Synergistic induction of promoters containing metal- and glucocorticoid-responsive elements

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ABSTRACT

We have shown that it is possible to synergistically activate gene transcription when several glucocorticoid responsive elements (GREs) and metal responsive elements (MREs) that coexist within the same promoter are induced simultaneously. To demonstrate this, additional GREs were introduced into a human metallothionein IIA (hMT-IIA) promoter in which some constitutive elements had been deleted. The transcriptional strength and inducibility of the modified hMT-IIA promoters were studied in transient expression experiments using the CAT gene as a reporter. As a result of synergistic activation of transcription by CdCl₂ and dexamethasone, the induced expression levels of the modified promoters were significantly higher than those obtained with wild-type hMT-IIA. Since the increase in inducible expression was not accompanied by a concomitant increase in basal levels, the inducibility of the modified MT promoters was up to 6-fold higher. The degree of transcriptional synergism was shown to depend on the position and the number of GREs introduced. Thus, the engineering of synthetic promoters that include both GREs and MREs should offer the opportunity to develop a new series of improved inducible mammalian expression vectors.

INTRODUCTION

Inducible promoters are particularly useful for the expression of proteins that are cytotoxic when produced above certain levels. They are also essential for comparative studies on the biological effect of different proteins since they allow investigation of the phenotype of a given cell clone before and after induction of those proteins (1). The 5' regulatory regions of the metallothionein (MT) gene family have been extensively used as inducible promoters (2). The best inducers for these promoters are heavy metal ions, particularly Cd⁺⁺ and Zn⁺⁺. Their induction is mediated by metal-activated factors (MAFs) which recognize metal responsive elements (MREs) located within the MT promoters (Figure 1). These promoters also contain elements which are recognized by

transcription factors that are constitutive and generate a significant basal level of gene expression (3,4). As a consequence, the uninduced level of expression of vectors that use MT promoters are generally high and the induction ratio poor, usually no greater than 5- to 10-fold (1).

The MT promoters can also be induced by other agents such as glucocorticoids (5), progesterone (6), interleukin-6 (7) and interferon (8). Induction of gene expression by glucocorticoids has been extensively studied (9–13). The hormone binds to the glucocorticoid receptor (GR) which becomes activated and stimulates transcription by direct interaction with glucocorticoid responsive elements (GREs) located in gene regulatory regions (9,11). Promoters that are induced by glucocorticoids usually contain more than one GRE. It has been shown that glucocorticoid induction of promoters that contain only one GRE is generally low, particularly when the GRE is located far from the TATA box (14). The presence of two GREs, on the other hand, synergistically enhances glucocorticoid induction of gene expression (14,15). This synergistic induction of expression has also been shown to occur between GR and other transcription factors such as NF1, OTF-1 and SP1 (15,16). Experiments performed with different modified promoters have indicated that various eukaryotic transcription factors can cooperate promiscuously to synergistically enhance gene expression (17,18). To explain this phenomenon, it has been proposed that eukaryotic transcriptional activators can interact simultaneously with different parts of the transcriptional machinery (18–20). However, this synergism in activation of gene expression has its limits. For a given expression system, the addition of more regulatory 'elements' recognized by the same transcriptional activator may not always result in a significant increase in gene expression (14). This could happen, for example, when the target for a given transcriptional activator becomes saturated.

To develop promoters with improved inducibility and transcriptional strength we decided to modify the 5' regulatory region of the human MT-IIA gene. This region contains a very strong promoter and has been extensively characterized (3,4). Besides four MREs and at least four constitutive elements (two AP2, one AP1 and one SP1), the MT-IIA promoter also contains

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one GRE. Its degree of inducibility with glucocorticoids varies with the cell type but is usually lower than with heavy metal ions (21). A reduction in the basal activity of hMT-IIA has been achieved by removing some of its constitutive elements. However, this removal also reduced the inducible level of expression which was only moderately increased by the insertion of additional MREs (22). An alternative way to reduce the basal level of expression of hMT-IIA promoter was recently described by Hu and Davidson (23). Incorporation of the lac operator-repressor into this promoter resulted in a significant decrease in the basal level of gene expression. Although this modification significantly improved the inducibility of the wild-type promoter, the levels of induced gene expression achieved in the presence of IPTG, Cd⁺⁺ and dexamethasone were lower than those obtained with the unmodified hMT-IIA.

