0013-7227/02/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 87(11):5312–5324

doi: 10.1210/jc.2002-020318

PAX3/Forkhead Homolog in Rhabdomyosarcoma Oncoprotein Activates Glucose Transporter 4 Gene Expression *in Vivo* and *in Vitro*

MICHAL ARMONI, MICHAEL J. QUON, GILA MAOR, SMADAR AVIGAD, DAVID N. SHAPIRO, CHAVA HAREL, DIANA ESPOSITO, YAACOV GOSHEN, ISAAC YANIV, AND EDDY KARNIELI

Institute of Endocrinology, Diabetes and Metabolism (M.A., C.H., E.K.), Rambam Medical Center, and Department of Morphology (G.M.), Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel; National Center for Alternative and Complementary Medicine (M.J.Q., D.E.), National Institutes of Health, Bethesda, Maryland 20892; Pediatric Hematology Oncology Department (S.A., Y.G., I.Y.), Schneider Children's Medical Center of Israel, Sackler Faculty of Medicine, Petach Tikva 49100, Israel; and Boehringer Ingelheim Pharmaceuticals Inc. (D.N.S.), Ridgefield, Connecticut 06877

Increased levels of glucose uptake and increased expression of the glucose transporter (GLUT) genes are characteristic features of tumors. In the muscle-derived tumor alveolar rhabdomyosarcoma (ARMS), a chromosomal translocation t(2:13) generates the PAX3/forkhead homolog in rhabdomyosarcoma (FKHR) oncoprotein. In muscle tissues, glucose transport is primarily mediated by GLUT4. However, the mechanisms that regulate GLUT4 gene expression in tumor tissues are largely unknown. Therefore, we evaluated the role of PAX3/FKHR in the regulation of GLUT4 gene expression in muscle tumorigenesis. GLUT4 mRNA and protein were detected in ARMS-derived human biopsies and in ARMS-derived RH30 myoblasts, which both express the PAX3/FKHR chimeric protein, but not in either C2C12 or embryonal rhabdomyosarcoma-derived myoblasts. GLUT4 was functionally active in RH30 cells, because insulin induced a 1.4-fold stimulation of basal 2-deoxyglucose uptake rates. Coexpression of PAX3/FKHR increased basal transcriptional activity from a GLUT4 promoter reporter (GLUT4-P) in C2C12, SaOS-2, and Chinese hamster ovary-K1 cells in a dose-dependent and tissue-specific manner. PAX3/FKHR mutants with deletions in either the homeodomain (ΔHD) or the FKHR-derived activation domain (ΔFKHR), or in which the PAX3-derived paired domain (PD) was point-mutated (PD-R56L), were unable to activate GLUT4-P. Progressive 5′-deletion analysis of GLUT4-P further identified a specific region of the promoter, −66/+163 bp, which retained about 65% of the full transactivation effect. EMSA studies established that the PAX3/FKHR protein directly and specifically binds to this region and to a shorter fragment, −4/+36 bp, that contains potential binding sites for HD and PD, but not to a −4/+36-bp fragment whose HD and PD sites have been mutated. Thus, the functional interaction of PAX3/FKHR with GLUT4-P appears to require all of the functional domains of PAX3/FKHR, as well as a −4/+36-bp region within the GLUT4 promoter. Taken together, the data suggest that the GLUT4 gene is a downstream target of PAX3/FKHR and that GLUT4 is aberrantly transactivated by this oncoprotein both *in vivo* and *in vitro*. (*J Clin Endocrinol Metab* 87: 5312–5324, 2002)

THE GLUCOSE TRANSPORTER (GLUT) family of membrane transport proteins mediates glucose uptake in eukaryotic cells. To date, six GLUT isoforms have been identified and cloned, and their tissue expression patterns have been extensively studied (for review, see Ref. 1). Thus, although GLUT1 and GLUT3 are widely expressed, tissue distributions of the other isoforms are much more restricted. GLUT2 is primarily present in liver, kidney, intestine, and pancreatic β-cells; GLUT5 is expressed in the small intestine; and GLUT7 is detected in gluconeogenic tissues (another isoform, GLUT6, is a pseudogene). The insulin-responsive GLUT4 isoform is expressed almost exclusively in insulin target tissues, *i.e.* in fully differentiated brown and white adipose, and in skeletal and cardiac muscle tissues. In these tissues, GLUT4 mediates the increase in glucose uptake that occurs in response to acute insulin stimulation (2, 3). Because glucose transport is a rate-limiting step in glucose metabolism, GLUT4 expression is tightly regulated at both mRNA and protein levels (4).

Accelerated glucose metabolism has long been recognized as a hallmark feature of transformed cells (5). Furthermore, the activation of GLUT genes represents one of the earliest events in oncogenesis (6, 7). The accelerated glucose uptake that is observed in tumor cells has been generally ascribed to GLUT1 overexpression (6–9). However, the aberrant expression of other GLUT isoforms has also been implicated in enhancing glucose uptake in tumor cells. For example, in gastric tumors, the amounts of GLUT1, GLUT2, and GLUT3 transcripts were higher than those in the respective normal tissues (10). Moreover, under normal physiological conditions, glial and mammary cells do not show GLUT4 and GLUT5 expression. However, the abnormal presence of mRNA and protein for both isoforms was detected in 20–50% of human astrocytic and breast tumors (11–13). Recently, we have detected GLUT4 mRNA in human papillary carci-

Abbreviations: ARMS, Alveolar rhabdomyosarcoma; CHO, Chinese hamster ovary; ERMS, embryonal rhabdomyosarcoma; FKHR, forkhead homolog in rhabdomyosarcoma; FOX, forkhead box; GLUT, glucose transporter; GLUT4-P, GLUT4 gene promoter; HA, hemagglutinin; HD, homeobox domain; MEF2, myocyte enhancer factor 2; PD, paired domain; PI3-K, phosphatidylinositol 3′-kinase; PKB, protein kinase B; PRS, paired response sequence.

noma but not in normal thyroid or goiter cells (Fishman, B., M. J. Quon, A. Fusco, G. Maor, O. Ben-Izhack, M. Armoni, and E. Karnieli, unpublished data). However, the molecular mechanisms that regulate GLUT4 transcription in general, and in muscle tumorigenesis specifically, are still unclear.

Solid tissue tumorigenesis is frequently associated with chromosomal translocations. Such events can result in the expression of novel oncogenic fusion proteins that act as aberrant transcription factors (14). In the skeletal muscle-derived tumor alveolar rhabdomyosarcoma (ARMS), a t(2:13)(q35;q14) chromosomal translocation juxtaposes the DNA binding domains of the PAX3 gene in frame with the DNA activation domain of the forkhead homolog in rhabdomyosarcoma (FKHR) gene (15, 16). Although both the PAX3 and the forkhead gene products are essential for normal cellular growth, the chimeric protein has acquired oncogenic potential, perhaps in part due to inappropriate activation of normal PAX3 target genes (17, 18).

The winged helix/forkhead family of transcription factors is a large group of proteins characterized by the presence of a highly conserved DNA binding domain termed the forkhead box (FOX) domain (19). The *Drosophila melanogaster* gene forkhead was the first member of this gene family to be identified (20). Since then, over 100 members of the winged helix/forkhead gene family have been found in numerous species, ranging from human to yeast. The human FOXO family includes, among others, the FOXO1a, FOXO3a, and FOXO4 genes, previously known as FKHR(alv), FKHRL1, and AFX, respectively (21). The human FOXO1a gene was originally identified in the chromosomal translocation PAX3/FKHR as a gene associated with human ARMS. A large body of evidence now suggests that there is a functional connection between members of the FOX family and insulin regulation of glucose homeostasis. Recent works have identified the Foxo1 protein as a downstream target of protein kinase B (PKB)/Akt, a serine-threonine protein kinase that lies downstream of phosphatidylinositol 3′-kinase (PI3-K) in the insulin-signaling cascade (22, 23). Thus, Foxo1 was suggested to act as a mediator of insulin-regulated gene expression, functioning as a transcription factor that binds to insulin response elements in a variety of downstream target genes, and thereby conferring an inhibitory effect of insulin on gene transcription (23).

The paired box (PAX) genes are members of a large superfamily of transcription factors that control development (24). Nine PAX-encoded factors have been identified in humans and mice, and each includes a conserved 128-amino acid DNA binding domain, termed the paired box (25). The PAX-3 gene encodes a 479-amino acid protein containing two conserved DNA binding motifs: an amino terminus paired domain (PD) of 128 amino acids and a paired-type homeodomain (HD) of 60 amino acids. PAX-3 controls regulatory events associated with myogenesis and is essential for the proliferation, specificity, and migration of limb precursor cells (26). Mutations in the PAX3 gene have been linked to Waardenburg syndrome in man and to the mouse mutant *Splotch*, which develops no limb musculature (24).

To date, several transcriptional targets have been identified for either native PAX3 (24, 27) or the chimera PAX3/FKHR, whereas only the antiapoptotic protein BCL-XL (28) and the proto-oncogene c-Met (29) were shown to be activated by both PAX3 and PAX3/FKHR. In this study, we provide data suggesting that the GLUT4 gene is a downstream target for transactivation by PAX3/FKHR as well as by PAX3. Our data also suggest that the GLUT4 promoter has novel *cis*-elements that may be involved in its activation in ARMS tumor cells. The identification of regulatory elements within the GLUT4 promoter that regulate its aberrant expression may provide important clues for understanding the molecular basis of tumorigenesis.

Materials and Methods

Materials

Luciferase Reporter assay kit, calf intestine alkaline phosphatase, and restriction endonucleases were obtained from Promega Corp. (Madison, WI). T_4 polynucleotide kinase was obtained from Boehringer-Mannehaim (Ottweiler, Germany). ATP-γ-^{32}P (6000 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). Cell labeling grade L-^{35}S-methionine and ^{125}I-labeled protein A were obtained from Amersham (Buckinghamshire, UK). Cell culture reagents were from Biological Industries (Beth Hemeek, Israel). Synthetic oligonucleotides were ordered from Eisenberg Brothers, Inc. (Tel Aviv, Israel). Rabbit polyclonal antibody directed against the C terminal of GLUT4 (clone no. R1288) was donated by Dr. Michael P. Czech (Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA). Mouse antihemagglutinin (anti-HA) monoclonal antibody (clone 12CA5; isotype IgG2b/k; 25 mg/ml) was from BabCo (Berkeley, CA).

