Regulation of tyrosine aminotransferase gene expression by glucocorticoids in quiescent and regenerating liver

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Following 70 % hepatectomy, the induction of tyrosine aminotransferase mRNA by glucocorticoids was marginal at 1.5 h, significantly impaired between 3 and 8 h and, at 16 h post-hepatectomy, reached a value approx. 5-fold the basal level, similar to the level observed in quiescent liver. The fold induction of the mRNA was accounted for by a similar fold activation of transcription of the gene by glucocorticoids in regenerating but not in quiescent liver; in the latter, activation of transcription was marginal in spite of glucocorticoid-induced hypersensitivity to cleavage by DNase I at the glucocorticoid-dependent enhancer of the gene. The possibility that in quiescent liver glucocorticoids

act at a transcriptional step beyond initiation, increasing the rate of elongation or overcoming a blockage in elongation, was excluded. However, a similar fold induction was determined for total and nuclear tyrosine aminotransferase mRNA in the presence of glucocorticoids, suggesting that in quiescent liver glucocorticoids promote efficient maturation of the tyrosine aminotransferase primary transcript. Thus a glucocorticoid-induced nuclear post-transcriptional up-regulation apparently compensates for impaired activation of transcription of the tyrosine aminotransferase gene by glucocorticoids in quiescent liver.

INTRODUCTION

Expression of the gene coding for tyrosine aminotransferase (TAT; EC 2.6.1.5) is restricted to parenchymal cells of the liver. Basal expression of the gene has been reported to depend on: (a) promoter sequences binding to liver-enriched and ubiquitous transcription factors [1,2], (b) a constitutive liver-specific enhancer located 11 kb upstream of the start site of transcription [3] and endowed with binding sites for ubiquitous and liver-enriched transcription factors, the latter of the hepatocyte nuclear factor 3 (HNF3) family [4], and (c) a cAMP-responsive element at –3.6 kb [3,5], apparently rendered liver-specific by coupling to an element binding the liver-enriched factor HNF4 [6].

TAT gene expression is under composite hormonal control, being activated by glucocorticoids and glucagon (via cAMP) [7] and repressed by insulin [8,9]. In primary hepatocytes, as well as in randomly growing hepatoma cells, glucocorticoid-receptor-mediated activation of gene transcription accounts for the induction of TAT mRNA [10,11] and is closely correlated with the appearance of a DNase I-hypersensitive site over the glucocorticoid-dependent enhancer at –2.5 kb [11,12], due to displacement of two specifically phased nucleosomes [11,13]. In fact, glucocorticoid-induced hypersensitivity at –2.5 kb was also detected in the liver [14]. Glucocorticoid receptor binding to a glucocorticoid-responsive element (GRE) within one of the nucleosomes is thought to be the first of a series of events leading to nucleosomal disruption and recruitment of transcription factor binding to regulatory elements in the disrupted chromatin [11,15]. Significantly, in addition to the GRE, the glucocorticoid-dependent enhancer contains a CACCC element and a CCAAT box, protein binding to both of which is established and maintained only in the presence of glucocorticoids [11], as well as binding sites for members of the CCAAT enhancer binding protein [16], HNF3 [6,15,16] and Ets [17] families of transcription

factors. The activity of the glucocorticoid-dependent enhancer is stimulated by two remote elements, one corresponding to a constitutive DNase I hypersensitive site at –5.4 kb [18] and the other to a glucocorticoid modulatory element at –3.6 kb, enhancing glucocorticoid responsiveness at subsaturating, but not receptor-saturating, concentrations of glucocorticoids [19].

Glucocorticoid induction of TAT gene expression has been reported to vary during the cell cycle [20–22]. Using synchronized hepatoma tissue culture cells these studies showed that, although the enzyme could be synthesized in all phases of the cell cycle, induction by glucocorticoids occurred only during the middle and late G1 phases and the entire S phase [20]. Experiments with pre-induced synchronized cells revealed rates of enzyme synthesis similar to induced levels prevailing during early G1 phase even in the absence of inducing steroids; these rates started to decrease as cells entered the glucocorticoid-responsive period 2–3 h into G1 [21,22]. It is well known, however, that the phenotype of hepatoma cells in culture deviates from that of liver cells in tissue. We investigated, therefore, whether glucocorticoid induction of TAT gene expression is perturbed during hepatocyte proliferation following 70 % hepatectomy. In this *in vivo* system, more than 90 % of the hepatocytes progress synchronously to S phase at 16 h and to mitosis at 30–34 h post-hepatectomy (reviewed in [23]). We have previously reported a rapid increase in the TAT mRNA level within 1 h of 70 % hepatectomy, primarily due to efficient nuclear processing of the primary transcript; levels had returned to that in quiescent liver at 16 h post-hepatectomy, largely due to changes in the rate of transcription of the gene [24]. The present work shows that glucocorticoid induction of TAT mRNA in regenerating liver is marginal for 1–2 h following hepatocyte transition from quiescence to proliferation and that induction progressively becomes more pronounced, as basal TAT mRNA levels begin to decline. Regulation of TAT mRNA levels by glucocorticoids is primarily

Abbreviations used: TAT, tyrosine aminotransferase; GAPDH, glyceraldehyde-phosphate dehydrogenase; GRE, glucocorticoid-responsive element; HNF, hepatocyte nuclear factor.

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exerted at the transcriptional level in regenerating, but not in quiescent, liver; in the latter, glucocorticoids appear to act through nuclear post-transcriptional mechanisms.

EXPERIMENTAL

Animals

Male Wistar rats (body wt. approx. 150 g) were adrenalectomized 4 days before the experiment and maintained thereafter on standard pellet diet and 0.9% NaCl. Removal of 70% of the liver was performed under ether anaesthesia, using the procedure of Higgins and Anderson [25]. Where indicated, rats were injected intraperitoneally with triamcinolone acetonide (Sigma) in ethanol (15 µg/100 g body mass), dexamethasone (Sigma) in ethanol (10 µg/100 g body mass) or cycloheximide (Sigma) in 0.9% NaCl (5 mg/100 g body mass). At the indicated times rats were killed, and livers were perfused with ice-cold 50 mM Tris, pH 8.0, 0.14 M NaCl and either directly homogenized (preparation of nuclei) or immediately frozen in liquid N₂ and stored at -70 °C (for preparation of total RNA).

