Transcriptional Control of the Htf9-A/RanBP-1 Gene during the Cell Cycle

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Abstract

The mouse Htf9-a gene encodes a small hydrophilic protein, named Ran-binding protein 1 (RanBP-1), for its interaction with the Ras-related Ran protein; RanBP-1 binds the biologically active form of Ran. Ran has been implicated in a variety of nuclear events including DNA replication, RNA export and protein import, and monitoring completion of DNA synthesis before the onset of mitosis; its biological activity is thought to be regulated in S phase. We have investigated whether expression of the Htf9-a/RanBP-1 gene is linked to cell proliferation. Expression of the Htf9-a/RanBP-1 transcript appeared to be dependent on the proliferating state of the cells and peaked in S phase. We have identified a putative region required for Htf9-a cell cycle-dependent expression and for responsiveness to cell cycle regulators. An E2F-binding site was identified within the cell cycle-regulated promoter region. Expression of the E1A oncprotein prevented Htf9-a down-regulation in quiescent cells; in addition, both pRb and its relative p107 inhibited transcription from the Htf9-a promoter. These results link the control of Htf9-a/RanBP-1 expression to the cell cycle progression.

Introduction

The mouse Htf9 locus was originally identified for its association with a CG-rich DNA sequence and was found to contain two unrelated genes, Htf9-a and Htf9-c, divergently transcribed from a common bidirectional promoter (Refs. 1 and 2; see map in Fig. 1). The locus was mapped to a region of mouse chromosome 16 subsequently identified in a genome-wide tumor screening for putative tumor suppressor genes and termed LOH2 (LOH1 region 2; Ref. 3). Although both Htf9-associated genes are ubiquitously expressed, the Htf9-a mRNA is particularly abundant in tissues having a high proliferation index compared to tissues mostly composed of quiescent cells (1).

The Htf9-a cDNA encodes a predicted protein of M 23,500, is highly hydrophilic, and contains overlapping sites for protein kinase C and for the calmodulin-dependent multi-protein kinase, which are used in alternative phosphorylation pathways in mammalian cells (2). The Htf9-a gene was also newly isolated from a mouse embryo cDNA expression library (4), and its product was named RanBP-1 for its association with the Ran/TC4 protein, a nuclear member of the Ras family sharing extensive similarities with Ran in the GTP-binding domain (5, 6). Accordingly, we now refer to the gene using the double nomenclature Htf9-a/RanBP-1.

Studies in yeast and in higher eukaryotic cells have shown that Ran regulates a variety of cellular events by transducing a GTP signal in the nucleus; Ran is implicated in RNA export and protein import (reviewed in Ref. 7), in DNA replication (8), and in monitoring completion of replication before the onset of mitosis (9). The involvement of Ran in premitotic control was first suggested by the finding that the guanine exchange factor for Ran corresponds to the product of the RCC1 gene (10); RCC1 was originally identified in a conditional hamster cell line, because inactivation at the restrictive temperature prevented the function of the mitotic checkpoint, leading to the appearance of prematurely condensed chromosomes undergoing mitotic contraction before completion of DNA synthesis (reviewed in Ref. 11). Expression of mutant Ran proteins inhibited replication of both transfected plasmid and of host cell chromatin (8). In addition, cells expressing Ran constructs lacking the GTP-binding domain were blocked in G2 and, to a lesser extent, in G1, suggesting that Ran was also required for the cell cycle progression; deletion of six carboxy-terminal residues DEDDDL in the Ran protein blocked this inhibitory activity (12). It is not clear whether these aspects reflect a primary role of Ran in the formation and maintenance of nuclear structures or whether they represent distinct pathways on which Ran acts as a signaling molecule (reviewed in Ref. 13).

By analogy with the GTP transduction pathway mediated by Ras in the cytoplasm, the function of Ran is exerted and transmitted via the interaction with partner molecules. Ran is active in the GTP-bound state and can self-inactivate through its intrinsic GTPase activity, which is rather low. Several Ran-interacting factors are beginning to be identified, which act as positive or negative modulators; RCC1, the best characterized partner of Ran, stimulates its activity by accelerating GTP turnover (10). On the other hand, Ran

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4 The abbreviations used are: LOH, loss of heterozygosity; RanBP-1, Ran-binding protein 1; RCC1, regulator of chromosome condensation; pRb, retinoblastoma gene product; CR1 and CR2, conserved regions 1 and 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FELC, Friend erythroleukemia cells; HMBA, hexamethylbenzamide; FACS, fluorescence-activated cell sorting; BrdUrd, bromodeoxyuridine; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus.
function is inhibited by proteins activating GTP hydrolysis, such as RanGAP1, a stimulator of the GTPase activity of Ran (14). The RanBP-1 product of the Htf9-a gene binds the GTP-bound, i.e., active, but not GDP-bound form of Ran in mouse (4), hamster (15), human (16), and yeast cells (17), where the gene was found to be vital.4 RanBP-1 is a major regulator of Ran activity and controls GTP hydrolysis on Ran by increasing RanGAP1 activity by one order of magnitude while inhibiting RCC1-mediated GTP exchange on Ran (16). Interestingly, RanBP-1 associates with the same residues of Ran (15) required to antagonize the inhibition of the cell cycle progression exerted by Ran proteins lacking the GTP-binding domain (12). It has been suggested that the activity of Ran is coupled to the cell cycle through signals marking “Start” and “Finish” of S phase (8, 12, 13). However, analysis of the mature Ran transcript in teratocarcinoma cell lines induced to differentiate (5) suggest that Ran transcription is unlikely to be regulated in relation to cell division.

