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A Novel *cis*-Acting DNA Element Required for a High Level of Inducible Expression of the Rat P-450c Gene

ATSUSHI YANAGIDA, KAZUHIRO SOGAWA, KEN-ICHI YASUMOTO, AND YOSHIAKI FUJII-KURIYAMA*

Department of Chemistry, Faculty of Science, Tohoku University, Aobayama Sendai 980, Japan

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A novel *cis*-acting regulatory element (designated BTE for basic transcription element) was found in the region proximal to the TATA sequence of the P-450c gene by the use of deletion mutations. This DNA element is considered to be involved in the basic transcription of the gene and does not show distinct enhancer activity in itself. Together with the XRE sequence (A. Fujisawa-Sehara, K. Sogawa, M. Yamane, and Y. Fujii-Kuriyama, Nucleic Acids Res. 15:4179–4191, 1987), however, this sequence is required for a high inducible expression of the P-450c gene in response to xenobiotic inducers. The BTE sequence contained the GC box consensus sequence and half of the NF-1-binding consensus or CAT box sequence, but their synthetic oligonucleotides, used as competitors in the gel mobility shift assays, did not compete with the BTE sequence for the binding protein, suggesting that the BTE sequence functions as a different recognition sequence from that for Sp1 or NF-1. Analogous sequences to BTE are found in the region promixal to the TATA sequence of other genes, especially other P-450 genes with different modes of regulation, suggesting that the BTE sequence plays a common regulatory role in basic transcription of genes including a group of the P-450 superfamily. The ubiquitous distribution of nuclear factor(s) binding to this element supports this suggestion.

A group of hemoproteins which catalyze the monooxygenase reaction of a wide variety of lipophilic substances of exogenous and endogenous origins constitutes the cytochrome P-450 superfamily. Most of them, if not all, are inducible enzymes (1). Synthesis of some specific forms of P-450 is known to be induced in response to either exogenous or endogenous inducers. In the process of studying the inducible expression of the drug-metabolizing P-450c gene, a *cis*-acting DNA element responsible for the inducibility of the gene has been identified and designated as the xenobiotic responsive element (XRE) (8). This DNA element or its homologs are found five times in the 5′ flanking region of the gene and exert strong inducible enhancer activities on P-450c gene expression in response to added inducers such as 3-methylcholanthrene(3-MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

In recent years, it has been demonstrated that several different kinds of *cis*-acting DNA elements and their related *trans*-acting factors are necessary to work in a concerted manner for the expression of genes (14). In further studies of a series of deletion mutants, we have found another regulatory DNA element essential for a high level of expression of the P-450c gene in the region proximal to the TATA sequence. Here we describe the characterization of the novel *cis*-acting regulatory element and some properties of the factor(s) binding to this element. A computer-aided search for sequences similar to BTE was performed in the relevant regions of various genes including other P-450 genes and suggests that this regulatory element is not specific to the P-450c gene but rather is common to several other genes, such as genes for other species of P-450s, the mouse apolipoprotein A gene, and others.

MATERIALS AND METHODS

Construction of deletion mutants in the 5′ upstream regulatory region. Plasmid pMC6.3k and its external deletion mutant pMC1033 were constructed as described previously (7, 19). A series of deletion mutants in pMC1033 (Fig. 1) were constructed as follows and designated pMC1033Δ(A/B) (A and B are the positions of the 3′ and 5′ ends of the fused sequences, respectively, numbered negatively from the transcription initiation site). Plasmid pMC1033 was cleaved by *Nhe*I at −537 base pairs (bp) and then treated with exonuclease Bal 31 for 1 to 5 min. The digested plasmid, which had been ligated with *Xho*I linker by T4 DNA ligase, was treated with *Xho*I to be sealed intramolecularly by the ligase.

pSV/MC53 and pSV/MC44 were constructed by inserting a simian virus 40 (SV40) enhancer sequence excised from pIFN^{EX1} (2) by *Xho*I between the *Sal*I and *Xho*I sites of pMC1033Δ(715/53) and pMC1033Δ(686/44) described above. To construct plasmids pMC6.3kΔ(96/53) and pMC6.3kΔ(96/44), pMC6.3k was linearized by partial digestion with *Mst*II at −96 bp and filled in at the cutting sites with *Escherichia coli* polymerase I (the Klenow fragment). After being ligated with the *Sal*I linker, the resulting plasmid was digested by *Sal*I to isolate the *Sal*I/−96 (the −96 end was modified to the *Sal*I end) fragment. The isolated fragment was inserted into the 5′ end of the −44 and −53 fusion genes, pMC1033Δ(686/44) and pMC1033Δ(715/53), respectively, by replacing the *Sal*I-*Xho*I fragment of the fusion genes.

