and that the only requirement for function is a properly oriented or clustered set of stem structures. In this context regions of RNA containing the Rex-responsive sequences of HTLV-I and HTLV-II (*19*) also may be folded into multiple stem/loop structures with "per base" folding energies within 10% of the structures predicted for CAR, yet they lack obvious sequence homology with CAR (*7, 20*). A requirement only for clustered stem loops may underlie the observation that Rex of HTLV-I can substitute for Rev of HIV-1 (*21*).

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Differential Effects of *nef* on HIV Replication: Implications for Viral Pathogenesis in the Host

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Stable lymphoid cell lines expressing the human immunodeficiency virus type 1 (HIV-1) *nef* gene product, p27, were established. The presence of p27 in the lymphoid cells suppressed replication of some strains of both HIV-1 and HIV-2. This observation indicates that *nef* could be important in the establishment of HIV latency. In contrast, fast replicating and highly cytopathic HIV-1 isolates recovered from patients with advanced disease states were not affected by the negative effect of *nef* present in these lymphoid cell lines. This lack of response to *nef* appears to constitute another viral feature that correlates with disease progression. Thus, manipulating expression of the *nef* gene in vivo might influence pathogenesis in the host.

THE GENOMES OF THE TWO SUBtypes of the human immunodeficiency virus, HIV-1 and HIV-2, contain genes coding for structural proteins (*gag*, *pol*, and *env*), regulatory sequences in the long terminal repeat (LTR), as well as at least six additional open reading frames. Three of these latter genes code for regulatory proteins (*tat*, *rev*, and *nef*). The *tat* and *rev* genes are positive regulators of HIV replication; in contrast, *nef* appears to act as a negative regulator of HIV replication (*1*). The *nef* gene encodes a protein, p27, which is myristylated and phosphorylated (*2*). The protein has biochemical properties similar to that of a G-binding protein, that is, guanosine triphosphate (GTP)–binding activity, GTPase activity, and autophosphorylation (*2, 3*). Furthermore, expression of *nef* via a vaccinia vector in the CEM cell line downmodulates the CD4 receptor molecule (*2*).

Mutational analysis has shown that mutants defective in expression of the *nef* gene product replicate faster (*4, 5*) and to several-fold higher titers than wild-type virus (*5, 6*). Recent studies indicate that *nef* suppresses HIV transcription by acting on the LTR, probably via an interaction with cellular factors (*6, 7*). We have previously proposed that *nef*, by suppressing HIV expression, could be involved in HIV latency (*5*). To further investigate this activity of the *nef* gene product on HIV replication and to evaluate its potential role in HIV pathogenesis, we constructed two plasmids for the expression of *nef* from HIV-1_{SF2} [formerly designated AIDS (acquired immunodeficiency syndrome)-associated retrovirus, ARV-2] (*8*). Its gene product has been shown in vitro to have GTP-binding and kinase activities (*3*). Expression of *nef* in plasmid p3R is under the control of the HIV-1–LTR, which contains sequences responsive to both viral (for example, *tat*) and cellular transcriptional activators (*1*). In plasmid pAM3, the expression of *nef* is under the control of the SV40 early promoter (*9–14*). In this latter plasmid, in contrast to the p3R construct that is autoregulated, the *nef* protein is made constitutively. Hu-

