*Proc. Natl. Acad. Sci. USA*
Vol. 93, pp. 9805–9809, September 1996
Medical Sciences

# The hybrid PAX3–FKHR fusion protein of alveolar rhabdomyosarcoma transforms fibroblasts in culture

(pediatric rhabdomyosarcoma/PAX protein/winged helix protein/oncogenic transformation/chicken embryo fibroblasts)

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*Contributed by Peter K. Vogt, June 3, 1996*

ABSTRACT **Pediatric alveolar rhabdomyosarcoma is characterized by a chromosomal translocation that fuses parts of the *PAX3* and *FKHR* genes. *PAX3* codes for a transcriptional regulator that controls developmental programs, and *FKHR* codes for a forkhead-winged helix protein, also a likely transcription factor. The PAX3–FKHR fusion product retains the DNA binding domains of the PAX3 protein and the putative activator domain of the FKHR protein. The PAX3–FKHR protein has been shown to function as a transcriptional activator. Using the RCAS retroviral vector, we have introduced the *PAX3–FKHR* gene into chicken embryo fibroblasts. Expression of the PAX3–FKHR protein in these cells leads to transformation: the cells become enlarged, grow tightly packed and in multiple layers, and acquire the ability for anchorage-independent growth. This cellular transformation *in vitro* will facilitate studies on the mechanism of PAX3–FKHR-induced oncogenesis.**

The pediatric solid tumor alveolar rhabdomyosarcoma shows a consistent translocation between chromosomes 2 and 13 t(2;13) (q35;q14) (1, 2). This translocation juxtaposes a truncated *PAX3* gene of chromosome 2 to the 3′-terminal region of the *FKHR* gene on chromosome 13 (3–6). The hybrid gene codes for a fusion protein that contains the amino-terminal portion of the PAX3 protein, including the paired box and homeo domains joined to the carboxyl region of the FKHR protein that is truncated within the winged helix DNA binding motif but still contains a putative transactivation domain. The PAX and winged helix proteins are families of developmental regulators that bind DNA in a sequence-specific manner, regulating the transcription of specific sets of genes (7–9). In the PAX3–FKHR fusion product, the intact and presumably functional DNA binding domains of PAX3 have been linked to a region of the FKHR protein that contains, like other winged helix proteins, proline- and alanine-rich regions that may function as transactivation domains (10). Alveolar rhabdomyosarcoma cells contain a chimeric *PAX3–FKHR* RNA transcript of 7.2 kb and express a 97-kDa fusion protein localized in the nucleus (11). The PAX3–FKHR fusion protein is a stronger transactivator than is wild-type PAX3 (11, 12).

The consistency and specificity of the translocation in alveolar rhabdomyosarcoma and the fact that the fusion protein is a transcriptional regulator suggest that the PAX3–FKHR chimeric sequence may act as a dominant oncogene and may be responsible for the initiation and for the maintenance of the oncogenic phenotype in alveolar rhabdomyosarcoma cells. To test the oncogenic potential of the PAX3–FKHR fusion protein, we cloned the chimeric gene in an avian retroviral vector and expressed it in chicken embryo fibroblasts (CEFs). We observed induction of oncogenic transformation in conjunction with expression and nuclear localization of the PAX3–FKHR fusion protein.

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## MATERIALS AND METHODS

**Cell Culture and Viruses.** CEF cultures were prepared from White Leghorn embryos as described (13). Cells were cultivated in cloning medium consisting of Ham's F-10, 10% calf serum, and 4% chicken serum, supplemented with vitamins, folic acid, and 0.4% dimethyl sulfoxide. DNA-transfection and focus assays were carried out as described (14). Agar colony assays were as described (15).

**Recombinant Plasmids.** The mouse–human *PAX3–FKHR* cDNA was inserted as a *Bam*HI–*Xho*I fragment in the pcDNA3 vector (Invitrogen). This fragment contains coding information for the full-length mouse–human PAX3–FKHR fusion protein using the second ATG in PAX3 (11). The *Bam*HI–*Xho*I fragment of *PAX3–FKHR* was cloned into the *Bam*HI–*Sal*I site of the adaptor plasmid Cla12 to create Cla12*PAX3–FKHR*. Partial *Cla*I digestion of the Cla12*PAX3–FKHR* plasmid yielded an approximately 2.4-kb fragment that was cloned into the *Cla*I site of the retroviral expression vector RCASBP(A) (16, 17) to create RCASBP(A)*PAX3–FKHR*.

