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# Infectious cDNA Clones of Echovirus 12 and a Variant Resistant against the Uncoating Inhibitor Rhodanine Differ in Seven Amino Acids

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**Determination of the complete sequences of echovirus 12 and a rhodanine-resistant variant revealed seven amino acid deviations and two additional exchanges not confirmed in all clones. In rhodanine sensitivity assays with infectious cDNAs, it was shown that the biological markers of the original viruses are maintained.**

We present the first sequence of echovirus 12 prototype Travis, a human enterovirus, and of a variant resistant against the uncoating inhibitor rhodanine. Despite their clinical importance, little is known about the pathogenic mechanisms and molecular biology of echoviruses. In order to study early events in picornavirus infection, antiviral compounds stabilizing the virus by interacting with the capsid turned out to be a valuable tool. Rhodanine (2-thio-4-oxothiazolidine) blocks selectively the uncoating of echovirus 12, whereas virus adsorption, penetration, virus macromolecular synthesis, and assembly are not affected (2, 4).

Echovirus 12 (Travis) RNA was cloned and sequenced (Fig. 1). Excluding the poly(A) tract, the RNA genome is 7,422 nucleotides (nt) in length and encodes a single polyprotein of 2,193 amino acids. The genome comprises a 5′ noncoding region of 741 nt, an open reading frame of 6,579 nt, and a 3′ noncoding part of 102 nt. Comparison with other picornaviruses exhibited a close relationship to echovirus 11 and the coxsackie B viruses. Capsid proteins VP1, VP2, and VP3 were separated as described previously (6) and sequenced at their N termini in order to determine the posttranslational cleavage sites of the P1 region (Fig. 1).

To generate a rhodanine-resistant variant of echovirus 12, we isolated plaques appearing on plates containing 100 μg of rhodanine per ml in the overlay. After five passages in cell culture with rhodanine-containing medium, we selected a virus stock with infectious titers of $8 \times 10^7$ PFU/ml without and $6 \times 10^7$ PFU/ml in the presence of rhodanine. RNA of this variant was cloned, and the sequence was determined. The polyprotein of the resistant variant differs from that of the wild type in at least 7 amino acids (Fig. 2). Six differences are located in the capsid proteins: two in VP2, one in VP3, and three in VP1. The seventh amino acid exchange is located in the P3 region close to the 3′ end of the virus. Furthermore, we found an additional exchange at position 2748 (Fig. 2, no. 5a) in four of six clones and in one of the remaining two clones an exchange at position 2650 (Fig. 2, no. 4a), both located in VP1.

A full-length wild-type cDNA clone (pT7E12wt) was constructed by ligating a PCR fragment spanning the very 5′ end of the genome to the longest cDNA clone. The RNA transcribed in vitro was identical to the viral genome except for three indispensable G's at the 5′ end due to the internal starting point of the T7 promoter and four additional nucleotides (GGGC) flanking the poly(A) tail of 109 adenine residues.

A rhodanine-resistant clone (pT7E12rhod-res) containing the seven nucleotide exchanges present in all clones tested and two additional clones (pT7E12rhod-res4a and pT7E12rhod-res5a) including the additional exchanges 4a and 5a (Fig. 2) were constructed by exchanging corresponding restriction fragments of pT7E12wt.

In vitro-transcribed RNA copies of the four clones described (Fig. 3) were shown to be infectious. The resulting recombinant viruses were neutralized by echovirus 12-specific antiserum to the same titer as the original virus.

Rhodanine sensitivity assays of the virus stocks were done as follows: confluent GMK cells were infected with 100 50% tissue culture infective doses and every 12 h the cytopathic effects were evaluated (Fig. 3) (4). Both the wild-type and the resistant recombinant viruses respond to rhodanine as their original counterparts (Fig. 3). It is shown that seven amino acid exchanges are sufficient to turn the sensitive virus into a resistant one. A further exchange (labelled 5a), a Val-to-Ala substitution found in four of six clones, does not influence the growth characteristics of the virus in the presence or absence of rhodanine. Another point mutation (labelled 4a), found in only one of the six clones investigated, introduces a drug-dependent character (Fig. 3).

