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THE HORMONAL CONTROL
OF GENE TRANSCRIPTION

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Hormonal and liver-specific control of expression of the tyrosine aminotransferase gene

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1. The tyrosine aminotransferase gene as a model system to study the interplay of controlling genes and hormone-signalling pathways

From a single cell a variety of cell types and tissues with differential structures and functions develop. To understand the underlying processes of differentiation it is necessary to elucidate the mechanisms of selective activation of those genes that give cells their characteristic structures and functions. Of particular importance for differentiation processes is the establishment of patterns of gene activity by regulatory genes. As a model system we have chosen the tyrosine aminotransferase (TAT) gene [1] and analysed the regulatory processes which determine its cell- and developmental-specific expression. We are characterizing the control sequences that determine cell-specific expression and the proteins that interact with these regulatory elements in order to understand their mode of action. TAT gene expression is an attractive system with which to analyse the molecular mechanism of a developmental process for the reasons summarized and shown in Fig. 1:

(1) Expression of the TAT gene is cell type-specific. As far as is known, only the parenchymal cells of the liver are able to synthesize the enzyme [1].

(2) Expression of the gene is regulated developmentally: the enzyme is not expressed before birth. Enzyme synthesis starts around birth and attains its maximal level within a few hours [2]. This developmental activation is likely to be dependent on glucocorticoids and glucagon, the effect of which is mediated by cAMP [3].

(3) A particularly interesting aspect of this system is the fact that the activity of the gene is influenced by two genetically defined trans-acting loci as deduced from analysis of a set of mouse mutations and by analysis of expression in somatic cell hybrids.
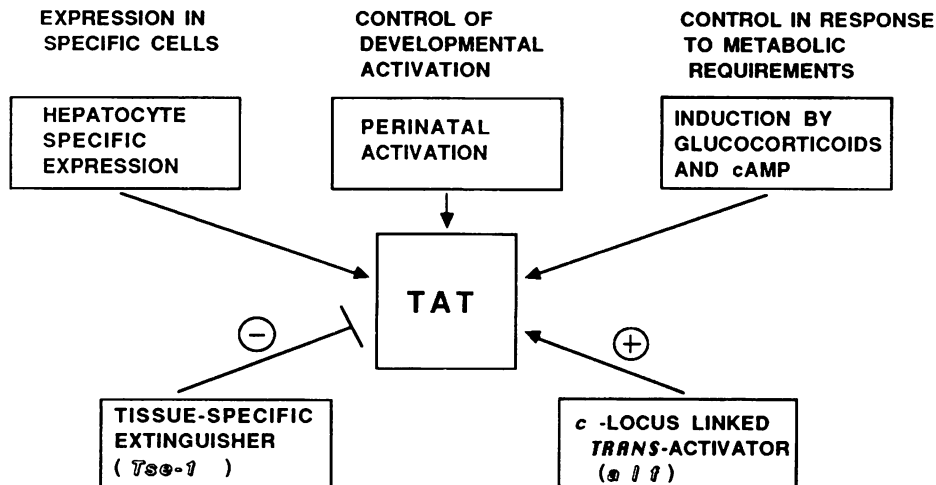
Fig. 1. Control of expression of the tyrosine aminotransferase gene.

(a) The albino lethal mutations of mice are characterized by a set of overlapping deletions within the region of the albino locus (c-locus) on chromosome 7 [4]. It has been shown that several liver-specific enzymes are expressed at a strongly reduced level in homozygous mutant animals [4]. One of the genes affected by the mutation is the TAT gene. The transcription rate of the TAT gene is severely reduced [5], suggesting that the factor indicated by the mutation is involved in cell-specific expression of the gene [6]. Since the structural gene for TAT is not deleted and has been mapped to chromosome 8 [7], it has been concluded that the albino lethal deletions remove a regulatory locus [6], which we denote as *alf* (factor indicated by the albino lethal mutations).