**Preparation of RNA and Northern Blot Analysis.** Total cellular RNA was isolated from $2 \times 10^7$ cells by the RNAzol method (Tel-Test, Friendswood, TX) as described (14). Total cellular RNA (15 μg per lane) was separated on 1% agarose gels containing 2.2 M formaldehyde and transferred onto Hybond-N membranes (Amersham). Hybridization was carried out in a solution containing 50% formamide, 5× standard saline citrate (SSC), 5× Denhardt's solution (1% bovine serum albumin/1% polyvinylpyrolidone/1% Ficoll 400), heat-denatured herring sperm DNA at 0.1 mg/ml, and 0.1% SDS at 42°C overnight. After hybridization, the filters were washed three times at 55°C in 0.1× SSC/0.1% SDS and exposed to x-ray film for autoradiography. The probe used for hybridization was a *Bam*HI–*Xba*I fragment from pcDNA3 m*Pax3* encompassing the complete coding region of the mouse *Pax3* gene. It was labeled with $^{32}P$ by the random-primer method (Boehringer).

**Immunofluorescence.** Immunofluorescence was carried out as described (14). Polyclonal rabbit serum directed against amino acids 280–479 of mouse Pax3 was used at a 1:500 dilution.

**Immunoprecipitation.** Cells used for immunoprecipitation were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 2 mM L-glutamine and 10% calf serum. Cells were starved for 30 min in methionine-depleted medium and then labeled for 4 hr with $^{35}S$ methionine (1175 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). Labeled cells were lysed in RIPA buffer (150 mM NaCl/10 mM Tris·HCl,

Abbreviation: CEF. chicken embryo fibroblast.

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pH 7.4/0.1% SDS/1.0% Triton X-100/1.0% sodium deoxycholate) containing the following protease inhibitors: 1.0 mM EDTA, leupeptin at 2 μg/ml, aprotinin at 10 μg/ml, and 1.0 mM phenylmethylsulfonyl fluoride.

After labeling, cells were washed twice, scraped off the dish with a rubber policeman into phosphate-buffered saline, and centrifuged. The cell pellet was lysed in 1 ml of RIPA buffer and incubated for 10 min on ice. The lysate was centrifuged for 10 min at 4°C and then incubated with a polyclonal antiserum against amino acids 280–479 of mouse Pax3 (2 μg of IgG per ml) (11) for 1 hr on ice. Immune complexes were collected with protein A-Sepharose CL4B (Pharmacia) by rotation for 30 min at 4°C. They were washed five times with RIPA buffer. The pellet was dissolved in 30 μl of 2× Laemmli SDS/PAGE buffer (120 mM Tris·HCl, pH 6.8/20% glycerol/6% SDS/10% 2-mercaptoethanol/0.01% bromophenol blue), boiled for 5 min, and centrifuged. The supernatant was resolved on a 7.5% polyacrylamide/SDS gel. Gels were fluorographed in EN$^3$HANCE (New England Nuclear), dried, and autoradiographed at −80°C overnight.

## RESULTS

**The PAX3–FKHR Expression Vector.** To test for oncogenic potential of the hybrid PAX3–FKHR fusion protein, we expressed the protein in CEFs. The RCASBP(A)*PAX3–FKHR* plasmid is an avian retroviral expression vector developed from the genome of Rous sarcoma virus (16). It contains the coding region of the PAX3–FKHR fusion protein starting with the second PAX3 ATG. Transfection of the vector into CEFs results in the expression of retroviral proteins and of the PAX3–FKHR protein as well as replication of the retroviral genome. Transfected cells produce infectious avian retrovirus that contains the RCASBP(A) *PAX3–FKHR* construct as its RNA genome. Fig. 1 shows a map of the RCASBP(A)*PAX3–FKHR* vector.

**The PAX3–FKHR Fusion Protein Transforms CEFs.** Two to 4 weeks after transfecting the RCAS vector with the *PAX3–FKHR* insert into CEFs, foci of transformed cells appeared. These transformed cells were spindle-shaped and grew in whorl-like aggregates. From these transfected cultures, single foci of transformed cells were picked with a capillary pipette and cultivated in individual wells of 12-well microdishes. They were then grown into mass cultures on 60- and 100-mm dishes (Fig. 2). These transformed cells were plated in soft nutrient agar. They formed colonies (about 50 per 1000 cells seeded; Fig. 3), while CEFs infected with and expressing only the vector failed to become transformed or produce agar colonies, even at cell densities of more than $10^6$ cells per dish. The PAX3–FKHR-transformed CEFs were expected to produce infectious retrovirus. Supernatant medium harvested from these cultures was, therefore, plated onto fresh CEFs for a focus assay. These supernatants induced foci of transformed cells with a morphology identical to that of the original PAX3–FKHR transfectants.

