Transcription Factors Stat3 and Stat5b Are Present in Rat Liver Nuclei Late in an Acute Phase Response and Bind Interleukin-6 Response Elements*

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Proteins binding at the interleukin-6 response element of the rat α2 macroglobulin gene were purified by a combination of chromatographic procedures including binding site-specific DNA-affinity chromatography as the principal step. Three polypeptides of 92, 91, and 86 kDa were enriched approximately 6,300-fold from nuclei of rat livers excised 12 h after the induction of an experimental acute phase response. Amino acid sequence analysis identified the 86- and 91-kDa species as two forms of the transcription factor Stat3 and the 92-kDa species as the factor Stat5b. This identification was confirmed by gel mobility shift-supershift experiments using specific antisera for Stat3 and Stat5. Unexpectedly, activated Stat5 was also detected in the nuclei of untreated control rats. cDNA clones representing Stat3 and two isoforms of Stat5b were isolated from a cDNA library prepared with mRNA from rat livers excised at the peak of an experimental acute phase response. Full-length Stat5b, predicted from cDNA, consisted of 786 amino acids, while the variant Stat5bΔ40C lacked 41 amino acids at the COOH terminus. The amino acid sequence of rat Stat5b showed 26.7% overall identity with rat Stat3, 87.3% with sheep Stat5a, 92.5% with murine Stat5, and 98.7% with murine Stat5b.

Hepatic acute phase genes have been divided into two classes according to the cytokines that are their main inducers. Type 1 genes are activated by interleukin-1 (IL-1), interleukin-6 (IL-6), and glucocorticoids; type 2 genes by IL-6 and glucocorticoids, but not by IL-1. Other IL-6-like cytokines such as leukaemia inhibitory factor, IL-11, and oncostatin M have similar characteristic dual function: the transport of the hormonal signal from the cell-surface receptor to the nucleus, and the transcriptional induction of a specific set of target genes. In the absence of an activating signal, Stat factors are present in a functionally latent, monomeric form in the cytoplasm. After ligand binding to a cell-surface receptor, the factors are activated by specific tyrosine phosphorylation through receptor-associated kinases from the JAK/STAT family (14–17). The name Stat factors (signal transducers and activators of transcription) points to a characteristic dual function: the transport of the hormonal signal to the nucleus, where they bind specific hormone response elements (REs) in the control regions of their target genes, and thus induce the transcription of these genes. Development of the full transcription-inducing potential of Stat factors was reported to require additional serine/threonine phosphorylation (18, 19).

The factor Stat3 dimerizes and binds at the IL-6 response element (IL-6 RE) of the rat α2 macroglobulin gene in response of hepatic cells to IL-6 or IL-6-like cytokines (7, 12, 13). In rats and mice, Stat3 was present in liver nuclei in an active DNA-binding state as early as 15–60 min after intravenous injections of IL-6 or lipopolysaccharides (7, 12, 13). It was activated equally fast in cultured hepatoma cells by treatment with IL-6 or IL-6-like cytokines (7, 20). Stat3 was purified from mouse livers 15 min after intravenous injection of IL-6, and the resulting sequence information led to the cloning of murine Stat3 cDNA (12).

In other well studied animal models of the acute phase response, which avoid intravenous injection of proinflammatory agents and thus more closely reflect naturally occurring acute phase responses, the response developed much more slowly. When turpentine or complete Freund’s adjuvant (CFA) were injected intramuscularly or intraperitoneally, then the transcription rates of the rat α2M gene reached maximum
values in vivo at 12–15 h, αM mRNA concentrations at 18 h, and αM plasma protein concentrations at 24–48 h after the injection (21–24). Under these conditions, the response of the αM gene was characteristically slower than that of other type 2 acute phase genes, such as thiotatin and β-fibrinogen (22). Similarly, the αM gene also behaved as a characteristically slow responding gene in cell culture experiments using rat and human hepatoma-derived cell lines (25). Therefore it had been suspected that a particular principle may be responsible for the slow response of the αM gene, which may not be applicable for the faster responding type 2 acute phase genes.

