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Molecular cloning and sequence analysis of the promoter region of mouse cyclin D1 gene: implication in phorbol ester-induced tumour promotion

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Abstract. Cyclin D1 is a cell cycle regulatory protein, which acts as a growth factor sensor to integrate extracellular signals with the cell cycle machinery, particularly during G1 phase of the cell cycle. Previous study using promotion-sensitive JB6 mouse epidermal cells, an *in vitro* model of the promotion stage of multistage carcinogenesis, showed that the expression of cyclin D1 is stimulated in the presence (but not in the absence) of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in these cells maintained under anchorage-independent culture conditions. In the present study, to explore the molecular basis of this observation, the promoter region of mouse cyclin D1 gene was cloned and sequenced (GenBank accession number AF212040). Dot matrix comparison of mouse, human and rat promoter sequences indicated that the mouse promoter is homologous to the human and more so to the rat promoters. The mouse promoter, like human and rat promoters, lacks canonical TATA-box or TATA-like sequence, but it has one or possibly two initiator (Inr) or Inr-like sequences. Energy dot plot analysis predicted that the mouse promoter consists of three domains: (1) the 3′ domain contains NF-κB response element, cAMP-response element (CRE), Inr or Inr-like elements, Sp1 binding site and Oct 1 (2) the middle domain contains another Sp1 binding site, E-box and E2F binding site and (3) the 5′ domain contains TPA-response element (TRE) and a tandem silencer element. The cyclin D1 promoter sequence of either promotion-sensitive or resistant JB6 mouse epidermal cells was, except for a few minor differences, essentially identical to the sequence determined for a mouse genomic clone. Since TPA is capable of stimulating the expression of cyclin D1 not only through TRE but also through CRE and NF-κB response element in the promoter, we tentatively propose a sequence of events that possibly leads to TPA-induced, anchorage-independent synthesis of cyclins D1 and A in the promotion-sensitive JB6 mouse epidermal cells.

INTRODUCTION

Cyclin D1 plays an important role in regulating the progress of the cell during the G1 phase of the cell cycle. It acts as a growth factor sensor to integrate extracellular signals with the

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cell cycle machinery (for a review article, see for example Sherr 1996). Cyclin D1 has been found to be frequently overexpressed in a variety of human cancers (Barnes & Gillett 1998; Donnellan & Chetty 1998; Hosokawa & Arnold 1998) and during chemically induced multistage carcinogenesis in a variety of rodent systems including mouse skin (Bianchi *et al.* 1993; Robles & Conti 1995; Zhang *et al.* 1997). Mechanistic studies have suggested that cyclin D1 may play a critical role in carcinogenesis (Weinberg 1996; Weinstein *et al.* 1997; Frame *et al.* 1998; Rodriguez-Puebla, Robles & Conti, 1999).

The process of carcinogenesis has been divided into three operationally distinct stages: initiation, promotion and progression (for a review article, see Sirica 1996). The mouse model of multistage skin carcinogenesis has for many years served as the experimental prototype for demonstrating such stages. The initiation stage reflects a permanent and irreversible change in the initiated cells. The promotion stage is characterized by the selective proliferation of initiated cells resulting in their clonal expansion. The two most distinguishing characteristics of promotion stage are the operational reversibility and the fact that promoting agents exhibit a threshold effect.

The initiated, promotion-sensitive JB6 mouse epidermal cells in culture have been serving as an *in vitro* paradigm to complement the *in vivo* model of the promotion phase of multistage mouse skin carcinogenesis (Colburn *et al.* 1978, 1979; Colburn, Koehler & Nelson, 1980). JB6 cells constitute a series of genetic variants that include promotion-sensitive (*P+*), promotion-resistant (*P–*) and transformed (Tx) cells. In *P+* (but not in *P–*) cells, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces the formation of tumorigenic anchorage-independent colonies in soft agar at high frequency. The most noteworthy difference between *P+* and *P–* cells involves activator protein-1 (AP-1) (Bernstein & Colburn 1989; Bernstein *et al.* 1991). The *P–* cells express a low level of mitogen-activated protein kinase (MAPK) (extracellular signal-regulated protein kinase, ERK) which contributes to the lack of AP-1 activation and cell transformation responses to TPA in these cells (Huang *et al.* 1998).

Previous study of expression of cyclins (A, B1, D1 and E) and p27Kip1 in these cells maintained under anchorage-independent culture conditions (Eto 1998) showed that (a) the expression of cyclin D1 is stimulated in the presence (but not in the absence) of TPA in *P+* cells and (b) the TPA-induced expression of cyclin D1 is significantly reduced in *P–* cells. A straightforward interpretation of these results is that the promoter region of mouse cyclin D1 gene contains TRE (TPA-response element) that binds AP-1 and thereby stimulates the expression of cyclin D1. In fact, human cyclin D1 gene is known to contain a functional TRE in its promoter (Albanese *et al.* 1999).

In this study, the promoter region of mouse cyclin D1 gene was cloned and sequenced (GenBank accession number AF212040). Analysis of the sequence revealed a TRE stie in its promoter. In addition to TRE, it also contains a CRE (cAMP-response element) and an NF-κB response element. Since TPA is able to stimulate cyclin D1 promoter not only through TRE but also through the latter two elements, this might provide an explanation as to why the TPA-induced expression of cyclin D1 is significantly reduced, but not completely eliminated, in *P–* cells.

MATERIALS AND METHODS

Library screening

Approximately 4×10^6 plaque-forming units (pfu) of a mouse genomic library 129SV (containing 2.0×10^6 primary plaques) in λ FIX II vector (Stratagene, La Jolla, CA, USA) were screened using a nonradioactive PCR-based method (Amaravadi & King 1994). The primer

sequences used were as follows: sense (30-mer), 5′-CCTCCAGAGGGCTGTCGGCG CAGTAGCAGA – 3′; antisense (30-mer), 5′-TCTTACCTCCAGCATCCAGGTGGCCAC GAT – 3′. This primer set covers entire 5 untranslated region and coding sequence of mouse cyclin D1 exon 1 (Smith, Peters & Dickson, 1995). The 5′ and 3′ flanking sequences of the exon 1 were included (underlined) in the sense and antisense primers, respectively, to enhance the specificity of the PCR product. The expected size of the product was 348 bp.

In the primary screening, 20 150-mm plates were used and approximately 200 000 plaque-forming units (pfu) were plated on each plate using XL1-Blue MRA (P2) as host *E. coli*. Plates were incubated until plaques began to contact each other (6–8 h). To soak phage from the plates, SM buffer (10 ml) was added to each plate. The plates were then placed on a slowly rotating shaker and incubated overnight at 4 °C. One milliliter of the SM buffer was collected from each plate and centrifuged at 5000 × g for 10 min to remove debris. *E. coli* were lysed by the addition of $CHCl_3$ to a final concentration of 0.3% (v/v), and the phage stocks were stored at 4 °C.

A 1-μl aliquot from each phage stock was added to a 12.5-μl PCR master reaction mixture and PCR amplifications were performed on a TwinBlock System (ERICOMP, San Diego, CA) using the following procedure: 35 cycles with a denaturation step at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 60 s, followed by a final extension at 72 °C for 7 min. For each amplification, *Taq* DNA polymerase (0.5 U; Promega Corporation, Madison, WI, USA) was used with 5 pmol of each primer, 0.2 mM of dNTPs and 1.5 mM of $MgCl_2$. An aliquot (8 μl) of each amplification reaction was analysed by electrophoresis in a 1.8% agarose gel stained with ethidium bromide.

The positive phage stocks from the primary plates were subjected to additional rounds of phage plating (initially on 150-mm and 100-mm plates and then on 60-mm plates) and analysis by PCR. Final purification of the positive plaques was performed by three rounds of phage plating (on a 100-mm plate), picking individual plaques, soaking each in a 1-ml SM buffer, and analysing by PCR. The purified positive phage stocks were stored at –80 °C in SM buffer containing 7% (v/v) DMSO.

