Binding Site Specificity and Factor Redundancy in Activator Protein-1-driven Human Papillomavirus Chromatin-dependent Transcription*§

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Background: Biochemical activity of dimeric AP-1 family proteins remains to be characterized.

Results: 1) Binding to canonical and non-canonical sequence elements in HPV-11 is different among dimeric AP-1 family members. 2) p300-mediated acetylation is important for AP-1-dependent HPV chromatin transcription.

Conclusion: Non-canonical binding sites and p300 are important for AP-1-regulated HPV transcription.

Significance: Binding-site specificity and redundancy among AP-1 family members and the molecular action of p300 are now defined in vitro and in cell-based assays.

Activator protein-1 (AP-1) regulates diverse gene responses triggered by environmental cues and virus-induced cellular stress. Although many signaling events leading to AP-1 activation have been described, the fundamental features underlying binding site selection and factor recruitment of dimeric AP-1 complexes to their target genes remain mostly uncharacterized. Using recombinant full-length human AP-1 dimers formed between c-Jun and Fos family members (c-Fos, FosB, Fra-1, Fra-2) for DNA binding and transcriptional analysis, we found that each of these AP-1 complexes exhibits differential activity for distinct non-consensus AP-1 sites present in human papillomavirus (HPV), and each AP-1 complex is capable of activating transcription from in vitro-reconstituted HPV chromatin in a p300- and acetyl-CoA-dependent manner. Transcription from HPV chromatin requires AP-1-dependent and contact-driven recruitment of p300. Acetylation of dimeric AP-1 complexes by p300 enhances AP-1 binding to DNA. Using a human C-33A cervical cancer-derived cell line harboring the episomal HPV type 11 genome, we illustrate binding site selectivity recognized by c-Jun, JunB, JunD, and various Fos family members in a combinatorial and unique pattern, highlighting the diversity and importance of non-canonical binding site recognition by various AP-1 family proteins.

Activator protein-1 (AP-1)** is a family of transcription factors containing a basic leucine-zipper (bZIP) motif that binds as a dimer to a seven-nucleotide consensus DNA element, TGA(C/G)TCA, classically defined as the 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE; Ref. 1). TREs are frequently found in the control region of many cellular and viral genes and serve as the binding platform for Jun and Fos family members as well as certain bZIP-containing proteins. In humans, three members in the Jun family (c-Jun, JunB, and JunD) can form homodimers or heterodimers within the same family members or with members in the Fos family, which consists of c-Fos, FosB, Fra-1, and Fra-2. Members of the Fos family are not able to form homodimers (2). Conceptually, a total of 18 AP-1 dimeric complexes can be generated between Jun and Fos family members, thus creating a regulatory circuit modulating transcription of a diverse set of genes implicated in cellular proliferation, differentiation, and apoptosis (3–6). Although the presence of TREs indicates a potential involvement of AP-1-regulated transcription, some degenerate sequences that are similar but not identical to the TRE seem to be recognized by selective AP-1 or bZIP complexes (7). This situation is further complicated by the fact that not every putative binding site is recognized by its cognate trans-acting factors, considering the dynamics of nucleosomal histones that may or may not present the sequence element in the right configuration accessible for direct protein-DNA interaction (8). Genome-wide surveys of transcription factor binding sites also uncover many cis-acting elements that do not conform to the consensus of factor binding sites (9). Accordingly, the likelihood and nature of protein-DNA contacts need to be examined on an individual basis even though the development of mathematical modeling and algorithms in computational biology has greatly facilitated the identification of putative cis-acting elements likely to be crucial for gene regulation.
In human papillomaviruses (HPVs), two AP-1 binding sites located in the upstream regulatory region (URR) of the E6 promoter driving the expression of virus-encoded E6 and E7 oncoproteins have been identified (10–16). The distal AP-1 site is critical for enhancer function (17, 18), whereas the promoter-proximal AP-1 site seems to be 12-O-tetradecanoylphorbol-13-acetate-responsive only in certain cell types (14, 16, 19). Although the importance of these two AP-1 sites in HPVs has long been recognized, the identities of protein factors acting through these two AP-1 sites and the molecular action of these trans-acting factors in HPV chromatin-dependent transcription have not been convincingly established. This is due to the difficulty in obtaining naturally assembled full-length AP-1 complexes for mechanistic studies in vitro and the limitation and variability of antibody detection of AP-1 association with its in vivo targets in native chromatin environments. Further complications in the analysis of AP-1 function lie in the dependence of epithelial cell differentiation for HPV propagation in vivo (20, 21) and a unique spatial and temporal distribution pattern seen with each of the Jun and Fos family members through various skin layers (22–24). To date it is still unclear which forms of AP-1 complexes are functionally important in HPV transcription and the specific target sites in the HPV URR that serve as the platform for transcription complex assembly.

Using an in vivo chromatin transcription system reconstituted with purified HeLa core histones, recombinant nucleosome assembly protein 1 (NAP-1) histone chaperon, and the ATP-utilizing chromatin assembly and remodeling factor (ACF), we have demonstrated that a specific form of AP-1 complex, c-Jun/c-Fos, is able to switch on transcription from an HPV type 11 (HPV-11) URR-containing chromatin template (25). With our recent adaptation of a bacterial co-expression system that enables us to purify full-length recombinant human AP-1 dimers (26), we are now able to define the role of each AP-1 complex in HPV chromatin-dependent transcription and reveal novel AP-1 sites that have not yet been characterized because of their deviation from the consensus TRE. All of these non-canonical AP-1 sites are also present in different types of HPVs in various combinations, indicating the existence of an intricate interplay between cis-elements and trans-acting factors in generating the regulatory circuit diversity to modulate HPV transcription in response to constantly changeable cellular environments.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Polycistronic bacterial expression plasmids used for purification of individual c-Jun-containing human AP-1 complexes, including c-Jun/c-Fos, c-Jun/FosB, c-Jun/Fra-1, c-Jun/Fra-2, and c-Jun/c-Jun as well as JunB/c-Fos and JunD/c-Fos, have been described (26). The accession numbers for AP-1 cDNA sequences are J04111 (c-Jun), BC009466 (JunB), NM_005354 (JunD), K00650 (c-Fos), NM_006732 (FosB), X16707 (Fra-1), and X16706 (Fra-2). Mutations introduced to each AP-1 binding site in pGL7072–161 (27) that contains HPV-11 URR nucleotides 7072–7933/1–161 at #1 (AP-1-1M), #2 (AP-1-2M), #3 (AP-1-3M), #4 (AP-1-4M), and #5 (AP-1-5M) sites were generated by PCR amplification using primer pairs listed in supplemental Table S1. The resulting AP-1 site-mutated reporter constructs were all confirmed by DNA sequencing.

