6. C. T. O'Konski and B. H. Zimm, *Science* **111**, 113 (1950).
7. C. T. O'Konski and A. J. Haltner, *J. Am. Chem. Soc.* **79**, 5634 (1957).
8. F. S. Allen and K. E. Van Holde, *Biopolymers* **10**, 865 (1976).
9. B. R. Jennings and H. G. Jerrard, *J. Chem. Phys.* **44**, 1291 (1966).
10. S. Fraden, A. J. Hurd, R. B. Meyer, M. Cahoon, D. L. D. Caspar, *J. Phys.* **46**, C385 (1985).
11. R. Oldenbourg and W. C. Phillips, *Rev. Sci. Instrum.* **57**, 2362 (1986).
12. M. H. J. Koch and J. Bordas, *Nucl. Instrum. Methods* **208**, 461 (1983).
13. The geometry of the cell is identical to that used for conventional solution scattering experiments without field. Its thickness (1 mm) is imposed by the optimal thickness of aqueous solutions at a wavelength of 0.15 nm, and its width (7 mm) by the maximum cross section of the x-ray beam at the sample position. The platinum electrodes forming the top and bottom walls of the cell are separated by 4 mm. The resistance of the cell when filled with distilled water is about 1 megohm. The windows consist of 15-μm-thick mica.
14. C. Boulin *et al.*, *Nucl. Instrum. Methods* **201**, 209 (1982).
15. C. Boulin *et al.*, *ibid.* **A249**, 399 (1986).
16. A. Gabriel and F. Dauvergne, *ibid.* **201**, 223 (1982).
17. J. Hendrix, A. Gabriel, C. Boulin, unpublished.
18. A. Gabriel, C. Boulin, M. H. J. Koch, *Nucl. Instrum. Methods*, in press.
19. A helium-neon laser is used as source of polarized light and the mica windows of the cell are replaced by quartz.
20. C. T. O'Konski and A. J. Haltner, *J. Am. Chem. Soc.* **78**, 3604 (1956).
21. M. A. Lauffer, *J. Phys. Chem.* **42**, 935 (1938).
22. J. Torbet, *Trends Biochem. Sci.* **12**, 327 (1987).
23. We thank J. Witz (IBMC-Strasbourg) for his gift of the TMV sample, E. Mandelkow for the oriented fiber, H. J. Brosch (MKI, DESY) for lending us the Cober generator, and D. J. Thompson (SERC Daresbury Laboratory) for lending us a DL100 memory for the area detector. R.M. was supported by a fellowship from the Federation of European Biochemical Societies (FEBS).

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Absence of TGF-β Receptors and Growth Inhibitory Responses in Retinoblastoma Cells

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The responses of retinoblastoma tumor cells and normal retinal cells to various growth inhibitory factors were examined. Whereas fetal retinal cells were highly sensitive to the antimitogenic effects of transforming growth factor β1 (TGF-β1), retinoblastoma tumor cell lines were all resistant to this factor. Binding assays and affinity labeling of these cells with radioiodinated TGF-β1 revealed that the cells did not have TGF-β receptors. The retinoblastoma cells lacked the three affinity-labeled proteins of 65, 95, and 300 kilodaltons typically seen in human cell lines and thus differed from normal retinal cells and from other types of neuroectodermal tumors that display the normal pattern of receptors. Loss of TGF-β receptors, which is a rare event among tumor cells, may represent one mechanism through which these cells escape from negative control and form retinoblastomas.

DURING THE PAST DECADE, substantial advances have been made in understanding the genes and proteins that act to promote normal cell growth. Aberrant forms of these genes, the oncogenes, participate in the formation of various types of tumors. Less well developed is the study of the molecular elements that act to constrain cell growth. Negative growth control depends on two types of components: signals that pass from cell to cell, often carried by diffusible molecules, and a receptor signal-transducing mechanism that enables a cell to recognize and respond appropriately to extracellular signals.

