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How Transcriptional Activators Bind Target Proteins*

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The product of the proto-oncogene c-*myc* influences many cellular processes through the regulation of specific target genes. Through its transactivation domain (TAD), c-Myc protein interacts with several transcription factors, including TATA-binding protein (TBP). We present data that suggest that in contrast to some other transcriptional activators, an extended length of the c-Myc TAD is required for its binding to TBP. Our data also show that this interaction is a multistep process, in which a rapidly forming low affinity complex slowly converts to a more stable form. The initial complex formation results from ionic or polar interactions, whereas the slow conversion to a more stable form is hydrophobic in nature. Based on our results, we suggest two alternative models for activation domain/target protein interactions, which together provide a single universal paradigm for understanding activator-target factor interactions.

c-Myc protein, the product of the proto-oncogene c-*myc*, is an important regulator of cell proliferation and apoptosis (Ref. 1 and references therein). These functions are mediated in part by its activity as a transcriptional activator. Through its C-terminal basic helix-loop-helix/leucine zipper domain, c-Myc can heterodimerize with Max (2–4). The Myc/Max heterodimer binds to the E-box sequence CACGTG and is believed to regulate genes important for cell cycle progression, including ornithin decarboxylase, Cdc25, cyclin D, and cul1 (Refs. 5–10 and reviewed in Ref. 11). The transactivation domain (TAD)[1] of c-Myc has been mapped to the N-terminal 143 amino acids of the protein (12). This region of the protein includes the "Myc boxes," sequences that are conserved among members of the Myc protein family and that have been implicated in Myc's transforming activity. One or both Myc boxes have also been implicated in the rapid turnover of the c-Myc protein (13–15) as well as c-Myc's transrepression function (16, 17). The isolated c-Myc TAD is mostly unstructured in solution (18), a property it shares with many other activation domains (19–25). Interaction with target factor induces secondary structure (18), which has been shown to be important for the function of some transactivators (26–28).

Transcriptional activators act by recruiting other transcription factors to target genes through protein interactions (29–33). They can act at multiple steps during transcription, such as regulation of chromatin accessibility (34–36), preinitiation complex (PIC) formation (32, 37–40), or post-initiation steps (41, 42). Thus, the formation of interactions between activators and target proteins is critical for the regulation of all cellular genes. Despite extensive investigations, the molecular basis of activation domain function is still poorly understood, and thus activation domains are still classified according to their amino acid composition as acidic, glutamine-rich, or proline-rich. One of the reasons for slow progress may be that many existing observations appear intrinsically paradoxical. For example, mutagenesis studies of several highly acidic activation domains have revealed a predominant importance of hydrophobic amino acids (43–47). Furthermore, although transactivation domains apparently need to make specific interactions with several different target factors, they are usually composed of short amino acid segments with a poor intrinsic propensity for structure formation (21, 25, 48). Indeed, fortuitous activation domains have even been found within proteins that are not involved in transcription (49). Early models suggested that the unstructured acidic activation domains ("acid blobs") recruited components of the transcriptional machinery via nonspecific ionic interactions (50). These models, however, do not account for the important role of hydrophobic residues, and more recently it has been shown that some activation domains are structured when bound to target factors (18, 20, 51, 52).

Here, we characterize the binding of the c-Myc TAD to one of its target proteins, TBP. We show that TBP needs an extended c-Myc sequence for interaction. Binding proceeds in two steps, where an initial weak complex forms rapidly by electrostatic interactions. This weak complex slowly converts to a more stable form in a reaction that has the thermodynamic characteristics of protein folding. We present two alternative models in which single-copy transactivators bind their target proteins in a stepwise fashion, whereas multicopy transactivators might bind in a simple one-step reaction. Taken together, these results provide a molecular framework for understanding how transcriptional activator proteins can make apparently specific interactions with a large number of different target proteins.

EXPERIMENTAL PROCEDURES

All standard laboratory chemicals were obtained from Sigma or through Merck Eurolab (Stockholm, Sweden).

Plasmids and Proteins—Plasmid pT7yD (53) was a kind gift of Diane Hawley (University of Oregon).

Plasmid pGEXTNBZ was obtained by replacing the *Bam*HI/*Sal*I fragment of pGEX4T1 (Amersham Pharmacia Biotech) with a double-stranded oligonucleotide of the following sequence.

