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Trithorax and dCBP Acting in a Complex to Maintain Expression of a Homeotic Gene

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Trithorax (Trx) is a member of the trithorax group (trxG) of epigenetic regulators, which is required to maintain active states of Hox gene expression during development. We have purified from *Drosophila* embryos a trithorax acetylation complex (TAC1) that contains Trx, dCBP, and Sbf1. Like CBP, TAC1 acetylates core histones in nucleosomes, suggesting that this activity may be important for epigenetic maintenance of gene activity. dCBP and Sbf1 associate with specific sites on salivary gland polytene chromosomes, colocalizing with many Trx binding sites. One of these is the site of the Hox gene *Ultrabithorax* (*Ubx*). Mutations in either *trx* or the gene encoding dCBP reduce expression of the endogenous *Ubx* gene as well as of transgenes driven by the *bxd* regulatory region of *Ubx*. Thus Trx, dCBP, and Sbf1 are closely linked, physically and functionally, in the maintenance of Hox gene expression.

In *Drosophila*, trxG genes are required to sustain appropriate levels of Hox gene expression during embryogenesis. Several proteins of the trxG are constituents of SWI/SNF-type chromatin remodeling complexes (*1, 2*), which suggests that some of these proteins exert their regulatory effects through alterations in chromatin structure. The mode of action of other trxG proteins, including Trx, is not known.

To investigate the function of trxG proteins, we purified a Trx protein complex from *Drosophila* embryos. To monitor the purification, we used the Trx antibody N1, which has been characterized previously (*3, 4*). Nuclear extract was fractionated using several chromatographic steps (*5*) (Fig. 1A). Three prominent bands are seen in the purified material, which contains most of the Trx found in the starting extract (Fig. 1B). We refer to this complex as trithorax acetylation complex 1 (TAC1). We estimate the size of TAC1 to be 1 MD, as determined from size fractionation of the highly purified 0.25 M Q Sepharose material on a 20 to 45% glycerol gradient (Fig. 1D). All three TAC1 components co-migrated on this gradient, as assayed by silver staining (*6*). No lower molecular weight proteins were detected in the purified material. The higher molecular weight band in TAC1 was recognized by N1 (Fig. 1C). In addition to Trx, two similar sized bands of about 300 kD were detected (Fig. 1B). The purified material was resolved by SDS–polyacrylamide gel electrophoresis (PAGE), and the protein bands were excised, digested with trypsin, and analyzed by mass spectrometry (*5*). This analysis confirmed that the upper band is Trx. Results from other bands showed that the upper band in the doublet is the *Drosophila* acetyltransferase dCBP and the lower band is Sbf1. Sbf1 is a mammalian anti-phosphatase that was previously found to interact with the SET (Suvar3-9, Enhancer-of-zeste, Trithorax) domain of ALL-1/HRX/MLL (*7*), the human homolog of Trx, which is known to be involved in acute leukemia.

To confirm these results, we raised specific antibodies to dCBP (C2) and Sbf1 (S1) (*8*), which detected single protein bands of the expected sizes in the purified material (Fig. 1C). Using these antibodies, we found that a portion

