Characterization and gene structure of a novel retinoblastoma-protein-associated protein similar to the transcription regulator TFII-I

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Retinoblastoma protein (Rb) is an important regulator of vertebrate cell cycle and development. It functions through a direct interaction with protein factors involved in cell cycle progression and differentiation. In the present study we characterized a novel Rb-associated protein, Cream1, which bound to Rb specifically through a C-terminal region. Cream1 contained 959 amino acid residues and migrated as a protein of approx. 120 kDa on SDS/PAGE. It was a widely expressed nuclear protein with a nuclear localization signal resembling that of the large T antigen of simian virus 40. Its primary sequence was characteristic of five direct repeats that were similar to, but distinct from, those of TFII-I, a multifunctional transcription regulator. Three additional regions were also highly conserved in

both proteins. Cream1 exhibited an activation activity that was attributed to its N-terminal portion when assayed in yeast. Its relationship with the muscle-enhancer-binding protein MusTRD1 further suggests a role in regulating gene expression. The structural gene, *CREAM1*, contained 27 exons and spanned more than 150 kb. It was located at human chromosome 7q11.23 in a region deleted for Williams' syndrome, a neurodevelopmental disease with multisystem abnormalities, implying its involvement in certain disorders. Taken together, our results suggest that Cream1 might serve as a positive transcription regulator under the control of Rb.

Key words: MusTRD1, nuclear protein, Williams' syndrome.

INTRODUCTION

Retinoblastoma protein (Rb) was the first identified tumour suppressor [1]. During the cell cycle, Rb functions as one of the key components of the G₁ checkpoint for the faithful regulation of the rhythm of the cell cycle. Disruption of Rb function might result in tumorigenesis, deregulated apoptosis and certain deficiencies during the developmental process [2–4]. Transforming proteins of several DNA tumour viruses, including the large T antigen of simian virus 40 (SV40), E1a of adenovirus and E7 of papillomavirus type 16, interact directly with the T-antigen-binding domain of Rb on virus transfection. Such a step is critical for the subsequent transformation of host cells. These interactions lead to the dissociation of Rb-associated cellular factors that are important for cell cycle progression, e.g. E2F-1, to bypass the normal mechanism in which these factors are released after the phosphorylation of Rb by cyclin-dependent kinases in late G₁. The cell cycle is therefore deregulated, which is a hallmark of tumour cells. A similar strategy has been adopted by naturally occurring tumour cells. As a result, various mutations impairing Rb function have been found in all retinoblastomas and with varying penetrances in tumours such as osteosarcoma, lung carcinoma and bladder cancer [1].

Recent studies have unravelled novel functions of Rb in early embryonic development and tissue differentiation. Rb-deficient mice die between embryonic days 14 and 15 with abnormalities

in neurogenesis and haemopoiesis [3]. Rb is also required for adipogenesis in mouse; in contrast with its general inhibitory effects in cell cycle control, Rb seems to regulate adipocyte differentiation by activating CCAAT-enhancer-binding proteins ('C/EBPs') through direct interaction [5]. Moreover, murine skeletal muscle cells are not fully differentiated without functional Rb because differentiated myocytes can re-enter the cell cycle [6,7]. Rb might exert its function in myogenesis by activating the myogenic transcription factor MyoD [6]. In addition, the stoichiometry of Rb and the transcription repressor HBP1 might also contribute to myoblast differentiation [8].

TFII-I is a multifunctional transcription regulator. It is initially identified as an initiator-binding factor involved in transcription initiation through initiator sites in TATA-less promoters [9]. TFII-I contains six conserved repeats [10,11]. Although the physiological roles of the repeats are currently unknown, the presence of helix–loop–helix-like domains implies their potential for binding DNA [10]. TFII-I has been shown to bind distinct DNA sequences. Among them are the initiator sites of adenovirus major late promoter [10] and murine V β 5.2 promoter [12], the E-box element [10], and c-sis-platelet-derived growth factor-inducible element (SIE) and serum-responsive element ('SRE') of the c-fos promoter [11]. There is evidence for at least two independent DNA-binding domains, for the initiator and the E-box element respectively [10]. TFII-I also interacts directly with various transcription factors such as c-myc [13], USF1

Abbreviations used: BD, DNA-binding domain of Gal4; Btk, Bruton's tyrosine kinase; Ccr1–Ccr3, Cream's conserved regions 1–3; Cr1–Cr5, Cream1 repeats 1–5; EST, expressed sequence tag; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Lys; ILF, indirect immunofluorescence; mAb, monoclonal antibody; MBP, maltose-binding protein; NLS, nuclear localization signal; SIE, c-sis-platelet-derived growth factor-inducible element; Rb, retinoblastoma protein; SV40, simian virus 40; Tcr1–Tcr3, TFII-I's conserved regions 1–3; Tnl_g, slow-muscle-fibre-specific troponin I; Tr1–Tr6, TFII-I repeats 1–6.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF089107.

[10], SRF and Phox1 [11], and STAT1 and STAT3 [14]. In addition, TFII-I (also named BAP-135) binds to Bruton's tyrosine kinase (Btk), a cytoplasmic tyrosine kinase required for normal B-cell development, and is tyrosine-phosphorylated depending on the activation of Btk [15]. The tyrosine phosphorylation of TFII-I is important for its initiator-dependent transcriptional activity [16]. These intriguing properties suggest that TFII-I might function to co-ordinate the basic transcription machinery and a group of upstream regulatory factors in response to certain signalling pathways. Finally, hemizygoty for TFII-I is associated with Williams' syndrome, a neurodevelopmental disorder [17].

Our previous work on the identification of Rb-associated proteins has led to the molecular cloning of E2F-1 and other candidate genes [18]. Some of these gene products, including lamin [19,20], UBF [21] and mitotin [22], have been studied further with regard to their relationship with Rb. Here we report the characterization of another candidate, Cream1, a widely expressed nuclear protein of molecular mass 120 kDa. Our results suggest that Cream1 might function as a ubiquitous transcription regulator under the control of Rb. Moreover, the gene for Cream1 is located within a region frequently deleted in patients with Williams' syndrome. This gene might therefore contribute to this multisystem disease.

EXPERIMENTAL

cDNA cloning

The original cDNA fragment, Ap-2, was isolated from a HeLa expression cDNA library by using purified Rb as a probe [18]. It was used to screen further for longer cDNA clones. Owing to the low abundance of Cream1 cDNA in the libraries used, the 5' coding sequences were obtained by PCR with plasmid DNA from a placenta cDNA library (Clontech) and the Marathon cDNA from human fetal spleen (Clontech) as templates. PCR fragments were subcloned into pGEM-T Easy Vector (Promega) and selected by Southern blotting. To avoid the possibility that mutations were introduced by PCR, at least three independent clones for each product were sequenced. cDNA fragments with sequences shared by the majority of clones were then selected to construct the full-length cDNA.

