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Recognition of the CDEI motif GTCACATG by mouse nuclear proteins and interference with the early development of the mouse embryo

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ABSTRACT

We have reported previously (1) two unexpected consequences of the microinjection into fertilized mouse eggs of a recombinant plasmid designated p12B1, carrying a 343 bp insert of non-repetitive mouse DNA. Injected at very low concentrations, this plasmid could be established as an extrachromosomal genetic element. When injected in greater concentration, an early arrest of embryonic development resulted. In the present work, we have studied this toxic effect in more detail by microinjecting short synthetic oligonucleotides with sequences from the mouse insert. Lethality was associated with the nucleotide sequence GTCACATG, identical with the CDEI element of yeast centromeres. Development of injected embryos was arrested between the one-cell and the early morula stages, with abnormal structures and DNA contents. Electrophoretic mobility shift and DNAse foot-printing assays demonstrated the binding of mouse nuclear protein(s) to the CDEI-like box. Base changes within the CDEI sequence prevented both the toxic effects in embryos and the formation of protein complex *in vitro*, suggesting that protein binding at such sites in chromosomal DNA plays an important role in early development.

INTRODUCTION

Plasmid p12B1 carries a non-repetitive 343 bp sequence from the telomeric region of mouse chromosome 5. This DNA fragment appears responsible for two peculiar properties of the construct. When microinjected into fertilized mouse eggs in low copy numbers (less than 50–100 molecules per egg), it could be maintained as an autonomous genetic element in the somatic tissues and the germ line of transgenic animals (2). On the other hand, injection at the concentration commonly used to generate transgenic animals (*ca* 2,000 molecules per egg (3)) leads to an early arrest of embryonic development (1).

Rosner et al. recently demonstrated that microinjection into one-cell embryos of oligonucleotides carrying the Oct-3 binding site could efficiently arrest development (4). We used the same approach to identify the sequences of p12B1 that are responsible for its toxic effect on embryonic development, by microinjecting double-stranded oligonucleotides with 15–20 bp sequences from the mouse DNA insert. Studies were first focused on a region of the plasmid where a significant similarity with the centromeres of *Saccharomyces cerevisiae* (*CEN* elements) had been previously noticed (2). It contains a completely conserved CDEI box (GTCACATG), followed at a distance of 40 bp by a sequence similar to the central part of the CDEIII element (Figure 1). Our present results indicate that high intracellular concentration of the CDEI motif arrests development between the one-cell and the early morula stages, and that single base substitutions in the sequence abolish this effect. We also report that the same nucleotide sequence is recognized by mouse nuclear protein(s), with a consistent match between the effects of mutations on the binding of protein and on the development of injected embryos.

METHODS

Plasmids and oligonucleotides

Plasmid DNA was prepared in *Escherichia coli* according to standard procedures (5). The plasmid p12B1 is identical with the construct described under the name pCG4 (6). DNA fragments were purified on low-melting point agarose gels and labeled with α-^{32}P-deoxyribonucleotides and DNA polymerase I Klenow fragment. Oligonucleotides were synthesized with the Pharmacia Gene Assembler Plus apparatus and purified by thin layer chromatography. Complementary oligonucleotides were annealed (7) and labeled with polynucleotide kinase and γ-^{32}P-ATP (5). All oligonucleotides used are listed in Table 1.

Microinjection in fertilized mouse eggs

Microinjection of DNA fragments and of chemically synthesized oligonucleotides into fertilized eggs, intra-uterine reimplantation, and embryo cultures were performed essentially as described (3). C57BL/6×DBA2 F1 hybrid parents kept on light cycle from 4 AM to 6 PM were mated overnight. Unless otherwise indicated, fertilized eggs were harvested and injected between 2 and 4 PM the next afternoon (estimated time after fecondation: 15 hrs). Following injection, embryos were either immediately reimplanted into hormonally competent foster mothers, or cultured in M16 medium under 5% CO_2 atmosphere at 37°C.

Cytophotometric analysis of DNA content

Cytophotometric measurement of DNA content after staining the nuclei with Feulgen-Azur was performed with a CAS200 Dual Color Image Analysis System (Becton Dickinson).

Cell extracts

Whole cell extracts were prepared either from mouse C127 cells grown to a density of 10^7 cells/90 mm-plate in Dulbecco modified Eagle's medium supplemented with 10% newborn calf

serum, or from mouse FM3A cells grown in suspension in the same medium. Cells were rinsed twice with phosphate buffered saline, harvested and resuspended in 1 ml of lysis buffer per 10^7 cells (Tris-HCl 50 mM pH 8, NaCl 150 mM, 2% Nonidet P-40, phenylmethylsulfonylfluoride 1 mM). The lysate was kept at 4°C and vortexed every 5 min during 30 min, then centrifuged at 15,000 rpm during 20 min. 200 µl aliquots of the supernatant were frozen at −70°C. Protein concentration estimated by the method of Petterson (8) was in the range of 5 µg/ml.

