[CANCER RESEARCH 53, 5535-5541, November 15, 1993]

Coamplification of the *CDK4* Gene with *MDM2* and *GLI* in Human Sarcomas[1]

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ABSTRACT

The 34-kilodalton cyclin-dependent kinase, $p34^{cdk4}$, is a major catalytic subunit of mammalian D-type cyclins, which act during the G_1 phase of the cell cycle to enforce the decision of cells to enter S phase. A murine complementary DNA clone was used to clone the cognate human *CDK4* gene, which was localized to human chromosome 12, band q13, by fluorescence *in situ* hybridization. Because this chromosomal band contains the *GLI* and *MDM2* genes, which are frequently amplified in human sarcomas, we analyzed *CDK4* copy number and expression in a panel of sarcoma cell lines. An osteosarcoma cell line, OsACL, manifested a 25-fold increased copy number of *CDK4*, amplified concordantly with both *GLI* and *MDM2*, whereas a rhabdomyosarcoma cell line, SJRH30, was found to have an amplicon that included *CDK4* and *GLI* but not *MDM2*. *CDK4* mRNA and protein were overexpressed in both cell lines, and nucleotide sequencing analysis indicated that the gene had not sustained mutations. These observations provide the first evidence for amplification of a gene encoding a cell division cycle protein kinase, complement recent data indicating that genes encoding D-type cyclins are targets of chromosomal rearrangement and gene amplification in tumor cells, and suggest that *CDK4* amplification might contribute to oncogenesis.

INTRODUCTION

Key cell cycle transitions in all eukaryotes are regulated by cyclins and their catalytic subunits, the CDKs.[3] The prototypic CDK, $p34^{cdc2}$ ($p34^{cdk1}$), together with its regulatory subunit, cyclin B, govern mitotic entry and exit (reviewed in Ref. 1), whereas cyclin A associates both with $p34^{cdc2}$ and a related catalytic partner, $p33^{cdk2}$, during the S and G_2 phases of the cell cycle and may coordinate the events that couple DNA replication to cell division (2-5).

Three recently described classes of mammalian cyclins (designated types C, D, and E) are expressed during G_1 phase (6-10), and both cyclins D and E have been inferred to regulate G_1 progression and entry into S phase (reviewed in Ref. 11). Active enzyme complexes consisting of cyclin E and $p33^{cdk2}$ accumulate periodically at the G_1-S phase boundary, and their appearance temporally precedes the formation of predominantly S phase-specific complexes between $p33^{cdk2}$ and cyclin A (12, 13). Both cyclin E and $p33^{cdk2}$ appear to regulate the G_1-S transition because the activity of $p33^{cdk2}$ is required for entry into S phase (14, 15), and enforced overexpression of cyclin E can shorten the G_1 interval (16).

In contrast, the D-type cyclins are encoded by three genes (6, 17-19), the expression of which is usually manifested earlier during G_1 phase than cyclin E. Cyclins D1, D2, and D3 are differentially expressed in various cell lineages in response to growth factor-induced signals (6, 17, 20-24), and they form complexes with a novel catalytic subunit, $p34^{cdk4}$, to activate its kinase activity (25, 26). Like cyclins A and E, cyclins D2 and D3, but not D1, can productively interact with $p33^{cdk2}$ (27, 28), whereas cyclins D1 and D3 also form complexes with $p31^{cdk5}$ in fibroblasts (28). Thus, the interactions of D-type cyclins with catalytic subunits are highly combinatorial and may serve to target the holoenzymes to different substrates, the phosphorylation of which are required for G_1 progression (11). For example, the D-type cyclins, but not cyclins E or A, can bind directly to pRb and to the pRb-related protein, p107 (26, 27, 29), and these interactions can direct the $p34^{cdk4}$ kinase toward pRb, enabling $p34^{cdk4}$/cyclin D complexes to phosphorylate pRb much more efficiently than other canonical CDK substrates, such as histone H1 (25, 26). Because pRb phosphorylation inactivates its growth suppressive function and accelerates G_1 exit (reviewed in Ref. 30), the D-type cyclin-dependent kinases may function in part in this manner. Data implicating D-type cyclins in G_1 progression have remained largely inferential, but recent results indicate that overexpression of cyclins D1 and D2 in fibroblasts can shorten their G_1 interval and decrease their dependency on serum growth factors (31). It may therefore prove that the D-type cyclins act upstream of cyclin E to commit cells to DNA synthesis.

Several lines of evidence implicate overexpression of the cyclin D1 gene, located on human chromosome 11, band q13 (8, 18, 19), in oncogenesis. Cyclin D1 was independently isolated (as *PRAD1*) at the breakpoint of a chromosome 11 inversion in human parathyroid adenomas, where its intact coding sequences are fused to the parathormone promoter, resulting in its deregulated overexpression (8, 32). Cyclin D1 (alias *BCL1*) is overexpressed as a result of a chromosomal translocation, t(11;14)(q13;q32), that is commonly found in centrocytic B-cell lymphomas (33-35) and pursuant to gene amplification in 15-20% of human breast cancers and squamous cell tumors of the head and neck (36-38). Expression of cyclins D1 and D2 can also be inappropriately induced by retroviral insertions in rodent lymphoid malignancies (39, 40). A reasonable hypothesis is that deregulated expression of D-type cyclins can contribute to tumor formation by interacting with CDKs, the kinase activities of which are rate limiting for G_1 progression.

