for recombinant selection on minimal plates, as in the first experiment. The envelopes of the cells on the other filter were removed before suspending the cells and plating for selection on minimal medium. A 100-fold increase in recombinant recovery was observed when cell envelopes were removed before plating. All 24 of the recombinant colonies recovered from the second filter contained both pHV2 and pHV11, as shown by colony hybridization (Fig. 3). Of the 24 recombinant colonies recovered from the control filter, 16 contained both plasmids and 8 did not contain any of them. This result shows that (i) removal of the envelopes of cells grown on solid support allowed the cells to fuse, probably as a result of destabilization of the structure of the cytoplasmic bridges; (ii) cells originating from WR344 were fused to cells originating from WR358, suggesting that cytoplasmic connections were formed de novo between cells originating from the two strains, and (iii) the increase in recombination frequency due to cell fusion shows that the bridging frequency is higher than the DNA transfer frequency. The same conclusion could be made by comparing the bridging frequency in the SEM images to the efficiency of the genetic transfer.

From the data described above, the following model for the mechanism of DNA transfer in *H. volcanii* is proposed: cytoplasmic bridges are formed between the cells. In this process the membranes and envelopes of the cells fuse to form cytoplasmic continuity between the two cells. The dimensions of these bridges are up to 2 μm long and 0.1 μm in diameter. The cells are not fully fused and their cytoplasms do not mix, as is the case in the sexual fusion of eukaryotic cells. The cytoplasmic continuity between *H. volcanii* cells seems to be restricted to the movement of certain molecules. Although chromosomal DNA moves through the bridges, the plasmids pHV2 and pHV11 do not move through the bridges.

The *H. volcanii* mating system has a functional resemblance to the eubacterial conjugation system in that cytoplasmic markers cannot be transferred in the process. However, the cytoplasmic bridges are a distinctive property of the halobacterial system.

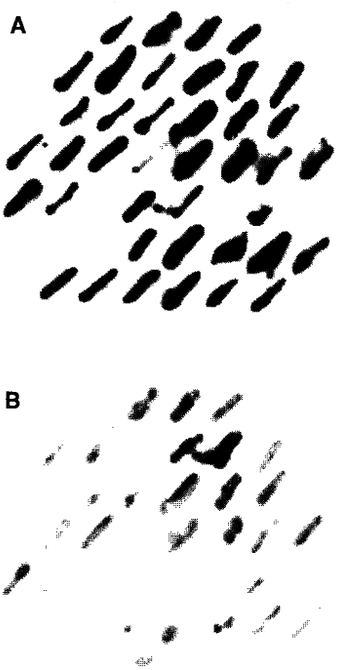

Fig. 3. The presence of (**A**) pHV2 and (**B**) pHV11 in 48 recombinant colonies generated by crossing WR358 with WR344 was examined by colony hybridization (*13*). Crosses were performed as described in the legend of Fig. 1 on two separate filters. The culture on one of the filters was immersed in 1*M* NaCl for 30 min before the cells were suspended and plated. This treatment caused the cell envelopes to dissociate and the cells to become spheroplasts (*8*). The upper 24 colonies in (A) and (B) originated from this treated filter; the lower 24 colonies in (A) and (B) serving as a control originated from the untreated filter.

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10. The frequency of recombinant generation by the mating system was 10^{-5} to 10^{-6}; the reversion frequencies of the markers that were used in this study were as follows: $<10^{-8}$ for Ser 3, 10^{-8} for Met 10, 10^{-9} for Pur 4, and $<10^{-9}$ for Arg 11.
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13. Colony lifting and hybridization were performed as described in *Methods of Transfer of DNA, RNA, and Protein to Nitrocellulose and Diazotized Paper Solid Supports* (Schleicher & Schuell, Keene, NH, 1981). Cloned pHV2 and pHV11 (7) were used as probes. They were labeled by ^{32}P by using "prime time" kit (International Biotechnologies, New Haven, CT).
14. The cover slips were coated by immersing them for 1 hour in 1% polylysine (BioMakor, Rehovat, Israel) in water.

