Suppression of the Neoplastic Phenotype by Replacement of the RB Gene in Human Cancer Cells

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Mutational inactivation of the retinoblastoma susceptibility (RB) gene has been proposed as a crucial step in the formation of retinoblastoma and other types of human cancer. This hypothesis was tested by introducing, via retroviral-mediated gene transfer, a cloned RB gene into retinoblastoma or osteosarcoma cells that had inactivated endogenous RB genes. Expression of the exogenous RB gene affected cell morphology, growth rate, soft agar colony formation, and tumorigenicity in nude mice. This demonstration of suppression of the neoplastic phenotype by a single gene provides direct evidence for an essential role of the RB gene in tumorigenesis.

"CANCER SUPPRESSION" WAS originally defined as a loss of tumorigenicity observed in fusion cells made between tumor cells and normal fibroblasts, lymphocytes, or keratinocytes; this effect was presumed to be mediated by dominant suppressive factors in normal cells (*1*). Evidence indicated that these factors were in part genetic, as a tight correlation existed between suppression of tumorigenicity and the presence of certain chromosomes in fused cells (*2*). For example, introduction of a normal chromosome 11 into Wilms' tumor cells by microcell fusion-mediated transfer suppressed their tumorigenicity, whereas chromosomes X and 13 did not have this effect (*3*). However, since entire human chromosomes were transferred, cancer suppression could not be attributed to molecularly defined genetic elements.

Another meaning for cancer suppressor genes arose in connection with genetic studies of certain childhood neoplasms (*4*) and adult tumor syndromes (*5*). Genes contributing to the formation of these tumors appear to become oncogenic by loss of function rather than by the activation typical of classical oncogenes (*6*). Retinoblastoma, a childhood eye cancer, is associated with loss of a gene locus, called RB or RB-1, located in chromosome band 13q14 (*7–9*). A gene from this region was molecularly cloned that had properties consistent with the RB gene (*10–12*). A 4.7-kb mRNA transcript of this gene was present in all normal tissues examined but was absent or altered in retinoblastoma cells (*11*), and deletions within the gene have been detected in many retinoblastoma tumors (*12, 13*).

A protein product of the RB gene was previously identified as a nuclear phosphoprotein of about 110 kD that has DNA binding activity (*14*). DNA sequences homologous to RB cDNA, and proteins antigenically related to RB protein, have been found in all vertebrate species examined (*11, 14*). The RB protein was recently shown to associate with large T antigen and E1A, the transforming proteins of DNA tumor viruses SV40 and adenovirus, respectively (*15*). These studies indirectly suggested that the RB protein has a role in regulating the expression of other cellular genes, and may also mediate the oncogenic effects of some viral-transforming proteins.

Inactivation of the RB gene has been observed in different types of tumors including osteosarcoma, synovial sarcoma and other soft-tissue sarcomas, small-cell lung carcinoma, and breast carcinoma (*16*). Only a subset of cases of any tumor type has demonstrable RB mutations, and the significance of RB gene inactivation in their genesis is not yet clear. A biological assay for RB gene function would be the ultimate proof of the significance of RB gene inactivation in these natural human tumors.

We therefore undertook to develop an assay system for RB gene function by introducing the gene into cultured tumor cells that contain inactivated endogenous RB genes. Two amphotropic retroviruses were constructed as shown in Fig. 1. One, Rb, consisted of the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTRs) (*17*) coupled to a modified RB cDNA and the neomycin-resistance gene under Rous sarcoma virus (RSV) promoter control (*18*). The other, Lux, was identical to Rb except that RB was replaced by the luciferase gene (*19*). The luciferase gene served not only as a control for specific effects of the RB gene but also as a means to examine expression efficiency of the viral construct in different cell types. These two plasmids were then transfected into PA12 cells, which carry a packaging-deficient provirus and express all the necessary components for virus production including synthesis of amphotropic envelope glycoproteins (*20*). Because this step produced very little infectious virus, supernatants harvested from transfected PA12 cells were used to infect the ecotropic helper line ψ-2 (*21*). Individual G418-resistant colonies were iso-

