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Protein binding regions of the mRNAs for the 55 kDa tumor necrosis factor receptor and the glucose transporter 1: sequence homology and competition for cellular proteins

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Abstract Gene expression is influenced by mechanisms regulating mRNA degradation. Knowledge on regulatory RNA elements involved and on proteins interacting with them is still limited. A 33 nucleotide (nt) region of the 55 kDa tumor necrosis factor receptor (TNFR-55) mRNA, previously reported by us to engage in such interaction with proteins from U-937 cells, exhibits homology to a 38 nt regulatory region of the glucose transporter GLUT-1 mRNA. Labeled RNA fragments comprising these two regions bind similar sets of proteins. Upon phorbol ester-induced differentiation into macrophage-like cells, protein binding to both fragments is changed similarly. Furthermore, both compete with each other for protein binding. This suggests that GLUT-1 and TNFR-55 RNA share a novel protein binding RNA motif involved in regulation of their half life.

Key words: mRNA; TNF receptor; GLUT-1; RNA binding protein

1. Introduction

Gene expression is regulated in part by changes in RNA stability [1]. The mechanisms that affect the half life of a certain transcript involve interaction between a protein binding sequence element and the respective trans-acting factor. Cis-acting elements within the mRNAs have been identified and characterized to very different extents, varying from allocation within several hundred nucleotides, frequently the 3′-UTR, to identification of the minimum target structure for proteins that bind to them (e.g. [2–4]). A well-characterized example for regulation of RNA degradation is the interaction between the iron responsive element (IRE) in the 3′-UTR of the transferrin receptor mRNA and the protein that binds to it (reviewed in [5]), thereby protecting the RNA from endonucleolytic attack [6]. Another type of regulatory domains are the AU-rich elements (AREs) found in the 3′-UTR of many cytokine and oncogene mRNAs [7]. Transcripts containing AREs often are short-lived, and AREs are thought to play a key role for their stability by interacting with binding factors, some of which have been purified [8] or cloned [9–12].

Recently we observed that a change in RNA stability contributes to regulation of expression of the 55 kDa tumor necrosis factor receptor (TNFR-55) [13]. By triggering this receptor, its ligand TNF activates signal transduction pathways which cause a multiplicity of effects in the various target cells. Thereby TNFR-55 plays a central role in orchestrating inflammatory responses [14]. Its expression is tightly controlled, keeping the cell surface number of receptors in the range of hundreds to a few thousands per cell. The necessity to restrain its number is apparent from the drastic effects that TNF can have in the organisms as well as on individual cells. Most notably, overexpression of TNFR-55 can lead to ligand-independent oligomerization, signaling and, consequently, cell death [15].

We have reported that in myelomonocytic cell lines cell surface expression of TNFR-55 as well as the amount of its mRNA are down-regulated during phorbol ester-induced differentiation into macrophage-like cells [16]. The decrease was largely due to destabilization of the mRNA in the differentiated cells [13]. A concomitant change in the binding of cytoplasmic proteins to the receptor RNA was noted. The protein binding region, located within 33 nt of the coding sequence, does not contain an ARE and no apparent homologies to the IRE or other classical protein binding RNA regions. Recently, distinct regions in the bovine and human glucose transporter GLUT-1 mRNAs were reported to bind proteins and to affect RNA stability [17–19]. We observed a significant homology between the protein binding regions of the GLUT-1 and TNFR-55 mRNAs and provide evidence that the protein binding regions of both RNAs can interact with the same or overlapping sets of proteins.

2. Materials and methods

2.1. Cell culture

The human histiocytic lymphoma cell line U-937 [20] was obtained from the ATCC and grown in RPMI 1640 medium supplemented with antibiotics and 5% fetal calf serum. Differentiation into macrophage-like cells was induced by incubation with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (10 nM) for 48 h [16].

