Characterization of Distant Enhancers and Promoters in the Albumin-α-Fetoprotein Locus during Active and Silenced Expression*

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The albumin and α-fetoprotein genes are adjacent and express closely related serum proteins. Both genes are strongly expressed in fetal liver, primarily through activation by distant enhancers, but the AFP gene selectively undergoes developmental silencing. We used chromatin immunoprecipitation to study enhancers and promoters during active and silenced gene expression. In adult phenotype cells, the silenced AFP gene was actively repressed at the promoter and two proximal enhancers, characterized by the absence of coactivators and acetylated histone 4, and the presence of corepressors and K9-methylated histone 3. Specific transcription factors, TBP, and RNA polymerase II were all detected on both active and silenced genes, indicating that both states were actively regulated. Surprisingly, promoter-specific factors were also detected on enhancers, especially with reduced chromatin shearing. Under these conditions, an enhancer-specific factor was also detected on the albumin promoter. Association of promoter- and enhancer-specific factors was confirmed by sequential immunoprecipitation. Because no binding was detected on intervening segments, these promoter-enhancer associations suggest looping.

The albumin-AFP locus is a well-known model of developmental and neoplastic gene regulation (1, 2). The two genes diverged from a single 15-exon gene 300–500 million years ago, but unlike most genes with a similar degree of relationship, they have remained next to each other, on rat chromosome 14, mouse chromosome 5, and human chromosome 4 (3, 4). Such conservation of locus structure is generally an indicator of common regulation. Albumin is more specific for liver, because AFP is also expressed in other endodermal tissues of the embryo, but the expression patterns of both genes mirror hepatic development and maturation (5–7). Albumin expression begins at hepatocyte specification, rises to adult levels by E14, and then persists in mature liver where albumin gene transcripts are the most abundant mRNA. AFP expression initially parallels albumin, although low-level expression activates somewhat earlier than albumin in the foregut. The high level of AFP expression in fetal liver selectively and rapidly silences after birth. Because this silencing reverses in liver cancer, AFP is the prototype oncofetal antigen (2). Reactivation of AFP suggests that the tumor cell transcription controls recapitulate those of the fetal hepatocyte.

The albumin-AFP locus is an ideal model system for study of long distance enhancer-promoter interactions during developmental regulation. In both genes, promoter-proximal controls mediate developmental regulation while distant enhancers drive high level expression (8–11). The AFP gene has three characterized enhancers. These do not appear to be developmentally regulated, because they are strongly active in adult hepatocytes when combined with an active promoter (11–13).

The extensively characterized albumin and AFP promoters bind a variety of liver-enriched and housekeeping transcription factors. Each promoter is dominated by HNF1 and C/EBP binding sites (14–16). The housekeeping factor NF-Y binds only the albumin promoter, while factors FTF and Nkx2.8 are specific for the AFP promoter. The enhancers have not been studied as thoroughly as the promoters, but several transcription factor binding sites have been well characterized in the albumin gene enhancer at −9.9-kb enhancer and the AFP gene enhancer (E3) at −6.1-kb. Both enhancers bind multiple C/EBP and Foxa isoforms, and HNF6 (10, 17, 18). Computer analysis also predicts that these factors bind the AFP gene at −4.3-kb (E2) and −2.7-kb (E1) enhancers. Thus C/EBP binds all of the enhancers and promoters. In contrast, Foxa predominantly binds the enhancers while HNF1 is confined to promoters.

The vast majority of cell lines derived from hepatocellular carcinomas either express AFP or have a null phenotype. The albumin-positive AFP-negative adult hepatocyte phenotype is quite rare in cell lines from all species, except in those derived from the rat Reuber hepatoma. AFP repression is mediated by elements that extend from about −900 to −250 bp (12). In the mouse, an upstream part of this repressing region, the SBE/p53RE (−878 to −762) binds p53, p73, and Smad4 (19, 20). However, a less well defined region closer to the promoter mediates most repressive activity in Reuber cells (11).
To exploit the phenotype of Reuber hepatoma, cell line H-4-II-E (H4E) was paired with another rat cell line, McA-RH8994 (8994). The latter has a fetal phenotype and expresses high levels of both albumin and AFP. A previous study showed that the genes were intact in both cell lines and characterized the transcripts in detail (21). Other articles described transient assays of promoter and enhancer constructs in these cells (11, 22) and we also characterized the relevant transcription factors (below).3 This resource of cell lines and characterization is the basis of a model system of enhancer and promoter function. The new experiments demonstrated that the albumin promoter and enhancer were active in 8994 but repressed in H4E cells. However, transcription factor binding was detected in both the active and silenced AFP gene. Although transcription factor binding patterns were gene-specific, we detected promoter-specific factors on enhancers, where binding sites for these factors had not been demonstrated. The clarification of these observations led us to rule out the presence of binding sites in detected regions and investigate the relationship of chromatin fragmentation to localized detection by immunoprecipitation.

