Geldanamycin Restores a Defective Heat Shock Response in Vivo*

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Induced expression of heat shock proteins (Hsps) plays a central role in promoting cellular survival after environmental and physiological stress. We have previously shown that scrapie-infected mouse neuroblastoma (ScN2a) cells fail to induce the expression of Hsp72 and Hsp28 after various stress conditions. Here we present evidence that this impaired stress response is due to an altered regulation of HSF1 activity. Upon stress in ScN2a cells, HSF1 was converted into hyperphosphorylated trimers but failed to acquire transactivation competence. A kinetic analysis of HSF1 activation revealed that in ScN2a cells trimer formation after stress was efficient, but disassembly of trimers proceeded much faster than in the uninfected cell line. Geldanamycin, a Hsp90-binding drug, significantly delayed disassembly of HSF1 trimers after a heat shock and restored stress-induced expression of Hsp72 in ScN2a cells. Heat-induced Hsp72 expression required geldanamycin to be present; following removal of the drug ScN2a cells again lost their ability to mount a stress response. Thus, our studies show that a defective stress response can be pharmacologically restored and suggest that the HSF1 deactivation pathway may play an important role in the regulation of Hsp expression.

Stress response mechanisms are essential for the maintenance of cellular integrity and viability. Diverse stress conditions converge to enhance the synthesis of heat shock proteins (Hsps),1 many of which function as molecular chaperones in protein biosynthesis, folding, assembly, translocation, and degradation (1–6). In addition to preventing proteotoxic damage, Hsps also appear to be involved in antiapoptotic pathways (7–9). In animal models as well as in vertebrate cell culture models, overexpression of specific Hsps decreases cytotoxicity induced by different environmental stress conditions, including thermal and oxidative challenges, ischemia, or exposure to toxic chemicals (reviewed in Refs. 10–12).

In the nervous system, Hsps are thought to play an important role in a variety of pathophysiological states, including neurodegenerative diseases, cerebral ischemia, epilepsy, and trauma. Aged cells exhibit a decreased ability to induce Hsp72 in response to stress (13). This compromised induction of Hsp expression may reflect an adaptive cellular response. Aged cells and cells affected by age-related diseases (e.g. Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, and prion diseases) are characterized by an increased amount of abnormally folded proteins. If the stress response were functional in these cells, Hsp72 would be expressed permanently at high levels and may interfere with the apoptotic program and thereby with the elimination of diseased cells (14). Notably, the threshold for stress induction is significantly raised in cells exposed to a prolonged (moderate) stress (Ref. 15 and references therein).

At the molecular level, the different physiological and environmental stressors are integrated through the activation of a single transcription factor, the heat shock transcription factor 1 (HSF1). In unstressed mammalian cells, HSF1 exists in an inert nontrimeric form. In response to stress HSF1 assembles into homotrimers, binds to specific heat shock element (HSE) sequences present within inducible Hsp genes, and becomes hyperphosphorylated (reviewed in Refs. 16–19). Acquisition of HSE binding activity by trimer formation, however, is not sufficient to render HSF1 transactivation-competent (20–25), suggesting that factor activation occurs at least in two steps: trimerization and acquisition of DNA binding activity followed by induction of transcriptional competence. HSF1 trimer assembly is negatively regulated by the molecular chaperone Hsp90 (26–28) that may interact with the three hydrophobic repeat sequences of the transcription factor (29). Repression of the last step of HSF1 activation that ultimately leads to transactivation-competent factor appears to be mediated through a regulatory domain defined by Green et al. (30) and Zuo et al. (24). Although this final regulation step is not yet understood, it appears to involve chaperone interactions (28, 31, 32) as well as phosphorylation/dephosphorylation events (33–38).

Scrapie-infected mouse neuroblastoma (ScN2a) cells have proved useful to study certain aspects of prion diseases in cell culture. In contrast to uninfected N2a cells ScN2a cells propagate mouse prions and accumulate intracellular protein aggregates composed of protease K (PK)-resistant PrPSc. We previously reported that stress-induced expression of Hsp72 and Hsp28 is impaired in a ScN2a cell line, whereas uninfected N2a cells can mount an effective stress response (39). In this study, we show that HSF1 trimers formed in ScN2a cells after stress are rapidly disassembled and lack transactivation competence. We demonstrate that the Hsp90-binding drug geldanamycin corrects the defective regulation of HSF1 activity after heat stress in ScN2a cells.

