Evaluation of transfection methods for RAW264.7 murine macrophage cells

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Introduction:

Macrophages occupy a pivotal position in regulating numerous aspects of the immune response. They are notoriously difficult to transfect and many different approaches have been applied including electroporation (1), DEAE dextran (2) and use of cationic liposomes (3). Whilst high transfection efficiency ***per se*** is an important aspect for enabling studies, it is not the only consideration. The responses of the transfectant cells are vitally important if valuable information is to be gleaned from any experiment.

For a number of years we have been interested in the ***Nramp1*** gene with particular consideration to its function and regulation. ***Nramp1*** encodes a macrophage-expressed divalent cation transporter and is regulated by members of the Myc family of transcriptional regulators both negatively, by Myc, and positively by Miz-1 and interacting cytokine-induced factors (4,5). The murine RAW264.7 cell line has been an important cell for both functional studies, as it expresses a functionally null ***Nramp1*** allele, enabling complementation-based studies and for studying regulation, as it responds to inflammatory cytokines and bacterial endotoxin by transcriptional responses through the JAK-STAT pathway. The core promoter of the murine ***Nramp1*** gene contains a tandem duplication of initiator elements, to which Miz-1 binds (6). Myc represses transcription via Miz-1 by displacing bound co-activator such as p300 (7).

In this experiment we have compared the responses of the ***Nramp1*** promoter-luciferase construct for regulation by Miz-1 and Myc and another less-well characterised protein of the Myc family, Mnt using Metafectene^{TM} PRO (MF), DEAE dextran (DD) and a polyplex (PP) compound (8) purchased from Sigma-Aldrich.

Materials and methods:

Materials

Metafectene PRO, a polycationic liposomal transfection reagent, was obtained from Biontex Laboratories GmbH (Munich, Germany), DEAE dextran was from Promega (Southampton, UK) and polyplex from Sigma Aldrich. RAW264.7 cells were grown as described previously on Nunclon tissue culture-treated plastic (3). The ***Nramp1*** promoter Luciferase reporter was prepared as described (4,5) and Myc, Mnt and Miz-1 expression plasmids were from (pEF-c-Myc (provided by Yongfeng Shang, Harvard Medical School, pCMV MIZ-1 expression plasmid was provided by Frank Hanel, Hans Knoll-Institut for Naturstoff-Forschung, Heidelberg, and Mnt from Eisenman RN. Fred Hutchinson Cancer Research Center, Seattle, USA.). Plasmid DNAs used for transfection were prepared using Machery-Nagel Maxi Prep kits, and the total quantity of DNA in any transfection was normalized with empty vector plasmid.

Experimental procedures / transfection protocol:

On approaching confluence, RAW264.7 cells were harvested by scraping, viable cell number determined in the presence of trypan blue and cells were plated out at $0.5x10^6$ cells per ml in 12 well tissue culture plates (Greiner), 1ml/well. Cells were allowed to adhere overnight before transfection. Each transfection was performed in triplicate and experiments shown are representatives of at least two. Values of DNA and transfection reagents are for triplicate wells. ***Nramp1*** promoter reporter plasmid DNA (1.5µg) was mixed with 100µl of DMEM without any additions, mix A. To this was added Miz-1, Myc, Mnt or empty vector plasmid DNA (1µg). Transfection reagent MF (5µl, 5µg), DD (5µl, 50µg) or PP (5µl, 5µg) was added to another 100µl of DMEM, mix B. Mix A and mix B were combined in a single 1.5ml eppendorf tube, and allowed to stand for 20 mins at RT. To ensure consistency between transfections DNA/DMEM was made in bulk then split for the various transfection reagents. Meanwhile 12 well dishes containing RAW264.7 cells were removed from the incubator, media removed by aspiration and cells carefully washed x2 with DMEM without additions. Subsequently, 0.5ml of DMEM, no adds was replaced. The transfection mix, 60µl per well was carefully pipetted into each well and the plates were returned to the 37^oC incubator for 4 hours. After which time an additional 0.5ml of DMEM without antibiotics containing 20% endotoxin free newborn calf serum was added and the cells were incubated overnight, 20-24 hours. Cells were harvested for Luciferase assay by standard procedures and Luciferase activity was determined using a Turner Designs luminometer. Luciferase reagent was purchased from Promega Corp (Southampton). All Luciferase Units (LU) were normalised to the amount of protein in the cell extract, determined using the Bio-Rad protein assay reagent.

