The pancreas-specific transcriptional enhancer of the rat elastase I gene was modified by substituting, in turn, each of its three individual constitutive elements with the tetO element, which confers regulation by exogenous tetracycline in the presence of the hybrid tetO binding transactivator (tTA). Whereas the unmodified enhancer was active in transfected acinar tumor cells, substitution of individual elements with the tet-responsive element abolished activity. The modified enhancers were reactivated in the presence of the tTA and, upon addition of tetracycline, were silenced. Thus, substitution of individual enhancer elements renders the enhancer responsive to regulation by tetracycline. Moreover, the tTA-activated levels were 2–8-fold greater than the unmodified enhancer. The acinar cell specificity of the unmodified enhancer was retained; none of the tetO-substituted enhancers were activated by tTA in a variety of nonacinar cell lines. These results show that a foreign and artificial transcriptional activator, tTA, can be incorporated into an enhancer to create a novel, efficient, and regulatable transcriptional control region whose cell specificity is retained.

Mammalian transcriptional enhancers and promoters are composed of multiple functional elements of distinct function that contribute to overall transcriptional specificity and strength. In some instances the collection of elements and their bound factors act highly cooperatively; alteration of individual elements or their spacing can dramatically affect the overall activity of an enhancer (1, 2). A precise arrangement of the elements is necessary for the cooperative assembly of DNA binding transcription factors in an ordered nucleoprotein complex required for transcriptional activity. Interactions between bound transcription factors appear crucial as is the presence of DNA-binding proteins whose primary role is to bend DNA to facilitate those interactions (3, 4). The cooperative interaction of transcription factors within this class of enhancer creates a transcriptional activity different than the simple sum of the activities of its individual elements (2, 4). Other enhancers appear to have much more flexible organizational requirements; individual elements play only incremental roles (5, 6) and spacing requirements are much less stringent (7, 8). When examined, the separate activities of these enhancers are evident in the individual elements (7–10).

One approach to testing the organizational requirements of an enhancer is to examine the activity of each of its elements independently (e.g. Refs. 9 and 11–13). We have used this approach to dissect the functional elements of the transcriptional enhancer of the pancreatic elastase I (EI) gene. The EI enhancer (14) comprises only three mutation-sensitive elements, termed A, B, and C (15, 16) (Fig. 1). By analyzing the elements individually as homomultimers or pairwise combinations of heteromultimers in transgenic mice, we have elucidated the role of each element in controlling the pancreas specificity exhibited by the complete enhancer (10, 16, 17). The 21-bp A element directs expression selectively to acinar cells. The 12-bp B element has a dual function; it directs expression itself selectively to pancreatic islet cells, whereas in acinar cells it augments the acinar specific activity of the A element approximately 20-fold. The 30-bp C element has no intrinsic ability to act on its own, but in combination with either A or B it augments their activity an order of magnitude without affecting cell specificity (10, 17). Consequently, the specificity and strength of this enhancer is based on the assembly of individual elements of unique function. In animals, the specificity of the enhancer is the sum of that of the A and B elements, whereas its overall strength includes synergy between all three elements. In transfected pancreatic acinar tumor cells in culture, the elements play similar roles except that each element is essential, whereas in acinar tissue in situ the B and C elements are largely redundant (15, 16).

Another strategy to probe the functional organization of complex enhancers is to substitute endogenous elements with an ectopic one to examine whether the enhancer complex is sufficiently flexible to integrate the function of the new element. In this report we describe the use of this strategy to test whether the tetracycline regulatory element, tetO, and its binding transactivator (tTA) (18) can integrate into the nucleoprotein complex of the EI enhancer to complement the transcriptional activities of the endogenous elements. tTA is a hybrid protein comprising the DNA binding portion of the bacterial tetracycline repressor that is fused to the transactivation domain of viral protein 16 of herpes simplex virus (18). In the absence of tetracycline tTA can bind to the tet repressor DNA binding site, tetO, and activate an adjacent promoter in vivo. The transactivation of the promoter can be inhibited by the presence of tetracycline, which binds to the tet repressor domain of tTA and causes its release from the tetO site (18).

