Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7594–7599, July 1996
Biochemistry

DP-2, a heterodimeric partner of E2F: Identification and characterization of DP-2 proteins expressed *in vivo*

(transcription/retinoblastoma/cell growth regulation)

KATHLEEN T. ROGERS*, PETER D. R. HIGGINS*, MARIA M. MILLA, RUTH S. PHILLIPS, AND JONATHAN M. HOROWITZ†

Departments of Molecular Cancer Biology and Microbiology, Duke University Medical Center, Durham, NC 27710

Communicated by W. K. Joklik, Duke University Medical Center, Durham, NC, May 6, 1996 (received for review September 22, 1995)

ABSTRACT **E2F is a heterodimeric transcription factor that regulates the expression of genes at the G1/S boundary and is composed of two related but distinct families of proteins, E2F and DP. E2F/DP heterodimers form complexes with the retinoblastoma (Rb) protein, the Rb-related proteins p107 and p130, and cyclins/cdks in a cell cycle-dependent fashion *in vivo*. E2F is encoded by at least five closely related genes, E2F-1 through -5. Here we report studies of DP-2, the second member of the DP family of genes. Our results indicate that (*i*) DP-2 encodes at least five distinct mRNAs, (*ii*) a site of alternative splicing occurs within the 5′ untranslated region of DP-2 mRNA, (*iii*) at least three DP-2-related proteins (of 55, 48, and 43 kDa) are expressed *in vivo*, (*iv*) each of these proteins is phosphorylated, and (*v*) one DP-2 protein (43 kDa) carries a truncated amino terminus. Our data also strongly suggest that the 55-kDa DP-2-related protein is a novel DP-2 isoform that results from alternative splicing. Thus, we conclude that DP-2 encodes a set of structurally, and perhaps functionally, distinct proteins *in vivo*.**

E2F is a cell-cycle-regulated, heterodimeric transcription factor that coordinates the expression of a variety of viral and cellular genes (for reviews, see refs. 1–4). First identified as an adenovirus E1A-induced DNA-binding protein that was essential for the transcription of the viral E2 gene (5), E2F has subsequently been shown to play an important role in the expression of a cadre of cellular genes at the G1/S boundary. E2F associates with a number of cellular proteins *in vivo*, forming protein complexes whose composition fluctuates in concert with cell-cycle progression. Proteins associated with E2F in such complexes include the product of the retinoblastoma (Rb)-susceptibility gene (pRb) and p107 and p130, two Rb-related proteins (6–13). pRb and Rb-related proteins inhibit E2F-mediated transcription by binding to and sequestering the E2F trans-activation domain (14–18). In addition to Rb family members, the cell-cycle-regulated kinase cdk2, in association with cyclins A or E, has been found in complexes with E2F (9–11, 19–22). Phosphorylation of E2F complexes by cyclins/cdks inhibits DNA-binding activity and E2F-dependent transcription (23, 24).

Transcription factor E2F is composed of two distinct proteins, termed E2F and DP (25–27). The E2F component of this protein complex is encoded by a family of genes consisting of at least five members (E2F-1 through -5; refs. 1–4). E2F family members are highly conserved proteins; however, functional differences between family members have been noted. For example, E2F-1, -2, and -3 form complexes with pRb, whereas E2F-4 and -5 appear to preferentially associate with p107 and p130 (28–32). Additionally, E2F-1, -2, and -3 encode a cyclin/cdk binding site that is not apparent in E2F-4 or E2F-5 (28, 30). It is not yet clear whether E2F complexes differ in their promoter specificity or in their regulation by upstream signals (33).

Until recently, DP-1 was the only identified heterodimeric partner of E2F. DP-1 is a ubiquitously expressed 410-aa phosphoprotein that is structurally related to E2F yet devoid of an E2F-like pRb-binding domain (26, 27). DP-1 has been noted in complexes with E2F-1, -2, and -3 *in vivo*, and such complexes activate transcription in an E2F-site dependent fashion (25, 32, 34, 35). DP-1 is a substrate of cyclin A/cdk2 and is phosphorylated in a cell-cycle-regulated manner *in vivo* (23, 24). Finally, DP-1 has recently been shown to be bound by mdm2, a p53-associated protein, *in vivo* and *in vitro*, and the formation of such complexes appears to increase E2F-dependent transcription (36).

Given that E2F is encoded by a family of related genes, we hypothesized that additional DP-related molecules might also exist. To identify such proteins, we used a functional assay, a yeast "two-hybrid" screen, to search for novel dimerization partners of E2F. This screen resulted in the cloning of DP-2, the second member of the DP family of proteins. While this work was in progress, two identical cDNAs were cloned and their characterization and functional analysis have recently been reported (32, 37). Here, we extend these studies by identifying and characterizing DP-2 proteins synthesized *in vivo*. These studies show that at least three DP-2-related proteins (of 55, 48, and 43 kDa) are synthesized *in vivo* and that each is a phosphoprotein. Interestingly, differential phosphorylation cannot account for the distinct DP-2-related proteins observed. Instead, our results indicate that DP-2 encodes structurally distinct proteins, one of which, 43 kDa, carries a truncated amino terminus.

MATERIALS AND METHODS

Yeast Two-Hybrid Cloning of DP-2. The "bait" for a two-hybrid screen for E2F-dimerization partners was prepared by cloning a fragment of the E2F-1 dimerization domain (encoding amino acids 122–284) into plasmid pAS2 (a yeast vector generously provided by Stephen J. Elledge, Baylor College of Medicine, Houston, TX), creating plasmid pAS2-ESB, such that E2F-coding sequences are in frame with the GAL4 DNA-binding domain. Yeast cells were analyzed for GAL4-dependent transcriptional activity following transformation with pAS2-ESB and a unidirectional HeLa cell cDNA library cloned into plasmid pGADGH (CLONTECH). A single "pos-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Rb, retinoblastoma; CMV, cytomegalovirus.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U35117).

