Involvement of Template-Activating Factor I/SET in Transcription of Adenovirus Early Genes as a Positive-Acting Factor

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The adenovirus genome complexed with viral core protein VII (adenovirus DNA-protein VII complex) at least is the bona fide template for transcription of adenovirus early genes. It is believed that the highly basic protein VII, like cellular histones, is a negative regulator for genome functions. Analyses with in vitro replication and transcription systems using the adenovirus DNA-protein VII complex have revealed that remodeling of the complex is crucial for efficient DNA replication and transcription. We identified host acidic proteins, template-activating factor I (TAF-I), TAF-II, and TAF-III as stimulatory factors for replication from the adenovirus DNA-protein VII complex. Recently, it was reported that the adenovirus DNA interacts with TAF-I and pp32, another host acidic protein (Y. Xue, J. S. Johnson, D. A. Ornelles, J. Lieberman, and D. A. Engel, *J. Virol.* 79:2474–2483, 2005). We found that TAF-I interacts and colocalizes with protein VII in adenovirus-infected cells during the early phases of infection, but pp32 does not. Although pp32 had the potential ability to interact with protein VII, pp32 did not remodel the adenovirus DNA-protein VII complex in vitro. Small interfering RNA-mediated knockdown of TAF-I expression leads to the delay of the transcription timing of early genes. These results provide evidence that TAF-I plays an important role in the early stages of the adenovirus infection cycle.

The adenovirus (Ad) genome is a linear double-stranded DNA of about 36,000 base pairs that is condensed with basic core proteins V and VII and polypeptide X in virions (1, 7). The copy number of protein VII in a virion particle is estimated to be 800 to 1,000, while that of protein V is approximately 160 (36). Although the precise structure of protein VII complexes with DNA is not clear, it is believed that protein VII binds strongly to DNA to maintain the condensed structure of the Ad DNA, while protein V associates with DNA-protein VII complexes less tightly (3, 6, 9).

The Ad genome DNA is imported into the nucleus as nucleoprotein complexes containing protein VII through nuclear pore complexes (11, 33). Protein VII remains associated with the Ad DNA during early phases of infection (5, 31, 39). Thus, the template of transcription of early genes forms a nucleoprotein complex composed of the Ad DNA and protein VII at least. Protein VII is likely to be a negative factor of genome functions. Experiments using cell-free transcription (37) and replication systems (16) with the Ad DNA-protein VII complex prepared from virions as template indicate that protein VII is inhibitory during elongation processes. The viral gene transcription occurs preferentially from the right and left ends of the Ad genome DNA, when the Ad DNA-protein VII complex is used as template (37). It has been suggested that this is likely due to the lower density of core proteins around both ends rather than in the middle region of the Ad genome (38). However, the biological significance of the interaction between

DNA and protein VII during the early phases of infection is not well clarified. It is hypothesized (14, 39) that protein VII would be involved in recruiting viral and host factors, which play a role in transcription and replication. For instance, a viral immediate-early gene product, E1A, which is an activator of other early and late gene promoters, is shown to be associated with protein VII in vitro (14), and this interaction is suggested to direct E1A to the viral DNA-protein VII complex to promote the transcription.

Host acidic proteins, template-activating factor I (TAF-I) (20, 21, 24, 29), TAF-II/nucleosome assembly protein 1 (NAP-1) (15), and TAF-III/nucleophosmin/B23 (27) were identified from HeLa cell extracts as stimulatory factors for a cell-free Ad DNA replication assay using the Ad DNA-protein VII complex as template. TAF-I also stimulates transcription from the complex in vitro (21). Biochemical analyses revealed that TAF-I remodels the Ad DNA-protein VII complex by formation of a stoichiometric ternary complex composed of DNA, protein VII, and TAF-I (12, 29). TAF-I, -II, and -III have acidic clusters rich in aspartic acids and glutamic acids, which are crucial for their TAF activity. TAF-I consists of a homo- or heterodimer between TAF-I α and TAF-I β , alternatively designated SET. TAF-I α differs from TAF-I β only at the short amino-terminal region. TAF-I binds to histones (19) and shows the histone chaperone activity (29). TAF-I promotes cell-free transcription also from cellular-type chromatin templates (10, 29). Thus, it is suggested that TAF-I plays a role(s) in assembly and disassembly of the cellular chromatin structure in uninfected cells.

Recently, it has been reported that pp32, another host acidic protein, associates with protein VII in pull-down assays using glutathione *S*-transferase (GST)-fused protein VII and cell

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lysates (39). Further, chromatin immunoprecipitation (IP) assays showed that pp32 associates with the viral DNA. Both TAF-I and pp32 have highly acidic regions at their carboxyl-terminal regions. In addition to this similar property, both TAF-I β and pp32 have been identified as herpes simplex virus type 1 VP22 binding proteins (35), HLA class II associated proteins (34), and intracellular inhibitors of PP2A (17, 18). A more intriguing finding is that TAF-I β and pp32 are identified as components of the multisubunit protein complexes inhibitor of acetyltransferase (INHAT) (30), SET (2), and Hur (4). These observations altogether raise the possibility that pp32 cooperates and functions with TAF-I to regulate transcription and replication of the Ad genome.

Here, we have tried to clarify the *in vivo* function of TAF-I and pp32 during early phases of Ad infection. pp32 could not remodel the Ad DNA-protein VII complex *in vitro*. Immunoprecipitation and indirect immunofluorescence assays showed that TAF-I interacts and colocalizes with protein VII during the early phases of infection. We could not detect the interaction between pp32 and protein VII in infected cells, although we found that pp32 potentially interacts with protein VII *in vitro* as previously described (39). Small interfering RNA (siRNA)-mediated knockdown (KD) of TAF-I expression caused the delay of the expression timing of early genes. These results strongly suggest that TAF-I plays an important role in the early stage of the Ad infection cycle.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of HeLa cells were maintained at 37°C in minimal essential medium (MEM; Nissui) containing 10% fetal calf serum (FCS; Vitromex) and used for all experiments in this study. Human adenovirus type 5 (HAdV5) was purified by the CsCl centrifugation procedure, and the virus titer was measured by plaque assay (32). Cells to be infected were plated in culture dishes 1 day prior to infection and maintained in MEM containing 5% FCS. Cells were infected with HAdV5 in MEM for 1 h at the appropriate multiplicity of infection (MOI) as indicated in each figure legend. After washing with MEM, cells were cultured at 37°C in MEM containing 5% FCS.

