The Acetyltransferase Activity of CBP Is Required for *wingless* Activation and H4 Acetylation in *Drosophila melanogaster*

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CBP is a critical coactivator of transcription, but little is understood about the importance of its intrinsic acetyltransferase (AT) activity in gene activation in vivo. We show that the intrinsic AT function of CBP in *Drosophila melanogaster* (dCBP) is necessary to maintain a dCBP overexpression phenotype in the eye, for the in vivo activation of a specific target gene, *wingless*, and for the global acetylation of histone H4. These findings indicate that a point mutation which alters the intrinsic AT activity of CBP (only one of many CBP functions) has profound effects on CBP-induced gene activation in a physiologically intact transcription system. Furthermore, the effects of CBP AT activity are not limited to a few specific promoters, but rather CBP AT activity may play a role in regulating global histone acetylation throughout the developing organism.

The coactivator CREB-binding protein (CBP) (9, 21) interacts with a multitude of signal-responsive transcription factors (12, 29, 34) supporting its putative role as an integrator of converging gene-regulatory pathways. The acetylation of histones and some transcription factors has been linked to the control of gene activation (for reviews, see references 31 and 32), and CBP has a potent intrinsic acetyltransferase (AT) activity (24). These results suggest a model where CBP is recruited to the promoter and aids the remodeling of the chromatin through acetylation of the core histones, thus allowing an increase in the accessibility of the chromatin to additional transcription factors. CBP may then modulate the activity of the transcription complex by acetylating the proteins recruited into the complex. However, little is known about the relative contribution of the intrinsic enzymatic AT activity of CBP to its overall function as a multifunctional coactivator of transcription.

Previous studies of the AT function in CBP have been limited to examinations of the expression of target genes at specific promoters in reconstituted chromatin assays in vitro or in cell culture. For example, prior studies of reconstituted chromatin that looked at the regulation of the estrogen receptor gene (14) or at the VP16 activator domain (15) found that the intrinsic AT activity of CBP/p300 appears to be important for transcriptional activation. Similarly, the interaction of CBP/p300 with components of a higher-order nucleoprotein complex is necessary for virally induced hyperacetylation of histones H3 and H4 and for activation of the beta interferon gene (25). By contrast, the enhancement of NF- κ B activity requires CBP to recruit an associated protein, p/CAF, to the promoter, and it is the intrinsic AT activity of p/CAF, not that of CBP, that stimulates NF- κ B-dependent transcription (27). Recently,

using selective hypoxanthine-aminopterin-thymidine (HAT) inhibitors for p300 and p/CAF (Lys-coenzyme A [CoA] and H3-CoA-20, respectively), Lau et al. (17) demonstrated that the HAT activities of p300 and p/CAF were additive in several in vitro systems and showed that specific inhibition of p300 mediated transcription on naked DNA and on chromatin templates. They furthermore demonstrated that Lys-CoA selectively blocked p300 transcription of the *myoD* promoter in a frog oocyte transcription system. However, one of several fundamental questions remaining is whether the intrinsic AT activity of CBP plays an important role in developing organisms with physiologically intact transcription systems. If so, then is the role of the CBP AT activity limited to specific promoters of target genes or does it affect the global acetylation of chromatin throughout the developing organism? Addressing these questions is the main thrust of this paper.

To determine whether the intrinsic AT activity of CBP contributes to endogenous gene expression in development, we introduced point mutations in the AT domain of *Drosophila melanogaster* CBP (dCBP). Homologous sites in a similar region of murine CBP (mCBP) have been shown to reduce AT activity in vitro (22). Two of these dCBP mutations (Y2160A and F2161A) reduce or eliminate acetyl (Ac)-CoA binding and AT enzymatic activity. By introducing dCBP genes containing these mutations into flies under the control of GAL4 and the upstream activation sequence (UAS), we show that the AT domain of dCBP is necessary for the smooth-eye phenotype produced by dCBP overexpression, *wingless* (*wg*) gene expression, and the acetylation of lysine 8 of histone H4. These findings indicate that a single point mutation that eliminates intrinsic dCBP AT activity produces profound and varied effects ranging from the loss of dCBP-induced activation of a specific target gene (*wg*) to the global loss of dCBP-induced histone H4 acetylation throughout the developing organism. Thus, the AT activity of dCBP, which represents only one of the proposed functions inherent to dCBP, appears to exert its

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effect on a large proportion of cellular chromatin rather than only at specific promoters.

MATERIALS AND METHODS

Plasmid vectors and protein purification. Full-length dCBP cDNA (1) was subcloned into the pMT-V5/His(C) vector (Invitrogen) in frame with the 3' V5 epitope tag sequence (the V5 epitope originated from paramyxovirus SV5). The dCBP-V5 gene was then subcloned into the pcDNA3 vector (Invitrogen) for expression in COS7 cells. The Q2146A, L2152A, L2158A, Y2160A, and F2161A mutations were introduced into the dCBP sequence with a 300-bp cassette containing the AT domain.

To purify a dCBP protein fragment containing the AT domain, a segment of the dCBP gene (coding amino acids [aa] 1853 to 2339) was subcloned into pET-28a (Novagen) in frame with the 3' six-His tag sequence. We also introduced a dCBP tag sequence at the 5' end of the dCBP segment that resulted in increased protein stability. The Y2160A and F2161A mutations were again introduced into the sequence through a 300-bp cassette. The protein fragments were expressed in bacteria and purified by nickel-nitrilotriacetic acid affinity chromatography (QIAGEN).

