YB-1 as a cell cycle regulated transcription factor facilitating cyclin A and cyclin B1 gene expression

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Running Title: YB-1 and cell cycle regulation
SUMMARY

Expression of the Y-box protein YB-1 is increased in proliferating normal and cancer cells, but its role in cell proliferation and cell cycle progression is unclear. We have identified a cell cycle dependent relocalization of YB-1 from the cytoplasm to the nucleus at the G1/S phase transition and demonstrate that both the charged zipper and the cold shock domain are involved in regulating this process. Using cell lines which constitutively overexpress YB-1, we show that nuclear accumulation of YB-1 is associated with increased cyclin A and cyclin B1 mRNA and protein expression. We provide evidence that deregulated YB-1 expression is linked to adhesion-independent cell proliferation through the induction of cyclin A. Thus, we have identified YB-1 as a cell cycle stage specific transcription factor important for cell proliferation.

INTRODUCTION

Y-box proteins belong to a family of evolutionary conserved proteins which function as transcription factors, regulators of RNA metabolism, and protein synthesis (reviewed by 1,2). Y-box proteins might play important developmental roles in early embryogenesis (3). In non-dividing germ cells, Y-box proteins regulate the utilization of maternal and paternal stores of mRNA by the translational machinery (4-7). YB-1 was first identified as a DNA-binding protein that interacts with the Y-box sequence in MHC class II promoters (8). High levels of YB-1 are present in human fetal tissues of the heart, muscle, liver, lung, adrenal gland and the brain (9). In contrast, the YB-1 expression patterns in most adult human tissues are unknown. A role of Y-box proteins in regulation of cell proliferation has been discussed, and high expression of
Y-box proteins occurs under conditions of cell proliferation, e.g., in certain embryonal tissues, in fetal liver, in the regenerating liver after tissue damage (10) and in the proliferating compartment of colorectal mucosa (11). YB-1 is induced in T lymphocytes by interleukin 2 (IL-2) stimulation, where it is involved in stabilizing IL-2 mRNA (12). Several cell cycle-regulated genes contain Y-box sequences in their promoter or enhancer regions (10). YB-1 interacts with p53 and it was discussed that this interaction is a mechanism for regulating gene expression (13). p53 is directly involved in regulating centrosome duplication (14), and in view of this result it is interesting to note that YB-1 associates with the centrosome in mitotic cells (15). YB-1 is overexpressed in malignant tissues of certain breast cancer patients where it facilitates the expression of the mdr1 gene (16). We have shown that YB-1 predicts drug resistance and patient outcome in breast cancer independent of clinically relevant tumor biologic factors HER2, UPA and PAI-1 (17). In addition to breast cancer, YB-1 is overexpressed in non-small cell lung cancer (18), ovarian serous adenocarcinomas (19), human osteosarcomas (20), colorectal carcinomas (11), and malignant melanomas (21).

Here we report that YB-1 changes its intracellular localization in a cell cycle dependent fashion. While located in the cytoplasm in G1 phase, YB-1 accumulates in the nucleus at the G1/S transition of the cell cycle. YB-1 consists of the evolutionary conserved cold shock domain and a charged zipper which is characterized by alternating negatively and positively charged subdomains. We find that this charged zipper region mediates nuclear accumulation of YB-1 and we show that the cold shock domain and the charged zipper domain are needed for cell cycle controlled nuclear targeting. In addition, we show that nuclear accumulation of YB-1
is associated with transcriptional activation of the cyclin A and cyclin B1 genes. Thus, YB-1 is a hitherto unrecognized cell cycle stage specific transcription factor important for cell proliferation.

EXPERIMENTAL PROCEDURES

Cell culture and cell transfection. HeLa cells were maintained in DMEM, HBL100, HBL100-mock, Y1 and Y17 cells were maintained in RPMI (Gibco BRL, Gaithersburg, MD) containing 10% heat-inactivated FCS (Gibco BRL, Gaithersburg, MD) and penicillin-streptomycin (Seromed-Biochrom, Berlin, Germany). Stably transfected HBL100 cells (HBL100-mock, Y1, Y17) were grown in the presence of 5 μg/ml blasticidin. Transient transfections were done using DOTAP (Roche Diagnostics, Mannheim, Germany) according to the protocol of the producer. For synchronisation experiments, HeLa cells were treated with 20 μM lovastatin for 24 hours. To release the cells from the lovastatin-block the medium was changed and synchronously growing cells were maintained in as described above. In certain experiments we added 2 mM sodium mevalonate for the lovastatin block release.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts of HBL100 cells and Y17 cells were prepared as described recently (22). 5 μg of each nuclear extract was incubated for 30 min at 30 °C with the 32P-endlabeled single or double stranded oligonucleotide. The following oligonucleotides were used: 5'-GCT TTC ATT GGT CCA TTT CA-3' derived from the human cyclin A promoter, 5’-ACG AAC AGG CCA ATA AGG AG-3' derived from the cyclin B1 promoter (proximal CCAAT-box), 5’-CGG CAG CCG CCA ATG GGA AG-3’ derived from the cyclin B1 promoter (distal CCAAT-box), 5’-TGA GGC TGA TTG GCT GGG CA-3’ derived from the human mdr1 promoter sequence -86 to -67 (Y-box -82 to -73, 23), and the non-specific competitor-
oligonucleotide 5´-CCC TGT CAC TTG GCC CCG CC-3´ derived from the human cyclin E promoter (Accession number: L48996, nucleotides 987-1006). The DNA-protein complexes were resolved by electrophoresis on a non-denaturing (native) 4% polyacrylamide gel. For autoradiography, X-Omat AR film (Kodak GmbH, Stuttgart, Germany) was exposed overnight.

Cell fractionation and immunoblotting. Cells were harvested using a solution of trypsin/EDTA and immediately lysed in ice cold buffer containing 10 mM Tris-HCl, pH 7.6, 250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 0.6% NP40, 1 mM DTT, 0.5 mM PMSF and protease inhibitors. After centrifugation for 10 minutes (6100 rpm) at 4 °C in an Eppendorf centrifuge, the residual nuclear pellets were washed with the buffer described above and lysed in sample buffer. For immunoblotting, standard procedures were used. Immunoblots were developed with the ECL system (Amersham, Little Chalfont, Buckinghamshire, UK).

