were identical for any one species. The GenBank accession numbers of the nine intron sequences are M36884 to M36886, M36888 to M36890, M36892, M36893, and M55288.
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Complementation of the Mitotic Activator, p80^{cdc25}, by a Human Protein-Tyrosine Phosphatase

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The onset of M phase requires the activation of the pp34 protein kinase in all eukaryotes thus far examined. In *Schizosaccharomyces pombe*, pp34 is phosphorylated on Tyr^{15}, and dephosphorylation of this residue regulates the initiation of mitosis. In this study, it is shown that dephosphorylation of Tyr^{15} triggered activation of the pp34-cyclin complex from fission yeast, that a human protein-tyrosine phosphatase can catalyze this event both in vitro and in vivo, and that activation of fission yeast pp34 does not require threonine dephosphorylation. The complementary DNA that encoded the tyrosine phosphatase replaced the mitotic activator p80^{cdc25}, closely associating the *cdc25*$^+$-activating pathway with tyrosine dephosphorylation of pp34.

THE FISSION YEAST *CDC2*$^+$ GENE IS required for cell cycle progression at both the G_1/S and G_2/M phase transitions (*1*). The product of the *cdc2*$^+$ gene is a 34-kD protein–serine/threonine kinase (pp34) that has been highly conserved throughout evolution (*2*). Homologs of this protein kinase appear to regulate the onset of mitosis in all eukaryotes examined, as well as meiotic M phase in amphibian, sea urchin, and mollusk oocytes (*2*). Activation of pp34 and entry into mitosis is dependent on physical association between cyclin, which in *S. pombe* is the product of the *cdc13*$^+$ gene (*2*), and pp34. Activation of pp34 in fission yeast also requires the product of the *cdc25*$^+$ gene, p80^{cdc25}(*3*), which accumulates during the G_2 period of the cell cycle (*4*).

Dephosphorylation of pp34 on tyrosine, threonine, or both residues has been correlated with activation of the pp34-cyclin complex in many systems (*5*–*7*). *S. pombe* pp34 is phosphorylated in a cell cycle–dependent manner on a single detectable threonine residue (*8*) and on a single tyrosine residue, Tyr^{15} (*5*). We expressed a mutant pp34 in which Tyr^{15} had been replaced with phenylalanine in *S. pombe*, and concluded that phosphorylation of Tyr^{15} normally inhibits pp34 activation and that Tyr^{15} dephosphorylation is a rate-limiting step for the initiation of mitosis (*5*). Here we show that in *S. pombe*, tyrosine dephosphorylation is the trigger for pp34-cyclin activation. Moreover, we show that a human protein-tyrosine phosphatase (PTPase) expressed in *S. pombe* can replace the mitotic activator p80^{cdc25} and drive entry into mitosis.

Prevention of Tyr^{15} phosphorylation causes advancement of fission yeast cells into M phase prematurely, and thus, probably represents the last step in the activation of the pp34-cyclin complex. However, it was possible that although tyrosine dephosphorylation was rate-limiting, other downstream events were required before activation of pp34 protein kinase activity. If tyrosine dephosphorylation alone is sufficient to activate the pp34-cyclin complex, then protein kinase activation should be achieved by enzymatically removing phosphate from Tyr^{15} in vitro. First we tested whether a purified PTPase, termed the T cell PTPase (*9*), could dephosphorylate ^{32}P-labeled pp34. We isolated ^{32}P-labeled pp34

