a common location. Although the role of glandular secretions in the Chrysomelidae as antipredator defenses is firmly established (*1, 2, 5, 16*), these chemicals may also mediate interactions within the second trophic level. Insect defensive secretions may play multiple ecological roles.

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Binding of the Sp1 Transcription Factor by the Human Harvey *ras*1 Proto-oncogene Promoter

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Members of the *ras* gene family encode proteins that when overproduced or mutated can transform immortalized mammalian cells. It is therefore important to understand the mechanisms by which the *ras* genes are regulated. The promoter region of the human Harvey *ras* proto-oncogene c-Ha-*ras*1 initiates RNA transcription at multiple sites and contains repeated copies of the hexanucleotide GGGCGG and its inverted complement CCGCCC, referred to as GC boxes. These GC boxes consist of sequences identical to those found in the SV40 early promoter, where the human cellular transcriptional factor Sp1 binds. Footprinting analysis with deoxyribonuclease I was used to show that Sp1 binds to six GC box sequences within the c-Ha-*ras*1 promoter. An in vivo transfection assay showed competition between the 21–base pair repeats of the SV40 promoter and the c-Ha-*ras*1 promoter for common regulatory factors. In this system the presence of Sp1 is apparently required for c-Ha-*ras*1 transcription. Analysis of deletions of the c-Ha-*ras*1 promoter region by means of a transient expression assay revealed that the three Sp1 binding sites closest to the RNA start sites were sufficient for full transcriptional activity.

THE *ras* GENES HAVE ATTRACTED A great deal of attention because of their possible role in the development of human cancers. There are three functional *ras* genes in the human genome, c-Ha-*ras*1 (cellular Harvey *ras*) (*1, 2*), c-Ki-*ras*2 (cellular Kirsten *ras*) (*1, 3*), and N-*ras* (*4, 5*); two other *ras* genes, c-Ha-*ras*2 and c-Ki-*ras*1, are pseudogenes (*6, 7*). The three functional genes code for proteins (p21) that are very closely related to each other. Because p21 proteins have guanosine triphosphatase (GTPase) activity (*8*) it has been suggested that their normal function may be as G-like regulatory proteins involved in the normal growth control of cells (*9*). Several groups of investigators have shown that a normal c-*ras* gene can be activated by a single point mutation that changes the glycine residue at position 12 to a valine (*10–12*). If the expression of a normal *ras* gene is enhanced by placing it under the control of a strong promoter, it can transform NIH 3T3 cells (*13*); however, there are as yet no examples of human tumors expressing very high levels of normal *ras* products. We examined the regulation of transcription of the c-Ha-*ras*1 gene to help define its role in controlling cellular growth.

We had earlier identified the promoter region of the human c-Ha-*ras*1 gene (*14*) and found that it contains multiple RNA start sites and ten repeats of a GGGCGG sequence and its inverted complement CCGCCC (called GC boxes). However, neither a TATA box nor a CAAT box were found in their characteristic positions upstream from the transcriptional start sites (*14*). These specific features of the promoter region are very similar to those found in the promoter region of the human epidermal growth factor (EGF) receptor gene (*15*). The GC box sequence was originally found in the 21–base pair (bp) repeats of the SV40 early promoter (*16*). This sequence binds the human HeLa transcription factor Sp1 (*17–20*). It was reported that five Sp1 molecules can bind to the tandemly repeated GC boxes of the SV40 early promoter and stimulate transcription in vitro (*17–20*). The GC boxes of some other viral genes [for example, herpes "immediate-early" [IE]3 and herpes *tk* genes (*21, 22*)] and cellular genes [mouse dihydrofolate reductase gene and the monkey β region (*23, 24*)] are also recognized by Sp1 protein. Recently the consensus sequence $^{G}_{T}$GGGCGG$^{GGC}_{AAT}$ for Sp1 recognition was deduced by the comparison of the sequences of 19 Sp1 strong binding sites (*25*). We found that the promoter region of the c-Ha-*ras*1 gene contains eight 10-bp GC boxes resembling this consensus sequence (Fig. 1).

