Multiple Mutations in HIV-1 Reverse Transcriptase Confer High-Level Resistance to Zidovudine (AZT)

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Human immunodeficiency virus (HIV) isolates with reduced sensitivity to zidovudine (3′-azido-3′-deoxythymidine, AZT) from individuals with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex were studied to determine the genetic basis of their resistance. Most were sequential isolates obtained at the initiation of and during therapy. Comparative nucleotide sequence analysis of the reverse transcriptase (RT) coding region from five pairs of sensitive and resistant isolates identified three predicted amino acid substitutions common to all the resistant strains ($Asp^{67} \rightarrow Asn$, $Lys^{70} \rightarrow Arg$, $Thr^{215} \rightarrow$ Phe or Tyr) plus a fourth in three isolates ($Lys^{219} \rightarrow Gln$). Partially resistant isolates had combinations of these four changes. An infectious molecular clone constructed with these four mutations in RT yielded highly resistant HIV after transfection of T cells. The reproducible nature of these mutations should make it possible to develop rapid assays to predict zidovudine resistance by performing polymerase chain reaction amplification of nucleic acid from peripheral blood lymphocytes, thereby circumventing current lengthy HIV isolation and sensitivity testing.

THE CLINICAL USE OF ZIDOVUDINE has become widespread since the demonstration that it is of benefit for the treatment of patients with AIDS and advanced AIDS-related complex (ARC) (*1*). In these individuals with underlying immune deficiency, long-term continuous zidovudine therapy is required to suppress chronic HIV infection. However, we have shown that most isolates from patients with AIDS or ARC who were treated for 6 months or more showed reduced sensitivity to zidovudine, whereas isolates from untreated individuals and those treated for less than 6 months showed uniform sensitivity to the drug (*2*). The relatively small numbers of patients in this study made it difficult to assess the clinical significance of these observations, although a substantial decline in long-term survival of zidovudine-treated individuals with AIDS or ARC has been observed after 15 to 18 months of therapy in a large group of patients (*3*). To determine whether this decline is related to zidovudine resistance and to assess the possibility of transmission of resistant virus, large numbers of individuals need to be studied. This is relatively difficult because conventional methods for HIV isolation and sensitivity testing are labor-intensive and involve lengthy culture procedures. Furthermore, the procedures we used to isolate virus suitable for accurate sensitivity testing resulted in only a 30% virus isolation rate from patients (*2*). It would thus be desirable to develop a rapid drug sensitivity assay that could be applied to large numbers of infected individuals.

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Zidovudine is believed to inhibit HIV replication by virtue of the ability of its triphosphate form (AZT-TP) to bind to HIV RT and cause chain-termination of viral DNA synthesis (*4*). We thus reasoned that resistance was most likely due to mutation in the RT gene and decided initially to examine a number of resistant isolates for mutation in RT. The complete RT coding region (1.7 kb) was obtained from sensitive and resistant isolates by polymerase chain reaction (PCR) amplification of infected cell DNA (*5*), and was inserted into an M13-based vector (mptac18.1) (*6*) (Fig. 1). Clones able to express functional RT enzyme (*7*) were sequenced by the dideoxynucleotide chain-termination method (*8*). The complete sequence of six RT clones was obtained (sensitive and resistant isolate pairs from three individuals), and partial sequence data were obtained from additional isolates (Table 1).

Comparison of predicted complete amino acid RT sequences from sensitive and resistant isolate pairs obtained from the same individuals (patients A012, A018, and P022) revealed differences of between 14 and 17 residues. In each case, however, we identified amino acid changes at four residues ($Asp^{67} \rightarrow Asn$, $Lys^{70} \rightarrow Arg$, $Thr^{215} \rightarrow$ Phe or Tyr, and $Lys^{219} \rightarrow Gln$) in the highly resistant isolates not seen in the sensitive counterparts (Fig. 2). Analysis of sensitive and highly resistant isolate pairs from a further two individuals (patients A036 and P026) revealed the same mutations at positions 67, 70, and 215, while the wild-type Lys at residue 219 was retained (Fig. 2). In cases where multiple M13 clones were sequenced from an isolate, only minor sequence variation was seen. Published HIV type 1 (HIV-1) RT sequences showed absolute conservation of all four residues in every strain (*9*), strongly implicating these mutations in zidovudine resistance.