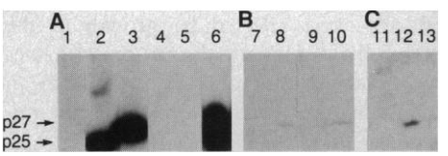

Fig. 1. Identification of cells expressing *nef*: 4×10^6 cells each of control HUT 78 and Jurkat cells, HUT 78 and Jurkat cells expressing *nef*, and a cell line chronically infected with HIV-1_{SF2} were lysed in virus disruption buffer (50 m*M* tris-Cl, *p*H 7.5, 0.1% Triton X-100, and 0.15 g of dithiothreitol per milliliter) and subjected to immunoblot analysis as described (*17*). Fractionated proteins were reacted with a pool of sera from HIV-1–positive individuals that had been screened for reactivity to the p27 protein or with a polyclonal rabbit antibody to p27 (anti-p27) antiserum. (**A**) The specificity of the sera used for screening p27 expression. Lanes 1 and 4, control HUT 78 *neo* cell lysate; lanes 2 and 5, HUT 78 chronically infected with HIV-1_{SF2}; lanes 3 and 6, p27 protein expressed in yeast (1 μg of protein per lane). Lanes 1 to 3 were reacted with HIV-1 positive human sera at a 1:50 dilution; lanes 4 to 6 with a polyclonal rabbit anti-p27 antiserum also at 1:50 dilution. (**B** and **C**) P27 reactivity in HUT and Jurkat *nef*-expressing cell lines as detected by the same pool of HIV-1 positive human sera. In (B): lane 7, control HUT 78 *neo* cell lysate; lane 8, HUTpAM3 *neo* cell lysate; lane 9, HUTp3R *neo* cell lysate, lane 10, lysate prepared from HUTp3R *neo* cells infected with HIV-1_{SF2}. In (C): lane 11, control Jurkat *neo* cell lysate; lane 12, JURpAM3 *neo* cell lysate; lane 13, JURp3R *neo* cell lysate. The p27 reactivity was absent in HUT 78 cells chronically infected with HIV-1_{SF2} (lanes 2 and 5). In lane 2, only reactivities to the *gag* p25 and *pol* p31 proteins were detected. The polyclonal rabbit anti-p27 showed a much stronger reactivity to the yeast p27 protein (lane 6) as compared to the positive HIV-1 human sera (lane 3).

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	HUT	HUTpAM3	HUTp3R	JUR	JURpAM3	JURp3R
CD4 (%)	>90	15 - 20	>90	56	58	63
CD3 (%)	100	100	100	90	100	100

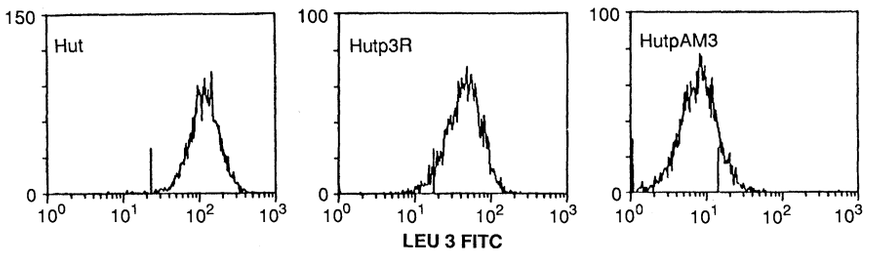

Fig. 2. Surface expression of CD4 and CD3 antigens in *nef*-containing lymphoid cells was determined with an FITC-labeled murine monoclonal antibody specific for the respective antigens (Leu 3a and Leu 4; Becton Dickinson) and then analyzed by FACS (*22*). The percent of fluorescent cells is indicated for each cell type listed. None of the cell lines reacted with FITC-labeled control mouse monoclonal antibody specific for human immunoglobulin G (IgG). HUT, HUT 78 T cell line; JUR, Jurkat T cell line.

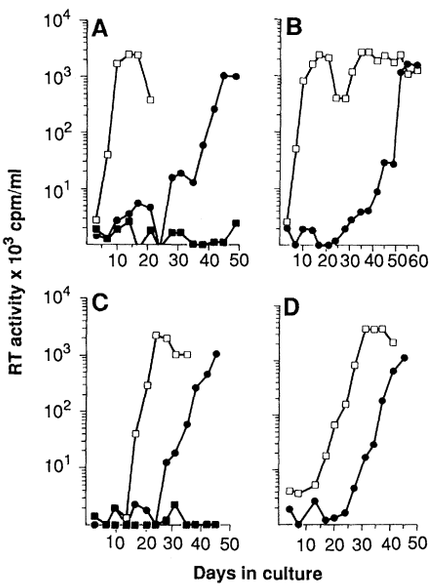

Fig. 3. Transfection (**A**) and infection (**B**) of HUT 78 cells (HUT) expressing *nef*. For transfection experiments, 10 μg of DNA from a biologically active molecular clone of HIV-1_{SF2} were added to 10^6 cells each of HUTp3R (●), HUTp3AM3 (■), and control HUT 78 (□) cells, as previously described (*13*). For infection of HUTp3R and HUT 78 cells, 5×10^5 cells each were inoculated with an infectious virus obtained after transfection of cells with the molecular clone of HIV-1_{SF2} (RT activity = 10^6 cpm/ml). Infections and transfections were performed in the absence of G418. The transfected and infected cells were maintained in medium without G418 and the particle-associated RT activity in the culture supernatant was measured at 3- to 4-day intervals (*24*). Similar numbers of cells per culture were present at the time of RT measurement. Transfection (**C**) and infection (**D**) of Jurkat cells (JUR) expressing *nef*. JURp3R (●), JURpAM3 (■), and control Jurkat cells (□) were transfected and infected with HIV-1_{SF2} as described above (A and B). Representative data of three independent experiments are shown.

