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5′UTR Sequences of the Glucocorticoid Receptor 1A Transcript Encode a Peptide Associated With Translational Regulation of the Glucocorticoid Receptor

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Abstract We have recently reported that glucocorticoid receptor (GR) transcript 1A, one of the five mouse GR splice variants (1A–1E), encodes membrane GR (mGR), which subsequently participates in mediating the apoptotic effects of glucocorticoids (GCs); all transcripts vary at their 5′UTR. Computer analysis of the entire1026 bp comprising the 5′UTR of transcript 1A identified five putative translation start sites at positions 85, 217, 478, 628, and 892 with the potential to encode peptides of 33, 93, 6, 18, and 41 amino acids, respectively. We then separately generated point mutations at these five upstream AUG codons of the GR 1A cDNA and performed in vitro transcription/translation experiments to investigate the regulatory effects of these sites on GR synthesis. GR translation products were immunocaptured with BUGR-2 antibody (Ab), then subjected to Western blot analysis. Mutation of the uAUG codon-2 completely inhibited GR synthesis, while mutations at the other four uAUG codons had no significant effect on the translation of transcript 1A. Antibodies (Abs) against the uORF-2 and uORF-5 protein products were used to perform Western blot analysis on cytosolic proteins from S-49 cells (which express GR transcript 1A), U937 cells transfected with GR 1A cDNA, or in vitro translation products from this cDNA. This assay identified an intense immunoreactive band of ~8.5 kDa recognized only with Ab to the uORF-2 peptide; this size is consistent with the computer-predicted size of the uORF-2 product, suggesting that the uORF-2 product is indeed synthesized in cells. No peptide was identified with Ab to uORF-5 peptide. Indirect fluorescent Ab staining, confocal microscopy and FACS analysis all showed that the ORF-2 peptide is localized both in the interior of the cell and at the plasma membrane. Using Ab to ORF-2 peptide for immunoadsorption we then asked whether cellular factors interact with the product of uORF-2. Immuno-captured uORF-2 peptide levels correlated with the concentrations of several salt-wash-sensitive cellular proteins, suggesting that protein–protein interactions occur between this upstream open reading frame (uORF) product and other factors. The uORF-2 product, however, does not appear to directly interact with GR, since there was no reciprocal immuno-capture between these two proteins. In summary, our results show that cells can synthesize the uORF-2 peptide, blocking of the synthesis of the uORF-2 peptide product abolishes translation of GR from the GR 1A transcript, and the peptide product of uORF-2 interacts with other cellular factors which might be involved in translation of GR. J. Cell. Biochem. 81:149–161, 2001.

Key words: UORF; membrane glucocorticoid receptor; apoptosis; GR 1A; splice variant

The ability of glucocorticoids (GCs) to inhibit growth and cause involution of lymphoid tissues has been known for more than four decades, and is the basis for using these steroids in treating leukemias and lymphomas [Ingle, 1940; Munk and Young, 1975; Stevens and Stevens, 1985; Kaspers et al., 1994]. However, it was recognized that not all patients responded favorably to corticosteroid, and that the initial benefits of steroid therapy were usually followed by resistance to further hormone administration [Greaves, 1981; Zawydiwski et al., 1990; Pieters et al., 1991]. Treatment with GCs often produces unfortunate side effects [Cline, 1974; Melby, 1977; Quddus et al., 1985]. Therefore, it is highly desirable to

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identify non-responsive patients and spare them from receiving an ineffective and potentially harmful agent. Further improvements in treatment and side-effect reduction strategies using GCs or their analogs will only be possible if the complexities of the mechanisms are understood.

The simple presence of high numbers of intracellular or whole cell receptor sites in patients' cells or in vitro tests do not guarantee a response [Stevens and Stevens, 1985; Gametchu et al., 1994]. Consequently, although it is agreed that these effects are mediated through some form of the GR, subforms of the GR with different subcellular locations have not been fully explored. Most research has focused on the intracellular GR (iGR), a transcription factor belonging to the nuclear receptor superfamily, that alters the expression of target genes in response to a specific hormone signal (reviewed in Evans, 1988; Simons et al., 1992; McEwan et al., 1997). Work conducted in our laboratories with several murine and human lymphoid cell lines produced evidence that mGR is more strongly correlated with GC-evoked lymphocytolysis than is the iGR [Gametchu, 1987; Gametchu et al., 1991a,b, 1994, 1995; Gametchu and Watson, 1995; Sackey et al., 1997]. That is, iGR was always present, but mGR levels varied considerably, and more precisely predicted the lymphocytolytic behavior. Therefore, lymphocytolytic mechanisms are likely to involve a combination of membrane-initiated and transcription factor-mediated activities of GR.

Evidence is accumulating that 5′ and 3′ UTRs modulate translational efficiency and mRNA stability [Jackson, 1993; Zimmer et al., 1994; Geballe, 1996] for some eukaryotic genes. Some of these eukaryotic mRNAs have one or more AUG translational initiation codons, or small upstream open reading frames (uORFs) that precede the major ORFs (reviewed in [Geballe and Morris, 1994]). The presence of these uORFs usually inhibits translation from downstream AUGs, although some cases have been described where uORFs stimulated translation of the major ORFs [Jackson, 1993]. The retinoic acid and androgen receptor members of the steroid receptor family are examples of this phenomenon [Mizokami and Chang, 1994; Zimmer et al., 1994]. In the former case, uORFs have been implicated in tissue-specific receptor expression [Zimmer et al., 1994]. Some have speculated that uORFs produce functional peptides [Morris, 1995].

The murine *GR* gene encodes at least five transcripts (named 1A–1E) with different 5′ ends that are alternatively spliced onto a single splice acceptor site 13 bp upstream of the ATG codon in exon 2 [Strähle et al., 1992; Gearing et al., 1993; Chen et al., 1999a]. More recent work indicates that there are 11 exon 1 GR splice variants in the rat [McCormick et al., 2000]. The physiological significance of these variant transcripts is not known, but all transcripts contain information for the same coding region and differ only in the 5′UTR. Very recently, we reported that the cellular presence of mGR is highly correlated with the expression of one of the alternative GR transcripts (1A) in this system [Chen et al., 1999a, b]. We constructed a plasmid expressing full-length (~7.3 kb) GR transcript 1A containing its long 5′ and 3′ UTRs, and also distinguished from the wild-type GR by single base substitutions at positions +1310 and +1637 in the coding region (both substitutions resulting in amino acid changes). Expression of this transcript in an in vitro transcription/translation system allowed comparison of the translation products with the size of purified iGR and mGR, showing that transcript 1A encodes both the normal sized GR (94 kDa) and the high M_r mGR [Chen et al., 1999a,b]. Transfection into several mGR-less and lysis-resistant heterologous cells resulted in both mGR-expression and acquired function—GC-evoked apoptosis [Chen et al., 1999b]. These results suggest that transcript 1A encodes mGR, which appears to be necessary for GC-sensitivity leading to apoptosis.

