

A rapid method of cloning functional variable-region antibody genes in *Escherichia coli* as single-chain immunotoxins

(*Pseudomonas* exotoxin/recombinant immunotoxin)

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ABSTRACT We have devised a strategy based on polymerase chain reaction (PCR) for the rapid cloning of functional antibody genes as single-chain immunotoxins. RNA from a hybridoma producing an antibody (OV3) that reacts with ovarian cancer cells was used as a template to make the first strand of a cDNA. Then a second strand was synthesized and amplified by using two sets of DNA primers that (i) hybridized to the ends of the light- and heavy-chain variable regions, (ii) encoded a linker peptide, and (iii) contained appropriate restriction enzyme sites for cloning. After 30 cycles of PCR, the DNA fragments containing sequences encoding the light- and heavy-chain variable regions were cloned into an *Escherichia coli* expression vector containing a portion of the *Pseudomonas* exotoxin gene. Clones encoding recombinant single-chain immunotoxins were expressed in *E. coli* and the protein product was assessed for its ability to bind to or kill cells bearing the OV3 antigen. By using this approach it should be possible to rapidly clone the functional variable region sequences of many different antibodies from hybridoma RNA.

“Targeted toxins” represent a different approach to cytotoxic therapy (1, 2). For this purpose, protein toxins have been chemically attached to monoclonal antibodies (mAbs) to make immunotoxins (1, 2), or genes encoding toxins have been fused to growth factor genes to create growth factor–toxin fusion proteins (3). Recently, fragments of antibodies containing the antigen binding site(s) have been produced in *Escherichia coli* either as two chains or as single-chain antibodies (4–7). We have produced a recombinant single-chain immunotoxin, anti-Tac (Fv)-PE40, which consists of the variable domains of a mAb directed at the interleukin 2 receptor linked to a modified 40 kDa form of *Pseudomonas* exotoxin (PE40) (8). To construct single-chain immunotoxins, only the DNA sequences encoding the variable domains of the light and heavy chains of an antibody and that of a toxin are required.

Polymerase chain reaction (PCR) is now widely used for genomic and cDNA cloning (9–11). Recently, PCR has been used to amplify genes encoding variable domains of antibodies (12–14). Here we describe a PCR-based method of cloning the variable light- and heavy-chain (V_L and V_H) genes of an antibody directly from total RNA. The genes are expressed in *E. coli* fused to PE as a functional single-chain immunotoxin. We have now used this method to clone the V_L and V_H of mAb OV3, an antibody that reacts with many human ovarian cancers (15). This immunotoxin selectively kills OVCAR3 cells, which express the OV3 antigen.

METHODS

Identification of Rare Restriction Enzyme Recognition Sites and Primers for Amplification. The nucleotide sequences

encoding V_L and V_H of murine antibody were extracted from the Kabat's nucleotide data base (16) and subjected to the computer program FPAT (Chuck Buckler, National Institutes of Health, personal communication) to analyze the presence of restriction enzyme recognition sites (see Table 1). Two sets of two primers each were devised for the amplification of the V_L and V_H of murine mAb OV3 (see Fig. 1A). V_L -5' was based on the N-terminal amino acid sequence of the OV3 light chain and contained 23 nucleotides encoding the N-terminal amino acids of the light chain and an *Nde* I site 5' to these nucleotides. V_L -3' contained a *Sal* I site, nucleotides complementary to those encoding a linker peptide, and its 3' end was complementary to the joining (J) region of a mouse κ light chain. V_H -5' contained a *Sal* I site and 20 nucleotides encoding the N-terminal amino acids of the heavy chain. V_H -3' contained a *Hind*III site and its 3' end was complementary to the J region of the heavy chain.

RNA Isolation, cDNA Synthesis, and Amplification. RNA was prepared from $\approx 5 \times 10^7$ hybridoma cells (17). Total RNA was used for first strand cDNA synthesis using random primers at 37°C for 1 hr in a 200- μ l reaction mixture containing 40 μ g of total RNA, 6.6 μ l of RNase- and DNase-free bovine serum albumin (3 μ g/ μ l; Pharmacia), 4 μ l of 25 mM dNTPs, 4 μ l of RNasin (40,000 units/ μ l; Promega), 120 ng of random primer (New England Biolabs), 40 μ l of 5 \times reaction buffer, and 20 μ l of reverse transcriptase (Moloney murine leukemia virus reverse transcriptase, 200 units/ μ l from BRL). (The 5 \times reaction buffer = 250 mM Tris-HCl, pH 7.5/375 mM KCl/50 mM dithiothreitol/15 mM MgCl₂.)

