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FUNCTIONAL GLUCOCORTICOID INDUCIBLE ENHANCER ACTIVITY IN THE 5′-FLANKING SEQUENCES OF THE RAT GROWTH HORMONE GENE

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Summary—Glucocorticoid regulation of rat growth hormone (rGH) gene expression has been investigated in a series of gene transfer studies into cells in culture. It has been established that sequences (−12 to −523) immediately flanking the start site for rGH gene transcription behave as a functional glucocorticoid inducible enhancer when associated with a heterologous promoter (RSV), displaying independence of orientation and position in mediating the glucocorticoid effect. The induction of chloramphenicol acetyl transferase (CAT) gene expression in these constructs by dexamethasone was established at the enzyme and mRNA levels and was inhibited in the presence of the antiglucocorticoid, RU 38486. The glucocorticoid inducible enhancer activity was not restricted to pituitary cells. The constructs containing the rGH-5′-flanking sequences, associated with the RSV promoter, also mediated glucocorticoid induction of CAT gene expression when transiently transfected into MH_1C_1 cells, a hepatoma cell line. The effect was similarly demonstrable on co-transfection of these constructs with a glucocorticoid receptor expression vector into receptor deficient COS cells.

Two elements within these rGH sequences (−97 to −111 and −250 to −264) display partial homology with a consensus sequence computed for a group of glucocorticoid regulatory elements. Mutation of both of these elements or of the more proximal element alone (−97/−111) led to a complete loss of ability to mediate glucocorticoid induction of gene expression. However, the rGH sequences still mediated glucocorticoid induction of gene expression when the distal GRE-like element was mutated or deleted. Thus, the proximal rGH GRE-like element is absolutely required to mediate this glucocorticoid inducible enhancer activity.

INTRODUCTION

It is long established that glucocorticoids stimulate rat growth hormone (rGH) production in pituitary cells, for example, in GH_1 and GH_3 rat pituitary tumour cells [1–3]. In parallel with this increase in rat GH production, glucocorticoids increase $mRNA_{GH}$ levels in GH_3 and related cell lines [4, 5]. This increase may arise in part from glucocorticoid-induced stabilization of $mRNA_{GH}$ [6] but there is evidence that it involves a significant induction of rat growth hormone gene transcription [7]. While the mechanism of this specific glucocorticoid induced transcriptional event remains undescribed other such events have been defined in some detail. From DNA transfection studies it has been established that murine mammary tumour virus (MMTV) contains sequences within the viral LTR that mediate inducibility by glucocorticoids in steroid responsive cells [8–10]. Furthermore, purified glucocorticoid receptor binds with specificity to these sequences within the LTR [11, 12]. Thus, sequences within the LTR act as a functional glucocorticoid inducible enhancer. A similar mechanism of control has been described for a number of cellular genes e.g. metallothionein IIa [13] and tyrosine aminotransferase [14]. In these genes, sequences in the 5′-DNA flanking the structural gene are sufficient to confer glucocorticoid inducibility. Indeed in these and other genes studied, purified glucocorticoid receptor binds with specificity to multiple short DNA sequences [glucocorticoid responsive elements (GREs)] which show significant homology between the various genes (reviewed in Ref. [15]). In fact, one such GRE was sufficient to confer glucocorticoid inducibility on an unresponsive heterologous promoter [16].

Against this background it was of interest to investigate the mechanism of glucocorticoid in-

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duction of rGH gene transcription. We report for the first time, on the basis of gene transfer studies, that sequences immediately flanking the start site for rGH transcription (−12 to −523) behave as a functional glucocorticoid inducible enhancer and would provide a means for transmitting this glucocorticoid transcriptional stimulus.

EXPERIMENTAL PROCEDURES

Cell culture

GH_3 cells [17] were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 12.5% horse serum (HS) and 2.5% foetal calf serum (FCS) or in the same medium which had been steroid-depleted by treatment with activated charcoal [18]. MH_1C_1 cells [19] were maintained in DMEM supplemented with 10% FCS and 7.5% HS. COS cells [20] and CV-1 cells [21] were maintained in DMEM supplemented with 10% FCS.

Plasmid construction

prGH (−523)CAT [22] was a gift from Tom Lufkin and Carter Bancroft, prGH (−235)CAT was obtained by ligating the prGH (−523)CAT *Bgl* II-*Bam H*1 fragment containing 235 bp of rGH 5′-flanking sequence linked to the CAT gene into the *Bam H*1 site of pGEM 2 (Promega Biotech). Enhancer sequences [23] were deleted from pRSVCAT [24] by *Nru* 1 cutting, fitting an *Sph* 1 synthetic linker, *Sph* 1 cutting, relegating and selecting plasmid in which the enhancer sequences had been deleted. This yielded pRSV(−)CAT. (Fig. 1B). rGH sequences (−523/−12) were then isolated, blunted, fitted with *Sph* 1 linkers and cloned into pRSV(−)CAT both in the sense (pR(−)G(+)CAT) and antisense (pR(−)G(+A)CAT) orientation (Fig. 1B). rGH sequences (−310 to −236) were deleted from pR(−)G(+)CAT to generate pR(−)G(+Δ)CAT (Fig. 1B). In addition, the rGH gene sequences (−523/−12) were provided with *Bam H*1 and *Bgl* II ends and were introduced into the unique *Bam H*1 site of pRSV(−)CAT, 3′—to the end of the CAT gene. This yielded pR(−)G(+3′)CAT and pR(−)G(+3′A)CAT), containing the rGH sequences in the sense and antisense orientation, respectively. For RNase protection analysis of RNA transcribed from these heterologous promoter containing constructs a plasmid pSR(−) was generated by ligating the 339 bp *EcoR*1 fragment from pRSV(−)CAT (−89 to +290) to the *EcoR*1 site by pGEM2 (Promega Biotech) in the antisense orientation relative to the SP6 promoter. *Sma*1 linearized pSR(−) and SP6 RNA polymerase were subsequently used to generate the appropriate 364 nt antisense RNA probe (see Fig. 2A).

Deletion mutations were introduced into the two potential GRE-like sequences in the rGH promoter. The sequence −111..AGCACAAGCTGTCAGT..−96 was mutated to −111..AGCACAAGCT----T..−96 and the sequence −250..CACCCAATGTGTCCTT..−265 was mutated to −250..CACCCAA-G----CTT..−265. In both cases the deletion-mutation generated a *Hin*dIII site. The deletions were generated by oligonucleotide directed mutation using a method based on the gapped duplex DNA approach and the pMa/c phasmid vectors constructed by Stanssens *et al.* [25]. The mutations were introduced into the rGH −523/−12 promoter fragment which was employed in the other studies reported here. Plasmids containing the mutation were selected by screening for the new *Hin*dIII restriction sites and were verified by DNA sequencing. The mutated rGH fragments bounded by *Sph*1 restriction sites were introduced into the *Sph*1 site in pRSV(−)CAT (see above) to generate pR(−)G(+*pm*)CAT, containing the mutation in the proximal GRE-like sequence (−111/−96), pR(−)G(+*dm*)CAT, containing the mutation in the distal GRE-like sequence (−250/−265) and pR(−)G(+*p*/*dm*)CAT, containing both mutations.

