The differences in substrate specificity between Moloney murine leukemia virus protease (MuLV PR) and human immunodeficiency virus (HIV) PR were investigated by site-directed mutagenesis. Various amino acids, which are predicted to form the substrate binding site of MuLV PR, were replaced by the equivalent ones in HIV-1 and HIV-2 PRs. The expressed mutants were assayed with the substrate Val-Ser-Gln-Asn-Tyr and Ile-Val-Gln-NH₂ (indicates the cleavage site) and a series of analogs containing single amino acid substitutions in positions P₄(Ser) to P₉(Val). Mutations at the predicted S₂/S₉ substrate of MuLV PR have a strong influence on the substrate specificity of this enzyme, as observed with mutants H37D, V39I, V54I, A57I, and L92I. On the other hand, substitutions at the flap region of MuLV PR often rendered enzymes with low activity (e.g. W53I/Q55G). Three amino acids (His-37, Val-39, and Ala-57) were identified as the major determinants of the differences in substrate specificity between MuLV and HIV PRs.

Retroviral maturation involves the proteolytic cleavage of viral precursor polyproteins by an aspartyl protease (PR) encoded within the virus genome (1). Since the discovery of the human immunodeficiency virus (HIV) as an etiological agent causing the acquired immunodeficiency syndrome (AIDS), these proteases have been widely studied as potential targets of antiviral drugs. As a result, many PR inhibitors have been synthesized and a number of them are currently undergoing in vitro screening or clinical evaluation (2, 3).

Retroviral PRs are homodimeric enzymes (4). Each subunit contains the conserved sequence Asp-Thr(Ser)-Gly, which provides the aspartyl group necessary for catalysis. Crystal structures of retroviral PRs in their native form and complexed with inhibitors have been determined (2, 5–10). These studies have shown that the substrate is bound to the PR in an extended conformation, maintained by hydrogen bond interactions between PR residues and the amide and carbonyl groups of the peptidic substrate. The PR dimer forms a series of subsites (termed S₁, S₂, S₂’, S₃, S₃’, S₄, and S₅), which correspond to the binding sites of the P₄, P₃, P₂, P₁, P₀, P₋₁, P₋₂, and P₋₃ residues of the substrate, where the scissile bond is located between the P₁ and P₋₁ positions. Oligopeptide substrates are useful tools to define the specific requirements of the binding site and therefore to analyze the substrate specificity of the retroviral PRs.

The model peptide VSQNYPIVQ (indicates the cleavage site), which derives from the MA/CA processing site in HIV-1 Gag, and a series of analogs containing single amino acid substitutions at P₄ to P₋₃ positions have been used previously to compare the substrate specificity of various retroviral PRs (11–14). The MuLV PR has a strong preference for analogs with hydrophobic residues, such as Val or Ile at P₄, and Ile or Leu at P₋₁ in contrast to HIV-1 and HIV-2 PRs, which prefer smaller or more polar residues at both positions (14). The amino acid sequences of MuLV and HIV-1 PR share 27% identical residues (Fig. 1). A molecular model of MuLV PR was built on the basis of the crystal structure of HIV-1 PR (14). Although the general topology of the MuLV and HIV PRs was predicted to be very similar, only 6 of the 22 residues found in subsites S₁ to S₃ of MuLV PR are conserved in both HIV PRs, based on our proposed model. The differences in specificity between both enzymes were attributed to the greater hydrophobicity of the S₄ subsite and the larger size of the S₅ subsite in the MuLV PR.

In this paper, site-directed mutagenesis has been used to identify critical residues which determine the substrate specificity of MuLV PR. Amino acids forming the MuLV PR subsites were replaced by the equivalent residues found in HIV-1 and HIV-2 PRs. The mutant PRs were assayed for proteolytic activity using VSQNYPIVQ analogs. The results of this analysis indicate that residues in subsites S₂/S₉ are important for substrate specificity, while the flap region is very sensitive to mutations. We identified His-37, Val-39, and Ala-57 as the major determinants of the differences in substrate specificity between MuLV and HIV PRs.