Isolation of RNA and Northern blot analysis

Isolation of total and nuclear RNA, separation on formaldehyde/agarose gels, blotting on to nylon membranes and hybridization to ³²P-labelled probes were carried out as described previously [24]. The following probes were used: a mixture of rat TAT clones EH 0.94, EH 2.45 and EE 1.05 [7], and the rat full-length glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA clone pRGAPD.13 [26]. After autoradiography, the mRNA bands were cut out and radioactivity was counted in a liquid scintillation counter. Nuclear TAT mRNA levels were quantified in arbitrary densitometric units using a laser scanning densitometer (Molecular Dynamics).

Isolation of nuclei and run-on transcription assay

Isolation of cytoplasm-free nuclei, run-on transcription, isolation of ³²P-labelled transcription products and hybridization to filter-bound DNA were performed as already described [24]. Equal amounts of radioactivity from the radiolabelled run-on transcription products (between 4 × 10⁶ and 12 × 10⁶ c.p.m.) were hybridized to 4.5 µg of GAPDH cDNA, 4.5 µg of pUC8 plasmid DNA and a mixture of 1.5 µg of each of the three TAT probes EH 0.94, EH 2.45 and EE 1.05, separately immobilized on to the same nylon filter. Following hybridization and extensive washing, the radioactivity bound to each of the immobilized probes was counted in a liquid scintillation counter. ³H-labelled TAT RNA transcribed from plasmid SP6-cTAT3 [10] was used to correct for hybridization efficiency. The transcription rate was calculated as:

$$\text{Rate (p.p.m.)} = \frac{\{(\text{c.p.m. of TAT}) - (\text{c.p.m. of pUC8})\}}{(\text{c.p.m. input})} \times (100/\text{efficiency}) \times (11/4.45)$$

where 11 kb is the size of rat TAT gene and 4.45 kb is the length covered by the probes used [7].

Mapping of DNase I hypersensitive sites

For DNase I digestion, nuclei were pelleted for 2 min at 1800 g, washed once with 15 mM Tris, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA and 1 mM PMSF containing 2 mM MgCl₂, and resuspended in the same buffer containing 6 mM MgCl₂ at a concentration of 300 µg of DNA/ml. DNase I digestion was performed at 4 °C for 10 min

using increasing concentrations of the enzyme, and the reaction was stopped by addition of EDTA to a final concentration of 10 mM. DNA was purified as described by Fritton et al. [27], recovered by three ethanol precipitations and digested overnight with 3 units of *Eco*RI/µg of DNA. For indirect end-labelling, DNA was electrophoresed in a 1.4% agarose gel at 2 V/cm and transferred to a nylon membrane (GeneScreen Plus; NEN) using the salt transfer protocol recommended by the manufacturer. Genomic DNA, prepared according to Sambrook et al. [28] and digested with the appropriate restriction enzymes (see legend to Figure 3C), was used as electrophoresis marker. Following transfer, the membrane-bound DNA was denatured, neutralized and fixed according to the manufacturer's instructions and then hybridized to a ³²P-labelled DNA fragment of the 5'-flanking region of the TAT gene (*Xba*I positions -2950 to -2561 of pTAT-CAT [12]). Prehybridization, hybridization and washing conditions were as previously described for Northern blots [24].

Quantitative immunoblotting analysis of the glucocorticoid receptor

PAGE analysis, electroblotting of rat liver nuclear proteins and detection of the glucocorticoid receptor using rabbit anti-receptor serum III and ¹²⁵I-labelled Protein A were carried out as previously described [24,29]. Following autoradiography, the nitrocellulose membrane was developed with peroxidase-conjugated second antibody and the receptor was quantified as already reported [30] using a calibration curve obtained with known amounts of rat liver cytosolic receptor, as determined from the amount of specifically bound [³H]triamcinolone acetonide.

RNase H digestion

Digestion was performed as described by Paek and Axel [31] with minor modifications. Total or nuclear RNA and a synthetic oligonucleotide complementary to the TAT mRNA sequence from nt 1747 to nt 1766 [32] were mixed (2 µg of RNA plus 0.1 µg of oligonucleotide per µl) and heated to 65 °C for 10 min. An equal volume of 2 × RNase buffer (80 mM Tris, pH 8.0, 8 mM MgCl₂, 2 mM dithiothreitol, 60 ng of BSA) was then added and the samples were incubated for 15 min at 42 °C in the presence or absence of oligo(dT) (0.5 µg/µl). RNase H digestion was performed for 30 min at 37 °C with *Escherichia coli* RNase H (0.05 unit/µl). The RNase-resistant fragments were phenol/chloroform-extracted, ethanol-precipitated, electrophoresed in 1.5% agarose/0.66 M formaldehyde gels and subjected to Northern blot analysis using probe EE 1.05 [7].

