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The Antiviral Compound Enviroxime Targets the 3A Coding Region of Rhinovirus and Poliovirus

BEVERLY A. HEINZ* AND LORI M. VANCE

Infectious Disease Research, Lilly Research Laboratories, Indianapolis, Indiana 46285

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Enviroxime is an antiviral compound that inhibits the replication of rhinoviruses and enteroviruses. We have explored the mechanism of action of enviroxime by using poliovirus type 1 and human rhinovirus type 14 as model systems. By varying the time of drug addition to virus-infected cells, we determined that enviroxime could be added several hours postinfection without significant loss of inhibition. This suggested that the drug targeted a step involved in RNA replication or protein processing. To identify this target, we mapped 23 independent mutations in mutants that could multiply in the presence of 1 μg of enviroxime per ml. Each of these mutants contained a single nucleotide substitution that altered one amino acid in the 3A coding region. Using oligonucleotide-directed mutagenesis of cDNA clones, we have confirmed that these single-amino-acid substitutions are sufficient to confer the resistance phenotype. In addition, we conducted two experiments to support the hypothesis that enviroxime inhibits a 3A function. First, we determined by dot blot analysis of RNA from poliovirus-infected cells that enviroxime preferentially inhibits synthesis of the viral plus strand. Second, we demonstrated that enviroxime inhibits the initiation of plus-strand RNA synthesis as measured by the addition of [^{32}P]uridine to 3AB in poliovirus crude replication complexes. To our knowledge, this is the first evidence that 3A can be targeted by antiviral drugs. We anticipate that enviroxime will be a useful tool for investigating the natural function of the 3A protein.

Rhinoviruses (RVs) and enteroviruses (whose prototype is poliovirus [PV]) are members of the *Picornaviridae* family. These viruses have a positive-sense, single-stranded RNA genome which encodes four capsid proteins (VP1 through VP4) and seven nonstructural proteins that are involved in viral RNA replication and/or protein processing. Upon infection into susceptible cells, the viral RNA is first translated into one large polyprotein that is cleaved at different times during the life cycle into various final and precursor forms by two virus-encoded proteases. Because processing of the polyprotein has been extensively studied in vitro, the characteristics of the two proteases, 2A and 3C (and its precursor, 3CD), are understood in some detail (reviewed in reference 38). In addition to their known proteolytic functions, both 2A and 3C appear to participate in the shutoff of host cell metabolism (15, 34) and viral RNA replication (1, 2, 43); additionally, recent results indicate that 2A can enhance cap independent translation (29).

The remaining five proteins (2B, 2C, 3A, 3B, and 3D) and their precursor forms are involved primarily in viral RNA replication. It has been shown that replication occurs within the cytoplasm in virus-induced lipid-containing vesicles known as replication complexes (8, 12). These membranes are apparently required for the production of single-stranded RNA both in vivo (28) and in vitro (3, 18, 61, 62). In contrast to minus-strand RNA, which can be synthesized in vitro with isolated viral components in the absence of membranes (75), it has not yet been possible to reconstitute the machinery required for plus-strand RNA replication in vitro. Presumably, this restriction exists because many of the required viral proteins cannot be supplied in *trans* (7, 25). An alternative approach has been to investigate the replication of these viruses in vitro by using a system which couples translation and RNA replication in the presence of uninfected HeLa S10 extracts (5, 44, 45). The advantage of this technique is that it permits direct access to the components of replication which are normally shielded by the cellular membranes. It is likely, however, that this cell-free system will be limited by the same *cis*-acting requirements seen in infected cells (to date, only full-length virion RNA has served as transcript).

Therefore, although much is known about the general mode of RNA replication in these viruses, the role of several individual proteins during replication is still poorly understood. Polypeptide 3D is the primer-dependent RNA polymerase required for elongation of nascent RNA chains (24, 69). In vitro genetic studies (65) indicate that 3D is also responsible for adding the initial uridines to its primer, VPg (the uridylylation step), and may contain an RNA duplex-unwinding activity (13). Protein 2C and its precursor, 2BC, may be involved in membrane proliferation preceding vesicle formation (10) and are postulated to attach the growing RNA strands to the vesicular membranes and so maintain the correct spatial orientation of the replication complex (11). PV mutants resistant to or dependent on guanidine map to 2C (52, 53). In addition, recent studies have shown that 2C contains ATPase and GTPase activities (55). There is ample evidence indicating the importance of 2B in viral replication as well. Several noncomplementable mutants with mutations in PV 2B exhibited a dose-dependent dominance over the replication of wild-type virus, suggesting that 2B plays a structural role in replication complexes (33). Moreover, variants of RV2 and RV39 which could replicate in normally nonpermissive mouse cells were found to contain altered 2B proteins (40, 73).

A small, highly polar polypeptide, 3B (also known as VPg), is found covalently linked to all newly synthesized viral plus- and minus-strand RNAs and to nascent strands of the replicative intermediate (48). 3B has been proposed to serve as a primer for plus-strand RNA synthesis (46, 48, 61–63). Several observations support this idea. The strongest evidence is that uridylylated 3B (3B-pUpU) can be immunoprecipitated from

* Corresponding author. Mailing address: Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285-0438. Phone: (317) 276-6911. Fax: (317) 276-1743. Electronic mail address: Heinz_Beverly_A@Lilly.com.

PV-infected cells (16). This nucleotidyl-protein can be generated in vitro in membranous fractions from infected HeLa cells, and preformed 3B-pU can be chased into 3B-pUpU and nucleotidyl proteins containing nine or more of the PV 5′-proximal nucleotides (61). In addition, mutations in 3B cause a primary defect in RNA synthesis (35, 54).