We therefore reasoned that *CDK4*, a unique D-type cyclin partner, might be a target for genetic alterations leading to aberrant cell proliferation in some human cancers. As a first step in investigating its potential role in tumorigenesis, we determined the chromosomal location of *CDK4* and assigned the gene to band q13 of human chromosome 12. This region, which also contains the *GLI* and *MDM2* protooncogenes, is rearranged or amplified in a variety of human sarcomas, such as malignant fibrous histiocytoma, rhabdomyosarcoma, osteosarcoma, and liposarcoma (41-45). Our results now demonstrate that *CDK4* is included in the 12q13 amplicon and is overexpressed in sarcoma cell lines with gene amplification.

MATERIALS AND METHODS

Genomic DNA Cloning. Bacteriophage and cosmid human genomic DNA libraries were screened with a ^{32}P-labeled 1.3-kilobase *Eco*RI fragment of the murine *CDK4* cDNA (25). Hybridization was performed overnight at 42°C in buffer containing 5× standard saline citrate (1× standard saline citrate is 0.15 NaCl-0.015 sodium citrate, pH 7.0), 50% formamide, 1.0% sodium dodecyl

Received 7/9/93; accepted 9/14/93.

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[1] Supported in part by Grants PO1-CA-23099 (A. T. L., D. N. S.), R35-CA-47064 (C. J. S.) and Cancer Center Support (CORE) Grant CA-21765 from the National Cancer Institute, NIH, and by the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital.

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[3] The abbreviations used are: CDK, cyclin-dependent kinase; cDNA, complementary DNA; PCR, polymerase chain reaction; pRb, retinoblastoma protein.

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sulfate, 4 × Denhardt's reagent, salmon sperm DNA (40 μg/ml), and 0.01 Tris HCl (pH 7.5). The membranes were washed twice in 0.1 × standard saline citrate and 0.1% sodium dodecyl sulfate at 42°C for 20 min, and autoradiograms were developed after exposure for 16 to 72 h at -70°C. Overlapping genomic clones were isolated, and a 4.0-kilobase *MboI-BamHI* fragment that hybridized with the cDNA probe was subcloned into a Bluescript SK+ plasmid vector. A portion of the 4.0-kilobase fragment was amplified by PCR, and the product was sequenced and used as a human *CDK4* probe. The PCR amplimers, prepared based on the human *CDK4* cDNA sequence (46), were 5'-CATGTAGACCAGGACCTAAGG-3' (sense strand) and 5'-GGAGGTCGG-TACCAGAGTG-3' (antisense strand), and reactions performed with templates comprising either the cloned 4.0-kilobase *CDK4* fragment or total human DNA yielded a fragment of 534 base pairs, which was designated pK4-PCR. Amplification was performed in a 100-μl reaction volume containing 200 μmol/liter deoxynucleotide triphosphates, 200 ng of each primer, 2.5 units of Taq DNA polymerase (added after heating the reaction mix to 95°C for 1.5 min), and 50 ng of either human genomic DNA or the subcloned *BamHI-MboI* genomic fragment in PCR buffer (Perkin-Elmer Cetus, Norwalk, CT) for 40 cycles (denaturation at 95°C for 1 min; annealing at 60°C for 2 min; extension at 72°C for 3 min) in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The nucleotide sequences of PCR products were determined by the dideoxynucleotide chain termination method (47) as applied to double-stranded plasmid DNA templates.

Cell Lines. Childhood sarcoma cell lines, derived at St. Jude Children's Research Hospital, included six rhabdomyosarcomas (SJRH1, SJRH4, SJRH5, SJRH18, SJRH28, SJRH30), five Ewing's sarcomas (SJES1, SJES4, SJES5, SJES7, SJES8), two osteosarcomas (OsA-CL and SJOS2), and one undifferentiated sarcoma (SJSA2). The histopathological and cytogenetic features of lines OsA-Cl (an atypical osteosarcoma resembling a multipotential tumor of bone) and SJRH30 (formerly called RH13) were previously reported (44); SJES4, SJES7, and SJES8 were described as cases 1, 2, and 12 (48); SJES1 as case 22 (49); and SJRH4, SJRH18, SJRH28, and SJRH30 as cases 11, 3, 4, and 13, respectively (50). An established human lymphoblastoid cell line, CJTW, was used as a normal control.

Southern and Northern Blotting. DNAs were digested with *EcoRI* and quantitated by a fluorimetric assay using 4,6-diamidine-2-phenylindole to ensure equal loading in all lanes (44). Electrophoresis was performed in 0.8% agarose gels, and the DNA samples were transferred to nylon membranes by capillary blotting. Probes radiolabeled by random priming with [^{32}P]CTP (NEN/Dupont, Wilmington, DE) were the human *CDK4* genomic PCR product (pK4-PCR), a 1.3-kilobase *GLI* fragment from the plasmid pKK36P1, and a human *MDM2* cDNA (both provided by Dr. B. Vogelstein). The degree of amplification of *CDK4* was determined by densitometric analysis of the autoradiogram with a Bioimage Visage II analyzer (Eastman Kodak, Rochester, NY). Total cellular RNA samples (25 μg/lane) were separated by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde, transferred to nylon, and hybridized with the pK4-PCR and β-actin probes.

Protein Analysis and Antiserum. Subconfluent adherent cells in T-75 flasks (Falcon) were starved for 30 min in 3 ml of methionine-free medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and glutamine) and metabolically labeled for 2 h with 200 μCi/ml [^{35}S]-methionine (1200 Ci/mmol; NEN/Dupont, Wilmington, DE). Cells were rinsed with phosphate-buffered saline and lysed with 1% Nonidet P-40 detergent, and the cleared lysates were precipitated with rabbit antiserum. Radiolabeled proteins separated on denaturing polyacrylamide gels containing sodium dodecyl sulfate were detected by autoradiography of the dried gels (51). Immunoprecipitates prepared with antiserum to CDK4 or to the D-type cyclins were separated on gels, transferred to nitrocellulose, and immunoblotted with the same antiserum at 1:250 dilution followed by ^{125}I-labeled *Staphylococcus aureus* protein A (Amersham) (52).

