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Expression and Stabilization of Microinjected Plasmids Containing the Herpes Simplex Virus Thymidine Kinase Gene and Polyoma Virus DNA in Mouse Cells

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To observe the effects of polyoma virus DNA on the expression of the herpes simplex virus (HSV) thymidine kinase (TK) gene early after transfer into TK-deficient mouse cells and the subsequent development of stable TK-positive transformants, we constructed a series of recombinant plasmids containing the herpes simplex virus TK gene joined with various segments of the polyoma virus genome and microinjected them into the nuclei or cytoplasm of LTK-A cells (TK^-, $APRT^-$). The frequency of nucleus-injected cells expressing TK after 1 day, measured by autoradiography of cells incubated with [^{3}H]thymidine, increased approximately 30-fold when the plasmids contained the polyoma virus origin of replication. The origin includes sequences with homology to the simian virus 40 origin of replication and adjoining sequences, including a recently defined transcription-enhancing sequence. After microinjection of a single origin-containing plasmid molecule per cell, TK expression was detected in approximately 50% of the injected cells. When a larger number of origin-containing plasmid molecules were injected per cell, all cells showed early TK activity. When the entire polyoma virus early region was present, neighboring uninjected cells became TK positive. When plasmids were injected into the cell cytoplasm, approximately 400 times as many molecules per cell were needed to cause early TK activity. The frequency of stable transformation observed 2 weeks after nuclear injection of 10 to 20 polyoma virus origin-containing plasmid molecules per cell was at least 2 orders of magnitude greater than with plasmids containing the TK gene alone. The greatest enhancement of stable TK transformation was obtained with plasmids containing the origin alone, when the maximum frequency of stable transformation was 5%. The addition of the coding regions for the small and medium T antigens or the entire early region significantly decreased TK transformation frequency in a copy-dependent fashion. The timing of stabilization of TK-positive transformation was analyzed by releasing hypoxanthine-aminopterin-thymidine selection pressure at various times after microinjection, culturing the cells in nonselective medium, and assaying for TK activity. Stabilization was found to occur between 3 and 6 days after nuclear injection. Cells injected with a plasmid containing the origin and the early region were examined for expression of the large T antigen with polyoma virus antitumor serum and immunofluorescent staining. The expression of the large T antigen was clearly associated with a cytopathic effect. TK-positive clones observed 2 weeks after injection of the plasmid were uniformly T antigen negative. Cytotoxicity may be the result of plasmid replication and toxic levels of T antigen or TK. In addition, expression of the large T antigen may block stabilization by preventing the integration of origin-containing plasmid molecules.

Gene transfer techniques have been used to introduce exogenous DNA into a variety of recipient cell types, including cultured somatic cells (2, 23, 36), frog oocytes (7, 21, 25), and mammalian eggs (13). Somatic cells in vitro have been most commonly used as recipients because the methods for cell culture are well established and a convenient method for introducing DNA by calcium phosphate precipitation has been developed (15). With this method, however, the

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frequency of transformation is very low—usually 1 in 10^4 to 10^6 cells—despite the use of specific cell lines that take up DNA efficiently and allow selection of transformants (22). Furthermore, carrier DNA is usually added to increase the efficiency of calcium phosphate-mediated transformation and forms concatemers in recipient cells with the transferred DNA under investigation (20, 28, 29), complicating later analysis. Direct microinjection of DNA can circumvent these problems.

Anderson et al. (1) succeeded in direct microinjection of a mixture of cloned human β-globin and herpes simplex virus (HSV) thymidine kinase (TK) genes into somatic cells and achieved a high frequency of DNA transformation. They selected TK-positive cells in hypoxanthine-aminopterin-thymidine (HAT) medium and showed transcription of the β-globin gene in the transformants. Capecchi (5) also studied the expression of microinjected DNA and observed that when the injected plasmid contained a segment of simian virus 40 (SV40) DNA that included the region of the origin of replication, the frequency of stable expression was increased by more than 2 orders of magnitude. Contained within this region is a 72-base-pair direct repeat sequence located in the noncoding segment in the proximal portion of the late region. Banerji et al. (3) showed that the 72-base-pair sequence enhanced the transcription of a β-globin gene positioned remotely on the same plasmid after calcium phosphate-mediated DNA transfer. This may at least in part explain the increased frequency of expression observed by Capecchi.

Recently, de Villiers and Schaffner (8) showed that a 244-base-pair fragment derived from a position in the polyoma virus genome corresponding to that of the SV40 72-base-pair sequence had similar effects on the transcription of β-globin. The polyoma virus sequence shares no extensive homology with the SV40 sequence. Tyndall et al. (35) analyzed the 244-base-pair "enhancing" region of polyoma virus with deletion mutants and found that the region is required for polyoma virus early region expression and virus DNA replication. These experiments were carried out by calcium phosphate-mediated gene transfer.

We were interested in the effects that polyoma virus DNA might have on the frequency of transformation and expression of linked genes in plasmids introduced into cells by microcapillary injection. Specifically, when various polyoma virus sequences are linked with the HSV TK gene, do sequences containing the enhancer region increase the frequency of early TK expression or stable TK-positive transformation? What happens when the entire polyoma virus early region is also present, as in lytic viral infection? What effect does the malignant transforming region have on transfer frequency, and, in particular, does it enhance stable TK-positive transformation?

We tested the effects of the various polyoma virus functions by using a series of plasmids containing various segments of the polyoma virus genome joined to the HSV TK gene. These plasmids were microinjected into LTK^- mouse cells. Expression of the TK gene was studied 1 day postinjection and again after 2 weeks, when stable TK-positive colonies appeared. The timing and process of stabilization of the TK-positive phenotype were also examined.

