Establishment of an autocatalytic conditional mammalian system for expression of stringently regulated genes

Iris Dotan, Idit Kopatz, Tova Naiman, Boris Perelman, Naomi Dafni and Dan Canaani*

Department of Biochemistry, Tel Aviv University, Ramat Aviv 69978, Israel

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Analysis of the biological roles and regulation of isolated genes in mammalian cells often requires inducible expression systems, particularly when the studied gene product is essential for viability and/or cytotoxic upon overexpression. Notable among these systems is the LAP267-regulated expression system developed by the groups of A. Levine and T. Shenk (1). It is based on converting the *lacI* repressor into a mammalian transcriptional activator protein (LAP267) by inserting the herpes simplex virus VP16 transactivator domain after amino acid 267 of the *lac* repressor protein, and including a nuclear localization signal from SV40 large tumor antigen. The insertion generates a temperature-sensitive transactivator which induces expression from an enhancerless simian virus 40 early promoter linked to *lac* operators at 32°C, but not at 37 or 39.5°C. LAP267 activity can be rescued at the nonpermissive temperatures by IPTG addition.

Previously, the LAP267 gene was transcribed off the constitutive human β -actin or cytomegalovirus IE promoter (1). The constitutive production of the transactivator might represent a drawback. In the initial phase of the generation of stable transfectants, as well as in transient transfection, there is a massive entry of DNA molecules into the fraction of competent cells among the recipient population. It is possible therefore that transfection of a toxic gene under the inducible promoter into constitutively LAP267-producing cells is accompanied, prior to integration, by an inducer-independent expression (leak) which eliminates those clones from the population of stable transformants obtained. One way to circumvent this potential problem would be to engineer the transactivator itself under an inducible transcriptional control, such that only its basal level is expressed in the cells prior to induction.

We now report the generation and characterization of mouse NIH 3T3 cell lines expressing the LAP267 transcriptional activator conditionally from the IPTG inducible LAP267-responsive L7 promoter (ILAP267). We compare the conditional system to the former (1) constitutive one (CLAP267) and demonstrate that the autocatalytic conditional expression system is superior to the former in its absolute level of activity, as well as its faster kinetics of induction.

A mouse fibroblast cell line characterized by a very low spontaneous rate of transformation (S. Aaronson's NIH 3T3/2) was transfected by a LAP267 gene construct transcribed off the human β -actin constitutive promoter—pH β ALAP267 (CLAP267),

together with a plasmid containing a dominant selectable marker gene—mpRSV*neo*. Following selection for G418 resistance (2), 41 cell clones were propagated for further analysis. Each was transiently transfected at 37°C by pRSVCAT as a reference for high level CAT expression, contrasted with transfection by pL7CAT—a CAT coding region transcribed from seven spaced *lac* operator domains localized upstream of an enhancerless SV40 early promoter (3), in the presence or absence of the IPTG inducer. Extracts harvested 43 h after transfection were assayed for CAT activity (4). The expression of CAT in the transfectants was LAP267-dependent and IPTG-dependent. Two independent clones—NIH 3T3/CLAP267-5 and -8, each containing a single copy of integrated LAP267 DNA, were identified as having both a very low basal activity at 37°C and high inducibility upon addition of 5 mM IPTG (data not shown). Induction upon IPTG addition almost approached the levels obtained by the strong RSV long terminal repeat promoter. It is noteworthy that in cases where the transfection efficiency of pL7CAT into the two recipient cells mentioned above was exceptionally high, a LAP267-dependent CAT synthesis was observed even in the absence of IPTG (data not shown).

To quantify the LAP267-dependent induction from stably integrated CAT target genes, the NIH 3T3/CLAP267-5 cell line was cotransfected by pL7CAT and pTK*hygro*, while selecting for resistance to hygromycin B. From the surviving colonies, 10 were isolated for further analysis. Most of the cell transfectants exhibited an appreciable IPTG-dependent CAT induction at 37°C. Quantitation of the data by PhosphorImager demonstrated that in the absence of IPTG, less than 2-fold induction was observed at 37°C. Upon addition of IPTG, CAT activity increased, reaching up to 560-fold in cell clone CLAP267-5/CAT-7.

As outlined above, constitutive production of the transactivator could potentially eliminate transfectants expressing toxic gene products. We therefore decided to make the synthesis of the transactivator itself inducible. Moreover, construction of the LAP267 coding region under the L7 promoter may generate an inducer-dependent autocatalytic system. For this purpose we substituted the CAT DNA fragment in the pL7CAT plasmid with the LAP267 coding region (by inserting the 1.6 kb *HindIII* LAP267 DNA fragment in between the *StuI* and *MscI* sites of

* To whom correspondence should be addressed

pL7), giving rise to pL7LAP267 in which the transcription of the transactivator is driven by a LAP267-responsive promoter. Generation of stable clones was initiated by cotransfection of NIH 3T3 cells with pL7LAP267 and pTK $hygro$ plasmid DNAs followed by selection for hygromycin B resistance. Screening of the $hygro^R$ colonies for LAP267 production, in the presence or absence of the IPTG inducer, was performed at 37°C by transient transfections with pL7CAT. CAT synthesis was shown to be LAP267—and IPTG—dependent. Surprisingly, most of the tested clones produced CAT levels which exceeded the ones produced by the two best constitutive LAP267 producers—NIH 3T3/CLAP267-5 and -8 (data not shown).

