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20. It has been suggested that the short (47-nucleotide) leader RNA that is made early in VSV infection plays a role in host cell shutoff (*17*), but evidence that it directly affects nuclear gene expression is lacking.
21. The mechanisms of transport in *X. laevis* oocytes are the same as those of other eukaryotic cells (*1*). Oocytes were injected and RNA and protein transport was analyzed as described [(*22, 23*); E. Lund and J. E. Dahlberg, *EMBO J.* **8**, 278 (1989)]. ^{32}P-labeled RNA export substrates were synthesized in vitro [A. Pasquinelli, J. E. Dahlberg, E. Lund, *RNA* **1**, 957 (1995)] with the use of DNA templates as described (*21–24*). The template for synthesis of M mRNA was made by inserting into pSP64-poly(A) (Promega Inc., Madison, WI) a Hind III fragment of pSV-OM (*19*) that corresponds to the full-length cDNA of VSV Orsay M protein. Preparation and analysis of ^{35}S-labeled nuclear proteins for import assays have been described (*23*). The immunoblot analysis was done by standard techniques with monoclonal antibodies to M protein [L. Lefrancois and D. S. Lyles, *J. Virol.* **121**, 157 (1982)] and monospecific antibodies to transport proteins as noted in the legend to Fig. 5; bound antibodies were detected by ECL (Kirkegaard & Perry Laboratories, Inc., Gaithersberg, MD).
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29. Ratios, rather than absolute amounts, of the proteins in the nuclear and cytoplasmic fractions are significant. Both the quantitative and qualitative changes in the distribution of importin-α in the treated oocytes were highly reproducible (*25*) and minor variations in amounts of Ran and importin-β between control and treated cells were not observed with other batches of oocytes. The persistance of multiple forms of importin-α even after phosphatase treatment (Fig. 5C) indicates that inaccessible phosphates or additional modifications may be involved. By co-immunoprecipitation we were unable to detect any interaction between M protein and Ran, or importins-α or -β (*25*).
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35. Although M protein alone is sufficient to inhibit RNA export, a role for the VSV leader RNA in shut-off of nuclear gene expression of virus infected cells cannot be excluded (see *19*).
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37. Inhibition of protein import by M protein may secondarily inhibit intranuclear functions such as transcription (for example, through depletion of transcription factors), but these effects are unlikely to be as immediate as a direct block of RNA export.
38. We thank members of our laboratory for helpful criticisms, D. Lyles for providing clones of the VSV M protein gene and monoclonal antibodies to M protein; M. Moore for antibodies to importins-α and -β; M. Dasso for antibodies to Ran and RCC1; T. Munns for antibody to m^{7}G-caps; and A. Grandjean and M. Barr for technical assistance. Supported by NIH grant GM30220.

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Transformation of Chicken Cells by the Gene Encoding the Catalytic Subunit of PI 3-Kinase

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The avian sarcoma virus 16 (ASV 16) is a retrovirus that induces hemangiosarcomas in chickens. Analysis of the ASV 16 genome revealed that it encodes an oncogene that is derived from the cellular gene for the catalytic subunit of phosphoinositide 3-kinase (PI 3-kinase). The gene is referred to as v-*p3k*, and like its cellular counterpart c-*p3k*, it is a potent transforming gene in cultured chicken embryo fibroblasts (CEFs). The products of the viral and cellular *p3k* genes have PI 3-kinase activity. CEFs transformed with either gene showed elevated levels of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate and activation of Akt kinase.

