β-ACTIN mRNA EXPRESSION IS MARKEDLY UPREGULATED WHEREAS ITS PROTEIN LEVELS ARE UNCHANGED IN PRIMARY HEPATOCYTE CULTURES FROM THE LITTLE SKATE, *RAJA ERINACEA*

David J. Seward[1], Rachel E. Anderson[2], Catherine S. Bennet[3], Jesse McCoy[4], Shi-Ying Cai[5], James L. Boyer[5], Ned Ballatori[1]
[1]Dept. of Environmental Medicine, Univ. of Rochester School of Med., Rochester, NY 14642
[2]Harvard College, University Hall, Cambridge, MA 02138
[3]Choate Academy, Wallingford, CT 06492
[4]Durham H.S., Durham, NC
[5]Dept. of Medicine and Liver Center, Yale University School of Med., New Haven, CT 06520

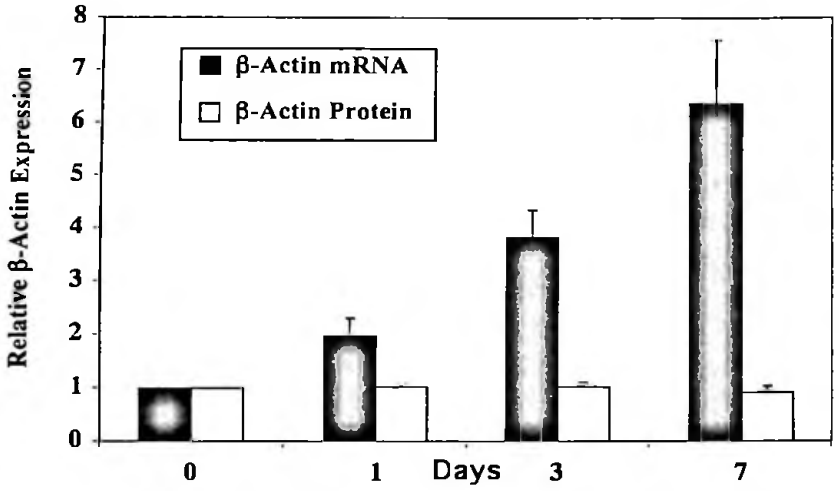
β-Actin is widely considered cell biology's quintessential housekeeping gene. This protein, a ubiquitous structural component of the cytoskeleton, is constitutively expressed in all cell types. Its expression levels within a particular cell type are stable, predictable and internally consistent and can thus be used to normalize RNA and protein data for other genes. To assess whether β-Actin might be used as a housekeeping gene in our primary skate hepatocyte cell culture model we measured its mRNA and protein levels over time in culture.