Characteristic early- and late-appearing protein-DNA complexes were assembled between the IL-6 RE and nuclear proteins from hepatoma cells after exposure to IL-6 or IL-6-like cytokines (25, 26). The early-appearing complexes contained tyrosine-phosphorylated Stat3 (7, 12, 13), and their appearance was not prevented by treatment with inhibitors of protein synthesis (cycloheximide; Refs. 7 and 20). However, assembly of the later-appearing complexes was inhibited by cycloheximide and thus was dependent on intermediate protein synthesis (25). The components of the late-appearing complexes that require intermediate protein synthesis have not yet been identified. We hypothesized that the late-appearing complexes may differ in their composition or their posttranslational modifications from the early-appearing complexes, which may provide an explanation for the slow kinetics of induction of the αM gene.

To test this hypothesis, nuclear proteins specifically binding at the IL-6 RE of the αM gene, which were present in rat livers 12 h after intraperitoneal injection of CFA, were studied. At this time the transcription rate of the αM gene was known to reach maximum values in vivo (21). As anticipated, activated, DNA-binding Stat3 was found at this late time, but in addition, DNA-binding Stat5b was also discovered. This finding raised the new hypothesis that Stat5b may also act as a transcription factor in mediating IL-6 effects for some of the late-responding type 2 acute phase genes, including the rat αM gene.

**EXPERIMENTAL PROCEDURES**

Preparation of Nuclear Extracts—Male Sprague-Dawley rats (Charles River; 350–400 g) were injected intraperitoneally with 0.4 ml of CFA (Life Technologies, Inc.) per 100 g of body weight (23). Nuclei were isolated from homogenized tissue by centrifugation through a 2M sucrose cushion containing 10% (v/v) glycerol, 10% (v/v) glycerol (27, 28). Proteins were eluted from purified nuclei with 0.4 M LiCl containing 0.01% Nonidet P-40 and were precipitated with ammonium sulfate. The proteins were resuspended in CP buffer (10 mM Hepes, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol) supplemented with 100 mM KCl, and applied to a 1 ml HR-grade phenyl-Sepharose column (Pharma- macia). Bound proteins were eluted first with CP buffer and subsequently with CP buffer containing 20% (v/v) n-propanol and pooled. The sample was subjected to the iodine strength of DNA-binding buffer and mixed with 1 ml of nonspecific DNA-binding matrix, consisting of the double-stranded synthetic oligonucleotide mTB2 coupled to Sepharose washed with amogram broide and described (33). mTB2 was a functionally inactive mutant of the IL-6 RE, unable to bind specific IL-6 RE binding proteins (31). Flow-through material from the nonspecific DNA-affinity matrix was loaded onto a specific DNA-binding matrix prepared with the double-stranded synthetic oligonucleotide TB2, a tandem dimer of the IL-6 RE core element of the rat αM gene (25, 31). After a wash step with CP buffer containing 250 mM KCl, bound proteins were eluted with CP buffer containing 500 mM KCl and extracted with Stratagene™ resin (Stratagene). At this point, material from 96 rat livers was combined for a large scale preparation. The resin was then mixed with SDS sample buffer (34) and boiled for 2 min, and the sample was loaded onto a 7.5% SDS-polyacrylamide gel. The proteins were separated by elec- trophoresis and then stained with Coomassie BrBlue. Relevant proteins were excised, destained, and partially digested inside the gel with lysyl-endopeptidase (35). Resulting peptides were extracted and separated by reverse-phase high pressure liquid chromatography. Well resolved peptides were sequenced by automated Edman degradation using an Applied Biosystems model 477A sequencer.

Cloning of Stat Factor cDNAs—Portions of rat Stat3 and Stat5 cDNAs were amplified by polymerase chain reaction (PCR) using first-strand cDNA prepared from rat liver mRNA and the following set of primers derived from published Stat3 and Stat5 sequences of other animal species (12, 36): Stat3 (sense, 5′-GACAGAGAAGAGCCGATGATG; antisense, 5′-ACGATCTCTTCTCTCCAGC) and Stat5 (sense, 5′-TTCTCATCAGAGAAGCCGAC; antisense, 5′-ACTCCGAAATGTAGTATCGT). PCR reactions were performed for 35 cycles with the steps (94°C, 1 min; 56°C, 1 min; 72°C, 2 min) for each cycle. PCR products were cloned in the plasmid vector pUC18 using a blunt-end ligation kit (Pharma- macia), and the identity of the products was confirmed by double-stranded plasmid sequencing. Probes specific for rat Stat3 and Stat5 were obtained by recovering the cloned fragments from the plasmid vectors and labeling them with [32P]dCTP using a random hexamer priming kit (Pharma- macia). These probes were used to screen two cDNA libraries prepared from rat liver RNA obtained 4 or 12 h, respectively, after intraperitoneal injection of CFA (37). The libraries were produced in the phage vector λ-ZapI using Gigapack Gold packaging mix (Stratagene) and the supplier's protocols.

