Infections of bacteria and viruses induce host defense reactions known as innate responses including the activation of interferon regulatory factor-3 (IRF-3), for the activation of type I interferon system. Upon immediate early signals triggered by the infection, IRF-3 is phosphorylated and a homodimer results. The homodimer complexes with the coactivator CREB-binding protein (CBP)/p300 in the nucleus; thus, holocomplex of IRF-3 competent in DNA binding is generated. We showed CBP/p300 to be indispensable for the DNA binding activity of the holocomplex and to aid the binding through direct interaction with the DNA. We demonstrated that p300 binds with the IRF-3 homodimer via a Q-rich domain and that an intact histone acetyltransferase (HAT) domain is indispensable for the DNA binding of the holocomplex along with a CH3 domain, which connects the HAT and Q-rich domains. These results highlight a novel function of CBP/p300: direct involvement in sequence-specific DNA binding. Furthermore, the critical function of these domains in virus-induced gene activation was demonstrated in vitro by using p300 mutants.

Bacterial and viral infections induce a series of host responses known collectively as innate immunity, in which a set of genes encoding proteins crucial to the primary phase of host defense are activated. Infection by a virus or treatment with double-stranded RNA (dsRNA) induces the activation of an array of genes including the gene for type I interferon (IFN-α and -β) in various cell types (1, 2). In certain cells such as macrophages, however, bacterial endotoxin triggers a signal leading to the activation of type I IFN genes along with other cytokine genes. Type I IFN genes are directly activated by the immediate early response, and the secreted IFN expands the response by activating additional sets of genes involved in antiviral responses and the modulation of cellular functions. Therefore, the activation of type I IFN genes is crucial to the amplification of the signal triggering late responses. A very low basal expression and a rapid reversal to efficient expression after activation are characteristics of type I IFN genes.

It has been shown that the versatile transcription factors NF-κB, ATF-2, and c-Jun are involved in the activation of the IFN-β gene (3–5). However, when these factors were activated by nonviral/dsRNA stimuli such as tumor necrosis factor-α or interleukin-1, no significant activation of the IFN-β gene was observed, indicating that activation of these factors alone are not sufficient (6, 7). Promoter analysis of IFN-α and -β revealed the involvement of the interferon regulatory factor (IRF) family of proteins (8). IRF-1–9 contain a conserved DNA binding domain at their N termini and potentially bound to the IRF motif in the promoter (9). Although some of the IRFs specifically bound to the promoter of the IFN-β gene or a synthetic IRF element in vitro, the exogenous expression of these IRFs is not sufficient to activate the gene as efficiently as viral infection (10).

Studies from different laboratories using dominant negative mutants, gene disruption techniques, and specific ribozymes show that IRF-3 plays a critical role in the viral induction of type I IFN genes (11–13). IRF-3 is expressed ubiquitously and accumulated in cytoplasm in an inactive form. Viral infection or treatment with dsRNA triggers a signal, which results in the specific phosphorylation of serine residues of IRF-3 (13–15). The phosphorylated IRF-3 becomes a homodimer and then forms a complex with the coactivators CBP/p300 in the nucleus (16, 17). This holocomplex of IRF-3 is conferred DNA binding activity for the IRF motif and possibly the potential to initiate gene activation (13, 16, 18, 19). CBP/p300 interacts with various DNA-binding transcription factors with its respective domains and forms a multimeric complex on promoter DNA (20). Because CBP and p300 are histone acetyltransferases, their involvement in the activation of nucleosomal loci by histone acetylation is suggested (21). In vitro, CBP/p300 does not significantly alter the DNA binding affinity or specificity of the DNA-binding transcription factors, except in some cases (for example p53 and c-Myb) where the DNA binding is modulated by acetylation (22–25). It has been observed in vitro that the complex of IRF motif DNA and the activated IRF-3 invariably associates with CBP/p300, suggesting an unusually high affinity of CBP/p300 for the phosphorylated IRF-3 and/or a direct association of CBP/p300 with the DNA in the context of an IRF-3 holocomplex (13, 15, 18, 26). This efficient recruitment of
CBP/p300 appears to be unique to IRF-3 and may be of physiological significance. In the present study, we analyzed molecular mechanisms behind the formation of the IRF-3 holocomplex. The biochemical reconstitution of the holocomplex from IRF-3 homodimers and CBP/p300 and the use of various p300 mutants allowed us to identify the critical domains of p300. The physiological importance of these domains in gene expression was shown in cells using the corresponding mutants of p300.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, DNA Transfection, and Preparation of Cell Extracts—** L929, 293T, and HeLa cells were maintained in Eagle’s minimum essential medium supplemented with 5% fetal bovine serum. DNA transfection of L929 cells and Newcastle disease virus (NDV) infection were performed as described previously (8). DNA transfection of 293T cells was performed by calcium phosphate methods as reported (15). Cell extracts were prepared as described previously (27).

