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Two E2F Sites Control Growth-regulated and Cell Cycle-regulated Transcription of the *Htf9-a/RanBP1* Gene through Functionally Distinct Mechanisms*

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The gene encoding Ran-binding protein 1 (RanBP1) is transcribed in a cell cycle-dependent manner. The *RanBP1* promoter contains two binding sites for E2F factors, named E2F-c, located proximal to the transcription start, and E2F-b, falling in a more distal promoter region. We have now induced site-directed mutagenesis in both sites. We have found that the distal E2F-b site, together with a neighboring Sp1 element, actively controls up-regulation of transcription in S phase. The proximal E2F-c site plays no apparent role in cycling cells yet is required for transcriptional repression upon growth arrest. Protein binding studies suggest that each E2F site mediates specific interactions with individual E2F family members. In addition, transient expression assays with mutagenized promoter constructs indicate that the functional role of each site is also dependent on its position relative to other regulatory elements in the promoter context. Thus, the two E2F sites play opposite genetic functions and control *RanBP1* transcription through distinct molecular mechanisms.

The murine *Htf9-a/RanBP1* gene encodes Ran-binding protein 1 (RanBP1)[1] (1), a major partner of the Ran GTPase (2, 3), which cooperates with members of the Ran signaling network in control of a variety of functions, including DNA replication, mitotic entry and exit, chromatin condensation, and nucleocytoplasmic transport (reviewed in Refs. 4 and 5). The *RanBP1* gene, unlike other members of the network, is expressed in a cell cycle-dependent manner: *RanBP1* transcription is activated at the G_1/S transition and peaks in S cells (6, 7). Up-regulation of *RanBP1* transcription during S phase is an important functional requirement for the Ran network activity: we have previously shown that replacing the *RanBP1* endogenous promoter with cell cycle-independent regulatory sequences yielded deregulated production of the RanBP1 protein and caused abnormalities in further cell cycle progression, including inhibition or delay in S phase, impaired mitotic exit, and failure of chromatin decondensation at the mitosis-to-interphase transition (8).

In previous work, we identified two *RanBP1* promoter regions that control separate aspects of *RanBP1* transcription (see map in Fig. 1*A*). Basal transcription requires a proximal region encompassing the major transcription start site, TS-1 (9). That region harbors two prominent genomic footprints (10), termed Sp1.2, acting as a bona fide Sp1-binding site (11), and *Htf9* footprinted element (HFE), a basal control element that can interact either with retinoid X receptor family members in quiescent cells or with single-stranded DNA-binding proteins in cycling cells; the latter determines the assumption of an active conformation around the transcription start site (9). The HFE is flanked by a recognition site for E2F/DP factors, termed E2F-c, which is not footprinted in proliferating cells (10). G_1/S up-regulation of *RanBP1* transcription is controlled by a region located from −150 to −90 relative to TS-1 (6), henceforth referred to as the distal promoter region. That region includes target sites for E2F/DP (E2F-b site) and Sp1 (Sp1.3 site) factors, both of which are footprinted *in vivo* in cycling cells (10). Thus, the E2F sites in the *RanBP1* promoter display different protein binding properties *in vivo*.

E2F factors control expression of many cell cycle genes through responsive promoter elements that confer either positive or negative control; precise temporal regulation of these genes by E2F is a prerequisite for ordered cell cycle progression. The E2F DNA binding activity is shared by heterodimeric complexes, in which both subunits are encoded by gene families: one heterodimeric component is synthesized from one of five related E2F-encoding genes and dimerizes with one of three related DP dimerization partner proteins. Control of transcription by E2F/DP dimers is subjected to various levels of complexity (see Refs. 12–14 for reviews). First, E2F-encoding genes are differentially induced during the cell cycle: E2F-1, -2, and -3 appear in mid- or late G_1, whereas E2F-4 and -5 are expressed relatively constantly. Second, transcription of cell cycle genes is controlled by the molecular balance established at any given time between E2F/DP complexes and repressor pocket proteins. Transactivation by E2F/DP heterodimers can be differentially antagonized in the interaction with members of the pocket protein family: E2F-1, E2F-2, and E2F-3 preferentially interact with the pRb retinoblastoma gene product (15), whereas E2F-4 preferentially associates with p107 and p130, and E2F-5 with p130 only (15–18); however, pRb can also interact with E2F-4 (15). The loss of particular pocket proteins affects distinct sets of genes (19), thus pinpointing specific roles for particular E2F/DP/pocket complexes in transcriptional con-

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[1] The abbreviations used are: RanBP1, Ran-binding protein 1; TS, transcription start; HFE, *Htf9* footprinted element; FCS, fetal calf serum; FACS, fluorescence-activated cell sorting; CAT, chloramphenicol acetyltransferase.

trol. These interactions are themselves temporally regulated: the p130 protein is essentially active in G_0 cells (Refs. 18 and 20; reviewed in Refs. 14 and 21); pRb and p107 are instead transcriptionally induced during G_1 and inactivated by phosphorylation as cells approach S phase (reviewed in Refs. 14 and 22). The interactions of activating complexes with target genes is also dependent upon regulated nuclear transport of particular E2F (23–26) and DP (27) members, which determines which complexes will assemble in the nucleus (28). Finally, a further level of control resides in the promoter structure, within which E2F/DP/pocket complexes may productively interact with positive (29, 30) or negative (31, 32) factors; recent experiments with synthetic reporter constructs indicate that the repressing or activating function of E2F elements depends in part on their position relative to neighboring regulatory sequences (33, 34).

In this work, we have sought to establish the role of the E2F sites in cell cycle-regulated transcription of the *RanBP1* gene. We have induced site-directed mutagenesis of both E2F sites to assess their individual contribution to cell cycle regulation of *RanBP1* promoter activity. We have found that the distal E2F-b, together with the neighboring Sp1.3 site, controls upregulation of transcription at the G_1/S boundary. In contrast, the proximal E2F-c site is dispensable for expression in cycling cells, yet is absolutely required for transcriptional repression in G_0 cells. Thus, both E2F sites independently contribute to cell cycle-regulated activity of the *RanBP1* promoter and mediate genetically distinct control mechanisms.

EXPERIMENTAL PROCEDURES

Cell Cultures and FACS Analysis—Murine NIH/3T3 fibroblast cultures (ATCC CRL 1658) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS) under 5% (v/v) CO_2 at 37 °C. Cell samples to be analyzed were harvested in phosphate-buffered saline, fixed in acetone:methanol solution (1:5 v/v), and incubated with RNase (10 μg/ml) for 5 min at 0 °C. Propidium iodide (0.5 μg/ml) was added and the DNA content of cell samples was measured in a FACStar Plus cytofluorometer (Beckton Dickinson). In experiments designed to monitor S phase, cells were incubated with 45 μM bromodeoxyuridine for 30 min before harvesting. Harvested cells were incubated in 1 N HCl for 45 min, neutralized, incubated with anti-bromodeoxyuridine antibody (Ig G clone BU5.1, Ylem) for 30 min and then with a fluorescein-conjugated secondary anti-IgG antibody (Ylem), and finally subjected to biparametric FACS analysis for simultaneous determination of the DNA content and of bromodeoxyuridine incorporation using the WinMDI software (10,000 events/sample).

Expression Constructs—The pA10 and pE1 clones carry the SV40 minimal promoter and a chimeric derivative containing the E2F-b site, respectively (for details, see Ref. 6), upstream of the chloramphenicol acetyltransferase (CAT) reporter sequence. The pTS-A clone carries the wild-type *RanBP1* promoter upstream of the CAT gene (11). Derivative clones were synthesized by site-directed mutagenesis, by either ligating synthetic oligonucleotides mutated in the E2F-b and/or Sp1.3 sites and terminating with a Sau96 end to the Sau96 unique site of the *RanBP1* promoter (GenBank™ accession number X05830), or using the QuickChange site-directed mutagenesis kit (Stratagene). Resulting clones carried the following mutations (underlined): pmE, mutation in the E2F-b site (sequence TTTGGCGGGA mutated to TTTACTCAGA); pmS, mutation in the Sp1.3 site (sequence GGGGCGGGC mutated to GAGATGGGC); pmES, simultaneous mutations of both the E2F-b and Sp1.3 sites; pGi, mutation in the proximal E2F-c site (sequence TTTCCCGCCGC mutated to TTTACTCACGC); pQl, double E2F-b and E2F-c site mutations; pmBB was derived from pmE and hence carries the mutated version of the distal E2F-b site, whereas the proximal E2F-c site (TTTCCCGCCGC) was replaced by a wild-type E2F-b site (TTTGGCGGGA) by site-directed mutagenesis; pBB was similarly derived from pTS-A and carries site E2F-b in both the distal and the proximal locations. Effector constructs were synthesized by cloning the coding sequences for the E2F-1, E2F-4, DP1, pRb, p130, and p107 proteins under the control of the cytomegalovirus promoter/enhancer region in a pBluescript vector. A construct carrying the *lac Z* gene under the cytomegalovirus promoter was used to control the efficiency of transfection.

