HNF-4 increases activity of the rat Apo A1 gene

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ABSTRACT

Apolipoprotein A1 (Apo A1) is the major protein component of high density lipoprotein (HDL) particles. HDL particles mediate the removal of cholesterol from extra-hepatic tissues via a process known as reverse cholesterol transport. Augmented production of Apo A1 will likely be beneficial to those who suffer from the consequences of hypercholesterolemia. One approach to increase expression of the protein is to identify nuclear factor(s) that enhance Apo A1 promoter activity. Therefore, we have used transient transfection to study a limited portion (−474 to −7) of the gene and showed that a cis-regulatory element, site C had a permissive effect on the ability of an adjacent site B to increase promoter activity by 30-fold. The importance of element C prompted us to identify the factor(s) that interact with this site. Results showed that HNF-4, a new member of the thyroid/steroid hormone receptor superfamily interacts with site C to enhance activity of the promoter. Based on this observation and that of the known inhibitory effects of ARP-1 on site C, we postulate a model which may account for the tissue-specific expression of the rat Apo A1 gene.

INTRODUCTION

Apolipoprotein A1 (Apo A1) is the major protein component of plasma high density lipoprotein particles. These particles mediate a process known as reverse cholesterol transport (1,2). In this process, excess cholesterol from peripheral tissues is shuttled to the liver, converted to bile salts, and eventually excreted from the body (reviewed in 3). Results from numerous studies have demonstrated an inverse correlation between plasma Apo A1 levels and the risk of atherosclerosis (4–6). This inverse correlation is likely attributed to activities of Apo A1 in reducing total body cholesterol. Since the number one cause of premature death in our society is coronary artery disease that is frequently attributed to hypercholesterolemia, we are attempting to develop ways to prevent the consequences of this disorder by increasing the plasma levels of Apo A1. One step towards achieving this goal is to try and identify factor(s) that will enhance the expression of Apo A1.

MATERIALS AND METHODS

Construction of plasmids

A 1.6-kb DNA fragment containing the chloramphenicol acetyltransferase (CAT) gene was excised from pSV2-CAT (17) by digestion with Hind III and Bam H1. This DNA fragment was blunt-ended using the Klenow fragment of DNA pol I, and inserted into the Hinc II site of pUC18 to construct the vector pUC-CAT. Cloning and insertion of the rat Apo A1 DNA fragment spanning nucleotides −474 to −7 into pUC-CAT to yield the construct pAI.474-CAT was described recently (18). The constructs that were derived from pAI.474-CAT using convenient restriction sites included: pAI.47-CAT, pAI.145-CAT, pAI.186-CAT, and pAI.232-CAT. These templates contained rat Apo A1 DNA fragment spanning nucleotides −47 to −7 into pUC-CAT to yield the construct pAI.474-CAT was described recently (18). The constructs that were derived from pAI.474-CAT using convenient restriction sites included: pAI.47-CAT, pAI.145-CAT, pAI.186-CAT, and pAI.232-CAT. These templates contained rat Apo A1 DNA fragment spanning −47 to −7, −145 to −7, −186 to −7, and −232 to −7, respectively.

Four templates were constructed to measure the activity of site B alone or in combination with other elements attached to the

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the transcription factor of interest. To construct the vector pAI.TATA-CAT, Apo A1 DNA between -47 and -7 was subcloned into pUC-CAT. Sites B and A+B were excised from pAI.186-CAT and pAI.232-CAT, respectively by digestion with Ava I and then blunted-ended before inserting into the Sma I site of pAI.TATA-CAT to yield pAI.B-CAT and pAI.AB-CAT, respectively. The pAI.BCD-CAT was derived from pAI.BCD-CAT by Hae III digestion to remove the -79 to -48 DNA fragment.

