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Identification of a Cis-Acting Element in the Rat α-Fetoprotein Gene and Its Specific Binding Proteins in F9 Cells During Retinoic Acid-Induced Differentiation

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Abstract Mouse F9 embryonic teratocarcinoma stem cells can be induced to differentiate into visceral endoderm. Following retinoic acid (RA) treatment, alpha-fetoprotein (AFP), a differentiation marker, is expressed and secreted. The mechanism by which RA regulates AFP expression during differentiation is not clear. The relatively late induction of AFP indicates that the AFP gene may not be a primary target of RA activity during F9 cell differentiation. In this study, a CAT reporter plasmid containing the rat AFP 5′-regulatory region (−7040 to +7) adjacent to the CAT gene (pAFPCAT) was stably transfected into F9 cells and used to delineate a cis-acting element which associates with AFP gene activation. Similar spatial and temporal expression patterns between the transcriptional activity of the recombinant AFP gene and the endogenous AFP gene demonstrate that this stably transfected F9 system can be used to dissect both cis-elements and trans-acting factors responsible for RA-induced AFP expression. Using a series of deletion mutants of the pAFPCAT, the region between −2611 to −1855 was found to be important in AFP-induction. Subsequent analysis identified a functional sequence (−1905 to −1891, 5′-ACTAAAATGGAGACT-3′) that differentially binds nuclear proteins from undifferentiated and differentiated F9 cells. This sequence, designed as differentiation-associated sequence (DAS) for its unique binding of a nuclear protein (DAP-II) that appears during RA-induced F9 differentiation, acts as a regulatory protein factor in AFP gene activation. J. Cell. Biochem. 72:25–34, 1999. © 1999 Wiley-Liss, Inc.

Key words: F9 cells; AFP gene; differentiation; retinoic acid

The alpha-fetoprotein (AFP) is an oncodevelopmental protein that is expressed in the visceral endoderm of embryonic yolk sac and fetal liver at high levels, and at a low level in the fetal gut. Synthesis of AFP decreases dramatically shortly after birth, primarily due to transcriptional repression. Only trace amounts of AFP mRNA are accumulated in adult liver and gut. However, expression of the AFP gene is reactivated by cell proliferation during liver regeneration, and in certain hepatocellular carcinomas. The AFP gene can also be induced in other oncogenic states, such as in hepatoid adenocarcinomas occurring in tissues other than the liver, and in teratoblastomas [Abelev, 1968; Adinolfi, 1979; Ruoslahti, 1979; Tilghman, 1985; Chen et al., 1996, and refs therein]. Therefore, the AFP gene provides an excellent model to study transcriptional regulation during mammalian development and oncogenic transformation.

Retinoic acid (RA), the major and most active physiological metabolite of vitamin A (all-trans-retinol), has profound effects on embryonal development, cell proliferation and differentiation [De Luca, 1991]. It has been proposed that retinoid activity is mediated by nuclear retinoid receptors which are members of the steroid/thyroid hormone receptor superfamily [Wahli and Martinez, 1991]. Two families of nuclear

Abbreviations used: AFP, alpha-fetoprotein; CHAP, 3-[(Cholamidopropyl)dimethyl-ammonio]-propanesulfonate; DAS, differentiation-associated sequence; DAP, DAS-associated protein; pAFPCAT, a CAT reporter plasmid containing the rat AFP 5′-flanking region between −7040 to +7; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor.

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retinoid receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), have been identified in mammals [Chambon, 1995; Mangelsdorf and Evans, 1995; Means and Gudas, 1995]. Retinoid receptors primarily act through direct association with specific sequences known as retinoic acid receptor response elements (RAREs) or retinoid X receptor response elements (RXREs) in the regulatory regions of the targets [De Luca, 1991; Glass et al., 1991]. Recently, 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 elements were demonstrated to enhance the transcriptional activities of both the rat AFP promoter and a heterogeneous TK promoter [Liu and Chiu, 1994; Liu et al., 1994a,b], indicating that RA participates in the regulation of the AFP gene expression.

F9 cells are one of the most extensively studied embryonal carcinoma cells that originate from a teratocarcinoma [Artzt et al., 1973]. In response to several types of physical and chemical stimuli, these nondifferentiated, multipotential 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 and allowed to grow in suspension, F9 cells form aggregates. Most of the cells on the outer surface of the aggregates differentiate into visceral endoderm and produce AFP as a marker of differentiation [Strickland and Mahdavi, 1978]. The capacity of F9 cells to differentiate in a controlled manner in culture provides a useful system for studying certain aspects of mammalian development and differentiation, and for investigating the mechanisms by which RA affects AFP gene expression. In the present study, we identified and characterized a cis-acting element which associates with the AFP gene activity in F9 cells during RA-induced cell differentiation. This element forms a specific complex with a nuclear protein that is expressed in response to RA in F9 cells, indicating that some trans-acting factors might be involved in mediating RA-induced AFP expression. Understanding the involvement of this cis-element and its trans-acting factors in the temporal expression of the AFP gene may shed light on the mechanisms governing AFP expression during development and the oncogenic process.

