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Proline for Alanine Substitutions in the C-Peptide Helix of Ribonuclease A†

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ABSTRACT: The effect on overall α-helix content of substituting proline for alanine has been determined at 5 positions (1, 2, 4, 5, and 13) of a 13-residue peptide related in sequence to residues 1–13 of ribonuclease A. The helix content falls off rapidly as proline is moved inward, and the proline residue effectively truncates the helix. No helix-stabilizing effect of proline is found at positions 2 or 4 within the first turn of the helix. Proline substitution at either end position (1, 13) has little effect on overall helix content, in agreement with an earlier study of glycine for alanine substitutions. The two end residues of the helix appear to be strongly frayed.

The proline ring is not easily accommodated in the interior of an α-helix, and proline lacks the NH group needed to maintain the H-bonding pattern of the α-helix backbone. Thus, it is not surprising that proline is the most strongly helix-breaking amino acid when helix propensity is measured either by host–guest studies using random copolymers (Altmann et al., 1990) or by single proline substitutions in a coiled-coil helix (O'Neil & DeGrado, 1990) or in an isolated α-helix (Merutka et al., 1990). Nevertheless, proline is found fairly often within α-helices in proteins: it occurs in 8% of the helices analyzed by Richardson and Richardson (1988). The proline ring can be accommodated comfortably within the first turn of an α-helix, and since the NH groups of the first four residues of an α-helix are not H-bonded, proline's lack of a free NH group presents no problem at positions 1–4. Indeed, proline is found more frequently in the first four residues of protein α-helices than in their interiors and strikingly so at the first helical position (N1, or N-cap plus one) (Richardson & Richardson, 1988). A free energy simulation study has found that proline is actually helix-stabilizing at N1 (Yun et al., 1991).

The basic aim of our study is to determine how the helix content of an isolated α-helix is affected by substituting proline for alanine at different positions. These substitutions are made singly in individual peptides. The reference peptide is related to the C-peptide (residues 1–13) of RNase A. The reference peptide, Suc-AATAAAKYLAAHA-NH_2, differs only by the substitution F8 → Y from a peptide studied earlier, RN 44, which is known to be a strong helix-former and which was used in a study of the Glu-2^-–Arg-10^+ salt bridge of C-peptide (Fairman et al., 1990). Both Glu-2 and Arg-10 have been replaced by Ala in RN 44; the purpose here of eliminating the Glu-2^-–Arg-10^+ salt bridge is to avoid steric hindrance between the salt bridge and a substituted proline residue. The substitution F8 → Y allows the peptide concentration to be determined by tyrosine absorbance (Marqusee et al., 1989), which is needed for accurate measurement of the mean residue ellipticity. The reason for using a unique-sequence peptide rather than one of the repeating sequence peptides studied by Marqusee and Baldwin (1987), or by Marqusee et al. (1989), is to facilitate NMR analysis of the system.

Our second aim is to investigate fraying of the end residues (1,13) of the helix. An earlier study of glycine for alanine substitutions (Strehlow & Baldwin, 1989) revealed that glycine substitution at either end position has little or no effect on the

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helix content of the whole peptide, in marked contrast to glycine substitution at interior positions. The explanation given as likely was that the end residues of the helix are frayed, and therefore that substitution of a helix-breaking residue has little effect at these positions. This explanation is tested here by substituting a strong helix-breaking residue, proline, at either end position.

The results of proline substitition at different positions are compared to predictions based on the Lifson and Roig (1961) theory of the helix–random-coil transition, using a computer program written by Dr. John A. Schellman [see Chakrabartty et al. (1991)]. In the simplified form of the program used here, the helix-breaking effect of proline is ascribed solely to its very low value of s, the helix propagation parameter.