To test whether multiple mutations in the RT gene could account for high-level resistance, we constructed an infectious molecular

Table 1. Properties of HIV isolates and M13 RT clones derived from them. HIV isolates derived from untreated and treated individuals are shown with duration of therapy at the time they were obtained and the zidovudine sensitivity. Where multiple isolates were obtained from the same individual, isolates are lettered in temporal order. The ID_{50} values were obtained by plaque-reduction assay with HT4-6C cell monolayers (*2, 16*). Some of the sensitivity values were taken from a previous report (*2*). The relative RT activity expressed by a representative M13 RT clone is shown for each virus isolate (RT was expressed in *E. coli*, strain 5KCPolA^{ts}F′). Values are percentage of control RT activity that was derived in each experiment by using the clone mpRT4, which expresses wild-type HIV-1 RT (*7*). RT assays were performed with [^{3}H]TTP and poly(rA) · oligo $(dT)_{18}$ as described (*7*). The amino acid residues at four positions in RT that are important for zidovudine resistance are illustrated for HIV isolates. Wild-type residues at the positions of interest are as follows: Asp^{67}, Lys^{70}, Thr^{215}, and Lys^{219}. Residues in italics represent mutations. Isolation of M13 RT clones and nucleotide sequence analysis are described in the legends to Figs. 1 and 2.

HIV isolate	Duration of therapy (months)	Zidovudine sensitivity (ID_{50} μ*M*)	RT activity of M13 clone (percentage of control)	Amino acid residues in RT			
				67	70	215	219
A012B	2	0.01	20	Asp	Lys	Thr	Lys
A012D	26	2	85	*Asn*	*Arg*	*Phe*	*Gln*
A018A	0	0.01	62	Asp	Lys	Thr	Lys
A018C	14	2.3	79	*Asn*	*Arg*	*Tyr*	*Gln*
A036B	2	0.01	47	Asp	Lys	Thr	Lys
A036C	11	0.6	52	Asp	Lys	*Tyr*	Lys
A036D	20	5.6	46	*Asn*	*Arg*	*Tyr*	Lys
P022A	1	0.01	20	Asp	Lys	Thr	Lys
P022C	16	1.4	67	*Asn*	*Arg*	*Phe*	*Gln*
P026A	0	0.01	76	Asp	Lys	Thr	Lys
P026B	11	2.8	78	*Asn*	*Arg*	*Tyr*	Lys
P035A	6	0.56	55	Asp	Lys	*Tyr*	Lys

clone of HIV containing only the four mutations described above (in isolate A012D) and assessed the sensitivity of virus produced from this clone by transfection of T cells. A 2.55-kb fragment of the HIV *pol* gene from infectious clone HXB2-D (*10*), inserted into the M13 vector mp19, was used as a target for site-directed mutagenesis (*11, 12*). Specific nucleotide changes were simultaneously introduced into the RT gene by using two synthetic oligonucleotides, and mutations were confirmed by nucleotide sequencing (*8*). To reconstruct the full-length clone, a 1.9-kb Bal I restriction fragment containing the mutations in RT was removed from the *pol* gene M13 clone and transferred into HXB2-D (*11*). DNA prepared from wild-type and mutant infectious clones was then used to transfect the T cell lymphoblastoid line, MT-4 (*13*), by electroporation (*14*). Virus-induced cytopathic effect was observed in each culture at similar times (after 2 to 4 days), and virus stocks were prepared 6 to 7 days after transfection (*15*). Wild-type and mutant HIV isolates (HXB2-D and HIVRTMC, respectively) were titrated by plaque assay in the HeLa-CD4$^+$ cell line HT4-6C and then tested for sensitivity to zidovudine by plaque-reduction assay in HT4-6C cells (*2, 16*). The results of these experiments (Table 2) demonstrated that the mutant virus constructed by site-directed mutagenesis was highly resistant to zidovudine. The median inhibitory dose (ID_{50}) value for HIVRTMC increased about 100-fold compared to wild-type virus, and the magnitude of resistance was similar to that of naturally occurring HIV isolates containing similar mutations in the RT gene (Table 2).

