Chloramphenicol Acetyltransferase Expression as a Sensor for Fusion Activity

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Membrane fusion is an indispensable biological process that still needs additional study at the molecular level. Fusogenic viral envelope proteins that mediate viral entry into host cells are a useful tool to study the molecular biology of this process, and precise quantification of fusion events is needed for this purpose. A classical method, with limited precision, however, is to monitor formation of polycaryons as a result of cell-cell fusion by light microscopy (11). More sensitive are gene activation assays, and several modifications of this approach have been developed, most of them using β-galactosidase as a reporter (1,2,7,9).

As an alternative approach, we describe here a new simplified assay using induction of the chloramphenicol acetyltransferase gene (CAT) as a sensitive indicator of cellular fusion events. In this system, two stably transfected human astrocytoma cell lines are used; both cell lines were derived from U373 MG (ATCC, Rockville, MD, USA). One cell line encodes a chimeric protein of the GAL4 DNA binding and herpes simplex virus (HSV) VP16 activation domains (including a GAL4 nuclear localization sequence) under the control of the simian virus 40 (SV40) early promotor. The other encodes five consensus GAL4 binding sites and the adenoviral E1b promotor upstream of the CAT gene. Fusion between these cell lines leads to transactivation of the reporter gene and is quantified by determination of CAT activity.

Cell lines were established following liposome-mediated co-transfection (Life Technologies, Karlsruhe, Germany) of subconfluent U373 MG cells 24 h after seeding (9 cm petri dishes), using 10 µg of constructs pM3-VP16 or pG5CAT (both from CLONTECH Laboratories, Heidelberg, Germany) and 2 µg of pIG-1 (8), a plasmid coding for neomycin resistance. Two days after transfection, selection was initiated with geneticin (G418) at 800 µg/mL. Resistant clones emerging after 2–3 weeks were isolated and, in the case of U373 MG, transfected with the GAL4/VP16 gene (U373-VP16). Expression was tested by western blotting with specific Polyclonal Antibody (CLONTECH Laboratories). Clones stably transfected with the CAT gene (U373-CAT) were identified by cocultivation with U373-VP16 cells followed by polyethylene glycol (PEG)- or virus-mediated fusion. For this purpose, trypsinized cells were mixed at a ratio of 1:1 and seeded into 35-mm-diameter, 6-well petri plates to obtain a subconfluent monolayer the next day (10^6 cells). For PEG-mediated fusion, the