man T lymphoid cells (HUT 78 or Jurkat) were cotransfected with one of these *nef* gene expression plasmids and a plasmid containing *neo* as a selection marker by the electroporation method (*13, 15*). As controls, the different cell types were transfected with only the SV40/neo plasmid. Transfected cells were selected by growth in the presence of G418, and the resistant cell lines were examined for expression of p27 by an immunofluorescence assay (IFA) and immunoblot analysis (*16, 17*).

The results of immunoblot analysis of transformed cells with a pool of HIV-1 positive sera or a polyclonal rabbit antibody to *nef* are shown in Fig. 1. The specificity of the sera used was demonstrated by their reactivity with p27 protein expressed in yeast (Fig. 1A, lanes 3 and 6). The p27 reactivity was not detected in control HUT 78 cells (lanes 1 and 4) or in HUT 78 cells chronically infected with HIV-1_{SF2} (lanes 2 and 5). This latter finding suggests that in chronic HIV-1 infection, the *nef* gene product is not expressed or it is produced in very low amounts. Different levels of p27 expression were detected in the G418-resistant *nef* plasmid–transformed HUT 78 and Jurkat cell lines. In two separate analyses of the transformed HUT 78 and Jurkat cells, lower levels of *nef* were expressed in cells transfected with p3R as compared to pAM3 (Fig. 1B, lanes 8 and 9, and Fig. 1C, lanes 12 and 13). This result is not unexpected since the HIV-LTR, in the absence of *tat*, is a weak promotor in lymphoid cells (*18, 19*). Over 90% of the cells in these transformed cell lines expressed *nef* as revealed by IFA. The protein, as reported by others (*20*), was cytoplasmic and membrane-associated.

The expression of the CD4 receptor molecules on the cell surface of these *nef*-expressing HUT 78 and Jurkat cell lines was examined and compared to control cells. Our results show that only in the HUTpAM3 cell line is the CD4 receptor molecule downmodulated (15% to 20% as compared to >90% in control HUT 78 cells) (Fig. 2). CD4 downmodulation was not observed in continuous cultures of the JURpAM3 cell line that had the highest level of p27 *nef* expression (Fig. 1, lane 12). No effect on CD3 cell surface expression was observed in any of the transformed cell lines. When five different randomly selected clones derived from each of the control and *nef*-expressing cell lines were examined, the percentage of cell surface CD4 expression ranged from 24% to 99% for the HUTp3R and HUTpAM3 cell clones and 35% to 60% for the JURp3R and JURpAM3 cell clones. All these clones express comparable levels of p27 as determined by IFA. Furthermore, the same range of CD4 expression was observed in the control HUT 78 and Jurkat cell clones containing just the neomycin plasmid. These data indicate that downmodulation of the CD4 receptor molecule is independent of p27 *nef* expression.

The effect of p27 *nef* production in T lymphoid cells on HIV replication was examined by infection or transfection of the plasmid-containing cells (*8, 13*). The latter procedure was used because of the reduction in CD4 receptor protein expression on the HUTpAM3 cells (Fig. 2). Virus production was suppressed in HUTp3R for over 30 to 40 days before the appearance of reverse transcriptase (RT) activity in the culture supernatant, as determined by infection and transfection of HUTp3R cells (Fig. 3, A and B). No viral antigens were produced in these cells at 8 and 14 days after transfection or after infection when examined by p25 enzyme-linked immunosorbant assay (ELISA) or immunoblot analyses (*17*). However, the expression of *nef* protein in the cells was increased when compared to uninfected HUTp3R cells (Fig. 1B, lanes 9 and 10). The suppression of virus replication was most dramatic in HUTpAM3 cells in which no RT activity was observed in the culture supernatant even after 50 days in culture (Fig. 3A). Low levels of RT activity were detected in these transfected cells after 70 days in culture. In contrast, HIV-1_{SF2} reached high levels of replication in the control HUT 78 cells within 12 days (Fig. 3, A and B). At peak RT activity (>10^6 cpm/ml), over 20% of the infected control cells showed HIV-1 antigen by IFA.

