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Genetics

Cloning of the human homolog of the *CDC34* cell cycle gene by complementation in yeast

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ABSTRACT **In a screen designed to isolate human cDNAs that complement a yeast G_2 phase checkpoint mutation (*mec1*), we isolated a cDNA homologous to the *Saccharomyces cerevisiae CDC34* gene. The human *CDC34* cDNA can functionally substitute for the yeast *CDC34* gene and represents a mammalian homolog of the group of yeast genes required for the late $G_1 \rightarrow S$ phase transition. The human *CDC34* gene is expressed in multiple cell lines as a unique species and Southern blot analysis reveals evidence for a single gene that is highly conserved in higher eukaryotes. The human gene is located on the far telomeric region of 19p13.3 in a location that defines a region of homology between human chromosome 19p and mouse chromosome 11.**

There are several types of mutations in yeast that result in deregulation of the cell cycle. Temperature-sensitive mutations in *CDC* genes result in cell death at defined points in the cell cycle when strains are shifted to the nonpermissive temperature (1). In many cases, most notably the *CDC2/CDC28* genes, these genes have been found to be functionally conserved between yeast and humans (2). The CDC28 kinase activity of *Saccharomyces cerevisiae* is essential for START, the commitment to enter the cell cycle during the G_1 phase of the cycle.

A second set of three essential *CDC* genes are required late in G_1 phase for the entry in S phase. Mutation in *CDC4* (3, 4), *CDC34* (1, 5), or *CDC53* (6) results in arrest in G_1 before the initiation of DNA synthesis and formation of a pseudobud morphology. The *CDC34* gene (7) is one member of the large family of ubiquitin ligases (UBC) that are capable of targeting a ubiquitin polypeptide onto recipient proteins. In yeast, the ubiquitin ligases regulate diverse cellular processes including cell cycle progression (*CDC34*), radiation resistance and sporulation (*RAD6*), and protein degradation (*UBC4*) (8). The specific *in vivo* target of the CDC34 ubiquitin ligase is unknown but has been proposed to be the G_1 cyclins (9). To our knowledge, no functional homologs of *CDC34* have been identified in other organisms.

A second class of cell cycle regulatory mutations has been defined and labeled checkpoint mutations (10). Although some of these "checkpoint" genes are not essential for viability, all mutant strains demonstrate increased lethality after DNA damage due to their failure to alter the cell cycle in response to damage. *rad9* (11) and *mec1* (T. Weinert, personal communication) are checkpoint mutations of *S. cerevisiae* that result in loss of G_2 delay after DNA damage and greatly increased radiation sensitivity. Damage to mammalian cells that results in DNA strand breaks, by ionizing radiation or drugs, also causes the cell to delay in both the G_1 and G_2 phases of the cell cycle to allow time for repair (12, 13). However, none of the mammalian G_2 checkpoint genes have been identified.

During initial screening for human cDNAs that would complement the *mec1* checkpoint mutation, we obtained a partially active cDNA that further analysis reveals is the human homolog of *S. cerevisiae CDC34*. We report here the cloning and functional and physical characterization of this human gene.‖

MATERIALS AND METHODS

Yeast and Bacterial Strains. The *S. cerevisiae* strains described in these experiments were isogenic with A364a. Source of the strain other than this laboratory are indicated: 171-10-2 (*MAT***a**, *cdc9-8, mec1-A401, leu2, ura3, ade2, ade3, trp1*—T. Weinert, University of Arizona, Tuscon), 9085-1-8-3 (*MATα, cdc9-8, rad9::HIS3, leu2, ura3, trp1*), 9085-1-10-4 (*MATα, cdc9-8, leu2, his3*), SJ1098-3d (*MAT***a**, *cdc34-2, leu2-3, ura3, trp1*—B. Byers, University of Washington, Seattle). All bacterial transformations were performed in the SURE strain (Stratagene).