MATERIALS AND METHODS

Materials

Retinoic acid (all trans), coenzyme A, S-acetyl coenzyme A synthetase, diethyl pyrocarbonate, and 3-[(Cholamidopropyl)dimethl-ammonio]-1-propanesulfonate (CHAP) were obtained from Sigma (St. Louis, MO). Dimethyl sulfate (DMS) and piperidine were from Aldrich. Cell culture materials were purchased from Gibco (Grand Island, NY), except α-Minimum Essential Media (α-MEM; Irvine Scientific, Santa Ana, CA). Erase-a-Base system and Altered sites in Vitro Mutagenesis System were from Promega (Madison, WI).

Construction of Plasmids

pAFPCAT was constructed by cloning the rat AFP 5′-flanking region (−7040 to +7) into pGEMCAT [Dong et al., 1989]. To construct deletion mutants, pAFPCAT was partially digested with EcoR I and completely digested with Sph I. The linearized DNA was used to generate deletion mutants by the Erase-a-Base system. Altered Sites in Vitro Mutagenesis System was used to construct pAFP*CAT. All mutants used in these studies were confirmed by sequencing.

Cell Culture and Treatment of Cell

Undifferentiated F9 cells were maintained as stem cells by culturing on 0.1% gelatin-coated tissue culture dishes in α-MEM, supplemented with 10% fetal bovine serum and 1 mM glutamine. For experiments involving cell differentiation into visceral endoderm, F9 cells were seeded at 5×10^5 cells per 100-mm bacteriological petri dish in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 medium, supplemented with 10% fetal bovine serum and 1 mM glutamine. On the day after seeding, cells were treated with 5×10^{-8} M RA. Cell aggregates were refed daily with the appropriate medium and RA concentration. For controls, undifferentiated F9 cells were grown as in the same medium except that no RA was added.

Transfection

Transfection was performed using the calcium phosphate coprecipitation method [Gorman et al., 1982] with minor modifications [Zhang et al., 1991]. Briefly, undifferentiated F9 cells were cotransfected with a total of 20 μg of pAFPCAT or its different mutants and pRSV-neo at a 10:1 ratio in a cell density of 5×10^5/10

cm diameter culture dish. Every 4 days thereafter, transfected cells were selected for stable transfectants by the addition of 500 μg/ml of G418. After 20–30 days, neomycin-resistant colonies were pooled. These cells were grown under differentiation culture conditions with 5×10^{-8} M RA for different periods of time. The chloramphenicol acetyltransferase (CAT) assay was performed as described previously with a correction for the amount of protein [Dong et al., 1989].

Northern Analysis of Total RNA

Total RNA was prepared by the guanidinium thiocyanate method, electrophoretically separated on formaldehyde-agarose gels, and transferred to nitrocellulose filters [Cook and Chiu, 1986]. Prehybridization, hybridization, and washing conditions were conducted according to Thomas [1980]. The AFP probe was a Pst I restricted DNA fragment digested from pRAF87 [Cook and Chiu, 1986] and it was labeled with ^{32}P-dCTP by a random primer labeling procedure [Feinberg and Volgelstein, 1983].

Gel Mobility Shift Assay

DNA probes were end-labeled by a Klenow fragment or T4 kinase [Sambrook et al., 1989]. Nuclear extracts were prepared and used for mobility shift assays as described [Zhang et al., 1991]. In competition experiments, different amounts of unlabeled DNA fragment were added with labeled probe. In experiments with detergents, CHAP was added either before or after the DNA-protein interactions and the reaction mixtures were incubated at room temperature for 5 min before loading the gel.

Chemical Interference Assays

The 5′-end ^{32}P-labeled 131 bp DNA fragment was modified by either dimethyl sulfate (DMS) or diethyl pyrocarbonate as described by Wissmann and Hillen [1991]. Methylated or carbethoxylated DNA fragments were then incubated with nuclear extracts from differentiated F9 cells as in the gel mobility shift assay. Protein bound fractions of the modified DNA were separated from free DNA by running on a 5% polyacrylamide gel. Corresponding bands were visualized by autoradiography and the DNAs were eluted by electroelution. Recovered DNAs were then cleaved by either piperidine or NaOH and analyzed on sequencing gels.