The GR 1A isoform's unusually long 5′UTR (1026 nt [Chen et al., 1999a,b]) could form stable secondary structures and/or translate five small uORFs preceding the major ORF. Because the most unique region of transcript 1A lies at the 5′UTR, distinguishing characteristics of the mGR (including M_r, subcellular location, function in apoptosis, and developmental timetable of expression) are likely to be associated with this region. The experiments described in this manuscript were designed to determine which sequences of the 5′UTR are responsible for translational regulation of the GR, both iGR and mGR. We disabled putative translational initiation sites for the uORFs by site-directed mutagenesis and then tested the

influence of these mutations on GR expression. We then determined whether the products of uORF-2 and uORF-5 are actually made in cells and interact with other cellular factors.

MATERIALS AND METHODS

Cell Selection, Cell Culture, and Production of Antibodies (Abs)

S-49 mouse lymphoma and U937 human promyelocytic cells were purchased from the ATCC (Rockville, MD), grown at 37°C in 5% CO_2 in Dulbecco's MEM medium and 10% bovine calf serum. Membrane GR-enriched (mGR^{++}) and mGR-deficient (mGR^{--}) S-49 cells were produced by sequential cell-separation techniques (immunopanning, fluorescent cell sorting, and soft agar cloning) and grown in RPMI 1640 in the presence of 10% bovine calf serum, as we have reported [Gametchu, 1987; Gametchu et al., 1991b, 1993; Sackey et al., 1997].

Using the FAST program in the GCG software suite, we analyzed the 1026 bp comprising the entire 1st exon of transcript 1A which codes for its 5′UTR. We found five potential translation initiation sites (AUG codons). Polyclonal Abs for two of the five uORF putative peptide products (uORF-2 and uORF-5) were commercially prepared by Animal Farm Services, Inc. (Healdsburg, CA). FITC-conjugated goat anti-rabbit IgG was purchased from Toga (Camarillo, CA). Our anti-rodent GR monoclonal Ab (BUGR-2) was produced as previously described [Gametchu and Harrison, 1984].

Construction of Transcript 1A cDNAs With Point Mutations at the uATG Codons

Construction and cloning of the full-length GR 1A cDNA were previously described [Chen et al., 1999b]. The full-length 7285 bp cDNA contained the 1026 bp 5′-untranslated region. We used Promega's GeneEditor in vitro site-directed mutagenesis system to introduce single point mutations separately at each of the five uAUGs, disabling potential translation initiating from each of these sites. Oligonucleotides designed with one point mutation at each of the ATG translational initiation codons (changing A to T, shown in bold) are sequentially (uORFs 1–5) listed: 5′-CCCCCAACCCCC**T**TGTCTCTCTCTC; 5′-CTGCTGAGGAA**T**TGAGAGCATGC; 5′-CCCTCTTGCT**T**TGGTTTCTATTTG; 5′-CTCTGGTCTGAA**T**TGGTCTCTTGTG; 5′-CTCTGCGTAAGA**T**TGGAGAAGAGAG). These mutant oligos, and an oligonucleotide provided by the kit (which codes for a mutation in the ampicillin resistance gene of any plasmid) were used in the simultaneous annealing reaction to the full-length GR 1A cDNA template cloned in pCR3.1 Uni vector. The rest of the mutant strand was then filled-in with T4 DNA polymerase and the ends ligated with T4 ligase. This heteroduplex DNA was then transformed into the repair deficient *E. coli* strain BMH 71-18 mutS and the cells were grown in GeneEditor Antibiotic Selection Mix formulated to select for the survival of bacteria containing the double-mutant plasmid. Resistant plasmids were then isolated and transformed into the final host strain, DH5a, for final plasmid isolation. All mutation sites were verified by sequence analysis.

In Vitro Transcription/Translation of GR 1A cDNA Plasmid Constructs With Point-Mutations at the uAUGs

To investigate the effects of the 5′UTR on GR synthesis, we performed coupled in vitro transcription/translation using these uORF-mutated GR 1A cDNA constructs. The cDNAs were transcribed with T7 RNA polymerase and translated in the TNT T-7-coupled reticulocyte lysate system (Promega), labeling the products with [^{35}S]methionine according to the manufacturer's instructions.

Transfection of Cells

S-49 cells contain GR transcript 1A and its products, so transfection was not required. We performed electroporation transfections with GR1A cDNA plasmid or empty parent vector in transcript 1A-less U937 cells. Briefly, suspended cells (5×10^6 cells/0.4 ml) were placed into an electroporation cuvette and DNA (25–30 μg) was added. The cells were then subjected to a high-voltage electrical pulse of 200V and 1000 capacitance and allowed to recover briefly before being placed in normal growth media for two to three days. Cells were then cultured in growth media supplemented with 8-mg/ml geneticin for stable transfectant selection.

Western Blot Analysis

To detect peptide products synthesized in cells or in vitro, we performed immunoblot analysis with cytosol prepared from S-49 or

transfected U937 cells, or with in vitro trancription/translation products of full-length GR 1A cDNA constructs. Translation products were analyzed for GR (BUGR-2 Ab added to a final concentration of 1:50) or uORF-2 product (anti-uORF-2 peptide Ab diluted to 1:200). After incubation for 2–16 h at 4°C, the samples were immunocaptured with protein A Sepharose 4B. Washed immune complexes were separated by 7.5% SDS-PAGE for GR and 15% SDS-PAGE for the uORF-2 product, transferred to nitrocellulose membranes, and processed by Western analysis as previously described [Gametchu, 1987]. To test the specificity of uORF-2 product antigen-antibody interaction, we added a 20-fold excess of synthetic uORF-2 peptide to some samples. To investigate the possibility of protein-protein interactions between GR and the product of uORF-2, cytosol preparations immuno-captured with BUGR-2 Ab were then immunoblotted with Ab against the uORF-2 product, and vice versa. All samples were then resolved by SDS-PAGE and transfered onto nitrocellulose filters. Blocked membranes were incubated for 1 h at room temprature with primary Abs, then with alkaline phosphatase-conjugated secondary Abs (goat anti-mouse IgG for use with BUGR Ab and goat anti-rabbit Ab for the polyclonal anti-peptide Abs to the uORF-2 and -5 products). Then membranes were washed and developed according to the manufacturer's instructions (Immune-Star, Bio-Rad Labs, Hercules, CA).

