Retinoic acid receptor β2 (RARβ2) is often down-regulated during the multistep process to cervical cancer. In that way, its inhibitory function on the transcription factor AP-1, indispensable to maintain human papillomavirus (HPV) gene expression is relieved. Using HPV-18 positive HeLa cells as a model system, we show that ectopic expression of RARβ2 is able to down-regulate HPV-18 transcription by selectively abrogating the binding of AP-1 to the viral regulatory region in a ligand-independent manner. This resulted in down-regulation of the viral mRNAs at the level of initiation of transcription. Decreased oncogene expression was accompanied by a re-induction of cell cycle inhibitory proteins such as p53, p21CIP1, and p27KIP as well as by a cessation of cellular growth. Reduced transcriptional activity as a consequence of AP-1 reduction by selective c-Jun degradation apparently targets the HPV-18 regulatory region for epigenetic modification such as de novo methylation and nucleosomal condensation. This mechanism is otherwise counterbalanced by active and abundant viral transcription in malignant cells, because RARβ2 itself becomes inactivated during cervical carcinogenesis. Hence, our study shows that the temporal co-existence of a potential repressor and viral oncoproteins is mutually exclusive and provides evidence of a cross-talk between a nuclear receptor, AP-1, and the epigenetic machinery.

Cancer is a multistep process that is characterized by accumulation of various cell-damaging events such as chromosomal instabilities, inactivation of cell cycle regulatory proteins, and epigenetic modifications (1, 2). In the case of cervical cancer, the second leading malignancy in women worldwide, certain types of human papillomaviruses (HPV) have been identified as etiological agents being involved both in initiation and maintenance of the transformed phenotype (3). The transcription of the viral oncoproteins E6 and E7 is controlled by the viral upstream regulatory region (URR), harboring the tissue-specific enhancer and promoter elements (4). Here, beside other cellular transcription factors, activator protein-1 (AP-1) is the main regulator determining the efficiency of expression and in turn the intracellular net amount of the viral oncoproteins E6/E7 (5).

As shown for many malignant cells, including HPV-positive cervical cancer cells, the retinoic acid receptor (RAR) β2 gene (RARβ2), the most potent RAR involved in suppression of tumor-related phenotypes (6, 7), is epigenetically silenced via de novo methylation and chromatin remodeling (8). In other words, the absence of RARβ provides a selective advantage during multistep progression to cervical cancer, because the gene is obviously not compatible with unscheduled cell proliferation (9, 10). Indeed, re-introduction of RARβ into malignant cells interferes with anchorage-independent growth (6, 11) and diminishes tumor formation upon heterotransplantation into immunocompromised animals (12). Together with other isotypes of retinoid acid receptors (α and γ) or retinoid X receptors (RXR), which are encoded by different genes (13), the biological and therapeutic outcome of retinoids (e.g. all-trans-retinoic acid or 9-cis-retinoic acid) is either dependent on RAR homodimers or heterodimer formation with RXRs, which in turn bind to retinoic acid response elements or retinoid X response elements within their respective target promoters (14).

Both RARs and RXRs possess trans-repressive function on the AP-1 in a ligand-dependent manner. AP-1 is a key element in a regulatory network, playing not only a fundamental role in transcriptional regulation of various HPVs (15, 16), but also in cell proliferation and tumor induction (5, 17). In the presence of ligands, RARs or RAR/RXR can negatively affect AP-1 either by a direct interaction with Jun/Fos family members (18) or by disrupting Jun-Fos dimerization (19). Retinoic receptors can also compete with the recruitment of transcriptional co-activators (20) or inhibit the c-Jun N-terminal kinase (JNK), in turn...
preventing phosphorylation-dependent activation of c-JUN (21).

Moreover, as previously reported, we have unraveled a mechanism that explains how RARβ can also diminish AP-1 activity even in the absence of any ligand. Ectopic re-expression of RARβ abrogates AP-1 binding by a post-transcriptional mechanism where c-JUN is selectively degraded and not substituted in the AP-1 complex by other Jun family members. This uncovers a cross-talk between AP-1 and non-ligated RARβ and explains how AP-1 is turned off in the absence of an exogenous stimulus, but can be activated by the mitogen-activated protein (MAP) kinase pathway upon stimulation (22).

