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Antioxidant-Induced Changes of the AP-1 Transcription Complex Are Paralleled by a Selective Suppression of Human Papillomavirus Transcription

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Considering the involvement of a redox-regulatory pathway in the expression of human papillomaviruses (HPVs), HPV type 16 (HPV-16)-immortalized human keratinocytes were treated with the antioxidant pyrrolidine-dithiocarbamate (PDTC). PDTC induces elevated binding of the transcription factor AP-1 to its cognate recognition site within the viral regulatory region. Despite of increased AP-1 binding, normally indispensable for efficient HPV-16 transcription, viral gene expression was selectively suppressed at the level of initiation of transcription. Electrophoretic mobility supershift assays showed that the composition of the AP-1 complex, predominantly consisting of Jun homodimers in untreated cells, was altered. Irrespective of enhanced c-*fos* expression, c-*jun* was phosphorylated and became primarily heterodimerized with *fra-1*, which was also induced after PDTC incubation. Additionally, there was also an increased complex formation between c-*jun* and *junB*. Because both *fra-1* and *junB* overexpression negatively interferes with c-*jun*/c-*fos* *trans*-activation of AP-1-responsive genes, our results suggest that the observed block in viral transcription is mainly the consequence of an antioxidant-induced reconstitution of the AP-1 transcription complex. Since expression of the c-*jun*/c-*fos* gene family is tightly regulated during cellular differentiation, defined reorganization of a central viral transcription factor may represent a novel mechanism controlling the transcription of pathogenic HPVs during keratinocyte differentiation and in the progression to cervical cancer.

Specific types of human papillomaviruses (HPVs) such as HPV type 16 (HPV-16) (18) and HPV-18 (6) are etiologically involved in the development of cervical cancer (84, 86). The oncogenic potential of the virus can be attributed to the E6 and E7 open reading frames (46), whose gene products can functionally interfere with cell cycle control in part by interacting with presumptive tumor suppressor proteins such as p53 (82) or the retinoblastoma protein (Rb) (20).

Although E6 and E7 themselves possess intrinsic *trans*-activation capacity on their homologous promoters (14, 52), constitutive expression of E6 or E7 in immortalized or malignantly transformed human keratinocytes is mainly dependent on the availability of a defined set of transcription factors derived from the infected host cell. AP-1, for example, normally consisting of a heterodimer between c-Fos and c-Jun (for a review, see reference 4), seems to play a central role in transcriptional regulation of viral oncogene expression, since point mutations of the corresponding consensus sequences within the upstream regulatory region (URR) of HPV-16 or HPV-18 almost completely abolish the expression of URR-driven reporter plasmids in transient transfection assays (7, 12, 77).

Recent studies have shown that *trans*-activation and the DNA-binding affinity of AP-1 (1, 44) as well as that of other transcription factors such as NF-κB (65, 78) or p53 (29) can be modulated not only via posttranslational modifications such as phosphorylation or dephosphorylation (for a review, see reference 31) but also by alterations of the intracellular redox status. This can be achieved by certain cytokines, which are able to induce a prooxidant state within the cell by generating reactive oxygen intermediates (ROIs) (26, 44). Following the binding to their respective receptors on the cell surface, intracellular ROIs in turn act as second messengers to transduce the signal into the nucleus (65). A paradigm for such a transduction-activation mechanism is the transcription factor NF-κB. Reactive oxygen triggers the release of the cytoplasmic inhibitory component (IκB) of NF-κB, leading to nuclear translocation of the p50 and p65 subunits and subsequently to the activation of a whole set of NF-κB-responsive effector genes (65, 68).

Similar prooxidant conditions can also be generated either by exposing cells directly to hydrogen peroxide (40) or by genotoxic stress after UV irradiation (60), leading in both cases to the activation of AP-1 (74) and NF-κB (44). While such intracellular redox changes are normally counterbalanced by antioxidant enzymes like Cu-Zn-superoxide dismutases (48) or oxidoreductases such as thioredoxin (30, 43), it is also possible to interfere directly with the redox regulation of a given gene by treating the cells with antioxidative drugs such as pyrrolidine-dithiocarbamate (PDTC) (67).

In a previous study, we showed that tumor necrosis factor alpha-induced transcription of a chemokine gene encoding monocyte chemoattractant protein 1 (MCP-1) could be completely abrogated when nonmalignant HPV-positive cells were incubated with PDTC prior to cytokine addition (57). Consistent with the aforementioned model of redox modulation of specific genes, detailed promoter analysis has revealed that NF-κB and AP-1 are indeed the predominant transcription factors involved in the regulation of this particular chemokine (70, 80).

Since AP-1 is also a central transcription factor for efficient

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gene expression of HPVs (12, 49), we investigated the effect PDTC on the transcription of HPV-16 in human keratinocytes. In the present study, we show that PDTC increases AP-1 binding affinity to the corresponding binding site within the viral regulatory region. However, despite the fact that AP-1 is required for efficient HPV-16/18 transcription (7, 12, 77), the observed enhanced AP-1 binding is accompanied by a selective suppression of viral gene expression on the level of initiation of transcription. Although the antioxidant elicits up-regulation of c-Fos and c-Jun, both at the transcriptional level and at the protein level, band-shift assays in combination with specific antibodies revealed that only c-Jun but not c-Fos becomes a major component in the AP-1 complex. While JunB is also slightly elevated, JunD binding is not affected.

PDTC not only induces c-Jun overexpression but also causes its phosphorylation. Most notable is the observation that instead of c-Fos, another member of the Fos family, Fra-1, was found to be the major binding partner of c-Jun and JunB after PDTC application. Since overexpression of Fra-1 has an inhibitory effect on the *trans*-activating efficiency of prototype AP-1 (75), known to be obligatory for HPV16/18 gene expression (7, 12, 77), our results suggest that the suppression of viral transcription is mainly a consequence of a PDTC-induced alteration in the heterodimerization pattern of AP-1.

MATERIALS AND METHODS

Cell culture. HPV-16-positive human keratinocytes (19), the HPV-18-positive cervical carcinoma cell line HeLa, and the HPV-negative cervical carcinoma cell line C33a were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. PDTC was obtained from Sigma Chemical and was freshly dissolved in medium immediately before use. Actinomycin D and cycloheximide (Sigma) were stored as stock solutions at −20°C.

