widely expressed but is highly localized in all cortical neurons that project to striatum (*6*). Here, we show that full-length wild-type huntingtin increases the transcription of the BDNF gene, influencing production and delivery of cortically derived BDNF to striatal targets, and that a cortical dysfunction occurs in HD involving the loss of huntingtin-mediated BDNF production.

We suggest that selective vulnerability of a subset of neurons in HD (and in other CAG-repeat diseases) may result from the loss of the activity of the wild-type protein(s) crucial for the functioning of these cells. In HD, a major defect in wild-type huntingtin activity is localized in the cortical afferents to the striatum.

Loss of cortical BDNF combined with the recent successful treatment of HD patients with neural cell therapies (*47*), provide support for the notion that increasing intracerebral BDNF levels may be beneficial, and suggest that therapeutic drugs aimed at mimicking or increasing the normal activity of wild-type huntingtin may provide an effective strategy for treating HD.

References and Notes

1. M. R. Hayden, *Huntington's Chorea* (Springer, New York, 1981), pp 140–189.
2. P. S. Harper, *Huntington's Disease* (Sounders, The University Press, Cambridge, 1996), pp. 317–358.
3. Huntington's Disease Collaborative Research Group, *Cell* **72**, 971 (1993).
4. A. H. Sharp *et al.*, *Neuron* **14**, 1065 (1995).
5. Y. Trottier *et al.*, *Nature Genet.* **10**, 104 (1995).
6. F. R. Fusco *et al.*, *J. Neurosci.* **19**, 1189 (1999).
7. S. Zeitlin, J. P. Liu, D. L. Chapman, V. E. Papaioannou, A. Efstratiadis, *Nature Genet.* **11**, 155 (1995).
8. J. Nasir *et al.*, *Cell* **81**, 811 (1995).
9. M. P. Duyao *et al.*, *Science* **269**, 407 (1995).
10. J. K. White *et al.*, *Nature Genet.* **17**, 404 (1997).
11. J. R. O'Kusky *et al.*, *Brain Res.* **818**, 468 (1999).
12. I. Dragatsis, M. S. Levine, S. Zeitlin, *Nature Genet.* **26**, 300 (2000)
13. D. Rigamonti *et al.*, *J. Neurosci.* **20**, 3705 (2000)
14. B. R. Leavitt *et al.*, *Am. J. Hum. Genet.* **68**, 313 (2001).
15. J. Velier *et al.*, *Exp. Neurol.* **152**, 34 (1998).
16. K. B. Kegel *et al.*, *J. Neurosci.* **20**, 7268 (2000).
17. R. Ventimiglia, P. E. Mather, B. E. Jones, R. M. Lindsay, *Eur. J. Neurosci.* **7**, 213 (1995).
18. K. Mizuno, J. Carnahan, H. Nawa, *Dev. Biol.* **165**, 243 (1994).
19. S. Ivkovic, M. E. Ehrlich, *J. Neurosci.* **19**, 5409 (1999).
20. J. M. Canals *et. al.*, *J. Neurosci.* **21**, 117 (2001).
21. M. Hofer, S. R. Pagliusi, A. Hohn, J. Leibrock, Y. A. Barde, *EMBO J.* **9**, 2459 (1990).
22. J. M. Conner, J. C. Lauterborn, Q. Yan, C. M. Gall, S. Varon, *J. Neurosci.* **17**, 2295 (1997).
23. C. A. Altar *et al.*, *Nature* **389**, 856 (1997).
24. 1.5×10^5 conditionally immortalized CNS cells (*13*) were plated in 60-mm dishes and grown in Dulbecco's Modified Eagle's medium supplemented with 10% of fetal calf serum at 33°C in a 5% CO_2 atmosphere. Cell medium and lysates were collected after 96 hours. Cell lysates were prepared in lysis buffer consisting of glycerol 10%, 25 mM Tris HCl, pH 7.5, 150 mM NaCl, Triton X100 1%, 5 mM EDTA, 1 mM EGTA supplemented with 1:100 of Protease Inhibitor Cocktail (Sigma, St. Louis, MO). Samples were homogenized, sonicated, and centrifuged (15 min at 4°C max speed Biofuge). The supernatants were collected and stored at −30°C. Samples were assayed for BDNF, NGF, NT3, NT4, and CNTF content. BDNF, NGF, NT3, and NT4 by ImmunoAssay System (Promega, Madison, WI). CNTF assay kit was from R&D ImmunoAssay Systems (Minneapolis, MN). Assays were performed as described by the manufacturer.