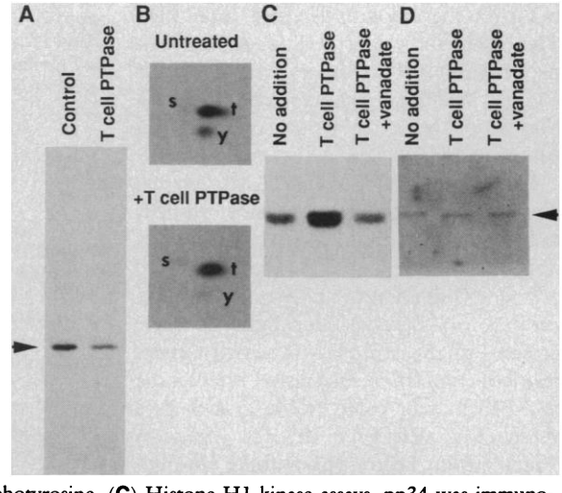

Fig. 1. Activation of pp34 by tyrosine dephosphorylation. (**A**) Treatment of pp34 with T cell PTPase. pp34 was immunoprecipitated with antiserum to pp34 (Ab 4711) (*5*) from nondenatured lysates (*16*) of *cdc25-22* cells labeled with [^{32}P]orthophosphate and growth-arrested at 36°C as described *17*). One-half of the immunoprecipitate was treated with PTPase buffer alone (*18*) and the other half with buffer and T cell PTPase for 30 min at 30°C (*19*). The arrow indicates pp34. The autoradiograph was exposed for 16 hours. (**B**) Phosphoamino acid analyses (*20*) of pp34 from ^{32}P-labeled–untreated and PTPase-treated immunoprecipitates similar to those in (A) (6-day exposure). S, phosphoserine; t, phosphothreonine; y, phosphotyrosine. (**C**) Histone H1 kinase assays. pp34 was immunoprecipitated with Ab 4711 from unlabeled nondenatured lysates of *cdc25-22*–arrested cells and split into three equal portions. One-third was treated with PTPase buffer, one-third with buffer and T cell PTPase, and one-third with buffer, T cell PTPase, and sodium orthovanadate (1 mM). After washing with kinase buffer (*16*), one-half of these immunoprecipitates was assayed for histone H1 kinase activity as described (*16*). The protein bands on the autoradiograph were quantified by densitometry. (**D**) pp34 immunoblot with antibody to pp34 COOH-terminal peptide, PN24, (*21*) of the remaining halves of the immunoprecipitates described in (C). The arrow indicates pp34.

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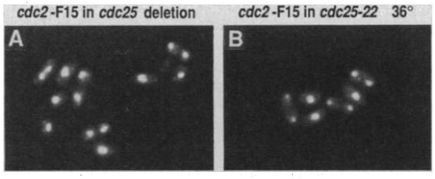

Fig. 2. Staining of yeast cells with diamidinophenylindole (DAPI). (**A**) *S. pombe* strain *cdc25*$^+$/*cdc25*::*ura4*$^+$, *leu1-32/leu1-32*, *ura4*-D18/*ura4*-D18, h$^+$/h$^-$ was transformed with a *S. pombe* expression vector (pIRT-2) that contained the *cdc2* promoter, *cdc2*-F15 coding sequences, and the *S. cerevesiae LEU2* gene (*17*). Diploid transformants were sporulated and haploid Leu$^+$Ura$^+$ clones were grown in minimal media at 32°C, fixed, and stained with the DNA-specific fluorescent dye DAPI as described (*17*). (**B**) *S. pombe* strain *cdc25-22 leu1-32* h$^-$ was transformed with the plasmid in (A). Transformants were grown in minimal media at 36°C, fixed, and stained with DAPI.

by immunoprecipitation from cells that were arrested in late G_2 because of a temperature-sensitive (ts) mutation in the mitotic activator p80^{cdc25}. Treatment with the T cell PTPase led to a decrease in ^{32}P-labeled pp34 (Fig. 1A), and, as expected from the known specificity of PTPases (*10*), phosphate was removed only from tyrosine residues, although the reaction was never complete (Fig. 1B). Unlabeled pp34 immunoprecipitates from *cdc25*^{ts}-arrested cells were then treated with the T cell PTPase and subsequently assayed for protein kinase activity towards the exogenous substrate, histone H1. The histone H1 kinase activity of pp34 was activated threefold by PTPase treatment relative to an untreated sample (Fig. 1C). Activation was prevented by the inclusion of the PTPase inhibitor vanadate (Fig. 1C), and PTPase treatment did not alter the amount of pp34 (Fig. 1D). These data show that in *S. pombe* tyrosine dephosphorylation activates the pp34-cyclin complex and that threonine dephosphorylation is not required for protein kinase activation.