For studies of site C we used the pUC-CAT vector to construct 5 templates. These templates pAI.CM1-CAT, pAI.CM2-CAT, pAI.CM3-CAT, pAI.CM4-CAT, and pAI.CM5-CAT contained Apo A1 sequences spanning -176 to -7 with the following mutations (indicated by the underlined nucleotides) -132 CACGCTCTAGGC-122, -119 ACGCTACAGGA-109, -119 ACGGTTTTCAC-109, -119 CTAGA-CAGGA-109, and -132 CACGCTCTAGGCTTAAAGACAGGA-109, respectively. DNA sequences between -176 and -105 were amplified by the PCR using pAI.BCD-CAT as template, the M-13 reverse primer and Apo A1 primers -105 CTAGAGCGTGAGGGCGTGCTTGTCCGAGGC-148 for F1, -105 ACGGATGTTCCAAGGATCAGCT-136 for F2, -105 CTAGAGTTCAGGATCAGGC-134 for F3, DNA sequences between -104 to -7 were amplified by the PCR using the same template, a CAT primer, and Apo A1 primers -104 TTTCCATCATGCCCAGCA-99 for F4, and -104 ACAGGAATGCTCGCCAGAGA-91 for F5. The PCR products F1, F2, and F3 were digested with Sac I, while F4 and F5 were digested with Xba I. To reconstruct the site C mutants CM1 to CM5, combinations of the digested PCR products; F1 + F4, F2 + F5, F3 + F4, F3 + F5, and F1 + F5 were subcloned between the Xba I and Sac I sites of pAL.BCD-CAT to yield pAL.CM1-CAT, pAL.CM2-CAT, pAL.CM3-CAT, pAL.CM4-CAT, and pAL.CM5-CAT, respectively. All constructs were verified by nucleotide sequence analysis using the Sequenase kit from USB.

To delineate site D, pAI.BD[-101/-48]-CAT and pAI.BCD[-101/-48]-CAT (described above) containing the -176 to -102 and -176 to -80, respectively, were inserted' of pAI.TATA-CAT. The construct pAI.BD[-101/-48]-CAT was derived from pAI.B-CAT by inserting the PCR product F1 between the B-site and TATA-element.

Eukaryotic expression plasmid pLEN4S containing HNF-4 cDNA (20) was kindly provided by Dr J.E.Darnell, Jr. (Rockefeller University) for cotransfection studies. The HNF-4 cDNA was excised from pLEN4S by digestion with Eco R1 and subcloned into the expression vector pMT2 (21) for transfection into COS cells. The other expression vector pMT2 carrying ARP-1 cDNA (14) was a generous gift from Dr M.-J.Tsai (Baylor College of Medicine, Houston).

Cell cultures and transfections

Human hepatoma cell lines, HuH-7 (22) and huH-1 (23) were grown in ISE-RPMI (24), with the addition of 1% fetal calf serum only for huH-1 cells. COS cells, HeLa cells, and bladder carcinoma J-82 cells (25), were cultured in ISE-RPMI containing 5% fetal calf serum. Calcium phosphate mediated DNA transfections were performed according to procedures described previously (24). Cells were transfected with 20 μg of plasmid DNA. In cotransfection studies, cells were treated with 10 μg each of a reporter CAT plasmid and an expression vector carrying the transcription factor of interest.

Assay for CAT activity

Transfected cells were harvested 48 h after glycerol shock and CAT activity was assayed according to methods described previously (24). Efficiency of DNA uptake into cells was monitored by including 2 μg of RSV-βGal in all transfections. β-Galactosidase activity in transfected cells was measured using a standard procedure (26). The percentage of acetylation of [14C]-chloramphenicol (ICN) was determined by scintillation counting of spots corresponding to substrate and acetylated products following separation by thin layer chromatography.

Preparation of nuclear extracts and COS cell extracts

Nuclear extracts from rat liver were prepared according to procedures described previously (27). Whole cell extracts were prepared from untransfected or transfected COS cells. Cells harvested by gentle scraping were pelleted, resuspended in five packed-cell volumes of buffer A (10 mM Hepes, pH 7.6, 15 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% trasylo on ice. After 15 min, cells were disrupted using a Dounce

