Activation of protein kinase C (PKC) and protein kinase A (PKA) in rat C6 glioma cells increases the half-life of short-lived lactate dehydrogenase (LDH-A) mRNA about 5- and 8-fold, respectively. PKA and PKC act synergistically and prolong LDH-A mRNA half-life more than 21-fold. Similar effects were observed after transfection and transcription of a globin/lactate dehydrogenase minigene consisting of a β-globin expression vector in which the 3′-untranslated region (UTR) of β-globin had been replaced with that of LDH-A. Synergism was only obtained by transfection of minigenes containing the entire 3′-UTR and did not occur when truncated 3′-UTR fragments were analyzed. Additional mutational analyses showed that a 20-nucleotide region, named PKC-stabilizing region (PCSR), is responsible for mediating the stabilizing effect of PKC. Previous studies (Tian, D., Huang, D., Short, S., Short, M. L., and Jungmann, R. A. (1998) J. Biol. Chem. 273, 24861–24866) have demonstrated the existence of a CAMP-stabilizing region in LDH-A 3′-UTR. Sequence analysis of PCSR identified a 13-nucleotide AU-rich region that is common to both β-globin and PCSR. These studies identify a specific PKC-responsive stabilizing element and indicate that interaction of PKA and PKC results in a potentiating effect on LDH-A mRNA stabilization.

During recent years we have established evidence for a bimodal mechanism of LDH-A1 regulation involving transcriptional as well as post-transcriptional modulation of LDH-A expression (1–6). Both mechanisms are regulated by a number of agonists of intracellular signaling pathways. For instance, agonists of PKA and PKC can modulate glucose and lactate metabolism of cells by inducing an altered program of LDH-A mRNA regulation. These mechanisms allow the cell to respond rapidly to changes in the physiologic environment of the cell and to cover a potential energy deficit through conversion of pyruvate to lactate. Clues to mechanisms underlying this dual mode of control were provided by the identification of cis-acting promoter elements instrumental in PKA- and PKC-mediated transcriptional regulation (2, 6). However, less information is available about the mechanism of protein kinase-mediated LDH-A mRNA stability regulation resulting in higher levels of intracellular LDH-A mRNA (3).

Attention has recently focused on the modulation of mRNA stability in response to a variety of physiological signals. For instance, it is known that activators of PKA and PKC are important effectors of mRNA stability regulation in a number of gene systems (1, 3, 5, 7–14). Our own studies identified a synergistic interaction between PKA and PKC in regulating the stability of LDH-A mRNA (3). Whereas the molecular basis for the synergistic effect remains unknown, we have recently identified a cAMP-stabilizing region (CSR) within the 3′-UTR of LDH-A mRNA (5) that in combination with specific CSR-binding proteins (4) is required to achieve LDH-A mRNA stabilization in response to PKA activation. The binding activity of the proteins to the CSR and the effect on LDH-A mRNA stabilization are regulated through a phosphorylation/dephosphorylation mechanism by PKA and as yet unknown protein phosphatases. Thus, it is now clear that the CSR, in concert with CSR-binding proteins, is absolutely required to achieve increased LDH-A mRNA stability in response to PKA activation. However, the molecular mechanism of mRNA stabilization by PKC is unknown.

The present paper describes the identification of a cis-regulatory element within the 3′-UTR of LDH-A mRNA that is required for PKC-mediated mRNA stability regulation. The identification was accomplished using a strategy previously developed for the identification of the cAMP-stabilizing region (5). Using deletion, mutation, and replacement analysis, we constructed chimeric β-globin/ldh 3′-UTR minigenes that were stably transfected and expressed in rat C6 glioma cells. Applying ribonuclease protection assays, we studied the effects of PKC activation on the rate of decay of chimeric β-globin/ldh 3′-UTR mRNAs. Using this methodology, we demonstrated the presence of an uridine-rich region within the LDH-A 3′-UTR, named PKC-stabilizing region (PCSR), capable of stabilizing LDH-A mRNA in response PKC activation. It is of particular interest that the sequences of CSR and PCSR overlap and possess a common 13-nucleotide region.