In the present work, we set out to investigate whether Htf9-a/RanBP-1 expression was dependent upon cell division, and if so, which factors were involved in this control. We have found that expression of the Htf9-a/RanBP-1 gene is indeed dependent on the proliferating state of the cells. In synchronized cells, the transcript appears at the G1-S transition and accumulates throughout S phase, consistent with a possible role of RanBP-1 in regulating Ran function during S phase. Up-regulation was found to be exerted at the transcriptional level and to require an E2F recognition site for members of the E2F transcriptional activator family. The E2F family, whose target sites are found in the promoter of many genes involved in cell proliferation (reviewed in Refs. 18 and 19), comprises several factors related in their DNA-binding specificity and expressed at specific phases of the cycle (reviewed in Ref. 20). The association of E2F factors with proteins encoded by genes capable of suppressing cell proliferation, such as the pRB product of the retinoblastoma susceptibility gene (21–23) and the related p107 (24–27) and p130/pRB (28) proteins, first suggested that E2F factors linked control of the cycle progression to the transcriptional activation of distinct target genes. The growth-suppressive activity of the Rb protein family is exerted at least in part by blocking the expression of genes that are otherwise the targets of E2F activation. The present results implicate both the pRB product of the retinoblastoma susceptibility gene and the related p107 protein in negative control of Htf9-a transcription. These findings might provide a link between the retinoblastoma-related tumor suppressor genes and the nuclear GTP transduction network via the control of the Htf9-a/Ran BP-1 gene.

### Results

**Transcription of the Htf9-a Gene Is Associated with Cell Proliferation.** The RanBP-1 product of the Htf9-a gene is a member of the nuclear GTP network that regulates several cell cycle-related functions; thus, it is conceivable that the RanBP-1 product is itself expressed in a cell division-dependent manner. To investigate this possibility, we used cellular systems in which the exit from and entrance into the cycling state could be modulated with the appropriate stimuli.

Myogenic C2 cells proliferate as undifferentiated myoblasts in the presence of high serum concentrations (growth medium, C) and are induced to differentiate in the absence of mitogens (differentiation medium, D); thus, the C2 line provides cells in alternative states of proliferation and differentiation. Fig. 2 shows that accumulation of the Htf9-a mRNA transcript was down-regulated as cells differentiated to myotubes and exited the cell cycle (Fig. 2, compare Lane G to D). The E1A oncoprotein overrides the block imposed upon proliferation by serum starvation because of its ability to sequester several negative regulators of the cycle, including the pRB product of the retinoblastoma susceptibility gene, to which E1A associates through the conserved CR1 and CR2 domains (29). In a study of the interference exerted by E1A upon myogenic differentiation, C2 cellular clones were constructed which stably expressed E1A proteins mutated in various domains, and their potential to undergo differentiation was characterized (30). Northern analysis of the Htf9-a transcript in these clones showed that expression of wild-type E1A oncoprotein counteracted Htf9-a down-regulation in differentiating conditions. Mutant E1A proteins maintaining functional sites of interaction with pRB (i.e., pm 563) also prevented Htf9-a down-regulation; on the other hand, expression of E1A proteins defective in either CR1 (dl 646N) or CR2 (dl 922–947, pm 928), and thus incapable of sequestering the pRB protein, did not interfere with down-regulation of the Htf9-a transcript in differentiating conditions. For comparison, the GAPDH mRNA was not significantly affected by the expression of functional or mutant

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E1A proteins; thus, the interference of E1A and its pRb-interacting derivatives with Htf9-a transcription can be regarded as a selective effect.

We next analyzed Htf9-a expression in a cell line in which the extent of induction of differentiation and cell cycle arrest could be quantified. FELC are virus-transformed erythroid precursors that can indefinitely proliferate, yet retain the ability to differentiate after exposure to inducing agents such as HMBA. Expression of the erythroid differentiation program follows the induction of cellular commitment, triggered by the inducing agent and transmitted to daughter cells through one cell division; once committed, FELC initiate their terminal division and express erythroid functions (Ref. 31 and references therein).