**Plasmid Constructs—** Expression constructs of pEFP50IRF-3, pEF-HA300 and pEFGSTIRF-3 were described previously (13, 17). Expression plasmids for p300 deletion mutants were obtained by deletion of SpeI (143–957), BamHI (5599–1240), BspHI and SmaI (1749–1572), or PstCI (1–1940) fragments from pEHAP300, pEHAP500 (1235–2412), pEHAP300 (1235–2221), and pEHAP300 (1235–2412ΔHAT) were obtained by PCR with oligonucleotides corresponding to the end point sequences. To generate pEHAP300 (143–957MutAT2), XbaI and NdeI fragment from pBluescript KS containing p300 (MutAT2) was inserted into pEHAP300 (143–957) (28). To obtain an expression construct of pEFHAP300 (143–957ΔZZ), a PCR fragment corresponding to amino acids 1902–1863 was inserted into XbaI/MluI-digested pEHAP300 (143–957). The expression vector of pEHAP300 (143–957TAZ) was constructed by insertion of two appropriate PCR fragments into pEHAP300 (143–957) to delete amino acids 1725–1806, and a MluI site was introduced to join these fragments without mutation. To obtain constructs of pEHAP300 (143–957ΔZn1/2, 143–957ΔZn2/3, and 143–957ΔZn1), appropriate fragments were inserted into the MluI site of pEHAP300 (143–957TAZ). To generate pEHAP300 (143–957ΔAT1), the Gene Editor in vitro site-directed mutagenesis system (Promega) was used. The vector pLNCX-FLAG-CBP was used for expression of human CBP (29). The reporter construct p-55CIB-CAT was described previously (7).

**Antibodies and Recombinant p300—** Anti-p50 epitope monoclonal antibody (3A8) was obtained from Santa Cruz Biotechnology, Inc., anti-HA mouse monoclonal (12CA5), anti-p300 (N-15, Santa Cruz), anti-GST (B-14, Santa Cruz), and anti-CBP (A-22, Santa Cruz) polyclonal antibodies were obtained commercially. Recombinant full-length histidine-tagged human p300 was a gift from Dr. T. Ita of Saitama Medical University. The protein was expressed by a baculovirus system and purified to homogeneity on a nickel column.

**Isolation of Phosphorylated IRF-3 Free of p300 by Sodium Deoxycholate (DOC) Treatment and Glycerol Gradient Fractionation—** L929 cells were transfected with pEFP50IRF-3 and infected with NDV for 12 h. The whole cell lysate prepared by a standard procedure (150 μl) was treated with 1% DOC on ice for 10 min, then separated on a 5–20% glycerol–sodium deoxycholate (10–40% glycerol containing 1% DOC, 50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 1% Nonidet P-40, and 1 mM sodium orthovanadate) by centrifugation for 16 h at 50,000 rpm in a Hitachi p55ST2 rotor. After fractionation of the gradient into 20 fractions, Nonidet P-40 was added to give a final concentration of 2%. Each fraction was assayed for IRF-3 and p300 by immunoblotting. The IRF-3 fraction free of p300 was pooled.

**GST Pull-down Assay—** L929 cells were transiently transfected with pEFGSTIRF-3 and infected with NDV for 12 h. The cell extract and HA-tagged recombinant p300 proteins generated from 293T cells were mixed and rotated with glutathione (GT)-Sepharose 4B (Amersham Biosciences) at 4 °C for 30 min. After an extensive wash with lysis buffer, the bound proteins were eluted with SDS loading buffer, separated by SDS-PAGE, and immunoblotted.

**EMSA—** EMSA was performed as described previously (13). To examine the DNA binding activity of homeric IRF-3, L929, or HeLa cell extract was treated with DOC (final 1%) on ice for 30 min and then subjected to EMSA. To investigate the DNA binding activities of p300 deletion mutants, L929 cells were transiently transfected with pEFP50IRF-3 and infected with NDV for 12 h. The extract and HA-tagged recombinant p300 mutants derived from 293T cells were incubated for 10 min at room temperature, and then the binding mixture was added.

