Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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16. The sequence of the 67-bp PCR product was 5′-GAGAGAAGCTT(T/C)TG(T/C)GG(A/T/G)AC(G/C)-GA(G/A)AA(T/C)CACTCGGCCGCCTA(T/C)(A/C)-G(A/C)GT(A/C/T)GA(C/T)CA(A/G)GGAATTC-TCTCTC-3′. This product was obtained with two degenerate PCR primers, 5′-GAGAGAAGCTT(T/C)-TG(T/C)GG(T/C/G/A)AC(T/C/G/A)GA(A/G)AA(T/C)-CA-3′ and 5′-GAGAGAGAATTCC(T/C)TG(A/G)TC-(T/C/G/A)AC(T/C/G/A)C(G/T)(A/G)TA-3′, with αTC-6 cell randomly primed cDNA as template. A 47-bp oligonucleotide, 5′-TTTTGCGGGACGGAGAATCA-CTCGGCCGCCTACCGCGTCGACCAAGG-3′, was designed based on the center of the 67-bp PCR product and was used to screen λ libraries.
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Mutations in the Sulfonylurea Receptor Gene in Familial Persistent Hyperinsulinemic Hypoglycemia of Infancy

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Familial persistent hyperinsulinemic hypoglycemia of infancy (PHHI), an autosomal recessive disorder characterized by unregulated insulin secretion, is linked to chromosome 11p14-15.1. The newly cloned high-affinity sulfonylurea receptor (*SUR*) gene, a regulator of insulin secretion, was mapped to 11p15.1 by means of fluorescence in situ hybridization. Two separate *SUR* gene splice site mutations, which segregated with disease phenotype, were identified in affected individuals from nine different families. Both mutations resulted in aberrant processing of the RNA sequence and disruption of the putative second nucleotide binding domain of the SUR protein. Abnormal insulin secretion in PHHI appears to be caused by mutations in the *SUR* gene.

PHHI is an autosomal recessive disorder of glucose homeostasis characterized by unregulated secretion of insulin and profound hypoglycemia (*1*). The pathophysiology of this disease remains obscure, but in vitro studies have suggested a defect of glucose-regulated insulin secretion in pancreatic islet β cells (*1, 2*). The *PHHI* gene was assigned to chromosome 11p14-15.1 by linkage analysis (*3, 4*). Candidate genes for this disorder include those involved in the β cell glucose sensing mechanism and in insulin secretion. Localization of *PHHI* to chromosome 11p14-15.1 excluded previously mapped genes involved in β cell function, such as the *glucokinase*, *islet glucose transporter*, and *glucagon-like peptide-1 receptor* loci (*5*). We considered as a candidate the newly cloned high-affinity *SUR* gene, a member of the adenosine triphosphate (ATP)–binding cassette superfamily (*6*). The SUR protein is a putative subunit of the β cell ATP-sensitive potassium channel (K$_{ATP}$), a modulator of insulin secretion (*7*).

We used the fluorescence in situ hybridization (FISH) technique to localize the *SUR* gene on chromosome 11. Partial complementary DNA (cDNA) clones that constituted 3.8 kb of the coding sequence of the human homolog of *SUR* were obtained (*8*) and were used for the analysis. A specific hybridization signal was detected in 85% of metaphases at band 11p15.1 on both chromatids of the two chromosomes 11. No other hybridization sites were detected.

Genomic DNA samples from affected individuals from three families were analyzed by Southern (DNA) blot; human cDNA (approximately four-fifths of full length) was used as a probe. Because the restriction fragment patterns of affected and unaffected samples appeared to be similar, major insertions or deletions of the *SUR* locus were unlikely to have occurred in the affected individuals (*9*). We then used direct sequence analysis to screen for small deletions, insertions, or missense mutations. The first region evaluated was the putative second nucleotide binding fold (NBF-2) of the human *SUR* homolog (Fig. 1), which is the most highly conserved region of the *SUR* gene (*6*). In other superfamily members, NBF-1 and NBF-2 have functional importance in the control of channel activity through their interaction with cytosolic nucleotides (*10, 11*). In cystic fibrosis, an autosomal recessive disease caused by mutations in another ATP-binding cassette member [the cystic fibrosis transmembrane conductance regulator (CFTR)], the more frequent and severe disease alleles are located in the regions of the two NBFs (*12*).

