Transcriptional regulation of the α -fetoprotein gene by SWI/SNF chromatin remodeling complex

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Abstract

α -Fetoprotein (AFP) is a fetal serum protein abundant in fetal liver whose expression is downregulated in adult liver. The promoter–proximal region of the AFP gene contains two binding sites for CCAAT/enhancer-binding protein α (C/EBP α), two for hepatocyte nuclear factor-1 α (HNF-1 α) and one for nuclear factor-1 (NF-1). Luciferase reporter assays showed that a combination of C/EBP α , HNF-1 α , NF-1 and coactivator p300 gave the maximal activity but the BRG1 and BRM, ATPase subunit of the chromatin remodeling complex SWI/SNF, repressed the transactivation of the AFP gene by these transcription factors. Deletion analyses of C/EBP α binding sites suggested that C/EBP α recruited SWI/SNF complex and caused the repression. This repression may also play important roles in the downregulation of the AFP gene in adult hepatocytes.

Abbreviations: AFP – α -Fetoprotein; C/EBP α – CCAAT enhancer binding protein α ; COUP-TF – chicken ovalbumin upstream promoter–transcription factor; HNF-1 α – hepatocyte nuclear factor-1 α ; HNF3 – hepatocyte nuclear factor-3; NF-1 – nuclear factor-1

Introduction

It has been reported that the α -fetoprotein (AFP) gene is activated in fetal liver, but developmentally silenced after birth (Tilghman and Belayew 1982; Vacher and Tilghman 1990; Hamamoto et al. 1999). Since AFP level in blood increased in most of liver tumors, the control of AFP expression has been extensively studied (Li et al. 2002; Semenkov et al. 2003; Osawa et al. 2004). In the rat AFP control region spanning to 7 kb upstream of the transcription start site, distal enhancers, pro-

motor–proximal elements and silencers are identified (Figure 1). Precise location and detailed structure of these elements are different between rat, mouse and human, but overall structure of the control region of the AFP gene shows considerable similarity between these species. The enhancer region, from –7 kb to –1 kb, can be divided into 3 regions (enhancer 1–3), and these enhancers confer liver-specific and developmental regulation of the AFP gene (Watanabe et al. 1987; Group et al. 1994; Ramesh et al. 1995; Lazarevich 2000). In rat, chicken ovalbumin upstream promoter–transcrip-

Figure 1. The structure of the promoter and enhancer regions of the rat AFP gene. Numbers in the figure show the position from the mRNA start site as +1 (P, promoter region; S, silencer element; E, enhancer).

tion factor (COUP-TF), hepatocyte nuclear factor 3 (HNF3) and CCAAT/enhancer binding protein α (C/EBP α) bind to enhancer 3, and these factors are likely responsible for the liver specific enhancer 3 activity (Thomassin et al. 1996). A major component of activating enhancer 1 is C/EBP α in rodents (Zhang et al. 1992; Bernier et al. 1993).

The promoter region ($\sim$ 250) also plays important roles in the liver-specific expression of the AFP gene (Zhang et al. 1990). There is considerable similarity in the organization of the promoters between the AFP and albumin genes, in particular, the presence and localization of C/EBP α and HNF-1 α binding sites (Bernier et al. 1993). In the rat AFP promoter, two HNF-1 α binding sites at $-65/-51$ and $-131/-117$ bp, and two C/EBP α binding sites at $-78/-69$ and $-115/-107$ bp have been identified (Zhang et al. 1990; Zhang et al. 1991; Bernier et al. 1993; Bois-Joyeux and Danan 1994) (Figure 1). Overlapping with the distal C/EBP α binding site, a nuclear factor-1 (NF-1) binding site was also found (Zhang et al. 1990; Bernier et al. 1993).