Work over the past years has provided examples of both types of components. Thus, interferons, tumor necrosis factors, and the transforming growth factors β1 and β2 (TGF-β1 and TGF-β2) are examples of secreted factors that may act physiologically to restrain normal cell proliferation after they interact with specific cell surface receptors (*1, 2*). The antimitogenic effects of these factors have been associated in some target cells with a selective reduction in expression of the c-*myc* nuclear proto-oncogene, which may be one mechanism through which the cell responds to encounters with these factors (*3*). Moreover, a genetic deregulation that led to resistance to one of these polypeptides, an autocrine interferon, disrupted the cessation of cell growth that normally occurs during terminal differentiation; this result indicated that loss of response to negative factors might in turn have an oncogenic effect on cells (*4*).

In parallel with this work, studies of hereditary and somatically induced tumors have suggested the existence of a group of genes and gene products that are normally involved in the negative regulation of cell growth, perhaps by conferring cellular responsiveness to growth inhibitory signals (*5*). The best studied example is the model of retinoblastoma, in which the homozygous loss of the RB gene function on human chromosome 13q14 appears to trigger tumor formation (*6–8*). We investigated the possibility that retinoblastoma cells have also lost the ability to respond to one or more of the well-studied growth inhibitors responsible for cessation of growth. We now report that retinoblastoma cells fail to respond to TGF-β1.

We initially examined the sensitivity of human fetal retinal cells to the antimitogenic effects of human interferons α, β, and γ, human tumor necrosis factor–α (TNF-α), and TGF-β1. Two primary retinal cell cultures were obtained by dissection of fetal eyes (14 and 21 weeks of gestation). The cultures were exposed to these factors during the exponential phase of their growth. Highly purified preparations of the various growth inhibitors (*9*) were added at increasing concentrations to subconfluent monolayers of the retinal cells and tested for their effects on DNA synthesis and on cell number.

Neither the various interferons nor TNF-α, when applied in a wide range of concentrations (0.1 to 10 ng/ml), substantially reduced these two growth-related parameters (*10*). In contrast, human TGF-β1 reduced the growth rate of retinal cells. The dose-response curve (Fig. 1) indicates that TGF-β1 is potent in inhibiting the incorporation of [^{3}H]thymidine into DNA; the half-maximal effect is detected between 0.2 and 0.3 ng/ml (10 p*M*). When treated continuously with TGF-β1 (0.2 n*M*), these cultures remained arrested at subconfluent densities and showed no loss in cell viability even after 4 to 6 weeks of treatment. The retinal cells resumed proliferation upon removal of TGF-β1, an indication that this polypeptide exerts a reversible cytostatic effect on these cells.

In contrast to the strong sensitivity of retinal cells to TGF-β1, four retinoblastoma cell lines established from independent tumors (Y79, RB24, RB27, and Weri) were completely resistant to growth inhibition by this factor. No effect on DNA synthesis could be detected even at the highest concentrations of TGF-β1 (Fig. 1). In addition, prolonged incubation of retinoblastoma cells with TGF-β1 (0.2 n*M*) had no detectable effects on either cell number or cell morphology.

The resistance of retinoblastoma cells to

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the inhibitory effects of TGF-β1 on cell growth could result from deficiencies at the receptor or post-receptor level. Thus, either the absence of TGF-β receptors or the breakdown of intracellular transducing mechanisms could explain the lack of responsiveness to TGF-β1. We therefore examined the ability of these cells to bind and become affinity-labeled with ^{125}I-labeled human TGF-β1. As controls in these measurements, we used normal fetal retinal cells and other cell types of interest.

We had previously identified, in many different cell types, three cell surface glycoproteins that behave as high-affinity receptors for human TGF-β1 (*2, 11, 12*). Affinity labeling of the intact fetal retinal cells with ^{125}I-labeled TGF-β1 followed by cross-linking revealed the same pattern of TGF-β1–binding proteins as described previously in other cell types (Fig. 2). Three components of approximately 65, 95, and 300 kD were specifically labeled by picomolar concentrations of the ligand in the retinal cell cultures. This pattern of labeled receptors was also observed with human embryonic retinal cells immortalized by the adenovirus type 12 (*7*) (Table 1). These three receptor types have been operationally defined as TGF-β receptor types I, II, and III, respectively, on the basis of their distinct structural and functional properties (*2, 13*).