25. Cells were washed with cold phosphate-buffered saline (PBS) and subsequently scraped into lysis buffer, subjected to SDS–polyacrylamide gel (SDS-PAGE), and were blotted. Polyvinylidene difluoride (PVDF) membranes were incubated at 4°C overnight with the BDNF rabbit polyclonal antibody sc546 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). After being washed the membranes were incubated with antibody to rabbit immunoglobulin G (IgG) labeled with horseradish peroxidase (HRP) (Kirkegard & Perry Laboratories, Gaithersburg, MD) diluted 1:10,000 for 1 hour at room temperature. The membranes were washed again and developed with the chemiluminescence ECL PLUS Western Blotting system (Amersham, Milano, Italy).
26. Measurement of BDNF level in parental 3T3 cells and clonally derived cells stably overexpressing FLwt (3T3-FLwt) or FLmu (3T3-FLmu) huntingtin. Multiple clones were tested. BDNF levels (expressed as ng/mg protein lysates) were as follows: parental 3T3 cells, 22.9 ± 6.9; 3T3-FLwt, 24.2 ± 6.5; and 3T3-FLmu, 28.5 ± 4.7. Data are means ± SEM of three independent experiments.
27. F. Trettel *et al.*, *Hum. Mol. Genet.* **9**, 2799 (2000).
28. V. C. Wheeler *et al.*, *Hum. Mol. Genet.* **8**, 115 (1999).
29. J. H. Cha, *Trends Neurosci.* **23**, 387 (2000).
30. J. H. Cha *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6480 (1998).
31. R. Luthi-Carter, *et al.*, *Hum. Mol. Genet.* **9**, 1259 (2000).
32. P. Hilditch-Maguire *et al.*, *Hum Mol Genet.* **9**, 2789 (2000).
33. Total RNA from cells and tissues was isolated with TRIZOL Reagent (Life Technologies, Rockville, MD). RNA was treated with RQ1 DNase (Promega) at 37°C for 30 min. RPA was performed on 10 μg of total RNA. After ethanol precipitation, RNA was dissolved in 20 μl of hybridization solution (80% formamide, 40 mM piperazine-*N*,*N*′-bis 2-ethanesulfonic acid, pH 6.4, 400 mM sodium acetate, pH 6.4, and 1 mM EDTA) containing 150,000 counts per minute (cpm) of each [α-^{32}P]CTP-labeled cRNA probes. The Riboprobe In Vitro Transcription System (Promega) was used to generate cRNA probes. Samples were incubated overnight at 45°C. Five antisense cRNA probes were simultaneously used in the assay. Plasmid pSK-rB (kindly provided by G. Yancopoulos) containing rat BDNF cDNA was linearized with Sma I and used to generate an antisense cRNA probe. Bluescript II SK$^+$ plasmid, containing 670–base pair (bp) coding sequence of rat CNTF (kindly provided by M. Sendtner) was linearized with Eco RI and used as a template to generate an antisense cRNA probe. Plasmid BSrNGF (kindly provided by S. Whittemore), containing a 721-bp fragment of the rat NGF cDNA, was linearized with Nco I and transcribed in vitro to generate a 447-bp antisense cRNA probe containing 401 bp of NGF sequence and 46 bp of the polylinker region. Plasmid containing an 800-base portion of the rat NT3 cDNA (kindly provided by Dr. George Yancopoulos) was linearized with Eco RI and transcribed in vitro to generate a 579-base antisense cRNA probe containing 544 bases of NT3 sequence and 35 bases of the polylinker region. As internal standard pTRI-β-actin-125-rat antisense control template (Ambion, Austin, TX) was used. At the end of the hybridization, 200 μl of RNase digestion buffer (300 mM NaCl, 10 mM Tris HCl, pH 7.4, 5 mM EDTA, pH 7.5) containing 1:400 dilution of an RNase Cocktail (Ambion) were added. The samples were incubated at room temperature for 30 min and then treated with Proteinase K. After extraction with phenol/chloroform and ethanol precipitation, the pellet was resuspended in loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 2 mM EDTA) and separated on a 5% polyacrylamide gel under denaturing conditions (7 M urea). The protected fragments were visualized and quantified by autoradiography.
34. T. Timmusk *et al.*, *Neuron* **10**, 475 (1993).
35. T. Timmusk *et al. J. Cell Biol.* **128**, 185 (1995).
36. M. Metsis, T. Timmusk, E. Arenas, H. Persson, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8802 (1993).