Regulated alterations of chromatin structure are integral to activation and repression of genes. Transcription factors, chromatin remodeling complexes and histone modifiers combine to alter chromatin and regulate transcription. The structural changes in chromatin that accompany with transcriptional activation often require multi-protein complexes that manipulate the nucleosomal structure (Urnov and Wolffe 2001; Narlikar et al. 2002). These are two general classes of complexes that disrupt chromatin structure: those that covalently modify histones by altering acetylation, methylation and phosphorylation patterns and those that use ATP hydrolysis to disrupt histone-DNA interactions (Kingston and Narlikar 1999; Muchardt and Yaniv 1999; Hayes and Hansen

2001; Berger 2002; Neely and Workman 2002; Zhang 2003). Mammalian chromatin remodeling complex, SWI/SNF consists of $\sim$ 15 subunits and falls into two broad classes, depending on whether they contain BRG1 or BRM as the ATPase subunit (Martens and Winston 2003). SWI/SNF interacts with a wide variety of proteins including transcription factors and nuclear receptors (Yoshinaga et al. 1992; Muchardt and Yaniv 1993; Chiba et al. 1994; Ichinose et al. 1997; Armstrong et al. 1998; Fryer and Archer 1998; Kowenz-Leutz and Leutz 1999). In many cases, the chromatin remodeling complex is essential for the activation of a number of inducible genes (Muchardt and Yaniv 1993; Chiba et al. 1994; Armstrong et al. 1998; Fryer and Archer 1998; Kowenz-Leutz and Leutz 1999).

In this study, we demonstrated that chromatin remodeling complex, SWI/SNF bound to the proximal promoter of the AFP gene and repressed the expression of this gene.

Materials and methods

Cell culture

Adult rat hepatocytes were obtained from male Sprague-Dawley rats (6–7 weeks old; Japan SLC) by the collagenase perfusion method (Hamamoto et al. 1998; Dohda et al. 2004b). Williams' medium E (Sigma-Aldrich, W-4125), supplemented with $0.1 \mu\text{M}$ $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 25 nM Na_2SeO_3 , $1.0 \mu\text{M}$ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $0.1 \mu\text{M}$ insulin (Sigma, I-2767), $1.0 \mu\text{M}$ dexamethasone (Wako Pure Chemical Industries, 590-03371), 20 ng ml^{-1} of epidermal growth factor (EGF) (Sigma, E-9644), $48 \mu\text{g ml}^{-1}$ of gentamicin sulfate (Sigma, G-1264) and $100 \mu\text{g ml}^{-1}$ of chloramphenicol (Wako, 036-10571)

was used as the culture medium. Fetal rat hepatocytes were isolated from the fetus (17 embryonic days; E17) of Sprague-Dawley rats (Japan SLC) (Lorenzo et al. 1986; Hamamoto et al. 1999), and cultured in Williams' medium E containing 5% (v/v) fetal bovine serum (Biowest, S1820). Cells were cultured in collagen type I-coated 100 mm plastic dishes (IWAKI, 4020-010) and 24 well plates (IWAKI, 4820-010) under a 5% CO₂ atmosphere.

C33A cells (ATCC, HTB-31) are derived from human cervical cancer. The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, 31600-083) supplemented with 10% (v/v) fetal bovine serum. We used this cell line for luciferase assays because it is deficient in C/EBP α , HNF-1 α , BRG1 and BRM, but expresses other subunits of SWI/SNF complexes (Decristofaro et al. 2001; Dohda et al. 2004a).

Plasmids

To construct the AFP promoter luciferase reporter plasmid, the AFP promoter region (−225 to +13) was amplified from rat genomic DNA by PCR using a set of primers (direct, 5'-CC-CCAAGCTTCATTGTCAGCATTTGCAAG-3'; reverse, 5'-CCCCAAGCTTCTTCATGGTTGCTGGCTGC-3'; *Hind*III sites are underlined), and introduced into pGL3-basic vector (Promega, E1751). For amplification of 6.3 kb DNA fragment (−6254 to +13) containing the promoter and enhancers, direct primer (5'-ATAACGCGTGATGAGATGTGGTCTCAGTC-3'; *Mlu*I site is underlined) and reverse primer (5'-CCTCTCGAGATTTTAAGGACCCCTACC-3'; *Xho*I site is underlined) were used. For the distal HNF-1 α binding site mutation that has the *Eco*RI recognition sequence in the distal HNF-1 α binding site, PCR was conducted with the AFP promoter (−225 to +13) luciferase reporter plasmid as a template with primers; 5'-AATGAATTCCTTATTGGCAAATTGCCTAACTT-3', and 5'-AATGAATTCGACAGGGGTCACCTTCTATCTTA-3' (*Eco*RI sites are underlined). The proximal HNF-1 α binding site mutant, and the distal or proximal C/EBP α binding site mutant plasmids were obtained by similar way with sets of primers; 5'-AATGAATTCCTAACAGACATCACTTAAAAAGGATATAAAA-3' and 5'-AATGAATTCACCTTCAGTGGGCAACA-3' (*Eco*RI sites are underlined) for proximal

