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Differentiation-Dependent Cytoplasmic Distribution and *in Vivo* RNA Association of Proteins Recognized by the 3′-UTR Stability Element of α-Globin mRNA in Erythroleukemic Cells

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In this study, we analyzed subcytoplasmic distribution and *in vivo* RNA association of proteins with specific affinity to cytosine-rich stability determinant sequences of α-globin mRNA 3′-UTR in a MEL-707 erythroleukemic model. We took advantage of the possibility that these cells can be reversibly differentiated (as a continuous population, but not at the level of individual cells) which, therefore, allows analysis of various stages of erythroid differentiation within the same cell population. Label transfer experiments revealed four major complexes with molecular mass of 110-, 70-, 55- and 50-kDa in various cytoplasmic fractions. Using the combination of *in vitro* label transfer and *in vivo* UV-crosslinking techniques, we also demonstrated that subcytoplasmic distribution as well as *in vivo* RNA association of various complex-forming proteins is differentiation dependent in MEL-707 cells. These results indicate that changes in the cytoplasmic environment imposed by the differentiating stimulus might direct important biochemical signals as to how the stability determinant 3′UTR elements interact with their binding proteins. These data also suggest that stability complexes are dynamic macromolecular structures with high response capacity to various extra- and intracellular regulatory stimuli. © 2000 Academic Press

Key Words: **α-globin mRNA; α-complex; mRNA stability; 3′UTR; erythroleukemic cells UV-crosslinking.**

Normal cell growth and differentiation require tightly controlled expression of a variety of genes. Besides adjusting transcriptional activities, cells have the capability to posttranscriptionally regulate the expression of a given gene product (1). A fundamental and potent mechanism of posttranscriptional control is the modulation of mRNA stability (2). mRNA stability is measured in half-life ($T_{1/2}$) and cells possess mRNA subsets with $T_{1/2}$ ranging from minutes to days. One extreme includes short-lived mRNAs ($T_{1/2} \leq 15$–30 min) encoding for transiently expressed cytokines, lymphokines, certain proto-oncogenes and transcription factors (1). Selective stabilization of these mRNAs can rapidly and reversibly contribute to up to 10- to 100-fold excess of the gene product. This mechanism involves key AU-rich sequences (ARE) on the 3′-untranslated region (UTR) or stem-loop secondary structural elements within various locations on the mRNA as well as proteins that recognize and bind to them (3). Another class of mRNAs is characterized by long $T_{1/2}$ (comparable to or exceeds that of the life span of the cell) and usually encodes for proteins of structural and/or housekeeping role (4).

Globin gene expression proved to be a valuable and classical model to study processes related to the $T_{1/2}$ regulation of stable mRNAs. Maturation and differentiation of cells of the erythroid lineage is associated with mass production of hemoglobin, which takes up to 95% of total cell protein of terminally differentiated cells (5). Accumulation of globin mRNAs, necessary for hemoglobin assembly during this process, depends largely on the extraordinary stability of globin mRNA (5, 6). Specific mechanisms that regulate α-globin mRNA stability has been examined in detail and shed light to a number of important aspects. First, using a naturally occurring α2-globin mutant and scanning mutagenesis in a transient transfection system, specific pyrimidine-rich elements within the 3′UTR of the mRNA were identified which were necessary for normal message stability (6, 7). Additionally, a ribonucleoprotein complex, called the α-complex with high specificity to this pyrimidine-rich region (also known as

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cytosine-rich element, CRE) of the α-globin mRNA 3′UTR was demonstrated to assemble both *in vitro* and *in vivo* (8, 9). The α-complex was initially dissected as consisting of two closely related protein components, αCP1 and αCP2 (9). It was later demonstrated that neither of these components could bind directly to CRE but rather in conjunction with additional protein factors within the complex (10). Intriguingly, in search for additional protein components of the α-complex, AUF1 (also known as heteronuclear ribonucleoprotein D or hnRNP D), the probably best-characterized protein involved in ARE-mediated mRNA decay was also demonstrated to be integral part of the complex (10–12). The exact molecular principles as to how the α-complex is organized or assembles have not yet been fully resolved. Contradicting some of these earlier data, it has recently been demonstrated that a single αCP molecule directly binds to CRE to establish a binary scaffold for the whole complex to build up on (13).