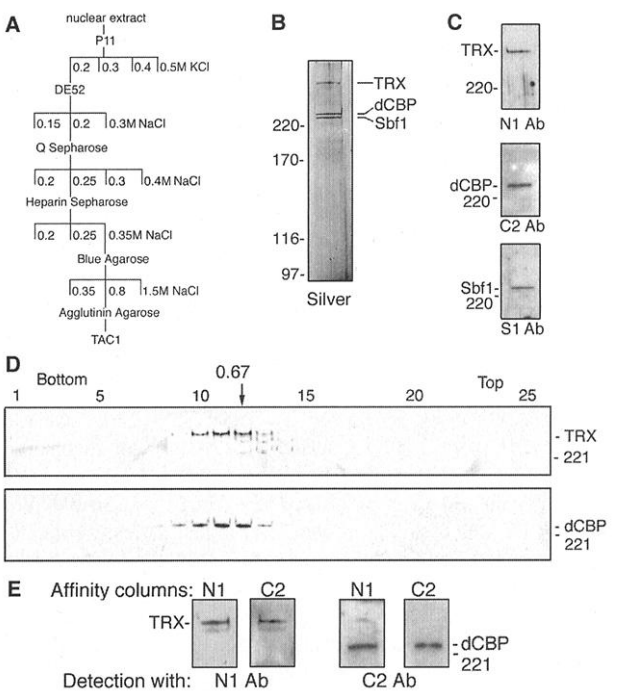

Fig. 1. Trx and dCBP are components of the TAC1 complex. **(A)** The TAC1 purification scheme. **(B)** Silver staining of material from an agglutinin–Agarose column. **(C)** Western blots of Trx, dCBP, and Sbf1 after fractionation on an agglutinin–Agarose column. **(D)** The 0.25 M NaCl Q Sepharose fraction was size-fractionated by glycerol gradient. Trx and dCBP proteins were visualized by Western blotting of the same filter. The position of the 670 kD protein marker is indicated. **(E)** The material from peak fractions of the glycerol gradient was applied independently to columns with attached antibodies to Trx (N1) and dCBP (C2). Eluted material was separated by 6% SDS-PAGE and used for Western blotting.

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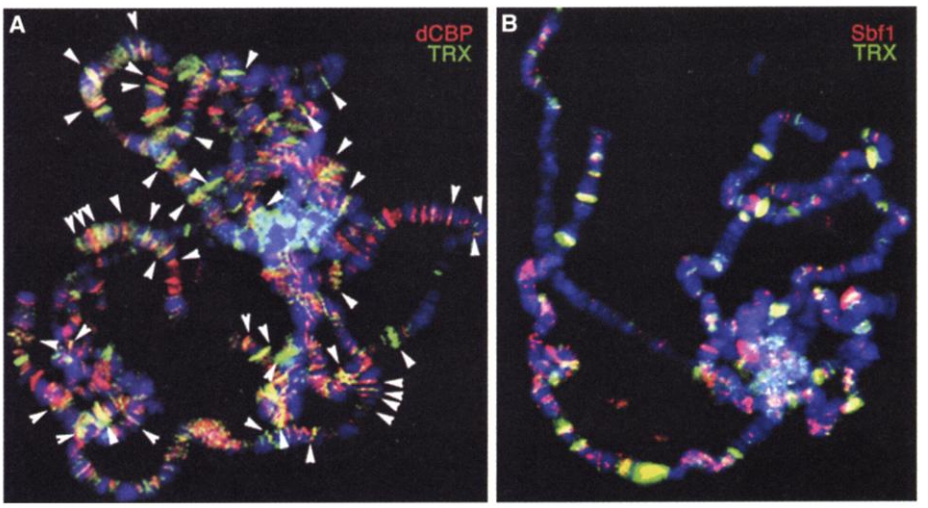

Fig. 2. Trx, dCBP, and Sbf1 colocalize on salivary gland polytene chromosomes (*22*). Merging of the red signal representing dCBP (**A**) or Sbf1 (**B**), with the green signal representing Trx, identifies sites where these proteins colocalize [the relative signal of Trx versus dCBP or Sbf1 varies to some extent among the sites; arrowheads in (A) indicate common sites for dCBP and Trx].

of dCBP and almost all Sbf1 cofractionated with Trx at all chromatographic steps, including size fractionation (Fig. 1D) (*6*). We estimate that roughly 90% of the total nuclear Trx and Sbf1 and roughly 5% of dCBP are found in TAC1. To further confirm the composition of TAC1, we applied highly enriched material after Q Sepharose to immunoaffinity columns containing either N1 or C2 (*8*). Both Trx and dCBP were specifically retained by each of these columns (Fig. 1E). The banding patterns of the C2 and N1 affinity-purified material were identical to those of the purified material shown in Figure 1B. Because the same set of proteins, including Sbf1, was obtained from both the C2 and N1 columns (Fig. 1E), we conclude that these proteins are components of a stable TAC1 complex.