HAT enzymatic analysis. HAT assays were performed as previously described (13). In brief, either full-length dCBP-V5 protein (the wild type [wt] or a protein with the F2161A or Y2160A mutation) immunoprecipitated from COS7 cells or 1 μ g of a dCBP protein fragment (aa 1853 to 2339; with the wt or the F2161A or Y2160A sequence) was incubated for 1 h at 30°C in the presence of 0.5 μ l of [³H]Ac-CoA (5.1 Ci/mmol), 1 μ g of fly core histones, and buffer containing final concentrations of 50 mM Tris (pH 7.4), 0.2 mM EDTA, 8% polyethylene glycol 8000, 50 mM NaCl, and 1 mM dithiothreitol in a 30- μ l reaction volume. The reaction mixture was applied to cellulose phosphate paper and washed, and the incorporated ³H was assessed by liquid scintillation spectroscopy or subjected to polyacrylamide gel electrophoresis and exposed to autoradiographic film.

Tissue culture, transfection, IP, and Western analysis. The tissue culture techniques employed have been described previously (7). Calcium phosphate transfections were carried out per the instructions of the manufacturer (Gibco BRL). Immunoprecipitation (IP) and Western analysis were performed as previously described (6, 7). V5 antibody was purchased from Invitrogen and used at a 1:5,000 dilution for both IP and Western analysis.

Ac-CoA binding. [³H]Ac-CoA binding assays were performed by incubating a 0.5 μ M dCBP fragment (aa 1853 to 2339; with the wt, F2161A, or Y2160A sequence) in the presence of various concentrations of free [³H]Ac-CoA (8 to 512 μ M) in 600 μ l of the above-mentioned buffer for 60 min at 4°C. The dCBP protein fragment (55 kDa) was then pelleted by centrifugation (28) at 100,000 rpm for 4 h with a Beckman TL-100 ultracentrifuge, washed quickly with cold binding buffer, and dissolved in glacial acetic acid. Control experiments demonstrated that insignificant amounts of [³H]Ac-CoA are pelleted in 4 h. Bound and free [³H]Ac-CoAs were assessed in a liquid scintillation counter. The reaction mixtures also contained a trace amount of [³⁵S]sulfate for assessment of the amount of free fluid trapped in the pellet. Nonspecific counts were determined by simultaneously performing parallel reactions in the presence of excess cold Ac-CoA.

Fly strains, germ line transformation, and whole-mount embryo in situ hybridization and immunostaining. Full-length dCBP-V5 (wt, F2161A, and Y2160A) constructs were cloned into the pUAST vector (4), and transgenic fly lines were generated as described previously (26, 30). At least three independent transformants were generated for each dCBP-V5 (wt, F2161A, or Y2160A) construct. G. Rubin (University of California, Berkeley) kindly provided the GMR-driven Gal4 line. This construct expresses GAL4 under the control of multimerized *glass*-binding sites, and its gene is referred to as *GMR* in this paper (33). For overexpression of dCBP in the eye, *dCBP-V5* (wt, F2161A, and Y2160A) females were crossed to *GMR/CyO* males and the eye phenotype of the non-*CyO* progeny was assessed. Mating the *GMR/CyO* flies to the *yw* injection stock generated *GMR/+* controls.

The *paired (prd)-GAL4* line (38) was kindly provided by D. Kalderon, Columbia University. The dCBP mutation used is null on the basis of RNA and protein expression (1) and has been designated *nej³*. For generation of dCBP-null embryos that express the different dCBP constructs in the *prd* domain, *nej³/Fm7c ftz-LacZ*; *dCBP-V5* (wt, F2161A, and Y2160A) females were crossed to *Fm7c ftz-LacZ*; *prd-GAL4/TM3* males and the progeny was stained for β -galactosidase, the V5 epitope, and wg transcripts. To visualize the expression of *even-skipped* (*eve*) and the levels of acetylation of histone H4 in the *nej³* and heterozygous control embryos, *nej³/Fm7c ftz-LacZ* females were mated to *Fm7c ftz-LacZ* males and the progeny was stained for both β -galactosidase and acetylated histone H4 or *eve* transcripts. The *Fm7c ftz-LacZ* chromosome was obtained from the

Bloomington Stock Center, and the *TM3* and *CyO* chromosomes have been described by Lindsley and Zimm (20).

The dual immunohistochemistry and in situ hybridization of whole-mount embryos were performed as previously described (6). The anti-acetylated H4 (K5, K8, and K12) antibodies were purchased from Upstate Biotechnology Company and were used at a dilution of 1:2,000. Chicken anti-dCBP antibody (3) was used at a 1:800 dilution. Anti- β -galactosidase antibody was used at a dilution of 1:1,000. Larval brains with eye antennal disks attached were dissected from third-instar larvae and subjected to immunostaining with the anti-V5 antibody (Invitrogen) at a 1:5,000 dilution. After being stained, eye antennal disks were further dissected away from larval brains and mounted for light microscopy. Nomarski optics and digital capture were used for the light microscope pictures, and a confocal laser scan microscope (Bio-Rad 1024ES laser and Nikon Eclipse TE300 microscope) was used for confocal microscopy.

Histology and SEM. Histological sections were prepared as described previously (18). In brief, the heads were halved, fixed in 75 mM cacodylate (pH 7.4)–2% paraformaldehyde–2% glutaraldehyde for 4 h at room temperature, and subsequently stained in 1% tannic acid–75 mM cacodylate (pH 7.4) at 4°C for 16 h. After they were washed (in 0.1 M cacodylate), the heads were postfixed (2% OsO₄–0.1 M cacodylate) for 2 h, dehydrated, mounted, and sectioned. To prepare them for scanning electron microscopy (SEM), the flies were treated with ether fumes, coated with SEM mount adhesive, and sputter coated with 20-nm-diameter gold particles. An Amray SEM was used for visualization and imaging.

