The Induction of Hepatic Selenium-Binding Protein by Aryl Hydrocarbon (Ah)-Receptor Ligands in Rats

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Selenium-binding protein (SeBP) is one of the cytosolic proteins which is believed to bind to selenium without containing selenocysteine. Although its physiological role remains to be identified, it has recently attracted interest due to its participation in the late stages of intra-Golgi protein transport. In a previous study, we found that SeBP in rat liver is significantly increased following administration of aryl hydrocarbon (Ah)-receptor ligands, 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) and 3-methylcholanthrene (MC). Little research has been carried out to examine the properties of the inducibility of SeBP by xenobiotics. The objectives of this study were to analyze the control of transcription of the message by Ah-receptor ligands. In this report, we cloned an inducible rat SeBP. The resulting PCR product consisted of the full coding sequence for the 472 amino acids. The deduced amino acid sequence exhibited homologies with mouse SeBP, mouse acetaminophen-binding protein and human SeBP of 92%, 93% and 87%, respectively. We also measured the mRNA level by quantitative reverse transcriptional (RT)-PCR. Treatment of rats with PCB126 resulted in significant induction of the mRNA of this protein in the liver compared with the control level. Our data represent the first report showing that SeBP is induced by an Ah-receptor ligand at the mRNA level.

Key words —— selenium-binding protein, acetaminophen-binding protein, PCB126, induction, cDNA cloning, quantitative RT-PCR

INTRODUCTION

Selenium-binding protein (SeBP) is one of the cytosolic proteins first reported by Bansal *et al*.[1)] and it is present in a variety of mouse and rat tissues. In particular, there are high levels in the testes, kidneys, ovaries, mammary glands and liver of mice.[2)] Recently, Yang and coworkers also reported that SeBP is one of the major non-heme proteins in erythrocytes from the subterranean mole rat.[3)] To date, the physiological role of SeBP has been unclear but, recently, Porat *et al*. have reported that SeBP participates in the late stages of intra-Golgi transport.[4)] Transport of proteins between intracellular membrane compartments is a highly regulated process that depends on several cytosolic factors. By using an intra-Golgi cell-free transport assay, they found that purified SeBP from bovine brain cytosol exhibits significant transport activity between intracellular membrane compartments.

Acetaminophen-binding protein (APBP) is known to be present in the liver[5)] and, although its physiological role is not yet completely clear, it is regarded as an important protein associated with hepatic necrosis in patients who have taken an acetaminophen overdose. SeBP is known to be highly homologous with APBP.[6)] In fact, the deduced APBP amino acid sequence differs from SeBP only at 14 residues.[6)] SeBP and APBP are also believed to bind selenium in a manner different from selenoproteins containing selenocysteine.[6,7)] The cDNA cloning of mouse SeBP and APBP has been reported by Bansal *et al*.[7)] and Lanfear *et al*.,[6)] respectively. The homologous human cDNA clone of SeBP has also been isolated by Chang *et al*.[8)] In that report, they described that the human cDNA sequences had 87.2 and 86.6% similarity when aligned with those of mouse SeBP and APBP, respectively.[8)]

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On the other hand, there has been little research into the regulation of SeBP expression. Yang and coworkers reported that the human SeBP gene showed differential expression in human prostate cancer cell lines.[9] In their report, they also clarified the downregulation of SeBP mRNA with treatment of dihydroxytestosterone, and suggested the possibility that the SeBP gene was under androgen regulation.[9] We have previously reported that aryl hydrocarbon (Ah)-receptor ligands, 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) and 3-methylcholanthrene (MC), and an antioxidant, butylated hydroxytoluene (BHT), increase the 54 kDa protein in rat liver using immunoblot analysis.[10–12] We have also shown that this 54 kDa protein is homologous with SeBP or APBP based on its partial amino acid sequence.[11] Therefore, one would expect that the 54 kDa protein is a counterpart of mouse SeBP and/or APBP.

In spite of those reports, the induction mechanisms of SeBP are not fully understood. The aims of this study are to analyze the nucleotide sequence of inducible rat SeBP cDNA and try to demonstrate upregulation at the mRNA level by an Ah-receptor ligand.

