ionophores can increase resorption in fetal rat long bones cultured in vitro. We believe this resorption, like that produced by PTH, is osteoclast mediated, since PTH, A23187, and Ionomycin all increase formation of multinucleated giant cells in bone and increase release of β-glucuronidase, an enzyme produced by activated osteoclasts (*9*). However, there are important differences between the action of calcium ionophores and PTH. For example, PTH had small effects on thymidine incorporation, whereas A23187 and Ionomycin markedly inhibited DNA synthesis. In addition, unlike PTH, both ionophores did not stimulate bone resorption at high concentrations. The effects of the ionophores on thymidine incorporation are similar to those observed by Dietrich and Paddock (*2*) in rat calvaria but differ from the effect of A23187 on lymphocyte cultures where DNA synthesis is increased (*10*).

The effects of high concentrations of ionophores on DNA and protein synthesis in bone could explain the inhibition of resorption observed by Dziak and Stern (*1*) and ourselves, as well as the inhibitory effects found by Ivey *et al.* (*3*) in mouse calvaria. Alternatively, these inhibitory effects on resorption could be due to a toxic action of the ionophores on mitochondrial function or adenosine triphosphate production (*11*). Feldman *et al.* (*12*) have recently shown that, in response to PTH, cultures of newborn mouse calvaria incorporate preexisting mononuclear precursors into functioning osteoclasts. Our results suggest that in cultures of fetal rat long bone, functioning osteoclasts can form without DNA synthesis and that divalent cation ionophores, like PTH, activate osteoclasts by stimulating precursor fusion through an effect which is probably mediated by an increase in intracellular calcium. The absence of any stimulatory effect on resorption by calcium ionophores in mouse calvaria could represent a species or tissue-specific difference from the rat long bone in the mechanism of osteoclast activation, particularly if mouse calvaria cultures were more dependent on cell replication for measurable osteoclastic bone resorption to occur.

Although our data do not rule out the possibility that ionophores cause an early initial stimulation of osteoclast precursor cell multiplication before inhibiting DNA synthesis, we expected that such an effect would also stimulate resorption in mouse calvaria. Moreover, in lymphocytes the mitogenic response produced by A23187 is not seen for 24 to 48 hours after treatment (*10*). It is also possible that multiplication of only a selected population of osteoclast precursors is necessary for resorption and that these cells are resistant to the inhibitory effects of calcium ionophores; however, this hypothesis seems unlikely because A23187 inhibited thymidine incorporation within 24 hours but did not produce resorption until 48 hours. At this time, A23187 at 1.0 μ*M* could still stimulate resorption, yet had reduced thymidine incorporation below our limit of detection. Thus, our results call into question the concept that cell replication is important for the stimulation of osteoclastic bone resorption by hormones, at least for fetal rat long bones. Not only did stimulation of resorption occur when DNA synthesis was inhibited by ionophores, but PTH had little stimulatory effect on thymidine incorporation in this system. We have also observed that the ability of PTH to stimulate bone resorption is not diminished in the presence of other inhibitors of DNA synthesis (*13*).

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DNA Sequence of Two Closely Linked Human Leukocyte Interferon Genes

Abstract. *A single recombinant lambda bacteriophage isolated from a human genome library contains two closely related human interferon genes of the leukocyte or α type. The two genes are separated by 12 kilobase pairs and are oriented in the same direction with respect to transcription. Comparisons of the DNA sequences of these two genes and interferon complementary DNA clones indicate that the two interferon genes lack intervening sequences.*

The human interferon multigene family consists of approximately ten leukocyte interferon (LeIF, IFN-α) genes (*1–3*) that have approximately 85 to 95 percent DNA sequence homology in their protein coding regions, and at least one fibroblast interferon (FIF, IFN-β) gene that is only about 50 percent homologous with the LeIF genes (*4–6*). The study of related gene families allows comparative analysis of nucleotide and amino acid sequences, which can help to identify features of importance for gene expression and protein function and can help elucidate modes of evolution of related genes. We therefore have determined the nucleotide sequence of eight distinct LeIF complementary DNA (cDNA) clones (*1*) and initiated DNA sequence analysis of LeIF genes isolated from a library of bacteriophage lambda clones containing overlapping fragments of the human genome. We now report the DNA sequences of two linked LeIF genes.

