The large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) is a multifunctional protein that contains a serine-threonine protein kinase (PK) activity (Nelson, J. W., Zhu, J., Smith, C. C., Kulka, M., and Aurelian, L. (1996) J. Biol. Chem. 271, 17021–17027). Phylogenetic analyses indicated that ICP10 PK belongs to a distinct subfamily of growth factor receptor serine-threonine PKs that are characterized by their ability to function with a limited number of conserved catalytic motifs (Hunter, J. C. R., Smith, C. C., and Aurelian, L. (1995) Int. J. Onc. 7, 515–522). Here, we report the isolation and characterization of a novel gene, designated H11, that contains an open reading frame of 588 nucleotides, which encodes a protein similar to ICP10 PK. The H11 protein has Mn$^{2+}$-dependent serine-threonine-specific PK activity as determined with a GST-H11 fusion protein and by immunoblotting assays of 293 cells transfected with a H11 eukaryotic expression vector. PK activity is ablated by mutation of Lys$^{131}$ within the presumptive catalytic motif II (invariant Lys). 293 cells stably transfected with H11 acquire anchorage-independent growth. Endogenous H11 RNA and the H11 phosphoprotein are expressed in melanoma cell lines and primary melanoma tissues at levels higher than in normal melanocytes and in benign nevi. Melanoma cell proliferation is inhibited by treatment with antisense oligonucleotides that inhibit H11 translation, suggesting that H11 expression is associated with cell growth.

Several herpes viruses including herpes simplex virus type 1 (HSV-1)$^1$ and herpes simplex virus type 2 (HSV-2) express a distinct ribonucleotide reductase activity formed by the association of a large (R1) and a small (R2) subunit. The HSV-2 R1 gene (also known as ICP10) differs from its counterparts in eukaryotic and prokaryotic cells and in other viruses in that it possesses a unique one-third 5′-terminal domain (1) that codes for a serine (Ser)-threonine (Thr) protein kinase (PK) (2–8) and causes neoplastic transformation of immortalized cells (9–11). Unlike other known PKs that have at least 12 conserved catalytic motifs (12, 13), ICP10 PK functions with only eight such motifs. They are clustered close to the N terminus, downstream of a transmembrane (TM) domain and surround a Src homology region 3-binding module (2, 4, 6, 14). The ICP10 PK activity is Mn$^{2+}$-ion-dependent, does not require monovalent cations, and is not inhibited by zinc sulfate, properties distinct from those of many cellular kinases such as casein kinase II (5). ICP10 is located on the cell surface and its PK activity is intrinsic. Mutants in which the TM domain or the conserved PK catalytic motifs were deleted or otherwise altered were rendered PK negative (4, 5, 7, 8), and the PK activity of a chimeric protein consisting of ICP10 PK and the ligand-binding domain of the epidermal growth factor receptor was activated by epidermal growth factor (15). Most significantly, the ICP10 PK activity was ablated by mutation of the invariant residues within the ATP-binding (Lys) or ion-binding (Glu) sites (6), a criterion used to establish that the kinase activity is intrinsic for all PKs studied to date (12, 13, 16–19). Similar conclusions were reached for the HSV-1 R1 (also known as ICP6) PK (20). Recent studies using a HSV-2 mutant deleted in the ICP10 PK domain indicate that the PK activity is required for timely onset of virus replication (8). This is apparently related to activation by ICP10 PK of the RAS/MEK/MAPK mitogenic pathway (21). However, ICP10 and ICP6 PKs are only 38% very closely related. Indeed, unlike ICP10 PK, ICP6 PK DNA does not have transforming potential (9), and the protein is not located on the cell surface (22) and does not activate the RAS/MEK/MAPK mitogenic pathway (23). Because the PK domains are only present in the HSV R1 proteins and sequences homologous to ICP10 PK DNA were cloned from human cells (HeLa)
heat shock protein 27 (hsp27) was the gift of Dr. Nose, Showa University.

LRRDPFRDSPLSSR; anti-H11–10) or 181–194 (SFNNELPQDSQEVT; H11 protein has Ser-Thr PK activity. It is expressed in melanoma cells and expression appears to be required for cell growth.

MATERIALS AND METHODS

Cells and Tissues—Human melanoma cell lines SK-MEL-31 and SK-MEL-3 and 293 cells (adenovirus E1a-immortalized human embryonic kidney) were grown in Eagle's modified minimal essential medium (MEM) containing 1 mM nonessential amino acids, 1% sodium pyruvate, and 10% FCS. HeLa and human melanoma G361 cells were grown in Eagle's modified minimal essential medium, 10% FCS and McCoy's, 10% FCS. Normal human melanocyte cultures were obtained from Dr. J. Burnett (Department of Dermatology, University of Maryland).

