Functional determinants for the tetracycline-dependent transactivator tTA in transgenic mouse embryos

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Abstract

Tetracycline-dependent transgenes promise to be an important tool for investigating the time dependence of gene function during mouse development. The pivotal element of this approach is the recombinant tetracycline-dependent transactivator tTA. Using a modified gene trap approach we successfully generated mouse lines expressing tTA in a wide spread manner during embryogenesis. The transgenic model system which we established allowed us to depict transactivator and target gene expression patterns with high resolution by histochemical means. Our data provide evidence that with decreasing concentrations of tTA protein the state of chromatin acetylation becomes an increasingly important determinant for tTA function. The observation of tTA-dependent position effects on tetO-linked target genes suggests that transcription patterns can be encoded at the level of promoter preactivation. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Inducible transgenes are important analytical tools in the developmental genetics of *Drosophila* and *C. elegans*. However the establishment of an equivalent instrument for the mouse is still in its infancy. One of the most promising approaches along this line is the tetracycline-dependent activator system (Gossen and Bujard, 1992). The pivotal element of this approach is the recombinant tTA gene which encodes a transcriptional activator (Gossen and Bujard, 1992), here called TetR-VP16. Its DNA binding function is conferred by the procaryotic Tet repressor protein (TetR) which has been fused to the VP16 activation domain.

TetR binds as a homodimer with a large free energy change ($K_A \approx 2 \times 10^{11}$ M^{-1}) to its palindromic operator sequences (Hillen et al., 1983; Kleinschmidt et al., 1988). The formation of the operator-TetR complex is efficiently inhibited by binding of tetracycline which is effective at very low, non-toxic concentrations due to its high affinity for TetR (Müller et al., 1995). Therefore, TetR-VP16 can activate a CMV core promoter coupled to seven TetR operator sites (tetO promoter) in a tetracycline dependent manner (Gossen and Bujard, 1992).

Initial attempts to establish the tetracycline-dependent activator system in the mouse have been principally encouraging (Furth et al., 1994). The system has already been used with success to address different physiological questions (Efrat et al., 1995; Ewald et al., 1996; Mayford et al., 1996). However, several problems have become apparent, which have hindered the usage of the system for developmental analysis. Up to now no mouse line is available which expresses tTA in early development. A high degree of variability in activator-dependent target gene expression has been observed using the CMV promoter and its enhancer to express tTA (Furth et al., 1994). Histochemical analysis of target gene expression, surprisingly revealed, that among the cells of the same tissue type only a subset of interspersed cells showed activator dependent target gene expression (Furth et al., 1994). Since expression of the activator was not analyzed concomitantly it remained obscure if the observed mosaicism is due to, a likewise, mosaic expression of the activator or due to particularities of the activation mechanism. Mosaic expression has also been observed using the CaMKIIα promoter to express tTA in postnatal brain (Mayford et al., 1996). In addition it was noticed that expression of tetO-linked transgenes

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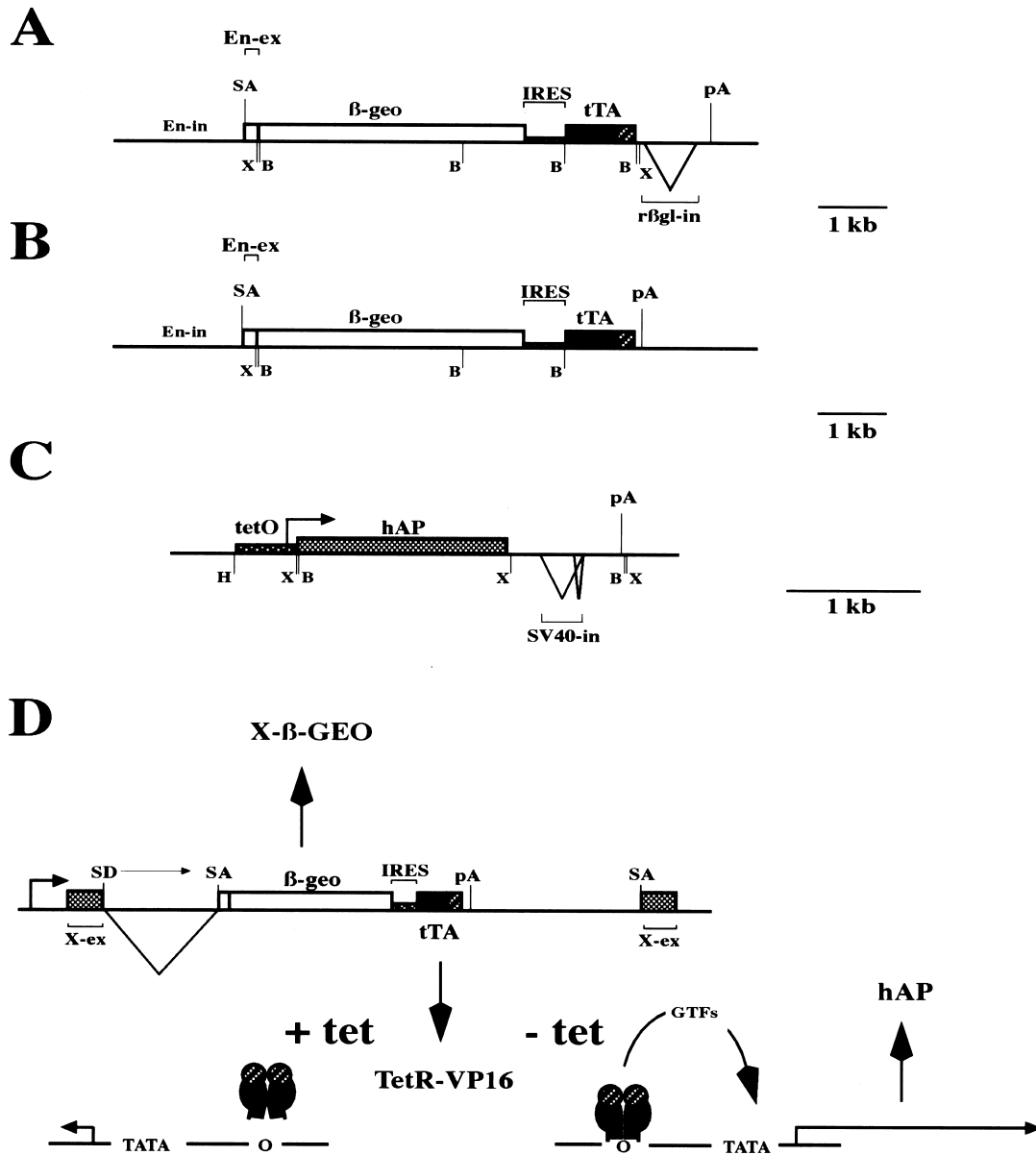
Fig. 1. Line M34 carries vector pM26.22 (A), line B35 vector pM41.1 (B) integrated into an endogenous gene locus. (C) Target gene vector pM42.7. (D) Experimental design. En-in, intron from mouse En2 gene; En-ex, part of En2 exon; β-geo, fusion of LacZ with neomycin phosphotransferase gene of Tn5; IRES, internal ribosomal entry site of encephalomyocarditis virus; tTA, tetracyclin-dependent transactivator gene; rβgl-in, rabbit β-globin intron 2, pA, polyadenylation signal of rabbit β-globin gene (A) and of SV40 (C), respectively; SV40-in, intron of SV40 (gives rise to two alternative transcripts); tetO, tetO promoter; X-ex, exons of an endogenous gene X. SA, splice acceptor; SD, splice donor; O, tet operator site; GTF, general transcription machinery, TATA, CMV core promoter, hAP, human placental alkaline phosphatase; tet, tetracycline; X, XbaI; B, BamHI; H, HindIII. Right angle arrows represent promoters.

appeared to be restricted to subregions of the tTA expression domain depending on the target gene's integration site (Mayford et al., 1996).

