treatment with chloroquine plus desipramine; 7 days of treatment with chloroquine is usually necessary to cure owl monkeys infected with chloroquine-susceptible *P. falciparum* (*20*). Therapy was stopped in this initial experiment after 3 days because of the striking reduction in parasitemia.

Although the doses of desipramine used in these studies appear to be high in comparison to the doses used in humans, the plasma desipramine concentrations were assumed to be in the same range of those seen with conventional doses of desipramine in humans because of a marked difference in plasma kinetics between monkeys and humans. In rhesus monkeys, a single oral dose of 25 mg of desipramine per kilogram yielded peak plasma concentrations of 50 ng/ml with a half-life of about 5 to 6 hours (*21*). Thus, steady-state plasma concentrations with three times daily administration of 25 mg of desipramine per kilogram (total daily dose, 75 mg/kg) would probably have been somewhat higher than 50 ng/ml. In humans, 0.4 mg of desipramine per kilogram given orally three times daily (total daily dose, 1.2 mg/kg) yielded a mean steady-state concentration of 42.7 ng/ml with a half-life of 18 hours (*22*). Detailed pharmacokinetic and toxicology studies must be conducted before desipramine can be used for the clinical treatment of chloroquine-resistant malaria. However, our present data suggest that the dose needed to obtain clearance of parasites in humans may be a small fraction of the dose administered to monkeys in this study, making clinical use of desipramine for treatment of malaria a realistic possibility.

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A c-*myb* Antisense Oligodeoxynucleotide Inhibits Normal Human Hematopoiesis in Vitro

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The nuclear protein encoded by the proto-oncogene c-*myb* has been hypothesized to play an important role in the process of hematopoiesis, but direct proof of this function has been lacking. To address this issue, normal human bone marrow mononuclear cells were exposed to c-*myb* sense and antisense synthetic oligodeoxynucleotides, and the effects on hematopoietic colony formation and maturation were examined. Exposure of these cells to c-*myb* antisense, oligodeoxynucleotides resulted in a decrease in both colony size and number, without apparent effect on the maturation of residual colony cells. Exposure to c-*myb* sense, or irrelevant antisense, oligonucleotides had no such effect. These results show that (i) c-*myb* plays a critical role in regulating normal human hematopoiesis and (ii) the combined use of antisense oligodeoxynucleotides and hematopoietic cell culture techniques will provide a powerful tool for studying the role of proteins encoded by proto-oncogenes, or other specific genes, in normal human hematopoiesis.

THE PROTO-ONCOGENE C-*myb*, THE normal cellular homolog of the avian myeloblastosis virus transforming gene v-*myb* (*1*), may play an important role in regulating hematopoietic cell proliferation, and perhaps differentiation (*2–4*). Evidence to support this hypothesis includes the observations that: (i) c-*myb* is preferentially expressed in primitive hematopoietic cell tissues and hematopoietic tumor cell lines of several species (*3*); (ii) as cells mature c-*myb* expression declines (*4*); and (iii) the constitutive expression of exogenously introduced c-*myb* inhibits the erythroid differentiation of a murine erythroleukemia cell line (MEL) in response to known inducing agents (*5*). Although these data clearly implicate the c-*myb* gene product as a potentially important regulator of hematopoietic cell development, this evidence is largely of an indirect nature. Accordingly, the function of the c-*myb* proto-oncogene in normal hematopoiesis remains speculative.

To directly determine whether c-*myb* has a role in regulating normal human hematopoiesis, at least in vitro, we used the antisense strategy (*6*). This approach to the study of specific gene function has been used successfully to study the effects of c-*myc* (*7, 8*) and proliferating cell nuclear antigen (PCNA or cyclin) (*9*) on cell proliferation and differentiation. We therefore synthesized (*9*) 18-bp oligodeoxynucleotides complementary to either the sense or antisense strands of the c-*myb* mRNA transcript starting from the second codon (*10*). We then exposed light-density, normal human bone marrow cells depleted of adherent macrophages and T lymphocytes to these synthetic oligodeoxynucleotides and assayed the effect of this treatment by analyzing colony formation in a plasma clot culture system (*11, 12*). As additional controls, we also synthesized antisense oligodeoxynucleotides of identical size and initiation point complementary to human myeloperoxidase (MPO) (*13*) and coagulation cofactor V (FV) mRNA transcripts (*14*). These proteins were chosen because decrements in the levels of such abundant proteins can be detected in single cells (see below), and the effect of blocking synthesis of relatively late appearing (*15, 16*), lineage-specific proteins on colony formation could also be determined.