**Experimental Procedures**

Plasmid Construction and Mutagenesis—The clone pMuLVPR3.2, which contains the Moloney MuLV PR coding region, has been described previously (17). The PR-coding region was cloned in the BamHI and HindIII sites of pALTER-1 (Promega). For such a purpose, the 395-base pair insert obtained after cleavage of pMuLVPR3.2 with BamHI and EcoRI was previously cloned in pTrChA (Invitrogen Corp.). Site-directed mutagenesis was done with the Altered Sites mutagenesis system kit from Promega, following the manufacturer’s instructions. This system uses a phagemid (pALTER-1) which contains two genes for antibiotic resistance. One of these genes, for tetracycline resistance, is maintained, together with the wild-type gene, by host selection, the other one being deleted by the presence of the insert. The insert vector, pTrChA (Invitrogen Corp.), contains the complete bacterial colE1 replicon, the MuLV PR coding region, the unique BamHI and HindIII sites of the insert, and the unique SalI and XhoI sites of pALTER-1. The inserts were cleaved at the unique BamHI and HindIII sites and the resulting phagemids were transformed into the E. coli strain DH5α, which was also used for propagation of the plasmid DNA. The plasmid DNA was isolated from the bacterial cells by a standard procedure.
cycline resistance, is always functional, while the other, which conveys ampicillin resistance, has been inactivated. During the mutagenesis reaction, ampicillin resistance is restored by using an oligonucleotide provided with the kit which is annealed to the single-stranded DNA template at the same time as the mutagenic oligonucleotide. Escherichia coli DH5α cells harboring the pALTER-1-derived construct were superinfected with the helper phage R408 and used for the isolation of a single-stranded DNA template to be used in the mutagenesis reaction. The PR mutations, oligodeoxynucleotides used in the mutagenesis reaction, and the restriction sites used for rapid screening of mutated clones are shown in Table I. The introduced mutations were confirmed by digestion with the appropriate restriction enzymes and by DNA sequencing (18). The pALTER-derived plasmids containing the mutated PRs were digested with BamHI and EcoRI to isolate the PR-coding region, which was then cloned into pGEX-2T, an expression vector which contains the gus gene of Schistosoma japonicum, encoding for a glutathione S-transferase (19). The double mutants V39I/V54I and V39I/A57I were obtained after ligation of the 1.1-kilobase pair fragments of A57I or V54I into the 4.1-kilobase pair fragment derived from V39I, after cleavage with PstI and StyI.

Expression and Purification of the Retroviral PRs—Expression and purification of wild type and mutant MuLV PRs was carried out essentially as described previously (17). Briefly, freshly prepared E. coli DH5α cultures containing the plasmid pMuLVPR32, or the mutated PRs, were grown at 37 °C in 1 liter of Luria broth medium containing 50 μg/ml ampicillin, to an A600 of 0.8–1.0. After induction with 0.4 μM isopropyl-β-D-thiogalactopyranoside (Life Technologies, Inc.) for 90 min, cells were harvested by centrifugation at 4,000 × g for 15 min at 4 °C. After removal of the supernatant, cells were resuspended in 50 ml of lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 0.1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride). Cells were then treated with lysozyme at 25 μg/ml for 15 min at 4 °C and deoxyribonuclease A (1 μg/ml) for 30 min at 37 °C. The incubation with deoxyribonuclease A was done in the presence of 5 mM MgCl2. Then, samples were sonicated, and the extract obtained was centrifuged at 12,000 rpm for 10 min at 4 °C using a Sorvall SS-34 rotor. The supernatant was loaded on a prepacked glutathione-Sepharose 4B column (Pharmacia LKB, Uppsala, Sweden). The immobilized chimeric protein was then cleaved with thrombin to excise the PR domain, as described previously (17). Purity of the PR was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% acrylamide gel). Protein concentrations were determined by densitometry of the corresponding Coomassie Blue-stained bands in polyacrylamide gels, using as a reference a PR, whose amount was determined previously by amino acid analysis as described (14).

Recombinant HIV-1 PR was prepared from E. coli inclusion bodies according to previously published procedures (20). E. coli strain DH5α was used for the mutagenesis of MuLV PR by the method of Inoue et al. (15), and the HIV PR sequences were from Cope and Oroszlan (16).