To follow up this functional interplay, the present study was aimed to investigate further consequences of ectopic RARβ expression in cervical carcinoma cells. Evidence is provided that AP-1 is the only factor involved in the transcriptional regulation of the HPV-18 URR that is quantitatively affected. Re-expression of RARβ was accompanied by a strong transcriptional down-regulation of the viral E6/E7 oncoproteins and by an induction of cell cycle inhibitory proteins. Continuous availability of transcription factors can protect CpG islands from DNA methyltransferase activity (23). Conversely, deficiency of activating proteins (e.g. AP-1), followed by a reduced transcriptional activity, enhances the probability that regulatory regions become targets for de novo DNA methylation. Such a scenario seems to be the case after ectopic RARβ expression and subsequent AP-1 reduction, where HPV-18 E6/E7 transcription becomes down-regulated. Monitoring epigenetic modifications of the viral URR, our results establish a link between RARβ-mediated AP-1 trans-repression, de novo DNA methylation, and transcriptional silencing of integrated copies of HPV-18 in cervical cancer cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Treatments**—The parental cervical carcinoma cell line HeLa and the stably transfected HeLa RARβ clones (12) were maintained in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% fetal calf serum (Invitrogen), 1% penicillin/streptomycin (Sigma), and 1% sodium pyruvate (Sigma), supplemented with 10% fetal calf serum (Invitrogen), to keep the selection pressure. HeLa and the stably transfected HeLa clones (12) were used: for matrix metalloproteinase-1 (MMP-1), 5'-TGC-TGAACACTGAGGGTTG-3' and 5'-CTGCTTGACCCTCA-GAGACC-3' (annealing temperature 55 °C); for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 5'-TGGATATTGT-TGCCATCAATGACC-3' and 5'-GATGCCATGGACTGTGTTGAT-3' (annealing temperature 65 °C). PCR products were analyzed by PAGE in 2% agarose gels.

**Hybridization Probes**—Plasmid pHV18 represents the unit-length genome of HPV-18, cloned in pBR322. pHF-Bα1 harbors an approximately full-length cDNA insert of the human fibroblast β-actin gene (5).

**Electrophoretic Mobility Shift Assays**—Oligonucleotides were generated in an Applied Biosystems (Foster City, CA) synthesizer using phosphoramidite chemistry and further purified by high performance liquid chromatography. For electrophoretic mobility shift assays (EMSA) the following oligonucleotides were used: AP-1 consensus, 5'-CCCTTGTAGACTAGCCG-GAA-3' (27); AP-1 (HPV-18 enhancer, position 7596–7620), 5'-CGCACCTTGGATTAGCTATTTCC-3'; AP-1 (HPV-18 promoter, position 7781–7805), 5'-GAATATATATGAC-TAAGCTTGCC-3'; keratinocyte-specific transcription factor-1 SDS-PAGE and Western Blotting—Protein extracts were prepared as described previously (22). Twenty-five μg of nuclear proteins were separated in 10% SDS-polyacrylamide gels, electrotransferred to Immobilon-P membranes (polyvinylidene difluoride, Millipore) and probed with the following antibodies: HPV-18 E7 (sc-1590, lot D228), RARβ (sc-552, lot F081), p21cip1 (sc-397, lot 1080), CDK-2 (sc-6248, lot L239), cyclin D1 (sc-246, Lot D129), p53 (sc-126, lot B261), and c-Jun (sc-1694, lot I3004) all from Santa Cruz Biotechnology. p27kip1 (catalog number k25020-050, lot 610241) was purchased from Transduction Laboratories. Bands were visualized with anti-rabbit, anti-goat, or anti-mouse IgG antibody, conjugated with horseradish peroxidase using ECL detection system (PerkinElmer Life Sciences). Equal protein transfer and loading was routinely monitored by re-incubating the blots with an actin-specific monoclonal antibody (69100, lot 7979E, ICN).

**DNA Analysis and Southern Blotting**—Total DNA was extracted according to standard methods. 5 μg was digested with HindIII (5 units/μg for 3 h), electrophoresed in a 1% agarose gel, blotted overnight on nylon membranes (Gene Screen, PerkinElmer Life Sciences), and hybridized with a radioactive labeled HPV-18 DNA probe (see below).

**RNA Analysis, Northern Blotting, and RT-PCR**—RNA was isolated with the “Absolutely RNA RT-PCR miniprep kit” (Stratagene) according to the manufacturer's instructions. Total RNA was separated on 1% agarose gels in the presence of ethidium bromide under non-denaturing conditions (5), blotted on nylon membranes (GeneScreen, PerkinElmer Life Sciences) and hybridized with radioactive labeled probes (25). Bands were quantified with a GE Healthcare PhosphorImage using the ImageQuant program as software. cDNA was obtained from 1 μg of RNA using random primers (Roche) and SuperScript II reverse transcriptase (Invitrogen) in a volume of 20 μl, following the manufacturer’s recommendations. One μl of the RT was amplified for 35 cycles consisting of 30 s at 94 °C, 45 s at the corresponding annealing temperature, and 30 s at 72 °C, with a final extension of 10 min. The following primers were used: for matrix metallproteinase-1 (MMP-1), 5'-TGC-TGAAACCCTGAAGGTG-3' and 5'-CTGCTTGACCCTCA-GAGACC-3' (annealing temperature 55 °C); for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 5'-TGATATTGT-TGCCATCAATGACC-3' and 5'-GATGCCATGGACTGTGTTGAT-3' (annealing temperature 65 °C). PCR products were analyzed in 2% agarose gels.

