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The conserved decanucleotide from the immunoglobulin heavy chain promoter induces a very high transcriptional activity in B-cells when introduced into an heterologous promoter

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A conserved decanucleotide (ATGCAAATNA) is present 45–60 nucleotides upstream from the transcription startpoint in all immunoglobulin heavy chain promoters (V_H promoters). We have introduced this decanucleotide (cd sequence) at a similar position into the upstream flanking sequence of the mouse Renin-1 gene. This gene is only transcribed in highly specialized tissues, and the fragment used here (−449 to +30 with respect to the main transcription startpoint) has little promoter activity in fibroblastic or myeloma cell lines, even if coupled to a functional enhancer. In contrast, after insertion of the decanucleotide, this fragment, while still inactive in non-lymphoid cells, becomes a potent promoter in B-cells when associated with SV40 or immunoglobulin heavy chain enhancer. In all respects, the engineered fragment behaves like an authentic V_H promoter isolated in this laboratory, except that it is even more active in B-cells. Deletion experiments show that all renin sequences are dispensable for the activity of the chimaeric promoter, except probably for the renin TATA box which defines the precise transcription startpoint. We conclude that the decanucleotide is sufficient to activate a promoter in B-cells but not in non-B-cells, and therefore that no other element is needed to account for the B-cell specificity of the V_H promoter. In addition, our results suggest that the lack of activity of the renin promoter in non cognate cells is not due to the binding of a repressor.

Key words: immunoglobulin genes/renin genes/tissue-specific upstream element/positive regulation/enhancer

Introduction

Increasing evidence shows that the tissue-specific transcription of specialized genes involves the interaction of activators and repressors which are only present in cognate or non cognate tissues, rspectively, with target DNA sequences. These target sequences, which are located within the transcription unit of the gene or in its immediate vicinity, have been operationally classified in two groups (Dynan and Tijan, 1985): (i) the enhancer elements (Khoury and Gruss, 1983) which were first identified in viral genomes, but are now known to modulate the activity of many cellular genes in a tissue-specific manner; these elements can potentiate transcription from a neighbouring promoter, largely independently of orientation and distance; and (ii) the promoter region itself, which often contains 'upstream elements' involved in the tissue-specific activation or repression of promoter activity; again, the orientation and, to some extent, the distance of these elements from the TATA box can frequently be varied without marked effect on promoter activity (Takahashi *et al.*, 1986).

These general principles are particularly well illustrated in the mouse immunoglobulin heavy chain gene family (IgH genes). The rearranged IgH genes are only transcribed in lymphoid cells (for a review, see Calame, 1985). This stringent tissue-specificity has formerly been attributed to the presence of the IgH enhancer, a regulatory element which is located in the J-Cμ intron and which activates transcription only in B-cells (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983). However, it has been recently realized that the V_H promoter itself also contributes to tissue-specificity. This promoter, which requires an associated enhancer for transcriptional activity, will allow specific initiation of transcription only in B-cells, even if the enhancer itself is not tissue-specific (Grosschedl and Baltimore, 1985; Mason *et al.*, 1985; Doyen *et al.*, 1986). All V_H promoters contain a conserved upstream element, corresponding to the consensus sequence ATGCAAATNA (Falkner and Zachau, 1984; Parslow *et al.*, 1984). This decanucleotide, hereafter noted cd according to Falkner and Zachau (1984), is found 45–60 nucleotides upstream from the cap site; it is the most prominent sequence element conserved between V_H promoters, and it has been shown to be necessary for promoter activity (Mason *et al.*, 1985; Ballard and Bothwell, 1986). Moreover, the same sequence, albeit in reverse orientation, occurs in the promoter of immunoglobulin κ light chain genes (dc sequence); again it is the most conserved element among these highly B-cell-specific promoters (Falkner and Zachau, 1984; Parslow *et al.*, 1984), and its deletion impairs promoter function (Queen *et al.*, 1986).