Recombinant proteins and *in vitro* assays. A supercoiling assay and Ad DNA replication assay using the Ad DNA-protein VII complex as template were carried out essentially as described previously (20, 28). Recombinant human TAF-I β and B23.1 proteins with or without a hexa-histidine (His-) tag were prepared as described previously (12, 24, 27). For construction of bacterial expression vectors for His-pp32 and His-NAP-1, the full-length cDNAs of human pp32 and NAP-1 were amplified by PCR from a cDNA library derived from HeLa cell using a set of primers: 5'-CGCGGATCCCATATGGAGATGGGCA GACGGATTTCATTAG-3' and 5'-GCGGCTCGAGACGTCAGTCATCATC TTCTCCCTCATCTTCAGGTTCTCGT-3' for pp32 and 5'-CCCGGGGCATA TGGCAGACATTGACAACA-3' and 5'-GGGCTCGAGTCACTGCTGCTTG CACTCTG-3' for NAP-1. Then, amplified cDNA fragments of pp32 and NAP-1 were digested with NdeI and XhoI and cloned into pET-14b vector (Novagen) that was predigested with the same enzymes. His-pp32 and His-NAP-1 generated in bacteria were purified by Ni-nitrilotriacetic acid column (Novagene) chromatography with the same method for preparation of His-TAF-I (12). His-pp32 was subjected to digestion with thrombin at 4°C and passed through an Ni-nitrilotriacetic acid column to remove the His tag and undigested proteins. Both His-pp32 and pp32 were further purified by MonoQ (Amersham Biosciences) column chromatography.

Indirect immunofluorescence assays. The double immunostaining of TAF-I and protein VII was carried out at room temperature as follows. HeLa cells were inoculated on coverslips in culture dishes 1 day before infection. Cells were mock infected or infected with HAdV5 at an MOI of 250 and incubated for 4 h. The cells were washed three times with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in PBS for 10 min. After washing with PBS, the cells were permeabilized in PBS containing 0.5% NP-40 and then incubated in TBS-T (25 mM Tris-HCl [pH 7.9], 137 mM NaCl, 3 mM KCl, 0.1% Tween 20) containing 5% skim milk for 30 min. Then cells were incubated with rat anti-

protein VII (12) and either mouse anti-TAF-I β (KM1720) (25) or goat anti-PP32 (I1PP2A [C-18]; Santa Cruz Biotechnology) antibodies for 30 min. After washing with PBS containing 0.5% NP-40, the cells were incubated with an appropriate concentration of donkey anti-rat immunoglobulin G (IgG) conjugated with Alexa 488 (Molecular Probes) and either goat anti-mouse IgG conjugated with Alexa 568 (Molecular Probes) or donkey anti-goat IgG conjugated with Alexa 568 (Molecular Probes), respectively. After washing with PBS containing 0.5% NP-40, the coverslips were mounted on slide glasses. The cells were then observed under a fluorescence microscope (Carl Zeiss).

Immunoprecipitation assays. HeLa cells were infected with HAdV5 at an MOI of 250. At 4 h postinfection (p.i.), cells were washed with PBS and collected in a test tube. Cells (2×10^7) were rinsed once with buffer A (10 mM HEPES-NaOH [pH 7.9], 10 mM KCl, 0.1 mM EDTA), suspended in 1 ml of buffer A, and left on ice for 10 min. The cells were lysed by using a glass Dounce homogenizer with a tight pestle. Nuclei were collected by centrifugation and washed twice with 1 ml of buffer A. Then nuclei were disrupted by sonication in 0.55 ml of IP buffer (10 mM Tris-HCl [pH 7.9], 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mg/ml bovine serum albumin), followed by centrifugation at 15,000 rpm for 10 min. The supernatant was recovered and used for the immunoprecipitation assay. The extracts (150 μ l) were incubated with preimmune serum or rat anti-protein VII antibody. The immunocomplex was recovered by the addition of protein A Sepharose Fast Flow beads (Amersham Bioscience). The beads were washed twice with 0.5 ml of IP buffer. Immunoprecipitated proteins were separated by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) and subjected to Western blot analysis using a mixture of goat anti-pp32 and mouse anti-TAF-I β (KM1720) antibodies. For *in vitro* interaction experiments, protein VII (100 ng) was incubated with pp32 (200 ng) in buffer D (20 mM HEPES-NaOH [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 0.5% NP-40, 20% glycerol, 1 mg/ml bovine serum albumin) or indicated amounts of nuclear extracts prepared according to the method described previously (8). Then immunoprecipitation assays were performed as described above except that buffer D was used instead of IP buffer.

Construction of KD HeLa cell line. We used the pU6 vector to express 21-nucleotide-long hairpin-type siRNA with a 9-nucleotide loop under the control of the U6 promoter (22). DNA fragments for targeting both TAF-I α and TAF-I β were prepared by PCR using a set of chemically synthesized oligonucleotides, 5'-GGCTCTAGAACCTGCCGCCACCGTAAGAAGTGATTGA ATATATTAGAATTACATCAAGGG-3' and 5'-GGCTCTAGAACCTGCTA GCGCATAAAAAGCAAGAAGCGATTGAACATATCTCCCTTGATGT AA-3', followed by digestion with BspMI. The fragment was inserted into the BspMI site of the pU6 vector. The resultant plasmid was designated pU6-siTAF-I. The nucleotide sequence of the insert was confirmed by the dideoxy sequencing method. HeLa cells in a 35-mm-diameter dish were cotransfected with pU6-siTAF-I and pSV2-neo using TransIT LT1 reagent (Mirus) and maintained in the presence of 1 mg/ml of G418 (Nacalai Tesque) for 2 weeks. G418-resistant cells were isolated, and the level of TAF-I in each clone was examined by Western blot analysis using anti-TAF-I antibodies. A drug-resistant cell line whose expression level of TAF-I is the same as that of untransfected cells was used as wild-type (WT) cells compared with pU6-siTAF-I-mediated KD cells. pp32 KD cell lines were constructed as described above. The insert DNA was prepared by PCR using a set of oligonucleotides, 5'-GGCTCTAGAACCT GCCGCCACCAAGAATTTGTCTTGATAATAGTTAGAATTACATCA AGGG-3' and 5'-GGCTCTAGAACCTGCTAGCGCATAAAAAAGAACTT GTCCTGGACAACAGTATCTCCCTTGATGTAA-3', followed by digestion with BspMI. To construct the plasmid for the expression of TAF-I β in mammalian cells, DNA fragments containing cDNA of TAF-I β were cloned in-frame into a pCHA vector containing a hemagglutinin epitope tag under the control of the cytomegalovirus enhancer and chicken β -actin promoter (25, 26).

