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A self-initiating eukaryotic transient gene expression system based on cotransfection of bacteriophage T7 RNA polymerase and DNA vectors containing a T7 autogene

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ABSTRACT

A novel cytoplasmic gene expression system has been developed. This system differs from other expression systems in that it relies on the co-delivery of plasmid DNA and T7 RNA polymerase (RNAP) during transfection. The plasmid contains a T7 RNAP gene driven by the T7 promoter (T7 autogene) and a functional/reporter gene driven by another T7 promoter (T7T7/T7-gene construct). Once this DNA – enzyme complex is introduced into eukaryotic cells, the transcription of the T7 RNAP and the functional/reporter genes is initiated by the co-delivered T7 RNAP. The T7 RNAP, which is responsible for the initiation and maintenance of expression of both T7 and functional/reporter genes, is replenished by translation of newly synthesized T7 mRNA. This T7 system was designed in such a manner that the expression of the functional/reporter genes can occur in the cytoplasm and does not require any nuclear involvement. When transfected by either a pT7T7/T7Luc or a pT7T7/T7hGH plasmids with the co-introduced T7 RNAP, mouse L cells were found to express high levels of luciferase immediately after transfection, apparently due to the cytoplasmic gene expression; the expression of human growth hormone (hGH) could be sustained for at least 6 days. Both T7 and hGH mRNA were expressed by the cells transfected with pT7T7/T7hGH. These results suggest that this cytoplasmic expression system may be used for certain targets of somatic gene therapy.

INTRODUCTION

Several DNA delivery and expression systems have been developed and successfully used to introduce and express foreign genes in eukaryotic cells. These systems basically consist of a DNA delivery method and a DNA vector. DNA delivery methods include calcium phosphate precipitation (1), DEAE–dextran (2), lipofection (3), electroporation (4), retroviruses (5), direct DNA injection (6, 7), specific receptor-mediated DNA uptake (8, 9), and more recently, aerosol DNA delivery (10). Expression vectors are in general a plasmid DNA encoding a gene of interest linked to a promoter sequence which dictates the expression of the gene once the gene is introduced into a given cell line.

Because of the use of eukaryotic or mammalian promoters, all these methods require nuclear localization of the introduced DNA for gene expression. Yet only a very small percentage of the DNA in the transfected cells is found within the nuclei of the cells. This problem becomes more pronounced when whole animal tissues are the targets of foreign gene expression because cell division, which either greatly enhances or is required for nuclear deposition of the introduced DNA (11, 12), does not occur at nearly as high a frequency in the cells of tissues as it does in cultured cells. Conversely, strong evidence has been provided for the efficient cytoplasmic uptake of foreign DNA by cells and animals (3, 7). And, the DNA introduced into the cells of these whole animal tissues has been observed to remain for periods of up to several months (7).

Bacteriophage T7 RNA polymerase (RNAP) has been used extensively *in vitro* and *in vivo* in transcription and expression studies both in bacteria and eukaryotic cells due to some of its unique biochemical characteristics: it is a single polypeptide enzyme capable of carrying out transcription with high promoter specificity and efficiency, without involvement of any other cellular transcription factors (13–15). When microinjected into monkey kidney cells, T7 RNAP was found to localize in the cytoplasm of the cells since it is a prokaryotic protein which does not contain signals for nuclear localization (16). Recombinant vaccinia viruses containing a T7 RNAP gene or a cell line which constitutively expresses T7 RNAP were used to express genes of interest in the cytoplasm of mammalian cells (17–20). When a chloramphenicol acetyltransferase (CAT) gene was inserted into a T7 promoter-containing mammalian vector and transfected into a stable cell line which expressed T7 RNAP, as high as 30% of cytoplasmic proteins were found to be the CAT enzymes in the transfected cells when the cells were coinfected with the recombinant vaccinia viruses during transfection (20). However, the expression of any gene using this T7 system has to be coupled with cell lines which express T7 RNAP in the first place and with coinfection of the recombinant vaccinia viruses, greatly

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limiting the usefulness and flexibility of the system. A T7 autogene construct, which contained a T7 RNAP gene under the regulation of a T7 promoter and could be expressed in bacteria, was previously described (21). Based on our knowledge of T7 RNAP and existing T7 expression systems, we hypothesized that a cell-type independent, eukaryotic vector for transient cytoplasmic expression could be constructed if T7 RNAP molecules could be co-delivered to eukaryotic cells along with T7 autogene DNA. During this study, transient gene expression using co-delivery of T7 RNAP and a T7 promoter sequence was described (22). In their study, Gao and Huang conclusively demonstrated that T7 RNAP was indeed able to be co-introduced to cells along with DNA, and to express a CAT gene driven by a T7 promoter in the cytoplasm (22). However, since no T7 autogene was involved, relative long-term gene expression could not be achieved and was not investigated.