**Protein Expression and Purification**—Distinct recombinant c-Jun-containing human AP-1 and JunB/c-Fos and JunD/c-Fos complexes were isolated by one-step Ni2+–NTA affinity tag purification following the described protocol (26). Purification of proteins used for chromatin assembly, including HeLa core histones, bacterially expressed recombinant human NAP-1, Sf9 insect cell-expressed recombinant Drosophila ACF, and p300 proteins has been described (28).

**Electrophoretic Mobility Shift Assay (EMSA)**—The DNA fragment (117 bp) containing a consensus AP-1 binding site, TGAGTCA, found in the upstream region (−901/−895) of the human cyclin D1 promoter (Ensembl gene name, CCND1; chromosome location 11, 69,455,873–69,469,241; transcript ID ENST00000227507) (29), was amplified from a human brain genomic DNA library with a sense primer (5′-AGGCA-GAGGGGACTAATA-3′) and an antisense primer (5′-TAAC-CGGGAGAAACACAC-3′). HPV-11 DNA fragments with individual AP-1 binding sites were generated by PCR amplification using p7072–70GLess/1t+ (27) as the DNA template and paired primers shown in supplemental Table S2. Each amplified DNA fragment was, respectively, end-labeled with [γ-32P]ATP by T4 polynucleotide kinase and purified by passing through a G-50 micro column (GE Healthcare). 1–5 fmol of 32P-labeled probe was used for EMSA as described (26). For oligo competions, 10- or 100-fold excess of unlabeled DNA fragments containing either wild-type or mutated AP-1 sequences were additionally included at the beginning of the reaction. For antibody supershift assay, 68 or 200 ng of rabbit polyclonal antibodies against the hexahistidine tag (sc-804, Santa Cruz Biotechnology) or the N-terminal 79 amino acids of human c-Jun (sc-1694a, Santa Cruz Biotechnology) were added 10 min before the termination of the reaction. The mixture was then analyzed by native polyacrylamide gel electrophoresis (26).

The *Kd* value for each AP-1 complex was determined as described (30) and briefly summarized below. 1) The intensity of the remaining free probe in each lane of the autoradiograph was quantified with ImageQuant software (Molecular Dynamics); 2) the fractional occupancy (Y) was calculated from the equation 100% (1 - [free probe]/[total probe]); 3) Y was plotted against the concentration (X) of AP-1 protein to obtain an XY trend chart using Microsoft Excel; 4) nonlinear regression trend lines were used to fit the curve; 5) a trend line equation with *r*2 > 0.90 was used to calculate *Kd* (i.e. the AP-1 concentration that binds 50% of the DNA probe).

For EMSAs performed with acetylated samples, different amounts of AP-1 complexes (as indicated in Fig. 5E) were mixed with 20 ng of p300 and 30 μM acetyl-CoA followed by the addition of 5 fmol of 32P-labeled DNA probe containing the HPV-11 #5 AP-1 site prepared as above described. The mixture was incubated at 30°C for 40 min and then processed as described (26).

**DNase I Footprinting**—A 32P-labeled DNA fragment spanning nucleotides 7688–7921 of the HPV-11 URR was incubated with various amounts (0, 3, 6, 10, or 30 ng) of c-Jun-containing...
AP-1 complexes followed by DNase I digestion and denaturing polyacrylamide gel electrophoresis analysis as described (26).

**Chromatin Assembly and in Vitro Transcription Assay**—The HPV-11 URR-containing G-less cassette p7072–70GLess/1⁺ and the control DNA template pMLΔ53, which is driven by the adenovirus major late promoter devoid of an AP-1 binding site, were described previously (27). In *in vitro* chromatin assembly using p7072–70GLess/1⁺ and transcription experiments performed with HPV chromatin with pMLΔ53 added as an internal control were conducted as described (25). Reactions performed with HPV-11 DNA as the template were similarly conducted without the addition of HeLa core histones, NAP-1 and ACF. The signal intensity of radioactive transcripts shown in autoradiography was quantified by using ImageQuant software with Typhoon 9200 PhosphorImager (GE Healthcare).

In the order-of-addition experiment (Fig. 5F), c-Jun/c-Fos (60 ng) was added before, during, or after p300/acyetyl-CoA addition at different time points after chromatin assembly but during the transcription complex assembly process. In the experiment comparing the transcription activity between full-length and truncated p300 proteins (Fig. 6C), 30 or 60 ng of histone acetyltransferase (HAT) was used to substitute for 30 ng of full-length p300 or ΔHAT. Desulfo-CoA (3.3 mM), when included, was added right before the addition of acetyl-CoA.