RT-PCR. HeLa cells were infected with HAdV5 at an MOI of 100 and collected at 4 h p.i. Total RNA was purified using the RNeasy mini kit (QIAGEN) and DNase I treatment. The concentration of RNA in each sample was determined using a spectrophotometer. cDNA was synthesized from the total RNA (2.5 μ g) using Superscript II reverse transcriptase (RT; Invitrogen) and a mixture of specific reverse primers complementary to β -actin, E1A, and E2 mRNAs. PCR was performed using synthesized cDNAs (1/40, vol/vol) as template and a set of primers by predetermined PCR cycles under which PCR products are logarithmically amplified. Primer sequences used were as follows: β -actin forward, 5'-ATGGGTCAGAAAGGATTCCTATGT-3'; β -actin reverse, 5'-GGTCATCTTCTCGCGGTT-3'; E1A forward, 5'-TTGAGTGCCAGCGA G-3'; E1A reverse, 5'-CAAAATGGCTAGGAGGTGGA-3'; E2 forward, 5'-TCGAAGGCGAGCTTAAGTGT-3'; and, E2 reverse, 5'-AGAAGAACATGCC GCAAGAC-3'. The PCR products together with those for the quantitative

standards were separated on a 6% PAGE gel, visualized by staining with SYBR Gold (Molecular Probes), and quantified with NIH Image.

Northern blot analysis. Total RNA (10 µg) was subjected to separation on a 1% formaldehyde agarose gel and blotted onto a Hybond N+ (Amersham Bioscience) membrane. DNA fragments containing nucleotides 1 through 1009 and 22955 through 23200 of HAdV5 (where nucleotide 1 indicates the 5' end of the left terminus of the genome) were ³²P-labeled using the Prime-It II random primer labeling kit (Stratagene) and used as probes for the detection of E1A and E2 RNAs, respectively.

Dot blot hybridization analysis. HeLa cells were infected with HAdV5 at an MOI of 100 and collected at every 3 h. The cells (1×10^5) were lysed in a buffer (0.1 M Tris-HCl [pH 6.8], 10% glycerol) containing 1% SDS and sonicated briefly to avoid the increase of the viscosity of the solution. Total DNA was purified by proteinase K treatment and phenol-chloroform extraction followed by RNaseA treatment. Purified DNA was boiled in 0.1 ml of 0.4 N NaOH and 10 mM EDTA for 10 min and rapidly chilled on ice. The denatured DNA was loaded on a Hybond N+ membrane by using a Dot-Blot apparatus (Bio-Rad). Viral DNA was detected by Southern hybridization using ³²P-labeled probe complementary to the HAdV5 genome as described above.

One-step viral growth assay. HeLa cells in 60-mm-diameter dishes were infected with HAdV5 at an MOI of 100. At 24 h and 48 h p.i., cells and medium were collected together by a scraper. The solution containing viruses was prepared by five freeze-thaw cycles in liquid nitrogen and a 37°C water bath, respectively, followed by centrifugation at 3,000 rpm for 5 min at 4°C to remove the debris. The virus titer of the virus solution was determined by plaque assay (32) using six-well dishes. Infection was performed in duplicate for each clone, and plaque assays were performed in triplicate for each virus solution.

RESULTS

Comparison of pp32 with TAF-I in the TAF activity. We identified TAF-I as a stimulatory factor for in vitro DNA replication from the Ad DNA-protein VII complex as template (20, 24). pp32 has structural properties similar to those of TAF-I (17, 18, 34, 35). Furthermore, pp32 interacts and plays a role with TAF-I as a variety of functional complexes (2, 4, 30). Here, we examined the nucleosome assembly activity and the TAF activity of pp32 in the supercoiling assay and the cell-free Ad DNA replication assay using the Ad DNA-protein VII complex as template, respectively (Fig. 1). We prepared recombinant human TAF-Iβ and pp32 without any tag and His-tagged recombinant human TAF-Iβ, pp32, NAP-1, and B23.1 (Fig. 1A). His-pp32 was shown to facilitate nucleosome assembly, as were His-TAF-I and His-NAP-1 (Fig. 1B), suggesting that pp32 binds to histones and functions as a histone chaperone. Then the stimulatory activity of each protein in DNA replication from the Ad DNA-protein VII complex (the TAF activity) was tested (Fig. 1C). TAF-I, NAP-1, and B23.1, those which had been identified as template-activating factors, stimulated the Ad DNA replication in a dose-dependent manner, although the stimulatory activities of NAP-1 and B23.1 were less than that of TAF-I. In contrast, pp32 did not stimulate DNA replication from the Ad DNA-protein VII complex within the doses examined here. This is in good agreement with the fact that pp32 was not identified as a protein containing the TAF activity (27). pp32 did not have any effect when the stimulatory activity of the mixture of TAF-I and pp32 was examined (lanes 14 through 16). These results indicate that pp32 does not have any efficient remodeling activity of the Ad DNA-protein VII complex, while it is capable of functioning as a histone chaperone.