Computer-assisted Collection and Interpretation of DNA Sequence Data—DNA sequence data were assembled into a data base using the Staden programs (38). For Stat3, the total data base contained 11,231 nucleotides of primary sequence data for a final length of the double- stranded cDNA sequence of 2,924 base pairs. Thus, each nucleotide of the final sequence has been determined on average 3.8 times. Ninety-eight percent of the sequence was determined on both strands. For Stat5b, the total data base consisted of 9,214 nucleotides of primary sequence data; the final length of the double-stranded cDNA sequence was 2,615 base pairs. Each nucleotide was sequenced 3.5 times on average, and 99% of the final sequence was determined on both strands. For Stat5a, 3400C, each nucleotide of the relevant portion of the cDNA done was sequenced, on average, 2.6 times. Sequence alignments were determined using the program package Geneworks for the Macintosh (IntelliGenetics).

**RESULTS**

Slow Induction of a Specific Protein-DNA Complex Containing the IL-6 RE of the αM Gene during an Acute Phase Response in Rats—A characteristic, sequence-specific protein-DNA complex (complex II, Fig. 1) had previously been reported to be assembled between the IL-6 RE of the rat αM gene and nuclear proteins from livers of rats undergoing an acute phase response (26, 31). A similar complex had also previously been obtained with nuclear extracts from cultured rat and human hepatoma cells treated with IL-6 or IL-6-like cytokines (25, 39). Complex II generated with extracts from rat livers excised at
various times after injection of CFA appeared with slow kinetics (Fig. 1) comparable to the slow kinetics of the transcription rate of the α2M gene, which reached maximum values 12 h after the injection of turpentine or CFA (21). The parallel slow increase of the transcription rate and of complex II in vivo led us to suspect that complex II may contain transcription factors mediating the cytokine-induced transcriptional increase of the α2M gene. In particular, the late-appearing complex II was expected to contain components that may provide an explanation for the characteristic slow induction of the α2M gene.

Purification of IL-6 RE-binding Proteins from Rat Livers—To test this prediction, it was necessary to analyze the protein composition of the late-appearing complex II. As a first step in this direction, nuclear proteins binding at the IL-6 RE were purified from rat livers excised late (10–12 h) after the injection of CFA. A purification scheme consisting of three conventional chromatographic steps and a final binding site-specific DNA-affinity step (Fig. 2) was designed. The conventional steps were needed to remove a nuclease activity, which otherwise damaged the DNA-affinity matrix. None of the three initial chromatographic steps alone (Sepharose SP, Sepharose Q, phenyl-Sepharose) was sufficient to remove this nuclease activity, but their combination was sufficient. Purification was monitored by assaying column fractions for their ability to form the characteristic complex II with TB2. Surprisingly, the ability to form complex II was retained after passage of the proteins over four different columns (Fig. 3A). SDS-polyacrylamide gel electrophoresis showed substantial enrichment of three polypeptides of 86, 91, and 92 kDa (Fig. 3B). The overall enrichment of these species was greater than 6,300-fold with a calculated final yield of 27% (Table I). A typical large scale experiment produced 10–20 μg of purified protein from 96 rat livers.
Purification and Cloning of Rat Liver Stat3 and Stat5b

TABLE I

| Protein          | Activitya | Specific activity | Yield | Purification |
|------------------|-----------|------------------|-------|--------------|
| Rat liver        | 330 (g)   | 19,500           | 82    | 100          | 1              |
| Nuclear extract  | 234       | 16,570           | 184   | 85           | 2.2            |
| Sepharose SP     | 91        | 14,430           | 656   | 74           | 7.9            |
| Sepharose Q      | 22        | 8,190            | 1,638 | 42           | 19.7           |
| Pool phenyl-Sepharose | 5.0  | 5,260            | >526,000 | 27 | >6,337   |

a Activity of DNA-binding proteins in arbitrary units. 1 unit is defined as the amount of protein required to shift approximately 10 fmol of radiolabeled double stranded oligonucleotide TB2 in a gel mobility shift assay.

