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The SV40 TC-II(κB) and the related H-2K^B enhansons exhibit different cell type specific and inducible proto-enhancer activities, but the SV40 core sequence and the AP-2 binding site have no enhanson properties

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The enhancer activity of the oligomerized SV40 TC-I and TC-II sequences has been investigated in lymphoid and non-lymphoid cell lines. While the TC-I sequence had no demonstrable enhanson activity, a class C enhanson (proto-enhancer), 5′-GGAAAGTCCCC-3′, overlapping the TC-II sequence and the GT-I enhanson was identified. This TC-II enhanson, which is identical to the κB motif from the κ chain enhancer, was active in both lymphoid and non-lymphoid cells, which contrasts with the previously reported lymphoid cell specificity of the κB motif. However, its activity in non-lymphoid cells is in agreement with our previous reports describing the effect of mutations in the 'TC region' within the total SV40 enhancer in lymphoid and non-lymphoid cells. The activity of the TC-II enhanson could be moderately increased in HeLa by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and cycloheximide treatment, indicating that the protein(s) mediating its activity may be partially repressed by the previously described inhibitor protein IκB. The TC-II related, H-2K^b element, 5′-TGGGGA-TTCCCCA-3′, of the histocompatibility class I H-2K^b gene promoter is also a class C enhanson which is active in both lymphoid and non-lymphoid cells. However, in contrast to the TC-II enhanson, the H-2K^b enhanson exhibits a very low activity in HeLa cells, but can be strongly induced by TPA and/or cycloheximide treatments which suggests that its cognate factor is inactivated (repressed) by an inhibitor protein. Interestingly, cycloheximide, but not TPA treatment, could induce the activity of both the TC-II and H-2K^b enhansons in F9 embryonal carcinoma cells, suggesting that these cells lack some component(s) of the protein kinase C signal transduction pathway. We also show that oligomers of the SV40 'core' sequence, which overlaps the TC-II enhanson, had no enhanson activity in any of the cell types studied, which questions the possible role of the AP-3 protein in SV40 enhancer activity in these cell types. In addition, oligomers of the AP-2 binding sites which are present in the SV40 TC region and in the human metallothionein IIA promoter show no enhanson activity, irrespective of whether the cells are treated with TPA. All of these results are in agreement with, and account for, those previously obtained with the total SV40 enhancer mutated selectively in the 'core' sequence or AP-2 binding sites, and confirm our previous proposals concerning the importance of the overlapping GT-I and TC-II enhansons in SV40 enhancer activity. Finally, we discuss the possible role of the TC-IIA/NF-κB like and TC-IIB/KBF1 like proteins [see Macchi *et al.* (1989) *EMBO J.*, 8, 4215-4227] in mediating the activity of the TC-II/κB and H-2K^b enhansons in the different cell types.

Key words: enhancer core sequence/HeLa cells/metallothionein AP-2 binding site/SV40 enhancer activity *in vivo*/TPA and cycloheximide treatment

Introduction

The transcriptional enhancer of simian virus 40 (SV40) (see Figure 1 in Macchi *et al.*, 1989) consists of a mosaic of functional elements (enhansons) which cooperate synergistically to generate enhancer function (Herr and Clarke, 1986; Zenke *et al.*, 1986; Nomiyama *et al.*, 1987.; Ondek *et al.*, 1987; Pettersson and Schaffner, 1987; Schirm *et al.*, 1987; Fromental *et al.*, 1988; see Macchi *et al.*, 1989, for additional references). Each enhanson displays a characteristic cell type specific activity which correlates with the presence of its cognate protein(s) (Davidson *et al.*, 1986; Landolfi *et al.*, 1986; Singh *et al.*, 1986; Staudt *et al.*, 1986; Wildeman *et al.*, 1986; Fletcher *et al.*, 1987; Rosales *et al.*, 1987; Scheidereit *et al.*, 1987; Sturm *et al.*, 1987; Xiao *et al.*, 1987a,b; Macchi *et al.*, 1989). The SV40 enhansons can be divided into three classes, each exhibiting distinct properties (Fromental *et al.*, 1988). Class A consists of enhansons GT-IIC and Sph (Fromental *et al.*, 1988; Ondek *et al.*, 1988) whose tandem repeats exhibit proto-enhancer activity, i.e. generate a functional enhancer when multimerized or associated with a second proto-enhancer (Fromental *et al.*, 1988). In contrast, the class B GT-I enhanson shows no enhancer activity when multimerized on its own, but can form a proto-enhancer element when associated with a class A enhanson. A class C enhanson, as exemplified by the octamer motif in lymphoid cells, possesses intrinsic proto-enhancer activity, since its oligomerization generates an enhancer which shows no stringent requirement for spacing between the enhansons within the oligomer (Gerster *et al.*, 1987; Fromental *et al.*, 1988; Tanaka *et al.*, 1988).

In addition to the above elements, studies in MPC11 lymphoid B cells of the effect of mutations within the total SV40 enhancer have indicated that the region containing the TC-II motif (Figures 1 and 2 in Macchi *et al.*, 1989) is also involved in the enhancer activity (Nomiyama *et al.*, 1987). The TC-II motif, 5′-(245)GGAAAGTCCCC(235)-3′, is identical to the κB motif from the κ chain gene enhancer (Sen and Baltimore, 1986a; Atchison and Perry, 1987; Lenardo *et al.*, 1987). Sequences homologous to the TC-II/κB motif are also found in the HIV long terminal repeat (Nabel and Baltimore, 1987), the cytomegalovirus promoter

(Boshart *et al.*, 1985), the β-interferon promoter (Goodbourn and Maniatis, 1988; Fan and Maniatis, 1989; Fujita *et al.*, 1989; Hiscott *et al.*, 1989; Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989), the interleukin-2 (IL-2) gene promoter (Hoyos *et al.*, 1989), the IL-2 receptor α promoter (Leung and Nabel, 1988. Cross *et al.*, 1989) and the serum amyloid A promoter (Edbrooke *et al.*, 1989). It has been reported that the activity of the κB motif is lymphoid B cell specific, which correlated with the lymphoid B cell specificity of its cognate protein NF-κB, (Sen and Baltimore, 1986a,b; Atchison and Perry, 1987; Lenardo *et al.*, 1987; Nelsen *et al.*, 1988; Pierce *et al.*, 1988; Wirth and Baltimore, 1988). In the SV40 enhancer, however, simultaneous mutation of the GT-I and TC-II motifs had a more detrimental effect in HeLa cells than mutation of the GT-I motif alone, suggesting that the TC-II motif may also be active in non-lymphoid cells (Nomiyama *et al.*, 1987). An enhancer element, 5′-TGGGGATTCCCCA-3′, with homology to the TC-II/κB motif has also been identified in the H-2K^b and β2-microglobulin gene promoters and shown to bind *in vitro* the KBF1/H2TF1 protein (Kimura *et al.*, 1986; Baldwin and Sharp, 1987, 1988; Israël *et al.*, 1987; Yano *et al.*, 1987), as well as the NF-κB protein (Baldwin and Sharp, 1988; Macchi *et al.*, 1989). However this element appears to be active in non-lymphoid cells (Kimura *et al.*, 1986; Baldwin and Sharp, 1987; Israël *et al.*, 1987).