Transient transfection, chloramphenicol acetyl transferase assay and RNase protection analysis

GH_3 cells in monolayer culture (60 mm dishes, 2×10^6 cells) were transiently transfected with 10 μg plasmid) using the DEAE-dextran technique [26]. MH_1C_1, COS and CV1 cells, also in monolayer culture (60 mm dishes, 2×10^6 cells) were transiently transfected by the $CaPO_4$ precipitation technique of Parker and Stark [27]. Following transfection, the cultures were treated with the synthetic glucocorticoid, dexamethasone, at the indicated concentrations (see figure legends) for 40 h. Treatment with the antiglucocorticoid RU 38486 followed the same protocol. Cells were harvested and extracts tested for chloramphenicol acetyltransferase activity essentially as described by Gorman *et al.* [28] with the following modifications. Cells were ruptured by two 10 s bursts of sonication. Extracts were incubated at 65°C for 5 min to

inactivate any possible endogenous transferase inhibitor and finally, incubation with 0.2 Ci [^{14}C]chloramphenicol (sp. act. 53 Ci/mmol) and acetyl co-enzyme A was at 37°C for 2 h.

For RNase protection analysis [29] total RNA was isolated from transiently transfected GH_3 cells (100 mm dishes) 40 h after transfection by the method of White *et al.* [30]. The 364 nt antisense RNA probe from pSR(−) (*Sma* I cut) was prepared as described by Melton *et al.* [29] and Zinn *et al.* [31]. Hybridization was carried out at 45°C, overnight, followed by treatment with RNase (A and T_1) at 32°C for 2 h. Carrier yeast tRNA (20 μg/tube) was always added in 200 μl H_2O. Products were analysed on urea saturated 5% acrylamide sequencing gels. Autoradiography was carried out at −70°C with intensifying screens for 1–5 days.

RESULTS

In Fig. 1A we show the structure of the proximal promoter of the rat GH gene and highlight the areas over which the somatotroph specific transcription factor binds [32], the position of the proposed thyroid hormone responsive element [33] and finally the coordinates of the two sequences which resemble glucocorticoid responsive elements and are the subject of this study. In Fig. 1B we present the structures of the rGH promoter/reporter gene constructs which were tested, by transfection, for glucocorticoid inducibility. We carried out a preliminary series of analyses on the glucocorticoid inducibility of the expression of prGH(−523)CAT (which conains 523 bps of rGH gene 5′-flanking sequence) when transiently transfected into GH_3 cells. A dose–response relationship (not shown) was observed between dexamethasone concentration and prGH-CAT expression, as reflected by CAT enzyme activity. Maximum CAT activity was observed in the presence of dexamethasone (10^{-6} M) and this represented an approx. 6-fold increase in expression over untreated cells (see Fig. 1C). Deletion of rGH promoter sequences in prGH(−523)CAT to −235 gave prGH (−235)CAT (Fig. 1B) whose expression was again dexamethasone inducible (Fig. 1C). These preliminary findings prompted us to analyse the 5′-flanking sequences of the rGH gene for the presence of functional glucocorticoid inducible enhancer activity.

Enhancer sequences [23] were deleted from pRSVCAT [24] to make pRSV(−)CAT (Fig. 1B). This gave an approx. 10-fold reduction in CAT activity on transient transfection into GH_3 cells (Fig. 1C). rGH Promoter sequences (−12 to −523) were then introduced into the unique *Sph* I site in pRSV(−)CAT, both in the sense (pR(−)G(+)(CAT) and antisense (pR(−)G(+A)CAT) orientations (Fig. 1B). These latter constructs exhibited basal CAT activity on transient transfection of less than 50% that of pRSV(−)CAT (Fig. 1C). While the expression from pRSVCAT and pRSV(−)CAT were not increased by dexamethasone treatment, that from both pR(−)G(+)CAT and pR(−)G(+A)CAT were (Fig. 1C). The increase in expression in both cases was approx. 10-fold. This, interestingly, would equate the promoter strengths of the RSV-LTR enhancer and the putative glucocorticoid regulated rGH enhancer under these conditions of study.

Deletion of rGH sequences from −310 (*Kpn* I site) to −236 (*Bgl* II site) in pR(−)G(+)CAT to generate pR(−)G(+Δ)CAT led to an approximate doubling in basal expression with dexamethasone inducibility being retained but at a lower level (4.9-fold) (Fig. 1B and C). The rGH 5′-flanking sequence fragment (−523/−12) was also introduced into the unique *Bam H*1 site lying 3′- to the CAT reporter gene in pRSV(−)CAT, again, in both the sense (pR(−)G(+3′)CAT) and antisense (pR(−)G(+3′A)CAT) orientation. This placed the putative rGH dexamethasone inducible enhancer a further 3.0 kb upstream from the *Sph* I site in pRSV(−)CAT (Fig. 1B). The basal CAT activity observed on transfection of these latter constructs into GH_3 cells was similar to that seen with pR(−)G(+)CAT and the level of inducibility with dexamethasone, was again, approx. 10-fold (Fig. 1C).

These studies were carried out with GH_3 cells maintained in medium containing serum. However, a similar pattern of dexamethasone inducibility was observed in duplicated experiments with pR(−)G(+)CAT (12.3-fold), pR(−)G(+A)CAT (19.1-fold), pR(−)G(+Δ)CAT (4.4-fold) and prGH(−523)CAT (26.2-fold) transiently transfected into GH_3 cells maintained in medium containing serum depleted of steroids by treatment with activated charcoal [18]. It was further demonstrated that the specific antiglucocorticoid RU 38 486 [34] could inhibit the dexamethasone induction of CAT activity observed on transfection of these enhancer deleted RSV constructs containing the rGH gene 5′-flanking sequence fragments (results not shown). Time-course studies of

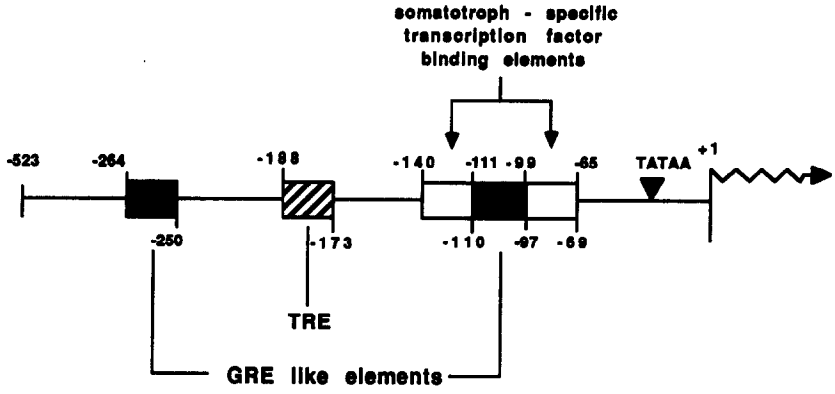
somatotroph - specific
transcription factor
binding elements
-523
-264
-188
-140
-111
-99
-65
TATAA
+1
-250
-173
-110
-97
-69
TRE
GRE like elements