Subcloning and DNA sequencing

A positive recombinant phage recovered after multiple rounds of screening and plaque purification carried an insert (approximately 23 kb in size) containing both exon 1 and promoter region of mouse cyclin D1 gene. This insert was subcloned into pBluescript vectors (Stratagene) as outlined in Fig. 1.

The recombinant λ FIX II phage DNA (λmCD1; see Fig. 1) was prepared by plate lysate method using a combination of λ Quick Spin Kit (BIO 101, Inc., Vista, CA, USA) and QIAGEN Lambda Midi Kit (QIAGEN, Inc., Valencia, CA, USA). Digestion of the entire λmCD1 DNA by *Eco*RI produced three fragments (E1, E2 and E3). E1 was the largest fragment containing λ FIX II phage vector. Of the other two fragments (E2 and E3 with approximately 14–15 kb and 7–8 kb, respectively, in size), E3 carried both exon 1 and the 5′ flanking region immediately upstream of the exon 1.

The fragment E3 was subcloned into *Eco*RI site of the pBluescript KS(+) vector (pmCD1E3; see Fig. 1) using chemically competent One Shot TOP10 cells (Invitrogen, Carlsbad, CA, USA). The cells transformed with pmCD1E3 were identified by ampicillin resistance, blue-white selection with X-gal, direct PCR screening of colonies (Lee & Cooper 1995) and restriction analysis. Direct PCR screening of the colonies was performed using the same PCR protocol described above except the reaction mixture was first incubated at 95 °C for 10 min prior to the standard 35-cycle PCR program.

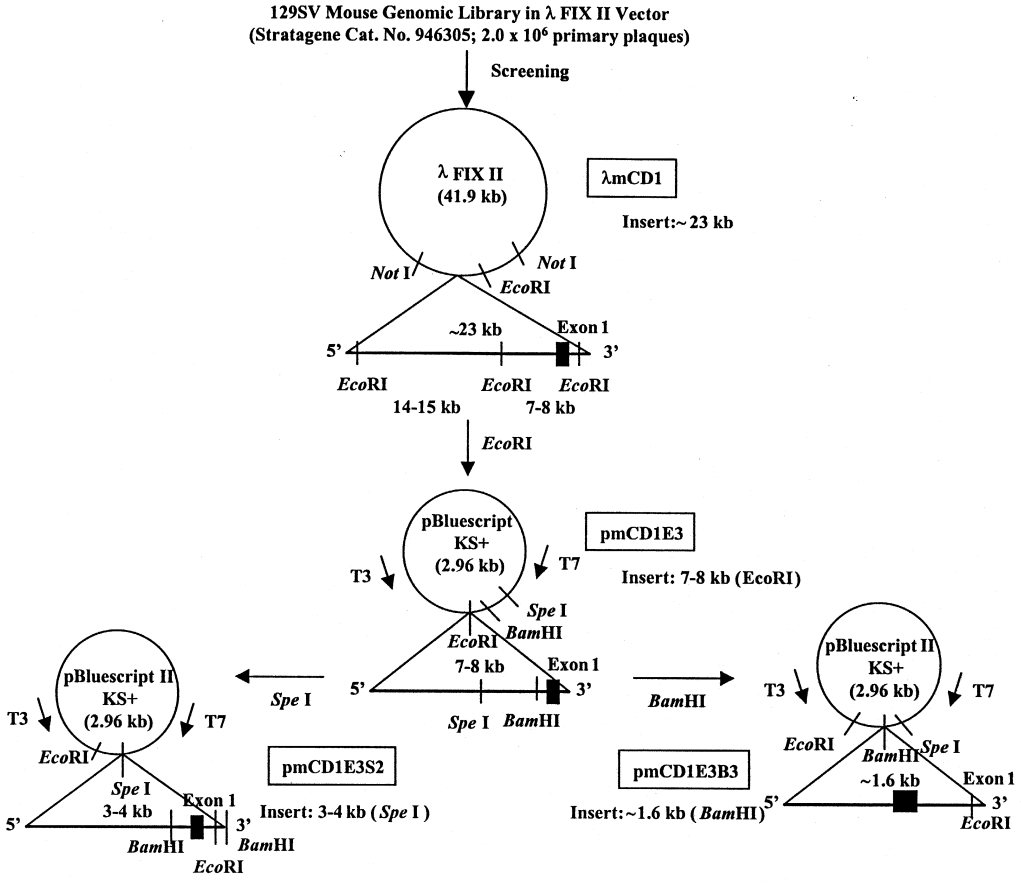

Figure 1. Strategy for screening, isolation and subcloning of mouse cyclin D1 promoter. A mouse genomic library 129SV was screened using a PCR primer set covering the entire 5′ untranslated region and coding sequence of exon 1. The positive phage DNA (λmCD1) was then digested with *Eco*RI and subcloned into pBluescript KS(+) (pmCD1E3). This plasmid was further digested with either *Bam*HI or *Spe* I and subcloned into pBluescript II KS(+) (pmCD1E3B3 and pmCD1E3S2, respectively).

The pmCD1E3 was then digested with either *Bam*HI or *Spe* I. The resulting fragments containing both exon 1 and the promoter region (approximately 1.6 kb in size after *Bam*HI digestion and 3–4 kb after *Spe* I digestion) were subcloned into pBluescript II KS(+) vector (pmCD1E3B3 and pmCD1E3S2, respectively; see Fig. 1).

The plasmid DNAs were isolated using a combination of QIAprep Spin Miniprep Kit and QIAGEN Plasmid Mini Kit (QIAGEN, Inc.) and dissolved in water. The exon 1 and the 5′ flanking region immediately upstream (approximately 1.02 kb in size) of the exon 1 was sequenced for both strands using automated ABI Prism Model 377 DNA sequencer available at the University of Alabama at Birmingham Automated DNA Sequencing Core Facility.

The sequence was submitted to the GenBank database under accession number AF212040.

Computer-aided analysis of DNA sequence

Genetics Computer Group (GCG) Sequence Analysis Programs (Version 10) (Genetics Computer Group, Inc., Madison, WI, USA) were used to analyse the DNA sequence. GCG

provides Web-based (SeqWeb) interface for access to its program suite. Default conditions were used to run all programs.

Two programs (Compare and MFold) were used to analyse the global structure of the DNA sequence. Compare Program was used to compare mouse and human or mouse and rat cyclin D1 promoter sequences and create dot plot graphs that show where the two sequences are similar. A window of 21 bases was used with a stringency of 14. MFold Program was used to analyse the DNA secondary structure of mouse cyclin D1 promoter. Default conditions consisted of the following: folding temperature of 37 °C, colour levels of suboptimality of 1, radius of circle of 45.0, maximum number of structures to display was 25, and plotted foldings differ by a distance of at least 3. The result was displayed graphically as an energy dot plot.

In order to locate and identify various promoter motifs, MotifSearch Program was first run to search the overall consensus segments between mouse and human or rat and human cyclin D1 promoter sequences. Once identified, the overall consensus segments were analysed further by visual inspection and using BestFit Program to locate and align individual consensus motifs.

PCR-TA cloning

The cyclin D1 promoter sequences of promotion-sensitive (*P*+, clone 41.5a) and resistant (*P*–, clone 30.7a) JB6 mouse epidermal cells were determined by PCR-TA cloning method.

The cells were grown as monolayer cultures (using 100-mm dish) as described previously (Eto 1998) in Eagle's Minimum Essential Medium containing 5% heat-inactivated fetal bovine serum, 2% L-glutamine and antibiotics. Genomic DNAs were isolated from these cells using Puregene Genomic DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) and digested with *Eco*RI.