**In Vitro HAT Assay**—HAT assay measuring the enzymatic incorporation of [³H]acetyl-CoA into histones or AP-1 components was performed as described (28) by incubating AP-1 (60 ng) and p300 proteins (10–90 ng in Fig. 6B or 30 ng in Figs. 5B and 6D) with or without 165 ng of HeLa core histones or HPV chromatin included in the reactions. After reactions, acetylated proteins were resolved by 10% (for AP-1) or 18% (for histones) SDS-PAGE and visualized after fluorography and film exposure.

**In Vitro Protein-Protein Interaction**—AP-1 (500 ng) was immobilized on Ni²⁺-NTA-agarose beads (10 μl) in BC100 (20 mM Tris-HCl, pH 7.9, at 4 °C, 20% glycerol, 0.2 mM EDTA, 100 mM KCl, 0.1% Nonidet P-40, 1 mM DTT, and 0.5 mM PMSF) for 2 h at 4 °C. The AP-1-containing beads were then washed twice with BC100 and incubated with 300 ng of FLAG-tagged p300 proteins in BC100 for 1 h at 4 °C. After washing the beads three times with BC100, the bound proteins were eluted by SDS-PAGE protein sample buffer and analyzed by Western blotting with α-FLAG M2 antibody (Sigma).

**Establishing HPV-11-containing C-33A Cell Line**—HPV-11 genomic DNA was released from pSVO10/HPV-11 (27) by BamHI digestion followed by self-ligation with T4 DNA ligase. 6 μg of re-circularized HPV-11 DNA and 0.5 μg of pBabe-neo (31) together with 50 μg of sheared salmon sperm DNA were electroporated into human cervical cancer-derived C-33A cells as described (32). Cells were selected with 1 mg/ml G418, and individual colonies were isolated and expanded. Positive clones were first identified by PCR amplification with HPV-11-specific primers followed by genomic Southern blotting to identify the physical status (i.e. episomal or integrated) of HPV-11 DNA. Briefly, 7.5 μg of genomic DNA isolated from C-33A-derived cell lines was digested overnight at 37 °C with 40 units of BamHI or ApaL1 and resolved on a 0.8% Tris-acetate-EDTA buffer-containing agarose gel. For copy number estimation, various amounts of pSVO10/HPV-11 were initially digested with BamHI and then mixed with 7.5 μg of BamHI-digested C-33A genomic DNA before electrophoresis. After a sequential 20-min depurination with 0.1 N HCl and 20 min of denaturation with 0.4 M NaOH at room temperature, genomic DNA was transferred from the gel to a Nylon membrane (Hybond-XL, GE Healthcare) by capillary action in 0.4 M NaOH. The membrane was rinsed with 2 × SSC, 0.2 mM Tris-HCl, pH 8.0, and then cross-linked with a Spectronic Spectrolinker (XL-1500 UV Crosslinker). Prehybridization was performed in Amersham Biosciences Rapid-Hyb buffer (GE Healthcare) for 4–6 h at 65 °C. 50 ng of linearized HPV-11 genomic DNA, isolated from pSVO10/HPV-11, was ³²P-labeled and used for hybridization overnight. Membranes were washed twice each in 2 × SSC (room temperature for 5 min), 1 × SSC, 0.1% SDS (65 °C for 15 min), 0.5 × SSC, 0.1% SDS (65 °C for 15 min), and 0.5 × SSC (room temperature for 5 min). Radioactive signals were imaged with a Typhoon 9200 PhosphorImager (GE Healthcare) and quantified with ImageQuant software.

**Luciferase Reporter Gene Assay**—C-33A, HCT116, and A549 cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and used for transfection at ~50% confluency with 40–250 ng of pGL7072–161 and its AP-1 site-mutated templates (i.e. AP-1-1M, -2M, -3M, -4M, and -5M), respectively, using FuGENE 6 (Roche Applied Science) as described (33). Cells were processed 24 h after transfection for measuring luciferase activity as described (33).

**In Vivo Chromatin Immunoprecipitation (ChIP) Assay**—In *vivo* ChIP assay was performed as described (34) with chromatin samples prepared from an HPV-11 episome-containing cell line (C-33A/HPV11–8). Protein-DNA complexes were immunoprecipitated with 1–5 μg of antibodies against each member of Jun and Fos family proteins. Quantitative PCR reactions were performed as described (34) using primer pairs that amplify AP-1 binding regions (see supplemental Table S3 for ChIP primer sequences). All ChIP signals were normalized against a control product amplified from the E2 open reading frame.

**Antibodies**—Antibodies used for protein detection and ChIP assay were from Active Motif® against c-Jun (#39309), JunB (#39326), JunD (#39328), c-Fos (#39008), FosB (#39022), Fra-2 (#39023), from Santa Cruz Biotechnology against Fra-1 (sc-22794), and from Sigma against β-actin (A5441).

**Sequence Alignment**—Promoter-proximal AP-1 binding sequences from HPV-11, -6, -16, -18, -31, -33, -58, and -59 were aligned manually together with the consensus AP-1 binding site (Fig. 9, highlighted in brown). The flanking sequences were similarly aligned with conserved nucleotides marked in yellow.