Protein Sequence Analysis—A first attempt to obtain NH2-terminal aminoacidic sequences for these three polypeptides failed, presumably due to blocked N termini. In a second attempt, these species were excised from preparative SDS-polyacrylamide gels and subjected to partial proteolysis. Resulting peptides were fractionated by high pressure liquid chromatography. Useful sequence was obtained from seven peptides (Table II). Computer-assisted comparison with public data bases revealed that five peptides from the 86- and 91-kDa proteins were derived from Stat3 and two peptides from the 92-kDa protein represented the rat homolog of a protein previously cloned and sequenced from sheep mammary gland epithelial cells, a factor called MGF/Stat5a (36).

Activated Stat5 Is Present in Nuclear Protein Extracts from Both Normal and Acute Phase Rat Liver Cells—Nuclear protein extracts were prepared from normal rat livers and from rats late in an experimentally induced acute phase response. The extracts were combined with radiolabeled oligonucleotide TB2 and antibodies specific for Stat1, Stat3, or Stat5, and gel mobility shift-supershift experiments were performed. As a result, the characteristic slowly migrating complex I, which was routinely seen with the TB2 probe and extracts from untreated rats (Fig. 1), was completely shifted to a slower mobility with two different anti-Stat5 sera, but not detectably with anti-Stat1 or anti-Stat3 sera (Fig. 4). The faster migrating complex II was assembled between nuclear extracts from rats undergoing an acute phase response and the TB2 probe. This complex showed a partial but reproducibly positive reaction with both anti-Stat5 sera, no reaction with anti-Stat3, and was shifted to completion with anti-Stat3. Thus, unexpectedly, activated, DNA-binding Stat5 was present in the nuclei of both untreated control rats and in rats undergoing an acute phase response. Complex I apparently consisted exclusively of activated Stat5 with no detectable Stat1 and Stat3, while complex II contained predominantly Stat3 and only a minor proportion of Stat5. Surprisingly, complex II was shifted completely with anti-Stat3, suggesting that it was not a mixture of two sub-populations, each consisting of homomultimers of either Stat3 or Stat5 alone, but that every complex contained at least some Stat3 molecules. These results confirm the protein purification and protein sequencing results.

Cloning and DNA Sequence Analysis of Stat3 and Stat5b cDNAs—Two cDNA libraries were prepared from mRNA of rat livers excised 4 and 12 h after induction of an experimental acute phase response. These libraries were screened with cloned PCR fragments of rat Stat3 and Stat5 cDNAs that had been generated from rat liver mRNA by using primers derived from the published sequences of the corresponding murine and ovine factors. Two sets of cDNA clones were obtained, which corresponded to Stat3 and Stat5b, respectively. They were identified by restriction mapping and cDNA sequence analysis. The complete cDNA and protein coding sequences of rat Stat3 and Stat5b are given in Figs. 5 and 6, respectively. Two species of Stat5, Stat5a and Stat5b, had previously been published for mice (40, 41). Sequence comparison suggested that the sequence of our rat clones matched the Stat5b sequence more closely than the Stat5a sequence, and therefore we concluded that our clones represented rat Stat5b.

Two Isoforms of Stat5b Differ in Their COOH-terminal Portion—The set of Stat5b cDNA clones contained a subset with a difference in the coding sequence for the COOH terminus. Full-length rat Stat5b had a predicted length of 786 amino acids (Fig. 6A). The variant clones had a predicted identical cDNA and protein sequence in the NH2-terminal portion, but contained a stop codon and then diverging cDNA sequence, and coded for an isoform of the protein that lacked the COOH-terminal 41 amino acids. This isoform was designated Stat5b (36).
between rat Stat3 and Stat5b, 98.7% between rat and mouse Stat5b, 92.5% between rat Stat5b and mouse Stat5a, and 87.3% between rat Stat5b and sheep Stat5a/mammary gland factor was calculated by computer-assisted sequence comparison (Fig. 7; Ref. 38). The SH2 Src homology domain, a characteristic functional domain of Stat factors, showed an even higher degree of identity between rat Stat3 and Stat5b than the overall sequence (42.1%). A sequence essential for the recognition of specific DNA binding sites has recently been identified between amino acid residues 400 and 500 of Stat3 (42). The corresponding region of rat Stat5b also showed a higher degree of sequence conservation with Stat3 than the molecules overall. Within this area, two subdomains, BD1 and BD2 (binding determinants 1 and 2), showed a particularly high degree of conservation. These areas had been defined by others as essential for the DNA recognition function (42).