RESULTS

Partial hepatectomy transiently impairs induction of TAT mRNA by glucocorticoids

Partially hepatectomized rats that had been adrenalectomized 4 days previously were injected with a fully inducing dose of triamcinolone acetonide at various times after hepatectomy and liver total RNA, isolated 2 h after injection, was subjected to Northern blot analysis using both TAT and GAPDH probes. Preliminary experiments established that, in accordance with published data [7], injection of the hormone leads to rapid accumulation of TAT mRNA in the livers of adrenalectomized rats which is near-maximal 2 h after treatment (results not shown). Figure 1(A) shows Northern blot analysis of basal and glucocorticoid-induced TAT mRNA levels at various times after hepatectomy. Evidently, a several-fold increase in TAT mRNA levels was observed in quiescent liver after glucocorticoid treatment of adrenalectomized rats. Following 70% hepatectomy,

Figure 1 Effect of 70% hepatectomy on the induction of TAT mRNA by glucocorticoids

Total RNA, isolated from two livers or liver remnants from triamcinolone acetonide (TA)-treated or untreated adrenalectomized rats at the indicated times after hepatectomy, was subjected to Northern blot analysis using TAT and GAPDH probes (**A** and **C**). The lower panel in (**A**) shows the part of the ethidium-bromide stained gel corresponding to 28 S rRNA. (**B**) The amount of radioactivity hybridized with the TAT mRNA band was normalized to that hybridized with the respective GAPDH mRNA band. Fold induction was calculated as the ratio of the hormone-induced TAT mRNA level to the respective basal, normalized, level from the same Northern blot. Values are means $\pm$ S.D. of determinations from at least three independent experiments.

basal TAT mRNA levels increased rapidly, reaching a level equivalent to the glucocorticoid-induced level in the quiescent liver at 1 h post-hepatectomy. Apart from a transient decline reproducibly observed at 3 h post-surgery, basal TAT mRNA levels remained elevated for up to 8 h and returned to the quiescent liver level 16–24 h after the operation. Interestingly, however, glucocorticoid-induced levels were practically unchanged during the first 16 h of the proliferative response. In addition, Figure 1(A) shows that GAPDH mRNA levels were unaffected by 70% hepatectomy or hormone treatment. TAT mRNA levels were normalized to the respective GAPDH mRNA levels, and fold induction was calculated as the ratio of the glucocorticoid-induced to the respective basal, normalized, TAT mRNA level at various times after partial hepatectomy. The mean fold induction values shown in Figure 1(B) were derived from several independent experiments. Evidently, induction of TAT mRNA by glucocorticoids was marginal at 1.5 h (depicted in Figure 1C) and 2 h post-hepatectomy, significantly impaired between 3 and 8 h and similar to that observed for quiescent liver at 16 and 24 h post-surgery.

Induction of TAT mRNA by glucocorticoids is primarily dependent on activation of TAT gene transcription in regenerating but not in quiescent liver

Nuclear run-on analysis of the rate of TAT gene transcription was carried out using liver nuclei isolated 1 h after injection of triamcinolone acetonide. We have previously reported that, during liver regeneration in the absence of glucocorticoids, the

rate of TAT gene transcription rapidly decreases within 1.5 h of hepatectomy and then transiently increases at 3 h post-hepatectomy before reaching comparatively low values 8–24 h after the operation [24]. In fact, gene transcription rates for quiescent, 1.5 h- and 3 h-regenerating liver were respectively 3.0-, 1.7- and 2.4-fold greater than the rate in 15 h-regenerating liver (Table 1). Injection of the hormone increased transcription rates in both quiescent and regenerating liver to values somewhat higher than that observed in quiescent liver in the absence of glucocorticoids. Consequently, transcriptional activation by the hormone, apparently determined by the basal rate of transcription, was marginal in quiescent liver and several-fold higher 15 h post-hepatectomy, when the basal rate of transcription declines (Table 1). Figure 2(A) shows the hybridization of TAT, GAPDH and vector probes to 32 P-labelled run-on products from hormone-treated and non-treated quiescent liver as compared with non-treated 15 h-regenerating liver. Hybridization to the GAPDH and PUC8 probes was unaffected by either hormone treatment or hepatectomy.

The 4.26-fold activation of TAT gene transcription by triamcinolone acetonide observed 15 h post-hepatectomy is comparable with the reported 6.8-fold transcriptional activation induced by glucocorticoids in primary hepatocytes randomly growing in culture [10], and fully accounts for the 4.6 ± 0.51 -fold ($n = 4$) induction of TAT mRNA by glucocorticoids measured 1 h later, i.e. 16 h after 70% hepatectomy (Figure 1B). Similarly, hormonal activation of transcription at 1.5 and 3 h post-hepatectomy is comparable with the induction of TAT mRNA expression 3 and 4 h after the operation (compare Figure 1B and

Table 1 Effect of glucocorticoids on the rate of TAT gene transcription in quiescent and regenerating liver

Nuclei, isolated from 3–6 livers or liver remnants of triamcinolone acetone-treated (+TA), dexamethasone-treated (+Dex) or untreated (—TA, —Dex) adrenalectomized rats at the indicated times after hepatectomy, were used for run-on analysis of the rate of TAT gene transcription. Relative rates of transcription are expressed in p.p.m., calculated as described in the Experimental section. Values are means of one experiment (Dex) or means $\pm$ S.D. of determinations from at least three independent experiments (TA; actual numbers of experiments given in parentheses). Fold activation was calculated as the ratio of the hormone-stimulated to the basal transcription rate from the same experiment. N.D., not determined.

Time after operation (h)	Transcription rate (p.p.m.)				Fold activation	
	+TA	—TA	+Dex	—Dex	TA	Dex
0	825 $\pm$ 74 (6)	667 $\pm$ 92 (6)	834 (1)	715 (1)	1.29 $\pm$ 0.24 (6)	1.16 (1)
1.5	807 $\pm$ 38 (3)	339 $\pm$ 64 (3)	N.D.	N.D.	2.15 $\pm$ 0.12 (3)	N.D.
3	789 $\pm$ 93 (5)	539 $\pm$ 86 (5)	788 (1)	590 (1)	1.44 $\pm$ 0.16 (5)	1.34 (1)
15	895 $\pm$ 30 (4)	229 $\pm$ 59 (4)	N.D.	N.D.	4.26 $\pm$ 1.07 (4)	N.D.

Figure 2 The rate of TAT gene transcription is marginally activated by glucocorticoids in quiescent liver

(A) Hybridization of the indicated filter-bound probes to 32 P-labelled run-on transcription products synthesized in liver nuclei from hormone-treated (lane 1) or untreated (lane 2) non-hepatectomized rats and untreated hepatectomized rats 15 h post-operation (lane 3). (B) Three different inputs of 32 P-labelled run-on products synthesized in liver nuclei from hormone-treated non-hepatectomized rats were hybridized to 4.5 μ g of immobilized TAT probes and radioactivity bound to the filters was corrected for hybridization efficiency and for the length of the gene covered by the TAT probes used. Results from four independent experiments are indicated by the four different symbols.