Experimental Procedures

Materials. Some peptides were synthesized by t-Boc chemistry on a RAMPS synthesizer (Du Pont); others were made by FMOC chemistry on a Milligen 9050 synthesizer. Purification procedures have been described (Marqusee et al., 1989). Peptide purity and amino acid composition were checked by analytical reverse-phase FPLC, amino acid analysis, and proton NMR spectroscopy. For reverse-phase FPLC, peptides were eluted from a 5 × 50 mm C-18 silica column (100-Å pore size, Pharmacia) with a 5–40% gradient of acetonitrile (J. T. Baker, Inc.) in water and 0.1% trifluoroacetic acid (Milligen/Biosearch). The flow rate was 1.0 mL·min^{-1}, and the total volume of eluate was 40 mL. More than 60% of the peptide eluted in the main peak, which always contained the desired peptide. The primary-ion molecular weight was determined by fast-atom bombardment mass spectrometry. Observed molecule weights were within 0.3 mass unit of the predicted values.

Methods. Peptide concentration was determined from 1 mM stock solutions by measuring the tyrosine absorbance at 275 nm, using an extinction coefficient of 1450 M^{-1} cm^{-1} for a single tyrosine residue in peptide linkage, in 6 M GuHCl at 25 °C, pH 7 (Brandts & Kaplan, 1973).

Circular dichroism measurements were made on an AVIV 60 DS instrument and have been described previously (Marqusee et al., 1989). The buffer used for recording the pH profile of $[\theta]_{222}$ is 0.01 M NaCl, 1 mM sodium citrate, 1 mM sodium phosphate, and 1 mM sodium borate; pH was adjusted with HCl and NaOH. For recording spectra, the buffer is 0.01 M KF, 1 mM potassium acetate, and 1 mM potassium phosphate; pH was adjusted to 5.3 with phosphoric acid. Peptide concentrations of CD measurements were 15–250 μM, and $[\theta]_{222}$ was independent of peptide concentration over the range studied. Data for pH profiles were collected with a 10-mm path-length cell while those for spectra were collected with a 1-mm cell. Spectra are the average of three scans collected from 195 to 300 nm in 0.25-nm intervals. The temperature was 3 °C for all experiments.

Proton NMR spectra were obtained on a General Electric GN-500 spectrometer operating at 500.12-MHz proton frequency. Sample solutions were ~1 mM peptide/3 mM sodium citrate, in D_2O (Cambridge Isotope Laboratories), and the pH* (not corrected for isotope effects) was adjusted to 5.3 with DCl and NaOD (MSD Isotopes). Sodium (trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP, ICN Biomedicals) was used as the internal chemical shift reference at 0 ppm. Urea-d_4 was prepared by repeated lyophilization of urea dissolved in D_2O. Data were collected on samples equilibrated for at least 10 min at the desired temperature. Free induction decays were the sum of 128 transients, each of which was 4096 complex points in size. The sweep width was 5400 Hz, the recycle delay was 1 s, and the residual HDO resonance was partially suppressed by irradiation during the recycle delay. Resonance assignments were based on standard amino acid results (Wüthrich, 1986) and on assignments made by Osterhout et al. (1989) on a closely related derivative of C-peptide.

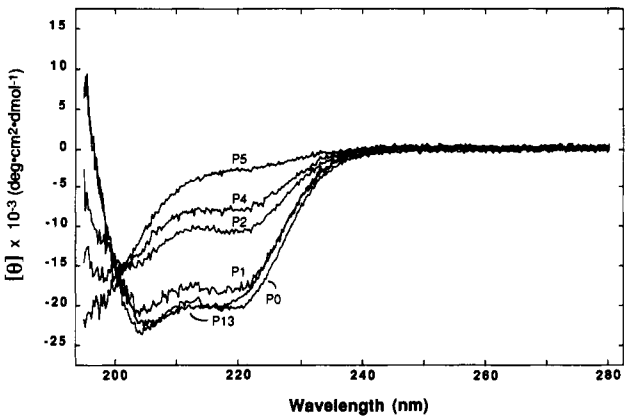

FIGURE 1: CD spectra of P0 and of the five proline-substituted peptides at 3 °C, in 10 mM KF, pH 5.3 (see Methods).