Figure 1. Deletional analysis of Apo A1 DNA from position -474. Panel A, shows the map of constructs containing deletions from -474 that were assayed for CAT activity in HuH-7 and huH-1 cells. Panel B, shows the autoradiograph of CAT activity in huH-1 cells containing the same constructs with increasing amount of 5' sequence pAI.47-CAT, pAI.145-CAT, pAI.186-CAT, pAI.232-CAT, and pAI.474-CAT are shown in lanes 1-5, respectively. The CAT activity of these constructs expressed as a percentage relative to that of pAI.474-CAT are; 8%, 11%, 33.5%, 70%, and 100%. Panel C, shows the autoradiograph of CAT activity in huH-1 cells containing the same deletional constructs. The activity of the constructs pAI.47-CAT, pAI.145-CAT, pAI.186-CAT, pAI.232-CAT, and pAI.474-CAT, are shown in lanes 1-5, respectively. The CAT activity of these constructs expressed as a percentage of pAI.474-CAT are; 14%, 17%, 45%, 75%, and 100%. 
homogenizer (Wheaton). Then five packed-cell volumes of buffer B (20 mM Hepes, pH 7.6, 1.5 mM MgCl₂, 0.2 mM EDTA, 1.0 M KCl, 20% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1% trasylol) was added, mixed, and incubated on ice for 30 min. The extract was centrifuged at 35,000 rpm in a Beckman TLA100.4 rotor for 30 min. The supernatant was removed and solid ammonium sulfate (0.36g/ml) was added. After 30 min on ice, the precipitated proteins were collected by centrifugation at 35,000 rpm in a TLA100.4 rotor for 20 min. The pellet was dissolved in dialysis buffer (25 mM Hepes, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) and dialyzed for 2 h with one change of buffer. Insoluble material was removed by centrifugation, and the supernatant was stored in aliquots at -70°C.

**Gel-retardation assays**

Apo Al sequences from -160 to -80 was subcloned into the Hinc II site of pUC18, then excised by digestion with Ava I to release a DNA fragment containing C-site from -145 to -80. This DNA fragment was radiolabeled by incubating with [γ-32P]ATP (NEN) and T4 polynucleotide kinase. Gel retardation assays were performed according to procedures described previously (28).

**RESULTS**

Sites A and B are common to both rat and human Apo Al promoter

We have previously described the isolation of a DNA fragment encompassing a limited portion (-474 to -7) of the rat Apo Al upstream region and used this sequence to study thyroid hormone regulation of the gene (18). To identify cis-regulatory elements that control the tissue specific expression of rat Apo Al we constructed a series of 5' deletions starting from position -474 and inserted these fragments in front of the CAT gene. CAT-activity was detected in hepatoma (HuH-7 and Huh-1) but not in nonhepatoma (Hela and J-82, bladder carcinoma) cell lines transiently transfected with these constructs. Results (Fig. 1, note striking increase from lane 2 to 3 and compare lanes 3 and 4) revealed the presence of two cis-regulatory elements A (-232 to -187) and B (-186 to -146) in rat DNA that were remarkably similar to sites A and B, respectively in the human Apo Al gene (13). Like their counterparts in the human gene, sites A and B in rat DNA had the identical effects on the promoter by enhancing activity 2- and 30-fold, respectively.

The -141 to -48 DNA fragment is required for maximal activity of site B

Since site B exerted a striking 30-fold increase in transcriptional activity of the rat Apo Al promoter, we wondered whether this site alone could activate the TATA-element to the same degree. To address this question, we constructed pAl.B-CAT and pAl.AB-CAT (Fig. 2) containing site B alone and sites A+B, respectively fused to the TATA-element. Unexpectedly, CAT activity of both constructs was the same as that of pAl.TATACAT (Fig. 2). A common feature in both pAl.B-CAT and pAl.AB-CAT was the deletion of the -141 to -48 sequence, thus indicating that the genetic information contained in this region was essential for the activity of site B.

To examine the -141 to -48 DNA fragment in detail, we constructed pAl.BCAD-CAT that differed from pAl.BCD-CAT by the removal of nucleotides -79 to -48 (Fig. 2). This deletion had low CAT activity, thus indicating an element is present in the DNA fragment between nucleotides -79 and -48. CAT activity (results not shown) of another construct pAl.BCA[-101/-48] containing a longer deletion (-101 to -48) was the same as pAl.BCAD-CAT, suggesting that the -102 to -80 did not contain additional cis-acting elements. These results show that the maximal effects of site B requires the presence of contiguous 3' sequences. Together these observations revealed the presence of at least two additional cis-acting elements that are essential for rat Apo Al promoter activity. These sites span nucleotides -141 to -102 and -79 to -48 and are referred to as sites C and D, respectively.