For the hydrophilic 3B protein to function as a primer within a membranous environment, a hydrophobic carrier is most probably required. The 3AB precursor polypeptide is widely believed to be the donor of VPg to the replication complexes (25, 27, 57, 63). Amino- and carboxy-terminal sequencing identified 3AB as the primary 3B-containing precursor immunoprecipitated from infected cells (57). Larger precursors of 3B, including 2BC3AB and 2C3AB, have also been isolated from membrane fractions of infected cells which are active in poliovirus RNA replication. However, these larger polypeptides are rapidly turned over and are presumed to be the precursor forms of 3AB (63). Moreover, 3A contains a conserved hydrophobic region of 22 amino acids near its C terminus. In membrane fractions from infected cells, this portion of 3AB was protected against proteinase digestion as a result of its association with membranes; this protection was abolished in the presence of mild, nonionic detergent (63). The authors suggested that the termini of 3AB were present on the same side of the phospholipid bilayer, an orientation known for other amphipathic membrane proteins such as cytochrome b_5. More recently, it has been shown that a significant portion of 3A and 3AB can be glycosylated in vitro and that an inhibitor of glycoprotein synthesis also inhibits viral RNA synthesis (19). Further evidence implicating 3AB as the 3B donor comes from genetic manipulation of the 3A protein. Bernstein and Baltimore generated a PV mutant that was cold sensitive for RNA replication by introducing a single-codon insertion into the N-terminal region of 3A (6). Moreover, Giachetti and Semler (27) demonstrated that a temperature-sensitive mutant of PV1 containing a single-amino-acid substitution in the hydrophobic domain of 3A (Thr-67 to Ile) showed altered RNA synthesis in vitro and in vivo. Their results indicated that the function provided by the hydrophobic domain of 3A could not be supplied in *trans*. In a subsequent study, the same authors introduced deletions, insertions, and amino acid substitutions into the hydrophobic domain of PV 3A. Several of these mutants were unable to replicate RNA despite normal protein processing (25). Taken together, these studies suggest that 3A is involved in sequestering the viral proteins needed for formation and function of the replication complexes.

In the late 1970s, the antiviral compound enviroxime [2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenyl ketone oxime] was reported to have potent activity against RVs and enteroviruses in cell culture (20, 21, 71). Experimental evidence suggested that the compound specifically inhibited a step in RNA replication (72). Furthermore, the compound was found to inhibit RV replication in human embryonic nasal organ cultures even when added to tissues at 26 h postinfection (22). In initial clinical trials, oral administration of enviroxime induced undesirable gastrointestinal side effects, while intranasal drug administration was well tolerated and gave indications of therapeutic efficacy (50). However, subsequent clinical trials against both natural and experimentally induced rhinovirus infections produced disappointing results (30, 42, 51), and clinical trials were halted.

Nevertheless, the unusual antiviral potency and broad spectrum of activity of enviroxime make it an interesting tool for exploring the function of viral proteins within the replication complex. We have been studying the mechanism of action of enviroxime by using human RV14 and PV1 Mahoney as models. In this report, we present genetic and biochemical evidence that enviroxime blocks the replication of plus-strand viral RNA by targeting the viral 3A coding region. Using in vitro uridylylation assays, we have learned that the drug prevents proper formation of the replication complex and can apparently target the functions of both 3A and its precursor 3AB.

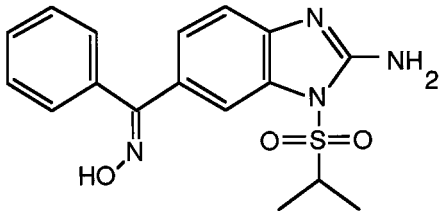

FIG. 1. Structure of enviroxime, 2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenyl ketone oxime.

MATERIALS AND METHODS

Cells and media. A cloned H-1 HeLa cell line, originally obtained from V. Hamparian, Ohio State University, was a gift from R. Rueckert, University of Wisconsin—Madison. Cell stocks were propagated in suspension culture by previously described methods (41). Monolayer cultures were grown in minimal essential medium supplemented with 10% newborn calf serum and 1% nonessential amino acids (medium A). Plaque assays were conducted in medium A supplemented with 12 mM $MgSO_4$ and 0.8% Noble agar.

Virus stocks and assay conditions. All viruses tested except for RV14 and coxsackievirus A21 (CA21) were obtained from the American Type Culture Collection. RV14 (strain 1059) was originally obtained from V. V. Hamparian, Ohio State University; we received it as a gift from R. R. Rueckert. CA21 was a Coe strain variant adapted to grow in 10-g mice (66). PV infections were conducted at 37°C; RV infections were conducted at 35°C. High-titer virus stocks were produced by inoculating suspension cell cultures (1.2×10^8 cells) at a multiplicity of infection (MOI) of 5 to 10 PFU per cell. Following a single cycle of replication (6 h for PV; 8 h for RV14), cells were pelleted and virus stocks were harvested as previously described (59). For plaque development, virus-infected monolayer cultures were incubated for 24 to 28 h (PV1) or 48 to 52 h (RV14). Viral plaques were visualized for selection by an additional 60-min incubation in the presence of 0.33 g of neutral red per liter in medium A.

Drug solutions. Enviroxime (Lilly Research Laboratories) (Fig. 1) was dissolved in dimethyl sulfoxide (DMSO) at 1,000 × concentrations and diluted 1/1,000 into culture media (final concentration of DMSO, 0.1%). Although DMSO had no detectable effect on virus replication, 0.1% DMSO was added to all no-drug control samples.

Spectrum of activity. The inhibitory effect of 0.1, 1.0, and 10 μg of enviroxime per ml was tested against seven representative RVs and enteroviruses in single-cycle infections in monolayer cultures. Cells were infected at an MOI of 1 and incubated in medium A supplemented with drug or just DMSO under the appropriate conditions: PV1, PV3, and coxsackievirus B3 (CB3) at 37°C for 6 h; PV2 and CA21 at 37°C for 8 h; and the RVs at 35°C for 8 h. Virus yields were quantitated by plaque assay. As a result of this experiment, we selected PV1 and RV14 in 1 μg of enviroxime per ml (2.9 μM) for further study.