Transfections—Cells were passaged at approximately 1×10^6 cells/25-cm^2 flask the day before transfection; on the following day, a mixture containing DOTAP reagent (Boehringer Mannheim) and DNA was added; 4 μg of CAT and 1 μg of β-galactosidase reporter constructs were routinely used. In cotransfection experiments, constructs expressing E2F-1, E2F-4, DP1, pRb, p130, and p107 were used. Various amounts (*i.e.* 0.2–2 μg) of effector plasmid DNA were initially tested, and routinely, 0.5–1 μg were used. In coexpression experiments, we used constructs expressing E2F-4 and DP1 (1 μg each), together with constructs encoding either p107 (1.5 μg) or p130 (1.5 μg or 3 μg). Vector DNA was added to equalize the total DNA amount in all experiments. The medium was replaced 6 h after transfection with either low serum (0.5% FCS) to induce growth-arrest or complete medium (10% FCS) to maintain asynchronous proliferation. To obtain S phase-enriched cultures, cells that had been starved for at least 48 h were stimulated to reenter the cycle by adding 15% FCS-containing medium and collected after 15 h of restimulation. Transfected cells were harvested and lysed by repeated freeze-thawing cycles. Proteins were extracted from each sample in 100 μl of Buffer A (100 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl); 10 μl were used for determining the extract concentration using the Bradford assay kit (Bio-Rad), 80 μl were used to measure synthesized CAT enzyme using the CAT enzyme-linked immunosorbent assay (Boehringer Mannheim), and 10 μl were used to measure the amount of β-galactosidase synthesized from the cotransfected construct, using the β-galactosidase enzyme-linked immunosorbent assay (Boehringer Mannheim). Promoter strengths were quantified by calculating the ratio of CAT/β-galactosidase activities from each transfected sample. To compare results from different experiments and calculate mean and S.D. values, promoter values are expressed relative to that of the wild-type *RanBP1* promoter (pTS-A construct), which was taken as 100% in most experiments unless otherwise indicated in the text.

Protein Extracts and Western Blot Assays—Extracts were prepared either from whole cells or after nuclei isolation as described in Ref. 23 with minor modifications. Briefly, cells were resuspended in two packed cell volumes of hypotonic buffer (10 mM Hepes, 10 mM NaCl, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM EDTA, and 1 mM EGTA), with freshly added aprotinin, leupeptin, and pepstatin A (1 μg/ml each), 1 mM sodium orthovanadate, and 1 mM sodium fluoride, incubated 30 min on ice and disrupted by repeated pottering while microscopically monitoring the incorporation of trypan blue (0.25% solution). The homogenate was centrifuged at $500 \times g$ for 20 min at 4 °C; the supernatant containing the cytoplasmic fraction was concentrated and adjusted to 150 mM NaCl (final concentration). The nuclear pellet was washed in five packed cell volumes of hypotonic buffer, resuspended in radioimmune precipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 1 mM EGTA) containing protease and phosphatase inhibitors as above, and lysed on ice. Gel electophoresis in SDS-polyacrylamide and electroblotting on polyvinylidene difluoride membranes were carried out as described elsewhere (9). Kaleidoscope molecular weight markers were from Bio-Rad. Membranes were blocked in 5% (w/v) low-fat milk in TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) at 4 °C overnight and then incubated for 2 h at room temperature with the following primary antibodies in 5% milk/TBST: anti-E2F-4 (C-20, Santa Cruz Biotechnology), anti-p27 (F-8, Santa Cruz Biotechnology), anti-α tubulin (Amersham Pharmacia Biotech) and anti-histone H1 (Upstate Biotechnology, Lake Placid). All primary antibodies were used at 0.5 μg/ml, except for anti-α tubulin, which was used 0.05 μg/ml. Bands were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and revealed using the enhanced chemiluminescence detection system (ECL-plus reagents, Amersham Pharmacia Biotech).

Gel Shift Assays—Protein extracts from NIH/3T3 cultures were prepared essentially as described in Ref. 35. All buffers and solutions contained 1 μg/ml each aprotinin, leupeptin, and pepstatin A; 4 mM sodium orthovanadate; and 4 mM sodium fluoride. The following oligonucleotides and their reverse complementary strands were used: Sp1–3, 5′-AATTCCGGCCCGCCCCGCGCTTG-3′; E2F-b, 5′-GCATCGCCGCGGGCGTTTTGGCGGGAAGCGC-3′; E2F-c, 5′-AATTCGCGTTTCCCGCCGCTG-3′; TATA, 5′-GCAGAGCATATAAGGTGAGGTAGGA-3′. All gel shift experiments were routinely performed using at least two independent extract preparations. Binding reactions with E2F oligonucleotides were set up as in Ref. 36 with 20–100 pg of [γ-^{32}P]ATP-labeled oligonucleotide and 7–10 μg of protein extract in a 20-μl reaction; 5 μg of protein were used in binding reactions using extracts enriched in E2F-1 and DP1. Gel shift conditions using the Sp1 and TATA oligonucleotides were described previously (9). For supershift experiments, antibodies (0.1 μg/μl of reaction) were added to the binding mixture for 3 h on ice. The following antibodies were used: pRb (C-15), p107 (SD9),

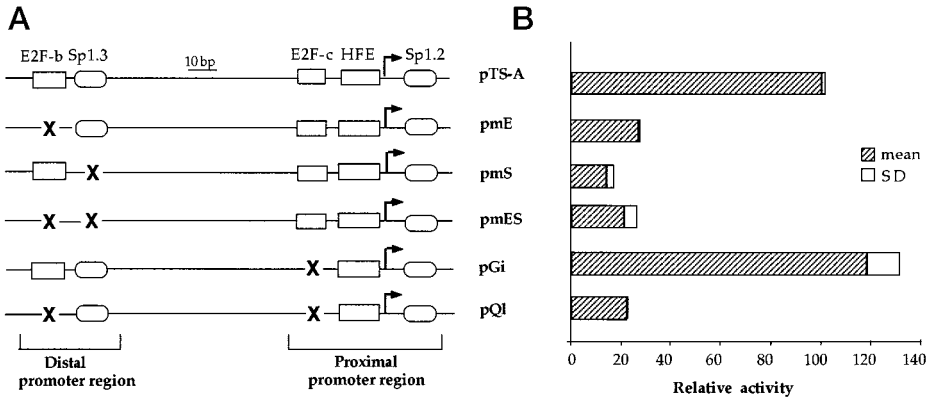

FIG. 1. **Activity of wild-type and mutated *RanBP1* promoter constructs in asynchronous NIH/3T3 cells.** *A,* map of assayed promoters; *crosses* indicate mutagenized sites, and *arrows* indicate the transcription start (TS-1). *B, bars* show the activity of reporter constructs relative to that of the wild-type promoter (pTS-A construct), which was taken as 100%. In these and all following experiments, absolute promoter strengths are calculated as CAT/β-galactosidase activity in each transfected sample. Mean and S.D. values were calculated from at least six independent assays for each construct.

p130 (C-20), E2F-1 (KH95), E2F-4 (C-20 sc866), E2F-5 (C-20), and Sp1 (PEP2) (all from Santa Cruz Biotechnology).

Northern Blot Experiments in E2F-overexpressing Cell Lines—Cell lines were all based on NIH/3T3 fibroblasts. Retroviral infections were used to establish stable E2F overexpressing cell lines. Expression constructs were based on the pBabe-puro vector harboring hemagglutinin-tagged versions of either E2F-1, E2F-2, E2F-3, E2F-4, or E2F-5 (see also Ref. 37). Cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn calf serum, starved by washing the cells twice with phosphate buffered saline and culturing them for 46 h in medium containing low serum, *i.e.* 0.25% (v/v) FCS, and restimulated for 15 h by applying high serum again. RNA was extracted as described in Ref. 38; 15–20 μg of total RNA/lane were used for Northern blot analyses. The probe was generated by polymerase chain reaction amplification of the p19.6 plasmid containing the *RanBP1* cDNA (GenBank™ accession X6045) and subsequent purification of the radiolabeled product on a Sephadex-G50 spin column. A glyceraldehyde-3-phosphate dehydrogenase cDNA probe was also used for control. Radioactive hybridization signals were both autoradiographed and quantified on a phosphorimager (Fuji).