In order to determine whether the cd/dc element is sufficient by itself to confer B-cell-specific transcriptional activity to a promoter, we decided to artificially introduce it into the promoter of a gene that does not normally contain it. This promoter should ideally be inactive in both B-cells and non-B cells such as fibroblasts in order to allow the detection of an eventual activating effect of the decanucleotide. Rather than designing an entirely synthetic promoter for this purpose, we decided to use the promoter of a highly specialized gene not normally transcribed in either of these cell types. We chose the mouse Renin-1 gene, which is only expressed in the convoluted tubules of the submaxillary gland and in the juxtaglomerular cells of the kidney (Field and Gross, 1985). This gene has been cloned in this laboratory and its putative promoter region has been analyzed (Panthier *et al.*, 1984). Preliminary experiments showed that, although this region contains a perfectly canonical TATA box and cap region (Bucher and Trifonov, 1986), it is unable to act as an efficient promoter in B-cells or in fibroblasts, even if coupled to a functional enhancer (see below). Here we show that, upon insertion of the cd sequence, the renin promoter coupled to the SV40 or IgH enhancer becomes highly active in B-cells, but not in fibroblasts. We conclude that the cd element *per se* is entirely responsible for the activity and B-cell specificity of the V_H promoter. Additionally, since the renin promoter can be activated by the insertion of the cd element, it seems unlikely that its lack of activity in non renin-producing cells is due to the binding of a repressor.

Table I. CAT activity of different constructions in X63Ag8 and 3T3 cell lines

Constructs		CAT activity in	
promoter	enhancer	X63Ag8	3T3
SV[a]	SV1[a]	100	100
R1	–	<0.3	<0.5
R1	IG	<0.3	1
R1	SV1	<0.3	9
R1	SV2	<0.3	10
R5	–	<0.3	2
R5	IG	<0.3	3
R5	SV1	<0.3	8
R5	SV2	<0.3	3
R1-cd	–	<0.3	2
R1-cd	IG	150	4
R1-cd	SV1	34	12
R1-cd	SV2	24	12
R5-cd	–	<0.3	4
R5-cd	IG	145	1
R5-cd	SV2	13	12
V1	–	<0.3	1
V1	IG	50	4
V1	SV2	5	5
–	–	<0.3	4
–	IG	<0.3	1
–	SV2	nd	2

For each plasmid, the percentage chloramphenicol conversion was measured. Each value is the arithmetic mean of two or three measurements after independent transfections, and is normalized to the value for pSV2cat[a]. nd: not determined. All other symbols as in Figures 1 and 2.

Table II. CAT activity in constructs containing deleted R1-cd promoters, in X63Ag8 cell line

Constructs		CAT activity
promoter	IgH enhancer	
R1	+	2
R1-cd[a]	+	100
R7	+	1
R7-cd	+	141
R5	+	1
R5-cd	+	143
R8	+	3
R8-cd	+	73
R6	+	1
R6-cd	+	100
R9	+	1
R9-cd	+	120
–	–	1

For each plasmid, the percent chloramphenicol conversion was measured. Each value is the arithmetic mean of two measurements after independent transfections, and is normalized to the value for pR1-cd catIG[a]. All these values correspond to transfections independent from those used in Table I.

Results

The renin promoter is not active in fibroblastic or myeloma cells

Recombinant phages containing the entire mouse kidney renin gene (*Ren-1* gene) have been obtained in this laboratory (Panthier *et al.*, 1984). We have isolated a fragment, noted R1, which corresponds to the immediate upstream flanking region of this

