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Nuclear localization and binding affinity of STAT5b for the α_2-macroglobulin gene promoter during rat liver development and the acute-phase response

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Expression of the rat α_2-macroglobulin (MG) gene undergoes dynamic changes throughout an individual's life and during the acute-phase (AP) response. Details of the participation of the STAT family of transcription factors in its control remain incompletely understood. Here we examined the involvement of STAT5b in MG gene expression during development and the AP response. Immuno-blot analysis revealed the highest nuclear level of STAT5b in the fetus and during postnatal development, whereas in the adult it decreased. Stimulation of MG expression during the AP response was accompanied by a decrease in STAT5b. Examination of STAT5b localization revealed that the relative concentrations of STAT5b were higher in the nuclear matrix than in the nuclear extract. Affinity chromatography with the extended promoter region of the MG gene (–825/+12), followed by immuno-blot analysis, revealed dynamic changes in STAT5b binding. The highest concentration of the promoter-binding form of STAT5b was observed in the fetus. As postnatal development progressed, the level of promoter-bound STAT5b decreased and in the adult liver it was the lowest. Stimulation of MG gene expression during the AP response in both the fetus and adult was accompanied by significantly decreased STAT5b binding to the MG promoter. The AP response was accompanied by lower levels of STAT5b serine and tyrosine phosphorylation in both fetus and adult. In the nuclear matrix derived from adult tissues, tyrosine phosphorylated species were completely absent. We conclude that developmental-stage differences in the mechanisms that determine STAT5b nuclear localization contribute to its activity *in vivo*.

Keywords: α_2-macroglobulin, STAT5b, rat liver development, phosphorylation, nuclear extract, nuclear matrix

INTRODUCTION

The synthesis of the serum protein alpha2-macroglobulin (MG) is initiated in the rat liver during embryogenesis. It lasts until about three weeks after birth when it starts declining to the very low level that is observed in the adult (Herbst & Babbis, 1990; Noda & Ichihara, 1993). It has been suggested that the increased serum MG level under basal conditions during embryogenesis and the early postnatal period is a reflection of its participation in tissue remodeling (Panrucker *et al.*, 1983; Fletcher *et al.*, 1988; Glibetić *et al.*, 1992). In the adult, the serum MG concentration dramatically increases during the acute phase (AP) reaction, a systemic, non-specific defense response of an organism to different injurious stimuli including infections, burns, neoplasia and trauma (Abbink *et al.*, 1991; Khan *et al.*, 1995; Schaefer *et al.*, 2004). The importance of MG is substantiated by the observation that normal adult rat

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Abbreviations: AP, acute-phase; ECL, enhanced chemiluminescence; DTT, dithiothreitol; INM, internal nuclear matrices; MG, α_2-macroglobulin; PMSF, phenylmethylsulfonyl fluoride; RE, response element; STAT, signal transducers and activators of transcription.

serum contains 32±4 μg /ml and that about 24 h after an injury its concentration increases 110–140-fold (Okubo *et al.*, 1981). During the AP response, the pro-inflammatory interleukins IL-1 and IL-6 rapidly induce in the liver the synthesis of a set of proteins (Baumann & Gauldie, 1994) referred to as the AP reactants, of which MG is an important member in the rat. The recognized ability of MG to inhibit proteases (Barrett & Starkey, 1973) is important in counteracting excessive proteolytic activity during the resolution phase of the AP response and during states of protracted AP response that characterize inflammatory states (Sottrup-Jensen *et al.*, 1989; Gonias, 1992). Thus, one of the roles that MG assumes is limiting the onset and progression of detrimental processes (Fritz *et al.*, 1986; Abbink *et al.*, 1991).

MG gene transcription is mediated by specific, cytokine-induced transcription factors. Following stimulation, the signal transducers and activators of transcription (STAT) family members that are present in a functionally latent, monomeric form in the cytoplasm of resting cells (Zhang & Darnell, 2001; Wesoly *et al.*, 2007) become tyrosine phosphorylated and as dimers translocate to the nucleus. In the nucleus, STAT3 binds to the IL-6 response element (RE) of the rat MG gene and stimulates its transcription (Wegenka *et al.*, 1994; Akira *et al.*, 1994; Zhang & Darnell, 2001). The STAT5b protein, another member of the STAT family, also displays a binding affinity towards the IL-6 RE of the rat MG and apparently participates in MG gene expression in the basal state rather than during the AP response (Ripperger *et al.*, 1995).

Inasmuch as the *de novo* synthesis and nuclear transport determines the functioning of gene regulatory proteins, an important contributing factor is the precise localization of critical concentrations of *trans*-factors in the nucleus (Lindenmuth *et al.*, 1997; Fackelmayer, 2004). The control of gene expression *in vivo* is integrated in the concept of a functioning nuclear architectural framework represented by the nuclear matrix. The nuclear matrix is a three-dimensional, mostly proteinaceous nuclear substructure. It consists of the peripheral nuclear lamina that circumscribes a complex anastomosing network of fibers that pervades the nuclear interior (Nickerson, 2001). Aside from its role in DNA metabolism (Berezney *et al.*, 1995), the nuclear matrix organizes subnuclear trafficking and localizes regulatory proteins at target sites (Jackson, 1997; Zeng *et al.*, 1998; Stein *et al.*, 2003). The association of different regulatory proteins and transcription factors with the nuclear matrix has been extensively documented (Getzenberg & Coffey, 1991; Hendzel *et al.*, 1994; Reyes *et al.*, 1997; Ivanović-Matić *et al.*, 2000; Dinić *et al.*, 2000; 2004). Consequently, it has been hypothesized that the nuclear matrix participates in gene expression by providing attachment points for transcription factors in close proximity to gene regulatory elements (Getzenberg, 1994; Lemon & Tjian, 2000; Sjakste & Sjakste, 2001).

In this work we examined the involvement of STAT5b in MG gene expression during different transcriptional states: throughout liver development and during the AP response. The binding affinity of STAT5b towards the MG gene promoter element was examined and correlated with its distribution in different nuclear protein fractions, the nuclear extract and the nuclear matrix. Our results revealed that decreased MG expression during liver maturation was accompanied by lowered levels of nuclear and MG promoter-associated STAT5b. In contrast, the stimulation of MG gene expression during the AP response was always accompanied by significantly decreased STAT5b binding to the MG promoter. We hypothesize that the interplay of STAT5b with the nuclear matrix represents an additional regulatory step of STAT5b activity and affects the rate of MG gene transcription.

