A promoter-linked coupling region required for stimulation of α -fetoprotein transcription by distant enhancers

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ABSTRACT

We previously demonstrated that the rat α -fetoprotein (AFP) gene has three upstream enhancers that stimulate the AFP promoter additively in HepG2 cells (1). In this paper, deletion analysis demonstrated that a promoter-linked segment from –178 to –155 was required for full activity when the enhancers were distant from the promoter, even at less than their normal genomic distances, but dispensable when the enhancers were moved close to the promoter. This 'promoter-coupling element' appears to interact simultaneously with all three enhancers. Deletion analysis also localized a transcription stimulatory and a negative region in the promoter. Though these latter regions controlled the strength of the isolated promoter, they did not affect 'coupling' to the distant enhancers, and transcription stimulation by these distal promoter elements was small compared to the distant enhancers. Overall, the distant enhancers, acting through the promoter-coupling element, accounted for 70% of the activity of the transfected AFP gene. Footprint analysis with HepG2 nuclear extracts demonstrated protein binding at two sites near the promoter-coupling element. The data indicate a positive transcription control mechanism by which distant enhancers stimulate the AFP promoter through a specific promoter-linked element.

INTRODUCTION

The α -fetoprotein (AFP) gene is an important model system for the study of tissue-specific gene expression. AFP transcription controls mediate developmental stage-specific expression, silencing, and probably coordinate AFP and albumin gene expression. AFP expression is regulated by transcription controls that include an unusually large promoter, three strong distant enhancers, and a repressor region (1–3). The number and size of these elements make it one of the most complex gene regulatory systems described.

In studies based on DNA transfection (1, 4), we mapped three enhancers and found them to be capable of acting from relatively long genomic distances (see Figure 1, below). Surprisingly, when

the three enhancers were combined on a single plasmid, their activity was the arithmetic sum of their separate activities on individual plasmids. The combined enhancers acting through a single promoter accounted for about 97% of the activity of the transfected AFP gene in HepG2 cells.

Since the AFP promoter region (i.e. the proximal transcription controls from –1 to about –250) can simultaneously interact with three separate enhancers, the studies in this paper were carried out to resolve promoter-region functions, particularly to characterize the elements responsible for enhancer interactions. Deletion analysis was employed to determine whether the enhancers interacted diffusely with the promoter, or with specific promoter elements. And, in the latter case, whether the different enhancers interacted with separate or common promoter segments. The promoter functions and elements so defined represent critical components of long distance AFP gene control.

MATERIALS AND METHODS

Cell lines

HepG2 cells were obtained from the American Type Culture Collection and maintained in Williams E medium plus glutamine and 5% fetal calf serum. Growth during transfection assays used hormone-free conditions, Williams E medium plus glutamine containing 0.5% charcoal-treated dialyzed fetal calf serum (1, 5).

Transfection assays

Our transfection conditions and CAT assay protocols for HepG2 cells have been described in detail (1). All assays used 15 μ g DNA, 1×10^6 cells grown for 2 days or 2×10^6 cells for one day, and 75 cm² plates, or proportional concentrations on smaller plates.

To compensate for varying plasmid sizes, all activities were expressed as relative CAT activity per mole of transfected plasmid. For matched comparisons, plasmids were always studied in groups that included a positive control (an intact enhancer) and a negative control (plasmid pSpe Δ X, which contains the AFP promoter deleted to –62). Since the series of plasmids analyzed were larger than could be accommodated in individual experiments, results were normalized to the common control plasmid used in each series.

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DNA plasmid construction

Plasmids pAFP7300, p1483, p1334, p3195ΔXN, pAFP1000, and pSpeΔX, used for construction of new plasmids and as expression controls, were described in Wen et al. (1). The construction of plasmids is summarized in Figure 1, and the exact sequences of the truncated promoters are listed in Figure 7, below.