MATERIALS AND METHODS

Construction of plasmids. Plasmids were constructed in two steps: first, a segment of the polyoma virus genome was inserted into pBR322; next, the HSV TK gene, carried on a *Bam*HI restriction fragment, was inserted into the *Bam*HI restriction site of the plasmid. Wild-type polyoma virus DNA was used as the starting material for construction of polyoma DNA-containing plasmids. HSV TK DNA was derived by cleaving plasmid pTKX1 (9) with the restriction enzyme *Bam*HI (Bethesda Research Laboratories). The HSV TK and polyoma virus DNA fragments used for plasmid construction were purified by electrophoresis through 5% acrylamide gels, followed by electroelution. DNA molecules were joined together and *Escherichia coli* K-12 strain HB101 was transformed as previously described (19). Bacterial colonies were screened for the presence of an inserted DNA segment by the method of Grunstein and Hogness (16), using as probe the inserted fragment radiolabeled by nick-translation. Plasmid DNA was prepared from positive clones by the method of Clewell and Helinski (6). Restriction analysis was then performed to confirm the presence of inserted DNA and in some cases to determine its orientation.

Specifically, plasmid por1TK was constructed by joining a segment of the polyoma virus genome that included the replication origin, contained between *Bam*HI (nucleotide 4,657, using the numbering system of Friedmann et al. [12]) and *Ava*I (nucleotide 672), with the large fragment of pBR322 obtained after digestion with *Bam*HI (nucleotide 375 by numbering of Sutcliffe [33]) and *Ava*I (nucleotide 1,424). The construction of plasmid pgt1TK was described previously (19). Plasmid pT4TK was constructed by joining a segment of the polyoma virus genome contained between *Bam*HI (nucleotide 4,657) and *Hin*cII (nucleotide 2,984), including the early region, with a segment of pBR322 contained between *Pvu*II (nucleotide 2,067) and *Bam*HI (nucleotide 375), including the gene encoding ampicillin resistance (see Fig. 1 for plasmid structures).

Cell cultures. Three derivatives of mouse L cells were used in these experiments and were cultured in Dulbecco modified Eagle minimal essential medium (DME) supplemented with 9% fetal calf serum. LTK-A cells, which were used in most experiments, lack both TK and adenine phosphoribosyltransferase activities. LTK-AJT is a cell line derived from LTK-A cells

that has an approximately 10-fold higher transformation efficiency with the calcium phosphate method. LMTK$^-$ cells lack only TK activity. In transformation experiments, injected cells were cultured in DME-HAT medium immediately after microinjection. The medium was changed every 3 days. In some experiments, cells were selected in α-minimal essential medium (MEM) with HAT. Transformants were tested for stability as previously described (20). The first stability test was usually performed approximately 1 month after microinjection.

Microinjection. The arrangement of the microscope (Leitz, Diavert) and micromanipulator (Leitz) was essentially as described by Graessmann et al. (14). Microcapillaries were made from Omega dot tubing (Glass Company of America) with a capillary puller (David Kopf Instruments, model 700C). Samples (5 μl) were spun at 60,000 × *g* for 45 min in a Beckman SW41 rotor to remove insoluble materials. Special adaptors were made for this purpose. Microinjection was carried out under 200× magnification. During microinjection, the culture medium was replaced with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered DME (pH 7.4) to maintain stable pH. After microinjection, recipient cells were cultured either in DME containing [^{3}H]thymidine or in DME-HAT. To determine the injection volume, a series of diluted diphtheria toxin solutions was injected into the cytoplasm of LTK-A cells. Because diphtheria toxin can kill a cell when a single molecule is introduced (38), the injection volume can be determined from the viability curve of the injected cells. For this purpose, individual cells, identified by their position on dishes marked with fine grids (made by scratching with a needle), were microinjected, and their viability was examined under a microscope 4 days later. Intoxicated cells died within 2 to 3 days, and cells without toxin divided several times. The injection volume was controlled by the change in the refractive index after microinjection. The injection volume was determined to be 4.2×10^{-11} ml per cell. For nuclear injection, we calculated the injection volume to be about 10^{-11} ml from the apparent increase in size of the nucleus of the recipient cell and by comparing the area of changed refractive index with that observed after cytoplasmic injection. The viability of the microinjected cells was greater than 90% in both cases. For the TK transformation experiments, cells were plated at 3×10^5 cells per 60-mm dish (Costar) in dishes marked with grids, and only cells within the ruled area were microinjected.

Autoradiography. Expression of the HSV TK gene was detected with autoradiography by a modification of the method of Stein and Yanishevsky (32). Single cells were cultured for 1 day on glass cover slips. After microinjection, these cells were cultured in DME with [^{3}H]thymidine (20 Ci/mmol; 1 mCi/ml) at 5 μCi/ml for 1 day after nuclear or 1 to 4 days after cytoplasmic injection. Cells were fixed with methanol-acetic acid (3:1) and diluted with distilled water (1:1) for 5 min and then with undiluted fixative for another 5 min. The cells were dehydrated in gradually increasing concentrations of ethanol (from 80 to 95%), dipped in Kodak NTB-2 emulsion, and exposed for 2 days at 4°C. After development and fixation, cells were stained with 5% Giemsa solution, and the TK-positive cells were counted under a microscope.

Immunofluorescent staining. Polyoma virus antitumor antiserum was an ascites obtained from 8-week-old Brown Norwegian rats injected intraperitoneally with 10^7 polyoma virus-transformed rat cells that had been adapted for ascites growth (pyB4A) (31). Fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulin G (IgG) antiserum, IgG fraction, was purchased from Cappel Laboratories. Both antibodies were adsorbed with LTK-A cells fixed with ethanol until nonspecific reaction with the cells was minimized. For the transient expression of large T antigen, LTK-A cells plated on cover slips for 1 day were injected and cultured for 1 to 6 days in normal medium. Stable TK-positive pT4TK transformants were plated on cover slips overnight. They were fixed consecutively with 50 and 95% ethanol for 5 min each at room temperature. The cover slips were stained for 30 min at 37°C with 18 μl of sixfold-diluted polyoma virus antitumor antiserum and then for another 30 min at 37°C with 18 μl of fivefold-diluted rabbit anti-rat IgG. Pictures were taken with a Zeiss Photomicroscope III.

