We have compared the activities of mouse α-fetoprotein (AFP) enhancers I, II, and III with their minimal enhancer fragments (Mers) I, II, and III and with the entire 7-kilobase pair enhancer domain by transient expression assay in primary fetal mouse liver cells. The level of expression directed by the AFP promoter [p(−1009)AFPcat] alone is stimulated at least 10-fold by the entire AFP enhancer domain (−1009 to −6983). Enhancer I can drive the level of chloramphenicol acetyltransferase activity equivalent to that of the entire enhancer domain, whereas the increase in activity by enhancers II and III is significantly lower (1.5-fold). Mers I, II, and III all mediate a greater increase in activity than their corresponding enhancer regions. The increase with MerI is 16-fold. Using DNase I protection analyses we identified 3 protein-binding regions in MerI; site Ia binds liver and brain nuclear proteins; site Ib binds liver, kidney, and brain nuclear proteins as well as purified C/EBP; site Ic binds liver and kidney nuclear proteins. Site-specific mutation of Ia, Ib, or Ic showed a 10–25% reduction in chloramphenicol acetyltransferase expression; deletion of the C/EBP-binding site in Ib showed a 45% reduction in activity and mutation of all 3 sites (Ia, Ib, and Ic) resulted in a 75% reduction in activity. Our studies indicate no single trans-acting factor is absolutely essential for enhancer activity, and that the enhancer activity of MerI is mediated via a combinatorial and additive mechanism.

The molecular basis of tissue-specific and developmental regulation of the rodent and human α-fetoprotein (AFP) genes has been localized to the cis-acting elements of the promoter and enhancer domains (1–9). These studies have shown that there are four distinct regulatory elements in the AFP gene beginning at the site of transcriptional initiation and extending to ~7.6 kilobase pairs upstream of the gene. The promoter region has been localized up to −1009 bp, although the proximal promoter which extends to ~202 bp contains all the information needed to mediate maximal tissue-specific expression in transient expression assays (10–12). The three enhancer domains, i.e. enhancers I, II, and III, have been localized within the region from −1 to −7.6 kilobase pairs. Their boundaries were initially established by transient expression assay of DNA fragments produced by BamHI restriction digestion (2, 9). Furthermore, this assay was used to identify minimal enhancer regions (Mers), which range in size from ~200 to 300 bp, and which are localized within each of the enhancer fragments (3). Hammer et al. (4) demonstrated that at least one of the three enhancer regions is required for tissue specificity as indicated by their function in transgenic mice. However, the postnatal repression of the AFP gene has been shown to be a function of the promoter, and/or portions of the structural gene (1, 6, 8). Thus, both tissue specificity and developmental repression are associated with AFP promoter function, whereas tissue specificity alone is associated with enhancer function. Although all three enhancers exhibit equal levels of activity in HepG2 cells, similar studies with transgenic mice have shown that enhancer I is more active in the livers of transgenic animals, than enhancer II and that enhancer III has very little activity (4).

The AFP promoter region contains protein-binding sites where trans-acting factors interact with cis-acting DNA sequences to mediate regulation of its tissue-specific expression (10, 12–15). Evidence for the existence of these multiple protein-binding sites in the proximal promoter of the AFP gene has been obtained by DNase I protection analyses (12–15) and by transient expression analyses using deletion mutations and site-specific mutations of each of the binding sites (2, 10, 14). Through these studies it has been shown that the proximal promoter alone, up to −202 bp, is sufficient to mediate tissue-specific transcription of a reporter gene in hepatoma cells (10, 11) and in primary cultures of fetal liver cells (14). By using site-specific mutation in each of the binding sites it was shown by transient expression assay that promoter-mediated regulation of the AFP gene requires the combinatorial action of these multiple cis- and trans-acting elements in the proximal promoter.