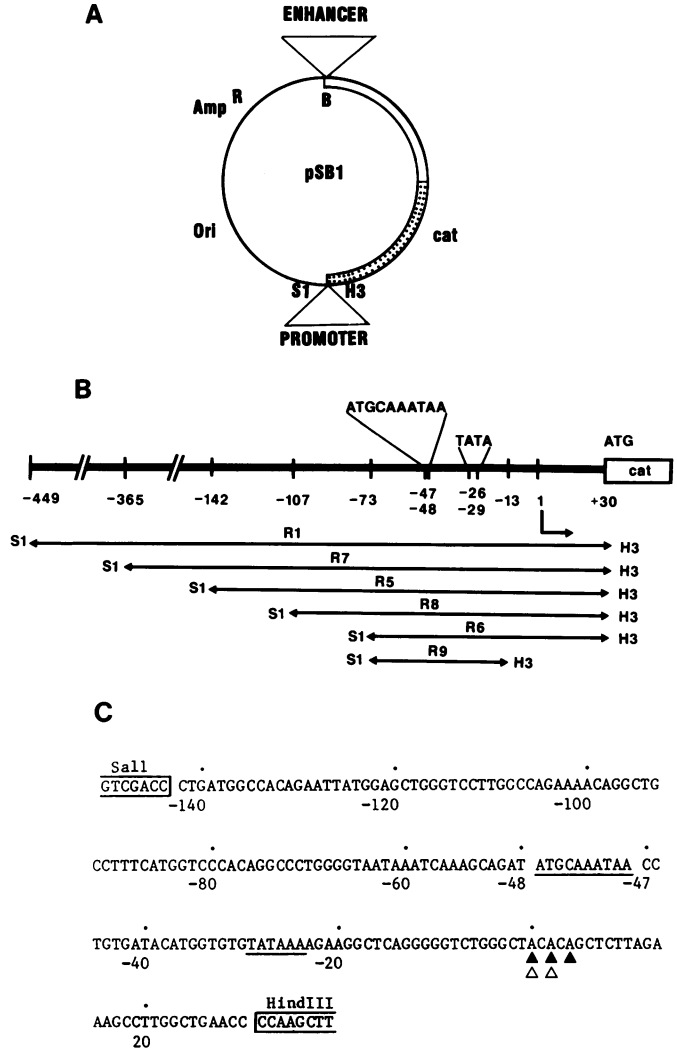

Fig. 1. **(a)** Structure of plasmids used in this work. The starting plasmid (pSB1) (Herbomel *et al.*, 1984) is similar to pSVOcat (Gorman *et al.*, 1982) except that it lacks the pBR322 'poison' sequences. Various promoter and enhancers fragments were inserted upstream and downstream from the *cat* gene, respectively. Thin line: pBR322 sequences. Stippled box: *cat* gene; open box: SV40 sequences corresponding to t antigen intron followed by early region polyadenylation signal. H3, S1 and B stand for *Hin*dIII, *Sal*I and *Bam*HI cleavage sites, respectively. **(b)** Schematic structure of the R1 to R9 promoter fragments. The sequence is numbered with respect to the main transcription startpoint (arrow). Fragments R1-cd to R9-cd result from the insertion of the indicated decanucleotide between positions −47 and −48 of R1 to R9, respectively. In each case, the initiation codon of the *cat* gene is the first ATG encountered downstream from the transcription startpoint. **(c)** Sequence of the R5-cd fragment. The decanucleotide and the TATA boxes are underlined. Open and black arrows correspond to the transcription startpoint from the R5-cd promoter (Figure 4), and to the renin capsite (Field *et al.*, 1984), respectively.

gene, from nucleotides −449 to +30 with respect to the main transcription start point (Field *et al.*, 1984). R1 was inserted upstream of the bacterial gene coding for chloramphenicol acetyl transferase (*cat* gene), in the plasmid pSB1 (Herbomel *et al.*, 1984) which is closely related to the pSVOcat described by Gorman *et al.* (1982). Four plasmids were constructed, containing either no enhancer, the IgH enhancer (in the same orientation with respect to transcription as in the IgH gene), or the SV40 enhancer in either orientation. These plasmids were transfected into mouse fibroblastic (3T3) or myeloma (X63Ag8) cell lines, and the CAT activity resulting from their transient transcriptional