Promoter deletion and construction of plasmids containing enhancer Complex 1. Constructs were based on plasmid p1483 (1), which contains AFP gene segments -2787 to -1303 , and -984 to -1 . A first series of deletions was constructed by single or double restriction enzyme digestion and religation, in some cases after blunting with Klenow DNA polymerase. Restriction sites used were at -2408 (NdeI), -2391 (BstEII), -2222 (AflIII), -2072 (NsiI), -652 (AvaI), -615 (NsiI), -378 (NsiI), -325 (HindIII), -82 (AflIII), and -62 (SpeI). A second series of deletions was made using bidirectional digestion with Bal31 nuclease from an NsiI digested plasmid (from sites at -2072 and -375). 20 plasmids were tested for activity and had deletion end-points sequenced; plasmids with promoters ending at -297 , -246 , and -191 were selected for the present studies. In a third series of plasmids, a 102 base pair synthetic oligonucleotide was inserted between the -82 AflIII site and the -2391 BstEII site. The oligonucleotide contained the intact region from -82 to -178 , except that a single A $\rightarrow$ T mutation was introduced at -155 to create an AflIII restriction site which was used for further deletion. Correct insertion and deletion was verified by DNA sequencing. Previous sequence analysis (4) suggested that this mutation would be neutral.

A fourth series of promoter deletion plasmids was constructed by inserting double-stranded oligonucleotides (-82 to -134 , -82 to -125 , or -82 to -116) between the AflIII site at -85 and the NsiI site at -2072 while conserving the NsiI site. Based on sequence analysis, these oligonucleotides were chosen to provide successive inactivation of individual binding sites in the promoter. Correct insertion was verified by DNA sequencing. These plasmids were designed for more convenient insertion of spacer or other DNA segments.

To produce plasmids containing only deleted promoter regions without enhancers, Complex 1 was deleted by double restriction enzyme digestion, from BstEII (-2391) or NdeI (-2408) to an NaeI (-7376) site in the vector.

Spacer addition. Spacers were added either by insertion of a 1097 bp segment (BstEII at -2391 to HpaI at -1294) into NdeI or BstEII sites at -2408 or -2391 respectively; or a 1775 bp segment (BstEII at -2391 to BamHI at -918 , plus BamHI at -918 to NsiI at -615) into BstEII (-2391) and NsiI (-2072) sites.

Complex 3 plasmids. For newer plasmids, an XbaI fragment (-6274 to -4939) was inserted at the -984 XbaI site; for older plasmids, a segment from BglII (-7054) to NdeI (-5596) was substituted at the NdeI (-2408) and BglII (-7054) sites. For more extensive deletion of spacing sequences in one plasmid (pAflΔ2), AflIII sites at -5861 and -87 were used. This deletion placed the enhancer about 100 bp from the TATA box.

Complex 2 plasmids. Plasmids containing Complexes 1 and 2 were made by substituting a fragment from NsiI (-2071) to XbaI (-4599) into compatible sites; Complex 1 was then deleted from XhoI (-3134) or HindIII sites (-3856).

Figure 1. Transcription control region of the rat α -fetoprotein gene and plasmid constructs. Above, Sequence analysis (4) and functional mapping of control elements (1) are summarized. Three upstream enhancer regions (Complexes 1–3) were found to be active in HepG2 and H4C3 cells. The repressor region near the promoter is approximated from our own mapping studies, some additional studies in this paper, and characterization by Vacher and Tilghman (2) in the mouse gene, which is almost identical to the rat gene in this region. Homology blocks are regions highly conserved between rat-mouse or rat-human AFP genes. Small repeated DNA segments, and repetitive DNAs (B1, B2, Alu, Line 1, and homopolymeric sequences) were also characterized. Z and A are the first two exons of the AFP gene. Restriction enzyme sites relevant to this paper are also illustrated. Below, A summary of plasmid constructs. The enhancer regions are numbered, spacer segments are shaded, and the variable promoter segments (P) are delimited by a dotted line. The specific promoters used with each enhancer-spacer combination are listed on the right.

Plasmids containing 3 enhancers. A segment that contained the entire enhancer region (-7054 to -2391) was substituted into plasmids using the unique BstEII site (-2391) and a unique NheI site (located at $+1786$ in pAFP7300). Substitutions were carried out in plasmids with and without spacers.

Spacer control plasmids. To demonstrate that the spacer sequences were neutral, the spacer segment from BstEII (-2391) to HpaI (-1294) sites was blunted and cloned into the NaeI site (-7376) of two enhancer-containing plasmids with the promoter region deleted to -155 . The blunt end cloning inserted this spacer in two orientations.

