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Cloning and Characterization of Chicken YB-1: Regulation of Expression in the Liver

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A cDNA expression library constructed from day 9 embryonic liver was screened with a previously identified protein binding site in the flanking region of the liver-specific, estrogen-dependent avian apoVLDLII gene. Two of the clones isolated were shown to encode the chicken homolog of the Y-box binding protein, YB-1 (dbpb), which we have designated chkYB-1. This protein was originally identified in avian extracts by virtue of its ability to bind to two reverse CCAAT motifs in the Rous sarcoma virus enhancer. Since its identification, additional nucleic acid binding properties have been ascribed to its homologs, or closely related proteins, in other species. We have determined the sequence of chkYB-1, investigated its ability to bind to sites known to be involved in tissue-specific expression in the liver, and examined factors influencing its hepatic expression. These studies have demonstrated that the level of chkYB-1 mRNA in the liver decreases steadily throughout embryogenesis and for several weeks posthatching until adult levels are attained. We present several lines of evidence that YB-1 expression in the liver is positively associated with DNA synthesis or cell proliferation. Its binding characteristics indicate that the protein can interact specifically with a number of binding sites for liver-enriched or specific factors. In addition, although it is not particularly asymmetric in terms of base composition, we find a marked preference in binding to the pyrimidine-rich strand of these sites regardless of the presence or polarity of an intact CCAAT box. The increased levels of expression of YB-1 during proliferation combined with its binding characteristics suggest that it may be involved in the reduced expression of liver-specific genes observed at early stages of development or during liver regeneration.

A large number of elements involved in the regulation of eukaryotic genes contain a variation of the motif CCAAT as a subsequence (33). Sites containing such motifs have been shown to be recognized by a number of different *trans*-acting factors, some of which display restricted tissue distribution and others of which are ubiquitous (2, 44). A requirement for sequences in addition to the CCAAT motif has been demonstrated for some factors, but others display an ability to bind an array of sites with apparently little structural similarity other than the motif itself (9, 18, 19, 29, 39, 61). A particularly striking example of the latter group is provided by the avian factor EF1a, which was initially identified in crude nuclear protein extracts by virtue of its ability to bind to a site in the Rous sarcoma virus (RSV) long terminal repeat (LTR) (21, 29, 51, 62). This protein has been purified and shown to bind to sites in the RSV LTR either as a homodimer or as a heterodimer formed with a second protein designated EF1b. Subsequently, EF1a was shown to be capable of interacting with a number of binding sites for *trans*-acting factors that contain the sequence CCAAT in the noncoding DNA strand.

DNA binding proteins that have characteristics very similar to those of chicken EF1a and that are believed to be the homologs of the avian protein have been cloned from several species, including rats, mice, humans, and frogs (10, 18, 25, 38, 43, 49, 51, 58, 59, 65, 66). Despite their very high degree of sequence similarity, particularly in the basic DNA binding domain, a diverse range of functions has been ascribed to them, including those of reverse CCAAT-binding proteins (18, 51, 66), positive or negative *trans*-acting factors (18, 66, 67), single-stranded DNA binding proteins (38, 65), factors binding to damaged DNA (30, 43), and messenger ribonucleoprotein components (30, 43). Although this class of proteins was first identified and characterized in chickens, the cloning of avian EF1a and its structural relationship to putative homologs in other species have not been described.

In this article, we report the cloning of cDNAs encoding a protein that appears to be EF1a. The clones were isolated by direct screening of an expression library constructed from liver mRNA obtained from 9-day-old chicken embryos. We have referred to the protein as chkYB-1, rather than EF1a, to be consistent with nomenclature used for other species. The oligonucleotide used for screening corresponded to a protein binding site in the 5′-flanking region of the estrogen-dependent very-low-density apolipoprotein II (apoVLDLII) gene, which, as we have shown previously, undergoes developmentally programmed demethylation (31).

Normally, the apoVLDLII gene is inactive in immature birds and roosters and is expressed exclusively in livers of mature hens. However, the gene can be activated by administration of hormone within a few days after differentiation of the liver begins (11, 70). We have shown previously that competence to activate the gene in the liver in response to exogenous estrogens is acquired some time between days 7 and 9 of embryogenesis (11, 20). Acquisition of this ability coincides with demethylation of an *Msp*I site located 2.6 kb upstream from the major transcriptional initiation site of the apoVLDLII gene. The *Msp*I recognition sequence has been shown to be part of a protein binding site containing two enhancer core sequences (11, 31). Gel mobility shift assays indicated that proteins binding to the site displayed a declining profile of hepatic expression during embryogenesis, suggesting that they may play a negative role in regulation of the gene. These studies also revealed a shift in mobility of the DNA-protein complex at a discrete stage of development corresponding to a period in which the efficiency of expression of the apoVLDLII gene increased markedly. These

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TABLE 1. DNA binding sites used in this study

Site	Sequence	Reference
	Half-site 1A	
Site 1	5′-TGGTACCGGTTTTGGTAAGATCTGGTCCACAAACCACTG-3′ 3′-TGGCCAAAACCATTCTAGACCAGGTGTTTGGTGACACCA-5′	31
	Half-site 1B	
D site (rat albumin promoter)	5′-TGGTATGATTTTGTAATGGGG-3′ 3′-ACTAAAACATTACCCCACCAT-5′	48
C site (rat albumin promoter)	5′-GTAGGAACCAATGAAATGCGAGGTAAGTAT-3′ 3′-TTGGTTACTTTACGCTCCATTCATACATCC-5′	48
High-affinity C/EBP site	5′-AATTCAATTGGGAATCAGG-3′ 3′-GTTAACCCTTAGTCCTTAA-5′	68
Chicken vitellogenin II promoter, nt −323 to −343	5′-AGCTTGAGACTGGTTTTGCCCAGG-3′ 3′-ACTCTGACCAAAACGGGTCCTCGA-5′	6
LF-A1 site	5′-AGCTCCACTGAACCCTTGACCCCTG-3′ 3′-GGTGACTTGGGAACTGGGGACTCGA-5′	54
Y box	5′-CTGATTGGCCAAAG-3′ 3′-GACTAACCGGTTTC-3′	18
EF1-binding site from RSV LTR	5′-ACCGTGCATGCCGATTGGTGG-3′ 3′-CGTACGGCTAACCACCTTCAT-5′	21
NF-1 site	5′-CCTTGGCAAGCTGCCAAGG-3′ 3′-GGAACCGTTCGACGGTTCC-5′	3

observations raised the possibility that different proteins may interact with the site in embryonic and adult liver or that the proteins involved may be modified during embryogenesis. Purification of the proteins from adult liver yielded two components, suggesting that a heterodimer is involved in binding to the site (32). The proteins have mobilities on sodium dodecyl sulfate (SDS)-polyacrylamide gels similar to that reported for EF1a (21, 32) and, as we demonstrate here, a similar developmental profile of expression in the liver. In addition, we describe the tissue distribution and developmental profile of expression of chkYB-1 mRNA, the DNA binding characteristics of a chkYB-1 fusion protein, and the identification of parameters that influence the levels of chkYB-1 mRNA in the liver.