Recently Zhang et al. (14) observed that by linking enhancer I to proximal promoter fragments (~202 bp) with C/EBP-binding site mutations, the reduced activity caused by these mutations is rescued, i.e. CAT gene expression is returned to normal levels. We hypothesized that enhancer I may provide site(s) for binding of trans-acting factor(s) which can rescue the C/EBP mutations in the promoter, possibly a C/EBP-binding site. On the basis of these observations we felt that it...
was important to elucidate the DNA-protein interactions of the AFP enhancers and their role in enhancer function. To do this we have sequenced the entire enhancer region (from -1009 bp to -6983 bp), assayed the enhancers I, II, and III as well as MerI, II, and III activities in primary fetal liver culture, and performed DNase I protection analyses to identify the binding sites in MerI using normal fetal liver and adult liver nuclear extracts. We also report on experiments to define the role of the trans-acting factors and cis-acting binding sites of MerI in augmenting AFP gene expression, using site-specific and deletion mutations.

MATERIALS AND METHODS

Constructing Plasmids for CAT Assay and Substitution and Deletion Mutagenesis—The p(-1009)AFPcat (BSID-CAT) and pAFPcat were as reported previously (9, 15), which contain AFP gene -1009 to +37 bp and -6983 to +437 bp upstream of the CAT gene, respectively. As shown in Fig. 1, pEnh(-1009)AFPcat contains AFP enhancer I DNA fragment (BanHI (-3835 bp) to BamHI (-1010 bp)) at the 5' end of AFP promoter (-1009 to +37 bp). The pEnhII(-1009)AFPcat contains AFP enhancer II DNA fragment (BanHI (-5333 bp) to BamHI (-3966 bp)) at the 5' end of AFP promoter. The pEnhIII(-1009)AFPcat contains AFP enhancer III DNA fragment (PoulI (-6981 bp) to BamHI (-5332 bp)) at the 5' end of AFP promoter. The orientation of all these enhancer fragments in the CAT constructs are as in the AFP genome.

In mutagenesis, the XhoI-EcoRI minimal enhancer region I (MerI) fragment from pUC19 was inserted into M13mp19, pmpMerI. Three substitution mutants mpMA, mpMB, and mpMC, in which the mutation sites correspond to the sites of nuclear protein protection regions Ia, Ib, and Ic on the AFP MerI were prepared by oligonucleotide-directed mutagenesis of the mpMerI with synthetic oligonucleotides that paired with 12 nucleotides of the wild-type sequence on either side of 6-8-nucleotide target sequence. The base sequence for each substitution (Fig. 3) consisted of a XhoI recognition sequence for each substitution. The mutations were confirmed by XhoI restriction endonuclease site analysis and dideoxy sequencing. All the substitution mutant fragments were isolated by digestion with BamHI and KpnI and used to replace the wild-type BamHI and KpnI fragment in the pMerI(-1009)AFPcat.

In pMid(-1009)AFPcat sequences from -2317 to -2275 bp of the MerI enhancer fragment were deleted. This construct was generated by digestion with the slow form of Bal31 exonuclease (International Biotechnologies, Inc.) from the XhoI site in pMPH(-1009)AFPcat. The DNA sequence of this construction was confirmed by dideoxy sequencing. The pMid(-1009)AFPcat and pmpMid(-1009)AFPcat are pMPH(-1009)AFPcat with oligonucleotide-directed site-specific mutations at regions Ia and Ic, respectively. The pMid(-1009)AFPcat is pMid(-1009)AFPcat with mutations in both regions Ia and Ic.

Plasmid Construction and End Labeling of DNA Fragments for DNase I Footprinting—The DNA fragment containing AFP MerI (AseI -2574 to -2190 bp NdeI), was excised from the AFP enhancer region did not show any activity in either fetal kidney cell cultures or in NIH3T3 fibroblast cells (18).