Because Trx is a chromatin-associated protein (*3*), one might expect dCBP and Sbf1 to colocalize with Trx on salivary gland polytene chromosomes. As Fig. 2 shows, Sbf1 associates with most Trx sites, and dCBP associates with many of them. These results strongly corroborate the finding that dCBP and Sbf1 are components of TAC1.

Mammalian CBP exhibits histone acetyltransferase activity (HAT) (*9, 10*), and we investigated whether or not the TAC1 complex is capable of acetylating histones. The 0.25 M Q Sepharose fraction, which contains highly enriched TAC1, contains HAT activity with respect to the four core histones (Fig. 3A). This activity is due to TAC1, because its level depends on the amount of added protein, and because this activity is reduced by depletion with either N1 or C2 (Fig. 3, A and B). TAC1 can also specifically acetylate the NH_2-terminal tail of histone H4 (Fig. 3C). Thus, the HAT activity of TAC1 is similar in specificity to that of mammalian CBP. To further assess the TAC1 HAT activity, we isolated mono- and oligonucleosomes (Fig. 3D). TAC1 also acetylates these substrates (Fig. 3E), which are physiologically more relevant than free histones.

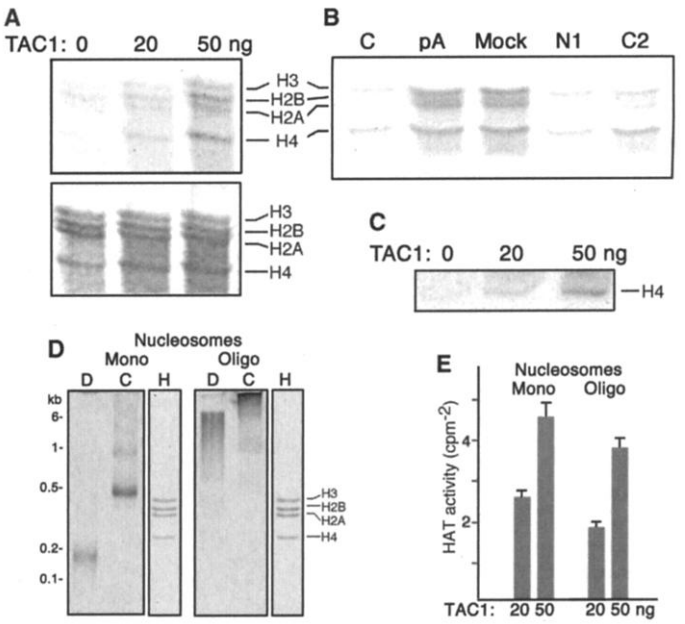

Fig. 3. Acetylation of core histones by TAC1. HAT activity was assayed as described (*10*). The indicated amounts (estimated from silver-stained gels) of the highly TAC1-enriched 0.25 M Q Sepharose fraction were added to the acetylation mix, containing either the four core histones (**A** and **B**) [In (A), Coomassie staining of a histone loading control is shown at the bottom], the NH_2-terminal peptide of histone H4 (**C**), or mono- and oligonucleosomes (**E**). The resulting material was either resolved by 18% SDS-PAGE (A to C), or analyzed by a filter binding assay [(E), the average and standard deviation from three independent experiments]. (B) HAT activity of the 0.25 M Q Sepharose fraction depleted with either N1 or C2 adsorbed to protein A before acetylation. C, histones only; pA, protein A with no antibodies; Mock, unrelated antibody. (**D**) Analysis of isolated nucleosomes (*23*) from chicken erythrocytes by native 4% PAGE. Ethidiumbromide–stained gels are shown as negative images. C, H1-stripped mono- and oligonucleosomes; D, isolated nucleosomal DNA; H, analysis of protein content of nucleosome core particles by 18% SDS-PAGE stained with Coomassie Blue.