To determine whether the transcription of this proto-oncogene is regulated by sequence-specific interactions of Sp1 to the promoter, as has been demonstrated for SV40, we tested the c-Ha-*ras*1 promoter for its ability to bind the Sp1 transcription factor in a deoxyribonuclease I (DNase I) footprint assay. An isolated DNA fragment end-labeled on either DNA strand was incubated with various amounts of partially purified Sp1, subjected to partial DNase I digestion, and analyzed as described in the

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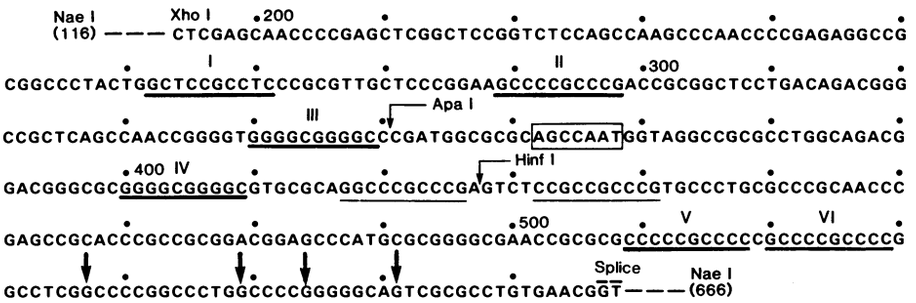

Fig. 1. Nucleotide sequence of the c-Ha-*ras*1 promoter region. The major transcriptional start sites (large arrows) and the splice site as determined previously (*14*) are shown. The GC box regions that Sp1 recognizes (I to VI) are underlined by a thick line. The GC-rich regions that do not bind Sp1 are underlined by a thin line. The 551-bp Nae I–Nae I DNA fragment (116 to 666) is the promoter fragment in the p*ras*CAT-1 plasmid construct. The Apa I (nucleotide 351) and Hinf I (nucleotide 427) sites used to construct p*ras*CAT-3 and p*ras*CAT-4 are shown. The CAAT box sequence is boxed (see text for details). Numbering of the c-Ha-*ras*1 sequence is that used by Reddy (*33*).

legend to Fig. 2. Six of the eight 10-bp GC box sequences located within the 350-bp region upstream from the RNA start sites were resistant to nuclease digestion (Fig. 2). Each binding site covers 18 to 20 bp and is centered about a single GC box, independent of the GC box orientation. Sp1 binding sites III (nucleotides 340 to 349), IV (nucleotides 400 to 409), and VI (nucleotides 520 to 529) all have the 10-bp sequence GGGGCGGGGC, which exactly matches the consensus sequence for Sp1 recognition reported earlier (*24*). Sites I (nucleotides 262 to 271, GAGGCGGAGC), II (nucleotides 289 to 298, CGGGCGGGGC), and V (nucleotides 509 to 518, GGGGCGGGGG) have only one base pair different (underlined) from the consensus sequence. The promoter region of the c-Ha-*ras*1 gene contains two 10-bp GC boxes that are not recognized by Sp1. Those GC boxes have the sequences CGGGCGGGCC (nucleotides 417 to 426) and CGGGCGGCGG (nucleotides 432 to 441), which have two or three base pairs that are different (underlined deoxynucleotides) from the consensus sequence. This confirms that the 6-bp GC box alone is not sufficient to specify a recognition site for Sp1.

These results show that Sp1 binds to the c-Ha-*ras*1 promoter region, but they do not prove any functional role for Sp1 in *ras* gene expression. The presence of cellular factors involved in transcriptional enhancement can be demonstrated with a competition assay. If Sp1 is a positive transcriptional regulator of the c-Ha-*ras*1 gene and present in a limited amount, then transfecting cells with a sufficient amount of DNA containing strong Sp1 binding sites should depress transcription by the c-Ha-*ras*1 gene promoter. A similar approach has been used to detect cellular factors that interact with molecules containing viral enhancer sequences (*26*). Cultured African monkey kidney cells (CV1) were transfected with a calcium phosphate co-precipitate of three different plasmid DNA's. (i) The test DNA plasmid consists of a 551-bp Nae I–Nae I (nucleotides 116 to 666, Fig. 1) human c-Ha-*ras*1 promoter fragment containing about 420 bp of DNA upstream from the RNA start site, as well as 40 bp of the first exon and 90 bp of the first intron, fused to the *Escherichia coli* chloramphenicol acetyltransferase gene [p*ras*CAT-1 (*14*)]. Transcription by the c-Ha-*ras*1 promoter is therefore quantitated by measuring chloramphenicol acetyltransferase (CAT) enzyme activity (*27*). (ii) The competitor plasmids, which are used to titrate out Sp1, contain various portions of the SV40 early promoter region. (iii) The carrier plasmid, used to keep the total DNA concentration constant in each precipitation mixture, is identical to the competitor plasmid DNA except that it lacks all DNA sequences of the SV40 promoter region and the *neo* gene. We used a constant amount of the test gene p*ras*CAT-1 (2 μg), added increasing amounts of competitor DNA, and used carrier DNA to reach the final amount of 22 μg per dish in all samples. The addition of increasing amounts of competitor pSV2neo DNA containing the SV40 early promoter region (*28*) led to a sequential decrease in CAT expression, but pSV0neo did not (Fig. 3a). These results show that some positive factor (or factors) binds to both the SV40 early promoter region and the c-Ha-*ras*1 promoter region.