**Histone Deacetylation Assays**—Epigenetic Silencing of HPV Expression by RARβ

**Immunocytochemistry**—Immunocytochemical analysis of cervical carcinoma cell lines was performed with the following antibodies: HPV-18 E6/E7 (clone 7B9, lot 5, BioGenex), p21 (22B2, lot 1057, BioGenex), p53 (BD Transduction Laboratories, catalog number 610241), and c-Jun (BD Transduction Laboratories, catalog number 69100, lot 7979E, ICN) using the manufacturer’s instructions. Immunoreactivity was visualized by an indirect immunofluorescence technique (12) and quantified by automated image analysis (NIH Image 1.61; National Institutes of Health, Bethesda, MD).
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(5')-GTG-3′ (intragenic E6-primer); 5′/H11032 TGCACAGC-3′ (25), annealed oligonucleotides were labeled with [γ-32P]ATP (Amersham Biosciences, 3000 Ci/mmol) and T4 polynucleotide kinase. Binding reaction was performed as described (22). In the case of AP-1 HPV-18 promoter, only 0.2 μg of poly(dI-dC) per reaction was employed. For monitoring AP-1 composition in supershift assays, 2 μg of the following antibodies were used: the phosphorylated form of 5′-CTGCCAGGTGCATACAGGA-3′ (c-JUN (sc-822X, lot H199), c-FOS (sc-52X, lot E286), and the following antibodies were used: the phosphorylated form of 7591–7620), 5′/H11032 HhaI recognition site(s) (29), enhancer oligos harboring the AP-1 binding site including the TGGACCGA-3′ (KRF-1, HPV-18 enhancer, position 7643–7672), 5′/H11032 TGGACTATTAGCTTTTCCTC-3′; oligo 2 (position 7580–7620), 5′/H11032 TTTGCGGCTTCTCCTCATGTTGCC-3′; oligo 3 (position 7602–7637), 5′/H11032 ATATAAGTTAATTTCTGAGGTTGG-3′. Annealed oligos were in vitro methylated using Hhal-methylase (New England Biolabs).

Methylation Assay—Analysis of CpG sites for methylation within the HPV-18 URR and Ha-ras 3′-VTR were performed by methylation sensitive restriction digestion of genomic DNA followed by PCR. Two restriction digestions per genomic DNA sample were prepared, one for Hhal (New England Biolabs) and one for HpaII (New England Biolabs) (see overview in Fig. 4, A and B). Samples were incubated 16 h at 37 °C. The digested DNA was purified and PCR was performed to analyze the methylation status. The following primers were used: 5′-CAATTGGGCTCTTGGCGCA-3′ and 5′-GCATAAATCTAGTATGCACAG-3′ (HPV-18 enhancer); 5′-GGAACTCTACAGGTATTTG-3′ and 5′-CTGGCCCTATAGTGCCC-3′ (intragenic E6-primer); 5′-GGAGCAAGTGAGGGAAGGTG-3′ and 5′-GCCCTGGGCTCTCCTCAGGC-3′ (Ha-ras 3′-VTR), 5′-GCTGCCAATCTCCCTCACTC-3′ and 5′-GCCAAGAATGGGCGACATAGGGACC-3′ (DNA loading control).