Antibodies—Polyclonal antibodies to peptides in ICP10 PK (3) and to peptides that respectively represent H11 amino acids 10–29 (CHYPSELRLLRDPFRDSPLSSR; anti-H11–10) or 181–194 (SFNNELPQDSQEVT; anti-H11–181) were generated as described (26). Polyclonal antibody to heat shock protein 27 (hsp27) was the gift of Dr. Nose, Showa University, Japan. GST antibody (rabbit polyclonal Z-5) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Library Screening and Plasmid Construction—An established pre-amplified HeLa cDNA (UniZap XR) library constructed in a lambda-based vector (Stratagene, La Jolla, CA) was screened with antibodies to ICP10 PK peptides. Preparation of host bacterial cultures (Escherichia coli XLI Blue MRβ), library infection/plating, cDNA expression and immunoblotting based screening were as per the manufacturer's instructions. A total of 1 × 106 plaques were screened, and positive plaques were subjected to three successive rounds of purification through plaque isolation and rescoring. Eight pure plaque isolates were obtained, which contained cDNA sequences. Single-stranded cDNA contained within a single-stranded phagemid (Stratagene) was rescued, isolated, and purified from lambda clones using freshly titered helper phage VCM13 according to the manufacturer's instructions. Single-stranded DNA was sequenced (UMAB Biopolymer Laboratory) using primers specific for either Bluescript II or cDNA sequences. cDNA was rescued as a double-stranded BlueScript II phagemid using the ExAssist/SOLR System (Stratagene) according to the manufacturer's instructions. The rescued phagemid was designated H11. H11 was subsequently cloned from a custom prepared library constructed from a primary human melanoma using oligo(dT)-primed cDNA and UniZap XR (Stratagene). Screening was with the 1.8-kilobase EcoRI/XhoI H11 cDNA fragment labeled with [α-32P]dCTP by random priming method using an oligonucleotide kit (Amersham Pharmacia Biotech) as per the manufacturer's instructions.

RT-PCR—RT-PCR was done as described previously (30) using SuperScript II reverse transcriptase (Gibco-BRL) and 10 μM random hexamers. PCR reactions were performed with 10 μm (0.5 μM) of the H11 sense and antisense primers (5'-ATCTGGCTGCGTGCTGATGTTTCCTCTCCTGTC-3'; 5'-CTCATGCAAAAGGCTACATCGACGACG-3') or β-actin sense and antisense primers (5'-GGTGGGCCCAGGAGAGGAGG-3' and 5'-CTCAGAGGAAGGAAAGGAAAG-3'). The PCR program included 1 min at 94°C for 1 cycle followed by 26 cycles of 94°C for 30 s and 68°C for 2 min and a final extension of 5 min at 68°C on a Perkin-Elmer thermocycler (model 9700). Relative abundance of H11 mRNA was estimated by first normalizing to the value of actin mRNA in each sample by densitometric scanning using a Bio-Rad GS-700 Imaging Densitometer.

Labeling of Cell Extracts and Immunoprecipitation—Cell extracts were labeled with [35S]methionine (100 μCi/ml; specific activity 1120 Ci/mmol; NEN Life Science Products) in methionine-free Dulbecco's modified Eagle's medium or with [32P]orthophosphate (500 μCi/ml; NEN Life Science Products) in phosphate-free Eagle's modified minimal essential medium with 10% dialyzed FCS. Whole cell extracts were prepared and used in immunoprecipitation exactly as described previously (25, 8, 10).

PK Assay and Phosphoamino Acid Analysis—DNA transfection was done in 293 cells by the calcium phosphate precipitation method using a glycerol boost (2, 4, 6, 10). Extracts of transfected and melanoma cells and glutathione-Sepharose beads coated with GST-H11 fusion protein (2 μg) were used in immunocomplex PK assays exactly as described previously (2, 4, 5–8, 10, 15).

Western Blot Assay—Proteins on SDS-polyacrylamide gel electro-
phoresis gels of cell extracts or immunoprecipitates were electrotransferred onto nitrocellulose membranes and immunoblotting was done with the respective antibodies followed by protein A-peroxidase (Sigma) for 1 h at room temperature each. Detection was with ECL reagents (Amersham Pharmacia Biotech), as described (8, 10).

**Anchorage-independent Growth**—293 cells were transfected with expression vectors, and clones were selected with G418 as described (4, 6, 10). For assay of anchorage-independent growth, cells were resuspended at a concentration of 5 × 10⁴ cells/ml in medium with 1.1% SeaPrep-agarose (FMC, Rockland, ME) and 0.1% Bacto-Peptone and layered on top of a solidified basal layer (5 ml) of Eagle’s modified minimal essential medium, 10% FCS containing 2.2% agarose and 0.2% Bacto-Peptone. After 12–22 days, large (>250 μm) colonies were scored. Results are expressed as cloning efficiency representing the number of colonies × 100/the number of seeded cells (10, 11).

