The Rat Liver Glutathione *S*-Transferase Ya Subunit Gene: Characterization of the Binding Properties of a Nuclear Protein from HepG2 Cells That Has High Affinity for the Antioxidant Response Element

Suxing Liu and Cecil B. Pickett*

Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, New Jersey 07033-0539

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ABSTRACT: A nuclear protein from HepG2 cells (YABP) that binds the antioxidant response element (ARE), which is required for activation of the rat glutathione *S*-transferase (GST) Ya subunit gene by planar aromatic compounds and phenolic antioxidants, was further characterized by quantitative competition binding experiments and DNA mutational analysis. The apparent dissociation constant of the YABP−ARE complex was estimated as ≤0.77 nM, suggesting that the YABP has very high affinity for the ARE. There is no difference in the affinity of the YABP for the ARE when HepG2 cells are treated with inducers that transcriptionally activate the GST Ya subunit gene. Quantitative competition binding analyses in conjunction with mutagenesis of the ARE revealed that an 11-nucleotide region in the 41-nucleotide ARE, 5′-GGTGACAAAGC-3′, is responsible for binding to the YABP. Eight nucleotides of this core sequence are in close proximity to the YABP, indicating that there is a broader spectrum of protein contact points than those required for the transcriptional activation. van't Hoff analysis of effects of temperature on binding has revealed that the binding reaction is governed mainly by entropy changes, which could result from conformational changes in the YABP and/or the ARE upon the formation of the complex. In addition, the native molecular weight of the YABP was determined to be 74 300 using gel filtration chromatography. These data together with previous UV cross-linking data suggest that the YABP exists as a heterodimer.

The glutathione *S*-transferases (GSTs)[1] are a family of phase II enzymes that catalyze the conjugation of glutathione with endogenous and xenobiotic substrates which have electrophilic functional groups (Pickett & Lu, 1989). A line of evidence from our laboratory has shown that a GST Ya subunit gene is transcriptionally activated in response to various xenobiotics. A cis-acting regulatory element, the antioxidant response element (ARE), in the 5′-flanking regions of a rat GST Ya subunit gene was identified by deletion constructs and transfection experiments and shown to mediate basal and inducible gene expression in response to metabolites of several planar aromatic compounds and phenolic antioxidants through a mechanism that does not require a functional Ah receptor (Rushmore & Pickett, 1990, 1993; Rushmore et al., 1991). The core sequence of the ARE, 5′-GTGACAAAGC-3′, is the minimal sequence required for inducible expression. DNase I footprinting experiments demonstrated that a nuclear protein(s) from HepG2 cells (YABP) bind(s) to a region of the Ya promoter that encompasses the ARE. DMS methylation experiments have demonstrated that the GpG dinucleotide preceding the core sequence and the G residue within the TGAC tetramer of the core sequence are in close contact with the YABP from HepG2 cells (Nguyen & Pickett, 1992). These observations support earlier functional data that demonstrated the ARE core sequence is required for basal and inducible expression (Rushmore et al., 1991). AREs have also been identified in the 5′-flanking regions of rat glutathione *S*-transferase P gene, mouse glutathione *S*-transferase Ya subunit gene, and human and rat NADP(H):quinone reductase gene (Okuda et al., 1989; Friling et al., 1992; Favreau & Pickett, 1991, 1993; Xie et al., 1995).

Interestingly, DNase I footprint and DMS methylation patterns of the ARE with nuclear extract from tBHQ-treated HepG2 cells were not changed compared to patterns obtained using nuclear extract from untreated cells. Similar data were obtained in qualitative binding assays (Nguyen & Pickett, 1992). Although data to date suggest that the ARE sequence is critical for binding, observations from DNase I protection and transient transfection experiments suggested that the upstream region of the core sequence also interacts with the YABP (Nguyen & Pickett, 1992; Rushmore et al., 1991). In this paper, we employed quantitative competition assays and DNA mutational analysis to further characterize the ARE−YABP interaction. Our data suggest that an 11-nucleotide core sequence of the ARE, 5′-GGTGACAAAGC-3′, is essential for high-affinity binding for the YABP but there is a broader spectrum of protein contact points than those required for transcriptional activation.