Here we describe the construction and expression studies of a novel eukaryotic expression vector. This vector contains a T7 RNAP gene driven by a T7 promoter (T7 autogene), and another sequence containing a functional or a reporter gene under the control of a second T7 promoter (T7T7/T7-gene construct). These two sequences can be either in the same plasmid (single plasmid system) or in two separate plasmids (dual plasmid system) when introduced to cells. However, the most important feature which distinguishes this system from other gene expression systems is that the initiation and maintenance of the gene expression depend upon the co-delivery of T7 RNAP and T7 autogene containing DNA to eukaryotic cells. Furthermore, this system does not require any nuclear involvement and should be able to function in the cytoplasm. Once the DNA–T7 enzyme complex is in the cytoplasm of the cells, the transcription is initiated by the co-transfected T7 RNAP from the T7 promoters in the plasmid. The transcription of both T7 and functional/reporter genes is subsequently maintained by newly synthesized T7 RNAP. In this study, gene expression using single or dual T7 plasmid systems was investigated and compared with conventional eukaryotic promoter-containing expression vectors. Two genes with different expression modes, hGH (as a secretory protein) and luciferase (an intracellular enzyme) were used in cultured mouse L cells. The T7 system described herein may provide a unique cytoplasmic gene expression vector for the rapid and efficient expression of RNA and protein products in a wide range of cultured cells and possibly in tissues as therapeutic targets.

MATERIALS AND METHODS

Enzymes

Bacteriophage T7 RNAP (50U/μl), restriction endonucleases, and the Klenow fragment were from New England BioLabs.

Cells and plasmids

Mouse L cells were provided by J.J.Kopchick, Ohio University (Athens, OH). *E.coli* DH5α was from Bethesda Research Laboratories. *E.coli* HMS174 with pLysE (15, 23); plasmids pAR1173 (13) and pT7AUTO-1 (21) were provided by F.W.Studier, Brookhaven National Laboratory (Upton, NY). Plasmid pTM1 (24) was provided by B.Moss, NIH. Plasmids pMThGH and pRSVLuc, the former contains a hGH cDNA (25) driven by a mouse metallothionein I promoter (26), and the latter contains a luciferase cDNA (27) driven by a Rous sarcoma virus (RSV) promoter, were constructed in our laboratory (X.C., unpublished data). The bacteria were grown in LB medium with appropriate antibiotics.

Constructions of plasmids

pTM1, a cytoplasmic expression vector which contains a T7 promoter with an EMC cap independent sequence at its 3′ end (24), and pAR1173, a T7 RNAP gene containing plasmid, were used to construct pT7T7 as shown in figure 1. However, unmodified T7T7 construct was found toxic to the host bacteria (X.C. and Y.L., unpublished data; 21, 23, 28). Several steps were subsequently taken to reduce the toxicity to bacterial hosts which results from the expression of the T7 gene while maintaining the ability of the construct to be expressed in eukaryotic cells.

First, a 1.6 kbp EagI/EcoNI Lac I gene which encodes a repressor was isolated from pT7AUTO-1 and inserted into ClaI and XbaI sites of pTM1 by a blunt-end ligation (figure 1). In addition, an operator sequence (28), which provides the binding site for Lac I repressor, was inserted immediately 3′ to the T7 promoter sequence (as shown in pTLO-1 in figure 1) where the operator was shown to have the maximal suppression of T7 promoter activity (28). Secondly, using a phagemid mutagenesis method (29, 30), the Shine–Dalgarno sequence (S–D box) of the T7 RNAP gene was removed from the gene to reduce unwanted translation of T7 RNAP mRNA which may be generated as a result of leaky Lac I suppression (figure 1). In addition, an NcoI site (CCATGG) was added to the T7 RNAP gene. A 2.6 kbp NcoI/BamHI modified T7 fragment was inserted into the gap between the NcoI and BamHI sites of pTM1 in such a manner that the optimized translation of the T7 RNAP gene would be initiated from the ATG in the NcoI site (figure 1). However, this modification altered the second amino acid residue of T7 RNAP from an Asn (AAC) to an Asp (GAC). Finally, the constructed pT7T7 was transformed into and prepared from HMS174 cells which contained a plasmid pLysE encoding a T7 lysozyme, a T7 RNAP inhibitor. All the mutations described in this and subsequent sections were confirmed by DNA sequencings (31).

