Loss of FBP function arrests cellular proliferation and extinguishes *c-myc* expression

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The *c-myc* regulatory region includes binding sites for a large set of transcription factors. The present studies demonstrate that in the absence of FBP [far upstream element (FUSE)-binding protein], which binds to the single-stranded FUSE, the remainder of the set fails to sustain endogenous *c-myc* expression. A dominant-negative FBP DNA-binding domain lacking effector activity or an antisense FBP RNA, expressed via replication-defective adenovirus vectors, arrested cellular proliferation and extinguished native *c-myc* transcription from the P1 and P2 promoters. The dominant-negative FBP initially augmented the single-stranded character of FUSE; however, once *c-myc* expression was abolished, melting at FUSE could no longer be supported. In contrast, with antisense FBP RNA, the single-stranded character of FUSE decreased monotonically as the transcription of endogenous *c-myc* declined. Because transcription is the major source of super-coiling *in vivo*, we propose that by binding torsionally strained DNA, FBP measures promoter activity directly. We also show that FUSE is predicted to behave as a torsion-regulated switch poised to regulate *c-myc* and to confer a higher order regulation on a large repertoire of factors.

Keywords: cell growth/*c-myc*/far upstream element/
FUSE-binding protein

Introduction

The *c-myc* proto-oncogene encodes an important member of the basic helix–loop–helix leucine zipper family of transcription factors and is involved in cell growth, proliferation, differentiation and apoptosis. The dysregulation of *c-myc* in a variety of neoplasms, which is necessary or contributory to the pathogenesis of the tumors, results largely from disruption of the normal transcriptional control mechanisms. *c-myc* responds to a host of intracellular and extracellular signals including metabolic state, DNA damage, hormones, cytokines, lymphokines, pharmacological agents and a variety of biological processes (Cole,

1986; Luscher and Eisenman, 1990; Spencer and Groudine, 1991; Marcu *et al.*, 1992; Potter and Marcu, 1997). These signals act through a large set of transcription factors operating through diverse elements flanking and embedded within the *c-myc* gene. The rules by which these signals are integrated to adjust the level of *c-myc* transcription are largely unknown. Transfection and transgenic studies using DNA fragments as large as 60 kb centered around *myc*-coding segments have failed to recapitulate fully the physiological pattern of *c-myc* expression (Lavenue *et al.*, 1994; Mautner *et al.*, 1996). Moreover, *c-myc* expression is dysregulated in Burkitt's lymphoma by translocations with break points as remote as hundreds of kilobases on either side of the gene. Together, these observations suggest two possibilities: either essential regulatory sequences operating over vast expanses embrace *c-myc* or the proper functioning of some proximal *cis*-sequences is determined by higher order features of *c-myc* chromatin.

Factors binding to particular sequences in single-stranded DNA are likely to operate most effectively within their natural chromosomal context where the appropriate physiological conditions exist to present the specific unpaired bases for molecular recognition. Hence the activity of these single-stranded *cis*-elements must be coupled with processes which stably or dynamically stress DNA (Rothman-Denes *et al.*, 1999). For example, transcriptionally generated torsional strain may recruit single-stranded DNA-binding transcription factors to their binding sites. The far upstream element (FUSE) is found 1.5 kb upstream of the *c-myc* promoter P1 and has been shown to be single stranded when this gene is expressed (Avigan *et al.*, 1990; Duncan *et al.*, 1994; Michelotti *et al.*, 1996). Moreover, when the *c-myc* gene is active, the regular nucleosomal array at FUSE is disrupted (Michelotti *et al.*, 1996). These structural alterations at the FUSE might be true architectural antecedents for subsequent regulatory steps controlling promoter activity or merely epi-phenomena of an activated *c-myc* locus, irrelevant to correct regulation. The FUSE-binding protein (FBP) complexes with FUSE in single-stranded or torsionally strained DNA *in vitro*. However, FBP will not bind to unstrained, duplex FUSE (Duncan *et al.*, 1994; Michelotti *et al.*, 1996). Therefore, FBP binding at FUSE *in vivo* may be coupled to processes destabilizing the double helix, such as transcription from a nearby promoter. Because FBP has an N-terminal repression domain and a C-terminal activation domain, it has the capacity, in principle, to modulate transcription directly (Duncan *et al.*, 1996) using FUSE as a single-stranded platform. FBP expression vectors stimulate the expression of transiently transfected *c-myc* promoter reporter plasmids, suggesting that FBP has the capacity to regulate *c-myc* *in vivo* (Duncan *et al.*, 1994). In this study, FBP's action on the endogenous *c-myc* gene was investigated by expressing

Fig. 1. The FUSE mediates activation by full-length FBP or repression by the DNA-binding central domain. Plasmids (5 μ g) directing the expression of either full-length FBP (lanes 3, 4, 9 and 10), the DNA-binding central domain (FBPcd, lanes 5, 6, 11 and 12) or the vector alone (lanes 1, 2, 7 and 8) were co-transfected with a *c-myc* promoter-CAT reporter gene into the B-cell line BJAB (A) or the osteosarcoma cell line U2OS (B). Reporters used the wild-type *c-myc* promoter (pMP-CAT, 5 μ g, lanes 1–6) or FUSE-deleted *c-myc* promoter (pMP-CAT- Δ FUSE, 5 μ g, lanes 7–12). TLC plates were quantitated using a phosphorimager and Image-Quant software.

in cells either a dominant-negative form of the protein or FBP antisense mRNA. Interfering with normal FBP function, even in the presence of many other *c-myc* regulatory proteins, establishes a direct and overriding role for this sequence-specific, single-stranded and supercoiled DNA-binding protein in the maintenance of proper gene expression at the *c-myc* locus. We propose that the response of the FUSE to the topological and architectural constraints at the *c-myc* locus controls FBP activity and explains the overriding influence of this protein.