EXPERIMENTAL PROCEDURES

Materials—Nucleic acid-modifying enzymes, acrylamide, and nucleoside triphosphates were from Roche Molecular Biochemicals. Radioisotopes were purchased from NEN Life Science Products. Other reagents were of molecular biology grade and purchased from Sigma. Cell culture products were purchased from Life Technologies, Inc.

Synthetic Oligonucleotides—Synthesis and processing of synthetic DNA oligonucleotides and their ligation into respective plasmid vectors were performed as described before (5).

Cell Culture—Rat C6 glioma cells (American Type Culture Collection...
CCL 107) were maintained as monolayers in Ham’s F-10 nutrient medium supplemented with 10% dialyzed fetal calf serum, 50 units/ml of penicillin, and 50 mg of streptomycin as described by us (5).

**Plasmids**—The LDH-A 3′-UTR was derived from plasmid pLDH-2 (kindly provided by Dr. Richard Breathnach) containing a full-length rat LDH-A cDNA insert. The mRNA consists of a 103-nucleotide 5′-untranslated region and a 510-nucleotide 3′-untranslated region (corresponding to nucleotides 1103–1610) (50). The 3′-UTR contains the classic polyadenylation signal AAUAAA 18 nucleotides before the poly(A) sequence. A HindIII/BamHI fragment containing the entire LDH-A 3′-UTR (with 28-base pair 5′ coding sequence and 100-base pair pLDH-2 vector sequence) was inserted into pGEM3Zf(−) at the BamHI site resulting in plasmid pLDH-5. To eliminate the 28-base pair ldh-a-coding and 100-base pair pLDH-2 vector sequences contained in pLDH-5, the complete 510-base pair 3′-UTR of LDH-A with 5′ BamHI and 3′ HindIII sites was amplified by polymerase chain reaction. The fragment was cloned into the BamHI-HindIII sites of pBluescript II KS (+) (Stratagene) resulting in pLDH-6 from which the various 3′-UTR fragments were prepared.

**Construction of Wild-type and Mutant Globin/ldh cDNAs**—The rabbit β-globin expression vector pRc/FBB (see Fig. 1) was constructed as described previously (5) by Dr. D. Chagnovich (Northwestern University) in two steps from plasmids pRcCMS (Invitrogen) and pBBB (kindly provided by Dr. M. E. Greenberg) (15). Plasmid pRc/FBB encodes a transcription unit consisting of β-globin coding region flanked by the β-globin 5′- and 3′-untranslated regions fused to the c-fos promoter.

To construct the chimeric globin/ldh expression vectors, various LDH-A 3′-UTR fragments were inserted into the BglII site or, alternatively, they replaced the BglII/Hind III fragment (β-globin 3′-UTR) of pRc/FBB. The fragments were constructed as follows. To construct the pRc/FBB expression vector containing the full-length LDH-A 3′-UTR, the 510-base pair 3′-UTR was polymerase chain reaction-amplified from plasmid pLDH-6 using 5′- and 3′-oligonucleotide primers with BglII and HindIII restriction sites, respectively. The polymerase chain reaction product was cloned into BglII and HindIII III sites of pBluescript II KS (+) (Stratagene) resulting in pLdH-6 from which the various 3′-UTR fragments were prepared.

**RESULTS**

**The Patterns of Decay and Regulation of Chimeric β-Globin/ldh mRNA Stability by Protein Kinases A and C Are Identical to Wild-type LDH-A mRNA**—Previous studies in our laboratory demonstrated that intracellular steady-state levels of LDH-A mRNA are regulated, in part, through modulation of mRNA stability. For instance, after treatment of rat glioma cells with activators of PKA or PKC, a dramatic but transient increase of LDH-A mRNA levels takes place (1, 3). The induced mRNA exhibits a markedly increased half-life as compared with the relatively short half-life of LDH-A mRNA in noninduced cells. This indicates that the LDH-A transcript in noninduced cells is targeted for rapid degradation through processes that can be modulated by effector agents capable of activating the PKA or PKC signal transduction pathways.