**RESULTS**

**Reconstituted Full-length Human AP-1 Complexes Are Fully Active in DNA Binding**—To define the functional properties of distinct AP-1 complexes, we first co-expressed FLAG-tagged human c-Jun (*Fc-Jun*) with each of the human Fos family members individually tagged at the N terminus with a hexahistidine (6His) tag (Fig. 1A). Co-expression of FLAG-tagged human c-Jun and 6His-c-Fos as well as the other members (6His:FosB, 6His:Fra-1, and 6His:Fra-2) was achieved by situating the FLAG-tagged human c-Jun-coding sequence preceding the
6His-tagged Fos-coding region, thus allowing isolation of dimeric AP-1 complexes formed in vivo based on affinity tag purification of the second cassette that generally has a lower expression level compared with the first cassette (26). After denaturation and renaturation of the inclusion bodies containing highly expressed c-Jun in complex with each Fos protein by nickel-NTA purification (Fig. 1B), we successfully purified four c-Jun-containing AP-1 heterodimers and one c-Jun/c-Jun homodimer in stoichiometric amounts ready for functional analysis (Fig. 1C). EMSA with a 117-bp DNA probe containing the TRE derived from the human cyclin D1 (hCyclin D1) gene, we found the DNA-bound c-Jun/c-Fos complex (Fig. 1D, lane 2) was efficiently competed away by the same wild type (WT) but not mutated (Mut) non-radiolabeled cold competitor (lanes 3–6) and could be supershifted by antibodies recognizing the hexahistidine tag in 6His:c-Fos or anti-c-Jun antibodies (lanes 7–10). All of the purified AP-1 complexes bound the TRE in a dose-dependent manner with heterodimers binding better than the c-Jun/c-Jun homodimer (Fig. 1E), indicating that our purified recombinant human AP-1 complexes all possess active DNA binding activity able to recognize the classically defined consensus TRE representing a high affinity AP-1 site.

**FIGURE 1.** Recombinant full-length dimeric c-Jun-containing human AP-1 complexes are all active in binding the consensus TRE sequence found in the human cyclin D1 gene. A, shown is a schematic drawing of human c-Jun and Fos family proteins tagged at the N terminus with the FLAG (F) or hexahistidine (6His) sequence. Numbers indicate the first and last amino acids of each protein. B, purification scheme of dimeric AP-1 complex is shown. C, purified AP-1 complexes are visualized by Coomassie Blue staining. Protein size markers (in kDa) are shown on the left. D, protein-DNA complexes formed on DNA fragments containing the TRE derived from the human cyclin D1 (hCyclin D1) gene are shown. The sequences of wild-type (WT) and mutated (Mut) TREs present in the non-radiolabeled cold competitors are shown on the right. Antibody supershift assay was performed with anti-6His (α-His) or α-c-Jun antibodies. E, each dimeric AP-1 complex binds the TRE in a dose-dependent manner. EMSA was performed as described under “Experimental Procedures” with the indicated amounts of protein complexes.

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**Differential Binding Activity among Various AP-1 Complexes with Degenerate Sequences**—The use of a strong binding site as seen with the TRE may not reveal the intrinsic difference of binding activity among various AP-1 complexes. To test whether five degenerate sequences (#1–#5) found in HPV-11 provide binding platforms for distinct AP-1 complexes (Fig. 2A), we carried out extensive EMSA for each purified AP-1 dimeric binding to each of these five putative AP-1 sites. Calculation of the equilibrium binding constant (Kd) for each set of binding reactions, according to the formulated equations (30), shows a clear distinction of binding affinities among various AP-1 complexes for each binding site (Table 1). A compilation of the binding affinity (1/Kd), shown in the bar graphs (Fig. 2B) with a representative EMSA set (Fig. 2C), reveals many interesting findings. First, for each non-canonical site, heterodimers still bind better than the homodimer (the first four bars versus the last one in each set) as seen with the consensus TRE. Second, the binding affinity for all AP-1 heterodimers follows the same order: #3/#11022 #2/#11022 #1/#11022 #5/#11022 #4. The c-Jun/c-Jun homodimer shows a similar trend but with a reversed order for #2 and #3 sites. It is noted that #2 and #3 sites have the same core sequence but are differentially recognized by different AP-1 complexes, suggesting that sequences flanking the core element also contribute to protein-DNA interaction. Third, deviation of three nucleotides from the consensus, as seen with the #4 site, creates a weaker site for AP-1 binding. Nevertheless, the weaker #4 site is preferentially recognized by a particular member (i.e. c-Jun/Fra-1) of the AP-1 family proteins, in contrast to other sites that are generally bound by many family members.
with comparable affinities. Fourth, all AP-1 complexes bind better to the #3 site, corresponding to the previously defined enhancer AP-1 site, than the promoter-proximal #5 site. The relative binding affinity by each AP-1 complex for the #3, #4, and #5 sites was independently verified by DNase I footprinting on a DNA fragment containing all three sites (Fig. 2D), which shows preferred protection of the #3 site over #5 and then #4 sites following the same descending affinity (#3 > #5 > #4) as measured by EMSA with individual binding sites.

Non-canonical AP-1 Sites Are Conserved and Universally Present in Genital HPVs—The identification of these five non-canonical AP-1 binding sites in HPV-11 prompted us to examine whether comparable elements could also be found in other HPVs causing either cervical cancer or genital warts. Indeed, all the genital HPVs analyzed contain degenerate AP-1 sites as found in HPV-11 but distributed in various combinations and, in general, are also spatially conserved (Fig. 3). This comparative sequence analysis highlights that the majority of HPVs uti-
lize non-canonical sequences recognized by various AP-1 complexes to control gene expression and that the consensus element for AP-1 binding is rarely found in this large family of human pathogens, except HPV-33 and HPV-58, both containing conserved promoter-proximal and -distal AP-1 sites, and HPV-6, containing one consensus AP-1 site situated further upstream.

Recombinant AP-1 Complexes Are All Active in Transcribing HPV Chromatin in a p300- and Acetyl-CoA-dependent Manner—To demonstrate whether these recombinant AP-1 complexes possess transcriptional activity, we performed an in vitro chromatin-dependent transcription experiment (26) using an HPV-11 chromatin template containing all five AP-1 sites and the TATA box of the E6 promoter linked to a 388-bp G-less cassette and a control DNA template driven only by core elements (i.e., TATA and Initiator) derived from the adenovirus major late promoter preceding a 280-bp G-less cassette (Fig. 4A). HPV chromatin was assembled by first incubating HeLa core histones and recombinant human NAP-1 histone chaperone followed by the addition of the HPV-11 DNA template, ATP, and the ACF chromatin assembly/remodeling factor (Fig. 4B). The assembled chromatin with nucleosome positioning recapitulating the in vivo chromatin pattern (25) was then used for transcriptional analysis according to the scheme outlined in Fig. 4C. As shown in Fig. 4D, transcription of HPV chromatin only occurred when an AP-1 complex was added together with p300 HAT and acetyl-CoA (lanes 3, 4, 6, 7, 9, 10, 12, 13, 15, and 16). Omission of p300 and acetyl-CoA led to an undetectable level of transcription signals from HPV chromatin (Fig. 4D, lanes 4 versus 5, 7 versus 8, 10 versus 11, 13 versus 14, and 16).