(b) The second trans-acting factor involved in TAT gene regulation is indicated by the phenomenon of extinction [8, 9]. In intertypic hybrids of TAT-expressing hepatoma cells and -non-expressing fibroblasts, TAT gene expression is shut off – a phenomenon termed 'extinction'. The work of Keith Fournier and his colleagues [9, 10] has shown that distinct loci are responsible for the extinction of specific gene expression. For extinction of TAT gene expression a locus named '*Tse-1*' (tissue-specific extinguisher 1) which maps on mouse chromosome 11 is operative as a dominant negative regulator [9]. Since the TAT gene is encoded on chromosome 8 [7], this clearly indicates that the extinguishing factor also acts in trans.

In order to understand these complex regulatory processes involved in TAT gene regulation and the interplay between controlling genes and signalling molecules, we are characterizing in detail those elements that are important for cell-specific expression as well as for responsiveness towards glucocorticoids and cAMP [11–13]. We are particularly interested in determining the targets for the factors indicated by the

albino lethal mutations and the tissue-specific extinguisher *Tse-1* and the mode of action of these two trans-acting factors. By gene transfer into hepatoma cells, by characterization of proteins interacting with regulatory sequences of the gene using in vivo and in vitro footprinting methods and finally by isolation and characterization of these proteins, we are dissecting the mechanisms underlying the cell- and developmental-specific expression of the TAT gene.

2. Two distinct trans-acting loci control overlapping sets of liver-specific genes

Mice homozygous for deletions around the albino locus on chromosome 8 fail to activate expression of a set of neonatal liver functions and die a few hours after birth. The perinatal lethal phenotype is associated with the reduction in activity of a number of enzymes expressed in liver (glucose-6-phosphatase, tyrosine aminotransferase, serine dehydratase and phosphoenolpyruvate carboxykinase), as well as with ultrastructural abnormalities of hepatocytes and cells of the proximal convoluted tubules of the kidney [4, 14]. However, a large number of other enzymes remain uninfluenced by the mutation, attesting to its specific nature [4]. In the case of phosphoenolpyruvate carboxykinase and TAT, it has been established that the decreased enzyme activities result from lower levels of steady-state mRNA [7, 15], which are due to a decreased transcriptional rate as deduced from nuclear run-on assays [5].

In order to understand the perinatal lethal phenotype more fully and to elucidate the nature and function of the product of the perinatal survival locus, we have attempted to identify additional genes influenced by the albino lethal mutations. Using differential cDNA screening we have collected and characterized genes whose cell type-specific transcription is affected in the mutants [5]. With this approach nine distinct genes could be identified, designated 'X1 to X9', none of which maps to the locus indicated by the albino lethal deletions [5]. This permits the conclusion that none encodes the transactivating protein indicated by the mutant phenotype. Some of the gene products encoded by these mRNAs have been identified by sequence analysis (see legend to Fig. 2). 15 genes, so far, have been identified whose expression is affected by *alf* (Fig. 2). Most of the genes are expressed in liver only. Some (X5, ASS and ASL), however, are expressed in all tissues analysed (liver, kidney, brain, heart, lung and skin), but the effect of the deletion is apparent only in liver. Interestingly, the mRNA for C/EBP, a transactivator with restricted tissue distribution [16], is also influenced by *alf* and is reduced in liver, but not in other tissues.

