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Characteristics of an SV40-Plasmid Recombinant and Its Movement into and out of the Genome of a Murine Cell

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Summary

A bacterial plasmid carrying the early region of SV40 (pOT) has been stably established in high molecular weight (hmw) DNA of mouse L cells by selection for the herpes virus thymidine kinase (tk) gene. DNA blotting has demonstrated that most cell lines contain multiple discrete copies of pOT, generally with an intact SV40 early region. No free copies of pOT have been detected. Both pOT and tk sequences may be amplified up to 20–200 copies of the SV40 early region. In contrast to the uniform staining pattern normally observed in SV40-transformed lines, indirect immunofluorescence using antiserum to the SV40 T antigen has demonstrated that the expression of the early region is heterogeneous in these cell lines. This fraction expressing T is characteristic of a given cell line, and varies from 0 to 99% positive. Several pOT cell lines have been fused to simian cells, and replicating low molecular weight DNAs were isolated from the heterokaryons. Transformation of E. coli with this DNA demonstrates that pOT can be rescued from hmw DNA in L cells and reestablished as a plasmid in E. coli. Excision is generally precise when pOT is introduced to the murine cells as a supercoiled molecule, and imprecise when pOT is introduced in linear form.

Introduction

DNA can be introduced into certain animal cell lines as a calcium phosphate/DNA co-precipitate (Graham and van der Eb, 1973). If a selectable marker is included along with nonselected DNA sequences, cells stably acquiring exogenous DNA can be identified and cloned by selection for that marker. This technique has been used for stably introducing the HSV thymidine kinase gene as well as nonselected procaryotic and eucaryotic sequences into high molecular weight (hmw) DNA of mouse Ltk⁻ cells (Mantei, Boll and Weissmann, 1979; Wigler et al., 1979a), a process we shall refer to as "convection."[1]

We have constructed bacterial plasmids which carry the early region of the virus SV40, and have stably established these DNAs in the genome of mouse L cells by selection for the herpes virus thymidine kinase gene. We have used these plasmids to examine the expression of genes *non*selectively introduced into somatic cells by the process of convection, as it is possible to follow the expression of SV40 sequences in individual cells by visualizing the viral T antigen by indirect immunofluorescence, and to study the feasibility of retrieving convected DNA sequences through SV40-mediated excision and replication of plasmid sequences upon fusion of the mouse cells to permissive simian cells or by a direct biochemical approach. The bacterial DNA sequence component allows the establishment of rescued molecules as plasmids in E. coli. (These experiments are represented schematically in Figure 2.) A number of surprising characteristics have been observed, relating both to the rescue of these plasmids from mouse cells and to the expression of the SV40 early region.

Results

Convection of pOT into Ltk⁻ Cells

Three recombinant pBR322 plasmids were constructed and used in these studies. pTK2 is a plasmid containing the Bam 3.4 kb herpes virus thymidine kinase gene (tk), pOT is a plasmid containing the entire early region of SV40 DNA and pOT-TK is a plasmid containing both the herpes virus tk gene and the SV40 DNA sequences contained in the pOT plasmid. The plasmid structures are diagrammed in Figure 1. To isolate cell colonies which had incorporated the SV40-containing plasmids into their genomes after convection, thymidine kinase selection was used as described by others (Maitland and McDougall, 1977; Wigler et al., 1977; Minson et al., 1978).

The plasmid pOT was convected into mouse Ltk⁻ cells as either a circular or a linear molecule in order to compare the characteristics of plasmids rescued from convected lines by the methods of SV40 rescue and plasmid rescue. SV40 rescue refers to excision mediated by the SV40 early region upon fusion with

[1] We define convection as the general process of gene transfer wherein DNA is introduced into eucaryotic cells and stably established in hmw DNA. The terms transfection and transformation each have distinct biological definitions that can be confusing when applied to describe the general acquisition of exogenous DNA sequences by the genome of a cell. Transfection has been used in the past to describe biochemically mediated absorption of viral DNA by cells, while transformation has a special meaning in the literature of mammalian cell culture, generally referring to the process by which cells change their normal patterns of growth regulation. Furthermore, convection techniques can be used to introduce DNA into cells without having any measurable phenotypic effect.

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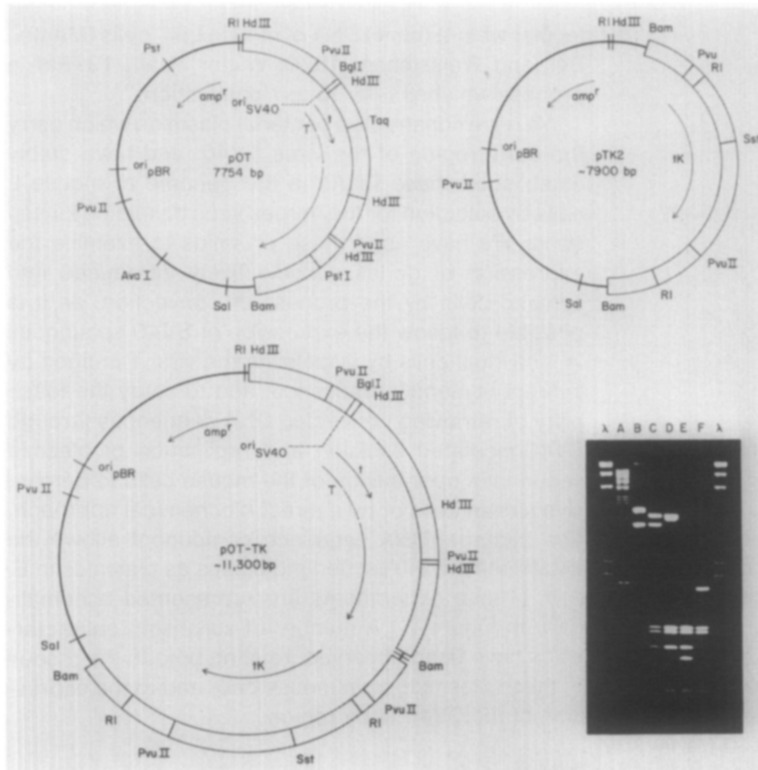

Figure 1. Restriction Maps of the Recombinant Plasmids pOT, pTK2 and pOT-TK

Characteristic restriction sites are indicated to describe the derivatives of pBR322 carrying the early region of SV40 (pOT), the HSV thymidine kinase gene (pTK2) or both (pOT-TK). At the lower right, an ethidium bromide-stained 0.7% agarose gel compares the cloned viral sequences to the viruses by digestion with restriction enzymes which are diagnostic for these particular sequences: (λ) λ DNA/Hind III; (A) HSV DNA/Bam; (B) pTK2/Bam; (C) pOT-TK/Hind III + Bam; (D) pOT/Hind III + Bam; (E) SV40 DNA/Hind III + Bam; (F) SV40 DNA/Hind III.

simian cells, followed by extraction of low molecular weight (lmw) DNA (Hirt extraction) and subsequent transformation of E. coli with this DNA (Figure 2). Plasmid rescue involves digestion of hmw cellular DNA with a restriction enzyme and ligation of the restricted DNA under conditions that effect cyclization of linear molecules, followed by transformation of E. coli, which selects those rare molecules that include the bacterial plasmid (Figure 2).