Results

The FBP central domain (FBPcd) dominantly interferes with *c-myc* expression

FBP has an N-terminal transcription repression domain, a C-terminal transcription activation domain and a central domain (FBPcd) which binds single-stranded nucleic acids of specific sequence (Duncan *et al.*, 1994, 1996). The FBPcd itself is composed of four distinct KH motifs, each followed by an amphipathic helix. KH motifs are found in a variety of proteins recognizing single-stranded nucleic acids. Just as zinc fingers comprise a module of protein architecture, which may be adjusted to recognize DNA or RNA (Clemens *et al.*, 1993; Siomi *et al.*, 1993; Caricasole *et al.*, 1996), so KH proteins may bind to a variety of

specific RNA or DNA targets determined by the exact structural features of each protein (Bomszyk *et al.*, 1997; Ostareck-Lederer, 1998; Baber *et al.*, 1999). *In vitro*, FBPcd and subdomains thereof bind specifically to FUSE. In contrast to the full-length FBP, FBPcd stripped of its effector domains should fail as a transcription regulator. Therefore, if overexpressed *in vivo*, FBPcd would be expected to compete with endogenous FBP for binding to DNA targets and subsequently to disrupt target gene regulation.

To test these predictions, plasmids directing the synthesis of FBPcd or full-length FBP were co-transfected with intact or FUSE-deleted *c-myc* promoter-CAT reporter plasmids. Whereas FBP stimulated CAT activity as expected, FBPcd depressed reporter activity below the level supported by the endogenous FBP (Figure 1A and B, lanes 3 and 4 versus 5 and 6). Both the activation by FBP and the depression by FBPcd required the FUSE (Figure 1A and B, lanes 3–6 versus 9–12) and were observed in both B- (Figure 1A) and osteosarcoma (Figure 1B) cell lines (BJAB and U2OS, respectively). These results indicated that FBPcd might provide an *in vivo* tool to explore the roles of FBP and FUSE in regulating the endogenous *c-myc* gene, at its native chromosomal locus.

If the FBPcd-mediated repression of the *c-myc* pro-

motor-CAT reporter mirrors the response of the endogenous *c-myc* gene, then the *c-myc* concentration should be lower in FBPcd-transfected than in untransfected cells. To mark transfected cells expressing FBPcd, a chimeric FBPcd-green fluorescent protein (GFP) was transiently

expressed in osteosarcoma cells (Chalfie *et al.*, 1994). 4',6-Diamidino-2-phenylindole (DAPI) staining identified all adherent cells in the areas analyzed, GFP fluorescence revealed transfected cells synthesizing the chimera, while immunostaining with anti-myc identified *c-myc*-expressing

A

GFP-FBP

GFP-FBPcd

c-myc

c-myc

DAPI

DAPI

cells. Representative fields illustrating these results are shown in Figure 2A. Examining several hundred transfected cells microscopically, 50–90% of the GFP-positive cells were judged either to be negative for *c-myc* or to stain at levels considerably lower than their untransfected (GFP-negative) neighbors (Figure 2A, right; Materials and methods). Of untransfected cells, only ~5% were judged to have low levels of *c-myc*, and staining with DAPI revealed that these were usually in mitosis. In contrast, *c-myc* staining was actually brighter in transfected cells expressing GFP–FBP (full-length) than in their non-transfected neighbors (Figure 2A, left). Apparently, expression of GFP–FBPcd interfered with the expression of *c-myc* protein, whereas FBP increased *c-myc* synthesis as predicted. Similar changes in *c-myc* staining were observed in cells expressing hemagglutinin (HA) epitope-tagged FBP or FBPcd. To quantitate the changes in *c-myc* expression in cells transiently synthesizing FBPcd-HA or over-expressing FBP-HA, transfected cells were immunostained for HA and *c-myc* and then analyzed by flow cytometry (Figure 2B). On average, the cells expressing FBP-HA almost doubled their *c-myc* content, whereas cells synthesizing FBPcd-HA had less than half the *c-myc* of their untransfected neighbors. These results indicate that FBP has the potential to adjust cellular *c-myc* levels.

To gain an indication of whether suppression of *c-myc* by HA-FBPcd might occur through the FUSE element,

HA-FBPcd was transfected into two Epstein–Barr virus (EBV)-positive Burkitt's lymphoma cell lines, Raji and Daudi. Whereas Raji has suffered a translocation 1398 nucleotides upstream of *c-myc* promoter P1, thereby removing the FUSE to another chromosome, in Daudi cells the pathogenic translocation occurred far 5' of *c-myc* and so did not disturb the arrangement of FUSE with the rest of the *c-myc* gene (Rabitts *et al.*, 1984; Dyson and Rabitts, 1985). The *c-myc* levels were not different when compared in HA-positive, HA-FBPcd-transfected and untransfected Raji cells. In contrast, many HA-positive Daudi cells had much lower levels of *c-myc* staining than untransfected or HA-negative cells. Most simply, this experiment suggests that FUSE directs FBP's action upon the *c-myc* gene, although definitive demonstration of this point will require the specific removal of FUSE through homologous recombination.

If FBPcd suppressed *c-myc* staining by interfering with endogenous FBP function, then other schemes blocking FBP action or decreasing FBP levels would be expected to yield similar biological effects. For example, expression of antisense FBP mRNA would be predicted to phenocopy the effects of FBPcd in many respects. To implement this strategy, an efficient means was sought to deliver and express FBPcd or antisense FBP mRNA (FBPAs) in cells in order to probe the response of candidate, endogenous FBP targets.