It has also been suggrested (Chiu *et al.*, 1987; Mercurio and Karin, 1989) that the activity of the region of the SV40 enhancer containing the TC motifs may be mediated in HeLa cells by the AP-3 protein which appears to bind to the 'core' sequence [5′(247)GTGG$^{AAA}_{TTT}$G(240)-3′] (Laimins *et al.*, 1982; Weiher *et al.*, 1983; see Figure 5), possibly in conjunction with the AP-2 protein which binds to two sites within this region (Mitchell *et al.*, 1987; Imagawa *et al.*, 1987; see Figure 1 and 2A in Macchi *et al.*, 1989).

Using oligomers of the SV40 enhancer region containing wild type and mutated TC-I and TC-II sequences, we have characterized here the sequence motifs which exhibit enhancer activity in a number of cell types. The enhanson activity of the related H-2K^b motif was also studied in the same cells. We show that both the TC-II/κB and H-2K^b motifs have proto-enhancer activity, i.e. generate enhancer activity when multimerized on their own. However, in contrast to the previously reported lymphoid cell specific activity of the TC-II/κB motif, the present results demonstrate that this motif, as well as the H-2K^b motif are active in lymphoid and non-lymphoid cells, notably in HeLa cells. Since it has been reported (Sen and Baltimore, 1986b; Baeuerle and Baltimore, 1988a,b) that the NF-κB DNA binding activity can be induced in nuclear extracts of non-lymphoid cells by the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and the protein synthesis inhibitor cycloheximide, we have also studied the effects of these agents on the activity of the TC-II/κB and H-2K^b proto-enhancers.

Results

The TC-II element, but not the TC-I motif, acts as a proto-enhancer in a variety of cells

The enhancer activity of the region containing the TC motifs was studied using recombinants containing the rabbit β-globin gene and tetramerized SV40 motifs inserted at either

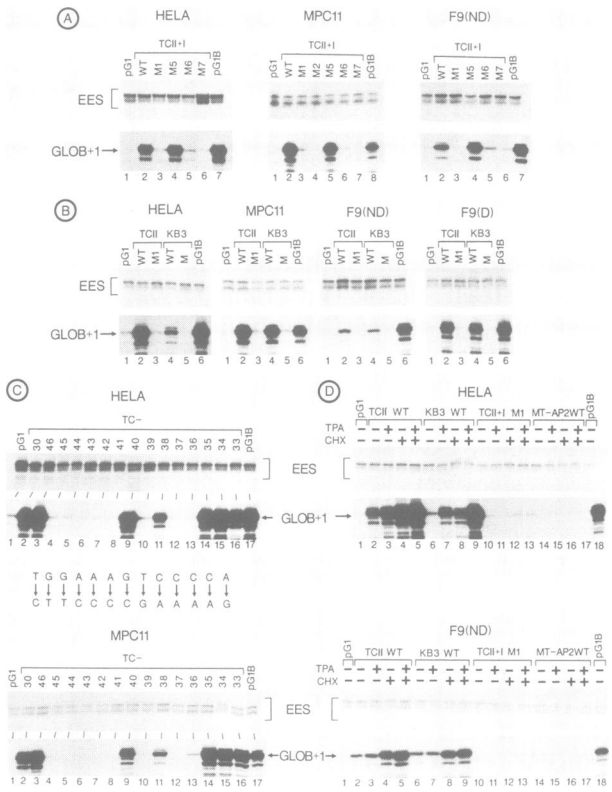
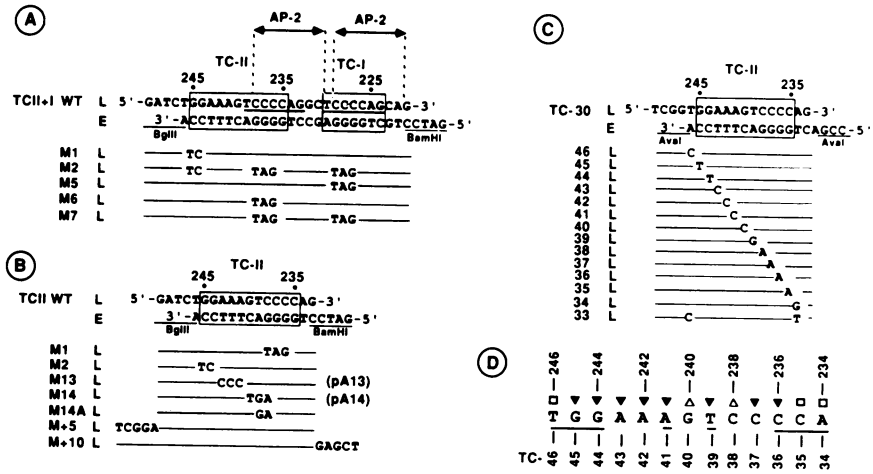


Fig. 1. Representative S1 nuclease analysis of RNA synthesized from pG1 recombinants containing oligomerized motifs and the reference plasmid pA56 following calcium phosphate mediated transfection into the HeLa, MPC11, F9(ND) and F9(D) cell lines. The quantities of the pG1 derived recombinants transfected were: 5 μg in F9(ND) and F9(D) cells, 2 μg in MPC11 cells and 1.6 μg in HeLa cells. For pA56, transfected quantities were 12 μg for HeLa and F9(D) cells, 9 μg for F9(ND) cells and 3 μg for MPC11 cells. Note that pA56, which contains the lymphoid specific purine box (Pettersson and Schaffner, 1987), is more active in MPC11 cells. S1 nuclease analysis was carried out with 20, 30, 40 and 40 μg of cytoplasmic RNA from HeLa, MPC11, F9(D) and F9(ND) cells, respectively. Protected fragments of 130−137 and 60 nucleotides correspond to RNA initiated at the SV40 promoter early-early (EES of the reference plasmid pA56) and β-globin promoter (Glob + 1 of pG1 and pG2 derivatives) startsites respectively. The oligomers present in pG1 recombinants are indicated above each lane along with the cell type transfected. (**A**) Activity of the TC-II and TC-I sequences. (**B**) Activity of the WT and mutant TC-II and H-2K^b (KB) enhansons. (**C**) Activity of the TC-30 to TC-46 series containing single point mutations in the TC-II enhanson. The L strand wild type (upper line) and mutated (lower line) sequences present in this series are indicated between the HeLa and MPC11 panels. (**D**) Activity of TCII WT, KB3 WT, MT-AP2 WT and TCII + I M1 after treatment with TPA or CHX (cycloheximide) or TPA and CHX, as indicated.

position −109 (in close apposition to the globin promoter, pG1 series) or −425 (at distance from the globin promoter, pG2 series) upstream of the globin gene capsite, in an orientation opposite to that existing in the SV40 early promoter (see Fromental *et al.*, 1988). These recombinants were transiently transfected into human HeLa cells, mouse MPC11 plasmocytoma B cells, LMTK$^-$ fibroblasts, F9 undifferentiated [F9(ND)] and retinoic acid differentiated [F9(D)] embryonal carcinoma (EC) cells. The enhancer activity was determined by quantitative S1 nuclease analysis of RNA transcribed from the β-globin gene promoter (Glob + 1 in Figure 1 in which typical autoradiograms are displayed). The plasmid pA56 (Zenke *et al.*, 1986) which



