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7. There are 24 identical sets of digital-to-analog converter (DAC)–amplifier combinations, one for each electrode. The electronics can switch between fields up to 1 kHz. There was no delay of voltage change among the electrodes when electric fields were switched. The voltage range for each electrode is +150 to −150 V and up to 75 mA of current can be supplied to each electrode. The voltage can be controlled in steps of 55 mV. The output voltages of the electrodes were measured at the beginning and near the end of each gel run with a high-impedance digital voltmeter. The components used for the control circuitry are as follows: DACs are all National Semiconductor DAC0830LCN, low-voltage operational amplifiers are all Precision Monolithics, Inc., OP-07, and high-voltage amplifiers are either Burr-Brown 3583JM or Apex Microtechnology PA83. All gels shown were cast with FMC SeaKem LE agarose and are 13 cm square and less than 1 cm thick. The conditions for sample preparation as well as casting and running the gels are as in (*8*). Temperature control during electrophoresis was accomplished by recirculating the electrophoresis buffer through a heat exchanger, which was immersed in a temperature-controlled water bath. All gels were run at 14°C as measured in the electrophoresis buffer.
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10. We thank C. F. Spence for software development, P. Arakelian for technical assistance, B. Jones for preparation of figures, and J. Kobori and R. Kaiser for their critical reading of the manuscript. B.W.B. is an NIH postdoctoral fellow (GM10974). Supported by grants from the NSF (DMB 85-00298) and the Baxter Foundation to L.H.

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# Two Mechanisms for the Extinction of Gene Expression in Hybrid Cells

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**When two different mammalian cell types are fused to generate a stable hybrid cell line, genes that are active in only one of the parents are frequently shut off, a phenomenon called extinction. In this study two distinct, complementary mechanisms for such extinction of growth hormone gene expression were identified. In hybrids formed by fusing fibroblasts to pituitary cells, pituitary-specific proteins that bind to the growth hormone promoter were absent. In addition, a negative regulatory element located near the rat growth hormone promoter was specifically activated.**

THE GROWTH HORMONE GENE IS EXpressed only in specialized pituitary cells called somatotrophs (*1*) and has provided an excellent system for studies of cell-type specificity of gene expression (*2–9*). Since neither fibroblasts nor hybrids between fibroblasts and pituitary cells express detectable levels of growth hormone mRNA (*10*), growth hormone also provides an excellent model for study of extinction of gene expression in cell hybrids.

Detailed characterization of the rat growth hormone (rGH) gene has identified both trans-acting factors and cis-acting DNA sequences thought to be responsible for cell type–specific expression. Extracts from rat pituitary cells contain proteins that bind specifically to sites in the rGH promoter between approximately −95 and −60, and between −140 and −115, relative to the start of the mRNA (*2–6*). A protein originally called GC1 (*2*) appears to bind to both sites. There is direct evidence from in vitro transcription experiments that this protein, which is found only in pituitary cells, is required for activation of the rGH promoter (*6*). An additional protein, found in both pituitary and nonpituitary cell types, binds only to the latter, distal site and will be referred to here as GC2 (*3*). Analogous results have been obtained from analysis of proteins binding to the human growth hormone (hGH) promoter (*7*), which is similar in sequence to the rGH promoter in this area.

A cis-acting, cell type–specific negative regulatory element, termed a silencer, has also been mapped just upstream of the rGH promoter (*8, 9*). The silencer acts to repress rGH promoter activity in nonpituitary cells, but has no effect on expression in pituitary cells. It seems that a combination of positive and negative regulatory elements generates the correct, cell type–specific expression of the rGH gene.

To investigate the mechanism of extinction of rGH expression, we used hybrid cell lines formed by fusing a hypoxanthine-guanine phosphoribosyl transferase–deficient ($HGPRT^-$) derivative of rat pituitary $GH_4$ cells to a thymidine kinase–deficient ($TK^-$) derivative of mouse fibroblast L cells ($LTK^-$). Several independent stable hybrids were subcloned and screened for the presence of the rat and mouse growth hormone (GH) genes by DNA blotting with a full-length rGH cDNA probe (*11*). Both genes were present in all of the subclones, demonstrating that they are true hybrids (Fig. 1A). The hybrids and parental cell lines were also analyzed for the presence of rGH mRNA. As expected (*10*), there was a high level of rGH mRNA in the $GH_4$ cells, but no detectable rGH mRNA in either the $LTK^-$ cells or the hybrids (Fig. 1B).