Immunofluorescence analysis. Cells were grown on slides, fixed with acetone/methanol (1:1), and preincubated for 30 min with phosphate-buffered saline (PBS) containing 1.5% horse serum (Vector Laboratories, Burlingame, CA). Cells were incubated for 30 min with the polyclonal anti-YB-1 antibody at a dilution of 1:200. To detect the V5 epitope cells were incubated for 60 min with a monoclonal anti-V5 antibody (Invitrogen GmbH, Karlsruhe, Germany) at a dilution of 1:50. To detect cyclin A and cyclin E the cells were incubated for 60 min with a monoclonal antibody specific for cyclin A (Santa Cruz Biotechnology, Inc. Santa Cruz USA) at a dilution of 1:50 or cyclin E at a dilution of 1:20 (Santa Cruz Biotechnology, Heidelberg, Germany). To detect bound immunoglobulins, we used an anti-rabbit
IgG-fluorescein F(ab’)2 fragment at a dilution of 1:200, or an anti-mouse Ig-rhodamine F(ab’)2 at a dilution of 1:100 (Roche Diagnostics, Mannheim, Germany). For nuclear staining, 4,6-diamidino-2-phenylindole (DAPI; Roth, Karlsruhe, Germany) was added in the last incubation step. Staining was evaluated in a fluorescence microscope (Leica, Bensheim, Germany).

Relative quantitative two step real time RT-PCR. Isolation of total RNA was carried out by using Trizol reagent (Invitrogen GmbH, Karlsruhe, Germany). The reverse transcriptase reaction as well as PCR was carried out as recently described (24). Expression of cyclin A, cyclin B1 and the house keeping gene glucose-6-phosphate dehydrogenase (G6PDH) was determined in duplicate from the same RT reaction. The G6PDH primer were obtained commercially (Roche Diagnostics, Mannheim, Germany). For cyclin A a 140 bp amplicon was designed using the forward-primer 5’-gagctatcctcgtggactgg-3’, the reverse-primer 5’-gccacaagctgaagttttc-3’, a FITC-labeled probe 5’-cctgcatttggctgtgaactacattgata-3’ and a LCRed-labeled probe 5’-gttcctgtcttccatgtcagtgctgag-3’ (TIB MOLBIOL, Berlin, Germany). For cyclin B1 a 140 bp amplicon was designed using a forward-primer 5’-acccaaatacctactggttgctg-3’, a reverse-primer 5’-gcataaaccgatcaataatgg-3’, a FITC-labeled probe 5’-tggtcaggtacagttcataatggaattcagg-3’, and a LCRed-labeled probe, 5’-tgttgcaggagaccagactgtactgt-3’ (TIB MOLBIOL, Berlin, Germany). The calibrator cDNA from HeLa cells was employed in serial dilutions simultaneously in each run.

FACS analysis. Cell samples were fixed in ice cold ethanol. After fixation, the cells were stained with propidiumiodide (50 μg/ml). Fluorescence intensity of 1 x 10^4 cells
was measured with a FACScan flow cytometer (BD GmbH, Heidelberg, Germany). The distribution of cell cycle phases was analyzed using the program Cylchred Version 1.0.2 for Windows 95 which is available online (University of Wales, UK).

*Reporter gene assays.* HBL100 and Y17 cells were transfected with the *cyclin A* reporter gene construct (pALUC) which contains a *cyclin A* promoter fragment fused to the *luciferase* gene. In all transient transfection assays, $10^5$ cells were transfected using the FuGene transfection reagent (Roche Diagnostics, Mannheim, Germany). For transfection, $3\mu l/2\mu g$ DNA and $0.2\mu g$ control DNA were used. The control DNA pRL-null vector was obtained from Promega (Promega, Mannheim, Germany). Luciferase activity was determined with the luciferase dualsystem kit (Promega, Mannheim, Germany). To examine *cyclin B1* we used the p240B1 plasmid which contains a *cyclin B1* promoter fragment fused to the *chloramphenicol acetyltransferase* gene. The reporter gene constructs and mutants are described in the text. To determine CAT activity, $10^5$ HBL100 and Y17 cells were transfected under conditions we have previously described (25). CAT activity was measured using the CAT-ELISA kit (Roche Diagnostics, Mannheim, Germany). All measurements were done in duplicate. Absorbance was measured at 492 nm and CAT values were calculated from the CAT-standard curve, using the EasySoftG200/Easy-Fit software (SLT-Labinstruments, Crailsheim, Germany). The amount of CAT-protein was normalized to the protein content in the lysate. Transfection efficiencies was controlled by cotransfection with the pCATBasic plasmid.
Breast tissue specimens and immunohistochemistry. All clinical specimens in this study were obtained during surgery. Breast tissues were fixed in formalin, embedded in paraffin and cut into sections. The sections were immunostained following a protocol provided by the producer of the APAAP dual system kit (Dianova, Hamburg, Germany). Specimens were coded by numbers and evaluated by three independent investigators.

RESULTS

The intracellular localization of YB-1 is cell cycle regulated

It has been previously reported that YB-1 is located predominantly in the perinuclear space of the cytoplasm (26, 27), and that certain cellular stresses like hyperthermia and UV irradiation cause nuclear accumulation of the protein (25, 28). We investigated the intracellular distribution of YB-1 in exponentially growing HeLa cells by indirect immunofluorescence using purified peptide-specific polyclonal antibodies directed against the N-terminus of YB-1 (Fig. 1A). While YB-1 was found in the cytoplasm of all cells, a subset of cells contained YB-1 in the nucleus (arrows). The frequency of exponentially growing cells with nuclear YB-1 was approximately 5%.

