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AUF1-Like Protein Binds Specifically to DAS *Cis*-Acting Element That Regulates Mouse α-Fetoprotein Gene Expression

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Abstract Alpha-fetoprotein (AFP) is one of the major serum proteins in the early life of mammals. We have previously identified a novel *cis*-acting element designated as DAS at the 5′-flanking region of the *AFP* gene and demonstrated that the DAS sequence can be specifically recognized by nuclear protein DAP-II in AFP-producing hepatoma cells and retinoic acid (RA)-induced AFP-producing F9 cells. In this study, we used DNA affinity chromatography to purify the DAP-II proteins from the nuclear extracts (NE) of RA-treated F9 cells. The purified DAP-II complex mainly contained five proteins, with molecular weights of 45, 42, 32, 30, and 20 kDa, respectively. The identification of these proteins was determined by MALDI-TOF mass spectrometric analysis and a database search. These proteins were found to belong to the AUF1 RNA-binding protein family. Protein (30 kDa), one of five proteins in an isolated DAP-II complex, was matched with amino acid sequence highly similar to muAUF1-3. The expression of this protein is inducible by RA, and the pattern of the protein expression is the same as DAP-II proteins in F9 cells after treatment with RA during differentiation. Our results suggest that the 30-kDa protein is a novel isoform of AUF1 family and is the main component of the DAP-II complex that binds to the DAS sequence. J. Cell. Biochem. 98: 1257–1270, 2006.

Key words: α-fetoprotein; RA-induced F9 cell differentiation; DAP-II-specific DNA-binding proteins; AUF1; *cis*-acting element

Alpha-fetoprotein (AFP) is an oncodevelopmental protein that is expressed in the embryonic yolk sac and fetal liver at high levels, and in the fetal gut at a low level [Cote and Chiu, 1984; Chen et al., 1997]. The synthesis of AFP decreases dramatically shortly after birth [Chiu et al., 1979; Huang et al., 1985]. However, the expression of the *AFP* gene can be reactivated in the adult liver upon inducing liver cell proliferation during liver regeneration, in certain hepatocellular carcinogenic states, and in hepatoid located in other tissues [Chen et al., 1997].

The expression of the *AFP* gene is at the level of transcriptional initiation by the interaction of transacting transcription factors with *cis*-acting DNA elements. In the past two decades, experiments with transgenic mice and DNA transfection studies have demonstrated that the *AFP* gene has a large and complex transcription control region. Three upstream enhancer regions have been defined. They are several hundred nucleotides long and are active in most tissue, although at various levels [Godbout et al., 1986; Muglia and Rothman-Denes, 1986; Widen and Papaconstantinous,

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1986; Hammer et al., 1987; Godbout et al., 1988; Wen et al., 1991]. The AFP promoter, covering a region of ~250 bp, is regulated by tissue-specific activators, such as HNF1 [Feuerman et al., 1989], C/EBP [Zhang et al., 1990], Nkx2.8 [Apergis et al., 1998], and FTF [Galerneau et al., 1996], and also by ubiquitous factors like NF1 [Berier et al., 1993]. AFP promoter is active in liver but not in non-liver cell lines, and therefore exhibits tissue-specificity [Scott et al., 1984; Henriette et al., 1997]. In addition, it has been suggested that a region in the first intron of AFP participates in the control of gene activity [Scohy et al., 2000]. The repressor loosely defined as a 600-bp region located between −250 and −850, is required for complete postnatal AFP repression; and the absence of this element results in the continued expression of AFP transgenes in the adult liver [Vacher and Tilghman, 1990].

F9 cells are one of the most extensively studied types of embryonic carcinoma cells that originate from a teratocarcinoma [Artzt et al., 1973]. In response to several types of physical and chemical stimuli, these non-differetiated, 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 retinoic acid (RA), a vitamin A derivative that exerts potent effects on many physiological processes 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 [Strickland and Mahdavi, 1978; Dong et al., 1990]. The capacity of F9 cells to differentiate in a control manner in culture provides a useful system for studying certain aspects of mammalian development and differentiation, and for investigating the mechanism by which RA affects *AFP* gene expression.

Previously, our laboratory [Dong et al., 1989] constructed a CAT reporter plasmid (pAFP-CAT) 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 was found to be important in AFP induction [Chen et al., 1999]. Subsequent analysis identified a functional sequence (−1,905 to −1,891, 5′-ACTAAAATG-GAGACT-3′) that differentially binds nuclear proteins (DAP-I or DAP-II) from undifferentiated and differentiated F9 cells. This sequence was designated as differentiation-associated sequence (DAS) for its specific binding activity of differentiation-associated protein (DAP) during the course of RA-induced F9 differentiation. 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 are 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. Our previous results strongly suggest 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 further characterize DAP-II protein(s) in this study, we purified the DAP-II protein(s) from nuclear extracts (NE) of RA-treated F9 cells by DNA affinity chromatography. We found that the purified DAP-II complex contained five proteins, which were identified by 2DE/MALDI-TOF mass spectrometric analysis as members of the AUF1 protein family, and that four of these could be recognized by anti-AUF1. A new 30-kDa AUF1-like protein which we designated p^{30AUF1} is one of the five proteins found in purified DAP-II complex. The protein p^{30AUF1} can bind to the DAS sequence and is an RA-inducible protein in F9 cells. We considered this protein to be the main component of the DAP-II complex, and suspected that it might also play an important role in regulating *AFP* gene expression during F9 cells differentiation induced by RA. This is the first report indicating that AUF1 or similar proteins are associated with the regulation of *AFP* gene expression.

MATERIALS AND METHODS

Cell Culture and Treatment

Undifferentiated mouse F9 EC cells (purchased from ATCC, Manassa, VA) were maintained 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 50 nM RA. Cell aggregates were resupplied daily with the appropriate medium and RA concentration until harvesting. For controls, undifferentiated F9 cells were grown under similar conditions but without the addition of RA.

Southwestern Analysis

Southwestern blotting of protein-DNA interaction was carried out according to a procedure described by Tully and Cidlowski [1993]. NE were separated on 12% SDS-PAGE gel and transferred to PVDF membrane. After renaturing the protein in a buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.0, 20 mM EDTA, 0.1 mM DTT, 4 M Urea), the membranes were blocked and incubated with ^{32}P-labeled DAS DNA to permit DNA binding of the proteins in the presence of 5% non-fat dry milk to minimize non-specific binding. Following washing four times in the same binding buffer and rinsing briefly in 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, the dried membranes were exposed to X-ray film at -80°C for at least 24 h under an intensifying screen.

Preparation of Nuclear Extracts for DAP-II Proteins Purification

NE were prepared as described by Zhang et al. [1991]. All manipulations were performed in the cold, and all buffers and centrifuges were chilled to $0 \approx 4^\circ$C.

