Glucocorticoids regulate the expression of the G₁ progression factor, cyclin D3. Cyclin D3 messenger RNA (CcnD3 mRNA) stability decreases rapidly when murine T lymphoma cells are treated with the synthetic glucocorticoid dexamethasone. Basal stability of CcnD3 mRNA is regulated by sequences within the 3'-untranslated region (3'-UTR). RNA-protein interactions occurring within the CcnD3 3'-UTR have been analyzed by RNA electrophoretic mobility shift assay. Three sites of RNA-protein interaction have been mapped using this approach. These elements include three pyrimidine-rich domains of 25, 26, and 37 nucleotides. When the cyclin D3 3'-UTR was stably overexpressed, the endogenous CcnD3 mRNA was no longer regulated by dexamethasone. Likewise, overexpression of a 215-nucleotide transgene that contains the 26- and 37-nucleotide elements blocks glucocorticoid inhibition of CcnD3 mRNA expression. These observations suggest that the 215-nucleotide 3'-UTR element may act as a molecular decoy, competing for proteins that bind to the endogenous transcript and thereby attenuating glucocorticoid responsiveness. UV-cross-linking experiments showed that two proteins of approximate molecular weight 37,000 and 52,000 bind to this 3'-UTR element.

Malignant lymphoid cells of thymic origin cease to proliferate and often die when exposed to glucocorticoids (1, 2), and glucocorticoids are an important tool for treatment of leukemias and other malignant and nonmalignant lymphoproliferative diseases. Glucocorticoids have also been proposed to be responsible for triggering apoptosis of CD4+/CD8+ cells in the thymus (reviewed in Ref. 3). We have analyzed glucocorticoid effects on murine T lymphoma P1798 cells in an attempt to elucidate the molecular mechanisms that account for glucocorticoid inhibition of cell proliferation and induction of cell death. The data indicate that glucocorticoids induce G₁/G₀ arrest by decreasing the expression of two critical cell G₁ progression factor, c-Myc (4) and cyclin D3 (5). P1798 cells utilize cyclin D3 as the principal D-type cyclin in G₁/S phase transition (6). Cyclin D2 is barely detectable and cyclin D1 is undetectable in P1798 cells (5). Consequently, inhibition of cyclin D3 blocks activation of Cdk4/Cdk6 and precludes progression through G₁ phase (5). Simultaneous overexpression of c-Myc plus cyclin D3 prevents cell cycle arrest and apoptosis of glucocorticoid-treated cells, although neither c-Myc nor cyclin D3 alone will suffice to protect cells (7).

Glucocorticoids control cyclin D3 expression in T-lymphoid cells by decreasing the stability of CcnD3 mRNA (5). CcnD3 mRNA is quite stable in mid-log phase P1798 cells, as determined by measuring mRNA abundance after treatment with actinomycin D. However, the rate of degradation of CcnD3 mRNA increases within 2 h after addition of glucocorticoids, to the extent that a 50% decrease in mRNA abundance occurs within 60–90 min after addition of actinomycin D to glucocorticoid-treated cells (5). This posttranscriptional mechanism of gene expression, coupled with the short half-life of the cyclin D3 protein, ensures a rapid response to glucocorticoids. There are several examples of glucocorticoid effects on mRNA stability (8–10), but the molecular mechanisms that underlie such events are largely unknown.

During the last decade, several RNA motifs have been identified that control the degradation rate of specific mRNAs. Iron-responsive elements (reviewed in Ref. 11) influence the stability of the transcripts of several genes that are involved in iron metabolism. AU-rich sequences (10, 12, 13), and polypyrimidine tracts (14–16) are known to control the stability of some mRNAs. Several families of RNA-binding proteins have been implicated in controlling the stability of mRNAs. The heterogeneous nuclear ribonucleoprotein (hnRNP) complex involved in maturation of mRNAs contain more than 20 protein species, many of which are involved in mRNA stability control (17, 18). Vertebrate homologues of the Drosophila embryonic lethal abnormal vision protein family regulate the stability of mRNAs encoding tumor necrosis factor-α (19), N-Myc (20, 21), and the cyclin-dependent kinase inhibitor p21Cip1 (22). Regulation of replication-dependent histone mRNAs involves specific RNA-protein interactions within the 3'-UTR. Processing and degradation of these mRNAs are mediated by a 26-base stem-loop structure and a M₅, 31,000 protein (23). The general conclusions that one may advance are that 1) mRNA stability is frequently determined by RNA-protein interactions, and 2) those interactions frequently occur within the 3'-UTR.