Plasmid construction

Plasmids for the expression of FLAG-tagged Cream1 in mammalian cells

To express full-length Cream1 tagged with the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys), the *NcoI*–*ScaI* restriction fragment (containing nt 6–2893) was ligated into the *NheI* site of pCEP4F vector [22] to create pFCream1 after fill-in reactions for both fragments with Klenow enzyme. pFAP32 was constructed in a similar way by using the *NcoI*–*ScaI* restriction fragment containing nt 762–2893 to express FLAG–Ap32 (containing residues 253–959). pFAP32N was constructed by PCR to express FLAG–Ap32N (containing residues 253–897).

Plasmids for the expression of FLAG-tagged Cream1 in *Escherichia coli*

To express FLAG–Ap32 in *E. coli*, pTrc-AP32 was constructed by inserting the 2.1 kb *NcoI*–*XhoI* restriction fragment from pFAP32 into pTrcHisA (Invitrogen) cleaved with the same restriction enzymes. To express full-length FLAG–Cream1, the full-length cDNA was ligated in frame into pTrcHisA to create pFLAP2. pFLAP2 was in turn cleaved with *PstI* and self-ligated to form pFLCream1p to express FLAG–Cream1p (containing residues 1–455).

Plasmids for assays in yeast

The *NcoI*–*ScaI* fragment (nt 6–2893) was inserted between the *NcoI* and *SaII* (filled in with Klenow) sites of pAS2-1 (Clontech) to express BD–Cream1 (containing residues 1–959; BD stands for the DNA-binding domain of Gal4). The *PstI* fragment was then deleted to express BD–Cream1p (containing residues 1–455). The *EcoRI* fragment of cDNA clone Ap-2 was introduced into pY1 [23] to express BD–Ap2 (containing residues 463–959).

Plasmids for expression of other proteins in *E. coli*

To express full-length TFII-I tagged with hexahistidine (His6–TFII-I), the 3.4 kb *NotI* (treated with mung bean nuclease)–*BamHI* restriction fragment from pI3-CX (kindly provided by Dr. R. G. Roeder, Rockefeller University, New York, NY, U.S.A.) was ligated to pTrcHisA (Invitrogen) linearized with *NheI* (filled in with Klenow enzyme) and *BamHI*. The predicted construct, pHisII-I, was identified by both restriction digestion and Western analysis. To express FLAG–E2F1 (containing residues 123–437), the 1.1 kb *BamHI*–*EcoRI* fragment from pGST-SH5 [18] was ligated into pFLAG1 (IBI) digested with *BglII* and *XhoI*; both the *EcoRI* and *XhoI* termini were filled in with Klenow enzyme before ligation. To express FLAG–mitotin10N, the 1.3 kb *EcoRI*–*HindIII* (filled in with Klenow enzyme) restriction fragment from pMBP-10/H [22] was cloned between *EcoRI* and *SaII* (filled in with Klenow enzyme) of pFLAG1 to create pF10N. To express His6–p56Rb, pGEX-2T-RBS, which expresses a functional 56 kDa form of Rb [1], was cleaved with *BamHI* and *EcoRI*. The cDNA insert was then introduced into pTrcHisA cleaved in the same way to create pTrcHisA-RBS. pGEX-2T-RBS was provided by Dr. W.-H. Lee (University of Texas Health Science Center at San Antonio, San Antonio, TX, U.S.A.).

Antibody production

The 0.5 kb *EcoRI*–*AvaI* (partly cleaved and filled in with Klenow enzyme) restriction fragment of Ap-2, containing nt 1394–1925, was inserted into pMAL-c vector (New England Biolabs) cleaved with *PstI* (treated with mung bean nuclease) and *EcoRI*. The resulting plasmid, pMAL-P05, was used to express maltose-binding protein (MBP) fusion protein MBP–P05. After affinity purification with amylose resin (New England Biolabs), the fusion protein was cleaved with Factor Xa (New England Biolabs). The immunogen (P05) was blotted to Westran PVDF membrane (Schleicher & Schuell) after SDS/PAGE [12% (w/v) gel]. The immunogen-containing portion was excised and used to immunize rabbits, as described [24]. In some cases, specific antibodies were further purified from antisera by using MBP–P05 immobilized on Hybond-C membrane (Amersham), as described [25].

Rb-binding assay *in vitro*

FLAG-tagged or His-tagged fusion proteins were expressed as described previously [22], with *E. coli* strain BL21 as host. His6–p56Rb was purified with 100 mM of EDTA (pH 8.0). Bacterial lysates containing FLAG–Cream1p, FLAG–Ap32, FLAG–mitotin10N and FLAG–E2F1 were subjected to SDS/PAGE [8% (w/v) gel] followed by electroblotting to Optitrans BA-S membranes (Schleicher & Schuell). The Rb-binding assay was performed *in vitro* as described previously [18] with minor modifications. The Rb sandwich was prepared by mixing 1 µg of His6–p56Rb, 1 µl of anti-hexahistidine monoclonal antibody

(mAb) (Sigma) and 1 μ l of alkaline phosphatase-conjugated goat anti-mouse IgG (Promega) into each 1 ml of binding buffer. Results were detected with CDP-Star chemiluminescent substrate (Promega).

Cell culture and transfection

Normal monkey kidney CV1, human gastric carcinoma SGC-7901, human lung adenocarcinoma SPC-A1 and human ovarian carcinoma HO-8901 cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) calf serum (Sijiqin Company, Hangzhou, China) in an air/CO₂ (19:1) atmosphere. Transfection was performed by the conventional calcium phosphate method.

Co-immunoprecipitation, immunoblotting and indirect immunofluorescence (IIF) studies

Approx. 2×10^7 CV1 cells co-transfected with pFCream1 and pCepRB (provided by Dr. W.-H. Lee) (1:1) were lysed in 1 ml of buffer A [20 mM Tris/HCl (pH 7.5)/100 mM KCl/0.1% (v/v) Nonidet P40/1 mM EDTA/1 mM dithiothreitol/10% (v/v) glycerol/50 mM NaF plus protease inhibitors]. After the removal of cell debris by centrifugation, half of the lysate was incubated overnight with 5 μ g of anti-Rb mAb 3C8 (provided by Dr. W.-H. Lee) at 4 °C; another half with the same amount of anti-CD4 mAb (Dako). Protein A-Sepharose (25 μ l) (Pharmacia) preincubated with 10 μ g of rabbit anti-mouse IgG (Anogen) was added for a further 2 h. The beads were then washed with 1 ml of buffer B [20 mM Tris/HCl (pH 7.5)/150 mM KCl/0.5% (v/v) Nonidet P40/1 mM EDTA/1 mM dithiothreitol/10% (v/v) glycerol/50 mM NaF plus protease inhibitors] three times. Samples were then subjected to SDS/PAGE [8% (w/v) gel] and immunoblotting.

