Distal enhancers commonly regulate gene expression. However, the mechanisms of transcriptional mediation by distal enhancers remain largely unknown. To better understand distal enhancer-mediated transcription, we examined the regulation of the FGF-4 gene. The FGF-4 gene is regulated during early development by a powerful distal enhancer located downstream of the promoter in exon 3. Sox-2 and Oct-3 bind to the enhancer and are required for the activation of the FGF-4 gene. Previously, we implicated the co-activator p300 as a mediator of Sox-2/Oct-3 synergistic activation of a heterologous promoter, suggesting that p300 may play a role in mediating enhancer activation of the FGF-4 gene. In this study, we provide both functional and physical evidence that p300 plays an important role in the action of the FGF-4 enhancer. Specifically, we show that E1a, but not a mutant form of E1a that is unable to bind p300, inhibits enhancer activation of the FGF-4 promoter. We also demonstrate that Gal4/p300 fusion proteins can stimulate the FGF-4 promoter when bound to the FGF-4 enhancer. Additionally, we present evidence that p300 mediation of the FGF-4 enhancer requires acetyltransferase activity. Importantly, we also show that Sox-2 and p300 are physically associated with the endogenous FGF-4 enhancer but weakly associated with the endogenous FGF-4 promoter. These results are consistent with a model of transitory interaction between the distal enhancer and the FGF-4 promoter. Our results also suggest that intragenic distal enhancers may use mechanisms that differ from extragenic distal enhancers.

Enhancers regulate the transcriptional activation of many, and perhaps most, genes. It is thought that activators bound to an enhancer located close to a promoter help recruit the preinitiation complex (PIC) to the promoter. This recruitment is likely to be mediated through the action of co-activators. Much is known about how proximal enhancers (located within 200–500 bp of a promoter) function; however, it is unknown how activators bound to distal enhancer sequences, located several kb away, are able to stimulate a promoter. Importantly, random interaction between a promoter and an enhancer is likely to decrease significantly when separated by distances greater than 200 bp. Several models have been proposed to explain the mechanism(s) of distal enhancers. These models can be categorized as “contact” and “noncontact” models. Contact models propose mechanisms of enhancer action that result in a direct interaction between the enhancer and promoter (1, 5–7), whereas noncontact models do not propose direct enhancer-promoter interactions (8, 9).

To better understand the mechanisms of distal enhancers, we are investigating the transcriptional regulation of the FGF-4 gene. The FGF-4 gene is regulated by a powerful distal enhancer located 3 kb downstream of the promoter within the untranslated region of the third exon (10–12). This gene serves as an excellent model as many of the critical cis-regulatory sites, and the factors that bind them have been identified in both the promoter (12–17) and its distal enhancer (12, 17–21). Mechanisms for enhancer action by distal enhancers located downstream of promoters within the transcribed region are not readily explained or addressed by current models of distal enhancer function.

Co-activators are believed to act as bridging molecules between promoters and proximal enhancer regions, and their roles in the activation of the proximal enhancer and promoter of the interferon-β gene have been characterized extensively (22, 23). One such co-activator is p300 and its homolog, CBP. p300/CBP interact with a variety of transcription factors and proteins of the PIC (reviewed in Refs. 24 and 25). p300/CBP is able to mediate the transcriptional activation of a variety of promoters (3, 26, 27) and has been found to be associated directly with several promoters (22, 28–30). Often, p300/CBP-mediated transcription is dependent upon its acetyltransferase (AT) activity (23, 31–35). p300/CBP also has been shown to stimulate several promoters when tethered to distal sequences (27, 36), and it has been demonstrated to associate endogenously with the LCR of the β-globin gene (28) and the distal enhancer of the PSA gene (37). Both the β-globin LCR and the PSA enhancer are located in extragenic regions upstream of the promoters they regulate. In contrast to promoters and proximal enhancers (3, 24–26, 29, 30), the roles of p300/CBP in the function of distal enhancers are poorly understood.

Previously, we identified p300 as a co-activator that can mediate the synergistic activation of transcription by the tran-
scription factors Sox-2 and Oct-3 (38). Importantly, FGF-4 expression requires Sox-2 and Oct-3 binding to its distal enhancer (12, 20, 21), suggesting a possible role for p300 in the regulation of the FGF-4 gene. However, the studies implicating p300 were performed using heterologous promoter/reporter constructs in cells where Sox-2 and Oct-3 are not expressed. To advance our understanding of the role of p300 in the regulation of the FGF-4 gene and the mechanisms of intragenic distal enhancers, we have employed F9 embryonal carcinoma (EC) cells. These cells biochemically and morphologically resemble the cells of the inner cell mass where Sox-2, Oct-3, and FGF-4 are expressed (39, 40). In addition, transcription of the FGF-4 gene decreases dramatically when EC cells undergo differentiation (10, 12, 42). Hence, these cells provide an excellent model for studying the regulation of FGF-4 expression. To this end, several questions are addressed in this study. First, can p300 mediate the action of the FGF-4 enhancer, and is its AT activity necessary? Second, does p300 associate physically with the endogenous FGF-4 enhancer and/or promoter? We demonstrate that p300 can stimulate the FGF-4 promoter when tethered to the enhancer, and full stimulation by p300 requires the CCAAT box and GC boxes in the promoter. This stimulation is dependent on the AT activity of p300. Importantly, we also demonstrate that both Sox-2 and p300 physically associate with the endogenous FGF-4 enhancer. Together, the findings described in this study provide both functional and physical evidence that p300 can mediate the effect of a distal enhancer. In the case of the FGF-4 gene, p300 may function both as an acetyltransferase and as a transitory bridging factor able to promote enhancer-promoter interactions.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—F9 embryonal carcinoma (EC) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) and 10% fetal bovine serum (Hyclone) as described previously (12, 43). For the promoter/reporter assays, F9 EC cells were seeded at 5 × 10^5 per 100-mm dish in DMEM + 10% fetal bovine serum and transfected 24 h later by the calcium phosphate precipitation method (12, 44). Cells were incubated with the precipitate for 14–16 h, washed with DMEM, and re-fed with DMEM + 10% fetal bovine serum. The following day, cells were harvested, and cell extracts were prepared. Chloroform:acetic acid:isopropanol (2:1:1, v/v/v) was added as described previously (21). Molar amounts of DNA were kept constant with the addition of null vectors. All transfections were normalized by addition of 1 μg of CMV-β-galactosidase (Clontech). Transfections were performed in duplicate or triplicate, and representative transfactions are shown.

