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Chapter 2

HOW RETINOIC ACID REGULATES GENE EXPRESSION IN DEVELOPING LIVER: THE HEPATOMA ALPHA-FETOPROTEIN GENE AS A MODEL

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ABSTRACT

Liver is the largest organ in the body and performs a wide variety of functions. The depth of understanding of control of gene expression in hepatocytes is better understood than any other cell type in the body. Retinoic acids (RA), including all-trans and 9-cis retinoic acids, are the bioactive components that bind and activate their cognate nuclear receptors to regulate target genes.

The RA signal is transduced by two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), which function as RXR/RAR heterodimers. Each family consists of three isotypes (α, β and γ) encoded by separate genes. RA receptors primarily act through direct association with specific DNA sequences, known as retinoic acid receptor response elements (RAREs) or retinoid X receptor response elements (RXREs), in the regulatory regions of target genes. In liver, RA regulates the expression of many liver-specific genes and plays a crucial role in hepatocyte differentiation, proliferation and apoptosis.

Alpha-fetoprotein (AFP) is a large serum glycoprotein belonging to the intriguing class of onco-developmental proteins. The AFP gene has been extensively studied to understand how its gene expression is controlled in liver and how it is modulated during development and cancerogenesis. During liver development, AFP expression starts in the visceral endoderm, in the presumptive territory of the liver, and is maintained in liver until birth, but resumes expression upon liver regeneration and cancerogenesis. It has been shown, in various experimental models, that AFP expression is regulated mainly at the transcriptional level. Five cis-acting elements that regulate AFP expression, the promoter, silencer, and three upstream enhancers, have been identified and characterized using transgenic mice and in vitro studies. The AFP promoter, covering a region of ~ 250 bp, is regulated by tissue-specific activators such as HNF1, C/EBP, Nkx2.8 and FTF, as well as by ubiquitous factors such as NF1. Promoter activity is limited to tissues producing AFP, indicating that this region contributes to tissue-specific expression. The three upstream enhancers are typical enhancers and can confer AFP promoter activity, as well as confer activity to heterologous promoters. Like the AFP promoter, the enhancers are tissue-specific and are not active in non-hepatic cells. Silencers are localized between the promoter and enhancers of the AFP gene, and play the critical role in suppression of the AFP gene transcription after birth.

RA regulates AFP gene expression in embryonic carcinoma and hepatoma cells. The influence of RA on AFP gene expression can be carried out both by means of HNF induction and through the hormone-receptor complex binding to the corresponding regulatory elements in the AFP promoter. Three RARE-like sequences, located in the 5´-flanking region of the rat AFP gene, have been identified that are specifically recognized by RARs and RXRs. These cis-acting elements enhance the transcriptional activities of the rat AFP promoter. In addition, a novel cis-acting element, designated as DAS, in the 5'-flanking region of the AFP gene has been identified. This DAS sequence can be specifically recognized by AUF1 RNA-binding proteins in AFP-producing hepatoma cells and RA-induced AFP-producing F9 cells.

1. GENES REGULATED BY RETINOIC ACID IN LIVER CELLS

Liver performs a wide variety of functions that are essential for the preservation of homeostasis in the organism. These functions include the synthesis of serum proteins and hormones, metabolism of nutrients, xenobiotics and systemic waste products, metabolism of lipids, bile acids and lipoproteins, storage and usage of glucose, bile formation, and neutralization of foreign antigens and microbes from the gut.

1.1. Liver Function and Gene Expression

During the latter half of the 20th century, facilitated by the explosion of molecular biology, our depth of understanding of the control of gene expression in hepatocytes became highly advanced. Many key technologies used to investigate cell function, including the production of cDNA libraries, electromobility shift assays (EMSAs) to identify DNA binding proteins, and in vivo DNA footprinting to identify the occupancy of transcription factors within promoters, were first established in studying liver function. Most of these advances, whether in understanding control of gene expression or cell proliferation, described fundamental mechanisms that are applicable to all aspects of biology.

Liver is second only to brain in transcriptome size, with 25% to 40% of human genes expressed in this multifunctional heterogeneous organ. However, the number of functionally significant transcripts in humans is unknown and may exceed 100,000 (Velculescu et al. 1999.). Probably the best estimate of the total number of mRNA transcripts is provided by the number of non-redundant GenBank UniGene clusters, currently greater than 96,000 (www.ncbi.nlm.nih.gov/UniGene/). At least 15,000 of these clusters contain expressed sequence tag sequences isolated from liver tissue. However, comparatively few genes have been characterized specifically in liver tissue or liver cells. Transcriptional profiling in liver

disease generally uses a global survey approach ensuring that the gene expression analysis will consider the diverse cellular interactions (Liotta and Petricoin 2000.). This approach is necessary because many forms of liver disease have no suitable in vitro models and the study of whole liver is commonly used to understand disease pathogenesis.

1.2. Retinoic Acid and Regulation of Gene Expression

Retinoic acid (RA), an active metabolite of vitamin A, belongs to the retinoid family and includes the isoforms all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cis-RA). RA is involved in various physiological processes, such as embryonic development, reproduction, vision, cell growth, differentiation, apoptosis, and inflammation (Marletaz et al. 2006; Zhou et al. 2012; Zhou et al. 2011.). The effects of RA are mediated through its binding to retinoid receptors, which are members of the nuclear receptor family. Retinoid receptors share the same modular structure as other nuclear receptors, with the main modules being the DNA binding domain (DBD), which confers sequence-specific DNA recognition, and a ligand-binding domain (LBD), which also harbors a ligand-dependent activation function AF-2 and a major dimerization interface (Laudet and Gronemeyer 2002). Retinoid receptors can be divided into two subgroups: RA receptors (RARs) and retinoid X receptors (RXRs). In vertebrates, there are generally three RARs and three RXRs (α, β, and γ) with RARs binding to ATRA and 9-cis RA, and RXRs binding to 9-cis RA with high affinity, but not to ATRA.

