Journal of Cellular Biochemistry 72:411–422 (1999)

Dual Posttranscriptional Targets of Retinoic Acid-Induced Gene Expression

Shehnaaz S. Manji,[1] Richard B. Pearson,[2] Marshall Pardee,[2] Vasilias Paspaliaris,[3] Anthony d'Apice,[3] T. John Martin,[1] and Kong Wah Ng[1]*

[1]Department of Medicine, University of Melbourne, St. Vincent's Institute of Medical Research, Fitzroy, Victoria 3065, Australia
[2]Trescowthick Research Laboratories, Peter MacCallum Cancer Institute, St. Andrews Place, East Melbourne, Victoria 3002, Australia
[3]Department of Clinical Immunology, St. Vincent's Hospital, Fitzroy, Victoria 3065, Australia

Abstract Retinoic acid-induced differentiation of the pre-osteoblastic cell line, UMR 201, is associated with a marked increase in the proficiency of posttranscriptional nuclear processing of alkaline phosphatase mRNA. In this study we attempted to correlate the posttranscriptional actions of retinoic acid with changes in phosphorylation, or abundance of spliceosome components, or both. Treatment with retinoic acid for periods of ≤4 h resulted in dephosphorylation of nuclear U1 70K protein without affecting its abundance. Peptide mapping showed that U1 70K dephosphorylation was related to the disappearance of one specific phosphopeptide out of four major U1 70K phosphopeptides. A twofold decrease in mRNA expression of an isoform of alternative splicing factor that inhibits splicing was also observed over the same period. Tumor necrosis factor-α, which enhances the posttranscriptional action of retinoic acid, reduced U170K mRNA expression, while an inhibition of retinoic acid action by transforming growth factor-β was associated with a marked increase in U1 70K mRNA levels. Our results draw attention to the complex interactions between short- and long-term alterations in the abundance and functional status of U1 70K, as well as SR proteins by growth and/or differentiation factors in the regulation of spliceosome formation and function. J. Cell. Biochem. 72:411–422, 1999.

Key words: U1 snRNP; splicing; posttranscriptional nuclear processing; SR proteins; TNF-α; TGF-β; differentiation; alkaline phosphatase gene regulation

Retinoic acid plays a major role in the differentiation of preosteoblasts. In previous studies using a clonal rat preosteoblastic cell line, UMR 201, we have shown that retinoic acid induced the expression of mRNAs normally associated with differentiated osteoblasts, such as alkaline phosphatase (ALP), osteopontin, osteonectin, and pro-α1(I) collagen [Ng et al., 1988; Zhou et al., 1991]. In these cells, we have also demonstrated a posttranscriptional action of retinoic acid in increasing the proficiency of nuclear processing of newly transcribed ALP mRNA. This action of retinoic acid probably plays a dominant role in determining the steady-state level of mature ALP mRNA [Manji et al., 1995; Zhou et al., 1994].

In this study, we examined the hypothesis that retinoic acid may exert its posttranscriptional effects by regulating precursor mRNA splicing. The catalytic reactions responsible for cleavage of introns and the splicing of exons occur within the spliceosome, a complex structure made up of snRNPs functioning cooperatively with non-snRNP splicing factors [Fu, 1995; Sharp, 1994]. U1 and U2 snRNPs bind to pre-mRNA early in the reaction, followed by binding of U4, U5, and U6 snRNPs [Green, 1991; Krainer and Maniatis, 1988]. U1 snRNP contains three specific proteins; the 33-kDa U1 A, the 22-kDa U1 C and the 70-kDa U1 70K proteins [Lührmann, 1988]. snRNPs by themselves are not sufficient to define splice sites. The essential splicing factor, alternative splicing factor/splicing factor 2 (ASF/SF2, also desig-

Contract grant sponsor: National Health and Medical Research Council.

*Correspondence to: Kong Wah Ng, Department of Medicine, University of Melbourne, St. Vincent's Hospital, Fitzroy, Victoria, 3065, Australia. E-mail: k.ng@medicine.unimelb.edu.au

Received 3 July 1998; Accepted 16 September 1998

nated ASF 1) is required for a very early step in spliceosome assembly [Krainer et al., 1990a, b]. It has been proposed that ASF 1 binds the pre-mRNA and helps recruit U1 snRNP to the 5′ splice site [Jamison et al., 1995; Kohtz et al., 1994]. The RNA binding region of ASF-1 interacts with 5′ splice site RNA in a sequence-specific manner [Zuo and Manley, 1994], while interaction between ASF-1 and U1 snRNP occurs through specific protein–protein interactions involving the RS domains of both proteins to form an exceptionally stable complex [Kohtz et al., 1994; Zuo and Manley, 1993]. The RS domain is characterized by several arginine-serine dipeptides. It is a hallmark of a family of splicing factors, the SR proteins, which include ASF-1, SC35, and $U2AF^{65}$, and is also present in the C-terminal portion of U1 70K protein [Fu, 1995].

U1 70K protein exists as a phosphoprotein in vivo and is the only heavily phosphorylated U1 protein in the cell [Woppmann et al., 1990]. The ability of the U1 snRNP-associated protein kinase to phosphorylate both ASF 1 and U1 70K in vitro suggests that the interaction between these two proteins may be regulated during splicing [Tazi et al., 1993; Woppmann et al., 1993]. The state of phosphorylation of U1 70K protein determines its functional status. When U1 70K protein was rendered resistant to the action of phosphatases through thiophosphorylation, splicing was completely inhibited, with no effect on the formation of the mature spliceosome [Tazi et al., 1993], implying that dephosphorylation of U1 70K is important in the initiation of the splicing reaction. By contrast, overexpression of U1 70K protein resulted in inhibition of splicing and nucleocytoplasmic transport of mRNA [Romac and Keene, 1995].

While it is recognized that the regulation of expression and function of spliceosome components that modulate splicing efficiency and splice site selection may result in control of gene expression in a cell- and tissue-specific manner [Fu, 1995], information on whether hormones and growth factors may influence these reactions is scarce. This study focuses on the effects of retinoic acid on U1 snRNP and ASF-1, two components essential to the formation and function of the spliceosome.

MATERIALS AND METHODS

Materials

Minimum essential medium-α modification (α-MEM) was purchased from Life Technologies (Grand Island, NY) Fetal bovine serum (FBS) was a product of Commonwealth Serum Laboratories Ltd (Parkville, Victoria, Australia). All-*trans*-retinoic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human transforming growth factor-β1 (TGF-β) was a gift from Genentech (San Francisco, CA) and recombinant murine TNF-α was purchased from Preprotech (Canton, MA). ^{32}P-orthohosphoric acid (spec act 37,000 KBQ per mCi) was obtained from Dupont/NEN Life Science Products (Boston, MA). All other reagents were of analytic grade obtained from standard suppliers.