Fluorescent Ab Staining, Confocal Microscopy, and FACS Analysis for the uORF-2 Product

Live intact or permeablized S-49 cells were incubated with 1.2 μg/ml of anti-uORF-2 peptide Ab for 1 h at 4°C as previously described [Van Houten et al., 1997; Chen et al., 1999b]. After two washes in PBS, $\sim 1 \times 10^5$ cells were cytospun onto slides (900*g* for 8 min), mounted in 50% glycerol-PBS, examined under a Nikon Labophot fluorescence microscope, and photographed using Kodak Tmax 400 film. In negative control experiments, cells were incubated with either pre-immune rabbit serum or epitope-saturated Ab (anti-uORF-2 product Abs previously incubated for 30 min at 37°C with the uORF-2 peptide). All preparations were then incubated with FITC-conjugated goat anti-rabbit IgG, washed with buffer, and examined both by fluorescent and confocal microscopy as previously described [Pappas et al., 1995]. A portion of these stained cell preparations were also analyzed by flow cytometry using a FACS analyzer (Benton/Dickeson) equipped with a FACS Lite laser [Gametchu et al., 1993, 1995].

Interaction of the uORF-2 Product With Other Protein Factors

To identify protein factors that interact with uORF products, we performed pull-down experiments with cell extracts via immunoadsorption with Ab against the uORF peptides [Powell et al., 1999]. Briefly, cytosol from S-49 cells was incubated overnight at 4°C with either normal serum or Ab to the uORF-2 epitope ± excess uORF-2 synthetic peptide. The next day, protein A-coupled Sepharose beads were added and after incubation, the preparation containing Ab-bound uORF-2 peptide and any interacting protein complexes was aliquoted equally into eight microfuge tubes. Each sample was then washed three times with buffer containing different molar concentrations of NaCl (0.05, 0.1, 0.3, 0.6). Each sample was split into two equal volumes and resolved by SDS-PAGE for both Western blot analysis and for Coomassie blue staining. Proteins released from unincubated protein A beads by boiling were resolved in a separate lane as a negative control.

RESULTS

Sequence analysis of the 1026 bp 5′UTR of the 1A transcript [Chen et al., 1999a,b] identified five uORFs starting at positions 85, 217, 478, 628, and 892 with the potential to encode peptides of 33, 93, 6, 18, and 41 amino acids, respectively (shown in Fig. 1, uORFs-2 and -3 are overlapping). To test the functional significance of uORFs in translational control, we separately generated point mutations at each of the five uATG codons of the GR 1A cDNA. In vitro translation products (synthesized in the presence of radioactive amino acids) and immuno-captured with BUGR-2 Ab are shown in Figure 2. The autoradiogram was deliberately overexposed to be sure that GR protein missing in lane ORF-2 was indeed absent. Mutations at the other four uATG codons (uATG-1, uATG-3, uATG-4, and uATG-5) had no significant effect on the translation of transcript 1A. The molecular sizes of identified GR synthesized from the other constructs were

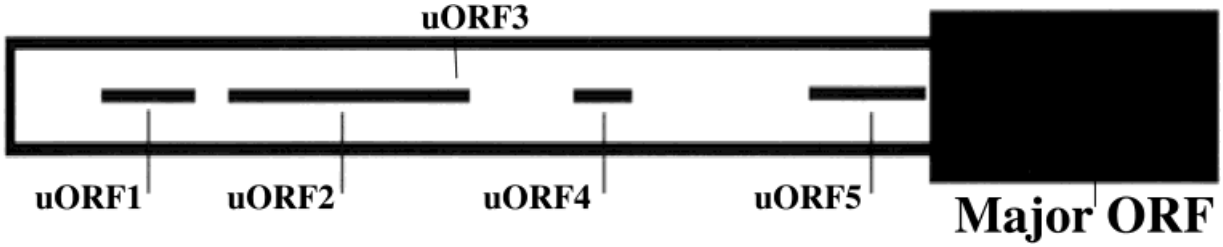

Fig. 1. The 5′UTR of the GR 1A transcript showing the five uORF sites (1–5, narrow black bars) which have potential to encode peptides of 33, 93, 6, 18, and 41 amino acids, respectively, in this 1,026 bp region. The start of the major ORF encoding GR (GR-ORF) is shown by the wide solid black bar.

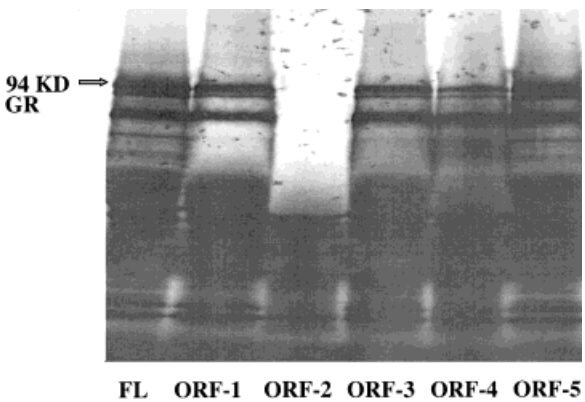

Fig. 2. In vitro transcription/translation of the full-length GR 1A cDNA after introducing mutations separately at each of the five uATG codons (at positions 85, 219, 478, 628, and 892) in the 5′UTR. FL = full-length GR 1A cDNA with no mutation introduced; lanes labeled ORF-1 through ORF-5 contain the translation products of cDNAs mutated at each of the 1st–5th uORF ATG codons, thus blocking the synthesis of the associated putative protein products, as indicated. The position of the 94 kDa GR is marked with an arrow. Only mutation at the 2nd uATG codon abolished GR in vitro synthesis.

94–97 kDa, the expected size for the intracellular GR, and smaller fragments (often seen with such overexposures), ranging in size from 45 to 85 kDa. We then immuno-captured in vitro translation products from GR 1A cDNA, uORF-2 mutant, or empty vector constructs using anti-uORF-2 peptide Abs. Western analysis with the same Ab revealed an 8.5 kDa immunoreactive peptide translatable only from the intact GR 1A cDNA (Fig. 3, FL, arrow), but not from the mutagenized uORF-2 construct (ORF-2), or empty vector (EV). The M_r of this product is consistent with the size predicted from the uORF-2 sequence. The reason for the large number of intense bands higher in the gel are not known, but they must be present due to the translation reaction mix (extracts made from rabbit reticulocytes) common between all samples. This may signify the presence of the uORF-2 peptide-related proteins in translationally competent preparations of multiple species. Since the uORF-2 product was now of interest as a possible regulator of GR synthesis, it was further examined using various immuno-identification techniques.