Nucleotide sequence data were also obtained from RT genes cloned from isolates of intermediate sensitivity, focusing on those regions containing mutations affecting zidovudine resistance. One of these (A036C) was part of a temporal sequence from the same patient from whom sensitive and highly resistant isolates were also sequenced for RT. In this case, and also for isolate P035A, only a single substitution at Thr^{215} (to Tyr) was observed (Table 1), suggesting partial resistance was conferred initially by this change. However, the situation was less clear when RT clones from other partially resistant isolates were sequenced, as these had combinations of all four amino acid changes found previously in highly resistant strains (*17*). Further extensive sequence analysis will be required to assess the extent of variation in these isolates, which most likely comprise mixed populations. However, since the changes observed at Thr^{215} (to Phe or Tyr) in resistant strains required the rare occurrence of two nucleotide transitions (ACC → TTC, or ACC → TAC), mutation at Thr^{215} might be the most critical precursor to develop-

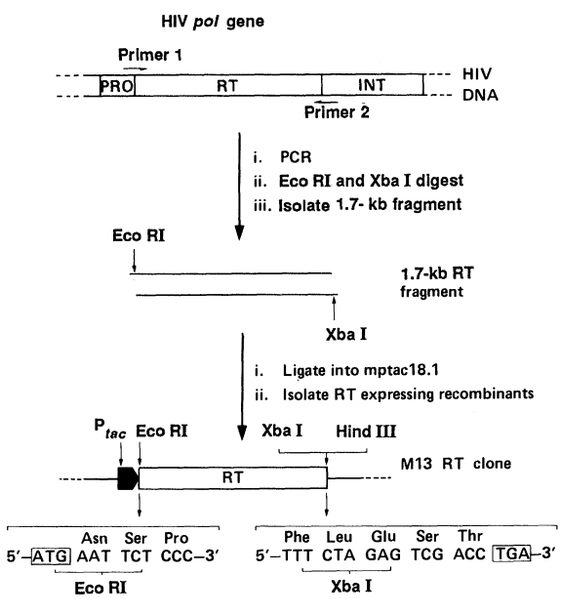

Fig. 1. A diagrammatic representation of the scheme used to amplify and clone HIV RT. Total DNA was extracted from cells infected with HIV isolates, and the entire 1.7-kb fragment of the RT coding region was obtained by PCR (*5*). After digestion with Eco RI and Xba I, whose recognition sites were built into the 5′ and 3′ ends by the PCR primers, fragments were purified from agarose gels. The fragments were ligated with the replicative form of M13mptac18.1, which had previously been digested with Eco RI and Xba I (*6*). Recombinant clones containing a 1.7-kb fragment were assessed for ability to express active RT by induction of M13-infected *Escherichia coli* (strain 5KCPo1A^{ts}F′) with isopropyl βD-thiogalactopyranoside and measurement of RT activity in *E. coli* lysates (*7*). The resulting RT clones were predicted to produce RT with two additional amino acids at the NH_2-terminus (Asn-Ser) and five additional residues at the COOH-terminus (Phe-Leu-Glu-Ser-Thr). Single-stranded DNA made from these recombinant M13 clones was used for nucleotide sequencing. Abbreviations: P_{tac}, *tac* promoter; PRO, protease; and INT, integrase.