Restriction Enzyme Accessibility Assay on Cross-linked and Native Chromatin—1 × 107 cells were fixed with 1% formaldehyde and cross-linking was stopped by adding 0.125 mM glycine and briefly washed with phosphate-buffered serum, trypsinized 20 min at 37 °C, and scrapped off. Cell pellets were resuspended in 1 ml of cell lysis buffer (10 mM Tris-HCl, pH 8, 10 mM NaCl, 0.2% Nonidet P-40) supplemented with proteases and phosphatases inhibitors (5, 29). 1.5 × 106 nuclei per ChIP were enzymatic sheared (Active Motif enzymatic shearing kit) according to the manufacturer’s instructions. After shearing, an aliquot of chromatin (equivalent to 25 μg of DNA) was removed and stored at −20 °C. Chromatin fractions were pre-cleaned adding 40 μl of protein A-agarose blocked with 40 μg of salmon sperm during 2 h at 4 °C on a rotating plate. The suspensions were then centrifuged to discard nonspecifically bound chromatin fragments. Supernatants (equivalent to 25 μg of DNA) were incubated with 1.5 μg of RNA polymerase II antibody (Abcam, ab-5131-50, lot 37712) or 2 μg of rabbit IgG antibody (Santa Cruz, sc-2027, lot C1605). Volumes were adjusted to 500 μl using ChIP buffer (16.7 mM Tris, pH 8, 167 mM NaCl, 1.2 mM EDTA, pH 8, 1.1% Triton X-100, and 0.01% SDS) supplemented with proteases and phosphatases inhibitors. Next day, ChIPs were incubated with 40 μl of blocked protein A-agarose beads for an additional period of 3 h. The immunocomplex containing chromatin fragments/α-RNA pol II/protein A-agarose was recovered by centrifugation at 13,000 × g for 2 min and washed once with buffer 1 (500 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, pH 8 and 0.5% Nonidet P-40), twice with buffer 2 (150 mM NaCl, 20 mM Tris, pH 8, 1% Triton X-100, and 0.1% SDS), twice with buffer 3 (250 mM LiCl, 10 mM Tris, pH 8, 1 mM EDTA, pH 8, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate), and finally twice with TE (10 mM Tris, pH 8, and 5 mM EDTA, pH 8). The immunoselected chromatin was eluted from protein A-agarose by adding 250 μl of elution buffer (1% SDS, 100 mM NaHCO3). The eluted chromatin was diluted 1:1 in a uncross-linking buffer (320 mM NaCl, 80 mM Tris, pH 8, 20 mM EDTA, pH 8, and 100 μg/ml protease K) and incubated overnight at 65 °C. DNA samples were purified and used for semiquantitative PCR. We routinely tested the specificity and linearity of chromatin immunoprecipitation by semiquantitative PCR. Utilizing RNA polymerase II antibodies for ChIP analyses of the GAPDH promoter (euchromatin) in comparison with the MYOD2 coding region (facultative heterochromatin), the following primers were used: GAPDH promoter, 5′-CCCAACTTTCGGCCCTTCTC-3′ and 5′-CAGCG-
RESULTS

Constitutive Expression of Non-liganded RARβ in HeLa Cells—
Reconstitution of RARβ expression under the control of the β-actin promoter in cervical carcinoma cells (Fig. 1A, right) resulted in a selective reduction of viral transcription (Fig. 1A, left) and a strong cessation of cellular growth (data not shown). Consistent with the decrease of HPV-18 on the RNA level, there was also a decline of the corresponding viral oncoprotein E7 (Fig. 1A, right). Viral down-regulation in the absence of any ligand occurred to the same extent as detected in non-transfected HeLa cells, incubated with pharmacological doses of all-trans-retinoic acid for 3 days. Note that viral transcription was not completely suppressed, because at least a certain threshold amount of oncogene expression is required to maintain cell proliferation (32). Moreover, RARβ restoration was also not altering the HPV-18 copy number due to the inherent karyotype instability of many cancer cells (33), because Southern blotting revealed no rearrangement or loss of viral genomes of the clones when compared with parental HeLa cells (see supplementary Fig. S1).

To examine the consequence of constitutive RARβ expression on cell cycle regulatory proteins, Western blot analyses were performed. The results depicted in Fig. 1B show not only an increase of p53 and the cyclin-dependent inhibitors p21<sup>CIP1</sup>
Epigenetic Silencing of HPV Expression by RARβ

A.

![URR/HPV-18 schematic](image)

- **URR/HPV-18**
  - Enhancer A
  - Enhancer B
  - Promoter

B.

![EMSA of Oct-1/KRF-1](image)

- **Control**
  - HeLa
  - Clone 1
  - Clone 2

- **3αtRA**
  - HeLa
  - Clone 1
  - Clone 2

C.

![EMSA of Sp-1](image)

- **Control**
  - HeLa
  - Clone 1
  - Clone 2

- **3αtRA**
  - HeLa
  - Clone 1
  - Clone 2

D.

![EMSA of c-Jun and IgG](image)

- **Control**
  - HeLa
  - Clone 1
  - Clone 2

- **3αtRA**
  - HeLa
  - Clone 1
  - Clone 2

E.