Immunoblotting and IIF microscopy were performed as described previously [22], with appropriate primary antibodies. Enzyme-conjugated secondary antibodies were purchased from Promega. FITC-conjugated secondary antibodies were from Sino-America Biotechnology Company (Shanghai, China). Anti-FLAG M2 and anti-hexahistidine mAbs were from Kodak and Sigma respectively. In competition experiments, primary antibodies were incubated with 5 μ g of MBP-P05 or MBP for 30 min before immunoblotting or IIF staining. IIF images were recorded on Kodak Gold III (ASA 400) film with an Olympus BX50 fluorescence microscope. Prints and other results were digitized with a U-Max vista-S6 scanner. Images for publication were organized with Adobe Photoshop software and exported from a Tektronix Phaser 450 image printer.

Yeast transformation and assay for β -galactosidase activity

We followed the protocols given by Clontech. Yeast strain Y190 (Clontech) was transformed with appropriate plasmids by using the lithium acetate method. Transformants were selected for their ability to grow on synthetic drop-out medium lacking tryptophan (SD/-Trp). β -Galactosidase activities were quantified by *o*-nitro- β -D-galactopyranoside assays of randomly picked colonies.

RESULTS

Cream1 is a novel protein related to TFII-I

The deduced amino acid sequence from clone Ap-2 (Figure 1) suggests a novel polypeptide with three conserved repeats of 86 residues each. We therefore named the corresponding full-length protein Cream1 (for containing repetitive eighty-six amino-acid

Figure 1 Primary sequence of Cream1

(A) Diagrams showing overlapping cDNA clones. Clone S22 has a G base deletion at position 1406 (indicated by an asterisk); the deletion of nt 1974–2018 in clone S21 is also shown. Filled boxes represent non-coding regions; restriction sites used for construction of plasmids are marked. (B) Deduced amino acid sequence. A potential MAPK phosphorylation site and the polyserine tract are both underlined. The putative NLS is boxed.

motif). Because Northern blotting indicated a ubiquitously expressed mRNA of approx. 3 kb (results not shown), a retinoblastoma Y79 cDNA library was screened with ³²P-labelled Ap-2 as a probe. The two longest clones, S21 (2.2 kb) and S22 (2.2 kb), were studied further (Figure 1A). Sequence analysis revealed that clone S21 contained a deletion of nt 1974–2018 that nevertheless did not disrupt the reading frame (results not shown), possibly owing to alternative splicing. Clone S22, in contrast, lacked a G base at position 1406, resulting in a shift in reading frame. Further screening with several other cDNA libraries did not result in longer fragments. We therefore used a PCR approach to exhaust a human placenta cDNA library (Clontech) by using one primer fixed on the vector and another primer located in the 5' region of S22. It seemed that the longest PCR product (B18, 0.6 kb) still lacked some coding sequences of approx. 200 bp in comparison with the expressed sequence tag (EST) sequence of mouse clone 555547 (GenBank accession no. AA111609; 483 bp). This EST probably represented the extreme 5' sequence of the murine counterpart owing to the existence of two stop codons upstream of the putative first methionine residue (results not shown). Then a 320-bp PCR fragment (clone A1) was obtained with Marathon cDNA from human fetal spleen (Clontech) as templates (Figure 1A). The longest overlapping sequences were therefore 3043 bp with an open reading frame encoding 959 amino acid residues (Figure 1B). According to the mouse EST, this sequence should contain the entire open reading frame for Cream1. A cDNA clone was then constructed accordingly, in which nt 1–289 were from clone A1, 290–762 from clone B18, 763–1239 from clone S22, and 1240–1522 from clone S21; the

Figure 2 Sequence characteristics of Cream1 and its gene structure

(A) Diagrams showing structural characteristics of Cream1 and TFII-I. Boxes with identical fill patterns represent homologous regions. (B) Sequence alignments for the repeats of Cream1 (Cr1–Cr5) and comparison with those of TFII-I (Tr1–Tr6). Similar residues (L = V = I, Y = F, S = T, K = R and E = D) are boxed. In the consensus sequence, residues appearing most frequently are listed on the first line; other similar residues are listed sequentially underneath. The most conserved region is underlined. (C) Sequence alignments between conserved regions Ccr1–Ccr3 (from Cream1) and Tcr1–Tcr3 (from TFII-I). The hydrophobic residues in the conserved hydrophobic zipper-like motifs are underlined. (D) Structure of *CREAM1*. The gene structure is depicted as four boxes with three intervals of unknown lengths. The first and last nucleotides in each exon are indicated by a pair of numbers referring to their positions in cDNA. The first exon was determined from the cDNA sequence of MusTRD1 [27]. Exons coding for Ccr1–Ccr3 and Cr1–Cr5 are indicated. The lengths of exons are not drawn to scale.

remaining nucleotides from clone Ap-2. All these fragments had been sequenced to exclude unexpected sequence changes.

Cream1 shared distinct similarity with the multifunctional transcription regulator TFII-I (also named BAP-135 and SPIN) (Figure 2) [10,11,15]. First, Cream1 contained a group of five conserved repeats, Cr1–Cr5, which were similar to the six repeats of TFII-I, Tr1–Tr6 (Figures 2A and 2B). In fact, only an 80-residue region within each repeat (underlined in Figure 2B) was conserved between both proteins. Secondly, the N-terminal portion of Cream1, residues 30 to 88 [i.e. conserved region 1 (Ccr1)], was also highly homologous to the corresponding Tr1 from TFII-I, residues 22–80 (Figures 2A and 2C); both Ccr1 and Tr1 contained a conserved hydrophobic zipper-like motif implicated in protein–protein interaction (Figure 2C) [10]. Moreover, Ccr2 (residues 499–510) and Ccr3 (residues 527–532) also had their counterparts, Tcr2 (residues 801–812) and Tcr3 (residues 224–229) respectively, in TFII-I (Figures 2A and 2C). On the basis of these similarities, both proteins apparently belonged to a novel protein family. Nevertheless, Cream1 also possessed other unique features. Among them were a tract of 12 serine residues (residues 912–923) and a putative nuclear localization signal (NLS) (residues 898–905; Figure 1B). This NLS closely resembled that of the large T antigen of SV40 [26].

Interestingly, a cDNA coding for a novel muscle-enhancer-binding protein, MusTRD1 [27], has emerged as being basically identical with Cream1 cDNA. However, MusTRD1 is only 458 residues long, mainly due to a reading frame shift: there is an

extra G base insertion within the region equivalent to 1307–1309 of Cream1 cDNA. MusTRD1 therefore contains only the first two repeats of Cream1. On the basis of sequence analyses of clone S21, clone S22 and all the available ESTs, none of the clones contained the G insertion characteristic of MusTRD1 cDNA (results not shown). This difference in MusTRD1 could be due to a specific form of mRNA in muscles, although we cannot at present exclude the possibility of a mutation or a sequencing artifact.