Retroviruses found in spontaneous animal tumors can be sources of oncogenes that reveal important aspects of cellular growth control (*1*). The avian sarcoma virus 16 has recently been isolated from a spontaneous chicken tumor. It induces hemangiosarcomas in chickens and transforms CEFs in cell culture (*2*). To characterize the oncogene of ASV 16, the viral genome was cloned from a λZAP cDNA library of ASV 16–transformed CEFs (*3*). The nucleotide sequence of the ASV 16 clone showed a nonviral insertion marking a possible oncogene of cellular origin. The 5′-terminus of the nonviral sequence was fused to viral *gag*, and the 3′-recombination junction was located within the viral *env* gene. A computer-assisted comparison revealed that the putative oncogene was homologous to the gene encoding the catalytic subunit, p110, of bovine PI 3-kinase (*4*). It was therefore named v-*p3k* (Fig. 1).

A clone of ASV 16 minus the 3′ *env* sequence was introduced into the avian retroviral expression vector RCAS (*5*), and the construct (RCAS-v-P3k) was transfected into CEFs, which resulted in the production of infectious retroviral progeny. After passage, the cultures became completely transformed and released a focus-forming RCAS retrovirus (Fig. 2, A and B).

The RCAS-v-P3k–transformed CEFs were tested for the presence of the Gag-v-P3k fusion protein by immunoprecipitation. A monoclonal antibody against avian retroviral Gag p19 (*6*) precipitated a protein of 150 kD from CEFs transfected with RCAS-v-P3k and from ASV 16–infected CEFs but not from CEFs transfected with the vector alone (Fig. 3A). The size of the protein corresponded to the predicted size of the Gag-v-P3k fusion. The 150-kD protein was

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also immunoprecipitated by a polyclonal antibody against a COOH-terminal sequence of the bovine PI 3-kinase p110α subunit (anti-p110) (*7*) (Fig. 3B). We performed tests for tumorigenicity by injecting 1-day-old SPAFAS (Storrs, Connecticut) chicks subcutaneously in the wing web with RCAS-v-P3k virus stock or with 1×10^6 CEFs transfected with RCAS-v-P3k. Both virus and virus-producing cells induced hemangiosarcomas at the site of injection after a latent period of 1 to 2 weeks (Fig. 2, D and E).

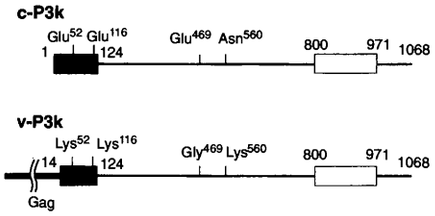

Fig. 1. Schematic structure of the gene products of c-*p3k* and v-*p3k*. The amino acid residues that are different between the c-P3k and v-P3k proteins are marked. The heavy line in v-P3k represents the retroviral *gag* gene product. The black box marks the domain of PI 3-kinase that binds to the regulatory subunit p85, and the open box represents the catalytic domain of PI 3-kinase. This sequence of ASV 16 has been deposited in the GenBank database [accession numbers AF001075 (v-*p3k*) and AF001076 (c-*p3k*)].

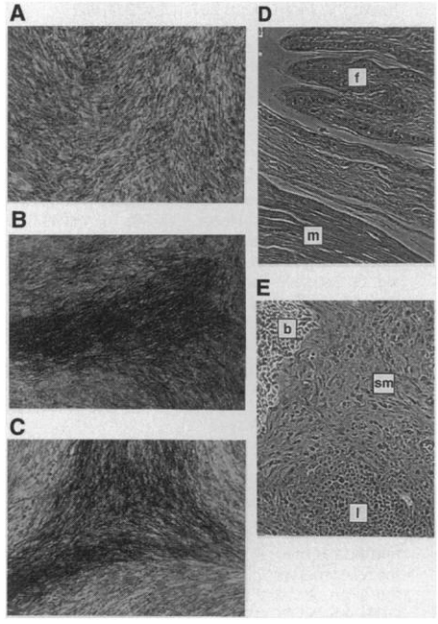

Fig. 2. Transformation in vitro and tumor induction in vivo by *p3k*. (**A**) CEF culture transfected with the RCAS vector. (**B**) Focus of transformed CEFs induced by v-*p3k* (*22*). (**C**) CEF focus induced by c-*p3k* (*22*). (A) through (C) were photographed at ×25 magnification. (**D**) Hematoxylin-eosin–stained histologic section of normal chicken wing web. f, feather follicle; m, muscle. (**E**) Hematoxylin-eosin–stained histologic section of *p3k*-induced hemangiosarcoma; b, blood-filled space; sm, sarcomatous infiltrate and muscle; l, lymphocytic infiltrate. (D) and (E) were photographed at ×250 magnification and phase contrast.

