Kinetically Defined Mechanisms and Positions of Action of Two New Modulators of Glucocorticoid Receptor-regulated Gene Induction*

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Most of the steps in, and many of the factors contributing to, glucocorticoid receptor (GR)-regulated gene induction are currently unknown. A competition assay, based on a validated chemical kinetic model of steroid hormone action, is now used to identify two new factors (BRD4 and negative elongation factor (NELF)-E) and to define their sites and mechanisms of action. BRD4 is a kinase involved in numerous initial steps of gene induction. Consistent with its complicated biochemistry, BRD4 is shown to alter both the maximal activity (A\text{max}) and the steroid concentration required for half-maximal induction (EC\text{50}) of GR-mediated gene expression by acting at a minimum of three different kinetically defined steps. The action at two of these steps is dependent on BRD4 concentration, whereas the third step requires the association of BRD4 with P-TEFb. BRD4 is also found to bind to NELF-E, a component of the NELF complex. Unexpectedly, NELF-E modifies GR induction in a manner that is independent of the NELF complex. Several of the kinetically defined steps of BRD4 in this study are proposed to be related to its known biochemical actions. However, novel actions of BRD4 and of NELF-E in GR-controlled gene induction have been uncovered. The model-based competition assay is also unique in being able to order, for the first time, the sites of action of the various reaction components: GR \text{EC}_{50} < \text{Cdk9} < BRD4 \leq \text{induced gene} < \text{NELF-E}. This ability to order factor actions will assist efforts to reduce the side effects of steroid treatments.

Steroid hormone action has been studied intensively since the discovery of steroid hormone receptors almost 50 years ago (1) for three reasons. First, the receptors are obligatory mediators for the biological responses of the five classes of endogenous steroid hormones. Second, steroids and their receptors provide an ideal system in which to define the molecular mechanisms that regulate the controlled induction of gene transcription. Such studies have resulted in the identification of many species or factors that are required for transcription initiation, elongation, and termination (2–4). Third, the clinical benefits of steroid hormones are often severely limited by undesired side effects (5–8). A better molecular understanding of steroid action should lead to fewer side effects, which would significantly expand the clinical applications of steroids in treating human pathologies.

The majority of factors identified to date for steroid-regulated gene induction participate in the early steps of transcription initiation (e.g. chromatin remodeling, assembly of the transcription preinitiation complex, and synthesis of the first oligonucleotides) (9). Two interesting classes of factors for steroid-regulated gene transcription are coactivators and corepressors (2–4). One of the earliest and most widely studied of these factors is TIF2/GRIP1, which is a member of the p160 family of coactivators with molecular masses of about 160 kDa (10, 11). Despite the detailed understanding of many of the factors involved in transcription initiation, much less is known about factors involved in elongation, termination, and release of the initially transcribed RNA. Furthermore, the overall number of molecular reactions between steroid binding to its cognate receptor and the appearance of product has yet to be determined. Because the precise sequence of events leading to the final product has not been identified, much less characterized, it is difficult to use classical biochemical or molecular biology approaches to determine the factors participating in each step. Similarly, the order of factor action, as opposed to binding, in the overall sequence of events is virtually unknown.

Here, we utilize an alternative approach to the problem of undefined reaction steps with unknown factors and the position of these steps in the overall reaction sequence. We first identify those agents that have any effect on receptor-mediated transcription (12). We then analyze pairs of these factors using a competition assay that is based on the analysis of a chemical kinetic scheme of gene expression. This assay yields mechanistic information about how and where each of the competing factors acts (13). This different approach is made possible by the observation that the dose-response curve for steroid-regulated gene expression is usually non-cooperative with a Hill coefficient of 1, as in a classical Michaelis-Menten curve. For
such regulated genes, an experimentally validated chemical kinetic model has been developed that explains ligand regulation of gene induction, gene repression, and the partial agonist activity of antisteroids (13–18).

This kinetic scheme models gene expression as a chain of complex building chemical reactions consistent with what is known about the process. The constraint that the dose-response curve be noncooperative is extremely stringent and renders the system to have a very specific form. The result is a mathematical formula for the dose-response curve as a function of the steroid in the presence of added cofactors. The formula shows that factors only affect the dose-response curve through the maximal activity ($A_{\text{max}}$) of gene expression and the steroid concentration required for half-maximal induction ($EC_{50}$) of steroid-mediated gene expression. These parameters vary as a function of how and where the factors act. Information about each factor is then obtained from a series of graphs, similar to Lineweaver-Burk graphs in biochemistry, that plot combinations of the $A_{\text{max}}$ and $EC_{50}$ values from the competition assays. Specifically, the graphs reveal the number of sites at which each factor acts, their kinetically defined mechanism of action, and their relative order of action in the overall sequence leading to changes in gene expression (12–21). This approach has been extensively validated, and the derived information is novel and not available from other methodologies.

Implicit in the model are new chemical kinetic and mechanistically descriptive alternatives to label the actions of a modulatory factor. The traditional terms of “coactivator” and “corepressor” consider only the ability of factors to increase and decrease, respectively, the total amount of gene product without reference to mechanism. In fact, chemical kinetics tell us that the final product can increase due to two opposite actions: (a) a species that accelerates an intermediate step or (b) a factor that decelerates a currently utilized step, thereby allowing a more productive but otherwise avoided step to be followed (19). Under the traditional terminology a coactivator could become a corepressor under different conditions, although its mechanistic action is invariant (17). Therefore, we adopt the terms “accelerator” and “decelerator,” which describe how a given species alters the reaction kinetics at that step where each species acts. An accelerator’s or decelerator’s local action is invariant, but it could increase or decrease the final gene product, depending on how and where it acts with respect to other factors involved. In the language of chemical enzyme kinetics, an accelerator acts like an activator, whereas a decelerator acts like an inhibitor. Like inhibitors in enzyme kinetics, decelerators can be competitive, uncompetitive, and noncompetitive.

A key feature of the chemical kinetic scheme for steroid hormone action is the concentration-limited step (CLS), which is the equilibrium analog of a rate-limiting step in enzyme kinetics. All reaction steps (known and unknown) in the scheme can be grouped according to whether they are positioned before the CLS, at the CLS, and or after the CLS. Accelerators are limiting in the reaction steps before and at the CLS, whereas, after the CLS, products are limiting, and accelerators are abundant (14). The chemical kinetic scheme shows that a competition assay between any two species can be used to classify the two species as accelerators or decelerators, to specify the number of steps at which they act, to provide the relative order in which they function, and to infer their positions of action relative to the CLS (13, 16–19, 21). These results are not obtainable by any other current methodology.

