Abnormalities in Structure and Expression of the Retinoblastoma Gene in Small Cell Lung Cancer Cell Lines and Xenografts in Nude Mice

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ABSTRACT

The putative retinoblastoma gene (Rb) is a tumor suppressor gene which is believed to cause retinoblastomas when both alleles are inactivated, leading to lack of the encoded M, 110,000-116,000 phosphoprotein. Inactivation of the Rb gene has also been found in several other tumor types, including small cell lung cancer (SCLC). Absence of the 4.7 kilobase mRNA has been found to be frequent in SCLC, and it has been reported that the Rb M, 110,000–116,000 protein product is always absent, even in tumors expressing Rb mRNA. Using Western blotting technique with a monoclonal antibody directed against the Rb protein, we investigated the expression of the M, 110,000-116,000 Rb protein in SCLC tumors grown as xenografts in nude mice and/or as cell lines. Rb messenger RNA expression was determined by Northern blotting, and gross structural gene alterations were investigated by Southern blotting. Tumors established from 23 patients were studied. Seven of the tumors did not express Rb protein, whereas expression was detectable in 13. Three tumors were not investigated for protein expression. Only two tumors expressed Rb mRNA without detectable Rb protein expression. Gross DNA alterations were found in four tumors, of which only one expressed Rb mRNA. Our results demonstrated frequent absence of Rb mRNA and protein in SCLC, but apparently normal Rb mRNA and protein were both expressed in more than one-half of the tumors.

INTRODUCTION

Much attention has recently been paid to the role of tumor suppressor genes in human carcinogenesis. The putative Rb3 gene (1), which has been cloned (2–4), is believed to cause retinoblastomas when inactivated. The tumors are thought to arise from cells in which both alleles are defective, leading to a stop in production of the presumed tumor suppressing Rb protein product.

The Rb gene is complex and contains at least 27 exons spanning more than 200 kb of DNA (5), which may make it vulnerable and a likely target for mutations.

Inactivation of the Rb gene has been demonstrated in a great proportion of retinoblastomas in several studies (2–4), but lower frequencies of inactivation have also been reported (6).

The availability of the cloned gene has made it possible to investigate the structure and mRNA expression of the Rb gene in other tumor types. It has been found that the gene is frequently structurally altered or that Rb mRNA and/or protein is absent or abnormal in tumor types other than retinoblastoma. Among these tumors are SCLC (7–9), breast cancer (10–12), osteosarcoma (2, 13, 14), and bladder cancer (15).

Antibodies directed against the retinoblastoma gene protein allow the investigation of its expression on the protein level (8, 10, 16–18). In one previous study using the immunoprecipitation technique, 6 of 9 SCLC tumors expressed apparently normal mRNA on Northern blots, but none of the tumors expressed detectable Rb protein (8). This has contributed to the assumption that Rb protein may be “missing from nearly all small cell lung carcinomas” (19).

We studied the expression of Rb mRNA and protein as well as gross structural DNA changes in a panel of SCLC established from 23 patients. Tumors were grown as cell lines and/or xenografts in nude mice. The panel included tumors originally established in four different laboratories in Europe and the USA (20–24) and may thus represent a wide spectrum of this common malignancy.

Rb protein expression was examined by the Western blotting technique using a monoclonal antibody specific for the Rb protein. Expression of Rb mRNA was studied on Northern blots, and possible underlying gross alterations in the gene were investigated by Southern blotting.

Seven tumors (35%) did not express Rb protein. All but two tumors with expression of Rb mRNA also expressed Rb protein. Gross structural changes in the Rb gene were demonstrable in four of the tumors, of which only one expressed the Rb mRNA.

Our findings show that, in SCLC, inactivation of the Rb gene leading to absence of Rb mRNA and protein is a frequent but not consistent finding.

MATERIALS AND METHODS

Tumors and Tissues. Tumors established from 23 patients were investigated. Five were grown as cell lines only, 6 as xenografts only, and 12 as both cell lines and xenografts.

