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A cell-specific activator in the *Xenopus* A2 vitellogenin gene: promoter elements functioning with rat liver nuclear extracts

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Transfection experiments using *Xenopus* vitellogenin A2 gene constructs allowed us to identify an activator which increases the activity of the thymidine kinase promoter. The activator is located between −121 and −87 of the A2 vitellogenin gene and is separated by a stretch of curved DNA from the estrogen-responsive DNA element at −331. The activator functions in a cell-specific manner, as it is active in human breast cancer cells (MCF-7) as well as hepatoma cells but not in fibroblasts or HeLa cells. The activator is composed of at least three elements: elements 1 and 2 which form a partial palindrome, function independently, but act synergistically when combined. Element 3 is not active on its own, but supports elements 1 and 2. A TATA box region derived from the *Xenopus* albumin gene is sufficient for the function of the activator. *In vitro* transcription experiments using rat liver nuclear extracts demonstrate that the activator interacts with transcription factors. These factors are distinct from those recognizing HP1, a regulatory element common to several genes specifically expressed in hepatocytes.

Key words: curved DNA/hepatocyte-specific promoter element/hepatoma cells/*in vitro* transcription/MCF-7 cells

Introduction

The vitellogenin genes of the frog *Xenopus* are tissue-specifically induced by estrogen in the liver. In a fully estrogen-stimulated female up to 70% of the polyadenylated liver RNA constitutes vitellogenin mRNA. This high level of expression is achieved by very efficient transcription as well as by a selective stability of the mRNA (reviewed in Shapiro *et al.*, 1983; Tata *et al.*, 1983; Wahli and Ryffel, 1985; Blume *et al.*, 1987). The high transcription rate may indicate that the vitellogenin promoter is efficiently utilized by RNA polymerase in hepatocytes. In several genes DNA sequences that activate the transcription have been identified. Such sequences can be classified into enhancers, which increase transcription over long distances, and upstream promoter elements, which are close to the transcription initiation site (reviewed in Maniatis *et al.*, 1987).

To identify regulatory elements in the *Xenopus* vitellogenin genes, we have previously transfected various A2 vitellogenin gene constructs into the human breast cancer cell line MCF-7. This approach allowed us to describe an estrogen-responsive element (ERE) as well as a hormone-independent activator in the 5′ flanking region of the A2 vitellogenin gene (Klein-Hitpaß *et al.*, 1986). The ERE as found in the vitellogenin genes has been characterized quite extensively, both in our group (Klein-Hitpaß *et al.*, 1988a,b) and in other laboratories (Seiler-Tuyns *et al.*, 1986; Martinez *et al.*, 1987), whereas the activator has not been further characterized. Analyzing deletion constructs of the 5′ flanking region of the A2 vitellogenin gene, we consistently observed that restriction fragments from this region migrate more slowly than would be expected from their size deduced from the nucleotide sequence (unpublished data). This observation suggests the presence of curved DNA, which is known to alter the electrophoretic mobility of DNA fragments (Marini *et al.*, 1982; Wu and Crothers, 1984). Curved DNA is formed by short runs of adenines and thymidines, which are 3−9 bp long and which are repeated with each helical turn, i.e. 10−11 bp (Diekmann, 1986; Koo *et al.*, 1986; Ulanowsky *et al.*, 1986). In this report we mapped the position of the curved DNA and show that it separates the ERE from the activator. Characterization of the activator reveals that it constitutes a cell-specific transcriptional regulatory unit that may be responsible for the high *in vivo* transcription of the vitellogenin genes.

Results

Curved DNA maps between the ERE and the activator

Previous experiments have shown that the 5′ flanking region of the A2 vitellogenin gene (position −260 to −87) contains an activator (Klein-Hitpaß *et al.*, 1986). The same region includes curved DNA, as restriction fragments containing this region migrate more slowly than expected during electrophoresis in polyacrylamide gels (unpublished data). To locate curved DNA in the 5′ flanking region of the A2 vitellogenin gene we analyzed progressive 5′ deletions of the sequences between −331 and −87 (see Figure 1). The deletion fragments were cloned between the *Hin*dIII and *Bam*HI restriction sites of pBLCAT8+ (Klein-Hitpaß *et al.*, 1986). Use of this cloning vector allowed us to test the same fragments for their potential to activate transcription (see below). The extent of deletion was analyzed by sequencing. Appropriate cloned fragments were cut out by *Hin*dIII/*Bam*HI digestion and the size of the insert determined on a polyacrylamide gel. The observed length for the fragment −331/−87 was 20% longer than expected from the sequence data, thus revealing curved DNA. Figure 2a demonstrates that this aberrant slow migration is maintained in deletions −317 and −278, but gradually decreases starting at −260 and is almost completely absent in the fragments −150 and −134. One might argue, however, that this observation rather reflects the fact that the curved DNA is shifted to the end of the tested fragment, where its effect is less pronounced