The pOT plasmid was introduced into Ltk$^-$ cells as a calcium phosphate DNA co-precipitate that included the 3.4 kb Bam fragment of pTK2 (in a ratio of 250:1, pOT:tk). Cells that stably acquired the exogenous tk gene were selected in HAT medium. A number of colonies were cloned and established as cell lines. These lines were examined by DNA blotting to determine whether pOT sequences had become established in hmw DNA. Figure 3 shows blots to six representative cell lines using Xba, a restriction enzyme that does not cut pOT. Duplicate blots were probed with nick-translated pBR322 or SV40 to allow some estimation of the extent of rearrangement occurring upon convection of pOT into these cells. pOT is 7754 bp in length, of which 52% is derived from pBR322 and 48% from SV40. In this experiment, equal amounts of probe, nick-translated to the same specific activity (2×10^8 dpm/μl), were used in the hybridizations, which were performed in parallel under identical conditions with the same preparations of restricted DNAs. All six cell lines contain sequences hybridizing to both pBR322 and SV40, and every line contains multiple discrete insertions.

Furthermore, it is apparent from the ethidium bromide-stained gels that equal amounts of the DNAs were analyzed. Yet the range of intensities of the hybridizing bands suggests that many of the integrants are present in multiple copies. For example, reconstructions have demonstrated that the insertion containing SV40 in D9 is present in single-copy amounts. Thus we conclude that the discrete insertions in lines such as C10 and D4 are present in many copies per cell (see Figure 5).

Many of the insertions appear to have equal quantities of SV40 and pBR. On the other hand, every line contains at least one band that hybridizes preferentially to SV40 or pBR, indicating that some of the pOT molecules have suffered appreciable rearrangement during convection. For example, D9 has a small Xba band which hybridizes very strongly to pBR and not at all to SV40 (see also structure of rescued plasmid C10, below). The fragmentation of some pOT molecules during convection is similar to that observed for SV40 viral transformations (Botchan et al., 1974).

In these analyses, the gels were treated with HCl prior to denaturation with NaOH, under conditions which have been shown to depurinate DNA partially and result in efficient blotting of supercoiled, circular and large DNAs (Wahl, Stern and Stark, 1979b). Since large DNA fragments are clearly blotting efficiently, we would expect to see any pOT that was persisting

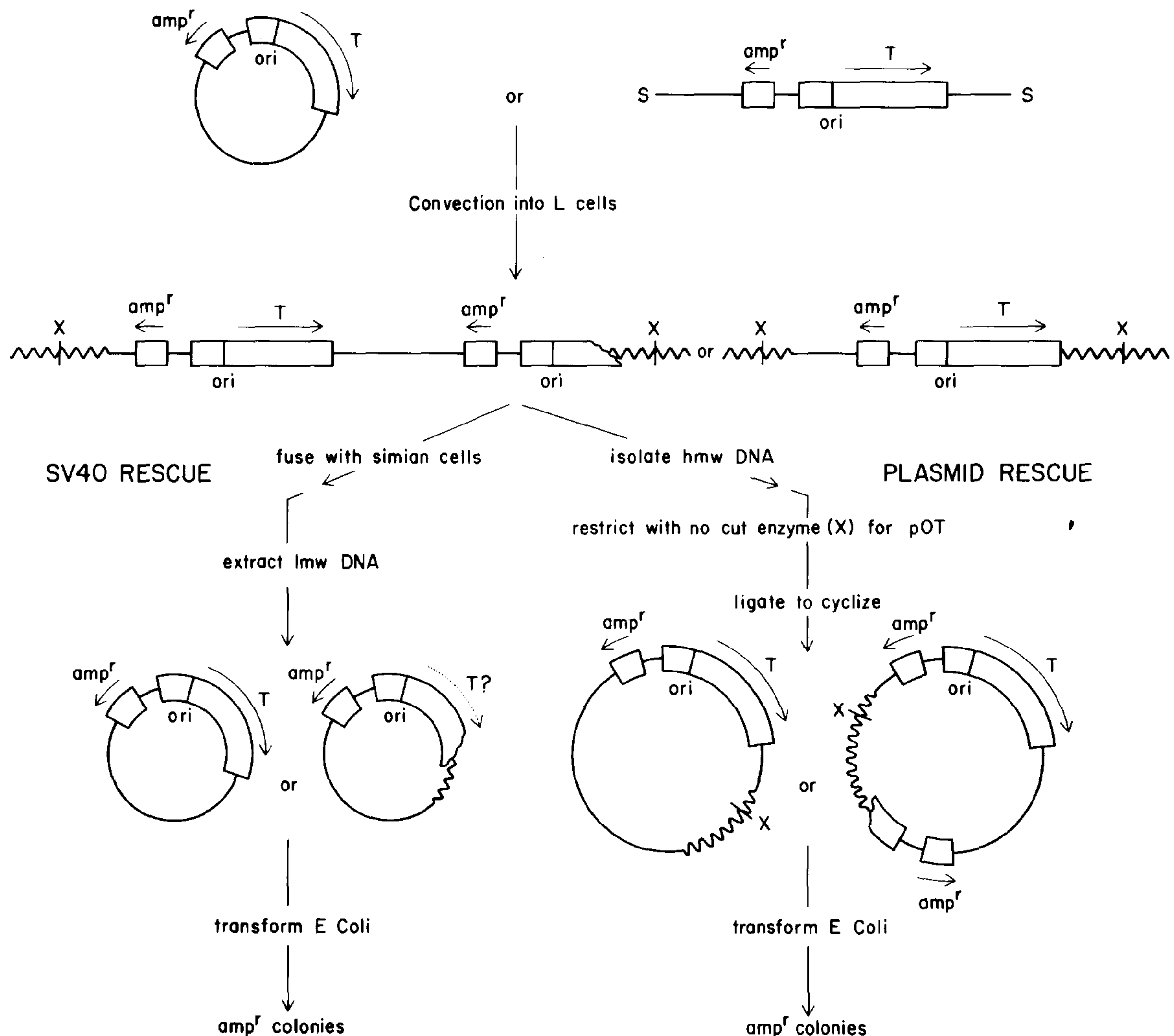

Figure 2. Schematic Illustrations of Alternative Strategies for Rescuing pOT from hmw DNA in L Cells

In analogy to studies on SV40 transformation, the scheme suggests that pOT may become integrated with or without a partial tandem duplication, which would then affect the structures of the plasmids obtained by either SV40 rescue or plasmid rescue. In the experiment diagrammed, the plasmid DNA is presented to the cell either as a circular molecule or as a molecule linearized by digestion with Sal I (S).

as a free circular molecule in these cloned cell lines. In addition, we have extracted lmw DNA from these cell lines using the Hirt extraction procedure (Hirt, 1967), analyzed the resultant DNA for pOT both by blotting and by transformation of E. coli, and have found no evidence for pOT existing in a free state.

Analysis of the Expression of T

Indirect immunofluorescence allows evaluation of the presence and distribution of antigenic determinants at the level of individual cells. In the present studies we have used a T antiserum raised in rabbits against the D2 protein, which is a 107 megadalton protein encoded in the genome of the adeno-SV40 hybrid virus Ad2⁺D2 (Tjian, 1978; Hassell et al., 1978). When rabbit antiserum to the D2 protein is applied to SV40-transformed cell lines and visualized with fluorescein-conjugated goat anti-rabbit gammaglobulin, bright nuclear fluorescence is observed. Nontransformed parental lines show no appreciable nuclear staining, although on occasion cytoplasmic fluorescence can be seen in parental cell lines or in transformed cells. The D2 antiserum provides a reasonable and sensitive assay for the presence of T in cells (D. Lane, manuscript in preparation). This antiserum has been used in all the studies described below. In addition, the results have been confirmed using monoclonal antibodies to specific determinants on the D2 protein, which specifically immunoprecipitate authentic T from transformed cells (D. Lane, manuscript in preparation).