To investigate the molecular basis of the protein kinase-stabilizing effect, initial studies were carried out to determine whether or not the decay of wild-type LDH-A mRNA and chimeric globin/ldh mRNA followed similar patterns, justifying the use of chimeric vectors for subsequent stability studies. By choosing an expression vector (pRc/FBB) with a serum-inducible c-fos promoter (15–17), we also avoided artifacts that potentially occur when commonly used transcriptional inhibitors (18, 19) are used to stop ongoing transcription. We modified pRc/FBB, which is under the control of a serum-inducible c-fos promoter, by replacing the BglII/HindIII III fragment (Fig. 1), containing the globin 3′-UTR, with the entire LDH-A 3′-UTR. The resulting chimeric minigene (pRc/FBB/LDH) was stably transfected into rat C6 glioma cells. After serum deprivation of cells for 25–30 h, the c-fos promoter was pulse-induced with fetal calf serum resulting in a brief pulse of transcription of chimeric β-globin/ldh mRNA. Nuclear run-off assays indicated a rapid induction of nuclear chimeric globin/ldh transcripts at 15 min (Table I). After 1 h the level of transcription had already decreased to levels seen before serum stimulation. Similar transient kinetics were observed without or with added DG or p-isomeric cyclic monophosphorothioate ((p)CAMPs), a potent activator of PKA, and DG, a membrane-permeable diazglycerol analog that activates PKC (20) and mimicks the effect of endogenous diazglycerol on PKC (21, 22). As shown in Fig. 2A, DG as well as (S)p-CAMPs achieved a marked stabilization of chimeric β-globin/ldh mRNA (Fig. 2A, exhibit a markedly increased half-life as compared with the relatively short half-life of LDH-A mRNA in noninduced cells. This indicates that the LDH-A transcript in noninduced cells is targeted for rapid degradation through processes that can be modulated by effector agents capable of activating the PKA or PKC signal transduction pathways.

**Stability of the Chimeric β-Globin/ldh mRNA Is Mediated Synergistically via the Protein Kinase A and C Signal Pathways**—To examine the effect of protein kinase activation on the rate of decay of β-globin/ldh mRNA, we used the S-isomeric form of adenosine 3′, 5′ cyclic monophosphorothioate ((S)p-CAMPs), a potent activator of PKA, and DG, a membrane-permeable diazglycerol analog that activates PKC (20) and mimicks the effect of endogenous diazglycerol on PKC (21, 22). As shown in Fig. 2A, DG as well as (S)p-CAMPs achieved a marked stabilization of chimeric β-globin/ldh mRNA (Fig. 2A, exhibit a markedly increased half-life as compared with the relatively short half-life of LDH-A mRNA in noninduced cells. This indicates that the LDH-A transcript in noninduced cells is targeted for rapid degradation through processes that can be modulated by effector agents capable of activating the PKA or PKC signal transduction pathways.

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globin/mRNA separate experiments. Synthesis results are given as the means was subtracted from the counts/min hybridized to globin filters. RNA action counting. Nonspecific hybridization to wild-type pBluescript filters was withdrawn for 24–28 h, after which serum and effector agents were allowed to incorporate [\( ^{32}P \)]UTP. RNA was isolated, purified, and hybridized to 2-aminophenylthioether filters carrying immobilized globin cDNA in Bluescript or wild-type pBluescript (1). Hybridized radioactivity was eluted from the filters and determined by liquid scintillation counting. Nonspecific hybridization to wild-type pBluescript filters was subtracted from the counts/min hybridized to globin filters. RNA synthesis results are given as the means ± S.E. determined from four separate experiments.

| Treatment | Rate of chimeric globin/ldh mRNA synthesis$^a$ ppm |
|-----------|-----------------------------------------------|
| None      | 448 ± 65                                      |
| 15 min    | 1077 ± 91                                     |
| 30 min    | 1166 ± 88                                     |
| 45 min    | 667 ± 48                                      |
| 60 min    | 456 ± 53                                      |
| DG (200 nM) | 1277 ± 94                                   |
| 0 min     | 1197 ± 123                                   |
| 30 min    | 1132 ± 88                                     |
| 60 min    | 510 ± 69                                      |
| (S$^3$)-cAMPS (100 μM) | 645 ± 58                                   |
| 0 min     | 1524 ± 132                                   |
| 30 min    | 1166 ± 210                                   |
| 45 min    | 955 ± 76                                      |
| 60 min    | 480 ± 41                                      |

$^a$ Rate of mRNA synthesis (expressed in parts per million) = [cpm globin/ldh mRNA - cpm pBluescript/cpm total 32P-RNA input] × 100/efficiency) × 1710/510. 1710 is the length of the chimeric globin/ldh mRNA, and 510 is the length of the LDH-A 3′-UTR in nucleotides.