![EMSA of Sp-1](image)

- **Control**
  - HeLa
  - Clone 1
  - Clone 2

- **3αtRA**
  - HeLa
  - Clone 1
  - Clone 2

FIGURE 2. AP-1 binding to the HPV-18 URR is selectively repressed in HeLa RARβ clones. **A**, upper part, schematic overview of location and distribution used transcription factors within the HPV-18 URR. **B**, lower part, EMSA of Oct-1/KRF-1. **C**, EMSA of Sp-1. Control, nuclear extracts from parental HeLa cells. 3αtRA, non-transfected cells treated with all-trans-retinoic acid (atRA) for 72 h. Clone B1 and B2, two representative cell clones constitutively expressing RARβ2 in the absence of all-trans-retinoic acid. **D**, Western blot of c-Jun. Equal loading and transfer was monitored by incubating the membrane with an anti-actin antibody. The molecular weights of the proteins are indicated. **E**, semiquantitative PCR analyses of the RNA pol II/P-c-Jun ChIP assays. Sequential chromatin immunoprecipitation was performed to confirm the absence of c-Jun at the HPV-18 integration locus in RARβ clones in comparison with parental HeLa cells. The GAPDH promoter, lacking functional AP-1 sites, was used as negative control. Each sample was titrated using increasing amounts of the recovered DNA (input) to show the linearity of the PCR. IgG, DNA immunoprecipitated with immunoglobulin G as nonspecific antibody alone. RNA pol II/c-Jun, DNA immunoprecipitated after sequential addition of phosphorylation specific polymerase II/c-Jun antibodies. PCR products were separated in 2% agarose gels. The sizes of the PCR fragments are indicated.

and p27KIP1, but also down-regulation of the mid-G1, cyclin D1. In contrast, no quantitative changes of the cyclin-dependent kinase-2 (CDK-2) could be discerned.

Decline of HPV-18-specific mRNA can be either due to lability and shortening of the cytoplasmic half-life or by direct suppression of viral URR-directed transcriptional activity (34). To discriminate between these possibilities and to quantify the actual transcription rate at the HPV-18 integration locus, chromatin immunoprecipitation against transcriptional active RNA polymerase II were carried out (RNA-Pol-ChIP assays) (35). Semi-quantitative PCR with three different sets of primers covering the coding region of the E6/E7 oncogenes (Fig. 1C) revealed that viral transcription was diminished up to 80% (as determined by quantitative PCR analysis, data not shown) in comparison with non-transfected cells (Fig. 1D). To demonstrate the selectivity of this mechanism, two additional genes either indicative for eu- or heterochromatin were examined. GAPDH represents a housekeeping gene that is constitutively expressed, whereas myogenic factor MYO D is only active during muscle differentiation, but not in epithelial cells (36). As depicted in Fig. 1E, GAPDH shows equal loading of RNA pol II in all cell lines, whereas the heterochromatized MYO D gene cannot be immunoprecipitated by pol II antibodies, because of absence of transcriptional activity. These data indicate that reconstitution of RARβ, even in the absence of all-trans-retinoic acid, leads to a selective suppression of HPV-18 E6/E7 oncogene expression at the level of initiation of transcription.

Enhanced. However, inspecting the RARβ clones under the same experimental conditions, binding was drastically reduced to levels usually only obtained after treatment of non-transfected cells with all-trans-retinoic acid (70–80% reduction, as determined by PhosphorImager analysis, data not shown). To confirm that decreased AP-1 binding at the HPV-18 URR was a selective process, EMSAs with other 32P-labeled URR-derived oligonucleotides were carried out. In fact, under conditions where AP-1 was reduced, neither the binding of enhancer binding factor Oct-1/KRF-1 nor Sp1 binding at the viral promoter was affected (Fig. 2, B and C). Depletion of c-Jun binding on native chromatin could be also demonstrated by RNA pol II/c-Jun double ChIP assays, where in contrast to parental HeLa cells, no HPV-18 E6-specific PCR fragment was obtained (Fig. 2E). The selectivity of this assay was confirmed using the GAPDH gene as control, because the corresponding promoter lacks functional AP-1 sites. Here, in contrast to the previous assay (see Fig. 1E), no PCR fragment could be amplified. These results demonstrate that decreased AP-1 binding at the HPV-18-URR was a selective process, without impairing the binding of other transcription factors involved in URR-directed E6/E7 expression.