RESULTS

Expression Patterns of the Endogenous AFP Gene and the Recombinant AFP Gene in Stably Transfected F9 Cells

In order to study the mechanism of AFP gene regulation in F9 cells during RA-induced differentiation, a segment of the rat AFP 5′-flanking region (−7.0 kb to +7 bp) was cloned adjacent to the bacterial CAT gene (designated as pAFPCAT, see ref. 18), and was stably transfected into F9 cells. The morphological appearance of the transfected F9 cells and the parental cells was similar during RA treatment (data not shown). Expression of the endogenous AFP gene also exhibited the same pattern in both untransfected and transfected cells. Little, if any, CAT activity was detected after 3 days of treatment with RA in pAFPCAT stably transfected F9 cells. A significant change in CAT expression was observed on the 6th day. It reached its highest level on day 9 and was maintained for up to 12 days. (Fig. 1A). Similarly, endogenous AFP mRNA increased 3 days after the treatment from an undetectable level, and achieved a maximal level on the 9th day (Fig. 1A). These data demonstrated that RA could induce expression of the CAT gene in pAFPCAT-transfected F9 cells in the same way as it induced the AFP gene in untransfected cells. Therefore, the stable transfection system can be used to delineate cis-acting elements that regulate RA-induced AFP expression in F9 cells.

Cis-Elements in the Rat AFP 5′-Flanking Region Responsible for RA-Induced AFP Expression in F9 Cells

To determine which cis-acting elements confer RA-mediated activation of the AFP gene in differentiating F9 cells, a series of deletion mutants of pAFPCAT were stably transfected into F9 cells. After selection, cells were tested by the CAT assay for their ability to respond to RA induction of differentiation. As shown in Figure 1B, CAT expression was induced to more than seven-fold in F9 cells transfected with p3.1AFPCAT, p2.9AFPCAT, or p2.6AFPCAT. This indicates that RA can induce AFP gene expression even after removing the 5′-end of the AFP gene up to −2.6 kb. However, deletion of the nucleotide sequence between −2611 to −1855, caused a five-fold drop in CAT activity (Fig. 1B). Insertion of this fragment into pAPCAT, an expression plasmid containing the AFP

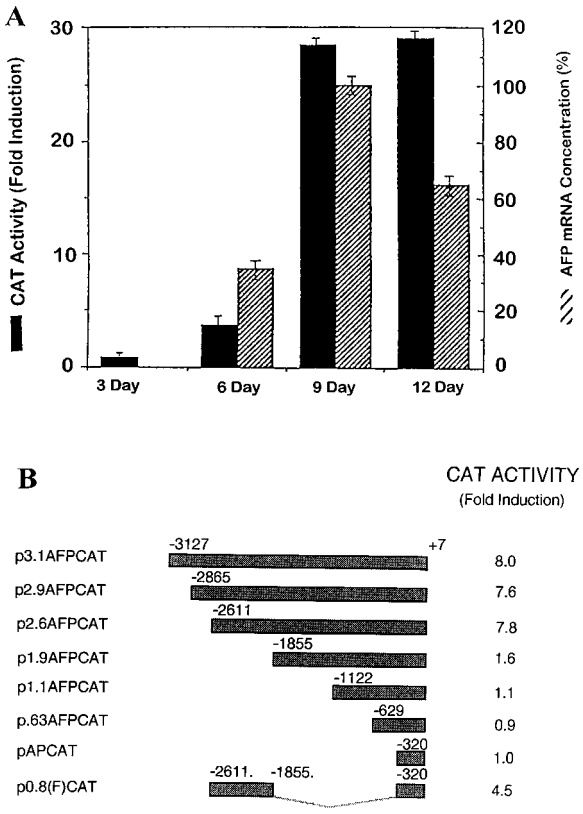

Fig. 1. Expression of stably transfected pAFPCAT and its deletion mutants in F9 cells during RA-induced differentiation. **A:** The pAFPCAT expression plasmid was stably transfected into F9 cells as described in the Materials and Methods. After selection, the transfected cells were treated with 5×10^{-8} M RA for 3, 6, 9, and 12 days. CAT assay and Northern analysis of total RNA were performed. Block bars represent the fold induction of CAT activity and hatched bars represent the levels of endogenous AFP mRNA in F9 cells during differentiation. **B:** Delineation of cis-acting elements that mediate the effect of RA on CAT expression. A series of deletion mutants of pAFPCAT were constructed and stably transfected into F9 cells, which were then induced to differentiation by 5×10^{-8} M RA for 9 days and assayed for CAT activity. Results are expressed as fold induction of CAT activity and represent the average of three to four different stable transfections.

promoter sequence (−324 to +7), restored about 60% of the CAT activity in differentiated F9 cells, suggesting that the nucleotide sequence between −2611 to −1855 plays a role in expression of the AFP gene. Other element(s) between −1855 to −1122 may also be needed to restore the full transcriptional activity of AFP.