DNase I footprinting

Crude nuclear extracts were prepared as described elsewhere (6, 7). Briefly, HepG2 cells were collected and lysed by incubation in a hypotonic solution (10 mM Hepes, pH 8.3, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 10 mM KCl, 0.5 mM spermidine, and 1 μ g/ml each of leupeptin, aprotinin, and antipain) for 10 min, followed by homogenization in a Dounce homogenizer. Nuclei were sedimented at $1000 \times g$ for 3 min,

Figure 2. Function of Complex 1 enhancer with deleted promoters. *Above.* Representative CAT assays. This matched series of assays shows plasmids which contain neutral DNA inserted between the deleted promoters and the Complex 1 enhancer. The -987-promoter-containing plasmid, p1483, is significantly larger than the other plasmids in the series. The assays used 20 μ g protein, 2 hr incubation, and 16 hr film exposure. *Below.* Expression of CAT plasmids. Net Activity is expression relative to the activity of the total AFP gene transcription control region (plasmid pAFP7300); Relative Activity is expression relative to a control plasmid with intact promoter and Complex 1 enhancer (p1483); ○, plasmids with simple promoter deletions; ●, plasmids with neutral DNA inserted between the enhancer and deleted promoter. All data points are based on at least two transfection experiments. The data points with error bars represent 3–4 separate transfection experiments.

washed with extraction buffer (30 mM Hepes, pH 8.3, 25% glycerol, 450 mM $MgCl_2$, 0.3 mM EDTA, 6 mM DTT, 1 mM PMSF, 0.5 mM spermidine, and 1 μ g/ml each of leupeptin, aprotinin, and antipain), resuspended in extraction buffer, and incubated at 4°C for 45 min with gentle mixing. Following centrifugation at 40,000 $\times$ g for 25 min, the solution was diluted with two volumes of extraction buffer without NaCl, rapidly frozen, and stored at -70°C. Protein concentration was determined according to Bradford (8).

DNA fragments with one 5'-overhang and one blunt end were labeled by filling in with reverse transcriptase (Stratagene, La Jolla, CA) and α - ^{32}P -dNTPs. DNA-binding reactions were carried out by mixing 25 μ l of protein mix (variable amounts of nuclear extract and incubation buffer [20 mM Hepes, pH 8.3, 25% glycerol, 0.2% NP-40, 20 mM $ZnSO_4$, and 12 mM $MgCl_2$]) and 25 μ l of DNA solution (0.5–1.0 ng end-labeled DNA, 2.4 μ g ds-poly(dI:dC), and 4% polyvinyl alcohol) (9–11). The mixtures were incubated on ice for 60 min followed by a 60 sec digestion at room temperature with 0.15–15 ng of freshly diluted DNase I solution. Digests were stopped by adding 100

Figure 3. Positive and negative elements in the AFP promoter region. Expression of CAT plasmids containing promoter deletions, but without upstream enhancers. Net Activity is expression relative to the intact gene (pAFP7300), and Relative Activity is expression relative to plasmid pAFP1000, which contains the promoter from -1 to -984. Only experimental background has been subtracted from these values. However, the activity of the plasmid deleted to -62 is indistinguishable from background.

μ l of 20 mM EDTA, pH 8.0, 1% SDS, 0.2 M NaCl, and 100 μ g/ml tRNA. The samples were extracted with phenol:chloroform, precipitated with ethanol, dried, redissolved in 5 μ l gel loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue), denatured by incubation at 90°C for 3 min, and resolved on a 6% acrylamide-urea DNA sequencing gel. The gel was then dried and autoradiographed.

RESULTS

Promoter deletion and complex 1

High resolution deletion analysis was carried out in several stages by different experimental strategies. A primary series of promoter constructs was generated in plasmid p1483 which contains enhancer Complex 1 and the full AFP promoter. Plasmids containing only deleted promoters, or other enhancers in combination with deleted promoters, were constructed by further deletion or addition of characterized DNA segments. The results presented here represent a synthesis rather than a sequential presentation of experiments.

Successive deletion of the promoter up to -82 had only moderate effect on expression of the Complex 1 enhancer (Figure 2B, open circles). These data suggest a weak negative effect of a segment from -297 and -246, because expression was moderately increased when this segment was deleted. Further deletion to -191 had no effect on expression, and there was moderate loss and then recovery of expression from successive deletions to -82. However, though the Complex 1 enhancer normally resides at approximately -2400, the deletions moved it progressively closer to the promoter. Further, preliminary analysis demonstrated that the -82 promoter had significant activity only when Complex 1 or Complex 3 enhancers were very close.