Using transgenic promoters we were not able to obtain early commencing, stable and widespread expression of tTA transgenes during mouse development. Since this problem was attributed to the usage of transgenic promoter elements we pursued a modified gene trap approach (Friedrich and Soriano, 1991) to express tTA under the control of endogenous, ubiquitously active promoters. Thus, we were

successful in establishing mouse lines which fulfill the above criteria. This allowed for the first time to study the relationship between tTA and target gene expression during mouse embryogenesis. The analysis suggests that below a critical concentration TetR-VP16 relies on endogenous *cis*-acting elements for promoter activation. These putative elements determine the pattern of TetR-VP16 action without activating the transgene promoter on their own. They are therefore appropriately described as promoter preactivating elements. Our data provide evidence that histone hyperace-
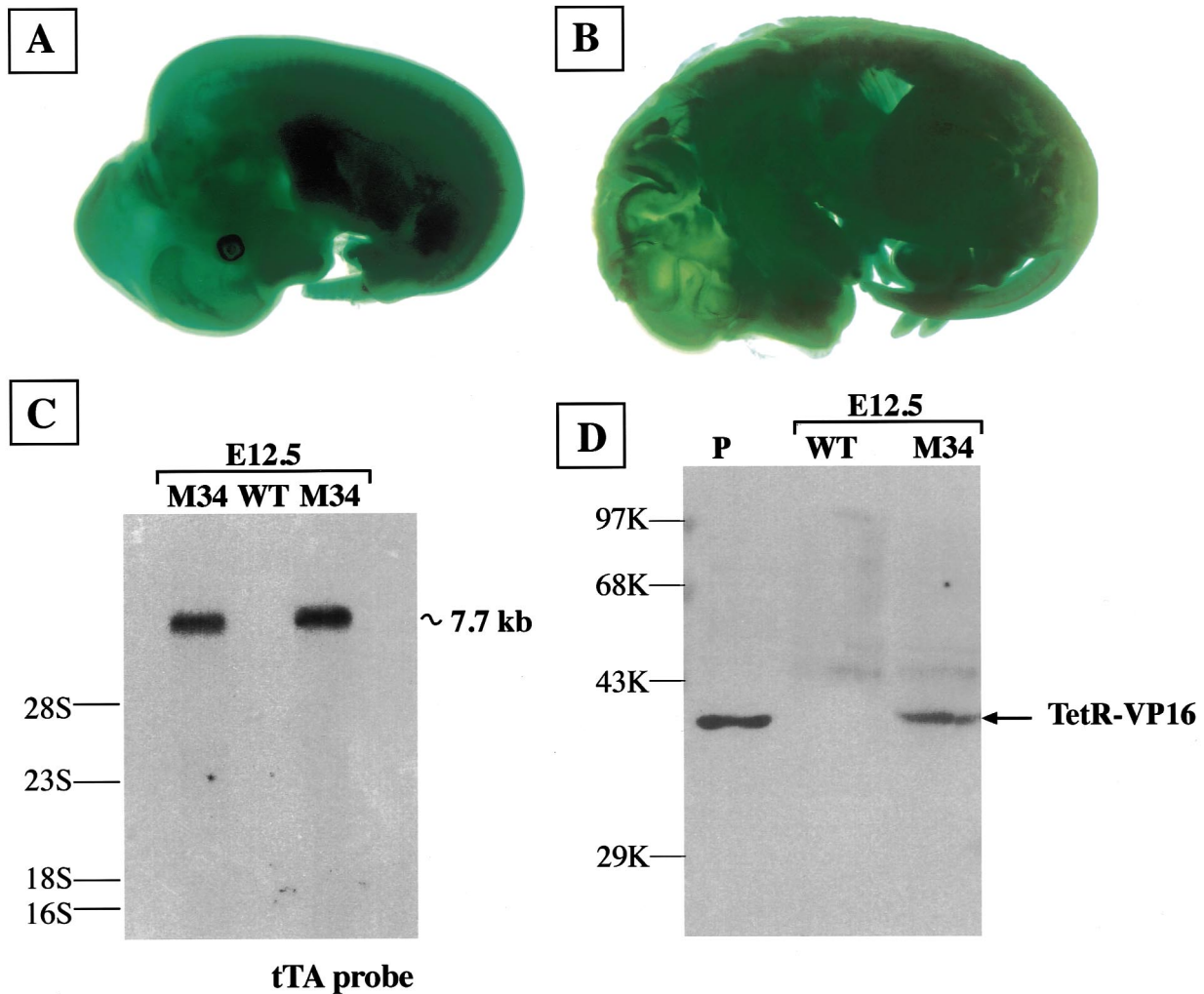
Fig. 2. (A–B) Embryos stained for β-galactosidase activity. (A) M34 embryo at E12.5. (B) M34 embryo at E15.5 cut sagitally. (C) Northern transfer of total RNA from E12.5 embryos (M34 and wildtype) hybridized with a tTA DNA probe. (D) Immuno transfer of protein from E12.5 embryos (M34 and wildtype) or using an anti-TetR antiserum. WT, wildtype; P, positive control. This was generated by transfecting mouse fibroblasts (Lt⁻) with activator plasmid pUHG15-1.

tylation provides a mechanism for promoter preactivation. Hence, activator concentration and the acetylation state of chromatin appear to be major determinants of TetR-VP16 function in transgenic embryos. This presumably accounts for many 'undesirable' properties of the tetracycline-dependent activator system in transgenic mice. The results suggest to search for constitutively hyperacetylated integration sites for tetO-linked transgenes in order to provide an activator responsive state.

2. Results

2.1. Experimental strategy

We used a modified gene trap approach to set tTA under the control of endogenous promoters which show little temporal and spatial restriction of their activity (Fig. 1D).

Different tTA-vectors were designed to trap endogenous promoters (Fig. 1A,B). These vectors carry downstream of the LacZ-neomycin-phosphotransferase fusion gene (β-geo) the tTA open reading frame fused to the internal ribosomal entry site (IRES) of the encephalomyocarditis (EMC) virus. The IRES element initiates translation by a mechanism different from the ribosomal scanning mechanism (Jackson et al., 1990; Kaminski et al., 1990). In frame integration of the tTA-vector into an active locus, therefore, gives rise to a trifunctional, dicistronic transcript (Fig. 1D). The distribution of the TetR-VP16 encoding transcript can be easily depicted with high resolution by staining for β-galactosidase activity. In order to reach the same resolution in the analysis of target gene expression, the tetO promoter was coupled to the cDNA of the human placental alkaline phosphatase (Fig. 1C). This permits the investigation of target gene expression by histochemical means.