MATERIALS AND METHODS

Animals. Male and female albino rats of the Wistar strain were used. Livers were isolated from: 20-day-old fetuses removed from 10-week-old dams; 1-, 7-, 14- and 21-day-old neonatals, and 10-week-old male adults. To obtain sufficient material for one experiment we pooled livers from up to five litters of 20-day-old fetuses (i.e. fetuses from up to five dams, depending of the number of the fetuses in each litter), and of 1- and 7-day-old neonatals, either from the control or the turpentine-treated group. Livers from three neonatals (14- and 21-day-old animals) and three adult (10-weeks-old) male rats were pooled for each group (control and turpentine-treated) per one experiment. For each group of rats 3–5 separate experiments were performed. The AP response was induced by a subcutaneous injection of turpentine oil (1 μl/g of body mass) in the lumbar region of the dams, neonatals and male adults (Baumann *et al.*, 1984). The animals were killed 12 h after turpentine injection. The rats were kept at constant temperature, humidity and dark/light intervals.

Measurement of serum MG concentrations. The concentration of MG was determined by rocket immunoelectrophoresis with anti-human MG antibody (Sigma) (Laurell, 1972). The concentration of MG was established by quantification of the areas under immunoprecipitation peaks after immunoelectrophoresis. The peak was drawn out on tracing paper and cut out, the areas calculated (P) and the concentration of protein expressed relative to the control adult samples (taken as 1).

Determination of mRNA concentrations during rat liver development. Total liver RNA from fetal, 1. 7. and 21. day of postnatal development and adult rats was isolated by a procedure based on the extraction of RNA with guanidine-HCl, as described by Cox (1968). Samples of RNA (2.5, 5, 10 μg/spot) were dot-blotted onto Schleicher-Schuell nitrocellulose filters and hybridized with a nick-translated plasmid for MG cDNA (Kafatos *et al.*, 1979). Relative changes in mRNA concentrations were determined by scanning the autoradiograms (Beckmann Microzone Densitometer, model 110) and expressed relative to the control adult samples (taken as 1).

Isolation of rat liver nuclear extract. Nuclear extracts were prepared from livers of control and turpentine-treated rats following the procedure elaborated by Gorski *et al.* (1986). Tissues were excised and homogenized in 10 mM Hepes pH 7.6, 25 mM KCl, 1 mM spermidine, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 M sucrose and 10% glycerol. The hepatocyte nuclei were collected by centrifugation through a cushion of the same solution at 72000×*g* in an SW 28 rotor (Beckman L7-55) for 30 min at 4°C. The nuclei were resuspended in lysis buffer (10 mM Hepes pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol), and chromatin was pelleted by centrifugation of the lysate (82000×*g*, Ti 50 rotor, 60 min, 4°C). Solid $(NH_4)_2 SO_4$ was added to the supernatant and the precipitated proteins were sedimented at 82000×*g* in a Ti 50 rotor for 30 min at 4°C. Nuclear extracts were dialyzed against 25 mM Hepes pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT and 10% glycerol and resuspended in dialysis buffer, frozen in small aliquots and kept at –80°C.

Isolation of the nuclear matrix proteins. Rat liver nuclei were isolated and purified according to Kaufmann & Shaper (1984). All steps were carried out at 4°C, unless otherwise indicated and all buffers contained 1 mM PMSF. Rat livers (0.5 g/ml) were minced in STM buffer (0.25 M sucrose, 50 mM Tris/HCl, pH 7.4, and 5 mM $MgSO_4$) and homogenized with several strokes in a Potter-Elvehjem Teflon-glass homogenizer. Homogenates were centrifuged at 1000×*g*, 15 min (Sorval SS-34 rotor) and washed once with STM. The crude nuclear pellet was resuspended in DS buffer (2.2 M sucrose, 50 mM Tris/HCl, pH 7.4, and 5 mM $MgSO_4$), layered over a cushion of the same buffer and centrifuged at 72000×*g* for 60 min (Beckman SW 28 rotor). The pellet was resuspended in STM buffer, layered over a cushion of DS buffer and centrifuged at 72000×*g*, 30 min. Nuclear matrix proteins were isolated by the procedure of Belgrader *et al.* (1991), with certain modifications. The purified nuclei were first stabilized by incubating at 42°C, 20 min, treated with 2 mM sodium-tetrathionate in STM buffer for 1 h and then washed twice with the same buffer without sodium tetrathionate by centrifugations at 5000×*g*, 10 min (Sorval SS-34 rotor) The nuclei were resuspended in STM buffer (10^8/ml), incubated with 100 μg/ml DNase I for 1 h at 30°C and subjected to consecutive extraction/centrifugation steps: twice with high salt buffer (0.25 M $(NH_4)_2SO_4$, 10 mM Tris/HCl, pH 7.4 and 0.2 mM $MgSO_4$), once with freshly prepared 1% Triton X-100 in low salt buffer (10 mM Tris/HCl, pH 7.4, and 0.2 mM $MgSO_4$) and washed twice with low salt buffer. Centrifugations were carried out at 8000×*g*, 15 min (Sorval SS-34 rotor). Aliquots of nuclear matrices (about 95% of its content are proteins) were resuspended in 0.25 M sucrose, 10 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.4, 0.5 mM PMSF to which an equal volume of sterile glycerol was added and were kept at –20°C. For the DNA affinity chromatography experiments, isolated nuclear matrices were subjected to solubilization in order to obtain internal nuclear matrices (INM). Preparation of INM proteins was carried out as described by Stuurman *et al.* (1990). Nuclear matrices were first resuspended in low salt buffer (2×10^8/ml) and then an equal volume of 2 M NaCl and 40 mM DTT in the same buffer was added to the suspension. After incubation for 20 min on ice, insoluble nuclear matrix proteins were removed by centrifugation at 10000×*g*, 15 min. The supernatant was dialysed against 10 mM ammonium acetate (pH 7.4) with several changes of buffer, lyophilized, and stored at –70°C.