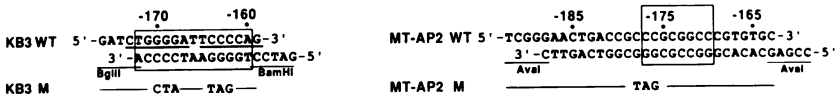
E

RELATIVE ENHANCER ACTIVITY

	HELA		MPC11		F9(ND)		F9(D)		LMTK⁻
	pG1	pG2	pG1	pG2	pG1	pG2	pG1	pG2	pG1
pG1B	100(400x)	—	100(100x)	—	100(100x)	—	100(300x)	—	100(150x)
pG2B	—	100(100x)	—	100(20x)	—	100(40x)	—	100(60x)	—
TCII+I WT	73(292x)	85(85x)	235(235x)	295(59x)	25(25x)	20(8x)	62(186x)	70(42x)	138(207x)
M1-M2	0	0	0	0	0	0	0	0	0
M5	75(300x)	80(80x)	218(218x)	290(58x)	62(62x)	52(28x)	74(222x)	80(48x)	140(210x)
M6	0	0	0	0	4(4x)	0	0	0	0
M7	0	0	0	0	10(10x)	0	0	0	0
TCII WT	85(340x)	76(76x)	210(210x)	315(63x)	12(12x)	10(4x)	65(195x)	72(43x)	137(206x)
M1-M2-M13 M14-M14A	0	0	0	0	0	0	0	0	0
M+5	69(276x)	ND	180(180x)	ND	10(10x)	ND	54(162x)	ND	99(148x)
M+10	51(204x)	|	110(110x)	|	4(4x)	|	25(75x)	|	64(96x)
TC-30	84(336x)		178(178x)		10(10x)		68(204x)		150(225x)
46	89(356x)		185(185x)		12(12x)		70(210x)		140(210x)
45-44-43 42-41	0		0		0		0		0
40	55(220x)		100(100x)		3(3x)		15(45x)		85(128x)
39	0		0		0		0		0
38	11(44x)		40(40x)		0		4(12x)		25(38x)
37	0		0		0		0		0
36	0		3(3x)		0		0		0
35	88(352x)		185(185x)		13(13x)		75(225x)		156(234x)
34	93(372x)		173(173x)		10(10x)		65(195x)		145(218x)
33	70(280x)		168(168x)		12(12x)		58(174x)		136(204x)

Fig. 2. Structure of the motifs oligomerized to study the activity of the TC-II and TC-I sequences, and a tabular representation of their enhancer activities. The nucleotide sequences of the double stranded wild type templates are shown along with the L strand sequence of the point mutations, the locations of which are shown below the wild type sequence. The SV40 coordinates (BBB numbering system; Tooze *et al.*, 1982) are indicated above the sequence, and the name of each template is indicated on the left. E and L designate the early and late SV40 coding strands respectively. The boxed sequences represent the TC-I motif and the TC-II enhanson. In each case, the sequences shown are those constituting the units which are tandemly repeated four times in pG1 or pG2. (**A**) Structure of the oligomers containing the TC-II and TC-I sequences. The TC-II and TC-I motifs as defined originally in Zenke *et al.* (1986) are underlined and the two AP-2 binding sites described by Mitchell *et al.* (1987) are indicated above the wt sequence. (**B**) Structure of oligomers containing only the TC-II enhanson. The names of the corresponding pA series mutants (pA13 and pA14) in the total SV40 enhancer (Zenke *et al.*, 1986) are indicated in parentheses. (**C**) Structure of the templates containing a systematic series of point mutations in TC-II enhanson. The TC-33 template contains two mutations converting the nucleotides flanking the TC-II enhanson into those flanking the $\varkappa$B enhanson in the $\varkappa$ enhancer. (**D**) Summary of the effects of the point mutations on enhancer activity. The positions where changes had no effect are shown by the open squares, while the filled triangles represent a fully deleterious effect and the open triangles a partially deleterious effect. The palindromic sequence of the TC-II $\varkappa$B motif is underlined. (**E**) Tabular representations of enhancer activities. The enhancer activity determined by densitometric scanning of several exposures of the autoradiograms from at least three different experiments are indicated. The values in parenthesis indicate the fold stimulation by each oligomer over the value for the enhancerless pG1 recombinant. The other values show the activity of each oligomer (indicated to the left) as a percentage of that of the total SV40 enhancer in pG1B or pG2B (taken as 100%). O corresponds to no stimulation over the pG1 basal level, and ND to not determined. No stimulation of transcription was observed in HeLa and MPC11 cells when a single TC-II enhanson was inserted in pG2.

contains the SV40 early promoter [early-early startsite signal (EES) in Figure 1] deleted for all of the enhancer motifs except the purine box of Pettersson and Schaffner (1987), was cotransfected to allow correction for variations in transfection efficiencies. Following densitometric scanning of autoradiograms corresponding to different exposures of the same gel, and correction for transcription from the reference pA56 recombinant, the results (see Figures 2–5) were expressed for each cell type as a percentage of transcription from the globin capsite (Glob + 1), taking as 100% either pG1B or pG2B which contain the SV40 enhancer with a single 72 bp sequence (see Fromental *et al.*, 1988). Alternatively, the results were expressed as the fold stimulation when compared with transcription from the parental recombinants pG1 or pG2 (numbers in parentheses in Figures 2, 3 and 5). In all cases the results presented



RELATIVE ENHANCER ACTIVITY

	HELA		MPC11		F9(ND)		F9(D)		LMTK⁻	
	pG1	pG2	pG1	pG2	pG1	pG2	pG1	pG2	pG1	pG2
pG1B	100(400x)	—	100(100x)	—	100(100x)	—	100(300x)	—	100(150x)	—
pG2B	—	100(100x)	—	100(20x)	—	100(40x)	—	100(60x)	—	100
KB3 WT	4(16x)	6(6x)	172(172x)	185(37x)	10(10x)	10(4x)	40(120x)	35(21x)	166(249x)	145
KB3 M	0	0	0	0	0	0	0	0	0	0
MT-AP2 WT	0	0	0	0	0	0	0	0	0	0
MT-AP2 M	0	ND	0	ND	0	ND	0	ND	0	ND

Fig. 3. Structure and enhancer activity of the KBF1/H2TF1 protein binding motif from the H-2K^b promoter (KB3) and the AP-2 binding motif from the human metallothionein IIA promoter (MT-AP2). The nomeclature used and expression of the results are as in Figure 2. The position of the motifs are indicated by the solid box. The numbers indicate the coordinates in the original promoters. The palindrome in the KB3 template has been underlined.

correspond to the average value (within 15%) of at least three independent transfection experiments, using at least two different plasmid preparations.