**Effect of Antisense Oligonucleotides**—Cells (5 × 10⁴) were incubated in triplicate in 96-well plates in 0.1 ml of culture medium supplemented with 10% heat-inactivated FCS for 30 min at 65 °C to destroy nuclease activity and were cultured (24 h) in the presence or absence of antisense, sense, or randomly scrambled phosphorothioate oligonucleotides (ODNs), as described (28, 31–33). The ODN sequences were first tested against sequences in the GenBank database. Two distinct antisense ODNs complementary to sequences that encompass the H11 translation initiation site were used. Their sequence was 5′-GGTACCGACTGGCATGT-3′ (aODN-1) and 3′-GGTTGACCGCTGCCC-5′ (aODN-2) respectively. The scrambled ODN sequence was 5′-CACTGGAATCCTGGTTACTG-3′ (scODN). To measure DNA synthesis, cell cultures were labeled with [3H]thymidine ([3H]TdR, 2.5 μCi/well; NEN Life Science Products) for 4 h and harvested as described (34). Anti-sense inhibition was calculated relative to that of scODN-treated cells, whose inhibition did not exceed 15% of the proliferation rate shown by untreated cells. Growth inhibition was calculated as [3H]Tdr incorporation of treated cells/[3H]Tdr incorporation of control cells) × 100 (33). For cell cycle analysis, cells (5 × 10⁶) were fixed in cold 70% ethanol and stored at 4 °C before analysis on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a 15-milliwatt argon laser with 488-nm excitation. DNA fluorescence was examined in propidium iodide-stained cells using a 560/25-nm band pass filter. Doublet discrimination was used to exclude doublets from cell cycle analysis. Percentages of stained cells and the mean fluorescence calculations were determined from the histogram and dot plot displays using CellQuest software (Becton Dickinson) (10).

**RESULTS**

**Isolation of H11 cDNA**—We chose to screen the HeLa and melanoma cDNA libraries, because immunoblotting with antibodies to ICP10 PK peptides identified a cross-reacting 25-kDa protein in HeLa (Fig. 1, lane 1) and melanoma (Fig. 1, lane 3) cells. The HeLa library was screened by immunoblotting with the ICP10 PK antibodies. This was followed by screening of the melanoma library with the αICP10 PK-CPTP-labeled H11 cloned from HeLa cells. The isolated cDNA, designated H11, consisted of 1835 base pairs (GenBank accession No. AF133207) (Fig. 2).

**Computer-assisted Predictions for the H11 Protein**—H11 cDNA contains one open reading frame that consists of 588 nucleotides. This would encode a protein of 196 amino acids with a calculated molecular mass of 21.5 kDa (Fig. 2). Significant homology was not registered to sequences in the non-redundant SwisProt database using BLASTX 2.0.13. However, computer-assisted analysis of the predicted amino acid sequence in the H11 ORF revealed the presence of potential conserved PK catalytic motifs, when allowing for conservative substitutions. A 30% level of identity to ICP10 PK was observed over the entire H11 protein using GCG GAP. When the PK motifs were anchored, the same identity was seen over the H11 PK catalytic domain (115 amino acids) using the ALIGN program (Genestream, Montpellier, France). A catalytic motif I followed by Val (amino acids 62–69), which anchors the ATP molecule to its binding site but is not conserved in all PKs (35) is partially conserved in H11. A catalytic motif II (residues 112–114) includes the invariant Lys residue which is required for ATP binding and is involved in phosphotransfer reactions (12, 13). In all PKs studied to date (12, 13, 16–19), including ICP10 PK (6), all substitutions at this site have resulted in the loss of kinase activity. Another potential catalytic motif in H11 is motif III, which includes the invariant Olu residue (Glu200). Motif III is involved in ion binding and, together with catalytic motif II, it is part of the stable scaffolding of PK active sites (12, 13, 19). Catalytic motifs IV and V are poorly conserved in all PKs (12, 13), but H11 has a potential catalytic motif VI at residues 132–136 which may be a strong indicator of Ser-Thr specificity. However, Ser-Thr PKs that do not conserve Lys or Asp have been identified (18, 25). A triplet similar to the consensus Asp-Phe-Gly (catalytic motif VII) is present in H11 at amino acids 150–152. A third motif, which is partially conserved (12, 13), is motif VII at residues 133–138, that retains the invariant Asn. Motif VI is involved in protein-protein interactions (37), is located between

**Fig. 1.** Protein in HeLa and melanoma cells cross-reacts with ICP10 PK. Immunoblotting of extracts from HeLa (lanes 1 and 2), G361 melanoma (lanes 3 and 4), and 293 (lanes 5 and 6) cells with antibody to ICP10 PK (lanes 1, 3, and 5) or preimmune serum (lanes 2, 4, and 6). Numbers on the right represent molecular size markers.