In our previous identification of a synergistic action of PKA and PKC on LDH-A mRNA stability (3), we examined whether such an effect also occurred when the chimeric β-globin/ldh 3′-UTR minigene was the transcribed template. Our data show that synergism was, indeed, demonstrable when a combination of DG and (S$^3$)-cAMPS was used as activators of the protein kinase pathways. The half-life of β-globin/ldh mRNA increased 18-fold to a half-life of about 2 h (extrapolated from Fig. 2B). We conclude from the data that the stability of β-globin/ldh mRNA in glioma cells is similar or identical to wild-type LDH-A mRNA. Furthermore, the decay patterns of chimeric β-globin/ldh mRNA in protein kinase-activated cells appear to follow mechanisms identical to wild-type LDH-A mRNA.

**Inhibitors of Protein Kinase C and A Abolish the mRNA Stabilizing Effect**—To further test the pivotal role of protein kinase activation in regulation of β-globin/ldh mRNA stability, we used inhibitors that prevent activation of the respective protein kinase pathway. Exposure of transfected cells to various activators and inhibitors of PKA and PKC markedly modified the half-life of β-globin/ldh mRNA. As shown in Table II, the phorbol ester TPA, which binds to and activates PKC irreversibly (23), caused an approximate 4-fold increase of β-globin/ldh mRNA half-life. Because TPA may possibly achieve this effect through mechanisms other than activation of PKC (24), we chose DG as activator. DG is a synthetic cell membrane-permeable analog of diacylglycerol, the endogenous activator of PKC, and mimicks the effect of endogenous diacylglycerol (21, 22). Indeed, DG treatment of transfected glioma cells increased the half-life of β-globin/ldh mRNA about 4-fold. The α-isomeric form of phorbol 12β,13a-didecanolate, which is unable to activate PKC (25, 26), lacked the stabilizing effect of TPA on globin/ldh mRNA, strongly suggesting that TPA exerts its effect through activation of PKC. Furthermore, to prevent activation of PKC, we used the specific PKC inhibitor bisindolylmaleimide GF 109203X (BIM) (27). The use of BIM alone did not change the half-life of globin/ldh mRNA. In combination with DG or TPA, BIM prevented stabilization of the mRNA. We have already previously shown that the use of (R$^3$)-cAMPS, which prevents activation of PKA, similarly failed to cause stabilization of chimeric globin/ldh mRNA (3, 5).

**Systematic Analysis of LDH-A 3′-UTR for the Presence of Protein Kinase C-stabilizing Region(s)**—LDH-A mRNA is char-
characterized by a moderately short half-life of approximately 55 min (1). Sequence analysis of its 3'-UTR identifies a 99-nucleotide domain (nt 1450–1549) that is relatively AU-rich when compared with the overall nucleotide composition of the 3'-UTR. Recently, we have shown that the 3'-UTR imparts a relatively short half-life to LDH-A mRNA because of the presence of three determinants of instability (5). Although two of the instability regions are not regulated, one of them, the 22-base 3'-UTR region comprised of nt 1478–1499, is subject to regulation by the PKA signal pathway. Its presence is an absolute requirement for cAMP-mediated stabilization of LDH-A mRNA. Using an experimentally similar approach, we now identified putative region(s) within the LDH-A 3'-UTR that are responsible for PKC-mediated stabilization of LDH-A mRNA. Our strategy consisted of the synthesis of two types of chimeric globin/ldh 3'-UTR vectors. First, we constructed a series of globin/ldh 3'-UTR vectors that contained (a) systematically truncated wild-type 3'-UTR fragments; (b) mutated 3'-UTR fragments; and (c) 3'-UTRs from which we had deleted short base regions. The deleted regions were of approximately similar size to prevent artifactual effects because of drastic variations in mRNA size. The fragments were inserted into the unique BglII site of pRe/FBB located at the junction of the β-globin translated and 3'-untranslated regions (Fig. 1) (28). In the second approach, the entire globin 3'-UTR was deleted by restriction at the BglII/HindIII sites (Fig. 1) and replaced with wild-type and various mutated LDH-A 3'-UTR fragments. Upon stable transfection and transcription of the appropriate vectors, unique chimeric globin/ldh mRNAs were produced whose stability was assayd by ribonuclease protection assay. Because in each vector the promoter (c-fos) and globin translation region are identical, the transcripts differ from each other only in their 3'-UTR sequence.