For a more rigorous analysis of distance relationships, spacer segments of neutral DNA were introduced between the promoter

Figure 4. Promoter interaction with enhancer Complexes 2 and 3. **A.** Expression of Complex 3 CAT plasmids. Complex 3 has been mapped from -6066 to -5896 . Net Activity is expression relative to the intact gene (pAFP7300) and Relative Activity is expression relative to p1334 which contains the region from -6274 to -4939 . $\circ$, plasmids with simple promoter deletions; $\square$, pAfl $\Delta 2$, which has a smaller enhancer-promoter distance; $\bullet$, plasmids with added spacers. **B.** Expression of Complex 2 CAT plasmids. Complex 2 has been mapped from -4490 to -3875 . Net Activity is expression relative to the entire AFP control region (pAFP7300) and Relative Activity is the comparison to a control plasmid (p3195 Δ XN) which contains the region from -4599 to -3134 .

and enhancer (Figure 2A, and 2B, closed circles). The spacers were from the region (-2408 to -614) which normally lies between the promoter and Complex 1. This DNA region has no significant positive or negative effect on the Complex-1-promoter combination (1). The segment from -613 to -246 was deliberately omitted from spacers, because this region has been shown to contain negative elements (1, 2, 12) that may contribute to developmental gene silencing. Even with addition of spacers, the promoter-enhancer distances were less than in the intact gene.

With the addition of spacer, it became apparent that the -155 and shorter promoters, but not the -178 promoter, had greatly attenuated activity. This region appears to be required for full activity of Complex 1 from a distance. The requirement for this promoter segment is abolished by moving the enhancer closer to the promoter. The results demonstrate critical distance effects

Figure 5. Activity of plasmids containing 3 enhancers. **A.** Expression of CAT plasmids with 3 enhancers. Net activity is relative to the entire AFP control region (-7054 to -1) in plasmid pAFP7300. **B.** Quantification of enhancer-promoter interactions. The activities of individual enhancers and promoters were measured on separate plasmids (Figures 2–4) and summed (the small promoter contribution of the promoter was subtracted from the values of enhancer-containing plasmids). All were directly measured except for Complex 2 combined with a -191 promoter, which was estimated to be 7% of total gene activity. The activity ratio was calculated by dividing this sum by the activity of comparable plasmids that contained all three enhancers (Figure 5B). A ratio of one would indicate simple additive activity. A ratio less than one would indicate interference, and greater than one, synergy.

which must be modelled in the test plasmids for proper evaluation of promoter function.

Enhancer-independent gene expression

The AFP promoter alone can direct transcription in HepG2 cells, but accounts for only 3% of total CAT activity of plasmids containing the entire upstream transcription control region in HepG2 cells. Nevertheless, this represents a level of activity that is readily analyzed in HepG2 cells. Accordingly, enhancers were deleted from a series of plasmids to enable direct mapping of positive and negative elements in the AFP promoter (Figure 3). Because a repressor had been demonstrated in the region from about -200 to -800 , deletions at -325 , -652 , -984 , -2071 , and -2391 were also studied. Activity in HepG2 cells was not

Figure 6. Sense-strand footprinting of the distal promoter. A 267 bp DNA fragment from the blunt XmnI site (–349) to the AflIII site (–82) was labeled at the AflIII end. End-labeled DNA fragments were prepared and footprinted as described in Materials and Methods. Lane designations: G+A, fragment cleaved at purines, showing base positions; DNase, control DNaseI cleavage without nuclear extract; HepG2-1 and HepG2-2, footprinting with 16 and 32 µg of extract protein, respectively. Footprinted regions are indicated by brackets and hypersensitive sites by arrows.

significantly affected by deletions from –2391 to –325, even though deletion to –375 caused partial derepression in H4C3 cells (1). However, a negative-acting region was largely eliminated by deletion from –325 to –297, and two positive regions were eliminated by deletions from –191 to –178 from –155 to –134.

The segment from –336 to –303 includes a block of sequence that we found to be conserved among human, mouse and rat genes ('homology block 19,' from –317 to –297, ref. (4)). Block 19 overlaps with a negative element described as a position-dependent silencer in the human AFP gene (12). Though deletion in this region increases activity of plasmids that contain Complex 1 as well as those that contain only the region near the promoter (Figure 3), this element has a very limited net effect in our experimental system. If promoter activity is subtracted from the total activity of Complex-1-containing plasmids, then the contribution of Complex 1 is almost the same whether or not the negative element is present (compare the profiles and net activities of Figure 2 and 3). Thus the negative region acts almost exclusively on a positive transcription control in the promoter,

but not on Complex 1, or on the ability of the promoter to interact with Complex 1. In addition, we have found that deletion of this region does not activate the gene in repressing H4C3 cells (data not shown). The positive elements in the promoter also seem to make only a small contribution to the total activity of the gene, and do not appear to be required for promoter interaction with enhancers.