The mutant pp34, *cdc2*-F15, which could not be phosphorylated on tyrosine, was able to bypass the requirement for p80^{cdc25} function, as *cdc2*-F15 was able to rescue growth of a *cdc25*^{ts} strain at the nonpermissive temperature (*5*). Because temperature-sensitive proteins might retain partial activity under restrictive conditions, we tested whether the *cdc2*-F15 mutant could rescue growth of a strain with a deletion in the *cdc25*$^+$ gene. A diploid strain heterozygous for a chromosomal deletion of *cdc25*$^+$ was transformed with a vector that contained *cdc2*-F15 coding sequences, and the transformants were sporulated. Spores were plated on medium that selected for haploid cells that contained both the chromosomal deletion of *cdc25*$^+$ and the plasmid. In the absence of *cdc25*$^+$ or gene sequences that bypassed the requirement for *cdc25*$^+$, no haploid cells would be expected to grow. However, haploid cells deleted for *cdc25*$^+$ were obtained and appeared identical to *cdc25-22* cells that were rescued by *cdc2*-F15 (Fig. 2). Both rescued strains were phenotypically "wee," indicating that the cells entered mitosis prematurely at a reduced cell size (Fig. 2). This evidence strengthens the hypothesis that p80^{cdc25} is involved in regulating tyrosine dephosphorylation of pp34.

The notion that p80^{cdc25} might be directly or indirectly controlling pp34 tyrosine dephosphorylation prompted us to ask whether expression in *S. pombe* of a known PTPase could substitute for p80^{cdc25} in the mitotic initiation process. We chose to express the T cell PTPase, which we had shown could remove phosphate from Tyr^{15} in vitro. The T cell PTPase cDNA (*9*) was integrated as a single copy (*8*) into the genome of the *S. pombe* strain *cdc25-22 leu1*-32 h$^-$ under the control of a thiamine-repressible promoter (*11*). This strain, which will be referred to as KLG13, could grow at 36°C in the presence or absence of thiamine (see below), demonstrating that the mammalian T cell PTPase even at low concentrations was capable of substituting for p80^{cdc25}. The T cell PTPase, like *cdc2*−F15, was also capable of rescuing growth of a *cdc25*$^+$-deletion strain (*8*).

In the absence of thiamine in the growth media, a band of the expected molecular size, 48 kD (*9*), was detected by immunoblotting with antibodies to the T cell PTPase, in lysates of KLG13 grown at both the *cdc25-22*–permissive (25°C) and *cdc25-22*–restrictive (36°C) temperatures (Fig. 3A). This protein was not observed in the parent strain and was barely detected when thiamine was included in the growth media (Fig. 3A).

KLG13 cells grown at 25°C in the presence of thiamine (low PTPase expression) were indistinguishable in length and growth rate from the parent strain (Fig. 3B, a). When KLG13 was grown at 36°C in the presence of thiamine, the cells were elongated, but they were not cell-cycle arrested (Fig. 3B, b). In the absence of thiamine (high PTPase expression) at 25°C, KLG13 cells entered mitosis at a detectably smaller cell size than in the presence of thiamine (Fig.3B, c). At 36°C with high PTPase expression, KLG13 cells were phenotypically wee (Fig. 3B, d). The T cell PTPase had more effect at 36°C than at 25°C, perhaps because it is a mammalian enzyme with a higher temperature optimum or because it is expressed at higher amounts at 36°C (Fig. 3A). The correlation of the sizes of cells with the amount of T cell PTPase expression suggested that the PTPase was removing phosphate from Tyr^{15} in vivo.