The experiments described in this paper were designed to test the hypothesis that the 3'-UTR of CcnD3 mRNA contains specific protein-binding elements that are involved in glucocorticoid regulation of degradation of the mature transcript. We have undertaken to analyze RNA-protein interactions within the 3'-UTR, to map the nucleotide components that are required for such interactions, and to characterize the RNA-binding proteins with which these elements interact. Our results indicate that there is a protein-binding element within

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‡ The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; UTR, untranslated region; β-gal, β-galactosidase.
the 3′-UTR of CcnD3 mRNA. This element interacts with at least two proteins, and this interaction is necessary for glucocorticoid-mediated destabilization of CcnD3 mRNA.

MATERIALS AND METHODS

Cell Culture, Treatment, and RNA Isolation—Murine T-lymphoma P1798-S20 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 3 mM glutamine, 25 mM HEPES, 20 mM 2-mercaptoethanol, and 2% fetal bovine serum, at 37 °C in 5% CO2. Mid-log phase cultures containing 5 × 106 cell/ml were used for all experiments. Dexamethasone was dissolved in 70% ethanol as a 0.1 mM stock, and this was diluted into medium to a final concentration of 0.1 μM dexamethasone and 0.07% ethanol in all experiments. Ethanol has no effect on P1798 cultures at this concentration. RNA isolation was performed using TRIzol reagent from Life Technologies Inc. Northern blotting was carried out under standard conditions, and all Northern blotting data were normalized to 18 S RNA. Autoradiographic data were quantified using a Lynx 5000 digital workstation.

Preparation of Cytosolic Protein Extracts (S100)—Cytosolic protein extracts were prepared using the method described by Sun and Antony (24). Cells were washed with 5 packed cell volumes of phosphate-buffered saline by centrifugation at 2000 rpm in a Beckman JA-14 rotor at 4 °C. Cells (5 × 106) were incubated at 4 °C in Buffer A (10 mM HEPES, pH 7.9, containing 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol) for 10 min at a ratio of 5 ml of Buffer A per ml of packed cell volume. The cells were centrifuged (5 min at 2000 rpm in a Beckman JA-14 rotor), suspended in 2 ml of Buffer A per ml of packed cells, and then lysed by 10 strokes of a glass Decon homogenizer (B pestle). Cell lysis was confirmed by microscopy. The lysate was centrifuged at 2000 rpm for 10 min at 4 °C. The resultant supernatant fraction was mixed with 0.11 supernatant volumes of Buffer B (300 mM HEPES, pH 7.0, containing 10 mM MgCl2, 1.4M KCl), and this solution was centrifuged for 60 min at 100,000 × g. The supernatant fraction was recovered and dialyzed for 8 h against 20 volumes of Buffer C (20 mM HEPES, pH 7.9, containing 100 mM KCl, 0.2 mM EDTA 20% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). Aliquots (100 μl) were frozen at –80 °C, and once thawed, they were never reused.

Transcription in Vitro—The MAXscript kit from AMBION Inc. was used for the synthesis of cyclin D3 3′-UTR probes. [α-32P]CTP (800 Ci:mmol, NEN Life Science Products, Inc.) was used to label the probes. The full-length 3′-UTR of murine CcnD3 mRNA from nucleotide 903 to 1871 was amplified by polymerase chain reaction and cloned into In Vitro T1 (which cuts at 1789), and run-off transcription was performed using the T7 promoter to generate the D3 3′-UTR probe (specific activity 217.8 Ci/μm). Nucleotides 933–1574 of the cyclin D3 mRNA were eliminated by digesting pCRII/D3UTR with SauI and EcoRV and ligating the blunt ends to yield a probe corresponding to nucleotides 1575–1789. This probe was named BC (specific activity, 54.4 Ci/μm). The probes were purified by electrophoresis in 8% urea on 5% polyacrylamide gels.