Rabbit antiserum to mouse D-type cyclins and $p34^{cdk4}$ were raised to bacterially expressed, full length polypeptides (6, 25). The antiserum to cyclins D1 and D2 readily precipitate the human proteins and cross-react with one another but do not immunoprecipitate cyclin D3, whereas the antiserum to cyclin D3 reacts only with the cognate mouse and human cyclins. Although the antiserum to D-type cyclins coprecipitate $p34^{cdk4}$, they react preferentially with the "free" regulatory subunits. None of these antisera react with cyclins A, B, C, or E. The antiserum to $p34^{cdk4}$ does not significantly cross-react with $p34^{cdc2}$, $p33^{cdk2}$, $p35^{cdk3}$, and $p31^{cdk5}$, but preferentially precipitates $p34^{cdk4}$ unbound to cyclins (25).

Fluorescence *in Situ* Hybridization. Bromodeoxyuridine-synchronized, phytohemagglutinin-stimulated peripheral blood lymphocytes from a normal donor were used as a source of metaphase chromosomes. Genomic DNA subcloned into phage or cosmid vectors was nick-translated with digoxigenin-11-UTP and hybridized overnight at 37°C to fixed metaphase chromosomes according to the method of Pinkel *et al.* (53), except for the inclusion of 0.33 μg/μl of highly reiterated human DNA self-annealed to C_0t 1 (Bethesda Research Laboratories, Gaithersburg, MD). Signals were detected by incubating the slides with fluorescein-conjugated sheep antidigoxigenin antibodies (Boehringer Mannheim, Indianapolis, IN) followed by counter staining in propidium iodide solution containing antifade [1,4-diazabicyclo(2.2.2)octane; Sigma Chemical, St. Louis, MO]. Fluorescence microscopy was performed with a Zeiss microscope equipped with fluorescein epifluorescence filters.

Somatic Cell Hybrids. Hybrid cell lines with the prefix "GM" in Table 1 were obtained from the National Institute of General Medical Sciences' Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ); the characterization and human chromosome content of these hybrids are described in the Repository catalogue. The preparation of the human ×

Table 1 *Assignment of the human CDK4 gene to chromosome 12 in human X rodent somatic cell hybrids*[a]

		Human chromosomes																							
Hybrid	CDK4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
GM 06317	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
GM 07297	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
GM 07299	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
GM 07300	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
GM 07301	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
GM 09927	-	+	+	+	+	-	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	-	-	-	-
GM 09934	+	-	+	-	-	+	+	-	+	-	-	+	+	-	-	+	-	+	+	-	+	+	-	-	-
GM 10027	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
GM 10114	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GM 10253	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GM 10324	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
GM 10449	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
GM 10479	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
GM 10498	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
GM 10611	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A3	+	-	+	+	-	-	+	+	+	-	+	-	+	+	-	-	-	-	-	+	-	+	+	+	-
A4	+	-	-	+	-	-	+	+	-	-	-	-	+	-	-	-	+	-	+	-	+	+	+	+	-
A5	+	-	-	+	-	-	+	+	-	-	-	-	+	-	-	-	+	-	+	-	-	+	+	+	-
A6	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-	+	-
C1	+	-	-	+	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-
C2	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	+	-	-	-	+	+	-
% Discordant		43	24	24	33	29	19	19	29	33	29	24	0	33	43	33	24	33	24	33	29	14	24	38	33

[a] + and - indicate the presence or absence, respectively, of the *CDK4* gene, as determined by Southern blotting, and each human chromosome in the hybrid cell lines. The origins of the hybrid cell lines are described in "Materials and Methods." % Discordant is defined as the sum of the hybrids containing the chromosome but lacking human *CDK4* and those lacking the chromosome but containing human *CDK4* divided by the total number of hybrid cell lines examined and multiplied by 100.

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hamster somatic cell hybrid lines A3, A4, A5, and A6 has been previously described (54). DNA (8 μg) from each hybrid line was digested with *Eco*RI and subjected to Southern blotting analysis using the pK4-PCR human *CDK4* probe, which identifies a 12-kilobase human restriction fragment that is readily distinguishable from hybridizing fragments (9.5 and 23.0 kilobases) derived from hamster DNA.

Sequencing of CDK4 cDNA. cDNA was synthesized from 1 μg of total RNA extracted from 2 cell lines exhibiting *CDK4* amplification. Murine leukemia virus reverse transcriptase (200 units) was added to the template in PCR buffer containing deoxynucleotide triphosphates (1 mM each), RNasin (3.5 units), and a specific antisense primer (5'-TCAGAGATAAAGGCA-3', derived from the 3' noncoding region of human *CDK4* cDNA) and incubated at 42°C for 90 min. The *CDK4* cDNA product was amplified by PCR as described above, in duplicate reactions using primers from the 5' and 3' untranslated regions of the human *CDK4* cDNA [5'-ACAGGATCCCGGGCTGGCGTG-3'(sense), 5'-AAGAATTCCCTCTCAGTGTCCAGAAG-3' (antisense)]; the primers contain *Bam*H1 or *Eco*R1 sequences, respectively, on their 5' ends to aid in subcloning the products). Amplified products were cloned into a Bluescript plasmid vector and sequenced by the dideoxynucleotide chain termination method (47).

