treatment. Thus Ras may be acting on K^+ channels via PKC activation. The PKC-independent nature of Ras-induced enhancement of Ca^{2+} conductance is consistent with a PKC-independent pathway for cell growth signal transduction (*16*).

A voltage-sensitive influx of Ca^{2+} has been implicated in the regulation of cellular processes such as those occurring during cell growth, development, and differentiation (*18–20*). Enzymatic programs that regulate cell growth are activated by intracellular Ca^{2+} concentration in the micromolar range, which can be brought about by regional clustering of L-type Ca^{2+} channels, leading to high local concentrations of Ca^{2+} (*19*). Similarly, enhancements of voltage-dependent Ca^{2+} conductances could provide an additional mechanism by which extracellular growth signals are initiated via *ras* oncogene activation.

Ras activation of voltage-sensitive Ca^{2+} channels in differentiated neurons may be an initial step in the expression of Ras function. Several proto-oncogenes are expressed in post-mitotic cells (*1*); our results are consistent with their potential role in transmembrane signaling. An analogous role in a learning context may be played by the low molecular weight G protein, cp20, which increases after classical conditioning (*21*) and causes marked K^+ current reductions quite similar to the reductions following Ras injection into type B cells in *Hermissenda* (*11*).

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22. The two-electrode voltage-clamp technique for *Hermissenda* neurons has been described (*21, 23*). After dissection, the *Hermissenda* pedal ganglion was treated with protease (2 mg/ml) (Sigma) for 30 min, and LP_1 was impaled with 2 to 2.5 megohm electrodes filled with 3 M $CsCl_2$ and then connected to a custom-made high-power voltage-clamp amplifier (Pelagic Electronics, Falmouth, MA). A third 1 megohm microelectrode was filled with Ras or control solutions, containing 5 mM tris, 2.5 mM $MgCl_2$, 0.5 mM EDTA, and 20 mM KCl; 1 M potassium acetate was used as charge carrier. The preparation was kept at 22°C and bathed in artificial sea-water solution containing 10 mM KCl, 50 mM $MgCl_2$, 10 mM $BaCl_2$, 327 mM tetramethylammonium (TEA), 10 mM tris (pH 7.4), 100 mM TEA, 3 mM 4-AP; osmolarity was 990 mosM. Pulse generation, data acquisition, and analysis was performed with pClamp (Axon Instruments, California). Immediately after impalement, a resting membrane potential of −45 to −55 mV was measured and then cells were voltage clamped at −60 mV. Current transients were low-pass filtered [corner frequency of 10 kHz, digitized at 200 μs per point and leak-corrected on line with a P/N protocol—a series of eight subpulses (P) of one-eighth the step amplitude (N)]. Inward currents, elicited with 150-ms, 10-mV depolarizing steps, were measured at a 30-ms time point before and every 10 min after iontophoretic injections (−10 nA for 10 min) of Ras or control solutions. H-Ras soluble proteins were purified from *Escherichia coli* (*14*). The prokaryotic vector (pJCL-30) places the murine *ras* genes under the control of the temperature-inducible λpL promoter. A 75% purity of the expressed proteins was first obtained on a AcA54 (molecular sizing) column, and 90% purity after a fast protein liquid chromatography (FPLC) mono Q column. After dialysis into 50% glycerol, proteins were stored at −20°C. Before use, proteins were solubilized in 10 mM tris (pH 8.0), 5 mM $MgCl_2$, and 1 mM EDTA.
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Hot Spots for Growth Hormone Gene Deletions in Homologous Regions Outside of Alu Repeats

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Familial growth hormone deficiency type 1A is an autosomal recessive disease caused by deletion of both growth hormone–1 (GH1) alleles. Ten patients from heterogeneous geographic origins showed differences in restriction fragment length polymorphism haplotypes in nondeleted regions that flanked GH1, suggesting that these deletions arose from independent unequal recombination events. Deoxyribonucleic acid (DNA) samples from nine of ten patients showed that crossovers occurred within 99% homologous, 594–base pair (bp) segments that flanked GH1. A DNA sample from one patient indicated that the crossover occurred within 454-bp segments that flanked GH1 and contained 274-bp repeats that are 98% homologous. Although Alu repeats, which are frequent sites of recombination, are adjacent to GH1, they were not involved in any of the recombination events studied. These results suggest that length and degree of DNA sequence homology are important in defining recombination sites that resulted in GH1 deletions.