Three sets of primers were used to amplify the cyclin D1 promoter region by PCR: (a) sense (30-mer), 5′-TCTGC CCGGC TTTGA TCTCT GCTTA ACAAC-3′ and antisense (30-mer), 5′-TCTTA CCTCC AGCAT CCAGG TGGCC ACGAT-3′; (b) sense (30-mer), 5′-AAATA ATGGC CACCA TCTTG AGCTG TTGCT-3′ and antisense (27-mer), 5′-GAGTT TTGTT GAAGT TGCAA AGTCC TG-3′ and (c) sense (20-mer), 5′-AATTC TAAAG GTGGG GGAAC-3′ and antisense (27-mer), 5-TTACA CGTGT TGATG AAATT GAAAG AA-3. The expected sizes of the products were 432, 727 and 525 bp, respectively. These three products overlap 81 bp (primer sets a and b) and 229 bp (primer sets b and c) in size and cover entire exon 1 and the 5′ flanking region immediately upstream (approximately 1.02 kb in size) of the exon 1. PCR amplifications were performed using the standard 35-cycle PCR protocol described above.

TA cloning of the PCR products was performed using TOPO TA Cloning Kit (Invitrogen) with pCR®2.1-TOPO as the plasmid vector and chemically competent One Shot TOP10 cells as the host *E. coli*. The linearized TA cloning vector provides 3′ T overhangs with covalently bound topoisomerase I. Screening of the transformed cells and the DNA sequencing of the inserts were performed as described above.

RESULTS

Genomic library screening, subcloning and DNA sequencing

Overall structure of the mouse cyclin D1 gene is shown in Fig. 2. A mouse genomic library 129SV was screened by a nonradioactive PCR method (Amaravadi & King 1994) using a primer set covering the entire 5 untranslated region and coding sequence of exon 1 (Smith *et*

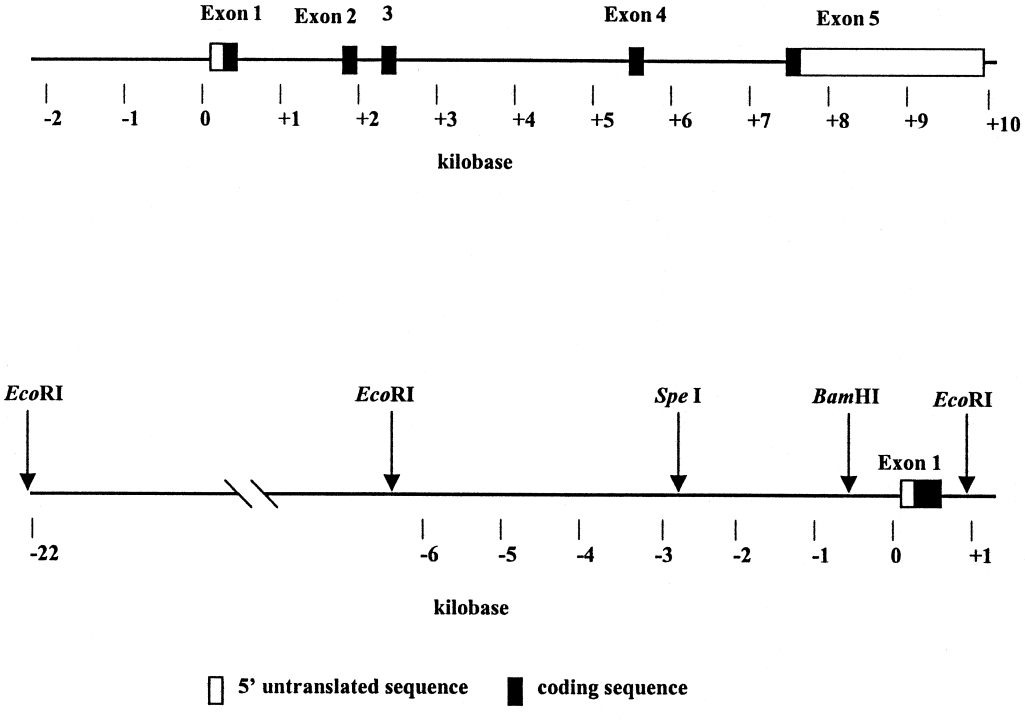

Figure 2. Overall structure of the mouse cyclin D1 gene. The mouse cyclin D1 gene extends for approximately 10 kb and is organized into five exons (Smith *et al.* 1995). The coding sequences are shown as closed rectangles. The first and the last exons contain 5′ and 3′ untranslated regions (open rectangles), respectively. The coordinates of the sites are relative to the putative initiator (Inr) or Inr-like element shown in Figure 3.

al. 1995). Only one positive clone was identified for each 2.0×10^6 primary plaques suggesting that cyclin D1 is a single copy gene. The positive phage DNA was digested with *Eco*RI and analysed by PCR to identify a fragment containing both exon 1 and the 5′ flanking region immediately upstream of exon 1. The positive fragment (approximately 7–8 kb in size) was subcloned into pBluescript KS(+) vector (pmCD1E3; see Fig. 1). The plasmid thus obtained was further digested with either *Bam*HI or *Spe* I and the positive fragments (approximately 1.6 kb and 3–4 kb, respectively, in size) were further subcloned into pBluescript II KS(+) vector (pmCD1E3B3 and pmCD1E3S2, respectively; see Fig. 1). The nucleotide sequence of the 5 flanking region approximately 1150 bp upstream of the translation initiation codon ATG was determined for both strands (GenBank accession number AF212040; Fig. 3).

Dot matrix homology analysis of the promoter sequence

The dot plot graphs of the similarities in sequence between mouse and human (Herber *et al.* 1994; Muller *et al.* 1994; Philipp *et al.* 1994) and mouse and rat (Yan & Ziff 1997; Kitazawa, Kitazawa & Maeda, 1999) cyclin D1 promoters are shown in Fig. 4(a),(b), respectively. A single, but broken stretch of similarity is evident along the diagonal between mouse and human sequences (Fig. 4a) and an almost unbroken stretch is visible between mouse and rat sequences (Fig. 4b). These graphs indicate that the mouse promoter sequence is homologous to the human and more so to the rat sequences.

Figure 3. Nucleotide sequence of the mouse cyclin D1 promoter region. The sequence is numbered relative to the putative initiator (Inr) or Inr-like element corresponding to that of the human promoter (Philipp *et al.* 1994). The ATG start codon is underlined. The GenBank accession number of the sequence is AF212040.

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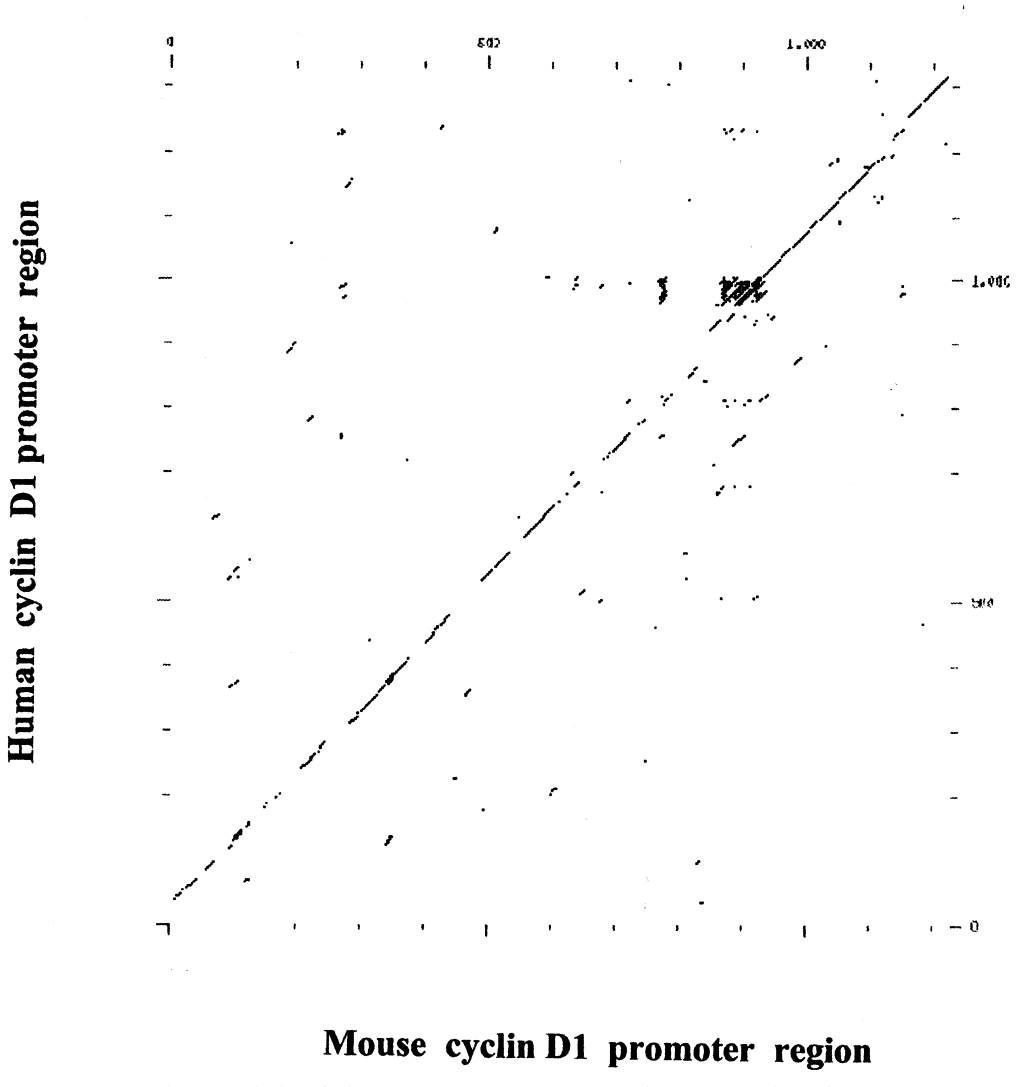