Table 1); notably, we have previously shown that changes in TAT mRNA levels after 70% hepatectomy lag approx. 1 h behind analogous changes in the rate of TAT gene transcription [24]. Interestingly, however, the 1.29-fold transcriptional activation in quiescent liver contrasts sharply with the 4.69 ± 0.70 -fold ($n = 6$) induction of TAT mRNA observed in this case. A similar fold activation was determined when dexamethasone, an equally strong agonist in mammalian cells, was injected (Table 1). The possibility that failure of the assay to detect a significant increase in the rate of transcription for quiescent liver was due to probe depletion was excluded, since an excess of filter-bound DNA probe was ascertained by two independent tests performed in each experiment: (a) the extent of hybridization was proportional to the input of 32 P-labelled RNA (Figure 2B), and (b) doubling the amount of filter-bound DNA had no effect on the amount of hybridized run-on products. Hence activation of transcription accounts for glucocorticoid induction of TAT mRNA in regenerating but not in quiescent liver. However, rather than increasing the rate of initiation of transcription, glucocorticoids could be primarily involved in either accelerating elongation of the primary transcript [33] or overcoming a blockage in transcript elongation [34]. To test this possibility,

transcriptional activation by the hormone in quiescent liver was determined using probes EE 0.94 and EE 1.05 from the 5' and 3' ends of the gene respectively [7]. Transcription rates at the 5' end of the gene (887 and 588 p.p.m. in the presence and absence of glucocorticoids respectively) were comparable with those at the 3' end (864 and 547 p.p.m. respectively). Transcriptional activation values thus determined were similar (1.51- and 1.58-fold respectively), suggesting that, in quiescent liver, glucocorticoids are not involved in TAT transcript elongation. The inference from these data is that glucocorticoid regulation of TAT gene expression in quiescent liver occurs primarily at the post-transcriptional level.

Induction of hypersensitivity at the glucocorticoid-dependent enhancer is not impaired in liver during quiescence or early regeneration

We investigated whether, in spite of the marginal activation of TAT gene transcription by triamcinolone acetone in liver during quiescence and early regeneration, the hormone is capable of promoting disruption of chromatin structure at the glucocorticoid-dependent enhancer of the gene. First, nuclei

Figure 3 Nuclear glucocorticoid receptor levels and glucocorticoid-induced DNase I hypersensitivity at the -2.5 kb TAT enhancer in quiescent and regenerating liver

(A) Glucocorticoid receptor (GR) levels in nuclei from triamcinolone acetonide-treated adrenalectomized rats. Approx. 1.5×10^6 nuclei isolated from quiescent (lane 6) or regenerating (lanes 7–9) livers from hormone-treated rats, 3 h (lane 7), 8 h (lane 8) and 15 h (lane 9) post-hepatectomy, were subjected to immunoblotting analysis followed by blot development with 125 I-labelled Protein A. A calibration curve obtained with samples of 50, 75, 100, 125 and 150 fmol of cytosolic glucocorticoid receptor (lanes 1–5 respectively) was used to determine the amount of nuclear glucocorticoid receptor, as described in the Experimental section. (B) Nuclear glucocorticoid receptor (GR) levels obtained following treatment with triamcinolone acetonide. Values are means $\pm$ S.D. of determinations from 3–5 independent experiments. (C) Mapping of DNase I hypersensitive sites in the TAT gene region from positions -3050 to $+570$. Nuclei were isolated from triamcinolone acetonide (TA)-treated (+) or untreated (–) quiescent liver (adx) and regenerating liver 3 h after 70% hepatectomy (3h-hep). Digestion with DNase I was performed at 4°C for 10 min with the indicated amounts of enzyme (units). After purification and digestion with *Eco*RI, the DNA was electrophoresed in a 1.4% agarose gel ($20 \mu\text{g}/\text{lane}$), blotted and indirectly end-labelled to the *Eco*RI site at position -3050 . A mixture of $14 \mu\text{g}$ of genomic DNA digested with *Eco*RI (E; -3050 to $+570$), and $3 \mu\text{g}$ each of *Eco*RI/*Xba*I (EX; -3050 to -2561) and *Eco*RI/*Tth*111 (ET; -3050 to -2308) double-digested genomic DNA was co-electrophoresed and co-hybridized to serve as an internal marker (IM). The positions of the DNase I hypersensitive sites are indicated by arrows.

isolated from the livers of untreated and hormone-treated rats 1 h after injection were analysed by quantitative immunoblotting for the amount of glucocorticoid receptor associated with the nuclear compartment. Figure 3(A) shows comparable nuclear receptor levels in quiescent and regenerating liver in the presence of the hormone, as assessed using an anti-receptor serum and 125 I-labelled Protein A followed by autoradiography. Quantification of several immunoblots established that similar amounts of receptor were associated with quiescent and regenerating liver nuclei following injection of the hormone (Figure 3B). Significantly, we have previously reported that undetectable amounts of the receptor are present in liver nuclei from adrenalectomized rats not treated with glucocorticoids [24], suggesting that residual endogenous glucocorticoids, if any, are not capable of causing association of the receptor with the nuclear compartment. In accordance with these findings, Figure 3(C) shows induction of hypersensitivity to digestion by DNase I at the glucocorticoid-dependent enhancer of the gene at -2.5 kb for nuclei isolated from quiescent and 3 h-regenerating liver 1 h after injection of triamcinolone acetonide. This suggests that glucocorticoid-induced alterations in chromatin structure, necessary for enhancer activation, apparently occur.