RESULTS

Chromosomal Assignment of the Human *CDK4* Gene. Human genomic libraries were screened with a murine *CDK4* cDNA probe (25), and one recombinant phage (λ-cdk4) and two cosmid clones (cos-10–3 and cos-14–3) were obtained that contained overlapping hybridizing sequences. Human sequences hybridizing to the probe were contained within a 6.7-kilobase *Bam*HI fragment, and a 4.0-kilobase *Mbo*I-*Bam*HI portion of this fragment was subcloned (Fig. 1). Specific oligonucleotide primers were prepared based on the previously published human *CDK4* cDNA sequence (46) and used to amplify a 534-base pair product by PCR, using the subcloned DNA as a template. The pK4-PCR product contained one complete and two partial *CDK4* exons (denoted by capital letters in Fig. 1) with nucleotide sequences identical to previously reported *CDK4* coding sequences (46), as well as two small introns, confirming that the genomic clones were derived from the human *CDK4* locus.

The chromosomal location of human *CDK4* was determined by fluorescence *in situ* hybridization, using probes prepared from each of the genomic clones (λ-cdk4, cos-10–3, and cos-14–3) which were hybridized to normal metaphase human chromosomes. The fluorescence signals obtained with each probe were localized to the long arm of chromosome 12, as indicated by cohybridization with the chromosome 12-specific centromeric probe, D12Z1 (Fig. 2*A*). The *CDK4* locus was sublocalized to band q13 based on the distance of the hybridization signal from the chromosome 12 centromere.

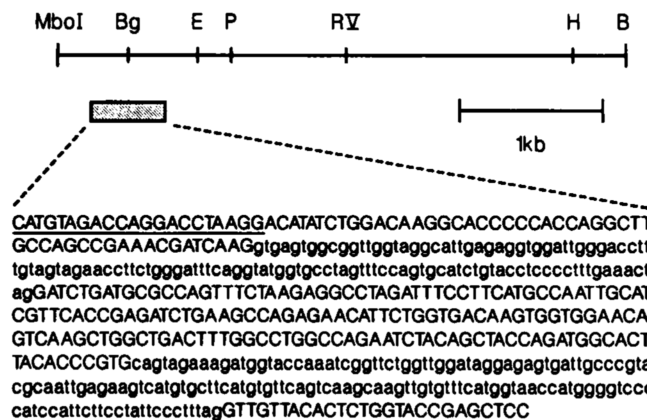

Fig. 1. Partial genomic map and sequence of human CDK4. The restriction map is shown for a 4.0-kilobase *Mbo*I-*Bam*HI human genomic fragment (*top*) that hybridized to the murine *CDK4* cDNA probe. The shaded box denotes a PCR product (pK4-PCR), prepared using internal *CDK4* cDNA primers. The pK4-PCR sequence is shown (*bottom*) in the 5' to 3' direction (exonic sequences in upper case letters and intronic sequences in lower case). The *underlined* sequences were used to prepare the oligonucleotides for PCR amplification.

The chromosomal assignment was confirmed by hybridizing the pK4-PCR probe to Southern blots containing DNAs extracted from a panel of rodent × human hybrid cell lines containing different combinations of human chromosomes. A 12-kilobase *Eco*RI restriction fragment contained within the authentic human *CDK4* genomic locus was detected concordantly with human chromosome 12 (Table 1), consistent with results obtained by fluorescence *in situ* hybridization.

***CDK4* Amplification in Sarcomas.** We and others have previously identified an amplicon at chromosome 12q13 involving the *GLI* and *MDM2* genes in soft tissue sarcomas (44, 45). To determine whether *CDK4* is included in this amplicon, we performed Southern blot analysis of *Eco*RI-digested DNAs extracted from 14 human sarcoma cell lines, including 2 osteosarcomas, 6 rhabdomyosarcomas, 5 Ewings' sarcomas, and 1 undifferentiated sarcoma, as well as a control lymphoblastoid cell line (CJTW) (Fig. 3). The Southern blot was sequentially hybridized with probes from the pK4-PCR *CDK4* fragment (Fig. 3*A*), the human *MDM2* cDNA (Fig. 3*B*), and the human *GLI* cDNA (Fig. 3*C*). As a control for DNA loading, we performed parallel experiments using a hematopoietic cell phosphatase cDNA probe, which detects a gene located on the short arm of human chromosome 12 (Ref. 55; Fig. 3*D*). We detected approximately 25-fold amplified levels of the 12-kilobase *CDK4 Eco*RI fragment in two cell lines, OsA-Cl and SJRH30 (Fig. 3*A*), which have previously been shown to have amplification of the *GLI* locus (Ref. 44; Fig. 3*C*). By contrast, no amplification of CDK4 was observed in 12 other sarcoma cell lines or in control CJTW cells (Fig. 3*A*). Our results also confirmed those obtained previously with *MDM2* in OsA-CL cells [Ref. 45; Fig. 3*B*]. Thus, all three genes were coamplified in OsA-CL, but only *CDK4* and *GLI* (not *MDM2*) were amplified in SJRH30.

To confirm these findings, we analyzed both metaphase and interphase cells from the two cell lines by fluorescence *in situ* hybridization with a *CDK4* cosmid probe (cos-10–3; Fig. 2, *B-D*). In metaphase cells from OsA-CL (Fig. 2*B*), we found increased levels of hybridization in a coalesced pattern, indicating amplification of this locus as an intrachromosomal homogeneously staining region located on an abnormal marker chromosome, which also hybridizes to *GLI* sequences (44). In interphase nuclei from OsACL cells (Fig. 2*C*) and SJRH30 (Fig. 2*D*), the *CDK4* cosmid probe also demonstrated localized areas of increased hybridization, consistent with amplification as components of homogeneously staining regions in each of the cell lines.