The α-complex has been proposed to confer α-globin mRNA stability by at least two distinct mechanisms. Through its direct interaction with the poly(A)-binding protein (PABP) this complex was demonstrated to interfere with deadenylation (14, 15). Additionally, it has also been shown that the α-complex confers α-globin mRNA stability by protecting the mRNA 3′UTR from a sequence-specific endonucleolytic cleavage activity (16). Moreover, the complexity of this latter pathway is substantiated by recent findings that the influence of the poly(A) tail is mediated by bound PABP facilitating αCP binding to CRE and, conversely, αCP also enhances PABP binding to the poly(A) tail (17). In any case, the growing number of mRNA substrates which are subject to αCP-related mRNA stability (18) and translational regulation (18–21) suggest a much broader role of α-complex and its protein components in regulated mRNA stability than in that of α-globin mRNA accumulation during erythroid differentiation. Indeed, more recently, direct binding of αCP1 to CD81 ligand (TAPA-1) and cytochrome *c* oxydase subunit II (coxII) mRNAs has also been reported (22).

Much less have been uncovered of how components of the α-complex are distributed within the cytoplasm as well as how they associate with RNA during the course of erythroid differentiation. To gain insight into some of these questions, in this study, we were aiming to analyze subcytoplasmic distribution and *in vivo* RNA-association of proteins with specific affinity to α-globin 3′-UTR stability determinant sequences in MEL-707 mouse erythroleukemic cells. More specifically, we were interested to see if changes in these parameters could be detected at various stages of differentiation within the same cell population. Using sequential detergent extraction as well as the combination of *in vitro* label transfer and *in vivo* UV-crosslinking techniques developed in our laboratory (23), we demonstrate that subcytoplasmic distribution as well as *in vivo* RNA association of various complex-forming proteins is differentiation dependent in MEL-707 cells. These results indicate that changes in the cytoplasmic environment imposed by the differentiating stimulus might direct important biochemical signals as to how the stability determinant 3′UTR elements interact with their binding proteins. These data also suggest that stability complexes are dynamic macromolecular structures with high response capacity to various extra- and intracellular regulatory stimuli.

EXPERIMENTAL PROCEDURES

Cell culture and induction of differentiation. MEL-707 murine erythroleukemic cells were grown at a density of 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum. Differentiation was induced with 1 or 2% dimethyl sulfoxide (DMSO, Sigma) essentially as described (24). Hemoglobin positivity was assessed by the acid benzidine reaction as described elsewhere (25). De-differentiation was reached by replacing DMSO-containing medium with fresh medium without the inducer and cultures were maintained continuously until hemoglobin positivity was comparable to that of control cells. Viability of cells was ensured using the trypan blue dye exclusion test throughout the differentiation–de-differentiation cycle.

Preparation of cytoplasmic subfractions. Cytoplasmic subfractions were prepared as described (26) with minor modifications. Briefly, 2–3 × 10^7 cells were washed in ice cold serum-free medium, pellets were resuspended in buffer A containing 10 mM Pipes, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed on ice for 3 min by addition of Triton X-100 to a final concentration of 1%. Following centrifugation for 3 min at 900*g*, supernatants were collected, aliquoted and frozen immediately. Triton-insoluble pellets were extracted in buffer B containing 10 mM Hepes, pH 7.4, 15 mM NaCl, 1.5 mM $MgCl_2$, 1% Tween 20, 0.5% sodium deoxycholate and 1 mM PMSF for 3 min on ice. Supernatants were collected after centrifugation with 1200*g* for 3 min at 4°C and frozen immediately. Protein concentration of the cytoplasmic fractions was determined by standard techniques.