Similar results were obtained after infection or transfection of Jurkat cells containing the p3R and pAM3 *nef* constructs (Fig. 3, C and D). Peak RT activity for HIV-1_{SF2} was delayed for 20 days in the JURp3R cells and for over 30 days in JURpAM3 cells. To determine if p27 *nef* provided by the plasmid

DNA was sufficient to suppress HIV replication, we inoculated the same *nef*-containing cell lines with infectious virus derived from the molecular clone of HIV-1_{SF2} deleted in *nef* expression (*5*). No viral replication took place in these cells for over 30 days, whereas control cells showed high production of this HIV-1 mutant virus within 10 days. This finding indicated that *nef* introduced as part of the viral genome via infection or transfection is not required for the observed virus suppression. All these results with HIV-1_{SF2}, originally obtained with uncloned populations of *nef*-expressing HUT or Jurkat cells, were reproducible in randomly selected clonal cell lines with consistently high CD4 expression that were derived from each of the parental cell lines.

To examine whether *nef* from HIV-1_{SF2} had the same suppressive effects on heterologous HIV isolates, we infected HUTp3R and control HUT 78 cells with HIV-1_{SF2}, HIV-1_{SF33}, or HIV-2_{UC1}. HIV-1_{SF33} is an extremely cytopathic and fast-growing HIV-1 isolate that replicates in a wide variety of different cell types (*21*). HIV-2_{UC1} is a noncytopathic HIV-2 isolate recovered from a patient from the Ivory Coast (*22*). Plasmid HIV-1_{SF2} p27 expression was associated with a substantial reduction in the replication of HIV-1_{SF2} and HIV-2_{UC1} (Fig. 4). In contrast, the presence of *nef* did not lead to suppression of the highly cytopathic HIV-1_{SF33} strain; the same kinetics of viral replication were observed in both the control HUT 78 and the HUTp3R cells infected with HIV-1_{SF33}. Similar results were obtained in Jurkat, JURp3R, and JURpAM3 cells.

To determine whether the nonresponsiveness of HIV-1_{SF33} to the "negative" effect of *nef* is a common feature of the more cytopathic and fast replicating viruses, the ability of HIV-1_{SF2} *nef* to suppress the replication of a group of HIV-1 strains, sequentially isolated from infected individuals, was determined (Table 1). These viruses were used in previous studies showing that, as disease develops in an individual, HIV-1 variants emerge that are more cytopathic in vitro and replicate more efficiently in a wide variety of different human cells (*23*). An example is HIV-1_{SF13}, which was isolated from the same individual as the HIV-1_{SF2} strain, but 5 months later when he had developed Kaposi's sarcoma and *Pneumocystis carinii* pneumonia. Unlike HIV-1_{SF2}, HIV-1_{SF13} replicates to high titers in both T and B cell lines and primary macrophages (*23*). Furthermore, characteristic of most cytopathic HIV-1 strains (*21*), HIV-1_{SF13} forms plaques in the MT-4 cell line. The data show that, in contrast to the observations with the earlier HIV-1 isolates, HIV-1_{SF2} *nef* in HUTp3R cells had no substantial effect on replication of the later isolates, HIV-1_{SF13} and HIV-1_{SF665} from subjects 1 and 3, respectively. It also was less effective in suppressing replication of the later sequential isolate HIV-1_{SF216} from subject 2. Similar findings were observed in infections of Jurkat and JURp3R cells. These results suggest that progression of disease in the host correlates with appearance of HIV-1 variants, which are also less responsive to the negative regulation of *nef*.

These data obtained from studies with T lymphoid cell lines expressing p27 elaborate on our earlier observations (*5*) suggesting that the *nef* gene is important in the delay of HIV production and hence in latency. The observed suppressive effect of *nef*-containing cells on HIV replication could not result from the presence of *neo* or competitive promotor sequences in the expression vectors since, as noted above, some viral strains (for example, HIV-1_{SF13}, HIV-1_{SF33}, and HIV-1_{SF665}) replicated with similar kinetics in the HUT 78 and Jurkat cells with or without the *nef* expression plasmids (Fig. 4 and Table 1).

The mechanism by which *nef* functions is unknown; however, it has been suggested that *nef* is a transcriptional silencer (*6, 7*).

