

Molecular Cloning and Expression Pattern of the DP Members of the Chicken E2F Transcription Factor

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The DP proteins are components of the E2F transcription factor. They form heterodimers with the E2F proteins and these complexes bind efficiently to E2F response elements in promoters of genes that are involved in cell cycle regulation. The properties of the DP proteins are less documented than those of their E2F counterpart and the present work was aimed at characterizing avian DP genes (named chDP) and their products. Here we describe the cloning of the chicken homologues of the mammalian DP-1 and DP-2 proteins. This work also suggests that DP-2 isoforms have an additional 60 amino acid extension at the N-terminus compared to its human counterpart. Gel-shift assays and coimmunoprecipitation show that both DP-1 and DP-2 dimerize to chE2F-1 and activate transcription efficiently, as demonstrated by transient expression assays. However, contrary to the expression patterns exhibited by E2F-1 during the cell cycle or during neuroretina development, DP member's expression appears more invariant, suggesting that E2F activity is limited by the availability of the E2F proteins.

DP proteins E2F transcription factors Molecular cloning Avian DP genes

MANY genes that control G₁ to S phase transition of the cell cycle are transcriptionally regulated by the E2F/DP transcription factor [for a review, see (45)]. The first member of the E2F family, named E2F-1, was described as a protein that binds the 5'-TTTCGCGC-3' site in the adenovirus E₂ early promoter, and promotes its expression in the presence of the viral E4 protein (14,22,32,33). Most of the cell cycle regulation of E2F/DP activity relies on the interaction, during the G₁ phase, of the E2F/DP heterodimers with the retinoblastoma antioncogene product, p105^{Rb}, resulting in inactive transcriptional complexes (17,24) on promoters. Similar interactions have also been documented with the family of related pocket proteins: p107 and p130 [reviewed in (6,42,44,48,50)]. As a consequence of the phosphorylation of p105^{Rb} by cyclin A or cyclin E/cdk2 complexes, at discrete

steps of the G₁ phase of the cell cycle (6,18,27,29), dissociation of the complexes leads to E2F/DP-dependent transactivation of promoters of genes involved in the control of S phase progression and DNA synthesis such as DNA polymerase α , thymidine kinase, DHFR, *c-myc* or *B-myb* [reviewed in (10,45)]. The same dissociation process can be obtained by displacement with viral oncoproteins such as adenovirus E1A protein, SV40 large T antigen, or human papilloma virus E7 [for reviews see (26,34)]. At the end of the S phase, the phosphorylation of E2F/DP by the cyclin A/cdk2 complex, together with the reassociation with "pocket" p107 and p130 proteins, inactivates the transcription factor, ultimately allowing reentry of cells in G₁ phase (6,9,23,44).

On the basis of their sequence homology and of their property to interact with the "pocket" pro-

Received November 12, 1996; revision accepted January 27, 1997.

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teins, five members of the human E2F family, called E2F-1 to -5, have been cloned during the last few years (3,4,11,16,19,21,28,40,43). All these proteins are supposed to combine as heterodimers with the DP proteins through their dimerization domain, which should yield a large repertoire of E2F factors (26). The prototype DP protein was isolated as a differentiation-regulated transcription factor in F9 cells and it was named DRTF-1/DP-1 (12,13). DP-2, a protein with 65% amino acid (aa) identity with DP-1, has been cloned recently (49). It shares biochemical and functional properties with DP-1, but its expression seems restricted to fewer cell types or tissues. Data suggesting additional forms generated by alternative splicing have been reported for five mouse relative DP-3 proteins, indicating that the DP family could be even more complex (36). DP proteins have also been characterized in *Xenopus laevis* and they show high homology with their human counterparts (12).

DP-1 is frequently isolated in complex with E2F-1 from cell extracts, indicating a privileged interaction between the two molecules (2). The DP proteins are thought not to contain a transactivation domain nor to bind to pocket proteins or cyclins, but, interestingly, DPs increase the DNA binding activity and consequently they enhance the transactivation power of the E2F factor (1,15,25). In addition, they are necessary to the recognition of the E2F/DP factor by pRb and by the adenovirus E4 protein (2). Although they do not exhibit transcriptional capacity on their own, the DP proteins bear important regulatory cell cycle function as demonstrated by the cell growth arrest induced by dominant-negative mutant DP-1 proteins (46) and by the proto-oncogenic activity of deregulated genes (20).

Because E2F-1 has been shown to be tightly regulated during specific stages of embryonic development of avian tissues, like the chicken neural retina (35,37), better insight into the role of the E2F factors in this process required the characterization of the physiological partners of E2F-1, the DP proteins. To date, these latter proteins are known only in mammals and amphibians. The present report describes the molecular cloning and the characterization of two avian DP proteins, named chDP-1 and chDP-2. They share high homology (around 90% amino acid identity in the functional domains) with their human counterparts. This work has also unraveled the unexpected finding that DP-2 is actually 60 amino acids longer, in its amino-terminal domain, than suggested from previous work with the human

protein. This revised sequence leads to a better alignment of both DP1 and DP2 proteins and increases interspecies homology. chDP-1 or chDP-2 do not seem to be regulated during the cell cycle or in response to serum-induced proliferation in quiescent cells. We have observed that their expression, although at low levels, in embryonic chicken tissues remains detectable in newborn and adult animals. Remarkably, expression is not modified in late stages of the developing neuroretina, which is in marked contrast to the downregulation of E2F-1 transcripts. This could suggest that E2F activity is mostly regulated by the availability of the E2F rather than the DP members of the E2F/DP transcription factor.

MATERIALS AND METHODS

Cell Cultures

Primary cultures of chicken embryonic fibroblasts (CEF) were prepared from 9-day-old chick embryos and passaged as previously described (5). In the cell cycle study, cell synchronization was achieved with cycloheximide, aphidicolin, and demecolcine as described in Pasteau et al. (37). Cell cultures were starved by incubation in medium containing 0% serum for 24 h and then reinduced, for varying periods of time, by addition of fresh medium, containing 10% serum.

Molecular Cloning of chDP-1 and chDP-2 cDNA

A chicken cDNA probe was generated by RT-PCR. Briefly, a reverse transcription reaction, with CEF total RNA, was primed by oligo dT and PCR was carried out from the cDNA with the following degenerate oligonucleotides (5'-ATGA ARGNTG YGARAARG-TN-3' and 5'-RTTYTT RAANGCDATYTYGTG-3'). These primers were devised on the basis of the peptide sequence of the conserved DNA binding and dimerization motifs of human DP-1 protein. The obtained 344-bp fragment was then subcloned into the pGEM-T Vector (Promega Biotec) and sequenced before use. This PCR probe was used to screen 5×10^5 recombinant phage plaques obtained from a cDNA library of CEF transformed by *v-src* in Lambda Uni Zap vector (Stratagene). This resulted in the isolation of chDP-1 and chDP-2 cDNA clones. In addition, 5' extended longer chDP-1 cDNAs were isolated from a chicken Heart 5'-Stretch cDNA library in λ gt 10 (Clontech).