We isolated the cellular *p3k* gene (c-*p3k*) from a chicken embryonic brain cDNA library by using the v-*p3k* coding sequence as a probe. The full-length cDNA clone encoding c-*p3k* was 3452 base pairs, and it contained a 1068–amino acid open reading frame. The in vitro–translated protein had a molecular mass of 110 kD and was recognized by anti-p110 (*7*) (Fig. 3C). The sequence differences between the c-P3k and v-P3k proteins are summarized in Fig. 1. When compared with the cellular protein, the viral version shows a 14–amino acid deletion at the NH_2-terminus and is fused to viral Gag sequences. There are also four amino acid substitutions in the v-P3k sequence.

To determine if c-*p3k* has transforming activity, we cloned the gene in the retroviral expression vector RCAS and transfected it into CEFs. The RCAS-c-P3k construct produced foci of transformed cells that were morphologically indistinguishable from v-*p3k*–induced foci (Fig. 2C). The efficiency of focus formation was ~100 foci per microgram of DNA for RCAS-c-P3k versus ~1000 foci per microgram of DNA for RCAS-v-P3k. RCAS-c-P3k also induced hemangiosarcomas in chickens at the site of injection after 1 to 2 weeks. When compared with v-*p3k* tumors, tumors induced by the cellular gene appeared to be smaller and to grow more slowly, but they were histologically identical to the ones represented in Fig. 2E. The mutations in v-*p3k* responsible for the enhanced tumorigenicity of the viral gene have not yet been identified.

Immunoprecipitation of extracts from c-*p3k*–transformed CEFs with anti-p110 (*7*) revealed a protein of 110 kD that comigrated with the in vitro–translated c-P3k (Fig. 3C). The immunoprecipitates of c-P3k expressed from RCAS had PI 3-kinase activity, as did the anti-p110 immunoprecipitates from RCAS-v-P3k–transformed cells (Fig. 4A). No activity was detected in anti-p110 immunoprecipitates from CEFs producing RCAS, which suggested that endogenous p110α is expressed at low levels in CEFs.

The levels of the PI 3-kinase lipid products phosphatidylinositol 3,4-bisphosphate (PI-3,4-P_2) and phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P_3) were higher in serum-starved CEFs infected with the RCAS-v-P3k or RCAS-c-P3k retroviruses than in RCAS-infected CEFs. The addition of the platelet-derived growth factor (PDGF) to the starved cells increased the levels of PI-3,4-P_2 and PI-3,4,5-P_3 in all cultures but had a greater effect on *p3k*-transformed CEFs (Fig. 4B). These results