The enhancer activity of a tetramer of the wild type TC region (TCII + I WT in Figures 1 and 2) inserted in either pG1 or pG2 was close to, or higher than, that of the whole SV40 enhancer (pG1B and pG2B, respectively) in all cell lines tested, except in F9(ND) cells where it was much lower (20–25%, Figure 2E). The mutation present in TCII + I M6, which affects three bases of the TC-II motif which have previously been shown to be important for the activity of whole SV40 enhancer notably in MPC11 cells (mutants pA14 and PA15 in Nomiyama *et al.*, 1987; see figure 5) abolished enhancer activity, whereas the analogous mutation in the TC-I motif (TCII + I M5; Figure 2A) which had little or no effect on the activity of the whole SV40 enhancer (mutants pA17 and pA18 in Nomiyama *et al.*, 1987), did not affect the activity of the TCII + I tetramer, except in F9(ND) cells where a 2-fold increase was observed (Figures 1A and 2E). In the case of F9(ND) cells only, the combination of M5 and M6 mutations in TCII + I M7 (Figures 1A and 2A and E) resulted in an increase of activity (when compared with M6) in the pG1, but not in the pG2 derivative, suggesting that this stimulation in F9(ND) cells may not correspond to a real enhancer activity. Since previous studies using mutants containing the whole SV40 enhancer had suggested that bases centered on coordinate 245 may be important for the activity of the TC-II motif (mutant pA12) in Nomiyama *et al.*, 1987; see Figure 5), the tetramer of TCII + I M1 was constructed (Figure 2A). Its total lack of activity in all cell lines (Figures 1A and 2E) indicated clearly that the TC-II enhanson is larger than the TC-II motif as originally defined in Zenke *et al.* (1986) (underlined sequence in TC-II + I WT in Figure 2A).

From the above results, we concluded that sequences containing the TC-II motif were sufficient for generating enhancer activity when oligomerized, whereas the TC-I motif had no activity of its own [with a possible exception of F9(ND) cells], nor cooperated with the TC-II motif. Tetramers lacking the TC-I motif were then tested to further support this conclusion (TC-II series in Figure 2B). As expected, the wild type tetramer, TCII WT, exhibited the same activity as TCII + I WT, except in F9(ND) cells where it was less active (Figures 1B and 2E). A number of mutations, spanning the TC-II motif (as defined above)

ENHANCER ACTIVITY RELATIVE TO pG1B (100%)

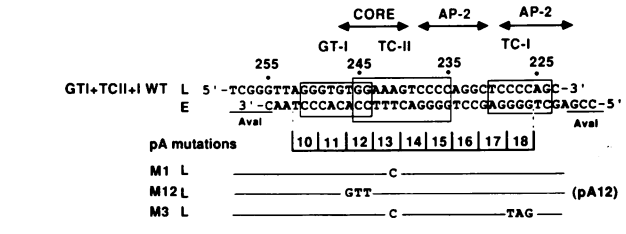
	HELA				F9(ND)			
TPA	–	+	–	+	–	+	–	+
CHX	–	–	+	+	–	–	+	+
TCII WT	85	136 (x1.6)	210 (x2.5)	600 (x7)	12	12 (x1)	60 (x5)	60 (x5)
KB3 WT	4	40-80 (x10-20)	52 (x13)	280-600 (x70-150)	10	10 (x1)	60 (x6)	60 (x6)
MT-AP2 WT	0	0	0	0	0	0	0	0
TCII+I M1	0	0	0	0	0	0	0	0

	MPC11				F9(D)				LMTK⁻			
TPA	–	+	–	+	–	+	–	+	–	+	–	+
CHX	–	–	+	+	–	–	+	+	–	–	+	+
TCII WT	210	273 (x1.3)	250 (x1.2)	410 (x2)	65	65 (x1)	460 (x7)	460 (x7)	150	195 (x1.3)	255 (x1.7)	600 (x4)
KB3 WT	175	227 (x1.3)	227 (x1.3)	350 (x2)	40	40 (x1)	280 (x7)	250 (x6)	180	234 (x1.3)	330 (x2)	720 (x4)
MT-AP2 WT	0	0	0	0	0	0	0	0	0	0	0	0
TCII+I M1	0	0	0	0	0	0	0	0	0	0	0	0

Fig. 4. Relative enhancer activity of the TCII WT, KB3 WT, MT-AP2 WT and TCII + I M1 templates after treatment with TPA or CHX, or CHX and TPA. The values in parenthesis indicate the fold stimulation induced by each treatment over the values of the same recombinant in untreated cells. The other values show the relative activity following each treatment (indicated at the top) as a percentage of that of the total SV40 enhancer in pG1B in untreated cells (taken as 100%). O is no stimulation over the pG1 basal level. Note that TPA and CHX treatments have little effect (< 1.5-fold) on the activity of the total SV40 enhancer in all of the cell types tested.

suppressed the enhancer activity, including TCII M13 and TCII M14 (Figure 2B and E) which contain mutations previously shown to be deleterious to the activity of the whole SV40 enhancer in MPC11 cells (pA13 and pA14 in Nomiyama *et al.*, 1987; see Figure 5).

It is important to note that no enhancer activity could be detected when a single wild type TC-II motif (identical to that tetramerized in TCII WT was inserted in pG2 (data not shown), indicating that the TC-II element is a class C enhanson (Fromental *et al.*, 1988) which possesses proto-enhancer, but not enhancer activity, on its own. Note also that the enhancer activity of TC-II tetramers does not appear to depend critically on the spacing of the constituent TC-II motifs, since adding 5 or 10 bp to each copy of the TC-II motif (TCII M + 5 and TCII M + 10. Figure 2B and E) did not decrease markedly their activity (the higher activity of the TCII + I WT compared with TCII M + 10 suggests that the nature of the 'spacing sequences' may not be neutral).



ENHANCER ACTIVITY RELATIVE TO pG1B

	HELA	MPC11	F9(ND)	F9(D)	LMTK$^-$
	pG1	pG1	pG1	pG1	pG1
pG1B	100(400x)	100(100x)	100(100x)	100(300x)	100(150x)
GTI+TCII+I WT	33(132x)	45(45x)	<2	ND	45(67x)
M1-M12, M3	0	0	0	ND	0

Fig. 5. Structure and enhancer activity of templates containing the GT-I and TC-II enhansons and the TC-I sequence, the positions of which are indicated by the solid boxes, in their natural SV40 configuration. The nomenclature and the expression of the results are as described in Figure 2. The positions of the AP-2 binding sites and the 'core' sequence are indicated above the sequence. The locations of the mutations present in pA10 to pA18 within the whole SV40 enhancer (Zenke *et al.*, 1986; Nomiyama *et al.*, 1987) are aligned below the sequence.

The same sequence is responsible for the activity of the TC-II proto-enhancer in the various cell lines

Since the activity of the TC-II enhanson may be due to the binding of different enhancer factors, possibly exhibiting cell specificity (see Introduction), we constructed a systematic series of point mutants (TC-33 to TC-46, Figure 2C) to characterize the TC-II enhanson at the base level and to investigate whether the same bases were similarly involved in different cell types. Mutations at positions 245, 244, 243, 242, 241, 239, 237 and 236, all resulted in a complete loss of enhancer activity in all cell types (Figures 1C and 2C–E). In contrast, a mutation at position 235 did not affect the enhancer activity, whereas mutations at positions 238 and 240 resulted only in a partial loss of activity. Mutations at positions 234 (TC-34), 246 (TC-46) or simultaneous mutations at both positions (TC-33) did not affect the enhancer activity. Note that the mutated bases in TC-33 are those flanking the TC-II enhanson in the $\varkappa$B enhancer motif of the immunoglobulin kappa chain (5′-*C*GGAAAGT CCCC*T*-3′) (see Sen and Baltimore, 1986a; Lenardo *et al.*, 1987). We conclude that very similar, if not identical, sequences are responsible for the activity of the TC-II element in the various cell types, notably in non-lymphoid cells (HeLa and LMTK$^-$) and MPC11 lymphoid B cells.