Mutational analysis was done on samples from 16 affected progeny of nine consanguineous matings (*13*). Because of the consanguineous matings and the autosomal recessive inheritance pattern of PHHI, affected individuals were expected to be homozygous by descent at the disease gene locus (*14*). Sequencing of a cloned pancreatic cDNA product, isolated from an

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affected child of family 6, revealed a 109–base pair (bp) deletion within the NBF-2 region of *SUR*, which corresponded to skipping of exon χ (Fig. 2, A and B). Such an event causes disruption of the NBF-2 consensus sequence and leads to premature truncation of the SUR protein as the result of production of a frame shift and inclusion of a stop 24 codons later. The splice sites of the skipped exon were evaluated at the genomic DNA level. A homozygous G to A point mutation was found at the 3′ end of the skipped exon (Fig. 2C). In other disorders, G to A point mutations at this position have been observed to result in skipping of the exon containing the mutation (*15*). A recognition site for the restriction endonuclease Msp I is destroyed by this base change, which provides a means to confirm and test for the presence of the mutation. Both affected children of family 6 were homozygous, whereas the parents and two unaffected siblings were heterozygous for the mutation (Fig. 2D). Preliminary semiquantitative analysis did not reveal *SUR* mRNA in the pancreatic tissue sample from the affected child of family 6; because this transcript was detected in normal pancreatic tissue samples under the same conditions, the mutant mRNA may be unstable (9).

Twelve other affected children (from six families of Saudi Arabian origin and one family of German origin) were homozygous for the G to A point mutation, as demonstrated by loss of the Msp I recognition site. In all families, homozygous loss of the Msp I site cosegregated with disease phenotype, and in families 1, 2, 3, and 5, the results of genotype analysis for this mutation were in agreement with the haplotype data (*4*). Direct sequencing of polymerase chain reaction (PCR)–amplified genomic DNA from a representative affected member of each family confirmed

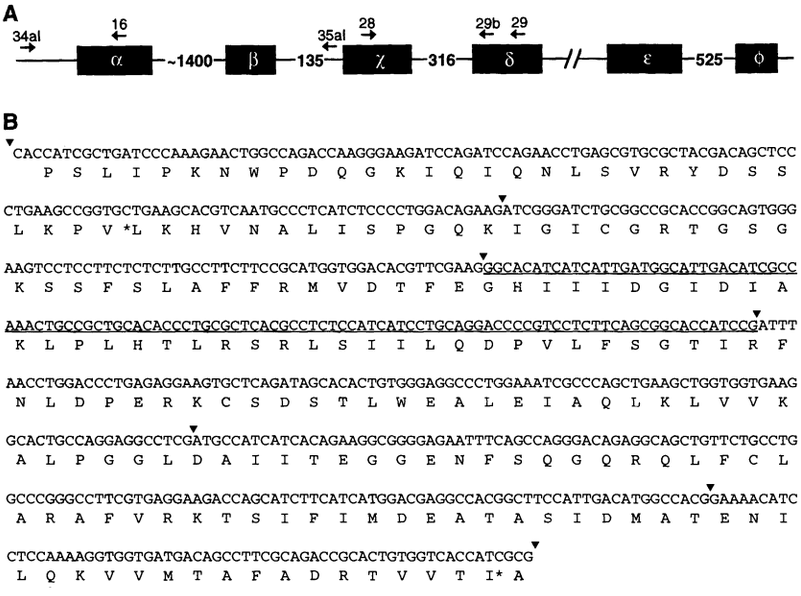

Fig. 1. Genomic organization and cDNA sequence of the NBF-2 region of the human *SUR* homolog. (**A**) Genomic organization of the exons containing the NBF-2 region of the human *SUR* homolog (*24*). Solid rectangles represent exons, which are arbitrarily labeled α through φ for identification. The numbers between rectangles represent intronic sizes. Arrows indicate the relative positions of the primers used in mutational analysis (*23*). (**B**) Nucleotide sequence and translation of the exons containing the NBF-2 region of the human *SUR* homolog. The NBF-2 region is denoted by asterisks; downward-pointing triangles indicate exon-intron boundaries. The χ exonic sequence is underlined. Abbreviations for the amino acid residues below each line are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