**Fig. 1. Sequence comparison of Mooney MuLV, HIV-1, and HIV-2 PRs.** Amino acids, which are identical in MuLV PR and either HIV-1 or HIV-2 PRs, are boxed. The elements of secondary structure observed for the crystal structure of HIV-1 PR are indicated by letters a through d, a' through d', and g for β-strands, and for the only α-helical segment of the PR monomer (5). Residues 43–58 in HIV-1 PR form the flap, which includes β-strand a, part of β-strand b, and the residues between both β-structures. The MuLV PR amino acid sequence was taken from Yoshinaka et al. (15), and the HIV PR sequences were from Cope and Oroszlan (16).

**Table I**

| Mutation | Oligodeoxynucleotide | Restriction enzyme |
|----------|----------------------|-------------------|
| NT       | CCACCTATAGTGGTACGTCAGGTCAGGACGCC | BamHI (+) |
| E15R     | GAGCAGGCCCCCTCAGACGATAACCCT | Styl (-) |
| H37D     | ACTGCGGCAAGATCTGCTGACC | Styl (-) |
| V39I     | GGGGCGCAACTCCATCTGACCGAAAATCTGG | KpnI (+) |
| W53I     | GAAGTCTGCTGATGTCGACG | PvuI (+) |
| V54I     | AAGTCTGCGATAGGATCAGCTGG | BamHI (+) |
| W53I/J55G | GATAATACGCTGATGTCGACG | BamHI (+) |
| A57I     | GGCCTGCTGCTGACGATGTCGACG | BamHI (+) |
| G60F     | GAGGCGCACAGTACGACGATGTCGACG | BamHI (+) |
| Y63V     | GAGAAGAAGGACGGTACGACGATGTCGACG | BamHI (+) |
| C88T     | CCAGTACGAGCTACGACGATGTCGACG | BamHI (+) |
| L92I     | CTTTGCCTATCTCAGATGTCGACG | BamHI (+) |

**a** Mutations are identified by amino acid and position number in MuLV PR followed by the substituted amino acid. Amino acids are denoted by the single letter code. NT corresponds to a deletion mutant lacking the amino-terminal extension of MuLV PR. Its amino-terminal sequence is Gly-Ser-Gly-Glu. Double mutants are indicated with a slash.

**b** Underlined nucleotides correspond to mutations introduced in the PR coding region.

**c** Restriction sites were either introduced (+) or eliminated (−) to identify the mutated clones.

**d** These oligodeoxynucleotides were synthesized by Isogen Bioscience bv (Amsterdam, Holland).
Mutational Analysis of MuLV PR Subsites

The comparison of the crystal structures of HIV PR-inhibitor complexes with the molecular model of Moloney MuLV PR revealed many amino acid differences at the substrate binding sites of both enzymes (Fig. 2). Only 6 of the 22 residues found in subsites S2 to S3 of MuLV PR (Leu-30, Asp-32, Gly-34, Ala-35, Gly-56, and Pro-89) are conserved in both HIV-1 and HIV-2 PRs. The remaining nonconserved 16 amino acids, which are predicted to be part of MuLV PR subsites, are scattered throughout the substrate binding pocket. None of the PR subsites is conserved in the three enzymes. Site-directed mutagenesis was used to generate a large number of mutants having single amino acid substitutions at the substrate binding pockets of MuLV PR. All the amino acids which were predicted to be part of MuLV PR subsites were systematically replaced by those found in the equivalent positions of HIV-1 or HIV-2 PRs. Enzymatic characterization of these mutants was limited to those that could be expressed and purified in significant amounts. Approximately half of the constructs were excluded from the analysis, since they rendered proteins which were poorly expressed, insoluble, or not stable (data not shown). Mutated MuLV PRs with single or double amino acid replacements at each of the PR subsites (S2 to S3) were then used to investigate the molecular basis of the substrate specificity differences between MuLV and HIV PRs. The introduced mutations were located in both subunits due to the homodimeric nature of MuLV PR. The activity of the PR mutants was tested by using the peptide VSQNYPIVQ and a series of substrate analogs having substitutions at the P4 to P3 positions.

