YB-1 Relocates to the Nucleus in Adenovirus-infected Cells and Facilitates Viral Replication by Inducing E2 Gene Expression through the E2 Late Promoter*

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The adenovirus early proteins E1A and E1B-55kDa are key regulators of viral DNA replication, and it was thought that targeting of p53 by E1B-55kDa is essential for this process. Here we have identified a previously unrecognized function of E1B for adenovirus replication. We found that E1B-55kDa is involved in targeting the transcription factor YB-1 to the nuclei of adenovirus type 5-infected cells where it is associated with viral inclusion bodies believed to be sites of viral transcription and replication. We show that YB-1 facilitates E2 gene expression through the E2 late promoter thus controlling E2 gene activity at later stages of infection. The role of YB-1 for adenovirus replication was demonstrated with an E1-minus adenovirus vector containing a YB-1 transgene. In infected cells, AdYB-1 efficiently replicated and produced infectious progeny particles. Thus, adenovirus E1B-55kDa protein and the host cell factor YB-1 act jointly to facilitate adenovirus replication in the late phase of infection.

Adenoviruses have developed efficient strategies to force infected cells into the S phase of the cell cycle (1). This process involves the adenoviral E1A and E1B proteins, which are the first viral proteins to be expressed after infection, and both are essential for viral replication (2, 3). Replication of adenovirus DNA depends directly on interactions between the host cell replication factors NFI, NFII, and NFIII (4) and the three viral replication proteins encoded by the E2 region. The adenovirus E2 transcription unit consists of the E2A and E2B genes, which encode precursor terminal protein pTP, DNA polymerase, and DBP, a multifunctional DNA-binding protein (5). E2 gene expression is driven from two promoters. At early times of infection, E2 gene transcription is under control of the E2 early promoter. At intermediate stages of infection, E2 gene expression is controlled by the E2 late promoter (6). E2 early promoter activity is regulated by adenovirus E1A protein, which controls the activity of the E2F transcription factor by targeting the tumor suppressor protein pRB (7, 8). In contrast, activity of the E2 late promoter is repressed by E1A (9). The E2 late promoter is characterized by the presence of a TATA box, two SP1 recognition sites, and three CCAAT boxes. Two of the inverted CCAAT boxes are located at positions −72 and −135 relative to the E2 late cap site in a 157-bp sequence of the E2 late promoter, which is sufficient for efficient E2 gene transcription (10, 11).

Inverted CCAAT boxes have been identified as sites for Y box proteins, which are highly conserved through evolution from prokaryotes to eukaryotes, and they can function as transcriptional, translational, and developmental regulators (12–14). In eukaryotes, increased expression of Y box proteins in somatic cells is associated with drug resistance and a malignant phenotype (15), and it was discussed that Y box proteins are involved in activating certain genes that are expressed in the S phase of the cell cycle (16). Recently, it has been reported that a major protein in messenger ribonucleoprotein particles in somatic cells is a member of the Y box-binding transcription factor family that acts either as a repressor or an activator of protein synthesis (17). YB-1 is also involved in regulating mRNA stability (18). In addition, it has been shown that YB-1 interacts with p53 (19), represses fas gene expression (20), and promotes splicing of the adenoviral E1A pre-mRNA (21). This suggests that YB-1 plays a significant role in the coordinated control of transcription, splicing, and translation in mammalian cells. Furthermore, Y box proteins are important host cell factors for several animal and human viruses. For example, it was shown that a Y box protein interacts specifically with the long terminal repeat of Rous sarcoma virus (22). In addition, YB-1 is a transcriptional regulator of human polyomavirus JC (23) and human immunodeficiency virus type 1 (24).

Here we report a novel function of E1B-55kDa in adenovirus replication. We show that E1B-55kDa facilitates nuclear accumulation of YB-1, which is associated with an induction of E2 gene transcription. We demonstrate a specific interaction of YB-1 with the promoter proximal Y box of the E2 late promoter and show that YB-1 controls E2 late promoter activity. Our data reveal that the viral E1B-55kDa protein and the host cell factor YB-1 act jointly to control E2 gene expression at later stages of infection.\n
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Experimental Procedures

Cell Lines—293 cells were kindly provided by F. Graham (McMaster University, Hamilton, Ontario, Canada). HeLa, SKOV3, A549, U2OS, and 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. All media were supplemented with 200 μg/ml l-glutamine, 100 μg/ml penicillin, and 25 μg/ml streptomycin.