Plasmids and Cloning—The promoter/reporter constructs kFGF427T+E (referred to as 427T+E) and pC30S3 have been described previously (12, 38, 45). pBLCo6E is based on a previous construct, pBLCo6 (45), and contains the FGF-4 enhancer from 427T+E ligated into the SacI site downstream of the CAT gene. The remaining promoter/reporter constructs are based on 427T+E. 427T+E + EnSp3 was constructed using the following primers in PCR with 427T+E to amplify the entire plasmid but containing a SacI site in place of the SacI-H (HMG) and Oct-3 (POU) sites of the enhancer: upstream primer, 5′-cggaggGAGAAGACCGCTCCAGGATCTTAAATATCATTCTG-3′; downstream primer, 5′-ctaGGAAGATTTGGAAGAAC-3′. Lowercase sequence denotes a SacI site used for screening purposes, sequence in bold denotes the GC box of the promoter but lacks the enhancer and was constructed by removing the enhancer of 427T SpDP (15) by digestion and ligation of the SacI sites flanking the enhancer. The enhancer containing the Gal4 site was isolated from 427T+E + EnSp3 by digestion with SacI and ligated into the SacI sites of 427T SpDP-E and 427T CCATmut (containing a mutant CCAAT box and lacking the enhancer) (46) to produce 427T GCmut + EnSp3 and 427T Cmut + EnSp3, respectively. A 2.4-kb BssH1 fragment from 427T SpDP encompassing the GC box mutations of the promoter was ligated to a 3.55-kb Bsal fragment isolated from 427T Cmut + EnSp3 to produce 427T GCmut + EnSp3. This construct contains mutations in the GC boxes and CCAAT box of the promoter and an enhancer with a Gal4 site in place of the HMG and POU sites.

Expression plasmids utilized include the following: pCMV 12SE1a (E1a) and pCMV 12SE1a 32–36 (mE1a), a mutant E1a unable to bind p300/CBP, (47); Gal4/p300 1–2414 and Gal4/p300 964–1922 (48); Gal4/Tip60, which was received from John Lough (Medical College of Wisconsin); and pCMVFLAGSos-2 (38). Gal4/p300 expression vectors, which lack AT activity, Gal4/p300 ATmut, and Gal4/p300 964–1922 ATmut, were constructed as follows. The plasmid pBSp300 AT2 was obtained from Lee Kraus. This construct contains full-length p300 with six or more changes in the AT domain that have been shown to reduce the AT activity to <1% of wild-type (35). The p300 AT2 mutant in pBSp300 AT2 was amplified by PCR with the upper primer 5′-TATACggcatGA- cTTGAGATTTGCGGTGTTG-3′ and the lower primer 5′-GAACAAAAGCTGGG-3′. The upper primer contains a SacI site (lowercase), and the lower primer is specific for sequence within the pBS vector downstream of the inserted 3′-flanking sequence. Following amplification, the fragment was digested with SacI and Nhel and ligated into the SacI and XbaI sites of CMVgala4 (38). The resulting expression vector, Gal4/p300 ATmut, contains an in-frame fusion between the Gal4 DNA binding domain (DBD) and p300 AT2. The vector Gal4/p300 964–1922 ATmut expresses a fusion between the Gal4 DBD and an 964–1922 of p300 lacking AT activity. This construct was produced by ligating a PorhAl-BstEI fragment from pBSp300 AT2, encompassing the six amino acid changes in the AT domain, into the PorhAL and BstEI sites in Gal4/p300 ATmut. All engineered constructs were confirmed by DNA sequencing. All primers were synthesized, and sequencing was performed by the Eppley Cancer Institute Molecular Biology Core Facility.