MATERIALS AND METHODS

Materials —— Ex Taq polymerase was purchased from Takara (Tokyo, Japan). PCB126 was synthesized by the method of Saeki *et al*.[13] The purity of PCB126 was confirmed to be at least 98% by GC using an electron-capture detector. All materials and reagents were of analytical grade and commercially available.

cDNA Cloning of Inducible SeBP from Rat Liver by RT-PCR —— Male Wistar rats (4 weeks old) were given MC intraperitoneally at a dose of 5 mg/kg body weight/day, dissolved in corn oil, for 3 days. The mRNAs were isolated from the liver using a Bio-Mag mRNA purification kit (Perspective Ltd., Japan, Tokyo). cDNA was synthesized from a part of the prepared mRNA (about 100 ng) using an RT-PCR High kit (TOYOBO Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. Oligo(dT)$_{20}$–P7 primer was used to prime on the first strand of cDNA synthesis. PCR for rat SeBP cDNA was performed using specific primers. The designed and used primers are shown in Fig. 1 and Table 1.

The sense primer p54F1, which corresponds to nucleotide positions –6 to +14, was designed from mouse and human SeBP, and the antisense primer, P7, was used against Oligo(dT)$_{20}$–P7 primer. Briefly, 5 μl synthesized cDNA solution was diluted to 25 μl with 1 × Plus buffer containing 100 nM of each specific primer, and 1.25 units Takara Ex Taq™ polymerase instead of the r-Taq DNA polymerase in the kit. These primers produced about a 1.6 kbp cDNA when used under the following cycling conditions: (1 min 30 sec at 94°C-1 min 30 sec at 60°C-3 min at 72°C) × 30 cycles. The PCR product was separated on 1% agarose gel, then the 1.6 kbp fragment was extracted using a StrataPrep® DNA Gel Extraction Kit (Stratagene, La Jolla, CA, U.S.A.). The extracted fragment was so faint that we performed a second PCR. After extraction, the extracted sample was diluted ten-fold with sterilized water. For the second PCR, 1 μl of the diluted products from the first PCR was diluted to 25 μl with 1 × Ex Taq™ buffer containing 100 nM specific primer as in the first PCR,

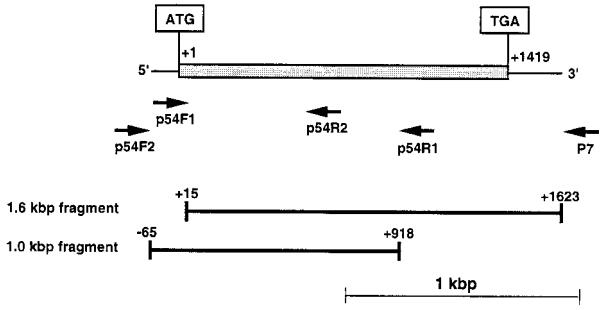

Fig. 1. Schematic Representation of Rat SeBP cDNA Clones

The initiation and stop codons are depicted at their positions according to the cDNA sequences in Fig. 1. The 1.6 and 1.0 kbp fragments were isolated by RT-PCR from liver mRNA of a rat treated with 3-methylcholanthrene. The sense primers R54F1 and R54F2 were designed from mouse and human SeBP cDNA, and the antisense primers R54R1 and R54R2 were designed from the sequence of newly cloned rat SeBP cDNA.

Table 1. Sequences of Primers

Primer name	Sequence
Selenium-binding protein	
p54F1	5′-AGCAGCATGCTACAAAATG-3′
p54F2	5′-GCACTTAATACCAGCACTGG-3′
p54R1	5′-ATGTCGGTGATCAAACCAGG-3′
p54R2	5′-TCCCTTTCACCTCGAAGGTC-3′
β-actin	
ACTF1	5′-CACCATGTACCCAGGCATCGC-3′
ACTR1	5′-AGGGGCCGGACTCATCGTACT-3′
P7	5′-CGCCAGGGTTTTCCCAGTCACGAC-3′
Oligo(dT)$_{20}$–P7	5′-CGCCAGGGTTTTCCCAGTCACGACT$_{20}$-3′

200 nM of each dNTP and 1.25 units of Takara Ex Taq™ DNA polymerase. The second PCR amplification was carried out as follows: 2 min at 94°C-(1 min 30 sec at 94°C-1 min 30 sec at 60°C-3 min at 72°C) × 30 cycles-3 min at 72°C.