RESULTS

Generation of Ac-CoA binding mutations in dCBP. To develop a system with which to study the role of the intrinsic AT activity of dCBP in vivo, we generated point mutations in dCBP which were targeted to a region of putative Ac-CoA contact points (Fig. 1a). These mutations were designed to reduce or eliminate AT activity without affecting the overall structure of dCBP. AT activity is known to exist within highly homologous regions of p300 (aa 1195 to 1810) (13) and mCBP (aa 1099 to 1877) (22), and point mutations in this region have been shown to reduce AT activity in vitro (22). Some of these mCBP mutations align in a best-fit model with the known Ac-CoA contact points of *Saccharomyces cerevisiae* (yeast) HAT1 (yHAT1), as determined from its crystal structure (10, 23) (Fig. 1a). Based on these observations, we generated single alanine substitutions in five sites in dCBP (Q2146A, L2152A, L2158A, Y2160A, and F2161A).

To test the effects of the five point mutations on AT activity, full-length dCBP that was tagged with a C-terminal V5 epitope was transiently transfected into COS7 cells, immunoprecipitated with a V5 antibody, and subjected to AT assays with fly core histones as the substrate (13). As shown in Fig. 1b, dCBP (F2161A) lost all measurable AT activity and the AT activity of dCBP (Y2160A) was reduced by 44% compared to that of the wt. Western blot analysis of the immunoprecipitates confirmed the presence of equal amounts of each full-length dCBP-V5 protein subjected to AT activity analysis.

To confirm that the reductions in the AT activities in the F2161A and Y2160A mutant proteins were due to the loss of intrinsic AT activity rather than to a disruption of dCBP interactions with other AT-containing factors, we expressed 55-kDa fragments (aa 1853 to 2339) of dCBP (with the wt, F2161A, and Y2160A sequences) in bacteria and subjected the purified proteins to AT activity analysis. As shown in Fig. 1c, the AT activities of the dCBP protein fragments containing the F2161A and Y2160A mutations were reduced by 94 and 32%, respectively, compared to that of the wt. All specific activity in this experiment was due to the incorporation of [³H]Ac into histones and not to autoacetylation of the dCBP fragment.

FIG. 1. Point mutations in the AT domain of dCBP specifically reduce AT activity and Ac-CoA binding. (a) Alignment of AT domain motifs A and B of yHAT1, p/CAF, mCBP, and dCBP. The black circles on top indicate the Ac-CoA contact points of yHAT1 (10). The gray circles at the bottom indicate mutated sites in dCBP. (b) HAT assay of various full-length dCBP-V5 constructs after IP from COS7 cells. The mutant and wt dCBP proteins were tagged at the C terminus with the V5 epitope, and IP experiments were performed with V5 antibodies. (c) HAT assay of purified 55-kDa (aa 1232 to 1711) wt, Y2160A, and F2161A dCBP protein fragments. (d) Saturation isotherm of [³H]Ac-CoA binding to wt dCBP protein (prot) fragment. Lines represent total binding (solid), binding in the presence of excess unlabeled Ac-CoA (dotted), and binding to heat-denatured dCBP (dashed). (e) Ac-CoA binding affinities for wt, Y2160A, and F2161A dCBP protein fragments. NSB, no specific binding.

This conclusion was evidenced by the lack of acetylation in control HAT reactions in which the histone substrate was omitted and was further confirmed by visualization of ³H-labeled HAT assay products on a polyacrylamide gel (only histones and not the 55-kDa dCBP fragment were acetylated [data not shown]). These findings confirm that the reduction in AT activity of the F2161A and Y2160A mutant proteins was due to the loss of intrinsic AT activity in dCBP.

The 55-kDa dCBP protein fragments (with the wt, F2161A, and Y2160A sequences) were tested for [³H]Ac-CoA binding by using a high-speed sedimentation binding assay. In the presence of increasing concentrations of [³H]Ac-CoA, saturable binding to wt dCBP was obtained (Fig. 1d). To determine specific binding to the dCBP fragments, the assay was performed in the presence of either excess unlabeled Ac-CoA or

heat-denatured dCBP; only low levels of nonspecific binding were observed under these conditions. As shown in Fig. 1e, the F2161A mutant protein lacks the ability to specifically bind Ac-CoA, suggesting that the mutation disrupts an Ac-CoA contact point. The Y2160A mutant dCBP only modestly reduced Ac-CoA affinity ($K_d = 140 \mu\text{M}$ compared to $50 \mu\text{M}$ for wt dCBP), suggesting that its loss in AT activity was due, at least in part, to a reduction in Ac-CoA binding. To ensure that the mutations did not affect the overall protein structure, we determined whether the mutant dCBP proteins could bind the adenoviral transforming factor E1A, which binds the region abutting the AT domain of CBP (3, 16) and is acetylated by CBP (39). Full-length (wt, F2161A, and Y2160A) dCBP-V5 proteins were expressed in COS7 cells and subjected to glutathione *S*-transferase-E1A pull-down analysis. Equal levels of

binding of the wt and mutant proteins to E1A were detected (data not shown). A similar series of experiments confirmed that the changes in the AT activities of the various (wt, F2161A, and Y2160A) dCBP constructs were not due to a disruption of dCBP with glutathione *S*-transferase-p/CAF, an AT-containing factor which interacts with CBP (37) (data not shown).

Characterization of dCBP Ac-CoA-binding mutants in vivo: dCBP AT activity is required for a dCBP overexpression phenotype. To test the function of the dCBP mutants in vivo, we cloned full-length wt, F2161A, and Y2160A dCBP-V5 into the pUAST vector (4) and generated transgenic fly lines as previously described (26, 30). This construct places dCBP under GAL4-UAS control. We overexpressed the dCBP constructs in the eye antennal disks of flies containing the GMR enhancer-GAL4 construct. Scanning electron micrographs of the resultant fly eyes are shown in Fig. 2. *GMR/+* control eyes have a virtually normal phenotype of regularly arrayed ommatidia and mechanosensory bristles. The eyes from *GMR/+; UAS-dCBP-V5/+* flies, in which wt dCBP is overexpressed, have a smooth-eye phenotype, are morphologically atrophic, and completely lack ommatidia and mechanosensory bristles. In contrast, eyes from *GMR/+; UAS-dCBP(F2161A)-V5/+* flies, in which the F2161A mutant dCBP is overexpressed, exhibit irregularly arrayed ommatidia and mechanosensory bristles. *GMR/+; UAS-dCBP(Y2160A)-V5/+* eyes, in which the Y2160A mutant dCBP is overexpressed, have a phenotype intermediate between the wt and F2161A mutant dCBP overexpression phenotypes.