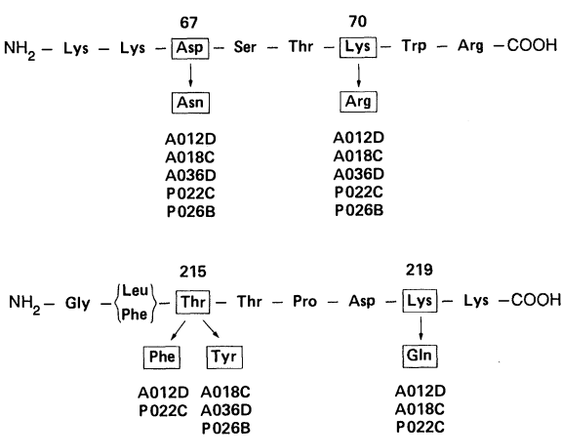

Fig. 2. Mutations in HIV RT that confer zidovudine resistance. The two areas of amino acid sequence from HIV-1 RT containing residues significant for zidovudine resistance are illustrated (residues 65 to 72 and 213 to 220). RT gene fragments were amplified by PCR from infected cell DNA, and the entire coding region of each isolate (1.7 kb) was cloned into the M13 vector mptac18.1 (*5, 6*). Nucleotide sequencing was performed by the dideoxynucleotide chain-termination procedure (*8*) with the use of single-stranded DNA from these clones and a set of ten specific oligonucleotide primers homologous to HIV-1 RT. The boxed amino acids depict wild-type residues and mutant residues derived from those that were common to all highly resistant isolates sequenced. Shown below the boxed amino acids are each of the resistant HIV isolates found to contain these specific mutations. All residues in the two areas illustrated are invariant in wild-type HIV-1 isolates except residue 214, being a Leu or Phe (*9*). Amino acid sequence numbers are relative to the NH_2-terminal proline of RT (*9*).

Table 2. Zidovudine sensitivity of HIV variant created by site-directed mutagenesis. The infectious molecular clone pHIVRTMC was constructed with mutations in the RT gene that give rise to four amino acid changes identical to the zidovudine-resistant isolate A012D (Table 1 and Fig. 2). Transfection of MT-4 cells with pHIVRTMC resulted in the HIV variant HIVRTMC, which was tested for susceptibility to zidovudine by plaque-reduction assay in the HeLa-CD4$^+$ cell line HT4-6C (*2, 16*). Control virus (HXB2-D) was obtained from MT-4 cells transfected with wild-type infectious clone HXB2-D (*10*). Multiplicity of infection for each virus was controlled by plaque titration in HT4-6C cells (*16*). In plaque-reduction assays, similar plaque-forming units were added to HT4-6C cells, and ID_{50} values were derived from plots of plaque reduction versus zidovudine concentration. The zidovudine-sensitive and zidovudine-resistant clinical isolates, A012B and A012D, respectively, were used as additional controls in these assays. Results of three separate plaque reduction assays (with each virus isolate) were used to derive the mean ID_{50} values (SD values are in parentheses).

HIV isolate	Zidovudine sensitivity (mean ID_{50} μ*M*)	Fold increase
HXB2-D	0.013 (0.005)	1
HIVRTMC	1.28 (0.24)	98
A012B	0.013 (0.005)	1
A012D	2.56 (1.03)	197

ment of high-level resistance. Construction of infectious clones with different combinations of mutations would define those conferring a partially resistant phenotype. Similarly, the significance of mutation at Lys^{219} for high-level resistance could also be tested by this strategy (as the highly resistant isolates A036D and P026B did not have this change). Multiple mutations are required to confer high-level zidovudine resistance, which might explain why attempts to isolate resistant virus in culture have been unsuccessful (*11, 18*). Furthermore, this is different from the situation with herpes simplex virus, as only single amino acid changes in the viral thymidine kinase or DNA polymerase are sufficient to confer drug resistance (*19*).

The four amino acid residues discussed (Asp^{67}, Lys^{70}, Thr^{215}, and Lys^{219}) are located in the NH_2-terminal domain of RT, which is believed to be responsible for nucleotide recognition and polymerase function (*20*). Although these residues are not found precisely within highly conserved regions A to F, previously examined by site-directed mutagenesis (*11, 21*), Asp^{67} and Lys^{70} are adjacent to region A, and Thr^{215} and Lys^{219} lie between regions E and F. In view of the narrow cross-resistance profile of zidovudine-resistant isolates, restricted to 3′-azido–containing nucleoside analogs (*2*), these residues might be involved in accommodating the 3′-azido group and recognizing the authentic 3′-OH group of nucleotides.

