Drosophila tissue-specific transcription factor NTF-1 contains a novel isoleucine-rich activation motif

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The *Drosophila* tissue-specific transcription factor NTF-1 provides a useful model system for studying the mechanisms by which promoter-selective factors control the development of a multicellular organism. A number of promoters that may be targets of NTF-1 regulation have been identified. For example, NTF-1 plays a critical role in the tissue-specific expression of the *Drosophila Dopa decarboxylase* gene. Additionally, by using in vitro assays, it has been possible to characterize the mechanism of NTF-1 activation, revealing its dependence on specific coactivators, or TAFs. Here, we report the use of both in vivo and in vitro assays to identify the functional domains of NTF-1. These consist of an unusually large, unique DNA-binding and dimerization domain, as well as a novel, isoleucine-rich activation domain. This 56-amino-acid activation region fails to interact with the putative Sp1 coactivator, dTAF_{II}110, and thus appears to use a mechanism distinct from the glutamine-rich activation domain of Sp1. Additionally, NTF-1 appears to activate transcription in a species-specific manner, utilizing distinct domains in *Drosophila* and yeast.

[Key Words: Tissue-specific transcription factor; NTF-1; *Drosophila*; activation domain]

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Cellular diversity is generated in large part by unique combinations of transcription factors interacting to specify distinct patterns of gene expression in different cell types. Both biochemical and genetic studies have led to the identification of developmentally regulated transcription factors that act in a combinatorial fashion to govern *Drosophila* embryogenesis. One such developmentally programmed transcription factor is NTF-1 (neurogenic element binding transcription factor), first identified by virtue of its ability to bind to a *cis*-acting element critical for neuronal expression of the *Drosophila Dopa decarboxylase* (*Ddc*) gene (Bray et al. 1988). NTF-1, also known as Elf-1 or *grainyhead* (Bray and Kafatos 1991), was found to bind specifically to promoters of several other developmentally regulated *Drosophila* genes, including *fushi tarazu*, *engrailed*, and *Ultrabithorax* (*Ubx*), suggesting a role in the expression of various tissue-specific genes (Soeller et al. 1988; Dynlacht et al. 1989). A cDNA-encoding NTF-1 was subsequently isolated, and expression of NTF-1 was shown to be spatially and temporally regulated during embryonic stages of *Drosophila* development. In addition, purified NTF-1 protein was shown to be a potent activator of the *Ddc* and *Ubx* promoters in vitro (Dynlacht et al. 1989). Thus, NTF-1 provides an ideal model system to examine the role of a tissue-specific transcription factor at both the mechanistic level in vitro and at the developmental level in vivo.

Despite intensive studies, little is known about how sequence-specific transcription factors actually alter the rate of initiation of mRNA synthesis by RNA polymerase II. Transcriptional activation is minimally a two-step process: (1) The factor is targeted to the promoter by binding to specific DNA sequences, and (2) the factor must somehow communicate with the general transcriptional machinery to alter its activity. Activators such as *Drosophila* NTF-1 and the mammalian factor Sp1 appear to require a defined set of auxiliary factors termed coactivators to activate transcription (Dynlacht et al. 1991). These coactivators, which are part of the TFIID complex, are known as TAFs (TATA-binding protein-associated factors) and apparently serve to bridge activators and components of the general machinery. The identification of specific interactions between activators and coactivators has therefore become a focal point for studies aimed at unraveling the mechanisms of activation. For example, the glutamine-rich activation domain of Sp1 has been shown to interact directly with dTAF_{II}110 (Hoey et al. 1993). An important step toward understanding mechanisms of activation by site-specific transcription factors, therefore, is the careful analysis of functional domains.

Structure-function relationships of few *Drosophila* site-specific transcriptional activators have been characterized in detail. Here, we report the mapping of NTF-1 domains involved in DNA binding, dimerization, and

transcriptional activation, by a combination of both *in vivo* and *in vitro* assays. The DNA-binding and dimerization motifs were mapped by generating NTF-1 mutants and testing their activity by gel mobility-shift and chemical cross-linking assays. A transcriptional activation domain was identified by examining the activity of NTF-1 mutants in a cotransfection assay, as well as by an *in vitro* transcription assay. A minimal domain containing specific isoleucine residues important for activation suggests that NTF-1 may represent a novel type of activator. This motif appears functionally distinct from the well-characterized glutamine-rich activation domain of Sp1. We also present evidence that activation by NTF-1 may occur via different mechanisms in *Drosophila* and yeast cells, emphasizing the importance of carrying out transcriptional studies in homologous systems.

Results

NTF-1 binds DNA as a dimer

Transcription factors frequently bind DNA as dimers, which can serve both to regulate the affinity or specificity of DNA binding and to modulate activation properties via formation of heterodimers with other partners. The twofold symmetrical nature of the NTF-1 recognition site suggested that it might bind DNA as a multimer. Also, a carboxy-terminal domain of NTF-1 contains a region of similarity to helix-loop-helix (HLH) proteins, suggesting a possible role in DNA binding and dimerization (Bray et al. 1989; Dynlacht et al. 1989). We used two independent assays to determine the oligomeric state of NTF-1. First, gel mobility-shift assays were used to assess the nature of NTF-1 complexes bound to DNA (Fig. 1A). To this end, full-length NTF-1 and an amino-terminal deletion derivative, NΔ197, were translated *in vitro*, mixed, and incubated with a labeled NTF-1-binding site derived from the *Ubx* promoter (Fig. 1A, lane 5). Three shifted species were observed: a slow mobility band corresponding to full-length NTF-1 homodimer (see lane 2), a faster migrating band corresponding to the truncated protein NΔ197 homodimer (see lane 4), and one intermediate band, consistent with the binding of a heterodimer. All of the complexes were specific, as they were competed with an excess of unlabeled NTF-1 site oligonucleotide (lane 6). Second, solution cross-linking studies were performed with the bifunctional cross-linker ethylene glycolbis(succinimidylsuccinate) (EGS) to confirm the size of the NTF-1 multimer as well as to determine whether DNA was required for the formation of multimers (Fig. 1B). [³⁵S]Methionine-labeled, *in vitro*-translated NTF-1 was incubated with increasing amounts of EGS and analyzed by SDS-PAGE. Upon addition of EGS, a novel species of approximately twice the molecular weight expected for a monomer of NTF-1 was detected. These experiments demonstrate that NTF-1 not only binds to DNA as a dimer but that it also forms dimers in solution.