The chDP-1 and chDP-2 cDNAs were trans-

ferred into the pSPUTK bacterial plasmid (Stratagene) and into the pLTR expression vector (5). The DP-1 incomplete cDNA was fused in-frame with the ATG codon provided by a *NcoI* site resulting in a 86-amino acid truncated peptide. Dideoxy sequencing was carried out on both strands of double-stranded DNA templates using sequenase 2.0 (United States Biochemical) or using the automatic sequencing PCR procedure with the fluorescent Dye Deoxy Terminator kit from Applied Biosystems. These sequences have been registered in the EMBL data library under the accession number X99071 and X99072 for DP-1 and DP-2, respectively.

In Vitro Translation

The chDP-2, ch(1-62)DP-2, and ch(*NcoI*)DP-1 cDNAs were transcribed and translated with [¹⁴C]leucine in a reticulocyte coupled-TNT transcription/translation lysate (Promega). The labeled protein samples were electrophoresed on 10% SDS polyacrylamide gels, transferred to Optitran BA-S 85 (Schleicher and Schuell), and autoradiographed.

Immunoprecipitations

In vitro coupled transcription/translation reticulocyte extracts programmed with the adequate cDNA were precleared by centrifugation and the supernatants from in vitro radiolabeled E2F reactions were incubated with either labeled DP-1 or DP-2 equivalent reactions for 2 h at 4°C and for 2 h at room temperature. Human anti E2F-1 antibodies (C-20 from Santa Cruz Biotechnology), which cross-react with the chicken protein, were used for immunoprecipitations in conditions recommended by the supplier.

Gel Mobility Shift Assays

The general procedure has been described previously (35,37). The probe was a 22-mer double-stranded synthetic (Genset) oligonucleotide 5'-TA GTTTTCGCGCTTAAATTTGA containing the E2F-1 binding site of the -72 to -50 region of the E2 adenovirus promoter. It was 5' end-labeled, with T4 polynucleotide kinase and [γ -³²P]ATP, at a specific activity of $2-4 \times 10^8$ cpm/ μ g. For each assay, 2.5 μ g of proteins from in vitro translated extracts were incubated for 10 min at 4°C in 20 μ l of the binding buffer [20 mM HEPES (pH 7.9), 25 mM KCl, 0.05 mM EDTA, 1 mM MgCl₂, 0.5 mM Pefabloc-SC 5 Pentapharm AG, 1 mM TCEP (Pierce), 0.5 mg/ml leupeptin,

0.1 mg/ml aprotinin, 20% glycerol], with 0.05-0.1 ng of the labeled probe and 1 μ g of the nonspecific competitor DNA, poly(dI-dC). The complexes were then fractionated onto 4% nondenaturing polyacrylamide gels and the gels were processed as described previously (35,37).

RNA Northern Blot and RT-PCR Determination

Total RNAs were isolated either from 8-day-old or newborn embryonic chicken tissues or from CEF in culture as indicated. The guanidium thiocyanate isolation procedure as described by Sambrook (39) was used. Total RNAs (20 μ g) were separated on 1.2% agarose/14% formaldehyde gels and blotted onto Hybond N membranes. The filter was prehybridized for 4 h and then hybridized at 42°C for 18 h in the $5 \times$ SSPE, 50% formamide, $5 \times$ Denhardt's solution, 20 μ g/ml sheared heat-denatured salmon sperm DNA, 0.1% SDS, and [³²P]dCTP labeled cDNA probe. The probes were chDP-1 cDNA from nucleotide position 1 to 390 (probe A), and for chDP-2 a 397-pb long fragment from nt 190 to 587 (probe B). The chicken GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was used as a control (probe C). Each filter was washed at 42°C in $0.1 \times$ SSPE buffer containing 0.1% SDS and exposed to Kodak Biomax film for 15 days at -80°C.

For RT-PCR, 0.3 μ g of total RNA was reverse transcribed for 45 min at 37°C with 100 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL), 250 μ M of each dNTP, and 5 μ M of poly-dT(12-18) primers. A 20th of these reverse transcriptions was used as templates for PCR amplifications on a Perkin Elmer Cetus thermal cycler. PCR reactions were performed using 0.75 μ M of the indicated specific primers, 250 μ M of each dNTP, and 2 U Taq DNA polymerase (Appligene) in a final volume of 100 μ l. After denaturation at 95°C for 5 min, the 30 cycles of amplification consisted of a denaturation step at 95°C for 30 s, an annealing step at 50°C for 30 s, and a primer extension step at 72°C for 40 s.

RNAse Protection Analysis

RNA was isolated from CEFs in culture as described in the RNA blotting section, and chE2F-1, chDP-1, and chDP-2 mRNAs were quantified by an RNAse protection assay. RNA probes were synthesized, in vitro, from the following recombinant plasmids: pSPUTK/chDP-1, pBSK/chE2F-1, pBSK/chDP-2, and pGEM1Zf/GAPDH. These plasmids were linearized with *PvuII*, *ApaI*, *SacI*, and *PstI* to provide, respectively, templates for

273, 370, 257, and 174 nucleotides long [^{32}P]UTP-labeled riboprobes in the presence of the T7 RNA polymerase (Riboprobe kit, Promega). The probes were purified by denaturing gel electrophoresis prior to use. Ribonuclease protection assay was carried out using the RPA II kit (Ambion) according to the manufacturer's instructions. The products were analyzed by electrophoresis in 5% polyacrylamide/8 M urea gels followed by autoradiography.

Transfections and CAT Assays

The complete chDP-2 cDNA and a partial chDP-2(ch(1-62)DP-2) cDNA corresponding to a truncated form of the 62 N-terminal amino acids of DP-2 and the ch(*Nco*I)DP-1 have been cloned in the pLTR eukaryotic expression vector. Plasmid DNA (0.5–3 μg) was transfected with lipofectamine (Gibco-BRL, Life Technologies) into NIH3T3 cells (0.5×10^5 per 35-mm dish) together with a chimeric plasmid containing the CAT reporter gene under the control of the adenovirus E2 promoter. In all cases, 1 μg of the β -Gal plasmid was transfected to calibrate for transfection efficiency. CAT expression was measured 36 h after transfection using a CAT Elisa procedure as recommended by the supplier (Boehringer). β -Galactosidase activity was determined from the same extracts using X-Gal (Boehringer).

In Situ Hybridization

For in situ hybridization analyses, sense and antisense riboprobes were generated from the chDP-1 cDNA (nt 1 and 390) and chDP-2 cDNA (nt 190 and 587) cloned in PBSK. The ^{32}P -labeled riboprobes were synthesized using the Boehringer in vitro transcription kit with T3 or T7 primers according to the provided instructions. The labeled riboprobes were purified by Sephadex G-50 filtration.