The effect of exposing normal human hematopoietic progenitor cells to different oligodeoxynucleotides was assessed by two

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criteria. First, we visually monitored the actual process of myeloid and erythroid colony formation in the cultures. Colonies were defined by standard criteria (*17*). Second, we enumerated the number of colonies that formed under the different culture conditions at the end of the incubation period.

The c-*myb* antisense oligodeoxynucleotide had immediate effects on the process of colony formation, which appeared to be secondary to an inhibitory effect on cell proliferation (Fig. 1). By day 4, for example, one observed that cells exposed to c-*myb* sense or MPO antisense oligodeoxynucleotides (Fig. 1, A and C, respectively) had already undergone numerous cell divisions with the consequent formation of rudimentary myeloid colonies. In contrast, cells exposed to the c-*myb* antisense oligomers were markedly delayed in their development and appeared to have undergone only one division during this observation period. Cell doublets, not aggregates, were uniformly observed in these cultures (Fig. 1B, arrows). This inhibitory effect on cell proliferation persisted throughout the culture period; when culture plates were harvested on day 12, fully developed colonies were observed in the c-*myb* sense–treated or MPO antisense–treated cultures (Fig. 1, D and F), but colonies in the c-*myb* antisense–treated plates were uniformly small, and more consistent with cluster, rather than true colony formation (Fig. 1E). An equivalent effect was also observed in the erythroid colonies (Fig. 1, G, H, and I); colony formation in the presence of the c-*myb* sense oligodeoxynucleotide (Fig. 1G) and FV antisense oligodeoxynucleotide (Fig. 1I) appeared to be normal. In contrast, exposure to the c-*myb* antisense oligomer almost uniformly led to the development of distinctly small, yet clearly hemoglobinized (confirmed by benzidine staining), colonies (Fig. 1H); normal-appearing colonies were rarely observed. Exposure to c-*myb* antisense oligodeoxynucleotide also resulted in the generation of megakaryocyte colonies composed of small numbers of individual cells.

The effect of the various oligomers on the number of colonies in the culture dishes in six separate experiments, each performed with a different preparation, is shown in Table 1. The exposure of a population of progenitor cells to the c-*myb* antisense oligomer, at concentrations similar to those employed by other investigators (*7–9*), resulted in a statistically significant (Student's *t* test) decrease in colony formation in every study. In studies 1, 2, 3, and 4, the decrease in granulocyte-macrophage colony-forming units (CFU-GM) was 86% ($P = 0.018$), 70% ($P = 0.040$), 43% ($P = 0.001$), and 74% ($P = 0.001$), respectively. Similar data were generated in the erythroid and megakaryocyte studies. In experiments 2, 5, and 6 the c-*myb* antisense oligomer inhibited erythroid colony-forming units (CFU-E) by 96% ($P = 0.003$), 54% ($P = 0.011$), and 71% ($P = 0.001$), respectively, and in studies 1, 3, and 4, megakaryocyte colony-forming units (CFU-Meg) were inhibited 77% ($P = 0.040$), 84% ($P = 0.001$), and 74% ($P = 0.001$), respectively. If the study data are combined, mean colony formation from CFU-GM was decreased 68% ($P = 0.002$, Student's *t* test), from CFU-E by 67% ($P = 0.044$), and from CFU-Meg by 76% ($P = 0.010$) in comparison to control.

In contrast, the c-*myb* sense oligomer had no consistent effect on the formation of hematopoietic colonies of any lineage. Even in those studies in which colony formation was inhibited after exposure to c-*myb* sense or unrelated oligomer, the colonies that formed were almost always morphologically normal, and rarely bore any resemblance to the small cluster-type aggregates shown in Fig. 1, E and H. If these data are combined, colony formation in control and c-*myb* sense–containing cultures showed no significant difference ($P = 0.778$, $P = 0.796$, and $P = 0.375$ for CFU-GM, CFU-E, and CFU-Meg, respectively).