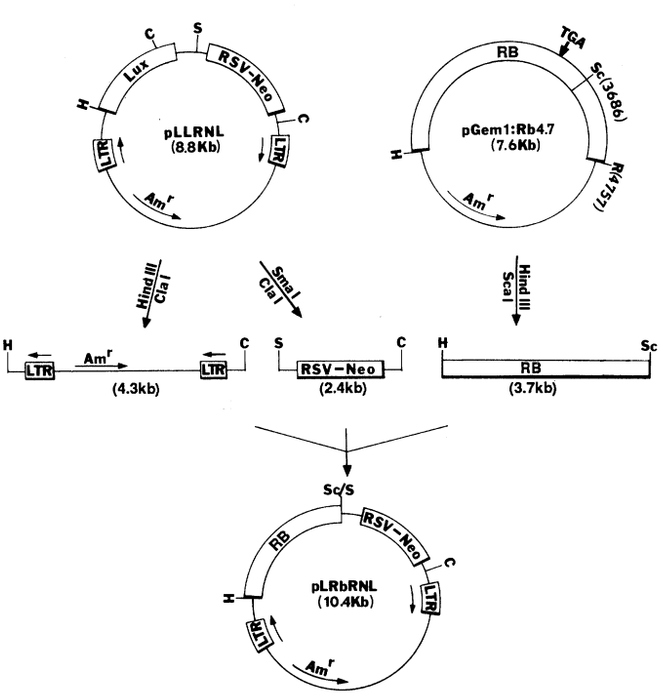

Fig. 1. Construction of Rb and Lux viruses. Plasmids pLLRNL (*20*) and pGem1:Rb4.7 (*14*) were digested with restriction endonucleases, and appropriate fragments were ligated to form pLRbRNL. Only selected restriction sites are shown (H, Hind III; C, Cla I; S, Sma I; Sc, Sca I; R, Eco RI). LTR, long terminal repeat of Moloney murine leukemia virus; Lux, luciferase gene; RSV, Rous sarcoma virus promoter; Neo, Tn5 neomycin-resistance gene; Am^r, ampicillin resistance gene; RB, RB cDNA; TGA, stop codon.

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lated and screened for virus production and RB protein expression. Among ten clones screened, eight produced 10^3 to 10^5 G418-resistant colony-forming units (cfu) per milliliter when assayed with 208F rat fibroblasts as indicator cells. Both parental clones and infected rat 208F cells expressed normal-sized human RB protein (~110 kD) in addition to rodent RB protein (~125 kD) when labeled with ^{32}P-orthophosphate and immunoprecipitated with a polyclonal antibody to RB (anti-fRB) (*14, 22*). Finally, ecotropic virus was used to infect PA12 cells, and G418-resistant clones were screened for virus production and for expression of human RB protein. Five of ten clones expressed RB protein and produced viral titers up to 4×10^4 cfu/ml. Lux virus was obtained by an identical procedure except that colonies were screened for luciferase expression; the highest titer was 1×10^5 cfu/ml.

Retinoblastoma cell line WERI-Rb27 and osteosarcoma cell line Saos-2 carry inactivated RB genes, as shown by the absence of normal RB protein (*14, 23*). Both cell lines have partial deletions of the RB gene (*13, 23*) and are referred to as RB$^-$ cells. Another osteosarcoma cell line, U-2OS, expresses normal-sized RB protein and has apparently normal RB alleles (*14, 23*). The low titer and efficiency of infection by amphotropic retrovirus made it necessary to include selection with G418 to remove uninfected cells in all assays. After infection, RB$^-$ cell lines expressed normal-sized RB protein when labeled with ^{32}P-orthophosphate (Fig. 2A, lanes 2 and 4); Lux-infected RB$^-$ cells expressed no RB protein. As expected, RB protein expression in U-2OS cells was not detectably altered after infection with Rb because of the presence of endogenous RB protein. However, G418 resistance of the selected clones indicated that viral infection had occurred.

To further verify the presence of newly expressed RB protein in tumor cells, its cellular localization was examined. Most of the endogenous RB protein is found in nuclei of U-2OS cells, as indicated by both cell fractionation and immunostaining (*14*). The nuclei of Rb-infected Saos-2 cells could be immunostained with anti-fRB (*14*) (Fig. 2B), whereas Lux-infected cells were entirely unstained under the same conditions. Therefore the newly expressed RB protein was indistinguishable from native RB by molecular weight, cellular localization, and phosphorylation. In all subsequent infections, RB gene expression was monitored by immunoprecipitation with anti-fRB.