RNA Mobility Shift Assays—RNA-protein binding reactions and electrophoresis of the complexes formed were carried out using a variation of the method described by Leibold and Munro (25). Binding reactions were carried out with various amounts of cytosolic extract and 15 pg of 32P-labeled RNA probe in 30 μl of Buffer D (containing 10 mM HEPES, pH 7.6, 3 mM MgCl2, 40 mM KCl, 2% glycerol, 1 mM dithiothreitol, and 5 mg/ml heparin). Cytosolic protein extracts were diluted in Buffer C (described above) to equalize the volumes among different reactions in each experiment. Reactions performed with the CcnD3 3′-UTR probe were incubated for 20 min at 30 °C, and then 20 units of ribonuclease T1 were added and the reaction was incubated for another 20 min at 30 °C. Under these conditions, the ribonuclease T1 digestion is incomplete. In one experiment, proteinase K was added to a final concentration of 2 mg/ml for 20 min at 30 °C. Reactions performed with the BC probe (1575–1789) were incubated for 30 min at 30 °C and contained 3.5 mg/μl of yeast tRNA as a nonspecific nucleotide. There was no ribonuclease T1 digestion of the BC-protein complex prior to electrophoresis. Binding conditions were otherwise identical to those described for the full-length 3′-UTR. In competition experiments, labeled probe was mixed with the unlabeled probes before addition of the cytosolic extracts. Electrophoresis of RNA-protein complexes was carried out in 5% nondenaturing polyacrylamide gels. Autoradiography was performed at –80 °C.  

Ribonuclease T1 Mapping of RNA-Protein Complexes—RNA-protein complexes were resolved by electrophoretic mobility shift. These complexes were excised from polyacrylamide gels, and the labeled RNA was isolated by phenol-chloroform extraction. The isolated RNA fragments were digested in 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 20 mM Tris-HCl at 37 °C. Ribonuclease T1-resistant fingerprint fragments were separated by electrophoresis in 8% urea and 0.1%SDS on 20% polyacrylamide gels.

UV Cross-linking of RNA/Protein Complexes—The method used to cross-link the BC probe to the RNA-binding proteins was performed as described by Chang et al. (26) with some modifications. Binding reactions were carried out with various amounts of cytosolic extract and 32P-labeled RNA probe in Buffer D containing 0.1 μg/μl of yeast tRNA. After 30 min at 30 °C the samples were transferred to ice and irradiated for 30 min with a UV light source (254 nm, 15 W) at a distance of approximately 4 cm. After irradiation, RNA was digested with 1 mg/ml of ribonuclease A for 1 h at 37 °C. The UV cross-linked products were separated on a 12% SDS-polyacrylamide gel and detected by autoradiography.

Tetracycline-repressible Expression Vectors and Cell Lines—pUHD10–4 was constructed by replacing the polylinker of pUHD10–3 (26) with a multiple cloning site that contains unique SacII (442), EcoRI (449), EcoRV (457), SalI (462), AccI (463), PstI (475), BglII (475), XhoI (481), and BamHI (487) sites. Numbers in parentheses are positions relative to the XhoI site of the parental pUHD10–3. pUHD10–5 was made by replacing the polylinker of pUHD10–3 with a multiple cloning site containing unique sites for SacII (446), NotI (446), EcoRV (455), SphI (469), AvrII (470), and PstI (480). pUHD2zeo4 was made by ligating the Xhol/PvuII fragment of pZeo (In Vitrogen) with the Xhol/PvuII fragment of pUHD10–4. pUHDzeo5 was made by ligating the Xhol/NcoI fragment of pUHD10–5 into the Xhol/PvuII fragment of pZeo. These pUHDzeo plasmids convey stable resistance to the antibiotic zeocin. Modified tetracycline-repressible expression vectors and their nucleotide sequences are available upon request.