MATERIALS AND METHODS

Screening the expression library. A λgt11 expression library was constructed from cDNA prepared by random priming of liver mRNA isolated from 9-day-old embryos (60). An aliquot of the unamplified library (3.7×10^6 plaques) was screened by the direct screening approach described by Singh et al. (64). The probe used consisted of the site 1 oligonucleotide (Table 1), which was 5′ end labeled with [γ-^{32}P]ATP by using polynucleotide kinase and ligated into concatemers with T4 DNA ligase. The ends of the concatenated DNA were then filled in with [α-^{32}P]dATP and unlabeled deoxynucleoside triphosphates (dNTPs) by using the Klenow fragment of *Escherichia coli* DNA polymerase.

Screening was done essentially as described by Singh et al. (64), except that duplicate nitrocellulose filters were made for each plate of phage. One filter was screened directly, while the protein bound to the other filter was subjected to a cycle of denaturation with 6 M guanidine hydrochloride followed by renaturation before screening. Poly(dI-dC) · poly(dI-dC) at 5μg/ml was used as a nonspecific competitor DNA. In addition, all screening steps were carried out at 4°C with the buffer pH adjusted to 7.5 at 21°C.

PCR cloning methods. The polymerase chain reaction (PCR)-based rapid amplification of cDNA ends (RACE) procedure was used to clone the 3′ end of the mRNA (24). The gene-specific primer 5′-CGCAACGAAGGTTTTGGG-3′ was located 287 nucleotides (nt) from the 3′ end of the cloned cDNA sequence. The PCR product was purified through a Centricon 100 microconcentrator (Amicon) before digestion with *Xmn*I, which has a restriction site in the cDNA sequence, and *Xho*I, which has a restriction site in the 3′ RACE primer. The products were then directionally cloned between the *Eco*RV and *Xho*I sites of pBluescript vectors (Stratagene).

DNA sequencing. Both the cDNAs from the λgt11 library and the PCR products were subcloned into pBluescript vectors and sequenced with the modified T7 polymerase of a Sequenase II kit (Stratagene). Because of the relatively high G+C content of the clones, it was necessary to use single-stranded DNA for sequencing and to include sequencing reaction mixtures containing 7-deaza-2′-dGTP. Both strands of DNA were sequenced independently. The sequences of the PCR products were confirmed by sequencing several subclones.

Tissue culture. The chicken hepatoma cell line LMH was cultured in Waymouth's medium supplemented with 15% chicken serum and 0.01 mg of insulin per ml (36). Before treatment, cells were plated on 100-mm-diameter dishes and grown to approximately 80% confluence. Cycloheximide was used at 25 μg/ml, dexamethasone was used at 1 μM, and 17β-estradiol was used at 1 μM.

Northern (RNA) analysis. Poly(A)$^+$ RNA was isolated from adult chicken tissues, embryonic chicken liver, and the chicken hepatocyte cell line LMH (36) with a Fastrack or Microfastrack RNA isolation kit (Invitrogen). The RNA (4 μg per lane) was electrophoresed in a 1.2% formaldehyde gel (60). Ethidium bromide was added to each RNA sample to ensure that equivalent amounts of RNA were loaded onto the gel (56). After electrophoresis, the RNA was transferred to a Zetaprobe membrane (Bio-Rad) by capillary blotting in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene). The blots were hybridized at 42°C in a mixture of 50% formamide, 4× Denhardt's solution, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 100 μg of sheared herring testis DNA per ml, and 100 to 200 ng of cDNA radiolabeled by random priming in the presence of [α-^{32}P]dATP (22). The

filters were washed at 52°C in a mixture of 0.1× SSC and 0.1% SDS. The autoradiographs were analyzed with a Hoefer GS300 scanning densitometer.

Southwestern (DNA-protein) blot analysis. Lysogens were prepared by infection of *E. coli* Y1089 (60). Cell samples (1.5 ml) before and after induction of the β-galactosidase fusion protein were concentrated by brief centrifugation and resuspended in a mixture containing 0.1 ml of extraction buffer and 0.1 ml of 2× SDS sample buffer (60, 64). After the samples were heated at 90°C for 5 min, 20-μl samples of uninduced and induced proteins were resolved on SDS–10% polyacrylamide gels. The proteins were transferred to nitrocellulose filters in 25 mM Tris–190 mM glycine at 120 mA for 3 h. Binding assays were done essentially as described above for the library screening with the following modifications. Oligonucleotides used for binding studies were not concatenated. All binding assays were carried out in a volume of 50 ml with 10 pmol of oligonucleotide and 250 μg of poly(dI-dC) · poly(dI-dC) as a nonspecific competitor DNA. Double-stranded oligonucleotide probes (10 pmol) were labeled with [α-^{32}P]dATP, unlabeled dNTPs, and the Klenow fragment of *E. coli* DNA polymerase, with the exception of the Y box, which was 5′ end labeled with T4 polynucleotide kinase. The oligonucleotides corresponding to site 1, C/EBP, D-site, EF1, Y-box, and LF-A1 binding sites were all labeled to a specific activity of 5 × 10^6 to 8 × 10^6 dpm/pmol. Those corresponding to the vitellogenin site, the C site, and half-sites 1A and 1B were all labeled to 2 × 10^6 dpm/pmol (±10%). The single-stranded oligonucleotide probes were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The specific activities obtained ranged from 1 × 10^6 to 2 × 10^6 dpm/pmol.

Nucleotide sequence accession number. The nucleotide sequence of the chkYB-1 gene (not presented here) has been submitted to GenBank and can be retrieved by using accession number L13032.