RNA probes and in vitro label transfer assay. The SP64(poly(A))/α-3′UTR construct containing the pyrimidine-rich stability determinant element of α-globin mRNA was generously provided by Dr. Xiaoming Wang. The sequence of α-globin mRNA 3′UTR with the instability sequence elements highlighted is the following: UAA^{ter}(n19) UUCCUUCCU UGCCCGCUGGG CCUUCCC AACGGG CCCUCCUCCCCCUCCUU GCACC (n17)AAUAAA$^{poly(A)}$ GUCUGAG-UGGGCGGC. [^{32}P]CTP-labeled RNA was transcribed from *Eco*RI-linearized construct using the SP6 RNA polymerase as described (27). 8 × 10^4 cpm probe RNAs (~10 fmol) were incubated with 0.5–2 μg proteins from cytoplasmic subfractions in 12 mM Hepes, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA and 10% glycerol for 10 min at 30°C. Protein–RNA complexes were fixed with UV light on ice using the UV Stratalinker Model 1800 (Stratagene) (5 min, 3000 μW/cm^2) and exposed to RNase treatment (15 units of RNase T1 and 30 μg of RNase A/sample) for 15 min at 37°C. Samples were then separated by 12.5% SDS-PAGE under reducing conditions, gels were dried and analyzed by autoradiography.

In vivo UV-crosslinking analysis. UV irradiation of intact cells was combined with subsequent label transfer analysis essentially as described (23). Briefly, control and differentiated cells were pelleted, washed once in PBS and resuspended in ice-cold PBS. Cells were distributed into 6-well tissue culture plates (1.6 × 10^7 cells/well) and placed on ice in the UV Stratalinker 1800 (Stratagene). The Stratalinker was placed on a horizontal shaker to provide continuous gentle

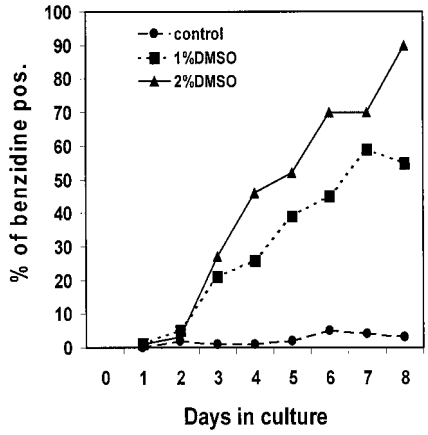

FIG. 1. Chemical induction of differentiation in MEL-707 cells. Cells were induced to differentiate in the presence of 1 or 2% of DMSO for lengths of time indicated at the bottom of the figure. Benzidine positivity was assessed periodically to monitor the percentage of differentiated cells in the culture. Viability was ensured using the trypan blue dye exclusion test. To reverse differentiation, DMSO was washed out and cells were further cultured under the same conditions but in the absence of the inducer. This figure is a representative of a typical experiment and all data presented in subsequent figures of this report are derived from cells of this culture.

stirring of the cell suspensions during exposure to monochromatic ($\lambda = 254$ nm) UV light (3000 μW/cm^2). Cells were then harvested and cytoplasmic subfractions were prepared as described above. By comparing band intensities of fractions from 0 to 8 min of *in vivo* UV crosslinking in a subsequent label transfer assay, it was possible to estimate the portion of proteins which crosslinked in the cell and, thus, failed to be RNA-protective *in vitro*. We have demonstrated earlier that this technique is capable to distinguish between RNA binding protein pools with various degree of *in vivo* association with specific RNA sequences (23).

RESULTS AND DISCUSSION

Induction of reversible differentiation of MEL-707 cells. Initially, we were aiming to construct a model system which would consist of the same cell population but at different stages of erythroid differentiation. In order to obtain this, we exposed murine MEL-707 cells to various concentrations of the known chemical inducer, DMSO. Cells were cultured under these conditions and the percentage of benzidine positive cells was determined at different time points. Figure 1 illustrates a typical differentiation curve of cells induced by 1 or 2% DMSO. It was seen that over 90% of cells became benzidine positive (a sensitive measure of hemoglobin production and, thus, of erythroid differentiation) by the eighth day of culture. When the inducer was removed from these cultures, cells were dedifferentiated and lost hemoglobin positivity within nearly the same time that was required for differentiation (not shown). Thus, we generated a reproducible model system in which we could maintain the same cell population at various stages of differentiation.