RESULTS

Description of plasmids containing the TK gene and segments of the polyoma virus genome. Three plasmids were constructed containing the polyoma virus replication origin and overlapping segments of the early regions. All these sequences were joined to the HSV TK gene in pBR322. These plasmids were compared in the context of microinjection experiments with plasmid pTKX1, which carries the TK gene on a 3.5-kilobase (kb) *Bam*HI restriction fragment inserted into pBR322 at the *Bam*HI restriction site (Fig. 1b).

The three polyoma virus DNA-containing plasmids included the origin of replication segment (Fig. 1a). The origin includes sequences homologous to the SV40 origin, to the early and late viral promoters, which promote the transcription of the T antigen genes and the virion genes, respectively, and to the recently defined transcriptional enhancer region located in the noncoding portion of the proximal late region. The polyoma virus plasmid constructs all included these elements. The late promoter in all three constructs was placed upstream from the TK promoter and oriented in the same direction (9, 24).

Plasmid por1TK contained the origin region of polyoma virus and short segments of the early and late coding regions (Fig. 1c). It did not contain sufficient information for the expression of T antigens or virion proteins. Plasmid pgt1TK contained the origin, a short segment of the late region, and the coding regions for the small and medium T antigens (Fig. 1d). The segment of the polyoma virus genome present in pgt1TK can malignantly transform mouse cells (18, 27), and experiments suggest that the medium T function alone may be sufficient for malignant cellular

transformation (34). Plasmid pT4TK contained the origin, a small segment of the late region, and the entire early region, which encodes the three T antigens (Fig. 1e). The large T antigen encoded by this plasmid should permit its replication in mouse cells, as occurs with the virus genome during the late phase of lytic viral infection.

Frequency of expression of the HSV TK gene 1 day after microinjection. The frequency of expression of the HSV TK gene was examined by autoradiography 24 h after nuclear or cytoplasmic injection of LTK-A cells. If the HSV TK gene was expressed in the TK-deficient LTK-A cells, the cells could then incorporate [^{3}H]thymidine into their DNA. Cells were microinjected, cultured for 1 day in medium containing [^{3}H]thymidine, and then prepared for autoradiography. Figure 2 shows the relationship between the copy number of injected plasmids and the percentage of cells that expressed HSV TK activity. When por1TK, pgt1TK, or pT4TK, each of which contained the origin of replication as a common polyoma virus DNA segment, was injected, about 0.7 copies per cell were needed to yield 50% TK-positive cells. When the polyoma virus DNA segments were omitted, as in pTKX1, approximately 20 copies per cell were required to obtain the same proportion of TK-positive cells. The 3.5-kb HSV TK fragment purified from pTKX1 required an intermediate number of plasmid molecules, approximately seven copies per cell, to obtain 50% TK-positive cells. These results indicate that the DNA segment containing the origin of replication of polyoma virus increased the frequency of early expression of HSV TK. In addition, by comparing the results for pTKX1 with those obtained for the TK segment alone, we concluded that pBR322 sequences may have an inhibitory effect on the frequency of expression of HSV TK. We cannot, however, exclude the possibility that the use of a linear DNA segment instead of a circular plasmid molecule may account for the difference observed in the frequency of expression.

Cell populations injected with pT4TK gave an unexpected result: more cells were TK positive than were injected. Cell proliferation as an explanation could be ruled out by direct observation. We believe that DNA, mRNA encoding TK, the TK enzyme itself, or metabolized nucle-