Libraries and DNAs. The U118 cDNA library (14) in the ADANS vector was obtained from J. Collicelli (University of California at Los Angeles). A human placental cosmid library in pWE15 (15) was provided by G. Evans (Salk Institute for Biological Studies, La Jolla, CA). The S.c. CDC34 plasmid was constructed by subcloning a PCR-amplified 1.0-kb piece of the *CDC34* gene downstream of the *Hin*dIII site in the ADANS plasmid. The *MEC1* and *RAD9* plasmids were provided by T. Weinert. Somatic cell hybrid DNAs were obtained from the Coriell Cell Repository (Camden, NJ). Both the human chromosome 19 hybrid (GM10449, no. 5HL9-4) and the human chromosome 17 hybrid [GM10498, no. MN-22.6 (16)] contain >90% of cells with a single human chromosome, and the chromosome 19 hybrid was reported to be negative by Southern blot analysis for a known chromosome 17 marker. The sequence of both strands of the cDNA insert were determined by dideoxynucleotide sequencing using Sequenase Version 2.0 (United States Biochemical).

Yeast Transformation. Logarithmic-phase cultures of the indicated strain were transformed by a modification of the method of Schiestl and Gietz (17), in which the DNA and 50% (wt/vol) polyethylene glycol solution are added directly to the yeast in lithium actetate without any preincubation. Plasmid DNA from yeast was extracted by glass-bead disruption (18) and transformed into *Escherichia coli* by electroporation (Bio-Rad). Plasmid DNA from a single colony was retransformed into the parent yeast strain to determine plasmid dependence.

Northern and Southern Blot Analysis. Total genomic DNA was digested with restriction enzymes by the manufacturers' recommendations and separated on 0.7% agarose gels with 89 mM Tris borate/2.5 mM EDTA. Transfer to GeneScreen*Plus*

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. L22005).

and hybridization were performed by the manufacturers' recommendations (NEN). The most stringent wash was 0.2× standard saline citrate/1% SDS at 65°C. The human *CDC34* probe was a 784-bp PCR product labeled by random-oligonucleotide-primed synthesis (Boehringer Mannheim). The oligonucleotides used to generate the PCR product are 5′-AACACCTACTACGAGGGCGGC-3′ and 5′-GCCCGTC-CACCGAGCCCCGAG-3′. Poly(A)$^+$ RNA, a gift of Carol Thiele (National Cancer Institute, Bethesda, MD), was separated on 1% agarose/formaldehyde gels and also transferred to GeneScreen*Plus* membrane. The filter was sequentially hybridized with the human *CDC34* PCR probe and a rat *GADPH* cDNA. Quantitation of the hybridization signal on the Northern blot was performed by direct phosphor imaging of the hybridized filter (Molecular Dynamics).

Fluorescent *in Situ* Hybridization. As described (19), after hybridization, washes were performed at 42°C and 50°C for 34cos2 and 34cos4, respectively.

RESULTS

Complementation of a *MEC1, CDC9-8* Strain by a Human cDNA. We developed a simple genetic assay for selecting human genes by complementation of defined yeast mutations. In addition to radiation sensitivity, the presence of a checkpoint mutation increases the lethality of several temperature-sensitive cell cycle mutations (10, 20). The presence of either a *rad9* or *mec1* mutation decreases the maximum permissive temperature from 30°C to 25°C of a strain with a DNA ligase mutation (*cdc9-8*). Presumably, the increased lethality of the checkpoint mutation is a consequence of cells with multiple DNA strand breaks entering mitosis. After transformation with a human cDNA library, selection for growth at 30°C of a *mec1, cdc9-8* or *rad9, cdc9-8* strain will allow for selection of cDNAs that complement the MEC1, RAD9, or CDC9 function. In our attempt to isolate human genes that complement the yeast mutation, we used a human cDNA library constructed with the yeast expression vector ADANS containing the *ADH* promoter and first 14 aa of the *ADH* gene flanking the cDNA insert and a *LEU2*-selectable marker gene (14). The source of cDNA was the human glioblastoma U118 cell line, which maintains an intact G_2 arrest mechanism after irradiation (K. Russell, personal communication).

