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Genomic Organization, Promoter Analysis, and Chromosomal Mapping of the Mouse Gene Encoding Cdk9

Luigi Bagella,[1] Peter Stiegler,[1] Antonio De Luca,[2] Linda D. Siracusa,[3] and Antonio Giordano[1]

[1]Departments of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, Sbarro Institute for Cancer Research and Molecular Medicine, Philadelphia, Pennsylvania
[2]Institute of Topographical Anatomy, School of Medicine, 2nd University of Naples, Naples, Italy
[3]Kimmel Cancer Center, Thomas Jefferson University, Department of Microbiology and Immunology, Philadelphia, Pennsylania 19107

Abstract Cdk9, previously known as PITALRE, belongs to the Cdc2 family of protein kinases. We report the isolation and characterization of the complete gene coding for the murine Cdk9 protein. The gene contains seven exons spanning over 6 kb of genomic DNA, and the exon/intron boundaries conformed to the GT/AG rule. The Cdk9 gene mapped on mouse chromosome 2, which is consistent with the known region of synteny with human chromosome 9q34.1. The length of the individual exons ranged from 82 to 850 bp, and introns ranged from 452 to 1,465 bp. The further 5′ flanking region of the gene showed features of a housekeeping promoter, such as the lack of a canonical TATA box and the presence of a CCAAT box as well as several GC boxes, which are potential binding sites for numerous transcription factors. Additionally, we performed a basic analysis of the transcriptional activity of the promoter and found that the 364 bp of Cdk9 5′ flanking region were able to elicit high transcriptional levels of a luciferase reporter gene in NIH3T3 cells. This study provides the molecular basis for understanding the transcriptional control of the Cdk9 gene, and could serve to facilitate the molecular genetic investigation of Cdk9 function during mouse embryonal development. J. Cell. Biochem. 78:170–178, 2000.

Key words: Cdk9; gene coding; mouse gene promoter

Some members of the Cdc2/Cdk1 family of protein kinases have been shown to play a crucial role in eukaryotic cell cycle control [Maclachlan et al., 1995]. It has also been shown that some of these closely related kinases are involved in processes clearly distinguishable from mere proliferative functions [Tsai et al., 1994; Lew et al., 1994]. In addition to sharing structural motifs, regulatory sites, and similar mechanisms of activation, this family of protein kinases also seems to have a broad spectrum of functions.

A new Cdc2-related kinase, PITALRE, was cloned by polymerase chain reaction (PCR) using degenerate oligonucleotide primers derived from sequences that are conserved in other Cdc2-related kinases [Graña et al., 1994]. RNA transcripts for PITALRE are present at different levels in all human tissues examined. PITALRE's subcellular localization is primarily, if not exclusively, nuclear [Graña et al., 1994]. Unlike most of the Cdc2/Cdk1-like kinases, PITALRE kinase activity is not cell cycle regulated [De Falco and Giordano, 1998].

The rules of nomenclature state that those proteins most closely related to Cdc2/Cdk1 and binding to cyclins or complementing Cdc2/Cdk1 function are known as cyclin-dependent kinases and are numbered in order of their discovery. Other kinases sharing similarity with Cdc2 only in the PSTAIRE motif, but not having a known partner in the cyclin family

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*Correspondence to: Antonio Giordano, Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, 1020 Locust, Philadelphia, PA 19107. E-mail: agiordan@lac.jci.tju.edu

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and not complementing Cdc2 function, are named temporarily on the basis of the homology region, using the single-letter amino acid code, until more information can be obtained [Meyerson et al., 1992]. A novel 87-kDa cyclin, named cyclin T, has been isolated recently. Cyclin T is a partner of PITALRE, which subsequently has been named Cdk9 [Wei et al., 1998].

Several studies have identified Cdk9 as the kinase involved in the TAK complex (Tat-associated kinase). The Tat protein encoded by the human immunodeficiency virus (HIV) is essential for viral replication. The HIV tat proteins act to target host cell kinases. The primary effect of Tat is to increase the processitivity of the RNA polymerases that otherwise would terminate prematurely after the synthesis of short nascent transcripts [Zhu et al., 1997; Jones, 1997].