*The first two authors contributed equally to the work described in this report.

†To whom reprint requests should be addressed at: Departments of Molecular Cancer Biology and Microbiology, Duke University Medical Center, Box 3686, Durham, NC 27710. e-mail: jmh@galactose.mc.duke.edu.

itive" clone, designated CHE2, was identified and sequenced in its entirety in pGADGH by double-stranded dideoxy-sequencing (38). A BLAST search of the GenBank nucleotide data base revealed CHE2 to be related to DP-1. Comparison of the CHE2 sequence with the unpublished sequence of DP-2 (generously provided by Chin-Lee Wu and Ed Harlow, Massachusetts General Hospital Cancer Center, Charlestown, MA) showed the two cDNAs to be encoded by the same gene.

The amino terminus of DP-2 was isolated by a hybridization screen of a human keratinocyte cDNA library (kindly provided by Xiao-Fan Wang, Duke University Medical Center, Durham, NC) using CHE2 as probe. A single cDNA clone, LDP-2, carrying the DP-2 amino terminus was obtained and completely sequenced. A full-length DP-2 cDNA carrying coding sequences from LDP-2 and CHE2 was then reconstructed from the partial cDNAs. Oligonucleotide-mediated mutagenesis of an internal DP-2 methionine residue was performed using a commercially available kit (Mutagene; Bio-Rad).

Northern and Southern blotting. A Northern blot prepared with poly(A)$^+$ RNAs from a variety of human tissues was obtained (CLONTECH), examined with DP-1- and DP-2-specific radiolabeled probes (DP-1, nucleotides 1017–1334 relative to the start of translation; DP-2, nucleotides 882-1176 relative to the start of translation), and washed at high stringency (50°C, 0.1× standard saline citrate, 0.1% SDS) according to instructions provided by the manufacturer. Southern blots were similarly examined following the cleavage of normal human DNA with restriction enzymes and transfer to nitrocellulose paper.

Preparation of Antisera and Immunoprecipitations. To prepare polyclonal DP-2 antiserum, PCR was employed to amplify a segment of the CHE2 cDNA encoding amino acids 295–386. This cDNA fragment was cloned into pQE-30 (Qiagen, Chatsworth, CA) such that six histidine residues were linked in frame at the amino terminus of DP-2. A similar construction was prepared using PCR and a human DP-1 cDNA (kindly provided by Joseph R. Nevins, Duke University Medical Center) such that six histidine residues were linked to the amino terminus of DP-1 amino acids 340–410. Resulting fusion proteins were prepared in M15pREP4 bacteria following induction with isopropyl β-D-thiogalactoside and purified by nickel-affinity chromatography before immunization. BALB/c mice were immunized with purified DP fusion proteins, and sera were collected following two successive boosts with immunogen. ML-1 human myeloid leukemia cells were obtained from Stephen H. Friend (Massachusetts General Hospital Cancer Center). Additional cell lines were obtained from the American Type Culture Collection. Mammalian cell extracts and *in vitro*-translated proteins were immunoprecipitated as described (39). Where indicated, antisera were incubated for 15 min on ice with 5–10 μg of purified immunogens before incubation with radiolabeled cell extracts. *In vitro* dephosphorylation reactions were performed by incubation of antigen-antibody complexes in dephosphorylation buffer [40 mM Pipes, pH 6.0/1 mM DTT/1 mM phenylmethylsulfonyl fluoride/5 μg/ml each of aprotinin, leupeptin, and antipain/0.5 μg/ml pepstatin] with 2 μg of potato acid phosphatase (Grade I; Boehringer Mannheim) at 30°C for various lengths of time.

Construction of Expression Constructs and Transfection of COS Cells. A cytomegalovirus (CMV)-expression vector carrying a full-length DP-2 cDNA (CMV-DP-2) was prepared by subcloning a 1.3-kbp DP-2 cDNA fragment into pCMV-6 (40). A DP-1 expression vector (CMV-DP-1; ref. 27) was provided by Joseph R. Nevins. Transient transfections of COS-7 cells were performed as described (40).

RESULTS

Isolation of a cDNA Encoding DP-2 by Use of a Yeast Two-Hybrid Screen. We employed a yeast two-hybrid strategy to identify novel dimerization partners of E2F within a HeLa cell cDNA library. The sequence of one resulting positive clone, designated CHE2, revealed it to be closely related to DP-1, a previously characterized E2F dimerization partner (26, 27). Subsequently, we learned that low-stringency hybridization screens of cDNA libraries by Wu and coworkers (32) and Zhang and Chellappan (37) had resulted in the isolation of similar DP-1-like cDNAs that were designated DP-2. Comparison of all three sequences confirmed that, with the exception of a truncation of the CHE2 5′ end, all three clones carried cDNAs derived from the same gene. To construct a full-length DP-2 cDNA, we used CHE2 as a hybridization probe to isolate the DP-2 amino terminus from a human keratinocyte cDNA library. This screen resulted in the isolation of a single clone (designated LDP-2) encoding the DP-2 amino terminus. Surprisingly, we noted that the sequence of the 5′ untranslated region of LDP-2 is identical to only a portion of the analogous region of DP-2 previously reported (37). As shown in Fig. 1*A*, the DP-2 coding region and 101 nucleotides immediately upstream are identical, whereas the remaining portion of LDP-2 shows little homology to this previously reported DP-2 sequence. This observation suggested that DP-2 may be an alternatively spliced gene, and this supposition is supported by Northern blot analysis of a variety of tissue-specific poly(A)$^+$ RNAs (Fig. 1*B*). As previously reported (32, 37), a DP-2-specific probe spanning nucleotides 882-1176 (relative to the start of translation) detects up to five distinct messages (ranging from 9.5 to 1.4 kb) in various human tissues. The abundance of these DP-2 mRNAs varied amongst the tissues analyzed. Hybridization of this same DP-2 probe to a Southern blot of human genomic DNA cleaved with several restriction enzymes revealed DP-2 to likely be a single-copy gene (data not shown). From these data, we conclude that DP-2 is an alternatively spliced gene and that one site of splicing occurs within the DP-2 5′ untranslated region.