Gene structure and locus

We also determined the complete gene structure for Cream1 (Figure 2D). The structural gene, *CREAM1*, was found to lie in genomic clones DJ0665P05 (approx. 133 kb; GenBank accession AC004851) and DJ1186P10 (approx. 149 kb; GenBank accession AC005231). Both clones were sequenced to near completion and released to GenBank by Dr. R. H. Waterston (Genome Sequencing Center, Washington University School of Medicine, St. Louis, MO, U.S.A.). *CREAM1* was located in four separate contigs and the lengths of gaps between these contigs were at that time unknown. This gene was composed of 27 exons spanning at least 150 kb (Figure 2D and Table 1). Exon 2 contained the first ATG codon and, together with exon 3, encoded the hydrophobic zipper-like region Tcr1. Coding sequences for the conserved regions Ccr2 and Ccr3 resided in exons 13 and 14 respectively. In addition, each Cream1 repeat

Table 1 Exon–intron boundaries in *CREAM1*

Exon sequences are in upper case letters, and intron sequences are in lower case letters.

Exon	Sequence at exon/intron junction			
No.	Size (bp)	5' splice donor	3' splice acceptor	Intron size (bp)
1	> 31*	G ¹ /gtaggagaaa	ctccccacag/G ² CGACC	53 885
2	129	TCCATG ¹³⁰ /gtgagtgctc	cttccccacag/T ¹³¹ GCTCA	≥ 4 549†
3	142	TCTGCC ²⁷² /gtgagtagcc	tgccccacag/G ²⁷³ AGGGC	2 469
4	156	TTTATA ⁴²⁸ /gtaagatcct	tccccacag/G ⁴²⁹ CGAGG	2 544
5	184	CAAGAG ⁶¹² /gtgagtgagg	ctttactcag/G ⁶¹³ CCACT	1 088
6	311	GTTGTG ⁹²³ /glaacattgc	cctgttcacg/G ⁹²⁴ ACAGA	1 490
7	90	AGTCAG ¹⁰¹³ /gtaggacag	gttttgatg/A ¹⁰¹⁴ GAAGT	2 775
8	84	GATATG ¹⁰⁹⁷ /gtgagtgagg	gtctttccag/C ¹⁰⁹⁸ GGAAG	≥ 6 219†
9	184	TAAGAG ¹²⁸¹ /gtcgggtggg	ctctccccag/G ¹²⁸² ATGTT	5 197
10	26	TCACAG ¹³⁰⁷ /gtatgtgggg	ctccccacag/G ¹³⁰⁸ GAACA	1 029
11	109	TGCTCG ¹⁴¹⁶ /gtagggcccc	cccttcacag/G ¹⁴¹⁷ TCAGA	1 862
12	38	GGCCAG ¹⁴⁵⁴ /gtgagaagga	ctccttgacg/G ¹⁴⁵⁵ AACCT	508
13	81	TCCAG ¹⁵³⁵ /gtaagggacg	ttgtcttcag/A ¹⁵³⁶ CCCTT	1 120
14	90	TGACAG ¹⁶²⁵ /gtaagaaatg	tctcttttag/A ¹⁶²⁶ CAAAG	3 013
15	48	TGGAGG ¹⁶⁷³ /gtgagggcct	tgctcttcag/A ¹⁶⁷⁴ CAGCC	943
16	66	GATACG ¹⁷³⁹ /gtgagcaaga	gtctctgcag/C ¹⁷⁴⁰ CAAGG	1 295
17	184	CAAGAG ¹⁹²³ /gtaagggcca	tggtttccag/G ¹⁹²⁴ CCCGA	7 888
18	50	GTCAG ¹⁹⁷³ /gtacccagcg	tgggtcccag/G ¹⁹⁷⁴ AACTG	171
19	102	TTCAAG ²⁰⁷⁵ /gtaaggttga	cgctttgcag/A ²⁰⁷⁶ AAATT	1 155
20	84	AATACG ²¹⁵⁹ /gtaagcagtg	tcgccccacg/G ²¹⁶⁰ GGAAG	1 139
21	184	CACCAG ²³⁴³ /gtactcagtg	tctctcttag/G ²³⁴⁴ CCTTT	583
22	29	AGCCTG ²³⁷² /gtaagaggca	cctctgttag/A ²³⁷³ TGAAG	≥ 18 306†
23	78	AATACG ²⁴⁵⁰ /gtcagtgctct	tgcccttcag/G ²⁴⁵¹ TGAGG	898
24	193	GCTCCA ²⁶⁴³ /gtgagtgccc	tccttttcag/A ²⁶⁴⁴ CCCTT	3 996
25	38	TGCCAG ²⁶⁸¹ /gtgagtcaga	ctccttcacg/C ²⁶⁸² CAAAG	5 993
26	137	CTCGTG ²⁸¹⁸ /gtaaggttgc	ctctctcag/C ²⁸¹⁹ AATGG	1 186
27	> 216	AAAAAA ³⁰⁴³		

* Based on sequences matched between *CREAM1* genomic DNA and MusTRD1 cDNA.

† Introns containing gaps of unknown lengths.

was encoded by three adjacent exons. For instance, Cr1 was encoded by exons 4–6, Cr2 by exons 8–10, Cr3 by exons 16–18, Cr4 by exons 20–22 and Cr5 by exons 23–25. In each set of three exons, the length of the middle one was fixed to 184 bp except for exon 24, which was 193 bp (Figure 2D and Table 1).

Cream1 cDNA corresponds to human UniGene Hs.21075. The corresponding gene has been mapped to human chromosome 7q11.23 in the GeneMap'98 issued at the NIH web site (<http://www.ncbi.nlm.nih.gov>). The sequence-tagged site (sts-AA019591) used to map *CREAM1* lay partly in the intron between exons 26 and 27 and extended further into exon 27 (results not shown). Interestingly, the *CREAM1* locus was sandwiched between the genes for LIM-kinase and syntrophin 1A in GeneMap'98. The frequent deletion of these two genes in Williams' syndrome [28,29] suggests that *CREAM1* is also deleted in the disease.