HNF-1 α binding site mutant plasmid, 5'-CCCGAATTCACTTCAACATAAGGAAAAA-AAGTAACA-3' and 5'-CATGAATTCATT-TGCCAATAATTAACAGCACA-3' (*Eco*RI sites are underlined) for distal C/EBP α binding site mutant plasmid, and 5'-AATGAATTCCTGAAGTTACTAGTTAACAGACATCACT-3' and 5'-AATGAATTCAAACATGTTACTTTTTTTCCT-TATGTTG-3' (*Eco*RI sites are underlined) for proximal C/EBP α binding site mutant plasmid, respectively. For the luciferase vector containing both C/EBP α binding sites mutant, PCR was conducted using the AFP proximal C/EBP α binding site mutant vector as a template with primers; 5'-AAACTGCAGACTTCAACATAAGGAAAAA-AAGTAACA-3' and 5'-AGACTGCAGATTTGCCAATAATTAACAGCACA-3' (*Pst*I sites are underlined). All the constructs were checked by DNA sequencing. For the expression of C/EBP α and HNF-1 α , pcDNA4-C/EBP α and pcDNA4-HNF-1 α were used, respectively (Dohda et al. 2004a). For the expression of NF-1 and a coactivator p300, pCMV-SPORT6 (Invitrogen, 12209-011) and pCMV-p300 (Upstate Biotechnology, 21-178) (Eckner et al. 1994; Kwok et al. 1994; Dohda et al. 2004a) were used, respectively. The construction of BRM (pcDNA4-BRM) and BRG1 (pcDNA4-BRG1) expression plasmids were as reported previously (Inayoshi et al. 2005).

Luciferase reporter gene assay

Fetal hepatocytes and C33A cells were seeded in collagen type I-coated 24 well plates (IWAKI, 4820-010) and regular 24 well plates (IWAKI, 3820-024), respectively. Cells were transfected with expression and reporter plasmids using Lipofectamine 2000TM reagent (Invitrogen, 11668-019). In the case of C33A cells, 100 ng of the luciferase reporter plasmids containing the AFP promoter or the promoter-enhancer, and 5 ng of the *Renilla* luciferase plasmid driven by the promoter of the elongation factor 1 α were introduced with or without 100 ng of the plasmids expressing C/EBP α , HNF-1 α , NF-1, BRG1 or BRM, and p300. The amount of total DNA was kept constant by adding the empty expression vector. In the case of fetal hepatocytes, 900 ng of the luciferase reporter plasmids containing the intact or the mutated AFP promoters, and 100 ng of the *Renilla* luciferase plasmid were transfected

using Lipofectamine 2000TM reagent. Reporter gene assays were performed with a dual luciferase assay kit (Promega, E1910) according to the manufacturer's instructions. Luciferase activities were measured with a luminometer JNR II (Atto, AB-2300) and normalized by *Renilla* luciferase activity.

Chromatin immunoprecipitation (ChIP) assay

The chromatin immunoprecipitation (ChIP) assay was performed using salmon sperm DNA conjugated protein A agarose (Upstate Cell Signaling Solutions, 16-201). The immunoprecipitated DNA and the input DNA were analyzed by PCR using primers for the AFP promoter (direct, 5'-CTGAAGTGGTCTTTGTCCTTG-3'; reverse, 5'-ACTGTGAGCAGTAGCGCTG-3'), enhancer 1 (direct, 5'-CGGGGGTAGAGCCTTCGTATAC-3'; reverse, 5'-AAAGCATGTGATCAGAAAGGTGC-3') and enhancer 3 (direct, 5'-CCTTGACCTATATCGATGTTCTAGTGAC-3'; reverse, 5'-CTTGGTTGGTTTCTGTCAACTCT-3'). As a negative control, the coding region of the AFP gene was also amplified using primers (direct, 5'-TCTGTGTTTCTGGATGAAATTTG-3'; reverse, 5'-ACATCGCCCTGTTTCTTGG-3').