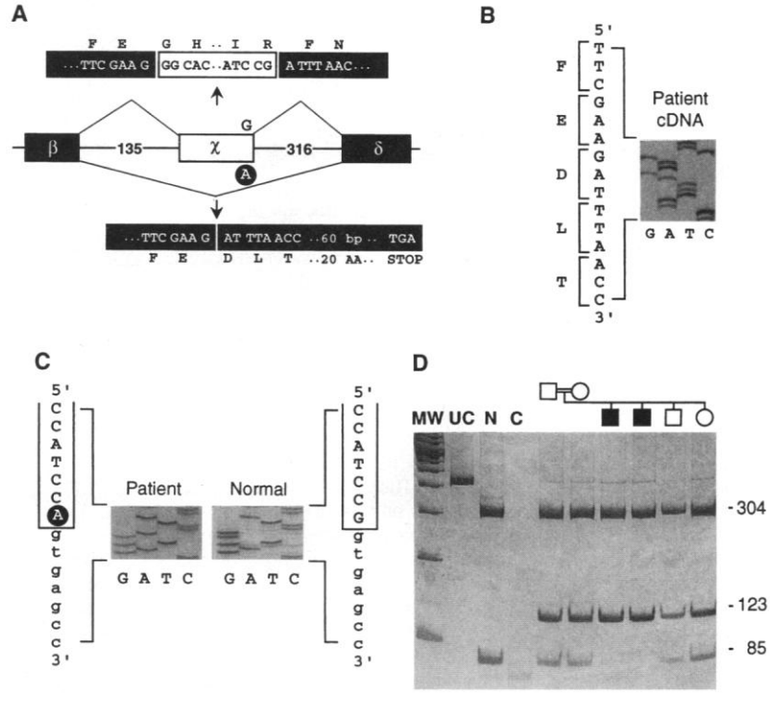

Fig. 2. Mutation of an exon in the *SUR* sequence encoding NBF-2. (**A**) Schematic representation of NBF-2 exons β, χ, and δ, illustrating the normal (upper) and mutant (lower) RNA splicing patterns and predicted cDNA sequences. The amino acids (abbreviated as in Fig. 1) provide the reading frame. The base within the solid circle denotes a point mutation. (**B**) Sequence of cDNA identified as the predominant mRNA in the pancreas of an affected child of family 6 (*25*). (**C**) Sequence of genomic DNA from the same patient. Comparison with normal genomic DNA revealed a G to A point mutation at the 3′ end of the skipped exon. Exon and intron sequences are shown as capital and lowercase letters, respectively. (**D**) Msp I restriction enzyme analysis of PCR-amplified genomic DNA from members of family 6. Solid symbols indicate affected individuals (squares denote males). The normal PCR product was digested into 304-, 85-, and 38-bp fragments, whereas that containing the mutation was digested into 304- and 123-bp fragments. The 38-bp fragment was run off the bottom of this gel. MW, molecular weight markers from a 100-bp ladder (Gibco BRL); UC, uncut sample; N, normal sample from an unrelated person without the disease; and C, control PCR reaction lacking template.

that all exhibited the homozygous G to A mutation.

Two of the three children of family 4 had a mutation in the 3′ splice site sequence preceding the NBF-2 region (Fig. 3A). This G to A mutation destroyed an Nci I restriction endonuclease recognition site, and homozygous loss of this site cosegregated with disease phenotype within the family. Again, genotype analysis of the members of this family supported the haplotype data (*4*); both parents were heterozygous for the mutation and the unaffected sibling was homozygous for the wild-type allele (Fig. 3B). Because a pancreatic tissue sample from an affected child of family 4 was unavailable and we were unable to recover the *SUR* transcript from transformed lymphocytes, a chimeric construct was created to examine the effects of this mutation on RNA splicing pathways (Fig. 3C) (*16*, *17*). With the construct containing the mutation, three cryptic 3′ splice sites occurred in place of the wild-type splicing pattern. These three cryptic splice sites resulted in a 7-bp addition, a 20-bp deletion, or a 30-bp deletion in exon α of the NBF-2 region (Fig. 3D). In the autosomal recessive disorder 21-hydroxylase deficiency, a similar intronic 3′ splice acceptor mutation also altered the splicing pattern, produced several cryptic splice products, and abolished normal protein activity (*18*). All PCR products prepared from the genomic DNA of 125 normal, unrelated individuals showed normal Msp I and Nci I restriction patterns; this finding indicates that neither mutation is a common polymorphism (*19*).