Differential Binding of the DNA Fragment (−1972 to −1842) With Nuclear Extracts From Differentiated F9 Cells and Undifferentiated F9 Cells

The DNA fragment (from −2611 to −1855) was digested with restriction enzymes into three smaller fragments and each of these fragments was used to analyze DNA-protein interactions. The protein binding pattern to DNA fragments between −2611 to −1972 showed no difference between differentiated and undifferentiated F9 cells, as indicated by gel retardation assays (data not shown). Specific protein binding bands were identified on the DNA fragment between −1972 to −1842. As shown in Figure 2, a predominant retarded protein complex (band I) was formed with nuclear extracts from both differentiated and undifferentiated F9 cells (Fig. 2, lanes 1 and 2). A second, faster migrating protein-DNA complex, designated as band II, was observed only in nuclear extracts isolated from RA-induced differentiated F9 cells (Fig. 2, lane 1, band II). Specific binding of the band II protein to this fragment (−1972 to −1842) was also detected with nuclear extracts from cells producing AFP, but was absent with nuclear extracts from cells where AFP expression was suppressed (data not shown).

Since the above 131 bp DNA fragment originated from a region that responded to RA, it is possible that this DNA fragment contains a RARE- or RXRE-like sequence that forms the specific binding pattern as described. However, sequence analysis failed to find any RARE- or RXRE-like sequences. No direct interactions between this DNA fragment and bacterial expressed RXRα or RARγ were detected using gel mobility shift assays. Binding competition assays with consensus RARE or RXRE also excluded the possibility that this fragment contained any RA receptor response elements (data not shown).

Identification of Specific Binding Sites of Nuclear Proteins

To determine the specific binding site of the nuclear proteins, we performed dimethylsulfate methylation interference assay. As illustrated in Figure 3A, binding of proteins on several guanine (G) nucleotides at positions −1894, −1896, and −1897 strongly protected these residues from methylation, while the G on the lower strand at position −1892 was weakly protected. All three G residues on the coding strand contribute equally well to protein binding, whereas on the non-coding strand only the G at −1892 forms close contact. To further characterize the bases within the binding site that are important in sequence specific recogni-

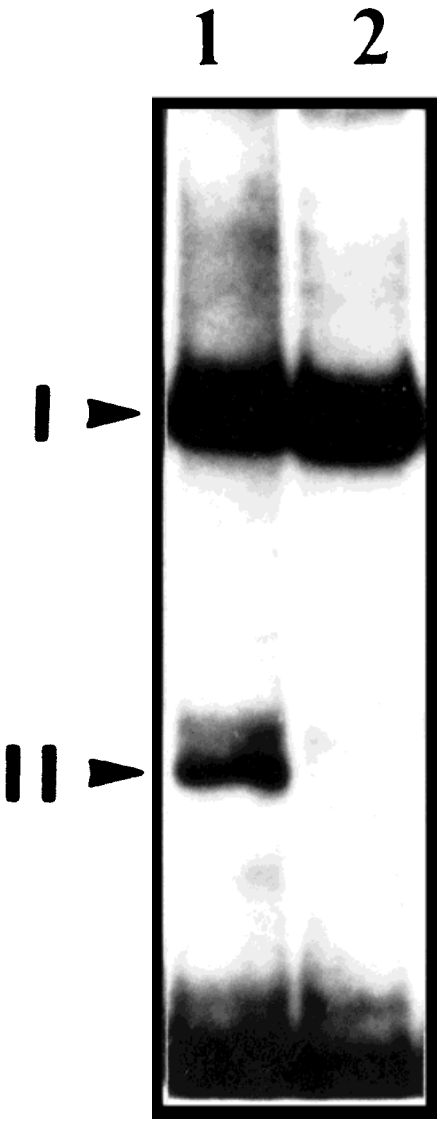

Fig. 2. Binding of a specific nuclear protein with a 131 bp DNA fragment in the 5′-flanking region (−1972 to −1842) of the rat AFP gene. Gel mobility shift assays were performed by incubating 5 μg of nuclear extract from differentiated F9 cells following 6 days of RA treatment (**lane 1**), and undifferentiated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