**Fig. 2.** Nucleotide sequence and predicted amino acid sequence of H11. Numbers on the left represent nucleotides. Numbers on the right represent amino acids. Putative PK catalytic motifs are boxed and identified by roman numerals (I, II, III, VI, and VII) listed underneath.
the PK catalytic motifs, consistent with the organization in ICP10 PK (6, 14). Additional computer-predicted H11 motifs similar to those in the ICP10 PK protein (which is actually myristoylated (3)) are: (i) two elements consistent with the consensus pattern for myristoylation (Gly-Xaa-Xaa-Ser/Thr; residues 62 and 132) and (ii) a potential N-glycosylation site (amino acid 138). H11 also has phosphorylation sites for casein kinase II (residues 47 and 176) and PKC (residues 27, 63, 76, 104, 122, and 140). The H11 sequence SPLSSSR (residues 24–29) is potentially similar to a core element (SPESER) in the peptides employed to generate the ICP10 PK antibodies used in library screening, particularly if the cross-reacting epitope is not contiguous. However, the exact identity of this epitope remains unclear.

The H11 Protein Has Ser-Thr-specific PK Activity—Having shown that the predicted H11 protein sequence contains potential conserved PK catalytic motifs, we wanted to determine whether it has PK activity. Two series of experiments were done. First, glutathione-Sepharose beads coated with extracts of bacteria induced to express GST-H11 (Fig. 3A) and uninduced bacteria (Fig. 3B), or bacteria induced to express GST (Fig. 3C), GST-H11–10 (N-terminal; lanes 1 and 6), H11–181 (C-terminal, lanes 2, 5, and 8), or GST antibody (lanes 3, 4, and 7). Numbers on the right represent molecular size markers.

Phosphorylation was not seen in the presence of Mg2+-dependent. However, activity was optimized in the presence of Mn2+-dependent and is not improved by monovalent cations (5). To further compare H11 to ICP10 PK, we used a checkerboard matrix of different Mg2+ and Mn2+ ion concentrations in immunocomplex PK assays with H11–10 antibody. Phosphorylated H11 was first seen at 2 mM Mn2+ (Fig. 5A, lane 4, densitometric units = 20), and its levels were minimally increased at 5 mM Mn2+ (Fig. 5A, lane 5, densitometric units = 32) or 10 mM Mn2+ (Fig. 5A, lane 6, densitometric units = 46). Phosphorylation was not seen in the presence of Mg2+ ions alone (Fig. 5A, lanes 1–3), indicating that the PK activity is Mn2+-dependent. However, activity was optimized in the pres-
Fig. 5. **Ion requirements of H11 PK.** A, immunocomplex PK assay with H11–181 antibody and H11 transfected 293 cells was done at different concentrations of Mg$^{2+}$ (lanes 1–3), Mn$^{2+}$ (lanes 4–6), or both Mg$^{2+}$ and Mn$^{2+}$ (lanes 7–9) ions. The effect of NaCl (250 mM) was also determined (lane 9). B, immunoblotting of precipitates from A with H11–10 antibody.

Fig. 6. **H11 is expressed in pYXH11 transfected 293 cells.** Extracts of mock transfected 293 cells (lane 1), lines established from independently selected clones (C16, 5, 3, 14) of pYXH11 transfected cells (lanes 2–5) and one line established from a clone of pCI-transfected 293 cells (lane 6) were immunoprecipitated with H11–10 antibody and immunoblotted with the same antibody. Numbers on the right represent molecular size markers.