The transcriptional activation by RAR is dependent on the formation of an RAR/RXR heterodimer, which occurs at two interfaces: (1) the LBD interface, which is independent of the DNA binding activity of the complex, and (2) the DBD interface, which is involved in the recognition of response elements on DNA. The dimerization interface found in the LBD further stabilizes, but does not change, the binding repertoire directed by the DBDs (Mader et al. 1993; Perlmann et al. 1996.). The precise heterodimerization surfaces of RAR and RXR in the DBD have been

determined by extensive structure–function analyses (Zechel et al. 1994; Zechel et al. 1994.). Within the RXR protein, the second zinc finger forms a surface that interacts with the second zinc finger of RAR bound to a retinoic acid response element (RARE) on the target DNA. The DNA response element for RAR/RXR consists of two or more degenerate copies of the (A/G)G(G/T)TCA or of the more relaxed (A/G)G(G/T)(G/T)(G/C)A half-site motif organized in direct repeats or palindromes normally separated by a nucleotide spacer (Balmer and Blomhoff 2005). In addition to DR5 (direct repeat with a five-nucleotide spacer), RAR/RXR heterodimers are able to regulate transcription from DR2 (two nucleotide spacer) and DR1 (one nucleotide spacer) elements (Durand et al. 1992; Smith et al. 1991.). When bound to DR2 and DR5 elements, the 5‘ half-site is occupied by RXR and the 3‘ half-site by RAR (Perlmann et al. 1993; Predki et al. 1994.). In contrast, when bound to DR1 elements, the polarity of the heterodimer is inverted (5‘-RAR-RXR-3‘) and the complex is unresponsive to RA stimulation, probably due to the inability of RAR ligands to induce the dissociation of co-repressors (Kurokawa et al. 1994.).

Over the last few decades, more than 500 genes have been identified as being regulatory targets of RA. In some cases, direct regulation has been demonstrated to be driven by liganded RAR/RXR heterodimers bound to RAREs. In most cases, though, the regulation of the proposed gene target is indirect, occurring through intermediate transcription factors or non-classical associations of receptors with other proteins. Of the target genes suggested, 27 were found to be direct targets of the classical RAR/RXR-dependent RARE pathway and another 100 were good candidates for being direct targets (Blomhoff R and Blomhoff HK 2006.). Moreover, a microarray-based screen in zebrafish embryos identified both positive and negative RA targets in vivo [Feng L et al. 2010.]. This screen identified genes previously reported to be regulated by RA, as well as novel RA-responsive genes, such as Dhrs3a (a member of the SDR family), which functions to limit RA signaling in the CNS by catalyzing the reduction of retinaldehyde to retinol (Feng et al. 2010.). Finally, using a chromatin immuno-precipitation on chip (ChIP on chip) approach in MCF-7 breast cancer cells, Hua et al. recently identified genomic RAR targets (Hua et al.

2009). With this ChIP on chip analysis, Hua et al. defined a total of 1,413 genes that were significantly regulated by either RA or paralog-specific RA agonists. Analysis of the distribution of RAR binding sites showed that the vast majority of these novel RAREs are located either in introns or in so-called promoter-distal intergenic regions locating distance from the actual RA target gene (Hua et al. 2009).

1.3. Retinoic Acid in Liver Cells

Liver is quantitatively the most important storage site for retinoids in the body (Blaner and Olson 1994; Blomhoff et al. 1991; Goodman and Blaner 1984). It is also quantitatively the most important tissue site of postprandial retinoid uptake in the body, accounting for uptake of 66–75% of all of dietary retinoid that is absorbed by the intestine (Blaner and Olson 1994; Blomhoff et al. 1991; Goodman and Blaner 1984), and liver is the major organ site for synthesis and secretion of retinol-binding protein (RBP), accounting for 70–80% of all RBP that is normally present in the circulation (Goodman and Blaner 1984; Soprano and Blaner 1994). In the fasting circulation of retinoid-sufficient animals, RBP maintains constant circulating levels of retinol, assuring continuous retinoid delivery to target tissues (Goodman 1984; Soprano and Blaner 1994; Tsutsumi, et al. 1992). Because of its large role in each of these processes, liver is the central organ in the body involved in retinoid storage and metabolism.

The liver is also an important target organ for retinoid action. The three retinoic acid receptors (RARα, RARβ, and RARγ) are expressed in the liver, as are the three retinoid X receptors (RXRα, RXRβ and RXRγ) (Hellemans et al. 2004). The importance of retinoid signaling for maintaining a healthy liver is evidenced by many observations. For instance, transgenic mice, which express in a hepatocyte-specific manner a dominant-negative form of RARα that ablates retinoic acid and RAR signaling, are predisposed to spontaneously developing hepatocellular carcinoma (Yanagitani et al. 2004). There also are established associations between hepatic disease development and impaired hepatic retinoid storage.

The progressively worsening stages of hepatic disease observed for alcoholic patients are associated with increasingly diminished hepatic retinoid stores. Thus, retinoids are needed for maintaining normal hepatic health, and hepatic retinoid stores are adversely affected by liver insult and injury.

2. AFP GENE AND PROTEIN STRUCTURE, AND TRANSCRIPTION PRODUCTS

In 1963, an antigen specific for a chemically-induced murine hepatoma and for human hepatocellular carcinoma (HCC) was first discovered (Abelev 1963; Tatarinov 1963). This antigen was not present in normal adult kidney, spleen or blood serum, but was detected in mouse and human embryonic liver, amniotic fluid, and blood serum. This antigen was shown to electrophoretically belong to the α-globulin fraction, and it eventually became clear that liver cancer cells produced and secreted into the blood an embryo-specific α-globulin that was initially named αF in mice and ESA-globulin in human. The newly revealed antigen was identical to the protein discovered in human fetuses (Bergstrand and Czar 1956), and later, in 1970, this antigen was named "alpha-fetoprotein" (AFP). Since then, AFP has been long recognized as the first oncodevelopmental biomarker. Currently, AFP is considered as a "golden standard" among tumor-specific molecular biomarkers (Debruyne and Delange 2008).

2.1. Protein Structure of AFP

AFP is classified as a member of an albuminoid gene family, which consists of four members to date: albumin (ALB), vitamin D-binding protein (DBP), AFP, and alpha-ALB (αALB), the latter termed afamin in humans (McLeod et al. 1989; Lichenstein et al. 1994.). Members of the albuminoid gene family are evolutionary closely related and originate from

common ancestor (Beatie et al. 1982). So far, the primary structure of full-length AFPs has been reported from thirteen mammalian species (UniprotKB/Swiss-Prot knowledge base), including human, mouse, rat, rabbit, and guinea pig (Deutsch 1991; Mizejewski 1995, Morinaga et al. 1983; Dudich et al. 1999). AFP is a glycoprotein with a molecular weight ranging from 68 to 73 kDa depending on carbohydrate content and biological origin. Being a secreted protein, AFP is synthesized as a precursor that undergoes post-translational processing with cleavage of the signal peptide, which is composed of 19 amino acid residues, and an additional 590 residues representing the mature protein (Morinaga et al. 1983, Pucci et al. 1991; Dudich et al. 1999).