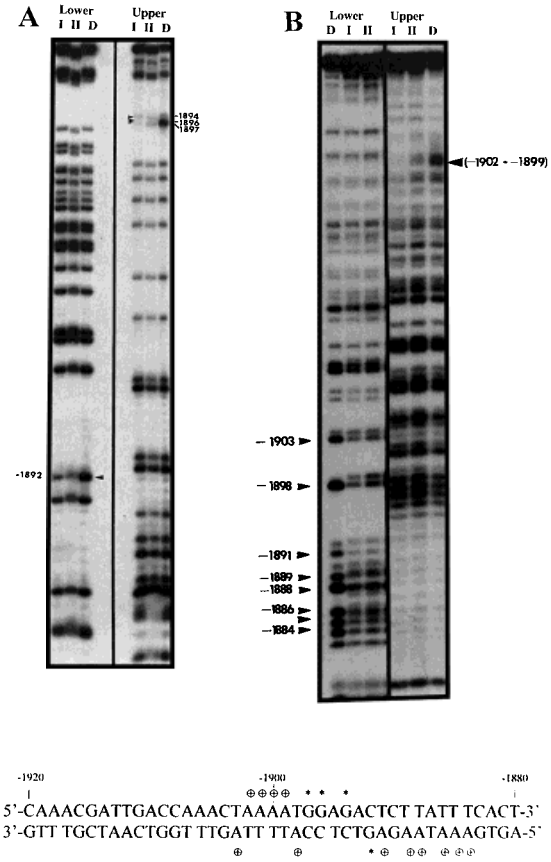

Fig. 3. Identification of a specific nuclear protein binding site by chemical interference assays. **A:** Methylation interference assay was performed by modifying G residues in the 5′-end labeled 131 bp DNA fragment with dimethyl sulfate (DMS). Modified bases that interfere with protein bindings are indicated by arrows. I, band I protein-DNA complex; II, band II protein-DNA complex; D, methylated DNA fragment. **B:** Carboxylation interference assay performed and analyzed as in (A) with diethyl pyrocarbonate (DEP) that modifies the A residues. **C:** A summary of results from both chemical interference assays on the DNA sequence from −1920 to −1880. Nucleotides that contact both I and band II proteins are indicated by * from methylation interference assay, and ⊕ from carbethoxylation interference assay.

tion by the nuclear proteins, we also performed carbethoxylation interference assay. Three adenines on the non-coding strand and four adenines on the coding strand showed protection from carbethoxylation by bound proteins (Fig. 3B). Figure 3C summarizes the bases identified by the two chemical interference assays. Obviously, band I protein and band II protein had the same base contacts within a 15 bp region from −1905 to −1891.

Based on the results of interference assays, we synthesized a 21 bp oligonucleotide from −1905 to −1891 (Fig. 4A). This oligonucleotide retained the same specific binding activity of the nuclear proteins as the 131 bp fragment (Fig. 4B, lane 1). The binding could be competed out by adding a nonradiolabeled 131 bp DNA fragment (Fig. 4B, lane 2). When critical bases at positions −1901, −1899, and −1896 were replaced by cytosines, the resulting mutant oligonucleotide (24*-mer) lost its ability to bind both nuclear proteins (Fig. 4C, lane 2). The binding sequence was thus identified as