The DNA binding/dimerization domain of NTF-1 is a large, novel motif

A region at the carboxyl terminus with similarity to the MyoD and myogenin HLH domains suggested that NTF-1 might use this common structural motif to mediate DNA binding and dimerization (Bray et al. 1989; Dynlacht et al. 1989). The presence of prolines in the putative helical stretches, however, cast doubt on the significance of this homology, as did the difference between the DNA recognition sites of NTF-1 and the HLH proteins (Murre et al. 1989). To map these domains without bias, amino- and carboxy-terminal deletions were generated by *in vitro* translation and assayed by a gel mobility-shift assay. These results are summarized in Figure 1C. Removal of 74 amino acids from the carboxyl terminus (CΔ74) abolished DNA-binding activity. In contrast, an amino-terminal deletion of 653 amino acids (NΔ653) was required before disrupting DNA binding. Furthermore, all DNA-binding forms of NTF-1 were shown to dimerize by the gel mobility-shift mixing assay described above. We were unable to separate the DNA-binding and dimerization functions, as mutants defective in DNA binding were not observed to dimerize by the EGS cross-linking assay (data not shown). Our results indicate that the DNA-binding and dimerization domain consists of an unusually large stretch of ~400 amino acids, encompassing the HLH similarity region but extending substantially beyond it. Apparently NTF-1 utilizes a novel structure, distinct from the bona fide HLH, for DNA binding and dimerization.

Mapping of the NTF-1 activation domain

Critical to understanding the mechanism of NTF-1 action is the identification and characterization of domains involved in transcriptional activation. NTF-1 contains several polyglutamine stretches, known as opa repeats, that could be related to the glutamine-rich activation domains of mammalian transcription factors such as Sp1 and Oct-2 (Mitchell and Tjian 1989). Determining whether NTF-1 contains a glutamine-rich activation domain would help us to establish whether it activates in a similar manner to Sp1, by virtue of interaction with dTAF_{II}110 (Hoey et al. 1993). Thus, it became essential to functionally define the activation domain of NTF-1. The transcriptional activity of wild-type and mutant NTF-1 proteins was assayed by cotransfection into *Drosophila* Schneider cells (Fig. 2), which lack endogenous NTF-1 (L.D. Attardi, unpubl.). The reporter gene, N₃BCAT, contains three NTF-1 sites upstream of the E1B TATA box fused to the CAT reporter gene (Fig. 2A). Full-length NTF-1 or various mutant versions were expressed under the control of the constitutively active actin 5C promoter. Cotransfection of wild-type NTF-1 and the reporter plasmid resulted in an efficient transcriptional activation of the CAT gene, in an NTF-1 binding site-dependent manner (Fig. 2B). Like many factors, NTF-1 activates synergistically from a template containing multiple binding sites, leading to transcrip-

Figure 1. NTF-1 dimerizes both when bound to DNA and in solution via a carboxy-terminal domain. (A) NTF-1 binds its site as a dimer in a gel mobility-shift assay. Full-length NTF-1 and an amino-terminal deletion mutant, NΔ197, were translated in vitro and incubated with a radiolabeled NTF-1-binding site derived from the *Ubx* promoter. All shifts are specifically competed with an excess of unlabeled oligonucleotide. (Lane 1) No protein; (lane 2) NTF-1 alone; (lane 3) NTF-1 plus specific competitor; (lane 4) NΔ197 alone; (lane 5) NTF-1 and NΔ197 mixed; (lane 6) NTF-1 and NΔ197 plus specific competitor. (B) NTF-1 forms a dimer in solution. Increasing amounts of the bifunctional cross-linker EGS [ethylene glycolbis(succinimidylsuccinate)] were added to in vitro-translated [³⁵S]methionine-labeled NTF-1. The products were detected by SDS-PAGE (6%) and fluorography. Arrows indicate monomeric and dimeric species. The migration of nonradioactive molecular weight standards is also indicated. (C) The NTF-1 DNA-binding and dimerization domain is a carboxy-terminal 400-amino-acid segment. Features of NTF-1 are marked as landmarks in this and subsequent figures, as follows:

Stippled areas represent glutamine-rich stretches, solid vertical bars represent alanine-rich stretches, and hatched areas represent the HLH similarity region. Amino- and carboxy-terminal deletion mutants of NTF-1 were generated by in vitro translation and tested for their ability to bind the NTF-1 site from the *Ubx* promoter in an electrophoretic mobility-shift assay. The results are summarized. The NTF-1 DNA-binding and dimerization domain resides between amino acids 632 and 1032.

tional levels that are several hundredfold above basal level transcription, thus providing a wide range over which to assess the activity of mutants. These experi-

ments establish that NTF-1 is a potent transcriptional activator in *Drosophila* cells.