EXPERIMENTAL PROCEDURES

Recombinant Plasmid DNAs— Expression constructs for wtHSF1, ΔHSF1, HSE-Luc, β-galactosidase, and GRE-Luc were described previ-
ously (24, 40, 41). The luciferase reporter vector pGL3 control was purchased from Promega.

**Antibodies**—Polyclonal anti-PrP antiserum A4 was raised against recombinant full-length PrP that was expressed and purified from baculovirus-infected Sf9 cells (42). The mouse monoclonal antibody specific for inducible Hsp72 (C92) has been characterized previously (43). The mouse monoclonal antibody N27 (clone N27F3-4) that identifies both the inducible and the constitutive form of Hsp70, the rabbit polyclonal antibody against Hsp90 (clone AC88) were purchased from StressGen, and the mouse monoclonal antibody specific for p23 (clone JJS-3) was purchased from Affinity Bioreagents.

**Cell Culture**—N2a and ScN2a cells were grown in minimum essential Eagle’s medium supplemented with antibiotics (1 unit/ml penicillin G and 1 mg/ml streptomycin) and 10% fetal calf serum. N2a cells are an immortalized neuroblastoma cell line (ATCC number CCl 131). ScN2a cells were established by infecting N2a cells with an enriched preparation of PrPSc isolated from scrapie-infected mouse brains (44). The mouse monoclonal antibody specific for inducible anti-PrPSc (Fig. 1A) was purchased from Amersham Pharmacia Biotech. Antibodies against Hsp73, Hsp90, and p23. As shown in Fig. 2A, PK-resistant, and infectious PrPSc, the same cells were electrophoresed for 2.5 h at 150 V, dried, and exposed for autoradiography. Indirect Immunofluorescence—N2a and ScN2a cells were grown on glass coverslips and fixed by immersion in cold methanol for 10 min. The fixed cells were incubated with the anti-Hsp72 antibody C92 (dilution of 1:50) or with the monoclonal anti-Hsp90 antibody (dilution of 1:100) that contains 1% bovine serum albumin for 45 min at 37°C. After extensive washing with PBS, the membranes were incubated with rhodamine-conjugated anti-mouse antibodies (Dianoiva) (dilution 1:200) followed at 37°C for 30 min. The washed coverslips were mounted on glass slides and examined by phase contrast and fluorescence microscopy.

**RESULTS**

Scrapie-infected N2a Cells Do Not Express Inducible Hsp72 and Hsp28 after Heat Shock—ScN2a cells provide a system to study the accumulation of infectious PrPSc in cell culture (48–50). In addition to productive propagation of detergent-insoluble, PK-resistant, and infectious PrPSc, the same cells were described by us to be impaired in the stress-induced expression of Hsp72 and Hsp28 (39). It is important to note that the deregulated stress response in ScN2a cells was restricted to the two most highly inducible stress proteins Hsp72 and Hsp28; exposure of ScN2a cells to various stress conditions resulted in increased expression of Hsp73, Hsp90, grp75, grp78(BiP), and grp94, as determined by two-dimensional gel electrophoresis (see Fig. 2 in Ref. 39). The deregulated expression of Hsp72 in ScN2a cells is again illustrated by the experiment shown in Fig. 1. Three independently established ScN2a cell lines were analyzed with respect to propagation of PrPSc and expression of Hsp72 subsequent to heat treatment. In contrast to uninfected N2a cells, all of the three ScN2a cell lines harbored significant amounts of detergent-insoluble (Fig. 1A, –PK) and PK-resistant PrPSc (Fig. 1A, +PK). After thermal challenge (44°C, 20 min), only N2a cells but none of the three ScN2a cell lines expressed inducible Hsp72 (Fig. 1A, a-Hsp72) and Hsp28 (data not shown). Thus, at least three different ScN2a cell lines showed derepression of the two inducible stress proteins Hsp72 and Hsp28.