Thirteen cell lines arising from the convection of

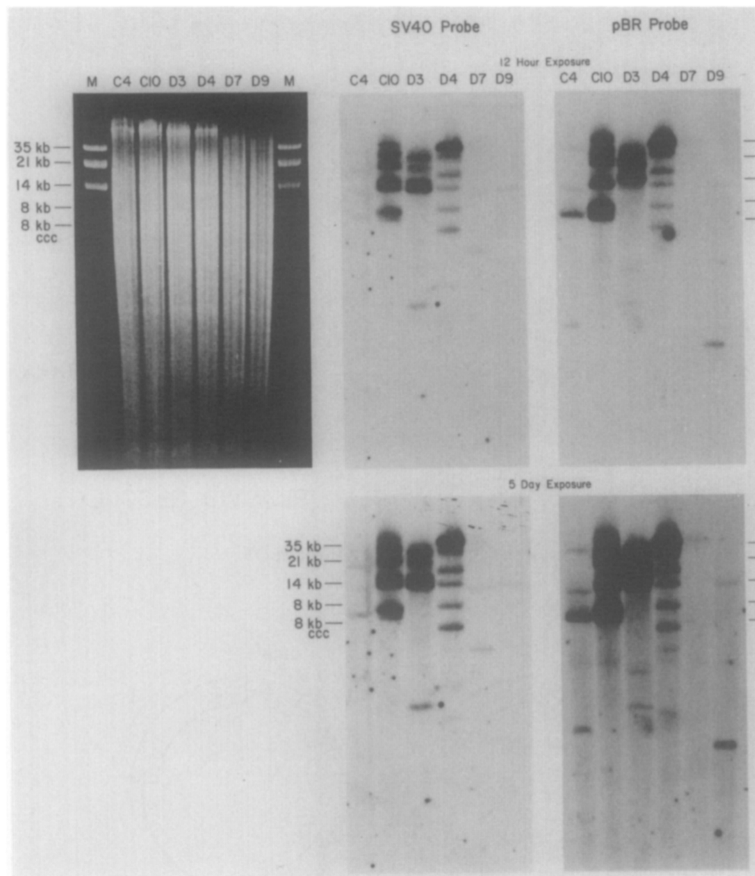

Figure 3. Analysis by DNA Blotting of the Physical State of pOT in Six Representative Lines

High molecular weight DNAs were extracted from cell lines arising from convection with supercoiled pOT (D lines) or linear pOT (C lines). 25 μg of each DNA were digested with Xba, and half of each digestion was fractionated on one of two 0.5% agarose gels which were run simultaneously. Both were treated with HCl prior to denaturation, and then the DNAs were transferred to nitrocellulose filters and hybridized with either an SV40 or a pBR probe (spec. act. 2×10^8 dpm/μl; 10 ng/ml final concentration). Autoradiography was performed using a single intensifying screen with XR film at $-70°C$ for the times indicated. One of the ethidium bromide-stained gels is shown at the left, aligned with the autoradiographs. The markers in channel M are Ad5 DNA (35 kb) and Ad5 DNA/Bam (21 kb + 14 kb). 8 kb/ccc denotes the position of supercoiled pOT. Xba does not cut within pOT. Therefore, the number of bands correlates with the number of discrete insertions. Many of the discrete insertions create intense hybridization bands after a 12 hr exposure, and are certainly present in multiple copies.

pOT into Ltk$^-$ cells were examined for T expression using indirect immunofluorescence. Surprisingly, expression of T was found to be heterogeneous—some nuclei stained distinctly while adjacent cells were completely negative for T-specific nuclear fluorescence (Figure 4).

Within a given cell line, considerable variation was observed in the intensity of nuclear staining. The spectrum ranged from a very bright intensity characteristic of that observed in cells selected for transformation by SV40 down to a very diffuse staining. The most striking feature of the T staining was the completely dark nuclei observed in some of the cells, comparable to the negative staining of parental Ltk$^-$ nuclei.

The fraction of nuclei scoring as negative varied considerably among the cell lines examined (Figure 4 and Table 1). Cells convected with either circular or linear pOT showed heterogeneity of T expression. All lines showed either heterogeneous expression or no expression of T. The extent of heterogeneity ranged from 0 to >99% positive.

At the time of the initial immunofluorescence assay, C4 and D3 were completely negative, D4 had 10% positive nuclei and C10, D7 and D9 were 50, 80 and 99% positive for T, respectively. Since DNA blotting of these six lines demonstrated that both D3 and D4 contain multiple distinct integrants that hybridize strongly to SV40, we examined whether the SV40 early region remained intact in these lines. DNAs from six lines were blotted and probed with SV40 after digestion with RI + Bam, which releases the SV40 sequences from pOT (see Figure 1). As shown in Figure 5, most lines contain at least one intact copy of the SV40 early region by this criterion. The detection of the pOT RI + Bam fragment, which contains SV40 DNA, provides a stringent test for the maintenance of an intact early region, since the sequences between RI and Pvu II are inessential for its expression (see Figure 1). Line D7 indeed contains an intact early region, as indicated by the immunofluorescence to T in Figure 4, and this has been confirmed both by other blotting experiments on this DNA and by direct immunoprecipitations of T antigen from these cells. A single-copy reconstruction of RI + Bam-cut pOT (channel R) indicates that C10, D3 and D4 have multiple copies of the early region, while D9 and probably D7 contain a single copy (Figure 5). From these results we conclude that fragmentation of the early region is not likely to be responsible for the heterogeneity of T expression, although lines such as C4 may be completely negative for these reasons.

One explanation for the heterogeneity of T expression is that the early region is being stably and perhaps