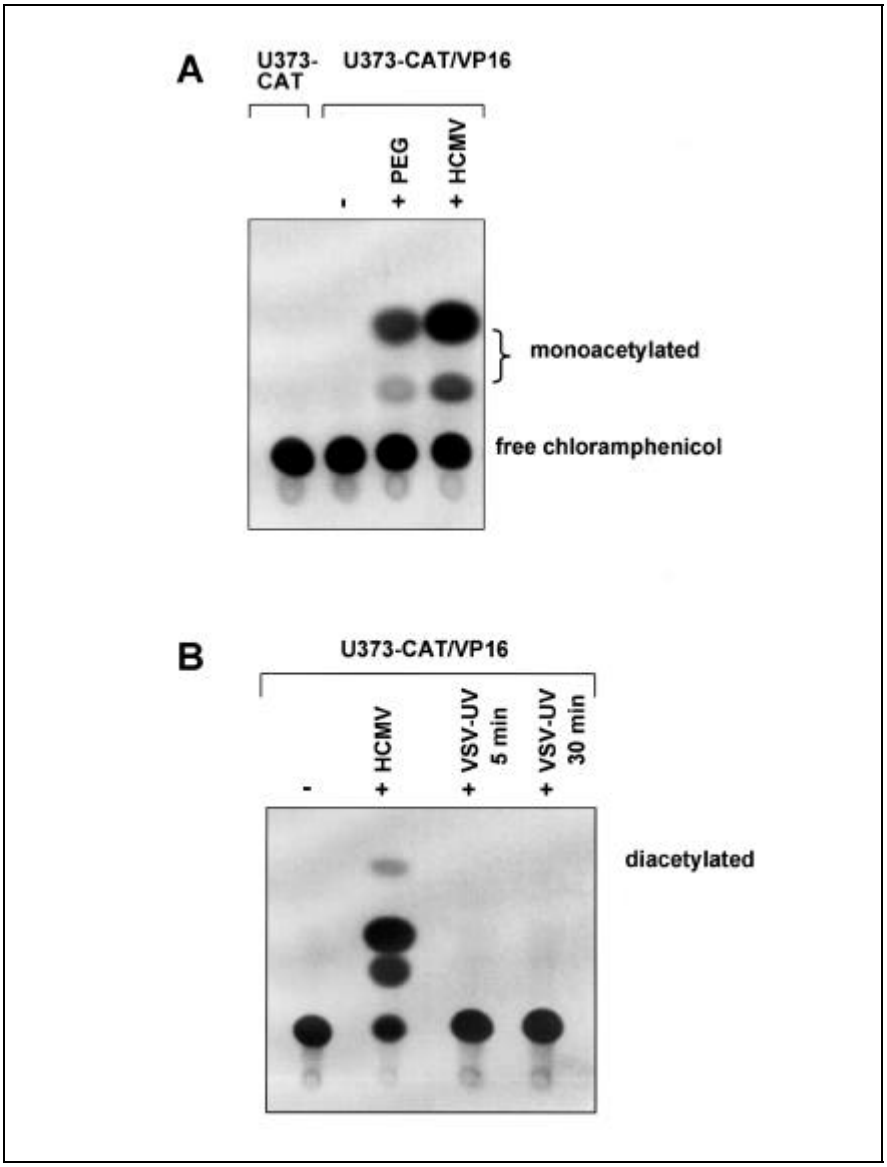

Figure 1. Reporter gene expression mediated by PEG- or virus-induced fusion of cocultivated U373-CAT and U373-VP16 cells. (A) U373-CAT cells or U373-CAT and U373-VP16 cells (ratio 1:1) were left untreated or fused either by treatment with 50% PEG solution in DMEM for 2 min at room temperature or by infection with HCMV at an MOI of 1.0 (final vol = 0.5 mL). Twenty-four hours after PEG treatment or p.i., cells (10^6 cells) were lysed in 150 µL lysis buffer, and CAT activity was determined using 75 µL cell lysate. Acetylated products were separated by TLC and autoradiographed. (B) U373-CAT and U373-VP16 cells (ratio 1:1) were left untreated or fused by infection with HCMV at an MOI of 1.0. UV irradiation of VSV was performed with a Sylvania F6T5 germicidal lamp at 254 nm for 5 or 30 min, respectively. U373-CAT/VP16 cells (ratio 1:1) were infected with VSV-UV at an MOI of 1.0. Twenty-four hour p.i. cells were lysed (150 µL lysis buffer per 10^6 cells) and analyzed for CAT activity.

culture medium was aspirated, and the cells were overlaid with 1 mL of a 50% (wt/vol) PEG 8000 (Sigma, Deisenhofen, Germany) solution in Dulbecco's modified Eagle medium (DMEM) adjusted to 37°C. After 2 min, the PEG solution was removed, and the monolayer was thoroughly washed three times with phosphate-buffered saline (PBS) before the addition of culture medium (DMEM supplemented with 10% fetal calf serum [FCS]) and incubation at 37°C.

For virus-mediated fusion, cell-free human cytomegalovirus (HCMV) strain AD 169 at a multiplicity of infection (MOI) of 1.0 was used. HCMV was grown and titrated in cultures of human foreskin fibroblasts (HF) as previously described (3). Twenty-four hours after PEG treatment or virus infection (p.i.), respectively, cell monolayers were solubilized in 150 µL of 1× lysis buffer (Promega, Mannheim, Germany) and analyzed for CAT enzyme activity using a thin-layer chromatography (TLC) assay according to a standard protocol (4). Under these conditions, PEG- or HCMV-mediated fusion with various U373-CAT clones resulted in comparable induction of CAT activity. Measurable enzyme activity was obtained neither for U373-CAT cells alone nor for cocultivated U373-CAT and U373-VP16 cells (Figure 1A). It is noteworthy that essentially similar results were obtained when the U373-VP16 cell line was replaced in the assay by a VP16 cell line derived from HeLa cells (data not shown).