Cream1 is a ubiquitously expressed protein of 120 kDa

To explore the biochemical properties of Cream1, we generated rabbit polyclonal antibodies by using bacterially expressed polypeptide antigen (P05) containing residues 463–640. An MBP fusion (MBP–P05) of approx. 70 kDa was expressed (Figure 3A, lane 1). After treatment with Factor Xa, MBP–P05 was cleaved into two portions: MBP alone (approx. 40 kDa) and P05 (Figure 3A, lane 2). P05 appeared as a doublet of approx. 30 and 31 kDa, possibly owing to one or more alternative cleavage sites of Factor Xa or to degradation. Because the cDNA insertion did not disrupt the reading frame of the *LacZ* gene designed for α -

Figure 3 Specificity of anti-Cream1 antibody T8

(A) Production of rabbit polyclonal anti-Cream1 antibody. Protein samples were subjected to SDS/PAGE [12% (w/v) gel] followed by either staining with Coomassie Blue (lanes 1 and 2) or immunoblotting (lanes 3 and 4). Purified MBP–P05 (5 μ g) (lane 1) was cleaved with Factor Xa to separate P05 from MBP (lane 2). Cleaved product (1 μ g) was immunoblotted with preimmune serum (lane 3) or antiserum T8 (lane 4) respectively at 1:2000 dilution. (B) Antiserum T8 is specific for Cream1. Total bacterial lysates containing a Cream1 mutant, FLAG–Ap32 (lanes 1, 3 and 5), and hexahistidine-tagged full-length TFII-I, His6–TFII-I (lanes 2, 4 and 6), were subjected to SDS/PAGE [8% (w/v) gel] and immunoblotting. Anti-Cream1 antibody T8 recognized only FLAG–Ap32 (lane 1), not TFII-I (lane 2). Identical samples in lanes 3 and 4 and in lanes 5 and 6 were immunoblotted with anti-hexahistidine or anti-FLAG M2 mAb respectively. The positions of molecular mass markers are indicated (in kDa) at the left of both panels.

complementation in the vector pMAL-c (New England Biolabs), P05 also contained a portion of β -galactosidase (approx. 60 residues). As shown in Figure 3(A), preimmune serum did not detect any proteins (lane 3). The immune serum specifically recognized both the doublet of P05 and the trace amount of intact MBP–P05, but not MBP despite its high abundance (Figure 3A, lane 4).

Because P05 contained both the third repeat and two additional regions homologous with TFII-I (Figures 1 and 2), we examined whether the antibody could cross-react with TFII-I. As shown in Figure 3(B), the Cream1 mutant FLAG–Ap32 was specifically detected as a protein of approx. 90 kDa in total bacterial lysate by either the anti-Cream1 polyclonal antibody T8 (lane 1) or anti-FLAG M2 mAb (lane 5). However, neither of these two antibodies recognized the full-length hexahistidine-tagged TFII-I (His6–TFII-I) (Figure 3B, lanes 2 and 6). In contrast, anti-hexahistidine mAb faithfully detected this TFII-I as a 120 kDa protein (Figure 3B, lane 4). These results indicate that the anti-Cream1 antibody T8 was highly specific.

This antibody was then used to detect endogenous Cream1 in African green monkey CV1 cells (Figure 4). It recognized both MBP–P05 in bacterial lysate (Figure 4A, lane 1) and a protein of approx. 120 kDa in CV1 total cell lysate (lane 2). To examine whether this 120 kDa protein was Cream1, the antibody was preincubated with either MBP–P05 or MBP expressed from

Figure 4 Cream1 is a 120 kDa protein in CV1 cells

(A) Identification of cellular Cream1. MBP-P05 was loaded as a positive control in lanes 1, 3 and 5. Lysates equivalent to 10^6 CV1 cells were loaded in lanes 2, 4 and 6. Blots containing lanes 1 and 2, 3 and 4, and 5 and 6 were immunoblotted sequentially with affinity-purified anti-Cream1 T8, T8 preincubated with $5 \mu\text{g}$ of MBP-P05, and T8 preincubated with $5 \mu\text{g}$ of MBP. The specific protein bands of approx. 120 kDa in lanes 2 and 6 represented cellular Cream1. (B) Co-migration of cellular Cream1 in CV1 cells (lane 1) with FLAG-Cream1 expressed in *E. coli* (lane 2). Additional bands in lane 2 were degradation products. The blot was immunoblotted with anti-Cream1 antibody T8 and detected with chemiluminescent reagent CDP-Star. The positions of molecular mass markers are indicated (in kDa) at the left.

pMAL-c vector. The antibody neutralized by MBP-P05 failed to detect either MBP-P05 (Figure 4A, lane 3) or the 120 kDa protein (lane 4). However, preincubation with MBP had no effect on the antibody (Figure 4A, lanes 5 and 6). These results indicated that Cream1 in CV1 cells migrated as a protein of approx. 120 kDa on SDS/PAGE. In addition, the same protein was also detected in a variety of human cell lines and tissues, including embryonic kidney HEK 293, gastric carcinoma SGC-7901, lung adenocarcinoma SPC-A1, ovarian carcinoma HO-8901, fetal and adult skeletal muscles, heart and fetal brain (results not shown). Generally, the protein levels in all the tested samples were quite low.

To examine whether the Cream1 cDNA contained the entire open reading frame, we also compared the migration rates of the cellular Cream1 and FLAG-tagged full-length Cream1 (FLAG-Cream1) expressed in *E. coli* (Figure 4B). FLAG-Cream1 (Figure 4B, lane 2) migrated closely to and a little more slowly than endogenous Cream1 in CV1 cells (lane 1) owing to the existence of the FLAG tag. These results confirmed that our cDNA contained the complete coding sequence for Cream1.

Cream1 binds to Rb both *in vitro* and *in vivo*

Cream1 was isolated as a candidate of Rb-associated proteins that was previously named Rbap2 [18]. To confirm the screening results, we tested its interaction with Rb in a similar assay. For convenience we used affinity-purified hexahistidine-tagged Rb (His6-p56Rb) to prepare the 'Rb sandwich' (Figure 5A). FLAG-mitosin10N, which contained residues 2484–2927 of mitotin (Figure 5B, lane 1), served as a negative control (Figure 5C, lane 1) [22]. FLAG-E2F1, which contained residues 123–437 (Figure 5B, lane 3), was used as a positive control (Figure 5C, lane 3) [18]. Two overlapping deletion mutants of Cream1, FLAG-Cream1p and FLAG-Ap32, were tested (Figure 5D).

Figure 5 Interaction of Cream1 with Rb *in vitro*

(A) The hexahistidine-tagged Rb used in this assay, His6-p56Rb, was purified from bacterial lysate and subjected to immunoblotting with anti-hexahistidine mAb after SDS/PAGE [10% (w/v) gel]. (B) A blot probed with anti-FLAG M2 antibody. Total bacterial lysates containing the indicated FLAG fusion proteins were subjected to SDS/PAGE [8% (w/v) gel] and immunoblotting. (C) An identical blot was probed with the Rb-sandwich, i.e. an immunocomplex formed by His6-p56Rb, anti-hexahistidine antibody, and alkaline phosphatase-conjugated secondary antibody. Arrows indicate positions of FLAG fusion proteins in both blots. Other unmarked bands are due to bacterial proteins in lysates. The positions of molecular mass markers are indicated (in kDa) at the left. (D) Diagrams of the Cream1 mutants used in this assay.