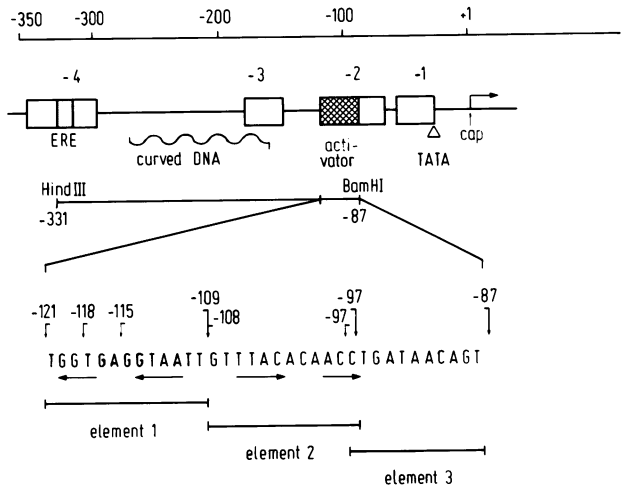

Fig. 1. Regulatory elements of the A2 vitellogenin gene of *Xenopus*. The four homology blocks that are conserved in the *Xenopus* and chicken vitellogenin genes (Walker *et al.*, 1984) are given. The scale at the top represents the nucleotide position upstream of the cap site (+1). The homology block −4 contains the estrogen-responsive element (ERE; Klein-Hitpaß *et al.*, 1986, 1988a), the block −2 contains the hormone-insensitive activator described in this work, whereas the block −1 includes the TATA region defining the transcription initiation at the cap site. The position of the curved DNA as defined in this paper is indicated. The *Hin*dIII−*Bam*HI deletion clone extending from −331 to −87 containing the ERE and activator is given. The sequence of the activator and its composition by elements 1, 2 and 3 is given. The arrows below the sequence correspond to the palindromic sequence as found in elements 1 and 2. The positions of the various 5′ and 3′ deletion clones within the activator are indicated.

(Wu and Crothers, 1984) than the removal of the curved DNA. Therefore we measured the size of restriction fragments which contain a part of the 5′ flanking plasmid DNA using the *Pvu*II site located 93 bp 5′ to the vitellogenin insert. Such fragments should contain the supposed curved DNA in the middle of the fragment, where the effect of curved DNA is most prominent (Wu and Crothers, 1984). In such digests, fragments of clone −176/−87 also reveal slow migration, whereas those derived from clone −150/−87 and −134/−87 migrate as expected from their size (data not shown). Therefore, the 3′ border of the curved DNA maps between −176 and −150. Taken together, curved DNA is located between −278 and −150 of the A2 vitellogenin gene. A similar conclusion has been derived from the analysis of the 3′ deletion clones (−331/−191, −331/−254, −331/−280; data not shown).

Our previous experiments located the activator between −260 and −87 (Klein-Hitpaß *et al.*, 1986). To test whether the curved DNA represents or at least includes the activator we used the various deletion fusion genes in transfection experiments. These constructs contain the vitellogenin 5′ flanking region linked to the thymidine kinase (tk) promoter of herpes simplex virus in front of the chloramphenicol acetyl transferase (CAT) gene and are essentially the same constructs as previously used (Klein-Hitpaß *et al.*, 1986). Transfection of these hybrid genes into the human breast cancer cell line MCF-7 allowed us to measure the increase in CAT activity due to the presence of the vitellogenin 5′ flanking sequences. Figure 2b illustrates that the deletion clones −331/−87 and −317/−87 yield some 70- to 80-fold higher activity than pBLCAT8+, the vector which contains only the tk promoter. Further deletion (−278 to −134) only

