prokaryotic and eukaryotic GAPDHs all have the same secondary structure and since the evolutionary distance (amino acid sequence similarity) between site 1 and site 2 of the same GAPDH is much greater than the distance between site 1 of prokaryotic and eukaryotic GAPDHs, this would indicate that the duplication that gave rise to the two NAD^+-binding sites 1 and 2 occurred before the divergence of prokaryotes and eukaryotes. Hence, the occurrence of introns in similar positions in the two sites implies that these introns existed in the progenitor GAPDH gene prior to the divergence of prokaryotes and eukaryotes, but some of these introns were subsequently lost from certain GAPDH species during evolution. The fact that one site has introns in the plant chloroplast (GapB, B4) and animal cytosolic (chicken, K6) genes, while its duplicated site has introns in plant (GapC, C4) and animal (chicken, K4) cytosolic genes gives strong support to this idea. We predict that, as more sequencing data are available, some GAPDH genes should have introns located between residues 20 and 30 in site 1 corresponding to C5/K5 in site 2 and between residues 120 and 125 in site 2 corresponding to B2/C3/K3 in site 1. Second, the introns always appear between, but not within, the structural domains of the NAD^+ binding region. This observation is consistent with the idea, as proposed by Blake (*20*), that introns might play important roles in bringing together small sequence units that encode potentially stabilizing secondary structures in the evolving protein.

The relationships between intron positions and the catalytic domain, which consists basically of nine-stranded antiparallel sheets and a long helical tail (*16*), is more difficult to interpret. However, based on the same lines of reasoning, Stone *et al.* (*19*) have argued that the chicken introns, K7 to K11, should also have existed in the progenitor GAPDH gene. Therefore, among a total of 18 intron positions in four GAPDH genes, 15 of them should have existed in the progenitor GAPDH gene. Whether the remaining three introns (B6, C6, and C8) are the result of deletional or insertional events remains to be elucidated.

In summary, we have provided evidence that some GAPDH introns existed before the divergence of prokaryotes and eukaryotes, consistent with the idea that introns played a role in the assembly of the progenitor GAPDH gene. In addition, a comparison of the intron positions with the structural domains of GAPDH indicates that the majority, if not all, of the introns that exist in the four GAPDH genes whose sequences are known should have existed in the progenitor GAPDH gene.

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HIV-Infected Cells Are Killed by rCD4-Ricin A Chain

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The gp120 envelope glycoprotein of the human immunodeficiency virus (HIV), which is expressed on the surface of many HIV-infected cells, binds to the cell surface molecule CD4. Soluble derivatives of recombinant CD4 (rCD4) that bind gp120 with high affinity are attractive vehicles for targeting a cytotoxic reagent to HIV-infected cells. Soluble rCD4 was conjugated to the active subunit of the toxin ricin. This conjugate killed HIV-infected H9 cells but was 1/1000 as toxic to uninfected H9 cells (which do not express gp120) and was not toxic to Daudi cells (which express major histocompatibility class II antigens, the putative natural ligand for cell surface CD4). Specific killing of infected cells can be blocked by rgp120, rCD4, or a monoclonal antibody to the gp120 binding site on CD4.

MOST INDIVIDUALS INFECTED with the human immunodeficiency virus (HIV) develop acquired immunodeficiency syndrome (AIDS) (*1*), which is characterized by the progressive depletion of T cells expressing CD4, the cellular receptor for HIV (*2*). A potential approach for preventing or delaying the onset of AIDS is to eliminate cells producing viral proteins early in the course of the disease. This may prevent the spread of infection and the release of viral proteins that may participate in the pathogenesis of the disease (*3*). HIV-infected cells could be eliminated with a toxic agent coupled to a targeting molecule that would bind only to cells expressing HIV-encoded proteins. An attractive targeting entity is recombinant soluble CD4 (rCD4) (*4, 5*) that binds to gp120, the envelope glycoprotein of HIV, with an affinity comparable to that of cell surface CD4 (*4*). Although gp120, which is expressed on the surface of many HIV-infected cells, shows extensive variability among different strains of HIV, its CD4 binding site is highly conserved (*6*).