The albuminoid gene family members display structural similarities, homologous amino acid sequence stretches, and similar cysteine disulfide bridge clusters. These proteins exhibit similar spatial organization and consist of three homologous domains (I–III), each of which, in turn, consists of two globular subdomains (IA, IB, IIA, IIB, IIIA, IIIB) linked by 15 regular-arranged disulfide bonds (Luft and Loscheider 1983; He and Carter 1992.).

2.2. Function of AFP

In a number of experimental models in vitro and in vivo, AFP from different biological species has been shown to exhibit various types of biological activity. AFP is known to bind and transport a multitude of ligands, including bilirubin, fatty acids, retinoid, steroids, heavy metals, dyes, flavonoids, estrogens, phytoestrogens, dioxins, and various organic drugs (Mizejewski 1995; Mizejewski 1997). Estrogen-binding activity of AFP is of crucial importance because this ability may represent a significant regulatory mechanism during embryonic development, since AFP may be involved in regulation of concentrations of free, active forms of hormones in vivo. The most successful results in studying AFP estrogen-binding activity were obtained by a group of French researchers, led by J. Uriel, who demonstrated high-affinity binding of both free and

immobilized estrogens to rodent AFPs (Soloff et al. 1972; Uriel et al. 1972; Uriel et al.1975; Uriel et al. 1976; Nishi et al. 1991; Milligan et al. 1998). This might protect fetal tissues from circulating maternal estrogens and prevent degradation of hormone molecules.

Evidence indicates that AFP can regulate the immune response. It has long been experimentally demonstrated that AFP is able to interact with macrophages to decrease their phagocytic activity and expression of Ia antigen (Lu et al. 1984; Atemezem et al. 2002.). Also, AFP inhibits activity of natural killer (NK) cells (Peck et al. 1982; Cohen et al. 1986.), reduces proliferation of T-lymphocytes stimulated by ConA and PHA with reduction of amount of total and CD4+ thymocytes (Yachnin 1976; Yachnin 1983; Matsuura et al. 1999), and also induces activity of T-suppressor cells (Alpert et al. 1978.). Treatment with recombinant human AFP reduces lymphocyte reactivity and the extent of neuroinflammation in mice with experimental autoimmune encephalomyelitis, due to involvement of AFP in immune cell apoptosis (Irony-Tur-Sinai et al. 2009).

AFP has been shown to be a dual regulator of cell proliferation and tissue growth, exhibiting both stimulatory and inhibitory effects. AFP exhibits stimulatory effects at low concentrations, and in estrogen-resistant tissues, both normal and tumor (Toder et al. 1983; Wang and Xu 1998; Wang and Xie 1999; Laderoute and Pilarski 1974). In estrogen-resistant HCC, intact AFP decreases expression of the cyclin-dependent kinase inhibitor p27 (kip1) both at the mRNA and protein levels, and increases expression of proliferating cell nuclear antigen (PCNA) (Shehata et al. 2006.), and stimulation of cell proliferation is accompanied by inhibition of apoptosis (Laderoute and Pilarski 1974.). The detailed mechanisms of regulation of cell proliferation and tumor growth by AFP remain unknown. However, specific binding of AFP to receptors located on the surface of normal and tumor cells has long been demonstrated.

Inhibition of cell apoptosis by AFP was demonstrated by cytological studies showing that TRAIL-induced apoptosis, occurring through the caspase-3-mediated apoptotic cascade, is abolished by AFP (Li et al. 2009.). In BEL-7402 cells undergoing apoptosis following co-treatment with both ATRA and TRAIL, caspase-3 co-localizes and interacts with

AFP in the cytoplasm and translocates into the nucleus. Knock-down of AFP gene increased the sensitivity of BEL-7402 cells to TRAIL, and thereby, triggered caspase-3 signaling. In human hepatoma cells, AFP complexes with caspase-3 and blocks transduction of apoptotic signals. ATRA- or TRAIL-resistance in AFP-producing hepatoma cells has been attributed to high levels of cytoplasmic AFP. Also, the capability of intracellular AFP to bind RAR and to block RA-RAR signaling by its co-repressor-like role (Li et al. 2009.). Fluorescence resonance energy transfer (FRET) techniques have shown that cytoplasmic AFP can function as a regulator in the phosphatidylinositol-3-kinase (PI3K)/AKT pathway in human hepatocellular carcinoma cells. All these results showed that AFP-positive human stomach cancer is characterized by significantly lower apoptotic index than AFP-negative ones and this might evidence inhibition of apoptosis by AFP (Goldstein et al. 1999).

2.3. AFP Gene Structure and Expression

All albuminoid family genes are located on the same chromosome, with albuminoid genes being located on chromosome 5 of mouse (Guan et al. 1996.), 14 of rat (Belanger et al. 1994), and on the long arm of chromosome 4 of human (4q11-q13) (Song et al. 1999). The AFP, ALB, and α-ALB genes are positioned near each other and have a common direction of transcription (Figure 2-1).

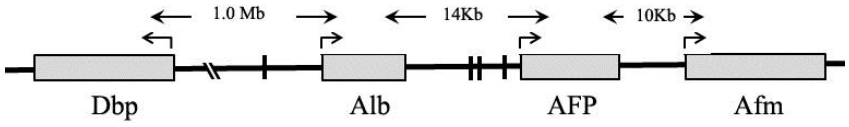

Figure 2-1. Map of the albumin gene family locus. The DBP, albumin (ALB), AFP and AFM genes are designated as boxes with arrows representing transcription start sites. The numbers above the intergenic regions represent the distance between genes. Black ovals between the DBP and albumin, and albumin and AFP genes represent the albumin enhancer and the three AFP enhancers, respectively.

The rat AFP gene is 19 kb. Like the ALB and α-ALB genes, it consists of 15 exons and 14 introns; exons 1 and 15 are non-coding. This structure is conserved among mice (Kioussis et al. 1981.), rats (Sargent et al. 1981), and humans (Dugaiczyk et al. 1985.).