We have taken an alternate approach to improve the hMT-IIA inducibility based on the hypothesis that the transcription activators that bind to MREs and GREs interact with different parts of the initiation complex and, therefore, may activate transcription additively or synergistically. We report that it is possible to generate synergistic activation of gene transcription when several GREs and MREs present in the same modified promoter are induced simultaneously. Consequently, we have been able to significantly improve the inducible transcriptional strength of the hMT-IIA promoter. This improvement was achieved without increasing the basal level of gene expression.

MATERIAL AND METHODS

Expression vectors

All MT expression vectors were derived from pSVOATCAT, a plasmid containing the chloramphenicol acetyl transferase (CAT) gene without any regulatory sequences (24). MT-CAT, our control plasmid in which the CAT gene is driven by the wild-type human MT-IIA promoter, was generated in the following way: an 800 bp KspI fragment of the promoter region of the human MT-IIA gene (bases -740 to +60) (Figure 1) was isolated (25). After generating blunt ends, HindIII linkers were added and the fragment was inserted into the HindIII site of pSVOATCAT, 5' to the CAT gene. Plasmid MT-CAT-ΔX was generated by removing the XmaIII fragment (bases -79 to -129) from the MT promoter of MT-CAT. To insert additional GREs, oligonucleotides containing the GRE consensus sequence, a 5'

BamHI site and a 3' BglII site were synthesized (5'-GATCTGGTACAGGATGTTCTAGCTACG-3') and multimeric head-to-tail elements were generated by self-ligating these synthetic sequences in the presence of BamHI and BglII. The multimeric GREs were then inserted at either the SacII or the XmaIII site of the MT-CAT-ΔX vector after the generation of blunt ends. The number of GREs inserted was confirmed by DNA sequencing. A similar procedure was used to insert synthetic MRE sequences (5'-GATCTTGCGCCCGGCCCG-3'). The MMTV-CAT vector was generated in the following way: the MMTV-LTR was removed from plasmid p201 (26) using PstI and, after the generation of blunt ends, inserted into the HindIII site of pSVOATCAT. The pCH110 eukaryotic expression vector containing the β galactosidase gene driven by the SV40 early promoter was purchased from Pharmacia.

Cell culture

Vero cells were obtained from the American Tissue Culture Collection and cultured in α-medium supplemented with 10% fetal bovine serum. The serum was preadsorbed with charcoal to remove glucocorticoids.

Transfections and CAT assays

Cells were transfected using the calcium phosphate method (27). Briefly, 10⁶ Vero cells were plated in 100 mm dishes and incubated for 6 h with 15 μg of precipitated DNA. After removing the DNA-containing medium, cells were shocked for 3 minutes with 15% glycerol in phosphate buffered saline, pH 7.2. The monolayers were then incubated with the different inducers (5 μM CdCl₂ and/or 1 μM dexamethasone) for 16 h and cell extracts prepared by three cycles of freeze and thaw (28). CAT activity was assayed using ¹⁴C-chloramphenicol as substrate and the radioactive acetylated product was extracted with xylene (28). Radioactivity was measured in a scintillation counter. Background CAT activity, measured on cells transfected with sheared salmon sperm DNA, represented around 10% of the CAT activity of the uninduced control MT-CAT plasmid and was systematically subtracted. In all the transfections 10 μg of a β-galactosidase expression vector were included. After cotransfection, an aliquot of the cell extracts was assayed for β-galactosidase activity. This

Figure 1. Map of the hMT-IIA promoter and the different modified versions used in this paper. The generation of the modified promoters is described in Materials and Methods.

Figure 2. Effect of the insertion of GREs and deletion of some constitutive responsive elements from the hMT-IIA promoter. Vero cells were transfected using the calcium phosphate method and treated with either 5 μM or CdCl₂, 1 μM dexamethasone or both, for 16 h. Transient CAT activity was assayed using ¹⁴C-Chloramphenicol as substrate and is expressed in relation to the activity obtained for the MT-CAT vector control and arbitrarily defined as 100.

activity was used to standardize the measurement of CAT levels in each experiment by taking into account the efficiency of transfection. CAT expression experiments were performed in quadruplicate and assays in which the standard deviation exceeded 10% were not taken into account and repeated. Induction ratio was defined as the ratio between induced and uninduced levels of CAT expression for each individual vector. A glucocorticoid expression vector (29) was also included in the transfection mixture since Vero cells do not express such a receptor.