To determine whether the titratable factor is specific for the GC boxes of the SV40 early promoter, we repeated the competition assay with two other competitor plasmid DNA's (pSVsdl41 and pSVsdl131), both inactive as promoters and both containing the 72-bp repeat enhancer element, but only one (pSVsdl41) containing the GC boxes of the 21-bp repeats of the SV40 early promoter (*29*). The addition of increasing amounts of competitor pSVsdl41 DNA led

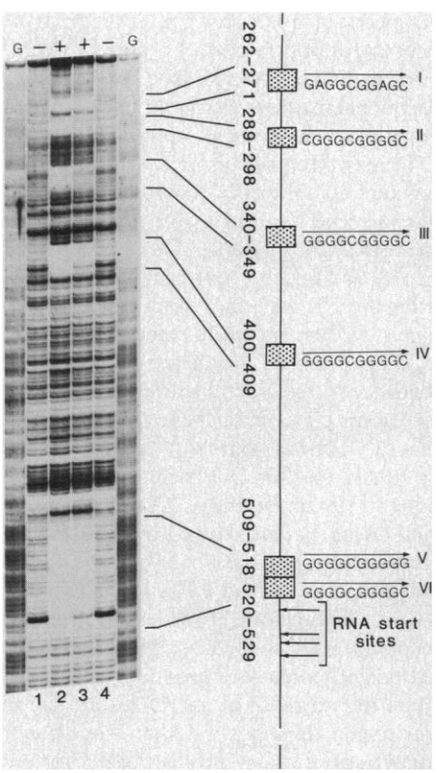

Fig. 2. DNase I footprint analysis of Sp1 binding sites in the c-Ha-*ras*1 promoter region. A 6% polyacrylamide sequencing gel was used to analyze the partial DNase I digests of the end-labeled c-Ha-*ras*1 promoter DNA fragment in the presence or absence of purified Sp1 (approximately 70% pure). The p*ras*CAT-1 plasmid was 5′ end-labeled at a Hind III linker site (nucleotide 666) and digested with Xho I; the 470-bp labeled fragment was isolated by gel electrophoresis. Incubation with Sp1 and partial digestion with DNase I were as previously described (*18, 19*). G refers to the guanine marker obtained by partial digestion with dimethyl sulfate of the same end-labeled DNA fragment. Lanes 1 and 4 (−) show the DNase I cleavage pattern in the absence of Sp1. Lanes 2 and 3 (+) show the DNase I cleavage pattern in the presence of 80 ng and 40 ng of Sp1, respectively. The relative positions of the areas of DNA protected from digestion (I to VI) are bracketed and represented by a shaded box (right). The major RNA start sites are shown at right. The arrows over the GC boxes indicate the 5′ to 3′ direction of the G-rich strand of DNA.

to a sequential decrease in CAT expression (Fig. 3b). The titration curve generated by pSVsdl41 addition is very similar to that by pSV2neo addition. When the competitor plasmid pSVsdl131 was used, however, no decrease in CAT activity was observed (Fig. 3b). These data are consistent with the hypothesis that the level of transcription of the c-Ha-*ras*1 gene is decreased when the level of cellular Sp1 is competitively reduced by introducing SV40 GC box DNA into the cells and that Sp1 is a positive transcriptional regulator of the c-Ha-*ras*1 gene.