Recent reports have demonstrated the utility of this competition assay in uncovering and characterizing actions of species previously thought to be unconnected to glucocorticoid receptor (GR)-regulated gene transcription, such as PA1 (Pax2 transactivation domain interaction protein-associated protein 1) (21) and assorted pharmaceuticals (12). In addition, several well known transcription factors have been found to modulate GR-regulated gene induction, such as components of the transcription negative elongation factor (NELF) complex (20) and CDK9 (16), which is the kinase subunit of P-TEFb. The NELF complex is a negative regulator of RNA polymerase II (Pol II), enforcing a pause in early elongation about 100 bp after transcription initiation. P-TEFb triggers the release of paused Pol II and the initiation of efficient elongation via phosphorylation of the NELF complex through the action of CDK9. P-TEFb/CDK9 also functions as a transcription elongation factor by phosphorylating Pol II on Ser-2 residues of its C-terminal domain (CTD), thus enabling the recruitment of necessary chromatin remodeling and RNA maturation factors (22, 23).

Another transcription factor, BRD4, has been identified as a kinase involved in the initial steps of gene induction. BRD4 is a bromodomain protein that binds to acetylated lysines of histones and remains associated with chromatin throughout mitosis, bookmarking actively transcribed genes. However, BRD4 also functions throughout the cell cycle. BRD4 interacts with transcription and chromatin-modifying factors and recruits them to enhancers and promoters (9). One of the major functions of BRD4 is to recruit P-TEFb to the transcription initiation complex. However, it was recently discovered that BRD4 is a kinase that also phosphorylates the Ser-2 of the RNA Pol II CTD, facilitating the transition of RNA Pol II from initiation to elongation (24, 25). Thus, BRD4 is an active participant in transcription (9, 24, 26). Furthermore, BRD4 participates in coordinating the early steps of transcription initiation and elongation through its interactions with and cross-regulations of P-TEFb and the general transcription factor TFIH (24, 26).

The purpose of the present study was to use the competition assay derived from the chemical kinetic model of steroid hormone action to investigate the possible roles of both BRD4 and the NELF-E subunit of the NELF complex in glucocorticoid induction of target genes. We report that both significantly modulate the $A_{\text{max}}$ and $EC_{50}$ of GR-regulated gene induction. Furthermore, BRD4 and the NELF-E subunit physically interact. In competition assays with CDK9, NELF-E, and the accelerator TIF2, BRD4 can act at a minimum of three sites with site-dependent activity as either a competitive decelerator or an accelerator. Surprisingly, the mode and relative positions of action of two of the three sites were unaffected by mutations.

4The abbreviations used are: CLS, concentration-limited step; GR, glucocorticoid receptor; NELF, negative elongation factor; Pol II, polymerase II; CTD, C-terminal domain; Dex, dexamethasone; BIC, Bayesian information criterion; LUC, luciferase; dnCDK9, dominant negative CDK9; wtCDK9, wild type CDK9; wtBRD4, wild type BRD4; mtBRD4, mutant BRD4.
that abrogate either the BRD4 interaction with CDK9 or CDK9 kinase activity, although one site of action was eliminated. By determining the kinetic behavior of various mutants of BRD4 and CDK9, we relate the kinetic properties to some of the multiple roles of BRD4, including recruitment and phosphorylation of P-TEFb and phosphorylation of RNA Pol II CTD. This new information will be helpful in reducing the side effects of glucocorticoid therapies.

**Experimental Procedures**

Unless otherwise indicated, all experiments were with U2OS cells maintained at 37 °C; all other operations were performed at room temperature.

**Chemicals**—Dexamethasone (Dex) was purchased from Sigma. The Dual-Luciferase reporter assay was from Promega (Madison, WI).

**Plasmids**—Renilla-TS reporter, rat GR (pSG5-GR), GREtk-LUC, and pSG5-TIF2 (27) and FLAG/BRD4 (28) have been described previously. FR-LUC reporter is from Stratagene (La Jolla, CA). Human mBRD4 with point mutations at F1357A/E1358A/E1359A (29) was donated by Eric Verdin (University of California, San Francisco, CA). Human FLAG/NEFL-E (30) was from Rong Li (University of Texas Health Science Center, San Antonio, TX). Rc/CMV-dnCkd9 (D167N) (Xavier Grana, Temple University, Philadelphia, PA) was generously provided. pSG5-FLAG-NEFL-E was prepared, probed, and visualized by ECL detection reagents as described by the manufacturer (Amersham Biosciences) or by using 0.7 µl of Lipofectamine (Invitrogen) or Fugene 6 (Roche Diagnostics) per well according to the manufacturer’s instructions. The total transfected DNA was adjusted to 300 ng/well of a 24-well plate with pBluescriptII SK+ (Stratagene, La Jolla, CA). The molar amount of plasmids expressing different protein constructs was kept constant with added empty plasmid or plasmid expressing human serum albumin (27).

**Antibodies and Immunoblotting**—Anti-BRD4, -CDK9, and -NELF-E antibodies. To test for direct interaction of BRD4 and NELF-E in vitro, purified FLAG-BRD4 was immobilized on anti-FLAG M2 agarose beads (Sigma) and incubated with increasing amounts of purified recombinant NELF-E protein for 2 h at 4 °C. The beads were washed twice with 50 mM Tris, pH 8.0, 150 mM NaCl, and 0.2% Nonidet P-40 and immunoblotted with anti-BRD4 and -NELF-E antibodies.

**Two-factor Competition Assays**—The basic protocol for gene induction (16) was followed except as noted for 4 × 4 (all 16 combinations of four concentrations of both factor 1 and factor 2), 3 × 6 (three concentrations of factor 1 and 6 of factor 2), and 2 × 8 (2 concentrations of factor 1 and 8 of factor 2) assays with four concentrations of Dex, all in triplicate, for a total of 192 or 216 wells. Briefly, triplicate samples of cells were seeded into 24-well plates at 20,000 cells/well and transiently transfected the following day with luciferase reporter (GREtkLUC) and DNA plasmids for rat GR plus the factors being examined by using 0.7 µl of Lipofectamine (Invitrogen) or Fugene 6 (Roche Diagnostics) per well according to the manufacturer’s instructions. The total transfected DNA was adjusted to 300 ng/well of a 24-well plate with pBluescriptII SK+ (Stratagene, La Jolla, CA). The molar amount of plasmids expressing different protein constructs was kept constant with added empty plasmid or plasmid expressing human serum albumin (27).