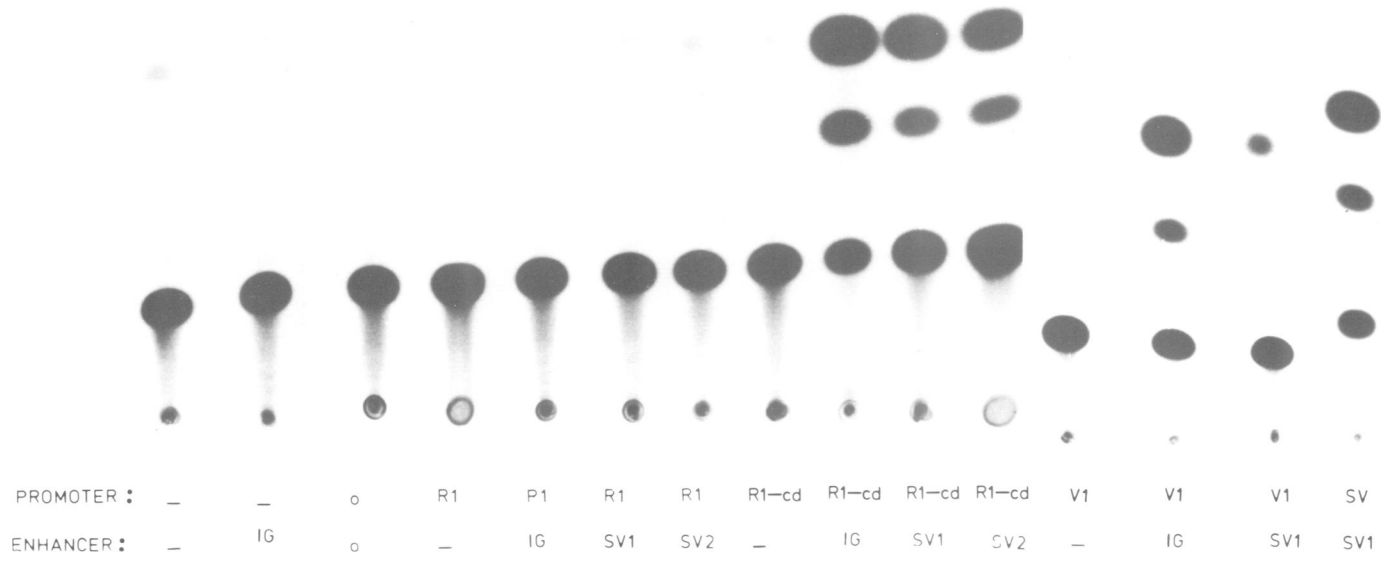

Fig. 2. CAT activity in extracts of myeloma cells (X63Ag8), harvested 48 h after transfection with various plasmids. The promoter (first line) and the enhancer (second line) present in each construction are indicated under the corresponding lane. Lanes (○,○) and (SV, SV1) correspond to no DNA (mock-transfection), and transfection with the plasmid pSV2cat of Gorman *et al.* (1982). The subscript following the SV40 enhancer refers to its two possible orientations with respect to the direction of transcription, that found in the SV40 genome being given the subscript 1.

activity was recorded 48 h later. None of these plasmids was found to yield detectable activity in myeloma cells (Figure 2 and Table I). In 3T3 cells, only the plasmids containing the SV40 enhancer in addition to the R1 fragment showed a slight activity compared to a similar construction lacking a promoter (Table I). This activity, however, did not exceed one tenth of that directed by the reference plasmid pSV2cat of Gorman *et al.* (1982). We conclude that the R1 fragment is unable to act as an efficient promoter in these two non renin-producing cell lines, even if coupled to a functional enhancer.

Several tissue-specific promoters are known to be regulated by repression in non-cognate cells. Progressive deletion of these promoters often results in transcriptional activation, presumably because of the removal of a repressor binding site (Larsen *et al.*, 1986; Muglia and Rothman-Denes, 1986). To assess whether this may be the case for the R1 fragment, we prepared shorter fragments derived from it by successive deletions from its 5′ and 3′ ends (Figure 1). These fragments, noted R5 to R9, were similarly inserted in pSB1 and their promoter activity was recorded in X63Ag8 or 3T3 cell lines, in the presence or in the absence of a functional enhancer. None of these plasmids, however, yielded more activity than their counterpart carrying the R1 fragment (Figure 3, Tables I, II, and results not shown).

The decanucleotide insertion strongly activates the renin promoter in myeloma cells

Next, we introduced the decanucleotide sequence ATGCAAATAA into the R1 fragment, to form the modified R1-cd fragment (Figure 1). The insertion was positioned 18 nucleotides upstream from the renin TATA box, a distance similar to that observed in some V_H promoters (Grosschedl and Baltimore, 1985; Mason *et al.*, 1985). The R1-cd fragment was then inserted into pSB1, with or without an associated enhancer (Figure 1), and the corresponding plasmids were similarly transfected into 3T3 and X63Ag8 cell lines. In the 3T3 cell line, we found that the engineered renin fragment, like the unmodified one, had little or no promoter activity (Table I). In myeloma cells, however, a strikingly different result was obtained: while the plasmids lacking an enhancer still showed no activity, those carrying both the modified renin fragment and the IgH enhancer were several hundred-fold more active than the corresponding constructs lacking the decanucleotide (Figure 2 and Table I). For comparison, we constructed similar plasmids in which R1-cd was replaced by an authentic V_H promoter (fragment V1 of Doyen *et al.*, 1986). In all respects, the V1 and R1-cd fragments behaved similarly, although quantitatively the chimaeric promoter was ~3-fold more active in B-cells than the natural one (Table I).