Cells lines were grown at 37°C in a humidified atmosphere containing 5% CO2. Media contained 10% fetal calf serum and the antibiotic gentamycin. CPH tumors were established in our laboratory (20) and grown in Eagle's MEM supplemented with MEM amino acids and MEM vitamins. CPH-54A and CPH-54B were subclones of the same original tumor and differ in DNA index and sensitivity to radiation therapy (25). CPH-136A and CPH-136B were established from the same patient before and after chemotherapy, respectively. DMS tumors (21), established at Dartmouth Medical School, Hanover, NH, were grown in Waymouth's medium with the exception of DMS-79, which was grown in RPMI 1640. NIH-H69 and NIH-N592 (22), from the National Cancer Institute and Naval Hospital, Bethesda, MD, and GLC-3 (23), GLC-14, GLC-16, and GLC-19 (24), from University Hospital of Groningen, The Netherlands, were grown in RPMI 1640. GLC-14, GLC-16, and GLC-19 were established from the same patient before and after the first and second reinuduction therapy, respectively.

Cells for investigation were harvested in mid- to late exponential growth phase. Harvested cells were washed in sterile buffer (150 mM sodium chloride-10 mM EDTA, pH 8.0-10 mM Tris, pH 8.0), spun down, immediately frozen in liquid nitrogen, and stored at −80°C.

Xenografts were established in the flanks of nude mice from cell lines by s.c. inoculation of 1014-1015 cells in 0.1 ml medium or directly from patients by inoculation of 2-mm-diameter tumor blocks (26). Serial transplantations were performed by s.c. inoculation of 2-mm-diameter tumor blocks under general anesthesia. The mice were of...
NMRI or BALB/c origin and in specific pathogen-free status; they were kept in laminar air flow clean benches. Sterile food and water were given ad libitum.

Tumor samples for investigation were cut free from visible necrotic tissue, immediately frozen in liquid nitrogen, and stored at −80°C.

Normal mouse tissue was taken from brain, heart, lung, liver, spleen, kidney, and muscle from mice of the same genetic background as above.

Normal human lung tissue was obtained from nonaffected parts of lungs removed at operation for lung cancer.

**Protein Extraction, Electrophoresis, and Blotting.** Frozen tissue clumps or cell pellets were homogenized in 3–5 volumes of lysis buffer [25 mM Tris, pH 7.5–50 mM NaCl-0.5% (v/v) sodium deoxycholate-1% Nonidet P-40-0.01% SDS-1 mM phenylmethanesulfonyl fluoride-500 KIE/ml aprotinin (Trasylol, Bayer)] in a manual glass-teflon homogenizer (27). The homogenates were transferred to microcentrifuge tubes and incubated on ice-water for 45 min, after which they were vortexed and spun at 13,000 x g for 20 min. The supernatant, except the lipid layer sometimes present on the top, was transferred to a fresh tube. The total concentration of protein was determined (28) using a commercial kit (Bio-Rad). Electrophoresis of 40 μg total protein/lane was carried out in denaturing 7.5% SDS-polyacrylamide 6–x-9-cm mini-gels (29). Transfer to nitrocellulose membranes (Schleicher & Schuell BA85) was done by the semi-dry method in a single buffer system (30).

Blots were blocked overnight in blocking buffer (4% bovine serum albumin-50 mM Tris, pH 8.0-150 mM NaCl-0.05% (v/v) Tween 20-10 mM sodium azide). Incubation with primary antibody PMG3-245 (17) (Pharmingen, La Jolla, CA), 5 μg/ml in blocking buffer, was for 3–4 h at room temperature. Blots were washed three times in TBST before addition of the secondary alkaline phosphatase-coupled rabbit antiserum (30). After washing in TBST as above, antibody binding was demonstrated by addition of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**RNA Extraction, Electrophoresis, and Blotting.** RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (31), and the concentration was determined by spectrophotometry. Electrophoresis under denaturing conditions was performed in 1% agarose gels and the concentration was determined by spectrophotometry. Electrophoresis of 15 μg total RNA/lane, the gels were viewed in UV light to confirm the integrity of the ribosomal bands and the equal loading of all lanes. Transfer to charged nylon membranes (GeneScreen Plus, NEN Du Pont) was done in 10× SSC as recommended by the supplier.

Prehybridization and hybridization were done in the presence of 50% formamide as recommended by the supplier of the membrane except that dextran sulfate was used at a concentration of 5%. Washing stringency was 2× SSC-1% SDS twice for 30 min at 60°C for the Rb probed and at 65°C for esterase D and β-actin.