Nuclear extracts: nuclei were prepared according to ref. 9, and nuclear extracts according to ref. 10. Protein concentration was between 20 and 30 µg/ml.

Heparin Ultrogel chromatography

Crude extracts were loaded onto a 250 ml column of Heparin Agarose (Pharmacia) equilibrated with 50 mM Tris pH 8, 150 mM NaCl, 2% NP40, 1 mM PMSF. The column was washed with 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 5% glycerol. Proteins were eluted by applying a linear gradient of NaCl concentration from 150 mM to 1 M in the same buffer. 4.5 ml fractions were collected and electrophoretic mobility retardation activity assayed.

Analysis of DNA-protein complexes by electrophoretic mobility retardation assay

Binding reactions (25 µl) contained 10 mM Tris-Cl pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, 5 µg poly(dI.dC) heteropolymer and 0.5 ng (15,000 cpm) of ^{32}P end-labeled probe. Protein fractions were added last (1−5 µl). After 15 min at 25°C, samples were loaded onto a 4% (29:1 crosslinked) polyacrylamide gel in Tris-HCl 40 mM, Na-acetate 20 mM, EDTA 1 mM, pH 7.2 and electrophorezed at 12.5 V/cm at 4°C. Gels were fixed for 15 min in 5% acetic acid, 5% methanol, dried at 80°C and exposed on X-ray film (Kodak XAR-5).

Footprint reactions

Standard reaction mixtures contained in a final volume of 20 µl: 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, 300 ng poly(dI.dC), 0.5 ng of labeled DNA fragment and up to 6 µl of nuclear extract. The extract was preincubated with 300 µg of poly(dI.dC) carrier DNA in the reaction mixture at 0°C for 15 min, after which the end-labeled fragment was added and the mixture was incubated for an additional 15 min at 25°C. DNase I (Sigma) freshly diluted in 50 mM $MgCl_2$ was added to give a final concentration of 5 mM $MgCl_2$ and digestion was allowed to proceed for 5 min at 25°C. The amount of DNase I was adjusted empirically for each extract to produce an even pattern of partial cleavage products. Reactions were stopped by the addition of 2 volumes of 50 mM EDTA, 0.2% SDS, 100 µg/ml tRNA, and 100 µg/ml proteinase K. Nucleic acids were extracted with 1 volume of phenol-chloroform (1:1), ethanol-precipitated, dissolved in 99% formamide, 10 mM EDTA with tracking dyes, heated at 90°C for 3 min and loaded on 7% polyacrylamide-7M urea gels.

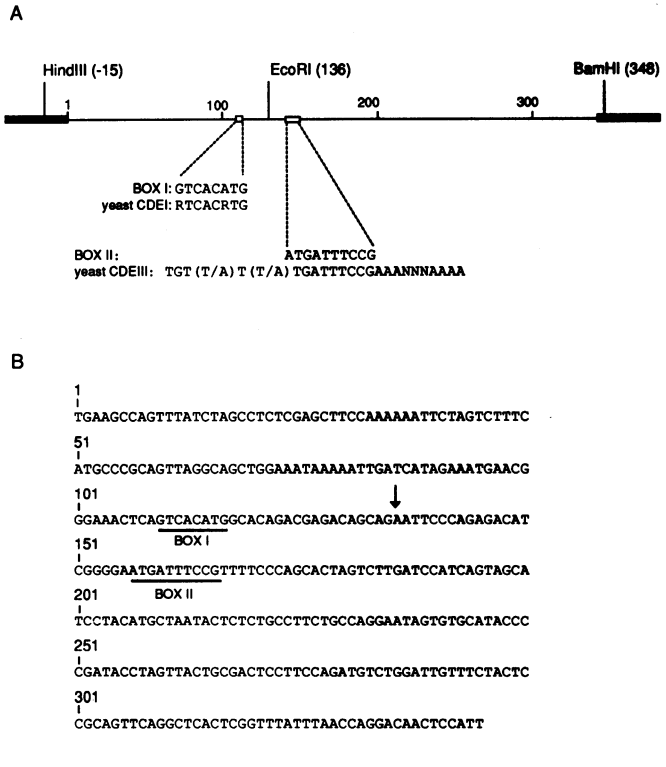

Figure 1. The p12B1 mouse insert is shown in A as a linearized map (thick line: pUC8 vector). Sequences indicated BOXI and II are respectively similar to the CDEI and CDEIII elements of the yeast *CEN*. Their positions are indicated in the complete sequence shown in B (1, 6).