Reversal of inhibition. To determine whether the inhibitory effect of enviroxime could be reversed, we incubated PV1- and RV14-infected monolayer cultures (MOI, 1) in medium A containing 1 μg of enviroxime per ml or 0.1% DMSO alone for 4 h. The cultures were rinsed four times with phosphate-buffered saline (pH 7.2) and reincubated in drug-free medium for an additional 6 h (PV1) or 8 h (RV14). Virus yields were harvested, and titers were determined by standard methods.

Enviroxime time-of-addition curves. To identify the approximate stage in the virus life cycle that is inhibited by enviroxime, we examined the effect of adding the drug to RV14- and PV1-infected cells at various times postinfection. Cell monolayer cultures were infected at an MOI of 1 and incubated for up to 6 h (PV1) or 8 h (RV14) in drug-free medium A. At 1-h intervals, the medium was replaced with medium A containing 1 μg of enviroxime per ml or just 0.1% DMSO and reincubated for the remainder of the growth cycle. Virus yields were harvested, and titers were determined by plaque assay.

Inhibition of plus- and minus-strand RNA. The inhibitory effect of enviroxime on plus- and minus-strand virion RNA was examined by dot blot analysis of total RNA isolated from virus-infected cells. Cell monolayers were infected with PV1 at an MOI of 1 and incubated at 37°C in serum-free medium A for 4 h. The medium was then replaced with fresh medium containing 1 μg of enviroxime per ml (or DMSO alone) in duplicate cultures: one culture was harvested immediately for 4-h baseline measurements, and the other was incubated for an additional 2 h at 37°C. Total RNA was harvested from all cultures by standard

procedures (56). To detect minus-strand RNA, 1 mg of total RNA was subjected to RNase protection as described by Novak and Kirkegaard (49). The remaining RNA was precipitated with tRNA and ethanol and resuspended in blotting buffer (20 μl of formamide, 7 μl of formaldehyde, 2 μl of 20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], and 10 μl of H_2O per sample). To detect plus-strand RNA, 1 mg of total RNA was subjected to poly(A)$^+$ selection by oligo(dT) cellulose chromatography. Prepacked oligo(dT) columns (Gibco/BRL, Bethesda, Md.) were primed with 0.1 M NaOH and equilibrated with 1× binding buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 0.5 M NaCl, 0.5% sodium dodecyl sulfate [SDS]). The RNA (in 250 μl of water) was diluted 1:1 in 2× binding buffer, heated at 70°C for 5 min, cooled on ice for 5 min, and loaded onto the column. The effluent was collected, and the column was washed with 500 μl of 1× binding buffer; these solutions were pooled and designated the (A−) fraction. The columns were then washed with 4 ml of 1× binding buffer, and the effluent was discarded. Bound RNA was eluted by washing the column three times with 500 μl of elution buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 0.2% SDS) and designated the (A+) fraction. The (A−) fractions were precipitated and subjected to RNase protection as described previously (49). The (A+) fractions were precipitated, combined with the RNase-treated (A−) fractions, and resuspended in blotting buffer. The RNA samples were heated at 68°C for 15 min, diluted in 2 volumes of 20× SSC, and blotted onto nitrocellulose membranes (Duralose-UV) with a vacuum manifold. Wells were washed twice with 10× SSC, dried for 5 min, and bound to the filters by UV cross-linking. As a control for the specificity of our oligonucleotide probes, 1 μg of viral RNA (extracted from wild-type PV1 as described below) was resuspended in blotting buffer and bound to membranes in the same manner.

The samples were probed for plus- or minus-strand RNA by hybridization with ^{32}P-labeled oligonucleotides at 42°C following standard procedures (56). To detect plus-strand RNA, we used a 20-nucleotide sequence corresponding to a portion of the VP1 capsid protein: 5′-CGGTGAGTGCCGGAATTTCC-3′; to detect minus-strand RNA, we used the complementary sequence. Individual dots were quantified by liquid scintillation counting.

Selection and characterization of spontaneous drug-resistant mutants. Wild-type plaques of PV1 and RV14 were individually amplified in monolayer cultures to provide independent stocks from which to select resistant mutants. These were first used to infect monolayer cultures that were incubated under agar overlay containing 1 μg of enviroxime per ml. However, no discrete plaques developed, despite a prolonged (72-h) incubation period. Therefore, resistant mutants were isolated in a four-step procedure. First, each virus stock was grown in monolayer culture in medium A containing 1 μg of enviroxime per ml for a three-cycle infection period (18 h for PV1; 24 h for RV14). Second, these virus preparations were grown in monolayer culture to cytopathic effect in drug-free medium A. In the third cycle, growth in 1 μg of enviroxime per ml was repeated, followed in the fourth cycle by another amplification to cytopathic effect in the absence of drug. The resulting virus preparations were overlaid for plaque development in drug-free medium, and isolated plaques were picked. Each mutant candidate was further amplified in drug-free medium to a final titer of 10^9 to 10^{10} PFU/ml. A total of 23 independent mutants were selected: 10 of PV1, and 13 of RV14.

Mutant infectivity was assayed by comparing virus yields following a three-cycle infection (18 h for PV1 or 24 h for RV14) at an MOI of 1 in serum-free medium A in the presence or absence of drug (mutants showed wild-type drug sensitivity when assayed in single-cycle infections). The harvested virus was quantitated by plaque assay in the absence of drug.

Viral RNA extraction and sequencing. Amplified PV1 mutants were pelleted (39), and PV1 RNA was extracted with phenol-chloroform and with isoamyl alcohol (31). Amplified RV14 mutants were immunoprecipitated with a monoclonal antibody (a gift from R. Rueckert), and viral RNA was extracted with guanidine isothiocyanate and acid-phenol (14). Selected regions of the genomes were sequenced by second-strand synthesis as described previously (31).