Cell cultures

Characterization of the endogenous expression of GLUT4 and *in vitro* transfection studies were performed in the following cell lines: human ARMS-derived RH30 (ATCC no. CRL-2061), human embryonal rhabdomyosarcoma (ERMS)-derived RD (ATCC no. CCL-136), human osteosarcoma-derived SaOS-2, murine skeletal muscle-derived C2C12 myoblasts, and Chinese hamster ovary (CHO)-K1 fibroblasts. Cell cultures were grown in 95% air/5% CO_2 atmosphere, in high-glucose DMEM (for RH30, RD, C2C12, and SaOS-2 cells) or Ham's F-12 (CHO-K1) medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

RT-PCR and Northern blot analysis

Total cellular RNA was prepared from the various cells using TriReagent (Molecular Research Center, Inc., Cincinnati, OH). For RT-PCR, sense and antisense primers specific for β-actin, GLUT1, GLUT4, and PAX3/FKHR mRNA were synthesized based on sequences obtained from GenBank, using the GCG program (Genetic Computer Group, http://www.accelrys.com), and are shown on Table 1. First-strand cDNA synthesis and PCR amplification were performed as described in detail by Zipris (30). Some experiments were performed in the absence of reverse transcriptase, to exclude the possibility of amplification from genomic contamination. PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. For Northern blot analysis, total RNA (20 μg) was subjected to electrophoresis on 1% formaldehyde/agarose gels and transferred to nylon membranes by vacuum pressure. Ethidium bromide staining of the 18S and 28S rRNA bands was used to control for equal loading, and the intensity of the bands was quantified by scanning densitometry under UV illumination. A 1.6-kb *Bgl*II-*Sal*I fragment from the human GLUT4 cDNA was used as a probe for Northern blots. Membranes were hybridized with 0.5–1.0 $\times$ 10^6 cpm of the ^{32}P-labeled GLUT4 cDNA probe in 10 ml buffer containing 1$\times$ SSPE, 2% dextran sulfate, 0.02% Denhardt's solution, and 0.2% SDS at 50 C for 48 h. Blots were washed twice for 30 min at 50 C in 2$\times$ SSPE with 0.2% SDS and once for 15 min at 60 C in 0.1$\times$ SSPE. Then, blots were dried and exposed to a PhosphorImager screen (Molecular Dynamics, Inc., Sunnyvale, CA). mRNA levels were quantified using a PhosphorImager and MacBas1000 software (Molecular Dynamics, Inc.), and the data were normalized to the intensity of 18S rRNA in each lane.

TABLE 1. Sequence of sense and antisense primers used in RT-PCR

	Sense	Antisense
β Actin	GACGAGGCCCAGAGCAAGAGCG	TCAGGCAGCATAGCTCTCCAGGG
GLUT1	GCCATACTCATGACCATCGC	AGCTCCTCGGGTGTCTTATC
GLUT4	CATCCTGATGACTGTGGCTC	TCTCATCTGGCCCTAAATACT
PAX3/FKHR	GCACTGTACACCAAAGCACG	AACTGTGATCCTGGGCTGTC

Sense and antisense primers specific for the detection of the human mRNA for β-actin, GLUT1, GLUT4, and PAX3/FKHR were synthesized based on sequences obtained from GenBank, using the GCG program (Genetic Computer Group, http://www.accelrys.com). Due to highest homology between the human and murine glucose transporters, primers for human species also recognize the mRNA species derived from murine C2C12 myotubes.

Immunodetection of GLUT4 proteins

Western immunoblotting analysis was performed to assess the expression of endogenous GLUT4 proteins in total membrane fractions prepared from the various cell sources, as we have described previously (31). Immunohistochemical localization of GLUT4 proteins was performed in deparaffinized sections prepared from muscle-derived biopsies that had been obtained from five patients previously diagnosed by histology as ARMS and shown by RT-PCR to bear the t(2;13)(q35;q14) chromosomal translocation (32). Endogenous peroxidase activity was blocked by incubating sections in a solution of 3% H_2O_2 (vol/vol) in methanol for 20 min. Sections were than incubated with a rabbit anti-GLUT4 antibody (1:200; AB1346, CHEMICON International Inc., Temecula, CA) for 90 min at room temperature, followed by incubation with a biotinylated goat antirabbit secondary antibody for 10 min at room temperature. The sections were then incubated with a streptavidin-peroxidase conjugate and with S-(2-aminoethyl)-L-cysteine as a substrate for 10 min following the instructions of the kit (Histostain-SP kit, Zymed Laboratories, Inc., South San Francisco, CA). Sections were counterstained with hematoxylin.

Cellular glucose uptake rates in myoblasts

Functional activity of GLUT4 transporters was assessed in RH30, C2C12, and RD myoblast cell lines by measuring the rates of 2-deoxy-D-glucose uptake exactly as described previously (33). Briefly, cells were incubated in Krebs-Ringer phosphate buffer (pH 7.4) in either the absence (basal state) or presence (insulin-stimulated state) of 10 nM insulin for 30 min at 25 C, followed by the addition of 2-deoxy-D-2,6-^{3}H-glucose to a final concentration of 0.1 mM (1 μCi/ml) for an additional 5 min at 25 C. Nonspecific 2-deoxyglucose uptake was measured in the presence of 20 μM cytochalasin B and was subtracted from each value to obtain specific uptake rates.

PAX3/FKHR expression vectors

A full-length cDNA of the human PAX3/FKHR and the related functional mutants (see *Results*) were obtained from Sublett *et al.* (18). All cDNAs were cloned into the BamHI/XhoI site of pcDNA3 (Invitrogen Corp., San Diego, CA), except for the parental PAX3 expression construct, which was cloned as a BamHI fragment. A 3′-HA-PAX3/FKHR expression vector, which includes a 3′-HA-tagged PAX3/FKHR cDNA, was used to verify the expression of PAX3/FKHR in EMSA. When expressed in cells (either by transfection of eukaryotic cells or by *in vitro* translation), this construct gave rise to a protein that was detectable by anti-HA antibodies (mAb HA-11, BabCo). A full-length cDNA of the human FKHR (current terminology, FOXO1a) in pcDNA3 was obtained from Tang *et al.* (34). Each of the mutants was generated by PCR mutagenesis and confirmed by DNA sequencing.

GLUT4-P-LUC promoter reporters

pGEM7-Luc was constructed by Quon *et al.* (35) by subcloning the firefly luciferase gene (taken as a HindIII/SmaI fragment from pRSV-Luc) into the pGEM7zf(+) plasmid (Promega Corp.). This construct has no eukaryotic promoter and was used as a negative control throughout the experiments. The GLUT4 promoter reporter (pGLUT4-P-Luc) was generated by subcloning the upstream 5′ region of rat GLUT4 gene (from −2213 to +163 bp relative to transcription start site) into pGEM7-Luc, upstream of the luciferase gene. GLUT4-P deletion mutants were generated from GLUT4-P-Luc by excising specific fragments with the appropriate restriction endonuclease enzymes, followed by religation.

Transient expression and GLUT4 promoter activity assays

Cells were plated in 100-mm dishes (750,000 cells per dish) and transfected 24 h later with a total of 15 μg affinity-purified plasmid DNA (QIAGEN Inc., Hilden, Germany) using the calcium-phosphate DNA precipitation method (36). Depending on specific experimental conditions, cells were transfected with 10 μg pGLUT4-P-Luc reporter DNA, 0–3 μg pcDNA3-PAX3/FKHR (parental or mutants) expression vectors, and 2.0 μg pCMV-β-galactosidase. In preliminary studies, we found that these conditions provide the optimal signal-to-noise ratio and are still nontoxic to the cells. To determine nonspecific activity, one set of dishes was cotransfected with 10 μg pGEM7-Luc reporter (which we named p0-LUC, because it is devoid of any eukaryotic promoter), and 0–4 μg pcDNA3-PAX3/FKHR. In each set of the experiments, this promoterless p0-LUC reporter exhibited only background levels of activity that were unaffected by PAX3/FKHR itself (data not shown), thus ruling out the possibility of global, nonspecific activity of the expression vectors tested. In each experiment, the total amount of DNA transfected was kept constant by adding the insertless pCDNA3 expression vector, to account for squelching by the CMV promoter itself. Five hours later, DNA-containing medium was replaced with complete medium, and the cells were incubated for an additional 48 h at 37 C. Luciferase activity was assessed using a Luciferase Reporter assay kit (Promega Corp.) and a Lumat LB9501 luminometer (Berthold Systems Inc., Nashua, NH) and was normalized for β-galactosidase activity (36). Within each experiment, values are expressed as percentage of the induced basal GLUT4-P activity, *i.e.* the activity obtained in cells transfected with the pGLUT4-P-Luc reporter alone. Each experiment was repeated three to four times, with each sample analyzed in triplicate.

In vitro translation and EMSAs

The Promega TnT T7 transcription/translation system with rabbit reticulocyte lysate was used to generate *in vitro* translated PAX3/FKHR protein from PAX3/FKHR cDNA. The resulting PAX3/FKHR protein lysate was used in EMSA. Protein production was confirmed by SDS-PAGE, followed by PhosphorImager analysis of proteins translated in the presence of ^{35}S-methionine. *In vitro* translation reactions generated sufficient protein to use in EMSA studies. For EMSA studies, a GLUT4 promoter-derived −66/+163-bp DNA probe was prepared by *Nco*I-*Hin*dIII digestion of pGLUT4-P-Luc. GLUT4 promoter-derived −4/+36-bp sequence (wild-type, and HD-PD mutated oligos) and paired response sequence (PRS)1 oligonucleotides were commercially synthesized (Table 2). Double-stranded DNA probes were end-labeled with γ-^{32}P-ATP in the presence of polynucleotide kinase. EMSA was then performed using Gel Shift Assay System kit (Pharmacia Biotech, Uppsala, Sweden) with minor modifications. Protein-DNA binding reactions were assembled in a total volume of 20 μl, which included 2–6 μl *in vitro* translated PAX3/FKHR protein lysate, radiolabeled probe (~50,000 cpm), and 4 μg poly(dI-dC) in a buffer containing 10 mM HEPES (pH 7.9), 1 mM dithiothreitol, 1 mM EDTA, 4% Ficoll, and 50 mM KCl. Competition experiments were performed in the presence of 10- to 100-fold molar excess of unlabeled probe DNA, which was added 10 min before addition of the radiolabeled probe. An OCT1-derived oligonucleotide

TABLE 2. Sequence of oligonucleotides used in EMSA

	Sense strand
PRS1	G**ATTA**GCACC**GTTCC**GCCGCTCTAGATATCTCG
−4/+36 (wt)	*GGATCC***ATTA**GATCCCGGAGAGCCTTGGTGCTCTCCG**GTTCC**TT*AAGCTT*
−4/+36 (HD-PD)	*GGATCC***TAGT**GATCCCGGAGAGCCTTGGTGCTCTCCG**GTTCA**TT*AAGCTT*

Synthetic sense and antisense oligonucleotide sequences were HPLC-purified and annealed, and the double-stranded DNA was end-labeled with γ-^{32}P-ATP prior to use in EMSA. The sequence of PRS-1 was obtained from Sublett *et al.* (18). The −4/+36 wt oligonucleotide is a promoter-derived oligonucleotide encompassing the 3′-proximal region of GLUT4-P spanning from −4 to +36 bp relative to the transcription start site. Positions of ATTA and GTTCC consensus motifs are underlined. The −4/+36 HD-PD oligonucleotide is similar to the −4/+36 wt, except that both PD and HD consensus motifs have been mutated according to Sublett *et al.* (18). Nucleotides that were added to −4/+36 oligo for future subcloning purposes, but are not derived from the GLUT4 promoter, are shown in *italics.*