RESULTS

Toxicity of CDEI-like oligonucleotides in fertilized eggs

When microinjected at the concentration commonly used to generate transgenic mice (6×10^{-9} µg per egg (3), corresponding to about 2,000 molecules of a 3 kb DNA fragment), p12B1 DNA prevented the development of the embryos (Table 2). This effect could be reproduced by microinjecting a 20 bp oligonucleotide containing the CDEI box

Table 1. Oligonucleotides used in microinjection and protein-binding experiments

Oligonucleotide[1]	Sequence[2]
PL01	5'-AAACTCAGTCACATG GCACAGAC
PL03	5'-AAACTCA . . . . . GCACAGAC
PL05	5'-GGAAA . . . . GTCACATG GCA
PL11	5'-agcttCTCAGTCACATG GCACA
PL13	5'-agcttCTCA . . . . . GCACA
PL14	5'-agcttCTCAGTCACAcG GCACA
PL15	5'-agcttCTCAGTCACATt GCACA
PL16	5'-agcttCTCAGTCACAgG GCACA
PL17	5'-agcttCTCAGTCACAaG GCACA
PL18	5'-agcttCTCAGTCACATa GCACA
PL19	5'-agcttCTCAGTCAtATG GCACA
PL20	5'-agcttCTCA . . CACATG GCACA
AP1[3]	5'-AAGTGACTCAG CGC
p12B1 insert (nt 101-125)	. . GGAAACTCAGTCACATG GCACAGAC . .

[1] All oligonucleotides were annealed with the complementary strand.

[2] Boldface capital letters: CDEI element; lowercase : a nucleotide that differs from the sequence of p12B1.

[3] Fos-Jun binding site (underlined) of the human *hMT-IIA* enhancer (11).

of the p12B1 insert plus a few nucleotides on each side (PL11, Table 1). Injections were first performed in one-cell embryos about 15 hrs after fecondation (see Methods). Embryos were then maintained in culture in M16 medium for 72 hrs. At that time, while untreated embryos had reached the 'compacted morula' stage (8–16 cells), the majority of the eggs that had received double-stranded PL11 DNA (usually more than 50%) was arrested at the two-cell stage (Table 3, Figure 2). A minority had reached stages between 3 and 8 cells, but with grossly abnormal features (cells of unequal sizes, fragmented cells).

Table 2. Toxicity of p12B1 DNA injected in mouse embryos

Injected DNA	Concentration (µg/ml)[1]	Eggs reimplanted[2]	Births
		10	0
		15	0
p12B1	6	20	0
		20	0
		25	0
		30	0
		8	5
		9	6
p12B1	0.1	10	0
		9	5
		11	7
		10	3
		10	6
pPyLT1[3]	6	12	8
		10	3

[1]ca 1 pl per egg injected into the male pronucleus 15 hrs after fecondation.

[2]8 to 12 eggs usually reimplanted per foster mother (up to 30 in the case of p12B1).

[3]this plasmid carries a modified polyoma virus genome in a bacterial vector (30), and is shown here as a representative example of the range of values obtained with any DNA but p12B1.

Quantitative DNA analysis by cytophotometric analysis after Feulgen-Azur staining showed abnormal and variable DNA contents (Figure 3). No toxic effect was seen in control experiments using DNA with different sequences, such as the oligonucleotide designated AP1 (Table 1). This sequence corresponds to the binding site for the Jun-Fos protein in the *hMT-II$_A$* promoter (11) and exhibits some similarity with the region of p12B1 containing the CDEI box. The minimal requirements for developmental arrest were delineated by experiments performed with a series of mutant oligonucleotides (Tables 3 and 4). No significant toxicity was seen upon injection of oligonucleotides PL03 and PL13, where the CDEI element had been entirely deleted. Mutations at positions 1 and 2, 6, 7 and 8, either abolished or significantly reduced toxicity. Thus the sequence required for toxicity spans from nucleotide 1 to 8 of the CDEI motif.

In these experiments, the majority of the embryos were arrested at the two-cell stage, the time when zygotic transcription starts. In view of the possibility that the injected nucleotide interferes with transcriptional regulation, we asked whether, under different experimental conditions, eggs might be predominantly arrested before the first cleavage. In support of this, we found that when microinjection was performed 9–10 hrs after fecondation instead of the usual 15 hrs, the majority of embryos remained as one-cell zygotes (Figure 2C).

Recognition of the CDEI-like element by mouse protein(s)

The simplest explanation for the observed sequence-specific effects of these short DNA fragments is a competitive inhibition of the binding of a protein to the same sites in chromosomal DNA. We were thus led to search for proteins in mouse cell extracts that would bind to the GTCACATG motif. A CDEI-binding protein has been well characterized in yeast (12–16).