Site-directed mutagenesis. To confirm that single amino acid substitutions in 3A were sufficient to confer the resistant phenotype, we introduced the mutations that had been identified in spontaneous isolates into cDNA clones that produce infectious transcripts (generous gifts from R. Rueckert [RV14] and R. Andino, Gladstone Institute for Virology & Immunology [PV1]). Mutagenesis was performed on full-length cDNAs with the Transformer Mutagenesis system (ClonTech Laboratories Inc., Palo Alto, Calif.) (23). We used Sequenase sequencing (United States Biochemical, Cleveland, Ohio) to identify cDNAs that had incorporated the desired 3A mutation. Mutant cDNAs were linearized by digestion with *Mlu*I and transcribed in vitro with T7 RNA polymerase under standard conditions (68). Mutant RNAs were transfected into HeLa cell monolayers by procedures described by Lee et al. (39). Isolated plaques were picked, amplified in monolayer cultures, and characterized for resistance to enviroxime as above.

Attempts to produce highly resistant RV14. We attempted in two ways to produce mutants of RV14 with a higher level of drug resistance than manifested by our initial set of mutants. First, we tried to select spontaneous multiple mutants capable of completing a growth cycle in 8 h in the presence of 1 μg of enviroxime per ml. Using the mutant viruses that had been constructed by site-directed mutagenesis as the inocula, we repeated the four-step selection procedure described above, this time limiting the period of virus growth in drug to 8 h; the amplifications to cytopathic effect were also conducted in the presence of the drug. Second, we constructed two RV14 mutants with multiple substitutions in the 3A region by site-directed mutagenesis. These transfections were incubated for plaque development at 32, 35, and 37°C to detect a temperature-sensitive phenotype.

TABLE 1. Inhibitory effect of enviroxime on representative picornaviruses

Virus	Relative resistance (% of survivors) in presence of drug at[a]:		
	0.1 μg/ml	1.0 μg/ml	10 μg/ml
PV1, Mahoney	32	0.05	0.04
PV2	45	0.3	0.3
PV3	1.3	0.02	0.02
RV14	4.9	0.63	0.67
RV16	6.8	0.84	1.1
CA21	32	0.02	0.02
CB3	21	0.04	0.04

[a] Virus yields in PFU following single-cycle infections in monolayer cultures at an MOI of 1. Numbers are percentage of survivors relative to no-drug controls.

In vitro uridylylation assays. To investigate the effect of enviroxime on the initiation of plus-strand RNA synthesis more closely, we conducted in vitro uridylylation assays with isolated crude replication complexes (CRCs) of PV1. In the first experiments, we determined whether enviroxime inhibited the uridylylation reaction if added either during the virus infection or after the complexes were isolated. Suspension HeLa cells (6.5×10^7 cells) were infected with PV1 at an MOI of 1, and serum-free medium A containing 1 μg of enviroxime per ml, 50 μg of 74385 (a control drug) per ml, or just DMSO was added to each culture. The compound 74385 [5-(3′,4′-dichlorophenyl)5-methylhydantoin; Lilly Research Laboratories] is an inhibitor that blocks PV1 RNA encapsidation without affecting RNA replication (67). In subsequent experiments, 1 μg of enviroxime per ml was added to PV1-infected HeLa cells at 0, 1, 2, or 3 h; cultures treated with DMSO only were harvested at each time point as controls. In all experiments, the cultures were incubated at 37°C for a total time of 5 h. CRCs were prepared as described by Takeda et al. (61). CRCs from drug-treated cultures were extracted in buffers containing 1 μg of enviroxime per ml or 0.1% DMSO (mimicking the infection conditions) and stored at −80°C. The CRCs were thawed, sonicated for 10 min, and allowed to stand for 1 h at room temperature. A 10-μl aliquot was removed, and membranes were collected by microcentrifugation at top speed for 15 min. Pellets were suspended in 7.5 μl of reaction buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH, 3.5 mM magnesium acetate, 10 mM dithiothreitol, 50 mM KCl, 10 μg of actinomycin D per ml, 2.5 μM creatine P, 50 μg of creatine P kinase per ml, 1 μM ATP, 30 μCi of [γ-^{32}P]UTP [800 Ci/mol]) supplemented with the compounds in 1% DMSO. As well as addition of drugs to previously untreated samples, drugs were maintained in those that had been treated during infection. Reactions were conducted at 30°C for 3 h. The reaction mixture was diluted with 20 μl of resuspension buffer, and radiolabeled proteins were immunoprecipitated as described by Semler (57, 58), using 10 μl of anti-3B antiserum, generated in rabbits by previously published procedures (4, 74). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide) and autoradiography.

RESULTS

Spectrum of activity. We compared the activity of enviroxime against several representative picornavirus serotypes by determining the percentage of survivors relative to that in no-drug control samples following single-cycle infections in the presence of 0.1, 1.0, and 10 μg of drug per ml (Table 1). For all viruses, there was no significant decrease in the surviving population by increasing the drug concentration from 1.0 to 10 μg/ml; this suggests that the surviving plaques at both concentrations are likely to be the same population of resistant mutants. In contrast, drug inhibition was considerably reduced at 0.1 μg/ml. On the basis of these results, we selected PV1 and RV14 at 1 μg of enviroxime per ml for further study.

Reversal of inhibition. By washing and reincubating drug-treated cultures in fresh medium, we were able to recover 3 to 4 log units of virus yield (compared with unrinsed samples); this corresponds to 2 to 30% of the yield for no-drug controls. Thus, it appears that the majority of the inhibitory effect can be readily reversed.