Fig. 1(A)

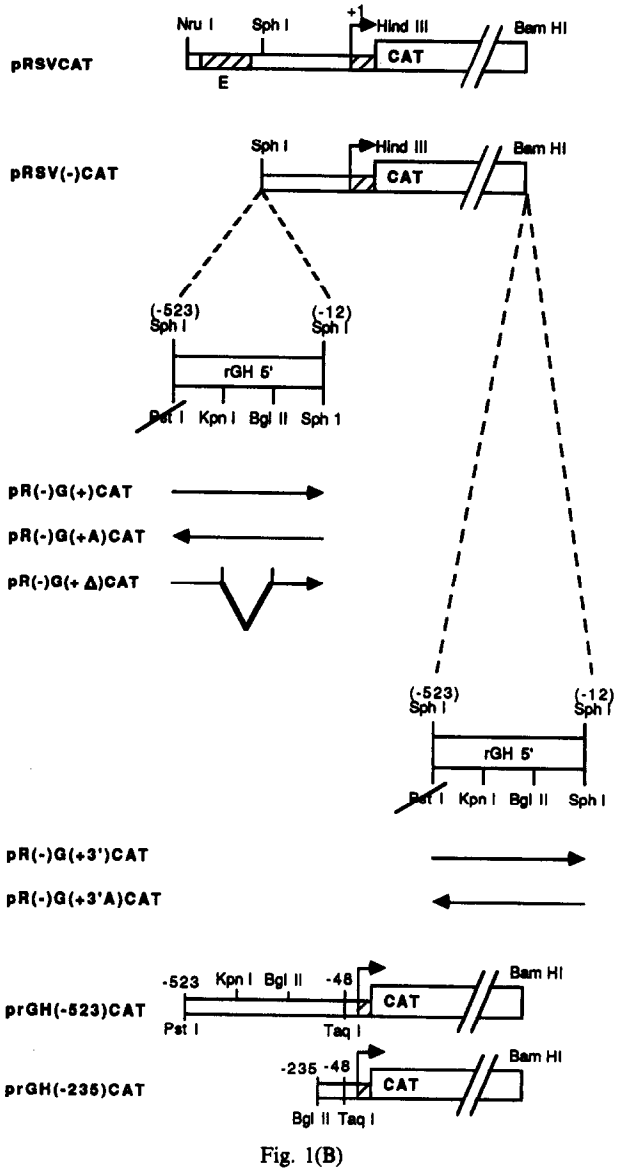
pRSVCAT
Nru I
Sph I
+1
Hind III
Bam HI
CAT
E
pRSV(-)CAT
(-523)
Sph I
(-12)
Sph I
rGH 5'
Pst I
Kpn I
Bgl II
Sph 1
pR(-)G(+)CAT
pR(-)G(+A)CAT
pR(-)G(+ Δ)CAT
pR(-)G(+3')CAT
pR(-)G(+3'A)CAT
prGH(-523)CAT
-523
Kpn I
Bgl II
-48
Pst I
Taq I
prGH(-235)CAT
-235
Bgl II
Taq I

Fig. 1(B)

Dex (1 μ M)	Mean Relative CAT Activity (%) –	+	Fold Increase
pRSVCAT	100	100	0
pRSV(-)CAT	12.8	12.3	0
pR(-)G(+)CAT	5.1	57.3	10.2
pR(-)G(+A)CAT	5.1	51.7	9.1
pR(-)G(+ Δ)CAT	9.1	53.6	4.8
pR(-)G(+3')CAT	4.6	48.9	9.6
pR(-)G(+3'A)CAT	4.4	49.5	10.2
prGH(-523)CAT	14.3	93.5	5.5
prGH(-235)CAT	9.8	39.1	2.9

Fig. 1(C)

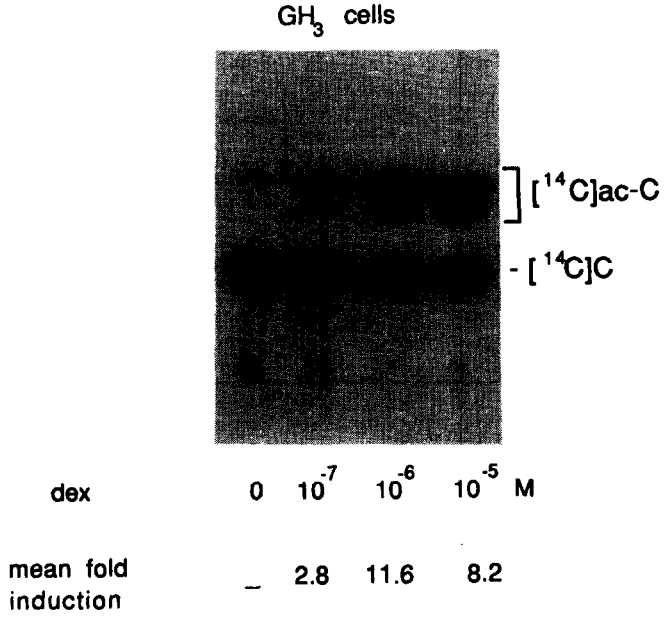

Fig. 1(D)

Fig. 1. Proximal rGH gene promoter sequences mediate glucocorticoid induced gene expression on transient transfection into GH_3 cells. (A) Structure of the proximal promoter of the rat GH gene highlighting the areas over which the somatotroph specific transcription factor binds [32], the position of the proposed thyroid hormone responsive element [33] and finally the co-ordinates of the two sequences which resemble glucocorticoid responsive elements that are the subject of this study. (B) Shown is the pRSV 'enhancer minus' −CAT construct, the pRSV(−)CAT constructs containing rGH promoter sequences and the prGH-CAT constructs which were transiently transfected into GH_3 cells. (C) Shown are the CAT activities (basal and dexamethasone-induced) obtained with the constructs illustrated in (B) above, on transfection into GH_3 cells. Values are expressed relative to that of pRSV CAT. Mean fold dexamethasone induced increases are also shown ($n \geq 5$). (D) Autoradiograph of TLC separation of [^{14}C]chloramphenicol and its acetylated metabolites showing the dose-dependent effect of dexamethasone on the expression of pR(−)G(+)-CAT (see Fig. 1B) transiently transfected into GH_3 cells. [^{14}C]C indicates the position of the [^{14}C]chloramphenicol substrate and [^{14}C]ac-C, of its acetylated products on the TLC plate. Calculated fold inductions over basal are also presented.