Of ≈200,000 LEU$^+$ transformants of the *mec1, cdc9-8* strain, 15 colonies grew at 30°C. One of these transformants (tx6) demonstrated plasmid-dependent growth at 30°C although with reduced viability compared to a *MEC1* control. The tx6 plasmid contained three independent cDNA inserts, only one of which was active (named tx61). Table 1 summarizes these results. Five days after transformation of the *mec1, cdc9-8* strain with the ADANS vector <0.1% of LEU$^+$ transformants formed a colony at 30°C. Transformation with the human tx6 cDNA or its subclone, tx61, resulted in 15% viability of LEU$^+$ transformants at 30°C; in comparison, transformation with the authentic *MEC1* gene resulted in near 100% viability at 30°C.

Table 1. Viability after transformation of yeast mutant strains by plasmid DNA

Strain	DNA	% viability at indicated temperature			
		23°C	30°C	34°C	36°C
mec1, cdc9-8	ADANS	100	<0.1		
mec1, cdc9-8	tx6	100	15		
mec1, cdc9-8	tx61	100	15		
mec1, cdc9-8	MEC1	100	100		
rad9, cdc9-8	ADANS	100	<0.1		
rad9, cdc9-8	tx61	100	<0.1		
rad9, cdc9-8	RAD9	100	100		
cdc9-8	ADANS	100	100	10	<0.1
cdc9-8	tx61	100	100	10	<0.1

ADANS, control vector; tx6, original cDNA clone; tx61, subclone of tx6 containing active cDNA; MEC1, plasmid containing genomic fragment of the *MEC1* gene; RAD9, plasmid containing genomic fragment of *RAD9* gene.

The tx61 cDNA was unable to complement the growth defect at 30°C of a *rad9, cdc9-8* strain (Table 1). Similarly, tx61 did not affect the growth characteristics of a *RAD*$^+$, *MEC*$^+$, *cdc9-8* strain. Thus, the activity of the tx61 cDNA appears to be specific to the *mec1*-mediated *cdc9-8* checkpoint and not a direct effect on the DNA ligase mutation.

However, in contrast to the partial complementation of the growth defect of a *mec1, cdc9-8* strain, there was no complementation of the radiation sensitivity and hydroxyurea sensitivity of a *mec1* strain by expression of tx61. The radiation sensitivity of a *mec1* strain containing the control vector or tx61 was identical, in contrast, to the radiation resistance of a *mec1* strain carrying a *MEC1* plasmid or a wild-type strain with the control vector. Similar data were obtained after exposure of the cells to various concentrations of hydroxyurea for 4 h (data not shown). Therefore, it seemed unlikely that tx61 was the human homolog of *MEC1*.

