Hairpin formation within the enhancer region of the human enkephalin gene

(cAMP response/cruciform/transcription)

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Communicated by K. E. van Holde, August 3, 1990 (received for review May 23, 1990)

ABSTRACT The 3',5'-cyclic adenosine monophosphate (cAMP)-inducible enhancer of the human enkephalin gene is located within an imperfect palindromic 23 base pairs. We have found that a 23-base-pair oligonucleotide duplex containing the enhancer undergoes a reversible conformational transition from the duplex to two individual hairpin structures each formed from one strand of the duplex. Each individual hairpin forms with mismatched base pairs, one containing two GT pairs and the other containing two AC pairs. The conformational transition is stabilized by proton transfer to the hairpin containing AC mismatched pairs. The unique physical and thermodynamic properties of the enkephalin enhancer DNA suggest a model in which DNA secondary structure within the enhancer region plays an active role in cAMP-inducible activation of the human enkephalin gene via formation of cruciform structures.

The enkephalin gene is one of many neuroendocrine peptide genes in which transcription is induced as a result of second-messenger production (1). Transcriptional induction of the gene by cAMP has been mapped to a short DNA element located between nucleotides −104 and −86, upstream of the transcriptional start site (1). Mutational analysis in this region has indicated that two elements, termed CRE-1 and CRE-2, working together constitute a cAMP-responsive enhancer (2). To understand the mechanism by which cAMP production can activate the transcription of genes, we have undertaken detailed physical studies of the DNA–protein interactions within the enkephalin enhancer region. In this report, we focus entirely on the unusual properties of the enhancer DNA itself. We show that, even in the presence of its hydrogen-bonded partner strand, a synthetic enhancer duplex will spontaneously convert into two hairpin structures under the appropriate solution conditions. The physical and thermodynamic properties of the unique enkephalin enhancer DNA suggest a model in which DNA secondary structure within the enhancer region plays an active role in cAMP-inducible activation of the human enkephalin gene by formation of a cruciform structure.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Oligonucleotides I (5'-GCTGGCCGCTGCAGC-3') and its complement II (5'-GCTGACGCGCCCTACGCCAGC-3') and III (5'-TGTACCCACCGTGAAGGACTGAGAGG-3') and its complement IV (5'-TCTTATGCCTCAGGTGGTGGTACA-3') were synthesized and purified by Genetics Design (Houston, TX). Oligomers were purified by gel electrophoresis and the sequence was verified by m13 DNA sequencing (3).

Gel Electrophoresis. Nondenaturing polyacrylamide gels were made according to standard methods described in ref. 4. Oligonucleotides were separated on 30 cm × 14 cm × 0.7 mm nondenaturing 12% polyacrylamide gels buffered in 10 mM Pipes/0.1 mM EDTA (Pipes 00) under the appropriate pH conditions, ranging from pH 5.5 to 7.0. Samples were diluted into the appropriate buffer containing 10% sucrose. No dye was added to any sample before loading on the gel. Each gel was run under constant current of 20–25 mA until the desired separation was achieved. The running buffer in all cases was identical to the buffer in the gel. All pH 5.5 samples were analyzed on gels at both pH 5.5 and at pH 7.0. In either cases, the results generated did not affect the conclusions of the experiment. After separation, each gel was dried and exposed to autoradiographic film.

The oligomer duplexes were separated from any excess single strands by electrophoresis on 12% polyacrylamide gels and the band corresponding to the duplex form was identified by UV shadowing (4). Oligomer duplexes were cut out of the gel, electroeluted, and further purified on a Sephadex G-25 column buffered in Pipes 00, pH 7.0.

32P-5'-End-Labeling and Column Chromatography. Oligonucleotides were labeled at their 5' termini according to standard methods (4). Free label was removed from the labeled oligonucleotides by precipitation with ammonium acetate (4).

32P-5'-end-labeled oligonucleotide duplexes and/or strands in Pipes 00, pH 7.0, were diluted into 10 mM Pipes at either pH 5.5 or pH 7.0. The samples were allowed to equilibrate at room temperature (23°C) for 12–24 hr and separated by size on a Sephadex G-50 column, equilibrated with the appropriate buffer. The bed volume was 10 ml and the column length was 11 cm. The flow rate of the column was 0.13 ml/min and 0.25-ml column fractions were collected.