Purification of DAP-II Proteins

DAS-DNA affinity resin was prepared as described by Kadonaga and Tjian [1986]. Two chemically synthesized 29-bp oligonucleotides containing nucleotides of complementary sequence to the DAS and having 4-bp cohesive ends (Fig. 2A) were annealed, subjected to 5′-phosphorylation, and then concatamerized in reactions in using T4 DNA ligase. The concatamerized DNA was coupled to cyanogen bromide-actived sepharose 4B.The DAS DNA coupled resin was used to purify DAP-II proteins as follow: whole NE were dialyzed into buffer Z (50 mM KCl, 20 mM HEPES (pH 7.9), 42 mM NaCl, 5 mM $MgCl_2$, 15% Glycerol, 0.1% NP-40) at 4°C for 3 h. After dialyzing, the NE was centrifuged for 10 min in 12,000*g* at 4°C and the supernatant was incubated for 10 min on ice in the presence of 60 μg/mg NE poly (dI-dC) and then centrifuged for 10 min in 12,000*g* at 4°C. The supernatant was loaded onto the DAS oligonucleotide affinity column pre-equilibrium with buffer Z. After loading, the column was washed extensively with buffer Z, and DAP-II activity subsequently eluted with a step gradient of 0.3 and 0.4 M KCl in buffer Z. Fractions of 2 ml were collected and assayed for DAP-II-binding activity by electrophoretic mobility shift assay (EMSA) after concentration and exchange of buffer C by YM-10 filter (Millpore). Active fractions were pooled, dialyzed in buffer Z, and applied to the same column.

Gel Mobility Shift Assay

Synthesized double-stranded normal and mutated DAS probes (Fig. 4A) were labeled with [r-^{32}P] ATP (6,000 Ci/mM; Amersham) and T4 DNA polynucleotide kinase. Binding reactions were carried out in 20 μl of binding buffer (10 mM HEPES (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT) containing 10,000 c.p.m of the ^{32}P-labeled probe, 1 μg of poly (dI-dC), 5 μg of NE, or 2–5 μl purified DAP-II solution. For competition experiments, unlabeled oligonucleotides were mixed with the NE and ^{32}P-labeled probe at the same time. After 20 min at room temperature, binding reaction mixture was resolved on non-denaturing 6% polyacrylamide gels (acrymide/bisacrylamide ratio 30:1). Gels were run at 200 V and at 4°C in 0.5× TBE buffer.

SDS-PAGE and Western Blotting

The protein samples were subjected to electrophoresis using 12% SDS-polyacrylamide gels and transferred to PVDF membrane with semi-dry transfer unit (Amersham). The membranes were blocked with 5% non-fat dry milk for 1 h and then incubated with polyclonal anti-human AUF1 antibody (Upstate) for 4 h and with horseradish peroxidase-conjugated secondary antibody for another 1 h prior to detection of antibody-reactive proteins with a chemiluminescent reagent (ECL, Amersham Pharmacia Biotech).

2D Electrophoresis and Trypsin Digestion of Gel-Separated Proteins

Purified DAP-II proteins were dissolved in solubilization buffer (9.5 M urea, 4% CHAPS,

2.5 mM DTT), 2% IPG buffer pH 3–10 (Amersham Bioscience), and 0.01% bromphenol blue, then loaded onto 13-cm IPG Drystrips (Amersham Bioscience). Samples were focused for 64,000 Vh at 20°C on an IPGhor (Amersham Bioscience). After focusing, IPG Drystrips were incubated in SDS-PAGE sample buffer containing 10 mg/ml DTT. They were then incubated in sample buffer containing 25 mg/ml iodoacetamide. Proteins were then separated on a 12% SDS slab gel and visualized by silver stain. DAP-II proteins were excised from the silver-stained 2D gel, cut into 1-mm squares, and placed into a 1.5-ml tube.

Protein Identification by Mass Spectrometry

Mass information of the peptides was obtained using a MALDI-TOF mass spectrometer Voyager-DE STR from PerSeptive Biosystems (Framingham, MA) according to our previous paper [He and Chiu, 2003]. The peptide mixture (1 μl) was crystallized with an equivalent volume of freshly prepared cyano-4-hydroxycinnamic acid matrix solution (10 mg/ml; Sigma) in 50% ACN/0.1% TFA onto the MALDI target plate. TOF spectra were acquired over the m/z range of 700–3,000 Da under the delayed extraction and reflector mode. All spectra were internally calibrated upon acquisition using two of the trypsin autodigest fragments (1,153.5741 and 2,163.0570 m/z peaks). Trypsin autolytic products and other contaminants (i.e. from keratins or matrix) were excluded from the peak list used for the database search. The resultant peptide masses were then searched against Swissprot and NCBI non-redundant databases using the MSFit software (http: //prospector.ucsf.edu/ucsfhtml3.4/msfit.htm; Protein Prospector, UCSF, San Francisco, CA). The MSFit search was performed with the following parameters: all molecular weight range, all p*I* range, oxidation of methionine, acetylation of N-terminus, carboxyamidomethylation of cysteine, and phosphorylation of serine, threonine, and tyrosine. Positive identification of the protein was assigned only if at least four peptide masses matched a particular hit in the database within a mass tolerance of 50 p.p.m. or lower, matched peptide masses were evenly distributed throughout its amino acid sequence, and the identified proteins' molecular weights and p*I* approximately corresponded to experimental values, with some exceptions.

RESULTS

Induction of DAP-II Proteins by RA During F9 Differentiation

Using normal synthesized 27-bp double-stranded DAS DNA as a probe (Fig. 4A), we performed the gel mobility shift assay with NE of F9 cells treated with RA on different days. As shown in Figure 1A, two specific DAS DNA-binding protein bands were identified. According to our previous studies [Chen et al., 1999], a predominant retarded protein complex is DAP-I and the faster migrating protein complex is DAP-II. The DAP-I proteins were detected in both undifferentiated and differentiated F9 cells, whereas, DAP-II protein(s) are only found in differentiated F9 cells. A specific DAP-II protein complex was initially detected on day 1 after RA treatment and expression of this protein increased and reached a peak on the sixth day after RA treatment. Accordingly, F9 cells treated with RA for 6 days were collected and used to purify DAP-II protein(s).