The basal half-lives of truncated wild-type chimeric globin/ldh mRNAs are shown in Table III. Whereas several chimeric mRNAs were stable over the time course of the decay period, others exhibited a relatively short half-live without being affected by DG. However, fragment 1471–1502 (and fragment 1463–1502) appears to contain a PCSR, because treatment of cells with DG resulted in a marked stabilization of the chimeric mRNA (Table III). Interestingly, although a synergistic stabilizing response was observed with chimeric mRNA in which LDH-A 3'-UTR replaced the entire globin 3'-UTR (Figs. 1 and 2), no synergism was observed with any of the mRNAs containing a truncated fragment (data not shown).

Whereas the above data were obtained by insertion of truncated 3'-UTR fragments into the BglII site of the globin gene, further analysis was carried out by replacement of the entire globin 3'-UTR with short overlapping fragments generated from the nt 1453–1527 region. This strategy was aimed at preventing any potential artifactual effects on chimeric mRNA stability because of the presence of the globin 3'-UTR. DG-mediated stabilizing activity was analyzed, and the half-lives of the resulting chimeric globin/ldh mRNAs are summarized in Table IV. Comparison of chimeric mRNA derived from insertion of fragment 1463–1527 into pRe/FBB (Table III) with chimeric mRNA in which fragment 1463–1527 replaced globin 3'-UTR (Table IV) showed not only similar half-lives but also similar stabilizing effects of DG. Identical results were obtained when the functional characteristics (insertion versus replacement) of other fragments were compared. Based on these data, the fragment consisting of nt 1463–1502 is the shortest base region so far identified required for PKC-mediated mRNA stabilization consistent with the data listed in Table III.

Identification of the Location of the Protein Kinase C Stabilizing Region by Deletion Analysis—To confirm and expand the above findings, we analyzed the stability of globin/ldh mRNAs in which short sequences had been systematically deleted from the 3'-UTR (Fig. 3). The resulting 3'-UTRs were inserted into the BglII site of pRe/FBB. The vectors were transfected and transcribed, followed by analysis of the decay characteristics of the chimeric mRNAs. The results are shown in Table V. Four 3'-UTRs lacking base regions located between nt 1286–1462 and 1527–1580 retained DG responsiveness. On the other hand, when fragments consisting of either nt 1453–1527, 1463–1482, or 1478–1506 were deleted, two of the chimeric globin/ldh mRNAs, with deletions at nt 1453–1527 and 1478–1506, exhibited relatively higher basal half-lives, whereas the globin/ldh mRNA with a deletion of nt 1463–1482 did not. However, none of the corresponding chimeric globin/ldh mRNAs were stabilized by DG, indicating that the deleted fragments contained the complete or at least part of the PKC-responsive site. These data allow location of the PKC-responsive site within nucleotides 1453–1506.

A 20-Nucleotide 3'-UTR Region Is Responsible for Stability Regulation—To achieve an even more precise delineation of the active PKC-responsive site, mutational analysis was carried out. We introduced several mutational changes into fragment
Effect of dioctanoylglycerol on the half-life of chimeric globin/ldh mRNAs

The BgII/HindIII fragment containing the entire globin 3′-UTR in pRC/FPBB (see Fig. 1) was deleted and replaced with the listed fragments of LDH-A 3′-UTR (with 5′ BgII and 3′ HindIII ends). Rat C6 glioma cells were stably transfected with these vectors and stimulated with 200 nM DG, and RNA decay was assayed as described under “Experimental Procedures.” Results are expressed as the means and S.E. of four separate experiments.