Cryosections (20 μm) from fresh frozen chick embryo retina or heads were thawed onto poly-L-lysine-coated slides, fixed in 4% paraformaldehyde (30 min at room temperature), and rinsed three times for 5 min in phosphate-buffered saline (PBS). The sections were then acetylated and dehydrated in ethanol. Following dehydration, tissue sections were incubated overnight at 50°C in hybridization buffer [50% formamide, 10 mM Tris (pH 8), 0.3 M NaCl, 1 mM EDTA, 1 \times Denhardt solution, 0.5 mg/ml tRNA] containing the radioactive probe (5×10^6 cpm/ml). After hybridization, sections were washed four times (5 min each) in 2 \times SSC and RNase treated (30 min

at 37°C) in a buffer containing 10 mM Tris-HCl (pH 8), 0.5 M NaCl, 1 mM EDTA, and 0.02 mg/ml RNase A (Sigma). Then sections were washed at room temperature as follows: 2 \times SSC, 5 min twice; 1 \times SSC, 10 min; 0.5 \times SSC, 10 min; a final bath was carried out at 60°C in 0.2 \times SSC. After a quick rinse in 0.1 \times SSC at room temperature, the samples were dehydrated in ethanol and air-dried. Slides were autoradiographed for 7–10 days. Once the film (β -max, Amersham) was developed, the slides were dipped into a photographic emulsion (Ilford K5) and revealed after 4 weeks of exposure.

RESULTS

Molecular Cloning of Chicken DP Proteins (chDP)

Isolation of the chicken DP protein genes was based on a reverse transcription-mediated PCR approach. A specific PCR avian nucleic acid probe was obtained with a couple of degenerate oligonucleotide primers whose sequences were designed from the conserved DNA binding and dimerization domains of the human DP-1 protein (see Materials and Methods section). A 344-bp DNA fragment containing a continuous open reading frame (ORF) was successfully amplified and used to screen, at high stringency of hybridization, a chicken cDNA library. Two different clone families have been isolated.

The first contained cDNA inserts, the longest of which is 2156 nucleotides long. It is highly homologous (90%) to the human DP-1 cDNA. From sequence comparison with the human gene we concluded that this clone is missing 30 nt at the 5' end of the coding sequence. Attempts to isolate the complete open reading frame by 5' RACE extension have been unsuccessful so far, indicating that possible mRNA tertiary structure might block extension by reverse transcriptase near the 5' end. For further use and for in vivo and in vitro translation of chDP-1, an artificial ATG, embedded in a *Nco*I restriction site of the pSPUTK (Stratagene) vector was fused in-frame, thus generating a chicken deletion mutant protein, named ch(*Nco*I)DP-1, which is devoid of the most N-terminal 86 amino acids at the end of the protein. The sequence of the protein encoded by the longest cDNA is lacking probably the 10 amino-terminal amino acids and it is displayed in Fig. 1A. chDP-1 protein sequence has 90% overall identity with the human DP-1 protein (Fig. 1B). The DNA binding and the dimerization domains are highly conserved (95%

identity) and all suspected phosphorylation sites in this region are also conserved between chDP-1 and hDP-1.

The second cDNA clone is highly homologous to the human DP-2 protein. The longest isolated molecular clone is 1819 bp long and contains a polyadenylation signal. Sequence analysis revealed a 1338-nt-long ORF, which predicts a product of 446 amino acids. This clone was called chDP-2. The chDP-2 protein appears 61 amino acids longer than its human counterpart, as a result of usage of a more upstream initiation codon (discussed later). The overall identity between DP-2 of both vertebrate classes is only 84%, but as in chDP-1, the DNA binding and the dimerization domain are particularly well conserved (96% and 88% identity, respectively). It is also of interest to note that, like their human counterparts, the two avian DP proteins contain a very acidic domain at the C-terminus end.

In addition to high interspecies homology, chDP-1 and chDP-2 also display significant intraspecies identity, like in their basic (96%), DNA binding (89%), and dimerization (81%) domains. The high conservation (90% identity) of the carboxy-terminal domains of both genes strongly suggests that it has an important role for the biological activity of the protein.

The chDP-2 Protein Has an Extension of 60 Amino Acids at the N-Terminus

The deduced chDP-2 protein is significantly longer (61 amino acids), at its 5' end, than the published human DP-2 sequence. Remarkably, the chicken gene contains a second in-frame ATG codon, 60 amino acids upstream from the purported equivalent initiation codon in the human protein. To investigate the capacity of the most upstream codon to initiate translation, two versions of the chicken DP-2 gene were generated. The first, corresponding to the normal situation, contains the two in-frame ATG codons, separated by 61 amino acids. The second version was deleted to remove the most upstream ATG codon. Figure 2 shows the proteins obtained in a coupled transcription/translation system in vitro. Lane 1 shows the products of translation of the complete cDNA form whereas lane 2 shows products obtained with the 5' truncated cDNA. A series of polypeptide bands corresponding to products whose size is reminiscent of initiation at the different methionine codons at positions 1, 62, and 88 (pointed by open arrowheads in Fig. 1A) is also observed. Clearly, deletion of the most upstream

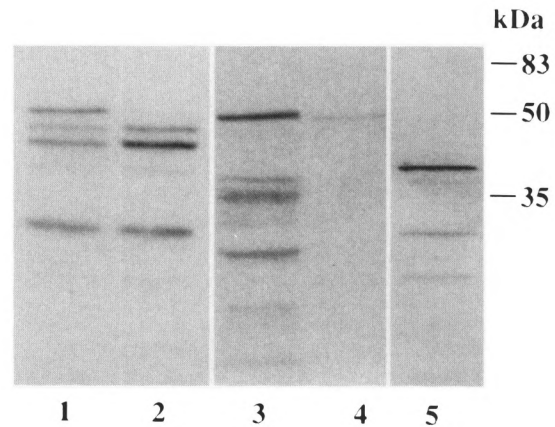


FIG. 2. In vitro translation of ch(*NcoI*)DP-1, chDP-2 and ch(1-62)DP-2 sequences. The experiment was performed as described in Materials and Methods with 0.5 μ g of the corresponding cDNA clone. Lane 1: 14 C labeled chDP-2; lane 2: 14 C ch(1-62)DP-2; lane 3: 14 C humDP-1; lane 4: 14 C ch-E2F-1; lane 5: 14 C ch(*NcoI*)DP-1. The faster migrating bands in lanes 1, 2, 3, and 5 correspond to initiation at internal AUG codons or to premature translation blocks.