The SV40 enhancer was also effective in activating the modified renin fragment as well as the V_H promoter, albeit 10-fold less than the IgH enhancer. It should be stressed that, in myeloma cells, the IgH or SV40 enhancers activated the promoters only when the cd sequence was present. In contrast, in 3T3 cells, a slight activity was observed with the SV40 enhancer, irrespectively of the presence or absence of the decanucleotide (Table I): experiments are currently in progress in order to determine the transcription startpoint in these cases.

Most renin sequences are dispensable for activity of the chimaeric promoter

Since the decanucleotide sequence is sufficient to activate the renin promoter in myeloma cells, it was of interest to determine whether this sequence *per se* constitutes the promoter, or whether the renin sequences contribute in some way. We progressively deleted the R1-cd fragments from both ends to produce the shorter fragments R5-cd to R9-cd, derived from R5 to R9 (Figure 1). The smallest fragment (R9-cd) retains only 60 nucleotides of renin sequence, bracketing the TATA box. All these fragments had

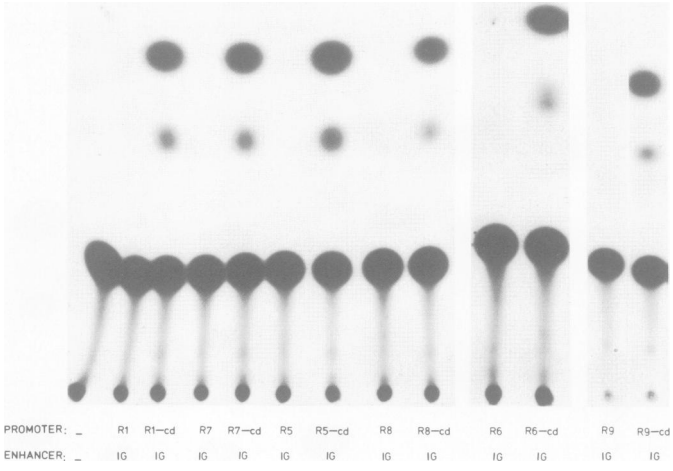

Fig. 3. CAT activity in extracts of myeloma cells transfected with plasmids carrying, in addition to the *cat* gene and the IgH enhancer (IG), various promoters derived from R1 and R1-cd by progressive deletion (Figure 1). All symbols as in Figure 2.

a promoter activity similar to that of R1-cd (Figure 3 and Table II).

Next, we mapped the 5′ end of the mRNA transcribed from the chimaeric promoter, using a primer extension technique (Figure 4). Rather than the R1-cd promoter, we used the shorter R5-cd promoter for this purpose (Figure 1). It was found that the startpoint of the mRNA corresponds precisely to the renin cap site (Field *et al.*, 1984; Panthier *et al.*, 1984) (Figure 1). It is probable, therefore, that the renin sequences, although almost completely dispensable for promoter activity, still contribute to promoter function by providing the functional TATA box which is necessary to define the precise transcription startpoint (Benoist and Chambon, 1981).

Discussion

In this work, we have shown that the introduction of the cd element into the renin promoter is sufficient to make this normally silent promoter active in B-cells, provided it is associated with a functional enhancer. In contrast, no activation was found in fibroblasts. Moreover, all renin sequences except for the TATA box seem dispensable for promoter activity, and thus the renin fragment presumably does not substitute for some V_H sequence element which would be important for activity. Therefore, aside from the decanucleotide and the TATA box, no additional element of the V_H promoter is needed for activity and cell-type specificity. This conclusion markedly extends previous reports showing that all the information necessary for the regulation of the V_H promoter is located between −154 and +57 (Grosschedl and Baltimore, 1985), or downstream of position −75 (Ballard and Bothwell, 1986) with respect to the transcription startpoint.