Cell Culture

UMR 201 cells were routinely grown in α-MEM containing 10% FBS. Incubation was carried out at 37°C in a humidified atmosphere equilibrated with 5% CO_2 in air.

ELISA

UMR 201 cells were seeded at 1×10^4 cells per well in 96 multiwell dishes containing α-MEM with 10% FBS. After an overnight incubation, medium was changed to α-MEM with 2% A-FBS (vitamin A-depleted FBS) and treated with 1 μM retinoic acid for 2, 4, and 24 h. At the indicated times, medium was aspirated and cells washed with phosphate-buffered saline (PBS) before fixing in acetone–methanol (1:1; v/v) for 5 min at room temperature. The plates were air-dried for 45 min and stored at −20°C.

In the enzyme-linked immunosorbent assay (ELISA), the plates were equilibrated and hydrated with PBS for 5 min at room temperature and then incubated with 5% skim milk powder in PBS as a blocking agent for 1 h at room temperature in a humidified container. The solution was aspirated and cells incubated for 2 h in the humidified container with 100 μl (10 μg) per well of the primary antibody—a serum polyclonal IgG fraction purified with protein G Sepharose beads and obtained from patients with mixed connective tissue disease who have high titer antibodies to U1 RNP. Nonimmune serum was obtained from normal subjects. The IgG fractions were diluted to a final concentration of 100 μg/ml. The plates were washed three times (10 min per wash) with 0.05% Tween in PBS, followed by a 10-min wash in PBS; 75 μl of rabbit anti-human peroxidase-conjugated immunoglobulins (1:1,000; Dako, CA) was added per well and incubated for 1 h before the washing step was repeated. Finally, 100 μl of sub-

strate (O-phenylenediamine dihydrochloride; Sigma) was added to each well for 15 min before the reaction was stopped with 50 µl 4 M H_2SO_4. Absorbance of the colorimetric assay was read at the dual wavelength of 490/630 nm.

Western Blotting

Cytoplasmic and nuclear protein extraction. UMR 201 cells were grown to a density of 5×10^7 cells in 500-cm^2 trays in α-MEM containing 10% FBS. Medium was changed to α-MEM containing 2% A FBS for the indicated incubation times with and without 1 µM retinoic acid. Medium was aspirated and cells rinsed three times with ice-cold PBS. A 1-ml lysis buffer (0.5% Triton X-100 in PBS, pH 7.0) containing a cocktail of protease inhibitors (Protease inhibitor cocktail kit, ICN Biomedicals, CA) was added to the each tray and cells were harvested using a rubber policeman. Cells from each tray were pipetted into, and resuspended in, a 1.5-ml Eppendorf tube, kept on ice for 30 min, centrifuged at 4,000 rpm for 15 min at 4°C, and the supernatant recovered as the cytoplasmic fraction. The pellet containing nuclear extract was resuspended on ice in 0.5 ml 0.1 M NaH_2PO_4, pH 7.0 containing protease inhibitors. The nuclear suspension was sonicated with 3- × 15-s bursts, with 1 min on ice between each sonication, before centrifugation at 13,000 rpm for 20 min at 4°C. The nuclear extract was recovered in the supernatant and stored in 100-µl aliquots at −70°C. Protein content of the nuclear extract and cytoplasmic fraction were determined with the BCA protein assay (Pierce, Rockford, IL).

Immunoprecipitation of U1 RNP-specific proteins. A total of 7.5 µl of primary antibody was added to 200 µg of nuclear protein in a 1.5-ml Eppendorf tube and incubated overnight at 4°C; 40 µl of protein A Sepharose beads in 50% PBS was added and the mixture gently rotated on a rotary mixer for 2 h at room temperature. The primary antibody was obtained from a patient with high titer autoantibody for U1 RNP as described above. Reference human anti-U1 RNP was obtained from Reference Serum Laboratory, Centers for Disease Control (Atlanta, GA). The tube was centrifuged at 13,000 rpm for 30 s and the supernatant aspirated. The beads were washed four times with 500 µl of wash buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 500 mM NaCl, 1% Triton × 100, 0.02% sodium azide), resuspended and spun for 30 s between washes. The beads were then washed twice with PBS. A total of 20 µl of sample buffer (consisting of 0.06 M Tris with 10% glycerol (v/v) and 2.3% SDS (v/v), pH 6.8, was added to the beads and boiled for 5 min to dislodge the immunoprecipitated proteins. Immunoprecipitated U1 RNP-specific proteins from the cytoplasmic fraction were similarly obtained. Samples were loaded onto a 4–15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Bio-Rad, Hercules, CA). The proteins were separated by electrophoresis at 200 V for 40 min and transferred onto nitrocellulose membrane (Hybond C-extra, Amersham). The membranes were probed with serum obtained from a patient with high-titer U1 RNP autoantibody and detected with ^{125}I-labeled protein A.

Phosphorylation of U1 snRNP-Specific Proteins

UMR 201 cells were subcultured in 150-cm^2 plates in α-MEM with 10% FBS to 80% confluency. The medium was aspirated and cells were rinsed with serum-free DMEM. A total of 20 ml of phosphate-free DMEM with 2% A FBS was placed in each tray and 2 mCi of ^{32}P-orthophosphoric acid added to the medium before incubation overnight at 37°C. The next day, cells were treated with 1 µM retinoic acid, 0.6 nM TNF-α or 1 ng/ml TGFβ. At the indicated times, tissue culture plates were placed on ice, radioactive medium aspirated, the cells rinsed twice with ice-cold PBS before harvesting with a rubber policeman in 300-µl lysis buffer containing a cocktail of protease inhibitors. The cytoplasmic and nuclear fractions were obtained as described above. The nuclear pellet was resuspended in 200 µl of 0.1M Na_2PO_4, pH 7.0.

A total of 7.5 µl of primary antibody was added to the nuclear (200-µl) and cytoplasmic (100-µl) fractions and incubated at 4°C overnight to permit immunoprecipitation of phosphorylated U1 RNP. The proteins were separated in a gradient gel as described above. The gel was wrapped in Gladwrap™ and subjected to autoradiography for 1 h at −70°C. After autoradiography, the phosphorylated bands were excised and ^{32}P-labeled protein quantified by Cerenkov counting using the ^{3}H channel. Results from four separate experiments were pooled.