We considered it possible that the subcellular distribution of YB-1 is cell cycle regulated. To test this hypothesis, we synchronized HeLa cells with lovastatin, which causes a cell cycle arrest in early G1-phase (29). The cells were released from the lovastatin block by replacement of the culture medium allowing the cells to proceed through the cell cycle. We analyzed cytoplasmic and nuclear fractions by immunoblotting at various time points after release from the lovastatin block (Fig. 1B).
At all time points, YB-1 was present in the cytoplasm (left panel), whereas it appeared in the nucleus 9 to 10 hours after the lovastatin block release (right panel, lanes 7 and 8). In some experiments we supplemented the fresh culture medium with a 100 fold excess of mevalonate to accelerate the release of the lovastatin block (29). In these experiments we detected a nuclear accumulation of YB-1 by indirect immunofluorescence about seven hours after the release (Fig. 1C, left panel). We observed that YB-1 moves to the nucleus at the same time as cyclin E (Fig. 1C, right panel). Thus, YB-1 is present in the nucleus at the boundary between G1 and S phase of the cell cycle. Based on these data we conclude, that YB-1 is a cell cycle stage specific transcription factor.
Cell cycle regulated nuclear accumulation of YB-1 depends on both the cold shock domain and the C-terminal tail

To investigate which region of the YB-1 protein is required for nuclear accumulation in S phase, we constructed a set of deletion mutants of YB-1 fused to green fluorescent protein (GFP). HeLa cells were transiently transfected with these constructs, and the localization of the fusion proteins was analyzed in living cells by fluorescence microscopy (Fig. 2A-D). The full length form of YB-1 was located in the cytoplasm, but also in the nuclei of a subset of transfected cells (Fig. 2A, c1). In contrast, the GFP control showed a homogeneous distribution within the cytoplasm and the nucleus (Fig. 2A, GFP). We noted that endogenous YB-1 was located in the nuclei of approximately 5% of exponentially growing cells (Fig. 1A), and a similar frequency was detected in the transfected cells (Fig. 2A, c1). This indicates, that the intracellular distribution of full length GFP/YB-1 molecules is cell cycle regulated just as the endogenous YB-1 protein. All transfection experiments with GFP constructs were repeated three times and approximately 100 individual transfected cells were examined. Next we investigated the intracellular distribution of an isolated cold shock domain (CSD) in transfected cells (Fig. 2A, c6). The figure shows that the CSD is predominant in the cytoplasm but also occurs in the nucleus with a diffuse distribution.

It was our next goal to determine the contribution of the C-terminal tail of YB-1 to nuclear targeting. In the following experiments, we have used a version of YB-1 which lacks 20 amino acids of the N-terminus. However, these amino acids are not required for cell cycle controlled YB-1 nuclear accumulation (Fig. 2B, c2).
carboxyl termini of Y-box transcription factors share a similar block distribution of basic and acidic amino acids, but little primary sequence homology (30). The positions of the four clusters of basic amino acids in the C-terminal tail of YB-1 are indicated by boxes in the schematic drawing of an GFP/YB-1 fusion protein (Fig. 2). In the GFP/YB-1 fusion protein constructs c3, c4, and c5 one or more of the basic amino acid clusters were deleted. A deletion of the basic amino acid cluster at the very end of the C-terminal tail of YB-1 was associated with a loss of cell cycle regulated nuclear accumulation of YB-1, as the GFP/YB-1 fusion protein was located either in the cytoplasm or the nucleus in 50% of the transfected cells (Fig. 2B, c3). A deletion of the adjacent cluster of basic amino acids was associated with a nuclear location of the GFP/YB-1 fusion protein in 100% of the transfected cells (Fig. 2B, c4). In contrast, a deletion of the third cluster of basic amino acids (Fig. 2B, c5) was associated with a diffuse cytoplasmic distribution of the GFP/YB-1 fusion protein in 100% of transfected cells.

Next we investigated the intracellular distribution of an isolated C-terminal tail domain of YB-1 (Figure 2C, c7). This molecule contains the four clusters of basic amino acids. In 100% of transfected cells the GFP-fusion protein was located in the nucleus with a speckled distribution. We generated additional GFP fusion proteins of the C-terminal tail domain YB-1 which are shown schematically in Fig. 2C. It is evident that the presence of two or three clusters of basic amino acids in the fusion protein is associated with a nuclear localization in 100% of transfected cell (Fig. 2C, c9, c11). We further divided the C-terminus of YB-1 into four single clusters according to the stretches of basic/aromatic aminoacids. Only the second cluster of basic amino acids at position 171-225 of the C-terminal tail of YB-1 was able to
direct the fusion protein into the nuclei of 100% of the transfected cells (Fig. 2D, c13), whereas the three other fusion proteins seem to be diffusely distributed in the cells (Fig. 2C, c15; Fig. 2D, c12, c14). This pattern is indistinguishable from the intracellular distribution of the GFP control (Fig. 2A, GFP). To discriminate between cytoplasmic retention and active nuclear import of GFP/YB-1, we fused a SV40 derived nuclear localization sequence to the GFP/YB-1 protein. In transfected cells, the fusion protein was located either in the cytoplasm or the nucleus. In these experiments 50% of the cells contained the fusion protein in the nucleus and another 50% in the cytoplasm (Fig. 2E). Taken together, these data indicate that at least two different regions of the C-terminus are responsible for the nuclear targeting of YB-1, and that both the CSD and the C-terminal domain are required for cell cycle regulated nuclear import.

Fig. 2

YB-1 interacts with the Y-box motif in the cyclin A promoter

We have shown previously that YB-1 acts as activator of transcription (16). Here we report cell cycle dependent subcellular movements of YB-1. Taken together these data demonstrate that YB-1 is a cell cycle regulated transcription factor. This finding raised the question which cell cycle-associated genes might be regulated by YB-1. Reports from the literature demonstrated the presence of Y-boxes in the promoters of the cyclin A and cyclin B1 genes (31, 32). Therefore, we decided to determine the significance of YB-1 for cyclin A and cyclin B1 gene regulation.