Plasmids pT7hGH and pT7Luc were made in a similar fashion to pT7T7 except that unmodified pTM1 was used as parental plasmid since protein products of hGH and luciferase genes are not toxic to bacterial hosts. The relative position of the ATG codon of hGH and luciferase genes to the T7 promoters was also adjusted to the optimal position by deletion mutations.

Following construction of pT7T7 and pT7hGH, pT7T7/T7hGH, was constructed from these sequences as shown in figure 2. pT7T7 was linearized at the EagI site, the EagI site was subsequently filled by Klenow fragment. A 2.1 kbp ClaI/EagI T7hGH fragment was isolated from pT7hGH, and the single stranded cohesive ends were also filled in by Klenow fragment. pT7T7/T7hGH was made by ligating these two sequences together followed by transformation using HMS174 pLysE cells. Plasmid pT7T7/T7Luc was constructed in a similar fashion.

Addition of poly(dT) tails to hGH and luciferase genes in pT7 plasmids

This T7 system was designed as a cytoplasmic expression system and therefore no natural poly(A) tails would be added to the hGH and luciferase mRNA generated by this system. Poly dT tails of 40 base pairs long were added to the 3′ ends of both hGH and luciferase genes in order to increase the stability and possibly the translation efficiency of the hGH and luciferase mRNA.

Transient transfections

Mouse L cells were grown in DMEM supplemented with 10% Nu serum (growth media), in 12-well cell culture dishes to 60–80% confluence. One μg of T7 plasmid DNA was diluted with DMEM in a total volume of 39 μl in sterile polystyrene tubes. One μl of T7 RNAP (50U/μl) was added to the DNA solution and the mixture was incubated at room temperature for 10 min, followed by addition of 10 μl (1μg/μl) of lipofectin (3). After gentle mixing, the lipofectin–DNA–enzyme solution was incubated at room temperature for another 10 min. Meanwhile, growth media were removed from L cells, the cells were washed twice with DMEM. One ml of DMEM was added to each well, followed by addition of 50 μl of lipofectin–DNA–enzyme solution. After gentle mixing, the dishes were incubated at 37°C in a cell culture incubator. Following 3 hours' incubation, the media were removed, 2 ml of growth media were added to each well, and the dishes were incubated at 37°C in the presence of 5% CO_2. Growth media from the transfected cells were collected every 8 hours for the first 24 hours and then every 24 hours and the cells were replenished with fresh growth media. Cells which were transfected with pT7T7/T7hGH alone (no T7 RNAP), pT7hGH + T7 RNAP, or pMThGH, served as expression controls. pMThGH, a conventional expression vector, also served as a reference for comparison of expression profiles. Transfection of pMThGH was performed using a standard lipofection protocol without adding T7 RNAP. In a similar manner, pT7Luc and pT7Luc + T7 RNAP were used as controls for luciferase assays, respectively.

hGH RIA

hGH RIA was performed using a commercially available RIA kit (Hybritech, San Diego).

Luciferase assays

Luciferase assays were performed using a luciferase assay system (Promega, Madison, WI). Briefly, after removal of the growth media, transfected cells in each well were washed with PBS, and lysed with 200 μl of luciferase lysis buffer. Five to 10 μl of the cell lysate from each sample were assayed for luciferase activity using a luminometer (Lumat LB 9501, Berthold).

Northern analysis of T7 and hGH mRNA

Twenty four, 48, or 72 hours after the transfection, transfected cells were washed in PBS and lysed by 1 ml of RNAzol (Cinna/Biotecx Laboratories). Total RNA was isolated as described (32). 20 μg of total RNA from each cell sample was subjected to 1% formaldehyde gel electrophoresis. Following the electrophoresis, resolved RNA was transferred from the gel to a nylon-based membrane (Gene Screen Plus from NEN), hybridized to a ^{32}P-labeled, 2.6 kbp T7 fragment and subsequently visualized by autoradiography (33). After being stripped the T7 probe, the same membrane was rehybridized to a 0.9 kbp hGH probe. Actin mRNA served as a reference control to standardize sample signal intensities.