Glucocorticoids affect an early step(s) in the nuclear processing of the TAT primary transcript in quiescent liver

In order to examine whether the pronounced induction of TAT

mRNA by glucocorticoids in quiescent liver, in the absence of a significant activation of gene transcription, is the result of a glucocorticoid-induced stabilization of cytoplasmic TAT mRNA, triamcinolone acetonide-treated adrenalectomized rats and untreated controls were injected with 0.5 mg of actinomycin D per 100 g body mass. Preliminary experiments have established that this dose of the inhibitor represses transcriptional activity in isolated nuclei by 80% , whether in the presence or the absence of glucocorticoids. Northern blot analysis established that both basal and hormone-induced TAT mRNA levels were comparably decreased within 1 h (62% and 64% of control levels respectively) and 2 h (37% and 36% of control levels respectively) of treatment with the inhibitor. Since the inhibitor was administered 2 h after hormone injection, the possibility that actinomycin D inhibited the synthesis of a glucocorticoid-inducible factor involved in the stabilization of TAT mRNA seems unlikely, particularly in view of results showing that inhibition of protein synthesis does not affect the inducibility of translatable [35] or nuclear TAT mRNA (see below). Thus, in agreement with a previous report [36], our data suggest that glucocorticoids do not affect the stability of liver TAT mRNA.

We have previously reported that, in regenerating liver in the absence of glucocorticoids, the increase in the level of total TAT mRNA observed within 1 h of 70% hepatectomy is regulated at a post-transcriptional step in the nucleus [24]. To examine whether glucocorticoid induction of TAT mRNA in quiescent liver is regulated at a similar step, total and nuclear RNA was

Figure 4 Induction of nuclear and total TAT mRNA by glucocorticoids

(A) Aliquots of total (T; 20 μ g) and nuclear (N; 30 μ g) RNA, isolated from the livers of three or four adrenalectomized rats at the indicated times after injection of triamcinolone acetate, were subjected to Northern blot analysis. The positions of TAT mRNA (arrowhead) and several TAT pre-mRNAs present in liver nuclei (arrows) are indicated. Part of the ethidium-bromide stained gel, showing 28 S rRNA and precursors, is depicted in the lower panel. (B) Time course of induction of nuclear (solid line) and total (broken line) TAT mRNA by triamcinolone acetate. TAT mRNA levels were quantified by laser scanning densitometry and are expressed relative to the level before hormone treatment from the same Northern blot. Values are means $\pm$ S.D. of determinations from three independent experiments (one experiment for the 2 h time point of nuclear RNA). (C) Northern blot analysis of 20 μ g of total (T) and 30 μ g of nuclear (N) RNA, isolated from the livers of three or four adrenalectomized rats; treatment with cycloheximide (CHX) was for 2 h and that with triamcinolone acetate (TA) was for 1 h. When combined, cycloheximide treatment preceded hormone treatment by 1 h. The positions of TAT mRNA (arrowhead) and TAT mRNA precursors (arrows) are indicated. Part of the ethidium-bromide stained gel, showing 28 S rRNA and precursors, is depicted in the lower panel.

isolated from the livers of untreated and triamcinolone acetate-treated rats 0.5, 1 and 2 h after treatment and analysed by Northern blotting. Figure 4(A) (upper panel) shows one such experiment, and Figure 4A (lower panel) shows the same gel stained with ethidium bromide. A pronounced induction of both nuclear and total TAT mRNA (2.4 kb) was observed after treatment with the hormone. In addition to the mature nuclear mRNA species, two distinct TAT-probe-specific bands (and a minor one seen in Figure 4C) of higher molecular mass were reproducibly observed under the high-stringency conditions used (arrows), either prior to or after hormone treatment, particularly on prolonged exposure of the autoradiographs. The absence of these bands from the samples of total mRNA suggests that they represent TAT mRNA precursor molecules. Notably, their amount relative to that of the mature-like mRNA was maintained following hormone treatment, suggesting that it is unlikely that a late step in pre-mRNA processing is affected by the hormone. We were unable to detect pre-mRNA transcripts of higher molecular mass, probably reflecting inefficient transfer of large RNA entities. Figure 4(B) compares the time course of accumulation of mature-like nuclear and total mRNAs after injection of the hormone, as deduced by scanning autoradiography data such as those of Figure 4(A) and expressing induced mRNA levels relative to non-induced levels in quiescent liver. A 5.12 ± 0.45 -fold ($n = 3$) induction was observed in the nucleus 0.5 h after hormone treatment, which compares well with a 5.07 ± 0.65 -fold ($n = 3$) induction of total mRNA 2 h after treatment. This excludes the possibility that glucocorticoids act by accelerating the efflux of mature TAT mRNA from the nucleus. Taken together, these data suggest that glucocorticoids affect an early step(s) in the nuclear processing of the TAT primary transcript.

To test the possibility that labile proteins may promote either the processing of nuclear TAT mRNA in the presence of glucocorticoids or the instability of the primary transcript in

their absence, we compared the inducibility of nuclear mature TAT mRNA in the presence and absence of ongoing protein synthesis (Figure 4C). Induction of mature TAT mRNA by glucocorticoids was not affected by inhibition of protein synthesis, excluding the possibility that the effect of glucocorticoids is mediated by either the synthesis of a positively acting factor or the inhibition of synthesis of a negatively acting one. The two TAT mRNA precursor bands detected in the experiment of Figure 4(A), as well as a faint band of higher molecular mass, were present in all samples. Although neither the size nor the relative abundance of any of these bands was affected by hormone treatment, inhibition of protein synthesis decreased the mobility of the higher-molecular-mass precursor, both in the presence and in the absence of glucocorticoids, suggesting that glucocorticoid-independent labile proteins are involved in this step of the maturation process.