RNA analysis. Cellular RNA was extracted according to the guanidinium-thiocyanate method (9). Between 2.5 and 5 μg of total RNA was separated on 1% agarose gels in the presence of ethidium bromide under nondenaturating conditions (33) and transferred to GeneScreen Plus membranes.

Nuclear run-on assay. Cells were washed twice with ice-cold phosphate-buffered saline, scraped from the plates with a rubber policeman, and lysed in 5 ml of 10 mM Tris-HCl (pH 7.4)–10 mM NaCl–2 mM $MgCl_2$–0.5 mM phenylmethylsulfonyl fluoride–5 mM dithiothreitol (DTT)–0.5% Nonidet P-40 (Boehringer, Mannheim, Germany). After cytoplasm removal, the nuclei were resuspended in storage buffer (50 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 40% [vol/vol] glycerol) and frozen in liquid nitrogen (55). The nuclear run-on assay was performed as described previously (39), with the only modification that the [^{32}P]UTP-labeled RNAs were recovered by the method of Chomczynski and Sacchi (9). Equal amounts of radiolabeled RNA were hybridized to plasmid and total cell DNA immobilized on small nitrocellulose strips as indicated in the figure legends.

Plasmids for transient transfection assays. pH16CAT and pH18CAT harbor the complete URRs of HPV-16 and HPV-18 cloned in pBLCAT3, a chloramphenical acetyltransferase (CAT) construct lacking any eukaryotic regulatory signals. The plasmids were kindly provided by A. Alonso and T. Bauknecht (Deutsches Krebsforschungszentrum, Heidelberg, Germany). pBLCAT2 consists of the thymidine kinase (*tk*) promoter of herpes simplex virus (HSV) cloned in pBLCAT3 (41). pSVCAT2 carries the complete promoter/enhancer region of simian virus 40 in front of the CAT gene (27). p3xNF-κB-CAT (64) and p3xAP-CAT (3) represent reporter plasmids where either three NF-κB consensus sequences or three 12-*O*-tetradecanoylphorbol-13-acetate-responsive elements (TREs) were cloned in front of the *tk* promoter of HSV. Both constructs were kindly made available by W. Dröge and P. Angel (Deutsches Krebsforschungszentrum). pTopoI-CAT harbors the regulatory region of the human topoisomerase I gene (35). pRSV-luc contains a luciferase gene under the control of the Rous sarcoma virus (RSV) long terminal repeat (LTR).

Transient transfections and CAT assays. A total of 5×10^5 C33a cells were transfected with 2.5 μg of the respective CAT reporter plasmids together with 0.1 μg of a RSV LTR-driven luciferase construct as an internal reference, using a lipofection protocol (Lipofectamine transfection kit; GIBCO, BRL). The cells were treated with 100 μM PDTC as mentioned in the figure legends and harvested to determine the luciferase and CAT activity. Cellular extracts corresponding to equal luciferase counts (16) were incubated for 1 to 2 h at 37°C as described by Gorman et al. (27).

EMSAs. For gel retardation tests, the following oligonucleotides were used: AP-1-#3 of HPV-16 (5′-ATAAAGGTTAGTCATACATTGTTC-3′; positions 7804 to 7827) (12), the enhancer-specific AP-1 site of HPV-18 (5′-CGCACCT GGTATTAGTCATTTTCC-3′; positions 7596 to 7620) (7), an AP-1 consensus sequence (5′-CGCTTGATGACTCAGCCGGAA-3′) (38), and an Oct-1 consensus oligonucleotide (5′-TGTCGAATGCAAATCACTAGAA-3′) (63). The DNA was synthesized in an Applied Biosystems synthesizer using phosphoramitide chemistry. For electrophoretic mobility shift assays (EMSAs), the annealed oligonucleotides were labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) by T4 polynucleotide kinase and gel purified from a 15% polyacrylamide gel (59).

Total cellular extracts were prepared as described by Dignam et al. (17). The binding was performed in a 25-μl reaction volume containing 50% glycerol, 60 mM HEPES (pH 7.9), 20 mM Tris-HCl (pH 7.9), 300 mM KCl, 5 mM EDTA, 5 mM DTT, 100 μg of bovine serum albumin per ml, 2.5 μg of poly(dI-dC), and 5 μg of nuclear extract. After 5 min, 10,000 cpm of the [γ-^{32}P]ATP 5′-end-labeled double-stranded oligonucleotide probe was added, and the incubation was continued for additional 25 min at room temperature.

For monitoring AP-1 composition in supershift assays, 2 μg of polyclonal antibodies (Abs) directed against the Jun/Fos family (Santa Cruz Biotechnology) was added, and the reaction mixtures was further incubated for 1 h at 4°C. The following Abs were used: c-Fos Ab (epitope corresponding to amino acids 3 to 16 mapping at the N terminus of human c-Fos protein), Fra-1 Ab (epitope corresponding to amino acids 3 to 22 mapping at the N terminus of the human Fra-1 protein), Fra-2 Ab (epitope corresponding to amino acids 3 to 22 mapping at the N terminus of the human Fra-2 protein), and FosB Ab (epitope corresponding to amino acids 102 to 117 of the mouse FosB protein). For the Jun family, we used c-Jun Ab (epitope corresponding to amino acids 91 to 105 mapping within the amino-terminal domain of the mouse c-Jun protein), JunB Ab (epitope corresponding to amino acids 45 to 61 mapping within the amino-terminal domain of the mouse JunB protein), and JunD Ab (epitope corresponding to amino acids 329 to 341 mapping within the carboxy terminus of mouse JunD). The DNA-protein complexes were resolved on a 4.5% nondenaturing polyacrylamide gel (29:1 cross-linking ratio), dried, and exposed overnight to Kodak X-Omat films.

SDS-polyacrylamide gel electrophoresis and Western blotting. Cellular extracts used for the band shift analysis (15 μg of protein per lane) were separated in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels, electrotransferred to Immobilon-P membranes (Millipore Corporation), and probed with polyclonal rabbit antibodies of the corresponding Fos/Jun members (see above for details). The incubation was carried out overnight in phosphate-buffered saline supplemented with 5% skim milk powder (Merck), 0.05% Tween 20 (Sigma), and a 1:5,000 dilution of the respective Ab. The bands were visualized with an anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase, using the Amersham ECL detection system.