Figure 4. Homology analysis of the promoter sequence. (a) (above) Homology between mouse and human (Herber *et al.* 1994) (GenBank accession number Z29078) cyclin D1 promoter sequences. The dot matrix was generated using Compare Program. A window of 21 bases was used with a stringency of 14. Horizontal and vertical axes represent mouse and human sequences, respectively. Homologous regions are indicated by dots. (b) (overleaf) Homology between mouse and rat (Yan & Ziff 1997) (GenBank accession number AF067056) cyclin D1 promoter sequences. Similar to Figure 4a, but horizontal and vertical axes represent mouse and rat sequences, respectively. Essentially the same plot was obtained with another rat promoter sequence (Kitazawa *et al.* 1999) (GenBank accession number AF148946).

Overall organization of mouse cyclin D1 promoter

Since several descriptions of the organization of human cyclin D1 promoter region are available in the literature (Herber *et al.* 1994; Muller *et al.* 1994; Philipp *et al.* 1994; Fukami-Kobayashi & Mitsui 1998), MotifSearch Program was first run to search the overall consensus segments between human and mouse cyclin D1 promoter sequences. These and other segments

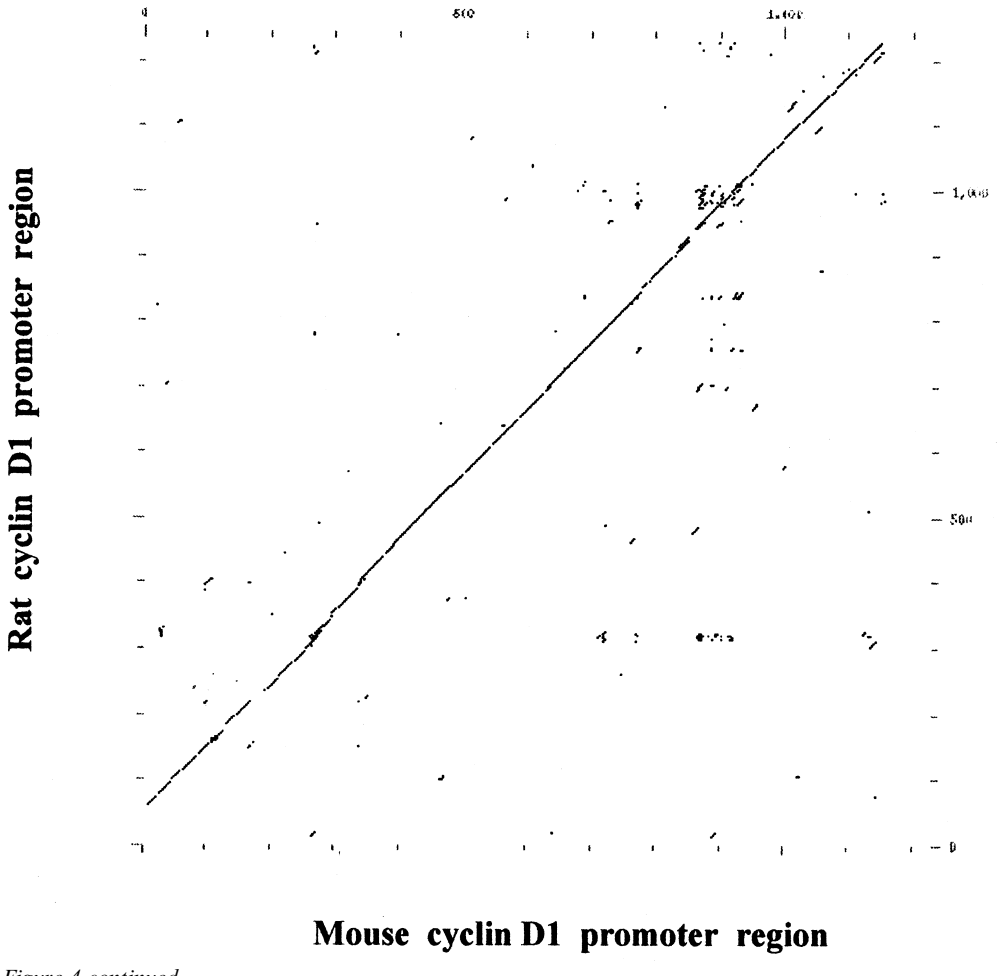

Figure 4 continued

were analysed further by visual inspection and using BestFit Program to locate and align individual consensus promoter motifs. The same strategy was also used to analyse the organization of rat cyclin D1 promoter region (Yan & Ziff 1997; Kitazawa *et al.* 1999).

As Fig. 5 shows, the overall organizations of mouse, rat and human cyclin D1 promoters are fairly similar to each other. All three promoters lack canonical TATA-box or TATA-like sequence, but they contain initiator (Inr) (human) or Inr-like element (mouse and rat). When the nucleotide position + 1 was assigned to this Inr element (Philipp *et al.* 1994; Yan & Ziff 1997), 5′ end of the ATG start codon in exon 1 was positioned at + 233 in mouse and rat and + 251 in human (due to the insertion of additional 18 bp at approximately + 230). The 5′ end of 5′ untranslated sequence of mouse exon 1 was located at + 96 (Smith *et al.* 1995).

Using the same nucleotide coordinate, all three promoters were found to contain NF-κB response element, CRE (cAMP response element) and Inr or Inr-like element at + 57 to + 65, + 38 to + 45 and + 1 to + 8, respectively (Figs 5 and 6b). In addition, all three promoters contain Oct 1, Sp1 and E2F binding sites (Figs 5 and 6c). They also contain TRE (TPA response element or AP-1 binding site) and a tandem silencer element (Figs 5 and 6a).