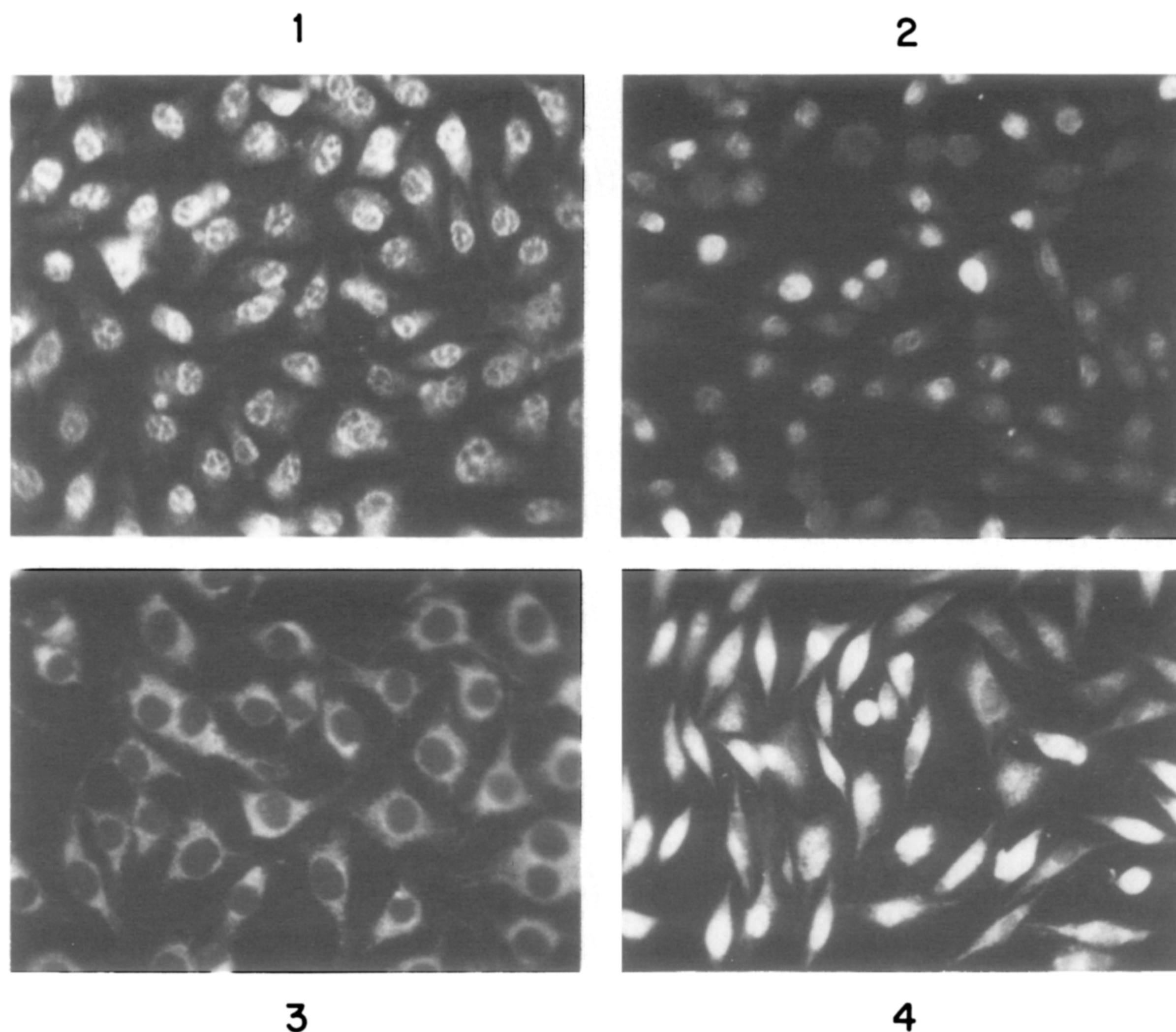

Figure 4. Representative Photographs of the Immunofluorescence Assay for T

(1) D9 at the initial staining (see footnotes to Table 1); (2) a D4 subclone (D4.14); (3) D3 at the initial staining; (4) P2, a pOT-TK line, at the second passage after the initial immunofluorescence assays.

irreversibly inactivated in negative cells. A clear prediction of this explanation is that it should be possible to subclone complete and stable negatives from a heterogeneous line. To test this possibility, we have subcloned cells from the heterogeneous lines D4, D7 and C10, which were originally 10, 80 and 50% positive. Individual cells from these lines were cloned in microwell dishes, and colonies arising from each were examined for T expression (Table 2).

D7, originally 80% positive, was subcloned, and all of the 33 subclones examined were positive and heterogeneous. The cell line C10 was sublconed and 41 subclones were scored. All were positive and heterogeneous, and the extent of heterogeneity ranged from 1 to 99% positive. Subsequent passages of C10 itself have also been scored, and the fraction of positive cells has risen to about 90% positive.

Sixteen subclones of D4 (originally 10% positive) were analyzed: 12 scored as completely negative, and 4 scored as positive and heterogeneous. Upon examination of the D4 negative subclones after several passages, one subclone was found to have a cluster of positive cells on the edge of an otherwise completely negative colony. Several other negative subclones were passaged further, and all of these have eventually begun to exhibit heterogeneous expression of T. One example is shown in Figure 4. All the above observations provide strong evidence that the heterogeneous expression arises from both switching on and switching off of the early region, and is not the result of irreversible gene inactivation or complete loss of all pOT sequences.

Further support for these conclusions comes from the examination of D3, which was completely negative at the initial scoring. Yet D3 has multiple copies (20–100) of the intact early region. After five passages,

Table 1. Expression of T as Assayed by Indirect Immunofluorescence

Cell Line	Form and Type of Convected DNA	Fraction of T-Positive Nuclei[a]			Excision[b] upon Fusion
		Initial[c]	2nd Passage[c]	5th Passage[c]	
C1	linear pOT	20	ND	ND	ND
C2	linear pOT	0	ND	ND	ND
C4	linear pOT	0	ND	ND	–
C5	linear pOT	5	ND	ND	+
C7	linear pOT	20	ND	ND	+
C9	linear pOT	0	ND	ND	–
C10	linear pOT	50	99	95	++
D3	supercoiled pOT	0	0	5	–
D4	supercoiled pOT		10	10	+
D6	supercoiled pOT	0	ND	ND	–
D7	supercoiled pOT	80	75	80	++
D8	supercoiled pOT	0	ND	ND	ND
D9	supercoiled pOT	99	99	99	+++
P2	supercoiled pOT-TK	ND	40	ND	ND
P4	supercoiled pOT-TK	5	~0	0	ND
P5	supercoiled pOT-TK	99	99	99	ND
P6	supercoiled pOT-TK	0	ND	20	ND
P7	supercoiled pOT-TK	5	10	5	ND
P8	supercoiled pOT-TK	90	99	80	ND

[a] An average value, obtained from the scoring of cells stained at various stages ranging from discrete colonies to confluence.
[b] As detected by DNA blotting one tenth of the Hirt supernatant from the extraction of three 100 mM plates of fused cells.
[c] tk^+ colonies were picked, established as cell lines and passaged (1:5) ten times. At this point the initial immunofluorescence and fusion experiments were carried out. 2nd and 5th passages refer to subsequent passages from this point.

this completely negative line was observed to become heterogeneous for T expression—about 5% of the cells now stain brightly for T. This again demonstrates that the control of expression arises from some complex mechanism and that the frequency of switching is irregular.

Heterogeneous expression may reflect either a specific cellular control of the early region or a general nonspecific effect arising from the environment or other aspects of the convected DNA. We have also examined the pattern of T expression when the early region was linked to tk in the plasmid pOT-TK. pOT-TK was convected into Ltk^- cells and tk^+ colonies were established as cell lines. Again, the expression of T is heterogeneous, as demonstrated by indirect immunofluorescence (Figure 4, Table 1). Thus proximity of the early region to the active tk gene in the input plasmid is not sufficient to assure high levels of continuous expression. The cell line P6, which was completely negative at the initial scoring, has subsequently become quite positive for T, confirming the observations and conclusions on D3 and the D4 negative subclones. These experiments on expression are necessarily incomplete and we do not know whether, on those molecules where SV40 is being expressed, the tk gene is being expressed actively or, for that matter, what the general level of tk expression is in relation to SV40. What we have demonstrated is that in the absence of direct selection for the SV40 T antigen, patterns of expression novel to this type of system are observed, and these peculiarities of heterogeneous expression are not overcome by direct linkage of the SV40 genes to the selected tk marker.

SV40 Rescue

A representative set of the pOT-convected L cell lines was tested for the capability to produce supercoiled pOT molecules when fused with simian cells. Generally, five plates were each seeded with 1×10^6 L cells and 2×10^6 CV1 cells, which were allowed to attach and then induced to fuse with polyethylene glycol. The heterokaryons were incubated for several days, and supercoiled lmw DNAs were collected using the Hirt extraction procedure. One fifth of each supernatant was run uncut on a 0.7% gel, and then blotted and hybridized to nick-translated pOT. A representative blot of the excision products obtained from several fusions is shown in Figure 6 (see also Table 1). Several observations can be made from analysis of the blots.