| 3′-UTR fragment (base no.) | Control | DG | DG/control |
|----------------------------|---------|----|------------|
| 1453–1471                  | 3.6 ± 0.5 | 3.2 ± 0.6 | 0.9 |
| 1453–1520                  | 1.4 ± 0.3 | 4.3 ± 0.3 | 3.1 |
| 1463–1482                  | 3.2 ± 0.4 | 3.1 ± 0.5 | 0.9 |
| 1463–1492                  | 2.9 ± 0.3 | 12.8 ± 1.3 | 4.4 |
| 1463–1502                  | 3.9 ± 0.7 | 13.9 ± 1.8 | 3.6 |
| 1463–1527                  | 3.1 ± 0.7 | 9.7 ± 1.1 | 3.1 |
| 1478–1506                  | 4.3 ± 0.6 | 4.5 ± 0.5 | 1.1 |
| 1503–1527                  | 3.8 ± 0.4 | 4.1 ± 0.4 | 1.1 |

Effect of dioctanoylglycerol on the half-life of chimeric globin/ldh mRNAs containing LDH-A 3′-UTR fragments with partial deletions

The BgII/HindIII fragment in pRC/FPBB (see Fig. 1) was deleted and replaced with LDH-A 3′-UTR from which the listed fragments had been deleted. Rat C6 glioma cells were stably transfected with these vectors. After stimulation of cells with 200 nM DG, the half-lives were determined as described under “Experimental Procedures.”

| Fragment deleted (base no.) | Control | DG | DG/control |
|-----------------------------|---------|----|------------|
| 1286–1351                   | 3.7 ± 0.5 | 11.6 ± 1.9 | 3.1 |
| 1343–1410                   | 2.9 ± 0.3 | 9.9 ± 0.8 | 3.4 |
| 1404–1462                   | 1.1 ± 0.2 | 2.6 ± 0.2 | 2.4 |
| 1453–1527                   | 9.8 ± 1.3 | 10.7 ± 1.9 | 1.1 |
| 1463–1493                   | 2.9 ± 0.3 | 3.1 ± 0.4 | 1.1 |
| 1478–1506                   | 8.5 ± 1.1 | 7.3 ± 0.9 | 0.8 |
| 1527–1580                   | 3.6 ± 0.4 | 8.9 ± 1.2 | 2.5 |

1453–1502, which, based on above data, contains the region required for PKC-mediated mRNA stabilization. The resulting chimeric mRNAs were analyzed for DG responsiveness. The data are shown in Table VI. Deletion of a -UUA- triplet at nt 1464–1466 (mut1) as well as deletion of two bases at nt 1470–1471 (-UUA-) (mut2) did not change the stabilizing effect of DG. Mutation of bases at 1486–1490 from -CUUGA- to -GACAG- (mut3) maintained the stabilizing effect although to a somewhat lesser degree. Mutation 4, synthesized from mut3 by deletion of a -UUA- doublet at 1484–1485 did not respond to DG stimulation and lacked the stabilizing effect. A change of the doublet -UU-at nt 1491–1492 (mut 5) to -GG- did not affect DG responsiveness. When wild-type fragment 1453–1520 was inserted into pRC/FPBB in the reverse orientation, the instability effect as well as DG responsiveness were not observed, indicating that the destabilizing and regulatory effects required a specific sequence polarity. Thus, mutations introduced upstream of nt 1472 (for instance at nt 1464–1466 and 1470–1471) (see Table VI) did not abolish stabilization. Similarly, deletion of sequences downstream of nt 1492 (see Table IV and V) showed no effect allowing the conclusion that the region encompassing bases 1472–1491 represents the nucleotide sequence required for stabilization of LDH-A mRNA via the PKC pathway.