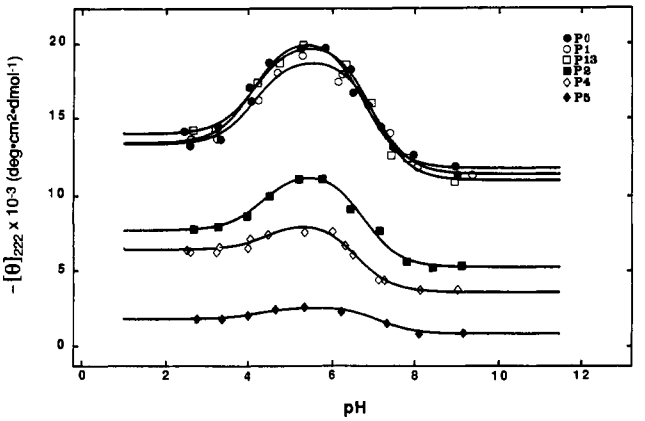

FIGURE 2: Comparison of the pH profiles of helix content for the reference peptide P0 and for the five proline-substituted peptides at 3 °C (see Methods for buffer); $[\theta]_{222}$ is the mean residue ellipticity at 222 nm. The solid line is a computer fit to a titration curve (Fairman et al., 1989) containing two adjustable pK_a's, one for the succinyl group and one for the histidine side chain.

Results

CD Spectra and pH Profiles of the Substituted Peptides. Figure 1 shows the CD spectra of the five substituted peptides (P1, P2, P4, P5, and P13) and of the reference peptide, P0. The abbreviation P1 means that the substitution Ala → Pro has been made at residue position 1, numbering from the N-terminus, and so on. All peptides show CD spectra indicative of mixtures of α-helix and random coil, with negative bands centered near 222 and 207 nm and with an isodichroic point at 201 nm. The CD spectrum of P5 is consistent with a small but significant α-helix content (see below).

Figure 2 shows the helix content of each peptide as a function of pH at 3 °C, measured by CD in a buffer containing 0.01 M NaCl. The increase in helix content between pH 2 and pH 5.3 reflects the ionization of the COOH group of the succinyl group at the N-terminus: the negatively charged COO^- group is close to the positive pole of the helix dipole, which increases helix stability (Fairman et al., 1989). The decrease in helix content between pH 5.3 and pH 9 reflects the titration of His-12$^+$, whose positive charge is required for a helix-stabilizing interaction between His-12 and the ring of Tyr-8 (Shoemaker et al., 1990). The fitted curves are made

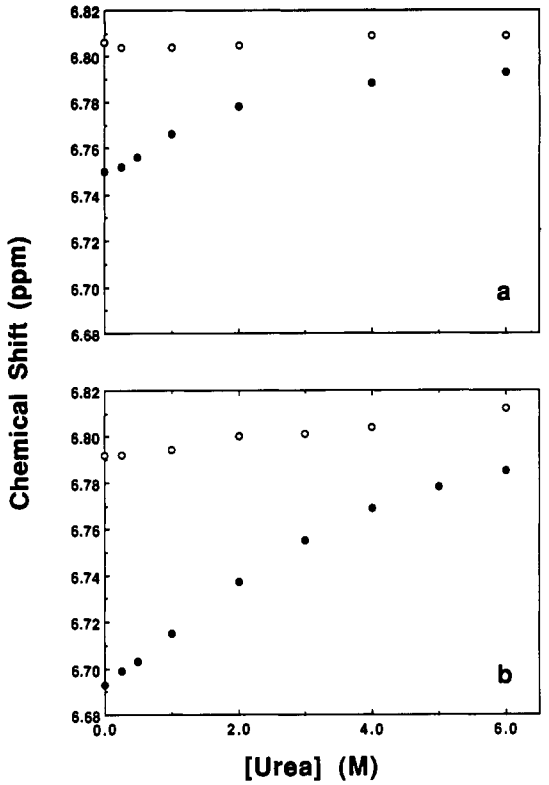

FIGURE 3: Chemical shift of the Tyr-8 Hϵ resonance in peptide P4 (a) and in the reference peptide P0 (b) as a function of urea concentration at 5 °C (●) and at 45 °C (○). The chemical shift is given as parts per million from a TSP standard (see Methods).