Immunoprecipitation and Western Blotting—F9 EC cells were transfected with 1 μg of CMV-β-galactosidase and 8 μg of the vectors expressing Gal4/p300 964–1922, Gal4/p300 964–1922 ATmut, E1a, or mE1a using LipofectAMINE in conjunction with the PLUS reagent (Invitrogen) (49). Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction Kit (Pierce) according to manufacturer’s directions. Immunoprecipitation (IP) of the Gal4/p300 fusion proteins or E1a proteins from nuclear extracts was accomplished by addition of Gal4 (DBD) antibody conjugated to agarose beads (Santa Cruz Biotechnology, Inc.) and Gal4/p300 964–1922 ATmut, and membranes containing E1a proteins were probed with Gal4 (DBD) antibody (Santa Cruz Biotechnology, Inc.), respectively. Following an overnight incubation at 4 °C, protein G beads (Santa Cruz Biotechnology, Inc.) were added to the anti-E1a samples and incubated for 1 h at 4 °C, and beads were collected and washed. Proteins were eluted by addition of sample buffer (62 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 0.1% w/v bromophenol blue) plus 10 mM dithiothreitol and boiling. Eluted proteins were run on a denaturing Tris-glycine gel (Invitrogen) and transferred to polyvinylidene fluoride membrane (Immobilon-P; Millipore). Membranes containing Gal4 proteins were probed with Gal4 (DBD) antibody (Santa Cruz Biotechnology, Inc.), and membranes containing E1a proteins were probed with the same E1a antibody used in the IP. Proteins were detected by enhanced chemiluminescence (Amersham Biosciences) as described previously (38). Membranes were scanned on a Storm PhosphorImager (Molecular Dynamics), and bands were quantified using the ImageQuant analysis software (Molecular Dynamics) where indicated. β-Galactosidase activities were determined (see above) using 5 μl of the cytoplasmic fraction of the extract. Transfection of the wild-type Gal4/p300 construct was 2–3-fold more efficient than the AT mutant construct. All IPs and Western analyses were performed at least three times with representative blots shown.

Chromatin Immunoprecipitation (ChIP)—ChIP was performed essentially as described previously (50) on non-transfected or pCMV-FLAG Sox-2-transfected F9 EC cells as indicated. Transfections were performed using LipofectAMINE PLUS (Invitrogen) as described by Nowling et al. (49). Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction Kit (Pierce) according to manufacturer’s directions. Immunoprecipitation (IP) of the Gal4/p300 fusion proteins or E1a proteins from nuclear extracts was accomplished by addition of Gal4 (DBD) antibody conjugated to agarose beads (Santa Cruz Biotechnology, Inc.) and membranes containing E1a proteins were probed with the same E1a antibody used in the IP. Proteins were detected by enhanced chemiluminescence (Amersham Biosciences) as described previously (38). Membranes were scanned on a Storm PhosphorImager (Molecular Dynanics), and bands were quantified using the ImageQuant analysis software (Molecular Dynamics) where indicated. β-Galactosidase activities were determined (see above) using 5 μl of the cytoplasmic fraction of the extract. Transfection of the wild-type Gal4/p300 construct was 2–3-fold more efficient than the AT mutant construct. All IPs and Western analyses were performed at least three times with representative blots shown.
p300 and the FGF-4 Distal Enhancer

E1a, but Not mE1a, Inhibits the Enhancer through the HMG/POU Sites—Co-activators, including p300, play important roles in many enhancer-promoter interactions (3, 26, 27, 51, 52). Previous results indicate that p300 can mediate the synergistic action of Oct-3 and Sox-2 in HeLa cells (38). Individually, Sox-2 and Oct-3 have been shown to stimulate expression (25- to 40-fold) (38, 53), and a further stimulation is observed in the presence of added p300 (38). FGF-4 expression in F9 EC cells is dependent upon Oct-3 and Sox-2 binding to a distal enhancer (12, 20, 21). This enhancer is located downstream of the FGF-4 promoter within the untranslated region of the third exon of the FGF-4 gene. To better understand the mechanism of the FGF-4 distal enhancer, we examined whether p300 is involved in mediating its action. E1a and a mutant E1a that is unable to bind p300 (mE1a) have been used previously to implicate p300 as a possible co-activator of gene expression (52). Previous results indicate that p300 can mediate the synergistic action of Oct-3 and Sox-2 in HeLa cells (38). Individually, Sox-2 and Oct-3 have been shown to stimulate expression (25- to 40-fold) (38, 53), and a further stimulation is observed in the presence of added p300 (38). FGF-4 expression in F9 EC cells is dependent upon Oct-3 and Sox-2 binding to a distal enhancer (12, 20, 21). This enhancer is located downstream of the FGF-4 promoter within the untranslated region of the third exon of the FGF-4 gene. To better understand the mechanism of the FGF-4 distal enhancer, we examined whether p300 is involved in mediating its action. E1a and a mutant E1a that is unable to bind p300 (mE1a) have been used previously to implicate p300 as a possible co-activator of gene expression (52). To test whether p300 can mediate the synergistic action of Oct-3 and Sox-2 in HeLa cells (38). Individually, Sox-2 and Oct-3 have been shown to stimulate expression (25- to 40-fold) (38, 53), and a further stimulation is observed in the presence of added p300 (38). FGF-4 expression in F9 EC cells is dependent upon Oct-3 and Sox-2 binding to a distal enhancer (12, 20, 21). This enhancer is located downstream of the FGF-4 promoter within the untranslated region of the third exon of the FGF-4 gene. To better understand the mechanism of the FGF-4 distal enhancer, we examined whether p300 is involved in mediating its action. E1a and a mutant E1a that is unable to bind p300 (mE1a) have been used previously to implicate p300 as a possible co-activator of gene expression (52, 54-57). To test whether p300 may be involved in mediating the action of the FGF-4 enhancer, we performed co-transfections with various FGF-4 promoter/reporter constructs and an expression vector for either E1a or mE1a. Initially, the promoter/reporter construct 427T+E (Fig. 1A), containing the FGF-4 enhancer placed downstream of the CAT reporter gene driven