UV Cross-linking—EMSA was performed with the lysate of infected L929 cells expressing p50-tagged IRF-3 and a [32P]-labeled ISRE probe in which thymines were substituted with hydroxymethyluridines (Brd-Urd). The probe sequence was: 5’-GAGGAGGAAACCCGGAACUGAATTACTTTCAGUUCCGGUUCCTTC3’ (positions of Brd-Urd are indicated by U, and the interferon-stimulated response element (ISRE) is underlined). After electrophoresis, the gel was irradiated (302 nm for 20 min) to cross-link the complex to the probe. The band corresponding to the holocomplex was excised from the gel, and the protein-DNA complexes were eluted with SDS loading buffer. Complexes were precipitated by acetone (80%) and dissolved with radioimmuno precipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.1% DOC, and 0.1% SDS). The samples were analyzed directly or subjected to immunoprecipitation followed by SDS-PAGE.

**In Vitro Acetylation Assay—** To detect the intrinsic HAT activity of p300 deletion mutants, whole cell lysates of 293T cells expressing HA-tagged p300 mutants were prepared. The lysates were subjected to immunoprecipitation by anti-HA monoclonal antibody and then dissolved with reaction buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 10 mM sodium butyrate, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonil fluoride). [3H]Acetyl-CoA (500 Bq) and 1 μg of histone were added to the reaction buffer and reacted at 30 °C for 1 h. SDS loading buffer was added to stop the reaction. After SDS-PAGE, proteins were transferred to PVDF membrane, and acetylated proteins were detected by autoradiography. The same membrane was reacted with anti-HA monoclonal antibody. To investigate the acetylation of IRF-3, L929 cells were transiently transfected with pEFGSTIRF-3 and mock-treated or infected with NDV for 12 h. The cell extracts were precipitated using GT-Sepharose 4B and then dissolved with reaction buffer as described above. After the in vitro acetylation assay was performed, the resin was washed with reaction buffer (20 mM Tris, pH 9.2, 150 mM NaCl, 2.5 mM CaCl2, and 5% glycerol) and incubated at 16 °C for 2 h with recombinant thrombin (Roche Molecular Biochemicals). SDS loading buffer was added to stop the reaction. After transfer to PVDF, acetylated proteins were detected by autoradiography and then reacted with anti-IRF-3 or anti-GST antibody.

**Immunoblotting and Immunoprecipitation—** Immunoblotting and immunoprecipitation were performed as described previously (13).

**CAT Assay—** The CAT assay was performed as described previously (7).

**RESULTS**

**CBP/p300 Is Essential for DNA Binding Activity of IRF-3—** It has been shown that viral infection or treatment with dsRNA results in the formation of a holocomplex of IRF-3, exhibiting activity to bind the ISRE or IRF motif (13, 15, 18, 26). The holocomplex is composed of a homodimer of IRF-3 and the coactivators CBP/p300 (17). A typical result of electrophoresis mobility shift assay (EMSA) with extract of mouse L929 cells expressing human IRF-3 and infected with NDV (Fig. 1A, lanes 1 and 2) or HeLa cells that had been treated with poly(I:C) for 60 min (Fig. 1A, lanes 5 and 6) are shown. Super-shift experiments with specific antibodies showed that the IRF-3 holocomplex contained endogenous IRF-3 and CBP/p300, as demonstrated previously (15), but not IRF-7 (data not shown). The generation of the IRF-3 holocomplex was dependent on induction by virus or poly(I:C) (lanes 2 and 6) and independent of overexpression of IRF-3 (lanes 5 and 6, untransfected HeLa cells), demonstrating that the inducible interaction of IRF-3 and CBP/p300 is not an artifact of transient overexpression. Furthermore, co-precipitation experiments with untransfected cells demonstrated that the interaction is physiologically relevant (Fig. 1B). It was shown that treatment of extract with 1% DOC results in a dissociation of CBP/p300 from IRF-3 homodimers (17, 19). Under these conditions, the activity to bind ISRE of the holocomplex induced...
FIG. 1. p300 is required for the DNA binding of the holocomplex. A, DOC inhibits the DNA binding activity of IRF-3. L929 cells were transiently transfected with expression vector for p50 epitope-tagged IRF-3 (lanes 1–4) and mock-treated (lanes 1 and 3) or infected with NDV for 12 h (lanes 2 and 4). HeLa cells treated with IFN-β overnight (lanes 5–8) were mock-treated (lanes 5 and 7) or stimulated with poly(I:C) for 60 min (lanes 6 and 8). Whole cell lysates were subjected to EMSA using ISG15 in the absence (lanes 1, 2, 5, and 6) or presence of 1% DOC (lanes 3, 4, 7, and 8). The bands corresponding to the holocomplex or to ISGF3 bound to the probe are labeled as IRF-3 holocomplex and ISGF3, respectively. ISGF3 is ambiguous in lane 6 because it is detectable as late as 2 h after poly(I:C) treatment. The asterisks indicate nonspecific complexes. B, inducible association of IRF-3 and CBP/p300. HeLa cells were metabolically labeled with 32P in the absence (lane 1) or presence of poly(I:C) (lane 2) for 1 h. Cell extracts were subjected to immunoprecipitation with anti-human IRF-3 NES (17). The precipitates were resolved by SDS-PAGE, lane 2 vector for CBP (pLNCX-FLAG-CBP (Ref. 29); lane 3 293T cells were transiently transfected with empty vector (lane 1) or 300 ng (lane 3) of recombinant p300 (rp300). For comparison, recombinant p300 alone (300 ng) was run in lane 4. The arrow indicates the holocomplex bound to the probe. The asterisks indicate nonspecific complexes. E, reconstitution of the holocomplex with IRF-3 homodimer and CBP/p300 derived from mammalian cells. 293T cells were transiently transfected with empty vector (lane 1) or expression vector for CBP (pLNCX-FLAG-CBP (Ref. 29); lane 2) or p300 (lane 3), and the extracts were subjected to EMSA using ISG15 probe in the presence of the homodimer of p50-tagged IRF-3 devoid of p300 (P-IRF-3).