Cells from various retinoblastoma lines displayed a completely different pattern of affinity labeling. No detectable receptors could be observed in the Y79 tumor cell line, even after long exposures of the autoradiograms; the Weri retinoblastoma cells displayed two bands of labeling. The lower band was due to nonspecific labeling, as it was not removed in the presence of excess unlabeled, competing TGF-β1, whereas the upper showed an anomalous migration rate, faster than that of type III receptor and of a type not seen in any previous labeling experiments (Fig. 2). These affinity labeling experiments were then extended to six additional retinoblastoma cell lines, all of which failed to display any detectable binding proteins (Table 1). Altogether, seven of eight retinoblastoma tumors tested showed no binding, and one displayed an unusual form of one of the TGF-β1–binding proteins (Table 1). A skin fibroblast cell line derived from a retinoblastoma patient (GM5877) also had a normal receptor profile when compared with a normal skin fibroblast line (GM316) (Fig. 2), an indication that the receptor deficiency in these patients is specific for tumor cells.

We contrast these data with our results on TGF-β1–binding proteins in more than 70 distinct cell lines and tissues of normal and neoplastic origin. These stem from seven different mammalian species and include a large number of cell types of mesenchymal, epithelial, hematopoietic, and neuroectodermal origin. In these cases, we have observed the presence of at least one or two types and most frequently the coexistence of all three types of cell surface TGF-β receptors. Other workers have also shown by binding experiments that high-affinity receptors for TGF-β were present on all the different normal and tumor cell types tested, whether derived from adult or embryonic tissues [see (*14*) for review]. The single exception to this has been PC12 rat pheochromocytoma cells in which we have been unable to detect the presence of TGF-β receptors (*15*). We also tested other cell lines established from neuroectodermal tumors and found that medulloblastoma, schwannoma, and astrocytoma cells had a normal profile of labeled TGF-β receptors (Fig. 2 and Table 1). Three neuroblastomas tested, IMR32, NGP-1, and SK-N-SH, exhibited normal type I TGF-β receptors. In addition, each showed receptors classified as type III, but these all had anomalously rapid electrophoretic migration rates of the sort seen only once before when studying the Weri retinoblastoma cells. The SK-N-SH cells also showed type II receptors (Table 1). In summary, we find that seven of eight retinoblastoma cell lines stand in sharp contrast to virtually all the cell types screened by us in that they have no detectable TGF-β receptors. An eighth retinoblastoma line lacks detectable expression of two of the TGF-β receptor types and has anomalous type III receptors.

The observed failure of most retinoblastoma cell lines to bind labeled TGF-β1 might be due to the continuous occupancy of their receptors by endogenously produced ligand. To test this possibility, we

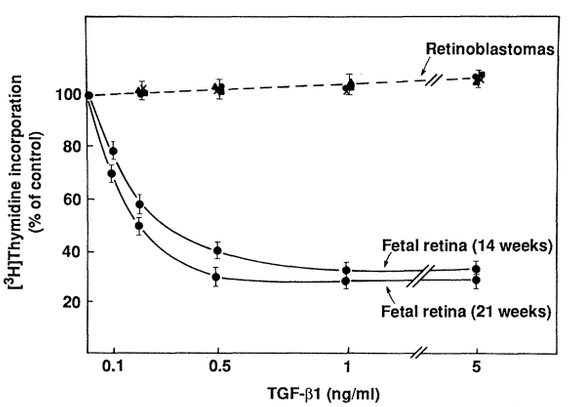

Fig. 1. Retinoblastoma tumor cell lines are resistant to the growth inhibitory effects of TGF-β1. The cells were analyzed for their ability to incorporate [^{3}H]thymidine into DNA in the presence of the indicated concentrations of human TGF-β1. The four cell lines established from independent retinoblastoma tumors (Y79, RB24, RB27, and Weri) (*7, 23*) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For growing the two primary fetal retina cultures (14 and 21 weeks of gestation), this medium was supplemented with vitamins and essential and nonessential amino acids (Gibco). TGF-β1 was added to cells growing at densities of 10^5 cells per milliliter and 10^4 cells per square centimeter for retinoblastoma cells and primary retina monolayers, respectively. After 72 hours, cells were pulse-labeled for 1 hour with [^{3}H]thymidine (5 μCi/ml) (Amersham). The ^{3}H-incorporated into macromolecular material was determined. The results are expressed as the percentage of incorporation of the ^{3}H in control cells incubated without TGF-β1. Results are the average of triplicate determinations ± SEM.