These studies provide evidence that Tat binds to the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II and to TAK. TAK is a complex of approximatively 110 kDa, whose kinase activity results from a 42-kDa protein corresponding to Cdk9 and its 87-kDa component cyclin partner, cyclin T. TAK hyperphosphorylates the carboxyl-terminal region (CTD) of the large subunit of RNA Pol II and affects transcription elongation. It is believed that phosphorylation of CTD converts RNA Pol II to an actively elongating complex. Thus, the phosphorylation of CTD by Cdk9 may represent activation of the elongation complex [Yang et al., 1997].

Additionally, a recent study reports that Cdk9 suppresses B-Myb autoregolation through direct interaction with the carboxyl-terminus of the B-Myb protein [De Falco et al., 2000].

We have cloned and characterized the mouse homologue of Cdk9. Murine Cdk9 cDNA is 98% identical with that of the human, and is expressed at high levels in brain and kidney tissues. The kinase activity and protein amount of Cdk9 was highest in terminally differentiated tissues such as muscle and brain. Using the C2C12 and P19 differentiation model systems, Cdk9 kinase activity peaks late in the differentiation process in cells induced toward the muscle and neural lineage, respectively. These results suggest that, among other roles, Cdk9 plays a role, not different from Cdk5, in the differentiation process of certain cell types [Bagella et al., 1998].

We report the characterization of the complete murine Cdk9 gene, and the identification and the functional analysis of its promoter region as an additional step in understanding the regulation of this protein kinase.

MATERIALS AND METHODS

Genomic λ Library Screening

A mouse 129SVJ Genomic Library Lambda (Stratagene, La Jolla, CA) was screened using a mouse cDNA corresponding with the fragment from the ATG to the stop codon labeled with [α -^{32}P]-dCTP by the random primer method (Boehringer Manheim, Indianapolis, IN). Briefly, λ phages were adsorbed to *Escherichia coli* XL1-Blue bacterial strain and plated in agar medium. Nitrocellulose filters were blotted and hybridized with the cDNA probe at a high stringency with the following hybridization mixture: 5X SSPE, 10X Denhardt's solution, 150 μg/ml herring sperm DNA, 50% formamide, and 2% sodium dodecyl sulfate (SDS), at 42°C with 1×10^6 cpm/ml of cDNA probe. Three washes of 20 min each at 42°C with 0.2 X SSC and 0.1% SDS were performed after 12 h of hybridization. After a tertiary screening, isolated clones were characterized by Southern blot (not shown).

Subcloning and Sequencing

Reactive clones were digested with HindIII or SacI, and restriction fragments were cloned into the pBluescript (Stratagene) plasmid vector. Resulting plasmid clones were sequenced with the chain termination method using the Applied Biosystems model 373A DNA sequencer [Sanger et al., 1977].

Primer Extension

The size of the 5′ untranslated region of the mRNA was determined by primer extension. Cytoplasmatic RNA was extracted using the RNAzol B method (Cinna/Biotecx., Friendswood, TX) from NIH3T3 cells.

A 21-mer oligonucleotide 5′-CGCCGCCCCCAGCCGCAGCTC-3′ complementary with the sequence starting at position −36 (Fig. 1) was end labeled with [γ-^{32}P]-ATP and hybridized with 20 μg of cytoplasmatic RNA at 55°C for 20 min. The primer-annealed RNA was converted into cDNA by an avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) in the presence of 2 mM deoxynucle-