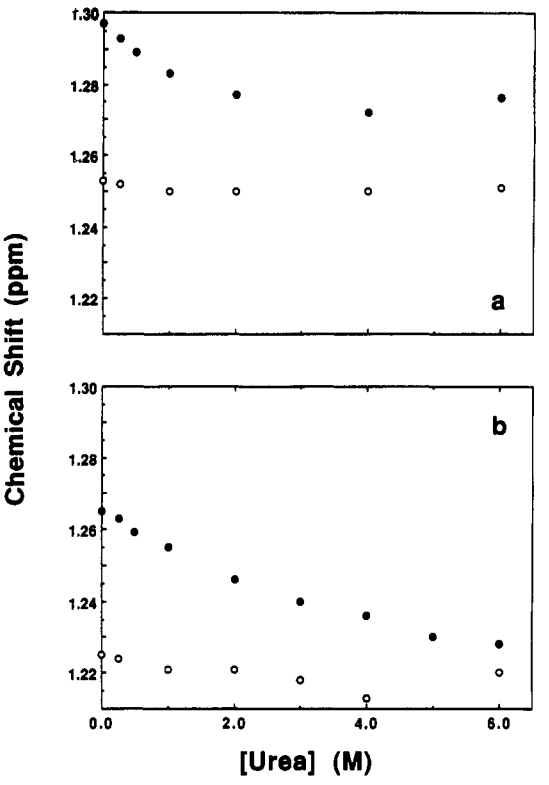

FIGURE 4: Chemical shift of the Thr-3 Hγ resonance in peptide P4 (a) and in peptide P0 (b) as a function of urea concentration at 5 °C (●) and at 45 °C (○).

by nonlinear least-squares fitting to two pK_a's. The pH profiles of P0, P1, and P13 are nearly identical. Since all charged residues are left unchanged in the substituted peptides, their pK_a's should be similar. The fitted curves shown in Figure 2 are given to facilitate visual inspection of the data, not to determine pK_a's. Because the plateau value of $[\theta]_{222}$ between the two pK_a's (near pH 5.3) cannot be measured directly, the pK_a determinations should be viewed with caution. Fitted values of the lower pK_a are 4.12 in P0 and 4.16 in P13, and for the higher pK_a, they are 6.77 in P0 and 6.96 in P13. As regards shape, the pH profiles of P2 and P4 are also similar to P0 but their helix contents are lower. The pH profile of P5 indicates that this peptide does have a measurable helix content, since it shows a significant pH dependence of $-[\theta]_{222}$ that is similar in shape to P0.

NMR Studies. No evidence was found for observable cis–trans isomerization of the proline residue in any of the substituted peptides. All spectra show a single pair of Pro Hδ resonances near 3.8 ppm, and no observable doubling of other resonances was found for peptide P4. This behavior remains the same when the helix is completely unfolded in 4 M urea at 45 °C. Consequently, the proline residue in P4 exists as a single isomeric form, probably the trans isomer, and the other proline-containing peptides appear to show the same behavior. In a 32-residue peptide containing chiefly Ala residues interspersed with Pro residues, the prolines were found to be exclusively in the trans conformation (Radford et al., 1989), and so the situation found here is not unique.

Since our results suggest that substitution of a proline residue effectively truncates the helix (see below), we tested whether residues 1–3 of peptide P4 are random coil by measuring the urea concentration dependences of side-chain chemical shifts of residues on either side of proline-4. This technique has been used in a study of the "helix stop" in the S-peptide of RNase A (Kim & Baldwin, 1984). The control experiment is to measure the urea concentration dependence of the chemical shift for the random-coil form, at a high temperature (45 °C), where the urea concentration dependence should be small if the peptide is truly a random coil and if urea has little effect on the chemical shifts of resonances in the random coil. Then melting-out of the helix by urea at a low temperature can be monitored from the nonlinear dependence of chemical shift on urea concentration.

Figure 3a shows the urea concentration dependence of the Tyr-8 Hϵ resonance of P4 at 5 and 45 °C, and Figure 3b shows the corresponding data for P0. At 45 °C, the data for P4 nearly fit a horizontal line, indicative of no measurable helix content, and the data for P0 at 45 °C approach the same line above 2 M urea. At 5 °C, a strong, nonlinear dependence of chemical shift on urea concentration is observed for P0 and a similar but smaller effect is seen for P4, in agreement with the smaller helix content of P4 found by CD. Similar results are found for the Leu-9 Hδ resonances and for an unassigned Ala Hβ resonance. Large changes in chemical shift are observed for the His-12 Hβ, β′, and ϵ resonances, but because the pH* (5.3) is too close to the range where His is titrated and His resonances show large titration shifts, no attempt was made to interpret these changes.