Histological samples of these four fly lines were prepared to determine the cellular structures of the eyes overexpressing the wt and mutant dCBP proteins (Fig. 3a). Again, the eyes from *GMR/+* adults are normal, with ommatidial arrays containing ordered photoreceptors. In the eyes from *GMR/+; UAS-dCBP-V5/+* adults, which overexpress wt dCBP, this architecture is completely lost and the photoreceptors are no longer detected. By contrast, the eyes from *GMR/+; UAS-dCBP(F2161A)-V5/+* adults, which overexpress the F2161A mutant dCBP, have intact ommatidial arrays, though sometimes an ommatidium may contain an abnormal number of photoreceptors. Again, overexpression of the Y2160A mutant causes an intermediate phenotype. Immunostaining (V5 antibody) and Western analysis (V5 antibody) of the expression levels of each dCBP-V5 construct (with the wt, F2161A, or Y2160A sequence) in the eye antennal disks of third-instar larvae (Fig. 3b and c, respectively) were performed to confirm equal levels of dCBP-V5 overexpression for each transformant. The overexpression of the three proteins does not cause any defect in the development of the eye antennal disk (Fig. 3b). When we stained the disks with the neuron-specific antibody 24B10 (11), we found that the ommatidia and their projections onto the brain were wt (data not shown). Thus, the overexpression of wt dCBP in the eye causes neural degeneration sometime during differentiation. Together, these findings demonstrate that the intrinsic AT domain of dCBP is necessary for the dCBP overexpression activity that leads to neural degeneration.

dCBP is expressed throughout embryogenesis in wt embryos but not in embryos homozygous for the *nej*³ (mutant dCBP) allele. After demonstrating that dCBP AT activity is required for the dCBP overexpression phenotype in the adult eye of

FIG. 2. The AT activity of dCBP is necessary to maintain the smooth-eye phenotype resulting from dCBP overexpression in adult fly eyes. Scanning electron micrographs of adult *Drosophila* eyes are shown. Low and high magnifications are shown in the left and right panels, respectively. (i) *GMR enhancer-GAL4/+* eye in which dCBP is not overexpressed; (ii) *GMR/+; UAS-dCBP(wt)-V5/+* eye; (iii) *GMR/+; UAS-dCBP(F2161A)-V5/+* eye; (iv) *GMR/+; UAS-dCBP(Y2160A)-V5/+* eye.

Drosophila, we chose to study the role of dCBP AT activity in the expression of endogenous developmentally regulated genes in dCBP-null embryos. For this system, we chose embryos homozygous for the *nej*³ allele. This allele is a dCBP molecular null, and *nej*³ embryos have no zygotic dCBP expression. Thus, in these embryos the only endogenous source of dCBP is maternal, and dCBP is significantly depleted by stage 9 of embry-

FIG. 3. The AT activity of dCBP is necessary to maintain dCBP overexpression. (a) Histology of adult *Drosophila* eyes. (i) *GMR/+* eye; (ii) *GMR/+*; *UAS-dCBP(wt)-V5/+* eye; (iii) *GMR/+*; *UAS-dCBP(F2161A)-V5/+* eye; (iv) *GMR/+*; *UAS-dCBP(Y2160A)-V5/+* eye. (b) Anti-V5 antibody immunostaining of eye disks from third-instar larvae overexpressing different full-length (wt, F2161A, and Y2160A) dCBP-V5 constructs under the control of the *GMR-GAL4* construct. The stripe of staining in each panel represents a wt, Y2160A, or F2161A dCBP-V5 construct expressed in the *glass* expression pattern under the control of the *GMR* enhancer-*GAL4* construct in the developing eye. (c) Western blot analysis (V5 antibody) of eye disks from third-instar larvae (each lane contains protein from 20 eye disks).

ogenesis. The *nej³* embryos that die at this stage often have a characteristic twisted phenotype. To confirm the absence of dCBP protein in *nej³* homozygotic embryos (from either zygotic or maternal contribution), we used a chicken polyclonal antibody to dCBP (3) and measured the expression of dCBP protein at various stages of embryogenesis in wt and *nej³* embryos. As shown in Fig. 4A and C to E, wt embryos at various stages of embryogenesis express dCBP. This immunostaining is specific to dCBP, since preincubation of the dCBP antibody with antigen reduces staining to background levels (Fig. 4B). In contrast to wt embryos, those homozygous for the *nej³* allele show no specific dCBP binding by stage 11 (Fig. 4F) and are usually not dCBP positive at stage 9.

dCBP AT activity is required for activation of *wg* expression. The transcription factor cubitus interruptus requires dCBP to activate *wg* in response to *hedgehog* signaling (8), and embryos

homozygous for *nej³* are no longer able to maintain *wg* gene expression by stages 9 to 11 (1). To examine the importance of dCBP AT activity for the expression of an endogenous developmentally regulated promoter, we examined the levels of *wg* gene expression in *nej³/Y; prd-GAL4/+* embryos (38) in which the three dCBP-V5 constructs were overexpressed in the alternate embryonic segments that express the pair-rule gene *prd*. As shown in Fig. 5, immunostaining with V5 antibody alone confirmed equal levels of dCBP-V5 overexpression for all constructs (wt, F2161A, and Y2160A). In segments expressing dCBP-V5, the *wg* gene is expressed in a distribution and intensity nearly identical to those seen in the wt embryo (8). By contrast, segments expressing F2161A dCBP-V5 do not stimulate *wg* expression. Segments that express Y2160A dCBP-V5 stimulate *wg* expression at a level that is intermediate between those of the wt and F2161A dCBP-V5 constructs. These results indicate that in vivo, dCBP AT activity is necessary for the induction of *wg* expression.