A Polyclonal Anti-DP-2 Antiserum Detects Several DP-2-Related Proteins *in Vivo*. To study DP-2 protein expression *in vivo*, we prepared a polyclonal mouse anti-DP-2 antiserum via immunization with protein prepared from a region of significant amino acid divergence between DP-1 and DP-2 (amino acids 295–386). Anti-DP-1 antiserum was also prepared using an analogous portion of DP-1 (amino acids 340–410). As a first step in the characterization of DP antisera, we established that each specifically precipitated DP-1 and DP-2 protein prepared in reticulocyte extracts programmed with appropriate cRNAs (Fig. 2*B*, lane 4; data not shown). To confirm and extend these observations, the vector CMV-DP-2 was constructed and used to overexpress DP-2 protein in transfected COS cells. In parallel, COS cells were transfected with a similar CMV-expression construct prepared with a full-length DP-1 cDNA (27). Transfected cells were radiolabeled with [^{35}S]methionine, nondenatured extracts were prepared, and immunoprecipitates were collected with appropriate antisera. In agreement with the predicted molecular weight of human DP-2 (43 kDa), anti-DP-2 antisera precipitates two major proteins, of 48 and 43 kDa, and two minor proteins, of 36 and 30 kDa, in extracts prepared from cells transfected with CMV-DP-2 (Fig. 2*A*, lane 1). As shown in Fig. 2*B* (lane 4), DP-2 proteins of identical size were also detected in immunoprecipitates of reticulocyte extracts programmed with DP-2 cRNA. In contrast, as shown in Fig. 2*A*, the DP-2-related proteins identified in extracts from transfected cells were not detected in (*i*) extracts prepared from mock-transfected COS cells (lane 9), (*ii*) immunoprecipitates that had been pre-incubated with homologous immunogen (lane 3), and (*iii*) anti-DP-1 immunoprecipitates (lane 6). Additionally, the recovery of DP-2-related proteins

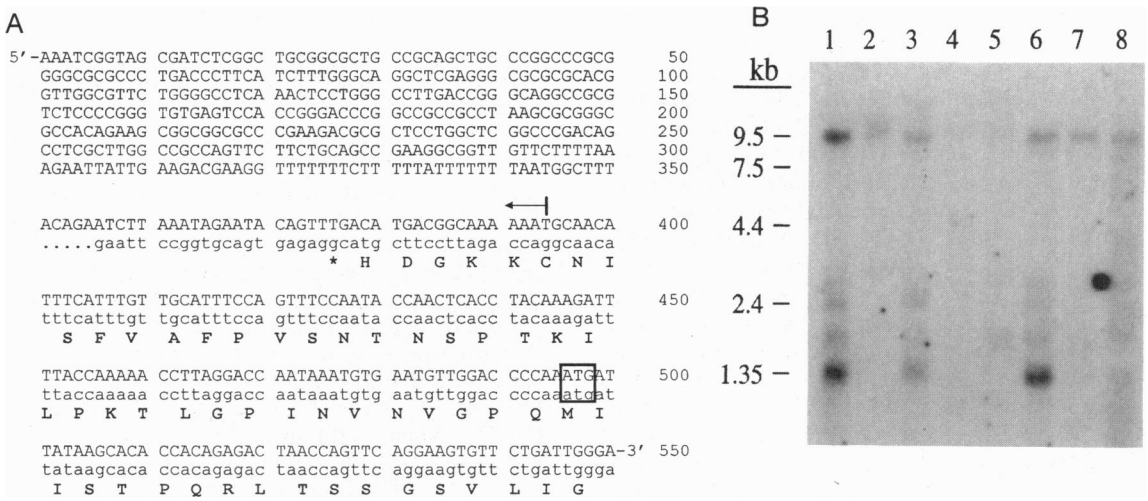

FIG. 1. Sequence comparison of DP-2 amino termini and tissue-specific DP-2 expression. (*A*) The nucleotide sequence of LDP-2 (capitalized nucleotides) is shown in comparison with the analogous portion of a previously published DP-2 cDNA (37). Divergent nucleotides are indicated by an arrow. Amino acids are indicated by bold letters, an upstream nonsense codon is denoted by an asterisk, and the initiating methionine is boxed. (*B*) A Northern blot of poly(A)$^+$ RNAs prepared from various human tissues was hybridized with a radiolabeled DP-2-specific probe. RNAs are as follows: lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. Molecular weight markers are indicated on the left. Autoradiography was for 8 days at −80°C.

was not inhibited by pre-incubation of antisera with heterologous immunogen (Fig. 2*A*, lane 2). In agreement with a previous report (25), anti-DP-1 immunoprecipitates prepared from cells transfected with CMV-DP-1 gave rise to proteins of 60 and 52 kDa (Fig. 2*A*, lane 4). DP-1 proteins were not immunoprecipitated by DP-2 antisera (Fig. 2*A*, lane 5). Taken together, these data indicate that the polyclonal anti-DP-1 and anti-DP-2 antisera we have prepared specifically recognize their cognate proteins. Our experiments also indicate that translation of CHE2-derived cRNA leads to the synthesis of several DP-2-related proteins *in vivo* and *in vitro*.