AP-1 Binding at the URR Can Be Restored upon Jun-N-terminal Kinase Pathway Stimulation—Transient transfection of a constitutive active mutant of the MEKK1Δ stabilizes c-Jun and reconstitutes AP-1 binding to its cognate AP-1 site at the hMT IIA promoter in HeLa RARβ clones (22). Analysis of AP-1 bind-
efficient transcription (15, 16), we therefore anticipated that both AP-1 elements within the URR is absolutely required for expression. Indeed, supershift EMSAs after MEKK1 transfection with MEKK1 completely restore AP-1 binding to the HPV-18 URR enhancer region. Using the presence of a 300-bp product covering the E6 region (after lack of DNA cleavage with HpaII sites within the VTR were protected in all cells, giving rise to a 292-bp PCR fragment. This indicates that there were apparently no differences in methylation in general between parental HeLa expressing clones. Notably, treatment of cells with decitabine did not induce demethylation of the Ha-Ras 3'variable tandem region (VTR) of the Ha-Ras protooncogene was analyzed. The VTR of Ha-ras is GC-rich and known to be targeted by DNA methylation (45). As depicted in Fig. 4B, the HpaII sites within the VTR were protected in all cells, giving rise to a 292-bp PCR fragment. This indicates that there were apparently no differences in methylation in general between parental HeLa cells or RARβ expressing clones. Notably, treatment of cells with decitabine did not induce demethylation of the Ha-Ras 3'VTR, which is consistent with previous reports showing that certain genes were refractory to demethylation (46, 47). In contrast, when RARβ expressing clones were treated under the same experimental conditions, the 254-bp fragment could no longer be amplified, indicating that demethylation of the HPV-18 enhancer was successful (Fig. 4A).

DNA methylation of a regulatory region may have principally two consequences: (i) interference with the binding of transcription factors (48) and/or (ii) being a step of a complex network of subsequent chromatin modifications, finally leading to a close chromatin structure and reduced accessibility of tran-
scription factors to their corresponding binding sites (42). To analyze whether de novo DNA methylation of the HPV-18 URR block AP-1 binding simply due to sterical hindrance, oligonucleotides were in vitro methylated by HhaI methylase. Using nuclear extracts from parental HeLa cells, EMSAs showed that the same binding efficiency could be discerned (data not shown). Moreover, to examine whether AP-1 differs in its affinity to methylated versus non-methylated DNA, cross-competition experiments with increasing molar excess of non-labeled oligos were carried out. Fig. 4C reveals that AP-1 binds with the same affinity suggesting that methylation per se was not sufficient to impair AP-1 binding.

To check whether de novo methylation within the URR was associated with a condensed nucleoprotein structure (49, 50), rendering the region more resistant to exogenously added restriction endonucleases (43, 51), isotonic nuclei were digested with MfeI. Fig. 4D shows a schematic overview of the MfeI restriction sites relative to the enhancer AP-1 binding site and the position of the primers used in this assay. Whereas the URR of HeLa RARβ clones exhibited much less accessibility to MfeI, as indicated by the appearance of a 254-bp amplification product, chromatin of HeLa parental cells was highly susceptible under the same experimental conditions. Here, no protected fragment could be amplified. The same results can be obtained when chromatin was digested with MfeI after cross-linking with formaldehyde (see supplementary Fig. S2). Control PCRs of an adjacent E6 coding region confirmed that comparable DNA inputs was used for each reaction. Because DNA methylation is mostly linked with nucleosomal condensation (52, 53), these results indicate that the regulatory region of most HPV-18 copies in the RARβ expressing cells were in a heterochromatic state.

Induction of E6/E7 Expression Requires Demethylation Prior to AP-1 Reconstitution—To test whether demethylation of the URR can restore HPV-18 E6/E7 gene expression, HeLa RARβ clones were treated for 4 days with the demethylating agent decitabine. However, even though the viral URR was demethylated (Fig. 4A), no quantitative changes of the HPV-18 E6/E7 mRNA steady-state levels could be observed (Fig. 5A, compare lanes 5, 6 and lanes 9, 10, respectively). This was further corroborated by RNA polymerase II ChIP assays (Fig. 5B), also demonstrating an absence of augmented transcriptional activity covering the E7 coding region. Although decitabine itself can potentially induce the MAP kinase pathway (54), accumulation of c-JUN was apparently not sufficient to increase AP-1 to threshold levels to enhance viral gene expression (data not shown). Conversely, AP-1 binding in RARβ clones could be readily induced by serum addition (Fig. 5C), but HPV-18 transcription was not elevated (Fig. 5, D and E). Only when cells were preincubated with decitabine and subsequently stimu-
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FIGURE 5. Decitabine treatment and AP-1 reconstitution by serum induction increases E6/E7 expression. A, Northern blot, HPV-18 expression after treatment with decitabine alone for 4 days (4d Dec) and decitabine after serum starvation for 24 h (+ FCS) or serum addition for 6 h (+ FCS 6h). The position of the 28S and 18S rRNA in the ethidium bromide-stained gel and the sizes of the corresponding mRNAs are indicated. B, semiquantitative PCR analysis of RNA pol II ChIP assays after decitabine treatment (1 μM) for 4 days. C, EMSA showing AP-1 binding reconstitution after serum addition in parental HeLa or RARβ expressing cells (clone β2). D, Northern blot, absence of HPV-18 induction after serum addition in parental HeLa or RARβ expressing cells. Order of treatments, see A for details. E, semiquantitative PCR analysis of RNA pol II ChIP assays after serum addition for 6 h. PCR products were separated in 2% agarose gels. The E7-specific PCR fragment is indicated. F, semiquantitative PCR analysis of the E6-specific PCR fragment is indicated. G, semiquantitative PCR analysis of the E7-specific PCR fragment is indicated. H, semiquantitative PCR analysis of the E6 specific PCR fragment is indicated.