The 1878-base pair EcoRI fragment containing full-length CcnD3 cDNA was cloned into pUHDzeo4 to create p4ZD3FL (full-length), which we have designated tetD3FL. p4ZD3FL was digested with XbaI to generate the 3′-UTR (which we have designated tetD3). p4ZD3FL was digested with XbaI to generate the 3′-UTR (which we have designated tetD3) and then ligated to the pCRII/D3UTR fragment from pCRII/D3FL and cloned into the EcoRV/XbaI sites of pUHDzeo to form p4ZBC. The p4ZBC transcript has a predicted size of about 250 nucleotides and is terminated by SV40 polyadenylation signals. Transcribed from the tet(Ohuman cytomegalovirus major immediate early TATA box (HCMV) chimeric promoter, the p4ZBC transcript is presumed to be capped. We have not been able to quantify this transcript, and we have not ascertained that the transcript is either capped or polyadenylated.

p5zβGal was made by ligating the NotI/AhoIIIII fragment of cytomegalovirus/βGal (Promega) into the NotI/EcoRV sites of pUHDzeo5. This expression vector is identified here as β-gal. p5BGDSUTR was made by inserting the SpeI/XbaI fragment from pCRII/D3UTR (containing the 3′-UTR) into the ApaII site of p5zβGal. This transgene is called β-gal/D3UTR herein.

P1798 cells were initially transfected to stably express the tetracycline transactivator from pUHD15–1neo (27). G418-resistant clones were screened for transient expression of pUHD13–3 (tet/luciferase). Secondary transfactions with appropriate expression vectors were carried out. Some of the expression vector harbors linked zeocin resistance genes; others were co-transfected with pZeo. Stable, zeocin-resistant clones were selected and analyzed for expression of the appropriate transgenes.

RESULTS

Cyclin D3 mRNA is very stable in P1798 cells treated with actinomycin D. As shown in Fig. 1A, there was little or no decrease in CcnD3 mRNA within the first 4 h after addition of actinomycin D to mid-log phase cells. In other experiments, we observed no decrease in CcnD3 mRNA after 24 h in the presence of actinomycin D (5), which leads us to suspect that actinomycin D affects the turnover of CcnD3 mRNA. Data that will be presented below are consistent with this supposition. Addition of dexamethasone accelerated CcnD3 mRNA degradation, to the extent that 50% decrease in CcnD3 mRNA abundance was observed within about 2 h after addition of actinomycin D to cells that had been exposed to dexamethasone for
The culture of cells that express tetD3FL was treated with 0.1 mM tetracycline to control cells (open circles) or to cells that were treated with dexamethasone after addition of tetracycline (filled circles). In contrast, the tetD3 derivative that was deleted of the 3′-UTR exhibited a slower rate of degradation (filled squares). We estimate a T1/2 of about 12 h for the 3′-deleted transcript. These data suggest that there are elements within the 3′-UTR of cyclin D3 mRNA that control basal stability of the transcript.

The rapid turnover of full-length transgenic tetD3 mRNA, (T1/2 of about 2 h) is inconsistent with the data shown in Fig. 1A, in which the abundance of endogenous CcnD3 mRNA remained relatively constant for several hours after addition of actinomycin D. These observations suggested that actinomycin D might interfere with degradation of cyclin D3 mRNA. The rate of degradation of transgenic tetD3FL mRNA was measured in cells that had been treated with actinomycin D for 2 h prior to addition of tetracycline. As shown in Fig. 1C (diamonds), no significant decrease in the abundance of the full-length tetD3 transcript was observed within 4 h after addition of tetracycline to actinomycin D-treated cells. This apparent stability should be contrasted with the rapid rate of degradation that occurs upon addition of tetracycline to tetD3FL-expressing cultures in the absence of actinomycin D (Fig. 1C, circles). We conclude that actinomycin D has an effect on the basal stability of cyclin D3 mRNA, but this phenomenon has not been pursued.