Phosphoamino Acid Analysis and Phosphopeptide Mapping

^{32}P-labeled U1 70K protein was electroeluted from the slice of gel in 100 mM NH_4HCO_3, 0.1%

(w/v) SDS using a model 422 Electro Eluter (Bio-Rad) and precipitated by the addition of 100% (w/v) trichloroacetic acid to 15% final concentration. The resultant protein pellets were washed twice with ethanol/ether (1:4, v/v) in preparation for partial acid hydrolysis or tryptic digestion.

Phosphoamino acid analysis. ^{32}P-labeled U1 70K protein pellet was redissolved in 50 µl 5.7 M HCl and incubated for 45 min at 110°C before lyophilization. The hydrolysate was relyophilized twice from 1 ml H_2O and resuspended in pH 1.9 buffer (1 vol formic acid–3 vol acetic acid–36 vol H_2O). Each sample was mixed with 0.5 µg of standard phosphoamino acids before separation by thin-layer electrophoresis (TLE) at pH 1.9 and 1,500 V for 60 min. The plates were dried and phosphoamino acids visualized by ninhydrin staining.

Tryptic phosphopeptide mapping. ^{32}P-labeled U1 70K protein pellet was redissolved in 10% (v/v) acetonitrile, 100 mM NH_4HCO_3, and digested with two additions of 0.5-mg sequencing grade, alkylated trypsin (Promega, Madison, WI) for 36 h at 37°C. After the digestion, tryptic peptides were lyophilized and relyophilized twice from 1 ml H_2O and resuspended in pH 1.9 buffer. Two dimensional tryptic phosphopeptide analysis was performed on cellulose thin layer plates essentially as described [Van der Geer and Hunter, 1994] with electrophoresis at pH 1.9 and 1,300 V for 35 min and chromatography in phosphochromatography buffer (1 vol glacial acetic acid–3.33 vol pyridine–4 vol H_2O–5 vol n-butanol). Plates were air-dried and labeled phosphopeptides visualized using a PhosphorImager and ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

Polymerase Chain Reaction Amplification of Reverse-Transcribed mRNA

First-strand cDNA was synthesized from 2 µg of total RNA by incubating for 1 h at 42°C with 15 U of AMV reverse transcriptase (Promega) after oligo (dT) priming. *Taq* DNA polymerase (Boehringer Mannheim) was added to 2 µl of the reaction mixture in a Perkin-Elmer (Norwalk, CT) 480 thermal cycler to amplify PCR products using specific primers. A total of 20 µl of each specific PCR reaction mixture was run on a 2% agarose gel and transferred to nylon. Products were authenticated by probing with ^{32}P-labeled oligonucleotides corresponding to sequences within each specific PCR product.

U1 RNP 70K gene. The primers were designed to encompass exons 7 and 8 [Hornig et al., 1989]. The 5′ primer (5′-TGGGGCAGATGTGAATATCAGG-3′) represents nucleotides 468–489 of exon 7 and a 3′ primer (5′-CCACCTCGAAGCTCCTCTTTTC-3′) represents nucleotides 776–797 of exon 8. The annealing temperature was 60°C, and amplification was carried out for 25 cycles to yield a 330-bp PCR product.

Alternative splicing factor/splicing factor 2 (ASF 1) gene. The 5′ primer (5′-TGATGTTTACCGAGATGGCACTG-3′) represents nucleotides 450–472 of the human gene while the 3′ primer (5′-AATAGCGTGGTGATCCTCTGCTTC-3′) represents nucleotides 689–712. The annealing temperature was 55°C, and amplification was carried out for 25 cycles. The oligonucleotide (5′-CAGTTTCTCCCTCATGAGAT-3′), which is complementary to nucleotides 543–560, is specific for the isoform ASF-1, while another oligonucleotide (5′-TCCATTGAAAGATCTAAGCT-3′), which hybridizes to nucleotides 160–179 contained in an intronic sequence that is differentially spliced, is specific for the isoform ASF-3 [Ge et al., 1991].

Alkaline phosphatase gene. Primers were designed to encompass exons 4 and 5 of the alkaline phosphatase gene [Zhou et al., 1994]. The 5′ primer (5′-AGAAAGAGAAAGACCCCAGTT-3′) represents nucleotides 1–21 of exon 4 and a 3′ primer (5′-CTTGGAGAGAGCCACAAAGG-3′) represents nucleotides 97–116 of exon 5.

GAPDH. To ensure equal loading of RNA in each sample, the reverse-transcribed material was also amplified using oligonucleotide primers specific for rat GAPDH [Tso et al., 1985].

Statistical Analysis

Statistics were performed using Student's t-test and expressed as mean ±SD.

RESULTS

Western Blotting for U1 70K Protein

The abundance of U1 snRNP within the cytoplasm and nuclei of UMR 201 cells was examined by Western blotting, using a U1 snRNP autoantibody present in the serum of a patient with mixed connective tissue disease as primary antibody. This antibody was authenticated with a human U1 snRNP reference serum obtained from the Reference Serum Labora-

tory, Centers for Disease Control (Atlanta, GA) (Fig. 1a). Autoantibodies to U1 snRNP which recognizes the specific U1 70K, A and C proteins are found in virtually all patients with mixed connective tissue disease, a clinical condition with features of systemic lupus erythematosus, scleroderma, and polymyositis [Tan, 1989]. The results shown in Figure 1 are representative of three to five separate experiments and demonstrate the presence of the U1 70K protein in the nuclei and cytoplasm of UMR 201 cells (Fig. 1b). Although the anti-U1 snRNP antibody identifies all three U1 specific proteins [Tan, 1989], U1 A and U1 C proteins were not detected in the Western blots probably because of their low abundance. Treatment with 1 μM retinoic acid for periods of ≤4 h did not alter the amount of U1 70K protein in the nuclei of treated UMR 201 cells compared to control cells (Fig. 1c).