To clarify the role of each Sp1 binding site in the c-Ha-*ras*1 promoter region, we constructed some deletion mutants of the promoter region and placed them upstream from the bacterial CAT gene. The original p*ras*CAT-1 construct contains six Sp1 binding sites. A second construct (p*ras*CAT-3) was made by deleting the region upstream from the Apa I site at nucleotide 351 while retaining Sp1 binding sites IV, V, and VI (Fig. 4). The third construct (p*ras*CAT-4) was made by deleting the region upstream from the Hinf I site at nucleotide 427 while retaining Sp1 binding sites V and VI (Fig. 4). The ability of those constructs to express CAT activity, a measure of promoter activity, was tested by DNA transfection. A typical result is shown in Fig. 4; cells transfected with p*ras*CAT-3 expressed CAT activity at essentially the same level as that obtained with p*ras*CAT-1. However, the level of CAT activity found in the extracts of CV1 cells transfected with p*ras*CAT-4 was one-fourth that obtained with p*ras*CAT-1 or p*ras*CAT-3. These results show that in this transient transfection assay, the three Sp1 binding sites proximal to the c-Ha-*ras*1 gene RNA start sites (IV, V, and VI) are sufficient for transcription. The three distal Sp1 binding sites (I, II, and III) are apparently not necessary for transcription in the transient expression system. A complete definition of the role of Sp1 in c-Ha-*ras*1 transcriptional regulation awaits a more detailed mutational analysis of this promoter region.

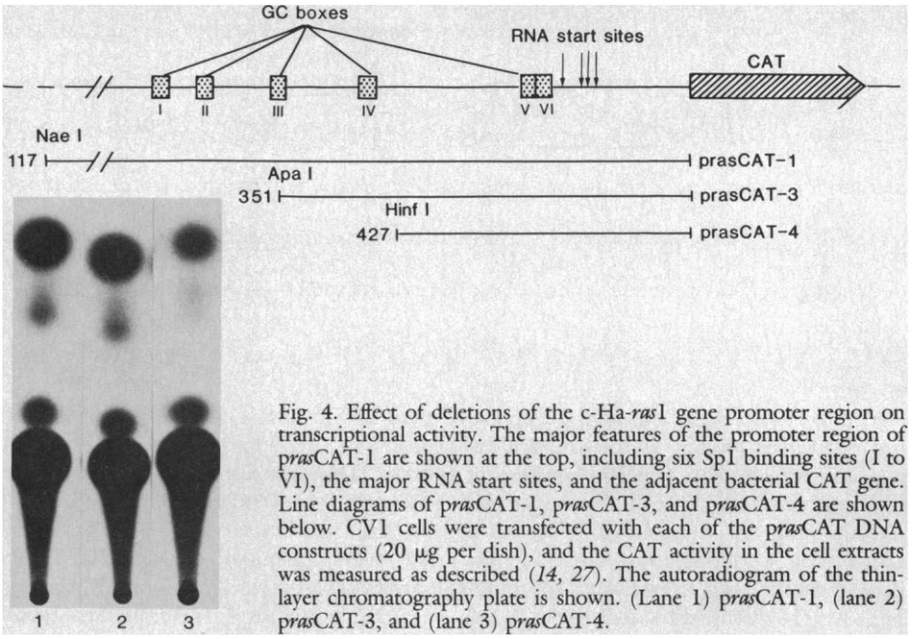

Fig. 4. Effect of deletions of the c-Ha-*ras*1 gene promoter region on transcriptional activity. The major features of the promoter region of p*ras*CAT-1 are shown at the top, including six Sp1 binding sites (I to VI), the major RNA start sites, and the adjacent bacterial CAT gene. Line diagrams of p*ras*CAT-1, p*ras*CAT-3, and p*ras*CAT-4 are shown below. CV1 cells were transfected with each of the p*ras*CAT DNA constructs (20 μg per dish), and the CAT activity in the cell extracts was measured as described (*14, 27*). The autoradiogram of the thin-layer chromatography plate is shown. (Lane 1) p*ras*CAT-1, (lane 2) p*ras*CAT-3, and (lane 3) p*ras*CAT-4.