Conjugates of toxins and cell-reactive ligands can specifically delete cells in vitro and in vivo (*7*). We and others have used the A chain of the plant toxin, ricin, conjugated to cell-reactive antibodies. Such conjugates kill cells after endocytosis of the conjugate-antigen complex and translocation of the A chain into the cytosol where it inhibits protein synthesis (*7*). Below we describe the coupling of soluble rCD4 to deglycosylated

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ricin A chain (dgA). Deglycosylation prevents a conjugate from binding to liver cells and, in vivo, results in more effective delivery to the target cell with significantly less hepatotoxicity (*7*). Thiolated rCD4 [derivatized with *N*-succinimidyl-*S*-acetyl thioacetate (SATA)] (*8*) was coupled to dgA derivatized with Ellman's reagent (*9*). The conjugate was purified by gel filtration on Sephacryl S-200 (HR) to remove free dgA and on a column of Sepharose anti-ricin A chain to remove free rCD4. The purified rCD4-dgA was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Two molecular species, each representing rCD4 coupled to one dgA chain, but with different mobilities on gels (75 and 97 kD), were present in the rCD4-dgA conjugate; no free rCD4 or dgA was detected (Fig. 1, lane B). Under reducing conditions (Fig. 1, lane C), the conjugate yielded two bands corresponding to rCD4 (Fig. 1, lane D) and dgA chain (Fig. 1, lane E); thus the two components of the conjugate are linked by a disulfide bond. In similar experiments, conjugates prepared with either ^{125}I-labeled rCD4 or ^{125}I-labeled dgA had label in both the 75- and 97-kD species, confirming the presence of rCD4 and dgA in each (*10*).

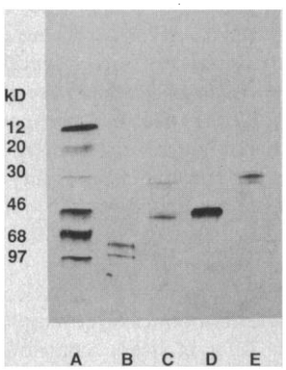

Fig. 1. SDS-PAGE of the rCD4-dgA conjugate and its components. Samples were separated on a Pharmacia Phast System with an 8 to 25% gel gradient. The gels were stained with 0.4% silver nitrate. Lane A, standards; lane B, rCD4-dgA; lane C, rCD4-dgA reduced with 5% 2-mercaptoethanol; lane D, thiolated rCD4 (rCD4-S-CO-CH_3); and lane E, Ellmanized dgA (dgA-S-TNB).

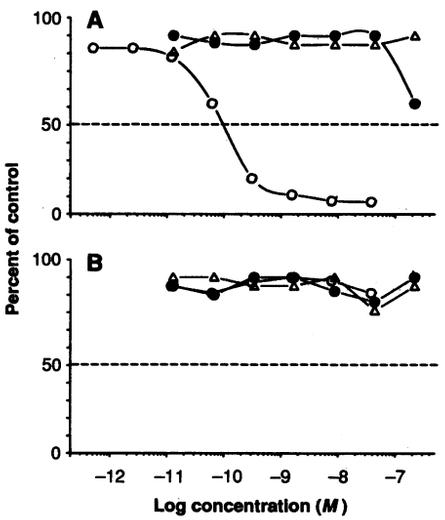

Fig. 2. The rCD4-dgA conjugate is toxic to HIV-infected H9 cells. (**A**) The human T cell line H9 chronically infected with HIV-1 (one representative experiment of four). (**B**) Uninfected H9 cells (one representative experiment of four). The rCD4-dgA (○), Fab′–MOPC-21–dgA (●) (*12*), and rCD4 (△) were plated in triplicate in 96-well microtiter plates in complete medium [RPMI, 12% fetal calf serum (FCS), and antibiotics]. Cells were added at a final concentration of 4×10^5 per milliliter and plates were incubated for 16 hours at 37°C (5% CO_2). Cells were pulsed for 6 hours with 1 μCi of [^{3}H]thymidine (*18*) and harvested on a Titretek automatic harvester. [^{3}H]thymidine incorporation was determined on an LKB Beta Counter. Results are expressed as a percentage of control (untreated cells).

The activities of the dgA and rCD4 components of the conjugate were evaluated. After reduction with dithiothreitol, the dgA released from the conjugate was comparable to native or Ellmanized dgA in its ability to inhibit protein synthesis in a cell-free rabbit reticulocyte assay (*11*) [IC_{50} (*12*) = $4 \times 10^{-11}M$ versus $2 \times 10^{-11}M$]. The rCD4-dgA had gp120 binding activity comparable to that of thiolated rCD4 and 25 to 50% that of native rCD4 in a solution or solid-phase binding assay (*4*).

Treatment of HIV-infected cells from the human T cell line H9 with rCD4-dgA inhibited DNA synthesis by >90% with an IC_{50} of $1.5 \pm 0.53 \times 10^{-10}M$ (mean ± SD of four experiments) (Fig. 2A). In contrast, free rCD4 or an irrelevant antibody–ricin A chain conjugate of the same size [Fab′–MOPC-21 (IgG_1-dgA (*13*)] were only 1/1000 as effective ($IC_{50} > 10^{-7}M$). Neither rCD4-dgA, rCD4, nor Fab′–MOPC-21–dgA killed uninfected H9 cells at a concentration of $>5 \times 10^{-8}M$ (Fig. 2B). Hence, the toxicity of rCD4-dgA is specific.