All the genes found to be affected by the albino lethal mutation are induced by cAMP and glucocorticoids, C/EBP being the only exception [5]. However, expression of the genes encoding proteins involved in mediation of hormonal signalling (the glucocorticoid receptor, the cAMP response element-binding protein (CREB) or protein kinase A) is not affected by the mutation [5].
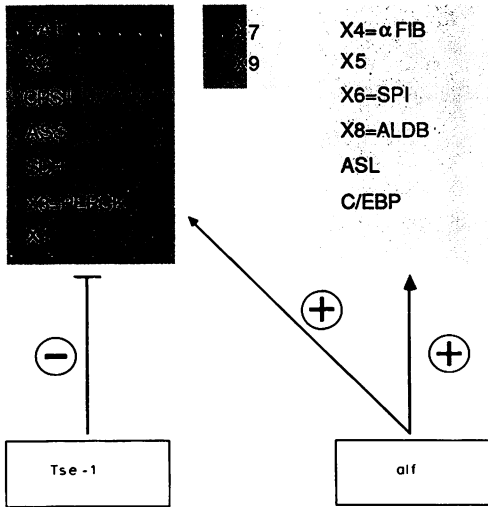
Fig. 2. Co-ordinate regulation of overlapping sets of genes by two distinct transacting loci. All the genes listed are regulated by the factor indicated by the albino lethal mutation (denoted *alf*). A subset of these genes is also regulated by the tissue-specific extinguisher 1 (*Tse-1*). Since the genes coding for X7 and X9 are not expressed in the hepatoma cell lines analysed, evidence for co-regulation by *Tse-1* cannot be established. X1–X9 designate the mouse cDNA clones isolated by subtractive cDNA hybridization. The following abbreviations are used: TAT (tyrosine aminotransferase), CPSI (carbamoylphosphate synthetase I), ASS (argininosuccinate-synthetase), SDH (serine dehydratase), PEPCK (phosphoenolpyruvate carboxykinase), αFIB (α-chain of fibrinogen), SPI (serine protease inhibitor), ALDB (aldolase B), ASL (argininosuccinate-lyase), C/EBP (CCAAT box/enhancer-binding protein).

Interestingly, a subset of the genes affected by the mutation is negatively controlled by the tissue-specific extinguisher locus *Tse-1* [5, 10]. As discussed in more detail below, *Tse-1*-dependent extinction can be relieved by cAMP and a *Tse-1*-responsive element co-resides with a cAMP response element (CRE) [12, 17]. This raises the interesting possibility that a functional antagonism between negative control mediated by *Tse-1* and hormonal induction might play a role in the hormone-dependent perinatal activation of this set of liver genes [2].

In summary, these results show that two trans-acting factors regulate expression of overlapping sets of liver-specific genes. These sets of genes are also characterized by the observations that most of them are activated shortly after birth and that their transcription rates are influenced by both glucocorticoids and cAMP. Since the ultimate demonstration of the function of the gene indicated by the albino lethal mutation depends upon its isolation, we are now attempting to clone it from the knowledge of its chromosomal location [18].

3. Alterations in chromatin structure indicate cis-acting regulatory sequences of the TAT gene

To understand the various levels of control of the TAT gene it is imperative to identify the regulatory regions of the gene. We have used DNaseI hypersensitive site analysis as a means to identify *cis*-regulatory elements in the TAT upstream region. By comparing the DNaseI-hypersensitive regions found in expressing and non-expressing cells with the enhancing activity of the sequences comprising the hypersensitive regions in gene transfer assays into these cells, we could establish that specific chromatin changes relate to the function of these sequences [11, 19, 20]. This correlative analysis of hypersensitive sites (HS) and transcriptional enhancers of the TAT gene was performed in hepatoma cells expressing the TAT gene (*Tse-1$^-$*), in hepatoma cells carrying a short segment of a human fibroblast chromosome with an active *Tse-1* locus which have a strongly reduced TAT mRNA level [10] and in fibroblasts in which the gene is inactive (Fig. 3).

DNaseI-hypersensitive sites 3.6 and 11 kb upstream of the initiation site indicate liver-specific enhancers, they are absent in fibroblasts [19]. Both sites are fully developed in *Tse-1$^-$* and *Tse-1$^+$* hepatoma cells. However, the enhancer at -3.6 kb is inactive in cells expressing *Tse-1* [12, 19]. As will be discussed below, the target for *Tse-1* action is the cAMP response element, which is crucial to enhancer function but which by itself does not constitute enhancing activity. Obliteration of its functional activity by *Tse-1* thus does not lead to disappearance of the HS, in agreement with the complex structure of this enhancer with binding sites for several proteins [12]. The activity of the enhancer at -11 kb is not affected by *Tse-1* [19]. This site is hypersensitive in foetal livers several days before the TAT gene is transcriptionally activated (F. Stewart and G. Yeoh, unpubl. data). This element might function during development to establish an active TAT locus, similar to the role of the dominant control region of the β-globin gene cluster [21].