Cells that express tetD3 derivatives were treated for 24 h with dexamethasone, as shown in Fig. 1B. Neither the full-length tetD3 transcript (lane 3) nor the 3′-truncated tetD3Δ-UTR derivative (lane 6) was regulated by glucocorticoids. The stability of both full-length and tetD3Δ-UTR transcripts was determined by measuring the abundance of the RNAs in dexamethasone-treated cells after addition of tetracycline. As shown in Fig. 1C, dexamethasone did not affect the stability of transgenic tetD3 transcripts (open circles and open squares). This result was unanticipated, because we had previously shown that glucocorticoids do not inhibit transcription of the cyclin D3 gene (5) and that endogenous CcnD3 mRNA is degraded more rapidly in dexamethasone-treated cells (Fig. 1A). We were also surprised to note that dexamethasone failed to inhibit the expression of the endogenous CcnD3 mRNA in cells that were transfected with the full-length tetD3 cDNA expression vectors (Fig. 1B, lane 3), whereas CcnD3 mRNA was inhibited when dexamethasone was added to cells that express the 3′-truncated derivative (lane 6).

The data shown in Fig. 1B suggest that overexpression of the cyclin D3 3′-UTR from a transgene (tetD3FL) interfered with glucocorticoid regulation of the endogenous CcnD3 mRNA expression. The abundance of the tetD3FL transcript, when maximally derepressed by withdrawal of tetracycline, never exceeded five times that of the endogenous CcnD3 mRNA (Fig. 1B), suggesting that the effect prevailed at relatively low concentrations of the transgenic 3′-UTR. We concluded that the transgenic D3 3′-UTR was acting in trans to block glucocorticoid-mediated destabilization of CcnD3 mRNA. To test this prediction, a number of P1798 cell lines were generated that stably expressed the β-galactosidase gene, with or without the cyclin D3 3′-UTR, under the control of a tetracycline-repressible promoter.
analyzed, and all showed essentially the same properties. The open bars in Fig. 2A illustrate the properties of those clones that express β-gal, whereas the filled bars illustrate the properties of those cells that express β-galactosidase fuse to the D3 3′-UTR chimera (β-galD3UTR). Initially, we noted that the level of expression of the β-galD3UTR chimeric gene was consistently lower than that of authentic β-gal. (Note that the scales are different in Fig. 2A.) We have shown that the D3 3′-UTR increases the turnover of tetD3 transgenic RNAs, and we suspect that this reproducible difference in β-galactosidase activity may be due to an increased turnover of β-galD3UTR transcripts; however, the rates of degradation of the β-galactosidase mRNAs have not been measured. We also observed that dexamethasone had no effect on β-galactosidase expression, irrespective of the presence or absence of the D3 3′-UTR. This observation is consistent with the data shown in Fig. 1B, which indicate that the presence or absence of the D3 3′-UTR did not affect glucocorticoid regulation of the tetD3 transgenes. Finally, tetracycline inhibition of β-galactosidase activity was >95% for the cell line illustrated in Fig. 2A and for all cell lines used in the experiments described below. Because tetracycline regulates the transcription of the transgene, it is reasonable to assume that the decrease in β-galactosidase activity that can be observed upon addition of tetracycline is associated with and attributable to a corresponding decrease in β-galactosidase mRNA.

Glucocorticoid regulation of CcnD3 mRNA was measured in several clones that express β-gal and β-galD3UTR transgenes, as shown in Fig. 2. Fig. 2, B and C, illustrates the properties of two clones that express authentic β-gal mRNA. The abundance of CcnD3 mRNA decreased rapidly upon addition of dexamethasone, indicating that expression of β-galactosidase had no effect on glucocorticoid-mediated inhibition of CcnD3 mRNA abundance (filled circles). Conversely, little or no decrease in CcnD3 mRNA was observed when dexamethasone was added to β-galD3UTR clones, as shown in Fig. 2, D–G (filled circles). These results indicate that cells that express the β-galactosidase/cyclin D3 3′-UTR chimera were resistant to glucocorticoid inhibition of cyclin D3 expression. The abundance of CcnD3 mRNA decreased rapidly when dexamethasone was added to β-galD3UTR cells that had been treated with tetracycline (Fig. 2, D–G, open circles), indicating that glucocorticoid inhibition of CcnD3 mRNA expression was restored when transcription of the β-galD3UTR chimeric transgene was repressed. Tetracycline had no effect upon glucocorticoid inhibition of cyclin D3 expression in cells that express β-galactosidase without the CcnD3 3′-UTR (Fig. 2, B and C, open circles).