Fig. 2. Expression of the FBP central domain (FBPcd) decreases endogenous *myc* protein, whereas overexpression of FBP increases *c-myc*.

(A) U2OS cells were transfected with GFP–FBPcd (left) or GFP–FBP (right). After 16 h, all cells were stained for DNA with DAPI (bottom), processed for immunostaining with anti-*c-myc* primary antibody and rhodamine-conjugated secondary antibody (middle) and transfected cells were identified by GFP fluorescence (top panel). The arrows indicate transfected cells identified by GFP fluorescence. (B) Plasmids directing the synthesis of FBP-HA or FBPcd-HA were transfected into U2OS cells, immunostained for *myc* and HA, and analyzed with FACS. HA-positive cells formed a discrete population with increased (middle) or decreased (left) *c-myc*. (C) FBPcd-HA was transfected into both FUSE-minus Raji and FUSE-positive Daudi cells (right panels) and immunostained as in (B). HA-positive (region B; top right panel) and HA-negative Raji cells (region A) displayed similar levels of *c-myc* whereas HA-positive Daudi cells (region B, bottom right panel) showed reduced levels of *c-myc* compared with cells not expressing FBP-interacting repressor (region A). Left panels show *c-myc* levels in mock-transfected cells. The region corresponding to FBPcd-transfected cells is enclosed by a red line and labeled B. The number of cells in regions B for control versus HA-FBPcd-transfected cells was 180 versus 2179 for Raji, and 92 versus 2907 for Daudi. The percentage of total cells transfected is indicated at region B.

Interfering with FBP function arrests proliferation

DNA segments directing the expression of either FBPcd or antisense FBPs were cloned into replication-defective adenoviruses, which are effective tools for introducing genes into cells (Bett *et al.*, 1994). The resulting viruses were used to infect osteosarcoma cells (U2OS). In order to visualize the reduction or elimination of target gene expression in infected cells, the residual expression from uninfected cells must be minimized. This requires that FBPcd or antisense FBP be expressed at sufficient levels and in enough cells to alter the observed expression levels of FBP target genes. At 20 p.f.u. per cell, 85–99% of the cultured osteosarcoma cells expressed virally encoded

Fig. 3. Adenoviruses expressing dominant-negative FBPcd or antisense FBP RNA down-modulate endogenous FBP. U2OS osteosarcoma cells were infected with replication-defective adenoviruses expressing dominant-negative FBP (AdFBPcd) or antisense FBP RNA (AdFBPAs). (**A**) Time course of FBP expression monitored by immunoblot with anti-FBP; the top panel shows the accumulation of FBPcd with the parallel decline of endogenous FBP (inset shows a shorter exposure). The middle panel shows the decline of FBP in response to antisense FBP RNA, and the lower panel shows that FBP levels do not respond to adenovirus vector. (**B**) Time course of FBPcd mRNA accumulation and FBP mRNA decline in response to AdFBPcd; RNA extracted from AdFBPcd-infected cells was analyzed by Northern blot.

Fig. 4. Dominant-negative FBP or antisense FBP RNA arrest cellular proliferation. Growth curve of U2OS cells infected with AdFBPcd, AdFBPAs or replication-defective adenovirus vector alone.

proteins (data not shown). Because B cells are resistant to adenovirus infection, even at high multiplicity of infection (m.o.i.), BJAB cells, though quite responsive to FBP and FBPcd (Figure 1), could not be tested. Northern blotting and immunoblots were used to verify the expression of FBPcd mRNA and protein in cells infected with the replication-defective adenovirus (AdFBPcd) encoding the candidate dominant-negative FBPcd (Figure 3). FBPcd mRNA was detected 6 h post-infection and the concentration of FBPcd mRNA exceeded that of endogenous FBP mRNA after 12 h (Figure 3B). FBPcd protein was first detected 10 h post-infection, was present at concentrations equal to FBP by 24 h, and by 72 h FBPcd was in vast excess compared with the endogenous protein (Figure 3A). As FBPcd expression increased, endogenous FBP mRNA and protein decreased, suggesting that FBP directly or indirectly up-regulates its own synthesis. This decrease in FBP levels would be predicted to enhance the ability of FBPcd to overwhelm its normal counterpart.

FBPcd rapidly prevented cell division, but preserved viability (Figure 4). Cessation of growth was also seen when SAOS2 osteosarcoma cells were infected with this virus. The distribution of non-dividing, FBPcd-expressing cells through the cell cycle was similar to that seen in the normally proliferating control, although late after infection a reduction in the S-phase fraction was noted (data not shown). The failure of the non-dividing AdFBPcd-infected cells to accumulate at either the G₁/S or G₂/M checkpoints suggests that continuous FBP activity is required for cells to progress through the cell cycle. These data are also compatible with the hypothesis that FBPcd causes an extreme prolongation of the cell cycle rather than a strict growth arrest. In fact, cell lines devoid of *c-myc* and lacking compensatory changes in one of the other *myc* proteins almost triple their doubling time. Prolongation of G₁ and G₂ accounts for the slower growth of these cells as the duration of S-phase is not increased in these *c-myc* null cells, hence there is a smaller S-phase fraction (Mateyak *et al.*, 1997, 1999), just as occurred late after infection with AdFBPcd and AdFBPAs.