In the experiment in Fig. 3, over 40% of FELC exposed to HMBA for 4 days resumed globin synthesis, as assessed by benzidine staining of the hemoglobin, and could be regarded as terminally differentiated erythroid cells. Differentiation was associated with the arrest of proliferation; accordingly, the FACS profile of the HMBA-exposed cell population was clearly shifted in comparison to that of uninduced cells. Control FELC showed a heterogeneous distribution of the DNA content, typical of rapidly dividing cells, with the highest proportion of cells in S phase, whereas after exposure to HMBA, most cells had a G0/G1 DNA content. Northern analysis of total RNA from both cell populations revealed that the Htf9-a mRNA was abundantly expressed in untreated erythroblasts while being barely detectable in the induced cell population. Together the results from both differentiating cell lines indicated that the Htf9-a gene was efficiently transcribed in cycling cells prior to differentiation while being down-regulated upon differentiation and exit from the cycle. In addition, Htf9-a down-regulation was prevented by expression of E1A oncoproteins functional in the domains of interaction with pRb.

We next attempted to assess whether down-regulation of the Htf9-a transcript in differentiating cell cultures mimicked a program of down-regulated expression upon cellular quiescence of specialized cells in vivo. To this aim, expression was investigated in adult mouse liver, an organ commonly regarded as paradigmatic of the quiescent and differentiated state, and in regenerating liver in which proliferation was induced following surgical hepatectomy. The Northern experiments in Fig. 4 show the level of Htf9-a mRNA expression in quiescent and in regenerating liver tissues from three different adult mice. Htf9-a expression, detectable at low levels in the quiescent tissue, was clearly up-regulated when G1 exit was experimentally induced in liver cells.

Expression of Htf9-a mRNA during the Cell Cycle. The information obtained from the analysis of Htf9-a expression thus far converged in the indication that the transcript was down-regulated in quiescent cells. We next wished to establish whether expression was actively up-regulated in cells committed to the division cycle. To address this question, we used NIH/3T3 fibroblasts that can be brought to the resting state by serum withdrawal; the proliferation block can then be released by supplementing serum again, thus stimulating cells to synchronously reenter the cycle. The appearance of the Htf9-a transcript was analyzed during the cycle progression as monitored by FACS analysis. The Htf9-a mRNA was barely visible in arrested fibroblasts and in cells harvested 4 h after the block release (Fig. 5A, Lanes
Fig. 4. Northern blot hybridization of RNA from mouse adult liver and after surgical hepatectomy. For each of the three animals, total RNA was extracted from tissues at the zero time (Q), quiescent and 24 h after operation (R, regenerating). Upper panel, pattern of hybridization obtained with the Htf9-a cDNA probe; lower panel, ethidium bromide-stained gel.

0 and 4). The transcript level was first seen to increase 7 to 9 h following serum stimulation (3- to 5-fold, as revealed by microdensitometric analysis of the autoradiographs); in BrdUrd incorporation assays designed to monitor progression from early G1 towards S, BrdUrd was first detected in the DNA of cells harvested 9 h after stimulation, indicating that at that time, cells were approaching the G1-S transition (Fig. 5B, compare 0, 4, and 7 h). The highest increase in the level of Htf9-a mRNA (15- to 20-fold) occurred 12 h after stimulation (Fig. 5A) when cells were in S phase (Fig. 5B). Cells were also synchronized at the G1-S transition by exposure to hydroxyurea for 16 h and subsequently allowed to progress into S phase in fresh drug-free medium. Northern blot analysis (Fig. 5C) confirmed that the highest level of Htf9-a mRNA was observed in S phase cells (5-h release) compared to cells arrested at the G1-S transition (no release) or progressing towards completion of the cell division cycle (9-h release). Together these results indicate that the Htf9-a gene is activated in late G1, and reaches its maximal expression in S phase.

Cell Cycle Regulation of Htf9-a mRNA Is Controlled at the Transcriptional Level. Having established that the Htf9-a mRNA was up-regulated as cells progressed towards S phase, we asked whether cell cycle control of Htf9-a expression occurred at the transcriptional level. In previous studies, the Htf9-a promoter was dissected by deletion mapping analysis, which had led to the identification of a 273-bp region sufficient for transcriptional activity (Ref. 32; Fig. 1B). The pTS-A construct, containing the entire Htf9-a 273-bp promoter linked to the CAT reporter gene, was transfected into NIH/3T3 cells, and its activity was measured during the cell cycle progression. The construct was transfected in cycling cell cultures; 6 h after transfection, the medium was replaced with serum-free medium to block proliferation. Arrested cells were subsequently stimulated to synchronously reenter the cycle by re-adding fresh serum and were allowed to reach S phase. For control, experiments were carried out in parallel cultures with the pE1A-CAT construct in which CAT gene expression is under control of the E1A promoter. As shown in Fig. 6, the activity of the pTS-A promoter was extremely low in arrested cells and was efficiently resumed 8 h following serum refeeding. Transcriptional activity was fully reestablished 13 h after stimulation, when cells reached S phase, and reached the level normally observed in asynchronously cycling cell cultures (see also Fig. 10). Thus, proliferation-dependent Htf9-a expression is indeed controlled at the transcriptional level. In contrast, E1A activity did not significantly vary in arrested or in restimulated cultures; these results indicate that transcriptional activation of the pTS-A promoter upon cell cycle reentry was specific and coincided with the timing of up-regulation of the endogenous RanBP1 mRNA during the cell cycle progression.