Using Southwestern blotting, we determined the size of proteins that bind to DAS sequence. Nuclear proteins from F9 cells treated with RA for various time periods were separated by SDS-polyacrylamide electrophoresis. Proteins were then blotted to PVDF membrane. After

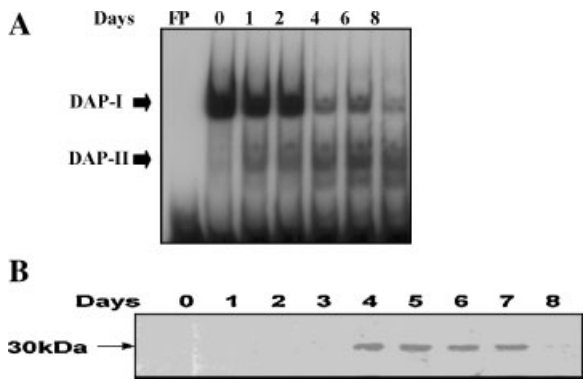


Fig. 1. **A**: Expressions of DAP-I and DAP-II proteins in F9 cell during RA-induced cell differentiation. F9 cells were treated with 50 nM RA for various days as indicated and harvested to extract nuclear proteins. Five micrograms of NE from each sample was analyzed for DAP-I and DAP-II expression levels by gel mobility shift assay. 'P' indicated free probe and 0–8 days represent the number of days treated with RA. **B**: Southwestern blotting analysis of DAS oligonucleotide-binding protein. Nuclear proteins were isolated from F9 cells treated with RA for various days as indicated and separated by SDS-polyacrylamide gel electrophoresis. Proteins were blotted to PVDF membrane and interacted with ^{32}P-labeled DAS oligonucleotide after renaturation. Twenty-five micrograms of NE from each sample was analyzed.

renaturation, the membrane-bound proteins were interacted with ^{32}P-labeled DAS and exposed to X-ray film. As shown in Figure 1B, a 30-kDa DNA-protein complex formed in F9 NE treated with RA from days 2 to 8. No such complex was detected in the NE without RA treatment. This result is consistent with our previous data [Chen, 1997], and suggested that the 30-kDa protein existing in NE of F9 cells treated with RA specifically binds to DAS sequence.

Purification of DAP-II Proteins by DNA Affinity Chromatography

We chemically synthesized two 29-bp complementary DAS DNA strands, which contain DAS core sequences and 5′-protruding end (Fig. 2A). These complementary DNA strands were annealed, 5′-phosphorylated, and then ligated to form oligomers of DAS oligodeoxynucleotides ranging from 3-mers to 70-mers (Fig. 2B). The ligated DAS oligonucleotides were then covalently coupled to cyanogen bromide-actived sepharose 4B. This resin was stable for at least 6 months and could be reused >20 times without any detectable loss of protein-binding capacity.

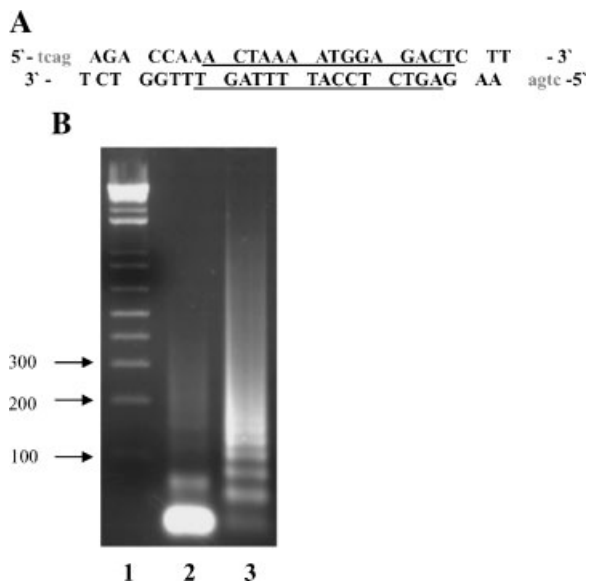


Fig. 2. Polymerization of complementary 5′-phosphorylated DAS oligodeoxynucleotides. **A**: Synthetic sequence of DAS oligodeoxynucleotides. **B**: Analysis of the ligated DAS DNA by 2% agarose gel electrophoresis. The DNA oligomers were visualized by ethidium bromide staining under ultraviolet light. **Lane 1**, linear double-stranded DNA molecular size markers (sizes of selected fragments are given in base pairs); **lane 2**, double-stranded DAS DNA before ligation; **lane 3**, oligodeoxynucleotides DAS after ligation. The size of multimers is indicated by arrows.

To purify the DAP-II proteins, F9 cells were treated with RA for 6 days, harvested and used for nuclear proteins extraction. After they were dialyzed against the buffer Z on ice for 3 h, white pellets were removed by centrifugation. Poly (dI-dC) was added to the sample solution as a competitor and the solution was then stood on ice for 10 min to block non-specific DNA-binding proteins. After centrifugation, supernatant was loaded onto DAS affinity resin column. The column was washed with buffer Z and then eluted with the buffer Z containing 0.2–1.0 M KCl. The DAS-binding activity of all fractions eluted from the column was monitored by EMSA. As displayed in Figure 3A, most of proteins with DAS-binding activity were eluted from the affinity matrix at fraction 0.3 and 0.4 M KCl. A low level of DAS-binding activity was also detected at 0.2 M KCl fraction. By comparing SDS-PAGE of fraction 0.2, 0.3, 0.4, and 1.0 M KCl, we found that fractions 0.3 and 0.4 contained more than one protein (see Fig. 3B), indicating that some non-specific binding proteins were still present in the fractions. We, therefore, mixed the proteins of fractions 0.3 and 0.4 M KCl eluants and then performed the second affinity chromatography by the same procedure. The results of the second purification are shown in Figure 3B. The eluants of 0.3 and 0.4 M KCl contained 30-kDa protein and proteins with molecular weights of 45, 42, 32, and 20 kDa. These proteins were designated as p45, p42, p32, p30, and p20 according to their molecular weight. However, the EMSA profiles demonstrated that the major DAS-binding activity was contributed by p30 protein (Fig. 3C), indicating that p45, p42, p32, and p20 proteins probably non-specifically co-purified in DNA affinity column chromatography.

Purified DAP-II Proteins Bind Specifically to DAS DNA Sequence

Although gel mobility shift assay had shown that the purified p30 protein could bind to DAS sequence, we carried out a competition experiment to further confirm its binding specificity. We chemically synthesized a mutant DAS (mDAS) DNA, in which the adenine bases at positions −1,901 and −1,899, and a guanine base at position −1,896 were replaced by cytosine (Fig. 4A). In the competition experiment, 50-fold excesses of unlabeled normal