EXPERIMENTAL PROCEDURES

Chromatin Immunoprecipitation—We used the Boyd and Farnham procedure (23) as described in Forsberg et al. (24). Solutions and sample volumes were according to the latter article. Fixed chromatin was sheared in capped Falcon 2059 tubes tubes, in a Branson 450 Sonifier equipped with an S-450 cup horn filled with water and refrigerated at 4 °C. Tubes were placed in the cup horn and sonicated from 4–8 min as 30-s pulses, using Power Setting 70%, Duty Cycle 25%. DNA was extracted from an aliquot, solubilized with proteinase K overnight, and resolved on an agarose gel. The DNA from these aliquots showed a broad distribution of molecular weight, and a preparation was considered suitable for further analysis if the maximum intensity was in a range of 400–800 bp. Such preparations generally gave successful ChIP assays, but with batch-to-batch differences in sensitivity of detection. We subsequently found that sonication varied with the number of tubes and position in the cup horn and added the following modifications. In the modified procedure, a single tube was suspended by a clamp in the center of the cup horn, 1 mm above the base. Sonication for 6 min was used for a standard assay, and other sonication times were studied for their differential effects on detection by ChIP.

Each preparation of formalin-fixed chromatin from 8994 was obtained from 2 × 10^7 exponentially growing cells while H4E chromatin was prepared from 3 × 10^7 cells in the same solution volumes. In a typical experiment, a preparation was divided into 5 separate immunoprecipitations. The final isolated DNA was resuspended in 30 μl. PCR amplifications were individually optimized and run for 30–40 cycles in various experiments. All illustrated reactions were amplified for 40 cycles to accentuate weak or to substantiate negative detections. A typical 20-μl PCR reaction contained 1 μl of isolated DNA, i.e. the input from 1.7 × 10^7 8994 cells or 2.6 × 10^6 H4E cells. Primers were designed to span 300–400 base pair regions.

For quantitative real-time PCR, 384-well plates were prepared with final reaction volumes of 8 μl, including 4 μl of SYBR Green PCR Master Mix (Applied Biosystems), primers at 0.25 μM, and sample or control DNA. Amplification and detection were carried out using an ABI PRISM 7900HT sequence detection system and data were analyzed using software provided with the system. All measured values were averaged from three separate reactions. A standard curve was obtained using normal rat DNA with 10-fold dilutions from 100 ng/ml (~30,000 gene copies) to 10 pg/ml (~3 gene copies). Experimental values were interpolated from the standard curve. Primers for this analysis were designed to amplify segments of 80–120 bp and are listed in Table 1.

For sequential immunoprecipitation, a protocol was modified from Hatzis and Talianidis (25), 2 × 10^7 cells were treated as above, but divided into two larger primary immunoprecipitations, each from about 1 × 10^7 cells. All volumes were proportionally enlarged. Following capture of immunoprecipitates by Protein A or G, the beads were suspended in 40 μl of 10 mM dithiothreitol for 37 °C for 30 min, in a solution also containing 26 mM Tris-HCl, pH 8.1, 3.6 mM EDTA, 120 mM NaCl, 0.8% Triton X-100, and 0.1% SDS. The samples were transferred to room temperature. 1 ml of fresh iodoacetamide buffer was added (10 mM iodoacetamide, 26 mM Tris-HCl, pH 8.1, 3.6 mM EDTA, 120 mM NaCl, 0.8% Triton X-100, 0.1% SDS) and the sample was equilibrated for 10 min. The suspension was then divided into 5 separate tubes for secondary immunoprecipitations, each from 2 × 10^6 cells. These were