GATCACCGCATATGGAATTCCCGGGATCCTGAC

TGGCGTATACCTTAAGGGCCCTAGGACTGAGCT

SEQUENCE 1

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[1] The abbreviations used are: TAD, transactivation domain; TBP, TATA-binding protein; GST, glutathione *S*-transferase; SPR, surface plasmon resonance; RU, resonance units.

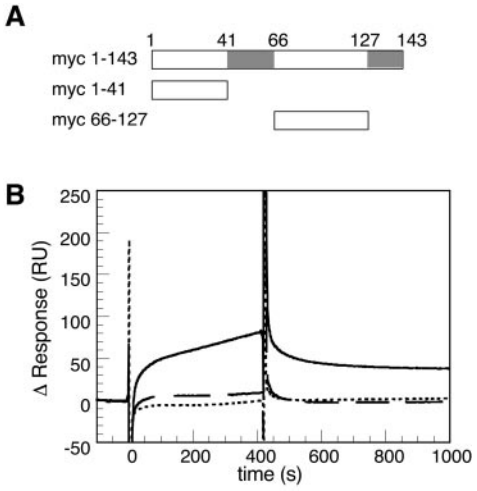

FIG. 1. **A large portion of c-Myc is needed to bind to TBP.** *A*, schematics of the protein constructs used. The *shaded areas* denote protein destruction sequences. *B*, about 2000 RU of anti-GST antibody were immobilized onto all four measuring areas of sensor chip B1. Equal molar amounts of GST, GSTmyc41, GSTmyc66–127, and GSTmyc143 were captured onto areas 1 to 4, respectively. 1.35 μM TBP was injected over all four measuring areas at a flow rate of 5 μl/min. Shown here are the differential responses to area 2-area 1 (GSTmyc41, *dashed line*), area 3-area 1 (GSTmyc66–127, *dotted line*), and area 4-area 1 (GSTmyc143, *solid line*).

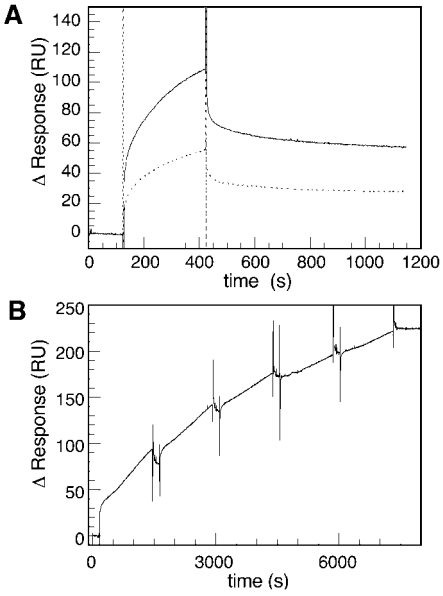

FIG. 2. **TBP binds to the c-Myc TAD in a multiphasic manner.** *A*, TBP at a concentration of 1.3 μM was injected over all four measuring areas of a sensor chip that had 94 RU, respectively, 205 RU of GSTmyc143 on areas 2 and 4, and corresponding molar amounts of GST on areas 1 and 3. The sensorgrams shown here represent the differential signals area 2-area 1 (*dotted line*) and area 4-area 3 (*solid line*). The spikes at the start and end of the injection result from this background correction. *B*, to prolong contact time between immobilized Myc protein and soluble TBP, the flow rate was reduced to 2 μl/min, and TBP at a concentration of 1.3 μM was repeatedly injected over areas 1 and 2 that had 273 RU GST and 376 RU GSTmyc143, respectively, captured onto them. The sensorgram shows the differential signal for area 2-area 1.

Plasmids pGSTTmyc41 and pGSTT66myc127, encoding the GST fusion protein of c-Myc amino acids 1–41 and 66–127, respectively, were constructed by polymerase chain reaction amplification of the corresponding fragments of c-Myc, including *Nde*I and *Bam*HI restriction sites in the amplification primers. The polymerase chain reaction fragments were ligated into *Bam*HI/*Nde*I restricted pGEXTNBZ. These modifications add six extra amino acids, G-S-P-H-M-A, between the GST and the Myc part of the fusion protein, as well as two amino acids, G-S, to the C terminus.