initiation codon results in the disappearance of the largest band, indicating that the ATG codon at position 1 is the actual initiation codon. This conclusion is supported by the comparative analysis of sizes of the different in vitro translated proteins [lane 1, ch DP-2 containing 419 aa; lane 2, chDP-2 initiated at codon 62 containing 385 aa; lane 3, human DP-1 containing 410 aa; lane 4 chE2F-1 containing 404 aa; lane 5, the products of in vitro translation of the ch(*NcoI*)DP-1 clone containing 319 aa]. It is also of interest to note that, on the contrary to the chDP proteins, chE2F-1, which has no other methionine in the N-terminal half of the molecule (37), is essentially translated as a single polypeptide. Thus, we have concluded that chDP-2 is actually 61 amino acids longer than the human DP-2 form as reported previously (49). In addition, this change leads to a better alignment with the different members of the DP protein family described so far (Fig. 1B).

chDP-1 and chDP-2 Cooperate With chE2F-1 for E2F-Dependent Transcriptional Activity

To test for transcriptional activity of the two avian DP proteins encoded by the cloned genes, transient expression assays were carried out in the presence of an expression vector of the chE2F-1 cDNA. Transcriptional activity was measured with a CAT reporter gene, placed under the control of the adenovirus E2 promoter containing E2F sites (reporter vector named pE2CAT). Preliminary experiments having shown that transacti-

vation efficiency of the CAT reporter gene is higher in the murine NIH3T3 cell line than in the embryonic chicken fibroblasts (CEF), experimental convenience led us to select NIH3T3 cells for further investigations.

Controls, with chDPs, in the absence of added chE2F-1, gave low background transcriptional activity (Fig. 3, lanes 5–8), whereas, in contrast, chE2F-1 alone increased CAT expression significantly (Fig. 3, lanes 3 and 4). When a constant amount of chE2F-1 expression vector was cotransfected with increasing amounts of chDP-1 or chDP-2 expression plasmids (Fig. 3, lanes 9–11 and 12–14), CAT transcription increased two to three times compared to basal levels of E2F-1 alone, indicating cooperative effect between chE2F-1 and each of the chDPs. This synergy indicates that DP and E2F-1 dimerize efficiently in these conditions. The only small difference noted for chDP-1- and chDP-2-mediated transactivation efficiencies might only reflect consequences of the N-terminal truncation of the chDP-1 clone.

Transactivator cooperative effects, in vivo,

strongly suggest that the chE2F-1 and chDP proteins encoded by the isolated cDNA clones interact with high efficiency. E2F/DP physical interaction was demonstrated by in vitro assays as shown in Fig. 4A and B. In vitro extracts of cotranslated chE2F-1 and DP-1 or DP-2 were tested by a gel mobility shift assay for retarded complexes bound to the E2F site. As shown in Fig. 4A, the specific E2F complex *a* is obtained only when either DP-1 (lane 2) or DP-2 (lane 5) is cotranslated with E2F-1. These complexes are specific because they are not competed (lanes 3 and 6) with a 100 molar excess of a mutant E2 site probe whereas they are competed out (lanes 4 and 7) with a similar excess of the unlabeled specific E2F probe. These competitions demonstrate that, in contrast, complex *b* is nonspecific and that E2F-1 homodimer complexes are not detected under these experimental conditions.

Interaction between the E2F-1 and the DP in vitro translated peptides was further demonstrated by immunoprecipitation, as shown in Fig. 4B. The in vitro translation products are shown in lanes 1–

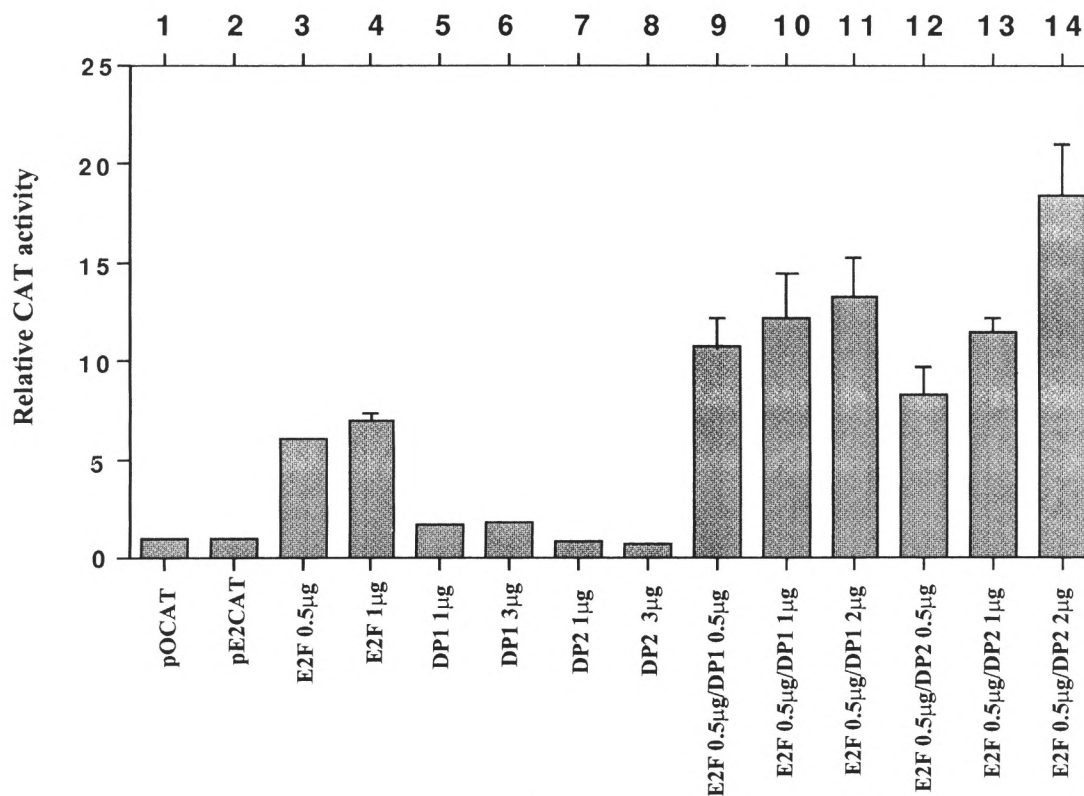


FIG. 3. Cooperation of chDP-1 and chDP-2 with chE2F-1 in E2F-dependent transcriptional activity. NIH3T3 cells were transfected with the pOCAT control plasmid (lane 1), or with pE2FCAT (lanes 2–14) together with expression vectors as indicated. Cell extracts were prepared 36 h later and processed for CAT Elisa determinations. The amounts of expression vector transfected were as follows: 0.5 μ g (lanes 3, 9–14) or 1 μ g (lane 4) of pLTR chE2F-1; 0.5 μ g (lane 9) or 1 μ g (lanes 5, 10) or 2 μ g (lane 11) or 3 μ g (lane 6) of ch(*Nco*I)DP-1; 0.5 μ g (lane 12) or 1 μ g (lanes 7, 13) or 2 μ g (lane 14) or 3 μ g (lane 8) of chDP-2. Values represent average from three separate experiments.