The phage λ Charon 4A recombinant library of the human genome constructed by Lawn *et al.* (*7*) was screened for leukocyte interferon genes (*7, 8*) with a radioactive probe derived from the cDNA clone LeIF A (*9*). Restriction endonuclease digestion and hybridization by the blot technique revealed that the recombinant designated λHLeIF2 contains an insert of 19 kilobase pairs (kb) of human DNA which, upon digestion with either of the restriction endonucleases Eco RI, Xba I, or Hind III, produces two fragments that hybridize to LeIF cDNA sequences. Subsequent analysis revealed two distinct LeIF genes in this 19-kb segment of the human genome (see below). A map indicating the location of the cleavage sites of several restriction endonucleases is pre-

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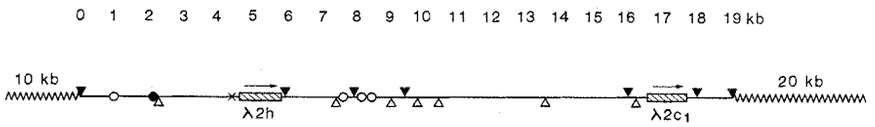

Fig. 1. Restriction endonuclease cleavage map of λHLeIF2. The recombinant bacteriophage λHLeIF2 was selected from a human genome library (*7, 8*) with a radioactive probe derived from fragments of LeIF clone LeIF A (*1*) labeled to ≥ 10^8 cpm/μg with ^{32}P (Amersham) by DNA polymerase (New England Biolabs) extension of small, random calf thymus DNA primers (*21*). The restriction map depicts all the cleavage sites of Eco RI, Hind III, and Xba I within the human genome derived portion of λHLeIF2. Bam HI does not cut the 19-kb human insert in this clone. One each of the several cleavage sites of Hpa II and Bgl II are also included in the map, as their location was employed in orienting the LeIF genes with respect to the direction of transcription. This map was derived chiefly by digestions of λHLeIF2 with restriction enzymes singly or in combination, followed by electrophoresis in agarose and polyacrylamide gels (*7*) and by isolation of single-enzyme digestion fragments by electroelution from gel slices and subsequent cleavage again, with different enzymes. The genes were oriented with respect to transcription by restriction endonuclease mapping and DNA sequencing. Restriction endonuclease sites within the human DNA portion of the recombinant DNA (horizontal line) are indicated as follows: (▼) Eco RI, (△) Hind III, (○) Xba I, (X) Hpa II, and (●)Bgl II. Restriction sites within the flanking λCh 4A "arms" (wavy lines) are not shown. A size scale in kilobases (*kb*) is shown above the map. The position of two interferon genes is indicated by the hatched box; arrows above these boxes indicate the direction of transcription of these genes.

sented in Fig. 1. The genomic clone λHLeIF2 contains two complete leukocyte interferon genes located 12 kb apart. The genes are located on 2.0- and 6.0-kb Eco RI fragments that were subcloned into pBR325 (*10*) for DNA sequence analysis. Both genes are oriented in the same direction with respect to transcription.

The complete DNA sequence (*11, 12*) of the two interferon genes in λHLeIF2 is shown in Fig. 2. The two gene sequences were aligned by introducing several deletions in the flanking regions to produce a best fit. The two genes are 91 percent homologous in the coding region, 71 percent homologous in the 5′ noncoding region from the presumed cap site to the initiator codon of the preinterferon gene, and 93 percent homologous in the 3′ noncoding region from protein termination to the presumed sites of polyadenylation (each deletion has been counted as equal to a single base change). These values fall within the range of homology found among the sequences of eight distinct LeIF cDNA clones derived from messenger RNA (mRNA) of cultured myeloid cells (*1*). The DNA sequence of the two genes becomes markedly divergent upstream of the vicinity of position minus 130. No deletions were introduced for alignment purposes preceding this location (Fig. 2). In contrast, the 3′ flanking regions of the two genes are very homologous for at least 580 nucleotides following the stop codon.