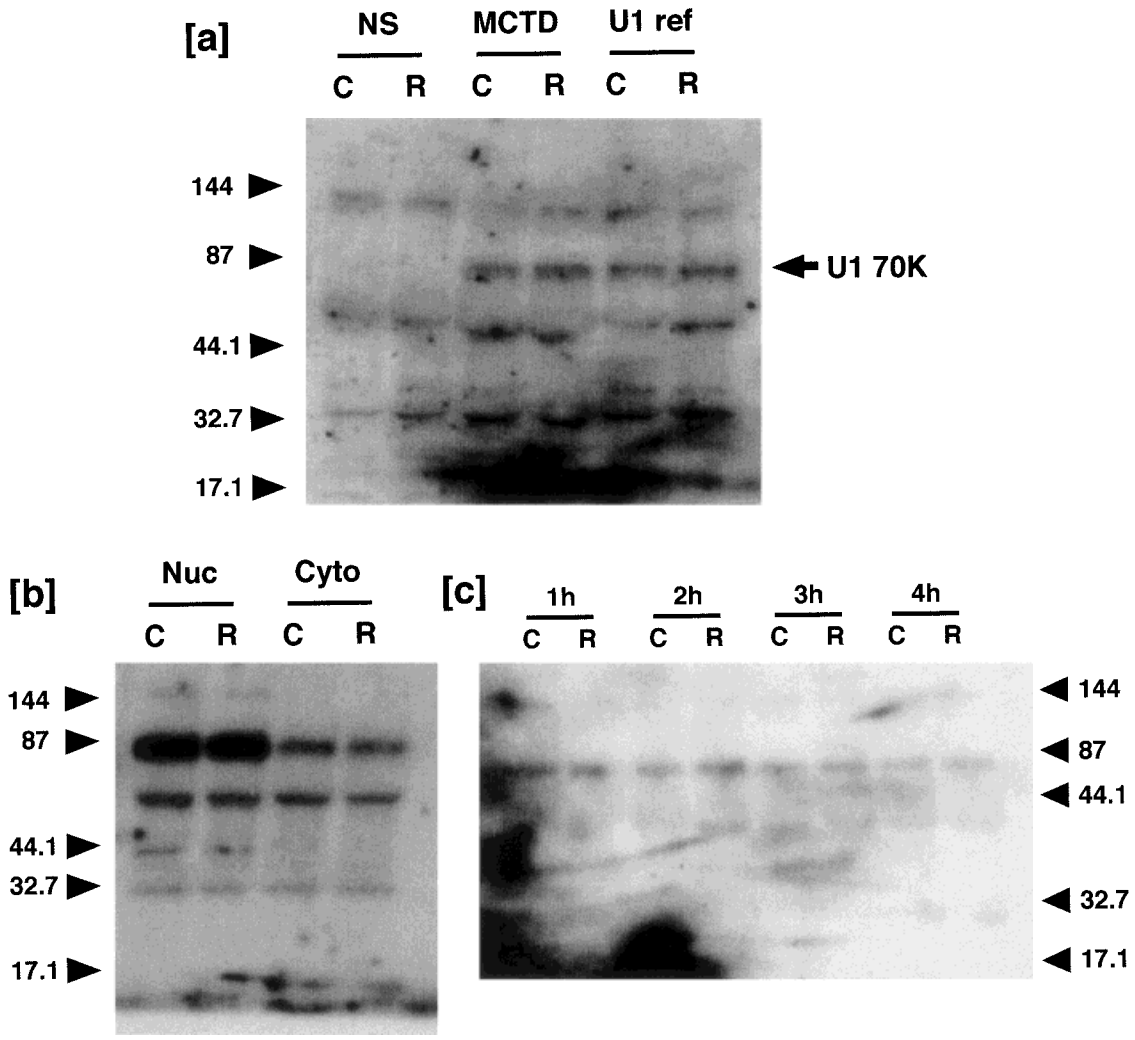

Fig. 1. Detection of U170K protein by Western blotting. Nuclear and cytoplasmic protein extracts from UMR 201 cells were prepared with and without treatment with 1 μM retinoic acid for 4 h. **a:** Nuclear extracts were immunoprecipitated with a human U1 reference serum (U1 ref) or serum from a patient with mixed connective tissue disease (MCTD) with a high titer of anti-U1-RNP autoantibody. Pooled serum from nonimmune subjects (NS) served as a negative control. Numbers indicate positions of protein size markers. C, control; R, retinoic acid treatment. **b:** Immunoprecipitation with human U1 reference serum demonstrated U1 70K protein in nuclear (Nuc), as well as cytoplasmic (Cyto) protein extracts. **c:** UMR 201 cells were treated for 1, 2, 3, and 4 h with 1 μM retinoic acid and nuclear extracts were immunoprecipitated for U1 70K protein with serum from a patient with MCTD shown to have a high titer antibody to U1 RNP. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto nitrocellulose membrane and probed with serum from a patient with MCTD. The detection system was ^{125}I-labeled protein A. These experiments were conducted three to five times; representative results are shown.