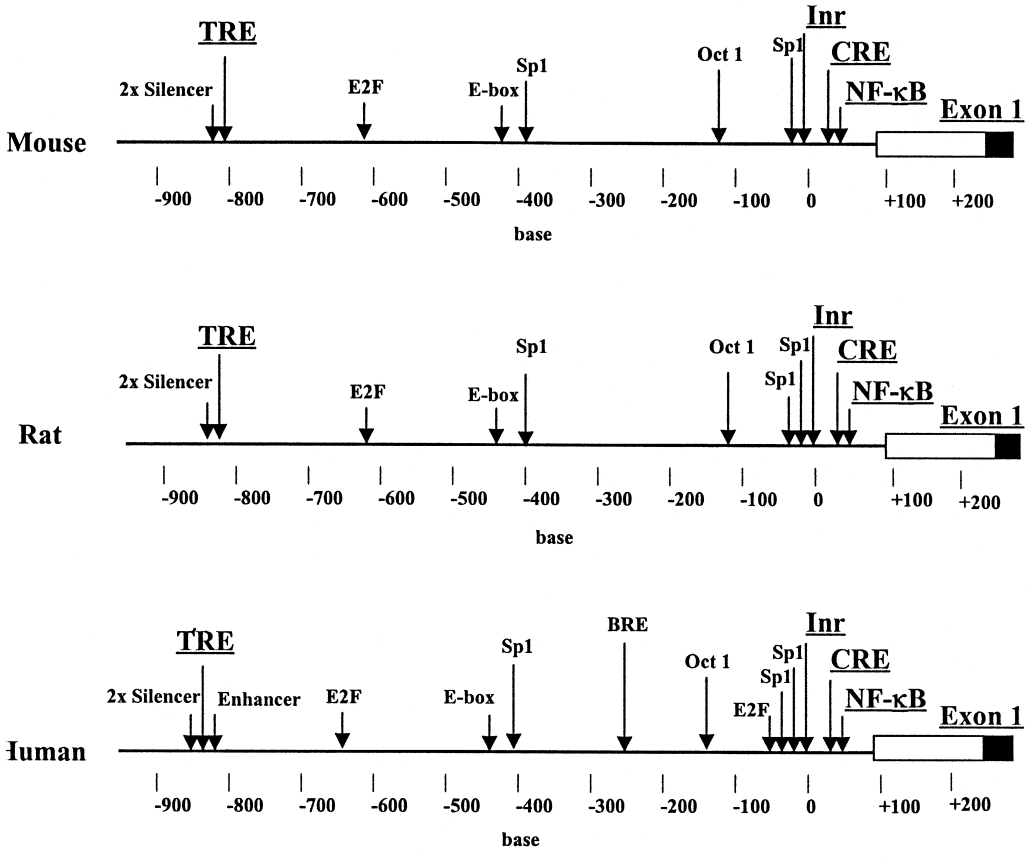

Figure 5. Comparative organizations of mouse, rat and human cyclin D1 promoters. The nucleotide position + 1 is assigned to the putative initiator (Inr) element corresponding to that of the human promoter (Philipp *et al.* 1994). The abbreviations used are: NF-κB, NF-κB response element; CRE, cAMP response element; Inr, initiator element; E2F, E2F binding site; BRE, butyrate response element; TRE, TPA response element (AP-1 binding site).

On the other hand, the BRE (butyrate response element) located at – 242 to – 252 in human promoter (Lallemand *et al.* 1996; Tran *et al.* 1998) could not be identified in either mouse or rat promoter. Also, the enhancer element or SRE (serum response element) located at either – 832 to – 839 (Herber *et al.* 1994) or – 836 to – 843 (Fukami-Kobayashi & Mitsui 1998) in human promoter could not be detected in either mouse or rat promoter (Fig. 5). In addition, E2F binding motif located within approximately – 43 to – 54 in human promoter (Herber *et al.* 1994; Phillipp *et al.* 1994; Fukami-Kobayashi & Mitsui 1998) was not found in mouse or rat promoter (Fig. 5). Finally, rat (Yan & Ziff 1997; Kitazawa *et al.* 1999) and human (Herber *et al.* 1994; Albanese *et al.* 1995; Fukami-Kobayashi & Mitsui 1998; Kim & Miller 1998; Watanabe *et al.* 1998) promoters contain two Sp1-binding sites between – 9 and – 24, but only one Sp1-binding site was identified within this segment in the mouse (Figs 5 and 6c)

Silencer and TRE

As shown in Fig. 6a, the silencer and TRE sequences are located within the most 5′ distal region (nucleotide positions from approximately – 789 to – 877) of all three promoters.

Mouse	-861	GGCGA	CTAAT	AACTT	GCAGC	GATTT	ACTTT	---TT	TTAAT	TAAAA	-820
Rat	-884	GGCAA	CTAAT	ACTTG	CA-GT	GATTT	ACTTT	---TC	TTAAT	TAAAA	-844
Human	-898	GGGGA	CTAAT	ATTTC	CAGCA	-ATTT	AATTT	CTTTT	TTAAT	TAAAA	-855
						TTT	AAT	T	TTAAT		
						Silencer		Silencer			
Mouse	-819	AATAA	ATTAG	G-AAG	GAGCC	TATCG	TGTCT	CAACC	TTTTC	TTCA	-777
Rat	-843	AATAA	ATTAC	ACAAA	GAGCC	TATCG	TGTCT	CAACC	TTTCC	TTCA	-800
Human	-854	AA-AA	-----	-----	-----	-----	TGAGT	CAGAA	TGGAG	ATCA	-832
							TGAGT	CA			
							TRE				

Figure 6 continued overleaf

		-1	+1		+11		+21		+31	
Mouse	-5	CCCCC	TTTTC	TCTGC	CCGGC	TTTGA	TCTCT	GCTTA	ACAAC	+35
Rat	-5	CTCCT	TTTTC	TCTGC	CCGGC	TTTGA	TCTCT	GCTTA	ACAAC	+35
Human	-5	GCTCC	CATTC	TCTGC	CGGGC	TTTGA	TCTTT	GCTTA	ACAAC	+35
			CATTC	TCT						
			Inr (Initiator)							
		AACGT	CATTC	GAATT	CCGTC	R1				
		GCCCT	CATTC	TGGAG	AC	TdT				
		-CACT	CATTC	TCC		N-CAM				
		GTCCT	CACTC	TCTTC	CG	AdML				
		-GCCT	CATTG	CCC		CD44				
		-CGAT	CACCT	TTC		Albumin				
		ACTCT	CACTT	TCTCAC		Vb				
		--CTG	GGTCT	CTCTG		HIV-1				

			+41		+51		+61		+71	
Mouse	+36	AGTAA	CGTCA	CACGG	ACTAC	AGGGG	AGTTT	TGTTG	AAGTT	+75
Rat	+36	AGTAA	CGTCA	CTCGG	ACTAC	AGGGG	AGTTT	TGTTG	AAGTT	+75
Human	+36	AGTAA	CGTCA	CACGG	ACTAC	AGGGG	AGTTT	TGTTG	AAGTT	+75
		TGA	CGTCA			GGGG	ACTTT			
		CRE				NF-κB RE				

			+81		+91		
Mouse	+76	GCAAA	GTCCT	GCAGC	CTCCA	GAGGG	+100
Rat	+76	GCAAA	GTCCT	GCAGC	CTCCA	GAGGG	+100
Human	+76	GCAAA	GTCCT	GGAGC	CTCCA	GAGGG	+100
						5' End of Untranslated Region of Exon 1 (at +96)	

Figure 6 continued opposite

The tandem silencer elements (Herber *et al.* 1994; Fukami-Kobayashi & Mitsui 1998) of mouse (5′-TTTAAT-3′ and 5′-TTTACT-3′), rat (5′-CTTAAT-3′ and 5′-TTTACT-3′) and human (both 5′-TTTAAT-3′) are embedded in a stretch of AT-rich sequence (Fig. 6a). Herber *et al.* (1994) suggested that this unusual structure might produce DNA bending in this region, thereby contributing to its inhibitory effect on transcription. Fukami-Kobayashi & Mitsui (1998) determined the consensus oligonucleotide sequence of this element and identified a 35-kDa protein binding to this site.

