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Decreased tumorigenicity of c-Myc-transformed fibroblasts expressing active USF2

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Abstract

USF is a small family of basic helix–loop–helix leucine zipper (bHLH-zip) transcription factors with DNA binding specificities similar to that of the c-Myc oncoprotein. Evidence for a role of USF in growth control includes inhibition of c-Myc-dependent cellular transformation in vitro and loss of USF transcriptional activity in many cancer cell lines. However, a direct effect of USF on the tumorigenicity of an established cell line has never been demonstrated. Here, cell lines derived from rat embryo fibroblasts transformed by c-Ha-Ras and either c-Myc or E1A were used as model system to investigate the tumor suppression ability of USF. Overexpression of USF2 stimulated transcription and inhibited colony formation in c-Myc-transformed, but not E1A-transformed, fibroblasts. Stable clones expressing high USF2 levels were constructed from c-Myc-transformed fibroblasts. In two of these clones, overexpressed USF2 did not activate transcription, and there was no significant change in the transformed phenotype. In contrast, a clone that expressed transcriptionally active USF2 exhibited altered morphology and a strongly decreased ability to proliferate in semisolid medium. The ability of these cells to form tumors in nude mice was also decreased by a factor of more than 30 as compared to the parental cell line or cells overexpressing transcriptionally inactive USF2. Cotransfection assays with USF- or Myc-specific dominant-negative mutants indicated that active USF2 inhibited cellular transformation by preventing transcriptional repression by c-Myc.

Keywords: c-Myc; USF; Transcription factors; Cellular transformation; Promoter repression

Introduction

USF is a family of basic helix–loop–helix leucine zipper (bHLH-zip) transcription factors that are similar to the c-Myc oncoprotein in both their structures and their DNA binding specificities [1,2]. In mammals, there are two different genes, *Usf1* and *Usf2*, both ubiquitously expressed [3,4]. Unlike c-Myc, which requires dimerization with another bHLH-zip protein, Max, in order to bind DNA [5], the USF1 and USF2 polypeptides form stable homodimers as well as USF1–USF2 heterodimers, all of which bind avidly to DNA elements containing a core CACGTG or CACATG sequence [2,6]. Together, the *Usf1* and *Usf2* genes are essential for embryonic development, as revealed by the early lethality of the double null mutation in mice [7]. Asymmetrical regulation of the two *Usf* genes, with *Usf1* repressing USF2 protein expression and *Usf2* stimulating USF1 expression, suggests distinct roles for the two proteins and that one function of USF1 is to precisely regulate USF2 expression level [7]. Accordingly, USF2-null mice display a more pronounced phenotype than USF1-null mice. USF1-null mice are essentially normal and fertile, while USF2-null mice display a severe growth defect and several other abnormalities in fertility, mammary gland function, and transcriptional response to glucose in liver [7–9].

Initial evidence for a role of USF in growth control came from experiments showing that cotransfection of either USF1- or USF2-expressing plasmids inhibited cellular transformation of primary rat embryo fibroblasts

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by c-Myc and activated Ras [10]. The inhibitory effect of USF1 in this assay was specific to the Myc pathway and did not affect E1A-dependent transformation. In contrast, USF2 overexpression inhibited focus formation induced by transfection of a variety of oncogenes and also prevented colony formation in certain transformed cell lines [10]. These differences between USF1 and USF2 can be explained if the two transcription factors have different, though perhaps overlapping, sets of downstream targets. This idea is also supported by the fact that USF1 and USF2, which are very conserved in their binding and dimerization domains, are quite divergent in their N-terminal transcription activation domains [2]. The downstream targets mediating the antiproliferative activities of the USF proteins remain to be determined. However, it is clear that many of the genes regulated by USF are implicated in the control of cellular proliferation and cancer. Among those are for example *Cox-2*, *BRCA2*, *cathepsin D*, *APC*, *IFG-2R*, and *CDK4* [11–16].