An Element of the Htf9-a Promoter Binds a Member of the E2F Family of Factors. We set out to identify promoter elements in Htf9-a that might have been responsive to cell cycle-dependent mechanisms of activation. In a previous study of the Htf9-a promoter, we had characterized several elements bound by transcription factors varying in different cell types and supporting ubiquitous Htf9-a transcription (33). To depict the actual nucleoprotein interactions taking place in
different cell types, the Htf9-a promoter had been examined by genomic footprinting (34). One in vivo footprint harbored an element identical in sequence to that of the E2F-binding site in the B-myb promoter; we called that site b-E2F. Another putative E2F site was also identified, which was identical to a c-myc promoter site (site c-E2F), yet no in vivo protection had been seen (34).

Since many cell cycle-regulated genes are controlled by members of the E2F family, the in vivo protection of the b-E2F site seemed particularly worth investigating. We first analyzed the binding of proteins from human HaCaT cells to an Htf9-a-derived oligonucleotide encompassing the b-E2F footprint; for control, complexes formed on the canonical E2F site from the adenosine 5′ E2 promoter (35) were also analyzed. Several nucleoprotein complexes were formed on the b-E2F oligonucleotide in a bandshift assay (Fig. 7A). Complexes were disrupted by the addition of unlabeled oligonucleotide from the adenosine E2 promoter as well as by the b-E2F oligonucleotide. The reciprocal competition experiment using the adenosine E2 site as the probe confirmed that the Htf9-a b-E2F site and the adenosine E2 site had essentially the same binding specificity (Fig. 7B). Addition of an oligonucleotide corresponding to a mutated E2F-binding site had no effect, indicating that the binding was specific for the E2F motif; thus, the b-E2F site is a bona fide E2F-binding site. The c-E2F site showed a lower affinity for proteins of the E2F group, as shown by its ineffectiveness as a competitor oligonucleotide versus both the Htf9-a b-E2F and the canonical adenosine E2 sites. The low binding affinity of E2F proteins for the c-E2F site is consistent with our previous finding that the b-E2F site, but not the c-E2F site, was footprinted in vivo.

The E2F group includes several transactivators of related DNA-binding specificity; this heterogeneity is further increased by the ability of E2F members to heterodimerize with the related DP proteins. In addition, individual E2F members associate with multiple regulatory proteins, including cyclin A, cyclin E, their associated kinases, and the related pRb, p107, and p130 proteins in a cell type- and cell cycle-specific manner (see the review in Ref. 20 and references therein). To analyze the nucleoprotein interactions established at the b-E2F site during the cycle, staged extracts were prepared from arrested/restimulated NIH3T3 cells as described above (Fig. 5A). One band of fast mobility was present throughout the cycle, as well as in resting cells, and was unaffected by the addition of the anti-p107 antibody; the mobility of that shift is compatible with that reported for the binding of free E2F to the DNA (36). Two additional complexes, both containing p107, could also be distinguished at different times of the cycle, designed here as E2FC1 and E2FC2 by analogy with the complexes described by Mudryj et al. (36). As shown in Fig. 8, the E2FC1 complex reached its highest abundance 6 h after stimulation. Between 6 and 9 h, that complex disappeared to be replaced by the more slowly migrating E2FC2 complex, also containing p107. S-phase-specific p107-containing complexes were characterized previously on E2F sites from the E2 and b-myb gene promoters (35–37). By analogy with those, the E2FC2 complex in Fig. 8 is likely to contain cyclin A. Thus, the timing of activation of the Htf9-a promoter in stimulated cells coincided with specific associations of regulatory molecules to the E2F complexes during the cycle progression.

The Htf9-a/Ran BP1 Gene Promoter Responds to Cell Cycle Regulators. As an initial step to identify the promoter region that might have been responsible for cell cycle control of RanBP1 transcription, we generated a promoter deletion that removed the b-E2F site. The resulting deletion was inserted upstream of the CAT reporter gene (construct pTS-d1), and its activity was compared to that of the full-length promoter (pTS-A) in transient expression assays. The activity of both promoters was first visualized by chromatography of CAT-modified products (Fig. 9); quantitative estimates were obtained by immunoenzymatic determination of the CAT protein. These experiments showed that the pTS-d1 construct, although retaining most target sites for transcription factors except the portion represented by the b-E2F binding oligonucleotide, had lost over 70% of the activity compared to the full-length promoter. Conversely, when the b-E2F oligonucleotide was inserted upstream of the pAl0-CAT2 minimal promoter (pE1 construct), transcriptional activity was increased by at least 4-fold; thus, the b-E2F oligonucleotide contains a positive control element in both deletion mapping and gain-of-function assays.