Substitution of any one of the three functional elements of the enhancer with the tet repressor DNA binding site tetO can indeed render the activity of the enhancer dependent on the presence of tTA. Whereas tTA activates the tetO-substituted enhancers in transfected acinar tumor cells, it does not in…

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1The abbreviations used are: EI, elastase I; tTA, tetO-binding transactivator; hGH, human growth hormone; bp, base pair(s); CMV, cytomegalovirus.
several of the nonacinar cell lines tested. Moreover, the acinar cell specific activity of the tetracyclineregulatable enhancers was regulatable by tetracycline. In addition to probing the nature of the enhancer, these results also demonstrate the potential to engineer novel enhancers by inserting ectopic control elements into existing cellular enhancers.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The enhancer and promoter test plasmids were constructed by standard recombinant DNA techniques (19). The plasmid T-tetO-CMV-hGH was made by first changing the unique XhoI site in plasmid pUHD10-3 (20) to a HindIII site by filling in the XhoI ends, adding a HindIII linker, and ligating blunt ends. The HindIII-to-BamHI fragment containing the seven tetO sites and the CMV promoter (P<sub>CMV</sub>−1) was subcloned into a pUC119-based plasmid immediately upstream of the human growth hormone (hGH) reporter gene. The −205EhGH gene construct containing the unmodified rat elastase I enhancer with the hGH reporter has been described (14). A single tetO site of 19 bp (TCCCTATCATGTGAGA) was substituted for the endogenous EI enhancer sequences at positions −189/−171 (C-tetO), −162/−144 (B-tetO), −114/−96 (A-tetO) by site-specific mutagenesis (21) and at −139/−121 (between the A and B elements) by polymerase chain reaction-directed mutagenesis and recombination (22). The hybrid TA expression plasmid pUG15-1 has been described previously (18). To test whether the VP16 transactivation domain was required, the VP16 coding region (codons 367–490) of the tTA hybrid gene was deleted from the TATA box, although at this time we cannot rule out that the transactivation domain is required within the context of the enhancer (data not shown). In other experiments, tTA did not further activate the unsubstituted enhancer above the normal level in 266-6 cells (data not shown); therefore, tTA exerts its effect through the tetO site. Thus, incorporation of the artificial tet repressor/VP16 protein into the nucleoprotein complex of the EI enhancer reconstituted an effective transcriptional enhancer.

The levels of tTA-augmented activity of the tetO-substituted enhancers were 2–8-fold greater (depending on which of the elements was substituted) than that of the unaltered enhancer. Therefore, in the context of the enhancer, the artificial tTA transactivator was even more effective than the cognate transcription factors that act through the A, B, or C elements.

Tetracycline Regulation of a Cell-specific Enhancer

To test whether the function of the tetracycline-regulatable transactivator (tTA) could be integrated into the EI gene enhancer, we replaced each of the three known functional elements of the enhancer in turn with the 19-bp tetO element (Fig. 1). The test constructs contained the substituted enhancer linked to the cognate EI promoter driving the hGH reporter gene. The effects of the substitutions were examined by transfection into the 266-6 pancreatic acinar cell line in which the intact enhancer is active (28) and in several nonacinar lines in which the intact enhancer is inactive. The 266-6 cell line was derived from a mouse pancreatic acinar tumor induced by an SV40 T antigen-transgene (29) and retains differentiated properties of acinar cells including the continued expression of pancreatic hydrolytic enzyme genes such as elastase I, trypsin I, and amylase (15). For each cell line the enhancer activity was measured (a) without coexpression of tTA and in the absence of tetracycline, (b) with coexpression of tTA, and (c) with tTA in the presence of tetracycline.

Incorporation of a Single tetO Site Confers Regulation by tTA and Tetracycline—Substitution of each element in turn with the tetO sequence inactivated the enhancer in transfected 266-6 acinar cells (Fig. 2). Replacement of the A element reduced activity 35-fold, of the B element 110-fold, and of the C element 60-fold. These effects are consistent with the mutational analysis of the enhancer (28), which demonstrated that each element is essential for the activity of the enhancer in these acinar tumor cells.