We demonstrate here that specific changes in the RT gene cause zidovudine resistance, although the actual biochemical basis for this phenotype is still obscure. We previously showed that virion-associated RT from these resistant isolates was as susceptible to inhibition by AZT-TP as RT derived from sensitive isolates (*2*). The Michaelis constant (K_m) value for thymidine triphosphate (TTP) and inhibition constant (K_i) for AZT-TP were also similar when purified RT cloned from a sensitive and resistant isolate pair were compared (*22*). In addition, experiments designed to assess ability of recombinant RT to incorporate AZT-TP into DNA revealed no differences between the same resistant and sensitive isolates (*23*). However, our previous studies, in which single amino acid substitutions were created in conserved domains of the HIV RT polypeptide, showed that the phenotype of RT cannot reliably predict the phenotype of HIV containing such mutations (*11*). Two RT mutants created by site-directed mutagenesis had reduced sensitivity to inhibition by AZT-TP, although viruses constructed from infectious molecular clones containing these mutations displayed hypersensitivity to zidovudine, not resistance, when tested in culture (*11*). Further experiments examining the phosphorylation and incorporation of zidovudine nucleotides into DNA in cells infected with sensitive and resistant isolates might help to explain the resistance mechanism.

Additional questions relating to the pathogenic potential of resistant isolates and their ability to replicate in vivo in the presence of zidovudine could be addressed, as it may now be possible to construct zidovudine-resistant animal retrovirus mutants. For example, the RT gene of a simian immunodeficiency virus (SIV_{mac}) has amino acid residues similar or identical to those of sensitive HIV-1 strains (*9, 24*) at the positions associated with resistance. Thus, mutation of these residues in an infectious molecular SIV_{mac} clone might create resistant SIV. The residue in SIV_{mac} and HIV-2 RT that is seemingly equivalent to Thr^{215} in HIV-1 in both cases is a conservative substitution to a serine (*24*). The $Thr^{215} \rightarrow$ Phe or Tyr changes in HIV-1 RT could both be achieved by initial mutation to a Ser residue that might not alter sensitivity to zidovudine.

In summary, we have identified common mutations that occur in the RT gene of zidovudine-resistant HIV isolates. Acquisition of a fully resistant phenotype appears to require accumulation of multiple mutations at specific amino acid residues. In view of the unusually high error rate of HIV RT and rapid genetic variation of the virus (*25*), the time period observed before fully resistant isolates appear seems relatively long (6 months or more of therapy). In part, this might be because one of the amino acid substitutions requires two nucleotide changes within the same codon, which would occur infrequently. The specific and seemingly predictable nature of these mutations should allow rapid assays based on PCR to be developed that would circumvent the need to culture virus and would enable large numbers of patients to be screened for resistance. Such studies might allow correlations to be made between emergence of resistance and clinical status. In addition, surveys could be initiated to determine whether transmission of resistant strains to untreated individuals is occurring. Finally, it may be possible with the use of these sequence data to construct zidovudine-resistant animal retrovirus isolates for pathogenicity studies.