It is widely believed that the mechanism by which overexpression of c-Myc causes cellular transformation involves the activation or repression of key transcriptional targets [17]. There is in particular a strong correlation between the ability of c-Myc to repress certain promoters and its ability to induce cellular transformation [18,19]. Repression of gene transcription by c-Myc is often associated with the presence of an initiator element in the promoter and is mediated by interaction with other transcription factors, including Miz-1 [19]. Interestingly, the activity of USF is also greatly influenced by the presence of an initiator element in its target promoter. However, in contrast to c-Myc, which represses initiator-containing genes, USF stimulates them [20–22]. This stimulation is mediated by a particular activation domain, the USF-specific region or USR, that is highly conserved and present in both USF1 and USF2 [23]. An attractive hypothesis is that opposing activities of USF and c-Myc on cellular transformation and proliferation result from their opposite effects on the expression of initiator-containing genes. However, as yet, no direct evidence supports this hypothesis.

The antiproliferative activities of the USF proteins and their functional antagonism with Myc suggest that loss of USF function could trigger uncontrolled cellular proliferation similar to that caused by c-Myc overexpression. In agreement with this hypothesis, we found that the USF1 and USF2 proteins, although present at similar levels in all cell types, lack all transcriptional activity in many cancer cell lines (e.g., Saos-2 and several breast cancer cell lines including MCF-7 and MDA-MB-231), while strong endogenous as well as exogenous USF activity can be demonstrated in nontumorigenic cells (e.g., MSC-1, MCF-10A, early passage human mammary epithelial cells) [15,24–26]. In other cancer cell lines, exemplified by HeLa and breast cancer cell lines such as Hs578T, MDA-MB-468, and T47D, endogenous USF is inactive, but some activity can be unmasked following USF overexpression [25]. These observations suggest different mechanisms contributing to the inactivation of endogenous USF in different cancer cell lines. Correlation between the loss of endogenous USF function and the tumorigenic potential of different cell lines also suggests that the genetic events leading to USF inactivation are selected because they confer a growth advantage to cells. However, the ability of USF to alter the malignant phenotype of an established cell line has never been directly investigated.

To explore the tumor suppression ability of USF, we chose as a model system cell lines established from foci of rat embryonic fibroblasts transformed by cooperating oncogenes. The goals of these experiments were to determine whether overexpression of USF can reverse cellular transformation and whether this reversal depends on the original transforming oncogenes. The results confirmed the previously noted selectivity of USF activity in Myc-transformed cells and suggested that USF inhibited transformation by preventing promoter repression by c-Myc.

Materials and methods

Plasmids

The USF-dependent reporter plasmids pU3MLLuc and pIGF2RLuc (also called pABCLuc), the mammalian expression vectors for full-length USF1 and USF2 (psv-USF1, psv-USF2, pCMV-USF1, and pCMV-USF2), and the dominant-negative mutants A-USF and A-Max were all previously described [15,23,25]. The tetracycline-inducible USF2 expression vector was constructed by subcloning the *Nco*I to *Xho*I fragment of murine *USF2* into the *Xho*I site of vector pUHD10.3 [27].

Cell culture and cell line derivation

Second-passage rat embryo fibroblasts were transfected by expression vectors encoding cooperating oncogenes as previously described [10]. Cell lines were established from individual foci of morphologically transformed cells on plates transfected with c-Myc and c-Ha-Ras (Myra2) or E1A and c-Ha-Ras (Era3) by expansion and propagation in DMEM medium containing 10% heat-inactivated fetal bovine serum. All cells were cultured in a 37°C incubator with 8% CO_2. USF2-overexpressing cell lines driven by the Tet-responsive promoter were constructed and tested as recommended for the Tet-off system (Clontech). Briefly, 3 μg of circular pTet-off plasmid were transfected into Myra2 cells plated at 2×10^5 cell/10-cm dish using Fugene 6 (Roche) as the transfection reagent. G418-resistant colonies were selected and tested by transient transfection of the pTRE-Luc reporter gene. The cell line with the lowest background and highest inducibility, designated MRO-32, was transfected with the pMT2 vector and pTK-Hyg, and the