Cotransfection of the substituted enhancers with an expression plasmid for the tTA increased expression of the reporter plasmids 120–280-fold greater than the level of the substituted enhancers in the absence of tTA (Fig. 2). The extent of tTA activation decreased with increasing distance of the tetO site from the TATA box, although at this time we cannot rule out that this might be due to context effects rather than distance. Co-transfection with a plasmid that expressed the tet repressor transactivator but was missing the VP16 transactivation domain did not reactivate the substituted enhancers, suggesting that the transactivation domain is required within the context of the enhancer (data not shown). In other experiments, tTA did not further activate the unsubstituted enhancer above the normal level in 266-6 cells (data not shown); therefore, tTA exerts its effect through the tetO site. Thus, incorporation of the artificial tet repressor/VP16 protein into the nucleoprotein complex of the EI enhancer reconstituted an effective transcriptional enhancer.

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Tetracycline effectively suppressed the activation by tTA (Fig. 2). The presence of tetracycline in the cotransfection of the tTA expression plasmid with the reporter plasmids reduced the level of enhancer activity 190–330-fold to levels similar to those in the absence of the tTA. Therefore, in the 266-6 acinar cells, tetracycline is capable of regulating the activity of the enhancer through the tetO binding site integrated at any of the

FIG. 1. Schematic of the promoter and enhancer regions of the rat EI gene. The positions of the three functional elements of the enhancer are shown together with the tetO substitutions. The tetO substitution (lowercase nucleotide lettering) extends beyond the boundaries of the B element whereas 11 bp and 2 bp are retained at the ends of the longer C and A elements, respectively. The tetO substitution for nucleotides −139 to −121 lies between the A and B elements. The fusion of a single tetO element to the EI promoter at −97 is shown at the bottom.
Tetracycline Regulation of a Cell-specific Enhancer

Tetracycline Regulation of the Substituted Enhancers Is Acellular

To demonstrate that tTA activation through the single tetO element must occur via cooperation with the endogenous factors that bind to the remaining enhancer elements, we tested whether tTA could activate a reporter gene without these additional enhancer elements. The reporter construct contained a single tetO element linked to the EI promoter at −97, similar to the position of the tetO substitution of the A element at −95 (Fig. 1). This truncated form of the EI promoter comprises TATA and other elements that are not sufficient on their own to activate transcription in transfected cells in culture (Ref. 15 and see below) or in transgenic animals (14). However, this promoter region can be activated by the addition of the EI enhancer (14), other enhancers (30), and artificial constructs of homomultimeric repeats of factor binding sites in both transfected cells (17) and transgenic animals (10, 16, 17). For the acinar 266-6 cells, the tetO-promoter construct was inactive even in the presence of cotransfected tTA. Therefore, tTA is not sufficient to activate through a single tetO site linked to the EI promoter but instead requires the participation of the other enhancer elements. Conversely, when only two of the three enhancer elements are intact, they require the participation of tTA for activity. This inactivity of a single tetO site can also be overcome by multimerization because a reporter construct (7-tetO-CMV-hGH) containing a heptamer of tetO sites linked to a truncated CMV promoter is activable by tTA and regulated by tetracycline in 266-6 cells and the other cell lines tested (see Table I and Refs. 31–33).

Tetracycline Regulation of the Substituted Enhancers Is Acinar Cell-specific—The unmodified elastase enhancer was inactive in all other cell lines tested, including HeLa cells, NIH3T3 fibroblasts, Rat2 fibroblasts, and RIN1046-38 insulinoma cells (Fig. 3, wt). Substitution of the tetO element for any of the endogenous enhancer elements had no effect when tested in these cells in the absence of tTA. Compared with the level of activation in 266-6 acinar cells, the presence of tTA also had little or no effect on the activation of the substituted enhancers. Curiously, in Rat2 fibroblasts there was low but significant tTA-induced activity that was responsive to tetracycline. The absence of tTA-induced activity in the nonacinar cell lines was not due to an inherently high activity of the EI promoter, the enhancer, or the substituted enhancers in the nonacinar cells because their activity in these cells was barely more than mock-transfected cells treated in parallel (data not shown). These results show that the substituted enhancers as well as their regulation by tetracycline remain largely acinar cell-specific.