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Type I and Type II $GABA_A$-Benzodiazepine Receptors Produced in Transfected Cells

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$GABA_A$ (γ-aminobutyric acid A)-benzodiazepine receptors expressed in mammalian cells and assembled from one of three different α subunit variants (α_1, α_2, or α_3) in combination with a β_1 and a γ_2 subunit display the pharmacological properties of either type I or type II receptor subtypes. These receptors contain high-affinity binding sites for benzodiazepines. However, CL 218 872, 2-oxoquazepam, and methyl β-carboline-3-carboxylate (β-CCM) show a temperature-modulated selectivity for α_1 subunit–containing receptors. There were no significant differences in the binding of clonazepam, diazepam, Ro 15-1788, or dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) to all three recombinant receptors. Receptors containing the α_3 subunit show greater GABA potentiation of benzodiazepine binding than receptors containing the α_1 or α_2 subunit, indicating that there are subtypes within the type II class. Thus, diversity in benzodiazepine pharmacology is generated by heterogeneity of the α subunit of the $GABA_A$ receptor.

BENZODIAZEPINES POTENTIATE THE activity of the GABA-gated chloride channel intrinsic to $GABA_A$ receptors (*1*). Although most benzodiazepines

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bind to these receptors with similar affinities and Hill coefficients of 1 throughout the brain, the pharmacological properties of several compounds (*2–4*), most notably CL 218 872 (*3*), have demonstrated heterogeneity of $GABA_A$-benzodiazepine receptors. Type I receptors are more sensitive to CL 218 872 than type II receptors and constitute the predominant $GABA_A$ receptor class in the central nervous system. The less sensitive type II receptors are enriched in only a few brain areas such as the hippocampus, striatum, and spinal cord as well as in neonatal cortex (*5–8*). In behavioral tests CL 218 872 was an effective anxiolytic agent with reduced ataxic and depressant effects as compared to other benzodiazepines such as diazepam (*9*). Therefore, these subtypes may mediate the different psychodynamic and physiological effects of benzodiazepines.

The electrophoretic properties of affinity-purified $GABA_A$-benzodiazepine receptors had suggested the presence of only two subunits, termed α [relative molecular mass (M_r) ~53,000] and β (M_r ~58,000) (*10*). Accordingly, receptor subtypes were thought to result from subunit modifications or different conformational states of a single receptor (*11*). However, cloning experiments have shown the existence of three different α (*12, 13*) and β subunit variants (*14*) as well as of members of other subunit classes, for example, the γ_2 subunit, important for benzodiazepine pharmacology (*15*). These results complement those of photolabeling experiments with [^{3}H]flunitrazepam, which reveal the presence of several labeled proteins with unique sizes and tryptic patterns (*16–18*) that are capable of reacting with α subunit–specific antibodies (*17, 18*).

To test the hypothesis that α subunit variants provide the structural basis for type I and type II benzodiazepine receptors, we coexpressed each of the three α variants with a β_1 and a γ_2 subunit in human embryonic kidney 293 cells (*15, 19, 20*). This transient expression system has been used to study recombinant $GABA_A$ receptors by both ligand binding and electrophysiology (*15, 20*) and to characterize the assembly of a glycine receptor subunit (M_r ~48,000) into functional channels (*21*). The pharmacological analyses of recombinant receptors on cell membranes (Fig. 1) demonstrate that [^{3}H]flunitrazepam binding to the α_1 subunit–containing receptors is more sensitive to displacement by CL 218 872 than to receptors containing α_2 or α_3 subunits. The inhibitory constant (K_i) values of 130 n*M* and 1.6 μ*M* derived from this competition are in good agreement with values of 100 n*M* and 2 μ*M*, respectively, estimated for the type I and type II benzodiazepine receptors in the rat hippocampus (*22*). Saturation

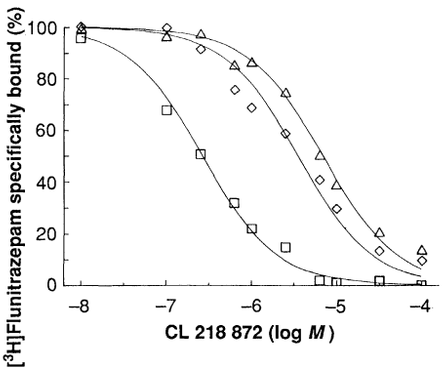

Fig. 1. CL 218 872 competition of [^{3}H]flunitrazepam (2 n*M*) binding to membranes of 293 cells transfected with expression vectors (*19*) encoding the following $GABA_A$ receptor subunit combinations: $\alpha_1\beta_1\gamma_2$ (□), $\alpha_2\beta_1\gamma_2$ (△), and $\alpha_3\beta_1\gamma_2$ (◇). The curves were generated by nonlinear least-squares fit of triplicate data, and each curve is representative of four experiments.

binding data for [^{3}H]flunitrazepam yielded a dissociation constant (K_d) of 2 n*M* for all three expressed receptor types.