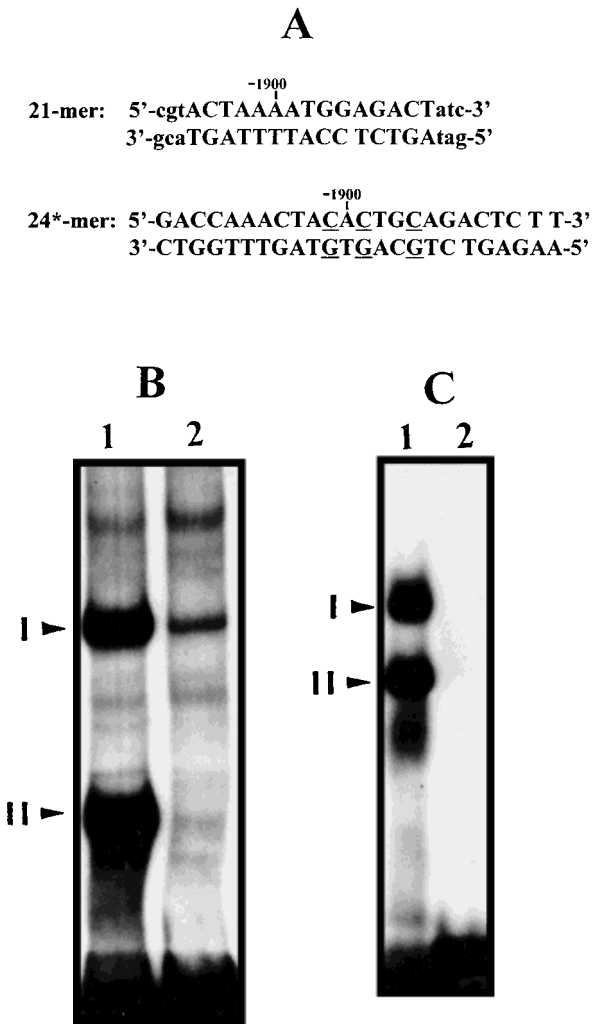

Fig. 4. DAS element retains the binding specificity with nuclear extracts from diffrentiated F9 cells. **A:** A 21-mer double stranded oligonucleotide (−1905 to −1891, DAS) were synthesized according to the chemical interference assays. 24*-mer is a synthesized mutant oligonucleotide in which bases at position −1901, −1899, and −1896 were replaced by cytosines (underlined). **B:** Gel mobility shift assay shows that the 21-mer retained the binding specificity with nuclear proteins from differentiated F9 cells (**lane 1**), and the binding could be competed out by cold 131 bp DNA fragment (**lane 2**). **C:** Mutations of three critical nucleotides in 24*-mer abolished the binding ability to both the band I and band II nuclear proteins from differentiated F9 cells (**lane 2**), as compared to the 21-mer (**lane 1**).

5′-ACTAAAATGGAGACT-3′. We have designated it as the differentiation-associated sequence (DAS) because of the band II protein binding during F9 cell differentiation.

Characterization of DAS-Binding Proteins

It has been extensively documented that many transcription factors are present as dimeric forms, either as a homodimer or as a heterodimer. Nuclear extracts from differentiated F9 cells always bind to the DNA fragment in two forms, a larger band I complex (DAP-I) and a faster migrating band II (DAP-II) complex. Co-appearance of these two proteins even on the minimal required sequence (i.e., the DAS) suggested that DAP-I could be a complex of DAP-II. To analyze the relationship between DAP-I and DAP-II binding proteins, we used CHAP to dissociate protein-protein interactions before or after incubating nuclear extracts with the DNA probe and analyzed the protein-DNA complexes by gel mobility shift assays. When concentration of CHAP was increased to more than 0.5%, the amount of DAP-I protein bound to the DNA fragment started to decrease (Fig. 5). However, there was no apparent effect on the DAP-II protein binding at any concentration of CHAP. The same results were obtained regardless of whether detergent was added before or after incubating the nuclear extracts with the DNA probe (data not shown). Since we did not observe any increase in the intensity of DAP-II-DNA complex after the addition of CHAP, we excluded the possibility that DAP-I was a homo- or heterodimer of DAP-II. However, because we failed to detect any new protein binding bands after the addition of the detergent it is still possible that DAP-I is a protein complex and that it requires protein-protein interaction for binding to DAS. We therefore concluded that DAP-I is either a single protein or a protein complex that is not related to DAP-II protein.

Activation of DAP-II Protein by RA During F9 Differentiation

The presence of DAP-II in differentiated F9 cells raised the possibility that expression of DAP-II protein could be regulated during F9 cell differentiation. Nuclear extracts were therefore prepared from F9 cells treated with RA on different days and used for DAS-binding assays. As shown in Figure 6A, specific DAP-II protein complex was initially detected on day 1 after the treatment. Expression of this protein reached a peak on the 4th day and stayed at a high level until day 8, after which it decreased dramatically and remained at a low level. DAP-I protein, on the other hand, was expressed at a constant level throughout differentiation. Endogenous AFP mRNA was also determined in parallel by Northern blot analysis. As shown in

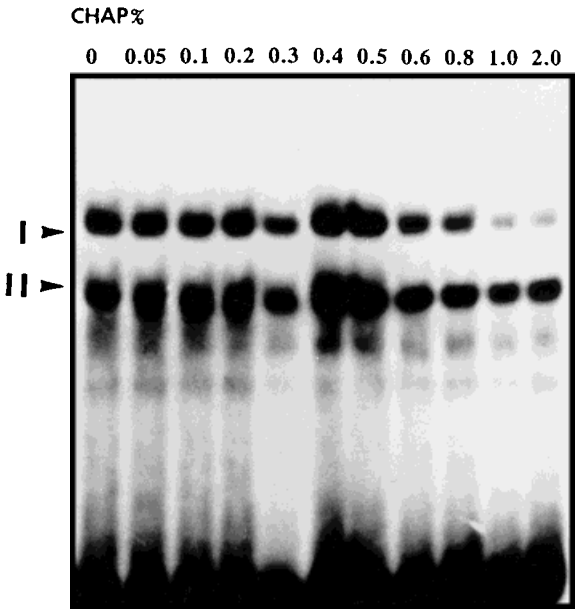

Fig. 5. DAP-1 is not a protein complex formed with DAP-II. Increased concentration of zwitterionic detergent CHAP was used to disrupt protein-protein interactions either before or after protein-DNA complex was formed. The effect of CHAP on binding of nuclear proteins to the DAS element was determined by gel mobility shift assay.