Next we used fluorescence microscopy to investigate cellular localization of the ORF-2 product in permeablized and unpermeablized live S-49 cells (Fig. 4). Large patches of specifically fluorescing labeled uORF products were detected. Comparison of specific fluorescence in live unpermeablized cells (B) and permeabilized cells (A) showed staining both on the membrane and inside the cell. The specificity of ORF-2 peptide-Ab interaction was verified by parallel experiments in which cells were incubated with preimmune rabbit serum (showing negligible staining, not shown) or after competitive blocking of the Ab by prior incubation with the ORF-2 synthetic epitope (C, D). The corresponding phase microcrographs are shown below each fluorescent photograph (A′–D′). It is clear from these comparisons that not all cells are stained, so these S-49 cells are heterogenous for ORF-2 peptide expression.

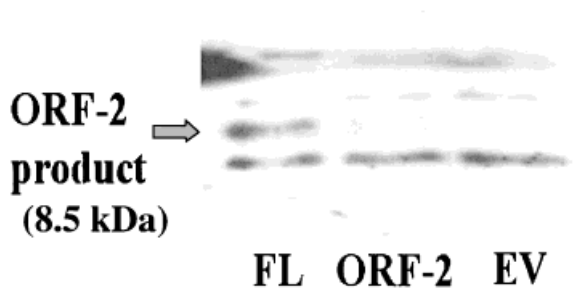

Fig. 3. Comparison of in vitro translation products from the full-length GR 1A cDNA and the uORF-2 mutated plasmid construct by Western analysis using Abs against the uORF-2 product. FL = full-length GR 1A cDNA; uORF-2 = GR 1A plasmid construct with mutation at the uAUG codon 2, and EV = empty vector.

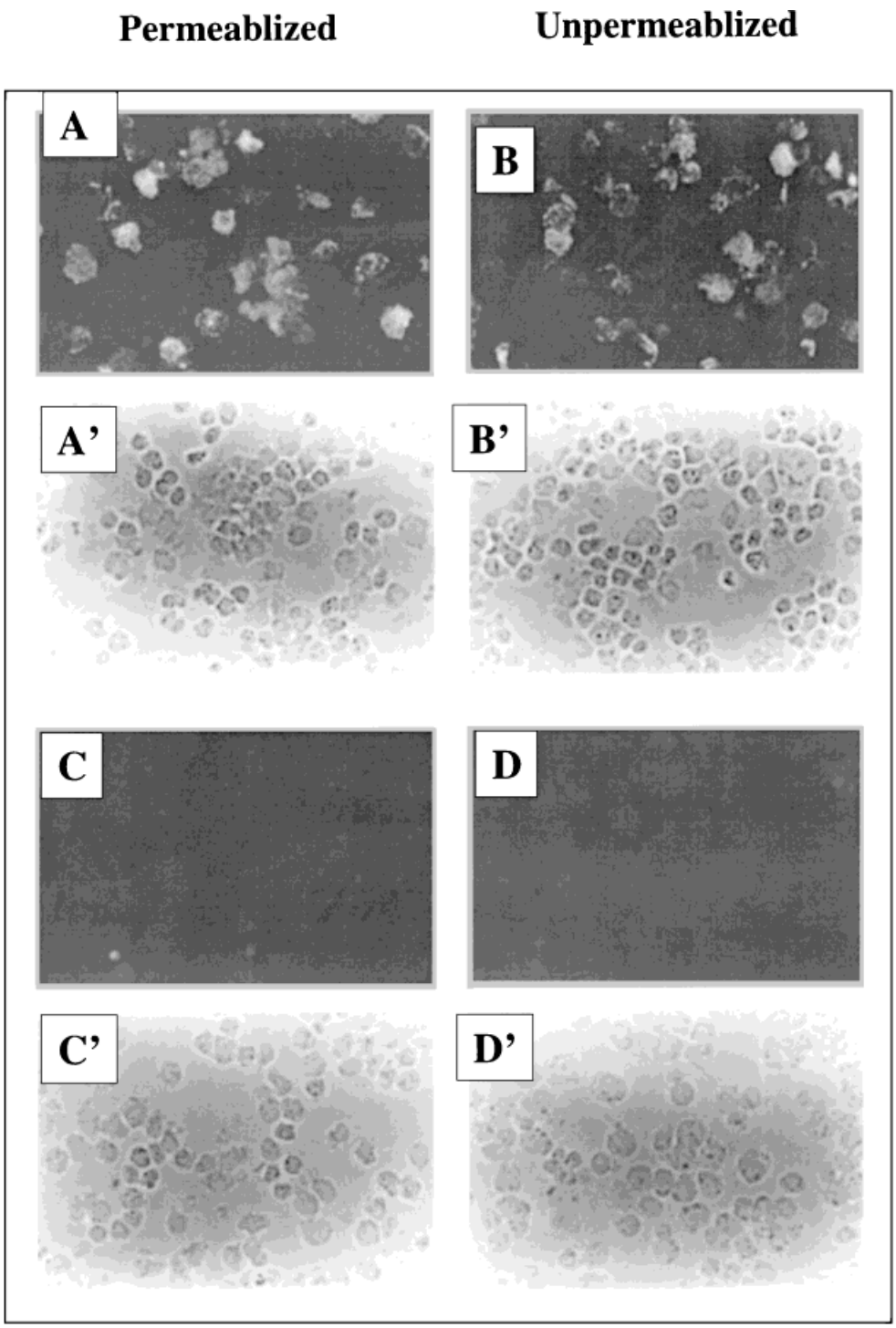

Fig. 4. Analysis of the uORF-2 products by indirect fluorescent Ab staining of cells. All cells are treated and then incubated first with anti-uORF-2 product Ab, then with FITC-conjugated anti-rabbit secondary Ab. **A**: Permeablized S-49 cells. **B**: Live S-49 cells. **C**: Permeablized cells incubated with ORF-2 specific Abs that were competitively blocked by prior incubation with the uORF-2 synthetic peptide. **D**: Live cells incubated with specific ORF-2 Abs competitively blocked by prior incubation with the uORF-2 synthetic peptide. A′–D′ are phase contrast micrographs of the correspondingly labeled fluorescence micrographs (A′D).