The cytotoxic effect of the rCD4-dgA could be effectively blocked in a concentration-dependent manner by soluble rgp120 (Fig. 3A), by monoclonal antibodies (MAbs) to the gp120 binding site of CD4 [Leu-3a (*14*)] (Fig. 3B), or by soluble rCD4 (Fig. 3C). In contrast, bovine serum albumin (BSA) (Fig. 3, A and C) or MAbs to another CD4 epitope not involved in gp120 binding [MAb 456 (*15*)], did not inhibit the cytotoxicity of rCD4-dgA (Fig. 3B). Thus, the toxicity of rCD4-dgA to HIV-infected H9 cells occurs by specific binding of the rCD4 component of the conjugate to gp120.

We next determined whether rCD4-dgA could kill cells expressing major histocompatibility (MHC) class II antigens, the putative natural ligand for cell surface CD4 (*16*). The IC_{50} of rCD4-dgA for Daudi cells, which express high levels of class II antigens (*17*), was $>5 \times 10^{-8}M$ (similar to that of free dgA) (Fig. 4) (*13*). In contrast, Daudi cells were killed efficiently with MAb to class II-dgA with an IC_{50} of $2.6 \pm 1.1 \times 10^{-10}M$

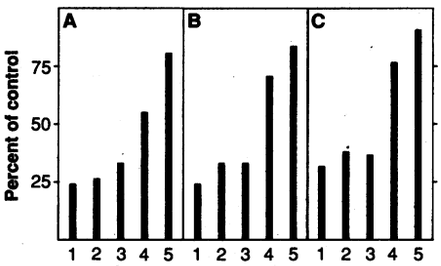

Fig. 3. The killing of HIV-infected H9 cells by rCD4-dgA is blocked by an excess of rCD4, rgp120 (*19*), or MAbs to the gp120 binding site of rCD4 (Leu-3a), but not another epitope on CD4 (MAb 456) (*15*). rCD4-dgA was used at a final concentration of $4 \times 10^{-10}M$. Microtiter plates containing HIV-infected H9 cells were incubated, [^{3}H]thymidine was added, and the cells were harvested as described in Fig. 2. Results are expressed as the percentage of [^{3}H]thymidine incorporation in untreated cells. The bars represent the average of four experiments. The SD among experiments was 20%. (**A**) Cells were treated with rCD4-dgA, which had been incubated for 1 hour at 37°C with (1) medium; (2) BSA, 25 μg/ml; and (3 to 5) rgp120 at 0.125, 2.5, and 25 μg/ml, respectively. (**B**) Cells were treated with rCD4-dgA, which had been incubated for 1 hour at 37°C with (1) medium; (2) MAb 456, 25 μg/ml; and (3 to 5) Leu-3A at 0.00625, 0.125, and 2.5 μg/ml, respectively. (**C**) Cells were incubated with rCD4 or controls at 37°C for 1 hour. rCD4-dgA was then added at a final concentration of $4 \times 10^{-10}M$. Cells were incubated, pulse-labeled, and harvested as described in Fig. 2. rCD4-dgA–treated cells were first cultured with (1) medium; (2) BSA, 25 μg/ml; and (3 to 5) rCD4 at 0.125, 2.5, and 25 μg/ml, respectively.

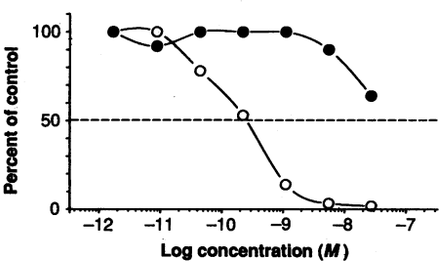

Fig. 4. The rCD4-dgA conjugate does not kill class II$^+$ Daudi cells. rCD4-dgA (●) or MAb to class II-dgA (*20*) (○) was titrated into 96-well microtiter plates. Daudi cells were added to a final concentration of 4×10^5 per milliliter. The plates were incubated for 16 hours at 37°C. The cells were centrifuged, resuspended in leucine-free medium, pulse-labeled with [^{3}H]leucine (5 μCi per well), and harvested as described in Fig. 2. One representative experiment of three that were performed is shown.