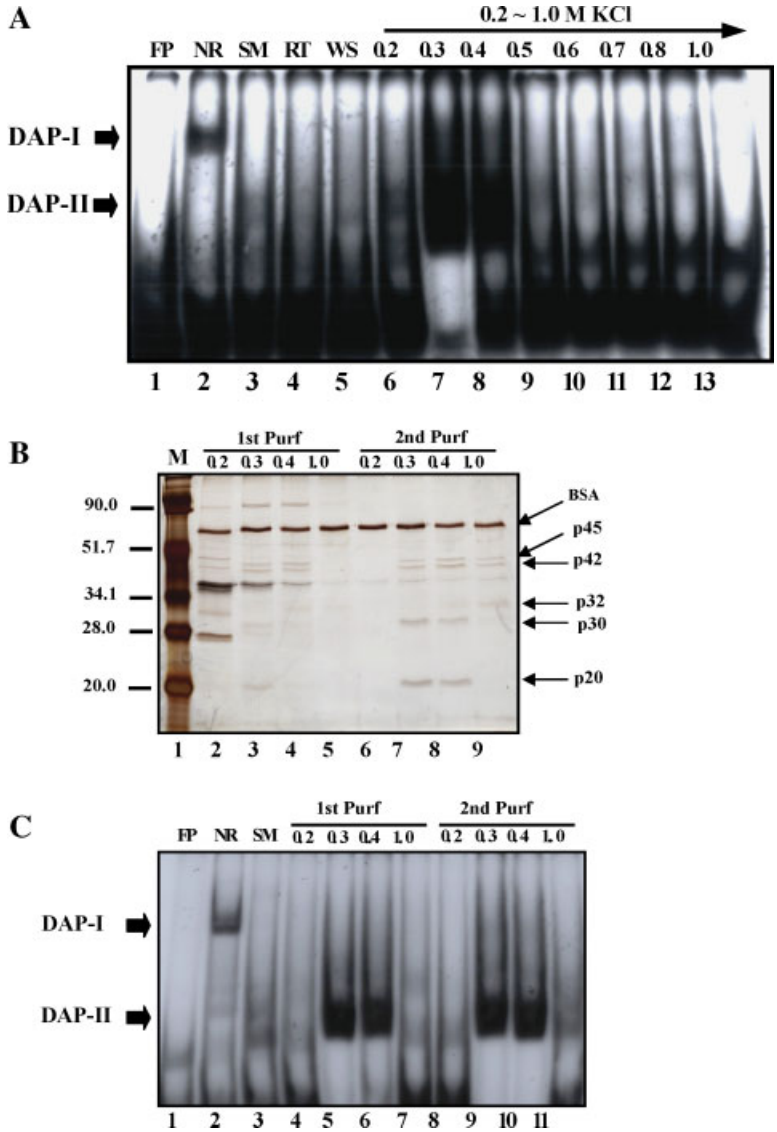


Fig. 3. **A**: DAP-II purification on DAS-DNA affinity column. NE of RA-induced F9 cells was applied to a first cycle of purification on DAS-DNA affinity column. The bound proteins were eluted with a 0.2–1.0 M KCl gradient across eight fractions. ^{32}P-labeled DAS double-stranded oligonucleotides were used as a probe for EMSA. FP, free probe; NR, non-RA-treated F9 NE; SM, starting material of RA-treated F9 NE; RT, runthrough (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 YM-10 filter (Millipore) prior to EMSA analysis. All samples were loaded 2 ul, except NR and SM that loaded 5 ug protein each. **B**: SDS-PAGE analysis of protein samples eluted from affinity chromatography. **Lanes 2–9** show the fractions eluted from different KCl concentrations at two purification cycles. Twenty microliter of each protein sample were loaded on 12% SDS-PAGE gel. Bove Serum Albumin (BSA) displayed on the gel was added to each fraction prior to concentration step to prevent non-specific absorption by YM filter. 'M' is molecular marker. **C**: EMSA analysis on DAS-binding activity of protein fractions. **Lanes 4–7** represent the fractions eluted from 0.2, 0.3, 0.4, and 1.0 M KCl in the first cycle purification, and **lanes 8–11** represent the fractions in the second purification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

or mDAS DNAs were used to compete with ^{32}P-labeled normal DAS probe. As shown in Figure 4B, unlabeled normal DAS can efficiently compete out the binding of the purified proteins to the ^{32}P-labeled DAS probe, whereas, unlabeled mDAS has no effect on the binding of purified proteins. This data confirm that the purified proteins are DAP-II proteins that can specifically bind to DAS sequence.

Identify the Purified DAP-II Proteins by 2D Electrophoresis and MALDI-TOF MS Analysis

To identify the DAP-II complex proteins, samples of 0.3 and 0.4 M KCl eluants from

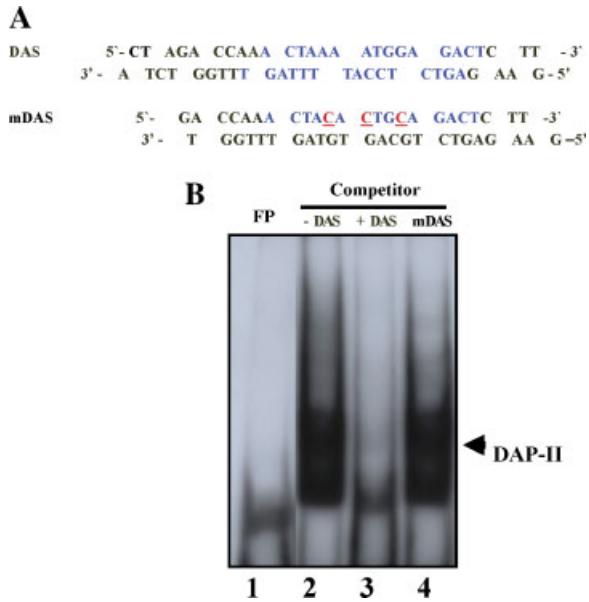
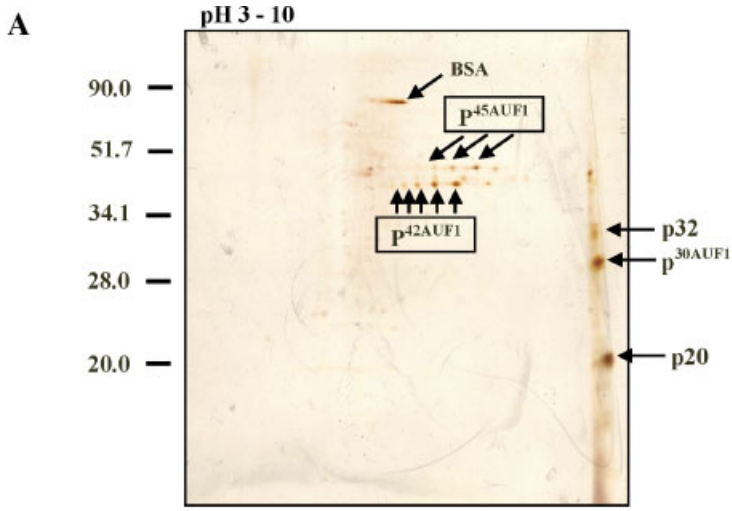


