Synergistic transcription activation is a key component in the generation of the spectrum of eukaryotic promoter activities by a limited number of transcription factors. Various mechanisms could account for synergy, but a central question remains of whether synergy requires transcription factor functions that differ from those that direct independent activation. The rat growth hormone promoter is synergistically activated by the pituitary-specific transcription factor, Pit-1, and the thyroid hormone receptor (TR). Mutations that disrupted the previously described DNA binding and transcriptional activation domains of both Pit-1 and TR reduced Pit-1/TR synergy in parallel with their effects on the much weaker, independent Pit-1 and TR activations of the rat growth hormone promoter. Thus, Pit-1 and TR amplify each other’s intrinsic activities. Mutations of Pit-1 that selectively inhibited synergism with the TR were identified. Pit-1/TR synergy is therefore a consequence of a novel synergism-selective activity and synergism-independent Pit-1 and TR functions.

The rGH gene resembles many other tissue-specific genes in that its transcription is regulated by several factors, both tissue-specific and tissue-general (1–3), that bind to discrete sites within the rGH promoter. The various factors appear to act in synergy since the collective contributions of these sites to rGH promoter activity exceed the sum of their independent contributions (2). Changing the relative positions of the binding sites within the rGH promoter alters the rGH promoter activity (4–6). Further suggesting that each factor is influenced by factors binding to adjacent sites.

The rGH promoter is much more active when rat Pit-1 and human β-1 TR are coexpressed in human monocyte U937 cells than when each factor is expressed individually (7). This synergism was observed only when the U937 cells were cultured with forskolin and phorbol 12-myristate 13-acetate (PMA), activators of protein kinases A and C, respectively (8, 9). Synergism also displayed by the thyroid hormone (triiodothyronine)-dependent and -independent components that were differentially dependent on the thyroid hormone response element but required either one of the two more Pit-1 binding

sites in the rGH promoter (7). Thus, Pit-1 and TR bind to separate sites and synergistically activate the rGH promoter. Pit-1 also synergizes with Zn15 (10), Ets-1 (11) or the estrogen receptor (12, 13), and a splicing variant of Pit-1 (14) to activate the growth hormone, prolactin (PRL), and thyroid-stimulating hormone β subunit promoters, respectively. Both the PRL and thyroid-stimulating hormone β subunit promoters are synergistically activated by Pit-1 and P-Lim expression (15). Some of the prolactin receptors of the PRL are synergistic with components of the Gαif/kinase A signal transduction cascade (11, 16) and are inhibited by protein kinase C activation (11, 16).

The undoubted importance of transcriptional synergy to the regulation of the rGH and other, natural promoters ultimately will require a better understanding of the activities required for synergistic activation. To determine if Pit-1/TR synergism requires any of the Pit-1 and TR domains known to be essential for independent Pit-1 or TR activity, mutations in TR and Pit-1 were examined for their effects on the synergistic activation of the rGH promoter in U937 cells. Mutations within the DNA binding, ligand binding, and amino-terminal domains of the TR reduced or eliminated Pit-1/TR synergistic activation. Similarly, synergistic activation was disrupted when the DNA binding domain or the previously described transcriptional domain of Pit-1 was deleted. Intriguingly, deleting a proline-rich region of Pit-1 strongly reduced TR-synergistic activation of the rGH promoter without affecting the ability of Pit-1 to independently activate the rGH promoter or a minimal promoter to which Pit-1 binding sites were appended. This synergism-selective effect was also observed for Pit-1/TR synergistic activation of the rGH promoter in mouse pituitary GHFT1-5 cells that are transformed at a developmental stage immediately preceding activation of the rGH promoter (18). Thus, synergistic activation of the rGH promoter by Pit-1 and TR not only was dependent on Pit-1 and TR functions that are essential for their independent activities but also required an activity that operates only in the synergistic context.