Plasmid pGSTTmyc143 was constructed by cloning the *Nde*I/*Bam*HI fragment from pET19myc (18) into *Nde*I/*Bam*HI restricted pGEXTNBZ.

The identity of all fusion plasmids was verified by DNA sequencing.

Yeast TBP was overexpressed in *Escherichia coli* BL21/DE3(pLysS) transformed with pT7yD and initially purified on heparin-agarose and DEAE-cellulose as described previously (53) and further purified on CM-Sepharose (linear gradient 75–400 mM KCl) and phosphocellulose. Purified TBP was dialyzed into TC100 buffer (20 mM Tris, pH 7.9, 100 mM KCl, 2 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol) and stored in small aliquots at −80 °C.

GST fusion proteins were overexpressed from the corresponding plasmids in *E. coli* BL21/DE3(pLysS). Cells were resuspended in phosphate-buffered saline supplemented with Complete Protease Inhibitor (Roche Molecular Biochemicals), lysed by freeze-thawing and sonication, and the fusion proteins were purified by affinity chromatography on glutathione-agarose (Sigma) according to standard protocols. GSTmyc143 was further purified and separated from shorter proteolytic products (mainly free GST) by ion-exchange chromatography on DEAE-Sephacel (Amersham Pharmacia Biotech), where GST is in the flow-through fraction, and GSTmyc143 can be eluted from the column with 300 mM KCl. The GST fusion proteins were dialyzed into TC100 and stored in small aliquots at −80 °C.

GST was overexpressed from plasmid pGEXTNBZ, purified on glutathione-agarose, dialyzed into TC100, and stored at −80 °C.

His-tagged Myc143 was purified as described previously (18).

Protein concentrations were measured with Coomassie Protein Reagent (Pierce) using bovine serum albumin (Pierce) as a standard, and by UV absorption. Both techniques gave similar results. All proteins were at least 95% pure, as judged by SDS-polyacrylamide gel electrophoresis.

Surface Plasmon Resonance (SPR)—SPR analysis of binding was performed using a BIAcore2000 instrument under the control of BIAcore control 3.0 software (BIACORE, Uppsala, Sweden). This setup allows to simultaneously monitor the binding of one protein dissolved in the mobile phase to four different proteins immobilized onto different areas of the sensor chip. Anti-GST antibodies were immobilized onto all four measuring areas of a Pioneer B1 sensor chip (both from BIACORE) according to the manufacturer's instructions, with the flow rate and dual-injection modifications described by Wu and co-workers (54). For the initial binding experiments, about 3000 RU of antibody were immobilized. For the kinetic experiments, areas 1 and 2 were targeted with 500 RU and areas 3 and 4 with 1000 RU of antibody. The binding capacity of the antibodies was tested by capturing GST protein. Unless otherwise noted, all binding studies were carried out at 25 °C with HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20; BIACORE) supplemented with 10 mM $MgCl_2$ as running buffer.

For kinetic experiments, the measuring areas were regenerated with two short (1 min) pulses of regeneration buffer (10 mM glycine, pH 2.2; BIACORE). Subsequently, GST was captured onto areas 1 and 3, and freshly thawed GSTmyc143 was captured onto areas 2 and 4. TBP was thawed on ice, diluted with running buffer to the appropriate concentration, and injected over all four measuring areas at a flow rate of 20 μl/min. The recorded data were on-line corrected for background binding to GST (area 2-area 1, area 4-area 3).

Data analysis was performed with BIAevaluation 3 software (BIACORE), except for the calculation of rapid equilibrium constants. Here, Kaleidagraph 3.0 (Abelbeck Software) was used to fit the binding data to the following equation,

$$\theta = \frac{[T_t]}{K_D + [T_t]} \quad \text{(Eq. 1)}$$

where θ is the fractional saturation and $[T_t]$ is the total concentration of TBP.

Sensorgrams were simulated using Hopkinsim (Johns Hopkins University), which is based on the kinsim algorithm (55).