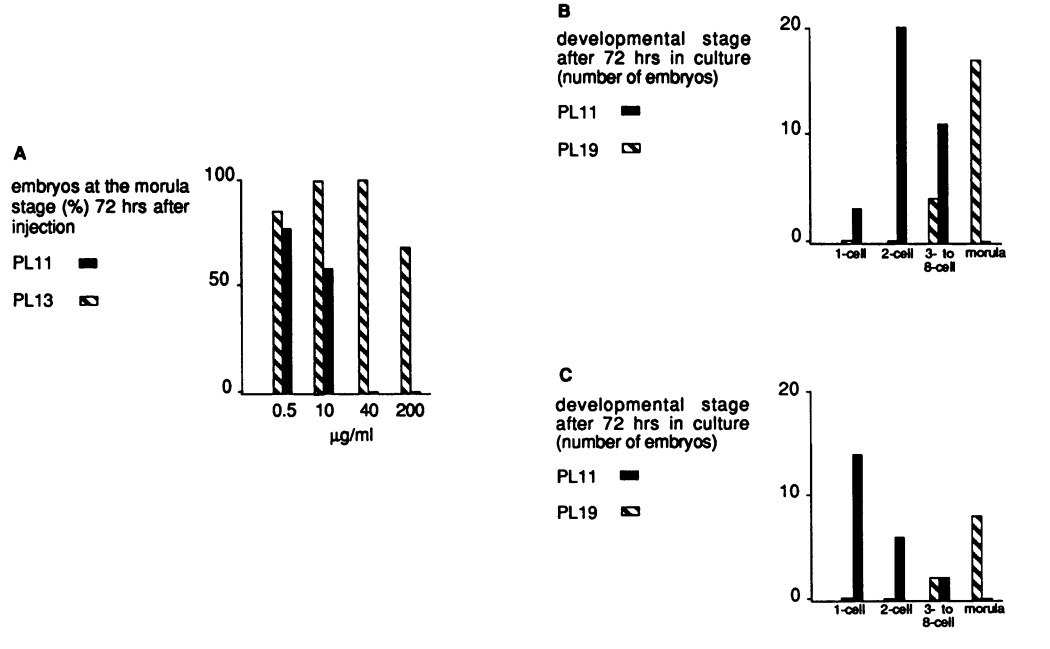

Figure 2. Development is arrested at early stages upon microinjection of the CDEI oligonucleotide PL11, but not of mutants PL13 (complete deletion of the CDEI motif and PL19 (C to T mutation at position 5). Microinjection was performed 15 hrs after fecondation, and embryos were maintained in culture for an additional 72 hrs. Data in A and B are from Table 3. The same experiment is presented in C, except that the time of injection was only 9 hrs.