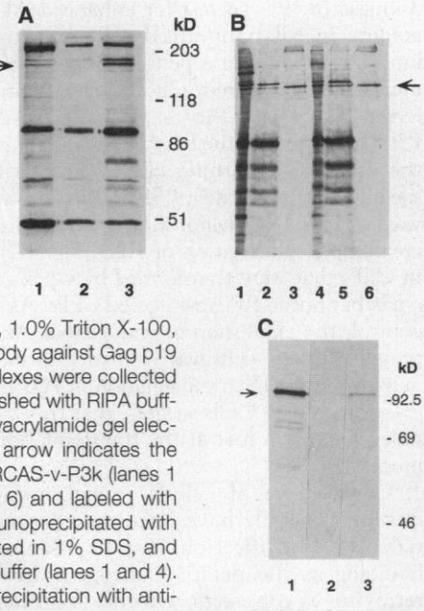

Fig. 3. (**A** and **B**) Expression of v-P3k protein in ASV 16–infected CEFs and CEFs transfected with RCAS-v-P3k. (**C**) Immunoprecipitation of in vitro–translated c-P3k or of *p3k*-transfected CEFs with anti-p110 (*7*). (A) CEFs were infected with ASV 16 (lane 3) or transfected with RCAS-v-P3k (lane 1) or RCAS (lane 2). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (high glucose) supplemented with 2 mM L-glutamine and 10% calf serum. Cells were starved by incubation in DMEM without methionine for 30 min, labeled with ^{35}S-methionine for 2 hours, and then lysed in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 10 mM tris-HCL (pH 7.4), 0.1% SDS, 1.0% Triton X-100, and 1.0% sodium deoxycholate]. A monoclonal antibody against Gag p19 was used for immunoprecipitation (*6*). Immune complexes were collected with protein A–Sepharose CL 4B (Pharmacia) and washed with RIPA buffer, and the proteins were resolved by 7.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. The arrow indicates the Gag-v-P3k protein. (B) CEFs were transfected with RCAS-v-P3k (lanes 1 through 3) or infected with ASV 16 (lanes 4 through 6) and labeled with ^{35}S-methionine as in (A). Cells were lysed and immunoprecipitated with antibody to Gag. Immune complexes were dissociated in 1% SDS, and 20% of the sample was diluted with 2× SDS-PAGE buffer (lanes 1 and 4). The rest was diluted with RIPA buffer for reimmunoprecipitation with anti-Gag (lanes 2 and 5) or anti-p110 (*7*) (lanes 3 and 6). Proteins were resolved by SDS-PAGE electrophoresis. The arrow indicates the Gag-v-P3k protein. (C) The c-P3k cDNA clone was transcribed and translated in vitro with T3 RNA polymerase and rabbit reticulocyte lysate in the presence of ^{35}S-methionine. Immunoprecipitations of in vitro–translated c-P3k (lane 1) or of RCAS- (lane 2) or RCAS-c-P3k– (lane 3) transfected and ^{35}S-methionine–labeled CEFs were performed with anti-p110 (*7*). The precipitates were dissolved in 2× SDS-PAGE buffer and separated by SDS-PAGE electrophoresis. The arrow indicates the c-P3k protein.