Fluorescence—Fluorescent analysis of binding was performed using a Shimadzu RF5000 spectrofluorimeter in a buffer similar in composition to SPR running buffer, except that it did not contain any detergent. The slitwidth for both excitation and emission was set to 4 nm. The excitation wavelength was set to 270 nm, and the emission spectrum from 305 to 400 nm was recorded at a scan speed of 0.8 nm/s. Assuming a 1:1 binding stoichiometry, an equilibrium constant was estimated using the equation of Swillens (56).

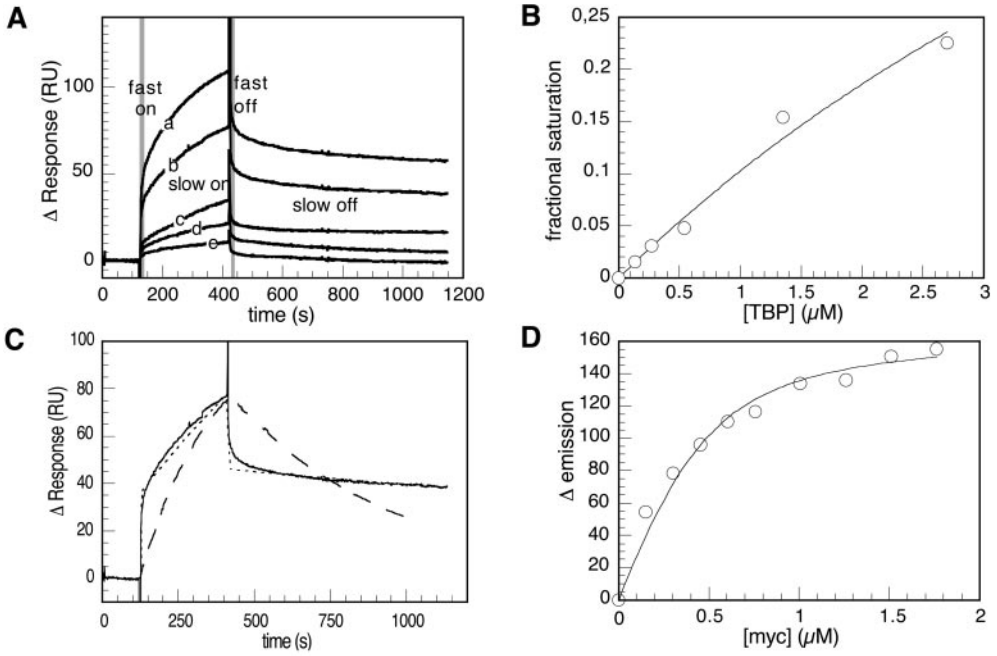

FIG. 3. **The binding can be modeled as a rapid equilibrium followed by a slow conversion step.** *A*, several concentrations of TBP were injected over all four measuring areas. Areas 2 and 4 had different amounts of GSTmyc143 captured, areas 1 and 3 carried the corresponding amounts of GST. Shown here are the differential signals for area 4-area 3. The response signal was divided into four separate phases: fast on, slow on, fast off, slow off. For the slow off phase, a rate constant of 2.6×10^{-4} s^{-1} was obtained by global analysis. The fast on and fast off phases were treated as rapid equilibrium, and an equilibrium constant could be determined (see *B*). The rate constant for the slow on phase could be obtained by first calculating the concentration of rapid equilibrium complex over time and using this concentration as a parameter for the conversion reaction. The slow on-rate was thus estimated to be 4.7×10^{-3} s^{-1}. The apparent overall equilibrium constant could be calculated to be $K_0 = 1.9 \times 10^6$ liters/mol. *B*, to estimate a binding constant for this rapid equilibrium, the response 10 s after the start of the injection was recorded. The expected response at saturation was calculated from the known amount of GSTmyc143 on the surface and the ratio of molecular weights. The fractional saturation θ was plotted *versus* the TBP concentration, and a binding curve was fitted to the data. The equilibrium constant for the fast phase was estimated to be 1.1×10^5 liters/mol. *C*, *curve b* from *A*, corresponding to a TBP concentration of 1.35 μM, was converted to a fractional saturation θ (*solid line*). The TBP + Myc $\Leftrightarrow$ C1 $\leftrightarrow$ C2 mechanism (Equation 2) was modeled using the parameters obtained above (*dotted line*). A simple one-step Langmuir binding isotherm (*dashed line*) does not fit the data. *D*, TBP changes its fluorescent properties when binding to target protein (data not shown). A constant amount of TBP was titrated with small aliquots of His-tagged Myc TAD-(1–143). Mock-titrations were performed for background correction, titrating TBP with buffer and titrating buffer with c-Myc. The differential emission between 305 and 315 nm was averaged and plotted *versus* the concentration of added HisMyc143. The titration curve was analyzed analogous to Swillens (56), and the apparent overall equilibrium constant was estimated to be $K_0 = 2.3 \times 10^6$ liters/mol.