Fig. 4. Competitive EMSA analysis of the affinity purified DAP-II proteins from F9 NE. **A**: A double-stranded oligonucleotides (−1,905 to −1,891, DAS) and mutant DAS (mDAS) were synthesized. The nucleotides at position −1,901, −1,899, and −1,896 were replaced by cytosines (underlined) in mDAS. **B**: Labeled double-stranded DAS probe was incubated with 2 ul purified DAP-II sample in the absence of competitors (**lane 2**), or in the presence of 50-fold excess (w/w) of unlabeled double-stranded DAS (**lane 3**) or mDAS (**lane 4**). **Lane 1** is free probe. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

second purification were concentrated and then exchanged to the dehydration buffer. The protein samples were then resolved on 2DE gel with IEF on 13 cm Immobiline Drystrip, pH ranges of 3–10 in the first dimension, and 12.5% SDS-PAGE in the second dimension. Silver staining was used for protein detection on the gel because it is one of the most sensitive visualization methods and is compatible with MALDI-TOF mass spectrometry. As shown in Figure 5, two major and several protein spots were seen on the 2D gel in addition to the BSA protein (BSA was added to the samples as a carrier). They can be classified into five groups according to their molecular weights; 45, 42, 32, 30, and 20 kDa. These results are all consistent with the results of 1D SDS-PAG (Fig. 3B). From the location of proteins on the 2D gel, p32, p30, and p20 are basic proteins while p45 and p42 were resolved into four or more spots with different p*I* ranging from 5 to 8. All protein spots were excised and subjected to tryptic digestion, and peptide mass fingerprint spectra were recorded on a MALDI-TOF mass spectrometer. By using the ProteinProspector website (http//prospector.ucsf.edu) MSFit, p30, p42, and p45 were identified as heterogeneous nuclear ribonucleoproteins (hnRNPs), which are mRNA-binding proteins with important roles in the biogenesis of mRNA. Protein p30 was matched precisely to the mouse AUF1 of the hnRNP family. The p32 and p20 failed to match because of weak MS signals. Figures 5B–D show the MALDI-TOF mass spectrum of the p30 tryptic peptides and amino acid sequences that matched to the mouse AUF1. The correlation of the matches is very significant, with a maximum difference in mass of <11 p.p.m. between the submitted and matched peptides. Nineteen percent of the matched protein sequence is covered by these peptides. The GenBank accession number for the matched p30 protein is AAA64654, and mRNA is from the clone muAUF1-3 submitted by Ehrenman et al. [1994a].

DAP-II Proteins Contain AUF1

From the MS analysis, p45, p42, and p30 belong to the hnRNP family, and p30 was identified as a member of AUF1 family. To confirm the results, Western blotting was used to analyze the samples of F9 NE and the purified DAP-II proteins with anti-AUF1 antibody. As shown in Figure 6A, four bands could be identified with the anti-AUF1 antibody in NE from RA-untreated or RA-treated F9 cells as compared with the positive Hela NE control. They are probably four AUF1 isoforms including p^{45AUF1}, p^{42AUF1}, p^{40AUF1}, and p^{37AUF1}. Two additional bands (p32 and p30) were identified in the NE of RA-treated F9 cells (Fig. 6A). In the purified DAP-II protein samples, we found that total four positive bands on the Western membrane could be identified; they are p45, p42, p32, and p30 (Fig. 6B). Apparently, the proteins p45 and p42 are AUF1 proteins p^{45AUF1} and p^{42AUF1} isoforms. AUF1 proteins have been shown to be phosphorylated [Nakamaki et al., 1995]. We noted that both the p45 and p42 proteins were resolved into four or more spots on 2DE gel (Fig. 5A), suggesting that both proteins were phosphorylated. The other two proteins on blot membrane are located at molecular weights of 30 kDa and 32 kDa. We designated the p30 protein as p^{30AUF1}, because it has been precisely matched to muAUF1-3 by MSFit and recognized by anti-AUF1 antibody in Western blot. As shown in Figure 6B, both p^{30AUF1} and p32



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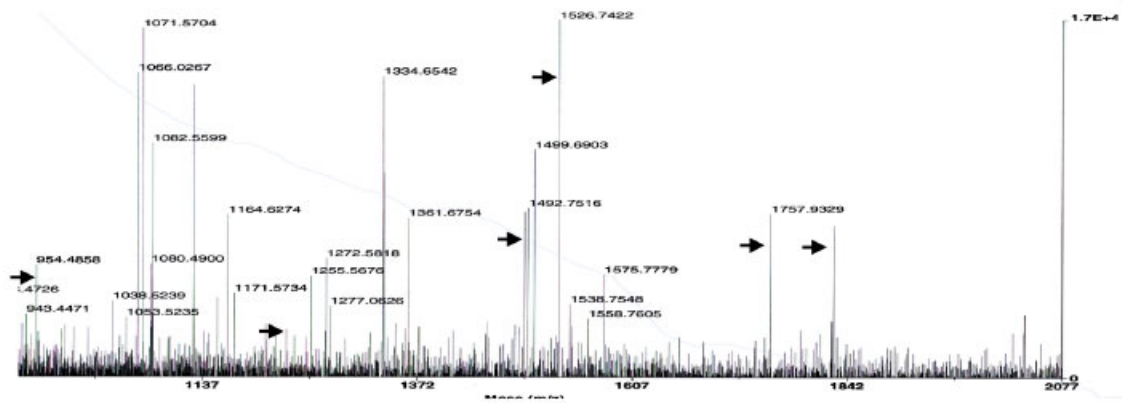

C

	m/z submitted	MH+ matched	PPM	start	end	Peptide Sequence
1.	954.4858	954.4757	11	55	63	(R)HTEAAAAQR(E)
2.	1237.6005	1237.6135	-10	106	115	(R)SRGFGFVLFK(E)
3.	1488.7503	1488.7586	-5.6	153	166	(K)IFVGGLSPDTPEEK(I)
4.	1526.7422	1526.7352	4.6	55	67	(R)HTEAAAAQREEWK(M)
5.	1757.9329	1757.9438	-6.2	153	168	(K)IFVGGLSPDTPEEKIP(E)
6.	1824.8452	1824.8283	9.2	193	206	(R) GFCFITFKEEEPVK(K)

Fig. 5. **A**: Silver-stained 2DE profile of purified DAP-II proteins from F9 cells treated with RA. The purified proteins of two purification cycles at 0.3 M and 0.4 M KCl fractions of 125 mg NE were combined and concentrated. The concentrated sample was exchanged to rehydration buffer by YM-10 filter prior to 2DE analysis. **B**: The results of ProteinProspector search. Thirty tryptic peptides resulting from MALDI-TOF mass spectrometric analysis were submitted to search for protein ID in the NCB protein database using the ProteinProspector website. **C**: Six submitted peptide masses were found to match precisely with virtually tryptic peptide masses of AUF1. The table shows the masses of corresponding peptides, including the difference in mass between the submitted and matched peptides (P.P.M). Matches within 50 p.p.m. are considered significant. **D**: The amino acid sequences of AUF1 (underlined) that matched to p30 tryptic peptides. These peptides covered 19% of the matched protein sequence. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