RESULTS

Isolation of the chicken homolog of YB-1. Our studies were designed to isolate cDNAs encoding regulatory factors that interact with site 1, a previously characterized DNA binding site located in the 5′-flanking region of the chicken apoVLDLII gene. Since the level of site 1 binding activity is high in early embryonic liver and decreases throughout development until it reaches adult levels (31), we screened a day 9 embryonic liver λgt11 expression library to increase the probability of detecting factors binding to the site. After several library equivalents were screened, four clones displaying strong binding to site 1 were plaque purified, and the cDNA inserts were subcloned to pBluescript vectors for further analysis. Preliminary sequence data suggested that two of the clones contained a total of 670 bp with an overlap of more than 600 bp, indicating that they were derived from the same mRNA. Additional sequence information was obtained by using the PCR-based RACE procedure to clone the 3′ end of the mRNA (24). We obtained 880 bp of new sequence which included a putative poly(A) tail preceded by a consensus polyadenylation signal. Primer extension analysis (26) indicated that we were missing only 20 nt from the 5′ end of the mRNA, although a minor extension product that terminated an additional 130 nt upstream was obtained (data not shown). The total sequence corresponded to an mRNA of 1,507 nt, not including the poly(A) tail or the 5′-proximal 20 nt, which is consistent with the size of the species detected by Northern blotting (Fig. 1). Analysis of

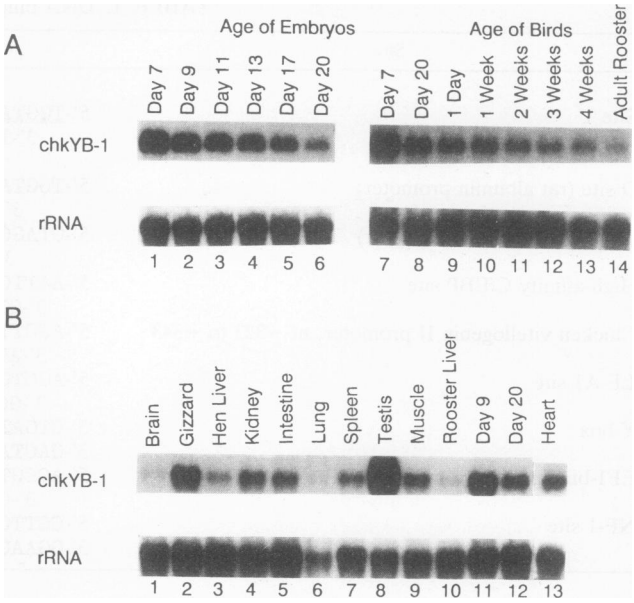

FIG. 1. Expression pattern of chkYB-1 mRNA. Northern blots of poly(A)$^+$ RNA were probed with an approximately 600-bp fragment of chkYB-1 cDNA. (A) The levels of expression of chkYB-1 mRNA in livers isolated from embryos, chicks, and adult roosters were compared. The ages of the embryos and birds are indicated above the lanes. (B) The levels of expression of chkYB-1 mRNA in selected tissues from adult roosters and in the livers of hens and 9- and 20-day-old embryos were compared. The amount of RNA in each lane was monitored by ethidium bromide staining and by hybridization to a 500-bp chicken 28S rRNA clone.

the sequence revealed a single open reading frame encoding a polypeptide of 321 amino acids, plus 133 bp of 5′ untranslated sequence and 428 bp of 3′ untranslated sequence. The nucleotide sequence was compared with the sequences in the GenBank data base by the program FASTA (52). A high degree of similarity was found with the sequences of human YB-1, which was originally cloned by virtue of its ability to bind to a Y box in the human major histocompatibility complex class II gene (18), and dbpb, which binds to the epidermal growth factor enhancer (59). The matches between our sequence and those of YB-1 and dbpb are 86 and 88%, respectively, for the mRNAs and 89 and 91%, respectively, for the proteins. This degree of identity suggests that the cloned mRNA is the chicken homolog of human YB-1 and dbpb. Therefore, we have designated this clone chkYB-1. The amino acid sequence of chkYB-1 is shown in Fig. 6.

Tissue specificity of chkYB-1 mRNA and regulation of chkYB-1 mRNA levels. The developmental profile of chkYB-1 mRNA expression in the liver and the levels of expression in other tissues were examined by Northern blotting. The mRNA was detected at variable levels in all tissues as a single 1.6-kb species. It was relatively abundant in the liver at day 7 of embryogenesis and decreased steadily throughout embryogenesis, so that levels of the mRNA had declined approximately 10-fold by day 20 (Fig. 1A). After the chicks hatched, the levels of the mRNA in the liver decreased a further three- to fivefold until they reached the levels found in adult roosters. This pattern of expression was similar to that of the site 1 binding activity, as measured previously by gel retardation assay (31). To address the possibility that the levels of mRNA and active protein may

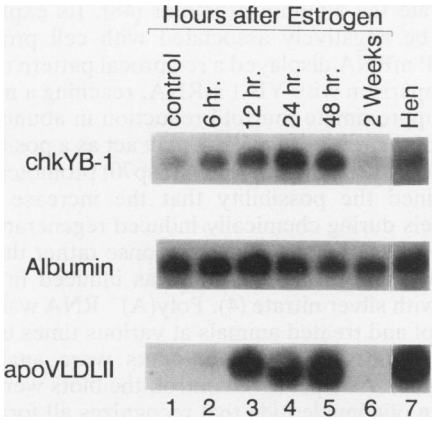

FIG. 2. Effect of estrogen treatment on chkYB-1 expression. Nine-week-old male chickens were injected with 1 mg of diethylstilbestrol per ml in polypropylene glycol and sacrificed 6, 12, 24, or 48 h or 2 weeks after treatment. A Northern blot of poly(A)$^+$ RNA isolated from the livers of these birds was probed with fragments of both the cloned chkYB-1 cDNA and the chicken serum albumin cDNA (27). As a positive control for the response of the birds to the estrogen treatment, expression of the chicken apoVLDLII mRNA was monitored (70).

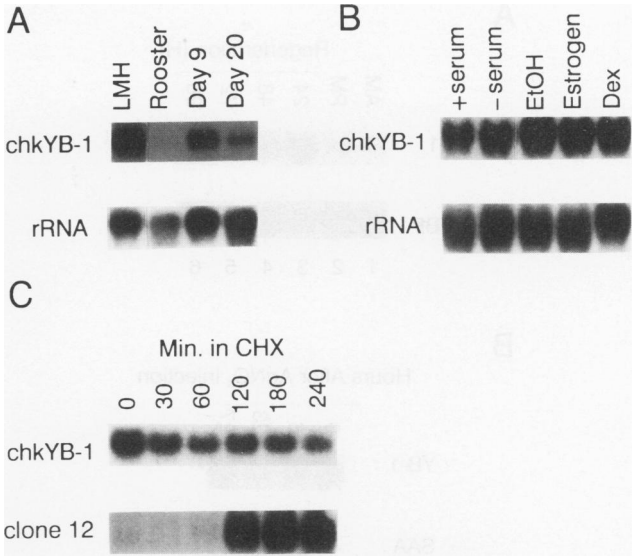

FIG. 3. Northern hybridization of poly(A)$^+$ RNA isolated from the chicken hepatoma cell line LMH (36). (A) The level of chkYB-1 mRNA in LMH cells was compared with the levels in 9- and 20-day-old embryos and in adult roosters. (B) LMH cells were incubated for 8.5 h with (+) or without (−) 15% chicken serum or treated for 16 h with 1 μM 17β-estradiol (estrogen), 1 μM dexamethasone (Dex), or 0.1% ethanol (EtOH), which was used as a solvent for both 17β-estradiol and dexamethasone. As in the experiment whose results are shown in Fig. 1, the blots were probed with a fragment of the chkYB-1 clone, and the amount of RNA loaded in each lane was monitored by ethidium bromide staining and by hybridization to a chicken 28S rRNA probe. (C) LMH cells were treated with 25 μg of cycloheximide (CHX) per ml for the times indicated above the lanes. As a positive control for the cycloheximide treatment, the expression of an mRNA identified as belonging to the interferon regulatory factor family is shown (clone 12) (28).