TABLE I
Summary of the measured affinity constants

Temperature	[NaCl]	K_1	K_2	K_0
°C	mM	liters/mol		liters/mol
5	150	$2.1 \pm 0.5 \times 10^5$	2.3 ± 0.7	4.8×10^5
10	150	$6 \pm 3 \times 10^5$	2.0 ± 0.6	1.2×10^6
15	150	$3 \pm 1 \times 10^5$	3.4 ± 0.4	1.0×10^6
25	150	$1.1 \pm 0.5 \times 10^5$	18 ± 2	1.8×10^6
30	150	$6 \pm 1 \times 10^5$	376 ± 200	2×10^8
25	50	$4.7 \pm 0.9 \times 10^5$	22 ± 2	1×10^7
25	300	$1.2 \pm 0.9 \times 10^4$	105 ± 43	1.3×10^6
Room temperature	150	Fluorescence		2.3×10^6

RESULTS

TBP Requires an Extended Myc Sequence for Interaction—Many TADs can interact with their target factors via short stretches of amino acids (54, 57). To find the minimal sequence of c-Myc that interacts with TBP, we tested shorter fragments of the c-Myc TAD. In a yeast transactivation assay, two such constructs, residues 1–41 and 66–127, were able to activate transcription, whereas residues 42–65 and 128–149 did not.[2] We therefore prepared GST fusion proteins containing residues 1–41 and 66–127, respectively, as well as the full TAD (1–143), and tested them for their ability to interact with TBP, monitoring the interaction in real time by SPR (Fig. 1). To our surprise, while TBP interacted efficiently with the full TAD, no significant interaction with the shorter fragments could be detected. This suggests that an extended range of residues is needed for efficient interaction of Myc with TBP and that efficient interaction requires more than an abundance of acidic residues and/or glutamines within the TAD.

TBP Interacts with c-Myc in a Biphasic Manner—When analyzing the data presented in Fig. 1, we noticed that the interaction appeared to be biphasic, a very fast initial binding followed by a much slower increase in response signal. To determine whether the fast phase had any significance or was an artifact caused by the bulk signal subtraction (58), we captured different concentrations of GSTmyc143 onto two measuring areas and corresponding amounts of GST onto the remaining measuring areas. TBP was injected over all four measuring areas at an increased flow rate to minimize the time

[2] E. Flinn and A. P. Wright, manuscript in preparation.