—Molecules related to pOT can be detected in some of the Hirt supernatants.

—The relative amounts of DNA present were variable,

and roughly paralleled the relative heterogeneity of T expression in the cell lines (Table 1). Thus D3, D6, C4 and C9 gave no detectable plasmids, D4, C1 and C5 gave moderate amounts and C10, D7 and D9 showed considerable amounts of low molecular weight species hybridizing to pOT.

—In fusions to the C lines, which were convected with linear pOT, a distinctly heterogeneous population of molecules arises, whereas in the D lines, convected with supercoiled pOT, bands co-migrating with pOT are observed.

—Finally, the yields of DNA from these fusions are about 20–100 fold less than that observed when wild-type SV40 is similarly rescued from transformed cells. For example, D9 releases about 0.5–1.0 ng of pOT per 100 mM plate, while pOT-D4 releases about one tenth of that amount. In comparison, SV40-transformed cells release about 10–20 ng of SV40 DNA per plate in a similar fusion.

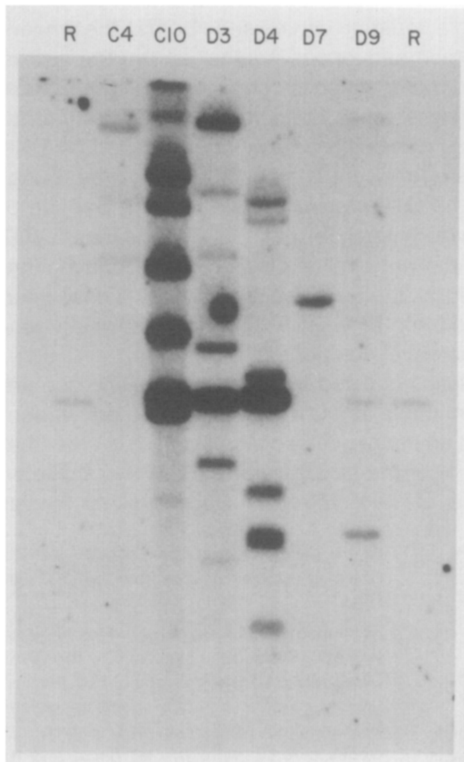

Figure 5. Detection of the SV40 Early Region

12 μg of each DNA were digested with RI + Bam, which releases the early region from pOT (Figure 1). About 10 pg of pOT were similarly digested in order to approximate a single-copy reconstruction (R). The DNAs were fractionated on a 0.7% agarose gel and blotted, using nick-translated SV40 as a probe. As indicated by optical density as well as ethidium bromide staining of the gel, all six cellular DNAs were present in equal amounts. RI + Bam digestion provides a stringent test for the maintenance of an intact early region, since the sequences between RI and Pvu II are inessential for its expression (see Figure 1).

Figure 6. Blotting Analysis of Excision following Fusion

Representative evaluation for the presence of pOT-related molecules in the Hirt supernatants that were extracted following fusion of pOT lines with simian cells. One tenth of the total isolate from three 100 mM plates was run uncut in a given lane on a 0.7% agarose gel, and blotted using nick-translated pOT DNA as a hybridization probe. The tracks are as follows: (R) reconstruction with 10 pg of pOT DNA; (A) a C10 fusion; (B) another C10 fusion; (C) a C5 fusion; (D) a D4 fusion; (E) a C7 fusion.

Table 2. Subcloning Experiment

Parent Cell Line	T-Positive Nuclei in Parent Line (%)	Number of Subclones	Number of T-Positive Subclones at Initial Scoring	Notes on Subclones
D4	10	16	4	Four negatives were randomly chosen for subsequent passage. All became ~10% T-positive after a few passages.
C10	50	41	41	Considerable variation in extent of heterogeneity among subclones (range 1–99%, most about 50%).
D7	80	33	33	Variation both in extent of heterogeneity and intensity of staining among subclones.

The excision products of the fusions have been introduced into E. coli and thus tested for the maintenance of biological activity of bacterial plasmid functions upon passage through L cells. The Hirt supernatants were treated extensively to remove all SDS and cellular matter. The DNAs were then used to transform E. coli χ1776 using a high efficiency transformation procedure (D. Hanahan, manuscript in preparation). In some cases, ampicillin-resistant colonies arose, and these colonies contained plasmids that hybridized to nick-translated SV40. Hirt supernatants from heterokaryons of C4, C9, D3 and D6 were unable to transform E. coli, while lines C10, D4 and D9 transformed E. coli to ampicillin resistance. Surprisingly, the transformation efficiencies were at least 100 fold down from predictions based on the amounts of plasmid DNA present in the Hirt supernatants as estimated from DNA blotting. For example, D9 was estimated by hybridization to produce about 1 ng of pOT per 100 mm plate, yet transformation of E. coli with 0.1 ng of this DNA results in about 10 colonies under conditions when 0.1 ng of pBR322 results in about 10^4 colonies. Repeated attempts to remove possible inhibitors in the DNA preparation have failed to improve the efficiency.

The structures of the cloned plasmids rescued from C10, D4 and D9 have been compared on agarose gels, both uncut, and after digestion with Hind III + Bam, which is diagnostic for the SV40 sequences in pOT. Thirty C10 plasmids were examined; eleven of these are compared in Figure 7. Since C10 has about seven discrete integrants, precise excision should be reflected in the isolation of some identical plasmids. The analysis indicates that no two plasmids are identical; thus excision from C10 is imprecise. All but one of the C10 plasmids have the SV40 Hind III-C band, which contains the origin of replication. Beyond that, considerable variation can be seen both in the amount of early region sequences present and in the size of the plasmid band, which is consistent with the pattern of hybridizing species observed in the Hirt supernatants. These conclusions have been verified by DNA blotting, using nick-translated SV40 as probe. In addition, all the C10 plasmids have lost the Sal site of pBR322, which implies that the Sal-linearized pOT molecules did not cyclize cleanly during convection. In all likelihood, the Sal-linearized plasmids have been incorporated into hmw DNA by a process which generated substantial deletions at both ends.

The plasmids isolated from D9 by SV40 rescue are clearly identifiable as pOT (Figure 7). The one variant may reflect an imprecise excision event. It is clear that some aspect of the structure of pOT in D9 facilitates the release of wild-type pOT upon fusion. Similar