The catalytic parameters ($k_{\text{cat}}$ and $K_m$) for the cleavage of VSQNYPIVQ by the wild type MuLV PR and 14 mutant enzymes are given in Table II. It should be noted that the wild type PR, as well as the mutant enzymes were expressed in fusion with the S. japonicum glutathione S-transferase and then excised with thrombin to release the PR, which contained two extra amino acids (Gly-Ser-) at the amino-terminal sequence, not found in the viral protease isolated from purified virus (15). These residues are required to maintain the integrity of the thrombin cleavage site. The influence of the amino-terminal extension on the proteolytic activity is apparently negligible. Thus, a mutated MuLV PR (NT), that lacks its natural amino-terminal extension TLDD (see Fig. 1), was found to cleave the reference oligopeptide substrate with similar $k_{\text{cat}}$ and $K_m$ values to those of the wild type enzyme (Table II). Similar kinetic parameters were also observed for mutants E15R, V39I, V54I, and for the double mutant V39I/V54I. The mutant H37D shows a significant decrease of the $K_m$ value. The double mutant W53I/Q55G, which involves amino acids located at the flap region of the PR, did not cleave the oligopeptide VSQNYPIVQ and showed negligible activity when the analogs P3' Val or P3' His were used as substrates ($k_{\text{cat}} < 0.001 \text{ s}^{-1}$). Other replacements involving residues at the flap (e.g. W53I or G60F) rendered PRs showing a decreased affinity for the substrate. The flap region is very sensitive to changes which can alter the catalytic rate, as well as the specificity, as shown for

[Fig. 2. Residues forming the substrate binding pocket of MuLV and HIV PRs. Left, schematic representation of the HIV-1 MA/CA substrate, VSQNYPIVQ (one-letter amino acid code), from P4 to P3. Right, diagram showing the contribution of residues of the substrate binding pocket to subsites S2 to S3. Equivalent residues in MuLV and HIV PRs that contribute to the same subsite are shown in black. Residues contributing only to MuLV PR subsites are horizontally dashed, while those contributing only to HIV PR subsites are vertically dashed.]
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| Kinetic parameters determined with wild type and mutant MuLV PRs using the synthetic peptide substrate VSQNY ↓ PIVQ |
|---|
| Protease | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|---|---|---|---|
| Wild type | 261 ± 26 | 0.55 ± 0.02 | 2.11* |
| NT | 324 ± 33 | 0.44 ± 0.05 | 1.88 |
| E15R | 265 ± 47 | 0.30 ± 0.02 | 1.13 |
| H37D | 41 ± 10* | 0.075 ± 0.007 | 1.83 |
| V39I | 268 ± 31 | 0.74 ± 0.03 | 2.76 |
| W53I | 2046 ± 638 | 0.30 ± 0.07 | 0.15 |
| V54I | 234 ± 57 | 0.35 ± 0.03 | 1.50 |
| V39I/V54I | 287 ± 38 | 0.46 ± 0.02 | 1.60 |
| W35I/J55G | ND* | ND* |
| A57I | 356 ± 45 | 0.23 ± 0.02 | 0.65 |
| V39I/A57I | 456 ± 50 | 0.072 ± 0.003 | 0.16 |
| G60F | >3000 | ND* |
| Y63V | 290 ± 56 | 0.20 ± 0.02 | 0.67 |
| C88I | >3000 | ND* |
| L92I | 1284 ± 92 | 0.042 ± 0.002 | 0.03 |

*Values taken from Ref. 14.

a When the concentration of substrate was increased well above the apparent $K_m$ value, an inhibitory effect was observed.

b ND, not determined.

d Turnover values for G60F and C88I were 0.031 and 0.0012 s$^{-1}$, respectively, when calculated at 0.2 mM concentration of VSQNYPIVQ.

Other retroviral PRs, such as HIV-1 or Rous sarcoma virus PRs (20, 24–26). Mutations C88T and L92I, which affect substrates S$2$–S$3$, also rendered PRs with a low catalytic efficiency for this substrate.

All MuLV PR mutants were examined for changes in substrate preference using a series of VSQNYPIVQ analogs with substitutions in the P$4$, P$3$ and P$2$ positions. The proteolytic activity obtained using the substituted analogs relative to that observed with VSQNYPIVQ was determined for all mutant PRs and compared with the wild type MuLV and HIV-1 PRs (Fig. 3). Large differences in substrate specificity were detected with analogs having large hydrophobic amino acids at P$4$, P$3$, and P$2$ positions. Less variation was produced by the substitutions tested at P$1$, P$3$ and P$2$, respectively, when calculated at 0.2 mM concentration of VSQNYPIVQ.