The data shown in Figs. 1 and 2 suggest that there are sequences within the D3 3′-UTR that influence glucocorticoid regulation of the stability of CcnD3 mRNA. These elements appear to act in trans, to the extent that overexpression of the 3′-UTR from a transgene (tetD3FL or β-galD3UTR) interferes with regulation of the endogenous CcnD3 mRNA. The data are consistent with the hypothesis that sequences within the transgenic 3′-UTR are acting as molecular decoys, titrating cellular proteins that would otherwise interact with the endogenous transcript to affect glucocorticoid regulation of CcnD3 mRNA stability.

RNA mobility shift assays were used to identify potential RNA-protein interactions within the CcnD3 3′-UTR. The full-length CcnD3 3′-UTR was transcribed in vitro in the presence of α-32P-labeled nucleoside triphosphates. The full-length, labeled 3′-UTR transcript, ~1000 bases long, was incubated with S-100 cytoplasmic extracts, which were prepared from exponentially growing P1798 cells. Ribonuclease T1 was added to degrade those regions of the transcript that were not protected.
by stable RNA-protein complexes. The T1 resistance complexes were then resolved by electrophoresis on nondenaturing polyacrylamide gels, as shown in Fig. 3. Lane 1 of Fig. 3A contains a ribonuclease T1-resistant RNA-protein complex (arrow). Formation of this complex was precluded by addition of 120 ng of unlabeled E. coli β-galactosidase mRNA. In the experiment shown in B, 15 pg of labeled 3'-UTR probe was incubated with increasing concentrations of S100 protein. Lane 1 contains no protein. In lane 4, probe was incubated with 180 µg of protein under the same conditions as used in lane 3. However, the ribonuclease T1-resistant products of the binding reaction were digested with proteinase K, as described under “Materials and Methods,” before being loaded onto the gel.

An RNA mobility shift assay was performed, and the RNA-protein complex was excised from the gel. The labeled RNA was isolated and digested to completion with ribonuclease T1. The products of ribonuclease T1 digestion were separated by electrophoresis on 20% polyacrylamide gels containing 8 M urea, as shown in Fig. 4A. Lane 1 contains a 10-nucleotide DNA ladder, the largest fragment shown being 50 bases. The RNA gel shift entity contains three predominant ribonuclease T1 resistant fragments, as shown in lane 2. The sizes of these fragments, estimated by comparison to the DNA ladder, correspond to a fragment of 35–40 bases and two fragments comprising a doublet of 25–30 bases. These fragments may be precisely identified by comparison to the limit ribonuclease T1 digestion of the labeled 3'-UTR (lane 3), and consist of ribonuclease T1 fingerprint fragments of 25, 26, and 37 nucleotides. The ribonuclease T1 pattern of digestion of the 3'-UTR can be predicted by searching for sequences that contain no GMP residues. Therefore, we can assign the ribonuclease T1-resistant fragments.
from the gel shift entity to specific positions in the CcnD3 3′-UTR. The three major fragments of 25, 26, and 37 nucleotides, which were consistently obtained by this fingerprinting assay, were designated A, B, and C, respectively. These fragments map to T1-resistant sequences at 1168–1192 (A), 1611–1636 (B), and 1686–1722 (C). From the relative intensities of the ribonuclease T1-protected fragments, we estimate that about 90% of the binding activity is associated with the B element that centers around the 26-nucleotide T1-resistant fragment and the C element that contains the 37-nucleotide T1-resistant fragment. These two elements are located adjacent to each other, within about 100 nucleotides (1611–1722). The proximity of the B and C elements and the intensity of binding to these sequences focused our attention on this part of the 3′-UTR. The nucleotide sequence of the murine CcnD3 3′-UTR from 1574 to 1754 is shown in Fig. 4B. The 26- and 37-nucleotide T1 fingerprint fragments (B and C) are underlined. This region of the 3′-UTR is highly conserved among mouse, human, and rat cyclin D3 mRNAs. Comparison of the mouse and human sequences revealed 84% identity from 1561 to 1714 nucleotides, compared with 87% identity within the coding regions. The complete 3′-UTRs of mouse and human CcnD3 mRNAs (including the BC domain) are 63% identical.