Effects on additional upstream enhancers

The interactions of promoter and enhancer Complexes 2 and 3 were also analyzed (Figure 4). Complex 3 is the strongest and most distal enhancer in HepG2 cells, accounting for about 60% of the total AFP expression, despite its location near –6 kb. Promoter deletion to –178 had little effect, but deletion to –155 reduced activity by 70%. Addition of spacer had no significant effect on –178 and –155 promoters. However, the –82 promoter showed a strong distance effect, so that moving Complex 3 to about 100 base pairs from the TATA box (plasmid pAflΔ2) restored most of the promoter activity. We had previously reported that pAflΔ2 is expressed at high level in H4C3 cells, which repress the intact AFP promoter (1). This apparent derepression appears, however, to be due to the close approximation of enhancer and promoter rather than loss of a repressing region.

Deletion analysis also showed that the –178 to –155 promoter upstream region was required for Complex 2 activity. Because Complex 2 was found to be the weakest enhancer in HepG2 cells, accounting for only 7% of total AFP expression, a more limited series of plasmids was studied (Figure 4B).

The combination of all three enhancers was studied with an extensive series of plasmids (Figure 5A). Constructs containing the promoter-upstream region to –191 showed full activity. Surprisingly, with simple deletions, the –178 and –155 promoters showed activity much higher than the intact promoter. However, when spacer DNA was introduced, the same promoters showed reduced activity compared to the intact gene. Shorter promoters also showed a marked distance effect. Notably, even with added spacer DNA, enhancer Complex 1 was still placed closer than in the intact gene. The shortened promoters were extremely sensitive to distance and attenuated if the enhancers were removed to near normal distances.

To evaluate the contributions of individual enhancers, we compared their separate and combined activities (Figure 5B). For a simple additive relationship, the ratio of the sum of 3 enhancer activities on separate plasmids to their combined activity on one plasmid should be 1.0. Promoters to –191 or larger, showed a ratio of 0.9, and the –178, –155, –134, and –125 promoters with spacers also showed ratios approximating 1. Though these promoters had reduced activity, the ratios indicated that the effect was uniform for all three enhancers. The shortened promoters that lacked spacers showed a striking difference. Their activity on a single plasmid was synergistic, much higher than the sum of individual enhancer activities. It thus appears that Complex 1 can complement a partially deleted promoter to restore and even augment its activity. This effect is distance dependent and does not occur at normal genomic distances, nor does it occur with a full length promoter (–191 or greater) even at reduced distances.

Additional control plasmids were constructed to rule out an adventitious effect of the spacer on the deleted promoter region or on the enhancers. The –155-promoter-region deletion was chosen because it had full activity when the enhancers were close,

Figure 7. Summary AFP gene promoter region studies. Shaded regions in the promoter region DNA sequence represent segments highly conserved among rat, mouse, and human AFP genes (4). The deletions studied in this paper are marked (Δ) above the line, as are the sites of footprinting (solid bars) and induced hypersensitivity (arrows). Transcription control elements are boxed. Elements indicated with bold type names (GRE (13), HNF1 (14, 16), CAAT/NF1 (16), C/EBP (15, 16), and TATA) have been verified by experimental studies or are obvious from sequence studies and promoter position. Other possible control elements are indicated with italic names (NF3, CORE, HNF4, PANC ENH, and LTFE); these sites represent significant similarities detected by computer analysis (4, 22).

but was greatly attenuated when the enhancers were separated by spacer. The spacer sequence (–2391 to –1294) was blunted and cloned into a plasmid site (–7376) upstream of the enhancers, in combination either with Complex 1 alone, or all three enhancers. Each combination was tested with the spacer in two orientations. For Complex I (full activity 30%), these upstream-spacer plasmids had activities of 34% (forward) and 30% (reverse), compared to an activity of 9.5% when the same spacer was inserted between Complex 1 and the –155 promoter (Figure 2). For the three enhancers (full activity 100%), the upstream-spacer plasmids had activities of 97% (forward) and 93% (reverse) compared to an activity of 28% when the spacer was inserted between the enhancer and deleted promoter (Figure 5). These additional controls further rule out a direct inhibitory effect of the spacer on the promoter or enhancers and verify that the effect of the spacer is positional.