Although the three enhancer fragments and their minimum enhancer regions showed very strong activity in the HepG2 hepatoma cell line, analysis of livers of transgenic mice indicated that the three enhancer fragments were not functionally equivalent in vivo (4). In the transgenic liver, enhancer I has higher activity compared to enhancer II, and enhancer III has little activity. Since studies of AFP enhancer activity in tissue culture have been limited to hepatoma cells, we conducted experiments to compare the in vivo activities with activities in normal fetal cells in culture. To do this, we determined the activity of the entire AFP enhancer domain and each subfragment by transient expression analysis in primary fetal mouse liver cells in culture (Fig. 1). The results indicate that enhancer I stimulates the promoter (-1009 bp) 9.8-fold; that enhancers II and III stimulate the promoter 1.5- and 1.6-fold, respectively, and that the whole enhancer from -7 to -1 kb stimulates promoter activity 10.7-fold. The data also show that MerI, II, and III stimulate the promoter 15.6-, 2.7-, and 3.4-fold, respectively. These data indicate that the enhancer I fragment can augment the activity of the CAT gene to a level equivalent to the activity of the entire 7-kb AFP enhancer region, and that the minimum enhancer fragment, MerI, can augment activity at a level that is 1.5-fold greater than that of the enhancer I fragment. Transient expression assay in the mouse hepatoma cell line (BWTG3) gave results similar to those obtained with primary fetal liver cells in culture (Fig. 1). In contrast, results from experiments in which HepG2 cells were used as host cells indicate no significant differences in the activities of the three enhancer and Mer fragments (Fig. 1 and Refs. 2 and 3). Furthermore, the 7-kb AFP enhancer region did not show any activity in either primary fetal kidney cell cultures or in NIH3T3 fibroblast cells (18).
Functional Analysis of Mouse AFP Enhancers

Since enhancer I and MerI showed the strongest enhancer activity in primary fetal liver cells and in transgenic mice, we conducted experiments to identify the protein-binding sites of MerI and the role of these DNA-protein complexes in MerI enhancer activity.

Localization of Fetal and Adult Nuclear Protein-binding Sites in AFP Minimum Enhancer I (MerI)—The ability of cis-acting enhancer or promoter regions to regulate gene expression involves the interaction of trans-acting factors with their specific DNA-binding sites. Using site-specific mutations we have shown that hepatocyte nuclear factor-1 (HNF-1), C/EBP, and nuclear factor-1 are essential for maximal activity of the AFP promoter (14). To study the DNA and protein components that contribute to AFP enhancer activity, fetal and adult liver nuclear protein extracts were used to perform DNase I footprinting with the MerI DNA fragment. These analyses revealed that there are three protein-binding regions in MerI, which we named regions Ia, Ib, and Ic (Fig. 2). The DNA sequence of each site identified by DNase I protection is indicated by the double underlined sequences in Fig. 3. The footprinting with adult and fetal mouse liver nuclear proteins showed similar protection patterns indicating there are no apparent developmental differences in protein binding patterns detected within this enhancer region.

Sequence analysis of the DNase I-protected regions of MerI (Ia, Ib, and Ic) revealed homologies with consensus sequences of well characterized trans-acting factor-binding sites as well as binding sites not previously described. The DNA sequence (−2238)CTCATGTTGCAA(−2228), in region Ia (Fig. 3) is homologous with the E3 site of the immunoglobulin H(μ) heavy-chain enhancer and immunoglobulin κ light chain promoter (20–23). Furthermore, the sequence TGGCA, also in region Ia is one-half of the nuclear factor-1 consensus sequence. Both of these sequences are potential binding sites for ubiquitous proteins reported to be important for enhancer and promoter activity. The sequence CACACAAA, immediately upstream of region Ia, is one of several that is also repeated in MersII and -III. However, our protection analyses show that these sequences are not protein-binding sites in any of the Mers.