RESULTS

Identification of RanBP1 Promoter Elements Required for Promoter Activity in Asynchronously Cycling Cells—Previous deletion mapping analysis established that a 273-base pair fragment from the *RanBP1* promoter, schematically shown in Fig. 1*A,* carries all the information required for control of transcription (11). Two sites match the E2F consensus: site E2F-c (positions −31 to −20 relative to the TS-1 transcription start) flanks an element termed HFE, acting as the target of retinoid receptors and required for basal transcription (9); site E2F-b (position −115 to −106), near a putative Sp1-binding site (Sp1.3, position −100 to −92), falls in a region previously identified for conferring G_1/S up-regulation of transcription (6). To investigate the contribution of each element to *RanBP1* promoter activity, several promoter constructs directing transcription of a CAT reporter sequence were synthesized by site-directed mutagenesis: the distal elements were mutated both individually in the E2F-b (pmE) and Sp1.3 (pmS) sites, or simultaneously (pmES); the proximal E2F-c site was mutated in the pGi construct, which maintained both wild-type distal sites; and finally, the pQl construct carries mutated versions of both E2F sites. Promoter reporter constructs were transfected in asynchronously cycling NIH/3T3 cells, and levels of synthesized CAT enzyme were measured (see under "Experimental Procedures" for details). Results in Fig. 1*B* show that transcriptional activity of all clones mutated in the distal sites was drastically reduced compared with the wild-type promoter; destruction of the Sp1.3 site impaired promoter activity somewhat more significantly than that of the E2F-b site; the simultaneous inactivation of the adjacent Sp1.3 and E2F-b sites did not amplify the effect of single mutations, indicating that both the Sp1.3 and E2F-b distal sites are important promoter elements in asynchronous cell cultures. In contrast, transcription from the E2F-c mutated construct (pGi) was comparable to, or slightly more efficient than, that of the wild-type promoter (Fig. 1*B*). Thus, efficient *RanBP1* promoter activity in cycling cells requires the integrity of both distal E2F-b and Sp1.3 sites.

The E2F Sites Exert Independent Roles in Cell Cycle-regulated RanBP1 Transcription—RanBP1 transcription is up-regulated after 15 h of cell cycle entry, *i.e.* when cells progress beyond the G_1/S transition (6, 7). To assess the role of particular promoter elements in up-regulation of transcription, the constructs shown in Fig. 1*A* were transfected in cell cultures that were serum-starved and either maintained in conditions of growth arrest or restimulated and harvested 15 h after release of the proliferation block; cell cycle arrest and progression through S phase were assessed by FACS analysis (Fig. 2*A*). We found that the activity of the wild-type pTS-A promoter construct was up-regulated after 15 h of restimulation (Fig. 2*B*) and ranged from 20 to 30% above the recorded level in asynchronously growing cells. None of the mutated promoters in the distal E2F-b or Sp1.3 sites (pmE, pmS, pmES, and pQl) reached the activity level of the wild-type construct in S phase: thus, G_1/S up-regulation of transcription requires the distal sites and cannot be sustained by the E2F-c site alone, as also seen in asynchronously proliferating cells (Fig. 1*B*). However, inactivation of site E2F-c (pGi construct) was not neutral, but resulted in failure of G_0-associated repression of transcription. Thus, formally distinct functions are exerted by each E2F site: E2F-c mediates transcriptional repression in arrested cells, whereas the neighboring E2F-b and Sp1.3 sites are both required for G_1/S activation of transcription. To further verify that site E2F-b acted as an activating promoter element, a 60-base pair long fragment from the *RanBP1* promoter retaining site E2F-b but not site Sp1.3 (6), was cloned upstream of the SV40 minimal promoter, formed by two copies of the 21-base pair repeat and a TATA box (39); as shown in Fig. 3*A,* in the pE1 chimeric derivative, site E2F-b is again immediately 5′ of a functional Sp1 site, yet is now inserted in a TATA-dependent context. These experiments (Fig. 3*B*) show that site E2F-b confers up-regulation to the SV40-derived promoter in an S phase-dependent manner.

The Distal E2F-b Site Is Responsive to Exogenously Expressed E2F Factors—The finding that each E2F site contributes to cell cycle-regulated *RanBP1* transcription through formally distinct mechanisms suggests that they respond differently to E2F factors. Co-transfection experiments were designed to assess the responsiveness of the wild-type *RanBP1* promoter and mutated derivatives to exogenously expressed E2F factors. These experiments were carried out in growth-arrested cultures, in which the basal activity of the *RanBP1* promoter is low. Both the E2F-1 and E2F-4 members of the family were chosen for this analysis: E2F-1 carries its own nuclear localization signal and is efficiently transported to the

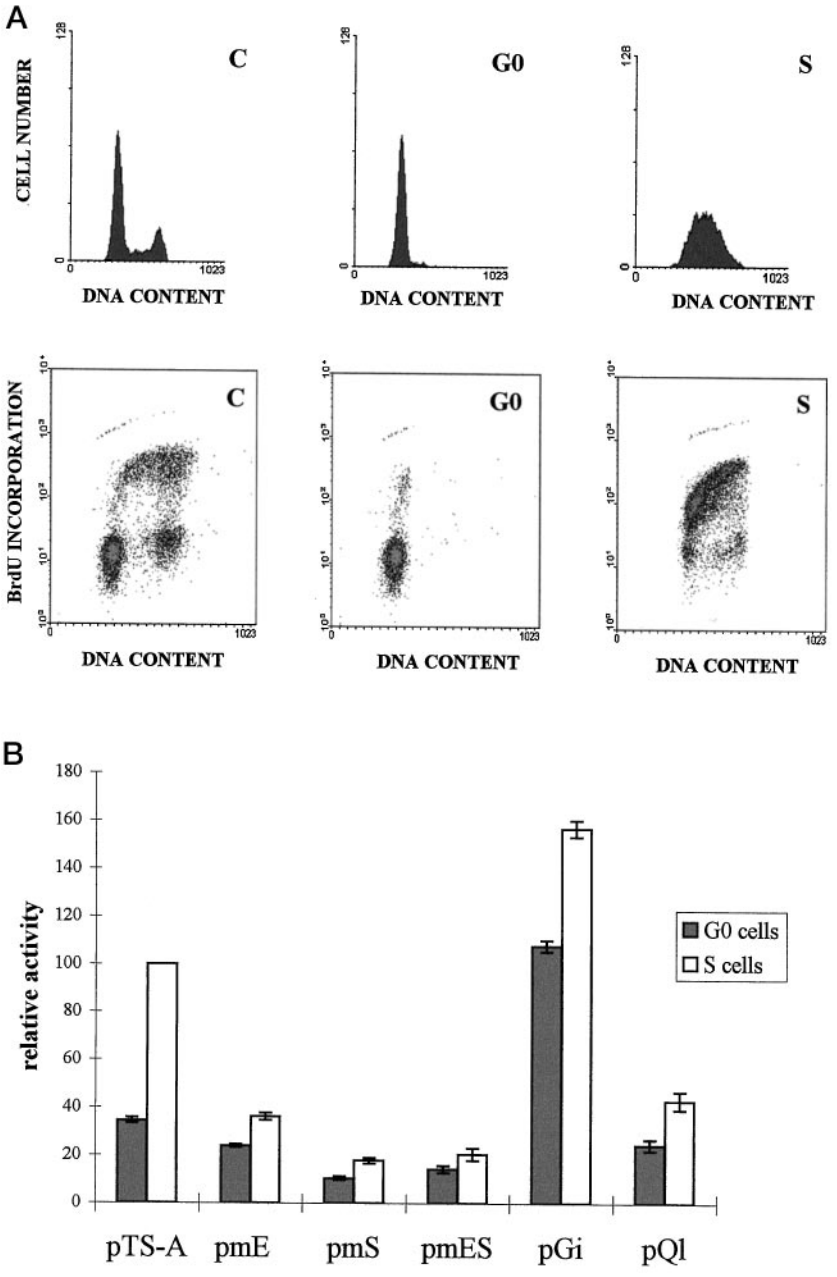

FIG. 2. **Activity of the wild-type and mutated *RanBP1* promoter constructs in growth-arrested and S phase cells.** *A,* FACS analysis of asynchronously cycling (*C*), serum-starved (*G0*) and S phase (*S*) NIH/3T3 cells harvested 15 h after cell cycle reentry. *Upper panels* show the DNA content of the cell populations as determined by Pr I incorporation; *lower panels* show a biparametric analysis of the cell cycle, in which bromodeoxyuridine incorporation (indicating the extent of DNA replication) is plotted *versus* the DNA content. *B,* relative activity of the wild-type and mutated promoter constructs in growth-arrested (*shaded histograms*) and S phase restimulated (*open histograms*) cell cultures. The mean value obtained for the wild-type promoter (pTS-A construct) in S phase-cells was taken as 100%; mean and S.D. (*bars*) values were calculated from the following number of experiments: 10 for pTS-A and pmE; 7 for pmS, pmES, and pGi; and 4 for pQl.