Enviroxime time-of-addition curves. By adding enviroxime

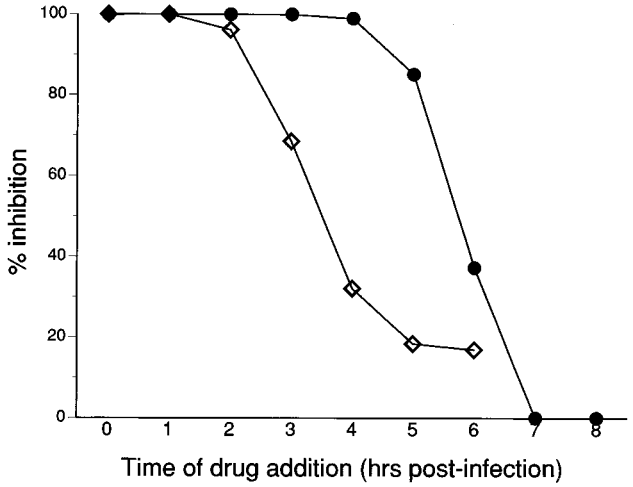

FIG. 2. Inhibitory effect of enviroxime added to infected cells at hourly intervals postinfection. HeLa monolayers were inoculated at an MOI of 1 with either RV14 (circles) or PV1 (diamonds), and the virus was allowed to attach for 30 min at room temperature. Medium A containing 1 μg of enviroxime per ml (zero-time samples) or 0.1% DMSO alone was added, and the cultures were incubated at either 37°C (PV1) or 35°C (RV14). Enviroxime was added to the remaining cultures when indicated. Virus yields were harvested after 6 h (PV1) or 8 h (RV14) and quantitated by plaque assay in the absence of drug. Yields are relative to those for drug-free controls assayed at each time point.

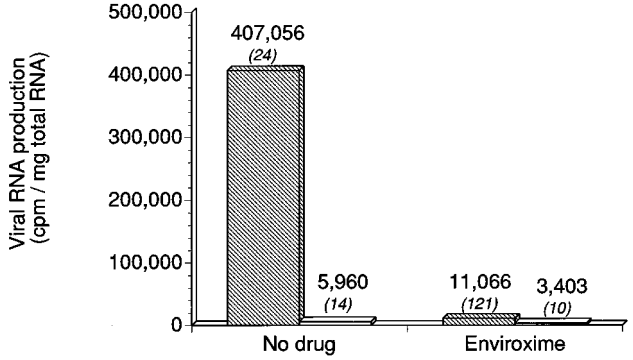

FIG. 3. Inhibitory effect of enviroxime on the production of plus- and minus-strand viral RNA. HeLa cell monolayers were infected with PV1 at an MOI of 1 and incubated at 37°C in drug-free medium A for 4 h. The medium was replaced with fresh medium containing 1 μg of enviroxime per ml (or just 0.1% DMSO) in duplicate plates: one was harvested immediately for 4-h baseline measurements; the other was incubated for an additional 2 h at 37°C. Total RNA was harvested from all plates, and 1 mg was used for the detection of plus- or minus-strand RNA. Minus-strand RNA was subjected to RNase protection, and plus-strand RNA was subjected to oligo(dT) chromatography before being blotted onto nitrocellulose and probed with ^{32}P-labeled strand-specific oligonucleotides. Radioactivity was quantitated by liquid scintillation counting. Results shown indicate the production of plus-strand RNA (hatched bars) and minus-strand RNA (open bars) during the period from 4 to 6 h postinfection (i.e., 4-h cpm values were subtracted as background). Values are the mean of three experiments; results found for mock-infected samples are shown in parentheses.

at various times postinfection, we determined that the drug targets a step in the middle of the replication cycle (Fig. 2). In PV1-infected cultures, drug addition could be delayed 1 to 2 h without significant loss of inhibitory activity. In RV14-infected cultures, drug addition could be delayed for 3 to 4 h before inhibition was significantly affected. These periods correlate to peak periods of RNA replication (9).

Inhibition of plus- and minus-strand RNA. We used dot blot analysis of total RNA from PV1-infected cells to determine whether enviroxime inhibits the production of viral RNA. Plus and minus strands were assayed separately to detect any preferential effect. Because the concentration of viral RNA in the first 3 h of infection was below our level of detection (data not shown), we postponed drug addition until 4 h postinfection (at which point the stage of maximal inhibition was passed [Fig. 2]). Nevertheless, sufficient RNA was produced during the last 2 h of infection (from 4 to 6 h postinfection) for us to detect a significant inhibitory effect in drug-treated samples compared with mock-infected samples (Fig. 3). Values shown are the mean of three experiments. As an additional control, we assayed virion RNA to determine the specificity of the oligonucleotide probes: counts detected by the probe for plus-strand RNA were 230-fold higher than those detected by the probe for minus-strand RNA. Virus infections conducted in drug-free medium showed a ratio of plus- to minus-strand RNA of approximately 68:1; in the presence of enviroxime, this ratio dropped to 3:1. Therefore, enviroxime inhibited the production of plus-strand RNA at least 35-fold, whereas it inhibited the production of negative-strand RNA less than 2-fold.

Selection and mapping of spontaneous drug-resistant mutants. Twenty-three independent mutants able to replicate in 1 μg of enviroxime per ml were isolated and examined by sequence analysis. Of these, 13 mutants were derived from RV14 and 10 mutants were derived from PV1. Initially, several proteins within the P3 region of the genome were sequenced; later, we sequenced only the 3A and 3B regions. Each mutant was found to contain a single nucleotide substitution that altered one amino acid in 3A (Table 2). Mutations in 7 of the 13 RV14-resistant mutants mapped to Glu-30; 5 of the 6 remaining RV14 mutants displayed identical substitutions at Ile-42. In PV1, 7 of the 10 mutants contained the identical substitution at Ala-70; the other three sites (Ala-71, Asn-45, and Asn-57) were represented by only 1 isolate each.