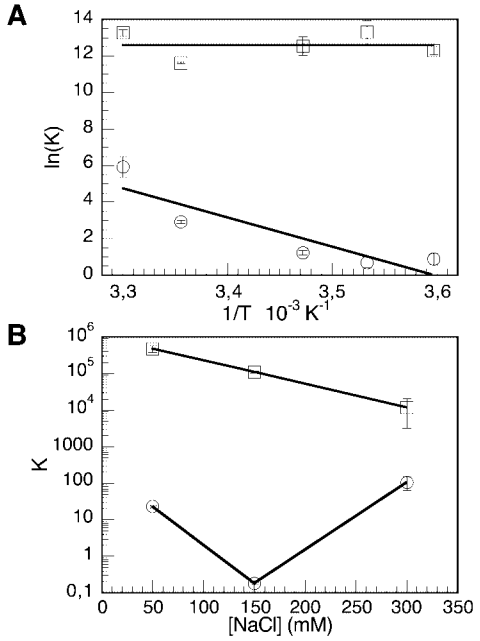

FIG. 4. **Binding of TBP to c-Myc is entropy-driven.** *A*, the binding of TBP to c-Myc was monitored by SPR at different temperatures. Binding constants were determined as described in the legend to Fig. 3. The logarithm of the estimated binding constants for the first step (*squares*) and second step (*circles*) was plotted against the inverse temperature, and a straight line fitted to the data. The first step, the rapid equilibrium, has no clear temperature dependence. For the second, slow, step, van't Hoff analysis revealed an entropy-driven process, with ΔH = 133 kJ mol^{-1}, and ΔS = 479 J mol^{-1} K^{-1}. The *error bars* represent the S.E., averaged over two measurements for the initial binding, or the S.D. from eight local fit measurements for the conversion. *B*, the binding of TBP to c-Myc was monitored at different salt concentrations. Equilibrium constants were estimated as described in the legend to Fig. 3 and plotted against the salt concentration. The rapid equilibrium (*squares*) shows a clear trend, with weaker binding at elevated salt concentrations. The slow step (*circles*) is less affected by salt. *Error bars* are the same as in *A*.

delay. The differential signal GSTmyc143-GST still showed a fast phase (Fig. 2*A*). Moreover, the magnitude of the fast phase was proportional to the surface concentration of GSTmyc143, indicating that it was indeed caused by the interaction between the immobilized Myc protein and TBP. In addition, the wash-out phase also exhibited a biphasic behavior: a rapid initial drop followed by a slower reduction. The magnitude of the fast signal loss was consistently slightly smaller than the fast binding (Fig. 2*A* and data not shown).

Such a biphasic response can have several causes. For example, heterogeneity in the immobilized surface protein, *i.e.* a population of GSTmyc143 that binds fast and a population that binds slowly or a population that binds strongly (slow off-rate) and a population that binds weakly (fast off-rate) would lead to the observed behavior. Alternatively, a conformational change in the c-Myc-TBP complex, transforming it from a fast-dissociating into a slow-dissociating complex, could also explain the binding data. To distinguish between these possibilities, we repeatedly injected TBP over the surface, with the shortest intervening time interval permitted by the instrument (Fig. 2*B*). The closer the response approached saturation, the smaller the relative magnitude of the fast phase became, both for the binding and the wash-out phases. Such a behavior is consistent with a conformational change model, but not with models relying on surface heterogeneity. Importantly, the response extrapolates to a numeric value consistent with a 1:1 binding stoichiometry.

To be able to estimate rate constants for the reaction, we monitored the binding of TBP to GSTmyc143 at various TBP concentrations (Fig. 3*A*). Global analysis using BIAevaluation, and the pre-defined conformational change model did not give reliable results, since minor changes in various reaction parameters caused major changes in the rate constants calculated for the fast phase. To circumvent this problem, we treated the binding reaction as a rapid equilibrium followed by a slow conversion step.

$$\mathrm{TBP} + \mathrm{Myc} \overset{K_A}{\rightleftharpoons} C_{\mathrm{initial}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C_{\mathrm{final}} \qquad \text{(Eq. 2)}$$

To estimate an equilibrium constant K_A for the fast phase, we recorded the magnitude of the fast phase, approximated by the response 10 s after injection, as a function of TBP concentration. Since Fig. 2*B* showed a 1:1 stoichiometry, indicating that all the Myc molecules on the surface are available for binding, the fast phase response could be converted to a fractional saturation θ (Fig. 3*B*) and an equilibrium constant calculated according to Equation 1. An off-rate for the slow phase can be calculated directly from the data using a simple binding model ($\mathrm{d}[C_{\mathrm{final}}]/\mathrm{d}t = k_{-2} \times [C_{\mathrm{final}}]$). An estimate for the rate constant of the slow binding phase can be obtained with the following differential equation.

$$\frac{d[C_{\mathrm{final}}]}{\mathrm{d}t} = k_2 \times K_A \times [\mathrm{TBP}] \times [\mathrm{Myc}] - k_{-2} \times [C_{\mathrm{final}}] \qquad \text{(Eq. 3)}$$

Finally, K_0, the equilibrium constant for the overall reaction, can be calculated from the equilibrium constant for the fast step and the kinetic constants for the slow step. The parameters thus obtained are $K_A = 1.1 \times 10^5$ liters/mol, $k_{-2} = 2.6 \times 10^{-4}$ s^{-1}, $k_2 = 4.7 \times 10^{-3}$ s^{-1} and $K_0 = 1.9 \times 10^6$ liters/mol. To test the validity of this approach, we simulated this model (Equation 2) using the thermodynamic and kinetic constants obtained above (Fig. 3*C*). The simulated curve correlates very well with the data, indicating that the model is a close approximation of reality, whereas a simple one-step Langmuir binding isotherm does not fit the data (Fig. 3*C*).