Transfection and assay of CAT activity. Transfection was performed by the calcium phosphate precipitation method, as described previously (7). Chloramphenicol acetyltransferase (CAT) activity was assayed by the method of Gorman et al. (9). Cell supernatants were prepared by centrifugation of cell lysates which were made by freezing and thawing, and aliquots were used for the CAT assay. All assays were performed within the range of a linear relationship of CAT activity to the time of incubation or concentration of extracted proteins. The acetylated forms of [^{14}C]chloramphenicol were separated from the substrate [^{14}C]chloramphenicol by high-pressure liquid chromatography (4.6 mm by 25 cm column; 5-μm TSK gel silica-60 particles [Tosoh Co., Tokyo, Japan]; mobile phase, chloroform-methanol, 92.5:7.5 [vol/vol]) and then quantified by scintillation spectropho-

* Corresponding author.

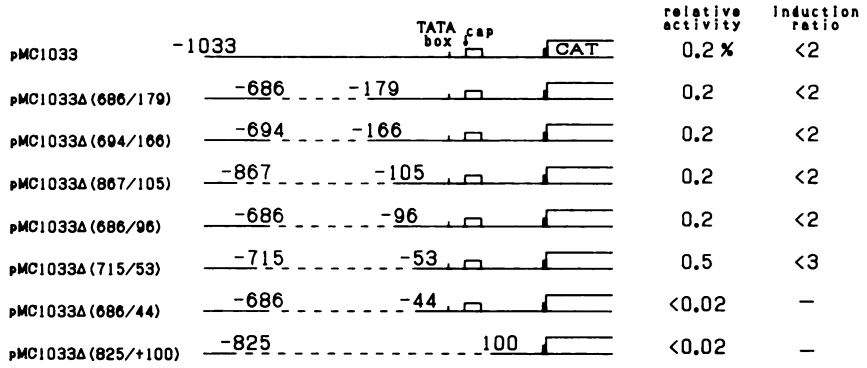

FIG. 1. Expression of CAT activity driven by various mutants with deletions in the 5′ flanking region of the P-450c gene. Dashed lines indicate the deleted sequences. The numbers on the lines show the boundary nucleotides of the deleted sequences counted negatively from the transcriptional start site. Large open boxes indicate CAT structural genes, and small open and hatched boxes indicate the untranslated leader sequence in exon 1 and a part of exon 2, respectively. The solid lines indicate the 5′ flanking and the intron 1 sequences. The fusion genes were constructed as described in Materials and Methods. Cap indicates the transcription start site. CAT activity was determined by separating acetylated products by high-pressure liquid chromatography and is expressed as the percentage of activity relative to that of the original plasmid pMC6.3k induced with 3-MC. Each value is an average of three or four experimental results. <0.02, less than the limit of detection. The inducibility of the genes is shown by induction ratios of the expressed CAT activities in the presence and absence of 3-MC.

tometry or detected by autoradiography after separation of the reaction products by thin-layer chromatography (9).

DNA footprint analysis. A DNA fragment used for DNA footprinting analysis was prepared as follows. pMC1033 (694/166) was digested by *Cla*I and *Ava*II, and the *Cla*I-*Ava*II fragment of 561 bp was isolated by agarose gel electrophoresis. The isolated fragment was filled in with a Klenow enzyme and subjected to digestion with *Xho*I at −166 bp. The resultant fragment from −166 to +42 bp with a blunt-end and a *Xho*I-cleaved end was subcloned into pUC18 between the *Sal*I and *Sma*I sites. The hybrid plasmid was first cleaved by *Eco*RI or by *Hin*dIII (for labeling the minus or plus strand, respectively) to be end labeled with ^{32}P (specific activities, 3.6×10^7 cpm/μg for the minus strand and 2.7×10^7 cpm/μg for the plus strand). After treatment with *Hin*dIII or *Eco*RI for the labeled minus and plus strands, respectively, the labeled fragment was isolated by agarose gel electrophoresis and used for the footprinting experiments.