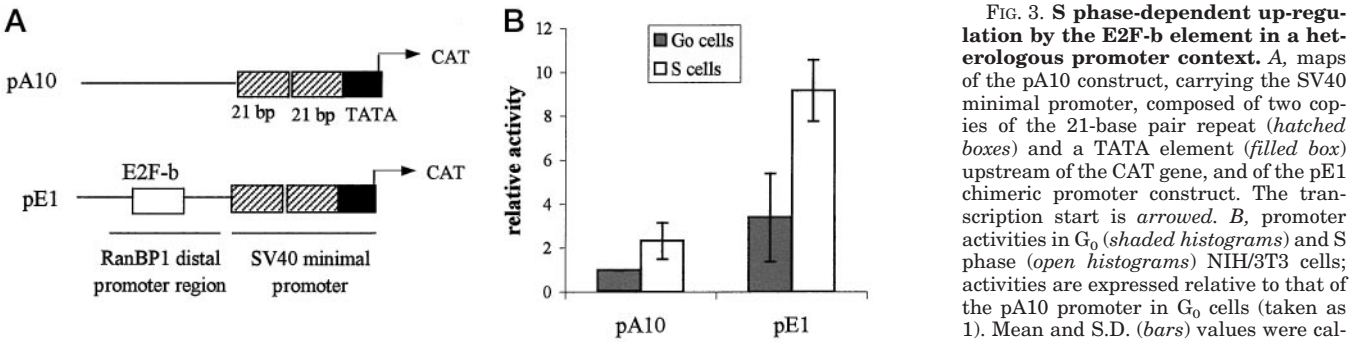

FIG. 3. **S phase-dependent up-regulation by the E2F-b element in a heterologous promoter context.** *A,* maps of the pA10 construct, carrying the SV40 minimal promoter, composed of two copies of the 21-base pair repeat (*hatched boxes*) and a TATA element (*filled box*) upstream of the CAT gene, and of the pE1 chimeric promoter construct. The transcription start is *arrowed. B,* promoter activities in G_0 (*shaded histograms*) and S phase (*open histograms*) NIH/3T3 cells; activities are expressed relative to that of the pA10 promoter in G_0 cells (taken as 1). Mean and S.D. (*bars*) values were calculated from three independent assays.

nucleus; in contrast, E2F-4 has no nuclear localization signal and is only transported to the nucleus when complexed with other proteins (23, 25). In our experiments, E2F-4 was used in combination with a construct expressing the DP1 dimerization partner.

Various molar ratios of E2F and DP expression constructs to CAT reporters were preliminarily assayed; FACS analysis revealed that transfected cells using high ratios of E2F-1, alone or with DP1, to reporter construct underwent significant apoptosis in response to a threshold level of E2F-1, as indicated by

the appearance of a hypodiploid cell population of high cellular density (data not shown). In our experiments, transfection of 0.5–1 μg of E2F-1 construct alone or of 1 μg of both E2F-4 and DP1 expression constructs per 10^6 cells induced a low level or no apoptosis and no significant change in the FACS profile of transfected compared with control cultures. Western blot experiments were carried out with extracts from transfected and control cultures: although the endogenous E2F-1 and E2F-4 factors were found to be expressed at different basal levels, with E2F-1 being virtually undetectable in G_0 cells, both plasmids yielded overexpression of both E2F members in transfected cells (data not shown). Under these conditions, repression of the wild-type *RanBP1* promoter (pTS-A construct) during growth arrest was fully relieved (Fig. 4). The E2F-4/DP1 combination restored promoter activity to a comparable level to that measured in S phase cells, and E2F-1 up-regulated it above S phase levels; transfection of E2F-4 had instead a minor effect in the absence of a co-transfected DP partner (data not shown).

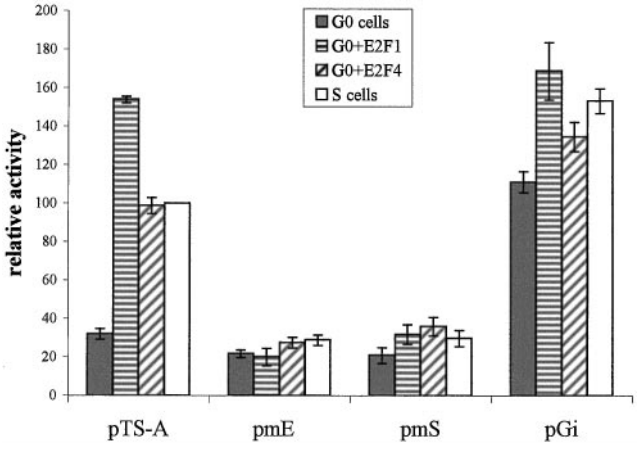

FIG. 4. **Activity of the wild-type and mutated *RanBP1* promoter constructs in the presence of exogenous E2F-1 or E2F-4 plus DP1, factors.** *Histograms* represent the relative activity of reporter promoter constructs in the presence or absence of constructs expressing either E2F1 or the E2F4/DP1 combination in G_0 cells; for comparison, promoter activities were also assessed in restimulated cells collected during S phase. pTS-A activity during S phase was taken as 100%. Mean and S.D. (*bars*) values were calculated from nine (for pTS-A) or four (for pmE, pmS, and pGi) cotransfection experiments with each set of constructs.

The relief of repression in G_0 cells was dependent on the integrity of the distal sites, because the pmE construct failed to respond to exogenous E2F factors. The pmS construct also showed a very low level of basal transcription, which was only modestly increased by exogenous E2F factors; thus, Sp1.3 site disruption impaired the responsiveness of the neighboring E2F-b site to exogenous E2F factors. Conversely, the pE1 construct, in which the E2F-b site flanks the SV40 Sp1 sites (see Fig. 3*A*), was up-regulated by exogenous E2F factors in growth-arrested cells (data not shown). Finally, the pGi construct, which is mutated in the proximal E2F-c site yet maintains both functional E2F-b and Sp1.3 sites, was highly expressed in G_0 cells; the overall activity further increased above (with exogenous E2F-1) or close to (with the E2F-4/DP combination) S phase levels, further confirming that responsiveness to E2F factors was essentially conferred by the distal site. Therefore, the data in Fig. 4 depict a specific responsiveness of the E2F-b site to exogenous E2F factors.

Regulation of RanBP1 Promoter Activity by Pocket Proteins—We next asked how the *RanBP1* promoter would respond to pocket proteins by cotransfecting wild-type or mutagenized promoters in the presence of constructs expressing pRb, p107, or p130 proteins. We firstly examined cells stimulated to cycle and harvested during S phase, when *RanBP1* promoter activity is highest. All three pocket proteins clearly affected the wild-type promoter (pTS-A subclone) and reduced its activity by 50–60% (Fig. 5*A*). However, repression by exogenous pocket proteins (particularly p130) in S phase cells was less effective than that induced by endogenous factors in G_0 cells. Incomplete repression may reflect the partial inactivation of pocket proteins by cyclin/kinase complexes in S phase cells. Indeed, the dose of transfected pocket proteins used in our experiments did not significantly alter the cycling profile of transfected, compared with control, cultures, as indicated by FACS analysis (data not shown); in those conditions, exogenous pocket proteins underwent S phase-specific modifications similar to their endogenous counterpart. In particular, Western blot experiments showed that p130, although overexpressed from the plasmid vector in G_0 cells, underwent degradation in S phase cells to a similar extent than the endogenous protein (data not shown). Indeed, overexpression of pocket proteins in G_0 cultures effectively repressed the *RanBP1* promoter (Fig. 5*A*). Mutagenized promoters in both distal sites (pmE, pmS, and pmES constructs) showed a low basal activity, and it was difficult to appreciate whether further repression by exogenous