Phosphatase treatment of the cellular extracts. To reduce the concentration of NaCl present in the Dignam extract, 20 μg of cellular extract was 10-fold diluted in 1× phosphatase buffer (50 mM Tris-HCl [pH 7.8], 2 mM $MnCl_2$, 5 mM DTT, 100 μg of bovine serum albumin per ml) and treated with 400 U of λ-phosphatase (New England Biolabs) for 30 min at 30°C. As a control, the same extracts were incubated under identical conditions without any enzyme. After acetone precipitation, the phosphorylation status of c-Jun was monitored by Western blotting with a monoclonal Ab specifically raised against c-Jun, phosphorylated on serine 63 (epitope corresponding to amino acids 56 to 69) (Santa Cruz Biotechnology).

DNA hybridization probes. pHPV16 and pHPV 18 represent unit-length of HPV-16 (18) and HPV-18 DNA (6) cloned in pBR322. pHF-A1 (28), harboring an approximately full-length insert of the fibroblast β-actin gene, was a generous gift from L. Kedes (Medical Center, Palo Alto, Calif.). Plasmid pc-myc, containing the third exon of the human c-*myc* gene, was kindly made available by G. Bornkamm (Institut für Klinische Molekularbiologie, Munich, Germany) (53). The cDNAs for c-*jun* (2), c-*fos* (45), and the *WAF* gene (22) were kindly provided by P. Angel (Deutsches Krebsforschungszentrum) and B. Vogelstein (John Hopkins University, Baltimore, Md.). The DNA harboring the human homolog for the *fra-1* gene (25) was a generous gift from M. Seiki (Cancer Research Institute, Kanagawa, Japan). All probes were labeled by the random-priming technique (23).

RESULTS

PDTC selectively suppresses HPV-16 transcription in human keratinocytes. Incubation of HPV-16-immortalized human keratinocytes (HPK Ia) (19) with the antioxidant PDTC for 2 h reveals only a slight reduction of the virus-specific mRNA steady-state level (Fig. 1A; compare lanes a and e). However, HPV-16 gene expression becomes almost completely down-regulated between 4 and 6 h (Fig. 1, lanes d and c) and is no longer detectable after overnight treatment (16 h) (lane b). With the half-life for the HPV-16-specific mRNA estimated to be approximately 2.5 h (Fig. 1B, where the HPV 16 transcripts decline between 120 and 240 min after actinomycin D addition), PDTC interference seems to act at the level of

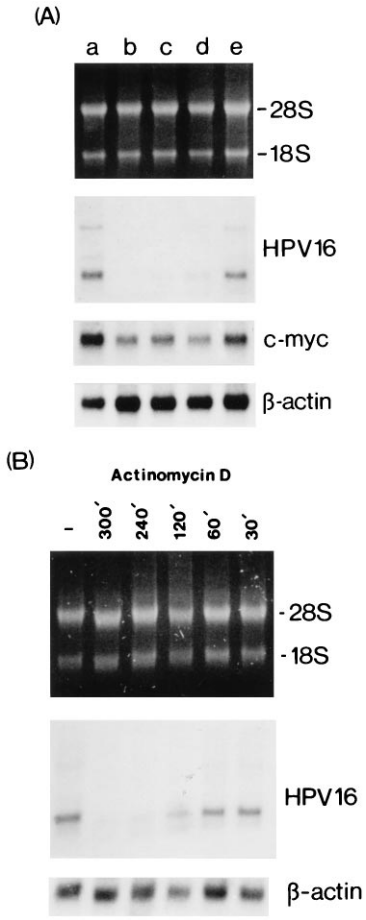

FIG. 1. (A) Time-dependent down-regulation of HPV-16 transcription in the presence of PDTC. HPV-16-immortalized human keratinocytes (HPK Ia) were treated with PDTC for different periods of time. Lane a, untreated control; lanes b to e, overnight and 6, 4, and 2 h of incubation in the presence of 100 μM PDTC, respectively. The filter was consecutively hybridized with HPV-16, c-*myc*, and β-actin probes. (B) Determination of the half-life of HPV-16 RNA. HPK Ia cells were treated with 10 μg of actinomycin D per ml, and RNA was extracted 30, 60, 120, 240, and 300 min after drug addition. −, RNA from untreated cells. The filter was rehybridized with a β-actin probe. The positions of the 28S and 18S rRNAs are indicated.