INDIVIDUALS WITH FAMILIAL ISOLATED growth hormone deficiency (IGHD) type 1A have a complete absence of growth hormone (GH) and frequently develop immune intolerance to exogenous GH (*1–3*). The molecular basis of this autosomal recessive disorder is the deletion of both GH1 alleles. Although the incidence of IGHD type 1A is unknown, ~35 unrelated cases from 15 countries have been reported. Differences in geographic origin of patients and the heterogeneity seen in restriction fragment length polymorphism (RFLP) haplotypes in nondeleted sequences that flank GH1 suggest that these deletions represent independent recombinational events.

The GH1 locus has been mapped by in situ hybridization to chromosome 17q22–24 and is normally present at the 5′ end of the 66.5-kb human GH gene cluster. The GH gene cluster includes (5′ to 3′) GH1, chorionic somatomammotropin pseudogene–1 (CSHP1), chorionic somatomammotropin–1 (CSH1), growth hormone–2 (GH2), and chorionic somatomammotropin–2 (CSH2), as well as 48 Alu repeats (*4,*

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5). A high degree of sequence homology (92 to 98%) exists within the immediate flanking, intervening, and coding sequences. Furthermore, DNA sequence analysis of this gene cluster suggests that it arose from multiple homologous but unequal recombinational events between Alu repeats. As a means of determining the role of the Alu repeats and other flanking DNA in GH1 gene deletions, we have characterized the breakpoints associated with ten different GH1 gene deletions.

DNA was isolated from the nuclei of peripheral leukocytes of ten unrelated patients, from eight countries, with IGHD type 1A. In all cases, a 6.7-kb deletion that encompassed GH1 was confirmed by Southern (DNA) blotting. To localize the breakpoints of the deletions, we examined the DNA sequences that normally flank GH1. The restriction endonuclease digestion patterns of the two 2.24-kb segments of highly homologous DNA sequences (located 4140 bp 5′ and 2477 bp 3′ to the GH1 transcription initiation site) were compared. Unequal homologous recombinations between these two segments resulted in 6.7-kb deletions that encompass GH1 (Fig. 1) (*6*). DNA from the ten patients had 2.0- and 5.6-kb junction fragments, after digestion with Mst II and Eco RI, respectively, and hybridization to the indicated probe (Fig. 1) (7). The exact crossover sites contained within these junction fragments were then determined (Figs. 1 and 2).

Table 1. Origin and characterization of GH1 deletions. An explanation of the nature and consequences of the studies was provided to each of the patients. All deletions were 6.7 kb in length (Fig. 1).

Patient	Country	Haplotype	
		Bgl II	Msp I
1	Argentina	+−	−−
2	Argentina*	−+/++	++/++
3	Austria	++	++
4	Brazil	+−	−−
5	China	+−	−−
6	Japan†	−+	++
7	Japan†	−+	++
8	Mexico	+−	−−
9	Switzerland	+−	−−
10	Turkey	−+	++

*Although the patient had both GH1 genes deleted, she was heterozygous for two different RFLP haplotypes immediately adjacent to her deletions. †The patients were not related by family history.

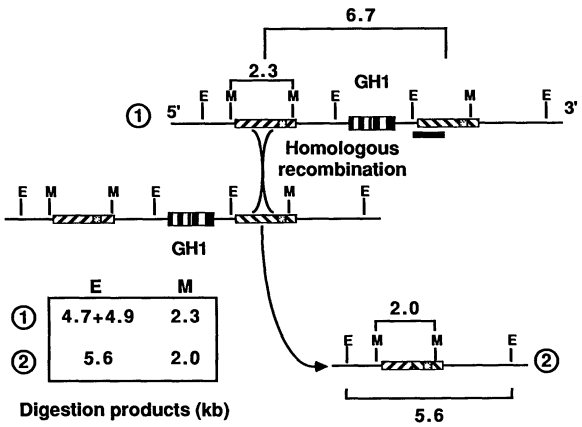

Fig. 1. Homologous recombination flanking the GH1 locus occurring between two homologous chromosomes. Two sequences flanking GH1 recombined at regions of homology (denoted as opposing hatched rectangles, both of which contain Alu sequences indicated by stippled boxes) to generate the common 6.7-kb deletion. The numbers shown above and below the restriction fragments are in kilobases. In order to sequence the crossover sites, DNA (100 μg) from two patients (numbers 2 and 9, Table 1) were digested with Eco RI (E) and subjected to gel electrophoresis, and fragments that ranged in size from 5.4 to 6.0 kb in length were excised from the gel. The 5.6-kb Eco RI fragments that contained the 2.0-kb Mst II (M) junction fragments were gel purified and ligated to λgt11 Eco RI–digested arms. Recombinant plaques (~40,000 to 65,000) were screened with the ^{32}P-labeled, 1.1-kb fragment (solid black rectangle). Positive recombinant clones were subcloned into the Eco RI site of pBR322, and a restriction endonuclease map of the cloned insert was generated. The 731-bp Sst I–Sma I fragments that represented fusions of the 5′ and 3′ homologous segments (see Fig. 2) were subcloned into M13-18 and M13-19. Both strands of these fragments were sequenced with standard dideoxy sequencing techniques and the universal primers for M13 (New England Biolabs), as well as an internal 20-nucleotide primer (5′-TGGGTGCTCCTTCCACTCTC-3′) (*30*).