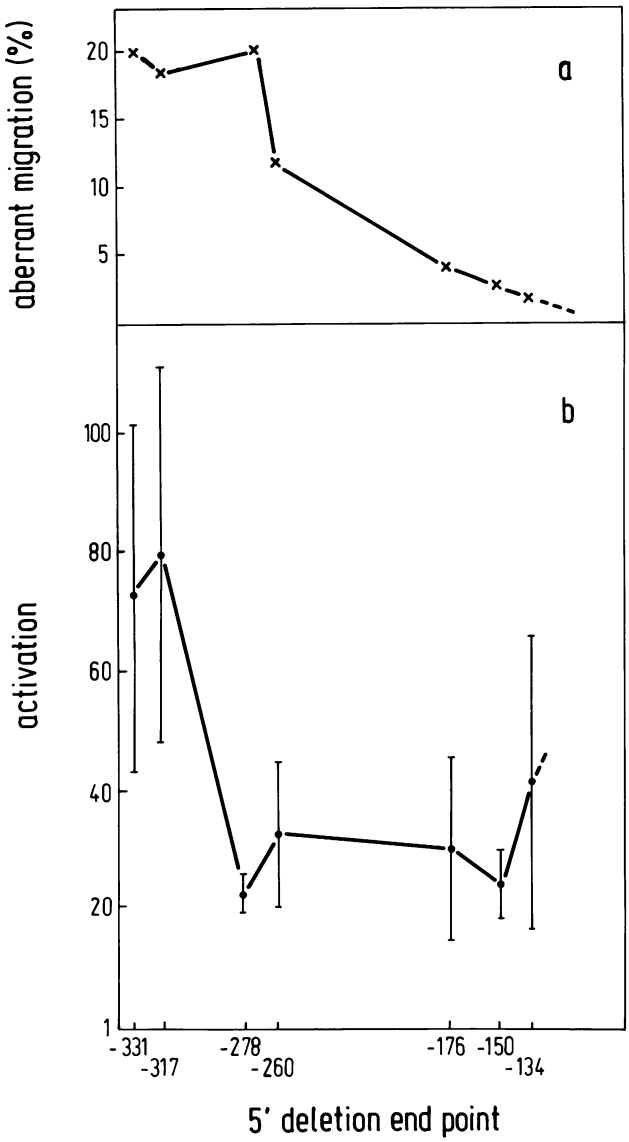

Fig. 2. The activator is outside of the curved DNA region. (**a**) *Hin*dIII−*Bam*HI fragments of various 5′ deletions within the 5′ flanking region of the A2 vitellogenin gene were sized by electrophoresis on polyacrylamide gels using *Hae*III-digested pBR322 DNA as marker. All clones used had the *Bam*HI linker at position −87, whereas the *Hin*dIII linker was at the indicated 5′ position. The aberrant migration is given for all the clones analyzed. (**b**) The same fragments as in (a) were tested for their potential to activate the promoter derived from the herpes simplex virus thymidine kinase gene (−105 to +51) as cloned in pBLCAT8+. The activation factor for each deletion fragment was calculated from the CAT activity obtained after transient transfection into MCF-7 cells using the activity of the vector pBLCAT8+ as reference. The vertical bars represent the SD determined in at least two independent transfections.

slightly reduced the activation (to ~20- to 40-fold on average, Figure 2b), thus demonstrating that most of the activating potential is still present. Clearly the activator is separated from the curved DNA, as deletion clones −150 and −134 retain high activating potential, but lack curved DNA.

The activator maps between −121 and −87 and consists of several elements

To delinate further the sequences involved in activation we analyzed smaller segments of the vitellogenin 5′ flanking

region for their activating potential in MCF-7 cells. Figure 3 shows that the sequence −121 to −87 yields the highest activation (160-fold). This value is 4-fold higher than the one obtained with clone −134/−87, suggesting the removal of a negative element between −134 to −121. We define the sequence between −121 to −87 as the activator of the A2 vitellogenin gene since further deletion reduces its activity significantly: a 3-bp 5′ deletion, i.e. clone −118/−87 retains only ~20% of the activity of −121/−87, and a 3′ deletion, i.e. clone −121/−97, is only 30% as active as the entire activator (Figure 3). This gradual loss in activity upon removing part of the activator is further supported by the reduced activation observed with the 5′ deletion clones −115/−87 and −108/−87 as well as with the 3′ deletion clones −121/−97 and −121/−109. A composite structure of the activator is also revealed by the fact that separate elements such as −121/−109 (element 1 in Figure 1) and −108/−97 (element 2 in Figure 1) give 7.5- and 3.3-fold activation, respectively. In contrast, element 3 (−97/−87 in Figure 1) has no clear activating potential. However, element 3 increases in combination with elements 1 and 2 from 60- to ~160-fold activation (compare −121/−97 with −121/−87 in Figure 3). The combined elements 1 and 2 (clone −121/−97) reveal an activation factor (60-fold) which is much higher than the addition of the value of each element (clone −121/−109 and −108/ −97). This suggests a synergistic mechanism and may correlate with the fact that the sequence in elements 1 and 2 forms a partial palindrome (see Figure 1).

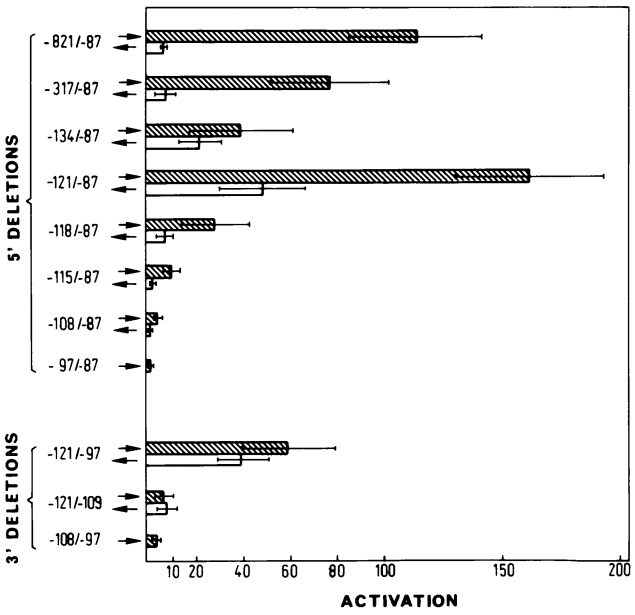

Fig. 3. Sequence requirements and orientation independence of the activator. Various vitellogenin A2 gene fragments were tested for their activation potential in syn (→) or anti (←) orientation using pBLCAT8+ and pBLCAT9+ as vectors respectively. The numbers refer to the deletion endpoints of the vitellogenin gene fragments tested. The activation factor for the various constructs is given with the SD derived from at least four independent transfections into MCF-7 cells. The values found for constructs containing the vitellogenin fragment in syn (→) or anti (←) orientation are given by striped or open columns, respectively.