**Protein Similar to ICP10 PK in Human Melanoma**

**Has PK Activity—** Three series of experiments were done to determine whether the H11 protein is expressed in melanoma cells and has PK activity. First, cells were labeled with $[^{35}S]$methionine for 4 h and immunoprecipitated with antibodies H11–10, H11–181, or preimmune serum control. The 25-kDa protein was precipitated by both H11 antibodies from G361 (Fig. 9A, lanes 1 and 2), SK-MEL-2, and SK-MEL-31 (data not shown) cells. It was also precipitated from melanocytes, but its levels were significantly lower (Fig. 9A, lane 7). Proteins were not precipitated from 293 cells (Fig. 9A, lanes 4 and 5), and preimmune serum was negative (Fig. 9A, lanes 3 and 6). The experiments were then repeated with cells labeled with $[^{32}P]$orthophosphate for 4 h. A 25-kDa phosphoprotein was precipitated from G361 cells by both H11 antibodies (Fig. 9B, lanes 1 and 2), but not by preimmune serum (Fig. 9B, lane 3). The phosphoprotein in the G361 precipitates was recognized by antibodies H11–181 (Fig. 9C, lanes 1 and 2) and the cross-reactive ICP10 PK (Fig. 9D, lanes 1 and 2), but not hsp27 (Fig. 9D, lanes 1 and 2) in immunoblotting. The precipitates obtained with preimmune serum (Fig. 9, C–E, lane 3) or from 293 cells (Fig. 9, C–E, lanes 4–6) were negative. In the third series of experiments we used immunocomplex PK/immunoblotting assays to examine whether the H11 protein in melanoma cells has PK activity. The phosphorylated H11 protein was seen in G361 cells when the PK assay was done with H11–181 antibody (Fig. 10A, lane 1), but not with preimmune serum (Fig. 10A, lane 2). 293 cells were negative (Fig. 10A, lanes 3 and 4). The phosphoprotein was recognized by H11–10 antibody in amplification, because it was not seen in 293 cells (Fig. 7B, lane 7) studied in parallel, nor in RT-PCR assays with unrelated primers (data not shown). The data are also not an artifact due to the use of cultured cell lines, because similar results were obtained with RNA extracted from three primary melanoma tissues, as shown for two of these in Fig. 7C, lanes 2 and 3. The H11/actin ratios in these tissues were 4.7, 9.6, and 10, respectively. This compares to H11/actin ratios of 0.1–0.2 in the benign nevi (shown for one of these in Fig. 7C, lane 5) and 0.1 in the normal melanocytes (Fig. 7C, lane 4). By arbitrarily assigning a value of 1.0 to the degree of expression in normal melanocytes (28), the expression levels in the three melanoma tissues were 47, 96, and 100, respectively. Those in the melanoma cell lines were 92, 150, and 120 for G361, SK-MEL-31, and SK-MEL-2 cells, respectively. Significantly, increased H11 expression in melanoma cells is not due to gene amplification, because similar signals (densitometrically estimated to represent 1 copy) were seen by Southern blot hybridization in melanoma, normal melanocytes, and placenta genomic DNA (Fig. 8).

**The H11 Protein in Melanoma Cells Is Phosphorylated and Has PK Activity—** Three series of experiments were done to determine whether the H11 protein is expressed in melanoma cells and has PK activity. First, cells were labeled with $[^{35}S]$methionine for 4 h and immunoprecipitated with antibodies H11–10, H11–181, or preimmune serum control. The 25-kDa protein was precipitated by both H11 antibodies from G361 (Fig. 9A, lanes 1 and 2), SK-MEL-2, and SK-MEL-31 (data not shown) cells. It was also precipitated from melanocytes, but its levels were significantly lower (Fig. 9A, lane 7). Proteins were not precipitated from 293 cells (Fig. 9A, lanes 4 and 5), and preimmune serum was negative (Fig. 9A, lanes 3 and 6). The experiments were then repeated with cells labeled with $[^{32}P]$orthophosphate for 4 h. A 25-kDa phosphoprotein was precipitated from G361 cells by both H11 antibodies (Fig. 9B, lanes 1 and 2), but not by preimmune serum (Fig. 9B, lane 3). The phosphoprotein in the G361 precipitates was recognized by antibodies H11–181 (Fig. 9C, lanes 1 and 2) and the cross-reactive ICP10 PK (Fig. 9D, lanes 1 and 2), but not hsp27 (Fig. 9D, lanes 1 and 2) in immunoblotting. The precipitates obtained with preimmune serum (Fig. 9, C–E, lane 3) or from 293 cells (Fig. 9, C–E, lanes 4–6) were negative. In the third series of experiments we used immunocomplex PK/immunoblotting assays to examine whether the H11 protein in melanoma cells has PK activity. The phosphorylated H11 protein was seen in G361 cells when the PK assay was done with H11–181 antibody (Fig. 10A, lane 1), but not with preimmune serum (Fig. 10A, lane 2). 293 cells were negative (Fig. 10A, lanes 3 and 4). The phosphoprotein was recognized by H11–10 antibody in...
immunoblotting (Fig. 10B, lane 1), and proteins were not seen in the other assays (Fig. 10B, lanes 2–4). The data suggest that the H11 protein expressed in melanoma cells is phosphorylated immunoblotting (Fig. 10B, lane 1), and proteins were not seen in the other assays (Fig. 10B, lanes 2–4). The data suggest that the H11 protein expressed in melanoma cells is phosphorylated
and has PK activity. However, the phosphorylation pattern of the in vitro labeled protein remains unknown.