The promoter region −79 to +100 relative to the predominant transcription initiation site facilitates cell cycle controlled expression of the cyclin A gene (31), and the Y-box located at position -55 to -50 mediates adhesion-dependent cyclin A gene
activation at the G1/S boundary (33). To test whether YB-1 binds to the Y-box of the 
*cyclin* A gene, we wished to use YB-1 isolated from the nuclear compartment of 
cells for electrophoretic mobility shift assays (EMSA). For this purpose, HBL100 cells 
were stably transfected with a YB-1 cDNA construct whose expression is controlled 
by the CMV promoter (28). This cell line, termed Y17, constitutively overexpresses 
YB-1. The YB-1 cDNA construct contained a V5 epitope which appears at the C-
terminus of YB-1. This permits detection of the exogeneous YB-1 protein with a 
monoclonal antibody specific for the V5 epitope. To demonstrate expression of the 
epitope tagged YB-1 protein, Y17 cells were analyzed by indirect 
immunofluorescence microscopy. The exogeneous YB-1 protein is localized in both 
the cytoplasm and the nucleus in the majority of cells (Fig. 3 bottom, left panel). The 
lack of any V5-related signal in HBL100 cells demonstrates the specificity of 
immunostaining (Fig. 3 top, left panel). Nuclear accumulation of YB-1 in Y17 cells 
was also demonstrated by immunoblotting using a nuclear extract and antibodies 
specific for the V5 epitope (unpublished data). Nuclear accumulation of 
overexpressed YB-1 protein is not affected by the V5 tag, because overexpressed 
YB-1 without a V5 tag is also located in the nucleus (16). Thus increased levels of 
YB-1 are associated with nuclear accumulation independent of the cell cycle.

For EMSA, a radiolabeled single-stranded 20-bp oligonucleotide comprising the Y-
box motif of the *cyclin* A gene promoter was used (Fig. 4). Three major retarded 
DNA:protein complexes were formed (arrows) with the extract from Y17 cells (lane 
2). We denominate the slow moving complex as complex A, and the two faster 
moving complexes as B and C. A report from the literature demonstrated by UV-
crosslinking that the same *cyclin* A promoter fragment which we have used for EMSA
here binds two proteins with a molecular weight of 40- and 115-kDa (33). Formation of the retarded complexes A, B and C was less prominent with a nuclear extract from exponentially growing HBL100 cells (lane 3). Specificity of complex formation was demonstrated by competition with a molar excess of unlabelled oligonucleotides comprising the Y-box sequence from the cyclin A promoter (lanes 4-6) and the mdr1 gene (lanes 7-9). To demonstrate that YB-1 is present in retarded complexes, we performed an immunoshift with an affinity purified YB-1 antibody (lanes 13 and 14). The results demonstrate that formation of complex A, B and C was inhibited by the antibody specific for YB-1. Antibodies specific for HA or p53 were used as controls in the immunoshift and they did not affect formation of complex A, B or C (not shown). An investigation of the protein composition of each of the retarded complexes will be part of future work. Note that YB-1 in complex B has a higher binding affinity to the Y-box of the cyclin A promoter as YB-1 in complex A or complex C (Fig. 4, lanes 4-6). Our data demonstrate that YB-1 specifically interacts with the Y-Box motif in the cyclin A promoter.

Fig. 3, Fig. 4

YB-1 specifically interacts with the proximal and distal CCAAT-box of the cyclin B1 promoter

S phase induction of cyclin B1 gene expression is mediated by a proximal and distal CCAAT box within the first 90 bp of the cyclin B1 promoter (32). Since CCAAT boxes are inverted Y-boxes, these motifs are potential binding sites for YB-1. Preliminary results obtained by EMSA demonstrated an interaction of YB-1 with both, the sense and antisense strands of the distal and proximal CCAAT motifs from the cyclin B1
promoter (data not shown). To show specificity we used EMSA and competition with an excess of unlabeled oligonucleotides comprising the CCAAT sequence motifs from the distal and proximal CCAAT boxes and a Y-box motif from the multidrug resistance gene (mdr1) promoter. First we investigated the proximal CCAAT box (Fig. 5A). This oligonucleotide formed a major retarded DNA:protein complex (lane 3, arrow) with nuclear extracts from Y17 cells. This complex corresponds to complex B (Fig. 5A). The formation of this complex was efficiently inhibited by a molar excess of the CCAAT motif from the proximal promoter (lanes 4-6) and by the Y-box from the mdr1 promoter (lanes 7-9). Complex formation was not affected by an unrelated control oligonucleotide (lanes 10-12). The presence of YB-1 in this retarded complex was demonstrated by an immunoshift (lanes 13 and 14). We then analyzed the YB-1 interaction with the distal CCAAT box from the cyclin B1 promoter (Fig. 5B). In this case two major retarded complexes were formed with nuclear extracts from Y17 cells (lane 3). These complexes correspond to complex B and C (Fig. 5A). Similar complexes were formed with nuclear extracts from exponentially growing HBL100 cells (lane 2). Competition with a molar excess of oligonucleotides comprising the distal CCAAT motif (lanes 4-6) or the Y-box from the mdr1 promoter (lanes 7-9) abolished complex formation, whereas the control oligonucleotide had no effect (lanes 10-13). The presence of YB-1 in these retarded complexes was confirmed by an immunoshift (lanes 13 and 14). Note that formation of complex C was inhibited more efficiently by the antibody than formation of complex B, indicating that YB-1 in complex B has a higher affinity to the distal Y-box than YB-1 in complex C. These data show that the transcription factor YB-1 specifically interacts with the proximal and distal Y-box of the cyclin B1 promoter.
YB-1 facilitates cyclin A and cyclin B1 gene expression

The data we have presented here strongly suggest that YB-1 may play a hitherto unrecognized role in regulating activity of the cyclin A and cyclin B1 genes. To address this question, we used Y17 cells with a high level of nuclear YB-1 and compared the mRNA levels of the cyclin A and cyclin B1 genes with those in exponentially growing HBL100 cells where YB-1 is cell cycle regulated (Figure 6A). Quantitative PCR shows that both the cyclin A and cyclin B1 mRNA levels are clearly increased in Y17 cells. As controls we analyzed HBL100 cells and a HBL100 cell line which contains a stably transfected empty expression vector (HBL100-mock). To exclude clonal variability we used another cell line termed Y1 which constitutively overexpresses YB-1 in the nucleus. These cells were created exactly as described for Y17 cells (28). Fig. 6A shows that the expression levels of cyclin A and cyclin B1 mRNA in Y1 cells were as high as in Y17 cells. These data demonstrate that YB-1 is involved in the regulation of both, the cyclin A and cyclin B1 genes.