To confirm that the amount of pp34 tyrosine phosphorylation was altered by T cell PTPase expression, we examined the abundance, the phosphorylation state, and the phosphoamino acid composition of

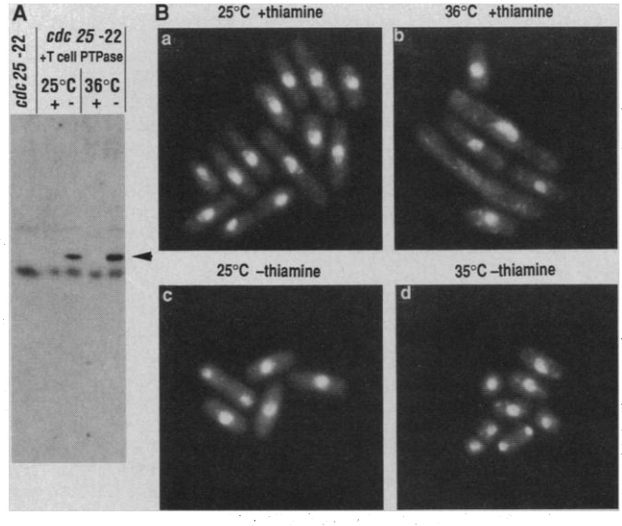

Fig. 3. T cell PTPase expression in *S. pombe*. (**A**) Immunoblot with antibodies to PTPase. The Eco RI–Hind III fragment of the T cell PTPase cDNA (*9*) was subcloned into the unique Bam HI site of the fission yeast expression vector pMNS21L, such that the T cell PTPase mRNA expression was driven by the thiamine-repressible *nmt1*$^+$ promoter (*11*). After transformation into *S. pombe* strain *cdc25-22 leu1*-32 h$^-$, an integrant was selected visually on the basis of plasmid stability and named KLG13. A single integration event was confirmed by Southern (DNA) blotting. Lysates were made from the parent strain and from KLG13 grown in minimal media at 25° and 36°C both in the presence and absence of thiamine (5 μg/ml). Equal amounts of protein from these lysates were resolved on an SDS-polyacrylamide (15%) gel, transferred to Immobilon-P (Millipore), and probed with an anti-PTPase peptide serum (*9*) followed by ^{125}I-labeled protein A (5-day exposure). The plus and minus refer to the addition of thiamine to the media. The arrow indicates the position of the 48-kD T cell PTPase. Addition of excess peptide does not reduce the amounts of the other protein bands on the autoradiogram, and these proteins are not recognized by affinity-purified anti-PTPase. (**B**) DAPI staining of KLG13. KLG13 was grown in minimal media at 25°C (a and c) or 36°C (b and d) in the presence (a and b) or absence (c and d) of thiamine (5 μg/ml), fixed, and stained with DAPI.

pp34 in KLG13. The amount of pp34 did not change appreciably when the T cell PTPase was expressed (Fig. 4A). However, pp34 phosphorylation was reduced up to 50% in KLG13 cells that expressed the T cell PTPase at 36°C (Fig. 4B). Phosphoamino acid analyses of ^{32}P-labeled pp34 immunoprecipitates revealed that the reduction in ^{32}P occurred on tyrosine residues (Fig. 4C). Thus, the amount of phosphotyrosine in pp34 was dependent on the amount of T cell PTPase expression and correlated with the size of the cells as they entered mitosis.