2.2. Templates used in in vitro transcription

Full length RNA of the TNFR-55 was transcribed from cDNA clone E13 in Bluescript KS plasmid [21]. Templates for the generation of fragments of human GLUT-1 mRNA (as specified in Fig. 2) were obtained by reverse transcription PCR using RNA from TPA-differentiated U-937 cells. To enable in vitro transcription from the cDNA templates, sense primers contained the T7 RNA polymerase start site (5′-T7, for details see [13]). Templates for the 33 nt and 38 nt fragments of TNFR-55 and GLUT-1 RNA were prepared by annealing and extension of overlapping primer pairs (TNFR-55/sense: 5′-T7-CAAGGAAAATATAT, antisense: 5′-CGAATTATTTTGAGGGT-

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Abbreviations: ARE, AU-rich element; GLUT-1, glucose transporter 1; nt, nucleotides; TNFR-55, 55 kDa tumor necrosis factor receptor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; UTR, untranslated region

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GGATATATTTTCCTTGCCCT; GLUT-1/sense: 5′-T7-TCTCTTC-CTACCCAAC; antisense: 5′-TAAAGAAAGATTAATTTGAGTG-GTTGGGTAGGAAGAGA). Nucleotide positions in the cDNAs are given according to GenBank accession numbers X55313 (TNFR-55), K03195 (human GLUT-1), M23384 (murine GLUT-1) and M60448 (bovine GLUT-1). RNA of the neomycin resistance gene and a 112 nt fragment of 18 S rRNA were transcribed from the control templates supplied in the T7 in vitro transcription kits from Boehringer Mannheim and Ambion, respectively.

2.3. In vitro transcription

RNAs were synthesized according to the instructions of the T3/T7 in vitro transcription kit (Boehringer Mannheim) or the T7-MEGA-shortskript kit (Ambion). After template digestion the RNAs were extracted with phenol/chloroform, precipitated, reconstituted with autoclaved water and stored at 70°C (for further details see [13]).

For the synthesis of labeled RNA, 50 μCi of [α-^{32}P]UTP (400 Ci/mmol, Hartmann Analytic) and unlabeled UTP were included in the reaction mixture at a final concentration of 24 μM. Following phenol/chloroform extraction, transcription mixtures were passed through a NucTrap push column (Stratagene) to remove free nucleotides. In the electrophoretic mobility shift assays (see below), similar amounts of radioactivity (0.5–1.5×10^5 cpm) were used, corresponding to 2–10 fmol of full length TNFR-55 or GLUT-1 3′-UTR transcripts and 250–750 fmol of the 33 or 38 nt RNA fragments.

2.4. Preparation of cytoplasmic extracts and detection of protein-RNA complexes

Preparation of cytoplasmic extracts and electrophoretic mobility shift assays were carried out as described [13]. Briefly, cells were lysed in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.9, 15 mM KCl, 0.6% (v/v) Nonidet P-40, 0.5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 2 mM benzamidine and 0.2 mM dithiothreitol). The cytoplasmic extract was cleared by centrifugation and aliquots were stored at −70°C. The radiolabeled RNA probes (see above) were incubated with cytoplasmic extracts (3 μg protein per sample) in 25 mM Tris-HCl, pH 7.9, 15 mM KCl, 5% (v/v) glycerol, 0.2 mM dithiothreitol, 0.5 mM EDTA and 4 μg tRNA in a total volume of 20 μl for 10 min at 30°C. The mixture was then digested with RNase T1 and electrophoresed on a non-denaturing polyacrylamide gel. Competition experiments with unlabeled RNAs and UV cross-linking of RNA-protein complexes were performed as described [13].

3. Results

As shown previously, radiolabeled TNFR-55 mRNA forms specific complexes with cytoplasmic proteins from U-937 cells induced to differentiate into macrophage-like cells by phorbol esters ([13] and Fig. 1A). To disclose a possible relationship of this protein-RNA interaction to already identified RNA binding proteins and their target sequences, several RNAs known to be regulated on the level of stability were tested for competition with the TNFR-55 RNA. As shown in Fig. 1A, the 3′-UTR of GLUT-1 RNA effectively competed with TNFR-55 RNA in complex formation. An unrelated transcript, of the neomycin resistance gene, dit not compete, indicating that the effect of the GLUT-1 RNA was specific (Fig. 1A). The labeled GLUT-1, 3′-UTR also formed complexes with protein (Fig. 1B). Furthermore, unlabeled TNFR-55 transcript competed with the GLUT-1 RNA for protein binding. These data suggest that the two mRNAs are recognized by the same or overlapping sets of proteins. The 3′-UTR of the GLUT-1 mRNA contains an AUUUA motif within an ARE [22] and two additional regions most recently reported to be implicated in regulation of its stability [17,18]. One of them (G-38 in Fig. 2) harbors a stretch of nucleotides with significant homology to the 33 nt protein binding region of the TNFR-55 mRNA (T-33 in Fig. 2). To locate the sequence elements within the GLUT-1 3′-UTR responsible for binding U-937 proteins, fragments of the transcript were tested for their competition with labeled GLUT-1 3′-UTR RNA. Only fragment B, which contained the G-38 region, effectively competed in formation of all four complexes (Fig. 3). Fragment C competed only weakly in formation of complexes C^G and D^G, and fragment A did not compete.

