The Yin Yang-1 (YY1) protein undergoes a DNA-replication-associated switch in localization from the cytoplasm to the nucleus at the onset of S phase

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Summary

The essential Yin Yang-1 gene (*YY1*) encodes a ubiquitous, conserved, multifunctional zinc-finger transcription factor in animals. The YY1 protein regulates initiation, activation, or repression of transcription from a variety of genes required for cell growth, development, differentiation, or tumor suppression, as well as from genes in some retroviruses and DNA viruses. Among the specific functions attributed to YY1 is a role in cell-cycle-specific upregulation of the replication-dependent histone genes. The YY1 protein binds to the histone alpha element, a regulatory sequence found in all replication-dependent histone genes. We therefore examined the abundance, DNA-binding activity and localization of the YY1 protein throughout the cell cycle in unperturbed, shake-off-synchronized Chinese hamster ovary and HeLa cells. We found that, whereas the DNA-binding activity of YY1 increased dramatically early in S phase, the YY1 mRNA and protein levels did not. YY1 changed subcellular distribution patterns during the cell cycle, from mainly cytoplasmic at G1 to mainly nuclear at early and middle S phase, then back to primarily cytoplasmic later in S phase. Nuclear accumulation of YY1 near the G1/S boundary coincided with both an increase in YY1 DNA-binding activity and the coordinate up-regulation of the replication-dependent histone genes. The DNA synthesis inhibitor aphidicolin caused a nearly complete loss of nuclear YY1, whereas addition of caffeine or 2-aminopurine to aphidicolin-treated cells restored both DNA synthesis and YY1 localization in the nucleus. These findings reveal a mechanism by which YY1 localization is coupled to DNA synthesis and responsive to cell-cycle signaling pathways. Taken together, our results provide insight into how YY1 might participate in the cell-cycle control over a variety of nuclear events required for cell division and proliferation.

Key words: YY1, DNA synthesis, Cell cycle, Replication-dependent histone genes, Nucleus

Introduction

Histone genes are vital to progression of proliferating cells into a new cycle and are classified as replication independent or replication dependent (Zweidler, 1984). The former include genes for histone variants that are typically expressed at low levels throughout the cell cycle. The latter encode the majority of histone proteins in the cell. These genes are up-regulated at the G1/S boundary of the cell cycle. The replication-dependent histone genes are regulated at transcriptional, post-transcriptional and post-translational levels (Zambetti et al., 1990; Osley, 1991).

As cells commit to S phase, common regulatory processes probably mediate the coordinate up-regulation of all classes of replication-dependent histone genes. For example, the NPAT protein, localized to discrete nuclear foci in Cajal bodies, has been implicated in this up-regulation (Zhao et al., 1998; Liu et al., 2000; Ma et al., 2000; Zhao et al., 2000). In addition, our laboratory has previously defined the cis-acting DNA sequences common to all of the replication-dependent histone genes (H2a, H2b, H3, H4) as the coding-region activating sequence (CRAS) (Hurt et al., 1991; Bowman et al., 1996). These sequences were most recently identified in a linker histone gene, rat histone H1d (Horvath et al., 2003). The rat histone H1d gene has intragenic activating sequences that are absent from the testis-specific variant H1t. Within the 110 nucleotides of the CRAS are two conserved 7-bp DNA sequence elements called the histone alpha (CATGGCG) and histone omega (CGAGATC) elements. Mutation in either causes a 4-fold drop in the steady-state levels of mRNA from a mouse histone gene (mH3.2) stably transfected into CHO cells (Hurt et al., 1989b; Hurt et al., 1991; Bowman and Hurt, 1995; Kaludov et al., 1996b). These experiments established the requirement for the histone alpha and omega elements as key components in the regulation of the replication-dependent histone genes.

A yeast one-hybrid screen using the histone alpha element as bait resulted in the identification of the transcription factor Yin Yang-1 (YY1) as the DNA-binding component of the histone alpha complex (Eliassen et al., 1998). All replication-dependent histone genes in metazoans contain the alpha elements in their coding regions, whereas replication-independent histone genes carry mutations in alpha-like elements within their coding regions. YY1-containing nuclear extracts bind specifically to H3.2 alpha elements but not to the

corresponding H3.3 alpha-like elements. Because the alpha element is conserved in all replication-dependent histone genes and is essential for the up-regulation of replication-dependent histone genes at the onset of S phase, we proposed that YY1 plays a major role in the coordinate up-regulation of histone genes at the G1/S boundary of the cell cycle (Bowman and Hurt, 1995; Bowman et al., 1996; Kaludov et al., 1996a; Eliassen et al., 1998). Others have also demonstrated a role for YY1 in regulation of the histone H4 (van der Meijden et al., 1998; Last et al., 1999).

Besides its role in histone-gene regulation, YY1 represses, activates, or initiates transcription for a variety of genes, hence the name Yin Yang (Seto et al., 1991; Shi et al., 1991; Safrany and Perry, 1993; Shrivastava and Calame, 1994; Austen et al., 1997; Galvin and Shi, 1997; Shi et al., 1997). The YY1 protein also interacts with a variety of other nuclear proteins, including Sp1, TFIIB, c-myc, B23, Rb, E1A, p300 and poly(ADP-ribosyl) polymerase (Inouye and Seto, 1994; Shrivastava and Calame, 1994; Guo et al., 1995; Oei et al., 1997; Sikorski, 2000; Oei and Shi, 2001; Petkova et al., 2001). YY1 regulates transcription of many retroviruses and DNA viruses, including poxvirus (Broyles et al., 1999), herpesviruses, papillomaviruses, adenoviruses and parvoviruses (Shi et al., 1991). YY1 has recently been shown to down-regulate CCR5, a co-receptor for HIV-1 infection, and is involved in regulation of HIV LTR activity (He and Margolis, 2002; Moriuchi and Moriuchi, 2003). Counter regulation of chromatin deacetylation and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) is affected by the HIV-1 repressor YY1 and HIV-1 activator Tat (He and Margolis, 2002). Furthermore, it has been shown to control cell growth (Austen et al., 1997), development and differentiation (Brown et al., 1998; Yao et al., 1998; Donohoe et al., 1999; Brown et al., 2003). The *Drosophila* pho-like gene encodes a YY1-related DNA-binding protein that is redundant with pleiohomeotic in homeotic gene silencing (Brown et al., 2003), tumor suppression (Lichy et al., 1996) and DNA repair (Griesenbeck et al., 1999). YY1 is a phosphoprotein, and some studies have shown that its DNA-binding activity depends on its phosphorylation (Becker et al., 1994). Others have shown that YY1 is a nuclear-matrix attachment protein, possibly partitioning the nucleus (Guo et al., 1995; McNeil et al., 1998).

Less well known is the distribution of YY1 in cells as they progress through the cell-division cycle. Its subcellular localization in asynchronous cell populations has been described as nuclear or nucleolar (Guo et al., 1995; Austen et al., 1997; McNeil et al., 1998). We speculated, consistent with observations that the cellular localization of YY1 changes during embryo development in mouse and *Xenopus* (Donohoe et al., 1999; Ficzycz et al., 2001), that differential subcellular localization of YY1 is involved in its regulatory role in histone-gene expression. Similarly, subcellular localization of some transcription factors changes under the control of the cell-cycle regulatory machinery. These changes are mediated by interactions with other proteins, change in phosphorylation state, or presence of regulated nuclear localization signals (Hutchison and Glover, 1997; Aerne et al., 1998; Kaffman and O'Shea, 1999; van Hemert et al., 2001).