The cotransfection assay was then utilized to map the

Figure 2. NTF-1 requires its amino terminus to activate transcription in *Drosophila* Schneider cells. (A) Schematic diagrams depicting the expression vector and reporter plasmids used in the cotransfection assay. NTF-1 or mutant derivatives were inserted into the pPac vector, under the control of the actin 5C promoter. The reporter plasmid BCAT consists of the E1B TATA box fused to the CAT gene, whereas N₃BCAT contains, in addition, three NTF-1-binding sites upstream of the TATA box. (B) NTF-1 activates transcription several hundredfold in Schneider cells. Either no expression vector or the pPacNTF-1 expression vector was transfected into Schneider cells with the BCAT or the N₃BCAT reporter genes. CAT activity assay is shown. NTF-1

activity in the presence of NTF-1 sites is actually 20-fold greater than depicted, as the extract was diluted 20-fold to keep activity in the linear range of the assay. (C) The amino terminus of NTF-1 is essential for transcriptional activity in Schneider cells. Amino-terminal truncation derivatives of NTF-1 are shown diagrammatically. These derivatives were tested for their ability to activate the N₃BCAT reporter construct in Schneider cells. The activities of each mutant are given relative to the full-length protein. The expression and nuclear localization of these proteins was verified by Western blot analysis (data not shown).

NTF-1 activation domain by testing a battery of amino-terminal deletions for transcriptional activity (Fig. 2C). Deleting 114 amino acids from the amino terminus had a minimal effect on NTF-1, reducing activity to 67% of wild type. In contrast, removal of the amino-terminal 197 amino acids resulted in the complete loss of transcriptional activity. More extensive deletions were also inactive. The expression levels and nuclear localization of these mutant proteins were verified by Western blot analysis (data not shown). These results indicate that the amino terminus of NTF-1 is necessary for *trans*-activation. This information, however, does not define the carboxy-terminal boundary of the activation domain.

To delineate the carboxy-terminal boundary of the transcriptional activation domain, NTF-1 fusions to a heterologous DNA-binding domain were employed. These fusions allowed identification of NTF-1 domains that were sufficient for activation. A series of carboxy-terminal deletions of NTF-1 were fused to the DNA-binding and dimerization domain of GAL4 (residues 1–147). The resulting fusion proteins were assayed in Schneider cells for their ability to activate a reporter plasmid, G₅BCAT, consisting of 5 GAL4 sites upstream of the E1B TATA box and the CAT gene (Fig. 3A). Full-

length NTF-1 fused to GAL4(1–147) was a strong activator from GAL4 sites, whereas GAL4(1–147) alone was inactive. Assays of the other fusion proteins indicated that all but one protein activated transcription efficiently. As few as 284 amino acids of the amino-terminal domain was sufficient to confer transcriptional activity on GAL4(1–147) (GAL4–NTFCΔ748). A smaller 102-amino-acid fragment, in contrast, was not sufficient for activation (GAL4–NTFCΔ930). These results, taken together with the data obtained with the amino- and carboxy-terminal deletion mutants, establish the importance of the amino terminus of NTF-1 for activation.

The minimal activation domain of NTF-1 is 56 amino acids

To define the minimal region of NTF-1 sufficient for activation, the 284-amino-acid activation domain was subdivided by creating a series of NTF-1 fragments by PCR, which were then fused to GAL4 (1–147). These fusion proteins were again tested for their ability to activate the G₅BCAT reporter in a cotransfection assay. Only a few of these fusion proteins were transcriptionally active (Fig. 3B). GAL4(1–147), fused to a 114-amino-

Figure 3. The NTF-1 minimal activation domain is a 56-amino-acid segment near the amino terminus of the protein. (A) A 284-amino-acid amino-terminal segment of NTF-1 is sufficient for activation. Full-length NTF-1 was fused to the DNA-binding and dimerization domain of the yeast activator GAL4 (amino acids 1–147). Several carboxy-terminal deletions in NTF-1 were made and tested for activity in a cotransfection assay using the reporter plasmid G_5BCAT . Activities are given relative to the fusion of full-length NTF-1 to GAL4(1–147). The expression of these fusion proteins was confirmed by a gel mobility-shift assay using transfected cell extracts (data not shown). (B) The minimal activation domain of NTF-1 is 56 amino acids in length. Fragments from the region of NTF-1 encompassing the 284-amino-

acid activation domain were generated by PCR and fused to GAL4(1–147). Activities of these fusions in a cotransfection assay with the reporter gene G_5BCAT are given relative to GAL4(1–147) fused to the 284-amino-acid activation domain, GAL4-NTFCΔ748. The activity of each mutant is normalized for DNA-binding units, which were determined by gel-shift assays using extracts from the same transfections (data not shown). (C) The 56-amino-acid minimal activation domain is necessary for activation in the context of full-length NTF-1. Wild-type NTF-1 and a series of internal deletion mutants spanning the activation domain are shown schematically. These constructs were cotransfected with the N_3BCAT reporter gene into Schneider cells. CAT assay activity of each mutant is given relative to wild-type NTF-1. The amounts of DNA-binding activity of each mutant were determined by gel mobility-shift assay to ensure that effects were not the result of differences in protein levels (data not shown). (D) The domain structure of NTF-1 is summarized.

acid stretch (GAL4-PCR2), had nearly the same activity as GAL4-NTFCΔ748 (75%). The minimal region capable of efficient activation was even smaller, consisting of only 56 amino acids (GAL4-PCR4). Its activity was 36% of the GAL4-NTFCΔ748 protein, which represents a level of activation between 50- and 100-fold above basal levels. This minimal activation region contains a preponderance of isoleucine residues (17%). The amino-terminal half of this 56-amino-acid region was essentially inactive (GAL4-PCR11) for transcription, suggesting

that the entire domain is important. Interestingly, addition of the glutamine stretch to this minimal 56-amino-acid region (GAL4-PCR9) did not significantly enhance transcriptional activity, nor did the glutamine-rich stretch have activity alone (GAL4-PCR7). In contrast, 58 additional residues amino-terminal to the minimal activation domain (GAL4-PCR2) contributed some transcriptional activity. These 58 amino acids, however, did not serve as an activation domain alone, because the GAL4-PCR1 fusion was inactive and, thus, seemed to

play an enhancement role. Finally, addition of 56 amino acids to the carboxy-terminal end of the minimal activation domain diminished its activation potential (GAL4-PCR5). Whether this resulted from a fortuitous improperly folded fusion protein or from a bona fide negative regulatory element is currently unknown. In summary, these analyses have allowed us to define a minimal 56-amino-acid region of NTF-1 sufficient for transcriptional activity in *Drosophila* cells.