Our results imply that the loss-of-function mutations in the NBF-2 region of the *SUR* gene are a cause of familial PHHI. The SUR protein would therefore appear to have a central role in the regulation of insulin secretion. According to current models of insulin secretion, increases in the intracellular ATP/adenosine diphosphate (ADP) ratio inhibit K_{ATP} channels and lead to β cell membrane depolarization, the opening of voltage-gated calcium channels, and, ultimately, an increase in the exocytosis of insulin (*20*). Although it remains unclear whether SUR has intrinsic channel activity (*6*), it is believed to be closely associated with K_{ATP} channels in β cell membranes (*21*). We postulate that mutations of the *SUR* gene that disrupt NBF-2, or lead to the production of little or no full-length protein, result in inappropriately low K_{ATP} channel activity and excessive insulin secretion. The role of SUR in other disorders of insulin secretion, including some forms of diabetes mellitus, remains to be determined.

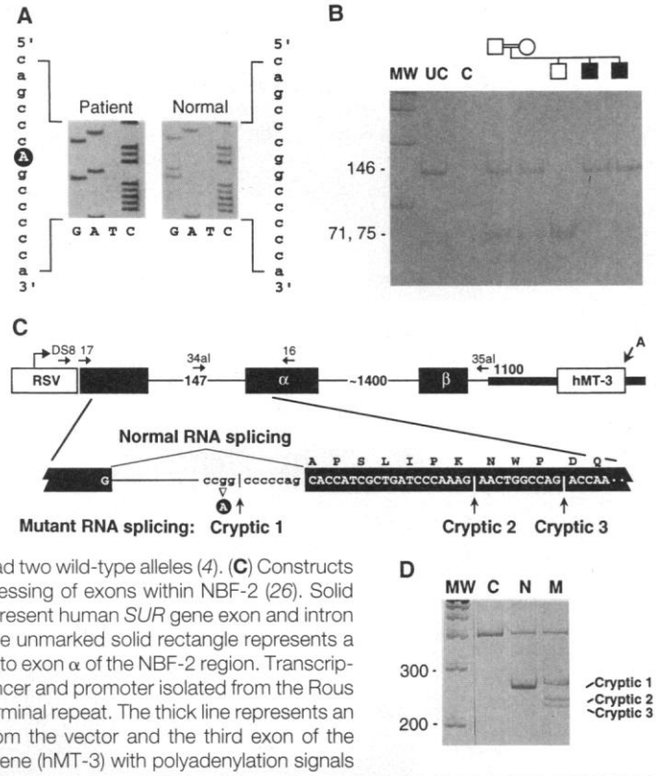

Fig. 3. A mutation in the intron preceding NBF-2 exon α activates cryptic 3′ splice site usage. (**A**) Sequence of genomic DNA from an affected member of family 4. Comparison with normal genomic DNA revealed a G to A mutation in the splice site preceding the first NBF-2 exon. (**B**) Nci I restriction enzyme analysis of genomic DNA from members of family 4. Solid symbols indicate affected individuals. The normal PCR product was digested into 71- and 75-bp fragments, whereas that containing the mutant sequence was not cut. The unaffected sibling in this family had two wild-type alleles (*4*). (**C**) Constructs used to examine RNA processing of exons within NBF-2 (*26*). Solid rectangles and thin lines represent human *SUR* gene exon and intron sequences, respectively. The unmarked solid rectangle represents a portion of the exon that is 5′ to exon α of the NBF-2 region. Transcription was driven by the enhancer and promoter isolated from the Rous sarcoma virus (RSV) long terminal repeat. The thick line represents an intron sequence derived from the vector and the third exon of the human metallothionine IIA gene (hMT-3) with polyadenylation signals (A, polyadenylation site). Normal and mutant RNA splicing patterns, including the locations of the three cryptic splice sites, are diagrammed in the lower portion. The open triangle marks the position of the mutated base within the splice site. Arrows indicate the relative positions of the primers (*23*). (**D**) PCR amplification across the splice sites of normal (N) and mutant (M) cDNA sequences isolated 48 hours after transfection with the splicing constructs; C, control cDNA amplified from untransfected cells. Subcloning and sequencing of these products revealed their identity as diagrammed in (C).