***CDK4* Expression in Sarcoma Cell Lines.** Northern blot analysis was performed with total cellular RNAs extracted from OsACL and SJRH30 cells, along with those from control rhabdomyosarcoma (SJRH28) and Ewings sarcoma (SJES8) cell lines (Fig. 4). The two cell lines containing the *CDK4* amplicon each had 8- to 10-fold increased levels of *CDK4* mRNA expression (Fig. 4, *Lanes 1* and *2*) when compared to controls (Fig. 4, *Lanes 3* and *4*), indicating that the increased *CDK4* copy number resulted in augmented levels of gene expression.

Metabolically labeled lysates from the same four sarcoma cell lines were immunoprecipitated with anti-$p34^{cdk4}$ antibodies to determine the levels of $p34^{cdk4}$ expression (Fig. 5, *Lanes 2*, *7*, *12*, and *17*). The levels of synthesis $p34^{cdk4}$ were approximately five-fold higher in OsA-CL and SJRH30 cells (Fig. 5, *Lanes 2* and *7*) than those in the control cell lines (Fig. 5, *Lanes 12* and *17*) but were somewhat lower than that predicted from the augmented *CDK4* mRNA levels detected by Northern blotting. The same lysates were precipitated with antisera raised against cyclins D1, D2, and D3. Although the antisera to cyclins D1 and D2 are cross-reactive, they preferentially precipitate the cog-

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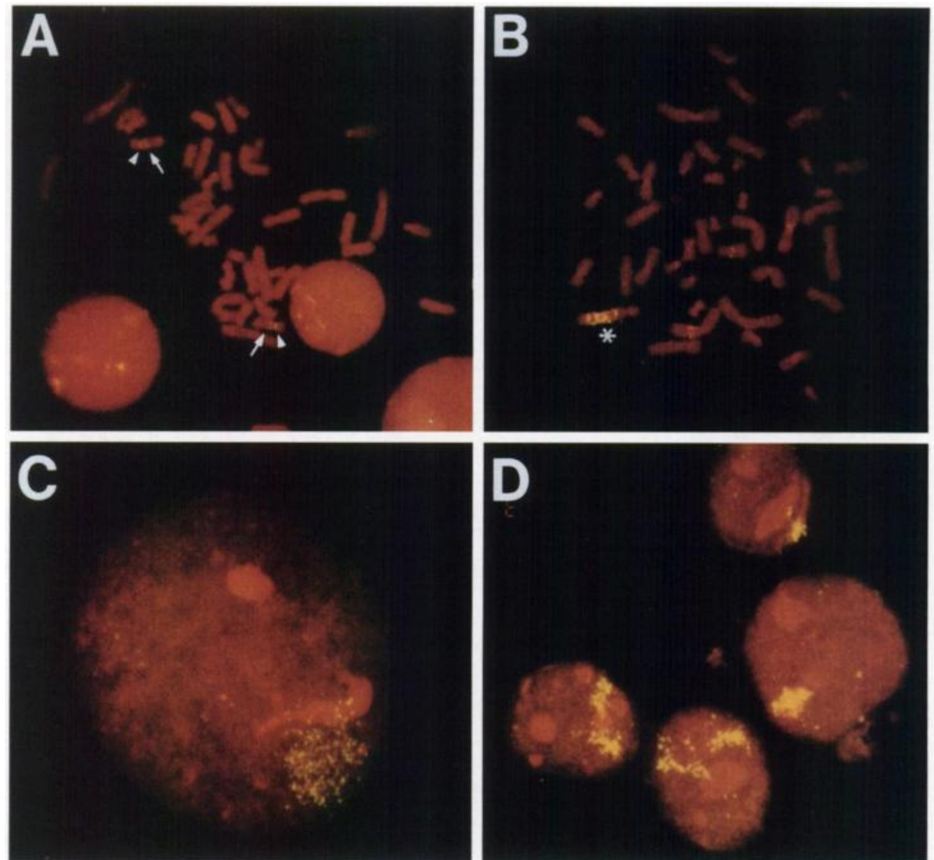

Fig. 2. Fluorescence *in situ* hybridization analysis of *CDK4*. Fluorescence signals were localized to human chromosome 12, band q13, in normal metaphase cells using a digoxigenin-labeled *CDK4* genomic probe (cos-10–3) (*A*). *Arrows*, the *CDK4* signals; *arrowheads*, the chromosome 12 centromere detected using a chromosome 12-specific centromeric probe, D12Z1. Fluorescence *in situ* hybridization analysis of the OsA-CL (*B*, metaphase; *C*, interphase) and SJRH30 (*D*, interphase) cell lines showed amplified copies of *CDK4* with patterns consistent with homogeneously staining regions (indicated by * in *B*).

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nate isoforms; note also that the three D-type cyclins can be clearly distinguished by differences in their electrophoretic mobilities on denaturing gels (25, 26) (Fig. 5), as predicted from their molecular masses (17). OsA-CL cells synthesized mostly cyclin D1 (Fig. 5, *Lane 3*), whereas SJRH30 and SJRH28 predominately expressed cyclin D2 (Fig. 5, *Lanes 9* and *14*). The control cell line, SJES8, expressed detectable levels of all three proteins, with cyclin D1 observed at high, D2 at intermediate, and D3 at comparatively low levels (Fig. 5, *Lanes 18–20*). Given that cells of different lineages express varying amounts of the three D-type cyclin proteins, it is not surprising that the cell lines examined here had unique patterns of cyclin D expression.