2.2. Establishment of the transgenic model system

In order to trap endogenous promoters, embryonic stem (ES) cells were electroporated with tTA gene trap vectors. ES cells were selected for neomycin resistance and β-galactosidase expression and were finally aggregated with mouse morulae. From 11 aggregated ES cell clones two gave rise to chimeras transmitting the mutation to the next generation allowing establishment of lines M34 and B35. Embryos of both lines exhibit a widespread LacZ expression which is fully developed not later than E 12.5 (Figs. 2A,B and 3A,B). Northern transfer and immunoblotting directly demonstrated expression of the activator in mouse lines M34 and B35 (Figs. 2C,D and 3C,D). We will refer to the promoter trap generated activator genes as M34::tTA and B35::tTA, respectively.

Transgenic animals carrying the target gene were generated by microinjection of zygotic pronuclei. Mouse lines that have thus been generated are called TOPj, where j is a natural number referring to the individual line. The respective genes will be designated as tetO::hAP-j.

2.3. Target genes are refractory to transactivation in the majority of integration sites

Fourteen TOP lines were crossed with activator lines M34 and B35, respectively. Embryos were genotyped and stained for hAP activity between E12.5 and E15.5. Only one target gene integration site represented by line TOP47 allowed for tTA-dependent expression (Figs. 4 and 5). This shows that TetR-VP16 is expressed in a functional manner in transgenic embryos but also indicates that most of the target loci are refractory with respect to activation by TetR-VP16.

2.4. Reliance on promoter preactivating position effects

Line TOP47 represents an enhancer trap event (Wilson et al., 1990). Embryos depict a specific expression pattern of the target gene even in the absence of the activator. The endogenous enhancer function drives hAP expression in cartilage anlagen of the limbs, vertebrae, ribs and in an intricate craniofacial pattern including patches in the
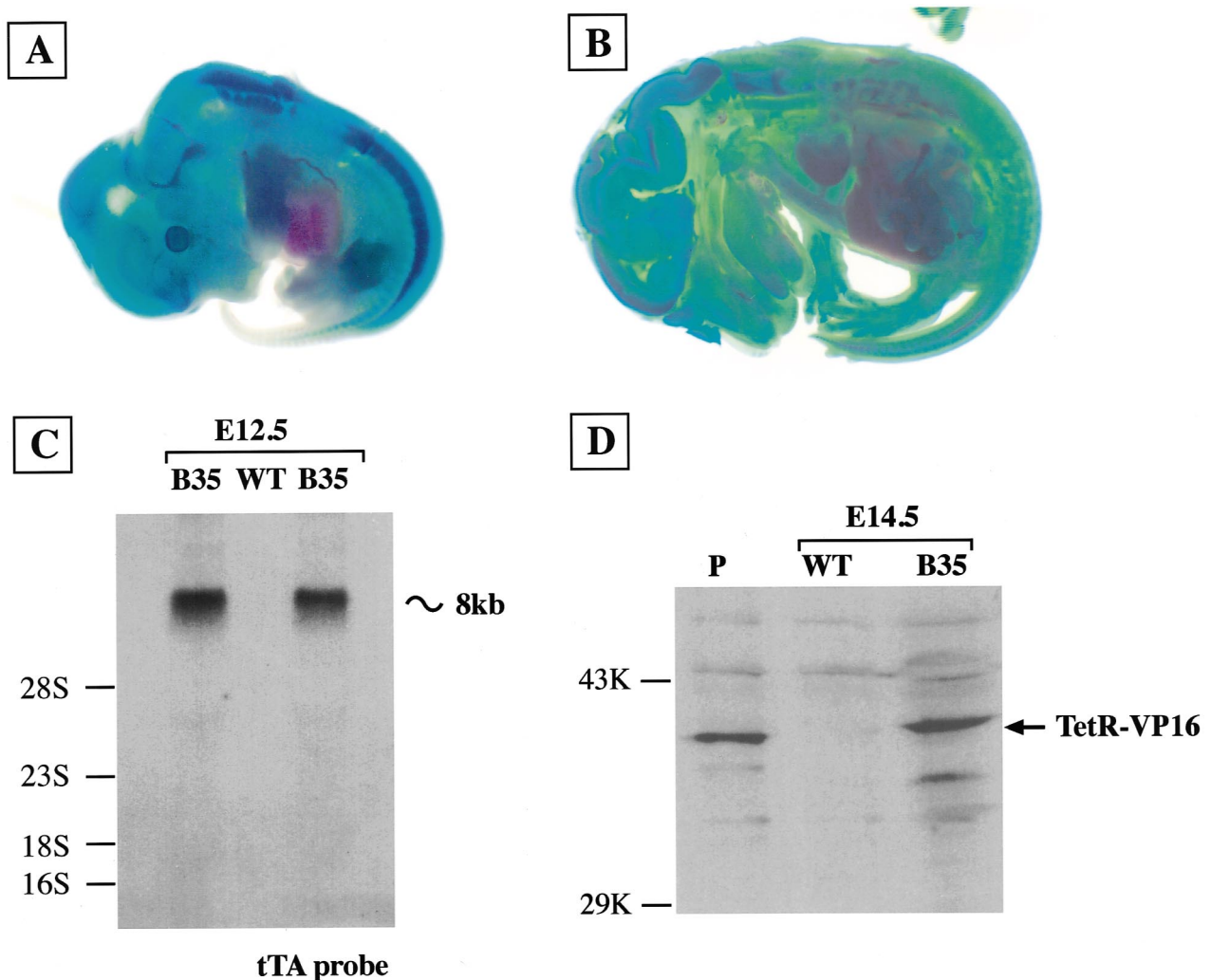

Fig. 3. Characterization of line B35. (A–D) like Fig. 2, except that immuno transfer (D) was done with protein of E14.5 embryos.