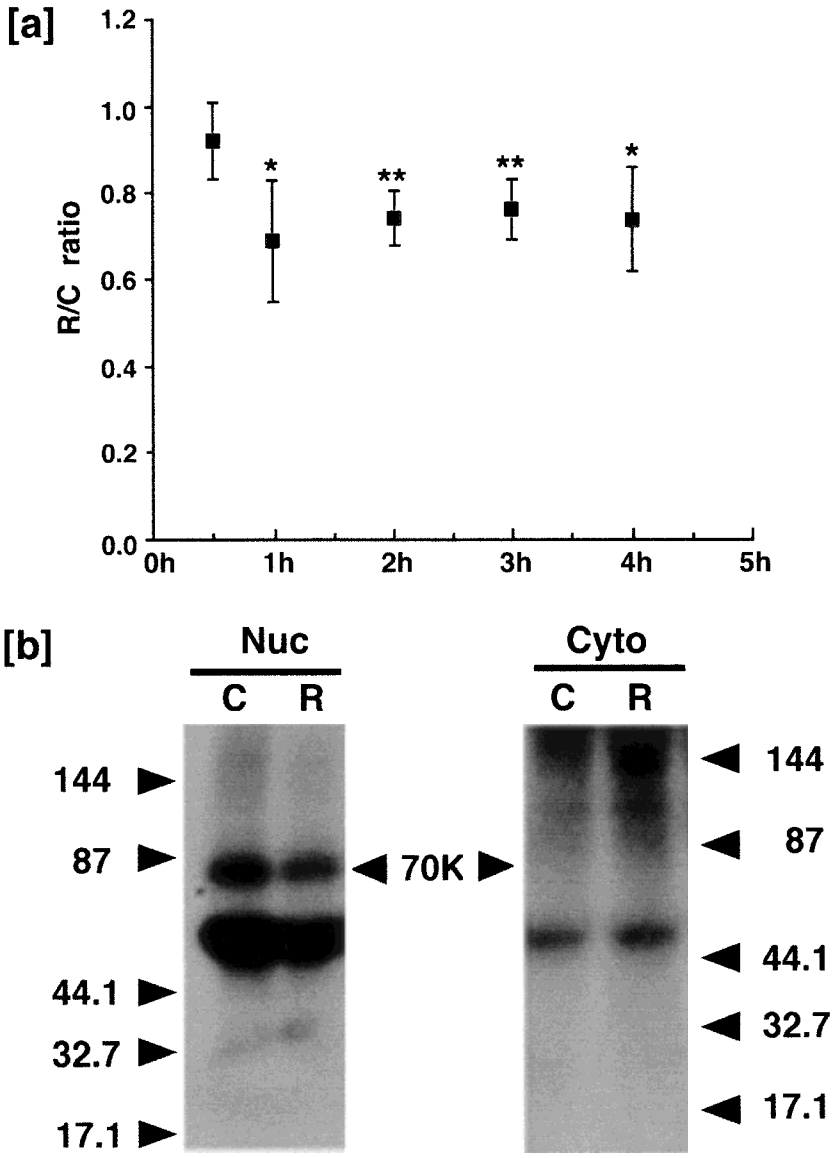

Fig. 2. Phosphorylated U1 70K protein in UMR 201 cells. UMR 201 cells were treated with 1 μM retinoic acid for 0.5, 1, 2, 3, and 4 h, after an overnight incubation with 2 mCi ^{32}P-orthophosphoric acid. Phosphorylated proteins were immunoprecipitated and separated in a 4–15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, as described under Materials and Methods. **a:** The amount of phosphorylated U1 70K protein within the nucleus at each time point was estimated by pooling the results from four separate experiments and expressing them as a retinoic acid-to-control ratio (R:C ratio). Bars = SD. *$P < 0.025$; **$P < 0.01$. **b:** Phosphorylated U1 70K protein within the nucleus, demonstrating dephosphosphorylation of U1 70K protein after treatment with retinoic acid for 1 h. C, control cells; R, retinoic acid-treated cells. **c:** At the same time, phosphorylated U1 70K protein was not detected in the cytoplasm of control or treated UMR 201 cells despite the presence of U1 70K protein in the cytoplasm as shown in Fig. 1b.

In all experiments, UMR 201 cells were treated with 1 μM retinoic acid because it has previously been shown that differentiation of UMR 201 cells was maximally induced at that concentration [Ng et al., 1988; Zhou et al., 1991]. Furthermore, posttranscriptional actions of retinoic acid were also reported at that concentration [Zhou et al., 1994; Manji et al., 1995].

Phosphorylation of U1 70K Protein

We then determined whether retinoic acid could change the state of phosphorylation of U1 70K protein because of its influence on U1 70K function [Romac and Keene, 1995; Tazi et al., 1993]. In a typical time course experiment, performed four times, treatment with 1 μM retinoic acid for 0.5–4 h resulted in significant dephosphorylation of nuclear U1 70K protein, which was first detected 1 h after treatment (Fig. 2a). Retinoic acid reduced the amount of phosphorylated U1 70K protein by approximately 25%, but not the overall amount of U1 70K (Fig. 1c). It should be noted that phosphorylated U1 70K protein was detected only in the nuclei and not the cytoplasm of UMR 201 cells (Fig. 2b) despite the demonstration in Figure 1b that U1 70K protein was clearly present within the cytoplasm.

Phosphoamino Acid Analysis and Phosphopeptide Mapping of U1 70K

As shown in Figure 3a, analysis of the phosphorylated amino acids in U1 70K protein of UMR 201 cells confirmed that phosphorylation

only occurred on serine residues. Tryptic phosphopeptide analysis of U1 70K protein confirmed the presence of four major phosphopeptides, as previously described [Woppmann et al., 1993], within the nuclei of control cells. Treatment with retinoic acid resulted in the specific dephosphorylation of phosphopeptide 3 (arrow, Fig. 3b) by 4 h. A similar phosphopeptide pattern was observed at 2 h in two separate experiments (data not shown). Some dephosphorylation of phosphopeptide 1 was observed after 4 h of retinoic acid treatment in Figure 3b. However, this effect was much less striking than that observed with phosphopeptide 3 and was not observed consistently in repeat experiments.

Expression of Alkaline Phosphatase mRNA

A time course of the effects of retinoic acid on ALP mRNA levels was performed to show an increase in ALP mRNA expression, first observed at 1 h (Fig. 4). This correlated with the time when dephosphorylation of U1 70K protein was first observed in Figure 2a.

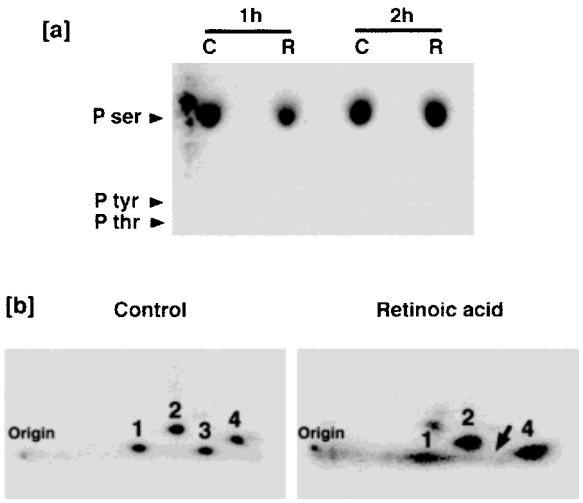

Fig. 3. Phosphoamino acid analysis and peptide mapping of phosphorylated U1 70K protein. Phosphorylated U1 70K protein from nuclei of UMR 201 cells were prepared for phosphoamino acid analysis and peptide mapping, as described under Materials and Methods. **a:** Analysis of the phosphorylated amino acids in U1 70K protein showed that only serine residues were phosphorylated. C, control; R, retinoic acid treatment. **b:** Tryptic phosphopeptide analysis of phosphorylated U1 70K protein demonstrated the presence of four major phosphopeptides (labeled 1, 2, 3, and 4) in the nuclei of control cells. Retinoic acid treatment for 4 h resulted in the specific dephosphorylation of one phosphopeptide (phosphopeptide 3, arrow). A similar picture was obtained after treatment with retinoic acid for 2 h (data not shown).