**Summary of Theory and Application of Dual Action Factors**—As shown previously (13, 14, 17, 18) the dose-response curve has the following form,

\[
[P] = \frac{A_{\text{max}} [S]}{E_{50} + [S]} \quad (\text{Eq. 1})
\]

where [P] is the concentration of the final gene product and [S] is the steroid concentration. Added factors can affect \(A_{\text{max}}\) and \(E_{50}\) depending on their mechanism and site of action with respect to the CLS. For a factor acting at site \(i\) before the CLS, \(A_{\text{max}}\) and \(E_{50}\) have the following formulas.

\[
\frac{A_{\text{max}}}{E_{50}} = \frac{q_i X_i (1 + \alpha_i \beta_i [l_i])}{1 + \gamma_i [l_i]} \quad (\text{Eq. 2})
\]
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\[
\frac{1}{EC_{50}} = C_2 + C_3 \frac{q_i C_i + \alpha_i q_i [I_i]}{1 + \gamma q_i [I_i]} + C_4 \frac{q_i X_i' (1 + \alpha_i \beta_i q_i [I_i])}{1 + \gamma q_i [I_i]}
\]

(Eq. 3)

For the factor acting at the CLS, they have the form,

\[
\frac{A_{max}}{EC_{50}} = C_2 + C_3 \frac{q_i C_i + \alpha_i q_i [I_i]}{1 + \gamma q_i [I_i]}
\]

(Eq. 4)

\[
\frac{1}{EC_{50}} = C_2 + C_3 \frac{q_i [C_i + \alpha_i q_i [I_i]]}{1 + \gamma q_i [I_i]}
\]

(Eq. 5)

and for the factor acting after the CLS, they have the form,

\[
\frac{A_{max}}{EC_{50}} = C_2 + C_3 \frac{q_i [C_i + \alpha_i q_i [I_i]]}{1 + \gamma q_i [I_i]}
\]

(Eq. 6)

\[
\frac{1}{EC_{50}} = C_2 + C_3 \frac{q_i [C_i + \alpha_i q_i [I_i]]}{1 + \gamma q_i [I_i]}
\]

(Eq. 7)

where \(X_i'\) is the total concentration of the accelerator, and the \(C\) constants are context-dependent parameters that depend on the unobserved factors in the system.

The formulas have some notable features that distinguish them. For example, the plot of \(A_{max}/EC_{50}\) always goes through the origin for accelerators before or at the CLS, \(1/EC_{50}\) is not affected by an accelerator at the CLS, and the only type of decelerator allowable after the CLS is a competitive decelerator. For a competitive decelerator before the CLS, \(EC_{50}/A_{max} \propto 1 + \gamma [I]\) (i.e. it is a linear function of the competitor).

The formulas can be generalized to multiple factors. For one factor acting before or at the CLS and one after the CLS, the formula for \(A_{max}/EC_{50}\) is as follows,

\[
\frac{A_{max}}{EC_{50}} = \left( C_1 + C_3 \frac{q_i X_i' (1 + \alpha_i \beta_i q_i [I_i])}{1 + \gamma q_i [I_i]}) \right)
\]

(Eq. 8)

and a similar formula can be written for all the possible locations of two factors with respect to the CLS. Hence, if a single factor \([B]\) acts as a competitive decelerator before or at the CLS and as an accelerator after the CLS, \(A_{max}/EC_{50}\) has the form,

\[
\frac{A_{max}}{EC_{50}} = \left( C_1 + C_3 [B] \right) \frac{1}{1 + \gamma [B]}
\]

(Eq. 9)

Therefore, \(EC_{50}/A_{max}\) has the functional form, \(EC_{50}/A_{max} \propto (a + bx)(c + x)\), which we call linear fractional.

Statistical Analysis—Unless otherwise noted, all experiments were performed in triplicate multiple times. KaleidaGraph version 4.1 (Synergy Software, Reading, PA) was used to determine a least-squares best fit \((R^2\) was almost always \(\geq 0.99)\) of the experimental data to the theoretical dose-response curve for a first order Hill plot, from which the values of \(A_{max}\) and \(EC_{50}\) were obtained. The values for \(1/EC_{50}\) \(A_{max}/EC_{50}\), and \(EC_{50}/A_{max}\) versus each factor for \(n\) independent experiments were normalized, averaged, and then plotted and analyzed as described throughout for Figs. 1–5, 7, and 8. The Bayesian information criterion (BIC) was used to determine the best of various types of fits for a particular graph (e.g. linear versus linear fractional versus quadratic).

Results

**BRD4 Is a Decelerator at Moderate Concentrations**—Cofactors in steroid hormone action are readily identifiable by their ability to change the \(A_{max}\) and \(EC_{50}\) of steroid-regulated gene induction (5, 31). To this end, we co-transfected U2OS cells with a constant, low amount of both GR plasmid and the widely used, synthetic reporter, GREtkLUC, which contains a GRE upstream of the thymidine kinase promoter driving expression of the luciferase (LUC) gene. The response to three concentrations of Dex plus an EtOH control was then measured. Best fits to Michaelis-Menten plots (i.e. first-order Hill plots) of LUC activity above background versus Dex concentration were constructed to obtain the \(A_{max}\) and \(EC_{50}\) parameters. As reported previously (16), the fits of the data to a first-order Hill plot were very good \((R^2 \geq 0.99)\).

In this system, increasing concentrations of BRD4 plasmid generally provoke a decrease in \(A_{max}\) and an increase in \(EC_{50}\). Preliminary experiments at low concentrations of added BRD4 (between 0 and 2 ng of BRD4 plasmid) indicated no statistically significant change in plots of \(A_{max}/EC_{50}\) versus BRD4. Similarly, Western blots detected negligible amounts of expressed protein from \(\leq 2\) ng of BRD4 plasmid (data not shown). However, the expression of transfected BRD4 was linear up to 40 ng of BRD4 plasmid (Fig. 1A, top). Therefore, no corrections in BRD4 concentration for non-linear protein expression need to be made before constructing the graphs to analyze the data (13). Extrapolation of these curves gives an \(x\) axis intercept of \(-9.0 \pm 3.7\) ng of BRD4 plasmid (mean \(\pm\) S.D., \(n = 4)\). This means that the level of endogenous BRD4 is equivalent to \(9.0 \pm 3.7\) ng of BRD4 plasmid.

For the above reasons, we chose to use \(\geq 2\) ng of added BRD4 plasmid in most experiments. BRD4 concentrations of 2–20 ng afforded a curvilinear, decreasing curve in plots of \(A_{max}\) versus BRD4 (data not shown). This graph is diagnostic of BRD4 acting as a competitive decelerator (Tables S1 of Refs. 13 and 16).