Fig. 4. Embryos stained for hAP activity with X-phosphate at E 15.5. Embryos were cut sagittally before staining. The embryos shown are littermates and have been stained under identical conditions. (A) TOP47 embryo. (B) TOP47 × B35 embryo. Note the enhanced hAP-expression in the cranial dermis and the ribs. (C) TOP47 × M34 embryo. (D) TOP47 × B35 × M34 embryo.

head dermis (Fig. 4A). Until E 14 double transgenic embryos carrying M34::tTA in addition to the target gene show an expression pattern that is indistinguishable from the pattern of littermates possessing the target gene alone (data not shown). Between E14 and E15, however, double transgenic and only double transgenic embryos start to express the target gene in the epidermis in a mosaic pattern (Figs. 4C and 5C). This expression was not observed in embryos from crossings of any other TOP line with line M34. It has to be concluded that the tTA-dependent epidermal expression in TOP47 × M34 embryos relies on a specific regulatory quality of the target gene's integration site. The endogenous *cis*-acting elements which must account for this position effect are functionally different to enhancers since they do not activate the transgene on their own. However, they are required for tTA-dependent activation and are therefore, best described as promoter preactivating elements.
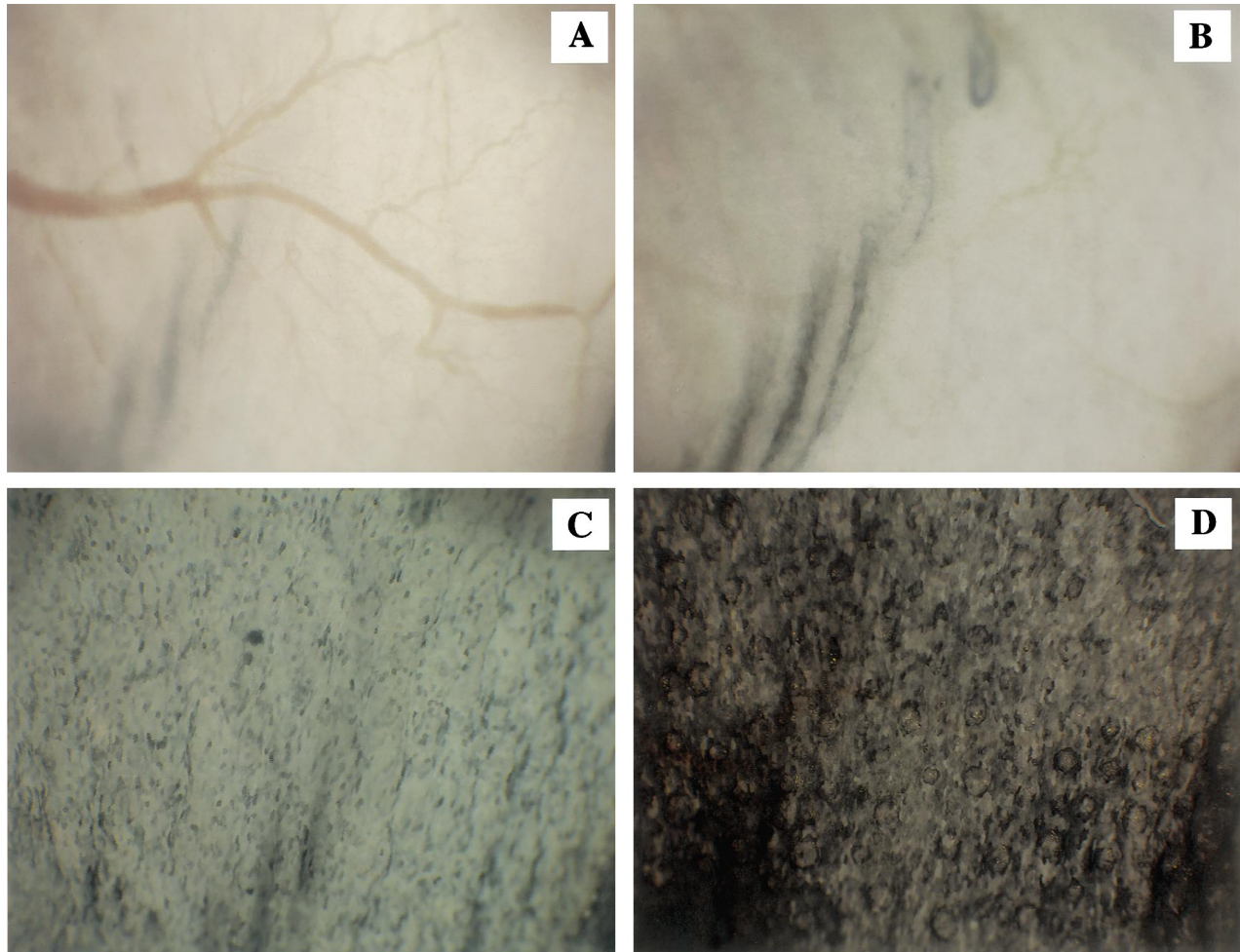
Fig. 5. Abdominal epidermis of X-phosphate stained embryos at E 15.5. (A–D) like Fig. 4. In (B) and (C) expression in the ribs can be seen through the epidermis.

2.5. Concentration is a critical parameter for TetR-VP16 function

We analyzed target gene expression in embryos from crossings between line TOP47 and activator line B35. With the exception of an enhanced cranial dermis expression B35::tTA does not stimulate target gene activity (Fig. 4B). Especially, TOP47 × B35 embryos lack any target gene expression in the epidermis. (Figs. 4B and 5B). This indicates that transactivation of tetO::hAP-47 by M34::tTA requires a feature that distinguishes the activator locus M34::tTA from B35::tTA. The most likely assumption is that the two activator loci are different with respect to their epidermal expression levels. This is supported by the observation that the epidermis of B35 embryos exhibits a fainter staining for β-galactosidase activity in comparison with M34 embryos (data not shown). To test for the effect of activator concentration on target gene expression we increased the activator concentration by generating triple-transgenic TOP47 × M34 × B35 embryos. These embryos show an almost uniform target gene expression in the

epidermis in contrast to the mosaic expression of TOP47 × M34 embryos (Figs. 4D and 5D). The synergism of the activator loci indicates that the concentration of the activator is a critical parameter for its ability to activate tetO::-hAP-47. Tripletransgenic embryos, however, do not exhibit new activator-dependent expression domains outside the epidermis at E 15.5 nor is the temporal frame of this expression altered. Therefore, we wanted to know if a further increase in activator concentration could result in activation of prior refractory promotor states. To answer this question we isolated primary embryonic fibroblasts (MEFs) from TOP97 × M34 embryos. The target gene of line TOP97 does neither exhibit tTA-dependent nor tTA-independent activity in vivo as judged by histochemical analysis of single and double transgenic embryos (data not shown). TOP97 × M34-MEFs were transiently transfected with an expression plasmid for TetR-VP16 and alkaline phosphatase activities were subsequently measured in extracts of transfected cells. The results show that increasing amounts of transfected activator plasmid lead to increasing phosphatase activities until a maximum is reached at 1600 ng of

activator plasmid (Fig. 6). With this amount of transfected activator plasmid a ~190-fold stimulation of alkaline phosphatase activity can be measured compared with the background level, whereas endogenous activator concentrations lead to a four- to five-fold increase (Fig. 6). A similar result was obtained using line TOP96 which, likewise, does not show tTA-dependent nor tTA-independent target gene activity in vivo (data not shown). The achieved increase in phosphatase activity is dependent on activator binding at the tetO-promoter since it can almost completely be repressed by culturing transfected cells in tetracycline containing medium (Fig. 6A, compare columns 1, 8 and 9). Immuno transfer experiments show that increasing

amounts of transfected activator plasmid used in our experiments, lead to increasing activator concentrations as expected (Fig. 6B). Although the transfection procedure itself potentiates activator function (Fig. 6, compare columns 2, 3 and 9) the dramatic increase in target gene activity observed in transfected cells compared with mock transfected cells can be mainly attributed to increased intracellular activator concentrations.