The activity of pTSd-1 did not appear to vary in conditions of proliferation or arrest (Fig. 10B) and was comparable to the low activity of pTS-A in arrested cells (Fig. 10A); we concluded that the full-length promoter contains a proliferation-responsive element removed in the pTS-d1 deletion. We had observed previously that E1A and derivatives retaining functional sites of interaction with pRb prevented down-regulation of the endogenous Htf9-a transcript in differentiating C2 cells (Fig. 2). We wished to further ascertain whether transient expression of the E1A oncoprotein could relieve the repression associated to the proliferation block. An expression construct producing the E1A protein under the control of its own promoter was assayed for its effect on both the pTS-A and pTS-d1 reporters. In cotransfection experiments, the pTS-A construct, but not pTS-d1, was activated by the E1A oncoprotein in both cycling and in arrested cells (Fig. 10), indicating that the E1A responsiveness mapped to the same promoter region that was already identified for being proliferation dependent. In arrested cells, E1A completely relieved the repression undergone by pTS-A and restored the promoter activity to the same level seen in cycling cells. The E1A P5s mutant, producing a nonfunctional E1A protein, had no effect (data not shown).

Both the pRb and p107 proteins are possible targets of E1A, as they both contain a "pocket" domain to which E1A can associate; therefore, we assayed the effect of constructs expressing either pRb or p107 over pTS-A activity in cycling and in resting cell cultures. The results in Fig. 10A show that overexpression of both pocket proteins resulted in repression of the pTS-A construct in cycling cells (3-fold reduction); repression was even more effective in arrested cells (over 6-fold), where virtually no CAT synthesis was detected in the presence of either protein. In a final set of cotransfection experiments, a construct encoding the E2F-1 factor was assayed for its effect on the pTS-A construct; promoter activation could be appreciated with relatively low amounts (i.e., 2 μg) of cotransfected E2F-1 expression construct (Fig. 10A), yet decreased when higher amounts were used (data not shown). Induction of activity was more effective in quiescent cells in which the promoter repression usually associated with exit from the cell cycle was completely rescued by the exogenous E2F-1.

Discussion

The Htf9-a gene encodes the RanBP1 protein, a major regulator of the nuclear Ran GTPase (16–17), which controls several nuclear functions, including DNA replication, monitoring
A

B

Serum withdrawal

Serum addition

0 4 7 9 12 24 30

C

Hydroxyurea

Medium replacement

0 5 9

FL2-H/DNA CONTENT

FL2-H/MONOCEN CONTENT

FL2-H/MONOCEN CONTENT

FL2-H/DNA CONTENT

FL2-H/MONOCEN CONTENT

FL2-H/MONOCEN CONTENT

FL2-H/MONOCEN CONTENT

DNA content

BUDR incorporation
of replication completion prior to maturation promoting factor activation, and progression of the cycle through the G1-M transition. Thus, Ran activity is largely related to events marking the start and end of S phase. The functional state of Ran, like that of many GTP-binding proteins, is modulated by its interacting partners. We report here that the Htf9-a transcript encoding RanBP-1, although being ubiquitously expressed, is down-regulated as cells exit the cycle; thus, the Htf9-a/RanBP-1 gene is the first of three identified genes coding for proteins involved in the nuclear GTP network (i.e., Ran, RCC1, and RanBP-1 itself) for which expression is clearly dependent upon cell division. This was observed in different cell lines and tissues, irrespective of whether proliferation was blocked upon terminal differentiation, in response to mitogen withdrawal, or by physiological control, as in the case of liver cells in vivo. The finding that expression of the Htf9-a endogenous transcript was also repressed in arrested fibroblasts shows that RanBP-1 function becomes generally inactivated as cells stop dividing, even temporarily, although the cell division program may not be irreversibly switched off, as in terminally differentiated quiescent cells. This is consistent with the observation that modulation of the mRNA level in hepatocytes from normal and regenerating liver is effective upon induction of proliferation and is unrelated to the differentiated state of the cells. Time course experiments indicated that Htf9-a mRNA synthesis was up-regulated as cells progressed towards S phase and placed the timing of up-regulation near the G1-S transition; expression of the Htf9-a/RanBP-1 mRNA peaks in S phase and thus coincides with the timing of Ran activity in S phase.

Different mechanisms might regulate expression of the Htf9-a transcript in relation to proliferation; it is possible that cell types with different proliferation abilities give rise to mRNA of different stability, as suggested by the presence of alternative polyadenylation signals in independent cDNA clones (2). However, in all analyzed cell types, down-regulation affected the overall transcript abundance rather than the relative ratio of mRNAs of different size, suggesting no differential polyadenylation during differentiation. Transient expression assays of the Htf9-a promoter in arrested cells, which were subsequently stimulated to reenter the cycle, indicated that expression was controlled, at least in part, at the transcriptional level by the activation of proliferation-dependent promoter elements. Transcription from the Htf9-a promoter was activated at the G1-S transition and thus coincided with the timing of up-regulation of the endogenous mRNA.