Interaction of protein VII with TAF-I but not with pp32. Next, we examined the binding efficiency of TAF-I and pp32 with protein VII. To try to confirm the previously reported result (39) that pp32 interacts with protein VII in vitro, we

FIG. 1. Stimulatory activity for DNA replication of the Ad DNA-protein VII complex. (A) Purified recombinant proteins. Recombinant TAF-Iβ and pp32 and recombinant His-tagged TAF-Iβ, pp32, NAP-1, and B23.1 (200 ng each) generated by bacterial expression systems were separated by SDS-10% PAGE and visualized by staining with Coomassie brilliant blue. (B) Supercoiling assay. Core histones (200 ng) preincubated without (lane 1) or with 100 ng (lanes 2, 5, and 8), 200 ng (lanes 3, 6, and 9), or 500 ng (lanes 4, 7, and 10) of His-tagged TAF-Iβ (lanes 2 through 4), pp32 (lanes 5 through 7), or NAP-1 (lanes 8 through 10) were mixed with closed circular DNA (200 ng) relaxed by topoisomerase I (1 U; TaKaRa) and further incubated at 37°C for 1 h. The DNA was purified and separated by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. Positions of relaxed and partially supercoiled (R), supercoiled (S), or nicked (N) circular plasmid DNA are indicated. (C) Cell-free Ad DNA replication assays using the Ad DNA-protein VII complex as template were performed in the absence (lane 1) or presence of 50 ng (lanes 2, 4, 6, 8, 10, and 12) or 200 ng (lanes 3, 5, 7, 9, 11, and 13) of TAF-Iβ (lanes 2 and 3), pp32 (lanes 4 and 5), His-tagged TAF-Iβ (lanes 6 and 7), His-tagged pp32 (lanes 8 and 9), His-tagged NAP-1 (lanes 10 and 11), or His-tagged B23.1 (lanes 12 and 13). For lanes 14 through 16, either 50 ng (lane 14), 100 ng (lane 15), or 200 ng (lane 16) of pp32 in the presence of 100 ng of TAF-Iβ was added to the reaction mixture. Replication products labeled with [α -³²P]dCTP were purified, digested with KpnI, separated on a 0.8% agarose gel, and visualized by autoradiography. KpnI fragments of HAdV5 DNA are indicated underneath.

FIG. 2. Interaction of protein VII with TAF-I and pp32. (A) Interaction between recombinant pp32 and protein VII. Protein VII (100 ng) was incubated with pp32 (200 ng). Next, the samples were subjected to immunoprecipitation with control (lanes 1 and 3), anti-protein VII (lane 2), or anti-pp32 (lane 4) antibodies. Immunoprecipitated proteins were separated by SDS-10% PAGE and detected by Western blotting (WB) using anti-pp32 (lanes 1 and 2) or anti-protein VII (lanes 3 and 4) antibodies. (B) Immunoprecipitation analysis using infected cell extracts. Extracts were prepared from infected cells at 4 h p.i. as described in Materials and Methods and subjected to immunoprecipitation with control (lane 2) or anti-protein VII (lane 3) antibodies. Input (5%) (lane 1), immunoprecipitated proteins (lanes 2 and 3), and indicated amounts of recombinant TAF-I and pp32 as standards (Std) (lanes 4 through 7) were separated by SDS-10% PAGE and detected by Western blotting using a mixture of anti-TAF-I β and anti-pp32 antibodies. (C) Immunoprecipitation analysis using uninfected nuclear extracts (NE) and protein VII. Nuclear extracts prepared from uninfected HeLa cells (14, 70, and 350 μ g for lanes 2 and 3, 4 and 5, and 6 and 7, respectively) were mixed with purified protein VII (100 ng) and then subjected to immunoprecipitation with control (lanes 2, 4, and 6) or anti-protein VII (lanes 3, 5, and 7) antibodies. Lane 1 indicates input extracts (35 μ g), and lanes 8 and 9 show recombinant pp32 and TAF-I.

performed immunoprecipitation assays using purified recombinant proteins and antibodies against either pp32 or protein VII (Fig. 2A). Western blot analyses clearly showed that pp32 interacts with protein VII (Fig. 2A). Then we examined the pp32-protein VII interaction in Ad-infected cells during the early phases of infection (Fig. 2B). Extracts were prepared from nuclei isolated from Ad-infected cells at 4 h p.i. when the expression level of early genes was increasing. The extracts were subjected to immunoprecipitation assays using anti-pro-

FIG. 3. Colocalization of TAF-I with protein VII in infected cells during early phases of infection. Cells were mock infected (A through D) or infected (E through H) with HAdV5 at an MOI of 250 and fixed at 4 h p.i. TAF-I β (A and E) and protein VII (B and F) or pp32 (C and G) and protein VII (D and H) were stained by the indirect immunofluorescence method with specific antibody for each protein. Panels I and J show higher-magnified images of the regions marked by squares in panels E and F, respectively. Panel K is a merged image of panels I and J visualized with red and green, respectively. The dots inside the nucleus contain both protein VII and TAF-I, while some portion of protein VII is localized at the periphery of the nucleus.

tein VII antibody. To measure the amount of precipitated proteins, purified TAF-I and pp32 as quantitative standards were also separated on the same gel. Western blot analyses revealed that TAF-I was coimmunoprecipitated with protein VII, while pp32 was not at any detectable level (Fig. 2B). Based on the amount of standard proteins, an amount of TAF-I at least 20 times that of pp32 was present in complexes with protein VII. Since the interaction between pp32 and protein VII was not detected in infected cell extracts, we tried to examine the interaction between pp32 and protein VII in nuclear extracts prepared from uninfected cells (Fig. 2C). We found that TAF-I was efficiently associated with exogenously added protein VII, while the interaction between pp32 and protein VII was not detected under the assay condition employed here. This result could be interpreted as that pp32 has much less affinity to protein VII than TAF-I and/or other protein VII-binding proteins possibly present in extracts.