Construction of YB-1-expressing Recombinant Adenovirus—A recombinant adenovirus expressing the YB-1 cDNA was constructed using the shuttle plasmid pHVad2 containing the human YB-1 cDNA under the control of the CMV promoter and a SV40-poly(A) recognition sequence. The shuttle plasmid and the adenoviral packaging plasmid pHVad1 were cotransfected into Escherichia coli. Recombinant plasmid was isolated and cotransfected into the E1-depleted adenovirus carrying the E1B gene using DOTAP (Roche Molecular Biochemicals). Individual clones were obtained by plaque purification and propagated in 293 cells according to standard methods. Evaluation of plaques was accomplished by PCR using specific primers for YB-1 (5’-TGGTAGATAGACGCTATG-CACTG-3’ and 5’-TCAGCTCCGGAGCGGGAATTCTCT-3’). The viral titers were determined by plaque assays using 293 cell monolayers. The E1B mutant virus Ad338 contains a 524-bp deletion in the 55-kDa E1B protein region (25). The E1A mutant virus Ad312 has been described (26).

Adenovirus Infection—Subconfluent cells were infected with adenoviruses at a m.o.i. of 0.1–200 in infection medium (Opti-MEM containing 2% fetal calf serum) after incubation for 1 h at 37°C in a 5% CO2 atmosphere with agitation every 15 min. Subsequently, the medium was replaced with Dulbecco’s modified Eagle’s medium.

Preparation of Nuclear Extracts and Western Blot Analysis—107 adenovirus-infected and control cells were washed twice in ice-cold phosphate-buffered saline and permeabilized by incubation for 5 min in 3 ml of ice-cold hypotonic lysis buffer (10 mM Tris-Cl, pH 8.0, supplemented with Complete™ protease inhibitor mixture (Roche Molecular Biochemicals)). For the preparation of nuclear extracts, we followed our published procedure (27). 30 μg of proteins/lane was subjected to electrophoresis on 10% SDS-polyacrylamide gel. For immunoblotting, standard procedures were used. For the detection of YB-1 we used a polyclonal affinity-purified antibody (15). Western blots were developed with the ECL system (Amersham Biosciences, Inc.).

Electrophoretic Mobility Shift Analysis—Electrophoretic mobility shift assays were performed following a published protocol (28), which also describes the preparation of nuclear extracts. To detect YB-1 binding to the E2 late promoter, a labeled synthetic oligonucleotide from the adenovirus E2 late promoter nucleotides –88 to –40 (5’-TGGTAGATAGACGCTATG-CACTG-3’ and 5’-TCAGCTCCGGAGCGGGAATTCTCT-3’) was used (10). Competing oligonucleotides from the E2 late promoter, the adenovirus E1A gene, and a 242-bp fragment containing the 5'- and 3'-flanking sequences of the human CYP2C9 gene (15), were used as a competitor for staining. For staining and counterstaining with uranyl acetate and lead citrate. Sections were examined on a Zeiss EM-1090 transmission electron microscope at 60 kV.

Immunofluorescence Analysis—Cells were grown on slides, fixed with acetone/methanol, and preincubated with phosphate-buffered saline containing 1% horse serum (30 min; Vector Laboratories, Burlingame, CA). An affinity-purified YB-1 antibody (15), a monoclonal antibody against YB-1 (clone B6-8) against the 72-kDa Ad DNA-binding protein (30), and monoclonal antibodies against EIA (Santa Cruz Biotechnology) were used for immunofluorescence analysis. Immunofluorescence analysis was performed as described (25). For 3D visualization of subcellular distributions was performed by confocal laser scanning microscopy using the LSM 410 (Zeiss, Jena, Germany, software version 3.80) at magnification of ×1,000.

Plaque Assays—To determine virus yield, plaque assays were performed. 72 h postinfection A549 and HeLa cells were scraped into the culture medium and centrifuged at 3,000 rpm for 10 min. In brief, virus was harvested from cells by multiple cycles of freezing and thawing. The cell lysates were centrifuged at 3,000 rpm for 10 min, and the supernatants of the cell lysates were tested for virus production by plaque assays using 293 cells as indicator cells.