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8. The partial cDNA clones were obtained as follows: A human pancreatic islet cDNA λ phage library (provided by G. Bell, University of Chicago) was screened with a 1.2-kb hamster cDNA probe, which spans the *SUR* NBF-2 sequence. Hybridizations and washes were done at reduced stringency (55°C) with standard methods (*22*). Subsequent screening was done at higher stringency (65°C), with the human cDNA sequence obtained from the first screen as a probe. Sequencing and production of the predicted translation product for these clones revealed an overall homology of 95% with the predicted translation product of the rat *SUR* cDNA.
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13. In each case, diagnosis of PHHI was based on established criteria (*7*). In six families the parents were first cousins, in two families they were second cousins, and in one family they were more distantly related. Eight families, recruited from the patient population of the Arabian-American Oil Company Hospital Medical Services Organization after institutional approval was received, were of Saudi Arabian origin, and one was of German origin. Members of families 1 through 5, 7, and 8 were included in previous reports (*4*) [P. M. Mathew *et al.*, *Clin. Pediatr.* **27**, 148 (1988)]. Family labels are unchanged from those in (*4*).
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23. The following primers were used in mutational analysis: primer 17, 5′-TCAACTGGATGGTGAGGA; primer 34al, 5′-ACAAGGAGCCTGGGGAT; primer 16, 5′-CACGCTCAGGTTCTGGAT; primer 35al, 5′-TGCA-

TGGGTCCCAGTGA; primer 28, 5′-TGACATCGCCAAACTGC; primer 29b, 5′-TCCTCTCAGGGTCCAGGTTA; primer 29, 5′-TCCTGGCAGTGCCTTCA; primer 22, 5′-ACCATCGACCAGCACATC; and primer DS8, 5′-TTGACCATTCACCACATTGGTGTGC.

24. To obtain the genomic structure of the human *SUR* homolog, we screened a normal human lymphocyte genomic bacteriophage library (provided by M. B. Humphrey, Baylor College of Medicine, Houston, TX) (*22*) with a human partial *SUR* cDNA probe, which encompassed 2470 bp at the 3′ end of the cDNA, including the polyadenylation site. Inserts in the bacteriophage clone λG4 were subcloned into pBluescript II (Stratagene). Exon-intron boundaries were defined by comparison of the nucleotide sequences of the human *SUR* gene and the cDNA; these sequences were obtained by means of the dideoxy chain termination method (Sequenase; U.S. Biochemical, Cleveland, OH).
25. Messenger RNA was directly isolated (Oligotex; Qiagen, Studio City, CA) from a freshly frozen pancreatic tissue sample. Reverse transcription (RT) into cDNA was accomplished with the use of random primers (Invitrogen, San Diego, CA) and Superscript II (Gibco BRL). For cloning of the NBF-2 region, an initial PCR amplification with primers 22 (located 5′ of 17) and 29 (*23*) was followed by a second amplification of a portion of the reaction with primers 17 and 29, as described (*4*). The amplified product was cloned into pCR II vector (Invitrogen) and sequenced as above. For detection of the mutation in genomic fragments, 100 ng of genomic DNA was amplified with the use of primers 28 and 29b as above but in the presence of PCR buffer N (Invitrogen), and was then either directly sequenced [S. Khorana, R. F. Gagel, G. J. Cote, *Nucleic Acids Res.* **22**, 3425 (1994)] or cut with 5 U of Msp I (Gibco BRL) at 37°C for 2 hours and run on a 10% polyacrylamide gel. Products on the polyacrylamide gel were visualized by silver staining.
26. Genomic DNA from affected and normal individuals was PCR-amplified with the use of primers 17 and 35al (*23*). Details of the cloning of these PCR products into pRSVhMT2A (*17*) will be provided on request. Constructs were transfected into the human glioblastoma cell line SNB 19 with the use of Lipofectamine (Gibco BRL). RT-PCR analysis was done with primers DS8 and 16, as described (*17*). The plasmids and their cDNA products were sequenced with primer 34al (*23*). Genomic DNA fragments were PCR-amplified with primers 34al and 16 and digested with Nci I, as described above.
27. We thank A. Shenker for helpful discussions and review of the manuscript; M. J. Haddad and G. Cheriyan (both of Saudi ARAMCO) for the surgical pancreatic specimen and for clinical care of their patients; Saudi ARAMCO for samples from Saudi Arabian patients and their families; E. Huang, Y. Yang, G. Gonzalez, and H. Herrera-Sosa for technical assistance; M. B. Humphrey for helpful discussions; and the participating family members. Supported in part by an M. D. Anderson Cancer Center Clinical Research Grant (P.M.T.), NIH grants DK38146 (R.F.G.) and DK44311 (J.B.), and a Juvenile Diabetes Foundation Career Development Award (L.A.B.). P.M.M. was employed by Saudi ARAMCO during the time of this study.