The interpretation of these experiments depends on tTA being capable of activation in the nonacinar cell lines tested. Others have shown that tTA transactivation can be cell line-specific (31–33). We examined the ability of tTA to transactivate in each of the cell lines by cotransfection of the tTA expression plasmid with a reporter gene containing a heptamer repeat of the tetO element linked to the CMV minimal promoter. A similar construct has been shown to be responsive to tTA transactivation in several cell lines (32, 33). In all five cell lines tested here, the tTA activated the reporter gene effectively (Table I) although induction ratios varied from 24 (in Rat2 cells) to 1500 (in HeLa cells). Moreover, the activation by tTA was prevented by tetracycline. Therefore, the selective regulation of the tetO-substituted enhancer in 266-6 cells by the tTA and tetracycline was not due simply to the inability of tTA to transactivate in the nonacinar cell lines.

**Table I**

| Cell lines | −/− | +/− | +/+ |
|------------|-----|-----|-----|
| 266-6      | 1   | 2   | 490 |
| HeLa       | 1   | 5   | 1500|
| NIH 3T3    | 1   | 3   | 34  |
| Rat2       | 1   | 1.5 | 24  |
| RIN 1046-38| 1   | 2   | 230 |

* a Without tTA and without tetracycline.
* b With tTA and without tetracycline.
* c With tTA and with tetracycline.
Addition of tetO to the Normal Three-element Enhancer Augments Activity in the Presence of tTA. To test whether binding of the tTA would increase the activity of the complete three-element EI enhancer, we inserted the tetO site between the A and B elements, a region largely insensitive to mutation (16). The enhancer with the tetO site insertion was still 35% as active as the unmodified enhancer (Fig. 4). The presence of tTA induced activity of this modified enhancer 35-fold, and the addition of tetracycline in the presence of tTA suppressed the activity nearly to the level in the absence of tTA. These effects were not due to the extraneous effects of tetracycline or the tTA because tetracycline alone had no effect on the activities of the substituted or unsubstituted enhancers, and cotransfection of the tTA expression plasmid had no effect on the unsubstituted enhancer (data not shown). In the presence of tTA the activity of the enhancer with the added tetO site is 12-fold greater than the unmodified enhancer (Fig. 4). Therefore, autoactivation through a tTA binding site should be one useful way to augment the activity of the cell-specific promoter used to drive the expression of tTA in transfected cells or transgenic animals.

DISCUSSION

In transient transfection assays, substitution of any of the three elastase enhancer elements with the tetracycline operator element, tetO, renders the activity of the enhancer responsive to regulation by tetracycline through the tTA chimeric transactivator in an acinar cell-specific manner. The activity of the modified enhancers is dependent on the presence of the tTA and the absence of tetracycline. The activity of the substituted enhancers in the presence of tTA and the absence of tetracycline is severalfold greater than the activity of the unmodified enhancer. Thus, the artificial transactivator created by fusion of the tetracycline repressor binding domain with the VP16 transactivator can integrate into the enhancer complex to efficiently complement the activity of the endogenous transcription factors that bind to and mediate the activity of the remaining elements.

The ability of a foreign and artificial transcriptional activator like tTA to integrate into the nucleoprotein complex of an enhancer and incorporate its transcriptional regulatory properties indicates the flexibility of organization of at least one class of enhancer. The binding of tTA to tetO at any of three sites within the enhancer compensates for the absence of the transcriptional activator that normally occupies those sites. Substitution at the position of the A element replaces the binding of the Ptf1 complex (16, 34), which contains DNA binding proteins of the basic helix-loop-helix class (35). Substitution of the B element displaces the binding of a pancreas-specific homeodomain factor complex2 (36). The proteins that bind the C element have not been characterized, except that they do not include the A or B element-binding proteins. Thus, tTA is able to complement the activity of distinctly different classes of factors at any of at least three different positions.

The strength of the tetO-substituted enhancers in the acinar cell line decreased nearly 4-fold with increasing distance from the basal promoter. This effect may be due to a decreasing effectiveness of tTA farther from the basal promoter. Alternatively, if the strength of the normal transcriptional activators is greatest for the C element and least for the A element, then replacing each in turn with tTA could give the same apparent effect.

These results also demonstrate the potential to create new transcriptional regulatory schemes through the integration of ectopic control elements into existing cellular enhancers. The ability of tetracycline to control transactivation by tTA within the context of the complex physiology of animals has been demonstrated by transgenic experiments (37–40). Similar analyses will be necessary to determine whether the regulatory properties of the tetO-substituted EI enhancers are retained in animals.

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