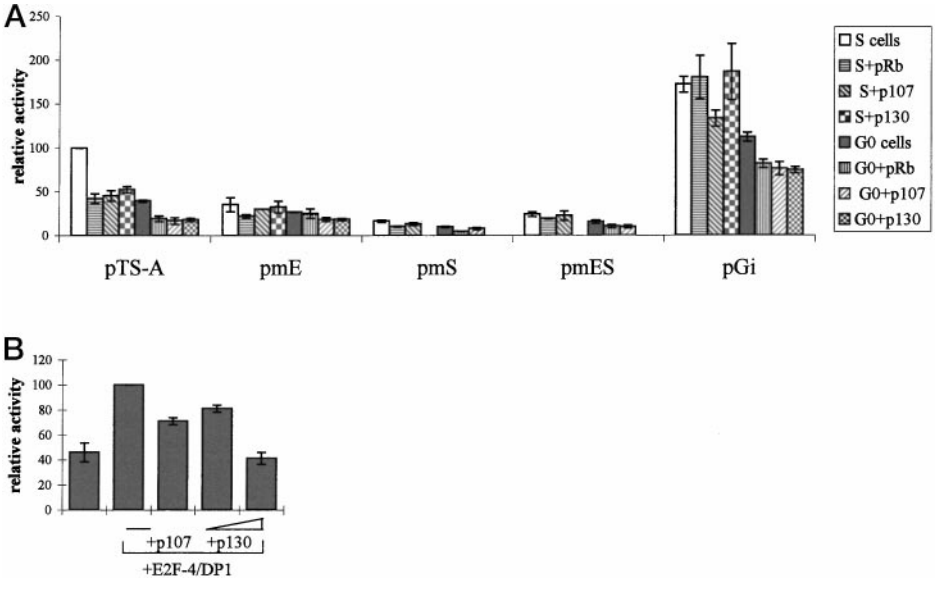

FIG. 5. **Activity of the wild-type and mutagenized *RanBP1* promoter constructs in the presence of exogenous pocket proteins in S phase and G_0 cells.** *A, histograms* represent the relative activity of the wild-type (pTS-A) and mutagenized constructs in S phase and G_0 cells, in the presence of constructs expressing pocket proteins as indicated. The activity of pTS-A in S phase cells was taken as 100%. Mean and S.D. (*bars*) values were calculated from four cotransfection experiments for each construct. Activity of pmS and pmES were not assayed in the presence of p130. *B, histograms* represent the relative activity of the pTS-A promoter in G_0 cells alone or in the presence of the E2F-4/DP1 combination (1 μg each), with or without constructs expressing pocket proteins: 1.5 and 3 μg of p130 construct, or 1.5 μg of p107 construct were used. The activity of pTS-A with E2F-4/DP1 was taken as 100%. Mean and S.D. (*bars*) values were calculated from three cotransfection experiments.

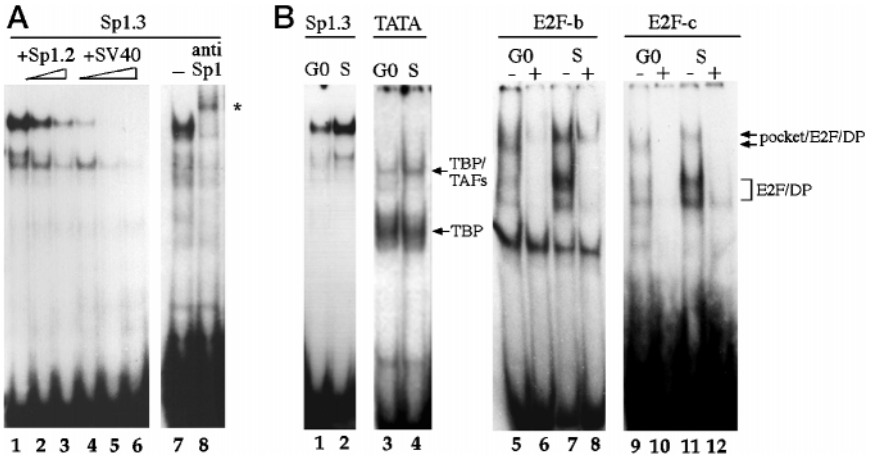

FIG. 6. **Protein binding features of the *RanBP1* promoter elements.** *A,* gel shift assays of the Sp1.3 oligonucleotide with 5 μg of NIH/3T3 extract from asynchronously growing cells (*lanes 1* and *7*) and increasing amounts of site Sp1.2 from the proximal *RanBP1* promoter (*lanes 2–3*) or of SV40-derived Sp1 site (SV40, *lanes 4–6*), and in the presence of anti-Sp1 antibody (*lane 8*). The *asterisk* marks the Sp1 supershift. *B,* gel shift experiments using extracts from growth-arrested (*G0*) and S phase (*S*) NIH/3T3 cells, with Sp1.3 (*lanes 1* and *2*), TATA (*lanes 3* and *4*), E2F-b (*lanes 5–8*), and E2F-c (*lanes 9–12*) oligonucleotides; 5 μg (*lanes 1–4*) or 10 μg (*lanes 5–12*) of protein extracts were used. The association of TAFs (TATA box-binding protein (*TBP*)-associated factors) in *lanes 3* and *4* marks the assembly of transcriptionally competent complexes, whereas the formation of the TATA box-binding protein-TATA complex is a relatively constant event. In *lanes 5–12,* − and + indicate the absence and presence, respectively, of homologous competitor DNA (50-fold excess).

pocket proteins was statistically significant. Interestingly, the pGi construct, the basal activity of which was high in S phase and which might therefore have been susceptible of undergoing significant variations in the presence of repressing factors, did not show the 50–60% reduction typical of the wild-type promoter and was essentially unaffected by pocket proteins. Pocket proteins caused indeed some repression in G_0 cells, which was evidently mediated by the distal region; however, pGi activity in the presence of all three pockets remained substantially higher than that of the wild-type promoter in the same conditions, indicating that integrity of site E2F-c is required for effective repression by pocket proteins.

Quiescence-associated repression by pocket proteins is largely exerted by interacting with E2F promoter elements through E2F factors, particularly E2F-4, which represents the most abundant family member in G_0 cells (reviewed in Ref. 14). In order to rule out the possibility that the results in Fig. 5*A* simply reflected a nonspecific inhibition of transcription by pocket factors, E2F-4 and DP1 were coexpressed with pocket-encoding constructs in cotransfection experiments with the *RanBP1* promoter. In these experiments, we analyzed both antagonistic partners of E2F-4, *i.e.* p107 and p130. As shown in Fig. 5*B,* both proteins counteracted relief of G_0 repression by E2F-4/DP1; p130, which normally acts as the preferential E2F-4 partner in G_0 cells (reviewed in Refs. 12 and 14), actually antagonized E2F-4/DP1-mediated activation in a dose-dependent manner and eventually reduced *RanBP1* promoter activity down to the low basal level normally seen in G_0 cells. Thus, the *RanBP1* gene is indeed a regulatory target of pocket proteins during growth arrest, which act at least in part through the interaction with E2F-4.

The Regulatory Elements in the RanBP1 Promoter Show Cell Cycle-regulated Interactions with DNA-binding Factors—We next examined the interactions established between DNA-binding factors and genetically identified promoter elements. Preliminary experiments were carried out to characterize site Sp1.3 using extracts from asynchronously cycling cells, where the site showed *in vivo* occupancy by protein factors (10). The highest proportion of the assembled complex migrated with an electrophoretic mobility compatible with that of Sp1 (Fig. 6*A, lanes 1* and *7*) was competitively inhibited by excess of canonical Sp1 sites, including site Sp1.2 from the *RanBP1* proximal promoter (*lanes 2–3*) and an SV40 Sp1 site (*lanes 4–6*), and was supershifted by the addition of anti-Sp1 antibody (*lane 8*), indicating that Sp1.3 is indeed a bona fide Sp1-binding site.