The same Northern blots were probed sequentially with Rb, β-actin, and esterase D.

**DNA Extraction, Electrophoresis, and Blotting.** DNA was extracted with phenol and chloroform by standard methods (27). Digestion with restriction endonucleases BamHI or HindIII was performed as recommended by the supplier (Bethesda Research Laboratories). Ten μg/lane were electrophoresed in 0.8% agarose gels and transferred to charged nylon membranes (GeneScreen Plus). Prehybridization, hybridization, and washing were as recommended by the supplier; washing stringency was 2× SSC-1% SDS twice for 30 min at 60°C. Southern blots were reprobed with a human β-actin probe.

**Probes.** The Rb probes used were a 3.8 kb and a 0.9 kb EcoRI fragment (called Rb 3.8 R and Rb 0.9 R) of the cloned 4.7 kb cDNA Rb gene and were kindly provided by Dr. T. P. Dryja (2). The esterase D probe was a 1.3 kb cDNA kindly provided by Dr. Y.-K. T. Fung (4).

Radioiodinated probes were prepared by the random priming method (33) using a commercial kit and [α-32P]dCTP (both from Amersham).

### RESULTS

In at least one of the two model systems, Western blotting analysis was performed on 20 of the tumors, Northern blotting on 23, and Southern blotting on 18. The results are summarized in Table 1.

Expression of Rb protein was not demonstrable in 7 tumors (Fig. 1), whereas 13 tumors did express it in variable amounts. Three were not analyzed for protein expression.

In tumors with Rb protein expression, additional more or less confluent bands in the range from $M_r$ 110,000 to 116,000, most likely representing varying levels of phosphorylation (16), were sometimes observed (Fig. 1).

The 4.7 kb Rb mRNA was not detectable in 5 of 23 tumors (Fig. 2). The level of expression varied widely from abundant amounts to barely detectable levels. The expressed Rb mRNAs were all of apparently normal size (4.7 kb).

Reprobing of Northern blots with a human β-actin probe demonstrated even loading of all lanes and confirmed the integrity of the RNA (Fig. 2).

All tumors and normal tissues expressed a 1.3 kb esterase D

### Table 1

**Summary of gross DNA changes and of expression of Rb mRNA and protein in SCLC cell lines and xenografts.**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Cell line or xenograft</th>
<th>mRNA changes</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPH-49</td>
<td>Line</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>CPH-54A*</td>
<td>Line</td>
<td>+++++</td>
<td>++</td>
</tr>
<tr>
<td>Xeno</td>
<td>None</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CPH-54B*</td>
<td>Line</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Xeno</td>
<td>None</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CPH-75-5</td>
<td>Line</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>Xeno</td>
<td>None</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>CPH-84</td>
<td>Line</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>CPH-123</td>
<td>Line</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>CPH-128</td>
<td>Xeno</td>
<td>(+)</td>
<td>ND</td>
</tr>
<tr>
<td>CPH-136A*</td>
<td>Xeno 5' (absent bands)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CPH-136B*</td>
<td>Xeno 5' (absent bands)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CPH-137</td>
<td>Line</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Xeno</td>
<td>None</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>CPH-167</td>
<td>Xeno</td>
<td>5' and 3' (major loss)</td>
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</tr>
<tr>
<td>CPH-186</td>
<td>Xeno</td>
<td>+</td>
<td>–</td>
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<tr>
<td>CPH-187</td>
<td>Xeno</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>DM-53</td>
<td>Line</td>
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<td>++</td>
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<tr>
<td>DM-79</td>
<td>Line</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DM-92</td>
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<td>++</td>
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<tr>
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<tr>
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<td>++</td>
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</tr>
<tr>
<td>Xeno</td>
<td>ND</td>
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<td>+++</td>
</tr>
<tr>
<td>DM-153</td>
<td>Line</td>
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<td>++</td>
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<tr>
<td>Xeno</td>
<td>5' (new band)</td>
<td>–</td>
<td>–</td>
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<tr>
<td>DM-273</td>
<td>Line</td>
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<tr>
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<td>+</td>
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<tr>
<td>DM-406</td>
<td>Line</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>DM-456</td>
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<td>(+)</td>
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</tr>
<tr>
<td>Xeno</td>
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<td>(+)</td>
<td>–</td>
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<tr>
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</tr>
<tr>
<td>Xeno</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>GLC-16*</td>
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</tr>
<tr>
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<td>–</td>
<td>–</td>
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<tr>
<td>GLC-19*</td>
<td>Line</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Xeno</td>
<td>None</td>
<td>(+)</td>
<td>–</td>
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<tr>
<td>NIH-H69</td>
<td>Line</td>
<td>None</td>
<td>–</td>
</tr>
<tr>
<td>Xeno</td>
<td>(+)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NIH-N592</td>
<td>Line</td>
<td>None</td>
<td>(+)</td>
</tr>
<tr>
<td>Xeno</td>
<td>None</td>
<td>(+)</td>
<td>–</td>
</tr>
<tr>
<td>Human lung</td>
<td>ND</td>
<td>None</td>
<td>++</td>
</tr>
<tr>
<td>HFF</td>
<td>None</td>
<td>None</td>
<td>++</td>
</tr>
</tbody>
</table>