Because X-ray crystallographic studies have not yet been performed, the precise basis for rhodanine resistance or dependence is still not known. Except VP4, all capsid proteins of wild-type echovirus 12 differ from those of the rhodanine-resistant variant. The fact that six of seven amino acid substitutions are located in P1 supports the concept that rhodanine stabilizes the virion by direct interaction with the capsid (2), as has also been demonstrated with radioactively labelled rhodanine (3).

The amino acid changes in VP1 are of special interest. By X-ray diffraction investigations it was shown that the two antiviral compounds WIN 51711 and WIN 52084 as well as the

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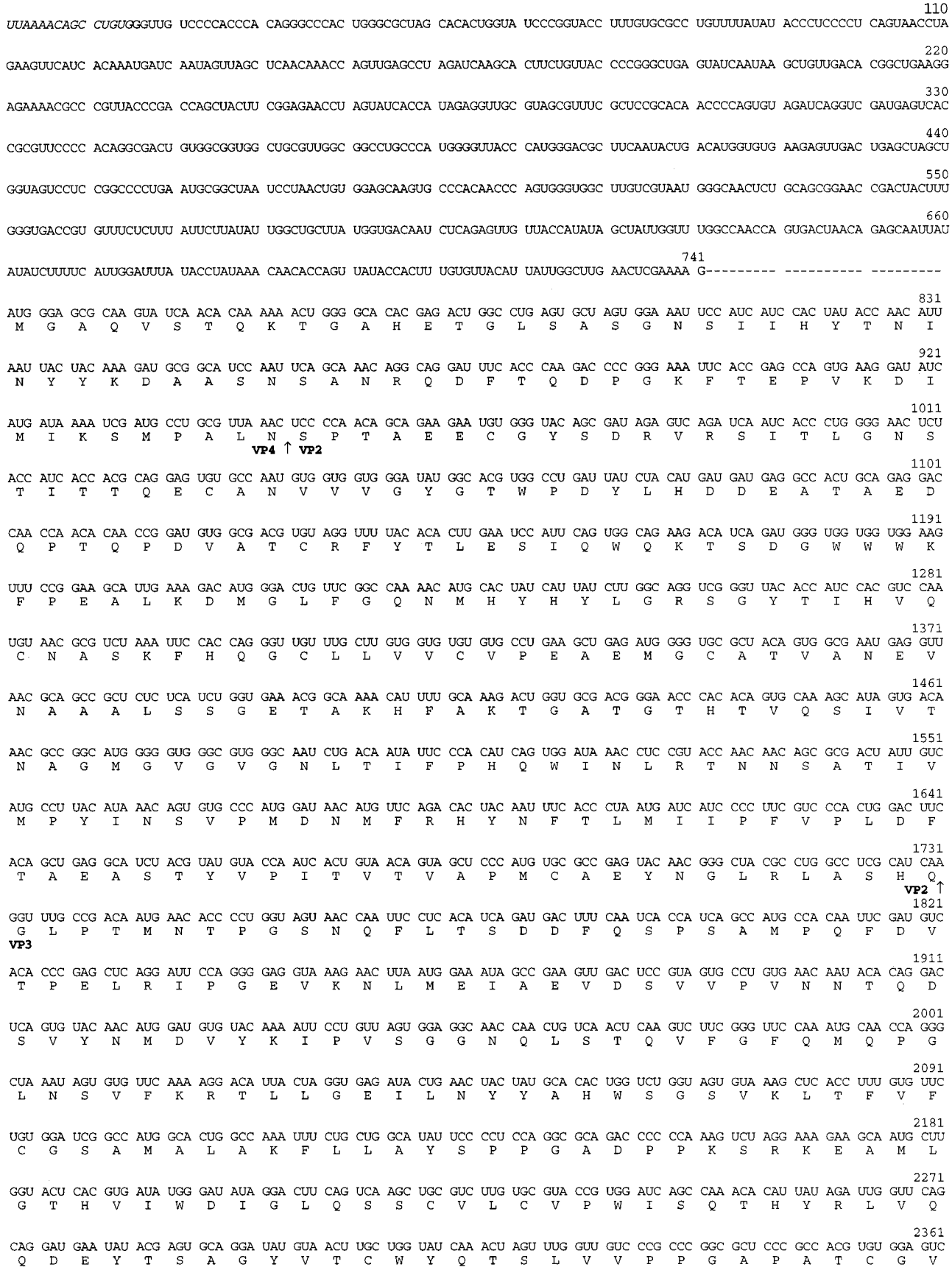