Sequence analysis by Godbout et al. (3) revealed an 18-nucleotide sequence, Box 1, in all three Mers and a 10-nucleotide sequence, Box 2, in MerII and II. Our DNase I footprinting data indicate that Box 1 of MerI is in protected region Ib. Furthermore, Box 1 of MerII is also protected, whereas there is no protein binding detected in the Box 1 region of MerIII. Sequence analysis also revealed the pres-
FIG. 3. The sequence of MerI and the binding sites for liver (L), kidney (K), and brain (B) nuclear proteins, and the C/EBP-binding site. The lines below the sequence denote the sequences of the coding and noncoding strands protected by liver nuclear proteins; the lines above the sequence denote the sequences of the coding strand protected by kidney and brain nuclear proteins and by purified C/EBP. The mutations introduced into regions Ia, Ib, and Ic are shown immediately below the sequence they replace.

FIG. 4. DNase I protection of the mouse AFP MerI by nuclear proteins from adult mouse liver, kidney, and brain. Panel A, the labeled coding strand of minimum enhancer region I (MerI). DNA fragments were incubated with 0, 30, and 60 μg of nuclear proteins from the different tissues as indicated at the top of each lane in the presence of 4000 ng of double stranded poly(dI-C). Lanes G and +A are the probe DNAs subjected to guanosine and guanosine + adenine-specific Maxam-Gilbert cleavage, respectively. All the other labels are the same as in Fig. 2.

were not cultured, there is the possibility that the levels (or activity) of trans-acting factors may be altered in cultured cells, thus affecting the levels of transient expression. Xanthopoulos et al. (28) reported, for example, that there is a gradual decrease of C/EBP mRNA levels in primary adult rat hepatocyte cultures. Since we were not able to extract nuclear proteins from the cultured mouse hepatocytes, to address this question we analyzed C/EBP mRNA levels in these cultures to determine whether levels of this trans-acting factor are altered. Total cellular RNA was isolated from adult rat liver, adult mouse liver, and spleen, from primary fetal liver cells in culture, and from mouse hepatoma cells (BWTG3) in culture. These RNAs were hybridized to rat [32P]C/EBP cDNA. As shown in Fig. 6 the level of C/EBP mRNA, in primary cultures, is the same throughout the 96-h culture period (and higher than the levels in BWTG3 cells). DNase I footprinting analyses using nuclear protein from BWTG3 cells exhibit strong protection of C/EBP-binding sites in both AFP and albumin promoters. These data support the use of fetal liver cells in culture for studies of the role of nuclear protein in the function of the AFP enhancer. To study the importance of factors binding to this region in enhancing AFP promoter activity, oligonucleotide-directed site-specific mutagenesis was used to generate mutations at each of the three binding sites. The wild-type protection pattern is shown in panel W of Fig. 7 and the mutated sequences are shown in Fig. 3. DNase I footprinting (as shown in panels A and C of Fig. 7) shows that these mutations abolish binding of the corresponding nuclear protein(s) to their binding sites.

3 C-C. Hsieh, D-E. Zhang, and J. Papaconstantinou, manuscript in preparation.
Fig. 5. DNase I protection of the mouse AFP MerI by purified trans-acting factor C/EBPα. DNA probe used is the labeled coding strand of MerI. Lane F is the DNase I-digested DNA probe in the absence of any protein. Lane C/EBPα the DNase I-digested probe in the presence of 4,000 ng of double stranded poly[d(I-C)] and 2 μl of protein solution which contains the full-length C/EBPα expressed in Escherichia coli. Lanes G and G+A are the probe DNAs subjected to guanosine and guanosine + adenine-specific Maxam-Gilbert cleavage, respectively. Numbers on left indicate the nucleotides upstream of the site of transcription initiation (+1). Sequences protected from DNase I digestion are indicated by brackets and numbers on right.

binding of proteins to regions Ia and Ic were fully and specifically abolished by the mutation in the corresponding DNA sequence. The 8-nucleotide substitution mutation in the middle of region Ib was observed to partially abolish binding (Fig. 7B) of its protein leaving the region from −2290 to −2276 still protected. MerI fragments with each mutation were inserted into the plasmid p(−1009)AFPcat, which contains the AFP promoter and transfected into cultures of primary fetal mouse liver cells as mentioned above. The CAT activities from transfection experiments, shown in Fig. 8, indicate that mutation in region Ia (Fig. 8B) resulted in a 10% reduction of MerI activity while the mutations in regions Ib (Fig. 8C) and Ic (Fig. 8D) resulted in a 25% reduction of MerI activity, but none of the single site mutations of MerI exhibited a dominant effect on its enhancer activity. Since the mutation of region Ib did not completely abolish binding to that region, a deletion mutation was prepared in which sequences from...