It has been reported that differential nuclear processing of α_1 -acid glycoprotein RNA during the acute-phase response is correlated with changes in the length of its poly(A) tail [37]. To test the possibility that glucocorticoids affect the length of the poly(A) tail of TAT mRNA, we performed site-directed RNase H digestion of total as well as nuclear TAT mRNA, in the presence or absence of an oligonucleotide complementary to the TAT mRNA sequence from nt 1747 to nt 1776, and compared the lengths of the 3' polyadenylated fragments (depicted in Figure 5A). Figure 5(B) shows Northern blot analysis of the RNase H-resistant 5' (thick arrow) and 3' (thin arrows) TAT mRNA fragments produced in the presence (lanes 2–7) but not in the absence (lane 1) of the oligonucleotide. As expected, several minor bands were produced upon digestion of precursor TAT mRNAs (lanes 4, 5 and 7). No discernible difference in the lengths of the 3' polyadenylated fragments of total (compare lanes 2 and 3) and nuclear (compare lanes 4 and 5) TAT mRNA was detected as a result of glucocorticoid treatment. However, oligo(dT)-directed RNase H-mediated deadenylation of total

Figure 5 Northern blot analysis of RNase H-digested TAT mRNA

Total or nuclear RNA, isolated from glucocorticoid-treated or untreated adrenalectomized rats, was hybridized to a 20 nt oligonucleotide, complementary to the TAT mRNA sequence from nt 1747 to nt 1776, in the presence or absence of oligo(dT), and the hybrids were digested with RNase H. The RNase H-resistant fragments were electrophoresed in a 1.5% agarose/formaldehyde gel and subjected to Northern blot analysis using probe EE 1.05, complementary to the TAT mRNA sequence from nt 1250 to nt 1918. (A) Schematic representation of the experimental protocol. (B) Northern blot analysis of 5 μ g of RNase H-treated, non-digested (lane 1) and RNase H-digested total RNA from triamcinolone acetonide (TA)-treated rats (lanes 2 and 6), 10 μ g of digested total RNA from untreated rats (lane 3), 5 μ g of digested nuclear RNA from TA-treated rats (lanes 4 and 7) and 20 μ g of digested nuclear RNA from untreated rats (lane 5). The positions of the 5' (thick arrow) and 3' (thin arrows) fragments, as well as of the non-digested TAT mRNA (open arrow), are indicated.

(lane 6) and nuclear (lane 7) TAT mRNA resulted in faster migrating fragments of similar length in both cases. These data suggest that glucocorticoids have no apparent effect on the length of the poly(A) tail of TAT mRNA.

DISCUSSION

It has been reported that TAT activity, although readily inducible by glucocorticoids in randomly growing cells, undergoes a glucocorticoid-insensitive period in the cell cycle of synchronized hepatoma cells, during which the enzyme is synthesized at relatively high rates independently of the presence of glucocorticoids [20–22]. The present study expands these observations to hepatocytes synchronously proliferating *in vivo* and, by focusing on the late part of the insensitive period, shows that impaired induction of TAT mRNA in the presence of glucocorticoids during the early G1 phase of hepatocyte replication is the result of limited activation of TAT gene transcription (Table 1), in spite of an appreciable amount of nuclear glucocorticoid receptor (Figures 3A and 3B) and of induction of hypersensitivity at the glucocorticoid-dependent enhancer of the gene (Figure 3C) detected in their presence. Surprisingly, although glucocorticoid induction of TAT mRNA in quiescent liver is

pronounced (Figure 1B and [7]), transcriptional activation by glucocorticoids was limited (Table 1 and Figure 2A) and in discordance with a similar induction of enhancer hypersensitivity (Figure 3C and [14]). That glucocorticoid receptor binding to the enhancer is necessary but not sufficient for transcriptional activation is also supported by reports showing that hypersensitivity at promoter and enhancer regions can result from antagonist-occupied or truncated steroid receptors binding to enhancers without activating transcription from nearby chromatin-integrated promoters [38,39]. Furthermore, it has been reported that phorbol ester impairment of glucocorticoid induction of TAT mRNA occurs in the absence of any discernible changes in GRE occupancy and hypersensitivity at the glucocorticoid-dependent enhancer of the gene, indicating that a step beyond glucocorticoid receptor binding to the enhancer is affected [40]. However, phorbol ester treatment was found to alter the chromatin structure upstream from and near to the start site of TAT gene transcription, suggesting that an interaction between a transcription factor crucial to the function of the glucocorticoid-dependent enhancer and the basal transcription complex is probably thus prevented.

We have previously reported that a constitutive activator of TAT gene transcription expressed in quiescent liver is rapidly debilitated following 70% hepatectomy, leading to a decrease in the basal transcription rate within 1.5 h of hepatectomy [24]. Interestingly, our present results show that the activation of TAT gene transcription by glucocorticoids is proportional to the decrease in basal transcription rate, and that activation is marginal in quiescent liver, in which the basal rate is relatively higher (Table 1 and Figure 2A). This suggests that, in this latter case, most of the TAT templates are engaged by the constitutive activator and that, in such actively transcribed TAT templates, an interaction between the receptor (or another factor binding to the glucocorticoid-dependent enhancer) and the basal transcription complex is probably prevented. The fact that the glucocorticoid-induced rate of TAT gene transcription in regenerating liver is similar to the basal rate in quiescent liver indicates that a comparable stabilization of the transcription initiation complex is brought about by either the glucocorticoid receptor or the constitutive activator.

Our results show that glucocorticoid induction of TAT mRNA during the post-hepatectomy period of hepatocyte proliferation *in vivo* is transcriptionally regulated. However, the induction of TAT mRNA by glucocorticoids in quiescent liver cannot be accounted for by the 1.3-fold activation of transcription (Table 1), suggesting that post-transcriptional mechanisms are involved. To our knowledge, this is the first report on the effect of glucocorticoids on the rate of TAT gene transcription in either intact or regenerating liver. Previous studies with isolated randomly growing primary hepatocytes revealed that induction of TAT mRNA by glucocorticoids is regulated at the transcriptional level [10]. It is known, however, that, despite their common origin, hepatocytes in culture are different from those in intact tissue. The maintenance of a high level of liver-specific expression depends on an intact mature tissue structure [41], disruption of which results in a sharp decline in the transcription of several liver-specific genes, whereas the transcription of common genes continues at normal or elevated rates [41,42]. In addition, hepatocytes in liver are strictly quiescent, but the disruption of cell-cell contacts is itself sufficient to promote the G0 $\rightarrow$ G1 transition, even prior to plating, as shown by the activation of the genes *c-fos*, *c-jun*, *c-myc* and *jun-B* and the decrease in the levels of CCAAT enhancer binding protein- α mRNA [43,44]. Plating of the cells in the presence of complete medium promotes further progression through the cell cycle

(reviewed in [45]). It is, therefore, reasonable to assume that the impact of several cell-cycle-regulated factors on gene expression may differ between cultured hepatocytes and intact tissue. The significantly lower basal transcription rate of the TAT gene in primary hepatocytes [10] as compared with that in intact liver [7], previously reported, complies with this hypothesis.