To address the protein binding capacity of the G-38 region directly, the corresponding 38 nt labeled RNA fragment was synthesized. Its complex formation with U-937 proteins was compared to that of TNFR-55 RNA (full length or 33 nt fragment) upon cross-linking by UV light to allow direct comparison of their sizes in SDS-PAGE. As shown in Fig. 4, all three RNAs underwent complex formation with proteins from untreated as well as from TPA-differentiated cells. Complex formation with the different RNAs resembled each other in two ways. (1) The capacity of proteins to bind G-38 RNA, as well as TNFR-55 RNA and its T-33 region, changes upon TPA-induced differentiation of the cells. Incubation of both RNA species with proteins from the differentiated cells resulted in much stronger formation of the larger complexes.

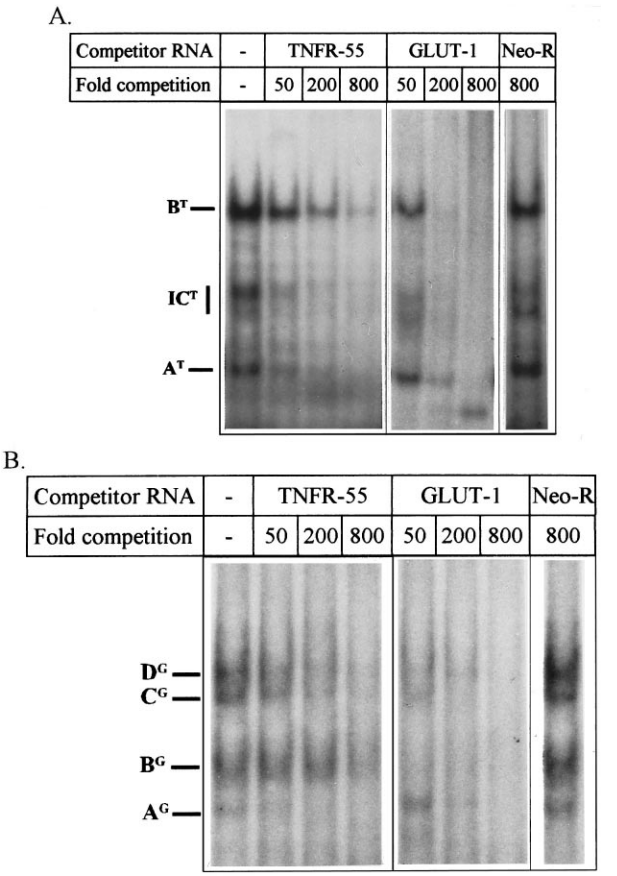

Fig. 1. Formation of complexes between proteins from U-937 cells and transcripts of TNFR-55 and GLUT-1. ^{32}P-labeled RNAs transcribed from a human full length TNFR-55 cDNA (A) or from a human GLUT-1 cDNA fragment (nt 1650–2540 of its 3′-UTR) (B) were incubated with extracts from U-937 cells cultured in the presence of TPA (10 nM) for 48 h. Where indicated, unlabeled RNA of TNFR-55, GLUT-1 3′-UTR, or the neomycin resistance gene (Neo-R) was included in the incubation at 50-, 200-, or 800-fold molar excess. Following RNase T1 digestion, the samples were analyzed by electrophoresis through a 5% non-denaturating polyacrylamide gel (see Section 2).