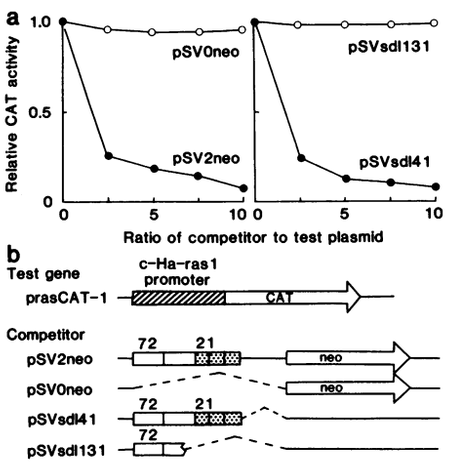

Fig. 3. Competition between the SV40 promoter and the c-Ha-*ras*1 gene promoter. African Green monkey kidney cells, line CV1, were transfected with 2 μg of p*ras*CAT-1 test plasmid DNA, 5 to 20 μg of the indicated construct as competitor, and sufficient carrier DNA to give a total of 22 μg. Transfection and the CAT enzyme assay were as described (*14, 27*). (a) After autoradiography of thin-layer chromatography plates, the spots of the acetylated form of the chloramphenicol were cut out and the CAT enzyme activities were quantitated by liquid scintillation counting. The resulting data were normalized relative to the value obtained for cells with no competitor added (1.0). (b) The structures of the test gene and the competitor DNA's. The c-Ha-*ras*1 promoter region, CAT gene, *neo* gene, 72-bp repeat of the SV40 enhancer, and the 21-bp repeats of the SV40 early promoter are indicated.

Transcription of the c-Ha-*ras*1 gene may be quite complex. We reported earlier that this *ras* gene has four major transcriptional start sites (*14*) (Fig. 2). There is a perfect CAAT box 170 bp upstream (362 to 368) from the 5′-most transcriptional start site, much farther upstream than the typical −80-bp position. Furthermore, when the sequence at the 5′ end of the T24 bladder carcinoma c-Ha-*ras*1 gene is compared with that of v-Ha-*ras*, homology can be detected up to 440 to 450 (*2*), which is about 80 bp downstream from the CAAT box. If the viral oncogenes are derived by reverse transcription of an RNA molecule, these data are consistent with an RNA start site in the 440 to 450 region. However, S1 nuclease mapping analysis of this region at various hybridization temperatures did not demonstrate the existence of an RNA start site in this region. It is possible that other minor transcriptional start sites are used for *ras* RNA production; however, we have found no direct evidence to support this possibility.

The apparent transcriptional regulation of the c-Ha-*ras*1 gene by Sp1 is, to our knowledge, the first evidence for a transcriptional regulator of a proto-oncogene. Other *ras* genes appear to have similar structural features. Hall and Brown (*30*) reported that the 5′ portion of the human N-*ras* gene has four 6-bp GC boxes and neither a TATA box nor a CAAT box. The 5′ portion of the c-Ki-*ras*2 gene also has four GC boxes and neither a TATA box nor a CAAT box (*7*). When the 10-bp consensus sequence (*25*) is compared to the N-*ras* and Ki-*ras* genes, the decanucleotide GC boxes in these *ras* genes can be seen to differ from the consensus by 1 to 4 bp. Although the promoter regions of the human EGF receptor (*15*), human c-*myc* (*31*), and mouse c-*myc* (*32*) genes also have GC boxes, the DNA sequences around those GC boxes contain 1-, 2-, or 3-bp differences from the 10-bp consensus sequence for the Sp1 binding site. It will be of interest to determine whether Sp1 or related factors bind to other proto-oncogene promoters and what role such proteins may have in the regulation of the promoters.

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The Role of Chloride Transport in Postsynaptic Inhibition of Hippocampal Neurons

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Hippocampal inhibitory postsynaptic potentials are depolarizing in granule cells but hyperpolarizing in CA3 neurons because the reversal potentials and membrane potentials of these cells differ. Here the hippocampal slice preparation was used to investigate the role of chloride transport in these inhibitory responses. In both cell types, increasing the intracellular chloride concentration by injection shifted the reversal potential of these responses in a positive direction, and blocking the outward transport of chloride with furosemide slowed their recovery from the injection. In addition, hyperpolarizing and depolarizing inhibitory responses and the hyperpolarizing and depolarizing responses to the inhibitory neurotransmitter γ-aminobutyric acid decreased in the presence of furosemide. These effects of furosemide suggest that the internal chloride activity of an individual hippocampal neuron is regulated by two transport processes, one that accumulates chloride and one that extrudes chloride.