We also determined whether the inhibition of colony formation correlated with the concentration of oligodeoxynucleotide. After progenitor cells were exposed to 0.3, 1.4, 7, and 14 μ*M* of c-*myb* sense and antisense oligodeoxynucleotides, erythroid and megakaryocytic colony formation was examined. As expected, the c-*myb* sense oligodeoxynucleotide had no significant effect on colony formation, even at the highest dose tested [CFU-E–derived colonies: control (no oligodeoxynucleotide added) versus experimental = 526 ± 81 versus 618 ± 31; CFU-Meg–derived colonies: control versus experimental = 24 ± 2 versus 25 ± 9). In contrast, there was a clear dose-dependent response between increasing amounts of the c-*myb* antisense oligodeoxynucleotide and the degree of colony inhibition. Exposure to the c-*myb* antisense oligodeoxynucleotide at the concentrations mentioned above led to the formation of 21 ± 3, 17 ± 1, 10 ± 2, and 4 ± 2 megakaryocyte colonies, respectively; exposure to 1.4, 7, and 14 μ*M*

Table 1. Effect of sense and antisense oligodeoxynucleotides on human hematopoietic colony formation in vitro. Light-density bone marrow mononuclear cells (MNC) were obtained from consenting normal volunteers, enriched for hematopoietic progenitor cells, exposed to oligodeoxynucleotides, and then cultured in plasma clots (studies 1, 2, 5, and 6, 2×10^5 MNC per milliliter; studies 3 and 4, 1×10^5 MNC per milliliter) as described (*11, 12*). Cultures used to assay for CFU-E contained either recombinant human erythropoietin (5 U/ml, studies 5 and 6) or aplastic anemia serum (study 3). Cultures used to assay for CFU-GM and CFU-Meg contained recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, 5 ng/ml) and recombinant interleukin-3 (~10 U/ml). Study 4 was resupplemented with the oligonucleotides noted in the table at 72 and 120 hours after clot formation. Hematopoietic colonies were identified in situ: CFU-E and CFU-GM by visual criteria (*17*), and CFU-Meg by immunofluorescent staining (*11, 16*) with a platelet glycoprotein IIb/IIIa–specific monoclonal antibody (*19*). Each study was carried out on a separate occasion with different oligomer preparations and MNC derived from a different donor. Colony numbers are reported as mean ± SEM of quadruplicate control cultures (no oligodeoxynucleotide added) and duplicate experimental cultures.

Study	Oligodeoxynucleotide	Colonies or clusters formed		
		CFU-GM	CFU-E	CFU-Meg
1	Control (no oligomer added)	110 ± 16		
	c-*myb* sense	52 ± 9		
	c-*myb* antisense	16 ± 2		
2	Control	152 ± 7		17 ±3
	c-*myb* sense	186 ± 8		12 ± 1
	c-*myb* antisense	46 ± 6		4 ± 4
3	Control	164 ± 6	68 ± 7	24 ± 2
	c-*myb* sense	133 ± 8	64 ± 2	25 ± 9
	c-*myb* antisense	95 ± 1	3 ± 1	4 ± 2
4	Control	355 ± 27		57 ± 4
	c-*myb* sense	474 ± 27		38 ± 7
	c-*myb* antisense	92 ± 11		15 ± 1
	MPO antisense	372 ± 18		
	FV antisense			41 ± 9
5	Control		526 ± 40	
	c-*myb* sense		616 ± 31	
	c-*myb* antisense		244 ± 25	
6	Control		1445 ± 59	
	c-*myb* sense		1608 ± 1	
	c-*myb* antisense		415 ± 4	
	FV antisense		1120 ± 20	

concentrations of the antisense oligodeoxynucleotide led to the formation of 775 ± 127, 515 ± 9, and 244 ± 25 colonies, respectively. In addition, we examined the effect of delayed addition of the oligomers on colony formation. When the c-*myb* antisense oligodeoxynucleotide was added to the cultures after 5 days of incubation, only slight (16%) inhibition of the formation of myeloid colonies and no inhibition of the formation of erythroid colonies was noted; addition on day 10 had no effect on the number of myeloid colonies formed. These results suggest that c-*myb* protein synthesis is most critical during the early stages of colony formation.