Results and discussion:

We have compared a variety of transfection methods for expression of an ***Nramp1*** promoter luciferase reporter construct and its transactivation/repression by Myc and interacting family members. Myc has a well established inhibitory function on promoters that lack a TATA box. One inhibitory mechanism is associated with the Miz-1 protein that binds to an initiator element. On binding Miz-1, Myc prevents or leads to the displacement of of co-activators including p300, thereby repressing transcription. Miz-1 strongly activates transcription when tethered to an initiator element in the absense of exogenous Myc. A role for Mnt in initiator-dependent transcription has not been described previously. In the experiment shown in Figure 1, we observed that the response to expression of the particular transcription factor is dependent upon the transfection procedure used. Thus MF provides strong transactivation by both Miz-1 and Mnt. The level of repression seen by Myc is not significant and this is common as it can be influenced by levels of endogenous Myc and usually a dose response curve is required to show repression. For the other transfection reagents a significant increase in reporter gene activity was observed with PP with Mnt transactivation, however the magnitude of increase was lower than that observed for MF. With DD transfections a significant decrease in transcription was observed with Miz-1 ($P=0.04$), however this is the opposite direction to that expected.

In Table 1 are the results for the yields of protein recovered from the various wells for cells treated with the 3 transfection reagents. Inspection by eye revealed no difference of appearance of the cells, however the yield of extract recovered from MF transfections was significantly greater than both PP and DD transfections indicating lower toxicity. This table also includes the absolute values of LU/µg for cell transfected with empty vector control plasmid. MF transfected cells yielded significantly lower activities than the DD transfection and higher activities than the PP transfection.

Conclusion / summary:

The results from this experiment have shown that absolute transfection efficiency is not the only parameter that is important for any transfection procedure. The MF transfection reagent did not yield the highest level of transfection and was intermediate between that achieved with PP and DD. However, transfection using MF led to the highest recovery of cell protein, which we interpret as arising from reduced toxicity. In addition and of more relevance for these studies was that the MF transfection reagent allowed for greater responses with both activating and repressing transcription factors.

References:

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Appendix: Tables and/or figures:

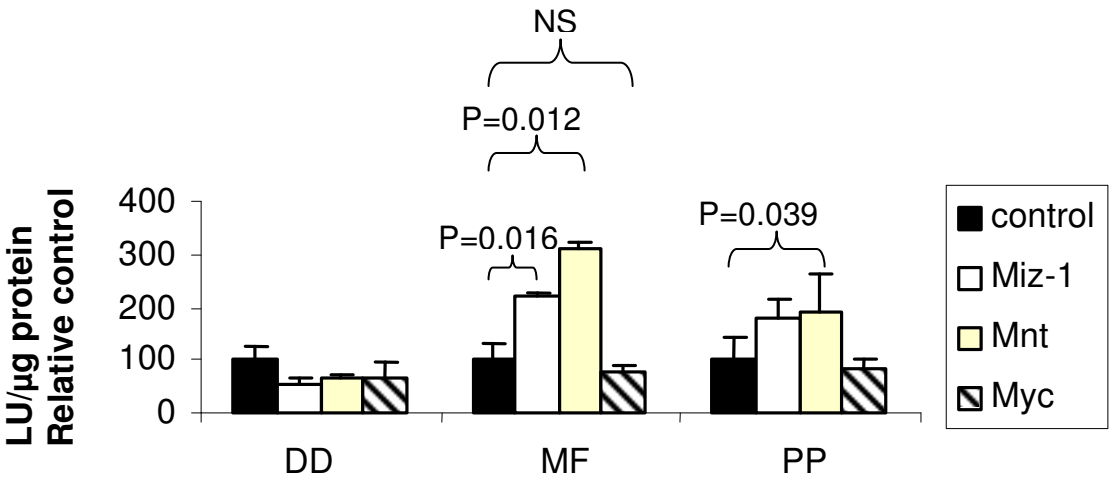

Figure 1: Influence of transfection reagent on the regulation of ***Nramp1*** promoter function by the Myc family of interacting proteins. Results are presented as Luciferase Units (LU)/µg cell extract protein and normalised to the control transfection, which is set at 100%. P values for significant differences are indicated on the bar chart.

Table 1: Yield of cellular protein recovered from the transfections with MF, DD and PP. Also shown are the absolute values of luciferase activity for the control transfections with MF, PP and DD and P values shown are for comparisons with MF.

	Protein µg/ml	t-test vs MF	LU/µg	t-test vs MF
MF	1.0±0.1		20.9±3.2	
DD	0.5±0.1	$P=2.6x10^{-07}$	39.4±10.2	P=0.026
PP	0.7±0.1	$P=1.6x10^{-05}$	12.3±5.2	P=0.006