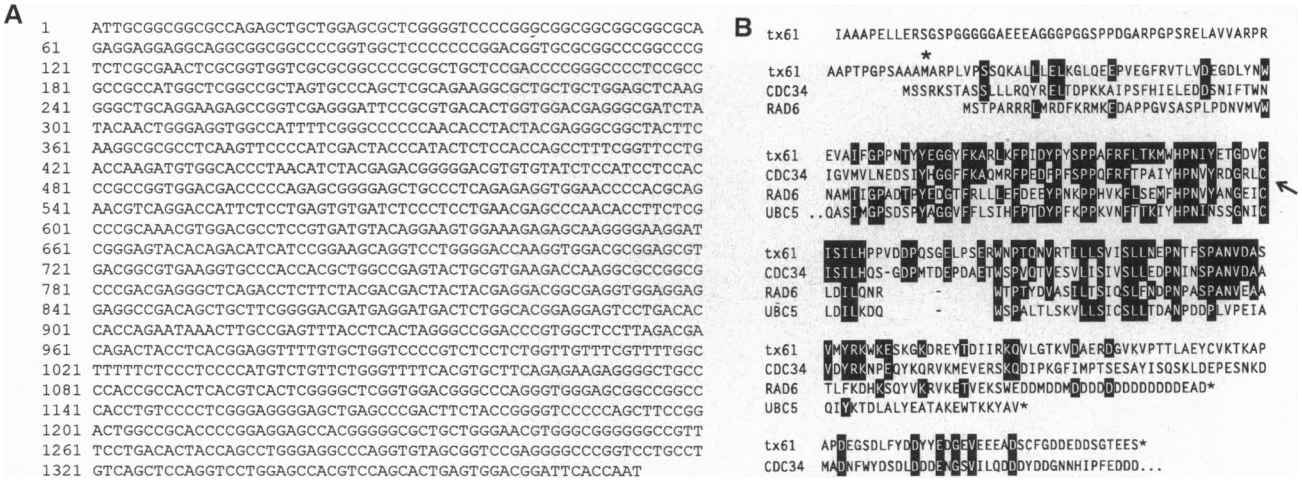

FIG. 1. (*A*) Sequence of tx61 human cDNA. (*B*) Alignment of predicted protein sequence of tx61 with three members of the ubiquitin ligase family (UBC) of yeast proteins. Identical amino acids are white letters on a dark background. The arrow points to the conserved active site cysteine of this family of proteins; the asterisk denotes the first methionine encoded by the human cDNA.

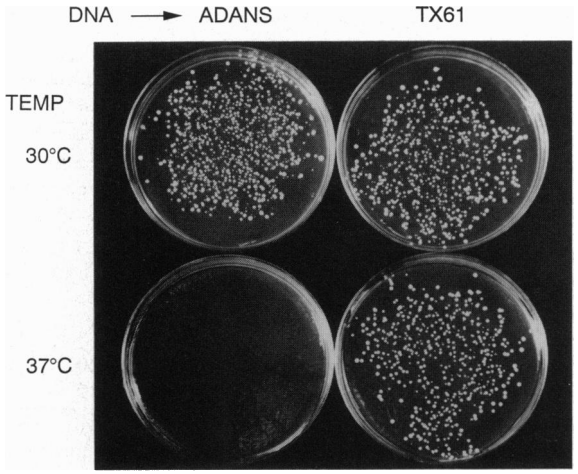

FIG. 2. Results of transformation of a *cdc34-2* temperature-sensitive strain with plasmid DNAs, ADANS and tx61. After transformation, cells were spread onto plates containing leucine-deficient medium and kept at 23°C for 24 h, after which they were shifted to the indicated temperature and incubated for 3 days. Plasmids are described in Table 1.

tx61 Is the Human Homolog of the *CDC34* Gene of *S. cerevisiae*. The sequence of the 1374-bp tx61 cDNA (Fig. 1*A*) encoded one long open reading frame of 298 aa that was in-frame with the first 14 aa of the *ADH* gene, resulting in a fusion protein. Surprisingly, analysis of the translated sequence with the PATMAT homology program (21) revealed a high degree of homology to the *S. cerevisiae* cell cycle gene *CDC34* (Fig. 1*B*) and several other members of the ubiquitin ligase (UBC) family (22). There is 50% perfect homology between tx61 and *CDC34* in the 108 aa flanking the active-site cysteine. Multiple alignment analysis (23) of tx61 with *CDC34* (*UBC3*), *RAD6* (*UBC2*), and *UBC5* revealed that the human tx61 is most closely related to yeast *CDC34* and yeast *CDC34* is more related to tx61 than the other yeast members of the family. For example, there is an insertion in CDC34 (12 aa) and tx61 (13 aa) proteins between the two highly conserved regions surrounding the active site that is not found in most other members of the family (Fig. 1*B*). The alignment also suggests that the first methionine of the tx61 cDNA may be the normal start site of translation. Class II ubiquitin-conjugating enzymes including *CDC34* and *RAD6* contain a highly charged C terminus (12). The tx61 translated sequence also contains an acidic C terminus but does not have the polyaspartate stretch found in the other two proteins. However, only the first 74 aa of the C terminus of CDC34, which does not include the polyaspartate region, is required for its activity *in vivo* (24, 25). The tx61 C terminus spans those amino acids but with little amino acid homology (see below). tx61 is also very homologous to wheat-germ *UBC7* gene in the area around the active site, but *UBC7* does not have the acidic C-terminal end (26).

