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Graded transcriptional response to different concentrations of a single transactivator

ANDREW M. KRINGSTEIN*, FABIO M. V. ROSSI*, ANDREAS HOFMANN†, AND HELEN M. BLAU‡

Department of Molecular Pharmacology, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305-5332

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ABSTRACT **Threshold mechanisms of transcriptional activation are thought to be critical for translating continuous gradients of extracellular signals into discrete all-or-none cellular responses, such as mitogenesis and differentiation. Indeed, unequivocal evidence for a graded transcriptional response in which the concentration of inducer directly correlates with the level of gene expression in individual eukaryotic cells is lacking. By using a novel binary tetracycline regulatable retroviral vector system, we observed a graded rather than a threshold mechanism of transcriptional activation in two different model systems. When polyclonal populations of cells were analyzed at the single cell level, a dose-dependent, stepwise increase in expression of the reporter gene, green fluorescent protein (GFP), was observed by fluorescence-activated cell sorting. These data provide evidence that, in addition to the generally observed all-or-none switch, the basal transcription machinery also can respond proportionally to changes in concentration of extracellular inducers and trancriptional activators.**

An issue of major importance to development is the mechanism by which a continuous gradient of an inducer or morphogen is converted into sharp transcriptional borders (1). For example, in *Drosophila* embryos the boundaries of the expression domains of *twist* and *hunchback* are thought to be sharper than would be expected if these boundaries resulted from a dose-dependent transcriptional response of these genes to their activators, dorsal and bicoid, respectively (2–5). As a result, it has been postulated that in response to an inducer, a certain "threshold" concentration of transcriptional activator is required to elicit a significant response from the general transcription machinery (1).

Several lines of evidence for threshold responses in transcription have been provided by *in vitro* transcription assays. By using a GAL4-VP16 hybrid transactivator, Carey and coworkers (6) showed that in the presence of saturating amounts of activator, increases from two to five in the number of GAL4 DNA-binding sites fused to a minimal promoter led to nonlinear increases in transcription of the reporter gene. Similarly, Laybourn and Kadonaga (7) showed that relatively small increases in the concentration of the GAL4-VP16 hybrid transactivator resulted in large increases in transcription from a chromatin-coated reporter construct containing five tandem GAL4-binding sites, leading to the conclusion that a shallow gradient of activator can be converted into a steep increase in transcription. Both of these studies suggest that once a minimal number of molecules of transcription factor are bound to a promoter, transcription is triggered maximally. An extreme interpretation of this model would be that a promoter is either off or on, precluding intermediate levels of transcription from single templates.

Patterns consistent with all-or-none responses have been observed *in vivo* in every instance in which reporter gene expression has been analyzed at the single cell level. An examination of dose-dependent gene expression induced by endogenous glucocorticoid receptors in response to dexamethasone revealed a heterogeneous distribution of cells that either did or did not express the reporter gene (8). Single cell expression patterns have been assayed by fluorescence-activated cell sorting (FACS) by using promoters fused to NF-AT- or NF-KB-binding sites or the entire IL-2 enhancer (9–11). In these systems, the response to stimulation of the T-lymphocyte antigen receptor revealed bimodal distributions of reporter gene expression, with some cells not expressing the reporter, and others expressing high levels, a pattern indicative of all-or-none transcriptional activation. In each case, an increase in the amount of inducer led to an increase in the percentage of the cells expressing the reporter, rather than an increase in the levels of reporter expressed per cell (8–11). Similar conclusions were drawn from the analysis of cells transfected with reporter constructs with or without the Simian virus (SV)40 enhancer. The presence of the enhancer resulted in a larger proportion of cells expressing the reporter, but not in an increase in expression levels within individual cells (12–16). These data all provide support for an all-or-none transcriptional activation mechanism. As a result, it has been proposed that thresholds set by the concentration of transcription factors may be a common property of inducible genes (10).

Here, we describe the first report of a graded response to increasing amounts of transactivator at the single cell level in eukaryotes. Until recently, investigation of these threshold effects was hindered by both the absence of a means for analyzing gene expression in individual live cells and by the lack of a simple methodology for controlling the amount of transcriptional activator in direct proportion to the inducer. To address the first problem, we used green fluorescent protein (GFP) as the readout of transcriptional activity, which allowed a quantitative single cell analysis by FACS. The second problem was overcome by the use of the VP16-based tetracycline (tet)-responsive transactivators (tTA and rtTA) (17, 18). These transcription factors can only bind DNA in the absence (tTA) or presence (rtTA) of the inducer tet. A great advantage of tet-regulatable systems for studying transcription is that the inducer becomes a component of the transcription factor, eliminating potential indirect effects. Since tet is known to

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Abbreviations: GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; hGH, human growth hormone; LTR, long terminal repeat; dox, doxycycline; tet, tetracycline; CMV, cytomegalovirus; SIN, self-inactivating.