The main product of AFP gene transcription in fetal liver is a 2.1 kb mRNA. In addition, AFP mRNAs of 1.7, 1.4, and 1.0 kb have also been detected in fetal and regenerating liver and in carcinogenesis (Petropoulos et al. 1985; Wan and Chou 1989), with the shorter 1.4 and 1.0 kb mRNAs dominating in adult liver (Lemire and Fausto 1991). Apparently, expression levels of the multiple mRNA forms are controlled by different mechanisms and can be changed independently (Lemire and Fausto 1991; Watanabe et al.1992.). All the products of AFP gene transcription can be translated. The 2.1 kb mRNA corresponds to polypeptides weighing 68 and 70 kDa (Wan and Chou 1989; Lemire and Fausto 1991). Functions of the different AFP forms are insufficiently studied; however, it is known that the shortened translation products maintain the transport properties (Lemire and Fausto 1991).

During embryogenesis AFP can be detected in the visceral endoderm of the yolk sac at embryonic gestation days 6-7 in the mouse (Dziadek and Adamson 1978); at this stage AFP is a dominant serum protein. Later the maximum level of its expression is observed in fetal liver and, at significantly lower levels, in the embryonic gut (Tyner et al. 1990) and in some other organs (Nahon et al. 1988; Cooke et al. 1991). At the end of the embryonic period of development, a drastic decrease in AFP expression takes place. This switch is carried out at the transcriptional level (Belanger et al. 1981). Shortly after birth, the AFP concentration in blood decreases 104-fold (Sala-Trepat et al. 1979; Tilghman and Belayew 1982).

Expression of the AFP gene in adult liver can be restored during the course of liver regeneration. An elevation of AFP serum level is also observed in the case of acute viral hepatitis, primary liver tumors, teratocarcinomas, and gut tumors (Abelev et al. 1967; Abelev 1971.). In the case of embryonic carcinomas, teratocarcinomas, yolk sac tumors, and hepatoblastomas, an increase in AFP level is observed in 80-90% of cases and appears to be an important diagnostic marker.

3. MECHANISMS OF AFP GENE REGULATION

AFP is an ideal model system for studying the temporal and tissue-specific regulation of developmental gene expression. Expression of these types of genes is often at the level of transcriptional initiation by the interaction of trans-acting transcription factors with cis-acting DNA elements. Therefore, it is important to understand these factors and their binding regions on the gene.

3.1. Regulatory Cis-Acting Elements of the AFP Gene

The location of the cis-acting elements present in the AFP gene has been investigated in a transgenic mouse model as well as mouse F9 teratocarcinoma cells (Scott and Tilghman, 1983; Scott et al. 1984; Camper and Tilghman, 1989; Godbout et al. 1988; Hammer et al. 1987). An AFP minigene containing the first three and last two exons, 7.6 kb of 5′ flanking DNA, and 400 bp of 3′ flanking DNA from the mouse AFP gene was constructed and used to investigate the sequences required for normal developmentally regulated tissue-specific expression of AFP. The AFP minigene was expressed in a tissue-specific manner in transgenic mice and was developmentally expressed in the F9 teratocarcinoma cell model, indicating that the sequences that are required for regulation are present in the minigene. These studies in cell lines and mice have identified five distinct regulatory cis-acting elements in the mouse gene, including a promoter, a silencer, and three distinct enhancer elements (Figure 3-1). These elements are located within the region from -7.6 kb to the transcription start site. There is a high homology between the AFP gene 5‘-regulatory regions of the mouse, rat, gorilla, and human (Chevrette et al. 1987; Ryan et al. 1991.).

The AFP promoter, localized within the first 250 bp upstream of the transcriptional start site, is active only in tissues where AFP is normally transcribed (Godbout et al. 1986.). Extensive analysis of the AFP promoter from mice, rats, and humans has shown binding sites for ubiquitous and

tissue-specific transcription factors (Chen H et al. 1997). In vivo, in the absence of the enhancer, the AFP promoter is inactive (Hammer et al. 1987). A considerable similarity of AFP gene promoter organization with other albumin genes can be noted, in particular, the presence and localization of the CCAAT-box and HNF1 binding sites within rat AFP and DBP gene. (Belanger et al. 1994; Song et al. 1998).

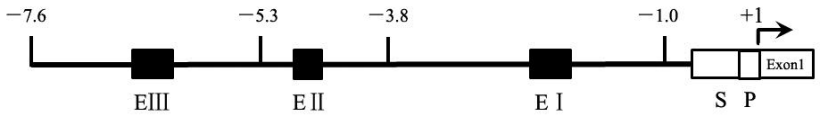

Figure 3-1. Structure of the AFP gene regulatory region. The AFP gene contains five distinct elements that are located within 7.6 kb upstream of the AFP transcription start site (+1) that defines the beginning of exon 1. The promoter (P) is from+1 to -250 bp, the silencer (S) is from -250 to -838 bp. The three distinct AFP enhancers (E I-III) were originally defined as fragments from -1.0 to -3.8, -3.8 to -5.3, and -5.3 to -7.6 kb.

The silencer, between -250 and -838 bp, is required for the postnatal repression of AFP expression (Vacher and Tilghman 1990). Deletion of the silencer sequence from -800 to -250 bp in transgenic mice leads to persistence of AFP gene expression in a manner controlled by the enhancers and the promoter in liver and gut of the adult mice (Vacher and Tilghman 1990). The mouse silencer activity is low in fetal liver and high in adult liver (Camper and Tilghman 1989; Vacher and Tilghman 1990), indicating that the silencer plays a major role in suppressing AFP expression during liver ontogeny, although this is not the only mechanism involved in developmental regulation of the AFP gene (Emerson et al. 1992). The possibility exists that the silencer may serve as a molecular switch to redirect the action of the AFP enhancer to stimulate the albumin promoter as the liver matures (Nakata et al. 1992).

At least two silencers, from -1822 to -951 and from -402 to -169 bp, have been identified in the human AFP gene regulatory region. The distal silencer is more powerful, with the ability to inhibit the activity of homologous and heterologous enhancers according to their localization, and independently from their orientation, but does not actually influence the AFP promoter function (Nakabayashi et al. 1991).

Within the mouse and rat AFP gene regulatory region, three independent enhancers (EI-EIII) each 200-300 bp, have been identified (Godbout et al. 1988; Wen et al. 1991; Groupp et al. 1994.). These elements are typical enhancers and are able to stimulate the AFP promoter as well as heterologous promoters. Like the AFP promoter, the enhancers are tissue-specific and are not active in non-hepatic cells. Each of the enhancers is able to stimulate the SA, as well as AFP promoter. Probably, at some stages of development and in some hepatoma lines, in which the upstream SA enhancer is inactive, intergenic enhancers control the expression of the two genes independently, and the corresponding promoters do not compete with each other due to their interaction with the different enhancer sites (Jin et al. 1995.).