RESULTS

Construction of plasmids

Figures 1 and 2 schematically demonstrate how the plasmids used in this study, pT7T7, and pT7T7/T7hGH, were constructed. The pT7T7 and pT7T7/T7hGH plasmids were grown in the presence

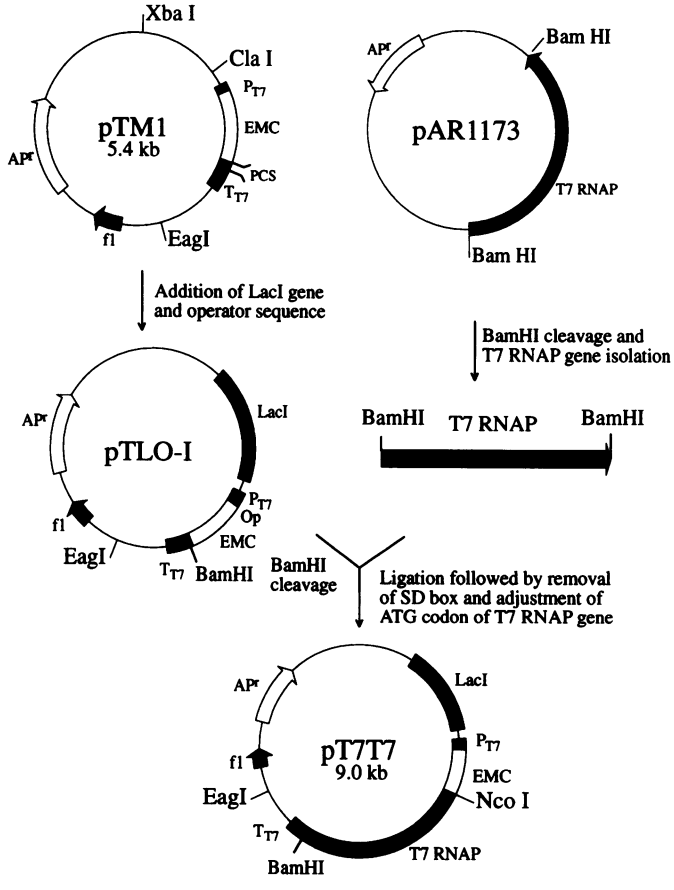

Figure 1. Construction of pT7T7 plasmid. pTM1 and pAR1173 were used as starting plasmids. pTM1 contains a bacteriophage T7 (Φ10) promoter (P_{T7}), a cap independent (EMC) sequence which facilitates translation of uncapped transcripts, as well as a T7 transcription termination sequence (T_{T7}). Polycloning site (PCS) in pTM1 contains several restriction sites for cDNA insertions and pAR1173 provides a T7 RNAP gene (Bam HI fragment). In pTLO-1, Lac I and Op represent the Lac I gene and the operator sequence, respectively. pT7T7 was constructed as described in Materials and Methods.

of the pLysE plasmid to reduce the toxicity of the T7 enzymes to *E.coli*. Therefore, the preparations of these plasmids were always contaminated with some pLysE plasmid (≤ 10%, Y.L. and K.X., unpublished data).

Transient expression of the functional/reporter genes

hGH expression. Expression of pT7T7/T7hGH (single plasmid) in L cells compared to that of pT7hGH and pMThGH is shown in figure 3. Cell culture fluids from transiently transfected L cells were collected and assayed for hGH every 8 hours for the first 24 hours and then every 24 hours for 5 days. Detectable levels of hGH could be found as early as 8 hours after transfection in cell culture fluids collected from cells transfected by pT7T7/T7hGH + T7 RNAP. Gene expression for sample pT7T7 + pT7hGH + T7 RNAP revealed an expression profile both qualitatively and quantitatively similar to that of pT7T7/T7hGH (X.C., unpublished data). However, hGH expression by pMThGH (which serves as positive control as well as a reference for expression profiles in this study) lagged behind that of pT7T7/T7hGH. It took pMThGH more than 24 hours to express hGH at levels similar to that expressed by pT7T7/T7hGH + T7 RNAP 8 hours after the transfection. Also, the hGH levels