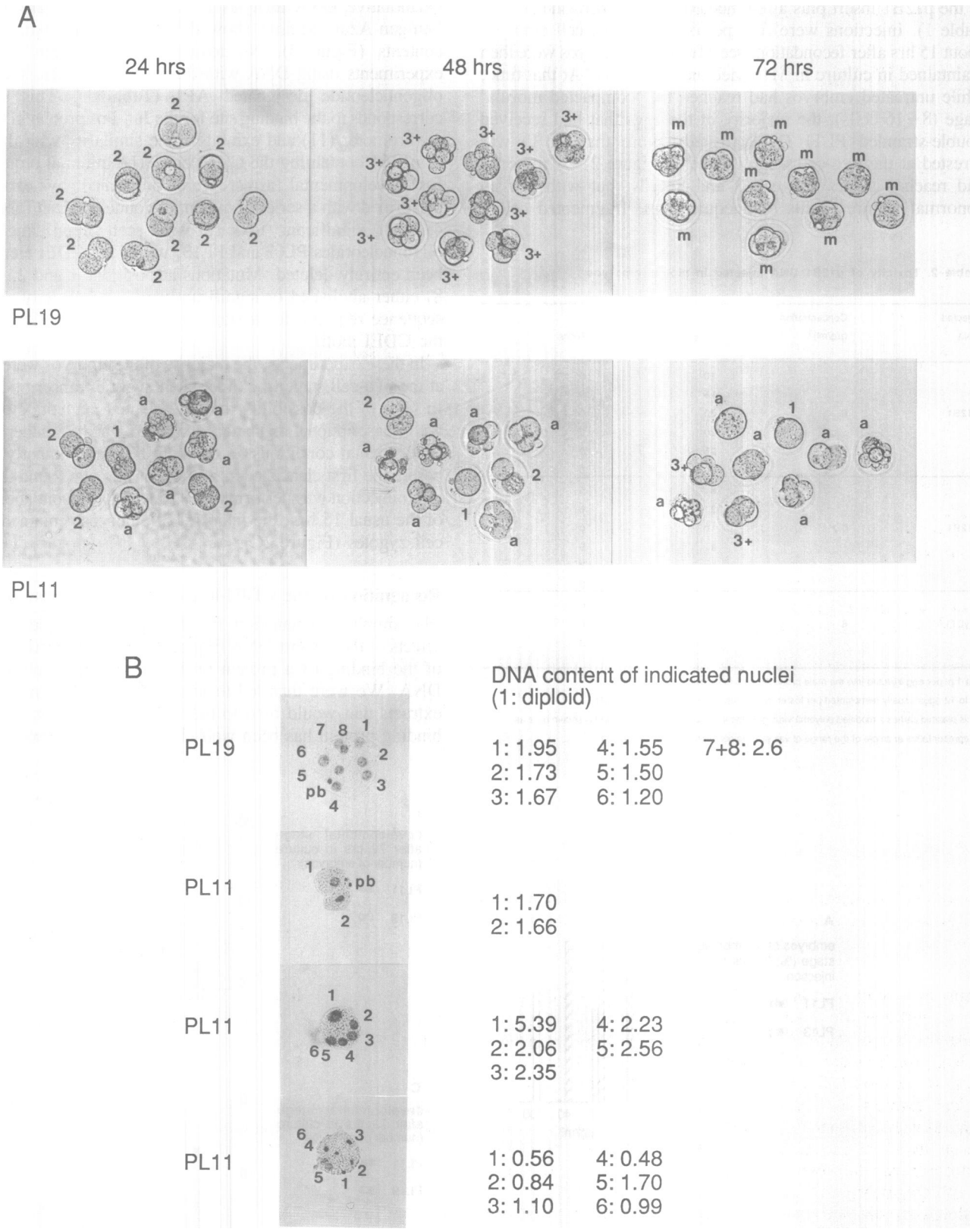

Figure 3. Embryos injected with oligonucleotide PL11 (complete CDEI) show abnormal structures and DNA contents. Injection was performed at the one-cell stage (15 hrs). Shown in panel A are pictures of the same group of embryos taken at 24 hrs intervals after injection of either PL11 or PL19 (C to T mutation at position 5): '2': two-cell stage; '3+': three- to eight-cell; 'm': morulas; 'a': abnormal. Panel B: a normal eight-cell embryo after injection with PL19, a two-cell embryo arrested, but apparently normal after injection of PL11 and abnormal embryos at later stages. DNA content of individual nuclei was estimated by cytophotometry and image processing on the Becton-Dickinson Cell Analysis System. Values indicated are relative to measurements (not shown) performed on mouse spleen cells (1) and sperm (0.5).

Table 3. Developmental stage of embryos 72 hrs after injection

Exp	DNA	µg/ml	Eggs injected[1]	One-cell	Two-cell	Three- to eight-cell	Compacted morula	Abnormal[2]
1	PL11	200	10	0	5	0	0	5
	PL03	200	11	0	1	0	7	3
2	PL11	200	14	0	7	0	0	7
		40	13	0	5	8	0	0
		10	17	0	4	2	10	1
		0.5	14	0	1	0	11	2
	PL13	200	9	0	1	0	6	2
		40	15	0	0	0	15	0
		10	8	0	0	0	8	0
		0.5	14	0	1	0	12	1
	PL17	40	15	0	1	0	13	1
	PL18	40	10	0	0	0	9	1
3	PL11	40	6	0	1	4	0	1
	PL17	40	10	0	0	0	8	2
4	PL11	200	6	0	4	1	0	1
	PL19	200	2	0	0	0	2	0
5	PL11	200	9	2	2	3	0	2
	PL19	200	10	0	0	0	10	0
6	PL11	200	8	0	3	2	0	4
	PL19	200	6	0	0	1	5	0
	AP1	200	10	0	0	2	8	0
7	PL11	200	10	0	2	1	1	6
	AP1	200	9	0	0	2	7	0

[1]ca 1 pl per egg injected into the male pronucleus 15 hrs after fecondation.

[2]cells and/or nuclei of abnormal sizes (see Figure 3).

Table 4 Mutations at positions 1, 2, 5, 7, 8 of the CDEI sequence abolish or reduce its toxic effects

DNA	CDEI box	Compacted morulas (per cent) 72 hrs after injection[1]
PL11	**GTCACATG**	0
PL20	**caCACATG**	90 - 100
PL19	**GTCAtATG**	90 - 100
PL17	**GTCACAaG**	90 - 100
PL18	**GTCACATa**	90 - 100

[1]injections performed at a concentration of 200 µg/ml 15 hrs after fecondation; values represent the extent of variation in at least two independent experiments (10 to 15 eggs per experiment).

A similar activity was previously reported in HeLa cells (12), but has not been otherwise characterized.

Electrophoretic mobility retardation assays identified mouse nuclear proteins that specifically interacted with the mouse DNA insert in plasmid p12B1. Most experiments were performed with nuclear extracts of FM3A cells, but the same results were obtained with every mouse cell extract tested (C127, Balb/C 3T3, mouse liver, mouse embryos). The first experiments were performed with the complete cellular sequence of p12B1 (BamHI-HindIII fragment, see Figure 1). They evidenced several protein binding sites (data not shown), and studies were first concentrated on one major site within the HindIII-EcoRI 136 bp sub-fragment that contains the CDEI-like box. After incubation of the labeled fragment with nuclear extracts, gel electrophoresis revealed one main band with retarded mobility (Figure 4A). Complex formation was not affected by the non-specific competitor poly(dI.dC) at molar ratios relative to the probe up to 10,000:1, or by the same high concentrations of unlabeled pUC plasmid