not be concordant, we compared the levels of EF1 binding activity in liver throughout embryogenesis by gel retardation assays using the original binding site from the RSV LTR (21, 28). The results of these experiments indicate that binding activity and mRNA levels correlate well and thus provide no evidence of modulation of YB-1 binding activity by translational or posttranslational mechanisms. However, we cannot exclude the possibility that the levels of binding activity determined by gel retardation assays are affected by the presence of proteins other than YB-1 which may be able to interact with the EF1 site. Examination of a variety of tissues revealed a wide range of expression of chkYB-1 mRNA, with higher levels being present in adult gizzard, adult testis, and fetal liver (Fig. 1B). The mRNA was particularly abundant in testis tissue.

To determine whether activation of the apoVLDLII gene was associated with a change in chkYB-1 mRNA levels, we inoculated roosters with estrogen and determined the levels of apoVLDLII and chkYB-1 mRNA in the liver 6, 12, 24, and 48 h and 2 weeks after treatment. The level of serum albumin mRNA was also determined (Fig. 2). As expected, induction of apoVLDLII mRNA was detectable within 6 h and the mRNA abundance was maximal between 24 and 48 h, consistent with previously reported data (70). chkYB-1 mRNA also increased in abundance after treatment with estrogen. The levels of mRNA doubled within 6 h and reached a maximal eightfold induction by 24 h in comparison with untreated rooster liver. In contrast, the level of serum albumin mRNA declined approximately twofold between 12 and 24 h after treatment. Expression of both apoVLDLII and chkYB-1 mRNAs returned to pretreatment levels by 2 weeks after administration of estrogen. However, despite this response to acute stimulation with a hormone, a comparison of chkYB-1 mRNA levels in the livers of normal roosters and hens indicated that they were very similar (Fig. 1B).

The similarity between the levels of chkYB-1 mRNA in rooster and hen livers suggested that induction by estrogen may be a secondary consequence of acute stimulation with hormone rather than a normal physiological response. Initial treatment of roosters with estrogen results in a round of DNA replication during the first 24 h following treatment (35). This raised the possibility that levels of chkYB-1 mRNA are increased during cell proliferation or DNA synthesis. Such a possibility would be consistent with the observation that levels of the mRNA in the liver decline throughout embryogenesis and for several weeks following hatching (Fig. 1A). This suggestion was examined further by monitoring chkYB-1 mRNA expression in the chicken hepatoma cell line LMH under various culture conditions. chkYB-1 mRNA levels in proliferating LMH cells were elevated severalfold relative to levels in liver from day 9 embryos and at least 30- to 40-fold relative to levels in normal adult liver (Fig. 3A). The cells were also treated with a pharmacological concentration of estrogen to determine whether the induction observed in vivo could be obtained in vitro. The level of chkYB-1 mRNA increased approximately twofold after estrogen treatment. However, similar increases in concentration were observed when the cells were treated with dexamethasone or with ethanol, which was used as a solvent for both hormones (Fig. 3B). Essentially the same results were obtained when we used a derivative line of LMH cells, LMH2a, that had been stably transfected with an estrogen receptor expression vector under the control of the RSV promoter (28). These cells contain levels of receptor comparable to those found in estrogen-treated rooster liver and are capable of activating the endogenous apoVLD-

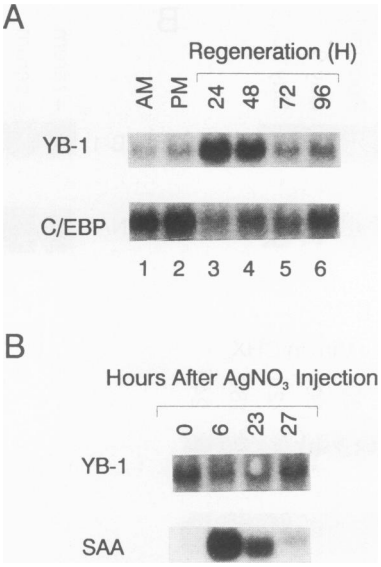

FIG. 4. Expression of YB-1 mRNA in rat liver during regeneration (A) and in mouse liver after induction of an inflammatory response (B). (A) Poly(A)$^+$ RNA was isolated from the livers of control rats at 8 a.m. (AM) and 8 p.m. (PM) and from rats in which liver regeneration was induced by a single intragastric dose of CCl_4 (48). All livers from CCl_4-treated rats were excised at 8:00 a.m. 24, 48, 72, or 96 h after treatment. (B) Eight-week-old mice received a 0.5-ml subcutaneous injection of 2% silver nitrate solution (4). Liver poly(A)$^+$ RNA was isolated 6, 23, and 27 h after treatment. Northern blots were probed with chkYB-1 cDNA at reduced stringency. As positive controls for the induced physiological response, the blots were stripped of the YB-1 probe and rehybridized with a rat C/EBP cDNA (A) (39) or an oligonucleotide specific for mouse serum amyloid A (SAA) mRNA (B) (4). In addition, loading of the RNA in panel A was assessed by densitometry after the blot was probed with a cloned fragment of 28S rRNA. Variation between the lanes was found to be less than ±10%.

LII gene in response to hormone. The results of these experiments support the suggestion that the elevation of chkYB-1 mRNA is associated with proliferation or DNA synthesis rather than being a direct response to estrogen.

The effect of cycloheximide on the levels of chkYB-1 mRNA was also examined, since it has been shown to cause superinduction of mRNAs for several *trans*-acting factors involved in cell proliferation (1, 8, 42, 57). The mRNAs for these factors typically contain instability sequences of the general form AU_nA in their 3′ untranslated regions (7, 63). Human, rat, mouse, chicken, and *Xenopus* YB-1 mRNAs contain several such sequences that are highly conserved. However, cycloheximide treatment of LMH cells did not increase chkYB-1 mRNA levels; rather, the levels declined approximately twofold over a 4-h period (Fig. 3C). This contrasted with induction of the mRNA for another *trans*-acting factor belonging to the interferon regulatory factor family (28), which was monitored as a positive control.