It is reported that protein VII is localized in the nucleus as discrete dots during the early phases of infection. These dots represent protein VII associated with Ad DNA (11, 39). It is possible that TAF-I accumulates in the dots containing protein VII. To confirm the relevance of the interaction between TAF-I and protein VII, the localization of TAF-I during the early phases of infection was examined by indirect immunofluorescence assays (Fig. 3). Protein VII was found to be localized as nuclear dots (Fig. 3F and H). In uninfected HeLa cells, TAF-I was localized throughout the nucleoplasm except for the nucleolus (Fig. 3A). However, when cells were infected with Ad, TAF-I was detected in not only the nucleoplasm but also nuclear dots (Fig. 3E). Double staining of TAF-I and protein VII clearly showed the colocalization of these proteins in the dots (Fig. 3E, F, and I through K). In contrast, the localization pattern of pp32 was not altered upon Ad infection (Fig. 3C, D, G, and H). These observations are consistent with those of the immunoprecipitation experiments (Fig. 2). Taken

altogether, it is strongly suggested that TAF-I, but not pp32, interacts with protein VII during the early phases of infection.

TAF-I knockdown-mediated delay of the accumulation of early gene transcripts. To further confirm that TAF-I is involved in Ad multiplication, we established stable HeLa cell lines in which the expression of TAF-I is knocked down by siRNA. A target sequence for siRNA was selected within the common sequence between TAF-I α and TAF-I β open reading frames in order to decrease the expression level of both TAF-I α and -I β . We obtained stable TAF-I KD cell lines whose expression level of both TAF-I α and -I β proteins was reduced to approximately 10% (clone 4) and 15% (clone 13) relative to WT-like cell lines (clones 7 through 9) (Fig. 4A). Although TAF-I is suggested to be involved in several biological processes including mRNA stability, cell death, development, and cell cycle, as well as transcription regulation, TAF-I KD cells grow like WT cells under the conditions used for our experiments (data not shown).

The effect of TAF-I KD on the transcription of early genes E1A and E2 was examined. WT (clone 7) and TAF-I KD (clones 4 and 13) cell lines were infected with HAdV5 at an MOI of 100 and collected at 4 h p.i. Total RNA was isolated from infected cells, and the amount of E1A, E2, and β -actin RNAs was measured by semiquantitative RT-PCR (Fig. 4B, upper panel), and results are summarized in the lower panel of Fig. 4B. The expression level of both E1A and E2 RNAs in TAF-I KD cells was less than that in WT cells (Fig. 4B), while that of β -actin RNA remained unchanged in both WT and KD cells. To confirm that the decrease of the amount of E1A and E2 RNAs in KD cells compared to that in WT cells is due to the down-regulation of TAF-I, rescue experiments by overexpression of TAF-I were performed (Fig. 4C). WT and TAF-I KD cells were transfected with indicated amounts of empty pCHA vector and pCHA-TAF-I β vector to transiently express HA-TAF-I β . Forty-eight hours after transfection, cells were infected with HAdV5. The amount of E1A RNA at 4 h p.i. was measured by semiquantitative RT-PCR (Fig. 4C). Transfection of HA-TAF-I β in the TAF-I KD cell line (clone 4) generated the protein level of HA-TAF-I β up to that of endogenous TAF-I β found in WT cells (Fig. 4C, bottom panel). Under this condition, the level of E1A RNA in pCHA-TAF-I β -transfected cells became higher than that in TAF-I KD cells transfected with empty vector. These results suggest that TAF-I is involved in the synthesis and/or accumulation of early gene transcripts. We next examined the effects of pp32 KD on the level of E1A and E2 RNAs using stable pp32 KD cell lines (Fig. 4D). The accumulation level of pp32 protein in KD cell lines was reduced to approximately 30% (lane 2) and 10% (lane 3) relative to that of WT-like cell lines (lane 1). The expression level of E1A and/or E2 RNAs in pp32 KD cells did not become reduced but rather, slightly increased, suggesting that pp32 may be a negatively acting factor. This result was unexpected, since the interaction between pp32 and protein VII was not found (see Discussion). To examine the time course of viral early gene expression in WT (clone 7) and TAF-I KD (clone 4) cells, total RNA was isolated from infected HeLa cells every 2 h after infection up to 8 h p.i., and E1A and E2 RNAs were examined by Northern blot analysis (Fig. 4E). The E1A RNA was detected from 4 h p.i. in WT and KD cells, but the expression level of E1A RNA in KD cells was

threefold lower than that in WT cells at 4 h p.i. The time course of accumulation of the E1A transcript in KD cells was delayed about at least 2 h compared with that in WT cells. Similarly, the time course of accumulation of the E2 transcript in KD cells was delayed 2 to 3 h compared with that in WT cells. The level of E2 RNA in KD cells was much less than that in WT cells (see lanes for 8 h p.i.). These results suggest that KD of TAF-I delays the timing of the transcription of early genes. This finding was confirmed at the protein expression level by Western blotting (data not shown).

We next examined the effect of TAF-I KD on the viral DNA replication (Fig. 4F). The template of the first round of the viral DNA replication is considered to be associated with protein VII. WT (clone 7) and TAF-I KD (clone 4) cell lines were infected with HAdV5 at an MOI 100 and collected every 3 h, as indicated in Fig. 4F. Next, total DNA was purified and dot blotted on a membrane, and the amount of the Ad genome DNA was analyzed by Southern blot analysis using a 32 P-labeled probe complementary to the HAdV5 genome DNA (Fig. 4F). The dramatic increase of the Ad DNA initiated at 12 h p.i. in WT cells, whereas the increase of the viral DNA was delayed 1.5 to 2.5 h in KD cells.

These results altogether strongly suggest that the interaction of TAF-I with protein VII on the viral genome during immediate-early phases of infection is important for viral early gene transcription. In late phases of infection, we have found by immunoprecipitation assay that TAF-I interacts with the precursor of protein VII (pre-VII) (data not shown). Thus, it is possible that TAF-I is involved in the events in late phases, for instance, in the regulation of transcription and replication and/or assembly of the viral DNA-protein VII complex for progeny virions. We examined the virus titer produced in WT (clones 7 and 8) and TAF-I KD (clones 4 and 13) cell lines (Fig. 4G). TAF-I KD cell lines had defects in viral growth to a certain extent compared with WT cell lines. The number of virus particles in WT cell lines reached the maximal level between 24 and 48 h p.i., while that in the TAF-I KD cell line, clone 4, was 1.5- to 3-fold less than that in WT cell lines.