The activator functions in inverted orientation and over long distance

To investigate whether the activator of the A2 vitellogenin gene has the properties of an enhancer, we inverted the various A2 vitellogenin 5′ flanking sequences in front of the tk promoter using pBLCAT9+ (Klein-Hitpaß *et al.*, 1988a). Figure 3 illustrates that the activator (−121/−87) is still active in its inverted orientation, but at a 3-fold reduced level. The same holds true for the 5′ deletions of the activator (−118/−87 to −108/−87). However, the effect of the activator which is truncated at its 3′ end, i.e. clones −121/−97 (elements 1 and 2) and −121/−109 (element 1), is not influenced by inversion. Based on this observation we assume that element 3 contributes to the performance of the activator only in its normal orientation.

Inversion of larger fragments of the 5′ flanking region containing the entire activator leads to a drop in the activating potential but even inversion of the sequence −821/−87 retains 6-fold activation. We conclude that the activator may act over longer stretches of DNA although at a much reduced level.

The activator is cell-specific for MCF-7 and hepatoma cells

As the vitellogenin genes are exclusively expressed in the liver we expected that the activator is also active in hepatoma cells. Therefore we transfected the activator-containing construct (−121/−87) into various hepatoma cell lines. Table I shows that the activator functions in both human HepG2 cells and murine BW1J cells but at a 3- and 15-fold reduced level, respectively. Activation similar to that found in BW1J is also observed in a rat hepatoma cell line (FTO-2B; T.Weimar-Ehl, unpublished). In contrast the activator is not significantly active in three cell lines of non-hepatic origin, i.e. murine Ltk$^-$ cells, human primary skin fibroblasts (NFB München) and human cervix carcinoma cells (HeLa). Based on these experiments the activator function is restricted to breast cancer and hepatoma cells. Testing separate elements of the activator reveals that element 1 is the most powerful element in all cell lines. Clearly, the combination of elements 1 and 2 (−121/−97) is the most active part of the activator in HepG2 cells. This finding agrees with the observation made in MCF-7 cells (Table I). In non-hepatic cell lines some of the elements even give clear inhibition.

By transfection of the clones −821/−87, −134/−87 and −121/−87 into HepG2 cells, we verified that the limits of the activator are identical to the ones as defined in MCF-7 cells (Döbbeling, 1988). From these experiments we can exclude the presence of an additional regulatory element specific for hepatoma cells.

The TATA box region is sufficient for the function of the activator

All experiments made so far used gene constructs with the tk promoter (position −105 to +51), which is known to contain two SP1 and one CTF-1 binding site (Jones *et al.*, 1985). To exclude the possibility that the activator requires such a complex promoter, we inserted the construct in front of a synthetic TATA box derived from the *Xenopus* albumin gene (position −50 to −27; Schorpp *et al.*, 1988a). This element is believed to contain just the TATA box function and has been demonstrated to be useful to test regulatory elements (Schorpp *et al.*, 1988b). Constructs containing the

Table I. Cell specificity of the activator[a]

Cell line	Activator sequences tested					
	−121/−87 (1,2,3)	−121/−97 (1,2)	−108/−87 (2,3)	−121/−109 (1)	−108/−97 (2)	−97/−87 (3)
MCF-7	163 ± 37	60 ± 18	4.5 ± 2.5	7.5 ± 4.1	3.3 ± 2.0	1.6 ± 0.7
HepG2	61 ± 9	38 ± 18	2.9 ± 1.4	5.2 ± 2.6	2.8 ± 2.7	2.7 ± 0.8
BW1J	11.5 ± 0.6	ND	2.5 ± 0.4	5.3 ± 2.6	1.6 ± 0.8	1.6 ± 0.2
Ltk$^-$	1.5 ± 0.5	0.4 ± 0.3	0.8 ± 0.6	0.7 ± 0.5	0.4 ± 0.2	0.2 ± 0.1
NFB-Mü	2.1 ± 0.4	0.6 ± 0.3	1.0 ± 0.9	0.5 ± 0.3	0.2 ± 0.1	0.7 ± 0.1
HeLa	1.7 ± 0.8	1.9 ± 1.5	1.4 ± 0.8	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1

[a]The activation factor for the various constructs is given with the SD (see Figure 3). The numbers in parentheses refer to the element contained in the construct.
ND = not determined.