For amplification of V_L and V_H , 5 μ l of cDNA was subjected to 30 cycles of PCR using reagents as per the manufacturer's instructions (Gene Amp; Perkin-Elmer/Cetus) in two separate tubes with 1 μ M each either V_L -5' and V_L -3' or V_H -5' and V_H -3' primers. Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 90 sec, and polymerization at 72°C for 2 min with a 10-sec extension in each cycle. The amplified V_L and V_H fragments were purified on 2% low-melting-point agarose (SeaPlaque; FMC).

Cloning of Amplified Fragments into a Bacterial Expression Vector. Plasmid pVC387 (18) was modified so that it contained a *Hind*III site between transforming growth factor type α and PE40. The purified PCR products were digested with *Nde* I and *Sal* I for the fragment encoding V_L and linker at its 3' end, or with *Sal* I and *Hind*III for V_H (see Fig. 1B). Expression vector pVC 38H was digested with *Nde* I and *Hind*III and dephosphorylated with calf intestinal phosphatase. V_L and V_H fragments were purified on 1.8% SeaPlaque agarose. The vector was separated on 1.2% SeaPlaque agarose and a 3.6-kilobase (kb) fragment was isolated. A three-fragment ligation was performed with the 3.6-kb vector, V_L and V_H fragments. The recombinants were screened with

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Abbreviations: PE, *Pseudomonas* exotoxin; PE40, 40-kDa PE; mAb, monoclonal antibody; V_H , heavy-chain variable region; V_L , light-chain variable region; PCR, polymerase chain reaction; J, joining.

Xba I and *Sal* I. Several positive clones were always checked for protein expression. To produce recombinant immunotoxin with a full-length PE clone pOVB3158-2 was digested with *Xba* I and *Hind*III to isolate an 800-base-pair (bp) fragment and ligated to a 4.9-kb fragment of *Xba* I/*Hind*III digested, dephosphorylated pVC45 f+T (19).

Expression and Analyses. Plasmid DNA from several clones of pOVB3-158 was transformed in BL21(ΔDE3) (20). Transformant colonies were inoculated in 5 ml of LB medium containing ampicillin (100 μg/ml) and induced with 1 mM isopropyl β-D-thiogalactopyranoside at an OD₆₅₀ of ≈0.8. After 90 min at 37°C, 200 μl of the culture was saved for SDS/PAGE. The rest was centrifuged and the pellet was suspended in 1 ml of extraction buffer (7 M guanidine/100 mM Tris-HCl, pH 8.0/5 mM EDTA), mixed in a Vortex, kept on ice for 1 hr, and then spun at 12,000 rpm in a microcentrifuge. Five hundred microliters of the guanidine extract containing proteins was diluted in 10 ml of rapidly stirred phosphate-buffered saline (PBS) and was allowed to stand overnight. A sample was dialyzed against 20 mM Tris-HCl (pH 7.4) in a microdialyzer unit (BRL), centrifuged at 12,000 rpm for 10 min, and tested for ADP ribosylation activity and binding to OVCAR-3 cells by an immunofluorescence assay.

OVB3 (Fv)-PE was expressed from plasmid pOVB3154-2. The protein was extracted from the inclusion bodies as described (21, 22) and was further purified on a Mono Q and a TSK-250 column.

Binding, Cytotoxicity, and Other Analytical Assays of Recombinant Proteins. Binding of chimeric toxins to OVCAR3 cells was evaluated by immunofluorescence. Proteins were diluted in Dulbecco's PBS containing 0.2% bovine serum albumin and incubated with OVCAR3 cells at 4°C for 1 hr, followed by a polyclonal rabbit antibody to PE and rhodamine-conjugated affinity-purified goat anti-rabbit IgG. The cells were fixed and viewed in a fluorescence microscope. ADP ribosylation assays were performed as described (21, 22). Cytotoxicity on human ovarian carcinoma cell line OVCAR3, murine Swiss 3T3 cells, and human leukemia T cells (HUT 102) was determined by assaying inhibition of protein synthesis (19, 21). SDS/PAGE was performed on 10% gels and immunoblotting was performed with polyclonal rabbit antibodies to PE (21). N-terminal amino acid sequencing of light and heavy chains of OVB3 was performed after their separation on SDS/polyacrylamide gel and transblotting onto Immobilon membranes (Millipore).