These experiments suggested that the inhibition of colony formation was due to a specific effect of the c-*myb* antisense oligodeoxynucleotide. The effect of antisense oligodeoxynucleotides against irrelevant, or relatively late, maturation marker proteins was therefore tested simultaneously in several studies. Antisense oligodeoxynucleotide to MPO and FV had no effect on myeloid or megakaryocyte colony formation, respectively (Table 1, study 4). Similarly, an antisense oligodeoxynucleotide to FV had no effect on erythroid colony formation (Table 1, study 6). Since neither the sense nor unrelated antisense oligodeoxynucleotides had any consistent effect on colony formation (Fig. 1 and Table 1), we conclude that the failure of hematopoietic colony development was due to a specific biological effect of the c-*myb* antisense preparation.

To provide more evidence that inhibition of hematopoietic colony formation was due to an intracellular effect of the c-*myb* antisense oligonucleotide, we determined whether the oligomers were indeed taken up by the target cells. Demonstrating uptake into hematopoietic progenitor cells was deemed technically unfeasible due to the rarity of these cells and the difficulty in purifying large numbers. However, we reasoned that if the cells took up the MPO antisense oligodeoxynucleotide, they should also be capable of taking up an identically sized (18-bp) c-*myb* oligodeoxynucleotide. Accordingly, we examined the intracellular synthesis of MPO protein after prolonged exposure (Table 1, study 4) to the MPO antisense oligodeoxynucleotide and a similar exposure to unrelated oligodeoxynucleotides. Myeloperoxidase synthesis by individual cells within colonies was monitored by indirect immunofluorescence (*11, 16*) with an antibody to MPO. The exposure of cells to either the c-*myb* sense or antisense oligodeoxynucleotide did not effect the synthesis of MPO as shown by the bright fluorescence of cells that make up these colonies (Fig. 2, A and B). Also, despite the fact that the antisense oligodeoxynucleotide inhibited cell proliferation (Fig. 2B), maturation, at least as demonstrated by an ability to synthesize MPO, was unaffected. In contrast, MPO antisense oligodeoxynucleotide exposure resulted in a clear decrease in synthesis of this protein (Fig. 2C). Quantitation of these data by computer-assisted microspectrophotometry (*9*) on an arbitrary fluorescence intensity scale of 0% to 100% showed that cells arising in cultures containing c-*myb* sense, c-*myb* antisense, and MPO antisense oligodeoxynucleotides had mean (± SEM) fluorescence intensities of 71.3 ± 1.3%, 77.3 ± 1.6%, and 22.6 ± 0.4%, respectively (number of colonies examined from each group was 10, 25, and 10, respectively; total cells examined per group was 200). The decrease in fluorescence intensity in the MPO antisense–treated cultures was highly significant ($P < 0.001$). Of note, inhibition of MPO synthesis had no apparent effect on cell proliferation, as there was no difference in the number of cells comprising MPO antisense– versus c-*myb* sense–treated colonies (Fig. 2B versus 2A).