DNase I footprinting was performed essentially as described previously (10). Nuclear extracts were prepared from various cultured cells according to the published procedure (5). Nuclear extracts (about 20 μg) were preincubated with poly(dI-dC) (1 μg) for 15 min at 0°C and then incubated with the end-labeled probe DNA (20,000 cpm) in 10 μl for 10 min at 20°C. The reaction mixtures were digested with DNase I at various concentrations and then analyzed by electrophoresis in a 7 M urea–6% polyacrylamide gel. The gel was autoradiographed at −70°C.

Gel mobility shift assay. Nuclear extracts were prepared from Hepa-1(8), P19(6), EL4 (ATCC TIB39), and L929 (ATCC CCL1) cells, as described by Dignam et al. (5). Probes for the gel mobility shift assay were as follows:

(i) the BTE sequence

```
      −59          −40
5′-GATCGAGAAGGAGGCGTGGCCAAC
     CTCTTCCTCCGCACCGGTTGCTAG-5′
```

(ii) the mutated BTE sequence

```
5′-GATCGAGAAGGAGGCCTCGCCAAC
     CTCTTCCTCCGGAGCGGTTGCTAG-5′
```

(iii) the CAT box or NF-1-binding sequence (18)

```
5′-GGGAGTCAAACAATTTTTTGGCAAGAATATTATGAATCCC
    TCAGTTTGTTAAAAAACCGTTCTTATAATACTTAGGGCCC-5′
```

(iv) the GC box sequence (13)

```
5′-GATCGGGGCGGGGC
     CCCCGCCCCGCTAG-5′
```

Each was synthesized by using a DNA synthesizer (Applied Biosystems). Sometimes these oligonucleotides were concatenated by T4 ligase for use as either a binding probe or a competitor. The double-stranded probe DNA (specific activity, 1.6×10^8 cpm/μg) was labeled at the 5′ end with [γ-^{32}P] ATP. The gel mobility shift assay was performed essentially as described previously (2).

RESULTS

Mutants with deletions in the region proximal to the TATA box. In addition to the XRE sequences, we searched further for another regulatory DNA element for the expression of the P-450c gene by deleting the sequence in the region proximal to the TATA box. A set of hybrid plasmids with various deletions were constructed (Fig. 1) and transfected into Hepa-1 cells for the assay of CAT expression.

The 5′ upstream regions with various deletions from about −866 to −53 bp displayed practically the same expression of CAT activity, although the hybrid genes practically lost the ability to be induced by 3-MC. pMC1033 contains a single XRE sequence around −1,010 bp, but only one element did not appear to be strong enough to express the inducibility of the gene. The expressed CAT activity was low compared with the fully induced activity of the original fusion gene, pMC6.3k, but it was significant and reproducible because the level of activity was about 10-fold higher than that of the negative control plasmid, pMLCAT (8). The deletion extending beyond positions −53 to −44 bp clearly reduced the expressed CAT activity to the level of the negative control. These results suggest the presence of a transcriptional regulatory element or at least a part of it for the basal expression of the gene between −53 and −44 bp. To confirm this suggestion, we constructed two pairs of fusion genes with

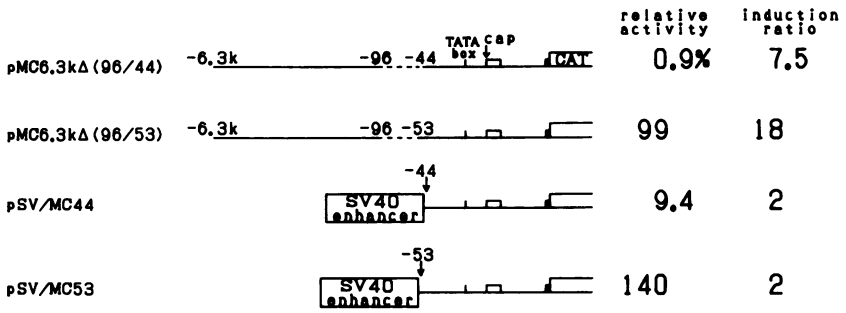

FIG. 2. Effect of the BTE region on expression of the CAT fusion gene. The test hybrid genes were constructed as described in Materials and Methods. Relative activities and induction ratios are as described in the legend to Fig. 1. Dashed lines indicate deleted sequences.