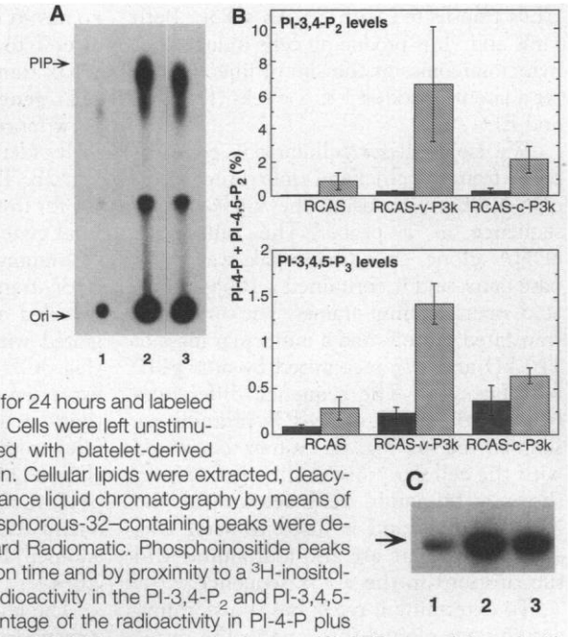

Fig. 4. (**A**) In vitro and (**B**) in vivo PI 3-kinase activity of CEFs infected with RCAS-c-P3k or RCAS-v-P3k retroviruses. (**C**) Activation of Akt kinase in CEFs infected with RCAS-c-P3k or RCAS-v-P3k retroviruses. (A) CEFs were infected with RCAS (lane 1), RCAS-c-P3k (lane 2), or RCAS-v-P3k retroviruses (lane 3) and lysed. Immunoprecipitations were performed with anti-p110 (*7*). After being washed, the pellet was assayed for PI 3-kinase activity (*4*). PIP, phosphoinositol phosphate; Ori, origin. (B) CEFs infected with RCAS, RCAS-v-P3k, or RCAS-c-P3k retroviruses were serum-starved for 24 hours and labeled with ^{32}P-orthophosphate for 3 hours. Cells were left unstimulated (solid bars) or were stimulated with platelet-derived growth factor (hatched bars) for 5 min. Cellular lipids were extracted, deacylated, and separated by high-performance liquid chromatography by means of an anion-exchange column (*23*). Phosphorous-32–containing peaks were detected and quantitated with a Packard Radiomatic. Phosphoinositide peaks were identified on the basis of retention time and by proximity to a ^{3}H-inositol-1,3,4-trisphosphate standard. The radioactivity in the PI-3,4-P_2 and PI-3,4,5-P_3 peaks is expressed as the percentage of the radioactivity in PI-4-P plus PI-4,5-P_2. Values represent the mean ± SEM from three experiments. (C) CEFs were infected with RCAS (lane 1), RCAS-c-P3k (lane 2), or RCAS-v-P3k (lane 3) retroviruses. Cells were lysed, and Akt was immunoprecipitated with a rabbit polyclonal antibody against a peptide composed of the 15 COOH-terminal amino acids of Akt (*8*). In vitro assays of Akt activation were performed as in (*8*) with histone H2B as substrate. The arrow indicates phosphorylated histone H2B.

demonstrate that the P3k and Gag-v-P3k proteins have catalytic activity in intact cells.

Recent studies have suggested that the serine-threonine protein kinase encoded by the *akt* proto-oncogene is a target of PI 3-kinase (*8*, *9*). To test for enhanced Akt activity in *p3k*-transformed cells, in vitro kinase reactions were performed with immunoprecipitates from CEFs that were infected with the RCAS-c-P3k or RCAS-v-P3k retroviruses or the RCAS virus alone in the presence of histone H2B, which is a specific Akt substrate (*9*, *10*). Akt kinase was activated, as demonstrated by the increased phosphorylation of H2B (Fig. 4C), in CEFs that were transformed by v-*p3k* or c-*p3k* but not in RCAS-infected CEFs. As a control, the expression of endogenous Akt protein in these cells was verified with immunoblotting. The up-regulation of Akt in *p3k*-transformed CEFs suggests that this kinase may play a role in the transformation process.

Components of cellular signal transduction generally have oncogenic potential. The identification of a PI 3-kinase homolog as the putative oncogene in a retrovirus is consistent with the fact that PI 3-kinase occupies an important nodal position in cellular signaling. PI 3-kinase is a heterodimeric enzyme consisting of a 110-kD catalytic and an 85-kD regulatory subunit (*11*). The regulatory subunit contains several modular protein binding domains, including two *src* homology 2 (SH2) domains and an SH3 domain, as well as proline-rich and phosphotyrosine target sequences for SH3 and SH2, respectively (*12*). These domains mediate regulatory signals addressed to and issued by PI 3-kinase. PI 3-kinase and its lipid products have multiple functions, including cell survival (protection from apoptosis) (*13*), secretion (*14*), vesicle trafficking (*15*), differentiation (*16*), regulation of cytoskeletal structure (*17*), and cell growth (*18*). A role for PI 3-kinase in oncogenic transformation is suggested by analyses of several viral oncoproteins (*19*). Mutant forms of the polyomavirus middle T protein, Src, and Abl that fail to bind to PI 3-kinase are also impaired in oncogenesis. Our observation that the catalytic subunits v-P3k and c-P3k are oncogenic provides the basis for further studies on the function of PI 3-kinase in the regulation of normal and cancerous cell growth.

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