DNA footprint analysis

In view of the critical importance of the promoter upstream region from –155 to –178, we carried out DNase I protection analysis in an attempt to demonstrate binding of HepG2 nuclear proteins to this region. Previous studies had characterized glucocorticoid receptor binding over this region (13) and HNF1, C/EBP, and NF1 binding to more proximal sites (14–16). Additional studies showed a weak footprint of this region, primarily from adult (11), but also from fetal liver extracts (16).

Crude nuclear extracts gave strong footprinting over the –45 to –69 (not shown) and –98 to –127 regions, corresponding to two known HNF1 binding sites, and the CAAT box region. Weaker footprinting was observed from –153 to –167 and

Figure 8. Models of simultaneous coupling by multiple enhancers to the intact (A) and truncated (B) AFP promoter. The model has been simplified by showing only enhancer Complexes 1 and 3. Promoter region elements are black and the large arrow is the TATA box-initiation region; coupling is represented by three lines, and local scanning by a small arrow. Coupling between enhancers might also occur when the promoter region is intact. Experiments in this and our previous study (1) do not rule out this latter possibility.

–177 to –184, and increased DNase sensitivity at –168 and –169 (Figure 6). Thus, binding was confirmed over the regions disrupted by the –178 and –155 deletions (Figure 7). The weak –153 to –167 HepG2 footprint corresponds almost exactly to the weak footprint designated NP-IV that was characterized from liver extracts by Zhang et al. (11, 16).

DISCUSSION

Functional regions in the intact AFP promoter

As modelled in this experimental transfection system, each of the three AFP enhancers can independently stimulate the AFP promoter, and the stimulation is additive when the three are combined on one plasmid. Rather than a diffuse interaction with the intact promoter, deletion analysis demonstrated that a discrete promoter segment was required for strong promoter stimulation by each enhancer. Mapping with each enhancer independently defined the same promoter region; the promoter to –178 had almost full function, while deletion to –155 reduced net CAT expression by about 70% for all enhancers. Thus, 70% of the total activity of the gene could be attributed to this one promoter segment. However, the AFP expression plasmids showed very strong expression in HepG2 cells, and the shortened promoters were still clearly stimulated by the enhancers. Our results are thus fully compatible with the observation that the HNF1 site near –120 also has a significant role in AFP promoter action (14). Indeed, enhancer-stimulated transcription acting through a more upstream site could also require the participation of elements like the –120 HNF1 site.

For convenience, the function eliminated by the –178 to –155 deletion will be described as ‘coupling’ to the distant enhancers, a function that appears to involve looping out of DNA segments between the enhancers and the promoter. Hence, the segment

approximated by the -178 to -155 deletion is the 'promoter-coupling element.' Coupling appears to be a distinct function that can be discriminated from other promoter region functions. Our data suggest that the ability to couple is independent of the strength of the promoter. Transcription factor Sp1 mediates promoter stimulation from a 1.8 kb distant enhancer, by binding to specific sites in the enhancer and promoter and then associating as multimers to loop the DNA between the sites (17, 18). Thus Sp1 appears to be an example of a specific coupling element, though there are no apparent Sp1 binding sites in the AFP (or albumin) promoter.

Coupling might also be related to HMG domain proteins like LEF-1, which bends DNA to 'facilitate communication between widely separated protein binding sites (19)'. Coupling is likely to result from specific interactions between several distinct transcription factors, but is probably not a general feature of all transcription factors.

Besides initiation of transcription, other promoter functions can be defined in our experimental system and discriminated from coupling. The promoter region alone has a distinct transcription stimulatory effect and behaves as an enhancer when combined with other promoters (1, 20). We mapped the main promoter-associated transcription stimulatory effect by deletion from -191 to -178 , just upstream of the promoter-coupling element. There is also a negatively acting region localized by the -325 to -297 deletion, a region which has also been characterized in the human AFP gene (12). In HepG2 cells, the negative region appears to act only on the stimulatory region from -191 to -178 . These two promoter-linked regions control the level of transcription from the isolated promoter, but have no effect on the distant enhancers, either positive or negative. The contributions of these two elements are a small fraction of the stimulation by the distant enhancers.