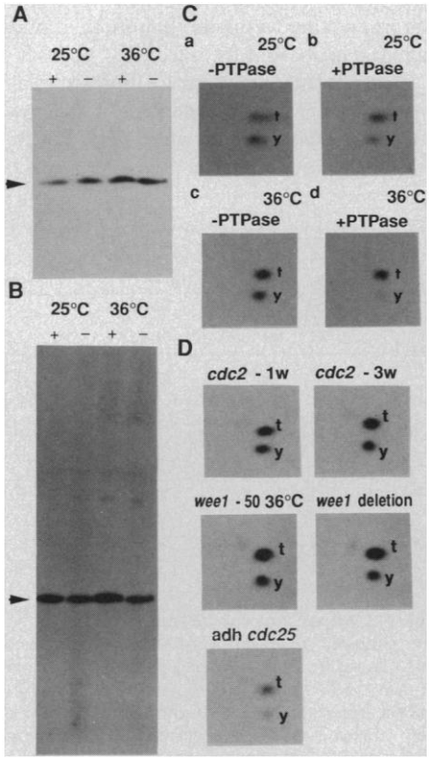

Fig. 4. Phosphorylation state of pp34 in KLG13. (**A**) Immunoblot of pp34. Lysates used in Fig. 3A were resolved by electrophoresis on an SDS-polyacrylamide (15%) gel, transferred to Immobilon-P, and probed with Ab 4711, followed by ^{125}I-labeled protein A (16-hour exposure). The first lane was inadvertently underloaded by half. The plus and minus refer to the addition of thiamine; the arrow indicates the position of pp34. (**B**) ^{32}P-labeled pp34 immunoprecipitates. pp34 was immunoprecipitated with PN24 from denatured lysates of KLG13 grown and labeled at 25° or 36°C in the presence or absence of thiamine (5 μg/ml). The immunoprecipitates were resolved on an SDS-polyacrylamide (15%) gel, transferred to Immobilon-P, and subjected to autoradiography (16-hour exposure). The arrow indicates the position of pp34. The bands were quantified by Cerenkov counting. (**C**) Phosphoamino acid content of pp34 in KLG13. The immunoprecipitates in (B) were subjected to partial acid hydrolysis (6-day exposure). (**D**) Phosphoamino acid content of pp34 in wee strains. *S. pombe* strains *cdc2-1*w h$^-$ (32°C), *cdc2-3*w h$^-$ (32°C), *wee1-50* h$^-$ (36°C), *wee1*::*ura4*$^+$ *leu1-32 ura4*-D18 h$^-$ (32°C), and adh *cdc25*$^+$ *cdc25*::*ura4*$^+$*ura4*-D18 *leu1*-32 h$^-$ (32°C) were labeled with [^{32}P] orthophosphate at the temperatures indicated in parentheses and lysed under denaturing conditions. pp34 was immunoprecipitated with PN24 and the immunoprecipitates were resolved on an SDS-polyacrylamide (15%) gel, transferred to Immobilon-P, and subjected to partial acid hydrolysis (6-day exposure).

A similar reduction in the amount of phosphotyrosine in pp34 was not observed in other phenotypically wee cells. The amount of phosphotyrosine relative to phosphothreonine in pp34 was not reduced in two strains that contained wee mutations of *cdc2*$^+$, *cdc2-1*w, and *cdc2-3*w (*12*) (Fig. 4D). Furthermore, in two strains that are phenotypically wee because of inactivation of the mitotic inhibitor p97^{wee1} (*13*), there was no apparent change in the amount of pp34 tyrosine phosphorylation (Fig. 4D). Thus, the reduction in pp34 phosphotyrosine content observed in KLG13 cells that expressed the T cell PTPase was a direct effect of T cell PTPase action and not an indirect effect of reduced cell size. In a fifth example, where p80^{cdc25} overexpression causes premature entry into mitosis (*3*), the amount of phosphotyrosine in pp34 appeared to be reduced (Fig. 4D). This would be expected if p80^{cdc25} positively regulated tyrosine dephosphorylation.

We have shown here that tyrosine dephosphorylation of *S. pombe* pp34 in vitro triggers protein kinase activation. Previous reports had concluded that tyrosine dephosphorylation alone was insufficient to activate pp34 protein kinase activity when pp34 was isolated from starfish or murine cells (*7*). This apparent discrepancy can now be explained by the identification of an additional site of phosphorylation at the adjacent residue, Thr^{14}, which serves in conjunction with phosphorylation at Tyr^{15} to inhibit pp34 protein kinase activation in higher eukaryotes (*14*). Phosphorylation at Thr^{14} does not ordinarily occur on pp34 in *S. pombe* (*5, 8*), and thus we were able to detect activation of the pp34-cyclin complex simply by dephosphorylating Tyr^{15}.