*A.M.K. and F.M.V.R. contributed equally to this article.
†Present address: Institut für Biochemie I, Medizinische Fakultät der Universität zu Köln, 50931, Germany.
‡To whom correspondence should be addressed. e-mail: hblau@cmgm.stanford.edu.

enter vertebrate cells by passive diffusion (19), the concentration of the inducer inside the cells, and thus the amount of transcription factor competent to bind DNA in these cells, correlates with the concentration of the inducer added to the culture medium. Moreover, because the components of the tet system are derived from prokaryotes, interactions with endogenous vertebrate proteins that could complicate the interpretation of transactivation studies are unlikely to occur. By using this simplified model system, we determined that transcription in individual cells can be tuned to distinct levels by different amounts of inducer (and hence transactivator), indicative of a graded transcriptional response.

METHODS

Cell Culture. C57 primary mouse myoblasts were isolated as previously described (20). Cells were grown on collagen-coated dishes in F10-DMEM myoblast growth medium (GIBCO/BRL) supplemented with 20% fetal bovine serum (FBS, HyClone), basic fibroblast growth factor (bFGF, 2.5 ng/ml), penicillin G (200 units/ml), and streptomycin (200 mg/ml).

Vector Construction. To create the Hermes-HRIgfp-human growth hormone (hGH) reporter construct (Fig. 1), a cassette containing the gene for hGH (*Sal*I–*Sma*I fragment from the p0GH plasmid, Nichols Institute, San Juan Capistrano, CA) followed by an encephalomyocarditis virus internal ribosomal entry site (from the pCITE 2a plasmid, Novagen) and the coding sequence for "red shift" GFP (S65T, CLONTECH) was assembled by using standard cloning techniques (21) and inserted in the self-inactivating (SIN)-Retrotet vector (22) downstream of the O7-cytomegalovirus (CMV) promoter. To generate the RTAb(−) and RTAb(+) viral vectors, both the tTA- and rtTA-coding sequences (kind gift of Hermann Bujard, Zentrum für Molekulare Biologie, Heidelberg, Germany) were amplified by PCR to insert an *Nco*I site at the ATG and a *Bam*HI site downstream of the stop codon. The PCR products were verified by sequencing and cloned into the *Nco*I and *Bam*HI sites of the MFG vector (23).

Transfections and Transductions. Infection of the C57 myoblasts with the RTAb(−) or RTAb(+) virus was carried out as described (24). Subsequently, both the C57 RTAb(−) and C57 RTAb(+) myoblasts were infected with the reporter virus by using the same infection protocol. After infections, both cell populations were cultured under induced conditions [no doxycycline (dox), RTAb(−); 1 μg/ml dox, RTAb(+)] for 4 days and then sorted based on GFP expression on a Becton Dickinson FACStar. All subsequent FACS analysis was performed on a Becton Dickinson FACScan.

hGH Protein and RNA Analysis. Quantitation of hGH secretion was performed by radioimmunoassay with a Nichols Diagnostic kit. All hGH measurements were normalized to total cell protein determined by Bradford assay (Bio-Rad). Kinetics of induction and deinduction (Fig. 4) were performed by culturing cells in fully inducing dox conditions [0 μg/ml RTAb(−); 1 μg/ml RTAb(+)] and fully deinducing conditions [0.001 μg/ml RTAb(−); 0 μg/ml RTAb(+)], after which the dox conditions were reversed in each sample. After this reversal, hGH values were measured at 0, 3, 8, 16, 24, 48, and 72 hr. The dose response data (Fig. 3) were collected from cells maintained in the indicated dox conditions for three days [0, 0.0001, 0.0002, 0.0005, 0.001, 0.005, 0.01, and 0.1 μg/ml dox, RTAb(−); 0, 0.001, 0.01, 0.05, 0.1, 0.5, 1, and 5 μg/ml dox, RTAb(+)].

Experimental design of the Northern analysis of hGH kinetics was the same as for hGH protein kinetics. Total RNA was collected (RNeasy Kit, Qiagen) and separated on a 1% denaturing agarose gel containing 17% formaldehyde. Separated RNA was transferred to a nylon membrane and hybridized with an [α-^{32}P]deoxycytidinetriphosphate-labeled probe complementary to the hGH sequence. Quantitative data of hGH RNA kinetics were collected by PhosphorImaging analysis (Molecular Dynamics).