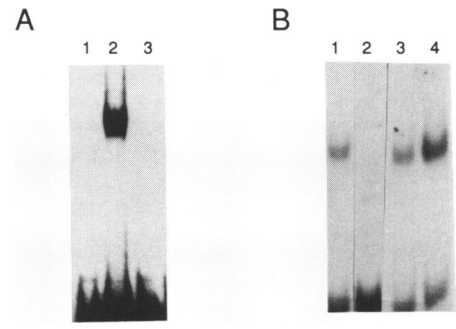

Figure 4. Nuclear proteins bind the CDEI region of p12B1. A: electrophoretic mobility retardation assays were performed on the 136 bp HindIII-EcoRI fragment of the p12B1 mouse insert (see Figure 1). Lane 1: labeled fragment, no protein; lane 2: labeled fragment, Heparin-fractionated protein from FM3A cells; lane 3: same as 2, but reaction was performed in the presence of a 50-fold excess of the unlabeled HindIII-EcoRI fragment. B: assay performed on end-labeled double-stranded PL11 oligonucleotide (see Table 1), in the presence of poly(dI.dC) as a sole competitor (lane 1), of a 50-fold excess of unlabeled PL11 (lane 2), PL03 (lane 3), PL13 (lane 4).

DNA. By contrast, it was extensively reduced in the presence of a 50-fold molar excess of either complete p12B1 DNA, or of the 136 bp HindIII-EcoRI sub-fragment. Both binding and efficient competition were observed (Figure 4B) using oligonucleotides PL01, 05 and 11, whose sequences include the CDEI-like motif plus only a few base pairs of the p12B1 sequence on each side (Table 1), but not with PL03 and 13, which were deleted of the 8 base pairs of the CDEI-like element. The activity could be partially purified by chromatography on Heparin Ultrogel, where it was recovered as a single peak eluting between 400 and 450 mM NaCl, with an estimated purification factor of about 20-fold (data not shown).

These results were independently confirmed by DNase I protection experiments performed on a sub-fragment of the p12B1 cellular sequence. They showed a protected region centered on the eight nucleotides of the CDEI box and spanning over 4 bp on each side (Figure 5). Oligonucleotide PL11 competed for the binding of nuclear proteins when present in a 50-fold molar excess. Oligonucleotide AP1, with a binding site for the Jun-Fos protein, started to act as a competitor only in a 5,000-fold excess indicative of non-specific binding.

The complete CDEI sequence appears to be required for protein recognition as well as for inihibiting development. Effects of mutations on complex formation *in vitro* (Figure 6) correlated with the effects on early development (Table 4). Mutations on the 1st and 2nd, 5th, 6th, 7th and 8th base pairs that reduced or abolished toxicity scored as negative when assayed either by electrophoretic retardation of the mutant oligonucleotides, or by competition for binding to labeled PL11 DNA.

DISCUSSION

In order to study the conditions and possible causes of the developmental arrest induced by microinjection of p12B1 DNA, we attempted to reproduce this effect using oligonucleotides of a short size (15−20 bp), thus of a lesser order of complexity than the whole mouse DNA insert. Since one of its striking features is a region that shows a clear similarity with the yeast *CEN* elements, our studies first concentrated on this part of the sequence. In addition, toxicity in eggs was reminiscent of a similar