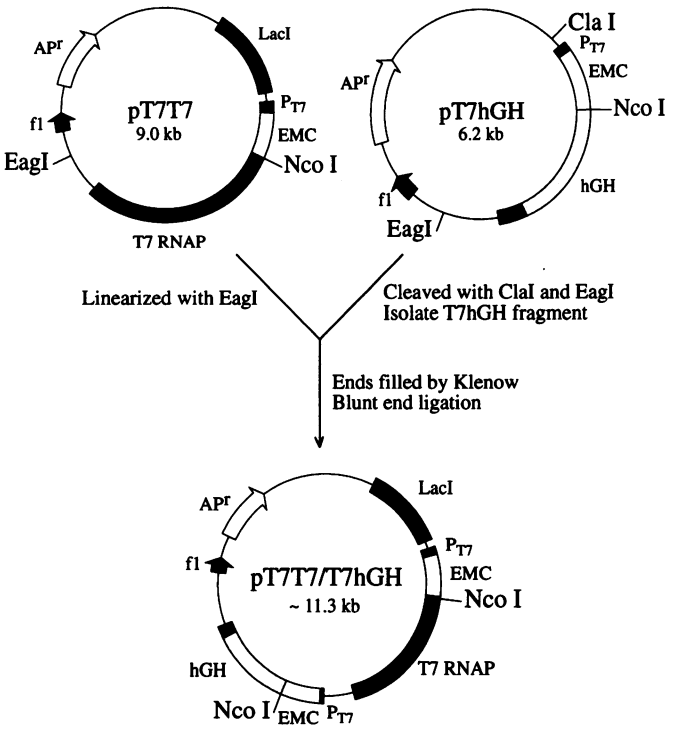

Figure 2. Construction of pT7T7/T7hGH. The smaller ClaI/EagI fragment from pT7hGH was isolated and inserted into the EagI site of pT7T7 by a blunt end ligation as described in Materials and Methods. In the clone used in this study, the orientation of the T7hGH fragment in pT7T7/T7hGH is the same as that of the T7T7 sequence. In this plasmid, the T7hGH sequence has a structural arrangement similar to T7T7 except that the operator is absent from T7hGH sequence.

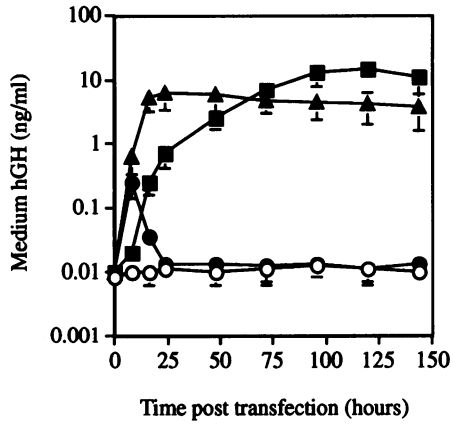

Figure 3. Expression of hGH in mouse L cells transfected by T7hGH systems. Mouse L cells were transfected by either pT7T7 + pT7hGH (dual plasmids) or pT7T7/T7hGH (single plasmid) with co-delivered T7 RNAP as follows: One μg of plasmid DNA (0.5 μg+0.5 μg for dual plasmids) was coincubated with 50 U of T7 RNAP at room temperature for 10 minutes and 10 μl of lipofectin was mixed with the DNA–enzyme complexes to a final volume of 50 μl. Following 10 min room temperature incubation, the liposome–DNA–enzyme mixture was added to mouse L cells in a 12-well cell culture dish. The transfection and protein assays were performed as described in Materials and Methods. Cell samples transfected by pT7T7/T7hGH alone, pT7hGH + T7 RNAP, or pMThGH (1 μg DNA/well) served as negative and positive controls, respectively. Each point in the curves represents an average value of at least three individual measurements. Error bars represent standard deviations of the measurements. ○ = pT7T7/T7hGH alone, ● = pT7hGH+T7 RNAP, ■ = pMThGH, and ▲ = pT7T7/T7hGH +T7 RNAP. For clarity of the graph, the expression curve for pT7T7 + pT7hGH + T7 RNAP, which is qualitatively and quantitatively similar to that of pT7T7/T7hGH + T7 RNAP, is not shown.

expressed by pMThGH was not higher than that of the pT7T7/T7hGH+T7 RNAP until 72 hours or later. For cell samples transfected by pT7hGH plus T7 RNAP, only the culture medium collected 8 and 16 hours after the transfection demonstrated low levels of hGH (figure 3). The expression disappeared after longer incubations. No hGH could be detected at any time in the culture fluids collected from the cells transfected by pT7T7/T7hGH alone. The expression of the T7 systems reached their peaks (~ 8 ng/ml) approximately 24 to 48 hours after the transfection whereas expression of pMThGH reached its peak (~ 15 ng/ml) at about 96 to 120 hours. Therefore, the kinetic profile of the hGH expression by the T7 system seemed to be quite different from that of a traditional expression vector.