Northern Blot Analysis—Total RNA was isolated using the Trizol system (Invitrogen) according to the manufacturer’s instructions. 10 μg of total RNA was size fractionated on 1% agarose-formaldehyde gels, transferred to a nylon membrane (Amersham Biosciences, Inc.), and hybridized with labeled cDNA probes. An E2A DNA generated by PCR using WT-Ad5 DNA and specific primers from the E2A gene (29). To generate cDNAs with a high-affinity E2 promoter, each is located between the early E2 promoter and the E2 late promoter, an appropriate primer pair was selected: 5’-AGCTGATTCCTCGTCTT-3’ and 5’-AGGATAGAACGCTTCACAAAC-3’ for PCR amplification. Cycling conditions were 30 cycles consisting of 60 s at 95°C, 60 s at 55°C, and 60 s at 72°C. A 1.8-kb cDNA β-actin probe served as an internal control (CLONTECH).

Detection of Adenovirus DNA Replication—DNA was isolated from infected cells (m.o.i. of 50 pfu/cell) 72 h after infection using the Qiangen purification system according to the manufacturer’s instructions. 5 μg of DNA was digested with the restriction endonuclease KpnI, size fractionated on 1% agarose gel, transferred to a nylon membrane, and hybridized with a labeled E2A cDNA probe.

Electron Microscopy—For ultrastructural analysis, confluent monolayers of infected cells were washed three times with 0.1 M sodium phosphate buffer, pH 7.2, and fixed with 2.5% glutaraldehyde in the same buffer for 30 min at room temperature. Cells were washed again with buffer and postfixed for 30 min with 1% osmium tetroxide. For further processing, the fixed cells were scraped from the culture dishes, collected by centrifugation, embedded in low melting agarose, and cryo-sectioned on ice. Cytoskeletal and nuclear protein fractions were analyzed according to standard procedures. Finally, thin sections were cut from resin blocks, mounted on 200-mesh copper grids, and stained with uranyl acetate and lead citrate. Sections were examined on a Zeiss EM1090 transmission electron microscope at 60 kV.

RESULTS

YB-1 Relocates to the Nucleus in Adenovirus-infected Cells—To test whether YB-1 protein YB-1 has a role in lytic infection with adenovirus type 5 (Ad5), we analyzed YB-1 in infected cells. HeLa cells were infected with Ad5 at a m.o.i. of 50 pfu/cell, and YB-1 was examined by immunofluorescence using an affinity-purified antibody that was raised against an amino-terminal peptide of YB-1 (15). Antibody specificity was controlled by immunofluorescence and peptide competition (15). The YB-1 antibody decorates a unique band in Western blot experiments which indicates the presence of YB-1. This signal was competed efficiently by preabsorbing the antibody with the immunizing amino-terminal peptide of YB-1 (data not shown). Figure 1 shows that in infected HeLa cells YB-1 protein was located predominantly within the nucleus in the perinuclear space (Control panels). In contrast, after infection with Ad5, YB-1 accumulated in the nucleoli of HeLa cells, where it was distributed in speckles (WT-Ad5 panels). When HeLa cells were infected with a recombinant, replication defective (E1-minus) adenovirus vector containing the E. coli β-galactosidase gene

Footnote:

1 The abbreviations used are: CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein; m.o.i., multiplicity of infection; pfu, plaque-forming unit(s); WT, wild type.
(lacZ), the subcellular distribution of YB-1 did not change (AdlacZ panels). In addition, even at high m.o.i. (>100 pfu/cell), a condition in which adenoviral genes are expressed in the absence of E1 proteins, AdlacZ did not induce any detectable nuclear accumulation of YB-1 (data not shown). These results were confirmed at a biochemical level by immunoblotting using cytoplasmic and nuclear extracts of infected cells (data not shown). Thus the E1 region controls nuclear accumulation of YB-1.