To study endogenous DP-2 protein expression, we performed a series of immunoprecipitations of extracts prepared from metabolically labeled cells. Immunoprecipitates prepared from ML-1 human myeloid leukemia cells contained two proteins, of 48 and 43 kDa (Fig. 2*B*, lane 2), that are identical in size with DP-2-related proteins detected in transfected COS cells (Fig. 2*A*, lanes 1, 2, and 7) and reticulocyte lysates programmed with DP-2 cRNA (Fig. 2*B*, lane 4). In addition to these proteins, two novel proteins, of 55 and 110 kDa, were also precipitated by anti-DP-2 antisera (Fig. 2*B*, lane 2) but not control antisera. Precipitation of these four

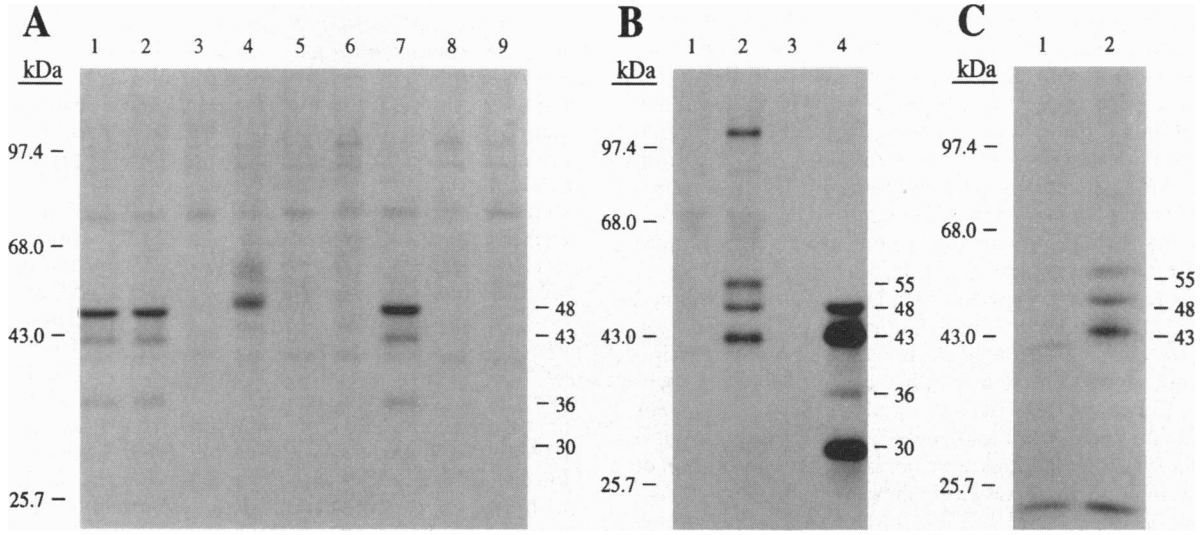

FIG. 2. Identification of DP-2-related proteins in COS cells transfected with CMV-DP-2, reticulocyte extracts programmed with DP-2 cRNA, and ML-1 cells. (*A*) COS cells transfected with CMV-DP-2 (lanes 1, 2, 3, 6, and 7) or CMV-DP-1 (lanes 4 and 5) or mock-transfected cells (lanes 8 and 9) were incubated with [^{35}S]methionine, and immunoprecipitates were prepared with anti-DP-2 (lanes 1, 2, 3, 5, 7, and 9) or anti-DP-1 (lanes 4, 6, and 8) polyclonal antisera. Immunoprecipitation of DP-2-related proteins was blocked with DP-2 immunogen (lane 3) but not DP-1 immunogen (lane 2). Immunoprecipitates were resolved on a 10% polyacrylamide gel. Apparent molecular weights of DP-2-related proteins are indicated on the right, and molecular weight markers are indicated on the left. Autoradiography was for 14 hr at −80°C. (*B*) [^{35}S]methionine-labeled ML-1 cell extracts (lanes 1 and 2) and reticulocyte extracts programmed with DP-2 cRNA (lanes 3 and 4) were immunoprecipitated with normal mouse serum (lanes 1 and 3) or anti-DP-2 antiserum (lanes 2 and 4). Immunoprecipitates were resolved on a 10% polyacrylamide gel. Apparent molecular weights of immunoprecipitated proteins are indicated on the right, and molecular weight markers are indicated on the left. Autoradiography was for 14 hr at −80°C. (*C*) [^{35}S]methionine-labeled ML-1 cell extracts prepared in RIPA buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) were immunoprecipitated with normal mouse serum (lane 1) or anti-DP-2 antiserum (lane 2). Apparent molecular weights of immunoprecipitated proteins are indicated on the right.

proteins from radiolabeled ML-1 extracts is specific since precipitation was blocked by pre-incubation of DP-2 antiserum with DP-2, but not DP-1, immunogen (see Fig. 4*A*, lanes 2 and 3). These four proteins were also noted in anti-DP-2 immunoprecipitates prepared from other mammalian cell lines; however, each precipitated protein was noted to be significantly less abundant (data not shown). This latter observation is consistent with previously published evidence that DP-2 mRNA is highly expressed in ML-1 cells (32).

To determine whether proteins harvested from ML-1 extracts were directly precipitated by anti-DP-2 antiserum or were coprecipitated by virtue of their physical interaction with DP-2, extracts were prepared under conditions that, as we have previously shown, dissociate DP-2 from its dimerization partner, E2F. As shown in Fig. 2*C* (lane 2), the 55-, 48-, and 43-kDa proteins precipitated with DP-2 antiserum were detected under these conditions, whereas the 110-kDa protein was not. Thus, we conclude that the 55-, 48-, and 43-kDa proteins expressed in ML-1 cells are likely to be directly precipitated by anti-DP-2 antiserum. Although not yet identified, Western analyses of DP-2 immunoprecipitates indicate that the 110-kDa protein is not encoded by Rb (data not shown).