Results

In fetal rat hepatocytes, the AFP gene expression is high level, but it decreases rapidly after birth as reported previously (Tilgman and Belayew 1982; Vacher and Tilgman 1990; Hamamoto et al. 1999). This developmental regulation of the AFP gene is known to be controlled by the liver enriched transcription factors such as C/EBP α and HNF-1 α , and the binding sites for these transcription factors have been identified in the AFP promoter (Figure 1). It is also reported that HNF-1 α physically interacts with the coactivator p300 (Souzoglu et al. 2000, 2001), and that C/EBP α interacts with BRG1 and BRM, ATPase subunits of chromatin remodeling complex, SWI/SNF (Pedersen et al. 2001; Inayoshi et al. 2006). We therefore analyzed the effect of transcription factors, C/EBP α , HNF-1 α and NF-1 in combination with p300 and BRG1 or BRM on the AFP promoter activity by conducting luciferase reporter gene assays in C33A cells. C33A cells are deficient in BRG1 and BRM but express other subunits of SWI/SNF complexes (Decristofaro et al. 2001).

The expressions of C/EBP α , HNF-1 α and p300 were not detected in C33A cells by Western blotting (data not shown). As shown in Figure 2a, the combination of transcription factors such as C/EBP α , HNF-1 α , NF-1, and p300 caused an increase in the expression level of the AFP promoter. Overall, the coactivator p300 upregulated the gene expression with these transcription factors. In contrast, BRG1 and BRM strongly reduced the expression level. Downregulation by BRG1 or BRM was observed with all combinations of transcription factors and p300. However, the extent of the inhibition decreased with an increase in the number of protein factors involved in the assay (Figure 2a). A similar downregulation by BRG1

Figure 2. Luciferase reporter gene assays. C33A cells were cotransfected with pGL3-AFP promoter and C/EBP α , HNF-1 α , p300, NF-1, BRG1 or BRM expression vectors as indicated (+). The bars represent standard errors from at least three independent experiments. (a) Effects of transcription factors, coactivator and chromatin remodeling factors on the expression of the AFP gene in C33A cells. (b) Effects of BRM or BRG1 on the AFP promoter activity mediated by C/EBP α and HNF-1 α .

and BRM were observed in SW13 cells, which lack BRG1 and BRM (data not shown).

SWI/SNF is usually recruited by various transcription factors at their binding sites through protein-protein interaction (Fryer and Archer 1998; Kowenz-Leutz and Leutz 1999). In fact, BRG1 and BRM can directly bind to C/EBP α and subsequently BRG1 or BRM based SWI/SNF complex was recruited to the region near the C/EBP α binding site (Pedersen et al. 2001; Inayoshi et al. 2006). We found that SWI/SNF complexes were able to inhibit C/EBP α mediated transcription but not HNF-1 α -mediated transcription on the AFP promoter (Figure 2b). Furthermore, the repression on the AFP promoter by BRG1 required its ATPase activity (data not shown). The mutant BRG1, lacking the ATPase activity, was not able to repress the AFP promoter activity. Together, these results suggested that the recruitment of SWI/SNF complexes might be mediated by C/EBP α and the repression on the AFP promoter by SWI/SNF may require its ATPase activity.