8, 167 mm NaCl), an aliquot was removed as "input" control, and the remaining sample was divided into two aliquots. M2 (anti-FLAG; Sigma) antibody or anti-p300 (N15; Santa Cruz Biotechnology, Inc.), alone or conjugated to agarose beads, or anti-p300 (C20; Santa Cruz Biotechnology, Inc.) was added to one aliquot, and anti-Gal4 (DBD) (Santa Cruz Biotechnology, Inc.), alone or conjugated to agarose beads, was added to the second aliquot as a nonspecific antibody control. Samples were incubated with antibody overnight with rotation at 4 °C. The following day, protein A beads were added to the samples with antibody not conjugated to beads and incubated for 1 h at 4 °C with rotation to collect DNA-protein-antibody complexes. Beads were washed, and DNA-protein complexes were eluted by two successive incubations at 65 °C for 10 min with freshly prepared elution buffer (1% SDS, 50 mM NaHCO3). NaCl to a final concentration of 0.3 M and incubations at 65 °C for 6 h to revert cross-links. Samples were precipitated overnight at –20 °C by addition of 2.5 volumes of 100% ethanol, resuspended in proteinase K buffer (10 mM Tris-Cl, pH 7.5, 5 mM EDTA, 0.25% SDS), and treated with proteinase K for 2 h at 45 °C. DNA was purified using the Geneclean turbo kit (Q Biogene) following the manufacturer's directions. ChiP experiments were repeated at least twice with similar results.

The following primer pairs were used for the indicated regions of the FGF-4 gene. FGF-4 promoter: FGFproU2, 5'-GTAAGGAAAGGACAGGAGAT-3' and FGFproL3, 5'-CAGACCTAATGCGGATAGCACA-3'; FGF-4 enhancer: FGFenhUp, 5'-AGACTTCTGAGCAGACTGGAA-3'; FGFenhDown, 5'-CAGACTTCTGAGCAGACTGGAA-3'; and FGFEnhDown, 5'-CAGACTTCTGAGCAGACTGGAA-3'.

RESULTS

E1a, but Not mE1a, Inhibits the Enhancer through the HMG/POU Sites—Co-activators, including p300, play important roles in many enhancer-promoter interactions (3, 26, 27, 51, 52). Previous results indicate that p300 can mediate the synergistic action of Oct-3 and Sox-2 in HeLa cells (38). Individually, Sox-2 and Oct-3 have been shown to stimulate expression (25- to 40-fold) (38, 53), and a further stimulation is observed in the presence of added p300 (38). FGF-4 expression in F9 EC cells is dependent upon Oct-3 and Sox-2 binding to a distal enhancer (12, 20, 21). This enhancer is located downstream of the FGF-4 promoter within the untranslated region of the third exon of the FGF-4 gene. To better understand the mechanism of the FGF-4 distal enhancer, we examined whether p300 is involved in mediating its action. E1a and a mutant E1a that is unable to bind p300 (mE1a) have been used previously to implicate p300 as a possible co-activator of gene expression (52). To test whether p300 may be involved in mediating the action of the FGF-4 enhancer, we performed co-transfections with various FGF-4 promoter/reporter constructs and an expression vector for either E1a or mE1a. Initially, the promoter/reporter construct 427T+E (Fig. 1A), containing the FGF-4 enhancer placed downstream of the CAT reporter gene driven

Fig. 1. Effects of E1a on enhancer-mediated stimulation of the FGF-4 promoter. A, physical map of FGF-4 promoter/reporter construct 427T+E, containing 427 bp of the FGF-4 promoter driving the CAT reporter gene. The FGF-4 enhancer (316 bp) is located downstream of the CAT gene to recapitulate its endogenous placement. GC and GT, Sp1/Gp3 binding sites; TATA, TATA box; HMG, Sox-2 binding site; POU, Oct-3 binding site. B, transfection of F9 EC cells with 15 μg of 427T+E alone or with increasing amounts of a CMV expression vector for 12S E1a (E1a) or 12S E1aΔ2−36 (mE1a), as indicated. A constant amount of DNA was achieved by addition of the null CMV vector pCMV5. Results are presented as CAT expression relative to the expression of 427T+E alone, which was set to 1. The mE1a vector expresses a mutant E1a unable to interact with p300/CBP. This experiment was repeated twice with similar results. C, protein expression in F9 EC cells. E1a proteins were immunoprecipitated from nuclear extracts and subjected to Western blot analysis as indicated under "Experimental Procedures.” *, E1a protein; #, mE1a protein. Approximate molecular mass is indicated on the left.
by 427 bp of the FGF-4 promoter, was co-transfected with an expression vector for E1a or mE1a into F9 EC cells. E1a caused a dose-dependent decrease in transcriptional activity, whereas mE1a had little or no effect (Fig. 1B). The lack of effect by mE1a, which is 35 amino acids smaller than E1a, was not because of a lack of expression as both E1a and mE1a are expressed at similar levels in F9 EC cells (Fig. 1C). Similar to previous observations with the same antibody (47), multiple bands were detected for both E1a and mE1a. It has been suggested that the multiple species are because of post-translational modifications (58). To determine whether E1a was acting through the FGF-4 enhancer, we utilized a promoter/reporter construct (TKCat+E) in which the FGF-4 promoter in 427T+E was replaced with the thymidine kinase (TK) promoter (Fig. 2A). Again, a dose-dependent decrease in transcriptional activity was observed in the presence of E1a but not with mE1a (Fig. 2B). To further define the region in the enhancer affected by E1a, the promoter/reporter construct pCatSO3 was co-transfected with expression vectors for E1a or mE1a into F9 EC cells. pCatSO3 contains six copies of the Sox-2 (HMG) and Oct-3 (POU) binding cassette (as it appears in the FGF-4 enhancer) upstream of the SV40 promoter driving the CAT reporter gene (Fig. 3A). E1a, but not mE1a, inhibited the Sox-2/Oct-3 activation of the SV40 promoter (Fig. 3B). As a control, E1a and mE1a expression vectors were co-transfected with the TKCat and SV40 parental plasmids to verify that E1a and mE1a do not affect the basal activity of the TK and SV40 promoters (data not shown). Together these results suggest that p300 can mediate the action of the FGF-4 enhancer, as well as transcriptional activation by Sox-2/Oct-3.

p300 Stimulates the FGF-4 Promoter When Tethered to the Enhancer—Previously, we demonstrated that the C-terminal region of Sox-2 is able to stimulate a heterologous promoter/reporter construct in HeLa cells identifying the C-terminal region as the transactivation domain (TAD) (38). The Sox-2 TAD is inhibited by E1a but not mE1a, and p300 recovers the E1a inhibition (38). Similarly, E1a, but not mE1a, inhibits Oct-3 stimulation of promoter/reporter constructs in HeLa cells.