The Htf9-a promoter contains two potential recognition sites for E2F, the most distal of which is identical to the "Rb-class I" consensus site, identified from a population of random DNA sequences as having the highest binding affinity for E2F/Rb complexes in vitro (22), and was previously characterized as a protein-binding site by genomic footprinting (34). The site, named b-E2F, is a

![Fig. 5.](image)

Fig. 5. A, Northern blot hybridization of RNA from synchronously cycling NIH/3T3 cells. Total RNA was extracted at various times (indicated above each lane) after serum stimulation of resting cells and hybridized with the Htf9-a cDNA probe. An aliquot was withdrawn from each sample before extraction to be processed for FACS analysis. The ethidium bromide staining of the RNA bands is shown. B, progression of the cycle after release of the proliferation block. Upper panel, the DNA content of cells harvested at the indicated times and analyzed by FACS as in Fig. 3. Lower panel, a time course analysis of BrdUrd incorporation designed to depict the G1-S transition. BrdUrd was added to the medium 30 min before harvesting the cells; in each graph, the anti-anti BrdUrd antibody fluorescence (ordinate) is plotted versus the propidium iodide fluorescence (abscissa). At 9 h, the increased BrdUrd level in the graph region corresponding to the G1 DNA content marks the start of DNA replication. C, Northern blot hybridization of RNA from cells harvested after synchronization at the G1-S transition. Hydroxyurea was added to the medium for 16 h, and cells were then grown in drug-free medium and harvested at the indicated times. The corresponding FACS profiles are shown. Total RNAs were hybridized with Htf9-a and GAPDH cDNA probes.

![Fig. 6.](image)

Fig. 6. Transient expression assays of the Htf9-a (pTS-A construct) and E1A promoters in arrested and restimulated NIH/3T3 cells. Culture conditions after transfection are schematically diagrammed at the top of the figure. Thirty min before harvesting, BrdUrd was added to the medium; transfected cells were then collected and split into aliquots, one of which was processed for FACS analysis, while the other one was extracted for immunoenzymatic determination of the CAT protein. The amount of CAT enzyme (mean values from three independent experiments; bars, SE) is expressed relative to the amount measured in asynchronously cycling cells (see also Figs. 9 and 10), which was taken as 100. The proportion of S-phase cells in transfected cultures is also shown.
cell cycle control of the Htf9-a promoter.\textsuperscript{5} Promoter constructs individually in each site are currently being assayed in a systematic study to establish whether the positive control defined by the deletion mapping analysis is mediated by the b-E2F site alone or in cooperation with the Sp1.3 site.

Growing evidence is accumulating that many cell cycle-regulated promoters are activated and inactivated at each cell division cycle, depending on the molecular balance established between particular activators of the E2F group and the antagonizing Rb-related proteins (41–43). E2F complexes also involve cyclin E, cyclin A, and cdk2 (40, 44, 45). The full-length pTS-A promoter construct could undergo E2F-1-dependent activation, repression mediated by Rb-related proteins, and E1A relief of repression, whereas the basal activity of the pTS-d1 deleted promoter did not significantly vary in relation to the proliferating state of the cells or in response to overexpression of cell cycle regulators; these findings formally identify a cell cycle-responsive element in the region encompassing the b-E2F site.

Repression in resting cells was prevented by E1A expression; this was observed both on the activity of the pTS-A promoter in cotransfection assays and on expression of the endogenous Htf9-a transcript in cell lines stably expressing E1A proteins. E1A can disrupt the association of pRb and its relative p107 with E2F factors (21, 23). The analysis of stable transfectants expressing particular E1A mutant proteins might identify regulators of Htf9-a expression because contact points for pRb or p107 interactions are not exactly coincident in E1A and are separated by individual mutations (29–30). All E1A mutant proteins that failed to counteract Htf9-a down-regulation in differentiating C2 cells were defective for binding to pRb, while their ability to interact with other cell cycle regulators was variably affected. Thus, removal of the endogenous pRb by E1A results in maintenance of Htf9-a transcriptional activity upon differentiation. This might either indicate a direct role played by pRb in repression of Htf9-a transcription or an indirect effect of E1A by preventing cell cycle arrest. Transient expression assays showed that direct expression of pRb repressed the Htf9-a promoter; the extent of repression was as effective or higher than reported for the Rb-1 and c-myc promoters (42, 46). Together, therefore, these independent lines of evidence converge to indicate that pRb plays a major role in negative control of the Htf9-a promoter, at least in differentiating C2 myogenic cell lines.

\textsuperscript{5} G. Guarguaglini and S. Moretti, unpublished data.
The experiments reported here suggest that p107 might also be implicated in cell cycle regulation of Hif9-a expression. We have found that overexpression of p107 repressed Hif9-a promoter activity. In addition, the timing of Hif9-a activation coincided with a specific change in the p107-containing complexes formed at the b-E2F site. The timing of Hif9-a promoter activation in relation to the cell cycle phases is similar to that reported for the B-myb gene and is associated with a change in the electrophoretic mobility of the p107 complex similar to that reported for the B-myb E2F site, which acquires cyclin A/cdk 2 at the G1-S transition (37). Mechanisms of transcriptional regulation involving p107 are not fully understood as yet. The recent cloning and characterization of p107-interacting partners has given some novel insight. Individual E2F members show distinct specificities in their pocket protein-binding ability; for example, E2F-1 binds to pRb but not p107 (47-49), whereas E2F-4 specifically associates to p107 (50, 51). The finding that certain growth-regulated promoters, such as those of the tk and B-myb genes, are responsive to p107 rather than to pRb has lead to the suggestion that pRb- and p107-containing complexes might control different subsets of E2F-responsive genes. It is also becoming increasingly apparent that different regulatory complexes are assembled in different cell types; for example, Saos-2 but not U2OS cells are sensitive to inhibition of E2F transactivation and suppression of cell growth by p107, a finding which is likely to reflect underlying differences between the interacting components in these two cell lines (27).