### TABLE 1

| Ap-1 dimer      | TRE                | #1          | #2          | #3          | #4          | #5          |
|-----------------|--------------------|-------------|-------------|-------------|-------------|-------------|
| c-Jun/c-Fos     | 1.23 ± 0.20        | 0.96 ± 0.06 | 0.90 ± 0.04 | 0.77 ± 0.14 | 4.95 ± 0.15 | 1.64 ± 0.05 |
| c-Jun/FoxB      | 1.02 ± 0.04        | 0.76 ± 0.04 | 0.57 ± 0.01 | 0.30 ± 0.02 | 5.27 ± 0.02 | 1.42 ± 0.17 |
| c-Jun/Fra-1     | 0.80 ± 0.04        | 1.04 ± 0.19 | 0.71 ± 0.05 | 0.46 ± 0.04 | 2.45 ± 0.27 | 0.97 ± 0.06 |
| c-Jun/Fra-2     | 0.87 ± 0.03        | 0.97 ± 0.06 | 0.60 ± 0.02 | 0.32 ± 0.03 | 5.95 ± 0.49 | 1.14 ± 0.09 |
| c-Jun/c-Jun     | 2.65 ± 0.27        | 3.64 ± 0.29 | 1.77 ± 0.06 | 2.75 ± 0.09 | 8.78 ± 0.13 | 4.28 ± 0.52 |

FIGURE 3. Non-canonical AP-1 sites are well conserved among genital HPVVs. The position of each non-canonical and consensus AP-1 site, whose sequence is shown in a unique color on the right, is indicated for eight prevalent genital HPV types. Four conserved E2 binding sites are also marked for position referencing. Accession numbers for the analyzed HPV sequences are listed in parentheses.
versus 17). This HAT-dependent transcription event was not observed when the HPV-11 DNA template (without chromatin assembly) was used (Fig. 4D, lanes 18–34), indicating that acetylation of chromatin by p300 was indeed necessary for activator-dependent transcription from chromatin templates (25, 28, 34). Transcription from the internal control DNA template (pMLΔ53) remained constant throughout the entire set of reactions (Fig. 4D, lanes 1–17 and 18–34), indicating that the inability to detect transcription signals from HPV chromatin in the absence of AP-1 (lane 2) or p300, and acetyl-CoA as indicated. Relative transcription (Rel Txn) is defined as the signal intensity, quantified by Typhoon 9200 PhosphorImager (GE Healthcare) from the HPV template relative to that performed in the presence of 60 ng of c-Jun/c-Fos, acetyl-CoA, and p300 (i.e. lane 4 for HPV chromatin and lane 21 for HPV DNA) after initial normalization with the signal derived from the internal pMLΔ53 control template.

**Acetylation of Dimeric AP-1 Complexes Enhances AP-1 DNA Binding Activity**—Because p300-mediated acetylation may alter the DNA binding activity of a transcription factor via direct interaction (35), we first examined whether p300 could interact directly with dimeric AP-1 complexes immobilized on Ni²⁺-NTA-agarose beads through the hexahistidine tag introduced at the N terminus of each Fos protein. Indeed, full-length p300, but not its HAT domain alone (see below), interacts directly with each dimeric AP-1 complex as detected by Western blotting with an antibody that recognizes the FLAG tag introduced at the N terminus of p300 and c-Jun (Fig. 5A). We then performed an *in vitro* HAT assay to see whether c-Jun and Fos family proteins were acetylated by p300. Incorporation of [³H]acetyl-CoA was detected on each AP-1 subunit as well as p300 itself, indicating that c-Jun and each Fos family protein were substrates for p300. Incorporation of [³H]acetyl-CoA was detected on each AP-1 subunit as well as p300 itself, indicating that c-Jun and each Fos family protein were substrates for p300. Incorporation of [³H]acetyl-CoA was detected on each AP-1 subunit as well as p300 itself, indicating that c-Jun and each Fos family protein were substrates for p300.
AP-1 complex was significantly enhanced by p300-mediated acetylation, suggesting that acetylation of AP-1 by p300 increases the DNA binding activity of AP-1 to its DNA target site.

AP-1 Enhances HPV Chromatin Transcription at a Step before or Concomitant with p300 Entry—To define the mechanism of AP-1-stimulated HPV transcription, we performed an order-of-addition experiment by dividing the transcription process into multiple steps and asked which step is regulated by AP-1.

As shown in Fig. 5F, the addition of AP-1 before (step 1) or together with (step 2) p300 significantly activated HPV chromatin transcription (lanes 4 and 5 versus lane 1), which only took place when both AP-1 and p300 were present (lanes 2–4). In contrast, inclusion of AP-1 subsequent to p300 entry (steps 3–5) but before nuclear extract addition led to gradually diminished transcription signals corresponding to the time gap extended between the additions of these two factors (lanes 6–8 versus lane 4). Transcription from HPV chromatin was barely detected if AP-1 was added concurrently (step 6) or after (step 7) nuclear extract addition (lanes 9 and 10). This order-of-addition experiment clearly demonstrated that AP-1 action takes place at a step before or concomitant with p300 access to HPV chromatin.
**AP-1 Regulates HPV Transcription**

**FIGURE 6.** p300-stimulated HPV chromatin transcription and acetylation of nucleosomal histones is an AP-1-dependent event requiring additional regions outside its HAT domain. 