This suggested that Sox-2 and Oct-3 activate the FGF-4 promoter by interacting with p300. To test this possibility functionally, we replaced the Sox-2 and Oct-3 binding sites of the enhancer in 427T+E with a binding site for the yeast transcription factor Gal4 (427T+EnGSp; see Fig. 4A). This promoter/reporter construct was transfected into F9 EC cells with an expression vector for a fusion protein, Gal4/Sox-2206–319, containing the Gal4 DBD and the transactivation domain of Sox-2. These transfections resulted in ~30-fold stimulation of the FGF-4 promoter whereas co-transfections with expression vectors for Gal4 alone or Gal4/Sox-2 constructs containing further deletions of the C-terminal end of Sox-2 failed to significantly stimulate the FGF-4 promoter (data not shown). These results indicate that the C-terminal region of Sox-2 functions as a TAD in the context of a natural promoter.

2 L. Johnson and A. Rizzino, unpublished results.
in cells where it is expressed. More importantly, these results, together with the E1a results above and earlier observations that p300 is able to mediate the TAD of Sox-2 (38), further suggest a role for p300 as a co-activator of Sox-2/Oct-3 action from the FGF-4 enhancer.

If p300 mediates the action of Sox-2/Oct-3 from the enhancer then tethering p300 directly to the enhancer should result in a stimulation of the FGF-4 promoter, bypassing the necessity of Sox-2/Oct-3 binding. Indeed, bypass experiments have implicated CBP, a homolog of p300, in the mediation of gene expression (27, 51, 59). Additionally, by directly targeting a factor to a gene, problems inherent in overexpression studies can be avoided. To test whether p300 is able to stimulate the FGF-4 promoter from the enhancer, 427T+EnGSp was transfected into F9 EC cells with expression vectors for fusion proteins containing the DBD of Gal4 and aa 1–2414 (full-length) or aa 964–1922 of p300 (Gal4/p300 1–2414 or Gal4/p300 964–1922, respectively). Both fusion proteins stimulated the FGF-4 promoter, bypassing the necessity of Sox-2/Oct-3 binding, although expressed at levels comparable with Gal4/p300 964–1922, which in includes the bromodomain and AT domain (data not shown). Tip60 is a co-activator with AT activity (60). Thus, not all co-activators with AT activity can mediate the action of the FGF-4 enhancer. Importantly, these results indicate that p300/CBP can stimulate the FGF-4 promoter when tethered to the FGF-4 enhancer downstream of the promoter, suggesting that Sox-2/Oct-3 binding may promote the association of p300 and the enhancer. Of particular interest is the observation that Gal4/p300 vectors with the promoter/reporter construct 427T+EnHPmut, which contains mutated HMG and POU sites and no Gal4 binding site (Fig. 4A), failed to stimulate 427T+EnGSp (data not shown). Tip60 is a co-activator with AT activity (60). Thus, it is unlikely that 427T+EnGSp may promote the association of p300 and the FGF-4 enhancer. Of particular interest is the observation that the fusion protein containing an aa 964–1922 of p300, which includes the bromodomain and AT domain, was itself able to stimulate the FGF-4 promoter when tethered to the enhancer. E1a is known to inhibit p300 by inhibiting its AT activity (61). Taken together with the E1a results, these observations suggest a possible role for the AT activity of p300 in the mediation

FIG. 4. Stimulation of the FGF-4 promoter by Gal4/p300 fusion proteins. A, physical map of 427T+EnGSp and 427T+EnHPmut promoter/reporter constructs. Both constructs are driven by the wild-type FGF-4 promoter. The FGF-4 enhancer in 427T+EnGSp contains a single Gal4 binding site (GALA) in place of the Sox-2 (HMG) and Oct-3 (POU) binding sites. The enhancer in 427T+EnHPmut contains mutated HMG and POU sites. GC and GT, Sp1/Sp3 binding sites; CCAAT, CCAAT box; TATA, TATA box. B, transfection of F9 EC cells with 15 μg of 427T+EnGSp or 427T+EnHPmut alone or with 5 μg of a vector expressing Gal4/p300 1–2414 (1–2414) or Gal4/p300 964–1922 (964–1922). Results are presented as relative CAT expression over the expression of 427T+EnGSp (solid bars) or 427T+EnHPmut (open bars) alone, which were set to 1. Gal4/p300 1–2414 and Gal4/p300 964–1922 are vectors expressing fusions between the DNA binding domain of Gal4 and full-length p300 (aa 1–2414) and the bromodomain and AT domain of p300 (aa 964–1922), respectively. This experiment was repeated at least twice with similar results.
of the FGF-4 enhancer. This possibility is addressed experimentally below.