SDS/polyacrylamide gel electrophoresis and Western immunoblot analysis. Protein concentrations were determined according to Lowry *et al.* (1951). For SDS/polyacrylamide gel electrophoresis (SDS/PAGE) 20 μg of proteins was loaded onto 4% stacking/12% separating slab gels as described by Laemmli (1970). Nucleoproteins separated by SDS/PAGE were transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech) and Western immunoblot analysis was performed by the procedure of Towbin *et al.* (1979) using polyclonal antibodies to rat STAT5b and phospho-STAT5b (Ser and Tyr) (Santa Cruz Biotechnology). After incubation with blocking solution (0.05% Tween 20, 50 mM Tris/HCl pH 7.6, 150 mM NaCl, 3% non-fat condensed milk), the membranes were incubated with specific antibodies for 2 h at room temperature. After rinsing, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin as a secondary antibody for 1 h. Immunoreactive bands were identified by an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions. Western immunoblots were quantified using TotalLab (Phoretix) electrophoresis software (v 1.10).

DNA affinity chromatography. In order to investigate whether STAT5b exhibited binding affinity towards the MG gene promoter, soluble nuclear proteins or internal nuclear matrix proteins were purified on a DNA affinity column and then examined by Western analysis. Affinity chromatography of sequence-specific DNA binding proteins from rat liver was performed by a slightly modified method of Kadonaga and Tjian (1986). A fragment of the MG gene (–825/+12), obtained from Dr. Peter Heinrch (Institute für Biochemie an der RWTH Aachen, Aachen, Germany) was annealed and ligated to obtain oligomers and then covalently coupled to Sepharose CL-2B with cyanogen bromide to yield the affinity resin. The DNA affinity resin was equilibrated in a Bio-Rad Econo-Column with dialysis buffer (25 mM Hepes pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.1 M KCl. Equal quantities (5 mg) of fetal or adult liver nuclear extracts or INM prepared from control and experimental animals were combined with competitor DNA (salmon sperm) and allowed to stand for 30 min and the protein-DNA mixture was then passed through the DNA-Sepharose resin by gravity flow ("flow-through" fraction). The resin was washed with dialysis buffer containing 0.1 M KCl ("rinse" fraction). The passage of buffer through the column was stopped and dialysis buffer containing 1 M KCl was added to the column. The resin was mixed with the buffer using a glass rod and allowed to stand for 10 min. After the passage of the buffer through the column, the eluate was collected. This step was repeated with dialysis buffer containing 1 M KCl. Since DNA affinity chromatography requires soluble protein fractions, nuclear matrices were not subjected to this analysis.

Identification of putative STAT5b binding sites in the MG gene promoter element (–852/+12) was performed by computer search using Alggen Promo software (available on web page www.alggen.lsi.upc.es/cgi/bin/promo).

Statistical method. The MG protein and mRNA concentrations are shown as means ±S.E.M. from three separate experiments, according to Hoel (1966).

RESULTS

MG expression during fetal and postnatal development was assessed and compared with that in the adult by examining changes in MG mRNA and serum protein concentrations. The highest level of MG mRNA and protein concentration in the serum were detected in the fetus and on the 1st postnatal day, after which it progressively decreased to the very low level generally observed in the adult (Fig. 1). The AP response induced by turpentine injection was characterized by increased serum MG concentrations in the fetus and during postnatal development. In the adult, a 40-fold increase was measured at the 24th hour after AP induction (Fig. 2), when maximal plasma concentrations of AP proteins are established (Ševaljević *et al.*, 1989).

Next, nuclear extracts and nuclear matrices were prepared from 20-day-old fetuses, at different times of postnatal liver differentiation (1st, 7th, 14th, 21st postnatal days), from the adult rat (75 days old), and from age-matched rats 12 h after induction of the AP response by turpentine injection. The proteins were examined by immunoblot analysis with an antibody to STAT5b (Fig. 3). The relative concentration of STAT5b was the highest in the nuclear extract (Fig. 3A) and nuclear matrix (Fig. 3B) prepared from the fetus (lanes 1). It progressively decreased with rat growth and maturation (lanes 3, 5, 7, 9, 11). Induction of the AP response slightly affected the concentration of STAT5b in the fetus (lane 2), as it decreased for 2.5% in the nuclear extract and 2.2% in the nuclear matrix compared to respective controls. However, in the postnatal (lanes 4, 6, 8, 10) and adult (lane 12) rat the levels of STAT5b decreased. In the adult nuclear extracts the level of STAT5b de-

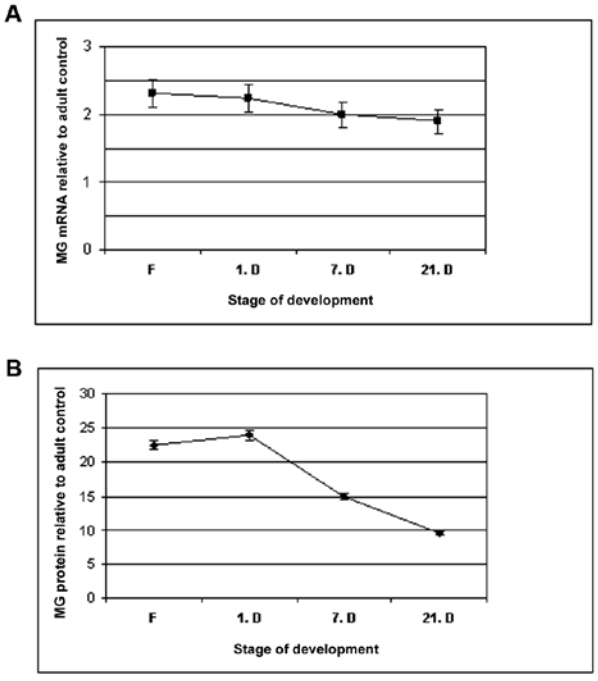

Figure 1. Developmental changes of relative concentrations of MG mRNA (A) and MG protein (B).
Total liver mRNA was isolated and dot-blot analysed. The relative concentrations of MG protein in the serum were determined by rocket immunoelectrophoresis with anti-MG antibody. The values were expressed relative to the control adult sample and represent means ±S.E.M. from three separate experiments. F, 20-day-old fetus; 1D, 1st postnatal day; 7D, 7th postnatal day; 21D, 21st postnatal day.

