Motor Neuron Degeneration in Mice That Express a Human Cu,Zn Superoxide Dismutase Mutation

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Mutations of human Cu,Zn superoxide dismutase (SOD) are found in about 20 percent of patients with familial amyotrophic lateral sclerosis (ALS). Expression of high levels of human SOD containing a substitution of glycine to alanine at position 93—a change that has little effect on enzyme activity—caused motor neuron disease in transgenic mice. The mice became paralyzed in one or more limbs as a result of motor neuron loss from the spinal cord and died by 5 to 6 months of age. The results show that dominant, gain-of-function mutations in SOD contribute to the pathogenesis of familial ALS.

Amyotrophic lateral sclerosis occurs in both sporadic and familial forms and results from the degeneration of motor neurons in the cortex, brainstem, and spinal cord. The disease typically begins in adults as an asymmetric weakness in two or more limbs and then progresses to complete paralysis (*1*). Familial ALS is inherited as an autosomal dominant trait (*2*). About 10% of ALS cases are familial and, of these, ~20% have mutations in Cu,Zn superoxide dismutase (SOD) (*3*–*5*). SOD catalyzes the dismutation of superoxide radical ($O_2^{\cdot -}$) into hydrogen peroxide and molecular oxygen. Familial ALS patients heterozygous for SOD mutations have 50 to 60% of the normal level of SOD activity in their red blood cells and brains (*4*, *6*).

To explore how mutations in SOD might selectively cause motor neuron degeneration, we produced transgenic mice that express wild-type or mutant forms of human SOD (*7*, *8*). Two mutations were analyzed: an $Ala^4 \rightarrow Val$ substitution (A4V) and a $Gly^{93} \rightarrow Ala$ substitution (G93A) (*3*, *4*). Previously described mice that express wild-type human SOD (NSOD) show no signs of overt motor neuron disease but do have mild pathologic changes in the innervation of muscle that are suggestive of premature aging (*8*, *9*).

Transgenic founder mice were produced by DNX (Princeton, New Jersey) or through the National Transgenic Development Facility (National Institutes of Health). Fertilized eggs for injection were obtained from crosses of (C57BL6 × SJL) F_1 hybrid mice. Founder mice were bred with C57BL6 mice, and their progeny were used for subsequent analysis (*10*). Transgenic mice were identified by polymerase chain reaction amplification of tail DNA (*11*) and were screened for expression of human SOD in red blood cells by an antigen capture enzyme immunoassay (EIA) that used a polyclonal antibody to human SOD and the mouse monoclonal antibody SD-G6 (*12*). The EIA detected human SOD in G93A and NSOD mice, but not in A4V mice. However, Northern (RNA) analysis (*13*) and immunoblots (*14*) developed with a different mouse monoclonal antibody (CZSODF2) demonstrated expression of human *SOD1* mRNA and protein in the brains of G93A, NSOD, and A4V mice (Fig. 1, A to C). Thus, the A4V mutation altered an epitope needed for recognition in the EIA.

The mutations of SOD found in familial ALS alter the stability of human SOD as shown by DNA transfection of cultured cells (*15*). Consistent with those results, we found that the mutant transgenic lines expressed only one-half as much human SOD as did NSOD mice expressing comparable amounts of mRNA (Table 1). In addition, we found that the G93A mutation had little discernible effect on human SOD activity, whereas the A4V mutation greatly reduced enzymatic activity (*15*, *16*). Although we detected enzymatically active mouse-human dimers in NSOD and G93A transgenic mice on SOD activity gels (*17*), we did not detect any active mouse-human A4V dimers. These results are compatible with the finding that recombinant human SOD bearing an $Ala^4 \rightarrow Gln$ substitution is enzymatically inactive (*18*).