[^{14}C]chloramphenicol acetylation by extracts of pR(−)G(+)CAT transfected cells maintained in the presence and absence of dexamethasone showed an elevated linear rate of acetylation on dexamethasone treatment, which was maintained for up to 4 h of incubation. Similar observations were made with pRSV-CAT and pRSV(−)-CAT. (These results are not shown.)

That the glucocorticoid induction of CAT gene expression in these rGH/RSV promoter constructs is dose-dependent is illustrated in Fig. 1D where approx. 3-fold and 12-fold increases in CAT activity were detected on treating GH_3 cells, transiently transfected with pR(−)G(+)CAT with 10^{-7} and 10^{-6} M dexamethasone, respectively.

Dexamethasone inducibility, after transfection into GH_3 cells of pR(−)G(+)CAT, pR(−)G(+A)CAT, pR(−)G(+)CAT, pR(−)G(+3′)CAT and pR(−)G(+3′A)CAT was also demonstrated to be evident at the mRNA level when examined by RNAse protection analysis (Fig. 2A–C). This analysis revealed that transcription from the transfected RSV-promoter and the heterologous rGH/RSV enhancer/promoter constructs was initiating at a single and correct site (as predicted by the protection of 290 nucleotides of the antisense CAT mRNA probe). From Fig. 2B it can also be seen that mean dexamethasone-induced increases in CAT mRNA levels, obtained with the rGH promoter containing constructs, paralleled

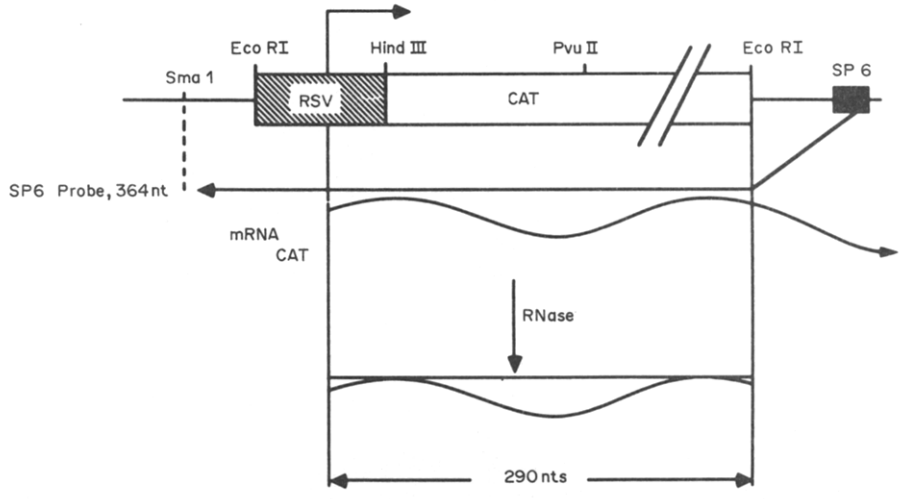

Fig. 2(A)

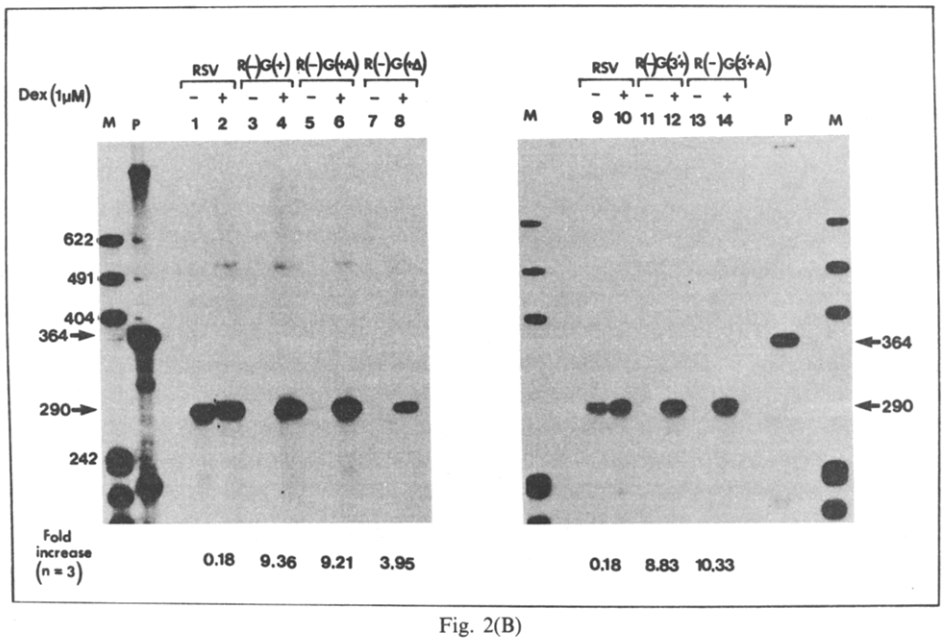

Fig. 2(B)

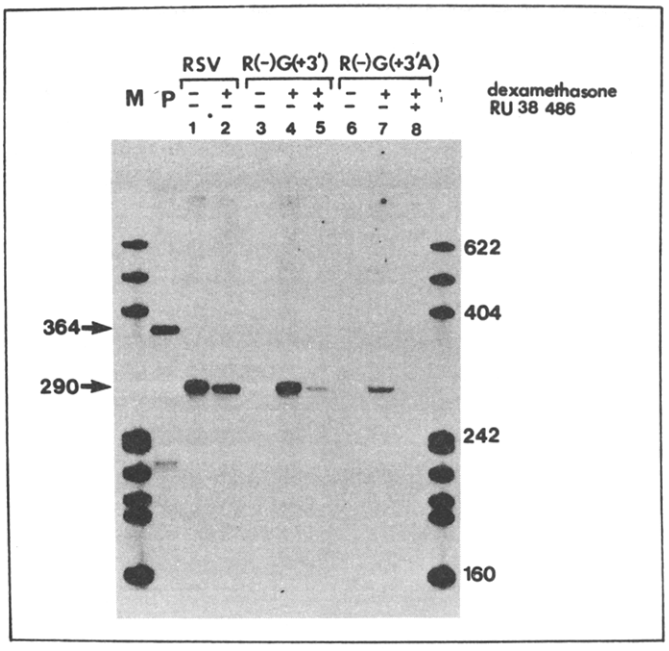

Fig. 2(C)

Fig. 2. Proximal rGH gene promoter sequences mediate glucocorticoid induced gene expression on transient transfection into GH_3 cells. RNase protection analysis. (A) Strategy for the RNase protection analysis. (B) Autoradiograph showing the effect of dexamethasone, on production of the correctly initiated 290 nt protected band from total RNA isolated from GH_3 cells transfected with the series of RSV-'enhancer minus'-CAT plasmid constructs containing rGH promoter sequences (illustrated in Fig. 1B). Band intensities were determined by densitometric scanning. Mean dexamethasone induced fold increase values, from 3 experiments, for each transfected plasmid construct are indicated. (C) Autoradiograph showing the effect of the anti-glucocorticoid RU-38486 on dexamethasone induced increases in the 290 nt protected band for two of the above constructs, pR(−)G(+3′)CAT and pR(−)G(+3′A)-CAT (see Fig. 1B).