Cytoplasmic distribution of α-globin mRNA stability sequence-binding proteins. In order to monitor complex formation between the pyrimidine-rich 3′-UTR stability determinant sequence of α-globin mRNA and cytoplasmic proteins of MEL-707 cells at various stages of differentiation, first, Triton X-100-soluble and insoluble cytoplasmic fractions were prepared from control and both differentiated and de-differentiated MEL-707 cells. These cytoplasmic fractions were then analysed in a label transfer assay using the α-globin mRNA 3′UTR probe described under Experimental Procedures. Four dominant complexes with approximate molecular mass of 110, 70, 55, and 50 kDa were detected in cytoplasmic fractions of MEL-707 cells regardless of their stage of differentiation (Fig. 2, "sol" lanes). We observed that the two lower MW complexes (50 and 55 kDa) were virtually absent from the detergent-insoluble compartments. These data indicate that the protein components of these complexes might largely be localized in the cytosol (i.e., not associated with cytoskeletal elements or residual cytoplasmic membrane-bound organelles). Although, we did not aim to determine the identity of these proteins in the current study, it is possible that the originally described αCP1 and αCP2 proteins are part of these smaller complexes.

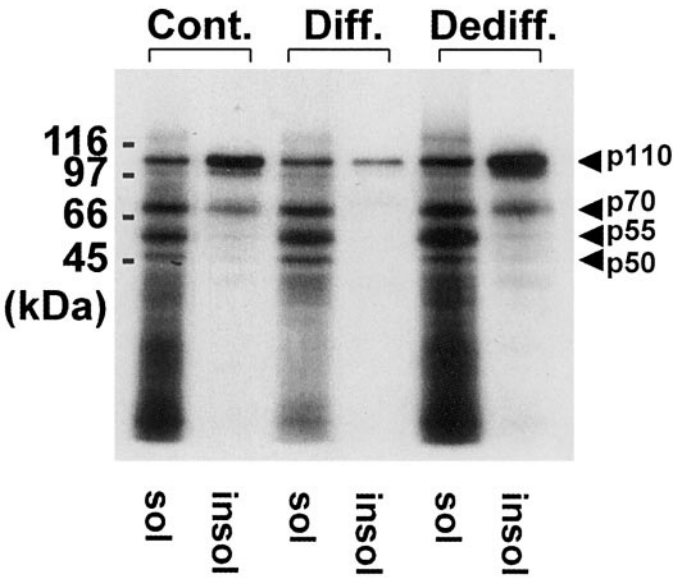

FIG. 2. Label transfer analysis of cytoplasmic fractions of MEL-707 cells. Triton X-100-soluble (sol) and insoluble (insol) cytoplasmic subfractions were prepared from control, differentiated and de-differentiated MEL-707 cells as described under Experimental Procedures. Equal amount of total protein of each fraction (0.5–2 μg) was incubated in the presence of radiolabeled RNA encoding for the C-rich stability determinant element (CRE) of the 3′UTR of α-globin mRNA. Reactions were exposed to monochromatic UV light ($\lambda = 254$ nm) to covalently fix formed RNA–protein complexes and RNase treated extensively to eliminate RNA sequences not protected by proteins. Complexes were then analysed by 12.5% SDS-PAGE and autoradiography. Arrowheads indicate major complexes with approximate molecular mass of 50, 55, 70, and 110 kDa.

In agreement with this, two isoforms of the 40-kDa αCP has been identified in a complex with erythropoietin mRNA 3′UTR stability element migrating as a 50-kDa activity (28). Alternatively, these complexes may contain yet unidentified proteins. This possibility is supported by a previously reported work, which, in addition to AUF1, described four protein components with molecular masses of 58, 55, 50, and 28 kDa (10). In our system, however, we were not able to detect complexes with molecular mass lower than 50 kDa. As of the possible identity of the 70-kDa activity, considering its size and sensitivity to poly(C) (see below), it is tempting to state that this complex might contain or consist of hnRNP K, yet another poly(C)-binding protein, which had been implicated in both α-globin and 15-lypoxygenase mRNA stability (13, 19).