DISCUSSION

In this report, we examined whether TAF-I and pp32 play a role in Ad multiplication, in particular, in the early phases of infection. TAF-I interacted with protein VII efficiently, while pp32 did not (Fig. 2). Down-regulation of the TAF-I activity by siRNA delayed the time course of accumulation of early gene transcripts. These results strongly suggest that TAF-I plays an important role in the synthesis of early gene transcripts by interaction with protein VII. In contrast, the experiments using pp32 KD cell lines indicate that pp32 may negatively regulate the synthesis of viral early gene transcripts.

Despite the similarity of TAF-I with pp32 in several structural and functional features, pp32 neither remodeled the Ad DNA-protein VII complex nor was associated with protein VII in our assay conditions. This may be explained by the difference of conformation between TAF-I and pp32. TAF-I functions as a dimer, while pp32 functions as a monomer. A TAF-I mutant protein that cannot form a dimer but contains the acidic tail does not have the remodeling activity of the Ad DNA-protein VII complex (23) and has less affinity for protein

FIG. 4. Knockdown of TAF-I expression. (A) The level of TAF-I in TAF-I KD cell lines derived from HeLa cells. Cell extracts prepared from WT (clones 7, 8, and 9) and TAF-I KD (clones 4 and 13) cell lines were separated by SDS-10% PAGE, and TAF-I proteins were detected by immunoblotting with anti-TAF-Iβ (top gel), anti-TAF-Iα (middle gel), or anti-TAF-Iα/β common (bottom gel) antibodies. Std, standard. (B) Semiquantitative RT-PCR. WT (clone 7) and TAF-I KD (clones 4 and 13) cell lines were infected with HAdV5 at an MOI of 100 and collected at 4 h p.i. Total RNA was isolated and analyzed by RT-PCR for semiquantitative detection of transcripts from *β-actin*, *E1A*, and *E2* genes. PCR products amplified by 18, 16, and 20 PCR cycles for *β-actin*, *E1A*, and *E2*, respectively, were loaded onto a 6% polyacrylamide gel. After separation, PCR products were visualized by staining with SYBR Gold (upper panel). The intensity of the band was measured using NIH Image software. Mean values $\pm$ standard deviations from four independent experiments are summarized in the lower panel, where the level of WT is set as 100%. (C) Rescue experiments. WT (clone 7) and TAF-I KD (clone 4) cells were transfected with indicated amounts of pCHA and pCHA-TAF-Iβ vectors, infected with HAdV5 at an MOI of 100 at 48 h after transfection, and collected at 4 h p.i. The level of *E1A* RNA was analyzed as shown in panel B (upper part of panel). Data are mean values $\pm$ standard deviations from five independent experiments. The levels of HA-TAF-Iβ and endogenous TAF-Iβ proteins were examined by Western blotting using anti TAF-Iβ antibody (bottom of panel). (D) KD of pp32. The level of pp32 protein in HeLa pp32 KD cell lines was examined by Western blotting (WB; top gel). Semiquantitative RT-PCR was performed as described above for panel B using pp32 KD cell lines (bottom gel for *β-actin*; graph for *E1A* and *E2*). Data are mean values $\pm$ standard deviations from three independent experiments. (E) Northern blot analysis. WT and TAF-I KD cells were mock infected (M) or infected with HAdV5 at an MOI of 100 and collected at indicated h p.i. Total RNA was isolated and analyzed by Northern blot hybridization using probes complementary to a part of *E1A* (top gel) or *E2* (middle gel) RNA. Ribosomal RNAs were also visualized by staining with ethidium bromide (bottom gel). (F) Time course of viral DNA synthesis in WT and TAF-I KD cells. WT and TAF-I KD cells were mock infected (M) or infected with HAdV5 at an MOI of 100 and collected every 3 h after infection. Total DNA was purified, dot blotted, and hybridized with ³²P-labeled probe complementary to the HAdV5 genome DNA. (G) One-step virus growth assay. WT (clones 7 and 8) and TAF-I KD (clones 4 and 13) cell lines were infected with HAdV5 at an MOI of 100. Infected cells and their culture media were collected at 24 and 48 h p.i., and the virus titer was measured by plaque assay.

VII compared with the WT protein (data not shown). Therefore, not only the acidic tail but also the region other than the acidic tail of TAF-I is important for the maximal remodeling activity of the Ad DNA-protein VII complex. Xue et al. showed the interaction between pp32 and protein VII using uninfected cell extracts and a large amount of GST-protein VII (39). We presume that pp32 binds to GST-protein VII only when the amount of GST-protein VII is much more than that of a protein VII-binding protein(s) including TAF-I. The amount of incoming protein VII (800 to 1,000 molecules/viral genome) into the infected cell even at an MOI of 100 is less than that of TAF-I (0.5×10^7 to 2×10^7 molecules of TAF-I β in a HeLa cell) (data not shown) in a nucleus. Results shown in Fig. 2 indicate that pp32 seems to have less affinity to protein VII than TAF-I. Thus, it is unlikely that pp32 interacts with protein VII stably in infected cells, although we cannot exclude the possibility that the transient interaction of pp32 with the Ad DNA-protein VII complex gives some effects on the viral genome function. In fact, KD of pp32 by siRNA slightly enhanced the accumulation of E1A and E2 RNAs (Fig. 4D). This result could be interpreted as follows: that although pp32 itself cannot remodel the Ad DNA-protein VII complex, pp32 competes with TAF-I to interact with protein VII or recruits an inhibitory factor(s) to the Ad DNA for transcription through protein VII. Alternatively, it is possible that pp32 may inhibit the synthesis and/or accumulation of RNA without its direct interaction with protein VII. It was reported that Ad E4orf6 interacts with pp32 and this interaction affects nuclear export and the stabilization of mRNAs containing an AU-rich element such as *c-fos* and *c-myc* mRNAs (13). However, at present, it is difficult to explain how the loss of this interaction lowers the expression level of E1A and E2 RNAs in pp32 KD cells.