Induction of YB-1 mRNA in regenerating liver. In order to test the suggestion that the abundance of chkYB-1 mRNA in the liver is related to cell proliferation, we determined the levels of the mRNA during chemically induced liver regeneration in rats. The level of YB-1 mRNA was elevated approximately 10- and 6-fold 24 and 48 h after administration of carbon tetrachloride, respectively (Fig. 4A). The levels of C/EBP mRNA were also monitored throughout the response as an example of a liver-enriched factor known to positively *trans* activate the albumin promoter (48). Its expression is known to be negatively associated with cell proliferation (46). C/EBP mRNA displayed a reciprocal pattern of expression in comparison with YB-1 mRNA, reaching a nadir at 24 h with an approximate fourfold reduction in abundance.

In view of the report that YB-1 may act as a positive *trans* activator of the heat shock protein (hsp70) promoter (34), we also examined the possibility that the increase in YB-1 mRNA levels during chemically induced regeneration is the result of a stress or acute-phase response rather than proliferation. An acute-phase reaction was induced in mice by treatment with silver nitrate (4). Poly(A)$^+$ RNA was isolated from control and treated animals at various times up to 72 h after stimulation, and equal amounts were analyzed on Northern blots. As a positive control, the blots were hybridized with an oligonucleotide that recognizes all forms of the mRNA encoding the acute-phase-responsive protein, serum amyloid A (4). Although induction of serum amyloid A mRNA was readily detectable, no alterations in the levels of YB-1 mRNA were observed (Fig. 4B).

DNA-binding specificity of chkYB-1. Mammalian and amphibian homologs of YB-1 have been isolated on the basis of their ability to bind sequences containing reverse CCAAT motifs (18, 51, 66). However, they have subsequently been shown to bind to a wide range of sites with no definable common motif. These studies have generated alternative suggestions that YB-1 binds to damaged DNA (30, 43) or single-stranded CT-rich DNA (38) or that it is an RNA binding protein (15, 49). The oligonucleotide we have used has a number of enhancer-like structural characteristics, but it does not contain a CCAAT box in either orientation (31). The site does contain several copies of the sequence CCA on both strands and a single reverse CCAA motif (Table 1).

The DNA binding specificity of chkYB-1 was examined by Southwestern blotting with the β-galactosidase fusion protein from the original λgt11 clone (Fig. 5) and oligonucleotides corresponding to known binding sites for liver-enriched or ubiquitous factors (Table 1). As described in Materials and Methods, all assays were carried out with the same molar concentration of monomeric binding sites in the presence of an approximately 1,500-fold weight excess of poly(dI-dC) · poly(dI-dC). Double-stranded binding sites were labeled by fill-in synthesis to ensure that any minor amounts of single-stranded oligonucleotides present would not be scored by the assay. The protein displayed a wide range of binding efficiencies that could not be explained by differences between the specific activities of individual oligonucleotides. The fusion protein bound very well to an oligonucleotide corresponding to the complete site 1 sequence (Fig. 5A, lane 1). We have shown previously by methylation interference studies that tight binding to site 1 primarily involves the left half of the site (31). However, the site contains copies of similar motifs in each half (5′-CCA CAAACCA-3′ on the sense strand and 5′-CCAAAAAC CA-3′ on the antisense strand), both of which may be potential binding sites for YB-1. Consequently, we examined binding to each half-site independently. Despite the similarity between the sequences and the broad range of binding specificities reported for YB-1, the fusion protein displayed highly selective binding to the left half of the site (lanes 9 and 10).

The protein bound less well to two oligonucleotides containing complete reverse CCAAT sequences that were used to clone mammalian homologs of YB-1. The first corresponded to the EF1 site of the RSV LTR, which was originally used to characterize the avian protein and subse-

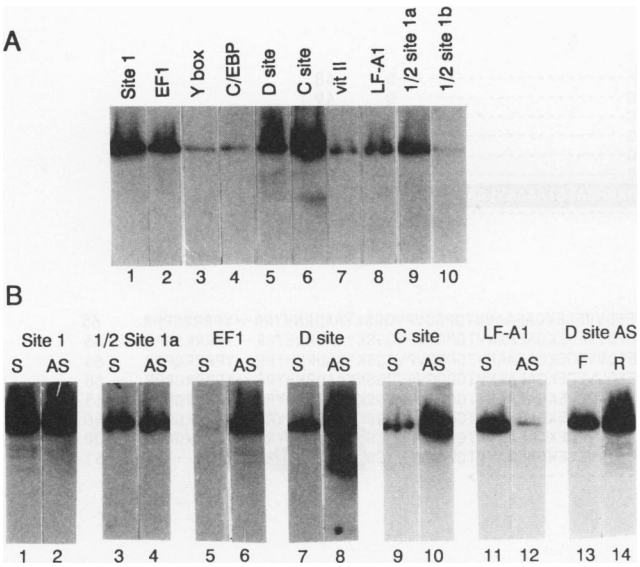

FIG. 5. Southwestern analysis of the double- and single-strand binding characteristics of the chkYB-1 fusion protein. The original λgt11 isolate of chkYB-1 was made lysogenic in *E. coli* Y1089 (64). This clone contains the most 5′ 576 nt of the cloned chkYB-1 sequence. The sequence encodes 198 amino acids which include the putative single- and double-stranded DNA binding domains (38, 67) and an additional 50 amino acids coded for in the linker and 5′ untranslated sequence of the clone. Western blots of the induced β-galactosidase fusion protein were denatured and renatured with 6 M guanidine HCl. They were then incubated either with double-stranded oligonucleotides labeled with Klenow fragment (A) or with end-labeled sense (S) and antisense (AS) strands of selected DNA binding sites (B). Binding of the top strand of the D site of the albumin promoter in the sense (F) orientation was also compared with binding of the same sequence with the reverse polarity (R). With the exception of the D site from the albumin promoter, the probes did not bind to either the β-galactosidase protein expressed from λgt11 or other *E. coli* proteins (data not shown). Both the double-strand D-site probe and the antisense single-strand probe did bind to a low-molecular-weight *E. coli* protein that is present in both induced and uninduced samples. vit II, vitellogenin II.

quently to clone the rat homolog (51). The second was the original Y box from the HLA class II genes, which was used to demonstrate the binding specificity of human YB-1 (18) (Fig. 5A, lanes 2 and 3). Binding to this sequence was extremely weak, an observation that was also made during studies with human NSEP-1, which may be identical or very similar to YB-1 (38).