Interactions established during cell cycle progression were analyzed using protein extracts from growth-arrested and S phase cells (Fig. 6*B*). Site Sp1.3 formed an abundant complex with S phase cell extracts, whereas a minor proportion of the probe interacted with factor(s) from growth-arrested cells (Fig. 6*B, lanes 1–2*); these results did not reflect a lower content of transcription factors in extracts from G_0, compared with S phase, cells, because the TATA box-binding protein had a comparable abundance in extracts from both sources (Fig. 6*B, lanes 3–4*). When the E2F sites were incubated with extracts from both starved and restimulated cells (Fig. 6*B, lanes 5–12*), site E2F-b was found to assemble more abundant nucleoprotein complexes (*lanes 5–8*) than did site E2F-c, the highest proportion of which migrated as free probe (*lanes 9–11*), indicating a generally higher avidity for DNA-binding proteins of site E2F-b compared with E2F-c. Regardless of their different affinity, both E2F sites interacted with proteins in a cell cycle-regulated manner: S phase complexes were more abundant than seen with growth-arrested cell extracts. Qualitative differences were also apparent between G_0 and S phase in the assembly of high molecular weight complexes, the migration of which was compatible with that of multimeric complexes containing E2F/DP/pocket proteins (Fig. 6*B,* compare *lanes 5* and *7* and *lanes 9* and *11*), indicating that G_0-specific interactions had been disrupted and replaced by S phase-specific complexes.

To define these interactions in more detail, binding reactions with both E2F sites were carried out in the presence of antibodies directed against particular E2F or pocket members. Representative panels are shown in Fig. 7. G_0 complexes assembled with both E2F probes essentially reacted with antibodies directed against E2F-4, p130, and pRb. When S cell extracts were used, however, the probes displayed a different antibody reactivity: E2F-b effectively interacted with pRb as indicated by the abundance of supershifted complexes, whereas the reactivity to anti-pRb antibody was significantly lower with the E2F-c probe (compare Fig. 7*A, lane 12,* and 7*B, lane 15*). Because the absolute amount of pRb protein in the binding reaction is identical, this observation suggests that reactivity to the preferential partner of pRb in S phase cells, *i.e.* E2F-1,

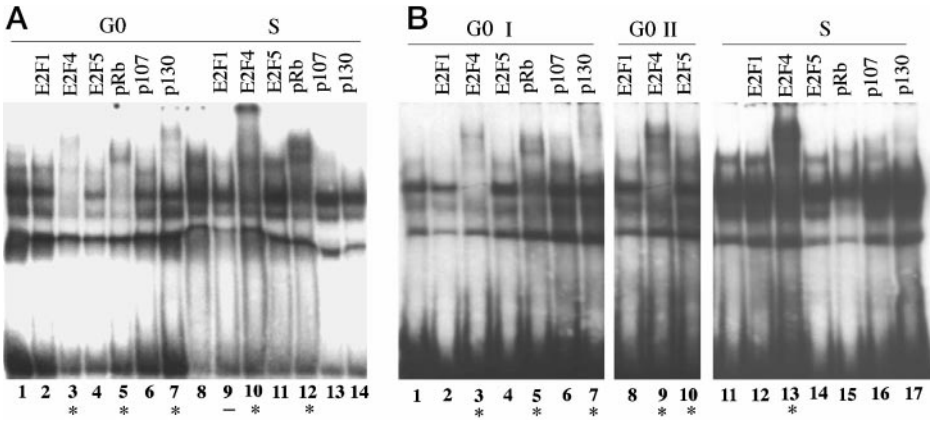

FIG. 7. **Immunological analysis of complexes interacting with sites E2F-b and E2F-c with growth-arrested (*G0*) and S phase (*S*) cell extracts.** Assayed antibodies are specified above each lane. Supershifts can be seen in lanes marked by the *asterisk*, whereas antibody interference is indicated by −. *A,* supershift assays of complexes binding to the E2F-b probe. *B,* supershift assays of complexes binding to the E2F-c probe; all panels correspond to a standard 15-h exposure, except for *lanes 8–11,* which show a longer exposure of *lanes 2–4* to visualize the anti-E2F-5 antibody reaction.

differed for the two probes. Indeed, E2F-c interacted with E2F-4 and, to a lesser extent, E2F-5 (as revealed on a prolonged gel shift exposure, see Fig. 7*B, lane 10*) among E2F family members. Complexes assembled with site E2F-b were also supershifted by anti-E2F-4 and, in addition, were recognized by anti-E2F-1, which interfered with the assembly of DNA-binding complexes with E2F-b, but not E2F-c, probe (compare Fig. 7*A, lane 9,* to Fig. 7*B, lane 12*). This differential interference of the anti-E2F1 antibody was confirmed in several independent experiments using different preparations of S phase cell extracts and different lots of antibody (data not shown). We reasoned that the absence of a discrete supershift might have reflected the low relative abundance of the E2F-1 species compared with other E2F family members. We therefore decided to assess enriched extracts from asynchronously cycling cells transfected with constructs expressing both E2F-1 and DP1: gel shift experiments (Fig. 8) showed that the E2F-b probe now assembled an abundant complex that was reactive to anti-E2F-1 (*lanes 1* and *2*); binding experiments were also carried out in conditions of partial competition with the heterologous E2F-c probe, with the expectation that a low molar excess of heterologous site would competitively inhibit those DNA-binding complexes that have similar affinity for both sites, yet would not interfere with the assembly of high affinity complexes. Indeed, a 10-fold excess of unlabeled E2F-c site competitively inhibited most DNA-binding complexes assembled with probe E2F-b, yet left a discrete E2F-1 supershift (Fig. 8, *lanes 3* and *4*), indicating that the interaction of factor E2F-1 with site E2F-b was substantially unaffected. In the reverse experiment using E2F-c as the probe, no DNA-binding component reactive to anti-E2F-1 antibody was visualized (Fig. 8, *lanes 5–8*). These results indicate that E2F-1 binds site E2F-b but not, or only very poorly, E2F-c. The reactivity of the distal site to anti-E2F-1 antibody was further increased when a longer probe was generated that included both adjacent E2F-b and Sp1.3 sites (data not shown), suggesting that the simultaneous binding of both factors to the DNA increases the complex stability.

Binding experiments thus far were carried out with whole cell extracts. However, E2F-4 is functionally regulated by compartmentalization (23–26). Therefore, it was important to assess whether the binding of E2F-4, depicted as the most abundant DNA binding activity in Fig. 7, was biologically significant. NIH/3T3 cells were brought to growth arrest and restimulated for 15 h; nuclei were isolated, and protein extracts were prepared from the nuclear and cytoplasmic fractions (23). E2F-4 was similarly abundant in whole cell extracts from both G_0 and S phases in Western blot assays; however, although it is nuclear in G_0 cells, the bulk of E2F-4 protein was found in the cytoplasm during S phase (Fig. 9). In order to ascertain that the

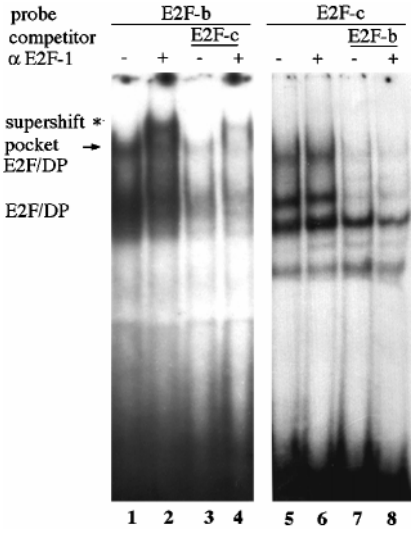

FIG. 8. **Supershift assays using extracts from cells transfected with E2F-1/DP constructs.** 5 μg of protein extract were used with probes E2F-b (*lanes 1–4*) and E2F-c (*lanes 5–8*) in the presence of anti-E2F1 antibody (*α E2F-1, lanes 2, 4, 6,* and *8*), with (*lanes 3, 4, 7,* and *8*) or without (*lanes 1, 2, 5,* and *6*) heterologous competitor site. A 10-fold molar excess of heterologous DNA was used to achieve a partial competition; the *asterisk* marks the E2F-1 supershift.

anti-E2F-4 reactive material depicted in Western assays was biologically active in our cell cultures, as previously shown in several cell lines (25, 26, 40), we assayed these extracts in gel shift experiments. Most DNA binding activity reactive to anti-E2F-4 antibody was found in extracts from G_0 nuclei; however, the distribution of anti-E2F-4 reactive protein was reversed in S phase extracts, with the highest proportion of E2F-4 DNA binding activity being detected in the cytoplasmic fraction (data not shown). These findings together suggest that the highest proportion, if not all, of the E2F-4 pool has exited the nucleus of S phase cells.

In summary, protein binding experiments indicate that all *RanBP1* promoter elements establish cell cycle-regulated interactions with factors. The binding of Sp1 to site Sp1.3 is quantitatively up-regulated in S phase. In addition, subtle sequence differences affect the assembly of S phase complexes with the E2F sites. Both sites interact similarly with complexes containing E2F-4 and pocket proteins in G_0 cells; however, assay of nuclear and cytoplasmic extracts indicate that E2F-4 is exported out of the nucleus in S phase; at the same time, newly assembled complexes containing E2F-1 preferentially interact with site E2F-b, which actively contributes to S phase up-regulation of the *RanBP1* promoter, but not with site E2F-c, which confers G_0 repression.