MATERIALS AND METHODS

Cell Culture and Preparation of Nuclear Extracts. Human heptoma cells, HepG2, were maintained as described previously (Nguyen & Pickett, 1992). The nuclear extracts from HepG2 cells were prepared from untreated cells or cells treated with 60 μM *tert*-butylhydroquinone (tBHQ, Aldrich) for 5 h at 90% confluence using the methods described (Dignam et al., 1983). After dialysis with buffer D [20 mM

* To whom correspondence should be addressed. Telephone: (908) 298-7300. Fax: (908) 298-7514. E-mail: cecil.pickett@spcorp.com.

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[1] Abbreviations: GST, glutathione *S*-transferase; QR, quinone reductase; ARE, antioxidant response element in the 5′-flanking region of rat glutathione *S*-transferase Ya subunit gene; YABP, ARE-binding protein from HepG2 cells; tBHQ, *tert*-butylhydroquinone; DMS, dimethyl sulfate; SD, standard deviation.

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HEPES, pH 7.9, 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.55 mM phenylmethanesulfonyl fluoride, and 0.5 mM dithiothreitol] for 4−5 h, nuclear extracts were centrifuged to remove the precipitates, aliquoted, and frozen at −70 °C. The concentration of nuclear proteins was determined using the protein determination kit (BioRad Laboratories, Inc.) according to manufacturer's instructions.

Partial Purification of the Nuclear Protein from HepG2 Cells. All operations were carried out at 4 °C. The nuclear extract from HepG2 cells was loaded onto a Superdex 200 Prep grade 60/600 column (Pharmacia, Inc.) preequilibrated with buffer D, and chromatographed at flow-rate of 1 mL/min. Fractions (2 mL/fraction) were collected and analyzed by gel mobility shift assays for specific DNA-binding activity (Figure 1B). The identity of the YABP was confirmed by competition experiments using the partially purified protein from fraction 57 (Figure 1B). Peak fractions were pooled and stored in small aliquots at −70 °C. The Superdex 200 Prep grade 60/600 column was calibrated with both low molecular weight and high molecular weight gel filtration calibration kits (Pharmacia, Inc.). Standards in the kits were prepared in buffer D. The void volume (V_o = 108.6 mL) of the column (total bed volume, V_t = 320 mL) was determined by the elution position of blue dextran 2000, the elution positions (V_e) of standard proteins were determined by A_{280nm}, and K_{av} was calculated using the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$ (Ackers, 1970). The K_{av} values were then plotted against the logarithm of their molecular weight and fit to a linear line, shown in Figure 1A.

Oligonucleotides. All oligonucleotides containing the ARE binding site or its mutants were synthesized and purified as described previously (Rushmore & Pickett, 1990, Nguyen & Pickett, 1992). Their sequences were confirmed by the dideoxy sequencing method. Concentrations of oligonucleotides were determined from the absorbance at 260 nm with base line correction and the base composition of the oligonucleotide on a Perkin-Elmer Lambda Bio UV/Vis spectrometer (Perkin-Elmer Corp.). To prepare an unlabeled double-stranded DNA for competition experiments, purified complementary strands were mixed in equal amounts, heated up to 70 °C for 10 min, and incubated at 37 °C for 30 min to permit the formation of a duplex DNA. The 41-nucleotide ARE coding and noncoding strands were end-labeled separately with [γ-^{32}P]ATP using T4 DNA polynucleotide kinase and annealed as described above, and then labeled oligonucleotides were purified by gel electrophoresis. The resulting labeled double-stranded DNA was quantitated from the absorbance reading at 260 nm multiplied by a factor of 50.

Electrophoretic Mobility Shift Assay for DNA Binding. Binding reactions were performed by incubating 0.6 μg of partially purified protein and 0.6 nM labeled probe at 30 °C for 2 h with 4 ng/μL of the random oligonucleotide for quantitative binding reactions or 0.1 μg/μL of poly(dI-dC) for determining the elution position of the YABP shown in Figure 1B. The binding buffer was 10 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 100 mM KCl, 50 μg of bovine serum albumin/mL, and 1 mM dithiothreitol. All proteins were freshly diluted into binding buffer just before use. Complexes were separated on a 6% polyacrylamide gel at 200 V in 0.5 × TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.3).