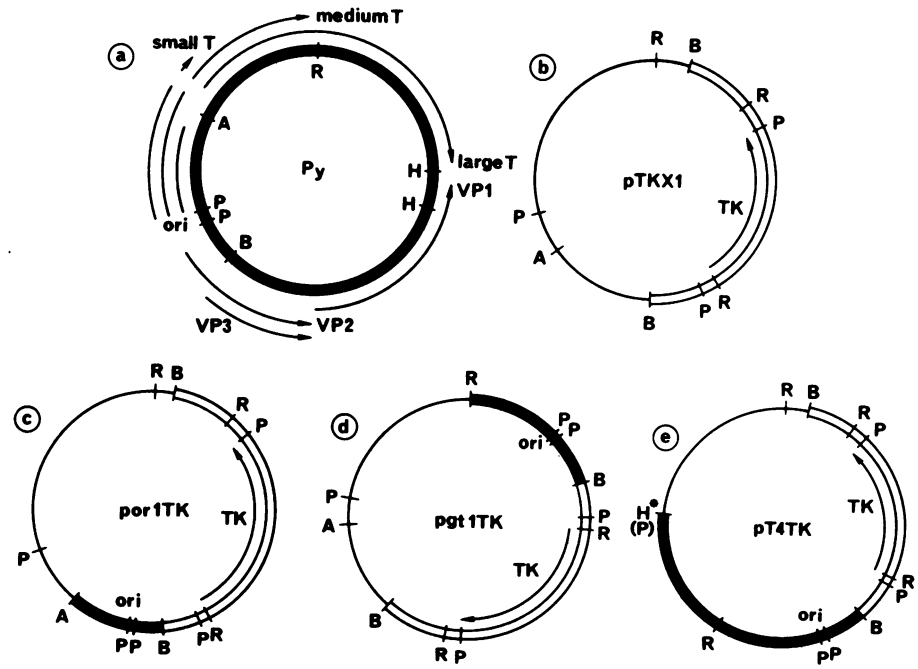

FIG. 1. Schematic representation of the structures of polyoma virus and the recombinant plasmids. (a) Polyoma virus (Py). The coding regions for the three T antigens and the three virion proteins (VP) are shown beginning in the area of the origin of replication (ori). Discontinuities represent introns. (b through e) Recombinant plasmids. Lines, pBR322 sequences; open bars, HSV sequences; solid bars, polyoma virus sequences. pT4TK lost the *Hin*cIII site where the polyoma virus segment is joined to the pBR322 segment [H*(P)] because a blunt-ended *Hin*cII end of polyoma virus was joined to a blunt-ended *Pvu*II end of pBR322 and neither restriction site was restored. A, *Ava*I; B, *Bam*HI; H, *Hin*cII; P, *Pvu*II; R, *Eco*RI.

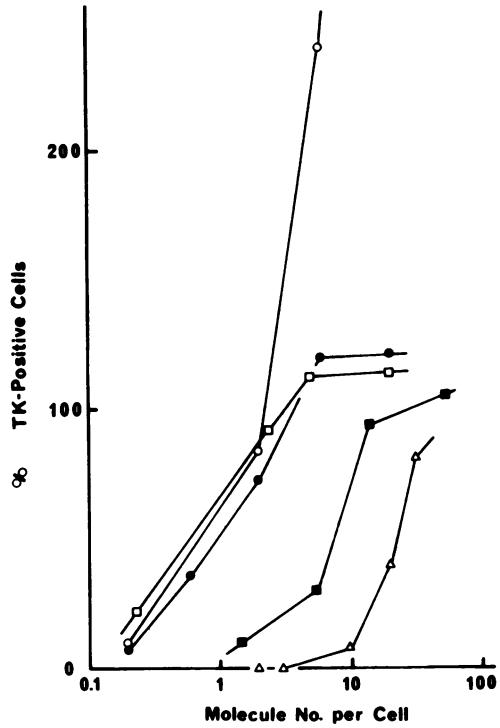

FIG. 2. Transient expression of the HSV TK gene in LTK-A cells after nuclear microinjection. Recipient cells were injected with different plasmids or the purified HSV TK gene and cultured for 1 day in normal medium containing [^{3}H]thymidine. The expression of TK was tested by autoradiography. For each point, 200 cells were microinjected. Symbols: □, porlTK; ●, pgtlTK; ○, pT4TK; △, pTKX1; ■, HSV TK gene purified from pTKX1.

otide leaked from the injected cells and was incorporated into neighboring uninjected cells. This is consistent with the fact that uninjected positive cells were always found clustered around injected cells that were invariably strongly positive. We believe that pT4TK replicated to high levels in injected cells, on the basis of their cytopathology as described below. High pT4TK replication is consistent with the presence of the intact early region and origin of polyoma virus in this construct, as is required for replication of the virus in infected mouse cells.

Injection of plasmids into the cytoplasm produced quite different results. In these experiments, the cells were cultured for 1 to 4 days after injection in medium containing [^{3}H]thymidine and then processed for autoradiography. The proportion of TK-positive cells increased with incubation time and reached a plateau 3 days after injection (Fig. 3A). TK-positive cells did not divide more than twice during this period, possibly because of irradiation by incorporated [^{3}H]thymidine. Polyoma DNA-containing plasmids produced a 10-fold greater frequency of TK-positive cells than did either pTKX1 or the 3.5-kb *Bam*HI TK insert. pT4TK gave a significantly greater enhancement than porlTK

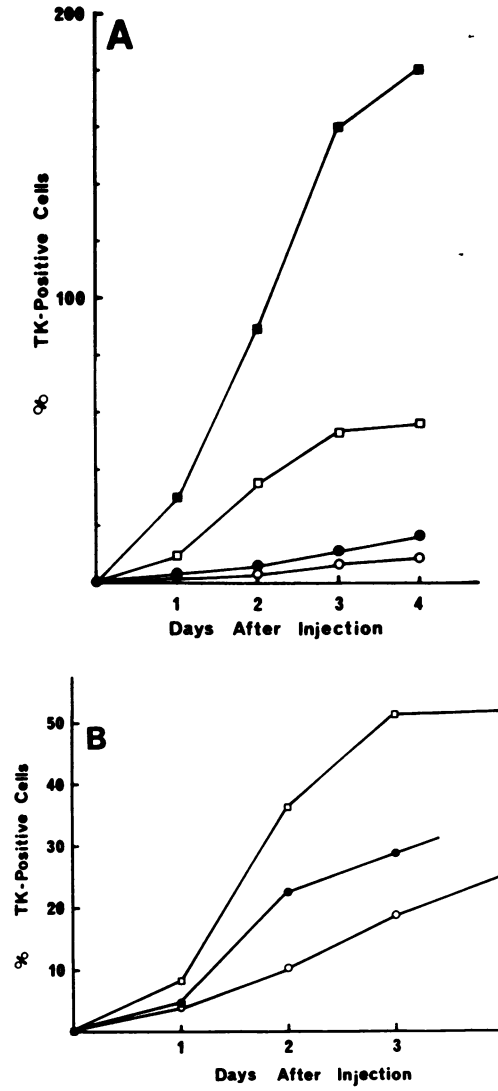

FIG. 3. Transient expression of the HSV TK gene in LTK-A cells after cytoplasmic microinjection. (A) Recipient cells were injected with different plasmids or the purified HSV TK gene in the cytoplasm and cultured for 1 to 4 days in normal medium containing [^{3}H]thymidine. The expression of TK was tested by autoradiography. For each point, 200 cells were microinjected. Symbols: □, porlTK (425 copies); ■, pT4TK (100 copies); ○, pTKX1 (400 copies); ●, HSV TK (300 copies). (B) Different numbers of porlTK were injected into the cytoplasm of LTK-A cells. Experimental procedures were the same as for panel A. For each point, 200 cells were microinjected. Symbols: ○, 100 copies; ●, 200 copies; □, 400 copies.

even when one-quarter as many pT4TK molecules were injected per cell. Moreover, pT4TK injection gave rise to more positive cells than were injected. We believe that this and the high levels of expression can be explained by the capacity of pT4TK to replicate. Thus, if only a few pT4TK molecules reach the nucleus there is a high probability for replication and hence expression. High levels of replication and transfer of products to adjacent cells would explain the expression in noninjected cells.