The GC Boxes and CCAAT Box of the Promoter Are Required for Optimal Stimulation by p300—Three cis-regulatory sites have been identified in the FGF-4 promoter (12, 14). Two GC boxes, which bind Sp1 and Sp3 in vitro (17), reduce expression of 427T+E by ~25% when mutated (15), and a CCAAT box, which binds NF-Y both in vitro and in vivo (13, 14, 16), results in an ~50% reduction of 427T+E activity when mutated (14).3 Both Sp1 and NF-Y have been shown to physically and functionally interact with and be acetylated by p300 (33, 62–64), suggesting that p300 may interact with these factors when bound to the FGF-4 promoter.

To test whether regulatory elements in the promoter are necessary for the stimulation by enhancer-tethered p300, mutations were introduced in the promoter within the context of 427T+E. 427T GCmut+EnGSp contains mutations in both GC boxes, which inactivate their function (15). 427T Cmut+EnGSp contains a mutation in the CCAAT box, which inactivates its function (46). 427T GC/Cmut+EnGSp contains the same mutations in both the GC boxes and the CCAAT box (Fig. 5A). When transfected into F9 EC cells alone, these constructs exhibited little activity compared with the wild-type construct 427T+E (Fig. 5B). This was expected, because the Sox-2 and Oct-3 binding sites in the enhancer were disrupted by replacement with a Gal4 binding site. When Gal4/p300 1–2414 was co-transfected with the constructs containing mutations in either the two GC boxes or the CCAAT box, a small decrease in stimulation (~25–30%) was observed when compared with the stimulation of 427T+EnGSp containing a wild-type promoter (Fig. 5B). Co-transfection of Gal4/p300 1–2414 with the construct containing all three sites mutated resulted in a 50% reduction in stimulation. These results argue that p300 requires interactions with NF-Y and the factors binding the GC boxes (Sp1/Sp3) for optimal stimulation. A complete reduction in stimulation of the mutated promoter was not observed. This suggests p300 may have another function in addition to interacting with factors that bind to the GC boxes and CCAAT box of the promoter. Interestingly, a similar decrease in activity of the promoter/reporter construct containing all three promoter sites mutated was observed when co-transfected in conjunction with the Gal4/p300 fusion protein containing the bromodomain and AT domain, Gal4/p300 964–1922 (data not shown).

p300 AT Activity Is Necessary for FGF-4 Expression—Based on the observations above and on prior observations of the necessity of AT activity in the regulation of other genes by p300 (23, 31–35, 65), we investigated the role of AT activity in the p300 stimulation of the FGF-4 promoter. Utilizing the 427T+E. EnGSp promoter/reporter construct, transfections were performed with Gal4/p300 constructs harboring several aa changes in the AT domain. These mutations have been shown to reduce the AT activity of p300 to <1% of wild-type (35). Gal4/p300 ATmut and Gal4/p300 964–1922 ATmut vectors express fusion proteins containing the Gal4 DBD and either full-length p300 or aa 964–1922 of p300 with the aa changes in the AT domain mentioned above. Compared with Gal4/p300 964–1922, which shows 50–200-fold stimulation of the FGF-4 promoter, the AT mutant fails to stimulate the FGF-4 promoter. To ensure that the failure to stimulate was not because of a lack of expression, nuclear extracts from cells transfected with the fusion proteins were analyzed for expression. A 2- to 4-fold lower expression of the AT mutant was observed (Fig. 6B). However, the differences in expression cannot account for

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3 L. Johnson and A. Rizzino, unpublished results.
the 50–200-fold difference in the ability of these proteins to stimulate the FGF-4 promoter. Similarly, Gal4/p300 1–2414 ATmut failed to stimulate 427T+EnGSp (data not shown).

To further test the necessity of the AT activity of p300 in the mediation of the FGF-4 enhancer, F9 EC cells were co-transfected with the promoter/reporter construct 427T+E, which does not contain a Gal4 binding site, with expression vectors for Gal4/p300 (1–2414 or 964–1922) AT mutants. Interestingly, both the Gal4/p300 1–2414 AT mutant and the Gal4/p300 964–1922 AT mutant inhibited the expression of 427T+E in a dose-dependent manner (data not shown). These results suggest that the AT mutants are acting as dominant-negatives to directly inhibit the FGF-4 promoter. Together, these results and those described in Fig. 6 argue that the AT activity of p300 is required for stimulation of the FGF-4 promoter.