One potential disadvantage of using SPR to monitor binding is that it is a method in which one reaction partner has to be immobilized close to a surface. This might change the binding parameters due to steric hindrance or other problems. While Fig. 2*B* extrapolates to the predicted saturation value, indicating that all GSTmyc143 immobilized onto the surface is available for binding, it was still a formal possibility that the binding characteristics were influenced by the immobilization. We therefore wanted to monitor the interaction with an independent method. The fluorescence emission spectrum of TBP undergoes spectral changes upon binding to DNA (59). A preliminary test showed that the interaction of TBP with c-Myc also lead to spectral changes, although of minor magnitude (data not shown). We utilized these spectral changes to monitor the binding of c-Myc to TBP. A constant amount of TBP was titrated with small aliquots of His-tagged c-Myc143, and after waiting for equilibrium, the emission spectra were recorded. Two mock-titrations were performed, where TBP was titrated with buffer and where buffer was titrated with c-Myc. The differential emission between 305 and 315 nm was averaged and plotted *versus* the total c-Myc concentration (Fig. 3*D*). When analyzed analagous to Swillens (56), an overall apparent equilibrium constant of 2.3×10^6 liters/mol was calculated. This corresponds very well with the K_0 value calculated from the SPR results, and thus we conclude that the c-Myc-TBP interaction is not seriously affected by immobilization of c-Myc to a surface.

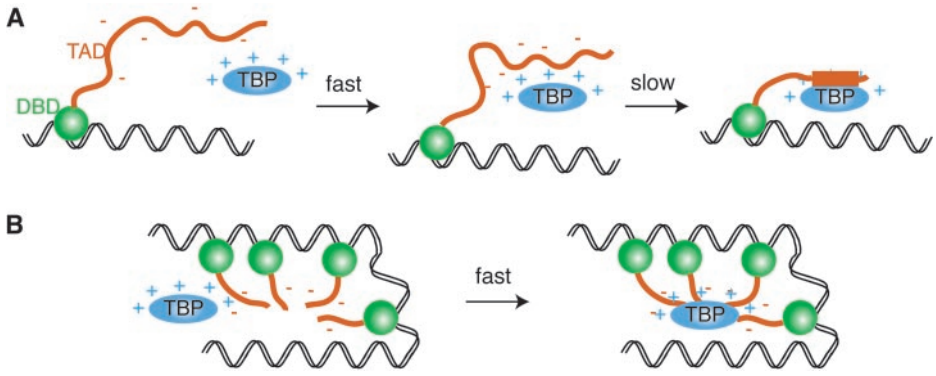

FIG. 5. **Model for the TAD-target interaction.** *A*, a single-copy unstructured TAD binds weakly to its target. Subsequently, the TAD folds into a specific three-dimensional structure to interact more stably with its target. *B*, multicopy TADs utilize multiple weak interactions to bind their target. Protein folding is not required.

The Interaction between c-Myc and TBP Is Entropy-driven—To further characterize the interaction between c-Myc and TBP, we monitored the binding at various temperatures and salt concentrations and analyzed the data as shown in Fig. 3. These and the previous results are summarized in Table I. It is obvious that the complex is favored at elevated temperatures and that it is mainly the second step that is affected by temperature (Fig. 4*A*). The initial binding, the rapid equilibrium, is not influenced. For the second step, the conversion from an unstable to a stable complex, van't Hoff analysis, reveals positive enthalpic and entropic contributions (ΔH = 133 kJ/mol, ΔS = 479 J mol^{-1} K^{-1}), indicating that this entropy-driven reaction involves hydrophobic interactions. In contrast, the initial binding step is less stable at elevated salt concentration (Fig. 4*B*), suggesting that polar and ionic interactions play a predominant role.