RESULTS

Identification of Rare Restriction Enzyme Sites and Primer Selection. To synthesize a cDNA encoding antibody genes, the restriction sites used for cloning the antibody genes cannot also be present in the variable region DNA sequences to be cloned. Therefore, a computer search of Kabat's nucleotide data base (16) was carried out to identify rare restriction sites in the genes encoding the murine V_L and V_H and enzyme sites that were either absent or rarely present were identified (Table 1). Because of the expression vector we used, *Nde* I and *Hind*III, which are rarely found in antibody genes, were chosen as cloning sites. In addition, restriction enzyme site *Sal* I was introduced at the 3' end of the linker in V_L and at the 5' end of V_H (Table 1 and Fig. 1A).

The sequences of the 5' primers for both V_L and V_H were based on the N-terminal amino acid sequence of the light and heavy chain of OVB3, respectively (Fig. 1A). In general, primers were designed so that 23 nucleotides at the 3' end of the primers would not have more than two to five mismatches. These mismatches occur because of variations in the N-terminal and J-region sequences and the degenerate nature of the codons. The base at the 3' end of each primer was either the first or second base of a codon or comple-

Table 1. Frequency of restriction enzyme recognition sites in the variable domains of mouse immunoglobulins

Restriction site	Frequency		Restriction site	Frequency	
	Light chain*	Heavy chain†		Light chain*	Heavy chain†
<i>Aat</i> I	0	1	<i>Hind</i> III	1	3
<i>Afl</i> II	0	1	<i>Hpa</i> I	0	0
<i>Ava</i> III	5	0	<i>Kpn</i> I	17	2
<i>Apa</i> I	0	0	<i>Mlu</i> I	0	2
<i>Apa</i> LI	1	1	<i>Nar</i> I	0	8
<i>Ase</i> I	9	16	<i>Nco</i> I	ND	10
<i>Bam</i> HI	12	3	<i>Nde</i> I	2	1
<i>Bcl</i> I	1	1	<i>Nhe</i> I	0	0
<i>Bgl</i> II	0	3	<i>Not</i> I	ND	0
<i>Bst</i> BI	0	0	<i>Nru</i> I	0	0
<i>Bsp</i> HI	0	2	<i>Pfl</i> MI	13	0
<i>Bst</i> EII	6	13	<i>Pst</i> I	29	46
<i>Bst</i> XI	19	3	<i>Pvu</i> II	0	35
<i>Bsu</i> 36I	2	8	<i>Sac</i> I	0	5
<i>Drd</i> I	ND	33	<i>Sac</i> II	0	1
<i>Cla</i> I	0	0	<i>Sal</i> I	0	0
<i>Eag</i> I	0	0	<i>Sma</i> I	0	0
<i>Eco</i> RI	0	6	<i>Spl</i> I	2	0
<i>Eco</i> RV	0	7	<i>Xba</i> I	ND	0
<i>Esp</i> I	0	32	<i>Xho</i> I	ND	0

ND, not determined.

*Fifty sequences from Kabat's data base were analyzed.

†One hundred sequences from Kabat's data base were analyzed.

mentary to the same. This was necessary because polymerization could only occur if the last base of the primer annealed to the template.

Amplification, Cloning, and Expression of Variable Domains. After first-strand synthesis, the cDNA was subjected to a 30-cycle PCR in two separate reactions. One reaction mixture contained primers V_L-5' and V_L-3' for amplifying V_L, while the other contained V_H-5' and V_H-3' for amplifying V_H. After amplification both reaction tubes showed a major band of ≈350 bp (data not shown). V_L and V_H were cloned into an expression vector containing a modified form of PE as shown in Fig. 1B. Analyses of recombinant clones revealed that 70% of the clones contain V_L, V_H, and PE40 (data not shown). Upon expression in BL21 (ΔDE3), 80% of these recombinants expressed a fusion protein of the expected size, as detected on SDS/polyacrylamide gel by Coomassie blue staining and immunoblotting using antibodies to PE (data not shown). Overall, 50% of the clones appeared to be correct because they expressed an immunoreactive protein of the expected size (63 kDa). The fusion protein bound to OVCAR3 cells when examined by immunofluorescence (Fig. 2A). Also, intact OVB3 competed for the binding (Fig. 2B). Two of the clones pOVB3 158-1 and pOVB3 158-2 were sequenced (Fig. 3). Both the clones had identical coding sequences, indicating that no major changes in sequence were produced in the PCR reaction. However, pOVB3 158-2 did not have an *Nde* I site; it was missing a C residue of the *Nde* I restriction site, which did not affect expression of the fusion protein.