We further characterized the benzodiazepine binding sites of each receptor by determining the K_i values of additional ligands (Table 1). Two other compounds known to be selective for type I receptors, the benzodiazepine 2-oxoquazepam and the β-carboline β-CCM (*2, 4*), display higher affinities for $\alpha_1\beta_1\gamma_2$ receptors than for $\alpha_2\beta_1\gamma_2$ and $\alpha_3\beta_1\gamma_2$ receptors. The four- to fivefold enhanced affinity of β-CCM and the tenfold enhanced affinity of 2-oxoquazepam to α_1 subunit–containing receptors is well within the range of reported differences for type I and type II receptors in brain membranes (*2, 23*). In contrast, four other compounds, clonazepam, diazepam, Ro 15-1788, and DMCM, known to be nonselective for the two receptor types (*22*), displayed no significant differences in their binding. Their binding affinities are close to those observed in brain membranes (*22*), suggesting that the recombinant receptors have benzodiazepine sites structurally similar to those in neurons. Notably, substituting the β_2 or β_3 subunit (*14*) for the β_1 subunit did not alter the type I, type II selectivity profile (Table 1), and use of the γ_1 subunit instead of γ_2 (*15*) resulted in reduced binding (maximum binding ratio $\gamma_2/\gamma_1 = 6$). These results indicate that different α subunits largely determine the pharmacological properties of type I and type II benzodiazepine receptors. In addition, type II receptors are heterogeneous, containing α_2 or α_3 subunits.

To further compare the recombinant receptors to those in brain membranes, we tested CL 218 872 competition of [^{3}H]Ro 15-1788 binding at 37°C. The selectivity of CL 218 872 is reduced at 37°C compared to that at 4°C (Table 1), an effect also observed for 2-oxoquazepam and β-CCM. This temperature-dependent selectivity change is also seen with brain membranes (*24, 25*), again suggesting that the recombinant receptors faithfully reproduce the structural properties of the type I and type II receptors. Because Ro 15-1788 is a specific benzodiazepine antagonist (*1*), our results indicate that the selectivity of CL 218 872 is the same, regardless of whether the receptor is in an agonist- or an antagonist-induced conformation.

Other evidence also suggests that α subunit variants are responsible for the properties of type I and type II benzodiazepine receptors in brain. Thus, the α_1 subunit mRNA predominates in cerebellum (*13, 26*), which contains mainly type I receptors

Table 1. Binding affinities of various benzodiazepines and β-carbolines to $GABA_A$ receptor subtypes. K_i ± SEM values for various compounds were determined by displacement of [^{3}H]flunitrazepam ([^{3}H]Ro 15-1788 where indicated by asterisk) binding to membranes of transfected 293 cells (*19*) and were calculated according to the Cheng-Prusoff equation (*30*) [$K_i = IC_{50}/(1+C/K_d)$, where C is the concentration used in the assay and K_d is the concentration of labeled ligand, which in the case of [^{3}H]flunitrazepam was 2 n*M*]. Values of the inhibition concentration IC_{50} for each compound were determined in at least three experiments similar to the one shown in Fig. 1 by linear least-squares fit of the data to the Hill equation. Values labeled with Δ were determined by averaging the K_i values for cells transfected with either β_1, β_2, or β_3 subunits in combination with the α and γ variants indicated in the column headings.

Compound	$\alpha_1\beta_1\gamma_2$ K_i (n*M*)	$\alpha_2\beta_1\gamma_2$ K_i (n*M*)	$\alpha_3\beta_1\gamma_2$ K_i (n*M*)
CL 218 872 (4°C)	131 ± 36	1820 ± 700	1530 ± 290
CL 218 872 (4°C)	108 ± 28$^\Delta$	1735 ± 432$^\Delta$	1620 ± 270$^\Delta$
CL 218 872 (37°C)	405 ± 75*	2002 ± 650*	1790 ± 370*
2-Oxoquazepam	19.6 ± 2.5	236 ± 12	205 ± 18
β-CCM	0.78 ± 0.12	2.9 ± 0.35	4.1 ± 0.6
DMCM	5.3 ± 1.5	14.4 ± 7.0	10.6 ± 6.3
Ro 15-1788	0.49 ± 0.24	1.2 ± 0.1	0.72 ± 0.22
Ro 15-1788	0.48 ± 0.2$^\Delta$	1.0 ± 0.3$^\Delta$	0.62 ± 0.2$^\Delta$
Clonazepam	1.27 ± 0.04	1.7 ± 0.4	2.0 ± 0.04
Diazepam	16.3 ± 0.5	16.9 ± 5.5	17.0 ± 1.8