dCBP AT activity is not required for the activation of *eve* expression. To determine whether dCBP AT activity at the *wg* gene has a direct, rather than a secondary, effect on alterations in global acetylation, we compared the levels of *eve* expression in wt and dCBP-null embryos. *eve* does not require dCBP for gene activation (S. M. Smolik, unpublished observation). As shown in Fig. 6a, *eve* mRNA is expressed in a normal alternating segment pattern in wt embryos. Similarly, as shown in Fig. 6b, *eve* is also expressed in a wt pattern and intensity in dCBP-null embryos.

Acetylation levels of histone H4 lysine 5, 8, and 12 are differentially regulated during embryogenesis. In order to study the role of the intrinsic AT domain of dCBP on histone acetylation, we first measured various anti-acetylated histone H4 lysine-specific antibodies in wt and dCBP-null embryos (Fig. 7). Anti-acetylated H4 (K8) staining in wt and dCBP-null embryos revealed that acetylation of H4 (K8) is ubiquitous on chromatin and requires the presence of dCBP for normal expression levels (Fig. 7a and b and 8a). The levels of H4 (K8)

FIG. 4. Chicken anti-dCBP antibody stains *Drosophila* embryos throughout embryogenesis. Shown are wt embryos at stages 4 to 5 (a), stage 6 (c), late stage 8 (d), and stage 12 (e) of embryogenesis stained with chicken anti-dCBP antibody; a wt stage 4 embryo stained with the anti-dCBP antibody that had been preincubated with antigen (b); and a dCBP-null (*nej³*) embryo (with the twisted phenotype) at about stage 11 stained with anti-dCBP antibody (f).

FIG. 5. The AT activity of dCBP is required for dCBP-induced *wg* gene expression in dCBP-null *Drosophila* embryos. *nej³/Y; UAS-dCBP(wt, F2161A, and Y2160A)-V5/+; prd-GAL4/+* embryos (stage 9) were immunostained with V5 antibody alone (left panels) or double stained with both V5 antibody and *wg* antisense RNA probe (right panels). In each panel, dCBP -V5 staining is apparent in alternate segments that express *prd*, but the V5 antibody staining in the right panels was intentionally left faint so as not to obscure the levels of *wg* staining in the segments overexpressing the exogenous dCBP proteins. The right panels show magnifications of embryos that show only three segments each. Segments of dCBP-null embryos overexpressing wt dCBP in the *prd* domains (arrow, top right panel) have significant *wg* expression, while the dCBP-null segment (between the *prd* segments) has minimal *wg* expression. Segments of dCBP-null embryos overexpressing the F2161A mutant dCBP protein (arrow, middle right panel) do not activate *wg* expression (all three segments have low levels of *wg* expressed). Segments of dCBP-null embryos overexpressing the Y2160A mutant protein (arrow, bottom right panel) have *wg* expression that is intermediate between the wt and F2161A mutant dCBP phenotypes.

acetylation were constant throughout embryogenesis (data not shown). By contrast, anti-acetylated H4 (K12) staining in wt (Fig. 7c) embryos revealed that the distribution of acetylated K12 changes differentially in the germ layers during development; the ectodermal nuclei exhibited a strong punctate staining while the mesodermal nuclei exhibited a weaker, more uniform staining. This staining also requires the presence of dCBP (Fig. 7d). Anti-acetylated H4 (K5) staining was most developmentally regulated. wt embryos at the syncytial and cellular blastoderm stages exhibited anti-acetylated H4 (K5) staining in a nuclear distribution (Fig. 7e). By the time the ventral furrow was formed, acetylated K5 of H4 was no longer detectable (Fig. 7f). Furthermore, staining with the anti-acety-

lated K5 antibody was not detected in nuclei during the remainder of embryogenesis. Because we did not have markers that would allow us to detect dCBP-null embryos at this early stage, we could not determine whether dCBP affects the early acetylation of H4 (K5). In *Drosophila*, acetylated lysine 16 of histone H4 is found only on the hyperactive X chromosome of the male and not the autosomes of either the male or female (35). Because these experiments could not distinguish control from mutant dCBP embryos, we did not assess the effect of dCBP on the acetylation of histone H4 (K16).

Mutations in the dCBP AT domain affect acetylation levels of histone H4 lysine 8. Because lysine 8 of H4 is the only one of the four H4 acetylation targets that has consistent levels and

FIG. 6. The AT activity of dCBP is not required for expression of *eve* in dCBP-null *Drosophila* embryos. The embryos were double stained with both the anti- β -galactosidase antibody and *eve* antisense RNA. (a) wt embryo (stage 9). The positive β -galactosidase staining (brown) indicates the presence of the *Fm7c* balancer chromosome that contains the β -galactosidase and wt dCBP genes. *eve* staining (blue) is present in a normal alternating segment pattern. (b) dCBP-null embryo (stage 9). The absence of β -galactosidase staining indicates that the embryo does not contain the *Fm7c* balancer chromosome and is therefore dCBP null (*nej³/Y*). In the absence of dCBP, *eve* staining (blue) is still present in a normal alternating segment pattern and at a wt level.