Although the GAL4-NTF fusion proteins allowed unambiguous identification of segments in NTF-1 with activation potential, it was important to confirm the relevance of these regions in the context of the intact NTF-1 protein. Internal deletions were constructed and assayed by the cotransfection assay (Fig. 3C). Strikingly, deletion of most of the 56-amino-acid minimal domain ($\Delta 176$ –226) severely crippled transcriptional activation, reducing activity 25-fold, and, thus, lending strong support to the conclusion that this region is the major activation domain in NTF-1. Removal of the carboxy-terminal half of this 56-amino-acid segment ($\Delta 204$ –226) also substantially abrogated activity (fivefold), establishing the importance of both halves of this region. Deletion of the 58 amino acids amino-terminal to the minimal 56-amino-acid activation domain ($\Delta 114$ –172) led to a modest decrease in transcriptional activation. These data are consistent with the idea that this domain, including the glutamine-rich stretch, may help to enhance transcriptional activation by NTF-1 but that it is not essential. The activation domain of NTF-1 thus appears to be a small, isoleucine-rich segment, rather than a glutamine-rich one like the mammalian transcription factors Sp1 and Oct-2 as hypothesized originally (Fig. 3D).

The NTF-1 minimal activation domain functions in vitro in Drosophila embryo extracts

To confirm the results obtained with activation domain fusions in transfected Schneider cells, we also performed in vitro transcription assays using extracts derived from *Drosophila* embryos (Fig. 4). It was important to show that the activities of the domains mapped in Schneider cells were similar in *Drosophila* cells that normally express NTF-1, to support the idea that similar activation mechanisms are used in both cases. GAL4-NTF fusion proteins were expressed as fusions to GST, purified by affinity chromatography, and incubated with the G₅BCAT template in embryo nuclear extracts. Using primer extension assays, we demonstrated that proteins with domains defined to be active by transfection, including GAL4-PCR4, GAL4-PCR9, and GAL4-NTF- $\Delta 748$, were also transcriptionally active in vitro. Full-length NTF-1 activates transcription in vitro ~5- to 10-fold above basal (data not shown). These GAL4-NTF-1 fusion proteins stimulated transcription between 4- and 8-fold above basal levels. As expected, GAL4(1–147) alone and GAL4-PCR1 also failed to activate transcription in vitro. We conclude that the minimal 56-amino-acid domain is sufficient for activation both in Schneider cells and in embryo extracts and is likely to represent a

Figure 4. The activities of GAL4-NTF fusion proteins in an in vitro transcription assay correlate with in vivo activities. GAL4-NTF fusion proteins were made as fusions to glutathione-S transferase (GST) and purified using glutathione-Sepharose. Two amounts (25 and 50 ng) were tested for transcriptional activity on the G₅BCAT template in a *Drosophila* nuclear extract. Products, which represent two transcripts initiated at two different start sites, were analyzed by primer extension. GAL4(1–147) and GAL4-PCR1 do not stimulate transcription, whereas GAL4-PCR 4, GAL4-PCR-9, and GAL4- $\Delta 748$ all activate transcription to levels that range from four- to eightfold above basal transcription levels. The ability of all of these GST fusion proteins to bind DNA was confirmed by gel mobility-shift assays (data not shown).

domain important for transcription in *Drosophila* cells normally expressing NTF-1.

Isoleucine residues are important for activation

The preponderance of isoleucines in the NTF-1 minimal activation domain represented a novel feature of an activation domain. To determine whether these isoleucine residues are important for the ability of NTF-1 to stimulate transcription, site-directed mutants in full-length NTF-1 were generated (Fig. 5). Substitution of all 10 isoleucine residues in the minimal activation domain with alanines (NTF-1 sdm1) severely diminished transcriptional activity in Schneider cells, an effect comparable to deleting the minimal activation domain. Changing these 10 isoleucines to alanines drastically affected transcriptional activity of both full-length NTF-1 and the minimal activation domain fused to GAL4 (data not shown), indicating that the effect of these mutations is not context dependent. To further dissect the activation domain, individual pairs of isoleucines were mutated. Changing one set of isoleucines to alanines (NTF-1 sdm2) or mutating a different set of isoleucines (NTF-1 sdm4 and NTF-1 sdm3) reduced transcriptional activity 2.5- and 6- or 8-fold, respectively. Altering either 3 or 4 isoleucines at a time reduced activity ~7- to 8-fold (NTF-1 sdm6, NTF-1 sdm7, and NTF-1 sdm8). Mutating different combinations of isoleucines inhibited activity substantially, suggesting that sequences important for the functional integrity of the domain are dispersed throughout the domain. These results highlight the im-

Figure 5. Isoleucine residues are important for maximal activation by NTF-1. NTF-1 point mutants were generated and tested for activation of the N₃BCAT reporter gene in transfected Schneider cells. Activities are given relative to full-length NTF-1. Isoleucine residues are underlined in the wild-type protein. Boxed residues represent amino acids that are either changed to alanines or deleted, as indicated. The activity of the deletion mutant of NTF-1 lacking the minimal activation domain is also shown, for comparison.

portance of at least the isoleucine residues for maximal transcriptional activity of NTF-1. None of these mutations affected DNA binding (data not shown) and are thus unlikely to exert their effects by causing gross conformational alterations in the folding of NTF-1, lending credence to the idea that these residues are important specifically for activation.