the increases in CAT enzyme activity, presented in Fig. 1C. The rGH/RSV promoter constructs containing rGH sequences −12/−523 showed an approximate 10-fold increase in mRNA levels, while the construct deleted of sequences −236/−310 (pR(−)G(+Δ)CAT) showed a mean 4-fold increase in mRNA levels. From Fig. 2C it can be seen that the specific antiglucocorticoid RU38486 inhibited the ability of dexamethasone to induce increases in CAT mRNA levels in GH_3 cells transfected with pR(−)G(3′+)CAT and pR(−)G(3′+A)CAT. This suggests strongly that the dexamethasone induction is being mediated by the glucocorticoid receptor.

The rGH gene promoter is tissue specific in its activity as judged by transient transfection studies. Transiently transfected prGH(−523)CAT is essentially inactive in C_6 (glioma), C127 (fibroblast), MH_1C_1 (hepatoma) and HeLa cells but active in somatotrophs, GH_3 and GC cells [35]. It was of interest, therefore, to establish if the functional glucocorticoid inducible enhancer defined in this study was also cell-type restricted. Its activity was therefore examined by transiently transfecting the RSV enhancer deleted/5′-rGH heterologous promoter constructs and prGH(−523)CAT into MH_1C_1 (hepatoma) cells. From Fig. 3A it can be seen that pR(−)(+)CAT, pR(−)G(+A)CAT and pR(−)G(+Δ)CAT were indeed dexamethasone inducible in respect of expressed CAT enzyme activity on transfection into MH_1C_1 cells. prGH(−523)CAT was essentially inactive while pMTCAT (containing mouse metallothionein promoter sequences (Ip, Lufkin and Bancroft, unpublished, used as a positive control) was, as expected [13], dexamethasone inducible.

The dependence of the dexamethasone inducibility, mediated by the rGH promoter, on the glucocorticoid receptor was then tested in a transient co-transfection study, where pR(−)G(+)CAT and a eukaryotic expression vector containing the rat glucocorticoid receptor cDNA (pRSV-GR [20], Fig. 3B) were introduced into, receptor-deficient COS cells [20]. In Fig. 3C (left hand panel) it can be seen that, in the absence of co-transfected pRSV-GR, dexamethasone failed to induce CAT formation from pR(−)G(+)CAT. However, on co-trans-

fection of pRSV-GR with pR(−)G(+)CAT, dexamethasone could induce CAT gene expression via the rGH promoter sequences. Thus, the mediation of glucocorticoid induced CAT gene expression in COS cells by rGH promoter sequences is dependent on the presence of glucocorticoid receptor. From other experiments it is clear that it is the rGH sequences and not the RSV-promoter that is the target for this GR mediated activation: (1) It is clear from Fig. 1C that the RSV- (enhancer deleted) CAT construct is glucocorticoid non-responsive; (2) As will be indicated below (Fig. 4C) mutation of the GRE-like elements in the rGH sequences of pR(−)G(+) CAT generates a dexamethasone non-responsive construct and (3) Camper *et al.* [36], for example, have also reported the RSV long terminal repeat to be glucocorticoid non-responsive. In Fig. 3C (right hand panel) the dependence of dexamethasone-induced CAT gene expression in COS cells from the established glucocorticoid-dependent enhancer/promoter system, the MMTV LTR [37], on co-transfection of pRSV-GR, is shown for comparative purposes.

The rGH promoter sequences −12/−523 which are demonstrated here to act as a functional glucocorticoid inducible enhancer contain two elements −111/−97 and −250/264 which show significant homology with a consensus glucocorticoid responsive element (GRE) derived by comparison of GREs in more than 20 glucocorticoid responsive promoters [15] (Fig. 4A). It was of interest to establish if these GRE-like elements were necessary for the mediation of glucocortioid inducibility of gene expression by the rGH promoter. Small deletion-mutations were therefore introduced into these two elements (Fig. 4A) and a set of RSV enhancer minus −CAT constructs identical to

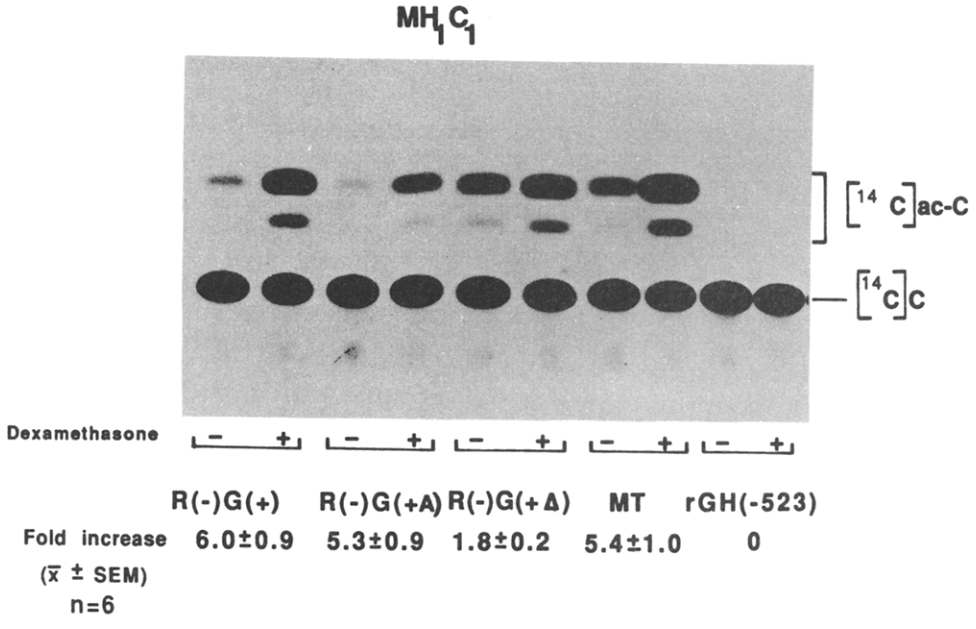

Fig. 3(A)

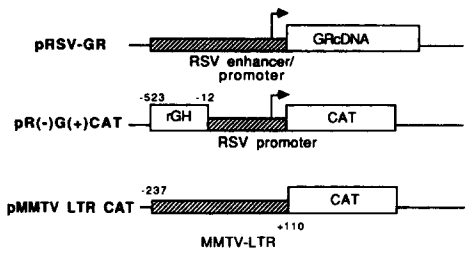

Fig. 3(B)