The morphology of Saos-2 cells, grown as a monolayer, was essentially unchanged after Lux infection but was markedly altered by Rb infection (Fig. 3). Starting 2 weeks after Rb infection, two populations of G418-resistant cells with distinct morphology were reproducibly observed. The majority (90 to 95%) of cells became flattened and greatly enlarged in average diameter (three- to tenfold) compared to Lux-infected or uninfected cells (Fig. 3). The remaining cells were smaller and resembled uninfected parental cells. After 4 weeks in culture and further passaging, the larger cells were replaced by smaller cells that resembled parental or Lux-infected Saos-2 cells. Suspension cultures of WERI-Rb27 cells were also unchanged by Lux infection. Four weeks after Rb infection, however, moderately enlarged cells appeared that became increasingly numerous up to 8 weeks. Large clumps of dead cells were also observed starting at 6 weeks (Fig. 3). After prolonged culture (>10 weeks), smaller cells that resembled parental or Lux-infected cells again became the predominant cell type. In contrast, monolayer cultures of U-2OS cells were unchanged after infection with either virus (Fig. 3).

Variation in numbers of these morphologically distinct populations suggested that the two cell types differed in rates of cell division or longevity in culture. This difference was quantified for monolayer osteosarcoma cells by means of a clonal growth assay. Infected Saos-2 and U-2OS cells were plated at low density, and individual colonies formed by these cells were identified (Fig. 4, B and C). The majority of colonies generated by Rb-infected Saos-2 cells either grew much more slowly than colonies of Lux-infected cells, or stopped growing completely after a few days (Fig. 4B). However, a few fast-growing colonies were always present. Infection by either virus did not change the growth rate of colonies of U-2OS cells (Fig. 4C). Subpopulations of WERI-Rb27 cells could not be separately tracked because cells grew in suspension. However, bulk population growth by WERI-Rb27 cultures was noticeably slower

Table 1. Soft agar colony formation of Rb- or Lux-infected osteosarcoma cells. Cells infected with Rb or Lux virus were grown in G418-containing medium for 10 days. Viable G418-resistant and uninfected cells were seeded in duplicate at various dilutions in 0.35% soft agar as described (*25*). Total colony numbers were scored (±SD)after 20 days of growth. Individual colonies of Saos-2 contained more than 50 cells, whereas U-2OS contained about 30 cells. TMTC, too many to count.

Cells	Colony number at initial seeding density (per plate) of	
	1×10^5	2.5×10^4
	Saos-2	
Uninfected	TMTC	124 ± 16
Lux-infected	396 ± 21	138 ± 10
Rb-infected	56 ± 10	7 ± 1
	U-2OS	
Uninfected	384 ± 19	109 ± 8
Lux-infected	340 ± 20	151 ± 11
Rb-infected	290 ± 24	90 ± 5