Surprisingly, similar results are also found for the Hγ resonance of Thr-3 (Figure 4a,b). The chemical shift of this resonance in the random-coil form (≥2 M urea, 45 °C) is different in P0 (1.22 ppm) and in P4 (1.25 ppm), and so there is an influence of the neighboring proline-4 residue on the Thr-3 Hγ resonance even in the random coil. The nonlinear dependence of chemical shift on the urea concentration of P4 at 5 °C (Figure 4a) shows clearly that the Thr-3 Hγ resonance is affected by the presence of the helix, although the analysis given below indicates that the helix is truncated at Pro-4 and Thr-3 should be in the unfolded segment.

Comparison with Helix–Random-Coil Transition Theory. Recent work in our laboratory shows that the Lifson and Roig

Table I: Predicted and Observed Changes in Helix Content Caused by Inserting Proline at Different Positions in the Helix

position (j)[a]	% helix[b]	truncation[c]	s = 0.01[d]
	62	62	
2	35	44	49
4	25	24	25
5	8	15	16

[a] The position of inserting a proline residue, numbered from the N-terminal residue. [b] The experimental percent helix measured by circular dichroism (see Methods), with $-[\theta]_{222}$ for 100% helix taken as 40000(1−2.57/13) = 32100 deg cm^2 dmol^{-1} (Chen et al., 1974) and with $-[\theta]_{222}$ for 0% helix taken as 0. [c] The percent helix calculated from the Lifson–Roig equation for a uniform helix, all of whose residues have the same value of s = 1.78 and σ = 0.0029, and with the number of residues in the helix equal to 13 − j. [d] The percent helix calculated from the Lifson–Roig equation for a uniform helix of 13 residues with proline inserted at position j. The value of s assigned to proline is s = 0.01 [which is the value found by Altmann et al. (1990) by the host–guest method]; other parameters are given in footnote c. It makes little difference if values of s smaller than 0.01 are used.

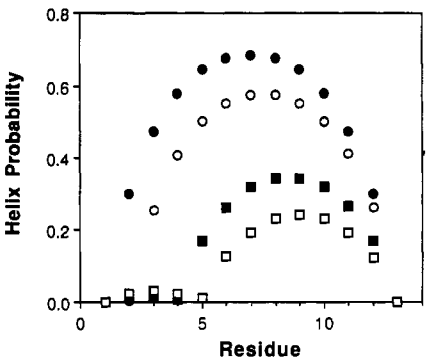

FIGURE 5: Helix content at each residue position predicted by the Lifson–Roig equation, for peptides P2 (○), P4 (■), P5 (□), and P0 (●), using a computer program written by Dr. John Schellman, with the helix–coil transition parameters given in Table I. The approximation is made that all residues except proline have the same helix–coil parameters.

(1961) theory successfully reproduces experimental results for helix formation by alanine-based peptides with simple repeating sequences (Chakrabartty et al., 1991; J. M. Scholtz, unpublished data). By assigning values to s, the helix propagation parameter, and to σ, the helix nucleation parameter, for different individual amino acid residues in the peptide, it is possible to use the Lifson and Roig (1961) theory to predict the helix contents of the proline-containing peptides. A single average value of σ = 0.0029 (which is preliminary) is taken from fitting experimental transition curves to theory for peptides of varying chain lengths (14–50 residues) that contain multiple repeats of the sequence AEAAKA. An average s value of 1.78 is required to reproduce the helix content of P0 at 3 °C. Table I compares the observed drop in helix content when proline is inserted at position j with the effect of (a) truncating a uniform helix between positions j and j + 1 and (b) assigning proline the very low value of s = 0.01. For proline at position 2, the observed drop in helix content is even larger than the predicted effect of truncating the helix. This large drop in helix content may result from interference between the proline residue and the helix-stabilizing interaction between the helix dipole and the negative charge on the succinyl group. There is not much difference between the predicted effects of truncating the helix or assigning proline s = 0.01, except at position 2.