Confirmation by site-directed mutagenesis. To confirm that the drug-resistant phenotype was due to the identified 3A mutations, we introduced single nucleotide substitutions into full-length viral cDNA clones by oligonucleotide-directed mutagenesis. These viruses were assayed for infectivity in the presence and absence of enviroxime (Table 3). All mutants grew normally in the absence of drug. In all cases, viruses containing only one mutation in 3A had at least 20-fold-higher resistance than did the wild-type virus when tested in 1 μg of drug per ml and at least 6-fold higher in 10 μg of drug per ml. For RV14, the resistance levels of cDNA-derived mutants correlated with levels found for spontaneous isolates (data not

TABLE 2. Spontaneous enviroxime-resistant mutants[a]

Virus	Amino acid substitution in 3A	Mutation	No. of isolates
RV14	Glu-30 to Asp	GAA → GAU	2
	Glu-30 to Val	GAA → GUA	4
	Glu-30 to Gln	GAA → CAA	1
	Ile-42 to Val	AUU → GUU	5
	Met-54 to Ile	AUG → AUC	1
PV1	Asn-45 to Tyr	AAC → UAC	1
	Asn-57 to Asp	AAC → GAC	1
	Ala-70 to Thr	GCC → ACC	7
	Ala-71 to Ser	GCA → UCA	1

[a] Mutants were selected for resistance to 1 μg of enviroxime per ml by following a four-step protocol that alternates growth in drug-containing and drug-free media.

TABLE 3. Confirmation of 3A mutations in RV14 and PV1[a]

Virus	3A mutation	Resistance (% of survivors) to drug at[b]:	
		1 μg/ml	10 μg/ml
RV14	Wild type	0.03	0.03
	Glu-30 to Gln	1.5	0.3
	Glu-30 to Asp	0.6	0.2
	Glu-30 to Val	1.1	0.2
	Ile-42 to Val	2.1	0.5
	Met-54 to Ile	5.5	1.4
PV1	Wild type	0.02	0.01
	Asn-45 to Tyr	21	8.8
	Asn-57 to Asp	18	10
	Ala-70 to Thr	30	11
	Ala-71 to Ser	6.1	1.3

[a] Single-amino-acid substitutions were introduced into cDNA clones by oligonucleotide-directed mutagenesis.

[b] Although the mutants grew normally in the absence of drug, all grew poorly in the presence drug. Thus, resistance was measured as PFU following one three-cycle (24- or 18-h, respectively) infection in monolayer cultures at an MOI of 1. Numbers are percentage of survivors relative to no-drug controls.

shown). In contrast, some of the spontaneous PV1 mutants exhibited a five- to eightfold-lower level of drug resistance than did their cDNA-derived counterparts (data not shown). This indicates the possible presence of a mutation which influences resistance in the PV1 cDNA relative to our parental stock outside of the 3A region. This difference was not observed when comparing parental and cDNA-derived PV1 stocks that contain no 3A mutations (that is, the wild-type viruses from both sources exhibited the same level of sensitivity).

Attempts to produce highly resistant RV14 mutants. Using cDNA-derived RV14 mutants as inocula, we attempted to enrich for mutants that could complete a growth cycle in 8 h in medium containing 1 μg of enviroxime per ml. These experiments were not successful (i.e., no survivors could be isolated). As an alternative approach, we used oligonucleotide-directed mutagenesis to construct two mutants of RV14 that contained more than one mutation in the 3A region. First, we constructed a mutant virus containing both the Glu-30-to-Gln and Ile-42-to-Val substitutions. RNA transcripts containing these two mutations failed to produce infectious virus upon transfection into cells at any temperature (several independent constructs were tested). Second, we constructed a triple mutant containing Glu-30-to-Gln, Ile-42-to-Val, and Met-54-to-Ile. These transcripts produced very small, diffuse plaques after incubation for 72 h at 35°C; there was no evidence of temperature sensitivity. Because this virus grows so poorly, we were unable to accurately determine its sensitivity to enviroxime.

In vitro uridylylation. To examine the inhibitory effect of enviroxime in RNA synthesis more closely, we conducted in vitro uridylylation assays on isolated CRCs of PV1. In uridylylation assays (61), the smallest band (approximately 3 kDa) corresponds to 3B covalently linked to one or two uridines; this represents the initiation step of RNA synthesis. The heavy smear of larger-molecular-mass RNA represents different lengths of single and duplex RNAs and the partially double-stranded replicative intermediate forms. In the first set of experiments, we tested whether enviroxime prevented the initiation and/or elongation reactions of plus-RNA synthesis (Fig. 4A). Enviroxime added to infected cells immediately after virus adsorption completely inhibited both the initiation and elongation reactions; in contrast, the control inhibitor 74385 had no effect on these processes. Inhibition was not observed, however, when enviroxime was added directly to isolated CRCs. In the second set of experiments, we examined the inhibitory effect of the drug when added to infected cells at various times postinfection (Fig. 4B). Drug added at 0, 1, and 2 h postinfection totally inhibited both RNA initiation and elongation. However, enviroxime added at 3 h postinfection showed a strong inhibition of elongation with minimal, if any, effect on RNA initiation. As a control, we also examined untreated CRCs harvested at the same time points. These CRCs showed a simultaneous gradual increase of both initiation and elongation (data not shown). In this assay, drug added at 4 h