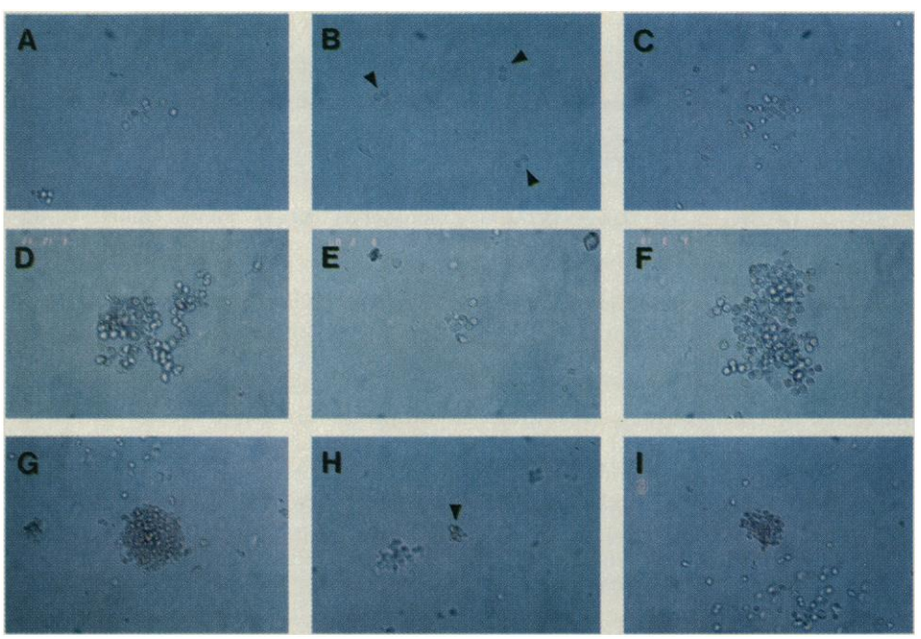

Fig. 1. Development of human hematopoietic colonies cloned in plasma clot cultures from normal bone marrow cells exposed to different sense and antisense oligodeoxynucleotides (see text and Table 1 for details). (**A**) to (**F**) are CFU-GM–derived colonies; (**G**) to (**I**) are CFU-E–derived colonies. Granulocyte-macrophage colony development after 4 days of exposure to c-*myb* sense (5′-GCC CGA AGA CCC CGG CAC-3′), c-*myb* antisense (5′-GTG CCG GGG TCT TCG GGC-3′), or MPO antisense (5′-AGA GAA GAA GGG AAC CCC-3′) oligodeoxynucleotides is shown in (A), (B), and (C), respectively. Arrows in (B) point to cell doublets. Granulocyte-macrophage colony development after 12 days of exposure to c-*myb* sense, c-*myb* antisense, or MPO antisense oligodeoxynucleotides is shown in (D), (E), and (F), respectively. Erythroid colony development after 7 days of exposure to c-*myb* sense, c-*myb* antisense, or FV antisense (5′-GCG TGG GCA GCC TGG GAA-3′) oligodeoxynucleotide is shown in (G), (H) (arrow), and (I), respectively.

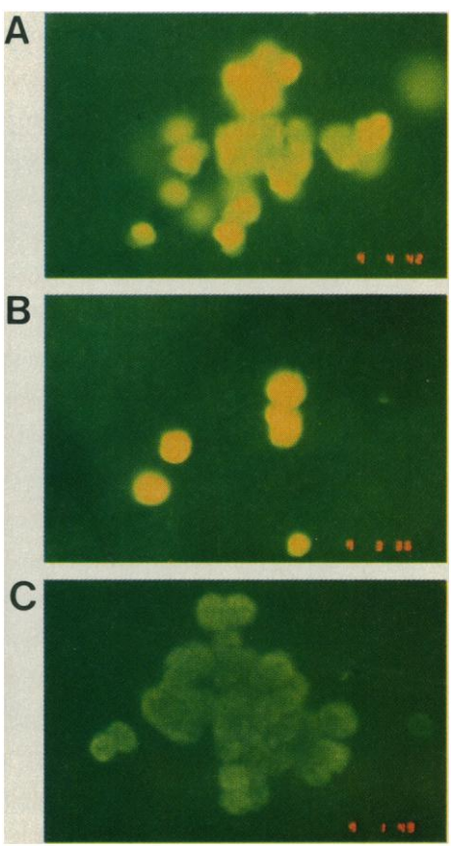

Fig. 2. Myeloperoxidase antigen expression, assessed by indirect immunofluorescence (*11*), in human CFU-GM–derived colony cells after prolonged exposure (see Table 1, study 4, for details) to c-*myb* sense (**A**), c-*myb* antisense (**B**), and MPO antisense (**C**) oligodeoxynucleotides.

Direct evidence that the c-*myb* gene product is required for the formation of normal human hematopoietic colonies of the myeloid, erythroid, and megakaryocytic lineages in vitro is provided by these studies. They suggest an important in vivo role for this nuclear protein as well. The function served by the c-*myb* proto-oncogene remains unclear. Based on the model that hematopoiesis is a developmental continuum (*18*), we hypothesize that the c-*myb* gene product is involved in regulating the very early stages of hematopoiesis, and is probably most relevant to initial progenitor cell proliferation events. We cannot exclude, however, more subtle effects on cells at either earlier (pluripotent hematopoietic stem cell) or later (precursor cell) developmental stages. These studies also imply that it is now possible to employ a powerful yet technically simple in vitro model for studying the effect of deleting any protein encoded by a proto-oncogene (or other specific gene) on the developmental biology of human hematopoietic cells. Finally, since blocking expression of relatively late differentiation markers has no discernible effect on either colony formation or the remainder of the cell's differentiation program, it is also now possible to develop in vitro models for the study of specific enzyme or protein deficiency disorders of hematopoietic cells.