Among the three enhancers, E1 and E2 are similar, suggesting they arose from a duplication event. E2 is present in all species whereas E1 is found only in rodents (Long et al. 2004). E3 is distinct from the other enhancers and has been the most extensively studied. In contrast to rodents, the human AFP enhancer contains E2 and E3 but does not have an E1 (Long et al. 2004.). In 2000, a novel enhancer and an alternative promoter were reported in the first intron of the mouse AFP gene (Scohy et al. 2000). The activity of the enhancer depends on a 44-bp sequence centered on a CACCC motif, and the alternative promoter is active in the yolk sac and fetal liver.

3.2. Trans-Acting Factors in AFP of Gene Regulation

The regulation of AFP gene expression occurs mainly at the level of transcription by the interaction of trans-acting transcription factors with cis-acting DNA elements. Whether the AFP gene is expressed and to what extent it is expressed depends on the presence of a distinct set of trans-acting protein factors, many of which interact with the AFP cis-acting elements. Some of these factors have an effect on AFP gene activity are briefly introduced below.

Hepatocyte nuclear factors (HNFs) play a critical role in regulation, which is carried out mainly at the transcriptional level (Tilghman and Belayew 1982; Powell and Suwanichkul 1993; Friedman et al. 1984). Several families of regulatory proteins (HNF1, C/EBP, HNF3, HNF4 and HNF6) can be attributed to HNFs, and their binding sites have been identified in the regulatory elements of the numerous liver-specific genes. Although, the expression of these factors is not restricted to liver cells, liver expresses the highest amounts.

HNF1 family proteins are the most widely distributed regulators of liver-specific gene expression; their potential binding sites have been found in regulatory regions of more than one hundred genes. Most often these sites are localized in promoter regions and form clusters with binding sites of other transcription factors (Tronche et al. 1997).

C/EBPα is the first identified member of the C/EBP family that binds to a CCAAT-box (Landschulz et al. 1988.). Family members contain a basic DNA-recognizing domain (so-called 'bZIP'), an amino terminal transactivation domain, and a helical structure of a leucine zipper type providing dimerization. C/EBP protein binding sites have been identified in the regulatory elements of AFP (Thomassin et al. 1992), SA (Costa et al. 1988), C/EBPα (Legraverend et al. 1993), and other liver genes.

HNF3 (FOXA) was originally identified as a liver-enriched factor that regulates the rat transthyretin gene (Costa et al. 1989; Lai et al. 1990). Additional analysis revealed three HNF3 isoforms, HNF3α, HNF3β, and HNF3γ, that are encoded by distinct genes (Lai et al. 1990). The HNF3 family of factors regulate numerous liver genes, and are among the earliest factors to be expressed during hepatogenesis (Kaestner. 2000).

HNF3β was first detected in the primitive streak and node on the seventh day of murine gestation (Ang et al. 1993). HNF3α follows the HNF3β dynamics, but at a lower concentration level. HNF3γ starts to be expressed on the twelfth day of gestation (Kaestner et al. 1994). In the adult organism HNF3α, β, and γ are localized in liver, gut, lung, and stomach, and in addition, HNF3β and γ occur in the ovary and HNF3γ in testicles (Xanthopoulos and Mirkovitch 1993). An increase in HNF3α level is observed in primary hepatocyte cultures grown on an extracellular

matrix. Apparently, HNF3α participates in extracellular signal transduction that determines hepatocyte differentiation (Cascio and Zaret 1991; DiPersio et al. 1991). In the HNF4 family of proteins, HNF4α is the most completely characterized. According to their structure HNF4 factors are related to the nuclear receptor superfamily. Like other nuclear receptors, they contain two DNA-binding 'zinc-finger' domains and a spacious carboxy-terminal region providing dimerization and ligand binding.

So far, the HNF families have been described that in some degree control the expression of the majority of the known liver-specific genes. These families are related to the earlier described broader superfamilies of transcription factors and are highly conserved among different species. The factors within one family recognize the same DNA sequence and often are able to form heterodimers, modulating their individual properties. Moreover, several forms of the same protein, but that differ in transactivation abilities, might be expressed within one cell. As far as these forms compete for the binding sites on a regulated gene, the efficacy of transcription depends on which form binds more successfully (Lazarevich 2000).

Zhx2 (formerly called Afr1) belongs to a small family of proteins, with Zhx1 and Zhx3 being found only in vertebrates. They all share a similar gene and protein structure (Spear et al. 2006). These proteins contain two C2H2 zinc fingers and four or five homeodomains, which suggests nucleic acid binding. All three Zhx proteins can form homodimers and heterodimers with each other and with NF–YA. Structural studies suggest that Zhx2 homeodomain 2 has an unusual conformation (Bird et al. 2010). Zhx2 and other family members have been called transcriptional repressors based on modest (up to 2-fold) activity in a one-hybrid assay (Kawata et al. 2003; Yamada et al. 2003). Similar repressive effects have been seen in other tissue culture models (Liu et al. 2006; Shen et al. 2008). However, this modest level of repression is unlikely to fully represent Zhx2 activity in vivo, since, in BALB/cJ mice, a reduction of Zhx2 results in about a 20-fold increase in AFP and H19 expression.

Zbtb20 (also known as DPZF, HOF, and Zfp288) was initially described in a screen of genes expressed in human dendritic cells (Zhang et

al. 2001), and is a member of a large family of proteins that contain an N-terminal BTB (broad complex, tram track, bric-a-brac) domain, involved in protein–protein interactions, and multiple C-terminal Kruppel-like C2H2 zinc fingers (four in the case of Zbtb20) that mediate interactions with nucleic acids. Proteins in this family often act as transcriptional repressors, but in some cases can activate target genes. Deletion of Zbtb20, accomplished by crossing Zbtb20 floxed mice with albumin-Cre transgenic mice, results in the persistence of AFP expression in adult mouse livers (Xie et al. 2008). Furthermore, adult livers appear normal in the absence of Zbtb20, indicating that this protein is not essential for normal liver development.