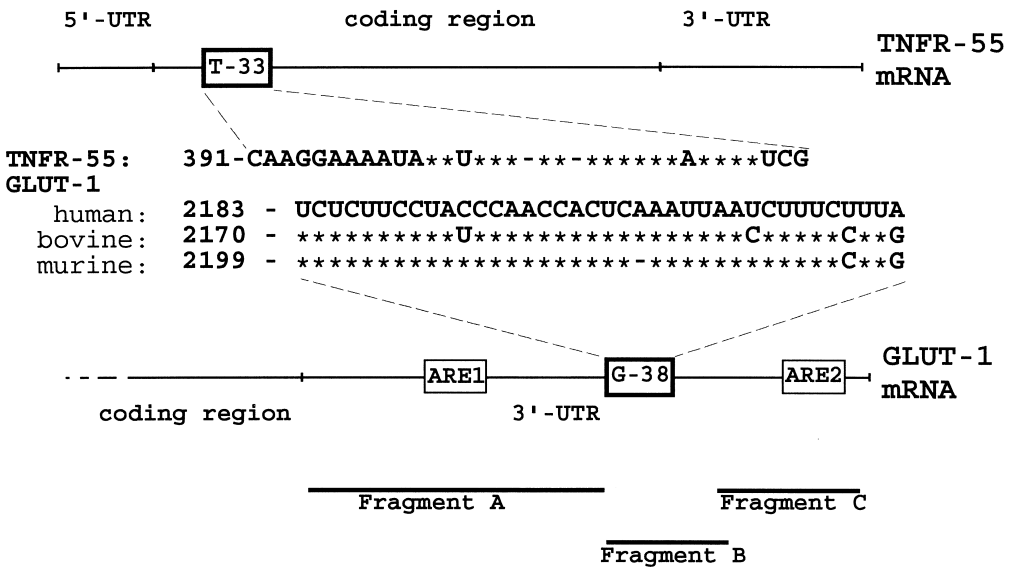

Fig. 2. Homology between protein binding regions of TNFR-55 and GLUT-1 mRNAs. Scheme of RNAs and positions of the 33 nt protein binding region of TNFR-55 mRNA ([13], designated T-33 in scheme), the 38 nt protein binding region of GLUT-1 RNA ([17] and this study, designated G-38) and its AREs ([18,22], designated ARE1 and ARE2, respectively). The sequence of the human G-38 region is compared to the T-33 region of TNFR-55 RNA, and to murine and bovine G-38. Asterisks (*) indicate identical base residues, (–) indicates a gap in the alignment. Also shown are the locations of GLUT-1 RNA fragments A to C used in competition experiments (Fig. 3).

(2) The complexes formed with the G-38 RNA fragment and full length TNFR-55 RNA or its T-33 fragment were of similar though not identical sizes: the protein binding element of TNFR-55 RNA yielded complexes of approximately 55 and 75–80 kDa in the same manner as the full length transcript [13], and an additional complex of about 120 kDa. Sizes of complexes with G-38 were about 51, 54, 73, 93, and 130 kDa. Considering that these differences could be due to differences in the way electrophoretic mobility of the complexes is affected by the GLUT-1 versus TNFR-55 RNA components, these results may indicate that the homologous fragments of TNFR-55 and GLUT-1 mRNA interact with the same cellular proteins. In support of this notion, specific cross-competition as observed between the full length TNFR-55 RNA and GLUT-1 3′-UTR (Fig. 1) was apparent between the two protein binding RNA fragments (Fig. 5).

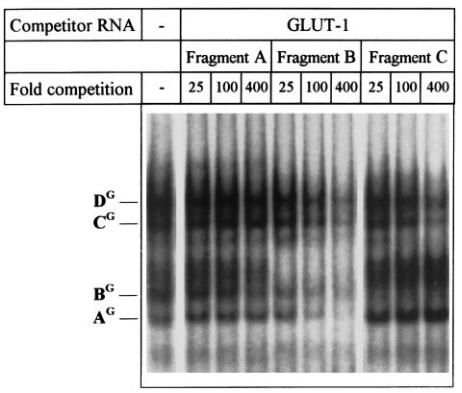

Fig. 3. Competition for protein binding between the GLUT-1 3′-UTR and fragments thereof. Protein-RNA complexes were analyzed as in Fig. 1, using extracts from TPA-treated U-937 cells and labeled GLUT-1 3′-UTR. Unlabeled RNA fragments of the GLUT-1 3′-UTR (see Fig. 2, fragment A, nt 1650–2182; fragment B, nt 2183–2363; fragment C, nt 2344–2540) were added at the indicated molar excess.