The NTF-1 activation domain fails to interact with dTAF_{II}110

The NTF-1 isoleucine-rich activation motif appeared distinct from the glutamine-rich activation domains of Sp1 (Courey and Tjian 1988). However, both NTF-1 and Sp1 require TAFs to activate transcription (Dynlacht et al. 1991), and it was of interest to know whether these apparently different activation motifs contact the same or different targets. To obtain evidence that these domains are functionally distinct, we attempted to determine whether NTF-1 contacts one of the targets of Sp1, dTAF_{II}110 (Hoey et al. 1993). A protein-protein interaction assay in yeast (Fields and Song 1989) was one means used to show that the activation domain of Sp1 interacts with dTAF_{II}110. In this assay the two proteins to be tested are fused to the GAL4 DNA-binding domain and the GAL4 activation domain, respectively. If these two test proteins interact in yeast, then a GAL4-responsive

β-galactosidase reporter is activated because the GAL4 activation domain is targeted to the promoter. We used this two-hybrid assay to test whether the NTF-1 activation domain interacts with dTAF_{II}110 (Fig. 6). As shown previously, cotransforming yeast with a GAL4 DNA-binding domain-dTAF_{II}110 fusion and an Sp1 activation domain-GAL4 activation domain fusion resulted in activation of the reporter gene. In contrast, cotransforming the GAL4-dTAF_{II}110 fusion with an NTF-1-GAL4 activation domain construct did not lead to stimulation of the reporter gene. Two different NTF-1 fusion proteins, one with the 56-amino-acid minimal activation domain (amino acids 173–228), and another with the larger 114-amino-acid activation domain (amino acids 115–228), both failed to interact with dTAF_{II}110 in this assay. Expression of these NTF-1 fusion proteins was verified by Western blot analysis using NTF-1 antiserum. These results are consistent with the proposal that distinct classes of activation domains may contact different target TAFs.

NTF-1 activates transcription via distinct mechanisms in Drosophila and yeast

In addition to being useful for performing protein-protein interaction assays, yeast also provides a convenient system for determining the critical elements of activa-

Figure 6. NTF-1 fails to interact with dTAF_{II}110 in yeast. Yeast containing an integrated *GAL1-lacZ* fusion gene were cotransformed with a plasmid encoding a fusion of dTAF_{II}110 to the GAL4 DNA-binding domain [G4(1-147)] and a plasmid encoding a fusion of the NTF-1 activation domain to the GAL4 acidic activation domain (AAD), and β -galactosidase activity was measured by blue color on X-gal plates. A fusion of Sp1 activation domain A to the AAD and a fusion of G4(1-147) to dTAF_{II}110 were cotransformed as a positive control. The AAD alone with G4(1-147)-dTAF_{II}110 and the G4(1-147) alone with NTF-1-AAD were used as negative controls. The β -galactosidase activity measured for the Sp1 and dTAF_{II}110 interaction is typically >500 U/mg of protein (+) whereas other activities are $\leq$ 3 U/mg of protein (-).

Figure 7. Distinct domains of NTF-1 activate transcription in *Drosophila* and yeast. GAL4-NTF fusion proteins tested previously in *Drosophila* were assayed in yeast for their ability to activate a reporter consisting of a GAL4 UAS upstream of the β -galactosidase gene. The minimal activation domain defined in *Drosophila* cells is marked by speckling and labeled I (for isoleucine-rich). Transcriptional activities of each protein in yeast and *Drosophila* are indicated. The expression of all fusion proteins that were inactive was verified by Western blot (data not shown). A map depicting the species-specific activation domains is shown.

tion domains through the use of genetic screens to identify mutants defective in activation (Gill and Ptashne 1987; Gill et al. 1990). We therefore tested the ability of NTF-1 to activate transcription in the yeast *Saccharomyces cerevisiae*, in hopes of further dissecting its mechanism of activation. For these studies, a GAL4-NTF-1 fusion protein containing the 284-amino-acid activation domain was tested in yeast for its ability to activate a reporter consisting of a GAL4 upstream activating sequence (UAS) linked to the β -galactosidase gene. This fusion protein strongly activated transcription in yeast, as it did in *Drosophila* cells (Fig. 7). Various GAL4-NTF-1 fusion proteins were then tested for their ability to activate transcription in yeast, to confirm that similar activation surfaces were utilized in yeast and *Drosophila*. Upon assaying these fusions in yeast, a surprising result was obtained. With the exception of GAL4-PCR2, all proteins that activated in *Drosophila* were inactive in yeast, and vice versa. These striking results indicate that the domains utilized in these two species are essentially mutually exclusive. This usage of distinct regions of NTF-1 for activation may reflect mechanistic differences in activation pathways used in yeast and *Drosophila*, perhaps because of the presence of different coactivator molecules in the two cell types.

Discussion

Here, we have dissected and characterized the functional domains of NTF-1. The NTF-1 DNA-binding and dimerization domain is an unusually large, 400-amino-acid region residing in the carboxy-terminal half of the molecule. Sequence analysis of this domain reveals that although it is distinct from other previously characterized motifs, including the zinc finger, helix-turn-helix, or basic leucine zipper domains, it does contain a region of similarity to the HLH motif of MyoD and myogenin (Bray et al. 1989; Dynlacht et al. 1989). It does not, however, contain the residues conserved between bona fide HLH proteins nor does the NTF-1 consensus DNA-bind-

ing site resemble the cognate HLH protein recognition site (Murre et al. 1989). A more significant similarity is observed between the NTF-1 DNA-binding/dimerization domain and the mammalian α -globin promoter-binding factor CP2. The most homologous region is one of 211 amino acids, which shows 32% identity and 48% similarity between the two proteins (Fig. 8A). The site to which CP2 binds is distinct from the NTF-1 site, suggesting that the two proteins may have different binding specificities (Lim et al. 1992). Perhaps CP2 and NTF-1 share a dimerization motif but have unique DNA recognition properties. Further analysis will be required to determine whether CP2 belongs to the same class of DNA-binding and dimerization domain as NTF-1.