We also observed that the most intense 110-kDa complex in the Triton-insoluble fraction showed significantly lower activity in differentiated cells and that the less intense 70-kDa complex entirely disappeared in the same fraction. Although an intracytoplasmic, Triton-insoluble to Triton-soluble shift of these activities cannot be ruled out, its detection might exceed the limit of our assay as no increase in the Triton-soluble fraction of differentiated cells was seen in any experiments, despite careful correction for loading using Coomassie-stained gels from which the autoradiograms were derived (not shown). It may also be possible that these proteins are modified or sequestered within the same cytoplasmic fraction in response to a differentiation-induced macromolecular rearrangement, which, in turn, results in decreased overall binding activity to CRE.

The relevance of the above possibilities may gain some support from a recent study where adhesion-dependent rapid and reversible modification of mRNA stability was observed in monocytes, a phenomenon that involved RNP complexes containing AUF1 (29). As adhesion/de-adhesion parallels with considerable rearrangements of various cytoplasmic structures, some of which may also be assumed during erythroid differentiation (changes in cell architecture, expulsion of picnotic nuclei, etc.), distribution and assembly of the components of AUF1-containing α-complexes may also be modified during this process. Nevertheless, complete restoration of the distribution profile in de-differentiated cells indicates that this feature is dynamic and reversible in nature.

Poly(C) competes for RNA-binding by most of the detected activities. To verify CRE specificity of the protein components detected in these experiments, we tested their sensitivity to the competitor poly(C) in label transfer reactions. As seen in Fig. 3, these experiments allowed us to conclude that, with the exception of the 50-kDa complex (which was not competed at even the highest concentration of competitor used), all complexes were poly(C)-sensitive (but not poly(U)-sensitive, data not shown) in this assay. This result shows that it is the C-rich context within CRE, which is recognized and bound by most of the protein components of the complexes. It is possible that in addition to the C-rich stretches of CRE, components of the 50-kDa complex recognize and require additional sequence elements to bind.

We found minor differences in both the degree and kinetics of poly(C)-sensitivity among the 55-, 70-, and 110-kDa complexes. The 70-kDa complex appeared to be most susceptible to competition (Fig. 3B). Although, the 55- and 110-kDa complexes competed off well, there always remained some visible activity after competing with the highest concentration of poly(C) (Fig. 3A). These data indicate that the biochemical properties of RNA–protein interactions are likely to be different within each complex. It is also feasible to assume that each protein component recognize and bind to different portions within CRE. These regions may partially overlap to provide close proximity for the various components to contact each other. The possibility, however, cannot be ruled out that there is more than one protein within one complex with similar or identical molecular mass. Detectable differences in their affinity to C-rich sequences could account for the discrepancies in the competition experiments. These possibilities can be resolved with additional studies. Our results also demonstrate that the CRE-binding affinity, revealed by poly(C) competition under these *in vitro* conditions, is independent of the stage of differentiation.

In vivo RNA association of α-complex proteins during differentiation. In our previous studies, we were able to detect changes in the *in vivo* RNA associations of AU-rich instability sequence binding proteins in response to various stimuli (30). Therefore, we became interested to see whether *in vivo* RNA association of the CRE-binding activities shown above could also be monitored and whether they differed in control vs differentiated cells. To test this, prior to *in vitro* label transfer assay, cells were exposed to monochromatic UV light ($\lambda = 254$ nm) for 0 or 8 min and Triton-soluble fractions were prepared from these cells. It was reasoned that if a protein molecule of interest is in close (binding) proximity to its specific target RNA sequence, then some of these complexes could be fixed by UV light within the cytoplasm and extracted subsequently together with nonbinding proteins. Analyzing this extract *in vitro* with a labeled RNA probe (carrying the target sequence for the protein) allows the detection of that protein pool which had not been crosslinked *in vivo* in a way that any decrease in RNA binding activity of the protein (from 0 to 8 min *in vivo* UV crosslinked samples) is inversely proportional with this protein pool.