Discussion

Predicted Effects of Proline Substitution. Table I shows that assigning a very low value of s to proline is sufficient to account for the changes in helix content produced by substituting proline at different positions. The Lifson–Roig theory allows prediction of the fraction helix at each residue position, and the predicted curves are shown in Figure 5, based on the same s and σ parameters as in Table I. The striking result is that proline substitution is predicted to divide the peptide into two parts: a helix-forming segment on the longer C-terminal side and a random-coil segment on the shorter N-terminal side [cf. Merutka et al. (1990)]. Thus, proline is predicted to be a highly effective helix stop, in agreement with the observation that proline occurs commonly at or next to the termini of helices (Perutz et al., 1965) and especially at the N-terminus (Richardson & Richardson, 1988).

We sought to test the prediction that residues 1–3 of peptide P4 are nonhelical by using NMR to monitor side-chain chemical shifts as the helix is unfolded with urea (Figures 3 and 4). The test showed, however, that the Hγ resonance of Thr-3 changes its chemical shift as the helix unfolds. Since the Lifson–Roig theory successfully predicts the changes in overall helix content as the position of the proline residue is varied (Table I), it is likely that the behavior predicted in Figure 5 is correct and that Thr-3 is nonhelical even though its chemical shift changes as the helix unfolds. A possible alternative explanation is that the inserted proline residue produces an altered conformation, which can be unfolded by urea or high temperature. The CD spectrum of P4 has the shape expected for a mixture of helical and random-coil residues. Merutka et al. (1990) found that insertion of a central proline residue in a 17-residue peptide completely destroyed its helicity, but gave a CD spectrum different from that of a random coil. The fact that the chemical shift of the Thr-3 Hγ resonance changes as the P4 helix is unfolded by urea need not mean that the Thr-3 residue is partly helical. Note that, in the random-coil forms of P0 and P4 (45 °C, ≥2 M urea), the chemical shift of the Thr-3 Hγ resonance has different values (Figure 4): thus, it is affected by a neighboring proline residue even when the helix is unfolded.

Fraying of the End Residues. The two-state equation, complete helix ⇔ random coil, is sometimes used to represent the unfolding of short helices, although calculations by Schellman (1958) indicate that it is a poor approximation. He found that the complete helix is practically not populated in the unfolding reactions of short helices and typically there is a distribution of helix lengths among the partly helical species. The predicted results shown in Figure 5 are in agreement with his conclusion: the fraction helix, which is shown separately for each residue, decreases rapidly as either end of a helical segment is approached. The physical reason is fraying of the helix at its ends. A residue at the end of a helical segment is more easily converted to the random-coil state, compared to residues in the interior of the helix (Zimm & Bragg, 1959), because of the interlocking H-bonds of the α-helix in which the CO of residue i is H-bonded to the NH of residue i + 4.

A stringent test of the two-state approximation is to substitute a helix-breaking amino acid at an end position and to compare the result with substitution at the center of the helix: if the two-state approximation is valid, the same decrease in helix content on substitition should be observed in both cases (Strehlow & Baldwin, 1989). It has been found earlier that glycine for alanine substitution at an end position has little effect on overall helix content (Strehlow & Baldwin, 1989), in contrast to the two-state-prediction, and the same result is now found for proline. The present results are more striking, because proline has a lower value of s than glycine. It is therefore clear experimentally, as well as from helix–coil

theory, that the two-state equation is a poor approximation to the unfolding of short helices and that the end residues of the helix are strongly frayed.

ACKNOWLEDGMENTS

We thank Dr. Harold Scheraga for a preprint of his manuscript (Altmann et al., 1990) describing measurement of the *s* value for proline by the host–guest method, Dr. J. Hermans for a preprint of his manuscript (Yun et al., 1991), and Dr. John Schellman for use of his computer program for analyzing measurements of helix–coil transitions by the Lifson–Roig theory.