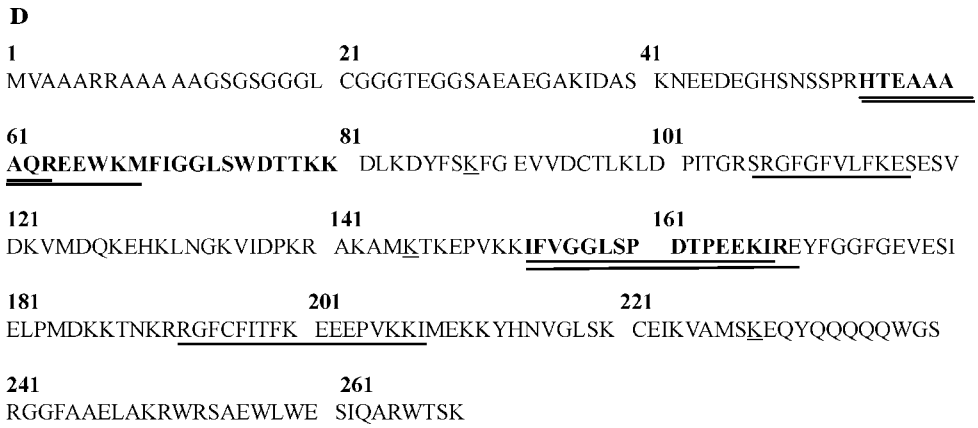

Fig. 5. (*Continued*)

bind to DAS sequence, indicating that they are the DAS-binding proteins in DAP-II complex.

The AUF1-Like Proteins can be Induced by RA

The p^{30AUF1} and p32 proteins were undetectable in NE of F9 cells without treatment with RA (Fig. 6C). This implies that these proteins are RA-inducible. We next performed Western blotting to detect the expression of these proteins in F9 cells treated with RA at various time periods. As we expected, these two proteins were induced by RA and could be detected on day 4 after RA treatment, whereas, the other four AUF1 isoforms (p^{45AUF1}, p^{42AUF1}, p^{40AUF1}, and p^{37AUF1}) had no visible change during the process of RA-induced F9 cell differentiation. Although very weak bands of these two proteins were detected in comparison with the AUF1 isoforms, the protein contents increased according to the length of RA treatment and reached a maximum around day 6. This was consistent with the EMSA data shown in Figure 1.

DISCUSSION

Our laboratory [Chen et al., 1999] has identified a *cis*-element located at the 5′-flanking region of the *AFP* gene that responded to RA treatment, and designated it as a DAS sequence. By using EMSA, specific DAS-binding protein(s), DAP-II, is detected in NE isolated from RA-induced differentiated F9 cells but not in undifferentiated cells [Chen et al., 1999]. In this study, DAP-II was purified by DAS-DNA affinity column chromatography and identified by 2DE/MALDI-TOF analysis.

To purify the DAP-II proteins, we used sequence-specific DNA affinity resin in which multiple copies of the DAS sequences were covalently attached to sepharose CL-4B. NE of the RA-treated F9 cells for 6 days, which displayed the 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 eluant fractions of 0.3 and 0.4 M KCl. Five silver-stained protein bands, p45, p42, p32, p30, and p20 were found in these fractions on SDS-PAGE gel.

Southwestern blotting analysis showed that one DNA-protein complex found at molecular weight of 30-kDa protein in F9 NE and this was consistent with our previous data [Chen, 1997]. The expression of 30-kDa protein was activated in F9 cells during differentiation induced by RA (Fig. 1B). The 30-kDa protein identified by Southwestern analysis strongly resembles the p30 of the purified proteins from DNA affinity column chromatography. On the 2D gel, p30 was found as a single spot with alkali p*I* and this protein was identified as a member of AUF1 family (see Fig. 5). Western blotting demonstrated that p30 could be recognized by anti-AUF1 antibody. AUF1 was shown to exist in four isoforms, namely p^{45AUF1}, p^{42AUF1}, p^{40AUF1}, and p^{37AUF1}.

AUF1 (also called hnRNP D0) is a member of the protein family that contains two highly conserved RNA-binding domains (RBDs) in tandem and a Gly-rich region in the C-terminus [Kajita et al., 1995]. 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]. Amino acids sequence of the p30 can be