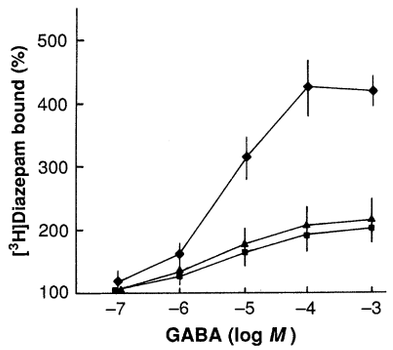

Fig. 2. Potentiation of [^{3}H]diazepam binding by GABA. Binding to $\alpha_1\beta_1\gamma_2$ (■), $\alpha_2\beta_1\gamma_2$ (▲), and $\alpha_3\beta_1\gamma_2$ (◆) transfected cell membranes is shown as the average of three separate experiments, with bars representing the SEM. The [^{3}H]diazepam binding in the absence of GABA is defined as 100% binding. The binding was determined as described (*19*) except that 100-μl samples of GABA at various concentrations in assay buffer were added to the 1-ml reaction volume.

(*5*), whereas significant amounts of α_2 and α_3 mRNA are only expressed in brain regions where type II receptors are found (*13, 26*). Further, $GABA_A$ receptor subunits photolabeled by [^{3}H]flunitrazepam, the molecular mass of which is greater (M_r 54,000 and 59,000) than that of α_1 subunits (53,000), correlate with the presence of type II receptors in a tissue-specific and developmental fashion (*27*). The greater mass of these subunits is consistent in the case of the α_3 subunit because the cDNA for α_3 predicts a mass of 52,000 for its polypeptide sequence, including four putative N-linked glycosylation sites. The addition of a mass of about 2,000 for the carbohydrate side chain to each glycosylation site would result in a mass of ~60,000 for the fully glycosylated α_3 subunit. In fact, the bovine α_3 subunit has a mass of 59,000 by protein immunoblotting (*28*). The predicted structure of the α_2 subunit specifies an additional N-linked glycosylation site relative to the α_1 subunit (*13*), and it could therefore migrate with a mass of 54,000.

To examine the effects of the physiological neurotransmitter GABA on the binding of benzodiazepines, we incubated membranes from cells expressing receptors assembled from α_x, β_1, and γ_2 subunits with [^{3}H]diazepam and increasing concentrations of GABA. The neurotransmitter clearly potentiates benzodiazepine binding to each of the receptor types (Fig. 2). However, potentiation is higher with receptors containing the α_3 subunit than with those containing the other α subunits. At saturating GABA concentrations, $\alpha_3\beta_1\gamma_2$ receptors showed an approximately fourfold increase in [^{3}H]diazepam binding, whereas $\alpha_1\beta_1\gamma_2$ and $\alpha_2\beta_1\gamma_2$ combinations bound only twice the [^{3}H]diazepam as compared to zero GABA controls. Approximately twofold stimulation of benzodiazepine binding by GABA has been observed with adult brain membranes (*25*). A higher stimulation has been seen with brain membranes from young animals or brain tissue in which the postsynaptic receptors had been destroyed. These conditions correlate with increased levels of type II receptors (*6, 29*), which, as shown above, constitute a heterogeneous receptor population. Our results indicate that the α_3 subunit–containing population is responsible for the elevated GABA-mediated stimulation of benzodiazepine binding in these latter studies.