| TABLE 1 | PCR primers and amplimers                                                                 |
|-----------------|------------------------------------------------------------------------------------------|
| Region | Position | Primers | Identifiers |
| Alb P | 315 to 7 | CTTCCCTGCAAACATGAAATGCC | Alb P 1 |
| | 115 to 6 | GTAGAACCAATGAAATGCC | Alb P 2 |
| Alb E | 9820 to 9862 | AGTTCTCGGTCGTTAGCTCCAGC | Alb E 1 |
| | 9588 to 9568 | CAGAGCTATCTAGACACACAAATG | Alb E 2 |
| Alb C | 880 to 517 | GTGAGAAGCAGCAGATGCACC | Alb C 1 |
| | 587 to 362 | ACAAATGTGAGGAGCGACTGAT | Alb C 2 |
| AFP P | 280 to 101 | AAATGCACATCCTACCTAATCACACAAATG | AFP P 1 |
| | 118 to 13 | GGCAAGAACATGTAGTAAAG | AFP P 2 |
| AFP E1 | 2702 to 2407 | CTAGAGAAGCAGCAGATGCACC | AFP E1 1 |
| | 2474 to 2370 | AAGGTCAGACAGGAAGCACC | AFP E1 2 |
| AFP E2 | 4332 to 4024 | CGCTGCGAAGACGCGGACG | AFP E2 1 |
| | 5882 to 5795 | TGGATGGATGGAACTGTTT | AFP E2 2 |
| AFP E3 | 6282 to 5882 | GTGACGAGACGAGCTG | AFP E3 1 |
| | 5882 to 5795 | TGGATGGATGGAACTGTTT | AFP E3 2 |
| AFP C | 1350 to 1042 | ACCTGACGTGCTGCTGCTGCTG | AFP C 1 |
| | 362 to 35 | ACAAGAGATACCAGGTCCCCACAAC | AFP C 2 |
| Ins P | 1042 to 5882 | AAGGTGCACAGGGTCACCGT | Ins P 1 |
| | 115 to 35 | GTTCGGCGGGCAGCTCG | Ins P 2 |
| BglO P | 207 to 5 | GTTCGGCGGGCAGCTCG | BglO P 1 |
| | 81 to 1 | CTCCTGAGCAAGCAGCAAC | BglO P 2 |

3 Position relative to the main transcription start site of each gene.
4 The first primer pair listed was used for conventional PCR, and the second pair for real-time PCR.
then treated by the standard immunoprecipitation protocol. Suitability of antibodies varied greatly. Those considered suitable showed a clear positive detection when secondary precipitation was carried out with the same antibody as the primary precipitation (i.e., primary epitopes were preserved), and no detection when precipitation was carried out without a secondary antibody (i.e., complete liberation from the primary antibody).

The Nkx2.8 antibody was characterized in Ref. 26 and the other antibodies specificities were: HNF1α (sc-6547), HNF1β (sc-7411), C/EBPβ (sc-150), FFT/BIF1 (sc-5998), Foxa1 (sc-6553), Foxa2 (sc-9187), Foxa3 (sc-5360), HNF6 (sc-6559), TBPl (sc-204), Pol II N terminus (sc-899), HDAC1 (sc-7872), HDAC2 (sc-7899), SMRT (sc-1612), MeCP2 (sc-5758), Brg1 (sc-8149), PCAF (sc-6300), Src1 (sc-6096), CBP (sc-7300), HP1 (sc-10217), CAF1 p60 (sc-10979), Histone 1 (sc-8030), SIRT1 (sc-15404) from Santa Cruz Biotechnology (Santa Cruz, CA); NF-Y A (556359) from BD-Pharmingen (San Diego, CA); and acetylated histone 4 (06–598), and monomethyl Histone 3 (K9) (07–395) from Upstate Biotechnology (Charlottesville, VA).