Experimental Procedures

Cell Transfection—cDNA expression vectors, rGH promoter constructs, electroporation, and data analysis were as described previously (7, 19, 20). 5 μg of the rGH/CAT construct was electroporated into U937 or GHFT1-5 cells. 1.5 μg of Pit-1 expression vector was used to enhance the activation of the rGH promoter by Pit-1 expression alone as shown in Fig. 2, whereas 10 μg of the Pit-1 expression vector was transfected as shown in Figs. 1 and 3–5. 15 μg of the TR expression vector was transfected as shown in Fig. 1, whereas 10 μg was used as shown in Figs. 2, 4, and 5. At 15 μg, the control expression vector lacking the TR weakly activated the rGH promoter in U937 cells to the level of the same expression vector containing TR mutations affecting DNA binding (data not shown). Thus, the C1275, 2–175, and 183–191 TR mutants in Fig. 1 more accurately reflect the absence of any TR activity than the “0 TR” data that, only in Fig. 1, represent points not cotransfected with control TR expression vector. Other empty cDNA expression vectors did not have this weak effect. 1 μg of the luciferase reporter gene under the control of the Rous sarcoma virus promoter was transfected together with control TR expression vector.
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This synergism index is higher than that shown in Fig. 2 in which there was more Pit-1 expression vector transfected, causing higher activity of Pit-1 alone.

RESULTS

The DNA Binding and Transactivation Domains of TR Contribute to Synergy with Pit-1—As previously reported (7), the activity of the rGH promoter was much greater when both wild-type TR and Pit-1 were co-expressed than when TR and Pit-1 were independently expressed. Domains within TR that were required for this synergy and for the much weaker activation of the rGH promoter by TR alone were determined by examining the effects of TR mutations on TR activation of the rGH promoter in U937 cells (Fig. 1). TR mutants with independent or synergistic activities significantly less than wild-type TR are indicated by * (0.01 < p < 0.05) or ** (p < 0.01).

Synergistic activation was disrupted when the DNA binding domain of the TR was eliminated (2–175) or disrupted by a point mutation (C127S) in the first zinc finger or by a short deletion of the α-helix at the carboxyl-terminal end of the DNA binding domain (183–191) (Fig. 1). The 183–191 mutation also deleted a nuclear localization signal between amino acids 185 and 191 (24). The deletion of the TR ligand binding/AF-2 transactivation domain (272–461) or the deletion of a shorter segment of the ligand binding domain (237–245) less severely affected but did reduce synergism with Pit-1. Deletion of the amino-terminal AF-1 transactivation domain of TR also reduced but did not eliminate synergistic activation (2–93). Thus, TR/Pit-1 synergistic activation depends on the TR DNA binding domain and utilizes activities within both the amino-terminal and ligand binding domains. The effects of TR mutations on the much weaker activation of the rGH promoter by TR alone paralleled the effects of the same mutations on Pit-1/TR synergistic activation (Fig. 1).

The DNA Binding and Transactivation Domains of Pit-1 Contribute to Synergy with TR—Mutations in Pit-1 were tested for their effect on independent and TR synergistic activation of the rGH promoter carried in two vector backgrounds that differ in the extent to which the rGH promoter is activated by Pit-1 expression alone (7). Pit-1 alone strongly activated the rGH promoter carried in the wild-type pUC vector (9.4 ± 2.7-fold; Fig. 2B) but had a relatively minor effect (2.9 ± 1.9-fold; Fig. 2A) on the rGH promoter inserted into a pUC vector with sequences known to activate a number of linked promoters deleted (19). All experiments other than those in Fig. 2B were conducted with the modified pUC vector to minimize the contributions of non-rGH promoter sequences. The degree of synergistic activation was expressed as a synergism index (see “Experimental Procedures”) in which a value of 1.0 is indicative of the simple summation of the independent TR and Pit-1 activations. Pit-1/TR synergy was observed in both vector backgrounds although the extent of synergy was somewhat blunted in the wild-type pUC background due to the high level of activity of Pit-1 alone (7). The effects of the mutations on independent or synergistic Pit-1 activation are similar in both vector backgrounds, suggesting that the pUC element did not alter the Pit-1 activities required for independent or TR-synergistic functions.