These two fusion proteins were minimally toxic to OVCAR3 cells, which was not surprising since chemical conjugates of OVB3 and PE40 have low cytotoxicity toward OVCAR3 cells, and conjugates of full-length PE with OVB3 are very cytotoxic (23). Therefore, we replaced the PE40 portion of pOVB3158-2 with full-length PE to create pOVB3 154-2 (Fig. 1B). Upon its expression, protein of the expected size (89 kDa) was produced (Fig. 4, lane A). OVB3 (Fv)-PE was purified from inclusion bodies (22, 23) by guanidine denaturation and Mono Q and TSK-250 chromatography (lanes B and C). Purified

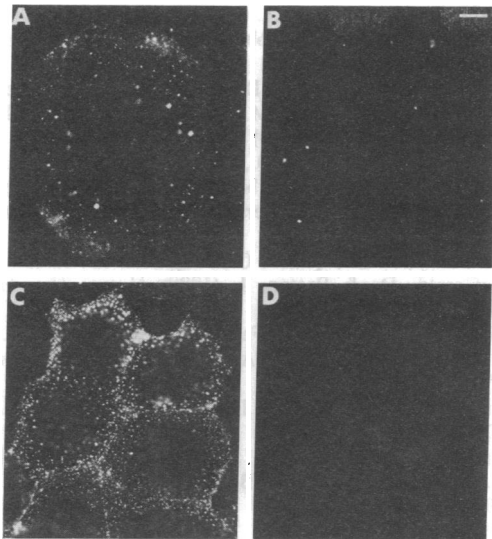


FIG. 2. Immunofluorescence detection of binding of OVB3 (Fv)-PE40 and OVB3 (Fv)-PE in OVCAR3 cells. OVCAR3 cells were incubated at 4°C either with crude extracts of recombinant OVB3 (Fv)-PE40 alone (A) or in the presence of excess OVB3 (B), or with purified recombinant OVB3 (Fv)-PE alone (C) or in the presence of excess OVB3 (D). Surface-bound proteins were detected with rabbit polyclonal antiserum against PE, followed by rhodamine-conjugated goat anti-rabbit immunoglobulin.

The nucleotide sequence of two separate clones of mAb OVB3 were identical except that OVB158-2 did not have an

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                                NdeI
                                CATATG
1(L)                               20
E H V L T Q S P A I M S A S P G E K V T
G A A A T G T G C T C A C C C A G T C T C C A C C A T C A T G T C T C C A G G G A A A A G G T C A C C
CDR1
M T C S A I S T V S Y M H W Y Q Q K S S
A T G A C C T G C A G T G C C A T C T C A A C T G T A A G T T A C A T G C A C T G G T A C C A G C A G A A G T C A A G C
CDR2
T S P K L W I Y E T S K L A S G V P G R
A C C T C C C C A A A C T C T G G A T T A T G A G A C A T C C A A A C T G G C T T C T G G A G T C C C A G G T C C C
F S G S G S G N S Y S L T I S S M E A E
T T C A G T G C A G T G G G T C T G G A A A C T T A C T C T C T C A C G A T C A G C A G C A T G G A G G C T G A A
88
D V A T Y Y C P Q G S G Y P Y T F G G G
G A T G T G C C A C T T A T A C T G T T T C A G G G A G T G G G T A C C C G T A C A C G T C G G A G G G G G
Linker
T K L E L K E G K S S G S G S E S K V D
A C C A A G C T G G A G C T C A A A G A G G T A A A T C C T C A G G A T C T G G C T C C G A A T C C A A A G T C G A C
1(H)                               20
D V Q L V E S G G G L V Q P G G S R Q L
G A T G T G A C G T G G T G A G T C T G G G A G G C T A G T C A G C C T G A G G G T C C C G G C A A C T C
30
S C A A S G F T F S S F G M H W V R Q A
T C T G T G C A G C T C T G G A T T C A C T T T C A G T A G C T T T G G A A T G C A C T G G G T C G T C A G G C T
CDR2 52 A 53
P E K G L E W V A Y I S R G G N T I Y Y
C C A G A A G G G C T G G A G T G G G T C G C A T A T A G T C G T G G C G G T A A T A C C A T C T A C T A T
A N T V K G R F T I S R D N P K N T L F
G C A A A C A C A G T G A A G G C C G A T T C A C C A T C T C C A G A G A C A A T C C C A A G A A C C C T G T T C
82 A B C 83
L Q M T S L R S D D T A M Y Y C A R S H
C T G C A A A T G A C C A G C T A A G G T C T G A C A C A G C G G C C A T G T A T A C T G T G C A A G A T C C C A T
100 A B C D 101
Y Y G Y F Y A M D Y W G Q G T T L T G S
T A C T A C G G C T A C T T C T A T G C T A T G G A C T A C T G G G G T C A G G A A C C A C T C T C A C A G T C T C C
113 HindIII
S
T C A A A A G C T T C
    