ELISA for U1 snRNP in UMR 201 Cells

In addition to the rapid change in phosphorylation of U1 70K protein observed above with retinoic acid treatment, we also determined whether the amount of U1 snRNP could be regulated. Using UMR 201 cells as substrate, we were able to show by immunofluorescence, that treatment with 1 μM retinoic acid for 24 h resulted in a fourfold increase in the amount of U1 snRNP present in nuclei of UMR 201 cells (data not shown). This observation was confirmed by ELISA, using sera from different patients with the same disease (Fig. 5). This result was reproducible in three separate experiments.

Expression of ASF 1 and ASF 3 mRNA

The interaction between U1 70K protein and ASF-1 led us to examine the regulation of mRNA expression ASF isoforms. ASF-1 is an essential splicing factor, and ASF-3 is an isoform generated by alternative splicing of ASF-1. Total RNA was prepared from UMR 201 cells treated with 1 μM retinoic acid for 1, 2, 4, and 8 h and specific RT-PCR primers were used to amplify mRNA fragments for ASF-1 and ASF-3. Figure 6 shows that 2 h after treatment with retinoic acid, there was a consistent twofold reduction in the expression of the inhibitory ASF-3 isoform mRNA in four separate experiments. The effect on expression of ASF-1 mRNA was more variable, and no consistent pattern was observed in each of the repeat experiments.

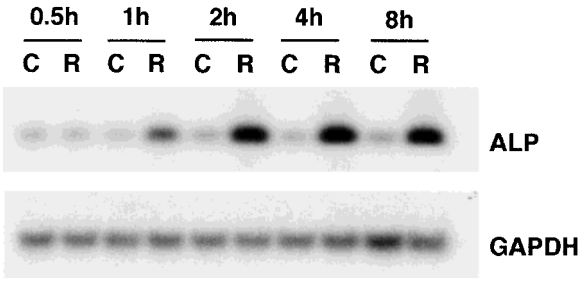

Fig. 4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of alkaline phosphatase mRNA expression by retinoic acid in UMR 201 cells. UMR 201 cells were treated with 1 μM retinoic acid for 0.5, 1, 2, 4 and 8 h. A PCR product for alkaline phosphatase gene was obtained using RT-PCR primers, as described under Materials and Methods. Treatment with retinoic acid to show an increase in the amount of spliced (mature) alkaline phosphatase mRNA present in the cytoplasm of UMR 201 cells. C, control; R, retinoic acid-treated cells. This is a representative result of an experiment carried out three times.

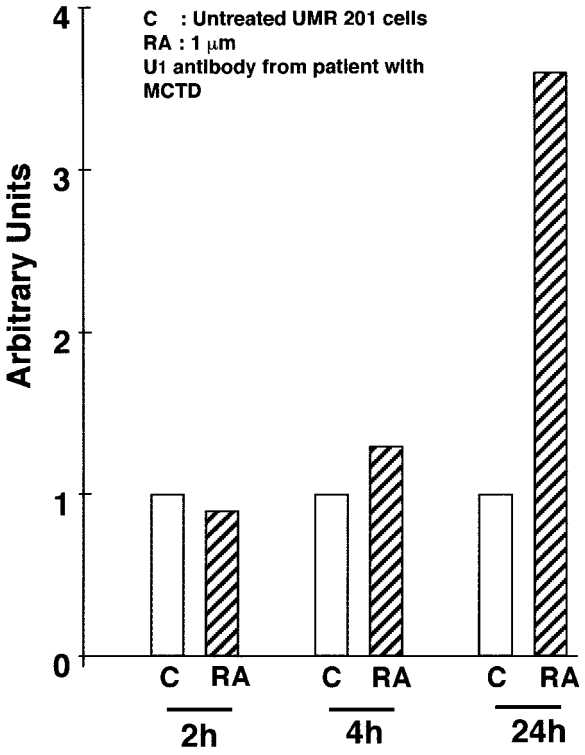

Fig. 5. Enzyme-linked immunosorbent assay (ELISA) for detection of U1 snRNP. Treatment of UMR 201 cells with 1 μM retinoic acid for 2, 4, and 24 h. The primary antibody was contained in serum from a patient with mixed connective tissue disease (MCTD) in whom antibody to U1 snRNP has been detected using a routine immunofluorescence assay with Hep2 cells as substrate. The experiment was performed three times; a representative result is shown. Treatment for each time point was performed in triplicate. The columns represent mean ±SD. *$P < 0.001$. C, control; RA, treatment with retinoic acid.

Phosphorylation of U1 70K and Regulation of U1 70K mRNA Expression by TNF-α and TGF-β

We have previously demonstrated that TNF-α enhanced the posttranscriptional action of retinoic acid on ALP mRNA expression in UMR 201 cells [Manji et al., 1995], whereas TGF-β had the opposite effect [Zhou et al., 1994]. The results shown thus far implicate modulation of U1 70K phosphorylation in the regulation of nuclear processing of ALP precursor mRNA. We therefore tested the effects of TNF-α and TGF-β on the phosphopeptide pattern of U1 70K. The results showed that a 2-h treatment with TNF-α resulted only in partial dephosphorylation of phosphopeptide 1 (Fig. 7). When combined with retinoic acid, dephosphorylation of phosphopeptide 1 was more complete but the degree of dephosphorylation of phosphopeptide 3 was similar to that observed with retinoic acid treatment alone (Fig. 3). In the same experiment, TGF-β on its own had no effect on the dephosphorylation of U1 70K phosphopeptides, and it did not modulate retinoic acid-induced dephosphorylation of phosphopeptide. Contrasting effects on expression of U1 70K mRNA were observed when the cells were treated for 8 h. 0.6 nM TNF-α markedly decreased U1 70K mRNA expression while treatment with 1 ng/ml TGF-β resulted in a marked increase in 70K mRNA expression (Fig. 8). An 8-h treatment was selected because our previous work showing the effects of interaction with retinoic acid was observed at this time point [Ng et al., 1988].

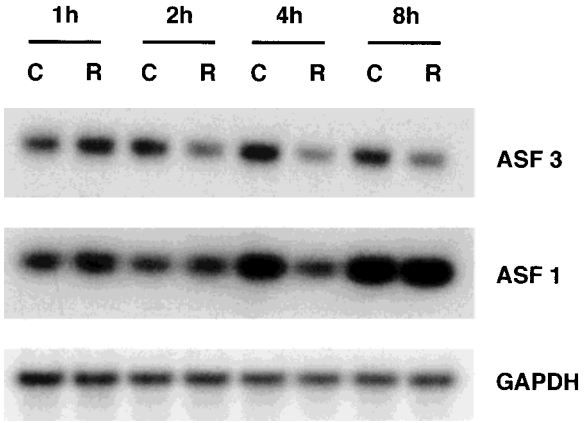

Fig. 6. Regulation of ASF isoform mRNA expression by retinoic acid. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA expression of isoforms of ASF by retinoic acid. UMR 201 cells were treated with 1 μM retinoic acid for 1, 2, 4, and 8 h. Specific RT-PCR primers were used to amplify mRNA fragments of ASF-1 and ASF-3, as described under Materials and Methods. This experiment was performed four times; a representative result is shown.