patterns of acetylation throughout embryogenesis (Fig. 7), we chose to monitor it in our experiments on the effect of dCBP AT mutations on histone acetylation. wt embryos labeled with anti-acetylated H4 (K8) antibodies revealed global histone H4 (K8) acetylation in the nuclei of developing embryos. By contrast, *nej³/Y* embryos from the same population as that of the wt embryos had significantly reduced levels of staining with the anti-acetylated H4 (K8) antibodies. To determine whether the intrinsic AT domain of dCBP is necessary to maintain wt levels of histone H4 (K8) acetylation, *nej³/Y; prd-GAL4/+* embryos expressing the wt, F2161A, and Y2160A dCBP-V5 constructs were double-labeled with antibodies to the V5 (red) and acetylated-H4 (K8-specific; green) epitopes (Fig. 8b). The levels of overexpression for the wt, F2161A, and Y2160A dCBP-V5 constructs were equal. In those segments overexpressing wt dCBP, H4 (K8) acetylation was higher. By contrast, segments expressing F2161A dCBP-V5 did not show augmented H4 (K8) acetylation. The segments expressing Y2160A dCBP-V5 affected H4 (K8) acetylation at levels intermediate between

those of the wt and F2161A dCBP-V5 constructs. Although we have not ruled out the possibility that the intrinsic AT domain of dCBP is required for the synthesis of H4, we find it unlikely because basal levels of histone H4 (K8) acetylation are visualized in the nuclei of the dCBP-null embryo (Fig. 8a) and dCBP-null segments (Fig. 8b). These findings suggest that the intrinsic AT activity of CBP is necessary for wt levels of histone H4 (K8) acetylation throughout the developing organism.

DISCUSSION

CBP is a multifunctional coactivator of signal-responsive gene expression. It has an intrinsic AT activity that is thought to affect the remodeling of transcriptionally active chromatin by acetylating not only the core histone proteins but associated coactivators as well (5, 14, 15, 19, 25, 27). CBP also acts as a scaffold to recruit additional transcriptional modulating pro-

FIG. 7. Anti-acetylated histone antibodies (K5, K8, and K12) have specific patterns of expression during embryogenesis in *Drosophila*. The red stripes identify β -galactosidase expression from the *FM7c* chromosome. The green staining identifies the different acetylated lysines of histone H4. Shown are wt (a) and dCBP-null (b) embryos (stage 11 and 9, respectively) stained with anti-acetylated H4 (K8) antibody; wt (c) and dCBP-null (d) embryos (stage 9) stained with anti-acetylated H4 (K12) antibody; and syncytial blastoderm (e) and early-gastrulation (f) wt embryos stained with anti-acetylated H4 (K5) antibody.

FIG. 8. The AT activity of dCBP is required for dCBP-induced histone H4 (K8) acetylation in dCBP-null *Drosophila* embryos. The embryos were immunostained with anti-V5 antibody (Texas Red secondary antibody) and acetylated-H4 (K8) antibody (fluorescein secondary antibody) and analyzed by laser confocal microscopy. All embryos shown were from the same experimental population and were visualized with the same fluorescence settings to allow for the comparison of relative expression levels. (a) wt (left) and dCBP-null (right) embryos labeled with anti-acetylated H4 (K8) antibody [α AcH4(K8)]. (b) *nej³/Y; UAS-dCBP(wt, F2161A, and Y2160A)-V5/+; prd-GAL4/+* embryos. The red stripes show staining for the expressed dCBP-V5 constructs (left), and the green stripes show staining for acetylated histone H4 (K8) (right). In each panel, a relatively small section (containing only a few segments) on the surface of the whole embryo was visualized.

teins, thus forming a bridge between transcriptional activators and the basal transcriptional machinery. How important each of these roles is to the function of CBP is unknown. Prior studies of reconstituted chromatin systems (14, 15) have suggested that the intrinsic AT domain of CBP/p300 plays a role in the activation of some genes. By contrast, Sheppard et al. (27) have shown that the recruiting activity of CBP, but not its AT activity, is required for the activation of p65-dependent transcription, suggesting that associated ATs, and not the intrinsic CBP/p300 AT activity, are necessary in this model of gene activation. Overall, it is unclear whether the AT domain of CBP is important at some promoters and not others or whether the difference observed in these studies is due to the technical constraints of the *in vitro* transcriptional assays. Furthermore, it is also not known (i) how important the intrinsic AT activity of CBP is for its transcriptional effects in physiologically intact systems and (ii) whether these effects are only gene specific or are global throughout the developing organism; addressing these two questions is the main thrust of this paper.

To address these questions, we generated single point mutations in the AT domain of dCBP that reduce or abolish the ability of the protein to bind the Ac-CoA substrate and determined the functions of the mutant proteins *in vitro* and *in vivo*. An earlier study described a series of mutations in mCBP that resulted in a loss of AT activity (22). Generous sequence alignments between mCBP and the cytoplasmic γ HAT1 suggested that these mutations might represent lesions near Ac-CoA contact points. However, none of the experiments demonstrated whether these mutations affected Ac-CoA binding. We generated the homologous mutations in dCBP and demonstrated that one of the mutant proteins (with the F2161A mutation) cannot bind the Ac-CoA substrate while another (with the Y2160A mutation) has approximately half the affinity for Ac-CoA that the wt protein has. It is likely that these mutations affect only Ac-CoA binding and not the local conformation of the proteins, since they are capable of binding a CBP acetylation target, the adenoviral E1A protein (39), at wt levels. We cannot rule out the possibility that an unidentified protein also interacts through these residues and affects the mutant phenotypes; however, we believe that this is unlikely.