**E1B-55kDa Facilitates Nuclear Accumulation of YB-1**

Next, we asked whether E1A or E1B is involved in nuclear translocation of YB-1. To test whether E1A plays a role, HeLa cells were infected with an E1B-55kDa-deleted adenovirus termed Ad338 (25) (Fig. 2A). E1A protein was detected in the nuclei of Ad338-infected cells (E1A panel); however, in these cells YB-1 remained in the cytoplasm (YB-1 panel). To test whether E1B-55kDa is responsible for nuclear accumulation of YB-1 we used an E1A-deleted adenovirus (Ad312) (2) and infected HeLa cells at a m.o.i. of 200 pfu/cell, a condition in which E1B-55kDa is expressed even in the absence of E1A (26). PCR analysis excluded an unintentional contamination with wild type adenoviruses in this experiment. Ad312-infected cells were identified by immunofluorescence assays with an E1B-55kDa-specific monoclonal antibody. Fig. 2B shows that YB-1 accumulated in the nuclei of Ad312-infected cells (YB-1 panel) with a diffuse distribution. Thus, the YB-1 nuclear distribution is distinctly different from that in WT-Ad5-infected cells shown in Fig. 1A (see below). Our data indicate that nuclear accumulation of YB-1 is E1B-55kDa-dependent.

**YB-1 and E1B-55kDa Co-localize to Nuclear Viral Inclusion Bodies in Ad5-infected Cells**

The speckled nuclear distribution of YB-1 in Ad5-infected cells (Fig. 1, WT-Ad5 panel) resembles nuclear viral inclusion bodies. Viral inclusion bodies are believed to represent viral replication and transcription centers, and it was reported that E1B-55kDa and the E4orf6 protein are present in these structures (32). Likewise, the E2A-72kDa DNA-binding protein is also a major component of viral inclusion bodies (33). To test whether YB-1 is associated with viral inclusion bodies, we infected HeLa cells at a m.o.i. of 20 pfu/cell with the Ad5 virus. The cells were then analyzed by immunofluorescence assays using the YB-1 antibody, a monoclonal antibody against the Ad DNA-binding 72-kDa protein (30), and a monoclonal antibody against the adenoviral E1B-55kDa protein (Fig. 3A). The E2A-72kDa and the E1B-55kDa antibodies mark the viral inclusion bodies in Ad5-infected cells (E2A and E1B panels, respectively). The figure shows that YB-1 co-localized with E2A and E1B-55kDa. Thus YB-1 is associated with viral nuclear inclusion bodies in Ad5-infected HeLa cells. To demonstrate a role of E1B-55kDa in the formation of nuclear viral inclusion bodies and the intracellular YB-1 localization, we infected HeLa cells with Ad338 where the E1B-55kDa region is deleted. Confocal laser scanning microscopy with the E2A antibody revealed that the E2A protein is dispersed diffusely in the nuclei, whereas YB-1 remained in the cytoplasm (Fig. 3B, E2A and YB-1 panels, respectively). These data show that E1B-55kDa facilitates nuclear YB-1 accumulation. However, the formation of viral nuclear inclusion bodies requires additional factors (see “Discussion”).

**YB-1 Controls E2 Late Promoter Activity**

Because the late E2 promoter contains several inverted CCAAT boxes (10) that are potential binding sites for Y box proteins, we wished to analyze whether YB-1 has a role in the E2 gene regulation. To test this, we constructed a replication-defective (E1-minus) recombinant adenovirus vector containing a YB-1 transgene under control of the CMV promoter (E1-minus AdYB-1). We then analyzed whether in AdYB-1-infected cells the transgene was...
expressed. Immunoblot analysis revealed high level YB-1 transgene expression in both the cytoplasm and the nuclei of infected HeLa cells (data not shown). Similar results were obtained in several different E1-minus AdYB-1-infected cell lines. Thus, overexpression of YB-1 is associated with nuclear accumulation. We have previously reported a similar result after overexpression of YB-1 using a tetracycline-dependent expression cassette (15).

To investigate whether YB-1 controls E2 gene expression, we infected HeLa cells with the AdYB-1 virus and as controls with an E1-minus AdlacZ and WT-Ad5 virus. RNA was extracted from the infected cells, and E2 gene expression was determined by Northern hybridization using a cDNA probe from the E2A gene (29). It is recognizable that after Ad5 infection the E2 gene was strongly expressed, which is indicated by a large amount of E2A mRNA (Fig. 4, lane 1). However after infection with the AdlacZ virus the E2 gene was not active (Fig. 4, lane 4). In contrast, strong E2 gene expression was observed after infection with the E1-minus AdYB-1-infected cells (Fig. 4, lane 3). Thus, YB-1 is involved in regulating transcription of the adenovirus E2 genes.