either by NDV or poly(I:C) disappeared (Fig. 1A, lanes 4 and 8). It has been shown that bacterially expressed recombinant IRF-3 could bind to IRF motif (30); however, we did not detect the faster migrating band corresponding to IRF-3 under these conditions (see below and “Discussion”). The bands indicated by asterisks were demonstrated as nonspecific bands by supershift experiments (data not shown). Because DOC treatment reversibly dissociates the tight association between the transcription factor NF-κB and its inhibitory subunit IκB (31), the result prompted us to dissociate the IRF-3 holocomplex into components and reconstitute them in vitro. To isolate IRF-3 homodimers, an extract of L929 cells expressing human IRF-3 and infected with NDV was treated with 1% DOC and subjected to glycerol density gradient fractionation. The fractionation allowed us to isolate human IRF-3 homodimers essentially free of p300 (Fig. 1C). The isolated homodimers exhibited no significant DNA binding on EMSA (Fig. 1D, lane 1). The faster mobility bands indicated by asterisks were shown as a nonspecific bands by supershift experiment (data not shown). However, the addition of recombinant p300, which was produced in insect cells and exhibited no significant ISRE DNA binding on its own (Fig. 1D, lane 4), generated DNA binding activity similar to the IRF-3 holocomplex (Fig. 1D, lanes 2 and 3). When the isolated IRF-3 homodimers were mixed with extracts of 293T cells, which had been transiently transfected with expression vectors for CBP or p300 (Fig. 1E, lanes 2 and 3) but not with vector alone (lane 1), DNA binding activity similar to that of the IRF-3 holocomplex was generated. A similar result was obtained using the IRF motif of IFN-β gene as a probe (data not shown). Thus, unlike most of the DNA-binding
transcription factors, which utilize CBP/p300, IRF-3 absolutely requires the coactivators for DNA binding. The results prompted us to investigate whether p300 physically interacts with DNA. CBP/p300 Interacts with DNA in the Presence of IRF-3—To test the contribution of p300 to the binding of IRF-3 to DNA, the ISG15 probe with thymine changed to BrdUrd was used. A complex containing 32P-labeled probe and the holocomplex of IRF-3 were subjected to EMSA using BrdUrd-substituted ISG15, which was labeled with 32P. Lane 1, uninfected cell extract; lane 2, NDV-infected (12 h) cell extract. The bands corresponding to the holocomplex or to ISGF3 bound to the probe are labeled as IRF-3 holocomplex and ISGF3, respectively. B, cross-linking of the holocomplex with probe DNA. The EMSA in A was performed on a larger scale. The gel was UV-cross-linked in situ, and the band of IRF-3 holocomplex as detected by autoradiography of the wet gel (NDV+) or corresponding area of the gel with uninfected extract (NDV−) was excised and extracted. The gel extracts, UV-cross-linked (lanes 3 and 4) or not (lanes 1 and 2), were subjected to SDS-PAGE, and an autoradiograph of the gel is shown. A portion of UV-cross-linked gel extracts (uninfected, lanes 6–8; infected, lanes 10–12) were subjected to immunoprecipitation with mixture of anti-CBP and anti-p300 antibodies (lanes 6 and 10), anti-IRF-3 antisera (lanes 7 and 11), or control serum (lanes 8 and 12). For comparison, one eighth of the extracts used for immunoprecipitation was run (uninfected, lane 5; infected, lane 9). The open and filled arrows indicate cross-linked complexes of 66 and >200 kDa, respectively.