The 43-kDa Form of DP-2 Carries a Truncated Amino Terminus. Although the 48- and 43-kDa proteins detected in ML-1 cells comigrate with DP-2 proteins expressed in transfected COS cells and in reticulocyte lysates programmed with DP-2 cRNA, we wished to determine whether these proteins were structurally identical. We also wished to establish whether the 55-kDa protein expressed in ML-1 cells is structurally as well as antigenically related to the 48- and 43-kDa DP-2 proteins. Consequently, DP-2 immunoprecipitates prepared from ML-1 cells and *in vitro* translations were subjected to Cleveland analysis. As shown in Fig. 3, analysis of partial peptides resulting from V8 protease digestion indicates that (*i*) the 48- and 43-kDa DP-2 proteins synthesized *in vitro* are virtually indistinguishable from their *in vivo* counterparts (compare lanes 8 and 9 of Fig. 3*A* with lanes 1 and 2 of Fig. 3*B*) and (*ii*) only one peptide (indicated by an arrow in Fig. 3*A*) distinguishes the 55-kDa protein from the 48- and 43-kDa DP-2 proteins precipitated from ML-1 cells. Since the 48- and 43-kDa DP-2 isoforms synthesized *in vivo* appear to be structurally identical to their *in vitro* counterparts, it became of interest to determine whether the 48- and 43-kDa proteins differed from each other in their primary sequence. Upon inspection of the DP-2 cDNA sequence, we noted an internal methionine residue 27 amino acids downstream of the initial methionine that is appropriately positioned to account for the synthesis of the 43-kDa DP-2 isoform. To determine whether translational initiation at this internal position might generate the 43-kDa DP-2 protein, we used oligonucleotide-mediated mutagenesis to change this residue to a leucine. Indeed, as shown in Fig. 3*C*, *in vitro* translation of cRNA prepared from this mutated DP-2 construct leads to the elimination of the 43-kDa DP-2 protein (lane 2). Taken together with evidence from our V8 mapping experiments, we conclude that the 43-kDa DP-2 protein synthesized in ML-1 cells lacks the amino-terminal 26 amino acids carried by the 48-kDa DP-2 isoform. We also conclude that the 55-kDa protein precipitated with DP-2 antiserum is closely related to the 48- and 43-kDa DP-2 isoforms.

DP-2-Related Proteins Are Phosphoproteins; However, Differential Phosphorylation Is Insufficient to Account for the 55-kDa DP-2 Isoform Detected *in Vivo*. Since DP-1 is a phosphoprotein, we reasoned that DP-2 was likely to be similarly modified. In addition, we speculated that differential phosphorylation might account for the synthesis of the 55-kDa DP-2-related protein detected in ML-1 immunoprecipitates. To determine if DP-2-related proteins are phosphoproteins, we radiolabeled ML-1 cells with ^{32}P-orthophosphate and prepared DP-2 immunoprecipitates in parallel with similar extracts prepared from [^{35}S]methionine-labeled cells. As shown in Fig. 4*A*, the 110-, 55-, 48-, and 43-kDa proteins precipitated by DP-2 antiserum were each noted to be phosphoproteins, with the 55-kDa protein being the most significantly labeled species. Precipitation of these four phosphoproteins, as well as two previously undetected proteins of 66 and 62 kDa, was blocked by inclusion of homologous, but not heterologous, immunogen (Fig. 4*A*, lanes 5 and 6). The identity of these latter proteins remains to be determined; however, given their apparent molecular weights, they are likely to be E2F-family members.

Given that the 55-kDa DP-2-related protein is phosphorylated *in vivo*, we wished to establish whether its distinct apparent molecular weight could be ascribed to differential