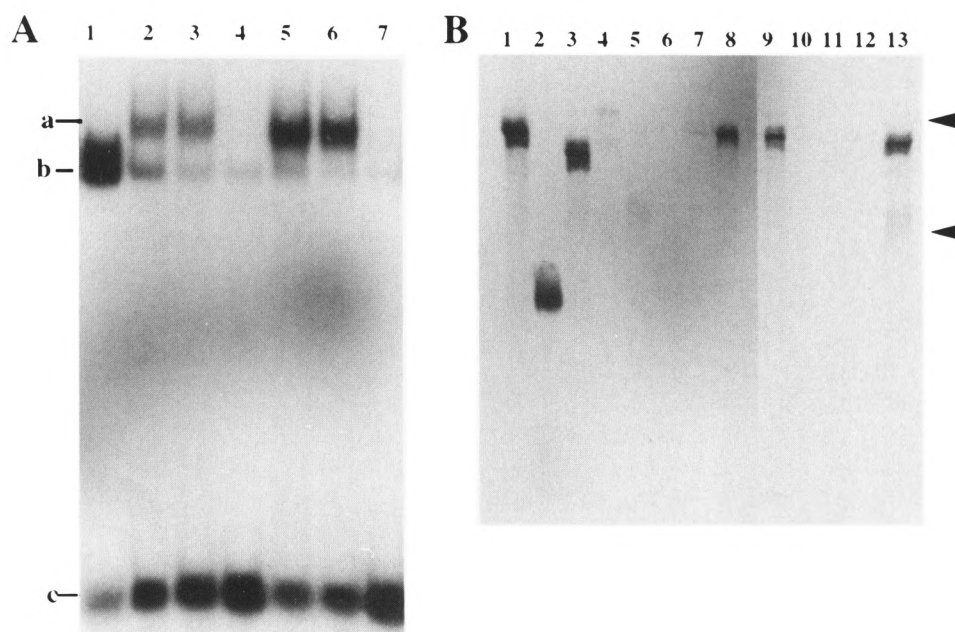


FIG. 4. Physical interactions between chE2F-1 and chDP-1 or chDP-2. (A) Mobility-shift assay with in vitro translated proteins as described in Materials and Methods. Lane 1 is a reticulocyte extract programmed with ch E2F-1, lanes 2 and 5 are mixtures (1:1) of extracts programmed with E2F-1 and DP-1 or E2F-1 and DP-2, respectively. Lanes 3 and 6 are as in lanes 2 and 5, respectively, except that a 100 molar excess of a mutated E2F probe was added prior to the incubation with the protein extract, and lanes 4 and 7 are with a 100 molar excess of the unlabeled normal E2F probe. a is a specific, b a nonspecific complex, and c is the free probe. (B) Immunoprecipitation of E2F-DP complexes. Lanes 1-5 show the in vitro translated proteins: 1, chE2F-1; 2, bHLH domain of chE2F-1; 3, chE2F-1 deleted of the Rb binding domain [see (37)]; 4, DP-2; 5, ch(*NcoI*) DP-1 as described in Materials and Methods. Lanes 6-8 are immunoprecipitations of the labeled E2F-1 proteins of lanes 3, 2, and 1, respectively, with anti-E2F-1 antibodies directed against the pRb binding domain of chE2F-1. Lanes 9-13 are immunoprecipitations of 1:1 mixture of different forms of in vitro translated chE2F-1 and either DP-2 (lanes 9-11) or DP-1 (lanes 12 and 13). Lane 9 is with full-length chE2F-1, lane 10 with bHLH E2F-1, and lane 11 with E2F-1 deleted of the Rb binding domain. Lane 12 is with bHLH chE2F-1.

5 whereas immunoprecipitation of mixtures of E2F and DP proteins is shown in lanes 9-13. To ensure the specificity of the immunoprecipitates chE2F-1 versions with different reactivities to anti-E2F-1 antibodies were used (full length, lane 1; bHLH domain only, lane 2; or Rb binding deleted domain, lane 3) was achieved at high levels from the pSPUTK translation vector (Stratagene). chDP-2 (lane 4) or the (*NcoI*) N-truncated DP-1 protein (lane 5) were translated independently. Anti-E2F-1 was used to coimmunoprecipitate the in vitro translated proteins alone (lanes 6-8) or as mixtures of E2F-1 and DP proteins (lanes 9-13). Lanes 6-8 show that, as expected, only full-length E2F-1 is immunoprecipitated by the antibodies raised against the Rb binding domain of the protein. Mixture of E2F-1 and DP in vitro translated extracts leads to coimmunoprecipitation of either DP-2 (lane 9) or DP-1 (lane 13). In contrast, absence of immunoprecipitation in lanes 10-12, corresponding to truncated versions of E2F-1, indi-

cates that immunoprecipitations of DP proteins are specific of E2F-1 antibodies and therefore of a physical interaction between E2F-1 and the DP polypeptides.

Cell Cycle Expression of chDP-1 and chDP-2

To get insight into the role of these chicken DP proteins in the regulation of the E2F/DP function during the cell cycle, we have analyzed, by RNase protection assay (RPA), expression of the chDP-1 and DP-2 genes in synchronized chicken embryonic fibroblasts (CEF). The cells were rendered quiescent by serum starvation and were restimulated by serum addition. The mRNA levels were quantified in G₁, S, and G₂/M phases of serum-stimulated cells (37) with chE2F-1 as a standard (Fig. 5A). A GAPDH probe was used for calibration and data were normalized by instant imager counting of the bands. The data are expressed as a ratio between the DPs signal and signal of