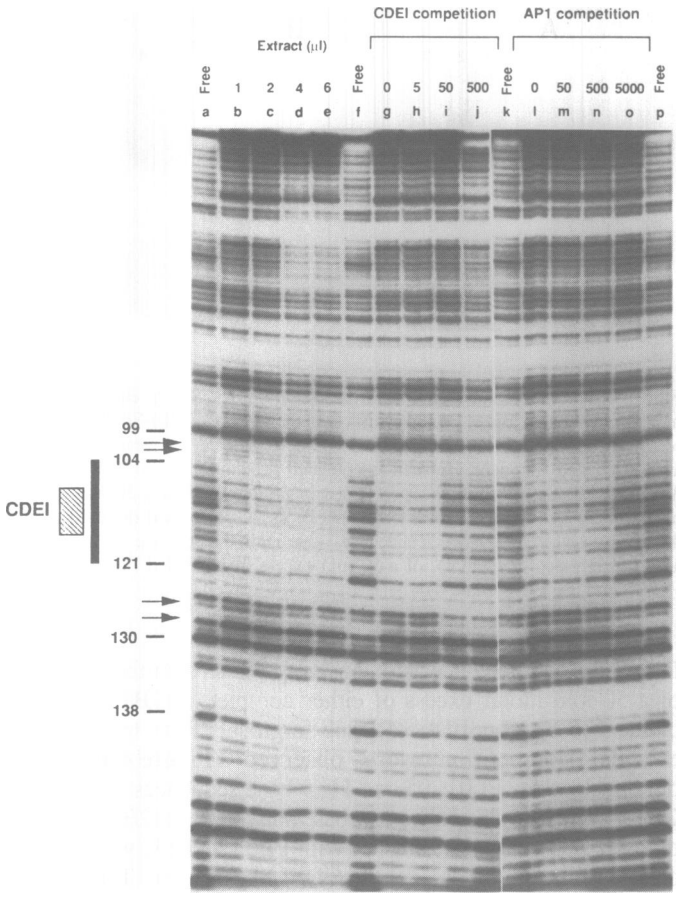

Figure 5. Recognition of the CDEI motif by mouse proteins. A subfragment of the p12B1 mouse insert (nt. 1−170) was incubated with a nuclear protein extract prepared from C127 cells and submitted to partial digestion by DNaseI (see Methods). Lanes a, f, k and p ('free'): electrophoresis of the digestion products in the absence of extract; lanes b−e: increasing amounts of extract; lanes g−j ('CDEI competition'): DNaseI digestion was performed in the presence of 2 µl of extract and of amounts of unlabeled PL11 oligonucleotide corresponding to 5-, 50- and 500-fold molar excess, respectively; lanes l−o: same as in g−j, but with 50- to 5,000-excess of unlabeled AP1 oligonucleotide (Table 1).

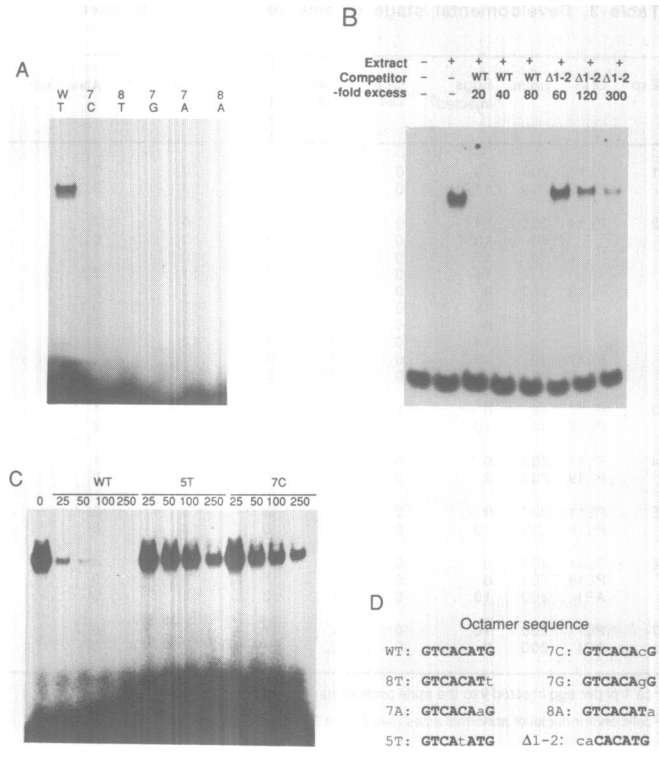

Figure 6. Efficient binding requires the integrity of the CDEI element. A: electrophoretic mobility retardation assays were performed on end-labeled oligonucleotides PL11 (WT), PL14 (7C), PL15 (8T), PL16 (7G), PL17 (7A), PL18 (8A). B: retardation of labeled PL11 in the presence of the indicated excess of unlabeled PL11 (WT) and PL20 (Δ1−2). C: same experiment as in B, with PL11 (WT), PL19 (5T), and PL14 (7C) as unlabeled competitors. D: sequence of the CDEI boxes of the injected oligonucleotides.

effect of high numbers of cloned *CEN* molecules in yeast cells (17). Microinjection of high concentrations of the double stranded oligonucleotide 5′-GTCACATG/3′-CAGTGTAC (CDEI element) efficiently arrested the development of mouse embryos during the very first zygotic divisions. Only a few injected embryos accomplished more than 2−3 cleavages, and all of them exhibited abnormal nuclear structures and DNA contents. Competitive inhibition of critical DNA-protein interaction(s) is a likely explanation for the toxic effects of short oligonucleotides (4), and we could obtain direct evidence of the binding of mouse nuclear protein(s) to the CDEI motif.

The molar concentrations of CDEI oligonucleotides required for arresting development were several orders of magnitude higher than for the whole p12B1 insert. Their effect was nonetheless specific, since at the same concentrations, oligonucleotides mutated in the CDEI motif had no detectable effect. Their lower efficiency may be due to a correspondingly low efficiency of complex formation with short oligonucleotides *in vivo*, since in a similar study (4), even higher concentrations of the Oct-3 binding site had to be used. Alternatively, the complete p12B1 sequence might contain additional motifs that interfere with early development, either separately or in combination with the CDEI element, a point that should be clarified by work presently in progress.

We cannot unambiguously conclude that the CDEI-binding protein that we detect in fibroblasts is the same as the one acting in the early embryo. The close match between the effects of mutations on *in vitro* binding and *in vivo* toxicity makes this plausible. We have isolated several overlapping cDNA clones by screening expression libraries with labeled CDEI oligonucleotides, and, once the complete sequence of the mRNA is established, microinjection of anti-sense oligonucleotides and/or antibodies might provide more definitive answers.