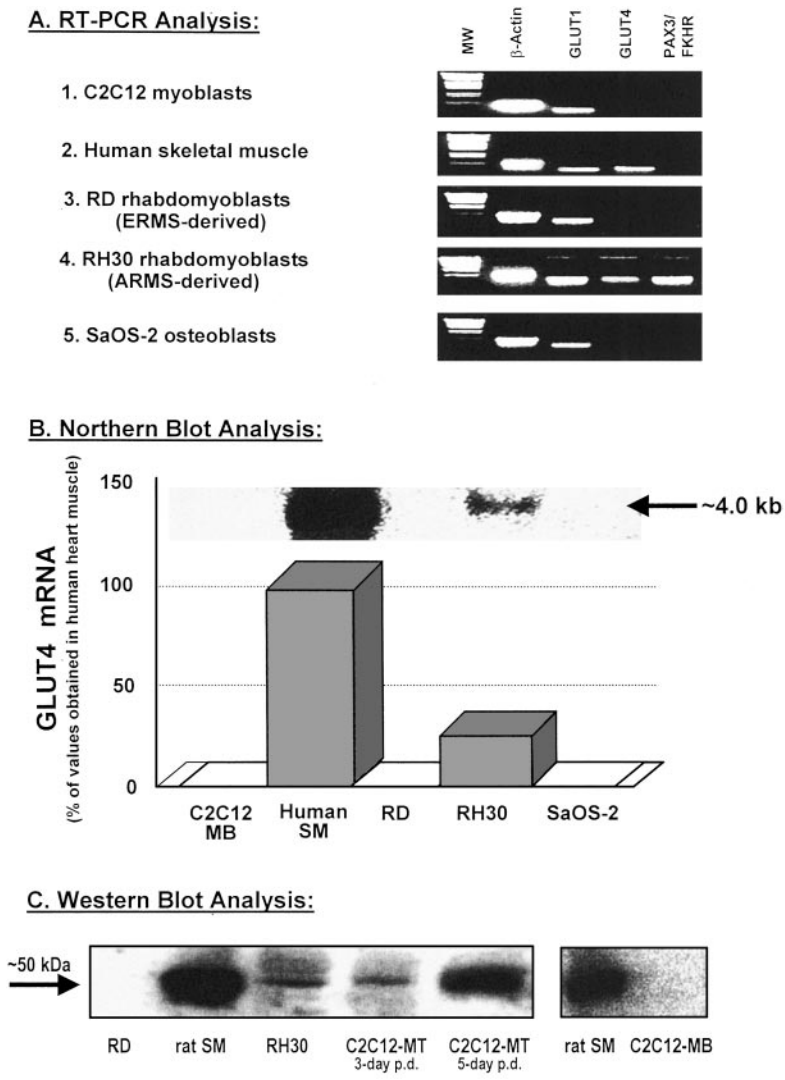

FIG. 1. Endogenous expression of PAX3/FKHR and GLUT4. Total RNA was isolated from the various cell lines (numbered 1–5 in panel A) and subjected to either RT-PCR (A) or Northern blot (B) analyses, as described in *Materials and Methods*. A, RT-PCR analysis. Specific primers corresponding to β-actin, GLUT1, GLUT4, or PAX3/FKHR were used to amplify these mRNA species, as indicated. Ten-microliter aliquots of PCR products were electrophoresed on 2% agarose gels and then stained with ethidium bromide. All PCR products were of the expected size, based on molecular weight (MW) markers shown in the *left lane* of each panel. B, Northern blot analysis. Total RNA samples (20 μg), prepared from the various cell lines as shown in panel A, were subjected to Northern blot analysis for GLUT4 using a ^{32}P-labeled GLUT4 cDNA probe. *Top*, One representative blot of three separate experiments using different membrane preparations. The size of GLUT4 mRNA bands (indicated by *arrow*) was estimated as approximately 4.0 kb, based on gel mobility relative to that of the 28S and the 18S rRNA bands (6.3 kb and 2.3 kb, respectively; data not shown). In each experiment, data were quantified, and the relative intensities of GLUT4 mRNA levels were expressed as percentage of the values obtained in human skeletal muscle. The mean ± SEM of the three separate experiments are presented in the histogram. C, Western blot analysis. GLUT4 immunoreactivity was assessed in total membrane fractions prepared from the various cell sources as indicated on the figure. Samples containing 50 μg of protein were subjected to Western immunoblot analysis exactly as described previously (31). Immunoreactive proteins were visualized by phosphor imaging, and the relevant bands are shown. The size of GLUT4 protein bands (indicated by *arrow*) was estimated as approximately 50 kDa on the basis of molecular weight markers (data not shown). Results from two (of three) representative experiments are presented. p.d., Post differentiation.

(supplied with the kit) that contains neither the ATTA nor GTPyA/CPy motifs (*i.e.* known recognition sites for HD and PD, respectively) was included as a nonspecific inhibitor. For supershift assays, 2 μl anti-HA antiserum (25 mg/ml) were preincubated with the 3′-HA-PAX3/FKHR protein lysate for 10 min before the addition of the labeled probe. After samples were incubated for 30 min at 25 C, DNA-protein complexes were resolved by electrophoresis on 6% nondenaturing polyacrylamide gels at 300 V at 4 C in 0.25× TBE buffer [25 mM Tris (pH 8.3), 25 mM borate, and 0.5 mM EDTA]. Gels were fixed in 10% acetic acid for 15 min, dried, and analyzed by PhosphorImager.

Results

Expression of functional GLUT4 in ARMS-derived RH30 myoblasts

The endogenous expression of PAX3/FKHR and GLUT4 genes was assessed in transformed ARMS-derived RH30 and ERMS-derived RD myoblasts, SaOS-2 osteoblasts, as well as nontransformed CHO-K1 fibroblasts and C2C12 myoblasts. The ubiquitously expressed β-actin and

GLUT1 genes were also studied. With RT-PCR analysis, the mRNA for GLUT1, but not GLUT4 isoform, was detected in predifferentiated C2C12 myoblasts (Fig. 1A). As previously shown (37, 38), we detected GLUT4 mRNA in fully differentiated C2C12 myotubes (data not shown), as well as in human skeletal muscle (Fig. 1A, panel 2). The RH30 cell line was originally developed by Shapiro and colleagues (18) from a human skeletal muscle ARMS tumor, and it was histologically characterized as undifferentiated rhabdomyoblasts that express the unique t(2;13)(q35;q14) chromosomal translocation. In this cell line, we found that GLUT4 mRNA was expressed in addition to PAX3/FKHR mRNA (Fig. 1A, panel 4). The presence of GLUT4 mRNA was unexpected, because these cells are in the undifferentiated proliferating stage (18). To exclude the possibility of a nonspecific effect of the transformed state *per se*, GLUT4 expression was studied in other transformed cell lines that resulted from different transforming events. We examined human osteosarcoma-derived SaOS-2 cells in which both p53 alleles are deleted (39) and RD cells, derived from a human ERMS tumor, that exhibit a mutant p53 allele Arg248Trp (40). Neither GLUT4 nor PAX3/FKHR mRNA was detected by RT-PCR analysis in these lines (Fig. 1A, panels 3 and 5), suggesting that the expression of GLUT4 is unique to the ARMS-derived tumor cells.

To characterize the extent of GLUT4 mRNA expression in the various samples, Northern blot analysis was performed (Fig. 1B). We used a 1.6-kb *Bgl*II-*Sal*I fragment from the human GLUT4 cDNA as a probe, because this fragment specifically recognizes the GLUT4 isoform and not the ubiquitously expressed GLUT1 isoform. These experiments confirmed that GLUT4 mRNA was expressed in RH30 myoblasts, at approximately 20–25% of the level found in normal human skeletal muscle. Furthermore, this mRNA was of the same size as that normally found in fully differentiated muscle cells.

Western blot analysis showed that GLUT4 mRNA, which is aberrantly expressed in ARMS-derived RH30 myoblasts (but not in normal nontumorigenic myoblasts like C2C12), is further translated to GLUT4 protein (Fig. 1C). Furthermore, and in accordance with our data regarding mRNA species, the translated GLUT4 proteins were of the same size as that observed in fully differentiated muscle cells, *i.e.* approximately 50 kDa.

To determine whether the aberrantly expressed GLUT4 protein functions as an active transporter, basal and maximally insulin-stimulated rates of cellular glucose uptake were assessed in RH30, C2C12, and RD myoblasts. As shown in Fig. 2, basal rates of ^{3}H-2-deoxyglucose uptake in RH30 cells were 50% lower than in C2C12 myoblasts, whereas their responsiveness to insulin was enhanced by 40% ($P < 0.01$). These findings suggest that there is an association between the unique presence of PAX3/FKHR in ARMS-derived RH30 rhabdomyoblasts and the induction of functional GLUT4 transporters in these cells. These data provide evidence for the role of PAX3/FKHR in GLUT4 gene expression under physiological conditions and emphasize the significance of GLUT4 in ARMS tumorigenesis.

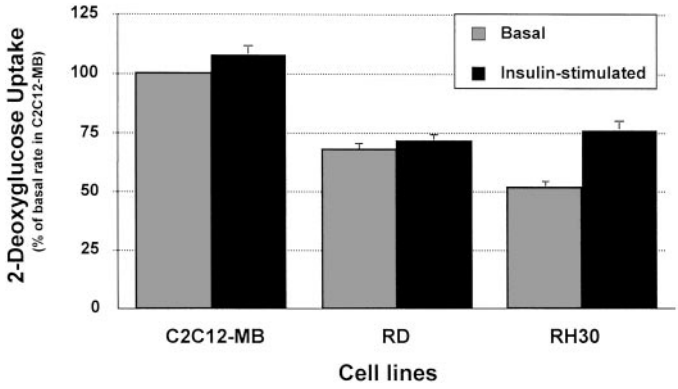

FIG. 2. Cellular glucose uptake rates in myoblasts. Murine C2C12, ERMS-derived RD, and ARMS-derived RH30 myoblasts were all cultured in 12-well plates. Twenty-four hours after plating, the cells were serum-starved for 2 h, then incubated in either the absence (basal, *gray bars*) or presence (insulin-stimulated, *black bars*) of 10 nM insulin for 30 min. Rates of ^{3}H-2-deoxyglucose uptake were measured as detailed in *Materials and Methods*. Within each experiment, data were normalized per cell number, and the results were expressed as a percentage of the basal uptake value obtained in C2C12 myoblasts. Data are expressed as mean ± SEM of three separate experiments.

Human ARMS tumors containing the PAX3/FKHR chimera express GLUT4 proteins

To further establish the role of PAX3/FKHR in the aberrant expression of GLUT4 *in vivo*, localization of GLUT4 proteins was assessed by immunohistochemical staining of deparaffinized sections prepared from muscle-derived biopsies that had been obtained from five patients previously diagnosed by histology with ARMS (32). All biopsies included in this study had been previously screened by RT-PCR at the Pediatric Hematology Oncology Department (Schneider Children's Medical Center of Israel, Sackler Faculty of Medicine, Petach Tikva, Israel) and found positive for the presence of the unique t(2;13)(q35;q14) chromosomal translocation, which gives rise to the PAX3/FKHR chimera (for original RT-PCR data, see Ref. 32). Biopsies were fixed in neutral buffered formalin and processed for paraffin embedding, and deparaffinized sections were immunostained to determine the distribution of GLUT4. As shown in Fig. 3, undifferentiated cells in the tumor tissues were positively stained for GLUT 4 (Fig. 3, A and B, *red staining*). Normal muscle tissue taken from an area adjacent to that shown in Fig. 3B, served as a positive control and exhibited intense staining for GLUT4, as shown in Fig. 3C. These results further suggest that there is an association between the presence of the PAX3/FKHR oncoprotein and the aberrant expression of GLUT4 *in vivo* in undifferentiated human ARMS tumors.