**RESULTS**

**Interactions in the Promoter Regions**—Analysis of the albumin and AFP promoters confirmed promoter binding of specific factors and established relationships of gene activity to local chromatin structure. We used a standardized qualitative ChIP protocol (26), amplimers that fully encompassed the conserved regulatory regions, and antibodies to binding factors present in H4E and 8994 cells (Fig. 1). HNF1α, HNF1β, and C/EBPβ were detected on both promoters, while Foxa isoforms and HNF6 were detected on neither. C/EBPβ, a liver-enriched transcription factor, is absent from H4E and most other hepatocellular carcinoma cell lines, so this antibody was not included in the study. NF-Y selectively bound the albumin promoter, while FFT and Nkx2.8 bound the AFP promoter. Nkx2.8 was absent in H4E cells, where it is not expressed, and was the only transcription factor that showed a cell-specific difference in any part of this study. All of the cis-binding factors showed the expected promoter localizations, which confirmed previous studies in other cell types as well as the specificity of the ChIP analyses.
Analysis of coregulators and histone modification demonstrated that the albumin promoter was active in both cell lines, while the AFP promoter was active only in 8994 cells. Activity was indicated by the presence of coactivators (BRG1, PCAF, SRC1, and CBP), and acetylated H4. In contrast, the AFP promoter in H4E cells showed H3 monomethylation but not acetylation, and a complete switch from coactivators to corepressors (HDAC1, HDAC2, and SMRT). These differences indicated active repression of the AFP promoter in the H4E cells, which was highly consistent with the known developmental regulation. Even so, active repression was surprising, because the known binding factors were all activators and their binding appeared to be unperturbed. Studies to elucidate repressing factors that bind the AFP promoter are underway, but outside the scope of the present article.

As additional markers of gene activity, we included TBP and Pol II in the ChIP analysis. The detections of these two preinitiation complex proteins showed characteristic differences between the two cell lines. TBP detections were weaker and pol II detections were stronger in H4E than in 8994 cells, differences that presumably reflect the growth and transcriptional dynamics of each cell line. However, both TBP and pol II were strongly detected on the repressed AFP promoter. Similar associations have been demonstrated in genes repressed by the Polycomb complex (29, 30).

Distant Regulators and Quantitative ChIP—Promoter region analysis was extended to the entire locus; a 70-kb region containing the rat albumin and AFP genes was cloned, sequenced, analyzed for repetitive DNA regions, and aligned with human sequences (Fig. 2A). The region was analyzed by both qualitative and quantitative PCR, with an enlarged panel of antibodies (Fig. 2, B and C). PCR detections were devised for all regulatory elements characterized in the rat (9, 11). Each analyzed region showed some positive detections, confirming that local chromatin structure was accessible to antibodies. As internal gene controls, we also examined neutral DNA segments (designated Alb C and AFP C in Fig. 2) with the following properties: intermediate position between promoters and enhancers, absence of function in deletion mapping studies, minimal conservation with the human, and absence of repetitive DNA. For controls external to the locus, we studied two genes unexpressed in 8994 and H4E cells. Insulin 1 has a close developmental relationship, because normal expression is in pancreas, a tissue that arises from foregut endoderm like liver. Moreover, the insulin-1 promoter proximal region had been shown to bind HNF1, C/EBP and NF-Y (31). β-Globin major, in contrast, is expressed only in
cells that are developmentally distant from liver. Altogether, ten gene regions were analyzed qualitatively in both cell lines. Six of these regions were also characterized with quantitative PCR.

Five additional immunoprecipitations enlarged the characterization of negative regulation, by detecting factors associated with inactive chromatin, repression, or heterochromatin: H1 (32); the p60 subunit of CAF1 (33); HP1 (34); SIRT1 (35); and MeCP2 (36).

The albumin gene showed essentially the same patterns of binding and histone modification in both cell lines. DNA binding transcription factors and coactivators were present on the promoter and enhancer, but not the neutral region between them. All three regions were positive for acetylated histone and basal transcription factors, and there was no detection of corepressors, negative chromatin factors, or methylated histone.

The AFP gene was active in 8994 but repressed in H4E, and this difference was reflected over a wide region that extended from the AFP promoter to E2. Like the albumin gene, the active AFP gene showed basal factors and acetylated histone over the neutral region (AFP C) between the promoter and enhancers, but not coactivators or transcription factors. In repression, however, ChIP of AFP C detected corepressors and two other silencing factors, MeCP2 and H1. Thus, coactivators were more localized around the promoter and enhancers during active gene expression than corepressors and negative chromatin factors during repression.

The AFP enhancers, especially E2 and E3, revealed additional complexities. In H4E cells, AFP E1 and E2 bound transcription factors, but also bound corepressors and heterochromatin factors. In contrast, E3 appeared active by these analyses. In 8994 cells, E1, but not E2 or E3, bound transcription factors. All three enhancers nevertheless bound coactivators and had acetylated histone in these cells. Significantly, the presence of transcription factors on the endogenous genes, but not activated or repressed chromatin, correlated with the function of the enhancers on transfected plasmids, because all three AFP enhancers were active in H4E cells when transfected in combination with other promoters (11). Conversely, only AFP E1, but not E2 or E3, was active in 8994–cell transfections (22). Basal transcription factors TBP and Pol II were not confined to the promoters but were instead detected at almost all targets in the locus regardless of transcriptional activity or histone acetylation. The localization appeared to be specific, because these basal factors were absent from the inactive insulin and β-globin promoters. This demonstration is consistent with recent studies of the β-globin LCR, in which Pol II (with transcripts) was detected over a wide region upstream of the e-globin gene (37).