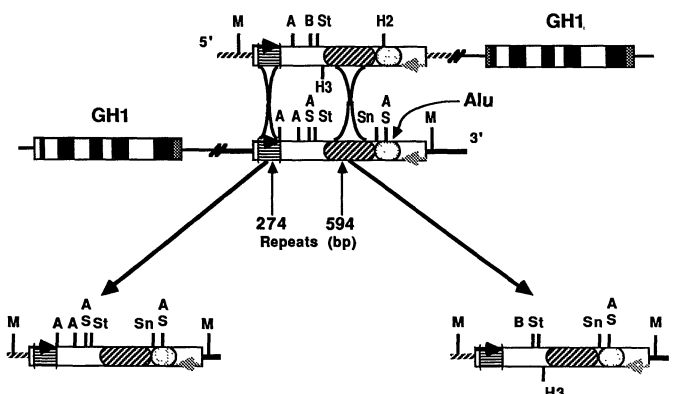

Fig. 2. The breakpoints were localized in DNA from the remaining eight patients after polymerase chain reaction (PCR) amplification of a segment of the novel 2.0-kb fusion fragments that were contained within Mst II sites (Fig. 1). Primer sequences lie 118-bp downstream from the 5′ Mst II site and 39 bp upstream from the 3′ Mst II site. These 5′ and 3′ primer sequences [5′-GGATCCAGCCTCAAAGAGCTTAC-3′ (solid arrow) and 5′-GAATTCCCAGAGCCTTGAGCAATGGA-3′ (stippled arrow)] included 5′ Bam HI and Eco RI cloning sites. The PCR-derived fragments from control samples were 1900 and 1921 bp in length, corresponding to the 5′ and 3′ blocks of homology (Fig. 1) (*31*). However, in IGHD type 1A patients, because GH1 is deleted by recombination between these homologous segments, only 1918-bp fusion fragments are amplified. For two samples (from patients 3 and 10 of Table 1) DNA sequencing was performed as in Fig. 1. The mismatches detected within the repeated regions compared to two controls allowed mapping of the 5′ and 3′ boundaries of the crossover sites. In the remaining six patients (numbers 1 and 4 to 8, Table 1), the breakpoints were localized by extensive restriction endonuclease mapping of the PCR-amplified fragments (7). Restriction endonuclease sites shown are: A, Ava I; B, Bgl I; H2, Hae II; H3, Hae III; M, Mst II; S, Sma I; Sn, Sna BI; and St, Sst I. Alu, 274- and 594-bp repeats are shown by stippled, horizontal, and hatched areas, respectively.

The crossover sites from four patients were determined by DNA sequencing and were localized to a 536-bp segment (position 1 to 5, Fig. 3). In each case, the 5′ end of the 536-bp segment is composed of DNA sequences derived from those lying 5′ to GH1, whereas its 3′ end is derived from DNA sequences lying 3′ to GH1. Because of single-base differences between these repeats, more precise localization of the breakpoints was not possible (Fig. 3).

The crossover sites that resulted in the deletions of the remaining six patients were localized by extensive restriction endonuclease mapping of the PCR-derived products (Fig. 2). In five of these subjects (numbers 1 and 4 to 7, Table 1), the breakpoints were within 594-bp (Hae III–Sna BI) fragments (Figs. 2 and 3). These 594-bp segments are 99% homologous and contain the longest perfect repeats found in the 2.24-kb segments of homology that flank GH1 (Figs. 1 and 3). Moreover, these 594-bp segments lie immediately upstream of Alu repeats of 292 and 297 bp, which are 85% homologous and have maximum perfect repeats of 23 bp (Fig. 2). The crossover site in the remaining patient (number 8 in Table 1) was localized to 454-bp (Mst II–Ava I) fragments, which lie 448 bp 5′ to the 594-bp repeats (Figs. 2 and 4). These 454-bp fragments are 82% homologous and contain 274-bp repeats that are 98% homologous and have maximum perfect repeats of 68 bp.