We also investigated whether YB-1 controls E2 gene transcription through the E2 late promoter. To determine promoter usage we used a second cDNA probe, termed E2 early, which detects transcripts from the E2 early promoter. A schematic drawing shows the relative locations of the probes is shown at the bottom. Lane 1, WT-Ad5-infected HeLa cells; lane 2, mock infected HeLa cells; lane 3, E1-minus AdYB-1-infected HeLa cells; lane 4, E1-minus AdlacZ-infected HeLa cells. The E2 early probe did not detect transcripts originating from the early promoter in E1-minus AdYB-1-infected cells, although the E2A probe detected an expressed E2 gene. As a control, a Northern blot was hybridized to a β-actin cDNA probe (bottom panel).
FIG. 5. YB-1 binds to the proximal Y box of the E2 late promoter. Sequence specificity of YB-1 DNA binding was determined by electromobility shift assay competition using an excess of specific and unspecific oligonucleotides. Specific oligonucleotides were an E2 late promoter Y box and a Y box from the mdr1 gene. The unspecific oligonucleotide was an unrelated oligonucleotide from the cyclin E gene. The unspecific Y box, two Sp1 sites, and a mutated promoter proximal Y box. Transfected cells were infected with either AdlacZ or AdYB-1 virus as indicated in the figure. Subsequently, adenovirus-infected cells were transfected with EGFP reporter gene constructs. CMV panels, EGFP reporter gene construct under control of the CMV promoter. E2-late panels, EGFP reporter gene construct under the control of an E2 late promoter fragment consisting of a TATA box, two Sp1 sites, and the promoter proximal Y box. E2-late mutated panels, EGFP reporter gene construct under the control of an E2 late promoter fragment consisting of a TATA box, two Sp1 sites, and a mutated promoter proximal Y box. Transfected cells were inspected by fluorescence microscopy and photographed.

YB-1 Specifically Interacts with the Proximal Y Box in the E2 Late Promoter—Next, we wished to investigate whether YB-1 interacts with the proximal Y box of the E2 late promoter at position −72 (10). Binding of YB-1 to this Y box was analyzed by an electromobility shift assay and a labeled E2 late promoter fragment, nucleotide positions −88 to −40 (10). As a source of YB-1 we used HeLa cells that were infected with AdYB-1 virus. As reported above, YB-1 is expressed in the nuclei of AdYB-1-infected cells. Fig. 5 shows that a major and a minor retarded DNA-protein complex were formed. Both of these complexes contained YB-1 as was demonstrated by an immunoshift (Fig. 5, lanes 13 and 14). The sequence specificity of DNA binding was assessed by competition with an excess of the E2 late promoter Y box (lanes 4–6), a Y box from the mdr1 gene promoter (lanes 7–9), and an unrelated promoter fragment from the cyclin E gene (lanes 10–12). Similar results were obtained using nuclear extracts of WT-Ad5-infected cells (data not shown). Thus the transcription factor YB-1 interacts specifically with the promoter proximal Y box of the E2 late promoter, suggesting that E2 late promoter activity is controlled at least in part through this interaction.

YB-1 Controls E2 Late Promoter Activity through the Promoter Proximal Y Box—It was reported that a minimum of −157 bp upstream from the Cap site is sufficient for the efficient transcription of the E2 late promoter in presence or absence of the E1A gene products (10). We wished to determine the functional role of the promoter proximal Y box for E2 late promoter activity and cloned a promoter fragment (position −22 to −87) into an expression vector encoding EGFP as a reporter gene (see “Experimental Procedures”). This fragment contains a TATA box and two Sp1 recognition sites (10). To test the significance of the promoter proximal Y box, the 5′-ATTG motif was replaced by an unrelated 5′-GCCT motif, and the mutant was cloned in the EGFP reporter vector. As a control we generated an EGFP reporter construct where EGFP is under control of the CMV promoter. All three reporter gene constructs were transfected into U2OS cells, which were infected with AdlacZ and AdYB-1 viruses prior to transfection (Fig. 6). The figure shows EGFP expression controlled by the CMV promoter in both AdlacZ- and AdYB-1-infected cells (CMV panels). In contrast, the E2 late promoter-driven reporter gene construct is expressed strongly in AdYB-1-infected cells but not expressed in AdlacZ-infected cells (E2-late panels). Furthermore, the E2 late reporter construct with the mutated promoter proximal Y box is not expressed in either AdlacZ- or AdYB-1-infected cells (E2-late mutated panels). Thus, YB-1 contributes to E2 late promoter activation through the promoter proximal Y box.