Fig. 2 demonstrates the consistency between the qualitative and quantitative PCR. Moreover, the positive quantifications, which varied over about three orders of magnitude, confirmed that even the lowest levels of detection represented hundreds of gene copies and were significantly different from the negatives.

Promoter Factors Bind to Enhancers—The detection of some DNA-binding factors on enhancers was unexpected. Foxa/HNF6 and C/EBP binding were predicted, but four factors thought to be promoter specific were also detected. Moreover, the enhancer detections selectively reflected the corresponding promoters (Fig. 2, B and C). The albumin enhancer bound HNF1 and NF-Y. AFP E1 bound HNF1, FTF, and Nkx2.8. Binding sites for these four promoter-specific factors were not detected in previous studies of the enhancers nor predicted by extensive motif analysis (16, 26, 38). We noted variation in these PCR detections from one experiment to another, but data displayed in Fig. 2 were confirmed in multiple experiments that had internally consistent controls. Because no site-specific DNA binding factors were detected on intermediate segments, the detection of these factors suggested enhancer-promoter looping. Looping would have been further substantiated by detection of enhancer-specific factors on promoters, but such binding by Foxa/HNF6 was not detected. Nevertheless, the detection of four different transactors on enhancers that did not have obvious binding sites for those factors led to a further analysis of binding sites and to a more rigorous approach to chromatin fragmentation (below).

A regional competition assay was designed to rule out cryptic binding sites (Fig. 3). Well characterized gel shifts were competed with a 50-fold molar excess of extended DNA segments containing entire promoter or enhancer regions, obtained as purified PCR products. All factors showed their expected localizations. HNF1 binding was competed away only by the two promoters, NF-Y only by the albumin promoter, FTF and
Nkx2.8 only by the AFP promoter, Foxa/HNF6 only by enhancers, and C/EBP by both promoters and enhancers. Foxa and HNF6 have overlapping binding specificities, and we used supershift analysis to demonstrate that both 8994 and H4E cells contain Foxa1, Foxa2, Foxa3, and HNF6 (not illustrated). The binding pattern designated Foxa/HNF6 in Fig. 3 is a composite of all 4 factors.

Chromatin Fragment Size and Demonstration of Looping—Because the competition assays ruled out HNF1, NF-Y, FTF, and Nkx2.8 binding sites in the enhancers, the ChIP detection suggested complexes containing both enhancers and promoters. Because combined enhancer-promoter complexes would be larger than solitary enhancer or promoter complexes, we examined the relationship of chromatin fragmentation to local factor detection. Our analysis focused on the albumin gene, because the substantial 9.8-kb distance between enhancer and promoter provided the most stringent test of looping, but parallel analysis was conducted on the 2.7-kb region that encompassed the AFP promoter and enhancer E1 (Fig. 5). We established more consistent conditions for sonication (see “Experimental Procedures”), then evaluated different degrees of chromatin shearing. Under these conditions, sonication times between 4.5 and 6 min gave strong immunoprecipitations and showed a progressive change in detections; these times provided two optimum conditions for detailed comparisons. Shorter sonication (e.g., 3 min) gave weak, inconsistent detections, suggesting that chromatin had not been sufficiently broken up to allow antibody penetration. Longer sonications gave the same, but progressively weaker detections than 6 min.

Chromatin preparations were made simultaneously with 4.5 and 6 min of sonication and analyzed with 5 selected antibodies. These were directed against HNF1α (promoter-specific), NF-Y (albumin promoter-specific), C/EBPβ (promoter- and enhancer-binding), Foxa3 (enhancer-specific), and HNF6 (enhancer-specific). The latter two were selected from the Foxa group because they gave the most consistent detections in all 8994 cell experiments.