cell line	- 11		- 3.6		-2.5 inducible		P	basal TAT mRNA level
	activity	HS	activity	HS	activity	HS	HS	
HEPATOMA (Tse-1 $^-$)	+	present	+	present	+	present	present	high
HEPATOMA (Tse-1 $^+$)	+	present	–	present	+	present	present, but modified	very low
FIBROBLAST	–	absent	–	absent	+	absent	absent	un-detectable

Fig. 3. Correlation of DNaseI-hypersensitive sites (HS) and enhancer function of the tyrosine aminotransferase gene. The enhancer activities were analysed by transient transfection assays in different cell lines as indicated on the left. Sequences at -11 and -3.6 kb act as liver cell-specific enhancers, the element at -2.5 kb as a glucocorticoid-inducible enhancer. P denotes the promoter.

The glucocorticoid-inducible enhancer [11] is characterized by a hormone-dependent HS at −2.5 kb. This site is rapidly induced following glucorticoid administration [11, 20] and maintained as long as the inducer is present. Removal of glucocorticoids leads to rapid disappearance of the site. The HS is not inducible in fibroblasts, but the enhancer is active in a transfected reporter plasmid [11], indicating additional controls in the endogenous gene.

The HS at −3.6 and −2.5 kb have been analysed in detail. As discussed below, this analysis reveals the complex nature of far upstream enhancers and gives an insight into the molecular interactions occurring between constitutive and inducible factors.

4. The glucocorticoid enhancer of the TAT gene

Our understanding of how steroid hormones affect gene expression has advanced considerably in recent years [22–24]. Our interest focused on the identification and characterization of elements required for steroid-dependent transcription of the TAT
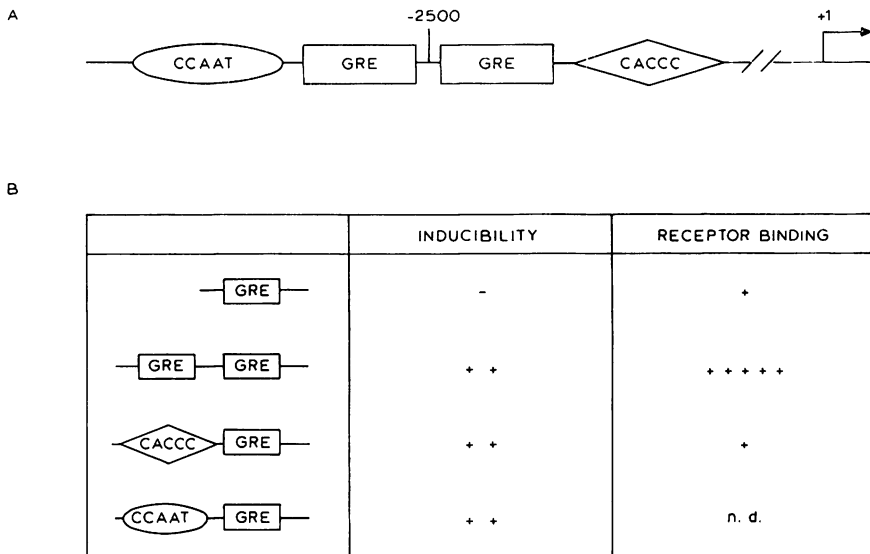
Fig. 4. The glucocorticoid-dependent enhancer of the tyrosine aminotransferase gene has a modular structure. The glucocorticoid-dependent enhancer of the TAT gene (schematically outlined in A) is located 2500 bp upstream of the start site of transcription and is composed of two GREs and sites for transcription factors recognizing the CCAAT and CACCC sequence. (B) A single GRE is not sufficient for induction from a distance. Multiple GREs or a combination of a GRE with binding sites for factors recognizing the CCAAT and CACCC elements are required to constitute a hormone-inducible enhancer. The receptor binds in a co-operative fashion to a GRE dimer when compared to a monomer (indicated by + + + + +). Binding affinity to a GRE is not influenced by the presence of a CACCC element.