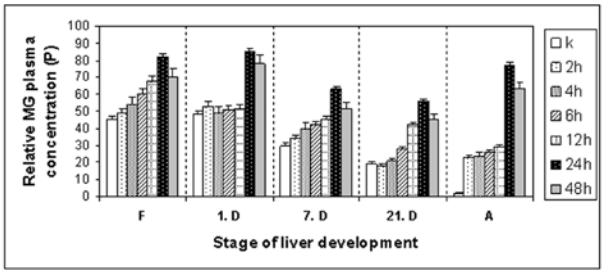

Figure 2. AP-related changes of MG serum concentrations.
The relative concentrations of MG in the serum were determined by rocket immunoelectrophoresis with an anti-MG antibody as described in Materials and Methods and are expressed in relation to adult control sample. The values represent means ±S.E.M. from three separate experiments. F, 20-day-old fetus; 1D, 1st postnatal day; 7D, 7th postnatal day; 21D, 21st postnatal day; A, 2.5-month-old adult.

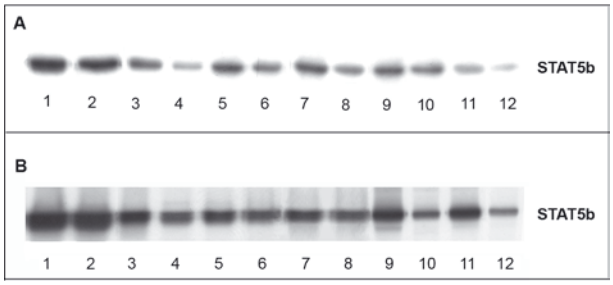

Figure 3. Western-blot analysis of rat liver nuclear extract (A) and nuclear matrix (B) proteins with anti-STAT5b antibody.
Twenty micrograms of proteins was separated by 12% SDS/PAGE, electrotransferred onto PVDF membrane and incubated with polyclonal rabbit antibody raised against rat STAT5b. The antigen–antibody complex was visualized by the ECL detection system. Lanes 1 and 2, 20-day-old fetus; 3 and 4, 1st postnatal day; 5 and 6, 7th postnatal day; 7 and 8, 14th postnatal day; 9 and 10, 21st postnatal day; 11 and 12, 2.5-month-old adult. Lanes 1, 3, 5, 7, 9 and 11, nuclear proteins isolated from control livers. Lanes 2, 4, 6, 8, 10 and 12, nuclear proteins obtained at 12 h after the induction of the AP response.

creased by 43% and in the nuclear matrix by 22% during AP response in relation to their mached controls. The relative STAT5b concentrations were consistently higher in the nuclear matrices compared to the respective nuclear extracts.

Whether the observed changes in nuclear STAT5b reflected its participation in the dynamic changes of MG gene expression was investigated by DNA affinity chromatography with the MG gene promoter fragment (–852/+12) containing the IL-6 re-

STAT consensus sequence -- TTN$_5$/N$_6$AA

MG gene promoter region (-852/+12)

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                                                    -860   CCAAGGAGCT
GCAGCAACGA  GAGGATGGAT  GCATGATTTA  ATGAAATTGC  CTCCAGGGCC  TGGAGACTAG
AGAGGGGAGT  GTTGGGCAAA  AGTGTTTGTG  TAGTGAATGG  CAAAGTCAGC  TGGGGTGTTG
GTTAGGCACA  GTAAAAAGGG  GTTATTTGAC  ACTTGTTACT  TTTCACCTTC  TTAAAACAGA
AATGAGTATA  TGTGAAGGTT  AAGGTCAATG  TTATGTTATA  CTGGAATTTA  GAAATTCACT
GTGAAGGTTA  CACTTACTGA  CCAGCAAGCC  CAAGACCCTC  CTGTCTCCTG  TCCTCCAGAA
CCCAGATTCT  AGCTAAGCAT  TAGGAAACCT  AATCATTTCT  CCTGGCCCCG  ACACTTCATT
TTCAGTCAAT  CTAATGTATA  ATAATACCTC  TAAAGTCAAT  ACCTACTTCC  TAGTTTGGTT
CAAAATGCTT  TCCATAGAAG  GGTGCTGATC  ATGGGCCTCA  CGTTAAAGAC  AGGCCTTATG
TGGCCCAGAG  CTTGCTGGCA  CACTCCGTTG  GAGTGAACTA  TTCAAGCCTC  CCAGGGCTGC
TTAAGCCACA  GCCTCCTTGC  CAACTATCCA  GACAGAAGCT  CAGAGCATCC  CTAAGAGGCT
GTGGGGGAGG  AGAAGCCGAT  TATCAAGTTT  CATGTACAAA  AGAGAAAAAG  TGAGCAGTAA
CTGGAAAGTC  CTTAATCCTT  CTGGGAATTC  TGGCTAACGG  GTCAGGAATT  AACCTTGGCG
GTAATTAGGC  CATCAGTGAC  TCTTTCAGAG  AATGTTCAAA  AACCTCAGCT  TTGTTTGGAG
AACTCGTGTG  GGTGGGAGCA  GCTGTTTGCA  CAAAGAACAG  CATAAAGTCT  AGCTGCTCCT
CACCACGTCC  AGGACCAGAT  CTC  +12
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STAT5b - _____

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-- ALLGEN PROMO predictions detail ----------
Factor name; Start position; End position; Dissimilarity; String; RE query
STAT5B [T04761]; -683; -672; 10.741335; TTCTTAAAACAG; 0.03121;
STAT5B [T04761]; -172; -161; 4.895795; TTCTGGGAATTC; 0.00677;
STAT5B [T04761]; -110; -99; 9.937531; TCTTTCAGAGAA; 0.01814;
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Figure 4. Potential STAT5b binding sites in the MG gene promoter region (–852/+12) predicted by Alggen Promo computer gene analysis.
Potential STAT5b binding sites are underlined in the MG gene promoter region (–860/+12). Shown are: STAT5b TRANSFAC database accession number; Start and End positions of putative binding sequences; Dissimilarity rate (%) between the putative and consensus sequences; String of potential binding nucleotides; Random Expectation (RE) as expected occurrence of the match in a random sequence of the same length as the query sequence (RE query, nucleotide frequencies as in the query sequence). **N,** any nucleotide.