Figure 6B, AFP mRNA started to appear after 4 days of treatment with RA and the level of AFP mRNA continued to rise to almost a maximum at day 6. Thus, the expression of DAP-II protein is regulated by RA, and occurs prior to AFP gene transcription.

DISCUSSION

Mouse F9 cells, the pluripotent stem cells of teratocarcinoma, provide an attractive system to study transcriptional regulation in response to extracellular stimuli [Martin et al., 1990]. Treatment of F9 stem cells with RA converts these cells into a new cell type that resembles primitive extraembryonic endoderm in the mouse embryo [Strickland and Mahdavi, 1978]. It leads to the activation of a set of genes that are also expressed in fetal liver, including AFP [Scott et al., 1984; Soprano et al., 1984]. 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]. Usually retinoic acid response elements (RAREs) can be found on the promoters of these genes. Three RARE-like sequences have been identified in the 5′-flanking region of the rat AFP gene [Liu and Chiu, 1994; Liu et al., 1994a,b]. Since the AFP transcripts are initially detected 4 days after the treatment with RA (Fig. 6B), the relatively late induction of AFP expression argues against the idea that the AFP gene is a primary target for activated retinoid receptors. It also implies that a hierarchy of steps is involved in activating the AFP gene in F9 cells during RA-induced differentiation. The F9 embryonal carcinoma cell system allows us to identify transcriptional regulators that are involved in the step by step de novo activation of the AFP gene.

To generate a system to study AFP gene expression in F9 cells, we constructed a CAT reporter plasmid (pAFPCAT) whose expression was under the control of the 7 kb 5′-flanking region of the rat AFP gene. This plasmid construct can be used to detect gene expression that mimics the endogenous AFP gene during differentiation induced by RA. This plasmid was stably transfected into F9 cells. The activation pattern of the CAT gene was identical to the expression pattern of endogenous AFP gene during RA-induced differentiation (Fig. 1). Cis-elements in this 7 kb region that are responsible for RA-activated AFP expression were identified using a series of deletion mutants. Previous data demonstrated that deletion of the sequences from −7 to −3 kb resulted a gradual decrease in AFP gene activity [Muglia and Rothman-Denes, 1986; Widen and Papaconstantinou, 1986; Godbout et al., 1988; Vacher and Tilghman, 1990; Wen et al., 1991]. These results suggest that the enhancer sequences were not restricted to a certain area of the gene, but rather were distributed over a wide range of the 5′-flanking region. Although the plasmid containing 2.6 kb of the 5′-flanking region (p2.6AFPCAT) had less CAT activity compared to the full length pAFPCAT, it was still sufficient for transcriptional stimulation of AFP by RA (Fig. 1B). However, deletion of 700 bp nucleotides from the 5′-end of the p2.6AFPCAT (resulting in plasmid p1.9AFPCAT) caused a dramatic decrease in CAT activity in differentiated F9 cells (Fig. 1B), indicating the existence of cis-elements in this region that are important for AFP expression. In this study, we identified a cis-element located from −1905 to −1891 (5′-ACTAAAATGGAGACT-3′) that responded to