YB-1 Facilitates Adenoviral DNA Replication—Next, we wished to investigate whether YB-1 plays a role in adenovirus DNA replication. To test this, we infected A549 cells with the AdYB-1 virus and analyzed viral DNA replication by Southern blotting. To detect replicated adenovirus DNA we isolated total DNA from infected cells, which was then digested with the restriction endonuclease KpnI and processed for Southern hybridization. The Southern blot is shown in Fig. 7. It is evident that the labeled E2A cDNA hybridized strongly to a 3646-nucleotide long KpnI DNA fragment from the adenovirus E2A gene, indicating efficient DNA replication of the E1-minus AdYB-1 (Fig. 7, lane 3). Please note that replication of AdYB-1 DNA was nearly as efficient as replication of WT-Ad5 DNA.
YB-1-mediated Adenoviral Replication

We report here that the Y box protein YB-1 is a previously unrecognized host cell factor facilitating adenoviral DNA replication.

**Production of Progeny Virus Particles in E1-minus AdYB-1-infected Cells**—Next, we asked whether E1-minus AdYB-1-infected cells produce adenovirus progeny particles. To investigate this we infected HeLa cells with AdYB-1 and analyzed adenovirus particle formation by transmission electron microscopy. We found by an inspection of ~600 infected HeLa cells that about 20% contained adenovirus particles (Fig. 8A). Typical crystalline arrays and randomly scattered individual particles are seen (Fig. 8A, arrows). However, characteristic morphological changes including nuclear morphology as marginal accumulation of fibrous material were visible in nearly all infected cells. In mock infected control and E1-minus AdlacZ-infected cells the cellular ultrastructure appeared normal (Fig. 8A, top panels). These data show that YB-1 permits the production of adenovirus particles even in the absence of the E1 region.

We then investigated whether the E1-minus AdYB-1 virus causes an adenovirus cytopathic effect. HeLa and SKOV3 cells were infected with the AdYB-1 virus at an m.o.i. of 50 or 200 pfu/cell, respectively. The cells exhibited rounded morphology and loss of adherence 3–5 days after infection, whereas an infection with AdlacZ had no effect (Fig. 8B). Similar results were obtained after infection of the human lung carcinoma cell line A549. The results show that the YB-1 transgene enables reproduction of the E1-minus AdYB-1 to such an extent that an adenovirus cytopathic effect is induced. To determine virus yield and to exclude YB-1-mediated toxicity we determined virus titers in supernatants of AdYB-1-infected cells by a plaque assay on 293 cells (Fig. 8C). The plaque assay shows that an AdYB-1 infection of A549 cells yields a virus titer of $10^6$ pfu/ml, about 2 orders of magnitude lower than an Ad5 virus. Similar results were obtained with HeLa cells (data not shown).

**DISCUSSION**

We report here that the Y box protein YB-1 is a previously unrecognized cellular factor involved in controlling adenovirus replication. Our data indicate that YB-1 induces E2 gene transcription by activating the E2 late promoter thereby permitting adenovirus DNA replication to a level comparable with a wild type adenovirus. We investigated how E2 late promoter activation by YB-1 is brought about. We have shown that YB-1 interacts specifically with the promoter proximal Y box at position −72 in the E2 late promoter, and we demonstrated that a mutation of the promoter proximal Y box abolished activity of an E2 late minimal promoter fragment. We have thus identified YB-1 as transcriptional activator of the E2 genes. Future experiments will reveal whether the other two Y boxes of the E2 late promoter at positions −135 and −229 interact with YB-1 and whether these Y boxes contribute to E2 late promoter activity. In this context, it is interesting to note that an unidentified factor has been described which binds to all three Y boxes of the E2 late promoter (11).