Quantitative Competition Binding Experiments and Data Analysis. Determination of IC_{50} (the concentration of unlabeled DNA at which the maximum binding of the labeled DNA is displaced by 50%) for a given competitor and a protein involves incubating various concentrations of unlabeled DNA while keeping concentrations of labeled probe and the partially purified protein constant. The binding assay was performed under standard conditions as described above, except that a competitor (wild-type ARE or its mutants) is added. Concentrations of a competitor were varied 4 orders of magnitude in order to give a fine shape to the competition curve. In all competition reactions, the total concentration of the labeled probe was in vast molar excess over the total concentration of the YABP. Time courses of the binding activity with labeled probe alone or with both labeled probe and a competitor have been determined to ensure that there is an adequate time for the binding reaction to achieve equilibrium and that the protein does not lose its binding activity during a period of incubation time. To quantify binding, gel areas corresponding to the retarded bands were excised, and the radioactivity retained was measured in a liquid scintillation counter in triplicate.

Binding occurring in the presence of the competitor was expressed as the percent of maximal binding, % B/B_0, where the amount of labeled bound DNA (total minus blank) in the absence of the competitor was taken as B_0. The percentage of maximal binding *versus* competitor concentration, [I], was fit to the equation:

$$\% \frac{B}{B_0} = \frac{100 \times IC_{50}}{IC_{50} + [I]}$$

with the computer program UltraFit (Biosoft, 1992). Since IC_{50} is the function of the concentration of labeled probe, measurements of IC_{50} for the wide-type ARE were made for every batch of labeled probe, with which all other IC_{50} values were normalized. In addition, the probe was used not longer than 2 weeks after labeling to minimize the background due to radiochemical nicking of the DNA. All IC_{50} values were determined from at least three independent experiments, and their standard deviations were all less than 20%.

RESULTS AND DISCUSSION

Determination of the Native Molecular Weight of the YABP. UV cross-linking of the YABP to the ARE suggested that the YABP consists of two polypeptides of apparent molecular weights of 28 000 and 45 000 (Nguyen & Pickett, 1992). To further elucidate the subunit structure and native molecular weight of the YABP, the native molecular weight of the protein was determined by gel filtration chromatography.

The elution position of the YABP was determined by gel mobility shift assays (lanes 1−11) and confirmed by competition experiments (lanes 12−14), shown in Figure 1B. The mobility of the complex formed with the partially purified protein is the same as that seen with the nuclear extract from HepG2 cells (data not shown). Binding activities peaked in fractions 58 and 59, corresponding to an average K_{av} of 0.42. Based on the calibration curve using known protein standards, the YABP appears to have a native molecular weight of approximately 74 300 (Figure 1A).