The timing of Hif9-a expression in NIH/3T3 cells is compatible with the activity of both the E2F-1 and E2F-4 factors, as they are both expressed in G1 and S phases (51-53). In our transfection experiments, expression of E2F-1 restored activity of the Hif9-a promoter in quiescent NIH/3T3 cells. Its effectiveness in cycling cells, however, was below that observed in other reports (52). In addition, activation of the Hif9-a promoter was effective upon cotransfection of relatively low amounts (2 μg) and decreased with higher amounts (4 and 10 μg) of E2F-1 construct. FACS analysis of transfected cells (data not shown) suggests that the apparent paradox in transcriptional activation that we observed with E2F-1 might in fact be ascribed to its activity as an inducer of apoptosis (54, 55). It is also possible that overexpression of E2F-1 is counteracted by the ability of endogenous cyclin A to directly bind and inactivate E2F-1 complexes in cycling NIH/3T3 cells (56), a phenomenon not expected to occur in resting cells.

In conclusion, the data reported here suggest that pRb complexes might be involved in negative control of Hif9-a expression upon exit from the cycle and differentiation, while p107 might control phase-specific regulation in cells committed to the cycle. In the light of the involvement of RanBP-1 in the nuclear GTP network that regulates several crucial cycle-related functions, it will be interesting to fully
dissect the regulatory mechanisms ensuring the coordination between expression of the RanBP-1 product and the cell cycle. Further understanding of the mechanisms of this control might help to recognize the hierarchy of events leading to control of cell cycle progression.

Materials and Methods

Cell Cultures. Mouse NIH/3T3 fibroblasts (ATCC CRL 1658) were grown in DMEM supplemented with 10% FCS in a 5% CO₂ atmosphere. For synchronization experiments, confluent cells were maintained in medium containing 0.5% FCS. After 36 h, FCS was added to 10%, and cells were harvested at regular intervals. For G₁,S synchronization, cells were exposed to 0.5 mM hydroxyurea for 16 h; cells were then washed, and fresh drug-free medium was added. Samples were collected immediately (G₁,S transition) and after 5 and 9 h. To monitor progression of the cell cycle, cells harvested by trypsinization were centrifuged, resuspended in a 5:1:4 PBS:acetone:methanol solution and incubated with 10 μg/ml RNase for 5 min on ice. Propidium iodide (50 μg/ml) was added, and samples were incubated in the dark for 30 min. Samples were analyzed in a FACSstar Plus cytofluorimeter using either the Multicycle (to determine the DNA content) or the WinMDI (for the simultaneous determination of the DNA content and of BrdUrd incorporation) softwares (10,000 events/sample). To analyze the G₁ to S progression, cells were exposed to 45 μM BrdUrd for 30 min at 37°C and then collected by trypanosimization and fixed with 1:5 acetone:methanol solution. Fixed cells were centrifuged, washed in PBS + 0.5% Tween 20, incubated in 1 N HCl for 45 min, neutralized, exposed to a monoclonal anti-BrdUrd antibody (IgG clone BU5.1, Ylem) for 30 min in the dark, washed, exposed to the secondary fluorescein-conjugated anti-IgG antibody (Ylem) and subjected to FACS analysis. The murine C2 myogenic cell line, bourin, Institut Curie, Paris, France) were grown in suspension in RPMI 1640 supplemented with 10% FCS. Differentiation was induced by exposure to 5 mM HMBA for 4 days, and hemoglobin synthesis was monitored by benzidine staining of the cells (31).

Mouse Tissue Preparation. For regenerating liver preparations, adult male C57 BL/6 X DBA/2 mice were anesthetized (xilazine; 0.05 mg/g body weight and ketamine, 1 mg/g body weight) and subjected to midventral laparotomy with 30–40% liver resection (left lateral lobe) as described (57). This tissue was used as the zero time (quiescent) control liver for RNA preparation. After 24 h, mice were sacrificed by cervical dislocation, and the remaining (regenerating) liver lobes were removed and processed for RNA extraction.

Northern Blot Hybridization Experiments. Total RNA was extracted following the acid guanidinium/phenol chloroform extraction protocol. Forty-μg aliquots of total RNA dissolved in formamide-formaldehyde buffer were loaded onto 1.5% agarose-formaldehyde gels and run in 1× 4-morpholinepropanesulfonic acid buffer. Gels were stained to visualize the 18S and 28S ribosomal bands and then blotted on GeneScreen membranes, UV cross-linked, hybridized, and washed as described (34). The Htf9-a probe was a gel-purified fragment corresponding to the Htf9-a cDNA (accession number X56045). Gels were controlled using a GAPDH cDNA subclone (30); probes were labeled by the random-priming method.