FIG. 1. Complete nucleotide sequence of echovirus 12 genome. The first 15 nt (italics) have not been sequenced but are assumed from the highly conserved 5′ end of enteroviruses sequenced so far. Cleavage sites of the viral capsid proteins determined by amino acid sequencing are indicated; the cleavage site between VP1 and P2 (in parentheses) has not been analyzed but is deduced from the coxsackievirus genome.

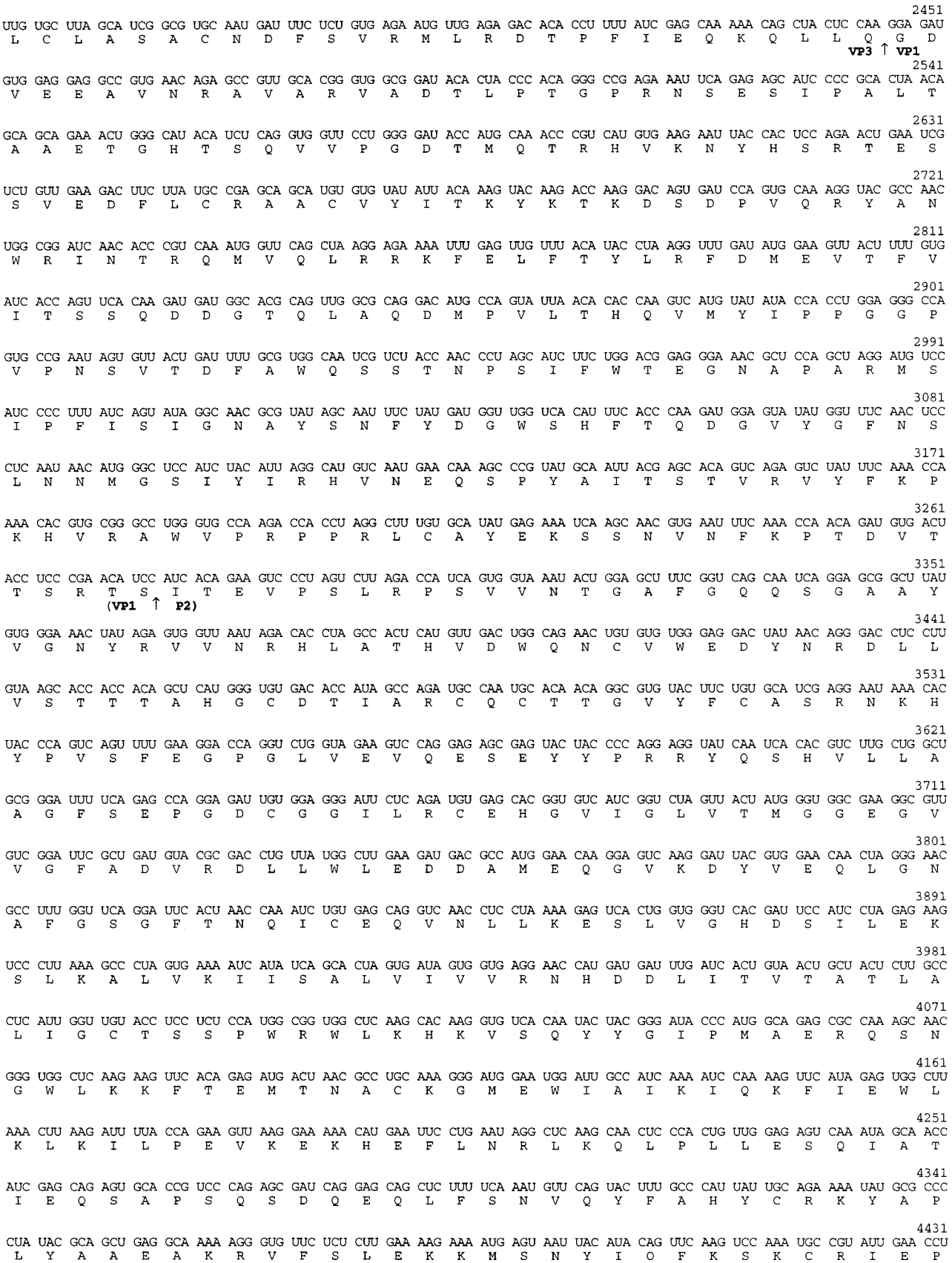


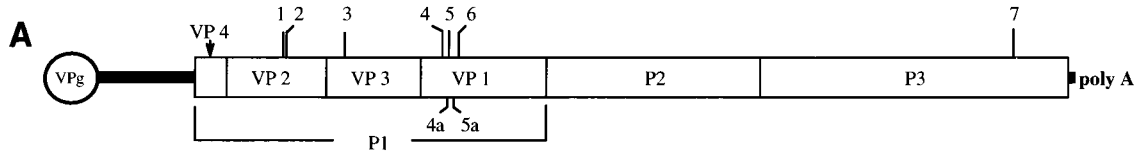

FIG. 1—*Continued.*

```
                                                                                                                    4521
GUA UGC UUG CUA CUA CAU GGG AGC CCC GGA GCA GGG AAA UCA GUU GCU ACC AGC CUC AUU GGG CGA UCA CUA GCU GAA AAA UUG AAU AGC
 V   C   L   L   L   H   G   S   P   G   A   G   K   S'  V   A   T   S   L   I   G   R   S   L   A   E   K   L   N   S

                                                                                                                    4611
UCA GUG UAC UCC UUA CCA CCA GAC CCA GAU CAC UUU GAU GGU UAC AAG CAA CAA GCU GUA GUG AUC AUG GAC GAU CUG UGC CAA AAU CCA
 S   V   Y   S   L   P   P   D   P   D   H   F   D   G   Y   K   Q   Q   A   V   V   I   M   D   D   L   C   Q   N   P

                                                                                                                    4701
GAU GGA AAA GAU GUG UCU UUG UUC UGU CAA AUG GUG UCU AGU GUA GAU UUU GUA CCA CCA AUG GCC GCG CUA GAG GAG AAA GGC AUU UUG
 D   G   K   D   V   S   L   F   C   Q   M   V   S   S   V   D   F   V   P   P   M   A   A   L   E   E   K   G   I   L

                                                                                                                    4791
UUC ACC UCU CCA UUC GUU CUA GCC UCA ACC AAU GCA GGA UCC AUC AAC GCA CCA ACA GUU UCG GAC AGC AGA GCC CUA GCU AGA AGG UUC
 F   T   S   P   F   V   L   A   S   T   N   A   G   S   I   N   A   P   T   V   S   D   S   R   A   L   A   R   R   F

                                                                                                                    4881
CAC UUU GAC AUG AAC AUU GAA GUU AUC UCC AUG UAC AGU CAA AAU GGA AAA AUA AAC AUG CCC AUG UCA GUC AAA ACA UGU GAU GAG GAG