Luciferase gene expression. Figure 4 demonstrates the result of expression profiles of pT7T7/T7Luc and pRSVLuc in transfected mouse L cells. Cells were lysed and assayed at 0, 1, 2, 4, 24, and 48 hours after 4 hours' transfection. As revealed by figure 4, luciferase expression by pT7T7/T7Luc immediately after the transfection was already as high as that generated by pRSVLuc 24 hours after the transfection. In addition, total luciferase protein produced by pT7T7/T7Luc in the first 4 hours post transfection was more than that produced by pRSVLuc for the first 48 hours.

Northern analysis of T7 mRNA

In order to prove that the sustained expression of the functional/reporter genes in the T7 systems was indeed due to the sustained expression of the T7 genes in the same systems, total RNA from the transfected cells was isolated and analyzed by Northern blots as shown in figure 5. Both T7 and hGH mRNA was found in the cells transfected by pT7T7/T7hGH + T7 RNAP, but not in the cells transfected either by pT7T7/T7hGH alone, or by pT7hGH+T7 RNAP (figure 5—I and II. Low levels of the hGH mRNA could be detected for pT7hGH+T7 RNAP samples after much longer exposure of the autoradiograph). The positions of the major bands on the blots correspond to the anticipated sizes for the T7 and hGH mRNA. Also, the levels of T7 and hGH mRNA were found to follow the same trend: the higher the T7 mRNA level, the higher the hGH mRNA level, and vice versa. Reduced mRNA levels for 72 hours' samples was partly due to diluting effect of cell divisions (reduced mRNA production per cell).

DISCUSSION

The importance of gene therapy to the future of medicine can not be underestimated. It is possible to envision gene therapy being applied not only to the treatment of permanent inborn disorders, but also as a means of genetic delivery of protein therapeutics over a discrete, controlled time period. We have described the construction and function of a novel eukaryotic gene expression system which may be useful in just such applications. This expression system has several unique features which make it ideal for transient gene therapy; it is a completely self-contained system independent of cell-provided nuclear transcriptases or transcription factors allowing functionality within the cytoplasm of the target cell, it enters target cells pre-activated and therefore

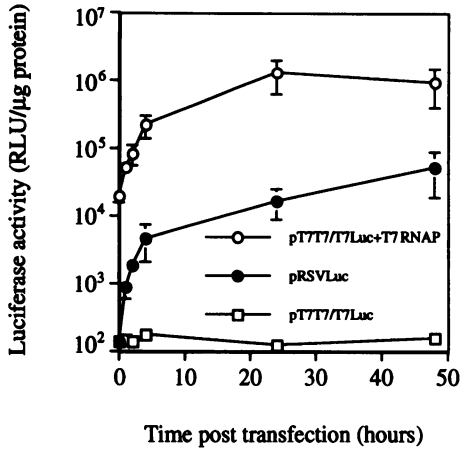

Figure 4. Rapid luciferase gene expression in mouse L cells transfected by the T7 system. Mouse L cells in 12-well plates were transiently transfected for 4 hours by 1 μg of either pT7T7/T7Luc ± T7 RNAP or pRSVLuc as described in Materials and Methods. After transfection, transfected cells were lysed at different time intervals to measure their intracellular luciferase activities. Expressed luciferase activities by the transfected cells were reported as relative light units (RLU) and normalized for 1 μg cellular proteins. Each data point represents an average of four independent measurements from two separate experiments. Error bars indicate standard deviations of the measurements.