RESULTS

To evaluate the inducible CAT activity of MT-CAT, an expression vector in which the CAT gene is driven by the wild-type hMT-IIA promoter (Figure 1) was transfected into Vero cells. The transient CAT expression was determined and efficiency of transfection assessed on the basis of β -galactosidase activity as described in Materials and Methods. All results were expressed relative to the basal CAT activity obtained with uninduced MT-CAT. As shown in Figure 2, CAT gene expression was induced 10-fold with 5 μ M CdCl₂. In contrast, CAT activity could not be induced with 1 μ M dexamethasone in transfected Vero cells. This result was unexpected since it has been previously shown that the hMT-IIA promoter is inducible by a similar concentration of dexamethasone in rat fibroblasts (5). It is possible, however, that the use of a different cell type may be responsible for this discrepancy (21). We did not try higher concentrations of dexamethasone since they inhibit the growth rate of Vero cells.

To reduce the basal level of gene expression and increase the inducible transcriptional strength of the wild-type hMT-IIA promoter, two major modifications were introduced: some of its constitutive regulatory 'elements' were removed and additional GREs inserted. The first modification was achieved by deleting the XmaIII fragment which contains two constitutive elements (AP2 and AP1) and one MRE (Figure 1). As expected, the

deleted vector (MT-CAT- Δ X) showed a reduction in both uninduced and induced levels of CAT gene expression (Figure 2). To confer dexamethasone inducibility to the MT-CAT- Δ X vector, two GREs were inserted at the SacII site to generate the SG2 expression vector. As shown in Figure 2, SG2 is significantly induced by 1 μ M dexamethasone (32-fold). Metal induced expression, on the other hand, is three times lower than with the wild-type promoter. Lastly, when dexamethasone and CdCl₂ were used simultaneously for induction of SG2-transfected cells a synergistic effect could be shown resulting in a 65-fold increase of CAT activity (Figure 2). As a consequence, the level of induced expression of SG2 was 2.3-fold higher than that obtained with the wild-type promoter. This higher inducible expression is accompanied by a 60% reduction in the level of basal CAT gene expression, thus conferring to SG2 a 60-fold induction ratio, six times better than that observed with the wild-type promoter.

The effect of varying the number of additional GREs inserted at the SacII site of the modified hMT-IIA promoter was studied. As shown in figure 3, the insertion of a single GRE (SG1) was sufficient to generate significant dexamethasone inducibility. The highest level of dexamethasone induction was achieved with three additional GREs (SG3). Both dexamethasone and metal inducibility were reduced when more than three elements were inserted. The synergistic induction with CdCl₂ and dexamethasone, originally observed in SG2, was also seen with all the other SG vectors except for the SG1 vector which contains only one additional GRE.

It is known that the relative position of different regulatory 'elements' can significantly alter the level of expression achieved by a given promoter (17). To study this phenomenon in our system, different number of GREs were inserted at the XmaIII site in the MT-CAT- Δ X vector, besides the Sp1 site (Figure 1). Vectors generated in this way (XG series) were tested in CAT assays as previously described. As for the SG vectors, the insertion of one additional GRE was sufficient to confer dexamethasone inducibility. In this case, however, maximum induction of expression was obtained with two additional GREs. Insertion of three or more elements produced a significant decrease in both dexamethasone and metal induction (Fig. 4 and data not shown). The level of dexamethasone-induced CAT gene expression obtained with XG vectors was lower than that

Figure 3. Effect of the number of GREs inserted at the SacII site of the modified hMT-IIA promoter on CAT gene expression. The effect of different inducers was compared in transient CAT assays as described in Figure 2. SG1, SG2, SG3, SG4, and SG5 represent expression vectors that contain 1, 2, 3, 4, and 5 GRE insertions, respectively.