Comparison of the activity of the TC-II and H-2K^b proto-enhancers: effect of TPA and cycloheximide treatments

The TC-II enhanson can bind efficiently and specifically two proteins, TC-IIA and TC-IIB, which have properties very similar to those of factors, NF-$\varkappa$B (originally identified by its binding to the $\varkappa$B enhancer motif, see above) and KBF1/H2TF1 (originally identified by its binding to the H-2K^b enhancer motif 5′-TGGGGATTCCCCA-3′) respectively (see Macchi *et al.*, 1989, and Introduction for references). To investigate which of the two factors may be responsible for the activity of the TC-II proto-enhancer, the enhancer activity of the tetramerized H-2K^b motif (KB3 WT in Figure 3) was studied in the various cell lines. KB3 WT activity was very similar to that of TCII WT in MPC11, F9(ND), F9(D) and LMTK$^-$ cells, but not in HeLa cells where it was ~20 times lower (Figures 1B, 2E and 3). Note that the mutant KB3 M had no activity in any of the cell lines (Figure 3), and also that a single H-2K^b motif inserted in pG2 had no detectable activity (data not shown), indicating that this motif acts also as a proto-enhancer element.

It has been reported that treatment of HeLa, pre-B and T cells with either a tumor promoter (TPA) or a protein synthesis inhibitor (cycloheximide, CHX) or both of them, could lead to an increase of the protein TC-IIA/NF-$\varkappa$B which can bind to the TC-II/$\varkappa$B element (for references see Sen and Baltimore, 1986b; Baeuerle and Baltimore, 1988a,b; Macchi *et al.*, 1989; and Introduction). These observations prompted us to study the effect of these agents on the activity of the TC-II and H-2K^b proto-enhancers in the various cell lines using the pG1 recombinants (Figures 1D and 4). In HeLa cells,the activity of the TC-II proto-enhancer (TCII WT) was increased ~1.6-fold by TPA, 2.5-fold by CHX and 7-fold by TPA + CHX, whereas the activity of the H-2K^b proto-enhancer (KB3 WT) was much more markedly increased ~10- to 20-fold with TPA, 13-fold with CHX and 70- to 150-fold with TPA + CHX, resulting in similar activities for the TC-II and H-2K^b proto-enhancers in the presence of TPA + CHX). To test the possibility that these differential increases could be due to 'saturation' of the globin promoter in the pG1 derivatives, the same experiments were carried out with the pG2 derivatives. Approximately 20-fold and 250-fold stimulations with respect to 'normal' HeLa cells were observed when TCII WT and KB3 WT respectively, were transfected into HeLa cells treated with TPA + CHX (data not shown), indicating that promoter saturation cannot account for the differential response of the TC-II and H-2K^b enhansons. In contrast, much weaker stimulations of activation by the H-2K^b element, similar to those obtained with the TC-II element, were observed with TPA and/or CHX-treated MPC11 and LMTK$^-$ cells (Figure 4). Similar stimulations were obtained using the pG2 recombinant series indicating again that these lower stimulations were not due to promoter 'saturation' (data not shown). In F9(ND) and F9(D) cells, CHX caused a 5- to 7-fold stimulation of the activity of both proto-enhancers, whereas TPA treatment had no effect.

The AP-2 binding site(s) located within the TC region does not act as an enhanson

The putative enhancer factor AP-2 interacts with the consensus sequence 5′-CCCCAGGC-3′ (Mitchell *et al.*, 1987). Individual sites which can vary substantially from the consensus are found in a number of viral and cellular transcriptional regulatory regions, e.g. in the SV40 (see Figures 2 and 5) and human metallothionein IIA (hMTIIA, MT-AP2 in Figure 3) enhancers (Haslinger and Karin, 1985; Imagawa *et al.*,1987; Lee *et al.*, 1987; Mitchell *et al.*, 1987). Moreover transcriptional activation by AP-2 may be positively regulated by phorbol esters (TPA) and cAMP (Imagawa *et al.*, 1987; Comb *et al.*, 1988; Hyman *et al.*, 1988). TCII + I M1 and TCII M2 (Figure 2A and B), which bear a fully deleterious mutation in the TC-II proto-enhancer while leaving intact the two or only one AP-2 binding sites respectively, did not show any enhancer activity when tetramerized as either a single AP-2 motif (TCII M2 in Figure 2A and E; see also GTI + TCII + I M3 in Figure

5) or a tandem repeat (TCII + I M1 in Figure 2A and E; see also GTI + TCII + I M12 in Figure 5).

The above results do not exclude that the AP-2 binding site may act as a class B enhanson (Fromental *et al.*, 1988; and Introduction) which can generate enhancer activity only when associated with another enhanson. To investigate this possibility, we constructed tetramers of GTI + TCII + I containing a mutation selectively deleterious to the activity of the TC-II proto-enhancer (A → C at coordinate 241), but having the GT-I motif and either one (GTI + TCII + I M3) or the two AP-2 binding sites intact (GTI + TCII + I m1) (Figure 5). None of these tetramers exhibited any stimulatory activity. The results obtained with GTI + TCII + I M1 and M3 confirm also our previous conclusion (Fromental *et al.*, 1988) that the GT-I motif is a class B enhanson which has no stimulatory activity on its own even when tetramerized.

The failure to reveal an enhanson/proto-enhancer activity of the SV40 enhancer sequences which bind the AP-2 protein prompted us to make a tetrameric construct containing the metallothionein hMTIIA AP-2 binding site, analogous to that previously made by Imagawa *et al.* (1987) and reported to have enhancer activity (MT-AP2 WT in Figure 3). Surprisingly, both the corresponding pG1 and pG2 derivatives were totally inactive in the various cell lines, in the absence (Figure 3) as well as in the presence of TPA (Figure 4). Transfecting the HeLa tk^- cells used by Imagawa *et al.* (1987) instead of our HeLa S3 cells also did not result in enhancer activity in the presence or absence of TPA (data not shown).

Discussion

The TC-II/κB element which overlaps the GT-I enhanson is a proto-enhancer in a variety of cell types, but the TC-I sequence is not an enhanson

The present results show that the SV40 TC-I and II sequences as originally defined in Zenke *et al.* (1986) on the basis of sequence homology (underlined in Figure 2A, 5′-TCCCCAG-3′) has no enhanson activity in any of the cell types tested. This conclusion is based on the inactivity of TCII M2 (Figure 2B and E) and TCII + I M6 (Figure 2A and E) which contain the intact sequences TC-II and TC-I respectively. Similarly TCII + I M1 (Figure 2A and E), in which both the TC sequences are intact, has no enhancer activity, indicating that these sequences show no enhanson activity even when present as a tandem repeat. Moreover, the TC-II and TC-I sequences cannot cooperate with the GT-I enhanson, since GTI + TCII + I M1 is inactive while these three sequences are intact (Figure 5). Oligomers of the region containing the TC-II motif, however, do exhibit enhancer activity. Analysis of a series of mutated templates (TCII + I M1, Figure 2A and E, and TC-33 to TC-46, Figure 2C, D and E) indicate that the TC-II enhanson sequence 5′-(245)GGAAAGTCCCC(235)-3′ overlaps with, but is larger than the previously defined TC-II sequence. Note that, while a C → A mutation at position C235 has no effect *in vivo* (TC-35 in Figure 2C and E) or on the binding of proteins TC-IIA or TC-IIB *in vitro* (Macchi *et al.*, 1989), the introduction of a methyl group in the DNA major groove at this position interferes with the binding of proteins TF-IIA and B (see Macchi *et al.*, 1989); thus this position has been included in the TC-II enhanson sequence. The TC-II enhanson exhibits class C (proto-enhancer) properties (Fromental *et al.*, 1988), since it requires no other enhansons for activity and has no stringent spacing requirements with respect to the distance between the repeats when oligomerized (compare TCII WT with TCIIM + 5 and TCIIM + 10, Figure 2B and E). Moreover, the same sequence is responsible for the enhanson activity in each cell type, notably in lymphoid and non-lymphoid cells, since the point mutations (TC-35−TC45) have the same effect in each cell type.