We used the deoxyribonuclease I (DNase I) protection assay to test nuclear extracts of both parents and the hybrid lines PT1CL5 and PT1CL6 for rGH promoter binding proteins (Fig. 2). As expected, extracts from $GH_4$ cells generated footprints at both the proximal GC1 binding site and the distal binding site for GC1 or GC2 (labeled GC2 in Fig. 2). In addition, a third footprint was seen between −240 and −220. We examined a number of cell types and found this protein, which we call GC3, only in pituitary cells (*12*), although there is evidence that some nonpituitary cells may contain a protein that interacts with this area (*4, 5*).

Extracts from $LTK^-$ cells and the hybrid PT1CL5 showed a footprint at the distal GC2 binding site, but not the proximal GC1 binding site or the GC3 binding site (Fig. 2). Essentially identical results were obtained with the independent hybrid line PT1CL6. The presence of GC2 in the $LTK^-$ cells was somewhat unexpected (*3, 5*), but is in agreement with results obtained with the hGH promoter (*7*). To determine whether the $LTK^-$ extracts contained an activity that prevented binding of the pituitary proteins, the two parental extracts were mixed in various proportions and used for footprinting. As would be expected if GC1 and GC3 were simply absent in the $LTK^-$ cells, both the proximal GC1 footprint and the GC3 footprint were observed at the expected $GH_4$ extract concentrations. We conclude that the expression or activity of

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both GC1 and GC3 is repressed in the hybrids, relative to the $GH_4$ parent.

To assay for activity of the rGH silencer, three chloramphenicol acetyltransferase (CAT)–expressing plasmids containing variable amounts of the rGH promoter and 5′ flanking sequences were transfected into $GH_4$ cells, $LTK^-$ cells, and the hybrid PT1CL5 cells (Fig. 3). The truncated rGH promoter in pRGH237 was slightly less active than the herpes virus TK promoter (pUTKAT1) in PT1CL5 and $LTK^-$ cells and slightly more active than TK in $GH_4$ cells. However, the additional rGH sequences present in both pRGH527 and pRGH387 strongly repressed rGH promoter activity in $LTK^-$ cells and in PT1CL5 (and PT1CL6, not shown) cells, but had no effect in $GH_4$ cells. These results are consistent with previous results obtained with the rGH silencer in pituitary cells and fibroblasts (*8, 9*).

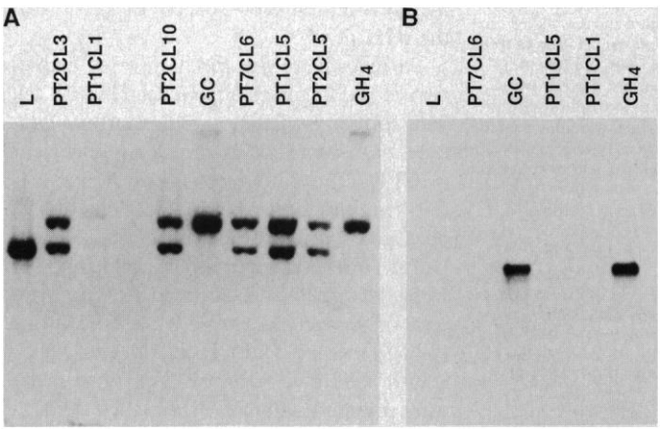


**Fig. 1.** Rat pituitary × mouse fibroblast hybrids. An $HGPRT^-$ subline of $GH_4$ cells was selected with 8-azaguanine and fused to $LTK^-$ cells by use of polyethylene glycol (PEG) (*19, 20*). Stable hybrids were selected with hypoxanthine, aminopterin, and thymidine (HAT) medium (*21*) and subcloned. (**A**) DNA blot. DNA from hybrids, both parental lines, and another pituitary cell line (GC), as indicated, was digested with Hind III, separated by electrophoresis, blotted, and probed with an rGH cDNA probe (*11*) by means of standard techniques (*22*). The molecular sizes of the hybridizing bands are 4.4 kb (mouse) and 5.6 kb (rat). (**B**) RNA blot. Total RNA from the indicated hybrids, parental cells, and GC cells (20 μg from each) was separated by electrophoresis on a formaldehyde agarose gel, blotted onto a nitrocellulose filter, and hybridized to an rGH cDNA probe (*11*), by use of standard techniques (*22*). The molecular size of the hybridizing band is approximately 900 nucleotides. Cells were propagated in the presence of 10% fetal bovine serum. Under these conditions, rGH expression is partially induced by the thyroid hormone present in the fetal bovine serum. Identical results were obtained with two independent preparations of RNA isolated at different times during the propagation of the hybrids.