Here, we have used both *in vivo* and *in vitro* assays to map the NTF-1 activation domain. NTF-1, in contrast to other factors such as Sp1 and Oct-2, has a single activation domain (Courey and Tjian 1988; Gerster et al. 1990; Muller-Immergluck et al. 1990), which is a discrete 56-amino-acid region both necessary for efficient activation by NTF-1 and sufficient to confer activation on a heterologous DNA-binding domain. It appears distinct from the previously described classes of activation domains, which include acidic, glutamine-rich, and proline-rich ones (Mitchell and Tjian 1989). A prominent feature of this activation domain is the high percentage of isoleucines, and we thus undertook to determine the relevance of these residues for transcriptional activity by changing them to alanines. Our results indicate that the isoleu-

cines are important for function, because changing as few as two in the context of full-length NTF-1 can significantly disrupt activity. These data are striking, as in many cases such mutations have little effect on activity, because there are often redundancies in activation domains of transcriptional activators.

The VP16 and the Epstein-Barr (EBV) Rta protein activation domains provide precedents for the importance of bulky hydrophobic amino acids for transcriptional activation function. (Cress and Triezenberg 1990; Hardwick et al. 1993; Regier et al. 1993). Like VP16 and Rta, the NTF-1 minimal activation domain contains a large number of charged amino acids interspersed with the hydrophobic ones. However, the NTF-1 domain does not align with these domains and seems to contain hydrophobic amino acid doublets rather than singlets; therefore, it may represent a new category of activation domain. The activation domain of another transcription factor, the mammalian factor NFY-A (Li et al. 1992), contains a region similar to half of the NTF-1 minimal activation domain (Fig. 8B) and could possibly be in the same class. Additional mutations should allow us to determine whether the NTF-1 activation domain is specifically an isoleucine-dependent motif or whether other bulky hydrophobic residues will substitute, as well as to determine the importance of other amino acids in this region.

Intriguingly, secondary structure predictions of the NTF-1 activation domain suggest a possible molecular

Figure 8. Features of NTF-1. (A) NTF-1 contains a region similar to the mammalian CP2 transcription factor. Identities are indicated by thick vertical bars, and conservative changes are indicated by thin bars. The amino-terminal boundary of the NTF-1 DNA-binding and dimerization domain begins approximately at the homologous region (amino acid 632), whereas the carboxy-terminal boundary extends beyond the homologous region. (B) The first 28 residues of the NTF-1 minimal activation domain show 32% identity and 53% similarity to a region in the activation domain of the NFY-A protein. (C) Secondary structure predictions by both the Chou-Fasman (Cf) and the Robson-Garnier (Rg) programs show that β -sheet (Sht) structures are lost upon changing the 10 isoleucines in the NTF-1 minimal activation domain to alanines. The wild-type minimal activation domain with the isoleucines underlined is shown at the top; the mutant domain with the alanine substitutions underlined is shown at the bottom.

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basis for the effect of the isoleucine mutants: Potential β -sheets present in the minimal activation domain are abolished upon substitution of the isoleucines by alanines (Fig. 8C) (Chou and Fasman 1978; Garnier et al. 1978). The importance of β -sheets in activation domains has been suggested recently in circular dichroism spectroscopic studies of the GAL4 and GCN4 proteins (Leuther et al. 1993; Van Hoy et al. 1993). Further analysis will be required to determine the actual structure of the NTF-1 minimal activation domain.

Complementary to studying the mechanism of activation by mapping the domains of a transcription factor is the identification of the targets that a transcription factor must contact to activate transcription. The coactivators or TAFs that NTF-1 and Sp1 require for activation in vitro are likely to be direct targets of these activators (Dynlacht et al. 1991). The glutamine-rich activation domain of Sp1 recently has been found to interact with dTAF_{II}110 (Hoey et al. 1993). In contrast, our studies suggest that NTF-1 does not interact with dTAF_{II}110. The NTF-1 isoleucine-rich activation motif is likely to contact one or more of the other TAFs in the TFIID complex to enhance transcription. Perhaps different families of transcriptional activation domains make contacts with different TAFs to allow the TFIID complex to integrate multiple regulatory signals.

To further elucidate the mechanism of activation by NTF-1, we had hoped to take advantage of yeast genetic screens. Upon assaying the 284-amino-acid activation domain of NTF-1 in yeast, we found that it was a potent transcriptional activator, as it was in *Drosophila* cells. However, when we assayed a variety of subdomains in yeast, we discovered that domains of NTF-1 capable of activation in *Drosophila* and yeast were mutually exclusive. These results suggested that NTF-1 is likely to be activating transcription via different mechanisms in yeast and *Drosophila*. These distinct functional domains could represent species-specific activation domains or perhaps even tissue-specific domains that function in different *Drosophila* cell types. These findings are unexpected because it is generally believed that the basic transcription machinery is highly conserved from yeast to mammals. For example, mammalian activator proteins such as the glucocorticoid receptor can function in yeast (Schena and Yamamoto 1988) and yeast proteins such as GAL4 can function in mammalian cells (Kakidani and Ptashne 1988; Webster et al. 1988). Consequently, transcriptional activators are often studied in yeast, under the assumption that they function via the same pathway as they do in homologous systems. In contrast, our results suggest that mechanisms of activation are not as conserved as is commonly believed and underscore the importance, certainly in some cases, of studying transcription factor activity in the proper cell type.