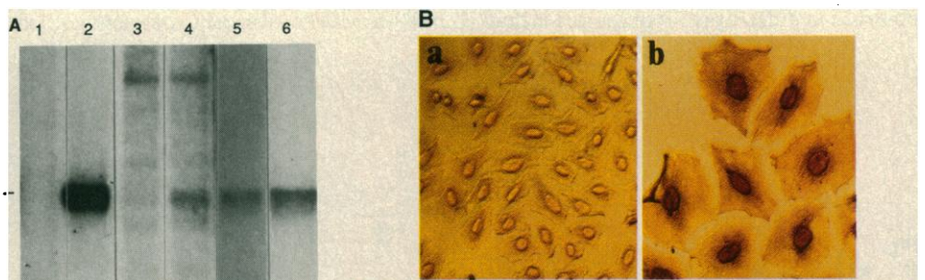

Fig. 2. Expression of RB protein in Rb-infected retinoblastoma and osteosarcoma cell lines. (**A**) WERI-Rb27 (lanes 1 and 2), Saos-2 (lanes 3 and 4) and U-2OS (lanes 5 and 6) cells were infected with Rb virus (lanes 2, 4, and 6) or Lux virus (lanes 1, 3, and 5) and grown in Dulbecco's modified essential medium (DMEM) + 10% fetal calf serum + G418 (800 μg/ml for Saos-2 and U-2OS and 1.0 mg/ml for WERI-Rb27) for 2 weeks (Saos-2 and U-2OS) or 4 weeks (WERI-Rb27). About 5×10^5 cells were then labeled with ^{32}P-phosphoric acid (0.25 mCi/ml) for 3 hours. Cellular lysates were immunoprecipitated with rabbit anti-fRB IgG (*14*) and analyzed on 7.5% SDS-polyacrylamide gels as described (*14*). (**B**) Saos-2 cells were infected with Lux (panel a) or Rb (panel b) and grown in G418-containing medium for 3 weeks in 60-mm dishes. Dishes were washed with phosphate-buffered saline (PBS) between each of the following steps: cells were fixed with 4% formaldehyde in 0.04*M* phosphate buffer (*p*H 7.4) for 20 minutes and immersed in 1% H_2O_2 in 0.04*M* phosphate buffer for 10 minutes. Fixed cells were preincubated with normal goat serum for 10 minutes and then incubated overnight with rabbit anti-fRB IgG diluted in 0.02% Triton X-100. After washing, biotinylated goat anti-rabbit IgG (TAGO, Burlingame, CA) was added to dishes. One hour later, cells were incubated with AB complex conjugated with horseradish peroxidase (Vector Labs, Burlingame, CA) for 30 minutes and then incubated with substrate (0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.05*M* tris-HCl, *p*H 7.6) (Polysciences, Inc). Reactions were stopped 3 to 5 minutes later by washing cells with PBS. Cells were photographed with a Nikon diaphotomicroscope (magnification is 80×). – Shows the position of the 110-kD RB protein.

Table 2. Tumorigenicity of RB or Lux virus-infected WERI - Rb27 cells. Cells infected with Rb or Lux were grown in G418-containing medium for 3 weeks (experiment 1) or 5 weeks (experiment 2). Cell viability was verified by trypan-blue exclusion and 2×10^7 viable virus-infected cells were injected subcutaneously into either flank of the same nude mouse. The same number of uninfected parental cells were injected into other mice. Tumor formation was scored after 1 month as presence or absence of a palpable tumor mass.

Experiment	Number with tumor/ number injected		
	Parental	Lux	Rb
1	5/5	5/5	0/5
2		2/2	0/2

after Rb infection (Fig. 4A). These results suggested that restoration of RB protein expression in RB^- cells influenced cell morphology and significantly inhibited growth.

We speculated that the Rb-infected tumor cells that divided rapidly despite G418 selection were nonsuppressed due to a defective viral RB gene. To test this proposal, fast-growing colonies of Saos-2 cells were cloned and grown into mass cultures, and expression of the RB protein was examined by immunoprecipitation. All nine clones had completely lost RB protein expression despite continued G418 selection (*22*). Similarly, the disappearance of enlarged cells and increased growth rate of the bulk population at 10 to 12 weeks in Rb-infected WERI-Rb27 cells was correlated with loss of detectable RB protein (*22*). Inactivation of the proviral RB gene in these tumor cells was not unexpected since native or recombinant retroviruses are prone to frequent mutations or epigenetic suppression of gene expression (*24*).

To test the influence of the RB protein on anchorage-independent growth, we assayed osteosarcoma cells infected with either Rb or Lux virus for their ability to grow in soft agar (*25*). Colony formation by Rb-infected Saos-2 cells was markedly reduced compared to that of uninfected or Lux-infected cells (Table 1). In contrast, colony formation by U-2OS cells did not vary significantly with regard to type of infection.

The most stringent experimental test of neoplastic behavior is the ability of injected cells to form tumors in nude mice. Conversely, loss of tumorigenicity would be a critical test of cancer suppression by the RB gene. Parental (uninfected) WERI-Rb27 and Saos-2 cells formed tumors in nude mice 3 weeks after injection of 2×10^7 cells; however, this quantity of Rb-infected Saos-2 cells could not be accumulated in culture due to the severe growth inhibition described above. The effect of RB expression on the tumorigenicity of WERI-Rb27 cells was studied by injecting Lux-infected and Rb-infected cells into opposite flanks of nude mice (Table 2). Palpable tumors formed only in the flanks injected with Lux-infected cells. Histopathological examination of a tumor confirmed its neoplastic character, whereas gross and microscopic sections of the opposite flank did not reveal any residual tumor cells. Thus expression of exogenous RB protein demonstrably suppressed tumor formation.