An alternative means of eliminating FBP was sought to confirm that the severe effects of FBPcd on growth were due to interference with proper FBP function rather than to an indirect or toxic effect of the overexpressed recombinant protein. If FBPcd blocks FBP action, then cells with decreased FBP levels should display similar changes to those seen in FBPcd-expressing cells. However, if FBPcd interferes with some other process, then reduced FBP levels should not phenocopy FBPcd expression. To reduce synthesis of endogenous FBP, cells were infected with a replication-defective adenovirus expressing FBP antisense mRNA (AdFBPAs). FBP levels decreased starting ~10 h after AdFBPAs infection (Figure 3A). The AdFBPAs-infected cells stopped dividing (Figure 4) and remained frozen throughout the cell cycle (data not shown), similarly to the AdFBPcd-infected cells. In contrast, cells infected with the control virus continued to proliferate. AdFBPcd and AdFBPAs seemed likely to prove effective tools to modify endogenous FBP activity in order to monitor the response of the native *c-myc* gene in infected cells.

Maintenance of *c-myc* RNA requires FBP

If FBP regulates *c-myc*, then manipulations interfering with levels or activity of the former should rapidly, if not persistently, perturb expression of the latter. Because *c-myc* mRNA has a short half-life of 20–30 min, its concentration serves as a good indicator of net *c-myc* promoter activity. Thus, when sufficient FBPcd accumulates to compete successfully with the endogenous FBP for binding at the FUSE, *c-myc* mRNA levels should

Fig. 6. FBPcd enhances and antisense FBP weakens melting at FUSE. (A) Cells infected with AdFBPcd (lanes 4, 7 and 10), AdFBPas (lanes 5, 8 and 11), adenovirus vector (lanes 3, 6 and 9) or uninfected cells (lane 2), as well as naked DNA (lane 1) were treated with KMnO_4 to oxidize single-stranded DNA *in vivo* and processed for ligation-mediated PCR. Infections proceeded for the time indicated prior to treatment. * indicates bases approximately equivalently reactive in all cells; < indicates bases relatively more reactive *in vivo* and hyperreactive due to FBPcd; v indicates bases becoming hyporeactive when *c-myc* expression shuts off. (B) Treatment with the transcription inhibitor α -amanitin gradually abolished the hyperreactivity of bases in FUSE to KMnO_4 in both HeLa and U2OS cells. Note that the same bases (marked a, b and c) made hyperreactive by FBPcd are made less reactive by α -amanitin.

Fig. 5. FBP function is required to maintain *c-myc* expression.

(A) Total RNA (10 μg) extracted from cells infected with AdFBPcd (lanes 6–10), AdFBPas (lanes 11–15), adenovirus vector (lanes 2–5) or uninfected cells at the indicated times was analyzed with a multiplex RNase protection assay using an SP6 RNA polymerase-generated probe for *c-myc* and T7 RNA polymerase-generated probes for cyclin G1, cyclin G2, cyclin I, ribosomal protein L32 and glyceraldehyde phosphate dehydrogenase (GAPDH). After hybridization and RNase digestion, the protected fragments were visualized by autoradiography after electrophoresis on a 6% denaturing polyacrylamide gel. (B) The intensity of the protected fragment for each RNA (and cyclins E and H, not shown) from AdFBPcd- and AdFBPas-infected cells was determined (Image-Quant Tools software) and expressed as a ratio relative to the same RNA from adenovirus vector-infected cells harvested at the same time. GAPDH levels were used to normalize for loading. (C) Expression of *c-myc* from a heterologous promoter partially reversed FBPcd-induced growth arrest in Rat1a cells. Rat1a or Rat1a-myc cells (Stone *et al.*, 1987; Hoang *et al.*, 1994) were infected with AdFBPcd, counted and scored for viability by the exclusion of Trypan blue at the indicated times post-infection.

decline rapidly. To test this hypothesis, total RNA from cells infected with AdFBPcd was harvested at selected intervals following infection and the abundance of particular transcripts was determined using a multiplex RNase protection assay in which antisense RNA probes for a variety of transcripts were hybridized with total cellular RNA.

The RNase protection analysis revealed three patterns of gene activity in response to AdFBPcd or AdFBPas. First, the level of *c-myc* transcripts rapidly and almost completely declined. The 650 nucleotide *c-myc* probe used in this assay discriminates between transcripts initiated at the P1 promoter, which generate a 500 nucleotide protected fragment, and P2-initiated transcripts that protect 350 nucleotides of the probe. For the first 12 h of AdFBPcd infection, when FBPcd protein levels were low, *c-myc* RNA was not changed dramatically (Figure 5A, lanes 6 and 7); however, as FBPcd accumulated, transcription from both P1 and P2 decreased. By 24 h post-infection, *c-myc* mRNA was greatly diminished and after 48 h was minimal or absent (Figure 5A, lanes 8–10). Similar multiplex RNase protection analysis of RNA extracted from AdFBPas-infected cells revealed the same dramatic fall of *c-myc* P1 and P2. The rapid and extensive loss of *c-myc* RNA in response to AdFBPcd and AdFBPas is consistent with a direct role for FBP in the maintenance of *c-myc* expression. Because cyclin E RNA levels declined rapidly but incompletely following infection with AdFBPcd or AdFBPas (Figure 5B), proper FBP function may be required directly or indirectly in order to maintain expression of this gene. A composite direct and indirect effect of AdFBPcd on rapidly responding genes cannot be excluded.

Several RNAs exhibited a gradual and partial decline throughout the course of infection with AdFBPcd or AdFBPas. Among these transcripts were ribosomal protein L32, cyclin I and cyclin H (Figure 5A and B, and data not shown). The slow and incomplete response of these genes to interference with FBP function and levels would seem to indicate that they are poor candidates to be direct targets for FBP action. Rather, the partial decline of these RNAs most likely reflects an indirect effect of AdFBPcd or AdFBPas, or may be secondary to growth arrest caused by these viruses.