a CPH-54A and CPH-54B were established from the same patient; this was also the case for CPH-136A and CPH-136B, and for GLC-14, GLC-16, and GLC-19.

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Fig. 1. Expression of Rb protein in a representative panel of SCLC cell lines (A) and xenografts (B). Forty μg/lane of total protein were electrophoresed in 7.5% denaturing SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with a monoclonal antibody against the Rb protein. Location of size markers in kD are indicated on the left.

Rb mRNA (Fig. 2). The Rb gene is located on chromosome 13q14, which also contains the esterase D gene (34). Demonstration of expression of esterase D therefore indicates that this region is generally intact. Three tumors (CPH-186, DMS-456, and xenografts of NIH-N592) had no demonstrable Rb protein on Western blots but did express Rb mRNA. NIH-N592 was also grown as cell line, in which case it expressed both Rb mRNA and protein. CPH-186 was not established as cell line. All other tumors expressing Rb mRNA also expressed Rb protein.

Southern blots of DNA digested with HindIII or BamHI were examined for gross DNA changes by hybridization with the Rb 3.8 R and Rb 0.9 R probes. Absent bands, indicating homozygous deletions, were found in CPH-136A, CPH-136B, and CPH-167, whereas abnormal bands were found in HindIII-digested DNA from CPH-187 probed with Rb 0.9 R and in BamHI-digested DNA from DMS-153 probed with Rb 0.9 R (Fig. 3). Reprobing of blots with a human β-actin probe showed only slight variation in the amount of DNA loaded into the different lanes (data not shown).

Normal human lung tissue and human foreskin fibroblasts were used as positive controls and did express Rb mRNA and protein (Figs. 1 and 2).

Since tumors grown as xenografts in nude mice contain varying proportions of murine stromal cells, we looked for possible expression of mouse proteins cross-reactive with the PMG3-245 antibody. A weak band of about M, 105,000 was detected in some mouse tissues (data not shown). The absence of disturbing murine proteins is also indicated by the total lack of bands in the M, 110,000 to 116,000 range in some of the xenografts, e.g., CPH-136A/B and CPH-167 (Fig. 18).

A nonspecific band of approximately M, 150,000 was seen on all Western blots of human proteins (Fig. 2).

DISCUSSION

In the present study, expression of the Rb protein product was undetectable in 7 of 20 SCLC tumors. Rb mRNA was not demonstrable in 5 of 23 tumors (Table 1). Only two tumors with Rb mRNA expression did not express Rb protein (CPH-186 and DMS-456); a possible explanation is that a small deletion or point mutation in the Rb gene rendered the Rb mRNA unfunctional as a template for translation.

Others (8) have found that 0 of 9 SCLC cell lines expressed Rb protein detectable by the immunoprecipitation technique even though 6 of the tumors expressed apparently normal Rb mRNA. Our findings thus differ from these previously published data, which has led to the assumption that Rb protein is "missing from nearly all small cell lung carcinomas" (19).

The difference in the proportion of tumors expressing detectable Rb protein in the present study and in the study of Yokota et al. (8) could result from selection of subpopulations with particular characteristics during establishment of the SCLC cells in culture. Another possibility is that Rb protein turnover in SCLC could be low, which would make detection of low levels of the protein difficult with the immunoprecipitation technique used in Yokota's study (8). The half-life of the Rb protein is reported to be about 6–10 h (18, 35).