To rule out that expression levels of proteins that are involved in the regulation of HSF1 activity were different in N2a and ScN2a cells, we performed a Western blot analysis with antibodies against Hsp73, Hsp90, and p23. As shown in Fig.
cells under all heat shock conditions tested (Fig. 2A). Initially, we were puzzled about the activation of HSE-Luc in ScN2a cells, especially under more severe heat shock conditions. This activation was most likely due to the presence of trimeric HSF1 that lacks the competence to transactivate the endogenous Hsp72 promoter but induces a weak transcription from the transfected HSE-Luc construct. Support for this notion was derived from experiments in which N2a and ScN2a cells were co-transfected with HSE-Luc and an expression construct for either wild type (wt)HSF1 or a HSF1 mutant denoted ΔHSEF. ΔHSF contains a deletion in the regulatory domain (Δ202–316) and is constitutively active (24). Co-transfection of HSE-Luc and wtHSF resulted in weak activation of HSE-Luc in both N2 and ScN2a cells (Fig. 2B, wtHSF). The luciferase activity in wtHSF-expressing ScN2a cells was comparable with the activity in ScN2a cells subjected to a severe heat shock (Fig. 2A). Of note, overexpression of wtHSF generates trimeric HSF1, which, however, is not sufficient to induce expression from the endogenous Hsp72 promoter (Fig. 3B). We therefore concluded that the weak activation of HSE-Luc in ScN2a cells after a severe heat shock resulted from trimeric HSF1, which is incompetent to transactivate the endogenous Hsp72 promoter. Indeed, HSF1 trimers were generated in ScN2a cells after a heat stress, as determined by electrophoretic mobility shift assays (see Fig. 5A).

Co-transfection of the ΔHSF construct with HSE-Luc resulted in an extremely high luciferase activity in N2a and ScN2a cells even without applying a heat shock. (Fig. 2B, ΔHSF). Notably, overexpression of ΔHSF was sufficient to induce expression of Hsp72 in ScN2a cells (Fig. 3B). To verify that the different levels of luciferase activity measured in our stress activation assay reflected stress-regulated promoter activity and not differences in transfection efficiencies or instability of luciferase, two different control experiments were performed. In the analysis shown in Fig. 2C, a plasmid encoding β-galactosidase under control of a cytomegalovirus promoter was co-transfected. Similar levels of β-galactosidase in N2a and ScN2a cells indicated similar transfection efficiencies. In addition, N2a and ScN2a cells were transfected in parallel with a plasmid coding for luciferase under the control of a constitutive SV40 promoter and treated exactly like the cells analyzed in Fig. 2A. The results of this experiment demonstrated that activity of luciferase was not different in the ScN2a background (Fig. 2D).

In conclusion, these experiments indicated that ScN2a cells are significantly impaired to induce expression from Hsp promoters using their own HSF1. It appears therefore that the virtual inability of ScN2a cells to induce expression of Hsp72 and Hsp28 during stress is caused by a defect in the activation of endogenous HSF1.