When the λ2h and λ$2c_1$ gene sequences were compared to eight LeIF cDNA clones (*1*), it was found that the λ2h gene corresponded to the cDNA clone LeIF H, and that λ$2c_1$ most closely resembled LeIF C. The differences between the genes and the message-derived clones are indicated in Fig. 2. The sequence of the gene λ2h differs by only one nucleotide from the sequence of the cDNA clone LeIF H, corresponding to one amino acid difference in the predicted protein sequences. The λ$2c_1$ gene differs in 18 nucleotides in the coding region from the LeIF C cDNA resulting in ten amino acid substitutions. The DNA sequences of the genes and the corresponding mRNA derived cDNA clones are colinear in the protein coding region and in the 5′ flanking region that are contained in the cDNA clones. Thus these two LeIF genes contain no introns in those regions. The minor differences in the 3′ noncoding regions are not likely to represent intron sequences (see below). The only remaining site for a possible intron would be in the region between the true 5′ terminus of the mRNA and the 5′ terminus of the corresponding cDNA clone. Experiments proposed to directly sequence this region of the corresponding mRNA's were complicated by the fact that the cell line used contains a mixture of at least eight related LeIF mRNA sequences. When a synthetic dodecanucleotide corresponding to the first 12 nucleotides of the mature protein sequence of most LeIF cDNA's was used to prime dideoxy sequencing of mRNA, a characteristic, readable sequence was not obtained. From the termination of the mixed sequencing ladder it was, however, possible to determine the distance of the 5′ end of the LeIF mRNA's from the primer position (data not shown). This placed the presumed capping site within two nucleotides of position minus 69 (Fig. 2). This region contains the dinucleotide AG (A, adenine; G, guanine), which corresponds to the presumed cap site of the LeIF gene described by Nagata *et al.* (*2*). The AG dinucleotide is found in a sequence of 16 nucleotides which are exactly conserved in the λ2h and λ$2c_1$ genes. If this indeed represents the 5′ terminus of the RNA's, these genes could only contain an intron within a limited region of about 20 nucleotides from the 5′ end of the message. The likelihood of this occurrence is reduced by the observation that the λ2h and λ$2c_1$ gene sequences are colinear (although not identical) to the one reported LeIF mRNA sequence (*2*) from 5′ terminus to the initiation codon. It is thus probable that these two LeIF genes, like the one described by Nagata *et al.* (*2*), contain no introns. This is in marked contrast to all other vertebrate nuclear genes examined so far.

Homology exists between the 5′ flanking regions of λ2h and λ$2c_1$, but is less than that found in the coding regions, as is the case with other related pairs of genes. Located approximately 30 bases upstream from the putative cap site of the two genes [as well as the gene IFN-α1 of Nagata *et al.* (*2*)] is the sequence TATTTAA (T, thymine). This may be a variant of the TATAAA or Goldberg-Hogness box (*13*), which is located about this distance upstream of the cap site in many eukaryotic genes and is postulated to play a role in the initiation of transcription by RNA polymerase II (*13*).

The sequence of the 3′ untranslated regions of gene λ2h is identical with that of LeIF H cDNA (*1*) and contains the sequence ATTAAA shortly before the site of polyadenylation, as does the gene λ$2c_1$. The hexanucleotide AATAAA precedes the site of polyadenylation of most eukaryotic genes (*14*); this particular variant has been reported in several cases (*1, 15*). Similar sequences can also be located in Fig. 2 further downstream from the polyadenylate [poly(A)] sites.

Gene λ$2c_1$ closely resembles its cDNA analog LeIF C in the 3′ untranslated region, except that it contains a stretch of 13 extra nucleotides approximately 260 base pairs after the protein termination codon. It seems unlikely that this represents an intron, since the sequence that is composed wholly of deoxyadenylate (dA) and deoxythymidylate (dT) bears no resemblance to any other introns at its possible "splice" junctions (*16*). In addition, these 13 nucleotides in λ$2c_1$, which are absent in LeIF C, can be found at a homologous location in other LeIF cDNA clones (*1*). Rather than indicating an intron, this sequence difference between λ$2c_1$ and LeIF C more likely represents a polymorphism or variation in the C type LeIF gene. The continued close sequence homology between λ2h and λ$2c_1$ beyond the poly(A) sites may

reflect recent gene duplication or conversion events such as those postulated for human α- and γ-globin genes (*17*).

The interferon genes comprise a multigene family. Their characterization should contribute to the understanding of gene control and functional relations between related genes and proteins, as well as of the processes of evolution by gene duplication and divergence. Interferons of slightly different protein sequence may respond differently to various inducers, modulate various secondary responses, be expressed preferential-