To demonstrate the role of YB-1 for cyclin A and cyclin B1 gene control directly, we used reporter gene constructs in transient transfection assays and compared the results of HBL100 with Y17 cells. To investigate the cyclin A gene, we used a promoter fragment from the cyclin A promoter which confers S phase specific cyclin A gene expression (31). This promoter fragment was fused to the luciferase gene, and the whole construct was denominated pALUC (31). Transient transfection assays with pALUC revealed that cyclin A promoter driven luciferase expression was strongly induced in Y17 cells demonstrating a direct involvement of YB-1 in cyclin A
gene regulation (Fig. 6B).

To investigate the *cyclin B1* gene, we used a promoter fragment fused to the *chloramphenicol acetyltransferase* gene (CAT). This reporter gene construct contains a 240 bp *cyclin B1* promoter fragment (p240B1CAT) which contains the distal and proximal Y-boxes which are important for S-phase dependent *cyclin B1* expression in proliferating cells (32, 34). This reporter gene construct was used in transient transfection assays of HBL100 and Y17 cells (Fig. 6B). It is recognized that the reporter gene was strongly expressed in Y17 cells demonstrating a direct role of YB-1 in controlling the *cyclin B1* gene. To further investigate the impact of YB-1 in *cyclin B1* gene regulation, we used several reporter gene constructs with mutated Y-boxes. In p240B1mXCAT the proximal Y-box is mutated, in p240B1mPCAT the distal Y-box is mutated, and in p240mPXCAT both Y-boxes are mutated (34). Transient transfection assays revealed that mutation of the proximal Y-box strongly reduced activity of the *cyclin B1* promoter, and a mutation of the distal Y-box reduced activity of the *cyclin B1* promoter even further (Fig. 6B). These data demonstrate that both Y-boxes of the *cyclin B1* promoter are important for controlling the activity of this promoter and they show that YB-1 is a transcription factor responsible for *cyclin B1* gene transcription.

Next we investigated whether increased mRNA levels correlate with cyclin A and cyclin B1 protein levels in Y17 cells. Whole cell lysates from Y17 and HBL100 cells were analyzed by immunoblotting using monoclonal antibodies specific for cyclin A and cyclin B1 (Fig. 6C, right panel). We found a significant higher amount of cyclin A and cyclin B1 protein in Y17 cells in comparison to HBL100 cells. As controls we
analyzed cyclin E, cyclin D1, and cdc2 protein expression. Here, no difference was seen between Y17 and HBL100 cells. To quantify these results, immunoblots were examined by densitometry and the data are shown (Fig. 6C, left panel).

Fig. 6

Cyclins are the regulatory subunits of the cyclin dependent kinases which are central cell cycle regulators. We therefore investigated the impact of cyclin A and cylin B1 overexpression on the cell cycle of Y17 cells. As control we included HBL100 cells in our analysis. Confluent Y17 and HBL100 cell were trypsinized and seeded in tissue culture flasks. We then cultivated the cells and analyzed the cell cycle satus by FACS analysis. We started to collect cell samples 6 hours after seeding and took further samples every six hours for a total of 48 hours. The results for HBL100 and Y17 cells are displayed in Fig. 7. Here we found a significant difference between the two cell lines. It appears that HBL100 cells grew like a synchronized cell population and we find a strong increase of S phase cells 12 and 38 hours after plating. In contrast Y17 cells grow as a more uniform cell population with constant ratios of cells in G1 and S phase. However, the population of cells in G2 phase is getting smaller over time indicating an accelerated passage through G2. The growth of epithelial cells in tissue culture is adhesion dependent and a link between cyclin A expression and adhesion-dependent cell cycle progression has been reported (35). Moreover, it was shown that the Y-box of the cyclin A promoter mediates the adhesion dependent transcriptional activation of cyclin A late in G1 (34), and deregulated cyclin A levels permit adhesion-independent growth (36). Our data show that overexpression of YB-1 in Y17 cells circumvents adhesion-dependent cell cycle oscillations which were observed in HBL100 cells. Thus YB-1 may contribute to adhesion-independent...
cell growth, which is a frequent hallmark of cancer cells.

Fig. 7

The expression of YB-1 correlates with the expression of cyclin A in primary breast cancers

A deregulation of cyclin A expression was observed in certain malignant tumors including breast cancer. High expression levels of either cyclin A or YB-1 have been linked to poor clinical outcome in women with breast cancer (17, 37). We wished to investigate whether YB-1 is also involved in controlling cyclin A expression in human breast cancer tissues. For this reason, we analyzed the expression of YB-1 and cyclin A in 18 untreated primary breast cancers by immunohistochemistry (Fig. 8). YB-1 was detectable in all tumor samples with different expression levels and a heterogeneous expression pattern. The expression levels were measured as reported previously (17). Our analysis revealed a low YB-1 score in 5/18 cases, a median YB-1 score 7/18 cases, and a high YB-1 score 6/18 cases. We then compared the YB-1 scores with the expression levels of cyclin A. We found that the YB-1 scores in 16/18 tumors were correlated with the expression levels of cyclin A. In only two cases we observed a difference in the scores. These data provide strong evidence that YB-1 facilitates cyclin A gene and protein expression in primary human breast cancer. Thus YB-1 might directly contribute to breast cancer proliferation and tumor aggressiveness in women with breast cancer. We are aware that larger clinical studies are needed to demonstrate the significance of these results.

Fig. 8

DISCUSSION

Y-box proteins are regulators of transcription and translation and play important roles
in development (reviewed in 1). Physiologically, increased expression of cold shock proteins in bacteria protects against the stresses of low temperature by sustaining the expression of genes necessary for cell growth (38). In eukaryotes, increased expression of Y-box proteins is also associated with cell proliferation and transformation (16). Here, we identify YB-1 as cell cycle regulated transcription factor facilitating cyclin A and cyclin B1 gene expression.

We report for the first time a cell cycle regulated nuclear targeting of YB-1 at the transition from the G1 to S phase. Cell cycle dependent nuclear accumulation of YB-1 was demonstrated by synchronization with lovastatin and a lovastatin block release (Fig. 1B). We determined that YB-1 moves to the nucleus at the G1 to S phase transition, at the same time as cyclin E (Fig. 1C, right panel). Future work will determine how cell cycle-dependent nuclear accumulation of YB-1 is brought about.