special attention to this small region and transfected them into Hepa-1 cells. A mutant with a small deletion from −96 to −53 bp in the original pMC6.3k showed the same high inducible activity as the original fusion gene (pMC6.3k) (Fig. 2). On the other hand, if the deletion proceeded a little further downstream to −44 bp, the deletion mutant lost most of the fully inducible activity. This reduced activity appears to be due to lowered basal expression rather than to decreased inducibility, because the induction ratio was not influenced very much in this deletion mutant. Reduced expression by the deletion from −53 to −44 bp was also clearly demonstrated by augmented constitutive expression when the SV40 enhancer was placed at the 5′ end of the fusion genes. The SV40 enhancer sequence placed at the 5′ end of the −53 bp fusion gene constitutively expressed enhanced CAT activity, while less than 1/10 of the activity was expressed from the −44 bp fusion gene with the SV40 enhancer sequence placed at its 5′ end. Thus, the result confirms the above suggestion that there exists another kind of *cis*-acting regulatory element responsible for supporting the basal expression between −44 and −53 bp of the P-450c gene. We designate this regulatory DNA sequence as the basic transcription element or BTE. In a pair of −53 and −44 bp fusion genes containing the SV40 enhancer, transcription was found to start from the same correct position of the transcription initiation site by S1 nuclease mapping analysis (data not shown). To investigate the transcriptional enhancer effect of the BTE sequence by itself, the synthetic BTE sequence was inserted tandemly three times at −847 bp (*Bam*HI site) of pMC6.3kΔ(96/44), which was deprived of BTE and the flanking sequences, and the resultant fusion gene was transfected into Hepa-1 cells. The expressed CAT activity in cells with or without treatment by 3-MC was assayed, only to find practically no distinct enhancer activity with the BTE sequence (data not shown). These findings may suggest that the BTE sequence does not function as an enhancer sequence but rather acts as a promoter element to support efficient transcription driven by enhancer sequences.

***trans*-Acting factor binding to the BTE sequence.** To investigate regulatory factors acting in *trans* on BTE sequences, DNA footprint analysis was performed with the sequence containing BTE and nuclear extracts from Hepa-1 cells. Nuclear extracts were prepared from Hepa-1 cells treated either with or without the inducer 3-MC, as described by Dignam et al. (5), and were incubated with the ^{32}P-end-labeled DNA fragment (−162 to +44 bp) containing the BTE sequence. After DNase I digestion, the DNA fragments were subjected to gel electrophoresis, the results of which are shown in Fig. 3. DNase I protection was clearly observed in the regions containing the BTE sequence from −61 to −41 bp in the plus strand and from −56 to −41 bp in the minus strand. The nuclear extracts from the cells treated with and without the inducer gave the same DNase I footprinting pattern. Both strands in another region from −147 to −122