The present study set out to determine the biological outcome of RARβ2 restoration in cervical carcinoma cells, known to become down-regulated during cervical carcinogenesis (9, 10, 55). Re-expression of physiological levels of non-ligated RARβ2 in HPV-18-positive HeLa cervical carcinoma cells (Fig. 1A, right) induced not only growth retardation in vitro and in vivo (12), but was also accompanied by a strong down-regulation of the E6/E7 mRNA at the level of transcription (Fig. 1, A and D). Reminiscent to the repressive function of the HPV E2 regulatory protein, predominantly lost after HPV integration into the host genome (56, 57), ectopic expression of RARβ2 resulted in an up-regulation of p53 and the cyclin-dependent kinase inhibitors (CKI) p21CIP1 and p27KIP1 as well as suppression of cyclin D1 (Fig. 1B). Similar to the reduction of HPV-18 URR-directed transcription, cyclin D1 down-regulation is consistent with the finding that c-JUN is selectively degraded upon RARβ2 restoration (see Fig. 2D). Cyclin D1 contains two AP-1 binding sites within its promoter and c-JUN has been found to induce its transcription (58). Referring to p53 and p21CIP1, two mechanisms may account for their increase: notably, c-JUN can also act as the negative regulator of p53 via a conserved atypical AP-1 site within the p53 promoter and in turn with the activation of its downstream target gene (59). In favor with this notion is the observation that c-jun-/- knock-out cells actually contain higher basal levels of p53 than their parental counterparts (58). In addition, reduction in the E6 expression (Fig. 1A) rescues p53 from E6-AP-mediated proteasomal degradation, therefore leading to its increase on protein level (60). p27KIP1 up-regulation has also been demonstrated after RARβ re-expression in lung cancer cells or after neuroblastoma differentiation (61, 62), but the exact mechanism is still elusive.

Regarding host regulatory proteins that bind to the viral URR, the transcription factor AP-1 plays an essential role in determining both the efficiency of HPV expression (4, 16, 63) and the phenotype of HPV-positive cells in nude mice (5, 29). Monitoring AP-1 in comparison with other transcription factors within the viral enhancer and promoter region, decreased binding was only detectable for AP-1, whereas Oct-1/KRF-1 and Sp-1, each participating in the transcriptional regulation of the E6/E7 promoter (4), were not affected (Fig. 2, A–C). This is in accord with the fact that ectopic re-expression leads to a

Related by serum treatment, could oncoproteins expression be restored (Fig. 5A, compare lanes 5, 8 and lanes 9, 12, respectively). To prove that combined treatment had a transcriptional effect, ChIP assays revealed increased RNA polymerase II activity spanning the coding region (Fig. 5F). In contrast, neither decitabine nor serum addition alone has a significant effect on RNA polymerase II loading (Fig. 5, B and E), which is consistent with the absence of HPV-18 mRNA induction (see Fig. 5, A and D). These data indicate that restored expression of RARβ2 not only reduces the amount of AP-1 (Fig. 2), but also leads to a cis-inhibitory effect on the viral upstream regulatory region by inducing de novo DNA methylation (Fig. 4A) and heterochromatinization (Fig. 4D).

DISCUSSION

The present study set out to determine the biological outcome of RARβ2 restoration in cervical carcinoma cells, known
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selective depletion of c-JUN (see Fig. 2D), whereas AP-1 binding can be restored when its proteasomal degradation is prevented (22).

The ability to phosphorylate and stabilize c-JUN cannot only be mediated by proinflammatory cytokines (64) but also by transient overexpression of a constitutive active mutant of the MEKK1Δ. A preferential substrate of MEKK1 is MKK4, in turn activating the JNK pathway (65). We have previously shown that JNK was not impaired in HeLa RARβ clones, because the MEKK1Δ mutant was also able to rescue c-JUN from degradation (22). EMSAs revealed that the amount of AP-1 binding to its two cognate cis-regulatory sites within the HPV-18 URR could be completely restored under these conditions (Fig. 3A).