Our present results support existing evidence implicating post-transcriptional mechanisms in the regulation of TAT gene expression by glucocorticoids. A 12-fold induction of TAT mRNA in FaO cells by glucocorticoids has been reported which is poorly correlated with the observed 2.5-fold increase in the rate of transcription [46]. Similarly, induction of TAT mRNA by glucocorticoids in KRC-7 cells is not fully accounted for by an increase in the rate of transcription [8]. Using synchronized KRC-7 cells we also found that activation of TAT gene transcription by glucocorticoids accounts for induction of TAT mRNA in proliferating but not in G0/G1-arrested cells (L. Baki and M. N. Alexis, unpublished work). Several other studies have shown that glucocorticoids can act at the post-transcriptional level to confer positive [31,47,48] as well as negative [49,50] regulation of gene expression, mainly by increasing or decreasing respectively the stability of mRNA. This action appears to be mediated primarily by sequences in the 3' untranslated region [47] and in exons of RNAs [31,49], and is occasionally related to an increase in the length of the poly(A) tail [31]. However, none of these appears to be the explanation in our case: glucocorticoid treatment did not seem to affect the stability of TAT mRNA, and the possibility that glucocorticoids act by influencing the length of the poly(A) tail was also excluded (Figure 5). In addition, an effect of glucocorticoids in relieving a blockage in the export of TAT mRNA from the nucleus seems unlikely, since accumulation of either pre-mRNA or mature mRNA in the nucleus was not detected in the absence of hormone (Figures 4A and 4B). Moreover, a similar fold induction was determined for nuclear and total TAT mRNA in the presence of glucocorticoids (Figure 4B). Hence, in quiescent liver, glucocorticoids appear to induce steady-state TAT mRNA levels by influencing a step in the nuclear maturation of the primary transcript. Such a glucocorticoid-sensitive step is likely to be an early one, since the levels of the processing intermediates detected were found to vary in direct proportion to those of the mature transcript. Lack of detection of the unprocessed transcript made further analysis of the exact step that is influenced by glucocorticoids a difficult task. Nuclear post-transcriptional regulation of gene expression by glucocorticoids has also been reported for α_1 -acid glycoprotein [48]. The observation that inhibition of ongoing protein synthesis had no impact on the induction of TAT mRNA (Figure 4C) is in accordance with a glucocorticoid-mediated regulation of the activity of a pre-existing TAT pre-mRNA processing factor. Such a post-translational modulation of a pre-existing RNA binding activity has also been proposed for the post-transcriptional regulation of phosphoenolpyruvate carboxykinase mRNA by glucagon and insulin [51]. Alternatively, the glucocorticoid receptor itself may affect the stability or maturation of TAT pre-mRNA. Significantly, the oestrogen receptor has been reported to co-localize with nuclear pre-mRNA particles [52].

Following 70% hepatectomy, the efficiency of nuclear processing of the TAT transcript increases [24]; this is possibly due to the synthesis of a cell-cycle-inducible factor, since induction of TAT mRNA by hepatectomy could be partially prevented by injection of cycloheximide 15 min before the operation (results not shown). Similarly, it has been shown that histone H4 pre-mRNA processing is down-regulated in serum-starved C127 cells due to limiting amounts of a specific processing component which is, however, present in excess in nuclear extracts from

exponentially dividing cells [53]. Our results support a model whereby, in quiescent liver, the TAT gene is actively transcribed, but the primary transcript is either rapidly degraded or inefficiently processed. In this case, glucocorticoids can influence a step in the nuclear maturation of the primary transcript, leading to increased accumulation of TAT mRNA. Following 70% hepatectomy, a factor is induced and/or activated that renders post-transcriptional processing of the primary transcript more efficient, also leading to induction of TAT mRNA. The fact that in this case glucocorticoids can no longer affect gene expression at the post-transcriptional level (compare Figure 1B and Table 1) suggests that both glucocorticoids and 70% hepatectomy influence the same step in the maturation process. The hepatectomy-activated post-transcriptional up-regulation, in conjunction with impaired activation of TAT gene transcription by glucocorticoids early in regeneration, results in glucocorticoid-insensitive TAT gene expression in hepatocytes during the early G1 phase of replication *in vivo*.