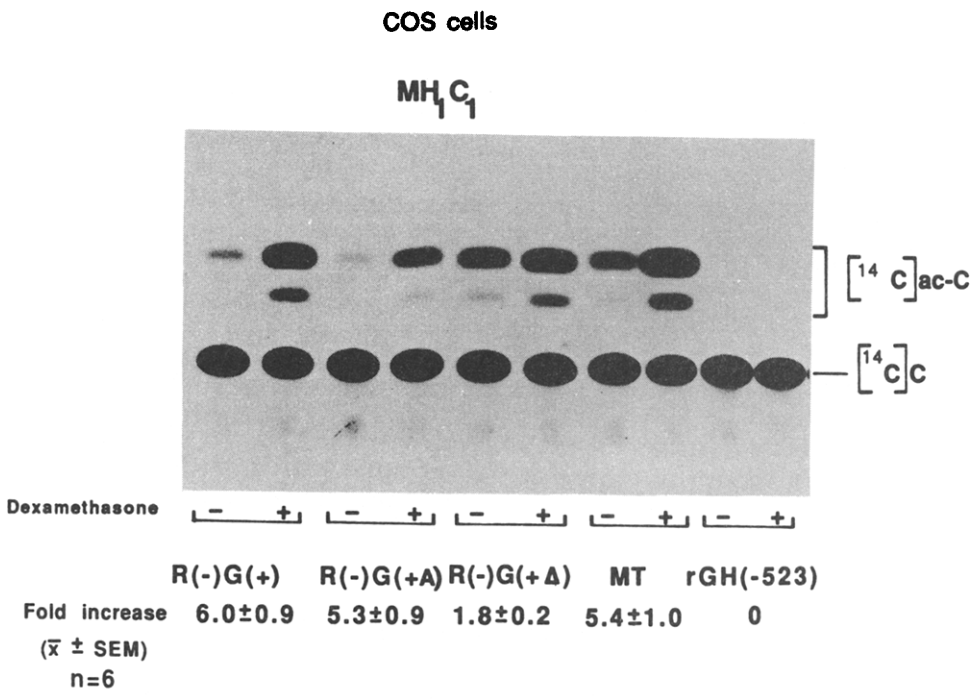

Fig. 3. Proximal rGH promoter sequences mediate glucocorticoid induced gene expression in non-pituitary cells. (A) Autoradiograph showing the effect of dexamethasone on the expression of pRSV-'enhancer-minus'-CAT constructs containing rGH promoter sequences transiently transfected into mH_1C_1 cells, a rat hepatoma cell line [19]. The promoter-CAT constructs used are depicted in Fig. 1. MT is pMt_{IIA}-CAT which contains promoter sequences of the glucocorticoid inducible mouse metallothionein IIA gene. rGH(−523) is prGH(−523)CAT (See Fig. 1). (B) Glucocorticoid receptor expression vector and reporter plasmids used in co-transfection studies. (C) Autoradiograph showing the effect of dexamethasone on the expression of pR(−)G(+)CAT transiently co-transfected with the glucocorticoid receptor expression vector, pRSV-GR [20], into the glucocorticoid receptor deficient COS-1 cell line. As a positive control, co-transfection of the glucocorticoid dependent pMMTV LTR CAT construct [37] with pRSV-GR was also investigated.

pR(−)G(+)CAT but containing the −111/−97 mutation (pR(−)G(+*pm*)CAT), the −250/−264 mutation (pR(−)G(+*dm*)CAT) or both mutations (pR(−)G(*p*/*dm*)CAT), generated. From Fig. 4B (left hand panel) it can be seen that mutation of the −250/−264 GRE-like element led to a reduction in glucocorticoid inducibility, but not a loss of inducibility, as judged by transient transfection of the pR(−)G(+*dm*) construct into GH_3 cells. In contrast, mutation of the more proximal element (−111/−97) led to a complete loss of dexamethasone inducibility under the same experimental conditions. The mutants were more extensively tested in receptor deficient CV-1 cells [21] in transient co-transfection studies with the glucocorticoid receptor expression vector, pRSV GR. The dependence of the cells on transfected pRSV GR to mediate dexamethasone induction of expression of the pMMTV LTR CAT construct was first established (Fig. 4C, left hand panel). In CV-1 cells the dexamethasone inducibility of the pR(−)G(+)CAT construct, on co-transfection with pRSV GR, was relatively low (mean induction 2.23, $P < 0.05$) but was reproducible and significant (Fig. 4C, right hand panel). The construct bearing both mutations lost its inducibility. The construct bearing the proximal mutation again lost its dexamethasone inducibility (Fig. 4C), as already noted in the GH_3 cell experiment. Furthermore, the construct bearing the distal mutation (−250/−264) retained its dexamethasone inducibility, as also seen in the GH_3 cells, but under these conditions, at the same level as seen with the wild type construct (Fig. 4C). That some sequence element(s) 3′ of −225 in the rGH promoter was capable of mediating glucocorticoid induction was already suggested by experimental data presented in Figs 1C, 2B and 3A. From Fig. 1C it can be seen that prGH(−235)CAT, which only contains rGH promoter sequences 3′ of −235 is still inducible by dexamethasone on transient transfection into GH_3 cells. From Figs 1C, 2B and 3A it can be seen that

pR(−)G(+Δ)CAT from which the rGH promoter sequences −310 to −235 have been deleted (and, thus, lack the GRE-like element at −250/−264) is also inducible by dexamethasone in GH_3 cells and MH_1C_1 cells. The data suggest that the GRE-like sequence −111/−97 must be intact for the rGH promoter sequences −12/−225 or −12/−523 to mediate glucocorticoid inducibility. However, while the more distal GRE-like element (−250/−264) alone is unable to mediate dexamethasone inducibility it may co-operate with the more proximal element to provide the higher levels of glucocorticoid inducibility seen, for example in GH_3 cells, when the more distal sequences were present.

DISCUSSION

Ligation of rGH gene 5′-flanking sequences to a heterologous promoter (enhancer deleted RSV-LTR) resulted in the transfer to pRSV(−)CAT of glucocorticoid inducibility, as reflected by increases in CAT enzyme activity and CAT mRNA levels in transient transfection studies on GH_3 cells (Figs 1 and 2). This glucocorticoid inducibility was dose-dependent and

rGH proximal (-111to -97)	AGCACAAGCTGTCAG
rGH distal (-250 to -264) anti-sense	CACCCAATGTGTCCT
hGH 1	GGCACAATGTGTCCT
CONSENSUS	GGTACANNNTGT$^{C}_{T}$CT
rGH proximal mutation	AGCACAAGCT-----T (-102) (-96)
rGH distal mutation	CACCCAA-G----CTT (-256) (-265)

Fig. 4(A)