A correlation existed between the percentage of TK-positive cells and the plasmid copy number for both cytoplasmic and nuclear injection (Fig. 3B). The limiting steps contributing to this linear relationship are unknown, but perhaps nuclear uptake is a limiting step in cytoplasmic injection. Dimethyl sulfoxide and glycerol have been shown to increase the efficiency of transformation in the calcium phosphate precipitation method (26) by approximately 1 order of magnitude. To see whether these agents enhanced the expression of TK in cells injected in the cytoplasm, we treated recipient cells with dimethyl sulfoxide, either immediately or 3.5 h after cytoplasmic injection, or with glycerol, 1.5 h after cytoplasmic injection, of porlTK. These treatments did not enhance TK expression, and our results are thus consistent with the idea that the enhancing effects of these agents are due to effects on the entrance of the precipitated exogenous DNA into the cytoplasm of recipient cells.

Stable transformation. Recombinant plasmids at various copy numbers were injected into the nuclei of LTK-A cells, and the cells were immediately cultured in HAT selective medium. From 20 to 50% of the cells injected with porlTK or pTKX1 divided several times and formed small colonies during the next few days, but subsequently most of the cells stopped dividing and died. A few surviving colonies gave rise to transformants, which were examined 2 weeks after injection. With pgtlTK and pT4TK, only 5 to 10% of the recipient cells formed small colonies, and a small number of these gave rise to transformants observed 2 weeks after injection.

Two weeks after microinjection, approximately 100 colonies were isolated, and more than 95 of these transferred colonies grew continuously in HAT medium. All (100%) of these transformants showed HAT resistance even after long cultivation in nonselective medium and were therefore called stable transformants. Based on these findings, we estimated the frequency of stable transformation by counting colonies still growing 2 weeks after microinjection (Fig. 4). The highest transformation frequency was 5%, obtained after injection of 40 copies of porlTK per cell. Transformation frequency decreased in the order porlTK > pgtlTK > pT4TK > pTKX1. With pTKX1, less than 0.5% of the recipient cells became stably transformed after injection of more than 100 copies per cell. With any of the plasmids, stable transformants were obtained only after injection of at least five copies of the plasmid per cell. The frequency of stable transformants increased rapidly with injected copy number and peaked at a copy number of 20 to 40 molecules per cell.

Although Capecchi (5) reported that 20% of the recipient cells became stable transformants after microinjection of SV40 recombinant plasmids, the maximal frequency in our system was 5%. We carried out the following experiments to

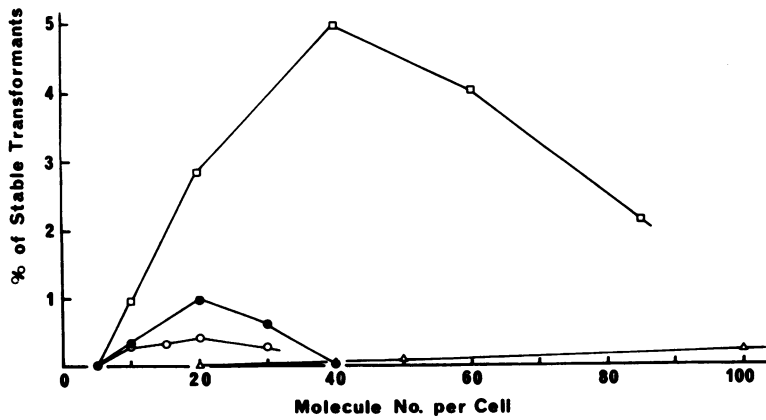

FIG. 4. Efficiency of stable transformation by nuclear microinjection. Different recombinant plasmids were microinjected into LTK-A cells. These cells were cultured in HAT medium immediately after microinjection. The medium was changed every 3 days. We counted the colonies that continued to grow in HAT medium 2 weeks after microinjection. For each point, 600 to 5,000 cells were microinjected. Symbols: □, porlTK; ●, pgtlTK; ○, pT4TK; △, pTKX1.

examine factors which might affect the frequency of transformation. To test the possibility that there is a subpopulation competent for stable transformation, we isolated six subclones from the original population of LTK-A cells. All of these subclones showed the same efficiency of stable transformation by porlTK found previously (Fig. 4). Wigler et al. (37) also found, for transformation by the calcium phosphate method, that their subclones all showed the same frequency of transformation as did the parent cell line. We used three different mutants of TK-deficient L cells as recipients, cell lines LMTK$^-$, LTK-A, and LTK-AJT. These lines had different frequencies of transformation in the calcium phosphate method with pTKX1 (unpublished data). The relative transformation frequency of LTK-A and LTK-AJT cells was roughly 10 and 100 times higher, respectively, than that of LMTK$^-$ cells. The transformation frequencies of these mutants were, however, the same after nuclear injection of porlTK. The differences in frequency by the calcium phosphate method may be due to differences in the frequency of entry of precipitated DNA. In another series of experiments, recipient cells were cultured in nonselective medium for 1 day after injection and then in HAT medium. This did not affect transformation frequency. The use of a richer medium, such as α-MEM, in place of DME did not increase the frequency. The frequency of stable transformation appears to be intrinsic to the plasmid construct in the nuclear microinjection system described here.