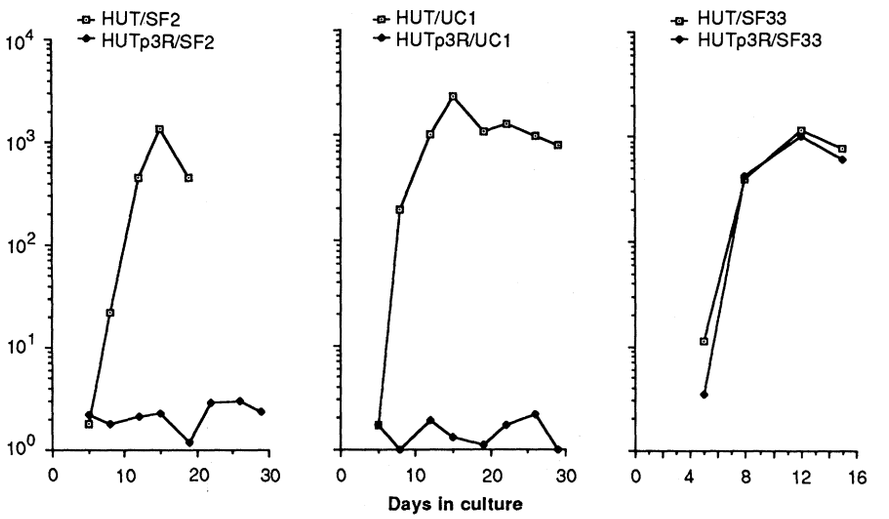

Fig. 4. Infection of HUTp3R and control HUT 78 cells with HIV-1 and HIV-2 isolates. HUTp3R and HUT 78 (HUT) cells (5×10^5) were infected with equal amounts (RT activity = 10^6 cpm/ml) of HIV-1_{SF2}, HIV-2_{UC1}, and HIV-1_{SF33} by the method described in the legend to Fig. 3 and maintained in culture medium without G418. Kinetics of virus replication in the two cell types, as determined by RT activity in culture supernatant, was compared to that observed with infection by HIV-1_{SF2}. A representative of three independent experiments is shown.

Table 1. Effect of *nef* on HIV-1 isolates sequentially recovered from infected individuals. HUT 78 and HUTp3R (5×10^5 cells) were infected with equal amounts (RT activity = 10^6 cpm/ml) of each sequential isolate from subjects 1, 2, and 3 as described in legend to Fig. 3. The time intervals of virus recovery for the different subjects ranged from 5 to 12 months (*23*). Virus replication was measured by level of RT activity detected in the culture supernatant at 3- to 4-day intervals (*24*). The data are representative of two independent experiments conducted. KS, Kaposi's sarcoma; LAN, lymphadenopathy; PCP, *Pneumocystis carinii* pneumonia. The isolation and characteristics of the HIV-1 strains used have been previously described (*23*).

HIV-1 isolate	Clinical state	Virus replication (RT activity $\times 10^3$ cpm/ml)			
		HUT 78		HUTp3R	
		Day 7	Day 30	Day 7	Day 30
		Subject 1			
SF2	Oral candidiasis	532.9	1556.8	2.2	18.7
SF13	KS, PCP	3177.8	2460.1	1518.8	1226.9
		Subject 2			
SF94	Asymptomatic	4.7	59.0	0.8	2.3
SF118	Asymptomatic	18.8	2028.1	2.1	2.7
SF216	LAN, diarrhea	1573.1	2739.7	28.0	1217.0
		Subject 3			
SF73	Asymptomatic	1.2	1.7	1.7	1.5
SF328	LAN	152.3	1449.6	1.3	262.9
SF665	PCP	1345.5	2338.4	1374.9	1554.1

The data showing that the *nef* product of one virus (HIV-1_{SF2}) can affect the replication of other HIV-1 as well as HIV-2 strains (Fig. 4 and Table 1) suggest that common sequences in the LTR of these viruses are responsive to the *nef* protein. In this regard, the observed lack of effect of HIV-1_{SF2} *nef* on the highly cytopathic and fast replicating HIV-1_{SF33} strain and the later sequential isolates from infected individuals (HIV-1_{SF13}, HIV-1_{SF216}, and HIV-1_{SF665}) is important. Since a functional *nef* is supplied in these infections by the plasmid, these more pathogenic HIV-1 variants that emerged over time in individuals may have mutated in the LTR sequences responsive to *nef*. Alternatively, other positive regulatory elements might be more potent in these strains and outweigh the *nef*-mediated suppression of replication. A comparison between the LTR regions of responsive and nonresponsive isolates should provide insight into this possible mechanism of HIV pathogenesis.