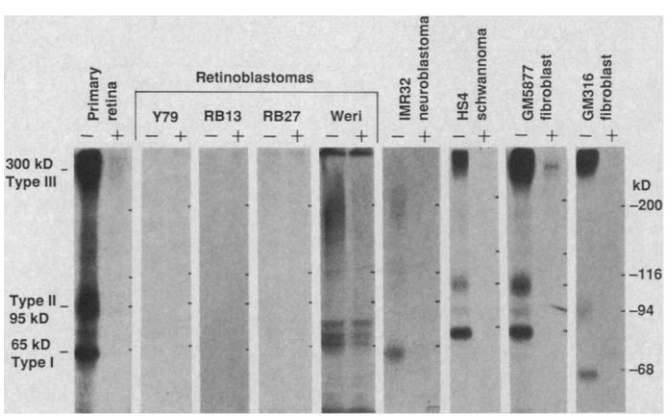

Fig. 2. TGF-β receptors in various normal and tumor cell lines. TGF-β–binding proteins were detected by incubation of the various cell lines with 50 p*M* ^{125}I-labeled TGF-β1 alone (−) or in the presence (+) of 10 n*M* TGF-β1. After binding had reached equilibrium, cells were cross-linked to receptor-bound ligand with disuccinimidyl suberate as previously described (*11, 16*). Affinity-labeled receptor components were displayed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography for 3 days (GM5877, GM316, schwannoma) or 6 to 7 days. The sample in each lane corresponds to between 2×10^5 and 4×10^5 cells, depending on the cell type. All samples were run in 5% to 8% polyacrylamide gradient gels except the GM316 samples, which were run on a 6% polyacrylamide gel. The positions of the molecular size markers are indicated on the right of each set of lanes. The origin of the cell lines is described in Table 1, except for the GM5877 and GM316 fibroblasts, which were supplied by the NIH Human Genetic Mutant Cell Repository.

assayed serum-free medium conditioned for 24 hours by 2×10^8 cells of the RB27 retinoblastoma line for TGF-β1 receptor–competing activity after acidification and 100-fold concentration of the sample. Using a TGF-β radioreceptor assay (*16*) and mink lung epithelial cells as indicators, we found that no detectable competing activity was present within the detection limits of 2 p*M* TGF-β1 or TGF-β2 (*17*). It is therefore unlikely that endogenous production of TGF-β–related molecules by retinoblastoma cells was interfering with the affinity labeling of any TGF-β1–binding protein. Instead, it appears that the TGF-β receptors on most of these retinoblastomas are totally absent or present as low-affinity receptors in amounts so small that they elude detection in our assay. We estimate that this assay can detect as few as 300 high-affinity receptor molecules per cell (*18*).

The receptor for another growth inhibitory factor, the type I interferon receptor, functions normally in these retinoblastoma cells. We have assessed the functioning of interferon receptors by measuring induction of expression of the gene that encodes 2′,5′-oligoadenylate synthetase (*19*). The dose-response induction of this enzyme by human interferon-α in two retinoblastoma cell lines was indistinguishable from that measured in primary fetal retinal cells (*20*). Retinoblastoma cells therefore display a selective receptor deficiency. Moreover, this deficiency can explain the resistance of the cells to growth inhibition by TGF-β1.

TGF-β is the prototype of a family of structurally related polypeptides that are expressed and may control the development of different tissues in organisms ranging from humans to *Drosophila* [for review see (*21*)]. It is therefore possible that some neuroectodermal cells may also be exposed to TGF-β–related molecules during early stages of their development. A source of TGF-β in the fetal retina remains to be defined. The absence of normal TGF-β1–binding activity on all the retinoblastoma cell lines tested presents a marked contrast to the expression of this activity on normal fetal retinal cells. We suggest that the apparent absence of TGF-β receptors from retinoblastoma cells represents a pathological change that occurs during tumorigenesis. This change may represent a general mechanism through which evolving tumor cells escape the control of growth inhibitory factors. Other mechanisms including defects in the production of active factors (*14*) and the breakdown of signaling pathways would also yield the same end result.