Mice from one of the G93A transgenic lines (G1) (Table 1) that expressed the largest amounts of mutant SOD in the brain

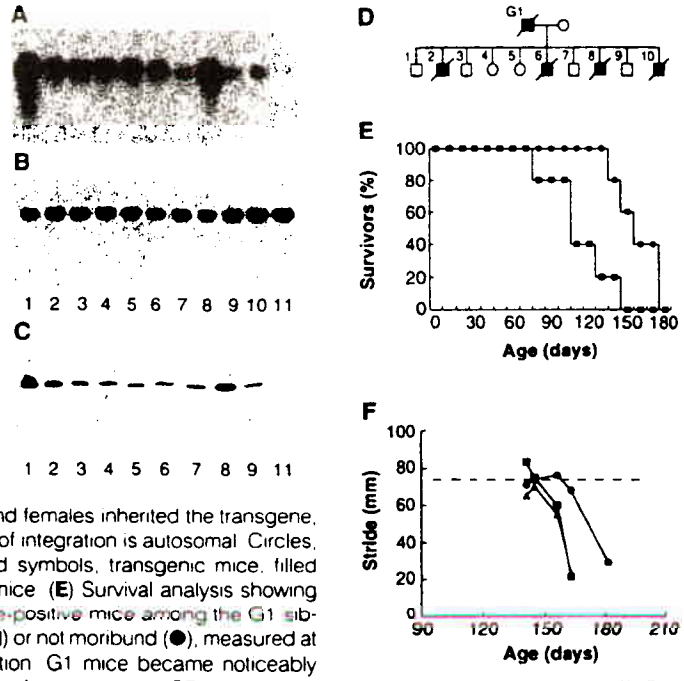

Fig. 1. (**A**) Northern analysis of human *SOD1* mRNA expression in transgenic mouse brain. (**B**) The same membrane hybridized with a probe for G3PDH. (**C**) Expression of human SOD in transgenic brain by immunoblotting. Lanes contain samples from the following mice: 1, G1; 2, G5; 3, G12.199; 4, G20; 5, A1073; 6, A1074; 7, N1026; 8, N1029; 9, N1030; 10, G12.15; and 11, nontransgenic littermate. (**D**) Partial pedigree of the G1 transgenic line. In the F_2 generation, both males and females inherited the transgene, which indicates that the site of integration is autosomal. Circles, female; squares, male; filled symbols, transgenic mice; filled symbols with bar, affected mice. (**E**) Survival analysis showing the percentage of transgene-positive mice among the G1 siblings that are not impaired (■) or not moribund (●), measured at 10-day intervals of observation. G1 mice became noticeably impaired by 121 ± 23 days of age (mean ± SD, n = 5) and moribund by 169 ± 16 days. (**F**) The condition of G1 transgenic mice deteriorated rapidly over the 2-week period before their death, as shown by the shortening of their stride (■, G1.2; ●, G1.6; ▲, G1.8; and dotted line, average stride of normal male mice).

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developed a stereotyped syndrome suggestive of motor neuron disease. The disease has not been observed in any line of NSOD mice expressing wild-type human SOD, nor have symptoms developed in any A4V mouse at comparable ages. At 3 to 4 months of age, G1 mice began to show signs of hind limb weakness (Fig. 1E). They extended their hind legs less than normal when lifted by the base of the tail, their coats developed a coarse appearance suggestive of impaired grooming, and they appeared thin along their flanks. Normal mice have a fairly constant stride of 74 ± 1.6 mm (95% confidence interval, n = 50 mice) when using an alternating gait (*19*). G1 mice had a normal stride at 3 to 4 months of age, but by 5 months of age it deteriorated rapidly (Fig. 1F). Over a span of 2 weeks, the mice became paralyzed in one or more limbs. The founder mouse and four of five transgenic F_1 progeny developed paralysis of one or more hind limbs. A fifth transgenic F_1 mouse (G1.6) retained use of his hind limbs but developed complete paralysis of his right forelimb. The six nontransgenic littermates of these mice showed no signs of disease. All affected mice developed a tremor of the hind limbs when suspended in the air. They had a normal posture when quiet with the hind limbs held in flexion, but after initiating movement, their hind limbs and toes frequently locked in a hyperextended position. Affected mice became moribund by 5 months of age and were killed when they were no longer able to forage for food or water.