Stability of the transformants. The stabilities of the transformants shown in Fig. 4 were examined. Injected cells were cloned and grown in HAT medium for approximately 1 month. The cells were then grown in nonselective medium (HT medium) for different periods and plated in HAT and HT media, and the ratio of the number of colonies formed in each medium was plotted. The 17 clones transformed by different plasmids showed constant ratios near 1 (Fig. 5). One

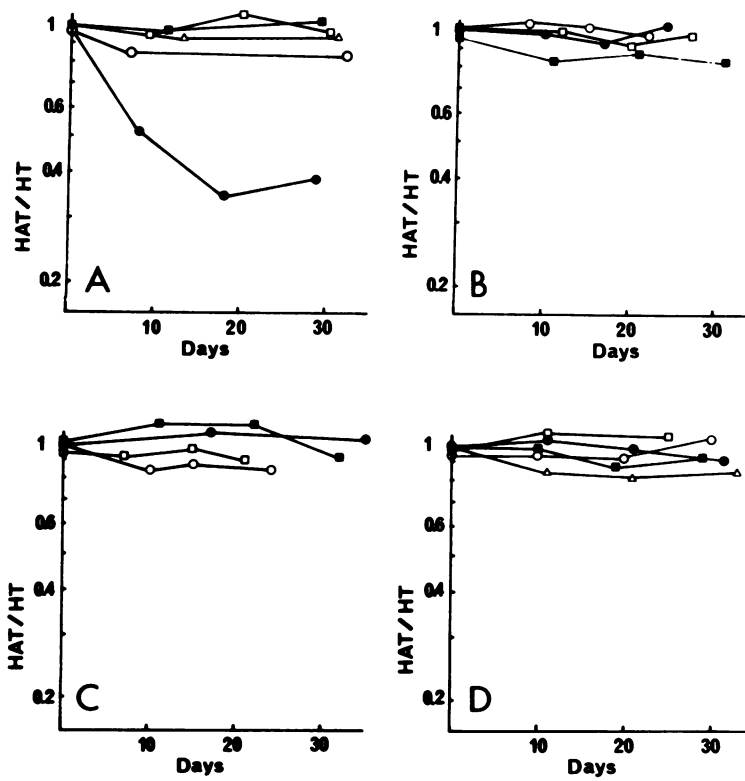

FIG. 5. Stability of the transformants. Transformants were cultured for about 1 month in HAT medium and then grown in normal medium (HT medium). The date that cells were transferred to HT medium was taken as day 0. On different days after the switch to HT medium, 500 cells were inoculated into duplicate T-25 Corning flasks in either HAT or HT medium. Ten days later, cells were stained with Wright solution. The ratio of the number of colonies in each medium was plotted against the duration of culture in nonselective medium. Transformants: (A) pTKX1, (B) porlTK, (C) pgtlTK, and (D) pT4TK. Symbols indicate separate clones.

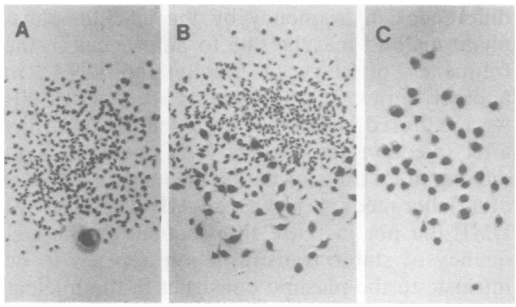

FIG. 6. Three types of subclones. (A) Negative subclone; (B) mixed type; (C) positive subclone. These subclones were derived from clone A-2-4 (Table 1). TK-positive cells swelled because of autoirradiation by the [^{3}H]thymidine incorporated over 2 days.

clone transformed with pTKX1 had an unstable phenotype initially, but became stable 20 days later. This clone might represent a mixed population of TK-positive and -negative cells. These results showed that all the clones that were capable of extended growth in HAT medium were stable in their expression of TK.

Time course of stabilization. To fix the time at which recipient cells became stably transformed, we cultured microinjected cells for different periods of time, ranging from 2 to 6 days, in HAT medium after microinjection and then in nonselective medium for 20 days. The cells in 1-cm dishes were trypsinized, plated on small cover slips, and cultured for an additional 4 days in normal medium and then for 2 days in medium containing [^{3}H]thymidine. If stabilization took place immediately after microinjection, all the subclones on the cover slip would be TK positive by autoradiography. On the other hand, if stabilization had not yet occurred when the HAT medium was changed to normal medium, the majority of subclones would be TK negative. If the stabilization process was in progress at the time of medium change, some subclones would be mixed and others totally positive or negative. In addition, on any given cover slip, a fraction of the cells would be TK positive and others TK negative depending on whether stabilization had occurred throughout a colony at the time of removal of selective pressure. Figure 6 shows autoradiographs of three groups of cells derived from three different colonies 3 days after HAT selection. All three patterns of TK expression were observed: TK-negative (Fig. 6A), mixed population of TK-positive and TK-negative (Fig. 6B), and TK-positive cells (Fig. 6C).

When cells were selected for 2 days in HAT medium, all five of the colonies studied produced TK-negative cells (Table 1). When cells were selected for 3 days in HAT medium, one of the six colonies studied produced 100% TK-positive cells (clone A-2-2). We concluded that that clone had stabilized wtihin the first 3 days after microinjection. Two colonies (clones A-2-4 and A-4-7) produced a mixed pattern of TK expression, and we concluded that these clones were in the process of stabilization on day 3 after microinjection. When cells were selected for 6 days in HAT medium, four of six colonies produced only positive cells, and the other two colonies produced a mixed cell population. These data indicate that stabilization of the microinjected DNA progressed over a period of several days in recipient cells incubated in HAT medium and was largely complete by day 6 after microinjection.