Earlier studies of site 1 demonstrated that it competed effectively with sites to which the liver-enriched factor C/EBP was known to bind, although reciprocal competition was not observed (31). Consequently, we examined the ability of chkYB-1 to bind to an oligonucleotide corresponding to a sequence optimized for binding to C/EBP and containing a reverse CCAAT sequence (39). This sequence does not correspond to a known naturally occurring site for this factor but is a synthetically generated high-affinity site that was used to optimize cloning of C/EBP by direct screening (68). Although binding was detected, the level was weak in comparison with the levels for site 1 (lane 4) and several other sites described below. We also examined a second binding site, the D site, known to play a major role in expression of the serum albumin gene and to bind several members of the bZIP family of transcription factors to which C/EBP belongs (13, 16, 17, 48). This site does not contain a CCAAT sequence but does contain the element CAAAA, which is also found in site 1. chkYB-1 bound this site more efficiently than the first C/EBP site but less well than Site 1 (Fig. 5A, lane 5). The C site from the albumin promoter was also examined. This site has been shown to be a binding site for NF-Y (45, 55), to which YB-1 was originally thought to be identical (21), and it contains a CCAAT motif on the sense strand. This site displayed the highest degree of binding of all sites tested despite the fact that the specific activity of the site was two- to threefold lower than those of, for example, site 1, EF1, and Y-box oligonucleotides (lane 6).

We examined two additional potential binding sites, both of which contained particular structural features of site 1 and have been implicated in the regulation of liver-specific genes. One corresponded to a sequence in the flanking region of the chicken vitellogenin II gene, which is coordinately regulated with the apoVLDLII gene (5, 20). This site contained the element CAAAA, which is common to the D binding site and to site 1, and also had an 11-of-14-nucleotide match with the core binding region of site 1 (32). The other was a binding site for the liver-enriched factor LF-A1 (HNF-4), which, like YB-1, may be expressed at elevated levels early during embryogenesis (12, 54). The vitellogenin sequence has been shown previously to bind weakly to the same factors as site 1 (32). This site also displayed a low level of binding with chkYB-1 (Fig. 5A, lane 7). chkYB-1 bound more efficiently to the LF-A1 oligonucleotide, although the level of binding was lower than those observed with site 1, C/EBP, D-site, and C-site oligonucleotides (lane 8). The LF-A1 sequence contains neither CCAAT or CAAAA motifs but has a high degree of strand asymmetry with respect to pyrimidine distribution, a feature that has been reported to be important for the binding of NSEP-1. Binding was also examined with an oligonucleotide corresponding to a site for the ubiquitous CCAAT box binding factor NF-1, but no binding was detectable (28). This site does not contain a CCAAT motif but does contain two copies of the sequence CCAAG.

Studies of human YB-1 have demonstrated that the factor has single-stranded DNA binding capability (30, 65). On the basis of its interaction with a binding site in the human papillomavirus type 18 enhancer, it was concluded that the protein bound specifically to the sense strand of this site (65). Similar studies with the closely related protein NSEP-1, whose amino acid sequence has been reported to differ from that of YB-1 in two locations in the COOH-terminal region and at the NH_2 terminus, also detected preferential binding to one strand of a number of binding sites (37, 38). On the basis of this study, the determining factor in strand selection appeared to be strong purine-pyrimidine asymmetry that has been shown to promote formation of H-DNA, a triplex structure which results in displacement of a pyrimidine-rich single-stranded region (23).

We examined binding of chkYB-1 to a number of sites to determine whether it also displayed a preference for the sense and/or pyrimidine-rich strand. Both strands of site 1 are equally CT rich, and both strands bound strongly and equally (Fig. 5B, lanes 1 and 2). Both strands of the left half-site, which maintained equal pyrimidine distribution, also bound, but they bound severalfold less well (lanes 3 and 4). The other sites tested had variable degrees of asymmetry in terms of pyrimidine distribution, and all displayed markedly asymmetrical binding, with a preference for the strand with higher pyrimidine content (lanes 5 to 12). However, the asymmetry in binding was not a simple function of differences in CT content. For example, the D and C binding sites