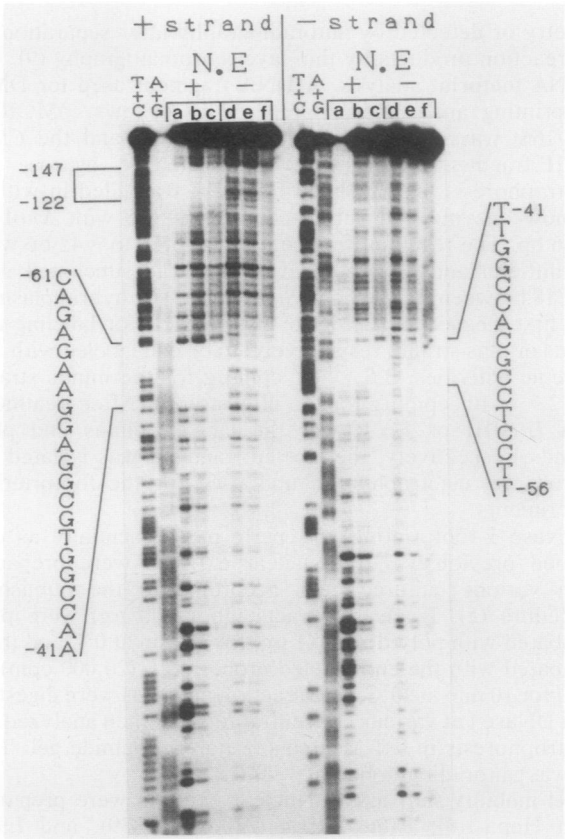

FIG. 3. Detection of the BTE-binding factor by DNase I footprint analysis. A DNA fragment from −166 to +42 bp of the P-450c gene was subcloned in pUC18 and used for DNase I footprint analysis, as described in Materials and Methods. Nuclear extracts from untreated Hepa-1 cells (about 20 μg on a protein basis) were preincubated with poly(dI-dC) (1 μg) for 15 min at 0°C and then incubated with the end-labeled probe DNA (20,000 cpm) of the plus or minus strand. The reaction mixtures were digested by various amounts of DNase I. Lanes: a to c, digestion by 0.6, 0.3, and 0.15 U of DNase I, respectively, in the presence of nuclear extract; e to g, digestion by 0.6, 0.3, and 0.15 U of DNase I, respectively, without nuclear extract.

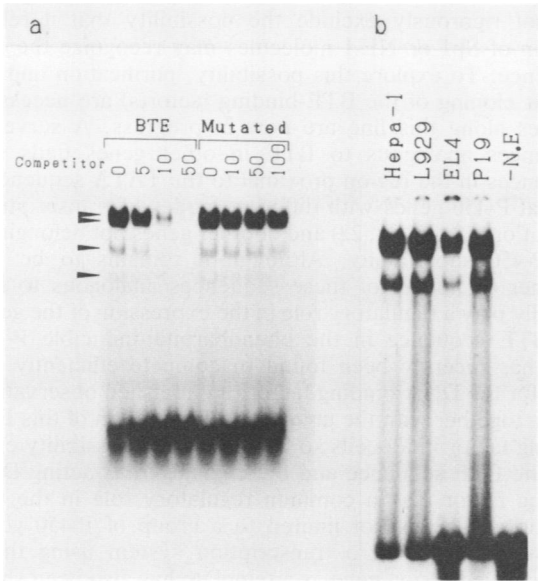

FIG. 4. Gel mobility shift assay. (a) Competition with various DNA fragments. A synthetic BTE sequence was used as a labeled probe. BTE and mutated BTE sequences (see Materials and Methods) were used as competitors. Binding reactions with Hepa-1 nuclear extract (4.2 μg on a protein basis) in 10 μl of the reaction mixture were carried out as described previously (6). After the reaction mixture was mixed with the probe DNA (20,000 cpm/120 pg), various amounts of competitor DNAs were added. The incubations were performed for 20 min at 20°C, and the reaction mixtures were subjected to gel electrophoresis. Fold molar excess of the cold BTE sequence and the mutated BTE sequence is indicated above each lane. The bands indicated by the fourth arrowhead from the top were not reproducible. (b) BTE binding activity of nuclear extracts from various culture cells. Nuclear extracts (8 μg) prepared from Hepa-1, L929, EL4, and P19 cells and used in the binding reaction, as described in Materials and Methods, are indicated.