Expression of the large T antigen in cells injected with pT4TK. pT4TK contained the replication origin and the entire early region of the polyoma virus genome. LTK-A cells injected with this plasmid were cultured for 1 day in normal medium, and the expression of the large T antigen was then examined by immunofluorescent staining. A double-antibody labeling technique was used, with polyoma virus antitumor antiserum in the initial adsorption, followed by rabbit anti-rat IgG antiserum. The percentage of fluorescent cells was studied in relation to the number of pT4TK molecules injected (Fig. 7). Injection of 20 plasmid molecules per cell was required to obtain 50% fluorescent cells; this contrasted with only one plasmid molecule being required for TK expression. Why a 20-fold-

TABLE 1. Colony type distribution

Days of culture in HAT medium	Clone no.	% of colonies that were[a]:		
		TK$^+$	Mixed	TK$^-$
2	n-3-1	0	0	100
	B-1-6	0	0	100
	B-1-7	0	0	100
	B-4-1	0	0	100
	B-4-4	0	0	100
3	A-2-2	100	0	0
	A-2-4	9	37	54
	A-4-7	3	28	69
	A-1-2	0	0	100
	A-3-1	0	0	100
	A-3-8	0	0	100
6	z-1-3	100	0	0
	z-1-4	100	0	0
	z-1-8	100	0	0
	z-2-8	100	0	0
	z-2-2	52	23	23
	z-2-4	38	36	26

[a] TK$^+$, All cells in subclone were TK positive; mixed, both TK-positive and TK-negative cells in subclone; TK$^-$, no cells in subclone were TK positive.

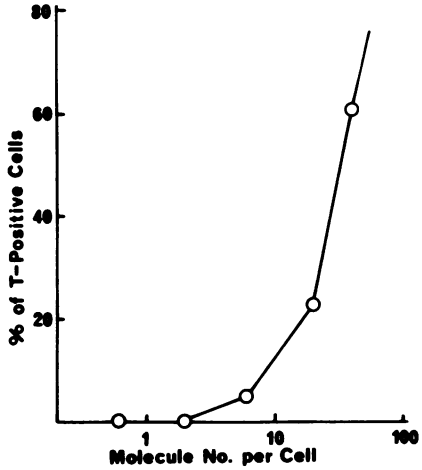

FIG. 7. Transient expression of the large T antigen of polyoma virus. LTK-A cells were microinjected in the nucleus with various numbers of pT4TK and cultured in normal medium for 1 day. The expression of the large T antigen of polyoma virus was examined by indirect immunofluorescent staining. For each point, 100 to 200 cells were microinjected.

higher injected plasmid copy number was required for large T antigen expression than for TK expression is unclear. The most likely explanation is that the assay for large T antigen is less sensitive than that for TK activity. However, it is also conceivable that fewer molecules of large T antigen than of TK are synthesized. This might reflect a difference in the activity of promoters controlling the transcription of the two coding regions. Finally, there is the possibility that turnover of the large T antigen is more rapid than that of TK.

To follow the expression of the large T antigen over time, we cultured cells injected with pT4TK for different periods of time and stained them for T antigen immunofluorescence (Fig. 8). The recipient cells were observed to change size for 5 days after microinjection, showing progressive enlargement of both the cytoplasm and nucleus. These cells subsequently died and became detached from the culture dish. T antigen-positive cells were examined continuously in culture dishes marked with grids. No dividing cells were detected. At 2 weeks after the injection of pT4TK, TK-positive transformants arose, and these were examined for T antigen immunofluorescence. Eleven independent TK-positive clones were examined, and in no case was T antigen fluorescence detected.

DISCUSSION

The experiments described here showed that the polyoma virus origin of replication sequence profoundly affected the transformational characteristics of the plasmids into which it was inserted. We observed a significant elevation in both TK expression and TK transformation. When expression of the TK gene was measured 24 h after injection, the frequency of expression was increased by a factor of 30: one in two of the cells injected with a single origin-containing plasmid expressed TK; every cell expressed it when four or more molecules were injected. The addition of the small and middle T antigen functions (plasmid pgt1TK) did not modify the properties of plasmids bearing the origin alone. However, the inclusion of the entire early region, including the large T antigen coding sequence (plasmid pT4TK), led to the expression of TK even in noninjected cells. The recruitment of noninjected cells to the TK^+ phenotype may be the result of the transfer of DNA, RNA, protein, or metabolized nucleotide from overproducing injected cells to neighboring cells. Overproduction may be the consequence of the high-level extrachromosomal replication of the pT4TK plasmid in mouse cells. Polyoma virus genome replication is known to be dependent on large T antigen expression (11).

The introduction of the polyoma virus origin clearly affected plasmid TK expression. Three functions may explain this: (i) replication, (ii) promoter function, and (iii) enhancer function.

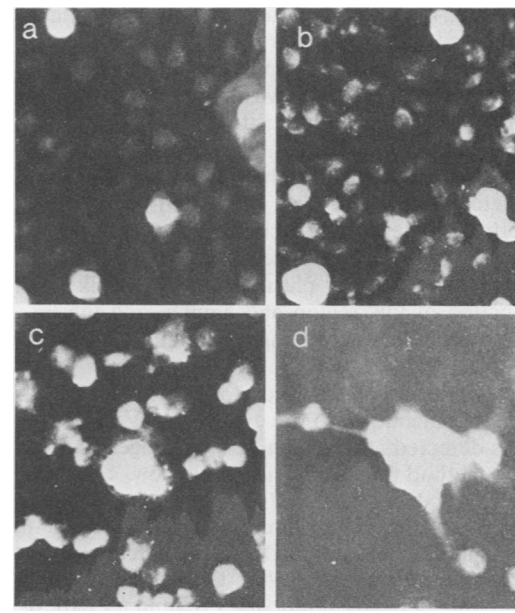

FIG. 8. Expression of the large T antigen in LTK-A cells. Cells were microinjected in the nucleus with 20 copies of pT4TK and cultured for 1 (a), 2 (b), 4 (c), or 5 days (d) in normal medium. They were fixed and stained by an indirect immunofluorescent staining method.