DISCUSSION

The discovery of the split gene in adenovirus during the late 1970s led to the concept of RNA splicing [Berget et al., 1977; Chow et al., 1977]. It is now universally recognized that precursor mRNA transcribed from eukaryotic genes require the removal of introns before spliced, mature RNA can be translocated from the nucleus into the cytoplasm for translation into functional proteins. RNA splicing also provides the opportunity to select different combinations of exons through alternative splicing, resulting in protein products with different functions. Much of our understanding of the biochemistry of the splicing reaction has been derived from in vitro studies using radioactive pre-mRNA substrates and soluble cellular components capable of processing pre-mRNAs [Green et al.,

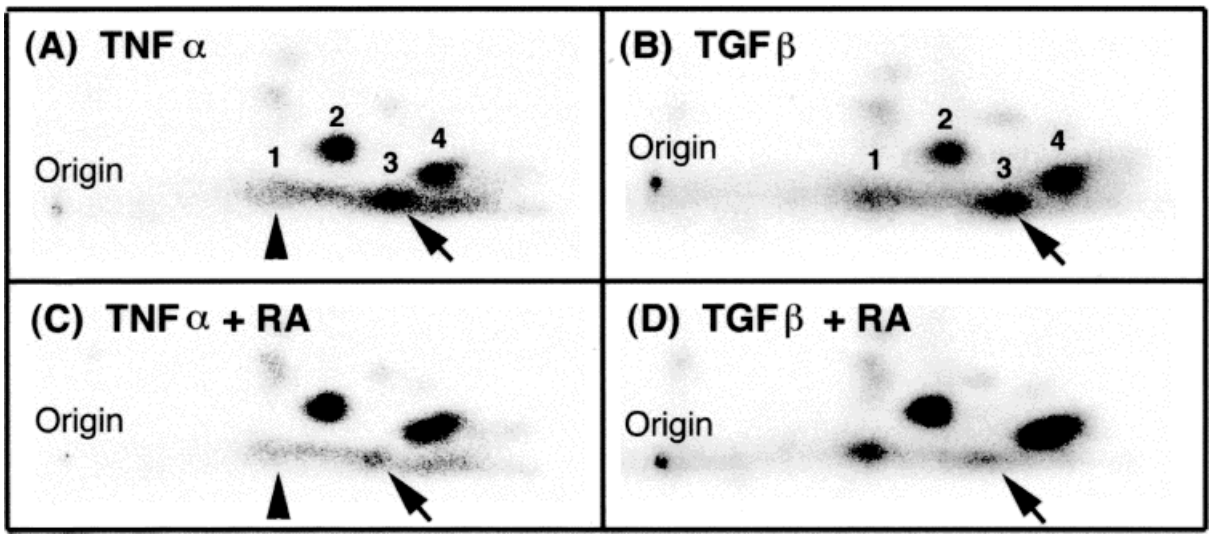

Fig. 7. Peptide mapping of phosphorylated U1 70K protein after treatment with tumor necrosis factor-α (TNF-α) or transforming growth factor-β (TGF-β). Tryptic phosphopeptide analysis of phosphorylated U1 70K protein to show the presence of major phosphopeptides in the nuclei of cells treated with 0.6 nM TNF-α **(A)** or 1 ng/ml TGF-β for 2 h **(C).** The same experiment was repeated when either TNF-α **(B)** or TGF-β **(D)** was combined with retinoic acid treatment for 2 h. Arrows, phosphopeptide (phosphopeptide 3), which is specifically dephosphorylated in response to retinoic acid action. The arrowhead points to phosphopeptide 1.

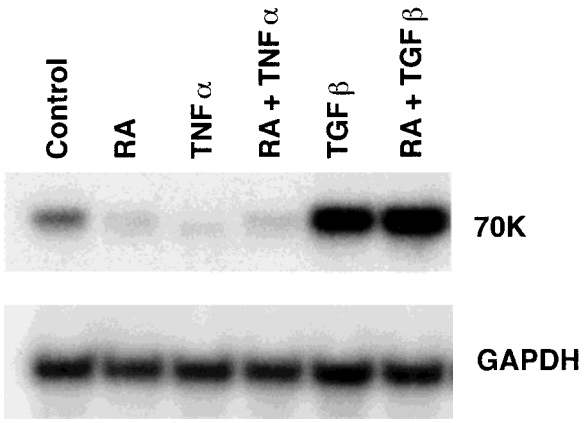

Fig. 8. Expression of mRNA for U1 70K protein after treatment with tumor necrosis factor-α (TNF-α) or transforming growth factor-β (TGF-β). UMR 201 cells were treated with 1 μM retinoic acid (RA), 0.6 nM TNF-α, 1 ng/ml TGF-β alone or in combination for 8 h. Total RNA was prepared and polymerase chain reaction (PCR) products for U1 70K protein and GAPDH were obtained using appropriate primers, as described under Materials and Methods. PCR products were hybridized with specific oligonucleotides for each mRNA.

1983; Hernandez and Keller, 1983; Padgett et al., 1983]. There is little current information on whether hormones and growth factors that modulate cell differentiation and function might regulate the expression and function of spliceosome components leading to modulation of spliceosome formation and function. The regulation of spliceosome assembly by phosphorylation (discussed below) provides an indication that it may be a target of signal transduction pathways.

We have previously reported that an increase in proficiency of posttranscriptional nuclear processing of ALP mRNA by retinoic acid in a preosteoblast cell line, UMR 201, plays an important part in its regulation of mRNA expression of ALP, a marker of differentiated osteoblasts [Manji et al., 1995; Zhou et al., 1994]. In this study, we examined the mechanism of action of retinoic acid with particular emphasis on its role in regulating both the amount of U1 snRNP and the phosphorylation state of the specific U1 70K protein in preosteoblasts because of its central role in initiating the splicing reaction.