Gel-Shift Assays. Double-stranded oligonucleotides encompassing the E2F-binding sites of the Htf9-a promoter, i.e., E2F2, 5'-GATCCGGCGGTTGCGGGAAAGC-3' and 5'-GATCGCGCTTCCGCGAATTGCCCAG-3', or c-E2F, 5'-GATCGAATTCGCGCGCTTCCGCGAATTGCCCAG-3' and 5'-GATCCAGGGCGGAAACCCACGCT-3', were 3'-end labeled and incubated with extracts from human HaCaT cells or from synchronized mouse NIH/3T3 fibroblasts as described (35). Oligonucleotides containing either the wild-type or a mutated E2F binding site from the adenovirus E2 promoter were used in control experiments. E2F-associated proteins were analyzed by incubation of the bandshift reaction with specific antibodies on ice for 50 min prior to electrophoresis. p107 was detected by the mAb SD15 (a gift from N. Dyson, Massachusetts General Hospital Cancer Center, Charlestown, MA).

Expression Constructs and Transfection Experiments. Construction of the subclone pTS-A, containing 273 bp of the Htf9-a promoter (accession number X05830) upstream of the CAT coding sequence in the pSV0 vector, was reported previously (32). The subclone pTS-d1 was constructed by generating a XmnII-Sau96I deletion, which removed the E2F-binding site. The deleted fragment was blunt-ended and inserted in the BglII site 5' of the SV40 21-bp repeats in the pA10.CAT2 vector (pE1 construct). The pUC 119 RPI subclone contained the promoter of the adenovirus E1A transcriptional unit upstream of the E1A coding sequence (nucleotides 1–1834 in the adenovirus genome; Ref. 29); E1A PSFs carries a deletion (nucleotides 624–877) shifting the reading frame, which gives a non-functional product; and the pE8-B-Rb construct (a gift from A. Felsani, Consiglio Nazionale delle Ricerche Institute of Biomedical Technology, Rome, Italy) contained the Rb coding sequence under the control of the Moloney sarcoma virus long terminal repeat. Constructs expressing p107 and E2F-1 were constructed by inserting the p107 cDNA (58) and E2F-1 cDNA (obtained from K. Helin, The Danish

conditions of growth or differentiation, and construction of C2 clones stably expressing wild-type or mutant E1A proteins were described (30). FELC (kindly given by P. Tam

Fig. 10. Transient expression assays of the Htf9-a-derived promoter constructs pTS-A and pTS-d1 in the presence of the indicated expression constructs in cycling and in resting cells. In each set of experiments, NIH/3T3 cells were transfected when subconfluent; 6 h after transfection, the medium was replaced, and the cultures were maintained either in FCS-containing or in FCS-free medium. Reporter pTS-A (A) and pTS-d1 (B) constructs were cotransfected either with pUC DNA or with the indicated expression constructs. The amount of CAT enzyme was determined by quantitative immunosays from each cotransfection experiment; values are shown relative to the activity of pTS-A in proliferating cell cultures, taken as 100. Histograms represent the mean value from five to seven independent experiments for each set of constructs.
Cancer Society, Copenhagen, Denmark; Ref. 47) inserts in the cytomegalovirus-driven expression vector pX. NIH/3T3 cells were lipofected using the DOTAP reagent (Boehringer), 5 to 10 µg of reporter CAT construct, and 10 µg of effector construct or of pUC DNA. For E2F-1 cotransfection experiments, 2, 4, or 10 µg of construct DNA were used.

For cell cycle analysis experiments, cells were passaged from one large culture the day before transfection to obtain cell aliquots with the same proliferation index. On day 2, constructs were lipofected in these identical cell cultures using DOTAP; 6 h after lipofection, the medium was changed and replaced either with fresh complete medium (15% FCS) to maintain proliferation or with low-serum medium (0.5%) to arrest the cell cycle. In experiments designed to follow promoter activity during G1 progression, 15% FCS was re-added 48 h after starvation, and individual cell aliquots were harvested at various intervals after refeeding. These conditions were chosen to maximize the homogeneity in transfection efficiency among cell samples, as assessed by Southern blot analysis of transfected cells using the amp gene as a probe to assess the plasmid copy number in transfected samples. Each transfection experiment was repeated three to seven times and was carried out each time on duplicate sets of cultures, which were subjected to determination of CAT activity and to FACS monitoring of the cycle progression. Promoter strengths were visualized by thin-layer chromatography of modified [3H]chloramphenicol products and quantitated by immunodetection of the CAT enzyme (CAT-ELISA kit; Boehringer). Control constructs included E1A-CAT (E1A promoter upstream of the CAT gene; obtained from A. Felsani), pSV0 (Promega), pRSV, and pA10-CAT2 (32).

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