PAX3/FKHR transactivates transcription from the GLUT4 promoter in vitro

To further investigate the role of PAX3/FKHR in aberrant GLUT4 induction, GLUT4 gene expression was studied *in vitro* at the promoter level, using transient cotransfection assays. Transformed SaOS-2 osteoblasts, nontransformed CHO-K1 fibroblasts, and C2C12 myoblasts (all devoid of endogenous GLUT4 expression), as well as ARMS-derived RH30 cells, were cotransfected with the GLUT4-P-Luc promoter reporter and

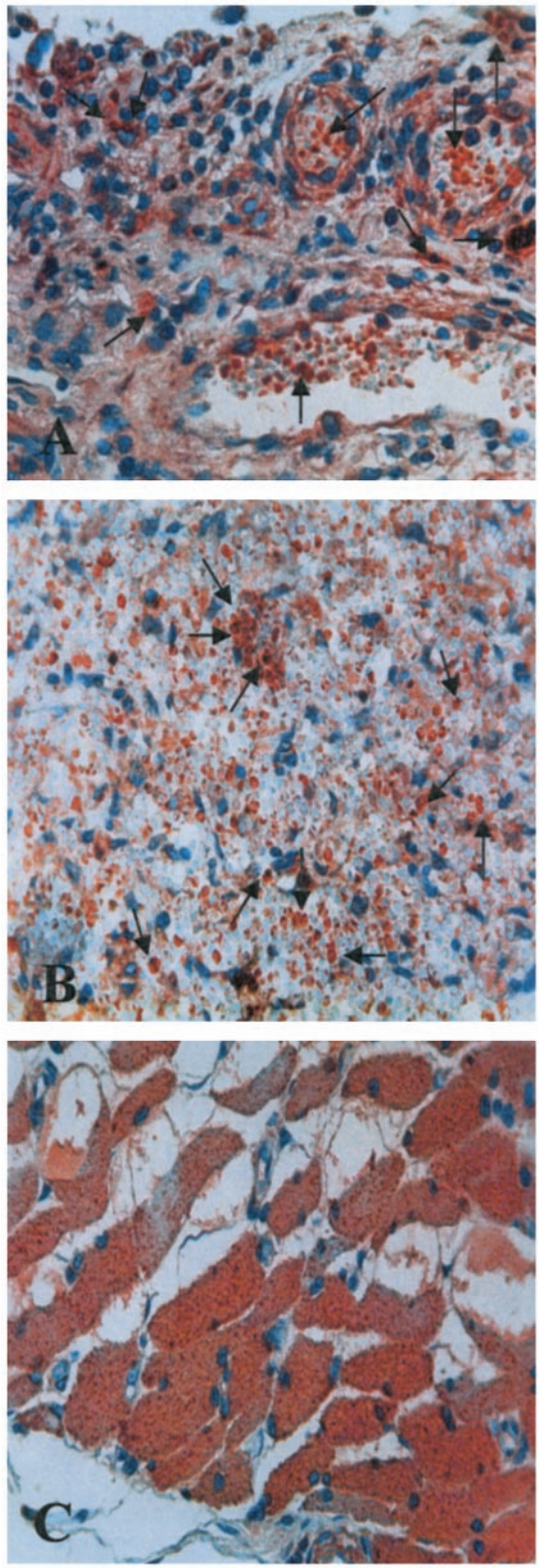

FIG. 3. GLUT 4 immunostaining of PAX3/FKHR-containing ARMS tumors. Skeletal muscle biopsies from PAX3/FKHR-expressing ARMS tumors were routinely processed for paraffin embedding. Deparaffinized sections (6 μm) were immunostained for GLUT4 and counterstained in hematoxylin, as described in *Materials and Methods*. Positive GLUT4 staining appears *red*, and nuclei are counterstained in *blue*. A and B, Two malignant tissues taken from two different ARMS patients. Positive GLUT4 staining is scattered throughout the tumor in undifferentiated cells, as indicated by *arrows*. C, A normal muscle sample, taken from tissue adjacent to the site of the biopsy shown in panel B, shows intense positive staining for GLUT4.

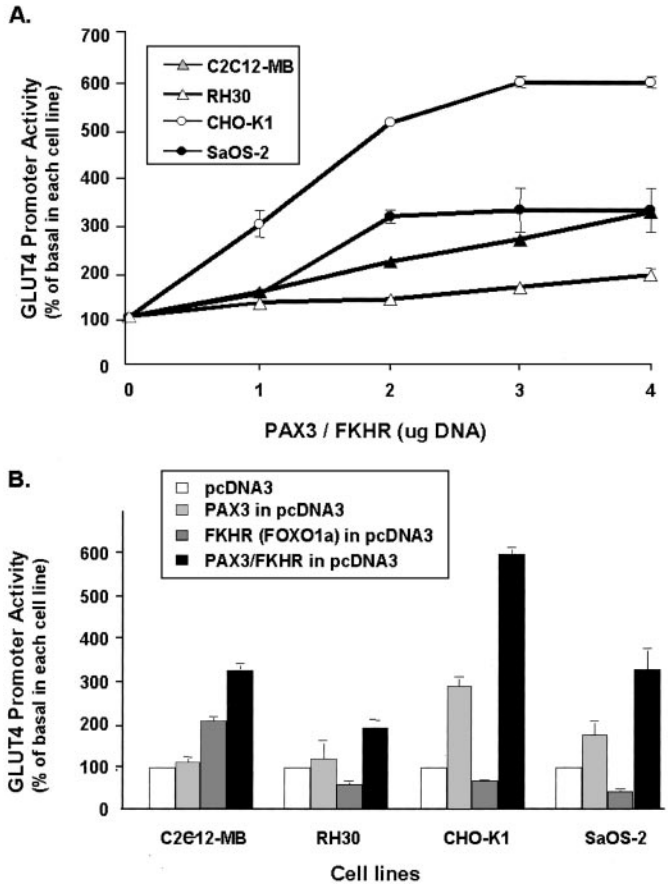

FIG. 4. PAX3/FKHR and PAX3 regulate GLUT4 promoter activity *in vitro*. C2C12-MB cells, RH30 cells, CHO-K1 cells, or SaOS-2 cells were plated in 100-mm dishes at a density of 750,000 cells per dish. A, Dose-dependent activation of GLUT4-P by PAX3/FKHR in various cell lines. Twenty-four hours after plating, the cells were cotransfected with 10 μg of a full-length pGLUT4-P-Luc promoter reporter, together with 0–4 μg of wild-type PAX3/FKHR in the pcDNA3 expression vector. GLUT4 promoter activity was determined by measuring luciferase and β-galactosidase activities, as described in *Materials and Methods*. Within each experiment, the results were expressed as a percentage of the basal GLUT4 promoter activity, *i.e.* the activity obtained from the expressed GLUT4-P-Luc reporter alone. The data are expressed as the mean ± SEM of three experiments performed in triplicate. B, Differential regulation of GLUT4-P by PAX3/FKHR, PAX3, and FKHR. The various cell lines were cotransfected with 10 μg of the pGLUT4-P-Luc promoter reporter, together with 3 μg of either wild-type PAX3/FKHR, PAX3, or FKHR (FOXO1a) in pcDNA3 expression vector, as described. GLUT4 promoter activity was assessed as described in panel A above. The data are expressed as mean ± SEM of three experiments, with each sample analyzed in triplicate.

with increasing amounts of expression vectors encoding the chimeric PAX3/FKHR protein or the native PAX3 protein (Fig. 4). In these cell lines, PAX3/FKHR expression enhanced the basal activity of cotransfected GLUT4-P in a dose-dependent manner to a maximum of 3- to 6-fold above basal levels. In

RH30, coexpression of PAX3/FKHR resulted in only a modest enhancement of GLUT4 promoter activity. This poor response of RH30 cells can be explained by the fact that these cells already express endogenous PAX3/FKHR, so GLUT4 promoter is already transactivated in the basal state, and addition of exogenous PAX3/FKHR can lead to only minor further transcription activation GLUT4. Coexpression of PAX3/FKHR had no effect on either the pCMV-β-galactosidase activity [driven by the cytomegalovirus (CMV) promoter], or the activity obtained from the p0-Luc reporter (the promoterless reporter serving as negative control). Thus, PAX3/FKHR-induced transactivation of GLUT4 gene is promoter-specific. Because PAX3/FKHR is the chimeric product of PAX3 and FKHR gene rearrangement, we further investigated the individual contribution of each of these gene products to GLUT4 promoter activity. Like PAX3/FKHR, PAX3 also transactivated GLUT4-P in CHO-K1 and SaOS cells, but this effect was approximately 50% lower than that induced by PAX3/FKHR (Fig. 3B).

We also assessed the effects of FKHR transcription factor (current terminology, FOXO1a) on GLUT4-P activity. Interestingly, our data show that FOXO1a regulated the transcription activity from the GLUT4 promoter in a tissue-specific manner, acting as a repressor of GLUT4 gene expression in SaOS-2, CHO-K1, and RH30 cells, whereas enhancing it in C2C12 myoblasts. These data show that PAX3/FKHR can transactivate the GLUT4 gene promoter *in vitro* in a dose-dependent and tissue-specific manner. In accordance with its known oncogenic potential (18), PAX3/FKHR was found to be a more potent transcriptional activator of GLUT4-P than was PAX3 in all cell types examined.

All PAX3/FKHR functional domains contribute to transactivation of GLUT4 promoter

To identify PAX3/FKHR *trans*-elements that contribute to GLUT4-P activation, cells were cotransfected with either the intact PAX3/FKHR or the related functional mutants, along with either the pGLUT4-P-Luc or pGEM7-Luc reporters. A schematic description of the native and mutant PAX3/FKHR expression vectors is shown in Fig. 5A. We used the following PAX constructs: 1) The human PAX3/FKHR, which encodes the full-length fusion protein, with intact PAX3-derived PD and HD DNA binding domains, and FKHR-derived transactivation domain. This cDNA was subcloned by Chalepakis *et al.* (41) from RH30 cells (GenBank accession no. U02368) and includes all three ATGs at the N terminal. 2) PD-R56L, which bears a specific point mutation in PD, that recapitulates a naturally occurring Arg56Leu mutation in PAX3 that has been reported in a family with Waardenburg syndrome type I (42). This construct has been found to be incapable of binding PD recognition sites or transactivating PD reporters (41). 3) ΔHD, a PAX3/FKHR deletion mutant lacking the entire HD binding domain of PAX3 (amino acids 219–289). This construct is transcriptionally inactive, with synthetic PRS1 targets, *i.e.* sequences that contain both PD and HD recognition motifs. 4) ΔFKHR, which is a PAX3/FKHR deletion mutant lacking the entire FKHR-derived DNA activation domain and is transcriptionally inactive. 5) PAX3, which encodes the murine PAX3 protein, is 98% homologous and 100% identical to human PAX3 in the DNA binding paired box and homeodomain (25). These mutants have been previously shown to be functionally altered (18). As