Overall, albumin gene detections were somewhat more robust, but both genes showed a similar sequence of changes. By quantitative PCR, some factors showed a moderate increase or decrease in binding between 4.5 and 6 min. This suggested that progressive break-up of chromatin might dislodge some factors but expose others. The results were consistent with the experiments of Figs. 1 and 2, because the degree of chromatin fragmentation in the earlier experiments was intermediate between the 4.5- and 6-min sonications of Fig. 5.

After 4.5 min of sonication, HNF1α was detected on both enhancers, but this detection was absent after 6 min of treatment. Moreover, Foxa3 was also detected on the Alb promoter, but only with the 4.5-min treatment. The findings suggest large complexes that contain promoter and enhancer factors, which were disrupted by longer sonication. They also suggest looping, since the intervening control segments were not detected at either sonication time.

Despite its close proximity to the promoter-region amplifier, the albumin intermediate segment (Alb C) was negative in both 4.5- and 6-min preparations. Promoter localization was further examined by amplification of a new intermediate segment, separated by only 65 bp from promoter target region (Alb C1). Even this amplification demonstrated only insignificant detection of factors. Thus, the lack of binding on promoter proximal regions cannot be explained only by DNA fragment size. For an immunoprecipitation by anti-HNF1 to be detected by the Alb C primer pair, a DNA fragment would have to extend from the HNF1 binding site at −64 to a distal primer site at −873. Analysis of fragment size distributions demonstrated that fragments large enough to accommodate this interval were abundant in the DNA preparations (Fig. 4A). Moreover, amplification of the Alb C1 region would have required only a −500-bp DNA fragment, and analysis of molecular weight distributions shows that fragments of this size were quite abundant in chromatin sonicated for either 4.5 or 6 min. The absence of detection in the promoter proximal region therefore suggests preferential breakage, presumably at a point of stress where the chromatin fiber extends from a large anchored promoter complex. The lack of detection by the Alb C and Alb C1 primer sets clearly demonstrates that precipitation of enhancer segments via promoter-region binding factors did not occur because the DNA between the two regions had remained intact.

Sequential Immunoprecipitation—A sequence of immunoprecipitations (“Re-ChIP”) was used to demonstrate that enhancers, promoters, and their site-specific DNA binding factors were present in the same complexes. We modified a system described by Hatzis and Talianidis (25) that utilized a high concentration of dithiothreitol to dissociate immune complexes. Stringent criteria were used; the complexes had to be fully dissociated from functional antibody, so that they no longer bound to protein A, but were still capable of reprecipitation with the same antibody. The published procedure worked well with a mouse IgG1 monoclonal antibody to NF-Y, but not with many of the polyclonal antibodies that were used for ChIP. A modified procedure, in which dithiothreitol treatment was followed by dilution and treatment with iodoacetamide, was successful with additional antibodies. However, the latter protocol still did not work with all antibodies, notably several different anti-HNF1 preparations. Nevertheless, the NF-Y antibody provided a promoter-specific target, and we also found an antibody to enhancer-specific Foxa3 that was compatible with the procedure.

The sequential precipitation demonstrated that Foxa3, HNF1α, and NF-Y were simultaneously present on promoters and enhancers in larger but not smaller chromatin complexes. A first series of experiments used anti-NF-Y as the primary antibody. After 4.5 min of sonication, anti-NF-Y precipitated both promoter and enhancer, but after 6 min, it precipitated only the promoter (Fig. 5, A and B, upper sections). At 4.5 min, the promoter and enhancer complexes could be reprecipitated with HNF1α or Foxa3 antibodies, demonstrating that all these factors were present in the same complexes on both promoters and enhancers. After 6 min of sonication, the promoter complexes could be reprecipitated with HNF1α but not Foxa3. The second series of experiments used anti-Foxa3 and gave a reciprocal pattern of detections (Fig. 5, A and B, lower sections), though this antibody produced weaker detections than anti-NF-Y.