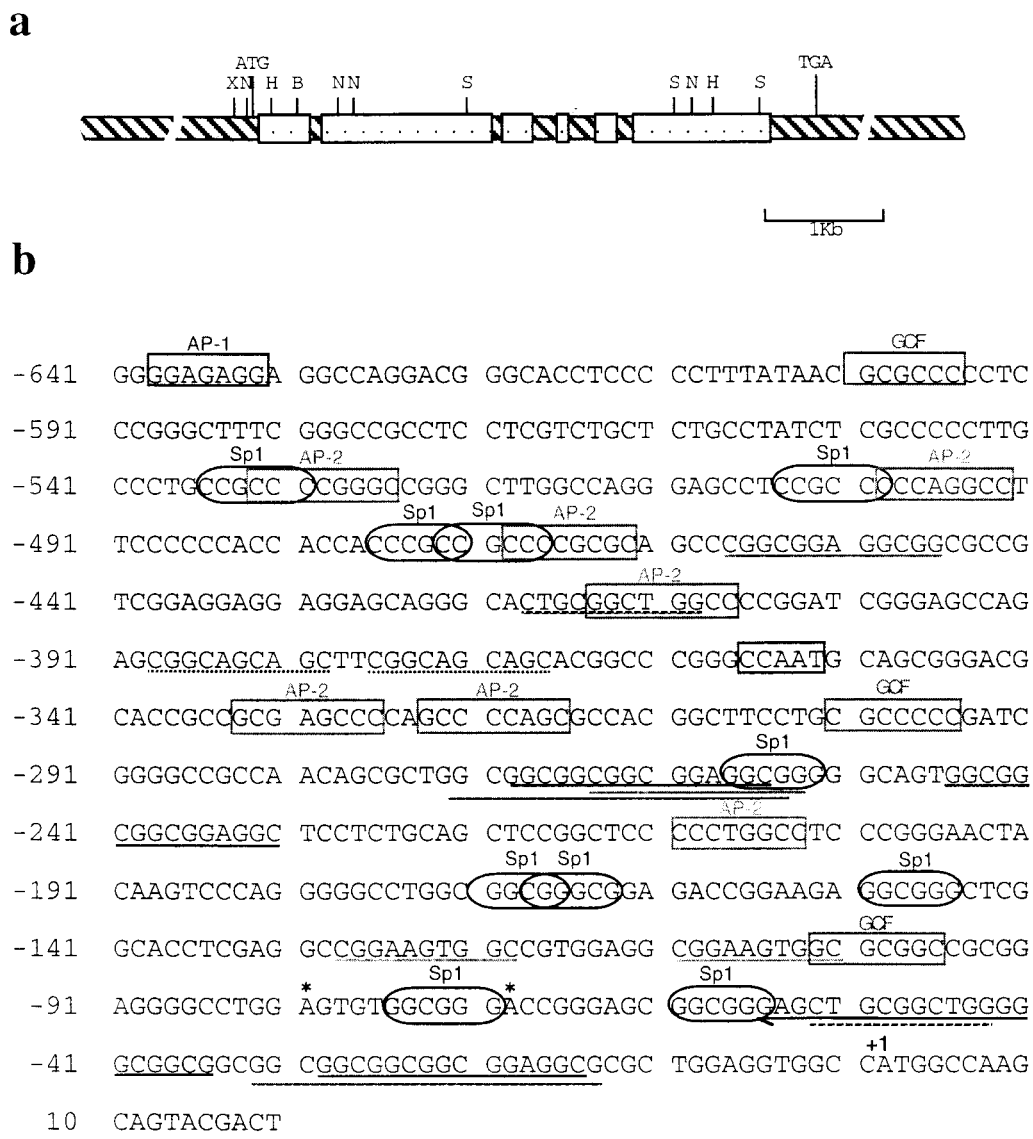

Fig. 1. **a:** Structural organization and restriction map of the mouse Cdk9 gene. Boxes indicate introns. Sites for BamHI (B), HindIII (H), NcoI (N), SacI (S), XhoI (X), and the position of the translation start codon and termination codon are indicated. **b:** Sequence of the mouse Cdk9 5′–flanking region. The putative CCAAT box is indicated by a box. The oligo used for primer extension is underlined by an arrow. The asterisks represent the major transcription start sites. Putative binding sites for regulatory elements are boxed with the factor name above the sequence. Short repeated sequences are marked by the same motif line. Numbers to the left refer to nucleotide position relative to the first nucleotide of the ATG initiation codon that is designated +1.

otides at 42°C for 45 min. The cDNA product was then analyzed on an 8% polyacrylamide denaturing gel containing 7 M urea.

Analysis of the Promoter Activity

The pGL2 expression vector system (Promega, Madison, WI) containing the firefly luciferase as a reporter gene was used to measure the promoter activity of cloned genomic fragments from the 5′ region of the Cdk9 gene. The 637-bp fragment from −637 to +1 relative to the first nucleotide of the ATG initiation codon (Fig. 1) was amplified by PCR. PCR was carried out for 30 cycles (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C) using as upstream oligonucleotide: 5′-TAGATCTAGAGGAGGCCAGGACGGGCA-3′ and as downstream oligonucleotide: 5′-TAAGCTTGGCCACCTCCAGCGCGCCTC-3′. The PCR product was subcloned into BglII and HindIII-digested pGL2-Basic

TABLE I. Listing of Restriction Fragment Length Polymorphisms Used for Interspecific Mouse Backcross Mapping of Cdk9 Locus