RESULTS

Design of Model Systems. A bicistronic reporter system was developed (Hermes-HRIgfp-hGH, Fig. 1) that allows both a sensitive overall determination of gene expression in cell populations by using hGH and flow cytometric analysis of the distribution of gene expression within individual cells by using GFP (25–27). The production of the two proteins from a single message was ensured by the use of an internal ribosome entry site (28). In all studies described herein, a more potent analog of tet, dox was used. A synthetic promoter consisting of heptamerized tet operator-binding sites (O_7) (17, 18) was cloned upstream of the CMV core promoter (from position −53 to +2) to direct transcription of the bicistronic message. Seven binding sites were included to allow detection of potential transcriptional thresholds in intact cells because at least five sites were required to observe this phenomenon using the same transcriptional activator, VP16, *in vitro* (7). Populations of cells were generated that stably expressed the transcription factors, tTA or rtTA, driven by the long terminal repeat (LTR) of a constitutively active retroviral MFG vector designated as RTAb(−) and RTAb(+) vectors (23). The tTA is a fusion protein of the viral transactivator VP16 and the classic *Escherichia coli* tet repressor that tightly binds the tet operator in the absence of dox [RTAb(−)] (17). The rtTA is a fusion protein of VP16 and a mutant tet repressor that only binds the tet operator in the presence of dox [RTAb(+)] (18). The bicistronic reporter construct was delivered to these cells in a SIN retrovirus (29). The use of a SIN vector avoided potential interference with the tet-sensitive CMV promoter because transcription from the viral LTR after integration was precluded due to deletions in the U3-enhancer region of the virus. The use of retroviruses ensured random integration of the reporter into the genome of the target cells. Viral integration appeared potentially important in view of previous findings by others that threshold responses *in vitro* were observed only with chromatin-coated templates (7).

Polyclonal Nature of Transduced Populations. To preserve polyclonality in the cell sample, we used retroviral vectors that could be produced at high titer and introduced into primary myoblasts (20), rather than immortalized cell clones, at high efficiency (>90%) (24). Myoblasts (4×10^5) were infected first with either the RTAb(−) or RTAb(+) retrovirus, followed by the reporter retrovirus. After infection, cells were maintained for 4 days in conditions that provided continuous induction and maximal transactivation of the reporter. The cells were then sorted by FACS on the basis of GFP expression to select for the subpopulation of cells (15%) that had integrated and expressed the reporter gene (Fig. 2 *A* and *B*), a frequency

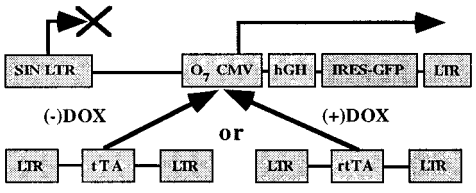

FIG. 1. Binary tet-inducible reporter system. A SIN retroviral backbone was used for the reporter virus (Hermes-HRIgfp-hGH) to eliminate transcriptional interference from the viral LTR. The tet-sensitive transactivators (either tTA or rtTA) are provided constitutively from a second retrovirus [Retrotet RTAb(−) or Retrotet RTAb(+)]. Under proper dox conditions, these transactivators bind to the tet-operator sites (O7) fused upstream of the CMV core promoter, inducing transcription of a bicistronic mRNA encoding hGH and GFP from the reporter vector.