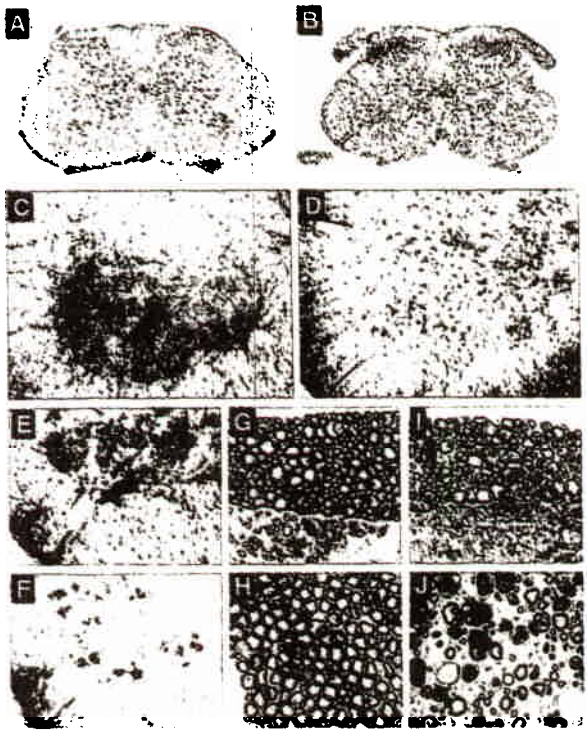

Fig. 2. Loss of spinal motor neurons in affected G1 transgenic mice. Spinal cords from a normal littermate (**A**) and a G1 transgenic mouse (**B**) show loss in the latter of lateral motor columns in the L4 spinal segment (cresylecht-violet stain). (**C** and **D**) Spinal cords from a normal littermate (C) and a G1 transgenic mouse (D), showing loss in the latter of ChAT-positive ventral horn motor neurons in the L3 spinal segment (*27*). Lumbar spinal cords from an N1026 mouse (**E**) and a normal littermate (**F**) show staining of ventral horn motor neurons with an antibody to human SOD (CZ-SODF2). (**G** through **J**) Normal littermate dorsal (G) and ventral (H) lumbar spinal roots and G1 transgenic dorsal (I) and ventral (J) lumbar roots (stained with toluidine blue). The dorsal sensory roots were relatively spared (I), whereas severe loss of myelinated axons, myelin debris, and infiltrating phagocytic cells were apparent in the ventral motor roots (J).