Motifs that are important for site C function

The indispensable role of the -141 to -48 fragment in maximizing Apo Al promoter activity provides a strong justification for identifying the nucleoprotein(s) that interacts with this region of the DNA. Inspection of the nucleotide sequence between -141 to -48 revealed a motif in site C (TGATCCCGAACCTCT) that shares 80% homology with a portion (TGACCCCGACCCCG) of site A. Recent studies showing that three members of the thyroid/steroid hormone receptor superfamily; ARP-1, RXRα, and TRα interact with a

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<th>Map of Construct</th>
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Figure 2. Maximal effect of B-site on Apo Al promoter activity requires the presence of sites C and D. Map of the pAl.BCD-CAT construct is shown at the bottom of this table. Deletional constructs are listed under 'map of construct'. All templates were transfected into HuH-7 cells and the relative CAT activities of transfection studies appear in the columns to the right, where each column represents a separate set of transfection studies.
motif in site A prompted us to think that other members of the same family may interact with site C (14, 15, 18). Close examination of the C site revealed a sequence (−119 CTAAAGTTCAC −109) that was similar to the binding motif (GGCTAAGTTCAC) for the transcription factor, HNF-4 (20). To determine whether the −119 to −109 sequence influenced promoter function we introduced mutations that encompassed the entire motif, the 5′, and the 3′ half of the −119 to −109 motif to yield the constructs, pAI.CM2-CAT, pAI.CM3-CAT and pAI.CM4-CAT, respectively (Fig. 3). Although all three templates had low levels of CAT activity, the reduction was most pronounced in cells transfected with pAI.CM2-CAT.

Previous results demonstrated that another portion of the C-site (−132 TGATCCTTGA ACT −120) binds ARP-1 (14). Selective mutations within this sequence gave rise to a template, pAI.CM1-CAT. CAT activity in cells transfected with pAI.CM1-CAT was drastically reduced compared to the wild type, pAI.BCD-CAT (Fig. 3). Together, these results indicate that the two motifs (−132 to −120 and −119 to −109) contained in site C are critical for the ability of this element to modulate Apo A1 promoter activity.

HNF-4 interacts with and binds to a motif in site C

In order to confirm the suggestion that HNF-4 is involved with regulating Apo A1 promoter activity, we measured CAT activity in cells cotransfected with pAI.BCD-CAT and a eukaryotic expression plasmid carrying the cDNA for either HNF-4 or ARP-1 (Table I). Compared with cells carrying pAI.BCD-CAT alone, CAT activity in HuH-7 and huH-1 cells cotransfected with HNF-4 increased 2.5- and 6.5-fold, respectively (Table I). To be certain that this observation is a reflection of HNF-4 activity and not an artifact of experimental design we showed that cotransfection of pAI.BCD-CAT and ARP-1 into huH-1 cells. These studies were performed twice and the values of both experiments appear in the table.

The construct pAI.BCD-CAT was cotransfected with pUC, HNF-4, or ARP-1 into both HuH-7 and huH-1 hepatoma cell lines. CAT activity of the transfected cells are shown in the columns on the right. All values shown represent the mean ± SD of at least 3 separate transfection studies, except for cotransfections of pAI.BCD-CAT and ARP-1 into huH-1 cells. These studies were performed twice and the values of both experiments appear in the table.
Figure 4. Binding of HNF-4 and ARP-1 to C-site DNA. Panel A, shows autoradiographs of gel-retardation studies with radiolabeled site C DNA and various extracts. The contents of lanes 1–4 are: rat hepatocellular extract (rHNE), WCE from untransfected COS cells, WCE from COS cells transfected with HNF-4, and WCE from COS cells transfected with ARP-1. Lanes 5–9 show competition studies in which all reactions contained HNF-4 enriched WCE and 100-fold molar excess of the various competitor DNA in lane: 5, no DNA; 6, site A; 7, site B; 8, site C; and 9, site D. Lanes 10–14 show competition studies in which all reactions contained ARP-1 enriched WCE and 100-fold molar excess of various competitor DNA in lane: 10, no DNA; 11, site A; 12, site B; 13, site C; and 14, site D. Panel B and C, competition for HNF-4 and ARP-1 binding, respectively with mutants of site C (sequences shown in Fig. 3). Panel B, shows HNF-4 binding to radiolabeled site C with the following competitor DNA in lanes 1–7; none, wild type, CM1, CM2, CM3, CM4, and CM5. Panel C shows ARP-1 binding to radiolabeled site C with the following competitor DNA in lanes 1–7; none, wild type, CM1, CM2, CM3, CM4, and CM5.