**Competition Assays of Wild Type BRD4 and Wild Type CDK9**—The above results with BRD4 and published data for CDK9 (16) show that both factors affect GR transactivation. BRD4 and CDK9 are also known to phosphorylate each other (26). In order to determine where and how each factor acts (i.e. accelerator or decelerator), we conducted a series of competition assays with four different concentrations of each factor in all 16 possible combinations. 2–20 ng of BRD4 plasmid was employed. The range of CDK9 plasmid used was 0–20 ng, which at its maximum is only a 2.2-fold excess over endogenous CDK9 and is also in the linear range of CDK9 expression (Fig. 1A, bottom) (16).

First, the \(A_{max}\) and \(EC_{50}\) for induction of a constant amount of transfected GREtkLUC by a constant amount of transfected GR plasmid with three different Dex concentrations plus EtOH (in triplicate) was determined for all 16 combinations of BRD4 and CDK9. Next, a series of plots was constructed using the averages of six independent experiments after first normalizing the data to that combination containing the lowest amount of both BRD4 and CDK9 (see “Experimental Procedures”). From
the characteristics of the resulting graphs, the mechanisms of action of both BRD4 and CDK9 were inferred. (For a detailed explanation of the mathematical basis for the different conclusions from each characteristic plot, see Refs. 14, 13, and 16; the most recent table relating graphical characteristics to the kinetic properties of competing factors is Table S1 of Ref. 16).

The chemical kinetic scheme shows that the most illuminating plots are those of $A_{\text{max}}/EC_{50}$ and $1/EC_{50}$ versus one cofactor for varying levels of the other cofactor. The shape of these curves and how they change with respect to the other cofactor give information about their action and position within the scheme. The nonlinear decreasing curves in $A_{\text{max}}/EC_{50}$ versus BRD4 (Fig. 1B) and versus CDK9 (Fig. 1C), with decreasing relative position as the amount of competing factor increases, are diagnostic of BRD4 and CDK9 acting as competitive decelerators (Tables S1 of Refs. 13 and 16). These results are consistent with the above data for BRD4 and the earlier report for CDK9 (16). Where each factor acts relative to the CLS is determined from plots of $EC_{50}/A_{\text{max}}$ (Tables S1 of Refs. 13 and 16). The kinetic scheme predicts that this will be a polynomial function, where the degree indicates the number of sites in which the cofactor acts. For CDK9, the plots in this graph are linear (data not shown), which means that CDK9 is a competitive decelerator acting at a single site before or at the CLS, just as observed previously (16) under different conditions. For BRD4, BIC analysis indicates that the plots are linear with low concentrations of CDK9 ($\leq 12$ ng), but the data are best fit by a quadratic equation at high CDK9 (20 ng of plasmid) (Fig. 1D). As described elsewhere (see Ref. 21 and Table S1 of Ref. 16), this behavior identifies BRD4 as acting as a competitive decelerator at two sites, each being before or at the CLS. Thus, we conclude that at BRD4 concentrations greater than 2 ng, the action of BRD4 as a competitive decelerator at a second site makes a significant contribution to the overall process in the presence of higher concentrations of CDK9.

The order of actions of BRD4 and CDK9 is determined from the graphs of $1/EC_{50}$ versus each factor (Figs. 1, E, and F). The plots in both graphs are nonlinear, declining, with the curves decreasing in position with added competing factor. However, the curves in $1/EC_{50}$ versus BRD4 approach a flat line with increasing CDK9 (Fig. 1E) faster than the curves in $1/EC_{50}$ versus CDK9 do with more BRD4 (Fig. 1F). When two decelerators compete, the graph of $1/EC_{50}$ versus factor 1 can become a horizontal line only if the competing factor (factor 2) acts before factor 1 (Table S1 of Ref. 16). Given that increasing Cdk9 can make the plots of $1/EC_{50}$ versus BRD4 flat and not vice versa, we conclude that CDK9 acts before BRD4 in this system, where both factors function as competitive decelerators before or at the CLS. Furthermore, no two competitive decelerators can act at the same step (e.g. the CLS). Because CDK9 acts before BRD4, we can further deduce that CDK9 acts before both the CLS and BRD4, whereas BRD4 acts before or at the CLS.

Most Actions of CDK9 Are Independent of CDK9 Kinase Activity—It is widely held that all CDK9 actions result from its kinase activity (22, 23), although exceptions have begun to appear (16, 32, 33). To define whether CDK9 kinase activity is required for the actions of Fig. 1, we repeated the competition assays with a dominant negative mutant of CDK9 (D167N) (dnCDK9) that lacks kinase activity (16, 34) but still interacts with BRD4. The only operational difference is that we used less dnCDK9 (Fig. 2A) and at about 3 times the level of wild type CDK9 (16). Therefore, the highest amount of dnCDK9 used (12 ng) is equivalent to 36 ng of wtCDK9 plasmid.

The graphs of the averages of six normalized experiments for $A_{\text{max}}/EC_{50}$ and $1/EC_{50}$ versus BRD4 (Fig. 2, B and E) and versus dnCDK9 (Fig. 2, C and F) are almost identical to the corresponding graphs with wtCDK9 in Fig. 1. Similarly, $EC_{50}/A_{\text{max}}$ versus dnCDK9 is again linear (data not shown), indicating that dnCDK9 is still a competitive decelerator acting at one site. An
important exception is the graph of $EC_{50}/A_{max}$ versus BRD4 (Fig. 2D). Here, there is no evidence of upward curvature in the presence of the highest level of transfected dnCDK9, although the amount of expressed dnCDK9 protein is 1.8-fold higher than that for 20 ng of wtCDK9 in Fig. 1D, where definite curvature is seen. We therefore conclude that the abilities of BRD4 and CDK9 to modulate GR-regulated gene induction in this system are independent of CDK9 kinase activity with one exception; BRD4 now acts at only one site in the presence of high concentrations of dnCDK9.

BRD4 and dnCDK9 are still competitive decelerators functioning at or before the CLS. The faster approach to a flat curve in Fig. 2E relative to in Fig. 2F again argues that dnCDK9 acts before BRD4. Thus, dnCDK9 acts at just one site again, and the order of action is still dnCDK9 < BRD4 ≤ CLS. Furthermore, the data suggest that CDK9 kinase activity is required for BRD4 to function at the second site seen in Fig. 1D with high levels of CDK9 but not at the first site with lower amounts of CDK9.