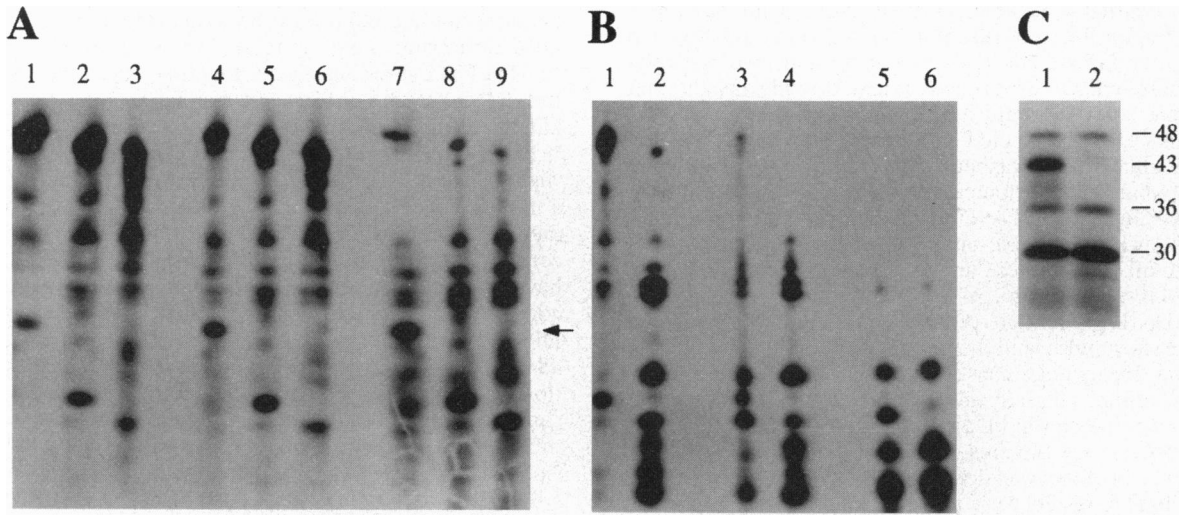

FIG. 3. Partial peptide mapping of DP-2 proteins synthesized *in vivo* and *in vitro*. (*A*) Radiolabeled DP-2-related proteins immunoprecipitated from ML-1 cells were individually excised from an SDS/PAGE gel and treated with increasing quantities of V8 protease (0.05 μg, lanes 1–3; 0.5 μg, lanes 4–6; 5.0 μg, lanes 7–9) before their resolution (55 kDa, lanes 1, 4, and 7; 48 kDa, lanes 2, 5, and 8; 43 kDa, lanes 3, 6, 9) on a second polyacrylamide gel. An arrow indicates a V8 peptide that is unique to the 55-kDa DP-2-related protein. (*B*) Radiolabeled DP-2 proteins synthesized in reticulocyte extracts (48 kDa, lanes 1, 3, and 5; 43 kDa, lanes 2, 4, and 6) were analyzed identically with V8 protease. (*C*) Immunoprecipitations of DP-2 proteins resulting from *in vitro* transcription/translation of wild-type (lane 1) and point-mutated (lane 2) DP-2 cDNAs. Precipitates were prepared as in Fig. 2*B*, and molecular weights of resulting proteins are indicated on the right.

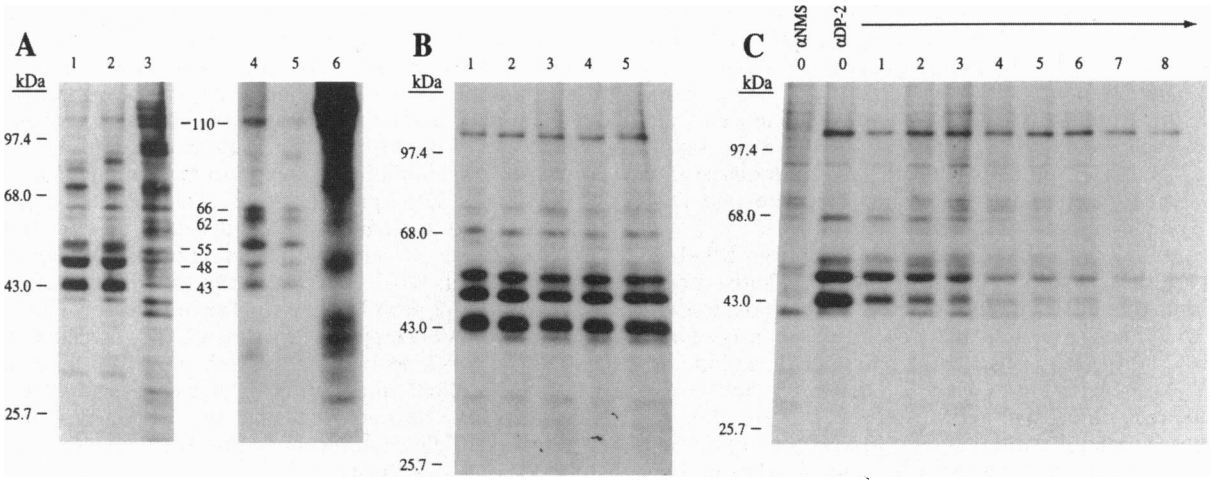

FIG. 4. DP-2-related proteins recovered from ML-1 cells labeled with [^{32}P]orthophosphate or [^{35}S]methionine and incubation with potato acid phosphatase, or following a [^{35}S]methionine "pulse-chase" experiment. (*A*) ML-1 cells incubated with [^{35}S]methionine (lanes 1–3) or [^{32}P]orthophosphate (lanes 4–6) were immunoprecipitated with anti-DP-2 antisera that had been pre-incubated with buffer (lanes 1 and 4), DP-1 immunogen (lanes 2 and 5) or DP-2 immunogen (lanes 3 and 6). Apparent molecular weights of immunoprecipitated proteins are indicated on the right, and molecular weight markers are indicated on the left. (*B*) ML-1 immunoprecipitates were resuspended in phosphatase buffer (lane 1) or phosphatase buffer containing 2 μg of potato acid phosphatase and incubated for 10 min (lane 2), 20 min (lane 3), 45 min (lane 4), or 60 min (lane 5) at 30°C before resolution on a 10% polyacrylamide gel. Molecular weight markers are indicated on the left. (*C*) ML-1 cells incubated with [^{35}S]methionine for 40 min were then extracted (0 hr) or washed and incubated for 1 to 8 hr in methionine-containing growth medium before extraction and immunoprecipitation with normal mouse serum (α-NMS) or anti-DP-2 antiserum (α-DP-2).

levels of phosphorylation. To this end, [^{35}S]methionine-labeled immunoprecipitates were incubated with potato acid phosphatase *in vitro* for varying lengths of time, antibody-antigen complexes were disrupted by boiling, and released proteins were resolved by electrophoresis. As shown in Fig. 4*B*, phosphatase treatment for up to 45 min resulted in only a slight decrease in the apparent molecular weight of each DP-2-related protein. Control phosphatase experiments using pRb immunoprecipitates as substrate revealed that under our standard assay conditions, dephosphorylation reactions were clearly complete by 10 min of incubation (data not shown). We conclude that the distinct apparent molecular weight of the 55-kDa DP-2-related protein in ML-1 cells cannot be accounted for solely by differential phosphorylation.