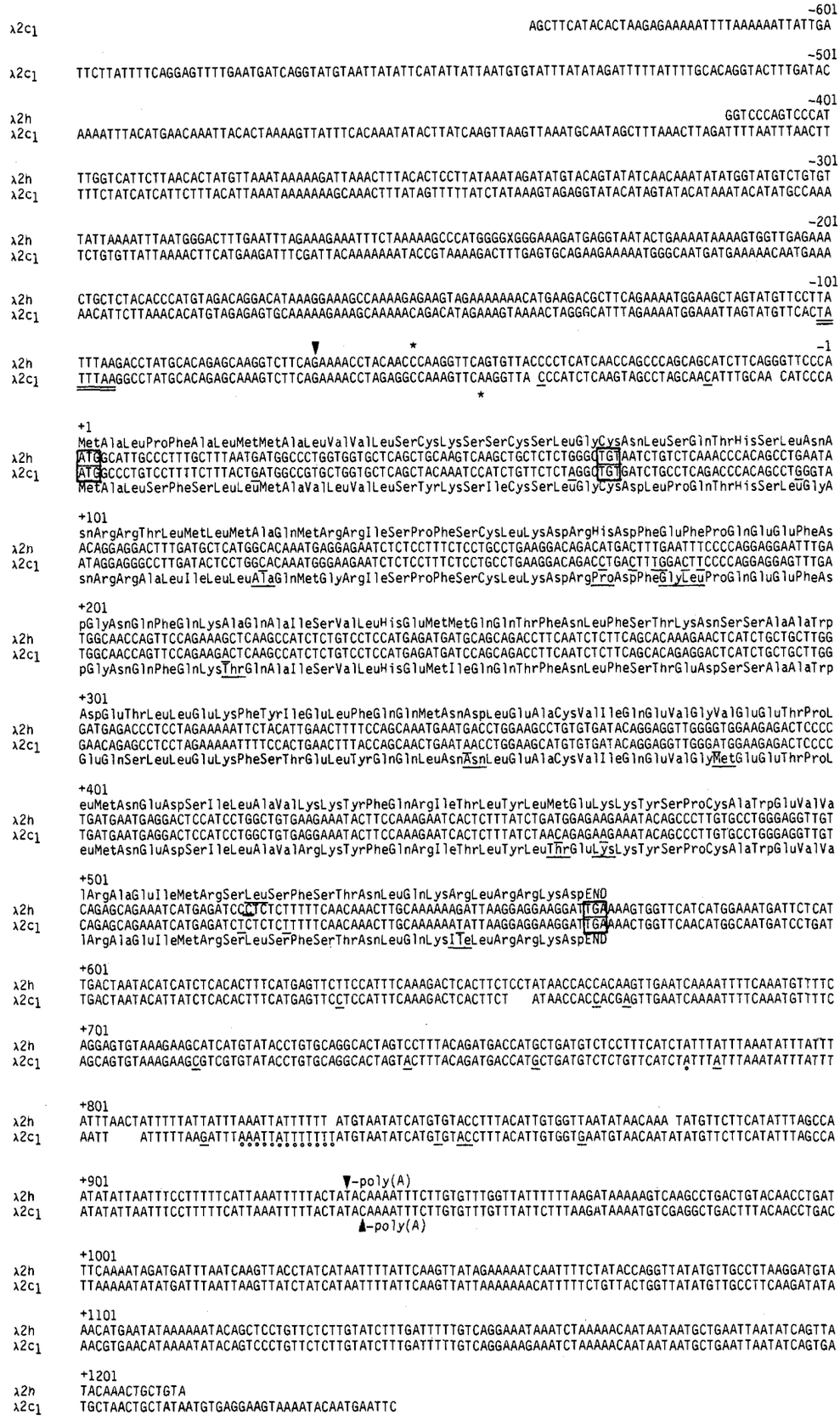

Fig. 2. DNA sequence of interferon genes. The DNA sequences of the λ2h and λ2c$_1$ LeIF genes contained in the recombinant phage λHLeIF2 are shown with several deletions introduced to achieve maximal sequence homology. No alignment for homology is attempted for regions preceding minus 130. The initiator codon of precursor interferon, the codon for the initial amino acid of the mature protein, and the termination codon are enclosed in boxes. Vertical arrows mark the putative capping site and polyadenylation sites of the corresponding mRNA's represented in cDNA clones LeIF H and LeIF C. The 5′ extent of those cDNA clones is indicated by asterisks, and nucleotides which differ at corresponding positions in those cDNA clones are underlined if there is a base substitution, or marked by a small circle underneath if a base is deleted in the cDNA sequence. The possible 5′ Goldberg-Hogness box is underlined twice. The predicted amino acid sequence for gene λ2h is shown above its nucleotide sequence, and for λ2c$_1$ below its nucleotide sequence. Predicted amino acids that differ from the respective cDNA clones are underlined. DNA sequencing was performed as described (*11, 12*). Synthetic DNA primers were prepared as described in (*22*).

ly in certain cells, or recognize different target cell types. The last-mentioned possibility is consistent with the findings that cloned human interferon proteins display varied activity in different animals and on cultured cells derived from various organs (*18*).