The introduction of these mutations in the MuLV PR resulted in an increased preference for hydrophobic amino acids at P$4$, P$3$, and P$2$ positions in VSQNYPIVQ. In the wild type MuLV PR, the catalytic efficiency was 6-fold higher with P$4$(Leu) and P$3$(Ile) than with VSQNYPIVQ (Table III). This increase was even more pronounced for the mutant enzymes of this group. For example, the $k_{cat}/K_m$ value obtained for L92I and VSQNYPIVQ was 0.03 mm$^{-1}$ s$^{-1}$ versus 4.59 mm$^{-1}$ s$^{-1}$, obtained with the P$2$(Leu) analog. Trp-53, Val-54, and Gly-60 are located in the flap region of the PR. In the model of MuLV PR, Trp-53 is predicted to lie close to P$4$ and would be expected to make the S$4$ subsite smaller than in HIV PRs. Accordingly, the substitution of Trp-53 by Ile favors the cleavage of analogs having larger residues at P$4$ (Fig. 3). Trp-53 is not predicted to be close to P$2$, unless it can insert into the PR structure in a very different conformation than the equivalent Met-46 of HIV-1 PR. The mutant V54I is predicted to reduce the size and increase the hydrophobicity of subsites S$4$, S$5$, and S$6$ so that smaller, more hydrophobic residues at P$4$, P$3$, and P$2$ will form better substrates, as observed in our assays. Gly-60 in MuLV PR is equivalent to Phe-53 in HIV PR which lies across the two antiparallel flap strands. The substitution of Gly by the hydrophobic Phe, as in G60F, would increase the hydrophobicity of the S$4$/S$5$ and S$6$/S$7$ subsites, consistent with the observed effects with P$4$ and P$2$-substituted analogs. In addition, MuLV PR Gly-60 is close to P$3$, but would be predicted to have an indirect effect since Gln-55 lies between Gly-60 and P$3$. Mutant G60F showed reduced cleavage of P$4$(Asn), C88T and L92I are predicted to influence binding at S$1$, S$2$, and S$3$ subsites (Figs. 2 and 3). The mutation C88T introduces a more bulky side chain that may increase the preference for hydrophobic residues at P$3$, as observed. In a similar way, mutant L92I involves the introduction of a more bulky residue which probably explains the preference for smaller P$2$(Met) over the larger Tyr and the increased selection of more hydrophobic residues at P$2$. Finally, a third group of mutants appears to be responsible for the major differences in substrate specificity between MuLV and HIV PRs. These mutations include H37D, V39I, A57I, and the double mutant V39I/A57I. In the model structure of MuLV PR, H37D is close to P$4$, P$3$, and P$2$ and is predicted to form a hydrogen bond interaction with Gln-36. In the crystal structures of HIV-1 PR, a hydrogen bond is formed between Asp-30 (His-37 equivalent) and Asn-88 (Asp-96 equivalent). The interaction seen in HIV PR and modeled for MuLV PR cannot occur in the H37D mutant, because the acidic Asp-37 would repel residue Asp-96 and instead may form an ion pair with His-84. H37D can still form a hydrogen bond interaction with Ser at P$4$ of the substrate and will probably decrease the hydrophobicity of S$4$, S$5$, and S$6$ subsites, giving rise to the observed preference for less hydrophobic residues at P$4$, P$3$, and P$2$. Despite the observed changes in specificity in the S$4$ subsite using the H37D mutant, the substitution of His-37 by Asp was not sufficient to obtain cleavage of the P$4$(Asp) analog. This oligopeptide was cleaved by the HIV-1 PR (relative activity: 0.23), but it was a poor substrate for MuLV PR, as well as for all the mutants tested (relative activity < 0.01). The mutant V39I is predicted to reduce the space available in S$3$, and S$4$ due to the presence of the larger Ile rather than Val. The presence of the larger Ile-39 is consistent with the decreased activity for substrates with the longer Leu at P$4$ and P$2$. The shorter but more bulky Ile at P$2$ can still fit in the subsite because of the presence of Leu-92 at the side of the subsite, rather than the more bulky Ile-84 as in HIV-1 PR. The mutation A57I lies at the tip of the flaps near substrate positions P$2$, P$3$, P$4$, and P$2$. The presence of the larger Ile is expected to reduce the size of the affected subsites, in agreement with the observed poor cleavage of the larger hydrophobic residues at P$2$. The double mutant V39I/A57I shows the simultaneous effect of both mutations in reducing the size of subsites S$2$ and S$3$. Substrate analogs having Leu, Ile, or Val at P$4$ are cleaved much less efficiently by V39I/A57I than by the single-amino acid mutants V39I or A57I. In addition, the oligopeptide having Ala at P$2$ position was not cleaved by the wild-type MuLV PR and by most of the mutants tested. In contrast, we observed cleavage of this peptide when mutants A57I and
Mutational Analysis of MuLV PR Subsites