Other promoters also contain coupling elements, because we previously showed that the albumin and tk promoters can also couple additively with AFP enhancers (1). Further, although the strongest AFP promoter coupling is localized to the -178 to -155 segment, analysis of truncated promoters implies that elements like HNF1 (-120) can also couple. We are currently investigating whether an intact HNF1 site is required for function of the more upstream promoter coupling element. Promoter architecture appears to be modular, and our analysis suggests that strong coupling is a specific function built into the promoter by the presence of specific elements. Most likely, other promoter elements can stimulate transcription without being able to couple to distant enhancers.

Distance effects and promoter complementation

Shortened AFP promoters showed greatly reduced activity with distant enhancers, but full activity when enhancers were moved closer. In addition, when close enough, enhancer Complex 1 can complement the deleted promoter, restoring coupling function. As is the case for coupling by the promoter itself, restoration of promoter coupling function by Complex 1 appears to be distinct from its transcription stimulatory properties. The latter is reflected in the direct measurement of transcription stimulation by Complex 1 (Figure 2), while coupling and promoter complementation are reflected by the synergy with the other enhancers (Figure 5B). Unlike coupling, promoter complementation is strongly distance dependent. Complementation occurred when Complex 1 was placed at -440 but not when placed at -1900 . Also, limited experiments with constructs that placed Complex 1 at -1300

showed weaker complementation (data not illustrated). These observations suggest that local transcription controls can reside over an extended region from the transcription initiation site, enlarging the region often referred to as a promoter. The full promoter-enhancer activation of AFP transcription probably involves a combination of long distance ('looping') and local ('scanning') mechanisms (Figure 8).

Identity of transcription factors that control AFP gene coupling

The promoter coupling element has been previously shown to include a glucocorticoid response element (GRE) that represses AFP gene expression in several cell lines (13). This GRE is unlikely to mediate coupling for several reasons: 1) We used experimental conditions that eliminated steroid hormones from the cell culture medium. 2) Addition of dexamethasone to the medium had a weak repressing effect on expression of transfected AFP-CAT genes in HepG2. HepG2 has low levels of the glucocorticoid receptor, but our cotransfection with a glucocorticoid-receptor-expression plasmid did not alter the dexamethasone effects (ref. (13), and our unpublished studies). 3) RU486 antagonizes the dexamethasone effect but has no other effect on AFP-CAT expression in this experimental system (our unpublished data); 4) The footprint we observed in this region differs from a GRE footprint (11, 13, 21). GRE-mediated-repression most likely occurs by modifying or blocking other interactions that occur at the promoter-coupling element. However, the fact that GRE binding at this site can strongly repress the AFP gene in some cells highlights the importance of positive gene controls at the promoter-coupling site.

Comparison of AFP promoter sequences to known transcription controls (4, 22, 23) has identified several possible binding sites that overlap the promoter-coupling element (Figure 7). These include 'enhancer core' or NF- κ B site similarities at -188 and -174 , an HNF4 similarity at -174 , and a pancreatic enhancer similarity at -142 . Since none of these factors has been shown to regulate the albumin gene (24–28), any could contribute to selective regulation of AFP expression. However, the observed footprints (approximately -184 to -177 and -166 to -153) coincide with none of these observed similarities. Further, extensive computer searching did not identify a relationship between the footprints and known transcription factor binding sites (22). The AFP promoter-coupling element binding factor therefore remains unidentified.

Overview of AFP gene and AFP locus regulation

The studies in this paper have dealt mostly with positive transcription control mechanisms linked to the AFP promoter. A more distal repressor (approximately -250 to -800) has been shown to mediate developmental silencing of transgenes (2, 29). The developmental regulation of the AFP gene apparently includes negative regulation by this upstream repressor as well as the positive coupling controls demonstrated in this paper. The repressor could act by perturbing more proximal controls in the promoter, perhaps the promoter-coupling element.

Most of the expression of the AFP gene is driven by the upstream enhancers, apparently through looping mechanisms. Since looping has the potential for acting over large genomic distances, both the controls and the mechanisms described in this paper have the potential for regulating a group of linked genes. The three enhancers can interact simultaneously with the AFP promoter, and the possibility that these enhancers could

simultaneously interact with two promoters (e.g. albumin and AFP) is intriguing. Alternatively, active repression, or down-regulation of a coupling factor, could release these enhancers from the AFP promoter and enable them to drive the albumin promoter. Elucidation of the AFP transcription controls might thus lead to understanding of the general regulation of linked gene families.

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