To corroborate our domain-mapping results obtained in Schneider cells, which may not be an accurate model for *Drosophila* embryonic cells, we took two approaches. First, we assayed NTF-1 fusion proteins in vitro, in *Drosophila* embryo nuclear extracts, to show that these proteins had activities similar to those in Schneider

cells. Second, we have also been able to show that this activation domain mapped by in vitro approaches is important in *Drosophila* embryos. Overexpression of an NTF-1 mutant lacking the amino terminus, and thus lacking the activation domain, acts as a dominant negative to inhibit activation by the wild-type protein, resulting in lethality (L.D. Attardi, D. Von Seggern, and R. Tjian, in prep.). In this manner, we have been able to corroborate our in vitro studies in a bona fide developmental context. These studies will ultimately be helpful in understanding the role of transcription factors in the expression of developmentally regulated genes.

Materials and methods

Plasmid construction for in vitro translation

Previously, we described the isolation of a partial cDNA encoding NTF-1 (Dynlacht et al. 1989). The 591 nucleotides missing at the 5' end were obtained by PCR of *Drosophila* genomic DNA, using primers based on the sequence of the full-length cDNA isolated by Bray et al. (1989). This PCR fragment was fused to the partial NTF-1 cDNA in BSSX (a derivative of BSSK⁺ with the *KpnI* site deleted) to create pBSSXNTF-1. An *NdeI* site was introduced at the ATG by site-directed mutagenesis (giving rise to pBSSXNTF-1Nde) to facilitate transfer of the NTF-1-coding sequences into other vectors.

The NTF-1 in vitro transcription/translation construct pT β StuNTF-1 was generated by inserting an NTF-1 fragment into the *StuI/EcoRI*-cut T β Stu vector (Norman et al. 1988). The NTF-1 fragment was generated from pBSSXNTF-1Nde by cutting with *NdeI*, blunting with mung bean nuclease, and digesting with *EcoRI*. Another in vitro transcription/translation vector was constructed to generate NTF-1 5' deletions. This vector was constructed by ligating an *NdeI/XhoI*-cut, and filled-in NTF-1 fragment from BSSXNTF-1Nde into *HincII*-restricted pT β STOP (Jantzen et al. 1992) to make pT β STOPNTF-1. Then, 5' deletions were generated by digesting pT β STOPNTF-1 with *NdeI*, chewing back with exonuclease III, repairing ends with S1 nuclease, cutting with *XbaI*, and recloning deleted fragments into *HincII/XbaI*-cut pT β STOP vector. Mutants were sequenced to select those in the correct reading frame.

In vitro transcription/translation and gel mobility-shift assays

pT β Stu or pT β Stop plasmids containing full-length or 5' deletion derivatives of NTF-1 were linearized 3' to the coding region at the *EcoRI* or *XbaI* sites, respectively. By linearizing the pT β StuNTF-1 construct at appropriate positions within the ORF, 3' deletion derivatives were made. CA74 was linearized at the *DraIII* site. In vitro transcription and translation were performed essentially as described (Turner and Tjian 1989). Briefly, in vitro transcription was performed using T7 RNA polymerase. The RNA was translated in a rabbit reticulocyte lysate (Promega) in the presence of [³⁵S]methionine. Gel mobility-shift assays were performed by incubating 3 μ l of reticulocyte lysate with 10,000–50,000 cpm of ³²P-labeled double-stranded NTF-1 site oligonucleotide derived from the *Ubx* promoter (Dynlacht et al. 1989) in a buffer containing 10% glycerol, 10 mM HEPES (pH 7.9), 25 mM KCl and 3.1 mM MgCl₂, 0.025% NP-40, 0.1 mM EDTA, 2 mM spermidine, and 1 mM DTT in the presence of 1 μ g of sonicated poly[d(I-C)]. Reactions were incubated on ice for 20 min. DNA-protein complexes were electrophoresed at 18 V/cm at 4°C on 4% (40 : 1) polyacrylamide gels containing 0.5 $\times$ TBE,

0.75 mM EDTA, and 0.05% NP-40. Gels were dried and autoradiographed.

Chemical cross-linking assays

In vitro-translated, [³⁵S]methionine-labeled NTF-1 was incubated with varying amounts of EGS under conditions described previously (Sorger and Nelson 1989). Samples were resolved by 6% SDS-PAGE and visualized by fluorography.

Plasmid construction for transfection assays

All forms of NTF-1 were inserted into the *Drosophila* expression vector pPACU+Nde (Biggin and Tjian 1989). An *Nde*I-*Xho*I fragment containing full-length NTF-1 from BSSXNTF-1Nde was inserted into the *Nde*I and *Xho*I sites of this vector to make pPACNTF-1. 5' Deletions of NTF-1 were subcloned by digestion of appropriate PTβSTOP NTF 5' deletion constructs with *Nde*I and *Sma*I and ligating these fragments into *Bam*HI-cut and repaired, *Nde*I-cut pPAC U+Nde. pPACGAL4-NTF was constructed by subcloning an *Eco*RI-cut and repaired, *Bgl*II-cut fragment containing GAL4(1–147) from pPACGAP (Yan-Fen Hu, unpubl.) into an *Nde*I-cut and repaired, *Bgl*II-cut pPACNTF-1 vector to create an in-frame GAL4-NTF fusion. Each GAL4-NTF 3' deletion was made by digesting pPACGAL4-NTF at the *Sau*I site in the 3'-untranslated region of NTF-1 and at an appropriate site in the coding region of NTF-1, repairing the ends with Klenow, purifying the fragment containing the vector and desired piece of NTF-1, and recircularizing this fragment, with the insertion of an *Xba*I linker containing a three-frame STOP codon. The following deletions correspond to the following restriction sites: CΔ92 = *Mlu*I; CΔ437 = *Bst*XI; CΔ707 = *Asp*718; CΔ748 = *Eco*RV; and CΔ930 = *Hinc*II.