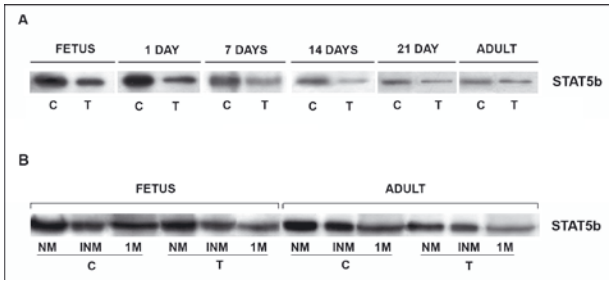

Figure 5. Western-blot analysis with anti-STAT5b antibody after DNA-affinity chromatography of nuclear extracts (A) and internal nuclear matrix proteins (B). Equal quantities of liver nuclear proteins were eluted from the DNA affinity column with 1 M KCl and Western blot analysis was performed with an anti-STAT5b antibody. Fetus, 20-day-old fetus; 1 day, 1st postnatal day; 7 days, 7th postnatal day; 14 days, 14th postnatal day; 21 day, 21st postnatal day; adult, 2.5-month-old adult; NM, nuclear matrix, INM, internal nuclear matrix, 1 M, nuclear proteins eluted from the DNA affinity column with 1 M KCl; C, nuclear proteins isolated from control livers; T, nuclear proteins obtained 12 h after the induction of AP response.

sponsive elements (RE) at positions –852/–777 and –404/–165, which is responsible for IL-6-dependent MG gene induction (Kunz *et al.*, 1989). Computer search of the promoter revealed three potential STAT5b binding sites — at positions –110/–99, –172/–161 and –683/–672 (Fig. 4). In the nuclear extracts obtained from fetal, postnatal and adult livers, STAT5b displayed binding affinity towards the MG promoter (Fig. 5A, lanes C). Compared to matched control samples, the AP response decreased the DNA binding of STAT5b (Fig. 5A, lanes T). To investigate the DNA binding potential of nuclear matrix-associated STAT5b protein, DNA affinity chromatography and Western analysis were performed with solubilized internal nuclear matrix protein fraction (Fig. 5B). The internal nuclear matrix was isolated from fetal and adult rat livers when the largest difference in STAT5b association with the nuclear matrix was observed (Fig. 3B, lanes 1, 2 and 11, 12, respectively). The internal nuclear matrix protein represents about 11% and 20% of the total nuclear matrix protein content in the fetus and in the adult under basal conditions and during the AP response, respectively. A significant amount of internal nuclear matrix-associated STAT5b displayed a binding affinity toward the promoter region of the MG gene in fetal as well as in adult control livers (Fig. 5B, lanes 1 M). Induction of the AP response was characterized by strikingly reduced amounts of promoter-binding STAT5b in both the fetus and adult (Fig. 5B, lanes 1 M).

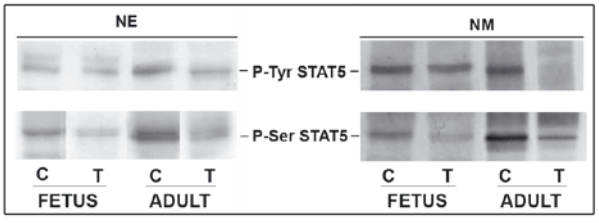

Figure 6. Western-blot analysis of rat liver nuclear extract (NE) and nuclear matrix (NM) proteins with anti-phospho-Tyr-STAT5 and anti-phospho-Ser-STAT5 antibodies. Fetus, 20-day-old fetus; adult, 2.5-month-old adult; C, nuclear proteins isolated from control liver; T, nuclear proteins obtained 12 h after the induction of the AP response.

The activity of members of the STAT family of transcription factors is fine-tuned by post-translational protein phosphorylation (Darnell, 1997). Phosphorylation profiles of STAT5b were examined by immunoblotting with phospho-STAT5-specific antibodies (Fig. 6). Compared to the respective controls, the AP response was accompanied by decreased Tyr phosphorylation in nuclear extracts that were prepared from fetal (by about 10%) and adult (by about 68%) livers (Fig. 6). Compared to the nuclear extract, the nuclear matrix was considerably enriched in Tyr-phosphorylated protein. In the fetus, the AP response was accompanied by decreased Tyr phosphorylation (by about 14%), while Tyr-phosphorylated protein was completely absent from the adult liver nuclear matrix during the AP response. Analysis for serine-phosphorylated STAT5b (Fig 6) revealed that the nuclear matrix was enriched in Ser-phosphorylated STAT5b, and that the AP response was consistently accompanied by a decreased Ser phosphorylation in both nuclear extracts and nuclear matrices in the fetus and adult. During the AP response, Ser phosphorylation of STAT5b decreased by about 47% and 48% in fetal and adult nuclear extracts, respectively, and by about 74% and 38% in the respective nuclear matrices. The observation of a decreased protein phosphorylation during the AP response, together with the established lower binding affinity of STAT5b for the MG promoter, suggests that STAT5b dephosphorylation may be involved in the decreased affinity of STAT5b for binding to the MG gene promoter.

DISCUSSION

Acute inflammation, tissue injury, pregnancy, birth and tissue remodelling are accompanied by proteinase release from macrophages and granulocytes. These enzymes cause damage of healthy tissues (Baumann & Gauldie, 1994). As MG inhibits a wide range of proteinases, it most likely assumes an important role in fetal development and during acute inflammation. Mechanisms that regulate its synthesis remain unclear, especialy during the differentiation process. The results presented here

show that under basal conditions, the relative serum concentrations of MG in fetuses and one day after birth are significantly higher than in the adults (Fig. 1). Compared to the adult, elevated MG levels continued until the third week after birth. They overlapped with the period of full rat liver differentiation. These results are in correlation with MG mRNA levels in the liver that were significantly higher during pre- and postnatal development compared to the adult (Fig. 1). Regardless of the stage of development, turpentine administration led to increased MG serum concentrations between the 2nd and 48th hour of the AP response. The maximal concentration observed at about 24 h (Fig. 2) resulted from an increased transcription of the MG gene occurring between 12 and 14 h (Northemann *et al.*, 1985; Ševaljević *et al.*, 1989).

Considering the potential importance of MG for appropriate tissue developmental remodelling, this work was performed to further elucidate the transcriptional aspect of MG gene expression. IL-6-directed transcriptional regulation of MG in hepatic cells has mostly been correlated with the activation of the DNA binding properties of STAT3 which assumes a principal role in MG regulation during the AP response (Fujitani *et al.*, 1994; Zhang & Fuller, 2000; Bode *et al.*, 2001; Sehgal, 2003). However, literature data suggests that STAT5b also takes part in the transcriptional regulation of MG gene activity. In luteal ovary cells, STAT5 is activated by prolactin and binds to specific sites present in the promoter of the MG gene, originally referred to as the IL-6 response element (RE), based on the activation of this gene by IL-6 in rat liver (Dajee *et al.*, 1998). Prolactin-mediated signalling events also transactivate the MG gene in the granulosa-luteal cells by mechanisms similar to those that regulate cytokine signal transduction of target gene activity in other tissues, such as the mammary gland (Standke *et al.*, 1994) and liver (Akira *et al.*, 1994; Ripperger *et al.*, 1995). STAT5 is also important in growth stimulation and cell differentiation.