We report that the localization of YY1 changes in CHO and HeLa cells when they enter into the S phase of the cell cycle,

in conjunction with the up-regulation of the replication-dependent histone-gene family at the G1/S boundary and subsequent down-regulation at the mid-point of S phase. Because of the multiple roles played by YY1 in regulation of transcription, YY1 is likely to play an important role in controlling viral growth in mammalian cells as well as playing a key role in regulation of normal cell growth and proliferation. Thus, YY1 must necessarily be involved in the events resulting in loss of regulation of cellular growth in the cancerous state. Furthermore, it is not surprising that the cellular machinery determining entry into another cell-division cycle also regulates subcellular localization of a key regulator of cell growth.

Materials and Methods
Cell culture and stable transfections
CHO cells were grown at 37°C with 5% CO_2 in McCoy's 5A medium (Sigma) supplemented with 10% calf serum (Gibco-BRL) and 1% penicillin-streptomycin (Gibco-BRL). Similar growth conditions were used for HeLa cells, except that they were grown in Dulbecco's Modified Eagle Medium, 10% fetal calf serum and MEM non-essential amino acids (Sigma M7145). Cells were grown in monolayer T-75 flasks with approximately 10 ml of medium until confluent. Confluent cultures were split every second day. Pools of stable transfectant CHO cells containing the mouse H3.2-614 gene (mH3.2) integrated into the genome were selected with G418 as previously described (Hurt et al., 1991).

Synchronization of cells by mitotic shake-off
Normal cycling CHO cells in unperturbed growth can be naturally synchronized by the mitotic shake-off method, because mitotic cells in culture round up and loosen their attachment to the growth substrate and can be harvested by vigorous agitation (Eliassen et al., 1998; Whitfield et al., 2000). CHO cells were seeded into flasks at a density of 2.5×10^6 cells/flask 24 hours before the experiment. Mitotic cells were collected as previously described (Terasima and Tolmach, 1963; Schneiderman et al., 1972; Kaludov et al., 1996a) with a large-scale automated mitotic shake-off machine that we built for cell-cycle studies. More than 95% of the cells collected by shake-off can be observed as mitotic pairs of daughter cells (Eliassen et al., 1998). Populations of cells in late mitosis were plated at a density of 0.5×10^6 cells/slide and allowed to progress out of mitosis at 37°C and 5% CO_2 in complete medium (McCoy's 5A containing 10% calf serum, 1× penicillin-streptomycin). Cells were incubated for 30 minutes, 1 hour, 3 hours, 5 hours and 7.5 hours after plating.

DNA synthesis/checkpoint experiments
CHO cells were trypsinized (as described above for transfections) and cultured on coverslips for 12 hours in McCoy's 5A medium supplemented with 10% calf serum and 1% penicillin-streptomycin (Gibco-BRL). Cells were then blocked for 30 hours with aphidicolin (Sigma) at 50 μg/ml plus an additional 15 hours with either 10 mM 2-aminopurine (Sigma) or 5 mM caffeine (ICN). At 30 minutes before fixation, cultures were pulse-labeled with BrdU (BrdU labeling and detection Kit, Roche Molecular Biochemicals/Boehringer Mannheim). Cells were fixed with cold 70% ethanol plus glycine (15 mM, pH 2.0) for 20-30 minutes at –20°C and prepared for immunocytochemistry.

For immunostaining, all incubations were done at 37°C. Fixed cells on coverslips were blocked with 1 mg/ml IgG-free BSA plus 5% normal goat serum (both from Jackson ImmunoResearch Laboratories) in 1× PBS for 1 hour. Solution was aspirated and coverslips were washed 3 times with 1× PBS for 10 minutes on a

shaker before addition of the primary antibody (1:30 dilution of anti-BrdU from the kit, and 0.4 μg/ml anti-YY1, c-20 from Santa Cruz Biotechnology) for 40 minutes. Cells were washed again and incubated with the secondary antibodies (from BrdU kit and 2 μg/ml Alexa-546 goat anti-rabbit antiserum from Molecular Probes). Fluorescent images were captured with a confocal laser scanning microscope (Zeiss LSM-510). Brightness and contrast adjustment were made with LSM 5 Image Browser and Adobe Photoshop 6.0.

RNA analyses

Synchronous populations of cells (3×10^6) were harvested by trypsinization at the appropriate time after mitosis, and total RNA was extracted with the UltraSpec RNA Isolation kit (BioTecx Laboratories), quantified by UV absorbance spectroscopy, and stored at –70°C.

The probe for the S1 nuclease-protection assay was prepared from the mouse histone H3.2 clone (Hurt et al., 1989a) by digestion with *Sal*I and subsequent labeling at the 3′ end with the Klenow fill-in reaction. The probe was mixed with mRNA from CHO cells that were stably transfected with a mouse H3.2 gene and was analyzed as previously described (Hurt et al., 1991). In these assays, two different protection products were visualized in each lane of the gel. The 286 nucleotide (nt) band ('mouse' in Fig. 1) resulted from protection by the 3′ half of the mouse H3.2 transcripts, which are homologous to the probe. The 240 nt band ('hamster' in Fig. 1) resulted from protection by the coding region of the endogenous hamster H3.2 transcripts, which lack sequence identity with the probe in their 3′ untranslated region.

For northern blots, total RNA (15 μg per lane) was fractionated by formaldehyde-agarose electrophoresis, capillary blotted onto a nylon membrane (Gene Screen Plus, New England Nuclear), UV cross-linked, and washed in 2× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate). Filters were prehybridized in buffer (50% formamide, 750 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA/NaOH, pH 8, 2× Denhardt's solution, 0.1% (w/v) SDS) at 42°C for 2 hours. Probes were prepared by random-primed labeling reactions with either the full-length mouse YY1 cDNA clone (Hariharan et al., 1991) or an EF1-alpha cDNA clone (Kreig et al., 1989; Gruber and Levine, 1997). The RNAs were hybridized by incubation of the blots in buffer (above) for 24 hours at 42°C. The filters were then washed with 1× SSC, 0.1% SDS for 20 minutes at room temperature, air dried, and subjected to X-ray film autoradiography at –70°C (Sambrook et al., 1989; Spector et al., 1998). For reuse with a different probe, the blot was stripped with 0.2 M NaOH plus 0.1% SDS for 20 minutes at 37°C and then used as described above. Radioactivity was quantified with a phosphoimager (Storm PhosphorImager, Molecular Dynamics).

Electrophoretic mobility-shift assay

Duplex histone alpha oligonucleotide probe (Eliassen et al., 1998) was radiolabeled by the Klenow reaction. Each binding reaction (1.5 ng probe, 2 μg dIdC, and 6 μg mouse myeloma nuclear extract or 12 μg for CHO nuclear extracts) was incubated in binding buffer (100 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 10 mM DTT, 50% glycerol) on ice for 30 minutes and analyzed as previously described (Hurt et al., 1991).

Protein extracts

For whole-cell extracts, cells were collected by centrifugation, and the cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM DTT), incubated at room temperature for 10 minutes and centrifuged at 14,000 rpm for 5 minutes at 4°C. The supernatant was stored at 4°C, and the protein concentration was determined by the Lowry assay (Lowry et al., 1951).