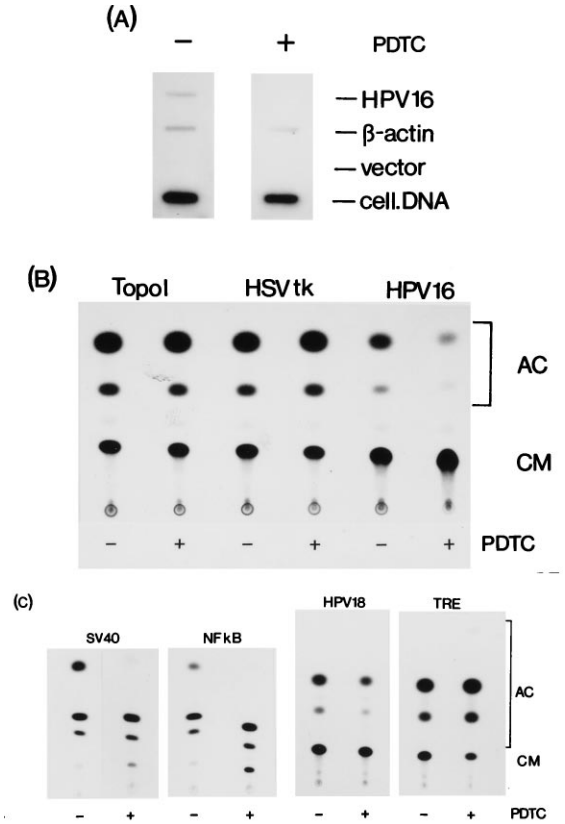

FIG. 2. (A) Nuclear run-on analysis of HPV-16 expression in HPK Ia. Equal amounts of ^{32}P-labeled nascent RNA were hybridized to nitrocellulose filters carrying slots of 2.5 μg of linearized heat-denatured DNA of HPV-16, β-actin, and an empty cloning vector as a negative control. As an internal positive control, 100 ng of total cellular DNA (cell.DNA) was applied. −, untreated cells; +, hybridization with ^{32}P-labeled RNA obtained from nuclei 8 h after treatment with 100 μM PDTC. (B and C) Transient transfection assays of C33a cells, using CAT reporter plasmids harboring the regulatory region of HPV-16 or HPV-18 or the human topoisomerase I gene (TopoI). TRE and NFκB represent constructs in which either three AP-1 consensus sequences from the collagenase promoter (p3xAP-CAT) or three NF-κB consensus sites (p3xNF-κB CAT) were cloned in front of the *tk* promoter of HSV (HSV tk). HSV tk is the basic cloning vector pBLCAT2 (41) lacking any oligomerized sequences. − or + indicates either the absence or the presence of 100 μM PDTC. Cells were cotransfected with 5 μg of the corresponding CAT construct and 0.5 μg of an RSV LTR-driven luciferase reporter plasmid, using the Lipofectamine transfection method. Extracts corresponding to equal amounts of luciferase counts were incubated for 2 h at 37°C to perform the CAT reaction. The acetylated products were finally separated by thin-layer chromatography. The locations of the [^{14}C]chloramphenicol substrate (CM) and the acetylated form (AC) are indicated.

initiation of transcription (see below), since the disappearance of viral mRNA follows roughly the same kinetics as detected with actinomycin D as a nonspecific transcriptional inhibitor (Fig. 1B).

Accordingly, to demonstrate that PDTC-mediated HPV suppression is a selective process and not the consequence of a general transcriptional reduction, the RNA filter was rehybridized with an endogenous reference gene such as c-*myc* or the β-actin gene. Although a two- to threefold reduction of the short-lived c-*myc* mRNA (13) can be noticed, the basic steady-state level of the transcript remains constant and is almost unaltered independent of incubation for 4 h (Fig. 1A, lane d) or 16 h (lane b) in the presence of the drug. Furthermore, subsequent hybridization with the human β-actin probe also demonstrates that the suppression effect is virus specific (see below) and not the result of a nonspecific transcriptional block mediated by the antioxidant.

PDTC-mediated down-regulation of viral transcription is transcriptionally regulated. To confirm the anticipation that the suppression of HPV-16 is regulated at the level of initiation of transcription, the elongation rates of nascent transcripts in isolated HPK Ia nuclei were monitored by using the nuclear run-on technique (39). Hybridization of identical counts of [^{32}P]UTP-labeled total RNA to defined amounts of DNA immobilized on a membrane permits a direct correlation between the degree of transcriptional activity at the HPV-16 integration locus in comparison to other reference genes. With the intensity of the hybridization signal of cellular DNA as an internal standard, ongoing RNA synthesis of the β-actin gene is not significantly altered (Fig. 2A). In contrast, the HPV-16-specific signal is completely absent after 8 h of incubation in the presence of PDTC, which is in agreement with our previous actinomycin D data (Fig. 1B), assuming that suppression of HPV-16 is regulated at the level of initiation of transcription.

Moreover, to confirm that the PDTC-mediated down-regulation of transcription is not a peculiarity of integrated HPV-16

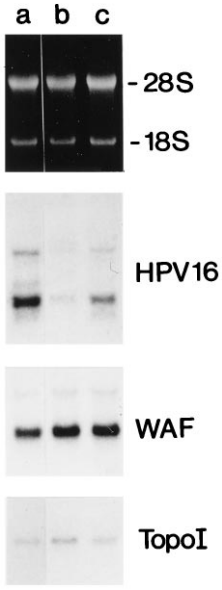

FIG. 3. Northern blot analysis of the p53-responsive *WAF* (p21) gene during PDTC treatment in HPK Ia cells. Lane a, untreated control; lane b, RNA from cells after 4 h of incubation; lane c, RNA obtained after 2 h of treatment in the presence of 100 μM PDTC. The filter was hybridized first with HPV-16 DNA and subsequently with a cDNA probe encoding the human topoisomerase I gene (TopoI) and the cyclin-dependent kinase inhibitor p21 (WAF).

genomes but represents a more general phenomenon also acting on episomal viral promoter elements, transient transfection studies were performed. Since we noticed that PDTC was unstable over prolonged periods of time, cell extracts were prepared 16 h after transfection and assayed for luciferase activity to correct the transfection efficiencies.

Examples of representative CAT assays are depicted in Fig. 2B and C. While PDTC treatment selectively reduces the transcriptional activity of HPV-16, HPV-18, and simian virus 40 URR-driven reporter constructs, CAT activities of other reference promoters such as the *tk* promoter of HSV (41) or the regulatory region of the human DNA topoisomerase I gene (35) are not influenced under the same experimental conditions. The negative interference of PDTC treatment with the transcription factor NF-κB (67) was confirmed by using a pBL2CAT construct harboring three copies of an NF-κB consensus sequence in front of the HSV *tk* promoter as a positive control (Fig. 2C). When the activity of a reporter construct with triplicated TRE sequences upstream of the HSV *tk* promoter is monitored, only a slight induction can be visualized (Fig. 2C; see also below).

PDTC does not affecting p53 or the expression of the p53-responsive gene *WAF-1*/p21. A recent study has shown that HPV transcription can be down-regulated by p53, apparently mediated through the epithelial cell-specific enhancer element within the viral URR (15). Since the DNA-binding affinity and biological activity of p53 can also be redox modulated under certain circumstances (29), we examined the expression level of the *WAF-1*/p21 gene, representing a known downstream target for altered p53 activity (22).

If p53 activation is directly involved in viral gene suppression, one should expect that *WAF*/p21 is induced before any E6/E7 down-regulation can be noticed. However, under conditions where HPV-16 suppression can be discerned (Fig. 3; compare lanes b and c), neither the p53 steady-state level itself (57a) nor the expression of the cyclin-dependent kinase inhibitor WAF/p21 is significantly altered. This finding argues against a direct involvement of p53 in the initial phase of HPV-16 down-regulation during PDTC treatment. Whether the lack of E6/E7 expression has some later downstream effects on p53 or Rb activity remains to be clarified in further experiments. Cross-hybridization with the cell cycle-regulated topoisomerase I gene (35) again shows no significant influence on its transcriptional activity, which is in agreement with the results of previous experiments using the corresponding promoter region in transient transfection assays (Fig. 2B).