We next studied the effects of HNF-1 α and C/EBP α binding precisely using the mutated promoters. In a series of the mutated promoter plasmids, either of two HNF-1 α binding sites, or either or both of the C/EBP α sites were destroyed by replacing them with the *EcoRI* or *PstI* recognition sequence by PCR. When the HNF-1 α mutated promoter luciferase vectors were introduced into primary fetal hepatocytes, the promoter activities were significantly reduced as reported (Figure 3a) (Feuerman et al. 1989; Bernier et al. 1993). However, the C/EBP α mutants showed less profound effects on the promoter activity; the C/EBP α mutant that lacked the distal C/EBP α binding site showed some activity. Furthermore, the mutant promoter, which lacked both of the C/EBP α binding sites, showed considerable activity; the level of activity was more than a half of that of the intact promoter. Similar results were obtained when the mutated plasmids were introduced into C33A cells with various combinations of the protein factors (Figure 3b).

It is known that the AFP gene is controlled by its enhancer regions (enhancer 1, 2 and 3), and both enhancer 1 and 3 contain C/EBP α binding sites (Lazarevich 2000). We thus studied the repressive activity of SWI/SNF complexes on the regulatory region containing these enhancers. As shown in Figure 4, the promoter activity in-

Figure 3. Mutational analysis of HNF-1 α and C/EBP α binding sites on the AFP promoter activities. The bars represent standard errors from at least three independent experiments. (a) Fetal hepatocytes (E17) were transfected with pGL3-AFP promoter, pGL3-luciferase plasmids with various mutations in C/EBP α and HNF-1 α binding sites, and pGL3-basic vector. (b) C33A cells were co-transfected with the wild type or mutant promoter plasmids of C/EBP α binding sites with C/EBP α , HNF-1 α , NF-1, p300, and BRM or BRG1 expression vectors as indicated (+). (black box: wild type promoter, dark gray box: proximal C/EBP α mutant, white box: distal C/EBP α mutant, light gray box: double C/EBP α mutant).

creased 1.6-fold in the presence of the enhancer regions by the reporter assay but the repression by BRG1 and BRM was not affected by the enhancers. Therefore, it is likely that the SWI/SNF mediated repression was caused by its short promoter-proximal region but not by the enhancer regions.

Figure 4. Effects of chromatin remodeling factors on the AFP promoter–enhancer regions. C33A cells were co-transfected with pGL3-AFP promoter or pGL3-AFP-promoter–enhancer, C/EBPα, HNF-1α and BRG1 or BRM expression vectors as indicated (+). The bars represent standard errors from at least three independent experiments. (black box: promoter–enhancer, white box: promoter).

The binding of protein factors to the AFP promoter region was then analyzed by the ChIP assay (Figure 5a). In both fetal and adult hepatocytes, the binding of transcription factors, C/EBPα, HNF-1α and NF-1 to this region was observed, although the extent of the binding differed between these two cell types. p300 was also recruited to this region in both fetal and adult hepatocytes but the band was faint in adult hepatocytes. This result is consistent with the finding that the expression of p300 is weak in adult hepatocytes compared to the fetal cells (Dohda et al. 2004a). The binding of BRM was observed only in adult hepatocytes since BRM was expressed in adult but not in fetal hepatocytes (Inayoshi et al. 2006). BRG1 was also detected only in fetal hepatocytes because of the limited expression of this ATPase subunit in the undifferentiated liver cells. In enhancer regions, C/EBPα bound to enhancer 1 and 3 in both fetal and adult hepatocytes. BRG1 but not BRM bound to

Figure 5. Binding of transcription factors to the control regions of the AFP gene. (a) ChIP assay for the endogenous AFP gene from fetal and adult liver cells. (b) ChIP assay for the enhancer 1 and 3 regions of fetal (E17) and adult hepatocytes. (c) ChIP assay for the AFP enhancer and promoter using C33A cells. C33A cells were transfected with pGL3-promoter–enhancer plasmid (upper) or the mutant promoter in both C/EBPα binding sites (bottom) and C/EBPα, HNF-1α, and BRG1 expression vectors. As a negative control, normal mouse IgG was used for immunoprecipitation. The coding region was also amplified as a negative control. The input sample (2%) was used as a positive control.

enhancer 1 weakly in fetal hepatocytes and both ATPase subunits did not bind to enhancer 3 (Figure 5b). Furthermore, similar tendency was observed with C33A cells that was transfected with the luciferase reporter vector, C/EBP α , HNF-1 α and BRG1 expression plasmids (Figure 5c). This result also suggested that the enhancer regions did not cause the repression by SWI/SNF. In addition, ChIP assay showed that BRG1 was not able to bind to the mutant AFP promoter vector, which lacked both C/EBP α binding sites (Figure 5c). This result suggested that the recruitment of BRG1 was mediated by C/EBP α .