Trx maintains expression of *Ubx*, and several *trx*-dependent trithorax response elements (TREs) have been mapped in the *bxd* regulatory region of *Ubx* (*11*). To address the functional significance of the Trx and dCBP association in TAC1, we asked whether dCBP is also required for regulation of *Ubx*. Figure 4B shows that embryos homozygous either for nej^{Q7} [nej^{Q7} and nej^{S103} are *dCBP* alleles that interact genetically with *Ubx* (*12*)] or for the null allele trx^{B11} show a similar decrease in the expression of *Ubx*. The earliest appearance of this defect in nej^{Q7} mutant embryos is about 5 hours after egg laying. This indicates that dCBP, like Trx (*13*), is not required for proper initiation, but rather for maintenance, of *Ubx* expression. Similar defects were also seen in both nej^{Q7} and trx^{B11} mutant embryos in the expression of the N2 lacZ reporter transgene carrying the *bxd* regulatory region (Fig. 4B). N2 contains several previously characterized TREs (*11*) (Fig. 4A). The expression pattern of N2 in nej^{Q7} mutant embryos (Fig. 4B) is similar to that seen in wild-type embryos carrying a transgene (C1) in which a characterized 124–base pair central TRE was deleted (*11*). Consistent with this, no changes in the expression of C1 in nej^{Q7} mutant embryos were observed (*6*), suggesting that *dCBP* may exert its function through this TRE-containing region of *Ubx*.