 H   F   D   M   N   I   E   V   I   S   M   Y   S   Q   N   G   K   I   N   M   P   M   S   V   K   T   C   D   E   E

                                                                                                                    4971
UGC UGC CCA GUC AAU UUC AAG AGA UGC UGC CCA CUG GUG UGU GGC AAG GCU AUU CAG UUC AUU GAC AGA AGA ACC CAA GUC AGA UAU UCA
 C   C   P   V   N   F   K   R   C   C   P   L   V   C   G   K   A   I   Q   F   I   D   R   R   T   Q   V   R   Y   S

                                                                                                                    5061
CUG GAU AUG UUA GUC ACC GAG AUG UUC AGG GAG UAC AAC CAC AGG CAC AGU GUG GGC GCC ACC CUC GAG GCU CUG UUC CAA GGU CCA CCG
 L   D   M   L   V   T   E   M   F   R   E   Y   N   H   R   H   S   V   G   A   T   L   E   A   L   F   Q   G   P   P

                                                                                                                    5151
GUC AUC AGG GAG AUC AAG AUC AGU GUU GCU CCA GAA ACA CCU CCC CCA CCA GCA AUC GCU GAU UUA UUA AAA UCA GUA GAC AGU GAA GCU
 V   I   R   E   I   K   I   S   V   A   P   E   T   P   P   P   P   A   I   A   D   L   L   K   S   V   D   S   E   A

                                                                                                                    5241
GUG AGA GAG UAC UGC AAG GAA AAA GGC UGG CUU GUA CCG GAA GUU AAC UCC ACC CUA CAG AUU GAG AAG CAC GUC AGC AGA GCA UUU AUC
 V   R   E   Y   C   K   E   K   G   W   L   V   P   E   V   N   S   T   L   Q   I   E   K   H   V   S   R   A   F   I

                                                                                                                    5331
UGU CUA CAA GCU CUG ACC ACU UUC GUC UCA GUA GCU GGC AUA AUC UAC AUU AUC UAC AAG UUG UUU GCC GGC UUU CAG GGC GCG UAU ACG
 C   L   Q   A   L   T   T   F   V   S   V   A   G   I   I   Y   I   I   Y   K   L   F   A   G   F   Q   G   A   Y   T

                                                                                                                    5421
GGG AUG CCA AAU CAG AAA CCC AAA GUG CCC ACU CUG AGA CAA GCU AAA GUG CAG GGC CCA GCA UUC GAG UUC GCU GUG GCA AUG AUG AAA