In the human promoter, a perfect TRE consensus sequence (5′-TGAGTCA-3′) (Herber *et al.* 1994; Albanese *et al.* 1995; Fukami-Kobayashi & Mitsui 1998; Watanabe *et al.* 1998; Albanese *et al.* 1999) is located immediately downstream (separated by 9 bp) of the tandem silencer element (Fig. 6a). In the mouse and rat promoters, TRE has the nucleotide sequence 5′-TGTCTCA-3′ and is located approximately 30 bp downstream of the silencer sequence. The AP-1 (activating protein-1) family of transcription factors consists of homodimers and heterodimers of Jun (v-Jun, c-Jun, JunB, JunD), Fos (v-Fos, c-Fos, FosB, Fra1, Fra2) or activating transcription factor (ATF2, ATF3/LRF1, B-ATF) bZIP (basic leucine zipper) proteins (Karin, Liu & Zandi, 1997). Jun-Jun and Jun-Fos dimers preferentially bind to the TRE. Albanese *et al.* (1999) has recently reported that the coactivator protein p300 activates the human cyclin D1 promoter through this site and that it was identified within a DNA-bound complex with c-Jun at this site.

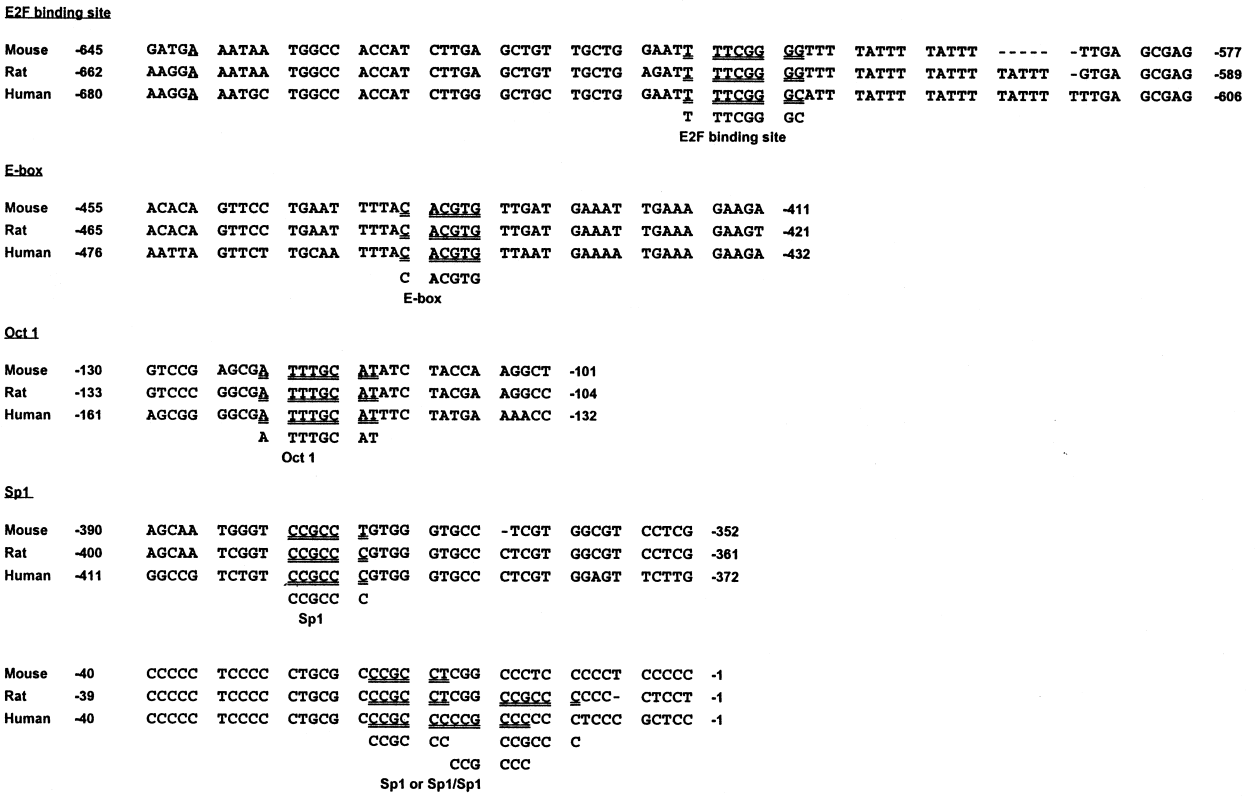

Figure 6. Alignment of (a) tandem silencer and TRE sequences (b) Inr, CRE and NF-κB response elements and (c) E2F, E-box, Oct 1 and Sp1. The consensus oligonucleotide sequences are underlined. Alignment was performed by visual inspection and using BestFit Program. The abbreviations used and the articles referenced for Inr element are: R1, mouse ribonucleotide reductase R1 (Johansson, Skogman & Thelander, 1995); TdT, mouse terminal deoxynucleotidyl transferase (Smale & Baltimore 1989; Roy *et al.* 1991; Garraway, Semple & Smale, 1996); N-CAM, mouse neural cell adhesion molecule (Akeson & Bernards 1990; Hirsch *et al.* **1990; Li** *et al.* 1994); AdML, adenovirus major late protein (Du *et al.* 1993; Marmillot & Scovell 1998); CD44 (Shtivelman & Bishop 1991); albumin (Li *et al.* 1994); mouse T-lymphocyte receptor Vb (Manzano-Winkler, Novina & Roy, 1996; Cheriyath, Novina & Roy, 1998); HIV-1, human immunodeficiency virus-1 (Roy *et al.* 1991; Weis & Reinberg 1992; Du *et al.* 1993).

Inr or Inr-like sequence, CRE and NF-κB response element

These three sites are clustered together within the most 3′ proximal region (nucleotide positions from approximately + 1 to + 65) of all three promoters (Fig. 6b).

The Inr sequence of human promoter has the sequence 5′-CATTCTCT-3′ (Phillipp *et al*. 1994). As shown in Fig. 6(b), this sequence is very similar to those from several other genes. The corresponding Inr sequence of the mouse and rat promoters (5′-TTTTCTCT-3′) does not contain CA at the 5′ end. Rather it contains TT in place of CA. This makes the mouse and rat Inr sequences somewhat different from that of the human promoter.

The CRE is located at nucleotide positions from + 38 to + 45 in all three promoters and has the same sequence 5′-TAACGTCA-3′ (Fig. 6b). The CRE-binding protein complexes include not only CREB (CRE-binding protein) but also AP-1 complex proteins such as Jun-ATF dimers, ATF homodimers or CREB-ATF dimers (Karin *et al*. 1997). cAMP and AP-1 appear to exert opposing effects on the expression of cyclin D1. Elevated levels of cAMP tend to downregulate cyclin D1 expression (L'Allemain *et al*. 1997; Cospedal, Lobo & Zachary, 1999; Fukumoto *et al*. 1999; Gagelin *et al*. 1999; Kronemann *et al*. 1999; Musa *et al*. 1999), whereas AP-1 complexes tend to upregulate it (Herber *et al*. 1994; Beier *et al*. 1999; Sabbah *et al*. 1999).

NF-κB response element is one of the latest motifs to be identified in the human cyclin D1 promoter (Guttridge *et al*. 1999; Hinz *et al*. 1999; Joyce *et al*. 1999). As Fig. 6b shows, in all three promoters, the NF-κB response element is located at nucleotide positions from + 57 to + 65 and has the same sequence 5′-GGGGAGTTT-3′.

Energy dot plot analysis of mouse cyclin D1 promoter

MFold Program (Zuker 1989; Jacobson & Zuker 1993; Zuker & Jacobson 1995) was used to predict secondary structure of mouse cyclin D1 promoter. This program produces a two-dimensional triangular energy dot plot in which predicted base-pairs are plotted as points. The energy dot plot is a useful representation of the folding or global domain structure of large DNAs or RNAs.

The plot (Fig. 7) shows three triangular regions above the diagonal, corresponding to base pairs within each of the following three domains: (a) nucleotide positions downstream of – 280 (b) from – 280 to – 700, and (c) upstream of – 700. The rectangles above and to the right of the middle triangle (the second domain) contain fewer dots, which means that base pairing between the three domains is less likely. Alternative structures are more likely to occur from alternative foldings within these domains.

Promotion-sensitive and resistant JB6 mouse epidermal cells

The cyclin D1 promoter regions of promotion-sensitive (*P*+ , clone 41.5a) and resistant (*P*–, clone 30.7a) JB6 cells were cloned using PCR-TA method. The three sets of PCR primers were designed to amplify the nucleotide positions from + 431 to – 937 with overlaps of 81 bp and 229 bp in size.