4. Discussion

Regulation of mRNA half life contributes to altered gene expression in inflammation, development and other situations of dramatic changes within an organism [1]. Knowledge on regulatory RNA sequence and factors interacting with them is still limited. We have previously observed destabilization of TNFR-55 mRNA during the TPA-induced differentiation of the histiocytic lymphoma cell line U-937 into macrophage-like cells and characterized the interaction between proteins from those cells and a 33 nt region of TNFR-55 mRNA [13]. The results of the present study suggest that the same or overlapping sets of proteins from U-937 cells can interact with TNFR-55 RNA and GLUT-1 RNA.

GLUT-1, the major glucose transporter molecule of the brain and of erythrocytes, can be expressed in a variety of other tissues. Its induction in macrophages exposed to endo-

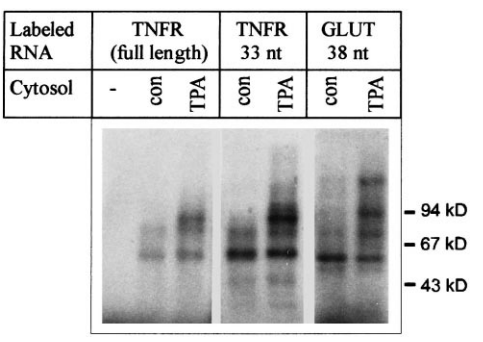

Fig. 4. Cross-linking analysis of the complexes formed with TNFR-55 and GLUT-1 RNA. Full length TNFR-55 RNA and the 33 nt TNFR-55 and 38 nt GLUT-1 RNA fragments were incubated with extracts from U-937 cells cultured for 48 h in the absence (*con*) or presence (*TPA*) of 10 nM TPA. After RNase T1 digestion, the samples were UV cross-linked for 15 min and analyzed by SDS-PAGE (10% acrylamide). The positions of molecular weight markers are indicated.

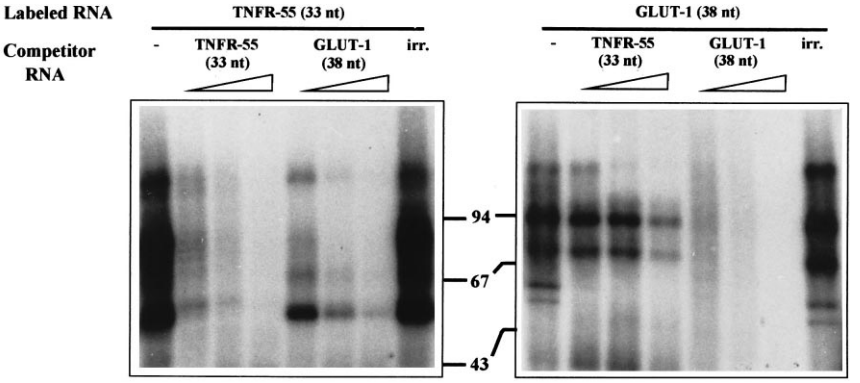

Fig. 5. Cross-competition between the protein binding regions of TNFR-55 and GLUT-1 mRNA. Complexes formed between extracts from TPA-treated U-937 cells and the labeled fragments of TNFR-55 RNA (33 nt) or GLUT-1 RNA (38 nt) were analyzed as described in Fig. 4. The same RNA fragments were included in the incubation as unlabeled competitors at a 50- to 500-fold molar excess (irr.: irrelevant RNA, consisting of 112 nt of the 18S-rRNA, added at 500-fold excess).

toxin [23] and in fibroblastoid cells treated with TNF [22,24] suggests that it plays a major role in the changes in glucose metabolism during inflammation. Mechanisms controlling its expression include alterations in RNA stability (reviewed in [25]).