When cells grow exponentially, approximately 30% are in S phase depending on cell type. However, nuclear YB-1 was detected in only 5% of exponentially growing cells, and this was true for the endogeneous and the transfected full length GFP-YB-1 molecule. This suggests that YB-1 prevails in the nucleus in early S phase and disappears from the nucleus later in S phase. Future experiments will have to clarify whether YB-1 is subject to proteolytic degradation in S phase or whether YB-1 moves back from the nucleus to the cytoplasm. The latter possibility is likely as YB-1 specifically binds to certain mRNAs in vitro (40). Note that a recent report demonstrated the association of cytoplasmic YB-1 with mRNA in endothelial cells (26).

We wished to identify domains of YB-1 which are required for cell cycle controlled nuclear accumulation in early S phase of the cell cycle. To address this issue we
generated a set of YB-1 deletion mutants fused to GFP (Fig. 2). The isolated CSD was predominantly located in the cytoplasm, whereas the C-terminal tail resided in the nucleus (Fig. 2A, C). Only the full length YB-1 or a version with a small deletion of the N-terminus displayed cell cycle dependent nuclear localization (Fig. 2A, B). Thus the CSD and the C-terminal tail region act cooperatively in this process. We have mutated the C-terminal tail region to identify elements which mediate nuclear accumulation. There are at least two responsible regions, one located between the amino acids 171-225 and another between 224-317. These regions display no similarities to known nuclear localization sequences, but share a common feature: the stretches of basic amino acids (41). The two subfragments of the second construct (c14 and c15) are distributed diffusely in the cells, indicating that a bipartite sequence or higher structures are necessary for the nuclear targeting of the fusion protein. The second deletion construct contains also a domain which was discussed as a cytoplasmic retention domain in a previous report (amino acids 247 – 267) (26). In contrast, we found this fusion protein predominantly in the nucleus, suggesting that the cooperation with other parts of the YB-1 protein is necessary for cytoplasmic retention. This conclusion is supported by the observation that the GFP/YB-1 fusion protein (c2) which consists of the subdomains c4 and c11 which are both located in the nucleus is now predominantly in the cytoplasm thus demonstrating a cytoplasmic retention. However, at the G1/S boundary this cytoplasmic retention mechanism is not active as the GFP/YB-1 fusion protein is found in the nucleus. The fusion of YB-1/GFP to a constitutive active SV40 nuclear localization sequence (NLS) resulted in a nuclear targeting of the construct in only about 50% of the cells, indicating, that YB-1 is strongly retained in the cytoplasm in a subpopulation of growing cells despite the
presence of the NLS (Fig. 2E). Future work will identify the underlying molecular mechanisms in detail. However, our data provide strong evidence that YB-1 is regulated by at least two different mechanisms, which are on the one side the retention in the cytoplasm and on the other side the activation of the nuclear targeting sequences.

Cyclin A and cyclin B1 are central regulators of cell cycle progression in S phase and the transition from the G2 phase to mitosis. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts (42), and it has been demonstrated that cyclin A induces DNA replication independently, but synergistically to cyclin E (43). Cyclin A overexpression in cultured cells affects cell cycle progression and leads to accelerated entry into S phase (44). The combined overexpression of cyclin A and cdk2 in mammary glands of transgenic mice results in the induction of focal areas of hyperplasia, demonstrating a role of both proteins in control of cell proliferation and tumor development in vivo (45).

Several factors were identified that either stimulate or repress transcription of the cyclin A gene. It has been described, that transcriptional activation occurs via variant E2F sites and cAMP-responsive elements (46, 47), whereas repression of transcription is mediated by ATF sites and CHR corepressor elements (48, 49). Recent data demonstrated a role for HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF complexes in the cell cycle dependent repression of cyclin A gene expression (36). Originally, the promoter region –79 to +100 relative to the predominant transcription start site was identified as being responsible for cell cycle regulation of cyclin A gene expression (31). Furthermore, a CCAAT box located in this region at position -55 to -50 was identified as an important cis-element for adhesion-dependent cyclin
A transcription at the G1/S boundary (33). A more recent report identified a heterotrimeric complex containing NF-YA and NF-YB and an additional 115kD protein which binds to this CCAAT box in a cell cycle dependent manner (50). We assume that the retarded complex A (Fig. 4A) contains YB-1 and, in addition the 115kD protein. Complex A was not detected using the distal or proximal CCAAT-box from the cyclin B1 promoter (Fig. 5). This demonstrates that the cyclin A promoter fragment containing the CCAAT-box contains another recognition motif for a DNA binding protein, which may be the 115kD protein. Future experiments will resolve this question. The YB-1 protein in retarded complex B and C may represent different forms of YB-1 (Fig. 4) which could be generated by certain modifications. Our results demonstrate that YB-1 specifically interacts with the CCAAT motif of the cyclin A gene. In addition, overexpression of YB-1 in Y17 cells was associated with increased cyclin A protein levels. As cyclin A is a major regulator of the cell cycle this shows that YB-1 is directly linked to the control of the cell cycle.

We have identified a hitherto unrecognized role of YB-1 in transcriptional control of the cyclin B1 gene (Fig. 6A). Cyclin B1 accumulates in the cytoplasm during the G2 phase of the cell cycle. The translocation of cyclin B1 into the nucleus is associated with a transition from G2 phase to mitosis (51, 52). The promoter region of the cyclin B1 gene contains binding sites for several transcription factors (32, 53) and it was intriguing to note that two CCAAT boxes are present. It has been reported that the CCAAT binding protein NF-Y either stimulates or represses cyclin B1 gene expression (34, 54). It has been shown that an inhibition of cyclin B1 gene expression in quiescent NIH3T3 cells is mediated by an E-box motif which is located at position −124 to -119 (55). p53 was identified as a negative regulator of cyclin B1 gene
transcription (56). In contrast, CIF150 stimulates cylin B1 gene transcription (57). Here, we show that YB-1 interacts specifically with the distal and proximal CCAAT-box within the cyclin B1 gene promoter, and we demonstrate that these CCAAT elements are important for YB-1 dependent cyclin B1 gene expression. Our results indicate that YB-1 is linked to the regulation of cell cycle progression from the G2 phase into mitosis via the cyclin B1 gene. This finding is supported by a recent report from the literature (58), where it was demonstrated that the chicken Y-box protein ChkYB-1b plays an important role in G2 and/or mitosis (G2/M).