The 1.6 kbp fragment contained an open reading frame (ORF) which corresponded to nucleotide positions at +15 to +1419, and a 3′-untranslated region. Next, to clarify the start codon of SeBP, we performed another PCR. The sense primer p54F2, which corresponded to nucleotide positions –86 to –66, was designed from mouse APBP, and the antisense primer p54R1 was designed from the analyzed cDNA sequence of rat SeBP (nucleotide positions 919 to 938) (Fig. 1 and Table 1). The two-step PCR was performed using the same method as for the 1.6 kbp fragment except for the primers used and cycling conditions. These primers produced about 1.0 kbp cDNA that contained a part of the open reading frame and 5′-untranslated region.

DNA Sequencing —— All PCR products were subcloned into the pGEM®-T Easy vector (Promega, Madison, WI, U.S.A.) and sequenced in both directions using the Applied Biosystems BigDye terminator DNA sequencing system (Applied Biosystems Inc., Foster City, CA, U.S.A.) and an automated sequencing system (ABI PRISM® 310 genetic analyzer). At least three independent clones of each cDNA fragment (the 1.6 and 1.0 kbp fragment) were sequenced in both directions to avoid any reading error.

mRNA Levels of SeBP Using Quantitative RT-PCR —— Firstly, the linearity of amplification of each RT-PCR was studied. The amplifications were carried out after serial dilution of cDNA samples and varying the number of amplification cycles. In each RT-PCR experiment, the first-strand cDNA sample containing the highest amount of SeBP cDNA expected was serially diluted and PCR-amplified to generate a calibration curve. A control experiment was carried out using β-actin specific primers with the same amount of cDNA. The band intensities of SeBP and β-actin at each cycle were estimated and the cycle numbers for quantitation of SeBP and β-actin were determined.

To study the increase in the SeBP mRNA level with PCB126 treatment, quantitative RT-PCR assay was performed. Male Wistar rats (4 weeks old) were given a single intraperitoneal injection of PCB126, dissolved in corn oil, at a dose of 10 mg/kg body weight. To compare the effects of reduced food consumption produced by PCB126 treatment, both free- and pair-fed controls were prepared, and the same volume of vehicle was injected. During examination, free-fed controls had free access to food and water, while pair-fed controls were only allowed the same amount of chow as that consumed by PCB126-treated rats. Then, 5 days after the injection, total RNA was isolated from each treated rat liver using the RNeasy Midi kit (QIAGEN, GmbH, Hilden, Germany). RT was then performed using the standard protocol of the RT-PCR high kit. Quantitative PCR reactions for SeBP and β-actin were performed on distinct sections. The quantitative PCR for SeBP was performed with specific primers, p54F1 and p54R2 (Fig. 1 and Table 1). Firstly, the RT product of each sample was diluted ten-fold with sterilized water. Then, 4 μl diluted RT product was used for PCR of SeBP, and amplification was performed as follows: 2 min at 94°C-(1 min at 94°C-1 min at 62°C-2 min at 72°C) × 27 cycles-3 min at 72°C. As a control, we measured the mRNA level of β-actin with specific primers (Table 1). One μl diluted RT product was used and the amplification schedule was as follows: 2 min at 94°C-(1 min at 94°C-1 min at 65°C-2 min at 72°C) × 27 cycles-3 min at 72°C. The identity of the PCR-amplified fragments was used to analyze each of the sequences.

Estimation of Band Intensity —— Estimation of the band intensity of PCR fragments was performed using a GT-7600U scanner (Epson, Tokyo, Japan) and NIH image software (version 1.52, Wayrve Rasband, Bethesda, MD, U.S.A.). Agarose gel stained with ethidium bromide was photographed then a digital image was computed using the scanner. The scan data were saved and imported into the NIH image software for subsequent analysis.

RESULTS

Cloning of Inducible SeBP cDNA from MC-treated Wistar Rat Liver

We previously reported that rat SeBP was increased following treatment with PCB126[10,11,14] which is an Ah-receptor ligand and one of the most toxic of the polychlorinated biphenyls.[15] To clarify this induction mechanism, we investigated the increase in the mRNA of rat SeBP produced by PCB126 treatment. To achieve the aim of this study, we tried to clarify the complete ORF sequence of inducible SeBP. Firstly, we performed PCR with p54F1 and P7 primers against the RT product from MC-treated rat liver. Then, a 1.6 kbp fragment was