For nuclear and cytoplasmic extracts, cell pellets were resuspended in 300 μl hypotonic buffer (10 mM Hepes-NaOH, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 15 minutes. The lysate was vortexed after addition of 15 μl of 10% NP-40, and the nuclei were collected by centrifugation at 2000 rpm for 15 seconds at 4°C. The supernatant was collected and stored as 'cytoplasmic extract' at –70°C. The nuclear pellet was resuspended in 140 μl nuclear resuspension buffer (20 mM HEPES-NaOH, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 0.4 M KCl, 1 mM DTT, 0.5 mM PMSF, 25% glycerol) and rocked vigorously for 20 minutes on ice. The lysates were cleared of debris by centrifugation at 10,000 rpm for 15 minutes at 4°C and stored as 'nuclear extract' at –70°C (Shapiro et al., 1988; Hurt et al., 1991).

Western analysis

Protein extracts were separated on 7.5% SDS-polyacrylamide gels, electroblotted onto nitrocellulose membranes (Hybond-C extra, Amersham Pharmacia Biotech), blocked for 1 hour at room temperature in blocking buffer [1× TBS, 0.5% (v/v) Tween 20, and 5% (w/v) Carnation non-fat dry milk], and washed twice, 20 minutes each, in wash buffer (1× TBS, 0.5% Tween 20). Blots were incubated with the 1:1000 dilution of primary antibody in blocking buffer for 1 hour at room temperature with anti-YY1 (anti-YY1 sc-281; Santa Cruz Biotechnology) or anti-tubulin (Asai et al., 1982). Next, the blot was washed twice in wash buffer at room temperature, for 20 minutes each. The secondary antibody, (anti-rabbit IgG-horseradish peroxidase, NA934, Amersham Pharmacia Biotech) was diluted 1:1000 in blocking buffer and incubated for 1 hour at room temperature, then washed twice in wash buffer, for 20 minutes each. Secondary antibody-HRP was detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

Fluorescence immunocytochemistry

Cells in S phase of the cell cycle were detected by incorporation of bromodeoxyuridine (BrdU), according to manufacturer's instructions (BrdU labeling and detection Kit, Roche Molecular Biochemicals/ Boehringer Mannheim). Ten nM BrdU was added to plated cells for the last 30 minutes of each time interval. For interval '0' mitotic cells were incubated for 30 minutes in BrdU then fixed. For all other times, BrdU was added 30 minutes before the end of the interval. After the 30-minute BrdU pulses, cells were washed 5 times with complete McCoy's 5A (described above), fixed with 70% ethanol plus glycine (15 mM, pH 2.0) for 1 hour at –20°C, and then washed 3 times with 1× PBS. Primary antibodies for detection of BrdU (clone 9318; Roche Molecular Biochemicals/Boehringer Mannheim) or YY1 (sc-281; Santa Cruz Biotechnology) were diluted 1:1000 in 1× PBS and applied to the slides for 30 minutes at 37°C, and the slides were then washed 3 times with 1× PBS. Cytoplasmic BrdU signals are found in cells that are not in S phase. Fluorescently labeled secondary antibodies (anti-mouse-Alexa488 or anti-rabbit-Alexa594; Molecular Probes) were diluted 1:1000 in 1× PBS and applied to the slides for 30 minutes at 37°C; the slides were then washed 3 times with 1× PBS. After washing, the slides were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 2 μg/ml in 1× PBS) at room temperature for 5 minutes and mounted in Vectashield (Vector Laboratories). As a negative control, we also stained cells with YY1 antisera that had been immunodepleted by passage over a YY1-coupled affinity column made from partially purified recombinant YY1. We also immunodepleted the sera by preincubation with the synthetic peptide (the blocking peptide for YY1 antisera sc-281; Santa Cruz Biotechnology). In both cases, these negative control slides gave bright DAPI and anti-BrdU signals but almost completely lacked fluorescence in the wavelength

corresponding to anti-YY1 (M.J.B. and M.M.H., unpublished observations).

Image data (except for Fig. 9) were collected with an Olympus IMT-2, Delta Vision (Applied Precision) deconvolution microscope with a 60× objective lens with 1.5× auxiliary magnification. Three-dimensional optical reconstructions were obtained and deblurred by deconvolution for each wavelength (DAPI, FITC and rhodamine filters). The figures show single optical sections or through-focus projections as indicated in the legends, obtained with the SGI/Unix-based Priism software (versions IVE3.2/IVE3.3) for image analysis as previously described (Bass et al., 2000).
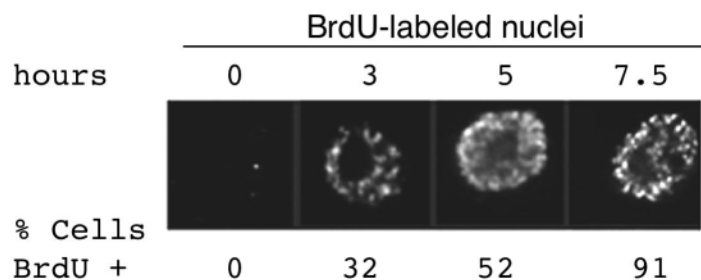
Results

Coordinate up-regulation of histone mRNA levels and YY1-binding activity upon S phase entry

Normal cycling CHO cells in unperturbed growth can be naturally synchronized by the mitotic shake-off method, because mitotic cells in culture round up and loosen their attachment to the growth substratum and can be harvested by vigorous agitation. With a large-scale shake-off machine that we built for cell-cycle studies, mitotic cells were harvested and plated for progression from mitosis into S phase (Eliassen et al., 1998; Whitfield et al., 2000). Entry into S phase was determined by BrdU incorporation into newly replicated DNA as shown in Fig. 1. Thirty minutes after plating, no cells were in S phase. Three hours later (early S phase), 32% of the cells were in S phase, and after 7.5 hours, 91% of the cells were in S phase. At least 500 cells were counted after each interval. The images in Fig. 1 show the immunofluorescence detection of BrdU incorporated into the nuclei of CHO cells.

Thirty minutes after plating, none of the CHO cells harvested by mitotic shake-off were in S phase. Three hours later (early S phase), 32% of the cells were in S phase, as determined by BrdU incorporation into newly replicated DNA as shown by immunofluorescence detection in Fig. 1. After 7.5 hours, 91% were in S phase. At least 500 cells were counted after each interval.
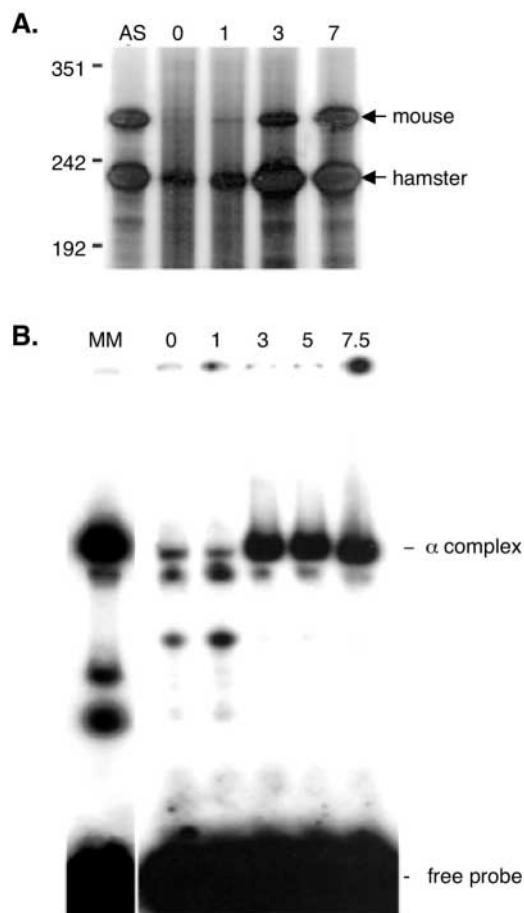
Results of the S1-nuclease protection assay employed to monitor the up-regulation of histone H3.2 genes in CHO cells stably transfected with a mouse H3.2 gene are shown in Fig. 2. In this assay, the endogenous hamster H3.2 histone mRNA (hamster arrow, Fig. 2A) and the transfected mouse H3.2 histone mRNA (mouse arrow, Fig. 2A) were assayed together, resolved elctrophoretically, and show the same pattern of gene up-regulation. Dramatic increases in histone H3.2 mRNA levels occur at the G1/S boundary (Fig. 2A, 3 hours), and the high levels of mRNA persist into middle S phase (Fig. 2A, 7 hours).