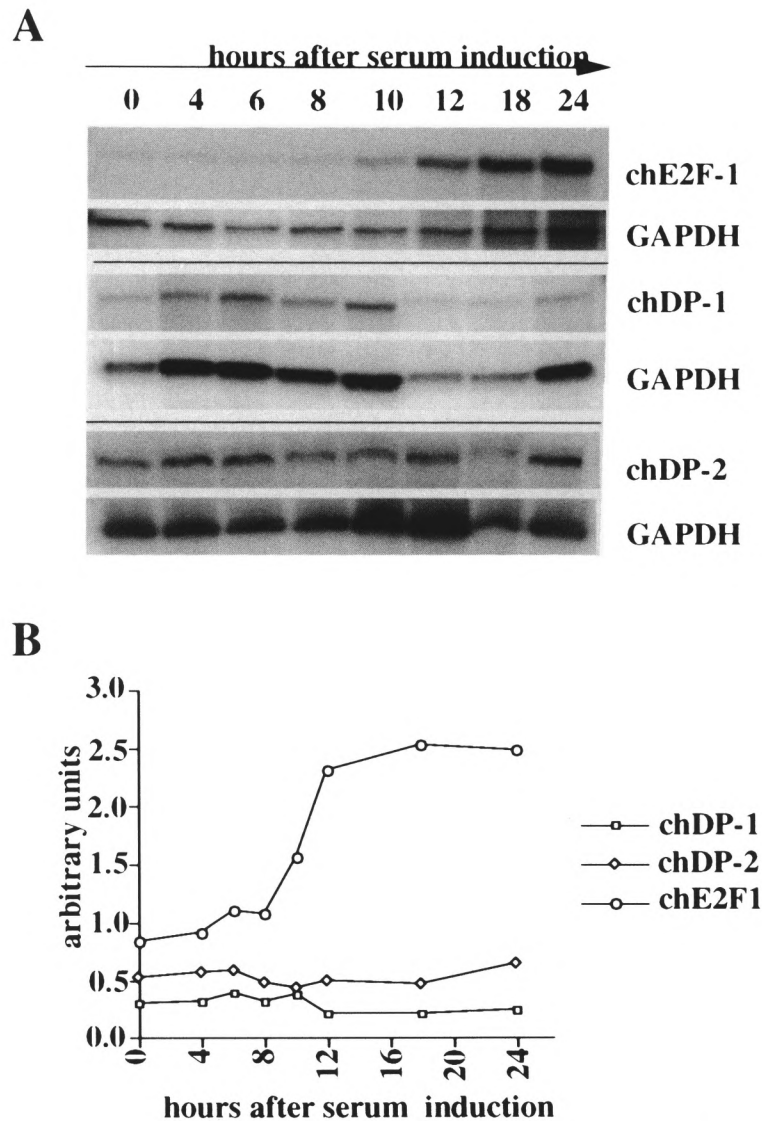


FIG. 5. Expression of chE2F-1, chDP-1, and chDP-2 mRNA following serum stimulation. RNase protection analysis of chDP mRNA accumulation in serum-stimulated CEF. Twenty micrograms of total RNA from samples, at different times following serum addition to arrested cells, was hybridized to either the chDP-1 273 nt, to the chE2F-1 370 nt, to the chDP-2 257 nt, or to the 174 nt GAPDH riboprobes and processed by RNase digestion. Only the entire protected fragments for each probe are shown (230, 305, 128, and 174 nt, respectively). Fragment length was estimated using DNA sequencing reaction ladders. Data from (A) were quantified by densitoscans, normalized to GAPDH, and expressed as arbitrary units, as a function of time, as presented in (B).

GAPDH (Fig. 5B). Confirming previous observations, the chE2F-1 transcript level was very low in quiescent fibroblasts and increased continuously after serum addition, to peak 12 h after induction, which corresponds to the entry of cells in S phase (Fig. 5A). In contrast, chDP-1 or chDP-2 mRNA levels remained more stable as indicated by the plot shown in Fig. 5B.

Similar conclusions were reached when cells

were analyzed after cell cycle blocks induced by different drugs (i.e., blockade at three positions: in early/mid G₁ phase with cycloheximide, at the G₁/S transition with aphidicolin, and during mitosis with demecolcine). Northern blotting (Fig. 6A), for DP-1 (a unique 3-kb transcript) and DP-2 (5 transcripts) messages, showed much less changes than E2F-1 when cells proceed from quiescence (lane 1) to G₁/S (lane 2), to S phase (lane 3), or to

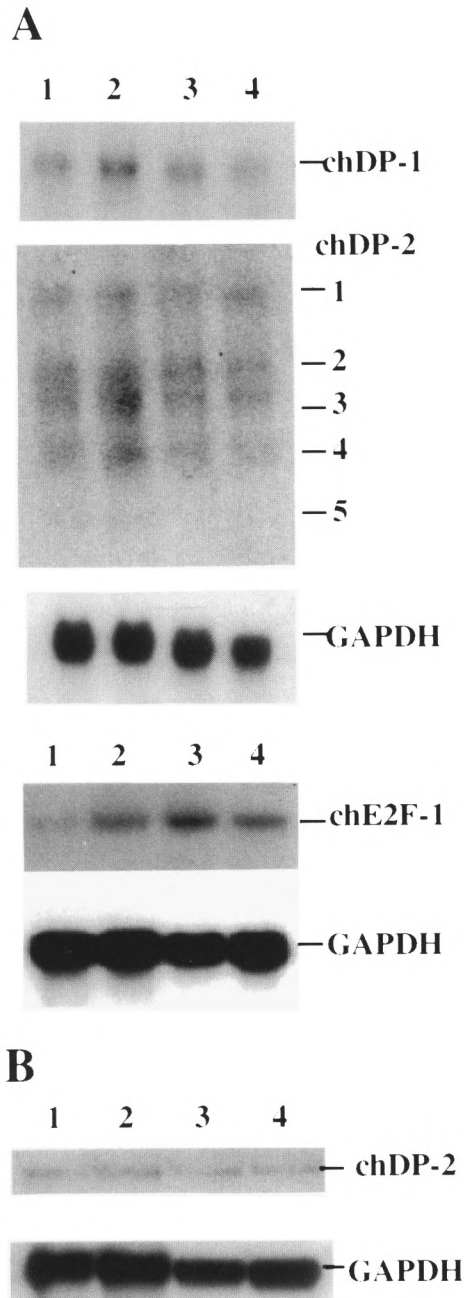


FIG. 6. Cell cycle regulation of chDP-1, chDP-2, and chE2F-1 mRNA accumulation. (A) Northern blot analysis of total RNA from synchronized CEFs cultured for 24 h in the absence of serum (lane 1) and, after 8, 12, or 24 h, respectively (lanes 2, 3, and 4), of serum addition. Synchrony was achieved by blocks at different stages of the cell cycle: lane 2, early to mid G_1 synchrony obtained by cycloheximide treatment for the last 4 h; lane 3, G_1/S synchrony with aphidicoline for the last 8 h; lane 4, M synchrony with demecolcine for the last 10 h. (B) RNAse protection analysis with a specific chDP-2 riboprobe. The same RNA templates as in (A) were analyzed using the same protocol and chDP-2 probe as described in Fig. 5.

mitosis (lane 4). The more specific and sensitive RNase protection assay carried out with a chDP-2-specific probe spanning amino acids 1 to 29 (Fig. 6B and Materials and Methods) confirmed these results, indicating that DP transcript levels have relatively constant patterns of expression both in cycling and in quiescent versus proliferative cells.

Regulation During Development and Tissue Expression of chDP-1

To examine the pattern of DP expression in different tissues, Northern blotting of RNA samples prepared from various embryonic and newborn chick tissues (heart, lung, liver, and brain) were carried out (Fig. 7). The results show that chDP-1 is expressed in all tissues tested, at similar levels in embryonic and newborn animals, indicating that the transcription level is independent of the proliferative state of the cells and of the development stage. This situation contrasts with the downregulation observed for chE2F-1 (37) in the same conditions. The study of chDP-2 has been hampered by the low levels of the transcripts, but data are also consistent with a persistent expression of the gene in adult organs (not shown).