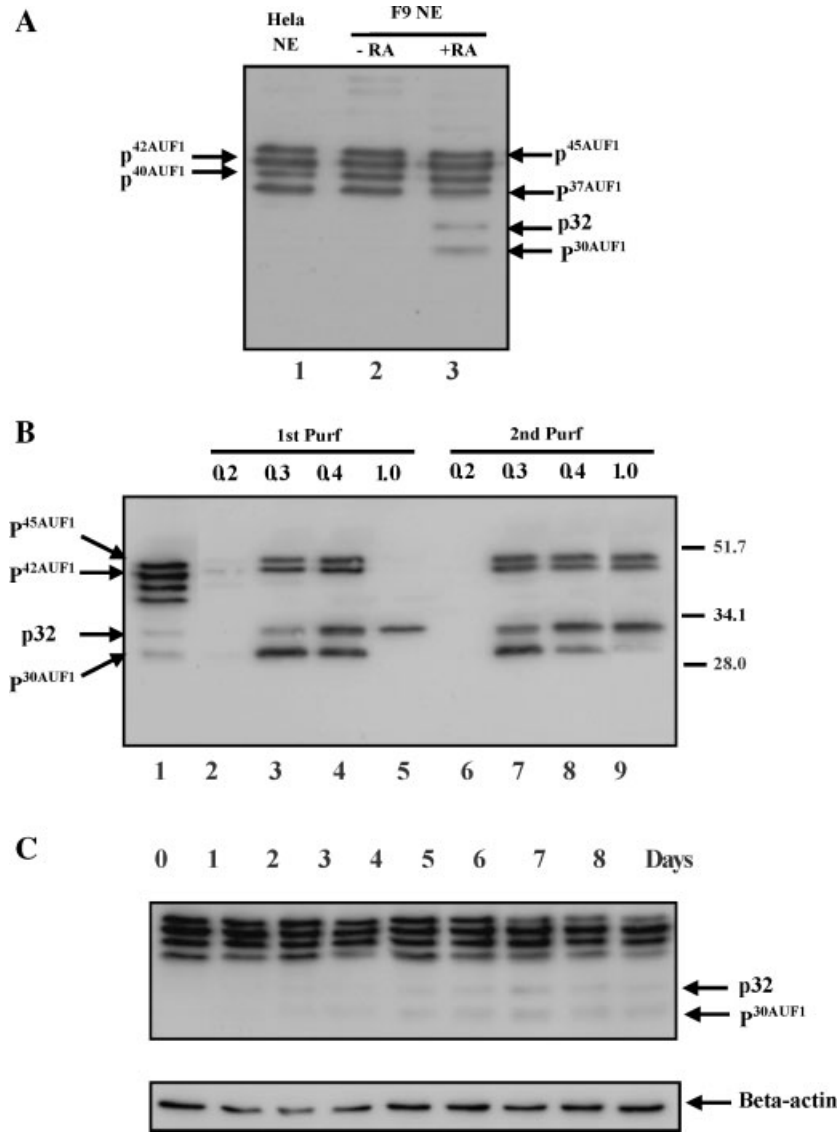

Fig. 6. Western blot analysis of AUF1 proteins in the F9 NE (**A**) and the purified DAP-II (**B**). The samples were fractionated by 12% SDS-PAGE gel and transferred to PVDF membrane. ECL was used to visualize the immunoreactive protein bands after incubation with anti-AUF1 polyclonal antibody. A: NE from F9 cells treated with RA (**lane 3**) or non-RA treated cells (**lane 2**), and positive control from the NE of Hela cells (**lane 1**). Twenty micrograms of each protein sample was applied to electrophoresis. B: The protein fractions from the two cycles of affinity chromatography were analyzed. Twenty microliter of each sample from fractions 0.2, 0.3, 0.4, and 1.0 M KCl were applied. **Lane 1** shows start material (SM) and addition of 20 μg protein. **C:** The expression patterns of 32 K and p^{30AUF1} proteins during F9 cells differentiation induced by RA. Equal amounts of F9 NE from the different days after treatment with RA were resolved by SDS-PAGE and then transferred to PVDF membrane. Anti-AUF1 antibody was used for immunoblotting. The blot was then stripped and reprobed with an antibody specific for beta-actin to verify equal loading. Zero days represents non-RA treated and undifferentiated F9 cells.

deduced from clone muAUF1-3, which was isolated from a murine fetal cDNA library [Ehrenman et al., 1994a]. Amino acid sequence comparison revealed a significant amino acids homology between p30 and other AUF1 isoforms. The optimal alignments are shown in Figure 7A. Two RBDs (RBD1 and RBD2) and a Gly-rich region in the C-terminus are 100% homologous. These results strongly suggest that the p30 is a new isoform of the AUF1 protein family and we have accordingly designated it as p^{30AUF1} according to its molecular weight on SDS-PAGE.

However, p^{30AUF1} has several characteristics not found in all other known AUF1 isoforms. Firstly, N-terminal 29 amino acids of AUF1