The sequence homology between tx61 and *CDC34* is of functional significance, as transformation of a *cdc34-2^{ts}* strain with the tx61 cDNA resulted in 100% of the colonies growing at 37°C, compared with <0.1% with the control vector (Fig. 2). Thus the C terminus of tx61 is functionally active even though there is little identity between the *CDC34* and tx61 C termini. However, tx61 did not complement the ionizing radiation sensitivity of a *rad6* strain, the next most homologous member of the yeast ubiquitin ligase family (data not shown). Similarly, yeast *CDC34* does not complement the radiation sensitivity of a *rad6* strain (24, 25). Based on the sequence homology, functional activity, and specificity, we conclude that tx61 is the human homolog of the *CDC34* gene of *S. cerevisiae*.

Given these results, we tested whether the yeast CDC34 protein has similar effects on the *mec1, cdc9-8* strain. Transformation with the *S. cerevisiae CDC34* gene under its own promoter demonstrated no effect on the *mec1, cdc9-8* strain (data not shown). In addition, we subcloned the entire open reading frame of the yeast *CDC34* gene downstream of the *ADH* promoter in the ADANS vector. This construct, S.c. CDC34, efficiently complemented the *cdc34^{ts}* mutation but also had little to no effect on the *mec1, cdc9-8* strain for growth at 30°C in numerous experiments (Fig. 3).

Characterization of the Human *CDC34* cDNA and Gene. Southern blot analysis using the human *CDC34* cDNA as a probe revealed specific hybridization to one or a few bands in human, mouse, and hamster genomic DNA (Fig. 4*A*). We also detected hybridization to chicken genomic DNA and weak hybridization to *Drosophila melanogaster* DNA but not to any lower species including *S. cerevisiae* (data not shown), implying that the human gene could not have been cloned by sequence complementarity.

Northern blot analysis of several human cancer cell lines revealed hybridization to a unique band of ≈1.4 kb (Fig. 4*B*), suggesting that our cDNA is nearly full length. We assayed poly(A)$^+$ RNA from two human neuroblastoma cell lines (SK-M-KCNR and SK-N-AS) and multiple hematopoietic

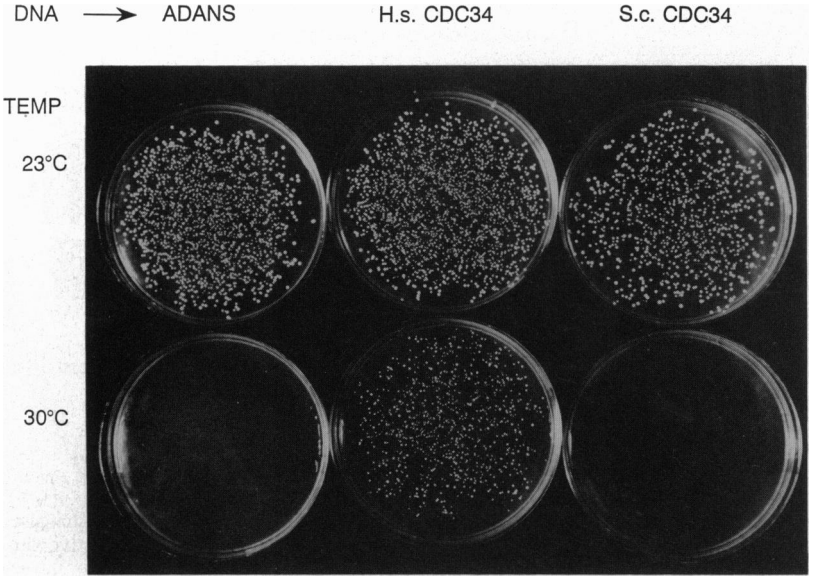

FIG. 3. Results of transformation of a *mec1, cdc9-8* strain with plasmid DNAs (see Fig. 2 for procedure, except that incubation was for 5 days at the indicated temperature). ADANS, plasmid vector; H.s. CDC34, human *CDC34* (tx61 cDNA); S.c. CDC34, *CDC34* gene of *S. cerevisiae* cloned into the ADANS vector.