The two RB^- cell lines used in this study differed in their response to replacement of the RB protein; in particular, growth in culture was more severely inhibited in Saos-2 cells. Similar growth inhibition effects have also been observed in RB^- breast cancer cell lines (*26*). The origin of this difference is as yet unknown. Since WERI-Rb27 cells expressed much more exogenous RB protein than Saos-2 cells, the quantity of RB protein could not directly explain the difference in growth inhibition.

In a previous study of Wilms' tumor cells (*3*) it was noted that reintroduction of chromosome 11 ablated tumorigenicity in nude mice but did not alter the cell morphology, growth rate, or colony-forming ability. These data suggested that growth rate in culture and tumorigenicity in nude mice are separable phenomena. In our experiments, the growth of retinoblastoma cells was retarded after replacement of the RB gene product. This could be attributed to possible functional differences between the RB gene and the "Wilms' tumor" gene, or to the different modes of expression control. However, slower growth of the Rb-infected retinoblastoma cells cannot entirely explain their loss of tumorigenicity in nude mice. The in vitro growth rate (doubling time) differed about threefold between Lux- and RB-infected cells, and the latter cells formed tumors after 3 weeks. Therefore, visible tumors would be expected from RB-infected cells at 2 months. However, even microscopic tumors were not formed by these cells. These results suggested that replacement of the RB protein specifically affected tumorigenic properties of RB^- tumor cells.

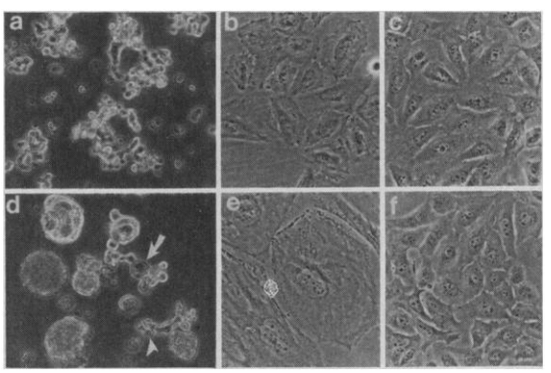

Fig. 3. Morphological effects of Rb or Lux virus infection in retinoblastoma and osteosarcoma cell lines. WERI-Rb27 (**a** and **d**), Saos-2 (**b** and **e**), and U-2OS (**c** and **f**) cells were infected with Lux (a to c) or Rb (d to f) and cultured in G418-containing media (concentrations as in Fig. 2A) for 8 weeks (WERI-Rb27) or 4 weeks (Saos-2 and U-2OS). Cells were photographed in phase contrast with a Nikon diaphotomicroscope (magnification is 105× in all panels). Arrow: enlarged WERI-Rb27 cell; arrowhead: normal-sized WERI-Rb27 cell.

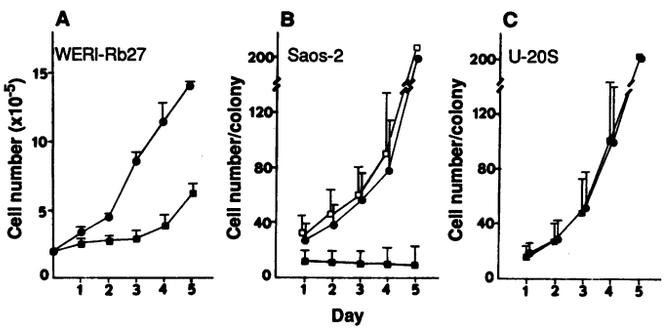

Fig. 4. Growth effects of Rb and Lux infection on retinoblastoma and osteosarcoma cells. Suspension cultures of WERI-Rb27 cells (**A**) were infected with Rb (squares) or Lux (circles) for 2 days and grown in the presence of G418 for 8 weeks. 2×10^4 cells were then seeded in 100 μl of culture medium in individual wells of 96-well microtiter plates (day 0). Three wells were harvested on each of day 1 to day 5 and counted in a hemacytometer. Average cell number per milliliter (+1 SD) is shown. Monolayer cultures of Saos-2 (**B**) and U-2OS (**C**) cells were infected with Rb (squares) or Lux (circles) for 2 days, then plated in 60 mm dishes and grown in G418-containing medium for 7 days. Similar numbers of neomycin-resistant colonies were present in each dish; about 50 randomly selected colonies were marked and the number of cells in each colony was determined under the microscope (day 1). Numbers of cells in the same colonies were measured during the next 4 days. Average cell number per colony (±1 SD) is shown. In dishes with Rb-infected Saos-2 cells, two subpopulations of colonies were observed, one slow-growing ($n = 41$, filled squares) and one fast-growing ($n = 6$, empty squares); these were plotted separately.