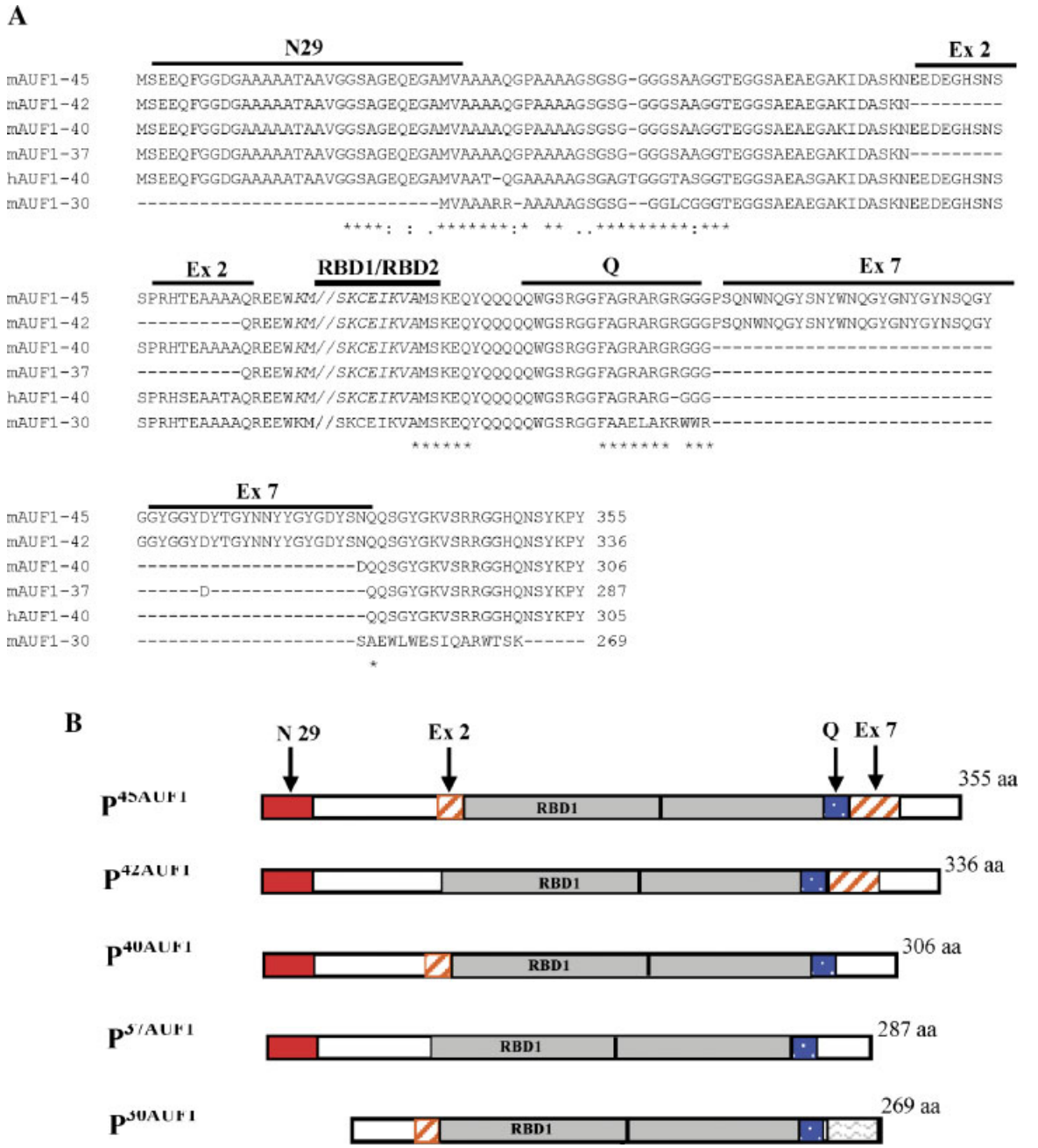

Fig. 7. **A**: Amino acid homologies between deduced $p30^{AUF1}$ and other AUF1 members from mice and humans. Alignment of amino acid sequence was computed by the Swissprot online multiple alignment software. For the proteins used for comparison, identical amino acids are indicated by asterisks (*) and gaps are indicated by dashes (–). Their common structural features are: conserved two consecutive RNA-binding domains, RBD1 and RBD2, a Gly-rich domain (Q). The 19-amino acid insert encoded by exon 2 (Ex 2) is at the N-terminal part of RBD1 in the $p30^{AUF1}$, $p40^{AUF1}$, and $p45^{AUF1}$ isoforms, while the 49-amino acid insert encoded by exon 7(Ex 7) is at the C-terminal part of the $p42^{AUF1}$ and $p45^{AUF1}$ isoforms. All AUF1 proteins except p^{30AUF1} contain a 29-amino acid sequence located at the N-terminal (N29). The C-terminal region of p^{30AUF1} is also different from all other AUF1 isoforms. The GenBank accession numbers for mouse p^{30AUF1} and human p^{40AUF1} are AAA64654 and NP_002129, respectively. The other amino acid sequences for mouse p^{37AUF1}, p^{40AUF1}, p^{42AUF1}, and p^{45AUF1} are obtained from the Swissprot database, and their accession numbers are Q60668-4, Q60668-3, Q60668-2, and Q60668-1, respectively. **B**: Schematic diagrams of five mouse AUF1 isoforms. The two RNA-binding domains (RBD1 and RBD2) are depicted as two gray-shaded regions at the central part of the protein molecules. A carboxy-terminal 49-amino acid encoded by exon 7 (Ex7) presents as a red hatched bar in p^{45AUF1} and p^{42AUF1}. A 19-amino acid encoded by exon 2 (Ex2) presents as grebe hatched bars at the amino terminus of p^{45AUF1}, p^{40AUF1}, and p^{30AUF1}. All the five proteins contain the Gly-rich region (a blue bar Q). A 29-amino acid sequence located at the N-terminal (a red bar N29) and C-terminal region (a brown cross bar for p^{30AUF1} 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.]

were not found to be present at the p^{30AUF1} probably because of difference in the start codon of translation [Ehrenman et al., 1994a]. This sequence was observed in all the other four AUF1 isoforms, and is also 100% identical in human and murine samples (Fig. 7B). Secondly, p^{30AUF1} contained a 19-aa insert at the N-terminal, which was encoded by exon 2 of the *AUF1* gene, and was not present in murine p^{42AUF1} and p^{37AUF1}. Thirdly, 24 amino acids at C-terminal revealed the greatest divergence between the p^{30AUF1} and other AUF1 isoforms (Fig. 7A). Based on these observations, we conclude that the p^{30AUF1} protein is encoded by the *AUF1* gene and is translated at a different start codon than the other known AUF1 isoforms (Fig. 7B).

AUF1 is an abundant, ubiquitous protein that can bind to RNA, as well as, to double- and single-stranded DNA sequences in a 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 protein during RA-induced cell differentiation. Purified DAP-II is mainly composed of AUF1, including p^{45AUF1}, p^{42AUF1}, and p^{30AUF1}, and AUF1-like protein p32. The reasons that the p^{30AUF1} protein was considered to be the major protein of DAP-II that plays a role in DAS DNA-binding are (1) Southwestern analysis showed that 30-kDa protein in F9 NE can specifically bind to DAS sequence, and this is consistent with our previous data [Chen, 1997], (2) in a comparison with SDS-PAGE and EMSA of purified proteins, p^{30AUF1} always accompanied with the DAS-binding activity, (3) the expression pattern of the p^{30AUF1} was the same as DAP-II proteins in F9 cells during RA-induced cell differentiation.

Although the p^{45AUF1} and p^{42AUF1} 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 the both RA-treated and RA-non-treated F9 cells. However, the role of the p^{45AUF1} and p^{42AUF1} isoforms in regulating *AFP* gene expression requires further study. The interaction between protein p32 with anti-AUF1 suggested that it is an AUF1-like protein or a protein with common immunoreactive epitope. P32 also shares some similarities with p30 (p^{30AUF1}). Its expression can be induced by RA, and the expression pattern is the same as p^{30AUF1}. It is one of DAS DNA-binding DAP-II proteins.

Previously, AUF1 proteins have been shown to be associated with mRNA processing [Zhang et al., 1993; Nakamaki et al., 1995]. Recent evidence suggests that AUF1 proteins can bind to double-stranded DNA and regulate gene expression. The molecular mechanism of human AUF1 (hnRNP D0B) binding to dsDNA has been studied [Tolnay et al., 1999, 2000]. This mechanism may be applied to explain the molecular basis of interaction between mouse p^{30AUF1} and double-stranded DNA. Tolnay et al. [2000] found that 24 amino acids of the C-terminus adjacent to the RBD2 of p^{40AUF1} impart sequence-specific DNA binding and this peptide sequence is present in all hnRNP proteins and was found to bind double-stranded DNA. We also found that this peptide exists in p^{30AUF1} indicating that p^{30AUF1} is capable of binding to dsDNA. Nineteen amino acids at N-terminal of p^{42AUF1} encoded by exon 2, are critical for the activity of transactivation [Tolnay et al., 2000, 2002]. This 19-amino acids sequence was also identified in p^{30AUF1} (Fig. 7). The presence of the exon 2-encoded region seems to determine relative affinity for binding double- and single-stranded DNA [Tolnay et al., 2000], whereas isoforms that lack it, have high affinity for AU-rich RNA sequence [Dreyfuss et al., 1993; Chen and Shyu, 1995]. Experiments have demonstrated that the sequence of 29 amino acids at N-terminal (N29) has no effect on the binding of double-stranded DNA. The N29 sequence is not present in p^{30AUF1} because of difference in translation start codon. We, therefore, conclude that p^{30AUF1} is a distinct member of the AUF1 family, whose expression can be induced in response to RA treatment.

Recently, several laboratories have shown that AUF1/hnRNP D can function as a transcription factor. AUF1 induces the Epstein–Barr virus C promoter in co-operation with a viral protein, EBNA2 [Fuentes-Panana et al., 2000]. E2BP, also known as AUF1, has been shown to bind to double-stranded DNA and

stimulates the hepatitis B virus enhancer II, as well as, the thymidine kinase promoter [Tay et al., 1992]. hnRNP D forms heterodimers with nucleolin in the B-cell-specific transcription factor LR1, which is implicated in transcriptional regulation of c-myc and EBVP Fp promoter activities [Gorlach et al., 1994; Demaria and Brewer, 1996; Hanakahi et al., 1997; Dempsey et al., 1998]. Human 42-kDa hnRNP is involved in the transcriptional regulation of the human *CR2* gene [Tolnay et al., 1997] and its coprecipitation with the TATA-binding protein (TBP) complex, suggesting that it may function as a transcription factor [Tolnay et al., 1999].

In conclusion, the data of this study demonstrated that p^{30AUF1} is the main component of the DAP-II complex. Since DAS sequence is an important element in activation of *AFP* gene expression in F9 cell during RA-induced differentiation [Chen, 1997; Chen et al., 1999], p^{30AUF1} may, therefore, play an important role in the regulation of *AFP* gene expression. It has been shown that DAP-II (p^{30AUF1}) was present in many AFP-producing cells, but not in non-AFP-producing cells [Chen, 1997; Chen et al., 1999]. We believe that p^{30AUF1} is a new AUF1 isoform capable of binding to double-stranded DNA and regulating the expression of the *AFP* gene. Further study to elucidate the mechanism of p^{30AUF1} in regulating gene expression is in progress.

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