Eliminating the Pit-1 DNA binding (124–201, 209–252, and 255–291) or transactivation (2–45 and 48–73) domains (22) abolished both independent and TR-synergistic activation of the rGH promoter by Pit-1 (Fig. 2). Therefore, as with the TR (Fig. 1), Pit-1/TR synergy depended on the integrity of both the DNA binding and transcriptional activation domains of Pit-1 required for independent activation (Fig. 2). The dependence of synergy on the Pit-1 and TR DNA binding domains is consistent with our previously reported dependence of synergy...
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Fig. 1. Both activation domains and the DNA binding domain of TR are required for TR synergy with Pit-1. CAT activity expressed from the −237/+8 rGH promoter co-transfected into human monocyte U937 cells with vectors expressing wild-type (wt) or mutated human β-1 TR cDNAs. The same panel of TR vectors was co-transfected with a vector expressing the rat Pit-1 cDNA. CAT activities that are statistically less than the activity observed when wild-type TR is expressed independently (TR only) or with Pit-1 (Co-transfected Pit-1) are indicated (*, p<0.01; **, p<0.001). The TR mutations were named according to the inclusive amino acid positions deleted, and the position of those mutations relative to previously identified landmarks (23) is depicted schematically. AF-1, amino-terminal domain including activation function 1; DBD, DNA binding domain; LBD, ligand binding domain including activation function 2. *X represents a mutation of Cys to Ser at amino acid 127 within the first zinc finger that destroys TR DNA binding. 10−8 M PMA and 10−5 M forskolin were added to all transfected cells described in this and subsequent figures unless otherwise noted. The data from six independent experiments were normalized to the CAT activity observed when Pit-1 and wild-type TR were co-expressed.

A Synergism-selective Activity in Pit-1—Interestingly, Pit-1/TR synergy also required a previously undetected, synergism-selective function. Deletion of amino acids 72 and 125 within Pit-1, shown previously to not affect Pit-1 activity (22), significantly reduced or eliminated synergism with the TR but had no effect on the activation by Pit-1 alone (Fig. 2, A and B, boxed). The equivalence of the wild-type (wt) and 72–125 mutant was also demonstrated on a minimal promoter to which a single Pit-1 binding site was attached (Fig. 3). Expression of neither the wild-type nor the 72–125 mutant Pit-1 activated a truncated rGH promoter containing little more than the TATA box (−33 to +8). In contrast, the wild-type and mutant Pit-1 equally activated the −33/+8 rGH promoter to which a single copy (1X) of the distal Pit-1 binding site, dGHF1, and overlapping Sp1 site (21) of the rGH promoter was appended. Thus, a deletion of amino acids 72 and 125 defines an activity within Pit-1 that is selectively required for TR-synergistic activation of the rGH promoter in U937 cells.

Pit-1/TR Synergism in Pituitary Cells—To test for the effects of the synergism-selective mutations in cells more closely related to those in which GH is normally expressed, we developed a Pit-1/TR synergism assay in a mouse pituitary cell line (Fig. 4). GHFT1–5 cells are immortalized at a stage during pituitary development when the Pit-1 promoter is active but before the growth hormone gene is expressed (18). In our hands, the transfected −237/+8 rGH promoter was somewhat active in GHFT1–5 cells (Fig. 4, 0, Pit-1; 0, TR; 0, PMA/forskolin (PF)) possibly because these cells contain a moderate amount of Pit-1 (18). There was no further activation of the promoter if the cells were transfected with vectors that express either Pit-1 or the TR. Co-transfection of both the Pit-1 and TR expression vectors also did not increase the activity of the rGH promoter unless the GHFT1–5 cells were cultured with 10−8 M PMA and 10−5 M forskolin (Fig. 4, +, PF), in which case the rGH promoter activity was 2.5 ± 0.9 times the sum of the increases by independent Pit-1 and TR expression. Thus, as in U937 cells, Pit-1/TR synergy in GHFT1–5 was sensitive to the cellular environment. Synergy was less dramatic than in U937 cells (Figs. 1 and 2A) possibly because of the presence of endogenous Pit-1 and/or the absence of other factors necessary for synergy.