Fig. 6. An analysis of the C/EBPα mRNA levels in fetal mouse liver primary cultures. Fetal mouse liver cells were plated immediately after dispersal (t = 0) and mRNA levels were determined at 24-h intervals for 4 days (t = 24–96). Agarose formaldehyde gels were run using 15 μg of total RNA and Northern hybridization analyses was performed as described under "Materials and Methods." C/EBPα mRNA levels were also determined for adult mouse liver and spleen and for adult rat liver. The hybridization signals were quantitated by densitometric scanning.

Fig. 7. The interaction of liver nuclear protein with wild-type or mutated DNA sequences of AFP MerI. DNase I protection of wild-type and mutated MerI by adult mouse liver nuclear protein. All experimental conditions and labeling are as designated in Fig. 2.

Fig. 8. The ability of mutated MerI to enhance AFP promoter activity. Regions A, B, and C of MerI were mutated by site-specific mutation, and both the wild-type and mutated Mers were inserted into the 5' end of the AFP promoter in the plasmid p(−1009)AFPcat. The plasmids were transfected into primary cultured fetal mouse liver cells as described under "Materials and Methods." A, pMerI(−1009)AFPcat contains the wild-type MerI; B, pMIA; C, pMIB; D, pMIC contain the Mers mutated in regions Ia, Ib, and Ic, respectively; E, pMID contains the Mer in which region Ib was deleted; F, pMIE contains deleted region Ib and mutated region Ia; G, pMIF contains deleted region Ib and mutated region Ic; H, pMIG contains deleted region Ib and mutated regions Ia plus Ic; I, p(−1009)AFPcat demonstrates the basal of AFP promoter activity in the absence of enhancer or Mer domains (see also Ref. 14 for a detailed promoter analyses). Enhancer activities were analyzed as described in the legend to Fig. 1.
—2319 to —2276 were removed. The CAT activity of this expression vector was reduced by ~40–45% (Fig. 8E). Site-specific mutation of either Ia (Fig. 8F) or Ic (Fig. 8G) in the Ib deleted MerI did not further reduce activity, indicating that approximately 50% of the enhancer activity can be mediated when 2 of the 3 binding sites are mutated. Finally, a triple mutation, i.e. Ia + Ic site-specific mutation plus deleted Ib, reduced activity by ~75% (Fig. 8H). We interpret these data to indicate that MerI enhancer activity is combinatorial and additive. The persistence of enhancer activity when protein binding at all three sites is abolished may be due to the presence of other important sequences. Alternatively, although mutations abolish protein binding in vitro, as indicated by DNase I protection, binding may not be fully abolished in vivo.

**DISCUSSION**

Transient expression analyses using primary fetal liver cell cultures have shown that: (a) the AFP promoter (~1009 bp) can direct transcription of p(-1009)AFPcat; (b) the level of expression directed by this promoter is stimulated at least 10-fold when linked to the entire AFP enhancer domain; (c) enhancer I and MerI can mediate the level of activity of the CAT gene equivalent to the entire enhancer domain (pAFPcat); (d) enhancers II and III only mediate a slight increase in the level of transcription (1.5-fold) above that of the promoter; (e) all the MerI fragments produce a greater increase in activity than their corresponding enhancer region. We conclude that in primary fetal liver cells the enhancer and Mer fragments do not exhibit equal levels of activity as reported for HepG2 or Hep3B cells (3). Furthermore, enhancer I and MerI exhibit the strongest enhancer activity in fetal liver cell cultures with the minimal element (MerI) showing approximately 1.5 times the activity of the enhancer I region. These results are consistent with the results obtained with transgenic mice (4).