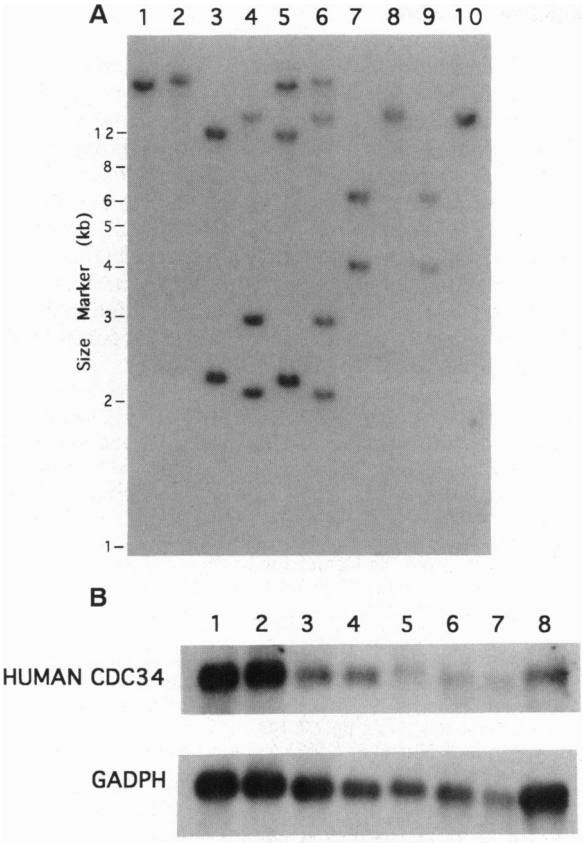

FIG. 4. (*A*) Southern blot analysis of genomic DNA probed with the human *CDC34* cDNA. Lanes: 1 and 2, normal human lymphocyte DNA; 3 and 4, chinese hamster ovary (RJK88) cell line DNA; 5 and 6, human chromosome 19–CHO somatic cell hybrid DNA; 7 and 8, mouse 3T6 cell line DNA; 9 and 10, human chromosome 17–mouse somatic cell hybrid DNA. Lanes 1, 3, 5, 7, and 9 were digested with *Bgl* II. Lanes 2, 4, 6, 8, and 10 were digested with *Xba* I. (*B*) Northern blot analysis of poly(A)$^+$ RNA from human cells probed with the human *CDC34* cDNA. Lanes: 1, SK-M-KCNR cells 5 days after treatment with solvent control; 2, SK-M-KCNR cells 5 days after treatment with retinoic acid; 3, SK-N-AS cells 5 days after treatment with solvent control; 4, SK-N-AS cells 5 days after treatment with retinoic acid; 5, primary human chronic lymphocytic leukemia cells; 6, primary peripheral blood lymphocytes from a Burkitt lymphoma patient; 7, HL60 human promyelocytic leukemia cell line; 8, ODA, an IgD-secreting plasmacytoma cell line.

tumor cell lines. *CDC34* was expressed in all of these lines as expected for a cell cycle regulatory gene, and quantitation revealed differences of only 2- to 4-fold among these lines. In addition, RNA from SMS-KCNR cells that have differentiated and exited the cell cycle after treatment with retinoic acid (27) showed no decrease in expression level (lane 2). SK-N-AS cells are resistant to retinoic acid and also showed no decrease after treatment with retinoic acid. Thus, we did not find any evidence for decreased transcription of human *CDC34* when cells were not cycling.