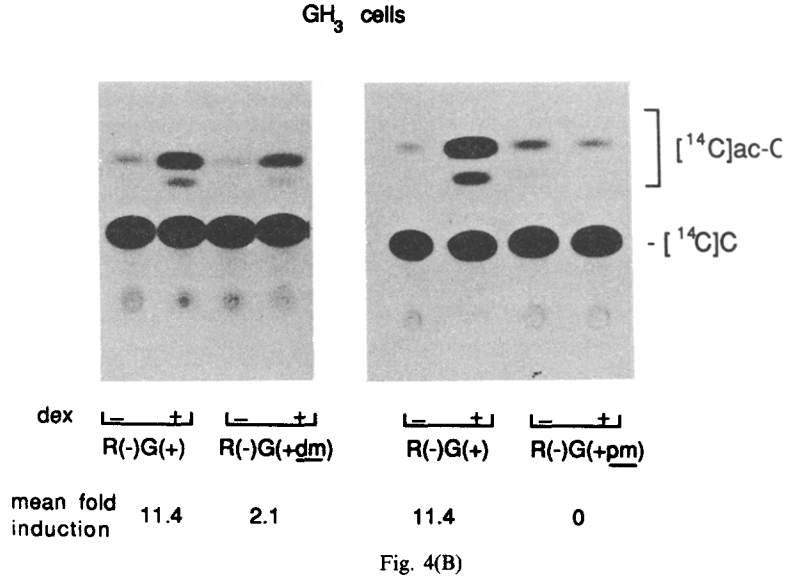

Fig. 4(B)

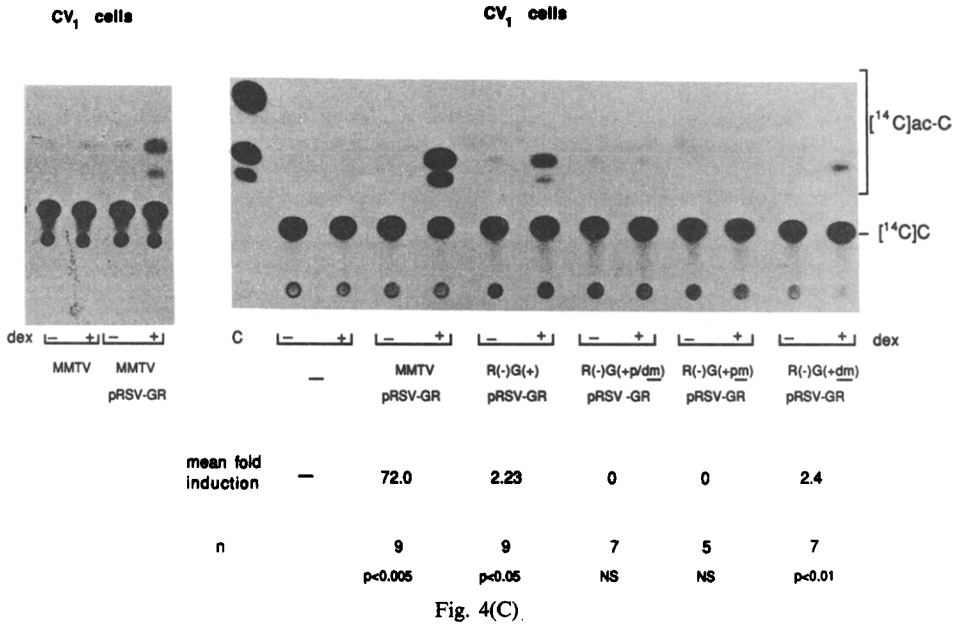

Fig. 4(C).

Fig. 4. Mutation of a glucocorticoid responsive element (GRE)-like sequence of the rGH promoter eliminates its ability to mediate induction by glucocorticoids. (A) GRE-like elements in the rGH promoter sequences (−523/−12). A GRE characterized in the first intron of the human GH gene [42] and the consensus sequence derived from GREs in 20 genes [15] is also shown. Mutations introduced into the GRE-like sequences of the rGH promoter are depicted. Plasmids bearing the mutation in the proximal GRE-like sequence (−96/−111) are termed *pm*, in the distal GRE-like sequence (−250/−264), *dm* and bearing both mutations, *p/dm*. (B) Autoradiograph showing the effect of dexamethasone on the expression of pRSV-enhancer minus-CAT constructs containing the mutated rGH promoter sequences (pR(−)G(+*dm*)-CAT and pR(−)G(+*pm*)-CAT, respectively) in transiently transfected GH_3 cells. (C) Autoradiograph showing the effect of dexamethasone on the expression of pR(−)G(+*p/dm*)-CAT, pR(−)G(+*pm*)-CAT and pR(−)G(+*dm*)-CAT co-transfected with the glucocorticoid receptor expression vector (pRSV-GR) into receptor deficient CV-1 cells. Mean fold glucocorticoid inductions, the number of independent observations and significance of differences (based on unpaired *t*-tests) are presented.

was independent of the orientation and position of the rGH 5′-flanking sequences indicating that they function as a glucocorticoid inducible enhancer. The expression of this dexamethasone inducibility was not confined to pituitary cells (Fig. 3A) suggesting a functional independence of the enhancer from the *cis*-acting elements residing in these sequences which mediate the tissue specific expression of the rGH gene [38–41]. Dexamethasone inducibility was further shown to be inhibited by the specific glucocorticoid antagonist RU 38486 which interferes with the binding of dexamethasone to the glucocorticoid receptor (e.g. Fig. 2C and results not shown). Furthermore, in receptor deficient COS cells, the glucocorticoid inducibility mediated by the rGH sequences was dependent on co-transfection of the glucocorticoid receptor expression vector pRSV GR. It is likely, therefore, that the effect is mediated by an interaction of the ligand occupied glucocorticoid receptor with a sequence element(s) within the rGH promoter fragment. Other groups [42, 43] have identified glucocorticoid inducible enhancing activity in GH gene constructs which lack 5′-flanking sequences. In the case of the human GH gene Slater *et al.* [42] characterized a functional glucocorticoid response element in the first intron of the gene. In other, limited, studies some groups [44, 45] have failed to detect glucocorticoid induction of reporter gene expression by the rat GH 5′-flanking sequences in rat pituitary tumour cell lines. It is not clear, at present, what the critical difference is between their experimental approach and our own. However, we clearly demonstrate glucocorticoid induction mediated by these sequences in pituitary cells (Figs 1 and 2) and in non-pituitary cells (Figs 3–5) when the sequences are associated with a permissive basal promoter.