The present *in vivo* results using TC-II enhanson oligomers are in complete agreement with those obtained with the whole SV40 enhancer [pA mutant series in Zenke *et al.* (1986) and Nomiyama *et al.* (1987); see Figure 5]. First, mutations within the TC-I sequence (pA17 and pA18) had no significant effect in any of the cell lines tested. Second, mutations spanning the whole TC-II enhanson (pA12−pA15), had a marked deleterious effect in MPC11 cells. In HeLa cells, the situation was more complex. Simultaneous mutations within the TC-II and GT-I enhansons had a detrimental effect on enhancer activity (pA1015, pA1115 in Nomiyama *et al.*, 1987), while mutations in either motif individually had little effect (pA10, 11, 13, 14 and 15; Zenke *et al.*, 1986; Nomiyama *et al.*, 1987), suggesting that in HeLa cells these two motifs could compensate for each other. The pA12 mutation, however, had a strong detrimental effect in HeLa cells analogous to that of the above double mutations, which led us to propose that the TC-II and GT-I enhansons overlap at these nucleotides (positions 244−246; Nomiyama *et al.*, 1987). The present demonstration that the TC-II enhanson, which extends to positions 244 and 245 (thus overlapping the GT-I enhanson), is strongly active in both MPC11 and HeLa cells, whereas the GT-I enhanson is inactive in MPC11 cells (Fromental *et al.*, 1988), is entirely consistent with the previously reported effect of the pA mutations within the whole enhancer and the above assumptions. Moreover, since the effect of mutations within the GT-I enhanson could be clearly seen in F9(ND) cells, we proposed (Nomiyama *et al.*, 1987) that the activity of the TC-II enhansion would be low in these cells, which is in agreement with the present low activity of the TC-II tetramers in F9(ND) cells. Thus, the effect of mutations in the region of the SV40 enhancer containing the GT-I and TC-II enhansons (pA10−pA15) can be fully accounted for, in each cell type, by the different cell specific activities of the overlapping GT-IIC + GT-I (Fromental *et al.*, 1988) and TC-II proto-enhancers.

The results obtained with oligomers of the TC-II/κB enhanson in MPC11 cells are in agreement with those of Pierce *et al.* (1988) and Wirth *et al.* (1988), showing that the κB sequence is active in lymphoid cells. However, the present strong activity of the TC-II/κB enhanson in HeLa cells contrasts with Pierce *et al.* (1988) and Nelsen *et al.* (1988), who could find no stimulatory activity in these cells. This difference cannot be explained by the different promoters used [globin gene in our case and *fos* gene in Pierce *et al.* (1988), or the enhancerless SV40 early promoter in Nelsen *et al.* (1988)], since insertion of TC-II tetramers upstream of the *fos* promoter gave the same results as with the globin promoter (our unpublished result). We note that our results are in complete agreement with those of two independent studies (Ondek *et al.*, 1987; Schirm *et al.*, 1987) which indicated that oligomers of the GT-I and TC-II enhansons (and not containing a complete AP-2 binding site) were active in a variety of lymphoid and non-

lymphoid cells, including HeLa cells. As we have previously shown (Fromental *et al.*, 1988) tht the GT-I class B enhanson requires the presence of a second enhanson for activity (this result is confirmed in this study; see the inactivity of GTI + TCII + I M1 in Figure 5), the activity of these latter oligomers must be due entirely to the TC-II enhanson. The present increase in TC-II enhanson activity in HeLa cells by treatment with TPA and cylcoheximide is however, consistent with the report that (at least some of) the TC-IIA/NF-$\varkappa$B-like protein(s) is sequestered in HeLa cell cytoplasm by an inhibitor, I$\varkappa$B, from which it can be released by TPA and cycloheximide treatments (Baeuerle and Baltimore, 1988a,b; see also Macchi *et al.*, 1989 in which the nuclear TC-IIA/NF-$\varkappa$B-like protein is increased ~4-fold by these treatments). At the present time we do not understand the reason for the discrepancy between our results *in vivo* [as well as those of Schirm *et al.* (1987) and Ondek *et al.* (1987)] and those of Pierce *et al.* (1988) and Nelsen *et al.* (1988), since it is clear that the TC-II/$\varkappa$B enhanson is very active in our untreated HeLa cells, whereas the activity of the H-2K^b enhanson is very low unless the cells are treated with TPA and/or cycloheximide (see Figure 4 and below), which demonstrates that our HeLa cells are responsive to these agents, and that our cell culture conditions do not mimic the effect of TPA.

The TC-II and GT-I enhansons overlap the SV40 'core' sequence which has no demonstrable enhanson activity

The region of the SV40 enhancer containing the TC-II and GT-I enhansons includes the 'core' sequence (5′(247)GTGG$^{AAA}_{TTT}$G(240)-3′) (Laimins *et al.*, 1982; Weiher *et al.*, 1983; see Figures 1 and 5 in Macchi *et al.*, 1989). Previous results (Fromental *et al.*, 1988; Ondek *et al.*, 1988) have demonstrated that oligomers of this sequence have no enhancer activity in HeLa cells *in vivo*. These results are confirmed here by the inactivity of mutants TC-36, 37 and 39, all of which contain an intact 'core' sequence (see Figure 2C and E). *In vitro* protein binding studies (Xiao *et al.*, 1987b; Macchi *et al.*, 1989) and genetic analysis *in vivo* (Fromental *et al.*, 1988 and this study), all indicate that while the GT-I and TC-II enhansons overlap the 'core', sequences 5′ or 3′ from the 'core' are absolutely required for the activity of GT-I and TC-II enhansons respectively.