Figure 6 Interaction of Cream1 with Rb *in vivo*

CV1 cells were co-transfected for 48 h with plasmids expressing Rb and FLAG-tagged Cream1. Cell lysates were subjected to co-immunoprecipitation (IP) and Western blotting with the indicated antibodies. Cream1 (lane 3) was readily co-immunoprecipitated with Rb (lane 1) when anti-Rb mAb was used. In contrast, control antibody anti-CD4 mAb failed to precipitate either Rb (lane 2) or Cream1 (lane 4). The positions of molecular mass markers are indicated (in kDa) at the left.

FLAG-Ap32 (Figure 5B, lane 2) clearly interacted with the Rb probe (Figure 5C, lane 2). However, FLAG-Cream1p (Figure 5B, lane 4) was negative (Figure 5C, lane 4). Because the

Figure 7 Identification of the NLS within Cream1

Plasmids expressing FLAG-Ap32 and FLAG-Ap32N were transfected into CV1 cells. At 48 h after transfection, cells were fixed in cold methanol and subjected to double IIF staining with anti-FLAG M2 [panels 1, 3 and 5 in (B)] and rabbit anti-Cream1 T8 [panels 2, 4 and 6 in (B)] antibodies. (A) Diagrams of the two Cream1 mutants. (B) Immunofluorescence images. Panels 1 and 2, cells expressing FLAG-Ap32. Panels 3 and 4, 5 μ g of MBP-P05 was included to neutralize anti-Cream1 antibody selectively. Panels 5 and 6, a typical cell expressing FLAG-Ap32N. Scale bar, 10 μ m.

Figure 8 Schematic diagrams for BD-Cream1 fusion proteins

The transactivation activities of these constructs are shown in Table 2.

polypeptide encoded by clone Ap-2 spanned residues 463–959, these results confirmed that Rb specifically interacted with this C-terminal portion of Cream1 *in vitro*.

Owing to low levels of endogenous Cream1, we co-expressed FLAG-Cream1 and Rb in CV1 cells to boost the local concentrations of these two proteins. The proper expression of FLAG-Cream1 was monitored by IIF staining (results not shown). When the lysate from these cells was subjected to co-immunoprecipitation with anti-Rb mAb (Figure 6, lane 1), FLAG-Cream1 was readily detected with anti-FLAG1 mAb M2 in multiple experiments (lane 3). In contrast, a control antibody (anti-CD4 mAb) failed to pull down either Rb (Figure 6, lane 2) or Cream1 (lane 4). However, reciprocal experiments were not successful because for some reason neither anti-Cream1 nor anti-

Table 2 Transactivation activity of Cream1

Yeast cells (strain Y190) transformed with indicated plasmids were subjected to assays with *o*-nitro- β -D-galactopyranoside. The β -galactosidase activity (1 unit = amount of enzyme hydrolysing 1 μ mol substrate/min per cell) for each transformation is presented as the mean $\pm$ S.D. for three randomly picked colonies. pAS2-1 and pY1 were control plasmids that expressed only BD. See Figure 8 for diagrams of the fusion proteins encoded by these plasmids.

Plasmid	β -Galactosidase activity	
	(units)	(Fold over background)
pAS-Cream1	1.99 $\pm$ 0.33	32
pAS-Cream1p	0.65 $\pm$ 0.05	10
pAS2-1	0.06 $\pm$ 0.02	0
pY1-Ap2	0.22 $\pm$ 0.03	1
pY1	0.12 $\pm$ 0.01	0

FLAG antibody was able to immunoprecipitate Cream1 or its FLAG fusion (results not shown).

NLS of Cream1 resembles that of the large T antigen of SV40

As a potential transcription regulator, Cream1 should be a nuclear protein. Nuclear localization is also essential for interaction with Rb. On the basis of the amino acid sequence, Cream1 contained a putative NLS similar to that of the large T antigen of SV40 (Figure 1B). However, cellular Cream1 was not detected by IIF microscopy in cell lines including CV1, HEK 293, SGC-7901, SPC-A1 and HO-8901 (results not shown), in agreement with the low expression levels implied by immunoblotting results (Figure 4, and results not shown). We then transiently expressed FLAG-Ap32 (Figure 7A) into CV1 cells. Indeed, FLAG-Ap32 exhibited typical nuclear localization when detected with both anti-FLAG antibody (Figure 7B, panel 1) and the anti-Cream1 antibody T8 (panel 2). To exclude a possible cross-reaction between antibodies during the labelling process, MBP-P05 was added to the mixture of primary antibodies to neutralize anti-Cream1 antibody selectively. This treatment did indeed significantly decrease the labelling by anti-Cream1 antibody T8 (Figure 7B, panel 4) without disturbing that by anti-FLAG antibody (panel 3), indicating that these two antibodies recognized their own epitopes independently. Nuclear localization was also observed when the full-length FLAG-Cream1 was expressed (results not shown). When the putative NLS was deleted, the corresponding mutant, FLAG-Ap32N (Figure 7A), became totally cytoplasmic (Figure 7B, panels 5 and 6), suggesting that Cream1 did indeed use the sequence as the NLS.

Cream1 has an intrinsic activating activity

To examine whether Cream1 contains an activation domain, we fused different portions of Cream1 with BD (DNA-binding domain of Gal4) and investigated the trans-activation activities of the resulting molecules in yeast. Yeast strain Y190 contains a reporter gene for β -galactosidase under the control of a Gal4-responsive promoter. As shown in Figure 8 and Table 2, the expression of BD-Cream1 in Y190 did indeed strongly stimulate the transcription of the reporter gene as judged by β -galactosidase activities. BD-Cream1p, which contained residues 1–455 of Cream1, was still active. In contrast, the fusion protein containing residues 463–959, i.e. BD-Ap2, showed little trans-activation activity over the background. An activation domain therefore seemed to reside in the N-terminal half of Cream1.

DISCUSSION

Cream1 and TFII-I are distinct members of a novel transcription regulator family

The 80-residue repeats and other conserved regions shared by Cream1 and TFII-I suggest that these two proteins fall into a novel protein family, possibly the TFII-I family. MusTRD1 [27] probably resulted from an alternatively spliced mRNA transcribed from *CREAM1*, although its existence still requires further investigation. TFII-I binds to a variety of DNA sequences including initiator and initiator-like (SIE and serum-responsive element) sites, and the E-box element [10–12]. It is important for the transcription regulation of several cellular genes, including *c-fos* and T-cell receptor [12,14]. MusTRD1, in contrast, reportedly binds to an enhancer element (USE B1) that is similar to SIE, with binding site requirements distinct from that of TFII-I [27]. From the sequence identity, Cream1 would also be a potential binding protein for USE B1.