Replication would seem to be an unlikely explanation, since the origin sequence does not permit detectable viral DNA synthesis in virus-infected mouse cells in the absence of functional large T antigen (11). A better case can be made for promoter or enhancer activity.

All three origin-containing plasmids had the polyoma virus early and late region promoter sequences. In all three plasmids, the TK gene was joined to polyoma virus DNA in the late coding region and oriented so that the direction of TK transcription was the same as polyoma virus late region transcription (Fig. 1). Thus, the late promoter could serve to increase TK activity. Against this notion is the observation that proteins encoded by the late region were generally detected after the synthesis of large T antigens. However, exceptions to this rule have been reported with SV40 (4, 10, 17) and may be extended to polyoma virus. Therefore, although the possibility that origin promoters are involved in activating TK is not compelling, it cannot be excluded.

The polyoma virus origin contains a transcriptional enhancer function (8, 34). De Villiers and Schaffner (8) reported that when a 244-base-pair sequence located in the proximal portion of the polyoma virus late region was introduced into a plasmid molecule containing the rabbit β-globin gene, transcription of the globin gene was significantly increased. The effect was observed between 24 and 60 h after calcium phosphate-mediated gene transfer. These effects were observed both when the polyoma virus sequence was placed contiguous with the globin gene and when it was remotely positioned in the plasmid molecule. Tyndall et al. (35) constructed mutants with portions of the 244-base-pair region deleted and found that the deletions affected both early region transcription and viral DNA replication. The enhanced early expression of the TK gene in our plasmids was most likely a function of this region and emphasized the importance of characterizing it in more detail in the future.

TK expression was monitored after the injection of plasmids into the cytoplasm. Expression was detected after a significantly longer time by this method than by nuclear injection. This may be the consequence of a barrier to nuclear uptake of the cytoplasmic DNA. With calcium phosphate-precipitated DNA, the calcium phosphate-DNA complex enters the nucleus rapidly (22), independent of mitosis. Preliminary experiments on our polyoma virus plasmid constructs indicated that cytoplasmically injected DNA was rapidly degraded. Radiolabeled plasmids showed a half-life of only 3 to 4 h as determined by autoradiographic studies. It should also be emphasized that stable transformants were not obtained from cells that had as many as 400 plasmids injected into the cytoplasm. All of these observations suggest the existence of a barrier to nuclear entry that few injected molecules pass. Dimethyl sulfoxide and glycerol treatments did not affect this barrier, supporting the idea that these agents affect the endocytotic uptake of calcium phosphate-DNA precipitates (22).

Polyoma virus DNA segments increased the frequency of stable TK transformation of mouse cells after nuclear injection. The frequency of stable transformation measured 2 weeks after the injection of origin-containing plasmid molecules was several orders of magnitude greater than that obtained with a plasmid containing the TK segment alone. The por1TK plasmid produced a significantly greater frequency of stable transformants than did plasmids containing in addition the coding regions for the small and medium T antigens (pgt1TK) or the entire early region (pT4TK). In all instances, the injection of at least five plasmid molecules per cell was required for stable TK transformation. Four mechanisms may be considered as explanations for the transformation-enhancing action of the origin segment. (i) We believe that replication itself can be ruled out, since it is unlikely that replication occurs in the absence of the large T antigen gene; the presence of the large T antigen was in fact correlated with low transformation efficiency. (ii) Promoter activity also seems unlikely, since a greater effect would be expected when the large T antigen is present, enabling activation of the late promoter, whereas the opposite was observed. (iii) There is the possibility that origin sequences foster plasmid integration in the host cell genome, but there is no direct evidence to support this possibility. (iv) Transcriptional enhancer sequences may contribute to transformation efficiency, but if this is the case, the low transformation efficiency of plasmids containing the origin plus additional segments must be explained. It is likely that the segments encoding the T antigens do in fact depress stable transformation. The middle T antigen has been reported to be involved in the malignant transformation of cells (34). In our studies, this function appeared to alter cell growth, since pgt1TK-injected cells became significantly larger than por1TK-injected cells.

The large T antigen permitted viral DNA replication to occur in the presence of the origin. We suspect that cells injected with the entire early region were injured by the overproduction of plasmid molecules, possibly as a consequence of TK toxicity. In somatic cells the synthesis of endogenous TK is strictly limited to the S phase of the cell cycle (30), whereas in the injected cells HSV TK was synthesized constitutively.

Toxicity from overproduction of TK is consistent with the observation that the frequency of stable TK transformants with any of the polyoma virus DNA plasmids declined when the plasmid copy number exceeded a certain point (Fig. 4). We also noted that pT4TK stable transformants invariably did not express the large T antigen, suggesting that it is incompatible with stable transformation. Although this may be due entirely to toxicity from plasmid replication, it is also likely that the large T antigen function is incompatible with the stable integration of origin-containing plasmid DNA into the recipient cell genome.

In conclusion, we showed that the origin sequence of polyoma virus had a profound effect on both the expression and transformation efficiency of plasmids microinjected into mouse cell nuclei. We believe it is likely that expression and transformation efficiency are correlated and that the success of transformation depends on appropriate levels of TK. It is likely that enhancer sequences in the origin segment control these properties in an impartial way. Future experiments will focus on a detailed analysis of the polyoma virus enhancer sequences and their effect on transformation and expression.

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