We investigated whether YB-1 and E1B-55kDa interact with each other in vivo and have addressed this question by immunoprecipitation studies. We were unable to detect any interactions by this method. Our data demonstrate that YB-1 relocates to the nucleus in adenovirus-infected cells in an E1B-55kDa-dependent manner. We have thus identified a previously unrecognized control function of E1B-55kDa. We think that targeting of YB-1 to the nucleus is required for Ad5 E2 late promoter activation in vivo. Our results with the transient reporter gene assays (Fig. 6) support this consideration strongly. Thus, E1B-55kDa is involved in controlling adenovirus DNA replication at later stages of infection. This is in contrast to the function of E1A which controls E2 gene expression early in infection through the E2 early promoter. It is well established that E1A is targeting the E2F transcription factor (7, 34), which is also regulated by products of the adenovirus E4 genes (35–37). Taken together the results from the literature and our results show that E1A and E1B act jointly in controlling timed E2 gene transcription during a lytic life cycle of an adenovirus.

In Ad5-infected cells YB-1 is associated with nuclear viral inclusion bodies (Fig. 3). However, in Ad312-infected cells YB-1 was distributed diffusely in the nuclei (Fig. 2B). Ad312 lacks the E1A gene, and it is thus tempting to speculate that E1A is required for the formation of viral inclusion bodies and for the association of E1B with these structures. This speculation is corroborated by the fact that the association of E1B-55kDa with viral nuclear inclusion bodies depends on the presence of adenovirus E4 gene products (32), and it is known that E4 gene expression is controlled by E1A. We observed in preliminary experiments using an E1-minus virus expressing E1B-55kDa as a transgene (AdE1B55k) that the sole expression of E1B-55kDa was not sufficient for nuclear YB-1 accumulation (unpublished data). In contrast, in 293 cells, which constitutively express E1A and E1B, YB-1 was diffusely distributed in the nuclei. These findings indicate that regulation of YB-1 intracellular movements in adenovirus-infected cells is apparently complex.

Adenovirus Ad5 E1B-55kDa is a multifunctional protein. During the early phase of infection, E1B-55kDa counteracts E1A functions that would otherwise lead to the stabilization of p53 and the induction of apoptosis (38–41). In the late phase, E1B-55kDa functions in complex with the E4orf6 gene product (42, 43) to stimulate accumulation and translation of the viral late mRNAs (25, 44–47). This is accomplished by shutting off host mRNA nuclear export and host protein synthesis (48, 49). It was originally believed that targeting of p53 by E1B-55kDa is an important step in adenovirus replication (50). For example, Ad5 E1B-55kDa interacts with the cell cycle regulator p53, inhibits the transactivation domain of p53 (51), and relocalizes p53 to the cytoplasm (52). However, several groups recently reported that adenovirus replication is independent of the status of p53 (53–57). We have shown here that YB-1 facilitates adenovirus DNA replication by controlling E2 gene transcription via the E2 late promoter. It thus appears that targeting of YB-1 by E1B-55kDa is a crucial step in the process of viral DNA replication later in infection. In light of our findings and the results from the literature (53–57), further work is...
needed to determine exactly the significance of p53 in the process of adenovirus DNA replication. It was reported that activation of the E2 early promoter is insufficient to promote the early to late phase transition during the life cycle of an adenovirus (58). We have identified here YB-1 as a cellular factor controlling the early to late phase transition during the life cycle of an adenovirus. Moreover YB-1 permits completion of a lytic viral life cycle. The E1-deleted AdYB-1 virus not only replicated its viral genome to almost the wild type level but also produced virus particles as shown by electron microscopy (Fig. 8A). We found that 20% of the AdYB-1-infected cells contained viral particles. Similar results were reported by Goodrum and Ornelles (59) using an E1B-55kDa-deleted adenovirus. We determined that virus yield in AdYB-1-infected cells was about 2 orders of magnitude lower than in Ad5-infected cells (Fig. 8C). These differences in virus yield are most likely because the E1-minus AdYB-1 adenovirus vector does not contain E1B-55kDa protein and does not express E4 (data not shown), which is induced by E1A (26). Proteins encoded by the E4 region and E1B-55kDa affect viral replication, viral and cellular RNA transport, and particle formation (44, 60–62).