Locus	Gene name	Restriction endonuclease	Size (kb) AEJ/Gn	Size (kb) *Mus spretus*[a]	Probe	Probe ref.
Abl	Abelson leukemia virus	*TaqI*	6.6, 5.7, 5.0, 3.5	6.6, 5.7, 5.0, 4.7, 2.9, 2.0, 1.1	v-ABL	Abelson and Rabstein, 1970
Cdk9	cdc2-related protein kinase	*SacI*	10.1, 3.1, 2.8, 0.7	10.1, 3.6, 0.7	mouseCdk9	Bagella et al., 1998

[a]The underlined restriction fragments identify the segregating *M. spretus* alleles followed in the N2 progeny by scoring for their presence or absence in each mouse.

vector. The construct obtained pGL2-B/637 was cleaved with SmaI, or PstI and BglII and blunted, or XhoI. The fragments were recircularized with T4 ligase, generating respectively the constructs pGL2-B/199, pGL2-B/222, and pGL2-B/136. The fragment of 165 bp obtained after cleavage of pGL2-B/637 with SmaI was subcloned in the SmaI site of pGL2-B/199 generating the construct pGL2-B/364. The correct orientation of this construct was confirmed by nucleotide sequencing.

NIH3T3 cells were propagated in Dulbecco Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Rockville, MD), 5 mM glutamine (Gibco BRL, Rockville, MD), in 5% CO_2 at 37°C.

C2C12 cells were maintained in DMEM supplemented with 20% FBS (Gibco BRL) and 5 mM glutamine (Gibco BRL), and differentiation was induced by switching to a medium containing 2% horse serum (Gibco BRL) for up to 96 h. Transient transfections were performed in three independent experiments and in duplicate using the lipofectamine transfection system (Gibco BRL) following the manufacture 's protocol. A *Lac-Z* gene under the control of a cytomegalovirus (CMV) promoter was used as a control for transfection efficiency.

Chromosomal Mapping

Mice of the AEG/Gn strain were purchased from The Jackson Laboratory (Bar Harbor, ME). AEJ/Gn females were crossed with *Mus spretus* males. The resulting F1 female offspring were backcrossed to AEJ/Gn males. Progenitors of the *M. spretus* mice were used at the F13 to F17 generation of inbreeding [Argeson et al., 1995]. The probes used for this study are listed in Table I. The mouse Cdk9 probe is a 1.6-kb embryonic mouse cDNA subcloned into pCR II (3.9 kb) (Invitrogen, Carlsbad, CA) at the EcoRI site [Bagella et al., 1998]. Except for D1Mit65, which was purchased from Research Genetics (Huntsville, AL) DNA primers for simple sequence length polymorphism analysis were made using an Applied Biosystems Model 394 DNA synthesizer. Genomic DNA from each N2 progeny was amplified with each of the primer pairs (Table II) using Taq DNA polymerase and buffer (Boehringer Mannheim, Indianapolis, IN). The PCR protocol used was an initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s [Argeson et al., 1995], except for D1Mit65, where the annealing temperature was 61°C. PCR products were electrophoresed on 3% Seakem agarose gels and visualized by ethidium bromide staining (Table II). High-molecular weight genomic DNA from AEJ/Gn and from *M. spretus* mice was isolated from frozen tissues as previously described [Jenkins et al., 1982]. Genomic DNA was digested with restriction endonucleases, transferred to nylon membranes, and hybridized to the mouse Cdk9 probes to screen for restriction fragment length polymorphisms (listed in Table I) and analyzed by Southern blot hybridization as described [Argeson et al., 1995]. The chromosomal localization and recombination frequencies [Green et al., 1981] of loci mapped in the interspecific backcross were determined and were calculated using the computer program Spretus Madness: Part Deux developed by C. Smalley, J. Averback, L. D. Siracusa, and A. M. Buchberg (Kimmel Cancer Center, Philadelphia, PA).