A. Coomassie staining of purified full-length (FL) p300 and its truncated mutants containing the wild-type (HAT) or acetylase activity-impaired (ΔHAT) HAT domain is shown. A schematic representation of protein domains in p300, HAT, and ΔHAT is shown on the left. B, a HAT assay performed with free core histones as substrates in the absence (−) or presence of different amounts of p300 (FL), ΔHAT, and HAT as indicated is shown. Protein size markers (in kDa) are shown on the left. C, in vitro transcription was performed as described in Fig. 4D with c-Jun/c-Fos in the absence (−) or presence of p300 (FL), ΔHAT, or HAT as indicated. D, *in vitro* HAT assay was performed with HPV chromatin as the substrate in the absence (−) or presence (+) of c-Jun/c-Fos and p300 (FL), ΔHAT, or HAT as indicated.

...gaged by post-translational modification of AP-1 and/or p300 is required to relieve these inhibitory events frequently encountered in a cellular environment.

**p300 Recruitment to HPV Chromatin Is AP-1-dependent**

To directly illustrate AP-1-dependent recruitment of p300 to HPV chromatin, as deduced from the above kinetic transcription study, we purified two truncated p300 domains with active (HAT) or impaired (ΔHAT) acetylase activity (Fig. 6A). As predicted, only p300 and the HAT domain, but not ΔHAT, were able to acetylate themselves and free core histones in a dose-dependent manner (Fig. 6B). Surprisingly, although the HAT domain could functionally substitute for full-length p300 in p53-dependent chromatin transcription (28), it could not support AP-1-dependent HPV chromatin transcription (Fig. 6C, *lanes 1–5*). The acetylase activity was absolutely essential, as the inclusion of desulfo-CoA, which is a dead-end analog of acetyl-CoA, completely abolished transcription signals from chromatin (Fig. 6C, *lanes 7 versus 2*) without affecting DNA template-driven transcription (Fig. 6C, see pMLΔ53 signals). The inability of the HAT domain to support HPV transcription is consistent with the fact that no acetylation of nucleosomal histones was observed after its incubation with AP-1 and that full-length p300 can acetylate both AP-1 components and nucleosomal histones (Fig. 6D, *lanes 6–8*). Without AP-1, even full-length p300 could not acetylate chromatin (Fig. 6D, *lanes 2 versus 6*), indicating that activator-dependent recruitment of p300 is crucial for transcription to take place from chromatin templates. The failure of the HAT domain to support HPV chromatin transcription and acetylate nucleosomal histones was attributed to its inability to interact with AP-1 complexes (see Fig. 5A). Thus, recruitment of p300 by AP-1 via direct contact is essential to modify chromatin structure, leading to productive transcription from HPV chromatin.

**Differential Patterns of AP-1 Complexes Assembled on Each Non-canonical Site in Living Cells**

To define whether AP-1 association with HPV chromatin could indeed be detected *in vivo* on non-canonical AP-1 sites, we established a stable C-33A-derived cell line harboring episomal HPV-11 genomes. The copy number of the HPV-11 episome was estimated to be ~0.5 copy per cell after prolonged passages (>20 times, Fig. 7A) as the extrachromosomal HPV-11 genomes tend to be lost over time without G418 selection for a drug-resistant plasmid cotransfected with the re-circularized HPV genome at the 1:10 ratio when the cell line was initially established (see "Experimental Procedures"). The presence of the HPV-11 genome does not alter the amounts of Jun and Fos family proteins detected in C-33A cells that generally exhibit comparable levels of proteins to those found in HeLa cells harboring integrated HPV-18 genomes, except for JunB, JunD, and c-Fos (Fig. 7B). The association of various AP-1 components with each AP-1 site was then monitored by ChIP with antibodies against each Jun and Fos family protein.

As shown in Fig. 7C, an unprecedented detail of distinct AP-1 complex association with each AP-1 site was unraveled by this comprehensive ChIP analysis. First, each non-canonical AP-1 site has a unique pattern of recognition by different AP-1 complexes; no two sites show the same binding profile of Jun and Fos family proteins, suggesting a context-dependent recruitment of various AP-1 components determined by flanking sequences extending beyond the heptanucleotide core. Second, the #1 site (*white box*) is bound by every family member, except Fra-2, and the #4–#5 cluster (*black box*).
box, too close to be resolved by ChIP, see the schematic drawing for each PCR-amplified fragment) is recognized by all Jun and Fos proteins. In contrast, the #2 site (light gray box) associates specifically with JunD and Fra-1, whereas the #3 site (heavy gray box) is strongly recognized by JunB, JunD, and Fra-2, with a weaker association with c-Fos. This finding not only supports a previous report (18) showing a specific recruitment of JunB/Fra-2 heterodimer to the enhancer region.

All the AP-1 Sites, Except #1, Are Crucial for HPV Transcription in Different Cell Types—The detection of five non-canonical AP-1 sites in HPV-11 raises an intriguing possibility of

FIGURE 7. Differential recruitment of Jun and Fos family members to individual AP-1 sites in HPV-11 episomes. A, establishment of a C-33A-derived cell line harboring HPV-11 episomes is shown. Genomic Southern blotting was performed by digesting 7.5 μg of genomic DNA, isolated from C-33A cells (−) or C-33A/HPV-11+ cells (HPV-11) with either a single-cutter (BamHI) or a triple-cutter (ApaI) of HPV-11 DNA (map shown in the box) and probed with 32P-labeled HPV-11 DNA as described under “Experimental Procedures.” BamHI-cleaved HPV-11 DNA representing different genomic copy numbers as indicated was mixed with 7.5 μg of C-33A genomic DNA and loaded as controls for quantification. B, detection of Jun and Fos family proteins in HeLa and C-33A cells with (+) or without (−) HPV-11 episomes by Western blotting with different anti-protein antibodies as indicated is shown. C, association of various Jun and Fos family members with different HPV-11 AP-1 sites as monitored by a ChIP assay performed with chromatin samples isolated from C-33A/HPV-11+ cells is shown. ChIP assay was performed with antibodies against different components of AP-1 family proteins as indicated. The locations of PCR-amplified DNA fragments containing specific AP-1 sites in HPV-11 are shown on the bottom.