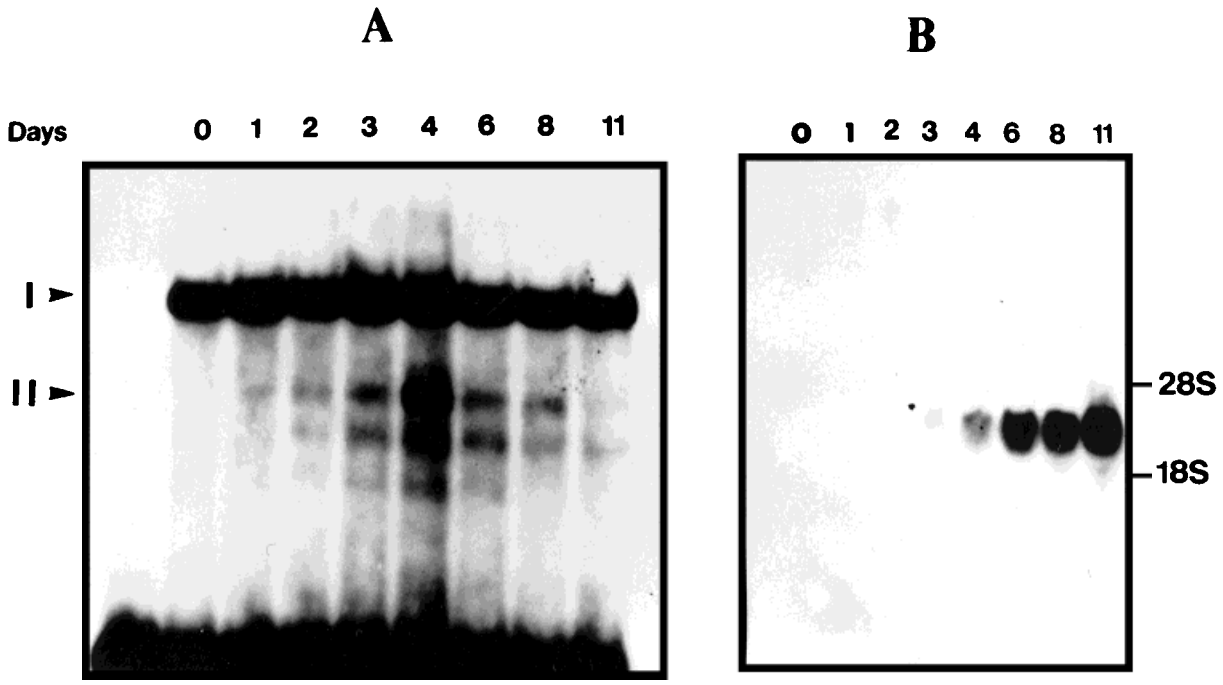

Fig. 6. Expression of DAP-II protein is regulated by RA during F9 cell differentiation. **A:** F9 cells were treated with 5×10^{-8} M RA for different days as indicated and harvested to extract nuclear proteins or total RNA. Four μg of nuclear extracts from each day was analyzed for DAP-I and DAP-II expression by gel mobility shift assay. 0 day represented undifferentiated F9 cells. **B:** 20 μg of total RNAs from each day were analyzed by Northern blot and the samples were shown to be equally loaded upon examining the 28S and 18S rRNA on ethidium bromide stained gel. A Pst I restricted DNA fragment of AFP clone pRAF87 was used as hybridization probe to detect AFP mRNA.

RA treatment, and designated it as a differentiation-associated sequence (DAS) for the unique appearance of its binding protein (DAP-II) during the course of RA-induced F9 differentiation (Fig. 6A). This demonstrated that DAS and its binding proteins were functional in regulating AFP expression. Although DAP-I protein expression remained constant regardless the differentiation status of F9 cells, the expression of DAP-II was regulated during F9 differentiation induced by RA (Fig. 6A). Since DAP-II was induced prior to any detectable AFP mRNA and accumulated to a maximum on day 4 (when endogenous AFP mRNA started to accumulate), we speculate that the gene encoding DAP-II could be a primary and an early response gene to RA. Subsequently DAP-II conveys RA's effect by directly participating the regulation of AFP expression.

A strong enhancer located near DAS at position −2845 to −2095 in rat AFP 5′-flanking region has been identified by Belanger et al. [1993]. This enhancer consists of several enhancer modules within a chromatin hypersensitive domain. Decreased in CAT activity following deletion of the region from −2611 to −1855 was due to the loss of several enhancer elements in this region. A similar enhancer has also been identified in mouse AFP regulatory region [Godbout et al., 1988]. The enhancers, however, seemed to be equally active regardless of AFP expression level because: 1) enhancer binding proteins were found in both undifferentiated and differentiated F9 nuclear extracts (data not shown), as well as in extracts from both newborn and adult rat liver nuclei [Bernier et al., 1993]; 2) Two of the three distal enhancers have been demonstrated to direct AFP expression constitutively in yolk sac, fetal liver, and fetal gut in transgenic mice [Hammer et al., 1987; Millonig et al., 1995]; and 3) Chromatin structure analysis of the gene throughout development also implies that mouse enhancers retain biological functions even after the transcription of AFP gene is repressed [Godbout and Tilghman, 1988]. One model to explain the postnatal suppression of AFP transcription involves a dominate negative regulator located upstream of the AFP promoter, between −1010 to −250 bp; it is only active in adult liver

[Millonig et al., 1995]. Our study also raises the possibility that: AFP regulation could be attained by the concerted actions of both enhancers and repressors. Maximal expression would be achieved when the functions of repressors were suppressed thus allowing enhancers to dominate. The DAS element may represent one such regulatory element. The DAP-I protein is a likely inhibitory protein since it is present at all differentiation state of F9 cells. DAP-II is induced by RA during differentiation may replace DAP-I binding to DAS and deactivate its inhibitory effect on AFP expression. The present study identifies the DAS as a cis-element in AFP gene regulatory region and implies that this element could regulate AFP transcription either positively or negatively, depends on the differentiation stage of F9 cells. However, further analysis of how DAS and its associated proteins participating in regulation of AFP gene expression, relies on cloning or purification of both DAP-1 and DAP-II proteins.

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