In the absence of the mitotic inhibitor p97^{wee1}, pp34 was phosphorylated on tyrosine (Fig. 4D). The simplest interpretation of this result would be that p97^{wee1} is not responsible for promoting tyrosine phosphorylation of pp34 and that it acts to inhibit mitosis through a different mechanism. More complicated interpretations of this result are possible but would have to invoke a duplication of the pathway promoting pp34 tyrosine phosphorylation.

Our data have implicated p80^{cdc25} as a potential regulator of Tyr^{15} dephosphorylation, as it is no longer required when either the *cdc2*-F15 protein or the T cell PTPase is expressed. Given that neither p80^{cdc25} nor its functional homologs from *S. cerevesiae*, *Drosophila melanogaster*, or human cells bear significant sequence similarity to mammalian PTPases (*3, 15*), it seems unlikely that p80^{cdc25} is itself a PTPase. Rather, because it accumulates just before the onset of M phase (*4*), p80^{cdc25} might serve as a positive regulator of a PTPase. Alternatively, the requirement for p80^{cdc25} action might be bypassed by constitutive Tyr^{15} dephosphorylation for unknown reasons; another *cdc2* mutant, *cdc2*-3w, independent of the requirement for p80^{cdc25}, has no obvious change in its phosphorylation pattern relative to wild-type pp34 (Fig. 4D).

The fact that expression of the T cell PTPase in yeast cells led to cell cycle alterations but no other growth or shape irregularities suggests either that there are few important tyrosine phosphorylation events in fission yeast (perhaps only one), or that the T cell PTPase specifically recognizes pp34 in these cells. Stable expression of the T cell PTPase in a heterologous system was first achieved in BHK cells. Under these circumstances, the 48-kD PTPase did not generate any obvious changes in cell growth or morphology, although the expressed enzyme was active and led to the dephosphorylation of tyrosyl residues in a variety of proteins (*9*). However, dephosphorylation of Thr^{14} is also required for the activation of vertebrate pp34 (*14*) and therefore overexpression of the T cell PTPase in BHK cells would not be expected to alter the timing of mitotic initiation. Thus, pp34 may be a physiologically relevant target for the T cell PTPase in human cells.

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Genetic Mechanisms of Tumor Suppression by the Human p53 Gene

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Mutations of the gene encoding p53, a 53-kilodalton cellular protein, are found frequently in human tumor cells, suggesting a crucial role for this gene in human oncogenesis. To model the stepwise mutation or loss of both p53 alleles during tumorigenesis, a human osteosarcoma cell line, Saos-2, was used that completely lacked endogenous p53. Single copies of exogenous p53 genes were then introduced by infecting cells with recombinant retroviruses containing either point-mutated or wild-type versions of the p53 cDNA sequence. Expression of wild-type p53 suppressed the neoplastic phenotype of Saos-2 cells, whereas expression of mutated p53 conferred a limited growth advantage to cells in the absence of wild-type p53. Wild-type p53 was phenotypically dominant to mutated p53 in a two-allele configuration. These results suggest that, as with the retinoblastoma gene, mutation of both alleles of the p53 gene is essential for its role in oncogenesis.

TUMOR-SUPPRESSOR GENES ARE DEfined as genes for which loss-of-function mutations are oncogenic (*1*). Wild-type alleles of such genes may thus function to prevent or suppress tumorigenesis. For example, introduction of wild-type copies of the retinoblastoma gene (*RB*), the prototype of this class (*2*), suppressed the neoplastic properties of human tumor cells with mutated endogenous RB, thereby providing direct evidence for tumor suppression by a single gene (*3, 4*). Another gene product, p53, was first identified as a 53-kD cellular protein that binds to SV40 T antigen (*5*), a property that is also shared by RB protein. The gene encoding p53 is commonly affected by deletions, rearrangements, or point mutations in human and murine tumor cells (*6, 7*). p53 was originally considered to be an oncogene because mutated p53 alleles could transform primary rat embryo fibroblasts in concert with an activated *ras* gene (*8*). However, cotransfection of wild-type murine p53 was shown to reduce transformation efficiency by many other oncogenes (*9*). These studies, and the observed diversity of mutations in human tumors, suggested that p53 might be a tumor suppressor gene, that is, a gene that is inactivated by mutation. The dominant transforming effect was presumed to be due to a "dominant negative" activity of mutated p53 protein that somehow blocked the growth-restricting function of wild-type p53 protein in cells. This model suggested that the relative quantity of mutated to wild-type p53 could determine the transformed phenotype, but gene dosages could not be tightly controlled in these transfection studies.