In samples immunoprecipitated with anti-$p34^{cdk4}$, some complex formation could be demonstrated between $p34^{cdk4}$ and the major D-type cyclin found in each cell line, as indicated by the open arrowheads in Fig. 5 (*Lanes 2*, *7*, *12*, and *17*). In reciprocal experiments, it was much more difficult to detect proteins with the mobility of $p34^{cdk4}$ in immunoprecipitates prepared with antisera to the cyclins. At least in part, these results may reflect the propensity of the antibodies to react with the free subunits (25) (see "Materials and Methods"). However, despite the preponderance of $p34^{cdk4}$ in the two cell lines exhibiting gene amplification, the levels of coimmunoprecipitated cyclin D proteins were not increased compared to those in control cell lines exhibiting much lower levels of $p34^{cdk4}$ expression.

Western blot analysis of lysates from OsA-CL or SJES8 cells immunoprecipitated with antisera to cyclin D1 and probed with the same antibodies revealed that the steady state levels of cyclin D1 in SJES8 cells were somewhat higher (Fig. 6, *Lanes 2* and *5*) in general agreement with the results obtained by metabolic labeling (Fig. 5, *Lanes 3* and *18*). Paradoxically, when immunoprecipitated proteins prepared with antiserum to $p34^{cdk4}$ were probed with antibodies to the cyclin, approximately 5-fold lower levels of cyclin D1 were complexed with $p34^{cdk4}$ in OsA-CL cells compared to control SJES8 cells (Fig. 6, *Lanes 3* and *6*, *open arrowheads*). Thus, the available evidence suggests that the levels of cyclin D1/$p34^{cdk4}$ complexes detected by coimmunoprecipitation were paradoxically decreased in the OsA-CL cell line, despite *CDK4* gene amplification and overexpression.

Sequence Analysis of *CDK4* mRNA. In view of the apparently lower levels of cyclin D-$p34^{cdk4}$ complexes in cell lines overexpressing $p34^{cdk4}$, it seemed possible that *CDK4* mutations might have resulted in the overexpression of a defective $p34^{cdk4}$ protein that was unable to interact with the D-type cyclins. Cellular mRNA extracted from OsA-CL and SJRH30 cells was therefore used as a template to prepare *CDK4* cDNAs by reverse PCR. DNA sequencing analysis of the complete *CDK4* cDNA coding sequences from two independent PCR reactions performed with each cell line did not reveal any mutations. Thus, the failure of the overexpressed $p34^{cdk4}$ proteins to form increased numbers of complexes with D-type cyclins must have another mechanistic basis.

DISCUSSION

Gene amplification is an important mechanism leading to the overexpression of specific protooncogenes in human solid tumors. Well studied examples involve the *N-MYC* gene in childhood neuroblastoma (56–58) and the *HER-2/NEU* gene in breast carcinoma (59, 60), which are amplified in subsets of patients who are more likely to have metastatic disease at diagnosis and a poor prognosis. Amplicons can be quite large, spanning more than a megabase (61), and it has frequently proven difficult to pinpoint target genes whose overexpression acts to maintain the amplicon and functionally contributes to the neoplastic phenotype. In human breast carcinomas and squamous cell carcinomas of the head and neck, the amplicon on chromosome 11, band q13, includes two genes (*INT2* and *HST1*) encoding fibroblast growth factors (62), the former being first identified as a target of retroviral insertion in murine breast carcinomas (63). Despite their potential to contribute to oncogenesis, these genes are not always