Hepatocytes from male skates (~1kg) were isolated by mild collagenase perfusion and cultured on standard plastic 6-well plates in a simple elasmobranch Ringer solution (containing in mM, 270 NaCl, 4 KCl, 2.5 $CaCl_2$, 3 $MgCl_2$, 0.5 Na_2SO_4, 1 KH_2PO_4, 8 $NaHCO_3$, 350 urea, 5 D-glucose and 5 Hepes/Tris, pH 7.5), supplemented with dexamethasone (0.1 μM) and penicillin/streptomycin (100 U: 0.1 mg/ml). Cells were kept in culture for 7 days with total RNA and protein samples isolated on days 0, 1, 3 and 7. Total RNA for each time point was isolated using Qiagen's RNeasy Mini Kit for RNA isolation from tissue culture. Preceding quantitative RT-PCR analysis, total skate hepatocyte RNA, 2 μg, was subjected to DNase I treatment using GibcoBRL Life Technologies Deoxyribonuclease I, Amplification Grade. Quantitative RT-PCR was conducted on a Rotor-Gene 2000 Real-Time Cycler employing Taqman chemistry and GibcoBRL Life Technologies SuperScript One-Step RT-PCR with Platinum Taq. A partial skate β-Actin sequence was cloned by degenerative PCR. Gene specific primers and Taqman probes were designed to that skate β-Actin sequence using Perkin-Elmer's Primer Express computer software. mRNA levels for the β-Actin gene were assessed by measuring the copy number in 10ng of total cell culture RNA. Day 0 values were assigned a value of 1 and successive time points were expressed as a ratio to day 0 values. Total cell protein was isolated via acid precipitation with homogenization buffer containing 5% PCA, 1mM EDTA, 1mM PMSF, and mammalian protease inhibitor cocktail 5μl/ml (Sigma P-8340). Cells were lysed completely in the homogenization buffer and then spun at high speed for 1 minute to pellet protein. The protein pellet was resuspended in a buffer containing 10 mM Tris (pH 7.4), 1 mM Na-orthovanadate, 0.1 mM leupeptin and 10% SDS. Final protein concentrations were determined using the Lowry protein assay. Protein samples were prepared for SDS-PAGE analysis by boiling in BioRad's Laemmli Sample Buffer containing β-mercaptoethanol. 50μg of total protein were separated on BioRad precast Tris-HCl polyacrylamide gradient gels (4%-20%) for 2 hours at 90-100V. The proteins were transferred to PVDF membranes where they were exposed to an anti-Actin monoclonal antibody (ICN, Clone: C4, cat# 691002). This antibody reacts with a conserved N-terminal region found in all actin monomers (Lessard, J.L., Cell Motility and the

Cytoskeleton, 10: 349-362, 1988). Reactive bands were visualized using an anti-mouse IgG_1 secondary antibody conjugated to HRP followed by chemiluminescence detection. Blots were analyzed and relative protein levels determined using a Kodak Image station. As with the mRNA data, day 0 protein values were assigned a value of 1 and successive time points were expressed as a ratio to day 0 values.

β-Actin mRNA expression increased 2.0±0.3 fold from day 0 to day 1. By day 3 levels had elevated to 3.8±0.5 fold over day 0 controls. On day 7, β-Actin mRNA levels had risen 6.4±1.2 fold over initial levels. Interestingly, these changes in transcript expression appeared to have no effect on β-Actin protein levels.

Figure 1. Relative β-Actin Expression in Primary Skate Hepatocyte Cultures Over Time. Total RNA and protein from skate hepatocytes in culture were isolated over a 7-day time course. Using gene specific Taqman probes and primers, relative mRNA expression levels for β-Actin were calculated for each time point. By day 7, β-Actin mRNA levels were 6.4±1.2 fold higher than those measured on day 0. Protein expression levels remained relatively constant over the 7-day culture period. All values are mean±standard error.



The reasons for the marked change reported in β-Actin mRNA expression are not fully understood. A recent paper by Otsu et al. (Cell Physiol. Biochem. 11:33-40, 2001) reports a similar phenomenon in primary rat hepatocyte cultures. Their data indicate a general trend in which liver specific gene mRNAs are downregulated while constitutively active genes, such as β-Actin, are upregulated. Their work suggests that the absence of an extracellular matrix could play an integral role in both the transcriptional regulation as well as the overall stability of gene transcripts. Our observations support these previous findings; however, because much of the skate genome has yet to be cloned we cannot assess whether this is a general trend in the expression of constitutive versus liver specific genes. Furthermore, it is unclear whether or not the increase in β-Actin transcript level is due to an increase in transcriptional activity or simply to an increase in transcript stability. The data presented by Otsu et al. in rat hepatocytes suggest the transcription rate for β-Actin is stable and as a result they speculate that increased transcript stability is the most likely explanation for the observed increases in β-Actin mRNA in their experiments. Despite the observed increases in β-Actin transcript in our skate hepatocyte RNA, western blot data indicate that β-Actin protein levels remain relatively constant. Thus, although β-Actin may still be useful for normalizing protein data, its role as a benchmark for evaluating changes in mRNA expression in primary culture models may require reassessment. (Supported by National Institute of Health Grants ES03828, ES01247, DK34989, DK48823, NSF DBI 9820400, and by the Burroughs Wellcome Foundation).