The unregulated overexpression of dCBP in the developing fly is a lethal event (1) that makes ubiquitous overexpression and rescue studies problematic. However, overexpression of dCBP in the eye under the control of the GMR-GAL4 construct leads to a viable fly with a smooth-eye phenotype due to the degeneration of both ommatidia and mechanosensory bristles sometime during pupariation. In the present study, we have demonstrated that the intrinsic AT activity of dCBP is necessary to maintain the atrophic-eye phenotype produced by the overexpression of dCBP in the adult fly eye. By utilizing this system, we were able to produce the first evidence that the AT domain of dCBP is necessary in a developmentally intact system. Furthermore, these studies suggest that the phenotype observed in flies overexpressing dCBP is functionally significant and not due to squelching effects that might arise from the overproduction of dCBP. If this phenotype were the result of transcriptional complex disruption or interference in pathways that would normally not involve dCBP function, then we would anticipate that the overexpression of all of these proteins

would lead to the same phenotype. In fact, the phenotypes produced by the mutant dCBPs correlate well with their Ac-CoA binding affinities and AT activities.

Because the full characterization of this overexpression phenotype would be complicated by the presence of endogenous dCBP in the genetic background, we looked at the role of the AT domain of dCBP in the expression of *wg*, a gene known to require an interaction between dCBP and the transcription factor cubitus interruptus for its late expression (8). We have shown that the AT activity of dCBP is necessary for *wg* expression, suggesting a promoter-specific role for the AT domain of dCBP. Although many mammalian genes are known to require mCBP for activity (for a review, see reference 12) and while *twist* expression and a *Mad* reporter gene are sensitive to mutations in dCBP (2, 36), only the *wg* promoter has been clearly shown to require dCBP for its regulatory control. Therefore, the determination of the spectrum of *Drosophila* genes activated and repressed by dCBP, and the determination of which of these genes require the AT activity of dCBP for regulation, will require a microarray analysis of dCBP target genes affected by the AT mutations.

Since CBP functions as a coactivator of transcription, most current models of CBP-mediated gene regulation generally describe CBP acting at specific promoters or altering chromatin structure locally. For this reason, the global effect of dCBP, and specifically the importance of its AT activity, on the histone H4 (K8 and K12) acetylation observed in this study was surprising and raises the possibility that the effects of the dCBP mutant on *wg* expression are a secondary effect of chromatin misregulation. We do not believe that this is the case, because a dCBP mutation does not affect *eve* expression or early *wg* expression (1). This early *eve* and *wg* expression is probably not due to rescue by the dCBP maternal component, because *twist*, which is expressed at the same time as *eve*, is markedly reduced in dCBP mutant embryos that have some maternal contribution (2). Furthermore, while zygotic dCBP mutant embryos often develop a twisted or segment polarity phenotype, they never have gap or pair-rule phenotypes, suggesting that dCBP does not affect the function or expression of gap or pair-rule genes. However, it may be that the early zygotic promoters are not sensitive to the dosage of dCBP or are less sensitive to the acetylation of histone H4 (K8) than are the late *wg* or other promoters.

It may be that dCBP AT activity indirectly affects chromatin acetylation by regulating the transcription or functions of proteins involved in elaborating higher-order chromatin structure. It is also possible that dCBP plays a complex role both as a promoter-specific transcriptional coactivator and as a general chromatin-regulatory factor. Further studies of the dCBP regulation of histone acetylation will discriminate between these possibilities.

In summary, these findings demonstrate that the intrinsic AT domain of dCBP is necessary for CBP-induced activation of an endogenous developmentally regulated gene in the developing *Drosophila* embryo and that associated AT proteins are not sufficient to maintain this effect. Furthermore, prior studies examining the role of CBP at individual promoters (5, 14, 15, 19, 25, 27) have suggested that CBP functions in part by acetylating promoter histones and/or associated transcriptional cofactors. Remarkably, this study raises the possibility that

dCBP is critical for the acetylation of histone H4 in the entire chromatin of the developing *Drosophila* embryo. Certainly, continued analysis of dCBP AT activity at specific promoters and in the remodeling of higher-order chromatin structure will aid our understanding of dCBP function as a coactivator of signal-responsive transcription.