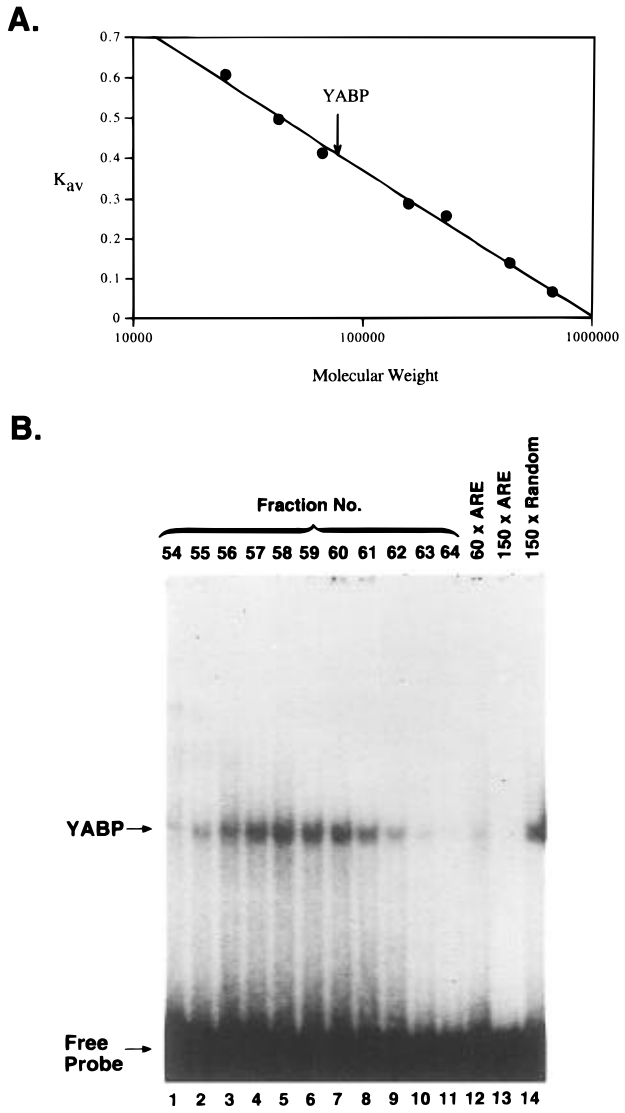

FIGURE 1: Determination of the native molecular weight of the YABP. (A) The calibration curve for Superdex 200 Prep grade 60/600 was determined as described under Materials and Methods. The K_{av} value for each protein standard (on the linear scale) was plotted against the corresponding molecular weight (on the logarithmic scale). The arrow indicates the position of the YABP. (B) Nuclear extracts of HepG2 cells were fractionated on the same column, binding activities of fractions with the ARE were determined by electrophoretic gel shift assays (lanes 1−11), and the identity of the YABP was confirmed by competition experiments (lanes 12−14) as described under Materials and Methods. Fraction numbers are indicated above lanes 1−11, and molar excesses of competitor DNA used in competition binding reactions are indicated above lanes 12−14. The arrows indicate the positions of the shifted YABP−ARE complex and free probe. The native molecular weight of the YABP was then estimated from the calibration curve as 74 300.

Together with the previous UV cross-linking data that showed the presence of two polypeptides of molecular weights of 28 000 and 45 000, the current data suggest that the intact YABP exists as a heterodimer.

Determination of the Binding Affinity of the YABP from tBHQ-Treated HepG2 Cells versus Untreated Cells with the ARE. In our previous experiments, we found that the footprint and methylation patterns of the ARE were not altered after tBHQ treatment (Nguyen & Pickett, 1992). To further address the question of inducible binding, we performed quantitative competition experiments with partially purified YABP from tBHQ-treated and untreated HepG2 cells. Using 0.6 nM labeled ARE as a probe and unlabeled ARE as a competitor, the IC_{50} values at 30 °C were 0.77 ± 0.03 nM with partially purified YABP from tBHQ-treated HepG2 cells and 0.70 ± 0.01 nM with partially purified YABP from untreated cells (Figure 2). The data suggest that there is no measurable difference in the affinity of the YABP for the ARE after treatment of cells with the inducer.

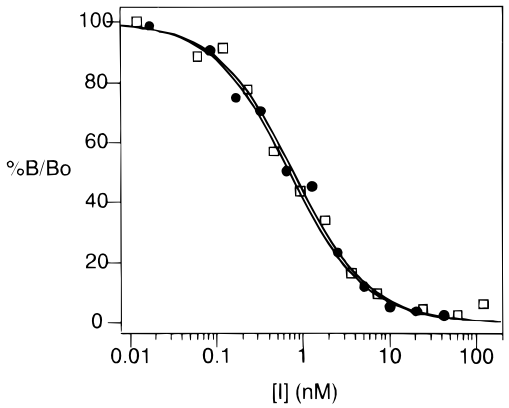

FIGURE 2: Competition curves using the 41-nucleotide ARE as a competitor with the YABP from tBHQ-treated cells and that from untreated cells. Experimental data are shown as circles for the YABP from untreated cells and as squares for the YABP from tBHQ-treated cells. The solid lines for the binding data are the computer fit of experimental data to the equation: $\% B/B_0 = 100IC_{50}/(IC_{50} + [I])$, using the program UltraFit (Biosoft, 1992). The IC_{50} values obtained from nonlinear regression analysis at 0.6 nM labeled ARE probe and 30 °C are expressed as the mean ±SD of at least three separate experiments: 0.77 ± 0.03 nM for the YABP from tBHQ-treated HepG2 cells; 0.70 ± 0.01 nM for the YABP from untreated cells.