We found that nine of ten (90%) of the crossover sites that resulted in 6.7-kb dele-

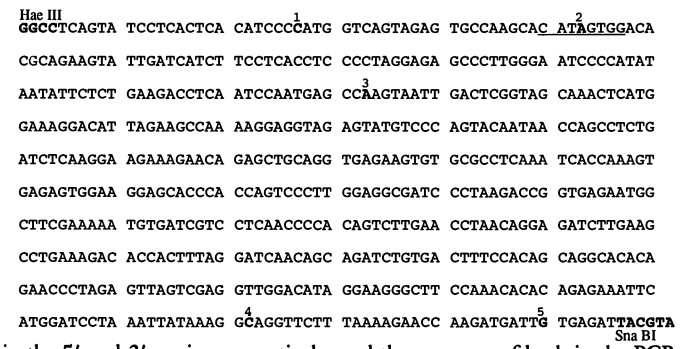

Fig. 3. The DNA sequence that contained the crossover sites of patients 1 to 7, 9, and 10 (Table 1 and Figs. 1 and 2). The repeats that flanked GH1 that contained this sequence are 3356 and 3261 bp 5′ and 3′, respectively, to the GH1 transcription initiation site. The underlined region indicates homology to the Chi sequence. The Hae III and Sna BI sites occurred only in the 5′ and 3′ copies, respectively, and the presence of both in the PCR products from patients 1 and 4 to 7 (Table 1) indicated that a crossover occurred between them (Fig. 1). In controls, the sequences were C, A/G, A/G, G, and C versus T, G, A, C, and G at positions 1 through 5 in the 5′ and 3′ repeats, respectively. In contrast, the sequence of the fusion fragments in patients 2, 3, 9, and 10 were C, A, A, C, and G at positions 1 through 5. The presence of a C at position 1 and 4 in these patients indicates that a crossover has occurred between these two sites to form the 536-bp fusion fragment.

tions that encompassed GH1 were localized to 594-bp repeats. Interestingly, none of the crossover sites were within the immediately adjacent Alu repeats. These findings are in contrast with numerous reports of gene deletions arising from unequal recombinational events in which one or both DNA segments involved contained Alu repeats (*8–17*).

Because patients studied were either not related or differed in race and RFLP haplotype of nondeleted regions that flanked GH1 (Table 1), we hypothesize that each deletion represents an independent and unequal recombinational event. We examined the sequence of the 594-bp apparent hot spot for recombination for properties identified in reported recombinagenic sequences (*18, 19*). However, this segment was not adenine-thymine (A-T) rich (52%) and did not contain significant direct repeats, palindromic regions, or tracts of alternating purine-pyrimidine sequences with potential to form Z-DNA. However, a sequence (CATGGTGG) with six of eight nucleotides homologous to the Chi sequence (GCTGGTGG) occurred in some 594-bp fragments (Fig. 3) (*20, 21*). Although Chi sequences promote recombination in *Escherichia coli*, it is not known whether they are frequent sites for recombination in mammalian cells.

Homologous recombination involves the exchange of nucleotide segments between two DNA molecules in regions of sequence homology, and results in two intact recombined DNA duplexes. Because of the technical difficulty in examining this process in vivo, relatively little is known about the mechanism of recombination in mammalian cells. Homologous recombination between DNA sequences in mammalian cells has been shown to be dependent on the extent of sequence homology (*22, 25*). In both intra- and extrachromosomal mammalian recombination systems, the rate of recombination between two sequences that share several hundred base pairs of homology is proportional to the degree of homology. When the length of homology is <200 bp, the rate of recombination decreases rapidly. However base-pair mismatch appears to have a greater effect on intra- versus extrachromosomal recombination. In *E. coli*, homologous recombination is also dependent on the length and degree of sequence homology (*26, 27*). For example, a single mismatch in a 53-bp sequence reduces the frequency of recombination. It was also shown that a reduction in homology from 100 to 92% in segments of ~170 bp decreases the frequency of recombination to <5% of the control. In mammalian cells, the dependence of homologous recombination on the length and degree of sequence homology has been substantiated through gene-targeting experiments (*28, 29*), in which a mutation within a gene is corrected by transferring the normal DNA sequence, contained as an insert in a cloning vector, to the desired chromosomal locus via homologous recombination. Because the success of gene targeting relies heavily on the fidelity of homologous recombination, understanding the basic sequence requirements is essential for characterization of the protein-DNA interactions that are involved in this process.

Although the hot spots for homologous recombination identified in this report are adjacent to Alu repeats, they preferentially participated in the recombinational events, probably because of their greater length and degree of sequence homology. Our findings suggest that Alu sequences in other genes may represent hot spots for recombination because they constitute the largest degree of sequence homology in the misaligned area.