Since differential phosphorylation does not distinguish the 55-kDa DP-2-related protein from other DP-2 isoforms, we hypothesized that additional posttranslational modifications might be responsible. Should a precursor–product relationship exist between individual DP-2-related proteins, we reasoned that a "pulse-chase" experiment might provide insight that steady-state analyses could not. To examine this hypothesis, ML-1 cells were radiolabeled with [^{35}S]methionine for 40 min (pulse), exhaustively washed with methionine-containing growth medium, and then incubated in growth medium for an additional 8 hr (chase). Protein extracts were prepared immediately following the pulse and each hour of the subsequent chase, and all extracts were analyzed by immunoprecipitation with anti-DP-2 antiserum. As shown in Fig. 4*C*, the 55-, 48-, and 43-kDa DP-2-related proteins were each radiolabeled during the short pulse, and their abundance rapidly diminished during the subsequent chase. The half-life of each DP-2-related protein was directly quantified by phosphoimaging and determined to be 50 (55 kDa), 70 (48 kDa), and 45 (43 kDa) min. Moreover, no obvious precursor–product relationship among these proteins was detected during the time of observation. Identical results were also obtained for cells that were incubated for 15 min with [^{35}S]methionine before a chase (data not shown).

DISCUSSION

The cloning and characterization of E2F-family members has revealed these transcription factors to be regulated by transcriptional and posttranslational mechanisms, including complex formation with Rb family members (pRb, p107, and p130) and cyclins/cdks (1–4). The observation that E2F dimerization partners, DP proteins, are members of a gene family has provided evidence for an additional level of regulation. Here we report the cloning of an alternatively spliced DP-2 cDNA and characterize DP-2 proteins expressed *in vivo*. Consistent with results reported by Wu and coworkers (32) and Zhang and Chellappan (37), our analysis of DP-2 indicates that, in contrast to the ubiquitous expression of a single DP-1 message, at least five distinct DP-2 mRNAs are synthesized in a tissue-specific fashion. We have extended this observation by reporting that (*i*) the DP-2 5′ untranslated region is a site of alternative splicing, (*ii*) at least three DP-2-related phosphoproteins (of 55, 48, and 43 kDa) are expressed *in vivo*, (*iii*) the 43-kDa form of DP-2 synthesized *in vivo* carries a truncated amino terminus, and (*iv*) the 55-kDa protein precipitated with DP-2 antiserum is closely related in structure to the 48- and 43-kDa DP-2 proteins. Taken together, these results indicate that DP-2 encodes at least two distinct proteins (of 48 and 43 kDa) that differ in their primary amino acid sequence.

DP-1 is phosphorylated in a cell-cycle-dependent fashion *in vivo*, and phosphorylation by cyclin A/cdk2 *in vitro* results in a reduction in E2F/DP-1 DNA-binding activity and E2F-mediated transcription (23, 24). Although it is not as yet certain that DP-2 is similarly regulated, the DP-2 proteins we have characterized are each phosphoproteins, and at least one consensus site of cdk phosphorylation is encoded within the DP-2 amino terminus. Interestingly, although present in the 48-kDa form of DP-2, this predicted site of cdk phosphorylation is not included in its 43-kDa counterpart. Thus, should some aspect of DP-2 function be regulated by phosphorylation of its amino terminus, a prediction of our studies is that DP-2 isoforms may be differentially regulated.

Five experimental results reported here support the hypothesis that the 55-kDa DP-2-related protein we have identified results from alternatively splicing. (*i*) This DP-2-related protein is not detected in COS cells transfected with a DP-2 expression vector nor in reticulocyte extracts programmed with DP-2 cRNA. (*ii*) ML-1 immunoprecipitates indicate that the 55-kDa protein is antigenically related to DP-2. (*iii*) Pulse-chase studies suggest that the 55-kDa protein does not

arise from the posttranslational modification of the 48- and/or the 43-kDa DP-2-related species. (*iv*) Partial peptide mapping experiments indicate that the 55-kDa protein is nearly identical in structure to the 48- and 43-kDa forms of DP-2. (*v*) Southern blot analyses using a DP-2-specific probe do not detect closely related DP-2-like genes. Consistent with this hypothesis, a recent report has documented the cloning of several cDNAs encoding alternatively spliced forms of murine DP-2, including one novel cDNA that carries 60 additional amino acids at its amino terminus (41). Although alternative splicing is strongly indicated by available data, we are mindful of the possibility that the 55-kDa DP-2-related protein may be encoded by a novel DP-related gene.

Given that E2F and DP proteins are families of related dimerization partners, it is becoming increasingly likely that E2F/DP heterodimers may not be entirely functionally redundant. Wu and coworkers (32) have demonstrated that DP-1 and DP-2 associate and functionally interact with E2F-1, -2, and -3, yet it is also clear that such heterodimers preferentially form complexes with pRb and not the Rb-related proteins p107 and p130 (28, 30). Complexes of E2F with p107 and p130 appear to be restricted to E2F-4 and -5, two recently cloned E2F-family members (28–31). It has not yet been established whether specific heterodimeric E2F/DP complexes govern the activity of distinct promoters and/or are responsive to distinct upstream stimuli. The synthesis of isoforms of DP-2 differing in their primary amino acid sequence looms as one further means by which E2F activity may be regulated.

This work was supported in part by National Cancer Institute Grant CA53248 and by American Cancer Society Faculty Research Award A-73970. J.M.H. is supported by the Pew Scholars Program in the Biomedical Sciences.