```

FIG. 3. Nucleotide sequence of OVB3 light and heavy chains. The sequence is shown as a single chain joined together with a linker. The top line shows amino acids in the single-letter code. Complementarity-determining regions (CDRs) and linker amino acids are boxed. 1(L), Beginning of the light chain; 1(H), beginning of the heavy chain. Cloning sites created by PCR are underlined. Sequences encoding PE40 (amino acids 253–613 of PE), which are present after the HindIII site, are not shown. Boldface amino acids were determined by N-terminal amino acid sequencing of the OVB3 antibody. Arrow, amino acids encoded within the V_L-5' and V_H-5' primers.

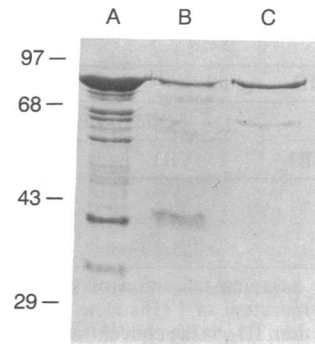


FIG. 4. SDS/PAGE of OVB3 (Fv)-PE at various stages of purification. Proteins were separated in 10% acrylamide gels and stained with Coomassie blue. Lanes: A, total cell pellet; B, pool after Mono Q column; C, pool after TSK250 column. Sizes of protein standards are shown in kDa.

Nde I site due to the loss of one base (C) from the site. We do not know whether this occurred during PCR or in a subsequent step of cloning. The strategy described here would not determine the real 5' and 3' sequences of V_L and V_H as these are dictated by the primers. However, work of Winter and his colleagues (24–26) have shown that transplanting the complementarity-determining regions from one antibody to another maintains the specificity of the antibody. Thus, framework regions, which may be slightly altered because of the choice of primers, are unlikely to affect the specificity of the reconstructed antibody or immunotoxin.

Expression of cloned variable domains as fusion proteins with a toxin such as PE is very advantageous, as the proteins from these clones can be directly tested for cytotoxicity or binding on the target cell line bearing the appropriate antigen. Depending on the nature of antigen, vectors capable of making fusion proteins with PE40 or PE can be used. The current immunotoxin was constructed initially by using a PE clone that encodes a form of PE (PE40) that does not contain domain I. When this fusion protein is bound to its antigen, which is poorly internalized, cell killing is minimal. However, the binding of OVB3 (Fv)-PE40 to antigen-bearing cells could be detected by using antibodies to PE, and immunofluorescence was used to identify clones binding to OVCAR3 cells. The PE40 vectors would also be useful for distinguishing between antibodies that bind to antigens that are internalized and to those that are not. A chimeric toxin of the former type is anti-Tac (Fv)-PE40 (8). If the antigen is not internalized or is poorly internalized, a fusion protein with PE may be useful because the binding of domain I of PE promotes internalization. It should also be possible to detect the antibody-toxin fusion protein by ELISA if the antigen is coated on a plate or nitrocellulose.

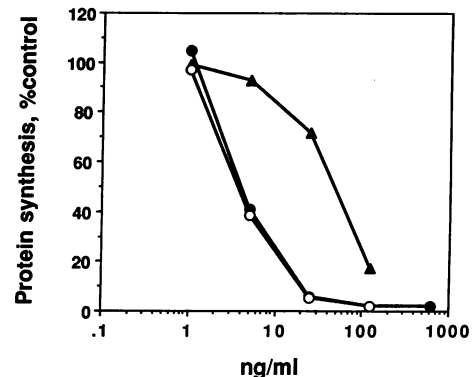


FIG. 5. Cytotoxicity of OVB3 (Fv)-PE on OVCAR3 cells. Various dilutions of OVB3 (Fv)-PE were added to OVCAR3 cells and incubated for 16 hr. Cytotoxicity was measured by determining [³H]leucine incorporation and results are expressed as percent of control with no toxin added. ●, OVB3 (Fv)-PE alone; ○, OVB3 (Fv)-PE + anti-Tac (50 μg/ml); ▲, OVB3 (Fv)-PE + OVB3 (50 μg/ml).

Table 2. Cytotoxicity of OVB3 (Fv)-PE and OVB3-PE on various cell lines

Cell line	ID ₅₀ , ng/ml			
	OVB3 (Fv)-PE		OVB3-PE	
	- OVB3	+ OVB3	- OVB3	+ OVB3
OVCAR3	4	46	1	1000
HUT 102	20	14	1200	1200
Swiss 3T3	7	8	53	50

Cytotoxicity was determined by assaying inhibition of protein synthesis as measured by the incorporation of [³H]leucine in the trichloroacetic acid-precipitable fraction. ID₅₀ is the concentration of the protein required to inhibit protein synthesis by 50% as compared to control, in which no toxin was added. + OVB3, Addition of mAb OVB3 at 50 µg/ml just prior to the addition of toxin. OVB3-PE is a chemical conjugate.