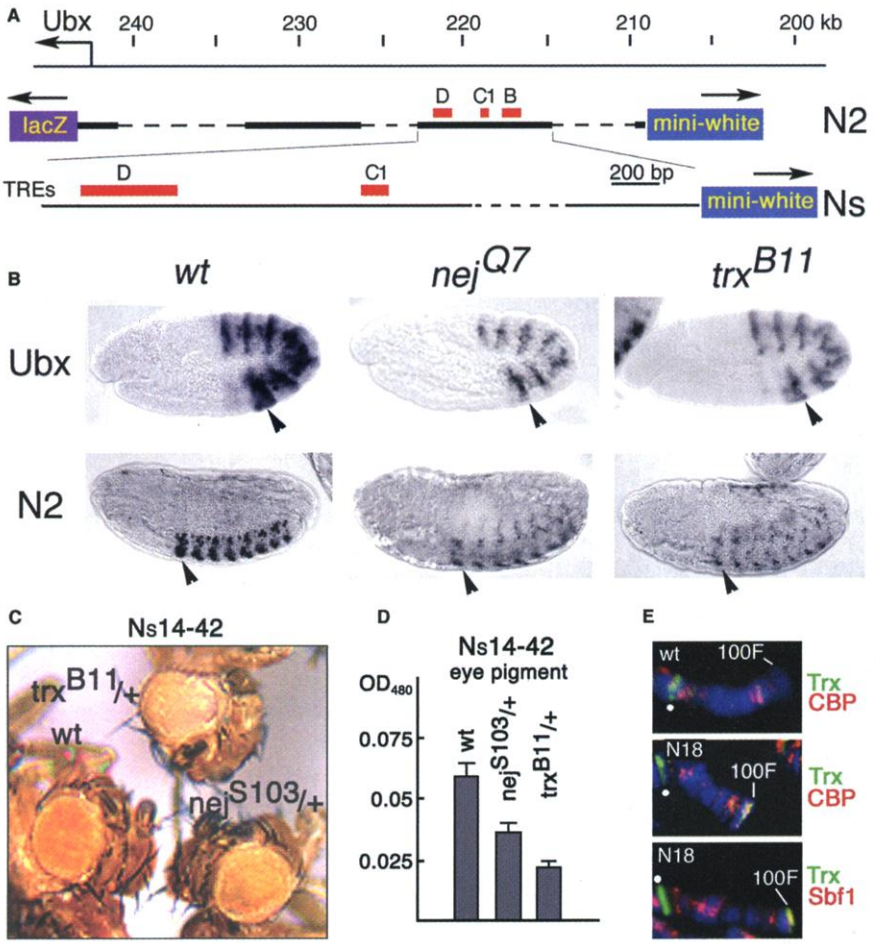

Fig. 4. Trx and dCBP coregulate *Ubx* expression through the TRE-containing *bxd* regulatory region. (**A**) Top: Partial map of the BX-C upstream of the *Ubx* promoter. Middle: Map of the N2 transgene used to assess dCBP and Trx requirements in embryos. Bottom: Map of the Ns transgene used to detect dCBP-dependent activity in transgenic flies and to show colocalization of TRX, dCBP, and Sbf1 on polytene chromosomes. Previously identified TRE modules ("D, C1, B") are indicated (*11*). (**B**) Effects of homozygous nej^{Q7} or trx^{B11} mutations (*24*) on expression of endogenous *Ubx* RNA (top panel, embryonic stage 10) and on lacZ reporter gene RNA from the N2 transgene (lower panel, embryonic stage 16). Arrows indicate parasegment 6. (**C**) Effects of the nej^{S103} and trx^{B11} mutations in heterozygotes on expression of the *mini-white* gene in the Ns14-42 construct. Eye color due to expression of the *mini-white* transgene in the Ns14-42 heterozygous line (wt) is decreased in nej^{S103} and trx^{B11} heterozygotes. (**D**) Quantification of eye pigment from Ns(14-42) flies (in three independent experiments red eye pigment was extracted from the eyes of 20 individuals and quantified by absorbance at 480 nm). Similar quantification results were obtained with four independent Ns lines. (**E**) Colocalization of Trx, dCBP, and Sbf1 on salivary gland polytene chromosomes at the site of insertion of the Ns(18) transgene. The site of insertion at 100F was determined previously by in situ hybridization (*11*). Top: wild-type 3R chromosome; no Trx, CBP, and Sbf1 (*6*) signals were observed at 100F. Trx and dCBP antibody staining (middle) and Trx and Sbf1 antibody staining (bottom), each showing binding at 100F. White dots indicate the same endogenous site.

To further investigate the functional significance of the Trx and dCBP association in TAC1, we examined 20 transgenic lines containing a smaller TRE-containing construct, Ns (Fig. 4A). Heterozygous mutations in genes required for the activity of *bxd* were previously shown to cause a lighter eye color in these transgenic lines (*11*). Sixteen of 20 such lines exhibit a decrease in eye color in nej^{S103} mutants, confirming that the activity of this TRE-containing region depends on the dosage of dCBP. An example of this effect is shown in Figure 4, C and D. These results are similar to those obtained previously with the same transgenic lines and with the null trx^{B11} allele (Fig. 4C) (*11*). These results imply that *trx* and *dCBP* exert their activity in a similar way, through previously identified TREs.

Trx was shown previously to bind in vivo to the site of insertion of the Ns transgene on polytene chromosomes (*11*). In such a transgenic line (N18), we observed new signals for all three TAC1 protein components at the site of insertion of the transgene (Fig. 4E). These results strongly suggest that Trx, dCBP, and Sbf1 directly associate in vivo on a *bxd* regulatory element.

In summary, we have purified a novel HAT trxG complex, TAC1, and we have shown that components of this complex act together in vivo through the same TRE in regulating *Ubx* expression. Because TAC1 exhibits intrinsic HAT activity, its mechanism of action may include acetylation of nucleosomes, an activity associated with transcriptional activation. Histones are not the only substrates of the CBP protein, and it can acetylate a number of other factors (*14–17*). The possibility of acetylation of proteins in addition to histones is intriguing because it might suggest other epigenetic mechanisms of maintaining gene activation by TAC1.