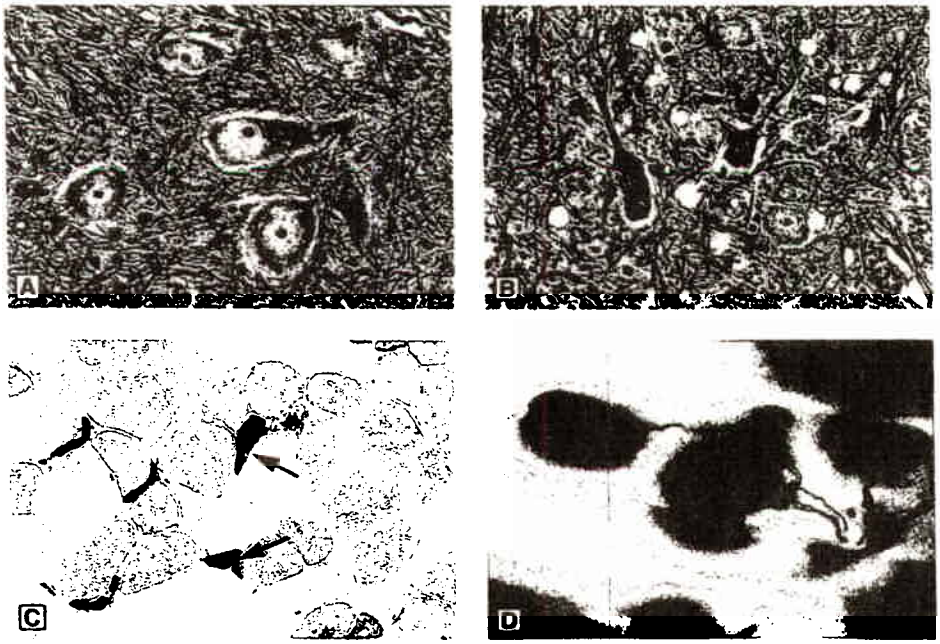

Fig. 3. Pathology in spinal cord and muscle of transgenic G1 mice. (**A** and **B**) Lumbar spinal segments from a normal littermate (A) and a G1 transgenic mouse (B), stained by the Bielschowski technique to reveal neurofibrils. (**C**) Nonspecific esterase stain of gastrocnemius, showing the low frequency of denervated, angulated muscle fibers (arrows) in G1 mice. (**D**) Sprouting and reinnervation of three denervated endplates in the gluteus muscle of a G1 mouse, revealed by a combined silver and cholinesterase stain (*28*).

The founder of the G1 line, all of his transgenic F_1 progeny, and at least one male F_2 mouse developed the same stereotyped syndrome suggestive of motor neuron disease affecting both upper and lower motor neurons. The other lines of G93A transgenic mice (Table 1) expressed smaller amounts of the mutant protein and so far have had normal motor behavior. In G1 mice as well as in humans with ALS (*2*), the onset of the disease is dependent on age, so it is conceivable that the other lines of G93A mice may develop the disease at a later age. However, because the disease is expressed in only one line of mice, we cannot exclude the possibility that the site of integration of the transgene caused the disease syndrome in these mice. Disease is not due simply to overexpression of SOD in the brains of G1 mice, because NSOD mice that express comparable or greater amounts of total brain SOD do not develop the disease (*10*) (Table 1).

Pathological analysis of G1 mice demonstrated a severe loss of choline acetyltransferase (ChAT)–containing spinal motor neurons (Fig. 2, A to D). A few motor neurons appeared normal, but most of the remaining neurons were filled with a neurofibrillar material (Fig. 3) that appeared to be phosphorylated neurofilaments (*20*). The most pronounced changes were observed in the ventral spinal cord, whereas the dorsal spinal cord, especially the substantia gelatinosa, was better preserved. Immunohistochemical staining revealed large amounts of human SOD in ventral horn motor neurons, best shown in NSOD mice (Fig. 2, E and F). In G1 mice, there was severe loss of large, myelinated axons from the ventral motor roots (Fig. 2, H and

Table 1. Expression in brain of human *SOD1* mRNA, human SOD protein, and total SOD enzymatic activity in different transgenic mouse lines. All values are the mean ± SEM (n = 3), except where indicated.

Line	Mutation	Gene copy number*	*SOD1* mRNA (ng)/10 μg of total RNA	Human SOD (ng)/total protein (μg)†	SOD (U)/total protein (μg)
G1	$Gly^{93} \rightarrow Ala$	18.0 ± 2.6	2.5 ± 0.5	4.1 ± 0.54	42.6 ± 2.1
G5		4.0 ± 0.6	0.8 ± 0.1	1.3 ± 0.21	27.0 ± 2.9
G12		2.2 ± 0.8	0.8 ± 0.1	1.1 ± 0.22	19.5 ± 0.8
G20		1.7 ± 0.6	0.8 ± 0.1	0.7 ± 0.06	16.9 ± 0.4
A1073	$Ala^{4} \rightarrow Val$	4.7 ± 0.4	1.1 ± 0.1	1.0 ± 0.21‡	14.6 ± 0.4
A1074		3.2 ± 0.2	0.7 ± 0.1	0.9 ± 0.21‡	9.1 ± 0.4
N1029	Wild-type	7.2 ± 2.4	1.5 ± 0.1	6.7 ± 0.76	37.3 ± 1.9
N1026		3.3 ± 1.0	0.4 ± 0.1	0.9 ± 0.11	18.6 ± 0.9
N1030		1.7 ± 0.7	0.3 ± 0.1	0.6 ± 0.16	11.8 ± 0.3
Nontransgenic		–	–	–	10.4 ± 0.5

*Per diploid genome. †The amount of human SOD was determined by EIA. ‡Determined by immunoblotting (mean ± SEM of regression).