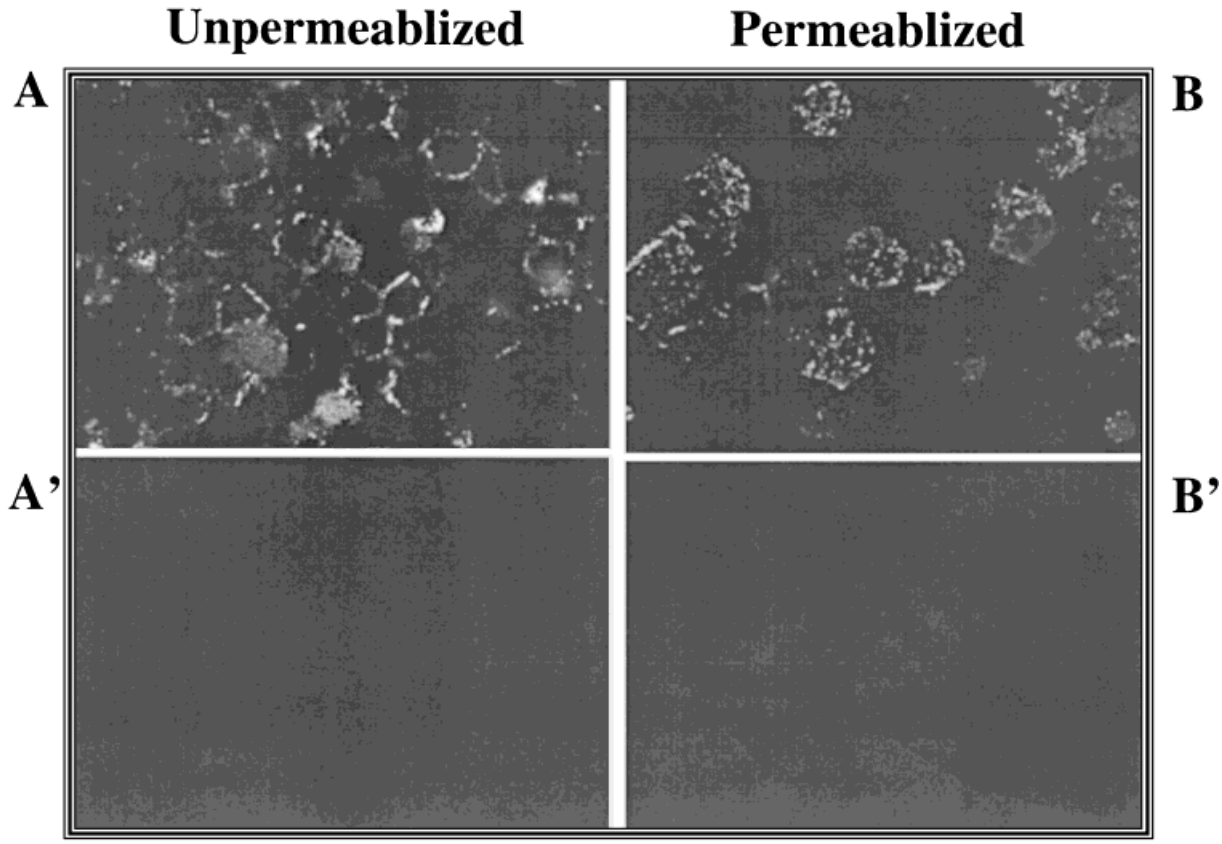

Fig. 5. Analysis of the uORF-2 products by confocal microscopy. In each case cells were incubated first with anti-uORF-2 product Ab, then with FITC-conjugated anti-rabbit secondary Ab. **A**: Live S-49 cells. **B**: Permeablized S-49. **A′** and **B′** are live and permeablized cells, respectively, after incubation with specific Abs competitively blocked by prior incubation with the uORF-2 synthetic peptide.

We next employed confocal microscopy to confirm the subcellular localization of uORF-2 product at both sites (membrane and intracellular) observed by conventional microscopy of intact and permiablized cells. Confocal cross-sections of unpermeablized live cells contained specifically fluorescing uORF antigens arranged in a circular pattern coincident with the plasma membrane contour (Fig. 5, panel A). Results from a permeablized cell group, however, showed large patches of ORF-2 products in plasma membrane, cytoplasm, and nucleus (panel B). The specificity of these antigen–antibody interactions was again confirmed in parallel experiments in which the specific Ab was saturated by prior incubation with the ORF-2 synthetic epitope, showing negligible fluorescence in both groups (A′ for unpermeablized B′ for permeablized).

To verify these observations by an independent method, we performed FACS analysis using an aliquot of the same batches of cells prepared for confocal microscopy. The representative results shown in Figure 6 illustrate high intensities of specifically fluorescing ORF-2 antigen in the plasma membrane of live unpermeablized cells (Fig. 6, unpermeablized +Ab). For the permeablized cells, the quantity of high intensity fluorescence did not vary significantly with the additional intracellular fluorescence detected, but it is possible that permiablization of the cell membrane to facilitate intracellular staining may destroy some of the membrane staining (Fig. 6, permeablized +Ab). Therefore, again the ORF-2 peptide antigen appeared to be localized both intra- and extra-cellularly. Negative control preparations (labeled with either normal serum (NS) or specific Abs competitively blocking by excess synthetic peptide epitope (EP-Ab)) did not exhibit significant high intensity fluorescence.

Next, cytosol preparations from S-49 cells and U937 cells transfected with the expression plasmid containing transcript 1A were exam-