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CORRECTION

Organization, Structure, and Polymorphisms of the Human Profilaggrin Gene, by Song-Qing Gan, O. Wesley McBride, William W. Idler, Nedialka Markova, and Peter M. Steinert*, Volume 29, Number 40, October 9, 1990, pages 9432–9440.

Page 9434. Figure 1 contains a sequencing error. The sequence should have an extra base, a T, at bp position 1303. As a result, this moves the *Eco*RV site to bp 1095. In addition, the open reading frame continues further upstream. Since there are now other in-frame ATG codons, we are no longer certain that the ATG codon at bp 1477 serves as the initiating codon. Additional functional assays are in progress to identify the correct initiation codon. A corrected portion of Figure 1 follows.

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gλHF5  tgtgattctgtctgatgcagtctccctctgtgacttccctctgtacagAATCCAGATGACCCAGATATGGTTGATGTCTTCATGGATCAC
λHF202                                                 AATCCAGATGACCCAGATATGGTTGATGTCTTCATGGATCAC  990

gλHF5  TTGGATATAGACCACAACAAGAAAATTGACTTCACTGAGTTTCTTCTGATGGTATTCAAGTTGGCTCAAGCATATTATGAGTCTACCAGA
λHF202 TTGGATATAGACCACAACAAGAAAATTGACTTCACTGAGTTTCTTCTGATGGTATTCAAGTTGGCTCAAGCATATTATGAGTCTACCAGA 1080

                  EcoR V
gλHF5  AAAGAGAATTTACCGATATCAGGACACAAGCACAGAAAGCACAGTCATCATGATAAACATGAAGATAATAAACAGGAAGAAAACAAAGAA
λHF202 AAAGAGAATTTACCGATATCAGGACACAAGCACAGAAAGCACAGTCATCATGATAAACATGAAGATAATAAACAGGAAGAAAACAAAGAA 1170

gλHF5  AACAGAAAAAGACCCTCAAGTCTGGAAAGAAGAAACAATAGAAAAGGGAATAAGGGAAGATCCAAGAGCCCAAGAGAAACAGGGGGGAAA
λHF202 AACAGAAAAAGACCCTCAAGTCTGGAAAGAAGAAACAATAGAAAAGGGAATAAGGGAAGATCCAAGAGCCCAAGAGAAACAGGGGGGAAA 1260

gλHF5  AGGCATGAATCTAGTTCTGAAAAAAAAGAAAGAAAAAGGATATTCACCTACTCATAGAGAAGAAGAATATGGAAAAAACCATCATAACTCA
λHF202 AGGCATGAATCTAGTTCTGAAAAAAAAGAAAGAAAAAGGATATTCACCTACTCATAGAGAAGAAGAATATGGAAAAAACCATCATAACTCA 1351

gλHF5  AGTAAAAAAGAGAAAAACAAGACTGAAAATACTAGATTAGGAGACAATAGGAAGAGGCTAAGTGAAAGACTTGAAGAGAAAGAAGACAAT
λHF202 AGTAAAAAAGAGAAAAACAAGACTGAAAATACTAGATTAGGAGACAATAGGAAGAGGCTAAGTGAAAGACTTGAAGAGAAAGAAGACAAT
λHF604                          AAAATACTAGATTAGGAGACAATAGGAAGAGGCTAAGTGAAAGACTTGAAGAGAAAGAAGACAAT 1441

                                          */M T  Q  K  W  I  Q  S  G  H  I  A  T  Y  Y  T  I  Q  ?/17
gλHF5  GAAGAAGGAGTATATGATTATGAAAATACAGGAAGAATGACTCAAAAATGGATACAATCAGGCCATATTGCCACATATTACACAATCCAG
λHF202 GAAGAAGGAGTATATGATTATGAAAATACAGGAAGAATGACTCAAAAATGGATACAATCAGGCCATATTGCCACATATTACACAATCCAG
λHF604 GAAGAAGGAGTATATGATTATGAAAATACAGGAAGAATGACTCAAAAATGGATACAATCAGGCCATATTGCCACATATTACACAATCCAG
λHF373        GGAGTATATGATTATGAAAATACAGGAAGAATGACTCAAAAATGGATACAATCAGGCCATATTGCCACATATTACACAATCCAG 1531
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