Research shows that the some oncoproteins affect the expression of the AFP gene. Within the AFP gene promoter, a binding site for transcription complex AP-1 has been localized. This site is bound by products of the oncogene families jun and fos (Zhang et al. 1991) both as Jun-Fos heterodimers and as Jun-Jun homodimers, in the latter case, the binding efficacy and activation properties being considerably reduced. In monkey kidney CV-1 and mouse teratocarcinoma F9 cells, the AFP promoter can be activated either by transfection with c-jun and c-fos oncogenes or with the glucocorticoid receptor in the absence of c-jun/c-fos products (Schule et al. 1990). In human hepatoma HuH-7 cells transfection of c-jun and c-fos oncogenes suppresses AFP promoter activity and decreases the activation that is observed under dexamethasone treatment of non-transfected cells (Schule et al. 1990).

Hormones involved in the regulation of AFP gene expression have been confirmed. At certain stages of development, glucocorticoids, retinoid acid and thyroid hormone can take part in the modulation of AFP gene expression. At different stages of ontogenesis and carcinogenesis, the same hormone may render either an activating or repressing influence on the same gene expression. Glucocorticoids and their synthetic analog dexamethasone considerably accelerate a reduction of AFP gene expression in rodent liver immediately after birth. Dexamethasone suppresses AFP gene expression in rat hepatoma McA-RH 7777 and induces it in hepatoma McA-RH 8994, which is characterized by relatively

low AFP synthesis levels (Dong et al. 1989). Glucocorticoid acts through its nuclear receptor binding to the glucocorticoid responsive element (GRE), which is located in the promoter region of the AFP gene [Guertin et al. 1988; Zhang et al. 1991; Thomassin et al. 1992.). The GRE binding sites partially overlap with binding sites for other transcription factors, e.g., nkx-2.8, FTF, LF, and AP-1 (Zhang et al. 1991; Bernier et al. 1993; Wen et al. 1993), which may compete with GRC for a site binding.

Thyroid hormone (T3) down-regulates the mouse AFP promoter, which contains a sequence resembling a T3-responsive element (T3RE-like) (Caturla et al. 1997). T3 acts through its nuclear receptors (T3R), which belongs to the nuclear hormone receptor superfamily (Ing and Malley 1995; Evans 1988). It has been observed that congenital hypothyroidism is correlated with high AFP levels in neonates, and that a several day thyroxin treatment shuts down AFP levels (Toublanc et al. 1994). T3 thus appears to play a role in the developmental regulation of AFP gene expression.

As for retinoid acid regulation of AFP gene expression we will focus on the following.

4. RETINOIC ACID REGULATES AFP GENE EXPRESSION IN EMBRYONIC CARCINOMA AND HEPATOMA CELLS

4.1. Retinoic Acid Regulates AFP Gene Expression by Direct Binding to RARE-Like Sequences within the AFP Gene Regulatory Region

Like other receptors in the steroid/thyroid hormone superfamily, ligand-bound receptors function as transcriptional regulators for a specific set of genes. Some of these genes respond rapidly to the action of RA, indicating that the regulatory sequences of these genes are direct targets for the receptors (LaRosa and Gudas 1988; Murphy et al. 1988). To analyze the cis-acting elements responsible for RA-induction of AFP gene activity,

Dong et al. constructed a CAT expression plasmid by cloning the rat AFP 5'-flanking region, from -7040 to +7 bp, into the pGEMCAT vector (Dong et al. 1989.). By using transient co-transfection of the RAR or RXR expression vectors and serial pAFPCAT deletion mutants, three cis-acting elements that regulate transcription of rat AFP gene were identified and characterized in the far upstream 5'-flanking region of the AFP gene (Liu and Chiu 1994; Liu et al. 1994 a, b) (Figure 4-1).

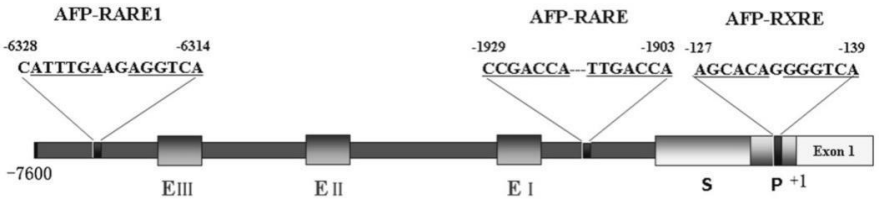

Figure 4-1. Cis-acting elements for the retinoic acid receptor identified in the regulatory region of the rat gene.

One of them, a retinoid X receptor response element (AFP-RXRE) located at position -139 to -127 bp of the rat AFP promotor in the 5'-flanking region of the AFP gene, conferred a marked RA responsiveness when co-transfected with the retinoid X receptor (RXR), but not with retinoic acid receptors (RARs). Chicken ovalbumin upstream promotor transcription factor (COUP-TF), an orphan member of the steroid/thyroid hormone superfamily (Sagami et al. 1986; Wang et al. 1989; Ladias and Karathanasis 1991), also demonstrated specific binding activity to the AFP-RXRE in vitro. In transfection assays, COUP-TF dramatically repressed the transactivation of RXR on AFP-RXRE. The mechanism of repression by COUP-TF may involve the mutual occupancy of the AFP-RXRE binding site between RXR and COUP-TF.

Another cis-acting element located between -6337 to -6266 bp in the 5'-flanking region of the AFP gene is AFP-RARE1. This cis-acting element is composed of a RARE direct repeat sequence of AGGTCA and RARE-like motifs ATTTGA at -6319 and -6327 bp, respectively. This cis-acting element showed strong RA responsiveness to RARα and RXRα with 15- and 20-fold increases in transactivation. In analogy to other

hormone response elements, AFP-RARE1 displays enhancer-like features in an orientation-independent fashion and functions at a heterologous promoter. The AFP-RARE1 identified far upstream of the AFP 5'-flanking region closely resembles the sequence found in other naturally occurring RAREs (Leid et al. 1992).

The third RA-inducible cis-acting element (AFP-RARE) is located between -2611 to -1855 bp in the rat AFP 5'-flanking region. Sequence analysis based on the nucleotide sequence for the 5'-flanking region of the rat AFP gene (Buzard and Locker 1990) revealed that the AFP gene from -1972 to -1905 bp contains the sequence TGACC (which is found in most RAREs), as well as three sequences that resemble TGACC: TGATT, TGAGT, and CGACC. They are located at -1912, -1948, and -1928 bp. These four TGACC or TGACC-like sequences form two groups of direct repeats with a spacing of 3 and 11 nucleotides, respectively. The retinoic acid receptor specifically binds to this AFP-RARE cis-acting element in mobility shift assays. Furthermore, this AFP-RARE cis-acting element function in exogenous TK promoter constructs in transient co-transfection assays. In addition to the direct effect of RA on AFP gene expression through RAREs, as described above, a study using F9 cells revealed that RA might also act through an indirect mechanism by regulating trans-acting factors on the AFP 5'-flanking region (Chen and Chiu 1994).