At present, only circumstantial evidence supports the importance of the TATA box in addition to the decanucleotide for proper initiation of transcription: thus, we have shown that the chimaeric renin-decanucleotide promoter uses the renin cap site, and therefore the renin TATA box is presumably functional in this promoter (Benoist and Chambon, 1981). Moreover, similar results by Mason *et al.* (1985) also emphasize the role of the TATA box: these authors placed a 1 kb V_H promoter fragment retaining the decanucleotide but not the TATA box, upstream of the β-globin TATA box, and they observed a B-cell specific

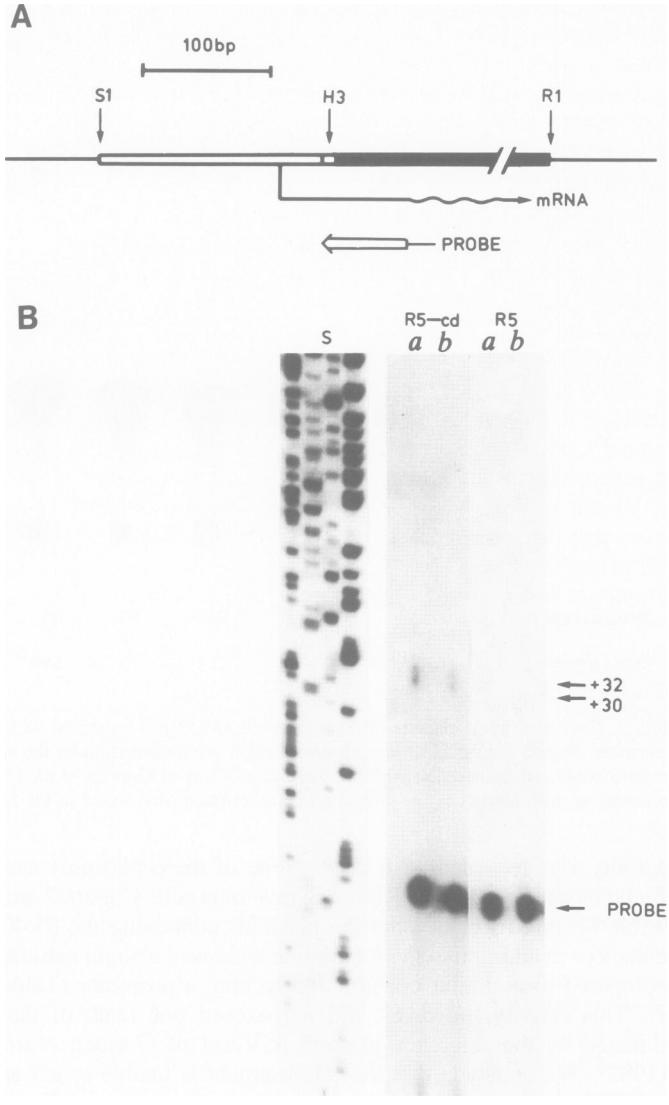

Fig. 4. Mapping of the transcription startpoint from the modified renin fragment. **(A)** Strategy for the preparation of the single-stranded DNA probe. A 400 bp *SalI*−*Eco*RI fragment corresponding to the R5 fragment followed by the beginning of the *cat* gene (Figure 1), was subcloned into M13mp9 and used to prepare a single-stranded, uniformly labelled DNA probe (open arrow) complementary to position 1−71 with respect to the most 5′ nucleotide of the *Hin*dIII site. Thin line: M13 DNA. Open box: R5 fragment followed by the *Hin*dIII linker. Black box: *cat* sequences. Wavy line: mRNA from the R5-cd fragment. R1 stands for *Eco*RI, other symbols as in Figure 1. **(B)** Primer extension mapping. Myeloma cells were transfected with CAT plasmids carrying either the R5 or the R5-cd promoter, in addition to the IgH enhancer (Figure 1), and poly(A)$^+$ RNA was prepared 48 h later. Samples (**a**: 1.5 μg, **b**: 0.75 μg) were hybridized with an excess of single-stranded probe, and the hybrids were elongated with reverse transcriptase in the presence of dNTPs and analysed on a sequencing gel. The figures +30 and +32 indicate the position of the transcription startpoint with respect to the *Hin*dIII site. S: calibrating sequencing lanes.

transcription originating at the globin mRNA cap site. However, since the TATA box region is quite poorly conserved among natural V_H promoters (cf. Ballard and Bothwell, 1986), this element may be dispensable for transcriptional activity, if not for the precise positioning of the mRNA start site.