A question provoked by the present results is the relations between the two peculiar attributes of retinoblastoma cells—the inactivation of the RB gene and the absence of display of the normal complement of TGF-β receptors. While the loss of the RB gene has a demonstrated causal role in tumorigenesis, the role of the TGF-β receptors in tumorigenesis remains a matter of speculation. The available data allow little insight into the mode of action of the RB gene product and its involvement in growth regulatory circuits. The RB gene product is a nuclear phosphoprotein associated with DNA-binding activity (*22*). As such, the RB gene product may act to regulate the expression of the TGF-β receptors. Another possibility is collaboration of the TGF-β receptors and the RB product in mediating growth inhibition of retinoblasts during normal development. This would require the participation of independent deregulating events in triggering tumor formation. The connections between these changes require extensive experimental investigation.

Table 1. Receptors for TGF-β in neuroectodermal tumor cells. Specific binding of 50 p*M* ^{125}I-labeled TGF-β was determined by subtracting the number of counts per minute bound in the presence of excess (10 n*M*) unlabeled TGF-β1. Affinity-labeled TGF-β receptors were detected as described in legend to Fig. 2. The retinoblastoma lines Y79, Weri, Rb 13-24, and Rb 27 were previously described (*7, 23*). Human neuroblastoma NGP-1 was provided by G. Brodeur. The neuroblastomas IMR32 and SK-N-SH and the medulloblastoma TEG71 were provided by the American Type Culture Collection. Astrocytoma cells were provided by D. Stravrou. The HS43OT schwannoma cells (referred to as HS4) were provided by the Cell Culture Laboratories, University of California in Oakland.

Cells	Specific binding (% of total)	Affinity-labeled receptors		
		Type I	Type II	Type III
Normal fetal retina (21 weeks)	86	+	+	+
Ad 12–transformed retinal cells	92	+	+	+
Retinoblastomas				
Y79	<3	−	−	−
RB13	<3	−	−	−
RB18	<3	−	−	−
RB20	<3	−	−	−
RB22	<3	−	−	−
RB24	<3	−	−	−
RB27	<3	−	−	−
Weri	19	−	−	Anomalous
Neuroblastomas				
IMR32	36	+	−	Anomalous
NGP-1	38	+	−	Anomalous
SK-N-SH	65	+	+	Anomalous
Medulloblastoma				
TE671	82	+	+	+
Schwannoma				
HS4	74	+	+	+
Astrocytoma				
79Hf-4	54	+	−	+