37. RNA was reverse-transcribed into single-stranded cDNA using Superscript II RNase H$^-$ Reverse Transcriptase (Life Technologies) as described by the manufacturer. PCR was performed in a total volume of 50 μl containing cDNA made from 0.25 μg of total RNA, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of the common reverse primer corresponding to exon V, 0.4 μM of one of the 5′ exon-specific primers, 0.4 μM of β-actin primers and 2 U Taq polymerase (Life Technologies). Primer sequences were as follows: RatBDNFIsense, 5′-GGTGGATGAGAGTTGAAGCTTGCGA-3′; RatBDNFIIsense, 5′-GGAGCGGAGCGTTTGGAGAG-CCA-3′; RatBDNFIIIsense, 5′-CAGGAGTACATATCG-GCCACCA-3′; RatBDNFIVsense, 5′-GGCTTTGATG-AGACCGGGTTCCCT-3′; RatBDNFVantisense, 5′-GTAGGCCAAGTTGCCTTGTCCGT-3′; MACT3′, 5′-CTCTTTGATGTCACGCACGATTTC-3′; MACT5′, 5′-GTGGGCCGCTCTAGGCACCAA-3′. Amplification was carried out for 35 cycles. Each cycle consisted of the following steps: 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. PCR products were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide.
38. RNase Protection Assay was performed as described in (*33*). Twenty micrograms of total RNA were used. Five antisense cRNA probes were used. Plasmid BDNF I KS contains a 0.35 kb Sac I–Hind III fragment of exon I cloned into the Eco rV site in pBSKS. Plasmid BDNF II GEM contains a 0.16-kb fragment of exon II extending 5′ of the exon II/intron II boundary cloned into Eco RI–Hind III site in pGEM4Z. Plasmid BDNF III contains 0.19 kb of exon III extending 5′ of the exon III/intron III boundary cloned into the Eco RI in pGEMZ4. Plasmid BDNF IV GEM contains 0.27 kb of exon IV ending at the Bam HI site in exon IV cloned into the Eco RI–Hind III site in pGEMZ4. While BDNF I KS, BDNF II GEM, and BDNF IV GEM were linearized with Eco RI, BDNF III GEM was linearized with Hind III, and all were used to generate antisense cRNA probe [T. Timmusk, N. Bellvardo, H. Persson, M. Metsis, *Neuroscience* **60**, 287, (1994)]. pTRI-β-actin-125-rat antisense control template (Ambion) was used as standard probe.
39. For transfection experimental, the following BDNF promoter constructs were used: BDNF II 1.1 CAT contains a 1.1-kb Hind III–Sac I fragment of exon II and its 5′ flanking region. The 5′ Hind III is at 988 bp and the Sac I is at 2086 bp (*34*); BDNF III 4.5 CAT is described in (*35*); BDNF IV 0.7 CAT contains a 0.7-kb Eco RI–Sac I fragment of exon IV and its 5′ flanking region. The 5′ Eco RI site is at 1148 bp and the 3′ Sac I site is at 1791 bp (*34*). psV2CAT vector was used as positive control. Cell lines were transfected with Lipofectamine PLUS (Life Technologies) as described by the manufacturer. Cells were collected 48 hours after transfection, subjected to four cycles of freeze and thaw and then were assayed for CAT activity. Reaction products were separated on thin-layer chromatography silica gel plates, and quantified in a scintillation counter. In addition, for quantitative analysis, plasmid with BDNF promoter-CAT construct (3.5 μg) and a control plasmid expressing β-galactosidase (0.5 μg) were used to co-transfect 2×10^6 cells in a 100-mm tissue-culture dish. In all the experiments results were normalized to protein content [determined according to M. Bradford, *Anal. Biochem.* **72**, 248 (1976)] and β-galactosidase activity. Data from any single histogram were obtained from experiments performed on cells that were simultaneously cultured, transfected, and assayed to minimize variability due to differences in experimental procedure.
40. J. G. Hodgson *et al.*, *Neuron* **23**, 181 (1999).
41. Lysates from brain tissues of YAC transgenic mice were prepared using the same buffer described for cells. BDNF protein levels (expressed as ng/mg protein lysate) were as follows: wild-type littermate, cortex (ctx) 45.4 ± 4.8, hippocampus (hip) 36.8 ± 2.3, striatum (str) 11.7; YAC18, ctx 66.8 ± 3.9, hip 69.6 ± 5.6, str 17.8; YAC72, ctx 32.01 ± 4.6, hip 23.4 ± 5.4, str 6.5. Duplicates were analyzed for striatum.
42. Web figs. 1 through 3 are available on *Science* Online at www.sciencemag.org/cgi/content/full/1059581/DC1.