These experiments revealed various degree of *in vivo* RNA association by the different activities in control

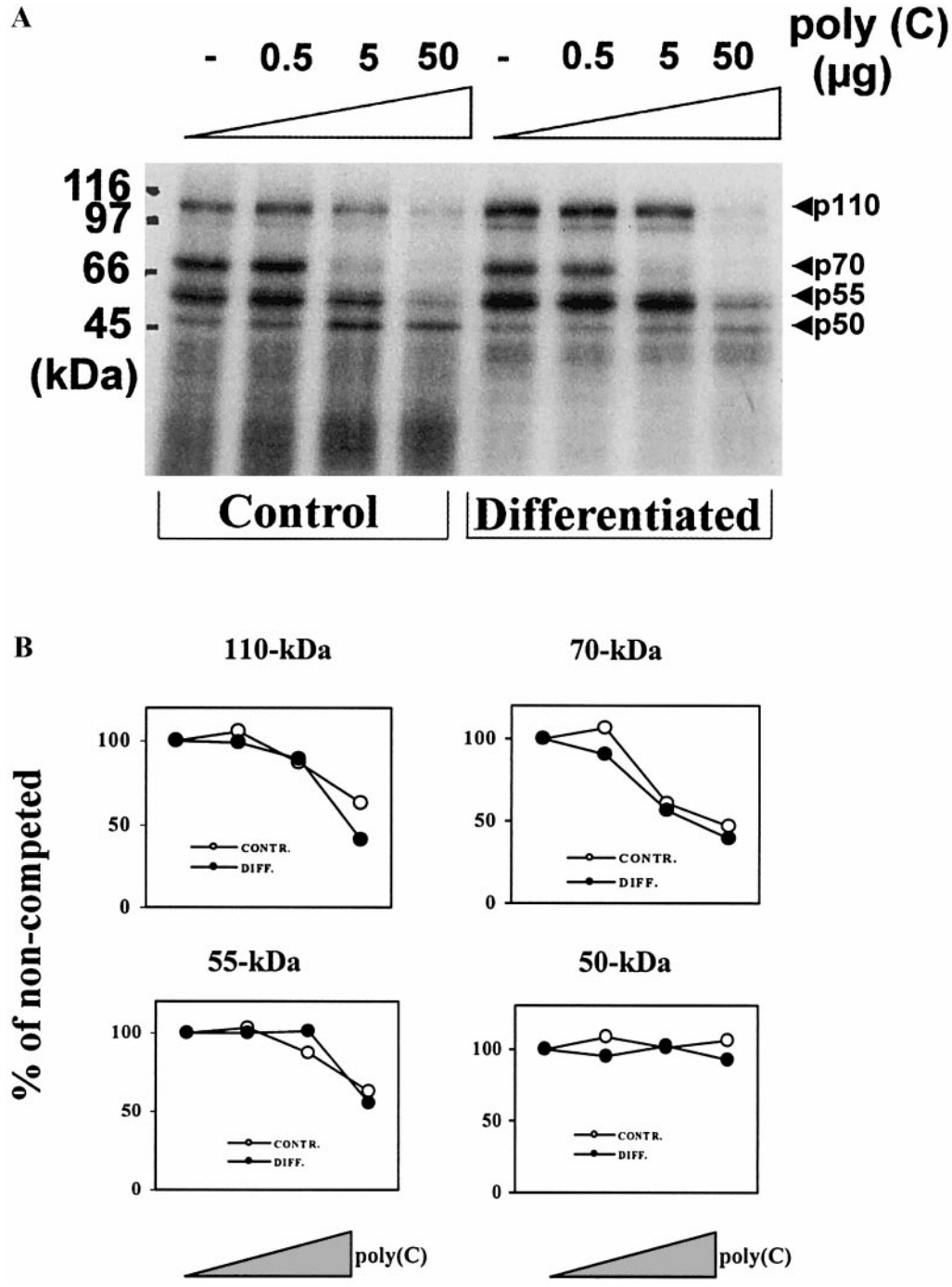

FIG. 3. Specificity of MEL protein binding to CRE. (A) Label transfer analysis of Triton X-100-soluble cytoplasmic fractions of control (left panel) and differentiated (right panel) MEL-707 cells was performed as described for Fig. 2, except that increasing amounts (indicated on the top of the figure) of the cold competitor poly(C) were added to the reactions simultaneously with the radiolabeled α-globin mRNA 3′UTR CRE. Samples were analyzed on 12.5% SDS-PAGE followed by autoradiography. Arrowheads indicate positions of complexes with approximate mass of 50, 55, 70, and 110 kDa. (B) Densitometric analysis of individual bands for each complex was performed using the very same autoradiogram shown in A. Arbitrary values of lanes with increasing poly(C) input were expressed as percentage of uncompeted lanes and plotted for both control and differentiated samples.

cells (Fig. 4). The only activity that crosslinked efficiently to RNA *in vivo* in control cells was the 70-kDa complex, showing about 50% reduction of radioactive signal in the label transfer assay after 8 min of *in vivo* UV crosslinking (Fig. 4B). While the 110-kDa activity did not show association to RNA *in vivo*, the smaller