Because of such questions, as well as the possibility of species-specific differences in p53 function (*10*) and the uncertain relevance of transformed animal cells to human neoplasia, we sought to reassess the biological properties of p53 in the human system. The human osteosarcoma cell line Saos-2 was chosen as a host cell because it has no endogenous p53, because of the complete deletion of its gene (*6*). We used recombinant retroviruses derived from Moloney murine leukemia virus (Mo-MuLV) to introduce mutated or wild-type p53 under the long terminal repeat (LTR) promoter control. Cell clones isolated after infection and selection carried only a single integrated provirus of each type, and multiple clones were analyzed to exclude positional effects. A comprehensive assessment of biological properties of these clones included morphology, growth rates and saturation density in culture, colony formation in soft agar, and tumorigenicity in nude mice.

As a reference standard for human wild-type p53, we used the genomic DNA sequence of Lamb and Crawford (*11*). Poten-

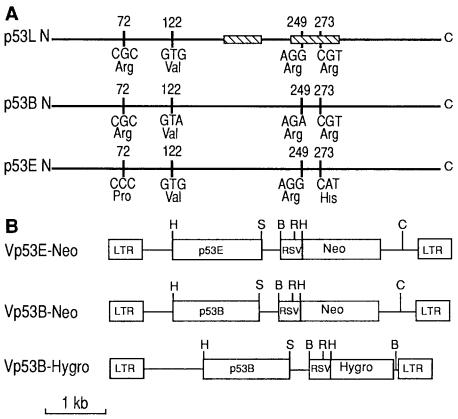

Fig. 1. Comparison of three human p53 cDNAs, and genomic organization of three recombinant retroviruses for expressing p53 protein. (**A**) Three human p53 cDNAs are diagrammed. The sequence reported by Lamb and Crawford (*11*), here labeled as p53L, was derived by sequencing clones from human fetal liver cDNA and genomic libraries, and is considered to be wild type. p53B is a cDNA clone derived from fetal brain RNA by the RT-PCR method (*12*). The deduced amino acid sequences of p53B and p53L were identical despite two silent nucleotide substitutions as indicated. p53E is a cDNA clone (*13*) that has amino acid substitutions at positions 72 and 273 relative to p53L or p53B. The Arg/Pro^{72} replacement represents a common amino acid polymorphism (*15*) without known functional significance, but the substitution of His for Arg at position 273 is found exclusively in tumor cells and is considered to be a mutation. Like many other p53 mutations, Arg^{273} → His lies within one of two conserved regions required for binding to SV40 T antigen (hatched boxes) (*23*). (**B**) Genomic organization of three p53 retroviruses are diagrammed. Vp53E-Neo was constructed by inserting a 1.5-kb Hind III–Sma I DNA fragment containing p53E into the plasmid pLRbRNL (*3*), replacing *RB* cDNA. A 1.35-kb p53B DNA fragment obtained by RT-PCR was inserted into the pLRbRNL vector to form Vp53B-Neo. The insert in one clone was entirely sequenced, as diagrammed in (A). Vp53B-Hygro was constructed by insertion of a Hind III DNA fragment containing p53B and the Rous sarcoma virus promoter into plasmid 477 (a MuLV-Hygro vector provided by W. Hammerschmidt and B. Sugden). These constructs were then used to produce the corresponding viral stocks as described previously (*3*). Some major restriction sites important for construction are indicated. H, Hind III; R, EcoR I; S, Sma I; B, Bam HI; and C, Cla I.

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