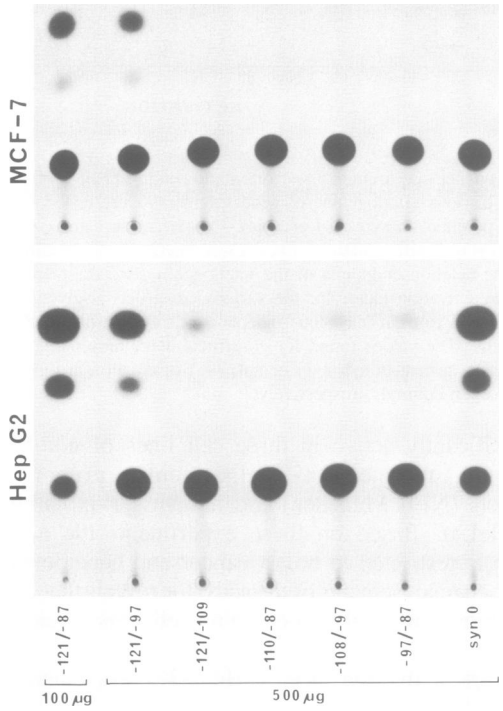

Fig. 4. The activator functions with a TATA box region. The activator as well as several subfragments (see Figure 1) were cloned in front of the TATA box region (−50 to −27) of the *Xenopus* albumin 68-kd gene (Schorpp *et al.*, 1988a) using the vector syn 0-T (Schorpp *et al.*, 1988b) containing a CAT gene as indicator. The constructs were transiently transfected into MCF-7 or HepG2 cells and the CAT activity determined using 100 µg protein for construct −121/−87 and 500 µg protein for all other plasmids. The construct syn0 contains the hepatocyte-specific promoter element (HP1, Schorpp *et al.*, 1988b).

activator in front of the TATA box linked to the CAT gene were transfected into HepG2 and MCF-7 cells. As Figure 4 illustrates, the presence of the activator yields high CAT activity in both cell lines. In HepG2 cells the activity is comparable to that found with a construct in which the activator is replaced by the hepatocyte-specific promoter element HP1 (syn0), known to confer liver specific gene expression (Schorpp *et al.*, 1988b). We also made constructs using only some of the elements of the activator. Of all combinations tested only elements 1 and 2, i.e. clone −121/−97, gives increased activity in comparison with constructs containing a polylinker insert (not shown). However, the activity is 5- to 10-fold lower than with the entire activator. These experiments confirm that element 3 contributes to the activator function and establish that the activator (−121/−87) and even the combined elements 1 and 2 (−121/−97) are efficient in activation of a TATA box region devoid of any other known regulatory element.

The activator enhances in vitro transcription in rat liver extracts

As the activator is located close to the TATA box, we assumed that it is a regulatory element that increases the transcription activity of the promoter. To verify this assumption, we tested the function of the activator in an *in vitro* transcription system using a rat liver nuclear extract (Gorski *et al.*, 1986). We essentially used the constructs described above in which the activator was inserted in front of the TATA box region of the albumin gene. To allow efficient measurement of *in vitro* transcription, we inserted the −G-cassette of 400 bp downstream of the TATA box, thus allowing direct quantification of [^{32}P]UTP-labeled transcripts synthesized in the absence of GTP (Sawadogo and Roeder, 1984). As an internal control (cont. in Figure 5) we added in all transcription assays a construct containing the adeno major late promoter in front of a shortened −G-cassette of 190 bp. As Figure 5 illustrates, the activator (Act, lane 4) allows *in vitro* transcription which is ~20-fold higher than the control (PL, lane 5), in which the activator is replaced by a polylinker sequence. The increase of *in vitro* transcription by the activator is similar to that found in constructs containing the hepatocyte-specific promoter element HP1, as found in the *Xenopus* albumin (syn0, lane 1) as well as in the human α_1-antitrypsin gene (AT, lane 3). HP1 of the α_1-antitrypsin gene has been initially identified in Cortese's group as the B-domain which is an essential promoter element for hepatoma-specific transcription (DeSimone *et al.*, 1987). Sequence comparison between the activator and HP1, which we have recently identified as a common promoter element in various liver-specific genes (Kugler *et al.*, 1988), suggested to us that the activator might also contain a functional HP1. This assumption has been disproved, however, as the addition of a large excess of HP1 oligonucleotide does not inhibit the function of the activator (lane 9), whereas the activity of HP1 as found in the albumin (lane 6) and α_1-antitrypsin gene (lane 8) is completely inhibited as previously reported (Kugler *et al.*, 1988; Schorpp *et al.*, 1988b). The activity of the adeno major late promoter (compare lanes 2 and 7, but also all the internal