Chromosomal Localization of *CDC34* in the Human and Mouse Genomes. To further characterize the human *CDC34* gene, we isolated two overlapping cosmids (34cos2 and 34cos4) that were homologous to the human *CDC34* cDNA by screening a human placental cosmid library. Demonstration of these cosmids as representing the human gene and not a homologous gene was confirmed by comparison of the restriction map of the cosmids and human genomic DNA when probed with the human *CDC34* cDNA.

These cosmids were used for chromosomal localization experiments by fluorescent *in situ* hybridization. Hybridization with cosmid 34cos2 in 41 of 42 metaphase human lymphocyte cells examined demonstrated signals on both chromosomes 19 at band p13.3 (Fig. 5). The signals were located at the telomeric end of band 19p13.3. One metaphase cell had signals on only one chromosome 19. Hybridization with cosmid 34cos4 demonstrated signals on both chromosomes 19 in 38 of 40 metaphase cells examined and on one chromosome 19 homolog in the other 2 cells. The signals from 34cos4 were also located at the very telomeric end of band 19p13.3 and were indistinguishable from the signals generated from hybridization with 34cos2. There was no significant hybridization to any other human chromosomes.

An independent confirmation of this chromosomal localization was obtained by Southern blot analysis of human–hamster somatic cell hybrids containing a single normal human chromosome 19. Hybridization with the human *CDC34* cDNA revealed hybridization to the same bands in total human genomic DNA and the chromosome 19 hybrid (Fig. 4*A*).

The human *CDC34* cDNA has also been used to map the location of the homologous gene in the mouse genome to chromosome 11D (N. A. Jenkins and N. G. Copeland, personal communication) by restriction fragment length polymorphism analysis of interspecific crosses (28) using four polymorphisms. Surprisingly, this region of mouse chromosome 11 is highly syntenic to human chromosome 17q not 19p. Given these results, we also probed genomic DNA from a human chromosome 17 mouse somatic cell hybrid with the human *CDC34* cDNA and did not find any hybridization signal (Fig. 4*A*) other than that expected for the mouse genome.

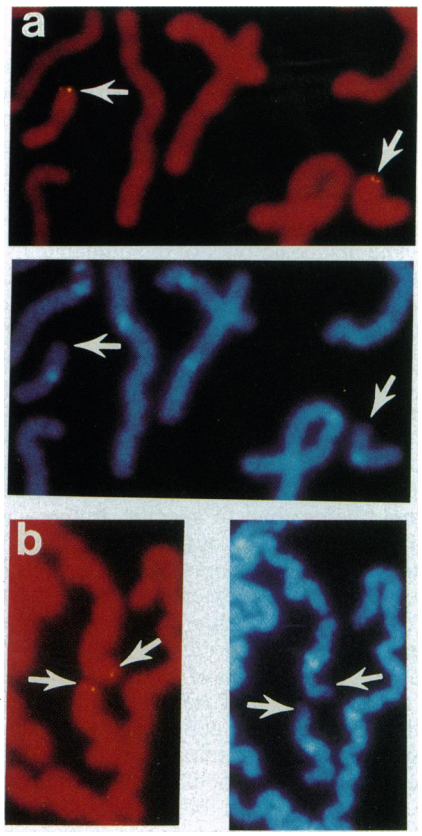

FIG. 5. Fluorescent *in situ* hybridization on human lymphocyte chromosomes using cosmids 34cos2 (*a*) and 34cos4 (*b*). The cosmid signals on the distal short arm of chromosome 19 are indicated with an arrow. The second micrographs for *a*, shown below, and *b*, shown to the right, present Hoeschst/actinomycin D-stained chromosomes for identification.