Computer analysis of the MG gene promoter (–852/+12) revealed three potential STAT5b binding sites. The predicted STAT5b binding site at position –172/–161 partly overlaps the sequence essential for MG gene transcriptional activity at –170/–165 (Kunz *et al.*, 1989) that has been observed in many AP protein gene promoters. In addition, another STAT5b-binding site overlaps the IL-6 RE which is required for basal MG gene expression and resides at position –209/–160 (Ito *et al.*, 1989) where sequences for several *trans*-factors such as C/EBP and STAT3 have also been found (Ripperger *et al.*, 1995; Bogojević *et al.*, 2003). Therefore, the extended MG gene promoter (–852/+12) that contained all of the sequences held to be important for MG gene expression was used for DNA affinity chromatography and investigation of STAT5b *trans*-activity.

STAT5b was detected in liver cell nuclei throughout development and in the adult (Fig. 3). DNA affinity chromatography followed by immunoblot analysis revealed dynamic changes in STAT5b binding to the extended promoter region of the MG gene. The highest concentration of the active, DNA-binding form of STAT5b was observed in the fetus when significant levels of MG gene expression normally occur. As postnatal development proceeded and MG expression decreased, the level of active STAT5b also decreased. In striking contrast, the stimulation of MG gene expression during the AP response was always accompanied by a decreased affinity of STAT5b for binding to the MG promoter. These results are in good correlation with the results of Ripperger *et al.* (1995) who showed that activated STAT5b was present in the liver of control rats. However, both STAT3 and STAT5b were present in rat liver nuclei during the AP response *in vivo*, suggesting that both regulatory proteins participate in the transcriptional induction of AP genes mediated by IL-6, to which the gene for MG belongs. The results presented in our paper are in agreement with this finding since STAT5b, although present at lower concentrations compared to the respective controls, was found in the nuclear extract and nuclear matrix at 12 h after the induction of the AP response. The results suggest that the interplay of STAT5b with different gene regulatory protein(s), together with its post-translational modification(s), determine the rate of MG gene transcription during constitutive MG expression in the basal state and also in the course of the AP response.

Tyr phosphorylation is essential for STAT nuclear translocation, DNA binding and subsequent transcriptional activation (Darnell, 1997; Aoki & Matsuda, 2000; Wesoly *et al.*, 2007). Immuno blot analysis with the phospho-Tyr antibody showed that the levels of Tyr-phosphorylated STAT5b in the nuclear extract and in the nuclear matrix were higher in both the fetus and in the adult under basal conditions than during the AP response. Considering the importance of Tyr phosphorylation for STAT activity, the observed lower level of Tyr-phosphorylated STAT5b species in the nuclear extracts during the AP response and their complete absence in the adult nuclear matrix are in agreement with the decreased binding of STAT5b to the MG gene promoter during the AP response. It also points to the existence of developmental-stage differences in the nuclear localization of Tyr-phosphorylated STAT5b species. A positive effect of Ser phosphorylation on the transcriptional activities of STAT1 and STAT3 was described by Wen *et al.* (1995). However, although Ser residues in STAT5 and STAT6 are also phosphorylated, a re-

sulting enhancement of transcriptional activity has not been demonstrated convincingly (Wen & Darnell, 1997). Our results suggest that the lower level of STAT5b Ser phosporylation observed during the AP response in the fetus and in the adult correlate with decreased transactivational activity. The lower levels of STAT5b Ser and Tyr phosphorylation during the AP response could partly be ascribed to the decreased amounts of nuclear STAT5b.

We propose that, in addition to post-translational phosphorylation, mechanisms that direct STAT5b nuclear localization contribute to its functioning *in vivo*. An important aspect of transcription factor activity is its subnuclear localization. The nuclear matrix is seen as a nuclear substructure that controls and coordinates gene transcriptional activity by concentrating regulatory factors near their target promoters (Getzenberg, 1994; Fackelmayer, 2004). Many different active regulatory proteins exhibit physiological-, developmental state- and tissue-specific association with the nuclear matrix (Getzenberg & Coffy, 1991; Bidwell *et al*., 1993; van Wijnen *et al*., 1993; Lindenmuth *et al*., 1997; Dinić *et al*., 2005). The observation of consistently higher relative STAT5b concentrations in the nuclear matrix compared to the nuclear extracts at every stage of development and during the AP response, together with the enrichment of the internal nuclear matrix in the active, MG promoter-binding form of STAT5b, lends support to the concept that interaction of STAT5b with the nuclear matrix is functionally important, representing an additional regulatory step that fine-tunes STAT5b activity.

The presented results suggest that, aside from the established mechanisms, molecular processes that control nuclear localization/distribution of STAT proteins influence its activity. Without a doubt, STAT5b participates in mechanisms that control MG gene regulation during development and the AP response. It remains to be established how STAT5b nuclear localization and activity are regulated in basal conditions.

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FREFERENCES

Abbink JJ, Nuijens JH, Eerenberg AJ, Huijbregts CC, Strack van Schijndel RJ, Thijs LG, Hack CE (1991) Quantification of functional and inactivated alpha 2-macroglobulin in sepsis. *Thromb Haemost* **65**: 32–39.

Akira S, Nishio Y, Inoue M, Wang XJ, Wei S, Matsusaka T, Yoshida K, Sudo T, Naruto M, Kishimoto T (1994) Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp 130-mediated signaling pathway. *Cell* **77**: 63–71.

Aoki N, Matsuda T (2000) A cytosolic protein-tyrosine phosphatase PTP1B specifically dephosphorylates and deactivates prolactin-activated STAT5a and STAT5b. *J Biol Chem* **275**: 39718–39726.

Barrett AJ, Starkey PM (1973) The interaction of alpha$_2$-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochem J* **133**: 709–724.

Baumann H, Gauldie J (1994) The acute phase response. *Immunol Today* **15**: 74–80.