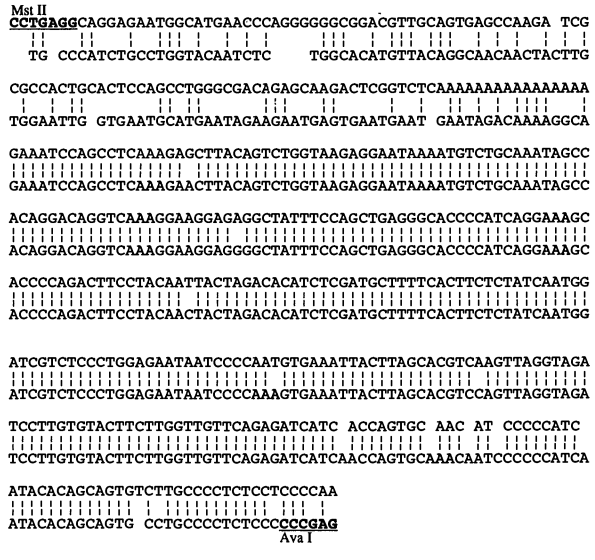

Fig. 4. The DNA sequence of the 454-bp repeats lying 4251 and 2368 bp 5′ and 3′ to the GH1 transcription initiation site is shown above and below, respectively. The presence of both the Mst II and Ava I sites in the PCR products of patient 8 indicated that a crossover occurred between them.

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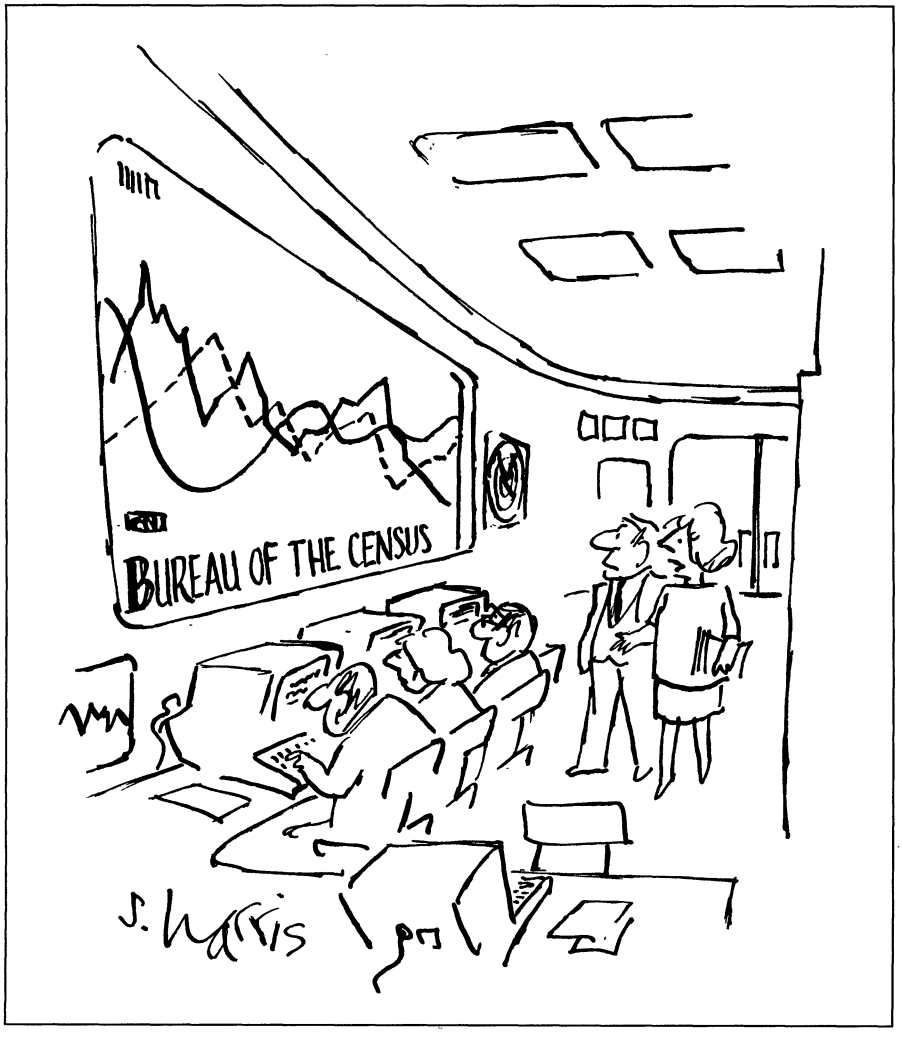

"We've come far. We used to just count."