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5. R. K. Saiki *et al.*, *Science* **239**, 487 (1988). MT-2 cells (*13*) (2×10^6) were infected with HIV (at a multiplicity of about 0.1 $TCID_{50}$ per cell) and incubated at 37°C for 3 to 4 days in RPMI 1640 medium supplemented with 10% fetal bovine serum, Polybrene (2 μg/ml), and antibiotics (called RPMI/10). Cells were disrupted by lysis in 0.4% SDS, 25 m*M* EDTA, 150 m*M* NaCl, and 25 m*M* tris-HCl, *p*H 8, followed by proteinase K (1 mg/ml) digestion for 1 hour at 50°C. Nucleic acid recovered by phenol/chloroform extraction and ethanol precipitation was treated with ribonuclease (10 μg/ml), and DNA was subjected to further phenol/chloroform extraction before ethanol precipitation. Approximately 1 μg of this DNA was used per PCR reaction to amplify the complete RT coding region. Each reaction mix (100 μl) contained 25 m*M* KCl, 2.5 m*M* $MgCl_2$, 50 m*M* tris-HCl, *p*H 8.3, bovine serum albumin (0.1 mg/ml), 0.2 m*M* each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and TTP, 0.25 μg of each primer, and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus). These mixtures were heated at 100°C for 2 min before addition of Taq polymerase, overlaid with 100 μl of light mineral oil, and subjected to 30 cycles consisting of a denaturation step (1 min and 30 s, 94°C), primer annealing (2 min, 37°C), and DNA synthesis (10 min, 72°C) with a Perkin-Elmer Cetus DNA thermal cycler. The oligonucleotide primers, made by using an Applied Biosystems 381A synthesizer, were as follows: at the 5′ end of RT, 5′-TTGCACTTTGAATTCTCCCATTAG-3′ and 5′-TGTACTTTGAATTCCCCCATTAG-3′ (two primers were used to accommodate sequence variation seen in this region) and at the 3′ end, 5′-CTTATCTATTCCATCTAGAAATAGT-3′.
6. HIV RT (1.7 kb) obtained by PCR amplification (*5*) was digested with Eco RI and Xba I (Bethesda Research Laboratories), purified from agarose gels, and ligated with the M13 vector mptac18.1 digested with Eco RI and Xba I (Fig. 1). This vector was constructed by replacing the *lac* promoter in M13mp18 with the *tac* promoter [H. A. De Boer, L. J. Comstock, M. Vasser, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 21 (1983)], providing an in-frame initiation codon. An appropriate termination codon was created by mutagenesis of the Pst I site in the polylinker region (CTGCAG → CTGAAG). The ligation mixture was used to transform *E. coli* (strain TG-1) made competent as described [D. Hanahan, *J. Mol. Biol.* **166**, 557 (1983)]. Recombinant M13 clones were screened for expression of functional RT (*7*), and single-stranded DNA was prepared from these constructs for nucleotide sequencing. The dideoxynucleotide chain termination method was used (*8*) with a set of ten oligonucleotides (17 mers) as primers and ^{35}S-labeled dATP (DuPont Biotechnology Systems).
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14. MT-4 cells (*13*) were transfected with 10 μg of DNA from each full-length HIV clone by electroporation [(*11*); A. J. Cann, Y. Koyanagi, I. S. Y. Chen, *Oncogene* **3**, 123 (1988)] by using a Bio-Rad Gene Pulser and were maintained in RPMI/10. When cytopathic effect was observed, cultures were expanded, and after 6 to 7 days virus stocks were

prepared from cell-free supernatants and stored at −70°C.
15. There was no evidence that the intrinsic infectivity of mutant virus stocks obtained from transfection experiments was different from that of wild-type virus. The median tissue culture infectious dose ($TCID_{50}$) determined by terminal dilution in MT-2 cells (*13*) was $10^{6.45}$/ml for HXB2-D and $10^{6.7}$/ml for HIVRTMC for stocks from a transfection experiment and $10^{6.95}$/ml and $10^{6.7}$/ml for HXB2-D and HIVRTMC, respectively, for stocks from a separate experiment. Furthermore, the titer of both viruses in HT4-6C cells (in plaque-forming units per milliliter) was also similar (5.2×10^4/ml for HXB2-D and 1.8×10^5/ml for HIVRTMC).
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22. RT cloned from zidovudine-sensitive isolate A012B and resistant isolate A012D was expressed in *E. coli* (Table 1) and purified as described [(7); M. Tisdale *et al.*, *J. Virol.* **62**, 3662 (1988)]. For K_m (TTP) and K_i (AZT-TP) determination, RT assays were performed as described (*7*) by using [^{3}H]TTP and poly(rA) · oligo(dT)$_{18}$ as primer template. RT from isolate A012B gave a K_m (TTP) value of 4.5 μ*M* and K_i (AZT-TP) value of 0.01 μ*M*; RT from A012D gave a K_m (TTP) of 13 μ*M* and K_i (AZT-TP) of 0.03 μ*M*.
23. Nucleotide sequencing reactions were carried out with single-stranded M13mp18 DNA and "universal" sequencing primer as described (*8*) except dideoxy-TTP was replaced by AZT-TP. Reactions contained 1 μ*M* TTP, 40 μ*M* each of dATP, dGTP, and dCTP, and either 0.01 μ*M*, 0.1 μ*M*, or 1 μ*M* AZT-TP. Reaction products were separated by buffer gradient polyacrylamide gel electrophoresis [M. D. Biggin, T. J. Gibson, G. F. Hong, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3963 (1983)]. Both enzymes (from sensitive and resistant isolates) showed equal ability to incorporate AZT-TP into DNA template over this 100-fold range of AZT-TP.
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1,25-Dihydroxyvitamin D–Responsive Element and Glucocorticoid Repression in the Osteocalcin Gene