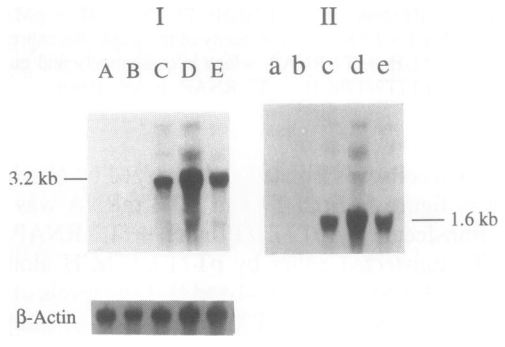

Figure 5. Northern analysis of the T7 and hGH mRNA expressed by mouse L cells transfected with the T7 system. The cells were transfected by the T7 systems, and total RNA was isolated as described in Methods. Following gel electrophoresis and transfer of resolved RNA on a membrane, the membrane was hybridized first with a 2.6 kbp ^{32}P-labeled T7 probe, then by a 0.9 kbp hGH probe after stripping off the first T7 probe. Actin mRNA in each sample served as a RNA concentration reference. I. Northern blot using a T7 probe. A = cells transfected by pT7T7/T7hGH, B = pT7hGH+T7 RNAP, A and B were isolated 24 hr after the transfection. C, D, and E = pT7T7/T7hGH+T7 RNAP; sample C was isolated 24 hr post transfection, D = 48 hr, E = 72 hr. II. Same membrane hybridized with a hGH probe. Lane order is the same as in I.

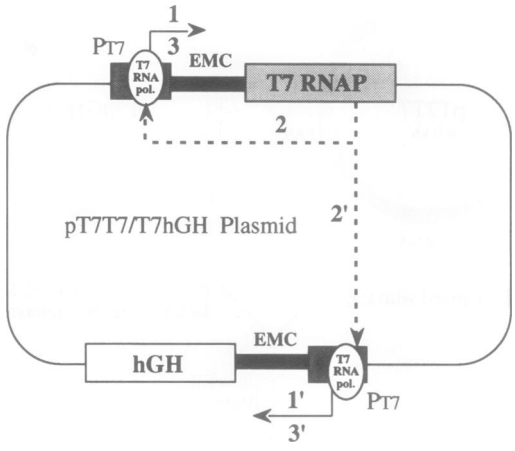

Figure 6. Schematic presentation of self-initiation and positive feedback loop features of the T7T7/T7-gene expression system. **A.** Cotransfected T7 RNAPs initiate transcription from T7 promoters (P_{T7}) on both T7T7 and T7hGH sequences (1 and 1'). EMC serves as a cap independent sequence in translation for the T7 and hGH transcripts; **B.** newly synthesized T7 RNAPs replenish the polymerase pool in the cytoplasm of the transfected cells (2 and 2'); and **C.** maintenance of the T7 and hGH gene expression in the cells (3 and 3'). In this plasmid, hGH cDNA can be replaced to express other cDNAs.

requires almost no lag time prior to maximal expression of protein gene product, it provides higher gene expression levels than the most commonly used eukaryotic expression vectors, it cannot function as a chromosomally integrated sequence within the nucleus and it is self-limiting due to gradual degradation of the unintegrated cellular plasmid DNA.

The expression system described herein is one of the first reported successful attempts to express genes of interest in mammalian cells using a transcriptase−DNA complex, in this case a T7 RNAP complex. The rationale for this unique design is presented and summarized in figure 6. During our study, Gao and Huang described a transient T7 expression system using co-delivery of T7 RNAP with a T7CAT construct (22). Our study has extended their work by including a T7 autogene sequence and demonstrated relatively long term mRNA and protein expression of the new vector systems. Relatively stable hGH and luciferase expression was found in cells transfected with either pT7T7/T7hGH + T7 RNAP (figure 3), or pT7T7 + pT7hGH + T7 RNAP (X.C., unpublished data), or pT7T7/T7Luc + T7 RNAP (figure 4). Human growth hormone which was secreted into the growth media by the transfected L cells was found biologically active in rat Nb2 cells (X.C. and Y.L., unpublished data). In contrast, extremely low levels of hGH activity was detected for just a very short period in cells transfected with pT7hGH + T7 RNAP (figure 3). These results suggest that it is the post-transfection expression of the T7 RNAP gene in either pT7T7 or in pT7T7/T7hGH or pT7T7/T7Luc which maintained the lasting expression of the luciferase and hGH genes. Since there was no T7T7 autogene sequence in the control plasmids to provide a functional T7 RNAP gene in the transfected cells, no T7 RNAP could be cellularly generated to replenish the enzyme pool established at the beginning of the transfection, resulting in low and temporary gene expression. The presence of the T7 and hGH mRNA in the cells transfected by pT7T7/T7hGH+T7 RNAP and the absence of both mRNAs in the cells transfected with pT7T7/T7hGH alone or pT7hGH+T7 RNAP (figure 5) strongly support the notion that the T7T7 positive feedback loop was indeed in operation in the transfected cells. A similiar result is observed in figure 4 where the expression profile of the pT7T7/T7Luc plasmid with cotransfected T7 RNAP is compared to pRSVLuc. Figure 4 shows that the amount of protein expression of pT7T7/T7Luc is greater in the first 4 hours following transfection than the total cumulative transient protein expression of pRSVLuc over a period of two days. These results are consistent with those of other