AP-1 binding affinity in HPK Ia cells is up-regulated after PDTC treatment. Since p53 seems to be not directly involved in the PDTC-mediated suppression effect (Fig. 3), we focused our attention on the transcription factor AP-1, whose binding affinity to its cognate recognition site is also known to be modulated by antioxidants (1, 44, 64). Extracts from PDTC-treated and untreated HPK Ia cells were prepared and analyzed in band-shift assays either with a ^{32}P-labeled probe harboring an AP-1 consensus sequence (38) (Fig. 4A) or with an

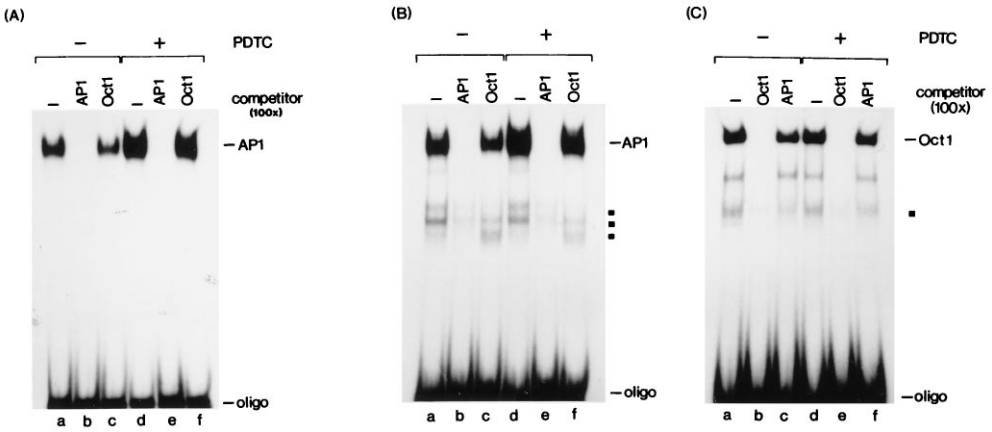

FIG. 4. EMSA using cellular extracts from untreated (−) and PDTC-treated (+) HPK Ia cells. Binding specificity was evidenced by preincubation with a 100-fold molar addition of the homologous unlabeled oligonucleotide (lanes b and e in each panel) in comparison with competition experiments using a heterologous oligonucleotide (lanes c and f in each panel). (A) EMSA with a ^{32}P-labeled oligonucleotide harboring an AP-1 consensus sequence; (B) EMSA using a ^{32}P-labeled oligonucleotide containing the proximal AP-1 site (AP-1#3) of the HPV-16 URR; (C) EMSA carried out with a labeled oligonucleotide (oligo) encompassing the consensus sequence of the Oct-1 transcription factor. The positions of the specific retarded bands are indicated. The squares mark the unspecific complexes.

oligonucleotide encompassing the proximal HPV-16-specific AP-1 recognition site of the viral regulatory region (AP-1#3, positions 7811 to 7817) (12) (Fig. 4B). Although there also exist two additional AP-1 sites within the HPV-16 URR (AP-1#1 and AP-1#2, positions 7631 to 7638 and 7648 to 7655) (12), we selected the proximal site for further investigations, since AP-1#3 has the highest binding affinity and confers the strongest *trans*-activating activity to heterologous promoters when c-*fos* and c-*jun* expression vectors are used in transient cotransfection assays (12).

Independently of whether a consensus binding site (Fig. 4A) or a virus-specific AP-1 site (AP-1#3) is applied (Fig. 4B), increased DNA binding affinity can be discerned (compare lanes a and d in each panel). The binding is specific, since the retarded complex disappears after competition with a 100-fold molar excess of a homologous (Fig. 4A and B, lanes b and e) but not with a heterologous probe, containing the consensus sequence for the transcription factor Oct-1 (Fig. 4A and B, lanes c and f). Furthermore, in agreement with previous reports (44, 67), PDTC treatment selectively increases AP-1 but not other transcription factors such as Oct-1 (Fig. 4C). Elevated AP-1 binding was also obtained with HeLa cell extracts, using an HPV-18-specific AP-1 oligonucleotide localized within the constitutive enhancer region (positions 7596 to 7620) as well as with C33a cells lacking any known HPV type (57a), which indicates that alteration in AP-1 binding by PDTC is not exceptional for HPV-16-immortalized human keratinocytes but reflects a more general phenomenon independently from the degree of malignancy.

PDTC influences the heterodimerization pattern of AP-1. To understand the conceptual problem of how elevated levels of a transcription factor, normally indispensable for efficient HPV expression (7, 12, 77), could be involved in the suppression mechanism of viral transcription, AP-1 complexes were dissected in their individual components in electromobility band shift assays using specific Abs raised against the different members of the Jun/Fos family.

While the c-Fos Ab fails to interact with AP-1 in untreated HPK Ia cells (Fig. 5A, lane c), most of the signal is supershifted after incubation with a c-Jun Ab (Fig. 5A, lane b). This finding suggests that under normal conditions, there exists a certain amount of AP-1 which mainly consists of c-Jun homodimers in HPV-16-immortalized human keratinocytes.

On the other hand, when a c-Fos Ab is used, PDTC treatment generates only faint supershift bands (Fig. 5A, lane f), which presumably reflect heterodimerization products with other members of the Jun family. Among those, only JunB becomes increased (Fig. 5B, lanes c and g), while the binding of JunD is not significantly altered (Fig. 5B, lanes d and h).

To determine whether inappropriate gene expression may account for the absence of c-Fos within the AP-1 transcription complex, RNA and protein analyses were performed. PDTC incubation results in a strong increase of both c-Jun (Fig. 6A, lanes b to d) and c-Fos (Fig. 6B, lanes b and c) transcription. While the steady-state level of the c-*jun* mRNA is only slightly reduced after overnight incubation (16 h) (compare lanes b and d in Fig. 6A), c-*fos* transcription declines almost to the basal level (Fig. 6B, lane d). In Western blot analyses, antioxidant treatment leads to a strong elevation of c-Jun and c-Fos also at the protein level (Fig. 6C and D, lanes b and c). Hence, inefficient gene expression cannot account for the underrepresentation of c-Fos within the AP-1 complex.