In summary we conclude that the concentration of TetR-VP16 is a critical parameter for its function irrespective of the large free energy change associated with TetR binding to DNA. The effect of activator concentration on the mosaicism of target gene expression suggests that variegating expression in the epidermis of TOP47 × M34 embryos is a phenomenon reflecting inefficient promoter activation.

2.6. Inhibition of histone deacetylases potentiates TetR-VP16 activity

Biochemical studies have shown that at least some transcription factors can bind to hyperacetylated nucleosome cores with affinity constants approaching the binding affinities for naked DNA (Lee et al., 1993; Vettese-Dadey et al., 1996). In order to test if the degree of histone acetylation influences the activity of TetR-VP16, TOP96xM34-MEFs were cultured in the presence and absence of tetracycline, at different concentrations of sodium butyrate. Butyrate inhibits histone deacetylases and thus causes global histone hyperacetylation (Candido et al., 1978; Sealy and Chalkley, 1978). Incubation of cells with butyrate leads to alkaline phosphatase activities exceeding background levels by more than a 550 fold at a concentration of 10 mM in the absence of tetracycline, whereas in the presence of tetracycline the activity did not increase by more than a factor of four (Fig. 7A). Similar results were obtained when TOP96 × M34- and TOP97 × M34-MEFs were treated with (R)-trichostatin A (TSA; Fig. 7B,C). TSA is an inhibitor of histone deacetylases which is believed to be much more specific than butyrate, since it is effective at concentrations
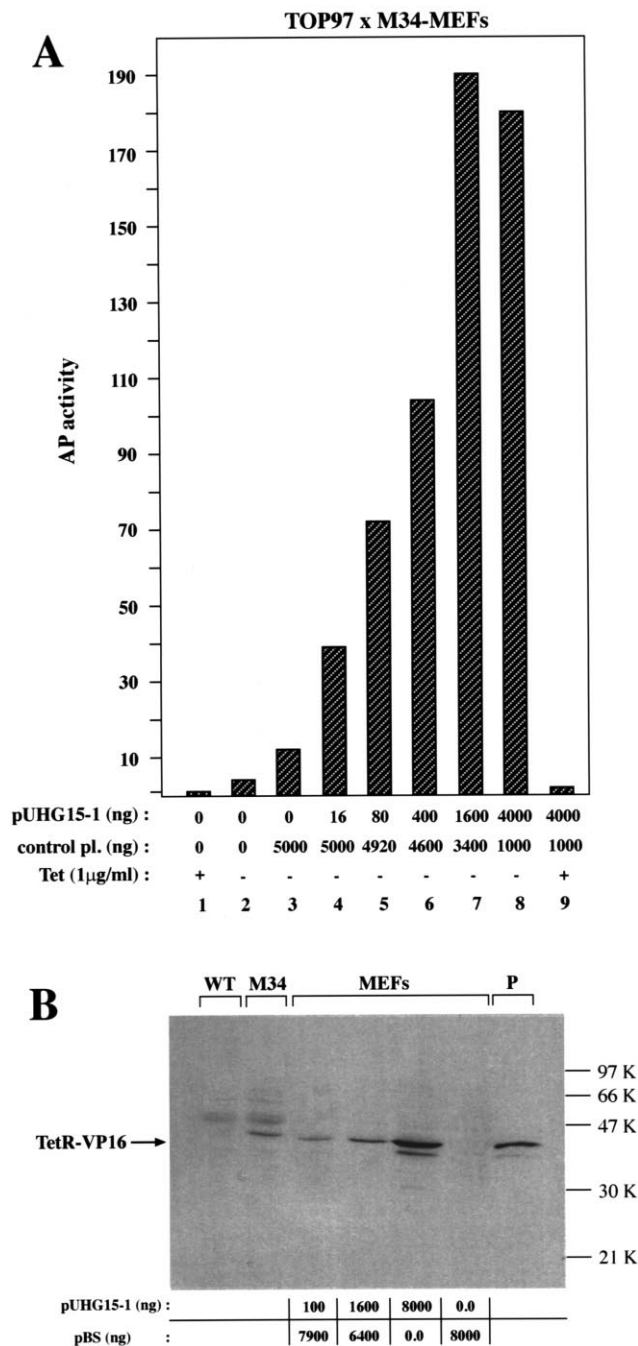

Fig. 6. (A) Transfection of TOP97 × M34-MEFs. Cells have been transfected with no DNA (columns 1 and 2) or a total of 5 μg of plasmid DNA (columns 3–9) containing different amounts of activator plasmid pUHG15-1 and control plasmid. AP, alkaline phosphatase. AP activity was normalized to the average CAT activity (see Section 4) measured in cells transfected with control plasmid only (column 3). AP activities are given on a relative scale. The value measured for mock transfected cells in the presence of tetracycline (column 1) was set to 1. Columns represent the average of two independent experiments done in parallel. (B) Immunoblot of protein from embryos and transfected MEFs. Equal amounts of protein of transfected TOP96-MEFs (proteins from two independent experiments done in parallel were pooled), and of a M34 and a wildtype embryo (E12.5) were loaded for comparison. The amounts of transfected plasmid are indicated below the photo. Note that < 1% of transfected cells contribute to the TetR-VP16 representing band, whereas presumably 100% of the embryo cells contribute to the TetR-VP16 representing band. P, see Fig. 2; pUHG15-1, activator plasmid; pBS, pBluescript (Stratagene).

five–six orders of magnitude lower than butyrate (Yoshida et al., 1995).

The induction of target gene activity by histone deacetylase inhibitors must be either due to the hyperacetylation of chromatin at the target promoter or due to a dramatic increase in TetR-VP16 concentration. To distinguish between these alternatives we compared transcript levels in TOP97 × M34 cells treated with different amounts of TSA. The results demonstrate that TSA does not influence the concentration of activator mRNA (Fig. 7D). However, it does lead to different transcript concentrations of the target gene (Fig. 7D), consistent with the induced changes in alkaline phosphatase activity (Fig. 7C). It is therefore most likely that the potentiation of activator activity by histone deacetylase inhibitors is due to the hyperacetylation of chromatin at the target promoter.

3. Discussion

To study the activation of tetO-linked transgenes in mouse embryos we used a modified gene trap approach to generate transgenic mice which ubiquitously express the tetracycline-dependent transactivator gene tTA during embryogenesis. Expression of the transgene is demonstrated by several means. Northern transfer analysis reveals a stable tTA-coding transcript for both activator lines investigated. Linkage of an IRES-tTA fusion with the β-Geo gene gives rise to a dicistronic mRNA, whose distribution can be monitored by histochemical analysis. This analysis suggests ubiquitous transcription of M34::tTA and B35::tTA, respectively. Immunotransfer analysis indicates successful translation of tTA sequences into TetR-VP16. Gene trap events isolated in our laboratory show ubiquitous expression of β-galactosidase from IRES-LacZ fusions (data not shown). This demonstrates that the IRES function used to direct the translation of tTA is not subjected to any obvious restrictions during mouse development. We therefore, conclude that the distribution of tTA-coding mRNA faithfully reflects the expression pattern of TetR-VP16.