Previous strategies for cloning variable-region genes of antibodies from RNA have had to reassemble the individual genes into a functional antibody by subcloning and expression in mammalian vectors (12). This approach is slow and tedious and requires several subcloning steps and the growth of antibody-producing cells in tissue culture. Using the method described here, from total RNA it is possible to isolate functional antibody genes in 1–2 weeks. Because the chimeric antibody-toxin molecule is made in *E. coli*, it is possible to make large amounts of the reagent for various types of biochemical and biological studies (8).

The choice of the linker used to make the single-chain immunotoxin may be very important. We have previously reported a very active immunotoxin, anti-Tac (Fv)-PE40, that contained a linker composed of glycine and serine residues (8). The linker used in the current construction contains several charged amino acids (Fig. 1B), some of which were introduced for convenience of cloning.

It has been previously pointed out that it should be possible to directly clone functional antibody genes from the spleens of immunized mice or from B cells of humans by using appropriate primers and PCR-based gene amplification (12, 14). The ability to clone such sequences as functional single-chain antibodies whose activity can be readily detected by fluorescence or in cytotoxic assays should make this goal more readily achievable.

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Biochemistry. In the article "Selectivity of phospholipase C phosphorylation by the epidermal growth factor receptor, the insulin receptor, and their cytoplasmic domains" by Shunzo Nishibe, Matthew I. Wahl, Philip B. Wedegaertner, Jae Jim Kim (sic), Sue Goo Rhee, and Graham Carpenter, which appeared in number 1, January 1990, of *Proc. Natl. Acad. Sci. USA* (87, 424–428), the following correction should be noted. The name of the fourth author is Jae Won Kim, not Jae Jim Kim.

Immunology. In the article "A rapid method of cloning functional variable-region antibody genes in *Escherichia coli* as single-chain immunotoxins" by Vijay K. Chaudhary, Janendra K. Batra, Maria G. Gallo, Mark C. Willingham, David J. FitzGerald, and Ira Pastan, which appeared in number 3, February 1990, of *Proc. Natl. Acad. Sci. USA* (87, 1066–1070), the authors request that the following change be noted. Ref. 14 contained a typographical error. The correct reference should read as follows:

14. Sastry, L., Alting-Mees, M., Huse, W. D., Short, J. M., Sorge, J. A., Hay, B. N., Janda, K. D., Benkovic, S. J. & Lerner, R. A. (1989) *Proc. Natl. Acad. Sci. USA* 86, 5728–5732.

Genetics. In the article "Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for *O*⁶-alkylguanine" by Keizo Tano, Susumu Shiota, Julia Collier, Robert S. Foote, and Sankar Mitra, which appeared in number 2, January 1990, of *Proc. Natl. Acad. Sci. USA* (87, 686–690), the authors request that the following error be noted. In the heading for the fourth column of Table 1 on page 687, the units for specific activity should read "pmol/mg of protein."

Neurobiology. In the article " γ -Preprotachykinin-(72–92)-peptide amide: An endogenous preprotachykinin I gene-derived peptide that preferentially binds to neurokinin-2 receptors" by Than-Vinh Dam, Yasuo Takeda, James E. Krause, Emanuel Escher, and Rémi Quirion, which appeared in number 1, January 1990, of *Proc. Natl. Acad. Sci. USA* (87, 246–250), the authors request that the following correction be noted. In Table 2, p. 249, the values for ¹²⁵I- γ -PPT-(72–92)-NH₂ binding in the substantia nigra should read 2.92 ± 0.36 .