The alpha-element binding activity (Hurt et al., 1991; Bowman and Hurt, 1995; Bowman et al., 1996; Kaludov et al.,

Fig. 2. Increases in histone mRNA levels in vivo and YY1-histone alpha-element-binding activity in vitro upon entry into S phase. (A) Total RNA was isolated from stable transfectant CHO cells for each time interval indicated across the top (AS, asynchronous; numbers are hours after mitotic shake-off). A fragment of the mouse H3.2-614 gene (3′-end-labelled) was used as a radioactive probe in S1-nuclease protection assays, with 3 μg of total RNA per reaction. The positions of the probe fragments protected by the mouse and hamster histone transcripts (arrows) and the positions of markers (nucleotide number) are indicated. (B) An electrophoretic mobility-shift assay using the duplex histone alpha oligonucleotides as a probe was carried out with nuclear extracts from synchronous populations of CHO cells (lanes 0, 1, 3, 5 and 7.5; numbers are hours after shake-off) or from mouse myeloma cells (lane MM) as a positive control. The position of the α complex (resulting from YY1 DNA-binding activity) and free probe are indicated.

Fig. 1. Incorporation of BrdU into synchronous populations of CHO cells at entry into S phase. Mitotic cells were pulse-labeled with BrdU for the last 30 minutes before the end of each interval. Fixed cells were subjected to fluorescence immunocytochemistry designed to visualize nuclear BrdU incorporation, indicative of S phase cells. FITC images across the top are from optical sections through individual cells, showing one example of a BrdU-negative cell (little to no fluorescence, 0 hours) and three examples of BrdU-positive cells with clear nuclear staining (3, 5 and 7.5 hours). The nucleus occupies most of the cell volume in these cells, and the cell periphery is not indicated. The percentage of intact cells (n=500 per time point) showing nuclear staining of BrdU is indicated at the bottom of the figure. The time after mitosis (in hours) is indicated at the top.

1996a) in mouse myeloma cell and CHO cell nuclear extracts can be seen here as the major shifted band (Fig. 2B, α complex). The alpha-element-binding activity was detectable in nuclear extracts from cells in G1 but increased by 20-fold between 1 and 3 hours after mitosis.
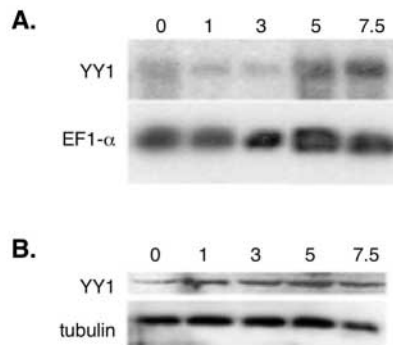
YY1 mRNA and protein are present throughout the cell cycle

Having shown that histone mRNA and YY1 binding activity both increase at the onset of S phase, we tested whether these increases correspond to increased expression of the YY1 gene. The results of the gel-blot analyses used to examine YY1 mRNA and protein levels in extracts prepared from synchronized cells appear in Fig. 3. Total RNA was hybridized with a full-length YY1 cDNA probe (Fig. 3A, YY1), and then stripped and rehybridized with EF1-α, a control gene that is active at relatively constant levels throughout the cell cycle (Kreig et al., 1989; Gruber and Levine, 1997; Last et al., 1999). Normalization of radioactivity in the YY1 band to the EF1-α band indicates that the relative levels of YY1 mRNA are fairly constant at the times examined. This pattern is significantly different from that observed for the histone genes (Fig. 2A).

Western blot analysis (Fig. 3B, YY1) revealed that the YY1 protein level is relatively constant from mitosis to middle S phase. Tubulin immunodection (Fig. 3B, tubulin) used to verify protein loading showed the expected pattern of constitutive levels across the times examined (Dumontet et al., 1996). Taken together, the results from Figs 1, 2 and 3 show that the binding activity of YY1 dramatically increases at the G1/S boundary but that this increase in activity is not reflected in changes in the levels of mRNA or protein abundance over the same time intervals.

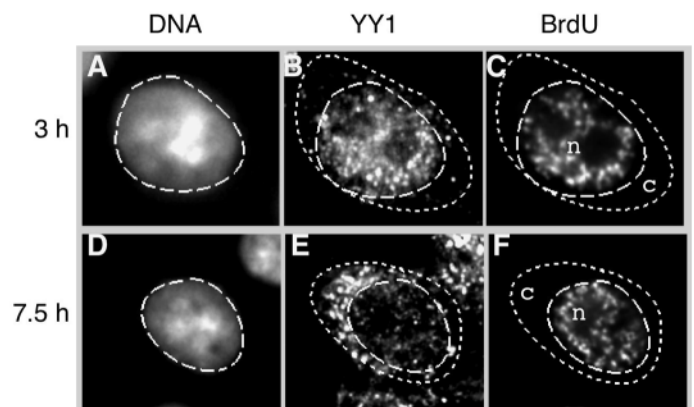## The localization of YY1 changes with the cell cycle