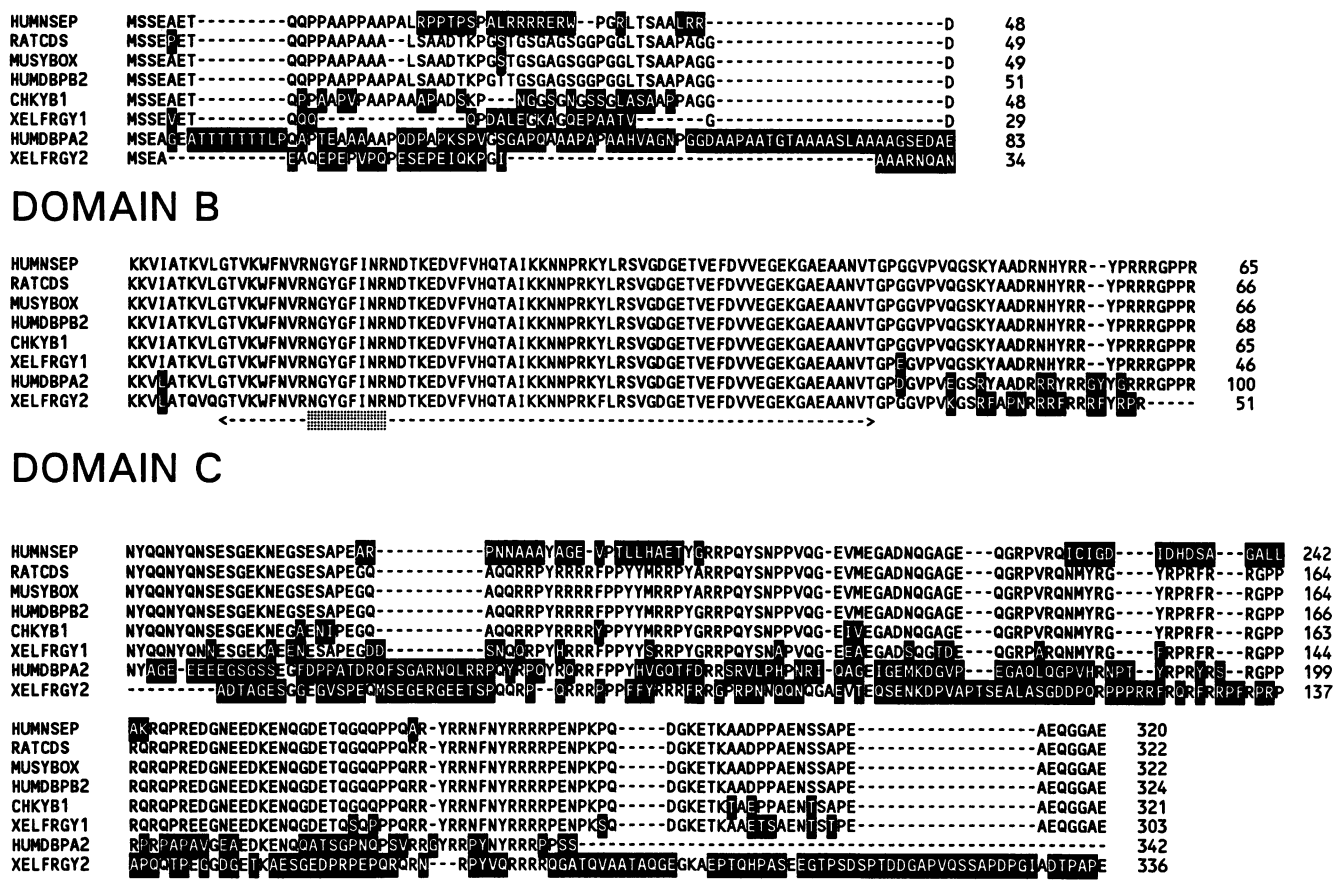

FIG. 6. YB-1 protein sequences. The computer program Clustal from the PC/GENE software system (Intelligenetics, Inc.) was used to align the amino acid sequences of the YB-1 homologs. The central domain (B) corresponds to the DNA binding domain (38, 67), and the COOH-terminal domain (C) is the putative multimerization domain (67). No function has as yet been ascribed to the NH_2-terminal domain (A). The regions with a black background indicate where amino acid sequences differ from the sequence of the human YB-1 protein, dbpb. The cold shock domain (71) and the conserved RNP-1 motif (40) are indicated by the dashed line and stippled box, respectively, under the sequence of domain B. The GenBank accession numbers for these sequences follow in the order in which they appear in the figure (top to bottom): M83234, M69138, M60419, M24070, L13032, M59453, M24069, and M59454. HUMNSEP, human NSEP-1; RATCDS, rat YB1 (EF1a); MUSYBOX, mouse Y box; HUMDBPB2, human dbpb2; XELFRGY1, *Xenopus laevis* FRGY1; HUMDBPA2, human dbpa2; XELFRGY2, *X. laevis* FRGY2.

from the serum albumin promoter display very similar levels of binding asymmetry. In the case of the C site, the CT contents of the sense and antisense strands are 30 and 70%, respectively, while in the case of D site, they are 43 and 57%. However, the D-site antisense strand contains a sequence (CCACCCC) which is similar to that found in the c-*myc* CT element (CCCCACCC), which has been shown to be capable of forming H-DNA in vitro (23). As a further test of the extent to which this element might account for the high level of asymmetrical binding seen with the serum albumin D site, binding was also examined with an oligonucleotide synthesized with reverse polarity (lanes 13 and 14). This increased the match with the c-*myc* element, since it converted the sequence to CCCCACC. Binding to this oligonucleotide was at least as efficient as binding to the oligonucleotide with normal polarity, suggesting that the pyrimidine-rich element, rather than surrounding sequences, was the major factor in determining binding. The polarity of a CCAAT sequence also did not determine the strandedness of binding, since in the case of the C site, the CCAAT sequence is on the poorly bound, purine-rich sense strand, while in the EF1 site it is on the strongly bound, pyrimidine-rich antisense strand. This suggests that in the case of single-stranded DNA binding, the presence of a CCAAT sequence is of secondary importance in comparison with pyrimidine content. Binding is also not simply a function of base composition, since the NF-1 site has approximately the same composition as site 1 and neither strand was bound by the protein.

Structural relationship of chkYB-1 to homologs and family members in other species. Figure 6 displays the predicted amino acid sequence of chkYB-1 aligned with putative human, rodent, and amphibian homologs. Mouse YB-1 has been independently isolated from three different sources. The sequences of the three are 98.4% identical and 0.6% similar. We have used a consensus sequence based on identity in two out of three of the sequences at each variant location. Similarly, rat YB-1 has been independently isolated from two different strains. The sequences differ in only one amino acid, a nonconservative change at position 306 from proline to serine. Since proline is found at the comparable

position of all other mammalian homologs and the chicken protein, we have included the rat sequence containing this amino acid in the comparison. The published sequence of human YB-1 differs in the COOH-terminal 11 amino acids from that of human dbpb and at one internal location (18, 59). Since the sequence of human dbpb matches the sequences of the other homologs, including the chicken homolog, at both variant locations, we have used the published sequence of dbpb rather than that of YB-1 for comparison. We have also included the sequence of human NSEP, since it is unclear whether it is identical to dbpb (YB-1) (37). The sequence of the chicken protein displays a high degree of homology with the human dbpb (YB-1) sequence in regions that differ significantly from the sequence of human NSEP-1 (Fig. 6). It has been suggested that the published sequence of human dbpb (YB-1) was incorrect on the basis of analyses of several NSEP-1 clones (37). However, comparison with both the chicken and frog sequences suggests that this is not the case. This leaves open the possibility that dbpb and NSEP-1 may be closely related members of the same family. This possibility is supported by the recent observation that the mouse genome may contain four closely related genes (65). In addition, the alignment includes two examples of other family members, human dbpa and *Xenopus* FRGY2, that contain the same or very similar DNA binding domains (59, 66).

On the basis of homology and the functional data available, the sequences have been divided into three domains (Fig. 6). Domain B is most highly conserved and is responsible for both double-strand and single-strand DNA binding activities (38, 67). The core region of the DNA binding domain displays significant similarity to a 70-amino-acid protein, CS7.4, from *E. coli* (71). The CS7.4 protein is rapidly induced by cold shock and is involved in regulation of the cold shock regulon of *E. coli* (41). Similar proteins have now been isolated from *Streptomyces clavuligerus* and *Bacillus subtilis* (69, 71). In addition, two glycine-rich proteins of unknown function from *Arabidopsis thaliana* and *Nicotiana sylvestris* contain a region with 65% similarity to the cold shock domain (14, 50). Members of the dbpb (YB-1) family and proteins containing just the cold shock domain have a highly conserved octamer motif known as the RNP-1 consensus that is also present in an extended group of otherwise unrelated RNA and DNA binding proteins (40). Kolluri et al. (38) have shown that deletion of this element from the human NSEP-1 protein eliminates single- but not double-strand DNA binding activity.

The COOH-terminal domain, C, is the most structurally distinct portion of the YB-1 (dbpb) proteins. It is composed of alternating positively and negatively charged regions and is also rich in proline, tyrosine, and glycine residues. The charged and extended nature of this domain may be responsible for the larger-than-expected apparent molecular weight of the YB-1 (dbpb) proteins in SDS-polyacrylamide gel electrophoresis (10, 15, 38, 49, 65). There is some evidence to suggest that this domain is responsible for protein-protein interactions and is involved in the extensive multimerization of YB-1 observed during purification (67).

No specific function has as yet been ascribed to domain A. It is the most variable domain among the YB-1 (dbpb) homologs and may influence the ability of the protein to interact with other nucleic acid binding proteins. The other two family members, dbpa and FRGY2, are highly variable in this region.