REFERENCES AND NOTES

1. P. Lengyel, *Annu. Rev. Biochem.* **51**, 251 (1982); A. Kimchi, in *Interferon*, I. Gresser, Ed. (Academic Press, London, 1987), vol. 8, pp. 85–110; P. Gray *et al.*, *Nature (London)* **312**, 721 (1984). D. Pennica *et al.*, *ibid.*, p. 724; R. Derynck *et al.*, *ibid.* **316**, 701 (1985).
2. S. Cheifetz *et al.*, *Cell* **48**, 409 (1987).
3. G. J. Jonak and E. Knight, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1742 (1984); M. Einat, D. Resnitzky, A. Kimchi, *Nature (London)* **313**, 597 (1985); *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7608 (1985); A. Yarden and A. Kimchi, *Science* **234**, 1419 (1986); K. Takehara *et al.*, *Cell* **49**, 415 (1987).
4. D. Resnitzky, A. Yarden, D. Zipori, A. Kimchi, *Cell* **46**, 31 (1986); A. Kimchi *et al.*, in preparation.
5. A. G. Knudson, *Cancer Res.* **45**, 1437 (1985).
6. W. F. Benedict *et al.*, *Science* **219**, 973 (1983); R. Godbout *et al.*, *Nature (London)* **304**, 451 (1983); W. K. Cavanee *et al.*, *N. Engl. J. Med.* **314**, 1201 (1986).
7. S. H. Friend *et al.*, *Nature (London)* **323**, 643 (1986).
8. W. H. Lee *et al.*, *Science* **235**, 1394 (1987); Y.-K. T. Fung *et al.*, *ibid.* **236**, 1657 (1987).
9. Natural human interferons α, β, and γ were prepared and purified to homogeneity as previously described [D. Novick, Z. Eshhar, M. Rubinstein, *J. Immunol.* **129**, 2244 (1982); D. Novick *et al.*, *J. Gen. Virol.* **64**, 905 (1983); D. Novick, Z. Eshhar, D. G. Fischer, J. Friedlander, M. Rubinstein, *EMBO J.* **2**, 1527 (1983)]. The specific activities were 2×10^8, 1×10^8, and 4×10^7 U/mg for interferon-α, -β, and -γ, respectively. Purified recombinant human tumor necrosis factor–α was supplied by Cetus at specific activity of 10^7 U/mg. TGF-β1 was purified to homogeneity from human platelets as previously described (*11*).
10. A. Kimchi, unpublished data.
11. J. Massagué and B. Like, *J. Biol. Chem.* **260**, 2636 (1985).
12. J. Massagué, *ibid.*, p. 7059; S. Cheifetz, B. Like, J. Massagué, *ibid.* **261**, 9972 (1986).
13. J. Massagué, S. Cheifetz, R. A. Ignotz, F. Boyd, *J. Cell. Physiol.* (suppl. 5) (1987), p. 43.
14. L. M. Wakefield, D. M. Smith, T. Masui, C. C. Harris, M. B. Sporn, *J. Cell Biol.* **105**, 965 (1987).
15. S. Cheifetz and J. Massagué, unpublished observations.
16. J. Massagué, *Methods Enzymol.* **146**, 174 (1987).
17. _____ and S. Cheifetz, unpublished data.
18. M. Ohta *et al.*, *Nature (London)* **329**, 539 (1987).
19. A. Kimchi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3208 (1979).
20. A. Kimchi, unpublished data.
21. J. Massagué, *Cell* **49**, 437 (1987).
22. W. H. Lee *et al.*, *Nature (London)* **329**, 642 (1987).

23. E. Bogenmann and C. Mark, *J. Natl. Cancer Inst.* **70**, 95 (1983); E. Bogenmann, *Int. J. Cancer* **38**, 883 (1986); R. C. McFall, T. W. Sery, M. Makadon, *Cancer Res.* **37**, 1003 (1977).
24. We thank D. Albert and R. Bernards for preparing the normal fetal retinal cell cultures. We thank E. Bogenmann for retinoblastoma cell lines and M. Rubinstein for human interferon-α, -β, and -γ. This work was supported by grants from the American Business Foundation for Cancer Research, by the Rowland Foundation, and by NIH grant CA39826 (all to R.A.W.) as well as by EMBO fellowship (to A.K.) and NIH grant CA34610 (to J.M.). A.K. is an incumbent of the Louis and Ida Rich Career Development chair, X.-F.W. is a fellow of the Damon Runyon–Walter Winchell Fund, and R.A.W. is an American Cancer Society research professor.

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Genetic and Crystallographic Studies of the 3′,5′-Exonucleolytic Site of DNA Polymerase I

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Site-directed mutagenesis of the large fragment of DNA polymerase I (Klenow fragment) yielded two mutant proteins lacking 3′,5′-exonuclease activity but having normal polymerase activity. Crystallographic analysis of the mutant proteins showed that neither had any alteration in protein structure other than the expected changes at the mutation sites. These results confirmed the presumed location of the exonuclease active site on the small domain of Klenow fragment and its physical separation from the polymerase active site. An anomalous scattering difference Fourier of a complex of the wild-type enzyme with divalent manganese ion and deoxythymidine monophosphate showed that the exonuclease active site has binding sites for two divalent metal ions. The properties of the mutant proteins suggest that one metal ion plays a role in substrate binding while the other is involved in catalysis of the exonuclease reaction.