In a study by Harbour et al. (7), Rb mRNA was undetectable in 15 of 26 SCLC cell lines. Thus, inactivation was somewhat more frequent in their study than in ours. Rb protein expression was not analyzed in their investigation. The two NIH cell lines analyzed in our study were also included in Harbour's paper, and our results are similar with regard to mRNA expression and absence of gross DNA changes. We extend their results by demonstrating Rb protein expression in these two cell lines.

The Rb mRNA demonstrated in the present study was all of apparently normal size. This was also the case in one previous study of SCLC (7), whereas in another, 1 of 9 SCLC tumors expressed an abnormal (4.8 kb) mRNA (8). Expression of abnormally sized Rb mRNAs has also been reported in other tumor types (3, 4, 10, 13). In a study of breast cancer (10), only cell lines with abnormal or absent Rb mRNA transcripts lacked expression of Rb protein.

Gross structural DNA abnormalities in the Rb gene were demonstrable in 4 of 18 investigated tumors. In one previous study (7), 4 of 22 SCLC cell lines had DNA rearrangements, whereas in another (8), differences in band intensities but no homozygous deletions or abnormal bands were observed. The extra 4.5 kb band seen in BamHI digests of DMS-153 probed with Rb 0.9 R may represent a previously described RFLP (36). The reason for the 4.5 kb band on HindIII digests of CPH-187 probed with Rb 0.9 R is unclear but may also represent an RFLP. The fact that the extra bands were seen only in digests with one restriction enzyme makes it probable that they represent RFLPs rather than deletions or insertions. Thus, only two tumors (CPH-136A/B and CPH-167) definitely had abnormalities (deletions) in the Rb gene. Of the tumors with DNA changes, only CPH-187 expressed Rb mRNA, making it likely that the extra band in this tumor resulted from alterations in
only one allele or represented an RFLP.

Variations in the procedures used in different laboratories when establishing cell lines from patients could result in the selection of subpopulations with distinct characteristics. The SCLC tumors investigated in this study were originally established in four different laboratories in Europe and in the USA (20–24) and are thus likely to be widely representative of the characteristics of this tumor type. Many of the tumors were propagated in two different model systems, which makes it probable that changes in Rb expression caused by particular conditions in one model system might be revealed.

It has been reported that amplification and expression of c-myc is sometimes higher when tumors are grown as xenografts than as cell lines (37, 38), and it was speculated that cell subpopulations with increased c-myc activity might have a growth advantage in nude mice. In analogy, lowered Rb protein expression might provide a growth advantage and make cell populations with decreased Rb expression dominant. Expression of Rb protein was not systematically higher in one model system than in the other, but generally Rb mRNA was expressed at higher levels in cell lines than in xenografts. Rb protein expression was not detectable in NIH-N592 grown as xenograft even though the corresponding cell line expressed the protein. Rb mRNA was detectable in both cell line and xenograft of NIH-N592.

The pattern of Rb protein bands with a mobility corresponding to a molecular weight of over 110,000 varied among the tumors. The bands in the Mr 110,000–116,000 area are likely to be caused by varying levels of phosphorylation (16). Phosphorylation may reduce the supposed growth-inhibitory function of Rb (39, 40), but the relevance of the presumed variation in the level of phosphorylation in the SCLC tumors is uncertain.
Phosphorylation of Rb varies through the cell cycle and is decreased in growth-arrested cells (35, 39–41). The cell lines were harvested during exponential growth, and xenografts do not stop proliferating, as judged by cell cycle distribution determined by flow cytometric DNA analysis (25), so factors other than growth arrest are likely to have contributed.

The function of the Rb gene in normal cells remains unknown, except that it is a nuclear phosphoprotein with DNA binding ability (16) and that it may play a role in cell cycle regulation (39–41). The Rb protein binds to proteins produced by tumorigenic viruses (17, 42, 43), but as yet no well characterized normal cellular protein has been demonstrated to interact with it. Hence, conclusions about the functionality of the Rb gene products detected in SCLC in the present study must await the advent of an assay of its biological function.

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