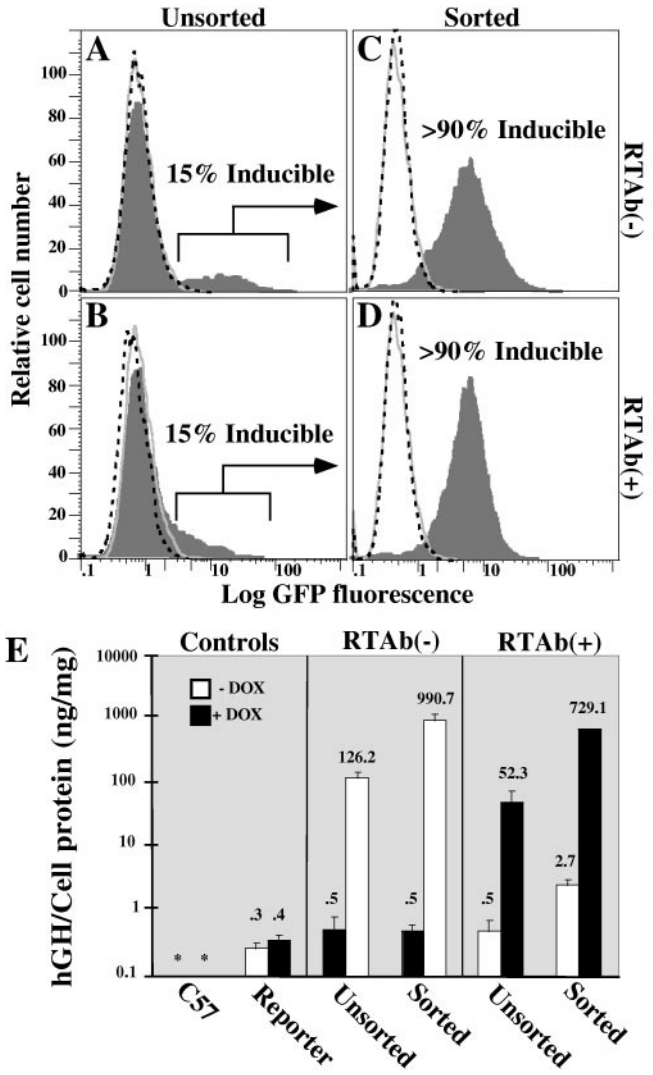

FIG. 2. Purification of inducible populations. (*A* and *B*) C57 myoblasts infected with both the Hermes-HRIgfp-hGH and either the RTAb(−) or RTAb(+) were analyzed by FACS after culture for 4 days under uninduced conditions (1 μg/ml dox for RTAb(−); 0 μg/ml dox for RTAb(+); dashed curves) and induced conditions (0 μg/ml for RTAb(−); 1 μg/ml for RTAb(+); filled dark gray curves). C57 cells transduced with the reporter only were analyzed as a measure of background fluorescence (unfilled light gray curves). For both RTAb(−) and RTAb(+) systems, 15% of the cells shifted to fluorescence levels above the background provided by the reporter construct alone. These inducible subpopulations were purified by sorting and then reanalyzed (*C* and *D*). After sorting, both RTAb(−) and RTAb(+) cells demonstrated >90% inducible subpopulations when cultured under induced conditions (filled dark gray curves), yet demonstrated low background fluorescence under uninduced conditions (dashed curves). Fig. 2*E* shows hGH secretion from the unsorted and sorted populations from *A-D*, verifying that the FACS-enrichment process based on GFP expression leads to increased inducibility.

suggesting that >97% of the cells harbored a single active copy, according to the Poisson distribution. Based on the substantial size of the isolated subpopulations (9.6×10^5) and the duplication time of primary myoblasts in our culture conditions (24 hr), at least 6.0×10^4 individual cells were infected. These conditions allow the generation within 1 week of a polyclonal population with a diversity of integration sites.

Transductions and FACS Enrichment for Inducible Populations. To obtain a population in which all of the cells responded to dox, the cells infected with either RTAb(−) or RTAb(+) were isolated under induced conditions based on GFP fluorescence by using the FACS, expanded in culture for 4 days, and then reanalyzed. Both of the selected populations showed a marked enrichment for expression of GFP, as >90% of the cells were GFP positive (Fig. 2 *C* and *D*). Under noninducing conditions, GFP fluorescence returned to the level detected in cells containing only the reporter construct, indicating that essentially all of the cells were responsive to dox. To verify that the FACS enrichment based on GFP provided a means of selecting for inducible cells, hGH secretion was measured from unsorted and sorted populations, under both uninduced and induced conditions. As shown in Fig. 2*E*, unsorted populations are already capable of inducing hGH up to 100-fold above background; however, the FACS enrichment process leads to an additional induction in both the RTAb(−)- and RTAb(+)-infected populations of ultimately 1,000-fold.

Dose Response, Kinetics, and Single Cell Analysis. A dose response curve revealed that accumulation of GFP and hGH protein levels, assayed by FACS and radio-immunoassay, respectively, correlated well in both RTAb(−) and RTAb(+) infected cells, although the hGH assay was more sensitive in detecting low levels (Fig. 3). In agreement with findings reported by others (17, 18), the concentration dependence of