p300 and Sox-2 Are Both Associated with the Endogenous FGF-4 Enhancer—The above observations are indicative of a mechanism whereby Sox-2/Oct-3 binding to the enhancer acts to recruit p300, possibly through interaction with the TAD of Sox-2 (38). p300 could then interact with and acetylate transcription factors and/or histones associated with the FGF-4 promoter. If this is correct then p300 would be expected to be associated with the FGF-4 enhancer and possibly the FGF-4 promoter. To address this possibility, ChIP analyses were performed using antibodies that recognize p300. Specifically, F9 EC cells were treated with formaldehyde to cross-link DNA-protein complexes, nuclear extract was prepared, and DNA was sheared. Samples were split into two aliquots. One aliquot was immunoprecipitated with an antibody that recognizes the N terminus of p300, and the other aliquot was immunoprecipitated with a nonspecific antibody to serve as a control. Primers were used to specifically amplify regions of the enhancer, the promoter, or exon 2 of the FGF-4 gene (Fig. 7A). In each case, the same DNA preparations of immunoprecipitated chromatin fragments were used in the PCR reactions. We observed a 9-fold enrichment of the endogenous FGF-4 enhancer in DNA-protein complexes immunoprecipitated with antibodies that recognize the N terminus of p300, and the other aliquot was immunoprecipitated with a nonspecific antibody and serve as a control. Primers were used to specifically amplify regions of the enhancer, the promoter, or exon 2 of the FGF-4 gene (Fig. 7A). In each case, the same DNA preparations of immunoprecipitated chromatin fragments were used in the PCR reactions. We observed a 9-fold enrichment of the endogenous FGF-4 enhancer in DNA-protein complexes immunoprecipitated with antibodies that recognize the N terminus of p300 (Fig. 7B). In contrast, the promoter and exon 2 region of the FGF-4 gene show approximately a 2- to 4-fold and less than a 2-fold enrichment, respectively, in DNA-protein complexes immunoprecipitated with antibodies that recognize p300, compared with DNA-protein complexes immunoprecipitated with a nonspecific antibody (Fig. 7B). In the case of exon 2, PCR with exon 2 primers was allowed to continue until amplification was observed. This illustrates that no significant enrichment of exon 2 fragments occurred with p300 antibodies. Moreover, the experiment with the antibody that recognizes the N terminus of p300 was performed twice, and similar results were obtained. To confirm these findings, this experiment was repeated with a second p300 antibody that recognizes its C terminus. In this experiment, an ~7-fold enrichment of the endogenous FGF-4 enhancer and no enrichment of exon 2 were observed (data not shown). The enrichment of the enhancer region but lack of enrichment of the exon 2 region with p300 antibodies demonstrates that p300 is associated specifically with the enhancer region of the FGF-4 gene. However, the association of p300 with the FGF-4 promoter appears to be weak. Results with the 427T+EnHPmut, which show that Gal4/p300 can stimulate the promoter when the enhancer is nonfunctional (Fig. 4B), argue that p300 may be interacting with the enhancer, as well as the enhancer. Hence, if p300 associates with the FGF-4 promoter, it appears to do so transiently. Alternatively, the weak association of p300 with the promoter may be a result of poor cross-linking because of an indirect interaction between p300 and the FGF-4 promoter.

If the interaction between the enhancer and promoter is transitory, then Sox-2 would also be expected to associate with the enhancer and transiently with the promoter. To test this possibility, we performed ChIP assays with an antibody that recognizes Sox-2. A Sox-2 antibody sensitive enough for ChIP is not available commercially. Therefore, a vector expressing FLAG-tagged Sox-2 (FSox-2) was transfected into F9 EC cells, and the M2 (anti-FLAG) antibody was utilized. A significant enrichment of the endogenous FGF-4 enhancer was observed with the M2 antibody compared with a nonspecific antibody (Fig. 7C). No enrichment of a 5′ region (3.6 kb upstream of the FGF-4 promoter) was observed, indicating that the enrichment of the enhancer is specific. Again, similar results were obtained when this experiment was repeated. Thus, these results formally demonstrate that Sox-2 binds to the endogenous FGF-4 enhancer. As with p300, only a slight enrichment (~1.6-fold) of the promoter region was observed (Fig. 7C). The weak association of FSox-2 with the promoter is consistent with either a transitory enhancer-promoter interaction or is indicative of a mechanism by which p300 recruitment to the enhancer merely initiates a series of events that do not involve a direct interaction between the enhancer and promoter.
p300 and its homolog CBP interact with a wide array of proteins, implicating them in the regulation of many genes (24). Previous studies in this laboratory (38) demonstrated that p300 can mediate Sox-2/Oct-3 synergistic activation, specifically through the TAD of Sox-2, as well as through Oct-3. Based on the studies reported here, p300 appears to play a major role in mediating the enhancer activation of the FGF-4 promoter. We demonstrate that E1a, but not a mutant form of E1a that is unable to bind p300, blocks the function of the FGF-4 enhancer. This inhibition appears to be mediated primarily through the Sox-2/Oct-3 binding sites of the enhancer. We also demonstrate that p300/CBP, when tethered to the enhancer, can stimulate the FGF-4 promoter from a distance. Previously, p300/CBP was shown to stimulate the \( \beta \)-globin promoter when tethered to an upstream LCR (27) and a synthetic promoter from a downstream distal position (36). In this study, we demonstrate that p300 can stimulate a natural promoter from a downstream distal position. Importantly, we provide evidence that acetylation plays a significant role in the transcriptional regulation of the FGF-4 gene, as is the case with other genes (31, 37). In this regard, p300 lacking AT activity fails to stimulate the FGF-4 promoter when tethered to the FGF-4 enhancer but acts as a dominant-negative to decrease transcription from the FGF-4 promoter. Together, these results argue strongly that p300 and its intrinsic AT activity are important in mediating enhancer-regulated transcription of the FGF-4 gene.

Previous studies suggested that Sox-2 binds to the FGF-4 enhancer (20, 21). However, the studies described in this report provide formal proof for Sox-2 association with the endogenous FGF-4 enhancer. Importantly, our studies also provide direct evidence of p300 association with a downstream, intragenic distal enhancer in the endogenous FGF-4 gene. This directly implicates p300 in the mediation of enhancer regulation of the FGF-4 gene. Association of p300 and Sox-2, albeit a weak association especially of Sox-2, with the FGF-4 promoter was also observed. Interestingly, although some Gal4/CBP fusion proteins were able to stimulate the FGF-4 promoter when tethered to the enhancer, CBP was not observed to be associated with either the FGF-4 enhancer or promoter in ChIP assays with two different CBP antibodies (data not shown). However, it is possible that the epitopes of CBP recognized by the antibodies used were masked.