The AFP enhancer domain and its subfragments exhibit the same pattern of activity in primary fetal liver cells, in mouse hepatoma cells (BWTG3), and in transgenic mice. In contrast, no significant differences were seen with the same expression vectors in HepG2 cells. Although we do not understand the basis for this difference with the HepG2 cells, preliminary gel shift analyses indicate that there are significant differences in binding activities of HNF-1 and C/EBP from nuclear extracts of HepG2 cells. These data suggest that the altered activity of essential trans-acting factors may be a basis for the ability of all enhancer subfragments to exhibit similar high levels of activity.

Sequence analysis of MerI has revealed a variety of potential binding sites for trans-acting factors (3). We have identified those sequences which exhibit protein binding activity, and we have attempted to correlate the functional roles of these protein-DNA complexes through mutation analysis. Box 1, for example, binds purified C/EBP as well as a protein(s) from liver nuclei, and both site-specific or deletion mutation of this site reduced enhancer activity by approximately 25 and 40%, respectively. The purified C/EBP used to localize this site is C/EBPα (29). There are other members of the C/EBP family of trans-acting factors, and we have attempted to correlate the functional activities of these two functional sites can contribute 75–90% of the enhancer activity. Mutation of region Ia (C/EBP-binding site) resulted in a 25% reduction of enhancer activity, and its deletion appears to reduce tissue specificity (3). Whether this is a functional HNF-3 binding site remains to be determined.

Our site-specific and deletion mutation analyses showed that none of the MerI protein-binding sites exhibited dominant tissue-specific enhancer activity and that these regulatory sites work in a combinatorial mechanism. Even though mutations totally abolish protein binding to regions Ia and Ic, these mutations only resulted in a 10 and 25% reduction of activity indicating that the other two functional sites can contribute 75–90% of the enhancer activity. Mutation of region Ib (C/EBP-binding site) resulted in a 25% inhibition of CAT expression. However, since protection of this site was only partly abolished, this might account for the low level of inhibition seen. Upon deletion of the entire region Ib (mutation Id), enhancer activity was reduced by ~45% indicating that site-specific mutation did not fully inactivate enhancer activity of that region.

To further understand the role of the binding sites we constructed MerI fragments with multiple site-specific mutations. Mutation of Ia or Ic in the Ib deleted MerI did not significantly affect the level of inhibition above that of the...
deletion of Ia alone. These results suggest that as long as one of the three binding sites of MerI remains intact, this functional site can augment transcription at one-half the control level. Finally, when both Ia and Ic are mutated in the Ia deleted MerI, the enhancer activity was reduced by ~75–80%. Thus, through the use of both site-specific and deletion mutation we were not able to identify a dominant trans-acting factor essential for enhancer activity. Similar results have been reported with the IgH(μ) (30, 31) and SV40 (32, 33) enhancers.

Our studies suggest that the activity of MerI is achieved through the combinatorial action of the binding of multiple trans-acting factors. The stepwise decline in activity with multiple mutations is indicative of such an additive mechanism of action. Interestingly, persistence of ~20–25% of enhancer activity by the triple mutation of MerI indicates that other sequences may play an important role in enhancer activity, or a significant percentage of the remaining activity may be due to promoter driven expression alone.

Using purified C/EBP protein in DNase I protection assays we identified C/EBP protected regions in all three Mers which also correspond to their liver-specific binding sites (Fig. 5 and data not shown). However, MersII and III exhibit relatively low activity, while MerI exhibits a 16-fold enhancement in a homologous system. These data clearly indicate therefore that C/EBP alone is not sufficient to drive the maximal level of transcription of the mouse AFP promoter. Thus the activity of enhancer I and MerI is not totally dependent on C/EBP sites of the AFP enhancer domains using recombinant pro-

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