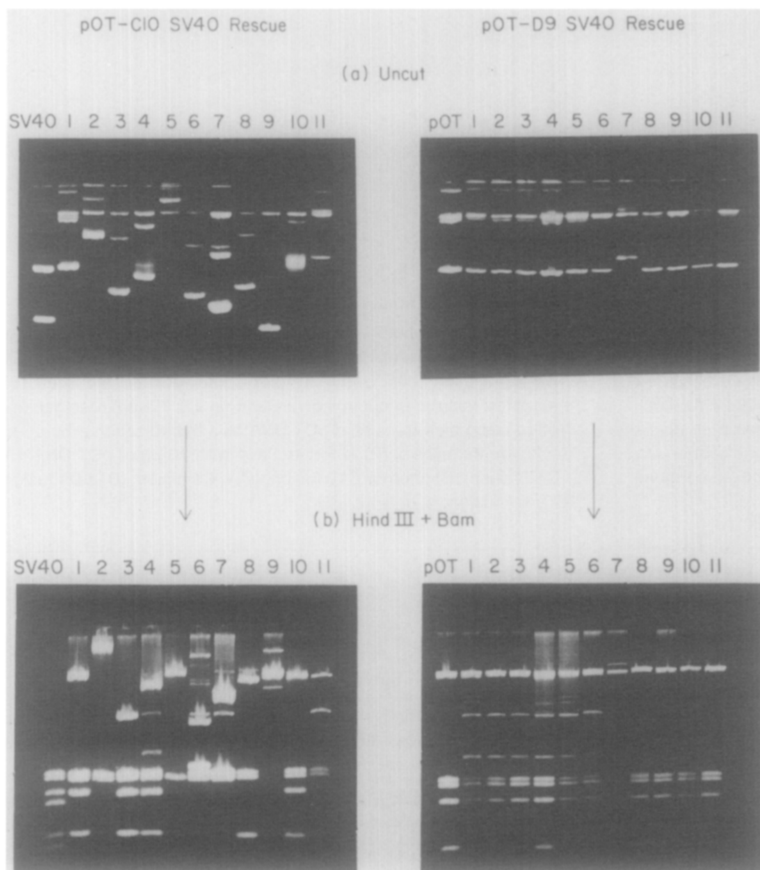

Figure 7. Analysis of pOT-Related Plasmids Obtained by SV40 Rescue from Two pOT Cell Lines

Hirt supernatants were collected following fusion of C10 and D9 to simian cells. The DNAs were used to transform E. coli χ1776. Plasmid DNAs were isolated from all resulting colonies and analyzed on gels. In this analysis, 11 clones of each were run (a) uncut and (b) digested with Hind III + Bam on 0.7% agarose gels, and visualized both by staining with ethidium bromide (shown) and by blotting (not shown). The left-hand track in each gel is either pOT DNA or SV40 DNA, as indicated. C10 and D9 arose from the convection of linear pOT and supercoiled pOT, respectively.

results were obtained after analysis of the plasmids rescued from D4.

Plasmid Rescue

To analyze directly the nature of the integrated state of convected pOT, we sought to plasmid-rescue the integrated structures from several cell lines. In each case, hmw cellular DNA was digested with a restriction enzyme that does not cut pOT. The DNA was then ligated under cyclization conditions and used to attempt transformation of E. coli.

Extensive attempts to rescue plasmids (from D7 and D9 using Bgl II, Sst I or Xba; from C10 with Bgl II or Xba; and from D4 using Bgl II or Sst I) all proved unsuccessful. It is possible that some of the integrated structures preclude plasmid rescue while allowing SV40 rescue, for example by having split plasmid functions. Three plasmids were rescued from D4 using Xba, and one was isolated from C10 with Sst I (see below). All the D4 rescuants hybridized to nick-translated SV40. Examination of the plasmids isolated from cultures of χ1776 showed multiple rearranged forms, some of which contained a site for Xba while others did not. Some feature of the structures of these plasmids appears to render them unstable in χ1776, and attempts to resolve their original structures have not succeeded.

Both C10 and D4 contain many copies of pOT sequences, yet the efficiencies of plasmid rescue are very low—at best one colony per 10 μg of cellular DNA, at a transformation efficiency of 10^8 colonies per μg of pBR322. DNA blotting data suggest that pOT DNA sequences are present in total cellular DNA of D4 and C10 at about 1 in 10^5, yet both rescue at about 1 in 10^9 (mass/mass). pOT is 8 kb in length, and any restriction enzyme used to release pOT by cutting in flanking cellular sequences can be expected to generate large plasmids (>10 kb). Decreased transformation efficiency due to size will certainly result. However, size alone is insufficient to account for the difficulties experienced in rescuing plasmids from mouse L cells.

One plasmid was rescued from C10, using Sst I to release the integrated structure. The plasmid (pC10S) contains a single restriction site for Sst I. Flanking the Sst site are sequences that do not hybridize to pOT. The plasmid co-migrates with one band in C10 cellular DNA when both are digested with Sst, distributed side by side on an agarose gel, and blotted using nick-translated pOT as probe. pOT was convected into Ltk⁻ cells as a Sal-cleaved linear molecule in the creation of the C10 cell line. pC10S lacks the Sal site of pOT, as well as the Bam site. This means that at least 275 bp have been lost from both ends of the linearized molecule in the process of integration. The structure of the integrant giving rise to pC10S is shown in Figure 8, as deduced from the restriction map of pC10S. In this case, pOT is clearly linked to the flanking DNA sequences in a manner most consistent with an integrated state that arose from imprecise end-on association with other DNAs.

Discussion

Rescue

Fusion of pOT-convected cell lines to permissive simian cells resulted in the excision of pOT-related molecules from hmw DNA and their subsequent replication in the heterokaryons. Analysis of these molecules indicates that lines convected with circular plasmids predominantly release molecules indistinguishable from the parental species, whereas lines convected with linear plasmids release a heterogeneous population of pOT-related molecules. These results bear comparison with studies on the integrated state and rescue upon fusion of cell lines resulting from the transformation of nonpermissive cells with SV40. Transformed cell lines arising from either viral infections or DNA transfections with supercoiled SV40 DNA will generally release wild-type SV40 when fused to permissive cells (Botchan, Topp and Sambrook, 1979; Schaffner, Topp and Botchan, 1979). It has been suggested that SV40 integrates with a partial tandem duplication, which can facilitate subsequent precise excision (by cyclization about the duplicated region) and hence rescue of wild-type SV40 (Kelly et al., 1974; Rigby, 1979; Basilico et al., 1979; Botchan et al., 1979). In lieu of a tandem duplication, cyclization will necessarily occur nonspecifically for lack of substantial region of homology, effecting imprecise excision and a heterogeneous population of supercoiled molecules. In one case, a transformed cell line

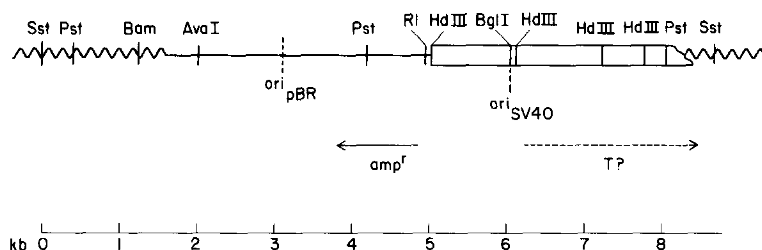

Figure 8. Physical Arrangement of a pOT Molecule in C10 DNA

The structure and restriction map of the integrated state of one of the copies of pOT in C10 is given as deduced from the structure of pC10S, which was isolated by plasmid rescue from hmw C10 DNA. High molecular weight cellular DNA was digested with Sst I, cyclized and used to transform E. coli χ1776, resulting in the appearance of pC10S. Analysis indicates that in this particular case sequences have been lost from both ends of the Sal-linearized pOT molecule that was convected into Ltk⁻ cells in the derivation of the C10 cell line. The cell/plasmid DNA junctions occur within the Pst-Bam fragment of the early region and within the Sal-Ava I fragment of pBR (see Figure 1).

that rescues SV40 imprecisely has been shown by cloning and sequencing studies to contain an integrated copy of SV40 that lacks any region of duplication (Botchan et al., 1980). Thus the rescues of plasmids from the lines convected with pOT mirror the patterns of rescue of SV40 from SV40-transformed lines. We suspect that upon convection the circular pOT molecules integrated with a partial tandem duplication whereas the linear molecules did not. In fact, the isolation by plasmid rescue and analysis of one integrant (pC10S) from a line convected with linear pOT (C10) demonstrates that in this case there was no appreciable duplication and that deletions have occurred at both ends of the linearized pOT molecule. The structure of this insertion is also consistent with the heterogeneous excision products observed after fusion of C10 cells to simian cells.