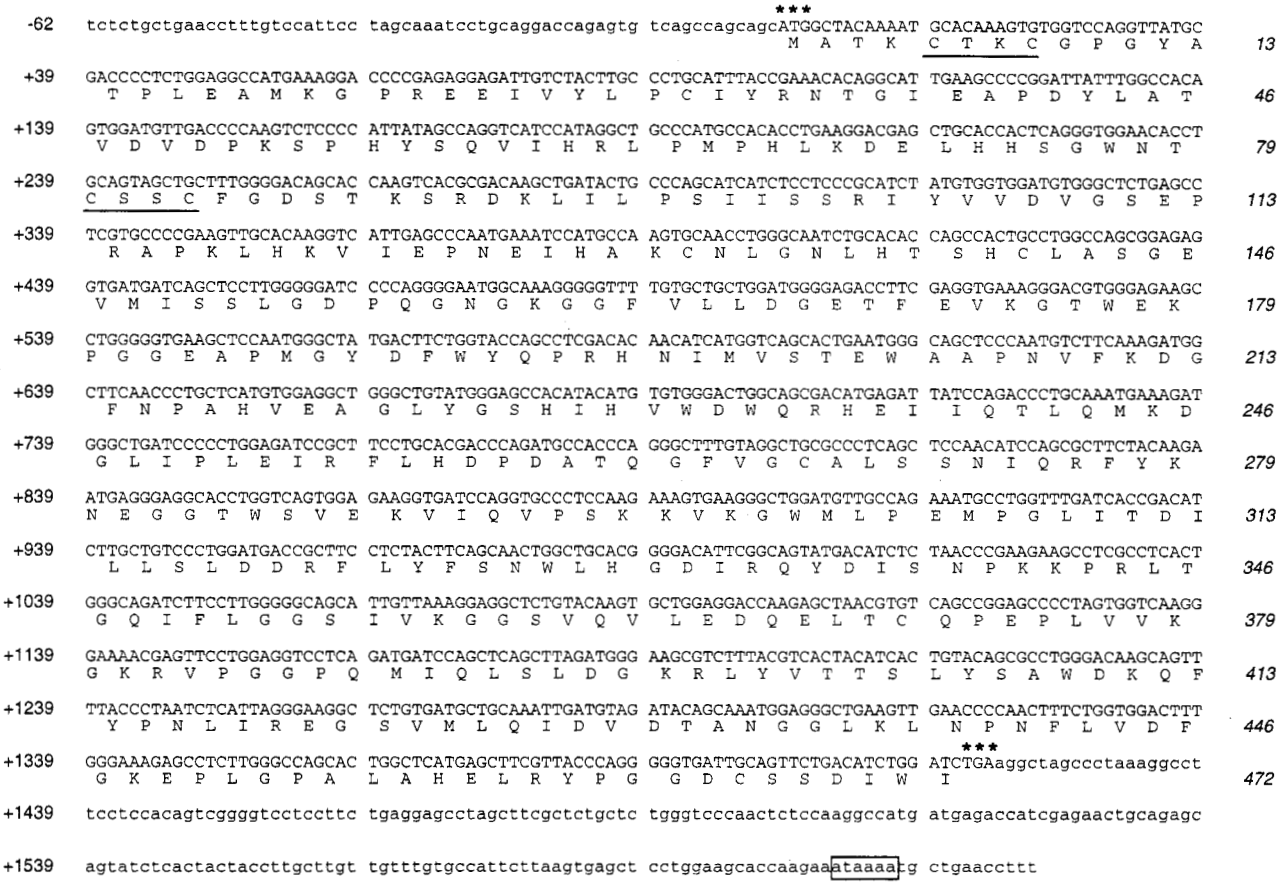

Fig. 2. cDNA and Deduced Amino Acid Sequence of Inducible Rat SeBP

The nucleotide sequence is numbered on the left, and numbering of the amino acid residues is on the right in italic numerals. Untranslated sequences are shown in lower case with the protein coding sequence in upper case. These include the putative ATG translation start codon and terminating TGA codon and are represented by asterisks. Also the putative AATAAA polyadenylation signal sequence is found at nucleotide position 1606 (represented by box). Two bis(cysteinyl) sequence motifs[14] are represented by underlines in the deduced amino acid sequence.

detected from PCR with those primers and it was subcloned into pGEM®-T Easy Vector was used to analyze the sequence. The 1.6 kbp fragments included the region from the 15th base of ORF to the 3′-untranslated region. Next, to clarify the start codon of SeBP, further PCR was performed. A 1.0 kbp fragment was detected from PCR with p54F2 and p54R1 primers, and the sequence was analyzed. The detected 1.0 kbp fragment included the start codon of the ORF of rat SeBP. Moreover, an overlapping sequence was identified with the analyzed 1.6 kbp fragment. From these two sequences, the complete ORF sequence of rat SeBP was identified.