Among metazoans, U1 70K is highly conserved and several functional domains have been identified in this peptide. It shares the C-terminus repeating arginine-serine dipeptide RS domain with the SR family of splicing factors [Birney et al., 1993; Cáceres and Kraine, 1993; Fu, 1995; Zamore et al., 1992]; the N-terminus RBD consensus domain contains the RNA binding motif; a glycine-rich region downstream of the RBD domain and made up of several RGG-like repeats has also been implicated in RNA binding [Dreyfuss et al., 1993]. U1 70K protein exists as a phosphoprotein in vivo [Woppmann et al., 1990]. In vitro, the

serine residues of the human U1 70K RS domain are phosphorylated by a U1 snRNP-associated kinase activity that phosphorylates U1 70K protein selectively in a reaction requiring ATP [Woppmann et al., 1993].

Both phosphorylation and dephosphorylation play important roles in splicing. A model proposed by Mermoud et al. [1994] suggested distinct steps where splicing may be regulated by a protein phosphorylation mechanism. Phosphorylation of SR proteins is required for early steps in spliceosome assembly. Once a spliceosome containing U1, U2, U4/U6.U5 snRNPs is assembled, at least two separate dephosphorylation events are essential for the splicing reaction to proceed. Dephosphorylation of U1 70K protein, probably by protein phosphatase 1 (PP1), plays an important role in the first catalytic step, while protein phosphatase 2A (PP2A) is required for the second catalytic step [Mermoud et al., 1992, 1994; Tazi et al., 1993; Umen and Guthrie, 1995].

There is very little information on the regulation of the phosphorylated state of U1 70K by local or systemic factors in intact cells. In this study, we showed that phosphorylation of U1 70K was specific to protein present within the nucleus, and not the cytoplasm. This observation may indicate that the relevant kinase is exclusively nuclear in location and/or only functions within the context of a spliceosome, consistent with the central role of U1 70K in initiating the splicing reaction taking place in the nucleus. To the best of our knowledge, these observations have not previously been reported. Furthermore, our results clearly show an early and unique action of retinoic acid in dephosphorylating one out of four major phosphopeptides present in U1 70K protein of UMR 201 cells. The observation that retinoic acid did not affect the amount of U1 70K protein in the first 4 h of treatment implies that in the short term, the major action of retinoic acid is likely to be mediated through an alteration in the functional status of U1 70K protein. The observation that retinoic acid-induced increase in the formation of spliced ALP mRNA from precursor mRNA in UMR 201 cells occurred within the same time course as dephosphorylation of U1 70K protein is not sufficient evidence that the two events are functionally linked. Whether retinoic acid-induced spliceosome changes are causally related to the increased in ALP mRNA upon treatment with retinoic acid remains to be determined. Furthermore, this result does not imply that U1 70K protein is the only target of retinoic acid action and neither does it rule out the possibility that retinoic acid may also have actions on other snRNP core proteins, SR proteins, or hnRNP proteins. A systematic study of the effects of retinoic acid on all factors that contribute to the formation or function of the spliceosome is obviously indicated. In our previous studies, we have shown that retinoic acid-induced increase in ALP mRNA in UMR 201 cells peaked at 24 h. This would imply that in addition to a possible role on splicing, retinoic acid may have other posttranscriptional effects on nuclear processing such as stabilization of ALP mRNA transcripts.

The function of the spliceosome may also be influenced by changes in the abundance of its components. For example, overexpression of ASF-1 [Cáceres et al., 1994] or U1 70K [Romac and Keene, 1995] may alter the selection of splice sites in cells or even the splicing reaction respectively. Our results show that retinoic acid treatment for 24 h increased the amount of U1 snRNP in UMR 201 cells. Although this may not have the immediate impact of dephosphorylation of U1 70K protein in spliceosome function, the effect may nevertheless influence U1 snRNP function in the longer term. In support of this argument, we have also shown that TNF-α and TGF-β treatment resulted in changes in the mRNA expression of U1 70K in directions which mirror their different effects on retinoic acid-induced nuclear processing of alkaline phosphatase mRNA [Manji et al., 1995; Zhou et al., 1994]. TNF-α enhancement of retinoic acid action is associated with a decrease in mRNA expression of U1 70K, while TGF-β antagonism of retinoic acid effect is associated with an increase in mRNA levels of the same protein. In the short term, a 2-h treatment with TNF-α resulted in partial dephosphorylation of phosphopeptide 1 and this may play an additional role in enhancing the posttranscriptional action of retinoic acid in nuclear processing. In contrast, TGF-β on its own did not have any influence on the dephosphorylation of U1 70K phosphopeptides.

The interaction between U1 70K protein and ASF-1 led us to examine the regulation of mRNA expression of ASF-1 isoforms. The full-length ASF-1 is a splicing factor required for a very early step in spliceosome assembly [Fu and Maniatis, 1992; Krainer et al., 1990a, b; Krainer

and Maniatis, 1985] and has a second function in the selection of alternative splice sites in a concentration-dependent manner [Ge and Manley, 1990; Krainer et al., 1990a]. The primary structure of ASF 1 shares similarity with known regulators of alternative splicing from *Drosophila*. Two of these proteins, Sex Lethal [Bell et al., 1988] and Transformer-2 [Amrein et al., 1988; Goralski et al., 1989], contain RBDs and each can bind specifically to target pre-mRNAs [Hedley and Maniatis, 1991; Inoue et al., 1990]. Small amounts of two isoforms of ASF 1, designated ASF-2 and ASF-3, can be generated by alternative splicing [Ge and Manley, 1990]. Both lack the RS region and about 12 residues immediately upstream of it. ASF-2 and ASF-3 inhibit ASF-1-activated splicing in vitro, possibly by interfering with the assembly or activity of functional ASF-1 multimers [Zuo and Manley, 1993]. The sustained decrease in expression of ASF-3 mRNA after treatment with retinoic acid for 2 h could conceivably facilitate the action of ASF-1 through an alteration in the ratio of the facilitatory and inhibitory isoforms. Nonetheless, the physiological significance of the effect of retinoic acid on ASF-3 mRNA expression remains uncertain because the existence of the protein in vivo is still to be proved.

In conclusion, we have demonstrated distinct mechanisms by which retinoic acid, TNF-α, and TGF-β can modulate spliceosome function. It is attractive to postulate that retinoic acid-induced differentiation of preosteoblasts proceeds, at least in part, through its coordinate effects on U1 70K phosphorylation state and the levels of the ASF-1 isoforms. It is our hypothesis that the short-term effectors are distinct from regulation by ASF-3 and changes in abundance of U1 70K protein that may dictate longer term, sustained effects. Finally, this data raises the possibility that the regulation of spliceosome assembly and activity may be a more general target for the effects of growth or differentiation factors, or both.

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