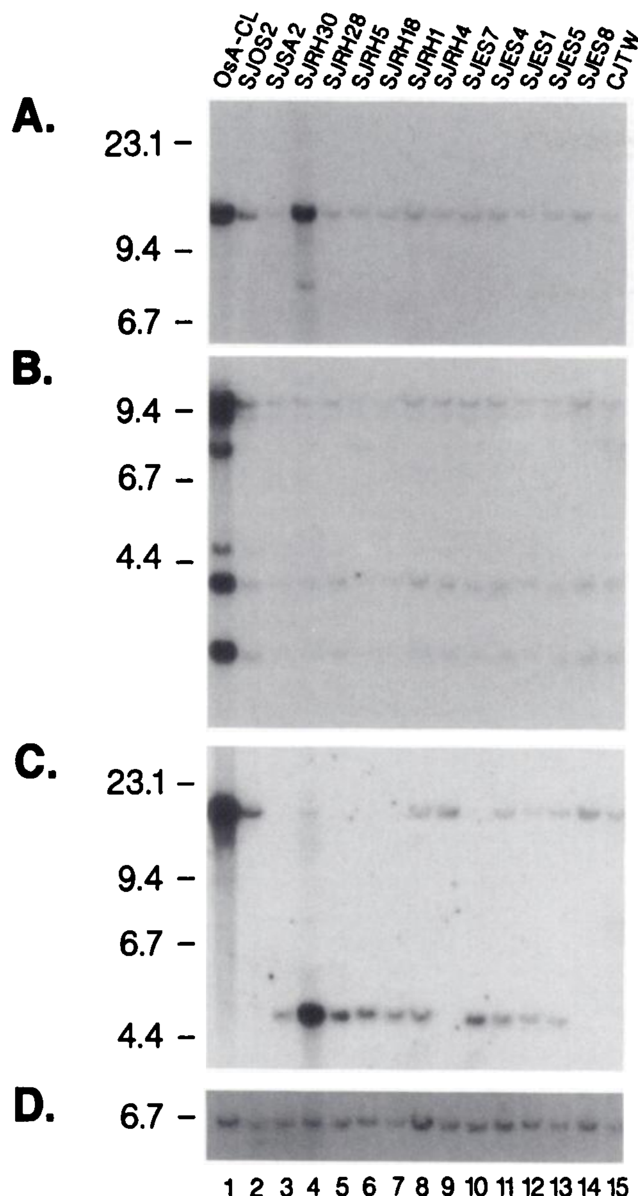

Fig. 3. Analysis of the 12q13 amplicon in childhood sarcomas. A Southern blot of *Eco*RI-digested DNAs (5 μg) from sarcoma cell lines, including two osteosarcomas (*Lanes 1–2*), one unspecified sarcoma (*Lane 3*), six rhabdomyosarcomas (*Lanes 4–9*), and five Ewings sarcomas (*Lanes 10–14*), and from one normal human lymphoblastoid cell line (*Lane 15*), was hybridized sequentially with a *CDK4* probe (*A*), an *MDM2* probe (*B*), a *GLI* probe (*C*), or a control chromosome 12p probe (hematopoietic cell phosphatase or *HCP*) (*D*). The DNAs shown in *C* exhibit heterozygosity for a restriction fragment length polymorphism detected with the *GLI* probe (44). The relative mobilities of bacteriophage λ *Hin*dIII fragments of known length (noted in kilobase pairs) are indicated. The exposure times were 72 h.

included in the 11q13 amplicon and are not invariably expressed in tumor cells, even when amplified (36, 64, 65). Indeed, the cyclin D1 gene now appears to be a more relevant candidate because it is both amplified and overexpressed in many such tumors (36–38).

A similar problem is presented by the amplicon in human sarcomas involving sequences at chromosome 12, band q13. This region was initially shown to be amplified in a human glioma, and positional cloning techniques were used to identify the *GLI* gene as a potentially relevant target within the amplicon (66). *GLI* encodes a potential transcriptional regulatory protein with five zinc finger DNA binding motifs and sequence similarity to the *Krüppel drosophila* segmentation gene (67, 68). *GLI* is both amplified and overexpressed in the two sarcoma cell lines, OsACL and SJRH30 (44), which also show amplification and overexpression of *CDK4*. Another potential protooncogene, *MDM2*, included in the same amplicon (45, 69), was originally identified in a spontaneously transformed subclone of BALB/c mouse fibroblasts (70). The *MDM2* product binds directly to *p53* and can interfere with the ability of wild-type *p53* to transactivate reporter gene expression (71, 72) and to suppress *ras*-induced transformation of rat embryo fibroblasts (73). Consistent with this hypothesis, *MDM2* has been found to be amplified in over one-third of human sarcomas tested (45), and tumors with *MDM2* amplification generally do not have inactivating *p53* mutations, which are frequently found in sarcomas with normal *MDM2* gene dosage (69). At least one other locus (*SAS*, for sarcoma amplified sequence) is included in the chromosome 12q13 amplicon (41, 74), but its nucleotide sequence remains unavailable and its physiological role is unknown. The amplicon in the SJRH30 cell line does not include *MDM2*, suggesting that other genes, such as *CDK4* or *GLI*, may be the relevant targets of gene amplification in some tumor subsets. An analysis of fresh tumor samples and further investigations of the biochemical properties of the *CDK4* gene product will be needed to determine whether it contributes directly to oncogenesis or whether it is simply coamplified with a more relevant target locus.

When complexes of different mammalian cyclins and CDKs were reconstituted *in vitro* or in insect cells, only the three D-type cyclins were found to interact with $p34^{cdk4}$ to activate its kinase activity (25, 26). Cyclin D proteins have a very short half-life in mammalian cells (half-life, <20 min), but $p34^{cdk4}$ is much more stable (half-life, 4–6 h) (6, 11, 25). These results imply that cyclin, rather than $p34^{cdk4}$, synthesis is both necessary and rate limiting for G_1 progression, and this

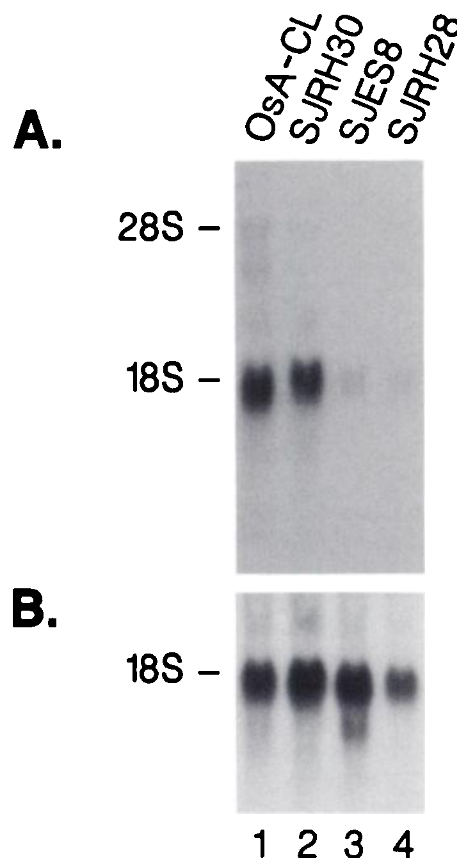

Fig. 4. *CDK4* mRNA expression in cell lines with gene amplification. Northern blot analysis was performed using total cellular RNA (25 μg/lane) extracted from the osteosarcoma line OsA-Cl (*Lane 1*), rhabdomyosarcoma cell lines SJRH30 and SJRH28 (*Lanes 2* and *4*, respectively), and Ewings sarcoma SJES8 (*Lane 3*). The blot was hybridized with a radiolabeled *CDK4* probe, pK4-PCR (*A*). Approximately equal amounts of RNA were analyzed for each cell line, as shown by hybridization with a β-actin probe (*B*). The relative mobilities of 18S and 28S ribosomal RNAs are indicated. The exposure time was 16 h.