FIGURE 8. Non-canonical #2–#5 AP-1 binding sites are crucial for HPV transcription in different cell types. A, shown are reporter plasmids containing HPV-11 wild-type or mutated AP-1 sites used for luciferase assay. Mutated nucleotides (underlined) are #1: TACGTAA; #2: TACCGAA; #3: TACCGAA; #4: TACGTAA; #5: TACGTAA (see supplemental Table S1). B, #2–#5 AP-1 sites are crucial for HPV transcription in C-33A cervical cancer cells. Different amounts of reporter plasmids as indicated were transfected into C-33A cells, and luciferase activity was measured 24 h later as described under “Experimental Procedures.” C, #2–#5 AP-1 sites are important for HPV transcription in HCT116 colon cancer cells and A549 lung adenocarcinoma-derived cells. Transfection of HCT116 and A549 cells was conducted with 250 ng of each reporter plasmid. Luciferase activity was measured 24 h post-transfection.
functional cooperativity among these cis-acting elements in HPV transcription. To examine whether each AP-1 site contributes to HPV transcription, we performed a luciferase reporter gene assay by transfecting a reporter plasmid driven by the HPV-11 URR containing either wild-type or site-specific mutation of each AP-1 site (Fig. 8A) into HPV-negative C-33A cells. Clearly, all AP-1 sites, except #1, contribute to the URR-driven promoter activity with the #5 site dominantly modulating the promoter activity (Fig. 8B). The importance of #2–#5 sites is not uniquely seen in cervical cancer-derived C-33A cells but is also true in colon cancer-derived HCT116 cells and lung adenocarcinoma-derived A549 cells (Fig. 8C), suggesting a general requirement of these AP-1 sites in driving HPV transcription in different cell types. It should be mentioned that although the mutation study generally supports the importance of these non-canonical AP-1 sites in HPV transcription, we could not exclude a potential involvement of other cellular factors acting through adjacent or overlapping binding sites that may be accidentally disrupted by our mutations.

**DISCUSSION**

AP-1 belongs to a bZIP-containing family of transcription factors regulating basal and induced expression of diverse cellular and viral proteins implicated in proliferation, differentiation, apoptosis, immortalization, inflammation, organogenesis, and tumorigenesis. The ability of AP-1 to control these broad and often counteracting biological processes lies in the wide-ranging compositions of dimeric AP-1 complexes generated not only between Jun and Fos family members but also extending to other related bZIP proteins (7). In addition, the transcriptional activity of AP-1 is fine-tuned by its association with distinct interacting partners, post-translational modification, redox-potential changes, and signaling events triggered by cytokines, growth factors, and viral and bacterial infection frequently in a promoter-dependent and stimulus- and cell-type-specific manner. Although the biological roles of Jun and Fos family proteins have been extensively investigated by gain- and loss-of-function studies in animals and cultured cells (2, 36–40), the biochemical characterization of AP-1 activity and the molecular understanding of its action have significantly lagged behind. This deficiency is in part attributed to the difficulty in obtaining full-length dimeric AP-1 complexes active in DNA binding and transcription for mechanistic studies in vitro.

Our successful purification of homodimeric c-Jun/c-Jun and heterodimeric c-Jun/Fos complexes made it possible for the first time to directly compare the functional properties of these dimeric AP-1 complexes in recognizing non-canonical AP-1 sequence elements found in a native HPV genome and define their transcriptional activity from in vitro-reconstituted HPV chromatin that faithfully recapitulates nucleosomal phasing seen in vivo (25). We found that all c-Jun-containing complexes have intrinsic trans-activating activity in initiating transcription from silenced HPV chromatin (Fig. 4D), including Fra-1 and Fra-2 that were previously thought to be incapable of activating transcription due to the lack of comparable activation domains as found in the C-terminal region of c-Fos and FosB (41). Our data, however, are consistent with knock-in studies showing Fra-1 can functionally replace c-Fos in mice (42), indicating factor redundancy among Fos family members with conserved biological function, and are also in agreement with transfection assay illustrating artificially tethered c-Jun/Fra-1 and c-Jun/Fra-2 single-chain dimers are able to stimulate AP-1-dependent reporter gene activity (43). It is conceivable that dimerization with c-Jun allows Fra-1 and Fra-2 to employ the activation domain in the partner protein to activate transcription. From this point of view, it is not surprising that c-Jun/c-Fos is generally considered a more potent activator when compared with c-Jun/FosB, c-Jun/Fra-1, and c-Jun/Fra-2 (Fig. 4D), as two distinct activation domains in c-Jun/c-Fos cooperatively (44) and more efficiently recruit components of the general transcription machinery (45) to the regulated promoters. The thermostability of c-Jun/c-Fos and other heterodimers is significantly higher than that of the c-Jun/c-Jun homodimer (2), resulting in more protein-DNA complexes (see Fig. 1E and Table 1) for subsequent transcription complex assembly. Nevertheless, dimerization clearly generates cooperative activation domains that compensate for the weak DNA binding activity as further exemplified by the c-Jun/c-Jun homodimer that has strong transcriptional activity (Fig. 4D) even though its DNA binding potential is generally low, particularly at low concentrations (Fig. 5E). Considering these, it is interesting to note that c-Jun, c-Fos, and Fra-2 are detected primarily in terminally differentiated skin cells, whereas JunB, JunD, and Fra-1 seem to express early in basal cells and gradually diminish upon differentiation (23). Clearly, AP-1 activity is required for every stage of the differentiation process that is tightly linked to the HPV life cycle, and the exchange of Jun and Fos family members ensures a smooth transition of AP-1 activity to selectively modulate cellular and viral gene expression necessary for different stages of the life cycle. Identification of reprogrammed transcriptome and proteome components triggered by the switch of distinct AP-1 members throughout the epithelial cell growth and differentiation process will be critical to unravel the role of cellular factors implicating in the propagation of HPV.