DISCUSSION

Homologs of YB-1 have been isolated from a number of species by using oligonucleotides corresponding to quite different protein binding sites as well as depurinated DNA (10, 18, 30, 38, 43, 51, 59, 65, 66, 73). The results of subsequent investigations have generated a number of hypotheses concerning the functions of YB-1. Several groups have demonstrated that the binding of YB-1 to double-stranded DNA is sequence specific and that it also displays specific, single-stranded DNA binding activity (10, 18, 38, 51, 65–67). In addition, the protein has been shown to display a relatively nonspecific preference for depurinated DNA relative to undamaged DNA (30, 43). Original studies with avian nuclear protein extracts indicated that the binding displayed by EF1 (YB-1) was sequence specific and involved the reverse CCAAT element present in the high-affinity binding site in the RSV LTR (21, 29, 62). Although additional sequences were clearly required for efficient binding to this site, mutagenesis failed to define a consensus sequence beyond the CCAAT box (21).

The DNA binding specificity of chkYB-1 supports the observation, made during studies with human NSEP-1, that members of this protein family display a marked preference for the pyrimidine-rich strand of the sites to which they bind (38). We observed that this preference supersedes the requirement for, and polarity of, the CCAAT motif. The sequences of the sites used to examine the binding specificity of chkYB-1 were significantly less asymmetric than those tested with NSEP-1 and do not appear to be likely candidates for the formation of H-DNA. Nevertheless, preference for the pyrimidine-rich strand was maintained. In addition, we found that strong binding to single-stranded DNA is not a simple function of pyrimidine content. Both strands of site 1 bound equally well and are only approximately 50% C+T, while neither double nor single strands of the NF-1 site, which have the same base composition, yielded detectable binding. Furthermore, sites such as the C/EBP site bound very poorly, despite an asymmetric distribution of pyrimidines including a run of 10 out of 12 nucleotides on the antisense strand. Although mutational studies with NSEP-1 indicate that single- and double-strand binding are separable functions, we have not found any example of a site that displayed strong binding as a duplex without detecting strong binding to one of its strands. It has been suggested that high, nonspecific background binding to DNA and the presence of incorrectly renatured protein may reduce the ability to detect specific binding by techniques such as Southwestern blotting (72). All binding studies reported here were carried out in the presence of a vast excess of poly(dI-dC) · poly(dI-dC) to minimize such background problems. Although we have been unable to define a consensus recognition sequence, the wide range of binding observed indicates a high degree of sequence selectivity with both double- and single-stranded sites.

The broad range of binding specificities displayed by YB-1 has prompted the suggestion that it may be a relatively low-affinity, low-specificity factor involved in a chromatin structural role or in binding to damaged DNA (30, 38, 43). However, such a role is difficult to reconcile with the exceptionally high degree of conservation of the whole protein and in particular the DNA binding domain. Comparison of the structure of chkYB-1 with the structures of mammalian and amphibian homologs indicates that the DNA binding domain defined by studies of human NSEP-1 (identified as domain B in Fig. 6) is 100% conserved in the avian

protein, with only one amino acid substitution being present in the *Xenopus* protein. Comparison of the whole protein indicates that there are only three amino acid differences between the mouse and human homologs, corresponding to less than a 0.9% difference. This difference is comparable to those observed for NF-YA (0.6%) and NF-YB (1.0%), which are among the most highly conserved of transcription factors (44). By analogy with arguments presented with respect to the high degree of conservation of NF-Y, this suggests that YB-1 may be involved in numerous, but specific, protein-DNA interactions and consequently may play a role in the regulation of many genes.

Expression of both FRGY1 and FRGY2 mRNAs and proteins has been shown to be developmentally regulated, with high levels being expressed in germ line tissues and in oocytes through stage III (66). chkYB-1 mRNA was also particularly abundant in testis tissue. It has been suggested that one or both of the *Xenopus* proteins may act as positive transcription factors for germ cell-specific and tissue-specific genes that contain a Y box in their promoters (66, 67). Functional studies of *Xenopus* YB-1 indicate that the factor can positively regulate expression from the hsp70 promoter (67). An evolutionary relationship with the *E. coli* cold shock protein, CS7.4, which has been shown to be a *trans*-acting factor involved in regulating expression of DNA gyrase, has also been detected (41). In addition, the bacterial protein has been shown to bind to CCAAT sites recognized by YB-1, suggesting that these proteins may be members of an evolutionarily conserved group of factors involved in regulation of stress response genes. Stress induces a number of alterations in the pattern of hepatic gene expression, including the induction of several stress response protein genes and a decrease in the transcription of genes characteristic of normal differentiated hepatocytes, such as the serum albumin gene (43, 56). Several of the experimental conditions under which we observed increased expression of chkYB-1 mRNA in the liver, such as acute stimulation with pharmacological doses of hormone and chemically induced regeneration, may generate a stress response as well as cell proliferation. However, the deliberate induction of an acute-phase reaction in murine liver demonstrated that hepatic expression of YB-1 mRNA is not stress inducible. The high levels of YB-1 mRNA in germ line tissues, its decline in the liver during embryogenesis, and the increase observed during regenetration and in cultured hepatocarcinoma cells are all consistent with YB-1 expression being associated with cell proliferation. Only one other example of the modulation of YB-1 mRNA levels has been reported. This example also involves a proliferative response after lymphokine-induced activation of T cells (58).

In contrast to the profile of hepatic expression of YB-1 mRNA, the ability to efficiently express liver-specific genes such as apoVLDLII and serum albumin develops relatively late during embryogenesis and is decreased in proliferating hepatocytes (20). The levels of positive *trans*-acting factors involved in regulating transcription of the serum albumin gene, such as C/EBP, DBP, and LAP, are also relatively low during early embryogenesis and decrease in regenerating liver and cultured hepatoma cells (13, 16, 17, 47, 48, 53). This suggests that any role that YB-1 may have with respect to tissue-specific gene expression in the liver is likely to be a negative one. We have demonstrated that the chkYB-1 fusion protein binds extremely well to a number of sites for *trans*-acting factors essential for efficient expression of the serum albumin gene. It is possible, therefore, that the high level of YB-1 expression in early embryonic liver, regenerating liver, and proliferating hepatocarcinoma cells serves to complement the decline in positive *trans*-acting factors involved in expression of tissue-specific genes like the albumin gene. However, several characteristics of YB-1 are consistent with an role alternative to that of a negative regulatory factor. Of particular interest in this respect is the ability of YB-1 to bind both single- and double-stranded DNA corresponding to the binding sites for a variety of *trans*-acting factors. In addition, work done with the *Xenopus* protein has shown that YB-1 has the potential to form multimeric complexes (67). Combined with its developmental profile of expression, the data suggest that YB-1 could function in maintaining a potentially active chromatin configuration during DNA replication.

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