Figure 2 shows the nucleotide sequence of the newly analyzed cDNA and the deduced amino acid sequence (The nucleotide sequence data will appear in the DDBJ/EMBL/GenBank nucleotide databases with the accession number "AB036799"). The cDNA was a 1685 bp sequence and contained the full length of ORF and part of the 3′- and 5′-untranslated region. The deduced amino acid sequence consisted of 472 amino acids. The ORF sequence in cDNA of rat SeBP has a homology of 93%, 94% and 87% when aligned with those of mouse SeBP, APBP and human SeBP, respectively. The amino acid sequence homology of rat SeBP, mouse SeBP, APBP and human SeBP was 92%, 93% and 87%, respectively. Unique amino acid motifs, the bis(cysteinyl) sequence motifs,[16] were found in the deduced amino acid sequence of rat SeBP. The first motif which was located between amino acid position 5 and 8 was well conserved among rats, mice and human. The second one found between amino acid position 80 to 83 was also aligned identically to that found in mouse SeBP and APBP, whereas the second motif in human SeBP was located at different region. These results support the finding that the 1685 bp cDNA that we obtained is the rat homologue of mouse SeBP.

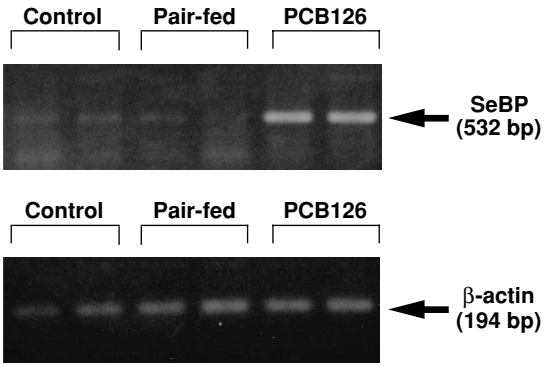

Fig. 3. Total mRNA Expression of Rat SeBP Analyzed by Quantitative RT-PCR

A 532 bp fragment encodes the open reading frame of SeBP and was amplified by RT-PCR. As a control, a 194 bp fragment of β-actin was also amplified. Both PCR products were separated on 1% agarose gel and stained with ethidium bromide. Stained bands were scanned and measured using quantification software. Details are described in Materials and Methods.

Table 2. Relative Abundance of SeBP mRNAs Estimated by RT-PCR of the Liver Total RNAs of Treated Rats

Treatment	SeBP/β-actin	X-fold induction
Control (2)	0.29	1.00
Pair-fed control (1)	0.18	0.62
PCB126 treatment (2)	1.13	3.90

The bands of the 532 bp (SeBP) and 194 bp (β-actin) PCR products on the gel shown in Fig. 3 were analyzed and quantified. The relative intensities of the bands are shown in the table. The data from the controls and PCB126 treated animals are the means of two independent experiments, but the data from pair-fed controls represent the value from only one experiment. The numbers in parentheses represent the number of samples quantified.

Increase in SeBP mRNA in Rat Liver Following PCB126-Treatment

Analysis of the increase in rat SeBP gene expression was performed at the mRNA level employing rat SeBP-specific RT-PCR. Total RNA was isolated from the liver of control-, pair-fed- and PCB126-treated rats, then RT-PCR was performed. The mRNA level of β-actin was measured as a control. Quantitative RT-PCR of rat SeBP with p54F1 and p54R2 primers designed from the analyzed cDNA sequence resulted in the expected band of 532 bp, and β-actin with primers ACTF1 and ACTR1 resulted in a band of 194 bp. When the PCR was terminated at 20, 22, 24, 26, 28, 30, 32 and 34 cycles and the band intensities were scanned and quantified, PCR amplification was found to be linear between 24 and 28 cycles (data not shown). Also, both bands were easily detected over 24 cycles. Based on these results, each subsequent analysis was conducted for 27 cycles. As shown in Fig. 3, mRNA of rat SeBP was notably induced by PCB126 treatment. Quantification was accomplished by measuring the level of each transcript relative to that of β-actin and the result is presented in Table 2.