Overexpression of the D3 3′-UTR attenuates dexamethasone-mediated destabilization of the endogenous CcnD3 mRNA, and the transgenic full-length tetD3 transcripts are not destabilized by glucocorticoids (Fig. 1). Consequently, we were unable to use deletion mutants to identify the RNA sequences that cause loss of stability in the presence of glucocorticoids. However, the observation that the 3′-UTR attenuated glucocorticoid regulation in trans suggested an alternative way to functionally map the response element. We reasoned that overexpression of the 3′-UTR might titrate the proteins responsible for the dexamethasone-mediated destabilization, thereby preventing their association with the endogenous CcnD3 mRNA. This hypothesis predicts that overexpression of the RNA response element should attenuate glucocorticoid regulation of CcnD3 mRNA. Our binding data suggest that the B and/or C sequences might be the binding sites for those proteins. If that is so, then overexpression of the BC domain should inhibit the dexamethasone effects on the stability of the endogenous CcnD3 mRNA. We constructed a cell line that was designed to overexpress the 215-nucleotide BC domain (nucleotides 1574 to 1789), as described under “Materials and Methods.” A mixed population of these cells, designated P1798p4zBC, was treated with dexamethasone for various periods of time. Total RNA was extracted and Northern blotting was carried out to measure CcnD3 and c-Myc mRNAs. These “BC” cells exhibited glucocorticoid inhibition of c-myc expression, as shown in Fig. 4C. However, glucocorticoids caused little or no change in the abundance of CcnD3 mRNA. Addition of dexamethasone to control cells that contain the “empty” expression vector (designated P1798p4z) caused a rapid decrease in the abundance of both CcnD3 and c-Myc mRNAs. Control and BC cells contained similar amounts of CcnD3 mRNA, and the basal turnover rates of CcnD3 mRNA (insofar as such rates can be estimated using actinomycin D) were similar in both populations of cells (data not shown).

Subsequent RNA gel shift experiments utilized a transcript that extends from nucleotide 1575 to 1789 in CcnD3 mRNA. This transcript contains both the B and C elements and is designated the BC probe. The 25-nucleotide A element has not been studied further. Given the small size of the BC probe, gel mobility shift assays could be performed without the need for ribonuclease T1 digestion after the binding reaction. In order to validate the use of the BC probe, a competition experiment was performed, as shown in Fig. 5A. Lane 1 contains free BC probe, without protein. Addition of S100 protein quantitatively shifted the BC probe, as shown in lane 2. This complex was not detected when a 100-fold molar excess of unlabeled BC transcript was added to the binding reaction (lane 3). However, addition of a 100-fold excess of β-galactosidase mRNA (lane 4) did not block formation of the RNA-protein complex. Note that the probe was completely shifted under the conditions that were employed in this experiment. If we add more probe to the binding reactions, we begin to observe another, more abundant binding activity that is probably due to poly pyrimidine tract binding protein (data not shown). If we add less S100 protein,
the signal becomes difficult to detect. Consequently, we have been unable to establish conditions under which we can perform mobility shift assays in probe excess.

A label transfer experiment was used to estimate the molecular weight of the proteins that bind to the BC elements of the CcnD3 3'-UTR. A binding reaction was performed using 32P-labeled BC probe and S100 cytoplasmic extract from control cells. The reaction was irradiated with UV light to cross-link the probe and any protein closely interacting with it. The probe was digested with ribonuclease A, and resolved by electrophoresis. The size of the labeled proteins was estimated by comparison to standards, and the estimated positions of 52,000 and 37,000 are indicated by the arrows.

The kinetics or thermodynamics of binding in the crude S100 extracts are not obvious similarities between the BC element and other polypyrzymidine-rich regulatory elements that would not be revealed by a sequence-based comparison. The BC element binds two proteins, of approximate molecular weights 37,000 and 52,000. The binding data suggest that the 52,000 protein either is more abundant or has a higher affinity. It is also possible that the formation of the BC-protein complex occurs by an ordered mechanism. Unfortunately, it is difficult to analyze the kinetics or thermodynamics of binding in the crude S100 preparations. These extracts contain abundant polypyrzymidine tract-binding protein, which binds to the BC element and interferes with detection of the 37,000/52,000/BC complex when binding experiments are performed in probe excess.