Merging with the second pattern, the third pattern of response to AdFBPcd and AdFBPas is exemplified by GAPDH and cyclins G1 and G2, whose transcripts remain at essentially constant levels throughout the infection. Infection with the replication-defective adenovirus vector did not alter the expression pattern of any genes analyzed with this assay (Figure 5A, lanes 2–5).

The growth arrest caused by AdFBPcd or AdFBPas might reflect the rapid loss of *c-myc* expression or the shut-off of unidentified FBP targets (not assayed with the RNase protection assay described above). To discriminate between these possibilities, Rat1a cells or Rat1a cells stably expressing *c-myc* were infected with AdFBPcd and monitored for growth. If *c-myc* were the only essential FBP target, then by restoring *myc* expression under the control of a heterologous, non-FBP-responsive promoter, cell growth should resume. AdFBPcd caused a rapid and complete growth arrest in Rat1a cells (Figure 5C). In contrast, growth was partially rescued in Rat1a-*myc*

(Figure 5C). These results suggest that FBPcd interferes with growth partly by blocking *c-myc* expression and partly by interfering with other FBP targets required to sustain normal growth.

FBP interacts directly with the FUSE of the endogenous *c-myc* gene

Does FBP operate on the endogenous *c-myc* gene directly through interactions with the FUSE? Previous studies have shown that the FUSE is single stranded *in vivo* when *c-myc* is expressed and is double stranded when *c-myc* expression is shut off irreversibly (Duncan *et al.*, 1994; Michelotti *et al.*, 1996). When the *c-myc* gene is silent, FUSE is closed even in the presence of FBP. If FBP braces the FUSE in an open, single-stranded configuration in *c-myc*-expressing cells, then enforced expression of FBPcd should maintain or augment the single-stranded character of the FUSE as long as *c-myc* is expressed. FBPcd would serve as an unregulatable strut hindering the reannealing of the unwound strands of the FUSE. [This model predicts that FBPcd sustains or enhances reactivity at FUSE only as long as sufficient superhelical stress persists because FBP can bind only with torsionally strained or single-stranded DNA (Bazar *et al.*, 1995; Michelotti *et al.*, 1996).] In contrast, depletion of FBP would leave the FUSE element vacant allowing the separated strands to reanneal to the more stable double helix, with diminished KMnO₄ reactivity. To test these predictions, cells were infected with AdFBPcd, AdFBPas or control virus, and treated with the selective agent KMnO₄ as a chemical probe for base unpairing. The reactivity of individual nucleotides at FUSE was monitored with ligation-mediated PCR.

As predicted from its dominant-interfering function, the permanganate reactivity of FUSE increased strikingly after AdFBPcd infection. FUSE melting was most prominent 16 h after infection (Figure 6, lane 4) and readily apparent even after 48 h (Figure 6, lane 7). However, by 72 h post-infection, FUSE hyperreactivity was lost, notwithstanding abundant FBPcd (Figure 6, lane 10). Thus FUSE hyperreactivity was seen only when *c-myc* RNA was also present, suggesting the coupling of FBPcd-facilitated FUSE melting with *c-myc* transcription. The ability of AdFBPcd to enhance the KMnO₄ reactivity at FUSE dramatically indicates that this element is melted incompletely in U2OS cells; were FUSE fully open, no further hyperreactivity would be seen after AdFBPcd infection. Compared with naked DNA, increased reactivity was noted at several positions within the FUSE of uninfected U2OS cells, indicating incomplete occupancy by cellular FBP. This pattern of increased FUSE reactivity was unaltered throughout the time course of infection with the replication-defective adenovirus control (Figure 6, lanes 3, 6 and 9); concurrently, *c-myc* RNA levels in these controls also remained unchanged.

Following infection with AdFBPas, the reactivity of FUSE with KMnO₄ decreased compared with uninfected cells. Oxidation at several bases within FUSE diminished monotonically, paralleling the declining levels of endogenous FBP (Figure 6, lanes 5, 8 and 11), and was completely abolished 72 h after AdFBPas infection. These data are consistent with reduced FUSE occupancy due to loss of FBP. Inhibiting transcription with α -amanitin in

Fig. 7. The sequence of the FUSE is tuned to melt biphasically between superhelical density -0.04 and -0.06 . A 3.2 kb segment of the *c-myc* sequence extending downstream of the *Hind*III site (-2329) was analyzed for melting probability as a function of superhelical density. Only the FUSE responded to the strain applied to the *c-myc* promoter. The sequence of FUSE is shown (bottom) along with the probability of melting (vertical) and the linking number deficit (ΔLk) starting at -12 , which corresponds to superhelical density $\sigma = -0.039$; subsequent (ΔLk , σ) are $(-13, -0.042)$, $(-14, -0.046)$, $(-15, -0.049)$, $(-16, -0.052)$, $(-17, -0.055)$, $(-18, -0.058)$, $(-19, -0.062)$ and $(-20, -0.065)$. Hyperreactive bases on the bottom strand are marked with arrowheads. The FUSE region is indicated by the line, which embraces hyper- and hyporeactive bases on both strands. (Duncan *et al.*, 1994; Michelotti *et al.*, 1996).

uninfected cells also decreased the $KMnO_4$ reactivity of these same bases compared with naked DNA. Inhibition of RNA synthesis might alter the conformation of FUSE either primarily due to loss of transcriptionally generated torsional strain or secondarily due to loss of a short half-life RNA necessary for FBP action or synthesis. HeLa cells possessing greater $KMnO_4$ reactivity throughout the FUSE region also display a more dramatic loss of FUSE melting upon treatment with α -amanitin. Loss of non-B DNA upstream of the *c-myc* gene following treatment with α -amanitin has been seen previously and supports the notion that ongoing transcriptional activity modifies upstream DNA structure (Wölfl *et al.*, 1997).