DISCUSSION

In this report we present results from our initial attempt to obtain human genes in the G_2 checkpoint pathway. The selection scheme was based on the increased lethality of DNA ligase mutations in the presence of a checkpoint mutation. We obtained an active clone that partially complements the *mec1, cdc9-8* strain for the growth defect at 30°C. Unexpectedly, by sequence and function, this clone appears to be the human *CDC34* gene (see below). It is unlikely that human *CDC34* acts in this assay by simply stabilizing DNA ligase at 30°C, since it has no effect on a *rad9, cdc9-8* strain at 30°C and on a *MEC*$^+$, *cdc9-8* strain at higher temperatures. However, the human *CDC34* cDNA complements only this specific phenotype of a *mec1* mutation; it has no effect on the radiation or hydroxyurea sensitivity. Similarly, we have obtained a second human cDNA that complements the effect of the *mec1* mutation on a *cdc9-8* strain and not the other two mec1 phenotypes (S.E.P., unpublished data). Thus, requirements for complementation of the cdc9 checkpoint may be less stringent than for other forms of DNA damage.

In contrast to human *CDC34*, the *S. cerevisiae CDC34* gene, even when overexpressed, does not have the same effect on the *mec1, cdc9-8* strain. Possible explanations for this difference include increased stability of the human protein compared with the yeast throughout the S or G_2 phase of the cycle. Alternatively, the human CDC34 protein may be a less-specific ubiquitin ligase than the yeast CDC34 in yeast. The specific G_1 target of yeast CDC34 is unknown but has been suggested to be one of the G_1 cyclins (9). In this assay a possible S or G_2 target of the human CDC34 protein is one of the B-type cyclins, CLB1-6, of *S. cerevisiae*, which contains a ubiquitin targeting signal (29). Several of these cyclins have been found to be expressed at a high level in both the S and G_2 phases of the cell cycle (30) and may play some regulatory role.

Sequence analysis of this cDNA revealed significant homology to the *CDC34* gene of *S. cerevisiae* and none to the *MEC1* gene itself (T. Weinert, personal communication). Subsequent experiments revealed that the human cDNA efficiently complements a *cdc34-2*^{ts} mutation and not *rad6*, the next most homologous member of the ubiquitin ligase family. Thus, this cDNA appears to be the human homolog of *CDC34*. The *in vivo* activity of the human cDNA provides insights into the structural requirements of the CDC34 protein. Previous studies have demonstrated that the first 74 aa of the C terminus of CDC34 attached to the RAD6 active site are sufficient for CDC34 complementing activity. This result suggested that the C terminus directs a specific interaction with another cellular protein (24, 25). The human CDC34 protein contains a C terminus that is highly charged but shares little homology to the yeast sequence. Thus the function of the C terminus may be more related to both it's structure and charge than to specific amino acid residues. This situation is reminiscent of the acidic activating domains of many transcription factors (31). Although the human *CDC34* does not contain as extensive a polyaspartate tract as both *CDC34* and *RAD6*, this region is not required for complementation of a *cdc34* mutant (24, 25).

Our data map the location of *CDC34* to the far telomeric region of the short arm of human chromosome 19. The telomeric location of this cell cycle gene in humans is intriguing given the role of telomeric shortening in cellular senescence (32). A recent model of senescence proposes that repression of essential genes found near the telomere occurs by a change in chromatin structure as telomeres shorten (33). Thus, it may be interesting to determine how close to the 19p telomere human *CDC34* is located and to examine the expression of this gene in cells as they near senescence.

In contrast to the human mapping data, mapping of the homologous gene in the mouse places it in a nontelomeric position on chromosome 11D. This region maps to a long region of synteny on human chromosome 17q, but we do not find any evidence for a *CDC34* homolog on human chromosome 17; thus *CDC34* defines a region of homology between mouse chromosome 11 and human chromosome 19.

The human *CDC34* homolog belongs to the group of genes (*CDC34, CDC4*, and *CDC53*) required for the late G_1 to S transition in budding yeast. Absence of any one of these functions results in cell cycle arrest before DNA synthesis is initiated and the formation of multiple pseudobuds. Identification of the components of the $G_1 \rightarrow S$ transition in human cells will be important for defining how the initiation of DNA synthesis is regulated and the mechanisms that control the $G_1 \rightarrow S$ transition after DNA damage.

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