Chicken neuroretina is an exquisite model for the analysis of molecular events leading to cell cycle block during early embryogenesis, at E7–E9. A coordinated downregulation of cell cycle control genes, like *cdc2* and cyclins (35) and E2F-1 (37), has been documented and it was therefore of interest to test for the behavior of chDP proteins in these conditions. Like chE2F-1, chDP-1 mRNA is very abundant, prior to cell division arrest, in the retina and in the optic tectum compared to others tissues of the embryo (Fig. 8A). However, on the contrary to the E2F-1 situation, no significant change occurs for chDP-1 when retinoblasts enter the postmitotic stage. The transcript remains abundant at E13, when no more chE2F-1 mRNA is detected (Fig. 8B). The hybridization signal is present within the whole thickness of the retina at days 7 and 13, indicating also that expression of chDP-1 is not restricted to a particular cell layer of this developing organ.

DISCUSSION

The Avian DP Gene Family Is Composed of Only Two Members: chDP-1 and chDP-2

We have cloned and characterized two members, chDP-1 and chDP-2, of the chicken DP transcription factor family. Search for other members

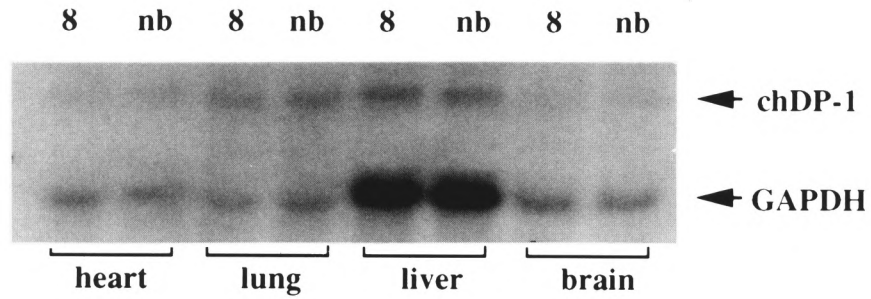


FIG. 7. RNA blot analysis of chDP-1 in different chicken tissues. Expression in 8-day-old chick embryo (8) or newborn chicken (nb). Each sample containing an equal amount (20 μ g) of total RNA from the indicated chicken tissues was hybridized with a chicken DP-1 cDNA probe (probe A, Materials and Methods) and the chicken GAPDH cDNA as a control (probe C, Materials and Methods).

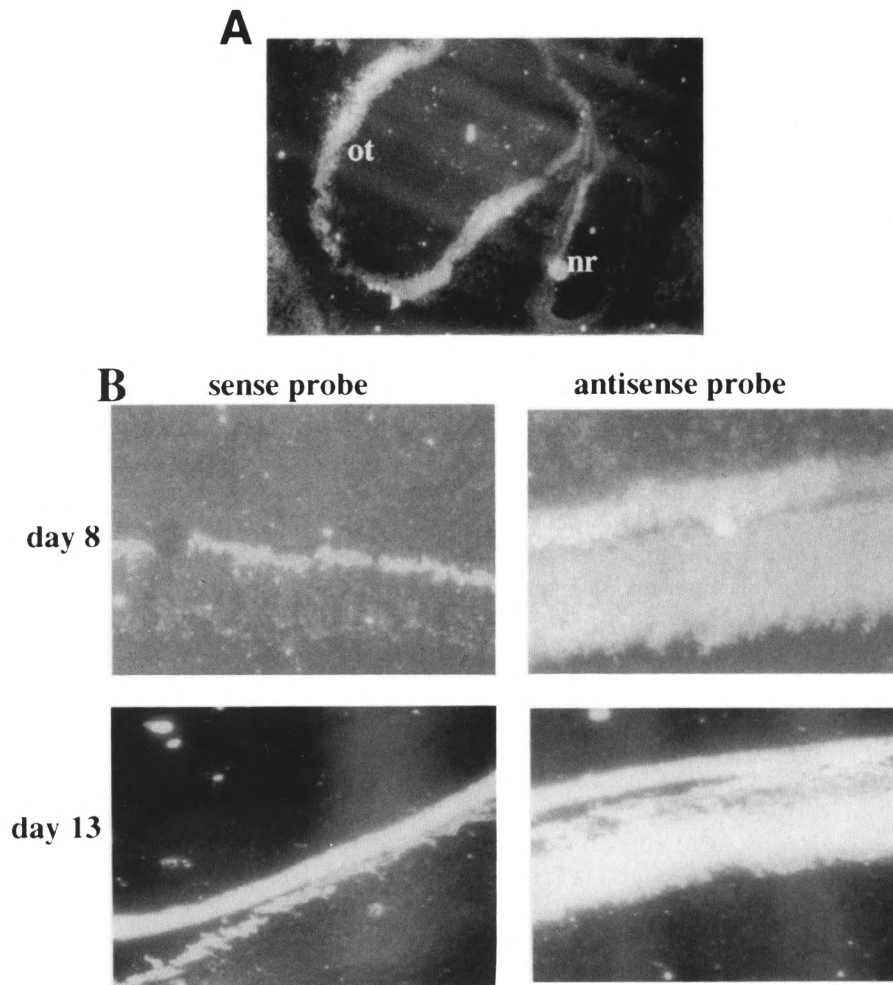


FIG. 8. Expression of chDP-1 at two stages of the developing chicken neuroretina. In situ hybridization (ISH) was performed on specimens as described in Materials and Methods. (A) Longitudinal section through a whole 4-day-old embryo hybridized with a specific chDP-1 probe. Magnification of the optic tectum (ot) and neuroretina (nr) regions with the eye lens visible on the bottom right of the picture. (B) Cryostat sections of the eyes from 8- and 13-day-old chicken embryo hybridized with either specific chDP-1 sense (control) or antisense probes as indicated. ISH with the chE2F-1 probe was carried out for control and it gave the results reported in Pasteau et al. (37).

of this family by screening chicken cDNA libraries from two different origins has failed. Although one cannot exclude existence of such additional putative members like the DP-3 gene, described recently in mice (36), isolation of more than 20 independent DP-1 and DP-2 clones suggests that transcripts of other genes, if any, should be highly underscored. In addition, genomic DNA analysis by Southern blotting, at low stringency of hybridization with the two DP-1 and DP-2 avian probes, confirms that the avian DP gene family could be restricted to only the two members that we describe here (data not shown).