Because the change in YY1 binding activity was not well correlated with changes in YY1 mRNA or protein levels, we examined the cellular distribution of the YY1 protein at different times in the cell cycle. We reasoned that, if changes in the subcellu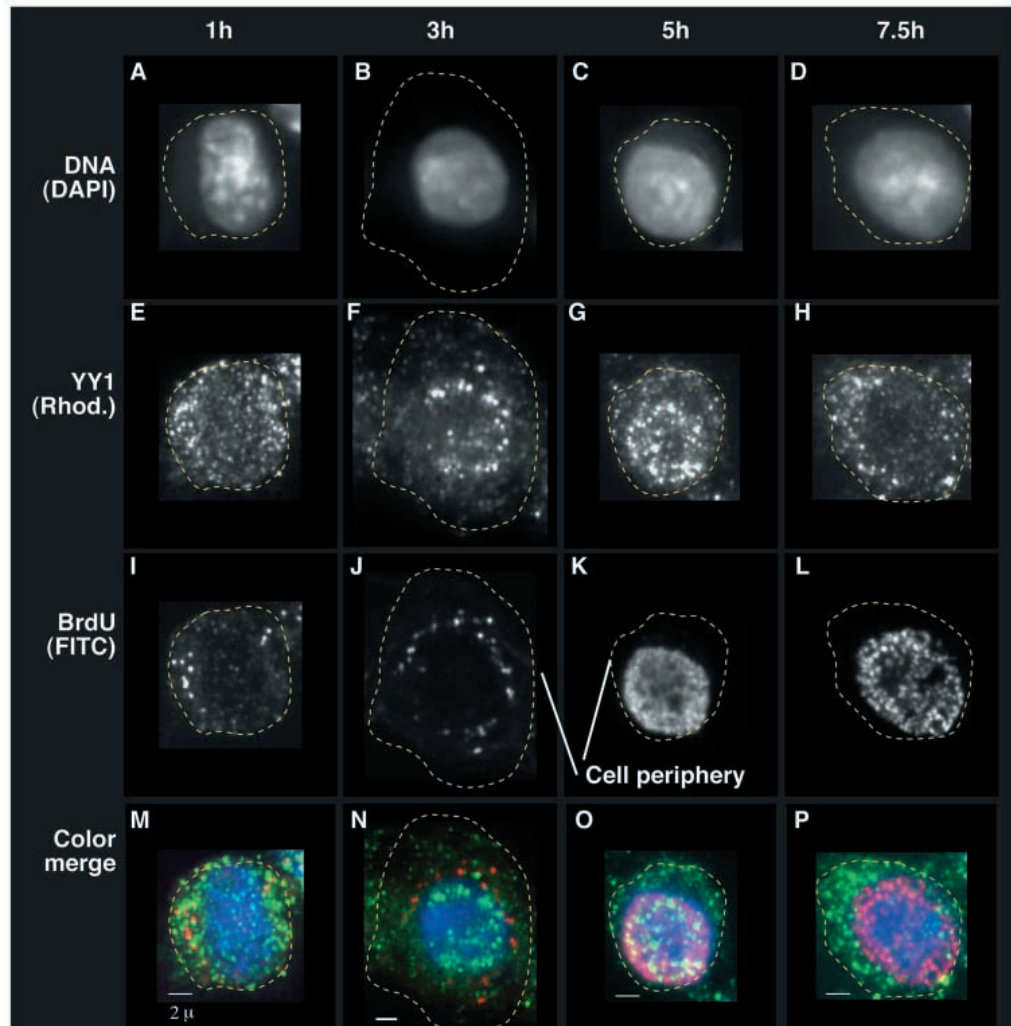lar localization of YY1 occurred, they might be detectable by microscopic or cell-fractionation analysis. To visualize the distribution of YY1 in the cell, we subjected synchronized cell populations to indirect immunofluorescence microscopy using commercially available, highly specific anti-YY1 polyclonal antibodies. Cell populations were pulsed with BrdU and then triple-labeled with antibodies and DAPI for imaging. The DAPI-stained chromatin images allowed distinction of nuclear from cytoplasmic compartments of the cell, whereas indirect immunofluorescence allowed detection of YY1 at one wavelength and BrdU-labeled nuclei at another. Optical-reconstruction deconvolution microscopy resulted in 3D, multiwavelength sets of data that provided a clear determination of the location of the YY1 staining within individual cells.

Surprisingly, most of the cells exhibited one of two distinct YY1 cellular-distribution patterns, primarily nuclear and primarily cytoplasmic. Images from two representative cells that illustrate these different patterns are shown in Fig. 4. Each row shows separate gray-scale images (DAPI, anti-YY1 or anti-BrdU) for an optical slab (1-1.2 μm thick along Z focus axis) through a single cell. At 3 hours after mitosis (Fig. 4A-C), most of the anti-YY1 fluorescence in the cell was from the nucleus (Fig. 4B), where it showed a patchy distribution throughout most of the nucleoplasm. At 7.5 hours after mitosis (Fig. 4D-F), anti-YY1 signals appeared to be mostly cytoplasmic (Fig. 4E), although some staining was still visible in the nucleus. The BrdU staining (Fig. 4C,F) shows that these cells were synthesizing DNA at the time of the BrdU pulse and were therefore in S phase. Two separate control experiments using immunodepleted anti-YY1 antisera revealed no fluorescence in the channel corresponding to the secondary antibody for YY1 at all time points examined (not shown). The different fluorescent staining patterns observed (cytoplasmic

Fig. 3. YY1 mRNA and protein levels throughout the cell cycle. Numbers above the lanes indicate cell-harvest time points (hours after shake-off). (A) RNA gel blots of equal amounts of total RNA from synchronized CHO cells were incubated with radiolabeled cDNA clones for YY1, then reprobed with EF1-α as a loading control. (B) Protein gel blots were incubated with anti-YY1 antibodies (YY1) or anti-α-tubulin (tubulin) and detected by ECL.

Fig. 4. Accumulation of YY1 in two distinct subcellular locations, cytoplasm and nucleus. Optical sections of triple-label images are shown for two cells fixed at different time points after shake-off (3 hours, A-C; 7.5 hours, D-F). Cells were stained by indirect immunofluorescence and data collected by serial optical sectioning microscopy. For each image, a projection (1-1.2 μm thick in Z comprising 4-6 optical sections) from a middle region of the data stack is shown. DAPI images (A,D) reveal the nucleus (boundary indicated by long-dashed lines). YY1 was visualized in the rhodamine channel (B,E), and BrdU was visualized in the FITC channel (C,F). The nuclear periphery (long-dashed lines) and the cell periphery (short-dashed lines) are indicated, and the nuclear and cytoplasmic areas are indicated by 'n' and 'c', respectively.

Fig. 5. Change in the localization of YY1 from cytoplasmic to nuclear upon entry into S phase. Synchronized CHO cells were fixed at the times indicated (top, hours after shake-off) and subjected to indirect immunofluorescence for visualization of the distribution of YY1 (E-H) and BrdU (I-L). 3D multiple-wavelength images were collected and are displayed as described in Fig. 4, and representative cells (one cell per column) are shown. Dashed lines indicate the cell periphery. YY1 signals appear mainly cytoplasmic at 1 hour (E), mainly nuclear at 3 (F) and 5 (G) hours, and then mainly cytoplasmic again at 7.5 hours (H). BrdU labeling of nuclei appears at 3 hours (J) and is strong throughout the nuclear volume at 5 (K) and 7.5 (L) hours. At the bottom (M-P), all three wavelengths are shown as a three-color (pseudo) merged images (blue for DAPI, green for Rhod/YY1, red for FITC/BrdU). Scale bars: 2 μm.
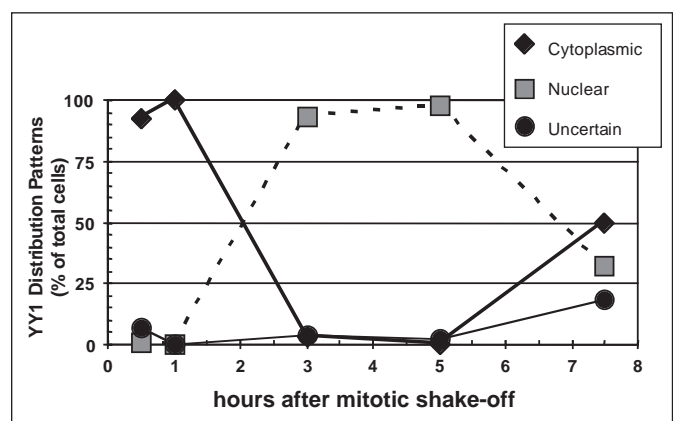
and nuclear) were therefore taken to reflect true differences in the overall localization of the YY1 protein at different times in the cell cycle.

YY1 switches from cytoplasmic to nuclear localization at early S phase in rodent and human cells

For better definition of the timing of these changing YY1 localization patterns, we examined the subcellular distribution patterns for YY1 in a large number of cells. Image analysis of 3D data collected from cells such as those shown in Fig. 5 revealed that, 1 hour after mitosis, in G1, the YY1 is predominantly cytoplasmic (Fig. 5E), whereas BrdU labeling (Fig. 5I) is not evident. After entry into S phase (3 hours and 5 hours), YY1 accumulates in the nucleus (Fig. 5F,G), and BrdU labeling is evident in the 5-hour example (Fig. 5K). After the midpoint of S phase (7.5 hours), the cytoplasmic YY1 reappears in most of the cells (Fig. 5H) and BrdU pulse labeling is still evident (Fig. 5L). Both the YY1 and BrdU staining revealed discontinuous nuclear staining patterns with apparent differences in substructure (compare, Fig. 5G with K and Fig. 4B with C, and E with F).