Reduction of the TAF-I expression by KD leads to the delayed accumulation of early gene products. This clearly indicates that TAF-I plays an important role in the viral early gene expression. However, the viral gene expression and the Ad genome DNA replication were not so severely impaired by TAF-I KD as expected. The most likely explanation is that the functionally redundant acidic proteins, such as TAF-II and TAF-III, could substitute for the TAF-I function. Alternatively, we should also take into account the possibility that the residual amount of the authentic TAF-I is enough to support the viral gene expression and the genome replication. On this line, it is possible that only a small amount of TAF-I is required for efficient remodeling of the viral DNA-protein VII complex, in particular, at/around the immediate-early gene promoter region. Indeed, we have shown that protein VII is relatively free at the E1A promoter compared with other regions of the Ad genome during the early phases of infection (12). Consistently, a cell-free transcription assay using the Ad DNA-protein VII complex as template demonstrated that TAF-I stimulates the transcription from the E1A promoter more efficiently than the major late promoter (21). We observed a 1- to 2-h prolonged delay of the E2 gene expression than the E1A gene expression by TAF-I KD. This could be due to not only the decrease of remodeling of the Ad DNA-protein VII complex around the E2 gene promoter by TAF-I but also the low accumulation level of the E1A gene product which is required for E2 gene expression. One of the interesting assumptions is

that transcription during the early phases of infection potentiates the template competency of the Ad DNA-protein VII complex. It is reported that nuclear dots formed by both protein VII and the virus genome disappear during RNA synthesis independent of DNA synthesis (39). This suggests that protein VII is dissociated from the virus DNA during transcription. Since the almost entire region of the Ad genome DNA is transcribed during the early phases of infection, it is likely that the Ad DNA-protein VII complex after transcription is less densely packed with protein VII.

The level of the virus growth in KD cells was not significantly reduced but distinctly lower than that in WT cells. This could be due to defects in events in not only early phases but also late phases. In fact, TAF-I interacts with pre-VII in infected cells at late phases. We have two kinds of working hypotheses to explain the meaning of the interaction between TAF-I and pre-VII in late phases of infection. One is that TAF-I would extend the period of transcription in late phases by preventing pre-VII from binding to the virus DNA. The other is that TAF-I is involved in the assembly of progeny DNA-pre-VII complex formation (as discussed in reference 40). These two hypotheses are supported by the fact that TAF-I dissociates pre-VII from DNA-pre-VII complexes, while DNA, protein VII, and TAF-I form stable ternary complexes (unpublished observation). Experiments are ongoing to find out a condition(s) that converts TAF-I-pre-VII complexes to DNA-pre-VII complexes, the process of which should occur during late phases in infection.

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REFERENCES

- Anderson, C. W., M. E. Young, and S. J. Flint. 1989. Characterization of the adenovirus 2 virion protein, mu. *Virology* **172**:506–512.
- Beresford, P. J., D. Zhang, D. Y. Oh, Z. Fan, E. L. Greer, M. L. Russo, M. Jaju, and J. Lieberman. 2001. Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J. Biol. Chem.* **276**:43285–43293.
- Black, B. C., and M. S. Center. 1979. DNA-binding properties of the major core protein of adenovirus 2. *Nucleic Acids Res.* **6**:2339–2353.
- Brennan, C. M., I. E. Gallouzi, and J. A. Steitz. 2000. Protein ligands to HuR modulate its interaction with target mRNAs in vivo. *J. Cell Biol.* **151**:1–14.
- Chatterjee, P. K., M. E. Vayda, and S. J. Flint. 1986. Adenoviral protein VII packages intracellular viral DNA throughout the early phase of infection. *EMBO J.* **5**:1633–1644.
- Chatterjee, P. K., M. E. Vayda, and S. J. Flint. 1986. Identification of proteins and protein domains that contact DNA within adenovirus nucleoprotein cores by ultraviolet light crosslinking of oligonucleotides 32P-labelled in vivo. *J. Mol. Biol.* **188**:23–37.
- Chatterjee, P. K., M. E. Vayda, and S. J. Flint. 1985. Interactions among the three adenovirus core proteins. *J. Virol.* **55**:379–386.
- Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475–1489.
- Fedor, M. J., and E. Daniell. 1983. Ionic and nonionic interactions in adenoviral nucleoprotein complexes. *J. Virol.* **47**:370–375.
- Gamble, M. J., H. Erdjument-Bromage, P. Tempst, L. P. Freedman, and R. P. Fisher. 2005. The histone chaperone TAF-I/SET/INHAT is required for transcription in vitro of chromatin templates. *Mol. Cell. Biol.* **25**:797–807.
- Greber, U. F., M. Suomalainen, R. P. Stidwill, K. Boucke, M. W. Ebersold, and A. Helenius. 1997. The role of the nuclear pore complex in adenovirus DNA entry. *EMBO J.* **16**:5998–6007.
- Haruki, H., B. Gyurcsik, M. Okuwaki, and K. Nagata. 2003. Ternary com-