Melting. UV spectral analyses and thermal melting experiments were performed on a Cary 2200 or on a Cary 219 spectrophotometer interfaced with an Apple-Ile microcomputer. The data collection conditions and analyses were as previously described (5).

RESULTS

Human Enkephalin Enhancer Undergoes a Reversible Duplex-to-Hairpin Conformational Transition That Is Usually Dependent on Concentration and pH. The cis-acting element responsible for cAMP induction of the human enkephalin gene is located 104 to 86 base pairs (bp) upstream of the transcriptional initiation site (1). Mutational analysis in this region has indicated that two elements, termed CRE-1 and CRE-2, work in concert to constitute a cAMP-responsive enhancer.

Abbreviation: CRE, cAMP-responsive enhancer.

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enhancer (2). Inspection of the DNA sequence in the enhancer region reveals that CRE-1 and CRE-2 are contained within a 23-bp imperfect palindrome and that each strand of the enhancer has the potential to form not only a 23-bp duplex but also the hairpin structures shown in Fig. 1. A unique feature of these hairpin structures is that each would form with mismatched base pairs, the top strand (GT) forming two GT pairs and the complementary strand (AC) forming two AC pairs. To examine the structure of the enkephalin enhancer region, the two 23-bp strands that compose the entire enhancer duplex were synthesized from residues -106 to -84, containing both CRE-1 and CRE-2 and flanking sequences. We observed that the individual strands, in the absence of their partner strand, formed stable hairpin structures, despite the fact that each hairpin forms with mismatched base pairs. Melting experiments utilizing these oligonucleotides demonstrate that increases in temperature result in a highly cooperative increase in absorbance, indicative of the large degree of base unstacking found in hydrogen-bonded structures upon melting. In Pipes 00 at pH 7.0 the temperature at the midpoint for the GT strand transition ($t_m$) is 45°C, the $t_m$ for the AC strand transition is 49°C, and the $t_m$ for the duplex is 63°C. The hyperchromicity for the melting of each strand is 15–16%, roughly half the value for melting of a 23-bp duplex molecule. These data are consistent with a structure in which roughly half the base pairs are hydrogen bonded. As expected, the $t_m$ values of both the AC and the GT strand transitions are independent of concentration over a 1000-fold range, conclusively demonstrating an intramolecular equilibrium (not shown).

To identify structural transitions within the human enkephalin enhancer, the conformational state of the native enhancer duplex (Duplex in Fig. 1) was examined under a wide range of solution conditions. An example of the gel-purified product is shown in Fig. 2A. The enhancer duplex form is easily distinguished from the single-strand forms by its greatly reduced mobility. Additionally, the strand forms are seen as doublets (Fig. 2A), with the faster migrating form (I) corresponding to the hairpin conformation and the slower-migrating form (II) corresponding to the linear strand conformation (unpublished results). We observed that, in solution, the duplex form of the 23-bp human enkephalin enhancer could spontaneously dissociate into the strand forms, and that this process was reversible. Starting with gel-
purified duplex molecules (Fig. 2A), we diluted the duplexes into Pipes 00 buffer, pH 5.5, and passed this sample over a Sephadex G-50 column equilibrated with the same buffer. The elution profile, shown in Fig. 2B, indicates that a single form of the enhancer oligonucleotide was present but that this form was no longer a duplex. Analysis of the column fractions revealed that each fraction contained sample which migrated as doublet bands (Fig. 2C) and that the doublet bands comigrated with the GT and AC strand markers (Fig. 2D). We observed no trace of the duplex form of the enhancer even after a prolonged period of autoradiographic exposure (Fig. 2D). Thus, dilution of the enhancer oligonucleotide duplex into lower-pH buffer resulted in total conversion of the duplex form into the strand forms. It is important to note that the lowest pH range included in the study is well above the pH range where significant base protonation generally occurs (6) and is within a pH range where nucleic acid duplexes are usually quite stable (see below). To demonstrate that the duplex-to-hairpin conformational change was reversible, we added an aliquot of the 32P-end-labeled material from selected column fractions (Figs. 20, 23, and 25) to a concentrated sample of unlabeled duplex (1.5 X 10^-7 M base pairs) in Pipes 00, pH 7.0. Increasing the concentration of the sample resulted in a reversible conformational change such that the strand forms of the enhancer enhancer were converted back into the duplex form (Fig. 2D). We concluded that the human enkephalin enhancer is capable of undergoing a reversible conformational change from the duplex to two individual hairpin structures. The relative level of the hairpin forms increases with increasing temperature and decreases with increasing ionic strength, as expected for normal duplex-hairpin equilibria (not shown). However, the enkephalin enhancer duplex-hairpin equilibrium is particularly dependent on concentration and unusually dependent on pH.