4.2. AUF1 Proteins Participate in the Regulation of AFP Gene Expression in F9 Cells

F9 cells are one of the most extensively studied embryonic carcinoma cells and originated from a teratocarcinoma (Artzt et al. 1973). In response to several types of physical and chemical stimuli, these non-differentiated, multi-potential stem cells can differentiate into somatic tissue corresponding to derivatives of the three germinal layers: endoderm, mesoderm, and ectoderm (Silver et al. 1983). When treated with RA, F9 cells form aggregates. Most of the cells on the outer surface of the aggregates differentiate into visceral endoderm and produce AFP

(Strickland and Mahdavi 1978; Dong et al. 1990). The capacity of F9 cells to differentiate in a controlled manner in culture has provided a useful system for studying certain aspects of mammalian development and differentiation, and for investigating the mechanism by which RA affects AFP gene expression.

Chiu's laboratory (Dong et al. 1989) constructed a CAT reporter plasmid (pAFPCAT) whose expression was under the control of the 7-kb 5'-flanking region of the rat AFP gene. The plasmid was transfected and stable F9 transfectants were isolated. Using a series of deletion mutants of the pAFPCAT, the region between -2,611 and -1,855 bp was found to be important for AFP induction (Chen et al. 1999). Subsequent analysis identified a functional sequence (-1,905 to -1,891 bp, 5'-ACTAAAATGGAGACT-3') that differentially binds nuclear proteins (DAP-I or DAP-II) from undifferentiated and differentiated F9 cells. This sequence was designated as DAS (for differentiation-associated sequence) for its specific binding activity of differentiation-associated protein (DAP) during the course of RA-induced F9 differentiation (Figure 4-2). The DAP-II protein complex was initially detected in F9 cells after treatment with RA and increased its expression during RA-induced differentiation. Since the AFP transcripts were initially detected 4 days after treatment with RA, the relatively late induction of AFP gene expression suggests that it is unlikely that the AFP gene is a primary target for activated retinoid receptors, which can then bind onto three RARE-like sequences in the 5′-flanking region of the AFP gene (Liu and Chiu 1994; Liu et al. 1994a, b) as has been suggested. Chiu hypothesized that the genes encoding DAP-II proteins may be primary and early response genes to RA, and that subsequently DAP-II conveys the effect of RA by directly participating in the regulation of AFP gene expression (Chen et al. 1999).

To purify the DAP-II proteins, we used a sequence-specific DNA affinity resin in which multiple copies of the DAS sequences were covalently attached to Sepharose CL-4B. Nuclear extracts of the RA-treated F9 cells, which displayed high DAP-II-binding activity and low DAP-I-binding activity by EMSA analysis, were applied to DAS-DNA affinity column. After two cycles of purification with the same affinity

resin, the highest binding activity was detected in the eluent fractions of 0.3 and 0.4 M KCl. The purified DAP-II complex mainly contained five proteins, with molecular weights of 45 (p45), 42 (p42), 32 (p32), 30 (p30), and 20 (p20) kDa (Figure 4-3). From MALDI-TOF mass spectrometric analysis and a database search, the p45, p42, p32 and p30 were found to be AUF1 proteins, which was confirmed by recognition with an anti-AUF1 antibody in a western blot.

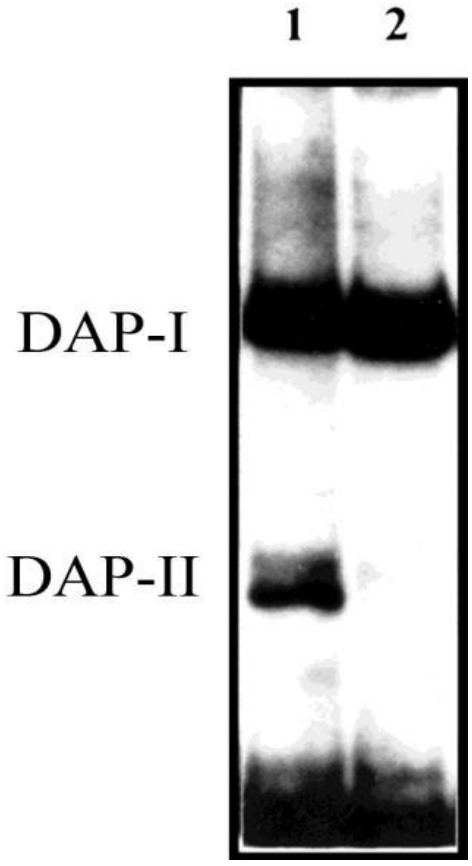

Figure 4-2. Binding of a specific nuclear protein with a 131 bp DNA fragment in the 5′-flanking region (-1972 to -1842) of the rat AFP gene. Gel mobility shift assays were performed by incubating 5 μg of nuclear extract from differentiated F9 cells following 6 days of RA treatment (lane 1), and undifferentiated F9 cells (lane 2), with a 32P labeled 131 bp DNA fragment. DNA-protein complexes band I and band II are indicated by arrows.

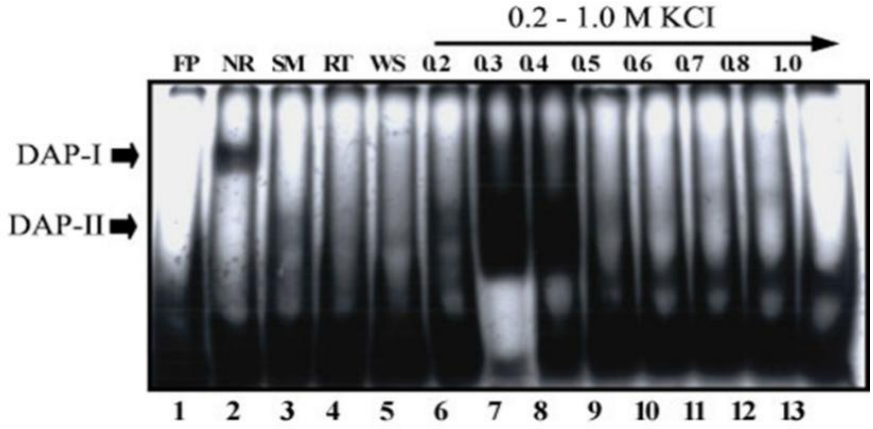

Figure 4-3. DAP-II purification on DAS-DNA affinity column. NE of RA-induced F9 cells was applied as a first cycle of purification on a DAS-DNA affinity column. Bound proteins were eluted with a 0.2–1.0 M KCl gradient across eight fractions. 32P-labeled DAS double-stranded oligonucleotides were used as a probe for EMSA. FP, free probe; NR, non-RA-treated F9 NE; SM, starting material of RA-treated F9 NE; RT, run-through (unbound) protein fraction; and WS, washing buffer solution. 0.2–1.0 represent protein samples eluted by 0.2–1.0 M KCl salt solution in washing buffer. The eluted protein samples were concentrated and exchanged to buffer C by a YM-10 filter (Millipore) prior to EMSA analysis. All samples were loaded with 2 µl, except for NR and SM, which were loaded at 5 µg protein each.