Interestingly, PDTC seems also to trigger phosphorylation of c-Jun, which is indicated by the slower-migrating band (51) just in front of the c-Jun-specific signal (Fig. 6C). Treatment of cellular extracts with λ-phosphatase results not only in a disappearance of this particular band but also in a reduction of the c-Jun signal in general. Since the monoclonal Ab used here is specifically raised against the phosphorylated N-terminal part of c-Jun (epitope 56-69), signal reduction demonstrates that obviously serine-63 is one of the major targets of the PDTC effect (Fig. 6E; compare lanes a and b with lanes c and d). Additionally, despite the fact that there is still detectable c-*fos* mRNA 8 h after PDTC addition (Fig. 6B, lane c), the protein becomes diminished (Fig. 6D; compare lanes b and c). Whether this reflects differences in the metabolic turnover rates of both proteins is presently not clear and awaits further elucidation (see Discussion).

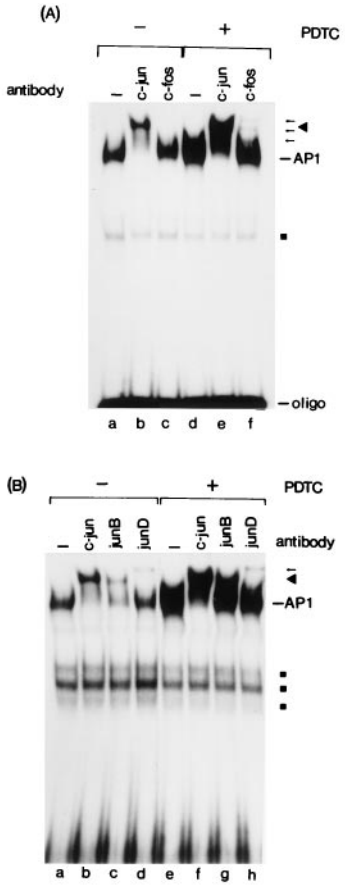

FIG. 5. Electromobility supershift assays using ^{32}P-labeled AP-1#3 oligonucleotides. Extracts from untreated (−) and treated (+) HPK Ia cells were incubated with specific Abs recognizing different members of the Fos/Jun family. (A) Lanes a and d, without Ab; lanes b and e, addition of a c-Jun Ab; lanes c and f, addition of a c-Fos Ab. (B) Lanes a, e, b and f, same as in panel A; lanes c and g, JunB Ab addition; lanes d and h, incubation with a JunD Ab. The position of the AP-1-specific complex is indicated. The arrowhead and the small arrows label the supershifted bands after Ab addition. The squares mark the unspecific complexes.

Additional experiments were carried out to detect further variations in AP-1 composition after antioxidant treatment. As demonstrated in supershift assays with Abs specific for members of the *Fos* family, it became obvious that only Fra-1 predominantly heterodimerizes with c-Jun and JunB after PDTC incubation (Fig. 7A, lane e). In parallel experiments in which Fra-2 (compare lanes c and f) or FosB (57a) antibodies were included, no supershifted protein-DNA complexes are detectable. Monitoring the corresponding RNA (Fig. 7B) and protein expression patterns (Fig. 7C), we found Fra-1 to be

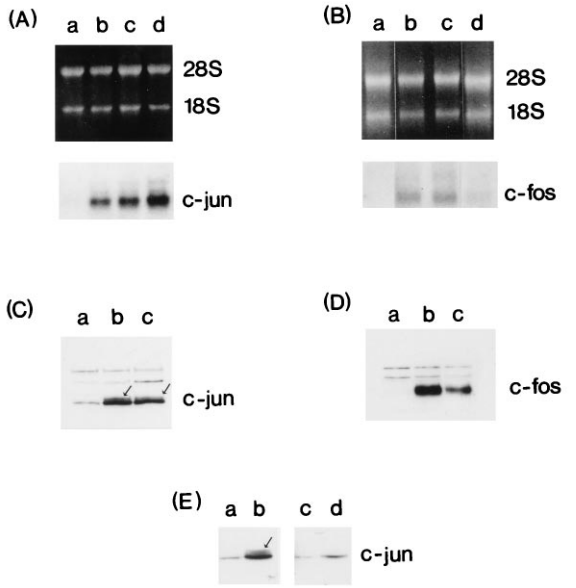

FIG. 6. PDTC induces c-*jun* and c-*fos* expression in HPK Ia cells. (A and B) Northern blot analysis after hybridization with *jun*/*fos*-specific cDNA probes. (A) Lane a, untreated cells; lanes b to d, 16, 8, and 4 h of incubation with PDTC, respectively. (B) Lane a, same as in panel A; lanes b to d, RNA from cells treated for 4, 8, and 16 h with PDTC. The positions of the 28S and 18S rRNAs are indicated. (C and D) Western blot analyses utilizing polyclonal c-Jun (C)- and c-Fos (D)-specific antibodies. Lanes a, untreated control; lanes b and c, protein extracts obtained from cells treated for 4 and 8 h with PDTC. (E) Lane a, untreated control cells; lane b, 8-h PDTC treatment; lanes c and d, same as lanes a and b but incubated with λ-phosphatase for 30 min at 30°C. (To compensate for the signal reduction after λ-phosphatase treatment, lanes c and d were exposed approximately five times longer than the signals shown in lanes a and b.)

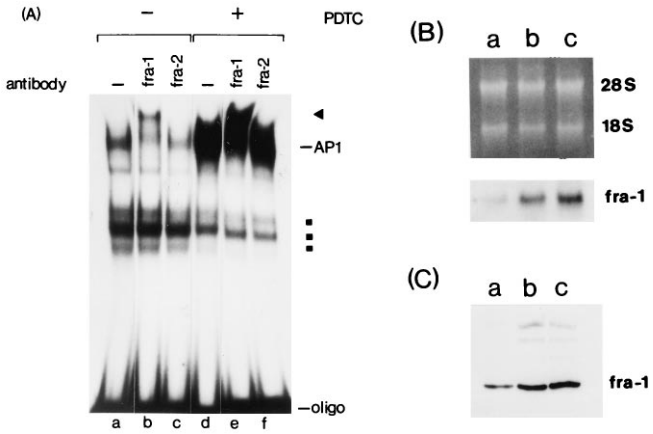

FIG. 7. Selective *fra-1* induction after PDTC treatment. (A) Supershift assays using ^{32}P-labeled AP-1#3 oligoprobes. Extracts from untreated (−) and treated (+) HPK Ia cells were incubated with a Fra-1- or Fra-2-specific Ab. Lanes a and d; without Ab; lanes b and e, after addition of a Fra-1 Ab; lanes c and f, after addition of a Fra-2 Ab. The arrowhead indicate the position of the complexes supershifted after Ab addition; the squares mark the unspecific complexes. (B) Northern blot analysis after hybridization with a *fra-1*-specific cDNA probe. The positions of the 28S and 18S rRNAs are indicated. (C) Western blot analysis using a Fra-1-specific antibody. Lanes a in panels B and C, untreated controls; lanes b and c, PDTC treatment for 4 and 8 h, respectively.