For a further control, the assay was performed with vesicular stomatitis virus (VSV), which enters host cells (unlike HCMV) by endocytosis. To preclude cytopathic host cell shut-off, UV-inactivated VSV (VSV-UV) was used (10). Figure 1B shows that only background CAT activity was obtained after VSV-UV infection.

Under the conditions used, transactivation of the CAT gene by HCMV infection was most likely effected by virion envelope glycoproteins of the input virus (fusion from without) (6) because biosynthesis of viral glycoproteins in infected cells is a late event during the 72-h infectious cycle. To further exclude that HCMV-mediated fusion was not initiated by transactivating HCMV immediate early proteins (5), U373-CAT cells were infected with HCMV at an MOI of 1.0 before the determination of CAT activity (Figure 2A, lane 1). In this case, signals equaled mostly background levels that were defined as CAT activity in the absence of the activation of GAL 4 sites or that resulted from cocultivation of U373-CAT and U373-VP16 cells (Figure 2A, lane 2).

To examine reliability of the assay for quantification of fusion events, subconfluent monolayers of seeded U373-

CAT and U373-VP16 cells were infected with HCMV at an MOI of 0.006–3.0. The virus inoculum (0.5 mL in culture medium) was aspirated at 1 h p.i., fresh growth medium (DMEM 2% FCS) was added, and the plates were incubated at 37°C. Twenty-four hour p.i. monolayers were lysed and analyzed for CAT enzyme activity. Quantification of the radioactivity was performed with a Fujifilm BAS-1000 Bio-Imaging Analyzer using the TINA™ software (both from Raytest, Straubenhardt, Germany). Figure 2, A and B, show increasing amounts of the input virus correlated with an increase of fusion events and consequently of CAT activity. Maximal CAT activity was reached at an MOI of 2.0; on the other hand, it was still detectable at an MOI of 0.06 when syncytia were hardly observed by light microscopy. The decrease at higher MOI possibly resulted from cytotoxicity of the viral inoculum as a consequence of an excessive formation of syncytia.

In conclusion, our results demonstrate that transactivation of the CAT gene by binding of GAL4-HSV-VP16 to GAL4 DNA-binding sites represents a sensitive and reliable method to measure cell-cell fusion by quantification of CAT reporter gene expression using a Bio-Imaging analyzer. In principle, quantification of CAT activity could be performed also by a nonradioactive CAT enzyme-linked immunosorbent assay (ELISA) technique using anti-CAT precoated microplates.