**PAX3–FKHR-Transformed CEFs Express the Chimeric Gene and the Corresponding Fusion Protein.** Mass cultures of PAX3–FKHR-transformed CEFs were tested for the presence of the *PAX3–FKHR* message by extracting total RNA and performing a Northern blot analysis using a *Pax3* probe (Fig. 4). Bands of 9.6 kb, 5.4 kb, and 3.1 kb hybridizing to the *Pax3* probe could be detected in all PAX3–FKHR-transformed cultures. These bands have the size calculated for the messages generated by the RCAS expression vector with the *PAX3–FKHR* insert.

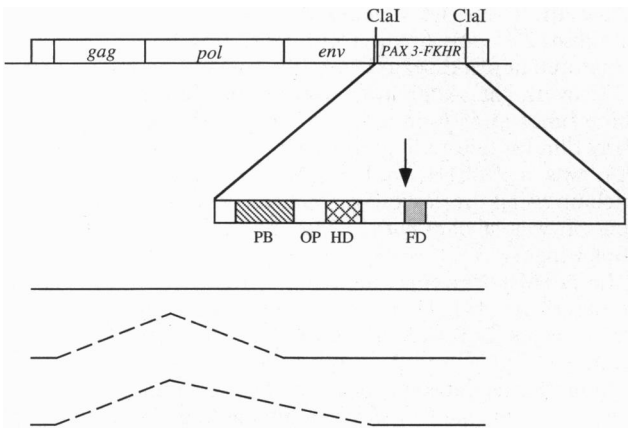


FIG. 1. Map of the avian retroviral expression vector RCASBP(A)-*PAX3–FKHR*. The PAX3–FKHR fusion protein consists of the amino-terminal paired-box and homeodomain of PAX3 and of the carboxyl-terminal truncated winged helix domain and presumed transactivation domain of FKHR. The breakpoint is indicated by an arrow. PB, paired box; OP, octapeptide motif; HD, homeodomain; FD, forkhead domain. The three expected transcripts derived from this construct are depicted below the map.

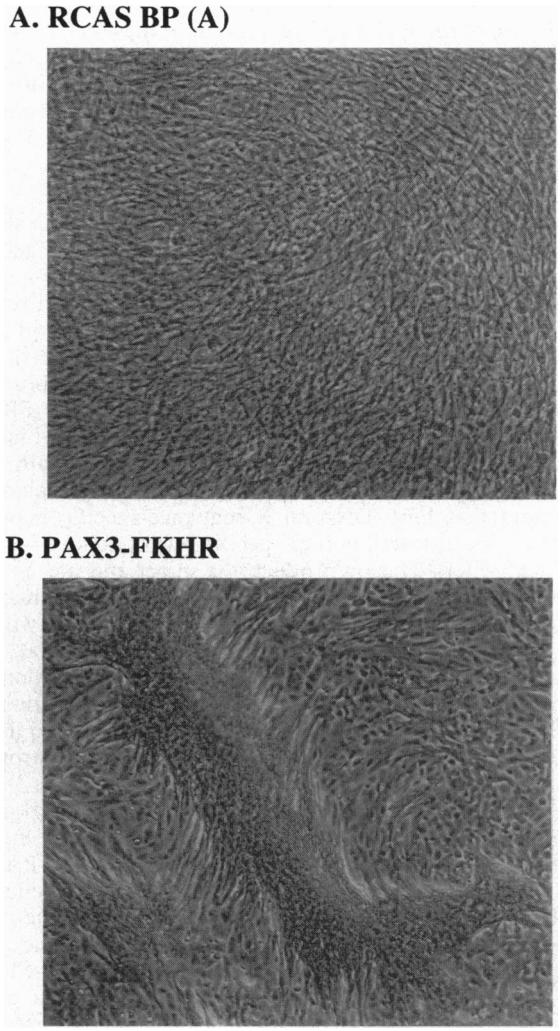


FIG. 2. Focus assay of PAX3–FKHR. Two to 4 weeks after transfecting the RCAS vector with the *PAX3–FKHR* insert into CEFs, foci of transformed cells appeared. These transformed cells were spindle-shaped and grew in whorl-like aggregates. (*A*) RCASBP(A). (*B*) RCASBP(A)*PAX3–FKHR*.