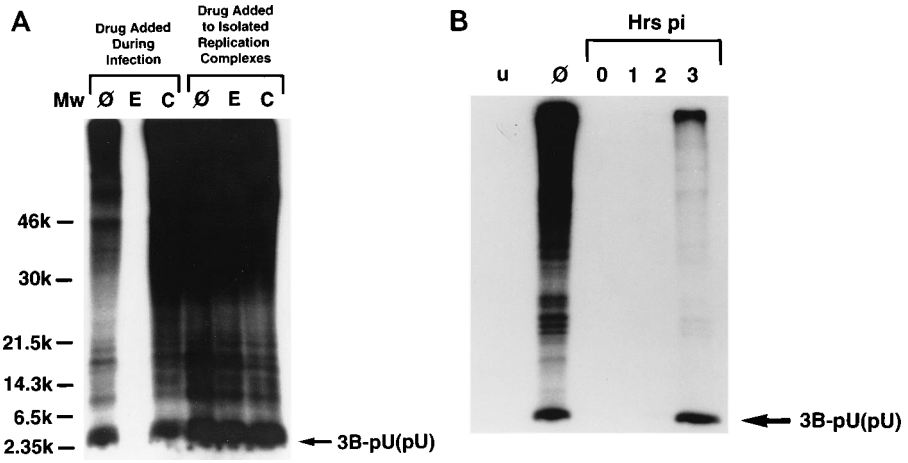

FIG. 4. (A) Uridylylation assay showing the effect of enviroxime addition on the initiation and elongation of plus-strand RNA. HeLa cell suspensions were infected with PV1 at an MOI of 1. Medium A containing 1 μg of enviroxime (E) per ml, 0.1% DMSO (Ø), or a control inhibitor that does not affect RNA synthesis (C) was added to half of the cultures (first three lanes); the other half were incubated in medium A without any additives. At 5 h postinfection, CRCs were harvested and drugs were added to the previously untreated samples (last three lanes); the CRCs were frozen, thawed, and sonicated to enhance drug diffusion into the complex. Uridylylation assays were performed by incubation for 3 h at 30°C with creatine P and creatine P kinase supplemented with [^{32}P]UTP, and the products were immunoprecipitated with anti-3B antiserum and electrophoresed on SDS-polyacrylamide gel electrophoresis (12% polyacrylamide) gels. (B) Uridylylation assay showing the effect of enviroxime addition at various times postinfection. HeLa cell suspension cultures were infected with PV1 at an MOI of 1 and incubated at 37°C in medium A. At 0, 1, 2, or 3 h postinfection (pi), 1 μg of enviroxime per ml or just 0.1% DMSO (Ø) was added to the cultures. CRCs were harvested at 5 h postinfection, and uridylylation reactions were conducted as described. u, uninfected controls.

post-infection had lost all of its inhibitory activity (data not shown).

DISCUSSION

The analysis of antiviral drugs and drug-resistant mutants has proven extremely valuable for studies of picornavirus function. Examples include virus attachment to cellular receptors (59), virus uncoating (47), and the role of 2C in viral RNA replication (52, 53). We have explored the mechanism of action of enviroxime, an inhibitor which at one time showed tremendous promise as an antiviral drug. Despite high potency and a broad spectrum of action, this drug failed the rigors of clinical development. Nevertheless, in this paper we provide evidence indicating that enviroxime may be a valuable tool for exploring the structure of the viral replication complex in general and the functions of proteins 3A and 3AB in particular.

We have demonstrated that single-amino-acid substitutions in the 3A region of PV1 and RV14 are sufficient to confer a resistance phenotype. Like other single-stranded RNA viruses, the picornaviruses have a spontaneous mutation frequency of 10^{-4} to 10^{-5} (32). Naturally occurring resistant viruses containing single-amino-acid substitutions are generally selected by picking plaques that develop in the presence of the selecting agent (31, 60). In this study, we were unable to isolate resistant plaques by this approach; single-site mutants are apparently not sufficiently resistant to form plaques in 1 μg of drug per ml. Instead, we used a four-step enrichment procedure that alternated growth in drug-containing and drug-free media, a procedure which is more likely to encourage the appearance of variants containing more than one mutation. Therefore, it was vital that we confirm that single 3A mutations were sufficient to confer the resistance phenotype by engineering these single mutations into the viral cDNA.

In all cases, drug-resistant mutants grew normally in the absence of drug. This demonstrates that the 3A polypeptide is surprisingly tolerant of mutations both in the hydrophobic domain, at least for PV1, and at N-terminal regions; in a previous study, many of the mutations introduced by genetic engineering turned out to be lethal (25). In contrast, all of the drug-resistant mutants were growth impaired in the presence of 1 μg of drug per ml. Resistance was manifested only by extending the incubation period beyond a single growth cycle. Rather than quantitate the length of a single cycle in drug for each mutant, we selected an incubation period equivalent to three infection cycles as a standard for comparison. Even then, the resistance levels demonstrated by the mutants were relatively low (Table 3). For example, the RV14 mutant isolated most frequently, containing the Ile-42-to-Val mutation, was only 70-fold more resistant than the wild type in 1 μg of drug per ml and 17-fold more resistant than the wild type in 10 μg of drug per ml. It is interesting that only one isolate of the most resistant RV14 mutant, containing the Met-54-to-Ile mutation, was selected. In PV1, the most resistant mutant, containing the Ala-70-to-Thr mutation, did predominate. This mutant was 1,500-fold more resistant than the wild-type virus in 1 μg of drug per ml and 1,100-fold more resistant in 10 μg/ml.

We attempted to select a highly resistant variant of RV14 that could complete a replicative cycle within 8 h by alternating 8- and 24-h infections in 1 μg of enviroxime per ml. Our inability to select a virus under these rigorous conditions implies that either several (more than two) simultaneous mutations are required or the necessary additional mutation(s) would severely compromise virus growth. The RV14 mutants constructed to contain multiple mutations in 3A were severely growth compromised. This suggests that high resistance is not likely to result from combining several 3A mutations in a viral variant.

Resistance to enviroxime can apparently be enhanced somewhat by additional mutations in other regions, however. Whereas some of the spontaneous isolates of PV1 exhibited the same resistance level as that of their cDNA-derived counterparts, a small number of them displayed a five- to eightfold-lower level of drug resistance (data not shown). This result suggests that the cDNA-derived PV1s differ from the parental PV1 used for mutant selection in some way that influences the resistance to enviroxime. However, this effect was not observed in the absence of 3A mutations, since both wild-type virus stocks showed the same degree of drug sensitivity. Thus, we believe that both our cDNA and some of our spontaneous isolates (i.e., those with the higher resistance levels) contain secondary mutations in other regions of the genome which affect resistance only when the primary 3A change is present. Efforts are under way to identify these additional mutations. We believe that their identity will provide unique insight into the structure of the replication complex. Although there is substantial evidence localizing all of the viral nonstructural proteins to the replication complex (8, 57, 63, 64), evidence for specific interactions between 3AB and other proteins is very limited. For example, it has been reported that 2C and 3AB are strongly associated (36); however, the biological significance of this interaction is not clear. More recently, Lama et al. (37) have demonstrated that purified poliovirus 3AB can enhance the in vitro activity of the 3D polymerase, suggesting that 3AB may serve as a cofactor for 3D in viral transcription.