RESULTS

Intron–Exon Organization of Genomic Mouse Cdk9

The mouse Cdk9 gene consisted of seven exons that spanned more than 6 kb of genomic

TABLE II. Listing of Simple Sequence Length Polymorphisms Used for Interspecific Mouse Backcross Mapping of Cdk9 Locus

Locus	Size (bp)[a] AEJ/Gn	*Mus spretus*[b]	Primer ref.
D1Mit4	200	170	Whitehead Institute/MIT, Center for Genome Research, Database Release 1995, Feb 7
D1Mit65	212	108	Whitehead Institute/MIT, Center for Genome Research, Database Release 1995, Feb 7
D2Mit9	187	174	Whitehead Institute/MIT, Center for Genome Research, Database Release 1995, Feb 7
D8Mit35	138	194	Whitehead Institute/MIT, Center for Genome Research, Database Release 1995, Feb 7
D8Mit42	144	168	Whitehead Institute/MIT, Center for Genome Research, Database Release 1995, Feb 7

[a]Exact size fragments for *M. spretus* polymerase chain reaction (PCR) products were taken from the Whitehead MIT database; the results from agarose gel electrophoresis appear consistent with the fragment sizes given. AEJ/Gn fragment sizes do not seem to vary from those reported for C57BL/6J.
[b]The underlined PCR products identify the segregating *M. spretus* alleles followed in the N2 progeny by scoring for their presence or absence in each mouse.

TABLE III. Exon–Intron Boundaries of the Mouse Cdk9 Gene[a]

Exon	bp	Sequence at exon–intron junctions 5′ Donor sequence	3′ Acceptor sequence	Intron	bp
1	>92	CACATTCGG^{92} gtaagattag	.....gtgcctgcag ^{93}GGAAGTCTT	1	452
2	82	AAGGAGGGG^{174}gtgagtatat	.....tcctccctag^{175}TTCCCCATC	2	1465
3	91	GGACCAAAG^{265}gtgtgttgct	.....tctgtatcag^{266}CCTCACCGT	3	26
4	167	AGGAACAAG^{432}gtggggcaga	.....ctcccttcag^{433}ATCCTGCAC	4	98
5	172	TGCTGCTCG^{604}gtgaggactc	.....ttcttctcag^{605}GAGAGCGGG	5	157
6	149	ACCCCAGAG^{753}gtgagtaacc	.....cctctcttag^{754}GTGTGGCCA	6	117
7	850	To the polyadenylation signal			

[a]Intron/exon organization of the mouse Cdk9 gene. Exon sequences are in capital letters; intron sequences are in lowercase letters. Also shown are nucleotide positions (superscript numbers) of exon/intron boundaries according to the published sequence [Bagella et al., 1998]. Intron sizes were determined by either direct sequencing, or amplification of intronic DNA within genomic clones by PCR using cDNA primers.

DNA. Subclones obtained by restriction endonuclease digestion were subjected to nucleotide sequencing. To characterize the position of the exons and introns-exons boundaries precisely, a set of oligonucleotides were synthesized at ≈150-bp intervals in the cDNA sequence from both strands, and were used as primers for sequencing genomic DNA clones. The intron–exon boundaries were identified in the positions where the genomic DNA sequence diverged from that of the cDNA. The DNA sequence of the introns was determined by using specific primers. Ultimately, seven exons were identified, that in sum, accounted for the entire cDNA sequence (Table III). The DNA sequences of all splice donor and acceptor sites contained the invariant GT-AG intronic dinucleotides [Mount, 1982]. All the exon sequences were identical to the cDNA sequence [Bagella et al., 1998]. Exon 2 is the smallest exon and contains only 82 nucleotides. Exons 1 and 3 were also small, containing 92 and 91 nucleotides, respectively, whereas exon 7 was the largest (829 nt). Introns 2 and 6 were relatively large, more than 1 kb (1,465 and 1,177 nt, respectively). Other introns were less than 0.5 kb long. The usual polyadenylation signal AATAAA [Proudfoot and Brownlee, 1976] is slightly modified (AGTAAA)

Transcription Start and Analysis of the Promoter Region

The transcription start site was identified by primer extension on RNA from mouse fibro-