The Synergism-selective Function Operates in Pituitary Cells and Maps to a Prolinerich Region of Pit-1—As in U937 cells (Fig. 2), the 72–125 mutant of Pit-1 did not synergize with TR to activate the rGH promoter in GHFT1–5 cells (Fig. 5A). Further deletion mapping showed that the synergism-selective activity required Pit-1 amino acids 72–100 but that amino acids 101–125 were dispensable for Pit-1/TR synergism on the rGH promoter. The wild-type and 72–125, 72–100, or 101–125 mutant Pit-1 proteins were equally capable of activating the minimal −33/+8 promoter under the control of the distal Pit-1 binding site, dGHF1, in GHFT1–5 cells (Fig. 5B). The poor activation of the 1X dGHF1 promoter by Pit-1 co-expression was possibly related to the presence of saturating, endogenous Pit-1. Activation by the exogenously expressed wild-type and mutant Pit-1 proteins became more obvious when an additional copy of the Pit-1 binding site was supplied (Fig. 5B, 2X dGHF1). Therefore, the 72–100 and 72–125 mutants were adequately expressed and were functionally competent in GHFT1–5 cells but were unable to synergize with the TR.

DISCUSSION

Despite a detailed understanding of the molecular mechanisms that underlie the activities of individual transcription factors, relatively little is known about the synergistic actions of transcription factors binding separately to distinct promoter sites (25–27). Changes in chromatin or DNA structure accompanying the binding of one factor could facilitate the binding or activity of the other factor (28–32). Alternatively, the factors could be synergistic through their concerted activities such as the recruitment to the promoter of the same, or complementary, rate-limiting components of the basal transcription apparatus (32–35). In the few examples studied, synergistic activation domains could not confer synergy when transferred to a single DNA binding domain (36, 37), suggesting that passive, simultaneous localization of multiple activation domains at the promoter is insufficient for synergy. A central issue becomes whether synergistic activation can be completely understood by the independent biochemical activities of the individual factors or whether the synergistic partners utilize distinct activities in the synergistic context.

To address whether the synergistic activation of the rGH promoter was due to mutual or complementary intrinsic activ-
ties of Pit-1 and TR, or whether Pit-1 and TR acquire or utilize new properties that they do not possess when operating in isolation, we analyzed whether domains previously identified for independent Pit-1 and TR transcriptional activity were also required for Pit-1/TR synergy. The previously described DNA binding and transactivation functions of both Pit-1 and the TR were required for the synergistic activation of the rGH promoter (Figs. 1 and 2), suggesting that the molecular mechanisms underlying synergistic and independent activation may at least partially overlap. Thus, synergy may require the amplification of independent Pit-1 and TR functions. The dependence of synergy on the intrinsic activation functions of both synergistic partners has also been observed in the few other examples in which required activities in both factorshave been investigated (15, 38, 39). Other studies have also demonstrated that at least one of the synergizing partners required their intrinsic activation functions (11, 40–45).

A novel activity defined by the Pit-1 72–100 and 72–125 mutations operated only in the TR-synergistic context in two different cell types (Figs. 2, 3, and 5). These Pit-1 amino acids were not required for independent Pit-1 activation of the rGH promoter (Fig. 2), suggesting that this synergism-selective activity depended on the specific combination of factors available to bind the rGH promoter. Amino acids 72 and 125 were also not required for activating a minimal promoter to which Pit-1 bindingsites were appended (Figs. 3 and 5B) or for cooperating with an ill defined site within the pUC vector (compare independent Pit-1 activations in Figs. 2, A and B). Synergistic activation of the PRL promoter by Pit-1 and estrogen receptor in CV-1 cells was also not affected by deleting Pit-1 amino acids 72 and 128 (38), confirming that the Pit-1/TR synergism-selective activity identified by deleting Pit-1 amino acids 72–125 and 72–100 (Figs. 2 and 5) is rGH promoter-specific.