Several reports from the literature have suggested that Y-box proteins could play a role in controlling cell proliferation (reviewed by 10). The levels of chicken (ChkYB-1) and rat (RYB-a) Y-box proteins are high in fetal- but low in adult liver and the expression ChkYB-1 is stimulated during liver regeneration (59). RYB-a is rapidly activated in serum-stimulated quiescent NIH/3T3 fibroblasts (60). Our data demonstrate that adhesion-dependent cell cycle oscillations in HBL100 cells were undetectable in Y17 cells, which express YB-1 in the nucleus. It has been reported that overexpression of cyclin B1 has no influence on cell cycle progression in Rat-1 fibroblasts (61), whereas overexpression of cyclin A stimulates entry into the S-phase (44) and facilitates adhesion-independent cell growth of epithelial cells (33, 36). We conclude that the observed cell cycle alterations in Y17 cells are best explained by the deregulated expression of cyclin A. We have thus identified YB-1 as a transcription factor controlling the cell cycle.

The control of cell proliferation plays an important role in the development of eukaryotic organisms and malignant diseases. Recently, we have reported that a
high expression of YB-1 is associated with poor clinical outcome in women with breast cancer (17). Our present data demonstrate that YB-1 is involved in the upregulation of *cyclin* A gene- and protein expression. We have found that increased YB-1 expression levels were associated with increased expression of cyclin A protein in human breast cancer tissues (Figure 8). This finding is important for tumorbiology, as increased levels of cyclin A in breast cancer are associated with poor clinical outcome (37). Furthermore, YB-1 is responsible for upregulation of the *mdr1* gene, resulting in a multidrug resistant phenotype (16). Moreover, YB-1 was identified as a regulator for the expression of matrix metalloproteinase gelatinase A which is involved in angiogenesis, tumor invasion/metastasis, and chronic inflammation (62). Thus, YB-1 is a cell cycle regulating transcription factor which affects a multitude of biological features of cancerous cells, which might cause a highly aggressive tumor phenotype.

Acknowledgments

We thank Ms. S. Metzner and Mrs. Inge Weinert for technical assistance and the Berliner Krebsgesellschaft (Interdisciplinary Research Project: Molecular Biology and Clinic of Breast and Ovarian Cancers), the state of Berlin for funding our work. We thank B. Henglein for donating pALUC.

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FIGURE LEGENDS

FIG. 1. The localization of YB-1 in HeLa cells is cell cycle dependent. Exponentially growing HeLa cells were stained using polyclonal peptide specific anti-YB-1 antibodies (panel A). In the majority of the cells, YB-1 is located in the cytoplasm, but in a subset it is found also in the nucleus (indicated by arrows). HeLa cells were synchronized with lovastatin and harvested at different time points after replacement of the culture medium. After cell fractionation, cytoplasmic and nuclear fractions were analyzed by immunoblotting using the polyclonal anti-YB-1 antibodies (panel B). Lane 1, HeLa cells treated for 24 hours with lovastatin; lane 2, 4 hours; lane 3, 7 hours; lane 4, 8 hours; lane 5, 8,5 hours; lane 6, 9 hours; lane 7, 9,5 hours; lane 8, 10 hours after replacement of the culture medium. Immunoblot of cytoplasmic extracts (left panel), immunoblot of nuclear extracts (right panel). Synchronized HeLa cells were analyzed seven hours after release of the lovastatin block by indirect immunofluorescence using antibodies specific for YB-1 and cyclin E (panel C). Note that sodium mevalonate was added in this experiment to accelerate the block release. YB-1 is detectable in the nuclei of the cells at this time point (left panel). The presence of cyclin E in the same nuclei indicates that the cells are at the G1 / S boundary of the cell cycle (right panel).
FIG. 2. **Identification of YB-1 domains important for cell cycle-dependent nuclear accumulation.** Localization of mutant GFP/YB-1 fusion proteins in HeLa cells. HeLa cells were transiently transfected with plasmids expressing fusion proteins of deletion mutants of YB-1 and GFP (*panels A – D*). GFP/YB-1 fused to a nuclear localization sequence from SV40 virus (*panel E*). Schematic drawings of deletion mutants, *left panels*. The localization of the fusion proteins in living cells were determined by immunofluorescence microscopy (*right panels*).

FIG. 3. **Overexpression of YB-1 is associated with cell cycle-independent nuclear accumulation.** To generate Y17, HBL100 cells were stably transfected with a plasmid expressing YB-1 fused to a V5-epitope. The expression of the ectopic YB-1 protein was determined using a monoclonal anti-V5 antibody (*left panel, bottom*). No signal was detectable in control HBL100 cells, indicating the specificity of staining (*left panel, top*). Nuclei were stained with DAPI (*right panels*).

FIG. 4. **Specific interaction of YB-1 with the CCAAT-box of the cyclin A promoter.** Equal amounts (5 µg) of nuclear extracts of HBL100 cells (lane 3) or Y17 cells (lanes 2 and 4-14) were analyzed using a radiolabeled single-stranded oligonucleotide originating from the Y-box of the human cyclin A promoter. For competition experiments, unlabelled oligonucleotides originating from the Y-box of the cyclin A promoter (lanes 4-6), from the Y-box of the human mdr1 promoter (lanes 7-9), or a non-specific oligonucleotide from the human cyclin E promoter (lane 10-12) were used in 50-fold and 100-fold molar excess, respectively. For immunoshift assays,
increasing amounts of anti-YB-1 antibodies were added to the incubation mixture (lanes 13 and 14). The three major retarded DNA:protein complexes were denominated A, B and C. The positions of these complexes are indicated.