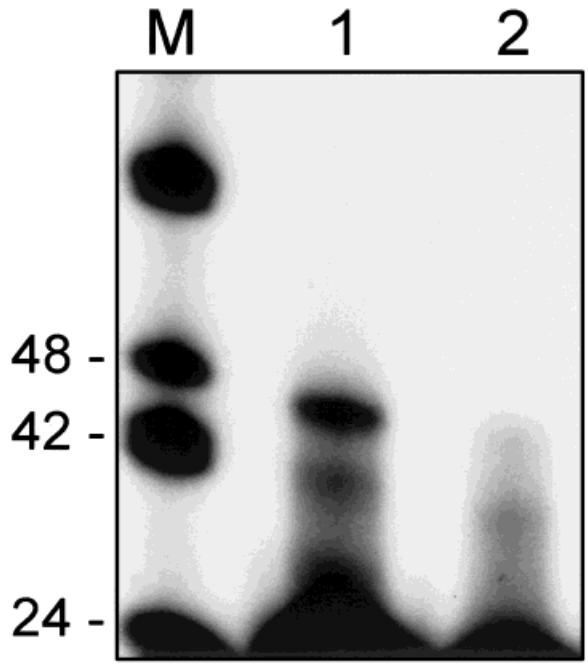

Fig. 2. Identification of the transcription initiation site of the murine Cdk9 gene by primer extension. The products resulting from extension with avian myeloblastosis virus (AMV) reverse transcriptase using, as template, cytoplasmatic RNA from NIH3T3 mouse fibroblast cell line (**lane 1**) or tRNA as a control (**lane 2**) were electrophoresed adjacent to an fX174 Hinf I DNA marker (Promega, Madison, WI).

blast cells using a 21-base antisense oligonucleotide primer homologous to the sequence between −56 and −36 bp upstream of the translation start site (Fig. 1). Two transcription start sites were identified (Fig. 2). The major transcription start point was mapped 81 nt upstream from the ATG that was assigned +1 as a reference for base positioning in the genomic sequence (Fig. 1). This result was in agreement with the 1.6-kb Cdk9 mRNA length estimated by Northern blot [Bagella et al., 1998]. As the initiation site of many eukaryotic genes, the mouse Cdk9 gene initiates at adenine [Breathnach and Chambon, 1981]. The promoter region showed features of a housekeeping gene; in fact, like other housekeeping promoters, the sequence of the Cdk9 promoter did not contain a consensus TATA box but contained a CCAAT box and had a high G/C content. The CCAAT box was much further from the initiation site. By using the GCG program to screen the 5′ region for potential transcription factor-binding sites [Gosh, 1990], GC boxes, potential Sp1-, AP-2-, GCF- binding sites, and a potential binding site for AP-1 were identified. G/C rich sequences play a critical role in the control of various genes, including housekeeping genes and cellular oncogenes [Melton et al., 1984; Reynolds et al., 1984; Ishii et al., 1985]. Regions with high G/C content can exhibit transcriptional enhancer activity when exposed to activating factors such as SP1 and ETF [Kageyama et al., 1988], and conversely exhibit repressor activity when bound to an inhibitory factor such as GCF [Kageyama and Pastan, 1989; Lania et al., 1997]

To test whether the regions of the transcription start site shows promoter activity, the mouse 5′ Cdk9 fragments of 637 bp and sequential deletions 364 bp, 222 bp, 199 bp, and 136 bp were cloned in front of the luciferase reporter gene. NIH3T3 cells were transiently transfected with these promoter constructs and were analyzed for luciferase activity. Considering the pGL2-B/637 luciferase activity 100%, transient transfections of NIH3T3 cells with the construct pGL2-B/364 elicited a luciferase activity of 158%. This result possibly identifies a Cdk9 gene repressor sequence within −637 and −364 bp (Fig. 3). A further deletion of 142 bp restored the original promoter activity. This indicates that within −364 and −222 bp, an activator site was lost. Computer analysis identified a CAAT box located at −357/−353 bp, as well as two AP-2 sites within this DNA stretch on the promoter. The pGL2-B/199 activates the luciferase gene similarly to the pGL2-B/222, demonstrating that this stretch of the promoter region, at least in this assay, does not contribute significantly to the expression of Cdk9 in the cell system analyzed. However, transfection of the pGL2-B/136 construct delivered an unexpected result: pGL2-B/136 activated the luciferase promoter only 16.5% of the activity of the pGL2-B/637. Two overlapping G/C rich sequences, most probably SP1 binding sites, flank another G/C rich SP1 site. These residues seem to provide an essential transcriptional activity of the Cdk9 promoter, because their deletion causes a tremendous loss in promoter activity. In conclusion, the stretch of 364 bp of Cdk9 5′ flanking region contains all the transcriptional regulatory elements to show full promoter activity in transient transfection using NIH3T3 cells.