**FIG. 3.**
the S2 subsite of HIV-1 PR, compared with Ile-32 and Val-47 in the S2 subsite of HIV-1 PR (9, 28, 29), Ile-47V (30), and Ile-84V (29, 30, 32–35). Furthermore, differences in specificity between HIV-1 and HIV-2 G48V (29, 31), I50V (29, 30), and I84V (29, 30, 32–35). These effects are even more pronounced in the double mutant V39/I50, whose catalytic efficiency with the P2(Leu) analog is about 10–20 times lower than with P4(Ile) or VSQNYPIVQ.

Interestingly, differences in specificity between the equine infectious anemia virus PR and the HIV-1 PR have been attributed to Thr-30 versus Asp-30 and Val-56 versus Ile-50 which increase the size and hydrophobicity of the S2 and S2' subsites. Accordingly, VSQNYPIVQ analogs with larger hydrophobic residues at P2 are less efficiently cleaved by mutant A57I than by the wild type MuLV PR, and analogs having Ala at P2' become better substrates of the mutant PR. These effects are even more pronounced in the double mutant V39/A57I, whose catalytic efficiency with the P2(Leu) analog is about 10–20 times lower than with P4(Ile) or VSQNYPIVQ. Analysis of the molecular model of MuLV PR suggests that the preference for larger hydrophobic residues at P2 can be correlated with the larger size of the S2 subsite of MuLV PR, compared with HIV PRs. The smaller Val-39 rather than Ile at the top of the subsite allows longer hydrophobic residues like Leu to fit at P2 and P2'. In a similar way, the presence of the larger Ile in the mutant A57I is expected to reduce the size of the S2 and S2' subsites. Accordingly, VSQNYPIVQ analogs with larger hydrophobic residues at P2 are less efficiently cleaved by mutant A57I than by the wild type MuLV PR, and analogs having Ala at P2' become better substrates of the mutant PR.

Apart from Val-54 and Ala-57, which contribute to S2/S2' subsites in MuLV PR, the mutation of other residues which are predicted to form the flap region (e.g. Trp-53, Gln-55, or Gly-60) led to a significant decrease of the PR activity. These results are in agreement with other reports showing that mutations at the flap regions of HIV-1 and Rous sarcoma virus PRs alter the catalytic rate of the enzyme (24, 37). Residues 47–56 at the top of the flap region of HIV-1 PR are highly conserved among clinical isolates (38, 39), and their substitution by site-directed mutagenesis often leads to the inactivation of the PR (37). In some cases, viral resistance is associated with flap mutations involving the replacement of Met-46 by Ile, Leu, or Phe (28–30, 39).
34, 40) or Gly-48 by Val (29, 31). Multiple substitutions at the flap region (e.g. M46I/I47V/I50V) have also been associated with resistance to PR inhibitors (30).

Although the subsites of the substrate binding pocket of the retroviral PR are capable of acting independently in the substrate selection, it will probably be necessary to combine a set of mutations to fully alter the specificity at any subsite. Interactions among different substrate-binding residues seem to be important for substrate specificity. Amino acids forming the $S_2/S_3$ subsites and those involved in the flap region appear to be critical determinants of specificity and catalytic activity in MuLV PR, as well as in other retroviral PRs. A better knowledge of the interactions between substrate and protease in various retroviral PRs would be helpful to design broad spectrum inhibitors, which would limit the emergence of drug resistant phenotypes in HIV.

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