AUF1, also called hnRNP D, is expressed as a family of four protein isoforms designated by their molecular masses as p37AUF1, p40 AUF1, p42 AUF1 and p45 AUF1 (White et al. 2013; Brewer 1991). Four isoforms of AUF1 are generated by alternative splicing of exon 2, which encodes 19 amino acids; and exon 7, which encodes 49 amino acids (Wagner et al. 1998). The proteins p45 and p42 are AUF1 proteins p45AUF1 and p42AUF1 isoforms. Although the p45AUF1 and p42AUF1 isoforms were components of the purified DAP-II proteins and present in different phosphorylated forms, they are not the active proteins of the DAP-II complex because they have no DAS-binding activity and are present in both RA-treated and untreated F9 cells. However, the role of the p45AUF1 and p42AUF1 isoforms in regulating AFP gene expression requires further study.

The p30 was identified as a member of the AUF1 family. To confirm the results, western blotting was used to analyze the samples, from F9

nuclear extracts and the purified DAP-II proteins, with anti-AUF1 antibody. Southwestern blotting analysis showed that one DNA-protein complex found at a molecular weight of 30 kDa in F9 nuclear extracts was consistent with our previous data (Chen et al. 1997). The expression of p30 protein was induced in F9 cells during RA-induced differentiation. The 30-kDa protein identified by Southwestern analysis strongly resembled the p30 resulting from purification by DNA affinity column chromatography. On 2D gels, p30 was present as a single spot with a basic pI, and was identified as a member of the AUF1 family. P30 was then designated as p30AUF1 because it precisely matched muAUF1-3 by MSFit and recognized by anti-AUF1 antibody in Western blot. The interaction between p32 with anti-AUF1 suggested that it was an AUF1-like protein or a protein with a common immunoreactive epitope. P32 also shared some similarities with p30 (p30AUF1). Its expression could be induced by RA, and the expression pattern was the same as p30AUF1. It was one of the DAS DNA-binding DAP-II proteins.

AUF1 is an abundant, ubiquitous protein that can bind to RNA, as well as to double- and single-stranded DNA sequences in a DNA sequence-specific manner. Its role in regulating the half-life of mRNA species containing an AU-rich element has been well characterized (Chen and Shyu 1995; Loflin et al. 1999; Wilson and Brewer 1999). Although several laboratories have shown that AUF1 can specifically bind to double-stranded DNA and regulate gene expression (Tay et al. 1992; Dempsey et al. 1998; Fuentes-Panana et al. 2000; Lau et al. 2000; Tolnay et al. 2000), no report has yet been made on the regulation of oncodevelopmental gene expression by AUF1 during RA-induced cell differentiation. Purified DAP-II is mainly composed of AUF1, including p45AUF1, p42AUF1, and p30AUF1, and AUF1-like protein p32. The reasons that p30AUF1 is considered to be the major protein of DAP-II that plays a role in DAS DNA-binding are (1) southwestern analysis showed that a 30-kDa protein in F9 nuclear extracts can specifically bind to the DAS sequence, consistent with our previous data (Chen 1997), (2) in a comparison with SDS-PAGE and EMSA of purified proteins, p30AUF1 always accompanied DAS-binding activity, and (3) the expression pattern of the

p 30AUF1 was the same as DAP-II proteins in F9 cells during RA-induced cell differentiation.

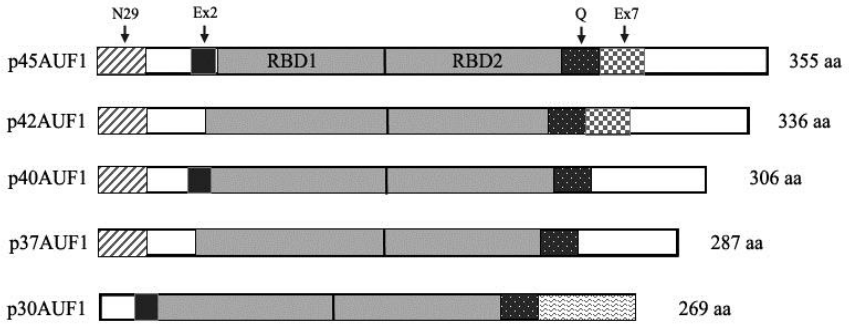

Figure 4-4. Schematic diagrams of the five mouse AUF1 isoforms. The two RNA-binding domains (RBD1 and RBD2) are depicted as two gray-shaded regions in the central part of the protein molecules. A carboxy-terminal 49-amino acid encoded by exon 7 (Ex7) presents as a hatched bar in p45AUF1 and p42AUF1. A 19-amino acid encoded by exon 2 (Ex2) presents as black bars at the amino terminus of p45AUF1, p40AUF1, and p30AUF1. All five proteins contain a Gly-rich region (barQ). The A29-amino acid sequence located at the N-terminal (slash bar N29) and C-terminal region (cross bar for p30AUF1 and open bars for all other AUF1 proteins) are also shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

The data described above demonstrated that p30AUF1 is the main component of the DAP-II complex. Since the DAS sequence is an important element for activation of AFP gene expression in F9 cells during RA-induced differentiation (Chen 1997; Chen et al. 1999), p30AUF1 may therefore, play an important role in the regulation of AFP gene expression. It has been shown that DAP-II (p30AUF1) is present in many AFP-producing cells, but not in non-AFP-producing cells (Chen 1997; Chen et al. 1999). It is believed that p30AUF1 is a new AUF1 isoform capable of binding to double-stranded DNA and regulating AFP gene expression (Figure 4-4). Further study is needed to elucidate the mechanism of p30AUF1 in regulating gene expression.

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