 G   M   P   N   Q   K   P   K   V   P   T   L   R   Q   A   K   V   Q   G   P   A   F   E   F   A   V   A   M   M   K

                                                                                                                    5511
AGG AAC GCC AGC ACA GUG AAA ACG GAA UAU GGU GAG UUC ACC AUG CUC GGC AUC UAU GAC AGA UGG GCA GUG CUA CCA CAC CAC GCC AAG
 R   N   A   S   T   V   K   T   E   Y   G   E   F   T   M   L   G   I   Y   D   R   W   A   V   L   P   H   H   A   K

                                                                                                                    5601
CCU GGA CCG ACU AUU UUG AUG AAU GAU CAG GAG AUC GGC GUG UUA GAC GCC AAA GAA UUA GUG GAC AAA GAU GGG ACA AAU CUG GAG UUG
 P   G   P   T   I   L   M   N   D   Q   E   I   G   V   L   D   A   K   E   L   V   D   K   D   G   T   N   L   E   L

                                                                                                                    5691
ACC CUU UUA AAG CUC AAC CGC AAU GAG AAG UUU AGG GAC AUC AGA GGG UUU CUG GCG AGA GAG GAA GCU GAA GUG AAU GAG GCU GUU CUG
 T   L   L   K   L   N   R   N   E   K   F   R   D   I   R   G   F   L   A   R   E   E   A   E   V   N   E   A   V   L

                                                                                                                    5781
GCA AUA AAC ACA AGC AAG UUC CCA AAU AUG UAC AUA CCC GUA GGU CAA GUC ACU GAU UAC GGU UUC CUG AAC CUG GGG GGA ACA CCC ACA
A   I   N   T   S   K   F   P   N   M   Y   I   P   V   G   Q   V   T   D   Y   G   F   L   N   L   G   G   T   P   T

                                                                                                                    5871
AAG AGG AUG CUC AUG UAC AAC UUU CCA ACU AGA GCA GGG CAA UGU GGU GGG GUA CUC AUG UCA ACA GGG AAA GUU CUU GGU AUA CAU GUA
K   R   M   L   M   Y   N   F   P   T   R   A   G   Q   C   G   G   V   L   M   S   T   G   K   V   L   G   I   H   V

                                                                                                                    5961
GGA GGA AAU GGA CAU CAA GGG UUC UCC GCU GCU CUU CUU AGG CAC UAC UUU AAU GAG GAG CAA GGU GAA AUA GAG UUU AUU GAG AGC UCA
 G   G   N   G   H   Q   G   F   S   A   A   L   L   R   H   Y   F   N   E   E   Q   G   E   I   E   F   I   E   S   S