TetR-VP16 is expressed in a functional form. This conclusion is based on the following observations: first, comparative histochemical analysis of TOP47 and TOP47 × M34 or TOP47 × M34 × B35 embryos reveals tTA-dependent epidermal expression of the target gene. Second, primary fibroblasts isolated from double transgenic embryos and analyzed by biochemical means show tetracycline-dependent target gene activity. Even in the absence of histone deacetylase inhibitors target gene activity is clearly dependent on tetracycline (Fig. 7A,B,C), though the stimulatory effect of the activator (four–five fold) is comparatively low and therefore most likely not detected by histochemical means in vivo.

3.1. Determinants for TetR-VP16 function

Our histochemical investigations surprisingly indicated that the tetO target gene is refractory with respect to transactivation by tTA loci in the majority of integration sites. The above discussion shows that this is not due to trivial reasons. Two possibilities can be envisaged to explain the refractory state of target genes in the presence of the activator: either the activator efficiently occupies its binding sites but transmission of the activation signal to the core promoter is inhibited, or alternatively, binding of the activator to DNA is hampered by chromatin factors. The observed dependence of promoter activity on the concentration of TetR-VP16 is only consistent with the second alternative. We therefore assume that activation of tetO-linked target genes is repressed at the level of activator binding to DNA. Repression of DNA binding in the context of chromatin has been demonstrated for several transcription factors (Taylor et al., 1991; Kwon et al., 1994). However, cell culture experiments have suggested that TetR-VP16 concentrations well below the detection level of immuno transfer analysis are sufficient for effective target gene activation (Gossen and Bujard, 1992). The difference between these and our results is most likely due to the fact that procedures for stable transfection always make use of cointegrating selection markers. This inevitably provides a bias for selecting accessible chromatin domains as integration sites for the transgene (Pikaart et al., 1998).

Mosaic expression of transgenes is frequently observed and has also been noticed implementing the tetracycline-dependent activation system in the mouse genome (Furth et al., 1994; Mayford et al., 1996). We show that increasing the activator gene dosage turns mosaic expression into uniform target gene activity. This suggests that increasing activator concentrations recruit an increasing number of target templates into the active state. Our observations are consistent with and provide the first in vivo evidence for the probabilistic model of activator function (Weintraub, 1988; Walters et al., 1995; Felsenfeld, 1996; Zlokarnik et al., 1998).

Since the activator depends for proper function on promoter preactivating position effects it seems that M34::tTA and B35::tTA express TetR-VP16 at a rather low level. Several experimental observations have suggested that cells cannot tolerate higher concentrations of TetR-VP16 (Gossen and Bujard, 1992; Baron et al., 1997; Gallia and Khalili, 1998). Since transcriptional activators have been shown to compete for low abundant coactivators (Kamei et al., 1996; Fryer and Archer, 1998), it is likely that expressing the multipotent VP16 activation domain can easily interfere with subtle equillibria of protein–protein interaction (Berger et al., 1990). Selecting for gene trap events that give rise to TetR-VP16 expression in ES cells and in a widespread manner during embryogenesis might, therefore, have introduced a bias for low abundant expression of the activator.

If increasing concentrations of TetR-VP16 can compensate for a chromatin-mediated repression of activator function, appropriate changes in chromatin structure might compensate for low activator concentrations. Several
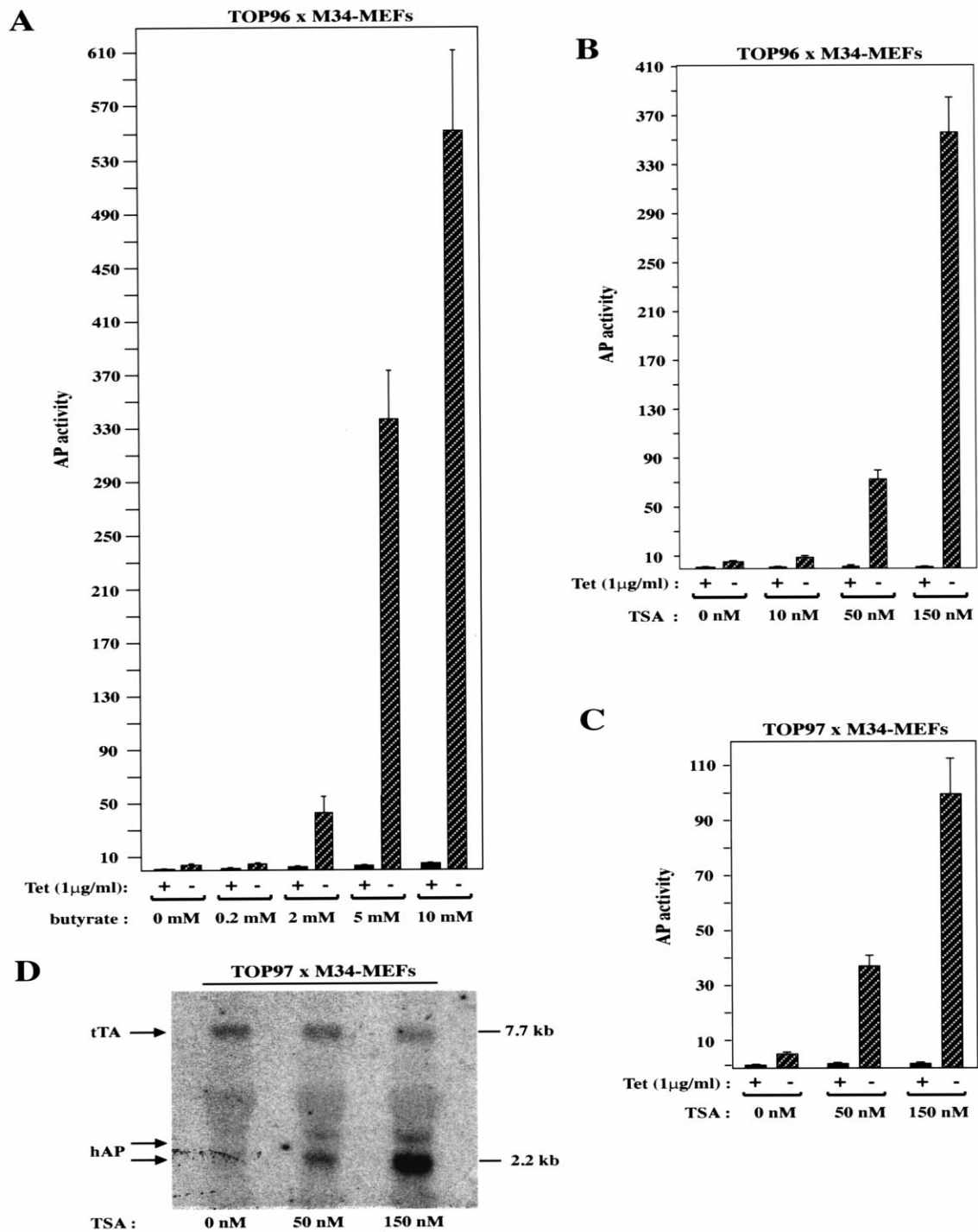
Fig. 7. MEFs were incubated with and without tetracycline at different concentrations of butyrate and TSA, respectively. (A) TOP96 × M34-MEFs treated with butyrate. (B) TOP96 × M34-MEFs treated with TSA. (C) TOP97 × M34-MEFs treated with TSA. Where indicated, cells were incubated with tetracycline for 2 days, before butyrate or TSA was added for 24 h. Subsequently cells were harvested and extracts were analyzed for alkaline phosphatase (AP) activity. Columns represent mean values of three independent experiments done in parallel. Bars represent standard deviations. AP activities are given on a relative scale. Values measured for cells, cultured in the presence of tetracycline but in the absence of histone deacetylase inhibitor, were set to 1. (D) TOP97 × M34-MEFs were treated with different concentrations of TSA as described above. Afterwards RNA was isolated and equal amounts were subjected to Northern blotting. The blot was co-hybridized with tTA and hAP probes. The hAP probe detects two different transcripts resulting from alternative splicing of the SV40 sequences (see Fig. 1).