Geldanamycin Restores the Deregulated Heat Shock Response in ScN2a Cells—To get insight into the regulation of HSF1 activity in ScN2a cells, we tried to modulate the heat shock response pharmacologically. Geldanamycin, a benzoquinone ansamycin, interacts with Hsp90 and interferes with Hsp90-dependent signal transduction pathways (51–59). Recently, Hsp90 has been described to be involved in the negative regulation of HSF1 activity (26–28). Consequently, we examined the effect of geldanamycin on the expression of Hsp72 in N2a and ScN2a cells. The cells were grown on glass coverslips at 37 °C and then subjected to treatment by heat (44 °C for 20 min), geldanamycin (5 μM for 3 h), or both. In the latter case the heat shock was applied 2 h after geldanamycin was added. After further incubation at 37 °C for 16 h in fresh medium, expression of Hsp72 was detected by indirect immunofluorescence. The results showed that after thermal stimulation the
entire population of N2a cells expressed Hsp72, whereas no ScN2a cell stained positive for Hsp72 under the same conditions. Interestingly, in N2a cells the effect of geldanamycin on the expression of Hsp72 was restricted to a small subpopulation of cells (Fig. 3A, N2a, GA). A similar discrete pattern of Hsp72 expression was detected in ScN2a cells subjected to combined thermal and geldanamycin treatment (Fig. 3A, ScN2a, GA/H11001/44°C). This restricted expression of Hsp72 might reflect a cell cycle-dependent action of geldanamycin, which has been shown to interfere with the expression of different cyclins (60–63). Unfortunately, it was not possible to verify this speculation because the ScN2a cells did not survive geldanamycin and heat treatment after synchronization. In summary, the ScN2a cell line was found to be a homogenous population with respect to the impaired stress response. Heat stress-inducible expression of Hsp72 could be restored by short term incubation of cells with the Hsp90-binding drug geldanamycin. Expression analysis indicated that the relative amount of Hsp90 was similar in N2a and ScN2a cells. (Fig. 1B). To investigate possible differences in the subcellular localization of Hsp90, we performed indirect immunofluorescence experiments. In N2a cells a diffuse cytosolic Hsp90 staining was observed, and we could not find differences between untreated and geldanamycin-treated N2a cells (Fig. 3C, N2a). In ScN2a cells, however, Hsp90 distribution was predominantly inhomogenous, reminiscent of aggresomal compartmentalization. Interestingly, after geldanamycin treatment this discrete pattern of accumulated Hsp90 disappeared, now resembling the uniform pattern found in N2a cells (Fig. 3C, ScN2a). Notably, in this experiment geldanamycin was used in the same concentration that restored the stress-induced expression of Hsp72. Additional proof of an impaired function of Hsp90 in ScN2a cells was obtained by analyzing the activation pathway of the glucocorticoid receptor, which is regulated similarly to HSF1 (65). After transfection of a GRE-Luc reporter construct, stimulation of the endogenous glucocorticoid receptor by dexamethasone resulted in a more than 55-fold luciferase activity in N2a cells, whereas in ScN2a cells luciferase activity was increased only 9-fold (Fig. 3D). Of note, there were no differences in the levels of endogenous glucocorticoid receptor between the two cell lines (data not shown).

Overexpression of ΔHSF and wtHSF Overcomes the Impaired Activation of Endogenous HSF1 in ScN2a Cells—The experiments described above supported the hypothesis that HSF1 regulation is defective in ScN2a cells. Moreover, they showed that the defect could be corrected by exposure to geldanamycin.

**FIG. 2. Activation of an exogenous HSE promoter by heat and ΔHSF.** A and C, N2a and ScN2a cells were transiently co-transfected with a reporter plasmid encoding luciferase under the control of a HSE promoter (HSE-Luc) and a plasmid encoding β-galactosidase under the control of a cytomegalovirus promoter. 24 h after transfection, the cells were subjected to a heat shock (44°C) for the time indicated, returned to 37°C, and harvested after additional 16 h. A, luciferase activity in the cell extracts was quantified as described under “Experimental Procedures.” Luciferase activity in cells cultivated constantly at 37°C was set as 1. B, N2a and ScN2a cells were transiently co-transfected with HSE-luc and a plasmid encoding either wtHSF1 or a constitutively active HSF1 mutant (ΔHSF) under the control of a cytomegalovirus promoter. After transfection cells were cultivated at 37°C for 24 h; luciferase activity was measured and graphically presented as described for A. C, β-galactosidase activity in the same extracts as shown for A was quantified as described under “Experimental Procedures.” D, N2a and ScN2a cells were transfected with a plasmid coding for luciferase under the control of a constitutive SV40 promoter and treated in parallel exactly like the cells analyzed for A. All graphs represent the quantitative analysis of at least three independently conducted experiments.
To further probe the defect in the stress response in ScN2a cells, we transiently transfected expression constructs for either constitutively active HSF or wtHSF. Hsp72 expression was detected by indirect immunofluorescence in unstressed cells or in cells exposed to a heat shock (44 °C for 20 min). In agreement with the co-transfection experiments discussed before, ScN2a cells transfected with the HSF construct expressed Hsp72 without stress (Fig. 3B, HSF, 37 °C). Subsequent to the stress treatment, the cells were further cultivated in fresh cell culture medium and analyzed after an additional 16 h at 37 °C. No further increase in Hsp72-positive cells was seen, corroborating our previous results that untransfected ScN2a cells could not mount a heat shock response. No Hsp72 expression was observed in unstressed ScN2a cells overexpressing exogenous wild type HSF (Fig. 3B, wt HSF, 37 °C). Taken together, we have established three ways of inducing Hsp72 expression in ScN2a cells. Overexpression of HSF resulted in a stress-independent expression of Hsp72, and overexpression of wtHSF or treatment with the Hsp90-binding drug geldanamycin restored the heat stress inducibility of Hsp72 expression.