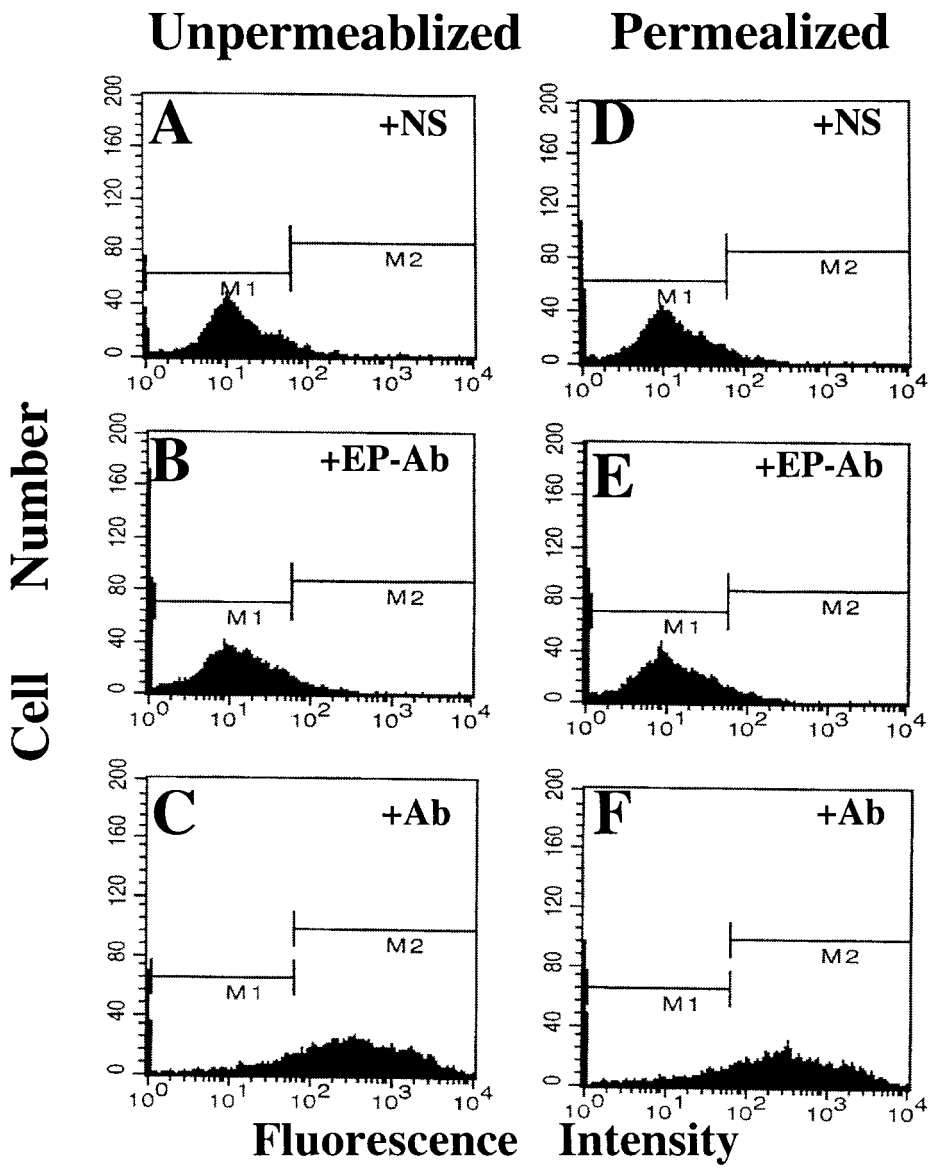

Fig. 6. Analysis of the uORF-2 Products by FACS. Cells prepared for confocal microscopy (as described in Fig. 5) were also used for the more quantitative FACS analysis. Panels **C** and **F** (+Ab) are histograms of live unpermeablized and permeablized cell preparations, respectively, after sequential incubations with anti-uORF-2 product and FITC-conjugated anti-rabbit secondary Ab. Panels **A** and **D** (NS) are live and permeablized cells, respectively, after incubation with preimmune serum. Panels **B** and **E** are live and permeablized cells, respectively, after incubation with specific Abs competitively blocked by prior incubation with the uORF-2 synthetic peptide (+EP-Ab).

ined by Western blot analysis for the presence of uORF-2 peptide; the results are shown in Figure 7. A single, intense immunoreactive band of ~8.5 kDa (lane 2, uncompeted) was observed for samples immunoprecipitated and immunoblotted with anti-uORF-2 peptide Abs (Fig. 7A); again the M_r of this band is consistent with the predicted size of translated uORF-2 protein product. Similar results were obtained with either stably transfected U937 cells (Fig. 7B) or with in vitro translated proteins from the 1A transcript (Fig. 3). The specificity of the Ab recognition of this protein was again verified by competition with excess synthetic uORF-2 peptide during immuno-capture (lane 1, competed). Higher M_r bands present in all samples are from the IgG molecules used for immunoprecipitations. Bands at the bottom of the epitope-competed lanes represent excess synthetic epitope peptide (which is smaller

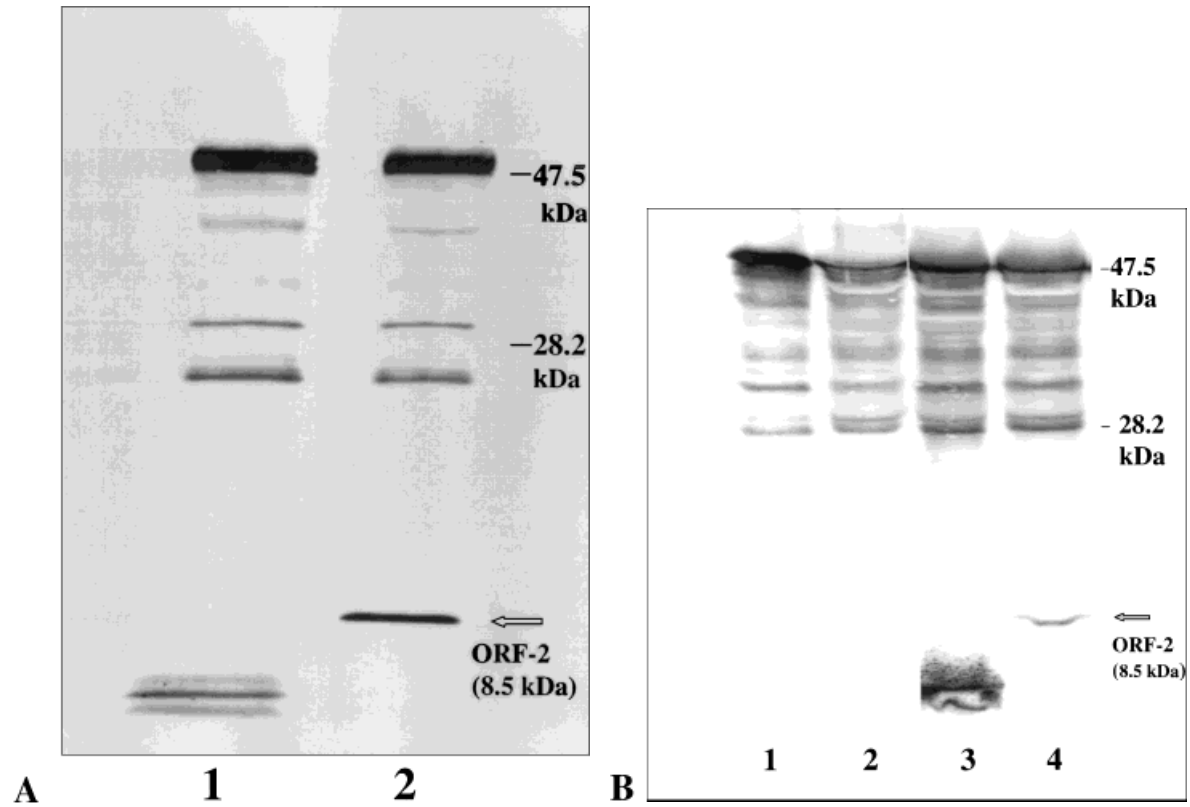

Fig. 7. Western analysis for the uORF-2 product. Cytosol from S-49 cells (panel **A**) and 1A cDNA-transfected U937 cells (panel **B**) was immunoadsorbed with anti-uORF-2 Ab in the presence (lane 1, panel A; lane 3, panel B) and absence (lane 2, panel A; lane 4, panel B) of a 20-fold excess of synthetic uORF-2 peptide. Cytosol untransfected (panel B, lanes 1) or transfected with GR 1A cDNA (panel B, lane 2) were immunoprecipitated with preimmune serum and resolved as controls. Western analysis was then performed with the same Ab. The arrows show the uORF-2 product. The bands from 28 to 47 kDa are signals from the immunoglobins used for immuno-capture.