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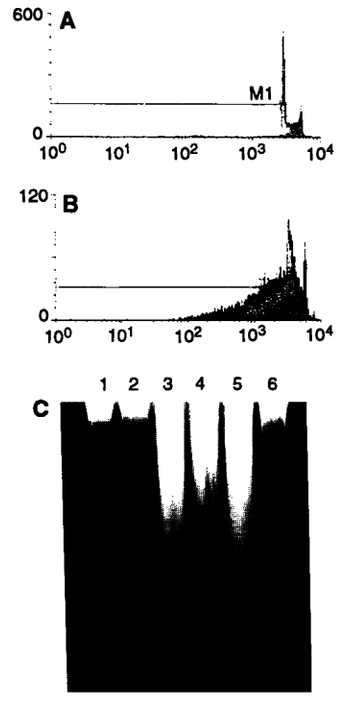

Fig. 1. Apoptosis in Jurkat-Tat cells under low serum conditions. Exponentially growing Jurkat (**A**) and Jurkat-Tat-34 (**B**) cells were shifted from 10% to 0.1% FCS, harvested after 48 hours, and subjected to flow cytometric analysis. Fractions of apoptotic cells in control Jurkat cells and Jurkat-Tat-34 were 9.0 ± 0.7% and 73.7 ± 11.4%, respectively. (**C**) Cellular DNA was extracted and subjected to electrophoresis on a 2% agarose gel to detect nucleosome laddering (*26*). Lane 1, HeLa-Tat; lane 2, HeLa; lane 3, Jurkat-Tat-42; lane 4, Jurkat-Tat-44; lane 5, Jurkat-Tat-34; and lane 6, Jurkat.

Induction of Apoptosis in Uninfected Lymphocytes by HIV-1 Tat Protein

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Infection by human immunodeficiency virus–type 1 (HIV-1) is typified by the progressive depletion of CD4 T lymphocytes and deterioration of immune function in most patients. A central unresolved issue in acquired immunodeficiency syndrome (AIDS) pathogenesis is the mechanism underlying this T cell depletion. HIV-1 Tat protein was shown to induce cell death by apoptosis in a T cell line and in cultured peripheral blood mononuclear cells from uninfected donors. This Tat-induced apoptosis was inhibitable by growth factors and was associated with enhanced activation of cyclin-dependent kinases.

Termination of the long latency of AIDS in patients infected with HIV-1 is marked by depletion of CD4 T cells. The mechanism by which HIV-1 kills immune cells remains unresolved (*1*), although a growing body of evidence points to a role for programmed cell death, or apoptosis (*1*, *2*). Increased apoptosis of lymphocytes has been detected in primate models of pathogenic lentiviral infections but not in HIV-1–infected chimpanzees that do not develop disease (*2*), suggesting that apoptosis is important in AIDS pathogenesis. Enhanced apoptosis has also been observed in lymph nodes of HIV-1–infected patients (*1*) and in lymphocytes isolated from AIDS patients (*3*).

The basis of HIV-1–enhanced apoptosis is not understood (*1*–*3*). Apoptotic signals generated by the virus must also be transmitted to uninfected cells, because massive T cell destruction can occur in HIV-1–infected individuals when only 1 in 1000 to 1 in 10,000 lymphocytes are productively infected with the virus (*4*). The mean production rate of HIV-1 virions in infected individuals was reported to be 1.1×10^8 to 6.8×10^8 per day, which was much less than the mean destruction rate of lymphocytes (1.8×10^9 to 2×10^9 per day) (*5*). Also, after administration of an antiviral drug to infected patients, lymphocyte depletion increased at a time when virion titer was still lower than prior to treatment (*5*). Thus, destruction of the infected cells by cytotoxic T lymphocytes appears to be insufficient to account for the observed death of T cells.

The viral envelope protein, gp120, has been suggested to activate T cells and prime them for apoptosis (*6*), although several studies do not support this model (*7*). We hypothesized that the HIV-1 transactivator protein, Tat, participates in the induction of lymphocyte apoptosis during infection. Several properties of Tat are compatible with this idea: (i) it is secreted by infected cells and has biological activity on uninfected cells (*8*); (ii) it is likely to accumulate locally in lymphoid tissue where HIV-1 replication is active even during clinical latency (*5*); (iii) it appears to affect cellular gene expression and function (*9*); and (iv) its role in HIV-1 pathogenesis extends beyond its transcriptional function (*10*).

Most lymphocytes in vivo are quiescent. We therefore investigated the effect

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