We have shown previously that Trx interacts directly with SNR1, a component of a *Drosophila* SWI/SNF chromatin remodeling complex (*18*), and ASH1 (*4*), a component of a distinct trxG complex (*2*). Although an ASH1 complex has not yet been purified, recent data suggest that ASH1 can directly interact with dCBP (*19*). Because neither SNR1 nor ASH1 are found in TAC1, their interactions with Trx may reflect interactions among components of different trxG complexes that cooperate in changing chromatin structure, possibly bringing together different mechanisms of nucleosome modification. PcG proteins act antagonistically to trxG proteins, repressing Hox expression in specific cells during development. Both the ESC/E(Z) *Drosophila* PcG complex and the homologous mammalian EED/EZH complex contain histone deacetylases (*20, 21*). Thus, the HAT activity of TAC1 suggests that trxG and PcG complexes may directly antagonize each other's effects on chromatin structure.

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5. Nuclear extract from wild-type 0 to 18 hour Oregon R embryos was prepared (*11*), dialyzed against chromatographic buffer C [20 mM Hepes (pH 7.6), 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride]; 700 mg of protein was applied to a 250-ml P11 Cellulose (Whatman) column, and bound proteins were eluted stepwise. The 0.2 M KCl fraction was applied to a 25-ml DE-52 Cellulose (Whatman) column. The 0.2 M DE-52 NaCl fraction was applied to a 5-ml Q Sepharose (Pharmacia) column. The 0.25 M NaCl fraction was applied to a 4-ml heparin Sepharose (Pharmacia) column, and the Trx-containing 0.35 M NaCl fraction was applied to a Cibarcon Blue 3GA Agarose (Sigma) column. The 0.8 M NaCl Blue Agarose fraction was passed through a wheat germ agglutinin–Agarose (Vector) column. The material from the 0.25 M Q Sepharose fraction was applied to a 20 to 45% glycerol gradient and centrifuged for 72 hours at 35,000 rpm. For immunoaffinity purification, affinity-purified antibodies (500 μg) were cross-linked to protein A–Sepharose (Pharmacia) using dimethylpimelimidate. Affinity resins were incubated with glycerol gradient protein fractions O/N at 4°C, the unbound proteins were removed by washing with C buffer containing 0.75 M NaCl, and bound proteins were eluted with 0.1 M glycine (pH 2.7). Eluates were resolved by 6% SDS-PAGE and visualized by silver staining or Western blotting. For protein analysis, TAC1 complex, purified on an agglutinin–Agarose column, was resolved by SDS-PAGE and stained with Coomassie Blue. Protein bands were excised and cleaved with trypsin, and samples were analyzed by SELDI mass spectrometry

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22. *Drosophila* polytene chromosome spreads were prepared from salivary glands of third-instar larvae and processed as described (*11*). Fluorescent double-labeling of proteins was carried out as described (*11*) using rat N1 (*4*) at 1:20 dilution and affinity-purified rabbit C2 or S1, each at 1:150 dilution. Image files of labeled chromosomes were acquired with an Olympus microscope equipped with a digital camera.
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24. Selection of mutant embryos was performed using GFP balancer chromosomes. RNA in situ hybridization of whole-mount embryos using digoxigenin-labeled *Ubx* and *lacZ* probes was performed as described (*4, 11*).
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Collaboration Between CC- and A-Adding Enzymes to Build and Repair the 3′-Terminal CCA of tRNA in *Aquifex aeolicus*

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The universal 3′-terminal CCA sequence of all transfer RNAs (tRNAs) is repaired, and sometimes constructed de novo, by the CCA-adding enzyme [ATP(CTP):tRNA nucleotidyltransferase]. This RNA polymerase has no nucleic acid template, yet faithfully builds the CCA sequence one nucleotide at a time using cytidine triphosphate (CTP) and adenosine triphosphate (ATP) as substrates. All previously characterized CCA-adding enzymes from all three kingdoms are single polypeptides with CCA-adding activity. Here, we demonstrate through biochemical and genetic approaches that CCA addition in *Aquifex aeolicus* requires collaboration between two related polypeptides, one that adds CC and another that adds A.