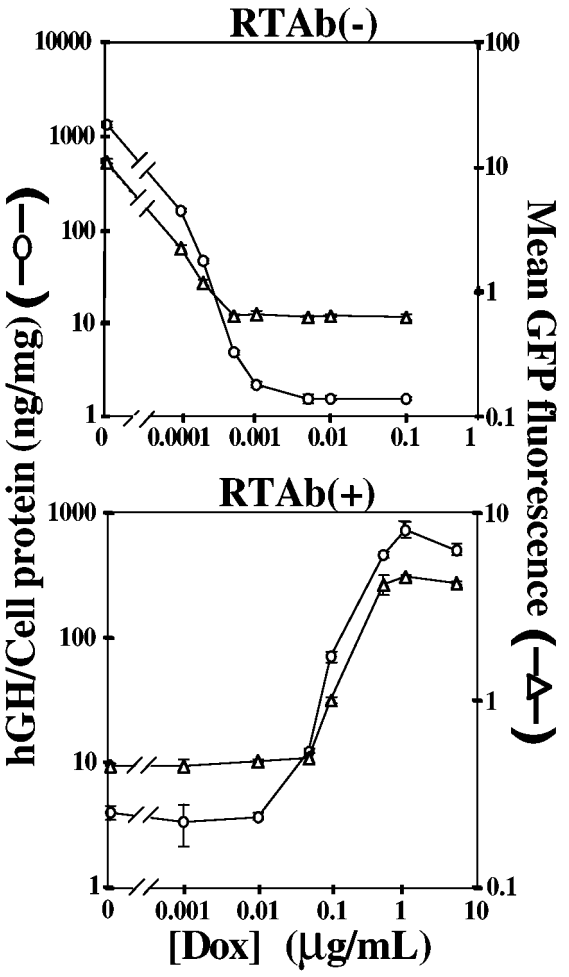

FIG. 3. Dose response of binary tet-inducible system. dox dose response of hGH expression (○) and GFP expression (△) for both RTAb(−) and RTAb(+) systems. hGH and GFP expression of RTAb(−) and RTAb(+) myoblasts cultured in eight different doses of dox were assayed after 3 days under these conditions [RTAb(−) dox doses: 0, 0.0001, 0.0002, 0.0005, 0.001, 0.005, 0.01, and 0.1 μg/ml; RTAb(+): 0, 0.001, 0.01, 0.05, 0.1, 0.5, 1, and 5 μg/ml]. Secreted hGH was measured by radioimmunoassay and normalized to total cell protein. GFP expression was determined by FACS.

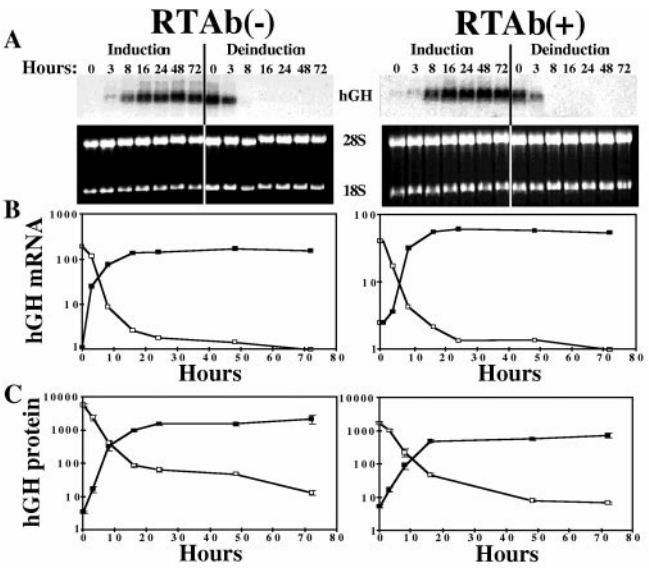

FIG. 4. Kinetic analysis of hGH inducibility. (*A*) Northern analysis of hGH transcript levels measured over time from the sorted C57 populations containing either the RTAb(−)- or RTAb(+)-based version of the reporter system. (*B*) Density values of the blots shown in *A* determined by PhosphorImaging analysis. Values are plotted as fold induction over lowest value. (*C*) Time course analysis of hGH protein secretion from the same cell populations as in *A*.