The GAL4-NTF PCR fusions were made as follows: Primers containing *Eco*RI sites at the 5' ends were used to amplify specific fragments of NTF-1 using PCR. These *Eco*RI fragments were fused in-frame to GAL4(1–147) in BSGAL4(1–147), a BS-derived vector containing GAL4(1–147) inserted into the *Eco*RI site. The GAL4-NTF fragments were removed by cutting with *Nde*I and *Xba*I and were subcloned into *Nde*I/*Xba*I-restricted pPACNdeXba, a derivative of pPACU+Nde, containing an *Xba*I, three-frame STOP linker in the *Bam*HI site.

To create internal deletions in NTF-1, segments of NTF-1 were generated by PCR and pairs of amplified fragments were fused so as to create deletions in the coding region, with the end points given in the text. Each pair of fragments was joined at an *Eco*RI site, thus introducing 2 amino acids into the coding region of NTF-1. These fragments were subcloned as *Nde*I-*Asp*718 fragments from BSSK into pPACNTF lacking the *Nde*I-*Asp*718 fragment of NTF-1.

Site-directed mutants were made as follows: To change 10 isoleucine residues to alanines, two mutated complementary oligonucleotides of ~80 bp, with *Eco*RI linkers at each end, were annealed and filled in with Klenow. This fragment was digested with *Eco*RI and subcloned into the *Eco*RI site within BSNTF-1(Δ176–226) [a form of NTF-1 lacking the minimal activation domain], and thus two *Eco*RI sites were introduced into the NTF-1-coding region. To change or delete only two isoleucines, standard protocols for site-directed mutagenesis were used to mutate NTF-1 in the BSSXNTF-1 vector. Once the mutant forms of NTF-1 were obtained, they were subcloned into pPACU+Nde.

The reporter plasmids BCAT (Lillie and Green 1989), G₅BCAT (Lillie and Green 1989), and N₃BCAT (Dynlacht et al. 1991) have been described previously. NBCAT (B. Dynlacht,

unpubl.) was constructed similarly to N₃BCAT, except that only one NTF-1 site was inserted into BCAT.

Cotransfection assays

Cotransfection of *Drosophila* SL2 cells and CAT assays was performed essentially as described previously (Pascal and Tjian 1991), except cells were plated at a density of 5 × 10⁶/6-cm plate. Each plate received 25–50 ng of expression vector, 2.5 μg of reporter plasmid, and an unrelated plasmid as carrier to bring the total amount of DNA to 7.5 μg. The data presented are averages of three to six transfections, each performed in duplicate.

Transfected cell extracts for gel-shift and Western blot analysis

Extracts from transfected cell extracts were prepared as follows: 2.5 μg of expression plasmid and 5 μg of carrier plasmid were transfected per 6-cm plate of Schneider cells, seeded as described above. After 36–48 hr, cells were spun down, washed with PBS, and spun down again. Cells were resuspended in 0.25 ml of TD buffer (25 mM Tris at pH 8.0, 2 mM MgCl₂, 0.5 mM DTT, and 0.01% PMSF) and incubated for 5 min at room temperature. Then 1.25 μl of 10% NP-40 was added, and cells were incubated at room temperature for 2 more minutes. Cells were spun at low speed (1700 rpm) and the supernatant was removed (cytosolic fraction). BL buffer (0.125 ml, consisting of 0.4 M LiCl, 10 mM Tris at pH 8.0, 0.5 mM DTT, and 0.01% PMSF) was added to each sample, and the samples were vortexed and incubated for 5 min at room temperature. Extracts were spun down at high speed, and the supernatant was removed (nuclear fraction). Protein concentrations were determined by Bradford assays. Approximately 80 μg of total protein was used for gel shifts and for Western blot analysis. For Western blots, polyclonal serum against NTF-1 (L.D. Attardi, D. Von Seggern, and R. Tjian, in prep.) was used as the primary antibody (1 : 1500 dilution), followed by alkaline phosphatase-conjugated anti-rat antiserum (Promega; 1 : 1500 dilution).

Primer extension assays

In vitro transcription assays were performed essentially as described previously (Dynlacht et al. 1991). Activator proteins were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusions and purified using glutathione-Sepharose beads. GAL4-NTF-1 fusion protein (25–50 ng) estimated by SDS-PAGE and silver staining was incubated with 200 ng of supercoiled G₅BCAT template and 30 μg of nuclear extract (Wampller et al. 1990), and products were analyzed by primer extension.

Yeast assays

Yeast expression vectors encoding GAL4(1–147) fused to dTAF_{II}110 and Sp1 domain A fused to the GAL4 acidic activation domain have been described previously (Hoey et al. 1993). The two constructs expressing the NTF-1 activation domain fused to the GAL4 acidic activation domain were made by excising the PCR2 and PCR4 NTF-1 fragments from pPACGAL4 PCR2 and pPACGAL4 PCR4 using *Eco*RI, repairing the ends, and inserting the fragments into *Bam*HI-restricted, filled-in pGAD3F (Chien et al. 1991). GAL4-NTF-1 fusion proteins were made in the pAS1 vector (Durfee et al. 1993), which contains the GAL4(1–147) DNA-binding domain. GAL4-PCR 1, -PCR2, -PCR4, -PCR5, -PCR6, and -PCR9 were made by subcloning the NTF-1 PCR products used in the *Drosophila* transfection

assays into *EcoRI*-restricted pAS1. Gal4-CA930 or -Δ748 was made by isolating either an *NdeI*-*HincII* fragment or an *NdeI*-*EcoRV* fragment of NTF-1 and ligating into *NdeI*/*SmaI*-digested pAS1.

Yeast assays were performed essentially as described [Hoey et al. 1993]. In brief, the yeast strain Y153 [a, *gal4*, *gal80*, *his3*, *trp1-901*, *ade2-101*, *ura3-52*, *leu2-3,112*, *URA3::GAL1:lacZ*, *LYS2::GAL1-HIS3* [Durfee et al. 1993]] was transformed using either the method of Shiestl and Gietz (1989) or Elble (1992). Transformed yeast were assayed qualitatively on plates containing X-gal.

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