The 3′-terminal CCA sequence (positions 74, 75, and 76) found on all mature tRNAs is required for tRNA aminoacylation (*1*) and for peptide bond formation by the ribosome (*2–4*). CCA-adding enzymes (*5*) have been identified in each of the three kingdoms (*6*) and are essential in organisms where not every tRNA gene encodes CCA (*7*). The CCA-adding enzyme is unique among nucleotidyltransferases (*6, 8, 9*) because it adds an ordered nucleotide sequence to a specific primer without using a nucleic acid template; moreover, the enzyme is sensitive to register, and can faithfully rebuild tRNAs with incomplete 3′ ends (tRNAD, tRNADC, and tRNADCC, where D is the discriminator base). Several models have been proposed to explain these properties (*10–14*), but the molecular details remain unknown. Compounding the mystery, all CCA-adding enzymes characterized to date consist of a single polypeptide with dual specificity for adenosine monophosphate and cytosine monophosphate incorporation.

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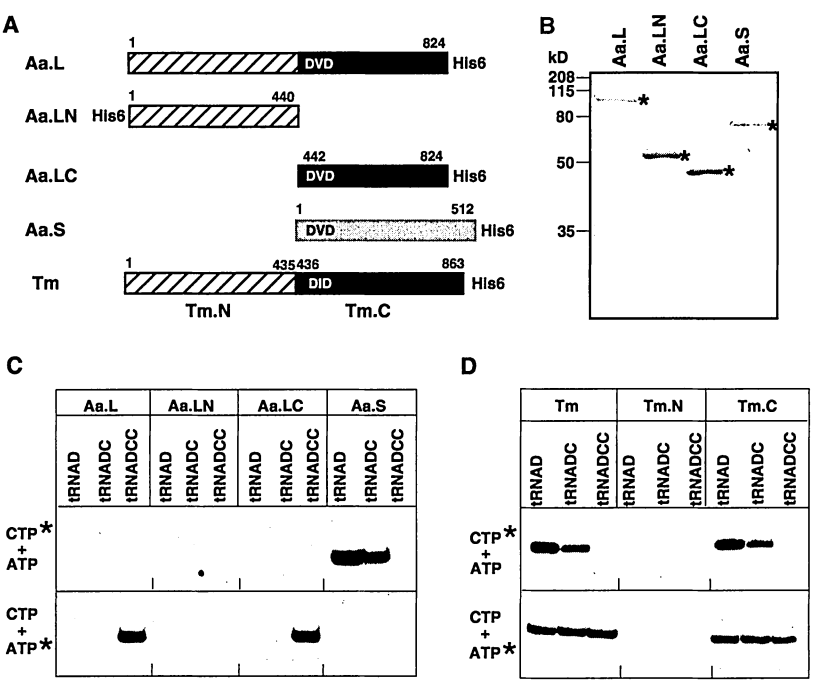

Fig. 1. C- and A-addition to tRNA are carried out by distinct, but related polypeptides in *A. aeolicus*. (**A**) *Aquifex aeolicus* nucleotidyltransferase homologs and the domains used in this study. Aa.L, the large polypeptide; Aa.LN, NH_2-terminal region of Aa.L; Aa.LC, COOH-terminal region of Aa.L; and Aa.S, the small polypeptide. The *T. maritima* nucleotidyltransferase is shown for comparison. DVD and DID indicate the active site signature found in all nucleotidyltransferase family members (*8*); His6 is the hexahistidine tag; striped boxes denote NH_2-terminal similarity, black boxes strong COOH-terminal similarity, and the gray box weaker COOH-terminal similarity. (**B**) Hexahistidine-tagged proteins were resolved by 12% SDS–polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. (**C**) Aa.L and Aa.LC have A-adding activity and Aa.S has C-adding activity. (**D**) The *T. maritima* nucleotidyltransferase is a CCA-adding enzyme and activity resides within the COOH-terminal region.