Geldanamycin Delays Disassembly of HSF1 Trimers after Heat Shock—To determine the mechanism by which geldanamycin restores the defective heat response, we analyzed transcription of the Hsp72-specific mRNA by Northern blotting. In N2a cells pretreatment with geldanamycin significantly prolonged Hsp72 transcription after a heat shock (Fig. 4A). After heat shock Hsp72-specific mRNA rapidly appeared in the cytosol and could be detected for about 2 h. If geldanamycin was applied 2 h prior to the heat shock, Hsp72-specific mRNA could now be detected for up to 8 h after the thermal stimulus (Fig. 4A, hs + GA). In ScN2a cells neither heat shock nor geldanamycin alone was sufficient to induce transcription; however, a combination of both stimuli resulted in the appearance of Hsp72-specific mRNA, corroborating our analysis of Hsp72 expression by immunofluorescence (Fig. 3A, ScN2a, GA + 44 °C). Thus, the Northern blot analysis was consistent with the results on the expression of Hsp72 obtained by immunofluorescence and indicated that geldanamycin prolonged transcription of Hsp72-specific mRNA in heat-treated N2a cells.
We next examined HSF1 trimerization and trimer disassembly in N2a and ScN2a cells exposed to a heat shock in the presence or absence of geldanamycin. The electrophoretic mobility shift assay is based on the HSE binding activity specific for HSF1 trimers. We had previously shown that the relative levels of HSF1 and the formation of hyperphosphorylated HSF1 trimers after stress was comparable in N2a and ScN2a cells (see Fig. 3 in Ref. 39). To extend these earlier studies, N2a and ScN2a cells were treated at 44 °C for 20 min, returned to 37 °C, and examined thereafter at various time points. Formation of HSF1 trimers was detected 20 min after the heat shock in both N2a and ScN2a cells (Fig. 5A, lane 2). However, whereas in N2a cells HSF1 trimers were still present 60 min after the heat shock, they had already been disassembled in ScN2a cells at this time point (Fig. 5A, lane 4). In the presence of geldanamycin, disassembly of HSF1 trimers was significantly delayed, and the kinetics of HSE binding activity were now essentially the same in N2a and ScN2a cells. In presence of geldanamycin, however, trimer disassembly was now essentially the same in N2a and ScN2a cells (data not shown).

**DISCUSSION**

Induced expression of stress proteins provides a powerful defense mechanism to ensure cellular survival after environmental and physiological stress conditions. HSF1, the transcription factor that mediates the stress response is known; however, some aspects of its regulation remain to be elucidated. The studies reported here indicate that the defective stress response in scrapie-infected neuroblastoma (ScN2a) cells is due to an impairment of HSF1 function and can be restored by the Hsp90-binding drug geldanamycin.

**Geldanamycin Corrects a Defect in HSF1 Regulation**—ScN2a cells are characterized by a defective stress-induced expression of Hsp72 and Hsp28. Inducibility of other stress-regulated Hsps as well as constitutive Hsps levels are comparable with those of uninfected N2a cells. In this study, the underlying mechanism of the impaired heat-induced expression of Hsp72 in ScN2a cells could be attributed to an impaired activation of endogenous HSF1. Upon heat treatment trimeric hyperphosphorylated HSF1 was formed in ScN2a cells; however, HSF1 trimerization and HSE binding was not sufficient to induce expression of Hsp72. Interestingly, in transfection experiments with a heat-regulated reporter construct (HSE-Luc), we observed a weak activation of the promoter. Co-transfection experiments with a constitutively active mutant of HSF1 (AHSF1) very efficiently induced expression of Hsp72, showing that the endogenous Hsp72 gene in ScN2a cells has a functional promoter. This is in contrast to some cell lines described earlier (20, 64–66) in which a chromatin-mediated effect accounted for an impaired Hsp72 expression after stress (67, 68).

Based on the finding that in ScN2a cells HSF1 activity after stress was sufficient to induce trimer formation yet insufficient to promote transactivation competence, we directed our attention to the activation/deactivation pathway of HSF1.