studies which showed that reporter genes were actively transcribed by T7 RNAP in the cytoplasm of cells (17, 20, 22). It is also consistent with the cytoplasmic expression ability of the T7 parental plasmid pTM1 (24). As shown in figures 3 and 4, the onset of expression of hGH and luciferase using the T7 systems is much more rapid compared to that of conventional eukaryotic promoter-containing plasmid pMThGH and pRSVLuc. This result is consistent with that of the study using a T7CAT vector coupled with recombinant vaccinia viruses, which showed that 48 hours after the transfection the cells transfected by the T7 system produced several hundred-fold higher CAT activities than those expressed either RSVCAT or SV40CAT (17). It is also consistent with that of a recent study in which T7CAT plasmid was shown temporarily expressed in the cytoplasm of the transfected cells by co-delivery of T7 promoter-containing DNA and T7 RNAP (22). The rapid expression and different kinetic profiles exhibited by the T7 systems (figures 3 and 4) suggest that an expression mechanism, which differs from the one used by pMThGH or pRSVLuc, was employed by the T7 system. It is very likely that the co-introduced T7 RNAP initiated transcription immediately after the plasmids were taken into the cytoplasm of the cells, followed by rapid protein synthesis which resulted in the shift of the expression curves to the left (figures 3 and 4). In contrast, pMThGH and pRSVLuc had to reach the nuclei of the transfected cells for hGH or luciferase gene expression. This may explain for the long delay of hGH and luciferase expression by these eukaryotic promoter containing vectors. The reason for the existence of the expression peak 24 to 48 hours after transfection and a gradual expression decline thereafter for pT7T7/T7hGH (figure 3) is not clear. It may result from a loss of DNA templates through cell division. Unlike in *E.coli*, no significant cytotoxicity was observed in the L cells transfected by the plasmids containing T7 autogene construct. One plausible explanation for this is that the direct competition between T7 and *E.coli* RNAPs for transcription substrates, which resulted in T7 cytotoxicity in *E.coli* (21, 23, 26), may not exist in the transfected L cells because cellular and the T7 transcriptions occur in the nucleus and cytoplasm, respectively. Also, the number of the cytoplasmic plasmids in each of the transfected L cells is reduced to about half with each cell division. Therefore, the putative balance between the number of the T7 autogene templates in a transfected cell and the autogene's 'self-amplifying' ability might have kept the quantity of T7 RNAP synthesized at levels not very toxic to the host cell. Cytoplasmic expression demonstrated by this and other studies may provide an alternative to nuclear expression, particularly in animals, for foreign gene expression. The rapid and efficient transcription demonstrated by this system suggest that the T7 system may also be utilized to produce RNA products such as antisense or ribozyme RNAs in cells. The successful expression of the functional/reporter genes with a DNA prebound enzyme suggests that other DNA binding proteins may also be cointroduced into cells with DNA.

Because of the unique self-sufficient and cellular factor-independent nature of the T7 RNAP, this T7 system is basically cell-type independent. It should allow expression of virtually any cDNA in a wide range of cultured cells and cells in animals. In addition to the features of this system already discussed, the T7 system has a safety feature (although it may be only partial) not present in other gene therapy vectors. It is unlikely that the T7 sequences, were they to integrate adjacent to a dominant acting gene such as an oncogene, would result in the aberrant expression of that gene, since the T7 RNAP required for the activation of the T7 system lack nuclear translocation signals and would be unlikely to enter the nucleus of mammalian cells (16) where the rare T7 plasmid might integrate.

The results described suggest the utility of the T7 expression system for rapid, directed transient gene expression in cells, and possibly living tissues. The cell and tissue independence of this system suggest broad laboratory and clinical applications for this novel gene expression system.

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