The various methods of introducing both the SV40 early region and linked DNA sequences into hmw DNA of nonpermissive cells—either nonselectively by convection, or by selection for transformation after viral infection or DNA transfection—all affect integrated states that allow for SV40-mediated excision upon fusion to cells permissive for SV40 replication. However, there are striking differences in the efficiencies of SV40 rescue. The amounts of pOT-related DNA in the Hirt supernatants are at least 20 fold less than that obtained from an equivalent lysate of a fusion involving an SV40-transformed line. At the moment we have no explanation for this observation. It is possible that the bacterial plasmid sequences in pOT inhibit the excision or replication of pOT in animal cells. While it is clear from our results that pOT can in fact both excise and replicate in the heterokaryons (see Figure 6), recent observations from several laboratories indicate that SV40-plasmid recombinants replicate very poorly (relative to SV40) when introduced into permissive simian cells (M. Botchan, D. Hanahan and K. Willison, unpublished results; W. Schaffner, personal communication; R. Tjian and R. Myers, personal communication). It is likely that the inefficient rescue of pOT and its poor replication in simian cells are related. Furthermore, the plasmid DNAs obtained either through SV40-mediated rescue or by the direct biochemical approach have proved to be surprisingly inefficient in retransforming E. coli. We suspect that some simple explanation such as DNA modification (or lack of it) links both of these observations together. For example, plasmid DNA may become heavily modified in the murine cells, which in turn may decrease its rate of replication in the eucaryotic nucleus and its ability to transform E. coli.

Expression

Two striking features of the expression of T antigen after convection of pOT into mouse L cells are the stable heterogeneity observed *within* a given cell line, where one fraction of cells shows no detectable T by indirect immunofluorescence, while another fraction exhibits intense nuclear staining and thus shows considerable expression; and its extremely wild variation *between* the cell lines examined, ranging from 0 to 99% positive in T. These may well be manifestations of the same phenomenon. We have no explanation for the mechanism by which the early region is switched on and off in such a perplexing fashion. Subcloning experiments and reexamination of the original lines on passage have demonstrated that heterogeneous expression is not simply a gradual decay process resulting from stable inactivation of the early region. The observations that completely negative lines can begin to express T on passage further indicate that changes in the regulation of T expression are occurring.

There is some precedent for these observations in the study of Risser and Pollack (1974), in which SV40 virions were used to infect mouse 3T3 cells at high multiplicity. Cells were replated under nonselective conditions at low density, and isolated colonies were picked randomly and examined for transformed properties as well as for T expression using indirect immunofluorescence. Risser and Pollack observed a class of cell lines that were stably heterogeneous for T expression. Attempts to subclone stable T negative clones from heterogeneous lines failed. Thus the patterns and stability of T expression in cell lines arising from an SV40 viral infection without selection parallel those arising from convection of the DNA of the early regions, also without selection. There have also been reports (Bacchetti and Graham, 1977; Scangos et al., 1979) detailing instability in the expression of the HSV thymidine kinase gene when the selection for expression is released. All these observations may well reflect some general property of the expression of convected genes analyzed without selection. Since the integration of exogenous genes does not appear to be site-specific, the ability of a convected gene to express may be dependent on variables in the integration process or on the chromosomal position the DNA becomes associated with.

The clonal variability of T expression is manifested not only in heterogeneous immunofluorescence patterns but also in the excision and replication of pOT in the heterokaryons of simian cells and pOT-convected cells. There is reasonable correlation between tbe extent of T expression and the relative efficiency of SV40 rescue. Lines that are predominantly positive for T rescue relatively well, whereas lines that are predominantly negative rescue poorly. Completely negative lines do not rescue pOT when fused to simian cells. However, there is no correlation between the apparent copy number of the SV40 early region in these cell lines and either the extent of T expression or the efficiency of SV40 rescue. Table 3 summarizes

the comparisons of these three characteristics among the six representative cell lines that have been studied in detail.

The wide range and apparent stability of heterogeneous expression among the convected cell lines may reflect some aspect of the environment of the early region in these cells. It is possible that convected DNA can become associated with domains of chromatin that are either relatively accessible or inaccessible, such that the ability of a convected gene to express is dependent on the state of chromatin it assumes. Recent evidence suggests that nonselected DNA becomes genetically linked both to the selected marker and to the carrier DNA used in the convection of DNA into mouse Ltk$^-$ cells (M. Perucho, D. Hanahan and M. Wigler, manuscript in preparation). Thus the multiple copies of the early region observed in cell lines such as D3 and D4 could all be genetically linked and in the same chromosomal domain, which might explain their poor expression and rescue. When the early region is directly linked to the thymidine kinase gene (in pOT-TK), heterogeneous expression of T is still observed. Thus it appears that linkage to the thymidine kinase gene in the input DNA is not sufficient to assure the continuous expression of the early region.

Selection for thymidine kinase demands expression of the exogenous tk gene in mouse Ltk$^-$ cells. If the tk gene became established in an inaccessible domain during convection, it is possible that the selection induced an amplification event in order to provide enough copies of the poorly expressing tk gene to satisfy the demands for sufficient thymidine kinase. In the process, the convected pOT plasmid may have been included in the amplification unit and thus fortuitously amplified. The evidence for amplification of pOT insertions follows directly from the comparison of the intensities with which discrete bands hybridize. For example, D9 has one insertion that appears to be single-copy, relative to the reconstruction shown in Figure 5. In contrast, D3 has five discrete insertions, and four of these are clearly present in many copies per cell when compared to the single-copy insertion in D9 (Figure 3). One prediction of the above hypothesis is that the tk gene should also be multiple-copy. Figure 9 shows a blot of the six lines characterized earlier, probed with the cloned thymidine kinase gene. Four of the five lines with multiple-copy pOT bands also appear to have multiple copies of the tk gene. In particular, the tk gene in D3 is multiple-copy and has an altered mobility, indicating that one of the HSV Pvu II sites was lost during convection. This strongly suggests that gene amplification after convection rather than initial acquisition of several genes is responsible for the multiple copies of the thymidine kinase gene observed in this particular line. Therefore, an amplification event may be occurring in response to the selection for thymidine kinase. Amplification induced by selection has been demonstrated clearly for the dihydrofolate reductase gene (Schimke et al., 1978, 1979) and the CAD gene (Wahl, Padgett and Stark, 1979a), and the amplification unit is on the order of 100 kb. There is no evidence to suggest that the mechanism of amplification is specific to these genes, and it is possible that any selection (including viral transformation) may induce amplification under certain circumstances. In fact, studies on rat cells transformed with adenovirus type 2 have shown similar apparent amplifications (Sambrook et al., 1977, 1980), as have adenovirus fragments convected into human tk$^-$ cells using a tk selection (Grodzicker and Klessig, 1980). Nevertheless, the relationship between the apparent amplification of pOT and the qualities of heterogeneous expression of the early region

Table 3. Comparison of the Observed Properties of Six Representative pOT Lines

Cell Line	Form of Convected DNA	Initial Scoring of T-Positive Nuclei (%)	Efficiency of SV40 Rescue	Apparent Copy Number of Intact Early Region
C4	linear	0	−	0–1
D3	supercoiled	0	−	>20
D4	supercoiled	10	+	>20
C10	linear	50	++	>30
D7	supercoiled	80	ND	2–3
D9	supercoiled	99	+++	1