Biphasic melting of FUSE by torsional strain

Opening of the FUSE is associated with FBP binding and *c-myc* expression, whereas closing of the FUSE marks FBP release and *c-myc* gene inactivity. What properties of FUSE might account for this relationship? If FUSE melting were coupled with transcriptionally generated superhelicity, then the possibility of a mechanical linkage between transcription and FBP operation could be envisaged. The potential for FUSE melting within a topological domain comprised of 3.5 kb of *c-myc* regulatory and coding sequences was analyzed using the method of Benham (Benham, 1992; Fye and Benham, 1999). This method calculates the destabilization free energy and the probability of base unpairing for each nucleotide within a defined topological domain as functions of superhelical

density. The results of these calculations have been shown to be in close quantitative agreement with experimental measurements (Benham, 1992). When applied to genomic DNA sequences, these methods find that DNA regulatory regions frequently contain segments with high melting propensities (Benham, 1993, 1996). Analysis of *c-myc* DNA extending from -2329 through exon I revealed a single sequence predicted to melt with near unit probability. A surface contour showing the response of this region to negative superhelicity is shown in Figure 7. Remarkably, the singular sequence extracted by this calculation is FUSE, and includes the target sequences for both the strong and weak sub-domains of FBP's bipartite DNA-binding domain. The FUSE element has little propensity for opening until a superhelical density of -0.04 . Between densities -0.04 and -0.05 , there is a sharp transition as the downstream segment of FUSE melts. By -0.06 , a second abrupt transition occurs as a shoulder on the first peak expands until the entire FUSE region is predicted to be single stranded. Starting essentially from first principles, these calculations predict that the FUSE behaves as a torsion-regulated switch primed to regulate FBP binding.

Discussion

The present studies show that proper levels of functional FBP are necessary to maintain *c-myc* expression in human cells. Overexpressing the DNA-binding domain of FBP as a dominant-negative protein or reducing FBP levels

with antisense FBP RNA have exposed functional coupling between FBP and *c-myc* expression; this linkage accounts for the parallel expression profiles of FBP and *c-myc* mRNAs reported previously (Duncan *et al.*, 1994; Bazar *et al.*, 1995a). The nexus between FBP and *c-myc* occurs at FUSE, a positive *cis*-element operating on the *c-myc* promoter *in vivo*. In transient transfections, FBP efficiently drives *c-myc* promoter reporters unless FUSE is deleted. *In vivo*, FUSE is melted and nucleosome free when *c-myc* is expressed, but is double stranded and nucleosome bound when the gene is silent (Michelotti *et al.*, 1996). These observations are mirrored *in vitro* where FBP binds to single-stranded FUSE, but not to duplex FUSE unless embedded in negatively supercoiled DNA (Duncan *et al.*, 1994; Bazar *et al.*, 1995; Michelotti *et al.*, 1996). The enhanced melting of FUSE in cells infected with AdFBPcd and the diminished melting in cells infected with AdFBPas reveal that the FBP DNA-binding domain interacts directly with FUSE *in vivo*.

The *c-myc* regulatory region includes the binding sites for many conventional transcription factors, yet without FBP this set is insufficient to sustain *c-myc* transcription. What does FBP add to the mix? Most directly, FBP binding at the FUSE of an active *c-myc* gene would regulate transcription using its positive and negative effector domains. Furthermore, DNA-bound FBP serves as a platform to recruit the FBP-interacting repressor (FIR) to the *c-myc* gene where the latter blunts TFIIF action to down-regulate promoter escape (Liu *et al.*, 2000). The FIR/FBP system operating late in the transcription cycle is well placed to endorse or override the activating signals delivered by conventional transcription factors. However, in order for FBP to act, the FUSE itself must first be activated. Because FBP only binds with single-stranded or torsionally strained DNA, an interesting scenario relating FBP's binding properties to FBP function may be possible. If silent, *c-myc* genes are relaxed, whereas transcribing *c-myc* genes are torqued as the transcription apparatus and template counter-rotate; thus, FUSE-FBP interactions would be restricted to active genes. Conventional factor binding to duplex *cis*-elements is determined by the amount and affinity of the particular protein involved; unless masked, the default state of the target sequence is permissive. In contrast, FUSE would be constitutively inaccessible, so FBP action would be coupled obligatorily with double-helical destabilization. The fact that FBPcd fails to sustain FUSE melting after *c-myc* expression ceases supports the notion that ongoing gene activity is a necessary condition for binding. FBP and similar single-strand DNA-binding proteins are therefore logical instruments for tuning the expression of active genes, but seem ill-contrived for primary switching between active and silent states.

FBP is required for *c-myc* expression, but *c-myc* expression must first be activated for FBP binding. How can these almost contradictory observations be reconciled? The presence of a paused RNA polymerase molecule and the hold-back of transcription downstream of *c-myc* promoter P2 may provide a neat solution to this dilemma (Bentley and Groudine, 1986; Strobl and Eick, 1992; Krumm *et al.*, 1995; Albert *et al.*, 1997). Following the action of conventional transcription factors to recruit the basal machinery and stimulate pre-initiation complex

formation, transcription to the point of hold-back could serve to generate enough torsional strain to prime FBP binding. Subsequent regulation by FBP would control the hold-back or release of paused RNA polymerases. Thus FBP would modulate, but not switch, *c-myc* activity by controlling a rate-limiting step for production of full-length transcripts.