CcnD3 mRNA that is deleted of the 3'-UTR is not destabilized by glucocorticoids. Overexpression of the BC element attenuates glucocorticoid regulation of CcnD3 mRNA in trans. These observations are consistent with the hypothesis that formation of a ternary RNA-protein complex is necessary for destabilization of CcnD3 mRNA in trans. Fig. 7 illustrates the working hypothesis that we have developed for our observations. We propose that the BC element interacts with the 37,000 and 52,000 proteins to form a ternary complex. When the BC element is overexpressed, it functions as a molecular decoy, titrating the 37,000 and 52,000 proteins from the endogenous transcript, thereby precluding formation of the ternary complex and attenuating glucocorticoid-mediated destabilization of CcnD3 mRNA in trans.
the BC element appears to be constitutive, because no change in binding was detected in extracts from glucocorticoid-treated cells. We propose that glucocorticoids induce another active principle, designated Dx in Fig. 7, that interacts with the ternary complex to form an unstable quaternary complex. Shoenberg and co-workers (28) have identified estrogen-induced ribonucleases that regulate mRNA stability in Xenopus liver, and it is conceivable that the principle that we have designated “Dx” could be a glucocorticoid-induced ribonuclease that is recruited to the ternary complex. Other hypotheses may be imagined, but the essential elements of any plausible hypothesis would seem to be that 1) two or more proteins must interact with each other and with the BC cassette to elicit dexamethasone-mediated destabilization, 2) the proteins must bind independently to the regulatory element and therefore distribute among the overexpressed BC cassettes, and 3) one or more of these proteins must be limiting in concentration. None of our data are inconsistent with any of these assumptions. Nonetheless, there is clearly a lot more to learn about how the Mf(37,000 and 52,000 proteins interact with the BC element.

Deletion of the 3′-UTR affects basal stability of tetD3 constructs, suggesting that there are elements within the 3′-UTR that control CcnD3 mRNA abundance in proliferating cells. However, overexpression of the BC element does not affect basal stability of endogenous CcnD3 mRNA. Actinomycin D blocks CcnD3 turnover in mid-log phase cells but does not prevent CcnD3 degradation in glucocorticoid-treated cells. These observations suggest that basal turnover and glucocorticoid-mediated degradation proceed by different mechanisms.

We have examined the properties of known RNA-binding proteins in an effort to identify p37 or p52. Our analysis has focused upon RNA-binding proteins of the appropriate size or those that are known to bind polypyrimidine tracts. Among these, hnrNP K (Mf 66,000), hnrNP I (62,000), hnrNP C1 (42,000), and hnrNP C3 (44,000) bind polypyrimidine tracks but are not of the right size (17, 18, 29–32). hnrNP A1 (Mf 36,000) binds AUUUA elements but not other polypyrimidine tracts in the 3′-UTRs of several mRNAs (33), and hnrNP H, with a molecular weight of 53,000, binds poly-G (34). Among the known poly-C-binding proteins (29, 30, 35), murine CUBP (Mf 48,000) may be a possible candidate for the Mf 52,000 CcnD3-binding protein that we have observed. This remains to be determined.

In summary, there is within the 3′-UTR of CcnD3 mRNA an element that appears to be necessary but not sufficient for glucocorticoid regulation of mRNA degradation. This element is conserved among mammalian CcnD3 mRNAs and contains, but is probably not restricted to, two polypyrimidine tracts. These two tracts form part of the binding sites for two proteins. We assume that formation of the BC/37,000/52,000 complex does not cause destabilization in the presence of glucocorticoids. Rather, we assume that this element is necessary for interaction with some other, as yet unidentified, glucocorticoid-induced protein(s). The mechanism is likely to be significantly more complex than that proposed in Fig. 7, and it may be that destabilization will require not only other proteins but also other nucleotide elements within the 3′-UTR or the coding sequence of CcnD3 mRNA.

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