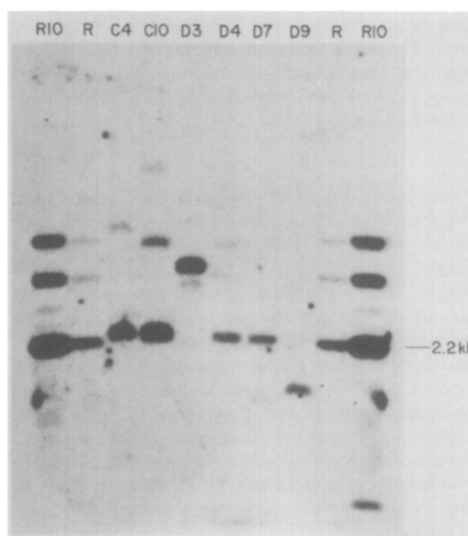

Figure 9. Blotting Analysis of Six pOT Lines for the tk Gene

12 μg of hmw cellular DNA from the six representative cell lines were digested with Pvu II, fractionated on a 0.9% agarose gel, blotted (no HCl treatment) and probed with 5 × 10^7 dpm of nick-translated tk DNA (the 3.4 kb Bam fragment, isolated from pTK2). Reconstructions of 1 (R) and 10 (R10) pg of Pvu II-cleaved pTK2 were included to approximate one and ten copies. Stringent hybridization conditions were used (3 × SSC, 72°C).

both between clones and within a given clone remain oblique.

Experimental Procedures

Plasmid Constructions

The tk gene had been localized to a 3.4 kb doublet in Bam HI-cleaved HSV-1 DNA (Wigler et al., 1977). To obtain a pure and plentiful supply of the tk gene, we cloned the HSV-tk gene in E. coli. The 3.4 kb Bam doublet was isolated by fractionation on an agarose gel and ligated to Bam-cleaved pBR322, and the ligation was used to transform E. coli strain χ1776. Resulting ampicillin-resistant colonies were screened by hybridization with a nick-translated HSV-1 Hpa I fragment that contains the tk gene but no sequences from the other 3.4 kb Bam fragment in the doublet (Wigler et al., 1977). Several hybridizing colonies were picked, and plasmid DNA was isolated and introduced into mouse Ltk$^-$ cells under HAT selection. All gave rise to tk$^+$ colonies. Suitable controls did not. One of the tk$^+$ plasmids was selected for further study and designated pTK2. This plasmid was mapped using several restriction enzymes; the map is shown in Figure 1. Comparable results have been reported by other investigators (Colbere-Garapin et al., 1979; Enquist et al., 1979; Wilkie et al., 1979).

The recombinant plasmid carrying the early region of SV40 is designated pOT for plasmid-SV40 origin of replication-*T* antigen, and was constructed as follows. SV40 DNA was digested to completion with Bam and then partially digested with Hind III. The DNA was fractionated on a 0.9% agarose gel. The band that included a 3737 bp fragment extending from the Hind III site at 0.86 map units through the SV40 origin and then through the entire early region to the Bam site at 0.14 map units was identified, cut out, electroeluted and ligated to pBR322 that had been digested with Hind III and Bam. The ligation was used to transform E. coli χ1776, and resultant colonies were screened with nick-translated SV40. Plasmid DNA was isolated from hybridizing colonies and analyzed with restriction enzymes. All such plasmids examined were found to have the structure predicted for pOT (Figure 1).

Finally, a plasmid (pOT-TK) was constructed that carried both the early region and the tk gene. pOT DNA was digested with Bam, ligated to a molar excess of the 3.4 kb Bam fragment of pTK2 and used to transform E. coli χ1776. DNA was isolated from several resulting colonies and analyzed with restriction enzymes. One contained the tk gene in the orientation shown in Figure 1 and was used in the studies described below.

Cell Culture

Culture of tk$^-$ cells under HAT selection (Szybalski, Szybalski and Ragni, 1962) for the acquisition of the HSV thymidine kinase gene has been previously described (Bacchetti and Graham, 1977; Maitland and McDougall, 1977; Wigler et al., 1977), as have the modifications (Wigler et al., 1979a, 1979b) to the calcium phosphate/DNA co-precipitation method (Graham and van der Eb, 1973) used to introduce exogenous DNAs into mouse Ltk$^-$ cells (Kit et al., 1963). Subcloning experiments utilized Costar microwells for establishing single cells as clones, and followed standard procedures. Fusions to CV1 cells were performed as described previously (Botchan et al., 1979). Transformation of E. coli χ1776 was accomplished using a high efficiency transformation procedure to be described elsewhere (D. Hanahan, manuscript in preparation). All experiments involving recombinant DNA were performed in accordance with the NIH Guidelines governing such research.

Fluorescent Assay for T

Expression of the SV40 early region can be monitored by indirect immunofluorescence using T antiserum, which exhibits strong and uniform nuclear staining in transformed cells (Pope and Rowe, 1964). Cells growing in plastic petri dishes or on glass coverslips were examined for the presence of the SV40 T antigen as follows. The cells were washed in PBS, fixed in 50% MeOH + 50% acetone for 1 min at room temperature, incubated with either rabbit antibody to the D2 protein or mouse monoclonal antibody (clone 3C5) to D2 protein for 30 min at room temperature, washed in PBS, incubated for 30 min with fluorescein-conjugated goat anti-rabbit or goat anti-mouse gammaglobulin (Capel Labs) and washed again in PBS. Cells were then mounted on gelvatol and examined in a fluorescence microscope. Cells were stained in various stages ranging from discrete colonies to confluence. At least 200 cells were scored for characteristic T nuclear fluorescence on any occasion. Both antibodies failed to give nuclear fluorescence to Ltk$^-$ cells, but stained SV40-transformed mouse cell lines (SV3T3). Both antisera immunoprecipitate T from SV40-infected cells.

DNAs

Plasmid DNAs were isolated from cultures of E. coli χ1776, grown to saturation in χb [2.5% tryptone, 0.75% yeast extract, 20 mM $MgCl_2$, 50 mM Tris (pH 7.6), 0.01% diaminopimelic acid, 0.01% thymidine], using a high salt/SDS lysis procedure (Tanaka and Weisblum, 1975). Other DNAs were prepared using methods that have been described previously: SV40 DNA was isolated from lytically infected CV1 cells (Trilling and Axelrod, 1970); HSV DNA (a gift from S. Silverstein) was isolated from infected CV1 cells (Pellicer et al., 1978); high molecular weight DNAs were extracted from confluent plates of L cells (Thomas, Berns and Kelly, 1966). Low molecular weight DNAs were collected using the Hirt extraction procedure (Hirt, 1967), as previously described (Botchan et al., 1979). The Hirt supernatants were purified further by phenol, chloroform and ether extractions; dialysis; treatment with RNAase; and several ethanol precipitations.

Nucleic Acid Analysis

DNA blotting followed standard modifications of published procedures (Southern, 1975; Botchan, Topp and Sambrook, 1976). After distributing the restricted DNAs on 0.5% agarose gels, the gels were soaked in 0.25 M HCl for ½ hour, washed twice in ddH_2O and then denatured with 0.25 M NaOH (Wahl et al., 1979b). Restriction enzymes and T4 DNA ligase were obtained from BRL or New England Biolabs and used under conditions recommended by the vendors.

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