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REFERENCES

1. Akimaru, H., Y. Chen, P. Dai, D. X. Hou, M. Nonaka, S. M. Smolik, S. Armstrong, R. H. Goodman, and S. Ishii. 1997. *Drosophila* CBP is a co-activator of cubitus interruptus in hedgehog signalling. *Nature* **386**:735–738.
2. Akimaru, H., D. X. Hou, and S. Ishii. 1997. *Drosophila* CBP is required for dorsal-dependent twist gene expression. *Nat. Genet.* **17**:211–214.
3. Bantignies, F., R. H. Goodman, and S. M. Smolik. 2000. Functional interaction between the coactivator *Drosophila* CREB-binding protein and ASH1, a member of the trithorax group of chromatin modifiers. *Mol. Cell. Biol.* **20**:9317–9330.
4. Brand, A. H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**:401–415.
5. Chen, H., R. J. Lin, W. Xie, D. Wilpitz, and R. M. Evans. 1999. Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* **98**:675–686.
6. Chen, Y., J. R. Cardinaux, R. H. Goodman, and S. M. Smolik. 1999. Mutants of cubitus interruptus that are independent of PKA regulation are independent of hedgehog signaling. *Development* **126**:3607–3616.
7. Chen, Y., N. Gallaher, R. H. Goodman, and S. M. Smolik. 1998. Protein kinase A directly regulates the activity and proteolysis of cubitus interruptus. *Proc. Natl. Acad. Sci. USA* **95**:2349–2354.
8. Chen, Y., R. H. Goodman, and S. M. Smolik. 2000. Cubitus interruptus requires *Drosophila* CREB-binding protein to activate *wingless* expression in the *Drosophila* embryo. *Mol. Cell. Biol.* **20**:1616–1625.
9. Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy, and R. H. Goodman. 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**:855–859.
10. Dutnall, R. N., S. T. Tafrov, R. Sternglanz, and V. Ramakrishnan. 1998. Structure of the histone acetyltransferase Hat1: a paradigm for the GCN5-related N-acetyltransferase superfamily. *Cell* **94**:427–438.
11. Fujita, S. C., S. L. Zipursky, S. Benzer, A. Ferrus, and S. L. Shotwell. 1982. Monoclonal antibodies against the *Drosophila* nervous system. *Proc. Natl. Acad. Sci. USA* **79**:7929–7933.
12. Goodman, R. H., and S. M. Smolik. 2000. CBP/p300 in cell growth, transformation, and development. *Genes Dev.* **14**:1553–1577.
13. Gu, W., and R. G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90**:595–606.
14. Kraus, W. L., E. T. Manning, and J. T. Kadonaga. 1999. Biochemical analysis of distinct activation functions in p300 that enhance transcription initiation with chromatin templates. *Mol. Cell. Biol.* **19**:8123–8135.
15. Kundu, T. K., V. B. Palhan, Z. Wang, W. An, P. A. Cole, and R. G. Roeder. 2000. Activator-dependent transcription from chromatin in vitro involving targeted histone acetylation by p300. *Mol. Cell* **6**:551–561.
16. Kurokawa, R., D. Kalafus, M. H. Ogliastro, C. Kioussi, L. Xu, J. Torchia, M. G. Rosenfeld, and C. K. Glass. 1998. Differential use of CREB binding protein-coactivator complexes. *Science* **279**:700–703.
17. Lau, O. D., T. K. Kundu, R. E. Soccio, S. Ait-Si-Ali, E. M. Khalil, A. Vassilev, A. P. Wolffe, Y. Nakatani, R. G. Roeder, and P. A. Cole. 2000. HATs off: selective synthetic inhibitors of the histone acetyltransferases p300 and PCAF. *Mol. Cell* **5**:589–595.
18. Lee, Y. J., S. Shah, E. Suzuki, T. Zars, P. M. O'Day, and D. R. Hyde. 1994. The *Drosophila* dgq gene encodes a G alpha protein that mediates photo-transduction. *Neuron* **13**:1143–1157.
19. Li, J., B. W. O'Malley, and J. Wong. 2000. p300 requires its histone acetyltransferase activity and SRC-1 interaction domain to facilitate thyroid hormone receptor activation in chromatin. *Mol. Cell. Biol.* **20**:2031–2042.
20. Lindsley, D. L., and G. G. Zimm. 1992. The genome of *Drosophila melanogaster*. Academic Press, San Diego, Calif.
21. Lundblad, J. R., R. P. Kwok, M. E. Lurance, M. L. Harter, and R. H. Goodman. 1995. Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. *Nature* **374**:85–88.
22. Martinez-Balbas, M. A., A. J. Bannister, K. Martin, P. Haus-Seuffert, M. Meisterernst, and T. Kouzarides. 1998. The acetyltransferase activity of CBP stimulates transcription. *EMBO J.* **17**:2886–2893.
23. Neuwald, A. F., and D. Landsman. 1997. GCN5-related histone N-acetyltransferases belong to a diverse superfamily that includes the yeast SPT10 protein. *Trends Biochem. Sci.* **22**:154–155.
24. Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**:953–959.
25. Parekh, B. S., and T. Maniatis. 1999. Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-beta promoter. *Mol. Cell* **3**:125–129.
26. Rose, R. E., N. M. Gallaher, D. J. Andrew, R. H. Goodman, and S. M. Smolik. 1997. The CRE-binding protein dCREB-A is required for *Drosophila* embryonic development. *Genetics* **146**:595–606.
27. Sheppard, K. A., D. W. Rose, Z. K. Haque, R. Kurokawa, E. McInerney, S. Westin, D. Thanos, M. G. Rosenfeld, C. K. Glass, and T. Collins. 1999. Transcriptional activation by NF- κ B requires multiple coactivators. *Mol. Cell. Biol.* **19**:6367–6378.
28. Sherrill, J. M., and J. Kyte. 1996. Activation of epidermal growth factor receptor by epidermal growth factor. *Biochemistry* **35**:5705–5718.
29. Shikama, N., J. Lyon, and N. B. La Thangue. 1997. The p300/CBP family: integrating signals with transcription factors and chromatin. *Trends Cell Biol.* **7**:230–236.
30. Spradling, A. 1986. P element-mediated transformation, p. 175–198. In D. B. Roberts (ed.), *Drosophila: a practical approach*. IRL Press, New York, N.Y.
31. Sterner, D. E., and S. L. Berger. 2000. Acetylation of histones and transcription-related factors. *Microbiol. Mol. Biol. Rev.* **64**:435–459.
32. Strahl, B. D., and C. D. Allis. 2000. The language of covalent histone modifications. *Nature* **403**:41–45.
33. Thomas, B. J., and D. A. Wassarman. 1999. A fly's eye view of biology. *Trends Genet.* **15**:184–190.
34. Torchia, J., C. Glass, and M. G. Rosenfeld. 1998. Co-activators and co-repressors in the integration of transcriptional responses. *Curr. Opin. Cell Biol.* **10**:373–383.
35. Turner, B. M., A. J. Birley, and J. Lavender. 1992. Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. *Cell* **69**:375–384.
36. Waltzer, L., and M. Bienz. 1999. A function of CBP as a transcriptional co-activator during Dpp signalling. *EMBO J.* **18**:1630–1641.
37. Yang, X. J., V. V. Ogryzko, J. Nishikawa, B. H. Howard, and Y. Nakatani. 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**:319–324.
38. Yoffe, K. B., A. S. Manoukian, E. L. Wilder, A. H. Brand, and N. Perrimon. 1995. Evidence for engrailed-independent wingless autoregulation in *Drosophila*. *Dev. Biol.* **170**:636–650.
39. Zhang, Q., H. Yao, N. Vo, and R. H. Goodman. 2000. Acetylation of adenovirus E1A regulates binding of the transcriptional corepressor CtBP. *Proc. Natl. Acad. Sci. USA* **97**:14323–14328.