The differential neurotransmitter-mediated potentiation of benzodiazepine binding to receptors containing different α subunit variants predicts different frequencies of channel openings (*1*) at these receptors and, hence, dissimilar potentiation of GABA-gated current flow. In fact, preliminary experiments show that $\alpha_3\beta_1\gamma_2$ receptors display higher current potentiation by benzodiazepine than do $\alpha_1\beta_1\gamma_2$ or $\alpha_2\beta_1\gamma_2$ receptors. However, a more thorough analysis of potentially differential rates of desensitization will be needed to correlate the pharmacological data with electrophysiological behavior. In previous studies on $GABA_A$ receptors formed by coexpression of each of the α subunit variants with the β_1 subunit, it was found that $\alpha_2\beta_1$ receptors are more sensitive to GABA than the other two subunit combinations (*13*). It should be kept in mind, however, that the pharmacological and electrophysiological properties of $GABA_A$ receptors composed of two subunits differ considerably from those of $GABA_A$ receptors containing three subunits (*15*), and, hence, the effect of GABA described here on benzodiazepine binding cannot be compared to apparent sensitivities to GABA in receptors formed from only α and β subunits.

Our findings suggest that the receptors we have constructed reveal the structural basis of type I and type II benzodiazepine receptor heterogeneity. That the α, β, and γ subunits in the combinations expressed in our experiments constitute the native type I and type II receptors awaits confirmation from biochemical experiments with subunit specific antibodies. However, the subunit combinations used in our experiments are largely consistent with the colocalization of the cognate mRNAs in neuronal subpopulations (*15, 26*).

We predict that the use of recombinant, pure receptor subtypes will lead to the identification of clinically useful compounds that differentiate between the type II receptors. The search for such compounds will be aided by knowing which of the few amino acid differences between the α subunit variants create the differential affinity.

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19. Cloned cDNAs encoding the human α_2 and α_3 subunits were inserted into the polylinker site of the PCIS 2 expression vector (*20*) by standard recombinant DNA methods. The human α_2 and α_3 subunit sequences are 97% the same as their bovine counterparts (*13*), and identical results were obtained with these bovine or rat cDNAs. Expression plasmid DNA was prepared by CsCl gradient centrifugation. Human embryonic kidney 293 cells (4×10^6) were transfected with plasmid DNA (20 μg) carrying the following subunit-encoding cDNAs: $\alpha_1\beta_1\gamma_2$, $\alpha_2\beta_1\gamma_2$, or $\alpha_3\beta_1\gamma_2$. Transfections with three cDNAs on separate plasmids (α, β_1, and γ_2) produced the same results as did transfections carried out with two plasmids ($\alpha_x + \beta_1$ cDNAs in tandem; γ_2). After 48 hours, cells were harvested by aspiration with phosphate-buffered saline and frozen at −70°C or homogenized immediately in 10 m*M* potassium phosphate buffer, *p*H 7.2. After two subsequent washes, the cell membrane pellet (100,000*g*) was homogenized in a mixture of 10 m*M* potassium phosphate, *p*H 7.2, and 100 m*M* potassium chloride. Incubations contained 800 μl of membranes, 100 μl of [^{3}H]benzodiazepine (New England Nuclear; [^{3}H]flunitrazepam, 90 Ci/mmol; [^{3}H]Ro 15-1788, 75 Ci/mmol, [^{3}H]diazepam, 83 Ci/mmol), and 100 μl of competing ligand (or 1 μ*M* clonazepam to determine nonspecific binding). After incubation for 60 min at 4°C or 30 min at 37°C, the membranes were collected by rapid filtration on GF/C filters. Radioactivity remaining after two subsequent washes with 5 ml of 10 m*M* potassium phosphate buffer, *p*H 7.2, was determined by liquid scintillation spectroscopy.
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The Protein Kinase Domain of the ANP Receptor Is Required for Signaling

MICHAEL CHINKERS AND DAVID L. GARBERS

A plasma membrane form of guanylate cyclase is a cell surface receptor for atrial natriuretic peptide (ANP). In response to ANP binding, the receptor-enzyme produces increased amounts of the second messenger, guanosine 3′,5′-monophosphate. Maximal activation of the cyclase requires the presence of adenosine 5′-triphosphate (ATP) or nonhydrolyzable ATP analogs. The intracellular region of the receptor contains at least two domains with homology to other proteins, one possessing sequence similarity to protein kinase catalytic domains, the other to regions of unknown function in a cytoplasmic form of guanylate cyclase and in adenylate cyclase. It is now shown that the protein kinase–like domain functions as a regulatory element and that the second domain possesses catalytic activity. When the kinase-like domain was removed by deletion mutagenesis, the resulting ANP receptor retained guanylate cyclase activity, but this activity was independent of ANP and its stimulation by ATP was markedly reduced. A model for signal transduction is suggested in which binding of ANP to the extracellular domain of its receptor initiates a conformational change in the protein kinase–like domain, resulting in derepression of guanylate cyclase activity.