Alternative splicing has been suggested as a mechanism giving rise to the multiple DP-2 murine transcript isoforms revealed by Northern blot (36). Such isoforms also exist in chicken (see Fig. 5). Thus, we were led to address the same question in the chicken system. Using PCR primers flanking the region equivalent to the deleted E zone of mDP-3b and g mRNA species (36), we amplified a unique product whatever the tissue tested. In addition, the screening of a chicken cDNA library with a large probe covering this putative spliced region resulted in five clones, all of which contain the E sequence. Therefore, it is likely that chicken DP-2 transcripts originate from a different mechanism than E sequence deletion. Thus, the complexity of the DP-2 gene transcript situation in avians remains to be clearly defined but neither alternative splicing nor differential polyadenylation signal usage can be excluded at that stage.

The modular organization of DP proteins is strictly conserved at intraspecies and interspecies levels. The avian DP proteins described here share the structural and functional domains that have been described in other species (2,12,13,36,49). Avians and mammals have more than 90% amino acid identity in the bHLH domain (Fig. 1A and B) and conservation is also remarkably high in another domain (aa 270-340), located in the carboxyl end of either DP-1 or DP-2 (Fig. 1B). This latter region has no obvious structural motif, and it has not yet received a defined function. It is likely to play an important role for the transactivation properties of the DP proteins.

Chicken DP-2 Has a 60 Amino Acid N-Terminal Extension, Compared to Its Human DP-2 Counterpart

Data concerning the DP proteins in human and rodents have reported the existence of a unique form of DP-1 and multiple forms of the DP-2 proteins. Recent data with the human DP-2 pro-

teins have shed insight on this diversity at the protein level. Three main polypeptides of 55, 48, and 43 kDa, respectively, have been identified *in vivo* (38) with one species (the 48 kDa one) being the predicted product deduced from the reading frame of the human cDNA (47,49). The relationship to transcript multiplicity and differential splicing as reported recently in another species [mouse (36)] remains to be established. The 43-kDa product was supposed to arise from translation from a downstream AUG codon, at 27 amino acids, whereas the 55 kDa remained unexplained, from sequence data, except if one assumes that splicing events occur in the 5' untranslated sequence of the gene. The present work shows that avian DP-2 protein is encoded by a cDNA with a 446 amino acid open reading frame. These data are consistent with a presumed molecular weight above 50 kDa, and *in vitro* transcription/translation experiments, with complete or 5' deleted versions of the ch DP-2 cDNA, support this conclusion. They clearly show that the AUG codon, located 60 amino acids upstream from the purported human DP-2, is used efficiently for *in vitro* translation. In addition, the two in-frame downstream Met codons, at 61 and 87 amino acids, are utilized efficiently and they give rise to proteins equivalent to the 48- and 43-kDa human DP-2 proteins (Fig. 2). Thus, we conclude that, at least in the chick situation, an uninterrupted reading frame of 446 amino acids encodes a protein with a 60-amino acid extension at its N-terminus compared to the human DP-2 described so far. This finding also leads to a better alignment of the DP-1 and DP-2 proteins, indicating that they share N-terminal domains of the same size.

chDP-1 and chDP-2 Cooperate to Similar Extents for E2F Transcriptional Activity

The high sequence and structural homology of the two avian proteins is reminiscent of their interaction with a common partner, the E2F family of proteins, to fulfill biological activity. Although DP-1 and DP-2 do not exhibit transactivation activity on their own, as demonstrated by transient expression assays, they synergize efficiently with chE2-1 activity in such transcriptional *in vivo* assays. Such observations, which support the heterodimer model of E2F active transactivator, have been confirmed by *in vitro* approaches showing physical interaction between the E2F and DP family of proteins. Figure 4 clearly shows that E2F site binding specific complexes can only be observed when E2F-1 and DP are present. Immunoprecipi-

tation analyses have confirmed this interaction. It is observed in the presence of low amounts of the DP proteins, suggesting that they have high affinity for their E2F-1 target site. Recent studies have suggested that the adenovirus E4 (8) and the cellular MDM2 (31) proteins increase the affinity and/or stability of heterodimeric E2F for its binding site. It would be of interest to check whether this viral protein or cellular homologues also synergize in the three assays (in vivo transactivation via E2F sites, gel retarded complex formation, and immunoprecipitation of dimers) that we describe here. It is of note that the ch(NcoI)DP-1 protein devoid of the N-terminal 86 amino acids has transcriptional efficiency in the same range as the long chDP-2 form, indicating that the amino-terminal domain is not essential for transcriptional activity. All these data are in good agreement with others obtained with the *Xenopus* DP-1 and DP-2 proteins (12).

Cell Cycle and Developmental Expression of chDPs genes. What About Posttranslational Mechanisms?

Expression of different DP-E2F complexes at defined steps of the cell cycle could provide functional selectivity for progression through the cell cycle. To test such a possibility, we have analyzed the expression of chDP-1 and chDP-2 and compared it to chE2F-1, in CEF stimulated by serum or synchronized at different stages of the cell cycle with drugs.

In contrast to the G₁/S phase regulation of chE2F-1 expression, chDP transcripts do not change significantly throughout the cell cycle. Similar conclusions have been obtained with DP-1 mRNA in mitogen-stimulated keratinocytes and NIH3T3 cells (30,41,42). Only limited DP-1 mRNA increase has been reported, in early G₁, in serum-stimulated NIH3T3 cells (46). Intriguingly the transcript pattern of the different species is quite different for chDP-1 (only one detectable species)

and for chDP-2 (five species at least), which is indicative of a more sophisticated function for DP-2 than for DP-1. So far, however, no proof exists for the occurrence of multiple DP-2 polypeptide species. The invariance of DP-2 transcripts during the cell cycle might suggest that DP-2 diversity is more related to tissue-specific roles than to cell-cycle specific regulation.

These observations are consistent with data showing that DP-1 expression persists in organs from embryos to newborn animals. The same conclusion has been reported in the mouse system (46). The behavior of the DP genes in response to growth or developmental signals contrasts with that described for E2F-1 (37), rendering at present their function difficult to assess. Recent investigations with truncated versions of the mouse DP-1 gene indicate, however, that it is a requisite for late G₁ to S phase progression (46). Answers to these questions require thorough investigation of the composition of DP-1- or DP-2-containing complexes in different biological situations. Questions concerning these proteins in the near future should also take into account their possible post-translational modifications and their interactions with members of the Rb pocket family of proteins known to play specific roles in quiescence, proliferation, or terminal differentiation (7).

ACKNOWLEDGEMENTS

The authors are indebted to Dr. K. Helin (Milano, Italy) for supplying the human DP-1 cDNA clone and to Dr. M. Castellazzi for providing a chicken cDNA library. The critical reading of the manuscript by Dr. E. Goillot is greatly acknowledged. This work was supported by funds from Association Pour la Recherche Contre le Cancer (ARC), Fédération Nationale des Ligues pour la Recherche contre le Cancer (FNLCC), Association Retinitis Pigmentosum, and the Région Rhone-Alpes.

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