If *c-myc* is essential, it is also dangerous. Stable 4-fold overexpression of *myc* is oncogenic (Aghib *et al.*, 1990), and even a pulse of unscheduled *myc* activity is tumorigenic, provoking genomic instability (Felsher *et al.*, 1999). Decreasing *myc* levels by just one-half prolongs the cell cycle (Shichiri *et al.*, 1993). Hence, *myc* must be tightly regulated. Basal levels of *c-myc* mRNA and protein are low in most cells, sometimes as low as 10 molecules per cell (Taylor *et al.*, 1986; Bresser and Evinger-Hodges, 1987; Evinger-Hodges *et al.*, 1988). Regulating levels of gene expression through the association and dissociation of factors with *cis*-elements is inherently stochastic. The number of copies of highly transcribed mRNAs, regulated by common factors, will be relatively stable, but wide fluctuations in transcript levels may be inevitable for genes with low transcription rates. Because some components of the basal transcription apparatus remain stably bound even after the first round of initiation (Zawel *et al.*, 1995), the statistical variation in promoter firing may be amplified by jackpot effects. Once activated, a promoter may initiate several times until regulation by upstream elements is re-established. Conventional feedback regulation may be too slow to achieve tight control; *c-myc* RNA must be transcribed, spliced, processed, transported and translated. Then *c-myc* protein must assemble with its appropriate partners, be modified appropriately, enter the nucleus and find its targets. Even if *c-myc* protein directly autoregulates, it is likely that the time required for this cycle would prove too long to suppress random fluctuations. These considerations suggest that maintaining correct levels of *c-myc* expression may require more immediate and rapidly responsive mechanisms, such as interactions between FBP and FUSE. If these considerations are valid, then FUSE-like elements and FBP-like proteins should participate in the regulation of other short half-life molecules where small changes in expression modify cellular activity.

Structural deformation of DNA due to transcriptional torque or forces generated by other mechanical, chemical or physical processes would be well suited to provide a real-time mechanism coupling the activity of particular regulatory factors with gene activity. A variety of structural adaptations to torsional strain are possible, including melting, hairpin extrusion and Z-DNA formation. In fact, proteins recognizing specific DNA elements adopting such unusual structures have been identified from phage to man (Rothman-Denes *et al.*, 1999). The dynamic interplay between these proteins and their target *cis*-elements promises to expand the regulatory repertoire conferred by the DNA sequences controlling gene expression.

Materials and methods

Plasmid and virus constructions

The expression vector for FBP includes a full-length FBP cDNA cloned between the *EcoRV* and *XhoI* sites of pcDNA I AMP (Duncan *et al.*, 1996). The central domain of FBP [residues 107–477 fused to the last

three residues, 642–644, thus preserving the 3'-untranslated region (3'-UTR) of FBP] was similarly cloned into pcDNA I-AMP to create FBPcd/pcDNA I-AMP. FBPcd and its 3'-UTR were excised with *HindIII* from FBPcd/pcDNA I-AMP and cloned into pEGFP-C2 (Clontech) to make GFP-FBPcd (Chalfie *et al.*, 1994; Cormack *et al.*, 1996; Yang *et al.*, 1996). HA-tagged FBP and FBPcd were amplified by PCR using primers 5'-GTAACGGCCGCAACCATGGTTCCAGATGGAATGGTTGGA-3' and 5'-TTATTAGTTCGACTTAACCAACGCGTAATCTGG-AACATCGTATGGGTACATTGCAGGGTTGTATGGTCCCAT-3' from FBP cDNA and ligated into pcDNA1/amp (Invitrogen) cut with *EagI* and *XhoI*. FBPcd and its 3'-UTR were isolated from FBPcd-pcDNA I-AMP after *BamHI* and *SaI* digestion, and cloned into the vector pACCMV.pLpA (Becker *et al.*, 1994). To clone FBPcd in the reverse orientation for antisense, the same DNA segment was isolated following cleavage with *BamHI* and *EcoRI*, and was cloned into pACCMV.pLpA (Bett *et al.*, 1994; Graham and Prevec, 1995). The resulting plasmids pAdFBPcd and pAdFBPas were recombined with the adenovirus packaging plasmid, transfected into 293 cells, plaque purified, expanded and virus was purified (Graham and Prevec, 1995). The control virus was Ad dl312 (Jones and Shenk, 1979). Reporter plasmids pMP-CAT-FUSE and pMP-CAT-ΔFUSE were described previously (Avigan *et al.*, 1990).

Cell culture, chemical treatment, viral infection and DNA transfections

U2OS cells were routinely grown in Dulbecco's modified minimal essential medium supplemented with 1 mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin G and 10% supplemented fetal calf serum (Life Technologies). The BJAB, Raji and Daudi cells were grown routinely in RPMI 1640 medium supplemented as described. α -amanitin (12.5 µg/ml) (Sigma) was used to inhibit transcription in both U2OS and HeLa cells (Casse *et al.*, 1999). Cells were treated with KMnO_4 and genomic DNA extracted at the indicated time and used for ligation-mediated PCR. Rat1a and Rat1a-myc cells were described previously (Hoang *et al.*, 1994; Stone *et al.*, 1987).

For viral infection, cells were seeded at 1×10^6 in a 10 cm dish. After overnight culture, the cells were incubated with recombinant adenovirus for 1 h in the serum-free medium at an m.o.i. of 10 per cell, and then supplemented with serum to 10%.