Actions of BRD4 Are Independent of BRD4 Binding to P-TEFb—BRD4 binds to both subunits of P-TEFb: CDK9 and cyclin T1 (29). To see if this binding is needed for the above actions of BRD4 in modulating GR transactivation, we conducted the competition assays detailed in Fig. 1 with a BRD4 mutant, (F1357A/E1358A/E1359A) (mtBRD4), which eliminates BRD4 binding to both CDK9 and cyclin T1 (29). The only procedural difference is that larger amounts of mtBRD4 protein are used. Western blots reveal that the mtBRD4 is linearly expressed up to 20 ng of plasmid (Fig. 3A) but with an efficiency that is 2.1 ± 0.7 (range, n = 2) times that of wild type BRD4 (data not shown). Thus, the 0–20 ng of mtBRD4 plasmid in these experiments corresponds to 0–42 ng of wtBRD4. Unexpectedly, the averaged graphs of three independent experiments are not appreciably different from those with dnCDK9 in Fig. 2. The nonlinear declining graphs of $A_{max}/EC_{50}$ (Fig. 3B) and $EC_{50}/A_{max}$ (Fig. 3D) versus mtBRD4 were obtained by first normalizing the data to the value for the lowest amount of BRD4 and dnCDK9 and then averaging and plotting the values (n = 6, mean ± S.E.).

FIGURE 2. Plots of dose-response parameters for varying concentrations of BRD4 and dnCDK9. Experiments were conducted as in Fig. 1. A, graph of density of Western blots with the indicated amounts of transfected dnCDK9 plasmid, after normalization to internal β-actin intensities, with subtraction of endogenous protein signal. Error bars, range of duplicate samples. Average plots of $EC_{50}/A_{max}$. B, of $EC_{50}/A_{max}$ versus BRD4 (D), and of $1/EC_{50}$ versus BRD4 (E) and dnCDK9 (F) were obtained by first normalizing the data to the value for the lowest amount of BRD4 and dnCDK9 and then averaging and plotting the values (n = 6, mean ± S.E.).

FIGURE 3. Plots of dose-response parameters for varying concentrations of mtBRD4 and CDK9. Experiments were conducted as in Fig. 1. A, graph of density of Western blots with the indicated amounts of transfected mtBRD4 plasmid, after normalization to internal β-actin intensities, without subtraction of endogenous protein signal. Error bars, range of duplicate samples. Average plots of $A_{max}/EC_{50}$ (B), $1/EC_{50}$ (C), and $EC_{50}/A_{max}$ (D) versus mtBRD4 were obtained by first normalizing the data to the value for the lowest amount of BRD4 and dnCDK9 and then averaging and plotting the values (n = 3, mean ± S.E.).

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mtBRD4 and versus wtCDK9 are insufficiently different to permit the identification of which factor acts first: mtBRD4 or wtCDK9 (data not shown).

Mutations of BRD4 and Cdk9 Each Affect the Same Site of BRD4 Action—The upward curvature with 20 ng of wtCDK9 in Fig. 1 indicates that there are two steps at which wtBRD4 acts in the presence of relatively high concentrations of wtCDK9. Mutations of wtBRD4 and of wtCDK9 each reduce the number of steps at which BRD4 acts to one, as seen by the absence of upward curving plots in Figs. 2 and 3. If each mutation is eliminating a different site of BRD4 action, then mtBRD4 should be relatively inactive in the presence of excess dnCDK9. We therefore looked at the net activities of mtBRD4 versus dnCDK9 in the competition assay. After averaging the normalized data of four independent experiments, the nonlinear decreasing plots of $A_{\text{max}}/EC_{50}$ versus BRD4 (Fig. 4A) and versus Cdk9 (Fig. 4B) show that the combination of mutant proteins has much the same qualitative and quantitative effects as with wild type proteins (compare with Fig. 1A and C). One difference is that dnCdk9 has little activity in the presence of high concentrations of mtBRD4. However, much of this is probably due to the largest amount of both proteins being used in Fig. 4 (20 ng of mtBRD4 and 36 ng of Cdk9) being considerably higher than that in Fig. 1 (20 ng each). Nevertheless, the combination of mutant proteins (and especially mtBRD4) is still active. This is consistent with each mutation affecting the same step of GR induction of gene expression in this system. The affected step is the second site of action of BRD4 requiring both BRD4 binding to P-TEFb and Cdk9 kinase activity. In support of this conclusion, the plots of $EC_{50}/A_{\text{max}}$ versus mtBRD4 are all linear (Fig. 4C), which indicates a single site of action at each concentration of dnCdk9. Furthermore, these graphs specify that both mutant proteins continue to act as competitive decelerators at or before the CSS.

Competition Assays of wtBRD4 with mtBRD4—In order to confirm the above conclusion that wild type and mutant BRD4 are equally active in the presence of endogenous Cdk9, a competition assay was performed with 2–20 ng of each BRD4 plasmid. If the actions of wtBRD4 and mtBRD4 are the same, then there should be no difference in the graphs, whether wtBRD4 or mtBRD4 is plotted on the x axis. In fact, the plots of $1/EC_{50}$ (Fig. 5A and B) and $A_{\text{max}}/EC_{50}$ (Fig. 5C)
and D) are indistinguishable for wtBRD4 (Fig. 5, A and C) and mtBRD4 (Fig. 5, B and D).

To further test the identical behavior of wtBRD4 and mtBRD4 in the absence of other added cofactors (such as CDK9), we looked at the plot of EC50/Amax versus total added BRD4 protein. As seen in Fig. 1D, these plots inform us of the number of sites at which a factor acts. Western blots reveal that the expression of mtBRD4 is 2.1-fold greater than that of wtBRD4. Therefore, the total amount of added BRD4 protein can be expressed as ng of wtBRD4 plasmid plus 2.1 times the ng of mtBRD4 plasmid. With this transformation, the plot of EC50/Amax versus total BRD4 is shown in Fig. 5E. If wtBRD4 and mtBRD4 are acting at two different sites, then the best fit would be an upward curving quadratic function, which is clearly not the case. BIC analysis was then used to distinguish between a linear fit, which would indicate a single site of action, and a linear fractional fit. The linear fractional fit was clearly preferred. The linear fractional curve has the form of \((a + bx)/(c + x)\), where all constants \((a, b, \text{ and } c)\) are positive. This is reflected in Fig. 5, C and D, by the \(A_{\text{max}}/\text{EC}_{50}\) plots decaying to a positive value with increasing BRD4. The mechanistic interpretation of the linear fractional fit is that BRD4 acts at two sites: \(a\) as a decelerator before or at the CLS and \(b\) as an accelerator after the CLS (see the “Experimental Procedures” for the underlying mathematics of this conclusion). The accelerator action only becomes apparent above 26 ng of total effective BRD4 plasmid. Thus, depending upon the conditions, BRD4 is seen to act at three sites: an accelerator after the CLS with high concentrations of plasmid (25–40 ng) and as a competitive decelerator at two sites before or at the CLS with intermediate concentrations (2–20 ng), where the appearance of the second decelerator site requires excess wtCDK9.