In summary, our observation on the differential effect of cell lines expressing the HIV-1_{SF2} *nef* protein on replication of HIV strains strongly suggest a key role for this viral gene in the establishment and maintenance of latent viral infection and in HIV pathogenesis. Further studies with these lymphoid cell lines should allow us to determine the mechanism by which *nef* exerts its negative effect. Moreover, they provide valuable cell culture systems for defining factors involved in activating latent infections. Finally, these results suggest that manipulation of *nef* in early stages of HIV infection may prove effective in therapeutic approaches.

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9. In plasmid pAM3, the SV40 early promoter and leader region cloned into the vector pML (*10, 11*), was positioned upstream of the HIV-1_{SF2} *nef* gene (*11*). A synthetic oligonucleotide adapter (GATCCAAGGCTTTTCCTATAA) joins the SV40 leader to the start codon for *nef*. Another synthetic oligonucleotide adapter encodes sequences from the start codon for *nef* to the Xho I site within the gene (*12*). The remainder of *nef* is contained in a DNA fragment derived from the biologically active, molecularly cloned proviral form of HIV-1_{SF2} (p9B-18) (*12, 13*). This DNA fragment has sequences from the Xho I site in *nef*, through the rightward LTR and into cellular flanking sequences up to an Eco RI site. The plasmid p3R has the HIV-1_{SF2} LTR positioned upstream from the *nef* gene. The DNA fragment bounded by BAM HI and Eco RI sites from pAM3 was cloned into a polylinker downstream from the LTR in the plasmid pLTR-1 (*14*).
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RNA Editing in Plant Mitochondria

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Comparative sequence analysis of genomic and complementary DNA clones from several mitochondrial genes in the higher plant *Oenothera* revealed nucleotide sequence divergences between the genomic and the messenger RNA–derived sequences. These sequence alterations could be most easily explained by specific post-transcriptional nucleotide modifications. Most of the nucleotide exchanges in coding regions lead to altered codons in the mRNA that specify amino acids better conserved in evolution than those encoded by the genomic DNA. Several instances show that the genomic arginine codon CGG is edited in the mRNA to the tryptophan codon TGG in amino acid positions that are highly conserved as tryptophan in the homologous proteins of other species. This editing suggests that the standard genetic code is used in plant mitochondria and resolves the frequent coincidence of CGG codons and tryptophan in different plant species. The apparently frequent and non–species-specific equivalency of CGG and TGG codons in particular suggests that RNA editing is a common feature of all higher plant mitochondria.

SEQUENCE ANALYSIS OF GENOMIC and cDNA clones from the mitochondrially encoded cytochrome oxidase subunits II (*coxII*) and III (*coxIII*), the cytochrome b (*cytb*), and the reduced nicotinamide adenine dinucleotide–dehydrogenase subunit I (*nadI*) loci revealed a number of discrepancies between the two respective sequences. One of these instances within the coding region of *coxIII* was reported earlier (*1*). Several independently derived cDNA clones contained two adjacent T residues where the genomic DNA encodes C's. The cDNA specifies phenylalanine instead of the proline specified by the genomic DNA, where phenylalanine is conserved in the human, yeast, and *Neurospora* proteins (Fig. 1).

Further analysis has now shown that such nucleotide exchanges are not a singular event at this locus, but occur at many positions in several different genes. Three additional nucleotide changes are found in the analyzed coding region of the *coxIII* cDNA sequence, all involving C to T transitions (Fig. 1). All four events are nonsilent and specify amino acids in the cDNA sequence that are better conserved in other species at the respective positions than the genome-encoded amino acids (Fig. 1C).

Artifacts of cDNA cloning had been assumed when the first sequence differences between genomic and the mRNA-derived DNAs were observed. This explanation now seems unlikely since a number of such events have been observed in apparently physically "normal" sequence surroundings that give no indication as to why reverse transcriptase or the bacterial amplification processes should introduce these particular modifications repeatedly. The conservation of the cDNA-specified amino acids between different species also indicates that the cDNA sequence is correct.

These nucleotide divergences between genomic and mRNA-derived sequences are not restricted to coding regions, but are also found, for example, in the trailer sequence of the *coxIII* locus (Fig. 1B), which presumably is not translated. The effect of these untranslated alterations is as yet unclear and needs further experimental evaluation.

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