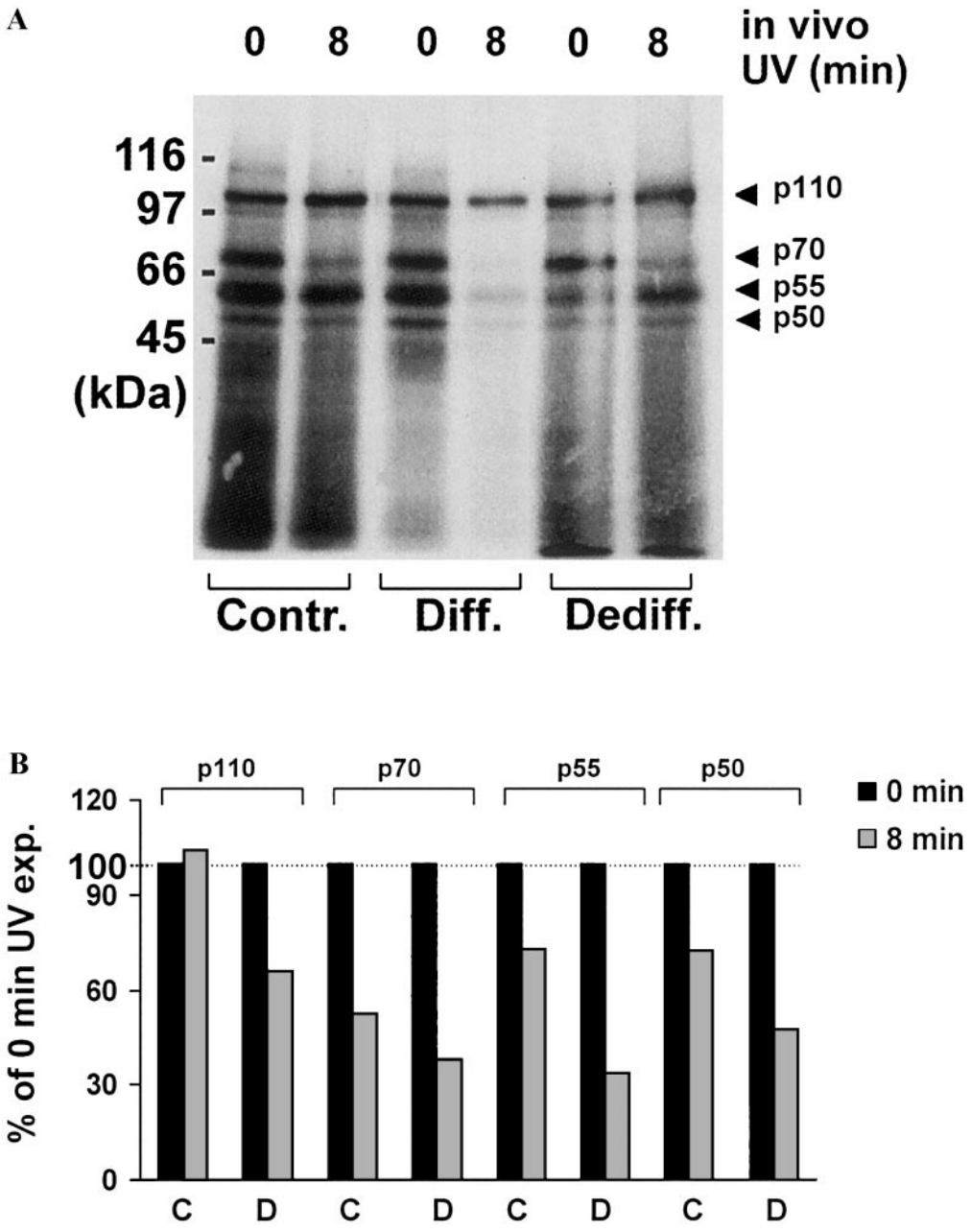

FIG. 4. *In vivo* RNA association of CRE-binding proteins in control and differentiated MEL-707 cells. (A) Prior to extraction of cytoplasmic fractions and subsequent label transfer assay, control and both differentiated and de-differentiated cells were exposed to 254 nm UV light for 0 or 8 min (indicated at the top of the figure) to fix RNA–protein complexes *in vivo*. Analysis of the formed complexes was as described for Figs. 2 and 3. (B) Values obtained from densitometric analysis of samples of 0 min of *in vivo* UV-crosslinking were chosen as 100%, whereas values for the 8 min samples were expressed as percentage of the 0 min samples for each complex (indicated at the top of the panel). "C" and "D" indicate samples of control and differentiated cells, respectively.

complexes could be crosslinked to RNA within the cell with a modest efficiency (about 30% reduction of *in vitro* detectable intensity at 8 min of *in vivo* UV crosslinking) (Fig. 4B). The intriguing finding was that, in differentiated cells, all complexes showed considerably higher RNA association *in vivo* when compared to control cells (the decrease of detectable binding intensity from 0 to 8 min of UV exposure was by 35, 15, 40, and 25% for p110, p70, p55 and p50, respectively) (Figs. 4A and 4B). When de-differentiated cells were analysed for the same parameters, we found that *in vivo* RNA association of all major activities appeared to be comparable to those of control cells (Fig. 4A).

In the light of previous studies implicating some of the α-globin CRE-binding proteins in the stability regulation of other mRNAs in the cell (18–22), it is possible that the