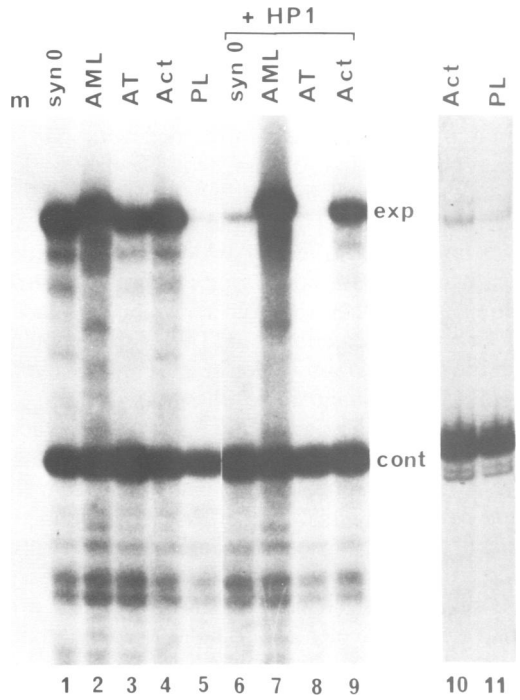

Fig. 5. The activator is a positive regulatory element in an *in vitro* transcription system derived from rat liver. Constructs containing the TATA box region of the *Xenopus* albumin gene linked to the −G-cassette were used in *in vitro* transcription assays to monitor the effect of regulatory elements inserted upstream of the TATA box. The constructs contained the following elements: syn0 (HP1 of the *Xenopus* albumin gene), AT (HP1 of the human α_1-antitrypsin gene, position −75 to −63; Kugler *et al.*, 1988), Act (the activator of the A2 vitellogenin gene, position −121 to −87) or PL (the *Hin*dIII−*Bgl*II polylinker sequence of pBLCAT3; Luckow and Schütz, 1987). As a control we also used the adeno major late (AML) promoter linked to the −G-cassette [pML(C_2AT)$_{19}$] as constructed by Sawadogo and Roeder (1984). The transcription assays in **lanes 6−9** contained in addition 100 ng of the HP1 oligonucleotide (Schorpp *et al.*, 1988b) as competitor. Rat liver nuclear extract and HeLa extract were used in **lanes 1−9** and in **lanes 10, 11** respectively. The signal migrating with a size of ~400 nt (exp) corresponds to the −G-cassette transcript derived from the promoter to be tested, whereas the signal of 200 nt (cont) represents transcription of the internal control.

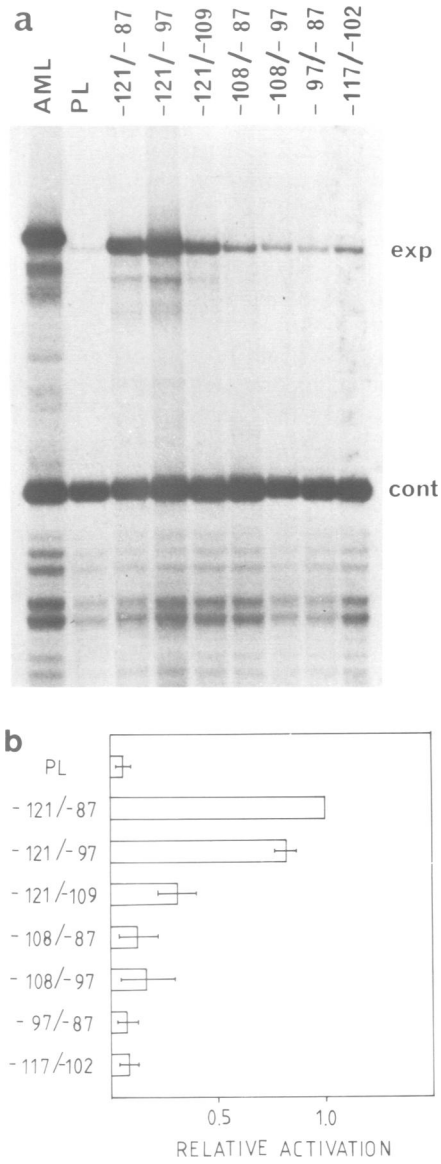

Fig. 6. Activity of the activator elements in the *in vitro* transcription system. (**a**) The activator (−121/−87) and several subfragments (see Figure 1) were inserted into the vector used for *in vitro* transcription and its activity measured in a rat liver nuclear extract. For further explanation see Figure 5. (**b**) The data of three independent experiments as shown in (a) were quantified by densitometry of the autoradiograms using the internal control pML cas9 for reference. The values were standardized to the activity found with the entire activator (−121/−87).

controls) is not significantly affected by the addition of HP1. To verify the cell specificity of the activator function we used a nuclear extract of HeLa cells, in which, based on transfection experiments, the activator is *in vivo* not functional (Table I). Clearly, the activator is not active in the *in vitro* transcription (compare lane 10 with lane 11 containing the polylinker construct PL) whereas the adeno major late promoter used as an internal control (cont. in lanes 10 and 11) gives high activity.