DISCUSSION

The minimal TAD of some activator proteins has been defined as a very short stretch of continuous amino acids. Examples of such simple transactivators include the yeast transcriptional activators Gal4p, where 17 residues are sufficient to activate transcription (54), and Gln3p, whose minimal transactivation domain has been mapped to 13 residues (57). On the other hand, there are more complex TADs like those from the mammalian transactivator pax 6 (60) or the human glucocorticoid receptor (61) for which a larger stretch of amino acid residues is necessary for efficient transactivation. Our results indicate that c-Myc is likely to belong to the second class of transactivators, since an extended activation domain is required for efficient interaction with TBP (Fig. 1). The interaction with TBP does not seem to be solely ionic, as has been suggested for some acidic TADs, since the two shorter fragments that do not interact efficiently have a net acidity that is higher than the intact TAD. The apparent contradiction with the finding that the shorter Myc fragments interact poorly if at all with TBP, yet can nonetheless activate transcription in yeast cells,[2] may be reconciled if the relative intracellular protein concentration is taken into account. The short fragments were present in concentrations that were orders of magnitude above the concentration of the construct carrying the full c-Myc TAD due to the lack of protein degradation signals that are present in the latter protein (13). This overexpression is likely to lead to increased occupancy of the c-Myc DNA binding sites in the reporter construct and thereby to a reduced dependence on high affinity interactions with at least some target proteins. Thus the findings presented here together with unpublished work[2] strongly suggest that at least two distinct regions within the c-Myc activation domain contribute to efficient target factor interaction.

Generally, TADs have little, if any, secondary or tertiary structure when they are isolated in solution (19–25). However, a small number of complexes between TADs and their target factors have been studied at atomic resolution, and all show that the TAD has a distinct tertiary structure in the complex (20, 52, 62). In many other cases, including c-Myc, the adoption of secondary structure has been suggested based on spectral changes or mutational studies (18, 21, 51, 63, 64). The question that has been unresolved so far is, when this postulated folding of the TAD takes place. Our results (Figs. 3 and 4) suggest that initial binding precedes the folding of the TAD. According to the model shown in Fig. 5*A*, the initial contact with target proteins occurs by electrostatic interactions, presumably between acidic residues of the TAD and positive charges on the target. This unstable complex has a dissociation constant in the supermicromolar range. It slowly converts to a more stable form in a process that involves folding of the TAD into a defined structure and presumably formation of specific contacts between the TAD and its target. Supporting evidence for this model comes from mutational analysis of the VP16 and glucocorticoid receptor (τ1) TADs. These studies revealed that individual acidic residues were of minor importance, but that the total acidity of the protein was important for activity (46, 65). In contrast, substitutions of individual hydrophobic amino acids did affect activity (46, 66). Interestingly, a group of such mutants that altered the activity of the τ1 TAD had similar effects on its interactions with a variety of target proteins (43). This suggests that the mutations affect a common mechanism such as protein folding that is an essential prerequisite for stable interaction of the TAD with all target proteins (43).

TADs frequently interact with more than one target factor. A strategy where the TAD does not fold into a specific structure until it encounters a target factor can be thermodynamically advantageous. The intermolecular protein-protein interaction surface can then be fitted closely to many structurally diverse target proteins, unrestricted by a pre-formed structure, thus increasing the stability of the complexes (67). A "bind then fold" strategy can also be advantageous kinetically, utilizing a postulated pathway that Shoemaker and co-workers (68) have termed the "flycasting mechanism." According to this model an unstructured protein has a larger interaction radius and thus encounters its target faster than a fully folded protein. The target can subsequently be "reeled in" through protein folding.

It may be that not all TADs rely on the mechanism proposed in Fig. 5*A*, despite its potential thermodynamic, kinetic, and specificity advantages. When an activator binds in multiple copies to its target genes, as is often the case with artificial reporter gene systems, multiple weak interactions could be sufficient to stabilize the complex and thus achieve transactivation (Fig. 5*B*). In such cases folding of the TAD into a specific three-dimensional structure might not be required. This mechanism might be physiologically relevant in enhancers of metazoan promoters, where many transactivators often bind adja-

cently. Transactivators working through this latter mechanism of multiple weak interactions might have a kinetic advantage, since they might be able to bind target proteins faster than transactivators relying on the bind then fold mechanism. However, they would lose the specificity achieved by specific hydrophobic interactions. On naturally occurring promoters, a "healthy" mixture of fast (but not very specific) and slow (but specific) activator-target interactions may contribute to the appropriate balance required for regulated gene expression.

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