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The active hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3[1,25(OH), which regulates cellular replication and function in many tissues and has a role in bone and calcium homeostasis, acts through a hormone receptor homologous with other steroid and thyroid hormone receptors. A $1,25(OH)_2D_3$-responsive element (VDRE), which is within the promoter for osteocalcin [a bone protein induced by $1,25(OH)_2D_3$] is unresponsive to other steroid hormones, can function in a heterologous promoter, and contains a doubly palindromic DNA sequence (TTGGTGACTCACCGGGTGAAC; −513 to −493 bp), with nucleotide sequence homology to other hormone responsive elements. The potent glucocorticoid repression of $1,25(OH)_2D_3$ induction and of basal activity of this promoter acts through a region between −196 and +34 bp, distinct from the VDRE.

THE IN VIVO EFFECTS OF STEROID and steroid-like hormones are mediated by specific receptors, which belong to a superfamily of trans-acting "zinc finger" proteins. After binding of the specific ligand, these receptor proteins influence gene expression via association with specific DNA elements known as hormone-responsive elements (HREs) (*1, 2*). Specific HREs are known for estrogen-, thyroid hormone–, progesterone-, and glucocorticoid-responsive genes (*1*). The estrogen-responsive element (ERE) is similar to, but distinct from, the glucocorticoid-responsive element (GRE) and the thyroid hormone–responsive element (T3RE) (*3–5*). The progesterone- and glucocorticoid-responsive elements are so similar as to be functionally identical (*1, 6*). $1,25(OH)_2D_3$ affects cellular replication and function in many cells and tissues through a typical steroidal hormone receptor (*7*) and is also thought to exert its effects through a vitamin D–responsive element (VDRE). It is not clear whether opposing effects on gene expression by different steroidal hormones are elicited through the same or different HREs.

Osteocalcin is the major noncollagenous bone protein, and its expression is stimulated by $1,25(OH)_2D_3$ and inhibited by glucocorticoids (*8*). We have therefore used promoter expression constructs of the human osteocalcin gene to investigate the influence of $1,25(OH)_2D_3$ and glucocorticoids on the activity of the osteocalcin promoter. We developed transient and stable transfection systems in a rat osteoblastic sarcoma cell line, ROS 17/2.8, which has an osteoblast (bone-forming cell) phenotype, expresses osteocalcin, and mineralizes in vitro (*9*). The promoter construct pOSCAT1 was made by exchanging the thymidine kinase (TK) promoter (*10*) of pTKCAT (*4*) for the −344- to +34-bp region of the osteocalcin gene (Fig. 1A), such that the osteocalcin promoter drives the bacterial chloramphenicol acetyl transferase (CAT) gene (Fig. 1B) (*11*). In ROS 17/2.8 cells, the promoter activity of pOSCAT1 was comparable to that of pTKCAT under the same conditions, that is, 30 times greater than background level as measured with a nonchromatographic CAT assay (*12*). This result demonstrated that the −344-bp region of the promoter was capable of directing significant CAT activity in the transfection system. However, pOSCAT1-derived CAT activity was not induced by $1,25(OH)_2D_3$, estrogen, synthetic progestin [Org 2058 (Organon International)], or thyroid hormones, but was repressed by 20 to 40% by $10^{-6}M$ dexamethasone. Dexamethasone had no effect on CAT activity derived from pTKCAT under the same conditions, indicating that the repression of pOSCAT1 by glucocorticoids is specific to the osteocalcin promoter and not an attribute of the parent plasmid.

In contrast to the lack of induction of pOSCAT1 by $1,25(OH)_2D_3$, the longer construct pOSCAT2, which contains an extra 1.0 kilobase pairs (kb) of further upstream sequences (Fig. 1B), showed a marked induction when transfected cells were treated with $1,25(OH)_2D_3$ at $10^{-7}M$. This induction was fivefold (5.0 ± 0.8) in the ROS 17/2.8 cells and up to 40-fold in the SAOS-2 human osteosarcoma cell line (*13*). Recently other workers, who have suggested that an equivalent 600- to 1000-bp region in the rat osteocalcin gene pro-

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