Concentration Dependence of the Reversible Conformational Change Is Specific for Structural Features Found Within the Human Enkephalin Enhancer. To show that the concentration dependence of the conformational change is specific for DNA duplexes that can form secondary structures, we synthesized a 23-bp oligomer duplex which was not palindromic (oligonucleotides III and IV). Thus, neither of the strands that constitute this duplex was capable of forming a stable hydrogen-bonded structure. The nonpalindromic (III/IV) 23-mer duplex was diluted into Pipes 00, pH 5.5, and passed over a Sephadex G-50 column in an identical manner as in experiments utilizing the human enkephalin duplex. In control experiments (Fig. 3, lanes A and B), we found that both the GT and the AC strands of the human enkephalin enhancer elute from the column as single peaks and comigrate with the strand markers. The results for the nonpalindromic 23-mer duplex and the human enkephalin duplex are shown in Fig. 3 lanes C-F. Under low concentration conditions (1.0 X 10^-9 M base pairs), 10-fold dilution of the nonpalindromic 23-mer duplex into Pipes 00, pH 7.0 (not shown) or 5.5 (Fig. 3, lane C), gives rise to essentially no strand dissociation. Thus, changes in concentration alone or pH did not induce significant alterations in the level of the duplex form for a nucleic acid whose strands could not assume a stable hairpin conformation. Under identical concentration conditions, the 23-bp human enkephalin enhancer duplex at pH 7.0 (Fig. 3, lane D) also remains in the duplex form. However, under the same low concentration conditions, dilution into pH 5.5 buffer resulted in total conversion to the strand form (Fig. 3, lane E). Under pH 5.5 conditions, increasing the concentration of the enhancer enhancer duplex to 1.5 X 10^-7 M base pairs shifts the equilibrium back towards the duplex form, although traces of the strand form can be observed (Fig. 3, lane F). We conclude that the enkephalin enhancer equilibrium displays a particular dependence on concentration, since changes in concentration alone at constant pH and ionic strength result in total conversion into the strand form, and the same effect is not observed for nonpalindromic DNA duplexes of identical length under the identical conditions. Thus, the concentration dependence is directly related to the palindromic nature of the enkephalin duplex and is the result of the small free energy difference between the duplex and hairpin states. However, the sensitive dependence on concentration is observed only under lower pH conditions. Consequently, we examined the nature of the pH effect.

pH Effect. The pH effect is mediated by the AC hairpin. To examine the nature of the pH effect, we measured the stability of the enkephalin enhancer duplex and both of the individual strands that constitute the duplex as a function of pH. Stability was monitored by the midpoint of the thermal transition (t_m) for each conformation. The results are shown in Fig. 4. For the native enkephalin enhancer, the stability of the duplex changes only 5°C over the pH range from 9.0 to 5.5 in Pipes 00. The duplex form is most stable in the range from pH 9.0 to 7.0, conditions under which the t_m changed very little, from 60°C to 58°C. Decreasing the pH from 7.0 to 5.5 resulted in a further decrease in the t_m to 55°C. Similarly the GT hairpin displays a roughly 5°C change within the same pH range. From pH 9.0 to 7.0, the t_m for the hairpin-to-strand transition remains unchanged at 45-46°C. Decreasing the pH from 7.0 to 5.5 results in a t_m decrease from 46°C at pH 7.0 to 41°C at pH 5.5. In contrast, the stability of the AC hairpin displays the opposite pH-dependent stability profile relative to the duplex form.