(mean ± SD of three experiments) (Fig. 4). The inability of rCD4-dgA to kill Daudi cells at concentrations $>5 \times 10^{-8} M$ may be due to a lower binding affinity of rCD4-dgA to class II molecules or to the inability of class II antigens to be internalized after binding rCD4-dgA.

If infected cells from HIV-positive individuals can also be killed by rCD4-dgA, it might be possible to prevent or delay the onset of clinical disease. The effective application of such a therapeutic strategy, however, must take into account the mode of latent HIV infection and the factors contributing to viral activation.

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Fidelity of HIV-1 Reverse Transcriptase

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The human immunodeficiency virus type 1 (HIV-1) shows extensive genetic variation and undergoes rapid evolution. The fidelity of purified HIV-1 reverse transcriptase was measured during DNA polymerization in vitro by means of three different assays. Reverse transcriptase from HIV-1 introduced base-substitution errors in DNA from the bacteriophage ϕX174 *amber*3 at estimated frequencies of 1/2000 to 1/4000. Analyses of misincorporation rates opposite a single template adenine residue showed that HIV-1 reverse transcriptase catalyzed nucleotide mismatches with a specificity of A:C >> A:G > A:A. The high error rate of HIV-1 reverse transcriptase in vitro translates to approximately five to ten errors per HIV-1 genome per round of replication in vivo. This high error rate suggests that misincorporation by HIV-1 reverse transcriptase is, at least in part, responsible for the hypermutability of the AIDS virus. The specificity of misincorporation may provide a basis for the systematic construction of antiviral nucleosides.

GENOMIC HETEROGENEITY IS A hallmark of HIV-1 (*1–3*). Studies on sequential HIV-1 isolates from persistently infected individuals suggest that HIV-1 evolves at a rate approximately a million times as great as that of eukaryotic DNA genomes (*2*). This hypermutability could be central to the pathogenesis of HIV-1 and could thwart efforts to develop effective vaccines.

The high rate of HIV-1 mutagenesis is shared by other retroviruses (*4*) and presumably originates in mechanisms unique to the retroviral life cycle. Replication of retroviral genomes proceeds by a series of enzymatic reactions involving virus-encoded reverse transcriptase (RT) and integrase, as well as host cell–encoded DNA polymerases and RNA polymerase II (*5*). The viral RT polymerizes deoxyribonucleotides by using viral RNA as a template and also acts as a DNA polymerase in converting the resulting minus strand DNA into double-stranded DNA (*6*). The high error rate of avian myeloblastosis virus RT in vitro, with both deoxyribonucleotide and ribonucleotide templates (*7*), implicates RTs as major contributors to retroviral mutagenesis. In contrast, provirus replication is not very error-prone (*8*), presumably because replication is achieved by the more accurate host cell DNA polymerase α (*9*).

We used three different methods to evaluate the fidelity of DNA synthesis by HIV-1 RT. First, as a qualitative assessment of misincorporations opposite multiple template sites, we used the "minus" sequencing gel assay (*10, 11*). In the presence of only three 2′-deoxyribonucleoside 5′-triphos-

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Table 1. Kinetic parameters for site-specific misincorporation. Apparent K_m and V_{max} values ± standard errors were determined by the method of Wilkinson (*22*) from the data in Fig. 2. The relative insertion frequencies were calculated from the V_{max}/K_m ratios and are expressed relative to dTTP.

RT	dNTP	K_m(app) (μ*M*)	V_{max}(app) (%/min)	V_{max}/K_m [%/(min *M*)]	Relative insertion frequency
HIV-1 (virion)	T	3.7 ± 1.0	80 ± 10	2.1×10^7	1
	C	290 ± 56	4.2 ± 0.2	1.4×10^4	1/1,500
	G	1200 ± 240	0.4 ± 0.05	3.2×10^2	1/66,000*
	A	ND†	ND	$<1.3 \times 10^2$	<1/170,000‡
HIV-1 (clone)	T	4.0 ± 1.7	133 ± 27	3.4×10^7	1
	C	370 ± 72	2.9 ± 0.27	7.8×10^3	1/4,300
	G	ND	ND	1.5×10^2	1/200,000‡
	A	ND	ND	$<1.3 \times 10^2$	<1/270,000‡
AMV (virion)	T	0.7 ± 0.2	32 ± 3.0	4.6×10^7	1
	C	1500 ± 370	2.7 ± 0.4	1.8×10^3	1/26,000
	G	ND	ND	$<1.3 \times 10^2$	<1/370,000‡
	A	ND	ND	$<1.3 \times 10^2$	<1/370,000‡

*Calculated from incubations containing excess RT (0.04 unit/μl). †Could not be accurately determined because of the low levels of misincorporation. ‡Estimated from the slope of the initial velocity versus [dNTP] plot.