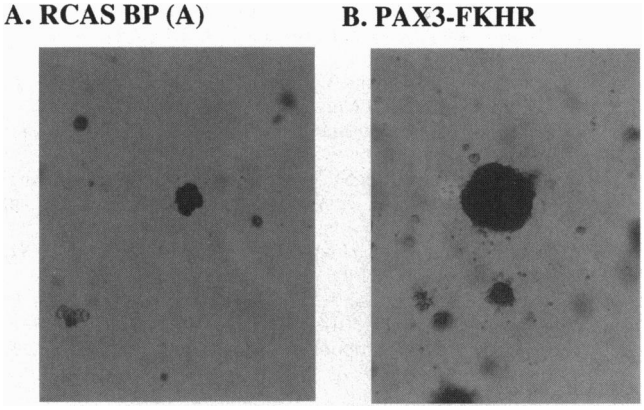


FIG. 3. Soft agar colony assay of PAX3–FKHR cells. Single foci of transformed cells were picked and cultivated. These transformed cells were plated in soft nutrient agar. They formed colonies (about 50 per 1000 cells seeded), while CEFs infected with and expressing only the vector failed to produce agar colonies. (*A*) RCASBP(A). (*B*) RCASBP(A)*PAX3–FKHR*.

The $^{35}$S-labeled PAX3–FKHR fusion protein was immunoprecipitated from transformed single focus-derived cultures with an antiserum against Pax3. Electrophoresis revealed a 97-kDa protein that was present in all PAX3–FKHR-transformed foci (Fig. 5) and absent from normal CEFs infected with vector alone. The expected size of the PAX3–FKHR fusion protein is 94 kDa. Immunofluorescent staining with Pax3 antibody localized the fusion protein in the nuclei of transformed cells (Fig. 6).

## DISCUSSION

Chromosomal translocations are seen frequently in human cancers. A given translocation is usually associated with a

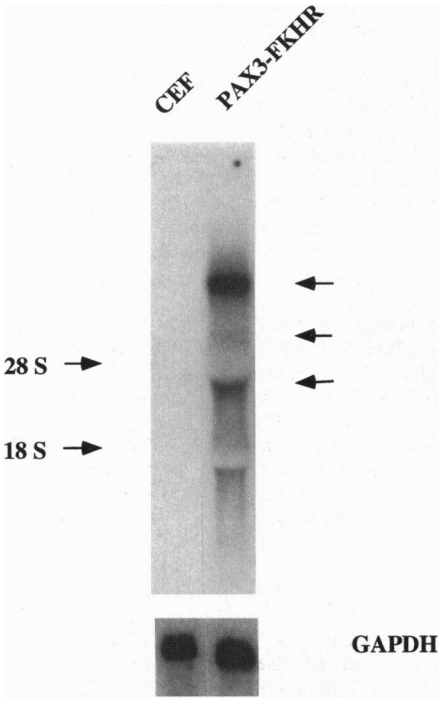


FIG. 4. Northern blot analysis of total RNA isolated from PAX3–FKHR-transformed CEFs. Three RNA species carrying the insert are expected from the RCASBP(A)*PAX3–FKHR* construct. The sizes are 3.1 kb, 5.4 kb, and 9.6 kb. They were detected with a *Pax3* probe and are marked by arrows. No signals are expected in normal CEFs. To show the integrity of the RNA, the blot was also probed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA.

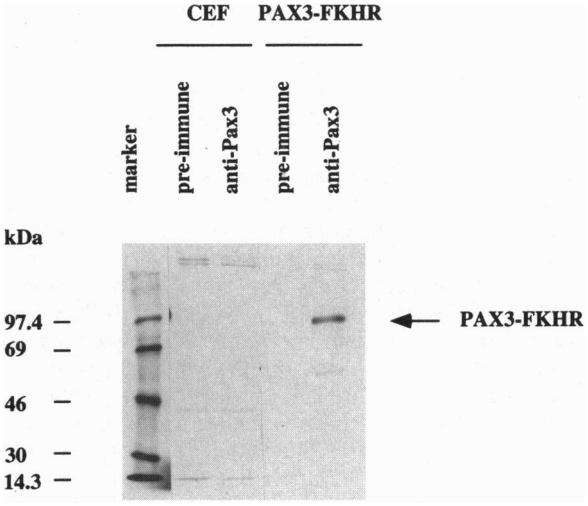


FIG. 5. Immunoprecipitation analysis of normal CEFs or cells derived from single foci of RCASBP(A)*PAX3–FKHR*-transformed cells. The $^{35}$S-labeled PAX3–FKHR fusion protein was immunoprecipitated from transformed single foci-derived cultures with an antiserum against Pax3. Electrophoresis revealed a 97-kDa protein that was present in all PAX3–FKHR-transformed foci.