studies have indicated that histone hyperacetylation can alleviate the repressive influence of chromatin proteins on transcription factor binding to DNA (Lee et al., 1993; Vettese-Dadey et al., 1996). Inhibition of histone deacetylases (HDACs) induces global hyperacetylation of histones (Candido et al., 1978; Sealy and Chalkley, 1978). This has been shown for many vertebrate cell types, including mouse fibroblasts (Candido et al., 1978). Incubating primary embryonic fibroblasts in the presence of HDAC inhibitors we observed a dramatic stimulation of target gene activity when and only when the activator was allowed to bind its operator site (Fig. 7). This suggests that TetR-VP16 is sensitive to changes in the acetylation state of chromatin. Alternatively it might be assumed that inhibition of HDACs gives rise to hyperacetylation of TetR-VP16 potentiating its function. Eukaryotic transcription factors have recently been found to be substrates of histone acetyltransferases and acetylation was shown to either positively or negatively affect their DNA binding affinity (Gu and Roeder, 1997; Munshi et al., 1998). However, it appears to be highly unlikely that the prokaryotic Tet repressor could accidentally be regulated this way in eukaryotic cells.

It has recently been demonstrated that sequences in the chicken β-globin locus control region protect a globin gene from inactivation by maintaining relative hyperacetylation of its chromatin in erythroid cells (Pikaart et al., 1998). Identification of *cis*-acting elements bestowing a sufficient degree of histone acetylation on juxtaposed chromatin in a broad range of cell types might help to establish tetracycline-dependent transgenes in mouse embryos.

In summary we conclude that concentration, as well as, the state of chromatin acetylation are likely to be important determinants for TetR-VP16 function. Hyperacetylation of histones can compensate for ineffective target gene activation at lower concentrations of TetR-VP16, whereas higher concentrations largely dispense the activator from influences of the acetylation state of chromatin. This perfectly agrees with observations in yeast showing that overexpression of transcriptional activators can overcome activational defects resulting from loss of function mutations in the histone acetyltransferase Gcn5 (Georgakopoulos and Thireos, 1992; Greogory et al., 1998).

3.2. Promoter preactivation and histone hyperacetylation

The tTA-dependent epidermal expression in TOP47 × M34 embryos relies on a specific regulatory quality of the target gene's integration site. The endogenous *cis*-acting elements which must account for this position effect do not directly activate the target gene but obviously synergize with tTA-loci in target gene activation. Hence, they are functionally different from those enhancer elements which activate the target gene in a tTA-independent manner, e.g. in the cartilage anlagen of the limbs. For this reason they are appropriately described as elements preactivating the tetO-promoter with respect to TetR-VPl6 function. Preactivation

most likely involves modified chromatin structures which facilitate the access of activator to DNA but, nevertheless, do not cause derepression of the core promoter. We demonstrate that histone hyperacetylation is a possible mechanism for promoter preactivation. For numerous transcriptional activators including nuclear receptors (Chakravati et al., 1996; Kamei et al., 1996; Kraus and Kadonaga, 1997; Blanco et al., 1998), MyoD (Yuan et al., 1996), NF-kB (Zhong et al., 1998) and cubitus interruptus (Akimura et al., 1997) there is strong experimental evidence that the interaction with histone acetyltransferases is a critical part of their activational function. The observation that histone hyperacetylation preactivates the tetO-promoter suggest that at least at the CMV core promoter other activator functions in addition to those recruiting histone acetyltransferase activity are necessary for efficient transcriptional activation. Uncoupling the necessary remodeling of promoter chromatin from the activation process proper, principally allows to encode expression patterns at the level chromatin structure. The modification of chromatin structure might therefore be one major goal of ontogenetic determination processes.

4. Experimental procedures

4.1. Vector constructions

Plasmid pGT1.8 (kindly provided by W.C. Scarnes) was completely digested with *Hind*III followed by partial digestion with *Xba*I. Resulting plasmid pieces were blunted using the Klenow fragment. A 5.8 kb DNA fragment carrying mouse En2 derived sequences fused to β-geo was cloned into the blunted XbaI site of IRES-tTA (kindly provided by Luc St-Onge) yielding vector pM26.22. Correct orientation of the insert was checked by digesting the vector with *Xba*I.

To construct pM4l.1 a 696 bp BamHI/MscI fragment was deleted from pUHG15-1 (Gossen and Bujard, 1992). The deleted fragment comprises the rabbit β-globin intron. After blunting the vector was religated to yield pM11.1. Finally a 3.45 kb NruI/ScaI fragment of pM26.22 was exchanged for a 2.76 kb NruI/ScaI fragment of pM11.1 to yield gene trap vector pM4l.1.

To produce pM42.7 a 474 bp XhoI/XbaI fragment from pUHG10-3 (Gossen and Bujard, 1992) was inserted into the singular HindIII site of phAP-SV (kindly provided by C. Neville and N. Rosenthal) using T4 ligase after blunting of vector and insert ends. The insert comprises the tetO promoter. Correct insert orientation with respect to the hAP open reading frame was examined by *Hind*III digest.

4.2. Cell culture

R1 embryonic stem cells (Nagy et al., 1993) have been kept and handled basically as described (Torres and Mansouri, 1994). Vector DNA was prepared using Qiagen columns. Prior to electroporation, vector DNA was linearized by digest with AseI (pM26.22) and ScaI (pM41.1),

respectively. Neomycin resistant and β-galactosidase positive clones were aggregated with morulae as described (Nagy et al., 1993). Primary embryonic fibroblast (MEFs) were basically prepared as described (Torres and Mansouri, 1994). MEFs were prepared from all embryos of one litter. To assure that MEFs of the desired genotype were included in the preparation, biopsies were taken from embryos for genotyping. MEFs were cultured at 37°C and 5% CO_2 in DMEM (1 g Glucose/l, Gibco-BRL) containing 100 U Penicillin/ml, 100 μg Streptomycin/ml, 10% heat inactivated FCS (Gibco-BRL), 2 mM L-Glutamin (Gibco-BRL). MEFs were transfected using the calcium–phosphate technique. In brief, DNA which included 1 μg of RSV-CAT (kindly provided by G. Chalepakis), different amounts of pUHG15-1 (Gossen and Bujard, 1992) and pBluescript (Stratagene) add 5 μg of total DNA was dissolved in 125 μl H_2O and mixed with 125 μl 0.5 M $CaCl_2$. Finally the mixture was dripped into 2 × HeBS buffer (50 mM HEPES, 1.5 mM Na_2HPO_4, 280 mM NaCl, pH 7.05) for a period of about 6 s while the buffer was gently vortexed. After an incubation of 20 min at room temperature the precipitates were dripped onto the cells which were grown on 9 cm plates. Two hours before and 20 h after transfection medium was changed. Cells were harvested at 37 h after transfection for preparation of extracts.