43. Lysates from frozen brain human tissue were prepared as in (*24*). Radioactive RT-PCR was performed in a total volume of 50 μl containing cDNA synthesized from 0.25 μg RNA, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.7 μCi [α-^{32}P]CTP, and 0.4 μM of the primers as follows: hBDNF5′, 5′-AGCCAGAATCGGAACCACGA-3′; hBDNF3′, 5′-GCACACCTGGGTAGGCCAAG-3′. PCR amplification was carried out for 30 cycles. Each cycle consisted of the following steps: 94°C for 30 s, 57°C for 30 s and 72°C for 30 s. The same amount of each cDNA was also amplified, independently, with SNAP-25 (synaptosomal associated protein 25, a presynaptic membrane-associate protein localized in grown cones, axons and presynaptic terminals) specific primers. SNAP-25 5′, 5′-CAAATGATGCCCGAGAAAAT-3′; SNAP25 3′, 5′-GGAATCAGCCTTCTCCATGA-3′. PCR products were separated by nondenaturing 8% polyacrylamide gel electrophoresis and visualized by autoradiography. BDNF levels were quantified and normalized relative to SNAP-25 levels.

44. V. O. Ona, *et al. Nature* **399**, 263 (1999).

45. Total cellular lysates from conditionally immortalized CNS cells (*13*, *27*) were obtained in a buffer containing Tris 50 mM pH 7.4, 5 mM NaCl, Triton X100 1%, 1 mM DTT, 15 mM EGTA supplemented with 1:100 of Protease Inhibitor Cocktail (Sigma). Immunoprecipitates were obtained by incubating the total cellular lysate (from 4 × 10^6 cells) with Mab2166 (1:1000) following conventional immunoprecipitation protocols and loaded. The blotted proteins were exposed to antibody to Htt Mab2166 (dilution 1:5000; Chemicon, Temecula, CA). RNA was reverse-transcribed into single-stranded cDNA using Superscript II RNase H$^-$ Reverse Transcriptase (Life Technologies) as described by the manufacturer. PCR was performed in a total volume of 50 μl containing 1 μg cDNA, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5% dimethyl sulfoxide (DMSO), 0.4 μM of Htt-specific primers (5′-CGACCCTGGAAAAGCTGATGAA-3′ and 5′-CACACGGTCTTTCTTGGTAGCTGA-3′), 2 U Taq polymerase (Life Technologies). Amplification was carried out for 25 cycles. Each cycle consisted of the following steps: 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. PCR products were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide.

46. E. Cattaneo *et al.*, *Trends Neurosci.* **24**, 182 (2001).

47. A. C. Bachoud-Levi *et al.*, *Lancet* **356**, 1975 (2000).

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Complete Genome Sequence of a Virulent Isolate of *Streptococcus pneumoniae*

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The 2,160,837–base pair genome sequence of an isolate of *Streptococcus pneumoniae*, a Gram-positive pathogen that causes pneumonia, bacteremia, meningitis, and otitis media, contains 2236 predicted coding regions; of these, 1440 (64%) were assigned a biological role. Approximately 5% of the genome is composed of insertion sequences that may contribute to genome rearrangements through uptake of foreign DNA. Extracellular enzyme systems for the metabolism of polysaccharides and hexosamines provide a substantial source of carbon and nitrogen for *S. pneumoniae* and also damage host tissues and facilitate colonization. A motif identified within the signal peptide of proteins is potentially involved in targeting these proteins to the cell surface of low–guanine/cytosine (GC) Gram-positive species. Several surface-exposed proteins that may serve as potential vaccine candidates were identified. Comparative genome hybridization with DNA arrays revealed strain differences in *S. pneumoniae* that could contribute to differences in virulence and antigenicity.

Streptococcus pneumoniae (pneumococcus) has played a pivotal role in the fields of genetics and microbiology. The pioneering studies of Avery, MacLeod, and McCarty in 1944 (*1*) demonstrated that DNA is the true hereditary material by transforming a noncapsulated, avirulent *S. pneumoniae* strain with DNA from a capsulated virulent strain. This work highlighted the importance of the bacterial polysaccharide capsule as a key pathogenicity factor.

As a human pathogen, *S. pneumoniae* is the most common bacterial cause of acute respiratory infection and otitis media and is estimated to result in over 3 million deaths in children every year worldwide from pneumonia, bacteremia, or meningitis (*2*). Even more deaths occur among elderly people, among whom *S. pneumoniae* is the leading cause of community-acquired pneumonia and meningitis (*3*). Since 1990, the number of penicillin-resistant strains has increased from 1 to 5% to 25 to 80% of isolates, and many strains are now resistant to commonly prescribed antibiotics such as penicillin, macrolides, and fluoroquinolones (*4*).

The complete genome sequence of a capsular serotype 4 isolate of *S. pneumoniae* [designated TIGR4 (*5*); TIGR indicates The Institute for Genomic Research] was determined by the random shotgun sequencing strategy (*6*) (GenBank accession number AE005672; see www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl). This clinical isolate was taken from the blood of a 30-year-old male patient in Kongsvinger, Norway, and is highly invasive and virulent in a mouse model of infection (*7*).

The genome consists of a single circular chromosome of 2,160,837 base pairs (bp) with a G + C content of 39.7%. Base pair 1 of the chromosome was assigned within the putative origin of replication. Of the 2236 genes identified (*8*), 1155 are located on the right of the

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