**Initial in vitro studies** established a physical interaction between Hsp90 and HSF1 (69, 70). More recently, Hsp90 was shown to act as a repressor of HSF1 trimerization in an *in vitro* HeLa cell extract system (27) and in the *Xenopus* oocyte model system (26, 28). To modulate Hsp90 function in ScN2a cells we used geldanamycin that has been described previously to promote formation of HSF1 trimers (27, 71, 72). After a short term treatment with geldanamycin, heat-induced expression of Hsp72 was restored in a subpopulation of ScN2a cells. This recovery was paralleled by a striking change in the subcellular distribution of Hsp90. Whereas in N2a cells Hsp90 was distributed homogeneously in the cytosol, in ScN2a cells Hsp90 was found predominantly in aggresome-like structures, which disappeared after geldanamycin treatment. These findings suggest a deregulation of Hsp90 function in ScN2a cells. Further support for a dysfunction of Hsp90 in ScN2a cells was provided by the analysis of another Hsp90-regulated pathway, the activation of the glucocorticoid receptor. In contrast to N2a cells, dexamethasone-induced activation of the glucocorticoid receptor was significantly suppressed in ScN2a cells.

The functional HSE binding assay indicated a possible molecular basis for the effect of geldanamycin on the heat-induced expression of Hsp72. Whereas in both N2a and ScN2a cells HSF1 gained DNA binding competence rapidly after a heat shock, HSF1 trimer disassembly occurred much faster in ScN2a cells. In presence of geldanamycin, however, trimer disassembly was delayed, and the kinetics of HSF1 trimer disassembly were now very similar in N2a and ScN2a cells. Notably, the relative amount of HSF1 trimers formed after a heat shock in ScN2a cells was not enhanced by geldanamycin.

**Implications for the Stress Response**—The study described above is, to our knowledge, the first demonstration that a defective heat shock response can be restored *in vivo* by a
specific modulator of Hsp90 function. It is important to note that Hsp72 expression was absent in ScN2a cells under all heat shock conditions tested as well as under other stressful conditions causing proteotoxic damage such as oxidative stress and exposure to sodium arsenite or amino acid analogs. Thus, the deregulation of HSF1 activity in ScN2a cells could not be compensated simply by increasing the severity of the stress. Our findings support previous reports indicating that Hsp90 is a key regulator of HSF1 activity and provide new insights into the stress-regulated expression of chaperones.

Two different models of how geldanamycin modulates regulation of HSF1 activity in ScN2a cells are supported by our results (Fig. 6). It has been suggested previously that the activated HSF1 complex resembles steroid receptor complexes
and that HSF1 activity is regulated similarly to steroid receptors (70). In a recent report Bharadwaj et al. (28) supported this hypothesis by showing that different constituents of the Hsp90 chaperone complex such as p23 and FKBP52 participate in the regulation of HSF1 activity in Xenopus oocytes. Thus, it is conceivable that HSF1 trimers in Scn2a cells lack transactivation competence because the HSF1-Hsp90 chaperone complex has a different composition. Binding of geldanamycin to the HSF1-Hsp90 chaperone complex might induce dissociation of regulators from this complex that suppress the transcriptional competence of trimeric HSF1. Alternatively, formation of HSF1 trimers after stress might be qualitatively the same in both cell lines, but the faster deactivation of HSF1 trimers in Scn2a cells interferes with transactivation competence. The fact that geldanamycin did not increase the relative amount of HSF1 trimers in Scn2a cells after stress but rather prolonged their lifetime might favor the kinetic model. The results obtained in transfection experiments with wtHSF1 are in line with both models discussed above. Overexpression of wtHSF1 could have provided a sufficient amount of trimeric HSF1 to compensate for the accelerated deactivation of HSF1 trimers in Scn2a cells. Alternatively, excessive HSF1 might absorb negative regulators, thereby shifting the equilibrium toward the transactivation competent form of HSF1. In summary, our data support a pivotal role of Hsp90 or a Hsp90 chaperone complex in the regulation of HSF1 activity (Fig. 6) and emphasize that the deactivation of HSF1 may play an important role in regulating Hsp72 expression.

Acknowledgments—We are grateful to F. Ulrich Hartl for stimulating discussions and continuous support. We thank William J. Welch for providing the anti-Hsp72 antibody C92, Richard I. Morimoto for the DNA probe specific for Hsp72 mRNA, and Theo Rein for the GRE-Luc plasmid.

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