Stock solutions of histone deacetylase inhibitors were prepared by dissolving butyrate (Sigma) in water and TSA (Sigma) in DMSO at a thousand-fold higher concentration than used in cell culture. Solutions were stored at −20°C. To cells treated with '0 nM TSA' 10 μl of DMSO/10 ml of culture medium were added. Tetracycline (Boehringer) was dissolved in ethanol at a concentration of 5 mg/ml and stored at −20°C. Cells were treated with TSA and butyrate for 24 h. Those cells which were additionally treated with tetracycline were incubated in tetracycline containing medium for 48 h in advance.

4.3. Microinjection and mice strains

To produce founder TOP founder animals a 3 kb NotI/SalI fragment from pM42.7 was microinjected into zygotes of FVB mice at a concentration of 2 ng/μl as described (Hogan et al., 1994). To isolate the 3 kb fragment from pM42.7 the vector was digested with *Not*I, *Sal*I and *Sca*I. The fragment was isolated from agarose gel after electrophoresis using Qiaex II (Qiagen).

Transgenic animals were bred to NMRI mice to build up and maintain colonies.

4.4. Northern blot analysis

Total RNA was isolated as described (Hogan et al., 1994). However, tissue homogenates were additionally sonicated after preparation. After phenol/chloroform extraction RNA was precipitated using NaCl. Precipitates were finally dissolved in DEPC treated H_2O and stored at −70°C. Total RNA (25 μg) was separated by electrophoresis at 0.9 V/cm in MOPS buffer (20 mM MOPS pH 7.0, 1 mM EDTA, 8 mM sodium acetate) through 1% agarose gels containing 2.2 M formaldehyde. RNA was transferred to Nylon membranes (Qiagen) in 20 × SSC by capillary flow and fixed by UV irradiation (0.3 J/cm^2, at 312 nm). Membranes were hybridized with P^{32}-labelled random primed DNA probes in hybridization buffer (0.5 M sodium phosphate pH 7.2, 7% SDS) at 68°C. The DNA template used for tTA probe preparation was a 1 kb BamHI fragment comprising the whole tTA open reading frame isolated from plasmid pM44.2 (unpublished data). The template used for hAP probe preparation was a 1.7 kb XbaI fragment isolated from plasmid pM42.7 (Fig. 1C).

4.5. Immunoblotting

About 5×10^6 MEFs were sonicated in 100 μl of HEPES buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 25% glycerol, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Embryos were homogenized in HEPES buffer prior to sonication.

Proteins were electrophoresed in a dicontinuous SDS-PAGE with a 5% stacking and 12% separating gel. Subsequently proteins were blotted to PVDF membranes (Millipore) in transfer buffer (39 mM Glycine, 48 mM Tris–Base, 0.03% SDS, 10% Methanol) at 1 mA/cm^2 for 1 h 30 min using a LKB 2117 Multiphor II electrophoresis cell. Antibody incubations were done in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM sodium phosphate, potassium phosphate, pH 7.4) with 5% milk powder and 1% Tween 20. TetR-VP16 was detected using a rabbit anti-TetR antiserum (kindly provided by W. Hillen) at a dilution of 1:2500 and horseradish peroxidase conjugated goat anti-rabbit antiserum at a dilution of 1:5000 (Cappel). Bound secondary antibody was visualized using the enhanced luminescence kit (Amersham).

4.6. Measurement of enzymatic activities

After culture on six well plates, fibroblasts were rinsed (3×) with PBS on the culture plate, scraped from the wells in 1 ml PBS, briefly spun down and finally lysed in 200 μl of ethanolamine buffer (10 mM ethanolamine pH9.5, 0.5 mM $MgCl_2$, 4% Chaps)/well. After a brief sonication in 1.5 ml caps the cell lysate was incubated for 30 min at 65°C, centrifuged at 25 000 × g and 4°C for 5 min. Fifty microliters of the supernatant were mixed with 150 μl 1 × Phospha dilution buffer (Tropix) and incubated for 1 h at 65°C. From this 100 μl were mixed with 100 μl of Phospha assay buffer (Tropix) and incubated for 5 min at room temperature. Finally 100 μl of Phospha reaction buffer (Tropix) were added. RLUs were measured using a Lumat LB 9501 (Berthold). Values measured at the point of maximal light emission were normalized according to protein concentration. Relative protein concentrations were determined after Bradford (Bradford, 1976).

Chloramphenicol acetyltransferase (CAT) activities were

measured as described elsewhere (Sambrook et al., 1989). Results of CAT assays were quantitated using an instant imager (Packard).

4.7. X-phosphate staining of embryos

Embryos were prepared from their deciduae in ample PBS/MgCl$_2$ (2mM) and visceral yolk sac epithelia isolated for subsequent DNA extraction. Embryos were fixed in 4% Paraformaldehyde (PFA)/PBS/MgCl$_2$ (2 mM) over night. The next day embryos were rinsed 3 × in PBS/MgCl$_2$ (2 mM) for 20 min each and afterwards incubated for 1 h at 65°C. Subsequently embryos were incubated in freshly prepared hAP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl$_2$, 0.1% Tween-20, 2 mM Levamisol) for 30 min at room temperature and finally stained in freshly prepared staining solution (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl$_2$, 0.1% Tween-20, 2 mM Levamisol, 0.1 mg/ml 5-bromo-4-chloro-3-indolylphosphate (X-phosphate), 1 mg /ml nitroblue tetrazolium chloride (NBT)) on ice over night. After staining embryos were carefully rinsed in PBS/EDTA (20 mM) and once again fixed in 4% PFA/EDTA (20 mM).

X-phosphate and NBT stock solutions were prepared at a concentration of 10 mg/ml of water and 50 mg/ml of 70% dimethylformamide and stored at −20°C.

4.8. X-Gal staining of embryos

Embryos fixed for lh on ice with 1% formaldeyde/0.2% glutaraldehyde in PBS, containing 2 mM MgCl$_2$. After rinsing 3 × with PBS, staining was performed in PBS containing 0.1% X-Gal, 2 mM MgCl$_2$, 5 mM K$_3$[Fe(CN)$_6$], 5 mM K$_4$[Fe(CN)$_6$] in the dark at 30°C overnight.

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