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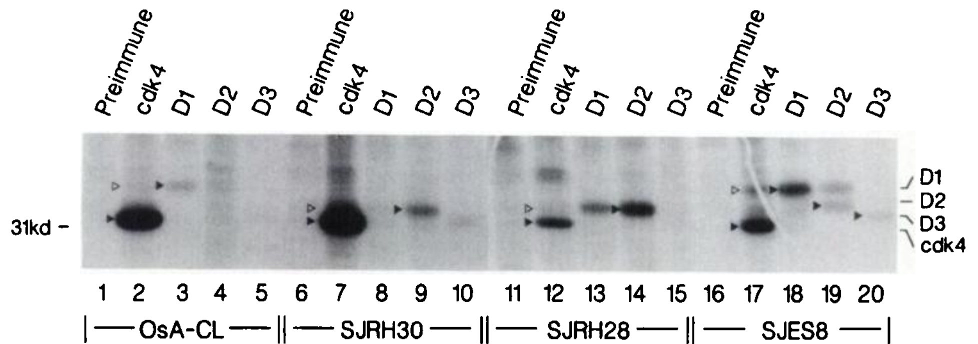

Fig. 5. Immunoprecipitation of $p34^{cdk4}$ and the D-type cyclins. [^{35}S]methionine-labeled cell lysates from the OsA-CL, SJRH30, SJRH28, and SJES8 cell lines were immunoprecipitated with antisera to $p34^{cdk4}$ or to cyclins D1, D2, and D3 as indicated. *Solid arrowhead*, the protein identified by the antiserum used in each lane. Bands corresponding to cyclin D1 were coprecipitated with antiserum to $p34^{cdk4}$ in the OsA-CL and SJES8 cell lines, whereas cyclin D2 coprecipitated with $p34^{cdk4}$ in lysates from SJRH30 and SJRH28 cells (*open arrowheads*). *Right*, the positions of the D-type cyclins and of $p34^{cdk4}$.

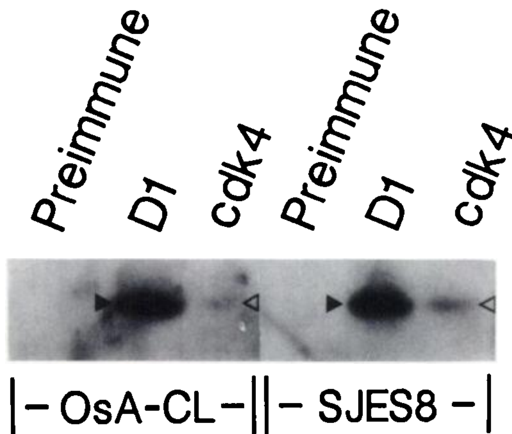

Fig. 6. Detection of $p34^{cdk4}$-cyclin D1 complexes. Immunoprecipitates prepared with antisera to cyclin D1 or to $p34^{cdk4}$ (*top*) were separated on a denaturing gel and immunoblotted with antiserum to cyclin D1. Approximately equal amounts of cyclin D1 were present in the cell lines OsA-CL and SJES8, as indicated by the *solid arrowheads* in *Lanes 2* and *5*. The levels of $p34^{cdk4}$-cyclin D1 complex detected by immunoblotting of the $p34^{cdk4}$ immunoprecipitates with anti-cyclin D1 are shown in *Lanes 3* and *6* (*open arrowheads*). The exposure time was 3 days.

has now proven to be the case, at least in fibroblasts (31, 75). However, in cells sensitive to the growth inhibitory action of transforming growth factor β, overexpression of $p34^{cdk4}$ overrides the transforming growth factor β block in late G_1 phase, resulting in activation of the cyclin E/cdk2 histone H1 kinase and entry into S phase (76). Based on immunoprecipitation and Western blotting analyses, the levels of cyclin D/$p34^{cdk4}$ complexes were paradoxically reduced in cell lines exhibiting *CDK4* amplification, although the amplified gene had not sustained detectable mutations. Similar results have recently been observed in other transformed cells, in which cyclin D/$p34^{cdk4}$ complexes undergo dissociation, and the catalytic subunits become bound to a series of low molecular weight proteins (28, 77). One possibility, then, is that the latter proteins act as stoichiometric inhibitors of $p34^{cdk4}$ kinase activity (*i.e.*, as anticyclins) and compete with D-type cyclins for binding to catalytic subunits. In principle, the overexpression of inactive, monomeric $p34^{cdk4}$ subunits might titrate out such negative regulators, resulting in the increased availability of D-type cyclin subunits and their reassortment into complexes containing other catalytic partners, such as cdk2, or their increased binding to pRb or pRb-related proteins (27, 29). Whatever the mechanism, our results provide the first example of amplification of a gene encoding a known cell division cycle kinase in human tumor cells.

ACKNOWLEDGMENTS

We thank Steven Hanks for access to unpublished human CDK4 cDNA sequence information and Sharon Nooner and Bart Jones for technical assistance.

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