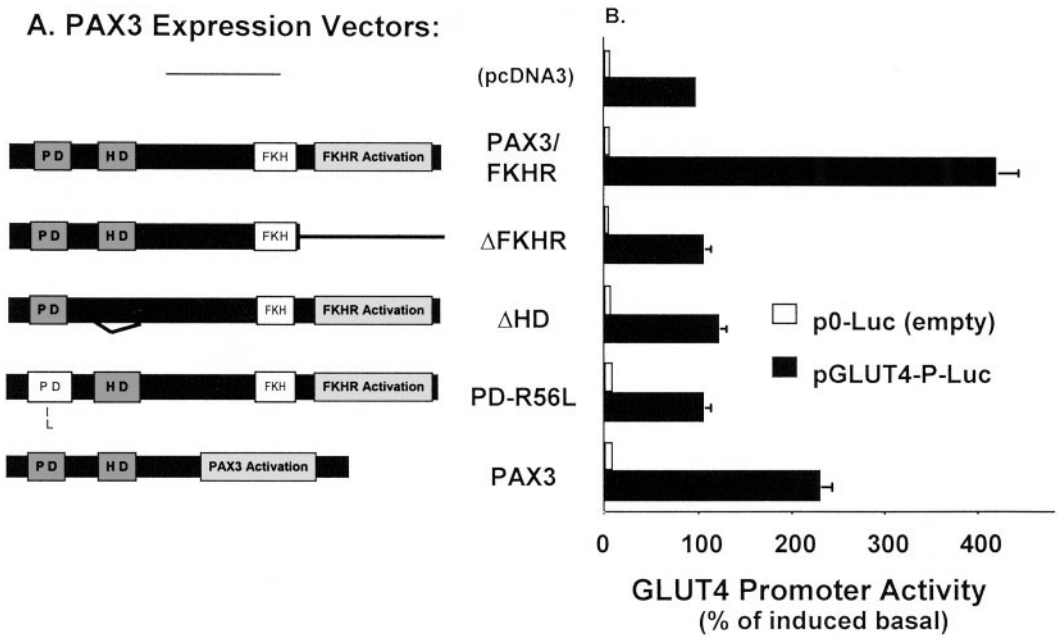

FIG. 5. PAX3/FKHR domains involved in GLUT4 promoter activation. A, Wild-type and mutated PAX3/FKHR expression vectors. A series of PAX3/FKHR mutants generated from the wild-type expression vector was used. In each mutant, a different functional domain was incapacitated either by a deletion (ΔFKHR, ΔHD) or by point mutation (PD-R56L). The structure of the native PAX3 cDNA is shown at the *bottom*. B, GLUT4 promoter activity. CHO-K1 cells were transiently cotransfected with 3 μg DNA of the various mutants (as indicated in panel A), together with 10 μg of either pGLUT4-P-Luc (*black bars*) or pGEM7-Luc (*white bars*). GLUT4 promoter activity was determined by measuring luciferase and β-galactosidase activities, as described in *Materials and Methods*. Data are expressed as mean ± SEM of three experiments, with each sample analyzed in triplicate.

shown in Fig. 5B, deletion of either the FKHR-derived DNA activation domain (ΔFKHR) or the PAX3-derived homeodomain (ΔHD) abrogated the ability of PAX3/FKHR to transactivate GLUT4-P. Similarly, the R56L point mutation in the PAX3-derived paired domain (PD-R56L) also abrogated the activation of GLUT4-P by PAX3/FKHR. In accordance with data shown in Fig. 4, coexpression of PAX3 also enhanced GLUT4-P activity, but this effect was approximately 50% less potent than that of PAX3/FKHR. These data suggest that all of the functional domains of the PAX3/FKHR protein are required to achieve a full transactivation of the GLUT4 promoter. Thus, it appears that, at least as the GLUT4 gene is concerned, impairment in any single domain, although still leaving all others intact, is sufficient to impair the ability of PAX3/FKHR to transcriptionally activate the GLUT4 promoter.

Cis-elements on GLUT4-P that are associated with its transactivation

Previous studies have identified several consensus sequences that are recognized by the PD and the HD DNA binding domains of PAX3. To date, the HD has been shown to recognize a single core motif, ATTA, whereas several recognition motifs have been identified for PD, including the classic GTTCC and GTTAC sequences (41, 42), a GTCAC motif found on the cMET promoter (27), and a newly identified GTTAT motif (43). However, although both the ATTA and the GTPyA/CPy motifs can be recognized by PAX3 (and by PAX3/FKHR), it is not yet known whether the simultaneous presence of both motifs is actually required for DNA binding. Our data suggest that all functional domains of PAX3/FKHR are required for full activation of GLUT4-P (Fig. 5). Thus, we proceeded to screen the 5′-flanking and promoter regions 1–2763 bp (−2213/+163 relative to transcription start site) of the rat GLUT4 gene (GenBank accession no. L36125) for the presence of PD and HD recognition motifs. We identified three ATTA (positions −1379, −144, and −3), four GTTCC (positions −1821, −726, −213, and +28), three GTTAC (positions −1426, −447, and −339), one GTCAC (position −353), and one GTTAT (position −1052) sequence motifs in this region of the GLUT4-P. The distribution of these motifs along the GLUT4 promoter is shown in Fig. 6A. To examine the contribution of these sequences to GLUT4-P transactivation, we prepared 5′-deletion promoter-reporter constructs, from which potential binding sites for PD and HD were progressively deleted (Fig. 6B). The relative ability of PAX3/FKHR to transactivate the various 5′-deletion reporter constructs was then compared. For clarity, PAX3/FKHR effect was expressed as percentage of the basal promoter activity in each construct. Analysis of the data (Fig. 6C) revealed that deletion of a region from −2155 to −1887 bp, relative to the transcription start site, resulted in an approximately 30–35% reduction in PAX3/FKHR-induced transactivation of GLUT4-P. Because none of the predicted HD or PD boxes lie in this region, we have generated additional deletion constructs that either include it or not. These constructs showed a similar 30%-reduced transactivation capacity toward GLUT4-P, and in fact, additional deletion of promoter regions up to −66 bp had no additional effect. A further deletion up to +163 bp led to complete loss of promoter transactivation. We verified that all PAX3/FKHR constructs used in the transactivation assays were capable of producing the correct protein at near equivalent amounts (Fig. 7A). Therefore, a minimal promoter region spanning −66/+163 bp was identified, whose presence was sufficient to retain approximately 65% of the full transactivation effect of PAX3/FKHR.

Physical interaction of PAX3/FKHR protein with GLUT4 promoter regions

A sequence analysis of the −66/+163-bp region of GLUT4-P identified potential binding sites for both the HD and the PD of PAX3/FKHR (ATTA at −3 bp and GTTCC at +28 bp, respectively). Thus, we used EMSAs to test whether this region can specifically and directly bind PAX3/FKHR proteins (Fig. 7). Full-length PAX3/FKHR and PAX3 proteins were translated *in vitro*, and were found to be expressed at the expected sizes of 97 kDa and 56 kDa, respectively, as determined by SDS-PAGE analysis (Fig. 7A). The *in vitro* translated PAX3/FKHR protein was functionally viable, as confirmed by its ability to form complexes with a ^{32}P-labeled PRS1 probe (Fig. 7B, lane 2). This oligonucleotide has been used as a "gold standard" for PAX3/FKHR (and PAX3) binding, because it contains binding sites for both the PD and the HD (18). The addition of increasing amounts of PAX3/FKHR protein (0–6 μl of PAX3/FKHR protein lysate) increased the formation of a complex with a ^{32}P-labeled probe derived from the −66/+163-bp region of the GLUT4-P (Fig. 7B, lanes 3–6). This binding could be progressively competed by the addition of increasing amounts of unlabeled probe, and was completely abolished by 100-fold molar excess of unlabeled probe, but it was unaffected by 100-fold molar excess of nonspecific DNA (Fig. 7B, lanes 7–10). These results suggest that PAX3/FKHR can bind directly and specifically to sequences within the −66/+163-bp region of the GLUT4 promoter. To more precisely identify these sequences, an oligonucleotide corresponding to a shorter region of the GLUT4-P, −4/+36 bp, which still contained the ATTA and the GTTCC recognition motifs, was synthesized (see Table 2 for detailed sequence). Increasing amounts of PAX3/FKHR protein (Fig. 7C, lanes 6–9; 0–6 μl of PAX3/FKHR protein lysate) increased the complex formation with this shorter ^{32}P-labeled probe derived from the −4/+36-bp region of the GLUT4-P. The binding could be competed by addition of 100-fold molar excess of unlabeled probe (Fig. 7C, lane 10), and it followed a pattern similar to that exhibited by complex formation in the presence of the PRS1 probe (Fig. 7C, lanes 1–5). To further establish the role of HD and PD recognition motifs, a −4/+36-bp HD-PD oligonucleotide was used, in which both ATTA and GTTCC sequences have been mutated to TAGT and GTTCA, respectively. According to Sublett *et al.* (18), reporter plasmid bearing these mutated sites (PRS10) could neither bind nor be transactivated by PAX3/FKHR. In accordance with that, our present data show that a double-mutant −4/+36 HD-PD oligonucleotide could not bind the PAX3/FKHR protein (Fig. 7C, lanes 11–14), thus indicating that the simultaneous presence of both the PD and the HD binding sites in the −4/+36-bp sequence is required for GLUT4-P activation.

To test the specificity of this interaction, supershift assays were performed using HA epitope-tagged PAX3/FKHR