J). The dorsal sensory roots appeared relatively spared when compared to the ventral roots; however, scattered swollen axons with dense axoplasm and occasional myelin-laden macrophages were observed at all levels of the spinal cord (Fig. 2, G and I). These changes extended into the central component of the afferent sensory fibers within the dorsal columns of the spinal cord, a pathology also seen in familial ALS (*21*). Severe loss of myelinated axons occurred in intramuscular nerves, but less than 10% of muscle fibers had the characteristics of denervated fibers—that is, a small, angular profile and an esterase-positive phenotype (Fig. 3C).

To investigate whether sprouting and reinnervation compensated for the destruction of motor units caused by the disease, we examined whole mounts of the gluteus muscle of a G1 mouse (Fig. 3D). The muscles showed severe loss of myelinated axons from the intramuscular nerves and consequent reinnervation of muscle fibers by primarily nodal sprouts. Ongoing reinnervation and remodeling of muscle innervation were indicated by the frequency of multiply innervated endplates and by the scarcity of denervated endplates. In one gluteus muscle, two surviving axons in the inferior gluteal nerve appeared sufficient to innervate more than 90% of the myofibers in the muscle. These data suggest that sprouting probably compensates for the loss of motor neurons until late in the course of the disease.

Toxicity by a free-radical mechanism is one plausible explanation for motor neuron death in the G1 transgenic mice and, by implication, in humans with familial ALS. This mechanism could involve the formation of the strong oxidant peroxynitrite ($ONOO^-$) from $O_2^{\cdot -}$ and nitric oxide ($NO\cdot$) free radicals (*22*, *23*). The formation of peroxynitrite and its decomposition into toxic chemical species have been linked to neurotoxicity in cell culture (*24*) and in brain ischemia (*25*). SOD mutations may facilitate this pathway of oxidative damage (*26*). Because formation of peroxynitrite is a second-order reaction that depends on the concentration of $O_2^{\cdot -}$ and $NO\cdot$, decreased SOD activity in familial ALS may also contribute to pathogenesis if the amount of $O_2^{\cdot -}$ in tissues is increased (*4*). Our results indicate that dominant, gain-of-function mutations in SOD play a key role in the pathogenesis of familial ALS.