q-rich Domain of p300 Binds with IRF-3 Homodimer but Is Not Sufficient to Confer DNA Binding of the Holocomplex—Next we examined the region of p300 required to induce the DNA binding activity of the IRF-3 holocomplex. A series of deletion mutants of p300 were expressed in 293T cells (Fig. 3A). The ability of these mutants to associate with phosphorylated IRF-3 was tested in vitro (Fig. 3B, top panel, left). The extracts of 293T cells overexpressing HA-tagged p300 mutants were reacted in vitro with those of L929 cells that had been transfected with GST-IRF-3 and infected with NDV to phosphorylate specific serine residues of IRF-3. The bound p300 mutants were, respectively, co-precipitated with GT-Sepharose and detected by immunoblotting using anti-HA (Fig. 3B, GST pull-down/αHA). In any case, GST-IRF-3 from uninfected cells did not form a complex with p300, indicating that phosphorylation is essential (data not shown). Because each of the mutants Δ143–957, Δ599–1240, Δ194–1572, 1235–2412, and 1235–2221 did form a complex with GST-IRF-3, the N-terminal region up to 1572 amino acids is dispensable for the association with IRF-3. Whereas 1–1946 did not bind to GST-IRF-3, indicating that the binding domain resides in the C-terminal region (1947–2221). This is consistent with our previous finding that GST-p300 (1752–2221) bound to IRF-3 in a phosphorylation-dependent manner (17), as well as with results from other laboratories (CBP, 1992–2441; Ref. 14). Thus, the Q-rich region is defined as the interface of the interaction with IRF-3 homodimer. Next we tested whether the complex of IRF-3 homodimers and p300 mutants binds to DNA (Fig. 3B, top panel, right). 293T cell lysate containing HA-p300 or the respective mutants was reacted with the lysate of L929 cells containing activated p50 tag-IRF-3. The mixture was subjected to EMSA using the ISG15 probe (EMSA). Under these conditions, the L929 extract exhibited DNA binding activity corresponding to the holocomplex composed of p50-IRF-3 and endogenous mouse p300 (EMSA, lane 1). However, the addition of excess p300 increased the intensity of the DNA-bound complex (lane 2), indicating that p300 is a limiting factor in the extract. Interestingly, the addition of p300 mutants Δ143–957, Δ599–1240, 1235–2412, and 1235–2221, which are smaller than the intact p300, generated a DNA-bound complex that migrated faster than the IRF-3 holocomplex (EMSA, arrows, lanes 3, 4, 7, and 8). Supershift experiments showed that the complexes binding DNA.
FIG. 3. The Q-rich domain of p300 is required for the association with IRF-3 homodimer but is not sufficient for the DNA binding. A, schematic representation of p300 mutants. Structures of wild type p300 (top) and mutants are shown. Locations of representative domains are indicated. Phenotypes as revealed by the analysis in B are summarized on the right. B, mutant analysis. The top scheme illustrates the assay for the association with IRF-3 homodimers (GST pull-down) and for the activation of DNA binding (EMSA). L929 cells were transiently transfected with the pEFGSTIRF-3 (left) or pEFp50IRF-3 (right) expression vector and infected with NDV for 12 h. 293T cells were transiently transfected with empty vector, expression vectors for the HA-tagged p300, or HA-tagged deletion mutants of p300 (middle). The cell lysates were prepared, mixed in vitro, and subjected to GST pull-down assay or EMSA. The expression constructs transfected to 293T cells are indicated above the lanes. Input: the whole cell lysate of 293T cells was subjected to immunoblotting using anti-HA polyclonal antibody. Asterisk indicates degradation product. GST pull-down, IB αHA: 293T cell extracts were incubated with L929 cell extract containing activated GST-IRF-3, then precipitated using GT-Sepharose 4B and subjected to immunoblotting using anti-HA antibody. GST pull-down, IB αGST: to compare the efficiency of GST pull-down, the above blot was probed with anti-GST antibody. EMSA: 293T cell lysates containing HA-p300 and the mutants were reacted with L929 cell lysate containing homodimer of p50-IRF-3, then subjected to EMSA using the ISG15 probe. The holocomplex composed of p50-IRF-3 and endogenous mouse p300 is labeled as IRF-3 holocomplex. Note the intensity of the holocomplex when HA-p300 was present (lane 2). The arrows show new bands.