Looping in the Albumin-AFP Locus

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FIGURE 4. **Progressive chromatin fragmentation and binding specificity.** Two identical chromatin preparations were prepared simultaneously from 8994 cells and sonicated for 4.5–6 min, under controlled conditions described under "Experimental Methods." Qualitative and real-time PCR were carried out as in Figs. 1 and 2. A, molecular weight distributions. DNA was extracted from the sonicated preparations, resolved on a 1.5% agarose gel, and analyzed by densitometry. Mass average (Mw) and number average (Mn) molecular weight were calculated from intervals. The ratio, \(M_n / M_w \), compares the interval-delimited regions that approximately reflect the minimum DNA fragment sizes necessary to amplify Alb P or Alb C1 from immunoprecipitations by promoter region factors. B, PCR targets near the albumin promoter. The white boxes show regions that are conserved with the human gene, and the promoter map includes specific binding sites relevant to the ChIP analysis. C, detection of promoter, enhancer, and control segments by semiquantitative and PCR. D, quantitative PCR. The values are presented as detected DNA copy number in 1 μl of input DNA.
DISCUSSION

A Looping Model—The ChIP analysis of site-specific transcription factors confirmed all of the established albumin and AFP promoter-binding specificities, but there was a striking difference in the analysis of enhancers; four promoter-specific factors, HNF1α, NF-Y, FTF, and Nkx2.8, were detected on distant enhancers. Since specific enhancer sites for these factors were ruled out, the analysis demonstrated that ChIP detection of a transcription factor on a specific DNA segment may not directly demonstrate a binding site in or near that segment. The factors were not detected on DNA segments between the promoter and enhancer of either gene. Moreover, each enhancer reflected the binding specificity of its corresponding promoter. HNF1 bound both promoters, but NF-Y was albumin-specific, while FTF and Nkx2.8 were specific for AFP. Taken together, these experimental results are most consistent with a chromatin structure in which enhancer and promoter have looped past an intervening region. Though strongly detected on enhancers, the promoter factors apparently occupied a more peripheral part of the enhancer chromatin complex, because they could be removed by increased shearing of chromatin under conditions that did not alter the binding of site-specific enhancer factors. The presence of looping was further confirmed by the eventual detection of an enhancer-specific factor, Foxa3, on promoter complexes. The lack of detection under our initial experimental conditions might have resulted from two differences between Foxa and the other factors in the study. 1) Foxa isoforms were detected at lower levels than the other site-specific factors, partly because binding was shared among different isoforms. 2) Cirillo et al. (39) demonstrated stable associations with between Foxa and nucleosome core particles, suggesting tighter integration into local chromatin than the other transcription factors that were examined. All of the findings are consistent with progressive fragmentation of a looped enhancer-promoter complex (Fig. 6).

Looping between distant enhancers and promoters is no longer controversial and has been clearly demonstrated in several other genes (reviewed in Ref. 40). Our findings therefore indicate that the albumin-AFP locus is a valuable model system for mechanistic studies of these interactions.

8994 and H4E cells both have stable phenotypes and all of our studies utilized rapidly growing cells. The enhancer-promoter associations were therefore consistently reassembled following DNA replication. Our observations are distinct from but complementary to the recent characterization of progressive establishment of enhancer-promoter linkage during differentiation of CaCo-2 cells (25). This latter study also showed looping asso-

FIGURE 5. Sequential immunoprecipitation of the albumin promoter and enhancer. Primary immunoprecipitation was carried out using an antibody to the promoter-specific factor NF-Y or enhancer-specific factor Foxa3, and the complexes were captured on protein A/G. The captured complexes were dissociated, and then the released supernatants were subjected to secondary immunoprecipitations using the same two antibodies, an antibody to HNF1α (promoter-specific), and a non-binding antibody to Nkx2.8 as a negative control. As an additional negative control, the released complexes were incubated with A/G in the absence of additional antibody. The analyses compared chromatin preparations sonicated for 4.5 or 6 min. A, qualitative PCR analyses. B, quantitative PCR. Values are expressed as detected DNA copy number in 1 μl of input DNA.
investigations. It might indicate that repression is superimposed on preexisting modification of the enhancer-promoter association over several days in differentiating post-mitotic cells. This suggests that enhancer-promoter associations were maintained in the presence of histone methylation and co-repressors. Moreover, a previous study showed E2 and E3, but not E1, were inactive on transfected plasmids in 8994 cells. This problem is exemplified by 8994 cells have a strong phenotype and other significant conserved regions (see Fig. 2A) in the AFP upstream region. 8994 cells must therefore lack an uncharacterized binding factor of critical importance, since it would appear to initiate assembly of entire regulatory complexes on E2 and E3. Despite the absence of site-specific transcription factors, however, the two enhancers demonstrated acetylated histone, coactivators, and preinitiation complex factors. This suggests that the enhancers are near other regions that recruit activators. Because no other enhancers are apparent, the presence of coactivators may result from linkage to an active chromatin hub that is independent of local transcription factor binding.

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