**Fig. 3.** PAGE analysis of column fraction 3 for the GT strand, the AC strand, a nonpalindromic 23-mer duplex, and the human enkephalin enhancer duplex. Column fractions at pH 5.5 were analyzed on PAGE gels (as in Fig. 2) at both pH 7.0 and pH 5.5. In the left three lanes, duplex standard is D; AC hairpin standard is AC, and GT hairpin standard is GT. Lane A, 5'-32P-GT strand in Pipes 00, pH 5.5 (1.0 X 10^-8 M base pairs) analyzed on a pH 7.0 gel; lane B, the 5'-32P-AC strand in Pipes 00, pH 5.5 (1.5 X 10^-8 M base pairs) analyzed on a pH 7.0 gel; lane C, a 5'-32P-labeled nonpalindromic 23-mer duplex in Pipes 00, pH 5.5 (1.3 X 10^-8 M base pairs) analyzed on a pH 7.0 gel; lane D, the 5'-32P-labeled human enkephalin enhancer duplex in Pipes 00, pH 7.0 (1.8 X 10^-8 M base pairs) analyzed on a pH 7.0 gel; lane E, the 5'-32P-labeled human enkephalin enhancer (1.8 X 10^-8 M base pairs) in Pipes 00, pH 5.5, analyzed on a pH 7.0 gel.

**Fig. 4.** pH dependence of melting transitions for the human enkephalin duplex (○), the GT strand (■), and the AC strand (▲). The A260 of each sample was 0.5; the concentration of each sample was 2.0 to 5.0 X 10^-5 M base pairs. The buffer used in all experiments was Pipes 00 at the indicated pH.
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to either the duplex or the GT strand. The AC strand undergoes a 30°C increase in stability as the pH decreases from 9.0 to 5.5. At pH 9.0, conditions under which both the duplex and the GT strand are the most stable, the AC strand is essentially denatured. As the pH is decreased from 9.0 to 7.0, the stability of the AC strand increases by 15°C, from 30°C to 45°C. Further decrease in the pH from 7.0 to 5.5 results in another 15°C increase in the \( t_{m} \), which reaches 60–61°C at pH 5.5. Between pH 6.0 and 5.5, the stability of the AC hairpin is greater than the stability of the duplex. At pH 5.5 under physiological ionic strength conditions, Na\(^+\) = 0.1 M, the \( t_{m} \) values of the duplex form and the AC hairpin are nearly identical at 65°C, and the \( t_{m} \) of the GT hairpin is 46°C. Thus, under slightly acidic conditions, the energy difference between the duplex and both the hairpin forms is small and, under low concentration conditions, the hairpin conformation becomes the favored form. The dramatic increase in stability arises, in part, from protonation of the AC strand, shown below.

The apparent pK\(_a\) of the AC strand is at physiological pH and is independent of ionic strength. From the pH titration curve, we were able to estimate the apparent pK\(_a\) of the AC strand (protonation) (Fig. 4). The midpoint for the protonation of the AC strand is pH 7.2, physiological pH. The importance of measuring the pK\(_a\) is that it allows identification of the relevant range of protonation. For example, the AC strand is 80% protonated at pH 6.6 and 20% protonated at pH 7.8. Thus, changes of only 0.5 of a pH unit create conditions under which the AC hairpin is either largely stabilized or largely destabilized. These small changes in pH dictate whether the enkephalin enhancer will remain as a duplex or form two hairpin structures from the individual strands. This conformational change is due, in part, to stabilization energy of the protonated AC strand. Protonation is involved in increased hydrogen bonding within the AC hairpin, since changes in pH from 7.9 to 6.2 result in a large increase in the apparent \( \Delta H^\circ \) from 52 to 53 kcal/mol (1 kcal = 4.18 kJ) (unpublished results). From melting data and the apparent \( \Delta H^\circ \) values, we were able to calculate \( \Delta n H^\circ \), the number of protons that are taken up by the AC strand in Pipes 00, pH 5.5, under a range of ionic strength conditions, using (ref. 7)

\[
\Delta n_{H} = \frac{-\Delta n_{H^+} R T_{m} / \Delta H^\circ}{[H^+]}
\]

[1]

The results are shown in Table 1. Independent of ionic strength, the number of protons which are taken up by the AC strand approaches 2.0. We conclude that the apparent pK\(_a\) remains at 7.2 under all ionic strength conditions. Thus, the AC hairpin can bind a maximum of two protons, which contribute to hydrogen bonding and stabilization of the AC hairpin form, at the expense of duplex formation. The biological significance of these results is discussed below.