Discussion

We report in this study that BRG1 and BRM repressed the AFP promoter activity. Since BRG1 or BRM formed the complex with other subunits of SWI/SNF in liver cells (Inayoshi et al. 2006), SWI/SNF based on either BRG1 or BRM may cause this inhibition. Furthermore, the recruitment of SWI/SNF by C/EBP α seemed to cause this inhibition, since the mutant lacking both distal and proximal C/EBP α binding sites was partially relieved from the BRG1 or BRM mediated repression and ChIP assay showed that BRG1 and BRM did not bind to the promoter region when both C/EBP α binding sites were mutated (Figure 5c). As shown in Figure 2a, C/EBP α activated the expression of AFP and was possibly a part of the pre-initiation complex (PIC) of RNA polymerase II. As well as being an activator, C/EBP α may inhibit the gene expression by recruiting SWI/SNF. Thus, C/EBP α seemed to be bifunctional and affect the AFP expression in different manner depending on the situation. Recently, the repression of gene expression by SWI/SNF has been reported for several genes. For example, BRG1 repressed the *c-fos* gene through the recruitment of the retinoblastoma tumor suppressor protein and a histone deacetylase (HDAC) (Murphy et al. 1999) and BRM repressed the cyclin A gene through the nucleosome sliding and the formation of a closed chromatin structure (Coisy et al. 2004). In the present case, trichostatin A (TSA) which is an HDAC inhibitor did not affect the SWI/SNF mediated repression (date not shown). This result suggests that HDAC was not involved in this repression.

A ChIP assay of the endogenous AFP gene in both fetal and adult hepatocytes indicated that C/EBP α , HNF-1 α , NF-1 and p300 bound to the proximal promoter element of the AFP gene. Although it is not clear that the ChIP assay gave quantitative results under the conditions, it is possible that decreased binding of positive regulators such as HNF-1 α and p300 may partly cause the repression of the AFP gene in adult hepatocytes. Furthermore, increased binding of C/EBP α in adult hepatocytes may facilitate the recruitment of SWI/SNF to the promoter-proximal region. In another word, the change in the binding of these transcription factors to the promoter-proximal elements of the AFP gene may partly reduce the repression by BRG1 in fetal hepatocytes.

ChIP assay showed that C/EBP α bound to the enhancer 1 and 3 in both fetal and adult hepatocytes as reported previously (Thomassin et al. 1996) and the recruitment of BRG1 was observed in enhancer 1 only with fetal hepatocytes. Furthermore, C/EBP α molecule bound to enhancer 3, but did not recruit BRG1. Similar results were obtained with the luciferase vector transfected to non-hepatic C33A cells. As judged from the ChIP experiments shown in Figure 5b, c, the binding of C/EBP α to enhancer 3 was much weaker than that to enhancer 1. This difference in C/EBP α binding may reflect the difference in the recruitment of BRG1. In enhancer 3, HNF3 and C/EBP α bind to the site 2 and 3, respectively (Thomassin et al. 1996) and these transcription factors are likely responsible for the liver-specific action of enhancer 3 (Thomassin et al. 1996). Thus, we cannot rule out the possibility that the binding of the transcription factors to the enhancer 3 blocked the recruitment of BRG1 or BRM in liver cells.

Furthermore, for the repression by BRG1, ATPase activity of BRG1 was required (date not shown). Together, these results suggest that SWI/SNF complexes containing BRG1 or BRM may alter the nucleosome structure nearby promoter site and inhibit the transcription initiation. In addition to enhancers and proximal promoter, the silencer, which resides between the enhancers and promoter, is believed to play crucial roles for developmental suppression of the AFP genes (Lazarevich 2000). By now, physiological importance of SWI/SNF mediated repression shown by the present study has not been clear, but it is possible that the repression

partly contributes to global regulation of the AFP gene during liver development.

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