proteins detected here might also be part of other α-complex related (or unrelated) assemblies and, therefore, they also crosslink to sequences in the cytoplasm which are similar to CRE. This could—with the exception of the 100-kDa activity—account for the efficient UV crosslinkability of these proteins in control cells. However, since in differentiated cells, the vast majority of anabolic activity is governed towards haemoglobin synthesis, we suggest that the considerable increase in UV crosslinkability *in vivo* reflects enhanced overall binding of these proteins to CRE and a consequently higher degree of α-complex formation. Thus, these results are consistent with the idea that protein binding to the CRE correlates with α-globin mRNA stability as well as show that in differentiated cells this binding is enhanced. It is possible that, in differentiated cells, proteins with CRE-binding capability have higher affinity to the stability determinant sequences. However, since we did not see reduced poly(C) competition of CRE-binding by complexes from differentiated cells *in vitro*, this explanation would assume an affinity property which is being imposed by factors in differentiated cells and which are apparent only *in vivo*. Such factors could be related to mRNA localisation, alterations in phosphorylation status or changes in protein–protein interactions within the complexes. In any case, again, in dedifferentiated cells this parameter was restored to a state comparable to that seen with control cells. In conclusion, our data demonstrate that in addition to α-complex formation, subcytoplasmic redistribution and enhanced *in vivo* RNA association of at least four major protein activities correlate with α-globin mRNA expression and stability during differentiation of murine erythroleukemic cells. Such studies may provide further insights as to how RNP complexes are dynamically modulated in response to environmental signals that direct changes in mRNA stability.

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