**DISCUSSION**

We have shown that a synthetic enhancer region of the human enkephalin gene can spontaneously undergo a reversible duplex-to-hairpin conformational change under acidic conditions at low DNA concentrations. From the standard relationship for a non-self-complementary duplex (8), we can calculate the free energy difference between an enhancer duplex and two hairpins, using the apparent \( \Delta H^\circ \) values of 92.0 kcal/mol of duplex, 46.0 kcal/mol of GT strand, and 52.0 kcal/mol of AC strand from calorimetric results. As a short oligonucleotide under physiological conditions, 37°C, 0.1 M Na\(^+\), pH 7.0, the free energy difference between the duplex and two stable hairpin structures, \( \Delta(\Delta G^\circ) \), is 18.6 kcal/mol. The equilibrium constant \( K \) for the duplex-to-hairpin conformational change under these conditions is \( 8.5 \times 10^{-14} \) M\(^{-1}\). Consequently, dissociation of the duplex into the strand form is never observed under these conditions. Decreasing the pH to 6.2 under the same ionic strength and temperature conditions results in a dramatic decrease in the free energy difference to 3.0 kcal/mol, \( K = 1.3 \times 10^{-3} \) M\(^{-1}\) (apparent \( \Delta H^\circ \) values at 6.2: duplex = 91.0 kcal/mol; GT strand = 51.0 kcal/mol; AC strand = 53.0 kcal/mol). Under these conditions, at high DNA concentration, the enkephalin enhancer remains as a duplex, but upon dilution, an increasing level of the hairpin conformation can be observed. At pH 5.5, we can obtain total conversion into the hairpin form under low-concentration conditions. Thus, considering only the formation energies of the DNA component, the cAMP-dependent enhancer region of the human enkephalin gene is poised for formation of a cruciform structure under slightly acidic conditions, needing very little energy input to induce such a conformational change.

An acidic environment is required to stabilize the AC hairpin. The chemistry of AC strand stabilization involves hydrogen bonding, evidenced by the large increase in the apparent \( \Delta H^\circ \) upon decrease in pH. The additional hydrogen-bonding probably occurs via protonation of the N1 of adenine within the AC mismatched pair, shown in Fig. 5. \(^1\)H NMR should allow conclusive assignment of the protonated residue. Since two mismatch pairs occur within the hairpin (see Fig. 1), a maximum of two protons can be taken up to stabilize the structure. This is exactly the value we calculate from the pH dependence of melting (Table 1). Measurement of the apparent pK\(_a\) of AC strand protonation indicates that protonation can be physiologically relevant. Since the apparent pK\(_a\) value is at physiological pH (7.2), changes of roughly 0.5 pH unit are enough to fully stabilize or destabilize the hairpin. A pK\(_a\) shift of this magnitude for protonation of ring nitrogens is easily mediated by the presence of a nearby charged group (6). For example, adding a single 5'-phosphate group to a nucleoside

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**Table 1.** Proton release at various ionic strengths for the AC hairpin

<table>
<thead>
<tr>
<th>Na(^+), M</th>
<th>pH 7.0</th>
<th>pH 6.0</th>
<th>pH 5.5</th>
<th>Slope, ( K^{-1} \text{pH}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>48.7</td>
<td>58.6</td>
<td>60.7</td>
<td>0.072</td>
</tr>
<tr>
<td>0.055</td>
<td>50.1</td>
<td>61.2</td>
<td>64.0</td>
<td>0.085</td>
</tr>
<tr>
<td>0.105</td>
<td>50.4</td>
<td>62.2</td>
<td>65.6</td>
<td>0.090</td>
</tr>
<tr>
<td>0.205</td>
<td>50.4</td>
<td>62.5</td>
<td>66.4</td>
<td>0.099</td>
</tr>
</tbody>
</table>

The slope was determined from the linear least-squares regression analysis of a \( 1/T \) (in kelvin) versus pH plot. \( \Delta H^\circ \) refers to the number of protons released from the AC strand during thermal denaturation under the indicated pH and ionic strength conditions. The number was calculated from the relationship derived from the van't Hoff equation, slope = \( \Delta n R / \Delta H^\circ \). The apparent \( \Delta H^\circ \) value used in the calculation was 52 kcal/mol of strand, the average energy of denaturation of strand AC from pH 5.5 to 7.0, determined in calorimetry experiments.