gene [11]. By gene transfer experiments, genomic footprinting analyses and in vitro binding studies with the purified glucocorticoid receptor we characterized glucocorti-coid-responsive elements of the TAT gene and defined the role of the hormone in the interaction of the receptor with its recognition sequence.

The element of the TAT gene required for glucocorticoid induction is located 2.5 kb upstream of the start site of transcription (Figs. 3, 4). These sequences contain two glucocorticoid receptor-binding sites which affect expression of the gene in a syn-ergistic fashion [11] and CCAAT and CACCC box-related sequences (Fig. 4) [25]. Although the proximal receptor-binding site has no inherent capacity to stimulate transcription, when present in conjunction with the distal glucocorticoid response el-ement (GRE), this element enhances glucocorticoid induction of gene expression synergistically [11]. Using the genomic footprinting technique we were able to show that the receptor binds to its target sequence only after induction with hormone. Concomitant with alterations in DNA–protein interaction in vivo at the GRE changes are seen at a CACCC box-related sequence in the vicinity of the GRE [26].

The observation that the GREs are found in close vicinity to binding sites for other transcription factors prompted a more detailed analysis of the dependence of recep-tor function on other transcription factors [25, 27]. It became clear that a single GRE is not capable of mediating hormone inducibility when positioned far upstream of the start site of transcription. Inducibility can, however, be attained by multimerizing the steroid-responsive sequences (Fig. 4), as found in the glucocorticoid-dependent enhancer of the TAT gene [25, 27]. Alternatively, a single steroid-responsive element can function in combination with a binding site for other transcription factors. We have demonstrated that the transcription factors interacting with the CCAAT and CACCC boxes which are found in the vicinity of the TAT GREs, as well as NF1- and Sp1-binding sites, can fulfil this synergistic action [25, 27]. Interestingly, these combinations of a GRE with different transcription factor-binding sites show strong cell type-dependent induction of expression. For example, the combination of a GRE with a CACCC box is most active in hepatoma cells. This correlates well with the observation that concomitant with receptor binding at the GRE in vivo, alterations in protein–DNA interaction occur at the CACCC box [26].

What is the molecular basis for the functional synergy of glucocorticoid-responsive sequences in vivo? By detailed analysis in vitro of receptor binding to one or two receptor-binding sites using various techniques, we demonstrated that the functional synergy of GREs is based on co-operative binding of the receptor to these elements (Fig. 4). Measurement of the relative affinity showed a > 10-fold higher affinity of the glucocorticoid receptor to a duplicated GRE when compared to a single element. Thus, co-operative binding of the receptor to two adjacent GREs is a basis for the synergism observed at the level of transcription [28]. However, no evidence for co-operative binding of the receptor and NF1 or the CACCC box-recognizing protein has been found in similar analyses (C. de Vack, unpubl. data).

The DNA elements of the TAT gene which mediate induction by glucocorticoids

are also capable of mediating response to progesterone and testosterone when transfected in appropriate reporter constructs into cells that contain the corresponding hormone receptors [27, 29]. This finding is surprising since these hormones control vastly different processes in different target cells. In view of the fact that the receptors mediating the effects of glucocorticoids, progesterone, testosterone and aldosterone interact with the same sequence it was of interest to determine whether the presence or absence of a steroid receptor is sufficient to control the transcriptional activity of genes that harbour a steroid response element. To address this question we introduced an expression vector for the progesterone receptor into hepatoma cells in which this receptor is not synthesized. By stably expressing the progesterone receptor in hepatoma cells we could demonstrate that the TAT gene becomes responsive to progesterone [30]. Thus, we conclude that steroid-specific effects, at least in part, are determined by differential expression of the respective receptors in target tissues. This is at least one mechanism by which steroid-specific gene activation is achieved.