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7. The A4V mutation was introduced into exon 1 of the human *SOD1* gene by two-primer mutagenesis with the polymerase chain reaction (PCR), the template for mutagenesis was a Sty I–Stu I fragment encompassing exon 1. The G93A mutation was cloned in a Hind III and Nsi I fragment encompassing exon 4 that was amplified from the genomic DNA of family 3-192 (*3*). These fragments were used to reassemble a complete 14.5-kb Eco RI–Bam HI fragment of the *SOD1* gene [R. A. Hallewell, J. P. Puma, G. T. Mullenbach, R. C. Najarian, in *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine*, G. Rotilio, Ed. (Elsevier, New York, 1986), pp. 249–256] in two more steps. Exons 1 and 4 of the transgenes were sequenced to verify that they contained only the desired mutation. The 14.5-kb Eco RI–Bam HI *SOD1* transgene directs tissue-specific expression of human SOD in mice under control of the endogenous human promoter (*8*).
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10. Mice were housed in microisolator cages within a barrier facility. Frequent monitoring revealed no evidence for infection by viral or bacterial pathogens.
11. The primers described (*3*) were used for identification of transgenic mice by PCR. Transgene copy number was estimated by Southern (DNA) DNA hybridization. Denatured DNA (10 μg) isolated from mouse tails or human placenta was transferred to a nitrocellulose membrane together with a dilution series of the cloned *SOD1* gene. The membrane was hybridized with a random-primed, ^{32}P-labeled probe to sequences within the 3′ untranslated region of the 0.9-kb human *SOD1* complementary DNA (cDNA); these sequences are specific to the human transgene. Bound radioactivity was quantitated by phosphor image analysis, and linear regression was used to calculate transgene copy number.
12. The EIA was constructed with a goat immunoglobulin G (IgG) antibody to human SOD (Chiron, Emeryville, CA) and a mouse monoclonal antibody designated SD-G6 (Sigma, St. Louis, MO). Recombinant human SOD (Chiron) was used as a standard. Samples were diluted to within the log-linear range of the assay (0.1 to 1.5 ng of human SOD per well). There was no cross-reactivity with mouse SOD.
13. Northern RNA hybridization was performed with 10 μg of total brain RNA. The membrane was hybridized with a random-primed, ^{32}P-labeled probe specific for the 3′ untranslated region of the human *SOD1* cDNA. Quantitation standards (a 0.9-kb sense human *SOD1* cDNA) were loaded on the gel with 10 μg of yeast RNA as a carrier, and the hybridization signal was analyzed by phosphor image analysis. To control for RNA loading variations we rehybridized the blot with a glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA probe.
14. Samples containing 2 μg of soluble brain protein were subjected to electrophoresis through 10% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, and probed with antibody CZSODF2. Bound antibody was detected with a biotinylated horse antibody to mouse IgG and a Vector ABC kit. The membrane was developed with an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL), and the chemiluminescence was quantitated by film densitometry. The amount of human SOD in brain extracts of A4V transgenic mice was determined by comparison to recombinant SOD standards in adjacent lanes.
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16. Mouse brains were homogenized in cold 10 mM tris HCl (pH 7.5) and 10 mM β-mercaptoethanol. After centrifugation at 50,000*g* for 15 min at 4°C, the protein content of the supernatant was measured by a bicinchoninic acid assay (Pierce, Rockford, IL). We assayed total SOD activity within brain extracts in microwells by measuring the inhibition of nitroblue tetrazolium reduction [D. R. Spitz and L. W. Oberley, *Anal. Biochem.* **179**, 8 (1989)]. Wells were monitored kinetically, and a $(V_{max}/V) - 1$ transform (where V is velocity) [K. Asada, M. Takahashi, M. Nagate, *Agric. Biol. Chem.* **38**, 471 (1974)] was used to linearize the data. Recombinant human SOD had an activity of 6 U per nanogram. The contribution of Mn SOD in the sample was determined in the presence of 5 mM sodium cyanide and was ~2% of the total SOD activity in the brain extract.
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19. Mice were trained to walk up a 75-cm, U-shaped ramp that was inclined at one end against the wire lid of their cage. Testing was performed in a horizontal, laminar flow hood to maintain barrier conditions. A bright lamp was placed at the base of the ramp, and the cage lid was left in semidarkness. The ramp obscured each mouse's view of the laminar flow hood and surrounding room. Testing was initiated by allowing the mice 1 to 2 min to explore the cage lid and the top of the

ramp. The hind feet of the mice were painted with children's poster paints of contrasting colors. The tracks left by the mice as they ran up the ramp were recorded on paper tape.
20 Degenerating neurons were positive for immunohistochemical staining with SMI-31 monoclonal antibody (Sternberger Monoclonal Antibodies, Baltimore, MD) to phosphorylated neurofilaments, although the small number of motor neurons remaining in affected spinal cords and their marked pathology require confirmation of this result.
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Molecular Genetic Analyses of the Tyrolean Ice Man

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An approximately 5000-year-old mummified human body was recently found in the Tyrolean Alps. The DNA from tissue samples of this Late Neolithic individual, the so-called "Ice Man," has been extracted and analyzed. The number of DNA molecules surviving in the tissue was on the order of 10 genome equivalents per gram of tissue, which meant that only multi-copy sequences could be analyzed. The degradation of the DNA made the enzymatic amplification of mitochondrial DNA fragments of more than 100 to 200 base pairs difficult. One DNA sequence of a hypervariable segment of the mitochondrial control region was determined independently in two different laboratories from internal samples of the body. This sequence showed that the mitochondrial type of the Ice Man fits into the genetic variation of contemporary Europeans and that it was most closely related to mitochondrial types determined from central and northern European populations.