- plex formation between DNA-adenovirus core protein VII and TAF-Ibeta/SET, an acidic molecular chaperone. *FEBS Lett.* **555**:521–527.
13. Higashino, F., M. Aoyagi, A. Takahashi, M. Ishino, M. Taoka, T. Isobe, M. Kobayashi, Y. Totsuka, T. Kohgo, and M. Shindoh. 2005. Adenovirus E4orf6 targets pp32/LANP to control the fate of ARE-containing mRNAs by perturbing the CRM1-dependent mechanism. *J. Cell Biol.* **170**:15–20.
 14. Johnson, J. S., Y. N. Osheim, Y. Xue, M. R. Emanuel, P. W. Lewis, A. Bankovich, A. L. Beyer, and D. A. Engel. 2004. Adenovirus protein VII condenses DNA, represses transcription, and associates with transcriptional activator E1A. *J. Virol.* **78**:6459–6468.
 15. Kawase, H., M. Okuwaki, M. Miyaji, R. Ohba, H. Handa, Y. Ishimi, T. Fujii-Nakata, A. Kikuchi, and K. Nagata. 1996. NAP-I is a functional homologue of TAF-I that is required for replication and transcription of the adenovirus genome in a chromatin-like structure. *Genes Cells* **1**:1045–1056.
 16. Leith, I. R., R. T. Hay, and W. C. Russell. 1989. Adenovirus subviral particles and cores can support limited DNA replication. *J. Gen. Virol.* **70**:3235–3248.
 17. Li, M., A. Makkinje, and Z. Damuni. 1996. Molecular identification of IPPP2A, a novel potent heat-stable inhibitor protein of protein phosphatase 2A. *Biochemistry* **35**:6998–7002.
 18. Li, M., A. Makkinje, and Z. Damuni. 1996. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J. Biol. Chem.* **271**:11059–11062.
 19. Matsumoto, K., K. Nagata, M. Okuwaki, and M. Tsujimoto. 1999. Histone- and chromatin-binding activity of template activating factor-I. *FEBS Lett.* **463**:285–288.
 20. Matsumoto, K., K. Nagata, M. Ui, and F. Hanaoka. 1993. Template activating factor I, a novel host factor required to stimulate the adenovirus core DNA replication. *J. Biol. Chem.* **268**:10582–10587.
 21. Matsumoto, K., M. Okuwaki, H. Kawase, H. Handa, F. Hanaoka, and K. Nagata. 1995. Stimulation of DNA transcription by the replication factor from the adenovirus genome in a chromatin-like structure. *J. Biol. Chem.* **270**:9645–9650.
 22. Miyagishi, M., and K. Taira. 2003. Strategies for generation of an siRNA expression library directed against the human genome. *Oligonucleotides* **13**:325–333.
 23. Miyaji-Yamaguchi, M., M. Okuwaki, and K. Nagata. 1999. Coiled-coil structure-mediated dimerization of template activating factor-I is critical for its chromatin remodeling activity. *J. Mol. Biol.* **290**:547–557.
 24. Nagata, K., H. Kawase, H. Handa, K. Yano, M. Yamasaki, Y. Ishimi, A. Okuda, A. Kikuchi, and K. Matsumoto. 1995. Replication factor encoded by a putative oncogene, set, associated with myeloid leukemogenesis. *Proc. Natl. Acad. Sci. USA* **92**:4279–4283.
 25. Nagata, K., S. Saito, M. Okuwaki, H. Kawase, A. Furuya, A. Kusano, N. Hanai, A. Okuda, and A. Kikuchi. 1998. Cellular localization and expression of template-activating factor I in different cell types. *Exp. Cell Res.* **240**:274–281.
 26. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**:193–199.
 27. Okuwaki, M., A. Iwamatsu, M. Tsujimoto, and K. Nagata. 2001. Identification of nucleophosmin/B23, an acidic nucleolar protein, as a stimulatory factor for in vitro replication of adenovirus DNA complexed with viral basic core proteins. *J. Mol. Biol.* **311**:41–55.
 28. Okuwaki, M., K. Matsumoto, M. Tsujimoto, and K. Nagata. 2001. Function of nucleophosmin/B23, a nucleolar acidic protein, as a histone chaperone. *FEBS Lett.* **506**:272–276.
 29. Okuwaki, M., and K. Nagata. 1998. Template activating factor-I remodels the chromatin structure and stimulates transcription from the chromatin template. *J. Biol. Chem.* **273**:34511–34518.
 30. Seo, S. B., P. McNamara, S. Heo, A. Turner, W. S. Lane, and D. Chakravarti. 2001. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell* **104**:119–130.
 31. Spector, D. J., J. S. Johnson, N. L. Baird, and D. A. Engel. 2003. Adenovirus type 5 DNA-protein complexes from formaldehyde cross-linked cells early after infection. *Virology* **312**:204–212.
 32. Tollefson, A. E., T. W. Hermiston, and W. S. M. Wold. 1998. Preparation and titration of CsCl-banded adenovirus stocks, p. 1–9. *In* W. S. M. Wold (ed.), *Adenovirus methods and protocols*, vol. 21. Humana Press, Totowa, N.J.
 33. Trotman, L. C., N. Mosberger, M. Fornerod, R. P. Stidwill, and U. F. Greber. 2001. Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. *Nat. Cell Biol.* **3**:1092–1100.
 34. Vaesen, M., S. Barnikol-Watanabe, H. Gotz, L. A. Awni, T. Cole, B. Zimmermann, H. D. Kratzin, and N. Hilschmann. 1994. Purification and characterization of two putative HLA class II associated proteins: PHAPI and PHAPII. *Biol. Chem. Hoppe Seyler* **375**:113–126.
 35. van Leeuwen, H., M. Okuwaki, R. Hong, D. Chakravarti, K. Nagata, and P. O'Hare. 2003. Herpes simplex virus type 1 tegument protein VP22 interacts with TAF-I proteins and inhibits nucleosome assembly but not regulation of histone acetylation by INHAT. *J. Gen. Virol.* **84**:2501–2510.
 36. van Oostrum, J., and R. M. Burnett. 1985. Molecular composition of the adenovirus type 2 virion. *J. Virol.* **56**:439–448.
 37. Vayda, M. E., K. Leong, and S. J. Flint. 1984. Transcription of adenovirus cores in vitro. *Virology* **139**:152–163.
 38. Wong, M. L., and M. T. Hsu. 1988. Psoralen-cross-linking study of the organization of intracellular adenovirus nucleoprotein complexes. *J. Virol.* **62**:1227–1234.
 39. Xue, Y., J. S. Johnson, D. A. Ornelles, J. Lieberman, and D. A. Engel. 2005. Adenovirus protein VII functions throughout early phase and interacts with cellular proteins SET and pp32. *J. Virol.* **79**:2474–2483.
 40. Zhang, W., and R. Arcos. 2005. Interaction of the adenovirus major core protein precursor, pVII, with the viral DNA packaging machinery. *Virology* **334**:194–202.