to the A motif. Thus HNF-4 and ARP-1 like proteins in rat liver nuclei bind to the same DNA fragment.

It is important to mention results of previous studies (29) showing that a nuclear protein, LF-A1 partially purified from rat liver bound to the ARP-1 binding motif in site A (−216 to −195). More recent information seems to indicate that HNF-4 and LF-A1 could be identical because of DNA-binding and antiserum cross reactivity (20). This observation suggests that HNF-4/LF-A1 binds to the rat Apo A1 A-site (−232 to −187) and is supported by results appearing in Fig. 4A, lane 6. In this lane site A competitor DNA displaced HNF-4 binding to radiolabeled site C.

Functionally inactive C-site mutants failed to bind HNF-4/ARP-1

Results summarized in a preceding section showed that mutations in site C affected the ability of this sequence to activate Apo A1 promoter activity. Therefore, correlating changes in functional activity with binding of HNF-4 and ARP-1 to site C by assessing the ability of each site C mutant to displace binding to the radiolabeled wild-type DNA would provide additional support for role of HNF-4 and ARP-1 in Apo A1 promoter regulation. Gel-retardation results showed that CM1, CM2, CM3, and CM5 could not displace HNF-4 binding to site C (Fig. 4B). Differences between wild type DNA and these mutants were confined to nucleotides spanning −132 to −109, thus suggesting that HNF-4 interacts with this region. Furthermore, we noted the close correlation between the inability of mutants CM1 and CM2 to compete for HNF-4 binding to site C (Fig. 4B) and drastic reductions in CAT activity of templates containing these mutations (Fig. 3). As mentioned above, CAT-activity failed to increase in HuH-7 and huh-1 cells when cotransfected with HNF-4 and pAl.CM1-CAT or pAl.CM2-CAT (data not shown). These results suggest that inhibition of HNF-4 binding to site C blocks its ability to enhance Apo A1 promoter activity.

To define the binding motif for ARP-1, we tested mutants CM1 to CM5 for their ability to displace ARP-1 binding to radiolabeled site C DNA. Results revealed CM1 and CM5 competed poorly for ARP-1 binding to site C, but CM2, CM3, and CM4 displaced ARP-1 from labeled DNA almost as effectively as wild-type DNA (Fig. 4C). Mutants CM1 and CM5 deviated from the wild-type sequence between nucleotides −132 to −120, thus localizing ARP-1 binding to this motif. Due to the low CAT activity of constructs containing the CM1 and CM2 mutations, it was not possible to measure the suppressive effects of ARP-1. Taken together, these results showed that HNF-4 increased Apo A1 promoter activity by binding to nucleotides from −132 to −109 in site C. In contrast, ARP-1 decreased Apo A1 promoter activity by interacting with the −132 to −120 motif. Although one expects mutations to the ARP-1 binding motif should increase promoter activity, it did not because the sequence overlaps the HNF-4 recognition site. Since the binding motifs of the two proteins overlap this would limit the interaction to either HNF-4 or ARP-1 at any given time.
DISCUSSION