To analyze whether elements 1, 2 and 3 play the same role *in vitro* as *in vivo*, we extended the *in vitro* transcription to the corresponding constructs. An example is given in Figure 6a and data from several independent experiments are quantitated in Figure 6b. Clearly, the combined elements 1 and 2 (clone −121/−97) have an activity which is nearly as high as found for the entire activator, whereas the separate elements 1 (−121/−109) or 2 (−108/−97) show much reduced activity which, however, is still higher than that observed with the control construct (PL). Element 3 (−97/−87) on its own is not functional. Comparing these *in vitro* transcription data with the results observed *in vivo*, we conclude that the elements function quite similarly *in vitro* as *in vivo*. A prominent difference is seen only with element 3 which combined with elements 1 and 2 hardly supports the activator function *in vitro*, whereas it increases the performance of the activator *in vivo* 3- to 10-fold (Figures 3 and 4). This documents further the importance of the palindrome sequence located in elements 1 and 2.

Based on the competition experiment with HP1 (Figure 5), we conclude that the activator is distinct from HP1. To confirm this finding we inserted into the transcription vector an oligonucleotide containing the part of the activator which is most similar to HP1 and analyzed this construct by *in vitro* transcription. Figure 6 shows that this construct (−117/−102) is inactive and therefore gives further evidence that the activator is distinct from HP1.

Discussion

Functional analysis of the 5′ flanking region of the A2 vitellogenin gene of *Xenopus* allowed us to identify a DNA element which confers cell-specific gene expression. This activator element is clearly outside the curved DNA region and therefore we cannot assign any clear function to the curved DNA. However, we believe that this stretch is of biological significance, as sequence analysis of the other vitellogenin genes (Walker *et al.*, 1984) suggests similar structures at homologous positions. In fact, electrophoretic analysis of restriction fragments of the 5′ flanking region of the chicken vitellogenin II gene reveals fragments with aberrant migration properties (data not shown). We propose the hypothesis that the curved DNA allows efficient simultaneous exposure of the ERE and activator in the chromatin, because curved DNA may be preferentially bound by a nucleosome (Satchwell *et al.*, 1986; Hsieh and Griffith, 1988).

Transfection into various cell types revealed that the activator is functional in three hepatoma cell lines as well as in human breast cancer cells (MCF-7), but is not active in fibroblasts and HeLa cells. The activity of an element derived from a frog gene in MCF-7 cells is obviously not of physiological relevance, as no corresponding cells exist in the frog. With the exception of MCF-7 cells the function of the activator is restricted to hepatic cells of various origin. This suggests that the element defined in this study is operative in the frog liver. We assume that the activator in combination with the ERE allows the high level of vitellogenin gene transcription found in an estrogen-stimulated liver. The activator of the frog vitellogenin A2 gene functions in human (HepG2) and mouse (BW1J) cells as well as in rat liver nuclear extracts. This indicates that factors involved in hepatocyte-specific expression have been conserved between different vertebrate species. This finding is not unusual since several elements present in frog genes function in mammalian cells (e.g. Klein-Hitpaß *et al.*, 1986; Mohun *et al.*, 1987; Schorpp *et al.*, 1988b).

Sequence comparison of *Xenopus* and chicken vitellogenin genes has revealed four blocks of sequence homology in their 5′ flanking regions (Walker *et al.*, 1984 as summarized in Figure 1). The activator is present at the 5′ border of block −2 and therefore has some homology in the other vitellogenin genes, although no clear consensus sequence can be deduced.

The activator is located at −121 to −87 of the A2 vitellogenin gene. This positioning classifies it as an upstream promoter element (UPE) as defined by Maniatis *et al.* (1987). Operationally the activator has the clear properties of an enhancer, since it can be turned around without destroying its function and is active over long distances (Figure 3). However, the long-range effect has only been measured in constructs containing large vitellogenin 5′ fragments in opposite orientation. Therefore, we cannot rigorously exclude that some other 5′ flanking sequences contribute to the activation observed. In fact, the various 5′ deletions give somewhat different activation factors: there is a clear drop by a factor of 3−4 by deletion of sequences from −317 to −278 (Figure 2b) and there is a significant increase by deleting from −134 to −121 (Figure 3). These observations suggest the presence of other minor regulatory elements. However, in comparison their effect is quite small and we have not further analyzed their properties.

The activator can be divided into at least three functional elements (see Figures 1 and 3). This division is somewhat arbitrary, as element 1 can be further subdivided. This is most evident in the 5′ deletion clones, in which gradual deletion of element 1 causes a corresponding drop in activity (clones −118/−87 and −115/−87 in Figure 3). Elements 1 and 2 are active on their own, whereas element 3 mainly supports the activity of the other elements. This is also observed in the constructs in which the tk promoter is replaced by the TATA box region (Figure 4). Elements 1 and 2 are able to give 3- to 7-fold activation each, and show clear synergism when combined (Table I). This holds true also for the *in vitro* transcription (Figure 6). Analyzing the elements of the enhancer we observed that removal of element 3 abolishes the orientation effect for the activator (Figure 3). This might mean that the activator is composed of an enhancer (elements 1 and 2) and an upstream element (element 3) that is active only in one orientation. In this context it is worth noting that element 3 has only weak effects in the *in vitro* transcription system (Figure 6). Sequence analysis reveals that elements 1 and 2 represent a partial palindrome in which elements 1 and 2 form the stem. This palindrome structure may explain why elements 1 and 2 function in the opposite direction. Furthermore one might expect that elements 1 and 2 interact with identical factors. Indeed, preliminary bandshift experiments using either labeled element 1 or 2 give similar shifted bands (data not shown). The complexity in band formation implies the binding of several proteins.