**Antisense H11 ODNs Inhibit Melanoma Cell Growth**—Having shown that H11 DNA has transforming potential and it is overexpressed in melanoma cells, we wanted to know whether its expression is related to melanoma cell proliferation. To test this possibility, we took advantage of previous findings from our (28, 31, 32) and many other (33, 38–41) laboratories that antisense ODNs specifically inhibit the translation of the targeted genes and thereby their function. G361, SK-MEL-31, and SK-MEL-2 cells were exposed (24 h) to different doses of antisense ODNs complementary to the H11 translation initiation site (aODN-1 and aODN-2) and analyzed for their ability to proliferate, as determined by [3H]TdR incorporation. sODN and scODN served as controls. Representative results are shown in Fig. 11 for treated G361 cells. Both antisense ODNs inhibited [3H]TdR incorporation. Inhibition was seen with as little as 10 μM of the ODNs and was maximal at 30 μM. [3H]TdR incorporation was minimally (10–15%) reduced by treatment with 30 μM of scrambled ODN (Fig. 11A). Growth inhibition (calculated relative to the scODN) was 98 and 86% for aODN-1 and aODN-2, respectively, whereas cell growth was not decreased in cells treated with the sODN (Fig. 11B). The difference between the proliferative rate of cells treated with the antisense and sense (or scrambled) ODNs was statistically significant (p < 0.001 by Student’s t test). In flow cytometry, G361 cells untreated or treated (24 h, 37 °C) with scODN or sODN were almost equally distributed between the G1 (43–46%) and S (37–39%) phases. By contrast, most (69–71%) of the cells treated with aODN-2 were in the G1 phase. Only 18–19% of the cells were in the S phase, suggesting that they are growth-inhibited and are arrested at/before the G1/S proliferative control checkpoints.

To examine the relationship between H11 expression and inhibition of cell growth, duplicate cell cultures were treated with aODN-2, scODN, or sODN (30 μM; 24 h) labeled with [35S]methionine (4 h; 37 °C), and cell extracts were immunoprecipitated with H11–10 antibody (N; lanes 1–4). Numbers on the right represent molecular size markers.

**DISCUSSION**

The salient feature of the data described in this report is the identification and cloning of a novel gene (H11) that has transforming potential and codes for a protein, which is similar to ICP10 PK and has Ser-Thr-specific PK activity. H11 expression appears to be required for the growth of human melanoma cells. The following comments seem pertinent with respect to these findings.

The HSV-2 R1 gene (ICP10) differs from its counterpart in eukaryotic and prokaryotic cells and in other viruses in that it possesses a unique one-third 5′-terminal domain that has transforming activity (9–11) and codes for a growth factor receptor Ser-Thr PK (2–9, 15). The ICP10 PK protein interacts with signaling proteins to activate the RAS/MEK/MAPK mitogenic pathway, both in transformed (10) and virus-infected cells.2 Because the R1 PKs function with only 8 catalytic motifs as compared with the 12 motifs conserved in most other PKs (12, 13), some voiced concern that the kinase activities are not...
Intrinsic (42). However, the ICP10 PK activity was ablated by mutation of the invariant residues within the ATP- or ion-binding sites (catalytic motifs II and III, respectively) (6), thereby fulfilling the same criterion for intrinsic activity as all other PKs studied to date (12, 13, 16–19). Based on insertion mutagenesis studies, similar conclusions were also reached for ICP6 PK (20). Phylogenetic analyses, based primarily on their limited number of conserved catalytic motifs and the presence of TM domains, indicated that the R1 PK proteins are members of a unique subfamily of Ser-Thr growth factor receptor PKs (14). However, ICP6 PK is only 38% identical to ICP10 PK (1) and it has different biological properties, including its failure to associate with the plasma membrane (22), activate the mitogenic RAS/MEK/MAPK pathway (23), and cause transformation (9). Another PK known to function with a similarly limited number of conserved catalytic motifs is FAST, which is only 12% identical to ICP10 PK (25). Because sequences homologous to ICP10 PK were previously cloned from human cells (24), we proposed that the unique PK domains in HSV R1 proteins arose from cellular gene(s) (9). However, these putative homologues were not previously cloned and their function is unknown. The studies described in this report were designed to address these questions.

We screened HeLa and melanoma cDNA libraries because these cells express a protein that cross-reacts with ICP10 PK in immunoblotting. H11 cDNA was cloned from both libraries. It codes for a protein that is 30% identical to ICP10 PK over the PK catalytic domain. This identity level is higher than that between FAST and ICP10 PK, but lower than that between the HSV-1 and HSV-2 R1 PK proteins (1). Potential conserved PK catalytic motifs are organized in a pattern similar to that in ICP10 PK and flanking an Src homology region 3-binding site (6, 14). Like ICP10 PK, which is myristoylated (3), H11 has consensus sites for myristoylation. However, the biologic and evolutionary significance of this level of identity is still unclear, particularly as it relates to the potential cellular origin of the R1 PKs.