| p300 mutants | DNA binding with IRF-3 | DNA binding with IRF-3 holocomplex |
|-------------|-----------------------|----------------------------------|
| Wild type   | +                     | +                                |
| Δ143–957    | +                     | +                                |
| Δ599–1240   | +                     | +                                |
| Δ194–1572   | +                     | +                                |
| 1–1946      | +                     | +                                |
| 1235–2412   | +                     | +                                |
| 1235–2221   | +                     | +                                |

contained both p300 mutants and IRF-3 (data not shown). Δ194–1572, which exhibited strong binding with the phospho-rlated IRF-3 (Fig. 3B, GST pull-down/αHA, lane 5), failed to promote DNA binding (Fig. 3B, EMSA, lane 5). Comparison of the structures of Δ143–957, Δ194–1572, 1235–2412, and 1235–2221 suggests the presence of a region between 1241 and 1573.
critical for DNA binding activity. Because this region corresponds to the HAT domain, several additional mutants were generated.

Requirement of HAT Activity of p300 for the Generation of DNA Binding Activity of IRF-3 Holocomplex—Two sets of mutants of the HAT domain were generated (Fig. 4A). The internal region of 50 amino acids was deleted from the mutant 1235–2412 to generate 1235–2412HAT. This deletion has been shown to remove the catalytic activity of HAT (32). Additionally, the substitution of 6 amino acids in a distinct region of HAT was shown to inactivate the enzyme (28). This substitution was introduced into the mutant Δ143–957 to generate Δ143–957MutAT2. These mutants were overexpressed in 293T cells, and cell extracts were subjected to a HAT assay using histone as substrate. The lack of HAT activity for these mutants as compared with the respective control was confirmed by the assay (Fig. 4B). These mutants were further analyzed for an association with IRF-3 and induction of DNA binding activity as in Fig. 3 (Fig. 4C). As expected, HAT activity was not essential for the association of p300 with IRF-3 homodimers (Fig. 4C, GST pull-down/oHA). However, the mutants lacking HAT activity failed to confer DNA binding activity (Fig. 4C, EMSA, lanes 2 and 4), suggesting the active and essential role of acetylation in the activation of the IRF-3 holocomplex.

Acetylation of IRF-3 in the Holocomplex—The DNA binding activity of several transcription factors was shown to be regulated through their acetylation catalyzed by CBP/p300 (20). Therefore, we examined whether IRF-3 can serve as a substrate of p300 for acetylation. GST-IRF-3 was transiently expressed in L929 cells, which were then either mock-treated or infected with NDV, and was isolated using GT-Sepharose affinity resin. The precipitated complex was reacted in vitro with [14C]acetyl-CoA, then subjected to SDS-PAGE and immunoblotting using anti-GST and anti-p300 antibodies. Fig. 5A shows that p300 specifically associated with GST-IRF-3 in the infected cells and both p300 and GST-IRF-3 were acetylated under these conditions. When monomeric IRF-3 was incubated with recombinant p300 and [14C]acetyl-CoA, no significant acetylation of IRF-3 was observed (data not shown), indicating that dimerization of IRF-3 and/or a close association between p300 and IRF-3 is conditional for the acetylation. To exclude the possibility that acetylation occurred at the GST moiety of the fusion protein, the acetylated GST-IRF-3 was cleaved into IRF-3 and GST by thrombin digestion (Fig. 5B). The result clearly shows that IRF-3 was specifically acetylated.

Involvement of the CH3 Domain of p300 in the Induction of DNA Binding Activity of IRF-3 Holocomplex—Both CBP and p300 contain a conserved region termed CH3 between the HAT domain and the Q-rich domain. Because CH3 has been shown to interact with a battery of proteins including viral E1A, which blocks the induction of IFN-β gene (33), we next investigated the function of CH3. CH3 contains ZZ motif and TAZ2 domains (Fig. 6A). ZZ domain is predicted to form zinc finger structure (34). The primary structure of TAZ2 domain is evolutionarily conserved between CBP and p300 (35). NMR analysis of the TAZ2 domain of mouse CBP showed that it forms a compact helical fold stabilized by three zinc ions (35).

An additional series of deletion mutants were constructed using the active Δ143–957 as prototype (Fig. 6A). These mutants were expressed in comparable amounts (Fig. 6B, Input) and retained the ability to bind to IRF-3 homodimers (Fig. 6B, GST pull-down/oHA) and exhibited histone acetyltransferase activity (Fig. 6B, In vitro HAT assay). However, their abilities to induce DNA binding activity of IRF-3 holocomplex were differ (Fig. 6B, EMSA). The parental Δ143–957 induced the DNA binding, but the CH3 mutants failed to do so, except...
that inactivates the DNA binding is deletion of amino acids
Viral Infected Cells
binding activity of the holocomplex. The results suggest the involvement of TAZ2 domain for the DNA
/H9004
with