                                                                                                                    6051
AAG GAU GCA GGA UUC CCU GUG AUC AAC ACC CCC AGC AAA ACA AAA CUG GAA CCA AGC GUG UUU CAU CAG GUG UUU GAA GGC AAU AAA GAG
 K   D   A   G   F   P   V   I   N   T   P   S   K   T   K   L   E   P   S   V   F   H   Q   V   F   E   G   N   K   E

                                                                                                                    6141
CCA GCA GUC CUU AGA AAU GGU GAU CCG CGU CUU AAA GUA AAC UUU GAA GAA GCC AUA UUU UCC AAG UAC AUC GGG AAC AUC AAC ACA CAU
 P   A   V   L   R   N   G   D   P   R   L   K   V   N   F   E   E   A   I   F   S   K   Y   I   G   N   I   N   T   H

                                                                                                                    6231
GUG GAU GAA UAC AUG CUC GAA GCA GUG GAU CAC UAC GCA GGG CAA CUG GCU ACU CUU GAC AUU AGC ACU GAA CCC AUG AAA CUA GAA GAU
 V   D   E   Y   M   L   E   A   V   D   H   Y   A   G   Q   L   A   T   L   D   I   S   T   E   P   M   K   L   E   D

                                                                                                                    6321
GCA GUG UAU GGC ACU GAA GGA CUA GAG GCU CUU GAC UUA ACA ACA AGU GCA GGG UAC CCG UAU GUC GCC AUA GGC AUC AAA AAG AGA GAC
 A   V   Y   G   T   E   G   L   E   A   L   D   L   T   T   S   A   G   Y   P   Y   V   A   I   G   I   K   K   R   D

                                                                                                                    6411
AUC CUA UCC AAA AAG ACU AAA GAU CUG ACU AAA UUG AAG GAA UGC AUG GAC AAG UAC GGU UUG AAC CUG CCG AUG GUG ACU UAU GUG AAG
 I   L   S   K   K   T   K   D   L   T   K   L   K   E   C   M   D   K   Y   G   L   N   L   P   M   V   T   Y   V   K

                                                                                                                    6501
GAU GAA CUU AGA UCA UCA GAG AAA GUG GCU AAG GGA AAA UCA AGA CUC AUU GAA GCA UCU AGU UUA AAU GAC UCU GUC GCG AUG AGG CAG
 D   E   L   R   S   S   E   K   V   A   K   G   K   S   R   L   I   E   A   S   S   L   N   D   S   V   A   M   R   Q
```