elevated in both cases. Although the *fra-1*-specific mRNA is also up-regulated, Fra-1 protein expression is not paralleled to the same extend as demonstrated for c-Jun and c-Fos (Fig. 6). Furthermore, the observation that c-Jun is already associated with significant quantities of Fra-1 even in untreated cells (Fig. 7A, lane b) is intriguing, since the basal level of Fra-1 expression as well as its ratio in the AP-1 complex seem to be inversely correlated with the steady-state level of virus-specific transcription (see Discussion).

DISCUSSION

During the past few years, it has become evident that there exist a whole variety of transcription factors whose *trans*-acting activity and DNA-binding affinity can be regulated by changes of the intracellular redox status (for a review, see reference 68). Certain viruses such as human T-lymphotropic retrovirus type 1 and Epstein-Barr virus use this strategy to induce proliferation and growth advantage of their host cells (81). Infection by these viruses consistently leads to an elevation of thioredoxin expression, which in turn protects the cells against cytokine-dependent cytotoxicity by neutralizing intracellular ROIs (42).

Another effective way to experimentally interfere with the redox status of a eukaryotic cell is by use of antioxidative drugs. PDTC is currently the agent best suited to modify the intracellular redox status of a given gene under defined tissue culture conditions, having the advantage of a low toxicity and an approximately 200-fold-higher efficiency than *N*-acetyl-L-cysteine to block NF-κB activity (67). PDTC can block not only the transcription of endogenous cellular NF-κB-responsive genes, such as the MCP-1 gene (57), but also the induction of viral genes. For example, Tax-mediated NF-κB activation of the LTR of human immunodeficiency virus type 1 is completely inhibited after use of micromolar concentrations of PDTC for a brief period of time (66).

The other important transcription factor which is also modulated by this particular antioxidant is AP-1 (44, 64). AP-1 is considered a central factor for tissue-specific transcription of HPVs (49). To determine whether changes in the intracellular redox status also influence HPV gene expression, we have investigated the effect of PDTC on viral transcription by using HPV-16-immortalized human keratinocytes (HPK Ia) (19) as an in vitro model system.

In this study, we demonstrated that HPV-16 transcription becomes selectively down-regulated upon PDTC treatment with approximately the same kinetics as when actinomycin D is used as a transcription inhibitor (Fig. 1). Nuclear run-on assays confirm the anticipation that the absence of the viral mRNA is not the result of a posttranscriptional control process as described recently for some cytokine genes after application of anti-inflammatory drugs (for a review, see reference 58) but rather the consequence of a regulatory pathway acting at the level of initiation of transcription (Fig. 2).

To gain a mechanistic insight into this effect, we first focused on the function of the tumor suppressor protein p53. The reasons why this particular protein was initially considered a potential candidate for the negative regulatory effect on HPV transcription can be summarized as followed. First, both the conformation and the affinity to DNA are also redox regulated through specific cysteine residues within the DNA-binding domain (29). p53 has to be in a reduced state for optimal binding to DNA in a sequence-specific manner (54). Second, as reported in a recent study, p53 overexpression alone is sufficient to down-regulate HPV-16/18 transcription through the epithelial cell-specific enhancer element within the viral URR (15). Finally, the function of p53 is not irreversibly impaired through the interaction with the E6 oncoprotein of HPV-16 or HPV-18 in cervical carcinoma cells (37, 62), since it can be reactivated under certain experimental conditions, for instance, after UV

irradiation (8). Functional reactivation of p53 can be easily monitored by elevated expression of the cyclin-dependent kinase inhibitor WAF/p21, which is a known target gene for p53 (22). Since WAF/p21 is not increased under conditions where diminished HPV-16 mRNA can be discerned (Fig. 3), one can exclude the possibility that p53 plays a significant role as a potential effector during the down-regulation process of viral transcription.

The finding that HPV-16 suppression is paralleled by an increased AP-1 binding to its cognate binding site within the viral regulatory region (Fig. 4) was initially unexpected, since mutational analyses have demonstrated that AP-1 is not only the major determinant for tissue specificity (49) but also absolutely required for efficient viral gene expression (7, 12, 77).

To delineate this apparent paradox in molecular terms, EMSAs in combination with specific Abs raised against different members of the Jun/Fos family were performed. As summarized in Fig. 5, PDTC treatment triggers mainly the induction of c-Jun binding (see also Fig. 6A and C), which represents the major component of AP-1 in untreated HPK Ia cells (Fig. 5A and B, compare lanes a and b). Although it has been reported that JunB is predominantly found in human keratinocytes (77) and sometimes even overexpressed in some cervical carcinoma cells (10), the amount of JunB in uninduced cells is at least 10-fold lower than that of c-Jun (Fig. 5B, lanes b and c). The weaker EMSA signal of JunB cannot be attributed to poorer affinity to the corresponding AP-1 site, since c-Jun and JunB have very similar DNA-binding properties (47). Western blot analysis also confirms that c-Jun is more highly expressed than JunB in our experimental system (57a). The c-Jun expression pattern is obviously independent of the degree of malignancy, because its level is reminiscent of that found in the cervical cancer cell line HeLa (5) harboring another high-risk HPV type, HPV-18.

Beside c-Jun binding, JunB binding at the proximal HPV-16 AP-1 site is also elevated upon antioxidant treatment (Fig. 5B, lane g). This is intriguing in the context of the observed suppression effect, since there exists an inverse relationship between JunB expression and the efficiency of c-Jun homodimers or c-Jun/c-Fos heterodimers to *trans*-activate AP-1-responsive indicator genes (11, 69). In contrast to JunB, JunD is not affected under the same conditions (Fig. 5B; compare lanes d and h).