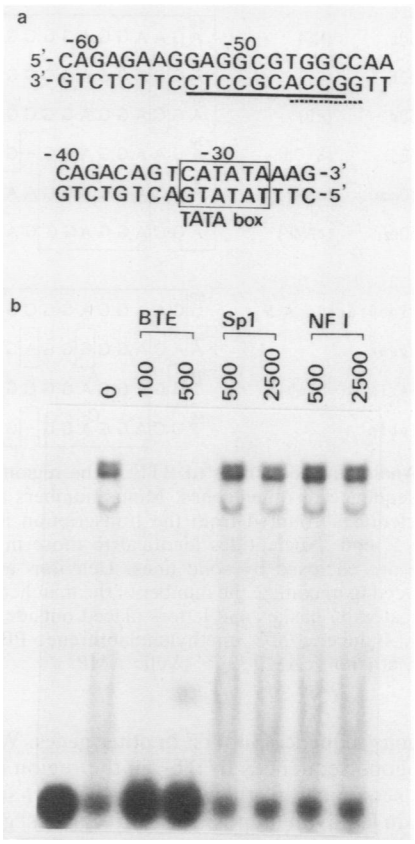

FIG. 5. Gel mobility shift assay of the BTE sequence with different competitors. (a) BTE region of the P-450c gene. Underlining and a broken line show the sequence satisfying the Sp1 and NF-1 consensus sequences, respectively. (b) Results of the gel retardation experiment obtained by using different oligonucleotides as competitors. A synthetic BTE sequence was used as a labeled probe. Sp1- and NF-1-binding sequences (see Materials and Methods) were synthesized and used as competitors. Nuclear extracts and assay conditions were the same as those described in Fig. 4. Fold molar excess of the cold BTE sequence, the Sp1 oligonucleotide, or the NF-1 oligonucleotide is indicated. Lane 0, No nuclear extract.

bp were also protected from DNase I digestion, suggesting the presence of another DNA-binding factor interacting with the sequences, but the role of this region in the transcriptional regulation of the P-450c gene remains to be seen.

To further characterize the binding factor(s) interacting with the BTE sequence, the gel mobility shift assay was carried out by using two synthetic oligonucleotides as probes. The sequences of the oligonucleotide probes are shown in Materials and Methods. One is the BTE sequence defined by DNase I protection analysis, and the other is the BTE sequence mutated at two nucleotides (see Materials and Methods). The BTE sequence gave approximately three retarded bands in the gel electrophoresis when incubated with the nuclear extracts from Hepa-1 cells (Fig. 4). Although the molecular mechanism giving rise to the three retarded bands has not yet been determined, it is clear that they were all retarded in the gel electrophoresis by the sequence-specific interactions between the BTE sequence and the binding factor(s) because excess amounts of the cold BTE probe competed efficiently with the labeled probe for the DNA-binding factor(s). In addition, it was demonstrated that the mutated BTE sequence neither competed with the labeled BTE sequence (Fig. 4) nor gave any retarded band with nuclear extracts from Hepa-1 cells (data not shown).

Then we investigated the distribution of the DNA-binding factor(s) in nuclear extracts from various cells, not only liver cells and a hepatocyte cell line but also lymphocyte-derived EL4, EC cell line P19, and L929 cells which do not express P-450c. These nuclear extracts were found to give retarded bands of the BTE sequence with the same mobilities as those observed with nuclear extracts from Hepa-1 cells (Fig. 4b). It is suggested, therefore, that the BTE sequence DNA-binding factor(s) is not unique to cells expressing the endogenous P-450c gene, but it may function as a sort of ubiquitous transcriptional regulatory factor for other genes as well. The BTE sequence contains a consensus sequence for the GC box $^{GG}_{TA}GGCG^{GGGC}_{TAAT}$ (4) and half of the consensus sequence for the NF-1-binding consensus sequence TTGGC (18) with one base substitution (Fig. 5). To clarify whether or not the BTE-binding factor(s) is a protein identical with Sp1 or NF-1, we performed a gel shift assay with synthetic probes for Sp1 and NF-1 as competitors. The oligonucleotide sequences for Sp1 and NF-1 are described in Materials and Methods. No competition was observed with either of the competitors (Fig. 5). These oligonucleotides were ligated separately in tandem to increase their binding affinity, but they did not yet show any competition with BTE (data not shown), indicating that the BTE-binding factor(s) is different from Sp1 and NF-1.