Recruitment of a HAT enzyme to targeted genes in chromatin is often an activator-dependent event (28, 46). Without AP-1, p300 cannot access HPV chromatin. Acetylation of nucleosomal core histones by p300 only takes place when AP-1 is present (Fig. 6D). This AP-1-triggered chromatin acetylation requires AP-1 action before or concomitant with the entry of p300 that also results in robust transcription from HPV chromatin (Fig. 5F). A direct protein-protein interaction between p300 and AP-1 is essential, as the HAT domain alone that is sufficient for mediating p53-dependent chromatin transcription (28) is incapable of supporting AP-1-dependent HPV chromatin transcription due to its failure to interact with AP-1 (Fig. 5A). Our data are consistent with the mapping of c-Jun interaction with an N-terminal region of p300 situated outside the HAT domain (47). It is important to note that the protein properties examined on a single polypeptide may or may not reflect its intrinsic activity in a protein complex. Our mechanistic studies performed with dimeric AP-1 complexes provide the first example for AP-1-regulated transcription in its native form and make it possible to directly compare the transcriptional activity of c-Fos, FosB, Fra-1, and Fra-2 in complex with c-Jun. The finding that p300-mediated acetylation of c-Jun,

**AP-1 Regulates HPV Transcription**
JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2 occurs in the dimeric complex (Fig. 5, B–D) is significant, suggesting that acetylation detected previously on c-Jun (48) is functionally relevant. Indeed, the DNA binding activity of dimeric AP-1 complexes is dramatically enhanced by p300-mediated acetylation, especially when AP-1 is limiting in the reaction (Fig. 5E). Clearly, that p300-mediated acetylation enhances AP-1 binding to DNA and AP-1 facilitates p300 recruitment to HPV chromatin illustrates how a synergistic effect can be generated from productive interaction between a sequence-specific targeting factor and a general HAT enzyme.

Our illustration of non-canonical AP-1 sites found in HPV-11 with similar motifs present in essentially every genital HPV in various combinations (Fig. 3) also highlights the importance of AP-1 in HPV transcription and the potential involvement of specific AP-1 members binding to individual sites (Figs. 2B and 7C). It is important to reiterate that the binding activity does not necessarily reflect the transcriptional activity of each dimeric AP-1 complex, as the functional outcome is also determined by the nature of activation domains and interacting proteins involved. Although the accessibility to individual AP-1 sites by distinct Jun/Fos complexes appears unhindered with DNA elements alone (Fig. 2B), differential recognition is evident in vivo when nucleosome assembly poses barriers for transcription factor binding to their cognate sites (Fig. 7C). Undoubtedly, the dynamics of factor recruitments to individual AP-1 sites potentially fluctuates in response to signaling events and the existence of particular Jun and Fos members in a specific cell type (16, 18, 49–52).

An interesting observation points to the fact that all functionally important AP-1 sites identified in HPV-11 in different cell types (Fig. 8) have nucleotide A at the sixth position (see Fig. 2A). These sixth A-containing non-canonical AP-1 motifs are dominant in every genital HPV examined without the consensus TRE (Fig. 3), especially at the promoter-proximal #5 site that is also spatially conserved (Fig. 9). It is likely that the promoter-proximal AP-1 site provides a housekeeping function to allow binding by every Jun and Fos family member, whereas the distal AP-1 sites specify regulatory activity and are thus recognized by only selective members of Jun and Fos proteins in a sequence context-dependent manner (Fig. 7C). Redundant factor binding at a sequence motif in the promoter-proximal region dominated in the housekeeping genes has also been observed in genome-wide binding studies of ETS family proteins (9). Clearly, our realization that functional elements are significantly lower than the pure existence of high affinity sequences in the genome for a given transcription factor (53) and that most degenerate low affinity binding sequences detected in vivo are juxtaposed to another transcription factor binding site (9, 54, 55) indicates that mechanistic studies unraveling the nature of protein-DNA interaction within family members with similar but non-redundant functions are of vital impor-

**FIGURE 9. A promoter-proximal AP-1 site is spatially conserved in genital HPVs.** A, shown is a schematic drawing of the promoter-proximal AP-1 site relative to the #2 E2 binding site and the TATA box of the E6 promoter in representative low-risk and high-risk genital HPVs. B, distance from the promoter-proximal AP-1 site to the #2 E2 binding site and the E6 TATA box is highly conserved among genital HPVs. C, sequence alignment of the heptanucleotide core (in brown) and conserved flanking nucleotides (indicated in yellow) is shown.
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tance. The association of selective family proteins at non-canonical sequence elements, as illustrated here with AP-1 binding to the HPV-11 URR, further ensures that binding flexibility and proper gene response can be readily achieved, and the similar feature is likely extended to AP-1-regulated cellular genes. Indeed, a recent genome-wide binding study performed with c-Jun indicates that non-canonical binding sites are indeed recognized by c-Jun (56). Clearly, the diversity of AP-1 binding and its regulatory mechanisms are likely conserved between viral and cellular systems even though the virus may sometimes modify the host machinery when co-existence becomes less beneficial.

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