The graph in Fig. 6, based on data from more than 100 cells at each of the four time points described above, illustrates the

Fig. 6. Cell cycle changes in YY1 distribution patterns. At least 100 cells per time point were chosen in the DAPI wave length, then classified by direct examination of YY1 staining as mainly cytoplasmic (solid diamond) or mainly nuclear (gray box).
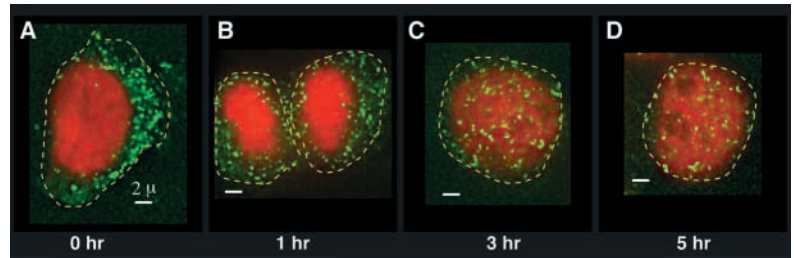
dynamic changes in YY1 distribution as a function of time after shake-off. Nuclear localization of YY1 is most pronounced in early and middle S phase.

To determine whether the localization was specific to CHO cells, we repeated these experiments using HeLa cells that were synchronized by mitotic shake-off and stained for YY1 and BrdU as described above. Representative results shown in Fig. 7 reveal a distinct change in YY1 localization, from primarily cytoplasmic at 0 and 1 hour (Fig. 7A,B) to primarily nuclear at 3 and 5 hours (Fig. 7C,D). Therefore, in HeLa cells, as in CHO cells, YY1 switches to accumulate in the nucleus near the G1/S boundary.

The results of the analysis of the nuclear and cytoplasmic fractions of synchronized cells, verification of the immunolocalization data, and testing of these fractions for the presence of YY1 by western blot assay are shown in Fig. 8. During telophase of late mitosis (0 hours), YY1 was located mainly in the cytoplasmic fraction. During G1 (1 hour), YY1 was mostly in the cytoplasm, but a small amount was in the nucleus. YY1 in the nuclear fraction was highest at 3 hours and 5 hours after mitotic shake-off, corresponding to early and middle S phase, but YY1 was still divided between the nuclear and cytoplasmic fractions, whereas the immunolocalization data, summarized in Fig. 6, would predict that most of the YY1 protein should be nuclear. This apparent discrepancy may arise because the cytoplasmic fraction is contaminated with protein from nuclei accidentally ruptured during the fractionation procedure, whereas the nuclear fraction is less likely to contain contamination. Epitope masking could also affect these results, but so far as we know, the epitope is not subject to post-translational modifications. Taken together, the microscopic and cell-fractionation analyses show that the subcellular distribution of YY1 changes from cytoplasmic to nuclear several hours after mitosis. The timing of this switch corresponds to late G1 and early S phase, such that YY1 accumulation in the nucleus precedes or coincides with entry into S phase.

Localization of YY1 in the cell is coordinately regulated with DNA synthesis

Because YY1 was found to be nuclear during S phase, we tested for linkage of this localization to DNA synthesis. We found that the DNA synthesis inhibitors aphidicolin and hydroxyurea blocked BrdU incorporation and that a concomitant loss of immunodetectable nuclear YY1 occurred. Confocal images from the aphidicolin experiments are shown in Fig. 9. Asynchronously growing CHO cells (untreated Fig. 9A-C) show the typical pattern of staining with nuclear YY1 detected in some cells (white arrows). The general staining pattern in asynchronous cells is consistent with the findings from shake-off-synchronized cells, where YY1 appears nuclear in a 2-hour window of a rather long S phase (Fig. 6). In CHO cells treated with 50 μg/ml aphidicolin for 30 hours, BrdU incorporation was blocked (Fig. 9E) and YY1 staining was dramatically reduced and not observed in nuclei (Fig. 9D). Similar results were obtained when the cells were immunostained after 45 hours with aphidicolin (data not shown). Next, a DNA damage checkpoint inhibitor, either 2-aminopurine or caffeine, was added to aphidicolin-blocked cells for an additional 15 hours. Evidence of DNA synthesis
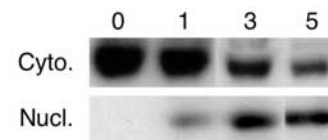

Fig. 7. Change in YY1 subcellular localization in HeLa cells. Synchronized HeLa S-3 cells were collected by mitotic shake-off at the time points indicated. Fixed cells were stained for YY1, imaged and presented as described in Fig. 4. Color-merge images show the nucleus (red, DAPI) and YY1 (green); dashed lines indicate the cell periphery. At late mitosis (A) and 1 hour after shake-off (B), YY1 appears mainly cytoplasmic. After 3 (C) and 5 (D) hours, most of the YY1 signals are located in the nucleus, where they appear as yellow-green in the merged images because of colocalization with red DAPI signal. Scale bars: 2 μm.