IRF-3 holocomplex. The

/H9004
lanes 7

mote DNA binding (stimulated by infection with NDV, and then reporter activity
/NDV

reporter vectors for IRF-3 and the representative p300
lanes 1, and

/H9251
participate in the formation of the holocomplex of IRF-3 and
shares domains with p300, we propose that CBP and p300

in the activation of IRF-3 in a similar fashion.
p300 plays a unique and critical role in the activation of IRF-3. First, p300 is necessary for the generation of a holocomplex with specific DNA binding activity. It is reported that recombinant CBP/p300 produced in bacteria binds to ISRE in vitro (30). However, a carefully calibrated experiment showed that nearly 100–1000-fold more protein is required to detect the DNA binding of IRF-3 monomer, thus indicating that, although the IRF-3 has intrinsic DNA binding activity, its ability is dramatically increased by holocomplex formation. Second, p300 physically interacts with the target ISRE motif in the context of this holocomplex. Remarkably, p300 appeared to be the major component cross-linked to the probe DNA (Fig. 2). Because neither p300 alone nor a complex of p300 and a mutant IRF-3 (58–427), which lacks the DNA binding domain of IRF-3, shows ISRE binding activity (19), both p300 and the DNA binding domain of IRF-3 must be responsible for the activity. It is tempting to speculate that the direct contribution of CBP/p300 to the binding of IRF-3 to DNA is not an exception and CBP/p300 may aid, at least in part, in the binding of other transcription factors to DNA in a similar fashion.

Our analysis by mutagenesis revealed three functional domains of p300. The Q-rich domain (amino acids 1947–2221) is required for physical association with IRF-3 homodimers through their C-terminal domains, including phosphorylated serine residues (17). While the present work was in progress, a CBP domain interacting with a portion of IRF-3 was identified (36). This residue (IBiD: 46 amino acids, comprises three α-helices) was included in the Q-rich domain we identified. It is worth to note that the report used truncated (amino acids 139–386) unphosphorylated IRF-3 for the interaction, unlike the phosphorylated homodimer of full-length (amino acids 1–427) IRF-3 used in the present work. Because the interaction between IRF-3 and CBP is dependent on the phosphorylation/dimer status of IRF-3, the significance of the interaction between the isolated IBiD and truncated IRF-3 must be corrobo-

DISCUSSION

Specific and Critical Role for CBP/p300 in the Formation of IRF-3 Holocomplex—CBP and p300 are shown to function as versatile coactivators for many DNA binding transcription factors. Although their overall level of homology is moderate, the two proteins share functional domains and interact with many transcription factors indistinguishably. It is hypothesized that CBP/p300 cross-interacts with multiple transcription factors facilitating the formation of a transcriptionally active complex including the promoter DNA. In a few cases, CBP/p300 increases DNA binding affinity of transcription factors by acetylation; however, in most cases, CBP/p300 does not appear to significantly alter DNA binding specificity and/or affinity (20).

In the context of the active complex, or enhanceosome, CBP/p300, through the HAT domains, is speculated to acetylate chromatin proteins including histones, resulting in a remodeling of the chromatin structure (21). In the present study, we extensively analyzed the function of p300. Because CBP also participates in the formation of the holocomplex of IRF-3 and shares domains with p300, we propose that CBP and p300 participate in the activation of IRF-3 in a similar fashion.

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p300 plays a unique and critical role in the activation of IRF-3. First, p300 is necessary for the generation of a holocomplex with specific DNA binding activity. It is reported that recombinant CBP/p300 produced in bacteria binds to ISRE in vitro (30). However, a carefully calibrated experiment showed that nearly 100–1000-fold more protein is required to detect the DNA binding of IRF-3 monomer, thus indicating that, although the IRF-3 has intrinsic DNA binding activity, its ability is dramatically increased by holocomplex formation. Second, p300 physically interacts with the target ISRE motif in the context of this holocomplex. Remarkably, p300 appeared to be the major component cross-linked to the probe DNA (Fig. 2). Because neither p300 alone nor a complex of p300 and a mutant IRF-3 (58–427), which lacks the DNA binding domain of IRF-3, shows ISRE binding activity (19), both p300 and the DNA binding domain of IRF-3 must be responsible for the activity. It is tempting to speculate that the direct contribution of CBP/p300 to the binding of IRF-3 to DNA is not an exception and CBP/p300 may aid, at least in part, in the binding of other transcription factors to DNA in a similar fashion.