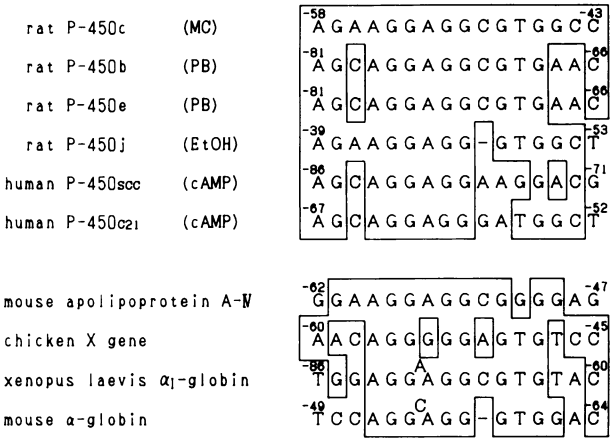

FIG. 6. Analogous sequences to BTE in the region proximal to the TATA sequence of other genes. Minus numbers are the positions of nucleotides counted from the transcription initiation site towards the 5′ end. Nucleotides identical to those in BTE of the P-450c gene are enclosed by solid lines. Deletions and additions were introduced to maximize the number of the matched nucleotides and are indicated by dashes and letters placed outside of the lines, respectively. Inducers: MC, methylcholanthrene; PB, phenobarbital; EtOH, ethanol; cAMP, 3′,5′-cyclic AMP.

Homologous sequences to BTE in other genes. We searched for homologous sequences to BTE in the region proximal to the TATA sequence of other genes and found several such sequences in equivalent positions on various genes, most prominently genes belonging to the P-450 gene superfamily which show different modes of gene regulation (Fig. 6); they are inducible by phenobarbital (P-450b and P-450e [21]), ethanol (P-450j [22]), and an endogenous effector, cyclic AMP (P-450SCC [15] and P-450C21 [12]). Some other genes (3, 11, 17, 20) also contain the analogous sequence in the promoter region. It remains to be seen, however, whether or not the sequences analogous to BTE in these genes are actually involved in transcriptional regulation.

DISCUSSION

It was clearly demonstrated that at least two kinds of *cis*-acting DNA elements with different regulatory activities are required for a high level of inducible expression of the cytochrome P-450c gene in response to xenobiotics such as 3-MC and TCDD. One is an inducible enhancer designated XRE, which occurs five times rather far upstream of the P-450c gene and drives specific transcription of the gene in response to the inducers, as reported previously (8). The other is located in the region proximal to the TATA sequence of the P-450c gene and plays a regulatory role in the basic transcription of the gene. It is therefore designated the basic transcription element, or BTE, because this DNA element does not appear to exhibit marked transcriptional enhancer activity by itself when placed in a different position upstream of the gene. This DNA element may function as a promoter element to help enhancer elements express their full activity. The importance of a fragment containing the BTE sequence in gene expression has been recently suggested in the corresponding mouse P-450 gene (16). Although the BTE sequence carries the consensus sequence of the GC box and a part of the NF-1-binding sequence, the BTE-binding factor seemed to be different from Sp1 and NF-1 by the competition assay (Fig. 5). This observation, however, did not rigorously exclude the possibility that a related protein of Sp1 or NF-1 molecules may recognize the BTE sequence. To explore this possibility, purification and molecular cloning of the BTE-binding factor(s) are necessary. Studies along this line are now in progress. A survey of sequences analogous to BTE in other genes finds such sequences in the region proximal to the TATA sequence of several P-450 genes with different modes of transcriptional regulation (12, 15, 21, 22) and of other genes not belonging to the P-450 superfamily. Although it remains to be seen whether or not all of these sequences analogous to BTE actually play a regulatory role in the expression of the genes, the BTE sequence in the phenobarbital-inducible P-450b gene has recently been found to compete efficiently with BTE for the DNA-binding factor (unpublished observation). Taken together with the ubiquitous distribution of this BTE-binding factor in all cells so far tested, the possibility exists that the BTE sequence and the cognate *trans*-acting DNA-binding factor play a common regulatory role in the transcription of genes not limited to a group of P-450 genes. Recently, our in vitro transcription system using the P-450c-derived fusion gene as a template has also been shown to require the BTE sequence for efficient transcription of the gene (unpublished data). This in vitro transcription system will elucidate in detail the regulatory role of the BTE sequence and the DNA-binding factor in the transcription of genes and how they work in combination with the XRE sequence and its related DNA-binding factor to achieve a high level of inducible expression of the P-450c gene in response to the xenobiotic inducers.

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