(BrdU incorporation) was observed (Fig. 9H,K), and YY1 nuclear localization was seen in all BrdU-positive cells (Fig. 9G,J). To quantify these effects, we determined the percentage of cells that were positive for BrdU incorporation or YY1 nuclear staining (Fig. 10). For each of the four treatments, at least 100 cells were scored. We found that either 2-aminopurine or caffeine resulted in a reappearance of BrdU labeling in most nuclei, consistent with their known mode of action as inhibitors of the DNA damage-signaling pathway. Remarkably, all cells with BrdU staining also showed nuclear YY1. These experiments show therefore that YY1 is lost from the nucleus when DNA synthesis is blocked but it appears to re-accumulate in the nucleus in cells where the block has been bypassed.
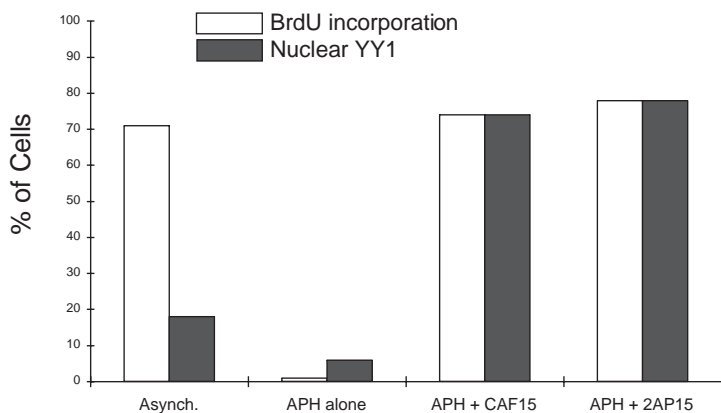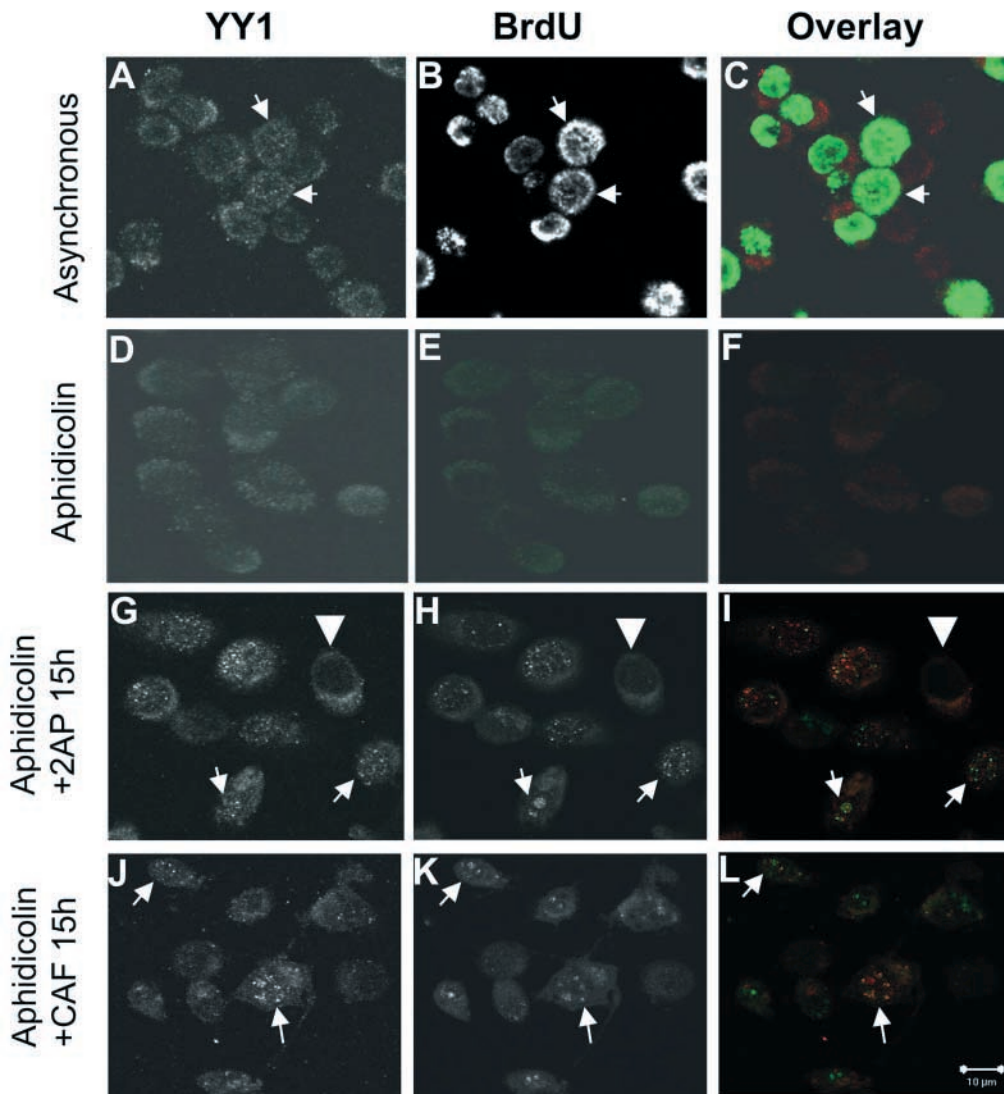
Discussion

We have shown that YY1 exhibits dynamic changes in localization within rodent and human cells during progression from G1 into S phase of the cell cycle. In CHO cells, YY1 is primarily localized in the cytoplasm in late mitosis and is primarily nuclear after cellular entry into S phase. The change in localization occurs at the time of up-regulation of the histone gene family at the G1/S boundary of the cell cycle. These findings are consistent with our hypothesis that YY1 plays a key role in the regulation of replication-dependent histone gene expression (Eliassen et al., 1998).

Our studies focused on the cellular processes through G1

Fig. 8. Cell-cycle changes in YY1 distribution observed by cofractionation assay. Cytoplasmic and nuclear extracts from synchronous populations of CHO cells were analyzed by western blot with anti-YY1 antibodies as described in Fig. 2B. Numbers above the lanes indicate the time of harvest (hours after shake-off). Cytoplasmic (Cyto.) or nuclear (Nucl.) extracts were loaded on an equal-protein basis (7 μg per lane).

Fig. 9. Loss of nuclear YY1 in response to inhibition of DNA synthesis, and recovery of nuclear YY1 with cell-cycle checkpoint inhibitors. Asynchronously growing CHO cells were fixed, immunostained and imaged with a confocal microscope. Images of immunodetectable YY1 (left column) and BrdU (middle column) are shown separately in gray scale and together as two-color overlay (red for YY1 and green for BrdU). Each row shows a field of cells from each of the four different treatments displayed at the same scale. (A-C) Untreated control cells. A subset of these cells are positive for both nuclear YY1 and BrdU staining (white arrows). (D-F) Aphidicolin-treated cells show loss of nuclear staining for both YY1 and BrdU. (G-I) Addition of the checkpoint inhibitor 2-aminopurine (2-AP) to aphidicolin-inhibited cells resulted in restoration of BrdU labeling and YY1 nuclear accumulation (white arrows). A few cells that did not overcome the block also failed to show YY1 (white arrowhead). (J-L) Addition of the checkpoint inhibitor caffeine (CAF) to aphidicolin-blocked cells had the same effect as 2-AP addition. Scale bars: 10 μm.

Fig. 10. Percentage of cells showing BrdU incorporation and nuclear localization of YY1 in response to aphidicolin and checkpoint inhibitors. The experiment was replicated three times, and for each treatment indicated at the bottom of the graph, at least 100 cells were scored. In the treatments with caffeine and 2-AP, all cells that showed BrdU incorporation also showed nuclear YY1.

into S phase of the cell division cycle. Cell cultures for such studies can be synchronized by different methods, and the method must be taken into consideration when such cell cycle data are interpreted. One group of methods involves synchronizing cells by means of arresting agents such as inhibitory drugs or growth-limiting media (Whitfield et al., 2002), but we used the substantially different mitotic shake-off, a method for selection of mitosis stage cells from a population. The selection is based on the phenomenon that substratum-attached cells round up and loosen their attachment to the substratum as they go through cytokinesis. Mitotic cells can therefore be selectively detached and collected from a growing culture by vigorous agitation. We have used this approach previously to examine histone gene regulation (Harris et al., 1991; Kaludov et al., 1996a; Whitfield et al., 2000) and the role of YY1 in the coordinate up-regulation of the histone gene family at the G1/S transition (Eliassen et al., 1998), and find that it consistently provides unperturbed, normally cycling cells for analysis of events following mitosis. Additional support comes from a recent DNA

microarray study involving the analysis of genes expressed throughout the cell-division cycle (Whitfield et al., 2002). For that study, mRNAs were isolated from cultured cells synchronized by mitotic shake-off (from the same large-scale shake-off system used in this study), double thymidine block, and thymidine-nocodozol treatment. Microarray data from these three approaches revealed that a common set of 874 genes in the human genome is periodically expressed in the cell cycle (Whitfield et al., 2002). Of particular relevance to our YY1 study was the observation that the shake-off technique gave optimal results for analysis of events following mitosis, in G1 and early S. Therefore, we believe that a major finding of the present study, the switch from cytoplasmic to nuclear YY1 at late G1 and early S, comes from a very reliable time point in these studies.

Evidence from other laboratories shows that the transcriptional activities of YY1 during embryogenesis and differentiation are correlated with changes in subcellular localization (Kalenik et al., 1997; Donohoe et al., 1999; Ficzycz et al., 2001). Austen et al. (Austen et al., 1997) identified a YY1 domain required for its localization to the nucleus in RK13 (rabbit kidney epithelium) cells. Specifically, the C terminus of YY1, the zinc-finger domain (zinc fingers 2 and 3), is necessary for nuclear localization (Austen et al., 1997; McNeil et al., 1998). Without the second and third zinc fingers, YY1's localization is cytoplasmic, as shown by deletion analyses (Austen et al., 1997). The zinc fingers have also been shown to be necessary for association with the nuclear matrix (Bushmeyer and Atchison, 1998).