BRD4 Binds to NELF-E—CDK9, BRD4, and the NELF complex are known participants in the release of paused RNA Pol II to the actively elongating and transcribing form (25). They have also been shown to act at various steps in GR transactivation (Figs. 1–4) (16, 20). We therefore asked whether BRD4 binds to any of the NELF components directly. Indeed, NELF-E co-immunoprecipitates with BRD4 just as efficiently as CDK9 from HeLa nuclear extracts (Fig. 6A). Further, an in vitro pull-down assay with recombinant FLAG-tagged BRD4 immobilized on beads efficiently bound purified recombinant NELF-E (Fig. 6B). Thus, BRD4 is capable of directly interacting with NELF-E. Although BRD4 is also able to co-immunoprecipitate NELF-A from HeLa nuclear extract, albeit less efficiently, we were unable to confirm this interaction in vitro (data not shown).
The increasing position of the plots with added NELF-E in the graph of $A_{\text{max}}/EC_{50}$ versus wtBRD4 (Fig. 7B) indicates that NELF-E is acting as an accelerator. The decreasing curves with increasing BRD4 concentration indicate that BRD4 is a decelerator. As determined by BIC analysis, the curves in Fig. 7B go to zero with infinite BRD4. The mechanism associated with this behavior is that wtBRD4 is a competitive decelerator before or at the CLS, whereas NELF-E is an accelerator (Tables S1 of Refs. 13 and 16).

To confirm the conclusion of NELF-E being an accelerator, we would normally examine the graph of $A_{\text{max}}/EC_{50}$ versus NELF-E (13), which is a series of linear plots (Fig. 7C). However, this approach can only be used to locate the position of factors that act at one site. As seen from Fig. 5, C–E, BRD4 has two modes of action in the concentration range of 5–40 ng of plasmid. This was confirmed in the present experiments by the BIC-preferred linear fractional fit to the plot of $EC_{50}/A_{\text{max}}$ versus BRD4 (Fig. 7D). A linear fractional fit (see above) describes a cofactor acting at two sites: one as a competitive decelerator before or at the CLS and the other as an accelerator acting after the CLS. Thus, under the conditions of the competition of wtBRD4 versus NELF-E, wtBRD4 is still a competitive decelerator before or at the CLS. However, at higher concentrations (>26 ng), the accelerator action of BRD4 becomes apparent. The linear fractional fit simultaneously identifies NELF-E as an accelerator after the CLS.

The kinetic scheme of steroid hormone action can further be employed to probe whether the accelerator action of BRD4 occurs before or after the accelerator action of NELF-E. Specifically, if the $y$ intercepts in Fig. 7C increase or decrease to a constant with added BRD4, then NELF-E acts after BRD4. If, however, the $y$ intercept of $A_{\text{max}}/EC_{50}$ versus NELF-E decreases to zero with increasing BRD4, then NELF-E acts before BRD4. Fig. 7C shows that the position of the $y$ intercept of the curves in Fig. 7C decreases to a non-zero value of about 0.20. Therefore, we conclude that NELF-E acts after wtBRD4. Confirmation of this interpretation comes from a alternative method of analyzing the data, which involves graphing the inverse value of the $y$ intercept versus exogenous BRD4 (Fig. 7E). A non-linear fit would be diagnostic of NELF-E acting after BRD4. A linear fit would indicate that NELF-E acts before BRD4. BIC analysis of the two different fits overwhelmingly preferred a nonlinear fit. Thus, by this second method, we again conclude that the accelerator action of NELF-E is expressed after the accelerator action of BRD4.

The present results with NELF-E would initially appear to contradict our earlier report that NELF-E decreases the $A_{\text{max}}$ of GR transactivation (20). However, this discrepancy was found to be due mostly to differences in the nature of the vectors employed. The current experiments use the pSG5 vector rather than the pcDNA3 vector of the earlier study as well as less GR and reporter plasmids. Furthermore, Western blots indicate that the expression of full-length NELF-E from pcDNA3 is about 4.0 ± 0.2 (range, n = 2) times greater than from the pSG5 vector. After adjusting for these different levels of NELF-E expression, both plasmids cause an increase in $A_{\text{max}}$ in the range of 0–80 units of NELF-E protein, which corresponds to 0–20 ng of NELF-E/pcDNA3 plasmid (data not shown). Thus, with 100 ng of plasmid under the conditions of the earlier report, the lower amount of NELF-E protein from the pSG5 vector increases the $A_{\text{max}}$, whereas the higher amount of protein from the pcDNA3 vector decreases the $A_{\text{max}}$.

Wild Type and Mutant BRD4 Actions Are the Same with TIF2—The site and mode of action of mutant BRD4 are the same as those of one site of action for wtBRD4 with two decelerators: CDK9 and dnCDK9 (Figs. 1–4). In the absence of additional cofactors, there is no difference in the site and mode of action of wtBRD4 and mtBRD4 (Fig. 5). To examine whether the same would be true with an accelerator, we competed both forms of BRD4 with the well known accelerator, TIF2 (13, 16, 19–21). Many concentrations of BRD4 were again used to focus on its mechanism. The amounts of TIF2 plasmid employed are in the known range of linear TIF2 protein expression in U2OS cells (16, 21). The graphs of $A_{\text{max}}/EC_{50}$ versus wtBRD4 (Fig. 8A) and mtBRD4 (Fig. 8C) show the now familiar nonlinear decreasing curves that increase in position with added TIF2. Graphs of $A_{\text{max}}/EC_{50}$ versus TIF2 in other competition assays have always given the linear plots with positive slope that are characteristic of an accelerator (13, 16, 19–21). Therefore, we conclude that the family of two-point curves with positive slopes for $A_{\text{max}}/EC_{50}$ versus TIF2 again represents linear plots (data not shown).