DNA sequence analysis of the clones indicated that, except for a few minor differences in the nucleotide sequence, mostly within the nucleotide positions from – 56 to – 85, the cyclin D1 promoters of both promotion-sensitive and resistant JB6 mouse epidermal cells (data not shown) were identical to the sequence determined for a clone isolated from 129SV mouse genomic library (Fig. 3).

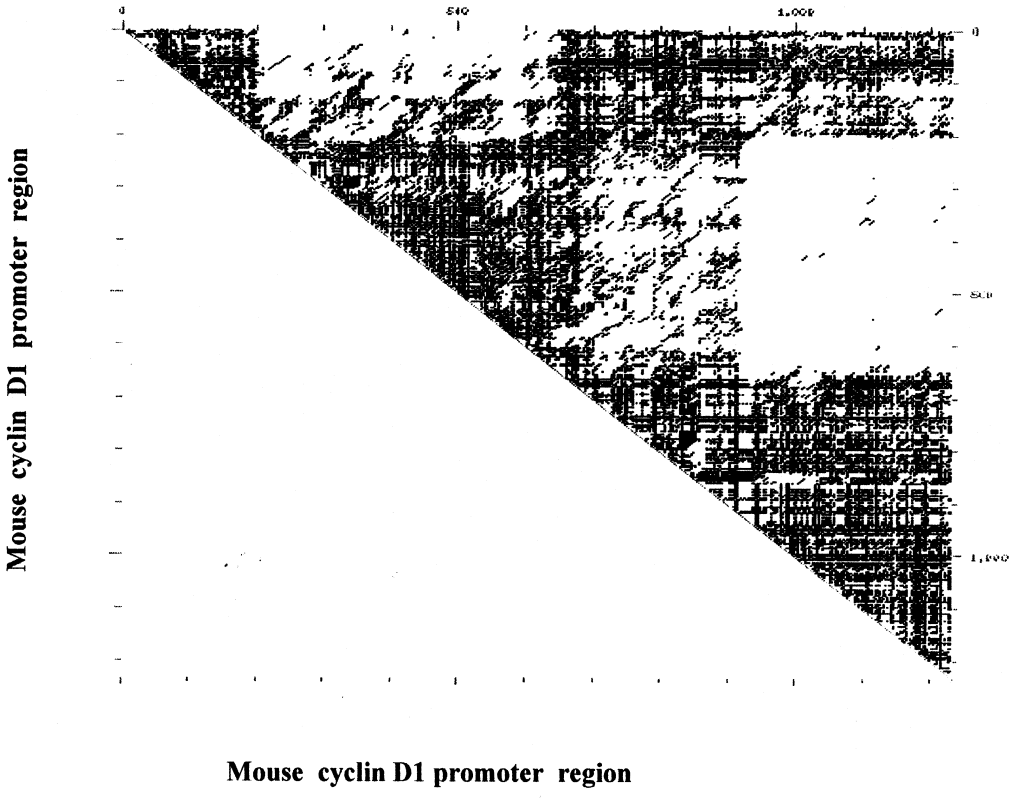

Figure 7. Energy dot plot of mouse cyclin D1 promoter region. MFold Program (under the default conditions as described in the Materials and Methods section) was used to generate this plot. Predicted optimal base pairs are plotted as black dots. The dots are evident only in the upper right triangle of the plot.

DISCUSSION

Comparative organizations of mouse, rat and human cyclin D1 promoters

The results show that all three promoters have similar organizations – at least within 1.15 kb immediately upstream of the ATG start codon in exon 1. In this region, the same promoter motifs are located at more or less comparable nucleotide positions in all three promoters. For example, using the three domain structure predicted by the energy dot plot analysis and assigning the nucleotide position + 1 to the Inr or Inr-like element, the most 3′ domain (nucleotide positions downstream of – 280) contains NF-κB response element, CRE, Inr or Inr-like elements, Sp1 binding site or sites and Oct 1. The middle domain (nucleotide positions between – 280 and – 700) contains an additional Sp1 binding site, E-box and E2F binding sequence. The most 5′ domain (nucleotide positions upstream of – 700) contains TRE and a tandem silencer element.

The results also show that the cyclin D1 promoter sequences of promotion-sensitive and resistant JB6 mouse epidermal cells are essentially identical to the sequence determined for a mouse genomic clone.

Inr or Inr-like sequences

The core promoter region of typical eukaryotic messenger RNA coding genes contains the minimal promoter elements required for initiation by RNA polymerase II. It consists

predominantly of two elements: the TATA box and/or the Inr (initiator) element, which can be present either alternately ($TATA^{+}Inr$ or $TATA^{-}Inr^{+}$) or in limited cases simultaneously ($TATA^{+}Inr^{+}$) (Roy *et al.* 1993a, 1993b). The cyclin D1 promoters of mouse, rat and human are $TATA^{-}Inr^{+}$. They contain Inr or Inr-like sequences, but, like those of many of the genes encoding the so-called 'housekeeping' enzymes, oncogenes, growth factors and their receptors, and transcription factors (Azizkhan *et al.* 1993), they do not contain a canonical TATA box.

The Inr of human cyclin D1 promoter has the sequence 5′-GCTCC C^{+1}ATTC TCTGC-3′ (nucleotide positions from – 5 to + 10). By primer extension analysis and alignment of Inr sequences from several other genes, Philipp *et al.* (1994) placed the nucleotide position + 1 at C^{+1}. The consensus, however, has been to place the nucleotide position + 1 at A^{+2}. For example, the mutant and computer analyses have defined preferred Inr sequence as $PyPyA^{+1}N(T/A)PyPy$ (where Py is T or C) with an A at + 1, a T or A at + 3 and a pyrimidine (Py) at – 1 as most critical for determining the strength of the Inr (Javahery *et al.* 1994; Kaufman & Smale 1994). Many Inr-containing genes appear to conform to this consensus motif with the nucleotide A assigned as the nucleotide position + 1, but there are exceptions. For example, in mouse fibulin-2 gene, the nucleotide position + 1 was assigned to two bases upstream of the nucleotide A (Grassel *et al.* 1999).

The Inr elements of mouse and rat promoters have the sequences 5′-CCCCC T^{+1}TTTC TCTGC-3′ and 5′-CTCCT T^{+1}TTC TCTGC-3′, respectively. These sequences are almost identical to that of human Inr, but they contain T^{+1} rather than C^{+1} and they lack the critical nucleotide A^{+2}. It should be noted, however, that the Inr of human immunodeficiency virus-1 (Roy *et al.* 1991; Weis & Reinberg 1992; Du, Roy & Roeder, 1993), mouse (but not human) KDR/flk-1 (Wu & Patterson 1999) and mouse c-*mos* (Pal *et al.* 1991) also do not contain the critical nucleotide A.

An alternative or possibly an additional Inr site was identified at nucleotide position + 90 in human cyclin D1 promoter by Herber *et al.* (1994) using RNase protection assay. This Inr element has the sequence 5′-AGC^{+90}CTCCAGAGGG-3′ (nucleotide positions from + 88 to + 100). The four nucleotides immediately 3′ to the nucleotide position + 90 (5′-CTCC-3′) conform to the consensus Inr sequence 5′-N(T/A)PyPy-3′. The mouse and rat promoters also have sequences identical to that of the human promoter. The nucleotide position + 90 is very close to the nucleotide position + 96, which was assigned as the 5′ end of the 5′ untranslated region (5′-UTR) of mouse cyclin D1 exon 1 (Smith *et al.* 1995).