The Activating Role of Site E2F-b Is Dependent on Its Position in the Promoter Context—We finally asked whether the DNA sequence preference depicted *in vitro* for factor E2F-1 was the sole determinant of the different genetic functions exerted by the two E2F sites in control of *RanBP1* transcription. To ask that question, we moved site E2F-b, *i.e.* the preferred target of the E2F-1 activator *in vitro*, to the proximal position that is normally occupied by site E2F-c. Two novel reporter constructs were generated (Fig. 10*A*): pBB carries duplicated E2F-b, but no E2F-c, sites, whereas pmBB carries a mutated E2F-b site in the distal region and a wild-type E2F-b sequence replacing the proximal E2F-c site. CAT assays of these constructs in growth-arrested and restimulated cells (Fig. 10*B*) showed that pBB, despite carrying two copies of the E2F-b site, was not up-regulated any more efficiently than the wild-type promoter in S phase cells. In addition, in the absence of a functional distal region (pmBB construct), the E2F-b site failed to reinstate S phase activity from the proximal position normally occupied by site E2F-c, contrary to what would have been expected if the activating function of the E2F-b element was exerted in a position-independent manner. These results suggest that the activating function of site E2F-b does not simply require the element integrity but has to be exerted from its natural position in the native promoter context and/or requires proximity with the flanking Sp1 site.

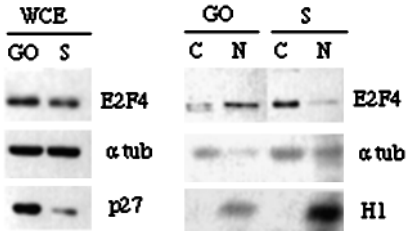

FIG. 9. **Distribution of the E2F-4 protein during the cell cycle.** Western blot of whole cell extracts (*WCE*) during growth arrest (*G0*) and S phase (*S*) analyzed with anti-E2F-4 antibody. Anti-α-tubulin and anti-p27 antibodies were used to control protein loading and cell cycle reentry, respectively. Subcellular compartmentalization was analyzed using cytoplasmic (*C*) and nuclear (*N*) extracts from synchronized G_0 and S phase cultures and analyzed with an antibody against E2F-4. Antibodies to α-tubulin and histone H1 were used to control the purity of the cytoplasmic and nuclear fractions.

E2F-1 Up-regulates Endogenous RanBP1 mRNA Transcription—Cotransfection assays in which both the promoter and the E2F expression constructs are overexpressed elicit the responsiveness of particular cis-active sequences to E2F family members, yet do not indicate which E2F factor actually regulates transcription of the endogenous *RanBP1* gene *in vivo*. That question was examined in NIH/3T3 cell lines that were infected with retroviral vectors directing the synthesis of individual E2F members. Cell lines overexpressing E2F-1 to E2F-5 factors were generated, and several independent clones were isolated for each E2F member. We ascertained that expression levels of the exogenous E2Fs were equal among cell lines; in

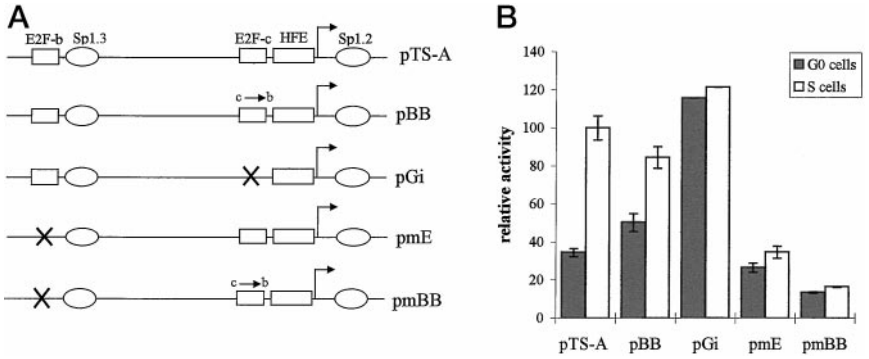

FIG. 10. **Activity of *RanBP1* promoter constructs carrying displaced elements in growth-arrested and S phase cells.** *A,* map of assayed promoters; *crosses* indicate sites inactivated by mutagenesis. In constructs pBB and pmBB, site-directed mutagenesis was employed to replace the E2F-c sequence with E2F-b (*c → b box*); the transcription start (TS-1) is *arrowed. B,* relative activity of promoter constructs in cells cultured as for Fig. 2*A* to induce growth arrest (*shaded histograms*) and synchronous S phase progression (*open histograms*). The mean value obtained for the wild-type promoter (pTS-A construct) in S phase-cells was taken as 100%; mean and S.D. (*bars*) values were calculated from four independent experiments.

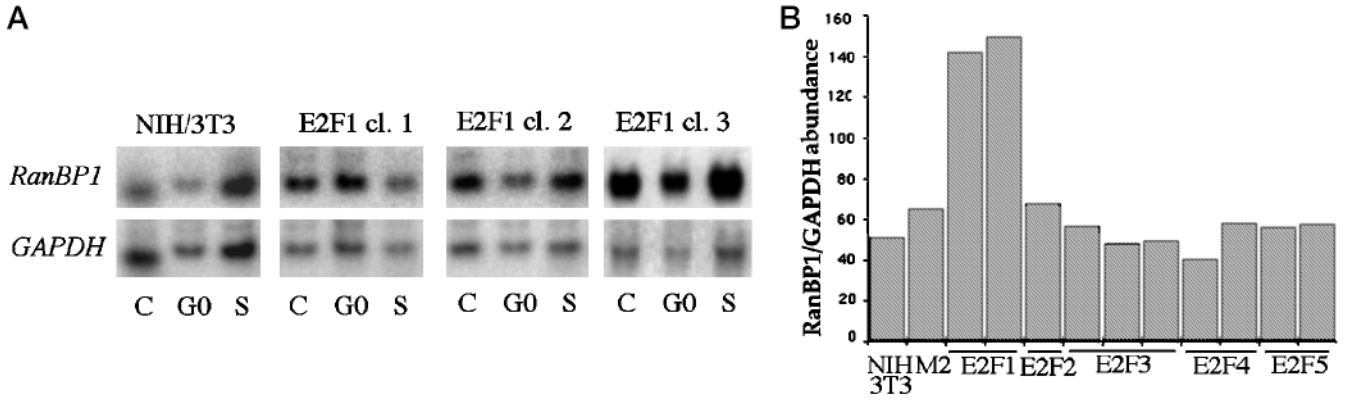

FIG. 11. **Expression of the endogenous *RanBP1* gene in cells overexpressing E2F factors.** *A,* Northern blot analysis of the *RanBP1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs in parental NIH/3T3 cell cultures and in three independently selected clones overexpressing E2F-1. *C,* asynchronously cycling cultures; *G0,* growth-arrested cultures; *S,* restimulated cells collected 15 h after cell cycle reentry. *B,* the *histograms* represent the *RanBP1*/*GAPDH* mRNA levels, as quantified by phosphorimager reading of Northern blots from different E2F-overexpressing cell lines in high serum, compared with control (NIH/3T3) and vector-infected (M2) cells. Overexpressed E2F factors are indicated *below* each lane.

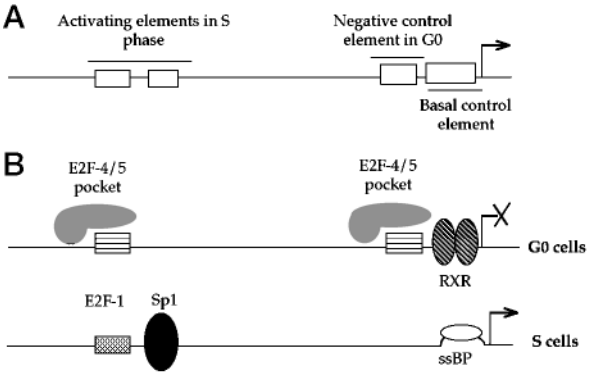

FIG. 12. **Growth control and cell cycle control elements in the *RanBP1* promoter.** *A,* genetic functions of *RanBP1* promoter elements identified in loss of function experiments; the *arrow* indicates the major start site of transcription. *B,* hypothetical model summarizing the interactions of positively and negatively acting factors with promoter elements in G_0 and in S phase cells. *ssBP,* single-stranded DNA-binding protein. × indicates transcriptional repression in growth-arrested cells.

addition, the gene encoding cyclin E, a known target gene of E2F-1, -2, and -3 factors (41–43), showed a 5–10-fold induction;[2] a more detailed characterization of the cell lines will be reported elsewhere. Northern blot experiments showed that *RanBP1* mRNA levels are indeed up-regulated in cell lines that overexpress E2F-1 compared with parental NIH/3T3 cells, or to cells infected with viral vector alone (M2 cell line). Whereas the *RanBP1* and *GAPDH* mRNA transcripts are expressed with comparable abundance in control NIH/3T3 cells, *RanBP1*, but not *GAPDH*, mRNA transcription shows significantly increased levels in three independently selected E2F-1 overexpressing clones (Fig. 11*A*). Cell lines expressing other members of the E2F family show no significant difference in the expression level of *RanBP1* mRNA compared with control cultures (Fig. 11*B*). Thus, *RanBP1* is indeed a regulatory target of activation by E2F-1 *in vivo*.