Baumann H, Held WA, Berger FG (1984) The acute-phase response of mouse liver. Genetic analysis of major acute phase reactants. *J Biol Chem* **259**: 566–573.

Belgrader P, Siegel AJ, Berezney R (1991) A comprehensive study on the isolation and characterization of the HeLa S3 nuclear matrix. *J Cell Sci* **98**: 281–291.

Berezney R, Mortillaro MJ, Ma H, Wei X, Samarabandu J (1995) The nuclear matrix: A structural milieu for genomic function. *Int Rev Cytol* **162A**: 1–65.

Bidwell JP, van Wijnen AJ, Fey EG, Dworetzky S, Penman S, Stein JL, Lian JB, Stein GS (1993) Osteocalcin gene promoter-binding factors are tissue-specific nuclear matrix components. *Proc Natl Acad Sci USA* **90**: 3162–3166.

Bode JG, Fischer R, Haussinger D, Graeve L, Heinrich PC, Schaper F (2001) The inhibitory effect of IL-1β on IL-6-induced α_2-macroglobulin expression is due to activation of NF-kB. *J Immunol* **167**: 1469–1481.

Bogojević D, Mihailović M, Petrović M, Dinić S, Ivanović-Matić S, Poznanović G (2003) Acute-phase-dependent binding affinity of C/EBPβ from the nuclear extract and nuclear matrix towards the hormone response element of the α_2-macroglobulin gene in rat hepatocytes. *Gen Physiol Biophys* **22**: 279–285.

Cox RA (1968) The use of guanidinium chloride in the isolation of nucleic acids. *Methods Enzymol* **12**: 120–129.

Dajee M, Fey GH, Richards JS (1998) Stat 5b and the orphan nuclear receptors regulate expression of the alpha$_2$-macroglobulin (alpha2M) gene in rat ovarian granulosa cells. *Mol Endocrinol* **12**: 1393–409.

Darnell JEJ (1997) STATs and gene regulation. *Science* **277**: 1630–1635.

Dinić S, Ivanović-Matić S, Bogojević D, Mihailović M, Poznanović G (2000) C/EBPα and C/EBPβ are persistently associated with the rat liver nuclear matrix throughout development and the acute phase response. *Cell Biol Int* **24**: 691–698.

Dinić S, Ivanović-Matić S, Mihailović M, Bogojević D, Poznanović G (2004) Expression of C/EBPδ in rat liver during development and the acute-phase response. *Gen Phys Biophys* **23**: 499–504.

Dinić S, Bogojević D, Petrović M, Poznanović G, Ivanović-Matić S, Mihailović M (2005) C/EBPα and C/EBPβ regulate haptoglobin gene expression during rat liver development and the acute-phase response. *Mol Biol Rep* **32**: 141–147.

Fackelmayer FO (2004) Nuclear architecture and gene expression in the quest for novel therapeutics. *Curr Pharm Design* **10**: 1–10.

Fletcher S, Thomas T, Schreiber G, Heinrich PC, Yeoh GCT (1988) The development of rat α_2-macroglobulin. *Eur J Biochem* **171**: 703–709.

Fritz H, Jochum M, Geiger R, Duswald KH, Dittmer H, Kortmann H, Neumann S, Lang H (1986) Granulocyte proteinases as mediators of unspecific proteolysis in inflammation: a review. *Folia Histochem Cytobiol* **24**: 99–115.

Fujitani Y, Nakajima K, Kojima H, Nakae K, Takeda T, Hirano T (1994) Transcriptional activation of the IL-6 response element in the junB promoter is mediated by multiple Stat family proteins. *Biochem Biophys Res Commun* **202**: 1181–1187.

Getzenberg RH (1994) Nuclear matrix and the regulation of gene expression: tissue specificity. *J Cell Biochem* **55**: 22–31.

Getzenberg RH, Coffey DS (1991) Interaction of tissue specific genes with tissue specific nuclear matrix proteins in rat sex accessory tissues. *J Cell Biol* **115**: 121A.

Glibetić M, Bogojević D, Matić S, Ševaljević Lj (1992) The expression of liver acute-phase protein genes during rat liver development and in response to inflammation of the dam. *Differentiation* **50**: 35–40.

Gonias SL (1992) α_2-Macroglobulin: a protein at the interface of fibrinolysis and cellular growth regulation. *Exp Hematol* **20**: 302–311.

Gorski K, Carneiro M, Schibler U (1986) Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell* **47**: 767–776.

Hendzel MJ, Sun JM, Chen HY, Rattner JB, Davie JR (1994) Histone acetyltransferase is associated with the nuclear matrix. *J Biol Chem* **269**: 22894–22901.

Herbst RS, Babbis LE (1990) Regulation of liver gene expression during development and regeneration. In *Mechanisms of Differentiation* (Fisher PB, Phil M, eds) pp 16–49. CRC Press, Boca Raton, FL.

Hoel PG (1966) *Introductions to Mathematical Statistics*. pp 402–403. John Wiley and Sons, New York.

Ito T, Tanahashi H, Misumi Y, Sakaki Y (1989) Nuclear factors interacting with an interleukin-6 responsive element of α_2-macroglobulin gene. *Nucleic Acids Res* **17**: 9425–9435.

Ivanović-Matić S, Dinić S, Vujošević M, Poznanović G (2000) The protein composition of the hepatocyte nuclear matrix is differentiation-stage specific. *IUBMB Life* **49**: 511–517.

Jackson DA (1997) Chromatin domains and nuclear compartments: establishing sites of gene expression in eukaryotic nuclei. *Mol Biol Rep* **24**: 209–220.

Kadonaga JT, Tjian R (1986) Affinity purification of sequence-specific DNA binding proteins. *Proc Natl Acad Sci USA* **83**: 5889–5893.

Kafatos FC, Jones CW, Efstradiadis A (1979) Determination of nucleic acid sequence homologies and relative concentration by a dot hybridization procedure. *Nucleic Acids Res* **7**: 1541–1552.

Kaufmann SH, Shaper JH (1984) A subset of non-histone nuclear proteins reversibly stabilized by the sulfhydryl cross-linking reagent tetrathionate. *Exp Cell Res* **155**: 477–495.

Khan MM, Shibuya Y, Kambara T, Yamamoto T (1995) Role of alpha$_2$-macroglobulin and bacterial elastase in guinea-pig pseudomonal septic shock. *Int J Exp Pathol* **76**: 21–28.