**DISCUSSION**

The main new results and conclusions drawn from this study were as follows.

1) An efficient purification scheme for Stat3 and Stat5b from rat liver nuclei was designed and optimized that allowed the isolation of Stat factors in their natural state in the 10–μg (100 pmoles).

2) Activated Stat5 was present in liver nuclei of control rats, prior to the onset of an inflammatory response.

3) Both Stat3 and Stat5b were present in rat liver nuclei in an acute phase response \textit{in vivo}, suggesting both may participate in the transcriptional induction of some type 2 acute phase genes mediated by IL-6. Stat1 was not enriched by our purification scheme.

4) cDNAs corresponding to two isoforms of Stat5b, the full-length form and a Δ40C variant lacking the COOH-terminal 40 C-terminal amino acids, were cloned.
amino acids, were isolated, suggesting each factor may play a functional role.

A few comments may be added to each of these points. Schibler and co-workers (27) established that it is an advantage for the purification of nuclear transcription factors, to start with purified nuclei rather than with whole cell extracts. Consequently, their procedures for the purification of nuclei from rat livers were adapted to our system. To reach the 100 pmol range of pure proteins needed for partial amino acid sequence analysis, it was necessary to start each preparation

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**Fig. 6.** cDNA and derived amino acid sequence of rat Stat5b. A. cDNA sequence for the coding region and portions of the 5'- and 3'-untranslated regions of the full-length Stat5b mRNA. The two peptide sequences obtained from the purified 92-kDa protein by amino acid sequence analysis (Table II) are underlined. N° position, at which a stop codon is found in the mRNA for the Stat5bA40C protein (B). cDNA sequence of the portion of the mRNA for the Stat5bA40C variant that covers the region containing the stop codon (*) and part of the sequence of the 3'-untranslated region. This sequence diverges from the corresponding sequence of the mRNA for full-length Stat5b, indicating that the variant most likely was generated by alternative splicing of the same initial transcript.
with 8–10 livers and to pool nuclear extracts from 50–100 livers, before continuing with the chromatographic steps. To process 8 or more livers in one batch, a custom-designed homogenizer was used (28). The procedures for the extraction of proteins from purified nuclei (27) were optimized to reach the highest possible yield of complex II-forming proteins, which resulted in the modifications of the published procedures described above.

Several chromatographic matrices were tested to remove nucleases contained in the initial nuclear protein extracts, but no single matrix achieved their complete removal. Reduction of the nuclease activity to acceptable levels that no longer damaged the DNA-affinity column required the combination of three conventional chromatographic steps.

The synthetic oligonucleotide TB2, representing a tandem repeat of the core IL-6 RE of the rat $\alpha_2M$ gene, preferentially enriched proteins with high binding affinity for this particular version of the IL-6 RE. This version differs in one nucleotide from the consensus IL-6 RE (42) and consequently shows a characteristically skewed spectrum of binding properties for DNA-binding factors. At the time, when this procedure was established, it was unknown which factors were to be expected in complex II. The procedure was designed to purify any factors binding at this IL-6 RE. It is now clear that the TB2 sequence introduced a bias in favor of Stat5b, which binds better at this sequence than at other known binding sites for Stat factors such as the gamma-activated site and serum-inducible element (11). However, this bias is not an artifact of our procedure, but rather reflects a natural preference of the IL-6 RE of the rat $\alpha_2M$ gene for Stat5b. The fact that Stat5b was isolated with our procedure from rodent livers, while it had escaped detection by other authors (7, 12, 13), is probably due to the choice of the TB2 oligonucleotide as the DNA-affinity reagent. In two cases an oligonucleotide was used as the affinity reagent that contained a mutated IL-6 RE sequence (7, 12). The sequence had been mutated to convert it to a palindrome, which is now known to bind Stat1 and Stat3 more strongly than Stat5b. This may explain why those authors only enriched Stat3 but not Stat5b. A second group of authors used the sequence corresponding to the IL-6 RE from the human $\alpha_2M$ gene as the