ATRIAL NATRIURETIC PEPTIDE (ANP), synthesized in cardiac atria and released into the bloodstream in response to increased central blood volume, lowers blood pressure by stimulating vasodilation, diuresis, and natriuresis (*1*). Two cell surface receptors for ANP have been described: a low molecular weight "clearance" receptor having a small cytoplasmic domain (*2*) and a high molecular weight, guanosine 3′,5′-monophosphate (cGMP)–coupled receptor, containing a much larger cytoplasmic domain (*3, 4*). The latter receptor, which is the subject of this report, appears to mediate the physiological effects of ANP through its intrinsic ANP-activated guanylate cyclase activity; cGMP is believed to act as a second messenger for ANP in many, but not all, circumstances (*5*). The intracellular portion of the ANP receptor has been divided into two domains on the basis of sequence analysis: one, adjacent to the membrane, has similarity to protein kinases; the other, at the COOH-terminus, is similar to regions of both a soluble guanylate cyclase and a brain adenylate cyclase (*3, 4, 6, 7*).

Mutants of the cDNA for the rat brain ANP receptor were prepared in which most of either the kinase-like domain or the COOH-terminal domain was deleted (*8*) (Fig. 1A). Mutant and wild-type receptor clones were inserted into an expression vector (pSVL) under the control of the simian virus 40 late promoter and transfected into COS-7 cells (*9*), which have low concentrations of endogenous ANP receptor and guanylate cyclase. Analysis of the transfected cells by cross-linking to ^{125}I-labeled ANP demonstrated the expression of mutant receptors of the predicted sizes (Fig. 1B). Comparison of ^{125}I-labeled ANP binding by cells transfected with mutant and wild-type receptor clones and the guanylate cyclase activity of particulate fractions of the cells, measured in the presence of nonionic detergent and Mn^{2+}, suggested that the wild-type receptor (pSVL-ANPR) and the kinase deletion mutant (pSVL-kin$^-$) had similar specific guanylate cyclase activities (Fig. 1C). That is, we have consistently observed five- to sevenfold higher expression of both ^{125}I-labeled ANP binding and guanylate cyclase activity in cells transfected with pSVL-kin$^-$ than in cells transfected with pSVL-ANPR. In the absence of an antibody with which to quantitate the expressed proteins, however, this comparison of "specific activities" must be interpreted with caution. In contrast, the COOH-terminal deletion mutant (pSVL-cyc$^-$) lacked enzyme activity (Fig. 1C). These experiments demonstrate that guanylate cyclase catalytic activity resides in the COOH-terminal domain and that deletion of the protein kinase–like domain does not interfere with cyclase activity.

These observations were surprising given that the protein kinase–like domain, but not the COOH-terminal domain, is well conserved in the membrane guanylate cyclase from the sea urchin *Arbacia punctulata* (Fig. 1A). It is possible that the structure of the catalytic domain of the enzyme from this primitive sea urchin is quite different from that of other known guanylate cyclases. The localization of guanylate cyclase activity to the COOH-terminal sequences suggests that similar sequences found in a bovine adenylate cyclase (*7*) and in a subunit of a soluble guanylate cyclase (*6*) represent the catalytic domains of those enzymes.

We next examined the effects of the receptor deletions on the regulation of cGMP concentration by ANP in intact cells. Cells transfected with wild-type and mutant ANP receptor clones were incubated in the absence or presence of ANP, and cellular cGMP content was determined (Table 1). In cells transfected with pSVL or pSVL-cyc$^-$, less than 0.2 pmol of cGMP per well was detectable in the absence or presence of

Table 1. Effect of ANP on cGMP content of transfected cells. COS-7 cells in 35-mm wells, transfected with the indicated plasmids, were incubated in the absence (−) or presence (+) of ANP for 5 min at 37°C (*15*). Medium was aspirated and cGMP was extracted with ice-cold 0.5*N* perchloric acid and assayed (*3*). Results are expressed as the mean of triplicate samples ± SE.

Transfection	ANP	cGMP (pmol per well)
pSVL	−	<0.2
	+	<0.2
pSVL-ANPR	−	<0.2
	+	19.0 ± 1.8
pSVL-kin$^-$	−	10.5 ± 0.4
	+	10.7 ± 0.9
pSVL-cyc$^-$	−	<0.2
	+	<0.2

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