Figure 4. Effect of the number of GREs inserted at the XmaIII site of the modified hMT-IIA promoter on CAT gene expression. The effect of different inducers was compared in transient CAT assays as described in Figure 2. XG1, XG2, and XG3 represent expression vectors that contain 1, 2, and 3 GRE insertions, respectively.

observed with the SG vectors, but the synergism between dexamethasone and metal induction was maintained when more than one additional GRE were inserted (Figure 4).

Since the removal of the XmaIII fragment from the wild-type hMT-IIA promoter reduced both the basal and induced levels of CAT gene expression, we wanted to investigate how the reinsertion of this fragment would affect the induction ratio of the SG vectors. For this purpose, the XmaIII fragment was reintroduced into SG2 and SG3 to generate vectors X-SG2 and X-SG3. As shown in Figure 5, the reinsertion of the XmaIII fragment produced a significant increase (2- to 5-fold) in the metal-induced level of gene expression and a 2- to 3-fold decrease in glucocorticoid induction. Synergism between cadmium and dexamethasone induction was still observed and the level of expression in the presence of both inducers was similar to those obtained with for SG2 and SG3. However, since an increase in the basal expression of X-SG2 and X-SG3 was observed, the induction ratio for these two vectors (25-fold and 30-fold, respectively) was not as good as for SG2 and SG3 (63-fold and 64-fold, respectively).

Finally, we compared the level of gene expression obtained with the SG3 vector with that of SM4, a vector with a modified promoter generated by the insertion of four MREs at the SacII site of MT-CAT-ΔX. As shown in Figure 6, SM4 had a much higher metal-inducibility than SG3. The basal level of expression, however, was also significantly increased and the induction ratio of SM4 was found to be 10-fold lower than that of SG3 induced by the simultaneous presence of CdCl₂ and dexamethasone. Similar results were observed when two or three MREs were inserted at the SacII site (data not shown). The level of CAT activity generated by modified MT promoters was further compared to that obtained with the widely used inducible MMTV promoter. To generate the MMTV CAT vector, the hMT-IIA promoter of MT-CAT was replaced by the promoter present in the long terminal repeat of the MMTV. This promoter contains four GREs and at least two other constitutive elements (30). As shown in Figure 6, the induced gene expression obtained with MMTV-CAT was twelve-fold lower than that observed with SG3

in the presence of Cd⁺⁺ and dexamethasone. The basal level of expression, on the other hand, was almost undetectable and, as a result, the induction ratio of MMTV-CAT was very high.

DISCUSSION

In this study we have shown that it is possible to achieve synergistic activation of transcription in the context of modified hMT-IIA promoters by inserting additional regulatory GREs. Addition of these regulatory elements did not increase the basal level of reporter gene expression and the inducibility and transcriptional strength of the modified hMT-IIA promoters were significantly improved over those of their wild-type counterpart. In contrast, the exclusive insertion of four extra MREs (vector SM4) resulted only in a moderate improvement in MT promoter transcriptional strength and this improvement was accompanied by a significant increase in basal expression. The uninduced level of CAT activity in the modified vectors with a deletion of the 60 bp XmaIII fragment was moderately but consistently lower than that observed with the wild-type MT-CAT vector. This can be explained by the fact that the XmaIII fragment contains two elements involved in basal level of expression and confirms previous observations in CHO cells (22). The increased basal gene expression seen when the XmaIII fragment was reinserted in two different SG vectors (Figure 5) is also consistent with this explanation.

We initially observed that the wild-type hMT-IIA promoter in the MT-CAT vector could not be induced by dexamethasone in Vero cells transfected with the GR gene. However, the insertion of at least one additional GRE was enough to confer glucocorticoid responsiveness.

To analyze the impact of the number of additional GREs inserted and the site of insertion, two series of modified promoters were generated by adding one or more GREs at either the SacII site (SG series) or the XmaIII site (XG series) of MT-CAT-ΔX. All vectors were inducible by CdCl₂ and glucocorticoids. However, a minimum of two adjacent GREs was necessary to generate synergistic inducibility by simultaneous treatment of transfected Vero cells with CdCl₂ and dexamethasone,

Figure 5. Effect of reinsertion of the XmaIII fragment into the SG2 and SG3 expression vectors. The effect of different inducers was compared in transient CAT assays as described in Figure 2. X-SG2 and X-SG3 are the expression vectors generated after reinsertion of the 60 bp XmaIII fragment from the wild-type hMT-II promoter into the XmaIII site of SG2 and SG3, respectively.