HYPERPOLARIZING INHIBITORY postsynaptic potentials (IPSP's) of cat motoneurons (*1*) and crayfish stretch receptor neurons (*2*) are generated by the influx of chloride ions down a gradient that is maintained by an outwardly directed chloride transport mechanism. Mammalian hippocampal neurons in vitro exhibit both hyperpolarizing and depolarizing responses to presynaptic stimulation (*3*) as well as to the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (*4*). We have used furosemide (*5*), which blocks chloride transport, to investigate the role of transport in the inhibitory responses of pyramidal cells in the CA3 region of the hippocampus and of dentate granule cells (GC).

We used guinea pig hippocampal slices (*6*) to record intracellularly from pyramidal cells in the CA3 region with electrodes containing K_2SO_4. Orthodromic and antidromic (*6*) stimulation always elicited a hyperpolarizing IPSP (*7*) that had a reversal potential (E_{IPSP}) of -70 ± 8.4 mV (mean ± SD, $n = 11$). The resting membrane potential (E_M) of these cells was -59 ± 6.9 mV. However, when we used KCl-containing electrodes, stimulation elicited a purely depolarizing response in most CA3 cells, although E_M of the cells was unchanged. A new E_{IPSP} for the response (-55 ± 7.5 mV, $n = 7$) was attained within about 5 minutes. Because the KCl electrode raised the chloride ion concentration in the cells, this result confirms the well-established role for chloride ions in the IPSP's of pyramidal neurons (*8*).

Granule cells in the dentate gyrus had a higher E_M (*9*) than CA3 neurons (-67 ± 5.9 mV) measured with both K_2SO_4- ($n = 24$) and KCl-filled ($n = 9$) electrodes. In contrast to CA3 cells, orthodromic and antidromic stimulation elicited purely depolarizing postsynaptic potentials in 21 of 24 neurons, even with K_2SO_4-filled electrodes. The apparent E_{IPSP} was -62 ± 8.4 mV. In addition, impalement of GC with KCl-filled electrodes did not depolarize the E_{IPSP} (-64 ± 8.8 mV, $n = 9$). The opposite directions of the driving forces for the IPSP's ($E_{IPSP} - E_M$) of CA3 pyramidal cells and GC paralleled the opposite responses to somatic GABA application (*10*); the response of CA3 cells to GABA was predominantly hyperpolarizing, that of GC was depolarizing.

Bath application of 0.5 to 2 m*M* furosemide (*11*) to GC or CA3 cells shifted the apparent E_{IPSP} in a depolarizing direction (Fig. 1A) and increased the amplitude of the depolarizing postsynaptic response (Fig. 1B) when we used a KCl-filled electrode. Depolarizing responses to GABA were also larger, which indicated that the observed increase in the postsynaptic response was due to a depolarizing action on the GABA-dependent inhibitory component. A new steady state was usually attained within about 20 minutes after furosemide application. The effect was reversible after a wash of about 1 hour (Fig. 1B, upper left and lower left). Furosemide enhanced the depolarizing effect of Cl^- injections (1 nA, 5 to 10 minutes) on the postsynaptic response (Fig. 1B). Under control conditions, the effect was short, with half-times of decay between 20 and 30 seconds ($n = 6$). Furosemide slowed the recovery by a factor of 20 to 30 (Fig. 1C). During washing, the time of recovery returned to control (Fig. 1C).

Furosemide might have impeded the recovery by reducing Cl^- permeability or blocking Cl^- transport, but several experiments indicated that the effect on Cl^- permeability was small. There was a slight increase in resting membrane resistance (control, 40.5 ± 18.4 megohms, furosemide, 49.8 ± 22.9 megohms, $n = 9$). Inward rectification was enhanced by furosemide, but the change in rectification ratio (*12*) was also small (control, 1.21 ± 0.43; furosemide, 1.65 ± 0.6). To test further for effects of furosemide on passive Cl^- permeability, we measured changes of E_{IPSP} in-

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