The structural genes of TFII-I (i.e. *GTF2I*) [17] and Cream1 (i.e. *CREAM1*) were related. Both gene loci were close together at human chromosome 7q11.23 according to GeneMap'98. Exon organizations were also similar: when multiple exons were required to encode a conserved region, their junctions were usually conserved in both genes. This was true for the junctions within sequences coding for Ccr1 and Tcr1, and all of the first junctions and most of the second junctions within sequences coding for Ccr1–5 and Tcr1–6 (Figure 2D and Table 1) [17]. In addition, each of the sequences coding for Ccr2, Ccr3, Tcr2 and Tcr3 was located within one short exon. Evidently the positions of these exons were subjected to independent rearrangements (exon shuffling) during the evolution of *CREAM1* and *GTF2I*, resulting in altered locations of Ccr2 and 3 in contrast with Tcr2 and 3 (Figure 2) [17].

Potential function of Cream1

Cream1 is expressed ubiquitously as a 120 kDa protein in cell lines and tissues of various origins (Figure 4, and results not shown). Its structural similarity to TFII-I further suggests itself to be a general transcription regulator, possibly a novel initiator-binding factor to mediate the transcription of certain TATA-less promoters. However, its identity with MusTRD1 also implies a function in slow muscle fibres [27]. To our surprise, the levels of Cream1 in skeletal muscles, including muscle samples from fetus and adults, were very low (results not shown). Such results were contradictory to the specific expression of the MusTRD1 gene (*CREAM1*) in skeletal muscles [27]. One possibility is that the half-life of Cream1 in muscles could be short. The steady-state levels of the protein were therefore low, despite high levels of synthesis. Another explanation could be that the major isoform in muscles is MusTRD1 instead of Cream1. After all, the extra G base that results in premature termination of the coding sequence in MusTRD1 cDNA could be due to alternative splicing because it lay at the junction between exons 10 and 11 of *CREAM1* (Figure 2 and Table 1) [27]. This explanation, however, still depends on the further characterization of MusTRD1 because no protein data for it are yet available to support the existence of such an isoform.

Both MusTRD1 and Cream1 might serve as positive transcription regulators. MusTRD1 cDNA was cloned by using the yeast one-hybrid system via its binding to USE B1, a regulatory DNA element located in the enhancer of slow-muscle-fibre-specific troponin I (*TnI_s*) promoter [27]. Moreover, Cream1 fused to BD was able to stimulate reporter gene transcription in the yeast system, indicating the existence of an activation domain

in the region shared by both MusTRD1 and Cream1. In contrast, our study showed that MusTRD1 lacks a valid NLS (Figure 7). A FLAG-tagged polypeptide resembling MusTRD1 also failed to enter the nucleus (results not shown). Consequently, MusTRD1, if it exists, has to find other ways in which to enter the nucleus. The relationship between these two proteins apparently still needs further investigation.

Regulation of Cream1 and TFII-I

The functions of Cream1 and TFII-I might be regulated by distinct mechanisms. TFII-I (i.e. BAP-135) binds to Btk, a B-cell-specific cytoplasmic tyrosine kinase of the Src superfamily [15]. On engagement of B-cell antigen receptors, TFII-I is transiently phosphorylated on tyrosine residues. Moreover, the stimulation of *c-fos* promoter by TFII-I depends on Ras-signalling pathways [14]. The Src-autophosphorylation sites of TFII-I are critical for the TFII-I-dependent transcription activation of *V β* promoter [16]. In contrast, Cream1 apparently lacked the Src-phosphorylation sites; neither did it interact with Btk in our hands (results not shown). Nevertheless, it could be regulated by MAP kinase through a potential phosphorylation site (Figure 1). In addition, our results suggest that Cream1 might be regulated through association with Rb. On the basis of its broad expression spectrum, Cream1 might act as a general transcription regulator involved in cell-cycle progression. However, Rb is also essential to terminal differentiation of skeletal muscles [6,7]. Depending on the function of MusTRD1 [27], Cream1 might have a role in muscle differentiation under the control of Rb.

Hemizyosity of *CREAM1* might contribute to Williams' syndrome

Williams' syndrome is a congenital disorder with characteristic elfin-like facial features and mental retardation, which is frequently associated with cardiovascular and musculoskeletal problems and many other traits [30,31]. Hemizyosity at chromosome 7q11.23 is responsible for the condition [30]. The breakpoints of common deletions are located approx. 1 cM on each side of the gene for elastin [32]. So far, almost a dozen genes have been reported to be hemizygous in most Williams' populations. Among them are genes for elastin [30], LIM kinase 1 [28], replication factor C [33], the human counterpart of the *Drosophila* frizzled Wnt receptor [34], syntaxin 1A [29], TFII-I [17], Wscr1 [35] and Fkbp6 [36]. Although a sufficient dose of LIM-kinase, a protein kinase mainly expressed in the brain, seems to be critical for the disease [28], the rest of these genes might also contribute to the pathogenesis of this syndrome.

Owing to its location between genes coding for LIM kinase 1 and syntaxin 1A in GeneMap'98, we speculated that one copy of *CREAM1* might also be deleted from the genotype of all people with Williams' syndrome hemizygous for these two genes. This speculation has been confirmed by the delineation of a gene, *WBSCR11*, in the region commonly deleted in Williams' syndrome [37]. *WBSCR11*, whose gene structure is also identified from the same genomic clones as we did for *CREAM1*, is apparently identical with *CREAM1*. However, the deduced amino acid sequence from the reported *WBSCR11* cDNA lacks some sequences encoded by exon 19 of *CREAM1*, a situation also found in cDNA clone S21 (Figure 1A). Both cDNA species therefore represent different mRNA isoforms of the same gene.

Owing to the expression of Cream1 in brain (results not shown), its decreased dose might affect the expression of one or more other proteins important for neuronal development. In contrast, *TnI_s* predominates in all muscle types during early fetal development and is restricted to both the slow muscle fibres and

the conductive tissues of heart after birth [38,39]. Given the role of MusTRD1 in regulating the expression of TnI_s [27], hemizygosity for *CREAM1* (WBSCR11) might also be correlated with certain cardiovascular and musculoskeletal abnormalities associated with Williams' syndrome.