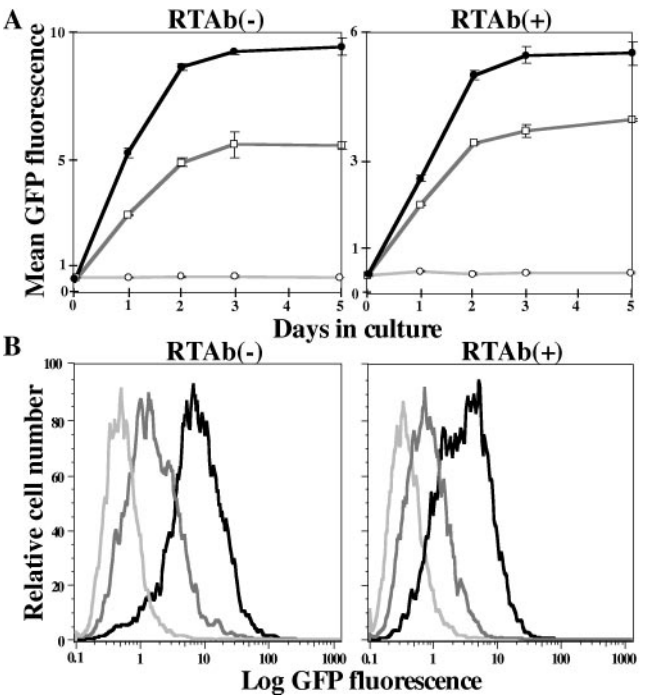

FIG. 5. Kinetic analysis of GFP accumulation and single cell analysis of expression. (*A*) GFP expression as measured by FACS has reached a plateau after 3 days in culture at low, intermediate, and high levels of dox [RTAb(−) dox doses: 0.001 μg/ml (light gray), 0.0001 μg/ml (dark gray), and 0 μg/ml (black); RTAb(+): 0 μg/ml (light gray), 0.33 μg/ml (dark gray), and 1.0 μg/ml (black)]. (*B*) Overlays of histogram plots of GFP fluorescence derived from FACS analysis of the dose response in Fig. 3 at three selected doses [RTAb(-): 0.1 μg/ml (light gray), 0.001 μg/ml (dark gray), and 0 μg/ml (black); RTAb(+): 0 μg/ml (light gray), 0.1 μg/ml (dark gray), and 5 μg/ml (black)]. These overlays demonstrate the uniform shift of the population to intermediate and high expression levels as would be predicted from an individual cell-graded response. The uniform shift observed in these plots is representative of the shifts observed at each concentration of dox used in the dose response experiment in Fig. 3.

the tTA and rtTA systems was found to differ by several orders of magnitude. At a concentration of 0.001 μg/ml dox, the cells expressing tTA had maximally shut off hGH and GFP expression; however, 1.0 μg/ml dox was required for a maximal response in rtTA-expressing cells. The kinetics of induction and deinduction of the system were determined by quantitation of hGH mRNA (Fig. 4 *A* and *B*) and hGH protein (Fig. 4*C*). Half-maximal induction was evident by 8 hr (mRNA) and 16 hr (protein), at which time the induction had reached steady–state level. The deinduction of gene expression was faster, reaching half-maximal levels after 3 hr. Because GFP is more readily assayed at the single cell level, it was used in subsequent assays.

To eliminate the possibility that the dose-dependent accumulation of reporter gene product was due to differential kinetics of induction upon treatment with suboptimal concentrations of inducer, we determined the kinetics of GFP induction without dox, at intermediate, and at high concentrations of dox. As shown in Fig. 5*A*, both tTA and rtTA-expressing cell populations reached maximal GFP expression simultaneously, but stabilized at markedly different levels. Thus, we decided to determine GFP expression on a cell by cell basis upon treatment with dox for 72 hr, a time at which gene expression had reached plateau in each case. We used the FACS to analyze the distribution of GFP expression within these samples. As shown in Fig. 5*B*, essentially the entire cell population shifted its GFP fluorescence in a uniform fashion in response to various doses of dox. Although results for only three concentrations are shown here, similar homogenous shifts were observed for all dox concentrations tested for which the mean values are shown in Fig. 3.

DISCUSSION

Our results provide the first unequivocal evidence that graded transcriptional responses can occur and that all-or-none responses are not a property shared by all inducible genes. Our data show that increases in the concentration of the inducer, and consequently active transcription factor, can result in a graded increase in transcription from an integrated reporter. This is apparent from the progressive shift in GFP expression by individual cells within a polyclonal population. Moreover, a single type of transactivator, either tTA or rtTA, present at different active concentrations can mediate such a graded response. This observation suggests that the number of binding sites occupied by either of these transcription factors is the major determinant of the level of transcriptional activity. Since in our experiments the transcriptional readout is the sum of many individual transcriptional initiation events occurring on single templates (within individual cells) over time, we cannot distinguish whether the responses we observed are mediated by either a graded change in the number of active complexes forming per template or a graded change in the probability that an individual template will be active at a fixed rate rather than off at any given moment. Resolution would require that the number of RNA polymerase molecules present on each single template at any instant in time be determined, an experiment that is not technically feasible at this time. Nonetheless, our findings provide the first *in vivo* evidence consistent with the hypothesis based on *in vitro* experiments that the primary function of a transcriptional activator is to modulate the rate of transcriptional initiation events from individual templates (30). Irrespective of the mechanism, we provide evidence for an alternative to the prevailing view that dose responses are mediated by the expression of a specific gene in an increasing number of individual cells, rather than by a graded increase in the expression of that gene within each cell.