MMTV [8–10], human metallothionein IIA [13], Moloney murine sarcoma virus [46] and tyrosine aminotransferase gene sequences [14] have previously been shown to function as glucocorticoid inducible enhancers. Receptor-DNA binding studies in each case

indicated the presence of more than one glucocorticoid receptor binding DNA sequence element although in some cases one of the elements identified was sufficient to confer glucocorticoid inducibility on a heterologous promoter [16]. Indeed, Klock *et al.* [47] have shown that a rotationally symmetrical 15-mer whose sequence was based on the tyrosine aminotransferase GRE sequence mediated a high degree of glucocorticoid inducibility when associated with a heterologous promoter. Inspection of a series of GREs identified in more than twenty known glucocorticoid inducible promoters led Beato [15] to propose a GRE consensus sequence: ...GGTACANNNTGTYCT... Inspection of the rat GH 5′-flanking sequences (−12/−525) for related elements showed that sequences from −111 to −97 and from −250 to −264 bear significant homology with the GRE consensus sequence. Partial deletion mutation of both these sequence elements led to a loss of glucocorticoid inducibility (Fig. 4C). Mutation of the more proximal element only, also led to loss of inducibility, both in transfected GH_3 cells and in CV-1 cells co-transfected with the glucocorticoid receptor expression vector pRSV GR (Fig. 4B and C). Thus, the proximal GRE-like element seems absolutely necessary for the mediation of glucocorticoid inducibility of gene expression by the rGH promoter sequences. That this element may be sufficient to allow the proximal rGH promoter sequences (−235 to +1) to mediate glucocorticoid induction of gene expression, is suggested by a number of the experiments reported here: partial deletion, by mutation, of the distal GRE-like element reduced but did not abolish the glucocorticoid inducibility in GH_3 cells (Fig. 4B) and did not abolish the effect on co-transfection with pRSV GR in CV-1 cells. Where sequences including the distal GRE-like element were deleted [i.e. in prGH(−235)CAT (Fig. 1C)] and pR(−)G(+Δ)CAT (Figs 1C, 2B and 3A) glucocorticoid inducibility was still detected, but at a level lower than encountered with the corresponding construct containing rGH promoter sequences out to −535. Thus, the proximal GRE-like element most likely confers on the −235 to +1 rGH promoter sequences the ability to mediate induction by glucocorticoids but with an efficiency less than that detected when both GRE-like elements are present. Thus, the more distal element which is insufficient to mediate induction in the absence of the proximal element (Fig. 4B and C), may co-operate with the latter to produce greater levels of induction. Similar observations have been made with glucocorticoid induction of the tyrosine aminotransferase gene [14]. This gene contains two GREs located 2.5 kb upsteam of its transcription initiation site. Of the two, the proximal element has no inherent capacity by itself to stimulate transcription. However, when present in conjunction with the distal GRE, this element synergistically enhances glucocorticoid induction of gene expression.

As outlined above the glucocorticoid hormone-regulated genes are characterized by the presence in their regulatory regions of short *cis*-acting sequences glucocorticoid response elements, GREs, which act as hormone inducible enhancers [13, 14, 42, 48, 49]. Target cells for the particular steroid hormone express specific receptors which bind the steroid with high affinity and subsequently activate transcription of regulated genes. Molecular biological analysis of the cloned glucocorticoid receptor has revealed the protein to consist of multiple separate and independent functional domains. The so-called E region specifically binds ligand [50–52] while region C specifically associates with particular GREs [53, 54]. Present knowledge suggests that ligand binding to region E can result in transcriptional activation of genes which is mediated through receptor binding (via region C) to the appropriate GRE [55, 56]. However, it is not yet clear how receptor binding to the GRE mediates transcriptional induction.

Several experimental observations with a variety of gene constructs suggest that multiple GREs, and possibly their co-operative glucocorticoid receptor binding, play a physiological role in the regulation of gene transcription. Multiple GREs (two or more) occur in the regulatory DNA sequences of a number of glucocorticoid inducible genes e.g. MMTV-LTR [57], rat tryptophan oxygenase [58] and the tyrosine aminotransferase genes [14]. There is, in addition, evidence to suggest that co-operativity between the multiple GREs in these promoters contributes to the extent of glucocorticoid-induced transcriptional activation. Both the distal and the proximal GR binding regions in the MMTV-LTR and the NF-1 binding site are all required to achieve maximal glucocorticoid induction of the MMTV-LTR [57]. Point mutations in or insertional mutations between GREs in the MMTV-LTR affected glucocorticoid receptor binding to the individual sites much less than they did glucocorticoid induc-

tion of transcription [57, 59]. This suggests that induction of transcription by glucocorticoids may require a defined steric interaction between GR molecules and adjacent GREs. Interestingly, a recent report from Schmid *et al.* [60] demonstrates that the glucocorticoid receptor binds co-operatively to adjacent glucocorticoid responsive elements.

Strahle *et al.* [61] have shown that in synthetic enhancer/promoter systems where glucocorticoid inducibility depends on the presence of two adjacent GREs, one of the GREs may be replaced by a general transcription factor binding motif (e.g. CACCC, NF1 or SP1 binding sites). Thus, in the rGH (−235) and R(−)G(+Δ) constructs, which contain only a single GRE-like element, glucocorticoid induction may be facilitated by the interaction of receptor molecules bound to the GRE-like element with tissue-specific or general transcription factors bound to other sequences in the GH or RSV promoters. In GH_3 and GC cells, the only cell type where the prGH(−235)CAT construct is functional, the somatotroph-specific transcription factor [32] binds to elements close to and either side of the GRE. These elements have been mapped to −68/−86 and −111/−129 [32]. Thus, interaction would be predicted between the bound receptor and the adjacent bound tissue-specific transcription factor molecules to generate the induced, activated transcription complex. In the RSV promoter containing constructs which are active in a range of cell types such interactions would be predicted with factors which bind to the RSV promoter sequences.

Evans *et al.* [7], employing a nuclear run-on transcription assay, confirmed the ability of glucocorticoids to up-regulate rGH gene transcription in a GH_3 related cell line, GC-cells. Diamond and Goodman [6] made similar observations but their results also indicated that dexamethasone also increases the stability of $mRNA_{GH}$ in GH_3 cells. The results of the present study suggest that the 5′-sequences (−12 to −523) immediately flanking the rGH gene, which show glucocorticoid inducible enhancer activity, most probably constitute the means by which glucocorticoids mediate induction of endogenous rGH gene transcription, *in vivo*. However, the levels of induction mediated by the rGH promoter sequences tested were low (e.g. 10-fold in GH_3 cells) relative to that reported for other glucocorticoid inducible promoters, in other systems (e.g. [36]). Thus, other GREs may co-operatively contribute to mediating the induction. In contrast to the rGH gene, the human GH gene contains a functional GRE in its first intron [42] but this is not conserved in the rat gene. However, Birnbaum and Baxter [43] have reported that sequences lying 3′ of the transcription start site can mediate glucocorticoid induction of rGH gene transcription: thus, downstream GREs may co-operate with those described in this study to mediate glucocorticoid induction of rGH gene expression. It is possible that additional functional GREs, as yet, unidentified but 5′ of -523 contribute to mediating glucocorticoid induction of rGH gene transcription, *in vivo*. A further possibility is that a combination of a weak glucocorticoid inducible enhancer and a post-translational increase in $mRNA_{GH}$ stability combine to provide satisfactory increments in $mRNA_{GH}$, *in vivo*.

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