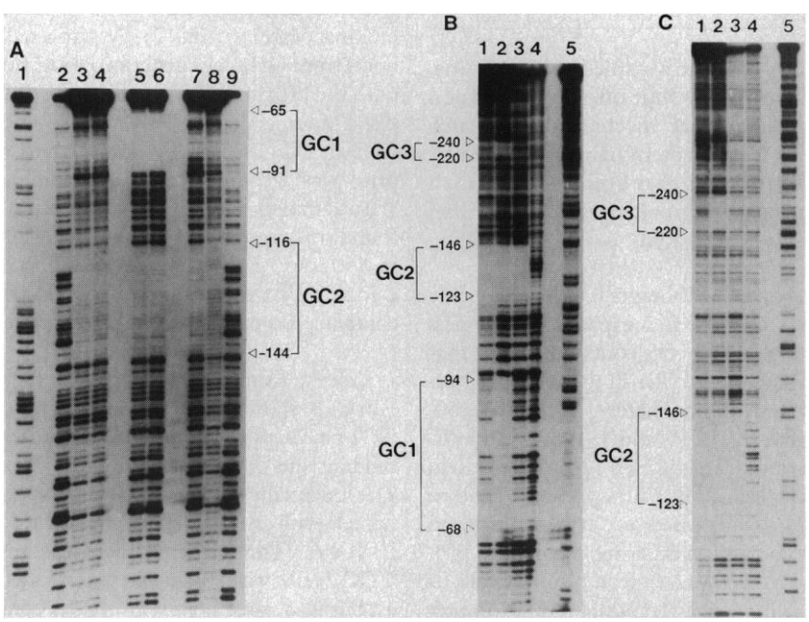


**Fig. 2.** Proteins binding to the rGH promoter. Vertical lines indicate extent of footprints for the three proteins. (**A**) Sense strand footprints were obtained by means of an end-labeled rGH promoter fragment extending from −237 (Bgl II) to −57 (Sty I). Lane 1, chemical sequencing guanine reaction; lanes 2 and 9, control DNase I with no added extract; lanes 3 to 8, DNase I footprinting with nuclear extract from $LTK^-$ cells (lane 3, 10 μg of extract; lane 4, 20 μg), $GH_4$ cells (lane 5, 10 μg; lane 6, 20 μg), and PT1C15 cells (lane 7, 10 μg; lane 8, 20 μg). (**B**) Antisense strand footprints were obtained by use of a labeled fragment extending from −20 (Sph I) to −455 (Ava II). Lane 1, 20 μg of $LTK^-$ cell extract; lane 2, 20 μg of $GH_4$ cell extract; lane 3, 20 μg of PT1C15 extract; lane 4, DNase I with no added extract; lane 5, chemical sequencing guanine reaction. (**C**) The same samples as in (B) analyzed by electrophoresis for a longer period. Nuclear extracts, containing 3 to 7 mg of protein per milliliter, were prepared from the different cell types as described (*23*). DNase I footprinting was carried out as described (*24*). The numbers next to each footprint represent the position of the protected regions relative to the rGH mRNA start site.

To confirm that the silencer is active in the hybrids, three additional plasmids containing the Rous sarcoma virus (RSV) enhancer were transfected. Insertion of the viral enhancer modestly increased activity of the 237-bp rGH promoter in all three cell types (Fig. 3). There was no effect in $GH_4$ cells when the silencer fragment was inserted either just upstream of the enhancer or between the enhancer and the promoter. In $LTK^-$ cells and the hybrids, insertion of the silencer between the promoter and the enhancer strongly decreased CAT expression (compare pRGH527R1 and pRGH237R), while insertion upstream of the enhancer (pRGH527R2) had relatively little effect. As a control for position effects on the enhancer, we inserted a 75-bp rGH fragment with no silencer activity either upstream or downstream of the viral sequences. As expected, expression directed by these plasmids was not significantly different from that directed by pRGH237R. We conclude that the rGH silencer is fully activated in the hybrids.

We have examined nuclear extracts for proteins binding to the silencer fragment. Although no DNase I footprints were seen with crude extracts, results with partially purified extracts suggest that both the parental and the hybrid cell lines contain a protein or proteins that bind to two similar sequences near −300 and −500. The role of this protein in silencer activity is under investigation.

The results described above identify two distinct but complementary mechanisms for extinction of rGH expression: (i) repression of expression or activity of the cell type–specific promoter binding proteins GC1 and GC3 and (ii) activation of the silencer element. In both aspects the hybrids behave very much like the $LTK^-$ parent. This strongly suggests that the process of extinction reflects basic mechanisms that prevent expression of genes in inappropriate cell types.