In an additional set of experiments, C2C12 cells were transiently transfected with pGL2-B/637 and induced to differentiate. Previously

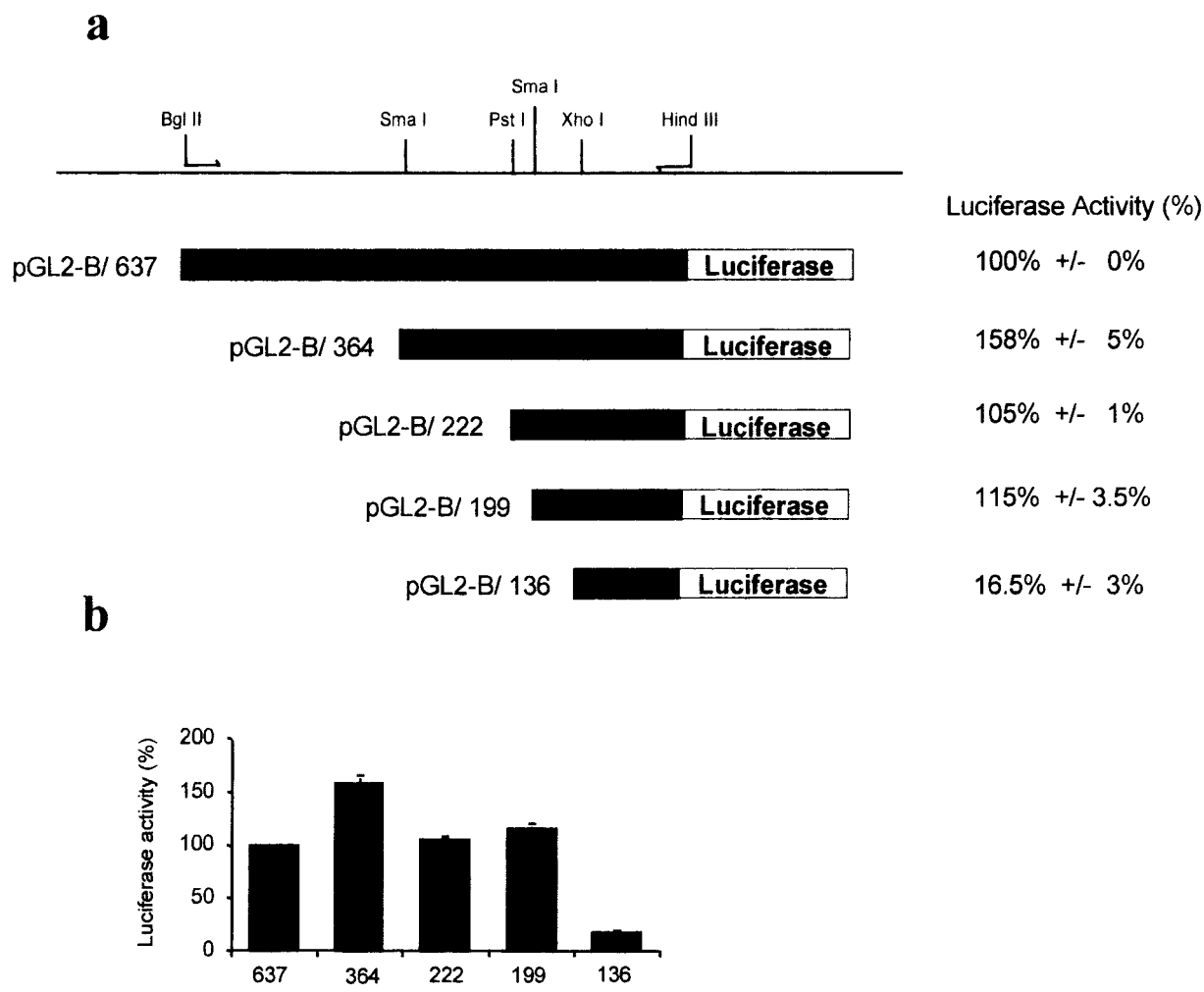

Fig. 3. Analysis of the promoter activity of the mouse Cdk9 5′ region. **a:** Schematic drawing of the investigated DNA fragments and vector constructs. Location of oligonucleotides and restriction sites used for cloning are indicated on the top. The length of mouse Cdk9 fragments subcloned in pGL2 basic and their relative luciferase activity are shown (see Materials and Methods section for the cloning strategy). **b:** One microgram of luciferase plasmid was cotransfected with 1 μg of CMV β-galactosidase. Black vertical bars represent the relative luciferase activity of the different luciferase constructs; all the data are in relation to the pGL2-B/637 luciferase activity that was set arbitrarily to 100%. Standard deviations are indicated. The control vector pGL2-B, without promoter or enhancer elements, showed a luciferase activity of 0.05% (data not shown).

measured unchanged Cdk9 protein expression levels matched the steady-state promoter luciferase activity levels (data not shown). This indicates that in this differentiation cell system, the promoter activity parallels the stable Cdk9 protein levels. Therefore, the high Cdk9-associated kinase activity measured in differentiating C2C12 cells could be caused, probably, by elevated levels of the cyclin partner, cyclin T.