THE LARGE PROTEOLYTIC FRAGMENT (Klenow fragment) of *Escherichia coli* DNA polymerase I is the only DNA-synthesizing enzyme for which high-resolution structural information is available (*1*). This makes it an ideal experimental system for a detailed molecular analysis of template-directed DNA synthesis. A large body of evidence (*2*) indicates that the two domains seen in the crystal structure correspond to separate polymerase and 3′,5′-exonuclease functions. The smaller (NH_2-terminal) domain was proposed to contain the exonuclease active site, since it binds deoxynucleoside monophosphate (dNMP) (*1*). A prominent feature of the dNMP complex with Klenow fragment is a divalent metal ion that is coordinated by the carboxylate groups of Asp^{355}, Glu^{357}, and Asp^{501}, together with the phosphate of dNMP (Fig. 1, site A). The binding of dNMP is accompanied by additional electron density that lies between the phosphate of dNMP and the carboxylate of Asp^{424}. The identity of the molecule giving rise to this extra density could not be established with certainty at 3.3 Å resolution, but the possibility that it corresponds to a second metal ion was raised. The proximity of Asp^{424} and the associated small molecule or ion to the phosphate of dNMP (and thus, by inference, to the phosophodiester bond to be cleaved in a DNA substrate) suggested a role for these groups in the exonuclease reaction. To investigate this possibility, we used site-directed mutagenesis to change Asp^{424} to Ala (D424A mutation). Further, to examine the role of the metal ion in site A, we eliminated two of its ligands by a double mutation changing both Asp^{355} and Glu^{357} to Ala (D355A,E357A mutation).

We made the mutational changes by using synthetic oligonucleotides to prime synthesis on uracil-containing M13 templates (*3*) containing portions of the *polA* gene. After characterization in M13, the mutations were transferred to an overproduction plasmid (Fig. 2). In each case the subcloned fragment was sequenced in its entirety to check that there were no additional mutations. Our first purification of the D355A,E357A protein indicated that this protein was substantially exonuclease-deficient. However, the sensitivity of our assay was affected by other cellular nucleases, including wild-type Klenow fragment from the chromosomal copy of *polA*. We therefore constructed a host strain for overproduction that was deficient in exonuclease III and carried the D355A,E357A mutant allele of *polA*. The overproduced proteins were purified to apparent homogeneity as described for wild-type Klenow fragment with one modification (*4*).

While the homogeneous mutant proteins have normal levels of polymerase activity, their exonuclease activity is reduced by about five orders of magnitude when compared with a wild-type preparation of equivalent purity (Table 1). The exonuclease activity present in the mutant protein preparations, although extremely low, is definitely above the background for the exonuclease assay. We do not know whether this residual activity is an intrinsic property of each mutant protein or is due to a very low level of contamination by cellular nucleases. Therefore, the values shown in Table 1 represent an upper limit for the 3′,5′-exonuclease activity of the mutant proteins.

To establish whether the loss of exonuclease activity in these two mutant proteins was due directly to the altered residues or was caused by some more general change in the protein structure, the two mutant deriva-

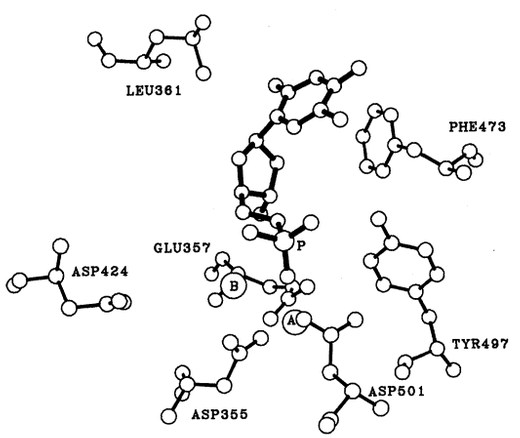

Fig. 1. The 3′,5′-exonuclease active site with the bound product molecule, deoxycytidine monophosphate (dCMP) (shown with thickened bonds). The side chains of residues interacting with the metal ions and dCMP are shown. The two binding sites for divalent metal ions are labeled A and B.

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