Dietary indiscretion in modern societies has magnified the potential dangers of hypercholesterolemia making it a risk factor in the pathogenesis of coronary artery disease. We want to develop useful approaches to lessen the effects of hypercholesterolemia by understanding the genetic control of the serum protein, Apo A1. Apo A1 plays a critical role in the excretion of cholesterol from the body because it mediates a process known as reverse cholesterol transport (1, 2). In this report, we have identified at least 4 cis-acting elements, A, B, C, and D, in the rat Apo A1 promoter by transient transfection assays. Sites A (-232 to -187), B (-186 to -146), and C (-141 to -102), like their counterparts in human DNA, increase Apo A1 gene expression in hepatic cells. Site D (-79 to -48), a novel element, increases rat Apo A1 promoter activity and shares 87% homology with the same nucleotides in human DNA. Although functional significance of the -79 to -48 sequence in human Apo A1 DNA is not known, there are three genetic polymorphism studies showing that a G to A transition at nucleotide -78 is associated with elevated concentrations of serum Apo A1 and high density lipoprotein cholesterol (30-32). In order to gain insight into how the nucleotide substitution affects human Apo A1 expression, it will be necessary to further characterize the D-element and identify the proteins that interact with this site.

Since site C is important for maximal promoter activity we have identified the factor(s) that interact with this element. Studies to date have shown that an inhibitory factor, ARP-1 from human placenta binds to sites A and C (14). However, site C functions as an enhancer (2-fold) rather than suppressor of Apo A1 activity and thus, points to the existence of additional factor(s) that interact with this element to increase promoter function. The fact that ARP-1 belongs to a superfamily of transcription factors prompted us to test another member of this family, HNF-4 for its ability to interact with site C (20). In this study we have presented evidence that site C is bound by a nuclear transcription factor, HNF-4. Our results showed that HNF-4 and ARP-1 interact with site C to stimulate and repress, respectively Apo A1 promoter activity in hepatoma cells.

One apparent inconsistency comes from mutational analysis of the ARP-1 binding motif in site C. Since ARP-1 is an inhibitory factor, we expect CAT activity of pALCM1-CAT, a construct containing a defect in the ARP-1 binding site to be higher than that of the wild type, pAL.BCD-CAT. Instead, we observed a reduction in CAT activity associated with pALCM1-CAT. A potential explanation is that the ARP-1 binding motif in site C may be bound by factors other than ARP-1 in the hepatoma cells and in the presence of these other factors rat Apo A1 promoter activity is intact. The observed repression by ARP-1 is evident only in the presence of co-transfections.

Whereas ARP-1 is found in a variety of tissues, HNF-4 is present in liver and small intestine, the same tissues that express Apo A1. We therefore, postulate that site C is preferentially occupied by HNF-4 in liver, thus leading to the activation of Apo A1 gene expression. Moreover, the overlap of the binding motifs for HNF-4 and ARP-1 led us to speculate that overall Apo A1 promoter activity is likely dependent on a balanced interaction of the two factors with site C. In extra-hepatic tissues where HNF-4 is absent, the effects of ARP-1 are dominant thus suppressing Apo A1 promoter activity.

HNF-4/ARP-1 interaction with the C3P element of another apolipoprotein gene, CIII was reported recently (33). Identical to the case of Apo A1, CIII gene activity is also stimulated by HNF-4 and repressed by ARP-1. Both Apo A1 and CIII genes are positioned immediately adjacent to each other on the same chromosome. The fact that both genes are expressed in the identical tissues adds support to the idea that HNF-4 and ARP-1 function to regulate tissue specific expression of Apo A1. Additional support for this possibility comes from studies of the BA1 and AIIJ elements present in the human Apo B and Apo AII gene, respectively. Results of these studies showed that both BA1 and AIIJ motifs interacted with HNF-4 and ARP-1 to increase and decrease, respectively expression of the Apo B and Apo AII genes (34). Another gene, involved in lipid metabolism, encoding the medium-chain acyl CoA dehydrogenase is also regulated in the same fashion by HNF-4 and ARP-1 (35).

In summary, rat Apo A1 DNA that span nucleotides -474 to -7 is comprised of at least four cis-acting elements A-D which enhance promoter activity. The novel findings in this report include: (1) HNF-4 and ARP-1 bind to and interact with site C to stimulate and repress, respectively Apo A1 expression in hepatocytes. (2) Tissue specific distribution and overlapping binding motifs for HNF-4 and ARP-1 may govern Apo A1 expression. (3) Site D is an essential component of the rat Apo A1 promoter. Identification of transcription factors that interact with proximal elements provide valuable insights into the regulation of Apo A1 gene expression. We expect this information to be useful in developing ways to manipulate the activity of the promoter.

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