A DNA binding activity, AP-3, has been indentified by DNase I footprinting in HeLa cells, which supposedly interacts with the 'core' motif (Chiu *et al.*, 1987; Mitchell *et al.*, 1987; Mercurio and Karin, 1989). The protein responsible for this activity has been variously described as a 57 kd (Mitchell *et al.*, 1987) or a 48 kd (Mercurio and Karin, 1989) polypeptide. It has been suggested (Mercurio and Karin, 1989) that this protein may mediate the activity of the 'core' sequence which would be active in the whole SV40 enhancer, and that there is a discrepancy between our results obtained with the total enhancer (Zenke *et al.*, 1986) and those seen with oligomers of the individual enhansons, in which the 'core' has been shown to be inactive (Fromental *et al.*, 1988). As discussed above, this suggestion is certainly not supported by an exhaustive comparison of the results obtained with either oligomers of the GT-I and TC-II enhansons or the total SV40 enhancer. In contrast, the propositions of Mercurio and Karin (1989) concerning the role of AP-3 and the 'core' sequence are inconsistent with all of the present results obtained *in vivo*. Moreover, in support for a possible role of AP-3 in the activity of the SV40 enhancer, Mercurio and Karin (1989) observe that the binding of AP-3 *in vitro* is impaired using our pA12 mutant (Zenke *et al.*, 1986; see Figure 5) which has a deleterious effect in HeLa cells *in vivo*; however, they overlook their own observation that the pA11 and pA13 mutations also impair AP-3 binding, although both have wild type activity in HeLa cells *in vivo* (Zenke *et al.*, 1986). Thus, it is impossible to account for the *in vivo* phenotypes of the pA10, pA11, pA12, pA13, pA14 and pA15 mutations (Zenke *et al.*, 1986; Nomiyama *et al.*, 1987) by invoking an enhanson activity of the 'core' sequence mediated by AP-3 (Mercurio and Karin, 1989), possibly cooperating with AP-2 (Mitchell *et al.*, 1987), whereas our model implicating differing cell specific activities of the overlapping GT-I and TC-II enhansons is entirely consistent with all of the *in vivo* data. Note finally that the only evidence supporting the contention that the AP-3 protein could be an enhancer factor interacting with the SV40 'core' sequence resides in its ability to stimulate transcription *in vitro* from a promoter containing oligomers of a sequence from the mouse IL-2 promoter which diverges notably from the SV40 'core' sequence (Mercurio and Karin, 1989).

The H-2K^b motif is a cell specific proto-enhancer

Deletion and *in vivo* competition experiments have suggested that the sequence 5′-TGGGGATTCCCCA-3′ (H-2K^b motif, KB3 WT in Figure 3) may be involved in the regulation of transcription of the histocompatibility H-2K^b gene (Kimura *et al.*, 1986; Baldwin and Sharp, 1987; Israël *et al.*, 1987). This motif shows sequence homology to the TC-II/$\varkappa$B enhanson and *in vitro* competition experiments have shown that the TC-IIA/NF-$\varkappa$B like protein(s) and the TC-IIB/KBF1/H2TF1 like protien(s) recognize both motifs (Baldwin and Sharp, 1988; Macchi *et al.*, 1989). The results of this study indicate that the H-2K^b sequence has class C enhanson properties since it acts as a cell specific proto-enhancer. The H-2K^b enhanson does not however, show the same cell specific activity as the TC-II/$\varkappa$B enhanson as indicated by the low activity of KB3 WT in untreated HeLa cells where the TC-II/$\varkappa$B enhanson exhibits high activity. As both the TC-IIA/NF-$\varkappa$B and TC-IIB/KBF1/H2TF1 proteins are present in HeLa cells (Macchi *et al.*, 1989; and references therein), these differences suggest that the activities of these motifs are mediated by distinct molecular mechanisms (see below).

Treatment of cells with TPA and cycloheximide has a differential effect on the activities of the TFC-II/$\varkappa$B and H-2K^b enhansons

Treatment of HeLa cells with TPA, a synthetic activator of protein kinase C, results in a 10- to 20-fold increase in the activity of the H-2K^b enhanson, but only in a minor stimulation (1.6-fold) of the TC-II enhanson activity (Figure 4). Cycloheximide treatment of HeLa cells also had a differential effect, stimulating the H-2K^b enhanson activity by 13-fold and that of the TC-II enhanson by only 2.5-fold. In contrast, TPA treatment of each of the other cell types had very little effect on the activity of each enhanson, while cycloheximide treatment stimulated both activities to the same extent (Figure 4). The results obtained using cyclo-

heximide suggest that existence in HeLa, F9(ND) and F9(D) cells, of inhibitor protein(s) which may repress the activity of the protein(s) mediating the activity of the TC-II/κB and H-2K^b enhansons. This repressing activity appears to be absent from MPC11 cells as cycloheximide treatment has no effect. These results are consistent with the reported existence of the NF-κB inhibitor protein IκB in non-lymphoid B cells (Baeuerøle and Baltimore, 1988a,b), although in the present study the TC-II/κB enhanson is evidently already active in untreated HeLa cells, where it could be further stimulated by cycloheximide. The possible existence of a fully potent IκB-like protein in untreated F9(ND) is indicated by the complete absence of the TC-IIA/NF-κB-like protein in nuclear extracts of these cells where its appearance can be induced by cycloheximide, but not TPA (see Macchi *et al.*, 1989 and below). The inability of TPA to induce the activity of the TC-II/κB and H-2K^b enhansons in both F9(ND) and F9(D) cells, which correlates with its inability to induced the TC-IIA binding activity in F9(ND) nuclear extracts, suggests that these cells lack the appropriate protein kinase C activity (Nishizuka, 1988) or another component of this signal transduction pathway which is present in HeLa cells. This absence of TPA inducibility in F9 cells was also observed for oligomers of the human metallothionein IIA AP-1 binding site whose activity was TPA-inducible in HeLa cells (Fromental, 1989).

Proteins mediating the in vivo effect of the TC-II/κB and H-2k^b enhansons

Several nuclear proteins have been described which recognize specifically the TC-II/κB and H-2K^b enhansons. In HeLa cells a protein(s) TC-IIA which has properties analogous to NF-κB has been identified (Macchi *et al.*, 1989). This nuclear DNA binding activity which has similar affinity for the TC-II/κB and H-2K^b enhansons, can be induced ≃4-fold in HeLa cells by treatment with TPA and cycloheximide (Macchi *et al.*, 1989). Furthermore it is not detectable in nuclear extracts of F9(ND) cells unless they are treated with cycloheximide (see Macchi *et al.*, 1989 and above). A 57 kd HeLa cell protein EBP-1 which may be identical to TC-IIA has also been identified and shown to interact specifically with the TC-II/κB enhanson (Clark *et al.*, 1988, 1989; Clark and Hay, 1989; see also Macchi *et al.*, 1989). A second binding activity, TC-IIB, with properties similar to the KBF1/H2TF1 protein(s), has been identified in all of the cell types tested here (Macchi *et al.*, 1989; and references therein). This protein(s) binds to the TC-II/κB and H-2K^b enhansons, but has an ~10-fold higher affinity for the latter enhanson (Baldwin and Sharp, 1988; Macchi *et al.*, 1989).

Since both proteins are present in nuclear extracts of MPC11, F9(D) and LMTK$^-$ cells and no differences can be seen in the *in vivo* activity of the TC-II/κB and H-2K^b enhansons, nor in their response to TPA and cycloheximide treatments, no conclusions can be drawn as to which protein(s) mediate the activity of these enhansons in these cells. However, the absence of the TC-IIA protein in the nuclear extracts of untreated F9(ND) cells (see above and Macchi *et al.*, 1989) suggests that the TC-IIB protein may mediate the activity of both the TC-II/κB and H-2K^b enhansons. On the other hand, the observation that TPA and cycloheximide treatment of HeLa cells increases the binding activity of the TC-IIA protein (but not that of the TC-IIB protein; see Macchi *et al.*, 1989) and the TC-II/κB enhanson activity to similar extents, suggests that the TC-IIA protein mediates the activity of this enhanson in these cells. Moreover the observation that both the TC-IIA and TC-IIB proteins are present in nuclear extracts of untreated HeLa cells, while the H-2K^b enhanson is inactive, suggests that, in contrast to the untreated F9(ND) cell case, the TC-IIB protein may be inactive in HeLa cells, and that the TC-IIA protein can mediate the activity of the TC-II enhanson, but not that of the H-2K^b enhanson. The induction of the H-2K^b enhanson activity in HeLa cells by TPA and cycloheximide treatment may then reflect a necessary activation of TC-IIB protein either by direct modification (e.g. phosphorylation of the TC-IIB protein itself) or by dissociation of an inhibitor protein.