Using rat liver and HeLa extracts we demonstrated that the activator interacts *in vitro* in a cell-specific manner with transcription factors. The stimulation observed (10- to 20-fold) is comparable to the activity of the hepatocyte-specific promoter element, HP1, which we have recently identified in the *Xenopus* albumin gene (Schorpp *et al.*, 1988b). Since part of the activator, i.e. position −115 to −103, is similar to HP1 found in several liver-specific genes (Kugler *et al.*, 1988), we performed a competition assay by adding a large excess of HP1 oligonucleotide to the *in vitro* transcription system. Based on the inability of the HP1 oligonucleotide to compete with the function of the activator (Figure 5), we conclude that the activator is not a HP1 homolog, but represents a distinct regulatory element. This is supported by the finding that the part of the activator most similar to HP1 cannot act as positive transcriptional element. These data correlated with our observation that the activator found in the vitellogenin gene has a broader spectrum in cell specificity in comparison with the HP1 present in the albumin gene (e.g. Figure 4 and cf. Schorpp *et al.*, 1988b). In this context it is worth noting that the albumin genes are already active in the liver of the tadpole (our unpublished data), whereas the vitellogenin genes are only inducible in the frog liver after metamorphosis (Huber *et al.*, 1979). This differential liver-specific expression clearly correlates with differences in the liver-specific regulatory elements. It seems likely that these differences contribute to the developmentally specific regulation of the albumin and vitellogenin genes.

Materials and methods

Plasmid constructions

5′ and 3′ deletion constructs were essentially prepared with *Bal*31 as previously described using *Hin*dIII- and *Bam*HI-digested DNA respectively (Klein-Hitpaß *et al.*, 1986). The exact deletion endpoints were determined by sequencing according to the method of Sanger *et al.* (1977) or by

super-coil sequencing (Chen and Seeburg, 1985). Some of the constructs containing small fragments of the activator were synthesized as double-stranded oligonucleotides with *Hin*dIII and *Bam*HI overhangs on a Gene Assembler (Pharmacia). The cloning was performed as previously described (Klein-Hitpaß *et al.*, 1988a).

Plasmids containing an inverted vitellogenin insert were obtained by cloning the *Hin*dIII–*Bam*HI fragment in pBLCAT9+ (Klein-Hitpaß *et al.*, 1988a).

Analysis of curved DNA

*Hin*dIII- and *Bam*HI-digested plasmid DNA was separated on a 6% non-denaturing polyacrylamide gel using 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3 as buffer. The gels were run at 5 V/cm at room temperature, or at 2 V/cm at 4°C. *Hpa*II- or *Pal*I-cut pBR322 was used as size marker. Restriction fragments containing repeating blocks of oligo(A) or oligo(T) were not used for the size determination.

Cell culture and media

MCF-7, Ltk$^-$, NFB München and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin/streptomycin. HepG2 cells were grown in a 1:1 DMEM/Ham's F-12 medium mixture supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. BW1J cells were grown in modified Ham's F-12 medium (Coon and Weiss, 1969) containing 5% FCS and 100 U/ml penicillin/streptomycin.

Transient transfection and CAT assay

Transient transfections were performed by calcium phosphate precipitation (Graham and van der Eb, 1973) as modified by Wigler *et al.* (1979). The transfections were carried out in the corresponding culture media except MCF-7 cells which were transfected in DMEM supplmented with 10% charcoal-treated calf serum, 100 U/ml penicillin/streptomycin and 10^{-6}M tamoxifen to suppress any estrogenic effects.

Cells were harvested at 40 h after transfection and assayed for CAT activity as previously described (Klein-Hitpaß *et al.*, 1986). pSV2-CAT was used as a control for transfection efficiency.

In vitro transcription

Rat liver nuclear extracts were prepared as described by Gorski *et al.* (1986); the conditions for *in vitro* transcription have been described by Schorpp *et al.* (1988b). Twenty microliters of the transcription mixture contained 12 μl rat liver nuclear extract and 70 ng of the plasmid to be tested. As an internal control all the reactions contained 35 ng pML cas9, a construct with a shortened −G-cassette of ~200 bp under the control of the adeno major late promoter. The experiments shown in Figure 6 were made with an independent rat liver extract using 7 μl extract and twice the amount of DNA template. HeLa nuclear extract was prepared according to a modification (M.Kaling *et al.*, in preparation) of the method described by Dignam *et al.* (1983). The *in vitro* transcription contained 4 μl extract, 400 ng template DNA and 200 ng pML cas9. In this case the *in vitro* transcripts were treated with 15 U T1 RNase for 30 min at 30°C to degrade G-containing transcripts made quite abundantly in the HeLa extract.

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