DISCUSSION

In the present work, we have addressed the mechanisms controlling cell cycle-regulated transcription of the *RanBP1* gene. We have concentrated on two E2F-binding elements in two previously identified promoter regions that control G_1/S up-regulation (6, 7) and basal transcription (9), respectively. We have found that each E2F site controls a distinct aspect of *RanBP1* transcription: the distal E2F-b site acts as an activating element and, together with the flanking Sp1.3 element, confers high levels of transcriptional activity during S phase. The proximal E2F-c site instead acts as a negative control element during quiescence. Conclusions drawn from the genetic dissection of the *RanBP1* promoter are schematized in Fig. 12*A*.

Although the dual role of E2F elements in positive and negative control of transcription is well documented (reviews in Refs. 12–14; also see Ref. 44), few promoters contain E2F sites in which the functions are clearly separated in genetic terms. Different functional properties were previously attributed to two E2F sites in the *p107* gene promoter (45): mutagenesis of the proximal site affected promoter activity more dramatically than that of the distal site, indicating a functional hierarchy between the sites, and the amplified effect of simultaneous compared with single site mutations suggested cooperation. In the *RanBP1* promoter, the E2F sites actually exert opposite functions; hence, we have asked which molecular mechanisms underlie these distinct roles.

Both sites showed cell cycle-regulated interactions with factors, yet their protein binding specificity was different. G_0 complexes formed with both sites showed a similar reactivity to antibodies, particularly against E2F-4 and p130. Most E2F-4 DNA binding activity exited the nucleus of NIH/3T3 cells during S phase, as revealed by Western blot assays (Fig. 9), in agreement with studies in different cell lines (23–26, 28, 40). At that stage, a differential reactivity to E2F-1 was depicted for each site, because antibody interference in crude extracts and supershift in overexpressing extracts indicated that only E2F-b, but not E2F-c, interacted with E2F-1 (Figs. 7 and 8). Thus, E2F-1 shows some DNA sequence preference, in agreement with results of recent CASTing experiments that pinpointed a differential affinity of specific E2F/DP heterodimers for particular E2F sequences (46). Preferences in E2F/DNA interaction are reflected by the composition of pocket-containing complexes: both sites showed a marked reactivity to the anti-p130 antibody with G_0 extracts; in S phase extracts, most p130-containing complexes had been disrupted as indicated by the weak antibody reactivity. Complexes assembled with site E2F-b, but not E2F-c, were now supershifted by anti-pRb, further supporting the idea that E2F-b is a target of E2F-1, which interacts with high affinity with pRb.

The different protein binding abilities *in vitro* are paralleled by differences both in site occupancy *in vivo* and in the activity of mutagenized promoters. E2F-b falls in an extended genomic footprint also covering site Sp1.3 (10). Transcriptional activation of *RanBP1* at the G_1/S transition (7) is concomitant with the synthesis of new E2F-1 (47, 48). The E2F-b site is a target of E2F-1 *in vitro*, and mutational inactivation of that site impairs transcriptional activation in S phase (Fig. 2*B*). Finally, overexpression of E2F-1, but not of other E2F factors, activates endogenous *RanBP1* transcription (Fig. 11). These data indicate that *RanBP1* G_1/S up-regulation is mainly controlled by E2F-1 via the E2F-b site.

The DNA sequence preference is not sufficient, however, to determine *per se* the functional role of E2F elements. The position in the promoter context is also relevant. Insertion of the E2F-b site away from the Sp1.3 site, near the HFE, did not support G_1/S up-regulation in the absence of a functional distal site (Fig. 10). In contrast, insertion of site E2F-b in the SV40, TATA-dependent promoter near an Sp1-binding site contributed to S phase activation (Fig. 3). Previous reports (29, 30) indicate that neighboring E2F and Sp1 elements cooperate in cell cycle-regulated transcription. Karlseder *et al.* (29) showed physical interaction between Sp1 and E2F factors and demonstrated cooperation in DNA binding by genomic footprinting analysis of target promoter elements. In our experiments, mutation of the Sp1.3 site affected the *RanBP1* promoter responsiveness to exogenous E2F factors, despite the integrity of the E2F-b site (Fig. 4), and inactivation of E2F-b (pmE), Sp1.3 (pmS), or both (pmES), similarly impaired transcriptional activity (Fig. 2*B*). These findings suggest that full S phase activity requires the simultaneous interaction of Sp1 and E2F-1 with their target sites in the distal region.

Site E2F-c acts instead as the target of repressing factors during growth arrest, because E2F-c mutation derepressed the *RanBP1* promoter and alleviated the requirements for exogenous activators in G_0 cells. Other E2F sites acting as repressing elements include those of the genes encoding B-myb (49), cyclin A (50), and E2F-1 itself (51), and mutagenesis at those sites renders these promoters constitutively active. In these instances, E2F factors essentially act as vectors of repressor molecules to the promoter. In our experiments, E2F-4/DP complexes can activate or inhibit *RanBP1* transcription depending

[2] R. M. Kerkhoven and R. Bernards, unpublished data.

on their ratio to pocket proteins: E2F-4/DP1 act as activating complexes when overexpression levels are such that endogenous pocket proteins are titrated (Fig. 4), yet exert an inhibitory role in the presence of co-expressed pocket proteins (Fig. 5*B*), implying that they can indeed bridge repressing factors to the *RanBP1* promoter.

Together, the results support the model in Fig. 12*B*: in the proposed model, E2F-4 would essentially act as a vector of repressors in G_0 cells. Although both E2F sites can interact with E2F-4, site E2F-c is essential for effective repression. E2F-c is flanked by the HFE, encompassing the TS-1 site. Previous characterization of that element showed binding by retinoid X receptor members in quiescent cells, associated with promoter inactivity (9). During transcriptional commitment, retinoid X receptors are displaced by single-stranded binding proteins that expose the TS-1 site, as depicted by the appearance of sensitivity to nuclease S1 *in vivo* (9). It is possible that pocket-bound E2F factor(s) interacting with site E2F-c and retinoid X receptors binding to the HFE stabilize each other and/or act in concert to determine promoter inactivity in G_0 cells. During G_1 progression, pocket protein phosphorylation and nuclear export of E2F-4 would free the promoter from repressing factors; at the same time, coordinated up-regulation of E2F-1 and Sp1 factors would yield a stable complex over the distal promoter elements and hence up-regulate *RanBP1* transcription.

These results contribute to identify two levels of control for the genes of the Ran signaling network. Examined members thus far are regulated by growth-dependent mechanisms: *Ran* is induced as an immediate-early responsive gene in the presence of serum (52), and *RCC1* is induced by the Myc protein through an E-box promoter element (53). In *RanBP1*, the alternative binding either of retinoid receptors in quiescent cells or of single-stranded DNA-binding proteins in cycling cells to the HFE act as mutually exclusive signals from the proliferation apparatus to the transcription machinery. These mechanisms may converge to coordinately down-regulate the genes of the Ran network in quiescent and terminally differentiated cells. In cycling cells, the Ran network is active in cell cycle coordination (reviewed in Refs. 4 and 5). It has been shown that the molecular balance between RanBP1 and RCC1 is crucial in this control (8, 54). We have shown here that this is essentially achieved by the binding of E2F-1 and Sp1 to upstream promoter sites, yielding high levels of *RanBP1* transcription during S phase. These findings further support the view that *RanBP1* may act as a pivotal gene linking cell cycle-regulated transcription by E2F factors and the coordination of cellular processes controlled by the Ran network.

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