To examine whether the higher activity of LAP267 in the autocatalytic conditional system is also maintained when expression is from integrated CAT target gene constructs, stable transformants were established by cotransfecting pL7CAT with mpRSV neo DNAs into the high producer NIH 3T3/ILAP267-16 (under conditions similar to those used for generating CAT transfectants in the constitutive CLAP267 producer). Following selection of stable clones, six randomly selected transfectants were assayed for CAT production in the presence and absence of IPTG. The results identified a single clone, NIH 3T3/ILAP267-16/CAT-3 that was superior in CAT production relative to others in its group. CAT activity in that clone was compared to the activity obtained by the best clone among the constitutive LAP267 producers (CLAP267-5/CAT-7). A summary of the data from a representative experiment carried out at 39.5°C is shown in Table 1. In the absence of IPTG, the basal level at 39.5°C was very low for both cell clones. Upon addition of IPTG, CAT synthesis was induced 900-fold in CLAP267-5/CAT-7 and up to 6167-fold increase in the ILAP267-16/CAT-3 cell line. The data therefore indicate that also in stable clones there is a higher production of CAT in the autocatalytic conditional LAP267 system, as compared to the one which constitutively produced the LAP267 transcriptional activator. It is noteworthy that our results with the constitutively expressed H β LAP267 system are comparable to those obtained by Baim *et al.* (1). These authors have employed the stronger L21 promoter (containing 21 tandem *lacI* repeats) in stable Ltk⁻ transfectants and achieved 340–1200-fold CAT induction after an expression period of 70 h.

Table 1. IPTG-dependent CAT synthesis in stable NIH 3T3 LAP267-producers of the constitutive and inducible type

Cell line	IPTG	CAT activity	Fold induction
CLAP267-5/CAT-7	–	20	
	+	18 010	900
ILAP267-16/CAT-3	–	10	
	+	61 670	6167

The two cell lines were incubated for three days at 39.5°C in the presence or absence of IPTG (5 mM) and cell extracts were prepared thereafter. CAT activity normalized to % conversion is expressed in arbitrary units as obtained through volume integration of the assay products by phosphorimaging. A background level exhibited by the respective parental cell lines at 39.5°C was subtracted from the values displayed by the two cell lines.

Figure 1. Time course of IPTG-dependent CAT synthesis in stable NIH 3T3 LAP267-producers of the constitutive and inducible type. The stable cell lines CLAP267-5/CAT-7 and ILAP267-16/CAT-3 were incubated at 37°C. IPTG (5 mM) was added to the cultures at defined points after time zero and cell extracts were prepared by 50.5 h (the staggered addition of IPTG excludes variations due to difference in cell number). CAT assays were performed as described (4), except that 1% CHAPS was added to the disruption buffer. A background level exhibited by an extract prepared from NIH 3T3 cells incubated for 50.5 h in the presence of IPTG, was subtracted from the values obtained for each time point.

The time course of CAT synthesis was analyzed by incubating the two cell lines mentioned above at 37°C with IPTG for increasing time length. Cell extracts were assayed for CAT production and the data was expressed as a percentage of chloramphenicol conversion to its acetylated products. As shown in Figure 1, both cultures display an initial lag of 4.5–6.5 h. By 19 h after IPTG addition, ILAP267-16/CAT-3 had synthesized 68% of the end point (50.5 h) level, whereas in the CLAP267-5/CAT-7 cell line the corresponding value had only reached 30%. The kinetics of induction is therefore certainly faster in the autocatalytic conditional system than in the constitutive LAP267 producer.

Although the activation of CAT synthesis in the conditional autocatalytic system is relatively fast, it is possible that the instability of the prokaryotic CAT mRNA (5) leads to an underestimation of the kinetics of induction. To evaluate the kinetics of induction for target mRNA other than CAT, a 1.44 kb segment of the cDNA for human casein kinase II- α' subunit was cloned under the L7 promoter. The NIH 3T3/ILAP267-16 cell clone was cotransfected by pL7CK II- α' cDNA with mpRSV neo while selecting for G418 resistant colonies. One of the clones shown to synthesize the exogenous CK II- α' mRNA in an IPTG-dependent manner was chosen for further studies. IPTG was added to a cell culture maintained at 37°C at different time points after zero time, and total RNA was harvested 46 h later (6). Uninduced cells displayed a very low level of the 2.8 kb exogenous mRNA (shown by an arrow in Fig. 2). At the first time point (4 h) after IPTG addition, there was already a very significant level of the 2.8 kb exogenous CK II- α' mRNA, and this level was sustained for the remaining 42 h of the experiment. Thus, for the mammalian CK II- α' subunit cDNA, the kinetics of induction in the autocatalytic expression system at physiological temperature is very fast, reaching plateau by 4 h after IPTG addition, or perhaps even earlier. It is certainly comparable to the kinetics displayed by two other inducible expression systems (8,9).

When the features of the autocatalytic conditional expression system are considered along with the potential to minimize the

Figure 2. Kinetics of transcription from an integrated CK II- α' target gene in the autocatalytic conditional system. NIH 3T3/ILAP267-16 cells in 90 mm petri dishes were cotransfected by 4.25 μ g mpRSVneo and 22.75 μ g pL7CK II- α' for 19 h at 39.5°C. Following selection to G418 resistance, several surviving colonies were analyzed for IPTG-dependent induction of exogenous CK II- α' RNA. One of the positive clones (NIH 3T3/ILAP267-16/pL7CK II- α' -16) was incubated at 37°C with IPTG (added in a staggered fashion) for the indicated times, and synthesis of CK II- α' RNA was monitored by Northern analysis. The probe was a radioactivity labeled 1.3 kb *Hha*I-*Hinc*II fragment of human CK II- α' cDNA (7). The positions of the 18S and 28S rRNAs are indicated. The arrow points to the position of the exogenous CK II- α' RNA.

extent of leakiness at the initial phase of transfection, it seems that it is most suitable for modulating the activity of genes whose products are stringently regulated within mammalian cells.

Studies of the controls of cell cycle progression and growth could particularly benefit from the mouse cell lines which we have established.

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