PDTC induces not only c-Jun and to a certain extend JunB expression but also c-Fos expression both at the RNA and protein levels (Fig. 6B and D). However, in contrast to the HeLa cell system, where transcription of these genes reaches a peak after 60 min and declines to the baseline level after 90 min (44), both genes remain expressed even 8 h after PDTC application (Fig. 6A and D). Remarkably, monitoring of the composition of AP-1 in the band shift assay showed that c-Fos did not participate in the AP-1 transcription complex formation, since supershifted bands were only barely detectable (Fig. 5A, lane f). Instead, as shown in Fig. 7A (lanes b and e), c-Jun and JunB mainly become associated with Fra-1, which is the only member of the Fos family found to be elevated during PDTC treatment (Fig. 7B and C).

Changes of AP-1 composition by antioxidants is not without a precedent. A recent study has demonstrated that the phenolic antioxidant *tert*-butylhydroquinone antagonizes the transcriptional effects of AP-1 during treatment with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate by preferential formation of c-Jun/Fra-1 heterodimers (83). Additionally, Fra-1 can efficiently negatively interfere with c-Jun and c-Fos in activating AP-1-responsive indicator genes (75). Hence, in addition to JunB, Fra-1 seems to be an essential key element in neutralizing the function of AP-1, known to be obligatory for initiation and maintenance of viral transcription at the URR of HPV-16 or HPV-18 (7, 12, 77). However, suppression of viral transcription obviously requires both a balanced stoichiometry of a defined set of AP-1 components and their posttranslational modification (e.g., c-Jun phosphorylation [see below]), since the mere overexpression of AP-1 components by effector plasmids failed to suppress HPV-16/18 URR-driven reporter constructs in transient transfection assays (57a). Stable introduction of a *fra-1* cDNA by using inducible expression systems may be an alternative approach to address such questions. On the other hand, besides HPV, c-*myc* (Fig. 1A, lanes b to d) seems to be another gene whose activity is reduced due to alterations of the AP-1 heterodimerization pattern. Analysis of the corresponding regulatory region has revealed that there exists an AP-1 binding site within a negative element of the P1 promoter, which is involved in the transcriptional modulation of this particular cellular proto-oncogene (76).

Furthermore, it is should be noted that the *fra-1* gene is localized in a chromosomal region (11q13) (71) which is often structurally affected in cervical carcinoma cells (32). This could be of particular relevance with respect to the tumor-suppressing function of chromosome 11 (34, 61) and the different patterns of regulation of HPV transcription in malignant and nonmalignant cells (55, 56, 85). In fact, preliminary experiments using somatic cell hybrids between tumorigenic HeLa cells and normal human fibroblasts (73) reveal the occurrence of a higher ratio of Fra-1 within the AP-1 transcription complex in nontumorigenic hybrid cells than in tumorigenic segregants or in parental HeLa cells (57a), both lacking the normal allele of chromosome 11 (72). Interestingly, tumor necrosis alpha treatment, which selectively suppresses HPV-18 transcription in nontumorigenic hybrids (57), makes this effect more pronounced. It remains to be seen whether there is a causal relationship between cytokine- and PDTC-mediated down-regulation of viral gene expression. The finding that dithiocarbamates induce cytokine release in human bone marrow cells (21) could support the suggestion of a potential link between the two phenomena.

Another important problem concerns c-Fos, which is strongly induced by PDTC at both the RNA and protein levels (Fig. 6B and D). Although complex formation is a very dynamic process consisting of defined association and dissociation events, c-Fos does not become heterodimerized with c-Jun and JunB (Fig. 5A, lane f). Since c-Jun is phosphorylated upon PDTC treatment (Fig. 6C and E), it is tempting to speculate that apart from simple competition with Fra-1 in heterodimerization, posttranslational modification of c-Jun may account for the exclusion of c-Fos from AP-1 complex formation. Indeed, there is accumulating evidence that the metabolic stability of c-Fos is regulated by c-Jun phosphorylation (50, 79). The targeted degradation of c-Fos is catalyzed by the 26S proteasome in an ubiquitin-dependent pathway, which is accelerated when phosphorylated c-Jun is present (79). Which kinds of protein kinases are involved in this process is currently under investigation.

Although c-Fos is underrepresented in the AP-1 transcription complex (Fig. 5A, lane f), it should be stressed that AP-1 activity is not completely neutralized. Consistent with this notion is the induction of c-*jun* transcription by PDTC (Fig. 6A), known to be autoregulated by its own gene product (3). Additionally, transient transfection assays with reporter constructs harboring tandem repeats of AP-1 sites in front of the HSV *tk* promoter are still *trans* activated after PDTC addition (44) (Fig. 2). The low but nevertheless inducible amount of c-Fos is obviously sufficient for proper AP-1 function, since

c-Jun/c-Fos heterodimers, albeit underrepresented (Fig. 5A), have a higher stability and a more effective *trans*-acting activity than Jun-Jun homodimers alone (for a review, see reference 4). Moreover, activation of AP-1 indicator plasmids after PDTC treatment is also in good agreement with our finding of higher JunB binding (Fig. 5B, lane g), because JunB is a better activator of promoters containing multimerized AP-1 sites. Promoters with only one AP-1 site were not *trans* activated (10). This finding in turn stresses the importance of the number and the locations of AP-1 sites in their natural context within a particular promoter (4, 10), an observation which is probably also true for the viral regulatory regions investigated here in this study.

Since AP-1 is a common and indispensable key regulator for tissue specificity and transcriptional activity for various HPV types (12, 36, 49), antioxidant-induced modification of such an important transcription factor is obviously sufficient to interfere with the architecture of an HPV-specific transcription complex, resulting in a selective suppression viral gene expression. How AP-1 is neutralized and by which mechanism modified AP-1 can disturb the interplay with other virus-specific factors during transcriptional initiation remain to be elucidated. Nevertheless, since dithiocarbamates have been pharmacologically evaluated in animal experiments (24), our study may provide a basis for a novel therapeutic approach to efficiently interfere with the expression of pathogenic HPVs by using antioxidative drugs such as PDTC.

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