Mutational analysis of response elements has also shown that the sequences required for estradiol- and ecdysone-dependent expression are closely related to those required for glucocorticoid/progesterone induction (Fig. 5) [27, 29, 31]. The similarity of the steroid response elements has its counterpart in the similarity of amino acid residues in the DNA-binding region of the corresponding receptor molecules [22–24]. In line with this close relationship of response elements is the recent demonstration that three amino acid substitutions in the estradiol receptor are sufficient to alter its target specificity to that of the glucocorticoid receptor [32].

5. Cell-specific expression of the TAT gene

5.1. The −3.6 enhancer is composed of a cAMP-responsive sequence and a liver cell-specific element

As summarized above we have identified two elements which are important for liver cell-specific expression of the TAT gene located at −3.6 and −11 kb. This conclusion is based on gene transfer experiments into hepatoma and non-hepatoma cell lines [19]. To characterize the enhancer at −3.6 kb in more detail a series of 5' and 3' internal deletion mutants was analysed which defined a sequence of 80 bp as absolutely essential for enhancer function of the element located 3.6 kb upstream [12]. To define the regulatory sequences a series of clustered point mutations was established. Analysis of these mutants showed that mutations in two regions destroyed enhancer function entirely [12]. Each of the two sequences was inactive by itself in front of a heterologous promoter. However, if multimerized, each element could function as a strong transcriptional activator (Fig. 6). One of these elements has a sequence similar to the cAMP response element (CRE) found in cAMP-inducible genes [33, 34]. This element confers strong cAMP induction and interacts in vivo

```
T -G- T -A- C -A- NNN - T -G- T -T - C - T        Glucocorticoid,  Progesterone
                                                  Testosterone,  Aldosterone
    .          . .          . .            . .

A - G- G- T - C - A- NNN - T -G - A- C - C - T    Estradiol

  . .  .                .  .             .  .

A - G- G- G - T - T - NNN - T -G - C - A- C - T   Ecdysone
```

Fig. 5. Sequence comparison of steroid response elements. The response elements for glucocorticoids, progesterone, testosterone and aldosterone are 15 bp palindromic sequences which mediate induction of gene transcription by the respective steroid. The response elements for estrogens and ecdysone are distinct, but closely related sequences. Identical bases in steroid response elements are indicated by dots.

with a protein in expressing hepatoma cells, but not in fibroblasts, as evidenced from genomic footprinting experiments [13]. The intensity of interaction seen at the CRE increases upon cAMP administration. This increase in footprinting activity is transient and is also inducible in the presence of cycloheximide [13]. These observations allow the conclusion that cAMP-dependent post-translational modifications lead to an increased binding activity of a protein, possibly CREB, to the TAT CRE.