Our results therefore suggest that the TC-IIB/KBF1/H2TF1-like protein can activate both TC-II/κB and H-2K^b enhansons, whereas the TC-IIA/NFκB like protein can activate only the TC-II/κB enhanson. Moreover, it appears that the activities of both proteins could be 'repressed' by inhibitor proteins exhibiting cell specificity. However, we cannot exclude at the present time more complex schemes in which the ability of the TC-IIA/NFκB-like protein to activate the H-2K^b enhanson would itself be controlled, e.g. by a distinct inhibitor protein. Cloning and expression of the genes encoding the TC-IIA/NF-κB and TC-IIB/KBF1/H2TF1 proteins will be required to elucidate these points.

The AP-2 binding sites in the SV40 and metallothionein hMTIIA enhancers do not exhibit enhanson activity

The inactivity of the TCII + I M1 (Figure 2A and E) tetramer, in which the AP-2 binding site present in the TC region of the SV40 enhancer is intact, shows that this AP-2 binding site has no proto-enhancer activity in any of the cell types studied here. Similarly the AP-2 binding site cannot cooperate with the GT-I enhanson, since the mutations M1 and M3 in GTI + TCII + I (both of which leave these sites intact) lead to a complete loss of enhancer activity (Figure 5). These results are in agreement with those obtained with the total SV40 enhancer, since mutations pA16 to pA19 (Zenke *et al.*, 1986; Nomiyama *et al.*, 1987; see Figure 5) which affect specifically the AP-2 binding sites (see Imagawa *et al.*, 1987; Mitchell *et al.*, 1987) have no effect on enhancer activity *in vivo*. Thus, while the AP-2 protein can moderately stimulate transcription *in vitro* from the SV40 AP-2 site (Mitchell *et al.*, 1987), this does not appear to be the case *in vivo*.

At present we do not understand the reason for the discrepancy between our results and those of Imagawa *et al.* (1987), who found that an oligomer of the hMTIIA AP-2 site could activate transcription *in vivo* in HeLa cells and act as a TPA responsive element. In the experiments described here, using oligomers analogous to those used by Imagawa *et al.* (1987), neither the SV40 nor the hMTIIA AP-2 sites activated transcription in the presence or absence of TPA. Even using the same HeLa tk$^-$ cells as those used by Imagawa *et al.* (1987), we observed no enhanson activity of the AP-2 sites. Thus, while over expression of the AP-2 protein can stimulate transcription from the hMTIIA site in

Drosophila Schneider cells (Williams *et al.*, 1988), both the SV40 and hMTIIA AP-2 sites do not appear to act as enhansons in the mammalian cells tested here.

Materials and methods

Recombinant plasmids

Complementary synthetic oligonucelotides containing the various sequence motifs were phosphorylated with T4 polynucleotide kinase, annealed and ligated into tandem repeats by virtue of the presence of an asymetric *Ava*I restriction enzyme recognition site at each end. For some constructions an alternative system using oligonucleotides with a *Bam*HI site at one extremity and a *Bgl*II site at the other was used. During the polymerization step, the *Bam*HI and *Bgl*II enzymes were added to prevent stable ligation of the *Bam*HI/*Bam*HI and *Bgl*II/*Bgl*II cohesive ends, thus favouring the tandum repeat organization. In each case the ligation products were separated on a 5% polyacrylamide gel, the tetramic fragments excised, and cloned into the *Bam*HI site in pUC19 or the *Ava*I site in vector pV2 (Fromental *et al.*, 1988). DNAs were transformed into *Escherichia coli* strain DH5 and in all cases the DNA sequence of the double stranded DNA from mini-preparations of insert containing clones was verified by dideoxynucleotide sequencing (Sanger *et al.*, 1977) using the universal primer. The inserts from recombinants containing the correct DNA sequence were excised using *Xba*I and *Sst*I and ligated into *Xba*I−*Sst*I digested pG1 or pG2 (Sassone-Corsi *et al.*, 1985; Hen *et al.*, 1986; Fromental *et al.*, 1988). Large scale plasmid preparation and purification of supercoiled DNA by two rounds of CsCl gradient centrifugation were as previously described (Zenke *et al.*, 1986). The DNA sequence of the insertions in pG1 and pG2 was rechecked before transfection.

Cell lines

HeLa cells were grown as described (Zenke *et al.*, 1986). MPC11, LMTK$^-$ and F9 cells were grown in Dulbecco's modified Eagle's minimal essential medium (GIBCO) containing 10% fetal calf serum and 500 units penicillin, 400 μg gentamycin and 100 μg streptomycin per ml. F9(D) cells were obtained by treatment of F9(ND) cells with retinoic acid (0.1 μM) for 4 days before transfection (Nomiyama *et al.*, 1987).

DNA transfection and quantitative S1 nuclease RNA analysis

Calcium phosphate mediated transfections of all five cell lines included the pA56 reference plasmid (Zenke *et al.*, 1986; Fromental *et al.*, 1988), the appropriate derivatives of pG1 or pG2, and plasmid carrier DNA to adjust the total quantity to 18 μg/9 cm dish. HeLa, LMTK$^-$ and F9(ND) cells were seeded 6−10 h before transfection and transfected at 50−60% confluency. The MPC11 cells were seeded at 40−50% confluency 20−24 h before transfection to allow attachment to the plates. TPA and cycloheximide (CHX) treatments were performed essentially as described by Imagawa *et al.* (1987) and Wan *et al.* (1987). HeLa and LMTK$^-$ cells were incubated with the calcium phosphate coprecipitate for 14 h, after which they were washed, and incubated in mediun containing 0.5% fetal calf serum (FCS) for 24 h. The cells kept in 0.5% FCS, were treated with 100 ng/ml TPA or 35 μg/ml CHX for 4 h, and total cellular RNA was harvested. The MPC11 and F9 cells were incubated in10% FCS supplemented medium with the DNA precipitate for 24 h, before washing and adding 100 ng/ml TPA for 16 h, or 35 μg/ml cycloheximide for 4 h before harvesting the RNA. Note that TPA treatment or MPC11 or F9 cells grown in low serum gave results similar to those obtained when the cells were grown in high serum. High serum conditions were thus used since cell growth was much better than in low serum.

Cytoplasmic RNA was prepared by lysing the cells with 0.5% Nonidet P40 (Groudine *et al.*, 1981) and hybridized overnight with an excess of single stranded ^{32}P-5′-end-labelled probe (Zenke *et al.*, 1986) at 68°C in10 mM PIPES (pH 6.5) and 400 mM NaCl. After S1 nuclease digestion [200 units (Appligene) per 64 μg of RNA at 25°C for 2 h; see legends to figures for quantities fo RNA analysed], the protected fragments were subjected to electrophoresis on an 8% denaturing polyacrylamide gel. Quantitation was performed by densitometric scanning of autoradiograms corresponding to different exposures of the same gel and an average value (to within 15%) from at least three independent experiments with at least two different preparations of plasmid was determined. The intensity of transcription signals from the internal enhancerless reference plasmid pA56 [as reflected by transcription from the EES of the SV40 early promoter] was used to correct for variations in transfection efficiency.

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