Our analysis by mutagenesis revealed three functional domains of p300. The Q-rich domain (amino acids 1947–2221) is required for physical association with IRF-3 homodimers through their C-terminal domains, including phosphorylated serine residues (17). While the present work was in progress, a CBP domain interacting with a portion of IRF-3 was identified (36). This residue (IBiD: 46 amino acids, comprises three α-helices) was included in the Q-rich domain we identified. It is worth to note that the report used truncated (amino acids 139–386) unphosphorylated IRF-3 for the interaction, unlike the phosphorylated homodimer of full-length (amino acids 1–427) IRF-3 used in the present work. Because the interaction between IRF-3 and CBP is dependent on the phosphorylation/dimer status of IRF-3, the significance of the interaction between the isolated IBiD and truncated IRF-3 must be corroborated by the present work. Because the interaction between IRF-3 and CBP is dependent on the phosphorylation/dimer status of IRF-3, the significance of the interaction between the isolated IBiD and truncated IRF-3 must be corroborated by the present work.
rated by elucidation of three-dimensional structure of the full-length protein.

The second domain corresponds to HAT. Two independent mutants (ΔHAT and MutAT2), which showed a marked reduction of HAT activity, simultaneously lost their ability to unmask the ISRE binding activity. The result with minimum amino acid substitution suggests that HAT activity, as well as the overall structure of the HAT domain, is critical for the DNA binding. We show that IRF-3 can be acetylated by p300 in the context of a holocomplex, suggesting that IRF-3 needs to be acetylated to cooperate with p300 and interact with DNA. In this regard, acetylation of IRF-1 and IRF-2 has been reported.

FIG. 6. The CH3 domain of p300 is necessary for the DNA binding activity of the IRF-3 holocomplex. A, schematic representation of p300 CH3 mutants. The parental construct Δ143–957 is shown at the top. The structure of a part of the CH3 domain (ZZ and TAZ2) is represented schematically. Zn1, Zn2, and Zn3 are zinc-binding sites. The putative secondary structures of the ZZ domain with its three β-sheets and the TAZ2 domain with its four α-helices are indicated underneath (34, 35). The names (left) and the constructs of deletion mutants are indicated. Phenotypes as revealed by the analysis in B are summarized on the right. B, phenotypic analysis of the mutants. 293T cells were transiently transfected with empty vector or expression vectors for the mutants indicated above the lanes. The experiments for GST pull-down and EMSA were performed as in Fig. 3B, and in vitro HAT assay was performed as in Fig. 4B. The arrow in EMSA shows new band.
however, these factors do not require CBP/p300 for their DNA binding, and its physiological significance remains to be elucidated. Alternatively, the observation that p300 acetylated itself under the assay conditions suggests the necessity of auto-acetylation for its function to facilitate DNA binding. Future analyses using HAT inhibitors and/or the identification and mutagenesis of acetylated residues will elucidate the significance of para- and auto-acetylation.

Our analysis further revealed that a third domain, termed CH3, located between HAT and the Q-rich region, is required for the ISRE binding activity. Deletion analysis of the CH3 domain showed that a minimum of 15 amino acid residues, which corresponds to a single helix of TAZ2 domain, is necessary for the induction (34, 35). We also demonstrated that recombinant TAZ2 protein fused with GST revealed nonspecific DNA binding activity (data not shown). It is tempting to speculate that the CH3 domain is involved in the direct interaction with DNA as well as interaction with other proteins. Alternatively, because CH3 physically connects the HAT and Q-rich domains, its regulatory role as a molecular hinge is suggested.

**Essential Role for p300 in the Gene Activation Mediated by IRF-3 Holocomplex.** A model for the gene activation mediated by the IRF-3 holocomplex is presented in Fig. 8. Signaling for the activation of IRF-3 kinase is not well elucidated. Recent reports demonstrated that Toll-like receptors (TLR) 3 and 4 act as the signaling receptors for dsRNA and lipopolysaccharide, respectively (38, 39). Although 293 cells require ectopic expression of TLR3 to respond to dsRNA (38), viral infection can abrogate the requirement of TLR3 to generate IRF-3 holocomplex, suggesting that viral infection may activate multiple pathways and poly(I:C) partly mimics it. Although different signals are triggered by different inducers, the signal is integrated at downstream resulting in the formation of IRF-3 holocomplex (27, 40). The activation of the holocomplex does not require de novo protein synthesis but is a result of multiple protein-protein interactions and specific phosphorylations, and possibly acetylation, enabling a rapid response from the detection of pathogens to the final specific gene activation through alteration of chromatin structure. The model indicates a regulatory function at multiple steps in the activation cascade. The tight regulation of each step is advantageous for maintaining a low basal expression level and for regulation of induced expression with a wide dynamic range.

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