Where and how TIF2 acts is determined from analyses of the $A_{\text{max}}/EC_{50}$ versus TIF2 graphs. The x and y axis intercepts in all linear graphs of $A_{\text{max}}/EC_{50}$ with positive slopes are mathematically restricted to the top left quadrant defined by $x \leq 0$ and $y \geq 0$. It should be noted that for this interpretation, one must first mathematically transform the position of $x = 0$ to correspond to zero total cofactor (exogenous plus endogenous) as opposed...
to zero added cofactor. If the plots intersect at the point where total TIF2 (exogenous plus endogenous) equals zero, then TIF2 is an accelerator at or before the CLS, whereas BRD4 is a competitive decelerator at any position. Convergence at any other point (\(x < 0\) and \(y = 0\), \(x = 0\) and \(y > 0\), or \(x < 0\) and \(y > 0\)) indicates that TIF2 is an accelerator after the CLS and that BRD4 is (a) a competitive decelerator before or at the CLS, (b) after the CLS and at the same position as or after NELF-E, or (c) after the CLS and before NELF-E, respectively (Tables S1 of Refs. 13 and 16). The intersection points were calculated from \(\delta\) versus \(\beta\) plots (see “Experimental Procedures”) to be 0.066 and 0.016 on the \(y\) axis and \(-14.2\) and \(-19.4\) ng of TIF2 plasmid on the \(x\) axis, respectively, for the graphs as a function of wtBRD4 and mtBRD4, respectively (data not shown). These \(x\) axis values are more negative than the value of no endogenous TIF2 (as determined from Western blots), which ranges between \(-1.1\) and \(-4.1\) ng of TIF2 plasmid (16, 21). As described above, this result is consistent with only one graphical interpretation (see Tables S1 of Refs. 13 and 16) (i.e. that TIF2 is an accelerator after the CLS and wtBRD4 is a competitive decelerator before or at the CLS). The graphs of \(EC_{50}/A_{\text{max}}\) versus wtBRD4 (Fig. 8B) and mtBRD4 (Fig. 8D) are nicely linear, which means that each form of BRD4 is acting at one site in this concentration range. Together, these data plus the graphs for 1/\(EC_{50}\) versus \(BRD4\) and \(TIF2\) (not shown) uniquely identify both wtBRD4 and mtBRD4 as competitive decelerators acting at one site before or at the CLS, whereas TIF2 is an accelerator functioning after the CLS. Thus, whereas the number of sites of wtBRD4 action as a competitive decelerator is reduced by one under some conditions (e.g. dnCDK9 with wtBRD4 and wtCDK9 with mtBRD4 reduce the total number of sites from two to one), the relative positioning of the remaining sites is indistinguishable between mtBRD4 and wtBRD4 under multiple conditions: with competitive decelerators, with no added cofactor, and with an accelerator.

**Discussion**

This study utilizes an experimentally validated model of steroid hormone action both to identify and to characterize the modes of action of two new modulatory factors (BRD4 and NELF-E). Several otherwise unobtainable properties of each factor are determined (i.e. their kinetically defined activities, the number of sites of factor action, and their position of action in the still poorly defined sequence of events in GR-regulated gene induction). The transcriptional cofactor BRD4 has numerous activities that impinge on gene expression. BRD4, CDK9, CDK7, and TAF7 all engage in cross-talk reactions that modulate their individual activities (26). For example, BRD4 phosphorylation of the kinase subunit of P-TEFB (CDK9) increases or decreases its RNA Pol II CTD kinase activity as a function of the BRD4/P-TEFB ratio, thereby regulating the post-translational modifications necessary for the recruitment of RNA biogenesis factors. We now report that BRD4 acts at three different sites, which is consistent with the known complexity of BRD4 biochemistry. BRD4 is also found to directly bind to the negative elongation factor component, NELF-E (Fig. 6). This raises the possibility that BRD4 alters the activity of the NELF complex, which is phosphorylated by P-TEFB (22) and is a key regulator for the release of paused Pol II (23). However, we further find that NELF-E modulates GR transactivation independently of the NELF complex. Finally, some of the previously documented, kinase-independent activity of CDK9 in modifying GR gene induction properties (16) is maintained with both wtBRD4 and a mutant BRD4 that is unable to interact with PTEF-b. Thus, several previously unsuspected activities of BRD4 and NELF-E have been divulged by our model-based assays (13–18).

BRD4 affects GR transactivation by altering both the \(A_{\text{max}}\) and the \(EC_{50}\) of the process. However, the mechanisms and sites of BRD4 action depend upon a variety of conditions. At concentrations of 2–20 ng, BRD4 acts as a competitive decelerator at or before the CLS in a manner that depends upon the level of CDK9. BRD4 functions at one site with low amounts of CDK9 but at two sites with higher CDK9 concentrations (Fig. 1) that are only 2.2-fold above the endogenous level of CDK9. The site of BRD4 action with high CDK9 disappears both with a mutant BRD4 that does not bind either component of P-TEFb (CDK9 and cyclin T1) and with a dominant negative CDK9 that lacks kinase activity. However, the other site of action persists with mtBRD4 and added dnCDK9, indicating that this site of action requires neither CDK9 kinase activity nor BRD4 binding to CDK9 (Fig. 4). With higher concentrations of BRD4 (i.e. between 26 and 40 ng of plasmid), two sites of action are observed with NELF-E, but only one site is apparent in the presence of added p160 cofactor, TIF2 (Figs. 7 and 8). Interestingly, with NELF-E and with even higher concentrations of just BRD4, this second site is that of BRD4 acting as an accelerator after the CLS and before NELF-E (Figs. 5 and 7). The number of sites for BRD4 action with the addition of just BRD4, of NELF-E, or of TIF2 does not change when mtBRD4 is used instead but does change in the presence of high CDK9. Thus, the sites at which BRD4 acts as a competitive decelerator depend not only on the concentrations of other factors but also on the absolute concentration of BRD4 and mutations to BRD4 itself.

NELF-E functioning as an accelerator after the CLS in competition assays with wtBRD4 (Fig. 7) was unexpected. Earlier results for two other components of the NELF complex (NELF-A and NELF-B) showed that both act as a competitive decelerator at two sites before or at the CLS (20). The results of Fig. 7 also support our earlier conclusion that the NELF complex subunits possess independent activities (20) for the following reasons. First, added NELF-A or NELF-B alters the \(A_{\text{max}}\) and/or \(EC_{50}\) of GR transactivation (20), as does added NELF-E. If all components are acting through the NELF complex, then only one component of the NELF complex can be limiting and could further modify the \(A_{\text{max}}\) and \(EC_{50}\). This is clearly not the case in our system. Second, NELF-A and -B act as competitive decelerators, whereas NELF-E is an accelerator. It is difficult to envisage how the addition of different components of the same complex can elicit opposite effects if the active species is the same (i.e. the NELF complex). Finally, the fact that NELF-A and -B act before the CLS, whereas NELF-E acts after the CLS, is direct evidence that these species are not acting through a common intermediate (i.e. the NELF complex).
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An additional benefit of the ordering of factor action sites is that it also reveals whether or not factor 1 influences the concentration of factor 2, or *vice versa*. Thus, if factor 1 acts downstream of factor 2, we know that factor 1 cannot alter the concentrations of factor 2. Such information is currently available only by the tedious method of conducting Western blots from parallel samples for each of the different reaction conditions. In contrast, the same information flows directly from analysis of the 192 samples of gene induction for the competition assay. For example, we previously reported that CDK9 acts downstream of GR (16), and the current study reveals that the site of action of NELF-E, BRD4, and TIF2 is, in each case, downstream of the site of action of CDK9. Thus, all of the factors examined in the current paper exert their activity downstream of GR. If any factor had functioned simply by changing the levels of active GR, then the competition assay would specify that that factor acts before CDK9. Because this was not observed, we can confidently conclude that the levels of active GR protein are not significantly modified by any of the factors overexpressed in this study. This same invariance of GR levels with added factor has been seen in competition assays of GR with GREtkLUC reporter in four different cell lines and with MMTVLUC reporter (19), with Ubc9 (14), with wild type and mutant NELF-A and NELF-B (20), and with Cdk9 (16). In every case, no factor acted before GR, which, according to our current methodology, means that the GR levels have not been changed. This is a significant conclusion because it is well established that the A<sub>max</sub> and EC<sub>50</sub> of GR-regulated gene induction are sensitive to differing levels of GR (14, 19, 35–37). However, the above results indicate that the observed modulation of A<sub>max</sub> and EC<sub>50</sub> by the various factors in the current study cannot be ascribed to any modulation of GR levels with the expression of exogenous factors.