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**Fig. 5.** Schematic diagram of the probable site of protonation within the AC base pair mismatch. The AC mismatch pair contains one hydrogen bond at pH 7.0 and binds an additional proton under acidic conditions. Protonation can occur at the N1 of adenine, giving rise to an additional hydrogen bond.
shifts the pK_a of the imino proton by roughly 0.4–0.7 pH units, depending on the base (6). The importance of the unusually high pK_a of AC strand protonation is the indication that only the vicinity of a charged group is enough to induce stabilization of the AC strand. While in vitro we have artificially induced an acidic environment by adjusting the pH, in vivo a similar change in pH can be induced locally by a charged protein. Therefore, the interaction of an acidic moiety with the enhancer region DNA, through proton transfer, is enough to determine the duplex or hairpin state of the enhancer. In vivo, the formation of two stable hairpins would constitute a cruciform structure. Thus, proton transfer and stabilization of the AC arm, at the expense of duplex formation, can be a biological switch for formation of a cruciform structure. The signal for AC strand stabilization could be proton donation from an auxiliary factor or a trans-acting factor containing regions of acidic or phosphorylated residues.

Several groups (2, 9, 10) have reported that multiple factors are capable of binding to the enkephalin enhancer region. One of these factors, the AP-1 complex, contains the jun protein, which is functionally homologous to the GCN4 protein of yeast, a classic acidic trans-acting factor (11). Formation of a cruciform structure not only retains the binding site for AP-1 and other factors known to bind to the enkephalin enhancer but also creates the potential for an additional AP-1 site within the AC arm (12). Thus, formation of a cruciform structure creates both the possibility for protein rearrangement and a statistical advantage for AP-1 binding. Finally, mutational analysis (2) has indicated that several point mutations alter basal and regulated expression of the human enkephalin gene. Within CRE-1 and CRE-2, two point mutations (positions −91 and −102) result in increased basal expression in vivo; another two point mutations (positions −90 and −92) eliminate enhancer function in vivo. In three of these cases (−90, −91, and −92), protein binding was examined and appears to be unaffected by the mutation (8). Since alterations in expression did not correlate with alteration of protein binding, the results, evaluated in terms of the linear duplex sequence, suggest that the in vitro binding of protein factors alone is not sufficient to account for the in vivo effects of the point mutations. However, all these results can be interpreted in terms of a cruciform structure. Although positioned within a cluster of down mutations, the two mutations at −102 and −91, which increase basal expression, are both base changes which make perfect base pairs out of the mismatched pairs. Thus, mutations which potentially stabilize the cruciform structure result in increased expression in vivo. The point mutations which result in elimination of enhancer function are base changes that would create further mismatched base pairs within the stem of the hairpin. Thus, in vivo, mutations which potentially destabilize the cruciform eliminate the ability to act as an enhancer. A common structural motif shared by many neuroendocrine genes which are responsive to cAMP is that the CREs of the respective genes are contained within or near stretches of imperfect palindromes, diagrammed in Fig. 6. All of these regulatory regions have the potential to form cruciform structures, all of which contain mismatched base pairs and, specifically, AC base pair mismatches. Thus, formation of cruciform structures may be a general feature of certain classes of CRE elements. Taken together with the unique physical and thermodynamic properties of the enkephalin enhancer DNA, these data suggest a model in which protein-mediated structural changes within the enhancer region DNA play an active role in cAMP-inducible activation of the human enkephalin gene via formation of cruciforms. Structural and functional analysis of enhancer mutations should provide information about the possible role of formation of
cruciforms as a biological switch for cAMP activation of gene expression.

The authors thank Ying Li, Craig Spiro, Jim Garrett, Bruce Godfrey, Mitch Martin, Kevin Walton, and Bob Rehfuss for critical evaluation and discussion of the experimental results. This work was supported by a McKnight Foundation award and National Institute on Drug Abuse Grant DA-04154 (to J.O.D.). C.T.M. was supported by Postdoctoral Fellowship PF-2997 from the American Cancer Society.