than the ORF-2 translation product). Abs against the uORF-5 peptide did not specifically recognize any protein in immunoprecipitates or on the immunoblots (not shown). Therefore the uORF-2 product is indeed synthesized in cells, while the uORF-5 peptide product is not.

To investigate if the cellular uORF-2 peptide interacts with GR, we did reciprocal coimmunoadsorption experiments (Fig. 8). As shown by this Western analysis, while both Abs specifically immuno-capture their cognate proteins (lanes 3 and 6 for uORF-2 product and GR, respectively, see arrows), they did not cross-immunoadsorb the heterologous protein, even under low salt-containing (0.1M) mild experimental conditions (lanes 2 and 5 for uORF-2 peptide and GR, respectively). The specificity of antigen-antibody interaction was shown by the lack of immunoadsorption with preimmune serum (lanes 4 and 7 for uORF-2 product and GR, respectively). Lane 1 contains proteins eluted from Sepharose 4B protein A beads only and lane 4 contains samples immuno-captured with normal serum; therefore, the multiple immunoreactive bands (ranging in M_r from 24 to 54 kDa) detected in most lanes are probably from protein A and IgG and their fragments released from the beads during sample boiling. These data suggest that no direct protein–protein contact occurs between the product of the uORF-2 and GR.

Using the same pull-down procedure, we then investigated whether other cellular factors interact with the product of uORF-2. The results described in Figure 9 show the same samples examined by Western analysis for identification of uORF-2 product, and then by Coomassie blue staining for the examination of the other interacting proteins from S-49 cell cytosolic preparations. The series of heavy immunoreactive and Coomassie stained-bands ranging from 27 to 47 kDa and at the gel bottom

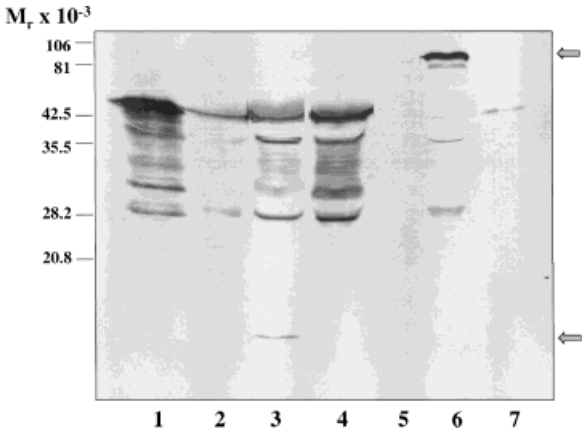

Fig. 8. Absence of direct protein–protein interactions between GR and the uOFR-2 product. Lane 1: Protein A beads alone; Lanes 2, 3, and 4 contain S-49 cell cytosol immuno-captured with BUGR-2, anti-uORF-2 peptide Abs, and preimmune rabbit serum, respectively, before Western analysis with Abs to the uORF-2 peptide; lanes 5, 6, and 7 contain S-49 cell cytosol immuno-captured with anti-uORF-2 peptide Ab, BUGR-2 Ab and preimmune serum, before Western analysis with BUGR-2, Ab. Bottom arrow = position of the uORF-2 product; Top arrow = position of GR.

are largely from protein A and heavy and light IgG chains and their fragments released from beads during the boiling of samples; most of these bands are detectable in the negative control samples containing only protein A beads alone (lanes 1 and 1′), or in samples immuno-captured with normal serum (lanes 2 and 2′). Therefore, the proteins that specifically interact with the uORF-2 product are mostly found in the lower M_r (<21 kDa) part of the gel.

Immunoreactive uORF-2 cellular product is recognized by its specific Ab, and the intensity of this band decreases with increasing salt concentrations (0.05–0.6M NaCl) in the wash buffer (lanes 5′ through 8′, see arrow) indicating high salt disruption of the Ab-uORF-2 cellular product association. The specificity of the uORF-2 product antigen–antibody interaction was verified by elimination of this band by displacement (where a 20-fold excess of synthetic uORF-2 was added during the incubation); as shown in lane 4′ the uORF-2 synthetic epitope competed successfully with the cellular uORF-2 peptide for Ab binding. The Coomassie-stained band intensity (reflecting associated protein concentration) of the low M_r interacting protein bands (<21 kDa) also decreases with increasing salt concentration in the wash buffer (lanes 5–8) as was observed for the uORF-2 product (lanes 5′–8′). The immunoreactive synthetic epitope peptide, which is a subdomain of ORF-2 peptide product, resolved as a ~2 kDa band (lane 3). Nevertheless, from the intensity of bands shown in lane 4, it appears to bind cellular proteins just as efficiently as the intact uORF-2 peptide product does (compare lanes 4 and 5).

DISCUSSION

The clinical importance of GC-evoked apoptosis requires that regulation of this process be thoroughly understood in order to provide avenues for improved therapeutic strategies against lymphoid cell proliferative diseases. Our laboratories have utilized several murine and human lymphoid cell lines to explore the participation of the membrane forms of the GR in this process. Expression of mGR is highly correlated with the expression of one of the five alternative GR transcripts (transcript 1A) in cells both naturally expressing the 1A transcript and in cells transfected with a 1A expression plasmid [Chen et al., 1999a,b]. Plasmid-driven expression of the full-length (~7.3 kb) GR transcript 1A caused acquisition GC-evoked apoptosis in several mGR-less and lysis-resistant cell lines [Chen et al., 1999b]. These results suggest that transcript 1A, which encodes mGR, appears to be necessary for GC-sensitivity leading to apoptosis. Since the unique portion of transcript 1A lies in the 1026 bp of exon 1 (the 5′UTR), we began to study this region more closely.