Although a dose-dependent transcriptional response to tet or dox has previously been reported in mammalian cells (17, 18), the potential of these experiments to determine the underlying mechanism of transcriptional activation was limited by problems that arise by interpreting results obtained from cell populations assayed in bulk. As illustrated in Fig. 6, a dose-dependent activation of gene expression in a cell population assayed biochemically en masse (Fig. 6*A*) could arise through two distinct single cell mechanisms (Fig. 6*B*): (*i*) a graded response or (*ii*) a threshold response. The mean level of expression of a gene by a cell population measured in a bulk assay could reflect either similar levels of gene expression in all cells (graded response), or the average of different subsets of cells that either express or do not express the gene (threshold response). To distinguish between these two mechanisms, a single cell assay of reporter gene expression is required (Fig. 6*C*). In this report, we used FACS analysis to examine induction of GFP by dox on a cell by cell basis within a polyclonal population. As shown in Fig. 5*B*, these findings were similar in two cases, tTA and rtTA, which are transcriptional activators that differ in their dox-binding domains. Treatment of cell populations harboring either transactivator with no dox or intermediate or high levels of dox led to a homogeneous increase in GFP expression within all of the cells. No evidence of a bimodal distribution of expression was observed at intermediate levels of dox, as would be expected in the case of an all-or-none threshold response (Fig. 6*C*).

There are several reasons why our findings contrast with previously published results showing threshold responses. *In vitro* results demonstrating nonlinear responses to increasing amounts of activators (6) could have resulted from the presence of a titratable repressor in the extracts used that is not normally available *in vivo*, or from the lack of active forms of relevant components of the basal transcription machinery. All-or-none responses have also been shown in intact cells for the glucocorticoid receptor, NF-AT and NFkB (8–11). The interpretation of these experiments, however, is complicated by indirect effects due to the use of inducers, such as hormones or cytokines, that have intracellular effects that are modulated by signal transduction pathways or lead to interactions of two or more transcription factors (31, 32). Strong evidence that indirect effects can yield threshold responses is provided by studies of kinases that act sequentially, such as the MAP kinase cascade (33, 34). Such complex indirect effects also could be the basis for the threshold response reported by Groudine and coworkers in (14, 16) single cell analyses, although these studies are not directly comparable with ours as they analyze the effects of distal enhancers on promoters rather than the effects of activators binding in close proximity to a transcription initiation site. Our studies circumvent the problem of indirect effects, as the concentration of transactivator, tTA or rtTA, available to bind the promoter is directly dependent on the concentration of the inducer, dox. Moreover, because all components of the two transcriptional systems studied here are prokaryotic, yet assayed in a eukaryotic cell background,