FIG. 7. Comparison of the amino acid sequences of the two isoforms of Stat5. Identical amino acid residues are boxed. Open bars, position of Src homology domains type 2 and type 3 (SH2 and SH3, respectively). Black mark, single tyrosine that is phosphorylated by JAK/tyk2 kinases upon activation of Stat factors. BD1 and BD2, binding determinants 1 and 2 (sequences within amino acids 400–500 of Stat1 and Stat3 that determine the specific DNA sequences at which these factors bind) (42). Within this overall region, BD1 and BD2 showed a particularly high degree of conservation among the different Stat factors, and these subdomains were shown by mutagenesis to directly affect the DNA binding specificity of Stat1 and Stat3 (42). -, no amino acid at this position; : , no consensus amino acid at this position; m, murine; r, rat; sh, sheep.
affinity reagent, which differs from that of the rat α2M gene (13). The human α2M gene is not a strongly responding acute phase gene. These authors enriched Stat1 and Stat3 from rat livers but not Stat5b, probably again because their affinity reagent was not binding Stat5b as strongly as TB2. The particular binding properties of the TB2 oligonucleotide also provide a partial explanation for the absence of Stat1 among the proteins enriched by our procedure.

This purification scheme can now be used to purify Stat5b in large quantities in its natural state, for example to study naturally occurring posttranslational modifications. In view of the less severe problems with nucleases encountered by other authors in the mouse system (12), the procedure presented here can probably be further improved by using magnetic beads coated with the affinity oligonucleotide to accelerate the purification. Overall, it is remarkable that in this procedure Stat3 and Stat5b co-purified over four chromatographic steps, demonstrating a great similarity of their chromatographic properties. It is further remarkable that after a 6,300-fold purification the yield was still as high as 27% (Table I). This estimation of the final yield may be an overestimate due to the difficulty of correctly assessing the content of these factors in the initial crude nuclear lysates.

Assuming the 27% figure for the yield to be correct, 10 μg of Stat5b purified from 100 livers would correspond to approximately 2,700 molecules of Stat5b/cell. If the yield was overestimated by 1 order of magnitude, then the content would be 27,000 molecules of Stat5b/cell. Thus a rat liver hepatocyte at the peak of an acute phase response contains at least 2,000–3,000 molecules of Stat5b/cell and possibly up to 10-fold more. This is a normal copy number for nuclear transcription factors. Copy numbers vary from 600 molecules/cell for hepatocyte nuclear factor 1 (HNF1) to several 100,000 molecules/cell for the general factor SP1 (4, 5).

We cannot yet definitively state that Stat5 was present in hepatocytes as opposed to other cell types of the liver. However, additional experiments demonstrated the presence of Stat5 in hepatoma cells by immunoreactivity with Stat5-specific antibodies. While we cannot exclude the presence of Stat5 in other cell types of the liver, the finding that it is present in hepatoma cells makes it very plausible that it should also be present in hepatocytes in vivo.

The purified new proteins were identified as Stat3 and Stat5b by sequence comparison. The cDNA sequence of our Stat5b clones (Fig. 6) showed a 98.7% match with the published mouse Stat5b sequence, but only a 92.5% and an 87.3% match with the mouse and ovine Stat5a sequences, respectively. The peptide sequences obtained for the purified proteins (Table II) showed a complete match with our cDNA-derived amino acid sequences for Stat3 and Stat5b (Figs. 5 and 6). It was possible to obtain distinct PCR products for both Stat5a and Stat5b by using PCR primers derived from the published murine Stat5a sequence in combined reverse-transcription PCR experiments with rat liver mRNA. Thus, rat livers contain mRNAs for both Stat5a and Stat5b, but so far peptide sequences and cDNA clones have been obtained only for Stat5b. Stat5a may have escaped detection or may have been less strongly enriched by affinity chromatography with the TB2 oligonucleotide than Stat5b. Most likely, Stat5a was present in lower copy numbers per cell than Stat5b, and therefore may not have been detected among the purified factors. The 86- and 91-kDa forms of Stat3, which were purified in this study, probably represented the same polypeptide in different secondarily modified states, most likely differing in their patterns of serine-threonine phosphorylation (18, 19). Until now, only one type of Stat3 mRNA has been found in mice, rats, and humans, and it is therefore unlikely that these two different proteins were derived from different mRNA species.