Chromosomal Mapping

To determine the chromosomal localization of Cdk9, female mice of the AEJ/Gn strain were crossed with *M. spretus* males. The resulting F1 female offspring were backcrossed to AEJ/Gn males. Progenitors of the *M. spretus* mice were used at the F13 to F17 generation of inbreeding [Argeson et al., 1995]. The probes used in this study are listed in Table I. The results of an interspecific mouse backcross provide the map location of Cdk9 genes in the mouse. The known regions of synteny between mouse and human chromosomes are consistent with our mapping results. The Cdk9 gene maps 1.2 ± 1.2 cM distal to the Abl gene on mouse chromosome

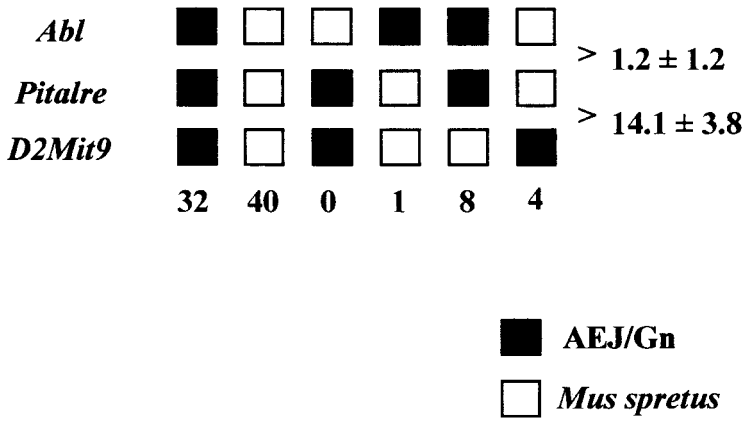

Fig. 4. Haplotype analysis of the N2 progeny from the interspecific backcross. The loci followed in the backcross are listed to the left. Each column represents the chromosome identified in the N2 offspring that was inherited from the (AEJ/Gn x *Mus spretus*) F1 parent. Black squares represent the AEJ/Gn allele, and white squares represent the *M. spretus* allele. The number of N2 progeny carrying each type of chromosome is listed on the bottom. The distances between the markers are given in centimorgans (± standard error) and are listed to the right.

2 (Fig. 4). These results are consistent with known regions of synteny with the human chromosome 9q34.1, where Cdk9 has been previously mapped [Bullrich et al., 1995].

DISCUSSION

In this report, we show the isolation and characterization of the genomic structure of the mouse Cdk9. This highly conserved gene spans more than 6 kb of genomic DNA and has seven exons that in sum accounted for the entire cDNA sequence. In addition, several putative regulatory elements have been identified in the 5′ flanking region, and two major transcription initiation sites have been mapped by primer extension. The Cdk9 gene promoter activity was tested in transient luciferase assays, and a rough map of the regions and important sites were established. Even though the analyzed murine Cdk9 approximate promoter region contains numerous binding domains, we show that the promoter architecture relies on G/C rich sequences and SP1 sites within a short stretch of 62 bp between −199 and −137, to generate almost all of its promoter activity. A deeper knowledge concerning the regulation of G/C rich region binding proteins will be useful to evaluate their significance for the expression of the Cdk9 gene in mouse development. Acetylation and deacetylation, mechanisms to activate and deactivate chromatin, have also been linked to some cellular factors, which are able to bind the Cdk9 promoter.

We have previously shown different steady-state levels of Cdk9 mRNA in various mouse tissues analyzed [Bagella et al., 1998]. Using a myoblast to myotube differentiation system (C2C12), we were unable to detect significant changes in transcriptional levels during differentiation, and the promoter activity paralleled the protein levels. However, this lack of regulation could be caused by the cellular system we used, and different systems could help to establish the different mRNA levels detected in various tissues. Future studies will widen the knowledge on the impact of the Cdk9 promoter region and its effector sites identified in this study and on the role of Cdk9 during development and homeostasis.

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