However, AP-1 reconstitution had no positive functional effect on HPV-18 E6/E7 transcription, but even more reduces viral gene expression in parental HeLa cells (Fig. 3B). This discrepancy is apparently due to the fact that MEKK1Δ transfection not only increased c-JUN incorporation into the AP-1 complex, but also FRA-1 (Fig. 3C), known to be involved in viral negative regulation (5, 29). Nonetheless, these data suggest that features other than AP-1 availability may regulate the response of HPV-18 URR after MAP kinase activation.

We therefore examined potential epigenetic modifications of the HPV-18 URR, monitoring the CpG sequences nearby the AP-1 binding site within the tissue-specific enhancer (43). These sequences coincide with cleavage sites of methylation sensitive restriction enzyme HhaI (Fig. 4A), which can be used as a tool to screen for cleavage protection followed by PCR in the case of de novo DNA methylation. Notably, hypermethylation of integrated HPV genomes can be considered as part of the multistep process to cervical cancer (43, 66, 67), particularly when tandem integration of unit-length HPV DNA occurred. De novo methylation obviously selects against the expression of those copies encoding the viral E2 repressor protein, known to negatively interfere with URR-directed E6/E7 transcription (44).

For instance, such a situation is found in the cervical carcinoma cell line CaSki, where most of the 600 unit-length copies of HPV-16 were extensively hypermethylated (43, 68). Although HeLa cells harbor between 30 and 50 genome equivalents of HPV-18, integrated at different chromosomes (69, 70), none of them express the E2 repressor and consequently the viral URR remains to be methylated (Fig. 4A). In contrast, RARβ expressing cells revealed that CpG sites around the AP-1 binding site within the viral enhancer were de novo methylated (Fig. 4A), forming a distinct nucleosomal structure with reduced accessibility against exogenously added nucleases (Fig. 4D, see also supplementary Fig. S2). Alterations in chromatin can therefore account for the inability of restored AP-1 binding to its cognate cis-regulatory sequences within the URR (Fig. 3A), because in vitro methylation of corresponding oligonucleotides did not prevent binding when EMSAs were performed (Fig. 4C). This argues against a simple steric hindrance of methylated DNA as described for E2 protein binding to the URR (44).

The induction/maintenance of gene silencing and heterochromatin formation is mediated by a concerted action between DNA methyltransferases, methyl-CpG-binding proteins, and accessory co-repressors (e.g. histone deacetylases) (for review, see Ref. 71). Whereas it has been reported that both de novo and maintenance DNA methyltransferases in conjunction with methyl-CpG-binding proteins can be up-regulated in certain cancers (72, 73), no increase of the respective genes could be noticed in RARβ expressing HeLa cells (data not shown). Moreover, because E7 was suppressed on the transcriptional level (Fig. 1A), an enhanced methyltransferase activity due to physical interaction between E7 and DNA methyltransferase 1 (74) can be excluded.

Removal of methyl residues after decitabine treatment had no beneficial effect on the transcription of HPV-18 oncogenes (Fig. 5, A and B), which can be explained by a still reduced AP-1 binding upon RARβ expression (Fig. 2A). Conversely, mere accumulation of AP-1 after serum stimulation (Fig. 5C) also did not induce viral transcription due to an impaired chromatin access to the URR, as revealed by the reduced susceptibility to exogenously added nucleases (Fig. 4D) and the lack of RNA polymerase II loading at the viral integration locus (Fig. 5E).

However, when demethylation was followed by serum addition, AP-1 binding was reconstituted and an increase in the oncogene expression could be achieved (Fig. 5, A and F).

In summary, we propose a model where HPV-18 gene silencing upon ectopic re-expression of RARβ is first triggering c-JUN down-regulation (22). This leads to a reduced transcriptional activity, which in turn labels the viral URR for subsequent epigenetic modification. De novo DNA methylation and following heterochromatinization (or vice versa) is then no longer counterbalanced by a functional availability of AP-1 and therefore an efficient viral enhanceosome cannot be built up (75). Hence, not only the binding strength but also the concentration of AP-1 seems to be a critical determinant. Experimental evidence that support this mode of regulation derives from artificially assembled episomes where binding of the lac repressor on operator sites prevents de novo methylation in cells overexpressing DNA methyltransferases 3a (23) (for review, see Ref. 71). Whether RARβ, similar to the orphan receptor germ cell nuclear factor (GCNF) (76), is directly involved in the recruitment of DNA methyltransferases to the URR remains to be elucidated.

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