The interferon gene family appears to be genetically linked. The fibroblast interferon gene and all of the approximately ten LeIF genes detectable by DNA cross-hybridization have been localized to human chromosome 9 by blot hybridization to DNA derived from mouse-human hybrid cell lines (*19*). At least some of these genes are closely linked. The λHLeIF2 clone described here contains two LeIF genes separated by 12 kb, while the clone λHLeIF1 (*20*) contains two other LeIF genes 5.0 kb apart. Nagata *et al.* (*2*) have also reported two λ clones isolated from the same human genome library which apparently contain two linked LeIF genes each. It remains to be seen whether all members of the IF gene family are clustered. Continued studies of interferon genes and their products may help to clarify the nature of interferon gene organization and expression and of interferon protein activity.

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Corticosterone Increases the Amount of Protein 1, a Neuron-Specific Phosphoprotein, in Rat Hippocampus

Abstract. *Corticosterone increased the amount of the neuron-specific phosphoprotein protein 1 in the hippocampus, a brain region rich in corticosterone receptors, but not in several brain regions that contain relatively few corticosterone receptors.*

Steroid hormones influence a variety of functions in the nervous system (*1*). The molecular mechanism of action of steroid hormones in nervous as well as in nonnervous system tissue appears to involve an increase or decrease in the synthesis of specific tissue proteins (*2*). Identifying those proteins in the nervous system that are regulated by steroid hormones may help elucidate the mechanism by which steroid hormones regulate various nervous system functions, including behavior.

One protein that is specific to nervous tissue and found throughout the central and peripheral nervous systems is a phosphoprotein referred to as protein 1 (*3–6*). Protein 1, which is present only in neurons (*3–5*), is concentrated in most, and possibly all, presynaptic nerve terminals (*5, 7*), where it appears to be associated predominantly with neurotransmitter vesicles (*5, 8*). This association suggests that protein 1 plays an important role in the functioning of those vesicles.

Protein 1 is a prominent endogenous substrate in nervous tissue both for cyclic AMP (adenosine 3′,5′-monophosphate)–dependent (*3*) and for calcium-dependent (*9*) protein kinases. Phosphorylation of protein 1 is stimulated in intact cell preparations by a variety of agents that regulate the cyclic AMP and calcium systems of neurons. For example, depolarizing agents, which induce the movement of calcium into nerve endings, phosphodiesterase inhibitors, which increase cyclic AMP levels, and derivatives of cyclic AMP stimulate the phosphorylation of protein 1 in the rat cerebral cortex (*10*). Serotonin and dopamine, neurotransmitters that increase cyclic AMP concentrations, increase the state of phosphorylation of protein 1 in well-defined regions of the central (*11*) and peripheral (*12*) nervous systems.

Since steroid hormones appear to act at least in part by altering the amount of specific proteins in target tissues, and since protein 1 may play an important role in the physiology of the synapse, we studied the possibility that steroid hormones might regulate the total amount of protein 1 in regions of the nervous system that contain steroid receptors. We report that corticosterone increases protein 1 in the rat hippocampus.

The ability of steroid hormones to regulate protein 1 in the brain was tested by treating adrenalectomized and ovariectomized rats with either corticosterone or estradiol under conditions that are known to elicit various physiological and behavioral responses (*1*). The effect of such treatment on protein 1 in four brain regions, measured by radioimmunoassay (*13*), is shown in Table 1. Corticosterone significantly increased protein 1 in the hippocampus, a brain region known to concentrate ^{3}H-labeled corticosterone in vivo (*14*) and to contain the highest concentration of cytosolic and nuclear corticosterone receptors in the brain (*15*). In contrast, corticosterone did not alter the amount of protein 1 in the preoptic area, medial basal hypothalamus, or parietal cerebral cortex, brain regions that contain relatively few corticosterone receptors. Estradiol did not alter the amount of protein 1 either in the preoptic area and medial basal hypothalamus, brain regions rich in estradiol receptors (*1*), or in the hippocampus and parietal cerebral cortex, brain regions that contain relatively few estradiol receptors.

The effect of corticosterone on protein 1 in the hippocampus and cortex is shown as a function of the duration of exposure in Fig. 1. A significant increase in protein 1 in the hippocampus was first apparent after 1 day. The amount was maximal after 7 days, at which time the protein 1 content of the hippocampus of corticosterone-treated rats was about 30 percent greater than that of control rats. After 14 days of exposure to corticosterone, protein 1 decreased somewhat but

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