		ENHANCER ACTIVITY IN			
		HEPATOMA TSE-1-		HEPATOMA TSE-1+	FIBROBLAST
		-cAMP	+cAMP	-cAMP	+cAMP	
CRE	-	-	-	-	-
CRE — CRE	+	+ + +	-	+ + +	-
(oval)	-	-	-	-	-
(oval)(oval)	+ + +	+ + +	+ + +	+ + +	-
CRE —(oval)	+ +	+ + +	-	+ + +	-
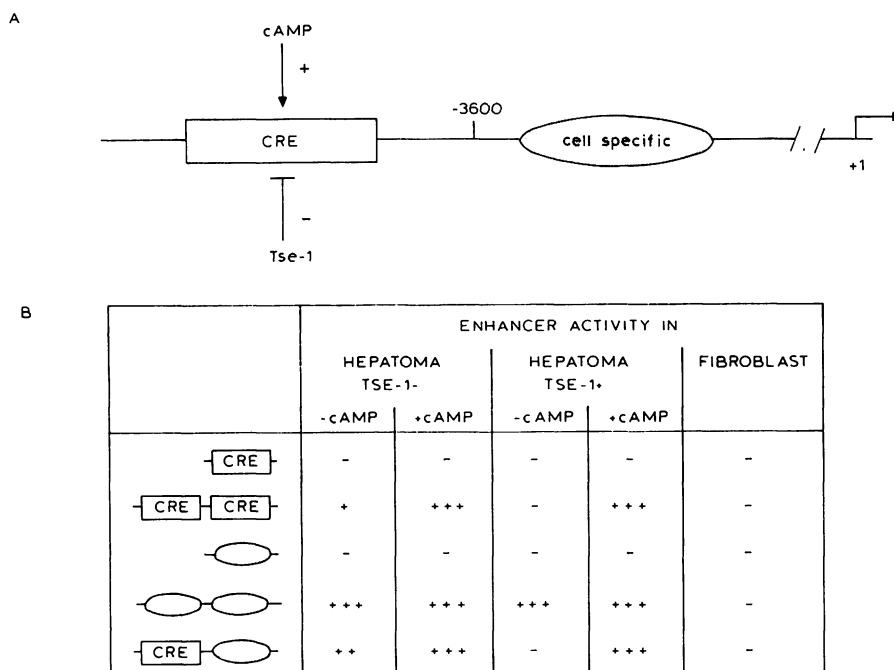
Fig. 6. Two distinct elements are essential for the function of the TAT enhancer at −3.6 kb. Two essential elements were defined by functional analysis of a series of clustered point mutants: a cell-specific module and a cAMP response element which is the target for *Tse-1* (A). Enhancer activity of plasmids carrying these elements alone or in combination was determined in transfection assays into hepatoma cells (*Tse-1⁻* and *Tse-1⁺*) and into fibroblasts (B). The two elements are functionally interdependent. Relief of extinction by cAMP demonstrates the dominant effect of cAMP over *Tse-1*.

The multimer of the second essential element behaves as a cell type-specific activator of transcription [12]. This element is active in liver cells, but inactive in any other cell type tested so far. In conjunction with the element carrying the CRE sequence, an enhancing element with all the regulatory properties of the TAT gene enhancer can be regenerated (Fig. 6) [12].

5.2. Reversal of extinction by cAMP

The cAMP response element of the -3.6 kb enhancer is also a target for negative regulation by *Tse-1* [12] (Fig. 6). In a hepatoma microcell hybrid line which contains only a small segment of the human fibroblast chromosome carrying *Tse-1* [10] administration of cAMP is able to overcome extinction of expression of the transfected reporter gene, thus revealing a functional antagonism between *Tse-1* and the cAMP signal transduction pathway. In vivo footprinting revealed characteristic changes in DMS reactivity at the CRE. In the presence of a functional *Tse-1* locus, protein–DNA interaction at the CRE sequence is abolished. This binding can be recovered by addition of cAMP [12]. Thus, the extinguished state of the TAT gene is characterized by the absence of binding at the CRE. As might be expected from the relief of extinction by cAMP, this footprint reappears after administration of the inducer.

As hormones acting via the cAMP pathway are thought to be critically involved in turning on TAT gene expression around birth, this suggests that the functional antagonism between *Tse-1* and the cAMP pathway might form the basis of a molecular switch governing the onset of TAT gene expression at birth. The strong increase of gluconeogenic hormones around birth may trigger the expression of the TAT gene [1, 2]. This is in line with previous experience that TAT enzyme activity can be prematurely induced before birth by administration of glucagon in utero [2].

In order to understand the mechanism involved in the cell-specific expression of the TAT gene, and in particular to understand the mode of action of the product of the *Tse-1* locus, we are presently isolating and characterizing the proteins involved in recognition of the CRE sequence and the liver cell-specific element. Analysis of possible modifications of the CRE-binding protein in response to cAMP and the tissue-specific extinguisher might give an understanding of the biochemical basis of activation of these controlling proteins.