In September 1991, the frozen mummy of a man was found in the Tyrolean Alps. Radiocarbon dates of skin and bone samples indicated an age between 5100 and 5300 years (*1*). Because no comparable archaeological discovery exists, this find has attracted considerable scientific and public interest. It has also been the subject of various rumors and even allegations of fraud (*2*). Molecular genetic investigations of the Ice Man could address some of the questions surrounding the find. Comparisons of DNA sequences from the body with contemporary populations may reveal aspects of his ethnic affiliation. Molecular studies of other organisms such as viruses or bacteria associated with the body may furthermore illuminate the evolution of these organisms. As a first step toward such investigations, we have analyzed the state of preservation of the DNA in the Ice Man and determined the sequence of a hypervariable segment of the mitochondrial control region from numerous samples removed from the body.

Ancient DNA has been retrieved from a variety of plant, animal, and human remains (*3*, *4*) that go back a few tens of thousands of years as well as from some fossils that are millions of years old (*5*–*7*), although the latter results are partially controversial (*8*). In most cases, work on archaeological DNA has been limited to mitochondrial DNA because its high copy number increases the chance of survival of a few molecules in the face of molecular damage that accumulates post mortem. Because the body of the Ice Man has been frozen with the exception of a short period after its discovery, its DNA may be preserved better than that of other finds. This unusual condition might allow nuclear markers such as microsatellites to be studied in addition to mitochondrial DNA and thus open several additional avenues of study.

A total of eight samples of muscle, connective tissue, and bone were removed under sterile conditions from the left hip region of the body, which had been damaged during salvage of the mummy. Additionally, parts of one sample that has been radiocarbon dated (*1*) were analyzed. Extracts of DNA were made from 10 to 200 mg of each sample by a silica-based method that is highly efficient in the retrieval of ancient DNA (*9*). Enzymatic amplifications from the mitochondrial control region were attempted. Because this region encodes no structural gene products and evolves faster than other parts of the mitochondrial genome, it is particularly suited for the reconstruction of the history of human popula-

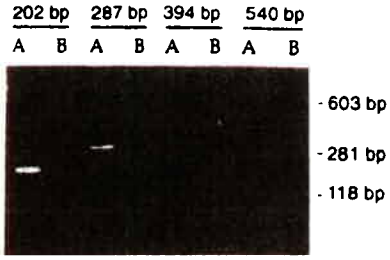

Fig. 1. Agarose gel electrophoresis of mitochondrial DNA amplification of different lengths from the Ice Man. For every primer pair, amplifications from (**A**) an extract of the Ice Man and (**B**) an extraction control are shown. The primer pairs used are as follows: L16055/H16218 (202 bp), L16055/H16303 (287 bp), L16055/H16410 (394 bp), and L15997/H16498 (540 bp), where L and H refer to the light and heavy strand, respectively, followed by the number to the nucleotide position (*14*) at the 3′ end of the primer. Migration positions of molecular size markers are given in numbers of base pairs.

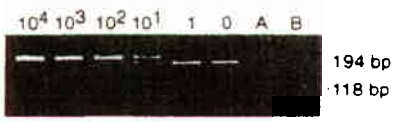

Fig. 2. Quantitation of mitochondrial DNA in an extract from the Ice Man. A dilution series of a competitor template, containing a 20-bp insertion in a mitochondrial fragment, was added to a constant amount of extract, and a PCR that used primers L16068/H16218 was performed as described in (*10*). The numbers above the lanes indicate the numbers of competition molecules added to the amplifications. (**A**) An extraction control and (**B**) a control where no template was added. Migration positions of molecular size markers are given in numbers of base pairs.

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