specific malignancy and is often found in most or all cases of that tumor. Cancer-specific translocations are common in various leukemias (18–20) and more recently have also been identified in pediatric soft tissue sarcomas (1, 2, 21). These translocations fuse parts of two unrelated genes leading to the generation of novel proteins. In typical examples, the fused genes code for transcriptional regulators; the fusion product then combines disparate modular protein domains determining DNA binding and transactivation. The result is an aberrant transcriptional regulator that interferes with normal transcriptional control and plays an important role in oncogenesis. Such chimeric transcription factors have been identified in a broad spectrum of leukemias (22–38) and pediatric sarcomas (21, 39–47). The cancer-specific chromosomal translocations are somatic genetic events; the cancer cell is heterozygous for the translocation and, therefore, codes not only for the chimeric transcription factor but also for the wild-type transcriptional regulators from which the chimeric molecule is derived. Cancer induction by a chimeric transcription factor then requires

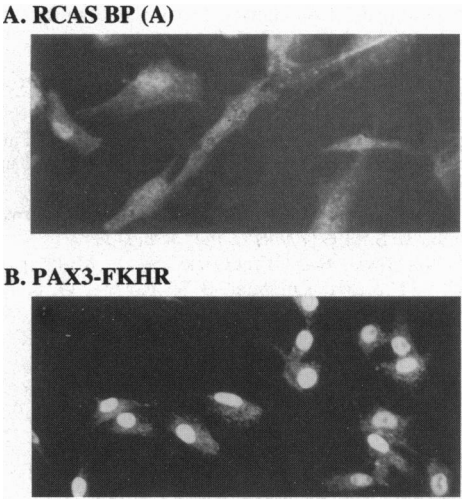


FIG. 6. Subcellular localization of the PAX3–FKHR fusion protein. Immunofluorescent staining with a Pax3 antibody localized the fusion protein in the nuclei of the transformed cells. (*A*) RCASBP(A) vector control. (*B*) RCASBP(A)*PAX3–FKHR*.

that it act in dominant fashion, as the product of an oncogene. Expression of the chimeric transcription factor in a susceptible normal target cell should then induce oncogenic transformation. Such oncogenic potential has been demonstrated, for instance, with the AML1–EVI-1 fusion protein (36), the E2A–Pbx fusion product (26), and the CBFβ–smooth muscle myosin heavy chain chimeric protein (48). The present paper documents oncogenic activity for the PAX3–FKHR chimeric transcription factor. The consequences of expressing PAX3–FKHR in CEFs include morphological transformation and anchorage-independent growth but they do not extend to tumorigenicity in the animal: The RCAS*PAX3–FKHR* virus does not cause tumors when injected in the wingweb of newly hatched chickens. This lack of oncogenicity *in vivo* may indicate that CEFs are an inappropriate target for the mammalian *PAX3–FKHR* oncogene or that tumor induction requires an additional genetic change that does not occur within the limited period of observation. The *in vitro* test for oncogenic transformation by PAX3–FKHR should, however, facilitate experiments on several important questions. It should allow a genetic analysis of the PAX3–FKHR protein, leading to a definition of the molecular domains required for transformation. The availability of PAX3–FKHR-transformed cells that can be compared with their direct normal progenitors should permit the determination of differentially expressed target genes. Over- or underexpression of such targets is presumably responsible for the malignant phenotype of the cell. Finally, the PAX3–FKHR transformation should be useful in testing the functions of proteins that bind to and interact with PAX3–FKHR.

The fact that PAX3–FKHR is a more potent transcriptional activator than is wild-type PAX3 suggests that the transforming activity of the fusion protein may reflect a gain in transactivation function on PAX3 targets. Indeed, wild-type PAX3 expressed from RCAS does not transform CEFs. However, preliminary experiments have demonstrated that a mutant form of PAX3 that may show increased transactivation potential can induce transformation in CEFs (S.S. and P.K.V., unpublished data).

We thank Ms. Susan Burke for help with the preparation of the manuscript and Jason Iacovoni for excellent technical assistance. This work was supported by U.S. Public Health Service Grants CA 42564 to P.K.V., CA 64202 to F.G.B., and a fellowship to S.S. from the Deutsche Forschungsgemeinschaft. F.J.R. is supported by U.S. Public Health Service Grants CA 52009, CA 10815, and DK 49210, the PEW Scholars Program, the I.A. Hansen Memorial Foundation, and the M.A. Rumsey Memorial Foundation.

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