It is difficult to judge the relative effects of the two mechanisms on expression of the endogenous GH genes in the hybrids. The effect of the silencer on expression of trans-

fected rGH promoters is strong in both the L cells (*8, 9*) and the hybrids. A similarly strong effect is exerted by GC1 on rGH promoter activity in vitro, although somewhat contradictory results have been obtained in transfection studies in which GC1 binding sites were deleted (*2, 4, 5*). It seems likely that both positive and negative mechanisms contribute to the extinction of expression of the rGH gene observed in hybrids. We have suggested (*8, 9*) that such a combination of positive and negative regulatory effects is required to generate the extremely strong (>$10^8$-fold) cell-type specificity of rGH expression (*13*).

Similar mechanisms may be involved in extinction of expression of other genes. This predicts, for example, that in lymphoid × fibroblast hybrids (*14*), the negative regulatory elements that flank the immunoglobulin heavy chain enhancer (*15*) should be activated. Similarly, expression of lymphoid-specific immunoglobulin gene promoter/enhancer binding factors (*16*) may be shut off in such hybrids.

Both positive and negative effects described here could arise from a single, simple mechanism. In particular, an L cell factor responsible for activating the negative effect of the rGH silencer could also act to shut off GC1 or GC3 expression, or both. Examples of such a factor may be provided by tentatively identified negative regulatory loci called tissue-specific extinguishers (*tse*'s). Expression of the appropriate *tse* is thought to be sufficient to shut off expression of tyrosine aminotransferase (*17*) or albumin (*18*) in liver × fibroblast hybrids. Similarly, a single *tse* could be sufficient for activation of the rGH silencer and repression of the rGH promoter binding proteins. Alternatively, if the two processes are mechanistically distinct, it might be possible to separate them genetically by approaches analogous to those used to identify the liver *tse*'s.

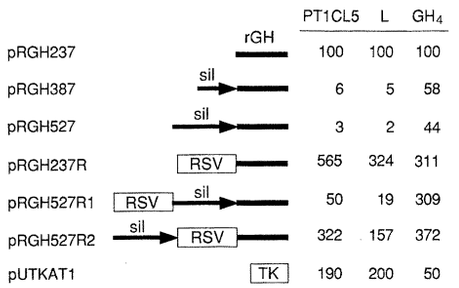


**Fig. 3.** Transient transfections of parental and hybrid cell lines. Relative CAT expression is presented as percent of the level of expression directed by pRGH237 (*8*). Results with pUTKAT1, which contains the herpes virus TK promoter fused to CAT (*25*), are included for comparison of promoter activity. The solid bar represents the 237-bp rGH promoter–containing fragment, the arrows represent additional 5′ flanking sequences (−237 to −387 in pRGH387, and −237 to −527 in other plasmids) that contain the silencer. The open boxes represent fragments containing the RSV enhancer or TK promoter, as indicated. $LTK^-$ (L) cells were transfected by use of DEAE dextran, and both PT1CL5 and $GH_4$ cells were transfected using calcium phosphate as described (*8*). Absolute levels of CAT enzymatic activity obtained with pRGH237 averaged 6.1, 9.7, and 2% of total chloramphenicol converted per 100 μg of protein per 4 hours, for $LTK^-$, pituitary cells, and hybrids, respectively. The effects of variations in transfection efficiency were eliminated by normalizing CAT expression levels to levels of hGH expression directed by the cotransfected control plasmid pXGH5 (*26*). Results represent averages of at least nine independent transfections, in which relative expression varied by less than 20%. Results similar to those obtained with PT1CL5 were also obtained with the independent hybrid line PT1CL6.


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# Binding of a Cytosolic Protein to the Iron-Responsive Element of Human Ferritin Messenger RNA


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**The human ferritin H chain messenger RNA contains a specific iron-responsive element (IRE) in its 5′ untranslated region, which mediates regulation by iron of ferritin translation. An RNA gel retardation assay was used to demonstrate the affinity of a specific cytosolic binding protein for the IRE. A single-base deletion in the IRE eliminated both the interaction of the cytoplasmic protein with the IRE and translational regulation. Thus, the regulatory potential of the IRE correlates with its capacity to specifically interact with proteins. Titration curves of binding activity after treatment of cells with an iron chelator suggest that the factor acts as a repressor of ferritin translation.**


FERRITIN IS AN INTRACELLULAR PROtein that sequesters iron. When cells are treated with iron, ferritin mRNA shifts from messenger ribonucleoproteins (mRNPs) to polysomes and becomes actively translated (*1, 2*). This regulation occurs in the absence of changes in ferritin mRNA levels (*3*). A 35-nucleotide region in the 5′ untranslated region (5′ UTR) of human ferritin H chain mRNA contains an element that is both necessary and sufficient for translational regulation by iron (*4, 5*). Placement of this IRE in the 5′ UTR of mRNAs encoding chloramphenicol acetyltransferase


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