E1B-55kDa mutant adenovirus vectors can function as oncolytic viruses in cancer therapy in cases where the host cell p53 gene is mutated or otherwise inactivated (50), and it was shown that these viruses do not replicate in normal tissues that express wild type p53 (63). Moreover E1B-55kDa mutant adenoviruses replicate less efficiently than a wild type adenovirus. Our results provide an explanation to these findings. We think that E1B-55kDa mutant adenoviruses replicate poorly because of a failure to induce nuclear accumulation of YB-1 in vivo. The results with AdYB-1 demonstrate this convincingly. In AdYB-1-infected cells YB-1 accumulated in the nuclei, and this was associated with E1-independent AdYB-1 replication. We also have created a breast epithelial cell line with constitutive nuclear overexpression of YB-1 and demonstrated efficient replication of E1-minus adenovirus vectors in these cells.2

The role of YB-1 in adenovirus replication is further strongly supported by our finding that the E1-minus adenovirus vector replicated efficiently in several multidrug-resistant cell lines in which YB-1 is located in the nucleus.3 These results are in line with a recent report by Ganly et al. (64), who showed that an E1B-55kDa-deleted adenovirus (ONYX-015) replicates with higher efficacy in cisplatin-resistant cell lines.

Deregulated nuclear YB-1 expression occurs in certain human malignant diseases such as breast cancer (15), osteosarcoma (65), ovarian serous adenocarcinoma (66), colorectal carcinoma (67), and glioblastoma multiforme (68). Furthermore, environmental stresses such as cytotoxic drug treatment (15, 69) and hyperthermia (70) cause nuclear accumulation of YB-1. In conclusion, we have identified YB-1 as an E1B-55kDa-deleted cellular factor that controls E2 late promoter activity and in consequence viral DNA replication at later stages of infection. Thus, our findings are fundamental for adenovirus

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Fig. 8. Production of infectious adenovirus progeny particles in E1-minus AdYB-1-infected HeLa cells. A, electron microscopy of HeLa cells infected with a replication-defective E1-minus AdYB-1 virus, which contains a YB-1 cDNA as an expressed transgene. Ultrathin sections of E1-minus AdYB-1-infected HeLa cells are shown in the top panels. The presence of viral inclusions indicates that the cells were infected (arrows, bottom left panel). Adenovirus progeny particles appear as single particles or crystalline arrays (arrows, bottom right panel). Ultrathin sections of mock infected and E1-minus AdLacZ-infected HeLa cells are shown in the top panels. Mock infected cells served as a control (top left panel). In E1-minus AdLacZ-infected cells no structural alterations indicative of viral replication were present (top right panel). B, cytopathic effect assay in E1-minus AdYB-1-infected cells. HeLa and SKOV3 cells were exposed to E1-minus AdLacZ and E1-minus AdYB-1 virus at a m.o.i. of 50 and 200 pfu, respectively. Cells were photographed 72 h after infection. E1-minus AdYB-1-infected HeLa and SKOV3 cells showed a cytopathic effect (right panels), whereas uninfected (left panels) and AdLacZ-infected HeLa and SKOV3 cells (middle panels) appeared normal. C, replication efficiency of E1-minus AdYB-1 and WT-Ad5 in A549 cells. The cells were infected at a m.o.i. of 10 pfu/cell with WT-Ad5 and 50 pfu/cell with E1-minus AdYB1 and Ad312, respectively, and virus production was measured in supernatants of infected cell cultures by plaque assay with 293 cells. The virus yield was obtained by averaging the results of three independent measurements.
biology and form a basis for the development of tumor selective adenovirus vectors for cancer gene therapy.

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REFERENCES

1. Boulanger, P. A., and Blair, G. E. (1991) Biochem. J. 275, 281–299
2. Jones, N., and Shenk, T. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3665–3669
3. Berk, A. J., Lee, F., Harrison, T., Williams, J., and Sharp, P. (1979) Cell 17, 935–944
4. De Jong, R. N., and van der Vliet, P. C. (1999) Gene 235, 245–251
5. Wolfe, A. P. (1998) Trends Cell Biol. 8, 239–245
6. Swamynathan, S., and Thimmapaya, B. (1995) J. Mol. Biol. 255, 2329–2359
7. Kovesdi, I., Christman, M., Kern, M. A., Dietel, M., Pick, M., Kaina, B., and Levine, A. J. (1984) J. Virol. 51, 480–484
8. Fessler, S. P., and Young, C. S. H. (1998) J. Virol. 72, 4049–4056
9. Reich, N. C., Sarnow, P., Duprey, E., and Levine, A. J. (1983) Virology 128, 337–371