For transient expression chloramphenicol acetyl transferase (CAT) assay, cells were transfected by electroporation with a cell-porator (Life Technologies) at 1180 µF and 180 V. The cells were harvested 16–20 h later; extracts were prepared and assayed for CAT as described (Gorman *et al.*, 1982). For GFP-FBPcd plasmid, cells were transfected using Lipofectamine Plus (Life Technologies). Cultures were plated routinely at 1×10^5 cells/well in 6-well plates overnight, transfected the following day, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 16–20 h later, and photographed.

Cell extracts and immunoblot analysis

The cells were lysed by RIPA buffer (1% w/w NP-40, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 0.15 M NaCl, 2 mM EDTA, 0.01 M sodium phosphate pH 7.2, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 100 U/ml aprotinin) for 30 min on ice and the debris was pelleted at full speed for 30 min. The supernatant (cell extract) was subjected to immunoblot analysis. The protein amounts in each sample were quantitated both directly by protein assay (Bio-Rad) and by estimating the intensity of Coomassie blue staining with SDS-PAGE. A 10 µg aliquot of total protein was separated by SDS-PAGE using a 4–12% gradient gel (NuPAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 4% non-fat dry milk in Tris-buffered saline (TBS) and then incubated overnight at 4°C with primary antibodies, washed three times, each for 5 min, with TBS containing 0.3% Tween-20, and incubated with 1:2500 diluted horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Membranes were washed as above, and HRP was detected using the enhanced chemiluminescence reagent (1.25 mM 3-amino-phthalhydrazide, 0.2 mM coumaric acid, 0.3 mM hydrogen peroxide in 0.1 M Tris pH 8.5). Membranes were then exposed on Kodak X-Omat AR X-ray film (Eastman Kodak). Affinity-purified rabbit antibodies against FBP were prepared in our laboratory and used at 1:1000 dilution in immunoblot analysis. Immunoblots were also analyzed with a 1:1000 dilution of c-myc antibody (Upstate Biotechnology) and 1:7500 dilution of actin antibody (Sigma).

Immunocytochemistry

The U2OS cells were grown up on coverslips overnight and then transfected with GFP-FBP and GFP-FBPcd using Lipofectamine Plus

reagents (Life Technologies). After 16 h, the cells were fixed in 4% paraformaldehyde in PBS for up to 20 min at room temperature and then washed twice with cold PBS. Immunocytochemistry was performed as described (Pihan *et al.*, 1998). The cells were then permeabilized by treatment with 80 mM HEPES pH 6.8, 5 mM MgCl_2 and 0.5% Triton X-100, for 5 min at room temperature. The blocking solution [PBS with 0.5% Triton X-100, 2% bovine serum albumin (BSA)] was added to the cells at 37°C for 30 min. The primary antibody (rabbit polyclonal against c-myc; Upstate Biotechnology) was added at a 1:1000 dilution to the blocking buffer, incubated for 1 h at 37°C and then washed twice in cold PBS. The secondary antibody conjugated to rhodamine (Roche) was added directly to the coverslip and covered with a plastic membrane for 1 h at 37°C. After immunostaining, cells were stained for 20 min with DAPI (1 µg/ml) and washed twice with PBS. Finally, the slides were mounted for photography. The intensity of GFP fluorescence and rhodamine staining were classified semi-quantitatively and/or qualitatively by multiple investigators, several of whom were blinded as to whether cells were transfected with GFP-FBP or GFP-FBPcd. Each investigator examined hundreds of transfected cells and there was unanimous agreement upon the experimental outcome by all observers.

Flow cytometry analysis

Cells were processed as for immunocytochemistry, but with the following changes. The cells were transfected with HA-tagged FBP or FBPcd. At 16 h post-transfection, the cells were trypsinized and fixed for immunostaining. The primary antibodies were rabbit anti-c-myc (Upstate Biotechnology) and mouse anti-HA (Santa Cruz Biotechnology, CA). The secondary antibodies, used at a 1:200 dilution, were fluorescein isothiocyanate (FITC)-conjugated anti-rabbit (Sigma) and R-phycoerythrin (PE)-conjugated anti-mouse (PharMingen). The samples were analyzed with FACScan; 10 000 cells of each sample were analyzed setting c-myc-FITC into FL1 and HA-PE into FL2. The transfected cells were identified as PE-positive cells shown on the x-axis.

Northern blot analysis

mRNA was oligo(dT) selected from the cell lysates with the mRNA isolation kit (Roche) as specified. A 1 µg aliquot of RNA was applied to a 1% agarose-formaldehyde gel, electrophoresed and transferred onto a HyBond N+ membrane (Amersham Pharmacia Biotech) for hybridization with DNA probes. The filters were hybridized and washed as described (Davis-Smyth *et al.*, 1996).

Ribonuclease protection assay (RPA)

Total RNA was isolated with Trizol (Life Technologies). A 10 µg aliquot of total RNA was processed for RNase protection assay as specified (PharMingen), except that the digestion was at 37°C. Exon 1 of human c-myc was cloned into PSV vector and its antisense transcripts were made with SP6 DNA-dependent RNA polymerase by incorporating [^{32}P]UTP. The labeled c-myc exon antisense probe was mixed with Cyc-2 probes (PharMingen) for RPA. Appropriately exposed gels were scanned and quantitated with Image-Quant Tools software. The effects of AdFBPcd or AdFBPas on transcript levels were compared with adenovirus vector alone and normalized to GAPDH for loading.

Potassium permanganate treatment and ligation-mediated PCR

KMnO_4 treatment and ligation-mediated PCR were performed as described (Michelotti *et al.*, 1996).

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