Since the IC_{50} for the ARE is similar to the concentration of radiolabeled probe, the apparent dissociation constant of the YABP−ARE complex can be estimated as $\leq 7.7 \times 10^{-10}$ M (Bennett, 1978). Therefore, there is an approximately -53 kJ·mol^{-1} favorable change in free energy upon formation of the YABP−ARE complex, suggesting that YABP binds to the ARE very tightly.

Major Recognition Site of the ARE. The functional ARE core sequence, 5′-gGTGACaaaGC-3′, was identified as a cis-acting element responsible for inducible expression by planar aromatic compounds and phenolic antioxidants (Rushmore et al., 1991). An oligonucleotide that had a mutated ARE core sequence could not compete with wild-type ARE in gel shift experiments using nuclear extracts from HepG2 cells. These data suggest that the ARE core sequence is the high affinity recognition motif for the YABP (Nguyen & Pickett, 1992). However, DNase I footprinting and transient transfection experiments suggested that the upstream region of the core sequence also interacts with the YABP and is required for full activity of the ARE (Nguyen & Pickett, 1992; Rushmore et al., 1991). In order to assess contributions of upstream and downstream regions of the core sequence to binding, a quantitative competition binding analysis and DNA mutational analysis were carried out.

We first determined the competition curve of a random oligonucleotide and demonstrated that it was unable to compete with the ARE throughout the entire concentration range analyzed (Figure 3). The AREM#5, which has a scrambled upstream sequence but maintains the ARE core sequence and downstream region intact, had an IC_{50} of 1.76

A.

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ARE:  GAGCTTGGAAATGGCATTGCTAATGGTGACAAAGCAACTTT
AREM#5:  TATTCGATGATCACACGATGTACAGGTGACAAAGCAACTTT
AREM#7:  TATTCGATGATCACACGATGTACAGGTGACAAAGCGGTCCC
Random:  ACTTAAAGACTCCGTACTTGGAGGATGTCGGTAGATAATGA
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B.

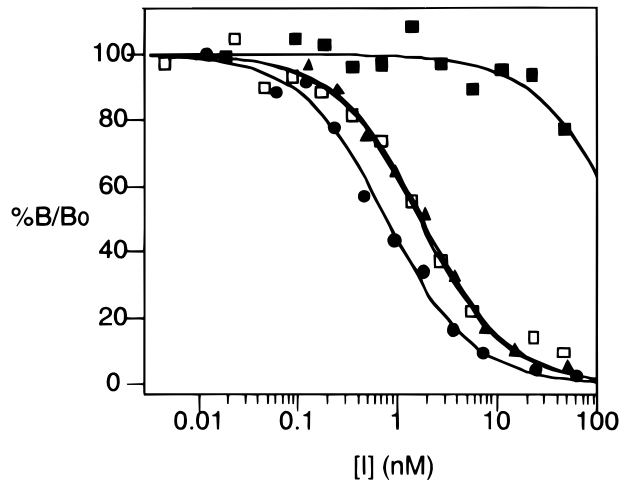

FIGURE 3: Major recognition site of the 41-nucleotide ARE. (A) Sequences of synthetic oligonucleotides used as competitors in quantitative competition binding experiments. Only the coding strand is shown. The mutated regions of AREM#5 and AREM#7 are underlined. (B) Competition curves for various competitors with the YABP employing the labeled ARE as a probe. Experimental data are shown as filled squares for the random oligonucleotide, circles for the ARE, open squares for AREM#7, and triangles for AREM#5. The solid lines for binding data are the computer fit of the experimental data to the equation: $\% B/B_0 = 100IC_{50}/(IC_{50} + [I])$, using the program UltraFit (Biosoft, 1992). The IC_{50} values obtained from nonlinear regression analysis at 0.6 nM labeled ARE probe and 30 °C are expressed as the mean ±SD of at least three separate experiments: 0.77 ± 0.03 nM for the ARE; 1.76 ± 0.22 nM for AREM#5; and 1.69 ± 0.06 nM for AREM#7. The protein used in the assays was partially purified from tBHQ-treated HepG2 cells.