Mutational analysis of the binding site shows a minimal requirement for the eight base pairs of the CDEI motif, which, on the other hand, were fully protected in DNase foot-printing experiments. They contain the CANNTG palindrome characteristic of the binding sites of the 'basic Helix-Loop-Helix' (bHLH) family of regulatory DNA-binding proteins (immunoglobulin enhancer binding factor, MyoD, Myc and Myc-associated proteins (18−21)). Recognition depends in each case on the nucleotides adjacent to the core sequence. With the exception of the yeast CBF1 protein (12−16), they all bind to sites distinct from the CDEI sequence (see ref. 16 for a review). Possible evolutionary relationships between the yeast and the mouse CDEI-binding proteins should become apparent once the complete amino-acid sequence of the mouse protein will be

available, as well as structural elements that may be common to the mouse protein and the known bHLH proteins.

The function of the GTCACATG motif in mouse cells remains a matter of speculation. Hypotheses may be developed from what is known of the functions of the yeast CDEI element (reviewed in refs. 22, 23) and of the CDEI-binding protein (12–16). CDEI was first defined as a sequence common to the centromeres of the *S. cerevisiae* chromosomes. A functional *CEN* element includes the CDEI box, an AT-rich spacer of variable length and sequence (CDEII), and a sequence designated CDEIII. Although not absolutely required, CDEI plays a role in the centromeric functions, which include a structural role in maintaining a tight association between sister chromatids during meiosis, and the kinetochore function that moves the chromosomes apart towards the poles of the spindle. Involvement of *CEN* elements in higher order structures is also suggested by their tight association with the nuclear matrix (24). On the other hand, CDEI blocks have been suspected to act in transcriptional regulation. The presence of isolated octamers was noticed in several promoters (12). More convincingly, a direct repeat in the upstream region of the yeast *MET25* gene was shown to be required for its efficient expression (25). Accordingly, disruption of the gene encoding the CDEI-binding protein CBF1 led not only to an increased chromosomal instability, but also to methionine auxotrophy (16).

In higher eukaryotes, centromeres have a more complex structure and a larger size than in *S. cerevisiae* (see refs. 26 and 27 for reviews). There is no evidence that CDEI motifs are present within centromeric regions, and the p12B1 sequence was in fact found by *in situ* hybridization to map in the telomeric region of chromosome 5 (MR, PL, J. Vailly, M.G. Mattei and FC, manuscript submitted for publication). On the other hand, the CDEI box of p12B1 is part of a more complex 'CEN-like' element, which also includes a region similar to the central part of CDEIII (2) (see Figure 1). An analogous structure was found in the genome of Bovine Papillomavirus Type 1 (BPV1), and preliminary results suggest that it may play a role in its extrachromosomal maintenance (AB, G.F. Carle, V. Pierrefite, MR and FC, manuscript submitted for publication; V. Pierrefite, MR and FC, unpublished). Since on the other hand, widely abnormal nuclear DNA contents were measured upon injection of oligonucleotide PL11, one possible hypothesis would be that protein binding on the CDEI site of the CEN-like region plays a role in the replication/segregation of the embryonic genome.

On the other hand, involvement of the CDEI box in transcriptional controls is clearly not excluded, in spite of the fact that experiments specifically designed to search for an effect of the p12B1 mouse sequence on gene expression yielded so far only negative results. Transfection of a construct where this sequence had been inserted upstream of a selectable *neo* marker produced the same number of drug-resistant colonies as that of the *neo* vector alone (MR et al., manuscript submitted for publication). Furthermore, no increase in chloramphenicol acetyl transferase (CAT) activity was evidenced upon transfection of constructs where the complete sequence had been inserted upstream of the CAT gene and of a minimal promoter (our unpublished results). It is clear also that competitive inhibition of the activity of a transcription factor is not a sufficient explanation. The Fos-Jun protein is a critical element of the control of the cell cycle, but injection under the same conditions of an oligonucleotide carrying its binding site had no effect on development (oligonucleotide AP1, Table 3).

It is also relevant to consider that, when the CDEI sequence was injected early enough after fecondation, development was arrested at the one-cell stage. At that time, zygotic transcription is not considered as having started, and the first division occurs even in the presence of high doses of α-amanitin (see ref. 28, 29 for reviews). As in the case of the Oct-3 protein-DNA interaction (4), one would have to conclude either that a limited, but essential α-amanitin-resistant transcriptional activity occurs in the one-cell embryo, or that the CDEI-protein interaction is important for a distinct function, for instance in the completion of the first S phase. A more detailed analysis of the time dependence of the first zygotic division on the time of injection of CDEI oligonucleotides should be informative with respect to the 'zygotic clock' that controls the first cell cycle in the absence of gene expression.

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