The basic shared functions common to all PKs are ion-dependent ATP binding and catalysis. The two conserved charged residues that respectively constitute catalytic motifs II (Lys) and III (Glu) presumably form an ion pair that, in the ternary complex, provides a docking site for MgATP (12, 13). In kinases that are expressed at low levels, replacement of the Lys residue in catalytic motif II is sufficient to show a null phenotype (13, 17). Our findings for H11 are consistent with these interpretations. Thus, a 25-kDa phosphoprotein was seen in extracts of 293 cells transfected with the H11 expression vector by immunocomplex PK/immunoblotting assays with antibodies specific for residues located at the N terminus or C terminus of the H11 protein. Like the ICP10 PK activity, the PK activity of H11 was Ser-Thr-specific, Mn2+-dependent, and it did not require monovalent cations. It was optimized in the presence of Mg2+ and Mn2+ ions. We do not exclude the possibility that H11 is a substrate for other cellular kinases. However, we conclude that it has intrinsic PK activity, because the activity was ablated (null phenotype) by mutation of Lys113 (catalytic motif II), which is the criterion used for all other PKs studied to date (12, 13, 16–19). The comparison between H11 and Lys113 in PK assays is justified, because the levels of the proteins in the assay were virtually identical, as evidenced by immunoblotting of the immunoprecipitates. Background activity was not observed in immunoprecipitates from mock-transfected cells or from cells transfected with H11 and precipitated with preimmune serum. If present, the contaminating activity in these assays is less than 0.1% of the specific activity of wild type H11, because PK negative mutants are sometimes detectable within this range (43), whereas the Lys113 mutant was not phosphorylated. The Lys113 mutant retains all known target sites for other PKs, and the H11 PK activity was not altered by mutation of Lys115 that is not located within a conserved catalytic motif (data not shown), suggesting that loss of PK activity by Lys113 mutation is not because of a nonspecific conformational alteration resulting from the mutation of a Lys residue. The conclusion that H11 has PK activity is also supported by the finding of PK activity in bacteria induced to express GST-H11, but not uninduced bacteria or bacteria induced to express GST. Background phosphorylation due to nonspecific protein binding to the glutathione-Sepharose beads was also not seen. However, H11 may be part of a hetero-oligomeric complex, in which it contributes much of the PK catalytic domain while another protein contributes other required sequences (35). Ongoing studies of the H11 PK activity include identification of H11 PK substrates and phosphorylation sites.

H11 resembles ICP10 PK in that 293 cells stably transfected with the H11 expression vector pYXH11 acquire anchorage-independent growth, a virtually absolute correlate of neoplastic potential in vivo. We conclude that H11 expression is required for anchorage-independent growth, because the latter was seen in at least four independently established lines that were positive for H11 expression but not in H11 negative cells (untransfected or transfected with the empty vector). In addition, cloning was determined at an early passage, before cells with a H11-unrelated growth advantage could be selected. The cloning efficiency of cells transformed with H11 was comparable or greater than that of such oncogenes as v-fo to (0.1–0.7% (44), int-2 (0.35%), c-Ha-ras (0.5%), c-erb B2 (1.0% (45), and v-sis (2.9–4.3% (46) in human cells, suggesting that H11 has a relatively strong transforming activity, as previously reported for ICP10 PK (9–11). When colonies similar in size to those scored for c-erb B2 and v-sis (60–80 μm) were counted, the cloning efficiency of H11-transformed cells was 10–20-fold higher than that reported for these oncogenes. However, the mechanism of H11-mediated transformation and the role of the PK activity are presently unknown. The presence of consensus myristoylation sites in the H11 protein suggests that it might be associated with the plasma membrane (47) and thereby function in the transduction of growth signals. Ongoing studies...
using cell lines established with the Lys113PK negative mutant are designed to examine the role of PK activity in H11-mediated transformation.

H11 RNA was seen in melanoma cell lines and in primary melanoma tissues, indicating that expression was not an artifact because of prolonged in vitro culture. RNA was also seen in normal melanocytes and benign nevi, but its levels were significantly lower (47–100-fold) than those in melanoma cells. Expression of the H11 protein was determined by immunoprecipitation/immunoblotting with antibodies that recognize epitopes located at the H11 N terminus or C terminus. A 25-kDa protein consistent with that seen in pYXH11-transformed 293 cells was recognized by both antibodies in melanoma cells labeled with [35S]methionine. The protein was also seen in normal melanocytes, but at significantly lower levels than those in melanoma cells. It was also precipitated from [32P]orthophosphate-labeled melanoma cells and, as predicted, it was recognized by ICP10 PK antibodies, but not by preimmune serum or antibody to the hsp27 protein. We conclude that the H11 protein in melanoma cells has PK activity because autophosphorylation was seen in immunocomplex PK/immunoblotting assays with the H11 antibodies. However, the in vivo versus in vitro phosphorylation patterns of the H11 protein in melanoma cells are still unknown.

Ongoing immunohistochemistry studies support the interpretation that H11 is overexpressed in melanoma cells and also in skin tissues. However, overexpression does not seem to result from gene amplification, at least as determined by Southern blot hybridization. This is consistent with previous findings for other overexpressed genes (viz. N-myc) for which the levels of expression are unrelated to gene amplification (27). Gene alterations that cause overexpression include deletion/mutation (48), altered regulation resulting from improved access to translational initiation sites or increased translational efficiency (49), increased RNA stability (50), or altered transcriptional regulation (51). The mechanism responsible for H11 overexpression in melanoma cells and the tissue distribution of H11 expression remain to be determined.