In transfection studies the GLUT-1 3′-UTR conferred instability to a stable reporter transcript. The chimeric RNA could be stabilized by TNF treatment [25]. Different regions of the GLUT-1 3′-UTR appear to contribute to regulation of its stability. Up-regulation of murine GLUT-1 mRNA half life after stimulation of 3T3-L1 cells was correlated to formation of a 44 kDa complex between the GLUT-1 3′-UTR and the adenosine-uridine binding factor (AUBF), and has been suggested to involve an ARE containing and AUUUA motif (ARE2 in Fig. 2) [22]. However, experiments with different RNA fragments indicated that a distinct ARE, located more upstream in the 3′-UTR (ARE1 in Fig. 2) engaged in complexes of 44 kDa [17,18], and according to a recent report the *elav*-like RNA binding protein Hel-N1 can bind to that region [26]. The results presented in Fig. 3 and additional experiments employing labeled RNA fragments failed to provide evidence that proteins from U-937 cells bind to the ARE1 region. The weak competition of fragment C in formation of complexes C^G and D^G (Fig. 3) indicates that binding of some proteins to the ARE2 region may occur. Yet the major domain for binding of proteins from U-937 cells is located within the G-38 region (Figs. 3 and 4).

That region is highly conserved among several mammalian species and contains the sequence homology to the protein binding region of the TNFR-55 RNA. The corresponding region of bovine GLUT-1 RNA has been identified as the RNA component of an 88 kDa complex between part of the bovine GLUT-1 3′-UTR and proteins from C6 rat glioblastoma cells [17]. Most recently, increased expression of a reporter gene upon its fusion with a partial cDNA of the bovine GLUT-1 3′-UTR was abolished by deleting 10 nt of that region [19], demonstrating its functional importance. In another study [18], evidence was provided that a sequence within the corresponding region of that human GLUT-1 transcript (nt 2186–2203) participates in forming 120 kDa complexes with proteins from human brain tumor extracts.

We show that an RNA fragment encompassing the human 38 nt GLUT-1 region (2183–2220) is sufficient to form several complexes with cytosolic proteins from U-937 cells (Figs. 4 and 5). Taking into account that due to different experimental setups these complexes contain RNA of 38 nt versus 52 nt in the studies of Tsukamoto et al. [18,19], it is likely that the 130 kDa and 93 kDa complexes detected by us correspond to the 120 and ~90 kDa complexes in those studies. Of note, it appears that the monocytic cells used in our study contain additional GLUT-1 RNA binding proteins whose relation to the proteins detected by others is not clear at present.

The GLUT-1 mRNA is strongly induced in TPA-differentiated U-937 cells (not shown). Though the barely detectable level in undifferentiated cells precluded direct comparison of mRNA half lives, the long half life in differentiated cells (~3 h, not shown), as compared to the basal half life in other cells, notably also in myeloid precursors ([25] and references therein), and the evidence for a regulatory function of the respective RNA region [19] lead us to assume involvement of the observed changes in protein-RNA interaction in regulation of GLUT-1 RNA degradation in U-937 cells.

The observations that TNFR-55 and GLUT-1 RNAs yield similar complexes with proteins from undifferentiated U-937 cells, that TPA-induced differentiation causes similar changes in protein binding to both RNAs, and that both compete with each other for binding proteins suggest that they might interact with the same cellular proteins. Yet, while GLUT-1 mRNA is induced during TPA-induced differentiation, the TNFR-55 mRNA is destabilized and down-regulated [13]. Possibly this inverse regulation is related to differences not reflected in the formation of protein-RNA complexes as observed here. Thus the proteins may serve as adaptors for different additional factors regulating RNA stability. Furthermore, the positioning of the homologous protein binding element within the respective transcript, in the N-terminal part of the coding region for TNFR-55 but in the 3′-UTR for GLUT-1 may be crucial.

In summary, it is suggested that the protein binding regions of the TNFR-55 and GLUT-1 RNAs contain a novel regulatory RNA element which may be present in additional, as yet unidentified transcripts. In the U-937 model of macrophage differentiation, the observed changes in the interaction of proteins with that regulatory element may coordinately affect the half life of a set of mRNAs, thereby contributing to altered gene expression. Gaining knowledge on the function of the proteins interacting with that element will be a major goal of future investigation.

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