Figure 6. Comparative induction of CAT gene expression in MT-CAT, MMTV-CAT, SG3 and SM4 vectors. The effect of different inducers was compared in transient CAT assays as described in Figure 2. In the MMTV-CAT vector the CAT gene is driven by the MMTV LTR promoter and the SM4 vector was generated by inserting four MREs at the SacII site of MT-CAT-ΔX.

regardless of the site of insertion. Maximum levels of both dexamethasone- and synergistic inducibility were obtained with the SG3 vector which harbours a modified hMT-IIA promoter containing three additional GREs and three MREs. This vector could be induced to express 3-fold more CAT than the wild-type hMT-IIA and 13-fold more CAT than the MMTV-LTR promoter, another widely used inducible 5' regulatory region (Figure 6).

The glucocorticoid receptor has been shown to bind cooperatively to adjacent GREs (31). Whether this cooperative DNA binding is necessary for the synergism found between GREs and MREs remains to be demonstrated. In this respect, it is important to note that the binding of GRs to adjacent GREs generates a significant conformational change in the DNA helix (31). The MT promoters in vectors like XG1 or SG1 which cannot be synergistically induced do not contain neighbouring GREs and thus, would not be subjected to this structural alteration. A surprising result was the decrease in inducible CAT gene expression observed when more than three additional GREs were inserted at the SacII site of MT-CAT-ΔX. A similar decrease was also seen when three or more GREs were added at the XmaIII site of the same vector. This reduction in gene expression was observed with both inducers acting either independently or simultaneously although synergistic induction was still evident. It is possible that the addition of too many regulating elements could destabilize the assembly of an optimally efficient transcription initiation complex.

The induction ratio calculated for the modified hMT-IIA promoters was increased up to 6-fold as compared to the wild-type promoter. The fact that the insertion of additional GREs did not increase the basal level of gene expression is an important factor in the improvement of this ratio. This observation emphasizes one of the advantages of generating synergistic transcription activation by adding certain inducible regulatory 'elements' rather than constitutive ones. Indeed, the addition of regulatory 'elements' that are recognized by constitutive factors would likely result in a higher basal expression level. In addition, the presence of two different types of inducible elements within a given promoter offers an alternative to regulate gene expression: addition of only one kind of inducer (in this case CdCl₂ or dexamethasone) will allow for an intermediate level of expression.

With respect to the mechanism of synergism between GREs and MREs reported in this study, at least two non-exclusive possibilities have to be considered. One is the existence of cooperativity in the binding between these regulatory DNA sequences and their respective transcription factors. This type of cooperative binding has mostly been described for systems in which only one type of transcription factor was involved (31,32). However, for certain promoters like those of the MMTV-LTR or the rat tyrosine aminotransferase gene, it has been proposed that the GR facilitates the binding of other transcription factors to DNA by changing the chromatin structure and interfering with nucleosome assembly (30,33). Another possible mechanism involves simultaneous protein-protein interaction between the transcription factors that bind to GREs and MREs and other components of the transcriptional machinery. This last mechanism has been suggested for situations in which synergistic activation of gene transcription by different types of transcription factors was observed (18). In the particular case of the GR, Wright and Gustafsson (34) have recently proposed that transcriptional synergism between two GREs can be explained just by cooperative DNA binding. Renkawitz et al.,

however, have suggested that such a synergism might also be generated by events that occur after DNA binding and could involve the interaction of different GR domains with other components of the transcriptional machinery (35,36). The deletion of different parts of the GR might allow us to investigate which receptor domains are necessary for synergism with MREs in the context of modified MT promoters.

Since the concentration of different transcription factors varies from cell type to cell type, our observations cannot be automatically extrapolated to other cell lines. However, preliminary experiments have revealed that the synergistic induction of gene expression described here for Vero cells, can also be observed in CHO, Hela, intestinal epithelial cells and RatII fibroblasts. It seems, therefore, that the genetic manipulation of promoters containing GREs and MREs will offer the opportunity to develop new series of improved inducible mammalian expression vectors that could be extremely useful for the biotechnology industry and basic research.

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