DISCUSSION

In this study, we have clarified the full length of the nucleotide sequence of cDNA of inducible rat SeBP and showed the induction of the mRNA with a high affinity Ah-receptor ligand, PCB126. We previously reported that SeBP was increased by treatment of PCB126 in rat liver.[10,11,14] Although the regulatory region of rat SeBP gene has not been identified, our result supports the existence of the region that is regulated by the Ah-receptor ligand. Further studies are needed to clarify the induction mechanism of SeBP following treatment with Ah-receptor ligands.

It has been reported that SeBP and APBP are expressed in mouse liver.[6] However, in other species like *C. elegance* [Burton, J. (2000) EMBL accession number "Z77668"], *A. Talliana* [Bevan, M. *et al*. (1999) EMBL accession number "Z97335"] and humans,[8] transcription of SeBP was present but not that of APBP. In this report, we have clarified the nucleotide sequence of the cDNA of inducible SeBP which was highly homologous with that of mouse SeBP. The primers used in this study, p54F1 and p54F2, were common on the cDNA of mouse SeBP and APBP. In this serial experiment, we could not find other highly homologous cDNA which differed from the cDNA of inducible SeBP. Thus, it is conceivable that the cDNA of the SeBP that we reported is the dominant one for induction by the Ah-receptor ligand.

Recently, participation of SeBP in the late stages of intra-Golgi transport has been reported.[4] Transport of proteins between intracellular membrane compartments is a highly regulated process that depends on several cytosolic factors. By using the intra-Golgi cell-free transport assay, these authors purified a 56 kDa protein from bovine brain cytosol that shows significant transport activity. Partial sequences of four tryptic peptides obtained from the 56 kDa protein revealed that is was identical to a cytosolic protein previously characterized as an SeBP. They proposed that SeBP is involved in regulating docking or fusion of vesicles. Considering the recent findings of fusion between vesicles and their

target, they suggested that the SeBP acts along with other soluble factors to accelerate and regulate this process. Although they were cautious when they suggested that this protein may have more than one physiological role, their initial finding is important in understanding the physiological role of this protein. In that report, they also showed that increasing amounts of recombinant SeBP significantly stimulated the cell-free intra-Golgi transport activity. It is believed that the Golgi apparatus is one of the key organelles for not only modification, intracellular transport and distribution of glycoproteins but also excretion of some endocrine proteins. In our report, it was shown that SeBP is induced by treatment of the Ah-receptor ligand, PCB126, at the mRNA level. Based on those results, although we could not elucidate the effect of induction of SeBP, it is to be expected that the induction of SeBP with treatment of Ah-receptor ligands might affect the physiological function of the Golgi apparatus.

Dioxin-related compounds like PCB126 are known to be important Ah-receptor ligands which markedly induce a number of drug metabolizing enzymes like cytochrome P4501A1.[17,18] The induction mechanism mediated by the Ah-receptor is considered to take place as follows.[19] The Ah-receptor is usually combined with a homodimer of heat shock protein (HSP) 90 that is displaced on ligand binding. When a ligand such as dioxin binds to the Ah-receptor, HSP90 dissociates and the ligand-Ah-receptor complex is translocated to the nucleus. Next, a complex between the ligand-Ah-receptor and Ah-receptor nuclear translocator (Arnt) is formed. Then, this complex transactivates the gene-encoding enzyme through binding to the xenobiotic responsive element (XRE) located 5′-upstream of the gene.

However, another possibility for induction by Ah-receptor ligands has been suggested. For example, CYP1A2 is one of a cytochrome P4501A subfamily and is known to be induced by environmental pollutants, such as halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons.[20] However, for the most part, the molecular mechanisms that control the expression of the CYP1A2 gene are unknown. Although regulation of the CYP1A2 gene by dioxin and MC in mice[21,22] occurs through transcriptional activation, DNA sequence analysis has not identified XREs in the noncoding region of the CYP1A2 gene from any species.

In this report, our data showed the inducibility of SeBP by Ah-receptor ligands. The relationship of the induction of SeBP and its involvement in *trans*-Golgi transport are still unclear. However, our results provide interesting new insights into some aspects of these processes.

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