Very few other examples of synergism-selective activities have been previously reported (15, 38, 40) but do include a deletion of Pit-1 amino acids 45–72 that selectively inhibited Pit-1/estrogen receptor synergy (38) without affecting independent Pit-1 activation of the PRL promoter. In our system, the very similar 48–73 deletion disrupted both Pit-1 independent activation as well as Pit-1/TR synergy of the rGH promoter (Fig. 2). Thus, the mechanism of synergistic activation of even the related (1–3), pituitary-specific GH and PRL promoters differs. Some promoter-specific differences in Pit-1 synergy may be at least partially mediated by dimeric status of Pit-1 dictated by the sequence of the Pit-1 binding sites (38).

Given the dependence of Pit-1/TR synergy on forskolin and PMA induction (7) (Fig. 4), the synergism-selective mutations may define a protein kinase A or C phosphorylation site that is
critical to synergy. However, the protein kinase A and C sites within Pit-1 have been well characterized both in vivo and in vitro (46), and no sites are located within amino acids 72–100. Unless altered folding of the 72–100 deletion mutant sterically blocks one of the protein kinase A or C sites in Pit-1, the Pit-1/TR synergism-selective mutant is unlikely to define a phosphorylation site selectively required for Pit-1/TR synergy.

One model of synergistic activation proposes that a novel complex formed between two synergistically active factors (15, 44, 45, 47) may possess properties not, or weakly, present in each of the individual factors. In such a model, synergism-selective activities could include amino acids required for complex formation. Indeed, a synergism-selective mutation recently reported within the pituitary-specific transcription factor P-Lim was observed to inhibit both Pit-1/P-Lim interac-

[Diagram of synergistic activation]
factors (48). It is possible that the 72–100 deletion identifies a proline-rich activator operating selectively in the synergistic context. Such structural correlations are, however, not very conclusive as demonstrated by the wild-type transcriptional properties of the 101–125 mutant Pit-1 protein (Fig. 5) that has a putative “acid blob” (49, 50) transcriptional activator deleted (Fig. 5C, -).

Regardless of the biochemical nature of the synergism-selective activator affected by deleting amino acids 72–100, it is obvious that elements of the classically defined, intrinsic transcriptional activation domain of Pit-1 (amino acids 1–72) are still required for TR-synergistic activation of the rGH promoter (Fig. 2). Whether the synergism-selective function is merely an extension of the classical transactivation domain or whether it defines a separable but co-dependent function remains unknown. The synergism-selective activator could complement a cryptic activity present in TR and therefore be observed only when TR is present, or the synergism-selective function may be independently active but not available or active until TR binds to DNA, chromatin, or to Pit-1. Numerous other mechanisms are possible, but it is evident from the current data and from synergism-selective mutations affecting Pit-1/P-Lim (15) and Pit-1/estrogen receptor (38) activation of the PRL promoter that the molecular mechanisms controlling the transcription of at least the rGH and rPRL promoters cannot be fully understood solely by studying the molecular functions of each factor in isolation.

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REFERENCES
1. Thell, L. E., and Karin, M. (1993) Endocrinology 132, 1017–1025
2. Endocrinology 132, 1017–1025
3. Endocrinology 132, 1017–1025
4. Endocrinology 132, 1017–1025
5. Endocrinology 132, 1017–1025
6. Endocrinology 132, 1017–1025
7. Endocrinology 132, 1017–1025
8. Endocrinology 132, 1017–1025
9. Endocrinology 132, 1017–1025
10. Lipkin, S. M., Naar, A. M., Kalla, K. A., Sack, R. A., and Rosenfeld, M. G. (1990) Mol. Endocrinol. 4, 1964–1971
11. Simmons, D. M., Voss, J. W., Ingram, H. A., Holloway, J. M., Brodie, R. S., Rosenfeld, M. G., and Swanson, L. W. (1990) Genes & Dev. 4, 695–711
12. Haugen, B. R., Wood, W. M., Gordon, D. F., and Ridgway, E. C. (1993) J. Biol. Chem. 268, 20818–20824