TRE, CRE, NF-κB response element: implication in phorbol ester-induced tumour promotion

The mouse, rat and human cyclin D1 promoters contain three elements (TRE, CRE and NF-κB response element) that are known to respond positively to the tumour promoter TPA. TPA may use all three elements to stimulate the expression of cyclin D1. To inhibit the expression of cyclin D completely, all three elements may need to be blocked because, otherwise, some elements may still be available for stimulation. One example bears noting in this respect. The promotion-resistant JB6 mouse epidermal cells express a low level of mitogen-activated protein kinase (MAPK) (extracellular signal-regulated protein kinase, ERK) which contributes to the lack of AP-1 activation at TRE and cell transformation responses to TPA in these cells (Huang *et al.* 1998). The results of our previous study (Eto 1998) indicated that, in these cells maintained under anchorage-independent culture conditions, TPA-induced expression of cyclin A was almost completely inhibited and the TPA-induced synthesis of DNA and cell proliferation was completely blocked. However, TPA-induced expression of cyclin D1 was not completely inhibited. TPA was still able to induce significant – albeit attenuated – expression of cyclin D1 in these cells.

Incorporating not only TRE but also NF-κB response element and CRE, we tentatively propose a possible sequence of events that leads to TPA-induced, anchorage-independent synthesis of cyclins D1 and A in the promotion-sensitive JB6 mouse epidermal cells (Fig. 8).

NF-κB response element is the latest element to be identified in the cyclin D1 promoter (Guttridge *et al*. 1999; Hinz *et al*. 1999; Joyce *et al*. 1999). NF-κB has been implicated in the regulation of cell proliferation, transformation, and tumour development. TPA has long been known to stimulate NF-κB transactivation in a variety of cells including JB6 cells (Li *et al*. 1997). In JB6 cells, NF-κB inhibitor efficiently inhibits not only TPA-induced NF-κB transactivation but also cell transformation. TPA appears to stimulate NF-κB transactivation in two ways: (a) TPA participates in the upstream NF-κB pathway and/or (b) TPA produces AP-1 proteins which, in turn, interact with NF-κB factors and bind to the NF-κB response element (Ahmad, Theofanidis & Medford, 1998). Recent discovery of the importance of NF-κB response element in the cyclin D1 promoter activity may provide evidence for a direct link between TPA-induced NF-κB transactivation, G1-to-S-phase transition and anchorage-independent growth of JB6 cells.

CRE appears to upregulate the expression of cyclin D1 when it interacts with AP-1 proteins. For example, Herber *et al*. (1994) reported that the cyclin D1 promoter is inducible by c-Jun and that this induction is mediated predominantly through the cyclin D1 CRE. Also, Beier *et al*. (1999) demonstrated that ATF-2, as a complex with a CRE-binding protein (CREB)/CRE modulator protein, binds to the cyclin D1 CRE and induces cyclin D1 expression. and Sabbah *et al*. (1999) provided evidence that ATF-2/c-Jun heterodimers bind to cyclin D1 CRE and mediate the activation of cyclin D1 promoter by the liganded oestrogen receptor.

Cyclins D1 and A: reversibility and threshold effects of TPA

The two most distinguishing characteristics of the promotion stage of multistage carcinogenesis are its reversibility and the threshold effects of tumour promoters. If we assume that these two characteristics are attributable to the TPA-induced, anchorage-independent expression of cyclins D1 and A, this appears to provide an explanation for some of the observations reported previously (Eto 1998). During the TPA-induced proliferation of promotion-sensitive JB6 cells maintained under anchorage-independent culture conditions, expression of cyclin D1 begins within 12 h after the addition of TPA and the level of cyclin D1 protein increases continuously afterward until it reaches maximum at about 48 h. In contrast, expression of cyclin A is not yet detectable even at 24 h after the addition of TPA. It begins sometime between 24 and 48 h and the level of cyclin A protein reaches maximum at about 48 h. This sequence of events suggests that, under anchorage-independent culture conditions, cyclin A protein cannot be synthesized unless the amount of cyclin D1 protein either reaches or exceeds a certain threshold level.

A corollary of this hypothesis is that, as in the case of the promotion-resistant JB6 cells, even a partial reduction in the expression of cyclin D1 such as inhibiting only one of the TRE, CRE and NF-κB response element may produce a nearly total attenuation of the anchorage-independent expression of cyclin A.

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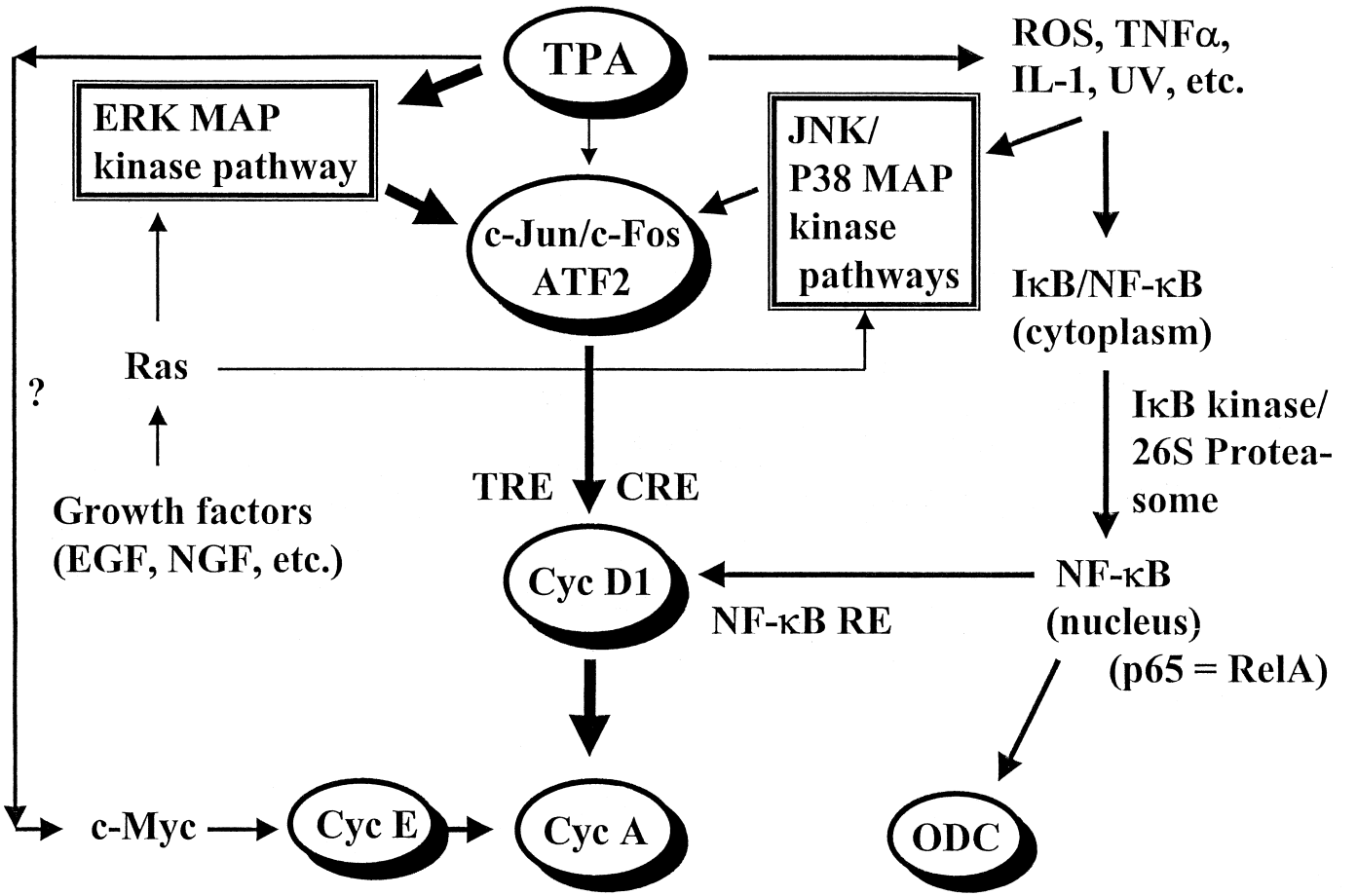

Figure 8. Proposed mechanisms underlying the TPA-induced, anchorage-independent expression of cyclins D1 and A in promotion-sensitive JB6 mouse epidermal cells.

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