On the other hand, osteosarcoma cells expressing apparently normal RB protein, such as U-2OS and TE85 (*22, 23*), were not significantly affected by infection with Rb virus. This implies that the RB gene is functional in these cells and that alternative pathways in osteosarcoma genesis do not involve RB gene inactivation. Our preliminary studies also support this concept in human breast cancers (*26*). Other proposed cancer suppressor genes, including those for Wilms' tumor on chromosome 11 (*27*), renal cell carcinoma and small cell lung carcinoma on chromosome 3 (*28, 29*), and colon cancer on chromosome 5 (*30*), may function analogously to the RB gene in suppressing different kinds of cancer.

It is not known whether inactivation of one or more cancer suppressor genes in a cell is sufficient to cause cancer. Regardless of this uncertainty, replacement of suppressor genes in tumor cells, as demonstrated here, could be a novel strategy for the treatment of clinical malignancy. Unlike conventional, cytotoxic cancer therapies, gene therapy would be based on permanent correction of an underlying defect in tumor cells (*31*). Therapy may not need to be targeted because cancer suppressor genes should not harm normal cells. The ultimate utility of this approach will depend on progress in obtaining other cancer suppressor genes, in understanding their involvement in human tumors, and in improving the technology of exogenous gene expression.

Note added in proof: We have established 12 retinoblastoma cell clones that stably express RB protein for more than 4 months. Thus, expression of the foreign protein is not lethal to the cells.

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Distinct Regions of Sp1 Modulate DNA Binding and Transcriptional Activation

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Sp1 is a sequence-specific DNA binding protein that activates RNA polymerase II transcription from promoters that contain properly positioned GC boxes. A series of deletion mutants of Sp1 were expressed in *Escherichia coli* and used to identify separate regions of the protein that are important for three different biochemical activities. The sequence-specificity of DNA binding was conferred by Zn(II) fingers, whereas a different region of Sp1 appeared to regulate the affinity of DNA binding. The *E. coli*–synthesized Sp1 was able to stimulate initiation of RNA synthesis in vitro, and at least two distinct segments of the protein contributed to its transcriptional activity.

TRANSCRIPTIONAL REGULATION OF genes in eukaryotes is mediated in part by sequence-specific factors that bind to promoter and enhancer elements (*1*). Many of these proteins have been characterized and, in some instances, the factors have been shown to participate directly in regulation of mRNA synthesis by in vitro transcription analysis. Promoters and enhancers typically contain multiple binding sites for several sequence-specific transcription factors, and it is likely that these factors act in conjunction with each other to specify a unique program of transcription for each of the thousands of genes in a eukaryote. Consequently, it is important to understand both how these factors modulate RNA polymerase II transcription as well as how these DNA binding proteins interact with each other.

Sp1 is a sequence-specific transcription factor that recognizes GGGGCGGGGC and closely related sequences, which are often referred to as GC boxes (*2*). Sp1 was initially identified as a factor from HeLa cells that selectively activates in vitro transcription from the SV40 early promoter (*3*) and binds to the multiple GC boxes in the 21-bp repeated elements in SV40 (*4*). The protein was then purified by sequence-specific DNA affinity chromatography (*5, 6*). Sp1 consists of two species of 95 and 105 kD (as determined by SDS–polyacrylamide gel electrophoresis), which appear to be variants of a single polypeptide (*7, 8*). More recently, by isolation of a partial cDNA encoding Sp1 and localization of the DNA binding domain, it was shown that Sp1 binds to DNA by interaction of contiguous Zn(II) finger motifs (*7*).

We had previously described isolation of a partial Sp1 cDNA, designated Sp1-1, that encodes the COOH-terminal 696 amino acid residues of Sp1 (*7*). To obtain the remainder of the Sp1 coding sequence, we prepared Sp1-enriched cDNA libraries by

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