An important as yet unresolved consideration is the role of H11 in melanoma. To begin to address this question, we used the antisense approach because numerous studies have shown antisense effects on virus- or cancer-related genes, and antisense therapeutics have begun to move into the clinical arena (28, 31–33, 38–41). We found that antisense ODNs that inhibit H11 translation also inhibited melanoma cell growth. Inhibition was specific, as evidenced by the findings that: (i) the effects of the antisense ODNs were dose-dependent, (ii) H11 expression and cell growth were not decreased in cells treated with sense or scrambled ODNs, (iii) antisense ODNs did not inhibit the translation of other genes (viz. actin), and (iv) antisense ODNs did not inhibit the growth of 293 cells that do not express H11.

Tumor development is a stepwise process involving the activation of oncoproteins and/or inactivation of tumor suppressor genes. Significant progress has been made in the identification of genes involved in melanoma progression/metastasis most of which function as tumor suppressors (52). With the possible exception of basic fibroblast growth factor and its cognate receptors (53), identification of oncoproteins involved in melanocyte transformation has been relatively slow. Because: (i) H11 has transforming potential, (ii) it is overexpressed in melanoma cells, and (iii) the majority of cells treated with H11 antisense ODNs are arrested in G1, it is tempting to propose that H11 is involved in melanocyte transformation, possibly by functioning at this stage of the cell cycle. This could involve activation of growth-associated transcription factors, such as E2F, and/or cyclin-dependent kinases (viz. cdk4) (54, 55). However, such conclusions are premature and must await the results of ongoing studies with H11 retrovirus vectors designed to examine whether H11 can also transform melanocytes. Elucidation of the mechanisms of H11-mediated transformation and H11 overexpression in melanoma cells, the identification of sequence and/or regulatory alterations in H11 cloned from melanoma as compared with normal melanocytes and the studies of appropriate transgenic mice, are ultimately needed to better define the role of H11 in melanoma development.

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REFERENCES

3 Gyotoku, T., Kokuba, H., Aurelian, L., Burnett, J. W., manuscript in preparation.
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41. Smith, J. B., and Wickstrom, E. (2000) Methods Enzymol. 314, 537–580
42. Langelier, Y., Champoux, L., Hamel, M., Guilhault, C., Lamarche, N., Gaudreau, P., and Massie, B. (1998) J. Biol. Chem. 273, 1435–1443
43. LaPorte, D. C., Thorsness, P. E., and Koshland, D. E., Jr. (1985) J. Biol. Chem. 260, 10563–10568
44. Lee, M.-S., Yang, J.-H., Salehi, Z., Arinstein, P., Chen, L.-S., Jay, G., and Rhim, J. S. (1993) Oncogene 8, 387–393
45. Basolo, F., Fiore, L., Ciardiello, F., Calvo, S., Fontanini, G., Conaldi, P. G., and Toniolo, A. (1994) Int. J. Cancer 56, 736–742
46. Yang, D., Kohler, S. K., Maher, V. M., and McCormick, J. J. (1994) Carcinogenesis 15, 2167–2175
47. Schultz, A. M., Henderson, L. E., Oroszlan, S., Gerber, E. A., and Hanafusa, H. (1985) Science 227, 427–429
48. Hosokawa, Y., Suzuki, R., Joh, T., Maeda, Y., Nakamura, S., Kodera, Y., Arnold, A., and Seto, M. (1998) Int. J. Cancer 76, 791–796
49. Child, S. J., Miller, M. R., and Gehalle, A. P. (1999) J. Biol. Chem. 274, 24335–24341
50. Bauer, S. R., Piechaczyk, M., Nordan, R. P., Owens, J. D., Nepveu, A., Marcu, K. B., and Mushinski, J. F. (1989) Oncogene 4, 615–623
51. Zachos, G., and Spandidos, D. A. (1998) Oncogene 16, 3013–3017
52. Keller-Melchior, R., Schmidt, R., and Piepkorn, M. (1998) J. Invest. Dermatol. 110, 932–938
53. Yayon, A., Ma, Y.-S., Safran, M., Klagsburn, M., and Halaban, R. (1997) Oncogene 14, 2999–3009
54. Bartkova, J., Lukas, J., Goldberg, P., Alsner, J., Kirkin, A. F., Zeuthen, J., and Bartek, J. (1996) Cancer Res. 56, 5475–5483
55. Halaban, R., Cheng, E., Zhang, Y., Mandigo, C. E., and Migliarese, M. R. (1998) Oncogene 16, 2489–2501