This work has benefited from the availability of an eucaryotic promoter, that of the renin gene, which is inactive both in B-cells and in fibroblasts. In the absence of any conflicting evidence, we shall assume that this lack of activity has the same origin in

the two cell lines. Conceivably, like many other systems (Killary and Fournier, 1984; Larsen *et al.*, 1986; Nir *et al.*, 1986; Colantuoni *et al.*, 1987) the renin promoter could be regulated by a repressor present in non-renin producing cells. The decanucleotide might activate the promoter by disrupting the repressor binding site; alternatively, it may be the target for an activator protein which overrides the repressor. However, the first hypothesis does not explain why the chimaeric promoter is active in B-cells only, while the second situation, to our knowledge, would be unprecedented. Moreover, the repressor hypothesis is unattractive because progressive deletion of the R1 fragment fails to activate the promoter (cf. Larsen *et al.*, 1986; Muglia and Rothman-Denes, 1986).

Thus, it is more probable that the renin promoter is inactive because it is unable to bind an activator. The decanucleotide would then be the target for a B-specific activator, which can compensate for this absence. Moreover, the fact that, in the chimaeric promoter, most renin sequences are dispensable, suggests that the binding of this activator *per se* may be sufficient for promoter activity in B-cells. Furthermore, the small size of the target DNA suggests that very few DNA-binding proteins, and possibly even a single one, are involved. Sensitive gel retardation techniques have recently been used to detect dc/cd binding factors in various cell types (Hromas and Van Ness, 1986; Mocikat *et al.*, 1986; Singh *et al.*, 1986). Two different factors seem to be present, one of them being ubiquitous while the other is found only in B-cells, and is therefore a possible candidate for the activator discussed here (Landolfi *et al.*, 1986; Staudt *et al.*, 1986). Nevertheless, whatever the exact nature of this activator, it is clear that the V_H promoter cannot be seen as the equivalent of the most simple positively controlled procaryotic promoters (Raibaud and Schwartz, 1984) because it requires an associated enhancer for activity. Enhancers are complex genetic elements which bind both repressors and activators (Schöler and Gruss, 1985; Augereau and Chambon, 1986; Davidson *et al.*, 1986; Kadesch *et al.*, 1986; Maeda *et al.*, 1986; Schlokat *et al.*, 1986; Wasylyk and Wasylyk, 1986; Weinberger *et al.*, 1986), and current models suggest that they influence promoter activity via long range protein−protein contacts, owing to the flexibility of the DNA (Ptashne, 1986). In this respect, it is intriguing that both the SV40 and the IgH enhancers contain sequences which are closely related to the decanucleotide, and which have also been shown to bind B-specific factors (Ephrussi *et al.*, 1985; Davidson *et al.*, 1986). Assuming that the same activator can bind cooperatively to the promoter and enhancer decanucleotides, it has been speculated that the presence of the nearby enhancer binding site can increase the occupancy of the promoter binding site, thereby contributing to enhancing (Ptashne, 1986). Experiments designed to test this hypothesis are now underway in this laboratory.

In addition to the immunoglobulin promoters and the heavy chain enhancer, several promoters known to be active in non B-cells contain cd/dc sequences (Falkner *et al.*, 1986; Mohun *et al.*, 1986). Moreover, in several of these cases, this sequence has been found essential for promoter activity (Ciliberto *et al.*, 1985; Krol *et al.*, 1985; Mattaj *et al.*, 1985; Sive *et al.*, 1986). Although the available data do not allow any quantitative comparisons, it is likely that these promoters, which are assayed without an exogenous enhancer, are much more active than the virtually silent, enhancerless, V_H or Renin-cd promoters. Therefore, it is evident that the simple presence of the dc/cd element, possibly complexed with the ubiquitous dc/cd binding factor mentioned above, is not sufficient for promoter activity in non-B-cells: evidently, these promoters contain additional regulatory elements to account for their high activity, as indeed demonstrated by deletion analysis (Ciliberto *et al.*, 1985; Krol *et al.*, 1985; Mattaj *et al.*, 1985; for a general discussion see Mizushima-Sugano and Roeder, 1986). From the present work, one would predict that promoters containing the cd/dc element even in the absence of other regulatory sequences should be active in B-cells but only when associated with a functional enhancer.