Kunz D, Zimmermann R, Heisig M, Heinrich PC (1989) Identification of the promoter sequence involved in the interleukin-6 dependent expression of the rat α_2-macroglobulin gene. *Nucleic Acids Res* **17**: 1121–1138.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Laurell CB (1972) Electrophoretic and electroimmunochemical analysis of proteins. *Scand J Clin Lab Invest* **29**: 21–37.

Lemon B, Tjian R (2000) Orchestrated response: a symphony of transcription factors for gene control. *Genes Dev* **14**: 2551–2569.

Lindenmuth DM, van Wijnen AJ, Hiebert S, Stein JL, Lian JB, Stein GS (1997) Subcellular partitioning of transcription factors during osteoblast differentiation: Developmental association of the AML/CBFα/PEBP2α-related transcription factor-NMP-2 with the nuclear matrix. *J Cell Biochem* **66**: 123–132.

Lowry OH, Rosebrough WJ, Farr AL, Randall RJ (1951) Protein measurements with the Folin phenol reagent. *J Biol Chem* **193**: 429–432.

Nickerson J (2001) Experimental observations of a nuclear matrix. *J Cell Sci* **114**: 463–474.

Noda C, Ichihara A (1993) Regulation of liver-specific gene expression. *Cell Struct Funct* **18**: 189–194.

Northemann W, Heisig M, Kunz D, Heinrich PC (1985) Molecular cloning of cDNA sequence for rat α_2-macroglobulin and measurement of its transcription during experimental inflammation. *J Biol Chem* **260**: 6200–6205.

Okubo H, Miyanaga O, Nagano M, Ishibashi H, Kudo J, Ikuta T, Shibata K (1981) Purification and immunological determination of rat α_2-macroglobulin in serum from injured rats. *Biochim Biophys Acta* **668**: 257–267.

Panrucker DE, Lai PCW, Lorscheider FL (1983) Distribution of acute-phase alpha$_2$-macroglobulin in rat fetomaternal compartments. *Am J Physiol* **213**: 138–142.

Reyes JC, Muchardt C, Yaniv M (1997) Components of the human SWI/SNF complex are enriched in active chromatin and are associated with the nuclear matrix. *J Cell Biol* **137**: 263–274.

Ripperger JA, Fritz S, Richter K, Hocke GM, Lottspeich F, Fey GH (1995) Transcription factor Stat 3 and Stat 5b are present in rat liver nuclei late in an acute phase response and bind interleukin-6 response elements. *J Biol Chem* **270**: 29998–30006.

Schaefer U, Brücker B, Elbers A, Neugebauer E (2004) The capacity of alpha$_2$-macroglobulin to inhibit an exogenous protease is significantly increased in critically ill and septic patients. *Shock* **22**: 16–22.

Sehgal PB (2003) Plasma membrane rafts and chaperones in cytokine/STAT signaling. *Acta Biochim Polon* **50**: 583–594.

Sjakste NI, Sjakste TG (2001) Transcription factors and the nuclear matrix. *Mol Biol* **35**: 627–635.

Sottrup-Jensen L, Sand O, Kristensen L, Fey GH (1989) The alpha-macroglobulin bait region. Sequence diversity and localization of cleavage sites for proteinases in five mammalian alpha-macroglobulins. *J Biol Chem* **264**: 15781–15789.

Standke G, Meier V, Groner B (1994) Mammary gland factor activated by prolactin in mammary epithelial cells and acute-phase response factor activated by interleukin-6 in liver cells share DNA binding and transactivation potential. *Mol Endocrinol* **8**: 469–477.

Stein GS, Zaidi SK, Braastad CD, Montecino M, van Wijnen AJ, Choi JY, Stein JL, Lian JB, Javed A (2003) Functional architecture of the nucleus: Organizing the regulatory machinery for gene expression, replication, and repair. *Trends Cell Biol* **13**: 584–592.

Stuurman N, Meijne AML, van der Pol AJ, de Jong L, van Driel R, van Renswoude J (1990) The nuclear matrix from cells of different origin. Evidence for a common set of matrix proteins. *J Biol Chem* **265**: 5460–5465.

Ševaljević Lj, Petrović M, Bogojević D, Savić J, Pantelić D (1989) Acute-phase response to scalding: changes in serum properties and acute-phase protein concentrations. *Circ Shock* **28**: 293–307.

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354.

van Wijnen AJ, Bidwell JP, Fey EG, Penman S, Lian JB, Stein JL, Stein GS (1993) Nuclear matrix association of multiple sequence-specific DNA binding activities related to SP-1, ATF, CCAAT, C/EBP, OCT-1, and AP-1. *Biochemistry* **32**: 8397–8402.

Wegenka UM, Lütticken C, Buschmann J, Yuan J, Lottspeich F, Muller-Esterl W, Schindler C, Roeb E, Heinrich PC, Horn F (1994) The interleukin-6-activated acute-phase response factor is antigenically and functionally related to members of the signal transducer and activator of transcription (STAT) family. *Mol Cell Biol* **14**: 3186–3196.

Wen Z, Darnell JJE (1997) Mapping of Stat3 serine phosphorylation to a single residue (727) and evidence that serine phosphorylation has no influence on DNA binding of Stat1 and Stat3. *Nucleic Acids Res* **25**: 2062–2067.

Wen Z, Zhong Z, Darnell JJE (1995) Maximal activation of transcription by Stat 1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* **82**: 241–250.

Wesoly J, Szweykowska-Kulinska Z, Bluyssen HAR (2007) STAT activation and differential complex formation dictate selectivity of interferon responses. *Acta Biochim Polon* **54**: 27–38.

Zeng C, McNeil S, Pockwinse J, Nickerson J, Shopland L, Lawrence JB, Penman S, Lian JB, Stein GS, Hiebert SW (1998) Intranuclear targeting of AML/CBFα regulatory factors to nuclear matrix-associated transcriptional domains. *Proc Natl Acad Sci USA* **95**: 1585–1589.

Zhang X, Darnell JEJ (2001) Functional importance of Stat3 tetramerization in activation of the α_2-macroglobulin gene. *J Biol Chem* **276**: 33576–33581.

Zhang Z, Fuller GM (2000) Interleukin 1β inhibits interleukin 6-mediated rat γ-fibrinogen gene expression. *Blood* **96**: 3466–3472.