Computer sequence analysis predicted five translation products from the 5′UTR of transcript 1A ranging in size from 5 to 93 amino acids; none of these predicted products had any sequence similarity to the downstream major ORF product, GR. Site-directed mutagenesis to disable the uAUG codon for each uORF, coupled with in vitro translation experiments, allowed us to examine regulatory effects on translation of the GR. Expression of the GR was positively modulated by uORF-2, as mutation at the uATG-2 codon completely inhibited GR synthesis. However, this is not a universal effect of transcript 1A uORFs because mutations at the remaining four uATG codons (uATG-1, uATG-3, uATG-4, and uATG-5) had no significant effect on the translation of GR from transcript 1A. Most other reports of uORF consequences have highlighted inhibitory effects of uORFs on downstream transla-

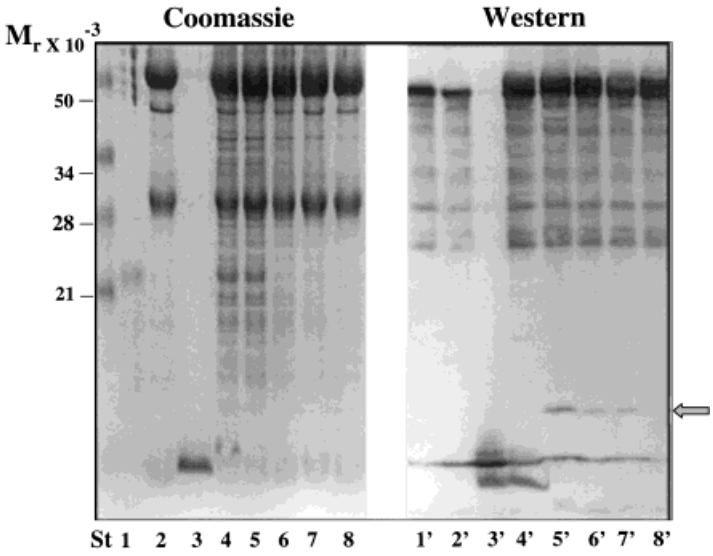

Fig. 9. Western and Coomassie staining analyses of proteins immuno-captured and coimmuno-captured by anti-uORF-2 peptide Abs. Protein A-coupled Sepharose 4B beads were allowed to bind antigen–antibody complexes, and then were washed with buffer containing increasing concentrations of NaCl: for lanes 4 and 4′—0.05M; 5 and 5′—0.05M; 6 and 6′—0.1M; 7 and 7′—0.3M; 8 and 8′—0.6M. Proteins boiled off the beads were resolved by 15% SDS-PAGE before Western analysis with anti-uORF-2 Abs (lanes 1′–8′) or Coomassie staining (lanes 1–8) of the gel. Lanes 1′–4′ and 1–4 represent controls: 1 and 1′ contain samples of protein A beads alone; lanes 2 and 2′ contain preparations immuno-captured with pre-immune rabbit serum; lanes 3 and 3′ contain synthetic epitope alone; lanes 4 and 4′ contain Ab binding for ORF-2 product competed with the uORF-2 synthetic epitope; std denotes M_r standards. Arrow shows the uORF-2 peptide product identified by Western blot, and lanes 5–8 show the unidentified cytosolic proteins interacting with this peptide.

tion. Nevertheless, there are some reports showing that uORFs augment downstream translation, or have a role in nontranslational events such encapsidation of viral RNAs [Geballe, 1996]. However, our demonstration that mutation at an uAUG codon eliminates translation of the downstream ORF does not necessarily prove that the mechanism of inhibition depends on the elimination of the uORF-2 peptide product initiated at that sequence. The AUG nucleotide sequence could be an essential component of another regulatory structure or translated sequence in some other reading frame or orientation. For example, although mutation of the seventh uAUG codon in the polio virus 5'UTR reduces downstream translation, this is not likely due to any effect of translation at this AUG codon [Pelletier et al., 2000]; because this uAUG codon is conserved among polio virus serotypes and has been shown to participate in forming a stable stem-loop, this is a more likely explanation for its effects on downstream translation in this case.

In theory, all transcripts containing uAUG codons have the potential to function as templates for translation of multiple polypeptides. To determine whether peptide products of GR regulatory uORFs are synthesized in cells, we prepared Abs to the products of uORF-2 and uORF-5 and performed immunocytochemical, FACS and Western blot analyses. We chose to examine these two peptides because of their larger size and relatively good resolution on SDS gels. In these studies a unique peptide of about 8.5 kDa was identified by Abs to the uORF-2 product, while no protein was observed with Abs to the putative uORF-5 peptide product (which would have been ~4 kDa). These data indicate that the uORF-2 peptide product (and not the uORF-5 product) is synthesized in cells which naturally contain the 1A transcript (S-49 cells) as well as in cells which have had this transcript introduced by transfection (1A plasmid-transfected U937 cells). It is possible that a ORF-5 peptide could have run off our gels, if it resolved at a different than predicted size. However, since the uORF-5 Abs did not precipitate any ^{35}S-methionine-labeled protein from the cell lysates or in vitro translation products (data not shown) we are fairly confident that this peptide was not made in our system. These data suggest not all uAUGs in the long 5′UTRs are used to initiate protein synthesis in the cells. Although in vitro translation experiments in others' labs have previously shown that short peptide products can be encoded by uORFs, ours is the only report of which we are aware to demonstrate synthesis of a uORF protein product in cells.

Our coimmuno-capture experiments investigated whether protein–protein interactions take place between the uORF-2 peptide product and GR and/or other cellular factors [Powell et al., 1999]. No direct protein–protein interaction could be detected between GR and the cellular product of uORF-2. On the other

hand, the uORF-2 product appeared to interact with several other cellular protein factors which could be variably released by incremental salt washing. The correlation between salt-induced declining intensity of the immuno-captured uORF-2 product level and the declining intensity of salt-wash-sensitive cellular protein factors also suggests that the concentrations of interacting cellular proteins is dependent upon the concentration of uORF-2 product remaining on the beads. This further supports the role of uORF-2 product in capturing those proteins by interaction. The identities and the specific role these proteins play are currently unknown.

In summary, we have identified several potential uORFs in the 5′UTR of GR transcript 1A, one of which is translated (initiated from the 2nd uATG codon) in vitro, in cells naturally expressing this transcript, and in transfected cells expressing the same transcript. Only mutation of transcript 1A at the 2nd uATG site abolished GR synthesis in vitro, suggesting that this sequence or the encoded peptide is important for GR regulation, and by extrapolation, for GC-mediated cell death. The mechanistic relationship of this peptide to GR translation and targeting of the mGR will be the subject of future investigations.

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