FIG. 1—*Continued.*

```
                                                                                                                    6591
ACG UUU GGC AAC UUG UAC AAA ACA UUC CAU CUG AAC CCG GGC AUU GUG ACA GGC AGU GCA GUU GGA UGU GAU CCG GAC CUU UUC UGG AGU
 T   F   G   N   L   Y   K   T   F   H   L   N   P   G   I   V   T   G   S   A   V   G   C   D   P   D   L   F   W   S

                                                                                                                    6681
AAA AUA CCC GUG AUG UUA GAC GGC CAC CUC AUA GCA UUC GAU UAC UCC GGA UAU GAU GCC AGC CUG AGC CCU GUG UGG UUC GCU UGU CUA
 K   I   P   V   M   L   D   G   H   L   I   A   F   D   Y   S   G   Y   D   A   S   L   S   P   V   W   F   A   C   L

                                                                                                                    6771
AAA CUG CUA CUC GAA AAA CUC GGG UAC ACG CAC AGA GAG ACA AAC UAC AUC GAC UAC CUA UGC AAC UCU CAC CAC CUG UAC AGA GAC AAA
 K   L   L   L   E   K   L   G   Y   T   H   R   E   T   N   Y   I   D   Y   L   C   N   S   H   H   L   Y   R   D   K

                                                                                                                    6861
CAC UAC UUU GUG CGU GGU GGC AUG CCA UCA GGG UGC UCC GGU ACC AGC AUU UUC AAU UCA AUG AUC AAU AAC AUC AUA AUC AGG ACA CUG
 H   Y   F   V   R   G   G   M   P   S   G   C   S   G   T   S   I   F   N   S   M   I   N   N   I   I   I   R   T   L

                                                                                                                    6951
AUG CUC AAA GUG UAC AAG GGA AUU GAC CUG GAC CAG UUU AGA AUG AUU GCA UAU GGU GAU GAU GUA AUU GCA UCC UAC CCU UGG CCU AUA
 M   L   K   V   Y   K   G   I   D   L   D   Q   F   R   M   I   A   Y   G   D   D   V   I   A   S   Y   P   W   P   I

                                                                                                                    7041
GAC GCU UCA CUA CUU GCU GAA GCU GGG AAG GGU UAU GGA CUG AUU AUG ACA CCA GCA GAC AAA GGG GAG UGC UUC AAU GAG GUU ACC UGG
 D   A   S   L   L   A   E   A   G   K   G   Y   G   L   I   M   T   P   A   D   K   G   E   C   F   N   E   V   T   W

                                                                                                                    7131
ACC AAU GUC ACC UUC CUA AAG AGG UAC UUU AGA GCU GAU GAG CAG UAU CCU UUC CUG GUU CAU CCA GUU AUG CCC AUG AAG GAC AUC CAU
 T   N   V   T   F   L   K   R   Y   F   R   A   D   E   Q   Y   P   F   L   V   H   P   V   M   P   M   K   D   I   H

                                                                                                                    7221
GAA UCU AUU AGG UGG ACA AAG GAU CCA AAG AAC ACU CAA GAC CAC GUU CGA UCG UUG UGC UUA UUG GCU UGG CAU AAC GGA GAA CAA GAA
 E   S   I   R   W   T   K   D   P   K   N   T   Q   D   H   V   R   S   L   C   L   L   A   W   H   N   G   E   Q   E

                                                                                                                    7311
UAU GAG GAA UUC AUC CGA AAG AUC AGA AGC GUC CCG GUU GGG CGC UGU CUG ACU CUC CCC GCG UUU UCA ACU UUA CGC AGA AAA UGG CUG
 Y   E   E   F   I   R   K   I   R   S   V   P   V   G   R   C   L   T   L   P   A   F   S   T   L   R   R   K   W   L

        7320                                                                                                        7420
GAU UCC UUU TAAATTAGAG ACAATTTGAA ATAATTTAAA TTGGCTTAAC CCTACTGTAC TAACCGAACT AGACAACGGT GCAGTAGGGG TAAATTCTCC GCATTCGGTG
 D   S   F

CG
```