6. Summary

Two different signal transduction pathways control the activity of the TAT gene via two inducible enhancers (Fig. 7). These enhancers are composed of constitutive and inducible elements which appear to be crucial for cell-specific and hormone-dependent transcription. The effect of glucagon via the cAMP transduction pathway most likely involves specific phosphorylation events [35]. As shown from genomic foot-
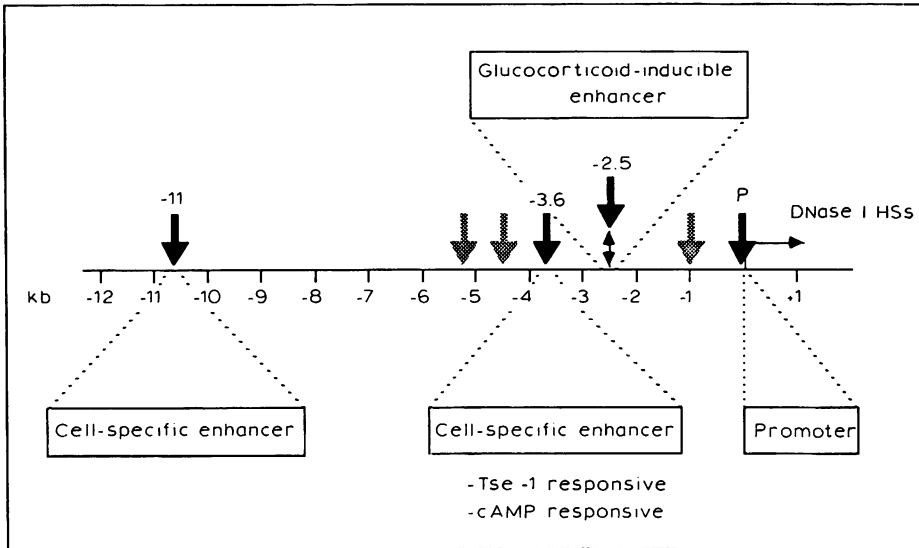
Fig. 7. Several enhancers determine the cell-specific and hormone-dependent activation of the TAT gene. The 5'-flanking region of the TAT gene is outlined with DNaseI-hypersensitive regions and enhancers indicated. Shaded and filled arrows point to DNaseI-hypersensitive sites observed in TAT gene-expressing hepatoma lines. The filled arrows indicate functionally identified elements; P indicates the promoter. The dependence of the DNaseI HS at the GRE on glucocorticoids is indicated by the double arrow.

printing experiments this phosphorylation may be the basis of the increased affinity of a DNA-binding protein recognizing the CRE. This CRE is essential for function of the liver-specific enhancer at −3.6 kb in hepatoma cells and it has to co-operate with a protein recognizing a liver cell-specific module. Since the cAMP response element is a target for the tissue-specific extinguisher it may contribute, in conjunction with a cell-specific enhancer, to the cell-specific and developmentally timed expression of the gene. The enhancer at −3.6 kb is characterized by a constitutive chromatin-hypersensitive site, possibly due to the fact that not only the CRE-binding protein determines the active state of this enhancer. The enhancer at −11 kb is not influenced in its activity by cAMP and glucocorticoids but is liver cell-specific. A DNaseI-hypersensitive region is present in fetal livers, possibly indicating that this distal element may function early in development to establish an activated locus.

The glucocorticoid-dependent enhancer is characterized by a hormone-dependent DNaseI-hypersensitive site which is induced by interaction of the glucocorticoid receptor with its binding site (Fig. 7). The glucocorticoid-dependent enhancer is characterized by the presence of two receptor-binding sites plus binding sites for additional transcription factors recognizing the CCAAT and the CACCC motif. This combination of glucocorticoid receptor recognition sequences with transcription factor binding sites might be involved in the cell-specific modulation of activity of the TAT gene. It is most remarkable that the sequences involved in hormonal control

function in conjunction with elements which display preferential activity in liver cells. This unique combination may be crucial for the precisely timed onset of expression of the TAT gene at birth in the parenchymal cells of the liver.

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