Mechanistically, p300 could mediate the effects of the enhancer by a noncontact or indirect mechanism that would involve p300 recruitment to the enhancer and the initiation of a series of events leading to activation of the promoter. Indirect mechanisms of enhancer action have been proposed in two different models, a DNA tracking model (9) and a linking model (8). The DNA tracking model proposes movement of co-factors recruited to the enhancer along the chromatin. Because Gal4/p300 can stimulate the promoter when tethered to the enhancer, we believe that p300 is unlikely to be mediating the action of the enhancer by a tracking mechanism. Because of the intragenic location of the enhancer, we believe a linking mechanism is also unlikely. The linking model (8) proposes that facilitator proteins (66) bind to the intervening sequences beginning at the enhancer and then spread toward the promoter to propagate a signal. Binding of proteins to the intervening (transcribed) sequences between the enhancer and promoter of the FGF-4 gene could impede transcription. Alternatively, p300 may be mediating the effects of the enhancer by a stable direct enhancer-promoter interaction that is undetectable using the ChIP assay or through a direct interaction that is transitory. Our functional and physical data are consistent with a contact model involving a sustained, direct interaction between the enhancer and promoter, as proposed in the facilitated tracking (7) and looping (5, 6) models.

In the facilitated tracking model, enhancer-bound factors
and associated co-activators track along the DNA in small steps, resulting in the formation of intermediate loops until the promoter is reached, and a stable loop is formed. One of the earliest models proposed to explain enhancer function, and the one for which the most evidence exists, is a looping model (5, 6, 67–72). In this model, chromatin between the enhancer and promoter forms a loop, allowing the enhancer-bound activators to contact factors bound at the promoter and recruit the PIC. Based on our ChIP analyses, if a direct enhancer-promoter interaction occurs, p300 is either not closely associated with the promoter or mediates a transitory interaction.

Based on the data presented here demonstrating p300 is strongly associated with the endogenous FGF-4 enhancer and weakly with the FGF-4 promoter and that enhancer-tethered p300 can stimulate the promoter from a distance, we currently favor a model involving a direct but transitory interaction between the FGF-4 enhancer and promoter. Evidence for transitory interactions, referred to as a “hit and run” mechanism, has been demonstrated in several cases (73–75). For example, p300 associates transiently at a single time point, and CBP associates in a cyclic nature with the CATD promoter following estrogen stimulation (73). Factors bound to the distal enhancer of the glnAp2 gene interact with the holoenzyme bound to the promoter, transcription is initiated, and the enhancer-promoter interaction is then destabilized (74). In addition, the glucocorticoid receptor was observed to continuously exchange between binding to regulatory sites and the unbound state (75). In the bound state, the glucocorticoid receptor is thought to recruit secondary factors such as chromatin remodeling factors, before dissociating from the chromatin. Based on the results presented here, we propose the following model for p300-mediated distal enhancer regulation of the FGF-4 gene (Fig. 8). Cooperative binding of Sox-2 and Oct-3 results in p300 associating with the enhancer. p300 interacts transiently with NF-Y and/or the factors binding to the GC boxes of the promoter bridging the enhancer and promoter. In this regard, p300 has been shown to interact with Sp1 (63) and NF-Y (33). p300 then acetylates proteins in the FGF-4 promoter in preparation for remodeling of chromatin and subsequent recruitment of other complexes such as chromatin remodeling complexes (76), mediator (77, 78), and the PIC. Recruitment of these secondary complexes would result in a dissociation of p300 and the FGF-4 enhancer from the promoter. An association of p300 with the enhancer would persist in anticipation of the next round of transcription.

Recent studies on the PSA gene have shed some light on mechanisms of distal enhancer transcriptional control. The PSA gene promoter is regulated by an enhancer located 4 kb upstream. Shang et al. (37) observed RNA PolII, p300/CBP, and histone acetylation associated with both the PSA promoter and enhancer in vivo but not with intervening sequences. These results are strongly indicative of a direct interaction between the enhancer and promoter through a stable looping mechanism involving p300/CBP. Similarly, we observe a strong association of p300 with the distal enhancer of the FGF-4 gene, supporting a role for distal enhancer mediation by p300/CBP. In contrast, only a weak association of p300 with the promoter is observed. Therefore, if direct interaction occurs, FGF-4 enhancer-promoter interaction is transitory, and a stable loop does not form. In this regard, the location of the PSA and FGF-4 distal enhancers are different, extragenic and intragenic, respectively. Hence, our results suggest different mechanisms may be used to mediate extragenic enhancers, such as that of the PSA gene, and intragenic enhancers located in introns and exons, such as that of the FGF-4 gene. Intragenic enhancers may employ a hit and run mechanism to avoid interference with transcription of the gene that could result from the formation of a stable interaction between the enhancer and promoter.

In conclusion, our findings provide both functional and physical evidence that p300 can mediate the effects of the FGF-4 enhancer. Given the recent findings described in two other studies, one involving an LCR and one involving an upstream distal enhancer (28, 37), our work adds further evidence that p300 and CBP can mediate the effects of regulatory sequences located at considerable distances from the promoters that they control. However, our studies differ from a previous report (37) dealing with the PSA gene. For this gene, recent studies suggest a mechanism involving stable enhancer-promoter interactions for extragenic distal enhancers. In contrast, our findings are suggestive of a mechanism involving transient enhancer-promoter interactions for intragenic distal enhancers. Hence, we suggest that different mechanisms are likely to mediate the effects of different classes of distal regulatory sequences. However, p300/CBP may be a common co-activator involved in mediating their influence on promoter activation.

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The Co-activator p300 Associates Physically with and Can Mediate the Action of the Distal Enhancer of the \textit{FGF-4} Gene

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