FIG. 5. **Specific interaction of YB-1 with the distal and proximal CCAAT-box of the **<i>cyclin B1</i>** promoter.** Equal amounts (5 µg) of nuclear extracts of HBL100 cells (lane 2) or Y17 cells (lane 3-14) were analyzed using radiolabeled single-stranded oligonucleotides originating either from the proximal CCAAT-box (panel A) or the distal CCAAT-box of the human <i>cyclin B1</i> promoter (panel B). For competition experiments, unlabelled oligonucleotides originating from the Y-box of the <i>cyclin A</i> promoter (lanes 4-6), from the Y-box of the human <i>mdr1</i> promoter (lanes 7-9), or a non-specific oligonucleotide from the human <i>cyclin E</i> promoter (lanes 10-12) were used in 50-fold and 100-fold molar excess respectively. For immunoshift assays, increasing amounts of anti-YB-1 antibodies were added to the incubation mixture (lanes 13 and 14). The positions of complex B and C are indicated.

FIG. 6. **YB-1 overexpression facilitates **<i>cyclin A</i>** and **<i>cyclin B1</i>** transcription.** Expression of <i>cyclin A</i> mRNA and <i>cyclin B</i> mRNA in HBL100, Y1 and Y17 cells (panel A). To generate YB-1 overexpressing cells, HBL100 cells were stably transfected with a YB-1 expression cassette (Y1, Y17). Quantitative real time RT-PCR for <i>cyclin A</i> (grey columns) and <i>cyclin B1</i> (black columns). The relative expression levels represent the ratios of <i>cyclin A</i> or <i>cyclin B1</i> expression relative to <i>G6PDH</i> expression. RT-PCR was performed as two step reaction using gene-specific primers and hybridization probes in the PCR step. Transient transfection assays with
reporter genes controlled by the cyclin A or cyclin B promoter (panel B). pALUC, promoter fragment from the cyclin A gene fused to the luciferase gene. The cyclin A promoter fragment contains the Y-box which mediates adhesion-dependent cyclin A gene activation. p240B1 promoter fragment from the cyclin B1 gene fused to the CAT gene. This promoter fragment contains the proximal and distal CCAAT-box. p240B1mP, the same fragment with a mutated proximal CCAAT-box. p240B1mX, the same fragment with a mutated distal CCAAT-box. p240B1mXP, the same fragment with mutated proximal and distal CCAAT-boxes. Relative activity was calculated as the ratio of luciferase or CAT expression between Y17 vs. HBL100. Expression levels of cell cycle related proteins in HBL100 and Y17 cells (panel C). Whole cell lysates of HBL100 and Y17 cells were examined by immunoblotting with antibodies specific for cyclin A, cyclin B1, cyclin E, cyclin D1, cdc2 and ß-actin. The latter was used as a control for equal loading and protein concentration (right panel). The immunoblots were quantified by densitometry and the ratio between Y17 and HBL100 is depicted (left panel).

FIG 7. Adhesion-dependent cell cycle kinetics in HBL100 and Y17 cells. To investigate the cell cycle kinetics of HBL100 and Y17 cells, confluent cell layers were trypsinized and plated. Cell samples were taken 6, 12, 18, 24, 30, 36, 42 and 48 hours after plating, and the cell cycle status was determined by FACS analysis. The graphic representation shows the percentage of cells in G1, S and G2 phase of HBL100 cells (top panel) and Y17 cells (bottom panel). G1 phase, blue ribbon, S phase, red ribbon and G2 phase, yellow ribbon.
FIG. 8. **Expression of YB-1 and cyclin A in human primary breast cancer.**

Immunohistochemical detection of YB-1 and cyclin A. Paraffin sections of human breast cancer specimens were immunostained with antibodies specific for YB-1 and cyclin A. Bound antibodies were visualized with the alkaline phosphatase method. Low YB-1 expression (*panel A*) correlates with no or low expression of cyclin A (*panel B*), whereas a high expression level of YB-1 (*panel C and panel E*) is associated with a high expression level of cyclin A (*panel D and panel F*).
A

GFP

c1) aa 1-317

B

c2) aa 21-317

c3) aa 21-262

c4) aa 21-225

c5) aa 21-172
Fig. 2

C

c7) aa 146-317

c9) aa 171-317

c11) aa 224-317

c15) aa 260-317
Fig. 2

D

c8) aa 146-262

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c10) aa 146-225

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c12) aa 146-172

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c13) aa 171-225

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c14) aa 224-262

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E

YB-1-GFP-SV40NLS

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Fig. 4

![Western blot experiment result]

- **YB-1 >**
- **YB-1 >**
- **free oligo >**

**Lanes:**
1. no protein
2. Y17
3. HBL 100
4. cyclin A-Y-box
5. mdr1-Y-box
6. control
7. anti-YB-1
Fig. 5

A

YB-1 >

free oligo >

1 2 3 4 5 6 7 8 9 10 11 12 13 14

B

YB-1 >

free oligo >

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fig. 6

**A**
Quantitative Real-Time RT-PCR

**B**
Reporter gene assay

**C**
Immunoblot

| Relative stimulation | HBL100 | Y17  | cyclin A | cyclin B1 | cyclin E | cyclin D1 | cdc2 | β-actin |
|----------------------|--------|------|----------|-----------|----------|-----------|------|---------|
| 1.60                 |        |      | cyclin A |           |          |           |      |         |
| 5.18                 |        |      | cyclin B1|           |          |           |      |         |
| 1.06                 |        |      | cyclin E |           |          |           |      |         |
| 1.00                 |        |      | cyclin D1|           |          |           |      |         |
| 1.15                 |        |      | cdc2     |           |          |           |      |         |
| 1.07                 |        |      | β-actin  |           |          |           |      |         |
Fig. 8

YB-1

A

C

E

cyclin A

B

D

F
YB-1-GFP + lovastatin
YB-1 as a cell cycle regulated transcription factor facilitating cyclin A and cyclin B1 gene expression
Karsten Jürchott, Stephan Bergmann, Ulrike Stein, Wolfgang Walther, Martin Janz, Isabella Manni, Giulia Piaggio, Ellen Fietze, Manfred Dietel and Hans-Dieter Royer

*J. Biol. Chem.* published online April 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212966200

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