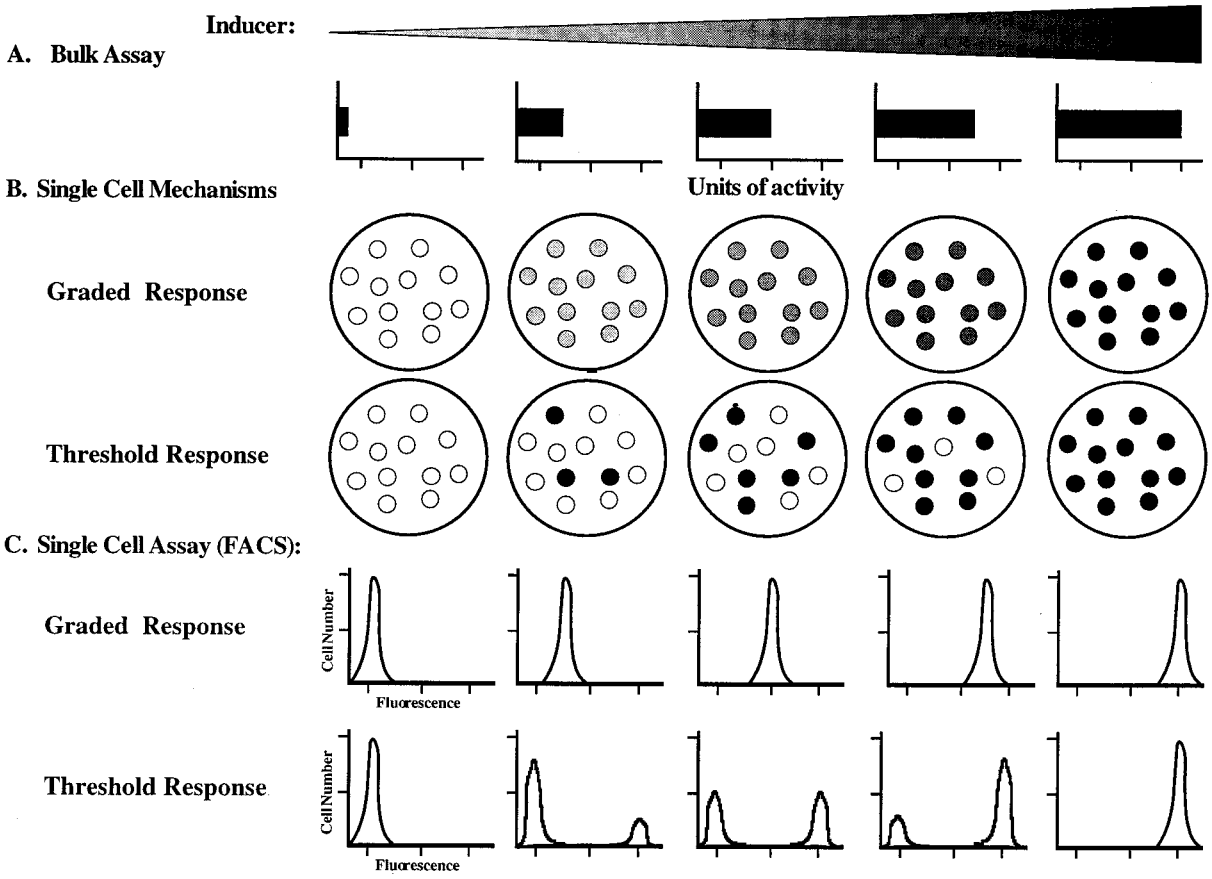

FIG. 6. Idealized mechanisms of graded vs. threshold transcriptional activation. An overall graded response of a heterogeneous population observed through a bulk assay of pooled cells (*A*) can be the result of two distinct underlying single cell mechanisms. (*B*) Each individual cell might have a graded response to increasing amounts of inducer, or each of the cells might respond in an on/off threshold manner, but vary with respect to the concentration of inducer necessary to cross the threshold. (*C*) These two mechanisms can be distinguished by assessing the distribution of single cell expression levels in a population exposed to different levels of inducer: In a population demonstrating a true graded response, the entire population should shift homogeneously as inducer is increased. This will result in a unimodal distribution of the population at all levels of inducer. A population demonstrating an individual cell threshold response will produce a bimodal curve at intermediate levels of inducer, with one mode centered at a level corresponding to "off," and one mode centered at "on."

components of the endogenous signal transduction machinery are not likely to be involved. In support of this view, evidence by others has shown that the VP16 activation domain common to both of these transactivators interacts directly with the basal transcription machinery and does not appear to require interaction with additional cofactors (35–38). Although Hop and colleagues (39) have reported the occurrence of all-or-none responses in cells expressing a gene under the control of tTA, this discordance with our results is likely to be due to the lack of sensitivity of the assays used (immunofluorescence and visual assessment), which cannot be used to assess a range of expression levels like the FACS used here. In summary, signal transduction pathways that mediate the effects of an inducer on transcription, as well as the interaction of activators with other cofactors on promoters, may be the basis of previously observed threshold responses, variables that are eliminated in the studies reported here.

In conclusion, we provide evidence that transcriptional activation need not proceed in a threshold manner. Although all-or-none responses have been well documented in some cases (6–13), our data raise the possibility that in the creation of sharp expression boundaries observed *in vivo* (1–5) the presence of a continuous gradient of a single factor may not suffice to produce this phenomenon. Instead, gradients of positive and negative factors may intersect to establish that boundary (40). The system presented here should allow an analysis of the cellular mechanism involved in converting a graded transcriptional response into a threshold response. Furthermore, our system is the first to be described that is characterized by a dose-dependent response at the single cell level. This facet opens the way to experiments designed to test the effects of gene dosage within single cells and the effects of the concentration of factors that mediate either differentiation or growth (41, 42), within the same population. Thus, predictions regarding the role of gene dosage in cell fate decisions (40, 44–49) can now be tested by inducing the expression of specific regulators at well controlled levels.

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