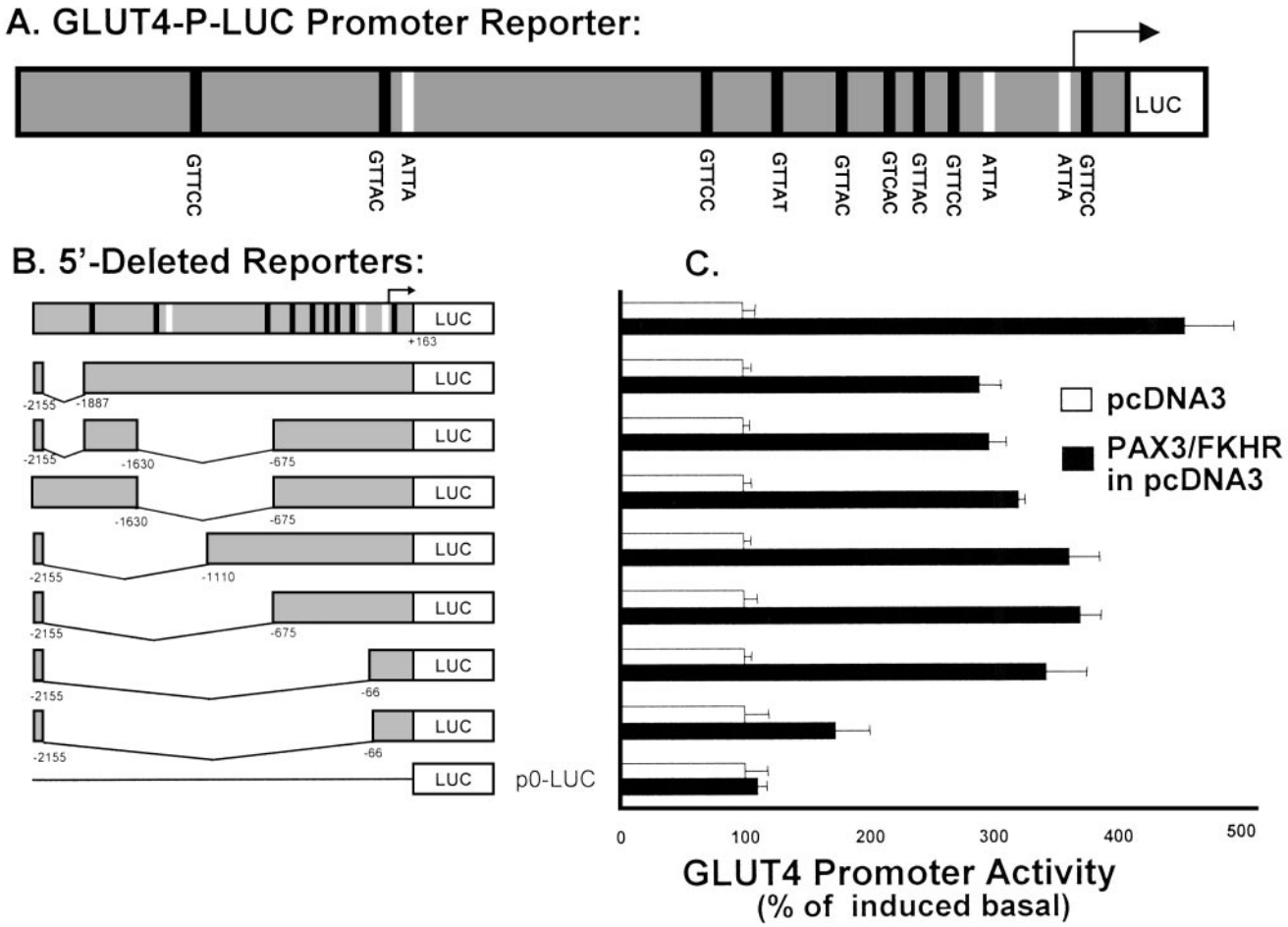

FIG. 6. Progressive 5′-deletion analysis of GLUT4-P. A, Schematic description of the rat GLUT4 promoter reporter. The full-length GLUT4 promoter reporter pGLUT4-P-Luc was generated by subcloning the upstream 5′ region of the rat GLUT4 gene (−2213/+163 bp) into pGEM7-Luc, upstream to the luciferase gene, as detailed in *Materials and Methods*. The transcription start site is indicated by an *arrow*. Distribution along the promoter of ATTA and GTPyA/CPy sequence motifs (which are recognized by HD and PD of PAX3/FKHR, respectively) is also shown. B, 5′-deleted GLUT4-P reporters. A series of progressive 5′-deletion mutants was used as detailed in *Materials and Methods*. The restriction sites used to generate each construct are indicated and numbered according to the first nucleotide in the site. C, GLUT4 promoter activity. Cells were transiently cotransfected with 10 μg of the various promoter-reporter constructs, as indicated, along with 3 μg of either the pcDNA3 expression vector alone (*white bars*) or with pcDNA3-PAX3/FKHR (*black bars*). GLUT4 promoter activity was determined by measuring luciferase and β-galactosidase activities, as described in *Materials and Methods*. For clarity, PAX3/FKHR effect was expressed as percentage of the basal promoter activity in each construct. The data are expressed as mean ± SEM of three experiments, performed in triplicate.

cDNA as a template for *in vitro* protein translation. As shown in Fig. 7D, HA-tagging of PAX3/FKHR protein did not affect its ability to specifically bind the −4/+36-bp probe. Incubation of the HA-tagged protein/DNA complexes with 2 μl of anti-HA antibodies resulted in further retardation of their electrophoretic mobilities, compared with probe and protein alone. These data indicate that the shorter (−4/+36 bp) region of the GLUT4 promoter retained the ability to form specific DNA-protein complexes with the PAX3/FKHR protein. Taken together, our data suggest that the PAX3/FKHR protein directly interacts with the GLUT4 promoter to mediate its transcriptional transactivation via a region that encompasses the transcription start site (−4/+36 bp). This region contains an ATTA and a GTTCC recognition motif, which may serve as potential binding sites for the HD and the PD, respectively. Thus, these elements may represent a PAX3/FKHR response element within the GLUT4 promoter.

Discussion

In the present study, we show that the ARMS-unique PAX3/FKHR oncoprotein induces the expression of the insulin-responsive GLUT4 gene both *in vivo* and *in vitro*, by binding to specific regions on the GLUT4 promoter and enhancing its transcriptional activity. The PAX3/FKHR protein is an aberrant transcription factor that is uniquely expressed in the muscle tumor ARMS. This protein has oncogenic potential and results from a t(2:13)(q35;q14) chromosomal translocation that juxtaposes the DNA binding domains of the PAX3 gene in frame with the DNA activation domain of the FKHR gene, thereby giving rise to a chimeric PAX3/FKHR gene (GenBank accession no. U02368; Refs. 15 and 16). Although PAX3/FKHR is an oncoprotein, both the PAX3 and the FKHR gene products are essential transcription factors that regulate normal cellular growth and differentiation.

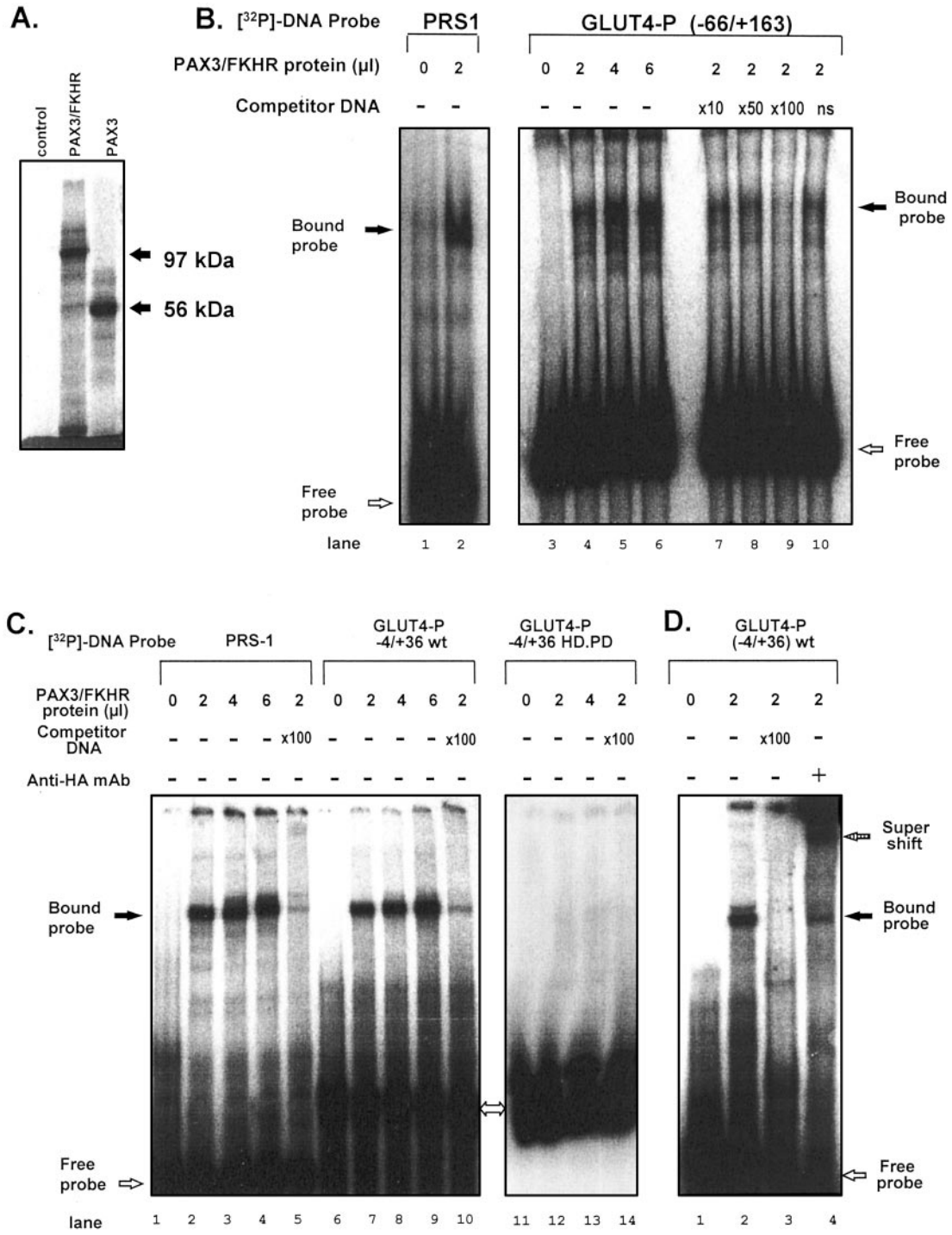

FIG. 7. EMSAs. A, *In vitro* translation of PAX3/FKHR. The integrity and correct size of the *in vitro* translated proteins for use in EMSA studies were confirmed in parallel reactions performed in the presence of ^{35}S-methionine. The resulting translation products were subjected to electrophoresis on 10% SDS-PAGE, followed by PhosphorImager analysis. PAX3 was included as a control but was not used for EMSA. B, PAX3/FKHR binding to GLUT4-P −66/+163-bp region. Binding reactions included either ^{32}P-labeled PRS1 (lanes 1–2) or the ^{32}P-labeled GLUT4-P −66/+163-bp restriction fragment (lanes 3–10) and the indicated volumes of PAX3/FKHR protein lysate. An unlabeled GLUT4-P −66/+163-bp fragment was used as specific DNA competitor (lanes 7–9), whereas an OCT1-derived oligonucleotide was used for a nonspecific competition (lane 10). C, PAX3/FKHR binding to GLUT4-P −4/+36-bp region. Binding reactions included either ^{32}P-labeled PRS1 (lanes 1–5) or synthetic oligonucleotide representing GLUT4-P sequence −4/+36 bp (wild-type, lanes 6–10; HD.PD mutant, lanes 11–14) and the indicated volumes of PAX3/FKHR protein lysate. Unlabeled PRS1 (lane 5) or −4/+36-bp (lane 10) fragments were used as specific DNA competitors. D, Supershift assays. Binding reactions included ^{32}P-labeled synthetic oligonucleotides corresponding to the GLUT4-P-derived sequence −4/+36 bp, in the absence (lane 1) or presence (lanes 2–4) of 2 µl of 3′-HA-PAX3/FKHR protein lysate. Complex supershifts were induced by the addition of 2 µl of anti-HA antibody 10 min before addition of the probe. *Black* and *white arrows* indicate motilities of bound and free probes, respectively. The fold molar excess of specific competitor DNA or the addition of nonspecific DNA (ns) is indicated above the relevant lanes.

Enhanced glucose metabolism and GLUT1 expression have long been recognized in transformed cells (6–9). However, the role of GLUT4 expression in tumorigenesis in general and in muscle tumors specifically is still undefined. In light of the central role played by GLUT4 in glucose uptake in muscle and the accelerated rate of glucose uptake in tumor cells, we hypothesized that the aberrant expression of PAX3/FKHR in ARMS tumors may be associated with the induction of the GLUT4 gene expression *in vivo*. Indeed, our data demonstrate that both mRNA and protein for GLUT4 are expressed in ARMS-derived human tissue biopsies and in RH30 cells. Under normal physiological conditions, GLUT4 is almost exclusively expressed in fully differentiated adipose and muscle cells, where glucose uptake is acutely responsive to insulin (4). The expression of GLUT4 that was found in ARMS-derived cells is aberrant because these cells are histologically characterized as undifferentiated rhabdomyoblasts (18). The physiological relevance of GLUT4 to cellular metabolism is further supported by our finding that, unlike normal myoblasts (*i.e.* C2C12), ARMS-derived myoblasts express functionally active GLUT4 protein, as demonstrated by the presence of insulin-responsive glucose uptake in these cells. In RH30 cells, insulin stimulated the basal rates of 2-deoxyglucose uptake by as much as 140% (Fig. 2). The magnitude of this effect is similar to that observed by Sarabia *et al.* (44) in primary cultures of human muscle cells. Although this effect of insulin on glucose uptake may seem low when compared with the 20- to 30-fold stimulation of glucose transport typically observed in rat adipocytes (for review, see Ref. 2), it is important to note that: 1) the responsiveness to insulin in human cells is far less than that observed in rat adipocytes in primary cultures; 2) insulin responsiveness is further decreased in immortalized cell lines, compared with cells in primary culture; and 3) lower levels of GLUT4 mRNA and proteins were detected in ARMS-derived cells compared with normal human skeletal muscle cells (Fig. 1B). Thus, we suggest that the unique presence of the PAX3/FKHR oncoprotein in human ARMS-derived rhabdomyoblasts is associated with aberrant yet functional expression of GLUT4 transport proteins in these cells. The ability of PAX3/FKHR to activate the GLUT4 promoter may be tissue- and/or tumor-type specific.