Materials and methods

Plasmid constructions

The R1 fragment was obtained from a recombinant λ phage (SW35) carrying most of the mouse Renin-1 gene (Panthier *et al.*, 1984). Briefly, a *Pvu*II−*Pvu*II fragment (−449 to +185 with respect to the main transcription startpoint) was digested with *Bal*31 from is 3′ end to remove the first exon, producing fragment R1 (−449 to +30). R1 was further deleted from its 5′ and 3′ ends, using *Bal*31 or adequate restriction endonucleases, to give the shorter fragments R7 (−365 to +30), R5 (−142 to +30), R8 (−107 to +30), R6 (−73 to +30) and R9 (−73 to −13). The isolation of a V_H promoter fragment (V1) was reported previously (Doyen *et al.*, 1986). Similarly the fragments carrying the IgH and SV40 enhancers (IG-SV) are the 1 kb *Xba*I−*Xba*I and 223 bp *Pvu*II−*Nco*I described in Doyen *et al.* (1986). All fragments were ligated with synthetic linkers for subcloning into the unique sites of pSB1 (Herbomel *et al.*, 1984).

For the insertion of the decanucleotide (cd sequence), R1 and R5 were subcloned between the *Sal*I and *Hin*dIII sites of M13mp8. We prepared a 30-mer oligonucleotide containing sequences −38 to −47 and −48 to −57 of R1 and R5, bracketing the decanucleotide of interest. Mutagenesis and selection of mutants were performed according to standard procedures (Carter *et al.*, 1985). After dideoxy sequencing, the modified fragments (R1-cd and R5-cd) were further deleted as above to produce R6-cd to R9-cd.

Cell culture and transfections

Myeloma cells X63Ag8 secreting IgG 1 (Köhler and Milstein, 1975), were grown in suspension in Dulbecco Modified Eagle Medium containing 10% Fetal Calf serum. The same medium was used for the mouse fibroblastic 3T3 cells. X63Ag8 (2.5×10^6 cells per experiment) and 3T3 (5×10^5 cells) were transfected in 10 cm Petri dishes using the DEAE-dextran and calcium phosphate procedures, respectively, with 10 μg of plasmid DNA (Doyen *et al.*, 1986).

For CAT assays, cells were resuspended in 250 mM Tris-HCl pH 7.8 and lysed by successive cycles of freezing and thawing. After centrifugation, the supernatants were assayed for CAT activity (Gorman *et al.*, 1982) using [^{14}C]chloramphenicol (Amersham). 0.5 mg of total protein were assayed in each case unless otherwise indicated. Unreacted chloramphenicol was separated from its acetylated derivatives by t.l.c.; the spots were visualized by autoradiography, excised, and counted. In each case, after blank subtraction, the ratio of acetylated versus unreacted chloramphenicol was expressed with respect to the value obtained with the reference plasmid pSV2cat (Gorman *et al.*, 1982).

Mapping of the 5′ end of the CAT mRNA

A single-stranded, uniformly labelled DNA probe complementary to nucleotides 1−71 of the *cat* gene (counted downstream from the *Hin*dIII site) was synthesized *in vitro* essentially as described in Davis *et al.* (1986): briefly, 0.2 μg of the single-stranded recombinant M13 DNA depicted in Figure 4A was hybridized with an excess of a 19-mer synthetic primer corresponding to nucleotide 52−71 of the *cat* gene. The hybrids were elongated with Klenow polymerase in the presence of dATP, dCTP, dGTP, and 20 μCi of [α-^{32}P]TTP (Amersham, 3000 Ci/mmol). The elongated hybrids were cleaved with *Hin*dIII, and the single-stranded probe was purified on a preparative sequencing gel.

For primer extension experiments, 2×10^7 X63Ag8 cells were transfected with 80 μg of each plasmid. After 48-h, the cells were collected and the poly(A)$^+$ RNA was prepared by guanidinium isothiocyanate/lithium chloride precipitation followed by oligo (dT)-sepharose chromatography as in Davis *et al.* (1986). The indicated amount of poly(A)$^+$ RNA was hybridized with 2500 c.p.m. of single-stranded probe in 10 μl of 40 mM PIPES pH 6.4, 1 mM EDTA, 0.5 M NaCl, 50% formamide, for 18 h at 42°C. After precipitation, the hybrids were elongated with reverse transcriptase (Genofit) and analyzed as in Field and Gross (1985) except that all four dNTPs were present in the reaction medium, and that the elongated hybrids were desalted by three successive ethanol precipitations before loading on a 6% sequencing gel.

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