**DISCUSSION**

Our results strongly support a role for the PKC signal pathway in the mechanism of LDH-A mRNA stabilization and identify a critical nucleotide sequence in LDH-A 3′-UTR that is required for PKC-mediated LDH-A mRNA stabilization. The most critical domain is comprised of nucleotides 1472–1486, although a somewhat larger region extending to nucleotide 1491 is required to express the full magnitude of the stabilizing effect. The region consists of a relatively U-rich (48%) 20-base sequence that CSR and PCSR contain a 13-nt region (nt 1478–1491; mut4, 5′-UU-UUAUGUGUCUGUAGUGUACAUUGCAAUA-3′; mut5, 5′-UU-UUAUGUGUCUGUAGUGUACAUUGCAAUA-3′) that is common to both regulatory elements (Fig. 4). Because the 13 nucleotides constitute 59% of the CSR and 65% of the PCSR, respectively, the question arises as to the significance of the overlapping arrangement of these two stabilizing regions within the 3′-UTR. One functional property common to both CSR and PCSR, namely destabilization of LDH-A mRNA, is also exhibited by a class of short-lived mRNAs that share consensus-like AU-rich motifs in their 3′-UTR (7). Although AU-rich motifs appear to mark mRNAs in general for rapid degradation, it is unlikely that relative nonspecific AU-rich sequences will participate in the selective regulation of mRNA stability, such as observed, for instance, for mRNAs that are under post-transcriptional control by certain effector agents (e.g., cAMP and DG). Rather, determinants in CSR and PCSR, other than the 13-nt common sequence, seem to be required to modulate stability specificity in response to the PKA and PKC signal pathways. In this fashion the stability and its regulation will be controlled in a dual mode by relatively nonspecific AU-rich sequences that determine the relative instability/stability of the mRNA and by sequence domains that will determine the specificity of the stabilizing response. Thus, we hypothesize that part of the LDH-A 3′-UTR is composed of two discrete module(s): (a) the 13-nt overlapping non-

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**Table IV**

Effect of dioctanoylglycerol on the half-life of chimeric globin/ldh mRNAs

| 3′-UTR fragment (base no.) | Control | DG | DG/control |
|-----------------------------|---------|----|------------|
| 1453–1471                  | 3.6 ± 0.5 | 3.2 ± 0.6 | 0.9 |
| 1453–1520                  | 1.4 ± 0.3 | 4.3 ± 0.3 | 3.1 |
| 1463–1482                  | 3.2 ± 0.4 | 3.1 ± 0.5 | 0.9 |

**Table V**

Effect of dioctanoylglycerol on the half-life of chimeric globin/ldh mRNAs containing LDH-A 3′-UTR fragments with partial deletions

| Fragment deleted (base no.) | Control | DG | DG/control |
|-----------------------------|---------|----|------------|
| 1453–1520wt                 | 1.4 ± 0.3 | 4.3 ± 0.4 | 3.1 |
| 1453–1520mut1               | 2.5 ± 0.3 | 0.2 ± 0.0 | 3.5 |
| 1453–1520mut2               | 1.3 ± 0.2 | 3.9 ± 0.2 | 3.0 |
| 1453–1520mut3               | 5.3 ± 0.7 | 11.1 ± 1.1 | 2.1 |
| 1453–1520mut4               | 2.5 ± 0.3 | 2.7 ± 0.4 | 1.1 |
| 1453–1520mut5               | 1.9 ± 0.2 | 4.9 ± 0.5 | 2.6 |
| 1453–1520rev                | 14.7 ± 2.1 | 17.8 ± 2.1 | 1.2 |
specific AU-rich determinant in PCSR and CSR and (b) specific site(s) that in concert with phospho/dephosphoproteins are instrumental in determining the specificity of the stabilizing response. These ideas are supported by our recent identification of four proteins whose specific CSR binding and mRNA stabilizing effects are achieved through their phosphorylative modification by PKA (4) in concert with phosphoprotein phosphatases. Efforts are underway to identify putative PCSR-binding protein(s) and to analyze their functional properties.

Our demonstration of a synergism involving PKA/PKC-mediated LDH-A mRNA stabilization is particularly intriguing (Fig. 2) (3). Importantly, the effect could be duplicated with a chimeric globin/ldh mRNA, provided the transcribed chimeric globin/ldh minigene contained the entire wild-type LDH-A 3'-UTR. Truncated 3'-UTR fragments, even though they contain both the CSR and PCSR, lacked the synergistic effect, and simultaneous activation of PKA and PKC achieved only an additive increase of globin/ldh mRNA half-life. Although the PCSR and CSR are necessary to achieve message stabilization, their presence alone appears not to be sufficient to obtain a cooperative response, and our results do not rule out the possibility that secondary structure or other sequences within LDH-A 3'-UTR may play a role in achieving a synergism. Nevertheless, synergism suggests a "cross-talk" between the two signal transduction systems involving trans-regulatory RNA-binding proteins that are substrates and targets for both PKA and PKC. Cross-talk between two major signal transduction pathways is a well recognized phenomenon known to occur in a number of systems (29–31). A mechanism for the two cell surface receptor coupled signal transduction pathways (PKA and PKC) has been suggested previously (32). It depends on the presence of serines and threonines that are specific sites of phosphorylation for different protein kinases. As mentioned above, our previous studies have identified several RNA-binding proteins (CSR-BP) that specifically interact with the CSR and are instrumental in regulating stability (4). Because CSR and PCSR possess a 13-nt region in common, the distinct possibility arises that one or more of the CSR-binding proteins are also substrates for PKC and may be mechanistically involved in the cooperative effect. However, the understanding of the molecular mechanism of PKA and PKC-regulated mRNA stability must come from the cloning of cDNAs encoding the RNA-binding proteins.