The tissue-specific regulation of GLUT4 expression contributes to the maintenance of normal glucose homeostasis. However, GLUT4 is also critically important in altered metabolic states. For example, in insulin-deficient states such as fasting and streptozotocin-induced diabetes, we and others have shown that GLUT4 protein is severely depleted and that this impairs insulin stimulation of glucose transport (45, 46). Similarly, insulin resistance in type 2 diabetes or aging was shown to be associated with a major reduction in the intracellular pool of GLUT4 (31, 47). Studies in transgenic animals show that GLUT4 overexpression results in a marked improvement in glycemic control (48). However, until now, activation of GLUT4 gene expression in predifferentiated myoblasts has not been described under any pathophysiological state, including tumorigenesis. Teleologically, the presence of a high-affinity functional GLUT4 isoform in tumor cells could confer them a metabolic advantage, because these cells need to maintain an increased rate of glucose metabolism.

To better understand the mechanism of abnormal GLUT4 expression in skeletal myoblasts, we studied the regulation of the GLUT4 promoter by PAX3/FKHR *in vitro*, using transient coexpression assays. We found that in transformed SaOS-2 osteoblasts, in nontransformed CHO-K1 cells, and in C2C12 myoblasts (which are all devoid of endogenous GLUT4 and PAX3/FKHR), the GLUT4 gene promoter was transactivated by PAX3/FKHR in a dose-dependent and tissue-specific manner (Fig. 4). Moreover, only minor further increases in GLUT4 promoter activity were observed in RH30 cells in response to exogenous expression of the chimera, possibly reflecting the relatively high basal levels of endogenous expression of PAX3/FKHR in these cells. Native PAX3 was also able to transactivate the GLUT4 promoter, but this effect was approximately 50% less than that of the chimeric oncoprotein. This difference may be due to the oncogenic potential of PAX3/FKHR. However, because downstream target genes for PAX3/FKHR have not been identified until now (other than the PDGFαR and BCL-XL), its transactivation potential has been assessed on synthetic target sequences derived from the even-skipped promoter of *Drosophila* (25). Using PAX3/FKHR mutants with deletions in either functional domain (Fig. 5), we found that the PAX3-derived PD DNA binding domain, the HD DNA binding domain, and the FKHR-derived DNA activation domain are all essential for transactivation of GLUT4-P. Similar findings have been previously demonstrated with other synthetic PD/HD targets (49).

Using progressive 5′ deletion analysis of GLUT4-P (Fig. 6), we identified a promoter region, −66/+163 bp, whose presence was sufficient to retain about 65% of the full GLUT4-P transactivation effect. A deletion of a region from −2155 to −1887 bp resulted in an approximately 30% reduction in PAX3/FKHR-induced transactivation of GLUT4-P, despite the fact that none of the predicted HD or PD boxes lie in this region. These results may be explained by a presence of an accessory or cooperating factor, that its binding to this upstream region may be required to achieve maximal transactivation effect. Alternatively, it may well be that deletion of this region leads to changes in the tertiary structure of the GLUT4 promoter that are sufficient to interfere with its transactivation. In this regard, studies by Pessin and co-workers (50) have shown that another upstream region in the rat GLUT4 gene (−522 to −422) contains a binding site for myocyte enhancer factor 2 (MEF2), and this MEF2 binding site confers GLUT4 myotube-specific expression. We have found that GLUT4-P constructs in which the MEF2 region was deleted can still be transactivated by PAX3/FKHR. Therefore, the relevance of the MEF2 binding site to the regulation of GLUT4 by PAX3/FKHR *in vitro* and to aberrant GLUT4 expression *in vivo* in ARMS cells warrants further investigation.

We found that the PAX3/FKHR protein binds specifically and directly to the −66/+163-bp promoter region of GLUT4, as well as to a shorter region spanning −4/+36 bp, using EMSA. Furthermore, a double-mutant oligonucleotide, in which both the ATTA and GTTCC sequences residing within the −4/+36-bp region have been mutated to TAGT and GTTCA, respectively, could not bind the PAX3/FKHR protein. These data indicate that the simultaneous presence of both an ATTA and a GTTCC recognition motif, which serve as binding sites for the HD and the PD respectively, is required for PAX3/FKHR binding to this promoter region. This is in line with our transactivation data, showing that both the PD and the HD of

PAX3/FKHR are indeed required for GLUT4-P transactivation (Fig. 5). Localization of the PD and HD recognition elements within 2–13 bp of each other has been reported to be essential for the high-affinity binding of PAX3 (42), although these elements are located 26 bp apart in the GLUT4 promoter. Therefore, defining the −4/+36-bp sequence as the PAX3/FKHR response element on GLUT4-P may be somewhat problematic. It may be that although both domains are required for binding, only one domain is actually involved in the transactivation, as suggested by Epstein *et al.* (51), for the PDGFα receptor. Phelan and Loeken (43) have shown that the GTS/PAX3 fusion protein will recognize each individual binding element in the absence of the other. However, these interactions with only one recognition element appear to be of low affinity, similar to those involving recognition elements that are either too close or too far apart (43). Alternatively, it is possible that the tertiary structure of DNA assumes a spatial position that permits the distant ATTA and GTTCC recognition motifs to be apposed in closer proximity to one another, thus allowing for high-affinity binding of PAX3/FKHR. However, whatever the case, our present work clearly demonstrates that a simultaneous presence of both the PD and the HD binding sites within the −4/+36-bp promoter sequence is required for PAX3/FKHR binding to and transactivation of the GLUT4 promoter.

The relevance of FKHR to the insulin signaling as well as to tumorigenicity has recently become apparent. It has been suggested that FKHR is the transcription factor that mediates the inhibitory effect of insulin on gene transcription, via its binding to insulin response elements on various downstream promoters (23). Recent studies now show that FKHR is negatively regulated by the human PKB/Akt via a rapid and hierarchic phosphorylation of FKHR on three PKB phosphorylation consensus sites, T24, S256, and S319 (for review, see Ref. 52). PKB is a serine/threonine kinase that lies downstream of PI3-K and is thought to mediate many of the intracellular actions of insulin and other growth factors, being an important member of the insulin signaling cascade as well as a factor in newly defined mechanisms of PI3-K-mediated tumorigenicity (52). The PAX3/FKHR protein contains two of the three consensus PKB phosphorylation sites that are present in FKHR, however neither the transcriptional activity of the chimeric protein nor its nuclear localization is altered by PKB phosphorylation, consistent with its tumorigenic potential (53). Because our data show that the level of GLUT4 transactivation by PAX3/FKHR differs depending on the cell line used, this may suggest that theoretically PAX3/FKHR would have an *in vitro* transactivation effect in all cells examined, but a repressor(s) that is present in normal nontumorigenic cells dampens this action. Providing novel data on GLUT4 promoter regulation by the human FKHR transcription factor (current terminology, FOXO1a), we now show that FKHR itself regulates transcription activity from the GLUT4 promoter in a tissue-specific manner (Fig. 4), acting as a repressor of GLUT4 gene expression in SaOS-2, CHO-K1, and RH30 cell lines, whereas enhancing it in C2C12 myoblasts. Thus, it is possible that the chromosomal translocation in ARMS results not only in the activation of PAX3 but also in the disruption of functional FKHR, which is essential for insulin-regulated gene repression and muscle cell differentiation. It could be argued that the loss of these functions of FKHR, rather than a gain of function by the PAX3/FKHR fusion, led to the abnormal derepression of GLUT4 gene expression, thus contributing to the development of ARMS. Our present work suggests that both options hold true. We show that a direct and specific effect of PAX3/FKHR oncoprotein itself exists, to transactivate GLUT4 gene expression both *in vivo* and *in vitro*. Concomitantly, we show that human FKHR transrepresses GLUT4 transcription in these tumors cells. The physiological relevance of PAX3 and FKHR proteins to the aberrant regulation of the GLUT4 gene is further exhibited by studies showing that mutations in the genes encoding for members of the large FKHR/hepatic nuclear factor family of transcription factors were found in patients with maturity-onset diabetes of the young, a genetically heterogeneous form of diabetes characterized by an autosomal dominant inheritance, early age of onset, and primary β-cell dysfunction (54). Along the same lines, Waeber *et al.* (55) have demonstrated that in pancreatic β-cells, the GLUT2 gene promoter is controlled by the islet-specific PDX-1 homeobox factor through an identified GLUT2 TAAT motif. Using cDNA microarray to elucidate the pattern of gene expression induced in ARMS, Khan *et al.* (56) have demonstrated the profound myogenic properties of PAX3/FKHR in NIH 3T3 cells. Although not investigating GLUT4 directly, this work clearly indicates that PAX3/FKHR activates a myogenic transcription program, including the induction of transcription factors like MyoD, Myogenin, Six1, and Slug as well as a battery of genes involved in major aspects of muscle function. However, to the best of our knowledge, the present study is the first to identify the GLUT4 gene as a downstream target gene that is transactivated by the chimeric oncoprotein PAX3/FKHR.

In summary, our studies suggest that the GLUT4 gene is a downstream gene target for the ARMS-derived PAX3/FKHR oncoprotein, and that GLUT4 is regulated by this aberrant transcription factor in a direct, specific, and dose-dependent manner. Although there is now a realistic possibility of cure in the majority of solid tumors, there remains a subset that is resistant to multimodality therapy. Therefore, characterization of the molecular mechanisms that lead to abnormal glucose metabolism may provide a better understanding of rhabdomyosarcoma tumorigenesis, which may ultimately result in novel therapeutic strategies that increase the overall cure. As such, the GLUT4 gene may serve as a novel potential target for gene therapy in patients with ARMS tumors, with respect to the treatment and prevention of tumor growth.

Acknowledgments

We thank Dr. Danny Zipris (Worcester, MA) for assistance with RT-PCR assays and Ms. Natalia Kritz for excellent technical assistance. We thank Dr. Dana Beitner-Johnson for providing scientific editing and valuable critical comments on this manuscript.

Received February 28, 2002. Accepted August 13, 2002.

Address all correspondence and requests for reprints to: Michal Armoni, D.Sc., Director, Unit of Molecular Endocrinology, the Institute of Endocrinology, Diabetes and Metabolism, Rambam Medical Center, Haifa 31096, Israel. E-mail: amichal@tx.technion.ac.il.

This work was supported in part by grants from the L. R. Diamond Fund, the San Francisco Diabetes Research Fund, and the Technion-Israel Institute of Technology's Vice President for Research Fund.