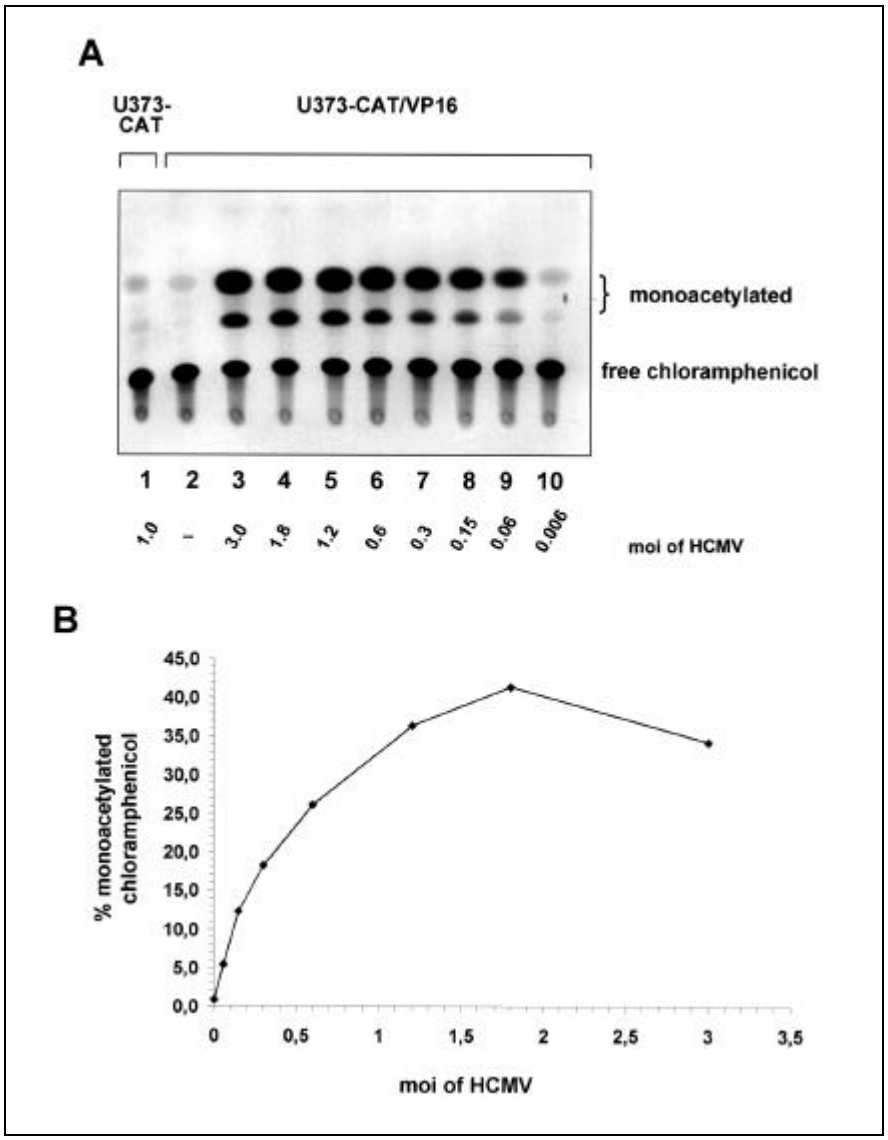

Figure 2. Quantification of virus-induced CAT reporter gene expression. (A) Autoradiogram of TLC analysis of CAT activity after HCMV-mediated fusion of cocultivated U373-CAT and U373-VP16 cells. Lane 1, U373-CAT cells infected with HCMV at a MOI of 1.0; lane 2, cocultivated U373-CAT cells and U373-VP16 cells (ratio 1:1) untreated; lanes 3–10, cocultivated U373-CAT and U373-VP16 infected with HCMV at MOI of 3.0–0.006. Twenty-four hour p.i. cells were lysed and analyzed for CAT activity. CAT assays were performed with 18 µL of the cell lysates (150 µL lysis buffer per 10^6 cells). (B) Correlation between HCMV-mediated fusion events at various input MOI (abscissa) in cocultivated U373-CAT /U373-VP16 cells and induced CAT activity (ordinate) that is expressed as relative percentage monoacetylated chloramphenicol as estimated by Bio-Imaging analyzer with the TINA software.

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Infecting Larval Arthropods with a Chimeric, Double Subgenomic Sindbis Virus Vector to Express Genes of Interest

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In a previous communication, we described how a double subgenomic Sindbis (dsSIN) expression system may be used to express green fluorescent protein (GFP) in mosquitoes, and we suggested that GFP expression might be useful as a reporter for mosquito transgenesis. This has subsequently been achieved (4). In our initial report, we infected mosquitoes by intrathoracic inoculation (5) because the available TE/3′2J dsSIN vectors were inefficient at orally infecting mosquitoes. Penetration of the cuticle during inoculation may have undesirable physiological consequences (e.g., increased expression of immune peptides).

Recently, a chimeric SIN virus has been engineered, designated MRE-1001, with improved oral infectivity for *Aedes aegypti* mosquitoes (6). The structural genes of a Malaysian strain of SIN, MRE-16, which efficiently infects *Ae. aegypti*, has recently been substituted into pTE/3′2J/GFP (2), yielding a double subgenomic vector MRE/3′2J/GFP (3). The leader sequence and 3′ nontranslated region of the 26S mRNA are of TE/3′2J origin. In this report, we describe a novel method by which larval mosquitoes may be infected with an MRE/3′2J virus expressing GFP. Arboviruses do not usually infect immature insect stages, and thus this report provides a technique by which gene expression may be achieved early during mosquito development and po-