A great number of examples of regulated mRNA stability (up- and down-regulation) in eukaryotic cells by phorbol ester or corresponding second messengers (such as DG) are known (7, 10, 33–44). Although these studies indicate that an increased level of phorbol ester is a sufficient signal for increased mRNA stability, the molecular mechanisms mediating the effects of phorbol ester have largely remained obscure. The fact that phorbol ester can activate PKC has led to the notion that mRNA stability regulation results from a cascade of events involving PKC isoenzymes and a stabilizing/destabilizing regulatory system such as cis- and trans-acting factors. Phosphorylative modification of specific RNA binding proteins would achieve the fine tuning of mRNA half-life. In the case of LDH-A mRNA, the use of a specific inhibitor of PKC, bisindolylmaleimide, can abrogate the effect of TPA or diacylglycerol on mRNA stability, indicating the involvement of PKC in the stabilization mechanism. These findings are in agreement with previous reports demonstrating that the stability of ribonucleotide reductase R1 mRNA is increased after phorbol ester treatment of cells. This phenomenon appears to be mediated through a 49-nt cis-elements in the 3'-UTR of ribonucleotide reductase mRNA and its interaction with specific binding proteins (45). A search for sequence similarities between PCSR
and the PKC regulated ribonucleotide reductase R1 has identified a 3'-UTR region (46) revealing marked sequence similarities (80% homology) (identical bases are printed in bold): LDH-A, UUGUGC - AUAAAAG - UUCUACAG - GAUA (nt 1457–1480), and R1, UUUUGAAUAAACAUUGU - AUAG (nt 2876–2902).

Protein kinase C is a family of at least 10 isoenzymes, all having closely related structure but differing in their individual functional properties (23, 24). Rat C6 glioma cells express mainly the α, δ, ε, and χ isofoms (47). The α, δ and ε forms are activated by phorbol ester and inhibited by bisindolylmaleimide. Thus, one or more of these three isoforms may mediate the stability regulation of PKC. We are presently investigating the precise nature of the isoenzyme(s) involved in mRNA stabilization.

Finally, it is appropriate to briefly discuss the physiological significance and implications of our findings. LDH plays a pivotal role in normal anaerobic glycolysis. Analysis of the LDH isoenzyme patterns in different cell types under a variety of physiological conditions suggests complex regulatory mechanisms. The patterns are subject to regulation by a number of different effector agents, including 17β-estradiol (48, 49), epidermal growth factor (50, 51), catecholamines (1, 52), phorbol ester- (2, 3), hypoxia (53, 54), and c-Myc (55). These agents almost exclusively change the isoenzyme pattern in favor of the homotetrameric (A4) isoenzyme LDH-5. The shifts are attributed to a need for increased A subunit-containing isoenzymes and increased glycolysis (56, 57). Complex interrelationships exist between these signals that regulate LDH-A expression. Torcitol, evidence has been provided for an interaction of C-J., Chung, K. C., and Jungmann, R. A. (1994) J. Biol. Chem. 269, 11547–11554. 11. Izzo, N., Jr., Tulenko, T. N., and Schueci, W. S. (1994) J. Biol. Chem. 269, 1705–1710. 12. Mitchell, R. L., Zokas, L., Schreiber, R. D., and Verma, I. M. (1985) Cell. Biol. 123, 1544–1552. 13. Morimoto, B. H., and Koshland, D. E., Jr. (1994) J. Biol. Chem. 269, 3065–3069. 14. Hod, Y., and Hanson, R. W. (1988) J. Biol. Chem. 263, 7747–7752.

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