We thank Qiongping Huang, Chi Zhang and Zhe Qu for technical assistance, Dr. R. H. Waterston (Genome Sequencing Center, Washington University School of Medicine, St. Louis, MO, U.S.A.) for releasing genomic clones containing *CREAM1* to GenBank, Dr. M. James (Wellcome Trust Centre for Human Genetics, Oxford, U.K.) for mapping sts-AA019591, R. G. Roeder (Laboratory of Biochemistry and Molecular Biology, Rockefeller University, New York, NY, U.S.A.) and S. Desiderio (Department of Molecular Biology and Genetics and Howard Hughes Institute, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.) for TFII-I and Bap-135 cDNA, and Dr. W.-H. Lee (University of Texas Health Science Center at San Antonio, San Antonio, TX, U.S.A.) for mentioned materials used in this study. This work was supported by grant no. 39870185 from the National Natural Science Foundation of China and research funds including KJ951-B1-608, STZ97-2-03, and the Genome Project (KJ95T-06) from the Chinese Academy of Sciences.

REFERENCES

- Goodrich, D. W. and Lee, W.-H. (1993) *Biochim. Biophys. Acta* **1155**, 43–61
- Weinberg, R. A. (1995) *Cell* **81**, 323–330
- Lin, S. C., Skapek, S. X. and Lee, E. Y. (1996) *Semin. Cancer Biol.* **7**, 279–289
- Tan, X. and Wang, J. Y. (1998) *Trends. Cell Biol.* **8**, 116–120
- Chen, P. L., Riley, D. J., Chen, Y. and Lee, W.-H. (1996) *Genes Dev.* **10**, 2794–2804
- Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V. and Nadal-Ginard, B. (1993) *Cell* **72**, 309–324
- Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J. D., Phillips, R. A. and Gallie, B. L. (1996) *Genes Dev.* **10**, 3051–3064
- Shih, H. H., Tevosian, S. G. and Yee, A. S. (1998) *Mol. Cell. Biol.* **18**, 4732–4743
- Roy, A. L., Malik, S., Meisterernst, M. and Roeder, R. G. (1993) *Nature (London)* **365**, 355–359
- Roy, A. L., Du, H., Gregor, P. D., Novina, C. D., Martinez, E. and Roeder, R. G. (1997) *EMBO J.* **16**, 7091–7104
- Grueneberg, D. A., Henry, R. W., Brauer, A., Novina, C. D., Cheriya, V., Roy, A. L. and Gilman, M. (1997) *Genes Dev.* **11**, 2482–2493
- Cheriya, V., Novina, C. D. and Roy, A. L. (1998) *Mol. Cell. Biol.* **18**, 4444–4454
- Roy, A. L., Carruthers, C., Gutjahr, T. and Roeder, R. G. (1993) *Nature (London)* **365**, 359–361
- Kim, D.-W., Cheriya, W., Roy, A. L. and Cochran, B. H. (1998) *Mol. Cell. Biol.* **18**, 3310–3320
- Yang, W. and Desiderio, S. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 604–609
- Novina, C. D., Cheriya, V. and Roy, A. L. (1998) *J. Biol. Chem.* **273**, 33443–33448
- Perez-Jurado, L. A., Wang, Y. K., Peoples, R., Coloma, A., Cruces, J. and Francke, U. (1998) *Hum. Mol. Genet.* **7**, 325–334
- Shan, B., Zhu, X., Chen, P.-L., Durfee, T., Yang, Y., Sharp, D. and Lee, W.-H. (1992) *Mol. Cell. Biol.* **12**, 5620–5631
- Mancini, M. A., Shan, B., Nickerson, J. A., Penman, S. and Lee, W.-H. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 418–422
- Ozaki, T., Saijo, M., Murakami, K., Enomoto, H., Taya, Y. and Sakiyama, S. (1994) *Oncogene* **9**, 2649–2653
- Cavanaugh, A. H., Hempel, W. M., Taylor, L. J., Rogalsky, V., Todorov, G. and Rothblum, L. I. (1995) *Nature (London)* **374**, 177–80
- Zhu, X., Mancini, M. A., Chang, K.-H., Liu, C. Y., Chen, C. F., Shan, B., Jones, D., Yang-Feng, T. L. and Lee, W.-H. (1995) *Mol. Cell. Biol.* **15**, 5017–5029
- Sadowski, I., Bell, B., Broad, P. and Hollis, M. (1992) *Gene* **118**, 137–141
- Knudsen, K. A. (1985) *Anal. Biochem.* **147**, 285–288
- Smith, D. E. and Fisher, P. A. (1984) *J. Cell. Biol.* **99**, 20–28
- Dingwall, C. and Laskey, R. A. (1991) *Trends Biochem. Sci.* **16**, 478–481
- O'Mahoney, J. V., Guven, K. L., Lin, J., Joya, J. E., Robinson, C. S., Wade, R. P. and Hardeman, E. C. (1998) *Mol. Cell. Biol.* **18**, 6641–6652
- Frangiskakis, J. M., Ewart, A. K., Morris, C. A., Mervis, C. B., Bertrand, J., Robinson, B. F., Klein, B. P., Ensing, G. J., Everett, L. A., Green, E. D. et al. (1996) *Cell* **86**, 59–69
- Osborne, L. R., Soder, S., Shi, X. M., Pober, B., Costa, T., Scherer, S. W. and Tsui, L. C. (1997) *Am. J. Hum. Genet.* **61**, 449–452
- Ewart, A. K., Morris, C. A., Atkinson, D., Jin, W., Sternes, K., Spallone, P., Stock, A. D., Leppert, M. and Keating, M. T. (1993) *Nat. Genet.* **5**, 11–16
- Lenhoff, H. M., Wang, P. P., Greenberg, F. and Bellugi, U. (1997) *Sci. Am.* **277**, 68–73
- Perez-Jurado, L. A., Peoples, R., Kaplan, P., Hamel, B. C. and Francke, U. (1996) *Am. J. Hum. Genet.* **59**, 781–792
- Peoples, R., Perez-Jurado, L., Wang, Y. K., Kaplan, P. and Francke, U. (1996) *Am. J. Hum. Genet.* **58**, 1370–1373
- Wang, Y. K., Samos, C. H., Peoples, R., Perez-Jurado, L. A., Nusse, R. and Francke, U. (1997) *Hum. Mol. Genet.* **6**, 465–472
- Osborne, L. R., Martindale, D., Scherer, S. W., Shi, X. M., Huizenga, J., Heng, H. H. Q., Costa, T., Pober, B., Lew, L., Brinkman, J. et al. (1996) *Genomics* **36**, 328–336
- Meng, X., Lu, X., Morris, C. A. and Keating, M. T. (1998) *Genomics* **52**, 130–137
- Osborne, L. R., Campbell, T., Daradich, A., Scherer, S. W. and Tsui, L. C. (1999) *Genomics* **57**, 279–284
- Sutherland, C., Elsom, V., Gordon, M., Dunwoodie, S. and Hardeman, E. (1991) *Dev. Biol.* **146**, 167–178
- Zhu, L., Lyons, G. E., Joya, J. E., Hardeman, E. C. and Wade, R. P. (1995) *Dev. Biol.* **169**, 487–503

Received 12 July 1999/18 October 1999; accepted 10 November 1999