It is interesting that the regions of 3A to which drug resistance mapped were different for RV14 and PV1 (Fig. 5). Examination of the protein sequence alignments suggests that the two viruses are converging on a common sequence. Mutations conferring resistance in RV14 were located exclusively upstream of the hydrophobic domain near patches of charged amino acids. Two of the three mutated residues were replaced by amino acids naturally found in PV1: Glu-30 to Gln and Met-54 to Ile. Some of these charged patches are likely to lie on the protein surface and so are putative areas of interaction with other proteins (17, 70). A variety of substitutions were observed, however, so it is difficult to envision how the protein-protein interactions may have been affected. In contrast to RV14, most of the PV1-resistant mutants contained an amino acid substitution within the hydrophobic domain: Ala-70 to Thr (the predominant mutation) or Ala-71 to Ser. It is tempting to speculate that resistance to enviroxime in both viruses is enhanced by the presence of a hydroxyl-containing amino acid in this region of 3A. In the analogous region in RV14, two hydroxyl-containing amino acids already exist (Ser-69 and Thr-70). If this hypothesis is correct, the introduction of a third residue of this type (Val-68 to Thr) analogous to the preferred mutation in PV1 should not confer drug resistance. We have confirmed this prediction by site-directed mutagenesis of the wild-type RV14 cDNA (data not shown).

Significant evidence exists which implicates 3AB as the VPg donor for the initiation of plus-strand RNA synthesis (25, 27, 57, 63). It is believed that 3AB is anchored to intracellular membranes via a conserved hydrophobic domain at its C terminus (26, 57). Mutations engineered into the hydrophobic domain of 3A cause defects in plus-strand synthesis which are noncomplementable or *cis* acting (25). Our data are consistent with this model. First, we have shown that enviroxime specifically inhibits plus-strand synthesis in infected cells: the effect of the drug on the production of plus strands was more than 20-fold greater than its effect on minus strands. Second, we demonstrated that enviroxime prevented the initiation reac-

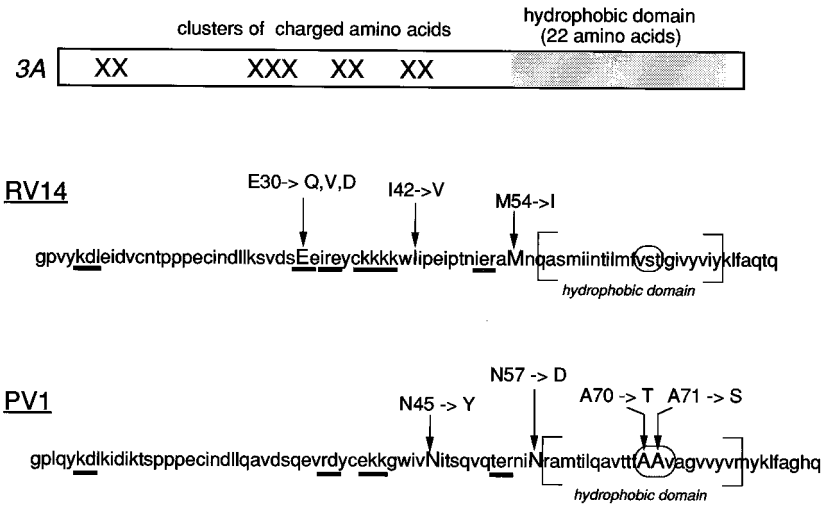

FIG. 5. Sequences of RV14 and PV1 3A showing the locations of mutations conferring resistance to enviroxime relative to patches of charged residues (underlined) and the hydrophobic domains (brackets). The presence of hydroxyl-containing amino acids in a region of the hydrophobic domain (circles) may be important for drug resistance.

tion, as measured by in vitro uridylylation of preformed replication complexes. The observation that enviroxime was active only if added to virus-infected cells suggests that the drug functions during formation of the replication complexes; apparently, the target of the drug remained inaccessible despite efforts to disrupt the complexes in vitro by freezing and thawing followed by sonication.

In addition to serving as donor for VPg, 3A may play a second, unrelated role during RNA replication. Bernstein et al. have described a cold-sensitive mutant containing a lesion in the N-terminal portion of 3A that could be complemented by mutants containing lesions in other viral proteins (7). This finding implies that 3A may function both in *cis* and in *trans* during the replicative cycle. The results from our in vitro uridylylation assays further support the idea that 3A serves multiple functions during replication. Adding enviroxime to infected cells at different times postinfection resulted in the ability to inhibit the initiation and elongation of viral RNA synthesis independently. This suggests that the role played by 3A during elongation is distinct from its participation in the priming event, perhaps via an association with another viral replicative protein. Thus, enviroxime added early in the replication cycle would prevent initiation by interacting with 3AB; drug added later would be more likely to inhibit a step involving 3A.

Taken together, our studies indicate that enviroxime has a unique mechanism of action among antiviral agents active against RVs and enteroviruses. Our data suggest that the drug may bind directly to 3AB and/or 3A, altering its conformation and thus preventing normal formation of the replication complex. Bound drug is likely to affect the association of 3AB both with membrane vesicles and with other viral proteins; both effects would result in the inhibition of plus-strand RNA replication. Drug resistance seems to involve the presence of a hydroxyl-containing residue in a specific portion of the hydrophobic domain and patches of charged amino acids which are likely sites of 3A-protein interactions. Since enviroxime is a hydrophobic molecule, it is possible that the drug binds directly to the hydrophobic domain of 3A. Therefore, mutations in this region may alter drug binding, whereas mutations near charged patches may functionally compensate for altered interactions with other proteins. Additional studies have been initiated to address these questions. Although single amino acid substitutions in 3A were sufficient to confer a low level of resistance, it is likely that high levels of resistance will require several mutations in different replicative proteins. As a result, 3A appears to be an excellent target for the development of antiviral drugs.

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