The current experiments have all been conducted with transiently transfected factors and a widely used reporter gene (GREtkLUC). Previous experiments looking at endogenous genes have yielded qualitatively identical results (13, 21, 37, 38). Therefore, the techniques described here can be used directly with endogenous genes and, so far, give qualitatively similar results. However, not all endogenous genes are amenable to analysis by our competition assay. This is because the dose-response curves of many endogenous genes are not described by a first order Hill plot (39), in which case the competition assay and our mathematical model cannot be applied.

Collectively, these chemical kinetic-based competition assays allow us to define not only the number of the sites where the four transcription cofactors (BRD4, CDK9, NELF-E, and TIF2) are functionally active but also their kinetic mode of action at each site and the relative positioning of their sites of action in the overall reaction sequence (Fig. 9A, top). Extension of this approach with new factors of unknown biochemical activity will permit their placement in sequence of reaction events of Fig. 9A to build up an extended series of factor actions. Such conclusions are not possible from classical biochemical experiments, which inform us only of when and where a factor binds and with what it interacts. The latter information is invaluable but cannot identify how or where a factor acts, which must be at or after the site it was bound. Clearly, the identification of those species acting close to the final outcome of a transcriptional event will be extremely valuable for modulating the final level of product. Furthermore, altering the activities at

![FIGURE 9. Correlation of kinetic and biochemical actions of factors contributing to GR induction of gene expression.](image-url)
steps closer to the final product should reduce the number of unwanted side effects because there should be fewer off-target pathways that are disturbed. A reduction of side effects has long been an objective of steroid hormone research (5–8) and would greatly increase the number of options for steroid hormone therapies.

A limitation of the competition assay is that it does not inform us of the biochemical nature of the step at which the factor acts. However, the results of the competition assays in Fig. 9A (top) nicely match several of the known actions of BRD4 in regulating transcription elongation in Fig. 9A (bottom). Furthermore, with the data from the mutant proteins of the current study, we can propose the ordered biochemical sequences of Fig. 9B for the factors discussed in this study. A major caveat, however, is that the arrows in Fig. 9B indicate only the relative positioning of factor actions and thus may include other currently unidentified steps or actions. It is known that BRD4 increases CDK9 kinase activity at molar ratios of BRD4/CDK9 \( \geq 1 \) but is an inhibitor at higher ratios of BRD4s (26), which is compatible with 2–20 ng of BRD4 being a competitive decelerator. The actions of each factor at low CDK9 concentrations (\( \leq 12 \) ng of CDK9 plasmid) are independent of both CDK9 kinase activity and BRD4 binding to CDK9 (Figs. 2–4). This result could derive from the known ability of BRD4 to directly phosphorylate the Pol II CTD (24). The present findings indicate that this phosphorylation by BRD4 would occur after the action of CDK9, which probably involves its kinase activity. However, because of the complexity of the interactions between BRD4 and CDK9, including recruitment from the Hexim complex, association with mediator, and phosphorylation of each other and the Pol II CTD, other biochemical sequences cannot yet be ruled out. Nevertheless, what is clear is that CDK9 acts before the competitive decelerator action of BRD4, which is at or before the CLS. The interaction between BRD4 and NELF-E, both physical and kinetic, is consistent with BRD4 regulating some activity of NELF-E other than pause release. Whether this action would be the result of the accelerator or decelerator activity of BRD4 is not yet known. However, given the closer proximity of BRD4 accelerator activity to the site of NELF-E activity, it is more likely that BRD4 as an accelerator is what directly affects NELF-E actions.

In summary, BRD4 is found to act at three different sites with two different, kinetically based mechanisms in the sequence of reactions for GR induction of gene expression. This multiplicity of sites reflects the diversity of BRD4 interactions in its regulation of transcription, which include recruitment of P-TEFb and regulation of its kinase activity, phosphorylation of the Pol II CTD, and interaction with NELF-E. However, results with mtBRD4 and dnCDK9 disclose that the action of wtBRD4 at one site occurs independently of BRD4 binding to CDK9 (and cyclin T1) and of CDK9 kinase activity. Future experiments are required to determine the exact biochemical mechanism(s) of this reaction step. Nonetheless, the present studies have further established that BRD4 binds to NELF-E but acts at two sites, one of which is before NELF-E in a manner that is again independent of BRD4 binding to CDK9 and cyclin T1. Whether one or both modes of action involve direct binding to NELF-E is presently unclear. Both wtBRD4 and mtBRD4 also act before TIF2. Where TIF2 acts relative to NELF-E remains to be determined. Thus, GR-mediated gene induction involves both conventional and non-conventional actions of BRD4, CDK9, and NELF-E. Identification of these new actions should significantly expand our understanding of, and capacity for greater selectivity and clinical applications in, GR- and steroid-regulated gene induction.

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**Author Contributions**—S. S. S. and D. S. S. conceived the study; C. C. C. performed the mathematical manipulations of the theoretical model; S. S. S. designed and, with C. C. C., analyzed the experiments; M. A. P. and J. A. B. performed and analyzed the experiments of Figs. 1–5 and 7–8; and B. N. D. and P. S. T. performed and analyzed the experiment of Fig. 6. Fig. 9 was the result of contributions by D. S. S., B. N. D., C. C. C., and S. S. S. and, S. S. S. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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