References

1. **Mueckler M** 1994 Facilitative glucose transporter. Eur J Biochem 219:713–725
2. **Czech M, Clancy B, Pessino A, Woon C, Harrison S** 1992 Complex regulation of simple sugar transport in insulin-responsive cells. Trends Biochem Sci 17:197–201
3. **Czech MP, Corvera S** 1999 Signaling mechanisms that regulate glucose transport. J Biol Chem 274:1865–1868
4. **James DE, Piper RC, Slot JW** 1994 Insulin stimulation of GLUT4 translocation: a model for regulated recycling. Trends Cell Biol 4:120–126
5. **Warburg O** 1931 The metabolism of tumors. New York: Richard R Smith; 29–169
6. **Flier JS, Mueckler M, Usher P, Lodish HF** 1987 Elevated levels of glucose transporter messenger RNA are induced by *ras* and *src* oncogenes. Science 235:1492–1495
7. **Birnbaum MJ, Haspel HC, Rosen OM** 1987 Transformation of rat fibroblasts by FSV rapidly increases glucose transporter gene transcription. Science 235: 1495–1498
8. **Salter DW, Baldwin SA, Lienhard GE, Weber MJ** 1982 Proteins antigenically related to the human erythrocyte glucose transporter in normal and Rous sarcoma virus-transformed chicken embryo fibroblasts. Proc Natl Acad Sci USA 79:1540–1544
9. **Younes M, Lechago LV, Somoano JR, Mosharaf M, Lechago J** 1996 Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers. Cancer Res 56:1164–1167
10. **Yamamoto T, Seino Y, Fukumoto H, Koh G** 1990 Over-expression of GLUT genes in human cancer. Biochem Biophys Res Commun 170:223–229
11. **Nagamatsu S, Sawa H, Wakizaka A, Hoshino T** 1993 Expression of GLUT isoforms in human brain tumors. J Neurochem 61:2048–2053
12. **Brown RS, Wahl RL** 1993 Over-expression of GLUT1 glucose transporter in human breast cancer. Cancer 72:2979–2984
13. **Zamora-Leon SP, Golde DW, Concha II, Rivas CI, Delgado-Lopez F, Baselga J, Nualart F, Vera JC** 1996 Expression of fructose transporter GLUT5 in human breast cancer. Proc Natl Acad Sci USA 93:1847–1852
14. **Cleary ML** 1991 Oncogenic conversion of transcription factors by chromosomal translocations. Cell 66:619–622
15. **Barr FG, Galili N, Holick J, Biegel JA, Rovera G, Emanuel BS** 1993 Rearrangement of the PAX3 paired box gene in the pediatric solid tumor alveolar rhabdomyosarcoma. Nat Genet 3:113–117
16. **Shapiro DN, Sublett JE, Li B, Downing JR, Naeve CW** 1993 Fusion of the PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyoblastoma. Cancer Res 53:5108–5112
17. **Fredericks WJ, Galili N, Mukhopadhyay S, Rovera G, Bennicelli J, Barr FG, Rauscher 3rd FJ** 1995 PAX3-FKHR fusion protein created by t(2;13) translocation in alveolar rhabdomyosarcoma is a more potent transcriptional activator than PAX3. Mol Cell Biol 15:1522–1535
18. **Sublett JE, In-Sang J, Shapiro DN** 1995 The alveolar rhabdomyosarcoma PAX3/FKHR fusion protein is a transcriptional activator. Oncogene 11:545–552
19. **Lai E, Clark KL, Burley SK, Darnell JE** 1993 Hepatocyte nuclear factor 3/fork head or "winged helix" proteins: a family of transcription factors of diverse biologic function. Proc Natl Acad Sci USA 90:10421–10423
20. **Hacker U, Grossniklaus U, Gehring WJ, Jackle H** 1992 Developmentally regulated *Drosophila* gene family encoding the fork head domain. Proc Natl Acad Sci USA 89:8754–8758
21. **Anderson MJ, Viars CS, Czekay S, Cavenee WK, Arden KC** 1998 Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. Genomics 47:187–199
22. **Rena G, Guo S, Cichy SC, Unterman TG, Cohen P** 1999 Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. J Biol Chem 274:17179–17183
23. **Nakae J, Barr V, Accili D** 2000 Differential regulation of gene expression by insulin and IGF-1 receptors correlates with phosphorylation of a single amino acid residue in the forkhead transcription factor FKHR. EMBO J 19:989–996
24. **Gruss P, Walther C** 1992 *Pax* in development. Cell 69:719–722
25. **Goulding MD, Chalepakis G, Deutsch U, Erselius JR, Gruss P** 1991 Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. EMBO J 10:1135–1147
26. **Stuart ET, Kioussi C, Gruss P** 1993 Mammalian Pax genes. Annu Rev Genet 27:219–237
27. **Epstein JA, Shapiro DN, Cheng J, Lam PY, Maas RL** 1996 Pax3 modulates expression of the c-Met receptor during limb muscle development. Proc Natl Acad Sci USA 93:4213–4218
28. **Margue CM, Bernasconi M, Barr FG, Schafer BW** 2000 Transcriptional modulation of the anti-apoptotic protein BCL-XL by the paired box transcription factors PAX3 and PAX3/FKHR. Oncogene 19:2921–2929
29. **Ginsberg JP, Davis RJ, Bennicelli JL, Nauta LE, Barr FG** 1998 Up-regulation of MET but not neural cell adhesion molecule expression by the PAX3-FKHR fusion protein in alveolar rhabdomyosarcoma. Cancer Res 58:3542–3546
30. **Zipris D** 1996 Evidence that Th1 lymphocytes predominate in islet inflammation and thyroiditis in the BioBreeding (BB) rat. J Autoimmun 9:315–319
31. **Armoni M, Harel C, Burvin R, Karnieli E** 1995 Modulation of the activity of glucose transporters (GLUT) in the aged/obese rat adipocyte: suppressed function, but enhanced intrinsic activity of GLUT. Endocrinology 136:3292–3298
32. **Tobar A, Avigad S, Zoldan M, Mor C, Goshen Y, Zaizov R** 2000 Clinical relevance of molecular diagnosis in childhood rhabdomyosarcoma. Diagn Mol Pathol 9:9–13
33. **Czech MP, Chawla A, Woon CW, Buxton JM, Armoni M, Tang W, Joly M, Corvera S** 1993 Exofacial epitope-tagged glucose transporter chimeras reveal COOH-terminal sequences governing cellular localization. J Cell Biol 123:127–136
34. **Tang ED, Nunez G, Barr FG, Guan KL** 1999 Negative regulation of the forkhead transcription factor FKHR by Akt. J Biol Chem 274:16741–16746
35. **Quon MJ, Zarnowski MJ, Guerre-Millo M, de la Luz Sierra M, Taylor SI, Cushman SW** 1993 Transfection of DNA into rat adipose cells by electroporation. Biochem Biophys Res Commun 194:338–346
36. **Sambrook J, Fritsh EF, Maniatis T** 1989 Molecular cloning: a laboratory manual, 2nd Ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory
37. **Richardson JM, Pessin JE** 1993 Identification of a skeletal muscle-specific regulatory domain in the rat GLUT4/muscle-fat gene. J Biol Chem 268:21021–21027
38. **Shimokawa T, Kato M, Ezaki O, Hashimoto S** 1998 Transcriptional regulation of muscle-specific genes during myoblast differentiation. Biochem Biophys Res Commun 246:287–292
39. **Fogh J, Wright WC, Loveless JD** 1977 Absence of HeLa cell contamination in 169 cell lines derived from human tumors. J Natl Cancer Inst 58:209–214
40. **Felix CA, Kappel CC, Mitsudomi T, Nau MM, Tsokos M, Crouch GD, Nisen PD, Winick NJ, Helman LJ** 1992 Frequency and diversity of p53 mutations in childhood rhabdomyosarcoma. Cancer Res 52:2243–2247
41. **Chalepakis G, Goulding M, Read A, Strachan T, Gruss P** 1994 Molecular basis of splotch and Waardenburg Pax-3 mutations. Proc Natl Acad Sci USA 91: 3685–3689
42. **Chalepakis G, Wijnholds J, Gruss P** 1994 Pax-3-DNA interaction: flexibility in the DNA binding and induction of DNA conformational changes by paired domains. Nucleic Acids Res 22:3131–3137
43. **Phelan SA, Loeken MR** 1998 Identification of a new binding motif for the paired domain of Pax-3 and unusual characteristics of spacing of bipartite recognition elements on binding and transcription activation. J Biol Chem 273:19153–19159
44. **Sarabia V, Ramlal T, Klip A** 1990 Glucose uptake in human and animal muscle cells in culture. Biochem Cell Biol 68:536–542
45. **Karnieli E, Armoni M, Cohen P, Kanter Y, Rafaeloff R** 1987 Reversal of insulin resistance in diabetic rat adipocytes by insulin therapy. Restoration of pool of glucose transporters and enhancement of glucose-transport activity. Diabetes 36:925–931
46. **Kahn BB** 1992 Alterations in glucose transporter expression and function in diabetes: mechanisms for insulin resistance. J Cell Biochem 48:122–128
47. **Garvey TW** 1992 Glucose transport and NIDDM. Diabetes Care 15:396–417
48. **Gibbs EM, Stock JL, McCoid SC, Stukenbrok HA, Pessin JE, Stevenson RW, Milici AJ, McNeish JD** 1995 Glycemic improvement in diabetic db/db mice by overexpression of the human insulin-regulatable glucose transporter (GLUT4). J Clin Invest 95:1512–1518
49. **Czerny T, Schaffner G, Busslinger M** 1993 DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. Genes Dev 7:2048–2061
50. **Liu M, Olson AL, Edgington NP, Moye-Rowley WS, Pessin JE** 1994 Myocyte enhancer factor 2 (MEF2) binding site is essential for C2C12 myotube-specific expression of the rat GLUT4/muscle-adipose facilitative glucose transporter gene. J Biol Chem 269:28514–28521
51. **Epstein JA, Song B, Lakkis M, Wang C** 1998 Tumor-specific PAX3-FKHR transcription factor, but not PAX3, activates the platelet-derived growth factor α receptor. Mol Cell Biol 18:4118–4130
52. **Kops GJ, Burgering BM** 2000 Forkhead transcription factors are targets of signaling by the proto-oncogene PKB (C-AKT). J Anat 197:571–574
53. **del Peso L, Gonzalez VM, Hernandez R, Barr FG, Nunez G** 1999 Regulation of the forkhead transcription factor FKHR, but not the PAX3- FKHR fusion protein, by the serine/threonine kinase Akt. Oncogene 18:7328–7333
54. **Hattersley AT** 1998 Maturity-onset diabetes of the young: clinical heterogeneity explained by genetic heterogeneity [published erratum appears in Diabet Med 15:437]. Diabet Med 15:15–24
55. **Waeber G, Thompson N, Nicod P, Bonny C** 1996 Transcriptional activation of the GLUT2 gene by the IPF-1/STF-1/IDX-1 homeobox factor. Mol Endocrinol 10:1327–1334
56. **Khan J, Bittner ML, Saal LH, Teichmann U, Azorsa DO, Gooden GC, Pavan WJ, Trent JM, Meltzer PS** 1999 cDNA microarrays detect activation of a myogenic transcription program by the PAX3-FKHR fusion oncogene. Proc Natl Acad Sci USA 96:13264–13269