1. Nevins, J. R. (1992) *Science* **258,** 424–429.
2. Chellappan, S. P. (1994) *Mol. Cell. Differ.* **2,** 201–220.
3. Lam, E. W.-F. & La Thangue, N. B. (1995) *Curr. Opin. Cell Biol.* **6,** 859–866.
4. Farnham, P. J., Slansky, J. E. & Kollmar, R. (1993) *Biochim. Biophys. Acta.* **1155,** 125–131.
5. Kovesdi, I., Reichel, R. & Nevins, J. R. (1986) *Cell* **45,** 219–228.
6. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M. & Nevins, J. R. (1991) *Cell* **65,** 1053–1061.
7. Bagchi, S., Weinmann, R. & Raychaudhuri, P. (1991) *Cell* **65,** 1063–1072.
8. Chittenden, T., Livingston, D. M. & Kaelin, W. G., Jr. (1991) *Cell* **65,** 1073–1082.
9. Cao, L., Faha, B., Dembski, M., Tsai, L.-H., Harlow, E. & Dyson, N. (1992) *Nature (London)* **355,** 176–179.
10. Devoto, S. H., Mudryj, M., Pines, J., Hunter, T. & Nevins, J. R. (1992) *Cell* **68,** 167–176.
11. Shirodkar, S., Ewen, M., DeCaprio, J. A., Morgan, J., Livingston, D. M. & Chittenden, T. (1992) *Cell* **68,** 157–166.
12. Cobrinik, D., Whyte, P., Peeper, D. S., Jacks, T. & Weinberg, R. A. (1993) *Genes Dev.* **7,** 2392–2404.
13. Li, Y., Graham, C., Lacy, S., Duncan, A. M. V. & Whyte, P. (1993) *Genes Dev.* **7,** 2366–2377.
14. Hiebert, S. W. (1993) *Mol. Cell. Biol.* **13,** 3384–3391.
15. Kaelin, W. G., Jr., Krek, W., Sellers, W. R., DeCaprio, J. A., Ajchenbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blanar, M. A., Livingston, D. M. & Flemington, E. K. (1992) *Cell* **70,** 351–364.
16. Helin, K., Harlow, E. & Fattaey, A. (1993) *Mol. Cell. Biol.* **13,** 6501–6508.
17. Flemington, E. K., Speck, S. H. & Kaelin, W. G., Jr. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6914–6918.
18. Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D. M., Dyson, N. & Harlow, E. (1993) *Genes Dev.* **7,** 1111–1125.
19. Bandara, L. R., Adamczewski, J. P., Hunt, T. & La Thangue, N. B. (1991) *Nature (London)* **352,** 249–251.
20. Lees, E., Faha, B., Dulic, V., Reed, S. I. & Harlow, E. (1992) *Genes Dev.* **6,** 1874–1885.
21. Mudryj, M., Devoto, S. H., Hiebert, S. W., Hunter, T., Pines, J. & Nevins, J. R. (1991) *Cell* **65,** 1243–1253.
22. Pagano, M., Draetta, G. & Jansen-Durr, P. (1992) *Science* **255,** 1144–1147.
23. Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., Jr. & Livingston, D. M. (1994) *Cell* **78,** 161–172.
24. Dynlacht, B. D., Flores, O., Lees, J. A. & Harlow, E. (1994) *Genes Dev.* **8,** 1772–1786.
25. Bandara, L. R., Lam, E. W., Sorensen, T. S., Zamanian, M., Girling, R. & La Thangue, N. B. (1994) *EMBO J.* **13,** 3104–3114.
26. Girling, R., Partridge, J. F., Bandara, L. R., Burden, N., Totty, N. F., Hsuan, J. J. & La Thangue, N. B. (1993) *Nature (London)* **362,** 83–87.
27. Helin, K., Wu, C.-L., Fattaey, A. R., Lees, J. A., Dynlacht, B. D., Ngwu, C. & Harlow, E. (1993) *Genes Dev.* **7,** 1850–1861.
28. Sardet, C., Vidal, M., Cobrinik, D., Geng, Y., Onufryk, C., Chen, A. & Weinberg, R. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2403–2407.
29. Beijersbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlee, L., Voorhoeve, P. M. & Bernards, R. (1994) *Genes Dev.* **8,** 2680–2690.
30. Hijmans, E. M., Voorhoeve, P. M., Beijersbergen, R. L., van 't Veer, L. J. & Bernards, R. (1995) *Mol. Cell. Biol.* **15,** 3082–3089.
31. Vairo, G., Livingston, D. M. & Ginsberg, D. (1995) *Genes Dev.* **9,** 869–881.
32. Wu, C.-L., Zukerberg, L. R., Ngwu, C., Harlow, E. & Lees, J. A. (1995) *Mol. Cell. Biol.* **15,** 2536–2546.
33. DeGregori, J., Kowalik, T. & Nevins, J. R. (1995) *Mol. Cell. Biol.* **15,** 4215–4224.
34. Bandara, L. R., Buck, V. M., Zamanian, M., Johnston, L. H. & La Thangue, N. B. (1993) *EMBO J.* **12,** 4317–4324.
35. Krek, W., Livingston, D. M. & Shirodkar, S. (1993) *Science* **262,** 1557–1560.
36. Martin, K., Trouche, D., Hagemeier, C., Sorensen, T. S., La Thangue, N. B. & Kouzarides, T. (1995) *Nature (London)* **375,** 691–694.
37. Zhang, Y. & Chellappan, S. P. (1995) *Oncogene* **10,** 2085–2093.
38. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
39. Sterner, J. M., Murata, Y., Kim, H. G., Kennett, S. B., Templeton, D. J. & Horowitz, J. M. (1995) *J. Biol. Chem.* **270,** 9281–9288.
40. Udvadia, A. J., Rogers, K. T., Higgins, P. D. R., Murata, Y., Martin, K. H., Humphrey, P. A. & Horowitz, J. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3265–3269.
41. Ormondroyd, E., de la Luna, S. & La Thangue, N. B. (1995) *Oncogene* **11,** 1437–1446.