FIG. 1—*Continued.*




**B**

| virus-variant | nucleotide exchanges (position within the echovirus 12 genome) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 (1408) | 2 (1423) | 3 (1869) | 4 (2641) | 4a (2649) | 5 (2669) | 5a (2747) | 6 (2915) | 7 (7021) |
| echovirus 12 | CAT [H] | GGT [G] | ATA [I] | GAC [D] | TTA [L] | TAT [Y] | GTT [V] | GTT [V] | TGC [C] |
| rhod.-res. | **T**AT [Y] | **A**GT [S] | AT**G** [M] | **A**AC [N] | TT**T** [F] | T**G**T [C] | G**C**T [A] | G**C**T [A] | **C**GC [R] |

FIG. 2. Sequence deviations of wild-type echovirus 12 and the rhodanine-resistant variant. (A) Schematic drawing of the echovirus genome. The nucleotide exchanges between the wild type and rhodanine-resistant variant are indicated. The translated part of the viral genome is shown as an open bar; the boxes VP (virus protein) 1 to 4 represent the segments coding for the capsid proteins. (B) Nucleotide exchanges are numbered according to panel A, and the position numbers within the echovirus 12 genome are given in parentheses. The codons affected by the exchanges are given, together with the corresponding amino acid (in brackets). The changed bases are underlined.

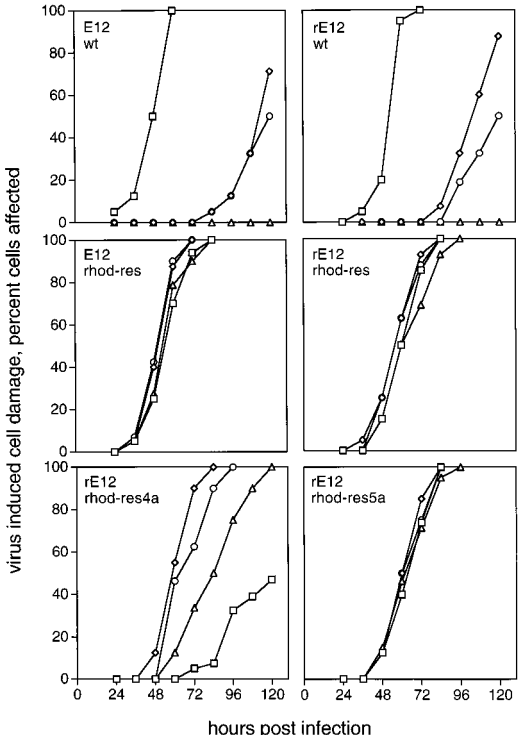



FIG. 3. Rhodanine sensitivity assay (4) of the original viruses (E12-wt and E12-res) and the recombinant ones (rE12-wt, rE12rhod-res, rE12rhod-res4a, and rE12rhod-res5a). Recombinant virus rE12-wt was derived from the plasmid pT7E12-wt, rE12rhod-res was derived from pT7E12rhod-res, rE12rhod-res4a was derived from pT7E12rhod-res4a, and rE12rhod-res5a was derived from pT7E12rhod-res5a (see the text). Cells were infected with 100 50% tissue culture infective doses of the above mentioned viruses in the presence of 0 (□), 50 (◇), 100 (○), and 150 (△) μg of rhodanine per ml. Every 12 h, the percentage of cells exhibiting cytopathic effects was determined.

pyridazinamine derivative R 61837 bind to a hydrophobic pocket located at the base of the canyon within the β-barrel (WIN pocket) of VP1 of rhinoviruses (1, 5).

We are currently investigating in detail which of the seven amino acid differences between echovirus 12, prototype strain Travis, and the rhodanine-resistant variant are responsible for the altered features of the two viruses.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the wild-type echovirus 12 and the rhodanine-resistant variant have been assigned EBI Data Library accession no. X79047 and X77708, respectively.

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