The transport of YY1 into the nucleus may involve a nuclear localization signal within the localization domain, or alternatively, YY1 may be cotransported into the nucleus with a protein that contains a nuclear localization signal (Austen et al., 1997). A nuclear shuttle factor that has been shown to associate with YY1 is B23/numatrin/nucleophosmin/NO38 (Inouye and Seto, 1994; Guo et al., 1995; Austen et al., 1997; McNeil et al., 1998; Thomas and Seto, 1999; Olson et al., 2000). B23 has two forms, one residing in the cytoplasm and the other in the nucleolus (Inouye and Seto, 1994). It is involved in rRNA transport between the cytoplasm and the nucleus (McNeil et al., 1998) and may also be part of the nuclear matrix. The interaction of B23 with YY1 might mediate some of the changes in YY1 distribution we have observed. The compartmentalization of YY1 in the cell or even within the nucleus may also reflect different regulatory functions of YY1 in gene expression (Lindenmuth et al., 1997; Bushmeyer and Atchison, 1998; McNeil et al., 1998).

Recently, Petkova et al. (Petkova et al., 2001) reported evidence of interaction between YY1 and the retinoblastoma protein Rb. YY1 colocalized with Rb in resting, differentiated cells (G0/G1) but not in cells in S phase, so the authors speculated that Rb regulates the S-phase activities of YY1 (Petkova et al., 2001). Rb is not functional in HeLa cells (Scheffner et al., 1991), and we have shown here that the cellular localization pattern of YY1 in HeLa cells changes from primarily cytoplasmic to primarily nuclear at the onset of S phase (see Fig. 7). We conclude that, although Rb may play a role in regulating the transcriptional activities of YY1 in the nucleus in S phase, Rb function is not required for the changes in cellular localization of YY1 near the G1/S boundary.

We have shown that the YY1-binding site in histone genes,

the alpha site, is necessary for correct up-regulation of genes in the cell cycle (Eliassen et al., 1998). Mutations or deletions of the alpha histone site result in a significant decrease in histone gene expression in vivo and abolishment of the alpha DNA-binding activity in vitro (Hurt et al., 1989b; Hurt et al., 1991; Bowman and Hurt, 1995; Bowman et al., 1996; Eliassen et al., 1998). The YY1-histone alpha interaction is regulated by phosphorylation/dephosphorylation (Kaludov et al., 1996a). YY1 DNA-binding activity is regulated by phosphorylation/dephosphorylation, either directly or indirectly through a YY1-interacting protein. The signaling process involved in regulating DNA binding may also regulate the subcellular localization of YY1 during the cell cycle.

Because of its involvement as a transcription factor in such a wide variety of genes, the nuclear localization of YY1 during the cell cycle was expected, but the cytoplasmic localization during G1 and later, after the mid-point of S phase, was unexpected because most of the activities attributed to YY1 involve interactions with proteins that function in the nucleus. The YY1 immunostaining data provide strong circumstantial evidence that nucleocytoplasmic shuttling of YY1 may be a key component of the coordinated up-regulation of the cell-cycle-dependent histone genes.

Interestingly, the nuclear YY1 does not appear to be strictly colocalized with the histone genes within the nucleus. The histone gene clusters should occupy only a small fraction of the nucleus, but the YY1 immunofluorescence appeared to be distributed throughout the nucleus. This distribution may reflect multiple functional pools of nuclear YY1, one associated with the histone genes and others associated with other nuclear activities. Because YY1 has been shown to act in many genetic contexts as either a positive or a negative regulator, multiple pools of YY1 might be expected to result from multiple interactions of the protein with DNA sequences or YY1-associating factors.

It seems clear from our evidence and that of others (Austen et al., 1997; Broyles et al., 1999; Ficzycz et al., 2001) that changes in the subcellular localization of YY1 play a key role in its activities as a transcriptional regulator. As we have shown here, obvious changes in the subcellular localization of YY1 are also observed at the time when cells enter the S phase of the cell cycle. A number of genes whose regulation is affected by YY1 have products that are required by a cell entering a new cell division cycle. Although many of these genes are not directly regulated by cyclin-dependent kinases, their products are essential for cell growth and proliferation. The timing of their expression may be due, in part, to the redistribution of YY1.

Because of the temporal link between the subcellular localization of YY1 and the initiation of S phase, we investigated the effects of inhibiting DNA synthesis upon the cellular localization of YY1. We found a direct relationship between the status of DNA replication and the subcellular localization of YY1. That is, localization of a substantial amount of cellular YY1 depends on normal, unblocked DNA replication. Blockage of DNA replication by aphidicolin or hydroxyurea caused YY1 to be primarily cytoplasmic, in direct support of our observations in synchronous cell populations. Those observations show that, early in the cell cycle, YY1 localization changes from primarily cytoplasmic to primarily nuclear in cells showing evidence of S phase entry. Further

evidence that YY1 localization is coordinately regulated with DNA synthesis is that inhibitors of the DNA damage checkpoint signaling processes restore a pattern of nuclear localization for YY1 in aphidicolin-blocked cells only in cells showing evidence of DNA synthesis.

Our experiments followed earlier studies showing that the addition of 2-aminopurine or caffeine to aphidicolin-inhibited cells restored DNA replication (Dimitrova and Gilbert, 2000). Aphidicolin, an antibiotic inhibitor of DNA polymerase (Huberman, 1981), generates stalled replication forks. The DNA checkpoint machinery recognizes these as a form of DNA damage, and checkpoint signaling processes are activated, arresting the cell in S phase until the block is removed or DNA damage is repaired (Bartek and Lukas, 2001). The aphidicolin block is mediated through the activation of the protein kinase ATR (ATM- and Rad3-related) (You et al., 2002). When 2-aminopurine, a purine analogue and a protein kinase inhibitor, was added to aphidicolin-arrested cells, YY1 reappeared in nuclear regions active in DNA synthesis. The same results were obtained with caffeine. By inactivating protein kinases involved in checkpoints, 2 aminopurine (2-AP) or caffeine allow the cells to override the block and progress aberrantly in S phase. Although the mechanisms underlying checkpoint inhibition are not completely known, supporting evidence from the literature suggests that 2-AP may bind directly to and affect the phosphotransferase activity of the protein kinase ATR (Mahadevan et al., 1990) and inhibit the downstream signaling needed to maintain the aphidicolin block. Caffeine was shown to inhibit the kinase activities of the proteins ataxia telangiectesia mutated (ATM) and ATR (Sarkaria et al., 1999). The aphidicolin-induced block is abrogated by the addition of caffeine or 2-AP, each of which results in reactivation of DNA synthesis consistent with the progression of the cell cycle (Fig. 9). Using these inhibitors, we have established a mechanistic link between DNA synthesis and nuclear localization of YY1. More specifically, we provide the first evidence that YY1 dynamics are responsive to some of the cell-cycle signaling pathways associated with DNA synthesis.

In summary, we have shown here that the subcellular localization of YY1 changes from primarily cytoplasmic to primarily nuclear at the G1/S boundary, when a cell commits to genome replication for cell division. We have provided evidence that the subcellular localization of YY1 can be regulated by the same signal processes regulating DNA synthesis. Given the multiplicity of nuclear functions attributed to YY1, these results provide new insights on which future investigations of the global role of YY1 in the cell cycle can be based.

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