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REFERENCES

- Schweizer-Groyer, G., Groyer, A., Cadepond, F., Grange, T., Baulieu, E.-E. and Pictet, R. (1994) *Nucleic Acids Res.* **22**, 1583–1592
- Becker, P. B., Ruppert, S. and Schütz, G. (1987) *Cell* **51**, 435–443
- Nitsch, D., Stewart, A. F., Boshart, M., Mestrlil, R., Weih, F. and Schütz, G. (1990) *Mol. Cell. Biol.* **10**, 3334–3342
- Nitsch, D. and Schütz, G. (1993) *Mol. Cell. Biol.* **13**, 4494–4504
- Weih, F., Stewart, A. F., Boshart, M., Nitsch, D. and Schütz, G. (1990) *Genes Dev.* **4**, 1437–1449
- Nitsch, D., Boshart, M. and Schütz, G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5479–5483
- Hashimoto, S., Schmid, W. and Schütz, G. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6637–6641
- Moore, P. S. and Koontz, J. W. (1989) *Arch. Biochem. Biophys.* **275**, 486–495
- Ganss, R., Weih, F. and Schütz, G. (1994) *Mol. Endocrinol.* **8**, 895–903
- Schmid, E., Schmid, W., Jantzen, M., Mayer, D., Jastorff, B. and Schütz, G. (1987) *Eur. J. Biochem.* **165**, 499–506
- Reik, A., Schütz, G. and Stewart, A. F. (1991) *EMBO J.* **10**, 2569–2576
- Jantzen, H.-M., Strähle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R. and Schütz, G. (1987) *Cell* **49**, 29–38
- Carr, K. D. and Richard-Foy, H. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9300–9304
- Becker, P., Renkawitz, R. and Schütz, G. (1984) *EMBO J.* **3**, 2015–2020
- Rigaud, G., Roux, J., Pictet, R. and Grange, T. (1991) *Cell* **67**, 977–986
- Grange, T., Roux, J., Rigaud, G. and Pictet, R. (1991) *Nucleic Acids Res.* **19**, 131–139
- Espinas, M. L., Roux, J., Ghysdael, J., Pictet, R. and Grange, T. (1994) *Mol. Cell. Biol.* **14**, 4116–4125
- Grange, T., Roux, J., Rigaud, G. and Pictet, R. (1989) *Nucleic Acids Res.* **17**, 8695–8709
- Oshima, H. and Simons, Jr., S. S. (1993) *J. Biol. Chem.* **268**, 26858–26865
- Martin, Jr., D., Tomkins, G. M. and Granner, D. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 248–255
- Martin, Jr., D., Tomkins, G. M. and Bresler, M. A. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 842–849
- Sellers, L. and Granner, D. (1974) *J. Cell Biol.* **60**, 337–345
- Michalopoulos, G. M. (1990) *FASEB J.* **4**, 176–187
- Baki, L. and Alexis, M. N. (1994) *Eur. J. Biochem.* **225**, 797–803
- Higgins, G. M. and Anderson, R. M. (1931) *Arch. Pathol.* **12**, 186–202
- Fort, P., Marty, L., Piechaczyk, M., El Sabrouly, S. E., Dani, C., Jeanteur, P. and Blanchard, J. M. (1985) *Nucleic Acids Res.* **13**, 1431–1442
- Fritton, H. P., Sippel, A. E. and Igo-Kemenes, T. (1983) *Nucleic Acids Res.* **11**, 3467–3485
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Alexis, M. N., Mavridou, I. and Mitsiou, D. J. (1992) *Eur. J. Biochem.* **204**, 75–84
- Mitsiou, D. J. and Alexis, M. N. (1995) *FEBS Lett.* **362**, 309–315
- Paek, I. and Axel, R. (1987) *Mol. Cell. Biol.* **7**, 1496–1507

- 32 Grange, T., Guenet, C., Dietrich, J. B., Chasserot, S., Fromont, M., Befort, N., Jami, J., Beck, G. and Pictet, R. (1985) *J. Mol. Biol.* **184**, 347–350
- 33 Yankulov, K., Blau, J., Purton, T., Roberts, S. and Bentley, D. L. (1994) *Cell* **77**, 749–759
- 34 Morello, D., Fitzgerald, M. J., Babinet, C. and Fausto, N. (1990) *Mol. Cell. Biol.* **10**, 3185–3193
- 35 Ernest, M. J. (1982) *Biochemistry* **21**, 6761–6767
- 36 Nickol, J. M., Lee, K.-L. and Kenney, F. T. (1978) *J. Biol. Chem.* **253**, 4009–4015
- 37 Shiels, B. R., Northemann, W., Gehring, M. R. and Fey, G. H. (1987) *J. Biol. Chem.* **262**, 12826–12831
- 38 Pham, T. A., Elliston, J. F., Nawaz, Z., McDonnell, D. P., Tsai, M.-J. and O'Malley, B. W. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3125–3129
- 39 Gilbert, D. M., Losson, R. and Chambon, P. (1992) *Nucleic Acids Res.* **20**, 4525–4531
- 40 Reik, A., Stewart, A. F. and Schütz, G. (1994) *Mol. Endocrinol.* **8**, 490–497
- 41 Clayton, D. F., Harrelson, A. L. and Darnell, Jr., J. E. (1985) *Mol. Cell. Biol.* **5**, 2623–2632
- 42 Clayton, D. F. and Darnell, Jr., J. E. (1983) *Mol. Cell. Biol.* **3**, 1552–1561
- 43 Xanthopoulos, K. G., Mirkovitch, J., Decker, T., Kuo, F. and Darnell, Jr., J. E. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4117–4121
- 44 Mischoulon, D., Rana, B., Bucher, N. L. R. and Farmer, S. R. (1992) *Mol. Cell. Biol.* **12**, 2553–2560
- 45 Fausto, N., Laird, A. D. and Webber, E. M. (1995) *FASEB J.* **9**, 1527–1536
- 46 Crettaz, M., Muller-Wieland, D. and Kahn, C. R. (1988) *Biochemistry* **27**, 495–500
- 47 Petersen, D. D., Koch, S. R. and Granner, D. K. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7800–7804
- 48 Vannice, J. L., Taylor, J. M. and Ringold, G. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4241–4245
- 49 Frost, G. H., Rhee, K. and Thompson, Jr., E. A. (1993) *J. Biol. Chem.* **268**, 6748–6754
- 50 Weiner, F. R., Czaja, M. J., Jefferson, D. M., Giambone, M.-A., Tur-Kaspa, R., Reid, L. M. and Zern, M. A. (1987) *J. Biol. Chem.* **262**, 6955–6958
- 51 Christ, B. and Nath, A. (1993) *Eur. J. Biochem.* **215**, 541–547
- 52 Vazquez-Nin, G. H., Echeverria, O. M., Fakan, S., Traish, A. M., Wotiz, H. H. and Martin, T. E. (1991) *Exp. Cell Res.* **192**, 396–404
- 53 Stauber, C. and Schimperli, D. (1988) *Nucleic Acids Res.* **16**, 9399–9414