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12. Oligodeoxynucleotides were purified by multiple washes and ethanol precipitations. They were lyophilized to dryness and then rehydrated in alpha medium (200 μg/50 μl; 654 μ*M*). The oligodeoxynucleotide solution (20 μl) was added to a polypropylene tube containing the marrow mononuclear cells suspended in growth medium (2.2 ml) (*11*). The cell suspensions were incubated at 37°C with gentle rocking for 15 to 18 hours, then another 20 μl of the oligonucleotide solution (final oligonucleotide concentration, 14 μ*M*) and 0.2 ml of citrated bovine plasma were added. The cell suspensions (1-ml aliquots) were then placed immediately into 35-mm plastic petri dishes and allowed to clot. Cultures were incubated (37°C, 5% CO_2) for either 7 or 12 days, to assay for erythroid or for myeloid and megakaryocyte colonies, respectively. To supplement established cultures with oligodeoxynucleotides, we diluted 10 μl of oligodeoxynucleotide solution (200 μg/50 μl) in 140 μl of alpha medium and layered it over the plasma clot (final concentration ~14 μ*M*). On three occasions, after enumeration of myeloid colonies in situ, the plasma clots were fixed in methanol:acetone (3:1) and then dried into a thin film. Megakaryocyte colonies, defined as three or more intensely fluorescent cells in aggregates (*11*), were identified in clots by direct immunofluorescence with a mouse monoclonal antibody to the megakaryocyte lineage-specific glycoprotein IIb/IIIa (*19*).
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Transient Expression Shows Ligand Gating and Allosteric Potentiation of $GABA_A$ Receptor Subunits

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Human γ-aminobutyric acid A ($GABA_A$) receptor subunits were expressed transiently in cultured mammalian cells. This expression system allows the simultaneous characterization of ligand-gated ion channels by electrophysiology and by pharmacology. Thus, coexpression of the α and β subunits of the $GABA_A$ receptor generated GABA-gated chloride channels and binding sites for $GABA_A$ receptor ligands. Channels consisting of only α or β subunits could also be detected. These homomeric channels formed with reduced efficiencies compared to the heteromeric receptors. Both of these homomeric GABA-responsive channels were potentiated by barbiturate, indicating that sites for both ligand-gating and allosteric potentiation are present on receptors assembled from either subunit.

THE $GABA_A$ RECEPTOR CONTAINS an intrinsic chloride ion channel that is opened (gated) by GABA, the major inhibitory neurotransmitter in mammalian brain. $GABA_A$ receptor activation stabilizes the neuron's resting potential (*1, 2*). Channel activity can be allosterically modulated by therapeutically useful drugs, for example, barbiturates and benzodiazepines (*1–3*). The affinity-purified receptor contains an α subunit of 53 kD, on which the benzodiazepine binding site is thought to be located, and a β subunit of 57 kD, which can be photoaffinity labeled by GABA agonists (*4–6*).

Human α_1 and β_1 subunit–encoding cDNAs were isolated from a fetal brain library (*7*). The DNA and predicted polypeptide sequences of these cDNAs reveal a high degree of conservation with the corresponding bovine sequences (*1*). The cloned α_1 and β_1 subunit cDNAs were inserted, singly or together (in tandem array), into a eukaryotic expression vector, pCIS2, that contained the human cytomegalovirus promotor-enhancer (*8*). Human embryonic kidney cells (*8*) were transfected with these constructs at high efficiencies, by using a modified $CaPO_4$ precipitation technique (*9, 10*). Cells were harvested 48 hours after transfection for RNA isolation, for pharmacological studies, or were used for electrophysiology. Analysis of total RNA from transfected cells showed high levels of $GABA_A$ receptor subunit mRNA but no such mRNA was seen in untransfected or mock-transfected cells (Fig. 1).

To characterize the transiently expressed heteromeric $\alpha_1 + \beta_1$ receptors, membranes

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