± 0.22 nM. These data indicate that mutation of the entire upstream region lowers the binding free energy by 2.1 kJ·mol^{-1}, which is approximately 4% of the wild-type ARE. Thus, some nucleotide(s) in the upstream region of the core sequence is (are) in contact with the YABP, but they are not essential for binding. These data are in reasonable agreement with the observations from footprinting experiments and transient transfection assays performed previously (Nguyen & Pickett, 1992; Rushmore et al., 1991). We further examined the downstream region of the core sequence by using AREM#7, which gives an IC_{50} of 1.69 ± 0.06 nM (Figure 3). AREM#7 has a similar IC_{50} to AREM#5, suggesting that nucleotides in the downstream region do not contribute significantly to binding. A comparison of the IC_{50} values for the random oligonucleotide, AREM#5, AREM#7, and ARE suggests that the 11-nucleotide sequence, 5′-GGTGACAAAGC-3′, is responsible for the tight association of the ARE−YABP complex. These data are consistent with previous functional and qualitative binding experiments which indicated that an 11-nucleotide core sequence was required for binding to the YABP.

Contributions of Individual Base Pairs in the Major Recognition Site to the Specificity of the YABP−ARE Complex. In order to investigate contributions of individual base pairs in the core sequence to the specificity of the YABP−ARE complex, a series of mutant oligonucleotides with single base substitutions were synthesized (Figure 4A). The complementary oligonucleotides used as competitors were annealed and quantitated, and their IC_{50} values were determined. The change of relative binding Gibbs free energy is presented as a function of mutated nucleotide in the 11 base pair core sequence of the ARE (Figure 4B). Changing any of the nucleotides within the tetramer, TGAC, which is required for both basal and inducible activity (Rushmore et al., 1991), resulted in a 2−5 kJ/mol decrease in the relative binding Gibbs free energy as compared with that for the ARE. These data suggest that they are required for binding. For the GC nucleotides at the 3′ end of the 11-nucleotide core sequence, which are required for inducible activity, mutant 10G/A had an increased IC_{50} whereas mutant 11C/A gives an IC_{50} value that is similar to that of the wild-type ARE. In transfection experiments, the point mutations of nucleotides at positions 7 through 9, AAA, were shown to have no effect on basal and inducible CAT activities as compared to the controls (Rushmore et al., 1991). Interestingly, in our quantitative experiments, mutant 7A/G showed

A.

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ARE:   GGTGACAAAGC
1G/A:  AGTGACAAAGC
2G/A:  GATGACAAAGC
3T/G:  GGGGACAAAGC
4G/A:  GGTAACAAAGC
5A/G:  GGTGGCAAAGC
6C/A:  GGTGAAAAAGC
7A/G:  GGTGACGAAGC
8A/G:  GGTGACAGAGC
9A/G:  GGTGACAAGGC
10G/A: GGTGACAAAAC
11C/A: GGTGACAAAGA
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B.