The gel mobility shift-supershift experiments (Fig. 4) confirmed the presence of Stat5 in rat liver nuclei. The surprising finding was that active Stat5, capable of binding at the IL-6 RE, was present also in the nuclei of untreated control rat livers. The signal causing its activation and the functional significance of activated Stat5 in the nuclei of normal liver cells are unknown. During an acute phase response, the major factor binding at the IL-6 RE clearly was Stat3. However, small amounts of Stat5 were present, and these probably represent the proteins purified in our procedure. The fact that complex II was shifted completely with anti-Stat3 serum and that every complex thus contained at least some Stat3 (Fig. 4) does not imply the formation of heterodimers between Stat3 and Stat5b. No evidence for Stat3/Stat5b heterodimer formation was obtained, when monomeric probes were used instead of the dimeric TB2 (43). Thus, the majority of complexes II probably consisted exclusively of Stat3, and the minority of mixed Stat3/Stat5b complexes most likely contained one homodimer each of Stat3 and 5b bound at each of the two tandemly repeated IL-6 REs of TB2.

The presence of Stat5b in liver cells was unknown until these data were prepared for publication. Recently, factors immunologically reactive with anti-Stat5 sera were found in liver cells and were reported to be activated by growth hormone or epidermal growth factor (44, 45). Our observation that Stat5b was present in an active, DNA-binding form in rat liver nuclei late in an acute phase response and that it preferentially bound at TB2 suggests that it may play a functional role in mediating the IL-6-induced transcription of certain target genes, including the α2M gene. Indeed, initial functional evidence has recently been obtained in transfection studies to support this view. The cDNA coding for rat Stat5b was placed into an expression vector and transfected into human hepatoma cells. A reporter construct carrying a chloramphenicol acetyltransferase reporter under the control of multiple copies of TB2 was co-transfected. After activation of the JAK/Stat signal cascade by treatment of the cells with suitable cytokines, a cytokine-induced increase of chloramphenicol acetyltransferase reporter activity was obtained, which was proportional within a certain range to the dose of the transfected expression construct.

These data were interpreted to show that Stat5b indeed can function as a transcription factor in rat liver cells, capable of mediating cytokine-induced transcription of particular target genes. These data are not proposed to constitute definitive proof of this hypothesis, but they provide preliminary evidence in its favor. Similarly, transfection studies with Stat3 expression constructs into human hepatoma cells were performed and provided evidence in support of the notion that our rat Stat3 cDNA clones code for a functionally active transcription factor capable of mediating cytokine-induced transcription of specific target constructs (46, 47).

A carboxyl-terminally truncated isoform of p91/Stat1α, referred to as p84/Stat1αδ, had previously been discovered and shown to be generated by alternative splicing (19, 48). This shorter variant has been discussed as a dominant negative inhibitor of Stat1, capable of competing with the full-length form for the same DNA-binding site, but no longer capable of transcriptional transactivation (48). Consequently, it was pro-

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3 G. Crabtree, personal communication.
4 R. Tjian, personal communication.
5 G. Hocke, unpublished data.
6 C. F. Lai, Y. Wang, K. Morella, J. Ripperger, G. Fey, and H. Baummann, unpublished data.
posed that the COOH-terminal 38 amino acids of Stat1 are essential for its transactivator function (19). A carboxy-terminally deleted version is now also known for Stat5b. Thus, the COOH-terminal 40 amino acids of both factors may share a common function. However, the XPXSP-motif conserved in the C termini of Stat1, Stat3, Stat4, and ovine Stat5a, which has been discussed as a potential target for mitogen-activated protein kinase-like activities (19), is absent in mouse and rat Stat5b. Potential specific functions for each isoform, their relative importance, and the mechanism of generation of the short isoform are currently unknown.

With the availability of these cDNA clones the initial question, whether Stat5b plays a specific role in the delayed transcriptional activation of the α2M gene in comparison with other type 2 acute phase genes, can now be addressed experimentally.

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Note Added in Proof—Since submission of this manuscript, the existence of Stat3, an carboxyl-terminally truncated isoform of Stat3, was reported. Schaefer, T. S., Sanders, L. K., and Nathans, D. (1995) Science 267, 285–288.

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