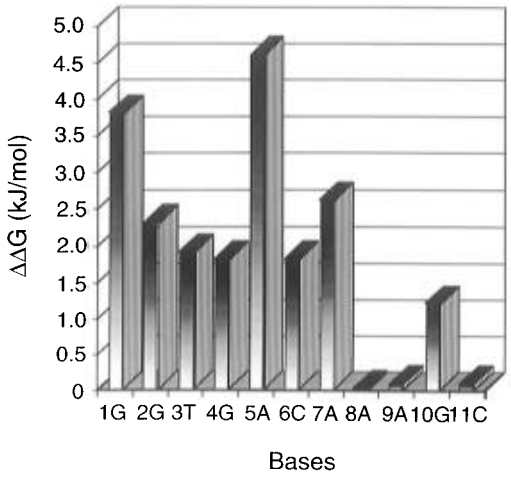

FIGURE 4: Influence of base substitutions within the core sequence of the ARE on YABP binding. (A) Sequences of point mutants used as competitors in quantitative competition binding experiments. Names of oligonucleotides indicate the position followed by the substituted base. Only 11 bases in the core sequence of the ARE and coding strands of 41-nucleotide competitors are shown. Mutated positions are shown in boldface. (B) The reduction in relative binding Gibbs free energy (kJ/mol), $\Delta\Delta G = RT \ln (IC_{50}^{mutant}/IC_{50}^{ARE})$, is presented as a function of mutated nucleotide in the 11-nucleotide core sequence of the ARE. The protein used in the assays was partially purified from tBHQ treated HepG2 cells.

an increased IC_{50} as compared to the ARE whereas those for the other two mutations at positions 8 and 9 were identical to that for the wild type ARE. Taken together, our data suggest that 8 out of 11 nucleotides in the core sequence of the ARE, 5′-GGTGACAaaGc-3′, are in close proximity to the YABP. Mutations at 1G and 5A in the core sequence appear to have the greatest effect on binding. Although they might not interact with the protein, 8A and 9A in the core sequence could affect their binding affinity for the YABP by influencing the conformation of the ARE. Therefore, the ARE core sequence is primarily responsible for tight binding, but there is a broader spectrum of protein contact points than those required for functional activity.

Although there is a similarity between the consensus AP-1 binding site (5′-TGACTCA-3′) and essential nucleotides within the ARE core sequence (5′-TGAC-3′), we have previously demonstrated that the ARE is not a high-affinity binding site for the Jun/Fos heterodimer (Nguyen et al., 1994). These results have been confirmed recently by Yoshioka et al. (1995).

Temperature Dependence of Binding. The standard enthalpy change of the binding reaction ($\Delta H°$) from the van't Hoff equation can be estimated from changes of the IC_{50} at different temperatures. The IC_{50} for the ARE is determined as 1.13×10^{-10} M^{-1} at 4 °C. By the van't Hoff equation, $\Delta H°$ for the binding is approximately $+10$ kJ·mol^{-1}. The standard entropy change ($\Delta S°$) can then be calculated as approximately $+208$ J·mol^{-1}·deg^{-1} at 30 °C since $\Delta G°$ is approximately -53 kJ·mol^{-1}. Therefore, the entropy change is the main driving force for the noncovalent binding processes. This result is consistent with the site-specific binding of other transcription factors, such as adenovirus major late transcription factor, whose binding is also governed primarily by entropy changes (Chodosh et al., 1986). It is quite possible that the change in entropy arises from changes in the conformation of YABP and/or the ARE upon the formation of the complex. These data are consistent with the change in binding affinity of the triple mutation in positions 7−9 of the core sequence with the YABP. The specificity of binding might result from a lack of steric hindrance between the YABP and the ARE. It has been reported recently that sequence-specific recognition of DNA by the serum response factor depends on inducible conformational changes of the serum response element (Pellegrini et al., 1995) and conformational changes of the transcription factor Ets-1 (Peterson et al., 1995).

Conclusions. A nuclear protein with a native molecular weight of 74 300 from HepG2 cells exists as a heterodimer and binds specifically to the ARE found in the 5′-flanking region of the rat GST Ya gene. The estimated equilibrium dissociation constant of the YABP−ARE complex indicates the high stability of the complex. Quantitative competition binding analyses in conjunction with mutagenesis of the 41-nucleotide ARE revealed that an 11-nucleotide core sequence is primarily responsible for the tight binding but there is a broader spectrum of protein contact points than those required for the transcriptional activation. The binding specificity of the YABP is the result of many independent interactions none of which is dominant. Finally, the binding affinity of the protein varied little following tBHQ treatment of HepG2 cells and transcriptional activation of the Ya subunit gene. The data suggest that the high-affinity binding to the ARE displayed by the YABP does not result in transcritional activation of the GST Ya gene.

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